Sustainable Food Technology

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Sustainable Food Technol.*, 2026, **4**, 350

Enhancing bioaccessibility and stability of carotenoids from pumpkin peel by nanostructured lipid carriers

Nicola Pinna,[a] Francesca Blasi, *[a] Federica Ianni,[a] Lara Manyes, [b] Lina Cossignani, [a] Maurizio Ricci[a] and Aurélie Schoubben[a]

Carotenoids were isolated from pumpkin peel powder (PP). Nanoencapsulation was studied to improve their bioaccessibility and stability. Carotenoids of PP showed the highest value of bioaccessibility for free xanthophylls, followed by β-carotene, di- and mono-esterified xanthophylls. The same trend was observed for carotenoid extract (CE). When CE was encapsulated into lipid nanoparticles and lyophilized (L-CE-NLC), free xanthophylls and β-carotene showed very similar bioaccessibility values (about 65%), which were higher than those observed for PP and CE. Similarly, the bioaccessibility of esterified carotenoids in L-CE-NLC was comparable and consistently higher than in PP and CE (20 *vs.* 3%). Then an accelerated stability study (dark and light conditions) was also carried out. In the dark, L-CE-NLC demonstrated a better preservation of antioxidant capacity than a commercial supplement of β-carotene. In the light, the L-CE-NLC preserved the antioxidant capacity of the CE between 65% and 74% of the initial value. These findings highlight the crucial role of nanoencapsulation in nutraceutical and pharmaceutical fields, especially when carotenoids are used.

Received 3rd July 2025
Accepted 27th October 2025

DOI: 10.1039/d5fb00353a

rsc.li/susfoodtech

Sustainability spotlight

In the context of sustainable food ingredients and environmentally friendly technologies, this study focuses on enhancing the bioaccessibility and stability of carotenoids isolated from agro-food waste byproducts, specifically pumpkin peel, applying the nanotechnology approach. The production of structured lipid carriers is sustainable thanks to both the green methods employed and the ingredients used. By utilizing materials that would otherwise be discarded, such as peels, the research promotes a zero-waste approach, which supports Sustainable Development Goal 12 (Responsible Consumption and Production). Carotenoids show interesting health benefits. The improvement of their chemical stability and solubility by nanocarriers enhances their use as nutraceuticals and functional ingredients. Therefore, our work underscores the importance of another UN Sustainable Development Goal: Good Health and Well-being (SDG 3).

Introduction

Carotenoids, abundant in vegetables and fruits, possess a polyene backbone in their chemical structure, responsible for their powerful antioxidant activity. This activity has a positive impact on the prevention and progression of chronic diseases, including cardiovascular and neurodegenerative disorders, as well as cancer.[1,2] However, the health benefits of carotenoids are limited by their high instability and poor solubility. Carotenoids contain multiple double-conjugated bonds in their structure, making them highly sensitive to heat, light, high temperatures, acidic pH, and oxygen. If exposed to any of these factors, their chemical structure can change, leading to a loss of their biological activities.[3,4] In particular, isomerization, cyclization, double-bond migration, and oxygen addition have been identified as structural changes that lead to compounds with low or no activity. Carotenoid poor solubility is another limitation to their beneficial effects in humans. The extent to which these bioactives can be solubilized during the digestion process will condition their absorption by intestinal enterocytes. The hydrophobic nature of carotenoids and their incorporation into food matrices hinder their dissolution. Additional factors, such as food processing methods (*e.g.*, cooking techniques), mastication and other dietary components consumed together, will affect the release and solubilization of carotenoids. After ingestion, an oil-in-water emulsion is formed in the stomach, where carotenoids are housed in the internal phase. Subsequently, in the intestine, bile salts and lipases are released, promoting the formation of mixed micelles. These structures are disc-shaped with diameter ranging from 40 to 400 nm. They are primarily composed of cholesterol, monoacylglycerols, phospholipids, fatty acids, and bile acids. In this scenario, dietary lipids play a positive role in carotenoid absorption by

[a]*Department of Pharmaceutical Sciences, University of Perugia, Via Fabretti 48, 06123 Perugia, Italy. E-mail: francesca.blasi@unipg.it*
[b]*Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de València, 46100 Burjassot, Spain*

promoting the secretion of bile salts and the formation of micelles. Several studies have shown that oils (such as fish, corn, orange, and Miglyol® 812) enhance the bioaccessibility of carotenoids. In particular, long-chain triglycerides are more effective than medium-chain triglycerides (MCT). Long-chain triglycerides form larger, swollen micelles with more effective hydrophobic regions than MCT, which produce smaller, less inflated micelles with reduced hydrophobic regions upon hydrolysis.[5]

Once carotenoids are solubilized in the mixed micelles, they become available for absorption through the epithelial cells of the small intestine. It is generally assumed that the absorption of carotenoid in the intestine primary occurs by passive diffusion, similarly to dietary lipids.[6] However, recent studies suggest that specific transporters located on the apical membrane of enterocytes may also help facilitate carotenoid absorption.[6,7]

Nanostructured Lipid Carriers (NLC) were chosen to encapsulate the carotenoid extract (CE) from pumpkin peel. This choice aims to enhance the stability and solubility of carotenoids, which are key factors that limit their bioaccessibility. NLC are characterized by a solid matrix that provides a physical barrier, protecting the carotenoids from harmful environmental factors, including the gastric environment during digestion.[8] Nanocarriers are an intriguing method for enhancing bioaccessibility and bioavailability due to their small size and large surface area.

In this study, the NLC were formulated using food-grade and sustainable ingredients, with careful attention given to the selection of lipids, as they significantly influence bioaccessibility. Nanoencapsulation was used as a strategy to improve both the bioaccessibility and stability of carotenoids. Specifically, the research examined the bioaccessibility of carotenoids after NLC *in vitro* digestion, as well as their stability under both dark and light storage conditions.

Materials and methods

Materials

Pumpkins belonging to the *Cucurbita maxima* species (Hokkaido variety) were collected in October 2022 from a local farm in Perugia (Umbria Region, Central Italy). The peel, removed using a sharp knife, was chopped into small pieces and placed into separate stainless-steel containers. Then, they were dried at 40 °C in a conventional ventilated oven (Binder, Series ED, Tuttlingen, Germany) until they reached a constant weight. After drying, they were ground using a grinder (Type KM28, Kenwood, Hampstead, UK) and then passed through a stainless-steel sieve with a mesh size of 200 μm (Controls, Milan, Italy). The resulting powder was stored at room temperature in amber glass vials, protected from light and humidity.

A commercial β-carotene supplement (Betacarotene 10.000; Natural Point, Milan, Italy) was purchased in a local pharmacy. The beads are made from food-grade gelatine and contain 7 mg of β-carotene (obtained through fungal fermentation) as reported on the label. They include other ingredients such as soybean oil, glycerol (as plasticizer), and mono- and diglycerides of fatty acids (as stabilizers).

D-(+)-Trehalose dihydrate was purchased from ThermoFisher (Kandel GmbH, Germany). Methanol (MeOH) and methyl-*tert* butyl ether (MTBE) for HPLC were from Carlo Erba Reagents (Milan, Italy). Talc, hexane, isopropanol, toluene, chloroform ($CHCl_3$), and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) were obtained from Sigma-Aldrich (Milan, Italy). Ultrapure water (H_2O) was generated by Synergy® UV Water Purification System (Millipore Sigma, USA). Lutein (≥92%) was purchased from Extrasynthese (Genay, France). β-Carotene (>97.0%) was purchased from TCI (Tokyo Chemical Industry) chemicals (Toshima, Tokyo, Japan). Zeaxanthin dipalmitate, used as a standard, was isolated from a hydroalcoholic extract of goji berries and purified as described in a previous work.[9] α-Amylase (930 U mg^{-1}; A3403), hydrochloric acid (HCl), sodium hydroxide (NaOH), pepsin A (674 U mg^{-1}; P7000), pancreatin (762 U mg^{-1}; P1750; CAS Number: 8049-47-6; various enzymatic components including trypsin, amylase and lipase, ribonuclease, and protease, produced by the exocrine cells of the porcine pancreas), and bile extract porcine (B8631; CAS Number: 8008-63-7; glycine and taurine conjugates of hyodeoxycholic acid and other bile salts, including hyodeoxycholic acid (1–5%), deoxycholic acid (0.5–7%), cholic acid (0.5–2%), glycodeoxycholic acid (10–15%), and taurodeoxycholic acid (3–9%)) were purchased from Sigma-Aldrich (St. Louis MO, USA). Hydrogenated sunflower oil (HSO) (VGB 5 ST, free fatty acids 0.07%) was kindly provided by ADM-SIO (Saint Laurent-Blangy, France). Soybean lecithin was purchased from GPR RECTAPUR VWR (Geldenaaksebaan, Leuven, Belgium), and cholic acid sodium salt (99% purity) from Acros Organics (Geel, Antwerp, Belgium). Size 3 (hydroxyl-propyl) methylcellulose (HPMC) capsules were a gift of Capsugel (Peapack, NJ, USA).

Carotenoid extraction from pumpkin peel

Pumpkin peel was selected as a source of bioactive molecules from agro-food waste. The procedure for carotenoid extraction (CE, carotenoid extract) from pumpkin peel powder (PP) was carried out following the conditions optimized in a previous paper.[9] In brief, the carotenoids were extracted for 30 min at 45 °C using hexane : isopropanol (60 : 40, v/v) through a sonication bath (model AU-65, ArgoLab, Carpi, Italy). An aliquot of CE was characterized by chromatographic analysis, while another aliquot was employed for NLC production (CE-NLC).

Production and characterization of carotenoid loaded NLC (CE-NLC)

The NLC used for this study were obtained following the production protocol reported in a previous study.[10] In particular, high pressure homogenization, a well-recognized eco-sustainable technique was exploited to prepare NLC.[11,12] Briefly, HSO (1.1% w/v), pumpkin seed oil extracted using petroleum ether (0.9% w/v) and CE (10% w/w) were melted in a water bath at ~75 °C and then soybean lecithin (0.8% w/v) was added to the lipid phase under magnetic stirring to reach

homogeneity. A buffer solution (pH = 7) was prepared by dissolving 4 mM monobasic sodium phosphate and 4 mM dibasic sodium phosphate in ultrapure water. Then, the molten lipid phase was added to the heated (~75 °C) aqueous buffer (20 mL) containing sodium cholate (0.3% w/v) and the mixture was processed with an Ultraturrax homogenizer (model T25 basic, IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 8000 rpm for 1 minute. The emulsion was subsequently homogenized for 5 cycles at 1000 bar using an Avestin EmulsiFlex C5 homogenizer maintained at 75 °C. The colloidal emulsion was collected and cooled in an ice bath to solidify the lipid and obtain the NLC suspension. NLC were characterized in terms of dimensions (after the encapsulation and the lyophilization processes, named S-CE-NLC and L-CE-NLC, respectively) using dynamic light scattering, applying the NICOMP distribution, based on the variation in the intensity of scattered light (INTENSITY-WT). The extract recovery (ER%) and extract content (EC%) were determined using previously described methods.[10] Before conducting the *in vitro* bioaccessibility and stability studies, the NLC were lyophilized (L-CE-NLC) using trehalose as cryoprotectant (10% w/v with a 1 : 10 dilution of the sample with ultrapure water). In addition to trehalose, for the stability study, talc (25% w/v) was also added before the lyophilization process to ensure that the resulting powder had sufficient flowability for filling size 3 HPMC capsules.

In vitro gastrointestinal digestion and carotenoid bioaccessibility evaluation

Preparation of samples. PP, CE, and L-CE-NLC were subjected to an *in vitro* simulation of gastric and intestinal (GI) digestion, carried out following the harmonized INFOGEST protocol.[13–15] Before digestion: (i) PP was rehydrated with ultrapure H_2O to its original moisture content, then the obtained mixture was homogenized and left overnight in a refrigerator; 1 g of the rehydrated PP was used for the *in vitro* digestion procedure; (ii) CE (40 mg) was directly introduced into the digestion vessel and stored at −40 °C until the day of the digestion; (iii) L-CE-NLC (2.5 g) were suspended in ultrapure H_2O to reconstitute the original NLC suspension.

Preparation of reagents. Electrolyte stock solutions were prepared at the following concentrations: KCl 0.5 M; KH_2PO_4 0.5 M; $NaHCO_3$ 1 M; NaCl 2 M; $MgCl_2(H_2O)_6$ 0.15 M; $(NH_4)_2CO_3$ 0.5 M; $CaCl_2{\cdot}(H_2O)_2$ 0.3 M. Simulated salivary fluid (SSF): 15.1 mL of KCl; 3.7 mL of KH_2PO_4; 6.8 mL of $NaHCO_3$; 0.5 mL of $MgCl_2$; 0.06 mL of $(NH_4)_2CO_3$. Simulated gastric fluid (SGF): 6.9 mL of KCl; 0.9 mL of KH_2PO_4; 12.5 mL of $NaHCO_3$; 0.4 mL of $MgCl_2$; 0.5 mL of $(NH_4)_2CO_3$; 11.8 mL of NaCl. SGF was adjusted to pH = 3 with HCl 1 M. Simulated intestinal fluid (SIF): 6.8 mL of KCl; 0.8 mL of KH_2PO_4; 85 mL of $NaHCO_3$; 0.33 mL of $MgCl_2$; 38.4 mL of NaCl. SIF was adjusted to pH = 7 with HCl 1 M. Enzyme solutions were freshly prepared and preincubated at 37 °C before use. α-Amylase 1500 U mL^{-1}: 30 mg of enzyme in 20 mL of SSF. Pepsin 20 000 U mL^{-1}: 600 mg of enzyme in 20 mL of SGF. Pancreatin 800 U mL^{-1}: 320 mg in 40 mL of SIF. Bile salts: 625 mg in 25 mL of SIF. Purchased enzymes were evaluated according to reference tests as specified in the literature and by the manufacturers:[14] (a) the determination of α-amylase activity was done with an enzymatic assay, which was based on spectrophotometric stop reaction using soluble potato starch as a substrate; (b) pepsin activity assay was based on spectrophotometric stop reaction using hemoglobin as the substrate; (c) pancreatin activity was evaluated for its trypsin and chymotrypsin activity based on continuous spectrophotometric rate determination using *p*-toluene-sulfonil-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosyne ethyl ester (BTEE) as substrates.[16]

***In vitro* gastrointestinal digestion.** *In vitro* digestion procedure was performed according to the INFOGEST protocol.[13,14] Digestive fluids were prepared for mouth (SSF), stomach (SGF), and small intestine (SIF) following the INFOGEST protocol guidelines.[13–15] The simulated fluids were prepared the day prior to the digestion and stored at 4 °C. The day of the digestion the simulated fluids were subjected to pH adjustment to 7.0 ± 0.2 for SSF, 3.0 ± 0.2 for SGF and 7.0 ± 0.2 for SIF and maintained at 37 °C. Digestion were performed in triplicate for each sample.

Oral phase. Samples (rehydrated PP, CE, and reconstituted CE-NLC suspension) were firstly combined with 4 mL of SSF, 25 μL of 0.3 M $CaCl_2$, 0.75 mL of α-amylase solution, and 0.255 mL of ultrapure H_2O. For CE that is a viscous liquid, vortex agitation was used to mix CE with SSF. The mixture was incubated in an orbital shaker (Infors AG CH-4103, Bottmingen, Switzerland) at 37 °C in the dark for 2 min under shaking (100 rpm).

Gastric phase. Oral bolus was combined with 8 mL of SGF, 5 μL 0.3 M $CaCl_2$, 0.667 mL of porcine pepsin solution in SGF. Ultrapure H_2O was added to reach a final volume of 20 mL and pH was adjusted to 3.0 ± 0.2 using 6 M HCl. Then the mixture was incubated in the dark for 2 h at 37 °C in the orbital shaker (100 rpm).

Intestinal phase. After two hours, gastric chyme was mixed with 8 mL of SIF, 5 mL of pancreatin solution in SIF, 3 mL of bile extract solution in SIF, and 40 μL of $CaCl_2$. Ultrapure water was added to reach a final volume of 40 mL, and the pH of the mixture was adjusted to 7.0 ± 0.2 with 1 M NaOH. The resulting mixture was then incubated again for the final step of the digestion, for 2 h at 37 °C under shaking (100 rpm) in the dark.

The value of pH during the digestion procedure was monitored and adjusted if values were different from target values beyond ±0.2 units. After the last incubation with SIF, samples were immediately transferred into 50 mL falcon tubes and place on ice to minimize enzymatic activity. Samples were then immediately centrifuged for 30 min at 5977×*g* at 4 °C using an Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany) to separate the aqueous supernatant (containing mixed micelles) from the solid portion of the raw digesta. The aqueous supernatant was then filtered with 0.45 μm Whatman® syringe nylon filter, to isolate the micellar aqueous fraction for the quantification of bioaccessible carotenoids. Once filtered, the total volume of the aqueous supernatant was divided into 5 mL aliquots and lyophilized overnight under exclusion of light and then stored at 4 °C in the dark until further analysis.

Carotenoid extraction from micellar aqueous fraction. Carotenoid extraction from the lyophilized aqueous supernatant was performed using a methodology previously described

with slight modifications.[17] Before proceeding with the HPLC-DAD (high-performance liquid chromatography with diode array detector) analysis described in the following section, the samples were solubilized in MeOH : MTBE (1 : 1, v/v) and filtered using PTFE filters (Millipore, 0.22 μm pore). The amount (μg) of each carotenoid group (free xanthophylls, mono- or di-esterified xanthophylls, and β-carotene) was used to calculate the carotenoid percentage composition (CC%) of the samples before the digestion protocol, as well as for the micellar aqueous fraction of the same samples after digestion. The following equation (eqn (1)) was employed to determine CC%:

$$\mathrm{CC\%} = \frac{\mu g \text{ of each carotenoid group(undigested sample or micellar fraction)}}{\text{total carotenoid content}(\mu g)} \quad (1)$$

Bioaccessibility of individual carotenoids and carotenoid groups from the micellar fraction of digested samples was calculated using the following equation (eqn (2)):

$$\text{Bioaccessibility}(\%) = \frac{[\text{carotenoid}]_{\text{supernatant}}}{[\text{carotenoid}]_{\text{digested sample}}} \times 100 \quad (2)$$

Accelerated stability studies

To understand the impact of nanoencapsulation on the preservation of CE, two different stability studies were performed: one in the dark and another in the light.[18,19] The stability of L-CE-NLC was compared with that of non-encapsulated CE and a commercially available β-carotene food supplement. CE was transferred into transparent vials, flushed with nitrogen to eliminate residual oxygen, hermetically sealed, and stored at −20 °C in the dark until the initiation of the stability studies. L-CE-NLC powder was sieved through a N° 3 sieve to obtain a fine and homogeneous powder, then distributed into size 3 HPMC capsules using a manual capsule filling machine. Finally, filled capsules were blistered using a blister machine (R. F. & Sanilab, Prato, Italy) and stored at 4 °C. For the stability study of the dietary supplement, the beads were removed from their packaging.

Dark storage conditions. The samples (CE, capsules with L-CE-NLC, and beads of β-carotene commercial supplement) were placed in triplicate in amber closed vials inside a climate chamber (KBFP720, BINDER GmbH; Tuttlingen, Germany) in the dark. Climate chamber was programmed to follow ICH guidelines parameters for accelerated study (6 months; 40 °C; 75% relative humidity, RH).[19] At each time point, at the beginning, after 15 days, 2, 3, and 6 months, samples were taken from the climate chamber and their antioxidant capacity was evaluated by ABTS assay (section ABTS assay).

Light storage conditions. The samples (CE, capsules with L-CE-NLC, and beads of β-carotene commercial supplement) were placed inside the climate chamber (KBFP720, BINDER GmbH; Tuttlingen, Germany) and kept under UV/Vis illumination (9 megalux per hours) for 30 days at 40 °C and 75% RH. Accelerated light storage conditions were chosen based on ICH guidelines[18] and Atencio *et al.*[20] Samples were taken from the climate chamber at each time point, at the beginning and every 5 days. Their antioxidant capacity was evaluated by ABTS assay (section ABTS assay). When the samples showed no antioxidant capacity, the test was stopped. To simulate a real-life scenario, the total amount of light used during the 30-day test was equivalent to the total light produced by a 600 lux light bulb in a supermarket operating 12 h a day for a total of 900 days (30 months).[20] This study allows to compare the stability of the CE-NLC formulation with the commercial supplement, in a simulated real-life scenario.

HPLC-DAD analysis

Chromatographic analysis of the carotenoid profile of the samples (PP, CE and L-CE-NLC) was performed accordingly to the procedure described in a previous paper.[9] For qualitative and quantitative analysis, a high-performance liquid chromatograph (HPLC-DAD) coupled with a UV 6000 LP diode array detector (Thermo Scientific, Waltham, MA, USA) was employed. Chromatographic separation was performed on a reverse-phase C-30 Develosil column (250 × 4.6 mm i.d., 5 μm, Nomura Co., Kyoto, Japan) using the following mobile phase solvents: A (MeOH : H_2O, 97 : 3 v/v); B (MTBE). The gradient program employed was previously reported in detail.[9] Quantification of carotenoids was performed using calibration curves of a standard solution lutein (0.4–13 μg mL^{-1}), zeaxanthin dipalmitate (0.48–155 μg mL^{-1}), and β-carotene (0.51–51 μg mL^{-1}). Lutein (μg LE) was employed for the quantification of free xanthophylls, zeaxanthin dipalmitate (μg ZDE) for mono- and di-esterified xanthophylls, and β-carotene for β-carotene. The identified carotenoids were divided into four groups (free xanthophylls, mono-esterified xanthophylls, di-esterified xanthophylls, and β-carotene). The results were expressed either as the quantification of individual carotenoids or as the sum of carotenoids belonging to one of the four groups. For PP, CE, and L-CE-NLC, the initial concentration of carotenoids was expressed as μg g^{-1} of PP, μg mg^{-1} of CE, and μg g^{-1} of L-CE-NLC, respectively. The method validation was reported in a previous paper.[9] The identity confirmation of carotenoids was carried out using an UHPLC system coupled to a quadrupole-time of flight mass spectrometer (HPLC-QTOF-MS), as reported in a previous paper.[9]

ABTS assay

The ABTS procedure for the determination of the antioxidant properties of samples was carried out using a method previously

developed.[9,10] The $ABTS^{\bullet+}$ stock solution was prepared solubilizing ABTS (7 mM) and potassium persulfate (2.45 mM) in ultrapure water. The solution was left to react in the dark at 4 °C for 18 hours to generate the radical cation $ABTS^{\bullet+}$. Then, the solution was frozen at −80 °C and freeze-dried overnight. The resulting $ABTS^{\bullet+}$ powder was reconstituted and diluted in $CHCl_3$: MeOH (1 : 1 v/v) to obtain an absorbance of 0.700 (±0.030) at 752 nm, using $CHCl_3$: MeOH (1 : 1 v/v) as the blank. The samples (CE, capsule with L-CE-NLC, and beads of β-carotene commercial supplement) were treated as follows. The vial containing CE was added with $CHCl_3$ (1 mL). The capsule with L-CE-NLC was completely emptied, and its content inserted into centrifuge tubes with $CHCl_3$ (1 mL) and centrifuged at 4000 rpm for 5 min; the supernatant was recovered. The bead, placed into an appropriate container, was pierced using a sterile scalpel; $CHCl_3$ was added and placed under magnetic stirring, away from light, until the content of the bead was dissolved. The samples were incubated for 10 min with the $ABTS^{\bullet+}$, and the absorbance was read in triplicate at 752 nm. Antioxidant capacity was calculated from a calibration curve obtained with α-tocopherol (α-TE) solutions in $CHCl_3$: MeOH (1 : 1 v/v) and treated using the same procedure. For each time point, antioxidant capacity of the samples was measured (expressed as μg α-TE/CE-NLC capsule, μg α-TE/β-carotene bead or μg α-TE mg^{-1} of CE, respectively for CE-NLC capsules, β-carotene supplement beads and non-encapsulated CE). Results were expressed as percentage of antioxidant capacity with respect to the initial antioxidant capacity, using the following equation:

$$\%\ \text{antioxidant capacity} = \frac{\text{measured antioxidant capacity at the time point}}{\text{measured antioxidant capacity at the beginning}} \times 100 \quad (3)$$

Statistical analysis

Data were expressed as mean value ± standard deviation of triplicate measurements ($n = 3$). The statistical analysis of the results was performed by Student's *t*-test for paired samples. Values of $p \leq 0.05$ were considered statistically significant. Microsoft Excel® for Microsoft 365 MSO (Version 2306 Build 16.0.16529.20164) was used as statistical software. GraphPad Prism version 9.3.1 for Windows (Boston, Massachusetts USA) was used as a graph's builder.

Results and discussion

CE-NLC characterization

Carotenoid extract (CE) obtained from pumpkin peel (PP) was employed for the production of the carotenoid extract loaded NLC (CE-NLC).

S-CE-NLC showed two nanoparticle populations: 91.1% of the sample had a mean diameter of 207.5 ± 44.4 nm, while the remaining part (8.9%) had a mean diameter of 61.7 ± 10.1 nm. A single L-CE-NLC population (100%) having a mean diameter of 206.4 ± 40.4 nm was observed. Dimensional analysis confirmed the achievement of nanoparticles with suitable dimensions (<300 nm).

In terms of loading properties, spectrophotometric analysis showed values of ER% and EC% equal to 87.12 ± 0.58% and 7.0 ± 0.2%, respectively. Furthermore, the chromatographic analysis revealed that 90 ± 2% of the initial carotenoid content used for the encapsulation process, was retained in the final lyophilized formulation. These data confirm the reliability of the production process, since similar results were also achieved with CE-NLC loaded with a different CE composition. This was demonstrated in a previous study that utilized hot high-pressure homogenization.[10] Overall, the NLC used in this work exhibited acceptable sizes and suitable loading properties.

Carotenoid *in vitro* bioaccessibility

Chromatographic characterization of samples before *in vitro* digestion. HPLC-DAD analysis was carried out to: (i) quantify the carotenoid species in all samples before digestion; (ii) understand the possible chemical changes after encapsulation; (iii) evaluate how the *in vitro* digestion process influences the carotenoid composition of the samples.

Fig. 1 shows the chromatographic profiles of carotenoids of CE and L-CE-NLC. As evidenced by the chromatograms, the encapsulation and lyophilization processes did not altered the qualitative carotenoid composition of the CE. Regarding the quantitative carotenoid composition of the samples (PP, CE and L-CE-NLC), calibration curves of the individual carotenoids were used, and the data shown in Table 1. The initial concentration of individual compounds grouped into four carotenoid groups (*i.e.*, free xanthophylls, mono-/di-esterified xanthophylls, β-carotene), and total carotenoid content were reported. Overall, samples before *in vitro* digestion showed a total carotenoid content of 20 511.96 μg g^{-1} for the PP, 348.77 μg mg^{-1} for the CE and 566.88 μg g^{-1} for L-CE-NLC. The content of esterified carotenoids was always higher than that of free xanthophylls and β-carotene ($p < 0.001$).

Profile and bioaccessibility of carotenoids in digested samples. Fig. 2(A–D) shows the bioaccessibility data of the four carotenoid groups (*i.e.*, free xanthophylls, mono-esterified xanthophylls, di-esterified xanthophylls, and β-carotene) and

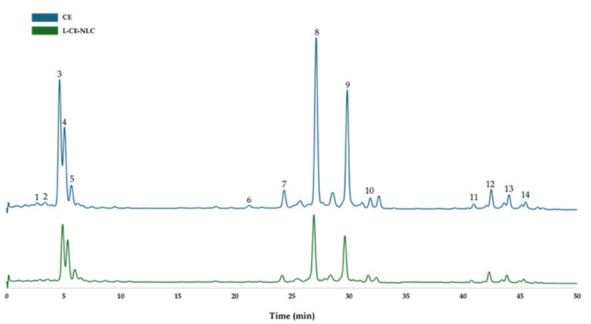

Fig. 1 HPLC-DAD profile of CE (blue) and of L-CE-NLC (green). (1) Neoxanthin; (2) violaxanthin; (3) antheraxanthin; (4) lutein; (5) zeaxanthin; (6) violaxanthin myristate; (7) lutein palmitate; (8) antheraxanthin myristate; (9) antheraxanthin palmitate; (10) β-carotene; (11) lutein laurate myristate; (12) lutein dimyristate; (13) lutein myristate palmitate; (14) lutein dipalmitate.

Table 1 Contents of free xanthophylls, mono-esterified xanthophylls, di-esterified xanthophylls, and β-carotene of PP, CE and L-CE-NLC before *in vitro* digestion[a]

Carotenoids		Carotenoid content		
		PP (μg g^{-1})	CE (μg mg^{-1})	L-CE-NLC (μg g^{-1})
Free xanthophylls (μg LE)	Neoxanthin	9.40 ± 0.18	0.16 ± 0.00	0.39 ± 0.01
	Violaxanthin	6.89 ± 0.00	0.12 ± 0.00	0.38 ± 0.04
	Antheraxanthin	310.34 ± 6.50	5.28 ± 0.11	7.72 ± 0.20
	Lutein	210.63 ± 6.75	3.58 ± 0.11	6.05 ± 0.62
	Zeaxanthin	55.11 ± 1.18	0.94 ± 0.02	1.55 ± 0.02
Total		592.36 ± 14.61	10.07 ± 0.25	16.09 ± 0.77
Mono-esterified xanthophylls (μg ZDE)	Violaxanthin myristate	261.35 ± 7.78	4.44 ± 0.13	4.90 ± 0.32
	Lutein palmitate	1148.43 ± 13.81	19.53 ± 0.23	28.38 ± 0.10
	Antheraxanthin myristate	9923.30 ± 197.84	168.73 ± 3.36	259.11 ± 10.75
	Antheraxanthin palmitate	6213.08 ± 150.47	105.64 ± 2.56	173.17 ± 7.31
Total		17 546.16 ± 369.91	298.34 ± 6.29	465.56 ± 17.65
Di-esterified xanthophylls (μg ZDE)	Violaxanthin dimyristate	96.45 ± 0.16	1.64 ± 0.00	ND
	Lutein laurate myristate	303.23 ± 5.47	5.16 ± 0.09	10.09 ± 0.05
	Lutein dimyristate	989.05 ± 7.26	16.82 ± 0.12	35.68 ± 1.65
	Lutein myristate palmitate	627.54 ± 26.04	10.67 ± 0.44	24.69 ± 2.09
	Lutein dipalmitate	281.30 ± 21.22	4.78 ± 0.344	12.42 ± 0.41
Total		2297.57 ± 60.15	39.07 ± 1.02	82.89 ± 4.21
β-Carotene	β-Carotene	75.86 ± 0.30	1.29 ± 0.01	2.34 ± 0.14
Total carotenoids		20 511.96 ± 444.37	348.77 ± 7.56	566.88 ± 22.48

[a] Data are reported as mean value ± SD on dry weight ($n = 3$). Individual and total free xanthophylls were quantified using lutein calibration curve and expressed as μg of Lutein Equivalents (μg LE) per sample. Individual and total mono-/di-esterified xanthophylls were quantified using zeaxanthin dipalmitate calibration curve and results are expressed as μg of Zeaxanthin Dipalmitate Equivalents (μg ZDE) per sample. β-Carotene were quantified using β-carotene calibration curve and results are expressed as μg of β-carotene per sample. Total carotenoids content results are expressed as μg of carotenoids per sample. ND, not detected.

of the individual carotenoids belonging to these groups for each sample (PP, CE, and L-CE-NLC).

Carotenoids of PP showed the following trend in terms of bioaccessibility: free xanthophylls > β-carotene > di-esterified xanthophylls > mono-esterified xanthophylls. Free xanthophylls (neoxanthin, violaxanthin, antheraxanthin, lutein, and zeaxanthin) showed the highest value of bioaccessibility among all carotenoids identified. The values of bioaccessibility ranged from 7.4 (antheraxanthin) to 15.0% (neoxanthin) in the group of free xanthophylls (Fig. 2A), from 0.3 (antheraxanthin palmitate) to 3.7% (lutein laurate myristate) for mono- and di-esterified xanthophylls (Fig. 2B and C), while for β-carotene it was 2.2% (Fig. 2D).

These results showed that the overall bioaccessibility of carotenoids from PP was severely limited primarily due to their chemical properties and the nature of the food matrix, but also related to the different chemical structures of carotenoids, as confirmed by other authors.[16] This result may be due to the presence of polar groups in their chemical structure, which makes them more prone to integration into mixed micelles, allowing a low-energy localization in the outer and polar sections of micelles.[16]

Recent studies have evaluated the bioaccessibility of carotenoids by applying different *in vitro* digestion protocols, including the INFOGEST protocol. Schmidt *et al.* evaluated the impact of high-pressure processing on kale to investigate its potential impact on carotenoid bioaccessibility.[21] In the case of untreated kale samples, lutein and β-carotene showed bioaccessibility values of 8% and 1.4%, data in line with the results obtained in the present study.

Iddir *et al.* evaluated the bioaccessibility of different xanthophylls in plant (spinach) and plant-based products (tomato and carrot juices) with and without the presence of proteins.[22] The bioaccessibility values of lutein (+zeaxanthin) showed considerable variability between spinach and the two tested juices (37.5% *vs.* 66.5/61.9%). This difference can also be explained by the different nature of the food matrix. For example, in orange fruits as pumpkins, carotenoids are dissolved into lipid droplets most bioaccessible when complexed to carotenoids located in chloroplasts, as in leafy-green vegetables.[23] The presence of pectins, key components of cell wall, contribute to the provision of a barrier between amylase and starch, in fact the digestion of pumpkin is strongly linked to pumpkin polysaccharide content and composition.[24]

Considering that PP was not subjected to any specific treatments besides the dehydration process used for its storage, the bioaccessibility values obtained for neoxanthin (15.0%), lutein (13.1%), zeaxanthin (10.2%) and β-carotene (2.2%) were in line with those observed for unprocessed spinach.[22] Concerning zeaxanthin bioaccessibility, similar findings were reported, showing values of 6.7% and 13.3% after the digestion of goji berries, without and with the addition of coconut fat (1% w/w), respectively.[25]

Moreover, the bioaccessibility values of β-carotene were lower than xanthophylls. This result can be explained by considering the chemical structure of carotenes and how they

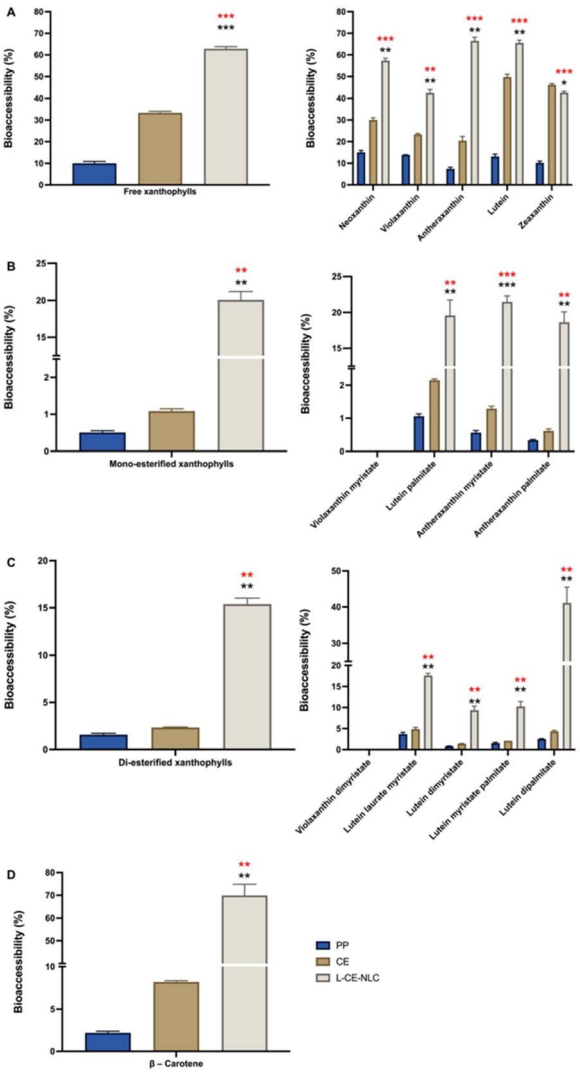

Fig. 2 Bioaccessibility (%) of free xanthophylls (A), mono-esterified xanthophylls (B), di-esterified xanthophylls (C) and the respective individual carotenoids of digested PP, CE and L-CE-NLC. Bioaccessibility (%) of β-carotene (D). Significant differences between samples are indicated as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***), with black asterisk indicating comparison between CE and L-CE-NLC and red asterisk comparison between PP and L-CE-NLC values.

occur in vegetable matrices. Similar bioaccessibility results for β-carotene were observed for fresh and dehydrated pumpkins with different moisture content ranging between 0.3% and 4.5%.[26] In contrast, higher bioaccessibility values for β-carotene (from 25.8% to 35.3%) were obtained for two pumpkin species (Delica variety *C. maxima* species and Violina variety *C. moschata* species). In this case, it should be taken into consideration that pulp was cooked (steam and oven cooking) before the *in vitro* INFOGEST digestion protocol. Thermal treatment improved carotenoid release from the food matrix, especially of more lipophilic carotenes.[15]

Mono- and di-esterified xanthophylls in PP samples showed the lowest bioaccessibility values (always lower than 3.7% of lutein laurate myristate) among the identified carotenoids, with di-esterified xanthophylls (0.8–3.7%) being slightly more prone to micellization than mono-esterified ones (0.3–1.1%). Bioaccessibility values similar to those obtained in this study were reported for esterified carotenoids from different fruits, *i.e.* cajà, lucuma, and papaya.[27–29]

The values of bioaccessibility obtained for PP are overall in line with those reported in the literature.[23]

Significant differences occurred in carotenoid bioaccessibility when CE was digested instead of PP. A significant increase in bioaccessibility for most carotenoids considered was observed, except for lutein laurate myristate and lutein myristate palmitate. Among all carotenoid groups considered, especially free xanthophylls (Fig. 2A) and β-carotene (Fig. 2D) showed a significant increase of bioaccessibility of 23% ($p < 0.01$) and 6% ($p < 0.001$), respectively. Among free xanthophylls, lutein and zeaxanthin showed an increment of 36 and 35%, respectively. However, even though a bioaccessibility increase was observed also for mono- and di-esterified xanthophylls in CE digestion, overall bioaccessibility values remained relatively low when compared to other groups. The absence of the food matrix during digestion ensures greater micellization of the carotenoids present in the CE, leading to an increase in bioaccessibility. Overall, bioaccessibility results of CE followed the same trend observed for PP (free xanthophylls > β-carotene > di-esterified xanthophylls > mono-esterified xanthophylls).

Bioaccessibility results for L-CE-NLC showed a slightly different trend than that observed for PP and CE. Free xanthophylls (Fig. 2A) and β-carotene (Fig. 2D) showed very similar bioaccessibility values, 63% and 70% respectively. For mono-esterified and di-esterified compounds, an inverse trend was observed, with mono-esterified xanthophylls (20%) showing higher bioaccessibility values than di-esterified xanthophylls (15%). In terms of individual carotenoids, encapsulation of the CE into NLC (L-CE-NLC) led to a significant increase in bioaccessibility, when compared to digested PP and CE, with the exception of zeaxanthin with a slight, but significant ($p < 0.05$), decrease of bioaccessibility for digested NLC with respect to CE. Comparing L-CE-NLC bioaccessibility values with those of digested PP, for all considered carotenoids a significant increase ($p < 0.05$) of bioaccessibility was observed, especially in the case of β-carotene (from 2% to 70%) ($p < 0.01$) and in the case of mono-esterified xanthophylls (from 0.5% to 20%) ($p < 0.01$), justifying the different trend of bioaccessibility previously observed in digested pumpkin peel and CE.

These results confirm the ability of NLC to improve carotenoids bioaccessibility. Higher bioaccessibility values can also be attributed to an improvement in the micellization process. This result is not only due to the size of the nanocarriers, but can also be related to the composition of the NLC themselves. NLC prepared in this work were composed of HSO, whose lipolysis during digestion can strongly influence the bioaccessibility of the encapsulated carotenoids.[30] Sunflower oil, primarily composed of unsaturated long-chain fatty acids, promotes micelle formation, thereby enhancing carotenoid bioaccessibility.[30,31] Similar findings were reported for β-carotene-loaded solid lipid nanoparticles composed of MCT and hydrogenated palm oil at different ratios or MCT and glyceryl stearate.[32]

In addition to bioaccessibility, another important aspect to consider is the carotenoid composition of analysed samples, as also reported in the paper of Rodrigues *et al.* (2016), even if it is known that the bioaccessibility of xanthophylls esters is a complicated topic, because a diester, by hydrolysis will be converted partially in a monoester and partially in the parent xanthophyll.[33]

The % of β-carotene changed from 0.4% before digestion to 0.9% in PP and 1.4% in CE and L-CE-NLC, respectively. Therefore, the percentage of β-carotene relative to the total carotenoid content increased significantly ($p < 0.01$ for PP, $p < 0.001$ for CE and L-CE-NLC) after *in vitro* digestion.

Before digestion (data not shown), as expected, PP and CE showed the same % composition (2.9% free, 85.5% mono-esterified, 11.2% di-esterified) where the micellar fraction was composed predominantly of esterified xanthophylls, reflecting the previously observed bioaccessibility trend.

Fig. 3 shows the relative composition (%) of PP, CE, and L-CE-NCL after the *in vitro* digestion. After digestion, the lower percentage of mono-esterified xanthophylls (47.5%) compared to the undigested PP sample (85.5%), can be attributed to various factors. Among these, the presence of liquid crystals within the original plant matrix, difficult to solubilize, combined with the high lipophilicity resulting from the esterification process.

However, a similar percentage composition can be observed in the micellar fraction of the digested CE (42.2%). This result can still be related to the lipophilicity of these compounds, as for β-carotene. Nevertheless, a possible degradation during the gastric phase of digestion (pH 2) cannot be ruled out. The entire simulated digestive process may cause the ester bond cleavage, allowing the release of the corresponding xanthophylls in their free form.

Further investigations will be necessary to better understand this phenomenon. Interestingly, the situation observed for the micellar fraction of the digested CE and PP did not occur in the case of L-CE-NLC (Fig. 3).

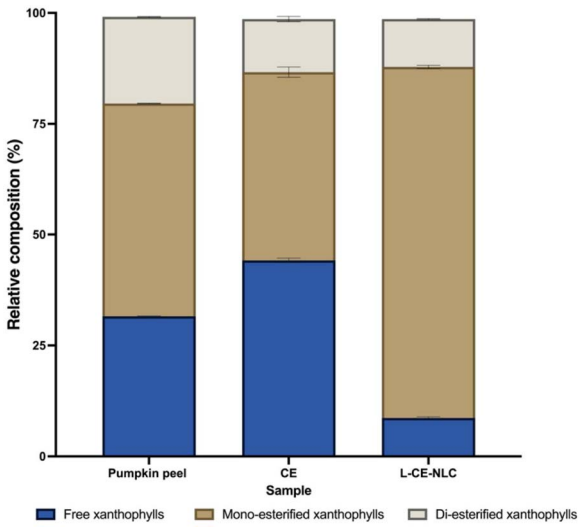

Fig. 3 Relative composition (%) of free, monoesters and diesters of carotenoids of pumpkin peel, CE, and L-CE-NLC after *in vitro* digestion.

After digestion, the relative composition (%) of the micellar fraction of L-CE-NLC showed an increase in the percentage of free xanthophylls (8.8%) and a slight decrease in the percentage of monoesters (79.0%) and diesters (10.8%), if compared to the initial sample was observed. The data obtained on the preservation of the percentage composition of the L-CE-NLC micellar fraction, compared to the initial sample, suggest that the encapsulation process contributed to increase the carotenoid stability during *in vitro* digestion. To the best of our knowledge, this is the first report of encapsulating pumpkin peel carotenoid extract in nanostructured lipid carriers using pumpkin seed oil as the liquid lipid. This encapsulation approach effectively preserved the carotenoid composition during digestion while simultaneously enhancing carotenoid bioaccessibility.

Accelerated stability study

The aim of this study was to evaluate and compare the stability profiles of carotenoids in L-CE-NLC and CE samples with that of a commercially available β-carotene supplement. Carotenoid stability in the various samples, under both dark and light conditions, was evaluated through the ABTS assay, and expressed as a percentage of the initial antioxidant capacity.

Fig. 4 shows the trend of the antioxidant capacity of the samples during 6-month storage in the dark. After 15 days of storage, the antioxidant capacity of the CE decreased by 53%. Over the following two months, there was an additional reduction of 15%. However, between the second and third months, the antioxidant capacity remained stable at approximately 31%. After six months of storage, no antioxidant activity was detected, indicating a complete loss of carotenoid antioxidant capacity in the extract. The results obtained confirmed the poor stability of carotenoids widely reported in literature.[32]

The results of the stability study for the L-CE-NCL formulation were noteworthy when compared to those of the commercial supplement.

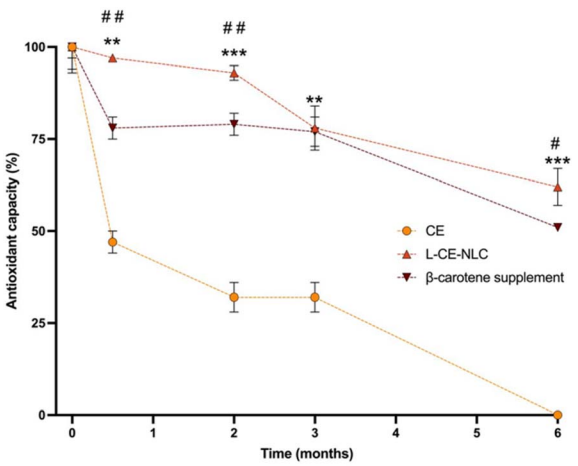

Fig. 4 Antioxidant capacity (%) of CE, L-CE-NLC and β-carotene supplement over 6-months storage in the dark at 40 °C and 75% RH. Significant differences between L-CE-NLC and CE are indicated as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***), while significant differences between L-CE-NLC and β-carotene supplement are indicated as $p < 0.05$ (#); $p < 0.01$ (##); $p < 0.001$ (###).

In fact, L-CE-NLC demonstrated a better preservation of antioxidant capacity over time ($p < 0.01$ after 1 and 2 months and $p < 0.05$ after 6 months). After 15 days and 2 months, the decreases in antioxidant capacity were only 3% and 7%, respectively, from the initial values. In contrast, the commercial supplement showed a significant reduction of 20% in antioxidant capacity after just 1 month of storage ($p < 0.01$). After 6-month storage, L-CE-NLC and commercial supplement showed a residual antioxidant capacity of 62% and 51% respectively, compared to the initial antioxidant capacity. Overall, the results of the accelerated stability study in dark conditions showed that NLC improved carotenoid stability and maintained their antioxidant capacity compared to CE ($p < 0.001$ after 6 months). Furthermore, the L-CE-NLC formulation stability was significantly higher than that obtained for the commercial supplement. This provides a promising outlook for the use of such nanocarriers in the production of nutraceutical/pharmaceutical formulations with good protection features for carotenoids.

Fig. 5 shows the results of the study concluded after 25 days, when the non-encapsulated CE no longer showed antioxidant capacity. After 5 days of the stability study, an important reduction in antioxidant capacity was observed for the CE, corresponding to 40% of the initial antioxidant capacity. The following checkpoints (10, 15, and 20 days) showed a decreasing trend in the antioxidant capacity of the CE, reaching 8% of the initial values by the 20th day.

The L-CE-NLC formulation showed interesting results. After 5 days, a decrease in antioxidant capacity was observed, reaching 60% of the initial value. In the following checkpoints, the L-CE-NLC preserved the antioxidant capacity of the extract between 65% and 74% of the initial value. These results indicated that encapsulating the extract within the NLC and filling the powder into capsules helped maintain antioxidant capacity of the extract above 50% throughout the study. This result also confirms the findings of the stability study performed in darkness.

The presence of light did lead to a faster reduction in antioxidant capacity compared to the initial stability study, as light itself is one of the primary environmental factors responsible for degradation of carotenoids in nature. Packaging will therefore be of fundamental importance to shield the formulation form light for its potential commercialization.[34] The commercial supplement showed a better preservation of antioxidant capacity from the 5th ($p < 0.01$) to the 25th day ($p < 0.05$), ranging between 77% and 86%, and consequently a greater stability of the β-carotene. However, the trend of antioxidant capacity values for the commercial supplement and the L-CE-NLC was very similar. The CE, unlike the other two samples, was in a hermetically sealed airtight transparent glass vial, receiving light irradiation over its entire surface, thus ensuring uniform exposure of the carotenoids. In contrast, the L-CE-NLC powder and the commercial supplement were in an HPMC capsule and a soft gelatine bead, respectively. Light exposure may have the greatest impact on the outer surface of these two oral forms, which can contain varying amounts of carotenoids depending on the individual unit. The unusual behaviour observed in the samples could therefore be attributed to differences in the distribution and concentration of carotenoids between the outer and inner layers, which may also vary from one capsule/bead to another.

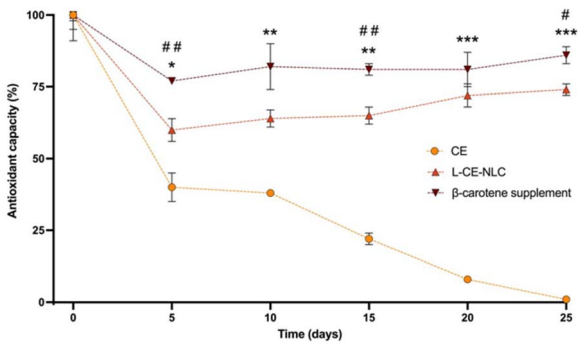

Fig. 5 Antioxidant capacity (%) variations of CE, L-CE-NLC and β-carotene supplement after 25 days of light storage. Significant differences between L-CE-NLC and CE are indicated as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***), while significant differences between L-CE-NLC and β-carotene supplement are indicated as $p < 0.05$ (#); $p < 0.01$ (##); $p < 0.001$ (###).

Conclusions

This study evaluated the impact of encapsulating carotenoids into NLC on their bioaccessibility and stability. The findings for both the bioaccessibility and stability assessments provide significant insights into the potential of nanoencapsulation for developing new nutraceutical/pharmaceutical supplements containing carotenoids. Specifically, the *in vitro* bioaccessibility study revealed that encapsulation significantly increase the bioaccessibility of all carotenoid groups, compared to the bioaccessibility of digested pumpkin peel and CE. Additionally, the study demonstrated that encapsulation into NLC not only improved the stability of carotenoids, but also more effectively preserved their initial percentage composition, both after the production and following *in vitro* digestion.

Both accelerated stability studies performed on the L-CE-NLC confirm the improvement of stability due to encapsulation and successive introduction into capsules, simulating a hypothetical commercial product. The data showed an improved antioxidant stability of the encapsulated carotenoids in comparison with non-encapsulated CE. These results underscore the important and emerging role of nanoencapsulation in the production of nutraceutical/pharmaceutical products enriched with bioactive compounds, particularly carotenoids. This work could contribute to further promote the use of nanocarriers, produced using an eco-friendly method, for the encapsulation of lipophilic bioactive compounds coming from agro-food waste, enhancing their physical and chemical properties.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Lara Manyes, Aurélie Schoubben and Francesca Blasi; formal analysis and investigation: Nicola Pinna and Federica Ianni; draft manuscript preparation: Nicola Pinna,

Francesca Blasi and Aurélie Schoubben; review and editing: Francesca Blasi and Aurélie Schoubben; supervision: Francesca Blasi, Lara Manyes and Aurélie Schoubben; resources: Maurizio Ricci and Lina Cossignani; project administration: Maurizio Ricci and Lina Cossignani. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

Data availability

All the data is presented within the manuscript itself.

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