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Preparation and Characterization of Nanostructured Lipid Carriers (NLCs) Formulated with Palm Kernel Stearin and Rice Bran Oil as Squalene Carriers Via Ultrasonication

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ABSTRACT

This study aimed to optimize the synthesis of squalene-loaded nanostructured lipid carriers (NLCs) using a mixture of palm kernel stearin and rice bran oil as the lipid matrix and Tween 80 as the surfactant. NLCs were prepared via ultrasonication. Optimization was performed using Box–Behnken Design response surface methodology. Ultrasonication parameters assessed included temperature, time, and amplitude. The effect of optimization on particle size (X1), polydispersity index (X2), zeta potential (X3), and encapsulation efficiency (X4) was determined. Nine tests were conducted on squalene-loaded NLCs and control samples over 28 days of storage. These NLCs were prepared at an ultrasonication time of 24.8 min amplitude of 80, and temperature of 43.7°C. NLC characteristics were evaluated before and after storage for 28 days at room temperature, which resulted in particle size of 38.6 and 40.3 nm, polydispersity index (PDI) of 0.282 and 0.213, zeta potential of −34.1 and −34.8 mV, and encapsulation efficiency of 85.69% and 84.04%, respectively; empty NLCs had a particle size of 37.4 and 38.5 nm, PDI of 0.240 and 0.276, and zeta potential of −41.5 and −32.9 mV.

Keywords: nanostructured lipid carriers, palm kernel stearin, rice bran oil, squalene, ultrasonication

INTRODUCTION

Natural bioactive compounds, such as vitamin E, phytosterols, and squalene, can effectively eliminate free radicals. Squalene is often used clinically in daily doses as a detoxifier, antioxidant, and moisturizer and a bactericidal, fungicidal, and antistatic agent in cosmetic and pharmaceutical preparations. However, ≥40% of natural compounds are estimated to have low solubility in water and high toxicity (Ramadon & Mun'im, 2016). A previous study (Gao et al, 2021) demonstrated the use of squalene as a promising adjuvant for an ovalbumin vaccine by modifying this in a chitosan-based nanostructured lipid carrier (NLC). The challenges associated with leveraging the potential of the food sector and the unmet need for advanced delivery systems, preferably lipid-mediated, can be met by delivering bioactive materials through incomplete lipid matrix systems with high-loading capacity (Banerjee & Pillai, 2019).

NLCs are cost-effective, modified solid lipid nanoparticles in which the lipid core contains both solid and liquid lipids with a poorly ordered crystalline structure or an amorphous solid structure at body temperature while the surfactant used is a generally recognized as safe material (Barabinta-Patrascu et al, 2014). Solid lipids first form crystals on the surface of the particle, and then liquid lipids are concentrated in the core of the particle together with the active ingredient to increase the stability of this biomaterial. The difference in the melting point between solid and liquid lipids is an important point in the selection of lipids in the NLC system and affects the crystallization process, which is directly related to the formation of a solid state on the surface of NLC particles when the temperature decreases. Many studies have shown that NLC improves encapsulation efficiency (EE), which is a valuable option for improving chemical stability, bioavailability, and controlled release of lipophilic compounds. Among their advantages, NLCs can

maintain stability over time because of their smallest size. The development of NLC manufacturing in recent years has achieved considerable progress because of the large application potential. Multiple methods have been proposed to form NLCs from various types of mixed materials. One method that is often used in making NLCs is high shear homogenization followed by sonication to produce nanoparticles that are then centrifuged (Chaudhari et al, 2021). However, the NLC manufacturing process is time-consuming and requires various methods. Ultrasonication is often used in preparing NLCs and is a fast and reproducible process that can be controlled via several parameters, such as time, temperature, and ultrasonication power. Moreover, ultrasonication is a practical technique that can minimize sample damage and be used for high-scale production (Gonzalez-Mira et al, 2011).

Ultrasonic-assisted extraction (UAE) is an extraction method that uses the principle of acoustic cavitation to produce spontaneous bubbles (cavitation) in the liquid phase below the boiling point. This subsequently damages cell walls so that the solvent can enter the biological material. UAE has advantages over the maceration extraction method as it can increase the penetration of a solvent into cell walls (Kanifah, 2015), afford faster mass transfer rate (Hartuti et al, 2013), and increases extraction results under low temperatures and short periods and using minimal solvent volumes (Dey & Rathod, 2013).

Therefore, this study aimed to determine the optimum ultrasonication method for NLCs prepared using a combination of palm stearin and rice bran oils as the squalene carrier and to determine their characteristics and stability during storage at room temperature for up to 28 days.

MATERIALS AND METHODS

Materials

Squalene was obtained from PhytoGaia Sdn. Bhd., (Kuala Lumpur, Malaysia), rice bran oil from Kasisuri Co. (Thailand), palm kernel stearin from PT. Wilmart Cahaya (Indonesia), and Tween 80 from KgaA, 64271 (Darmstadt, Germany). Equipment used included a Hielscher ultrasonicator (UP 200St, Teltow, Germany), dynamic light scatterer (Nano ZS, Malvern Instruments, UK), Genesys 10s UV-VIS spectrophotometer (Germany, France), transmission electron microscope (JEOL JEM-1400 Flash, New England, USA), hot plate, magnetic stirrer, and Shimadzu AUW 220 analytical balance.

Preparation of squalene-loaded NLCs

Squalene-loaded NLCs were prepared via sonication. In brief, the lipid phase comprising rice bran oil and palm kernel stearin as solid and liquid lipids, respectively, was melted at 60°C, and 0.5 mL (%w/w) of squalene was dissolved in the liquid lipid mixture. The surfactant phase of Tween 80 was heated until this reached the same temperature as the lipid phase. The lipid solution was poured into the hot surfactant phase and homogenized using a magnetic stirrer for 20 min. NLC was then sonicated at specified amplitudes, times, and temperatures.

Determination of NLC formulation

The formulation was determined based on the results of trials conducted by researchers by looking at the appearance of the NLC that had been made. The formulation used was a water:lipid ratio of 7:3 or 8:2 with a lipid:surfactant ratio of 1:3 or 1:3.5. Based on the experimental results, the formulation used in this research used a ratio of distilled water:lipid and surfactant (80:20) and a ratio of lipid:surfactant (1:3.5). The formulation used in this research was 80 g of aquabidest, 15.56 g of surfactant (in this case Tween 80), 1.74 g of rice bran oil as a liquid lipid, and 2.64 g of palm kernel stearin as a solid lipid.

Table I. Variables and their levels for the Box–Behnken Design

Variables, units	Factor	Levels		
	X	-1	0	1
Independent Variable				
Temperature (°C)	A	35	50	65
Time (min)	B	10	20	30
Amplitude	C	40	60	80
Dependent Variable				
PS (nm)	X1			Min
PDI	X2			Min
ZP (mV)	X3			Max
EE (%)	X4			Max

Note: particle size (PS), polydispersity index (PDI), zeta potential (PZ), and EE.

Determination of factors

The factors evaluated in this study were temperature, amplitude, and ultrasonication time. These factors were selected via screening and based on published studies. Screening determined the required ultrasonication time, while temperature and amplitude were selected based on previous studies (Table I).

Particle size, polydispersity index, and zeta potential

PS, PDI, and PZ were determined using dynamic light scattering by diluting the sample 10-fold with aquabidest and then stirring at 200 rpm at room temperature for 5 min before measurement (Lv et al, 2016).

Encapsulation efficiency (EE)

Squalene-loaded NLC (3 mL) was centrifuged at 10,000 rpm for 30 min at 25°C. The water phase was separated from the lipid phase, diluted again in ethanol, and then analyzed using a Genesys 10s UV-VIS spectrophotometer at 454 nm. A calibration curve with a linear regression equation ($y = 6.566x - 0.1625$; $R^2 = 0.9851$) was prepared for squalene quantification, where the x and y values were the concentration (mcg mL^{-1}) and absorption values, respectively (Rohmah et al, 2020).

Free fatty acid value

The amount of free fatty acids was measured using the AOAC method. A 5-mL volume of sample was placed in an Erlenmeyer flask, and then 50 mL of 95% ethanol was added and stirred until the solution was homogeneous. Then, 3–5 drops of 1% phenolphthalein indicator were added and a standardized 0.1 N NaOH solution was titrated until the solution turned pink. The results of the free fatty acid value were calculated using the formula:

Peroxide value

A standard curve was prepared for $FeCl_3$ by performing a series of four $FeCl_3$ dilutions to obtain a linear regression equation to calculate the POV value.

The blank solution was produced by mixing 1 mL of chloroform: methanol (7:1) into a test tube, then adding 9.8 mL of chloroform:methanol (7:3) and vortexing for 2–4 s. Then, 50 µL of ammonium thiocyanate was added and vortexed for 2–4 s followed by 50 µL of $FeCl_2$ with vortexing for 2–4 s. The solution was incubated in the dark for 5 min, and then the absorbance was measured at 500 nm using a spectrophotometer (Genesys 10s UV-VIS).

Sample analysis was performed using 0.05 g of the extracted oil diluted with 0.95 mL of chloroform:methanol (7:3). Then, 0.1 mL of this solution was added to chloroform:methanol (7:3) to a volume of 1 mL, which was then further diluted with 9.8 mL of chloroform:methanol (7:3) and vortexed for 2–4 s. Ammonium thiocyanate (50 µL) was then added, and the solution was vortexed for 2–4 s. Finally, 5 µL of $FeCl_2$ was added and vortexed for 2–4 s. The sample was incubated for 5 min in the dark, and then the absorbance was measured at 500 nm (IDF, 1991). The peroxide value was then calculated using the formula:

$$POV = \frac{(As - Ab)\ x\ \left(\frac{1}{slope}\right) x\ FP}{55.84\ x\ g\ sample\ x\ 2}$$

As is absorbance of sample, Ab is absorbance of blank, FP is dilution factor, and slope is obtained from linear regression equation of the standard curve.

Transmission electron microscopy

The morphology of NLC-TT was observed via transmission electron microscopy (TEM) by diluting the sample 10-fold with aquabidest. Next, one drop of the diluted sample was placed in a 200-mesh copper grid coated with carbon film and left for 3 min. One drop of contrast dye containing uranyl acetate was then added to the sample and left for 2 min. The copper grid was inserted into the specimen holder, which was inserted into a transmission electron microscope (1400 Flash TEM-JEOL USA) (Barri et al, 2023).

Anisidine value

A blank solution was prepared by adding 5 mL of isooctane solution to 1 mL of p-anisidine reagent and then homogenized via vortexing. The solution was then left for 10 min in the dark.

The sample solution was prepared by dissolving 1 mL of the extracted oil with isooctane in a 25-mL measuring flask. A 5-mL aliquot of the dissolved sample was homogenized with 1 mL of p-anisidine reagent. The sample was left for 10 min in the dark, and then the absorbance of the blank (Ab) and sample (As) solutions were determined at 350 nm using a spectrophotometer (AOCS, 1998).

Oxidation studies

Samples (0.2 mL) were added to a mixed solvent of isooctane/2-propanol (1.6 mL, 3:1 v/v) and stirred vigorously (10 s, three times) followed by centrifugation at 3400 ×g for 10 min. Then, 0.2 mL of the extract supernatant was then carefully removed and mixed with a solvent of 2.8 mL methanol/1-butanol (2:1, v/v) (Huang et al, 2017).

Table II. BBD matrix and experimental responses

Run	A	B	C	X1	X2	X3	X4
1	0	0	0	73.5	0.422	-34.3	82.2
2	-1	-1	0	112.0	0.479	-38.6	82.1
3	1	1	0	117.9	0.246	-42.0	82.3
4	0	0	0	62.4	0.432	-23.9	82.6
5	0	-1	1	135.4	0.264	-32.8	82.6
6	0	1	1	61.0	0.205	-30.9	85.2
7	0	0	0	58.0	0.351	-24.0	87.2
8	0	0	0	58.8	0.390	-30.4	85.9
9	0	1	-1	52.4	0.464	-35.1	83.8
10	1	-1	0	134.8	0.315	-36.5	83.0
11	1	0	1	124.6	0.387	-41.0	82.3
12	0	0	0	67.9	0.397	-31.0	83.4
13	1	0	-1	139.7	0.228	-25.0	82.1
14	-1	1	0	103.2	0.556	-37.9	83.5
15	-1	0	1	47.8	0.334	-29.3	84.1
16	0	-1	-1	93.5	0.337	-40.5	82.9
17	-1	0	-1	147.9	0.245	-33.7	83.9

Data are expressed as mean (n = 2). A: Temperature (°C); B: Time (min); C: Amplitude (%); X1: PS (nm); X2: PDI; X3: PZ (mV); and X4: EE (%)

Experimental design

A Box–Behnken experimental design (BBD) was used, and a three-level and three-factor experimental model with 17 processes was selected to optimize the formulation procedure. Extraction amplitude, time, and temperature were selected as independent variables for optimization. The dependent variables of this study included PS, PDI, PZ, and EE.

Statistical analysis

Data was analyzed using Design Expert® software, version 7.1.5 (Stat-Ease Inc., Minneapolis, MN, USA). Models, including intercepts, primary effects, and interactions for all response variables, were generated using a multiple nonlinear regression analysis approach. Each model was evaluated based on several statistical parameters, including the coefficient of determination (R2), adjusted coefficient of determination (Adj. R2), predicted coefficient of determination (Pred. R2), and adequate precision (Adeq. Prec). The influence of significant factors on the response was determined by the F test or ANOVA p-value, with a confidence level of 95% (p = 0.05).

RESULTS AND DISCUSSION

Effect of ultrasonication on squalene

Squalene is not damaged when exposed to waves produced by ultrasonication as confirmed by Kristina et al (2021) who showed that the time for which a sample is exposed to sonication has no effect on the sample value. In the present study, samples containing squalene were exposed to ultrasound for 5, 10, 15, 20, 25, and 30 min at 40 KHz.

BBD-response surface methodology optimization

Stable NLCs were analyzed for PS, PDI, PZ, and EE (Table II). This showed that longer sonication times affected the size of NLC particles. This result is consistent with previous results (Abbas et al, 2020), where a decrease in PS was observed with the increased sonication time due to particle decomposition.

No significant effect was found in EE or PDI with changes in factors ($p > 0.05$). PDI was directly related to PS of NLCs and ranged from 0.205 to 0.556, indicating a monodisperse distribution. Long sonication times produced a low increase in PDI values, although this was not significant ($p < 0.05$) (Figure 1 (e, f)). A prior study (Shete et al, 2023) stated that the increase in PDI values due to longer sonication times was caused by the aggregation of larger particles into particles of relatively smaller size due to the coalescence phenomenon, which dominates the particle breakdown process (Souza et al, 2019). Statistical analysis revealed significant changes in PS with changes in temperature.

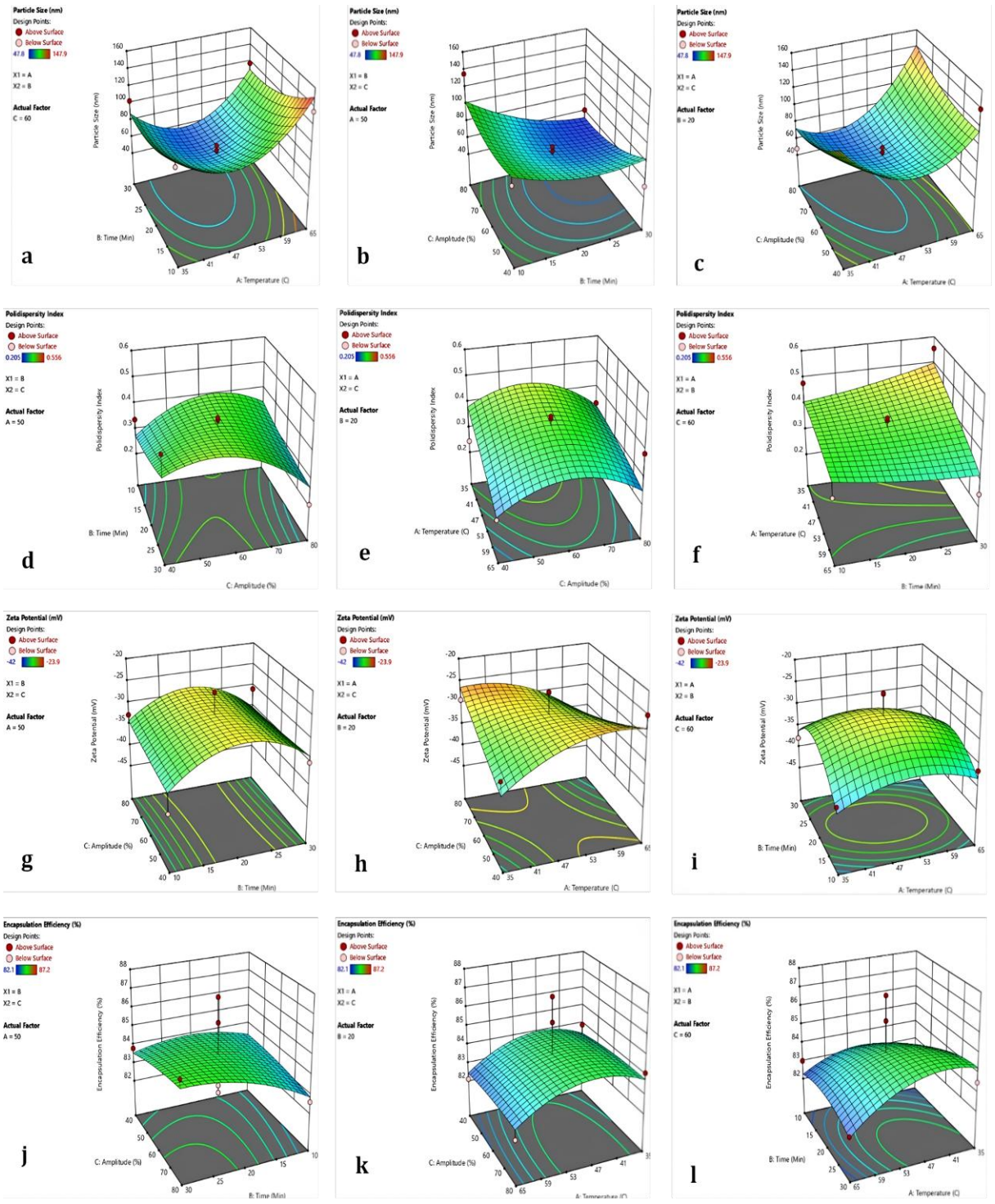

Figure 1. 3D surface plot showing the influence of independent variables on (A) PS (a–c), (B) PDI (d–f), (C) ZP (g–i), and (D) % EE (j–l)

EE analysis indicated the ability of lipids to encapsulate biomaterials. Encapsulation plays an important role in improving the function of bioactive compounds. The EE value in this study ranged from 82.1 to 87.2. The presence of hydrophobic compounds, such as squalene, with high solubility in oil can affect the EE value. This is similar to previous results (Poonia et al, 2016), which showed a higher lipid concentration with a higher proportion of bioactive components.

A prior study showed that PZ values ≥ 30 mV indicate the stability of the nanosuspension system (Patel & Agrawal 2011). Herein, NLCs were composed of lipids and nonionic surfactants, where the surface charge of NLCs was relatively low (−30mV). In addition, the low negative charge of NLCs was because of the presence of free fatty acids within the lipids (Rahman et al, 2013). Herein, The observed PZs ranged from −42 to −23.9. Rahman et al (2019) reported that NLC compounds had no significant effect on PZ (Table II).

Optimal factor levels were determined using expert Design software. Based on the response surface methodology (RSM-BBD) optimization results, the best optimization values for temperature, time, and amplitude were obtained for the response in PS, PDI, ZP, and EE (Table III).

Table III. RSM-BBD optimization results

Temperature(°C)	43.7
Time(min)	24.8
Amplitude (%)	80
Particle size (nm)	49.1
Polydispersity Index	0.303
Zeta Potential (mV)	-28.2
Encapsulation Efficiency (%)	84.8
Desirability	0.732
Selected	

Physicochemical characterization

After identifying the optimal conditions via RSM-BBD testing, physicochemical testing was conducted on samples over a storage period of 28 days. The tests were divided into physical tests, namely PS, PDI, ZP, EE, and TEM, which were performed on days 0 and 28 on control and NLCs, and chemical tests, namely free fatty acid, peroxide value, stability, and anisidine tests performed on days 0, 7, 14, 21, and 28. The control variable used in this study was NLC with the same formulation and manufacturer without the active compound (squalene).

PS in this study ranged from 37.4 to 38.6 nm. This small size is due to using Tween 80 as the surfactant as this can control PS and dispersion stability. PDI value for the control was lower than that for NLCs. The use of ultrasonication can affect PS because the sound waves applied to the sample cause particle degradation to smaller sizes. The increase in PS values during the storage period is caused by using palm kernel stearin as a constituent of NLC, which can prevent particle agglomeration and lead to an increase in PS and system instability (Mendes et al, 2019). Esadini et al (2022) stated that storage at room temperature produced a PS of <200 nm with a relatively homogeneous distribution characterized by a PDI value of <0.5.

During the storage period, PS increased in both NLCs and controls. PS of NLCs on day 0 was 38.6 nm and by day 28 had increased to 40.3 nm. The control had a smaller PS than NLC samples, and was 37.4 nm and 38.5 nm on day 0 of storage and on day 28, respectively. PDI of NLC samples also decreased from 0.282 at day 0 to 0.213 at day 28. Conversely, in the control, PDI increased with storage from 0.240 to 0.276. PDI ranged from 0 to 1. PDI value that is close to 0 indicates a homogeneous dispersion, whereas >0.5 indicates high heterogeneity (Marrissa, 2017). PDI of the NLC system was 0.240 (sd. 0.282) on day 0 and 0.213 (sd. 0.276) on day 28, indicating that no significant change occurred during storage. This was due to the level of uniformity in the sample, wherein smaller the PDI value, the more uniform the particle distribution in a monodisperse system is (Luo et al, 2017). PDI values that do not differ much during storage reflect a uniform size distribution, which is required for better stability during particle storage (Sharma et al, 2020).

In the ZP test, NLCs had a value of −34.1 mV on day 0 and −34.8 on day 28, whereas the control sample values were −41.1 and decreased to −32.9mV, respectively. Rahayu et al (2022) stated that based on statistical analysis, the ratio of polymer and surfactant does not have a significant influence on the ZP value, which is affected by storage temperature. In the EE test, NLC sample values were 85.7% and 84% on days 0 and 28, respectively, and were inversely proportional to the use of large amounts of surfactant.

The number of free fatty acids in the NLC sample increased with storage. This agrees with results in previous research (Cui et al, 2021), which stated that squalene oxidizes quickly after 30 days.

Table IV. Results of FFA, anisidine, peroxide, and stability tests for NLCs and control samples

Sample	Storage Time	Test			
		FFA Value	Anisidine Value	Peroxide Value	Stability
NLC-s	Day 0	0.044±0.011^{a}	0.261±0.144^{a}	0.048±0.024^{a}	0.624±0.211^{c}
	Day 7	0.045±0.006^{a}	0.416±0.189^{ab}	0.162±0.049^{b}	0.412±0.014^{b}
	Day 14	0.051±0.005^{a}	0.606±0.020^{b}	0.187±0.024^{c}	0.349±0.008^{ab}
	Day 21	0.055±0.006^{a}	0.661±0.301^{b}	0.248±0.037^{d}	0.269±0.004^{ab}
	Day 28	0.069±0.005^{b}	1.363±0.075^{c}	0.256±0.005^{d}	0.213±0.007^{a}
Control	Day 0	0.033±0.005^{a}	0.069±0.057^{a}	0.0052±0.003^{a}	0.363±0.026^{c}
	Day 7	0.068±0.008^{ab}	0.096±0.091^{ab}	0.060±0.016^{a}	0.211±0.006^{b}
	Day 14	0.075±0.005^{ab}	0.306±0.106^{bc}	0.111±0.009^{ab}	0.190±0.001^{b}
	Day 21	0.081±0.006^{b}	0.345±0.192^{c}	0.130±0.0016^{b}	0.149±0.018^{a}
	Day 28	0.091±0.044^{b}	0.579±0.126^{d}	0.216±0.066^{c}	0.126±0.001^{a}

Data are expressed as mean ± standard deviation

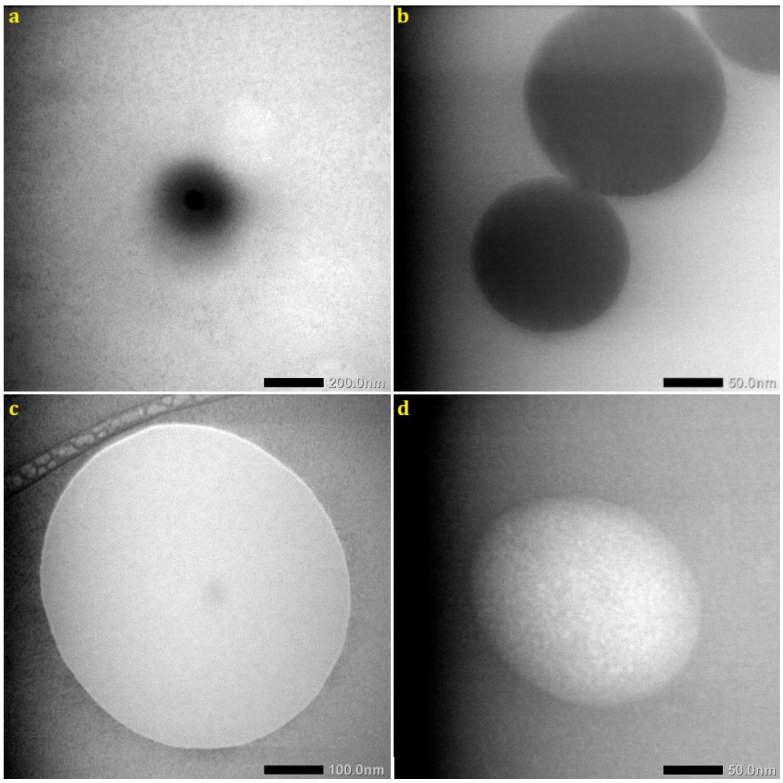

Figure 2. TEM images of NLC samples on day 0 (a) and day 28 (b) and control samples on day 0 (c) and day 28 (d)

NLCs stored over a long period can have different acid numbers from other NLC samples, which showed that a storage period of 28 days had a significant effect on this characteristic ($p < 0.05$). The control showed the same results as NLCs, namely that the number of free fatty acids increased with the length of storage. In the controls, the free fatty acid number ranged from 0.033 to 0.091.

The peroxide value increased during storage in the NLC samples. According to prior research (Lekshmi et al, 2021), results of the oxidative number are significantly influenced by the cell wall material. In this study, squalene based on

maltodextrin-whey protein isolate and GA had results of 0.50–3.23 meq peroxide/kg oil, which is still below the CODEX/FAO limit. A significant increase occurred in stored NLC samples by day 28 with a value of 1.363 meq peroxide/kg oil. This happened because oil is released during storage as indicated in prior research (Lekshmi et al, 2021), which stated that the uncapped oil fraction can easily undergo oxidation. The peroxide value in this study is also influential because of the active compounds used, with squalene been proven to be an effective singlet oxygen quencher that prevents lipid peroxidation, contributing to oxidative stability (Kohno et al, 1995).

The anisidine value also increased with the length of storage time. The anisidine number in the control was highest on day 28 at 1.363, which showed a significant increase ($p<0.05$). The control had lower anisidine value than NLC samples, which also had the highest anisidine value by day 28 of storage. Estiasih et al (2009) stated that temperature can influence secondary oxidation. Rancidity is caused by aldehydes and ketones produced from secondary oxidation in oil. Feryana et al (2014) stated that prolonged storage can cause the formation of p-anisidine compounds from the natural antioxidant content contained in NLC.

The decrease in stability in control samples and squalene-loaded NLCs was caused by the storage time and temperature used in the research, namely 28 days at 28°C. Sharma et al (2020) stated that NLCs are unstable at high temperatures but are stable at room temperature. PS parameters and surface properties are key characteristics used for evaluating the stability of colloidal systems (Table IV).

NLC materials can influence the shape of the particle and release of active compounds. Effective encapsulation ensures better oxidative stability due to lower permeation of prooxidants, such as moisture and oxygen. The ultrasonication method, which uses lower operating temperatures and shorter extraction times, is effective for minor unstable compounds (Chanioti & Tzia, 2019).

TEM images were obtained from NLC samples on days 0 and 28 as well as controls on the same testing days (Figure 2). TEM photos (a & c) show the presence of particles bigger than test PS, which ranged between 200 and 100 nm (Figure 2a and c). Based on the TEM results, the NLC and control had the same morphological characteristics, namely a round shape.

CONCLUSION

The optimum conditions for the preparation of NLCs containing squalene with a combination of palm kernel stearin and rice bran oil were as follows: sonication time of 24.8 min, sonication temperature of 43.7°C, and amplitude of 80%. Squalene-loaded NLCs and controls stored at room temperature for 28 days had increased peroxide, free fatty acid, and anisidine values, whereas the stability of NLCs and controls decreased. PS, PDI, ZP, and EE analysis showed stable results during 28 days of storage at room temperature (28°C).

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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