J Am Oil Chem Soc (2011) 88:717–725
DOI 10.1007/s11746-010-1714-7

ORIGINAL PAPER

Response Surface Modeling of Processing Parameters for the Preparation of Phytosterol Nanodispersions Using an Emulsification–Evaporation Technique

Wai-Fun Leong · Kok-Whey Cheong · Oi-Ming Lai · Kamariah Long · Yaakob B. Che Man · Misni Misran · Chin-Ping Tan

Received: 8 November 2009 / Revised: 5 October 2010 / Accepted: 5 November 2010 / Published online: 23 November 2010

Abstract The purpose of this study was to optimize the production parameters for water-soluble phytosterol nanodispersions. Response surface methodology (RSM) was employed to model and optimize three of the processing parameters: mixing time (t) by conventional homogenizer (1–20 min), mixing speed (v) by conventional homogenizer (1,000–9,000 rpm) and homogenization pressure (P) by high-pressure homogenizer (0.1–80 MPa). All responses [i.e., mean particle size (PS), polydispersity index (PDI) and phytosterols concentration (Phyto, mg/l)] fitted well to a reduced quadratic model by multiple regressions after manual elimination. For PS, PDI and Phyto, the coefficients of determination (R^2) were 0.9902, 0.9065 and 0.8878, respectively. The optimized processing parameters were 15.25 min mixing time, 7,000 rpm mixing speed and homogenization pressure 42.4 MPa. In the produced nanodispersions, the corresponding responses for the optimized preparation conditions were a PS of 52 nm, PDI of 0.3390 and a Phyto of 336 mg/l.

Keywords Phytosterol · Nanodispersion · Response surface methodology · High-pressure

Abbreviations

AAD	Absolute average deviation
A_i	Peak area of internal standard
A_s	Area of sample
A_{st}	Peak area of standard
R^2	Coefficient of determination
P	Homogenization pressure
PDI	Polydispersity index
Phyto	Phytosterol concentration
PS	Mean particle size
RRF	Relative response factor
RSM	Response surface methodology
t	Mixing time
v	Mixing speed
V_i	Amount of internal standard
V_{st}	Amount of the standard

Introduction

Dietary plant sterols or phytosterols are naturally occurring steroid alcohols found exclusively in plants. These fat derivatives are essential constituents of plant cell membranes, and they can be classified into three groups based on their biosynthesis and structure: 4-desmethyl sterols, 4-methyl sterols and 4,4-dimethyl sterols. Nevertheless, only 4-desmethyl sterols have been shown to reduce low

W.-F. Leong · K.-W. Cheong · Y. B. Che Man · C.-P. Tan (✉)
Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, UPM, 43400 Serdang, Selangor, Malaysia
e-mail: tancp@putra.upm.edu.my

O.-M. Lai
Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM, 43400 Serdang, Selangor, Malaysia

K. Long
Malaysian Agricultural Research and Development Institute (MARDI), P.O. BOX 12301, 50774 Kuala Lumpur, Malaysia

M. Misran
Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

density lipoprotein (LDL) and total serum cholesterol in humans [1, 2]. Some of the commonly found 4-desmethyl sterols include β-sitosterol (24α-ethylcholesterol), campesterol (24α-methylcholesterol), stigmasterol (Δ^{22}, 24α-ethylcholesterol), and brassicasterol (22,23-dihydrobrassicasterol). Although phytosterols have structures similar to cholesterol, they have distinct metabolic routes in the human body. Phytosterols are hypocholesterolemic agents that are well known for their efficacy in improving serum cholesterol profiles and reducing the risk of cardiovascular disease. The hypocholesterolemic effects of phytosterols in humans were first documented in the early 1950s [3], and numerous studies later revealed that the consumption of phytosterols and phytostanols can reduce total serum cholesterol and LDL [4, 5]. The human body is able to synthesize cholesterol, but not phytosterols. Supplementation is therefore warranted.

Due to its ability to lower cholesterol levels and the risk of cardiovascular disease, phytosterols have been recently incorporated into a growing spectrum of consumer food products. At present, the major commercial sources of phytosterols used in food enrichment are byproducts of vegetable oil refinement and from the wood pulp industry. Soybean oil and tall (pine tree) oil are the major sources of commercial phytosterols, although other plant oils are also sources of plant sterols, including corn oil, sunflower oil, rapeseed oil, safflower oil, olive oil, almond oil and milk thistle seed oil [6–8]. Phytosterols are present in most foods of plant origin, especially in their fat and oil portions. The amount of phytosterols in a normal diet is at a minimal amount that is hardly adequate to perform its cholesterol-lowering effects in humans. The consumption of phytosterols in a population ranges from 160 to 430 mg/day [7], but clinical studies have revealed that the minimum amount of phytosterols that can effectively reduce serum cholesterol ranges from 300 to 1,000 mg [9–11]. However, most of the studies focused on a higher intake level of 2–3 g/day, which has been shown to provide close-to-maximum cholesterol lowering effects [4, 12]. Phytosterols have been awarded the Generally Recognized as Safe (GRAS) status in United States. In 2000, the Food and Drug Administration (FDA) approved the use of phytosterol in foods, and cholesterol-lowering claims are allowed if the food contains at least 0.65 g of plant sterol esters or at least 1.7 g of plant stanol esters per serving.

The water insoluble and barely lipid soluble nature of phytosterols makes their incorporation into food tricky. Only a very small amount of pure phytosterols can be incorporated into the oil phase of a food product, or significant crystallization may occur, causing a waxy, gritty and unfavorable texture in food. Esterified phytosterols, which have increased solubility in fat, are more preferable for fortification in the food industry. However, phytosterols esters distributed in a small amount of fat adversely affected eating quality. In other words, incorporation of pure and/or esterified phytosterols is limited to high fat food products only. Consumers who wish to benefit from the serum cholesterol lowering effects had to consume high-fat food despite the health risks of a high fat diet. Phytosterols in their pure or esterified forms have different efficacies for lowering serum cholesterol. An intake of 6 g/day of free phytosterols is required to reduce serum cholesterol by 10% [13], while only 2–3 g of esterified phytosterols is required for the same effect [14]. Some recent studies have demonstrated that phytosterols in an emulsified form, which has increased water solubility, have a higher efficacy for lowering serum cholesterol. The cholesterol-lowering effect of a much smaller dose of emulsified phytosterols was similar to the esterified or non-esterified phytosterols [9, 11]. In addition, phytosterols prepared in a water-soluble form enable their use in a wider range of food products. Further development and optimization of the production of stable nanoparticulate phytosterols with small particle size and low polydispersity are critical to enhance the response dosage. In other words, lower doses of nano-sized phytosterols maybe required for a similar, or even greater, cholesterol lowering effect. Increasing the efficacy of phytosterols will lead to a dose reduction and the curtailment of costs and may make phytosterol-enriched food products more affordable.

The purpose of this study was to model and optimize the processing parameters for preparation of the phytosterol nanodispersions, including the minimum mean particle size (PS), the minimum polydispersity index (PDI) and the maximum retention of phytosterols, using the response surface methodology (RSM).

Materials and Methods

Materials

The phytosterols powder, Vegapure® FTE (containing a minimum 99% phytosterols) was a gift from Cognis (Duesseldorf-Holthausen, Germany). Analytical grade hexane, analytical grade dichloromethane, HPLC grade ethanol, potassium hydroxide and sodium sulfate were purchased from Merck (Darmstadt, Germany). Polyoxyethylene sorbitan mono-laurate (Tween 20) was supplied by Fisher Scientific (Pittsburgh, PA, USA). The standards of β-sitosterol (>95%), stigmasterol (95%), stigmastanol (97.4%), campesterol (65% crystalline), brassicasterol and 5α-Cholestane, which was used as an internal standard, were obtained from Sigma–Aldrich (St. Louis, MO, USA). The *N-O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Supelco (Bellefonte, PA, USA).

Experimental Design

RSM was applied to study the effects of three processing parameters on the preparation of phytosterol nanodispersions. A five-level, three-factor central composite rotatable design (CCRD) was used in this study. The independent variables were mixing time by conventional homogenizer (t, 1–20 min), mixing speed by conventional homogenizer (v, 1,000–9,000 rpm) and homogenization pressure of high-pressure homogenization (P, 0.1–80 MPa), while the responses measured were mean particle size (PS, nm), PDI and phytosterol concentration in the produced nanodispersion (Phyto, mg/l). The coded and uncoded levels of independent variables used are listed in Table 1. A total of 20 experiments consisting of 6 center points with a combination of different levels of each independent variable were generated using the software Design Expert version 6.0.6 (Minneapolis, MN, USA) (Table 2). The experimental runs were divided into three blocks and completed in three consecutive days. Individual experiments were carried out in a randomized order to minimize the effects of unexplained variability in the experimental responses due to extraneous factors.

Preparation of Phytosterol Nanodispersions

Phytosterols (0.5% w/v) in powder form were first dissolved in hexane to form the organic phase (dispersed phase). Before mixing with the aqueous phase, the organic phase was kept in a 45-°C water bath for 5 min to facilitate phytosterols dispersion. The aqueous phase (continuous phase) was prepared by dissolving Tween 20 (0.2% w/v) in deionized water. The organic phase was slowly added into the aqueous phase under conventional homogenization (Silverson L4R, Buckinghamshire, UK) to produce a coarse pre-emulsion. The pre-emulsions were immediately subjected to high-pressure homogenization (APV, Crawley, UK). Phytosterol nanodispersions were produced after the removal of hexane by rotary evaporation (NE 1001, Eyela, Tokyo, Japan) attached to a circulating bath (Ca 1111, Eyela Cool Ace, Tokyo, Japan), under reduced pressure at 0.17 ± 0.02 bar for 7 min to a concentration ratio (volume of nanodispersions after evaporation:volume of nanodispersions before evaporation) of 0.75 ± 0.005. The circulating bath was set at 5 °C. Twenty samples with different levels of processing conditions were prepared according to the designed RSM experimental runs (Table 2). The organic to aqueous phase volume ratio was fixed at 1:9. To study the effects of the processing parameters, the formulation parameters of the phytosterol nanodispersions were kept constant.

Table 1 Independent variables and their levels used for the central composite rotatable design (CCRD)

Factors	Symbol	Level				
		–2	–1	0	1	2
Mixing time	t (min)	1.00	5.75	10.50	15.25	20.00
Mixing speed	v (×1,000 rpm)	1	3	5	7	9
Homogenization pressure	P (MPa)	0.1	20.0	40.0	60.0	80.0

Table 2 Central composite design and the responses for the processing of phytosterol nanodispersion

Independent variables				Responses		
Run	t (min)	v (×1,000 rpm)	P (MPa)	PS (nm)	PDI	Phyto (mg/l)
1	10.50	5	40	59.63	0.421	317.8
2	5.75	3	20	119.60	0.294	374.9
3	10.50	5	40	64.54	0.440	329.1
4	5.75	7	60	97.57	0.386	294.2
5	15.25	3	60	91.18	0.515	304.0
6	15.25	7	20	91.22	0.541	370.8
7	10.50	5	40	65.40	0.389	325.6
8	5.75	7	20	141.60	0.440	378.3
9	5.75	3	60	67.39	0.422	310.8
10	15.25	3	20	178.80	0.493	408.4
11	15.25	7	60	57.67	0.243	301.2
12	10.50	5	40	65.54	0.382	322.4
13	10.50	5	80	52.68	0.355	259.8
14	10.50	5	40	57.79	0.370	330.4
15	10.50	9	40	129.90	0.421	339.8
16	1.00	5	40	74.38	0.271	331.7
17	10.50	5	40	53.67	0.251	354.2
18	10.50	1	40	151.40	0.513	354.7
19	10.50	5	0.1	165.90	0.444	485.2
20	20.00	5	40	52.21	0.328	354.7

PS mean particle size, *PDI* polydispersity index, *Phyto* phytosterol concentration in the produced nanodispersion. Other abbreviations refer to Table 1

Analysis of Particle Size and its Polydispersity

Measurements of PS and the particle size distribution of the nanoparticles in the phytosterol nanodispersions were conducted after the removal of hexane. Measurements were done using a dynamic light scattering particle size analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). The measuring range of the Zetasizer Nano ZS was 0.6–6,000 nm, and the measurement temperature was set at 25 °C. Measurements were made by placing the nanodispersion-containing cuvette into the module directly. A dynamic light scattering technique was used to measure the time-dependent fluctuation in the scattered light arising

from a suspension of particles undergoing Brownian motion. Droplets suspended in the nanodispersions scattered the light in a manner dependent upon their particle size. The PS of the nanodispersions was analyzed based on the cumulants means (*Z*-average) diameter, which is dependent on the intensity of scattered light that arises from the Brownian motion of the particles. PDI is a dimensionless estimate used to describe the broadness of particle size distribution and is scaled from 0 to 1. The PDI is calculated according to the size distribution graphic and the acceptable PDI values were 0.7 and less. The final nanoparticle diameter, the PS and PDI value were the average of two measurements; each measurement was the average of five runs.

Gas Chromatography Analysis of Phytosterols Content

Sample Preparation

A 300-µl sample was placed into a 250-ml flat-bottom flask. After adding 100 µl 5α-Cholestane (1 g/ml) and 5 ml 1 M ethanolic KOH, the sample was saponificated at 90 °C for 60 min. The reacted mixture was transferred into a 100-ml separating funnel for further extraction. First, 10 ml hexane was added to extract the unsaponifiable fraction. The organic phase was then washed 3 times with 5 ml distilled water to remove the soap of resins and fatty acids. The organic phase was collected and sodium sulfate was added to absorb moisture. The remaining amount of solution containing phytosterol was transferred to 15-ml tubes and purged to dryness under nitrogen stream. The dry residue was derivatized with 50 µl of BSTFA and incubated in a water bath at 60 °C for 60 min. One microliter of the mixture was analyzed by gas chromatography.

Preparation of Standards and Internal Standards

Standards of β-sitosterol, β-sitostanol, stigmasterol, campesterol and brassicasterol were prepared in ethanol at a concentration of 1 mg/ml. 5α-Cholestane was prepared in dichloromethane in a concentration of 1 mg/ml. All standards and internal standards were analyzed immediately upon dilution. A 100-µl standard and a 100-µl internal standard were placed in 15-ml tubes and purged to dryness under a nitrogen stream. The dry residue was derivatized with 50 µl of BSTFA and incubated in a water bath at 60°C for 60 min. One microliter of the mixture was analyzed by gas chromatography.

Gas Chromatography Analysis of Phytosterols

Analysis of the phytosterols was performed with Agilent technologies network GC system, series 6890 N (Wilmington, DE, USA) with a flame ionization detection (FID) controlled by the Agilent Chemstation software. Separation was conducted on a MDN-5 fused silica capillary column of 30 m length × 0.32 mm i.d. × 0.25 µm film thickness (Supelco, Bellefonte, PA, USA). Splitless injection was used. The oven temperature program was initially set at 250 °C and it was increased at 2 °C/min to 300 °C. The detector and injection port temperatures were set at 300 °C.

Calculation of Phytosterol Content

Concentrations of the types of phytosterols were evaluated by calculations using the following equation:

The relative response factor, RRF, of the internal standard to the standard for calculation of phytosterol content was calculated according to Eq. 1:

$$\mathrm{RRF} = (V_{\mathrm{st}}/V_{\mathrm{i}}) \times (A_{\mathrm{i}}/A_{\mathrm{st}}) \quad (1)$$

While the phytosterol content was calculated based on Eq. 2:

$$\text{Amount of phytosterol} = (A_{\mathrm{s}} \times V_{\mathrm{i}} \times \mathrm{RRF})/A_{\mathrm{i}} \quad (2)$$

Where V_{i} and V_{st} are the known amounts of the internal standard and standards used in the analysis, respectively; and A_{i}, A_{st} and A_{s} are the peak areas of the internal standard, standard and samples, respectively, that were obtained from the GC analysis.

Statistical Analysis

Experimental data were analyzed by RSM using the software Design Expert version 6.0.6 (Minneapolis, MN, USA). Multiple regressions were used to fit the responses to the independent variables. Analysis of variance (ANOVA) was performed to determine the significance of the model terms. The coefficients of determination (R^2) and adjusted R^2, the absolute average deviation (AAD), the ANOVA and the lack of fit test were calculated to evaluate the accuracy of the model. A chi-square goodness-of-fit test was carried out to verify the adequacies of the model established. The AAD values were calculated by Eq. 3 [15]:

$$\mathrm{AAD} = \left\{ \left[\left(\sum |y_{i,\mathrm{exp}} - y_{i,\mathrm{cal}}|/y_{i,\mathrm{exp}} \right) \right]/q \right\} \times 100 \quad (3)$$

where $y_{i,\mathrm{exp}}$ is the experimental value of the responses, $y_{i,\mathrm{cal}}$ is the predicted value of the responses and q is the total number of runs. All measurements were duplicated.

Optimization and Verification

The optimized processing parameters were generated using the optimizer function of the software Design Expert 6.0.6.

The PS and PDI were optimized to the lowest minimum values, while Phyto was set at the maximum value. For model verification, experimental data were subjected to a chi-square goodness-of-fit test. The chi-square values of the responses were calculated based Eq. 4:

$$\text{Chi-square}, \chi^2 = \sum \left[(O - P)^2 / P \right] \quad (4)$$

where O and P are the observed and predicted responses, respectively.

Results and Discussion

In the present study, phytosterol nanoparticles were prepared using an emulsification–evaporation method accompanied by high-pressure homogenization. This method combines chemical and mechanical processes that employ hexane as the hydrophobic organic phase and conventional homogenization followed by high pressure homogenization as the energy source to break down the larger particles. The emulsifier used in this study was Tween 20, a nonionic emulsifier, also named polysorbate 20 or polyoxyethylene (20) sorbitan monolaurate. It is frequently used due to its superior capability to reduce the interfacial tension of two immiscible liquid phases [8]. Emulsion particles stabilized by Tween 20 were based on steric stabilization and other short-range repulsive forces such as hydration interaction and thermal fluctuation interactions [11]. A preliminary study was previously done to identify the independent parameters and their levels that potentially affect the PS, PDI and Phyto. The mixing time and speed for the preparation of a coarse emulsion and homogenization pressure were the three independent variables selected for the optimization of processing of phytosterol nanodispersions using the RSM.

Fitting the Models

The PS, PDI and Phyto of the phytosterol nanodispersion from each generated experiment are shown in Table 2. The experimental values of PS and PDI were best-fit to a reduced quadratic model by multiple regression after the manual elimination of non-significant terms, excluding some non-significant terms that were required to retain the hierarchy of the model. The value of Phyto was best-fit into a linear model. The regression coefficient, R, F values and p values of the PS, PDI and Phyto are shown in Table 3. The values of R^2, adjusted R^2, AAD and lack of fit are shown in Table 4. The values of R^2 and adjusted R^2 ranged from 0.8878 to 0.9902 and 0.8014 to 0.9816, respectively. More than 80% of the variation in the models was explained by the regression model. The generalized response surface model for predicting the response variables is as Eq. 5.

Table 3 Regression coefficients, p-values and f-values for PS, PDI and Phyto after manual elimination

Variables	Regression coefficients	F-value	p-value
Mean particle size (PS, nm)			
Intercept	64.64	90.46	<0.0001
t	–3.23	3.34	0.0974
v	–6.99	15.71	0.0027
P	–27.74	247.06	<0.0001
t^2	–3.32	5.34	0.0461
V^2	21.42	235.79	<0.0001
P^2	13.58	94.80	<0.0001
tv	–21.66	75.30	<0.0001
vP	7.78	9.72	0.0109
Polydispersity index (PDI)			
Intercept	0.37	8.62	0.0029
t	0.02	5.44	0.0480
v	–0.02	3.65	0.0926
P	–0.02	5.93	0.0409
t^2	–0.01	1.93	0.2022
v^2	0.03	14.97	0.0047
P^2	0.01	3.06	0.1182
tv	–0.04	9.16	0.0164
tP	–0.04	10.06	0.0132
vP	–0.06	20.70	0.0019
Phytosterol concentration (Phyto, mg/L)			
Intercept	341.41	126.54	<0.0001
P	–48.31	126.54	<0.0001

Abbreviations: refer to Tables 1 and 2

Table 4 Coefficient of determination, R^2, adjusted R^2, AAD and lack of fit for the PS, PDI and Phyto of the phytosterol nanodispersion

	PS (nm)	PDI	Phyto (mg/l)
R^2	0.9902	0.9065	0.8878
Adjusted R^2	0.9816	0.8014	0.8807
Model (p-value)	<0.0001	0.0029	<0.0001
AAD (%)	0.8785	0.2136	0.2177
Lack of fit (F-value)	7.1100	0.4000	2.8600
Lack of fit (p-value)	0.0680	0.8245	0.2096

AAD absolute average deviation. Other abbreviations refer to Table 2

$$Y_i = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (5)$$

where Y_i is the predicted response variable, β_o is the intercept value, β_1, β_2 and β_3 are the linear terms, β_{11}, β_{22} and β_{33} are the quadratic terms and β_{12}, β_{13}, and β_{23} are the interaction terms of the regression equation. The correlations between the actual and predicted values for PS, PDI and Phyto were well-correlated. The AAD values describe the percent

deviation of the predicted and experimental data. Low AAD values (<0.9%) and high R^2 values (>0.80) indicated that the model equation that characterized the true behavior of the phytosterol nanodispersion system and the chosen model was accurate. The regression models for PS, PDI and Phyto were statistically well fit (p-values of <0.001, 0.0029 and <0.001, respectively). The ANOVA showed no significant lack of fit for the final reduced model for all three responses, indicating that the model was adequate. The developed model equation can be used for interpolation in the experimental domain, but it is not recommended for extrapolation.

The Main Effects and the Interaction Effects of the Independent Variables

The experiments demonstrated that the PS ranged from 52 to 179 nm, while the PDI varied from 0.243 to 0.541 (Table 2). The model targeted production of small PS and low PDI, as these parameters are major determinants of stability in the prepared nanodispersion. According to Stokes' law, the square of the radius of the particles is related to the velocity of sedimentation or creaming [8]. Flocculation, coalescence, creaming, sedimentation and Ostwald ripening are the major phenomena that cause instability in nanodispersions [8]. That is, smaller PS tend to have longer periods of stability. On top of the spectacular stability, nanoparticles of 100 nm or less have a broader spectrum of delivery systems compared to nanoparticles of larger sizes [16]. Three first order coefficients contributed significant effects to PS: t, v, and P ($p < 0.05$). The production of small PS and stable dispersion require a great amount of supplied energy and an adequate amount of emulsifier [17]. High-pressure homogenization is commonly used in the production of nanodispersions with narrow particle size distributions, as it facilitates the breakup of coarse particles into nano-sized particles by intense high sheer force and turbulence and/or cavitation [18]. By fixing the formulation parameters, this study agrees with previous findings that high-pressure homogenization significantly reduced the PS at $p < 0.001$ [19–22]. Homogenization pressure had the greatest negative effects among the three coefficients ($p < 0.001$). Although conventional homogenization was only able to produce large droplets with wide droplet size distribution, it is an important pre-preparation step before high-pressure homogenization. Without the formation of a coarse emulsion, high-pressure homogenization alone was not efficient enough to produce the desirable small PS with low PDI. Even though t had insignificant effects on PS, it was not removed by manual elimination to maintain the hierarchy of the model. Quadratic terms of all independent variables for PS had significant effects on PS ($p < 0.05$). A response surface plot was constructed to evaluate the interaction effects among the independent variables on PS (Fig 1a-b). The insignificant interaction between t and P was eliminated from the model. The increase of PS is tremendous only after achieving a v of 6,000 rpm, while PS increased linearly with increased t (Fig. 1a). In other words, by holding P constant, PS increased at higher v and t. PS is at its minimum at P and v values of approximately 60 MPa and 5,000 rpm, respectively (Fig. 1b).

Monodispersed or minimally polydispersed nanodispersions are satisfying conditions for long term stability. A polydispersed nanodispersion contains a mixture of different particles sizes and is not preferable due to instability caused by Ostwald ripening [23]. A newly produced nanodispersion may be stable for a very long time, but all polydispersed nanodispersions will eventually destabilize by the Ostwald ripening mechanism [24]. The rate of Ostwald ripening in a nanodispersions has a direct relationship with its polydispersity [8]. For this reason, it is important to ensure the lowest possible polydispersity of the produced nanodispersion system to reach the maximum shelf life. Table 3 shows the regression coefficients, p-values and F-values for PDI. Three insignificant effects, namely, v, t^2 and P^2, were not eliminated from the model to maintain the hierarchy of the model. The factors t and P had significant effects on PDI ($p < 0.05$), but not v. The interaction effects on PDI among the independent variables are as in Fig. 1c–e. The most significant interaction parameter for PDI was the interaction between v and P (Table 3). According to the response surface generated, PDI rises with v and t (Fig. 1c), P and t (Fig. 1d) and P and v (Fig. 1d). The lowest level of the three processing parameters observed was the most preferable to reach the lowest PDI. Increasing the level of any of the processing parameters observed increases the PDI. The possible cause of this observation may be that higher energy supplies promoted a higher rate of particle collision that causes the coalescence of the particles than breakage of the particles. Therefore, the numbers of particles that coalesce instead of break increases as v and/or P increase during processing, hence increases the PDI. Nevertheless, the lowest PDI may not agree with the smallest PS. Although the types and concentration of emulsifier used were kept constant in the optimization of processing parameter, their effects on PS and PDI should not be overlook.

Phytosterols are relatively stable to mild heating and light. As expected, the Phyto values were rather stable after 5–20 min of mixing at speed of 1,000–9,000 rpm. The phytosterol content (Phyto) in the final nanodispersion produced decreased with increasing high pressure (P) applied during homogenization. The P is the sole independent variable that had a significant linear relationship with the Phyto. The Phyto was significantly reduced after the application of a higher pressure of

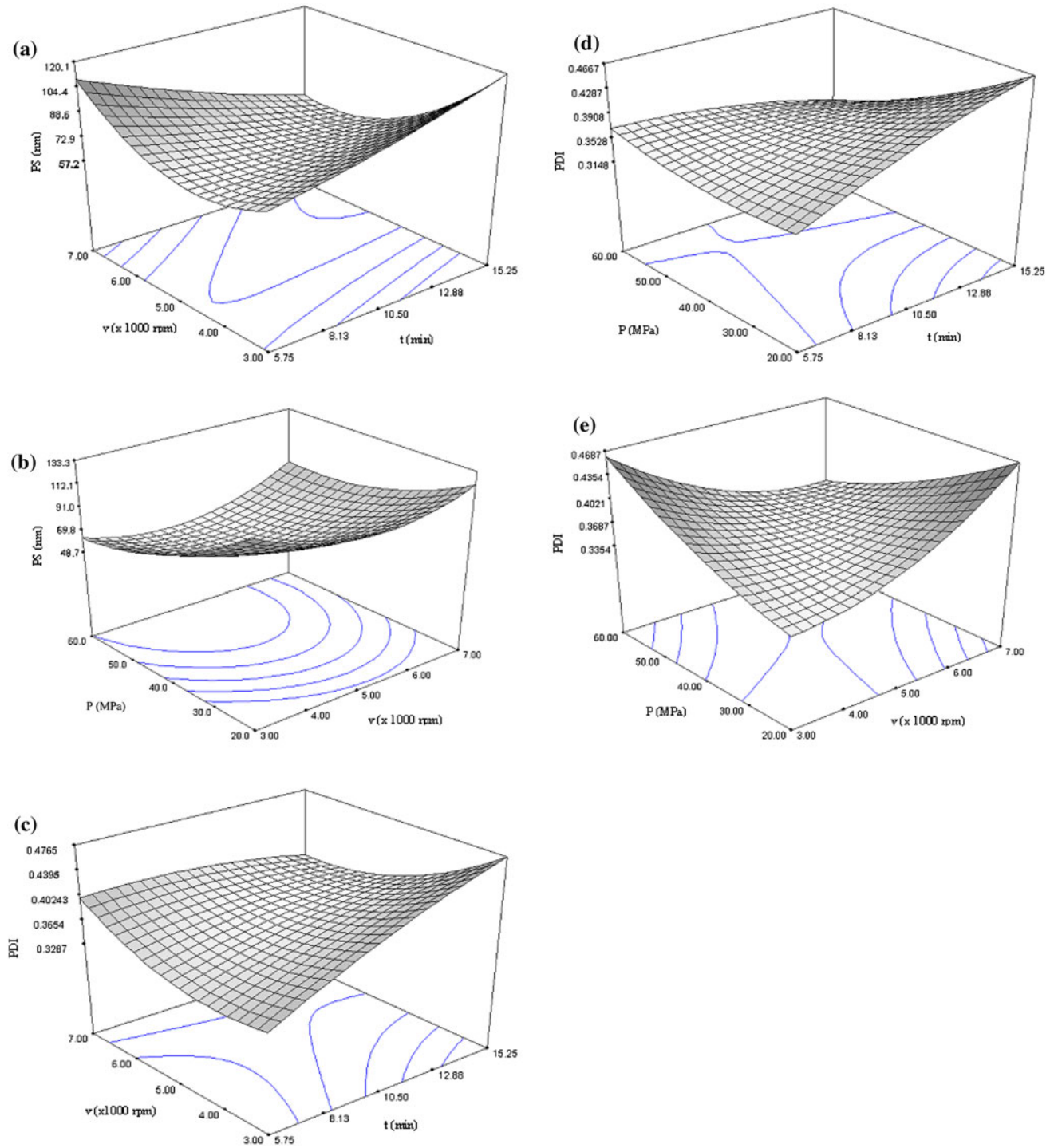

Fig. 1 Response surface plots of significant interactions between (**a**) mixing time and mixing speed on mean particles size; (**b**) mixing speed and homogenization pressure on mean particle size; (**c**) mixing speed and mixing time on polydispersity index; (**d**) homogenization pressure and mixing time on polydispersity index; and (**e**) homogenization pressure and mixing speed on polydispersity index ($p < 0.05$)

homogenization from 20 to 80 MPa. The cause of this has yet to be precisely clarified. Perhaps the high sheer force and cavitations produced during the application of high-pressure homogenization created short term, but intense, heating, triggering the degradation of phytosterols in the nanodispersion. In addition, cavitation produced during the high-pressure homogenization was reported to generate free radicals [25] that most probably prompted the loss of

Table 5 Optimized processing parameters and experimental runs for model verification

Independent variable			Response					
t (min)	v (×1000 rpm)	P (MPa)	PS (nm)		PDI		Phyto (mg/l)	
			Observed	Predicted	Observed	Predicted	Observed	Predicted
15.25	6.97	42.12	52.32	52.20	0.3390	0.3482	345.40	335.62
15.25	6.77	41.72	56.88	51.21	0.2891	0.3446	346.10	337.24
5.75	4.35	41.89	60.12	62.54	0.2633	0.3386	329.20	333.99
5.75	4.01	27.17	86.33	91.72	0.2541	0.3080	374.44	372.42
5.75	3.88	25.94	88.01	93.56	0.2891	0.3037	380.02	373.78
			$\chi^2 = 1.401$		$\chi^2 = 0.036$		$\chi^2 = 0.552$	

Abbreviations: refer to Tables 1 and 2

phytosterols in the prepared nanodispersion. While degradation triggered by high-pressure homogenization was minor for many functional compounds, studies have consistently reported the loss of functional ingredients incorporated into nanodispersions after applying high-pressure homogenization [19, 21, 26].

Optimization of Processing Parameters for the Production of Phytosterol Nanodispersions

The optimizer function of the software Design Expert was used to generate optimized processing parameters. PS and PDI were optimized at the lowest minimum values, while the Phyto was targeted at its maximum. The optimized processing parameters were 15.25 min mixing time, 7,000 rpm mixing speed and 42.41 MPa high-pressure homogenization processes. The optimized responses for the preparation of phytosterol nanodispersions are a minimal particle size (52.21 nm), PDI (0.3390) and phytosterol concentration at 335.6 mg/l.

Model Verification

A list of five sets of conditions was selected for modal verification by a Chi-square goodness-of-fit test (Table 5). The calculated chi-square value for PS, PDI and Phyto were less than the critical value of chi-square (9.488) at $\alpha = .05$ and $df = 4$. The chi-square test notably implied that the experimental values of PS, PDI and Phyto agreed with the predicted values at a 0.05 level of significance. The models were sufficient to predict PS, PDI and Phyto in the processing of phytosterol nanodispersions.

Conclusion

Generally, we successfully optimized the processing parameters for the production of phytosterols-containing nanodispersions using the RSM while keeping the formula parameters constant. Among the independent variables, P had significantly negative effects on all observed responses, namely, PS, PDI and Phyto ($p < 0.05$). Higher v values resulted in significantly lower PS values, and higher t values significantly increased PDI. The optimized processing parameters for the production of phytosterol nanodispersions were 15.25 min mixing time, 7,000 rpm mixing speed and a homogenization pressure of 42.41 MPa. The model was verified using a chi-square analysis. The established models for PS, PDI and Phyto are adequate for the prediction of responses. The values of R^2, adjusted R^2 and AAD were satisfactory. The model defines the true behavior of the phytosterol nanodispersion system produced. Interpolation in the experimental domain is achievable, but extrapolation beyond the experimental region is not recommended. RSM is an appropriate statistical tool for the experimental design of processing parameter optimization for the production of phytosterol nanodispersions using the emulsification–evaporation method.

Acknowledgments Financial support of this work by the Ministry of Science, Technology and Innovation of Malaysia through the Science Fund (05-01-04-SF0384) and the Ministry of Higher Education through Fundamental Research Grant Scheme (02-11-08-0619FR) is gratefully acknowledged. The first author thanks the Graduate School of Universiti Putra Malaysia for the Graduate Research Fellowship.

References

1. Vissers MN, Zock PL, Meijer GW, Katan MB (2000) Effect of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum lipoprotein concentrations in humans. Am J Clin Nutr 72:1510–1515
2. Sierksma A, Weststrate JA, Meijer GW (1999) Spreads enriched with plant sterols, either esterified 4, 4-dimethylsterols or free 4-desmethylsterols, and plasma total-and LDL- cholesterol concentrations. Br J Nutr 82:273–282
3. Pollak OJ (1953) Reduction of blood cholesterol in man. Circulation 7:702–706

4. Clifton PM, Noakes M, Ross D, Fassoulakis A, Cehun M, Nestel P (2004) High dietary intake of phytosterol esters decreases carotenoids and increases plasma plant sterol levels with no additional cholesterol lowering. J Lipid Res 45:1493–1499
5. de Jong A, Plat J, Mensink RP (2003) Metabolic effects of plant sterols and stanols. J Nutr Biochem 14:362–369
6. Fathi-Achachlouei B, Azadmard-Damirchi S (2009) Milk thistle seed oil constituents from different varieties grown in Iran. J Am Oil Chem Soc 86:643–649
7. Ostlund REJ (2002) Phytosterols in human nutrition. Annu Rev Nutr 22:533–549
8. McClements DJ (2005) Food emulsion: Principles, practices and techniques, 2nd edn. CRC press, London
9. Takeshita M, Saito S, Katsuragi Y, Yasunaga K, Matsuo N, Tokimitsu I (2007) Combination of plant sterols and diacylglycerol oil lowers serum cholesterol and lipoprotein (a) concentrations in postmenopausal women with mild-to-moderate hypercholesterolemia. Clin Nutr 2:4–11
10. Hallikainen MA, Sarkkinen ES, Gylling H, Erkkila AT, Uusitupa MI (2000) Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. Eur J Clin Nutr 54:715–725
11. Ostlund REJ, Spilburg CA, Stenson WF (1999) Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. Am J Clin Nutr 70:826–831
12. Mensink RP, Ebbing S, Lindhout M, Plat J, van Heugten MMA (2002) Effects of plant stanol esters supplied in low-fat yoghurt on serum lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations. Atherosclerosis 160:205–213
13. Becker M, Staab D, Von Bergman K (1992) Long-term treatment of severe familial hypercholesterolemia in children: effect of sitosterol and bezafibrate. Pediatrics 89:138–142
14. Plat J, van Onselen ENM, van Heugten MMA, Mensink RP (2000) Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters. Eur J Clin Nutr 54:671–677
15. Bas D, Boyacı İH (2007) Modeling and optimization I: usability of response surface methodology. J Food Eng 78:836–845
16. Acosta E (2009) Bioavailability of nanoparticles in nutrient and nutraceutical delivery. Curr Opin Colloid Interface Sci 14:3–15
17. Jafari SM, He Y, Bhandari B (2007) Optimization of nano-emulsions production by microfluidization. Eur Food Res Technol 225:733–741
18. Dickinson E (1992) An introduction to food colloids. Oxford University Press, New York
19. Leong WF, Che Man YB, Lai OM, Long K, Misran M, Tan CP (2009) Optimization of processing parameters for the preparation of phytosterol microemulsions by the solvent displacement method. J Agric Food Chem 57:8426–8433
20. Chu BS, Ichikawa S, Kanafusa S, Nakajima M (2007) Preparation of protein-stabilized β-carotene nanodispersions by emulsification–evaporation method. J Am Oil Chem Soc 84:1053–1062
21. Tan CP, Nakajima M (2005) β-Carotene nanodispersions: preparation, characterization and stability evaluation. Food Chem 92:661–671
22. Tan CP, Nakajima M (2005) Effect of polyglycerol esters of fatty acids on physicochemical properties and stability of β-carotene nanodispersions prepared by emulsification/evaporation method. J Sci Food Agric. 85:121–126
23. Tadros T, Izquierdo P, Esquena J, Solans C (2004) Formation and stability of nano- emulsions. Adv Colloid Interface Sci 108: 303–318
24. Gutierrez JM, Gonzalez C, Maestro A, Sole I, Pey CM, Nolla J (2008) Nano-emulsions: New applications and optimization of their preparation. Curr Opin Colloid Interface Sci 13:245–251
25. Lander R, Manger W, Scouloudis M, Ku A, Davis C, Lee A (2000) Gaulin homogenization: a mechanistic study. Biotechnol. Prog 16:80–85
26. Cheong JN, Tan CP, Man YBC, Misran M (2008) Tocopherol nanodispersions: preparation, characterization and stability evaluation. J Food Eng 89:204–209