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# DEVELOPMENT AND OPTIMIZATION OF ASIATICOSIDE NANOEMULSION FORMULATION BY BOX-BEHNKEN DESIGN

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**Abstract**

This study aimed to develop and optimize the nanoemulsion formulation of asiaticoside (AS) using Box-Behnken design. Nanoemulsion optimization was used to obtain the optimum formula for ideal characteristics. Nanoemulsions were formulated using a combination of high-speed homogenization and ultrasonication. The independent variables were virgin coconut oil (VCO) as the oil phase, sucrose ester with HLB 3 (SE) as a surfactant and sodium caseinate (SC) as a co-surfactant. Particle size, polydispersity index (PDI) and zeta potential were the dependent variables used as responses to determine the optimum formula. The best combination of the composition factors that gave the optimum response was found to be VCO (13.55%, w/w), SE (2.5%, w/w) and SC (2%, w/w). The obtained nanoemulsion optimum formula was evaluated according to polydispersity index, particle size and zeta potential with the results obtained at 0.146 ± 0.002, 262 ± 1.000 nm and -62.9 ± 1.365 mV, respectively. The developed nanoemulsion formulation using the Box-Behnken design could be used as the optimal formulation of asiaticoside and to determine the effects of the formulation variables.

**Rezumat**

Acest studiu și-a propus să dezvolte și să optimizeze formula de tip nanoemulsie pe bază de asiaticozidă (AS) folosind designul Box-Behnken. Optimizarea nanoemulsiei a fost utilizată cu scopul de a obține formula optimă pentru caracteristicile ideale. Nanoemulsiile au fost formulate folosind atât omogenizarea de mare viteză cât și ultrasunetele. Variabilele independente au fost uleiul de cocos virgin (VCO) ca fază uleioasă, esterul de zaharoză cu HLB 3 (SE) ca surfactant și cazeinatul de sodiu (SC) ca și co-surfactant. Dimensiunea particulelor, indicele de polidispersitate (PDI) și potențialul zeta au fost variabilele dependente utilizate ca răspunsuri pentru a determina formula optimă. Cea mai bună combinație a factorilor de compoziție care au dat răspunsul optim a fost VCO (13,55%, g/g), SE (2,5%, g/g) și SC (2%, g/g). Formula optimă de nanoemulsie obținută a fost evaluată în funcție de indicele de polidispersitate, dimensiunea particulelor și potențialul zeta cu rezultatele obținute la 0,146 ± 0,002, 262 ± 1,000 nm și, respectiv, -62,9 ± 1,365 mV. Formularea dezvoltată de tip nanoemulsie folosind designul Box-Behnken ar putea fi utilizată ca formula optimă de încorporare a asiaticozidei și pentru a determina efectele variabilelor de formulare.

**Keywords**: asiaticoside, nanoemulsion, Box-Behnken design, optimization

## Introduction

*Centella asiatica* (L.) Urban, known by the name gotu kola in India or pegagan in Indonesia, comes from the *Apiaceae* family and grows widely in humid areas with tropical and subtropical climates such as Asia, Africa and Oceania. The largest active metabolites in this plant are ursane-type triterpenes, namely asiatic acid, madecassic acid, asiaticoside and madecassoside. The largest metabolite from *C. asiatica* is asiaticoside [1]. Asiaticoside has many pharmacological activities, such as neuroprotective which protects neurons from oxidative damage, antidepressant, anti-inflammatory, anti-allergic, immunomodulatory, hepatoprotective, cardiovascular protective, antiaging and wound healing including burns and diabetic wounds [2-8].

The pharmacological potential of asiaticoside is limited by its physicochemical properties, including low solubility (250 μg/mL), low lipophilicity and high molecular weight (959.12 g/mol) [9-11]. The poor water solubility of asiaticoside limits the rate of dissolution in water, which ultimately results in poor *in vivo* bioavailability [12]. Since drug absorption in the gastrointestinal tract is strongly influenced by the drug's solubility, the main issues in pharmaceutical formulation for the oral route are solubility and dissolution of the drug compounds.

Various methods have been carried out to increase the solubility of compounds in water. One of the methods used is physical modification by forming inclusion complexes in the active ingredients with the addition of various excipients. A variety of formulation techniques, such as micronization, solid dispersions,

inclusion complexes with cyclodextrin, liposomes encapsulated drug and solid lipid nanoparticles have been investigated to increase the dissolution rate of the poorly water-soluble drug, but these techniques could not resolve the intestinal absorption issues [13-16].

Lipid-based formulations, particularly the emulsion formulations, have demonstrated their utility in enhancing the absorption of poorly water-soluble drugs based on the increased drug dissolution in the gastrointestinal tract system [17]. Forming an inclusion complex from the active substance with excipients will provide a greater dissolution rate than the physical mixture of active substances and excipients [18].

Nanoemulsions are thermodynamically stable isotropic systems of dispersion of two immiscible liquids, which become stable by the interfacial layer of surfactant molecules [19-21]. The advantage of nanoemulsion is that the very small droplet size prevents creaming formation or sedimentation during storage due to the large reduction in gravitational force. Furthermore, flocculation can also be prevented so that the system remains dispersed without separation. These very small droplets can also prevent coalescence [19]. A developed formula in nanoemulsions can overcome the solubility issue of hydrophobic compounds and it can be applied in other dosage forms [22].

Virgin coconut oil (VCO) has activity as an anti-oxidant, it also can reduce levels of total cholesterol, triglycerides, phospholipids, low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) and increase high-density lipoprotein (HDL) [23]. Medium-chain fatty acids (MCFAs) total content is between 60.5% and 63.6% [24]. The solubility of the drug in oil is an important factor in emulsion formulation due to the fact that the ability of the emulsion to retain the drug in dissolved form is greatly influenced by its solubility in the oil phase. In this research, VCO was chosen as the oil phase because of its health benefits and development as a nutraceutical [25].

Sodium caseinate (SC) is an emulsifier from milk protein that is commonly used in the food and beverage industry as a dissolved mixture of four main caseins namely $\alpha_{s1}$-casein, $\alpha_{s2}$-casein, β-casein and κ-casein [26]. Compared to other dietary proteins, SC can form a thick steric stabilization layer at the emulsion droplet interface, which protects newly formed droplets from flocculation and coalescence [27]. Using SC as a surfactant at a level of 7 - 9% gave quite stable and homogeneous nanoemulsion results while using sodium caseinate at less than 7% resulted in phase separation in the emulsion. In another case, SC levels above 9% gave the highly viscous emulsion resulting in a difficult spray-drying [28].

In recent years the use of sucrose esters (SE) has been widely used as surfactants. SE are non-ionic compounds, food-grade, surface-active agents and biocompatible agents with excellent biodegradability and low toxicity. This compound comes from natural ingredients which are derivatives of sucrose and vegetable oil. Several studies have been conducted using this sucrose ester as a surfactant or cosurfactant to stabilize oil-in-water emulsions [29, 30].

An experimental design approach can be used in the formula development and optimization to simplify and minimize the time and costs required in the experiment [31]. This study aims to develop and optimize the nanoemulsion formulation of asiaticoside (AS). The asiaticoside nanoemulsions were optimized by three factors at a three-level Box-Behnken statistical screening design (BBD). Compared to other surface response methodologies, such as full factorial design or central composite design, BBD is more efficient because it often requires fewer experiments to determine the influence of independent factors on the responses [21, 32, 33].

## Materials and Methods

*Materials*

Asiaticoside standards with 98% purity was obtained from PT EBM Saintifik dan Teknologi, Bandung, West Java, Indonesia. Asiaticoside with 95% Purity as an active ingredient was purchased from New Natural Biotechnology Co. Limited, Shanghai, China. SE was purchased from Wuxi Vland Biotech Co. Ltd, China. SC was purchased from Sigma-Aldrich, St. Louis, Missouri, USA. VCO was purchased from PT Trivico, Indonesia. Ethanol absolute, acetonitrile for liquid chromatography and methanol HPLC Grade were purchased from Merck, Germany. All other remaining solvents and chemicals were of analytical grade and procured from local vendors.

*Instrumentation*

The instruments used in this study included homogenizer (Ultra Turrax T25 easy clean IKA, Germany), sonicator (Qsonica Q500A Sonicator Ultrasonic Homogenizer, USA), particle size analyser (Malvern Zetasizer - Nano Zs, UK), HPLC Shimadzu SPD-20A (Japan) with Zorbax Eclipse Plus C18 4.6 x 250 mm, 5 µm (Agilent, California, USA), analytical balance (Sartorius Secura 225D-1S, Lab Instruments GMbH Goettingen, Germany), Ohaus PA 323 (China), vortex (Maxi Mix II, Thermo Scientific, USA) and magnetic stirrer (AM4 Multiple Heating, Velp Scientifica, Italy).

*Experimental designs*

*Nanoemulsions Optimization*

The Box-Behnken design result of Design-Expert soft-ware (DX ver.13, State Ease Inc., Minnesota) was utilized to improve the composition of asiaticoside nanoemulsions (AS-NE) based on three independent factors. Fifteen trial runs were conducted to determine the optimum formula of the effects of the independent variables. The selected independent variables were VCO (10 - 20 % w/w) as oil phase, SE (0.5 - 2.5 % w/w) as surfactant and SC (0.5 - 2 % w/w) as co-

surfactant. The polydispersity index (PDI) (Y1), particle size (D90) (Y2) and zeta potential (Y3) were selected as parameters to assess the asiaticoside nanoemulsions.

*Preparation of Nanoemulsion*

The AS-NE was prepared in two steps. First, all ingredients are mixed with a high-speed homogenizer before proceeding with a sonicator [34]. The procedure was carried out by heating the VCO at 70°C and adding SE as an oil phase surfactant. AS was weighed 0.5 g dissolved in 9.5 mL absolute ethanol, and mixed into the oil phase, while SC was dissolved in water. A low-speed homogenizer at 5,000 rpm was used for pre-homogenization for 5 minutes. The water phase was then added dropwise into the oil phase. After 5 minutes, the speed of the homogenizer was then increased to 10,000 rpm for 5 minutes. The AS-NE was then homogenized using a probe sonicator for five minutes at 15 W of pressure and 40% amplitude.

*Determination of polydispersity index, particle size (D90) and zeta potential*

The polydispersity index, particle size and zeta potential were analysed by diluting 10 µL of asiaticoside nano-emulsion in 10 mL of water for injection and homogenized using vortex. The mixture was analysed using Malvern Zetasizer (Malvern Instruments, UK) where light scattering was monitored at 25°C and at a scattering angle of 173°C [32].

*Validation of High-Performance Liquid Chromatography (HPLC) method for the determination of asiaticoside*

*Chromatographic conditions*

Liquid chromatography Shimadzu SPD-20A with a UV-Vis detector (Kyoto, Japan) was used as a quantitative instrument. Separations were performed using Zorbax Eclipse Plus C18 4.6 x 250 mm, 5 µm (Agilent, California, USA). Quantitative determination of asiaticoside was performed on a reversed-phase HPLC component system. The HPLC condition was as follows; mobile phase: acetonitrile: water (28:72 v/v); flow rate: 0.9 mL/min; oven temperature: ambient; UV detector wavelength: 210 nm; injection volume: 10 µL; and run time: 10 min. The HPLC method was compiled in method validation to measure system suitability, specification, linearity, precision, accuracy, the limit of detection (LOD) and the limit of quantification (LOQ) [35, 36].

*Preparation of standard solution of asiaticoside*

The stock solution of 200 µg/mL was prepared by accurately weighing 5 mg of asiaticoside diluted in 25 mL methanol. The stock standard solution was diluted with methanol to obtain seven calibration standards in the concentration range of 1, 5, 10, 25, 50, 75 and 100 µg/mL.

*Validation Procedure*

*Linearity*

Seven concentrations of the mixed standard were prepared: 1, 5, 10, 25, 50, 75 and 100 µg/mL. The standard solutions were filtered through a 0.22 µm-pore size nylon syringe filter and analysed using the HPLC instrument in triplicate. The calibration curve of asiaticoside was constructed by plotting the average peak area *versus* the corresponding concentration.

*Precision*

Precision was evaluated using three concentrations of the standard solutions. The 25, 50 and 75 µg/mL standard solutions were prepared and filtered through a 0.22 µm pore size nylon syringe filter. They were analysed by HPLC instruments in triplicate. The intra-day and interday precisions were reported as the percent relative standard deviation (% RSD). The interday precision was evaluated by repeating the procedure on two different days.

*Accuracy*

The three concentrations of the standard solutions (25, 50, 75 µg/mL) were prepared to determine the accuracy of the method. The procedure was repeated on two different days. The corresponding % recovery was calculated according to the following equation:

$$\%\ recovery = \frac{calculated\ concentration}{theoretical\ concecntraion}\ x\ 100. \quad [1]$$

*Limit of Detection (LOD) and quantification (LOQ)*

The seven samples (1, 5, 10, 25, 50, 75 and 100 µg/mL) were analysed to calculate the LOD and LOQ. The calculation was made with the following equations:

$$LOD = \frac{3.3\ \sigma}{S}, \quad [2]$$

$$LOQ = \frac{10\ \sigma}{S}, \quad [3]$$

where, σ was the standard deviation of the response (estimated from the standard deviation of *y*-intercepts of regression lines) and *S* was the slope of the standard curve.

*Determination of asiaticoside purity*

AS purity was determined using a validated method of HPLC. The stock solution of asiaticoside was prepared in 1 mg/mL concentration diluted in methanol. The test solution was prepared in 100 µg/mL. It was filtered through a 0.22 µm-pore size nylon syringe filter and then analysed by the HPLC instrument (n = 3).

*Statistical analysis*

The effect of formulation variables on polydispersity index, globule size and zeta potential were analysed using Design-Expert software. The optimum formula obtained was validated, and the response results were analysed. Analysis of variance (ANOVA) based on the optimum formula data was performed to assess the significant differences between each response using a one-sample t-test with SPSS 19 software. p-values of less than 0.05 is considered significant.

## Results and Discussion

SE is a highly suitable emulsifier for nanoemulsion formulation. The concentration of SE was used between 0.5% and 2.5% [37, 38]. SC is a promising option for emulsion preparation. However, it is highly responsive

to acidic pH near its isoelectric point and, when utilized as an emulsion stabilizer, this characteristic could have a detrimental impact on the stability of the emulsion [39]. To avoid this issue, SC was combined along with a non-ionic surfactant (SE) to perform as an emulsifier for oil/water nanoemulsions [40, 41]. The quantity of SC ranged from 0.5 to 2% (w/w) [42]. The high content of medium-chain fatty acids and antioxidant properties in virgin coconut oil (VCO) have been demonstrated to promote good health. VCO has been used in nanoemulsion preparation in the range of concentration from 10 to 20% [28, 43].

The components were optimized using a Box-Behnken design to obtain a concentration ratio that could produce the optimum formula. The Box-Behnken design was selected to reduce the number of formulas needed to determine the optimum formula using a statistical approach. By assuming that the best combination of factors is near the midpoint, this design can decrease the number of experiments needed, thereby avoiding the evaluation of extreme combinations to minimize undesirable results [44, 45]. The determination of the optimum formula was based on the response generated from each run of the formula. The responses investigated included polydispersity index, particle size and zeta potential. The optimum formula was determined based on the p-value of analysis of variance (ANOVA), the lack of fit, $R^2$ and adjusted-$R^2$ to determine the optimal model according to the experimental data [46].

Prior to the nanoemulsion formulation, screening should be performed to determine the most suitable ingredients to be incorporated in the nanoemulsion. For the selection of oils, surfactants and co-surfactants; the solubility of poorly soluble drugs in oil, surfactants and co-surfactants is one of the most important factors for the development of nanoemulsion. The amount of oils contained in nanoemulsions significantly affects the size and zeta potential [21, 32].

Fitting of data to the model, the effect of formulation variables on polydispersity index (Y1), particle size (Y2) and zeta potential (Y3) were investigated using three factors at a three-level Box-Behnken statistical design. The coded independent variables (oil, surfactant and co-surfactant) and dependent variables (polydispersity index, globule size and zeta potential) used in optimization were given in Table I. For response surface methodology, 15 experimental runs were conducted, including 5 runs with central points in which the responses were recorded and analysed. The best-fitted model parameters are quadratic polynomial models for the PDI factor and linear models for globule size and zeta potential factors. The obtained responses are listed in Table II. The observed value and the predicted value were found to be close to each other, indicating that the experimental data is accurate.

*Polydispersity index (PDI)*

PDI describes the uniformity of particle size distribution in a particular sample. For a perfectly uniform sample, the PDI numerical value is 0.0, and the greater the PDI value, the more heterogeneous the particle size. The largest PDI numerical value is 1.0, which indicates that the sample is very polydisperse [46, 48]. The polydispersity index is a ratio that furnishes information about the homogeneity of the particle size distribution in a given system [49].

**Table I**

Detail on the variables used for the preparation of asiaticoside nanoemulsion by Box-Behnken design

| Factors | Levels | | |
|---|---|---|---|
| Independent variables | Low (-1) | Medium (0) | High (+1) |
| A = VCO (% w/w) | 10 | 15 | 20 |
| B = Sucrose ester HLB 3(% w/w) | 0.5 | 1.5 | 2.5 |
| C = Sodium caseinate (% w/w) | 0.5 | 1.25 | 2 |
| Dependent variables | Goals | | |
| $Y_1$ = Polydispersity Index (PDI) | Minimize | | |
| $Y_2$ = Globule size (D90) | Minimize | | |
| $Y_3$ = Zeta potential | In range | | |

**Table II**

Result of the optimization formula using Box-Behnken design

| Formula | Independent variables | | | | | Dependent variables | | | |
|---|---|---|---|---|---|---|---|---|---|
| | VCO (%) | SE (%) | SC (%) | PDI | | D90 (nm) | | Potensial zeta (mV) | |
| | | | | Observed | Predicted | Observed | Predicted | Observed | Predicted |
| 1 | 15 | 1.5 | 1.25 | 0.151 | 0.1547 | 304 | 313.53 | -60.5 | -61.92 |
| 2 | 15 | 2.5 | 2.0 | 0.147 | 0.1486 | 260 | 255.41 | -62.4 | -62.63 |
| 3 | 10 | 1.5 | 2.0 | 0.156 | 0.1550 | 302 | 310.53 | -66.4 | -67.30 |
| 4 | 10 | 1.5 | 0.5 | 0.175 | 0.1763 | 346 | 348.28 | -62.5 | -62.72 |
| 5 | 20 | 2.5 | 1.25 | 0.151 | 0.1506 | 263 | 258.41 | -59.3 | -57.26 |
| 6 | 15 | 1.5 | 1.25 | 0.160 | 0.1547 | 312 | 313.53 | -62.1 | -61.92 |
| 7 | 15 | 0.5 | 2.0 | 0.171 | 0.1716 | 326 | 333.19 | -68.9 | -65.78 |
| 8 | 10 | 2.5 | 1.25 | 0.160 | 0.1594 | 294 | 290.16 | -65.1 | -63.43 |
| 9 | 15 | 1.5 | 1.25 | 0.153 | 0.1547 | 307 | 313.53 | -61.1 | -61.92 |

| Formula | Independent variables | | | Dependent variables | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | VCO (%) | SE (%) | SC (%) | PDI | | D90 (nm) | | Potensial zeta (mV) | |
| | | | | Observed | Predicted | Observed | Predicted | Observed | Predicted |
| 10 | 10 | 0.5 | 1.25 | 0.192 | 0.1924 | 381 | 368.66 | -65.7 | -66.58 |
| 11 | 15 | 2.5 | 0.5 | 0.154 | 0.1534 | 298 | 293.16 | -58.1 | -58.06 |
| 12 | 20 | 0.5 | 1.25 | 0.161 | 0.1616 | 345 | 336.91 | -61.9 | -60.41 |
| 13 | 15 | 0.5 | 0.5 | 0.176 | 0.1744 | 377 | 371.66 | -61.0 | -61.21 |
| 14 | 20 | 1.5 | 0.5 | 0.138 | 0.1390 | 303 | 316.53 | -55.8 | -56.55 |
| 15 | 20 | 1.5 | 2.0 | 0.154 | 0.1528 | 285 | 278.78 | -58.0 | -61.12 |

The polydispersity index showed values between 0.138 and 0.192 (Table II), indicating that all the nano-emulsions had a narrow size distribution. p-values less than 0.05 indicate a significant model term. In this case, A, B, AB, AC, $B^2$ are significant model terms. Meanwhile, the co-surfactant did not show any significant influence on the PDI value ($p > 0.05$). Interaction between factors of AB and AC showed a significant effect, but not for BC.

The Lack of Fit (F) of 0.18 implies the value is not significant relative to the pure error. Non-significant lack of fit is desirable. The model had predicted $R^2$ and adjusted $R^2$ of 0.8850 and 0.9382, respectively. It defined the values in reasonable agreement, with the differences between them less than 0.2. The adequate precision is 19.375, which indicates an adequate signal because a ratio greater than 4 was desirable. Therefore, this model can be used to navigate the design space.

The data fitting to the quadratic model provided the following equation which indicates the effect of % VCO as oil phase (A), % SE as a surfactant (B) and % SC as co-surfactant on the polydispersity index (PDI) of the nanoemulsion.

$$Y1 \text{ (Polydispersity index)} = 0.310 - 0.010A - 0.053B - 0.030C + 0.0011AB + 0.0023\,AC - 0.00067BC + 0.0001A^2 + 0.0088B^2 - 0.0026C^2, \quad [4]$$

A, B and C values imply that an increased concentration of oil, surfactant and co-surfactant leads to a decrease in the polydispersity index of nanoemulsions. Nevertheless, the surfactant was insufficient at higher oil concentrations to micellize the oil droplets, which led to the fusing of oil globules and an increase in the PDI [50].

In Figure 1, the model successfully captured the correlation between all independent variables, as evidenced by the $R^2 = 0.9779$ difference between the predicted PDI values derived from the model and the actual PDI values collected from the experimental data.

*Particle size*

Particle size is an important variable that significantly affects drug release patterns [32]. In reference to Table III, the model F-value of 76.40 implies the model is significant. p-values of A, B and C factors less than 0.05 indicate model terms are significant. In this case, A, B and C are significant model terms. The three factors indicate the significant influence on the particle size of nanoemulsion. The Lack of Fit F-value of 5.39 implies that it is not significant relative to the pure error. A non-significant lack of fit is desired.

The predicted $R^2$ of 0.9094 is in reasonable agreement with the Adjusted $R^2$ of 0.9417. The difference is less than 0.2. Meanwhile, the adequate precision value is 25.995 indicates an adequate signal.

The data fitting to the linear model provided the following equation which indicates the effect of % VCO as oil phase (A), % SE as surfactant (B) and % SC as co-surfactant on the particle size of the nano-emulsion.

$$Y2 \text{ (D90)} = 451.49 - 3.175A - 39.25B - 25.17C, \quad [5]$$

According to equation [5], factors A, B and C imply the negative influence on particle size that an increased concentration of oil, surfactant and co-surfactant leads to a decrease in particle size. According to Perugini L *et al*., when surfactant was incorporated into the nano-emulsion, it showed that the more emulsifier was added, the smaller the oil droplets in the dispersed phase formed. However, unlike the previous study where the presence of SC had no impact on the size of the dispersed phase aggregates, in this study, an increase in SC concentration led to a reduction in particle size [42]. The combination of low molecular weight emulsifiers with caseinate led to a reduction in particle size and an enhancement of viscosity in the emulsion. Furthermore, the nonionic emulsifier sucrose ester showed higher displacement of caseinate and greater absorption capacities for oil droplets compared to ionic and amphoteric emulsifiers [51]. The model's prediction of particle sizes compared to the experimental data's actual particle sizes demonstrates that it was successful in capturing the correlation between all the independent variables, with $R^2 = 0.9542$ (Figure 1b).

*Zeta potential*

The zeta potential measures the electrokinetic potential of a particle and was used to determine the stability of the nanoemulsion. A nanoemulsion with zeta potential values higher than +30 mV and lower than -30 mV was said to be stable [52].

The Model F-value of 15.04 implies the model is significant. A, B and C are significant model terms ($p < 0.05$). The three factors indicate the significant influence of the zeta potential of nanoemulsion. The predicted $R^2$ of 0.5960 is in reasonable agreement with the adjusted $R^2$ of 0.7505 because the difference is less than 0.2. The adequate precision measures gave the desirable ratio. The ratio is 11.905, indicating an adequate signal.

The data fitting to the linear model provided the following equation which indicates the effect of % VCO as oil phase (A), % SE as surfactant (B) and % SC as co-surfactant on the zeta potential of the nano-emulsion.

$$Y3 \text{ (Potensial zeta)} = -69.73 + 0.618A + 1.575B - 3.05C, [6]$$

Zeta potential is positively influenced by the A and B factors, as seen by equation [6]. However, the influence of factor C is negative. Zeta potential increases when oil and surfactant concentrations rise, on the other hand, zeta potential decreases as co-surfactant concentrations rise. There is a correlation ($R^2 = 0.8040$) between all the independent variables when the predicted zeta potential values from the model are compared to the actual zeta potential values obtained from the experimental data (Figure 1c).

Typically, an emulsion is considered to have good stability if it has an absolute zeta potential above 30 mV and those with a zeta potential above 60 mV indicate excellent stability and the potential for settling. An increase in the zeta potential demonstrates a reduction in the attraction between droplets and an increase in the repulsive force. The interaction of SE with sodium caseinate at the oil-water interface may involve electro-static forces, leading to an enhancement of the zeta potential [39].

The correlation between the experimental design variance and response is presented in a three-dimensional (3D) surface plot, which enables a quick and simple understanding of these correlations. Figure 2 displays the 3D plot of the zeta potential, particle size and polydispersity index.

Based on the desirability value that the model of all responses generated, the software Design Expert provides the optimal formula. The better the prediction of the intended response, the higher the desirability value, which is near 1 [52]. The software analysis showed that the highest desirability value is 0.907, containing a combination of VCO (13.55%), SE (2.5%) and SC (2%). Afterward, the optimum formula from the predictive software results was confirmed through triplicate experiments and then evaluated to validate the response predicted by the software (Table IV).

*Validation of the analysis method*

The content of asiaticoside was determined using High-Performance Liquid Chromatography (HPLC) with a validated method. HPLC samples were run in reverse phase isocratic using UV spectroscopy as the detector. The methods to determine the concentration of AS were also validated. The mobile phase consisted of acetonitrile (ACN) and water (28:72 v/v) at a flow rate of 0.9 mL/min.

*System suitability*

The system suitability test confirms the accuracy of the analytical method. It also serves to confirm the resolution among various interest peaks. The system suitability parameters of the validation method are shown in Table V.

The proposed method was assessed using statistical analysis and the results showed it has good linearity, reproducibility and has been validated for various applications. The parameters led us to the conclusion that it could be applied for the simple and reliable determination of asiaticoside[53].

The results of the analysis method validation in determining the concentration of AS are listed below.

**Table III**

Analysis of variance and lack of fit test of the model for the response

| Model fit parameters | F-value | p-value | Significance |
|---|---|---|---|
| ***(a) PDI*** | | | |
| **Model** | 24.62 | 0.0013 | significant |
| **A-VCO** | 68.53 | 0.0004 | |
| **B-Sucrose Ester - 3** | 85.04 | 0.0003 | |
| **C-Sod, Caseinate** | 2.47 | 0.1768 | |
| **AB** | 10.63 | 0.0224 | |
| **AC** | 26.90 | 0.0035 | |
| **BC** | 0.0878 | 0.7788 | |
| **$A^2$** | 2.10 | 0.2074 | |
| **$B^2$** | 25.07 | 0.0041 | |
| **$C^2$** | 0.6898 | 0.4441 | |
| **Lack of Fit** | 0.1828 | 0.9002 | not significant |
| **$R^2$** | | 0.9779 | |
| **$R^2$ adjusted** | | 0.9382 | |
| **$R^2$ predicted** | | 0.8850 | |
| **Adeq. Precision** | | 19.3753 | |
| ***(b) Globule size*** | | | |
| **Model** | 76.40 | < 0.0001 | significant |
| **A-VCO** | 26.88 | 0.0003 | |
| **B-Sucrose Ester - 3** | 164.88 | < 0.0001 | |
| **C-Sod, Caseinate** | 38.00 | < 0.0001 | |
| **Lack of Fit** | 5.39 | 0.1662 | not significant |
| **$R^2$** | | 0.9542 | |
| **$R^2$ adjusted** | | 0.9417 | |
| **$R^2$ predicted** | | 0.9094 | |
| **Adeq. Precision** | | 25.9946 | |
| ***(c) Zeta potential*** | | | |
| **Model** | 15.04 | 0.0003 | significant |
| **A-VCO** | 24.94 | 0.0004 | |
| **B-Sucrose Ester - 3** | 6.49 | 0.0271 | |
| **C-Sod, Caseinate** | 13.69 | 0.0035 | |
| **Lack of Fit** | 5.50 | 0.1633 | not significant |
| **$R^2$** | | 0.8040 | |
| **$R^2$ adjusted** | | 0.7505 | |
| **$R^2$ predicted** | | 0.5960 | |
| **Adeq. Precision** | | 11.9046 | |

*Linearity*

The standard solutions relative to each point in the calibration curve were injected in triplicate to establish the linearity study. Linearity is measured by plotting the area value (Y-axis) with the standard solution content (X-axis).

The calibration curve's $R^2$ value is required to be greater than or equal to 0.997 to confirm that the linearity achieved in this study with external standards

is suitable for the desired objective (Table VI) [36]. These findings are in close agreement with the previously published reports [35].

*Limit of Detection (LOD) and quantification (LOQ)*

The sensitivity of the test is expressed in the Limit of Detection (LOD) value, namely the smallest level of analyte in the sample that can still be detected. Meanwhile, the limit of quantification (LOQ) is a parameter in analysis that is defined as the smallest quantity of analyte in a sample that can still meet the criteria for accuracy and precision. The detection limit is the level of analyte that provides a response that is 3 times the standard deviation of the blank measurement. The LOD and LOQ values obtained were 4.57 µg/mL and 13.86 µg/mL (Table VI).

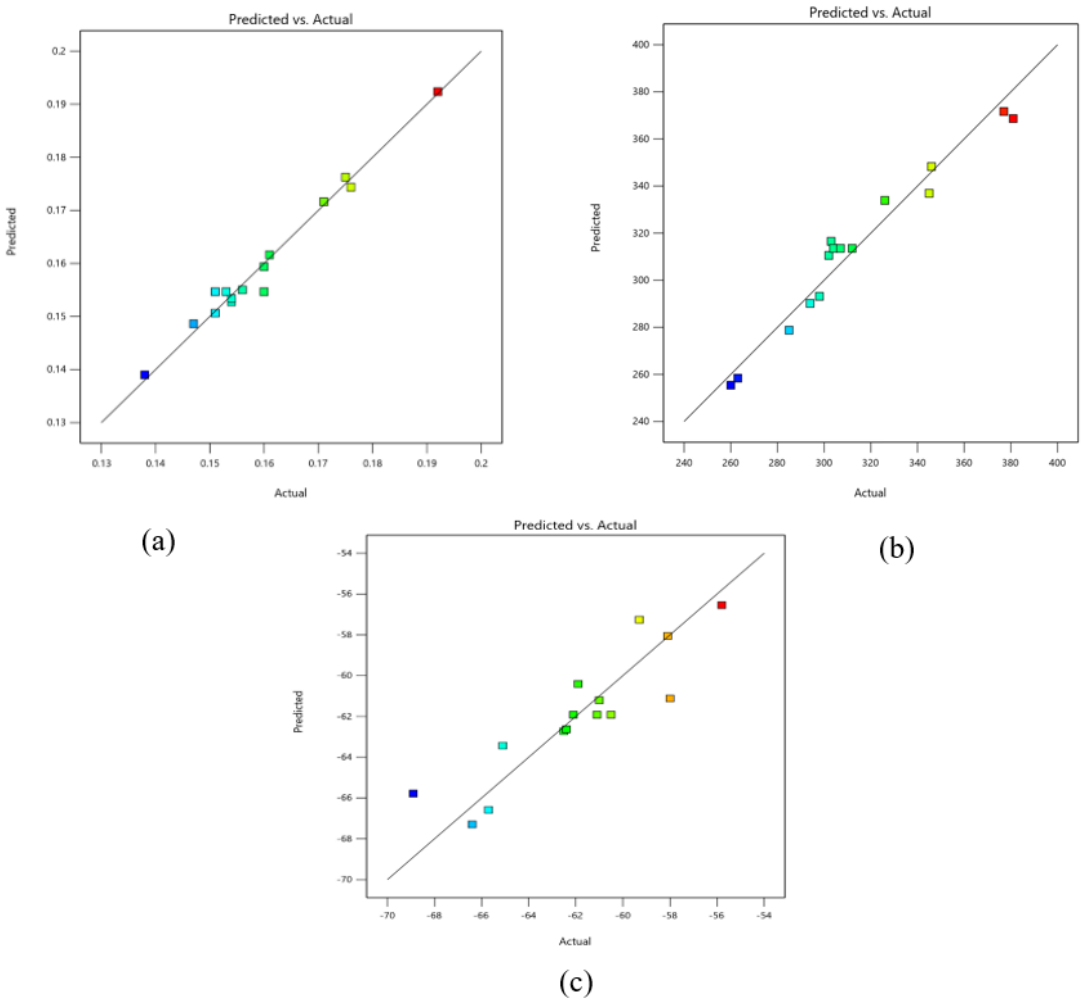



**Figure 1.**

The scatter plot comparing the predicted and actual from the three-factor Box-Behnken Design (BBD); (a) PDI, (b) particle size and (c) zeta potential

**Table IV**

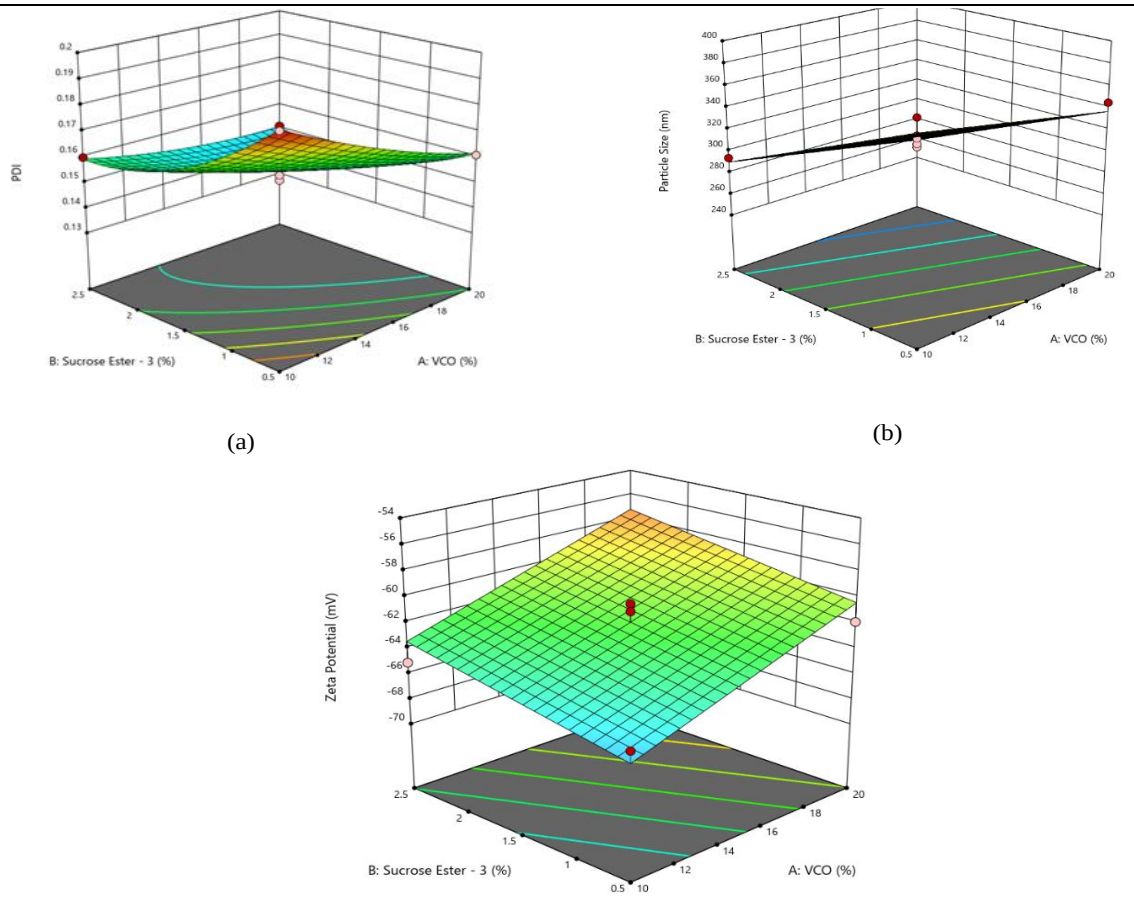
Comparison of observed experimental and predicted values of asiaticoside nanoemulsion

| Response | Observed value | Predicted value | p-value (one sample t-test) |
|---|---|---|---|
| **Polydispersity Index (PDI** | 0.146 ± 0.002 | 0.148 | 0.222 |
| **Particle size (D90)** | 262 ± 1.000 | 260 | 0.074 |
| **Zeta Potential** | -62.9 ± 1.365 | -63.5 | 0.547 |

**Table V**

System suitability parameters

| Parameter | Data | RSD | Requirement |
|---|---|---|---|
| **Retention time** | 9.114 ± 10.36 | 0.114 | RSD < 1 |
| **Peak area** | 249389 ± 2471.33 | 0.991 | RSD < 1 |
| **Tailing factor** | 1039 ± 2.61 | | T ≤ 2 |
| **Theoretical plate** | 9247 ± 123.13 | | N > 2000 |




(a) (b)

(c)

**Figure 2.**

Three-dimensional response surface plot illustrating the impact of independent factors on the responses: (a) zeta potential; (b) particle size; and (c) the Polydispersity index (PDI)

**Table VI**

Method validation parameters for determining asiaticoside content

| Parameters | Result |
|---|---|
| **Linearity equation** | Y = 2048.1x - 1339.6 |
| **Correlation coefficient (r)** | 0.9989 |
| **Retention time (min)** | 8.83 ± 0.09 |
| **LOD (µg/mL)** | 4.57 |
| **LOQ (µg/mL)** | 13.86 |

*Precision*

When measuring a sample, there may be an error or interference which can cause the results to be inaccurate. The accuracy of the analytical method used must be tested by observing whether the instrument's response to an analyte is constant or repeatable over time. In this study, measurements were carried out by repeating three times (triple). Precision results are evaluated based on the relative standard deviation (RSD) value. Based on Table VII, it was found that the RSD value for intraday precision was between 0.57% and 1.40%, and interday was between 0.87% and 1.50%. Following the requirements according to USP, this result shows that the analysis method used in this study has good accuracy because the value RSD < 2 %.

*Accuracy*

The accuracy value is expressed in recovery percentage. Based on the data shown in Table VII, the recovery values were in the range of 99.67 - 101.06%. This shows that the obtained recovery still meets the permitted requirements (98 - 102%).

**Table VII**

Precision and accuracy of asiaticoside analysis

| Standard | Conc. (µg/mL) | Precision (% RSD) | | Accuracy |
|---|---|---|---|---|
| | | intraday | interday | Recovery (%) |
| **Asiaticoside** | 25 | 0.57 | 1.50 | 101.61 ± 0.11 |
| | 50 | 1.40 | 0.87 | 99.67 ± 0.51 |
| | 75 | 1.06 | 1.26 | 100.82 ± 0.17 |

*Drug Purity*

The purity test was carried out on asiaticoside which is used as an active ingredient in the formula. It was determined using HPLC (high-performance liquid chromatography) with a validated method. The active ingredient contains 95.08 ± 0.63%. This result is in accordance with what is stated in the CoA.

## Conclusions

This study shows the nanoemulsions containing asiaticoside have been successfully optimized using the Box-Behnken design. It was found that the best combination of the composition factors that gave the optimum response was found to be VCO (13.554%, w/w), SE (2.5%, w/w) and SC (2%, w/w). The obtained nanoemulsion optimum formula was evaluated according to polydispersity index, particle size and zeta potential with the results obtained at 0.146 ± 0.002, 262 ± 1.000 nm and -62.9 ± 1.365 mV, respectively. The HPLC analysis method in determining the asiaticoside content in samples provides good linearity and quite good sensitivity. The optimized AS nanoemulsion formulation provides a promising platform for drug delivery by exhibiting good nanoemulsion properties such as particle size, polydispersity index and zeta potential for formulation stability. Furthermore, the optimized nanoemulsion can be explored for potential applications in cosmetics and nutraceuticals.

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## Conflict of interest

The authors declare no conflict of interest.

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