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Nanoencapsulation of carotenoid extract *via* the temperature-induced phase transition of triblock polymer in supercritical carbon dioxide

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Abstract

Objectives Carotenoids are increasingly explored as nutraceuticals, but their low bioavailability due to poor aqueous solubility limits their applications. This study discusses the development of a novel and organic solvent-free method to develop carotenoid-containing polymeric nanoparticles via temperature-induced phase transition (TIPT) of pluronic F-68 to obtain formulations with the improved dissolution of carotenoids.

Methods The nanoencapsulation of carotenoids in pluronic F-68 was performed in supercritical carbon dioxide ($scCO_2$) to avoid oxidative or temperature/solvent-induced degradation. The nanoencapsulates were prepared in $scCO_2$ at 40°C or 60°C and 10 MPa without the aid of any organic solvent. The formulations thereafter were characterised for particle size via dynamic light scattering, particle morphology via scanning electron microscopy and carotenoid content/release via high-performance liquid chromatography (HPLC).

Key findings HPLC results showed carotenoid degradation to be negligible in freshly prepared formulations when prepared in $scCO_2$ at 60°C and 10 MPa. The developed particles were spheroidal with sizes ranging between 150 and 250 nm depending on carotenoid content in the preparation. An improvement in the aqueous solubility and storage stability (5°C) of carotenoids was also observed for the formulations prepared in $scCO_2$.

Conclusions These results suggest that TIPT under $scCO_2$ can be applied to formulate nanoparticulates with improved dissolution rate and stability of thermosensitive molecules such as carotenoids without causing any degradation during the processing.

Keywords: carotenoid; polymer nanoparticles; carotenoid bioavailability; supercritical CO_2; HPLC; temperature-induced phase transition

Introduction

In the food industry, β-carotene is primarily applied as a food colourant but there is a growing demand for β-carotene-based food, drinks and beverages with the increasing interest in nutraceuticals.[1, 2] Carotenoids are natural pigments comprising 8 isoprene units joined to a skeleton of 40 carbon atoms classified as oxygen-containing (xanthophylls, e.g., lutein, astaxanthin, cryptoxanthin, etc.) and hydrocarbons (carotenes, e.g., α-, β-carotene, lycopene, etc.). The antioxidant properties of carotenoids are known to provide numerous health benefits from the regulation of various bodily functions to acting as anti-inflammatory agents. Their applications in the treatment and management of cardiovascular, ophthalmological, pulmonary, cancer prevention and neurodegenerative disorders are reported by numerous researchers.[3, 4]

Carotenoids are a vital component of human nutrition due to their important biological functions and these are regularly used as colourants, antioxidant supplements and precursors of aroma or flavour compounds.[5, 6] Due to these reasons, there is a clear interest in the food, chemical, pharmaceutical, cosmetics, personal care and nutraceutical sectors in utilising carotenoids for various applications, as functional and/or bioactive compounds. Animals including humans cannot synthesise them in the body; so, these must be included in the diet. The bioavailability of carotenoids obtained from natural sources differs significantly due to matrix variability, poor intestinal absorption and their limited aqueous solubility.[1] The applications of carotenoids remain limited because of their low bioavailability and stability concerns.[7–9] The discussion around the bioavailability of carotenoids is important as the only viable option to introduce them into the body is as dietary supplements.[10] Although fruits and vegetables are good sources of carotenoids, their limited bioaccessibility of around 10% of total ingested carotenoids is a major constraint and matrix variability of plant sources also makes effective carotenoid dosing difficult to achieve.[1, 11] Hence, solubility and/or dissolution rate improvement of carotenoids is essential to enhance their bio-efficiency and expand their applications. Furthermore, carotenoids are also highly sensitive to temperature changes, organic solvents and stresses normally applied in traditional formulation development processes. This study aims to identify an effective, solvent-free and novel approach to enhance the stability and aqueous solubility of carotenoids.

Nanoencapsulation is currently being increasingly explored to preserve the nutritional and bioactive properties

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and solubility of hydrophobic compounds in the pharmaceutical and food industries.[1] Nanoencapsulation involves coating a compound with a suitable material at the nanoscale (10–1000 nm) to produce particles with greater surface area per mass.[12] The formulation of carotenoids loaded in lipid-based nanoparticles such as solid lipid nanoparticles, nanostructured lipid carriers (NLCs) and micelles has been widely explored in recent years.[13–16] These formulations typically involve thermal treatments at atmospheric pressure or require organic solvents during formulation, which could cause the degradation of carotenoids. Polymer-based encapsulation may offer an alternative and is increasingly used to develop delivery systems in the pharmaceutical and food industries.[12] In general, polymer-based encapsulation offers high stability, high encapsulation efficiency and controlled release of encapsulated compound.[17] A diverse range of polymers either of biological origin (e.g., albumin, chitosan, dextran, gelatine, phospholipids etc.) or synthetic [e.g., polylactides (PLA), polyglycolide and block copolymers] can be used to develop stable and controlled release formulations.[18–21]

Polyethylene glycol (PEG) is a polymer of choice in drug delivery systems, and it is also commonly used in the food and cosmetic industries.[22] It is a synthetic, hydrophilic and biocompatible polymer that is synthesised via ring-opening polymerisation of ethylene oxide to obtain a broad range of molecular weights and geometries. It is a USFDA-approved excipient, and its popularity principally stems from its tuneable properties and favourable safety profile. Pluronics (trade name of poloxamers supplied by BASF) are triblock polymers consisting of hydrophobic polypropylene oxide (PPO) core with hydrophilic chains of polyethylene oxide (PEO) on each side.[23] Pluronic F-68 is a semi-crystalline polymer with a molecular weight of 8400 g/mol, containing on average 152 PEO and 29 PPO units.[24] Pluronics are listed excipients in the US and British Pharmacopoeias, considered generally regarded as safe and commonly used in the development of novel formulations.[25–27] Pluronics have various applications in food and pharma industries as emulsifiers, dispersants, thickeners and antifoaming/wetting agents.[28–30] Nanoencapsulation in this study was obtained using poloxamer or Pluronic and PEG, structures of both are presented in Figure 1.

Traditionally, polymeric nanoparticles are prepared via solvent-based processes usually involving the injection of an organic phase (e.g., polymers or triglycerides and the compound of interest in low boiling point solvents like acetone or ethanol) into an aqueous phase containing water and a surfactant under stirring. $scCO_2$-associated processes such as supercritical emulsions extraction (SEE) are also being explored for micro-/nanoparticle formation containing sensitive molecules.[31] These processes require organic solvent/s and are commonly performed in batch mode. The recent advancements in the continuous SEE to prepare nanoparticles reproducibly have made it more attractive but the use of a solvent in this process is unavoidable.[32–37] Nonetheless, encapsulates prepared via continuous SEE showed high encapsulation efficiency along with improved stability and antioxidant activity.[38, 39] There are a few solvent-free processes such as one reported by Sang *et al*. for nanoencapsulation by temperature-induced phase transition (TIPT) of Pluronic F-68.[40] In this study, the authors reported that heating a mixture of Pluronic F-68 and drug solution in PEG 400°C to 120°C followed by sudden cooling to 0°C resulted in TIPT of the polymer and the obtained mass-produced well-dispersed nanoencapsulates when suspended in water. TIPT results from the phase separation of copolymers at the nanometric scales caused by the incompatibility of different blocks within a copolymer [e.g., ABA (PPO–PEO–PPO) triblock polymers also commonly known as Pluronics]. However, the high temperature required to obtain nanoparticles in the method is not suitable to develop formulations containing carotenoids due to the thermal degradation of these compounds.[41] Pluronic F-68 has a melting temperature of 55°C at atmospheric pressure and PEG 400 is a liquid at room temperature. Significant to this study, Bhomia *et al*. studied the effect of pressure on the solid–liquid transition of pluronics in pressurised CO_2 and observed a substantial decrease (35°C at 100 bar) in their melting point in comparison to their actual melting point at atmospheric pressure.[24] The melting point depression caused by the dissolution of CO_2 in the polymer along with the prospect of working in an environment that is free from atmospheric water and oxygen can be favourable for the processing of sensitive molecules such as carotenoids at considerably low temperatures. The dissolution of CO_2 in polymer to produce a melt followed by spraying of the melt through a nozzle is commonly used for the particle formation in the process known as particles from the gas-saturated solution or PGSS.[42] This study utilises the capability of CO_2 to dissolve within pluronic to allow TIPT to occur at lower temperatures than 120°C as used previously for the encapsulation of paclitaxel. However, it cannot be classed as PGSS because spraying of the melt was not performed for the particle formation. Hence, this study entails the development of nanoencapsulates via TIPT containing carotenoid extracts in $scCO_2$ at considerably low temperatures to avoid thermal, oxidative, hydrolytic and/or solvent-based degradation of carotenoid.

Materials and Methods

Materials

The extracts and reagents used in this study were used without any further purification. Extracts of *Dunaliella* biomass prepared using supercritical CO_2 were obtained from Hopfenveredlung St. Johann GmbH ($NATECO_2$), Munich, Germany. Pluronic F-68 and PEG 400 were purchased from Sigma-Aldrich Ltd., Gillingham (Dorset), UK. HPLC grade ethanol, methyl *tert*-butyl ether (MTBE), methanol and HPLC vials were purchased from Fisher Scientific UK Ltd., Loughborough, UK. Liquid CO_2 with 99.9% purity was

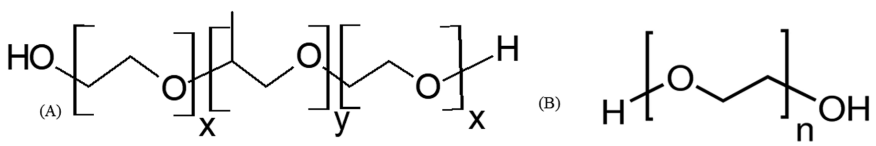

Figure 1 Generalised structure of a PEO–PPO–PEO triblock (a) polymer (*x* and *y* represent the length of PEO and PPO blocks, respectively) and polyethylene glycol (b).

supplied by BOC Ltd. The standards of *All-trans* β-carotene, α-carotene, lutein, zeaxanthin and phytoene were purchased from Sigma-Aldrich Inc. (Merck KGaA, Darmstadt, Germany).

Preparation of carotenoid-loaded pluronic nanoparticles

Carotenoid-loaded pluronic nanoparticles were prepared by TIPT. The method used for the preparation of carotenoid-polymeric nanoparticles (Car-PNPs) was adapted from the study published by Sang *et al.* but modified such that nanoparticles were prepared in $scCO_2$.[40] Car-PNPs were assigned descriptors based on formulation conditions as presented in Table 1.

The carotenoid extract (5–20 wt.% of total polymers) was thoroughly mixed with PEG under stirring at 200 rpm to form a sample-loaded phase. Subsequently, Pluronic F-68 was added under gentle mixing and the carotenoid extract-polymer blend was processed using an instrument (Thar Technology Inc., USA) detailed in Figure 2.

The instrument consisted of a temperature-controlled 150 ml high-pressure vessel with sapphire windows to visualise the experiment during processing. The temperature change in the vessel was achieved via cartridge heaters operated by a temperature controller. A beaker containing the mixture was placed in the high-pressure vessel and heated at temperatures specified in Table 1 either at atmospheric pressure or under pressurised CO_2 (10 MPa). The processing temperature and pressure were chosen according to the study published by Bhomia *et al.*[24] The mixture was kept at a specified temperature and pressure for 90 min before cooling it by switching the heat off and performing a sudden depressurisation within 2 min or by leaving it in an ice bath (samples prepared at 0.1 MPa) to induce phase transition. The exit port on the vessel was fitted with 0.45 µm frit to avoid possible loss of particles in the process. No spillage of the contents in the beaker was observed in the high-pressure vessel after depressurisation. The beaker was then removed from the vessel and the processed material was gently crushed prior to being stored at 5°C in an amber-coloured vial until required. Each preparation consisted of polymers in the ratio of 1:4 (PEG:pluronic F-68), except Car-PNP-1s which was prepared by mixing pluronic F-68 directly with the carotenoid extract in the absence of PEG. Each formulation was prepared in triplicate to ensure reproducibility. The nanoparticulates' preparation process is schematically depicted in Figure 3.

Table 1 Formulation descriptors

Formulation	Pressure (MPa)	Temperature (°C)	Polymer/s
Car-PNP-0a	0.1	120	PEG/Pluronic F-68
Car-PNP-1a	0.1	60	PEG/Pluronic F-68
Car-PNP-1s	10	60	PEG/Pluronic F-68
Car-PNP-2s	10	40	PEG/Pluronic F-68
Car-PNP-1s*	10	60	Pluronic F-68

The '*' signifies 'formulation prepared without PEG'.
Each formulation code comprises a generic indicator -Car-PNP, a temperature indicator (0, 1 or 2) and a pressure indicator (a-atmospheric or s-supercritical CO_2).

Carotenoid quantification

Carotenoids from the crude extract and formulations were extracted in ethanol by solid–liquid sonication method. Five milligrams of extract or Car-PNPs containing an equivalent amount of carotenoids were suspended in 10 ml ethanol by

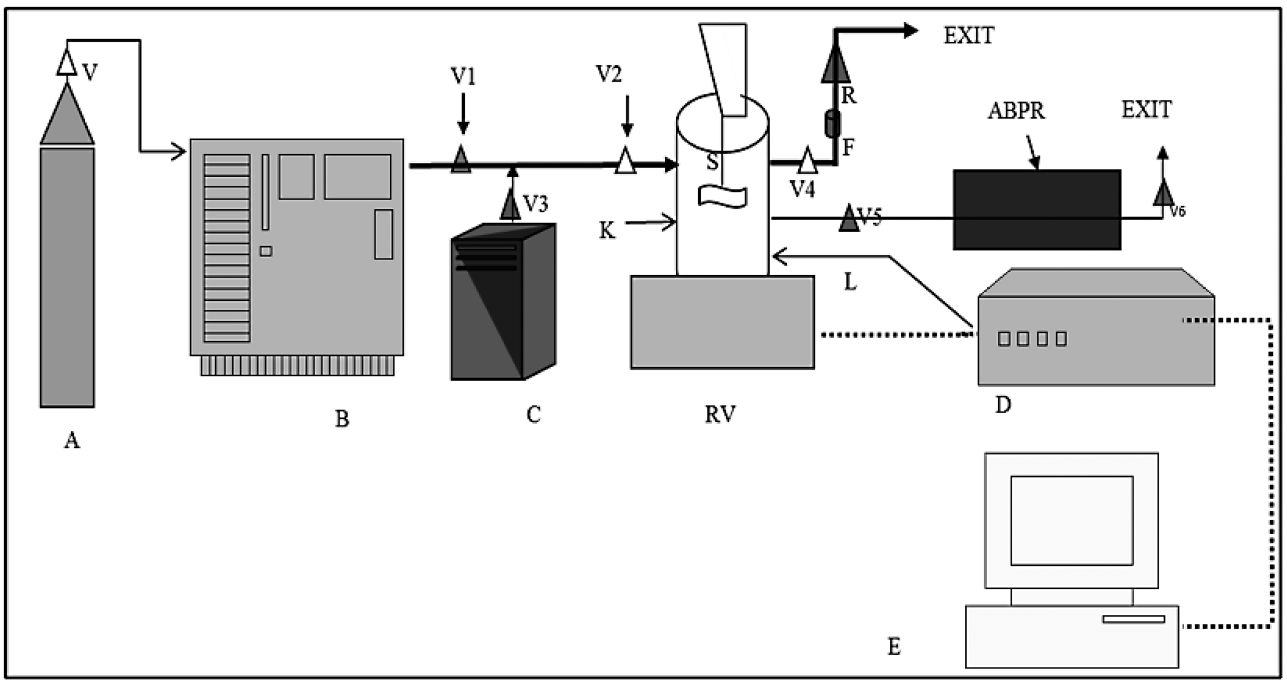

Figure 2 Schematic presentation of SCF instrument [CO_2 cylinder (a), chiller (b), CO_2 pump (c), high-pressure vessel, automatic back pressure regulator, temperature controller (d) and display unit (e)].

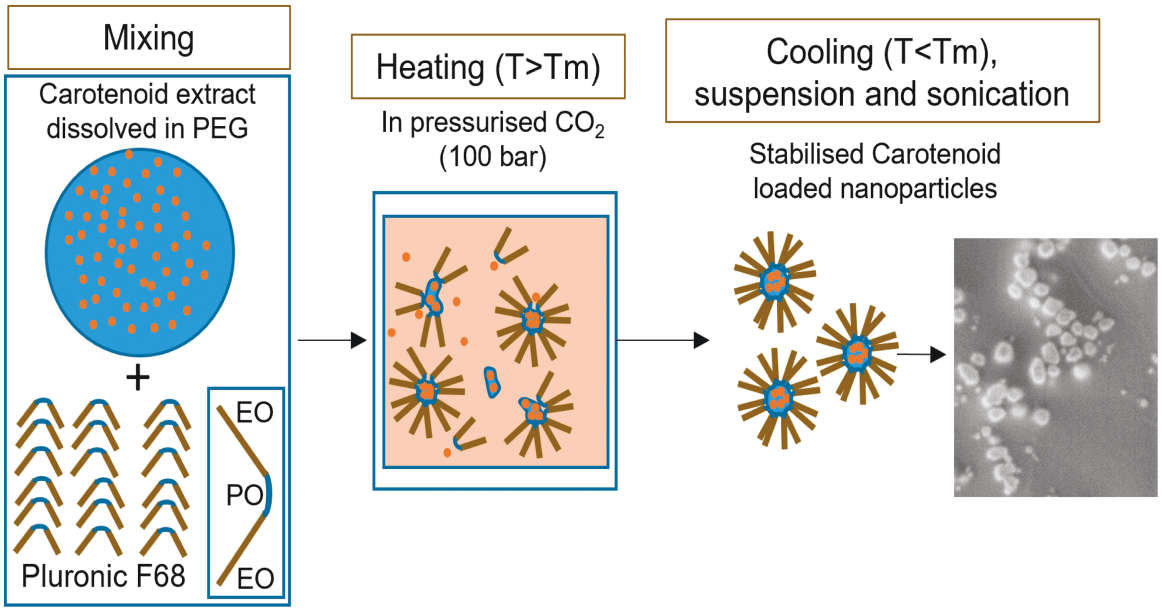

Figure 3 Schematic representation of the nanoparticulates' preparation process.

gentle mixing prior to sonication and vortexing for 1 min each. After sonication, each sample was centrifuged at 3000 *g* for 10 min at 15°C and then the supernatants were filtered (0.45 µm filter) into amber-coloured HPLC vials. Samples were analysed by HPLC using an Agilent 21000 series HPLC equipped with a YMC_{30} (250 × 4.9 mm I.D., S- 5 µm) analytical column and diode array detector. Ethanolic extracts (20 µl) were analysed at 25°C under an isocratic elution (mobile phase-80% MeOH: 20% MTBE) at a flow rate of 1 ml min^{-1}. Carotenoids were quantified by reference to standards after separation by HPLC. Based on the quantification results, it was decided to investigate Car-PNP-1s further due to consistently high values for all studied carotenoids.

Particle size of carotenoid-loaded pluronic nanoparticles

The formulations were analysed to determine the average size and polydispersity index (PDI) of the nanoparticles. The average diameter and size distribution of the Car-PNPs were measured via dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). Fifty milligrams of Car-PNP-1s or physical mixture (containing 10% w/w carotenoid extract) were dispersed in 10 ml of deionised water and sonicated for 2 min to produce well-dispersed nanoparticles in the media. Two minutes of sonication was found to be sufficient to disperse taken mass which also provided reproducible particle size results. Measurements were conducted in triplicate at room temperature and the average particle size was presented as the mean of three measurements.

Morphology of carotenoid-loaded pluronic nanoparticles

The shape and surface morphology of the nanoparticles was determined using scanning electron microscopy (SEM). One microlite of the nanoparticle suspension was placed on a microgrid coated with a thin carbon film and excess liquid was removed by capillary action. The grid was vacuum-dried overnight forming a monolayer of the sample. The stub (12 mm diameter, Agar Scientific, G3347N) was mounted onto the sample holder and placed inside the Hitachi triple detector CFE-SEM SU8030 (Hitachi Triple detector CFE-SEM SU8030, Roland Schmidt, Hitachi High-Technologies Europe, Germany) scanning electron microscope. Micrographs were obtained using the upper secondary electron detector at a voltage of 1.0 kV.

Aqueous solubility and stability

The aqueous solubility of carotenoids was determined on carotenoid extract, Car-PNP-1s and a physical mixture. An accurately weighed extract or Car-PNP/physical mixture containing 5 mg of carotenoids was weighed into a vial containing 10 ml of distilled water. The mixture was sonicated for 2 min to obtain well-formed nano-dispersion. It was then stirred at 200 rpm for 60 min and centrifuged thereafter at 3000 *g* for 10 min. The HPLC analysis on the filtered (0.45 µm filters) supernatant was performed (method detailed in carotenoid quantification section) at 25°C using a YMC_{30} column and under an isocratic elution (mobile phase—80% MeOH:20% MTBE) at a flow rate of 1 ml min^{-1}.

For stability analysis, carotenoid extract and Car-PNP-1s and extract were stored in amber-coloured vials at 5°C for 5 weeks. Each sample was extracted and analysed using HPLC weekly using methods detailed earlier.

Results

Characterisation

Carotenoids are susceptible to oxidation; therefore, it is important to ensure that they are not decomposed during the formulation process. Table 2 shows the recovery of carotenoids from freshly prepared formulations as estimated using HPLC.

The processing temperatures of 60° and 40°C were selected as these were 5°C above pluronic F-68's melting temperature at atmospheric pressure and in $scCO_2$ at 100 bar, respectively. The results point to the possible detrimental effect of

the processing temperature on carotenoid stability. In general, formulations prepared in $scCO_2$ had significantly higher carotenoid content than the ones processed at atmospheric pressure at 60°C. The TIPT was also performed at 120°C (Car-PNP-0a), as suggested by Sang *et al.* to compare the feasibility of Car-PNP formation according to their method.[40] Although it was possible to prepare nanoparticulate as seen from the DLS data but increase in temperature resulted in significantly higher degradation (>50% loss of total carotenoids) of carotenoids. The major cause of carotenoid decomposition during processing and storage is widely accepted to be due to oxidation which is known to increase with higher processing temperature and longer processing times.[43–45] The processing time was kept constant in the preparation of nanoencapsulates in this work; so, it could be suggested that temperature played an important role in the reduction of carotenoid content in the formulation listed in Table 2. A comparison of Car-PNP-1a to Car-PNP-1s and the samples processed at 60°C (Car-PNP-1a) and 120°C (Car-PNP-0a) allude to the impact of processing temperature on the stability of carotenoids. Although further experiments may be needed to confirm this hypothesis, these results suggest that the processing temperature accompanied by the absence of oxygen during $scCO_2$ processing may have had a positive impact in reducing carotenoid decomposition due to oxidation. Interestingly, Car-PNPs prepared at 40°C and 10 MPa resulted in lower recovery of carotenoids than Car-PNP-1s but higher than Car-PNP-1a. This could be attributed to the partial phase transition/encapsulation at 40°C down to the differences in density and mole content of CO_2 in the vessel along with the melt viscosity of the polymer that could provide better mixing at 60°C than at 40°C.

The $scCO_2$-extracted oil was composed of 47%, 35%, 17%, 0.1% and 0.1% of all-trans β-carotene, 9′-*cis*-β-carotene, all-*trans*-α-carotene, lutein and zeaxanthin, respectively. The HPLC analysis was performed for Car-PNPs and carotenoid extracts, and the ratios (Table 3) of individual carotenoids in each formulation were calculated by normalising the concentration of all identified carotenoids with the concentration of 9′-*cis* β-carotene in the extract. Table 3 confirmed that the ratio of individual carotenoids recovered from each formulation remained relatively the same as in the carotenoid extract regardless of formulation condition and carotenoid recovery percentage after formulation.

On the basis of these results, processing parameters of 60°C and 10 MPa were chosen to prepare further formulations via TIPT. Car-PNPs were formulated with 5, 10 and 20% of the carotenoid extract and particle size results are presented in Table 4.

All formulated Car-PNPs had a monomodal distribution with the particle sizes at different weight ratios of carotenoid extract varying within the range of ~150–250 nm. The average particle size increased with the extract content in the formulation. Similarly, PDI values for 10 and 20% formulations increased to ~0.5 from 0.3 (5% Car-PNP), indicating a broadening of particle size distribution with the increase in carotenoid content in the formulation.[46]

SEM analysis showed the formation of spheroidal particles along with irregularly shaped structures as presented in Figure 4. Although a few discrete spherical or spheroidal structures could be seen at the higher magnification, but particles also appeared to be agglomerated or merged which could have occurred during the drying of the suspension before SEM analysis.

The work reported by Oh *et al.* presented cryo-TEM micrographs of nanoparticles that were comprised of a dark core consisting of paclitaxel-loaded PEG surrounded by a much lighter Pluronic F-68 outer shell, suggesting the formation of a core/shell structure.[40] Although it is difficult to delineate this from the SEM micrographs shown in Figure 3, a comparable morphology could be expected for these nanoparticulates where the extract dissolved in PEG could be assumed to be enveloped by pluronic F-68. The particle sizes obtained by SEM micrographs also complimented the values obtained using DLS.

Solubility of carotenoids

The carotenoid solubility from each freshly prepared formulation was determined in deionised water and the results are presented in Table 5. A physical mixture of the polymers and crude carotenoid extract was also included in the study as controls. The recovery of carotenoids from each sample was obtained using HPLC and benchmarked against the carotenoids recovered in an ethanolic solution of the crude extract.

An improvement in the aqueous solubility of all carotenoids was seen when incorporated in Car-PNP formulations compared with the crude extract and physical mixture as shown in Table 5. The analysis of the recovery of individual carotenoids indicated the variability in the solubility of various carotenoids. For example, all-*trans*-β-carotene was consistently the carotenoid with the least recovery in all Car-PNP formulations except for the Car-PNP-0a, and amongst xanthophylls, the aqueous solubility of lutein from Car-PNP formulations was higher than zeaxanthin regardless of the formulation. The effect of the processing method was also clear where the Car-PNP-1s formulation prepared in $scCO_2$ resulted in the highest (≈650-fold) total carotenoid content in water than

Table 2 The percentage (%) recovery of carotenoids in Car-PNP formulations

Carotenoids	Car-PNP-0a	Car-PNP-1a	Car-PNP-1s	Car-PNP-2s	Car-PNP-1s[a]
9′-*cis*-β-carotene	44.0 ± 0.5	63.3 ± 0.3	95.9 ± 1.5	80.4 ± 0.3	100.9 ± 1.3
All-*trans*-β-carotene	43.2 ± 0.6	68.3 ± 0.2	98.5 ± 0.8	75.9 ± 0.5	100.0 ± ± 2.7
All-*trans*-α-carotene	55.5 ± 0.7	73.8 ± 0.3	99.1 ± 0.2	78.7 ± 0.6	101.1 ± ± 2.4
Lutein	29.2 ± 0.2	67.6 ± 0.8	92.2 ± 1.5	85.0 ± 1.4	89.3 ± ± 2.7
Zeaxanthin	28.0 ± 0.9	64.2 ± 2.3	97.5 ± 1.2	77.4 ± 1.2	87.9 ± ± 3.7
Total carotenoids	43.6 ± 0.1	66.3 ± 0.1	97.4 ± 1.5	78.5 ± 0.13	99.9 ± 4.7

[a]The values shown are the percentage of the corresponding carotenoid in the carotenoid extract that was recovered in each sample of Car-PNP. Values are the mean of three replicates ± SD.

Table 3 The ratio of individual carotenoids in each formulation normalised to 9′-cis β-carotene

	Retention time (min)	Car-PNP-0a	Car-PNP-1a	Car-PNP-1s	Car-PNP-2s	Car-PNP-1s[a]	Crude extract
9′–*cis*-β-carotene	34.1 ± 1.3	1.00	1.00	1.00	1.00	1.00	1.00
All-*trans*-β-carotene	29.3 ± 1.8	0.84	0.93	0.83	0.82	0.85	0.80
All-*trans*-α-carotene	22.9 ± 1.6	0.20	0.19	0.17	0.16	0.16	0.17
Lutein	6.8 ± 0.4	0.03	0.05	0.05	0.05	0.04	0.05
Zeaxanthin	7.9 ± 0.6	0.02	0.03	0.03	0.03	0.03	0.03

[a]The values shown are the percentage of the corresponding carotenoid in the carotenoid extract that was recovered in each sample of Car-PNP. Values are the mean of three replicates ± SD.

Table 4 The average particle size of carotenoid-loaded polymer nanoparticles

Car-PNP-1s	Average particle size (nm)	PDI
5%	158 ± 0.4	0.29 ± 0.02
10%	208 ± 3	0.45 ± 0.03
20%	234 ± 12.1	0.50 ± 0.08

any other. The recovery of total identified carotenoids in each formulation can be summarised as Car-PNP-1s > Car-PNP-2s > Car-PNP-0a > Car-PNP-1s* > Car-PNP-1a.

Stability of carotenoid-loaded polymer nanoparticles

The recoveries of 9′-*cis*-β-carotene, all-*trans*-β-carotene, all-*trans*-α-carotene, lutein and zeaxanthin in Car-PNP-1s and carotenoid extract during 5-week storage in the dark at 5°C are presented in Figure 5a–e. The carotenes were generally more stable in the formulation than in the pure extract for the duration of the study while, for lutein and zeaxanthin, improved stability was observed only in the first 2 weeks of storage.

The estimated degradation rates for carotenoids in Car-PNP-1s and the carotenoid extract were obtained from the regression equations and the degradation rate constants are presented in Table 6.

The stability of individual carotenoids can be summarised as follows: all-*trans*-α-carotene > lutein > all-*trans*-β-carotene > zeaxanthin > 9′-*cis*-β-carotene in the crude extract and all-*trans*-α-carotene > all-*trans*-β-carotene > 9′-*cis*-β-carotene > lutein > zeaxanthin in Car-PNP-1s. Although individual carotenoids exhibited degradation rate variability, the stability of carotenoids was higher in Car-PNP-1s, suggesting that the stability of carotenoids is improved after nanoencapsulation.

Discussion

The bioavailability of carotenoids is limited owing to their poor aqueous solubility. Thus, carotenoid-containing food, formulations and supplements typically require dispersion, emulsification or encapsulation to modify solubility.[10] The adaptation of the method reported by Sang *et al.* to the findings of Bhomia *et al.* allowed the preparation of formulations under pressurised CO_2 at 60°C with minimal carotenoid oxidation that was significantly lower than the temperature previously used (120°C).[24, 40]

The self-assembly of amphiphilic polymers such as poloxamers caused by temperature changes is a comparatively easy way to obtain nanoparticles but it generally requires high temperatures. The dehydration of PPO chains upon melting causes them to interact with the carotenoid solution in PEG resulting in a core/shell structure as reported by Oh *et al.*[40] There have been previous attempts to reduce the high TIPT temperature, for example, Lee *et al.* successfully prepared Pluronic-based nanoparticles loaded with either caffeine, ibuprofen or orlistat at 75°C under atmospheric pressure and Yuk *et al.* formulated docetaxel encapsulates at 60°C by changing the solubiliser from PEG to soybean oil/tween 80 mixture.[47, 48] However, these modifications still remain insufficient for carotenoid processing because the stability of carotenoids is not only influenced by high temperatures, but oxidation is also a major concern.[11] El-Tinay and Chichester observed that β-carotene solubilised in toluene and treated at 60°C with a stream of oxygen was oxidised, producing various epoxides.[49] In the present study, carotenoid decomposition was evident in the samples (Car-PNP-1a) formulated at 60°C, under atmospheric pressure. However, TIPT (Car-PNP-1s) at 60°C in $scCO_2$ produced carotenoid nanoparticles that resulted in the minimal carotenoid loss. It was assumed that the carotenoid stability was maintained in $scCO_2$ due to the exclusion of atmospheric oxygen leading to the reduced decomposition by oxidation.

Microscopic imaging of Car-PNPs confirmed the formulation contained nano-sized particles. The average size of nanoparticles for each formulation was <250 nm with PDI <0.5. Oh *et al.* also reported smaller particle sizes for corresponding sample loading.[40] Other studies have reported different particle size ranges (e.g. 144–249 nm) for β-carotene-loaded NLCs.[15] Particle size variability can occur due to the size/volume of the encapsulant, quantity of payload, formulation method, excipients used and other complexities of the compound. The carotenoid extract used in the present study is a complex crude concentrate containing carotenoids and other lipids that may explain larger particle sizes when compared with previously reported studies. Results also showed that particle size was proportional to the percentage content of the carotenoid extract in the formulation. This is expected as a high loading will require a larger encapsulation surface. For example, Govender *et al.* prepared PLGA nanoparticles by nanoprecipitation and found that the particle size increased with increasing drug concentration.[50] Although particle size changed with the carotene extract loading, but small PDI (<0.5) as obtained via DLS measurements indicated a monomodal and comparatively narrow size distribution of nanoparticles. In general, PDI values higher than 0.5 are indicative of broad distribution.[12]

Improved aqueous solubility of carotenoid from polymeric nanoparticles formulated via TIPT was also seen as

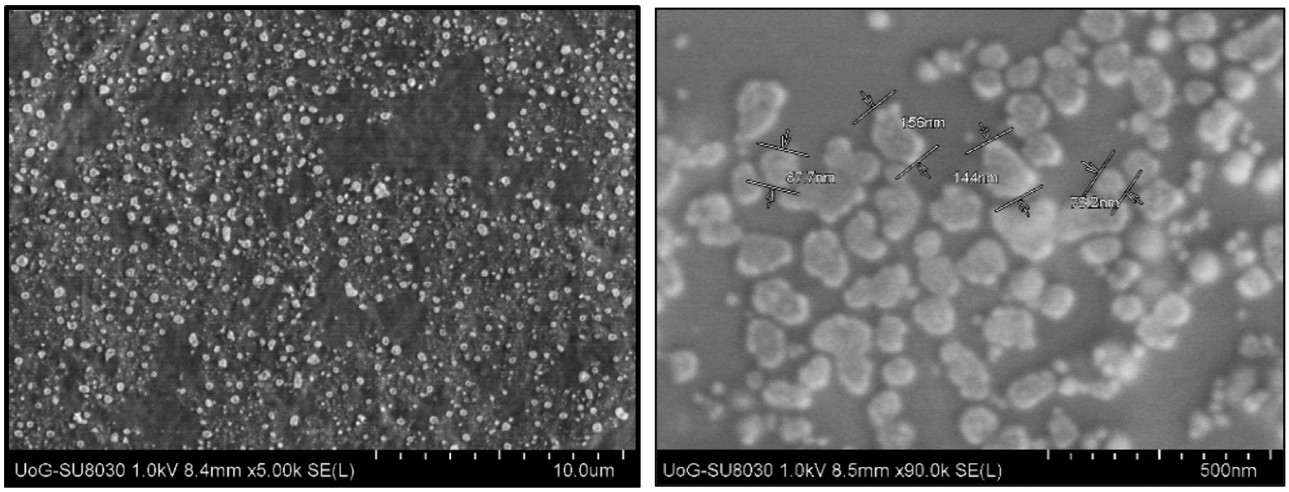

Figure 4 SEM micrographs of 5% carotenoid-loaded pluronic nanoparticles. (Micrograph of nanoparticles at 5k magnification (left) and 90k magnification (right).

Table 5 The percentage (%) recovery of carotenoids in deionised water

	Crude extract	Physical mixture	Car-PNP-0a	Car-PNP-1a	Car-PNP-1s	Car-PNP-2s	Car-PNP-1s[a]
9′–*cis*-β-carotene	0.08 ± 0.01	1.2 ± 0.2	28.5 ± 0.8	19.5 ± 1.5	57.0 ± 2.8	53.3 ± 4.8	30.9 ± 1.1
All-*trans*-β-carotene	0.5 ± 0.01	0.8 ± 0.1	20.7 ± 2.3	14.0 ± 1.5	27.1 ± 1.0	23.4 ± 1.3	13.1 ± 0.5
All-*trans*-α-carotene	0.1 ± 0.02	2.0 ± 0.4	28.3 ± 1.8	23.6 ± 3.0	71.7 ± 0.8	37.6 ± 4.4	23.0 ± 1.4
Lutein	0.2 ± 0.01	1.1 ± 0.1	22.0 ± 0.5	40.1 ± 4.8	63.4 ± 6.3	54.8 ± 7.6	50.6 ± 3.6
Zeaxanthin	0.4 ± 0.03	8.4 ± 0.3	16.2 ± 3.1	31.0 ± 3.6	51.2 ± 3.1	35.5 ± 4.3	36.0 ± 4.1
Total carotenoids	0.07 ± 0.01	1.2 ± 0.2	25.4 ± 1.2	18.6 ± 1.3	45.7 ± 2.6	40.8 ± 3.7	23.9 ± 0.8

[a]Values are the percentage of corresponding carotenoids originally quantified in an ethanolic solution of the carotenoid extract that was recovered from an aqueous solution of each test sample. Values are mean ± SD (n = 3).

presented in Table 5. Surfactants or surfactant-like molecules are known to significantly improve the solubilisation of hydrophobic compounds as observed in this study.[3] The solubility of natural high-value carotenoids extracted from *Dunaliella* increased by 650-fold (total carotenoid) after nanoencapsulation in PEG and Pluronic, but the sheer presence of these polymers was not sufficient to obtain the solubility improvement as was seen from the results obtained for the physical mixture. Therefore, nano-encapsulation of carotenoids by TIPT was important to obtain both highly soluble and comparatively stable carotenoid formulations. The formulation method, processing parameters and polymer composition had a clear influence on the solubility of the carotenoids. The aqueous solubility of carotenoids from the formulation containing PEG and prepared in $scCO_2$ (Car-PNP-2s and Car-PNP-1s) was significantly higher than Car-PNP-1s* (without PEG) which implies that PEG was important to obtain nanoencapsulates with the improved solubility of carotenoids. Also, phase transition temperature close to the melting point of Pluronic F-68 appeared to be insufficient to allow the required homogeneity of the mixture within the heating period used in this study. For example, the improvement in the aqueous solubility of carotenoids was obtained for the formulation prepared at 120°C/0.1 MPa (Car-PNP-0a) than 60°C/0.1 MPa (Car-PNP-1a). A similar effect was seen with the samples prepared at 40°C and 60°C in $scCO_2$ where the latter formulation resulted in the highest recovery of carotenoids. In some cases, it may be possible to resolve this issue by extending the processing time at lower temperatures.[47] However, processing for longer at any given temperature under atmospheric pressure may still result in higher degradation of thermosensitive and oxidation-prone molecules. Hence, it is compelling to develop formulation strategies that limit thermal and oxidative degradation, such as via processing in $scCO_2$ as described in the current study. The melting point depression of Pluronics caused by the dissolution of CO_2 in the polymeric matrix allowed a considerable reduction in the processing temperature to perform TIPT at 60°C (Car-PNP-1s) and also provided oxygen and solvent-free environment to reduce the decomposition of carotenoids during processing.

These types of strategies can have a significant impact on the development of formulations with improved shelf-life and the safe delivery of carotenoids or similar compounds. For example, the UK general population reportedly consumes, on average, ~3.1 mg of carotenoids daily.[51] About 6–15 mg day^{-1} of β-carotene is used for prophylactic treatment of conditions like psoriasis, vitiligo and hairy leucoplakia and a dose of 150–180 mg day^{-1} is used in the treatment of erythropoietic protoporphyria.[51] With increased aqueous solubility, carotenoid dose reduction may be

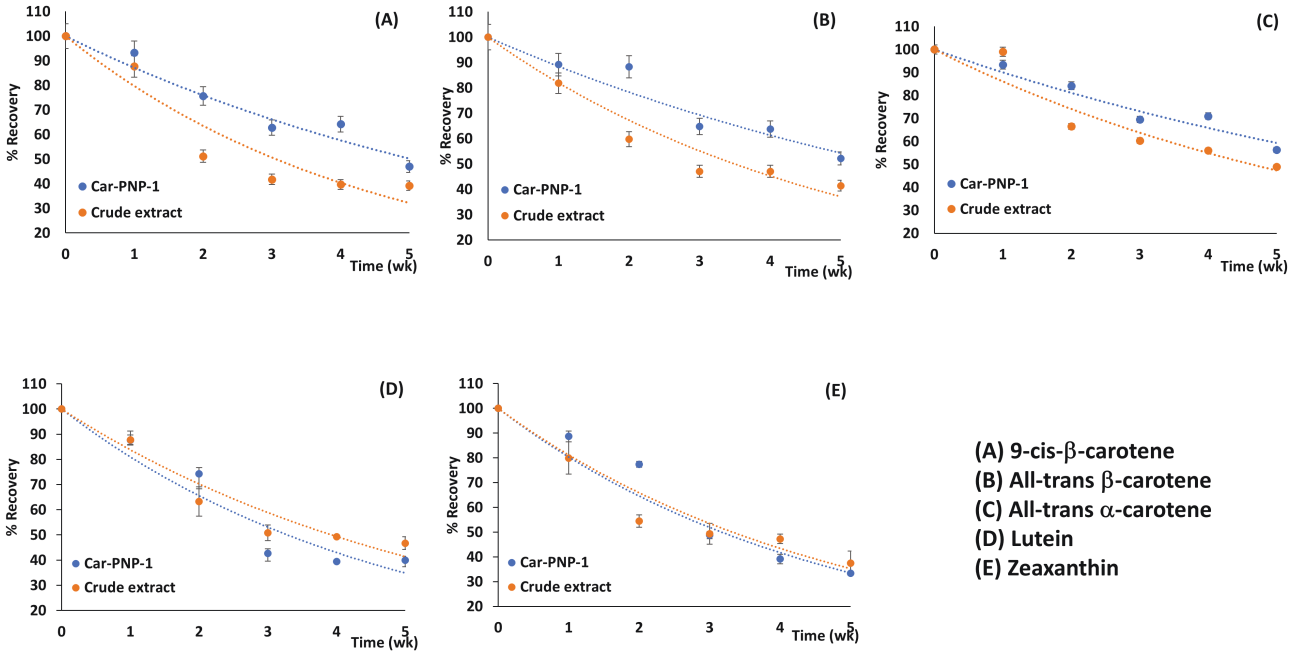

Figure 5 Stability of carotenoids in Car-PNP-1s and crude extract during 5-week storage.

Table 6 Estimated rate constants (week^{-1}) for first-order degradation of carotenoids in Car-PNP-1s and crude extract after 5-week storage in the dark at 5°C

Carotenoid	Car-PNP-1s	Crude extract
9′-*cis*β-carotene	0.138	0.227
All-*trans*β-carotene	0.122	0.198
All-*trans*-α-carotene	0.104	0.150
Lutein	0.211	0.176
Zeaxanthin	0.219	0.208
Total carotenoids	0.138	0.188

attainable. For instance, 20 and 26 mg of the crude carotenoid extracts would be required to attain 1 mg of 9′-*cis*-β-carotene and all-*trans*-β-carotene in solution. Whereas only 1.7 and 3.7 mg of Car-PNP-1s will be potentially required to achieve 1 mg of 9′-*cis*-β-carotene and all-*trans*-β-carotene based on the solubility data presented in Table 5.

The stability study showed that the stability of carotenoids is improved in Car-PNP-1s, although xanthophylls appeared to be more stable in the carotenoid extract than in Car-PNP-1s over the 5-week period. In Car-PNP-1s, lutein appeared to degrade quicker than carotenes whereas, in the carotenoid extract, lutein was the second most stable carotenoid. The poor stability of lutein identified in Car-PNP-1s conflicts with previous stability studies that found that lutein is more stable than β-carotene isomers.[52, 53] Therefore, this suggests that the degradation observed in Car-PNP-1s is not solely due to the inherent oxidative vulnerability of carotenoids. It is also possible that the site of loading of carotenoids may have influenced the stability of the carotenoids. For example, it could be possible that carotenes were deposited deep within the core while the xanthophylls are situated closer to the surface owing to the difference in their polarity. Consequently, it is possible that the xanthophylls (lutein and zeaxanthin) were more exposed and available for oxidation than carotenes. The solubility of carotenoids in $scCO_2$ is pressure dependent and usually increases with the increase in pressure at isothermal conditions. However, a review published by Shi *et al.* suggests that considerably higher pressures are required to obtain carotenoid solubilities of any significance in $scCO_2$.[54] This may indicate that the difference in the interaction of various components with the polymer could be negligible due to solubilisation and fractionation in the $scCO_2$ at 10 MPa. Nevertheless, the extract was presented as a solution in PEG so that could also impact how individual components may interact with the polymer and influence their encapsulation and stability in the studied system. Although Car-PNP1s improved the stability of some components of the extract, but these results are difficult to explain and certainly require further investigation.

Conclusion

Nanoencapsulation of natural high-value carotenoid extracts obtained from Dunaliella significantly improved the aqueous solubility of carotenoids. This study indicated that a temperature of 60°C in $scCO_2$ (10 MPa) for Pluronic F-68 was sufficient to perform TIPT for the nanoencapsulation of carotenoids with minimal carotenoid decomposition. Following encapsulation, a 650-fold improvement of carotenoid aqueous solubility was recorded. Encapsulates containing carotenoids also showed improved stability, particularly of carotenes when stored at 5°C. Conclusively, this study provides a novel solvent-free method to obtain formulations with improved aqueous solubility and shelf-life of carotenoids. However, further studies including encapsulation efficiency, release pattern and in-vivo bioavailability are required to establish the bioaccessibility of carotenoids from prepared polymeric nanoparticles.

Author contributions

Conceptualisation: V.T., P.J.H.; methodology: V.T., P.J.H.; formal analysis: V.T., C.I.W.; investigation: V.T., C.I.W.;

resources: V.T., P.J.H.; writing—original draft preparation: CIW, VT; writing—review and editing: C.I.W., V.T., P.J.H.; supervision: V.T., P.J.H.; project administration: V.T., P.J.H.; All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

None declared.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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