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## Ultrasound-assisted microalgal docosahexaenoic acid (DHA) nanoemulsion preparation using casein, chitosan, and pectin as emulsifiers for enhanced oxidative stability and shelf life for food fortification

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The current work examines the effect of three distinct emulsifiers, such as casein, chitosan, and pectin, on the oxidative stability and physicochemical characteristics of docosahexaenoic acid (DHA) nanoemulsions. The DHA nanoemulsion formulated using chitosan (CHDN) had a greater zeta potential value of  $42.2 \pm 0.75$  mV with a particle size of  $122.5 \pm 2.5$  nm initially and maintained electrostatic stability ( $>30$  mV) throughout storage (28 days). The DHA nanoemulsions were further characterized using SEM, FTIR-ATR spectroscopy, and micro-Raman spectroscopy to confirm the presence of DHA in the nanoemulsions. Rheological measurements revealed shear-thinning behavior among nanoemulsions with an increase in shear rate. From the shelf life studies (28 days), it was evident that the DHA nanoemulsion formulated using chitosan ( $4.94 \pm 0.1$  Meq O<sub>2</sub> per kg) had significantly ( $p < 0.05$ ) higher oxidative stability compared to casein ( $6.4 \pm 0.2$  Meq O<sub>2</sub> per kg) and pectin ( $5.7 \pm 0.12$  Meq O<sub>2</sub> per kg). Furthermore, the encapsulation efficiency of CHDN was found to be 87.33%, as confirmed by GC-FID. Kalakand (a dairy confectionary) fortified with CHDN retained a higher concentration of DHA (92%) compared to neat DHA oil-incorporated kalakand (20%) after 10 days of storage. The physicochemical and textural properties of CHDN-fortified kalakand were significantly higher ( $p < 0.05$ ) than plain kalakand. Moreover, CHDN-incorporated kalakand had higher sensory attributes. These results demonstrate the successful use of DHA nanoemulsions in food matrices and thereby offer a unique nanoencapsulated DHA-fortified food with increased DHA retention.

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### Sustainability spotlight

The study offers a promising and sustainable solution to the worldwide problem of omega-3 fatty acid deficiency by employing a green nanoencapsulation method for algal-derived docosahexaenoic acid (DHA). Utilizing microalgal oil, a plant-based, renewable, and ecologically friendly source of DHA, the study supports international initiatives to reduce reliance on overfished marine resources while maintaining nutritional equity. Casein, chitosan, and pectin, which are non-toxic and biodegradable natural polymers, are used as emulsifiers. By extending DHA's shelf life and sensory compatibility, the nanoemulsion technology used here makes it possible to include it into the dairy confectionary, kalakand. The study aligns with UN Sustainable Development Goals such as zero hunger, good health and well-being, responsible consumption and production, and life below water.

## 1. Introduction

Docosahexaenoic acid (DHA) is an omega-3 fatty acid important for brain function and visual development.<sup>1</sup> DHA is primarily sourced from fatty fish, cod liver, krill, tuna, and sharks. Each source varies in omega-3 composition and intestinal absorption. The alternatives, such as algal oil, chia seeds, and walnuts, are gaining popularity nowadays.<sup>2</sup> DHA from algal oil provides

a safe substitute for fish oil without compromising its bioavailability. Algal oil is a sustainable source of DHA, especially in the context of growing concerns about heavy metal poisoning in fish and overfishing.<sup>3</sup> Dietary intake of DHA is often insufficient, especially in populations with low fish consumption. Fortifying everyday food products, such as dairy, bread, beverages, and snacks, ensures that a larger segment of the population can meet recommended intake levels. Unfortunately, DHA is quite vulnerable to oxidative breakdown, which can reduce its nutritional benefits and also result in the development of an unpleasant aroma and taste.<sup>4</sup> The stability of the DHA can be improved by encapsulation using different wall

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materials and emulsifiers by various technologies, including ultrasonication, microfluidization, freeze-drying, and high-pressure homogenization.<sup>5</sup> The ultrasonication process involves the use of high-frequency sound waves, which create intense shear forces and cavitation within the liquid medium, breaking the oil droplets into nanoscale sizes and forming a stable emulsion. It is a commonly utilized encapsulation method that may produce tiny droplet sizes.<sup>6</sup>

Nanoemulsions, colloidal particulate systems, are widely used for the encapsulation and protection of bioactive compounds with droplet sizes between 20 and 200 nm.<sup>7</sup> Nanoemulsions have a high surface area and greater kinetic stability compared to microemulsions. Nanoemulsions prevent oxidative degradation of DHA by preventing lipid oxidation, which is a major challenge with traditional formulations. Encapsulation of DHA in nanoemulsions improves its sensory characteristics by masking the unpleasant odor, which is a limiting factor in food fortification.<sup>8</sup> Protein and polysaccharide-based materials are extensively used for their stabilizing properties.<sup>9</sup> Casein is an amphiphilic protein without a fixed secondary structure. It is composed of phosphoproteins with four sub-fractions, namely  $\alpha$ S1-,  $\alpha$ S2-,  $\beta$ -, and  $\kappa$ -caseins.<sup>10</sup> All these protein fractions have an isoelectric point between 4.1 and 5.3. Due to the presence of exposed hydrophobic regions in its structure, casein adsorbs quickly to the surface of droplets.<sup>11</sup>  $\kappa$ -Casein is a special stabilizing protein essential for preserving the stability and integrity of casein micelles. It creates stearic hindrance, which prevents the mixing of oil and water droplets in an emulsion.<sup>12</sup>

Polysaccharides such as chitosan and pectin migrate slowly in a solution, as they are more rigid and larger in size.<sup>12</sup> Chitosan, a natural polysaccharide, is extensively used as an encapsulating material known for its polycationic properties and can form electrostatic interactions with negatively charged components.<sup>13</sup> Chitosan is composed of  $\beta$ -(1→4)- linked D-glucosamine and *N*-acetyl-D-glucosamine units. Chitosan stabilizes emulsions by providing electrostatic repulsion and steric hindrance when the pH of the solution is below its  $pK_a$  for the amine group (-NH<sub>2</sub>).<sup>14</sup> The concentration, viscosity and degree of deacetylation are the important factors that enhance the emulsifying properties and reduce droplet aggregation and creaming.<sup>15</sup> Usage of chitosan as an emulsifier enhanced the stability of DHA-enriched fish oil along with sodium tri-polyphosphate by limiting the production of lipid peroxides, aldehydes (4-hydroxy-2-hexenal), and secondary oxidation products of fish oil.<sup>16</sup> Pectin, a plant-based polysaccharide, is composed of 1,4-linked  $\alpha$ -D-galacturonic acid units. It stabilizes the nanoemulsion by increasing viscosity and preventing phase separation.<sup>17</sup> Pectin contributes a negative charge, especially when the pH of the solution is above its  $pK_a$  for the carboxyl group (-COOH). Pectin as an emulsifier exhibits good electrostatic stabilization if the pH of the solution is above its  $pK_a$ .<sup>18</sup> Pectin containing ferulic acid and a higher degree of methoxy and acetyl groups is more efficient in reducing interfacial tension.<sup>12</sup>

Milk and milk-based products are widely consumed foods that can be the right medium for the delivery of bioactive

compounds.<sup>19</sup> Kalakand is one of the significant traditional milk products made by adding sugar and the coagulant to the hot milk. Kalakand is an excellent source of lactose, fat, minerals, and proteins.<sup>20</sup> In the present study, kalakand was selected as a food matrix for DHA fortification because of its favorable composition. Milk proteins, particularly casein found in kalakand, maintain the stability of the DHA nanoemulsion by inhibiting lipid oxidation and enhancing its bioavailability.<sup>11</sup> Since kalakand doesn't require additional heat treatment during fortification, its structural integrity is maintained while preserving DHA's stability. DHA-fortified kalakand serves as a nutritious snack and mitigates omega-3 deficiencies, especially in populations with low fish consumption.<sup>21</sup>

Fortification of DHA in a food matrix in its encapsulated form, such as nanoemulsion, protects it from oxidative degradation, masks its unpleasant odor, and increases its bioavailability.<sup>22</sup> In recent research, Estrada *et al.* fortified microencapsulated salmon oil in yogurt to develop an omega-3-fortified dairy product. The above fortified product was supplemented to pregnant and breastfeeding women, which resulted in increased PUFA (polyunsaturated fatty acid) content in the blood.<sup>23</sup> Similarly, flaxseed oil emulsions were fortified in butter to enhance its nutritional profile.<sup>24</sup> Other food products such as spaghetti, cheese, low-fat probiotic fermented milk, probiotic soymilk, and apple juice were fortified with omega-3 fatty acids, particularly DHA and EPA.<sup>21</sup> The novelty of the current study lies in the incorporation of a chitosan-stabilized DHA nanoemulsion in kalakand, a dairy-based confectionery. Even though fortification of nanoencapsulated omega-3 fatty acids has been widely studied in beverages and dairy matrices such as yogurt and milk, the application of DHA, especially as a nanoemulsion in dairy-based confectionery, remains unexplored. This innovation transforms conventional dairy products into functional foods.

In the current research, DHA nanoemulsions were prepared using three different emulsifiers: casein, chitosan, and pectin. The study investigates the impact of different emulsifier concentrations on the zeta potential, droplet size, morphology, and oxidative stability of DHA nanoemulsions and the fortification of DHA nanoemulsions in dairy food products. The findings aim to bridge the gap between nanoemulsion technology and traditional food fortification, contributing to both scientific knowledge and consumer health.

## 2. Materials and methods

Algal DHA oil (50% purity) was purchased from Ovegha Nutraceuticals Pvt Ltd, India. Casein and pectin were procured from Saatvic Foods, India. Tween 80 was procured from Sigma-Aldrich, USA. Food-grade chitosan with 90% deacetylation was purchased from Mark Nature, USA.

### 2.1. Oil holding capacity of emulsifiers

The oil-holding capacity of the emulsifiers was determined along with neat DHA oil to assess their ability to emulsify and retain DHA oil as described below.<sup>25</sup> The emulsifiers, such as



casein, chitosan, and pectin, were individually measured (1 g, w/v) and added to 10 ml of neat DHA oil. The mixture was vortexed and centrifuged at 1600 g for 10 minutes. The oil-holding capacity of the emulsifiers after removing the supernatant was measured using the following equation, as per the literature.<sup>25</sup>

$$\text{Oil holding capacity (\%)} = \frac{W_2 - W_1}{W_0} \times 100$$

where  $W_0$  is the weight of the dry emulsifier (g),  $W_1$  is the weight of the centrifuge tube containing the dry emulsifier (g), and  $W_2$  is the weight of the centrifuge tube containing sediment after centrifugation (g).

## 2.2. Formulation of DHA nanoemulsions

The concentration of the emulsifiers was fixed based on the preliminary studies (SI Table 1), and the range of concentration to be studied was fixed based on the literature. The oil-in-water nanoemulsion was prepared by dissolving casein (5, 10, and 15% w/v),<sup>26</sup> chitosan (1, 1.5, and 3% w/v),<sup>16</sup> and pectin (3, 3.5, and 6% w/v)<sup>27</sup> at different concentrations along with Tween 80 (1%) for the encapsulation of DHA (Table 1). Casein and pectin were dissolved individually in distilled water at 80 °C, and chitosan was dissolved in 1% acetic acid (w/v) at 800 rpm using a magnetic stirrer. Emulsifiers were allowed to hydrate overnight, which served as an aqueous phase. After complete hydration, DHA was added dropwise along with Tween 80 under stirring at 800 rpm for 40 to 60 minutes. The oil-to-aqueous phase ratio was maintained at 1:20. The emulsions formulated were sonicated (500 W) at 30 kHz using a probe sonicator with a 25 mm diameter probe size (VC-505-220, Sonics Materials, Portugal) for 10 minutes with a cycle of 10 s on and 10 s off. The formulated emulsion was stored under refrigerated conditions (4 °C) until analysis. All the experiments were carried out at ambient temperature (25 ± 2 °C) unless otherwise stated.

## 2.3. Stability studies of DHA nanoemulsions with various emulsifier concentrations

**2.3.1. Measurement of physical stability (creaming index%).** The fresh DHA nanoemulsions (10 ml) were transferred to clear glass tubes and sealed immediately to avoid oxidation. Emulsions were monitored for creaming or phase

separation throughout storage. The creaming index was calculated as described by Singh *et al.*<sup>28</sup>

$$\text{Creaming index (\%)} = \frac{\text{height of cream layer}}{\text{total height of emulsion}} \times 100$$

**2.3.2. Measurement of color stability.** The DHA nanoemulsion samples were determined using a Hunter LAB colorimeter (UK), considering the  $L^*$ ,  $a^*$ , and  $b^*$  indices, and the difference in color variation was expressed as  $\Delta E$ .

**2.3.3. Measurement of droplet size and electrostatic stability.** The measurement of droplet size and electrostatic stability (zeta potential) of nanoemulsions was performed using an SZ-100 (particle size analyzer), Horiba Ltd, Tokyo, Japan. The droplet size analysis was performed based on Brownian motion (dynamic light scattering) with a dynamic range between 0.3 nm and 8  $\mu\text{m}$ . The samples were diluted 50–60 times and homogenized using a bath sonicator for 5 minutes before analysis.<sup>29</sup>

## 2.4. Characterization of stable DHA nanoemulsions with selected emulsifier concentration

**2.4.1. Morphological measurements.** The morphology of the nanoemulsion samples was analyzed using a scanning electron microscope (SEM). For the study, 10–15  $\mu\text{l}$  of the emulsion samples were placed on copper grids and allowed to dry overnight. The samples were observed using a TESCAN VEGA3 (Czech Republic) scanning electron microscope at a voltage of 20 kV.

**2.4.2. Chemical characterization.** DHA nanoemulsions were analyzed for chemical composition and interaction among ingredients using FTIR (SHIMADZU, ITRACER 100, Japan) and micro-Raman spectroscopy (HORIBA, Japan). For FTIR analysis, the wavelength was recorded between 4000 and 500  $\text{cm}^{-1}$ . Raman spectra were recorded between 3500 and 400  $\text{cm}^{-1}$ .<sup>30</sup>

**2.4.3. Viscosity measurements.** The emulsion samples were determined for viscosity using a Brookfield viscometer (Brookfield Engineering Labs Inc., US). 8 g of the emulsion samples were taken and measured at various shear rates (0 to 100  $\text{s}^{-1}$ ) with spindle number SC4-21.<sup>31</sup>

## 2.5. Storage stability of DHA in nanoemulsions

The DHA nanoemulsions were developed and stored at 4 °C for 28 days. During storage, droplet size, electrostatic stability,

Table 1 Composition of DHA nanoemulsions formulated using different types of emulsifiers

Type of emulsifier	Emulsifier concentration%	T80%	DHA oil%	Water%
Casein	5	1	5	95
	10	1	5	95
	15	1	5	95
Chitosan	1	1	5	95
	1.5	1	5	95
	3	1	5	95
Pectin	3	1	5	95
	3.5	1	5	95
	6	1	5	95



oxidative stability, and concentration of DHA in nanoemulsions were measured.

**2.5.1. Measurement of droplet size and electrostatic stability.** The size and electrostatic stability of the emulsion were measured every 7th day as described earlier in Section 2.3.3 for 28 days. The change in zeta potential value and size of droplet was expressed as mean  $\pm$  standard deviation.

**2.5.2. Measurement of oxidative stability.** The pH of the nanoemulsions was measured using a Lab Friend (India) pH meter. The acid value (AV) was determined for the nanoemulsion samples throughout storage based on the AOAC 2000 method. Briefly, the DHA nanoemulsion samples were weighed (1 g) and mixed with ethyl alcohol (50 ml). Phenolphthalein (1 ml) was used as an indicator. The mixture (sample, ethyl alcohol, and phenolphthalein) was heated (80 °C) for 15 minutes. After that, the mixture was titrated against potassium hydroxide solution until dark pink became light in color.<sup>32</sup> The peroxide value (PV) was determined for the DHA nanoemulsion samples to measure their oxidative stability. Briefly, a 5 G nanoemulsion sample was suspended in 30 ml of acetic acid-chloroform solvent. Potassium iodide solution (saturated) (1 ml) was added and kept in the dark for one minute. The mixture was added with water (30 ml) and titrated against Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (0.1 N). The peroxide value (PV) of the samples was measured using the AOAC (2000) method.

**2.5.3. Concentration of DHA in nanoemulsions.** The concentration of DHA present in the CHDN nanoemulsion was measured using GC-FID (Agilent 7890B, US). In order to produce fatty acid methyl ester (FAME), nanoemulsion samples were first hydrolyzed for 20 minutes at 60 °C using a 0.5 M potassium hydroxide solution and then esterified for 5 minutes at 60 °C using a 14% boron trifluoride solution. Throughout the sample preparation process, nitrogen gas was utilized to avoid oxidation of DHA. FAME was extracted using *n*-hexane followed by centrifugation at 3000 rpm. The resulting supernatant was subsequently analyzed for fatty acid concentration (AOAC, 2019).

## 2.6. Fortification of DHA nanoemulsions in kalakand

The kalakand was prepared using the standardized milk with 3 g total fat, 4.7 g carbohydrates, and 3.4 g protein procured from a local market in Chennai, Tamil Nadu, India. Kalakand was prepared according to the method described by Jain *et al.*<sup>33</sup> The preparation was divided into three groups consisting of a negative control (plain kalakand, KCON), a positive control (kalakand with neat DHA oil, KDHO), and a test group (kalakand with DHA oil nanoemulsions, KCDN). Neat DHA oil and DHA nanoemulsions were added to the kalakand formulation at 40–45 °C and were immediately allowed to set at room temperature. The concentration of DHA present in the kalakand samples was 0.5 g per 100 g. CHDN was selected for food fortification based on its higher stability throughout storage. A detailed flowchart illustrating the preparation of kalakand is provided in the SI (SI Fig. 1).

**2.6.1. Sensory analysis.** The sensory analysis was performed using 20 semi-trained panelists. The panelists were provided with 50 g of kalakand samples for assessment and

rated the samples for color, flavor, taste, texture, and overall acceptability using a 9-point hedonic scale.<sup>29</sup>

## 2.6.2. Shelf-life analysis of kalakand

**2.6.2.1. Physicochemical analysis.** The prepared kalakand samples were analyzed for moisture, ash, protein, fat, and carbohydrates using the AOAC (2000) method immediately after preparation and throughout storage (day 10). The color, pH (AOAC, 2000), acidity (AOAC, 2000), and peroxide value (AOAC, 2000) of the samples were monitored throughout storage (day 10) as described earlier. The water activity ( $a_w$ ) of kalakand samples was determined using a water activity meter (AquaLab 3 TE, USA).

**2.6.2.2. Concentration of DHA in kalakand.** The concentration of DHA present in the fortified kalakand samples was measured using GC-FID (Agilent 7890B, US). The analysis was performed as described in Section 2.5.3.

**2.6.2.3. Texture profile analysis.** A texture analyzer (Stable Micro Systems, UK) was used to characterize the texture of kalakand samples (1.5 cm<sup>3</sup> size pieces) using probe P/75 compression platen with the speed of 5 mm s<sup>-1</sup>.

**2.6.2.4. Microbial analysis.** The microbiological analysis of samples was performed to assess their safety for consumption and to ensure the absence of microbial contamination. All the prepared samples were tested for yeast and mold count (Y&M), as well as total plate count (TPC). Kalakand samples (1 g) were homogenized using a mortar and pestle after adding sterile saline (9 ml). Then, 1 ml of the homogenized sample was taken, and serial dilution was performed using sterile saline. The homogenized kalakand sample, as such and after different dilutions, was plated on plate count agar (TPC) and potato dextrose agar (Y&M). For TPC, the plates were incubated at 37  $\pm$  1 °C for 48 h, and for Y&M at 25  $\pm$  1 °C for 3 to 5 days. 1 g of DHA nanoemulsion after preparation was also analyzed for TPC and Y&M, to check for any microbial contamination. The samples were analyzed throughout the storage period (10 days).

## 2.7. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 20. One-way ANOVA followed by Tukey's multiple comparison test was used to identify significant differences.

# 3. Results and discussion

## 3.1. Oil holding capacity of different emulsifiers

The oil holding capacity (OHC) of the emulsifiers such as casein, chitosan, and pectin was determined initially before the preparation of the nanoemulsion to determine its efficiency. The OHC of casein, chitosan, and pectin was found to be 19.3  $\pm$  0.57%, 34.5  $\pm$  0.68%, and 17.2  $\pm$  0.57%, respectively. The oil-holding capacity of chitosan was significantly ( $p < 0.05$ ) higher compared to casein and pectin.

## 3.2. Physical stability (creaming index%) of DHA nanoemulsions with various emulsifier concentrations

The casein DHA nanoemulsion (CADN), chitosan DHA nanoemulsion (CHDN), and pectin DHA nanoemulsion (PEDN)



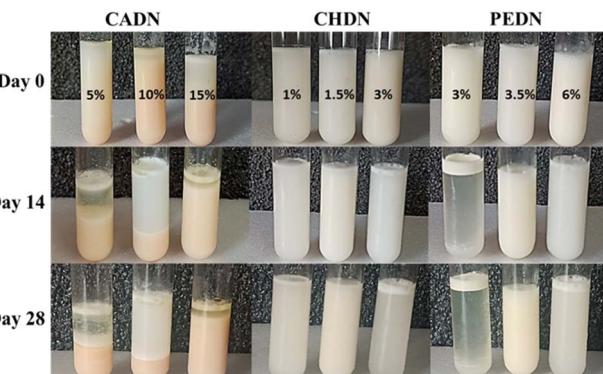


Fig. 1 Visual representation of the effect of emulsifier concentration on the creaming stability of DHA nanoemulsions. Where CADN represents the casein DHA nanoemulsion, CHDN represents the chitosan DHA nanoemulsion, and PEDN represents the pectin DHA nanoemulsion.

prepared at different concentrations of emulsifiers (10–20%, 1–3%, and 3–6%, respectively) were analyzed for phase separation over storage for 28 days at 4 °C (Fig. 1). The creaming index evaluates the physical stability of the nanoemulsion, as it is indirectly related to droplet aggregation.<sup>28</sup> All the prepared nanoemulsions were stable, and no phase separation was observed immediately after the preparation (Table 2). However, CHDN (1.5%) and PEDN (3.5%) exhibited higher physical stability without phase separation for up to 28 days compared to other combinations of nanoemulsions. The separation of the cream and serum layers occurred due to the Ostwald ripening effect.<sup>34</sup> Phase separation can also happen due to the difference in density of the oil and aqueous phase, coalescence, increased particle size, and flocculation in the emulsion.<sup>28</sup>

### 3.3. Color stability of DHA nanoemulsions with various emulsifier concentrations

The color analysis was performed for all the samples considering the  $L^*$ ,  $a^*$ , and  $b^*$  parameters. All the DHA nanoemulsion

samples were analyzed for color during storage (28 days) (Table 3).  $L^*$ ,  $a^*$ , and  $b^*$  indexes significantly varied ( $p < 0.05$ ) across samples immediately after preparation. All combinations of casein-based nanoemulsions showed a slight shift towards red. After storage (28 days), all the casein and pectin-based nanoemulsions varied significantly ( $p < 0.05$ ) in lightness and yellowness index with time, which may be due to particle aggregation.<sup>29</sup> Chitosan-based nanoemulsions showed a greenish tint that decreased over time. There was a continuous increase in the yellowness of all the nanoemulsions throughout storage. Among the formulations, CHDN1.5, PEDN6, and CADN5 showed significantly lesser ( $p < 0.05$ ) variations in color ( $\Delta E < 3$ ) upon storage. Major deviation in color was observed with PEDN3. Interestingly, the yellowness of the nanoemulsion with a chitosan concentration of 1.5% does not have much variation in yellow index from day 1 ( $14.6 \pm 0.1$ ) to day 28 ( $14.8 \pm 0.1$ ). This may be due to the uniform dispersion of particles in the nanoemulsion, which prevented particle aggregation and oxidation of the DHA oil. The particle size influences the color of the nanoemulsion, which occurs due to Ostwald ripening.<sup>29</sup> Decreased particle size with a higher zeta potential value increases the stability of the emulsion.<sup>34</sup> The DHA nanoemulsions with emulsifier concentrations of CADN (10%), CHDN (1.5%), and PEDN (3.5%) were selected for further analysis because of higher physical and electrostatic stability.

### 3.4. Droplet size and electrostatic stability of DHA nanoemulsions with various emulsifier concentrations

DHA nanoemulsions prepared using various concentrations of casein (CADN), chitosan (CHDN), and pectin (PEDN) as emulsifiers had zeta potential values in the range from  $-31.33 \pm 0.58$  to  $-16.37 \pm 0.55$  mV,  $42.2 \pm 0.75$  to  $11.5 \pm 0.44$  mV, and  $-38.63 \pm 1$  to  $-29.17 \pm 0.29$  mV, respectively (Table 4). Zeta potential measures the electrophoretic mobility and characterizes the surface charge.<sup>35</sup> Zeta potential values above 30 mV indicate higher electrostatic stability.<sup>36</sup> The droplet size of CADN, CHDN, and PEDN varied from  $192.3 \pm 7.51$  to  $225 \pm 5$  nm,  $185.1 \pm 4.46$  to  $258.6 \pm 2.28$  nm, and  $122.5 \pm 2.5$  to  $235.43 \pm 4$  nm,

Table 2 Effect of emulsifier concentration on the creaming stability of DHA nanoemulsions<sup>a</sup>

DHA nanoemulsion	Emulsifier concentration (%)	Creaming index (%)				
		Day 1	Day 7	Day 14	Day 21	Day 28
CADN	10	*	$25 \pm 0.01^c$	$47 \pm 0.01^b$	$48 \pm 0.05^b$	$50 \pm 0.05^a$
	15	*	$35 \pm 0.07^c$	$69.2 \pm 0.05^b$	$71.8 \pm 0.07^a$	$71.8 \pm 0.07^a$
	5	*	$10 \pm 0.05^c$	$20.6 \pm 0.01^b$	$25 \pm 0.05^a$	$25 \pm 0.01^a$
CHDN	1	*	*	$50 \pm 0.03^c$	$70 \pm 0.07^b$	$75 \pm 0.05^a$
	1.5	*	*	*	*	*
	3	*	*	*	$5.5 \pm 0.03^b$	$9.09 \pm 0.03^a$
PEDN	3	*	*	$12.5 \pm 0.07^a$	$12.5 \pm 0.01^a$	$12.5 \pm 0.05^a$
	3.5	*	*	*	*	*
	6	*	*	*	$5 \pm 0.05^b$	$10 \pm 0.07^a$

<sup>a</sup> CADN – casein DHA nanoemulsion; CHDN – chitosan DHA nanoemulsion; PEDN – pectin DHA nanoemulsion. \* indicates a stable nanoemulsion without phase separation. Data are articulated as mean  $\pm$  standard deviation ( $n = 3$ ). In an individual column, each value tailed by a different letter is significantly different ( $p \leq 0.05$ ) as determined by Tukey's test.



Table 3 Effect of the emulsifier concentration on the color stability of DHA nanoemulsions<sup>a</sup>

Number of days	Color parameters	CADN5	CADN10	CADN15	CHDN1	CHDN1.5	CHDN3	PEDN3	PEDN3.5	PEDN6
Day 1	<i>L*</i>	67.7 ± 0.5 <sup>a</sup>	63.9 ± 0.3 <sup>ab</sup>	63.9 ± 0.9 <sup>c</sup>	65.9 ± 0.1 <sup>ab</sup>	78.9 ± 0.2 <sup>a</sup>	72.3 ± 0.2 <sup>a</sup>	59.2 ± 1.0 <sup>b</sup>	68.7 ± 0.6 <sup>c</sup>	69.6 ± 0.7 <sup>b</sup>
	<i>a*</i>	0.66 ± 0.01 <sup>a</sup>	4.06 ± 0.05 <sup>a</sup>	1.42 ± 0.1 <sup>c</sup>	-2.09 ± 0.1 <sup>b</sup>	-1.75 ± 0.2 <sup>bc</sup>	-1.9 ± 0.09 <sup>c</sup>	-0.9 ± 0.2 <sup>a</sup>	0.32 ± 0.28 <sup>a</sup>	-0.98 ± 0.1 <sup>ab</sup>
	<i>b*</i>	18.6 ± 0.1 <sup>a</sup>	19.8 ± 0.04 <sup>a</sup>	14.01 ± 0.7 <sup>d</sup>	11.03 ± 0.2 <sup>d</sup>	14.6 ± 0.1 <sup>b</sup>	9.81 ± 0.2 <sup>c</sup>	9.35 ± 0.3 <sup>ab</sup>	14.05 ± 0.2 <sup>b</sup>	8.69 ± 0.2 <sup>ab</sup>
Day 7	<i>L*</i>	67.2 ± 3.4 <sup>a</sup>	67.9 ± 0.9 <sup>a</sup>	72 ± 0.06 <sup>a</sup>	69 ± 0.5 <sup>a</sup>	77.5 ± 0.8 <sup>b</sup>	71.4 ± 0.01 <sup>a</sup>	63.5 ± 0.9 <sup>a</sup>	77 ± 0.09 <sup>a</sup>	71.8 ± 0.06 <sup>a</sup>
	<i>a*</i>	0.65 ± 0.1 <sup>a</sup>	3.4 ± 0.05 <sup>a</sup>	0.41 ± 0.09 <sup>d</sup>	-2.1 ± 0.2 <sup>b</sup>	-1.8 ± 0.04 <sup>c</sup>	-2.2 ± 0.04 <sup>c</sup>	-1 ± 0.05 <sup>a</sup>	0.01 ± 0.09 <sup>a</sup>	-1.3 ± 0.1 <sup>c</sup>
	<i>b*</i>	18.6 ± 0.5 <sup>a</sup>	19.04 ± 0.5 <sup>a</sup>	15.6 ± 0.08 <sup>c</sup>	11.8 ± 0.4 <sup>c</sup>	14.3 ± 0.3 <sup>c</sup>	10.1 ± 0.05 <sup>c</sup>	8.6 ± 0.2 <sup>bc</sup>	14.7 ± 0.03 <sup>a</sup>	8.37 ± 0.1 <sup>b</sup>
Day 21	<i>L*</i>	66.1 ± 1.3 <sup>a</sup>	63.01 ± 0.2 <sup>c</sup>	67.04 ± 0.8 <sup>b</sup>	63.7±2 <sup>b</sup>	77.2 ± 2.6 <sup>a</sup>	69.9 ± 1.5 <sup>b</sup>	65.04 ± 0.2 <sup>b</sup>	71.7 ± 1.4 <sup>b</sup>	68.9 ± 0.4 <sup>b</sup>
	<i>a*</i>	0.9 ± 0.09 <sup>a</sup>	2.3 ± 0.2 <sup>ab</sup>	4.2 ± 0.2 <sup>b</sup>	-1.7 ± 0.07 <sup>b</sup>	-1.9 ± 0.2 <sup>b</sup>	-1.5 ± 0.1 <sup>b</sup>	-0.8 ± 0.2 <sup>a</sup>	0.16 ± 0.3 <sup>a</sup>	-0.7 ± 0.04 <sup>a</sup>
	<i>b*</i>	15.4 ± 0.3 <sup>c</sup>	18.5 ± 0.16 <sup>a</sup>	20.2 ± 0.14 <sup>a</sup>	13.8 ± 0.2 <sup>b</sup>	14.7 ± 0.4 <sup>b</sup>	10.9 ± 0.4 <sup>b</sup>	8.1 ± 0.4 <sup>c</sup>	14.4 ± 0.08 <sup>ab</sup>	8.9 ± 0.07 <sup>a</sup>
Day 28	<i>L*</i>	67.4 ± 0.2 <sup>a</sup>	64.9 ± 1.6 <sup>c</sup>	61.9 ± 0.7 <sup>d</sup>	63 ± 0.9 <sup>ab</sup>	77.3 ± 0.1 <sup>c</sup>	68.7 ± 0.2 <sup>b</sup>	68.8 ± 0.3 <sup>b</sup>	65.7 ± 0.4 <sup>d</sup>	72.2 ± 0.05 <sup>a</sup>
	<i>a*</i>	1.5 ± 0.4 <sup>b</sup>	1.2 ± 1.5 <sup>b</sup>	3.9 ± 0.08 <sup>b</sup>	-1.9 ± 0.2 <sup>a</sup>	-1.8 ± 0.1 <sup>a</sup>	-1.4 ± 0.2 <sup>a</sup>	-1 ± 0.2 <sup>a</sup>	0.1 ± 0.1 <sup>a</sup>	-1.1 ± 0.1 <sup>bc</sup>
	<i>b*</i>	16.8 ± 0.4 <sup>b</sup>	18.7 ± 1.5 <sup>a</sup>	18.1 ± 0.2 <sup>a</sup>	13.6±1 <sup>a</sup>	14.8 ± 0.1 <sup>a</sup>	12.1 ± 0.5 <sup>a</sup>	9.6 ± 0.5 <sup>c</sup>	14.5 ± 0.2 <sup>ab</sup>	8.8 ± 0.3 <sup>ab</sup>
	$\Delta E$	3.31 ± 0.06 <sup>b</sup>	3.6 ± 0.08 <sup>b</sup>	2.97 ± 0.1 <sup>bc</sup>	1.59 ± 0.2 <sup>c</sup>	3.91 ± 0.08 <sup>b</sup>	9.05 ± 0.09 <sup>a</sup>	3.48 ± 0.5 <sup>b</sup>	2.62 ± 0.41 <sup>c</sup>	

<sup>a</sup> CADN – casein DHA nanoemulsion; CHDN – chitosan DHA nanoemulsion; PEDN – pectin DHA nanoemulsion. The numbers next to the sample code denote the percentage of emulsifier used. Data are articulated as mean ± standard deviation ( $n = 3$ ). In an individual row, each value tailed by a different letter is significantly different ( $p \leq 0.05$ ) as determined by Tukey's test.

respectively. CHDN had a higher zeta potential value next to PEDN. The increased zeta potential of chitosan is due to its low pH and polycationic nature. In an acidic environment, the amino groups ( $-\text{NH}_2$ ) become protonated, forming  $-\text{NH}_3^+$  groups, which leads to an overall positive surface charge, which in turn increases the zeta potential value. The polycationic nature of the chitosan enhances the electrostatic repulsion between the particles, which is one of the major reasons for its increased stability.<sup>37</sup> In a study conducted by Esquerdo *et al.*,<sup>32</sup> a food-grade nanoemulsion was prepared using chitosan and gelatin as wall materials to encapsulate PUFA from carp oil. An increase in the zeta potential value (26.5 to 31 mV) was observed with an increased concentration of chitosan.<sup>32</sup> In a similar study, a double-layer emulsion was prepared to encapsulate capsaicin using chitosan and alginate, which had zeta potential values of 26.3 ± 12.7 mV and  $-8.97 \pm 0.43$  mV, respectively.<sup>38</sup> The increased stability of nanoemulsions improves the absorption of DHA in the intestinal membrane and also protects DHA from degradation during the gastric digestion.<sup>38</sup>

### 3.5. Morphological measurements of DHA nanoemulsions

The morphology and shape of the nanoemulsion were analyzed using SEM (Fig. 2). Among DHA nanoemulsions, CADN and CHDN exhibited a smooth and uniform microstructure, while PEDN showed an irregular shape with poor distribution. The surface morphology of the emulsions can be correlated with their particle size and polydispersity index (PDI), as a low PDI value indicates uniform dispersion and high homogeneity. The PDI values of CADN, CHDN, and PEDN were 0.4, 0.5, and 1, respectively. Even though PEDN has increased electrostatic stability ( $-38.6 \pm 1$  mV) and smaller droplet size ( $122.47 \pm 2.5$  nm) compared to CADN ( $-31.3 \pm 0.58$  mV;  $192.27 \pm 7.51$  nm), a spherical shape with uniform droplet distribution was attained in CADN. The morphology of CHDN aligns well with its electrostatic stability ( $42.2 \pm 0.75$  mV) and droplet size ( $185.1 \pm 4.46$  nm). Overall, from the morphological studies, it was evident that CHDN and CADN had uniform dispersion, which may be due to their ability to prevent Ostwald ripening and coalescence of droplets.

### 3.6. Chemical characterization

The FTIR spectra were analyzed to study the chemical interaction between DHA and emulsifiers within the nanoemulsion (Fig. 3). In neat DHA oil, the characteristic peaks of DHA were found around  $1740 \text{ cm}^{-1}$ ,  $2930 \text{ cm}^{-1}$ ,  $1160 \text{ cm}^{-1}$ ,  $1450 \text{ cm}^{-1}$  and  $713 \text{ cm}^{-1}$ .<sup>39</sup> A sharp peak at  $1740 \text{ cm}^{-1}$  corresponds to the carbonyl ( $\text{C}=\text{O}$ ) stretching vibration of ester functional groups present in fatty acids. Peaks around  $2800\text{--}3000 \text{ cm}^{-1}$  in neat DHA oil correspond to C-H stretching vibration of the  $-\text{CH}_2-$  and  $\text{CH}_3-$  groups of the lipid backbone, indicating its aliphatic nature.<sup>28</sup> The peak at  $1160 \text{ cm}^{-1}$  was due to C-O stretching. The appearance of a peak at  $1450 \text{ cm}^{-1}$  was due to the bending vibration of methylene groups, and a weak band around  $713 \text{ cm}^{-1}$  occurred due to the vibration of methylene groups present in long-chain fatty acids.<sup>39</sup> The above functional groups confirm the structural integrity of neat DHA oil.

Table 4 Influence of emulsifier concentration on the electrostatic stability and droplet characteristics of DHA nanoemulsions<sup>a</sup>

DHA nanoemulsion	Emulsifier concentration (%)	Zeta potential (mV)	Droplet size (nm)	Polydispersity index (PDI)
CADN	5	$-31.3 \pm 0.58^{\text{abc}}$	$192.27 \pm 7.51^{\text{bc}}$	$0.4 \pm 0.06^{\text{de}}$
	10	$-29 \pm 1^{\text{abc}}$	$225 \pm 5^{\text{abc}}$	$0.34 \pm 0.05^{\text{e}}$
	15	$-16.37 \pm 0.55^{\text{c}}$	$247.33 \pm 5.5^{\text{ab}}$	$0.59 \pm 0.04^{\text{c}}$
CHDN	1	$38.33 \pm 0.5^{\text{a}}$	$213.97 \pm 4.3^{\text{abc}}$	$0.83 \pm 0.06^{\text{b}}$
	1.5	$42.2 \pm 0.75^{\text{a}}$	$185.1 \pm 4.46^{\text{c}}$	$0.5 \pm 0.05^{\text{cd}}$
	3	$11.5 \pm 0.44^{\text{c}}$	$258.63 \pm 2.2^{\text{a}}$	$0.91 \pm 0.07^{\text{ab}}$
PEDN	3	$-32.7 \pm 1.13^{\text{ab}}$	$198.33 \pm 1.53^{\text{bc}}$	$0.61 \pm 0.06^{\text{c}}$
	3.5	$-38.6 \pm 1^{\text{ab}}$	$122.47 \pm 2.5^{\text{d}}$	$1 \pm 0.04^{\text{a}}$
	6	$-29.17 \pm 0.29^{\text{abc}}$	$235.43 \pm 4^{\text{ab}}$	$0.52 \pm 0.05^{\text{ed}}$

<sup>a</sup> CADN – casein DHA nanoemulsion; CHDN – chitosan DHA nanoemulsion; PEDN – pectin DHA nanoemulsion. Data are articulated as mean  $\pm$  standard deviation ( $n = 3$ ). In an individual column, each value tailed by a different letter is significantly different ( $p \leq 0.05$ ) as determined by Tukey's test.

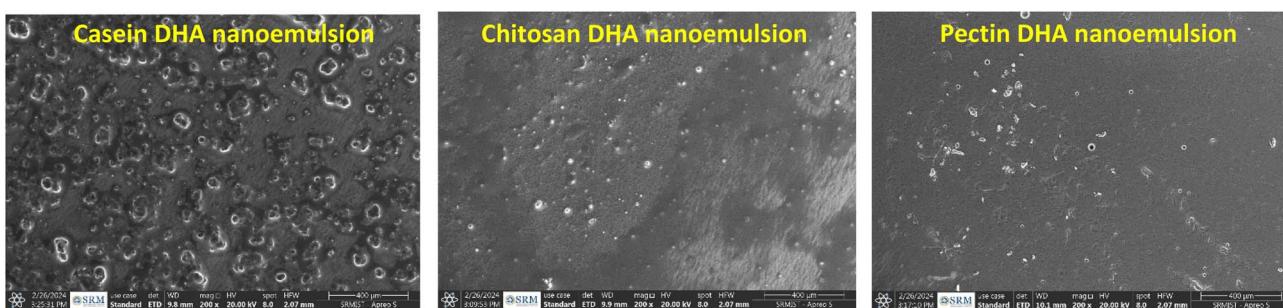


Fig. 2 Morphological examination of DHA nanoemulsions using scanning electron microscopy (SEM).

In neat casein, the bands between  $3300 \text{ cm}^{-1}$  and  $3000 \text{ cm}^{-1}$  indicate N-H stretching of the amide group, and the bands around  $1730 \text{ cm}^{-1}$  represent the vibration in the carbonyl (C=O) group of ester groups.<sup>40</sup> The band around  $1550 \text{ cm}^{-1}$  (amide I) indicates the presence of N-H group vibrations.<sup>40</sup> A shift in peak  $1650 \text{ cm}^{-1}$  was observed in CADN, which may be due to the interaction of ester carbonyl groups present in casein with DHA, causing changes in its secondary structure.<sup>41</sup> In neat pectin, the bands around  $1645 \text{ cm}^{-1}$  represent  $\text{COO}^-$  asymmetric stretching.<sup>42</sup> The bands around  $1400 \text{ cm}^{-1}$  represent the stretching of  $\text{CH}_3$ .<sup>43</sup> The bands between  $1200 \text{ cm}^{-1}$  and  $950 \text{ cm}^{-1}$  indicate the presence of carbohydrates from polysaccharides.<sup>42</sup> Bands between  $1000 \text{ cm}^{-1}$  and  $1150 \text{ cm}^{-1}$  indicate the presence of homogalacturonan, a structural component present in pectin.<sup>44</sup> The bands between  $3500 \text{ cm}^{-1}$  and  $3000 \text{ cm}^{-1}$  represent galacturonic acid present in pectin.<sup>44</sup> The peak at  $3313 \text{ cm}^{-1}$  (O-H stretching) in the pectin nanoemulsion represents strong hydrogen bonding between DHA and the hydroxyl (-OH) groups present in pectin, leading to a change in vibrational frequency.<sup>44</sup>

A broad band around  $3300 \text{ cm}^{-1}$  represents stretching of O-H in chitosan. The broadening of the peak around  $3300 \text{ cm}^{-1}$  (OH band) indicates hydrogen bonding and interaction of chitosan with DHA in nanoemulsions.<sup>45</sup> The occurrence of a band around  $1540 \text{ cm}^{-1}$  in chitosan indicates N-H bending of amino groups  $-\text{NH}_2$  and  $-\text{NH}_3^+$  (amide II) found in chitosan. There was a shift in the peak at  $1540 \text{ cm}^{-1}$  (chitosan) to a lower wave number of  $1420 \text{ cm}^{-1}$  in CHDN, indicating interaction of DHA

with amine groups present in chitosan.<sup>46</sup> But there was no formation of new covalent bonds, indicating physical interaction between DHA and chitosan at the interface.<sup>47</sup> Additionally, there might be a formation of a hydrogen bond between DHA (carboxyl or hydroxyl group) and chitosan (amide or carboxyl group) in CHDN. In similar research, Liu *et al.* observed a shift in the amide II band of DHA-loaded nanoparticles due to the electrostatic interaction of DHA with zein and PLGA.<sup>48</sup> Therefore, in the current study, the shift in amide II band was due to electrostatic interaction between chitosan ( $-\text{NH}_3^+$ ) and DHA ( $-\text{C=O}$  and  $-\text{COOH}$  groups). The occurrence of bands around  $1000 \text{ cm}^{-1}$  indicates C-O stretching skeletal vibrations, and around  $1100 \text{ cm}^{-1}$  indicates C-O-C antisymmetric and C-N stretching. The shift observed from  $1080 \text{ cm}^{-1}$  to  $1015 \text{ cm}^{-1}$  may be due to the adsorption of chitosan with DHA oil.<sup>46</sup> These interactions confirm the formation of nanoemulsions only by physical adsorption without the formation of additional new bands.

Raman spectroscopy was carried out to analyze the nonpolar molecular interactions between DHA and emulsifiers in the nanoemulsions (Fig. 4). DHA in CADN, CHDN, and PEDN was confirmed by its characteristic peaks around  $1650 \text{ cm}^{-1}$ , representing the C=C stretching vibrations of unsaturated fatty acids.<sup>49</sup> An intense peak around  $2850 \text{ cm}^{-1}$  (C-H stretching) was also observed in all the DHA nanoemulsions (CADN, CHDN, and PEDN). These results are in agreement with the findings of Killeen *et al.*, where intense bands were observed around



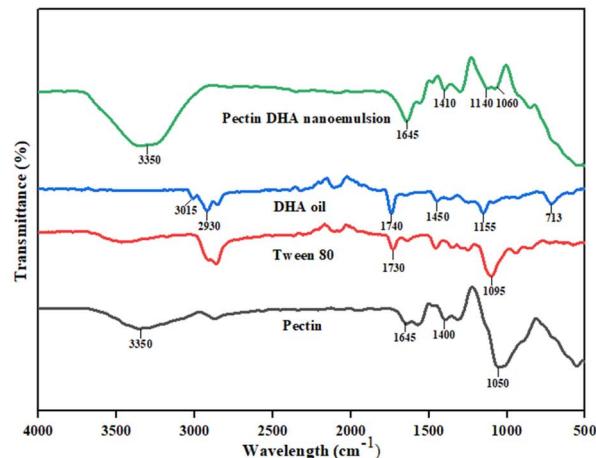
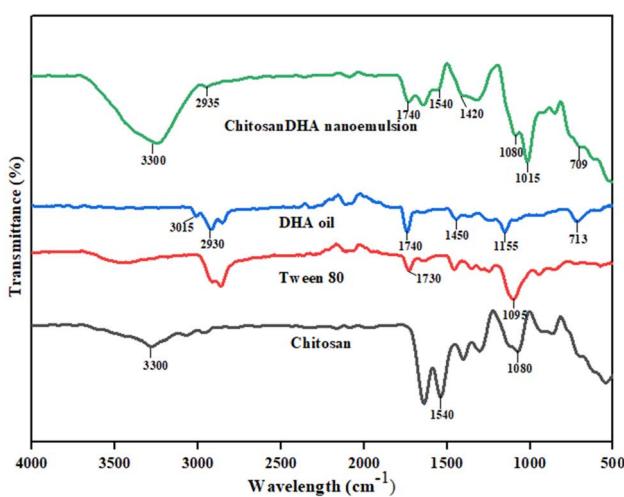
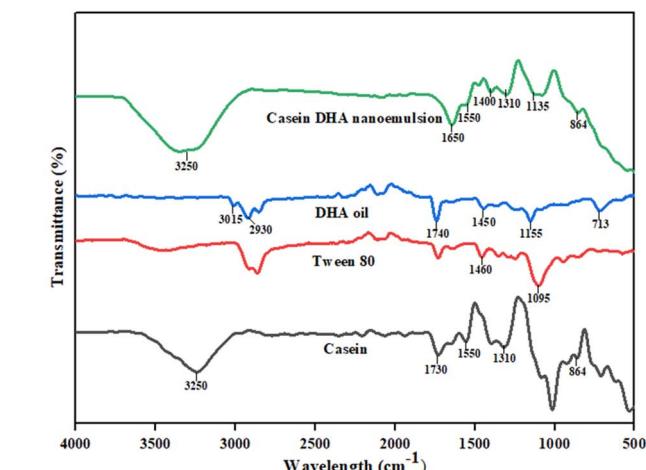


Fig. 3 FTIR spectra showing the chemical characterization of DHA nanoemulsions formulated with different emulsifiers.

1660  $\text{cm}^{-1}$  (C=C stretching) and 2839  $\text{cm}^{-1}$  (C–H stretching) in oil samples rich in omega-3 fatty acids.<sup>50</sup> The CADN spectrum confirms the presence of casein ( $\alpha$  helix structure) by exhibiting its characteristic bands around 1650  $\text{cm}^{-1}$  (amide I C=O stretch). The bands between 1200 and 1300  $\text{cm}^{-1}$  represent the

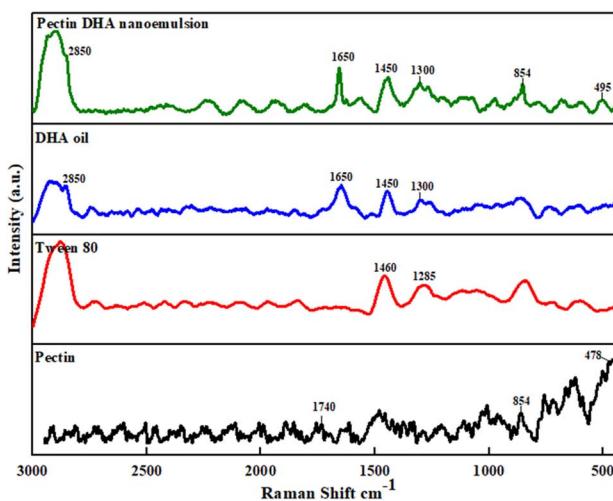
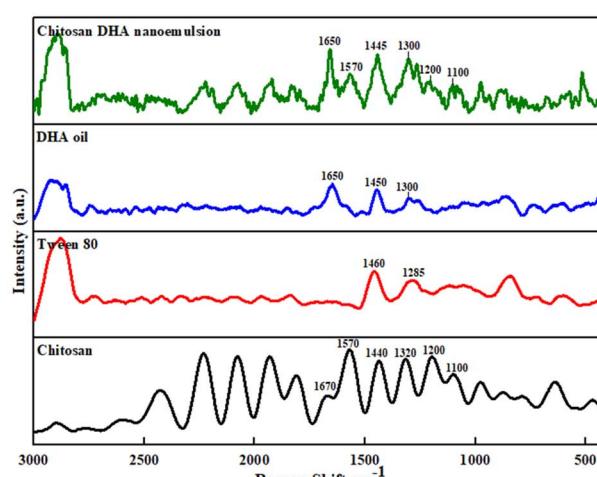
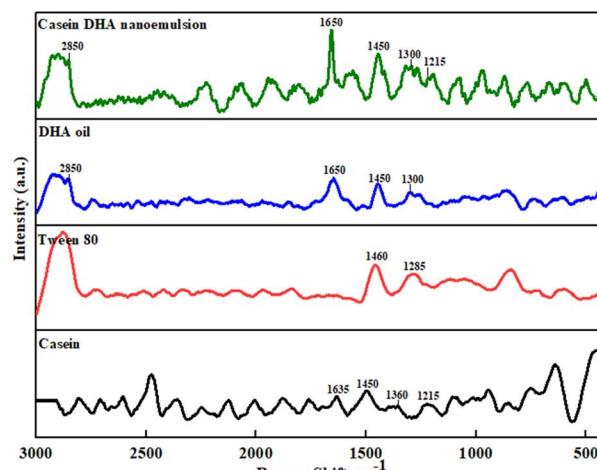


Fig. 4 Raman spectra showing the chemical characterization of DHA nanoemulsions formulated with different emulsifiers.

(amide III) C–N stretch and N–H bending of casein in CADN.<sup>51</sup> The characteristic bands between 1600 and 1750  $\text{cm}^{-1}$  correspond to C=O stretching vibrations of pectin.<sup>52</sup> The bands occurring around 850  $\text{cm}^{-1}$  indicate the presence of  $\alpha$ -glycosidic bonds in PEDN and pectin. Another characteristic band



around  $478\text{ cm}^{-1}$  was observed in pectin as well as in PEDN, corresponding to galacturonic acid present in pectin.<sup>53</sup> The characteristic spectra of chitosan were observed around  $1670\text{ cm}^{-1}$ ,  $1570\text{ cm}^{-1}$  and  $1440\text{ cm}^{-1}$  corresponding to amide I, amide II, and amide III bands, respectively.<sup>54</sup> Additional bands between  $1320$  and  $1200\text{ cm}^{-1}$  (C–N bending) and around  $1100\text{ cm}^{-1}$  (C–C) confirm the presence of chitosan in CHDN.<sup>54</sup>

### 3.7. Viscosity measurement

The DHA nanoemulsions were evaluated for viscosity measurements. The impact of the type of emulsifiers on the nanoemulsion's viscosity with respect to different shear rates is represented in Fig. 5. Along with CHDN, PEDN also exhibited favorable shear-thinning behavior, contributing to improved emulsion stability under varying shear rates. Significant changes ( $p < 0.05$ ) in the viscosity of CADN, CHDN, and PEDN were detected with an increase in shear rate. The initial viscosity (shear rate  $1\text{ s}^{-1}$ ) of CADN, CHDN, and PEDN was 80, 80 and 70.6 cP, which was decreased to 2.58, 39.38 and 42.23 cP, respectively, when the shear rate increased to  $100\text{ s}^{-1}$ , showing shear thinning behavior.<sup>55</sup> Compared to casein, nanoemulsions containing chitosan and pectin had a higher viscosity, which correlates with the findings of Tavares *et al.*<sup>55</sup> where garlic extract was encapsulated using whey protein isolate and chitosan. The viscosity of the coacervates showed non-Newtonian (shear thinning) behavior. The same behavior from the previous study was observed in chitosan with increased shear rate due to structural rearrangement or breakdown of molecules.<sup>56</sup> In a similar study, nanoemulsions were prepared using chitosan and alginate as wall materials using the self-assembly method.<sup>57</sup> It was found that the increase in chitosan concentration increased the viscosity of the nanoemulsions. The increase in viscosity is due to interactions in the hydrogen bonds, electrostatic bonds, and polymer chain. The addition of

food hydrocolloids such as chitosan and pectin increases the viscosity of the emulsion, whereas ionic strength, size of particles, molecular weight, charge of particles, temperature, and particle interactions are the factors affecting viscosity.<sup>56</sup>

### 3.8. Influence of emulsifiers on electrostatic stability over storage

Zeta potential is a critical component in determining the electrostatic stability of colloidal systems.<sup>29</sup> All three nanoemulsions (CADN, CHDN, and PEDN) had initial zeta potential values  $>30\text{ mV}$ , suggesting that there is greater electrostatic repulsion to keep particles from aggregation (Fig. 6A). CHDN had an initial zeta potential value of  $42.2 \pm 0.75\text{ mV}$ . The zeta potential value of CHDN after 28 days of storage was  $34 \pm 1\text{ mV}$ , which indicates higher stability. Higher levels of zeta potential often indicate more electrostatic repulsion between particles, which helps to avoid aggregation and preserve stability. The zeta potential may decrease with time due to the disintegration of the emulsifier, desorption, or rearrangement. The zeta potential of CADN dropped dramatically, indicating that emulsifier desorption may have happened more widely. Despite this reduction, CHDN maintained a zeta potential above  $30\text{ mV}$ , suggesting it remains relatively stable. Pan *et al.*<sup>58</sup> prepared nanocapsules using spray-dried technology with sodium caseinate to encapsulate curcumin, which had stable particles at a pH around 6.8 ( $-31.1 \pm 0.9\text{ mV}$ ) with a particle size of  $168.7 \pm 10.2\text{ nm}$ . Furthermore, casein was studied for its stability at various pH ranges from 4 to 8 to encapsulate  $\beta$ -carotene using spray-drying technology.<sup>59</sup> It was found that, between pH 5 and 7, casein had a negative charge and had an isoelectric point at pH 4.4. In the above study, there was a noticeable increase in the stability of the spray-dried powders with a decrease in pH. However, below the isoelectric point, precipitation of casein occurred due to aggregation in particles. At the same time,  $\beta$ -carotene was well preserved at pH 5.5 at different concentrations of casein: 0.5% w/v ( $-20$  to  $-30\text{ mV}$ ), 1% w/v ( $-25$  to  $-30\text{ mV}$ ), and 5% w/v (around  $-20\text{ mV}$ ). In a recent study, chitosan was modified using octenyl succinic anhydride to increase the stability of fish oil DHA nanoemulsions. The prepared nanoemulsion had an initial zeta potential value of  $36\text{ mV}$ , which decreased to  $32\text{ mV}$  upon storage (23 days).<sup>60</sup> However, in the current study, formulation of DHA nanoemulsions using chitosan along with Tween 80 as an emulsifier improved the stability of DHA ( $42\text{ mV}$ ) without any modification in the structure of chitosan.<sup>61</sup>

### 3.9. Influence of droplet size on emulsifiers over storage

Over storage (28 days), the droplet size of chitosan nanoemulsion ( $346.7 \pm 5.1\text{ nm}$ ) was stable. For casein and pectin nanoemulsions, the droplet size was increased ( $p < 0.05$ ) to  $578 \pm 4.16\text{ nm}$  and  $382.3 \pm 7.56\text{ nm}$ , respectively, over storage (Fig. 6B). This can be correlated with the study published by Guerra-Rosas *et al.*<sup>62</sup> where the phase separation occurred with an increase in the droplet size of the emulsion prepared using pectin as an emulsifier. The significant increase in size suggests that factors such as pH changes, ionic interactions, or the

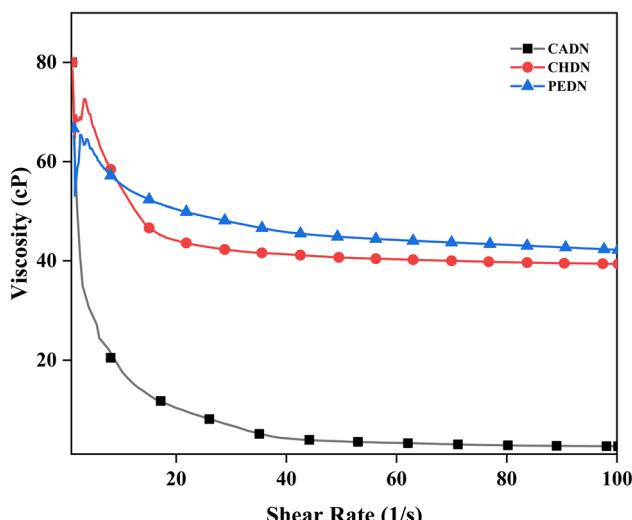


Fig. 5 Viscosity of DHA nanoemulsions as a function of shear rate. Where CADN represents the casein DHA nanoemulsion, CHDN represents the chitosan DHA nanoemulsion, and PEDN represents the pectin DHA nanoemulsion.



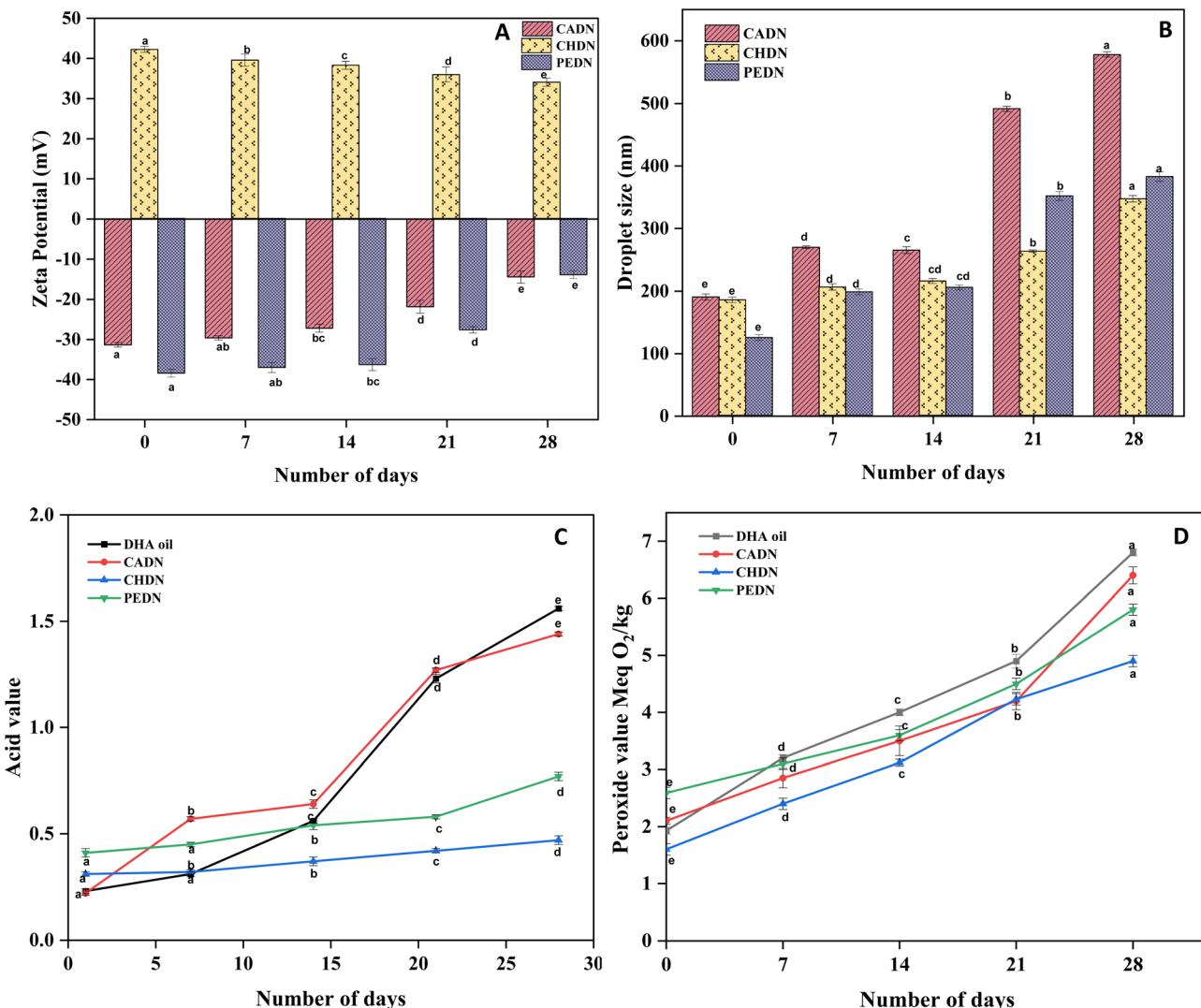


Fig. 6 Storage stability of the DHA nanoemulsion. Change in (A) zeta potential, (B) droplet size, (C) acid value, and (D) peroxide value of the DHA nanoemulsion.

presence of counter ions influence the stability, leading to droplet coalescence.<sup>63</sup> Pectin and Tween 80 were used to encapsulate essential oils using the micro fluidization technique.<sup>62</sup> The synthesized particles had a droplet size of less than 50 nm, which increased up to 1000 nm upon storage for 56 days. However, the zeta potential of the particles was very low, between  $-6$  and  $-15$  mV, depending on the type of essential oil used. The particle size affects the viscosity of an emulsion. Because the droplets in the nanoemulsion break with a higher shear rate, which in turn changes their molecular conformation. High shear rate, high-frequency vibration, and cavitation forces are some of the reasons for decreased viscosity.<sup>31</sup>

### 3.10. Oxidative stability of DHA nanoemulsions

The type of emulsifiers used in the emulsion influenced the final pH of the emulsion. The pH of casein, chitosan, and pectin immediately after preparation was  $6.75 \pm 0.02$ ,  $8.05 \pm 0.04$ , and  $2.16 \pm 0.02$ , respectively. The pH of the neat DHA oil was  $4.7 \pm$

$0.01$ . The pH of DHA nanoemulsions varied significantly over storage ( $p < 0.05$ ). The acid value of DHA nanoemulsions was determined to measure the rancidity. Free fatty acids are formed during storage, which is due to the decomposition of triglycerides present in the samples.<sup>32</sup> The acid value of neat DHA oil was  $0.23 \pm 0.01$  at the time of preparation. Geng *et al.*,<sup>64</sup> used various heat treatments to study the stability of various cooking oils at different intervals of time (1 to 5 minutes). The acid value of the above study varied between  $1.09 \pm 0.13$  and  $3.48 \pm 0.74$  g, indicating lesser stability at higher temperatures for prolonged durations. The method of emulsion formulation and storage temperature affects the stability of DHA oil. Higher stability was found in CHDN ( $0.48 \pm 0.03$ ) at day 28, with the least stability found in CADN ( $1.45 \pm 0.04$ ) over storage (Fig. 6C).

CADN, CHDN, and PEDN were determined for their primary oxidative products during storage. Lipid hydroperoxides produced by oil droplets quantify the extent of oxidation.<sup>32</sup> During storage, CHDN was found to have higher oxidative



stability ( $4.94 \pm 0.1$  Meq O<sub>2</sub> per kg) compared to PEDN and CADN (Fig. 6D). This could be achieved due to the strong electrostatic repulsion between the DHA droplets and emulsifiers in nanoemulsions.<sup>60</sup> Moreover, the oil becomes unacceptable (rancid) when the peroxide value is above 20 Meq O<sub>2</sub> per kg. A peroxide value below 10 Meq O<sub>2</sub> per kg indicates that the sample is fresh and acceptable for consumption. In a similar study, a peroxide value of 4.8 Meq O<sub>2</sub> per kg was obtained after 7 days of nanoemulsion preparation formulated using chitosan and gelatin to encapsulate carp oil.<sup>32</sup> Taken together, the higher oxidative stability attained by CHDN throughout storage was due to the antioxidant properties of chitosan to quench reactive oxygen species (ROS). The functional groups ( $-\text{NH}_2$  and  $-\text{OH}$ ) present in chitosan are responsible for maintaining oxidative stability by neutralizing ROS.<sup>65</sup> Encapsulation of DHA using chitosan forms a strong coating around DHA further protecting it from oxidation.<sup>66</sup> Moreover, these results correlate with the electrostatic stability of DHA nanoemulsions stabilized by chitosan (CHDN). In the current study, higher electrostatic stability was maintained in CHDN ( $34 \pm 1$  mV) even after 28 days of storage. This could be possibly due to strong electrostatic repulsion between droplets, which prevents coalescence and exposure of the DHA droplet's surface to oxygen.<sup>66</sup>

### 3.11. Concentration of DHA in nanoemulsions over storage

From the shelf life studies it was evident that CHDN had greater oxidative stability over storage and was further studied (SI Fig. 2A–C). The encapsulation efficiency of the CHDN nanoemulsion on day 1 and day 28 was found to be 87.33% and 78.6%, respectively. About 90% of the DHA was preserved in the nanoemulsion from day 1, even after storage of 28 days. The initial (day 1) concentration of DHA in CHDN was 2 g per 100 g. At the end of storage period (28 days), the concentration of DHA was 1.8 g per 100 g. The other predominant fatty acids present in CHDN were oleic acid (2.82 g per 100 g), palmitic acid (3.9 g per 100 g) and myristic acid (0.14 g per 100 g). The concentrations of the above-mentioned fatty acids at the end of storage were 1.55 g per 100 g, 1.81 g per 100 g and 0.1 g per 100 g respectively. Interestingly, the retention of 90% of DHA during storage suggests higher oxidative stability. The encapsulation of DHA in a chitosan matrix and storage of the DHA nanoemulsion under refrigerated condition (4 °C) prevented oxidative degradation, even without the use of additional antioxidants. The droplet size of the DHA nanoemulsion stabilized by chitosan ( $185.1 \pm 4.46$  nm) was also an important parameter in maintaining the stability of DHA. The encapsulation of DHA using the chitosan-stabilized nanoemulsion demonstrated improved shelf life compared to unencapsulated DHA oil.

The increased encapsulation efficiency of chitosan, the integrity of the interfacial layer created by chitosan and Tween 80, and the storage of DHA nanoemulsions in cold temperature (4 °C) were the factors that protected DHA from oxidation and reduced the formation of free radicals.<sup>67</sup> Similar results were observed by Rao *et al.*<sup>8</sup> where nanoemulsions containing omega-3 fatty acids were prepared, which had an encapsulation efficiency >80% with no phase separation for 4 weeks. Zeng *et al.*

has demonstrated that nanoencapsulation of DHA using zein (a corn protein) creates a stable core–shell structure, mimicking milk fat globules. This structure not only protected DHA during digestion but also facilitated its absorption across the blood–brain barrier and increased its bioavailability.<sup>68</sup> Nanoemulsions formulated using chitosan may improve DHA's bioaccessibility by enhancing lipid digestion and micelle formation, which are the key steps in the *in vivo* absorption process. The current research demonstrates that chitosan is the most effective emulsifier for producing stable DHA nanoemulsions, positioning it as a promising option for food applications.

### 3.12. Physicochemical analysis of kalakand

The chitosan-stabilized DHA nanoemulsion (CHDN) was fortified into kalakand to evaluate its stability in a dairy matrix during storage. Sensory analysis was performed immediately after the preparation of kalakand samples. Kalakand fortified with CHDN (KCDN) scored higher in overall acceptability compared to kalakand fortified with neat DHA oil (KDHO) in sensory analysis (Fig. 7A). Incorporation of DHA in the nanoemulsion masked the unpleasant fishy odor of DHA, whereas kalakand fortified with neat DHA oil had the fishy odor of DHA, making it unacceptable for consumption. In addition, KCDN showed a creamy mouthfeel due to the addition of the nanoemulsion and exhibited significantly higher ( $p < 0.05$ ) cohesiveness (discussed below in Section 3.15). These results correlate with recent research where lipid vesicles loaded with omega-3 fatty acids were prepared to mask their unpleasant fishy odor.<sup>69</sup> The formulated omega-3 lipid vesicles demonstrated higher sensory acceptance compared to the unencapsulated product. The nanoencapsulation process enhances the palatability of DHA and other omega 3 fatty acids, making them suitable candidates for food fortification.<sup>70</sup>

The moisture content of food products affects various biochemical and microbiological activities.<sup>71</sup> The moisture content of KCON has significantly decreased ( $p < 0.05$ ) on day 10 ( $47.41 \pm 0.06$ ) compared to day 1 ( $48.57 \pm 0.08$ ). KDHO showed a similar trend ( $48.26 \pm 0.2$  on day 1 to  $47.44 \pm 0.08$  on day 10) indicating minor dehydration over time (Table 5). KCDN exhibited the highest moisture retention over storage ( $49.57 \pm 0.28$  on day 1 and  $48.58 \pm 0.05$  on day 10). This implies that the increased emulsification stability and reduced droplet size of the DHA nanoemulsion led to an increase in water-holding capacity. The initial water activity of KCON, KDHO and, KCDN was same ( $0.77 \pm 0.01$ ). There was no significant difference ( $p > 0.05$ ) in the water activity across samples. The higher water activity ( $>0.95$ ) supports the growth of spoilage microorganisms. Water activity impacts lipid oxidation, which alters the flavor and appearance of food products over storage and shortens their shelf life.<sup>72</sup>

Ash content remains consistent across KCON and KDHO samples over storage, indicating no significant mineral loss (Table 5). KCDN samples consistently show the highest carbohydrate content ( $1.26 \pm 0.01$ ), followed by KCON ( $1.22 \pm 0.01$ ) and KDHO ( $1.22 \pm 0.01$ ). This may indicate the interaction of the nanoemulsion with residual sugars, promoting structural



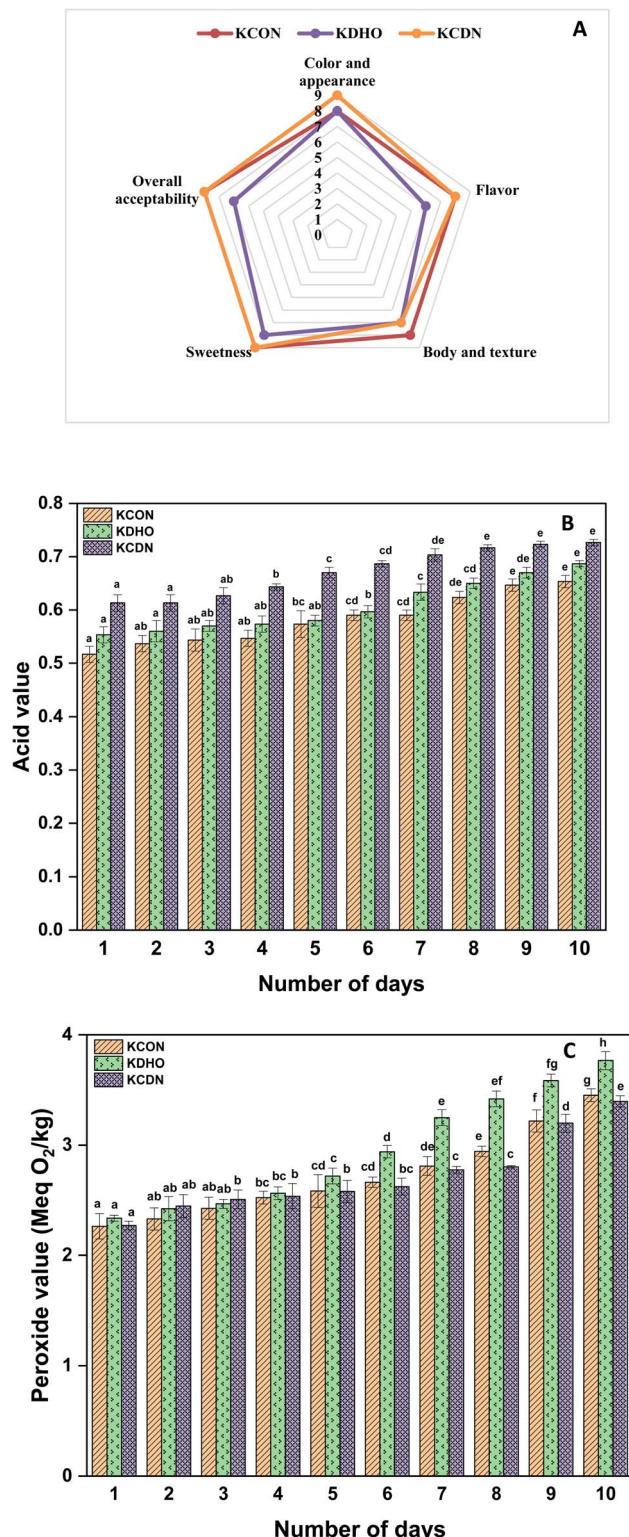


Fig. 7 Sensory and storage study of kalakand fortified with the chitosan DHA nanoemulsion. (A) Sensory analysis, (B) acid value, and (C) peroxide value of kalakand over storage.

integrity, or because of the presence of chitosan, which is a polysaccharide.<sup>73</sup>

KCON showed higher protein content ( $19.64 \pm 0.03$  on day 1 and  $19.16 \pm 0.03$  on day 10) compared to KDHO and KCDN.

Protein content decreased significantly over time in all samples due to enzymatic or chemical reactions during storage (Table 5). KCON has the lowest fat content ( $23.64 \pm 0.04$  on day 1 and  $23.5 \pm 0.05$  on day 10), reflecting the absence of DHA (Table 5). Higher fat content was observed in the KCDN sample ( $23.98 \pm 0.12$  on day 1 and  $23.87 \pm 0.02$  on day 10), likely because nanoemulsions increase the dispersion of DHA, enhancing fat incorporation. Fat stability between day 1 and day 10 in KDCN suggests that DHA in the nanoemulsion form improved lipid stability compared to oil form.

The pH of KCON, KDHO, and KCDN on day 1 was found to be  $6.56 \pm 0.01$ ,  $6.53 \pm 0.01$ , and  $6.39 \pm 0.01$  respectively. The pH and acid value of kalakand varied significantly ( $p < 0.05$ ) over storage (Fig. 7B). All the kalakand samples had good oxidative stability with a peroxide value  $<20$  Meq O<sub>2</sub> per kg. KDHO had a significantly higher peroxide value of  $3.77 \pm 0.08$  (day 10) compared to KCON and KCDN (Fig. 7C).

### 3.13. Color analysis of kalakand

KCDN has a significantly higher  $L^*$  index compared to KCON and KDHO on day 1 due to the incorporation of the DHA nanoemulsion (SI Table 2). Over storage (10 days), the lightness of the samples increased significantly ( $p < 0.05$ ). The increase in lightness of kalakand over storage is likely due to a combination of factors primarily related to moisture loss, crystallization of sugar, and potential changes in the texture of the product.<sup>74</sup> The increase in  $a^*$  and  $b^*$  over time for all three kalakand samples indicates a gradual shift towards a red hue, and yellow hue respectively. KCDN showed a similar increase in  $b^*$ , but the rate of increase is significantly less ( $p < 0.05$ ) compared to KCON and KDHO. All the kalakand samples showed a significant ( $p < 0.05$ ) color change upon storage. Compared to KCDN ( $\Delta E 3.95 \pm 0.1$ ), KDHO ( $\Delta E 4.41 \pm 0.1$ ) had significantly higher ( $p < 0.05$ ) color variation upon storage. The above findings show that chitosan nanoemulsion-incorporated kalakand was well preserved throughout storage.

### 3.14. Concentration of DHA in fortified kalakand

The initial concentration of DHA in KCDN and KDHO on day 1 was 0.5 g per 100 g. The concentration of DHA in KCDN and KDHO on day 30 was 0.46 g per 100 g and 0.1 g per 100 g, respectively (SI Fig. 3A and B). About 92% of DHA was preserved in KCDN, and 20% of DHA was preserved in KDHO over storage (10 days). The incorporation of DHA in the nanoemulsion form protected DHA from degradation. Compared to neat DHA oil-incorporated kalakand (KDHO), kalakand with DHA-encapsulated nanoemulsions (KCDN) had a 72% higher DHA retention.

In a study conducted by Ojagh *et al.*,<sup>75</sup> nano-liposomes encapsulating fish oil were produced and fortified in bread. It was observed that fish oil nano-liposomes significantly increased the volume of bread loaf with good texture compared to free fish oil fortified bread.<sup>75</sup> Moghadam *et al.* used gum Arabic to encapsulate fish oil to fortify fermented milk. It was found in the above study that the incorporation of nano-encapsulated fish oil increased the *Lactobacillus plantarum*

Table 5 Physicochemical analysis and textural properties of kalakand fortified with DHA nanoemulsions<sup>a</sup>

	KCON		KDHO		KCDN	
	Day 1	Day 10	Day 1	Day 10	Day 1	Day 10
<b>Physicochemical analysis</b>						
Moisture	48.57 ± 0.08 <sup>b</sup>	47.41 ± 0.06 <sup>b</sup>	48.26 ± 0.2 <sup>b</sup>	47.44 ± 0.08 <sup>b</sup>	49.57 ± 0.28 <sup>a</sup>	48.58 ± 0.05 <sup>a</sup>
Ash	2.15 ± 0.01 <sup>a</sup>	2.15 ± 0.02 <sup>a</sup>	2.15 ± 0.01 <sup>a</sup>	2.15 ± 0.01 <sup>a</sup>	1.97 ± 0.01 <sup>b</sup>	1.96 ± 0.01 <sup>b</sup>
Protein	19.64 ± 0.03 <sup>a</sup>	19.16 ± 0.03 <sup>a</sup>	18.21 ± 0.02 <sup>c</sup>	17.75 ± 0.05 <sup>c</sup>	18.51 ± 0.02 <sup>b</sup>	18.18 ± 0.05 <sup>b</sup>
Fat	23.64 ± 0.04 <sup>b</sup>	23.5 ± 0.05 <sup>c</sup>	23.86 ± 0.03 <sup>a</sup>	23.71 ± 0.02 <sup>b</sup>	23.98 ± 0.12 <sup>a</sup>	23.87 ± 0.02 <sup>a</sup>
Total carbohydrates	1.22 ± 0.01 <sup>b</sup>	1.21 ± 0.02 <sup>a</sup>	1.22 ± 0.01 <sup>b</sup>	1.21 ± 0.01 <sup>a</sup>	1.26 ± 0.01 <sup>a</sup>	1.26 ± 0.01 <sup>b</sup>
<b>Textural properties</b>						
Hardness (g)	270.88 ± 2.49 <sup>a</sup>	553.95 ± 4.12 <sup>a</sup>	272.56 ± 2.02 <sup>ab</sup>	557.99 ± 1.68 <sup>a</sup>	285.13 ± 3.65 <sup>b</sup>	542.98 ± 2.55 <sup>a</sup>
Springiness	0.73 ± 0.01 <sup>ab</sup>	0.72 ± 0.01 <sup>a</sup>	0.61 ± 0.09 <sup>b</sup>	0.52 ± 0.01 <sup>a</sup>	0.79 ± 0.07 <sup>a</sup>	0.77 ± 0.01 <sup>a</sup>
Cohesiveness	0.68 ± 0.06 <sup>a</sup>	0.7 ± 0.01 <sup>ab</sup>	0.67 ± 0.02 <sup>a</sup>	0.66 ± 0.02 <sup>b</sup>	0.75 ± 0.04 <sup>a</sup>	0.77 ± 0.01 <sup>a</sup>
Gumminess	137 ± 2.19 <sup>a</sup>	137.65 ± 4.6 <sup>b</sup>	140.15 ± 1.67 <sup>a</sup>	141.09 ± 6.63 <sup>ab</sup>	156.25 ± 2.86 <sup>b</sup>	155.12 ± 2 <sup>a</sup>
Chewiness	343.89 ± 2.66 <sup>b</sup>	344.79 ± 4.6 <sup>b</sup>	356.65 ± 2 <sup>a</sup>	358.65 ± 6.63 <sup>ab</sup>	373.08 ± 2.48 <sup>a</sup>	374 ± 2 <sup>a</sup>

<sup>a</sup> KCON – kalakand control; KDHO – kalakand with neat DHA oil; KCDN – kalakand with the DHA nanoemulsion. Data are articulated as mean ± standard deviation ( $n = 3$ ). In an individual row, each value tailed by a different letter is significantly different ( $p \leq 0.05$ ) between samples at day 1 and 10 as determined by Tukey's test.

count ( $8.41 \log \text{CFU mL}^{-1}$ ), and the concentrations of DHA and EPA were higher compared to free fish oil.<sup>76</sup> In another study, flaxseed oil and flaxseed oil nanoemulsion formulated using whey protein concentrate were fortified in butter. The fortified butter had 3.7 times more  $\alpha$ -linolenic acid compared to free flaxseed oil with good spreadability.<sup>77</sup> DHA-enriched egg products with unique compositions and varying structures (mousse, omelet, and hard-boiled egg) were developed to assess the recovery of DHA after preparation.<sup>78</sup> DHA transportation through the duodenum was faster in mousse compared to omelet and hard-boiled egg. The recovery of DHA in mousse after the heat treatment at the time of preparation was  $91.7 \pm 3.6$ . Rao *et al.*<sup>8</sup> fortified a rose hip essential oil nanoemulsion in yogurt and studied its stability over storage. From the above study, it was evident that yogurt fortified with a nanoemulsion was able to preserve omega-3 fatty acids (linoleic acid and oleic acid) compared to neat rosehip oil-fortified yogurt.<sup>8</sup> In the current study, the DHA nanoemulsion was effectively fortified in kalakand with higher retention of DHA (92%) at the end of storage (10 days).

### 3.15. Texture profile analysis of kalakand

Texture is an important parameter determining the acceptability and quality of products. Hardness measures the force required to compress the food sample to a certain extent.<sup>71</sup> During storage of kalakand samples (10 days), the hardness increased significantly ( $p < 0.05$ ) with time, depending on their moisture content. KCDN ( $553.95 \pm 4.12$ ) had significantly reduced ( $p < 0.05$ ) hardness over storage compared to KCON ( $557.99 \pm 1.68$ ) and KDHO ( $542.98 \pm 2.55$ ) due to its better moisture retention.

The kalakand samples showed no significant difference ( $p > 0.05$ ) in springiness throughout storage. Jain *et al.*<sup>79</sup> reported similar observations in kalakand samples stored under modified atmosphere packaging conditions for 10 days. Cohesiveness measures the extent to which the food holds together

during chewing.<sup>33</sup> KCDN ( $0.75 \pm 0.04$ ) had significantly higher cohesiveness compared to KCON and KDHO (Table 5). The incorporation of the DHA nanoemulsion improved the texture by well binding with the kalakand, with no significant difference ( $p > 0.05$ ) throughout storage, indicating retention of moisture. Furthermore, the gumminess of the kalakand fortified with the DHA nanoemulsion significantly increased ( $p < 0.05$ ) over storage. The chewiness of the kalakand samples didn't vary significantly ( $p > 0.05$ ) over storage.

### 3.16. Microbial analysis of kalakand

The total plate count (TPC) and yeast and mold count (YMC) of the chitosan-stabilized DHA nanoemulsion (CHDN), plain kalakand (KCON), kalakand fortified with neat DHA oil (KDHO), and kalakand fortified with CHDN (KCDN) were analyzed over storage. The TPC count of CHDN on day 1 of preparation was  $<10 \text{ CFU g}^{-1}$ . At the same time, on day 1 of storage, the TPC of KCON, KDHO, and KCDN was  $2.9 \log_{10} \text{CFU g}^{-1}$ ,  $2.8 \log_{10} \text{CFU g}^{-1}$ , and  $2.9 \log_{10} \text{CFU g}^{-1}$ , respectively. At the end of the storage period (10 days), the TPC of KCON, KDHO, and KCDN was found to be  $4.27 \log_{10} \text{CFU g}^{-1}$ ,  $4.19 \log_{10} \text{CFU g}^{-1}$ , and  $4.25 \log_{10} \text{CFU g}^{-1}$ , respectively. Before and after storage, there was no significant difference ( $p < 0.05$ ) observed among KCON, KDHO, and KCDN. As per the FSSAI standards, the TPC and Y&M counts of paneer (cottage cheese)-based confectionaries should be less than  $5.54 \log_{10} \text{CFU g}^{-1}$  and less than 150 CFU g<sup>-1</sup>, respectively, for safer consumption. In the current study, all the samples had a TPC count of less than  $5.54 \log_{10} \text{CFU g}^{-1}$ , which is under the recommended (FSSAI, 2015) microbial standard for paneer (cottage cheese)-based confectionaries. The Y&M count of KCON, KDHO, and KCDN at the end of storage was 13 CFU g<sup>-1</sup>, 9 CFU g<sup>-1</sup>, and 10 CFU g<sup>-1</sup>, respectively. Similar results were observed in a study where the TPC count of a kalakand sample on day 0 and day 7 of storage was  $3.2 \log_{10} \text{CFU g}^{-1}$  and  $3.75 \log_{10} \text{CFU g}^{-1}$ , respectively.<sup>80</sup> The observed increase in microbial count during storage may be attributed to



the initial microbial load in the kalakand samples. Preparation of kalakand under sterile industrial conditions may increase its shelf life further.<sup>81</sup> Overall, the findings from sensory, physicochemical, and shelf-life analyses indicate that the DHA nanoemulsion-fortified kalakand had higher acceptability compared to the kalakand fortified with neat DHA oil.

## 4. Conclusion

The current study presents the importance of choosing appropriate emulsifiers for improving the stability of the DHA nanoemulsion using ultrasound, a green technology, and its effective application in a dairy confectionary, kalakand. Among the three emulsifiers—casein, chitosan, and pectin—the DHA nanoemulsion stabilized using chitosan (CHDN) showed enhanced oxidative stability with a higher zeta potential ( $42.2 \pm 0.75$  mV) and smaller droplet size ( $122.5 \pm 2.5$  nm). This strong electrostatic repulsion of chitosan prevented droplet aggregation throughout the storage (28 days). The polycationic nature and enhanced oil-holding capacity of chitosan improved the emulsification and protected DHA against oxidative degradation with high encapsulation efficiency (87.3%). Furthermore, fortification of kalakand with CHDN (KCDN) enhanced the sensory and nutritional properties of kalakand and masked DHA's unpleasant odor compared to neat DHA oil-incorporated kalakand. At the end of the storage study, kalakand with CHDN retained the higher concentration of DHA (92%) with good textural properties under refrigerated conditions (4 °C). The above findings confirm the use of the DHA nanoemulsion as an efficient approach to improve the stability and retention of DHA in dairy products such as kalakand. In addition, a detailed investigation into the bioavailability of the DHA nanoemulsion and the cost feasibility of producing DHA nanoemulsion-fortified food on an industrial scale remains scope for future studies. Furthermore, upcoming research should focus on expanding the application of nanoencapsulated DHA in other food matrices such as beverages, bakery products, and infant formulas. Fortification of DHA nanoemulsion in food matrices opens the door for transforming conventional food products into functional foods.

## Ethical statement

No formal approval was required for conducting sensory analysis, whilst appropriate protocols were still followed for protecting the rights and privacy of all participants involved in the research. Informed consent was obtained from the panelists before performing the experiment.

## Author contributions

Sirajdeen Asmath Mubeena: conceptualisation, methodology, investigation, validation, visualisation, formal analysis, project administration writing – original draft; Radhakrishnan Preetha: conceptualisation, supervision, writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d5fb00388a>.

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