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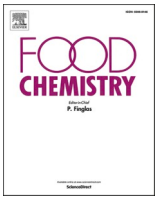

Recovery and encapsulation of *Dunaliella salina* β-carotene through a novel sustainable approach: Sequential application of an ionic liquid as naturally-derived solvent and emulsifier

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ABSTRACT

Dunaliella salina is a promising source of β-carotene, widely employed in the food industry. This study aimed to evaluate the sequential application of the Ionic Liquid (IL) cholinium oleate as an extraction solvent for *D. salina* β-carotene recovery and, sequentially, as emulsifier for emulsion-based products obtained therefrom. The IL was evaluated regarding its ability to permeabilize the cells and recover β-carotene at different temperatures (25–65 °C) and IL concentrations (0–46%). The use of the IL as solvent greatly improved β-carotene recovery (>84%). The IL already present in the obtained extracts loaded with recovered β-carotene was sequentially used as emulsifier in the production of nanoemulsions (NE). NE presented a β-carotene entrapment efficiency of 100% and were kinetically stable for 30 days and presented droplet size, size distribution, and ζ-potential of 220 nm, 0.21, and -67 mV, respectively. These results indicate that using IL sequential as solvent and emulsifier has potential applications in the food industry.

1. Introduction

Nowadays, there is an increasing trend for products obtained from natural sources, being carotenoids one of those examples. Carotenoids are bioactive compounds used to produce functional foods and can be obtained from microalgae biomass (Hamed et al., 2023; Sousa, Pereira, Vicente, Dias, & Geada, 2023). Some microalgae species, such as *Dunaliella salina*, are already well established as a source of β-carotene at the commercial scale (Monte et al., 2020; Sousa et al., 2023). β-carotene is currently widely used in the food industry as a colorant or as pro-vitamin A, and microalgal β-carotene has some advantages over the traditional chemically synthesized commercial sources. Synthetic β-carotene contains only *all-trans* isomers, whereas 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). Despite all the advantages of microalgal β-carotene over synthetic β-carotene, it is necessary to make it accessible for application in the food and pharmaceutical industries as it is inside of microalgae cells (Sousa et al., 2023). To overcome this, the utilization of Ionic Liquids (ILs) as a green extraction solvent has been considered (Desai, Streefland, Wijffels, & Eppink, 2016; Santos et al., 2018).

ILs are salts that melt below 100 °C (Pan et al., 2016; Praveenkumar, Lee, Lee, & Oh, 2015; Rogers & Seddon, 2003). They have been regarded as emergent solvents and some of them have an extraordinary capacity to dissolve biopolymers present in a wide range of biomass matrices, thus allowing easier access to the target compounds (Sousa et al., 2023). ILs have also presented the ability to efficiently extract a wide range of compounds from microalgae biomass, such as lipids (Pan, Muppaneni, et al., 2016; Y. Zhang et al., 2018), pigments (Chang, Show, Lan, Tsai, & Huang, 2018; Desai et al., 2016; Praveenkumar et al., 2015), polysaccharides(Santos et al., 2018), and proteins (Lee, Show, Ling, & Chang, 2017; Santos et al., 2018). In general, imidazolium-, phosphonium-, and pyridinium-based ILs are the most popular choice, despite the discussion about their toxicity and environmental persistence (Petkovic, Seddon, Rebelo, & Pereira, 2011). However, the divergence in literature on possible toxic effects of such traditional ILs limits their application in certain areas, namely food, cosmetics or pharmaceutical

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industries, being those mainly utilized for chemical and petrochemical applications (Toledo Hijo et al., 2022; Toledo Hijo, Maximo, Costa, Batista, & Meirelles, 2016). In order to overcome this challenge, a new concept of bio-based IL emerged as a potential solution for food applications.

The new generation of ILs are derived from natural and bioactive compounds that can be used as anion and/or cation precursors for IL synthesis, which are present in natural food sources such as vegetable or animal products (Farias et al., 2023; Toledo Hijo et al., 2016). The precursors of these novel ILs can include amino acids, choline, sugars, carboxylic acids, among other compounds (Toledo Hijo et al., 2016). In fact, this might be an interesting approach, considering that the food industry could offer healthier foods and food supplements containing essential nutrients, such as omega fatty acids (FAs) and vitamins (e.g., choline), which are required to reach a good nutritional balance. FAs are bioactive compounds widely applied as additives in the food industry in their free or salt form. In the group of FAs are included compounds such as oleic, linoleic, and linolenic acids, essential nutrients that are related to health benefits. These FAs can be obtained as by-products during the refining of vegetable oils, which presents a huge advantage in the circular economy context (Lavelli, 2021; Rodrigues, Peixoto, & Meirelles, 2007). Choline is a food additive belonging to the B-complex vitamins that can be naturally found in several foods (e.g., eggs, peanuts) and is considered an essential nutrient in the manufacture of food products (Spaggiari et al., 2020; Zeisel & Da Costa, 2009). In addition to the previously mentioned advantages associated with this new generation of IL, the emulsifier and encapsulation ability of FA-based ILs has been recently reported in the literature (Toledo Hijo et al., 2022; Toledo Hijo, Silva, Cristianini, & Meirelles, 2023; Toledo Hijo, Silva, Meirelles, Cunha, & Meirelles, 2023). Moreover, the possibility of customizing their emulsifying and functional properties, as well as to improve process sustainability, through the strategic selection of the precursors represents a great advantage over the traditional emulsifiers (Toledo Hijo et al., 2022; Toledo Hijo, Silva, Cristianini, & Meirelles, 2023; Toledo Hijo, Silva, Meirelles, et al., 2023). On the other hand, the incorporation of carotenoids, such as β-carotene, into emulsions is highly beneficial since these delivery systems contribute to avoid their chemical degradation through the formation of a protective barrier that isolates them from the external environment, contributing to increase the stability, handling, storage, and efficacy of such pigments (Hamed et al., 2023). However, the sequential application of ILs as solvent and emulsifier is still unexplored.

In this context, the present study evaluated the sequential application of a bio-based IL derived from oleic acid and choline as emerging extraction solvent and subsequently as emulsifier to produce emulsions that hold potential to be considered food-grade. To the best of our knowledge, this is the first study to evaluate such sequential application of ILs. Firstly, the effect of different process temperatures and IL concentrations on the extraction of β-carotene was assessed. Envisioning a more sustainable and cost-efficient strategy, the IL used as solvent in the β-carotene extraction and already present in the β-carotene-rich extract was sequentially used as emulsifier for its encapsulation through emulsification. The β-carotene entrapment efficiency was determined, and the resulting emulsions were thoroughly characterized. The proposed integrated process represents an advance in the field by transforming the difficulty in recycling ILs from extraction processes into a virtue. Furthermore, this strategy adds value to the final emulsion-based product, considering the bioactive properties of the IL precursors.

2. Material 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) was used in the formulation of emulsions as oil phase.

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/). The use of this strain is related to its ability to accumulate considerable amounts of β-carotene (Lou et al., 2020; Xi, Bian, Luo, Kong, & Chi, 2022), which was essential for the purpose of the current work. Microalgae culture was grown in a 2 L flat bottom flask under autotrophic conditions with a constant light supply of 240 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$, 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 mmol.L^{-1}; $NaH_2PO_4.H_2O$: 0.1 mmol.L^{-1}), trace metals solution ($Na_2EDTA.2H_2O$: 11.7 μmol.L^{-1}; $FeCl_3.6H_2O$: 11.7 μmol.L^{-1}; $MnCl_2.4H_2O$: 0.91 μmol.L^{-1}; $ZnSO_4.7H_2O$: 0.0765 μmol.L^{-1}; $CoCl_2.6H_2O$: 0.042 μmol.L^{-1}; $CuSO_4.5H_2O$: 0.0393 μmol.L^{-1}, and $Na_2MoO_4.2H_2O$: 0.026 μmol.L^{-1}), and vitamins solution (thiamine HCl: 0.296 μmol.L^{-1}; cyanocobalamin: 3.69 × 10^{-4} μmol.L^{-1}; and biotin: 2.05 × 10^{-4} μmol.L^{-1}). After 15 days of growth, the microalgae were submitted to extraction processes.

2.3. Synthesis of ionic liquid

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%. An ethanolic-aqueous solution of a Ch-based IL (without a purification/drying step) was obtained as described in Toledo Hijo et al. (2022) in order to obtain an IL solution with lower production cost and a more sustainable synthesis process.

Then, a second solution with IL at 23% was prepared by adding ethanol into the previously obtained IL ethanolic-aqueous solution. Thus, two IL solutions at different IL concentrations (46 and 23 wt%), were obtained and applied as solvent systems for β-carotene recovery.

[Ch][Ole] was characterized by proton Nuclear Magnetic Resonance (^{1}H NMR), using a Bruker 300 Fourier 300 MHz and CDCl3 as solvent, and by Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR ALPHA, Bruker Scientific, Banner Lane), in order to confirm the structure of the IL and the effectiveness of the synthesis reaction. The IL structure and effectiveness of the synthesis was confirmed by the spectra and the results are presented in the Supplementary Material (**Fig. S1 and S2**). According to NMR measurments, [Ch][Ole] presented a yield of 98%. Before characterization, the IL solution obtained was dried under vacuum conditions using a rotary evaporator at 50 °C for 24 h in order to obtain a gel-like IL. The vial containing [Ch][Ole] was properly sealed to avoid water contamination and stored at 25 °C. The IL's water content was determined in triplicate using an automated Karl Fisher titrator (Metrohm, 870 KF Titrino Plus model, Switzerland). [Ch][Ole] presented water content of 0.76%.

2.4. Extraction method

Microalgae aliquots were centrifuged for 10 min at 15314*g* (Allegra 64R, Beckman Coulter Inc., USA). After centrifugation, the supernatant was discarded. In each test, an aliquot of 10 mL of the microalgae biomass with a final concentration of 7.04 g.L^{-1} was used. The solid-liquid extraction of β-carotene from *D. salina* wet biomass was performed using different temperatures (room temperature (25 °C) and

65 °C) and different IL concentrations (0%, 23%, and 46%) based on the extraction procedure described by Farias et al. (2023) with modifications, as shown in Table 1. For that, the mixture of wet biomass and solvents were incubated for 60 min at 300 rpm using a magnetic stirrer (MS-51 M, Jeio Tech, Korea). The extraction process was carried out in closed reactors in order to prevent potential loss through evaporation. 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 were submitted to 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 their absorption spectrum using UV-VIS spectrophotometer (V-560, Jasco, USA) at 450 nm and a previously obtained calibration curve using n-hexane.

2.5. β-carotene quantification

The total β-carotene quantification was performed as described by Lüdtke et al. (2024) and Morowvat and Ghasemi (2016) with some modifications, as follows. 2.5 mL of *D. salina* culture were centrifuged for 10 min at 15314 *g* (Allegra 64R, Beckman Coulter Inc., USA) and then the supernatant was replaced with 15 mL of hexane/ethanol (1:2) solution. The obtained solution was vortexed for 5 min and distilled water was added until the final volume of 25 mL, being the mixture subsequently centrifuged at 15314 *g* (Allegra 64R, Beckman Coulter Inc., USA) at 4 °C for 5 min. The upper hexane phase was collected and analysed 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 $mg.L^{-1}$ was previously prepared with a determination coefficient of 0.997. Concomitantly, for all the samples evaluated, the spectrum from 350 to 700 nm was also recorded.

2.6. Surface electron microscopy

D. salina cells (raffinate) obtained after solid-liquid extraction for β-carotene recovery were morphologically analysed in an Ultra-high resolution Field Emission Gun Scanning Electron Microscopy (FEG-SEM) (NOVA 200 Nano SEM, FEI Company, USA). Topographic images were obtained with a secondary electron detector at an acceleration voltage of 10 kV. Before morphological analyses, samples were covered with a thin film (10 nm) of Au—Pd (80–20%, weight), in a high-resolution sputter coater, (208HR, Cressington Company, UK), coupled to an MTM-20 Cressington High-Resolution Thickness Controller.

2.7. Nanoemulsion preparation using extracts containing Ionic Liquid and β-carotene

Nanoemulsions (NEs) were prepared by pre-emulsification followed by high-intensity ultrasound, as described by Gonçalves et al. (2021) and Toledo Hijo, Silva, Meirelles, et al. (2023). For that, a pre-emulsion composed of water as aqueous phase (88%), sunflower oil (SO) as lipid phase (10%), and β-carotene- and IL-rich extract at 2% (*w*/w) (Table S1) was prepared at room temperature (25 °C) using an Ultra-Turrax (T18, Ika-Werke, Germany). Previously, the β-carotene-IL extract was dissolved into the aqueous phase and the lipid phase (SO) was added dropwise at 5.000 rpm. Subsequently, the Ultra-Turrax condition was established at 10.000 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.

Table 1
Treatment conditions tested and codes adopted.

Extraction temperatures	IL (wt%)		
	0%	23% ***	46% **
RT*	EtOH RT	EtOH+IL RT	IL RT
65 °C	EtOH 65	EtOH+IL 65	IL 65

*RT- room temperature (25 °C) and EtOH – Ethanol; ** referred to the IL ethanolic-aqueous solution containing IL at a concentration of 46% that was obtained after the synthesis reaction in ethanolic media, as previously explained; *** IL ethanolic-aqueous solution with IL at 23%, obtained by diluting the IL ethanolic-aqueous solution containing IL at 46% in ethanol.

2.8. Characterization of nanoemulsions

2.8.1. Droplet size, size distribution (PDI) and ζ-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).

2.8.2. Entrapment Efficiency of β-carotene

The extraction and quantification of β-carotene entrapped into the NEs was performed by liquid-liquid extraction and spectrophotometric analysis, based on the methodology described by Lüdtke et al. (2024) with some modifications. 2.5 mL of NE was 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 64R, Beckman Coulter Inc., USA) at 4 °C for 5 min. The upper hexane phase was collected and analysed 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/L was previously prepared with a determination coefficient of 0.997.

The Entrapment Efficiency (EE) of β-carotene into the NEs was calculated according to **Eq. 1** (Nguyen, Hwang, Park, & Park, 2012):

$$EE\ (\%) = \frac{Entraped_{\beta-carotene}}{Total_{\beta-carotene}} \quad (1)$$

2.9. Statistical analysis

All the experiments were performed in triplicate. Statistical analysis was carried out using GraphPad Prism 9 (GraphPad Software, La Jolla, 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 ± 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

Fig. 1 describes the novel integrated strategy developed in the present study, employing sequential applications of a naturally-derived IL obtained from Ch and a FA ([Ch][Ole]) as emergent solvent followed by its use as emulsifier to encapsulate β-carotene for emulsion-based products. Firstly, the [Ch][Ole] solution was applied as solvent for β-carotene recovery from *D. salina* wet biomass through a solid-liquid extraction. Then, the [Ch][Ole] already present in the β-carotene-rich extract obtained was further used as emulsifier on β-carotene encapsulation through a high-intensity ultrasound-assisted emulsification. It is worthy to note that this approach integrates two technological uses of a naturally-derived IL for a more sustainable and cost-efficient strategy. In terms of process, it is an alternative for IL recycling that is currently a

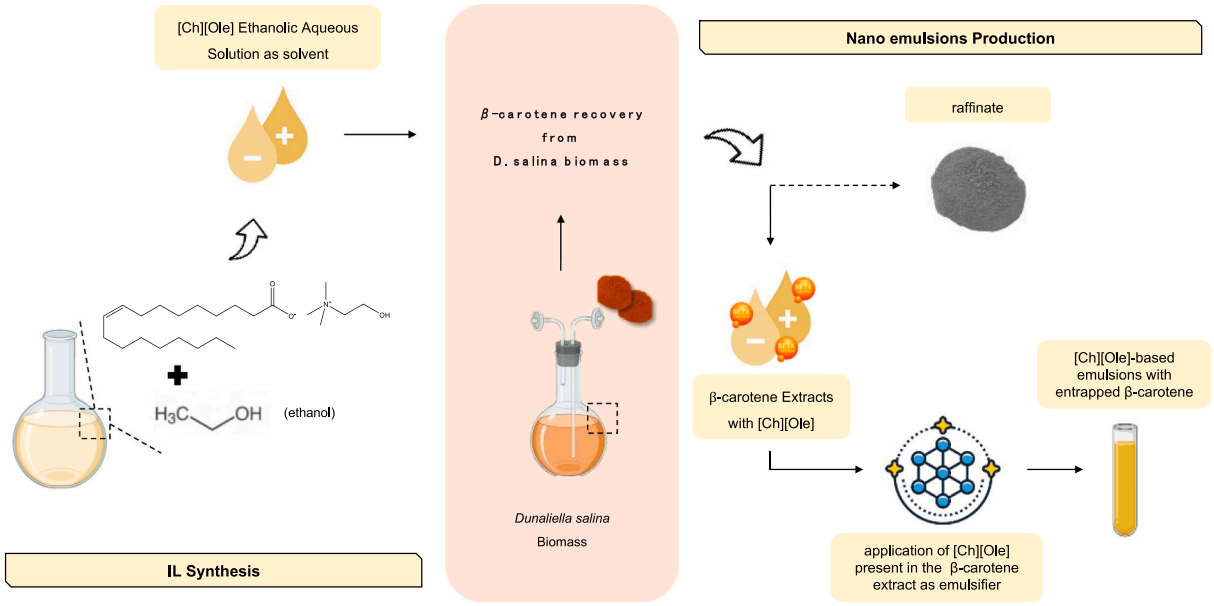

Fig. 1. Schematic representation of the proposed strategy.

major bottleneck of ILs' applications. Also, in terms of product formulation, it eliminates the additional need of an emulsifier or encapsulation agent, thus adding value to the final product.

3.1. Effect of IL concentration and process temperature on β-carotene extraction efficiency

The current experimental work was conducted in order to evaluate the influence of the IL concentration and extraction temperatures on the ability of the IL to both permeabilize *D. salina* cells and extract β-carotene. The use of ethanol (EtOH) as solvent was also evaluated as reference. Fig. 2 presents the β-carotene extraction efficiency as function of the different treatment conditions analysed.

The results indicate that for all treatment conditions, except for the individual use of EtOH as extraction solvent, the increase of process temperature from room temperature (25 °C) to 65 °C led to a significant increase ($p < 0.05$) in β-carotene extraction. This clearly shows that the application of temperature acts as an enhancer of the extraction capacity of the IL, which is aligned with some previous studies (Chang et al., 2018; Desai et al., 2016; Farias et al., 2023; Pan, Muppaneni, et al., 2016). Desai et al. (2016), for instance, reported increases higher than 30% in the amount of astaxanthin extracted from intact cells of *Haematococcus pluvialis* using ILs – 1-Butyl-3-methylimidazolium dibutylphosphate and 1-Ethyl-3-methylimidazolium dibutylphosphate – when elevating the treatment temperature from 25 °C to 45 °C.

Regarding the effect of IL concentration at the same temperature, there was a similar behaviour for both tested temperatures (RT and 65 °C). It was observed an increase in extraction efficiency as the IL concentration increased from 23 wt% to 46 wt%. Similar behaviour was reported on lutein extraction from *Chlorella* sp. using the IL choline dihydrogen citrate. The authors determined an extraction of 3.32 mg.g^{-1} of lutein using 26 wt% IL, whereas the extraction efficiency decreased to just over 2 mg.g^{-1} using 14 wt% IL (X. Zhang et al., 2024).

Based on the results from Fig. 2**a**, one can infer that applying a treatment temperature of 65 °C and an IL concentration of 23 wt% (EtOH+IL 65) resulted in a similar extraction efficiency when compared to the utilization of highly concentrated IL (46 wt%) at RT (no statistically significant differences, $p > 0.05$). This is very interesting as it makes it possible to adopt a strategy orientated towards the target compound. If the target compound exhibits high temperature sensitivity, these data show that it is possible to lower the treatment temperature by increasing the IL ratio, without compromising extraction performance. On the other hand, if the target compound is not too sensitive to temperature, these results also demonstrate that it would be possible to reduce the IL concentration and, consequently, reduce the cost associated with the extraction solvent maintaining the process efficiency.

Considering the application of IL (46 wt%) at 65 °C as the best β-carotene extraction condition in the present work, one was capable of extracting >84% of the total β-carotene present in *D. salina* biomass

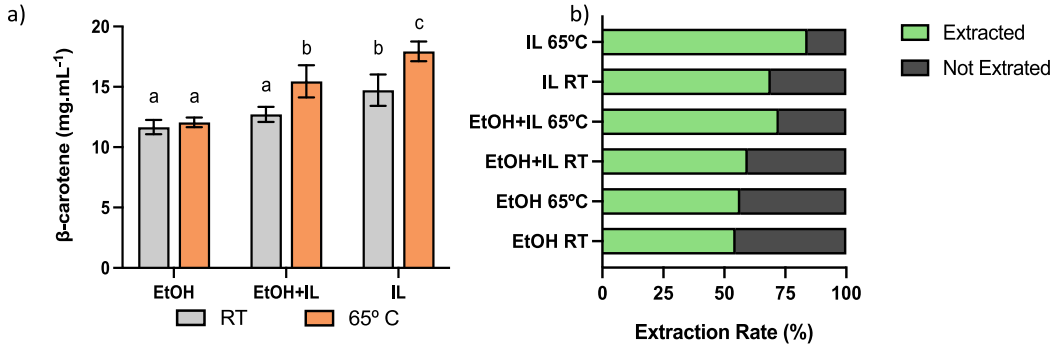

Fig. 2. β-carotene extraction efficiency under different temperatures and IL concentrations (a), extraction rate of the different treatment condition on test (b). According to Tukey's test, the assays presenting the same letter do not differ significantly (α = 0.05).

(Fig. 2b). The extraction efficiency is much higher than that found using ethanol as solvent (56%). Moreover, other approaches targeting the extraction of β-carotene from *D. salina* have revealed considerably lower efficiencies, as the case of super-critical CO_2 with an EtOH fraction of 10%(Ludwig, Rihko-Struckmann, Brinitzer, Unkelbach, & Sundmacher, 2021) or the use of a centrifugal partition with ethyl oleate–5% dichloromethane as a solvent (Marchal, Mojaat-Guemir, Foucault, & Pruvost, 2013), where 25% and 65% of the available β-carotene were extracted, respectively. The high extraction yields of β-carotene through the utilization of [Ch][Ole] achieved in the present study can be attributed to the greater aptitude of the developed IL to solubilize the intracellular matrix of the cells (Mussagy et al., 2020). Mussagy et al. (2022) evaluated the extraction of β-carotene from the yeast *Phaffia rhodozyma* and reported that the relative hydrophobicity of the IL [Ch][Ole] is a key factor for the β-carotene recovery since this is a very non-polar carotenoid. In general, the extraction strategy herein proposed is quite positive, helping to obtain an IL [Ch][Ole] enriched in β-carotene with capacity to be used as a novel emulsifier (see section 3.3) in further steps.

The measurement of the absorbance of the β-carotene extracts (Fig. 3) allowed to conclude that β-carotene spectrum remains in a normal range. An interesting indication, based on these data, is related to the fact that no changes have occurred in the spectra obtained when comparing the samples with IL subjected to different temperature conditions (RT and 65 °C). These findings indicate that the application of higher temperatures appear not to affect the β-carotene. Based on these observations, the impact of treatments on the structural properties of β-carotene should be object of further research. Particularly, in the case of the extract obtained using EtOH at 65 °C, the spectrum confirmed the presence of chlorophylls with a peak at ~660 nm, which was not observed when applying IL (**Fig. S3**).

3.2. Impact of processing conditions on microalgal cells

In order to understand the effect of the IL on the cell surface, *D. salina* biomass was examined by SEM (Fig. 4) after extraction. In EtOH treatments, there were no significant morphological changes compared to the untreated cells. EtOH acted as a cell permeabilizer, as evidenced by the appearance of pores on the surface of the cells (Fig. 4 **b** and **c**). In turn, the utilization of IL as extraction solvent (at 23% or 46%) contributed to the loss of the initial morphological characteristics of the cells. The use of [Ch][Ole] induces the disintegration of *D. salina* cells, which was expected since previous studies using this IL showed its capacity of solubilising the intracellular matrix from yeast biomass (Mussagy et al., 2020). Another interesting aspect that should be highlighted is associated with the IL concentration; as the IL content in the extraction solvent increased (from 23 to 46 wt%), a greater degree of disorganisation of the cell structures was observed, demonstrating the ability of [Ch][Ole] to swell or dissolve the cell membrane of *D. salina.* Similar effects have already been reported for other ILs, such as 1-butyl-3-methyl imidazolium hydrogen sulfate – in the extraction of lipids from *Galdieria sulphuraria* (Pan, Muppaneni, et al., 2016) – or 1-ethyl-3-methyl imidazolium hydrogen sulfate and 1-ethyl-3-methyl imidazolium thiocyanate – in the extraction of lipids from *Chlorella vulgaris* (Choi et al., 2014).

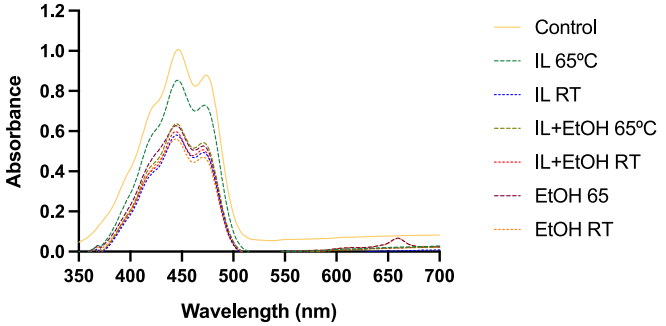

Fig. 3. β-carotene spectrum resulting from the different treatment conditions.

3.3. Nanoemulsions characterization

3.3.1. Droplet size, size distribution (PDI) and ζ-Potential

The same IL already present (at different concentrations) in the β-carotene extracts previously obtained from *D. salina* biomass, was sequentially used as emulsifier to produce different NEs. These NEs (labelled according to the extract composition) were evaluated for 30 days regarding their kinetic stability, droplet size, PDI, and ζ-potential (Table 2). All NEs had a homogeneous (one phase) aspect, did not present phase separation and were kinetically stable for at least 30 days (**Fig. S4**).

According to the results, regardless the composition, all NEs showed a downward trend in droplet size over time, with values <220 nm after 30 days of analysis. On the other hand, when comparing the performance of the different emulsifier compositions (extracts), there were statistically significant differences ($p < 0.05$) between those containing IL at 46% and 23% after 24 h, with a lower droplet size being observed for higher IL content. The decreasing droplet size values may indicate that the adsorption of the IL as emulsifier on the aqueous-oil interface probably did not achieve the saturation point yet, suggesting that a higher emulsifier concentration could improve emulsion properties. The variation in droplet size can be related to the nature and molecular structure of the emulsifier and its interaction with the lipid phase and with the bioactive compound incorporated into it (Lüdtke et al., 2023). In fact, EtOH might reduce the difference between aqueous and oil phases polarity, which is related to lower interfacial tension, favouring emulsion properties(Toledo Hijo et al., 2022). However, even when EtOH content decreased and IL concentration increased, the droplet size was still reduced, suggesting that such effect can be directly related to the IL concentration as effective emulsifier. Otherwise, higher droplet size observed for the NE produced with higher EtOH content can be related to a lower amount of IL molecules available to adsorb the interface (Hijo, Meirelles, Maximo, Cunha, & Meirelles, 2024). However, at the end of the storage period under study, unexpectedly, the differences between both emulsifiers tested were no longer statistically significant ($p > 0.05$).

PDI is a direct measure of the "spread" of the droplet size distribution and it allows the determination of the degree of homogeneity of the assessed droplets. Low PDI values indicate the production of droplets of homogeneous size, while higher values suggest the heterogeneity of the sizes of the droplets formed (Lakshmi & Kumar, 2010). As was observed in droplet size, the PDI also showed a downward trend over time. Based on the results from Table 2**,** it is possible to observe that all the NEs, except that resulting from the extraction treatment with EtOH+IL RT, presented a PDI valuable lower than 0.250 after 30 days of storage, which is an indication of homogeneous droplets and a narrow droplet size distribution of the system (Schaffazick & Guterres, 2003). Considering that lower droplet size and PDI values indicate a high kinetic stability during storage, the NEs produced in the present study exhibited a good stability by the end of the storage time (30 days). Interestingly, the extraction temperature did not affect the emulsifying ability of the IL. Based on the results obtained, with droplet size values lower than 220 nm and a PDI lower than 0.3 (except, as previously mentioned, in the case of EtOH+IL RT) after 30 days of storage, it is possible to conclude that the developed IL successfully acted as an outstanding emulsifier after being used as solvent. Additionally, the obtained NEs could be potentially suitable for oral delivery because they showed droplet size and PDI values suitable for these purposes (Gonçalves et al., 2021). Lüdtke et al. (2023) evaluated the production and characterization of β-carotene loaded nanostructured lipid carriers using soy lecithin, Tween 80, and whey protein isolate as emulsifiers. The authors

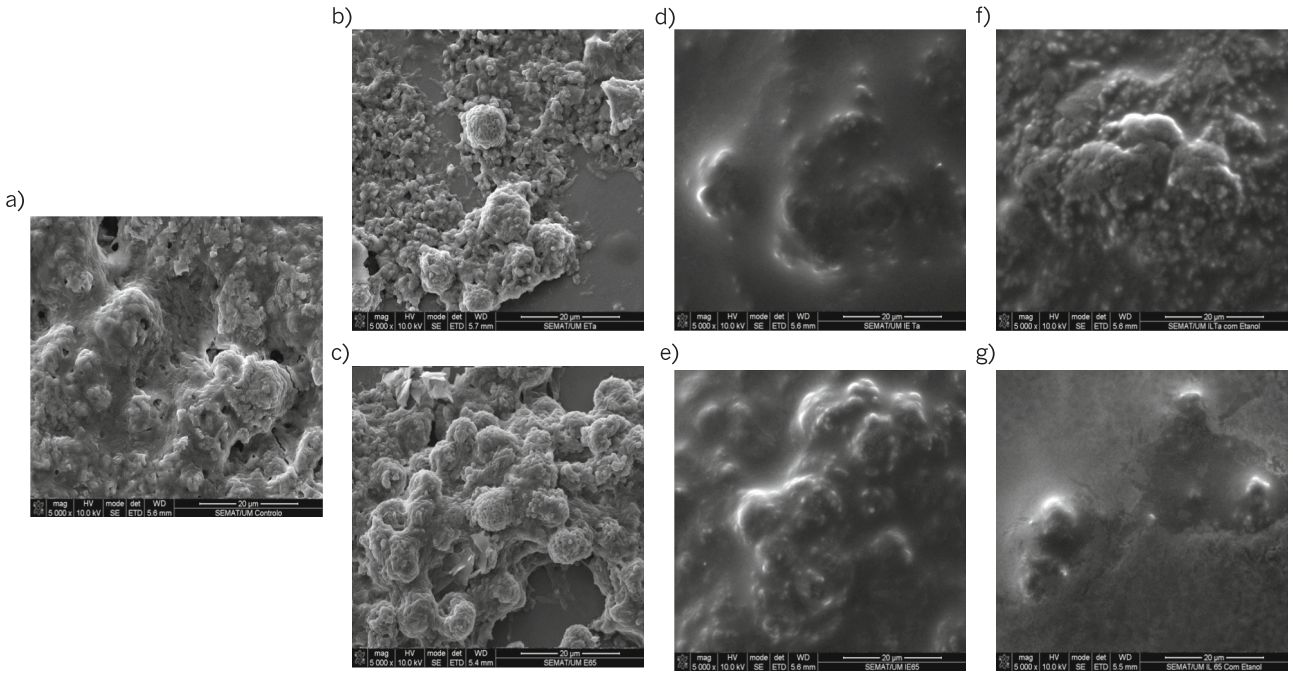

Fig. 4. SEM images of *D. salina* biomass without any treatment (a), EtOH at room temperature (b), EtOH at 65 °C (c), EtOH+IL at room temperature (d), EtOH+IL at 65 °C (e), IL at room temperature (f) and, IL at 65 °C (g)

Table 2
Droplet size, size distribution (PDI), and zeta potential (ZP) of the β-carotene loaded nanoemulsions (NEs) evaluated after 1 day, 7 days, 15 days, and 30 days. Different lower-case letters represent significant differences ($p < 0.05$) related to the evaluation of each parameter (droplet size, PDI, and ZP) in comparison to the different times of storage for the same NE. Different capital letters represent significant differences ($p< 0.05$) related to the evaluation of the same parameter (droplet size, PDI, or ZP) at the same time of storage between different NEs.

NE	Droplet size (nm)				
	1 day	7 days	15 days	21 days	30 days
EtOH+IL RT	361.67 ± 3.81^{a,A}	288.69 ± 28.00^{a,A}	279.52 ± 29.06^{a,b,A}	230.82 ± 15.84^{b,A}	220.48 ± 3.97^{b,A}
EtOH+IL 65	317.82 ± 15.08^{a,b,A}	322.82 ± 16.57^{a,B}	270.27 ± 2.22^{b,c,A}	250.06 ± +19.52^{c,d,B}	206.60 ± 1.32^{d,A}
IL RT	266.53 ± 14.07^{aB}	240.81 ± 18.42^{a,b,A}	269.22 ± 16.41^{a,A}	202.35 ± 5.07^{b,A}	213.70 ± 8,74^{b,A}
IL 65	280.42 ± 1.96^{aB}	250.18 ± 10.58^{a,b,A}	221.18 ± 0.26^{b,c,A}	203.41 ± 3.24^{c,A}	220.89 ± 25.57^{bc,A}
	Polydispersity index (PDI)				
NE	1 day	7 days	15 days	21 days	30 days
EtOH+IL RT	0.45 ± 0.01^{a,A}	0.39 ± 0.03^{a,b,A,B}	0.45 ± 0.06^{a,A}	0.36 ± 0.04^{a,b,A}	0.34 ± 0.01^{b,A}
EtOH+IL 65	0.40 ± 0.04^{a,A}	0.45 ± 0.03^{a,A}	0.27 ± 0.04^{b,B}	0.26 ± 0.01^{b,B}	0.24 ± 0.01^{b,B}
IL RT	0.36 ± 0.01^{a,A}	0.31 ± 0.02^{a,b,B,C}	0.33 ± 0.03^{a,A,B}	0.21 ± 0.01^{c,B}	0.23 ± 0.01^{b,c,B}
IL 65	0.36 ± 0.05^{a,A}	0.28 ± 0.01^{a,b,C}	0.25 ± 0.03^{a,b,B}	0.24 ± 0.01^{b,B}	0.21 ± 0.05^{b,B}
	ζ-potential (mV)				
NE	1 day	7 days	15 days	21 days	30 days
EtOH+IL RT	−70.42 ± 0.32^{a,A}	−69.06 ± 1.12^{a,b,A}	−69.23 ± 0.85^{a,b,A}	−70.96 ± 1.30^{a,A}	−63.59 ± 3.87^{b,A}
EtOH+IL 65	−67.06 ± 2.17^{a,A}	−69.36 ± 2.50^{a,A}	−71.36 ± 3.37 ^{a,A}	−66.82 ± 2.97^{a,A}	−69.31 ± 5.52^{a,A}
IL RT	−65.29 ± 1.77^{a,A}	−62.32 ± 5.38^{a,A}	−68.62 ± 2.04 ^{a,A}	−64.58 ± 4.76^{a,A}	−68.63 ± 4.22^{a,A}
IL 65	−66.18 ± 3.79^{a,A}	−68.43 ± 4.47^{a,A}	−70.64 ± 6.02 ^{a,A}	−66.96 ± 6.30^{a,A}	−67.02 ± 0.97^{a,A}

reported a droplet size of 218, 195, and 346 nm and a PDI of 0.225, 0.215 and, 0.155, respectively, after 30 days of storage.

ζ-potential is directly related to electrostatic interactions in the system and particle or molecule aggregation. Hence, it measures the droplet surface charge, which is related to the stability of the system during storage. According to the literature, it is possible to achieve good stability for an electrostatically stabilized nanosuspension with a minimum ζ-potential of |30 mV| (Gonçalves et al., 2021). Based on these considerations, the ζ-potential of the NEs produced in the present work supports the observed high kinetic stability, preventing droplet aggregation, coalescence, and phase separation. Regarding the ζ-potential, no statistically significant differences ($p > 0.05$) were found between the different emulsifier compositions tested. Furthermore, it was found that all the NEs, at all sampling times, presented a ζ-potential close to −70 mV. The NEs produced by Teixé-Roig, Oms-Oliu, Odriozola-Serrano, and Martín-Belloso (2023), using soybean lecithin and whey protein isolate as emulsifiers, presented a ζ-potential of approximately −50 mV and −36 mV, respectively. It is interesting to note that the use of IL as emulsifier resulted in more stable NEs (ζ-potential up to ca. -70 mV) than those previously reported using traditional emulsifiers such as lecithin, Tween 80, and whey protein isolate, with −58, −24, and − 61 mV, respectively (Lüdtke et al., 2023). The ζ-potential observed in the NEs under study is due to the arrangement of the IL that forms micelles with high negative charges, which in turn causes electrostatic repulsion and favours the high stability of the emulsion (Hijo et al., 2024).

The results obtained in the present work indicate that the use of IL as emulsifier that was already present in the β-carotene extract and previously used as solvent has performed well to form and stabilize NEs that present high kinetic stability. Considering that [Ch][Ole] contains bioactive compounds with biological functions, the approach herein presented contributes to the possibility of producing functional foods by incorporating these β-carotene-based NEs into food matrices, offering benefits for human health.

3.3.2. *Entrapment efficiency of β-carotene*

The concentration of loaded β-carotene in the NEs was measured to determine the entrapment efficiency. The EE of the NEs refers to the amount of β-carotene trapped in the NE structure. All β-carotene loaded

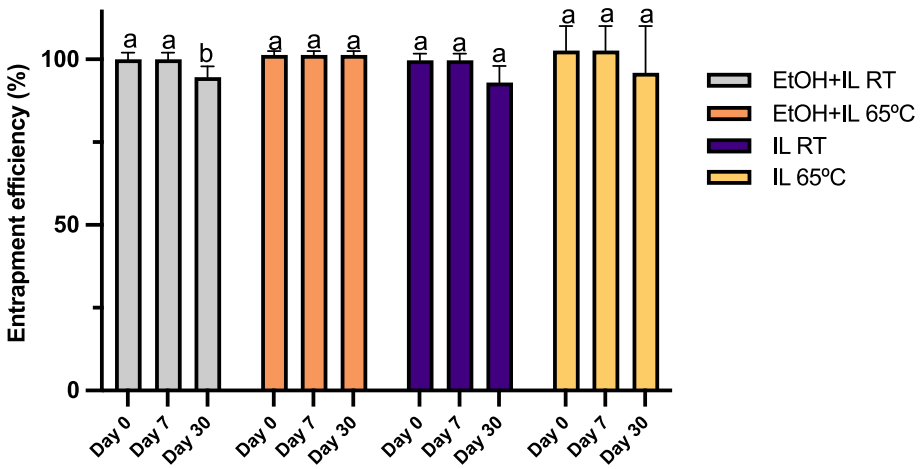

Fig. 5. Entrapment efficiency of the β-carotene loaded NEs considering the different extraction conditions tested. According to Tukey's test, the assays presenting the same letter do not differ significantly (α = 0.05).

NEs showed high EE, reaching 100% for all the evaluated compositions during the first 7 days (Fig. 5). On the 30th day of storage, a statistically significant decrease ($p < 0.05$) was observed only for EtOH+IL RT samples, presenting a decrease of <7%. No statistically significant differences ($p > 0.05$) were seen in the remaining emulsions over the storage period. This high β-carotene retention capacity might be related to the fact that the developed IL is an efficient encapsulation agent, as previously reported in the encapsulation of bioactive compounds obtained from rosemary essential oil (Toledo Hijo, Silva, Meirelles, et al., 2023). Pan, Tikekar and Nitin (2016) also have demonstrated the positive effect that lecithin has as emulsifier, achieving EE values close to 100%. Similarly, other studies reported high EE of β-carotene – values around 91.99% – using a whey protein hydrolysate-pectin soluble complex as emulsifier, as described by López-Monterrubio, Lobato-Calleros, Vernon-Carter, and Alvarez-Ramirez (2021).

4. Conclusions

The sequential application of the IL [Ch][Ole] for β-carotene recovery from *D. salina* microalgae and subsequent encapsulation has been investigated. The results of the current study highlight the potential of [Ch][Ole] to permeabilise *D. salina* and extract β-carotene. The use of the IL as solvent enhanced β-carotene recovery, with >84% of recovery, which is a significant increase when compared to the employment of ethanol (56% of recovery). The application of temperature and the increase in the IL concentration contributed to increase in the extraction efficiency due to its ability to dissolve biopolymers and disintegrate cells' structure, as evidenced by SEM analyses. This extraction strategy allows modulating the treatment conditions without compromising extraction efficiency, in order to make them suitable for the target compound. Furthermore, the IL [Ch][Ole] already present in the β-carotene extracts obtained from *D. salina* acted as a highly effective emulsifier to form and stabilize NEs, having the NE presented a β-carotene entrapment efficiency of 100%. Therefore, the outcomes of this study show that the IL [Ch][Ole] shows potential for the production of functional foods that offer benefits to human health.

CRediT authorship contribution statement

Vítor Sousa: 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, Investigation, Conceptualization. **Fernanda L. Lüdtke:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **António A. Vicente:** Writing – review & editing, Supervision, Resources. **Oscar Dias:** Writing – review & editing, Supervision. **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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140232.

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