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SHORT COMMUNICATION

β-Carotene nanodispersions synthesis by three-component stabilizer system using mixture design

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Abstract In current research, simple centroid mixture design was applied to evaluate the interaction effects between three selected food grade stabilizers, namely, Tween 80, gelatine and pectin as stabilizing system in the formation of carotenoid nanoparticles through solvent displacement process. Both, particle size and β-carotene loss of produced nanodispersions, as selected response factors, special cubic regression models with acceptable determination coefficient (>90%) was obtained. The multiple response optimization analysis showed that the overall optimum concentration for stabilizers will be 35% w/w Tween 80, 46% w/w gelatine and 19% w/w pectin, which led to the production of β-carotene nanoparticles of spherical shape with minimum particle size of 155.8 nm and carotenoids loss of 25.3% w/w.

Keywords β-Carotene nanodispersions · Food grade stabilizer · Particle size · Optimization · Mixture design

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Introduction

β-Carotene is the supreme common carotenoids, which originate the yellow-orange color in different plants and fruits such as carrot, pumpkin, mango and etc. It is a non-polar, two-cyclic carotenoid that holds 11 conjugated double bonds. The use of β-carotene can decrease the risks of heart attack deaths and motivate the production and differentiation of osteoblast cells (Ribeiro et al. 2010; Yin et al. 2009). However, as other carotenoids, it is water insoluble, and only slightly soluble in oil, that makes it problematic to be used in many food and pharmaceutical formulations. Additionally, it is prone to light, heat and oxygen, which restrictions furthermore its applications in pharmaceutical, nutraceutical and food products (Guo et al. 2014; Tan and Nakajima 2005).

In the last two decades, nanotechnology provides the prospective to significantly increase the solubility and bioavailability of various functional lipids (Joye and McClements 2013; Yerramilli and Ghosh 2017). The nanoparticles, with particle sizes in the nanometer ranges, are physically more stable in comparison to the particles with micrometer sizes (Jo and Kwon 2014). The solubility and bioavailability improvement of these compounds, is because of their fine particles, which decrease the intrinsic limitations of incomplete and slow dissolution of the functional lipids, due to their greater surface area and more dissolution pressure (Anarjan et al. 2010; Ribeiro et al. 2008). Particles are surrounded by stabilizer molecules that hinder their coalescence, which can also protect their bioactive components from induced oxidation by either metal ions or free radicals (Anarjan and Tan 2013b). Then, one of the important factors for making nanoparticles is the selection of stabilizer system. It has been shown that mixtures of stabilizers can improve the physicochemical properties of nanoparticles, more efficient than using them individually (Anarjan and Tan 2013b; Guo et al. 2014; Sen Gupta and Ghosh 2015).

Mixture design of experiment has been widely used to gain product formulations with optimized properties and to evaluate the antagonistic or synergistic effects in a multi-component system. By employing linear or non-linear regression models, the technique has been find very convenient in predicting the interaction effects extents on the characteristics of mixture systems (Anarjan and Tan 2013a). The mixture design is a distinctive type of response-surface design since the independent factors are the components of a multicomponent system, and the dependent variables are expected to rest just on the components' proportions of blend (not the amount of the blend). In this design, each constituent is considered at six levels, namely 0, 1/6, 1/3, 1/2, 2/3 and 1, with an experimental domain was inside an equilateral triangle which supports the fitting of special cubic model (Anarjan et al. 2011b; Montgomery 2001).

The aims of present study were to explore the interaction effects among three selected common small molecular green stabilizers, protein and polysaccharide namely, Tween 80, gelatine and pectin as stabilizer components on characteristics of obtained nanoparticles and to optimize their proportions in order to prepare the β-carotene nanoparticles with the smallest particle size and highest β-carotene concentrations.

Materials and methods

Materials

β-Carotene, gelatine and pectin were purchased from Sigma (*Missouri*, *USA*). Tween 80 (polyoxyethylene sorbitan monooleate) were acquired from Merck (Hohenbrunn, Germany). The used analytical-grade acetone and other solvents were obtained from Fisher Scientific (Leicestershire, UK).

β-carotene nanodispersions synthesis

Pure, binary and ternary blends of Tween 80, gelatine and pectin were prepared in triplicate, according to the augmented simplex-centroid design as are shown in Table 1. These ten blends were used as stabilizer systems in order to prepare β-carotene nanodispersions. To prepare the aqueous phase, stabilizer mixture with total concentration of 1% (w/w) was successively dissolved in deionized water. While mixing the aqueous phase using a conventional homogenizer (Silent Crusher, Heidolph, Germany), for 5 min at 3,000 rpm, the organic phase composed of

Table 1 Matrix of an augmented simplex-sentroid design

Run	Tween 80 (X_1, %)	Gelatine (X_2, %)	Pectin (X_3, %)
1	0.17	0.67	0.17
2	0.50	0.00	0.50
3	1.00	0.00	0.00
4	0.00	0.00	1.00
5	0.17	0.17	0.67
6	0.50	0.50	0.00
7	0.67	0.17	0.17
8	0.00	1.00	0.00
9	0.34	0.33	0.33
10	0.00	0.50	0.50

dissolved 0.2% w/w β-carotene in acetone, was slowly dispersed in the aqueous phase. The organic phase to aqueous phase weight ratio was set at 1:9 in all samples. Then, the acetone was totally evaporated from the system using by a rotary evaporator at 40 °C (Heidolph 2000, Germany). In fact, solvent displacement method was used to preparation of nanodispersions (Anarjan et al. 2013, 2014a).

Analytical methods

Average particle size

Average particle size of the β-carotene nanoparticles was assessed using a dynamic light-scattering particle-size analyzer (Nanotrac Wave, Microtrac., Montgomeryville, PA, US). The tests were conducted, after preparation, on undiluted samples. The data were reported as mean of two distinct injections with three readings, in the dimension of nano-meter.

β-carotene concentration

β-Carotene was absolutely extracted from the nanoparticles using dichloromethane as the organic solvents (three times). After extraction process, its concentration was measured at 450 nm wavelength using a spectrophotometer (Pharmacia Biotech Ultrospec 2000, NJ, US), according to the technique described by Britton, Liaaen-Jensen and Pfander (Anarjan et al. 2011a, 2014b; Ribeiro et al. 2008).

Morphology

A drop of the aqueous nanodispersion of the complex was deposited on a carbon-coated microscopy grid. It was negatively stained with a drop of 2% (w/v) uranyl acetate, dried at room temperature, and observed using a transmission electron microscope (Philips Tecnai 12, Eindhoven, Netherlands). The TEM image with magnification of 25000 was used in this study.

Experimental design and statistical analysis

An augmented simplex-sentroid mixture design with 10 points (Table 1) was used to determine the interaction effects between the stabilizer components on average particle size (Y_1) and β-carotene loss (Y_2) in each prepared nanoparticle. The experimental field consist of different proportions of components of X_1 (Tween 80), X_2 (gelatine) and X_3 (pectin) between zero and one ($0 \leq X_i \leq 1$; $\Sigma X_i = 1$). Mixture analysis was used to determine the significance of the model terms, probable coefficients and coefficient of determination (R^2). Multiple-regression coefficients were calculated to estimate the special cubic models as follow (Eq. 1):

$$Y_i = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (1)$$

where, Y_i is the predicted response, β_{123} and β_{12}, β_{13} and β_{23} are ternary and binary interaction effect terms, and β_1, β_2 and β_3 are the regression coefficients for each linear effect terms (Anarjan et al. 2011b). The significance of the found regression coefficients was evaluated by T-value at a probability (p) of 0.1 (Anarjan et al. 2011b; Montgomery 2001). The counter plots show how a response associated to three stabilizer component according to suggested models. All experimental design, data analysis, regression and optimization steps were completed by Minitab v. 14 statistical package (Minitab Inc., PA, USA).

Results and discussions

Analysis of response models

To empirically predict the particle size and β-carotene loss of produced nanoparticles, as a function of the stabilizers, mixture regression analysis with special cubic model was applied on experimental data. The regression coefficients, coefficients of determination (R^2 and R^2-adj) are shown in Table 2. Table 3 shows the interaction effects of stabilizer compounds and p value and T-value of regression coefficients in final reduced special cubic polynomial models on particle size and β-carotene loss.

In special cubic model of either particle size or β-carotene loss, the significant interaction terms, pointed out that these two responses would be affected by either the individual stabilizer compounds, or binary and ternary interactions among them. As shown in Tables 2 and 3 pectin has the most significant effect ($\beta_3 > \beta_2$ and β_1) on both studied responses. All terms of special cubic model, should remain in final reduced regression model fitted for particle size, but the binary interactions of Tween 80 with gelatine and pectin ($X_1 \times X_2$ and $X_1 \times X_3$) should be removed in final regression model fitted for β-carotene loss because they were insignificant ($p > 0.1$). The positive secondary effects of pectin with Tween 80 and gelatine ($X_1 \times X_3$ and $X_2 \times X_3$) (Tables 2 and 3) in the suggested model for average particle size showed that these two components act synergically towards one another. Then using this blend would increase the particle size. However due to the negative binary interaction of Tween 80 and gelatine, these two component are antagonistic toward each other and consequently reduce the particle size of nanoparticles without any significant effect on β-carotene loss. Considering the interaction terms in fitted model for responses, the high negative coefficient indicated that the strongest antagonistic effect could be given by ternary blend of all three stabilizers ($X_1 \times X_2 \times X_3$). Thus, using all three stabilizer components could produce nanoparticles with the most desirable characteristics. The comparison between the p-values of the interactions, also showed that binary effect of gelatine and pectin is more significant (smaller p) than other interactions in either particle sizes or β-carotene losses. These effects were evidently envisioned in the relevant counter plots (Fig. 1). According to obtained results from previous studies, using small molecular emulsifiers can produce nanoparticles with the smallest particle size (Fathi et al. 2014), which was in good agreement with our results.

The results also indicated that using binary mixture of Tween 80 and gelatine (Fig. 1a) caused a considerable reduction in average particle size of produced nanoparticles in applied processing conditions. Figure 1a also confirmed that the binary combination of pectin with gelatine or Tween 80, led to production of β-carotene nanodispersions with large particle size. The single response optimization indicated that the mixture of 95% w/w Tween 80 and 5% w/w gelatine would give the nanodispersions with least particle size (136.7 nm). The influence of the stabilizer components on chemical stability of nanoparticles is shown in Fig. 1b. Generally using all three stabilizer components would lead to high β-carotene concentration or less β-carotene lost in the prepared nanoparticles. There were some probable reasons for the degradation of β-carotene nanoparticles during processing. The high specific surface area of small particles was one of the reasons to why the β-carotene with less particle sizes were more susceptible to degradation in produced systems (Anarjan et al. 2013; Tan and Nakajima 2005). Rebeiro et al. reported that the β-carotene were most expected to be encapsulated inner the core of the produced particles, layered by the stabilizer at

Table 2 Regression coefficients, r^2, adjusted r^2 and probability values for the final reduced models

Regression coefficient[a]	Average particle size (nm)	β-carotene lost (% w/w)
b_1	136.7	59.4
b_2	160.8	37.0
b_3	218.3	69.8
b_{12}	−22.8	–
b_{13}	32.7	–
b_{23}	71.4	−113.5
b_{123}	−397.6	−518.8
R^2	0.973	0.937
p value (regression)	0.000[b]	0.003[b]
F value (regression)	703.2	18.48

[a] b_i, and b_{ij}, b_{ijk} are the linear, quadratic and special cubic interaction coefficients of the polynomial equation, respectively; 1: Tween 80; 2: gelatine; 3: pectin

[b] Significant at $p < 0.05$

Table 3 The significance probability (*p* value, *t* value) of interaction coefficients in final reduced polynomial models

Variables[a]		X_1X_2	X_1X_3	X_2X_3	$X_1X_2X_3$
Average particle size	*p*-value	0.036[b]	0.014[b]	0.001[b]	0.002[b]
(Y_1, nm)	T-value	−3.64	5.21	11.39	−9.67
β-carotene lost	*p*-value	–	–	0.01[b]	0.022[b]
(Y_2, % w/w)	T-value	–	–	−4.05	−3.26

[a] 1: Tween 80; 2: gelatin; 3: pectin

[b] Significant at $p < 0.05$

the outward, during process (Ribeiro et al. 2008). The greater particle sizes might also reveal that the precipitated β-carotene in the core of the particle was surrounded by a thicker layer. This thick exposure of stabilizer, offered the physically protection to the precipitated active compound against attack of free radicals. The nature of the stabilizer at the interface, also affected the stability of β-carotene in the nanoparticles. For instance, it was presented that the oxidation of lipoid compounds in emulsion systems could be improved by modifying the packing of the stabilizer molecules at the interface (Ribeiro et al. 2008, 2010). Tween 80, gelatine and pectin are different in terms of molecular weight and hydrophobicity, and later, they were anticipated to provide unlike molecular packing at the interface (Fathi et al. 2014). Furthermore, they have dissimilar affinity and mechanism of action towards the singlet oxygen and free radicals. Such differences partially described their enactment in protection the β-carotene, and providing the chemical stability in the samples (Anarjan et al. 2010, 2013). These characteristics of stabilizers were adjusted to produce the water dispersible nanoparticles with maximum β-carotene concentration by using the ternary blend of them with optimum proportions. The single response optimization showed that the mixture of 33% Tween 80, 39% gelatine and 28% pectin would give the highest β-carotene concentration in dispersion products (the loss of β-carotene would be 22.6% w/w).

Optimization of the three-component green stabilizer system

The overall optimum proportions were determined by superimposing all response-surface plots (Fig. 2). It was shown that using quite less proportions of pectin, would produce the most desirable nanodispersions with less particle size and β-carotene loss. Furthermore, the numerical optimization results, shown that the total optimum values for each stabilizer components, were predicted to be 35% w/w Tween 80, 46% w/w gelatine and 19% w/w pectin. The optimum β-carotene nanodispersion was compared with bulk microsized.

β-carotene solution (dispersed in deionized water) in similar concentration. The results indicated that as compared to the bulk solution, the size reduction process of β-carotene into nano-ranges with optimized stabilizer system could produce more clear and transparent system with high color intensity, which satisfied the common characteristics of nanodispersion systems (Anarjan et al. 2014a, 2015). The matching response values for average particle size and β-carotene loss obtained under the suggested optimum conditions were 155.8 nm and 25.3% w/w, respectively, with the composite desirability of 0.69. Desirability varies from zero to one, that one denotes the perfect case. To be far from one, points to that some responses are outside of their satisfactory limits.

The morphology of the obtained β-carotene nanodispersions with optimum formulation, was also determined by electron microscopy (TEM) technique (The image was not shown). The optimum formulated spherical β-carotene nanoparticles had same particle size which that was in agreement with the results of particle size analysis using dynamic light scattering theories.

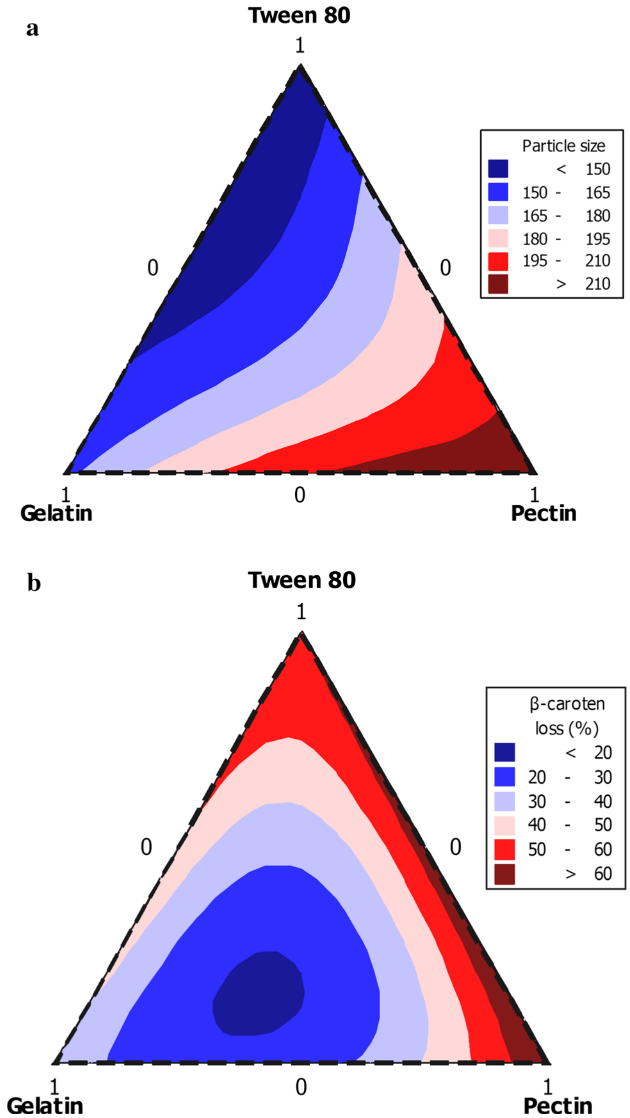

Fig. 1 Surface counter of estimated **a** average particle size (nm) and **b** β-carotene loss (%w/w) as a function of used stabilizer components according to the special cubic model

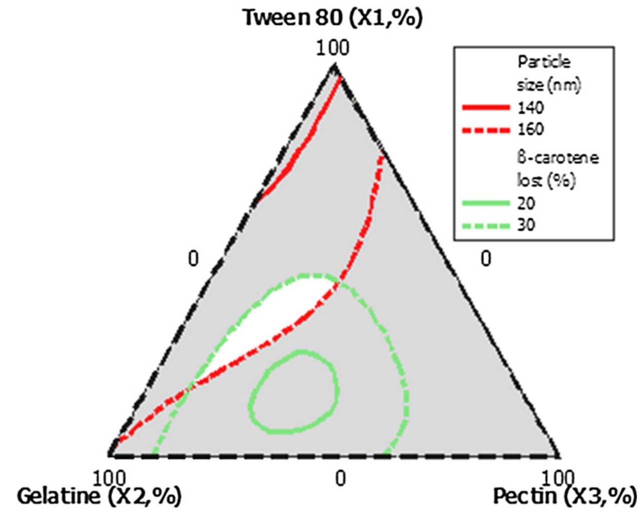

Fig. 2 Graphical optimization (overlaid surface counter) of stabilizer components to get the most desirable β-carotene nanodispersions (minimum average particle size and β-carotene loss)

Validation of the response models

To certify and validate the models of each response, the obtained multiple optimums blends for producing the optimum β-carotene nanodispersions in terms of particle size and β-carotene loss was chosen. The new nanodispersions were prepared in triplicate with these blend stabilizers and their particle size and β-carotene concentration were determined (Anarjan et al. 2015; Anarjan and Tan 2013a). A mean of responses was compared to predicted value obtained by equation model. The results indicated that the predicted values for particle size and β-carotene were 155.8 nm and 25.3% (w/w), respectively. While, the experimental values of these responses, were 150 ± 7.33 nm and 26.0 ± 1.9% (w/w). The comparison analysis results indicated that, there was no significant difference between the experimental and predicted data ($p > 0.05$). Thus, the sufficiency of the conforming special cubic models used for predicting the changes of average particle size and β-carotene loss as functions of stabilizer component proportions can be confirmed.

Conclusion

The results proved that the selected stabilizers can be used effectively to produce β-carotene nanodispersions with minimum particle size and carotene loss. The mixture design is a useful technique for optimizing the stabilizer components' proportions to obtain the carotenoid nanoparticles with the most desired physicochemical characteristics. The experimental values also were agreed to the predicted values confirming the suitability of suggested models. Consequently, developing the empirical equations was possible for predicting the changes of the dependent response variables in nanodispersion preparation processes.

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