



Development and characterization of niosomal delivery system for betalain-rich extract from prickly pear (*Opuntia ficus-indica* L.)

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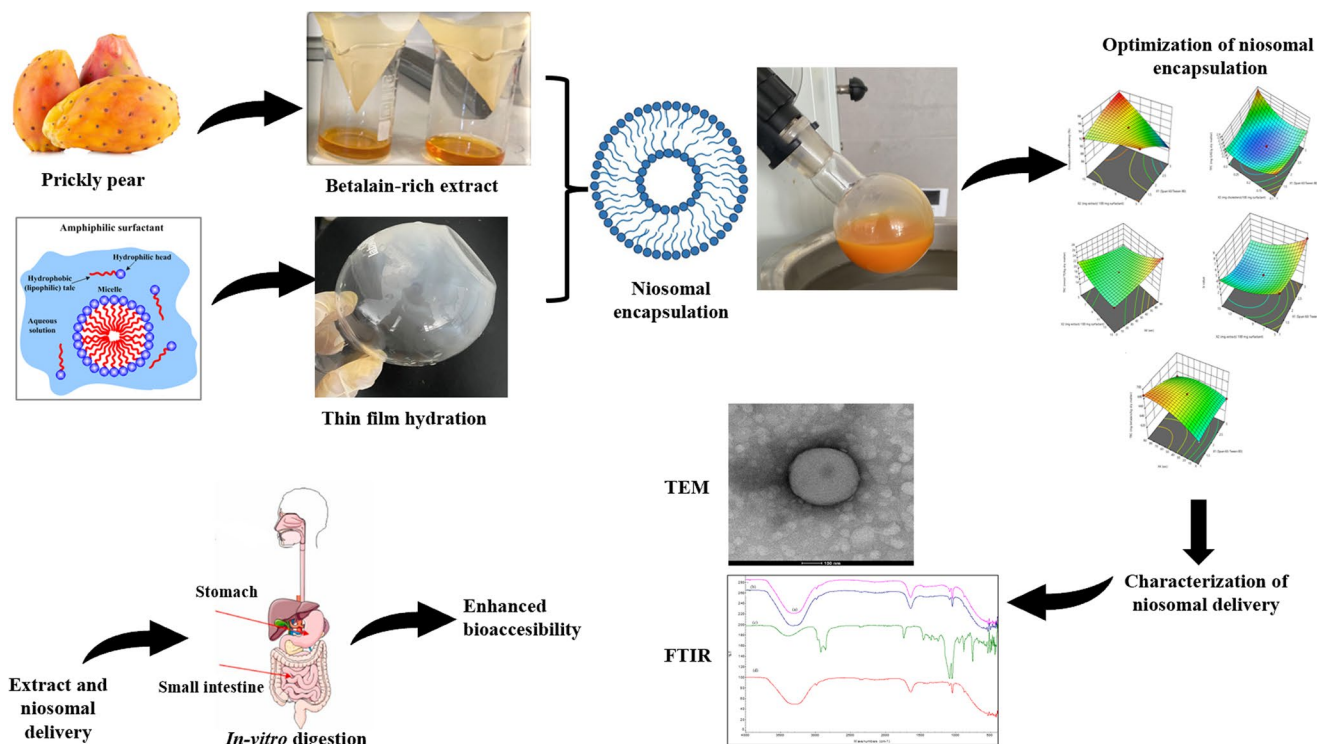
Abstract

Betalains are secondary metabolites that support their use as natural food colorants. Niosomes have been applied as encapsulation systems to enhance betalain stability. This study aimed to encapsulate betalain-rich extract from prickly pear using a niosomal system, with process parameters optimized by response surface methodology (RSM). Niosomes produced under optimum conditions were characterized. Optimum encapsulation was achieved at a Span 60:Tween 80 molar ratio of 1.161, 14.44 mg extract/100 mg surfactant, 0.1 mg cholesterol/100 mg surfactant, and 89.99 s ultrasonication. Under these conditions, encapsulation efficiency and TBC were 96.06% and 687.301 mg betalain/kg dry matter, respectively. Characterization results were consistent with model predictions. Following in-vitro gastrointestinal digestion, niosomal formulations enhanced the stability and controlled release of phenolics and betalains compared to the extract alone, rather than increasing their apparent total content. Overall, niosomal encapsulation represents a promising strategy for stabilizing betalain-rich extracts, offering potential as natural alternatives to synthetic food colorants while meeting consumer demand for functional ingredients.

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Graphical Abstract



Keywords *Opuntia ficus-indica* L. · Prickly pear · Betalains · Niosomal encapsulation · Optimization · Characterization

Abbreviations

ANOVA	Analysis of variance
BI	Bioaccessibility index
CTEM	High contrast transmission electron microscopy
EE	Encapsulation efficiency
EHS	Encapsulation heat stability
ES	Encapsulation stability
FTIR	Fourier transform infrared spectrophotometry
GAE	Gallic acid equivalence
HLB	Hydrophilic lipophilic balance
RSM	Response surface methodology
TEM	Transmission electron microscopy
TAC	Total antioxidant capacity
TBC	Total betalain content
TE	Trolox equivalence
TPC	Total phenolic content
VMAE	Vacuum microwave-assisted extraction

Introduction

Betalains are nitrogenous, water-soluble compounds that have emerged as promising natural food colorants [1]. Derived from the amino acid L-tyrosine, they are secondary

metabolites classified into two groups: betacyanins and betaxanthins [2, 3]. Due to the presence of betalamic acid in their structure, betalains act as effective electron donors with notable antioxidant activity [4]. The prickly pear (*Opuntia ficus-indica* L.) is a particularly rich source of betalains and can be utilized as a natural colorant in foods [5, 6].

A major challenge limiting betalain application is their instability under environmental conditions. Thus, stabilization strategies remain a central focus of betalain research [7]. Encapsulation using different matrices and techniques is an effective means to improve their stability [8]. Among these systems, niosomes—lipid nanovesicles composed of non-ionic surfactants—have gained attention [9]. Niosomes can encapsulate both hydrophobic and hydrophilic agents and are considered cost-effective and more stable alternatives to liposomes, which are less stable due to phospholipids and more expensive [10]. Furthermore, niosomes are biodegradable, biocompatible, and nontoxic [11].

Nanocarriers are extensively investigated in pharmaceutical delivery. For instance, Fahmy et al. (2023) encapsulated essential oils from *Pistacia lentiscus* resins in niosomes for cancer treatment [12]. However, food-grade applications of niosomes remain limited. In one study, niosomes with different surfactant combinations and additives were employed

to enhance quercetin absorption [11]. Gorjian et al. (2021) investigated niosomal encapsulation of myrtle (*Myrtus communis* L.) extract with medicinal properties, while Gutiérrez et al. (2016) entrapped iron in niosomes and applied the formulation to yogurt [13, 14]. These examples highlight the potential of niosomes in functional food systems, though research is still scarce.

From a nutritional standpoint, bioaccessibility refers to the fraction of compounds released from the matrix during gastrointestinal digestion [15]. In-vitro methodologies are widely used to assess this property. Parralejo-Sanz et al. (2023) reported that encapsulating phenolics and betalains from *Opuntia stricta* var. *dillenii* with a double emulsion method improved digestive stability and bioaccessibility [16]. Similarly, Cea-Pavez et al. (2024) compared different coating combinations for spray-dried propolis extract and found inulin/pectin and inulin/sodium alginate improved bioaccessibility during digestion [17]. Yeasmen and Orsat (2024) demonstrated that ultrasound-assisted phenolic extracts of sugar maple leaves, encapsulated via spray-and freeze-drying, achieved more than 43% higher intestinal bioaccessibility. Such findings emphasize the value of encapsulation in improving the digestive stability and release of bioactive compounds [18].

The present study aimed to encapsulate betalain-rich bioactive compounds extracted from prickly pear using vacuum microwave-assisted extraction, followed by niosomal encapsulation. To the best of our knowledge, this is the first study vacuum microwave assisted extracted of betalains from prickly pear being encapsulated in niosomal vesicles. Beyond introducing this novel application, the study systematically optimized encapsulation parameters. The optimized formulation was then characterized for encapsulation efficiency and thermal stability. Finally, the impact of encapsulation on betalain stability during simulated digestion and its effect on bioaccessibility were evaluated.

Table 1 The coded and actual values of the independent variables in niosomal encapsulation

Independent variables		Coded and actual values		
		−1	0	1
Molar ratio of surfactant (Span 60:Tween 80)	X ₁	1:1	2:1	3:1
Amount of prickly pear extract (mg extract/100 mg surfactant)	X ₂	5	10	15
Amount of cholesterol (mg cholesterol/100 mg surfactant)	X ₃	0.1	0.2	0.3
Ultrasonication duration (sec)	X ₄	0	45	90

Materials and methods

Plant material and chemicals

Orange-colored prickly pears (*Opuntia ficus-indica* L.) were supplied from a local producer (BECOS Agriculture, Mersin, Turkey). After separating the peels, the fruits were sliced, freeze-dried (Christ, Alpha 1–4 LD Plus, Osterode, Germany), ground (Fakir, Istanbul, Turkey), and stored at −20 °C until analysis and niosomal encapsulation.

Methanol, ethanol, gallic acid, and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Sodium carbonate, trolox, ammonium acetate, neocuprione, CuCl₂, Span 60, Tween 80, cholesterol, and chloroform were purchased from Sigma-Aldrich (Darmstadt, Germany).

Preparation of prickly pear extract

A vacuum microwave extractor (IFTech Food R&D Consulting, Ankara, Turkey) was used to extract betalain-rich bioactive compounds from prickly pear. The vacuum microwave-assisted extraction (VMAE) conditions were 110 mmHg, 2.5 min extraction time, 20% ethanol concentration, and a 1:30 solid-to-solvent ratio. After extraction, the solution was filtered twice, centrifuged at 14,000 rpm for 20 min (Sigma 3–18 K, Germany), and stored at −18 °C.

Formulation of niosomal encapsulation system

Niosomal encapsulation of prickly pear extract was carried out using the thin film hydration method with modifications [19]. Response surface methodology (RSM) was applied to optimize encapsulation parameters using Design Expert 13 (Statease Inc., Minneapolis, USA). The Box-Behnken design was used to evaluate four independent variables (X₁: surfactant molar ratio, Span 60:Tween 80; X₂: extract amount, mg/100 mg surfactant; X₃: cholesterol amount, mg/100 mg surfactant; X₄: ultrasonication duration, sec) on five dependent variables (Y₁: encapsulation efficiency, Y₂: TPC, Y₃: TAC, Y₄: b* value, Y₅: TBC). Levels of the independent variables were determined by preliminary experiments (Table 1). In total, 29 experiments were performed, including 5 replicated center points. Conditions of all experiments are shown in Table S1.

Span 60, Tween 80, and cholesterol were dissolved in chloroform: ethanol (2:1, v/v) and evaporated under vacuum at 50 °C and 120 rpm using a rotary evaporator (Heidolph, USA) to form a thin film. The prickly pear extract

was added to this film, followed by 30 min rotation without vacuum for complete mixing. To reduce and homogenize vesicle size, the mixture was ultrasonicated (Bandelin Sonoplus, Berlin, Germany) at 70% amplitude for different durations. The resulting niosomal vesicles loaded with extract were stored at 4 °C until analysis.

Analysis of total phenolics, antioxidant capacity and betalain content

The total phenolic content (TPC), total antioxidant capacity (TAC), and total betalain content (TBC) of niosomal vesicles were determined for each condition during optimization experiments. The niosomal samples were centrifuged at 14,000 rpm for 20 min, and analyses were performed using the supernatant [13, 20]. To calculate the amounts of bioactive compounds retained within the niosomal vesicles, TPC, TAC, and TBC values found in the supernatant fractions of the niosomal samples were subtracted from the values obtained during the optimum conditions of VMAE.

TPC was determined spectrophotometrically using the Folin–Ciocalteu method [21], and the results were expressed as mg gallic acid equivalent (GAE)/g dry matter. TAC was measured at 450 nm using a UV/VIS spectrophotometer (Thermoscientific, Genesys 10 S UV-Vis) [22]. The results were expressed as mmol trolox equivalent (TE)/kg dry matter. TBC was determined spectrophotometrically and calculated using the following Eq. 1;

$$\text{TBC (mg betalain/kg dry matter)} = \frac{A \cdot \text{df} \cdot M_w \cdot V_d \cdot 1000}{\epsilon \cdot L \cdot W_d} \quad (1)$$

where A: absorption value at 535 and 483 nm for betacyanins and betaxanthins, respectively, df: dilution factor, M_w : molecular weight (550 and 308 g/mol for betacyanin and betaxanthin, respectively), V_d : volume of sample (mL), ϵ : molar extinction coefficient (60000 and 48000 L/(mol cm) for betacyanin and betaxanthin, respectively), L: path-length (1 cm) of the cuvette and W_d : the amount of the extracted sample (g) [23].

Color analysis

The color analysis of each niosomal sample was conducted using a color spectrophotometer (CM-3600d, Konica Minolta, Netherlands). The results were expressed according to the CIELAB system, where b^* values range from +b (yellowness) to −b (blueness) [24].

Encapsulation efficiency

Encapsulation efficiency (EE %) was determined spectrophotometrically based on the amount of free and entrapped TBC of the niosomes [20]. EE% was calculated through the following Eq. 2;

$$\text{Encapsulation efficiency (EE\%)} = \frac{\text{TBC}_{\text{capsule}}}{\text{TBC}_{\text{capsule}} + \text{TBC}_{\text{free}}} \times 100 \quad (2)$$

Optimization and predictive analysis of niosomal parameters

Multiple regression analysis was performed to obtain quadratic model equations showing the effects of independent variables on the responses. The general form of this quadratic equation was given in Eq. 3, where y represents the response; β_0 , the constant coefficient; β_j , the linear coefficients; β_{jj} , quadratic coefficients; and β_{ij} , the interaction coefficients.

$$y = \beta_0 + \sum_{j=1}^k \beta_j x_j + \sum_{j=1}^k \beta_{jj} x_j^2 + \sum_{i < j} \beta_{ij} x_i x_j \quad (3)$$

The dependent variables were calculated and compared with the model predictions. Analysis of variance (ANOVA) was applied for each response, and the model's adequacy was assessed using the coefficient of determination (R^2), Fisher's test (F value) ($p < 0.05$), and lack-of-fit analysis. The combined effects of the independent variables on the responses were illustrated through response surface plots.

A desirability function approach was employed to determine the optimal formulation conditions for the niosomal system. The formulation condition in which the responses were maximized and the desirability value approached 1 was selected as the optimum. After conducting experiments under these predicted optimal conditions, the experimental data was used to validate the model by comparing the actual and predicted responses.

Characterization of optimized niosomal delivery systems

Fourier transform infrared (FTIR) spectrophotometry

Fourier transform infrared (FTIR) spectroscopies of the extract, solvent (20% ethanol solution), blank niosomal sample (without extract), and extract-loaded niosomal sample prepared under optimum conditions were determined

using an FTIR spectrophotometer (Thermo Scientific Nicolet iS50, USA) by utilizing KBr disc method [19, 25]. Spectra were recorded in the 500–4000 cm^{-1} spectral range with a nominal resolution of 4 cm^{-1} . The number of scans used in each analysis was 32.

Transmission electron microscopy (TEM) analysis

The size and the morphology of the niosome vesicles, prepared under optimum encapsulation conditions, were analyzed using high contrast transmission electron microscopy (CTEM) (FEI Tecnai G2 Spirit BioTwin, USA). The morphology of the vesicles was imaged on the first day of the encapsulation. Additionally, the same sample was stored in a dark place at +4 °C for 30 days, and morphological changes were examined after 1 month of storage. Before analysis, the vesicle suspension was stained with 1% phosphotungstic acid solution for 1 min. A drop of stained sample was placed onto a copper grid and allowed to dry for several minutes. Images were captured at an accelerating voltage of 120 Kv.

Size and zeta potential measurement

Particle size and zeta potential were measured using a Zetasizer Nano ZSP (Malvern Instruments Ltd, Malvern, UK). The niosomal suspension was diluted with distilled water at a ratio of 1:10. All measurements were performed in triplicate [19, 26].

Encapsulation and thermal stability evaluation

The niosome samples obtained under optimum conditions were stored in the dark at 4 °C for 30 days. To determine encapsulation stability (ES%) the TBC content of the samples was analyzed spectrophotometrically on the 0th, 14th and 30th days of storage [11]. The ES % was calculated using Eq. 4.

$$\text{Encapsulation stability (ES \%)} = \frac{\text{TBC}_{\text{nth day}}}{\text{TBC}_{0\text{th day}}} \times 100 \quad (4)$$

The encapsulation heat stability (EHS%) of the niosomal vesicles obtained under optimum conditions was also calculated. The niosomal samples were placed in a shaking water bath (Microtest, msc 30, Turkey) at 70 °C for 30 min. To determine the TBC content after heat treatment, the method of Elmi et al. (2021) was modified [19]. 2 mL of niosomal sample was mixed with 2 mL of 50% ethanol solution and centrifuged at 10,000 rpm for 10 min. The resulting pellet was treated with chloroform (1:1, v/v) to disrupt the vesicle

structure. The supernatant was then used for TBC analysis following heat treatment. EHS% was calculated using Eq. 5.

$$\text{Encapsulation heat stability (EHS\%)} = \frac{\text{TBC}_{\text{after heat treatment}}}{\text{TBC}_{\text{before heat treatment}}} \times 100 \quad (5)$$

Kinetic evaluation of betalain degradation

The TBC of the niosomal samples obtained under optimum encapsulation conditions was regularly measured during storage at 4 °C for 30 days. The TBC values were fitted by a first order kinetic model (Eq. 6) and the degradation rate constant was calculated from the slope of a plot of the natural log of TBC vs. time [27].

$$\ln(C) = \ln C_0 - k(t) \quad (6)$$

where C_0 is the initial TBC at day 0, and C is the TBC on the n^{th} day.

Simulated gastrointestinal digestion of extract and niosomal delivery system

In vitro digestibility of the extract and niosomal formulations prepared under optimum conditions was evaluated according to the method of Brodtkorb et al. (2019) with minor modifications [28]. Since the samples were in liquid form, the digestion process included gastric and intestinal phases. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as given in Table S2. For each sample, 5 mL was mixed with 5 mL SGF and 2.5 mL pepsin solution (12.5 mg/mL in 0.1 N HCl), and the pH was adjusted to 3. The mixture was incubated in a shaking water bath at 60 rpm and 37 °C for 2 h. Subsequently, 10 mL bile salt solution (10 mg/mL in SIF) and 2.5 mL pancreatin solution (10 mg/mL in distilled water) were added, and the pH was adjusted to 7.5. At the end of the intestinal phase, the temperature was rapidly reduced to stop enzymatic activity. The supernatants were collected by centrifugation at 10,000 rpm for 10 min at 4 °C. Total phenolic content (TPC), total antioxidant capacity (TAC), and total betalain content (TBC) were then analyzed as described in Sect. 2.4.

Statistical analysis

ANOVA results of each response during RSM were obtained using Design Expert 13 software. Means and standard deviations were calculated from triplicate analyses. One-way ANOVA was applied to the TPC, TAC, and TBC results from the in-vitro digestion study using MINITAB version

22 (State College, USA). Tukey's Multiple Comparison Test was used to identify significant differences ($p \leq 0.05$).

Results and discussion

Niosomal encapsulation

Five responses of the encapsulation experiments are summarized in Table 2. Encapsulation efficiency (Y_1) ranged from 87.66% to 97.62%. While X_2 and X_3 remained identical under the minimum and maximum efficiency conditions, changes in the surfactant molar ratio (X_1) and ultrasonication duration (X_4) were directly associated with efficiency [29]. A molar ratio of 2:1 (Span 60: Tween 80) resulted in higher encapsulation efficiency compared to a ratio of 3:1.

TPC values (Y_2) ranged from 2.17 to 2.86 mg GAE/g dry matter, showing little variation across formulations. TAC (Y_3) varied more substantially (14.96–26.24), with higher extract amounts (X_2) and longer ultrasonication times (X_4) increasing TAC, and a 2:1 surfactant ratio being favorable. Phenolic compounds are highly sensitive to heat, light, pH,

and oxygen but also exhibit strong antioxidant activity due to their unsaturated bonds [30]. Through niosomal encapsulation, these sensitive compounds can be protected from degradation, while the release rate is retarded [31]. In this study, the TPC and TAC of the prickly pear extract were effectively preserved.

The color parameter b^* (Y_4) ranged from 2.07 to 8.84, which can be directly related to the intense yellow pigmentation arising from the total betaxanthin content of niosomal samples [24]. Interestingly, the highest b^* value occurred at minimal extract content (X_2), producing a more intense yellow color. The hydrophilic-lipophilic balance (HLB) determines solubility, with values near 20 indicating hydrophilicity and values near 0 indicating hydrophobicity [32]. Span 60 has an HLB value of 4.7 and exhibits high solubility in the oil phase, while Tween 80, with an HLB value of 15, is more soluble in the aqueous phase [33]. Consequently, a surfactant ratio (X_1) of 3:1 slightly increased the overall HLB value, providing better preservation of water-soluble compounds.

TBC (Y_5) ranged from 627.17 to 698.45 mg betalain/kg dry matter, consistent with encapsulation efficiency (Y_1)

Table 2 The experimental results of the responses obtained from niosomal encapsulation (Y_1 : Encapsulation efficiency (%), Y_2 : TPC (mg GAE/g dry matter), Y_3 : TAC_{CUPRAC} (mmol TE/kg dry matter), Y_4 : b^* value in color analysis, Y_5 : TBC (mg betalain/kg dry matter))

Exp No.	Y_1	Y_2	Y_3	Y_4	Y_5
1	94.16±0.04	2.31±0.02	19.77±0.10	3.60±0.02	673.68±0.25
2	95.86±0.01	2.79±0.02	20.68±0.10	3.38±0.13	685.87±0.08
3	92.89±0.02	2.47±0.01	21.56±0.21	5.25±0.03	664.63±0.17
4	90.11±0.01	2.52±0.03	16.88±0.15	7.26±0.22	644.72±0.05
5	92.94±0.08	2.72±0.01	21.46±0.31	4.75±0.21	664.95±0.56
6	96.55±0.01	2.80±0.01	23.75±0.06	3.36±0.17	690.85±0.09
7	97.62±0.04	2.17±0.03	21.62±0.31	6.39±0.07	698.45±0.25
8	91.53±0.02	2.49±0.06	18.63±0.15	5.54±0.26	654.89±0.12
9	94.11±0.03	2.33±0.04	19.91±0.12	3.59±0.02	673.34±0.18
10	94.09±0.03	2.26±0.05	19.81±0.23	3.57±0.05	673.20±0.25
11	96.31±0.04	2.72±0.01	23.48±0.21	3.35±0.14	689.07±0.28
12	94.69±0.04	2.82±0.02	14.96±0.12	6.60±0.13	677.49±0.27
13	95.38±0.03	2.63±0.01	21.69±0.10	4.00±0.02	682.45±0.19
14	94.10±0.03	2.28±0.03	19.87±0.36	3.64±0.05	673.25±0.24
15	97.12±0.02	2.78±0.01	18.66±0.18	5.76±0.06	694.92±0.15
16	94.30±0.07	2.46±0.02	26.24±0.10	2.07±0.03	674.71±0.48
17	93.87±0.06	2.83±0.03	19.67±0.10	6.09±0.21	671.64±0.41
18	94.87±0.07	2.58±0.04	19.81±0.21	5.48±0.19	678.82±0.52
19	92.70±0.03	2.63±0.06	19.81±0.06	6.81±0.25	663.25±0.20
20	90.92±0.03	2.74±0.10	15.97±0.25	5.70±0.21	650.51±0.23
21	87.66±0.07	2.77±0.02	16.91±0.12	8.84±0.85	627.17±0.52
22	94.12±0.03	2.26±0.01	19.91±0.12	3.54±0.05	673.42±0.23
23	90.51±0.03	2.60±0.04	22.97±0.06	5.54±0.12	647.57±0.20
24	93.62±0.01	2.86±0.04	23.75±0.06	4.33±0.31	669.87±0.06
25	90.89±0.04	2.50±0.05	19.50±0.31	5.05±0.14	650.33±0.27
26	90.43±0.03	2.53±0.04	18.36±0.36	4.26±0.09	646.99±0.19
27	95.98±0.02	2.44±0.02	19.27±0.30	8.01±0.01	686.76±0.14
28	93.04±0.05	2.68±0.04	21.29±0.44	6.46±0.29	665.71±0.34
29	92.15±0.05	2.48±0.02	17.45±0.44	5.45±0.12	659.30±0.36

*Results were expressed as the mean and the standard deviation for three replicates

results. Surfactant molar ratio (X_1) and the ultrasonication duration (X_4) showed significant interactive effects. The coating composition is critical for stability, while ultrasonication and stabilizers affect release and membrane permeability [34]. Furthermore, cholesterol enhances the protection of the betalain-rich core material by positioning between the hydrocarbon chains of the surfactants. It strengthens the vesicle structure, reduces permeability, and prevents leakage [35].

Model fitting

For each encapsulation condition, the effects of four independent variables on the five responses were evaluated using multiple regression analysis. The effects were expressed as second-order polynomial model equations, and ANOVA was applied to confirm the significance, adequacy, and lack-of-fit. A coefficient of determination (R^2) above 0.75 was considered indicative of good model performance.

Response surface plots illustrated the binary effects of independent variables on the responses, and the coefficients of interaction terms in the polynomial equations corresponded well with the observed trends. Larger coefficients reflected more pronounced variations in the response surfaces.

Equation 7 demonstrates the effects of independent variables on encapsulation efficiency (Y_1).

$$Y_1 = 94.11 - 1.09 \times X_1 + 1.92 \times X_2 - 0.18 \times X_3 + 1.43 \times X_4 + 3.47 \times X_1 \times X_2 + 2.06 \times X_1 \times X_3 - 1.80 \times X_1 \times X_4 + 0.50 \times X_2 \times X_3 - 1.71 \times X_2 \times X_4 - 0.82 \times X_3 \times X_4 - 0.60(X_1)^2 - 0.19(X_2)^2 + 0.34(X_3)^2 - 1.26(X_4)^2 \quad (7)$$

As shown in Table S3, the ANOVA results confirmed that the model was significant ($p < 0.05$) with a non-significant lack of fit ($p > 0.05$). The adjusted R^2 value of 0.9996 indicated an excellent agreement between experimental and predicted Y_1 values. Equation 7 further revealed that X_1 , X_2 , X_3 , and X_4 exerted significant linear, quadratic, and interaction effects on encapsulation efficiency. Negative coefficients of X_1X_4 , X_2X_4 , and X_3X_4 reflected adverse effects of these interactions, whereas among quadratic terms, only cholesterol content (X_3) positively influenced Y_1 .

The model equation for TPC (Y_2) is presented in Eq. 8:

$$Y_2 = 2.29 + 0.12 \times X_4 - 0.16 \times X_1 \times X_2 + 0.16 \times X_1 \times X_3 - 0.16 \times X_2 \times X_4 + 0.16(X_1)^2 + 0.12(X_2)^2 + 0.23(X_3)^2 - 0.15(X_4)^2 \quad (8)$$

According to ANOVA (Table S4), the model fitted the experimental data well ($p < 0.05$) with a non-significant lack of fit, confirming adequacy. The R^2 value (0.9104) showed

good agreement between experimental and predicted values. In Eq. 8, the linear effects of X_1 , X_2 , and X_3 , and the interactions X_1X_4 , X_2X_3 , and X_3X_4 , were not significant for Y_2 . A positive interaction between X_1 (molar ratio of surfactants) and X_3 (cholesterol) indicated improved coating stability during encapsulation [29].

The second-order polynomial model (Eq. 9) describes the effects of the independent variables on TAC (Y_3):

$$Y_3 = 19.85 - 1.48 \times X_1 + 0.79 \times X_2 - 0.50 \times X_3 + 0.97 \times X_4 + 0.19 \times X_1 \times X_2 - 1.86 \times X_1 \times X_3 - 2.38 \times X_1 \times X_4 - 0.33 \times X_2 \times X_3 + 2.47 \times X_2 \times X_4 + 2.05 \times X_3 \times X_4 - 1.83(X_1)^2 + 1.34(X_2)^2 + 0.64(X_3)^2 + 0.80(X_4)^2 \quad (9)$$

This model was statistically significant ($p < 0.05$) with an adjusted R^2 of 0.9983 (Table S5). Unlike TPC, all linear, quadratic, and interaction coefficients were significant for TAC. Negative effects of X_1 (surfactant ratio) and X_3 (cholesterol) contrasted with positive contributions from X_2 (extract amount) and X_4 (ultrasonication). Positive interactions of X_1X_2 , X_2X_4 , and X_3X_4 highlighted their role in enhancing TAC, while the quadratic effect of X_1 reduced TAC.

Equation 10 describes the effect of the four independent variables on b^* value (Y_4).

$$Y_4 = 3.59 + 0.17 \times X_1 - 1.48 \times X_2 + 0.27 \times X_3 - 0.92 \times X_4 - 0.39 \times X_1 \times X_2 + 0.52 \times X_1 \times X_3 + 1.02 \times X_1 \times X_4 + 0.07 \times X_2 \times X_3 - 0.56 \times X_2 \times X_4 + 0.33 \times X_3 \times X_4 + 1.48(X_1)^2 + 1.00(X_2)^2 + 0.59(X_3)^2 + 0.42(X_4)^2 \quad (10)$$

As shown in Table S6, the model was significant ($p < 0.05$) with a non-significant lack of fit ($p > 0.05$). The adjusted R^2 of 0.9988 indicated excellent agreement. All terms were significant, with X_2 (extract amount) exerting the strongest effect. The positive interaction X_1X_4 improved coating stability and color retention. Since b^* represents chromaticity along the blue–yellow axis, higher values indicated greater betaxanthin content in niosomal formulations [36, 37].

Equation 11 represents the effects of independent variables on the TBC response (Y_5):

$$Y_5 = 673.38 - 8.96 \times X_1 + 10.46 \times X_2 - 2.12 \times X_3 + 11.52 \times X_4 + 20.89 \times X_1 \times X_2 + 13.11 \times X_1 \times X_3 - 9.88 \times X_1 \times X_4 - 1.04 \times X_2 \times X_3 - 8.71 \times X_2 \times X_4 - 7.93 \times X_3 \times X_4 - 5.06(X_1)^2 - 0.78(X_2)^2 + 2.36(X_3)^2 - 10.99(X_4)^2 \quad (11)$$

ANOVA results (Table S7) confirmed that the model was significant with no lack of fit ($p > 0.05$). The R^2 value of 0.9994 indicated strong agreement between experimental and predicted data. All independent variables significantly affected TBC, with similar trends to encapsulation

efficiency (Y_1) [13]. Positive effects of X_2 and X_4 suggested that increasing extract amount and ultrasonication enhanced TBC, while higher quadratic terms of X_1 , X_2 , and X_4 reduced it. These findings emphasize the importance of surfactant ratio, extract amount, and sonication in determining TBC.

Literature also confirms that variations in coating combinations, core-to-coating ratios, and encapsulation techniques strongly influence betalain retention [38–40].

All response surface plots are shown in Figures S1–S5, while Fig. 1 illustrates selected binary effects of the

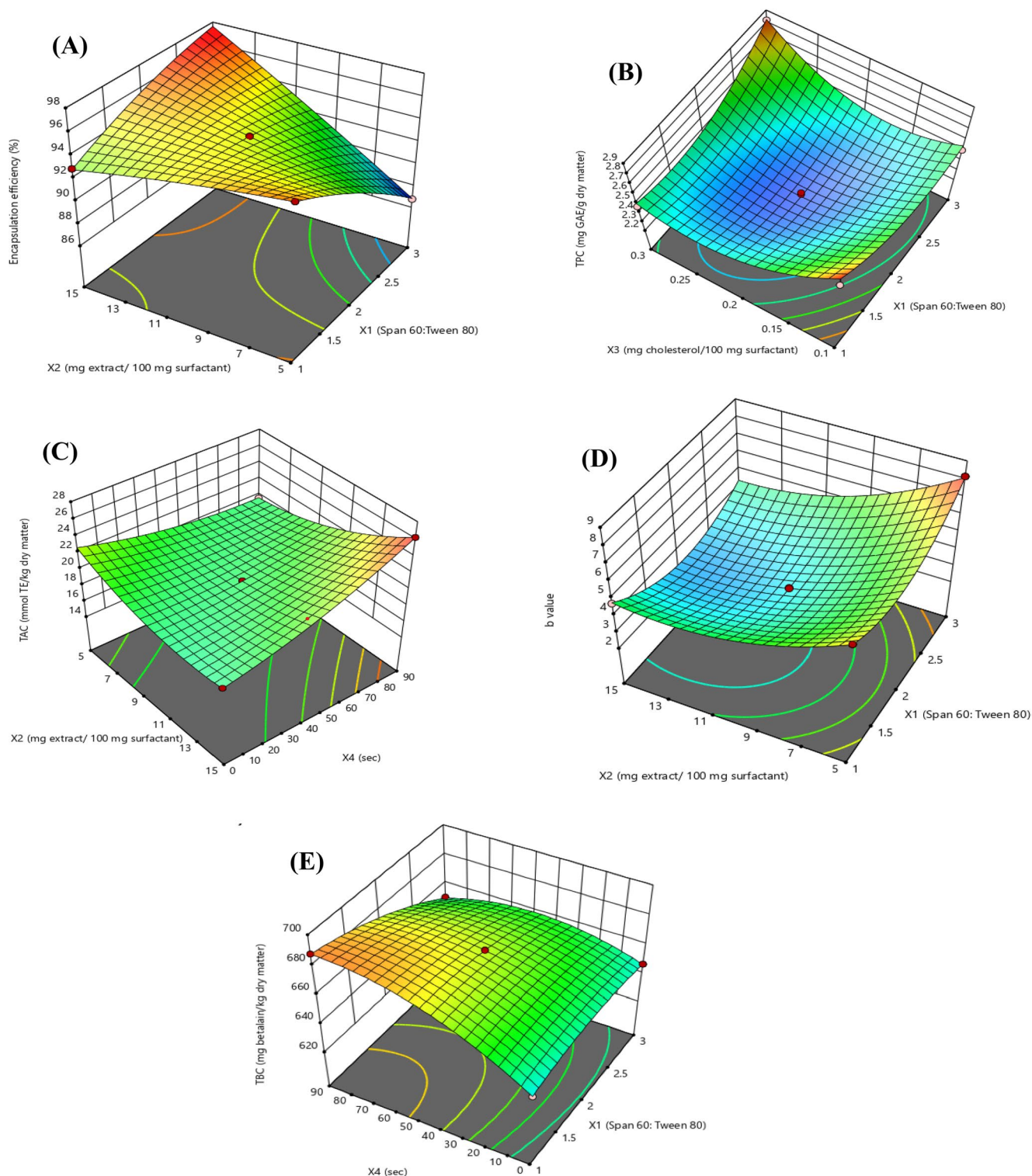


Fig. 1 Selected response surface plots for the effects of independent variables on Y_1 , Y_2 , Y_3 , Y_4 and Y_5

independent variables. As presented in Fig. 1A, encapsulation efficiency was maximized when X_1 was at its highest and X_2 at its lowest level. Achieving high encapsulation efficiency and chemically stable niosomes requires optimization of the surfactant molar ratio according to their hydrophilic–lipophilic balance (HLB). Since HLB values directly influence core entrapment, higher HLB leads to increased vesicle size and alkyl chain length, thereby affecting encapsulation efficiency [41]. Consistent with Eq. 7, the interaction between X_1 and X_2 showed the largest coefficient, confirming its dominant role in determining Y_1 .

In Fig. 1B, the binary effect of X_1 and X_3 on the TPC (Y_2) response is shown, with TPC predicted to reach its minimum at the central levels of these variables. The surfactant molar ratio (X_1) and cholesterol amount (X_3) were key parameters in forming a stable coating material in niosomal formulations. Incorporation of cholesterol or its derivatives enhances stability and regulates membrane flexibility through interactions with nonionic surfactant combinations [10]. Thus, selecting an appropriate X_1 – X_3 combination was crucial to preserve phenolic compounds within vesicles. As illustrated in Fig. 1C, TAC values increased when both ultrasonication duration (X_4) and extract amount (X_2) were at their highest levels, while other X_2 – X_4 combinations showed nearly constant values. The binary effects on TPC and TAC were consistent in the niosomal encapsulation process.

The binary effect of X_1 and X_2 on the Y_4 response is shown in Fig. 1D. The b^* value reached its maximum when extract content was at the lowest level and the Span 60: Tween 80 molar ratio at the highest. This was reasonable, as lower extract combined with a more concentrated coating promoted stronger color formation in vesicles, increasing yellow pigmentation and b^* value [37]. Figure 1E illustrates the Y_5 response, where TBC approached its maximum as ultrasonication duration (X_4) increased and the surfactant ratio (X_1) decreased. Although ultrasonication is not essential in encapsulation, it homogenizes particle size and reduces vesicle size. Previous studies also showed that optimized sonication improved encapsulation efficiency and stability of niosomal carriers [42].

Table 3 Experimental and estimated responses in optimum conditions of niosomal encapsulation (Y_1 : Encapsulation efficiency (%), Y_2 : TPC (mg GAE/g dry matter), Y_3 : TAC (mmol TE/kg dry matter), Y_4 : b^* value in color analysis, Y_5 : TBC (mg betalain/kg dry matter))

Y_i	Experimental response ^a	Estimated response
Y_1	96.060±0.014	96.356
Y_2	3.062±0.059	3.126
Y_3	24.405±0.263	25.197
Y_4	2.810±0.07	2.749
Y_5	687.301±0.010	695.848

^aExperimental responses were expressed as the mean and the standard deviation for three replicates

Process optimization of niosomal encapsulation

The optimal encapsulation conditions were determined using the desirability function, which yielded a value of 0.959. This method, ranging from 0 to 1, is useful for optimizing multiple responses simultaneously; values closer to 1, indicate strong agreement between experimental and predicted responses [43]. The optimal conditions for niosomal encapsulation were $X_1 = 1.161$, $X_2 = 14.440$ mg extract/100 mg surfactant, $X_3 = 0.1$ mg cholesterol/100 mg surfactant, and $X_4 = 89.99$ s. As shown in Table 3, the estimated responses under these conditions were $Y_1 = 96.356$, $Y_2 = 3.126$ mg GAE/g dry matter, $Y_3 = 25.197$ mmol TE/kg dry matter, $Y_4 = 2.749$, and $Y_5 = 695.848$ mg betalain/kg dry matter. The experimental results closely matched these predictions: $Y_1 = 96.060$, $Y_2 = 3.062$ mg GAE/g dry matter, $Y_3 = 24.405$ mmol TE/kg dry matter, $Y_4 = 2.810$ and $Y_5 = 687.301$ mg betalain/kg dry matter.

The hydrophilic–lipophilic balance (HLB) of nonionic surfactants, ranging from 0 to 20, directly influences niosome properties such as shape and stability. Low HLB values (< 9) indicate lipophilicity, while higher values (> 11) denote hydrophilicity. For spherical and homogeneous niosomes, an HLB range of 4–8 is recommended [44]. In a related study, ionic gelation was used to encapsulate betalains from red beet juice, optimized via RSM. Under optimal conditions, TBC decreased from 295.52 to 234.87 mg/100 g dry weight after six weeks of storage, underscoring the importance of optimized conditions for efficiency and stability [45].

Characterization of optimized niosomal delivery systems

Fourier transform infrared (FTIR) spectrophotometry

FTIR analysis was conducted to identify functional groups in the betalain-rich extract and to evaluate molecular interactions in the niosomal system. Spectra of extract-loaded niosomes, betalain-rich extract, blank niosomes, and the extract solvent (20% ethanol) were analyzed (Fig. 2). In Fig. 2d, absorption bands at 880.28, 1046.39, and 2983.05 cm^{-1} were attributed to O–H stretching of ethanol [46]. In the spectra of betalain-rich extract and extract-loaded niosomes (Fig. 2a and b), peaks at 1641.87 and 1455.49 cm^{-1} corresponded to asymmetric and symmetric C = O stretching [47]. The betalain-rich extract also showed an absorption band at 1080.70 cm^{-1} , assigned to C–O–C symmetric stretching, while C–H bond deformation appeared near 1046.26 cm^{-1} . Additionally, the band at 876.08 cm^{-1} was related to C–COOH stretching. These findings are consistent

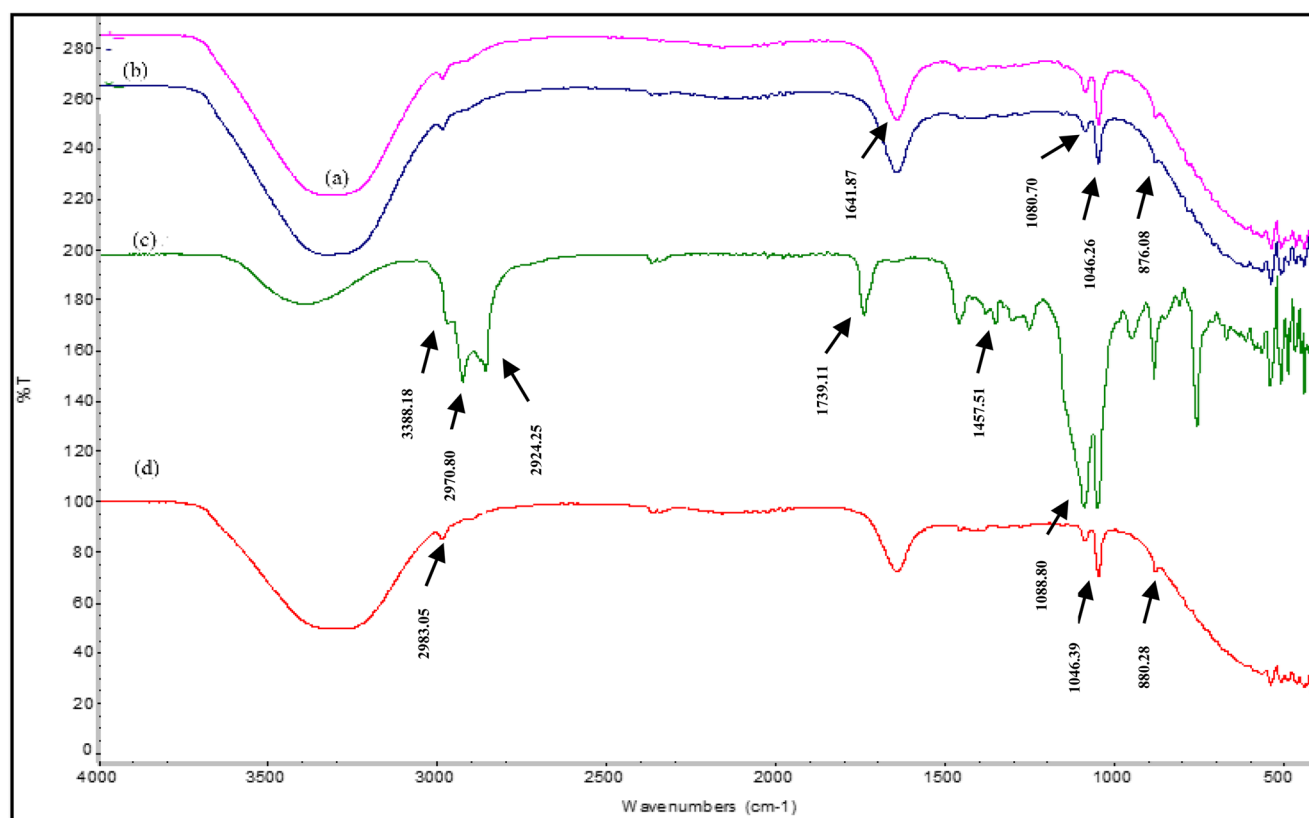


Fig. 2 FTIR spectra of (a) extract-loaded niosomal vesicle, (b) extract, (c) blank niosome, (d) solvent of the extract

with previous studies using red prickly pear extract in pH-indicator film production [48].

From the blank niosome spectrum, hydrogen bonding between cholesterol hydroxyl groups and Span 60 carbonyl groups was evident, enhancing vesicle rigidity [49]. Span 60 exhibited two characteristic peaks at 2924.25 and 3388.18 cm^{-1} , associated with hydroxyl and aromatic CH_3 vibrations. A peak at 1739.11 cm^{-1} was attributed to C–O stretching of ester groups [50]. Cholesterol showed characteristic peaks at 2970.85 and 2855.37 cm^{-1} due to C–H stretching of methylene and methyl groups [50]. In Fig. 2c, the band at 1088.80 cm^{-1} was assigned to C–O vibrations, while the 1457.51 cm^{-1} band corresponded to methylene groups of the fatty acid/acyl chain in Tween 80 [51].

Transmission electron microscopy (TEM)

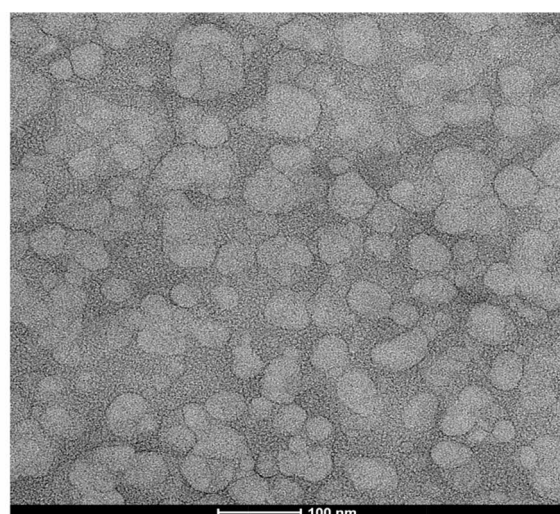
The transmission electron micrographs of niosomal vesicles under optimum encapsulation conditions are shown in Fig. 3. On day 1 (Fig. 3A and B), vesicles appeared compact and spherical, whereas after 30 days (Fig. 3C), their sphericity declined, indicating partial structural changes [52]. The particle size on day 1 was approximately 250 nm, consistent with particle size results, but homogeneity decreased after 30 days. The presence of cholesterol maintained

bilayer rigidity, reducing leakage and improving stability [29]. Similarly, Machado et al., (2021) reported that adding dodecanol as a membrane stabilizer enhanced stability and promoted spherical, homogeneous vesicles, as confirmed by their TEM images [53].

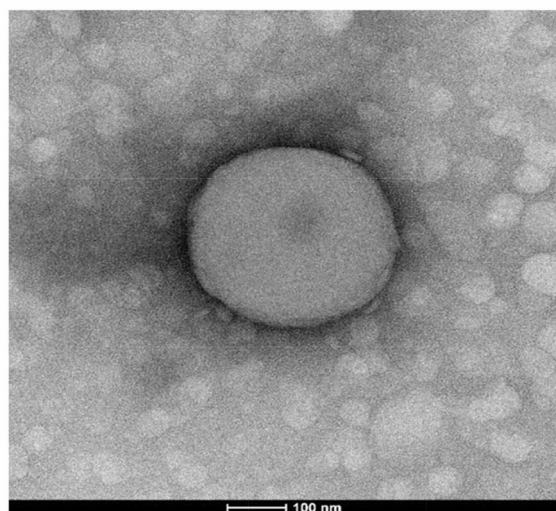
Size and zeta potential of niosomal vesicles

Niosomal samples can be categorized into three groups based on vesicle size: large unilamellar vesicles (> 100 nm), multilamellar vesicles (> 50 nm), and small unilamellar vesicles (25–50 nm) [29]. The particle size and zeta potential values on the 1st day of storage were 221.70 ± 1.78 nm and -19.30 ± 0.52 mV, respectively, corresponding to large unilamellar vesicles. Particle size and zeta potential varied depending on formulation parameters, such as surfactant type, molar ratio, the presence or absence of cholesterol, and the application of ultrasonication [44].

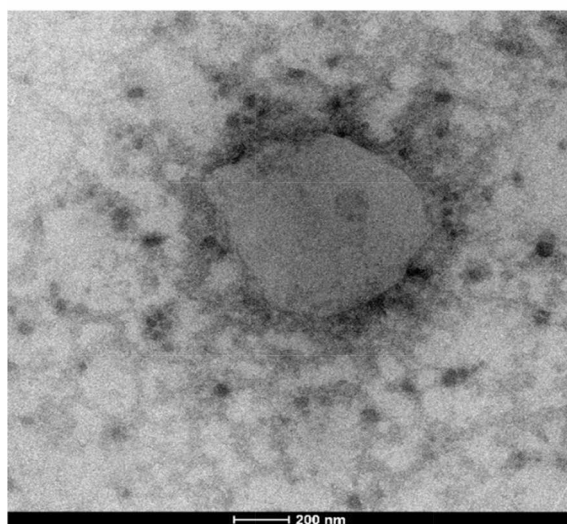
According to literature, increasing the HLB value is generally associated with larger nanovesicle size. In this context, a higher molar proportion of Span 60 compared to Tween 80 was a reasonable choice to ensure spherical morphology and smaller vesicle size in the optimized formulation [44]. Moreover, ultrasonication is essential to achieve homogeneous particle size distribution [54].



(A)



(B)



(C)

Fig. 3 Transmission electron micrographs of niosomal vesicles obtained from optimum conditions: (A) and (B) for 1st day at 23,000 x and 49,000 x magnifications, respectively and (C) for 30th day at 49,000 x magnification

Zeta potential reflects the degree of electrostatic repulsion between similarly charged particles. Low values may allow attractive forces to dominate, leading to aggregation, whereas higher magnitudes indicate stability [19]. In this study, the negative zeta potential confirmed that the lipid nanovesicles formed a stable dispersion, with surfaces carrying a net negative charge [55]. Murugesan et al. reported similar results, with niosomal vesicles showing a particle size of 194 nm and a zeta potential of -20 mV, consistent with our findings [56].

On the 30th day of storage, the particle size and zeta potential values were 143.17 ± 2.38 nm and -19.93 ± 0.98 mV, respectively. While the particle size decreased compared to day 1, zeta potential remained statistically unchanged. The slight reduction in size may be attributed to partial disruption and disintegration of vesicle integrity during storage.

The observed trend of the particle size reduction may be attributed to structural reorganization within the niosomal system. Over time, the surfactant bilayer may undergo rearrangement, leading to tighter molecular packing and the formation of compact and thermodynamically stable vesicles. Additionally, the release of loosely bound or surface-associated compounds, as well as the possible dispersion of initially aggregated vesicles, could contribute to the decrease in average particle size. Similarly, Talebi et al. (2021) reported a decrease in particle size after 40 days of storage for vitamin D₃ encapsulated in different niosomal formulations [57].

Encapsulation and thermal stabilities of niosomal delivery system

Encapsulation and thermal stability experiments were performed on niosomal formulations obtained under optimum conditions. The stability of the vesicles was strongly influenced by bilayer fluidity, which is affected by the phase transition temperature of the nonionic surfactants, the length of the hydrocarbon chain, as well as storage time and temperature [11, 58]. After 0, 14, and 30 days of storage, the encapsulation stability values were 100%, 93.85%, and 74.98%, respectively, under optimum conditions. Stability could be improved by modifying the surfactant type and molar ratio to increase the phase transition temperature. Surfactants with higher hydrophilicity may enhance membrane packing. The presence of Span 60 in the nonionic surfactant mixture reduces interactions between the polar head groups of amphiphilic molecules, thereby increasing bilayer rigidity [59].

The heat stability of the optimized niosomal formulation was 92.91% when stored at + 70 °C for 30 min. This high thermal stability can be attributed to the inclusion of cholesterol, which intercalates between the hydrocarbon chains of the surfactants, thereby reinforcing the bilayer, reducing leakage, and enhancing membrane rigidity [60]. Despite these improvements, colloidal nanovesicular systems such as niosomes remain thermodynamically unstable at elevated temperatures, and the use of additional stabilizers may further enhance their heat resistance.

Kinetic evaluation of betalain degradation

The degradation rate constant of betalains in niosomes under optimum encapsulation conditions was obtained from the slope of the plot of natural log (ln) of TBC versus time, with a coefficient of determination (R^2) of 0.9404 (Fig. 4). The degradation of TBC followed first-order reaction kinetics. The experiments were conducted under constant conditions (+4 °C, stable pH, and absence of light), indicating that degradation was mainly associated with oxidation.

According to the literature, betalain oxidation begins with the formation of betalain radicals by interaction with oxygen molecules. These radicals subsequently polymerize, leading to disruption of the betalain chromophore structure and progressive color fading during storage [61]. Similar results were reported by Güneşer (2016), who demonstrated first-order kinetics for betalain degradation in cow milk during heating [62]. Yang et al. also observed first-order kinetics for betalains from red beet used as colorants in white currant juice, whereas anthocyanins from grape did not exhibit a clear kinetic trend under the same conditions [63]. Likewise, Trishitman et al. investigated betalain stability in yogurt formulations containing beetroot juice concentrate,

and the degradation of betalains was also consistent with first-order kinetics [64].

In-vitro bioaccessibility of betalain-rich extract and niosomal vesicles

The variations in TPC, TAC, and TBC of the extract and niosomal formulations under optimum conditions before and after in vitro gastrointestinal digestion, together with the bioaccessibility index (BI), are shown in Table 4. BI (%) was calculated by dividing the amount of bioactive compounds after the intestinal phase by that before digestion and multiplying by 100 [65].

Before digestion, TPC, TAC, and TBC values of the extract were higher than those of the niosomal formulation, likely due to partial loss of bioactivity during encapsulation [66]. Phenolic compounds and antioxidant activity are strongly influenced by digestive enzymes, bile salts, and pH variations [67]. After digestion, both extract and niosomal samples showed a significant increase in TPC and TAC, attributed to enzymatic cleavage that released bound bioactive compounds and activated previously inactive phenolics and antioxidants [68]. Although niosomal values were initially lower, after digestion they reached levels like the extract. Consequently, BI values of the niosomal samples were higher, indicating improved bioaccessibility, consistent with Otálora et al. (2023), who reported higher TAC bioaccessibility in spray-dried dragon fruit extract compared to the free extract when incorporated into gummy formulations [69].

For TBC, the extract showed a greater increase than the niosomal formulation after digestion, reflected in higher BI values. This suggests that encapsulation forms a barrier around betalains, reducing drastic changes during digestion

Fig. 4 The equation for betalain degradation rate during 30 days of storage

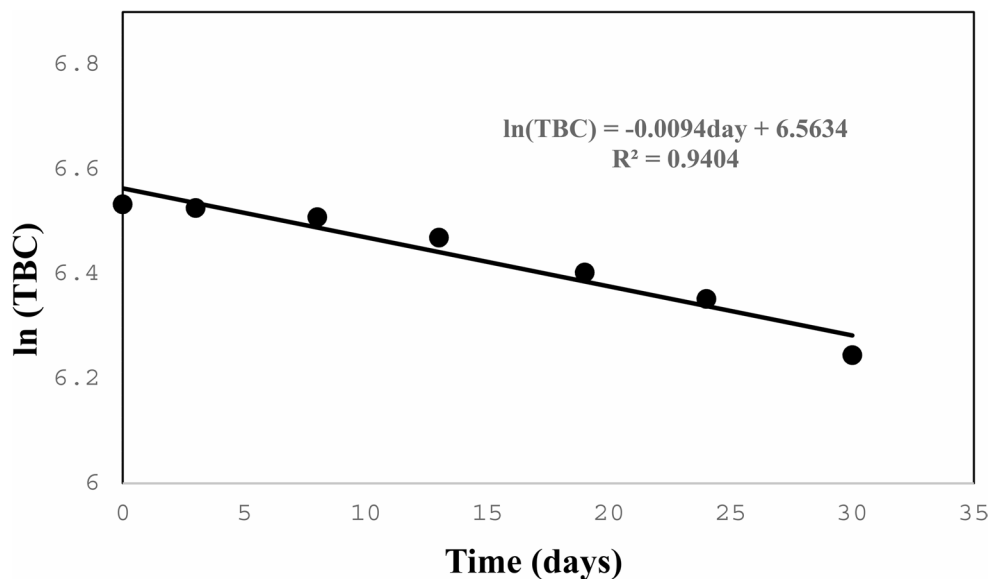


Table 4 In-vitro digestibility results of the extract and niosome samples obtained from optimum encapsulation conditions (TPC: Total phenolic content (mg GAE/g dry weight), TAC: Total antioxidant capacity (mmol TE/kg dry weight), TBC: Total betalain content (mg betalain/kg dry weight))

Extract	TPC	TAC	TBC
Before digestion	7.78 ^c ±0.13	47.06 ^b ±0.43	715.50 ^b ±5.50
After gastric digestion	10.38 ^b ±0.11	46.98 ^b ±0.96	645.47 ^c ±18.19
After intestinal digestion	16.75 ^a ±0.03	98.28 ^a ±1.13	2181.36 ^a ±17.76
Bioaccessibility index (%)	215.30	208.84	304.87
Niosome	TPC	TAC	TBC
Before digestion	3.06 ^c ±0.06	24.40 ^c ±0.26	687.30 ^b ±0.10
After gastric digestion	9.86 ^b ±0.24	91.04 ^a ±0.26	503.77 ^c ±3.52
After intestinal digestion	15.99 ^a ±0.34	88.42 ^b ±0.81	830.29 ^a ±18.51
Bioaccessibility index (%)	522.55	362.38	120.80

*Results were evaluated separately for extract and niosome, the different lowercase letters in the same column indicated statistically significant differences before and after each stage of digestion ($p < 0.05$). Results were expressed as the mean and the standard deviation for three replicates

[65]. Following the gastric phase, TBC values decreased in both samples but increased again in the intestinal phase, as betalains are more stable at pH 5–7 [4]. The intestinal environment favored stabilization and reactivation of betalains. Moreover, prickly pear betalains are mainly betaxanthins, which are more resistant to gastric and intestinal digestion than betacyanins due to their lower hydrophobicity. Similarly, Grace et al. (2025) showed that while betacyanins degraded after digestion, betaxanthins were fully preserved in beetroot betalains encapsulated by freeze- and spray-drying with pea protein [70].

Conclusion

Color is a key determinant of consumer acceptance of foods, and the growing demand for natural additives has promising interest in betalains due to their dual role as colorants and bioactive compounds. In this study, vacuum microwave assisted betalain-rich extracts obtained from prickly pear were successfully encapsulated for the first time using niosomal carrier systems, with formulation parameters systematically optimized by a Box–Behnken design within RSM. The influence of critical process variables on multiple response parameters was comprehensively evaluated, modeled and the optimized formulation of niosomal encapsulation was thoroughly characterized through FTIR, TEM, particle size, and zeta potential analyses, confirming the formation of stable vesicular structures. Furthermore, storage stability results

demonstrated a controlled degradation profile of betalains over 30 days at +4 °C. Simulated in vitro gastrointestinal digestion revealed that niosomal encapsulation significantly enhanced the retention and protection of betalains, thereby preserving their bioactive properties from degradation. Collectively, the optimized niosomal formulation enhanced the stability and functionality of betalain-rich prickly pear extract. These results offer a promising basis for the incorporation of natural colorants in diverse food systems, and future studies could further investigate the application of these formulations into different food matrices and evaluate their long-term stability and functional performance under industrial conditions.

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Declarations

Competing interest The authors declare that they have no conflict of interest.

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