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Ultrasonic Fabrication of Catechin Nanoemulsions from Green Tea with Enhanced Stability and Antioxidant Activity Optimized by Box-Behnken Design

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Abstract

Nanoemulsions are emulsions, either O/W (oil in water) or W/O (water in oil), with droplet diameters typically ranging from 50 to 1,000 nm. Research indicates that nanoemulsions are highly effective in enhancing the bioavailability, bioactivity, digestibility, stability, safety, quality and sensory attributes of food components. The study aimed to determine the optimum formulation and ultrasonic condition for fabricating catechin nanoemulsion (Ca-NE) from green tea with improved physical stability and antioxidant activity using response surface methodology. The effects of Medium Chain Triglyceride oil concentration (7.5 - 12.5 % w/w), Tween® 80 concentration (1 - 5 % w/w) and sonication time (5 - 15 min) on the droplet size, polydispersity index (PDI) and antioxidant activity presented by DPPH value of the Ca-NE were investigated using Box-Behnken design (BBD). The results found that droplet size and antioxidant activity of the Ca-NE were significantly ($p < 0.05$) affected by the proposed parameters, whereas the PDI value was slightly affected by those factors. The BBD results suggested that the Ca-NE fabrication condition was optimized with 7.5 % (w/w) oil concentration, 5 % (w/w) Tween® 80 concentration and a sonication time of 10.87 min. The optimum nanoemulsion showed good physical stability in terms of droplet size and PDI and had higher antioxidant activity than unencapsulated catechins when stored at 4 and 30 °C for 90 days. This study showed that catechin nanoemulsions fabricated by ultrasonication had good stability and antioxidant activity and, hence, had high potential for food and beverage applications in the food industry.
Keywords: Catechin, Nanoemulsions, Green tea, Ultrasonication, Box-Behnken design, Response surface methodology, Antioxidant

Introduction

Catechins are a group of polyphenolic compounds extensively contained in a variety of plants, especially tea leaves (*Camellia sinensis*). The major green tea catechins include (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (GC) and (−)-epicatechin (EC) [1,2]. These catechins, having strong antioxidant and free radical scavenging activity, are an essential bioactive ingredient widely used in the healthcare sector [3]. Catechins are susceptible to degradation mainly through autoxidation and epimerization during food processing and storage, as many factors affect their oxidation

and epimerization rate, such as temperature, pH, oxygen, metal ions and UV light [4]. Oxidation and epimerization are essential reactions that cause a decrease in the biological activity of catechins, resulting in the loss of their health benefits [5]. To overcome these issues, catechins in oil-in-water (O/W) nanoemulsions have been encapsulated to make catechins more stable [6]. Many researchers have suggested that nanoemulsions have higher stability, solubility and bioavailability due to their smaller particle size (< 200 nm) as compared to conventional emulsions [7,8].

Generally, nanoemulsion fabrication is divided into low-energy and high-energy techniques. High-energy approaches (e.g., ultrasonication, microfluidization and high-pressure homogenization) utilize high kinetic energy through mechanical instruments for size reduction of micro-droplets to nano-droplets of the emulsions [9,10]. Ultrasonication has gained interest in nanoemulsion fabrication due to its low production cost, higher energy efficiency, and lower instrumental requirements. Moreover, ultrasonication is considered an optimal technique that gives nanoemulsions higher stability, smaller droplet size and lower polydispersity index (PDI) compared to other high-energy techniques [11,12]. For ultrasonication, the ratio of the components of nanoemulsions, including oil, water and especially surfactants, significantly affects the quality and stability of the nanoemulsions [8,13,14]. Previous studies reported that sonication time and temperature have an essential impact on the stability of nanoemulsions [8,15]. The optimal compositions and processing conditions should be investigated to obtain the nanoemulsion with good stability and desirable functional properties.

Response Surface Methodology (RSM) is a practical statistical and mathematical approach that can be used to optimize multifactorial processes [16]. RSM can be used to study the relationships between 1 or more response variables and several independent variables by using univariate or multivariate models. It has been widely applied in such a way that the performance of the optimization of pharmaceutical formulation and food processing, as well as the fabrication of nanoemulsions, is successful [17-19]. Box-Behnken design (BBD) is one of the response surface designs based on 3-level incomplete factorial designs used for the second-order response surface model to obtain the optimum [20,21]. BBD is superior to other response surface designs (e.g., Central Composite designs, Doehlert matrix, 3-level complete factorial design) because it is not only effective in the optimization of variables with the least amount of experiments but also contributes to further analysis of the interactions between different variables [22].

Although nanoemulsions of catechins have been recently reported in the literature, such as catechin nanoencapsulation with enhanced antioxidant activity in high-pressure processed catechin-fortified coconut milk, optimizing the ultrasonic fabrication of catechin nanoemulsion using RSM-BBD has never been investigated. Information on the optimal condition of Ca-NE fabrication would benefit the food and pharmaceutical industry by allowing it to produce nanoemulsions with good stability and desirable quality practically. Therefore, this study aimed to investigate the optimum ultrasonic fabrication of Ca-NE by using RSM with BBD. The effects of independent variables, including oil concentration, surfactant concentration and sonication time, on droplet size, PDI and antioxidant activity of the Ca-NE were described in this study.

Materials and methods

Materials

Green tea catechins (Polyphenon 60) and Tween® 80 (polyoxyethylene sorbitan monooleate) were procured from Sigma-Aldrich Co. Ltd. (Stein Cheim, Germany). Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma-Aldrich Co. Ltd. (St Louis, MO, USA). Medium Chain Triglyceride (MCT) oil was obtained from Talent Co., Ltd. (Bangkok, Thailand). All the other chemicals used in the study were of analytical grade.

Optimization of the catechin nanoemulsions (Ca-NE) using RSM

The optimal oil concentration (MCT oil), surfactant concentration (Tween® 80) and sonication time of the Ca-NE formulation were investigated to obtain the Ca-NE with high stability and antioxidant activity. The RSM technique was used to determine the optimum Ca-NE formulation, which was fabricated by 7.5 - 12.5 % (w/w) MCT oil concentration, 1 - 5 % w/w Tween® 80 concentration, and 5 - 15 min sonication time. The effects of oil concentration (X_1), Tween® 80 concentration (X_2) and sonication time (X_3) on the 3 response variables, including droplet size (R1), PDI (R2) and DPPH value (R3), were investigated by RSM with BBD. The BBD with 3 independent variables and their combinations was used in this experiment to determine the optimum Ca-NE formulation to obtain the desirable droplet size, PDI and DPPH value. Independent variables, including oil concentration, Tween® 80 concentration and sonication time, were run at 3 levels in terms of BBD (**Table 1**) for each of the individual coded values (−1, 0 and 1). The BBD was performed to investigate the effect of each independent variable and the interaction between independent variables on the response. The quadratic regression model, Eq. (1), was used to explain the relationship between the independent variables and the response.

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 \quad (1)$$

where Y_i is the response function (i = 1, 2 and 3), a_0 is the constant, a_1, a_2 and a_3 are the linear coefficients, a_{11}, a_{22} and a_{33} are the quadratic coefficients and a_{12}, a_{13} and a_{23} are the interaction coefficients. The parameters' coded values (x_i) can be obtained using Eq. (2).

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (2)$$

where X_i is the actual value for the independent variable, X_0 is the actual value for the independent variable at the center point, and ΔX is the step of changing values for the independent variable.

RSM with 3 coded levels (−1, 0 and 1) was employed to examine the effects of MCT oil concentration (X_1: 7.5 - 12.5 % w/w), Tween® 80 concentration (X_2: 1 - 5 % w/w) and sonication time (X_3: 5 - 15 min) [23] on the droplet size (Y_1), PDI (Y_2) and DPPH value (Y_3) of the Ca-NE. Actual levels and coded factor levels of all 3 independent variables for optimizing the fabrication of the Ca-NE using RSM are shown in **Table 1**. **Table 2** shows the 3 coded levels, 3 independent variables and 20 experimental runs of the RSM with BBD used to optimize the Ca-NE formulation.

Table 1 Actual levels at coded factor levels of independent variables used in the RSM.

Symbol	Independent variable	Actual levels at coded factor levels		
		−1	**0**	**1**
X_1	MCT oil concentration (% w/w)	7.5	10	12.5
X_2	Tween® 80 concentration (% w/w)	1	3	5
X_3	Time (min)	5	10	15

Table 2 Response surface methodology design of different conditions.

Run	MCT oil (X_1)	Tween 80 (X_2)	Time (X_3)
1	10 (0)	3 (0)	10 (0)
2	10 (0)	3 (0)	10 (0)
3	10 (0)	3 (0)	10 (0)
4	7.5 (−1)	5 (1)	10 (0)
5	12.5 (1)	1 (−1)	10 (0)
6	10 (0)	1 (−1)	5 (−1)
7	10 (0)	3 (0)	5 (−1)
8	10 (0)	3 (0)	15 (1)
9	7.5 (−1)	1 (−1)	10 (0)
10	10 (0)	1 (−1)	10 (0)
11	12.5 (1)	5 (1)	5 (−1)
12	7.5 (−1)	3 (0)	15 (1)
13	12.5 (1)	5 (1)	10 (0)
14	12.5 (1)	3 (0)	15 (1)
15	10 (0)	5 (1)	10 (0)
16	7.5 (−1)	3 (0)	5 (−1)
17	10 (0)	5 (1)	15 (1)
18	10 (0)	5 (1)	5 (−1)
19	7.5 (−1)	5 (1)	15 (1)
20	12.5 (1)	3 (0)	10 (0)

The number in parentheses indicates the coded factor level of nanoemulsion formulation.

Preparation of the Ca-NE using ultrasonication

The aqueous phase was prepared by dissolving Tween® 80 (1 - 5 % w/w) and 0.4 % (w/w) Polyphenon 60 in deionized water. Coarse emulsions (100 g) were prepared by gradually dropping MCT oil (7.5 - 12.5 % w/w) into an aqueous phase at 25 °C using a rotor-stator homogenizer (Antrieb X10/25, Ystral GmbH Maschinenbau + Processtechnik, Germany) for 5 min at 16,000 rpm·min^{-1}. An ultrasonic processor (UP400S, Hielscher Ultrasonics GmbH, Germany) with a 22 mm diameter probe was used for ultrasonication. The coarse emulsion was sonicated at electric power, frequency and amplitude of 400 W, 24 kHz, and 50 %, respectively, with various sonication times (5 - 15 min). During the emulsification process, the sample was immersed in an ice bath (4 ± 2 °C) to avoid a sudden increase in temperature.

Measurement of droplet size and polydispersity index (PDI)

The droplet size and PDI of the Ca-NE were evaluated by dynamic light scattering with non-invasive backscattering using a particle size analyzer (Zetasizer Nano ZS90, Malvern Instruments Ltd., UK). The samples were diluted by deionized water at a 1:100 ratio to avoid multiple scattering effects [24]. The droplet size and PDI measurements were performed at 25 °C in triplicate. PDI can be calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) using Eq. (3).

$$PDI = \frac{M_w}{M_n} \quad (3)$$

where M_w is the mass average molecular weight, and M_n is the number-average weight.

Measurement of antioxidant activity

The antioxidant activity of the Ca-NE was evaluated by 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity [23]. Briefly, the nanoemulsions were diluted 50 times with deionized water. One hundred μL of diluted sample were mixed with 0.9 mL of 0.01 mM DPPH radical in methanol. The mixture was incubated in the dark for 30 min, and the absorbance was measured at 517 nm. The antioxidant activity was calculated as a percentage of DPPH radical scavenging activity using:

$$\text{DPPH (\% inhibition)} = \frac{A_c - A_s}{A_c} \times 100 \quad (4)$$

where A_s is the absorbance of the mixture of the sample, A_c is the absorbance of the mixture in which 100 μL distilled water was used instead of the Ca-NE.

Long-term storage stability

The stability of nanoemulsions (e.g., particle size, PDI) and retention of bioactive compounds within nanoemulsions have been related to the encapsulation process efficiency [13]. Since it was also hypothesized that encapsulation can preserve the bioactive compounds, the changes of antioxidant activity based on the DPPH method of Ca-NE was investigated in comparison to that of unencapsulated catechins. The particle size, PDI and antioxidant activity of the samples were measured under different storage intervals for a period of 90 days. The Ca-NE with 0.02 % (w/w) sodium azide as an antibacterial agent were kept in siliconized glass vials and stored at 4 and 30 °C under dark conditions. Unencapsulated catechins (0.4 % (w/w) catechin solution, pH 5.5) were also stored at the same conditions as nanoemulsions to investigate the efficiency of the protective barrier of emulsification. The antioxidant activity of the unencapsulated catechins was then analyzed and compared with those of the Ca-NE. Experiments were performed in triplicate.

Statistical data analysis

Multiple regressions analyzed experimental data to fit the second-order polynomial equation to all independent variables. Analysis of variance (ANOVA) was performed to evaluate significant differences between independent variables using IBM SPSS Statistics V22.0 (International Business Machines (IBM) Corporation, United States). To visualize the relationships between the responses and the independent variables, surface response plots of the fitted polynomial regression equations were generated using STATISTICA software (Version 7.0, Stat Soft Inc., Oklahoma, USA).

Results and discussion

Fitting the RSM model

RSM and BBD were used to optimize the oil concentration, Tween 80 concentration and sonication time. Analysis of variance (ANOVA) was used to determine the significance of the coefficient of the polynomial equations. A large F-value and a small *p*-value demonstrate a significant effect of each term [25]. The coefficient of multiple determination (R^2), the adjusted coefficient of multiple determinations (adjusted R^2) and the lack of fit indicate whether a regression model adequately represents experimental data. Smaller R^2 and adjusted R^2 values indicate that dependent variables are less relevant in explaining the variation of behavior [16,26]. The quadratic polynomial model for droplet size showed that it satisfactorily fit the model because the model was significant ($p < 0.01$), the lack-of-fit test was not significant ($p > 0.05$), and both R^2 and adjusted R^2 values were higher than 0.99. The variables with the largest to most miniature

effects on the droplet size were the linear effect of Tween® 80 concentration, linear effect of oil concentration, quadratic effect of Tween® 80 concentration, quadratic effect of sonication time and linear effect of sonication time, respectively. The interaction of all independent factors had no impact on the droplet size of the Ca-NE. Likewise, the quadratic polynomial model for antioxidant activity was highly efficient for fitting the data under the condition of experimental conditions with a significant effect ($p < 0.01$) on the model, no significant effect ($p > 0.05$) of lack-of-fit, and high R^2 and adjusted R^2 values which were higher than 0.96. The DPPH value was significantly ($p < 0.01$) affected by the linear effect of sonication time, the quadratic effect of sonication time and the interaction effect of oil concentration and Tween® 80 concentration, respectively. The second-order polynomial models' R^2 and adjusted R^2 values for PDI were 0.7708 and 0.7279, respectively, and the model was significant ($p < 0.01$). At the same time, the lack-of-fit test was not significant ($p > 0.05$), indicating that the model fits well. The linear effect of Tween® 80 concentration and sonication time significantly ($p < 0.05$) affected the PDI of the Ca-NE.

The regression coefficients of polynomial response surface models for droplet size, PDI and antioxidant activity are shown in **Table 4**. The results showed that increasing Tween® 80 concentration with a lower oil concentration and sonication time provided the Ca-NE with a smaller droplet size. The results suggested that a smaller PDI value can be obtained with a higher Tween® 80 concentration and shorter sonication time. Finally, higher antioxidant activity can be obtained at a lower sonication time.

Table 3 ANOVA of the regression coefficients of the fitted equations for droplet size (Y_1), polydispersity index (Y_2) and antioxidant activity (Y_3) of the Ca-NE.

Variable	Droplet size (Y_1)			PDI value (Y_2)			DPPH value (Y_3)		
	Mean square	**F-value**	***p*-value**	**Mean square**	**F-value**	***p*-value**	**Mean square**	**F-value**	***p*-value**
Main effect									
x_1	1316.61	760.85	< 0.0001	-	-	-	-	-	-
x_2	9591.63	5542.89	< 0.0001	0.0053	49.67	< 0.0001	-	-	-
x_3	25.49	14.73	0.0033	0.0032	2.82	0.0113	38.08	353.19	< 0.0001
Interaction effect									
x_1x_2	-	-	-	-	-	-	0.84	7.77	0.0092
x_1x_3	-	-	-	-	-	-	-	-	-
x_2x_3	-	-	-	-	-	-	-	-	-
Quadratic effect									
x_1^2	-	-	-	-	-	-	-	-	-
x_2^2	1103.80	637.87	< 0.0001						
x_3^2	93.02	53.76	< 0.0001				4.29	39.78	< 0.0001
Model	1898.25	1096.98	< 0.0001	0.0019	17.94	< 0.0001	5.73	53.11	< 0.0001
Lack of fit	58.09	0.5006	0.6895	< 0.0001	0.4380	0.7788	0.1265	0.5407	0.6643
R^2		0.9990			0.7708			0.9795	
Adjusted R^2		0.9981			0.7279			0.9611	

x_1 = coded value of oil concentration (% w/w), x_2 = coded value of Tween® 80 concentration (% w/w) and x_3 = coded value of sonication time (min).

Table 4 Model representing the equation of the droplet size and DPPH value of the Ca-NE.

Responses	Equations
Droplet size	Code: $Y = 168.25 + 11.06x_1 – 35.46x_2 – 1.52x_3 + 15.73x_2^2 + 5.21x_3^2$
	Actual: Droplet size $= 216.15 + 8.54X_1 – 41.17X_2 – 4.57X_3 + 3.93X_2^2 + 0.21X_3^2$
PDI value	Code: $Y = 0.20 – 0.022x_2 + 0.005x_3$
	Actual: PDI value $= 0.23 – 0.011X_2 + 0.001X_3$
DPPH	Code: $Y = 59.70 – 1.86x_3 – 0.45x_1x_2 – 1.12x_3^2$
	Actual: DPPH $= 59.31 + 0.63X_3 – 0.09X_1X_2 – 0.04X_3^2$

Effect of independent variables on response variables

Response surface graphs were drawn using the STATISTICA software to visualize the effects of oil concentration, surfactant concentration and sonication time on response variables. These graphs were generated by varying 2 independent variables under the experimental ranges (code −1, 0 and 1) and setting the remaining variable at a central point (code 0).

Droplet size

The surface plots of the 3-dimensional response for the droplet size of the Ca-NE are presented in **Figure 1**. The effects of oil concentration and Tween® 80 concentration on droplet size of the Ca-NE were shown in **Figure 1(a)**. At lower Tween® 80 concentration, an increase in droplet size was observed by increasing the oil concentration. The increase in size was attributed to insufficient surfactant to cover the small oil droplets produced during sonication, resulting in an initiation of the oil droplet coalescence [27]. **Figure 1(b)** illustrates the effects of oil concentration and sonication time on the droplet size of the Ca-NE. A decrease in the droplet size of the emulsions was observed by increasing sonication time at low oil concentrations, while big droplets were formed at higher oil concentrations. The increase in droplet size at higher levels of oil concentration and short sonication time may relate to the fact that the higher levels of oil concentration increased the viscosity of the emulsions [28]. Consequently, less shear force applied at a shorter sonication time was insufficient to reduce the size of oil droplets. From **Figures 1(a)** - **1(b)**, it can be concluded that the droplet size of emulsions increased with the increased oil content at lower surfactant concentration and sonication time. These results were consistent with the previous literature. The droplet size of nanoemulsions stabilized by Tween® 80 increased with increasing oil concentration [29]. The average droplet size of nanoemulsions with 25 - 53 % oil ranged from 84.75 to 131.37 nm. Similarly, regarding the effects of oil concentration on droplet size, it was found that the increase in the proportion of the oil phase of the emulsions led to an increase in droplet size of olive oil-based nanoemulsions stabilized by Tween® 80 [28].

There was a significant decrease in the droplet size with an increase in sonication time from 5 to approximately 11 min, whereas sonication time longer than 11 min led to a slight increase in droplet size of the Ca-NE. Increasing the sonication time tended to decrease the droplet size because it could generate more shear, shocks and severe cavitation, resulting in smaller particles and a homogeneous distribution [30]. On the contrary, a much longer sonication time led to a coalescence of emulsion droplets [31]. The time increment of ultrasonication may cause a rapid increase in the temperature of the emulsions, which is an important factor affecting the coalescence rate of emulsions [32]. In this study, although the temperature of the samples was controlled using an ice bath during the sonication, a gradual increase in temperature with increasing sonication time was observed. In this study, the temperature of the samples increased from

4.46 ± 0.56 to 31.43 ± 0.85, 42.37 ± 0.76 and 59.29 ± 0.86 °C, respectively, after 5, 10 and 15 min sonication. The increase in temperature up to 59.29 °C of the samples sonicated for 15 min led to a rapid coalescence of oil droplets. Similarly, the nanoemulsions prepared by a high-speed homogenizer were susceptible to droplet aggregation when the processing temperature of the nanoemulsions was above 50 °C [33].

Figure 1(c) illustrates the effect of time and Tween® 80 concentration on the droplet size of the Ca-NE. Surfactant concentration was the main effect on the droplet size of the Ca-NE. The results showed that the droplet size decreased with increasing surfactant concentration. Increasing surfactant concentration up to 3 and 5 % (w/w) reduced the droplet size by approximately 24.57 and 33.76 %, respectively, compared with the Ca-NE containing 1 % (w/w) Tween® 80. Larger droplets were observed in the samples with lower surfactant concentration, probably because insufficient surfactant covers the newly formed droplets [34]. As the surfactant concentration was increased, the droplet size decreased because the additional surfactant may be sufficient to envelope more of the oil-water interface, thus enabling small droplets to be formed [34]. These results were by those from Gadkari *et al.* [6] who reported that by increasing Tween® 80 from 0.5 to 9 % (w/v), the droplet size of green tea catechins nanoemulsions was reduced from 900 to 121 nm. It should be noted that the droplet size may increase if the emulsions contain an intense concentration of surfactant due to excessive coverage of crystalline particles by surfactant [28].

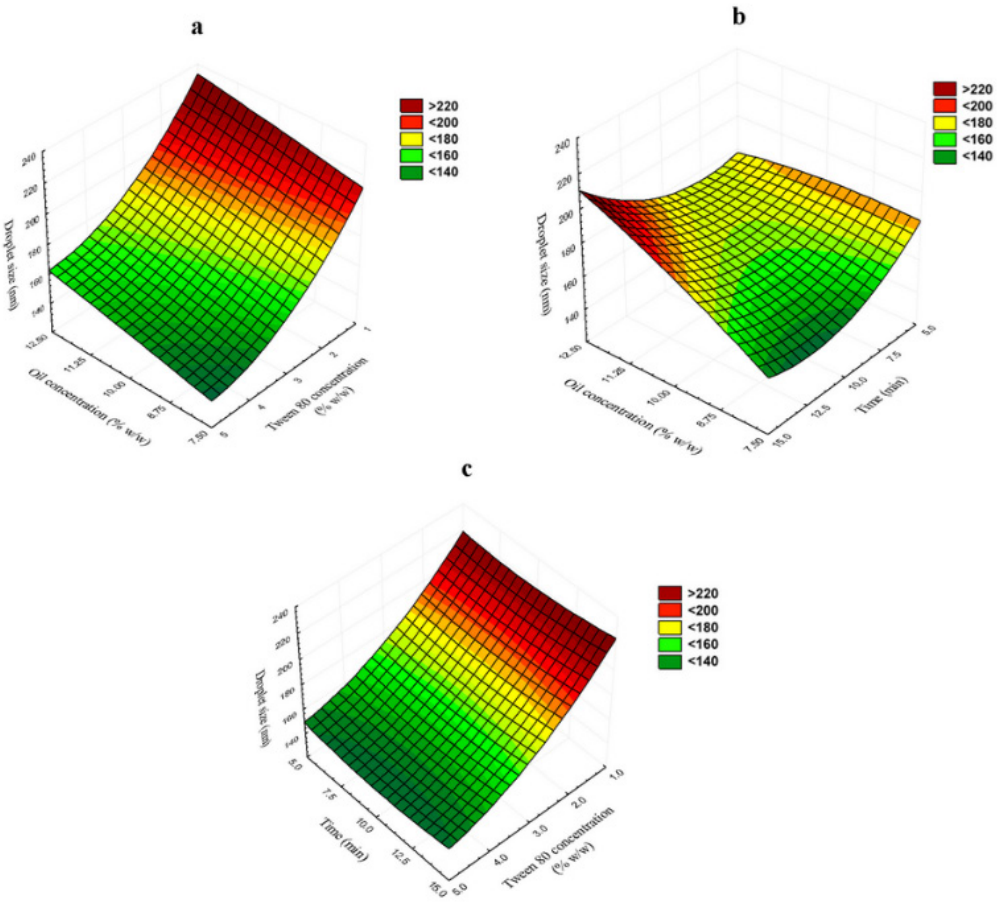

Figure 1 Response surface of the interactions between MCT oil concentration and Tween® 80 concentration (a), time and Tween® 80 concentration (b) and time and Tween® 80 concentration (c) on the droplet size of the Ca-NE.

PDI

PDI, as a measure of the width of the particle size distribution, is one of the important parameters to evaluate nanoemulsion stability. PDI value is usually reported in a range from 0 to1. PDI values less than 0.05 indicate nearly monodisperse sample while values greater than 0.7 are polydisperse sample with a very broad-size distribution [35]. Generally, PDI values of 0.2 and below are most commonly considered acceptable in practice for nanoparticle materials [36]. The PDI values of all samples were low ranging from 0.168 to 0.244. The narrow particle size indicated that the nanoemulsion fabrication using ultrasonication in this study is highly efficient.

The effects of independent variables on PDI value of the Ca-NE are presented as 3-dimensional response plots in **Figure 2**. The effect of oil and surfactant concentrations on the PDI of the Ca-NE is shown in **Figure 2(a)**. The 3D plot showed that the PDI decreased with increasing Tween® 80. In contrast, the PDI was relatively constant even when changing the oil concentration. For this reason, the more significant surfactant may increase the formation of a homogenous nanoparticle. This result was similar to the data reported by Pongsumpun *et al.* [8]. The effect of oil concentration and sonication time on the PDI of the Ca-NE is shown in **Figure 2(b)**. The 3D plot showed that increasing the sonication time resulted in a slightly increasing PDI value. This is probably because the longer sonication time could lead to the heterogeneity of the emulsion, resulting in an increase in the PDI value. The results in **Figure 2(b)** also showed that the oil concentration did not affect the PDI value of the Ca-NE. **Figure 2(c)** shows the effect of surfactant concentration and sonication time on the PDI of the Ca-NE. Increasing Tween® 80 concentration decreased the PDI of the Ca-NE significantly, whereas increasing the sonication time slightly increased the PDI value.

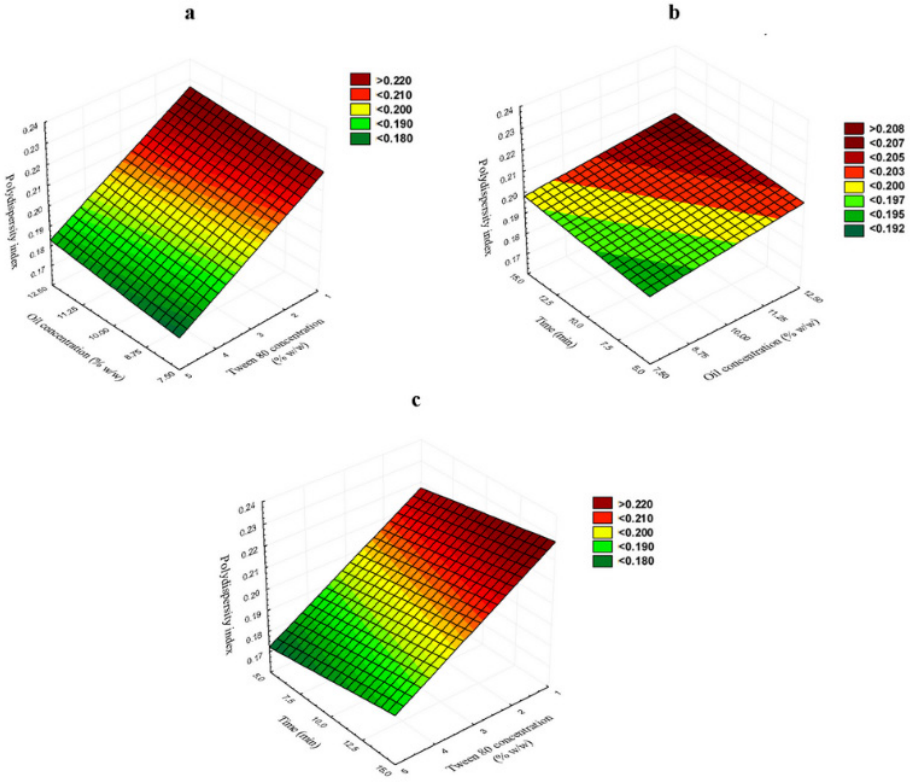

Figure 2 Response surface of the interactions between MCT oil concentration and Tween® 80 concentration (a), Tween® 80 concentration and time (b) and Tween® 80 concentration and time (c) on the PDI value of the Ca-NE.

Antioxidant activity

The antioxidant activity of the Ca-NE is associated with its ability to donate electrons to quench the free radical. Antioxidant activity of the Ca-NE was evaluated by DPPH assays, which provide information on the reactivity of tested compounds with a free radical and expressed as % (inhibition).

The interactive effect of oil concentration and surfactant concentration on the DPPH value of the Ca-NE is shown in **Figure 3(a)**. The 3D plot shows that the DPPH value of the Ca-NE decreased with increasing oil concentration (> 10 % w/w) and decreasing surfactant concentration (< 3 % w/w). It may be related to the protective barrier of emulsification. The antioxidant activity expressed as DPPH radical scavenging activity is related to the amount of the remaining catechins in the Ca-NE. The degradation of catechins in the Ca-NE may be caused by heat, light, oxygen and free radicals produced during ultrasonication, leading to a reduction of antioxidant activity. Conditions of high oil concentration and low surfactant concentration produced large oil droplets that were unstable and had low efficiency in the protective barrier [29]. Catechins are, therefore, easily destroyed by various oxidative stressors during the sonication. These results are in agreement with a previous work that studied the antioxidant activity and bioaccessibility of size-different nanoemulsions of lycopene-enriched tomato extract and found that the protectability of lycopene in smaller droplets of nanoemulsion was relatively better than that in the larger droplets [37]. In contrast, our finding did not agree with Zheng *et al.* [38], who reported that emulsions containing higher oil content had higher DPPH radical scavenging activity probably because the antioxidants in the oil may have higher local concentration in bigger droplets than smaller droplets. They were also more distributed at the relative interface of the bigger particles. However, reports about the particle size effect on antioxidant activity and oxidative stability of emulsions are frequently contradictory, depending on many factors, such as surfactant-to-oil ratio [39].

Figure 3(b) shows the effects of oil concentration and sonication time on the DPPH value, which explains that sonication time significantly ($p < 0.05$) affected the DPPH value of the Ca-NE. The DPPH value showed a downward trend with the passage of sonication time. The decrease in DPPH values may be caused by an increase in the temperature of the samples during the sonication. Catechins are highly sensitive to temperature, and their degradation rate is faster at higher temperatures [40]. Besides, an increase in temperature during the sonication can accelerate the formation of free radicals, which could react with the antioxidants in the emulsions, and thus, fewer antioxidants were available to scavenge DPPH radicals [38]. The effects of Tween® 80 concentration and sonication time on the DPPH value are shown in **Figure 3(c)**. The DPPH value decreased with increasing sonication time, whereas the DPPH value slightly increased with increasing Tween® 80 concentration. The decrease in the DPPH is probably because catechins were degraded by heat generated during the sonication.

Optimization of condition for nanoemulsions fabrication

The RSM was used to optimize the CaNE fabrication by finding the optimal oil concentration, Tween® 80 concentration and sonication time that provide the most desirable Ca-NE with minimum droplet size, PDI value and DPPH value. Eight different solutions were found that contained different levels of independent variables. As shown in **Table 5**, the optimum oil concentrations, Tween® 80 concentration and sonication time to provide the Ca-Ne with maximum desirability value were 7.5 % (w/w), 5 % (w/w), 10.87 min, respectively. The values of droplet size, PDI value and DPPH value for Ca-NE are 136.35 ± 2.42 nm, 0.183 ± 0.023 and 60.50 ± 0.98 %, respectively.

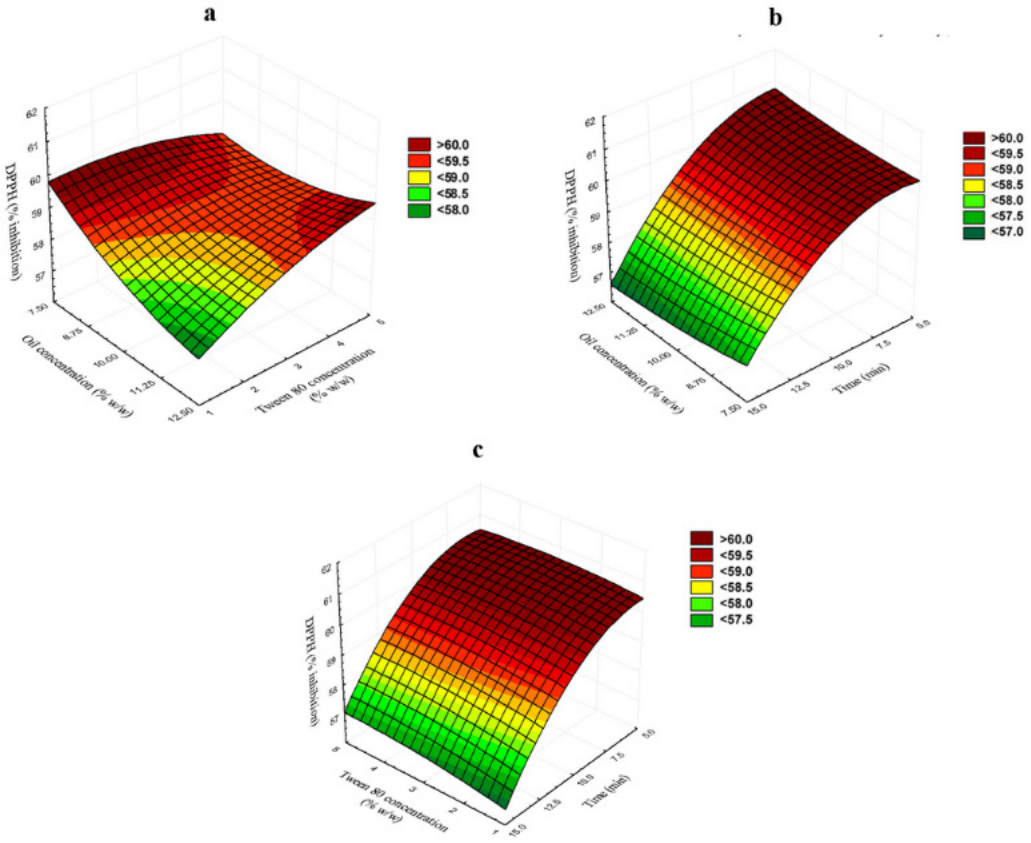

Figure 3 Response surface of the interactions between MCT oil concentration and Tween® 80 concentration (a), Tween® 80 concentration and time (b) and Tween® 80 concentration and time (c) on the DPPH value of the Ca-NE.

Verification of the predicted model

To verify the accuracy of the obtained optimal nanoemulsion fabrication, the model's accuracy was validated with experiments under the optimum conditions. The conditions used in the confirmatory experiment were 7.5 % oil concentration, 5 % Tween® 80 concentration and a sonication time of 10.87 min. The predicted response values at optimum condition levels were 135.98 nm droplet size, 0.176 PDI value and 60.33 % DPPH value. The actual response values for droplet size, PDI and DPPH were 136.35 ± 2.42 nm, 0.183 ± 0.023 and 60.50 ± 0.98 %, respectively. The results showed that the experimental response values agreed well with the predicted response values calculated using the RSM-BBD. The use of ultrasonic fabrication resulted in a smaller droplet size compared to the high shear homogenizer method, achieving a droplet size of 185.6 nm [11].

Table 5 Comparison of predicted and experimental response values of optimized conditions for the Ca-NE fabrication.

Optimum conditions	Coded levels	Actual levels
MCT oil concentration (% w/w)	-1	7.5
Tween® 80 concentration (% w/w)	1	5
Sonication time (min)	0.174	10.87
Response	**Predicted values**	**Experimental values**
Droplet size (nm)	135.98	136.35 ± 2.42
PDI	0.176	0.183 ± 0.023
DPPH (%)	60.33	60.50 ± 0.98

Long term storage stability

Droplet size and PDI

The physical stability of the Ca-NE was tested by evaluating the changes in its droplet size and the PDI at various temperatures (4 and 30 °C) and storage times (0, 30, 60 and 90 days). The changes in droplet size of the Ca-NE stored at 4 and 30 °C were shown in **Figure 4(a)**. The droplet size of the Ca-NE stored at both 4 and 30 °C showed an upward trend during the 90-day storage period. The droplet size of the samples stored at 4 and 30 °C gradually increased from 136.4 ± 2.42 to 144.7 ± 3.32 and 152.6 ± 4.01, respectively, at the end of storage. The droplet size of the Ca-NE significantly ($p < 0.05$) increased from the initial time when stored up to 90 and 60 days at 4 and 30 °C, respectively. During storage, Brownian motion and gravitational motion of the dispersed phase instigate collisions among the emulsion droplets, which can cause coalescence or agglomeration, increasing the size of the dispersed phase over time [41]. The droplet sizes of the samples stored at 30 °C were more significant than those stored at 4 °C, probably because the coalescence rate of the emulsion droplets at higher temperatures was higher than at lower temperatures [8]. However, the droplet size of the Ca-NE was less than 200 nm and thus is categorized as nanoemulsions typically in the range of 20 - 200 nm [42]. Moreover, no creaming, which is the movement of oil droplets to form a concentrated cream layer at the top of the emulsion, was observed during storage of 90 days, suggesting that the Ca-NE was relatively stable against the tested storage temperatures and times. Literature has reported that the creaming nanoemulsion is usually observed when the oil droplets exceed 200 nm [43].

The PDI values of the Ca-NE stored at 4 and 30 °C for 90 days were shown in **Figure 4(b)**. The PDI values of all samples slightly increased during storage of 90 days. The PDI values of the Ca-NE stored at 4 and 30 °C increased from 0.183 ± 0.024 to 0.189 ± 0.017 and 0.198 ± 0.019, respectively. However, nanoemulsions with a PDI value of less than 0.2 are considered to have a narrow distribution [7,8], indicating that the Ca-NE had good stability under the tested storage temperatures and times.

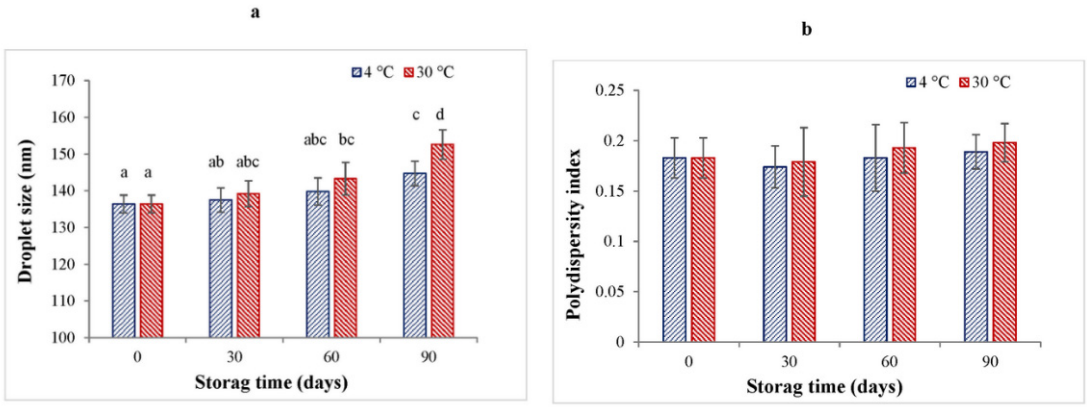

Figure 4 Physical parameters of the Ca-NE stored at 4 and 30 °C for 90 days: (a) droplet size and (b) PDI.

Antioxidant activity

In this study, it was hypothesized that nanoemulsion was an important tool to preserve the stability of bioactive compounds. Therefore, the changes in antioxidant activity of the developed Ca-NE in comparison to that of unencapsulated catechins were monitored during storage at various times and temperatures. The antioxidant activity of the Ca-NE was compared with unencapsulated catechins to investigate the efficiency of the protective barrier of emulsification. **Figure 5** represents the DPPH values of the Ca-NE and unencapsulated catechins stored at 4 and 30 °C for 90 days. The antioxidant activity of all samples significantly ($p < 0.05$) decreased by increasing storage time. The results showed that when the Ca-NE were stored at 4 and 30 °C, the DPPH values decreased from 60.53 ± 0.90 to 49.08 ± 0.86 and 43.45 ± 1.24 %, respectively. It suggested that the degradation rate of catechins increased by increasing the storage temperature [6,38].

The decrease in DPPH values of unencapsulated catechins was more significant than that of the Ca-NE when compared at the same storage temperature. At the end of storage time, DPPH values of the Ca-NE stored at 4 and 30 °C were lost by 18.91 and 28.22 %, respectively, whereas the DPPH values of unencapsulated catechins stored at 4 and 30 °C were lost by 25.99 and 36.89 %, respectively. These results showed good retention of the antioxidant activity of the bioactive compounds from the Ca-NE. The ultrasonic fabrication of Ca-NE results in better retention of antioxidant activity than unencapsulated catechins.

The nanoemulsion encapsulation provided a protective barrier to the catechin during storage. These results agreed well with those of Gadkari *et al.* [6], who studied the effect of temperature on the changes in catechins in green tea contained in nanoemulsions and found a significant reduction in polyphenol content and antioxidant activity of green tea catechin solution after heat treatment when compared to those of catechin nanoemulsions.

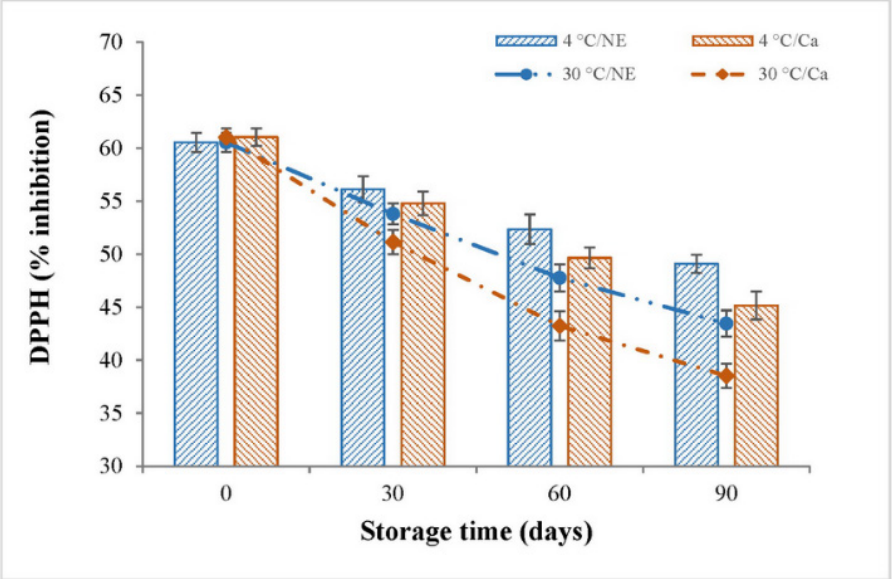

Figure 5 Antioxidant activity of the Ca-NE and unencapsulated catechins stored at 4 and 30 °C for 90 days; (NE = Ca-NE, Ca = Unencapsulated catechins).

Conclusions

This study showed that RSM and BBD can be successfully used to optimize the fabrication of nanoemulsion enriched with catechins. The second-order polynomial model was sufficient to predict and describe the responses of the Ca-NE's droplet size, PDI and antioxidant activity about the changes in emulsifying conditions (oil concentrations, surfactant concentrations and sonication time) within the given experimental ranges. The optimum condition, which gave the minimum droplet size, minimum PDI value and maximum DPPH value, were 7.5 % (w/w) oil concentration, 5 % (w/w), Tween 80 concentration and 10.87 min sonication. The optimum nanoemulsion had good physical stability regarding droplet size and PDI when stored at 4 and 30 °C for 90 days. The antioxidant activity loss of the Ca-NE stored at lower temperatures was less than that of the sample stored at higher temperatures. The ultrasonic fabrication of Ca-NE results in better retention of antioxidant activity than unencapsulated catechins. The ultrasonic fabrication provided the Ca-NE with good stability and high antioxidant activity. Moreover, the fabricated nanoemulsion had good stability and preservation of catechins during storage. The results suggest that the obtained nanoemulsion of catechins has great potential in food or pharmaceutical applications.

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