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*Research Article*

# Effect of Cholesterol to Vitamin D3 and Span 60 to Tween 60 Ratios on the Characteristics of Niosomes: Variable Optimization Using Response Surface Methodology (RSM)

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The main objective of this research was to evaluate the partial replacement of cholesterol (CSL) with vitamin $D_3$ (VD3) on the niosome structure. The effects of different molar ratios of Span 60 (SP60) : Tween 60 (TW60) and CSL : VD3 were investigated on the physicochemical characteristics of niosomes, including particle size, span (distribution width), stability, and encapsulation efficiency of VD3. The data were then optimized using response surface methodology (RSM). Larger particles were obtained as the ratios of SP60 : TW60 and CSL : VD3 were increased. The smallest particles were obtained at SP60 : TW60 and CSL : VD3 ratios of 40.46 : 59.54 and 38.06 : 61.94, respectively. Increasing the ratio of SP60 : TW60 led to higher values of span. As CSL : VD3 ratio was increased from 0 : 100 to 67.67 : 32.32, value of the span was decreased; however, increasing this ratio further led to the increased value of the span. The lowest values of the span were observed at SP60 : TW60 and CSL : VD3 ratios of 67.90 : 32.10 and 72.41 : 27.59, respectively. The increase in the SP60 : TW60 ratio led to lower values of encapsulation efficiency. The highest values for encapsulation efficiency were observed at ratios of 31.76 : 68.24 and 40.02 : 59.98 for SP60 : TW60 and CSL : VD3, respectively. The highest stability was observed at SP60 : TW60 and CSL : VD3 ratios of 31.72 : 68.28 and 14.65 : 85.35, respectively. The optimum conditions were achieved at ratios of 31.72 : 68.28 and 49.37 : 50.63 for SP60 : TW60 and CSL : VD3, respectively. Finally, it can be concluded that VD3 is a suitable replacement for CSL in terms of stability and encapsulation efficiency of niosome.

## 1. Introduction

Niosome is a novel delivery system patented by L'Oreal Company. It has high application in food, pharmaceutical, and cosmetic sectors [1]. These bilayer vesicles, which are prepared by the self-assembly of nonionic surfactants, have high encapsulation ability [2]. Encapsulation of both polar and nonpolar compounds could be accomplished by niosomes. They are biodegradable, nonimmunogenic, and nontoxic. They demonstrated some advantages over liposomes, such as higher chemical stability and low production costs [3]. Niosomes prepared with native biomolecules are suitable for human consumption. Niosomes developed from sorbitan monoesters are among the most widely evaluated vesicles [4].

Niosomes are fabricated by hydration of cholesterol (CSL) and single-alkyl chain nonionic surfactants [5, 6]. To reduce the energy interactions between the alkyl chain of surfactant and solvent, these molecules self-assemble in such a way that the hydrophobic tails face each other [7]. In this closed bilayer structure, hydrophobic parts of the molecule are oriented away from the aqueous solvent, whereas the hydrophilic head comes into contact with the aqueous solvent. Therefore, they are capable of entrapping the hydrophilic molecules in the core and hydrophobic molecules in the bilayer membrane [5, 7]. Nonionic surfactant vesicles can entrap the active material in a manner analogous to liposomes [5, 6]. More chemical stability, no requirement for special conditions for storage and handling, and low cost are some of the advantages of niosomes [8].

The formation of niosomes is not a spontaneous process. Arrangement of the lipid molecules happens as a result of energy input. The bilayer form of vesicles is formed to achieve a thermodynamic equilibrium in the aqueous phase. Different methods provide the required energy in different forms [9]. Sufficient amount of mechanical or thermal energy causes bilayer sheets to separate and form multilamellar vesicles. Modification of the particle composition or surface can improve the release rate of bioactive compounds on the target site [10]. Therefore, nanocapsules play an important role in the delivery systems. It is known that niosome properties such as size distribution, number of layers, encapsulation efficiency, and permeability of vesicle membrane are influenced by their method of preparation [2, 11].

CSL is usually applied as the main additive for niosomes and liposome production to increase the stability of vesicles. It is reported that CSL also plays an important role in niosomes formulation when hydrophilic surfactants are used as main membrane compounds. Also, CSL has a similar structure to VD3 and VD3 can probably increase the stability.

The main objective of this research was to evaluate the partial replacement of cholesterol (CSL) with vitamin D3 (VD3) in the niosome structure. Also, the influence of different molar ratios of SP60 : TW60 was investigated on the physicochemical properties of the niosomes, in terms of particle size, span, stability, and encapsulation efficiency.

## 2. Materials and Methods

*2.1. Materials.* Vitamin D3 (VD3), Span 60 (SP60), cholesterol (CSL), Tween 60 (TW60), sodium hydroxide, citrate buffered saline (CBS), hydrochloric acid, Amicon filter (MW Cut-off 10 kDa), ethanol, and methanol were obtained from Merck & Co. (Darmstadt, Germany) and Sigma Aldrich (St. Louis, MO, USA).

*2.2. Experimental Design.* Response surface methodology (RSM) (Design Expert-Version 11, Minneapolis, USA) was used to optimize the parameters in niosome production and to determine the influence of independent variables on the characterization of niosome. For two distinct variables, including SP60 : TW60 and CSL : VD3 ratio, RSM suggested 11 experimental runs consisting of additional 3 replicated center points (Table 1). The maximum and minimum levels of variables were selected based on preliminary studies and literature-reported values [11, 12].

*2.3. Sample Preparation.* Preparation of niosomes using the thin film hydration method was carried out according to the modified method described by Abaee and Madadlou [13]. Accurately weighed quantities of SP60 (100 mM), TW60

TABLE 1: Variables and levels and responses used in RSM.

| Run | Factor 1 SP60 : TW60 | Factor 2 CSL : VD3 | Responses Size (nm) | Span | EE (%) | Stability (%) |
|---|---|---|---|---|---|---|
| 1 | 60 : 40 | 0 : 100 | 76.13 | 4.19 | 1.12 | 0.462 |
| 2 | 100 : 00 | 50 : 50 | 335.77 | 0.59 | 81.14 | 0.456 |
| 3 | 88.28 : 11.72 | 14.65 : 85.35 | 142.10 | 3.17 | 21.42 | 0.467 |
| 4 | 31.72 : 68.28 | 85.36 : 14.64 | 136.33 | 1.07 | 23.25 | 0.576 |
| 5 | 88.28 : 11.72 | 85.36 : 14.64 | 443.33 | 0.25 | 60.46 | 0.422 |
| 6 | 60 : 40 | 50 : 50 | 95.50 | 0.23 | 89.56 | 0.455 |
| 7 | 60 : 40 | 50 : 50 | 99.90 | 0.15 | 90.36 | 0.447 |
| 8 | 60 : 40 | 100 : 0 | 332.10 | 1.16 | 2.63 | 0.379 |
| 9 | 31.72 : 68.28 | 14.65 : 85.35 | 71.23 | 1.67 | 71.13 | 0.611 |
| 10 | 20 : 80 | 50 : 50 | 77.50 | 0.09 | 91.17 | 0.656 |
| 11 | 60 : 40 | 50 : 50 | 97.50 | 0.16 | 90.56 | 0.441 |

SP60: Span 60, TW60: Tween 60, CSL: cholesterol, VD3: vitamin D3, and EE: encapsulation efficiency.

(100 mM), CSL (100 mM), and VD3 (100 mM) were dissolved in 100 mL of ethanol in a round-bottom flask. The organic solvent was then evaporated under vacuum and constant rotation at 60°C using a rotary evaporator (R3, BÜCHI Labortechnik AG, Flawil, Switzerland). The dried thin film was hydrated for 1 h with CBS buffer solution (1.13 mM) at 60°C under atmospheric pressure.

*2.4. Particle Size Analysis.* Particle size and span of niosomes were determined using dynamic light scattering (DLS) (Horiba SZ-100 system, Kyoto, Japan) at 25°C according to the method described by Ravaghi et al. [14]. The particle size distribution was measured as the span. For the measurement, niosome formulations were diluted (1 : 10) with water to avoid multiple scattering effects.

*2.5. Encapsulation Efficiency (EE).* The encapsulation efficiency (EE) of niosomes was evaluated by the spectrophotometry method according to the method proposed by Abaee and Madadlou [13]. Freshly prepared samples (1 mL) were centrifuged at 14000 g for 30 min at 4°C in Amicon filters to remove free VD3. The pellet was diluted in 50 mL of methanol to break the niosomal membrane. The obtained solution was mixed with methanol in a ratio of 1 : 9 (v/v) and analyzed with a spectrophotometer at 265 nm by using the standard curve of VD3 to determine the content of VD3 in niosomes. The EE was calculated by equation (1). Runs were carried out in triplicate, and results were expressed as mean ± standard deviation.

$$\text{Encapsulation efficiency (EE\%)} = \frac{\text{VD3 content in niosomes (mg)}}{\text{initial VD3 content used in niosomes preparation (mg)}} \times 100. \tag{1}$$

*2.6. Stability.* The absorbance of diluted aqueous dispersions (1 : 10) was measured at 600 nm using a UV-visible spectrophotometer (spec1650PC, Shimadzu, Osaka, Japan). The optical path length was 1 cm, and double distilled water (DDW) was used as the blank [15].

*2.7. Statistical Analysis.* The central composite design (CCD) is the most popular RSM. This is a good technique for optimizing the multiple process variables' influences on the sample properties using a combination of statistical and mathematical approaches. Optimization of data for extraction using various extractors was performed using the RSM method through Design Expert 11. A CCD consisting of 11 experimental runs were selected. In the process of optimization, experimental design and subsequent regression analysis of the experimental data were performed. Statistical analysis of the quadratic model was performed to evaluate the analysis of variance (ANOVA). The polynomial model equation's quality was statistically justified with respect to the determination coefficient ($R^2$), and its statistical significance was computed using an F-test approach.

The CCD approach generally consists of $2^k$ factorial runs with 2k axial runs and $n_c$ center runs, according to the following equation:

$$N = 2^k + 2k + n_c, \tag{2}$$

where $N$ represents the number of experiments and $k = 3$.

In this study, the independent variables that governed the production of niosome using CCD are grouped in Table 1.

# 3. Results and Discussion

The variables and levels of responses (including size, span, encapsulation efficiency, and stability) are summarized in Table 1.

*3.1. Particle Size Analysis.* The results of particle size as a function of preparation conditions are given in Table 2. Also, the fitted equation is shown in equation (3). The effects of quadratic parameters of SP60 : TW60 and CLS : VD3 ratio on the particle size were statistically significant ($P < 0.05$). The coefficient value of determination, $R^2$, of the predicted models was 0.999, and the $pp$ value for lack of fit of the model was 0.107 ($P > 0.05$) for particle size, respectively. These values provide a suitable fit to the mathematical model. According to Figure 1, increasing the SP60 : TW60 and CSL : VD3 ratios leads to higher values of particle size. The lowest particle size was observed at 40.46 : 59.54 for SP60 : TW60 and 38.06 : 61.94 for CSL : VD3 ratio. Similar results were reported by Basiri et al. [11]; they reported that with an increase in SP60 : TW60 ratio, the particle size of niosomes was increased. According to their results, the particle size of samples was between 106.8 and 190.2 nm, and the sample with the highest level of SP60 in the formulation had the highest particle size. Xu et al. [16] prepared curcumin-loaded niosome by solvent evaporation technique

and reported reducing the $z$-average and PDI by increasing HLB of the surfactant blend. Similar results were reported for ellagic acid niosomes [3]. Lower hydrophilicity and higher critical packing parameters of the SP60 versus TW60 led to the increase in the average volume sizes. Pando et al. [17] reported a clear relationship between the surfactant type and the particle size of the samples. CSL can align parallel to the hydrocarbon chains of the amphiphilic surfactants because of its unique geometrical packing characterization. The hydroxyl group of the sterol moiety of cholesterol forms a hydrogen bond with the ester group in nonionic surfactants, and thus, cholesterol can enhance the bilayer hydrophobicity, leading to a decrease in the surface free energy and therefore particle size reduction. Some properties of CSL, such as poor water solubility, rigid structure, and extreme hydrophobicity, create membrane-stabilizing properties for vesicles [18]. Attia et al. [19] reported that using high CSL in the formulation may lead to a large vesicle size in niosome.

$$Size = 289.09 - 7.58A - 5.08B + 0.059AB + 0.066A^2 + 0.04B^2. \tag{3}$$

Here, A is SP60 : TW60 and B is CSL : VD3.

*3.2. Span.* The results of span as a function of preparation conditions are given in Table 3. Also, the fitted equation is given in equation (4). The effect of quadratic parameters of SP60 : TW60 and CSL : VD3 ratios were statistically significant ($P < 0.05$) on the span. The coefficient values of determination, $R^2$, of the predicted models was 0.996, and the $p$ value for lack of fit of the model was 0.062 ($P > 0.05$) for span, respectively. These values provide a suitable fit to the mathematical model. According to Figure 2, the increase in the SP60 : TW60 ratio leads to higher values of span. The increase in CSL : VD3 ratio up to 67.67 : 32.32 resulted in decreasing span, but at a higher ratio, the trend was reversed. The lowest values for span were observed at ratios of 67.90 : 32.10 and 72.41 : 27.59 for SP60 : TW60 and CSL : VD3, respectively. The mean particle size and size distribution of niosomes play a key role in their biodistribution, and narrow size distribution is desired for nutraceutical carriers [8].

$$Span = +2.36 + 0.021A - 0.09B - 0.00057AB + 0.0001A^2 + 0.001B^2. \tag{4}$$

Here, A is SP60 : TW60 and B is CSL : VD3.

*3.3. Encapsulation Efficiency.* The aim was to substitute CSL with VD3 in the niosome. This may be possible due to the structural similarity of these two molecules. The research further examined the encapsulation of VD3. The results of encapsulation efficiency as a function of preparation conditions are given in Table 4. Also, the fitted equation was shown as follows: The effect of quadratic parameters of SP60 : TW60 and CSL : VD3 ratio was statistically significant ($P < 0.05$) on the encapsulation efficiency. The coefficient value of determination, $R^2$, of the predicted models was

TABLE 2: Estimated coefficients of multiple determinations ($R^2$) for particle size using coded values.

| Source | Sum of squares | df | Mean square | F-value | p value | |
|---|---|---|---|---|---|---|
| Model | $1.73 \times 10^5$ | 5 | 34592.0 | 1295.47 | <0.0001 | Significant |
| A-A | 69026.72 | 1 | 69026.7 | 2585.05 | <0.0001 | |
| B-B | 66307.10 | 1 | 66307.1 | 2483.2 | <0.0001 | |
| AB | 13939.74 | 1 | 13939.7 | 522.04 | <0.0001 | |
| $A^2$ | 15694.67 | 1 | 15694.7 | 587.77 | <0.0001 | |
| $B^2$ | 14954.34 | 1 | 14954.3 | 560.04 | <0.0001 | |
| Residual | 133.51 | 5 | 26.7 | | | |
| Lack of fit | 123.80 | 3 | 41.2 | 8.5 | 0.107 | Not significant |
| Pure error | 9.71 | 2 | 4.8 | | | |
| Cor total | $1.73 \times 10^5$ | 10 | | | | |

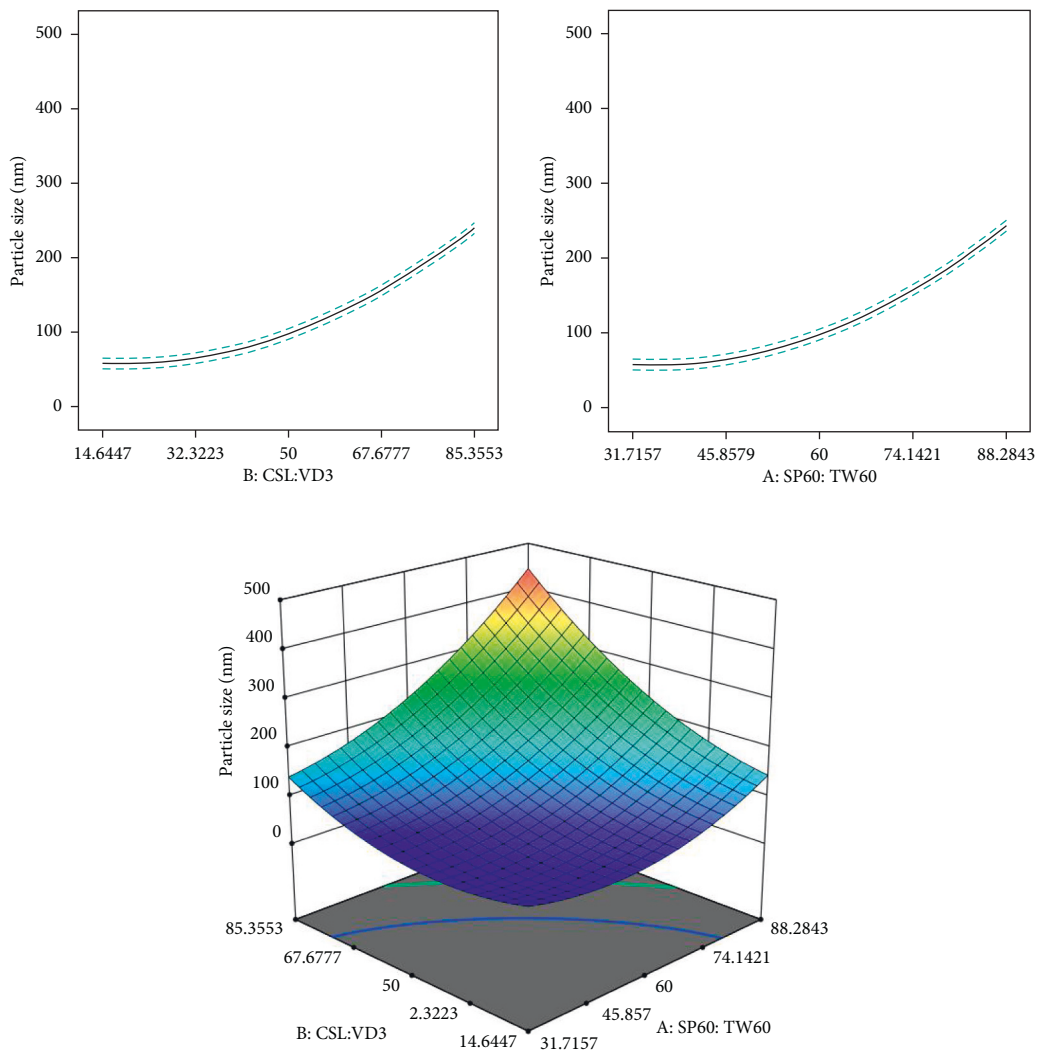$R^2$: 0.9992; adjusted $R^2$: 0.9985; predicted $R^2$: 0.9948.



FIGURE 1: Effects of Span 60: Tween 60 and cholesterol: vitamin D3 ratio on particle size of niosome.

0.998, and the $p$ value for lack of fit of the model was 0.052 ($P > 0.05$) for encapsulation efficiency, respectively. These values provide a suitable fit to the mathematical model. Entrapment of functional ingredients in a vesicle system may protect them from inactivation, help to store their activity for a prolonged time, and reduce their toxicity [20]. According to Figure 3, increasing the SP60 : TW60 ratio

leads to lower values of encapsulation efficiency. The highest values for encapsulation efficiency were observed at ratios of 31.76 : 68.24 and 40.02 : 59.98 for SP60 : TW60 and CSL : VD3, respectively. There is a direct relationship between niosome particle size and encapsulation efficiency. Similar results were reported by Noronha et al. [21] and Rovoli et al. [22]. These results are in good agreement with previous

TABLE 3: Estimated coefficients of multiple determinations ($R^2$) for span using coded values.

| Source | Sum of squares | df | Mean square | F-value | p value | |
|---|---|---|---|---|---|---|
| Model | 18.62 | 5 | 3.72 | 235.91 | <0.0001 | Significant |
| A-A | 0.2421 | 1 | 0.2421 | 15.34 | 0.0112 | |
| B-B | 7.65 | 1 | 7.65 | 484.50 | <0.0001 | |
| AB | 1.35 | 1 | 1.35 | 85.26 | 0.0003 | |
| $A^2$ | 0.0441 | 1 | 0.0441 | 2.79 | 0.1556 | |
| $B^2$ | 8.89 | 1 | 8.89 | 563.57 | <0.0001 | |
| Residual | 0.0789 | 5 | 0.0158 | | | |
| Lack of fit | 0.0756 | 3 | 0.0252 | 15.33 | 0.062 | Not significant |
| Pure error | 0.0033 | 2 | 0.0016 | | | |
| Corrected total | 18.69 | 10 | | | | |

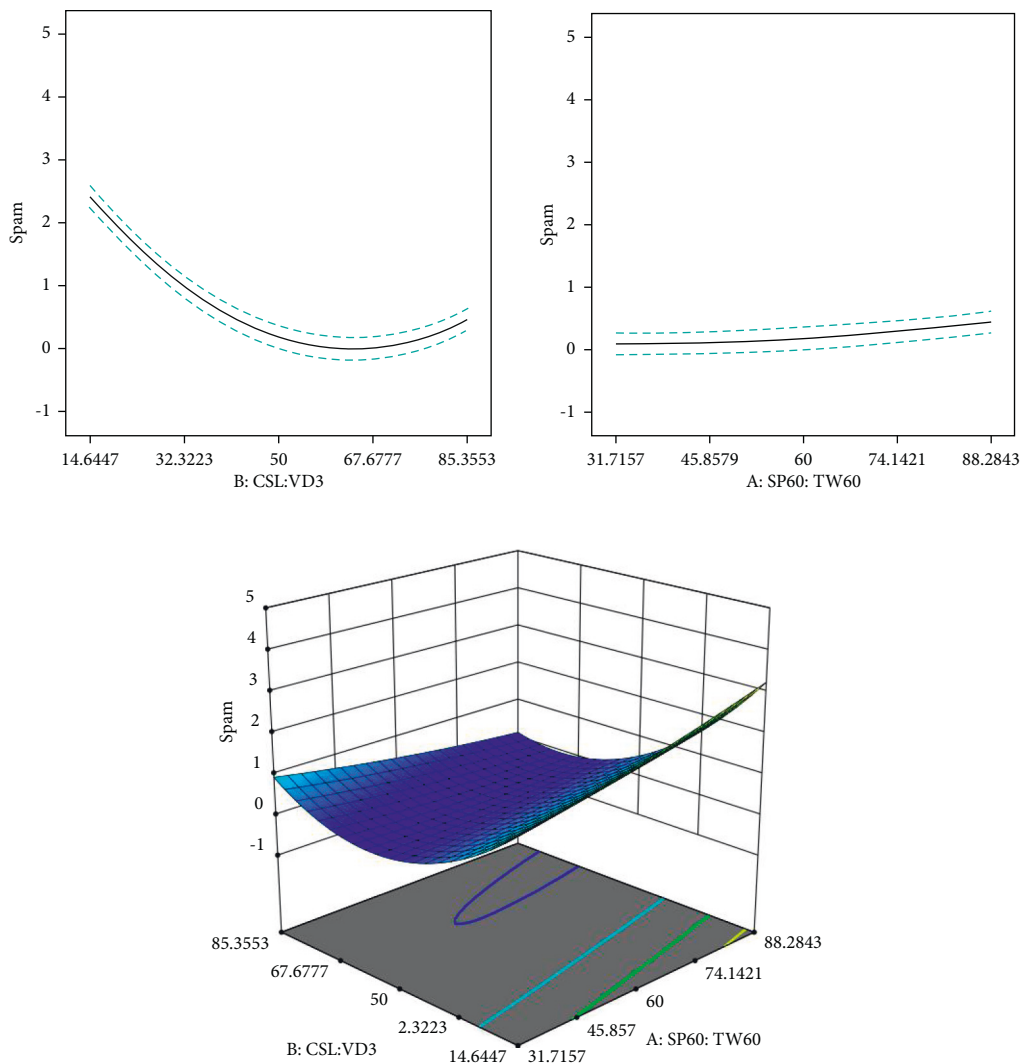$R^2$: 0.9958; adjusted $R^2$: 0.9916; predicted $R^2$: 0.9708.



FIGURE 2: Effects of Span 60: Tween 60 and cholesterol: vitamin D3 ratio on span of niosome.

research findings that encapsulation efficiencies are affected greatly by the type and content of the surfactants [4, 21, 23]. Mokhtar et al. [24] reported that the SP60 having the highest phase transition temperature provided the highest encapsulation of nutraceuticals. TW60 versus SP60 required considerably large amounts of CSL to obtain suitable entire

critical packing parameter values [25]. Therefore, it can be expected that the mixed component in the bilayer improves the membrane characteristic. Such a system is outstanding for entrapment of both lipophilic and hydrophilic compounds [5, 25, 26]. It is believed that CSL is one of the most important ingredients included in the formulation of

TABLE 4: Estimated coefficients of multiple determinations ($R^2$) for encapsulation efficiency using coded values.

| Source | Sum of squares | d$f$ | Mean square | $F$-value | $p$ value | |
|---|---|---|---|---|---|---|
| Model | 13727.9 | 5 | 2745.58 | 858.94 | <0.0001 | Significant |
| A-A | 89.02 | 1 | 89.02 | 27.85 | 0.0033 | |
| B-B | 5.62 | 1 | 5.62 | 1.76 | 0.242 | |
| AB | 1888.57 | 1 | 1888.57 | 590.83 | <0.0001 | |
| $A^2$ | 22.35 | 1 | 22.35 | 6.99 | 0.0457 | |
| $B^2$ | 10998.03 | 1 | 10998 | 3440.67 | <0.0001 | |
| Residual | 15.98 | 5 | 3.2 | | | |
| Lack of fit | 15.42 | 3 | 5.14 | 18.26 | 0.0524 | Not significant |
| Pure error | 0.5629 | 2 | 0.2814 | | | |
| Corrected total | 13743.89 | 10 | | | | |

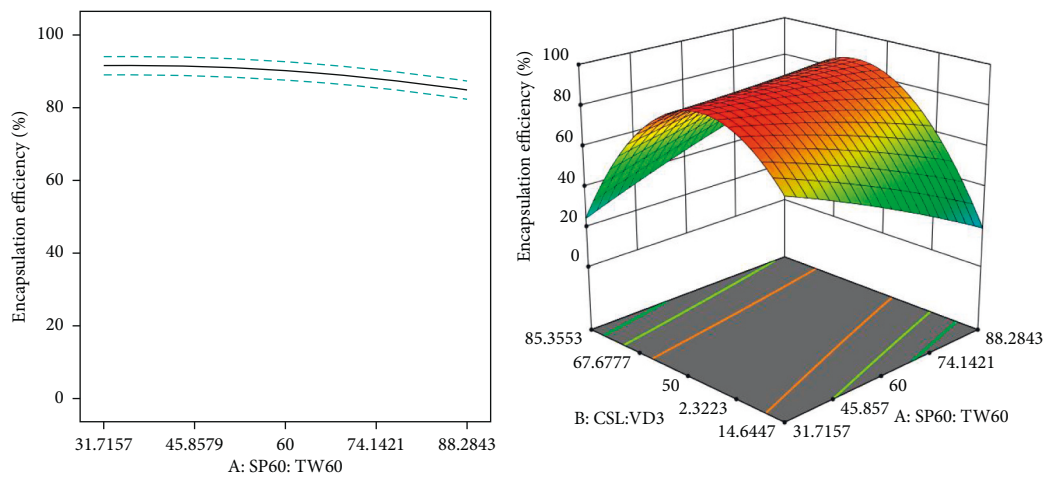$R^2$: 0.9988; adjusted $R^2$: 0.9977; predicted $R^2$: 0.9919.



FIGURE 3: Effects of Span 60: Tween 60 and cholesterol: vitamin D3 ratio on encapsulation efficiency of niosome.

niosome in order to prepare a stable system. CSL increases the chain order of liquid state bilayers. Therefore, CSL is known to abolish the phase transition of niosome systems [23, 27]. The positive effect of CSL could be due to the increased hydration of the thin lipid film, minimizing vesicle aggregation and incising the quality of the formed vesicle dispersion [18]. As shown in Figure 2, by increasing the ratio of TW60 in the surfactant mixture, CSL plays a prior role in increasing the encapsulation efficiency. Manosroi et al. [25] reported that niosomes with the 7 : 3 M ratio of SP60 : CSL gave the highest entrapment efficiency.

$$EE = +66.39 - 0.91A + 2.20B + 0.02AB$$
$$- 0.0025A^2 - 0.035B^2. \tag{5}$$

Here, A is SP60 : TW60 and B is CSL : VD3..

### 3.4. Stability.

The results of stability as a function of preparation conditions are given in Table 5. The quadratic parameters of SP60 : TW60 and CSL : VD3 ratios had a statistically significant ($P < 0.05$) effect on stability. The coefficient value of determination, $R^2$, of the predicted models was 0.970, and the $p$ value for lack of fit of the model was 0.066 ($P > 0.05$) for stability, respectively. These values

provide a suitable fit to the mathematical model. The stability of niosome suspensions has always been a crucial determinant for making use of these vesicles, a lasting alternative to the conventional delivery systems [28]. According to Figure 4, the increase in the SP60 : TW60 and CSL : VD3 ratios leads to lower values of stability. The highest values for stability were observed at 31.72 : 68.28 and 14.65 : 85.35 for SP60 : TW60 and CSL : VD3 ratios, respectively.

$$Stability = +0.89 - 0.012A - 7.61 \times 10 - 5 \times B - 2.49 \times 10 - 6$$
$$\times AB + 7.73 \times 10 - 5 \times A^2 - 4.72 \times 10 - 6 \times B^2. \tag{6}$$

Here, A is SP60 : TW60 and B is CSL : VD3.

### 3.5. Optimization.

Multiresponse optimization (MRO) was performed to investigate the optimal values of independent variables in order to reach the desired response goals. The objective was to acquire the lowest particle size and span and the highest stability and encapsulation efficiency at varying production parameters. The results show the optimal processing conditions based on a combination of all responses. The optimum conditions were 31.72 : 68.28 and 49.37 : 50.63 for SP60 : TW60 and CSL : VD3 ratios, respectively. The

TABLE 5: Estimated coefficients of multiple determinations ($R^2$) for stability using coded values.

| Source | Sum of squares | df | Mean square | F-value | p value | |
|---|---|---|---|---|---|---|
| Model | 0.0723 | 5 | 0.0145 | 32.95 | 0.0008 | Significant |
| A-A | 0.0422 | 1 | 0.0422 | 96.14 | 0.0002 | |
| B-B | 0.0049 | 1 | 0.0049 | 11.1 | 0.0207 | |
| AB | 0 | 1 | 0 | 0.057 | 0.8208 | |
| $A^2$ | 0.0216 | 1 | 0.0216 | 49.26 | 0.0009 | |
| $B^2$ | 0.0002 | 1 | 0.0002 | 0.4475 | 0.5332 | |
| Residual | 0.0022 | 5 | 0.0004 | | | |
| Lack of fit | 0.0021 | 3 | 0.0007 | 14.15 | 0.0667 | Not significant |
| Pure error | 0.0001 | 2 | 0 | | | |
| Cor total | 0.0745 | 10 | | | | |

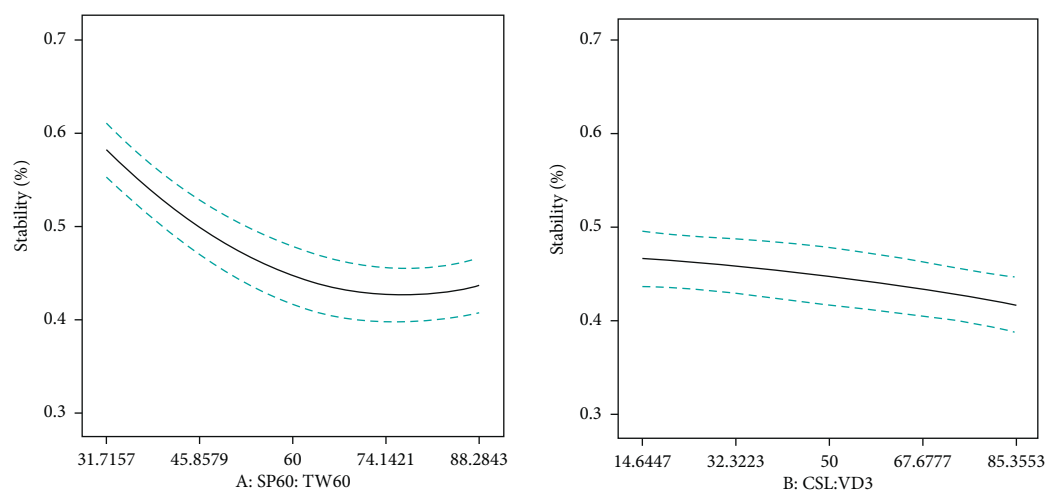$R^2$: 0.9705; adjusted $R^2$: 0.9411; predicted $R^2$: 0.7970.



FIGURE 4: Effects of Span 60: Tween 60 and cholesterol: vitamin D3 ratio on stability of niosome.

differences between the estimated and observed values were not significant ($P > 0.05$). These findings validated the model predictability for the niosome production from SP60 : TW60 and CSL : VD3 ratios for the designed experimental condition.

## 4. Conclusion

The main objective of this research was to evaluate CSL's lowering potential and its partial replacement with VD3 in the niosome structure. The results show that at high ratios of SP60 : TW60, higher values of particle size and span and lower values of encapsulation efficiency and stability are achieved. However, increasing the CSL : VD3 ratio led to stability being reduced. The optimum conditions were 31.72 : 68.28 and 49.37 : 50.63 for SP60 : TW60 and CSL : VD3 ratios, respectively. The findings indicate that partial replacement of CSL with VD3 improved technofunctional properties of niosome.

## Data Availability

No data were used to support this study.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

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