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Stable Nanoemulsion System Development Enabling the Topical Delivery of *Synechococcus*-Derived Peptides

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Abstract

Marine microalgae including *Synechococcus* sp. VDW offer the potential to serve as a source of bioactive peptides offering valuable antioxidant and antimelanogenic qualities for use in the pharmaceutical and cosmetics industries. At present, however, the use of such peptides is challenging due to their poor physicochemical stability. This research therefore sought to achieve the production and characterization of a stable nanoemulsion system based upon the use of synthetic *Synechococcus*-derived peptides through the process of high-pressure homogenization (HPH). Preparation of the nanoemulsions involved the use of Tween-80 and caprylic/capric triglyceride at a pressure of 7,500 psi to perform homogenization for varying durations of 15, 30 and 45 min. Using the optimized formulation with 0.1% w/w peptide for a time of 45 min resulted in 122.16 nm droplets while the zeta potential was −80.09 mV and the PDI (polydispersity index) value was 0.13. Colloidal stability could be considered high, while physical stability under thermal cycling, centrifugation and freeze-thaw cycles was very good, with no phase separation. For all testing intervals, the viscosity and refractive index were stable. It can thus be argues that the HPH approach is suitable to produce peptide-loaded nanoemulsions offering good stability and useful physicochemical characteristics. The developed nanoemulsion system has been optimized to offer potential for applications involving the transdermal delivery of marine peptides in the cosmeceutical sector and for a range of dermal therapies.

Keywords: Colloidal stability, Cosmetic formulation, High-pressure homogenization, Nanoemulsion, Peptide delivery, *Synechococcus*

Introduction

Many different bioactive compounds, such as lipids, pigments, polysaccharides and peptides, can be obtained from marine microalgae, providing significant benefits for producers of cosmetics as well as nutraceutical and pharmaceutical products [1].

Synechococcus, a type of marine cyanobacteria, is particularly valuable since it provides bioactive peptides suitable for a wide range of applications [2]. It has been shown that the peptides from *Synechococcus* sp. VDW in particular are strong antioxidants [3] which are also capable of inhibiting melanogenesis in a safe and effective manner [4]. The peptides in question are understood to downregulate the expression of MITF, TYR, TRP-1 and TRP-2, which are proteins involved in the process of melanogenesis. Accordingly, the peptides might find application in the production of agents offering skin-whitening and anti-aging properties. However, notwithstanding this bioactive potential, marine-derived peptides have not yet been widely employed for commercial purposes because they lack stability and can be highly sensitive to changing environmental conditions, including temperature and pH, leading to enzymatic degradation, and oxidation. Accordingly, the bioavailability of the peptides is not consistent and their effectiveness as a therapeutic agent can be significantly reduced. One approach which can potentially address these concerns is the use of encapsulation strategies, preventing degradation while mitigating any potential loss in bioactivity [5,6].

In the fields of cosmetics and drug delivery, nanoemulsions are playing increasingly prominent roles since they offer the potential to both encapsulate and stabilize ingredients which might otherwise be too sensitive for application. Nanoemulsions take the form of oil droplets dispersed in water (or vice versa), with surfactants and co-surfactants providing stability [7]. Their droplet sizes typically range from 20 nm to 200 nm, allowing excellent transdermal absorption and skin residence time. The high surface area allied to small droplets and good optical clarity result in superior kinetic stability, a greater capacity to be spread and improved bioavailability through the skin [8]. A number of approaches have been employed in the creation of nanoemulsions, among which are ultrasonication, phase inversion temperature (PIT), spontaneous emulsification, and high-pressure homogenization (HPH). It is HPH which has been most widely employed for industrial purposes since it allows the production of stable nano-sized emulsions for which the distribution of droplet sizes is relatively uniform. The HPH approach demands that the course emulsion be subjected to powerful mechanical forces as high pressure is applied, disrupting large droplets and forming small droplets in their stead. HPH can also be scaled and the process is easily reproduced. Both of these qualities are necessary for commercial operations [9,10]. This includes biplot and heatmap visualisation that are fundamental to understanding the complex relationships of different nanoemulsion samples (Metabolyte, a metabolomics data analysis platform). Biplot analysis supplies a strong class-related metabolite-signature reduction framework, characterizing group-wise contributions of relevant metabolites that permit the differentiation among metabolic phenotypes regarding typically shared data characteristics and variable interactions. Moreover, the use of heatmaps to depict complex data patterns and correlations and identify redundant metabolites with high dynamic ranges or the same template-derived statistical measures makes them a very helpful approach for identifying meaningful signals in an information-rich plant-association dataset. Integration of biplot and heatmap analyses provides a comprehensive map of the relationships between metabolites, enabling identification of important metabolic markers relevant to properties of nanoemulsion formulation—both advancing mechanistic understanding and optimization for design in both biomedical and industrial applications [11]. This research placed emphasis upon developing a nanoemulsion based upon synthetic peptides obtained from the *Synechococcus* marine microalgae via the HPH approach. In particular, it was necessary to establish the influence of both homogenization time and the concentration of the peptide upon the properties of the resulting nanoemulsion, such as the particle size, viscosity, zeta potential, refractive index and physical stability when exposed to various stresses. It was also necessary to determine the optimal formulation parameters to ensure the production of a uniform and stable bioactive nanoemulsion system which might be effectively applied in the cosmetics and pharmaceutical industries. Furthermore, metabolite analysis using bipilot and Heatmap was employed to investigate the correlation between different nanoemulsion formulations. The results should serve to expand our understanding of marine peptide applications along with the use of nanoemulsion-based delivery systems in developing new functional products.

Materials and methods

Materials

GenScript Biotech (Singapore) supplied the synthetic peptides with the sequence (Ala-Ile-Leu-Glu-Ser-Tyr-Ser-Ala-Gly-Lys-Thr-Lys; AILQSYSAGKTK; AK-12) with a purity of 28.25% obtained from *Synechococcus* sp. VDW. Chemipan Corporation (Thailand) provided the Tween-80 (polysorbate 80) and caprylic/capric triglyceride (CCT), while all chemicals used in the study were of analytical grade and required no additional purification. Equipment purchases included a centrifuge (Ortoalresa Biocen 22R, Spain), a particle size and zeta potential analyzer (NanoPlus 3, Micromeritics, USA), a viscometer (Brookfield LVT, USA), and a high-pressure homogenizer (Microfluidizer® M-110P, USA).

Peptide-loaded nanoemulsion preparation

The development of nanoemulsion formulations utilized a surfactant system composed of Tween 80 and caprylic/capric triglyceride (CCT) in a 7:3 ratio, which has been previously shown to produce the smallest and most stable nanoemulsion droplets [12]. The preparation process began by combining all components (surfactant, oil and water), followed by initial mixing using a vortex mixer to assess preliminary physical characteristics. AK-12 peptides were then incorporated at various concentrations (0.1% w/w, 0.25% w/w, 0.5% w/w and 0.75% w/w) for comparative evaluation. The formulation exhibiting the most favorable physicochemical properties was further processed using a Microfluidizer® (M-110P, USA) at 7,500 psi to reduce particle size. Size reduction was performed for durations of 10, 30 and 45 min. The optimal formulation was identified based on its translucent appearance and the absence of phase separation.

Nanoemulsion stability

The stability of the nanoemulsions was evaluated using three sequential tests. First, a centrifugation test was performed using a Biocen 22R centrifuge (Ortoalresa, Spain) at 3,500 rpm for 30 min, followed by visual inspection. The absence of phase separation indicated good stability. Second, heating-cooling cycles were conducted by alternately storing samples at 4 °C and 40 °C for 48 h each, over six cycles (totaling 24 days). The samples were examined visually for any physical changes [13]. Third, freeze–thaw testing involved subjecting the formulations to –21 °C for 48 h followed by 25 °C for 48 h, repeated over three complete cycles (12 days), with visual assessment for phase separation. Nanoemulsions that maintained their integrity and exhibited no phase separation throughout all testing procedures were considered thermodynamically stable.

Measurement of droplet size, polydispersity index (PDI) and zeta potential

The droplet size, polydispersity index (PDI), and zeta potential of the nanoemulsions were determined using a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, UK), following the method described by Lotfy *et al*. [14]. To minimize multiple scattering effects, each sample was diluted 100-fold with deionized water prior to analysis. Refractive indices were set at 1.47 for the oil phase and 1.33 for the aqueous phase. All measurements were conducted in triplicate at a temperature of 25 ± 2 °C.

Statistical analyses

All nanoemulsion measurements were conducted in quintuplicate, and the results are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS Statistics Version 23, GraphPad Prism 10 and MetaboAnalyst 6.0. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to determine statistically significant differences among the samples. Statistical significance was defined at four levels: p-value < 0.05, p-value < 0.01, p-value < 0.001 and p-value < 0.0001.

Results and discussion

Effects of peptide concentration upon pH and viscosity

The concentration of peptides derived from *Synechococcus* marine microalgae and the properties of the resulting nanoemulsions appears to show a relationship involving various physicochemical interactions which determine the eventual characteristics of the formulation. **Figure 1(a)** shows that as the concentration of the peptide climbs from 0.1%w/w to 0.75%w/w, the pH value drops significantly from 5.8 to 5.2, as a consequence of the release of protons from the acidic amino acid residues which are

found within the structure of the peptide [15]. Meanwhile, **Figure 1(b)** reveals that viscosity drops significantly from 15,000 cP to 15,000 cP as the peptide concentration increases from 0.1%w/w to 0.75%w/w. Peptide concentration and viscosity are therefore inversely related, revealing that as the acidity of the environment rises, intermolecular forces are disrupted within the nanoemulsion system. It seems that there is interference from the peptides with the hydrogen bonding networks and that the peptides are able to modify the electrostatic interactions taking place between surfactant molecules (Tween 80 and CCT), thus causing flow resistance to decline as the concentration of the peptide rises. Since the various nanoemulsion formulations have properties which depend upon the concentration of the Synechococcus marine microalgae peptides, it is apparent that accurate rheological property control will be necessary, whereby heightened peptide concentrations will be beneficial in applications which demand low levels of viscosity in nanoemulsions which can maintain their stability. These results are in concurrence with earlier studies examining the pH responses of cyanobacteria [16], in which it has been revealed that the pH value can have a significant impact upon the growth rates and metabolism of *Synechococcus* species [17]. Nanoemulsion properties are therefore significantly influenced by the physiochemical attributes of their bioactive compounds, with studies confirming that temperature, pH values and the concentrations of different components can affect both stability and viscosity.

To achieve the most effective size reduction in nanoemulsions, the formulation with the Synechococcus algae peptide concentration of 0.75% w/w appears superior since it offers low viscosity at around 15,000 cP, which was around 67% lower than the 0.1% w/w formulation. Low viscosity influences the homogenization process to develop smaller particle sizes within the nanoemulsion system. While there was a drop in pH to 5.2 as a consequence of the protons released by amino acids in the peptide, this value remains acceptable in a stable system. Furthermore, the effects of the peptide upon the surfactant molecules (Tween 80 and CCT) in terms of their hydrogen bonding and changes in electrostatic forces serve to lower flow resistance. This is beneficial by leading to the creation of nanoemulsions which have smaller particle sizes. Studies on nanoemulsions have shown that decreasing the viscosity not only affects leaflet thickness but also reduces the particle size within the system. Lower viscosity leads to smaller droplets and less probability of coalescence, all of which improves the stability as well as the efficacy of a nanoemulsion. Due to an increase in water content, a change in the ratios of its various components, or both together, would keep positive modification of droplet characteristics and enhance the overall aerial performance. For example, when viscosity is decreased amongst their formulations [18-20]. Peptides, particularly those containing negatively charged amino acids, can interact with surfactant molecules such as Tween 80 and MCT/CCT through hydrogen bonding and electrostatic interactions. These interactions disrupt the emulsion's flow resistance, leading to smaller particle sizes and improved stability of the emulsion system. Furthermore, when peptides or functional proteins associate with surfactants (such as Tween 80), they can alter the surface tension and critical micelle concentration (CMC) of the system. The generation of hydrogen bonds and electrostatic attractions promotes particle dispersion and reduces the viscosity of the emulsion, thereby enhancing its overall performance and stability [21-23]. While a decrease in pH to around 5.2 from protons released by the amino acids within the peptides, most nanoemulsions are unstable at (or below) pH 5, according to work done earlier—especially those containing whey protein or its hydrolysate. However, this pH is generally accepted for systems using peptide-based scaffolds, since the stability optimum is, in fact, higher or close to pH 5 - 6 [24].

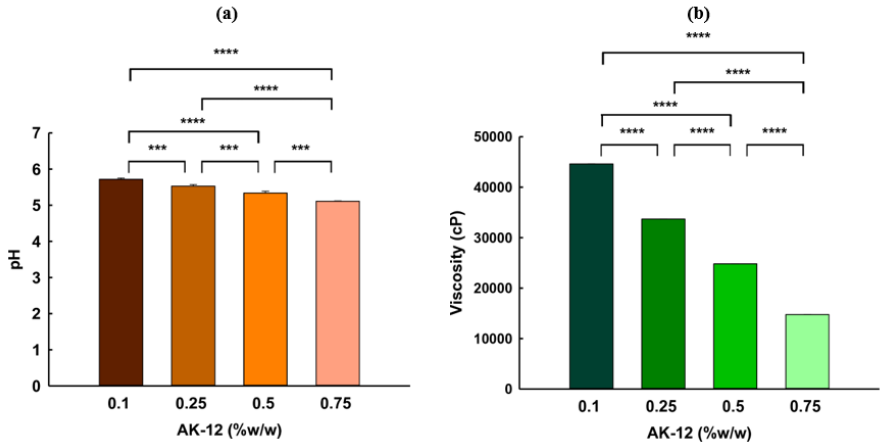

Figure 1 The influence of synthetic peptide concentration (%w/w) upon (a) pH and (b) viscosity (cP) of the resulting formulation. Asterisks indicate values with significant differences when comparisons are drawn between groups (***p-value < 0.001, **** p-value < 0.0001).

Effects of processing time upon the clarity of nanoemulsions

This research examined the effects upon nanoemulsion clarity and stability of using high-pressure homogenization (7,500 psi) with a Microfluidizer (M-110P, USA). The results are presented in **Figure 2**. Comparisons were drawn between processing times of 10, 30 and 45 min. The smallest particle sizes were obtained with the longest time, along with the greatest transparency and lowest viscosity, as a result of greater uniformity of dispersion. Formulations which had particles smaller than 100 nm showed lower levels of light scattering, leading to greater translucence. Where the particles were larger, a hsieh appearance was noted. Previous studies [25] reported similar findings for longer homogenization durations, suggesting that the repetition of mechanical shear forces results in narrower distributions of droplet sizes, and better kinetic stability via Brownian motion. The results align with expectations based upon well-known principles [26,27] which associate smaller particle sizes with greater optical clarity in systems lacking thermodynamic stability. It is thus the case that processing under high pressure for longer periods enhances the physicochemical properties of nanoemulsions [28].

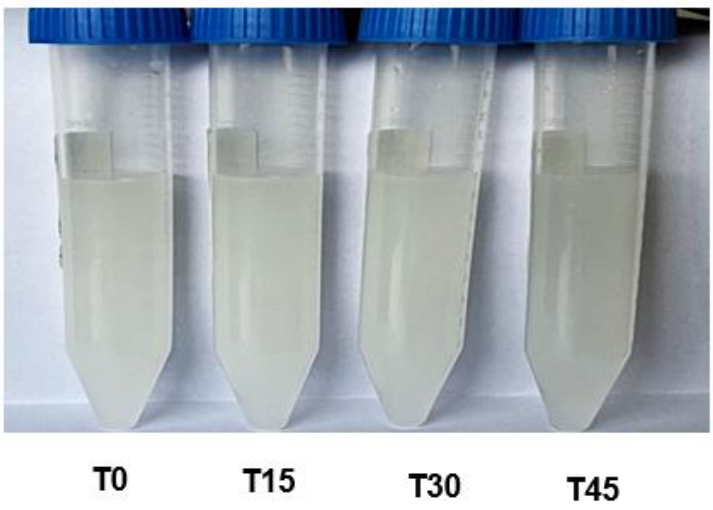

Figure 2 Clarity of the nanoemulsion over time, measured at 0, 15, 30 and 45 min.

Stability of the nanoemulsion

Table 1 shows the data concerning nanoemulsion stability, revealing consistency for each of the investigated time intervals, measured after 0, 15 30 and 45 min *via* different stress-inducing techniques. Centrifugation testing carried out at 3,500 rpm produced positive results (+) revealing the ability of the nanoemulsion to prevail when exposed to gravitational separation forces which might normally lead to creaming, sedimentation, or phase separation if the nanoemulsion system lacks stability. When exposed to a series of heating-cooling cycles (ranging from 4 °C to 40 °C, each for periods of 48 h, for 24 days in all) the formulation integrity was maintained, indicating the ability of the nanoemulsion to withstand destabilization mechanisms related to temperature, including Ostwald ripening or coalescence. Freeze-thaw testing revealed the ability of the nanoemulsion to tolerate large changes in temperature by subjecting the formulation to a range from –21 °C to 25 °C for a period of 12 days. This would normally lead to the destabilization of conventional emulsions as ice crystals are formed and interfacial disruption causes irreversible damage. This formulation, however, successfully passed the evaluations for all time periods and parameters, confirming its excellent thermodynamic stability. It can thus be concluded that the nanoemulsion retains its structural integrity when subjected to stressful changes in temperature and mechanical forces, confirming the findings reported by McClements [29], whose work demonstrated the need for multiple complementary stability techniques in order to predict the shelf-life of an emulsion as it is stored and distributed. Anton and Vandamme [30] followed a similar approach to testing, confirming that thermodynamic stability under laboratory conditions shows direct correlation to the long-term stability exhibited by nanoemulsions used in pharmaceutical applications. Furthermore, it was shown by Tadros *et al*. [31] nanoemulsions which demonstrate the ability to withstand centrifugal forces above 3,000 rpm along with challenging temperature cycles are likely to exhibit long-term stability when used in nanoemulsion systems, especially where the formulations are required to contain bioactive compounds which are highly sensitive to temperature. Since the outcomes are positive for all of the tested stability parameters, it can be argued that this particular nanoemulsion might easily find application for purposes demanding a stability profile.

Table 1 Nanoemulsion stability testing.

Time Parameter (Mins)	Centrifugation	H–C*	F–T**	Inference
0	+	+	+	Passed
15	+	+	+	Passed
30	+	+	+	Passed
45	+	+	+	Passed

Note: 0 = prior to particle size reduction; H–C* = Heating–Cooling cycle; F–T** =Freeze–Thaw cycle

Nanoemulsion droplet sizes and distribution

The inverse relationship between microfluidization time and nanoemulsion droplet size is shown in **Figure 3(a)**. At T0, prior to size reduction, the particle size was around 450 nm. This subsequently dropped to 410 nm at 15 min, 380 nm at 30 min and 120 nm by 45 min. For all time points the results represented statistically significant reductions (*** p-value < 0.001 and **** p-value < 0.0001), thus establishing the inverse relationship as particle sizes drop as the processing time lengthens. **Figure 3(b)** shows data concerning the polydispersity index (P.I.), whereby each of the formulations had acceptable distribution characteristics indicated by P.I. values lower than 0.4, meeting the standard criteria for nanoemulsions. For all of the time periods, the P.I. values were quite consistent, at around 0.29-0.30. Overall, the findings indicate that the ideal processing time was 45 min, since this achieved the best P.I. values and smallest particles. This conclusion matches those of earlier studies [29] in which it was stated that where the P.I. value is below 0.25, the nanoemulsion can be classified as stable and monodisperse. Jafari *et al*. [32] made similar observations, reporting that longer processing periods in

microfluidizers could enhance the qualities of nanoemulsions due to the increase in disruptive forces. Meanwhile, Tang *et al*. [33] argued that the decrease in size resulting from high-pressure homogenization occurs due to a mechanism based upon intense shear, cavitation, and turbulence forces. These forces cause disruption to large droplets, so a longer processing time allows this disruption sufficient time to create smaller droplets with greater uniformity in their distributions.

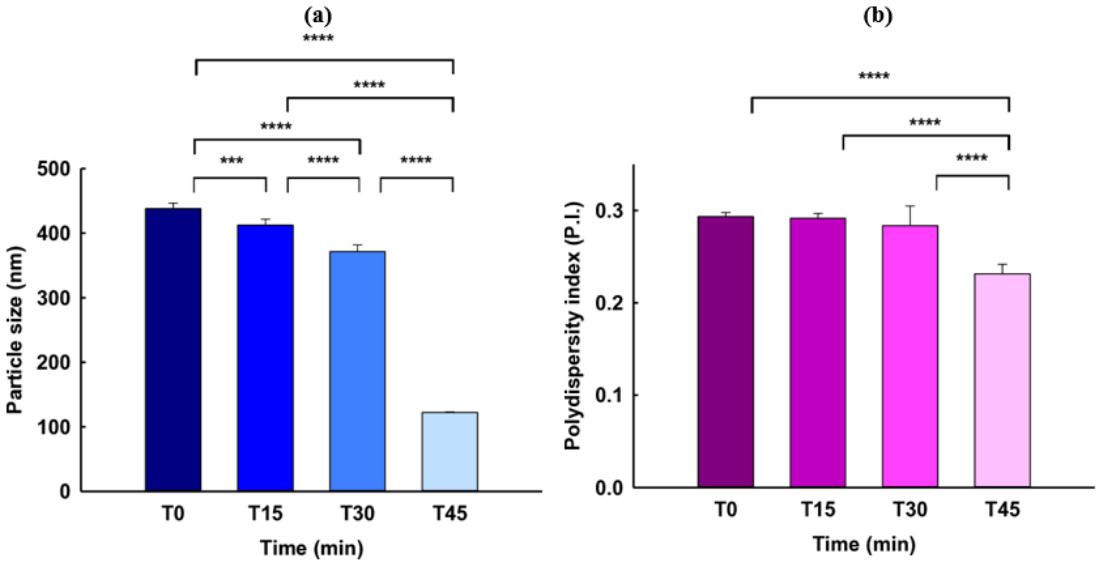

Figure 3 Changes in (a) particle size and (b) polydispersity index (P.I.) for various time intervals (T0, T15, T30, T45). Asterisks indicate values with significant differences between groups (*** p-value < 0.001 and **** p-value < 0.0001).

Measurement of zeta potential

Figure 4 presents data from experiments to measure colloidal stability, showing changes over time. In **Figure 4(a)**, the measurements for zeta potential are strongly negative, ranging from −70 to −80 mV at each of the time intervals and differing significantly between time points. A high degree of colloidal stability is indicated by these negative values, as a zeta potential measurement lower than −30 mV represents good stability [34]. Since the charge is strongly negative, this can be attributed to acidic functional groups dissociating upon the peptide surfaces, leading to greater electrostatic repulsion among particles. **Figure 4(b)** presents the conductivity measurements, which rise gradually from 0.01 mS/cm for the initial measurement to 0.023 after 45 min, from which it can be inferred that ionic mobility increases over time, possibly because the dissociation of charged species rises. **Figure 4(c)** shows the frequency parameter, which is non-linear, as it reaches its maximum after 15 min, before falling away at 30 and 45 min. Meanwhile, **Figure 4(d)** presents the intensity values, which tend to fluctuate while peaking at 45 min. It can be concluded, therefore, that T45 offers the best conditions to deliver colloidal stability, since the conductivity is maximized, the signal intensity is at its greatest, and there is a high level of negative zeta potential. The changes which occur over time might be attributed to peptide-surface interactions which appear similar to those reported by Alvares *et al*. [35], in which both the adsorption of peptides and distribution of the charge showed a tendency to evolve as time passed.

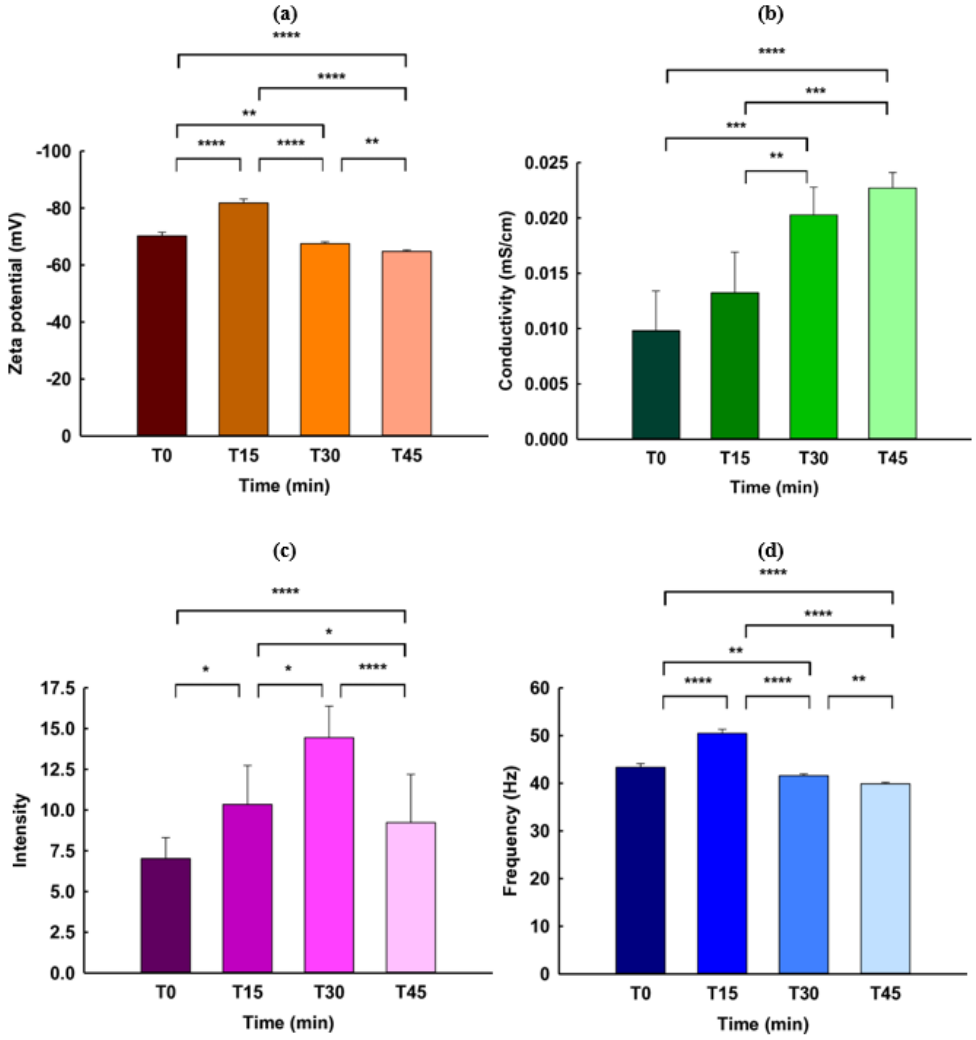

Figure 4 Changes in (a) zeta potential, (b) conductivity, (c) frequency and (d) intensity at various time intervals (T0, T15, T30, T45). Asterisks indicate significant differences between groups (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 and **** p-value < 0.0001).

Multivariate analysis of nanoemulsions

Figure 5(a) presents the Principal Component Analysis (PCA) biplot, exhibiting the changes as the nanoemulsion evolves over time. Together, PC1 and PC2 can account for 80.79% of the total variance. The patterns reveal distinct clusters. The first, shown in red, represents the T0 samples, with a greater polydispersity index that suggests heterogeneity as the initial formulation develops. Next, the T15 samples are shown in green, and these are strongly linked to frequency parameters and smaller droplet sizes, indicative of the initial stabilization taking place relatively quickly. The samples for T30 samples are shown in blue, and are closely correlated to intensity measurements. Finally, the T45 samples shown in cyan have the greatest association with the conductivity vectors and zeta potential, suggesting that electrostatic stabilization takes place progressively over time. PCA clearly reveals the various relationships arising over time between different physicochemical parameters, whereby the initial position of variables from the origin makes a considerable contribution to the eventual sample differentiation. In **Figure 5(b)**, the hierarchical clustered heatmap is presented, allowing the earlier findings to be visualized through an alternative perspective. In this view, one hierarchical cluster comprises T0 and T15,

while a second comprises T30 and T45, thus showing a pair of different stability phases. These parameter clusters indicate the similarities in behavior between conductivity and zeta potential, and between size and polydispersity index. From the original color gradient it can be observed that the sample for T45 shows the best combination of low polydispersity, a moderate droplet size, and greater zeta potential values (-60 mV). All of these qualities lead to better nanoemulsion stability. The results concur with those of McClements [29], whose work revealed that when nanoemulsions achieve electrokinetic equilibrium, their stability over the longer term is enhanced via combined electrosteric mechanisms, especially if the absolute value of the zeta potential is greater than 25 mV while the polydispersity index is lower than 0.3.

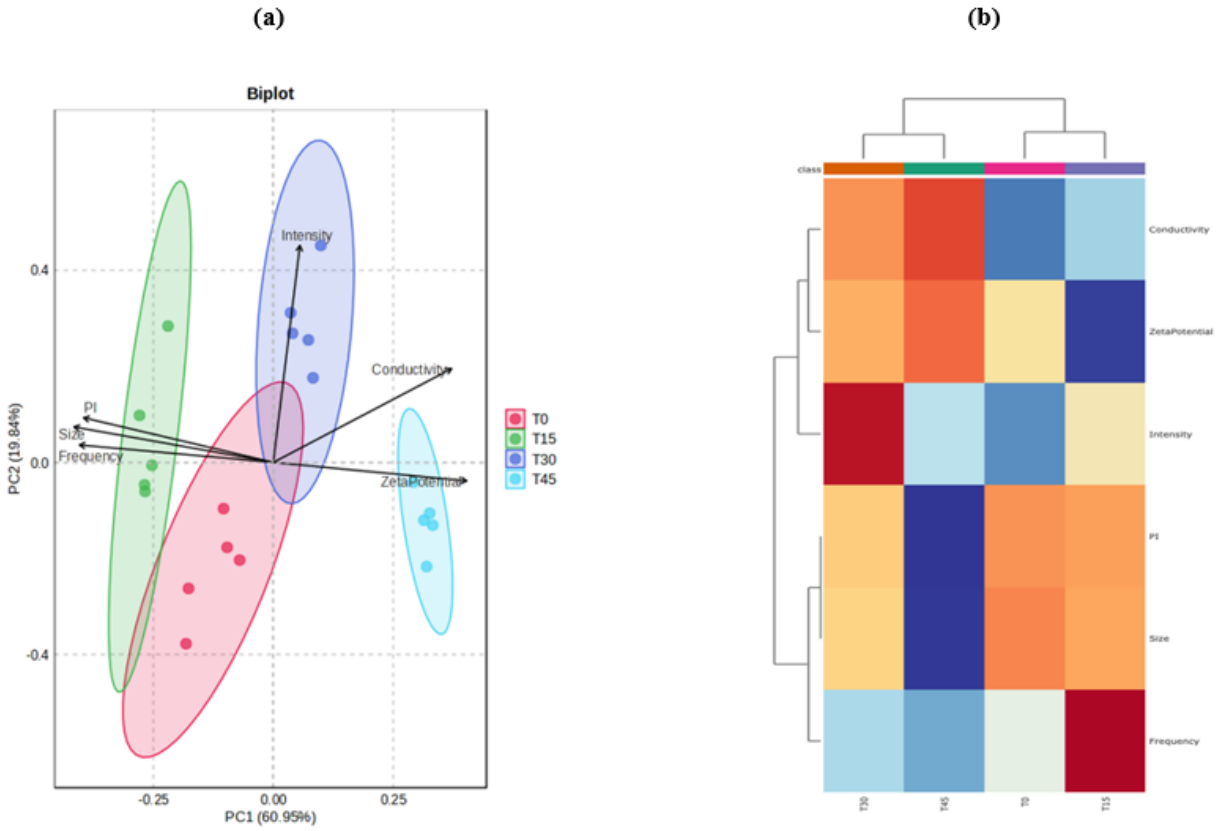

Figure 5 Correlation among variables at various time intervals (T0, T15, T30, T45) presented as (a) PCA biplot exhibiting sample clustering and variable contributions and (b) Heatmap exhibiting hierarchical clustering and relative variable intensities.

Recently, research has proven that PCA, when combined with Cluster Analysis within the framework of data analytics, is a straightforward method for rationalizing the study and development of catechin-SNEDDS/nanoemulsion formulations. Visualization tools such as biplot and heatmap tell the statistical story of how various formulation variables interact, so one can more easily interpret complex data sets with fewer dimensions [36]. More importantly, more recent studies emphasize the role of PCA in intuitively visualizing the associations and properties of nanoemulsions by generating graphical results, aiding in a better comprehension of the elements that control these systems [37]. Finally, principal component analysis biplots have been useful in the depiction of data points across principal components and clustering of features within nanoemulsion formulations to identify dominant trends and correlations [38].

Conclusions

In this research study, a peptide-loaded nanoemulsion was developed from synthetic peptides

derived from *Synechococcus* sp. The resulting nanoemulsion then underwent testing to determine the optimal formulation. It was found that the concentration of the peptide has a significant effect upon the corresponding physicochemical properties. The use of HPH at 7,500 psi for 45 min served to lower the particle size from an initial 438 nm to 122 nm, while polydispersity, transparency, and emulsion uniformity were all improved. This optimized formulation was physically stable and retained its physical integrity when subjected to centrifugation and to the thermal stresses involved in the heating-cooling and freeze-thaw cycles. The results indicate the suitability of the developed nanoemulsion to serve as a means of delivering bioactive peptides used in the cosmetics and pharmaceutical industries. The nanoemulsion offers small and highly uniform droplets with excellent stability, which provide strong potential for further applications in the areas of transdermal or topical delivery.

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Declaration of Generative AI in Scientific Writing

The authors declare that no generative AI technologies were used in the writing, editing, or preparation of this manuscript.

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