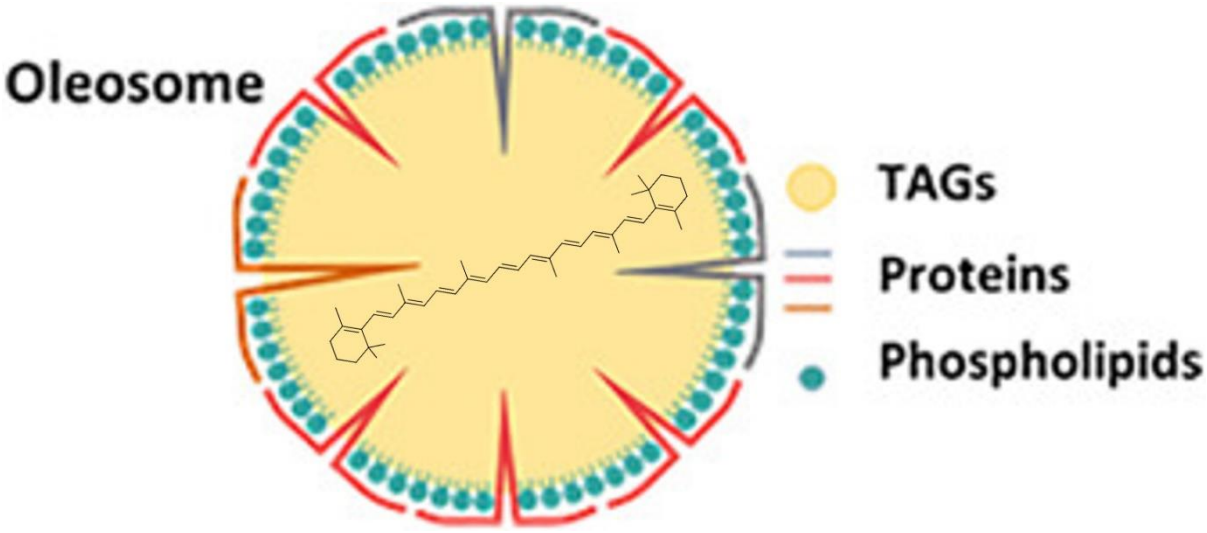
Gastrointestinal fate and oxidative stability of oleosome encapsulated β-carotene



MSC thesis- Food Quality and Design (FQD80436)

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Acknowledgements

I would like to express gratitude to my supervisors, Edoardo Capuano and Costas Nikiforidis, for their scientific inputs and encouragement throughout the thesis period. I would also like to thank the FQD laboratory technicians, Erik Meulenbroeks, Jelle Bos, Anastasia Louizaki and Charlotte van Twisk for their assistance in the lab. In addition, I would like to thank Lorenz Plankensteiner for his advice on the lipid oxidation experiments.

Abstract

β-carotene, a plant-derived lipophilic red-orange molecule, had been reported to have potential health benefits due to its antioxidant and pro-vitamin A activity. However, its low water solubility, chemical stability and bioavailability makes its incorporation into food system a challenge. The aim of this study was to investigate the encapsulation efficiency of β-carotene in a novel carrier, oleosome, using high pressure homogenization (HPH) and to investigate the subsequent gastrointestinal fate and oxidative stability of the encapsulated system. The encapsulation efficiency was determined by quantification of β-carotene and ranged between 65% to 88% due to variation in the dissolution of β-carotene in rapeseed oil. Particle size measurements showed no difference between the encapsulated and unencapsulated oleosome after HPH. The gastrointestinal fate was investigated by performing *in vitro* digestion and a similar digestive stability was observed between encapsulated (89.9% ± 10.7%) and unencapsulated β-carotene (57.0% ± 13.5%). The bioaccessibility, however, could not be determined due to the low amounts of mixed micelles in the system. The oxidative stability was determined by quantification of β-carotene and quantification of lipid oxidation products after storage at 40 ºC, pH 7, in the dark over a period of 21 days. Creaming was seen in both encapsulated and unencapsulated oleosome emulsion during storage. A higher amount of β-carotene remained after 21 days when encapsulated in oleosome (44.3% ± 2.70%) compared to unencapsulated (28.4% ± 3.87%), indicating higher oxidative stability. However, the presence of β-carotene did not result in a lower amount of oxidation products from the lipid in oleosome. Based on these results, encapsulation of β-carotene into oleosome had no clear improvement on the stability after digestion but had improvement on the stability during storage. The effect on bioaccessibility remains to be investigated, and the HPH encapsulation method must be optimized to improve the physical stability and oxidative stability of the oleosome emulsion before it is suitable to be used as a delivery system in food.

Keywords: oleosome, encapsulation, β-carotene, stability, oxidation, digestibility, bioaccessibility

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1. Introduction

β-carotene is a red-orange pigment found in many vegetables and fruits and is the most studied carotenoids due to its provitamin A activity and antioxidant capacity (Gul et al., 2015). This results in a lower oxidative stress environment which can then contribute to possible health benefits such as the prevention of certain cancers and cardiovascular diseases (Anand et al., 2022; Gul et al., 2015; Yang et al., 2022; Yuan et al., 2008). As such, it is of great interest within the food industry to incorporate β-carotene into their food products to make them into functional food (Acevedo-Fani et al., 2020). However, there are several challenges associated with the development of β-carotene enriched food due to its low chemical stability, low water solubility and low bioavailability.

β-carotene consists of conjugated double bonds and 2 β-rings (Fig. 1). This specific structure contributes to its beneficial effect, but the unsaturated hydrocarbon chain makes β-carotene highly susceptible to degradation by light, heat and oxygen (Britton, 1995). As such, it is likely that β-carotene will be degraded over time if directly supplemented into food products, resulting in the loss of bioactivity. Furthermore, as a hydrophobic molecule, β-carotene has limited water solubility and dispersibility, thus making incorporation into aqueous-based food products limited (Jalali-Jivan et al., 2022; McClements, 2018).

In addition, β-carotene must be bioavailable to elicit its potential benefits. Bioavailability is defined as the fraction of ingested molecule that reaches the bloodstream (Mourtzinos & Biliaderis, 2017). As a lipophilic molecule, β-carotene require liberation from the food matrix, followed by emulsification and incorporation into the mixed micelles before uptake by intestinal cells (Mcclements et al., 2015). However, this is affected by several host-related and dietary factors, including the state of β-carotene (crystalline or soluble) and the presence or interaction with other dietary components (Gibson, 2016; Lin et al., 2018). In addition, the presence and nature of lipids present is also important as it can affect the formation of sufficient mixed micelles for β-carotene to be incorporated for absorption. These factors thereby limit the bioavailability of β-carotene.

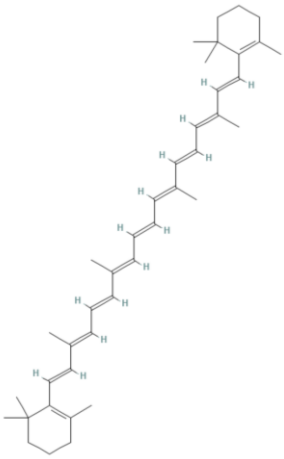

Fig. 1. Chemical structure of β-carotene (National Center for Biotechnology Information, 2022)

To address these challenges, encapsulation of β-carotene had been attempted using different delivery vehicles, whereby β-carotene is entrapped within the core of another substance (Mourtzinos & Biliaderis, 2017). Depending on the carrier, the solubility, bioaccessibility and/or bioavailability and chemical stability of β-carotene can be improved. Current carriers for β-carotene in food application can be distinguished into 2 classes: polymer-based or lipid-based (Maurya et al., 2021). Some examples of polymer-based carriers include micro-/nanocapsules

and hydrogels while some examples of lipid-based carriers include micro-/nanoemulsions and liposomes (Jalali-Jivan et al., 2022; Lin et al., 2018). However, these carriers have certain setbacks such as instability under certain ionic environmental conditions (eg. pH, ionic strength), low permeability to the encapsulated bioactive compound, poor consumer perception, and usage of strong and toxic organic solvents (Drosou et al., 2022; Jalali-Jivan et al., 2022). Therefore, oleosome is proposed to be an alternative novel carrier of β-carotene for food applications.

Oleosomes, or oil bodies (OB) are intracellular lipid storage organelles present in many plant tissues, particularly in seeds (Tzen et al., 1993). It consists of a triacylglyceride (TAG) core stabilized by a phospholipid monolayer embedded with structural proteins such as oleosins and caleosin (Nikiforidis & Kiosseoglou, 2011) (Fig.2). Oleosomes are easily extracted in the form of natural oil-in-water emulsion or cream from plant sources such as rapeseed without the use of toxic organic solvents (Nikiforidis, 2019). The emulsified oil droplet is stable due to electrostatic repulsion between charged proteins and polar lipids present on the membrane (C. Liu et al., 2020; Nikiforidis et al., 2012). The unique membrane structure also protects the TAG core from environmental stresses (Nikiforidis, 2019) and reduces degradation of the oil, which can affect the perceived quality and nutrition of the oil (Jacobsen, 1999). In addition, oleosome consists of α-Tocopherol, an antioxidant which can provide further oxidative stability to the TAG (Zhang & Omaye, 2000). As the membrane is permeable to hydrophobic molecules, encapsulation within the TAG core is possible and protection can be conferred to the encapsulated molecule (Acevedo et al., 2014; Boucher et al., 2008). Altogether, this makes oleosome a promising natural delivery system in food applications for unstable lipophilic bioactive compounds such as β-carotene.

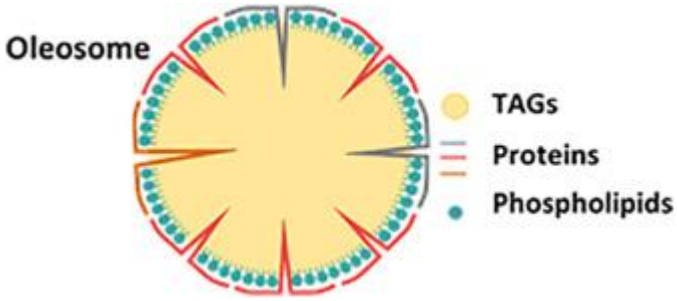

Fig.2. Structure of oleosome (Nikiforidis, 2019)

Encapsulation of astaxanthin, D-limonene and curcumin within oleosomes had been attempted, and a higher stability and/or bioaccessibility was reported (Acevedo et al., 2014; Fisk et al., 2013; Zheng et al., 2019). The techniques used in these studies for encapsulation include hydrophobic partitioning, spray drying and pH loading. As oleosomes are naturally emulsified, another possible encapsulation method is homogenization after dissolution of the compound in oil (Zheng et al., 2019). As such, it is likely that β-carotene can be encapsulated into oleosomes via homogenization, but the encapsulation efficiency and the subsequent effect on the bioaccessibility and stability of β-carotene requires investigation.

The aim of this study was to determine the encapsulation efficiency of β-carotene into oleosomes using high pressure homogenization (HPH) and the subsequent effect on the digestive stability, bioaccessibility and oxidative stability of β-carotene after encapsulation. In addition, since β-carotene is an antioxidant, its effect on the oxidative stability of the TAG core after encapsulation within the oleosome was also investigated. For this purpose, the encapsulation efficiency after HPH was determined by quantification of β-carotene. The encapsulated oleosome emulsion was subjected to *in vitro* digestion to determine the digestive

stability and bioaccessibility of β-carotene. An accelerated storage test was performed to determine the oxidative stability of β-carotene and the TAG core after encapsulation.

The aim of this study was achieved by addressing the following research questions:

What is the encapsulation efficiency of β-carotene in oleosome using high pressure homogenization and the size of the β-carotene encapsulated oleosome?

What is the effect of encapsulation within oleosome on the digestive stability and bioaccessibility of β-carotene after *in vitro* digestion?

What is the effect of encapsulation within oleosome on the oxidative stability of β-carotene during storage?

What is the effect of encapsulated β-carotene on the oxidative stability of oleosome emulsion during storage?

2. Materials and methods

2.1. Materials

Oilseed rape seeds were stored at -20 ºC until use. Rapeseed oil (Fusion brand) was purchased from a local supermarket. β-carotene (C9750-10G), butylated hydroxytoluene (BHT) (1082708-500MG), sodium azide (71290-100G), sodium bicarbonate (S5761-1KG), sodium chloride (S9888-1KG), sodium hydroxide (30620-1KG-R), potassium chloride (1.04933.0500), potassium dihydrogen phosphate (1048711000), magnesium chloride hexahydrate (1.05833.1000), ammonium carbonate (207861-500G), calcium chloride dihydrate (31307-500G), pancreatin from porcine pancreas (P7545-100G), pepsin from porcine gastric mucosa (P6887-5G), bile extract porcine (B8631-100G), cumene hydroperoxide (247502-100G), malonaldehyde bis(diethyl acetal) (T9889-100ML), *p*-anisidine (A88255-100G), ammonium thiocyanate (221988-500G), 37% hydrochloric acid (320331-2.5L) were purchased from Sigma Aldrich (St Louis, USA). Trichloroacetic acid (TCA) (1.00807.0100), 2-thiobarbituric acid (TBA) (88481), barium chloride dihydrate (1.01719.0500), iron(II) sulfate heptahydrate (1.03963.1000) were purchased from Merck (Darmstadt, Germany). High performance liquid chromatography (HPLC) grade solvents were purchased from Actu-ALL Chemicals (Oss, The Netherlands). All other solvents used were of analytical grade. Ultrapure water was used for the preparation of all solutions.

2.2. Oleosome extraction

The extraction method of oleosome was based on the method by De Chirico et al. (2018) with some modifications. Oilseed rape seeds were soaked at 4 °C for 16 h in 0.1 M $NaHCO_3$ pH 9.5 solution (adjusted using 1 M NaOH) at ratio of 1:7 (w/v). The seeds were then blended in the same solution for 60 s at high power using a blender (Waring commercial 7011HS, Torrington, USA). The slurry was filtered through three layers of cheesecloth and the filtrate was centrifuged at 10,000 *g* for 30 min at 4 °C (Avanti centrifuge J-20XP, Beckman Coulter, Brea, USA). The upper creamy layer, which was the crude oleosome fraction, was isolated using a spatula and drained on filter paper (Whatman, grade 1). This fraction was collected and dispersed in the 0.1 M $NaHCO_3$ pH 9.5 solution (1:4 w/v) and centrifuged at 10,000 *g* for 30 min at 4 °C. The upper layer was isolated again using a spatula and drained on filter paper. The cream was then suspended in water (1:4 w/v) and centrifuged at 10,000 *g* for 30 min at 4 °C. The cream layer was collected using a spatula and drained on filter paper. The oleosome cream was stored at 4 °C for up to 24 hours.

The dry content of the oleosome cream was determined by gravimetric analysis after leaving the cream at 105 ºC overnight, followed by 1 hour in a desiccator.

2.3. Preparation of oleosome emulsion for encapsulation

Oleosome cream was re-suspended in water (5% w/v dry weight basis) using a magnetic stirrer and thermally treated at 95 °C in a water bath for 10 min to reduce endogenous lipolytic activity without altering its physical properties (De Chirico et al., 2020). 0.02% (w/v) sodium azide was added to the emulsion to prevent microbial spoilage.

2.3.1. Encapsulation of β-carotene into oleosome

β-carotene was dissolved in rapeseed oil (0.02% w/v) through sonication and heating at 50 °C. The rapeseed oil with dissolved β-carotene was then added to the heat treated oleosome emulsion such that the final composition of the emulsion was 5% OB (w/v dry weight basis), 5% rapeseed oil (v/v) and 0.001% (w/v) β-carotene. Firstly, a coarse emulsion was prepared by blending the oil and aqueous phase (oleosome emulsion) together at 13,500 rpm for 1

minute (Ultra Turrax T25, IKA Works Inc, Wilmington, NC). Next, the coarse emulsion was passed through a high-pressure homogenizer 5 times at 150 mPa (NS 1001L Panda, GEA Process Engineering Inc., Columbia, USA). This β-carotene encapsulated oleosome emulsion was termed as EOB. An oleosome control was prepared using the same method but with 5% rapeseed oil without β-carotene and was termed as OBC. Both EOB and OBC emulsions were flushed with nitrogen, protected from light and stored at 4 °C for up to 3 days.

2.4. Particle size measurement

The average droplet size of the OB emulsions was determined before encapsulation, after encapsulation and at the end of each storage time point. Measurement was done using a light scattering instrument based on the Mie theory of light scattering by spherical particles (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK). The refractive index used for the calculation of OB size was 1.465, corresponding to the refractive index of rapeseed oil and the refractive index of the medium (water) was 1.333. Each sample was measured in triplicates and expressed with volumetric particle size distributions and volume mean particle diameter, $D_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of droplets with a diameter of d_i.

2.5. *In vitro* digestion

A simulated gastrointestinal tract that mimics the mouth, stomach and small intestine of the human gut was used to determine the digestibility of the encapsulated (EOB) and non-encapsulated β-carotene which serve as a control. The control, termed ROC, was prepared by dissolving β-carotene (0.01% w/v) in 10% rapeseed oil through sonication and heating at 50 °C. After which, water was added such that the final composition of ROC was 10% rapeseed oil, 90% water and 0.001% (w/v) β-carotene. The digestion protocol used was based on the harmonized INFOGEST protocol (Minekus et al., 2014) with some modifications. Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) used were modified with $NaHCO_3$ replaced by NaCl. Stock solutions of the enzymes were prepared based on the respective enzyme activities. A trial digestion was performed beforehand to determine the amount of HCl and NaOH needed to adjust the pH to 3 and 7 respectively. All solutions were heated to 37 ºC before usage and incubation of samples was done at 37 ºC on a rotating device in duplicates.

Briefly, 5 mL of the sample (EOB or ROC) was mixed with $CaCl_2$ (final concentration of 0.75 mM) and SSF to achieve a ratio of 50:50 (v/v) in the final mixture. Next, 10 mL of oral bolus was mixed (50:50 v/v) with SGF and $CaCl_2$ (final concentration of 0.075 mM). The pH was adjusted to pH 3 using 1 M HCl before the addition of porcine pepsin (final activity 2000 U/mL). Samples were incubated for 2 hours. After which, the 20 mL of gastric chyme was adjusted to pH 7 using 1 M NaOH and mixed (50:50 v/v) with SIF and $CaCl_2$ (final concentration of 0.3 mM). Bile salts were added to obtain a final concentration of 10 mM or 20 mM. Pancreatin solution was added to achieve a final concentration of 2,000 U/mL or 4,000 U/mL based on lipase activity. Samples were incubated for another 2 hours and immediately processed to quantify the amount of β-carotene remaining in the samples and present in the micelles.

2.6. Stability test

18 mL of ROC, EOB and OBC were prepared in 50 mL tubes with at least 50% headspace in triplicates and stored at 40 ºC in the dark for a period of 7, 14 and 21 days at pH 7. At the end of each time point, part of the emulsions was taken for quantification of β-carotene (5 mL) and particle size measurement (1.5 mL). The remaining solutions were stored at -20 ºC and subsequently freeze dried for at least 48 hours for lipid oxidation analysis.

2.7. Sample preparation to quantify β-carotene

2.7.1. β-carotene in EOB

Encapsulated β-carotene was extracted from the oleosomes using organic solvents in triplicates. EOB samples were centrifuged at 7,000 *xg* for 5 minutes at 4 ºC and the upper cream layer was collected into new tube using a spatula. 1:2 (v/v) Methanol (MeOH):chloroform was added to the oleosome cream and mixed by vortexing for 10 minutes. The tubes were centrifuged at 7,000 *xg* for 10 minutes at 4 ºC and the organic phase was collected in a new tube. Chloroform was added to the tube again, vortexed for 10 minutes and centrifuged at the same conditions. The organic phase was added to the same tube. The tubes with the collected organic solvent were dried under N_2 at ambient temperature and redissolved in MeOH:Tetrahydrofuran (THF) (1:1) + 0.01% BHT. Samples were filtered through 0.2 µm RC filter into amber vials for HPLC analysis.

The encapsulation efficiency was determined using Eq. (1):

$$Encapsulation\ efficiency\ (\%) = \frac{M_{encapsulated}}{M_{initial}} * 100\% \quad (1)$$

Where $M_{encapsulated}$ is the mass of the β-carotene present within the oleosome and $M_{initial}$ is the mass of the initial amount of β-carotene added to the system.

The storage stability for the encapsulated β-carotene was defined as the percentage of β-carotene remaining after storage using Eq. (2):

$$Storage\ stability\ (\%) = \frac{M_{storage}}{M_{encapsulated}} * 100\% \quad (2)$$

Where $M_{storage}$ is the mass of β-carotene present in the samples after each storage time point.

2.7.2. β-carotene in ROC

β-carotene in ROC was extracted in triplicates by adding 1:1 (v/v) hexane to the samples and mixed by vortexing for 10 minutes. The samples were centrifuged for 4,816 *xg* (Multifuge X3R centrifuge, Thermo Fisher Scientific, Waltham, USA) for 10 minutes at 4 ºC. The tubes were stored at -20 ºC for 30 minutes to eliminate water by freezing. The upper organic phase was collected into new tubes and dried under N_2 at ambient temperature. After which, the samples were redissolved in MeOH:THF (1:1) + 0.01% BHT and filtered through 0.2 µm RC filter into amber vials for HPLC analysis.

The storage stability for the control was determined using the following Eq. (3):

$$Storage\ stability\ (\%) = \frac{M_{storage}}{M_{initial}} * 100\% \quad (3)$$

2.7.3. β-carotene in digested samples

At the end of the intestinal phase, part of the final digesta was aliquoted out as the micelle sample to isolate the micellar phase. The remaining digesta in the tube was processed to determine the amount of β-carotene remaining after digestion. 5 mL of hexane was added to the tubes and mixed by vortexing for 10 minutes. The tubes were centrifuged at 4,816 *xg* for 10 minutes at 4 ºC and the upper organic phase was collected in a new tube. This was repeated twice. The collected upper organic phase was dried under N_2 at ambient temperature overnight. After which, the samples were redissolved in MeOH:THF (1:1) + 0.01% BHT and filtered through 0.2 µm RC filter into amber vials for HPLC analysis.

The micelle sample was transferred into a centrifuge tube and centrifuged at 8,000 *xg* for 30 min at 4 ºC (Sorvall Lynx 4000, Thermo Fisher Scientific, Waltham, USA) or 160,000 *xg* for 30 min at 18 ºC (Optima Ultracentrifuge XE-90, Beckman Coulter, Brea, USA). The clear supernatant was collected as the mixed micelle fraction and the β-carotene in this fraction was extracted in the same manner as the total digesta fraction. Both fractions were analysed in duplicates.

The bioaccessibility was defined as the percentage of β-carotene solubilized in the intestinal phase that was solubilized within the mixed micelle fraction and is available for absorption Eq. (4), while the digestive stability was defined as the percentage of β-carotene that remained after the intestinal phase Eq. (5):

$$Bioaccessibility\ (\%) = \frac{M_{micelle}}{M_{digesta}} * 100\% \quad (4)$$

$$Digestive\ stability\ (\%) = \frac{M_{digesta}}{M_{encapsulated}\ or\ M_{initial}} * 100\% \quad (5)$$

Where $M_{micelle}$ is the mass of β-carotene present in the micelle fraction and $M_{digesta}$ is the mass of β-carotene present in the digesta after the intestinal phase. $M_{encapsulated}$ was used for EOB and $M_{initial}$ was used for ROC.

2.8. Quantification of β-carotene using HPLC

Reverse phase HPLC (UltiMate 3000, Thermo Fisher Scientific, Waltham, USA) equipped with a photodiode array detector was used to quantify the concentration of β-carotene in the samples after encapsulation, after storage, mixed micelle fraction and total digesta fraction based on the method by Sadler and Dezman (1990) and Bushway (2006) with some modifications. β-carotene was chromatographed on a Phenomenex Onyx C18 column (100 x 4.6 mm) at 35 ºC with a solvent system of acetonitrile (ACN):MeOH:ethyl acetate (ETAC) (60:30:10) + 0.1% triethylamine (TEA) pumped at a flow rate of 1 mL/min for 10 min. The injection volume was 20 µL and the detection wavelength was 445 nm. A standard curve of β-carotene ranging from 0-100 µg/mL was prepared fresh for each run to calculate the concentration of β-carotene in the samples.

2.9. Oxidative stability of oleosome

2.9.1. Extraction of oil from freeze dried emulsion

5 mL of hexane:diethyl ether (1:1 v/v) was added to the freeze dried samples and incubated for 10 minutes while shaking. All content was transferred into a new tube. Additional 5 mL of hexane:diethyl ether (1:1 v/v) was added to the original tubes and incubated for 10 minutes with shaking. The content was transferred to the same tube and centrifuged at 4,816 *xg* for 5 minutes. The supernatant (with dissolved oil) was collected and dried under N_2 at room temperature overnight. The tubes with extracted oil were flushed with N_2, protected from light and stored at -20 ºC.

2.9.2. Determination of hydroperoxides

Lipid hydroperoxides was determined using the method by Matalanis et al. (2012) with some modifications. 5 mL of chloroform: methanol (2:1 v/v) were added to 0.1 mL of oil samples and vortexed for 30 seconds. 0.2 mL of this organic solvent was then added to 2.8 mL of chloroform:1-butanol (2:1 v/v), along with 15 µL of 3.94 M ammonium thiocyanate and 15 µL of ferrous iron solution which was prepared by mixing 0.132 M barium chloride and 0.144 M ferrous sulfate. A blank was prepared in the same manner but replacing the ferrous iron

solution with water. The mixture was vortexed and incubated in the dark at room temperature for 20 minutes. After which, the absorbance was measured at 510 nm in triplicates using a UV spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, USA). A standard curve of known amounts of cumene hydroperoxide ranging from 0.025 mM to 0.3 mM (coefficient correlation, R^2 = 0.9942) was prepared to calculate the concentration of lipid hydroperoxides in the samples.

2.9.3. Determination of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS was determined according to the method of Qiu et al. (2015) with some modifications. In a screw cap glass tube, 0.1 mL of oil sample was added to 5 mL of TBA solution which was made from 15 g of TCA, 0.375 g of TBA, 1.76 mL of HCl (37% w/w) and 82.9 mL of water. The mixture was vortexed and heated at 100 ºC for 10 minutes. The tubes were cooled on ice for 10 minutes and centrifuge at 10,000 *xg* for 15 minutes to remove any precipitate. After which, the absorbance was measured at 532 nm in triplicates using a UV spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, USA). A standard curve of known amounts of malonaldehyde bis(diethyl acetal) ranging from 0.04 mM to 0.2 mM (coefficient correlation, R^2 = 0.9887) was prepared to calculate the concentration of TBARS in the samples.

2.9.4. Determination of *p*-Anisidine value (*p*-AV)

p-Anisidine value (*p*-AV) in the oil samples was determined according to the standard method from the International Union of Pure and Applied Chemistry (IUPAC) (1979) with some modifications. 0.1 mL of oil samples were added to 2 mL of isooctane. 2 mL of this organic solvent solution was then added to 400 µL of 0.25% *p*-anisidine (in glacial acetic acid). A solvent blank was prepared in the same manner using water instead of oil and a sample blank was prepared in the same manner with glacial acetic acid instead of 0.25% *p*-anisidine to correct for the color from β-carotene. The samples were vortexed and incubated in the dark at room temperature for 10 minutes. After which, the absorbance was measured at 350 nm. The *p*-AV was calculated using Eq. (6):

$$p - AV = 2.1 * \frac{1.2 * A_S}{M_{oil}} \quad (6)$$

Where A_s is the absorbance of the sample (corrected for β-carotene sample blank if applicable) and M_{oil} is the mass of the oil added.

2.10. Statistical analysis

All experimental data were processed using Microsoft Excel and reported as mean ± standard deviation (stdev). Student's t-test (2-tail, unpaired, unequal variances) was used to determine any significant differences in the mean particle size between EOB and OBC after HPH and stability test, in the amount of β-carotene in EOB and ROC after digestion and stability test, and in the amount of oxidation products in EOB and OBC after stability test. Differences were considered significant at $p<0.05$.

3. Results & Discussion

3.1. Characterisation of β-carotene-loaded oleosome (EOB)

3.1.1. Encapsulation efficiency

The encapsulation efficiency of the EOB had a range of 65% to 88% between different batches (Table 1). However, when the EOB was centrifuged to isolate the oleosome layer, no sediments was seen at the base of the tube and no free oil layer was seen at the top (Appendix I). Thus, it was assumed that the dissolved β-carotene had been successfully encapsulated. The reason that encapsulation efficiency had a large variation and was not above 90% as seen in other papers (Acevedo et al., 2014; Zheng et al., 2019) that used oleosome as a carrier was probably due to some degradation of β-carotene during sample preparation and the variability of β-carotene that was dissolved in rapeseed oil. The duration of sonication and heating of β-carotene in rapeseed oil to dissolve it was not standardized, leading to large variability in the encapsulation efficiency. As such, this should be optimized to reduce variation and to maximize the encapsulation efficiency.

Table 1. Encapsulation efficiency of oleosome with β-carotene (EOB) from batch 1 (B1), batch 2 (B2) and batch 3 (B3). Data were reported as mean ± stdev (n=3).

	Encapsulation efficiency (%)
B1	87.4 ± 1.43
B2	73.0 ± 0.843
B3	65.0 ± 3.88

3.1.2. Particle size distribution

The volume mean diameter of the oleosome, $D_{4,3}$ and particle size distribution were reported after extraction and HPH (Fig. 3). The $D_{4,3}$ of the oleosome after extraction was found to be 1.71 ± 0.03 µm (Fig. 3A). This was within the size range of 2.0 µm as determined by transmission electron microscopy (TEM) (Katavic et al., 2006). After HPH, the mean diameter was 1.79 ± 0.16 µm and 1.76 ± 0.12 µm for OBC and EOB respectively and no significant difference was observed. All 3 samples had a monomodal particle size distribution (Fig. 3B). Acevedo et al. (2014) reported an increase in oleosome mean diameter after encapsulation of astaxanthin (AST). This could be due to the different encapsulation method used. The encapsulation of astaxanthin was based on partitioning of hydrophobic compounds, whereby AST intercalate into the membrane of the oleosome (Hama et al., 2012). For HPH, the membrane of the oleosome was broken up and the proteins and phospholipids subsequently reassembled naturally around the TAG core (with or without dissolved β-carotene). Thus, no difference was expected between loaded and unloaded oleosomes after HPH.

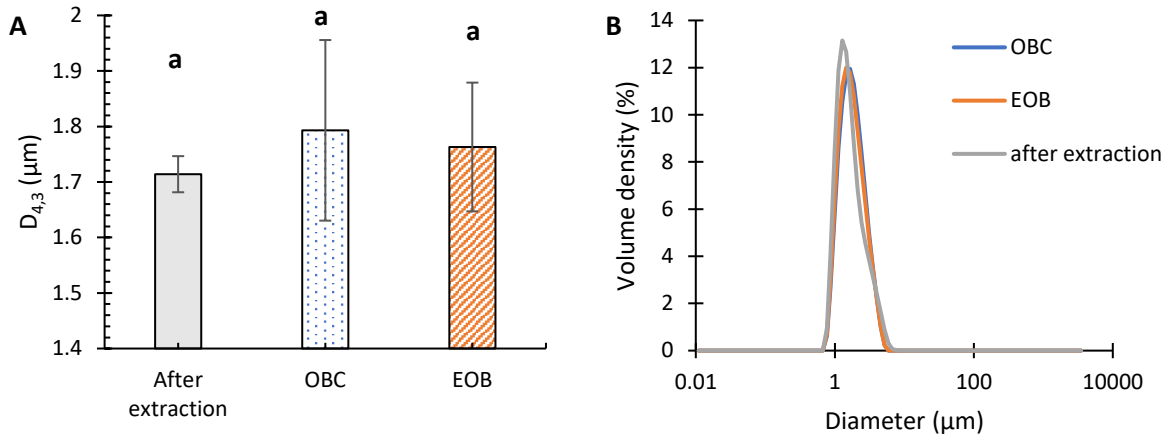

Fig. 3. Particle size (A) and particle size distributions (B) of the oleosome without (OBC) and with β-carotene (EOB) after high pressure homogenization (HPH) and oleosome after extraction. Data were reported as mean (n=3) ± stdev. Different lowercase letters mean significant difference (p<0.05).

3.2. Effect of oleosome encapsulation on the digestive stability and bioaccessibility of β-carotene

EOB and ROC samples were subjected to *in vitro* digestion and the β-carotene content remaining in the samples after digestion and present in the micellar phase were quantified (Fig. 4). The digestive stability indicates the amount of β-carotene that survived the transit through the oral, gastric and intestinal phases (Fig. 4A). The level of β-carotene in all samples were less than the initial amount present, ranging from 55%-90%. This suggests that there was some chemical degradation of β-carotene after simulation through the oral, gastric and intestinal phases. The digestive stability of EOB after digestion with 1x concentration of pancreatin (2000 U/mL) and bile salts (10 mM) was 89.9% ± 10.7%, which was slightly higher (p=0.11) than ROC (57.0% ± 13.5%). Based on a study by Zheng et al. (2019), a significantly higher digestive stability was achieved when curcumin, a similar lipophilic molecule with poor chemical stability, was encapsulated in soy oleosome compared to the control (curcumin in double distilled water). Since β-carotene is sensitive to pH changes and has poor stability especially at acidic pH (Qian et al., 2012; Shao et al., 2017), the encapsulation within the core of the oleosomes would have reduced the exposure of β-carotene to the different pH environment of the simulated GI tract and thus reduced its degradation during digestion. Taken together, the digestive stability of β-carotene should be significantly higher when encapsulated in oleosome compared to the unencapsulated form. The high p value (p=0.11) resulting in non-significant difference could be attributed to the small sample size (n=2). This resulted in a larger standard error compared to having a larger sample size of n≥3, and in turn resulted in a lower T statistic value and larger p value. As such, the experiment should be repeated with a larger sample size to determine if there is any significant difference in the digestive stability of β-carotene in EOB compared to ROC.

The bioaccessibility indicates the amount of β-carotene solubilized within the mixed micelles that is available for absorption (Fig. 4B). The micellar phase from the digesta was isolated through centrifugations and taken as the clear supernatant. Different centrifugation speed and time combinations had been used to isolate the mixed micelles in different studies (J. Liu et al., 2022; White et al., 2009), thus 2 different modified combinations were tested: 160,000 *xg* for 30 mins and 8,000 *xg* for 30 mins. After digestion with 1x concentration of pancreatin and bile salts, no β-carotene was detected in the supernatant after centrifugation at 160,000 *xg*. At 8,000 *xg*, the bioaccessibility was found to be 3.05% ± 4.32% in EOB and 0% in ROC (Fig. 4B). While the slight increase in bioaccessibility suggests that there was more β-carotene

available for absorption upon encapsulation compared to free β-carotene, this was only seen in 1 of the duplicates. It was noted that some orange oil droplet was present at the surface of the tube after centrifugation (Appendix I). Thus, it was possible that there were not enough emulsifiers present or limited lipid digestion resulting in few free fatty acids available to form mixed micelles. As such, the concentration of both pancreatin and bile salts were doubled. This was done to increase lipolysis to produce more free fatty acids from TAG and to increase the emulsifying capacity of the system to determine if the bioaccessibility could be increased as seen in Hou et al. (2014). While the amount of pancreatin and bile salts could not be increased as such *in vivo*, similar effect could be obtained through consumption of other food that contain fats and emulsifiers. Thus, the observation from these changes could still have some physiological relevance. However, after doubling the concentrations, the orange oil layer was still observed in the samples after both centrifugation and time combinations. As with 1x concentration, no β-carotene was present in the clear supernatant after centrifugation at 160,000 *xg.* At 8,000 *xg,* the bioaccessibility decreased to 0.68% ± 0.07% for EOB and increased slightly for ROC to detectable levels (0.97% ± 0.10%) (Fig. 4B). This could be due to the lower (73.9% ± 0.07%) and higher (78.9% ± 6.92%) digestive stability of EOB and ROC respectively compared to when 1x concentration was used (Fig. 4A). This then affected the amount of β-carotene that can be incorporated into the mixed micelles. The difference in digestive stability of β-carotene could be due to the difference in lipolysis rate. In EOB, the higher amount of pancreatin and bile salts likely increased the lipid digestion. This in turn increased the amount of β-carotene released from the TAG core to the environment and thus resulted in lower stability. In ROC, as the β-carotene was not encapsulated, changes in lipolysis rate should not affect the amount of β-carotene exposed to the environment and thus not affect its stability.

When soy oleosome in soymilk was used to encapsulate curcumin, the bioaccessibility of curcumin was 60% which was 50% more than the unencapsulated curcumin. However, this could be an overestimation as the soymilk contained other emulsifiers that could have contributed to the formation of mixed micelles (Zheng et al., 2019). In other static *in vitro* digestion studies where β-carotene was encapsulated in different carriers, variations in β-carotene bioaccessibility had been reported. Mun et al. (2015) reported a bioaccessibility of 55% (centrifugation at 4,000 rpm for 40 min at 25 ºC) when β-carotene (dissolved in corn oil) was encapsulated in hydrogel (Mun et al., 2015). Shao et al. reported a bioaccessibility of 24% to 38% (centrifugation at 10,000 rpm for 45 min at 4ºC) when β-carotene (dissolved in soybean oil) was encapsulated in emulsions stabilized by *Ulva fasciata* polysaccharides, gum arabic and beet pectin, with the control (soybean oil) having a bioaccessibility of 4.44% (Shao et al., 2017). Both studies used different buffer compositions and enzymes concentration. The pH for the gastric phase were also different at 2.5 and the pH for the intestinal phase was kept constant at pH 7 using NaOH with a pH-stat titration unit. In addition, the different matrices in the system could have resulted in different amounts and types of free fatty acids and emulsifiers available to form mixed micelles. Based on these studies, the oleosome encapsulated β-carotene was expected to have a much higher bioaccessibility than the amount quantified in this study. It could be that the micelles were not isolated from the centrifugation conditions used, or that there were insufficient free fatty acids to form mixed micelles.

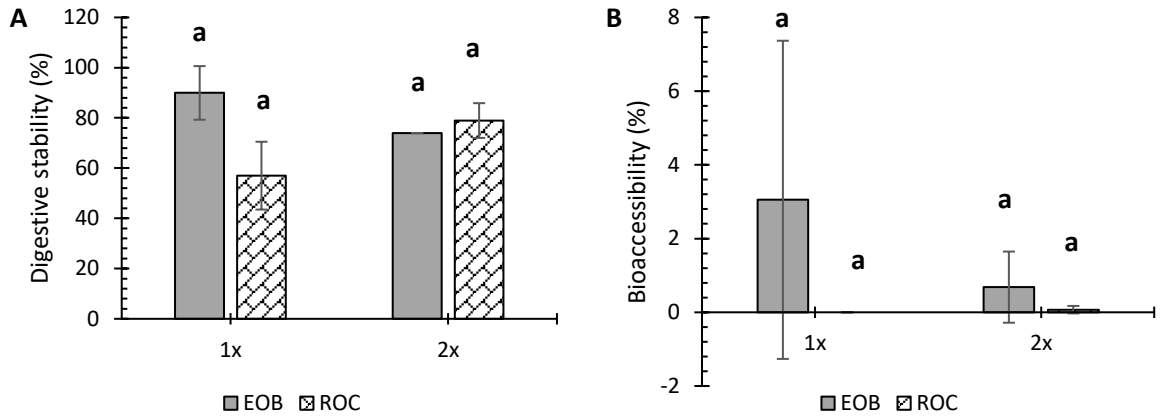

Fig. 4. Digestive stability (A) and bioaccessibility (B) of oleosomes with β-carotene (EOB) and β-carotene in rapeseed oil (ROC) after digestion with 1x or 2x concentration of pancreatin and bile salts. β-carotene present in the mixed micelles used for the quantification of bioaccessibility was isolated by centrifugation at 8,000 xg for 30 min. Data were reported as mean (n=2) ± stdev. Different lowercase letters mean significant difference (p<0.05).

3.3. Effect of oleosome encapsulation on the oxidative stability of β-carotene during storage

Accelerated shelf-life test was performed at 40 ºC in the dark for 21 days to determine the stability of β-carotene with and without encapsulation in oleosome. The temperature was fixed at 40 ºC as oleosomes are unstable above this temperature (Acevedo et al., 2014). The amount of β-carotene was quantified every 7 days (day 0, 7, 14 and 21) (Fig. 5). The level of β-carotene decreased with time in both EOB and ROC. This can also be observed in the tubes as the orange intensity decreased over time (Appendix I). However, the rate of degradation was different in both samples. β-carotene in EOB decreased gradually, while β-carotene in ROC had a sharp decrease after 7 days followed by a gradual decrease. The degradation in ROC was expected given that β-carotene is sensitive to oxygen and heat (Boon et al., 2010). The degradation observed in EOB could be due to partly exposed core of the oleosome from the HPH process, which then exposed β-carotene to thermal and oxidation stress leading to degradation. A significant difference ($p<0.05$) was found between the amount of β-carotene remaining in EOB and ROC at all time points (except day 14), with a higher amount of β-carotene remaining in EOB (44.3% ± 2.70%) compared to ROC (28.4% ± 3.87%) at the end of the storage period. This suggests that the encapsulation within the oleosome conferred a protective effect to the β-carotene against heat and oxidation during storage.

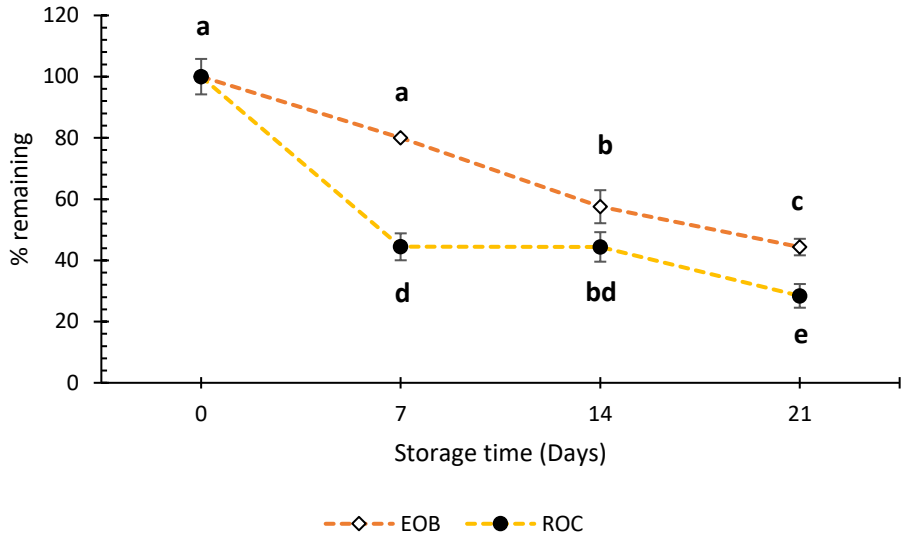

Fig. 5. Percentage of β-carotene remaining in the oleosomes with β-carotene (EOB) and β-carotene in rapeseed oil (ROC) after 0, 7, 14 and 21 days of storage at 40 ºC in the dark. Data (with dashed line to guide the eye) were reported as mean (n=3) ± stdev. Different lowercase letters mean significant difference (p<0.05).

3.4. Effect of encapsulated β-carotene on the physical and oxidative stability of oleosome during storage

3.4.1. Changes in particle size distribution during storage

Accelerated shelf-life test on EOB and OBC were performed at 40 ºC and the particle size was measured at the various time point to give an indication on the physical stability of the emulsion. As the isoelectric point of oleosins that are present on the membrane of the oleosomes are around 5.5 (Tzen et al., 1993; White et al., 2008), the pH of the emulsion has to be above or below that to maintain electrostatic repulsion. Thus, the stability test was done at pH 7 to represent neutral food systems. The volume mean particle diameter and particle size distribution were reported in Fig. 6 and Fig. 7. The mean particle size of both EOB and OBC increased with time, indicating that both emulsions were physically unstable towards flocculation and probably also coalescence. This also correlated with the creaming observation in the day 7, day 14 and day 21 samples (Appendix I). On day 7, the extent of aggregation was significantly higher in OBC than EOB as the mean particle size was larger in OBC (33.5 ± 0.49 µm) compared to EOB (27.7 ± 1.57 µm). This should likely be due to steric hindrance of β-carotene rather than changes in electrostatic repulsion between encapsulated and non-encapsulated oleosomes. As β-carotene is a non-charged molecule, the electrostatic interaction between oleosomes in both OBC and EOB should be similar (Ligia Focsan et al., 2019; Mascoli et al., 2021). However, this should be verified with zeta potential measurements. The increase in aggregation in EOB at later time points could be attributed to the loss of β-carotene as seen in section 3.3 which reduced the steric hindrance between particles. The large variation in mean particle size of samples at the later time points was due to the poor reproducibility in measurement of flocculated system as the aggregates are considered uniform spheres (Dickinson, 2010).

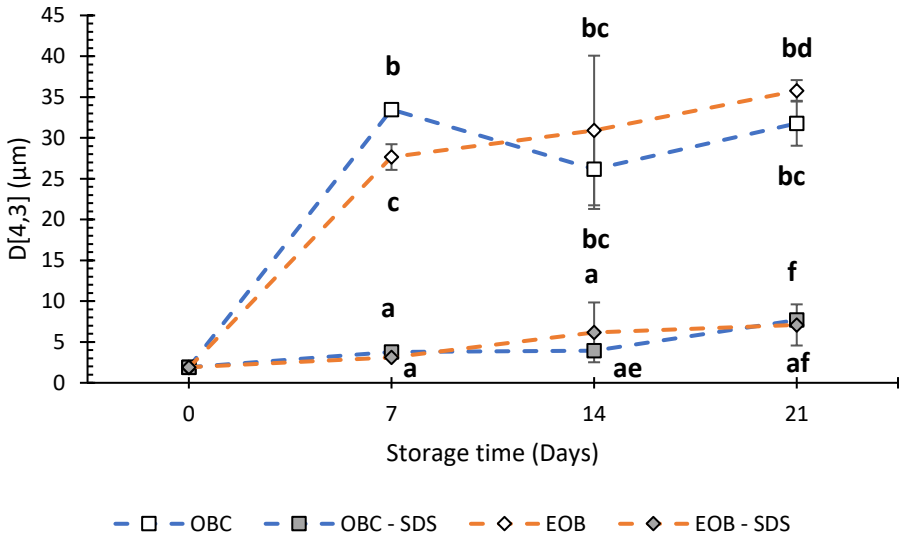

Fig. 6. Particle diameter of oleosomes without (OBC) and with β-carotene (EOB) with and without 1% SDS at pH 7 after different storage time at 40 ºC. Data (with dashed line to guide the eye) were reported as mean (n=3) ± stdev. Different lowercase letters mean significant difference (p<0.05).

1% sodium dodecyl sulfate (SDS) was added to the samples in 1:2 v/v ratio to disrupt the interactions within the aggregated oleosomes (Romero-Guzmán et al., 2020). After the addition of 1% SDS, the mean particle size in OBC decreased from 31.8 ± 2.75 µm to 7.69 ± 0.52 µm and 35.8 ± 1.31 µm to 7.09 ± 2.52 µm in EOB after 21 days. This means that the increase in mean particle size was mainly attributed to aggregation of oleosomes. However, since the particle size after 1% SDS addition was still higher than the initial particle size of <2 µm, coalescence also occurred in the emulsions. The aggregation and coalescence phenomenon were also reflected in the particle size distribution as seen by the polymodal distribution in both OBC and EOB (Fig. 7A and Fig. 7B) and monomodal distribution after the addition of 1% SDS (Fig. 7C and Fig. 7D). This could be due to insufficient repulsive forces and steric hindrance between oleosomes. At pH 7, the proteins on the surface of the oleosomes should be negatively charged and thus result in electrostatic repulsion between oleosomes. However, as the emulsions underwent HPH, the oleosomes were broken up and the membrane components were reassembled around the oil droplet. Thus, it could be that the surface of the oil was not completely covered with proteins, which resulted in coalescence when the droplets came into contact randomly due to Brownian motion (Dickinson, 2010). This could also explain why the increase in mean particle size was much higher than what was observed in Ding et al. (2014) when the oleosome emulsions were stored at the same pH of 7. However, in that study, the stability test was performed at room temperature and not at 40 ºC, and the difference in temperature could have contributed to the difference in the stability of the oleosome emulsion. From the particle size distribution, it seems that the extent of aggregation does not differ much with increasing storage time. On the other hand, the extent of coalescence increased with time as seen by the shift in the distribution of particle size from left to right.

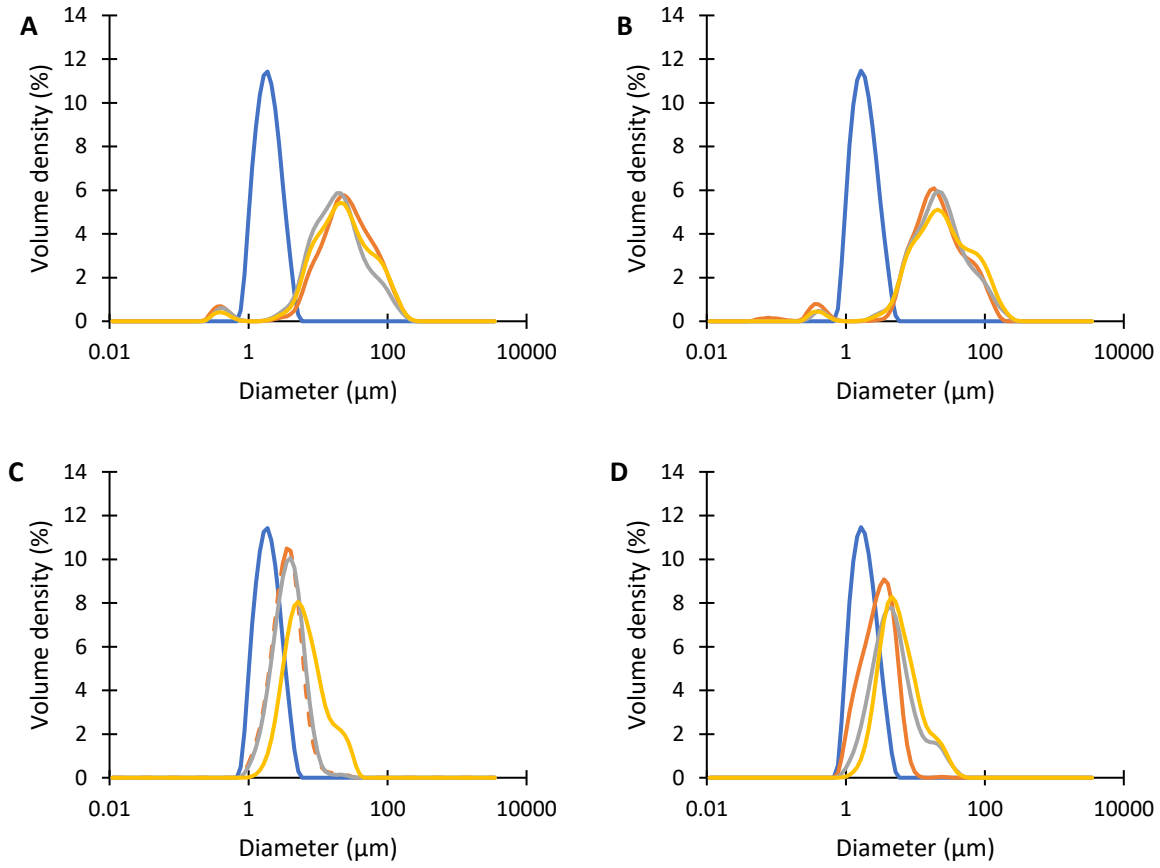

Fig. 7. Particle size distribution of oleosomes without (OBC) (A), oleosomes with β-carotene (EOB) (B), OBC with 1% SDS (C) and EOB with 1% SDS (D) at pH 7 and 40 ºC during storage at day 0 (——), day 7 (——), day 14 (——) and day 21 (——). Data were reported as mean (n=3).

3.4.2. Oxidative stability of β-carotene encapsulated oleosome

As β-carotene is an antioxidant, its effect upon encapsulation on the oxidative stability of oil (TAG) in the oleosomes was investigated by measuring the primary and secondary oxidation products formed at the end of each storage time point. The production of lipid hydroperoxides was measured as an indicator of primary oxidation (Fig. 8). On day 0, the hydroperoxides were 2.88 ± 0.73 mmol/kg oil in OBC and 2.34 ± 0.22 mmol/kg oil in EOB. For OBC, the hydroperoxides level decreased overtime to 1.10 ± 0.19 mmol/kg oil on day 21. For EOB, the amount of hydroperoxides increased to 2.98 ± 0.13 mmol/kg oil before decreasing to 1.23 ± 0.35 mmol/kg oil on day 21. The initial amount of hydroperoxides in both OBC and EOB were higher than what was reported in other studies. In Ding et al. (2018) and Kapchie et al. (2013), the hydroperoxides levels of soybean oleosomes at pH 7 was close to 0. The higher levels observed in this study could be due to the HPH process which exposed the oleosomes to heat, resulting in some lipid oxidation in the emulsions. In addition, the lipid core might not be fully covered by the phospholipids and proteins after HPH, thus exposing the lipids to more oxygen compared to intact oleosomes. On day 7, the hydroperoxides level was significantly higher in EOB compared to OBC ($p<0.05$). This could be attributed to the lower volume mean particle size of EOB compared to OBC on day 7 as seen in Fig. 6, resulting in a higher surface area/volume ratio and thus higher lipid oxidation rate.

As the hydroperoxides are unstable, they can be broken down into lower molecular weight compounds such as aldehydes (D. M. Shin et al., 2019). A low amount of hydroperoxides could either be due to little oxidation in the lipids or higher rate of decomposition of the

hydroperoxides into secondary products compared to the rate of formation of hydroperoxides. Therefore, measurement of the secondary oxidation products was also done to provide a better understanding of the oxidation state of the samples along with the primary oxidation data.

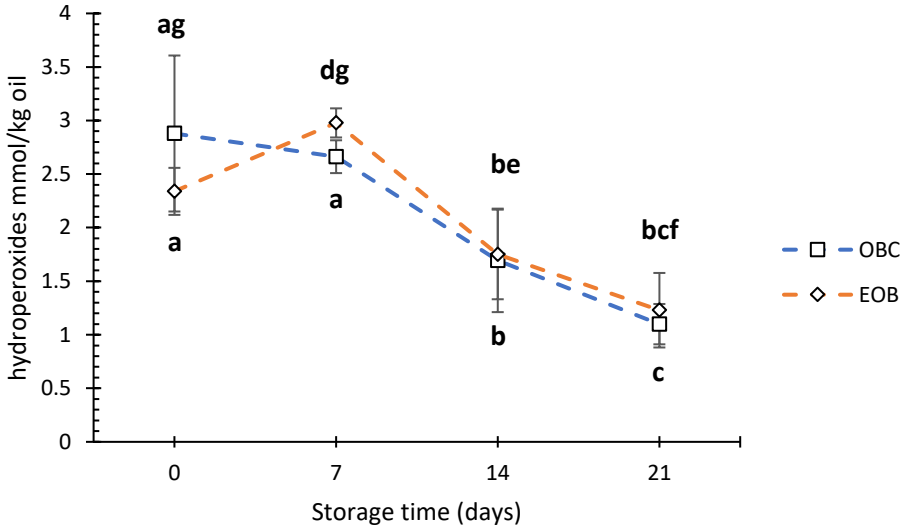

Fig. 8. Formation of lipid hydroperoxides in oleosomes without (OBC) and with β-carotene (EOB) with time during storage at 40 ºC and pH 7. Data (with dashed line to guide the eye) were reported as mean (n = 3) ± stdev. Different lowercase letters mean significant difference (p<0.05).

TBARS was measured as an indication of the secondary oxidation in the OBC and EOB at the various time points (Fig. 9A). The amount of TBARS quantified in both OBC and EOB were low (<0.05 mmol/kg) on day 0 and day 7 and was undetectable at the later time points. This was unexpected as a higher amount of TBARS and an increasing trend should be observed with time (Ding et al., 2018; Gray et al., 2010). This could be due to air bubbles present in the tubes from the boiling process during incubation which affected the absorbance measurement. While a significant difference ($p<0.05$) was observed in the amount of TBARS between EOB and OBC on day 7, the higher level of TBARS observed in EOB was likely an overestimation due to the orange pigment from β-carotene that was not corrected in the blank. As such, *p*-anisidine test was also performed to quantify the secondary oxidation products.

The *p*-AV increased with time in both EOB and OBC (Fig. 9B). The initial amount of secondary oxidation products was similar with OBC having a *p*-AV of 3.45 ± 0.14 and EOB having a *p*-AV of 3.65 ± 0.59. On day 7, the amount of secondary oxidation products was significantly higher ($p<0.05$) in OBC compared to EOB. At later time points, the formation of secondary oxidation products was faster in EOB than OBC, with EOB having higher significantly higher ($p<0.05$) *p*-AV than OBC after 21 days.

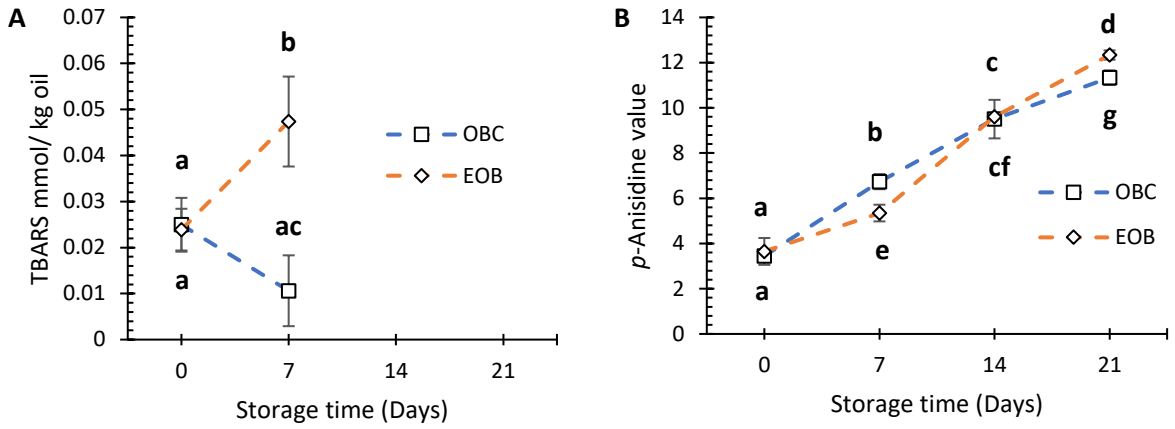

Fig. 9. Changes in TBARS (A) and p-AV (B) with time in oleosomes without (OBC) and with β-carotene (EOB) during storage at 40 ºC and pH 7. Data (with dashed line to guide the eye) were reported as mean (n = 3) ± stdev. Different lowercase letters mean significant difference (p<0.05).

As β-carotene is known for its antioxidant activity, it was hypothesized that the oil within the oleosome with encapsulated β-carotene would be protected against oxidation compared to without β-carotene. However, a lower amount of oxidation products in EOB was not observed in both primary and secondary oxidation data. One possible reason could be the location of β-carotene within the TAG core. If β-carotene is not present at the outer part of the TAG core, it could not exhibit its antioxidant property and oxidize itself in place of the lipids (Gray et al., 2010). Another possible reason could be that β-carotene is acting as a pro-oxidant rather than an antioxidant. If β-carotene is acting as an antioxidant, the increase in oxidation products could be due to the degradation of β-carotene over time as seen in Fig. 5. However, this does not justify the higher amount of primary and secondary oxidation products in EOB compared to OBC. Thus, it could be that β-carotene is enhancing instead of reducing lipid oxidation (Lee et al., 2003). In a high reactive oxygen species (ROS) environment, β-carotene may act as pro-oxidant (Shin et al., 2020). In this study, a high amount of lipid hydroperoxides was present on day 0, which constitutes a high ROS environment. β-carotene peroxy radicals can be formed from the lipid peroxy radicals which can contribute to the chain reaction of lipid oxidation (Burton & Ingold, 1984; Iannone et al., 1998). β-carotene can also become radical cation by donating elections to free radicals (Liebler, 1993; Mortensen et al., 2001). The β-carotene radical cation can oxidize tocopherols (Liebler, 1993) which then reduce the antioxidant capacity of the system, resulting in higher lipid oxidation in the TAG core. However, further studies are required to determine if the lack of protective effect on the oxidative stability is due to the location of β-carotene in the oleosome or the high ROS environment.

4. Conclusion

This study aimed to investigate the encapsulation efficiency of β-carotene into oleosomes using HPH and the subsequent effect on the gastrointestinal fate and oxidative stability of the β-carotene loaded oleosome emulsion. The encapsulation efficiency ranged between 65% to 88% which depended on the solubility of β-carotene in rapeseed oil. In addition, there was no difference in mean particle size and particle size distribution between the OBC, EOB, and the oleosome after extraction from oil seed. Thus, HPH is a feasible method to encapsulate β-carotene into oleosome.

No clear protective effect of encapsulation on the digestive stability of β-carotene can be observed between EOB (89.9% ± 10.7%) and ROC (57.0% ± 13.5%). In addition, the effect on the bioaccessibility cannot be concluded due to the limited amount of mixed micelles isolated. Therefore, further studies are required before determining if oleosome is a suitable carrier to potentially improve the bioavailability of β-carotene in food systems.

Based on the accelerated storage test, a higher oxidative stability of β-carotene was obtained when encapsulated in oleosome (44.3% ± 2.70%) compared to unencapsulated form (28.4% ± 3.87%). This suggests that encapsulation within oleosome can confer protection to β-carotene against degradation during storage, and thus could help preserve β-carotene when used as a delivery system in food.

Both OBC and EOB were physically instable during storage as indicated by creaming and the large mean particle size (>25 µm) which was likely due to the HPH process. No improvement in the oxidative stability of lipids in oleosome was obtained in the presence of β-carotene. On the contrary, a higher amount of lipid oxidation products was present with β-carotene. This could be due to the high ROS environment in the initial samples (>2 mmol hydroperoxides/kg oil) from the encapsulation method. As such, the HPH encapsulation method requires further optimization to improve the physical and oxidative stability of the emulsions before application in food.

5. Recommendations

While HPH was demonstrated to be able to encapsulate β-carotene into oleosomes, the encapsulation efficiency was highly dependent on the amount of β-carotene that was solubilized in rapeseed oil. Therefore, the sonication and heating step to solubilize β-carotene in rapeseed oil should be standardized for future work. Attempts should also be made to dissolve higher amounts of β-carotene.

No significant difference in the mean particle size and particle size distribution was observed between the native, unencapsulated and β-carotene encapsulated oleosomes after HPH. However, an electron microscope should be used to provide further understanding of the membrane structure of the oleosome after HPH which could also provide insights into the possible location of β-carotene in the oleosome.

The non-significant difference in digestive stability between EOB and ROC, could probably be due to the small sample size which affected the standard error of mean and in turn resulted in a larger p value. As such, it was suggested to perform the *in vitro* digestion with a larger sample size ($n \geq 3$) to determine if the difference observed was indeed non-significant. The effect of encapsulation on the bioaccessibility of β-carotene cannot be firmly concluded given that β-carotene was only detected in 1 of the duplicates in EOB. Despite the increase in the concentration of bile salts and pancreatin to facilitate the formation of more mixed micelles, no improvement in the bioaccessibility was seen. This suggests that there might not be enough free fatty acids in the system to form mixed micelles, or that the mixed micelles isolation conditions were not optimal. Thus, future studies should involve the addition of fats in the system and/or optimization of the centrifugation conditions to generate and/or isolate more mixed micelles to determine the effect of encapsulation on the bioaccessibility of β-carotene.

During the storage period, aggregation and coalescence occurred in both OBC and EOB. Given the poor reproducibility of the measurement of large flocs, it was suggested to visualize and measure the size of the flocs with a light microscope to complement the data obtained from the particle size measurement. In addition, it was recommended to measure the zeta potential of the samples to determine any differences between samples at various time points. Given that the physical instability of the emulsion was likely due to the HPH process, further research should be done to optimize the method to obtain droplets that are better covered with proteins and phospholipids. This would make the emulsion more stable against aggregation and/or coalescence, thereby, making it more applicable for use in food systems.

The oxidative stability of β-carotene was significantly higher in EOB than ROC during the accelerated storage test at 40 ºC. On the other hand, no protective effect on the oxidative stability of lipids within the oleosome was seen when β-carotene was encapsulated. Instead, based on the amount of primary and secondary lipid oxidation products, the extent of lipid oxidation appears to be higher in the presence of β-carotene. This could be attributed to the location of β-carotene in the oleosomes which limited the antioxidant capability towards the lipids. It is also possible that the high ROS environment in the sample (based on the initial lipid hydroperoxides level caused β-carotene to act as a pro-oxidant. If this is the case, the tocopherol amount from the oleosome should decrease. Thus, the tocopherol content should be measured to determine if β-carotene is indeed acting as a pro-oxidant. Alternatively, efforts could be made to optimize the HPH process to reduce the amount of lipid hydroperoxides in the initial starting sample before repeating the accelerated storage test.

6. References

Acevedo-Fani, A., Dave, A., & Singh, H. (2020). Nature-Assembled Structures for Delivery of Bioactive Compounds and Their Potential in Functional Foods. *Frontiers in Chemistry*, *8*, Article e564021. https://doi.org/10.3389/FCHEM.2020.564021/

Acevedo, F., Rubilar, M., Jofré, I., Villarroel, M., Navarrete, P., Esparza, M., Romero, F., Vilches, E. A., Acevedo, V., & Shene, C. (2014). Oil bodies as a potential microencapsulation carrier for astaxanthin stabilisation and safe delivery. *Journal of Microencapsulation*, *31*(5), 488–500. https://doi.org/10.3109/02652048.2013.879931

Anand, R., Mohan, L., & Bharadvaja, N. (2022). Disease Prevention and Treatment Using β-Carotene: the Ultimate Provitamin A. *Revista Brasileira de Farmacognosia*, *32*(4), 491–501. https://doi.org/10.1007/S43450-022-00262-W/FIGURES/5

Boon, C. S., McClements, D. J., Weiss, J., & Decker, E. A. (2010). Factors Influencing the Chemical Stability of Carotenoids in Foods. *Critical Reviews in Food Science and Nutrition*, *50*(6), 515–532. https://doi.org/10.1080/10408390802565889

Boucher, J., Cengelli, F., Trumbic, D., & Marison, I. W. (2008). Sorption of hydrophobic organic compounds (HOC) in rapeseed oil bodies. *Chemosphere*, *70*(8), 1452–1458. https://doi.org/10.1016/j.chemosphere.2007.08.065

Britton, G. (1995). Structure and properties of carotenoids in relation to function. *The FASEB Journal*, *9*(15), 1551–1558. https://doi.org/10.1096/FASEBJ.9.15.8529834

Burton, G. W., & Ingold, K. U. (1984). β-Carotene: an Unusual Type of Lipid Antioxidant. *Science*, *224*(4649), 569–573. https://doi.org/10.1126/SCIENCE.6710156

Bushway, R. J. (2006). Separation of Carotenoids in Fruits and Vegetables by High Performance Liquid Chromatography. *Journal of Liquid Chromatography*, *8*(8), 1527–l1547. https://doi.org/10.1080/01483918508067163

Chaijan, M., Srirattanachot, K., Nisoa, M., Cheong, L. Z., & Panpipat, W. (2021). Role of antioxidants on physicochemical properties and in vitro bioaccessibility of β-carotene loaded nanoemulsion under thermal and cold plasma discharge accelerated tests. *Food Chemistry*, *339*, Article e128157. https://doi.org/10.1016/J.FOODCHEM.2020.128157

De Chirico, S., di Bari, V., Foster, T., & Gray, D. (2018). Enhancing the recovery of oilseed rape seed oil bodies (oleosomes) using bicarbonate-based soaking and grinding media. *Food Chemistry*, *241*, 419–426. https://doi.org/10.1016/J.FOODCHEM.2017.09.008

De Chirico, S., di Bari, V., Romero Guzmán, M. J., Nikiforidis, C. V., Foster, T., & Gray, D. (2020). Assessment of rapeseed oil body (oleosome) lipolytic activity as an effective predictor of emulsion purity and stability. *Food Chemistry*, *316*, Article e126355. https://doi.org/10.1016/J.FOODCHEM.2020.126355

Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces*, *81*(1), 130–140. https://doi.org/10.1016/J.COLSURFB.2010.06.033

Ding, J., Xu, Z., Qi, B., Jiang, L., & Sui, X. (2018). Physicochemical and oxidative stability of a soybean oleosome-based emulsion and its in vitro digestive fate as affected by (−)-epigallocatechin-3-gallate. *Food & Function*, *9*(12), 6146–6154. https://doi.org/10.1039/C8FO01215F

Drosou, C., Krokida, M., & Biliaderis, C. G. (2022). Encapsulation of β-carotene into food-grade nanofibers via coaxial electrospinning of hydrocolloids: Enhancement of oxidative stability and photoprotection. *Food Hydrocolloids*, *133*, Article e107949. https://doi.org/10.1016/J.FOODHYD.2022.107949

Fisk, I. D., Linforth, R., Trophardy, G., & Gray, D. (2013). Entrapment of a volatile lipophilic aroma compound (d-limonene) in spray dried water-washed oil bodies naturally derived from sunflower seeds (Helianthus annus). *Food Research International*, *54*(1), 861–866. https://doi.org/10.1016/J.FOODRES.2013.08.024

Gibson, R. S. (2016). The Role of Diet- and Host-Related Factors in Nutrient Bioavailability and Thus in Nutrient-Based Dietary Requirement Estimates. *Food and Nutrition Bulletin*, *28*(1 SUPPL. 1). https://doi.org/10.1177/15648265070281S108

Gray, D. A., Payne, G., McClements, D. J., Decker, E. A., & Lad, M. (2010). Oxidative stability of Echium plantagineum seed oil bodies. *European Journal of Lipid Science and Technology*, *112*(7), 741–749. https://doi.org/10.1002/EJLT.200900280

Gul, K., Tak, A., Singh, A. K., Singh, P., Yousuf, B., & Wani, A. A. (2015). Chemistry, encapsulation, and health benefits of β-carotene - A review. *Cogent Food & Agriculture*, *1*(1). https://doi.org/10.1080/23311932.2015.1018696

Hama, S., Uenishi, S., Yamada, A., Ohgita, T., Tsuchiya, H., Yamashita, E., & Kogure, K. (2012). Scavenging of Hydroxyl Radicals in Aqueous Solution by Astaxanthin Encapsulated in Liposomes. *Biological and Pharmaceutical Bulletin*, *35*(12), 2238–2242. https://doi.org/10.1248/BPB.B12-00715

Hou, Z., Liu, Y., Lei, F., & Gao, Y. (2014). Investigation into the in vitro release properties of β-carotene in emulsions stabilized by different emulsifiers. *LWT - Food Science and Technology*, *59*(2), 867–873. https://doi.org/10.1016/J.LWT.2014.07.045

Iannone, A., Rota, C., Bergamini, S., Tomasi, A., & Canfield, L.M. (1998). Antioxidant activity of carotenoids: An electron-spin resonance study on β-carotene and lutein interaction with free radicals generated in a chemical system. Journal of Biochemical and Molecular Toxicology, *12*(5), 299-304. https://doi.org/10.1002/(SICI)1099-0461(1998)12:5<299::AID-JBT6>3.0.CO;2-G

IUPAC. (1979). Determination of the p-anisidine value (p-AV) (#2.504). Paquot, C. (Ed.), *Standard Methods for the Analysis of Oils, Fats and Derivatives* sixth edition (pp. *143-144).* Pergamon Press, NY.

Jacobsen, C. (2000). Sensory impact of lipid oxidation in complex food systems. *European Journal of Lipid Science and Technology, 101(12),* 484-492. https://doi.org/10.1002/(SICI)1521-4133(199912)101:12<484::AID-LIPI484>3.0.CO;2-H

Jalali-Jivan, M., Rostamabadi, H., Assadpour, E., Tomas, M., Capanoglu, E., Alizadeh-Sani, M., Kharazmi, M. S., & Jafari, S. M. (2022). Recent progresses in the delivery of β-carotene: From nano/microencapsulation to bioaccessibility. *Advances in Colloid and Interface Science*, *307*, Article e102750. https://doi.org/10.1016/J.CIS.2022.102750

Kapchie, V. N., Yao, L., Hauck, C. C., Wang, T., & Murphy, P. A. (2013). Oxidative stability of soybean oil in oleosomes as affected by pH and iron. *Food Chemistry*, *141*(3), 2286–2293. https://doi.org/10.1016/J.FOODCHEM.2013.05.018

Katavic, V., Agrawal, G. K., Hajduch, M., Harris, S. L., & Thelen, J. J. (2006). Protein and lipid composition analysis of oil bodies from two Brassica napus cultivars. *Proteomics*, *6*(16), 4586–4598. https://doi.org/10.1002/PMIC.200600020

Lee, J. H., Ozcelik, B., & Min, D. B. (2003). Electron Donation Mechanisms of β-Carotene as a Free Radical Scavenger. *Journal of Food Science*, *68*(3), 861–865. https://doi.org/10.1111/J.1365-2621.2003.TB08256.X

Liebler, D. C. (1993). Antioxidant Reactions of Carotenoids. *Annals of the New York Academy of Sciences*, *691*(1), 20–31. https://doi.org/10.1111/J.1749-6632.1993.TB26154.X

Ligia Focsan, A., Polyakov, N. E., & Kispert, L. D. (2019). Supramolecular Carotenoid Complexes of Enhanced Solubility and Stability—The Way of Bioavailability Improvement. *Molecules, 24*(21), 3947. https://doi.org/10.3390/MOLECULES24213947

Lin, Q., Liang, R., Williams, P. A., & Zhong, F. (2018). Factors affecting the bioaccessibility of β-carotene in lipid-based microcapsules: Digestive conditions, the composition, structure and physical state of microcapsules. *Food Hydrocolloids*, *77*, 187–203. https://doi.org/10.1016/J.FOODHYD.2017.09.034

Liu, C., Wang, R., He, S., Cheng, C., & Ma, Y. (2020). The stability and gastro-intestinal digestion of curcumin emulsion stabilized with soybean oil bodies. *LWT*, *131*, Article e109663. https://doi.org/10.1016/J.LWT.2020.109663

Liu, J., Liu, D., Bi, J., Liu, X., Lyu, Y., Verkerk, R., & Dekker, M. (2022). Micelle separation conditions based on particle size strongly affect carotenoid bioaccessibility assessment from juices after in vitro digestion. *Food Research International*, *151*, Article e110891. https://doi.org/10.1016/J.FOODRES.2021.110891

Mascoli, V., Liguori, N., Cupellini, L., Elias, E., Mennucci, B., & Croce, R. (2021). Uncovering the interactions driving carotenoid binding in light-harvesting complexes. *Chemical Science*, *12*(14), 5113–5122. https://doi.org/10.1039/D1SC00071C

Matalanis, A., Decker, E. A., & McClements, D. J. (2012). Inhibition of lipid oxidation by encapsulation of emulsion droplets within hydrogel microspheres. *Food Chemistry*, *132*(2), 766–772. https://doi.org/10.1016/J.FOODCHEM.2011.11.034

Maurya, V. K., Shakya, A., Aggarwal, M., Gothandam, K. M., Bohn, T., & Pareek, S. (2021). Fate of β-Carotene within Loaded Delivery Systems in Food: State of Knowledge. *Antioxidants 426*, *10*(3), 426. https://doi.org/10.3390/ANTIOX10030426

McClements, D. J. (2018). Enhanced delivery of lipophilic bioactives using emulsions: a review of major factors affecting vitamin, nutraceutical, and lipid bioaccessibility. *Food & Function*, *9*(1), 22–41. https://doi.org/10.1039/C7FO01515A

Mcclements, D. J., Zou, L., Zhang, R., Salvia-Trujillo, L., Kumosani, T., & Xiao, H. (2015). Enhancing Nutraceutical Performance Using Excipient Foods: Designing Food Structures and Compositions to Increase Bioavailability. *Comprehensive Reviews in Food Science and Food Safety*, *14*(6), 824–847. https://doi.org/10.1111/1541-4337.12170

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., MacIerzanka, A., MacKie, A., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function*, *5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Mortensen, A., Skibsted, L. H., & Truscott, T. G. (2001). The Interaction of Dietary Carotenoids with Radical Species. *Archives of Biochemistry and Biophysics*, *385*(1), 13–19. https://doi.org/10.1006/ABBI.2000.2172

Mourtzinos, I. & Biliaderis, C.G. (2017). Chapter 1 - principles and applications of encapsulation technologies to food materials. Krokida, M.K. (Ed.), *Thermal and nonthermal encapsulation methods* (pp. 1-37). CRC Press.

Mun, S., Kim, Y. R., Shin, M., & McClements, D. J. (2015). Control of lipid digestion and nutraceutical bioaccessibility using starch-based filled hydrogels: Influence of starch and surfactant type. *Food Hydrocolloids*, *44*, 380–389. https://doi.org/10.1016/J.FOODHYD.2014.10.013

National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 5280489, Beta-Carotene. Retrieved December 11, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Beta-Carotene.

Nikiforidis, C. V. (2019). Structure and functions of oleosomes (oil bodies). *Advances in Colloid and Interface Science*, *274,* Article e102039. https://doi.org/10.1016/j.cis.2019.102039

Nikiforidis, C. V., Biliaderis, C. G., & Kiosseoglou, V. (2012). Rheological characteristics and physicochemical stability of dressing-type emulsions made of oil bodies–egg yolk blends. *Food Chemistry*, *134*(1), 64–73. https://doi.org/10.1016/J.FOODCHEM.2012.02.058

Nikiforidis, C. V., & Kiosseoglou, V. (2011). Competitive displacement of oil body surface proteins by Tween 80 – Effect on physical stability. *Food Hydrocolloids*, *25*(5), 1063–1068. https://doi.org/10.1016/J.FOODHYD.2010.10.002

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry*, *132*(3), 1221–1229. https://doi.org/10.1016/J.FOODCHEM.2011.11.091

Qiu, C., Zhao, M., Decker, E. A., & McClements, D. J. (2015). Influence of protein type on oxidation and digestibility of fish oil-in-water emulsions: Gliadin, caseinate, and whey protein. *Food Chemistry*, *175*, 249–257. https://doi.org/10.1016/J.FOODCHEM.2014.11.112

Romero-Guzmán, M. J., Vardaka, E., Boom, R. M., & Nikiforidis, C. V. (2020). Influence of soaking time on the mechanical properties of rapeseed and their effect on oleosome extraction. *Food and Bioproducts Processing*, *121*, 230–237. https://doi.org/10.1016/J.FBP.2020.03.006

Sadler, G., Davis, J., & Dezman, D. (1990). Rapid Extraction of Lycopene and β-Carotene from Reconstituted Tomato Paste and Pink Grapefruit Homogenates. *Journal of Food Science*, *55*(5), 1460–1461. https://doi.org/10.1111/J.1365-2621.1990.TB03958.X

Shao, P., Qiu, Q., Xiao, J., Zhu, Y., & Sun, P. (2017). Chemical Stability and in vitro release properties of β-carotene in emulsions stabilized by Ulva fasciata polysaccharide. *International Journal of Biological Macromolecules*, *102*, 225–231. https://doi.org/10.1016/J.IJBIOMAC.2017.03.186

Shin, D. M., Kim, D. H., Yune, J. H., Kwon, H. C., Kim, H. J., Seo, H. G., & Han, S. G. (2019). Oxidative Stability and Quality Characteristics of Duck, Chicken, Swine and Bovine Skin Fats Extracted by Pressurized Hot Water Extraction. *Food Science of Animal Resources*, *39*(3), 446–458. https://doi.org/10.5851/KOSFA.2019.E41

Shin, J., Song, M. H., Oh, J. W., Keum, Y. S., & Saini, R. K. (2020). Pro-oxidant Actions of Carotenoids in Triggering Apoptosis of Cancer Cells: A Review of Emerging Evidence. *Antioxidants*, *9*(6), 532. https://doi.org/10.3390/ANTIOX9060532

Tzen, J. T. C., Cao, Y. Z., Laurent, P., Ratnayake, C., & Huang, A. H. C. (1993). Lipids, Proteins, and Structure of Seed Oil Bodies from Diverse Species. *Plant Physiology*, *101*(1), 267. https://doi.org/10.1104/PP.101.1.267

White, D. A., Fisk, I. D., Makkhun, S., & Gray, D. A. (2009). In vitro assessment of the bioaccessibility of tocopherol and fatty acids from sunflower seed oil bodies. *Journal of Agricultural and Food Chemistry*, *57*(13), 5720–5726. https://doi.org/10.1021/JF9003412/ASSET/IMAGES/LARGE/JF-2009-003412_0003.JPEG

White, D. A., Fisk, I. D., Mitchell, J. R., Wolf, B., Hill, S. E., & Gray, D. A. (2008). Sunflower-seed oil body emulsions: Rheology and stability assessment of a natural emulsion. *Food Hydrocolloids*, *22*(7), 1224–1232. https://doi.org/10.1016/J.FOODHYD.2007.07.004

Yang, J., Zhang, Y., Na, X., & Zhao, A. (2022). β-Carotene Supplementation and Risk of Cardiovascular Disease: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Nutrients*, *14*(6), 1284. https://doi.org/10.3390/NU14061284/S1

Yuan, Y., Gao, Y., Zhao, J., & Mao, L. (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Research International*, *41*(1), 61–68. https://doi.org/10.1016/J.FOODRES.2007.09.006

Zhang, P., & Omaye, S. T. (2000). β-Carotene and protein oxidation: effects of ascorbic acid and α-tocopherol. *Toxicology*, *146*(1), 37–47. https://doi.org/10.1016/S0300-483X(00)00160-8

Zheng, B., Zhang, X., Peng, S., & McClements, D. J. (2019). Impact of curcumin delivery system format on bioaccessibility: nanocrystals, nanoemulsion droplets, and natural oil bodies. *Food & Function*, *10*(7), 4339–4349. https://doi.org/10.1039/C8FO02510J

Appendix I - Images

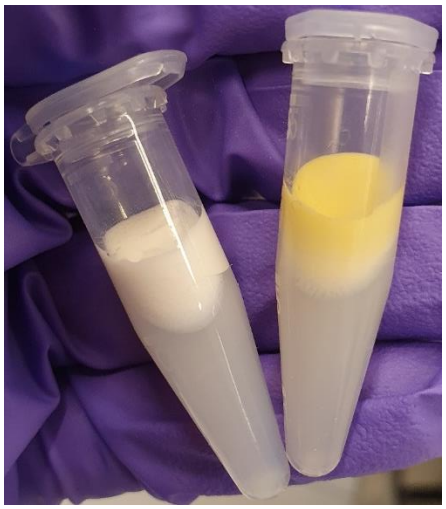

Fig. 10. Appearance of the oleosome cream from oleosome without (OBC) (left) and with β-carotene (EOB) (right) after centrifugation.

Fig. 11. Digested oleosome with β-carotene (EOB) after centrifugation to isolate the mixed micelles fraction.

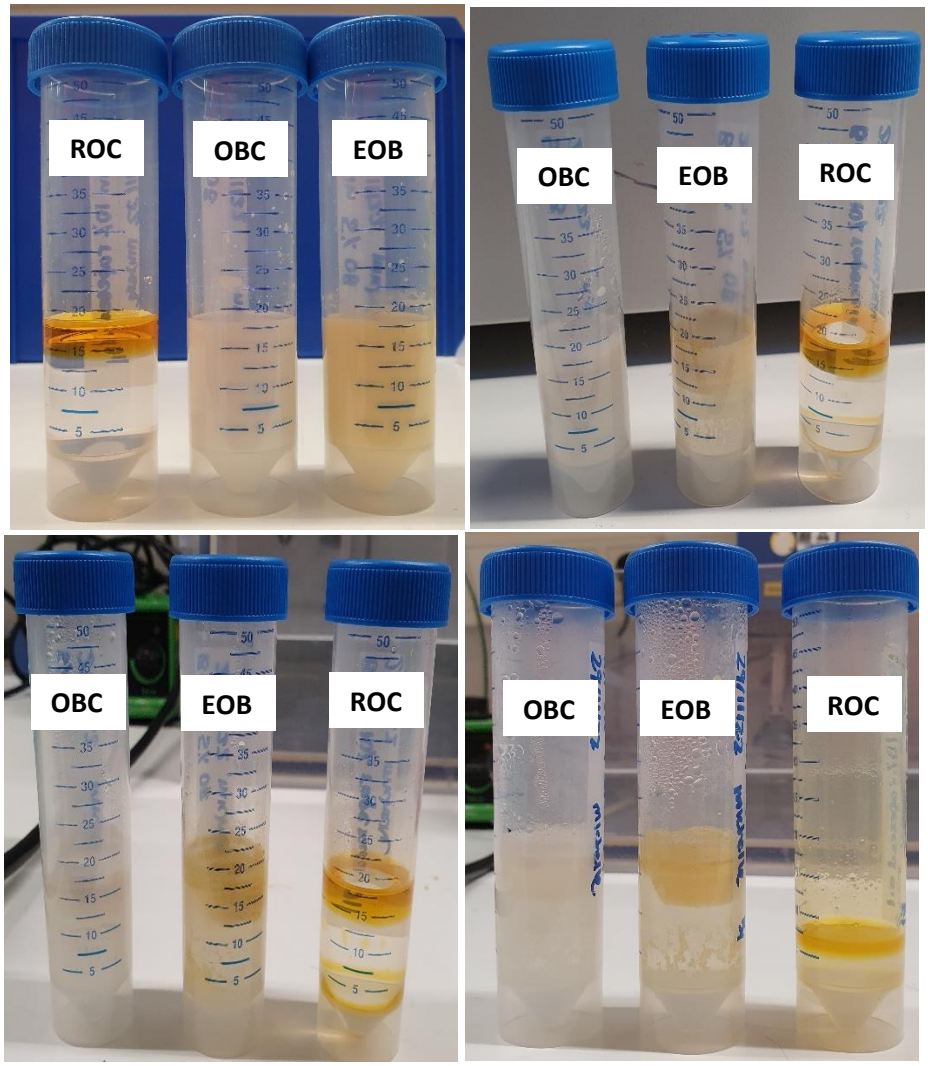

Fig. 12. Appearance of oleosome without β-carotene (OBC), oleosome with β-carotene (EOB), β-carotene in rapeseed oil (ROC) on day 0 (A), day 7 (B), day 14 (C), day 21 (d) of stability test at 40 ºC.

Appendix II – Data analysis

Table 2. T-test (unequal variance) analysis of the mean particle size of oleosome without (OBC) and with β-carotene (EOB) after high pressure homogenization (HPH). Presented are the p values. Values are considered significant when p≤0.05.

		Mean particle size
OBC	EOB	0.4147291

Table 3. T-test (unequal variance) analysis on the digestive stability of oleosome with β-carotene (EOB) and β-carotene in rapeseed oil (ROC). Presented are the p values. Values are considered significant when p≤0.05.

		Digestive stability
1x OBC	1x EOB	0.113586
2x OBC	2x EOB	0.492423857
1x OBC	2x OBC	0.280298
1x EOB	1x EOB	0.289424

Table 4. T-test (unequal variance) analysis on the oxidative stability of oleosome with β-carotene (EOB) and β-carotene in rapeseed oil (ROC) during storage at 40 ºC and pH 7. Presented are the p values. Values are considered significant when p≤0.05.

		Oxidative stability
D7 EOB	D7 ROC	0.00507
D14 EOB	D14 ROC	0.105
D21 EOB	D21 ROC	0.00424
D0 EOB	D7 EOB	0.0879
D0 EOB	D14 EOB	0.0118
D0 EOB	D21 EOB	0.000175
D7 EOB	D14 EOB	0.0186
D7 EOB	D21 EOB	0.00192
D14 EOB	D21 EOB	0.0324
D0 ROC	D7 ROC	0.0115
D0 ROC	D14 ROC	0.0139
D0 ROC	D21 ROC	0.00662
D7 ROC	D14 ROC	0.994
D7 ROC	D21 ROC	0.00543
D14 ROC	D21 ROC	0.0361

Table 5. T-test (unequal variance) analysis on the mean particle size of oleosome without (OBC) and with β-carotene (EOB) during storage at 40 ºC and pH 7 with and without 1% sodium doedecyl sulfate (SDS). Presented are the p values. Values are considered significant when p≤0.05.

		Particle Size
D7 OBC	D7 EOB	0.0257
D14 OBC	D14 EOB	0.488
D21 OBC	D21 EOB	0.109
D7 OBC - SDS	D7 EOB - SDS	0.0645
D14 OBC - SDS	D14 EOB – SDS	0.340
D21 OBC - SDS	D21 EOB - SDS	0.727

D7 OBC	D14 OBC	0.124
D7 OBC	D21 OBC	0.407
D14 OBC	D21 OBC	0.182
D7 OBC - SDS	D14 OBC - SDS	0.600
D7 OBC - SDS	D21 OBC - SDS	0.0015
D14 OBC - SDS	D21 OBC - SDS	0.00173
D7 EOB	D14 EOB	0.606
D7 EOB	D21 EOB	0.00236
D14 EOB	D21 EOB	0.459
D7 EOB - SDS	D14 EOB – SDS	0.284
D7 EOB - SDS	D21 EOB - SDS	0.112
D14 EOB – SDS	D21 EOB - SDS	0.741
D7 EOB	D21 OBC	0.888
D7 OBC	D21 EOB	0.0661

Table 6. T-test (unequal variance) analysis on the hydroperoxides level of oleosome without (OBC) and with β-carotene (EOB) during storage at 40 ºC and pH 7. Presented are the p values. Values are considered significant when p≤0.05.

		Hydroperoxides
D0 OBC	D0 EOB	0.132
D7 OBC	D7 EOB	0.00364
D14 OBC	D14 EOB	0.836
D21 OBC	D21 EOB	0.443
D0 OBC	D7 OBC	0.508
D0 OBC	D14 OBC	0.00893
D0 OBC	D21 OBC	0.00115
D7 OBC	D14 OBC	0.00340
D7 OBC	D21 OBC	2.15E-08
D14 OBC	D21 OBC	0.0306
D0 EOB	D7 EOB	0.000301
D0 EOB	D14 EOB	0.0158
D0 EOB	D21 EOB	0.000168
D7 EOB	D14 EOB	0.000481
D7 EOB	D21 EOB	2.64E-05
D14 EOB	D21 EOB	0.0411
D14 OBC	D21 EOB	0.0878
D14 EOB	D21 OBC	0.0103
D0 OBC	D7 EOB	0.757

Table 7. T-test (unequal variance) analysis on the TBARS level of oleosome without (OBC) and with β-carotene (EOB) during storage at 40 ºC and pH 7. Presented are the p values. Values are considered significant when p≤0.05.

		TBARS
D0 OBC	D0 EOB	0.0178
D7 OBC	D7 EOB	0.00689

D0 OBC	D7 OBC	0.863
D0 EOB	D7 EOB	0.0325
D0 OBC	D7 EOB	0.0421
D0 EOB	D7 OBC	0.0821
D0 EOB	D7 OBC	0.0821

Table 8. T-test (unequal variance) analysis on the p*-AV of oleosome without (OBC) and with β-carotene (EOB) during storage at 40 ºC and pH 7. Presented are the p values. Values are considered significant when p≤0.05.*

		p-AV
D0 OBC	D0 EOB	0.634
D7 OBC	D7 EOB	0.00845
D14 OBC	D14 EOB	0.849
D21 OBC	D21 EOB	0.0202
D0 OBC	D7 OBC	0.000569
D0 OBC	D14 OBC	0.00673
D0 OBC	D21 OBC	3.86E-05
D7 OBC	D14 OBC	0.0136
D7 OBC	D21 OBC	6.92E-05
D14 OBC	D21 OBC	0.0400
D0 EOB	D7 EOB	0.0246
D0 EOB	D14 EOB	0.00331
D0 EOB	D21 EOB	0.00174
D7 EOB	D14 EOB	0.00251
D7 EOB	D21 EOB	9.27E-05
D14 EOB	D21 EOB	0.00189
D17 OBC	D14 EOB	0.00454
D14 EOB	D21 OBC	0.0117
D0 OBC	D7 EOB	0.00367