

Ionic liquid-based nanoemulsion as a promising strategy to enhance the bioaccessibility of β -carotene obtained from *Dunaliella salina*

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ABSTRACT

Dunaliella salina is a unicellular halophile green microalga that presents the capacity to accumulate high amounts of β -carotene, a bioactive compound widely recognized by its health benefits. To fulfil its biological functions, however, β -carotene needs to be bioaccessible, which is challenging as its bioaccessibility is typically low. A promising strategy to overcome β -carotene's low bioaccessibility is its entrapment in nanoemulsions (NEs). This study evaluated the influence of a bio-based ionic liquid (IL) as an emulsifier and different lipid concentrations on both the *in vitro* gastrointestinal behavior of NEs and β -carotene bioaccessibility. NEs were obtained from the sequential application of the IL as a green solvent for β -carotene extraction from *D. salina* and as an emulsifier to stabilize the β -carotene-loaded NEs. The NEs were composed of sunflower oil (10, 20, and 30 %), 2 % extract from *D. salina* enriched with β -carotene and IL and water (88, 78 and 68 %), being produced by high-intensity ultrasonication. The produced NEs were evaluated regarding physical stability (droplet size, polydispersity index, and ζ -potential) and the entrapment efficiency of β -carotene during 30 days of storage. In addition, β -carotene bioaccessibility and NEs' behavior under *in vitro* static digestion were evaluated as well. It was observed that the increase in lipid content resulted in higher droplet size (295–337 nm), decreased ζ -potential (–66 to –50 mV) during all the storage time, and increased β -carotene bioaccessibility (11–92 %), with no significant changes observed in β -carotene entrapment. The results suggested that the entrapment of β -carotene in NEs has great potential for its protection and for increasing its bioaccessibility.

1. Introduction

Microalgae have emerged as one of the most promising sources of bioactive compounds. Among these compounds are carotenoids, which have a wide range of applications in the food industry due to their nutritional and functional properties (Sousa et al., 2023). Carotenoids are natural pigments that perform essential functions in photosynthesis and offer various benefits to human health (Novoveská et al., 2019; Sousa et al., 2023). Microalgae are a rich and diverse source of carotenoids, in particular, β -carotene, a precursor of vitamin A that has important biological functions in terms of vision, cell growth, and immune response (Hamed et al., 2023; Sousa et al., 2023; Teixeiro-Roig et al., 2023). β -carotene from microalgae, such as *Dunaliella salina*, is highly

valued for its high concentration and bioactive properties (Monte et al., 2020; Sousa et al., 2023; Teixeiro-Roig et al., 2023). Most of the carotenoids (80–90 %) currently used are produced synthetically; however, microalgal β -carotene is a natural mixture of all-trans and 9-cis isomers, thus possessing higher bioaccessibility and more pronounced antioxidant properties that result in better health benefits (Monte et al., 2020; Novoveská et al., 2019). Therefore, microalgae represent a valuable source of carotenoids and their market is growing rapidly, driven by the increasing demand for healthy and sustainable food. Their nutritional richness, sustainability, and versatility of applications make them a promising solution for meeting the food challenges of the future, contributing to healthier diets and a more sustainable environment (Hamed et al., 2023; Monte et al., 2020; Sousa et al., 2023).

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Despite the potential of β -carotene for food purposes, its instability due to high sensitivity to environmental factors, such as light, oxygen, and heat, leads to its degradation and, consequently, to low bioavailability (Hamed et al., 2023). One potential way to solve this problem is to encapsulate β -carotene in emulsion-based delivery systems (Hamed et al., 2023; Teixeiro-Roig et al., 2023), since they can contribute to avoid its chemical degradation through the formation of a protective barrier that isolates β -carotene from the external environment, contributing to increase its stability, handling, storage, and efficacy (Hamed et al., 2023; McClements & Öztürk, 2021). In addition, the encapsulation process allows for the controlled release of β -carotene, ensuring that it is released at the appropriate time and place in the gastrointestinal tract (GIT), contributing to increase its bioavailability (Gonçalves et al., 2021; Teixeiro-Roig et al., 2023; Vardanega, Lüdtke, Loureiro, Toledo Hijo, et al., 2024). Considering that some studies have shown that two thirds of the β -carotene in the diet is inaccessible for absorption or excreted and only half of the amount absorbed is converted into retinol (Thurnham, 2007), the encapsulation of β -carotene appears as a potential solution to overcome this problem (Lüdtke et al., 2024; Teixeiro-Roig et al., 2023). In this regard, nanoemulsions (NEs) have been used for the delivery of β -carotene as demonstrated by Teixeiro-Roig et al. (2023), where the authors studied the influence of two different natural-based emulsifiers (soybean lecithin and whey protein isolate) on the *in vitro* gastrointestinal stability and bioaccessibility of β -carotene NEs. However, their physicochemical characteristics and their potential to enhance bioaccessibility are strongly dependent on the vegetable oil and emulsifier used in their formulation (Lüdtke et al., 2024; Teixeiro-Roig et al., 2023).

There is a wide range of emulsifiers that can be used in the formulation of emulsions for food application, most of them synthetic (McClements et al., 2017). The growing trend towards natural compounds, coupled with some of the health problems associated with the use of non-sustainable synthetic emulsifiers, such as liver damage (Jimenez-Escobar et al., 2020) or intestinal barrier dysfunction (H. Zhang et al., 2003; Zhu et al., 2009), has fuelled the search for bio-based alternatives. Therefore, the development of bio-based food emulsifiers as an alternative to non-sustainable synthetic ones is increasing. One potential alternative is the use of a new generation of Ionic Liquids (ILs) that are derived from natural bioactive compounds (Sousa et al., 2024; Toledo Hijo et al., 2016, 2022; Toledo Hijo, Silva, Cristianini, & Meirelles, 2023; Vardanega, Lüdtke, Loureiro, Toledo Hijo, et al., 2024). A recent study showed that by using ILs as a naturally-derived emulsifier, it is possible to obtain lipid-based nanostructured vehicles to effectively deliver cannabidiol (Vardanega, Lüdtke, Loureiro, Toledo Hijo, et al., 2024). Other studies also reported that ILs derived from compounds obtained from natural sources may have synergetic potential as emulsifiers and antimicrobial preservatives (Sousa et al., 2024; Toledo Hijo et al., 2022).

Building on our previous work, where an ionic liquid (IL) derived from oleic acid and choline was successfully applied as a green solvent and bio-based emulsifier for β -carotene extraction and nano-encapsulation (Sousa et al., 2024), the present study aimed not to re-evaluate the encapsulation method itself but to advance this concept by addressing a critical knowledge gap: the gastrointestinal stability of IL-stabilized β -carotene nanoemulsions (NEs) and *in vitro* bioaccessibility of β -carotene. Specifically, we investigated the impact of three lipid concentrations (10, 20, and 30 %) on NEs' performance during simulated gastrointestinal digestion, providing new insights into their potential as oral delivery systems for carotenoids.

2. Materials and methods

2.1. Materials

Oleic acid (90 %), choline hydroxide (46 % w/w, aqueous solution), and β -carotene (95 %) were purchased from Sigma-Aldrich (St. Louis, USA). Ethanol (absolute ≥ 99.8 %) and n-hexane (HPLC grade) were

purchased from Fischer Chemical (Hampton, USA). Refined sunflower seed oil was purchased in a local market (Masterchef®, Lisbon, Portugal) and was used in the formulation of emulsions as oil phase. Pepsin (≥ 2500 U/mg, P7012), lipase (30–90 U/mg, L3126), pancreatin (8x USP, P7545), and bile salts (B8631) were purchased from Merck (Portugal).

2.2. Microalgae culture

The microalga *Dunaliella salina* CCAP 19/18 was acquired from the Culture Collection of Algae & Protozoa, Scotland (<https://www.ccap.ac.uk/>). Microalgae culture was grown in a 2 L flat bottom flask under autotrophic conditions with a constant light supply of $240 \mu\text{mol}_{\text{photons}}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, an air stream of 800 mL min^{-1} , and a CO_2 stream of 6 mL min^{-1} in f/2 medium under a salinity of 65 PSU. The chemical composition of f/2 medium was as follows: nutrients solution (NaNO_3 : $9.8 \text{ mmol}\cdot\text{L}^{-1}$; $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$: $0.1 \text{ mmol}\cdot\text{L}^{-1}$), trace metals solution ($\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$: $11.7 \mu\text{mol}\cdot\text{L}^{-1}$; $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$: $11.7 \mu\text{mol}\cdot\text{L}^{-1}$; $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$: $0.91 \mu\text{mol}\cdot\text{L}^{-1}$; $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$: $0.0765 \mu\text{mol}\cdot\text{L}^{-1}$; $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$: $0.042 \mu\text{mol}\cdot\text{L}^{-1}$; $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$: $0.0393 \mu\text{mol}\cdot\text{L}^{-1}$, and $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$: $0.026 \mu\text{mol}\cdot\text{L}^{-1}$), and vitamins solution (thiamine HCl: $0.296 \mu\text{mol}\cdot\text{L}^{-1}$; cyanocobalamin: $3.69 \times 10^{-4} \mu\text{mol}\cdot\text{L}^{-1}$; and biotin: $2.05 \times 10^{-4} \mu\text{mol}\cdot\text{L}^{-1}$). After 15 days of growth, the microalgae were subjected to extraction processes.

2.3. Synthesis of ionic liquids

The IL cholinium oleate ([Ch][Ole]) was synthesized according to the method previously described by Toledo Hijo et al. (2022). The IL was synthesized through an acid-base reaction between cholinium hydroxide (46 % w/w, aqueous solution) and oleic acid at x_1 (molar ratio) = 0.5 in ethanolic medium under constant agitation and nitrogen atmosphere at room temperature for 24 h. After the reaction, an IL ethanolic-aqueous solution was obtained with IL at 46 %, ethanol at 36 % and water at 18 %.

2.4. Extraction method

Microalgae culture was centrifuged for 10 min at $15314 g$ (Allegra 64 R, Beckman Coulter Inc., USA). After centrifugation, the microalgae pellet was resuspended to a final concentration of $7.04 g\cdot\text{L}^{-1}$. The solid-liquid extraction of β -carotene from *D. salina* wet biomass was performed for 60 min, at 300 rpm using a magnetic stirrer (MS-51 M, Jeio Tech, Korea), under a treatment temperature of 65°C and using the IL cholinium oleate (see section 2.3) as extraction solvent, based on the extraction procedure described by Sousa et al. (2024). After the extraction, the samples were centrifuged at $2817 g$ for 10 min (Hettich Mikro 120, Tuttlingen, Germany) and the supernatant samples containing β -carotene were collected as extracts. For β -carotene quantification, the extracts underwent a liquid-liquid extraction using n-hexane as solvent, following the protocol previously described by Lüdtke et al. (2024). β -carotene was quantified by measuring its absorption spectrum using a UV–VIS spectrophotometer (V-560, Jasco, USA) at 450 nm and a previously obtained calibration curve using n-hexane.

2.5. Nanoemulsions production

NEs were prepared by pre-emulsification followed by high-intensity ultrasound, as described by other authors (Gonçalves et al., 2021; Toledo Hijo, Silva, Meirelles, et al., 2023). For that, a pre-emulsion composed of water as aqueous phase, sunflower oil (SO) as lipid phase, and β -carotene- and IL-rich extract at 2 % (w/w) was prepared at room temperature (25°C) using an Ultra-Turrax (T18, Ika-Werke, Germany). In this study, the effect of the oil concentration in NEs was investigated, using three different concentrations: 10 % (NE₁₀), 20 % (NE₂₀), and 30 % (NE₃₀). First, the β -carotene-IL extract was dissolved

into the aqueous phase and the lipid phase (SO) was added dropwise at 5000 rpm. Subsequently, the Ultra-Turrax condition was established at 10000 rpm for 5 min. Then, the pre-emulsion was subjected to high-intensity ultrasound using a titanium microtip (3.0 mm diameter, 20 kHz, Vibra-cell VCX 500, Sonics & Materials, Newtown, USA) with an amplitude of 40 % for 5 min with pulses (4 s on; 2 s off). To prevent overheating, an ice bath was used during the ultrasonication procedure. Finally, the NEs were transferred to a light-protected tube and stored at 5 °C for 30 days.

2.6. NE characterization and stability

2.6.1. Droplet size, polydispersity index, and zeta potential

The mean droplet size (DS), size distribution (PDI), and the ζ -potential of NEs were measured at 25 °C by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). Prior to the analysis, the samples were diluted at 1:100 with ultrapure water. The oil refractive index and particle absorbance values used in Malvern software were 1.47 and 0.001, respectively. The mean DS was expressed as z-average (nm).

2.6.2. Fourier-transform infrared spectroscopy (FTIR)

The FTIR analysis was conducted using Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR, Bruker, Alpha II, Billerica, USA). The NEs samples were analyzed directly with a spectral resolution of 4 cm^{-1} , over 48 scans in the range of 375–4000 cm^{-1} .

2.6.3. Entrapment efficiency

The extraction and quantification of β -carotene entrapped into the NEs was performed by liquid-liquid extraction and spectrophotometric analysis, respectively, based on the methodology described by Lüdtke et al. (2024). Briefly, 2.5 mL of NEs were transferred to a light-protected tube and vortexed with 5 mL of acetone with BHT (0.01 %) for 30 s. Subsequently, 5 mL of hexane were added and vortexed for 10 s and distilled water was added until the final volume of 25 mL. The solution was centrifuged at 15314 g (Allegra 64 R, Beckman Coulter Inc., USA) at 4 °C for 5 min. The upper hexane phase was collected and analyzed by UV-VIS spectrophotometry (V-560, Jasco, USA) at 450 nm (absorbance peak). A standard curve of β -carotene in the concentration range between 1.25 and 10 mg mL^{-1} was previously prepared, with a determination coefficient of 0.997.

The Entrapment Efficiency (EE) of β -carotene into the NEs was calculated according to Equation (1):

$$\text{EE (\%)} = \frac{\text{Entrapped } \beta\text{-carotene}}{\text{Total } \beta\text{-carotene}} \quad \text{Eq. 1}$$

2.7. In vitro digestion

The behaviour of NEs samples under digestion was evaluated according to the harmonized INFOGEST static *in vitro* GIT protocol (Brodtkorb et al., 2019). This method replicates the electrolytic and enzymatic conditions that simulate the digestive parameters of GIT digestion during the oral, gastric, and intestinal phases. Initially, to perform oral phase, simulated salivary fluid (SSF), composed of KCl (15.1 mmol.L^{-1}), KH_2PO_4 (3.7 mmol.L^{-1}), NaHCO_3 (13.6 mmol.L^{-1}), $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.15 mmol.L^{-1}), $(\text{NH}_4)_2\text{CO}_3$ (0.06 mmol.L^{-1}), and HCl (1.1 mmol.L^{-1}), 25 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 mol.L^{-1}), and ultrapure water were added to 5 mL of sample to achieve a sample:SSF ratio of 1:1 (v/v). Amylase was not used since none of the samples contained starch. Samples were incubated at 37 °C under agitation (50 rpm) using an incubated tube rotator (RotoTherm Plus, H2024-E-UK, Benchmark Scientific, Sayreville, USA) for 2 min. Throughout the digestion process, the temperature and agitation were maintained at 37 °C and 50 rpm, respectively. In the gastric phase, simulated gastric fluid (SGF)

composed of KCl (6.9 mmol.L^{-1}), KH_2PO_4 (0.9 mmol.L^{-1}), NaHCO_3 (25 mmol.L^{-1}), NaCl (47.2 mmol.L^{-1}), $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.12 mmol.L^{-1}), $(\text{NH}_4)_2\text{CO}_3$ (0.5 mmol.L^{-1}), and HCl (15.6 mmol.L^{-1}), 5 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 mol.L^{-1}), and porcine pepsin solution (SigmaAldrich P7012, to achieve a final concentration of 2000 U.mL^{-1} in the final volume) were added. The pH was adjusted to 3.0 using HCl (1 mol.L^{-1}) and ultrapure water was added to achieve a bolus:SGF mixture ratio of 1:1 (v/v). Samples were incubated at 37 °C, under agitation (50 rpm), for 2 h. To simulate intestinal phase, simulated intestinal fluid (SIF) composed of KCl (6.8 mmol.L^{-1}), KH_2PO_4 (0.8 mmol.L^{-1}), NaHCO_3 (85 mmol.L^{-1}), NaCl (38.4 mmol.L^{-1}), $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.33 mmol.L^{-1}), and HCl (8.4 mmol.L^{-1}), 40 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 mol.L^{-1}), bile extract porcine solution (Sigma-Aldrich B8631, to achieve a final concentration of 10 mmol.L^{-1}), and pancreatin from porcine pancreas solution (Sigma-Aldrich P7545, to achieve a final concentration of 100 (TAME) U. mL^{-1}) were added. The pH was adjusted to 7.0 with HCl (1 mol.L^{-1}) or NaOH (2 mol.L^{-1}) and ultrapure water was added to achieve a chyme: SIF mixture ratio of 1:1 (v/v). Samples were incubated at 37 °C, under agitation (50 rpm), for 2 h.

Samples were collected at the end of each digestive phase (i.e., oral, gastric, intestinal, and micellar) to evaluate the morphological changes throughout *in vitro* digestion by DS, PDI, and ζ -potential (as described in section 2.7.1) and fluorescence microscopy (as described in section 2.7.2) analysis. The collected samples were maintained in an ice bath to reduce enzymatic activity until analysis were performed. Enzyme inhibitor Pefabloc® Sc (Sigma-Aldrich), 1 mmol/L (10 $\mu\text{L mL}^{-1}$ of sample), was added to the samples obtained at the end of intestinal phase (digesta) to stop the digestion reactions. Digesta samples were centrifuged at 18,500 $\times g$ at 4 °C for 30 min (Multifuge XR3, Thermo Fischer Scientific, Waltham, USA), and the supernatant was assumed as the micellar phase. Micellar samples were used to assess β -carotene bioaccessibility (as described in section 2.7.4). *In vitro* digestion experiments were performed in triplicate.

2.7.1. Droplet size, polydispersity index, and zeta potential

NEs size and stability throughout *in vitro* digestion were evaluated considering the measurement of DS, PDI, and ζ -potential by DLS, as previously described in section 2.6.1. Before analysis, all samples were diluted at 1:100 in a buffer solution with the same pH of each *in vitro* digestion phase.

2.7.2. Fluorescence microscopy

NEs morphology was observed before and throughout *in vitro* digestion using a fluorescence microscope (Olympus®, BX51, Japan). Nile red dye (0.25 mg mL^{-1} DMSO) was used to stain the samples at 1:10 ratio (dye:sample, v/v). The images were obtained with a 100x oil immersion objective lens (Gonçalves et al., 2021).

2.7.3. Free fatty acids release

The percentage (%) and the absolute amount (μmol fatty acids. mL^{-1} of sample) of free fatty acids (FFA) released from the lipid digestion of NEs was measured using an automatic pH-stat method. NEs samples were firstly subjected to oral and gastric phase of static *in vitro* digestion protocol, according to the methodology described in section 2.7. At the end of the gastric phase, the sample was transferred to a heated jacketed reactor at 37 °C under agitation, SIF and bile solution were added (Section 2.7), and the pH was adjusted to 7.0 with HCl (1 mol.L^{-1}) or NaOH (1 mol.L^{-1}). Pancreatin solution was added to the sample and the pH was maintained and monitored at 7.0 by the continuously addition of NaOH (0.05 mol.L^{-1}) using an auto-titration unit (Titrand 902, Metrohm, Herisau, Switzerland). The pH was controlled and monitored during 2 h. Blank assays, conducted without pancreatin, were performed to determine the volume of NaOH needed to achieve the pH of 7.0. The percentage of FFA released was calculated through Equation (2) (Li & McClements, 2010) and the absolute amount of FFA released through Equation (3) (Pinsirodom & Parkin, 2001):

$$FFA (\%) = \frac{(V_{NaOH \text{ sample}} - V_{NaOH \text{ blank}}) \times m_{NaOH} \times M_{Elipid}}{W_{lipid} \times 2} \times 100 \quad \text{Eq. 2}$$

where $V_{NaOH \text{ sample}}$ and $V_{NaOH \text{ blank}}$ are the NaOH titrated volume in the sample and blank assays, respectively; m_{NaOH} is the NaOH titrant molar concentration, in this case 0.05 mol.L^{-1} ; M_{Elipid} is the equivalent molecular weight of lipid phase; W_{lipid} is the mass of lipid (g) presented in NEs structures.

$$FFA (\mu\text{mol fatty acids per mL of sample}) = \frac{(V_{NaOH \text{ sample}} - V_{NaOH \text{ blank}}) \times C \times 1000}{V_{\text{sample}}} \quad \text{Eq. 3}$$

where $V_{NaOH \text{ sample}}$ and $V_{NaOH \text{ blank}}$ are the NaOH titrated volume in the sample and blank assays; C is the molar concentration of the NaOH titrant; $V_{NaOH \text{ sample}}$ is the final volume of the sample after intestinal digestion.

2.7.4. β -carotene bioaccessibility

β -carotene bioaccessibility was assumed as the β -carotene fraction present in the micellar phase and evaluated as described by Lüdtke et al. (2024), with some modifications. Micellar phase samples (2.5 mL) were transferred to a light-protected tube and evaluated according to the methodology described in section 2.6.3. β -carotene bioaccessibility was calculated according to the following equation (Teixé-Roig et al., 2023):

$$\text{Bioaccessibility } (\%) = \frac{C_{\text{micelle}}}{C_{\text{initial}}} \quad \text{Eq. 4}$$

where C_{micelle} is the β -carotene concentration (mg/mL) in the micellar fraction and C_{initial} is the initial β -carotene concentration (mg.mL^{-1}) in the nanoemulsion.

2.8. Statistical analysis

Statistical analysis was carried out using OriginPro 2018® Statistic Software (Origin Lab Corporation, Northampton, USA). One-way and two-way analyses of variance (ANOVA) with Tukey *post-hoc* test were applied to analyse all data. Data is represented as mean $\pm$ standard error of the mean (SD). Statistical significance was set for $p < 0.05$ and it is indicated by different superscript letters according to their significance.

3. Results and discussion

3.1. Enrichment of ionic liquid cholinium oleate with β -carotene from *D. salina*

The present study promoted the sequential application of a naturally-derived IL obtained from Ch and a FA ([Ch][Ole]) as green solvent and as an emulsifier to encapsulate β -carotene in NEs, which were subsequently evaluated in terms of their behavior during an *in vitro* digestion.

Firstly, after the growth stage, the *D. salina* biomass was subjected to a solid-liquid extraction for 60 min, at 300 rpm, and under 65°C (previously described in section 2.4). This extraction process allowed to recover $75.1 \pm 4.66\%$ of the β -carotene present in the *D. salina* biomass. Other methods for extracting β -carotene from *D. salina* have shown significantly lower efficiencies. For instance, using super-critical CO_2 with an EtOH fraction resulted in the extraction of 25 % of the available β -carotene, while using a centrifugal partition with ethyl oleate and 5 % dichloromethane as a solvent yielded a 65 % extraction (Ludwig et al., 2021; Marchal et al., 2013). It is worth noting that the approach proposed in the current study promoted a higher recovery of β -carotene by

using a naturally-derived IL, which is concomitantly a more sustainable and cost-efficient strategy. In this regard, the IL used during the current work contained 0.74 mg mL^{-1} of β -carotene.

3.2. Nanoemulsions characterization and stability

3.2.1. Particle size, polydispersity index, and ζ -potential

The same IL already present in the β -carotene extracts obtained from

D. salina biomass (section 3.1), was sequentially used as an emulsifier to produce different NEs. These NEs (with varying oil concentrations) were evaluated for 30 days regarding their kinetic stability, droplet size, PDI, and ζ -potential (Fig. 1). All NEs had a homogeneous (one phase) aspect, did not present phase separation and were kinetically stable for at least 30 days.

According to the results, all three different NEs tested in the present study did not present statistically significant differences ($p > 0.05$) in DS between the first and the last day of storage (30 days), being an indication of their stability during the storage time. On the other hand, there were statistically significant differences ($p < 0.05$) between NE₁₀ and NE₃₀ in all the samples taken at different times. Indeed, there was a tendency for the NEs' DS to increase as the oil content increases, which can be explained by the rise in the oil/water ratio. This leads to the formation of oil droplets with increased size (due to rationing of the IL), consequently increasing the contact surface between the dispersed oil droplets and the aqueous phase (Onaizi, 2022; R. Zhang et al., 2020). This outcome is in agreement with the literature as it has been widely described that as the oil/water ratio increases, the droplet size also increases (Fernandes et al., 2024; Onaizi, 2022; Teixé-Roig et al., 2023; R. Zhang et al., 2020). Fernandes et al. (2024) evaluated the effect of the oil/water ratio on the DS of emulsion and excipient emulsion using lecithin as an emulsifier. The authors reported lower DS for the lowest oil/water ratio (5 %) comparing to the samples with higher oil/water ratios (10 % and 15 %). In addition, Teixé-Roig et al. (2023) showed that the increase of oil content, from 10 to 30 %, in the production of β -carotene NEs results in greater DS of NEs that used whey protein isolate as an emulsifier, whereas no increase was observed for soybean lecithin emulsions.

Similar to the DS, considering each NEs individually, no differences were observed in PDI values throughout time ($p < 0.05$). Initially, after 24 h of storage, the PDI was similar between each NE; however, after 30 days, a difference was highlighted between NE₁₀ and NE₃₀ ($p < 0.05$). Based on the results of the present study, the PDI values, which are indicative of the homogeneity of the system (Lakshmi & Kumar, 2010), either showed no differences ($p < 0.05$) between the different NEs, or very slight differences.

According to the literature, it is possible to achieve good stability for an electrostatically stabilized nanosuspension with a minimum ζ -potential of $|30 \text{ mV}|$ (Gonçalves et al., 2021). Based on these considerations, the ζ -potential of the NEs produced in the present work ($>|50 \text{ mV}|$) supports the observed high kinetic stability, preventing droplet aggregation, coalescence, and phase separation. The ζ -potential observed for the cholinium oleate-based NEs is due to the arrangement of the IL, which forms micelles with high negative charges causing, in turn, electrostatic repulsion that favours higher stability of the emulsions (Hijo et al., 2024). In the present study, as the oil content of the emulsion increased, the ζ -potential became less negative ($p < 0.05$). Over time, there was a tendency for the ζ -potential values of NE₁₀ and

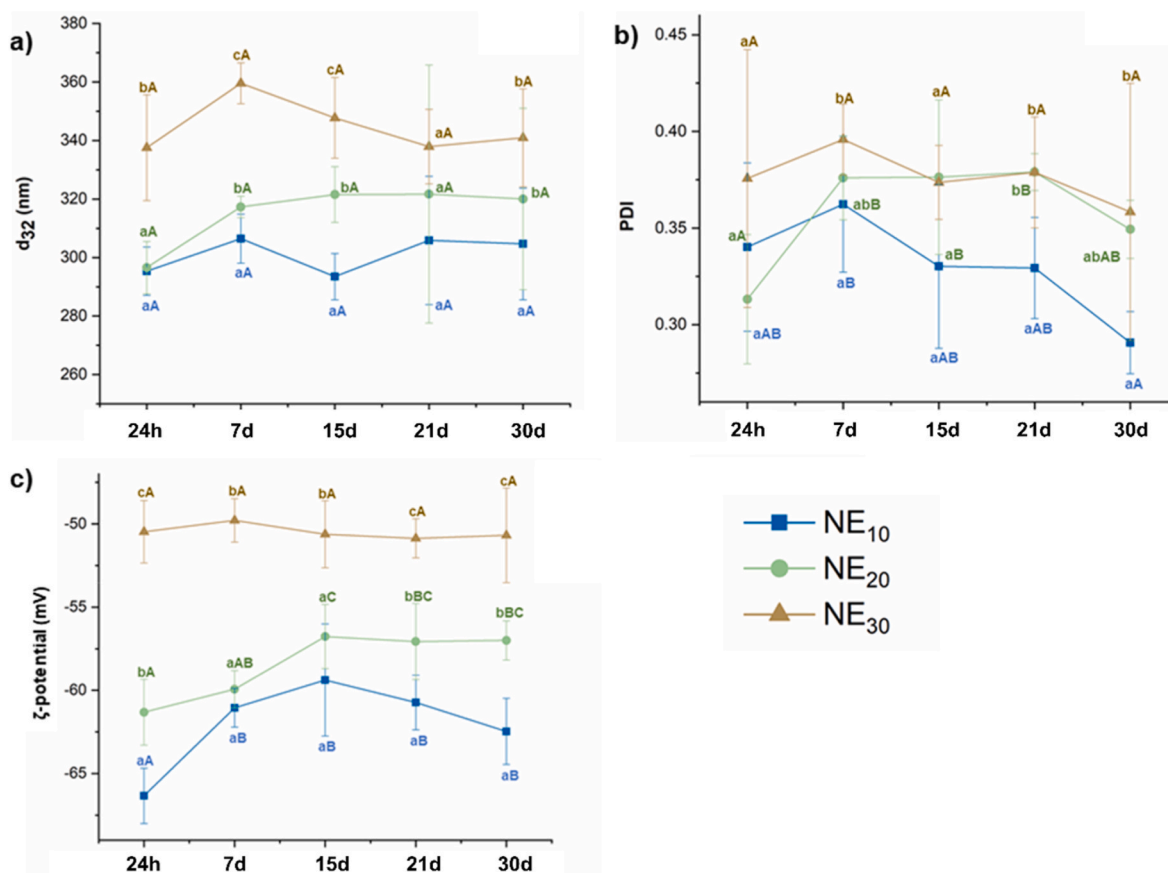


Fig. 1. Droplet size (nm) (a), size distribution (PDI) (b), and ζ -potential (mV) (c) of β -carotene loaded NEs. Error bars represent the standard deviation of $n = 3$ replicates. ^{A–C} Different capital letters indicate significant difference of the same NEs during the storage time ($p < 0.05$). ^{a–c} Different lower-case letters indicate significant difference between NEs in the same day of storage ($p < 0.05$).

NE₂₀ to become less negative (changing from -66.33 to -62.47 mV in NE₁₀ and from -61.32 to -57.00 mV in NE₂₀), indicating a decrease in its absolute value. In another work, [Teixé-Roig et al. \(2023\)](#) also incorporated β -carotene from *D. salina* into NEs. The NEs produced using soybean lecithin and whey protein isolate as emulsifiers presented a ζ -potential of approximately -50 mV and -36 mV, respectively, and no statistically significant differences were found by increasing the oil content from 10 % to 30 % in NEs stabilized with both emulsifiers, in

contrast to the behavior observed in the present work. It is interesting to note that the use of the IL as an emulsifier resulted in more stable NEs (ζ -potential up to ca. -70 mV) and these findings indicate that IL provided a higher electrostatic stabilization than other natural emulsifiers, such as soybean lecithin and whey protein isolate.

The results obtained in this study suggest that the use of the IL cholinium oleate as an emulsifier, already present in the β -carotene extract and previously used as an extraction solvent, was effective in

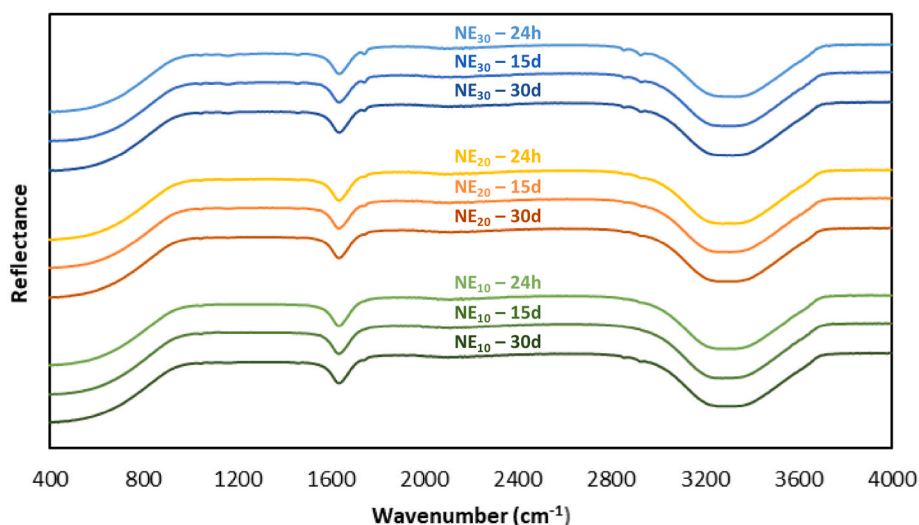


Fig. 2. FTIR spectra of β -carotene loaded NEs during 30 days of storage.

forming and stabilizing NEs, regardless of the oil content within the 10–30 % range.

3.2.2. Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra (Fig. 2) of the IL-based NEs were obtained to identify the interactions that occurred between the constituents of the systems and to assess potential changes over the storage time. Apparently, FTIR spectra were not affected by the storage time, as no changes were observed over time in the three different NEs produced. It was also important to note that the changes in the oil content (10–30 %) of the different NEs did not promote changes in the FTIR spectra. The peaks observed in the region of 1635–1750 cm^{-1} correspond to the stretching vibration of the carbonyl group ($\text{C}=\text{O}$), while the peaks in the region of 2865 and 2950 cm^{-1} correspond to aliphatic CH_2 symmetric and asymmetric stretching, which corresponds to a typical band of a lipidic backbone (Caldas et al., 2021; X. Shu et al., 2023). Lipid mixtures typically present these peaks. Finally, the peaks in the region around 3200–3600 cm^{-1} correspond to an O–H stretching, which are characteristics of systems with water content (X. Shu et al., 2023; Vardanega, Lüdtke, Loureiro, Gonçalves, et al., 2024). These were the characteristic peaks reported in a previous work (Vardanega, Lüdtke, Loureiro, Toledo Hijo, et al., 2024) where the IL cholinium oleate was used.

3.2.3. Entrapment efficiency

The EE of β -carotene in the NEs produced was above 80 % (Fig. 3) for NE₁₀, NE₂₀, and NE₃₀. However, during storage, a decrease in the retention rate was observed for NE₁₀ and NE₂₀, which may be related to the fact that β -carotene is a hydrophobic carotenoid and a higher oil content (more hydrophobic) may provide more suitable conditions for its solubilization, storage, and stability inside the NEs.

Based on the results presented in Fig. 3, it can be concluded that the IL cholinium oleate is an efficient encapsulation agent due to its high β -carotene retention capacity. Previous studies had already demonstrated the capacity of the IL cholinium oleate as a good encapsulating agent of bioactive compounds (Toledo Hijo, Silva, Meirelles, et al., 2023; Vardanega, Lüdtke, Loureiro, Toledo Hijo, et al., 2024). Indeed, Toledo

Hijo, Silva, Cristianini, and Meirelles (2023) have reported an EE of 77, 55 % using the IL cholinium oleate as an encapsulation agent for bioactive compounds naturally present in rosemary essential oil. Additionally, the good results of β -carotene EE and retention rate of β -carotene obtained in the present study are in line with β -carotene EE obtained using traditional emulsifiers, as described by López-Monterrubio et al. (2021). These authors used whey protein hydrolysate-pectin soluble complex as a β -carotene emulsifier and obtained an EE of 91.99 %.

3.3. In vitro digestion

The impact of lipid content in NEs formulations on their behavior during GIT passage was assessed by evaluating changes in their properties (DS, PDI, and ζ -potential) and morphology (via fluorescence microscopy). Additionally, the influence of lipid content on the release of FFA and β -carotene bioaccessibility was evaluated.

3.3.1. Droplet size, polydispersity index, and ζ -potential

Fig. 4 shows the DS (a), PDI (b), and ζ -potential (c) of the initial samples and samples collected from different phases of the *in vitro* digestion (gastric, intestinal, and micellar phases) for NE₁₀, NE₂₀, and NE₃₀.

Initially, as presented in Section 3.2.1, NE₃₀ displayed a significantly higher ($p < 0.05$) DS compared to NE₂₀ and NE₁₀. Regardless of lipid content, a drastic increase in DS was observed in the gastric phase (Fig. 4a and S1). This increase can be attributed to changes in the interfacial surface of the droplets due to the neutralization of electrostatic forces, along with aggregation, coalescence, or flocculation — processes likely influenced by the action of digestive enzymes, as well as alterations in pH and ionic strength (Gonçalves et al., 2021; Pinheiro et al., 2013). In the intestinal phase, a noteworthy decrease ($p < 0.05$) was observed for all NEs samples in the DS, as shown in Fig. 4a and supported by fluorescence microscopy images (Fig. S1). This reduction may result from the conversion of triacylglycerols within the oil droplets into free fatty acids and monoacylglycerols by lipase action (Y. Tan &

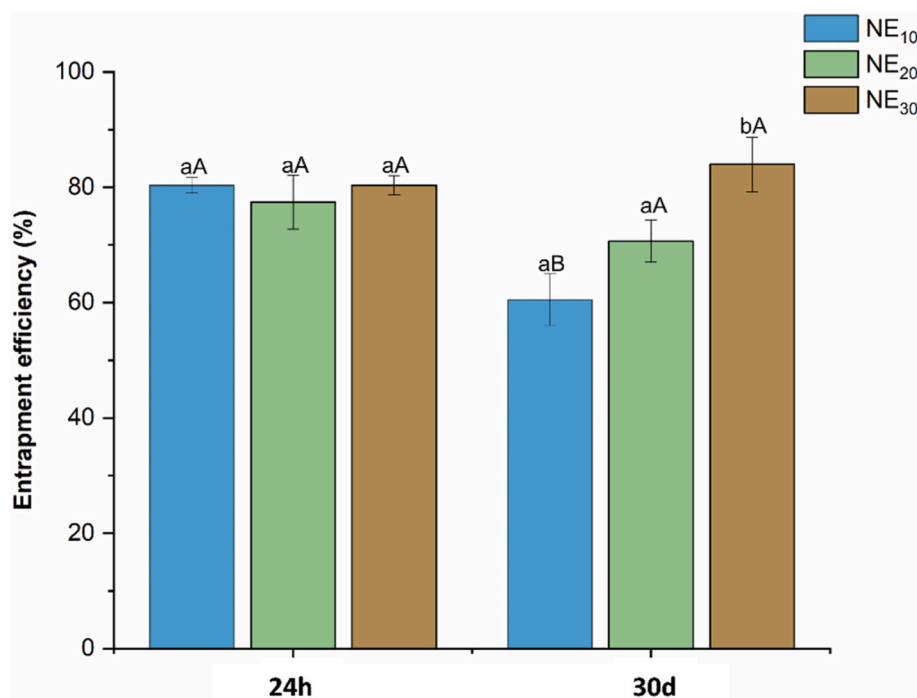


Fig. 3. Entrapment efficiency (%) of β -carotene loaded NEs. Error bars represent the standard deviation of $n = 3$ replicates. ^{A–B} Different capital letters indicate significant difference of the same NEs during the storage time ($p < 0.05$). ^{a–b} Different lower-case letters indicate significant difference between NEs in the same day of storage ($p < 0.05$).

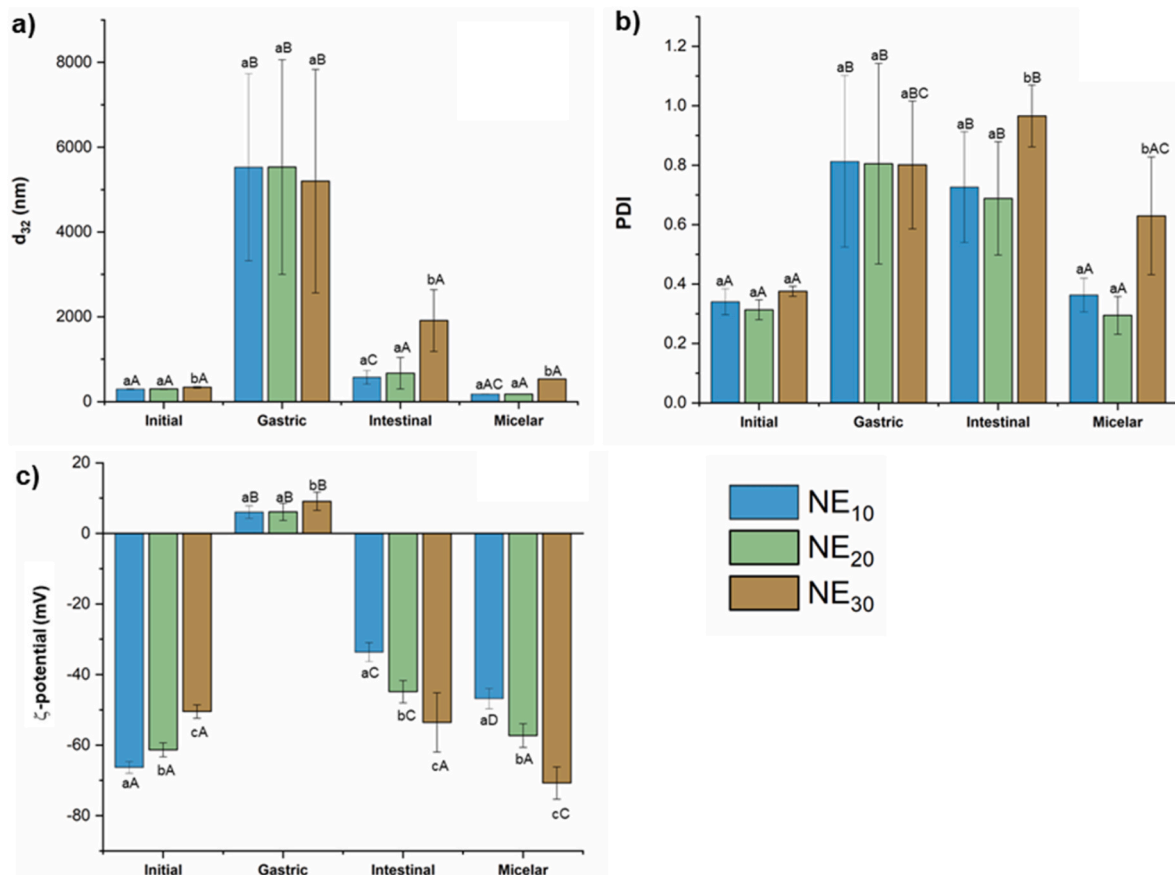


Fig. 4. Droplet size (nm) (a), size distribution (PDI) (b), and ζ -potential (mV) (c) of β -carotene loaded NEs samples collected at different stages of *in vitro* digestion. Error bars represent the standard deviation of $n = 3$ replicates. ^{A–C} Different capital letters indicate significant difference of the same NEs during the storage time ($p < 0.05$). ^{a–c} Different lower-case letters indicate significant difference between NEs in the same day of storage ($p < 0.05$).

McClements, 2021). Among the NE samples, NE₃₀ exhibited the largest ($p < 0.05$) DS in both the intestinal and micellar phases. In the intestinal phase, the largest DS of NE₃₀ can be attributed to its higher lipid content, which promoted the formation and coalescence of a greater number of mixed micelles. In the micellar phase, the highest DS of NE₃₀ may be related to the greater entrapment of β -carotene within the micelles formed during the digestion process, as confirmed by the bio-accessibility results (section 3.3.3). The β -carotene present in *D. salina* has a complex chemical structure that, when incorporated into mixed micelles during lipolysis, may have led to larger micelles. Similar findings were previously reported (Fernandes et al., 2024; Gonçalves et al., 2021; Teixeira-Roig et al., 2023; Vardanega, Lüdtke, Loureiro, Toledo Hijo, et al., 2024; Yao et al., 2021), where an expressive increase in DS was observed during the gastric phase, followed by a decrease in the intestinal phase.

The destabilization observed in the NEs droplets is confirmed by the significant increase ($p < 0.05$) in the PDI during the gastric phase (Fig. 4b). This high PDI value persists in the intestinal and micellar phases due to the presence of mixed micelles resulting from lipid digestion. Similar to DS, NE₃₀ sample presented the largest ($p < 0.05$) PDI in both the intestinal and micellar phases, probably due to the higher concentration of mixed micelles in this sample. Although all NEs have initially presented a highly negative electrical charge (ζ -potential $> |50$ mV|), under gastric conditions the electrical charge of all NEs became positive (Fig. 4c). The acidic conditions, the presence of ionic species, and the high ionic strength may have induced changes in the interfacial surface of NEs during the gastric phase (Gonçalves et al., 2021). In the intestinal phase, the ζ -potential of all NEs samples became negative again (Fig. 4c), and was also favoured by the adsorption of

lipase, bile salts, and phospholipids onto the lipid droplets surfaces and the increase in FFA concentration resulting from lipolysis (G. Shu et al., 2018; Yao et al., 2021). Additionally, the ζ -potential of NE₃₀, lower than that of NE₁₀ and NE₂₀ in the initial samples, became higher than the other samples in the intestinal phase. This can be explained by the increased concentration of anionic FFA which enhanced the negative charge on the droplet surface (Gonçalves et al., 2021; Mutsokoti et al., 2017).

3.3.2. Free fatty acids release

The amount of FFA increased rapidly within the first 10 min (data not shown) after entering the small intestine digestion phase. As the digestion time was prolonged, the release of FFA entered a phase of stabilization. Fig. 5a presents the percentage of FFA released from NE₁₀, NE₂₀, and NE₃₀ as a result of lipolysis during *in vitro* digestion. Some researchers associated the instability of NEs during the gastric phase to the release of FFA (Gonçalves et al., 2021; Lüdtke et al., 2024; Y. Tan et al., 2020). However, since the NEs exhibited similar ($p < 0.05$) DS during the gastric phase (section 3.3.1), the FFA release in our study was likely influenced by the lipid content of the NEs.

The increase in lipid content in the NEs formulation led to a decrease in the percentage of FFA released during *in vitro* digestion. These results are consistent with previous studies that reported an inverse relationship between initial lipid content in emulsion-based delivery systems and the percentage of FFA released at the end of lipolysis reaction (Teixeira-Roig et al., 2023; Yao et al., 2021). The reduction in the percentage of FFA release with increasing lipid content can be attributed to the inherent limitations of the static *in vitro* digestive system, such as the use of fixed amounts of lipase and bile salts (Y. Tan et al., 2020). The use

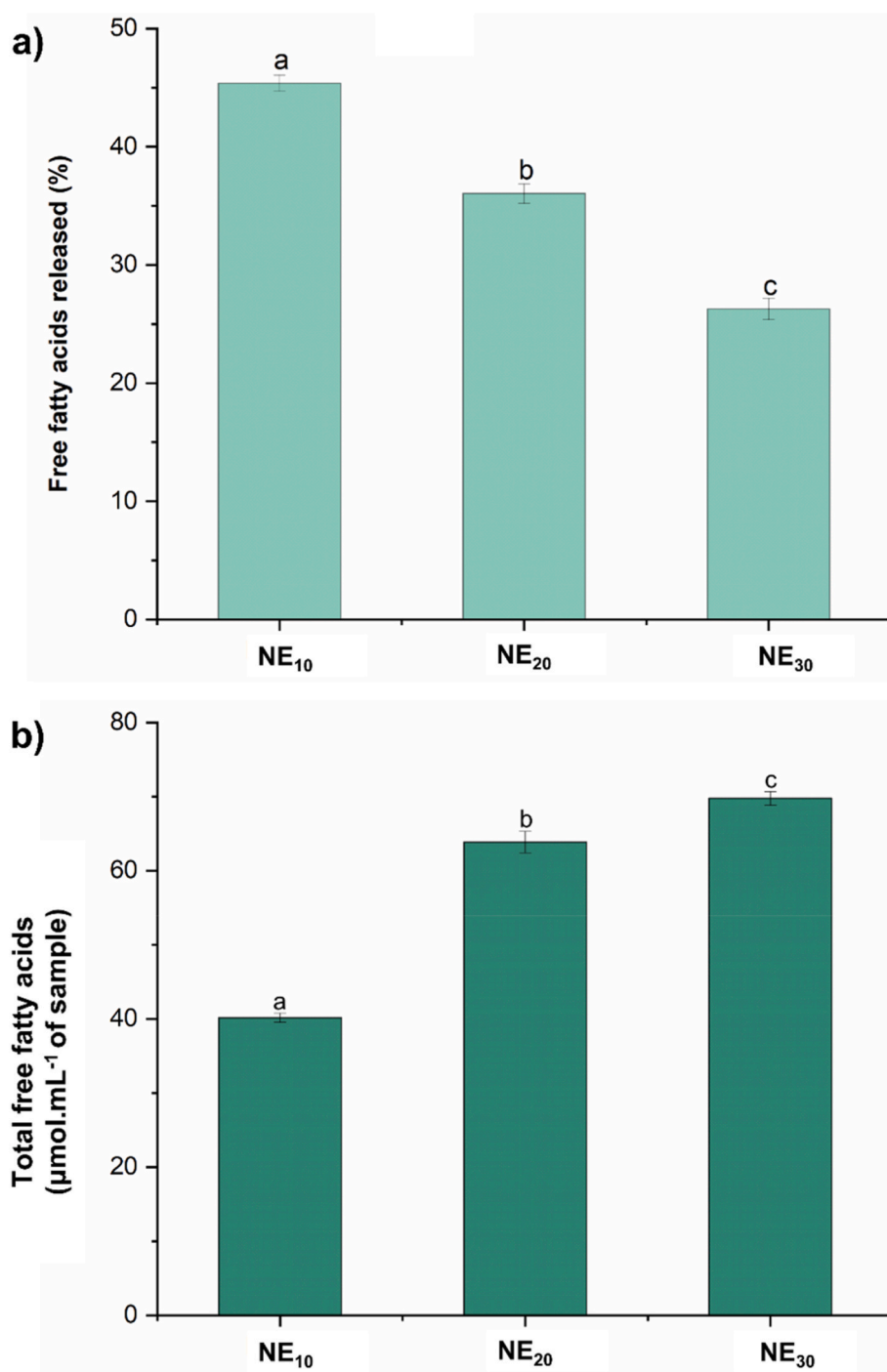


Fig. 5. Percentage (%) (a) and absolute amount (μmol fatty acids, mL^{-1} of sample) (b) of free fatty acids (FFA) released after *in vitro* digestion from β -carotene loaded NEs samples. Error bars represent the standard deviation of $n = 3$ replicates. ^{a-c}Different lower-case letters indicate significant difference between NEs ($p < 0.05$).

of a fixed amount of intestinal phase components, regardless of the lipid content of the sample, may not be sufficient to ensure the complete digestion of the triacylglycerols present (Yao et al., 2021). As lipid concentration increased, the ratio of lipase-to-fat and calcium-to-FFA decreased, leading to a reduced rate of lipolysis and greater accumulation of FFA at the droplet interface (Li et al., 2011). Additionally, higher lipid content may increase the medium's viscosity, which can hinder or reduce lipase activity (Teixé-Roig et al., 2023).

Moreover, a low percentage of FFA release does not necessarily indicate a low absolute amount of FFA released, as observed in Fig. 5b. In this case, it was found that the increase in lipid content in the NE

formulation resulted in a higher ($p < 0.05$) absolute amount of FFA released during the digestion process. These findings have also been reported in similar studies. For instance, Tan et al. (2020) reported that the absolute amount of FFA produced during lipid digestion increased with higher oil concentrations, as more triacylglycerol molecules were available for conversion into fatty acids. Similarly, Yao et al. (2021) observed that while the percentage of FFA released decreased with increasing total lipid content, the absolute amount of FFA released (mM) increased. In this regard, and considering other results from the present study, such as bioaccessibility, DS, and ζ -potential, the reduction in the percentage of FFA release not to imply a decrease in the production of

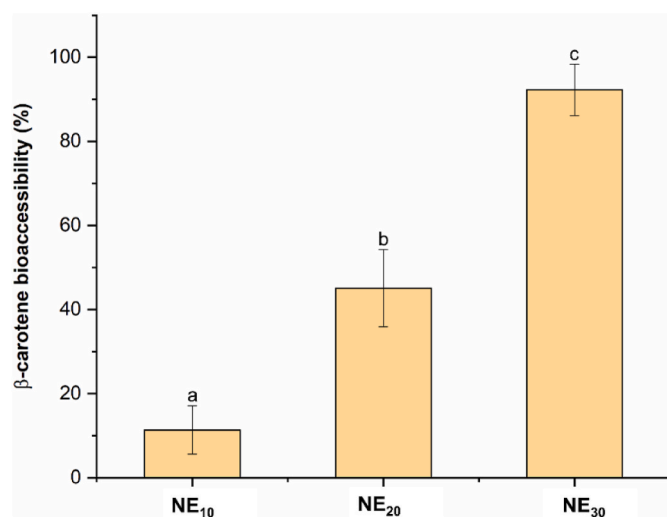


Fig. 6. Bioaccessibility of β -carotene loaded into NEs samples. Error bars represent the standard deviation of $n = 3$ replicates. ^{a-c}Different lower-case letters indicate significant difference between NEs ($p < 0.05$).

mixed micelles available to solubilize the β -carotene from *D. salina*, as supported by the results of the absolute amount of FFA released (Fig. 5b).

3.3.3. β -carotene bioaccessibility

At the end of the *in vitro* digestion process, the bioaccessibility of β -carotene entrapped in NEs was assessed. Fig. 6 illustrates the effect of lipid content in NEs formulation on β -carotene bioaccessibility. β -carotene bioaccessibility increased with higher lipid content in the NEs formulation. Consequently, NE₃₀ exhibited the highest bioaccessibility (92.22 ± 6.14 %), followed by NE₂₀ (45.11 ± 9.16 %) and NE₁₀ (11.36 ± 5.74 %).

Several factors can contribute to the increased bioaccessibility of lipophilic β -carotene, as the case of NE₃₀. One key factor is the enhanced EE, which correlates positively with higher β -carotene bioaccessibility. Free and non-entrapped β -carotene cannot efficiently transfer to the solubilized micelle phase for absorption (Tan et al., 2014). In our study, as detailed in section 3.2.3, all NEs samples demonstrated EE values close to 80 % 24 h after production (Fig. 3). However, after 30 days of storage, the NE₃₀ sample exhibited the highest EE, indicating its superior ability to solubilize and retain β -carotene. Also, after digestion, the percentage triacylglycerol was higher for NE₃₀, indicating that it was still present in the nanosystem and available to solubilize β -carotene (Fig. 5). Moreover, the role of the IL as a highly efficient encapsulating agent and emulsifier was fundamental to achieve these promising results, considering that the encapsulating systems and main physical parameters were maintained relatively stable during the digestion, favouring a higher retention and stability of β -carotene. The higher retention contributed to greater stability of β -carotene during its passage through the GIT, leading to enhanced solubilization into mixed micelles and, consequently, higher bioaccessibility. Additionally, the presence of long-chain monounsaturated fatty acids, such as oleic acid, has shown to enhance β -carotene bioaccessibility (Abreu-Martins et al., 2020). Therefore, the increased sunflower oil content in the NE₃₀ formulation increased the absolute amount of FFA released (Fig. 5b) and may have resulted in the release of higher concentration of oleic acid during lipolysis, further facilitating the solubilization of β -carotene in mixed micelles.

Similar trends have been reported in previous studies evaluating the β -carotene bioaccessibility in different lipid concentrations. Tan et al. (2020), for example, found that β -carotene bioaccessibility increased from 60.5 ± 5 % to 93.2 ± 4 % as the corn oil concentration rose from

2.5 % to 10 % in model emulsions. Yao et al. (2021) reported that both *in vitro* bioaccessibility and *in vivo* bioavailability of β -carotene increased with higher lipid content, following the order: 1.0 g > 0.6 g > 0.2 g > 0 g. On the other hand, Teixe-roig et al. (2023) evaluated *D. salina*-based NEs produced with soybean lecithin and whey protein as emulsifiers and observed a decrease in β -carotene bioaccessibility as lipid concentration increased (from 10 to 30 %). The authors attributed the lower bioaccessibility to the higher amount of emulsifier present in the 30 % oil NE, which may have increased β -carotene sedimentation thereby reducing its bioaccessibility.

High β -carotene bioaccessibility values in emulsion-based delivery systems have also been reported. Zarif et al. (2023) achieved a bioaccessibility of 94.8 ± 5 % of β -carotene encapsulated in milk phospholipid and buttermilk powder composite nanosystems. On the other hand, Zhang et al. (2023) reported a bioaccessibility of approximately 80 % for β -carotene in emulsions stabilized with whey protein isolate-Mesona chinensis polysaccharide. The high bioaccessibility value found in the present study highlights the great potential of *D. salina* microalgae as a source of carotenoids, as well as the potential of ionic liquids in extracting β -carotene and stabilizing NEs with different lipid contents.

Although some ILs may raise toxicity concerns, the one used in this study was synthesized from choline and oleic acid — two biocompatible and naturally occurring molecules — and has previously been reported to exhibit low toxicity *in vitro*. Specifically, cell viability assays using Caco-2 cells exposed to ILs and IL-based NEs showed values above 80 % (Vardanega, Lüdtke, Loureiro, Toledo Hijo, et al., 2024), while no signs of toxicity were observed in Wistar rats treated with fatty acid-based ILs, as reported by Toledo Hijo et al. (2020). However, any new food additive candidate, including compounds in the ionized form, such as salts/ILs, must be thoroughly evaluated by further toxicological/safety and efficacy studies to meet all the general requirements of food legislation. In addition, it should be noted that the technological stability of IL-based nanoemulsions was not assessed in terms of light and heat resistance or pH and salt concentration fluctuations. These factors are particularly relevant for real food processing and storage conditions, where carotenoids are prone to degradation. Therefore, future studies should address the impact of environmental and technological stresses on the stability and functionality of IL-based formulations, in order to better evaluate their practical applicability in food systems.

4. Conclusions

The use of ionic liquid-based nanoemulsions proved to be an effective strategy to enhance the bioaccessibility of β -carotene obtained from *D. salina*. The results obtained in this study showed that the lipid composition influenced the physical stability, the EE, and the behavior of *D. salina* β -carotene-loaded NEs under *in vitro* static digestion. In general, by increasing the lipid content in NEs formulation, higher β -carotene entrapment, stability, and delivery in GIT were achieved. Thus, the use of 30 % of lipid content to produce *D. salina* β -carotene-loaded NEs showed to be more efficient to entrap, solubilize and deliver this bioactive compound. These findings suggest that encapsulating β -carotene in NEs with 30 % oil content not only protects the compound but also enhances its release in the gastrointestinal tract, making it more available for absorption in the human body. Therefore, this study offers an innovative approach for the encapsulation and delivery of β -carotene in NE-based systems with improved stability and bioaccessibility throughout gastrointestinal digestion, contributing to the development of functional foods and biofortified ingredients aimed at improving human health. Nevertheless, it should be emphasized that the applicability of IL-based systems in food formulations is still dependent on further toxicological studies and subsequent regulatory approval.

CRediT authorship contribution statement

Vitor Sousa: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fernanda L. Lüttke:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ariel A.C. Toledo Hijo:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Luís Loureiro:** Writing – review & editing, Investigation, Formal analysis. **Oscar Dias:** Writing – review & editing, Supervision. **António A. Vicente:** Writing – review & editing, Supervision, Resources. **Ana C. Pinheiro:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Pedro Geada:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2025.112078>.

Data availability

Data will be made available on request.

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