

Article

Spirulina-Stabilized Emulsions as Sustainable Delivery Systems for β -Carotene-Rich Sunflower Oil Extracted from *Dunaliella salina*

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Abstract

The incorporation of lipophilic bioactive compounds into aqueous food matrices requires delivery systems capable of improving their dispersion and storage stability. This study developed oil-in-water emulsions containing β -carotene-rich sunflower oil extracted from *Dunaliella salina*, using a commercial blue *Spirulina* extract as a bio-based emulsifier. The *Spirulina* extract concentration was initially screened from 0.5 to 3 wt.%, and the processing conditions were subsequently optimized through a central composite design and response surface methodology. The systems were characterized in terms of droplet size distribution, physical stability, apparent encapsulation efficiency, microstructure, and β -carotene retention at 4 and 25 °C. A *Spirulina* concentration of 2.5 wt.% produced the smallest droplet size during the preliminary screening, whereas 3 wt.% resulted in larger droplets and broader distributions. The optimized formulation exhibited a $D_{3,2}$ of 0.680 μ m, an apparent encapsulation efficiency of $91.4 \pm 2.1\%$, and a Turbiscan Stability Index of approximately 4.8 after 30 days. β -Carotene retention after 30 days was 68.5% at 25 °C and 91.3% at 4 °C, compared with 41.8% and 80.7%, respectively, in the non-emulsified oil. These results support the potential of *Spirulina*-stabilized emulsions as sustainable carriers for carotenoid-enriched food products.

Keywords: ultrasonication; response surface methodology; clean-label emulsifier; encapsulation efficiency; carotenoid retention; physical stability



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1. Introduction

The advancement of functional foods increasingly depends on the integration of bioactive compounds into complex food matrices, with an emphasis on minimizing losses during processing and storage [1]. Emulsions, particularly submicron- and nanoemulsions, are among the most extensively studied food-grade colloidal delivery systems for this purpose [2]. These systems facilitate the dispersion of lipophilic compounds in aqueous products, can be engineered to alter appearance and texture, and can be customized to influence chemical stability and gastrointestinal fate [3]. In this context, the term “innovative food technologies” encompasses not only novel ingredients and formulations

but also processing methods that allow for precise control over microstructure, such as droplet-size distributions and interfacial architecture, while minimizing thermal impact [4]. High-energy emulsification is particularly pertinent as it facilitates the production of small droplets with relatively narrow size distributions, which are crucial for the physical stability of emulsions and certain aspects of their chemical stability, provided that formulation, device configuration, and processing conditions are optimally adjusted [5].

A significant and rapidly evolving trend is the transition towards “clean-label” and sustainability-focused ingredients, which involves substituting synthetic surfactants or animal-derived emulsifiers with materials derived from plants or microorganisms [6,7]. Proteins from microalgae and cyanobacteria have been identified as promising interfacial stabilizers due to their adsorption and film-forming capabilities at oil–water interfaces, with their functionality being highly dependent on extraction and purification methods as well as environmental conditions such as pH and ionic strength [8].

Carotenoids are esteemed in food products for their pigmentation and bioactive properties. Nonetheless, many carotenoids, particularly β -carotene, exhibit chemical instability due to their conjugated double-bond structures, rendering them susceptible to oxidative degradation and isomerization [9]. These processes can be exacerbated by exposure to light, oxygen, elevated temperatures, and pro-oxidant environments. Instability is a fundamental reason why encapsulation strategies, including emulsion-based approaches, are extensively researched for carotenoid delivery in food products. In emulsion-based delivery systems, β -carotene is typically dissolved in the oil phase and “protected” to the extent that the interfacial layer and surrounding continuous phase limit the access of pro-oxidants and mitigate environmental stresses [10]. However, encapsulation does not ensure chemical stability: factors such as droplet size, interfacial composition, emulsifier type, ionic environment, and storage conditions (pH, temperature, light) can significantly influence degradation kinetics [11]. A classic example is that β -carotene can be effectively entrapped in food-grade emulsions yet still undergo progressive chemical degradation during storage, as evidenced by color fading that depends on storage temperature and pH [12]. From a processing and scale-up perspective, high-energy emulsification techniques (such as high-pressure homogenization, microfluidization, and ultrasonication) are frequently employed to create carotenoid-loaded (nano)emulsions with enhanced physical stability [13]. For instance, β -carotene nanoemulsions have been produced using high-pressure homogenization, demonstrating resistance to coalescence during storage and significant retention after thermal treatments, although retention remains contingent on temperature and light exposure history [14]. Studies utilizing microfluidization similarly indicate that increased processing pressure and appropriate cycle numbers can markedly reduce droplet size for β -carotene emulsions, achieving physical stability over weeks at ambient temperature in model systems; however, formulation factors, notably emulsifier type, continue to govern degradation rates and storage outcomes [13]. Among carotenoid-producing microalgae, *Dunaliella salina* is particularly relevant because of its well-known ability to accumulate high amounts of β -carotene under stress conditions such as high salinity, intense light exposure or nutrient limitation [15]. This halotolerant green microalga has therefore been widely explored as a natural and sustainable source of β -carotene for food, nutraceutical and cosmetic applications [16]. In addition, the lack of a rigid cell wall in *D. salina* facilitates the release of intracellular carotenoid-rich structures during extraction, making it especially suitable for the development of mild and food-compatible extraction strategies [17]. In this sense, the direct extraction of β -carotene into an edible lipid phase represents an attractive approach, as it avoids the use of conventional organic solvents and generates a carotenoid-enriched oil that can be directly employed as the dispersed phase in emulsion-based delivery systems [18].

The constituents of “Blue Spirulina” are predominantly sourced from phycocyanin-rich extracts of *Arthrospira* biomass, which are esteemed for their inherent blue pigmentation and biological properties [19,20]. Nonetheless, these extracts are recognized for their susceptibility to environmental stressors, including heat and light [21]. The sensitivity of phycocyanin has led to research focused on processing and formulation strategies to enhance its stability and functionality, including the use of ultrasound treatment and encapsulation techniques [22]. From the perspective of colloid science, proteins derived from microalgae and cyanobacteria serve as emulsifiers by adsorbing at oil–water interfaces and forming viscoelastic interfacial films [23]. Notably, the processes of extraction and purification have a significant impact on their performance. Mechanistic evidence suggests that purified fractions of *Arthrospira* proteins can establish stronger interfacial networks and stabilize smaller droplets when normalized by protein concentration [24]. In contrast, co-extracted surface-active components in less refined extracts may hinder protein adsorption. These findings support the broader assertion that Spirulina-derived emulsifiers do not constitute a single material class but rather represent a spectrum whose interfacial behavior is contingent upon composition and processing history. Recent studies have identified phycocyanin as not merely a colorant [25] but also as a functional interfacial component [26]. Specifically, phycocyanin has been documented to function as a stabilizer and co-emulsifier in edible oil-in-water emulsions, in conjunction with plant proteins [27]. This dual role influences droplet charge and stability while concurrently providing blue coloration. The findings are particularly relevant to systems employing blue Spirulina extracts, where the functions of pigment and emulsifier may overlap.

Ultrasonication and microfluidization are two distinct methodologies employed to produce fine emulsions, each with unique operational mechanisms and associated challenges [28]. Ultrasonication utilizes sound waves to generate cavitation bubbles, which subsequently fragment droplets into smaller sizes. Critical parameters influencing droplet size and stability include the type of ultrasound device, power, duration, temperature, and pre-mixing procedures [29]. Repeated processing can further reduce droplet size; however, excessive processing may compromise emulsion stability. Both techniques are capable of producing minuscule droplets, yet they are optimized under different conditions. Research indicates that the selection of an appropriate method is contingent upon the specific ingredients and processing conditions. In ultrasonication, the use of a probe is often more effective than a bath for achieving smaller droplets. Factors such as processing time, emulsifier concentration, and temperature are pivotal for maintaining stability. Both methods are utilized in encapsulation and nutrient delivery within food systems. Microfluidization is closely associated with food processing, whereas ultrasonication is recognized as a low-heat, energy-efficient technique that can enhance emulsifier performance.

Droplet formation and stability are contingent upon a range of interacting variables, including oil fraction, emulsifier concentration and composition, pre-emulsification intensity, device settings, temperature history, and storage pH/ionic strength. Consequently, systematic multivariate optimization is generally more effective than one-variable-at-a-time approaches for developing predictive understanding and identifying robust optima. Emulsion research offers numerous instances where statistical designs quantify the significance of factors and their interaction structures, and where sequential strategies reduce experimental burden while enhancing model relevance [30]. Within this context, the present manuscript examines oil-in-water emulsions formulated with sunflower oil as the lipid carrier, water as the continuous phase, and a “blue Spirulina” extract as the emulsifier. In this system, the oil phase functions as a carrier for β -carotene (provitamin A) previously extracted from algal biomass. The central technological inquiry is how a high-energy emulsification method (ultrasonication) controls droplet size and physical stability when the

system is optimized using design of experiments (DoE) and response surface methodology (RSM) rather than one-factor-at-a-time experimentation. Furthermore, the apparent encapsulation efficiency of β -carotene within the developed systems was assessed to evaluate its potential as a delivery vehicle for lipophilic bioactive compounds.

2. Materials and Methods

2.1. Materials

An extract rich in β -carotene was derived from the microalgal biomass of *Dunaliella salina* through oil-based extraction utilizing sunflower oil procured from a local market. The resulting oily extract was used as the dispersed lipid phase in the emulsification experiments. A commercial phycocyanin-rich Blue Spirulina powder (Ecospirulina, Valencia, Spain), containing 49 wt.% protein, was used as the bio-based emulsifying ingredient. The material was obtained from a local supplier and used as received. Since the product was a commercial extract rather than purified phycocyanin, it may also have contained other Spirulina-derived components, including soluble proteins, pigments, carbohydrates, minerals, and residual solids. Detailed compositional information regarding these individual fractions was not provided by the supplier. Deionized water was used to prepare the continuous aqueous phase in all formulations.

2.2. β -Carotene Extraction from *Dunaliella salina*

β -Carotene was extracted from *Dunaliella salina* biomass utilizing an environmentally sustainable oil-based ultrasound-assisted extraction method. The microalgal biomass was sourced from natural salt production ponds situated in the Sfax region of southern Tunisia. The strain was morphologically characterized using optical microscopy (microscope CX43 (Olympus Life Science, Tokyo, Japan), revealing typical ovoid to ellipsoidal cells and the absence of a rigid cell wall, a structural attribute that enhances solvent penetration and facilitates the release of intracellular pigments. Following the harvest, the biomass was concentrated and maintained under low-temperature and dark conditions to mitigate carotenoid degradation. The extraction process was conducted in batch mode utilizing refined sunflower oil as an environmentally friendly lipophilic extraction medium. Specifically, 1 g of fresh *Dunaliella salina* paste was combined with 50 mL of sunflower oil in 100 mL Erlenmeyer flasks, resulting in a biomass-to-oil ratio of 1:50 (*w/v*). Sunflower oil was chosen due to the lipophilic properties of β -carotene, facilitating its direct transfer into the lipid phase and allowing the subsequent use of the enriched oil as the dispersed phase in emulsion formulation. Ultrasound-assisted extraction was conducted utilizing a probe sonicator operating (Ultrasonic Processor Model CV334 (Sonics & Materials, Newtown, USA) at approximately 20 kHz. The procedure was executed at 50% amplitude for a duration of 15 min in pulsed mode, employing 5-s ON and 5-s OFF cycles. Throughout the sonication process, the temperature was meticulously monitored, commencing at 30 °C and not exceeding 45 °C, to prevent thermal degradation and isomerization of β -carotene. The extraction mechanism is primarily attributed to acoustic cavitation, which generates microbubbles that collapse violently, resulting in localized shear forces, micro-turbulence, and partial cell disruption. The effect is particularly pertinent to *Dunaliella salina*, as the absence of a rigid cell wall facilitates the release of intracellular carotenoid-rich lipid globules. Following sonication, the mixture underwent centrifugation at 3000 rpm for 20 min to separate the residual biomass from the β -carotene-enriched oil phase. The supernatant oil fraction was meticulously recovered, shielded from light, and stored at a low temperature until further utilization. All extraction assays were conducted in triplicate. The β -carotene extraction yield was quantified spectrophotometrically and expressed as micrograms of β -carotene per gram of biomass. Under the specified extraction conditions, a

β -carotene yield of 823.3 $\mu\text{g/g}$ biomass was achieved, affirming the efficacy of this solvent-free oil-based strategy for generating a carotenoid-enriched lipid phase for subsequent emulsion development.

2.3. Sample Development

Prior to the multivariate optimization of the emulsion formulation and processing conditions, a preliminary investigation was conducted to determine the optimal concentration of Spirulina extract to be employed as a bio-based emulsifying and stabilizing agent. In this study, batches of 150 g oil-in-water emulsions were prepared with varying Spirulina extract concentrations, ranging from 0.5 to 3 wt.%, while maintaining constant formulation parameters, including an oil concentration of 10 wt.%. The continuous phase was formulated by dissolving the emulsifier in the requisite amount of distilled water. The dispersed phase was prepared using an appropriate quantity of β -carotene-rich sunflower oil. All samples were processed under fixed homogenization conditions, which were subsequently selected as the central point of the experimental design. The formulation of these emulsions was achieved through an initial homogenization process employing an Ultraturrax T25 rotor-stator homogenizer (IKA, Staufen, Germany). The procedure was conducted in a discontinuous mode at a rotational speed of 17,500 rpm for 120 s. To achieve an emulsion with reduced droplet size, the emulsion underwent a secondary homogenization process using a VCX750/VCX500 ultrasonic system (Sonics & Materials Inc., USA) equipped with an 830-00427 S-250D acoustic chamber. This secondary homogenization was performed at an amplitude of 50 with a 5s:5s pulse over 450 s. This preliminary approach facilitated the isolated examination of the effect of Spirulina extract concentration on droplet formation, droplet size distribution, and initial physical stability.

An experimental design methodology was subsequently employed to optimize the processing conditions, facilitating a systematic evaluation of the combined effects of the selected homogenization variables on emulsion droplet size and physical stability.

This procedure was conducted in a discontinuous mode at a speed of 17,500 rpm, with varying homogenization times. The independent variables investigated included the primary homogenization time, the amplitude and duration of ultrasonication and sonication time. A three-factor rotatable central composite design (CCD) was employed to map the combined influence of processing variables. The design space thus encompassed primary homogenization times from 29 to 211 s, sonication amplitudes ranging from 25 to 75% and likewise sonication times ranging from 149 to 751 s. A total of seventeen experimental runs were generated using Echip v11 (Experimentation by Design, Wilmington, DE, USA). The runs comprised eight factorial points, six axial points, and three center-point replicates (see Table 1). Two responses were measured: (i) the Sauter mean diameter $D_{3,2}$ (mm) and (ii) span, immediately after emulsification.

Table 1. Experimental design used for the secondary homogenization using sonication with indication of actual values of the independent variables.

Sample	Primary Homogenization Time (s)	Sonication Amplitude (%)	Sonication Time (s)
1	174	35	629
2	211	50	450
3	120	75	450
4	120	50	450
5	120	50	149
6	120	50	450
7	66	65	629

Table 1. Cont.

Sample	Primary Homogenization Time (s)	Sonication Amplitude (%)	Sonication Time (s)
8	120	50	450
9	174	35	271
10	120	50	751
11	120	25	450
12	66	65	271
13	66	35	629
14	174	65	271
15	174	65	629
16	66	35	271
17	29	50	450

2.4. Emulsion Characterization

The size distribution of the emulsion droplets was assessed at the time of preparation utilizing a Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments, Worcestershire, UK). Furthermore, the Sauter mean diameters ($D_{3,2}$) were calculated, which serve as a metric for characterizing a dispersed system by correlating the total volume of droplets with their surface area. Mathematically, it represents the diameter of a sphere that possesses the same volume-to-surface-area ratio as the entire droplet population:

$$D_{3,2} = \frac{\sum_{i=1}^N n_i d_i^3}{\sum_{i=1}^N n_i d_i^2} \quad (1)$$

where d_i is the diameter of droplets belonging to size class, n_i is the number of droplets in that size class, and N is the total number of size classes considered. The width of the droplet size distribution was evaluated using the span parameter, calculated as:

$$span = \frac{D(v, 0.9) - D(v, 0.1)}{D(v, 0.5)} \quad (2)$$

where $D_{(v,0.1)}$, $D_{(v,0.5)}$, and $D_{(v,0.9)}$ correspond to the droplet diameters below which 10%, 50%, and 90% of the cumulative dispersed-phase volume are found, respectively. Since droplet size was determined by laser diffraction, span was used as the descriptor of distribution width rather than the polydispersity index commonly obtained by dynamic light scattering. Lower span values indicate narrower and more homogeneous droplet size distributions. Samples for FESEM were prepared using a chemical fixation method, according to Vela-Albarrán et al. [31].

To evaluate and monitor the physical stability of the samples, multiple light scattering (MLS) measurements were conducted using a Turbiscan (Formulation, L'Union, France). This non-intrusive technique enables the detection of destabilization prior to its visibility to the naked eye. The procedure was repeated at various aging intervals to assess the temporal stability or instability of the different emulsions. Furthermore, the Turbiscan stability index (TSI) was calculated for all samples [32].

The apparent encapsulation efficiency and storage stability of β -carotene within the formulated emulsions were assessed by measuring the β -carotene content retained in the dispersed oil phase. The apparent encapsulation efficiency of β -carotene was determined by a mass-balance approach. The initial amount of β -carotene incorporated into the analyzed emulsion sample, C_0 , was calculated from the β -carotene concentration previously determined in the enriched sunflower oil and the mass of oil contained in the sample. After disruption of the emulsion matrix and two consecutive extractions with ethanol/n-hexane

(2:3, v/v), the organic phases were combined and analyzed by UV–Vis spectrophotometry at 450 nm. The recovered amount of β -carotene, C , was calculated from the concentration obtained using the calibration curve, the total volume of the combined organic extracts, and the corresponding dilution factor.

$$AEE(\%) = \frac{C}{C_0} \cdot 100 \quad (3)$$

where C_0 is the initial amount of β -carotene incorporated into the formulation and C is the amount recovered from the emulsified system. Since droplet-associated and free β -carotene were not physically separated before extraction, AEE should be interpreted as an operational recovery parameter rather than as the direct quantification of an independently isolated encapsulated fraction.

Specifically, 1 g of the sample was thoroughly mixed with an ethanol/n-hexane solution in a 2:3 ratio to facilitate the extraction of β -carotene from the emulsion matrix [33]. Following phase separation, the organic phase was meticulously collected, and the extraction process was repeated twice to ensure maximal recovery of the carotenoid. The combined organic fractions were subsequently analyzed using UV–Vis spectrophotometry at a wavelength of 450 nm, utilizing a pre-established β -carotene calibration curve. All procedural steps were conducted under conditions of reduced light exposure to mitigate photo-oxidative degradation. The degradation of β -carotene during storage (at 4 °C and 25 °C) was quantified as the relative concentration of β -carotene, C/C_0 , where C represents the β -carotene concentration at a specific aging time, and C_0 denotes the concentration measured immediately following sample preparation. This methodology facilitated the evaluation of the emulsion structure's protective effect on the chemical stability of β -carotene over time. The degradation kinetics of β -carotene during storage at 4 and 25 °C were evaluated using the relative pigment concentration and the experimental data were fitted to zero-order and first-order kinetic models. The zero-order model was expressed as:

$$C = C_0 - k_0 t \quad (4)$$

where k_0 is the zero-order degradation rate constant and t is the storage time. The first-order model was expressed as:

$$\ln\left(\frac{C}{C_0}\right) = -k_1 t \quad (5)$$

where k_1 is the first-order degradation rate constant. For each model, the corresponding rate constant was obtained by linear regression. The half-life was calculated as $t_{1/2} = C_0/(2k_0)$ for the zero-order model and $t_{1/2} = \ln 2/k_1$ for the first-order model. Model suitability was assessed by comparing the coefficient of determination (R^2) and the root mean square error (RMSE). The kinetic model presenting the highest R^2 and the lowest RMSE was considered the most appropriate for describing β -carotene degradation under each storage condition.

2.5. Statistical Analysis

To ensure the reliability and reproducibility of the results, all experiments were conducted in triplicate. Standard deviations are represented in the figures as error bars, when observable, or as percentage values in the figure captions. When significant differences were detected, Tukey's post hoc test was applied for pairwise comparisons at $p < 0.05$. Statistical analysis was conducted using one-way analysis of variance (ANOVA) to evaluate the significance of differences among the tested formulations. A confidence level of 95% ($p < 0.05$) was established as the criterion for determining statistical significance. Additionally, a central composite design (CCD) was employed to optimize the formulation variables and assess their combined effect on the responses of interest. The design, data

fitting, and generation of response surface plots were executed utilizing Echip 7.0 software (Experimentation by Design, Wilmington, DE, USA). This methodological approach facilitated the identification of optimal processing and compositional conditions by modeling the effects of independent variables and their interactions on the measured outputs. Model adequacy was evaluated using the overall model significance, coefficient of determination (R^2), adjusted coefficient of determination, residual standard deviation, cross-validation root mean square error, and lack-of-fit test. The lack of fit was assessed against the pure error obtained from the replicated central points.

3. Results and Discussion

Figure 1 and Table 2 illustrate that the concentration of Spirulina extract significantly influenced both droplet disruption and stabilization during emulsification. An increase in extract concentration from 0.5 to 2.5 wt.% resulted in a progressive shift in droplet size distributions towards smaller diameters. This was quantitatively corroborated by a reduction in the Sauter mean diameter, $D_{3,2}$, from 3.341 ± 0.219 to 1.397 ± 0.065 μm . The observed progressive decrease in $D_{3,2}$ with increasing Spirulina extract concentration from 0.5 to 2.5 wt.% aligns with the enhanced availability of surface-active constituents during emulsification. Previous studies on phycocyanin and other microalgal proteins [34] suggest that adsorption at the oil–water interface and a reduction in interfacial tension may contribute to this trend. However, as interfacial tension and adsorption kinetics were not measured in this study, the proposed explanations of improved interfacial coverage and reduced immediate recoalescence remain speculative rather than experimentally confirmed. Additionally, the span values indicated that droplet-size reduction did not consistently result in a narrower distribution. Based on the measured $D_{3,2}$ and span values, the formulation with 2.5 wt.% Spirulina extract achieved the optimal empirical balance between droplet-size reduction and distribution width within the examined concentration range.

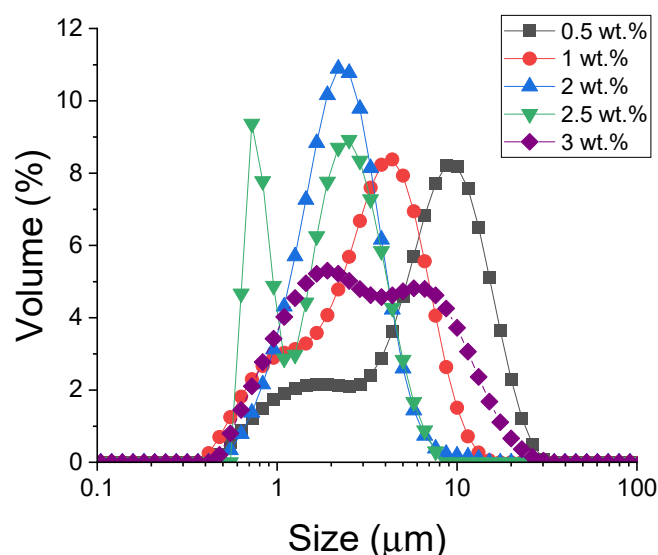


Figure 1. Effect of Spirulina extract concentration on the droplet size distribution of oil-in-water emulsions.

Increasing the concentration of Spirulina extract from 2.5 to 3 wt.% resulted in an increase in both $D_{3,2}$ and span, suggesting the development of a broader and coarser apparent droplet population. This observation may be explained by several mechanisms, including the presence of non-adsorbed macromolecular components in the continuous phase, alterations in continuous-phase viscosity and cavitation efficiency, or transient aggregation or recoalescence during processing. Depletion interactions offer one potential explanation; however, laser diffraction data alone cannot differentiate individual droplets

from flocculated structures or determine the cause of the increase in apparent size. Consequently, these mechanisms remain speculative, necessitating further measurements such as zeta potential, continuous-phase rheology, and interfacial characterization to differentiate among them.

Table 2. Sauter mean diameter ($D_{3,2}$) and span values as a function of Spirulina extract concentration.

Spirulina Extract Concentration (wt.%)	$D_{3,2}$ (mm) ¹	Span ¹
0.5	3.341 ± 0.219^a	1.917 ± 0.095^a
1	2.016 ± 0.126^b	3.341 ± 0.159^b
2	1.771 ± 0.105^b	1.355 ± 0.062^d
2.5	1.397 ± 0.065^c	1.661 ± 0.071^c
3	2.074 ± 0.159^b	3.051 ± 0.125^b

¹ Values are expressed as mean $\pm$ standard deviation. Different lowercase letters within the same column denote significant differences according to Tukey's test ($p < 0.05$).

Following the initial formulation screening, the concentration of Spirulina extract was determined to be 2.5 wt.% as this formulation produced the smallest Sauter mean diameter while maintaining a relatively narrow droplet size distribution. Consequently, this concentration was selected as the reference formulation for subsequent processing optimization. A central composite design was utilized to evaluate the combined effects of primary homogenization time, sonication amplitude, and sonication time on the droplet size parameters of the emulsions. The experimental results for $D_{3,2}$ and span across each trial are presented in Table 3.

Table 3. Sauter mean diameter ($D_{3,2}$) and span values as a function of independent variables for the experimental design.

Sample	$D_{3,2}$ (mm)	Span
1	1.265	2.435
2	1.735	1.440
3	1.966	1.959
4	1.397	1.661
5	1.195	2.498
6	1.459	1.596
7	2.316	1.994
8	1.669	1.417
9	1.811	1.797
10	2.239	1.411
11	0.680	2.077
12	1.976	1.768
13	1.575	2.380
14	2.030	2.010
15	2.622	1.972
16	0.920	2.212
17	1.783	2.046

The central design point was characterized by 120 s of primary homogenization, a 50% sonication amplitude, and 450 s of sonication time. This condition was exemplified by samples 4, 6, and 8, which exhibited $D_{3,2}$ values of 1.397, 1.459, and 1.669 μm , respectively, and span values of 1.661, 1.596, and 1.417. These findings demonstrate the good reproducibility of the central condition, particularly regarding distribution width. Consequently, the central processing condition serves as a reliable reference point, yielding emulsions with intermediate droplet sizes and relatively narrow droplet size distributions. Although the span values showed limited variation, the differences in $D_{3,2}$ suggest some degree of

experimental variability in droplet disruption under these central processing conditions. This variability may be attributed to the inherent heterogeneity of the *Spirulina* extract, the sensitivity of ultrasonic emulsification to localized cavitation phenomena, or minor variations in the initial coarse emulsion produced during the primary homogenization phase [35]. An examination of Table 3 reveals that the lowest $D_{3,2}$ values were recorded for samples 11 and 16. Sample 11, prepared using 120 s of primary homogenization, a sonication amplitude of 25%, and a sonication time of 450 s, exhibited the smallest droplet size within the experimental design, with a $D_{3,2}$ of 0.680 μm and a span of 2.077. Sample 16, prepared using 66 s of primary homogenization, a sonication amplitude of 35%, and a sonication time of 271 s, also exhibited a low $D_{3,2}$ value of 0.920 μm , with a span of 2.212. These results indicate that the smallest droplet sizes were not necessarily obtained under the highest sonication amplitude or the longest processing time, highlighting the nonlinear influence of the processing variables on droplet disruption. However, the moderate increase in span observed in these samples suggests that the formation of very small droplets was accompanied by a broader droplet size distribution. This broader distribution may reflect the coexistence of differently sized droplets and/or weakly associated structures. Nevertheless, laser diffraction alone cannot confirm the presence of flocculation or distinguish flocculated structures from individual large droplets [36].

Figure 2 illustrates the droplet size distributions of selected emulsions obtained under varying processing conditions. Consistent with the findings presented in Table 3, the curves demonstrate that processing variables influenced both the position and width of the droplet size distributions. However, these effects were complex, indicating that the final droplet population resulted from a balance between droplet breakup during ultrasonication and potential recoalescence or aggregation phenomena. Generally, samples with lower $D_{3,2}$ values in Table 3 are anticipated to exhibit distributions shifted towards smaller droplet diameters. This is particularly evident for conditions yielding the smallest mean droplet sizes, such as samples 11 and 16, with $D_{3,2}$ values of 0.680 and 0.920 μm , respectively. Nonetheless, these samples also displayed relatively high span values, indicating broader droplet size distributions. Thus, while these processing conditions favored the formation of small droplets, they also resulted in more heterogeneous systems. Preliminary analysis suggests that sonication amplitude and duration exerted a greater influence on droplet size distribution than primary homogenization time. In some instances, low or moderate energy input favored smaller droplets, whereas excessive sonication intensity or duration did not necessarily enhance droplet disruption and could lead to broader or coarser distributions. This qualitative interpretation will be further elucidated through mathematical modeling of the experimental design by response surface methodology.

The experimental values obtained for the Sauter mean diameter were fitted using the response surface model described in Equation (6):

$$D_{3,2} = 1.51 + 0.48 \cdot AMP + 0.28 \cdot SH + 0.17 \cdot PH \cdot AMP + 0.09 \cdot PH^2 - 0.06 \cdot AMP^2 + 0.07 \cdot SH^2 \quad (6)$$

where PH, AMP, and SH represent the coded values of primary homogenization time, sonication amplitude, and sonication time, respectively.

To assess the adequacy of the fitted response surface model, an ANOVA was performed including the overall model significance, the effect of each processing variable, the interaction terms, and the lack-of-fit test. The corresponding results are summarized in Table 4. The response surface model was statistically significant ($p = 0.0031$) and showed an R^2 of 0.928 and an adjusted R^2 of 0.835, indicating that it explained most of the experimental variability in $D_{3,2}$. Sonication amplitude and sonication time significantly affected droplet size ($p = 0.0003$ and $p = 0.0054$, respectively), whereas primary homogenization time was not

significant ($p = 0.3606$). This result suggests that, once a sufficiently homogeneous coarse emulsion had been produced by rotor–stator mixing, the final droplet size was mainly governed by the secondary ultrasonic treatment. Sonication amplitude determines the intensity of acoustic cavitation and the magnitude of the shear stresses acting on the droplets, whereas sonication time controls the duration of their exposure to these disruptive forces. Nevertheless, the final droplet population reflects a balance between cavitation-induced breakup and competing phenomena such as droplet collisions, transient coalescence, and insufficient adsorption of surface-active components onto the newly generated interface. None of the interaction terms were statistically significant within the studied domain, although the interaction between primary homogenization time and amplitude approached significance ($p = 0.0699$). The residual standard deviation and cross-validation RMSE were 0.205 and 0.334 μm , respectively. Furthermore, the lack of fit was not significant ($F = 2.48$, $p = 0.312$), supporting the adequacy of the model for describing and optimizing $D_{3,2}$ within the investigated processing range.

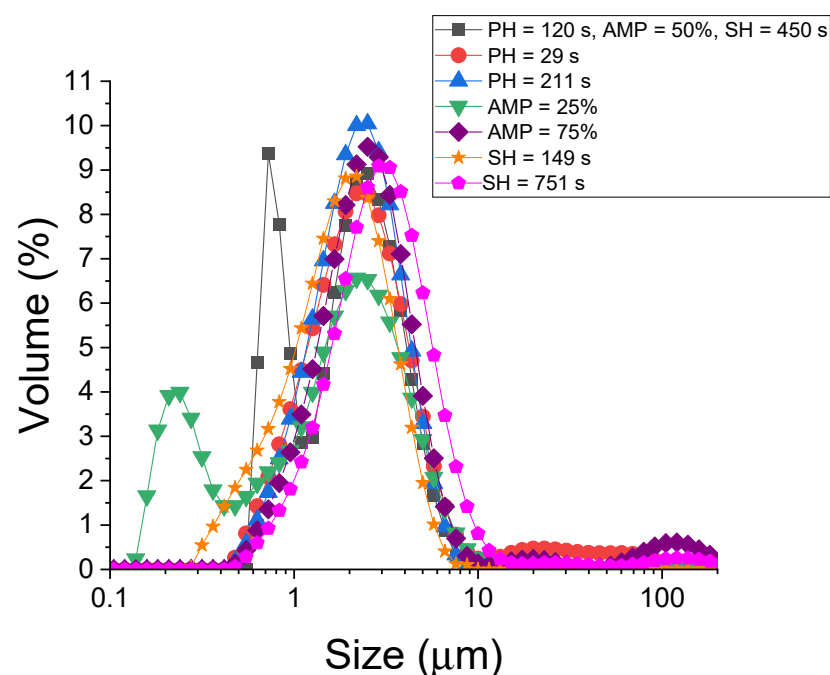


Figure 2. Effect of primary homogenization time (PH), amplitude (AMP) and sonication time (SH) on the droplet size distribution of selected oil-in-water emulsions.

Table 4. Analysis of variance for the response surface model fitted to $D_{3,2}$.

Source	Sum of Squares	df	Mean Square	F-Value	p-Value
Model	3.7589	9	0.4177	9.98	0.0031
PHt	0.0991	2	0.0496	1.18	0.3606
AMP	2.6310	2	1.3155	31.44	0.0003
SHt	1.0070	2	0.5035	12.03	0.0054
PHt · AMP	0.1912	1	0.1912	4.57	0.0699
PHt · SHt	0.0402	1	0.0402	0.96	0.3596
AMP · SHt	0.0583	1	0.0583	1.39	0.2763
Lack of fit	0.2523	5	0.0505	2.48	0.3116
Pure error	0.0406	2	0.0203	—	—
Residual error	0.2929	7	0.0418	—	—
Total	4.0518	16	—	—	—

$R^2 = 0.928$; adjusted $R^2 = 0.835$; residual SD = 0.205 μm ; cross-validation RMSE = 0.334 μm .

Through response surface analysis, optimal processing conditions were identified, aligning with the experimental point that achieved the smallest droplet size within the design framework. Consequently, this optimized emulsion underwent further characterization concerning its microstructure and physical stability. Scanning electron microscopy (SEM) was utilized to provide visual evidence of droplet morphology and to verify the formation of a finely dispersed structure, following methodologies previously applied to curcumin-loaded emulsion systems. Figure 3 shows the FESEM microstructure of the optimized emulsion, corresponding to the experimental condition that produced the lowest Sauter mean diameter within the design. The image provides qualitative evidence of a finely dispersed morphology and is broadly consistent with the laser diffraction results. However, FESEM analysis requires sample dehydration and exposure to vacuum, which may induce shrinkage, deformation, or aggregation of the dispersed structures. Consequently, the clustered regions observed in the micrograph cannot be unequivocally attributed to droplet flocculation, recoalescence, or incomplete interfacial stabilization. Likewise, the absence of large oil domains in the image should not be considered direct evidence of the absence of coalescence in the original hydrated emulsion. Therefore, Figure 3 should be interpreted as qualitative microstructural support for the formation of a finely dispersed system rather than as experimental confirmation of a specific stabilization or droplet-interaction mechanism.

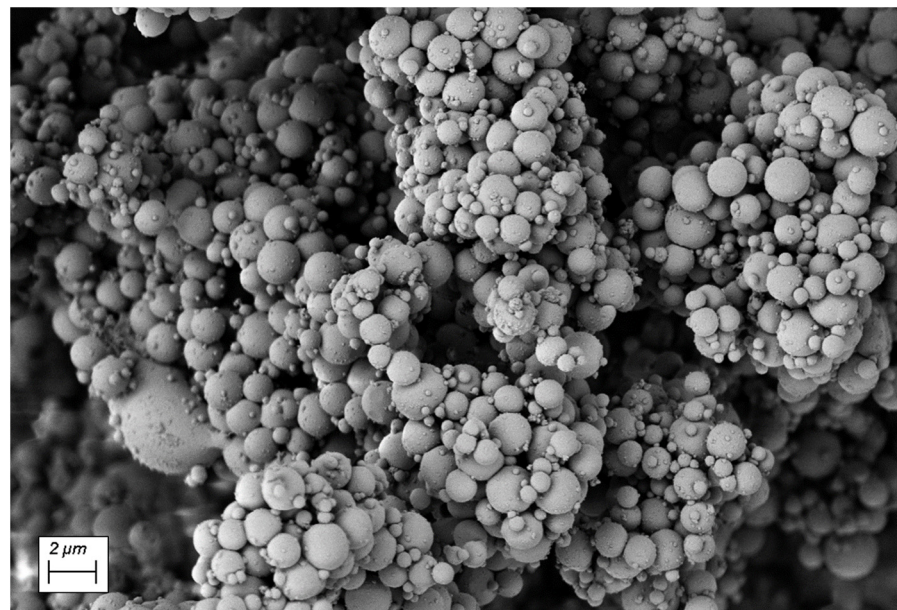


Figure 3. Microstructure observed by FESEM of the optimum emulsion.

The physical stability of the optimized emulsion was assessed through multiple light scattering during storage. The backscattering profiles exhibited only minor variations within the first month, indicating that the system maintained physical stability, with no significant clarification, sedimentation, or coalescence phenomena observed during this period. After approximately 30 days of storage, a slight decrease in backscattering was detected in the lower part of the measuring cell, accompanied by a modest increase in the upper region. This behavior is typically indicative of the onset of a mild creaming process, attributed to the upward migration of oil droplets rather than extensive droplet coalescence or phase separation. This limited destabilization was also reflected in the Turbiscan Stability Index (TSI), which remained low throughout the storage period, reaching approximately 4.8 after 30 days. This value confirms the good kinetic stability of the optimized emulsion, particularly given that the formulation was stabilized using a natural *Spirulina*-derived extract and contained β -carotene-rich sunflower oil as the dispersed phase.

The apparent encapsulation efficiency of β -carotene was evaluated immediately after preparation using the mass-balance procedure described above. The optimized formulation exhibited an AEE of $91.4 \pm 2.1\%$, indicating that most of the β -carotene initially incorporated through the enriched sunflower oil was recovered from the emulsified system after processing. Correspondingly, the non-recovered fraction represented approximately 8.6% of the initial amount. This fraction may include β -carotene lost during processing, degraded during emulsification, precipitated, incompletely recovered during solvent extraction, or located outside the retained dispersed oil phase. Since free and droplet-associated β -carotene were not independently separated, the result should be interpreted as an apparent incorporation and recovery efficiency rather than as direct evidence of the physical localization of all recovered β -carotene within individual droplets.

Subsequent evaluation of β -carotene storage stability was conducted at two temperatures, refrigeration ($4\text{ }^{\circ}\text{C}$) and room temperature ($25\text{ }^{\circ}\text{C}$), by monitoring the relative β -carotene concentration (C/C_0) in both the β -carotene-rich sunflower oil and the optimized emulsion (Figure 4). As anticipated, β -carotene degradation was strongly influenced by storage temperature and by whether the pigment was present in the non-emulsified oil or incorporated into the emulsion. The non-emulsified oil exhibited a pronounced decrease in β -carotene concentration, particularly at $25\text{ }^{\circ}\text{C}$, where C/C_0 declined to $54.6 \pm 3.2\%$ after 21 days and $41.8 \pm 2.9\%$ after 30 days. This behavior confirms the high susceptibility of β -carotene to oxidative degradation when dispersed directly in the oil phase. Refrigerated storage reduced the degradation rate, although the β -carotene-rich oil still showed a notable decrease, retaining 86.9% after 21 days and $80.7 \pm 2.7\%$ after 30 days. In contrast, the optimized emulsion showed a slower degradation profile at both temperatures. At $25\text{ }^{\circ}\text{C}$, the emulsion retained $74.2 \pm 2.4\%$ of the initial β -carotene content after 21 days and $68.5 \pm 2.6\%$ after 30 days. This protective effect was even more pronounced under refrigerated conditions, where the optimized emulsion retained 93.4% after 21 days and $91.3 \pm 1.9\%$ after 30 days of storage. These results indicate that both refrigeration and emulsification contributed to improving β -carotene stability during storage.

The degradation kinetics of β -carotene in the enriched sunflower oil and in the optimized emulsion were evaluated during storage at 4 and $25\text{ }^{\circ}\text{C}$ by fitting the experimental retention data to zero-order and first-order models. In general, degradation was strongly affected by both storage temperature and the physical state of the system. At $25\text{ }^{\circ}\text{C}$, the first-order model provided the best fit for both the optimized emulsion and the non-emulsified oil, as indicated by the higher R^2 values and lower RMSE values compared with the zero-order model. For the emulsion, the first-order degradation constant was 0.0127 day^{-1} , with an estimated half-life of 54.7 days. In contrast, the β -carotene-rich oil showed a markedly higher degradation constant of 0.0290 day^{-1} and a shorter half-life of 23.9 days. Therefore, emulsification reduced the apparent degradation rate by approximately 56% at room temperature. These kinetic results are consistent with the retention data shown in Figure 4, since 68.5% of the initial β -carotene remained in the emulsion after 30 days, compared with only 41.8% in the non-emulsified oil. Refrigerated storage substantially slowed β -carotene degradation in both systems. The emulsion stored at $4\text{ }^{\circ}\text{C}$ was also best described by the first-order model, with a degradation constant of 0.00311 day^{-1} and an estimated half-life of 222.5 days. For the oil stored at $4\text{ }^{\circ}\text{C}$, the zero-order model provided a slightly better fit, with an R^2 of 0.999 and an RMSE of 0.207 percentage points. The corresponding zero-order degradation constant was $0.634\text{ percentage points day}^{-1}$, resulting in an estimated half-life of 78.8 days. After 30 days of refrigerated storage, β -carotene retention was 91.3% in the emulsion and 80.7% in the oil. Overall, the kinetic analysis showed that refrigeration was the most effective condition for limiting β -carotene degradation. At both temperatures, the emulsion exhibited a lower apparent degradation rate and a longer estimated half-life

than the non-emulsified oil. These results demonstrate improved β -carotene retention in the emulsified system under the tested storage conditions, but they do not identify the mechanism responsible for this improvement. A contribution from the *Spirulina*-derived interfacial layer, altered accessibility of oxygen or other pro-oxidant species, and/or the antioxidant activity of components present in the commercial extract is plausible based on the literature. However, oxygen transfer, interfacial composition and properties, and antioxidant activity were not measured in the present study. Consequently, these possibilities should be considered proposed mechanisms rather than experimentally verified explanations. Similarly, the contribution of the observed physical stability to β -carotene retention remains possible but cannot be established from the available data.

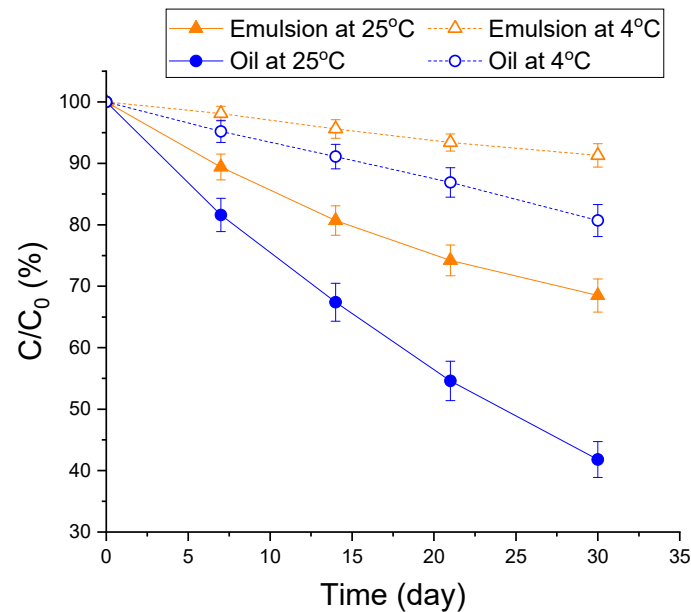


Figure 4. Relative β -carotene concentration (C/C_0) during storage for β -carotene-rich sunflower oil and the optimized emulsion at 4 and 25 °C. Values are expressed as mean $\pm$ standard deviation. C and C_0 correspond to the β -carotene concentration at each storage time and immediately after preparation, respectively.

To place the practical performance of the present formulation in context, comparison with previously reported emulsion-based delivery systems is necessary. Microfluidized emulsions formulated with 2 wt.% phycocyanin achieved droplet sizes as low as approximately 243 nm after six processing passes; however, the phycocyanin-only system underwent extensive creaming, and at least 5 wt.% fumed silica was required to inhibit this destabilization mechanism [37]. In contrast, the present formulation produced a larger, although still submicron, $D_{3,2}$ of 0.680 μm , but maintained a relatively low TSI of approximately 4.8 after 30 days without an additional thickener or particulate stabilizer. β -Carotene nanoemulsions formulated with a low-molecular-weight surfactant and guar gum previously exhibited an encapsulation efficiency of 87.2% and retained more than 80% of the carotenoid after 21 days, demonstrating the effectiveness of combined surfactant–polysaccharide stabilization [13]. The present *Spirulina*-based system achieved an apparent encapsulation efficiency of $91.4 \pm 2.1\%$ and retained 68.5% and 91.3% of β -carotene after 30 days at 25 and 4 °C, respectively. Recent studies have also demonstrated the potential of ultrasound-modified *Spirulina* proteins for stabilizing curcumin-loaded high-internal-phase emulsions [34]. The main practical advantage of the present approach is therefore the use of a commercial *Spirulina*-derived ingredient as the primary and sole emulsifying component, simplifying the formulation and avoiding additional synthetic surfactants or

auxiliary biopolymers [38]. Nevertheless, comparisons among studies should be interpreted cautiously because of differences in oil type, dispersed-phase fraction, emulsification technology, active compound, and storage conditions.

4. Conclusions

This study demonstrates the feasibility of developing Spirulina-stabilized emulsions as sustainable delivery systems for β -carotene-rich sunflower oil extracted from *Dunaliella salina*. Preliminary formulation screening identified 2.5 wt.% Spirulina extract as the most suitable concentration, since it produced the smallest Sauter mean diameter while maintaining a relatively narrow droplet size distribution. Response surface analysis showed that sonication amplitude and sonication time were the main processing variables affecting droplet size, whereas primary homogenization time had no significant effect within the investigated range. Under the selected conditions, a submicron emulsion with a $D_{3,2}$ of 0.680 μm was obtained.

The optimized emulsion exhibited good physical stability, with only slight creaming and a Turbiscan Stability Index of approximately 4.8 after 30 days. It also showed an apparent β -carotene encapsulation efficiency of $91.4 \pm 2.1\%$. Emulsification improved β -carotene retention compared with the non-emulsified oil at both storage temperatures. After 30 days, retention values were 68.5% at 25 °C and 91.3% at 4 °C in the emulsion, compared with 41.8% and 80.7%, respectively, in the oil. The kinetic analysis was consistent with improved β -carotene retention following emulsification, particularly under refrigerated conditions. These findings demonstrate enhanced storage stability under the investigated conditions. However, because zeta potential, interfacial properties, oxygen transfer, and antioxidant activity were not directly measured, the mechanisms responsible for this improvement remain to be established.

From a practical perspective, the use of a commercial Spirulina-derived ingredient as the primary emulsifying component offers a promising clean-label strategy for incorporating carotenoid-rich oils into aqueous functional foods, beverages, sauces, dressings, and other emulsified products. Nevertheless, the present study has several limitations. In particular, the absence of zeta potential and interfacial characterization measurements prevents a detailed assessment of the physicochemical mechanisms governing droplet stabilization and interactions. Furthermore, although β -carotene retention was monitored, complementary lipid oxidation indices, such as peroxide value, conjugated dienes, or secondary oxidation products, were not determined; therefore, the oxidative stability of the lipid phase could not be comprehensively evaluated. Future studies should address these limitations and should also evaluate energy consumption, process reproducibility, sensory acceptance, regulatory aspects, performance in complex food matrices, and scale-up using continuous-flow ultrasound, high-pressure homogenization, or microfluidization.

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