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Preparation of Curcumin-Loaded Egg Albumin Nanoparticles Using Ethanol as Desolvation Agent

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Curcumin-loaded egg albumin nanoparticles were prepared by desolvation method using ethanol as desolvation agent. In this study, effect of factors *viz*. egg albumin concentration (5-15 % w/v) and pH (5-7) of egg albumin solution on solubility, curcumin loading, curcumin entrapment efficiency, nanoparticles yield and particle size of the prepared nanoparticles were investigated. Preparation process was optimized by the response surface methodology-central composite design of experiments. Under the optimum conditions of 8.06 % (w/v) egg albumin concentration and pH of 5.34, maximum solubility of 30.54 %, curcumin loading of 5.78 %, curcumin entrapment efficiency of 56.78 %, yield of 71.65 % and minimum particles size of 263.5 nm are achievable. Response surface methodology was found to be an effective tool to describe the effect of independent variables on quality characteristics of prepared curcumin-egg albumin nanoparticles and optimize the process parameters.

Keywords: Curcumin, Egg albumin nanoparticles, Desolvation, Ethanol.

INTRODUCTION

Curcumin, a natural polyphenol bioactive compound, the main yellow pigment of turmeric has been used for its medicinal properties including anti-inflammatory properties, skin wounds and tumors [1]. It has many bioactive properties, including antioxidant, anti-inflammatory and anticarcinogenic activities. Even though curcumin possess these promising features its applications as a functional ingredient in food products and therapeutic agent has been limited due to its low water solubility and sensitivity to alkaline conditions, thermal treatment, light, metallic ions, enzymes and oxygen. A major limiting factor of curcumin is its low solubility in water (0.0004 mg/mL at pH 7.3) and soluble curcumin molecules are extremely sensitive at physiological pH [2]. These properties limit its bioavailability and clinical efficacy.

Nanotechnology based drug delivery systems can provide a unique solution to overcome this problem of low systemic bioavailability of curcumin. Nano-encapsulation of curcumin could increase the water solubility, drug efficacy, specificity and absorption of curcumin into a selected tissue, bioavailability, retention time and it could also improve the intracellular penetration. Nanoparticles are comprised of nanospheres and nanocapsules with 1 to 1000 nm average diameter [3]. Various techniques have been applied to increase water solubility, stability and bioavailability of curcumin. Such techniques are emulsification, chemical modification, encapsulation in polymer nanoparticles, cyclodextrins, hydrogels and nanogels, polymeric and surfactants micelles, lipid bilayers, liposome/phospholipid, solid lipid nanoparticles, polymer conjugates, self-assemblies, vesicles and other delivery systems [4].

Albumin-based nanoparticle carrier systems represent an attractive strategy, since a significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule [5]. Albumin is an attractive macromolecular carrier that has been shown to be biodegradable, non-toxic, metabolized *in vivo* to produce innocuous degradation products, non-immunogenic, easy to purify and soluble in water and thus an ideal candidate for nanoparticle preparation [6].

Commercially, albumins are obtained in significant quantities from egg white (ovalbumin), bovine serum albumin and human serum albumin and also available from soybeans, milk and grains. Ovalbumin a major globular protein in egg white has valuable functional properties such as surface activity, its gel forming ability and stabilization of emulsions and foams [7]. Hence ovalbumin could be chosen as a carrier for drug delivery due to its availability and low cost, compared with other proteins. Due to its pH and temperature-sensitive properties, ovalbumin has high potential for use as a carrier

for controlled drug release [8]. Generally albumin nanoparticles are prepared by emulsion formation, desolvation or coacervation methods. Desolvation process of albumin with organic solvents followed by cross-linking with glutaraldehyde is the commonly used method to assemble protein nanoparticles [9]. In this method, the protein undergoes conformational changes that lead to selective aggregation around poor solvent nuclei by adding a desolvating agent drop by drop into the protein solution.

Albumin desolvation by water miscible organic solvents such as ethanol results in well-defined nanoparticles with denaturated albumin forming the matrix of the spheres. However, process parameters have a great influence on size, size distribution and particle yield of the resulting albumin nanoparticles. On the other hand, the particle size is assumed to have an effect on the drug incorporation and the cellular uptake [10]. In previous studies, ethanol was mainly used as desolvating agent of choice for the preparation of albumin-based nanoparticles [11]. Using ethanol as desolvating agent, the preparation process led to nanoparticles in a size range between 150 and 200 nm [12]. Lin *et al*. [13] prepared human serum albumin nanoparticles containing noscapine using coacervation method, in which ethanol was used as a dissolving agent, followed by crosslinking using glutaraldehyde. Merodio *et al*. [14] prepared the bovine serum albumin nanoparticles containing ganciclovir by coacervation method, in which the dissolving agent used was ethanol, followed by a cross-linking step with glutaraldehyde.

Dubey *et al*. [15] prepared egg albumin microspheres of 5-flourouracil by chemical crosslinking technique. Li *et al*. [16] used ethanol as the desolvating agent and 0.25 % (by volume) glutaraldehyde solution to prepare folate conjugated-human serum albumin. Kim *et al*. [17] produced nanoparticles from curcumin-human serum albumin using albumin bound nanoparticle technology. Curcumin-human serum albumin nanoparticles improved curcumin solubility in water (300-fold) leading to improved biological activity of curcumin. The average particles size and the curcumin loading were 135.5 ± 2.9 nm and 7.2 ± 2.5 %, respectively. Ankarao *et al*. [18] prepared carvedilol loaded egg albumin nanoparticles by coacervation method using gluteraldehyde as the cross linking agent and ethanol as desolvating agent. Aziz *et al*. [19] evaluated the effect of ovalbumin on microencapsulation of various drugs and reported that the per cent yield, drug loading and entrapment efficiency of non-soluble drugs increased, when the amount of ovalbumin was increased. Namasivayam and George [20] synthesized the bovine serum albumin nanoparticles coated with ofloxacin drug nano conjugate using ethanol as desolvating agent. Sadeghi *et al*. [21] studied the effect of different desolvating agents on curcumin-bovine serum albumin nanoparticle properties. Mirzaee *et al*. [22] suggested that "protein-curcumin" complexes might be effective tools for curcumin delivery *in vivo* and recommend that curcumin consumption as albumin containing capsules may lead to more stability, bioavailability and therapeutic efficiency, *in vivo*.

Most of the investigations on protein-based nanoparticles for drug carriers have been carried out with albumin from bovine and human serum [23]. There are only a few studies on the application of egg albumin as a drug carrier to encapsulate curcumin. Therefore, this study mainly focused on the preparation of curcumin nanoparticles using egg albumin as a carrier material using ethanol as desolvating agent.

Response surface methodology is a useful statistical technique in optimization of processing conditions for a range of food processes where a particular response is influenced by different variables [24]. The aim of this study is to investigate and optimize important process parameters for the preparation of curcumin loaded egg albumin nanoparticles using desolvation method. Response surface methodology was applied in order to explore the relationships between independent and response variables and optimize the preparation of curcumin-loaded egg albumin nanoparticles.

EXPERIMENTAL

Egg albumin powder (minimum 80.5 % protein) was obtained as a gift from M/s SKM egg products, Erode, Tamil Nadu. Curcumin (minimum 95 % purity) was obtained from M/s Synthite Industries Ltd, Kolenchery, Kerala. Glutaraldehyde (25 % w/v solution) was purchased from M/s Astron Chemicals (India), Ahmedabad. All other reagents used in the experiments were of AR grade.

Selection of effective experimental factors: A wide variety of factors such as desolvation agent concentration, desolvation agent addition rate (continuous or intermittent), glutaraldehyde concentration, pH, albumin concentration, stirring rate and curcumin concentration, could influence the production process. Earlier researches used ethanol or acetone (as a desolvating agent) to produce nanoparticles from bovine serum albumin or human serum albumin [11,25]. Li *et al*. [26] observed the uniformity in size of bovine serum albumin nanoparticles when ethanol was added intermittently. Langer *et al*. [11] reported that glutaraldehyde concentrations had no influence on particle size. The pH is the most important factor in controlling the coagulation of the albumin molecules during the desolvation process. When pH is far from the isoelectric point (pI) that provides a highly electrostatic repulsive condition for the albumin molecules and concomitantly, coagulations by protein-protein interactions are limited. As a result, fine albumin particles could be formed [27]. Isoelectric point of egg albumin is 4.8. Lin *et al*. [13] reported that the suitable pH for the crosslinking of protein ranged from 7 to 9. Weber *et al*. [25] reported that the lowest glutaraldehyde concentration required for the production of stable nanoparticles was 40 % with a reaction time of 24 h for sufficient cross-linking of all amino groups.

Based on the earlier research studies, ethanol was selected as desolvating agent and intermittent addition of ethanol was chosen to obtain smaller size particles. Since curcumin is stable only at a pH < 6.5 [28], egg albumin was chosen as a drug carrier and was made into solution with a pH range of 5-7 and at 5-15 % (w/v) concentration. Curcumin at 0.25 % (w/v) of egg albumin solution was selected for encapsulation.

Experimental design: Response surface methodology (RSM) was applied in order to explore the relationships between independent and response variables and optimize the preparation of curcumin-loaded egg albumin nanoparticles using ethanol as desolvating agent. Response surface methodology can

reduce the number of experiments without neglecting the interaction among the parameters. The process was optimized by a two-factor, five-level central composite design (CCD), with egg albumin solution concentration (EAC, A) and pH of egg albumin solution (pH, B) as independent variables (factors) and solubility, curcumin loading, curcumin entrapment efficiency, powder yield and particle size as response variables. A central composite design model was used to statistically optimize the formulation parameters and evaluate the main effects, interaction effects and quadratic effects of the formulation factors on the response variables of curcumin loaded-egg albumin nanoparticles. The two variables were taken at two levels as low and high, which were represented by coded value of -1 and +1, respectively. Center point was coded as 0 and five runs were carried out at the center point, to estimate the experimental error and to check the overall curvature effect. Four additional runs called axial (star) points which are coded as $-\alpha$ and $+\alpha$ were added to the 2^2 factorial design to form a central composite design. The distance of the axial points to the center point was calculated in order to make the design rotatable and it was given by $\alpha = 2^{k/4}$ (k is the number of independent variables, in this study $\alpha = 1.41$). A rotatable design provides equally good predictions at points equally distant from the center, a highly desirable property for response surface methodology. This experimental design allows estimating a full second order model for each response with only $2^k + 2k + 5$ central experiments (in this study k = 2). For each factor, low and high levels were selected on the basis of the results of previous studies and the feasibility of preparing the curcumin-loaded egg albumin at the extreme values. Table-1 shows the effective factors and their levels considered for the experimental design. The central composite design consists of 13 runs (Table-2), which includes four factorial points, five central points and four extra axial points.

Preparation of curcumin-egg albumin nanoparticles: Curcumin nanoparticles were prepared using egg albumin as the polymer using desolvation technique. For preparation of curcumin nanoparticles the method described by Weber *et al*. [25] was followed with some modifications. Egg albumin solution of various concentrations % (w/v) and pH as per the experimental design was prepared in distilled water at room temperature (28 ± 2 °C). The pH of solution was directly measured using a digital pH meter (HANNA instruments, Woonsocket, Rhode Island) and it was adjusted by adding 0.1 N HCl or sodium chloride solution. Subsequently, 0.25 % (w/v based on egg albumin solution) curcumin was dissolved in 20 mL ethanol, which was added intermittently into the aqueous egg albumin solutions under magnetic stirring at 500 rpm at room temperature. Each 5 min interval 2 mL of curcumin dissolved ethanol was added in egg albumin solution and this addition was continued until the solution became just turbid. This resulted in the formation of an opalescent suspension spontaneously at room temperature. After this desolvation process, 0.11 mL of 8 % (by volume) glutaraldehyde in water was added to the turbid solution to induce particle crosslinking. Then this solution was stirred continuously at 500 rpm at room temperature for 1 h. The complete cross linking process of the colloidal suspension was performed over a time period of 24 h. After that formed nanoparticles were purified by three cycles of centrifugation (3000 rpm, 30 min) and re-dispersion with distilled water to remove unreacted chemicals and desolvating agents. Purified pellets were transferred to a Teflon plate and dried in an oven at 50 °C for 6 h. The resulting curcumin-egg albumin particles were gently triturated using the mortar and pestle to obtain the dried fine curcumin nanoparticles.

Characteristics of curcumin-egg albumin nanoparticles: Curcumin loaded egg albumin nanoparticles which are prepared using ethanol as desolvating agent were analyzed for its properties.

Solubility: Solubility of curcumin-egg albumin nanoparticles was determined by the method of Cortes-Rojas and Oliveira [29]. One gram of encapsulated powder was added to 100 mL of distilled water and this mixture was stirred using a magnetic stirrer at 500 rpm. After keeping the mixture at 37 °C for 30 min, it was centrifuged at 10,000 rpm for 15 min. The precipitation was transferred into a drying vessel and dried at 105 °C until constant weight. The solubility (%) was calculated as the ratio of the solids mass of supernatant to the amount of the original weight of sample.

$$\text{Solubility (\%)} = \frac{\text{Grams of solid in supernatant}}{\text{Grams of sample taken}} \times 100 \quad (1)$$

Curcumin loading: Curcumin loading of curcumin-egg albumin nanoparticles is the ratio of amount of curcumin present in curcumin-egg albumin nanoparticles to the total weight (yield) of the nanoparticles obtained at the end of desolvation process and was calculated using the following equation [19]:

$$\text{Curcumin loading (\%)} = \frac{\text{Weight of curcumin in nanoparticles (g)}}{\text{Total weight of nanoparticles (g)}} \times 100 \quad (2)$$

Amount of curcumin present in curcumin-egg albumin nanoparticles is the difference between the total amount of curcumin used (0.25 % w/v based on egg albumin solution) and amount of curcumin present in aqueous supernatant phase which was obtained at the end of desolvation process after centrifugation. Curcumin present in supernatant was determined by measuring absorbance in the UV-visible spectrophotometer 108 (Make: Systronics, Ahmedabad, India) at 425 nm. A standard calibration curve of curcumin concentration *vs*. absorbance was plotted for this purpose. Regression equation obtained from the standard curve was as follows:

$$y = 0.2461x - 0.0052 \quad (3)$$

where 'y' referred to the absorbance value and 'x' referred to the concentration of curcumin (μg/mL). Furthermore, its R^2 was 0.998. Concentration of curcumin in supernatant was calculated by substituting the absorbance value in the regression equation.

Curcumin entrapment efficiency: Curcumin entrapment efficiency of the prepared curcumin-egg albumin nanoparticles was calculated using the equation as previously reported by Park *et al*. [30].

$$\text{Curcumin entrapment efficiency (\%)} = \frac{\text{Curcumin (Total)} - \text{Curcumin (in Supernatant)}}{\text{Curcumin (Total)}} \times 100 \quad (4)$$

where, Curcumin (Total) – Total amount of curcumin used in desolvation process (g), Curcumin (Supernatant) – Amount of curcumin present in aqueous supernatant phase which was obtained at the end of desolvation after centrifugation (g).

Nanoparticles yield: Curcumin-egg albumin nanoparticles yield is the ratio of the amount of curcumin-egg albumin nanoparticles obtained at the end of desolvation to the total amount of egg albumin and curcumin used during desolvation and percent yield was calculated using the following equation [19].

$$\text{Nanoparticles yield (\%)} = \frac{\text{Weight of curcumin – Egg albumin nanoparticles (g)}}{\text{Total weight of egg albumin and curcumin (g)}} \times 100 \quad (5)$$

Particle size: Particle size ranges of the nanoparticles were determined by particle size analyzer (nanopartica SZ-100, Horiba, Japan) using the method suggested by Jun *et al*. [31] with some modifications. For the size measurement, prepared curcumin loaded egg albumin powders were dispersed in absolute alcohol at 1:200 ratio. The dispersions were stirred continuously at 500 rpm for 15 min prior to particle size measurement. Particle size was measured at a temperature of 25 °C and a scattering angle of 90°. Experiments were done in triplicate and the average value was recorded.

Optimization and statistical analysis: Response data obtained from the central composite design (Table-2) were fitted into the polynomial models, given by the equation as given below, which described the effect of variables as well as the combined effect of all the variables on the response Y and determined the interrelationship among the variables.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad (6)$$

where Y is response calculated by the model, b_0 is the estimated regression coefficient of the fitted response at the center point of the design, b_1 and b_2 are the regression coefficient for linear effect terms, b_{11} and b_{22} are quadratic effects and b_{12} is interaction effect. A few response graphs were generated based on the highest interaction between the variables in order to visualize the relationship between response and experimental levels of independent variables as well as to deduce optimum conditions. The adequacy of the polynomial models was determined using model analysis, lack-of-fit test and coefficient of determination (R^2) and significance of the model was determined by analysis of variance at $P < 0.01$.A good fit of a model was considered when R^2 value was more than 0.8. Numerical optimization was carried out by defining constraint criterion as maximum and minimum ranges of the responses to determine the optimum level of independent treatment. The experimental design matrix, data analysis and optimization procedure were generated using commercial statistical package, Design-Expert version 9.0.5.1 (Statease Inc., Minneapolis, USA, Trial version).All results were expressed as the average values of three independent trials.

RESULTS AND DISCUSSION

Responses of different combinations of experiments as described in experimental design (Tables 1 and 2) were evaluated using the analysis of variance (Table-3). Analysis of variance indicated that all responses were found to have significant ($P < 0.01$) sum of squares and a non-significant ($P > 0.01$) lack of fit. Non-significant lack of fit indicates that the models are sufficiently accurate for predicting these responses. The estimated regression coefficients (in coded units) of the quadratic polynomial models (eqn. 6) for various responses and the corresponding R^2 and CV values are given in Table-4. All the responses are having high coefficient of determination (R^2) ranged from 0.974 to 0.997 for the regression equations. This indicated an adequate fit of the polynomial models to the response data. As a general rule, the coefficient of variation should not be greater than 10 %. In this case, the coefficients of variation for

TABLE-1
FACTORS AND THEIR LEVELS IN CODED AND ACTUAL VALUES

Independent variables	Symbol	Coded values				
		-α (-1.41)	-1	0	+1	+α (+1.41)
Egg albumin solution concentration (% w/v)	X_1	2.93	5	10	15	17.07
pH of egg albumin solution	X_2	4.58	5	6	7	7.41

TABLE-2
EXPERIMENTAL DESIGN AND RESPONSE VALUES OF CURCUMIN LOADED EGG ALBUMIN NANOPARTICLES

Run No.	Egg albumin solution concentration (% w/v)	pH of egg albumin solution	Solubility (%)	Curcumin loading (%)	Curcumin entrapment efficiency (%)	Nanoparticles yield (%)	Particle size (nm)
1	17.07	6.00	40.98	0.92	64.13	82.55	286.7
2	10.00	6.00	35.86	3.98	56.72	76.84	267.0
3	2.93	6.00	14.63	14.92	48.89	40.71	225.3
4	10.00	6.00	36.95	3.43	56.32	75.53	267.1
5	10.00	6.00	36.15	4.05	56.86	75.96	266.3
6	15.00	5.00	39.77	1.17	62.50	81.66	285.4
7	10.00	7.41	38.36	1.88	54.70	74.07	265.4
8	10.00	6.00	35.89	3.86	55.46	77.98	268.5
9	15.00	7.00	40.65	1.05	60.99	80.37	283.8
10	10.00	4.58	35.33	4.37	57.38	79.13	273.4
11	10.00	6.00	37.75	3.13	56.50	77.35	267.9
12	5.00	7.00	26.45	9.20	50.76	52.63	240.1
13	5.00	5.00	22.51	10.16	54.24	54.48	245.6

TABLE-3
ANALYSIS OF VARIANCE OF THE RESPONSES FOR THE FITTED SECOND-ORDER POLYNOMIAL MODEL

Source	Solubility (%)		Curcumin loading (%)		Curcumin entrapment efficiency (%)		Nanoparticles yield (%)		Particle size (nm)	
	df	Sum of squares	df	Sum of squares	df	Sum of squares	df	Sum of squares	df	Sum of squares
Model	5	738.70 [a]	5	206.00[a]	2	210.06 [a]	5	2088.50 [a]	5	3905.33 [a]
A-EAC	1	590.38 [a]	1	170.56[a]	1	200.43 [a]	1	1627.09 [a]	1	3626.65 [a]
B-pH	1	10.36 [a]	1	2.65 [a]	1	9.64 [a]	1	13.25 [a]	1	42.38 [a]
AB	1	2.34	1	0.18	-	-	1	0.078	1	3.80
A^2	1	131.79 [a]	1	30.19[a]	-	-	1	446.16 [a]	1	204.64 [a]
B^2	1	0.20	1	0.69	-	-	1	1.91	1	11.33 [a]
Residual	7	6.92	7	2.29	10	5.52	7	14.96	7	8.62
Lack of fit	3	4.25	3	1.66	6	4.31	3	10.97	3	5.71
Pure error	4	2.67	4	0.62	4	1.21	4	3.99	4	2.91
Corrected total	12	745.61	12	208.29	12	215.58	12	2103.46	12	3913.95

[a]Significant at $p < 0.01$

all the responses except curcumin loading per cent were less than 3 % (Table-4). To investigate the integrated effect of egg albumin concentration and pH of solution on each responses, response surface methodology was used and results were given in the form of 3D (three dimensional) response surface plots.

Effect of factors on solubility: Solubility values of nanoparticles are presented in Table-2 and the values ranged from 14.63 to 40.98 % within the combination of variables studied. Regression equation describing the effect of process variables on solubility of curcumin loaded egg albumin nanoparticles in terms of actual level of variables is given as:

$$\text{Solubility (\%)} = -8.05 + 6.12 * EAC + 0.66 * pH - 0.15 * EAC * pH - 0.17 * EAC^2 + 0.17 * pH^2 \quad (7)$$

where EAC is egg albumin solution concentration (% w/v) and pH is pH of egg albumin solution.

The linear term of egg albumin solution concentration (A) was found to have significant ($p < 0.01$) and positive effect and quadratic term (A^2) have significant ($p < 0.01$) and negative effect on solubility of nanoparticles. Linear term of egg albumin solution pH (B) have significant ($p < 0.05$) and positive effect on solubility (Table-4). Quadratic term (B^2) and interactive term (AB) have no significant effect on solubility, implying that the interaction between the different factors did not influence the response. R^2 value of 0.990 showed that the quadratic model had a good fit with experimental data.

Interactive effect of factors on solubility of curcumin loaded egg albumin nanoparticles is shown in Fig. 1. It was observed that increasing the concentration and pH of egg albumin solution increased the solubility of nanoparticles. This may be due to the high solubility of albumin (up to 40 % w/v) at pH 7.4 [32]. Increasing the pH value of albumin solution reduced the particle size due to an increased ionization of the albumin above its isoelectric point (pI), which leads to repulsion of albumin molecules and aggregates during particle formation [13]. Reduction in particle size could be the reason for higher solubility of nanoparticles due to the increased surface area. Kim *et al*. [17] produced curcumin-human serum albumin nanoparticles and reported that curcumin-human serum albumin nanoparticles showed much greater water solubility (300-fold) than free curcumin.

Effect of factors on curcumin loading: Curcumin loading per cent of prepared nanoparticles are presented in Table-2. Highest curcumin loading of 14.92 % was obtained with egg albumin solution having a pH and concentration of 6 and 2.93 %, respectively. Egg albumin solution at 6 % pH and 17.07 % concentration yielded the lowest curcumin loading of 0.92 %. Regression equation describing the effect of process variables on curcumin loading per cent in terms of actual level of variables is given as:

$$\text{Curcumin loading (\%)} = 15.91 - 2.84 * EAC + 2.78 * pH + 0.04 * EAC * pH + 0.08 * EAC^2 - 0.31 * pH^2 \quad (8)$$

where EAC is egg albumin solution concentration (% w/v) and pH is pH of egg albumin solution.

From Table-4, it was observed that the linear and quadratic term of egg albumin concentration (A, A^2) were found to have significant ($p < 0.01$) negative and positive effect on curcumin

TABLE-4
REGRESSION COEFFICIENTS OF THE FITTED SECOND-ORDER POLYNOMIALS

Coefficients	Solubility (%)	Curcumin loading (%)	Curcumin entrapment efficiency (%)	Nanoparticles yield (%)	Particle size (nm)
Intercept	36.52	3.69	56.57	76.73	267.36
A	8.59[a]	-4.62 [a]	5.00 [a]	14.26 [a]	21.29 [a]
B	1.14 [b]	-0.57 [b]	-1.10 [a]	-1.29 [b]	-2.30 [a]
A^2	-4.35 [a]	2.08 [a]	–	-8.0085 [a]	-5.42 [a]
B^2	0.17	-0.31	–	-0.52	1.28 [b]
AB	-0.76	0.21	–	0.14	0.97
R^2	0.9907	0.9890	0.9744	0.9929	0.9978
Adj. R^2	0.9841	0.9812	0.9693	0.9878	0.9962
C.V. %	2.93	11.96	1.31	2.05	0.42

[a]Significant at $p < 0.01$; [b]Significant at $p < 0.05$

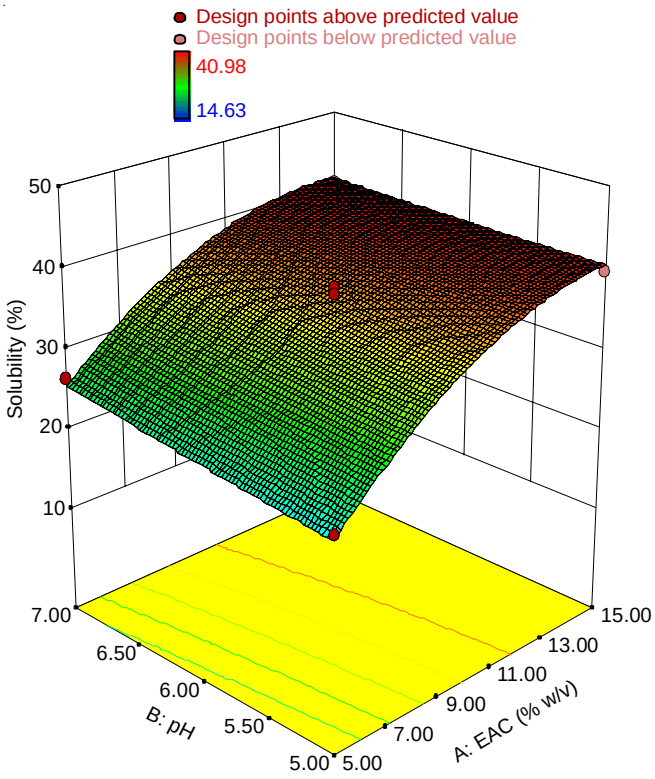

Fig. 1. 3D response surface plot for the effect of factors on solubility

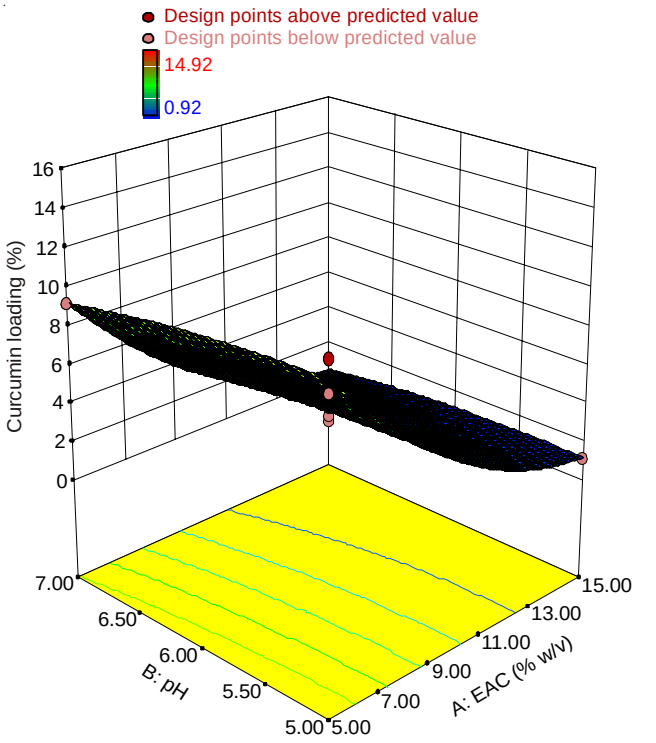

Fig. 2. 3D surface plot for the effect of factors on curcumin loading

loading, respectively. Linear term of pH (B) was significant at $p < 0.05$ and showed negative effect on curcumin loading. Quadratic term of pH (B^2) and the interactive term were not significant which indicated that the interaction between concentration and pH of egg albumin solution did not influence the curcumin loading. The coefficient of determination R^2 had a value of 0.989 indicating a good fit of the quadratic model with experimental data.

Fig. 2 shows the effect of factors on curcumin loading of prepared nanoparticles. It was observed that increasing the concentration of egg albumin decreased the curcumin loading. Increasing the egg albumin concentration might have increased the amount of nanoparticles. Higher quantity of nanoparticles with constant addition of curcumin will reduce the curcumin content and hence the loading percentage. Li *et al.* [26] observed reduction in drug loading when albumin:core ratio was increased from 2:1 to 16:1 (by mass) during the preparation of sodium ferulate entrapped bovine serum albumin nanoparticles using desolvation process. From Fig. 2 it was also observed that increasing pH of solution decreased the curcumin loading of nanoparticles. This may be due to the increased electrostatic repulsion between protein and drug at higher pH values. Reduction in pH value will increase the binding affinity because pH values nearer to the isoelectric point of the albumin molecule leading to a reduced electrostatic repulsion between protein and drug. Wilting *et al.* [33] reported that a decrease of the pH value led to an increase of antisense oligonucleotides (ASO) adsorption to human serum albumin.

Effect of factors on curcumin entrapment efficiency: Curcumin entrapment efficiency of the curcumin loaded egg albumin nanoparticles are shown in Table-2 and the values ranged from 48.89 to 64.13 %. The following is the regression equation describing the effect of process variables on curcumin loading per cent in terms of actual level of variables:

$$\text{Curcumin entrapment efficiency (\%)} = 53.15 + 1.00 * \text{EAC} - 1.10 * \text{pH} \quad (9)$$

where EAC is egg albumin solution concentration (% w/v) and pH is pH of egg albumin solution.

The linear terms of factor (A) and (B) were found to have significantly ($p < 0.01$) positive and negative coefficients, respectively (Table-4). This indicated that concentration of egg albumin solution had positive effect and the pH of solution had negative effect on curcumin entrapment efficiency. Coefficient of determination value of the regression equation was 0.974, indicating the good fit of model to response values.

Fig. 3 shows the effect of factors on curcumin entrapment efficiency during desolvation process. It can be observed that curcumin entrapment efficiency increased from 48.89 to 64.13 % with the increase in egg albumin concentration from 2.93 to 17.07 % (w/v). This may be the result from the fact that larger particles have larger volume, thus it can hold more drugs than smaller size particles. Increase in particle size was observed when egg albumin concentration was increased due to increase in viscosity of the droplet which resulted in the formation of larger particles. Further explanation could be that with the increase in the concentration of polymer in the aqueous phase, viscosity of the aqueous phase increased. The more viscous phase provides higher mass transfer resistance [34] and prevents drug diffusion towards the external phase, which in turn results in higher entrapment efficiency. Deveswaran *et al.* [35] studied the encapsulation of aceclofenac using albumin microspheres and they found that as the polymer concentration increased the drug encapsulation was found to be increasing in albumin microspheres and encapsulation efficiency was found to be between 36.3-65.2 %. Aziz *et al.* [19]

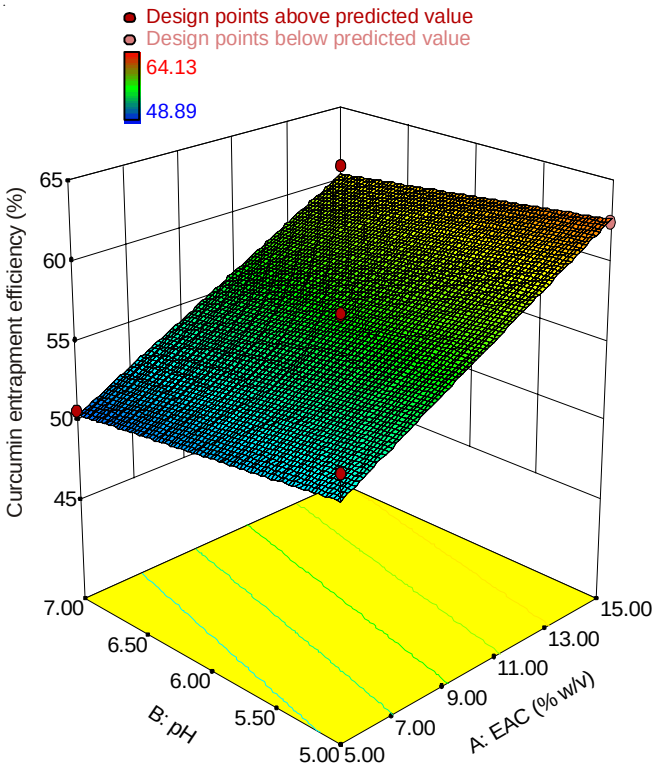

Fig. 3. 3D surface plot for the effect of factors on curcumin entrapment efficiency

observed that the per cent of yield, drug loading and entrapment efficiency of curcumin increased, when the amount of ovalbumin was increased.

From Fig. 3, it was also observed that increase in pH of solution decreased the entrapment efficiency due to the reduced particle size at higher pH values. It was found that with the increasing pH values of albumin solution, particle size was reduced due to an increased ionization of albumin, which leads to repulsion of albumin molecules and aggregates during particle formation [13]. Smaller particles have less volume to hold curcumin than larger one, thus increase in pH reduced the entrapment efficiency.

Effect of factors on curcumin nanoparticles yield: Yield of the curcumin nanoparticles are shown in Table-2. The highest yield of 82.55 % was obtained at egg albumin concentration of 17.07 % (w/v) and pH of 6 and the lowest yield of 40.71 % was recorded at the conditions of 2.93 % (w/v) of egg albumin solution and pH of 6. Effect of process variables on curcumin entrapment efficiency in terms of actual level of variables is given as:

$$\text{Nanoparticles yield (\%)} = 6.73 + 9.09 * EAC + 4.71 * pH + 0.03 * EAC * pH - 0.32 * EAC^2 - 0.52 * pH^2 \quad (10)$$

where EAC is egg albumin solution concentration (% w/v) and pH is pH of egg albumin solution.

Coefficients of regression equation representing the relationship between the variables and nanoparticles yield (Table-4) revealed that the linear and quadratic terms of egg albumin concentration (A, A^2) have significantly ($p < 0.01$) positive and negative effect on curcumin entrapment efficiency, respectively. Linear term of pH (B) showed significantly negative effect at $p < 0.05$ and the quadratic (B^2) and interaction (AB) had no influence on response. The coefficient of determination (R^2) had a value of 0.992 revealing that the quadratic model had a good fit with experimental results.

Effect of factors on nanoparticles yield is shown in Fig. 4 and it was observed that increase in egg albumin concentration increased nanoparticles yield. The more egg albumin used, the more desolvation of albumin was involved, which resulted in higher particle yield. Jithan *et al.* [36] prepared polymer-based curcumin nanoparticles using bovine serum albumin at drug: polymer ratios of 1:1, 1:2, 1:3, 1:4 and percent yield was found to be 51.9, 84.3, 85 and 85.2 %, respectively.

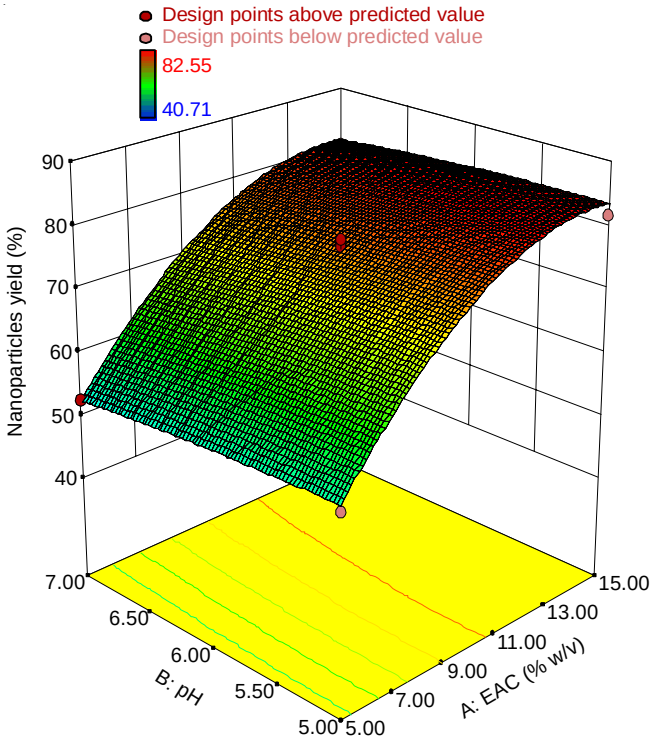

Fig. 4. 3D surface plot for the effect of factors on nanoparticles yield

Effect of factors on particle size: Particle size of curcumin loaded egg albumin nanoparticles ranged from 225.3 to 286.7 nm and the values are given in Table-2. It was recorded that smaller sized particles were obtained at the egg albumin concentration of 2.93 % (w/v) and pH 6. Egg albumin concentration of 17.07 % (w/v) and pH 6 of the solution resulted in large size particles. The relationship between factors and particle size in terms of actual level of variables is given by the following equation:

$$\text{Particle size (nm)} = 274.54 + 7.43 * EAC - 19.57 * pH + 0.20 * EAC * pH - 0.22 * EAC^2 + 1.28 * pH^2 \quad (11)$$

where EAC is egg albumin solution concentration (% w/v) and pH is pH of egg albumin solution.

From the coefficients of responses (Table-4), it was observed that the linear terms of egg albumin concentration (A), pH (B) and quadratic term of egg albumin concentration (A^2) had significantly ($p < 0.01$) positive, negative and negative effect on particle size, respectively. Quadratic term of pH (B^2) have significant positive effect at $p < 0.05$ on particle size. Interactive effect showed no significant effect on response. R^2 value of 0.997 indicated a good fit of the model.

Fig. 5 shows that the increase in egg albumin concentration increased the particles size when the concentration was increased from 2.93 to 17.07 % (w/v). Probably this might be due to the formation of aggregates with large diameter at higher concentrations of egg albumin solution. Langer *et al.* [11] reported that with increasing human serum albumin concen-tration the size of human serum albumin nanoparticles were increased. Aziz *et al.* [19] evaluated the effect of ovalbumin on microencapsulation process and reported that an increase in the amount of ovalbumin increased the particle size of curcumin particles.

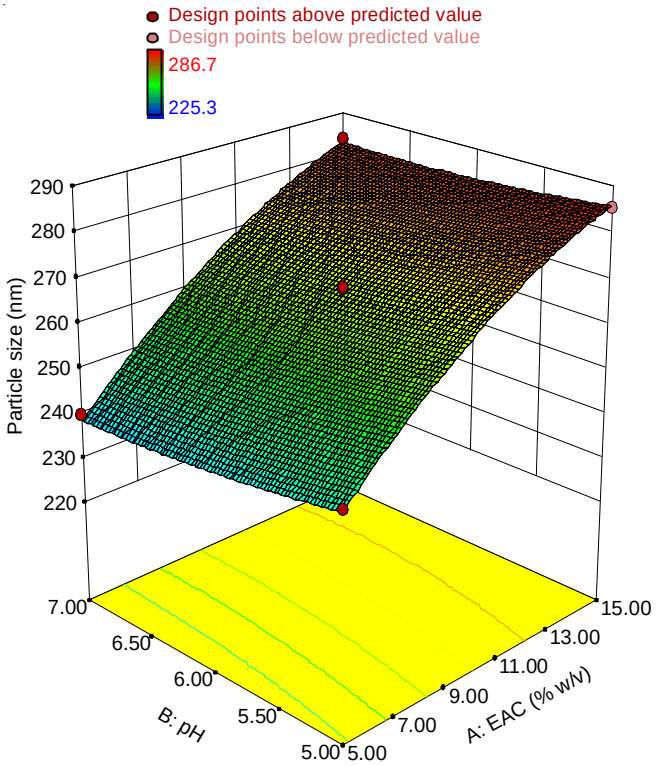

Fig. 5. 3D surface plot for the effect of factors on particle size

From Fig. 5 it was also found that increasing pH values of albumin solution, showed slight reduction in particle size due to an increased ionization of the serum albumin (at isoelectric point), which leads to repulsion of the serum albumin molecules and aggregates during particle formation [13]. On the other hand, at pH close to the isoelectric point electrostatic attraction could enhance the coagulation among albumin molecules; as a result, larger albumin particles are formed. When pH of the solution is close to its isoelectric point (pI), the enhanced protein-protein reactions lead to an increase of coagulation amongst albumin molecules [37]. Paik *et al.* [38] studied the effect of pH on size distribution of the bovine serum albumin nanoparticles and found that during the intermittent addition of ethanol, particle size decreased as pH increased from 6 to 9. At pH 6, the average size of the bovine serum albumin nanoparticles was about 300 nm.

Optimization and validation: Numerical optimization technique was adopted to optimize the process parameters for preparation of curcumin loaded egg albumin nanoparticles using ethanol as desolvation agent. The constraints criterion for numerical optimization was defined as maximum solubility, curcumin loading, curcumin entrapment efficiency, nanoparticles yield and minimum particle size. The desired goals for each factor and response were chosen and same weights were assigned to each goal (Table-5).

Egg albumin solution concentration of 8.06 % (w/v) and pH of 5.34 were found to be the optimum conditions. This predicted optimum condition had the maximum desirability of 0.499. Desolvation process was performed using the derived optimum process parameters and the quality characteristics of the resulting particles were determined. The experimental values (mean of 3 measurements) as well as the predicted values of various characteristics are presented in Table-6. Experimental values prepared under optimum conditions were close to the predicted values and with low error per cent. This suggested that the optimized preparation was reliable and reasonable and that the second-order polynomial models could be used to predict quality characteristics of curcumin loaded egg albumin nanoparticles at different levels of concentration and pH of egg albumin solution during desolvation process. The maximum solubility of 30.54 %, curcumin loading of 5.78 %, curcumin entrapment efficiency of 56.78 %, yield of 71.65 % and minimum particles size of 263.5 nm were obtained at the process condition of egg albumin solution concentration of 8.06 % (w/v) and pH of 5.34.

Conclusion

Response surface methodology approach was used to optimize the process parameters for the preparation of curcumin-egg albumin nanoparticles using ethanol as desolvating agent. Responses such as solubility, curcumin loading, curcumin entrapment efficiency, nanoparticles yield particle size were dependent significantly on the process variables namely, concentration and pH of egg albumin solution. The second-order polynomial model was well fitted to predict the experimental data for all responses with high values of R^2 (>0.9). The optimum condition was found to be egg albumin solution concentration of 8.06 % w/v and pH of egg albumin solution of 5.34. The experimental response values were found in close to the predicted values from fitted models. With the optimum conditions

TABLE-5
OPTIMIZATION CRITERIA FOR DIFFERENT FACTORS AND RESPONSES

Factors/Response	Goal	Lower limit	Upper limit	Importance
A: EAC	In the range	5	15	3
B: pH	In the range	5	7	3
Solubility (%)	Maximize	14.63	40.98	3
Curcumin loading (%)	Maximize	0.92	14.92	3
Curcumin entrapment efficiency (%)	Maximize	48.89	64.13	3
Nanoparticles yield (%)	Maximize	40.71	82.55	3
Particle size (nm)	Minimize	225.3	286.7	3

TABLE-6
PREDICTED AND EXPERIMENTAL VALUES OF RESPONSES AT OPTIMUM CONDITIONS

Response	Predicted value	Actual value	Error (%)
Solubility (%)	31.67	30.54	3.56
Curcumin loading (%)	6.08	5.78	4.93
Curcumin entrapment efficiency (%)	55.36	56.78	-2.56
Nanoparticles yield (%)	70.66	71.65	-1.40
Particle size (nm)	260.6	263.5	-1.11

Error (%) = (Predicted value-Actual value)/Predicted value × 100

given for the variables, the process may be scaled up for the preparation of curcumin loaded egg albumin nanoparticles as a functional ingredient.

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