DOI:10.1021/jf901853y

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Optimization of Processing Parameters for the Preparation of Phytosterol Microemulsions by the Solvent Displacement Method

Wai Fun Leong,[†] Yaakob B. Che Man,[†] Oi Ming Lai,[‡] Kamariah Long,[§] Misni Misran,[#] and Chin Ping Tan*,[†]

[†]Department of Food Technology, Faculty of Food Science and Technology and [‡]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, UPM, 43400 Serdang, Selangor, Malaysia, [§]Malaysian Agricultural Research and Development Institute (MARDI), P.O. Box 12301, 50774 Kuala Lumpur, Malaysia, and [#]Department of Chemistry, University of Malaya, 50603 Kuala Lumpur, Malaysia

The purpose of this study was to optimize the parameters involved in the production of water-soluble phytosterol microemulsions for use in the food industry. In this study, response surface methodology (RSM) was employed to model and optimize four of the processing parameters, namely, the number of cycles of high-pressure homogenization (1−9 cycles), the pressure used for high-pressure homogenization (100−500 bar), the evaporation temperature (30−70 °C), and the concentration ratio of microemulsions (1−5). All responses—particle size (PS), polydispersity index (PDI), and percent ethanol residual (%ER)—were well fit by a reduced cubic model obtained by multiple regression after manual elimination. The coefficient of determination (R^2) and absolute average deviation (AAD) value for PS, PDI, and %ER were 0.9628 and 0.5398%, 0.9953 and 0.7077%, and 0.9989 and 1.0457%, respectively. The optimized processing parameters were 4.88 (approximately 5) homogenization cycles, homogenization pressure of 400 bar, evaporation temperature of 44.5 °C, and concentration ratio of microemulsions of 2.34 cycles (approximately 2 cycles) of high-pressure homogenization. The corresponding responses for the optimized preparation condition were a minimal particle size of 328 nm, minimal polydispersity index of 0.159, and <0.1% of ethanol residual. The chi-square test verified the model, whereby the experimental values of PS, PDI, and %ER agreed with the predicted values at a 0.05 level of significance.

KEYWORDS: Phytosterols; microemulsions; solvent displacement method; response surface methodology; high-pressure homogenization

INTRODUCTION

Phytosterols, or plant sterols, are a group of naturally occurring functional lipid and steroid alcohols found exclusively in plants and are essential constituents of the cell membranes in plants. The phytosterols have structures similar to but different from that of cholesterol, as the phytosterols contain an additional hydrophobic carbon chain at the carbon-24 position. In contrast to cholesterol, phytosterols are hypocholesterolemic agents. They are not synthesized by the human body, and supplementation is therefore warranted. Phytosterols are widely applied as a food additive, in cosmetics, and in pharmacology. They are known for their efficacy in improving serum cholesterol profiles and reducing the risk of cardiovascular disease. Studies revealed that consumption of 2−3 g of plant sterols and stannols creates an approximately 10% reduction in total serum cholesterol and a 15% reduction in low-density lipoprotein (LDL) (*1*−*4*). Phytosterol-enriched food products are a promising dietary intervention to reduce the risk of cardiovascular disease in individuals with mild to moderate hypercholesterolemia (*5*).

In the past decade, industries have introduced phytosterols into a number of fat-derived food products, turning these low-nutritional-value products into more expensive, value-added, premium functional food products. Incorporating phytosterols into food products is not an easy job, as these sterols are water insoluble and hardly soluble in fats and oil. The high melting temperature of the pure phytosterols allows for only very small amounts of phytosterol enrichment. Otherwise, significant crystallization of these sterols will occur within the oil phase of the food products, resulting in a gritty, waxy, and unacceptable texture. Therefore, most, if not all, of the phytosterols currently used in food enrichment are esterified. Esterification of phytosterols permits maximal incorporation into minimal amounts of fat; however, the taste of the food products is adversely affected when phytosterol esters are distributed within a small amount of fat. Incorporation of esterified phytosterols is limited to high-fat food products only. In other words, consumers who expect to benefit from the hypocholesterolemic effect of phytosterols have

*Corresponding author (telephone +603-89468418; fax +603-89423552; e-mail tancp@putra.upm.edu.my).

 Published on Web 08/20/2009

had to consume high-fat food, despite the high calories and health risk of a high-fat diet.

Researchers have employed the emulsification technique to increase the water solubility of phytosterols (*6*−*10*). A study shows that consumption of lecithin-emulsified phytosterols at a dosage of 300 mg reduces cholesterol absorption 3 times more than unesterified phytosterols powder at a dose of 1 g (*6*). Takeshita reported the potential use of a combination of phytosterols and diacylglyceride (DAG) in lowering cholesterol. Significant reductions in serum cholesterol among mild to moderate hypercholesterolemic postmenopausal women were observed after the consumption of 0.3 g/day of phytosterols with DAG in rapeseed and soybean oil for 4 weeks (*11*). By converting the phytosterols into oil-in-water microemulsions, phytosterols become applicable to non-fat-based food products while also demonstrating higher cholesterol-lowering effects than esterified phytosterols. Further development and optimization of the production process used to create stable phytosterols microemulsions with small particle sizes and low polydispersity are critical, as the dose response for serum cholesterol reduction may be affected; that is, a lower dosage of phytosterols is needed to achieve a similar or even higher serum cholesterol reduction effect. Increased efficacies of phytosterols will lead to dosage reductions and lower costs; phytosterol-enriched food products may become more affordable.

Application of the microemulsions preparative methods in the fields of pharmaceuticals and cosmetics is rather common and well established, as compared to its application in food and functional ingredients. Several factors affect the particle size and stability of a prepared microemulsion and/or nanodispersions. These factors can be divided into processing factors, that is, temperature, time and speed of mixing (120), homogenization pressure and cycle (*13*, *14*), and formulation factors such as the type and concentration of organic solvent used as well as types and combination of emulsifiers used, the organic/aqueous ratio (*12*, *15*), and type and concentration of water-insoluble material loaded, such as drug, polymer, or bioactive ingredients. The solvent displacement method, also described as the nanoprecipitation method (*16*), that is used to prepare nanodispersions had been studied for 20 years. This method was first described for the preparation of nanocapsules by deposition of poly(D,L-lactide) biodegradable polymer following acetone displacement (*17*). It is also used in the preparation of polymer nanoencapsulated drugs (*18*, *19*)) and polycaprolactone nanoparticles (*20*) as well as in the preparation of nanoparticles of cosmetic ingredients (*21*). Recently, this method has also been used to prepare β-carotene nanodispersions (*22*−*25*). Although solvent displacement is one of the simplest methods of nanodispersion preparation, not many studies have been done comparing this method to other methods. Perhaps it is due to the complexity involved in selecting a compatible combination of solvent, active compound, emulsifier, and/or polymer.

The purpose of this study was to use response surface methodology (RSM) to model and optimize the processing parameters for preparing a phytosterol microemulsions of minimum particle size, minimum polydispersity index, and minimum acceptable solvent residual.

MATERIALS AND METHODS

Materials. Vegapure FTE (contains a minimum of 99% phytosterols) was a gift from Cognis (Duesseldorf-Holthausen, Germany). Polyoxyethylene sorbitan monolaurate (Tween 20) was obtained from Fisher Scientific (Pittsburgh, PA). HPLC-grade ethanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Headspace 20 mL vials and PTFE/silicon septa aluminum caps was obtained from Agilent Technologies (Wilmington, DE).

Table 1. Independent Variables and Their Levels Used for the Central Composite Rotatable Design (CCRD)

		level				
factor	symbol	−2	−1	0	1	2
cycles of high-pressure homogenization	Cy	1	3	5	7	9
pressure of high-pressure homogenization	Pr (bar)	100	200	300	400	500
temperature of evaporation	Te (°C)	30	40	50	60	70
concentration ratio	Cr	1	2	3	4	5

Experimental Design. Response surface methodology (RSM) was applied to study the effect of four processing parameters in the preparation of phytosterol microemulsions. A five-level four-factor central composite rotatable design (CCRD) was used in this study. The independent variables were cycles of high-pressure homogenization (Cy, 1−9), pressure of high-pressure homogenization (Pr, 100−500 bar), temperature of evaporation (Te, 30−70 °C), and concentration ratio of microemulsions (Cr, 1−5). The responses were particle size (PS, nm), polydispersity index (PDI), and ethanol residual (%ER). The coded and uncoded levels of independent variables used are listed in **Table 1**. A total of 30 experiments, consisting of 6 center points with combinations of different levels of each independent variable, were generated using the software Design Expert version 6.0.6 (Minneapolis, MN) (**Table 2**). The experimental runs were divided into three blocks, completed on three consecutive days. Individual experiments were carried out in randomized order to minimize the effect of unexplained variability in the experimental responses due to extraneous factors.

Preparation of Phytosterol Microemulsions. Microemulsions of phytosterols were prepared by modifying the preparative method described in previous studies (*17*, *23*). Phytosterols (1% w/w) in powder form were first dissolved in ethanol to form an 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 dispersion of phytosterol. The aqueous phase (continuous phase) was prepared by dissolving Tween 20 (0.2% w/w) in deionized water. The organic phase was slowly poured into the aqueous phase under conventional homogenization (Silverson L4R, Buckinghamshire, U.K.) at 5000 rpm for 5 min to produce coarse pre-emulsions. The pre-emulsions were immediately subjected to high-pressure homogenization (APV, Crawley, U.K.). Phytosterol microemulsions were produced after the removal of ethanol and concentrated to a particular concentration ratio of microemulsions 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 bar and rotation of 100 rpm. The circulating bath was set at 5 °C. Thirty samples with different levels of processing conditions were prepared according to the designed RSM experimental runs (**Table 2**). The volume ratio of organic-to-aqueous phase was fixed at 2:8. To study the effects of the processing parameters, the formulation of the phytosterol microemulsions sample was kept constant.

Analysis of Particle Size and Its Polydispersity. Measurement of mean particle size and particle size distribution of the particles in the phytosterol microemulsions was conducted after removal of ethanol, except for sample run 24. Measurements were done by using a dynamic light-scattering particle size analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, U.K.). The measuring range of the Zetasizer Nano ZS was 0.6−6000 nm. The measurement temperature was set at 25 °C. Measurements were done by placing the microemulsion-containing cuvette directly into the module. Droplets in the microemulsions scattered the light according to their particle size. The particle size of the microemulsions was analyzed on the basis of the cumulants mean (*Z*-average) diameter, which depends on the Brownian motion of the particles. PDI was used to describe the particle size distribution. The PDI was calculated according to the size distribution graph. The final particle diameter (PS) and polydispersity index (PDI) were calculated from the average of three measurements; each measurement was the average of five runs.

Determination of Ethanol Residual. *Headspace Solid-Phase Microextraction* (*HS-SPME*) *Analysis*. HS-SPME coupled with GC was the common method used in analyzing ethanol in alcoholic beverages (*26*, *27*) and human body fluid (*28*, *29*). A Supelco 85 μm polyacrylate (PA)-coated SPME fiber and a manual fiber holder were

Table 2. Central Composite Design and Responses for the Processing of Phytosterol Microemulsions[a]

	independent variables				responses		
run	Cy	Pr	Te	Cr	PS, nm (Y1)	PDI (Y2)	%ER (Y2)
1	3	200	40	4	322	0.227	0.0000 ± 0.0000
2	7	400	40	4	296	0.184	0.0520 ± 0.0016
3	5	300	50	3	322	0.186	0.0390 ± 0.0024
4	3	400	60	4	314	0.231	0.0300 ± 0.0007
5	3	200	60	2	359	0.180	0.4212 ± 0.0042
6	7	200	60	4	315	0.196	0.0180 ± 0.0011
7	3	400	40	2	341	0.164	0.1320 ± 0.0014
8	7	400	60	2	341	0.148	0.5419 ± 0.0010
9	5	300	50	3	323	0.182	0.0105 ± 0.0021
10	7	200	40	2	335	0.170	0.1429 ± 0.0044
11	7	400	40	2	326	0.177	0.1163 ± 0.0031
12	5	300	50	3	331	0.192	0.0850 ± 0.0032
13	7	200	40	4	314	0.213	0.0310 ± 0.0055
14	7	400	60	4	303	0.202	0.0350 ± 0.0021
15	7	200	60	2	354	0.158	0.7081 ± 0.0009
16	5	300	50	3	327	0.189	0.0850 ± 0.0000
17	3	400	60	2	355	0.138	0.8535 ± 0.0021
18	3	200	60	4	336	0.198	0.0660 ± 0.0044
19	3	400	40	4	328	0.193	0.0000 ± 0.0000
20	3	200	40	2	345	0.205	0.1206 ± 0.0042
21	5	300	50	3	325	0.164	0.0940 ± 0.0032
22	9	300	50	3	314	0.180	0.0264 ± 0.0000
23	5	300	50	5	308	0.251	0.0000 ± 0.0000
24	5	300	50	1	547	0.169	0.0277 ± 0.0052
25	5	300	70	3	323	0.177	0.0380 ± 0.0029
26	5	300	30	3	299	0.122	0.0800 ± 0.0014
27	5	500	50	3	373	0.204	0.0460 ± 0.0047
28	1	300	50	3	339	0.148	0.0154 ± 0.0015
29	5	100	50	3	337	0.168	0.0930 ± 0.0087
30	5	300	50	3	322	0.227	0.0000 ± 0.0000

[a] Abbreviations: PS, particle size; PDI, polydispersity; %ER, percentage of ethanol residual. For other abbreviations refer to **Table 1**.

employed for the SPME headspace analysis. The PA fiber was first conditioned in the GC injection port at 250 °C for 2 h. To a 20 mL headspace vial containing 4 mL of phytosterol microemulsions was added 1.5 g of NaCl. Acetonitrile (100 μL) was used as internal standard. The vial was immersed in a water bath for heating with stirring for 20 min at 60 °C. After 20 min, the fiber was exposed for 20 min at 60 °C for absorption and then immediately injected into the injection port of the GC for 10 min. Each analysis was carried out in duplicate. To determine the relative response factor (RRF), 4 mL of phytosterol microemulsions was replaced by deionized water containing Tween 20 (0.2% w/v) and ethanol (100 μL).

Gas Chromatography−Flame Ionization Detection (GC-FID) Analysis. The analysis was performed with an Agilent Technologies network GC system, series 6890N (Wilmington, DE) with flame ionization detection, controlled by the Agilent Chemstation and a DB-Wax column (J&W Science, 30 m length × 250 μm i.d. × 0.25 μm film thickness). A 0.75 mm i.d. linear was used to minimize the effect of peak broadening. Splitless injection was used. Helium was used as the carrier gas at a flow rate of 1.1 mL/min. The injector temperature was set at 250 °C, the maximum temperature recommended by the manufacturer to avoid significant carry-over effect. The oven temperature program was initially set at 40 °C for 3 min and then increased 4 °C/min to 60 °C for 2 min. The detector temperature was set at 270 °C.

Statistical Analysis. Experimental data were analyzed by RSM using the software Design Expert version 6.0.6 (Minneapolis, MN). Multiple regression was 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 chosen model. The AAD values were calculated with the following equation (*30*): AAD = $\{[\ (\sum|y_{i,\text{exptl}} - y_{i,\text{calcd}}|/y_{i,\text{exptl}})]/p\} \times 100$, where $y_{i,\text{exptl}}$ is the experimental value of the responses, $y_{i,\text{calcd}}$ is the predicted value of the responses, and p is the total runs. All measurements were duplicated.

Optimization and Verification. Optimized processing parameters were generated using the optimizer function of the Design Expert software. The PS, PDI, and %ER were optimized to the minimum value possible. For model verification, experimental data were subjected to the chi-square goodness-of-fit test. The chi-square values of the responses were calculated on the basis of the formula

$$\text{chi-square, } \chi^2 = \Sigma[(O-P)^2/P]$$

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

RESULTS AND DISCUSSION

In the present study, phytosterol microemulsions were prepared by the solvent displacement method accompanied by high-pressure homogenization. Phytosterols are slightly polar compounds that are hardly soluble in either water or lipids but are soluble in mildly polar solvent. Therefore, ethanol, a polar protic solvent, was selected as the organic phase, whereas the continuous phase was deionized water. The emulsifier used in this study was Tween 20, also known as polysorbate 20 or polyethylene (20) sorbitan monolaurate. It is a frequently used water-soluble nonionic emulsifier for its capability to reduce the interfacial tension of two immiscible liquid phases, thus reducing the energy needed to break down the particle to a smaller size (*31*). A preliminary study was previously done to identify the independent parameters and their levels that would potentially affect the PS, PDI, and %ER. The four independent variables selected for the optimization of phytosterol microemulsions processing using RSM were number of cycles of high-pressure homogenization, pressure of high-pressure homogenization, temperature used to remove ethanol during reduced pressure evaporation, and concentration ratio of microemulsions.

Fitting the Models. The PS, PDI, and %ER of the phytosterol microemulsions for each generated experiment are shown in **Table 2**. The experimental values of PS, PDI, and %ER were best fit with a reduced cubic model through multiple regression after manual elimination of nonsignificance terms. Manual elimination is commonly used to exclude some nonsignificant terms, but some of them are required to be preserved to retain the hierarchy of the model (*32*). Run 24 was excluded in modeling for all responses to improve the fit of the model (**Table 1**). The regression coefficients, F values, and P values of the PS, PDI, and %ER are shown in **Table 3**. The values of R^2, adjusted R^2, AAD, and lack of fit are given in **Table 4**. The values of R^2 and adjusted R^2 ranged from 0.9809 to 0.9989 and from 0.9503 to 0.9958, respectively. In other words, > 95% of the variation in the models was explained by the regression model. The correlation between the experimental and predicted values for PS, PDI, and %ER was satisfactory. The AAD values describe the percent deviation of the predicted and experimental data. A high R^2 value (> 0.95) and low AAD values (< 1.05%) indicated that the selected model was accurate and the model equation characterized the true behavior of the phytosterol microemulsion system. The regression model for PS, PDI, and %ER was statistically well fit with a significant P value of < 0.001. The ANOVA showed no significant lack-of-fit for the final reduced model for all three responses. Therefore, the developed model can be used for interpolation in the experimental domain.

Main Effects and Interaction Effects of the Independent Variables. PS ranged from 296 to 545 nm, whereas the PDI varied from 0.12 to 0.25 (**Table 2**). Small particle size and low polydispersity are major determinants for promoting stability in the prepared microemulsions. Stability of emulsion refers to stable

Table 3. Regression Coefficients, *P* Values, and *F* Values for PS, PDI, and %ER after Manual Elimination[a]

parameter	variable	regression coefficient	*F* value	*P* value
PS	intercept	327.50	32.06	<0.0001
	Cy	−4.75	10.15	0.0097
	Pr	−3.00	4.05	0.0719
	Te	4.92	32.62	0.0002
	Cr	−14.31	187.29	<0.0001
	Cy^2	3.44	17.68	0.0018
	Pr^2	−2.68	10.73	0.0083
	Te^2	1.32	2.59	0.1387
	Cr^2	1.79	2.66	0.1337
	Te−Cr	−3.38	10.25	0.0095
	Cy^3	−2.50	16.87	0.0021
	Pr^3	−1.75	8.27	0.0165
PDI	intercept	0.1800	77.15	<0.0001
	Cy	−0.0057	53.61	0.0002
	Pr	−0.0068	76.06	<0.0001
	Te	−0.0062	21.16	0.0025
	Cr	0.0103	13.71	0.0076
	Cy^2	0.0065	47.02	0.0002
	Pr^2	−0.0078	66.85	<0.0001
	Te^2	0.0018	3.41	0.1074
	Cr^2	−0.0013	0.16	0.7028
	Cy−Pr	0.0036	14.62	0.0065
	Cy−Te	0.0001	0.02	0.8988
	Cy-Cr	−0.0013	1.74	0.2288
	Pr−Te	0.0053	30.68	0.0009
	Pr−Cr	0.0039	16.71	0.0046
	Te−Cr	0.0064	45.23	0.0003
	Te^3	0.0010	3.62	0.0987
	Cr^3	0.0087	11.94	0.0106
	Cy−Pr−Te	−0.0030	10.02	0.0158
	Cy−Pr−Cr	−0.0064	45.23	0.0003
	Pr−Te−Cr	0.0075	62.60	<0.0001
%ER	intercept	0.0680	323.51	<0.0001
	Cy	−0.0007	0.07	0.8052
	Pr	0.0159	32.02	0.0008
	Te	0.1741	1276.27	<0.0001
	Cr	−0.0912	80.87	<0.0001
	Cy^2	−0.0143	17.28	0.0043
	Pr^2	−0.0115	11.04	0.0127
	Te^2	−0.0152	19.36	0.0032
	Cr^2	0.1903	277.37	<0.0001
	Cy−Pr	−0.0352	104.08	<0.0001
	Cy-Te	−0.0098	8.14	0.0246
	Cy−Cr	0.0036	1.11	0.3270
	Pr−Te	0.0151	19.15	0.0032
	Pr−Cr	−0.0156	20.38	0.0028
	Te−Cr	−0.1217	1246.87	<0.0001
	Te^3	−0.0442	492.86	<0.0001
	Cr^3	−0.0841	84.99	<0.0001
	Cy−Pr−Te	−0.0330	91.88	<0.0001
	Cy−Pr−Cr	0.0444	166.06	<0.0001
	Pr−Te−Cr	−0.0201	33.96	0.0006

[a] For abbreviations, refer to **Tables 1** and **2**.

particle size distribution over time with no particle flocculation, sedimentation, coalescence, crystallization, or phase separation. According to Stokes' law, the velocity of creaming/sedimentation (V_{Stokes}) is proportionate to the radius of the particles in an emulsion, $V_{Stokes} = [2gr^2(\rho_2 - \rho_1)/9\eta 1]$, where g is gravity, r is the radius of the particle, ρ_1 is the density of the aqueous phase, ρ_2 is the density of the dispersed phase, and η is the shear viscosity. In other words, smaller particle sizes are related to slower creaming rates, and longer periods of stability of the emulsion are expected (*31*). Owing to the small particle size in the microemulsions produced, Brownian motion is able to reduce gravity-related

Table 4. Coefficient of Determination, R^2, Adjusted R^2, AAD, and Lack of Fit for the PS, PDI, and %ER of the Phytosterol Microemulsions[a]

	PS	PDI	ER%
R^2	0.9628	0.9952	0.9989
adjusted R^2	0.9355	0.9823	0.9958
model (*p* value)	<0.0001	<0.0001	<0.0001
AAD (%)	0.5398	0.7077	1.0457
lack of fit (*F* value)	0.83	2.93	1.70
lack of fit (*P* value)	0.6527	0.2018	0.3451

[a] For abbreviations, refer to **Table 2**.

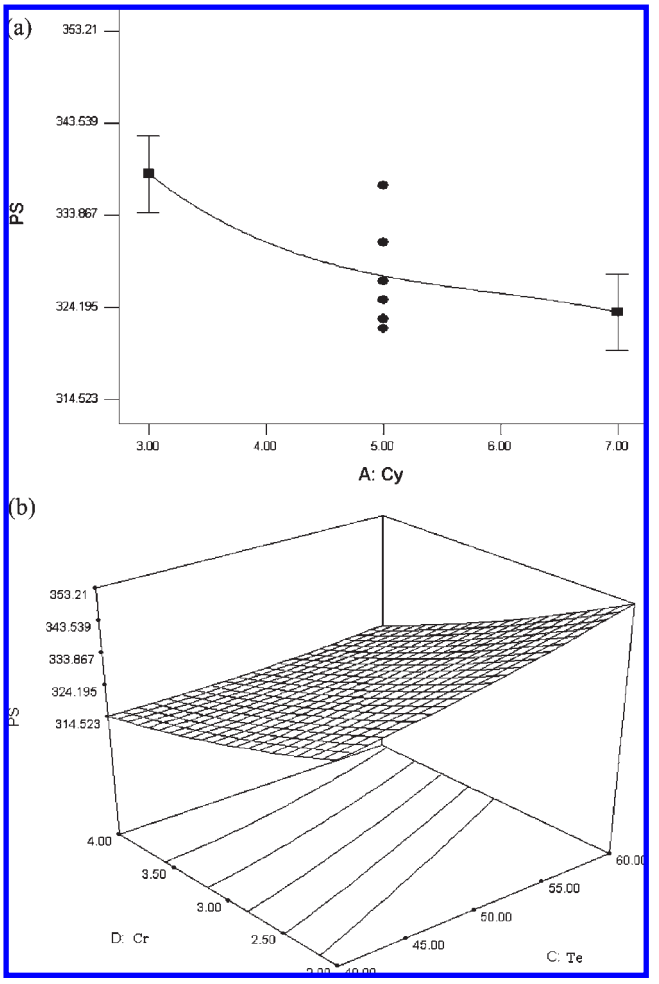

Figure 1. (**a**) Main effect plot of Cy on PS and (**b**) response surface plot of interaction between Cr and Te on PS. All interactions are significant at $p < 0.05$.

instabilities such as gravitational separation, sedimentation, and creaming (*33*).

Table 3 shows that Cy, Te, and Cr are three of the first-order coefficients contributing to PS, and Cr had the greatest negative effects among the three coefficients (**Figure 1**). Application of high energy and/or a large amount of emulsifier is required to produce small particles (*34*). High-pressure homogenization is one of the most commonly used methods to provide the energy input required to reduce the particle size and polydispersity of the emulsions and therefore increase the stability of the produced emulsion (*14*, *35*). This study confirmed that the number of cycles of high-pressure homogenization significantly ($P < 0.05$) affected the PS from three cycles to five cycles; higher than five cycles showed minimal reduction on PS (**Figure 1a**). The effect of second-order coefficients for Cy and Pr on PS were significant

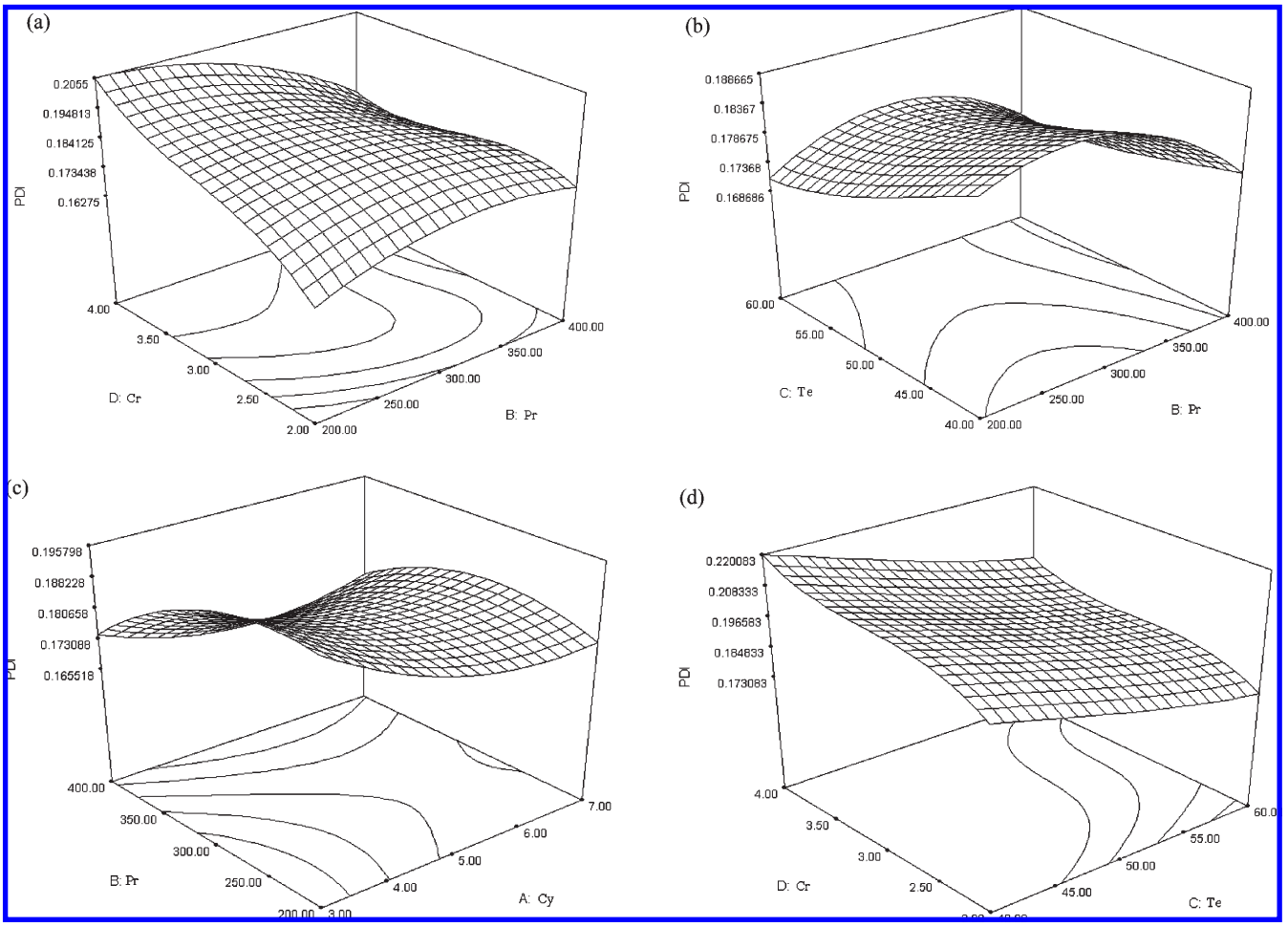

Figure 2. Response surface plot of significant interactions between (**a**) Cy and Pr, (**b**) Pr and Te, (**c**) Pr and Cr, and (**d**) Te and Cr on PDI ($p < 0.05$).

($P < 0.05$). The second-order coefficient for Cr was greatest on PS. The P values of all stated third-order coefficients were significant at $P < 0.05$. The only significant ($P < 0.01$) interactive effect on PS was Te−Cr. A response surface plot was constructed for a clearer evaluation of the interaction between Te and Cr on PS (**Figure 1b**). PS reduced as the Cr increased because removal of ethanol from the fine emulsion particles reduced the particle size. In contrast, increasing Te contributed to an increase in PS. In general, low evaporation temperature and high concentration ratio of microemulsions were two important factors in the production of small particle size.

Emulsion with monodispersed droplets or minimally polydispersed droplets is the most desirable condition for long-term stability (*36*). Owing to the technology available now, the phytosterol microemulsions produced in this study were polydispersed. For this reason, it is important to ensure the lowest possible PDI of the microemulsion system produced for maximum shelf life. **Table 3** shows the regression coefficients, P values, and F values for PDI after manual elimination. The Cy, Pr, Te, and Cr had significant ($P < 0.01$) effects on PDI. The interactions among the independent variables for PDI are shown in **Figure 2**. Cr and Te had the highest ($P < 0.05$) interaction effect on PDI compared to other interaction coefficients. As shown in the response surface plot (**Figure 2a**,**b**), PDI rises with an increase in Cr but decreases with increase in Te (**Figure 2a**). The PDI increased slowly for concentration ratios of microemulsions from 1 to 3, but not from three cycles onward (**Figure 2c**). Removal of ethanol from microemulsions produced small particle size when the microemulsions were first subjected to evaporation. Further evaporation led to concentration of the microemulsions, where water was removed together with ethanol. Dependent on the Cr value (except for Cr of 1), the process of concentrating the microemulsions causes an increase in the number of particles per unit volume. Cr values of 2, 3, 4, and 5 indicate that the total particles per unit volume increase by 2-, 3-, 4-, and 5-fold, respectively, after the evaporation process. This could increase the frequency of collision between particles and eventually lead to flocculation and/or coalescence of the particles. As a result, particle distribution broadening took place (**Figure 2d**). Although increases in Cr facilitate the formation of small particles, it also increased the PDI, which favors instability. Even though higher Te reduces PDI, it is also related to higher PS. At higher Te, the frequency of collision between particles increases, which is certain to promote coalescence of particles to form mostly large particles with less surface area and better stability. Both Cr and Pr were also significant ($P < 0.01$) interaction parameters for PDI. On the basis of the constructed response surface plot, PDI decreased as the Cy and Pr increased (**Figure 2c**). However, after approximately five cycles, the rate of reduction of PDI began to decrease. The Pr showed higher reduction of PDI from approximately 300 bar and above. Although PDI is low at Pr lower than 300 bar, lower Pr is related to higher PS values.

Complete removal of ethanol from aqueous-based food products is complicated because ethanol and water are highly miscible in one another, as both contain hydroxyl groups. Although ethanol and water have different boiling points, water tends to evaporate at any temperature, even below its boiling

point. For this reason, ethanol used in the preparation of phytosterol microemulsions was removed together with large amounts of water. The use of ethanol in food processing is acceptable only if the final ethanol content of the food ingredient is <0.5%, and more preferably 0.1%, in consumer products. Most, if not all, of the halal-certifying bodies allow small amounts of ethanol, generally <0.1% and sometimes up to 0.5% (*37*). Ethanol is reported to reduce the steric stabilization capacity of nonionic surfactants (*38*). Hence, removal of ethanol not only permits the final product to be labeled as halal, it also reduces the particle size and improves the stability of the final product. HS-SPME is a more sensitive method than the conventional headspace analysis for the determination of ethanol (*28*, *29*). The limits of detection of ethanol analysis using HS-SPME were reported to be as low as 0.1 mg/dL (*29*). A %ER ranging from 0 to 5.52% (**Table 2**) was observed in this study. The %ER value lower than 0.0001% was reported as 0 in this study. Three of the first-order coefficients, namely, Pr, Te, and Cr, showed significant ($P < 0.01$) effects on %ER (**Table 3**). The interaction effects between the independent variables on %ER are elucidated in **Figure 3**. The most significant ($P < 0.001$) interaction parameter for %ER was interaction between Te and Cr (**Figure 3b**). According to the constructed response surface plot, it was observed that as Te increased the %ER increased, whereas the %ER was lowest at a Cr of about 3. Higher Te was not as effective as lower Te in eliminating the ethanol residual. The %ER reduced as Cr increased. Although the lowest possible ethanol residual is preferable, complete removal of ethanol is not practical from the perspective of industries, as higher Cr is related to longer processing times and greater energy inputs, as well as higher production cost. An optimum level of Cr and Te is necessary for the cost-effective production of microemulsions.

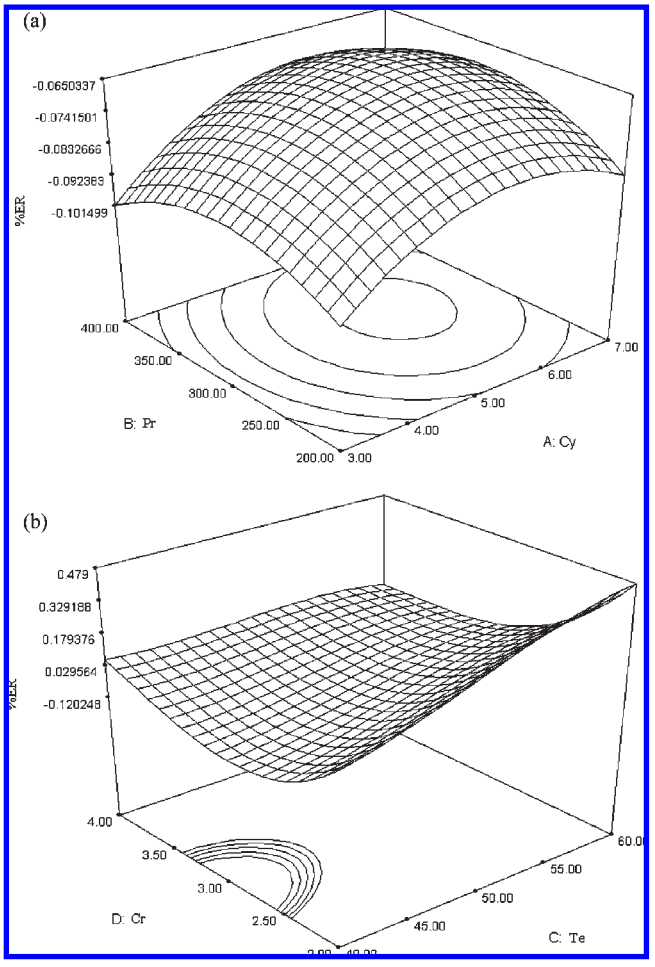

Figure 3. Response surface plot of significant interaction between (**a**) Cy and Pr and (**b**) Te and Cr on %ER ($p < 0.05$).

Optimization of Processing Parameters for the Production of Phytosterol Microemulsions. Optimized processing parameters were generated using the optimizer function of the Design Expert software. The PS and PDI were optimized at the lowest possible minimum value, whereas the %ER was targeted at <0.1%. It is not practical to optimize %ER to a minimum value, because extra energy and time may be required to concentrate the microemulsions, which may also sacrifice the stability of the microemulsions by promoting larger particle sizes and higher polydispersity. The optimizer generated nine sets of optimized parameters, of which five sets with the highest desirability were selected for model verification. The selected optimized processing parameters with highest desirability (0.788) were 4.88 Cy, Pr of 400 bar, Te of 44.5 °C, and Cr of 2.34. The optimized responses for the preparation of phytosterol microemulsions were minimal particle size (328 nm), polydispersity index (0.159), and %ER (<0.1%).

Model Verification. A list of five sets of optimized conditions was selected for model verification by the chi-square goodness-of-fit test (**Table 5**). Five sets of samples were prepared according to the optimized conditions, and analyses of particle size, polydispersity, and %ER were conducted. The chi-square values of the responses were calculated on the basis of the formula

$$\chi^2 = \Sigma[(O-P)^2/P]$$

where O and P are the observed and predicted responses, respectively. The calculated chi-square values for PS, PDI, and %ER were less than the critical value of chi-square (9.49) at $\alpha = 0.05$ and df = 4. The chi-square test notably implied that the experimental values of PS, PDI, and %ER agreed with the predicted values at $P < 0.05$, where the residual error between experimental value and predicted value was minimal. The models were sufficient to predict PS, PDI, and %ER in the processing of phytosterol microemulsions.

Table 5. Optimized Processing Parameters and Experimental Runs for Model Verification[a]

independent variables					response					
					PS (nm)		PDI		%ER	
Cy	Pr	Te	Cr	desirability	observed	predicted	observed	predicted	observed	predicted
4.88	400	44.45	2.34	0.7876	312	328	0.1660	0.1589	0.0840	0.1000
4.84	400	46.32	2.47	0.7869	341	327	0.1512	0.1596	0.0891	0.1000
5.09	400	42.94	2.23	0.7861	334	329	0.1700	0.1591	0.0919	0.1000
7.00	200	41.68	2.23	0.7337	321	332	0.1532	0.1695	0.1005	0.1000
5.96	200	40.00	2.14	0.7219	345	333	0.1800	0.1718	0.0900	0.1000
					$\chi^2 = 2.2528$		$\chi^2 = 0.0035$		$\chi^2 = 0.0054$	

[a] For abbreviations, refer to **Tables 1** and **2**.

In summary, the processing parameters for the production of phytosterol microemulsions were successfully optimized using RSM, while the formulation was held constant. Among the independent variables, the temperature used to evaporate or concentrate the microemulsions, as well as the concentration ratio of microemulsions, shows significant ($P < 0.05$) positive and negative effects, respectively, on all three responses (PS, PDI, and %ER). Whereas the pressure used in high-pressure homogenization had a positive effect on PDI and %ER, the number of cycles of high-pressure homogenization showed a negative effect on PS. Optimized processing parameters for the production of phytosterol microemulsions were 5 Cy, Pr of 400 bar, Te of 44.5 °C, and Cr of 2.34. Verification of the model using chi-square analysis showed the experimental value was in agreement with the predicted one ($P < 0.05$). The acceptable values of R^2, adjusted R^2, and AAD implied that the model described the true behavior of the phytosterol microemulsions system that was produced, and interpolation in the experimental domain is achievable. However, extrapolation beyond the experimental region is not recommended, as RSM is an empirical modeling approach. Although the model fits well in the region of the original data, it may not fit well beyond that region.

LITERATURE CITED

(1) Moghadasian, M. H. Pharmacological properties of plant sterols in vivo and in vitro observations. *Life Sci.* **2000**, *67*, 605–615.

(2) Noakes, M.; Clifton, P.; Ntanios, F.; Shrapnel, W.; Record, I.; McInerney, J. An increase in dietary carotenoids when consuming plant sterols or stanols is effective in maintaining plasma carotenoid concentrations. *Am. J. Clin. Nutr.* **2002**, *75*, 79–86.

(3) de Jong, A.; Plat, J.; Mensink, R. P. Metabolic effects of plant sterols and stanols. *J. Nutr. Biochem.* **2003**, *14*, 362–369.

(4) Clifton, P. M.; Noakes, M.; Ross, D.; Fassoulakis, A.; Cehun, M.; Nestel, P. High dietary intake of phytosterols esters decreases carotenoids and increases plasma plant sterol levels with no additional cholesterol lowering. *J. Lipid Res.* **2004**, *45*, 1493–1499.

(5) Lichtenstein, A. H.; Deckelbaum, R. J. Stanol/sterol ester-containing foods and blood cholesterol levels. A statement for healthcare professionals from the Nutrition Committee of the Council on Nutrition, Physical Activity, and Metabolism of the American Heart Association. *Circulation* **2001**, *103*, 1177–1179.

(6) Ostlund, R. E. J.; Spilburg, C. A.; Stenson, W. F. Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. *Am. J. Clin. Nutr.* **1999**, *70*, 826–831.

(7) Gremaud, G.; Dalan, E.; Piguet, C.; Baumgartner, M.; Ballabeni, P.; Decarli, B.; Leser, M. E.; Berger, A.; Fay, L. B. Effects of non-esterified stanols in a liquid emulsion on cholesterol absorption and synthesis in hypercholesterolemic men. *Eur. J. Nutr.* **2002**, *41*, 54–60.

(8) Engel, R.; Schubert, H. Formulation of phytosterols in emulsions for increased dose response in functional foods. *Innovative Food Sci. Emerging Technol.* **2005**, *6*, 233–237.

(9) Spernath, A.; Yaghmur, A.; Hoffman, R. E.; Garti, N. Self-diffusion nuclear magnetic resonance, microstructure, transititons, and solubilization capacity of phytosterols and cholesterol in Winsor IV food-grade microemulsions. *J. Agric. Food Chem.* **2003**, *51*, 2359–2364.

(10) Rozner, S.; Verkhovski, L.; Nissimov, Y.; Aserin, A.; Vilensky, R.; Danino, D.; Zouboulis, C. C.; Milner, Y.; Garti, N. Inhibition of cholesterol transport into skin cells in cultures by phytosterols-loaded microemulsion. *Chem. Phys. Lipids* **2008**, *153*, 109–118.

(11) Takeshita, M.; Saito, S.; Katsuragi, Y.; Yasunaga, K.; Matsuo, N.; Tokimitsu, I.; Yasukawa, T.; Nakamura, H. Combination of plant sterols and diacylglycerol oil lowers serum cholesterol and lipoprotein (a) concentrations in postmenopausal women with mild-to-moderate hypercholesterolemia. *Clin. Nutr.* **2007**, *2*, 4–11.

(12) Trotta, M.; Debernardi, F.; Caputo, O. Preparation of solid lipid nanoparticles by a solvent emulsification−diffusion technique. *Int. J. Pharm.* **2003**, *257*, 153–160.

(13) Tan, C. P.; Nakajima, M. β-Carotene nanodispersions: preparation, characterization and stability evaluation. *Food Chem.* **2005**, *92*, 661–671.

(14) Yuan, Y.; Gao, Y.; Mao, L.; Zhao, J. Optimisation of conditions for the preparation of β-carotene nanoemulsions using response surface methodology. *Food Chem.* **2008**, *107*, 1300–1306.

(15) Tan, C. P.; Nakajima, M. 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.* **2005**, *85*, 121–126.

(16) Galindo-Rodriguez, S.; Allemann, E.; Fessi, H.; Doelker, E. Physicochemical parameters associated with nanoparticle formation in the salting-out, emulsification-diffusion, and nanoprecipitation methods. *Pharm. Res.* **2004**, *21*, 1428–1439.

(17) Fessi, H.; Puisieux, F.; Devissaguet, J. P.; Ammoury, N.; Benita, S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. *Int. J. Pharm.* **1989**, *55*, R1–R4.

(18) Mosqueira, V. C.; Legrand, P.; Pinto-Alphandary, H.; Puisieux, F.; Barratt, G. Poly(D,L-lactide) nanocapsules prepared by a solvent displacement process: influence of the composition on physicochemical and structural properties. *J. Pharm. Sci.* **2000**, *89*, 614–626.

(19) Teixeira, M.; Alonso, M. J.; Pinto, M. M. M.; Barbosa, C. M. Development and characterization of PLGA nanospheres and nanocapsules containing xanthone and 3-methoxyxanthone. *Eur. J. Pharm. Biopharm.* **2005**, *59*, 491–500.

(20) Molpeceres, J.; Guzman, M.; Aberturas, M. R.; Chacon, M.; Berges, L. Application of central composite designs to the preparation of polycaprolactone nanoparticles by solvent displacement. *J. Pharm. Sci.* **1996**, *85*, 206–213.

(21) Alvarez-Roman, R.; Barré, G.; Guy, R. H.; Fessi, H. Biodegradable polymer nanocapsules containing a sunscreen agent: preparation and photoprotection. *Eur. J. Pharm. Biopharm.* **2001**, *52*, 191–195.

(22) Chu, B. S.; Ichikawa, S.; Kanafusa, S.; Nakajima, M. Preparation of protein-stabilized β-Carotene nanodispersions by emulsification−evaporation method. *J. Am. Oil Chem. Soc.* **2007**, *84*, 1053–1062.

(23) Chu, B. S.; Ichikawa, S.; Kanafusa, S.; Nakajima, M. Preparation and characterization of beta-carotene nanodispersions prepared by solvent displacement technique. *J. Agric. Food Chem.* **2007**, *55*, 6754–6760.

(24) Ribeiro, H. S.; Chu, B. S.; Ichikawa, S.; Nakajima, M. Preparation of nanodispersions containing β-carotene by solvent displacement method. *Food Hydrocolloids* **2008**, *22*, 12–17.

(25) Yin, L. J.; Chu, B. S.; Kobayashi, I.; Nakajima, M. Performance of selected emulsifiers and their combinations in the preparation of β-carotene nanodispersions. *Food Hydrocolloids* **2008**, *23*, 1617–1622.

(26) Jelen, H. H.; Wlazly, K.; Wasowicz, E.; Kaminski, E. Solid-phase microextraction for the analysis of some alcohols and esters in beer: comparison with static headspace method. *J. Agric. Food Chem.* **1998**, *46*, 1469–1473.

(27) Pinho, O.; Ferreira, I. M. P. L. V. O.; Santos, L. H. M. L. M. Method optimization by solid-phase microextraction in combination with gas chromatography with mass spectrometry for analysis of beer volatile fraction. *J. Chromatogr., A* **2006**, *1121*, 145–153.

(28) Lee, X. P.; Kumazawa, T.; Sato, K.; Seno, H.; Ishii, A.; Suzuki, O. Improved extraction of ethanol from human body fluids by headspace solid-phase microextraction with a carboxen−polydimethylsiloxane-coated fiber. *Chromatographia* **1998**, *47*, 593–595.

(29) De Martinis, B. S.; Martins Ruzzene, M. A.; Santos Martin, C. C. Determination of ethanol in human blood and urine by automated headspace solid-phase microextraction and capillary gas chromatography. *Anal. Chim. Acta* **2004**, *522*, 163–168.

(30) Bas, D.; Boyacı, İ.H. Modeling and optimization I: usability of response surface methodology. *J. Food Eng.* **2007**, *78*, 836–845.

(31) McClements, D. J. *Food Emulsion: Principles, Practices and Techniques*; CRC Press: London, U.K., 2005;

(32) Lo, S. K.; Cheong, N. Z.; Arifin, N.; Tan, C. P.; Long, K.; Yusoff, M. S.; Lai, O. M. Diacylglycerol and triacylglycerol as responses in a dual response surface-optimized process for diacylglycerol produc-

tion by lipase-catalyzed esterification in a pilot packed-bed enzyme reactor. *J. Agric. Food Chem.* **2007**, *55*, 5595–5603.
(33) Solans, C.; Izquierdo, P.; Nolla, J.; Azemar, N.; Garcia-Celma, M. J. Nano-emulsions. *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 102–110.
(34) Jafari, S. M.; He, Y.; Bhandari, B. Optimization of nano-emulsions production by microfluidization. *Eur. Food Res. Technol.* **2007**, *225*, 733–741.
(35) Dickinson, E. *An Introduction to Food Colloids*; Oxford University Press: Oxford, U.K., 1992; Vol. *1*.
(36) Taylor, P. Ostwald ripening in emulsions. *Adv. Colloid Interface Sci.* **1998**, *75*, 107–163.
(37) Riaz, M. N. Chaudry, M. M. *Halal Food Production*; CRC Press: Boca Raton, FL, 2004.
(38) Dickinson, E.; Golding, M. Influence of alcohol on stability of oil-in-water emulsions containing sodium caseinate. *J. Colloid Interface Sci.* **1998**, *197*, 133–141.

Received May 31, 2009. Revised manuscript received August 4, 2009. Accepted August 4, 2009. Financial support of this work by the Ministry of Science, Technology and Innovation of Malaysia through Science Fund (05-01-04-SF0384) is gratefully acknowledged.