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Original Article

Effect of Processing Parameters on Physicochemical Properties of β-Carotene Nanocrystal: A Statistical Experimental Design Analysis

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Abstract

Incorporation of functional foods and nutraceuticals such as carotenoids which suffer from poor water solubility and low bioavailability into nano-sized delivery systems can improve their solubility, stability, and oral bioavailability. The aim of this study was to prepare β-Carotene nanodispersion and investigate the effects of preparation parameters by means of response surface methodology using central composite design. Therefore, the impact of the preparation conditions namely the homogenizer speed, evaporation temperature, and rotation speed (as independent variables) on the mean particle size, particle size distribution, and β-carotene amount of the prepared β-carotene nanodispersion (as responses) were evaluated. A multiple-optimization procedure showed that the optimum conditions of homogenization speed as well as evaporation temperature and rotary speed were 15000 rpm, 32 °C , and 140 rpm, respectively. A statistical assessment showed insignificant ($p > 0.05$) differences between experimental and predicted responses values, verifying the fitness of the final reduced models for explaining the variation of nanoemulsion properties. Using statistical methods can reduce the number of experiments by optimizing of formulations during development and lead to significant save in time and cost.

Keyword: Nanodispersion, β-carotene, Response surface methodology, Central composite design, Factorial design

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1. Introduction

Functional food ingredients such as carotenoids are being extensively used in a great variety of food, cosmetic and pharmaceutical products as antioxidants and nutrition value enhancers [1-3]. β-Carotene is the most well-known carotenoid with distinguished antioxidant property. Its hydrophobic and bicyclic structure contains 11 conjugated double bonds which is responsible for its high antioxidative activity [4,5]. That is why β-Carotene is more prone for instability in the presence of light, oxygen, and heat, as well. Many health benefits have been reported for proper administration of β-Carotene. For instance, it can reduce the risks of heart disease and stimulate the proliferation of osteoblast cells differentiation. Furthermore, it may protect the skin cells against sunburn, prevent aging, macular degeneration, cancer, etc. [6,7]. However, the water insolubility of β-Carotene makes it less bioavailable and also difficult to incorporate in most food and pharmaceutical products [8-10]. Therefore, it is important to plan a strategy for improving β-Carotene dispersibility in water, chemical stability, and bioavailability [11,12]. Nanotechnology provides a good strategy to overcome the mentioned drawbacks of this lipophilic compound. For example, the incorporation of these compounds in nanosized-dispersion can increase their surface areas and dissolution rates and thus enhance their saturation solubility [13]. Moreover, it was shown that the absorption and particle uptake of nanoparticles with the mean particle size less than 500 nm is higher than particles larger than 500 nm. In addition, nanodispersions with sizes of less than 200 nm have the appealing aesthetic properties of low viscosity and transparent appearance [14,15]. Among all developed techniques for preparation of organic nanodispersion systems, emulsification–evaporation is the most prevalent for preparing the carotenoid nanodispersions. In this method, the active compound is dissolved in a non-polar solvent, and the oil-in-water emulsion is formed by emulsifying the non-polar phase into the aqueous one containing a suitable emulsifier. The particle size of dispersed phase can be decreased to nano ranges by high energy input applying into system using homogenizers [16,17]. The traditional methods for the formulation design are based on large number of experiments, time, and cost. Statistical experimental design is a well-established method for planning experiments. Response surface methodologies (RSM) have been successfully applied in both drug discovery and development. RSM is an empirical modeling approach for determining the relationship between various process parameters and responses. Its main advantage is the reduction in the number of experimental trials needed to evaluate multiple parameters and their interactions. Therefore, it is less laborious and time-consuming than other approaches required to optimize a process [18-20]. Therefore, the present work was aimed to optimize the most affective processing parameters, namely, homogenization speed and temperature as well as the rotary speed, as independent variables leading to the smallest particle size and the highest β-Carotene amount during processing steps using RSM.

2. Materials and Methods

2.1 Materials

β-Carotene (>97%) was purchased from Sigma-Aldrich Company (St Louis, USA). Polyoxyethylene sorbitan monolaurate (Tween® 20) was prepared from Merck Chemicals Company (Darmstadt, Germany). Dichloromethane and n-hexane were analytical and HPLC-grade purchased from Scharlau Chemie SA (Barcelona, Spain).

2.2. Preparation of β-Carotene Nanodispersion

Emulsification–evaporation method was used to prepare β-Carotene nanodispersion. β-Carotene and Tween® 20 were dissolved in n-hexane/dichloromethane mixture (1:1 v/v) and deionized water with the concentrations of 3 mg/mL and 10 mg/mL, respectively. The organic phase to aqueous phase ratio was set at 1:9 (v/v) and the aqueous phase was added into organic phase under the homogenization (Silent Crusher M, Heidolph, Germany) at different homogenizer speed at room temperature for 15 min to produce o/w nanoemulsions. The organic phase of the prepared nanoemulsions was evaporated at 0.25 bar for 20 min using a rotary evaporator (Laborata 4002, Heidolph, Germany) with different temperatures and speeds (Table 1) to form β-Carotene nanocrystals.

2.3. Particle Size Analysis

The mean particle size of nanodispersions was assessed by measuring the severity of laser beam scattered by the samples at the angle of 90° in room temperature with the dynamic light scattering technique using a laser diffraction particle-size analyzer (SALD 2101, Shimadzu, Japan). The experiments were carried out on the diluted nanodispersions after preparation. The particle size of the prepared β-Carotene nanodispersions was described by the volume mean diameter (VMD). The polydispersity of the particles was expressed by the span index:

$$\text{Span} = [dv,_{90} - dv,_{10}]/dv,_{50}$$

Where $dv,_{90}$, $dv,_{10}$ and $dv,_{50}$ are the equivalent volumes diameters at 90%, 10% and 50% cumulative volume, respectively. The measurements were reported as averages of three samples, with two readings of each experiment [21].

2.4. β-Carotene Nanocrystal Formation Yield

In order to measure the β-Carotene content of the prepared nanodispersions, the β-Carotene in the nanodispersions was extracted by adding 1000 µL dichloromethane to the samples (500 µL) and then vortexed-mixed (Heidolph, Germany). After appropriate dilutions with dichloromethane, the β-Carotene content was determined by spectrophotometry at 461 nm (Ultrospec 2000, Pharmacia Biotech, UK) [4]. The calibration curve of peak area versus β-Carotene concentration was linear in the concentration range of 0.5–10 µg/mL (R^2 = 0.9954, n = 8). Interday and intraday variabilities were calculated and found to be <3% of coefficient variations. Measurements were performed in triplicate for each sample and standard.

2.5. Experimental Design

A three-factor central composite design was employed to study the effects of the preparation procedure variables (rotary temperature, x_1; rotary speed, x_2; and homogenization speed, x_3) on average particle size (Y_1), size distribution (Span, Y_2) and β-Carotene nanocrystal formation yield (Y_3) of prepared formulations. The center point was repeated six times and individual experiments were randomized in order to decrease the effects of inexplicable variability in the actual responses due to extraneous factors (Table 1 and 2).

Response surface analysis is commonly used for predicting the response variables, changed by studied preparation conditions, and determining the optimum levels of the independent variables leading to the obtain desired response goals. Multiple-regression coefficients were determined by employing a least-squares technique to predict the linear and quadratic polynomial models for the response variables [22]. Analysis of variance (ANOVA) and regression-surface analysis were conducted to determine the statistical significance of model terms and fit a regression relationship linking the experimental data to the independent variables. The generalized polynomial model for describing the variation of the response variables is given below:

$$Y=\beta_0 + \sum\beta_i x_i + \sum\beta_i x_i^2 + \sum\beta_{ij} x_i x_j$$

where Y is the response value, b_0 is an offset value, and b_i, b_{ii} and b_{ij} are the main (linear), quadratic and interaction regression coefficients, respectively. The quality of the polynomial models was determined by the coefficient of determinations, namely, R^2 and Adj-R^2 [23]. The significance of each term was expressed in confidence level of 0.05. Therefore, the terms with larger absolute t value or smaller p-value were considered as more significant on selected response variations. The terms with p>0.05, were reported as statistically non-significant and dropped from the initial models. The experimental data were re-fitted only to significant (p<0.05) independent variables. However, non-significant linear terms were kept in the model if a quadratic or interaction term containing this variable was significant

Table 1. Levels of independent variables established according to the central composite design.

Variables	**Independent variable levels**				
	Low	**Center**	**High**	**-α**	**+α**
Rotary Temperature (ºC)	30	46	62	36	56
Rotary Speed (rpm)	0	135	270	52	218
Homogenizer Speed (× 1000 rpm)	11	14.5	18	12.5	17

Table 2. Matrix of the central composite design.

Treatment runs	**Blocks**	**Evaporator Temperature (^{0}C)**	**Evaporator Speed (rpm)**	**High-speed homogenizer Speed *1000 (rpm)**
1	1	46	270	5.14
2	2 (C)	46	135	5.14
3	3 (C)	46	135	5.14
4	4	46	135	18
5	5	30	135	5.14
6	6	62	135	5.14
7	7	46	0	5.14
8	8	46	135	11
9	9	56	218	17
10	10	36	52	17
11	11 (C)	46	135	5.14
12	12	36	218	12.5
13	13	56	52	12.5
14	14 (C)	46	135	5.14
15	15	56	52	17
16	16	36	52	12.5
17	17	56	218	12.5
18	18	36	218	17
19	19 (C)	46	135	5.14
20	20 (C)	46	135	5.14

(C): center point.

($p<0.05$). All obtained correlations are valid only within the selected range of variables. The experimental design matrix, data analysis and optimization procedure were performed using the Minitab v. 14 statistical package (Minitab Inc., PA, USA).

2.6. Fitting the Response-Surface Equations

Response-surface analysis provided empirically significant ($p<0.05$) models for estimating the variation of the average particle size, Span, and β-Carotene nanocrystal concentration as functions of the preparation condition of the nanodispersion. As a result, the optimization process predicted an optimum level for all independent variables that resulted in the desirable goals. The non-significant ($p>0.05$) terms were dropped from the initial model and then the experimental data were fitted again only to the significant ($p<0.05$) parameters to obtain the final reduced model. However, the non-significant ($p>0.05$) linear terms were included in the final reduced model if quadratic or interaction terms containing these variables were found to be significant ($p<0.05$) [24].

2.7. Response Surface and Contour Plots

This type of plots is very useful in formulations that contain three or more variables that affect the responses and used to show the relationship between them. To evaluate and compare the combined effects of variables, this type of plots gives useful information. Influential factors in the responses and the level of separated and combined effects of each factor are clear in these plots. In contour plots three-dimensional relationship is plotted in two dimensions with the axis Y, X (as a predictor) and response is shown as contour (like topographic maps). In these plots effect of variables on formulations parameters are analyzed.

3. Results and Discussion

Table 3 contains the corresponding R2 and adjusted R2 values of regression equations. The significance of each term, using F-ratio and p-value, is shown in Table 4. The response surface analysis indicated that the relationships between processing variables (x1, evaporation temperature; x2, evaporation speed and x3, homogenization speed) and the average particle size (Y1), Span (Y2), and β-Carotene concentration (Y3) could be explained by significant ($p<0.05$) second-order polynomial regression models. Rationally, high coefficients of determination (R2), ranging from 0.896 to 0.967, were obtained for the regression models (Table 3). As shown in Tables 3 and 4, all the main effects of processing variables should be retained in the final reduced models, while some of their quadratic and interaction effects must be deleted from the initial models for different responses. The main and quadratic effects of evaporation temperature and speed had a significant ($p<0.05$) effect on the variation of all studied nanodispersion properties, except for β-Carotene concentration, for which the quadratic effect of evaporation temperature and speed was found to be insignificant ($p>0.05$) (Table 3). As shown clearly in Tables 3 and 4, the interaction effects of evaporation temperature with other two independent variables had significant ($p<0.05$) effects on the particle size distribution and β-Carotene concentration. The variation of all studied nanodispersion properties were significantly ($p<0.05$) influenced by most of the processing variables. As stated by Montgomery (2001), the final reduced model fitted to the experimental data, was a valid statistical empirical model only in the selected ranges [24,25].

3.1. Effects of Variables on Average Particle Size

In the high energy emulsification-evaporation technique, severe mechanical forces of homogenizers are used to break the drops into

Table 3. Regression coefficients, R_2, adjusted R_2 and probability values for the final reduced models.

Regression coefficient*	Size (nm)	Span Value	β-Carotene nanocrystal (μg/mL)
B_0	7.23384	11.4821	220.69
B_1	-0.08078	-0.1506	-3.266
B_2	-0.00543	-0.0369	-0.395
B_3	-0.00068	-0.0005	-0.002
B_1^2	0.00092	-0.0016	-
B_2^2	0.00001	0.0000	-
B_3^2	0.00000	-	-0.0000
B_{12}	-	0.0004	0.004
B_{13}	-	0.0000	0.000
B_{23}	-	-	0.000
R^2	0.9626	0.9598	0.9678
R^2(adj)	0.9111	0.8968	0.9172
P-value (regression)	0.000[b]	0.001[b]	0.000[b]
F-value (regression)	22.79	15.99	22.24

*b_0 is a constant, b_i, b_{ii} and b_{ij} are the linear, quadratic and interaction coefficients of the quadratic polynomial equation, respectively. 1 Evaporator Temperature 2: Evaporator Speed; 3: High-speed homogenizer Speed.
**Significant ($p<0.05$).

smaller ones. In Silva et al.'s (2011) study the increase of homogenization rate due to the high intensity of the produced shear forces in the homogenization process showed a remarkable role in reducing the size of nanoparticles and improving the particle size distribution profile. In the present study, there was not more decrease in particle size in the homogenization rates higher than 15000 rpm. It is also worth mentioning that higher speeds of homogenization, helps the production of more air combination in prepared nanodispersions. This harmful phenomenon results in the rise of proper temperature of homogenization and dispersion system, and this temperature shock can cause a remarkable waste of active ingredients, especially in the case of β-Carotene which is sensitive to the temperature. The solvent evaporation rate also has main effect on characteristics of the prepared nanoparticles.

As shown in Table 4, all the main and quadratic effects of the independent variables were significant ($p<0.05$) for the mean particle size (Y1), and the variation of mean particle size was significantly ($p<0.05$) explained by a second-order regression equation ($R^2 = 0.96$; Table 3). These observations confirmed that the variation of the particle size could be accurately explained as a function of linear and quadratic effects of temperature and the speed of evaporator and homogenization. As also presented in table 3, the interaction effects had no significant effect on the mean particle size. Therefore, the particle size was explained by a second-order polynomial equation of independent variables without any interaction terms. As shown in the regression equation, all

Table 4. The significance probability (p-value, F-ratio) of regression coefficients on final reduced second-order polynomial models.

Variables		**Main effects**						**Quadratic effects**		
Interacted effects		X_1	X_2	X_3	X_1^2	X_2^2	X_3^2	X_1X_2	X_1X_3	X_2X_3
Average particle size (Y_1, nm)	**p-value**	0.004[b]	0.03[b]	0.000[b]	0.000[b]	0.0011[b]	0.000[b]	-	-	-
	F-ratio	15.64	6.96	39.04	32.04	10.86	39.56	-	-	-
Particle size distribution(Y_2)	**p-value**	0.04[b]	0.000[b]	0.146	0.012[b]	0.001[b]	-	0.002[b]	0.002[b]	-
	F-ratio	5.621	39.6	2.66	11.26	25.44	-	21.7	23.84	-
Beta-carotene concentration(Y_3, μg/ml)	**p-value**	0.002[b]	0.002[b]	0.631	-	-	0.014[b]	0.001[b]	0.001[b]	0.01[b]
	F-ratio	22.54	24.6	0.25	-	-	10.44	27.09	5.16	12.2

*Significant ($p < 0.05$).

coefficients of single effects of variables (b_1, b_2 and b_3) were negative, so at low levels, increasing the temperature and speed of evaporation as well as homogenization speed decreased the particle size, while the positive signs of all the quadratic terms (b_{11}, b_{22} and b_{33}) demonstrated that at high levels, the increases in variable values could lead to an increase in the particle size (Fig 1).

The individual optimum condition indicated that the minimum particle size (Y1 = 30.5 nm) was predicted to be obtained by the homogenization speed of 15000 rpm, evaporation temperature of 36 ºC and evaporation speed of 150 rpm.

3.2. Effects of Variables on Particle Size Distribution

As shown in tables 3 and 4, the variation in total particle size distribution was significantly ($p<0.05$) well-fitted by a second-order nonlinear regression equation ($R^2 = 0.95$). Thus, more than 95% of the variability of physicochemical properties in the dispersions could be explained by the RSM models as a nonlinear function of main processing conditions. Both evaporation factors (temperature and speed) had significant ($p<0.05$) main effects on the particle size distribution, so they were remained in the final reduced model. The homogenization speed was also remained in the final reduced model despite its insignificant main effect ($p>0.05$) due to its significant interaction effect with the evaporation temperature ($p<0.05$). Overall, among the quadratic effects, the evaporation temperature and speed showed significant ($p<0.05$) effects on the particle size distribution. As it can be seen in the regression equation, all of the coefficients had negative individual effects. Therefore, at lower levels, the increase in the homogenization speed as well as the speed and evaporation temperature resulted in the span value decrease. As shown in Tables 3 and 4, the interaction effect of evaporation temperature with the evaporation speed as well as the homogenization speed were significant on

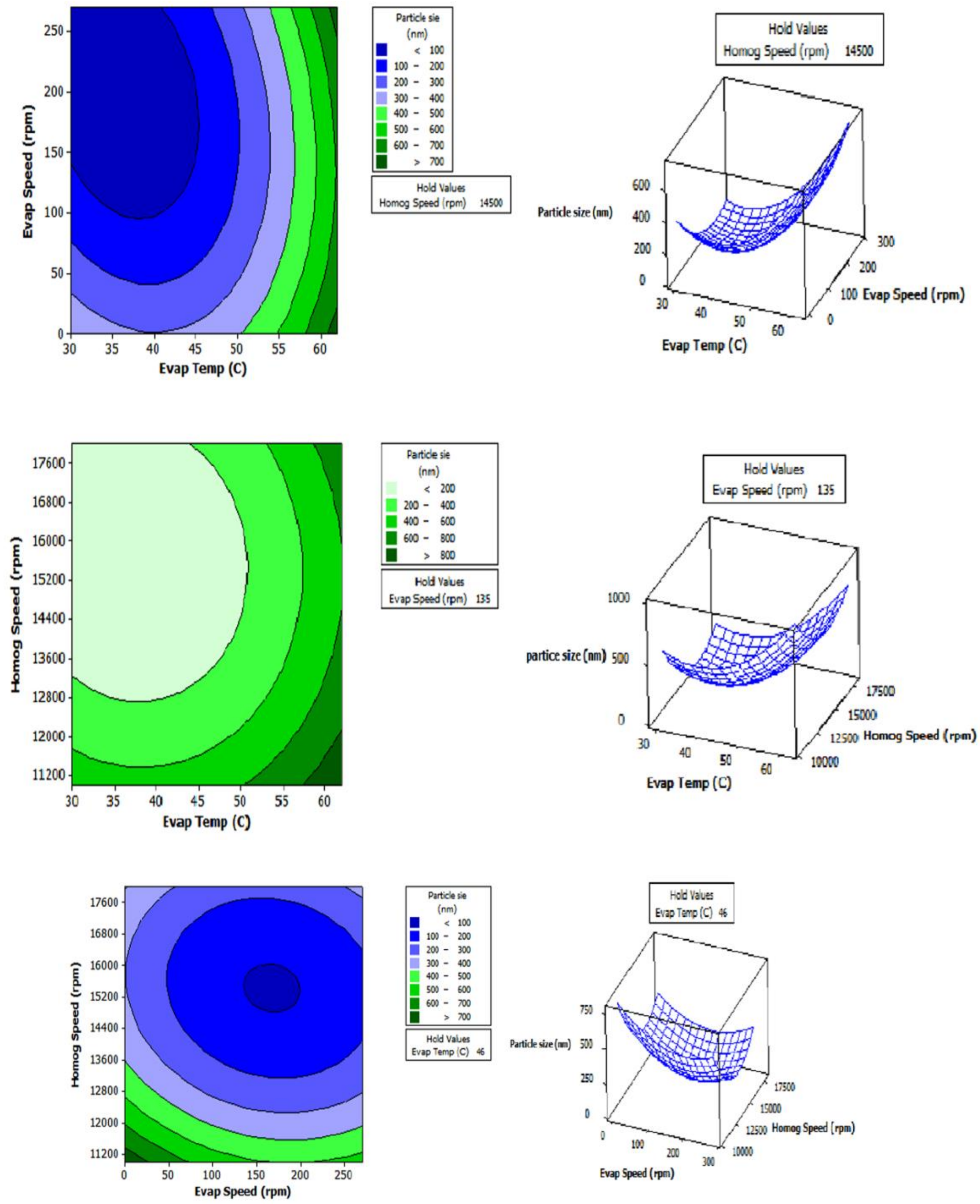

Figure 1. Response surface plots for particle size as function of significant ($p<0.05$) interaction effects between preparation variables.

particle size distribution ($p<0.05$). The increase in homogenization speed at low evaporation temperature or the rise of evaporation temperature at low speeds of homogenization, produced more homogenized nanodispersion. Generally, the influence of evaporation conditions on the particle size distribution was more significant (lower p-values) than homogenization speed. Linear and quadratic effects of evaporation speed were found to have the most significant ($p<0.05$) effects on the particle size distribution (Table 3). Therefore, this parameter was considered as a critical parameter to control the particle size distribution of the prepared nanodispersions. The evaporation variables (the evaporation speed and temperature) showed more meaningful effects ($p<0.05$) on the particle size distribution (Y2) in comparison with the homogenization variable. Besides, the main (linear) effect of the evaporation speed, showed the most effectiveness on the span value (the least p value and the most ratio of F), and the quadratic effect of the evaporation speed was the second main parameter that had remarkable effect on the span changes. Figure 2 shows that at high evaporation temperatures, increasing the evaporation speed up to about 190 rpm, reduced the particle size distribution, but further increasing in evaporation temperature caused increasing in the particle size distribution. This may be related to the nonequivalent distribution of energy and the nonequivalent rate of particles evaporation. On the other hand, as it can be seen in Figure 2, at high homogenization speed, increasing the evaporation temperature, increased the particle size distribution, but at low homogenization speed, increasing the evaporation temperature resulted in the reverse effect. Overall, the combinational results indicated that the upper range of the evaporation-emulsification speed and the low amount of evaporation temperature resulted in the most desired particle size distribution.

3.3. Effects of Variables on β-Carotene Concentration

The β-Carotene content of freshly prepared emulsion was approximately 100 μg/mL. As shown in Table 3, the variation of β-Carotene concentration (Y3) was significantly ($p<0.05$) explained by a second-order regression equation ($R^2 = 0.967$). Tables 3 and 4 showed that the variation of β-Carotene content was significantly ($p<0.05$) explained by nonlinear function of emulsification and evaporation variables. Both evaporation factors (temperature and speed) had significant ($p<0.05$) main effects on the β-Carotene concentration, so they were remained in the final reduced model. The emulsification speed was also remained despite its insignificant main effect ($p>0.05$) due to its significant ($p<0.05$) quadratic effect and its significant interaction effect with the evaporation temperature and evaporation speed ($p<0.05$). Among the quadratic effects, only emulsification speed was significant ($p<0.05$) on β-Carotene concentration. According to the significance probabilities of interaction terms of the studied independent variables (Table 4), the interaction of evaporation temperature with the evaporation speed, influenced the β-Carotene content of nanodispersions more significantly (i.e., with a

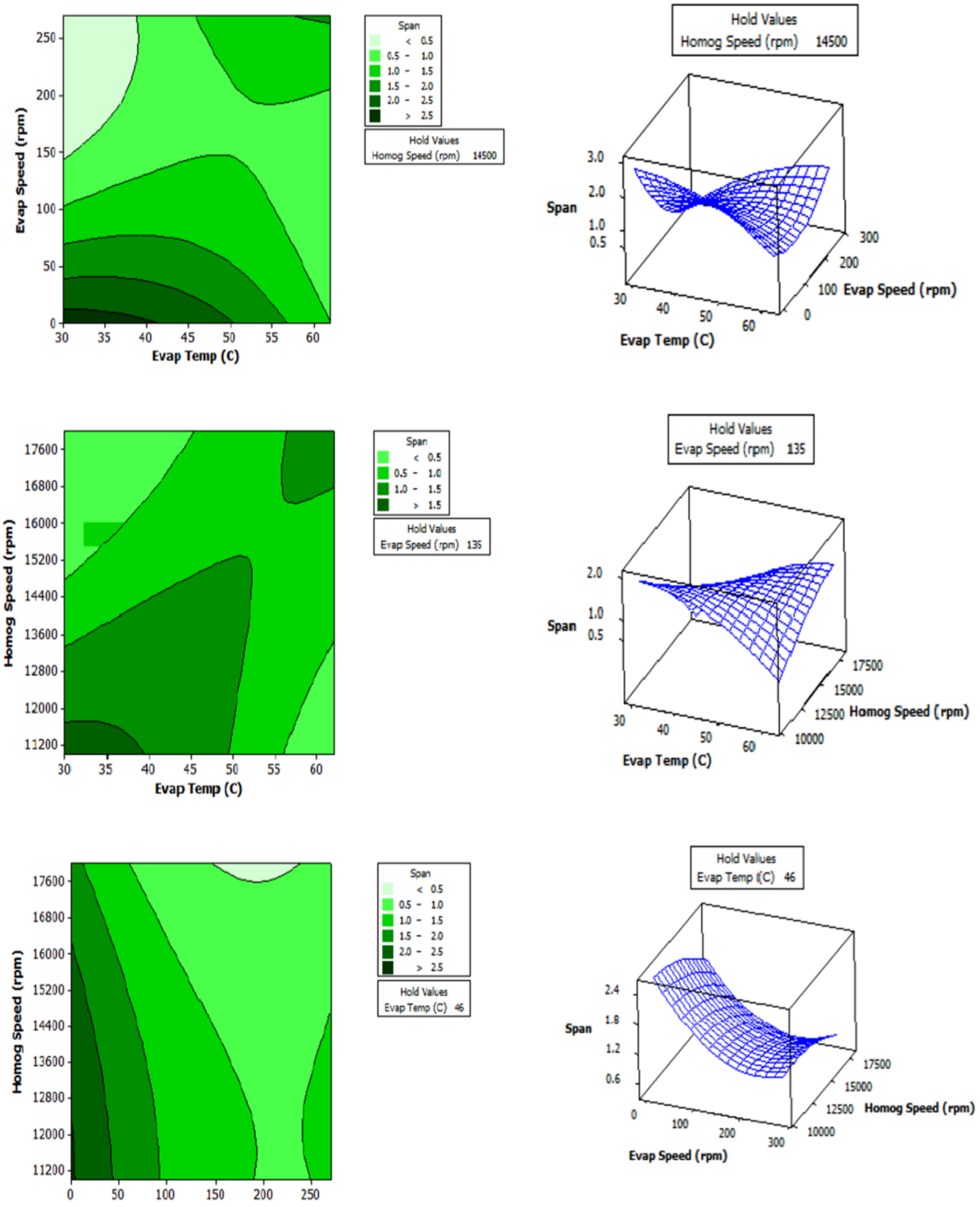

Figure 2. Response surface plots for particle size distribution as function of significant ($p<0.05$) interaction effects between preparation variables.

lower p-value and a higher F-ratio) than the other interaction terms.

As shown in figure 3, the effects of evaporation and homogenization speed on β-

Carotene concentration, depended on the used evaporation temperature. At low levels of evaporation temperature, increasing the evaporation and emulsification speed reduced β-Carotene concentration, but at high evaporation temperature in the system, increasing the evaporation and emulsification speed increased the β-Carotene concentration.

3.4. Optimization Procedure for Predicting the Processing Conditions to Produce the Most Desirable Β-Carotene Nanodispersions

Individual and multiple-optimization procedures were carried out to predict the optimum levels of three independent variables (x_1, x_2 and x_3) leading to obtain the desired response. Surface response of the quadric

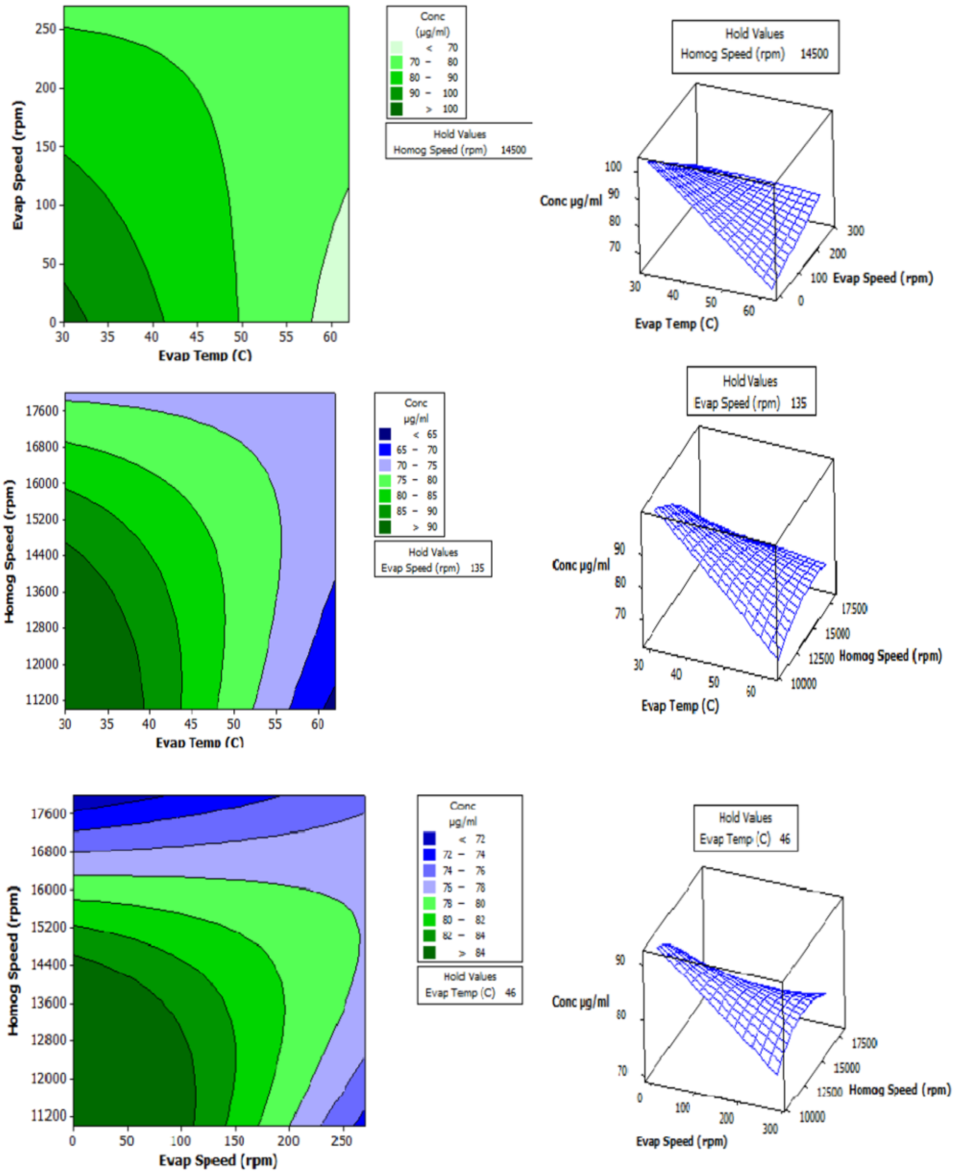

Figure 3. Response surface plots for β-Carotene concentration as function of significant ($p<0.05$) interaction effects between preparation variables.

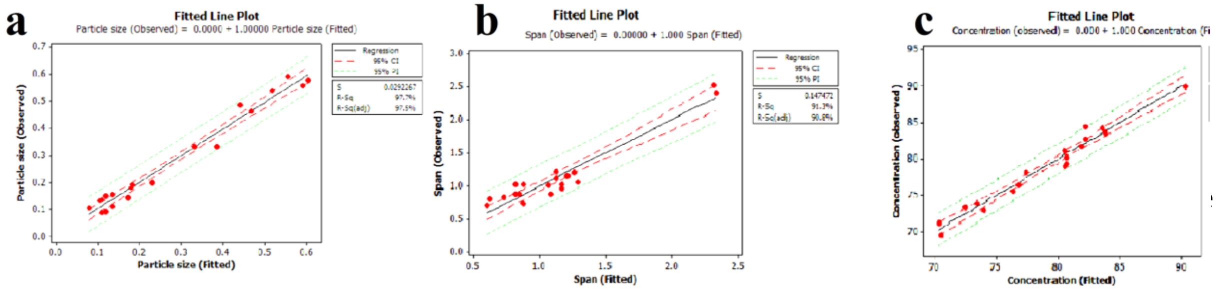

Figure 4. Fitted line plots between the experimental and predicted values of a) mean particle size, b) Span and c) β-Carotene concentration.

150 rpm. The corresponding response values for the average particle size, span and β-Carotene concentration were predicted under the recommended optimum conditions, 95.47 nm, 0.724, and 89.8 µg/mL, respectively. The suitability of obtained regression equations was checked by plotting the experimental values versus the predicted ones by the final reduced models. As shown in figure 4, the linear plots obtained with intercepts of zero and a slope of 1, as well as their high R^2 values (>0.91) confirmed the suitability of the models. Furthermore, the overall closeness between the predicted and experimental values of the responses could be concluded from the p values of t-test analysis (1.00 for all three responses). Moreover, three β-Carotene nanodispersions were prepared according to the recommended optimal levels by numerical multiple optimization and characterized in terms of studied physicochemical properties. The measured experimental values for the mean particle size, span and β-Carotene concentration of these three nanodispersion samples were 96.7 ± 25 nm, including, temperature, speed of evaporation and emulsification and their effect on average particle size, particle size distribution and β-Carotene concentration of β-Carotene nanodispersions. The results of this study clearly showed that the physiochemical properties of nanodispersions were significantly ($p<0.05$) influenced by these processing conditions. Fitting of the second-order polynomial regression models with the experimental data were found to be highly adequate to describe the relationship between the preparation conditions and the nanodispersion properties ($R^2 > 0.95$). The linear effects of evaporation temperature, as well as the evaporation speed, had significant ($p<0.05$) effects on all the studied response variables, and also interaction effects between the temperature of evaporation and two other studied independent variables also had significant ($p<0.05$) effects on the fitted models, except for the average particle size. Therefore, the evaporation step can be considered to be the most important stage influencing the final characteristics of nanodispersions. Concerning the desired goals, the β-Carotene

nanodispersions that were prepared at 33 0C and 150 rpm in the rotary evaporator, followed by emulsification at 14000 rpm, were predicted and shown to provide the optimal physicochemical properties.

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