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ORIGINAL PAPER

Investigation of storage stability and *in vitro* digestion of nanoemulsions loaded carotenoids from *Gracilaria dura*, *Sargassum acinarium* and *Ulva rigida* macroalgae

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Abstract

This research determined the potential utilization of carotenoids extracted from macroalgae collected in the coastal water of Türkiye in the food industry. Carotenoids were extracted from *Gracilaria dura*, *Sargassum acinarium* and *Ulva rigida* collected from Türkiye seas by using ultrasound-assisted enzymatic extraction. Then, physical and storage stabilities of nanoemulsions including carotenoid and bioaccessibility of β-carotenoid *during in vitro* digestion were investigated. According to the study findings, carotenoid nanoemulsions were prepared with an encapsulation efficiency ranging approximately between 87% and 94%. The encapsulated carotenoids exhibited a superior ability to maintain stability in terms of both carotenoid content and antioxidant activity during in vitro digestion as compared to their non-encapsulated counterparts ($p < 0.05$). Chromatographic analysis of carotenoid profiles revealed the presence of β-carotene in nanoemulsions derived from *Gracilaria dura*, *Sargassum acinarium* and *Ulva rigida*. A reduction in the amount of β-carotene was observed in the nanoemulsions during in vitro digestion ($p < 0.05$). Results from the storage stability study indicate that the oxidative stability of algal carotenoid nanoemulsions was better than that of the control ($p < 0.05$). Conversely, destabilization occurred in algal carotenoid nanoemulsions on the seventh day of storage due to creaming. Lightness (L^*) and color intensity (a^*, b^*) of the nanoemulsions gradually decreased over the 28-day storage period ($p < 0.05$). This study emphasizes the potential application of carotenoids from *Gracilaria dura*, *Sargassum acinarium* and *Ulva rigida* macroalgae in food products.

Keywords Carotenoids · Emulsion · Stability · Macroalgae · *In vitro* digestion

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Introduction

Macroalgae perform photosynthesis and create vast underwater forests in the oceans [1]. In this regard, they hold a significant advantage over terrestrial plants. For instance, they grow approximately 3 to 10 times faster than terrestrial plants such as corn and sugarcane [1, 2]. Due to their natural habitats, macroalgae are exposed to high oxidative and free radical stress from challenging environmental conditions. As a result of these effects, it is known that natural protective systems in algae such as pigments (carotenoids, chlorophyll and phycobiliproteins) with significant health effects such as antioxidant activity and polyphenols (catechins, flavonols and phlorotannins, etc.) develop [3].

Carotenoids, a group of pigments found in macroalgae, are synthesized not only by algae but also by terrestrial plants, bacteria and fungi [4–6]. Several carotenoids, including β-carotene, α-carotene and β-cryptoxanthin, are known to provide various health benefits in humans. For instance, zeaxanthin and β-carotene have been reported to possess anticarcinogenic effects and antioxidant activity [7, 8]. Christaki et al. reported the presence of various carotenoids in different types of algae. They determined α-carotene, neoxanthin, violaxanthin and siphonoxanthin in green algae; β-carotene, lutein, zeaxanthin in red algae; and fucoxanthin, β-carotene and violaxanthin in brown algae [7]. In a study by Xie et al. the effect of light on the concentration of β-carotene and zeaxanthin was investigated in the red macroalgae *Pyropia yezoensis* [9].

In general, it can be stated that in many cases, the extraction of carotenoids from algae with thick cell walls is not possible through simple solvent extraction [8]. I t has been reported that the use of some pre-processing techniques like ultrasonication, can increase the extractability of carotenoids. The application of ultrasound to an aqueous medium facilitates the mass transfer of components to be extracted by causing deformation in the cell wall through acoustic cavitation [10].

Due to their long-conjugated bond systems, carotenoids can lose their stability in various environments, including heat, light, oxygen, acidity, alkalinity and their combinations. Therefore, encapsulation is considered an effective method to preserve the stability of the antioxidant properties of bioactive compounds [11]. Typically, carotenoid encapsulation entails entrapping them within colloidal particles, the composition and structure of which may vary significantly based on formulation and preparation methods [12]. The size of these colloidal particles used for encapsulation can vary widely, ranging from nanoparticles (1–100 nm) to microparticles (100–1000 μm) [13]. Diverse nanoencapsulation techniques and types of nano-sized carriers, including polymeric carriers and lipid nanostructures, serve as delivery systems to achieve this goal [14, 15]. Emulsion-based encapsulating methods are typically used to create nanoemulsions, which are a means of encapsulating bioactive chemicals in aqueous solutions. Nanoemulsions are colloidal dispersions with droplet sizes ranging from 50 to 1.000 nm, made up of two immiscible liquids, one of which is being dispersed in the other [16]. The process of encapsulating bioactive compounds like carotenoids into nanoemulsions [17] represents the first effective approach to improve the dispersion and bioavailability.

The creation of different environmental conditions such as changes in temperature, pH level and salt concentration have a role in the physical stability of emulsions [18, 19]. These factors have an impact on how the continuous phase, dispersed phase and emulsifier molecules interact within the emulsion system [20]. The phase separation and emulsifier activity both are susceptible to temperature fluctuations [21]. The ability of the emulsifier molecules to stabilize the emulsion by electrostatic repulsion or attraction is affected by pH levels, which change the charge on the molecules [22]. The ionic strength of the continuous phase can be affected by salt concentrations, which can have an impact on the emulsifier's capacity to create and preserve a stable interfacial film [23]. Thus, observing emulsions at varying pH, temperature and salinity levels aids in comprehending how these variables impact the emulsion's physical stability in a range of applications, including food, medicine and cosmetics [18, 24]. Thus, encapsulated carotenoids within a well-designed colloidal delivery system has the potential to enhance their stability, handling, storage and effectiveness [25].

In recent years, the increasing of interest in clean-label and functional foods, the food industry is moving towards more naturally sourced and sustainable ingredients. Marine algae continue to become interesting candidates as they can yield products with active bioactive compounds, including pigments (e.g., fucoxanthin, β-carotene, phycobiliproteins), which provide not only a natural color in food products, but also antioxidant, anti-inflammatory, and health-promoting effects [26–29]. The best of our knowledge, there is no study investigating nanoemulsions of macroalgal carotenoids from the coastral waters of Türkiye. Therefore, this study aims to develop the potential use of macroalgae based carotenoid pigments-loaded emulsions and add to developing sustainable health formulations in the food industry. In this study, firstly, the carotenoids from red macroalgae *Gracilaria dura*, brown macroalgae *Sargassum acinarium and* green macroalgae *Ulva rigida* collected from Türkiye seas were extracted using ultrasound-assisted enzymatic extraction. Then, carotenoid extracts were encapsulated by using nanoemulsion methods. The storage, physical and oxidative stabilities and simulated in vitro digestion studies of the

nanoemulsions were performed. In this context, nanoemulsions were examined for physical stability under different temperatures (40–80 °C), pH (4–7) and salt concentrations (100–500 mM). In storage stability studies, the nanoemulsions were stored at + 24 °C for 28 days and changes in particle size and charge, color, creaming index, antioxidant activity and carotenoid content were investigated on 0th, 7th, 14th, 21th and 28th storage days. Within the scope of oxidation stability studies, oxidation tests including lipid hydroperoxide and thiobarbituric acid-reactive substances (TBARS) analysis were carried out on the 0th, 1st, 3rd, 5th, 7th, 10th, 12th, 15th and 20th storage days of nanoemulsions stored at 37 °C. Finally, changes in bioaccessibility, antioxidant activity and carotenoid content of nanoemulsions were determined during in vitro digestion.

Materials and methods

Collection and Preparation of macroalgae

Gracilaria dura (coordinates: 40°1'35.90"N and 26°19'49.49"E) and *Ulva rigida* (coordinates: 40°1'35.90"N and 26°19'49.49"E) macroalgae were collected from Çanakkale province of Türkiye at a depth of 1 m. *Sargassum acinarium* macroalgae was collected from Antalya province of Türkiye (coordinates: 36°27'31.90"N and 30°24'48.10"E) at a depth of 20 m. The collected algae were cleaned of epiphytes and any potential stones, sand, or mud and washed with freshwater. The macroalgae were identified by Dr. Emine Şükran Okudan who is also the one of the co-authors of this study and has an expertise in the field of taxonomy of marine macroalgae and seagrasses. After cleaning, the algae were dried at 30 °C without direct exposure to sunlight. Subsequently, they were ground using a laboratory-type grinder (Waring 8011 Eb Blender, Cole-Parmer Instrument Company, Illinois, USA) and further sieved to obtain samples with particle sizes of < 500 µm. These samples were then packaged to protect them from oxygen and daylight exposure and stored at -20 °C until further analysis.

Chemicals

Ethanol, butanol, methanol, acetone, hexane, ammonium thiocyanate, HCl, NaOH, Tween 80, $FeCl_2$, methyl tert-butyl ether, acetonitrile, thiobarbituric acid, trichloroacetic acid, Trolox, copper (II) chloride, neocuproine, ammonium acetate buffer, ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), potassium persulfate, phosphate buffer, bile salt, flaxseed oil, zeaxanthin (CAS No: 144-68-3, EC: 205-636-4), β-carotene (CAS No: 7235-40-7, EC: 230-636-6), fucoxanthin (CAS No: 3351-86-8), lycopene (CAS No: 502-65-8), astaxanthin (CAS No: 7542-45-2), α-amylase (CAS No: 9000-85-5, EC: 3.2.1.1), pepsin (CAS No: 9001-75-6, EC: 3.4.23.3), lipase (CAS: 9001-62-1, EC: 3.1.1.3) enzymes were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Pancreatin was obtained from Merck (Catalogue No: 1.07130.1000, Merck, Germany). Viscozyme L enzyme was provided as a gift by Novozymes A/S (Bagsvaerd, Denmark).

Ultrasound-assisted enzymatic extraction of carotenoids

The extraction process of carotenoids from *Gracilaria dura*, *Sargassum acinarium and Ulva rigida* involved an initial step of osmotic shock and subsequent ultrasonication of the samples in a buffered aqueous solution before the application of organic solvent extraction. This was done to break down the cell wall and enhance the efficiency of carotenoid extraction during the organic solvent extraction phase. The methods employed were modifications of the procedures outlined by Heffernan et al. [30], Fleita et al. [31] and Deenu et al. [32]. In the first extraction step, 1 g of powdered algal sample was subjected to osmotic shock by soaking it in 40 mL of acetate buffer solution (pH 4.5) at + 4 °C overnight. After the addition of enzyme Viscozyme L, the mixture was incubated in a shaking water bath (N-Biotek-303, Biotek Co., Ltd., Korea) at 37 °C with a shaking speed of 50 rpm for 30 min. At the end of the incubation process, the mixture was held in an 80 °C water bath for 10 min to stop enzyme activity and then centrifuged at 4100 rpm at 4 °C for 20 min. The supernatant was discarded and the remaining pellet was treated with a 1:20 ratio of hexane: acetone solution. Nitrogen gas was introduced into the headspace of the container, which was tightly sealed and the mixture was subjected to extraction in a shaking water bath at 4 °C with a shaking speed of 50 rpm for 24 h. Afterward, the suspension was filtered through Whatman No:1 filter paper. Organic solvent was added again to the remaining portion and the extraction was repeated under the same conditions. The resulting filtrates were combined. The solvent in the filtrate was completely removed under vacuum at 50 °C in a rotary evaporator (Hei-VAP Advantage, Heidolph, Germany) and the residue was stored at -20 °C under a nitrogen atmosphere until analysis [30–32].

Total carotenoid content

The absorbance of the carotenoid extracts was measured at 470 nm using a UV spectrophotometer (Scilogex Sci-UV1000 Spectrophotometer, Scilogex, USA) to determine the total carotenoid content [33, 34]. A calibration curve

was prepared using β-carotene and the total carotenoid content in the sample) was expressed as milligrams of carotenoid obtained per kilogram of dry matter (dm) in terms of β-carotene. The total carotenoid content was calculated using the Eq. 1 provided by Gu et al. (2008) [35]:

$$\text{Total carotenoid content (mg } \beta - \text{carotene/kg)} = \frac{A \times D \times V}{W} \quad (1)$$

A is absorbance of the diluted extract; D is dilution ratio; V is solvent volume (mL); W is weight of the dried algae used in the extraction (kg).

Determination of the carotenoid profile

Carotenoid profiles of the carotenoid extracts were determined by HPLC. A reverse-phase C18 column (5 µm, 250 × 4.6 mm id.) was used in a Shimadzu Prominence HPLC system equipped with a PDA detector. Separation was carried out with an isocratic solvent system of acetonitrile: methanol (70:30) at a flow rate of 1 mL/min, column temperature of 30 °C, detection wavelength of 470 nm and an injection volume of 20 µL. Firstly, samples were dissolved in 1000 µL of methanol and then subjected to HPLC analysis [36, 37]. The qualitative and quantitative analysis of carotenoids was performed using retention times and calibration curves of external standards (β-carotene, fucoxanthin, lycopene).

Preparation of nanoemulsions

Nanoemulsions of the carotenoid extracts were prepared using the method applied by Salvia-Turijillo et al. [38]. In summary, 1% (w/w) carotenoid extract was added to flaxseed oil and this solution was mixed at 600 rpm for 24 h at 4 °C. A 5% (w/w) portion of this oil phase and 95% aqueous phase (10 mM phosphate buffer (pH 7) containing 1.5% Tween 80) were mixed to prepare nanoemulsion. Then, the mixture was blended for 2 min using an ultra-high-speed hand blender (T18 digital, IKA, Germany) to prepare coarse emulsion. Subsequently, sonication process for 20 min at a frequency of 23 kHz and amplitude of 15 µm with ultrasonicator (Soniprep 150, MSE Scientific Instruments, UK) was performed to reduce the particle size, resulting in the formation of a final nanoemulsion containing algal carotenoids (Fig. 1). Also, the emulsion sample without the addition of algal carotenoid extraction was represented as the control sample [38].

Physical characterization of nanoemulsions

Encapsulation efficiency

The encapsulation efficiency of carotenoids in nanoemulsion was calculated with slight modifications based on the method reported by Fitri et al. [39], using the following formula Eq. 2:

$$\text{Encapsulation efficiency}(\%) = \frac{A1}{A2} \times 100 \quad (2)$$

A_1 is the amount of carotenoids in the nanoemulsion (mg/g); A_2 is the amount of carotenoids in the extract before nanoemulsion (mg/g).

Particle size and zeta potential

The particle size of the emulsions was measured using a static light scattering equipment mastersizer (Mastersizer 2000, Malvern Instruments, U.K.). Dynamic light scattering (DLS) equipment (Zetasizer Nano ZS-Zen3600, Malvern Instruments, U.K.) was used to determine the charge of particles [40].

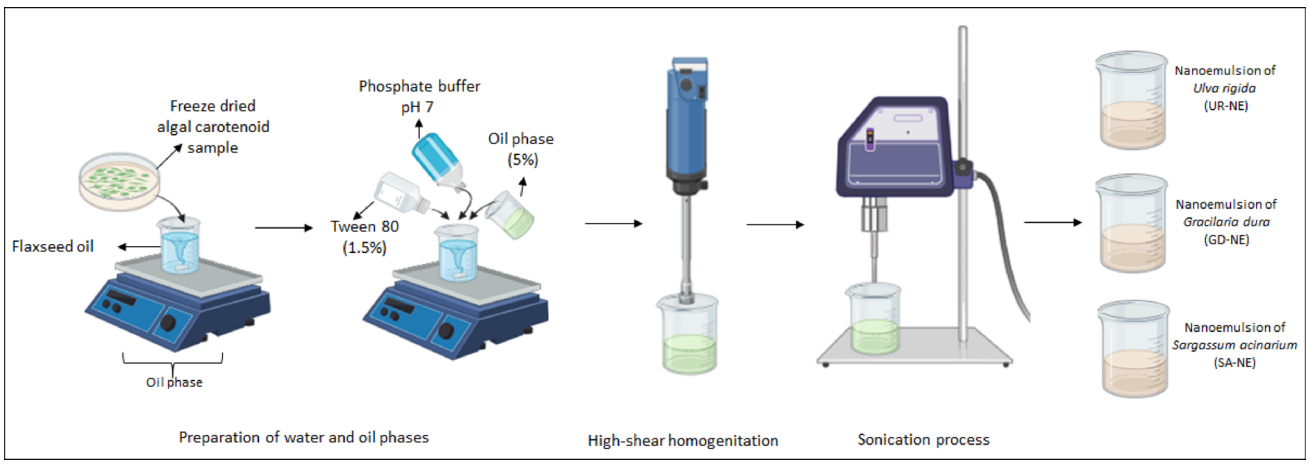


Fig. 1 Preparation of nanoemulsions

Visual appearance, creaming characteristics and color

The visual appearance and creaming characteristics of the nanoemulsions were evaluated by direct visual observation. The color properties (Hunter CIE L*, a*, b*) of the samples (carotenoid extracts and nanoemulsions) were determined using a Hunter colorimeter (LabScan XE, Hunterlab, Virginia, USA). The samples were placed in an optical glass cell with a path length of 1 cm and triplicate measurements of CIE L*, a* and b* values were taken in total transmission mode using illuminant C and a 2° observer angle.

Physical stability of nanoemulsions

Thermal stability

The nanoemulsions were subjected to temperatures of 40, 50, 60, 70 and 80 °C in a water bath for 30 min and then they were kept at room temperature for 24 h. After this period, the emulsions were diluted with a phosphate buffer (pH 7.0) and the particle size of the emulsions was measured using a static light scattering equipment mastersizer (Mastersizer 2000, Malvern Instruments, U.K.). Dynamic light scattering (DLS) equipment (Zetasizer Nano ZS-Zen3600, Malvern Instruments, U.K.) was used to determine the charge of particles [40].

pH stability

The pH of the nanoemulsions was adjusted to 4.0, 5.0, 6.0, 7.0 using 1.0 M HCl and 1 M NaOH and then incubated at room temperature for 24 h. After incubation, the emulsions were 100 fold diluted with phosphate buffer (pH 7.0), the particle size of the emulsions was measured using a static light scattering equipment mastersizer (Mastersizer 2000, Malvern Instruments, U.K.). The DLS equipment was used to determine the charge of particles [40].

Ionic strength stability

To measure the effect of different salt concentrations on emulsion stability, emulsions containing NaCl (100–500 mM) were prepared and transferred to glass test tubes. They were then incubated at room temperature for 24 h. After incubation, the emulsions were diluted with a phosphate buffer (pH 7.0) and the particle size of the emulsions was measured using the static light scattering equipment mastersizer. The DLS equipment was used to determine the charge of particles [40].

Storage stability of nanoemulsions

To determine the storage stability of nanoemulsions, the samples stored at 37 °C were subjected to particle size and zeta potential, color changes, creaming index, antioxidant activity, oxidative stability, total carotenoid content and carotenoid profile analysis on days 0., 7., 14., 21. and 28. of storage. Color, particle size, zeta potential, total carotenoid content and carotenoid profile analyses were performed as explained in previous sections.

Creaming index

The sensitivity of the emulsions to creaming was determined by measuring the total height of the emulsion, including the opaque droplet-rich serum layer on top of the test tube, using the following Eq. 3 [41]:

$$\text{Creaming Index (CI)} = 100 \times \frac{H_s}{H_T} \quad (3)$$

H_S is the height of the serum layer; H_T is the total height of the emulsion.

Determination of antioxidant activity

ABTS method The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) analysis was conducted according to Miller and Rice-Evans (1997). In short, 100 µL of the sample was mixed with 1 mL of the ABTS solution and the absorbance was measured at 734 nm. The results were expressed as milligrams of Trolox equivalent (TE) per gram of dry weight (dw) [42].

CUPRAC method In the cupric reducing antioxidant capacity (CUPRAC) method, 100 µL of the sample extract was mixed with the following solutions sequentially: 1 mL of 10^{-2} M copper (II) chloride solution, 1 mL of 7.5×10^{-3} M neocuproine solution (in ethanol), 1 mL of ammonium acetate buffer solution (pH: 7.0) and 1 mL of distilled water. The mixture was kept at room temperature for 30 min and then absorbance of the samples was measured at 450 nm using a UV spectrophotometer. The results were expressed as mg TE/g dw [43].

Oxidative stability

The nanoemulsions were transferred to vials with a volume of 2 mL, sealed and then placed in an incubator set at 37 °C for oxidation. The samples were periodically taken and

the peroxides, an indicator of oxidative deterioration, were determined using the ferric thiocyanate method [44]. For this purpose, 200 µL of the emulsion sample was diluted 15-fold with isooctane-2-propanol (3:1) mixture and vortexed for 10 s. Subsequently, it was centrifuged at 4000 rpm for 3 min and 200 µL of the supernatant was taken. Then, it was mixed with 2.8 mL of methanol: butanol (2:1) mixture, 15 µL of water containing 30% ammonium thiocyanate solution and 15 µL of water containing a 20 mM $FeCl_2$ solution. After 20 min, the absorbance was measured at 510 nm and the results were expressed in terms of cumene hydroperoxide.

Secondary oxidation products were determined using the modified TBARS method [45]. In summary, 1 mL of the emulsion and 2 mL of thiobarbituric acid (TBA) reagent (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 0.25 M HCl was mixed 2% BHT in ethanol solution) were mixed in a sealed tube and vortexed. The tubes were then kept in a 90 °C water bath for 15 min. Then, tubes were cooled to room temperature in an ice bath and centrifuged at 1.000 g for 15 min. Subsequently, they were left to stand for 10 min and absorbance was measured at 532 nm. The amount of secondary oxidation products was calculated in terms of 1,1,3,3-tetraethoxypropane (0–20 nM).

In vitro digestion

The in vitro digestion experiments were conducted to determine the changes in carotenoid content, carotenoid profile and antioxidant activity of the carotenoid extracts and nanoemulsions during in vitro gastric and intestinal digestion. The INFOGEST 2 method, developed by Brodkorb (2019), with some modifications, was used. This method mimics the oral, gastric and intestinal phases of digestion and involves the analysis of digestion products formed after incubation at specific pH, temperature and time. To simulate the salivary phase, a saliva simulating fluid (SSF) which consisted of 15.1 mL of 0.5 mol/L KCl solution, 3.7 mL of 0.5 mol/L KH_2PO_4 solution, 6.8 mL of 1 mol/L $NaHCO_3$ solution, 0.5 mL of 0.15 mol/L $MgCl_2(H_2O)_6$ solution and 0.06 mL of 0.5 mol/L $(NH_4)_2CO_3$ solution was prepared. To mimic the gastric phase, a gastric simulating fluid (SGF) was prepared by mixing 6.9 mL of 0.5 mol/L KCl solution, 0.9 mL of 0.5 mol/L KH_2PO_4 solution, 12.5 mL of 1 mol/L $NaHCO_3$ solution, 11.8 mL of 2 mol/L NaCl solution, 0.4 mL of 0.15 mol/L $MgCl_2(H_2O)_6$ solution and 0.5 mL of 0.5 mol/L $(NH_4)_2CO_3$ solution. For simulating the intestinal phase, an intestinal simulating fluid (SIF) was prepared by mixing 15.1 mL of 0.5 mol/L KCl solution, 0.8 mL of 6.8 mol/L KH_2PO_4 solution, 42.5 mL of 1 mol/L $NaHCO_3$ solution, 9.6 mL of 2 mol/L NaCl solution and 1.1 mL of 0.15 mol/L $MgCl_2(H_2O)_6$ solution. In the oral digestion phase, 5 g of the sample was mixed with 5 g of SSF solution and then salivary amylase enzyme (75 U/mL) and 0.5 µL of $CaCl_2$ (588 g/L) per mL of SSF were added. The mixture was incubated at pH 7 for 2 min. For in vitro gastric digestion phase, the solution resulting from the oral digestion phase (SSF mixture) was mixed with SGF in a 1:1 ratio. Pepsin enzyme (2000 U/mL) and 0.17 mM phospholipid were added to the mixture, which was then incubated at 37 °C and pH 2 with shaking at 250 rpm in a water bath for 1 h. After in vitro gastric digestion, the samples were immediately placed in a freezer at -80 °C to stop the activity of pepsin enzyme. Subsequently, in vitro intestinal phase digestion was carried out using the mixture obtained from in vitro gastric phase digestion (gastric mixture) and SIF + enzyme extract (pancreatin (100 U/mL) + bile (bile extract or fresh pig bile) mixture (1:1)). The mixture was incubated at 37 °C and pH 7 with shaking at 250 rpm in a water bath for 2 h [46]. The adjustment of pH to 7 in vitro intestinal phase allowed the inhibition of pepsin enzyme activity and the activation of pancreatin enzyme [47]. After in vitro intestinal digestion, the samples were immediately placed in a freezer at -80 °C to stop the enzyme activity and were stored until analysis (carotenoid content and antioxidant activity).

Statistical analysis

All analyses were conducted in triplicate and the data was presented as mean ± standard deviation. Data was analyzed using the Minitab statistical software program (Minitab, Version 17, Minitab Inc., State College, PA). Experimental data was analyzed using analysis of variance (ANOVA) and differences between means were determined using the Tukey test at a significance level of $p < 0.05$.

Result and discussion

Total carotenoid content and carotenoid profile of the extracts

In the present study, the carotenoid content of the macroalgal extracts from *Ulva rigida*, *Sargassum acinarium and Gracilaria dura* was determined to be 26.55 ± 0.40, 42.46 ± 0.01 and 10.97 ± 0.30 mg/kg dw, respectively. Similarly, in carotenoid extraction from three brown macroalgae (*Himanthalia elongate*, *Undaria pinnatifida*, *Laminaria ochroleuca*), one red macroalgae (*Porphyra* spp.) and one microalgae (*Spirulina* spp.), the highest carotenoid content was measured in *Laminaria ochroleuca* among brown macroalgae (27.0 ± 2.4 mg/kg) [47]. On the other hand, the total carotenoid contents of red algae *Porphyra* spp. and microalgae *Spirulina* spp. were found to be 74.5 ± 3.8 mg/kg

and 1263.9 ± 54.0 mg/kg, respectively [47]. In the research conducted by Machado et al. (2016) on the extraction of carotenoids from microalga *Haematococcus pluvialis*, the highest astaxanthin content in the extracts was achieved with the application of ultrasound together with the addition of Glucanex® (contains mainly β1,3-glucanase and some β1,6-glucanase) [48]. Similarly, in a study by Michelon et al. (2012) on the extraction of carotenoids from *Phaffia rhodozyma*, the highest carotenoid yield was obtained with the application of Glucanex® and ultrasound [49].

When compared with the carotenoid content of terrestrial plants, the carotenoid content of macroalgea is competitive with those of plant sources. For example, Singh et al. (2007) determined that the lutein concentration in 6 broccoli varieties ranged from 4.1 to 10.2 mg/kg and in 18 cabbage varieties, it ranged from 0.3 to 2.2 mg/kg [50]. In a study conducted by Strati et al. the content of carotenoids from tomato waste changed between 34.45 and 51.90 mg/kg depending on the solvent used in the extraction process [51]. On the other hand, in the study by Bozalan and Karadeniz β-carotene content of carrots was determined to be 41.60–71.2 mg/kg fresh weight [52]. In the study of Kopsell et al. the lutein content of spinach samples was determined to be in the range of 6.49–12.98 mg/kg [53].

According to the results of the carotenoid profile analysis of the extracts, carotenoid analysis was performed using retention times and calibration curves of external standards (β-carotene, fucoxanthin, lycopene) (Fig. S1) and only β-carotene was identified in the green macroalgae *Ulva rigida*, red macroalgae *Gracilaria dura* and brown macroalgae *Sargassum acinarium*. In this present study, β-carotene, broadly present in all the macroalgal carotenoid extracts and the other carotenoid compounds was not detected. According to Stinco et al. applications of ultrasound and enzymes in the extraction process lead to the breakdown of cell walls and enhance the carotenoids' exposure to oxygen, enzymes and other substances [54]. Salgado-Roman et al. stated that high extraction efficiency in the extraction of carotenoids from red pepper was achieved by enzymatic hydrolysis of the cell wall [55]. On the other hand, in ultrasound application, acoustic cavitation causes a destructive effect on the cell wall and pours the matrix component into the solvent medium with shorter extraction time and solvent consumption [56].

According to the literature, β-carotene, lutein, violaxanthin, neoxanthin and zeaxanthin are the most abundant carotenoids in green algae. The dominant pigments in red algae are lutein and zeaxanthin, along with α- and β-carotenes. Brown algae, while abundant in various carotenoids, also contain carotenoid derivatives such as fucoxanthin, peridinin, diatoxanthin, heteroxanthin, siphonaxanthin, astaxanthin and canthaxanthin [4, 6]. Moreover, Christaki et al. generally reported the presence of α-carotene, neoxanthin, violaxanthin and siphonaxanthin in green algae; β-carotene, lutein, zeaxanthin in red algae; and fucoxanthin, β-carotene and violaxanthin in brown algae [7]. In the study by Bianchi et al. β-carotene, violaxanthin and lutein were detected in green macroalgae *Cladophora glomerata* and *Enteromorpha intestinalis*, while fucocanthin and zeaxanthin were undetectable. Brown algae, including *Chorda filum*, *Dictyosiphon foeniculaceus*, *Fucus vesiculosus* and *Pilayella littoralis*, showed the absence of lutein and zeaxanthin, with the presence of fucoxanthin, β-carotene and violaxanthin. In red algae such as *Ceramium tenuicorne*, *Furcellaria lumbricalis*, *Phyllophora spp*., *Polysiphonia nigrescens* and *Rhodomela corifervoides*, the pigments fucoxanthin, violaxanthin and β-carotene were detected. In contrast, β-carotene, lutein and zeaxanthin were also found in some samples, but fucoxanthin and violaxanthin were not detected [57]. Kanda et al. stated that lutein is the main carotenoid in the green macroalgae *Monostroma nitidum* [58].

Table 1 Encapsulation efficiency, average particle size and zeta potential of emulsions

Sample	Encapsulation efficiency (%)	Particle size (μm)	Zeta potential (mV)
Control	–	0.189 ± 0.01^{a}	-17.60 ± 1.18^{c}
UR-NE	91.36 ± 0.95^{a}	0.178 ± 0.01^{c}	-23.01 ± 1.70^{a}
GD-NE	94.13 ± 1.08^{a}	0.176 ± 0.01^{d}	-20.55 ± 1.46^{b}
SA-NE	87.29 ± 2.48^{b}	0.181 ± 0.01^{b}	-24.35 ± 0.92^{a}

*UR-NE: Nanoemulsion of carotenoids from *Ulva rigida*, GD-NE: Nanoemulsion of carotenoids from *Gracilaria dura*, SA-NE: Nanoemulsion of carotenoids from *Sargassum acinarium*. The results represent the average of three repetitions (n:3). Averages indicated by different letters (a, b) in the same column are significantly different from each other at the $p < 0.05$ level among the samples

Physical characterization of nanoemulsions

Encapsulation efficiency

In the present study, the encapsulation efficiency of *Ulva rigida* nanoemulsion (UR-NE), *Gracilaria dura* nanoemulsion (GD-NE) and *Sargassum acinarium* nanoemulsion (SA-NE) was determined to be 91.36% ± 0.95, 94.13%±1.08 and 87.29% ± 2.48, respectively ($p < 0.05$, Table 1). Similarly, in a study investigating the stability of brown algae pigments, encapsulation with maltodextrin and Tween 80, including β-carotene, resulted in an encapsulation efficiency of 88.94% [59]. In another study, the efficiency of a nanoemulsion produced was determined to be 87.2% after β-carotene encapsulation in flaxseed oil [60]. According to Barbosa et al. increased emulsion stability is correlated with higher encapsulation efficiency, resulting in a decreased presence of non-encapsulated material on the particle surface [61]. Several studies have demonstrated that

decreasing the size of emulsion droplets, commonly associated with enhanced stability, leads to improved retention of active substances and consequently boosts encapsulation efficiency [62, 63]. A high zeta potential can facilitate the preferred development of the capsule wall during encapsulation, thereby enhancing encapsulation efficiency [64]. Consistent with our study, the low particle size and high zeta potential in emulsions yielded an increased encapsulation efficiency.

Particle size and zeta potential

The particle sizes and zeta potentials of droplets are provided in Table 1. The mean particle diameter of the droplet ranged from 0.176 ± 0.01 to 0.189 ± 0.01 µm ($p < 0.05$, Table 1). The mean particle diameter of all samples was lower than 0.2 µm, confirming that these delivery systems were nanoemulsions [41]. According to the results, emulsions containing carotenoid extract have smaller particle sizes compared to the control sample. This is considered to be due to the reduction in the structures of the emulsions caused by the interfacial tension occurring at the common boundary between the extract and the lipid membrane [65]. Particle charges varied between (-17.6 ± 1.18) and (-24.35 ± 0.92) mV. Lipid droplets are coated with anionic emulsifiers, leading to the determination of their negative particle charges [50].

Visual appearance, creaming characteristics and color

In emulsions visual qualities, such as color and creaming, typically play a crucial role in determining the overall product appeal. Visual appearance forms the consumer's initial impression of the product, providing information about its quality [41]. The perceived quality of emulsion-based products, such as those in the food, pharmaceutical, healthcare and cosmetic industries, is determined by a combination of physicochemical properties, including stability, rheology and appearance [66]. The visual appearances of nanoemulsions are shown in Fig. 2.

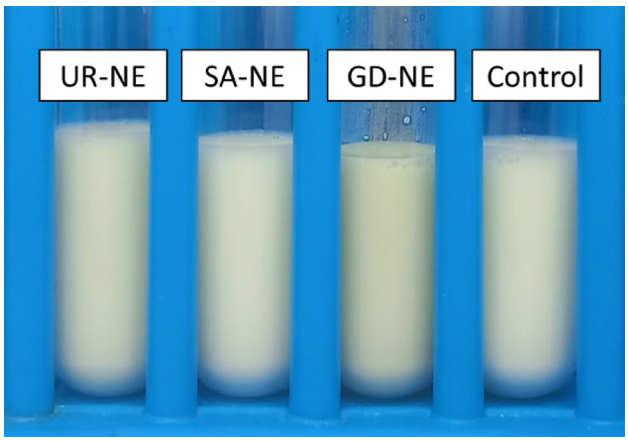


Fig. 2 The visual appearances of nanoemulsions. *UR-NE* Nanoemulsion of carotenoids from *Ulva rigida*, *GD-NE* Nanoemulsion of carotenoids from *Gracilaria dura*, *SA-NE* Nanoemulsion of carotenoids from *Sargassum acinarium*

Creaming index measurements showed that the initial values for all nanoemulsions were the same and no creaming signs were observed (Fig. 2). The sonication process delays flocculation and prevents creaming by reducing particle size, enhancing dispersion stability and promoting electrostatic repulsion among particles [67]. Ultrasonic treatment disrupts particle agglomeration, leading to a more uniform and stable dispersion, thereby inhibiting the formation of large aggregates and the formation of creaming [67]. In this study, all emulsions exhibited initial stability with no observable creaming on the first day (Fig. 2). The color characteristics of the nanoemulsions varied depending upon the algae species used. The L^*-values (brightness, lightness) of the emulsions for control, UR-NE, GD-NE and SA-NE nanoemulsions were measured as 79.78 ± 0.00, 76.04 ± 0.01, 74.36 ± 0.01 and 74.48 ± 0.02, respectively (Table 2 and 0th day). The lightness (L^* value) of emulsions containing algae carotenoid extracts is lower as compared to the control group. The a^*-values (redness) for control, UR-NE, GD-NE and SA-NE nanoemulsions were found to be - 5.06 ± 0.00, - 5.1 ± 0.01, − 4.64 ± 0.02 and − 4.3 ± 0.00, respectively. The b^*-values (yellowness) for control, UR-NE, GD-NE and SA-NE nanoemulsions were determined to be as 5.06 ± 0.05, 6.06 ± 0.01, 5.26 ± 0.00 and 7.64 ± 0.01, respectively (Table 2 and 0th day). The highest redness and yellowness values were recorded in SA-NE containing brown algae carotenoids ($p < 0.05$). It was observed that the yellowness (b^* value) of emulsions containing algae carotenoids was higher than the control group ($p < 0.05$).

Physical stability of nanoemulsions

Thermal stability

Emulsions are expected to maintain their desired functional properties under various conditions encountered during processing, storage, transportation and application. Therefore, understanding the impact of environmental stresses on the properties of emulsion-based delivery systems is important [68]. It was observed that the heat application did not cause any significant difference in the visual appearance of the emulsions and no phase separation occurred (Fig. S2(a)). A change in particle size was observed at 60 °C for the control sample, but no significant changes were observed ($p < 0.05$, Table S1, Table S2) in the particle sizes and particle charges of the other samples (Fig. 3a and b). Similarly, in the study by Fu et al. (2019), no phase separation was observed in

Table 2 Color changes of nanoemulsions during 28-day storage period

Value	Sample	Storage day				
		0th	7th	14th	21st	28th
L^*	Control	$79.78 \pm 0.22^{a, x}$	$76.17 \pm 2.19^{a, xy}$	$67.22 \pm 2.32^{b, y}$	$62.58 \pm 1.39^{c, z}$	$61.22 \pm 1.11^{b, z}$
	UR-NE	$76.04 \pm 2.37^{b, x}$	$64.82 \pm 3.52^{c, y}$	$68.14 \pm 3.52^{a, y}$	$66.78 \pm 2.64^{a, y}$	$62.04 \pm 2.12^{a, z}$
	GD-NE	$74.76 \pm 1.15^{c, x}$	$67.70 \pm 1.27^{b, y}$	$65.60 \pm 1.87^{b, z}$	$65.18 \pm 3.44^{b, z}$	$60.82 \pm 1.69^{c, t}$
	SA-NE	$74.48 \pm 5.12^{c, x}$	$67.66 \pm 2.87^{b, y}$	$62.68 \pm 2.19^{c, z}$	$62.56 \pm 2.79^{c, z}$	$60.84 \pm 2.07^{c, t}$
a^*	Control	$-5.06 \pm 1.92^{b, z}$	$-4.78 \pm 5.33^{a, y}$	$-5.74 \pm 3.14^{a, z}$	$-4.88 \pm 1.32^{a, y}$	$-4.02 \pm 1.42^{a, x}$
	UR-NE	$-5.10 \pm 0.97^{b, y}$	$-4.14 \pm 1.24^{a, x}$	$-4.70 \pm 1.19^{a, x}$	$-7.42 \pm 1.24^{c, z}$	$-5.06 \pm 1.22^{b, y}$
	GD-NE	$-4.64 \pm 2.71^{a, xy}$	$-4.04 \pm 2.18^{a, x}$	$-7.94 \pm 2.98^{b, z}$	$-5.08 \pm 1.36^{b, y}$	$-7.80 \pm 3.18^{c, z}$
	SA-NE	$-4.70 \pm 1.12^{a, x}$	$-4.02 \pm 1.36^{a, x}$	$-4.98 \pm 2.34^{a, x}$	$-5.62 \pm 2.13^{b, x}$	$-7.94 \pm 1.45^{c, z}$
b^*	Control	$5.06 \pm 1.56^{c, yz}$	$6.01 \pm 3.35^{b, y}$	$7.44 \pm 2.11^{a, x}$	$5.20 \pm 1.94^{b, yz}$	$4.00 \pm 2.91^{b, t}$
	UR-NE	$6.06 \pm 3.12^{b, x}$	$2.70 \pm 1.78^{c, z}$	$5.62 \pm 3.45^{b, y}$	$5.48 \pm 3.46^{b, y}$	$5.26 \pm 2.68^{a, y}$
	GD-NE	$5.26 \pm 0.88^{c, y}$	$1.64 \pm 1.42^{c, t}$	$7.28 \pm 1.36^{a, x}$	$5.88 \pm 1.23^{b, y}$	$4.20 \pm 2.18^{b, z}$
	SA-NE	$7.64 \pm 3.11^{a, y}$	$7.20 \pm 0.32^{a, y}$	$5.94 \pm 2.91^{b, z}$	$8.44 \pm 2.78^{a, x}$	$5.0 \pm 1.29^{a, z}$

*The results represent the average of three replicates (n:3). Different letters (a, b, c, d) in the same column indicate significant differences ($p < 0.05$) between measurements of different samples. Similarly, different letters (x, y, z) in the same row indicate significant differences ($p < 0.05$) between different day measurements of same sample. UR-NE: Nanoemulsion of carotenoids from *Ulva rigida*, GD-NE: Nanoemulsion of carotenoids from *Gracilaria dura*, SA-NE: Nanoemulsion of carotenoids from *Sargassum acinarium*.

emulsions containing encapsulated β-carotenoids under temperature applications of 65 and 90 °C [69]. Liu et al. also reported no significant changes in the appearances and particle sizes of astaxanthin-containing emulsion systems under temperature applications of 30, 50 and 80 °C [70]. According to the results, it can be said that all emulsions were stable between 30 and 80 °C.

pH stability

Emulsions can be exposed to various pH conditions during production and digestion and this plays a significant role in the degree of ionization of functional groups carried by biopolymers [71]. The changes in the average particle size and zeta potentials of emulsions (Fig. 3c and d) under different pH conditions were statistically significant ($p < 0.05$, Table S3, Table S4). However it was observed that no phase separation occurred according to results of visual evaluation of emulsions under different pH conditions (Fig. S2(b)). Additionally, none of the emulsions exhibited creaming after 24 h of different pH treatments (Fig. S2(b)). Similarly, in the study conducted by W. Liu et al. β-carotenoid emulsion remained stable against coalescence over a wide pH range (3–7) [72]. In contrast to our study by C. Liu et al. in emulsion system containing astaxanthin, stability loss occurred under temperature applications of 30, 50 and 80 °C at varying pH conditions from 3 to 8, due to changes in surface charges at high and low pH values [70].

Ionic strength stability

Stability against salt concentrations is crucial for emulsions as it affects their structure and can promote coalescence, potentially compromising their homogeneity and stability. Thus, proper control of salt levels is essential to prevent stability issues like phase separation or creaming [73]. In our study, generally, the addition of salt did not cause a significant change in the particle size of emulsions and even at high ionic strengths (Fig. 3e and f) they exhibited high stability ($p < 0.05$, Table S5, Table S6). Moreover, the high stability was confirmed by visual methods and there was no observable change in the macroscopic appearance of the emulsions (Fig. S2(c)). Similarly, in a study conducted by Liu et al. β-carotenoid emulsion remained stable against coalescence at high salt levels (0-1000 mM NaCl) [72]. In emulsion systems with added NaCl, positive ions (Na^+) in the aqueous phase tend to gather around the negatively charged groups (COO^-) on the surface of particles due to electrostatic attraction, further reducing their net charges [74]. Among the nanoemulsions produced in the study, the concentration of NaCl at which the net charge decreased the most was the emulsions containing 200 mM NaCl (Fig. 3f).

In the present study, after addition of different salt concentrations, very small changes were observed in droplet size. This indicates that the adsorbed polymer layer forms a steric repulsive barrier between droplets and the stabilization mechanism is likely related to electrostatic repulsive forces [75]. Surprisingly, carotenoid-loaded emulsions (Fig. S2(c)) remained stable against droplet aggregation at all salt concentrations, contrary to previous studies on similar systems [76]. Similarly, in the study by Liu et al. there was no significant change in the average particle size in the range of 0-1000 mM NaCl concentration in the carotenoid emulsion [72].

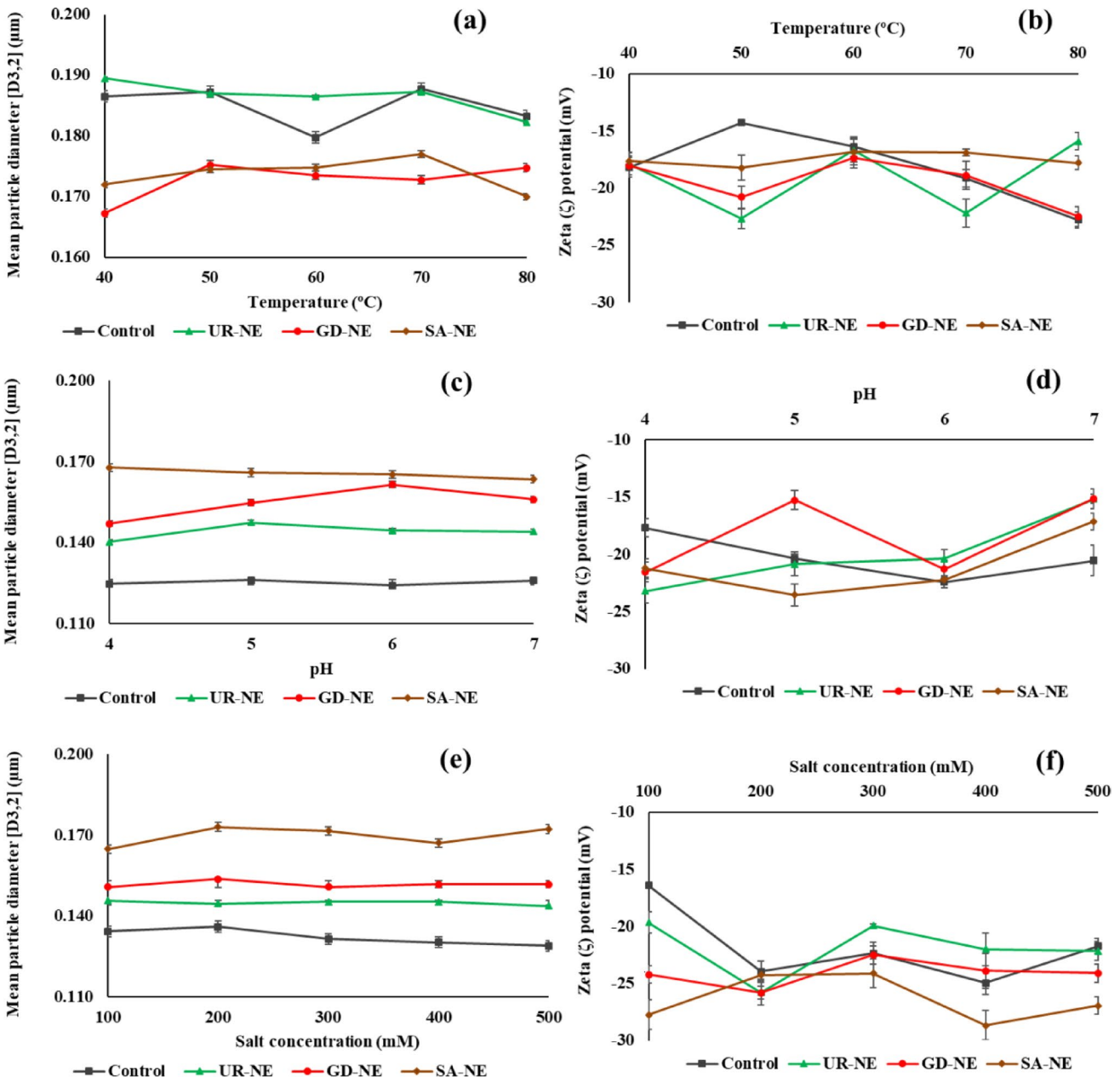


Fig. 3 The graphs illustrate the changes in mean particle diameter and zeta potential of nanoemulsions in response to environmental influences. *UR-NE* Nanoemulsion of carotenoids from *Ulva rigida*, *GD-NE* Nanoemulsion of carotenoids from *Gracilaria dura* and *SA-NE* Nanoemulsion of carotenoids from *Sargassum acinarium*

Storage stability of nanoemulsions

Particle size and charge

Emulsions with larger particle sizes tend to exhibit higher instability and a faster rate of creaming. Therefore, systems with smaller particle sizes are crucial for achieving better storage stability over an extended period [77]. According to the results, nanoemulsions showed particle sizes ranging between 0.16 and 0.17 µm over a 28-day storage period (Fig. 4a). Generally, there was no significant change or a slight decrease in size distribution parameters after a 28-day storage period ($p < 0.05$, Fig. 4a). In a similar study, β-carotenoid nanoemulsions exhibited a decrease in particle sizes after 21-days of storage [78]. Akhoond Zardini et al. suggested that the decrease in particle size observed in nanostructured lipid carriers containing lycopene, a type of carotenoid, is attributed to the ability of the surfactant to penetrate and completely cover the surfaces of droplets during the storage period [79]. The decrease was attributed

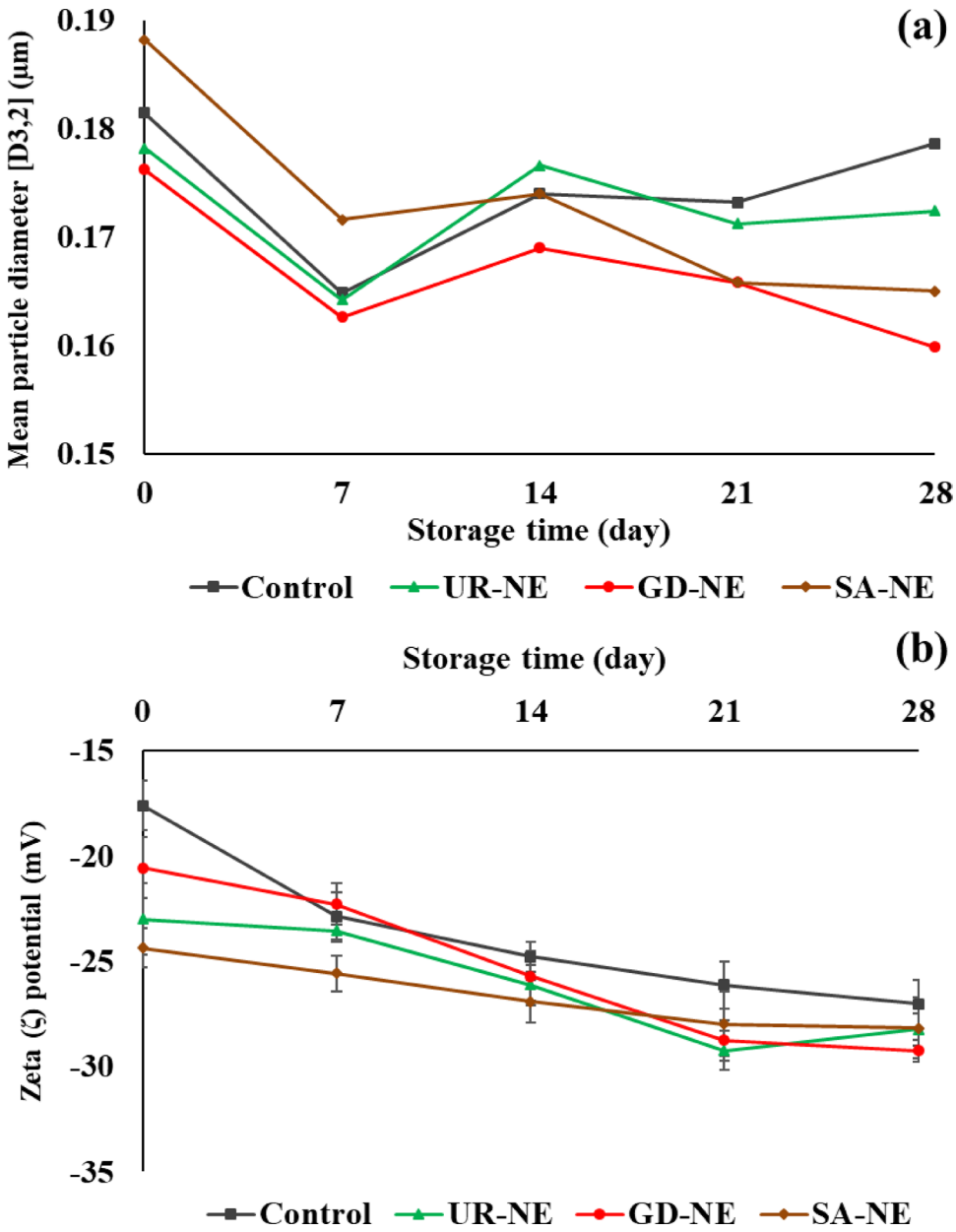


Fig. 4 The change graph of particle sizes (**a**) and particle charges (**b**) over 28-day storage period for the emulsions. UR-NE: Nanoemulsion of carotenoids from *Ulva rigida*, GD-NE: Nanoemulsion of carotenoids from *Gracilaria dura*, SA-NE: Nanoemulsion of carotenoids from *Sargassum acinarium*

to the inadequate or incomplete evaporation of hexane during the emulsification-evaporation stage, indicating that residual organic solvents like hexane from the extraction step might be present in the samples during storage, or hexane could be trapped in nano droplets [78]. In another study, particle size reduction after 7 days was reported in marine algae extract-loaded nanoemulsions, which was attributed to the progressive reorganization of the interfacial layer and improved surfactant alignment over time [80].

The results of particle charge are provided in Fig. 4b. Similar to particle size, reductions in zeta potential values occurred during the storage period ($p < 0.05$, Fig. 4b). After 28-day storage period, particle charges became more negative in all nanoemulsions ($p < 0.05$, Fig. 4b). This increase in negative charge is likely associated with an increase in free fatty acids and does not affect the stability of the sample [81].

The absolute value of the zeta potential larger than 30 mV indicates that the electric charge in the system is dominated by repulsive forces between droplets. This electric charge provides enhanced physical stability and bioactivity in terms of cellular binding and uptake [82, 83]. Similar to Fitri et al.'s study where β-carotenoid was encapsulated with nanocellulose and Tween 20, the low negative charge in the control emulsion might be attributed to the composition of this emulsion, which consists only of the non-ionic surfactant Tween 80 [80]. A similar trend was reported in another research article, where β-carotenoid nanoemulsions

formulated with corn oil, having an average size in the range of 300 nm, a negative charge (- 25 mV or more) and a monodisperse profile, showed statistically significant a decrease in particle charge and size after 90 days of storage [21].

Color

The color values for control, UR-NE, GD-NE and SA-NE were determined over 28 storage days (Table 2). According to the results, the lightness (L^*) and color intensity (a^* and b^*) of nanoemulsions gradually decreased during storage ($p < 0.05$, Table 2). In the present study, a slight decrease in lightness of nanoemulsions was observed during storage and L^* value of nanoemulsions containing carotenoid extract was found to be lower than that of the control group ($p < 0.05$, Table 2). This could be due to the changes in light scattering intensity, for example, due to alterations in particle size or environmental effects. Conversely, the increase in lightness is generally observed when color intensity decreases because more light is reflected from the surface of the emulsion [66]. Similarly, in the study by Santipanichwong and Suphantharika as the amounts of β-carotene and lutein added to mayonnaise increased, the L^* value of mayonnaise decreased. This was explained by the absorption of light by carotenoid molecules, resulting in less light being reflected in the emulsion structure [84].

A decrease in the a* value indicates a decrease in redness, whereas a decrease in the b* value signifies a decrease in yellowness [85]. In the present study, the highest values for redness (a^*) and yellowness (b^*) were observed in SA-NE containing brown algae carotenoids ($p < 0.05$, Table 2). As expected, the emulsions containing algal carotenoids have a higher b^* (yellowness) value than that of the control ($p < 0.05$, Table 2). In the study by Santipanichwong and Suphantharika as the amounts of β-carotene and lutein added to mayonnaise increased, the b* value of mayonnaise also increased [84]. In the present study, color discoloration of nanoemulsion occurred during the storage period of 28-days. The β-carotene degradation reactions (e.g., oxidation) and the distribution of particle sizes contribute to the color changes in nanoemulsions [78]. The decrease in color values is believed to be attributed to the oxidation of the oil. Similarly, Hagenimana et al. stated in their study that both enzymatic and non-enzymatic oxidation could lead to color losses [86].

Creaming index

Creaming Index (CI) provides indirect information about the degree of flocculation of oil droplets in an emulsion; higher the droplet size, faster the flocculation and creaming formation occurs [87]. When the visual appearance of the stored samples was examined, it was observed no phase separation in the samples during the storage period (Fig. 5).

On the first day, none of the nanoemulsions showed creaming. (Fig. 5). The creaming index further confirmed the correlation between zeta-potential and emulsion stability; low zeta-potential and high particle size increased the

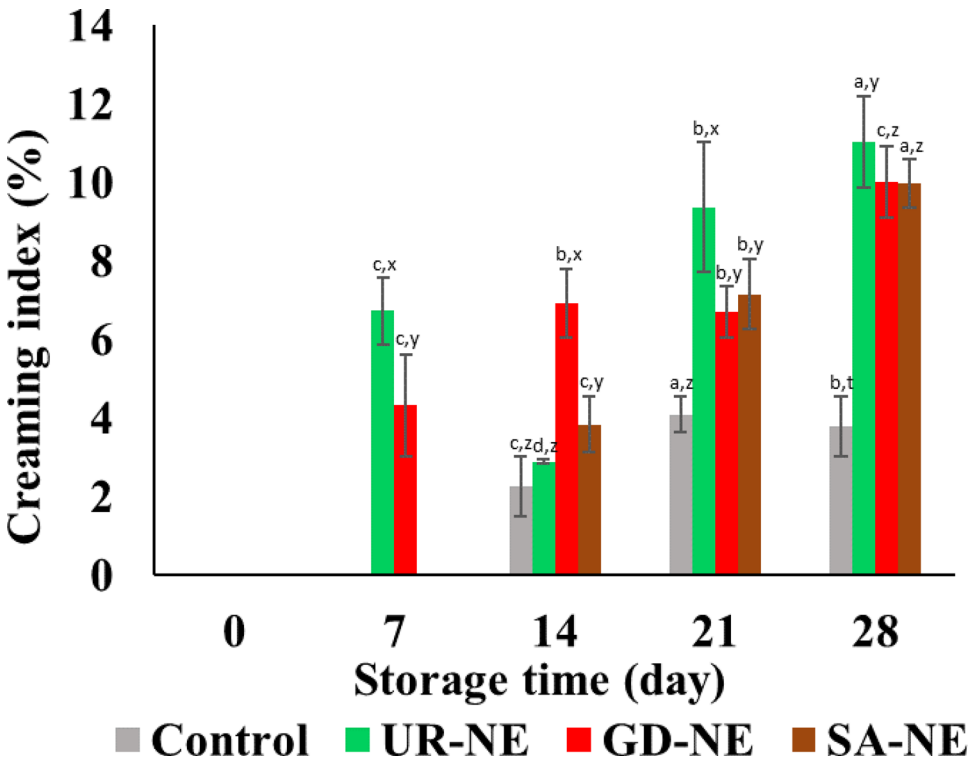


Fig. 5 Creaming indices measured over 28-day storage period for nanoemulsions. UR-NE: Nanoemulsion of carotenoids from *Ulva rigida*, GD-NE: Nanoemulsion of carotenoids from *Gracilaria dura*, SA-NE: Nanoemulsion of carotenoids from *Sargassum acinarium*. Different letters (a,b,c,d) across days indicate significant differences ($p<0.05$) in measurements between same sample on different days, while different letters (x, y, z, t) within the same day group denote significant differences ($p<0.05$) between measurements on the same day

Table 3 Changes antioxidant activity of nanoemulsions by ABTS method during the storage period

AOA_{ABTS} mg TE/100 mL emulsion					
Sample	Storage day				
	0th	7th	14th	21st	28th
Control	$4.85 \pm 0.11^{b, x}$	$4.46 \pm 0.21^{c, y}$	$4.01 \pm 1.03^{a, y}$	$3,88 \pm 0.09^{c, z}$	$3,12 \pm 0.12^{c, z}$
UR-NE	$6.80 \pm 0.09^{a, y}$	$7.29 \pm 0.07^{a, x}$	$6.10 \pm 0.18^{a, z}$	$6.42 \pm 0.21^{a, y}$	$6.36 \pm 0.24^{a, y}$
GD-NE	$6.76 \pm 0.05^{a, x}$	$6.65 \pm 0.32^{b, x}$	$5.73 \pm 0.06^{b, y}$	$5.32 \pm 0.11^{b, y}$	$4.92 \pm 0.06^{b, z}$
SA-NE	$6.39 \pm 0.10^{a, x}$	$5.32 \pm 0.15^{b, y}$	$5.62 \pm 0.16^{b, y}$	$4.58 \pm 0.20^{c, z}$	$4.29 \pm 0.18^{b, z}$

*Results were averaged over three replicates (n:3). Means indicated by different letters (a, b,c) in the same column are different from each other at $p < 0.05$ level. Means with different letters (x, y,z) in the same row indicate that the values measured in each week are different from each other at $p < 0.05$ level

Table 4 Changes antioxidant activity of nanoemulsions by CUPRAC method during the storage period

AOA_{CUPRAC} mg TE/100 mL emulsion					
Sample	Storage day				
	0th	7th	14th	21st	28th
Control	$1.89 \pm 0.43^{c, x}$	$1.08 \pm 0.08^{c, y}$	$1.22 \pm 0.19^{a, y}$	$0.91 \pm 0.09^{c, z}$	$0.69 \pm 0.02^{b, z}$
UR-NE	$2.53 \pm 0.06^{a, x}$	$2.3 \pm 0.09^{b, x}$	$2.13 \pm 0.06^{a, y}$	$2.17 \pm 0.04^{a, y}$	$1.82 \pm 0.07^{a, z}$
GD-NE	$2.74 \pm 0.12^{a, x}$	$2.69 \pm 0.08^{a, x}$	$2.23 \pm 0.12^{a, y}$	$2.13 \pm 0.06^{a, y}$	$1.61 \pm 0.22^{a, z}$
SA-NE	$2.24 \pm 0.12^{b, x}$	$1.92 \pm 0.05^{c, y}$	$1.98 \pm 0.14^{b, y}$	$1.9 \pm 0.03^{b, y}$	$1.89 \pm 0.07^{a, y}$

*Results were averaged over three replicates (n:3). Means indicated by different letters (a, b,c) in the same column are different from each other at $p < 0.05$ level. Means with different letters (x, y,z) in the same row indicate that the values measured in each week are different from each other at $p < 0.05$ level

creaming index and decreased stability [39]. Smaller oil droplet size was associated with a lower creaming index; therefore, SA-NE with the smallest particle size exhibited minimal creaming and a predicted lower creaming index for UR-NE and GD-NE nanoemulsions, destabilization started on the seventh storage day, after which UR-NE showed the fastest creaming trend (Fig. 5). Throughout the storage period, UR-NE consistently exhibited the lowest zeta potential among the nanoemulsions, indicating an earlier creaming trend. In Li et al. study examining the stability and release of β-carotene in egg protein gels, the creaming indices of emulsions increased rapidly in the first 3 days and gradually until the end of one week of storage. The reason reported for this is that emulsions with a higher degree of droplet size have a higher tendency to creaming [88]. Similarly, in study of Fan et al. where whey protein-cross-linked flavonoids obtained from *Lycium barbarum* L. leaves were used for the stabilization of β-carotene emulsions, the creaming value of emulsions increased rapidly in the first 2 h, then slowly increased and almost remained unchanged after 10 h. It was reported that the creaming index of the sample containing carotene was lower compared to the sample containing only whey protein [89]. In another study, it was found that the addition of β-carotene led to the formation of larger oil droplets by increasing the viscosity of the oil phase, making droplet coalescence more difficult. For corn oil emulsions, they observed an increase in the creaming index of emulsions, indicating negative effects on stability [90]. Similarly, in the present study, the faster creaming tendency observed in emulsions using carotenoids extracted from macroalgae compared to the control sample may be attributed to this negative effect (Fig. 5). The visual appearance of the nanoemulsions during the storage period is given in Fig. S3.

Antioxidant activity during storage

The antioxidant activity of the nanoemulsions was determined on the 0th, 7th, 14th, 21st and 28th day of storage. According to the results, the nanoemulsions retained their antioxidant activity determined by ABTS and CUPRAC method throughout their storage period ($p < 0.05$, Tables 3 and 4). The carotenoid extracts from *Ulva rigida*, *Sargassum acinarium* and *Gracilaria dura* had different carotenoid contents as discussed in 3.1 section. However, antioxidant activity of the nanoemulsions was statistically the same at the 28-day storage period by ABTS method ($p > 0.05$, Table 3). On the other hand, all nanoemulsions had a high AOA with ABTS and CUPRAC methods compared to control at the first storage day. While there may be a strong relationship between the carotenoid content and AOA on the 0th storage day, on other days this relationship may weaken or develop completely in the opposite direction (Tables 3 and 4). The protective effect of encapsulation on carotenoids seems to be lost after the 7th storage day according to the ABTS results (Table 3).

Oxidative stability

The lipid hydroperoxide formation and TBARS values of the nanoemulsions are shown in Fig. 6a-b. The sample without algae extraction was represented as the control sample

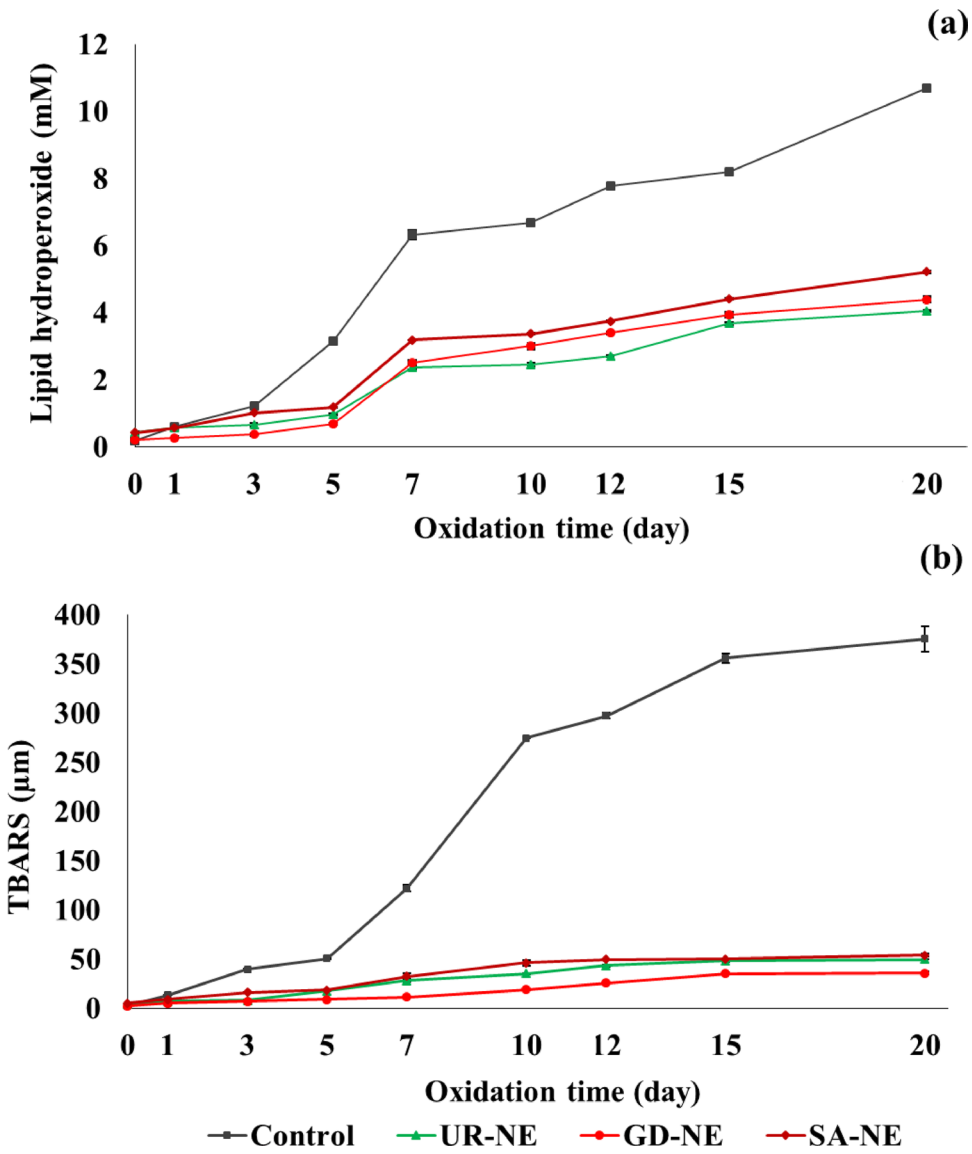


Fig. 6 The changes in lipid hydroperoxide levels (**a**) and TBARS (**b**) during the storage period of nanoemulsions

in this study. The lipid hydroperoxides formation of the control sample started to increase on storage day 3 and continued the oxidation period ($p < 0.05$, Fig. 6a). The lipid hydroperoxides formation of nanoemulsion samples stabilized with algae extract dramatically increased on storage day 5 and continued to increase during storage period ($p < 0.05$, Fig. 6a). The TBARS value of the control sample started to increase after the 3rd storage day and continued the storage time ($p < 0.05$, Fig. 6b). Emulsions stabilized with algal carotenoids exhibited an increase in TBARS values from the 5th day onwards, indicating the formation of secondary oxidation products, which continued throughout the oxidation period ($p < 0.05$, Fig. 6b). Similarly, extracts obtained from brown algae *Sargassum muticum* have been reported to be successfully used to enhance the oxidative stability of oil-in-water model emulsions [91]. In another study, emulsions containing 5% fish oil were created using 50% ethanolic extracts of *Polysiphonia fucoides* algae. Antioxidant tests revealed that the components responsible for the decrease in TBARS inhibition with increasing concentration were likely the *P. fucoides* polyphenol fractions, which presumably had higher reducing power [92]. In two different studies where extracts obtained from algae were used in food products, the potential antioxidant use of Icelandic *Fucus vesiculosus* extract was investigated and it was found to be extending the oxidation period in food products enriched with fish oil (mayonnaise, milk and granola bars with pre-emulsified fish oil) [93, 94]. When compared with the reviewed studies in the literature, the results stated that carotenoids obtained from algae, like in the literature, exhibit inhibitory properties against lipid oxidation. As a result, the produced nanoemulsions showed delayed spoilage during storage compared to the control sample.

Table 5 The change in β-carotene (mg/ 100 mL emulsion) levels of nanoemulsions during the storage period as determined by HPLC analysis

Sample	Storage day				
	0th	7th	14th	21st	28th
UR-NE	$49.84 \pm 0.42^{a, x}$	$44.04 \pm 3.54^{a, y}$	$43.32 \pm 0.15^{a, y}$	$42.54 \pm 2.66^{a, z}$	$42.32 \pm 0.12^{a, z}$
GD-NE	$47.03 \pm 0.29^{b, x}$	$43.74 \pm 0.78^{b, y}$	$43.03 \pm 2.65^{a, y}$	$42.82 \pm 0.98^{a, z}$	$42.06 \pm 3.12^{a, z}$
SA-NE	$47.15 \pm 1.26^{b, x}$	$44.09 \pm 0.93^{b, y}$	$42.64 \pm 1.38^{b, z}$	$42.40 \pm 4.44^{a, z}$	$42.11 \pm 2.46^{a, z}$

*The results represent the average of three repetitions (n:3). Different letters (a, b, c, d) in the same column indicate that the measurements between samples are significantly different at $p < 0.05$. Different letters (x, y, z) in the same row indicate that the measurements taken between days are significantly different at $p < 0.05$. UR-NE: Nanoemulsion of carotenoids from *Ulva rigida*, GD-NE: Nanoemulsion of carotenoids from *Gracilaria dura*, SA-NE: Nanoemulsion of carotenoids from *Sargassum acinarium*

Table 6 During in vitro digestion, the bioaccessibility of the encapsulated and non-encapsulated carotenoids (%)

Bioaccessibility (%)		
Sample	Encapsulated carotenoids	Non-encapsulated carotenoids
UR-NE	$93.21 \pm 0{,}88^{a, x}$	$61.94 \pm 1.42^{c, y}$
GD-NE	$94.15 \pm 2{,}63^{a, x}$	$59.47 \pm 1{,}28^{c, y}$
SA-NE	$70.24 \pm 0{,}26^{b, x}$	$44.21 \pm 0.34^{b, y}$

*The results represent the mean of three replicates (n:3). Different letters (a, b, c, d) in the same column indicate a significant difference between measurements of different samples at $p < 0.05$. Different letters (x, y) in the same row indicate a significant difference between values in different in vitro digestion phases at $p < 0.05$. UR-NE: Nanoemulsion of carotenoids from *Ulva rigida*, GD-NE: Nanoemulsion of carotenoids from *Gracilaria dura*, SA-NE: Nanoemulsion of carotenoids from *Sargassum acinarium*

Total carotenoid content and carotenoid profile

Table 5 shows the changes in the amount of β-carotene obtained through HPLC analysis during the storage period of nanoemulsions. The β-carotene losses increased from the first week onwards ($p < 0.05$, Table 5). Generally, various factors during food processing and storage, such as heat, acidity, light, oxygen, metal ions, accelerated oxidation and isomerization of carotenoids, can lead to impaired bioavailability and reductions in quantity [95]. In the present study, a significant decrease in the amount of β-carotene was observed during the 28-day storage period compared to the initial day ($p < 0.05$, Table 5). Similarly, in Igual et al. study monitoring the changes in β-carotene content in grapefruit peels over three months, a rapid decrease in β-carotene content was observed in the first 15 days [96]. Continuous reduction in β-carotene levels was observed in nanoemulsions prepared with the encapsulation of polyglycerol esters of fatty acids and as a result, undegraded β-carotene residues were detected in the samples ranging from 31% to 23% after 12 weeks of storage [97]. Fig. S4, Fig. S5 and Fig. S6 displays chromatograms related to the β-carotene profile of nanoemulsions during the storage period.

In vitro digestion

In the present study, the nanoemulsions stabilized with algal carotenoids were subjected to in vitro digestion tests and the percentage bioaccessibility of carotenoids was determined (Table 6). The encapsulated carotenoid bioaccessibility ranged from 70.24 ± 0.26% to 94.15 ± 2.63%, while the bioaccessibility of non-encapsulated carotenoids varied between 44.21 ± 0.34% and 61.94 ± 1.42% ($p < 0.05$, Table 6). The bioaccessibility of encapsulated carotenoids was higher than that of non-encapsulated carotenoids ($p < 0.05$, Table 6). Similarly, a work on the bioaccessibility of carotenoids from rosehip and tomato paste, bioaccessibility values were determined as an average of 67%±6 for β-carotenoid, 32%±5 for lycopene and 63%±2 for rubixanthin [98]. Bioaccessibility is known to depend on the type of microcomponent (lutein >β-carotene and α-carotene > lycopene and α-tocopherol generally >gamma-tocopherol), the food matrix and differences in food processing [99]. On the other hand, in the present study, amount of carotenoids in the nanoemulsion decreased with in vitro digestion ($p < 0.05$, Fig. 7). Similarly, Scrob et al. (2019) has been indicated that the carotenoid content of extracts from *Brassica oleracea* vegetable decreased with in vitro digestion. As a result, it was found that the amount of carotenoids was digested by 78.07% after in vitro digestion [100]. There are studies suggesting that the stability of carotenoids against acidic conditions during in vitro digestion cannot be maintained [100, 101]. However, the in vitro bioaccesibility of carotenoids varies not only for a specific carotenoid but also for different carotenoids in a specific food and for carotenoids in different foods [101]. In the present study, GD-NE, which has the smallest particle size, exhibited a higher bioaccessibility percentage (%94.15 ± 2.63) ($p < 0.05$, Table 6) as compared to SA-NE, along with UR-NE. In the study by Li et al. smaller lipid droplets were digested more rapidly than larger droplets due to their larger surface area exposed to lipase molecules [102]. Salvia-Trujillo et al. investigated the effect of particle size on lipid digestion and β-carotene bioaccessibility using corn oil emulsions with different initial droplet sizes of 23 µm (large), 0.4 µm (medium) and 0.2 µm (small) [38]. Similarly, they observed an increase in the amount of free fatty acids released during lipid digestion with a decrease in the average droplet size [38]. This increase was attributed to the larger lipid surface area exposed to pancreatic lipase accompanying the reduction in droplet size.

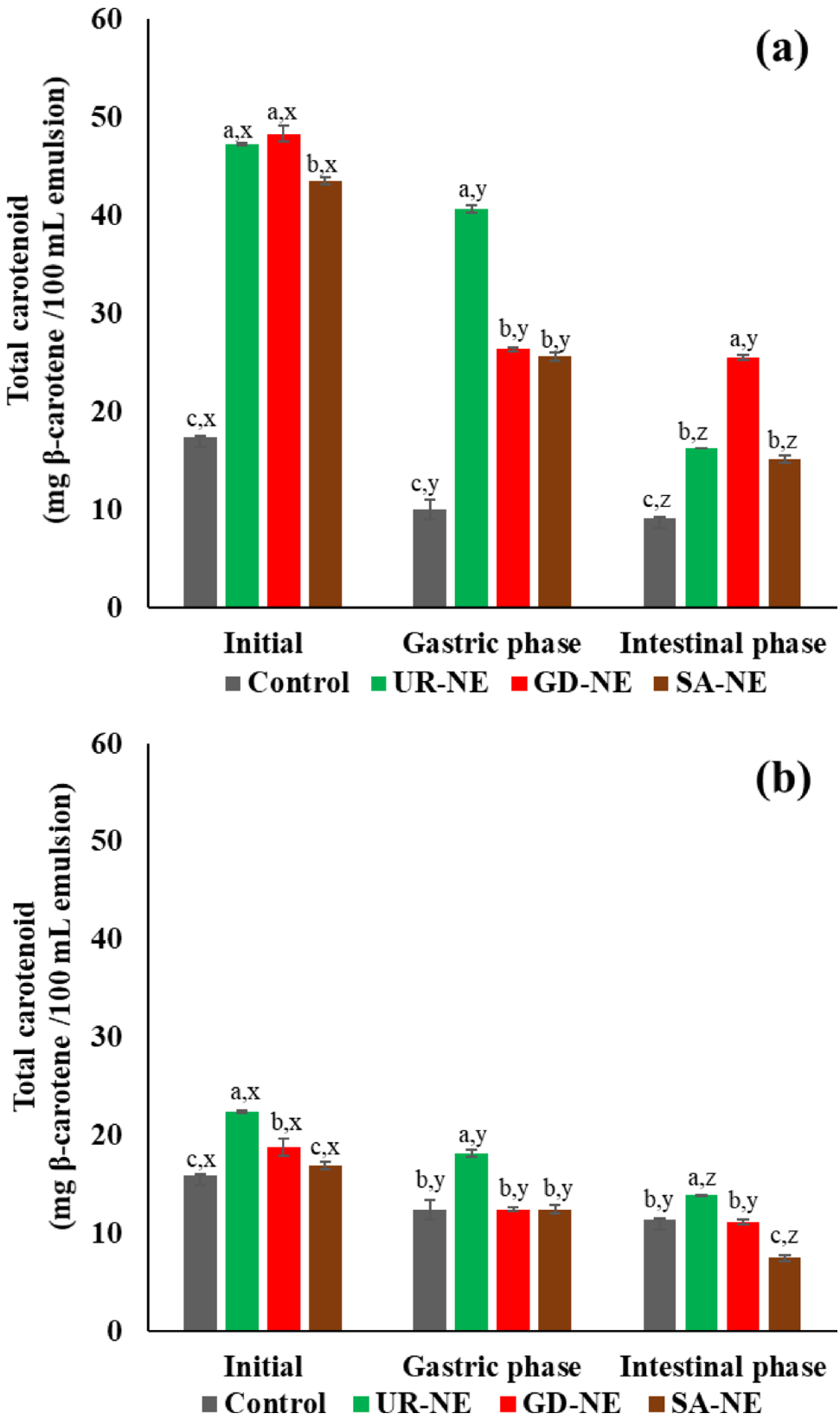


Fig. 7 The amount of β-carotene in encapsulated (**a**) and non-encapsulated (**b**) carotenoid extracts before and after in vitro digestion.*UR-NE* Nanoemulsion of carotenoids from *Ulva rigida*, *GD-NE* Nanoemulsion of carotenoids from *Gracilaria dura*, *SA-NE* Nanoemulsion of carotenoids from *Sargassum acinarium*. Different letters (a, b, c, d) within the same in vitro digestion phase indicated significant differences ($p<0.05$) in measurements between samples. Similarly, different letters (x, y, z, t) across different phases signify same samples significant differences ($p<0.05$) during in vitro digestion

Table 7 Antioxidant activity of encapsulated and non-encapsulated samples before and after in vitro digestion by ABTS method

AOA_{ABTS} mg TE/100 mL emulsion			
Encapsulated carotenoid emulsion			
Sample	Initial	Gastric	Intestinal
Control	$4.85 \pm 0.11^{b, x}$	$0.41 \pm 0.04^{b, z}$	$0.85 \pm 0.02^{b, y}$
UR-NE	$6.80 \pm 0.09^{a, y}$	$0.36 \pm 0.07^{b, z}$	$0.86 \pm 0.05^{b, y}$
GD-NE	$6.76 \pm 0.05^{a, x}$	$0.44 \pm 0.03^{b, z}$	$1.01 \pm 0.11^{a, y}$
SA-NE	$6.39 \pm 0.10^{a, x}$	$0.86 \pm 0.04^{a, y}$	$0.77 \pm 0.06^{b, y}$
Non-encapsulated carotenoid emulsion			
Control	$1.23 \pm 0.02^{a, x}$	$0.28 \pm 0.07^{b, z}$	$0.92 \pm 0.05^{a, y}$
UR-NE	$1.32 \pm 0.05^{a, x}$	$0.42 \pm 0.03^{ab, y}$	$0.41 \pm 0.10^{b, y}$
GD-NE	$1.27 \pm 0.04^{a, x}$	$0.38 \pm 0.01^{ab, y}$	$0.39 \pm 0.02^{b, y}$
SA-NE	$1.22 \pm 0.10^{a, x}$	$0.50 \pm 0.09^{a, y}$	$0.25 \pm 0.04^{c, z}$

*Results are the average of three replicates (n:3). Means with different letters (a, b,c) in the same column represent that the measurements between the samples are different from each other at $p < 0.05$. Means with different letters (x, y,z) in the same row represent that the values in in vitro digestion phases are different from each other at $p < 0.05$ level

Table 8 Antioxidant activity of encapsulated and non-encapsulated samples before and after in vitro digestion by CUPRAC method

AOA_{CUPRAC} mg TE/100 mL emulsion			
Encapsulated carotenoid emulsion			
Sample	Initial	Gastric	Intestinal
Control	$1.89 \pm 0.43^{b, x}$	$0.75 \pm 0.14^{a, z}$	$1.65 \pm 0.13^{a, y}$
UR-NE	$2.53 \pm 0.06^{a, x}$	$0.73 \pm 0.14^{a, z}$	$1.32 \pm 0.06^{a, y}$
GD-NE	$2.74 \pm 0.12^{a, x}$	$0.57 \pm 0.04^{b, z}$	$1.43 \pm 0.06^{a, y}$
SA-NE	$2.24 \pm 0.12^{a, x}$	$0.65 \pm 0.06^{b, z}$	$1,54 \pm 0,08^{a, y}$
Non-encapsulated carotenoid emulsion			
Control	$0,19 \pm 0,06^{b, y}$	$0.51 \pm 0.28^{b, x}$	$0.32 \pm 0.04^{c, z}$
UR-NE	$0.18 \pm 0.05^{b, z}$	$0.28 \pm 0.03^{c, y}$	$0.52 \pm 0.09^{b, x}$
GD-NE	$0.17 \pm 0.04^{b, y}$	$0.25 \pm 0.07^{c, y}$	$0.75 \pm 0.03^{a, x}$
SA-NE	$0.25 \pm 0.09^{a, y}$	$1.77 \pm 0.07^{a, x}$	$0.3 \pm 0.09^{c, y}$

*Results are the average of three replicates (n:3). Means with different letters (a, b,c) in the same column represent that the measurements between the samples are different from each other at $p < 0.05$. Means with different letters (x, y,z) in the same row represent that the values in in vitro digestion phases are different from each other at $p < 0.05$ level

Indeed, a decrease in droplet size was associated with an increase in β-carotene bioaccessibility during in vitro digestion [38]. Similarly, Hedren et al. stated that particle size influences the bioaccessibility of carotenoids during in vitro digestion [103].

In the present study, it is observed that encapsulated carotenoids are better preserved as compared to the non-encapsulated carotenoids (Fig. S7, Fig. S8, Fig. S9) during in vitro digestion ($p < 0.05$, Table 6). Emulsion-based delivery systems are particularly suitable tools to enhance the utilization of carotenoids by facilitating the easier dissolution, longer stability and better utilization of encapsulated bioactive compounds [104].

Antioxidant activity during in vitro digestion

Antioxidant activity during in vitro digestion of encapsulated and non-encapsulated carotenoid extracts and emulsions was determined. In our study, the antioxidant activities of carotenoids determined by both CUPRAC and ABTS methods decreased with in vitro digestion ($p < 0.05$, Tables 7 and 8). Before in vitro digestion, the antioxidant activity of the samples ranged between 5.85 ± 0.12–6.80 ± 0.09 mg TE/100 mL by ABTS method and between 1.89 ± 0.43–2.74 ± 0.12 mg TE/100 mL by CUPRAC method (Tables 7 and 8). In the present study, the differences in ABTS radical scavenging activity and CUPRAC assays may be due to antioxidant selectivity in response to the chemicals used in the methods. In the study of Fariman et al. it was reported that the CUPRAC activity of *Nizamuddinia zanardinii* ranged from 1.8 to 0.8 mg TE/g, while that of *Cystoseira indica* ranged from 1.04 to 0.72 mg TE/g [105]. The decrease in antioxidant activity observed after in vitro gastric digestion is considered to be due to the acidic conditions during in vitro gastric digestion (Tables 7 and 8). This decrease in antioxidant activity after in vitro digestion may be related to the decrease in the amount of carotenoids in the samples during in vitro digestion. Previous studies on the in vitro release properties of β-carotene indicate that the degree of degradation of β-carotene in an acidic environment (pH 3–7) increases with decreasing pH and reaches a maximum at pH 5 [106]. On the other hand, it was seen that the antioxidant activity is better preserved with the encapsulation of carotenoids than those of non-encapsulated (Tables 7 and 8). Durante et al. it was reported that emulsions prepared to improve the stability of tomato oil exhibited higher antioxidant activity and preserved β-carotene stability than those of non-emulsified ones [107]. Moreover, an increase in antioxidant activity by ABTS method of the control non-encapsulated carotenoid emulsion phase was recorded after gastric digestion (Table 7). According to Bouayed et al. compounds are extracted in vivo during gastrointestinal digestion, and depending on the food matrix, pH, temperature, the presence of inhibitors or absorption-enhancing factors and enzymes; the nature, stability and antioxidant activity of extractable phytochemicals may change [108]. In the study of Chen et al. AOA after in vitro intestinal digestion of 23 different edible flowers increased compared to AOA after in vitro gastric digestion. It is well known that a substance's pH can influence how molecules racemize, potentially producing two chiral enantiomers with varying reactivity in the respective reagent. Since racemization can rise with pH in other chemicals, this might theoretically change their biological reactivity and make the antioxidants less reactive at pH 7.4 in the duodenal phase and more reactive early in

the digestive process, especially at acidic pH in the gastric phase [109].

Conclusion

In the scope of this study, encapsulation of carotenoid extracts obtained from *Ulva rigida*, *Gracilaria dura and Sargassum acinarium* was achieved using the nanoemulsion method. It was observed that encapsulated carotenoids better preserved their stability in terms of carotenoid content and antioxidant activity during in vitro digestion as compared to the non-encapsulated carotenoids. According to the results of the storage stability study, algal carotenoid nanoemulsions exhibited better oxidative stability as compared to the control. Utilizing carotenoids extracted from algae in nanoemulsion formulations can enhance the absorption of bioactive compounds by cells. In future studies, the potential use of algal carotenoid emulsions in food products can be investigated by incorporating them into various food items to assess their impact on the physicochemical, sensory and biological properties of the food products. Additionally, nanoemulsions contribute to the stabilization of carotenoids, prolonging product shelf life by preventing unwanted reactions such as oxidation or degradation. Lastly, nanoemulsion production presents advantages such as improved bioaccessibility, increased stability and enhanced solubility of carotenoids, making it a promising approach for incorporating these compounds into various products.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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