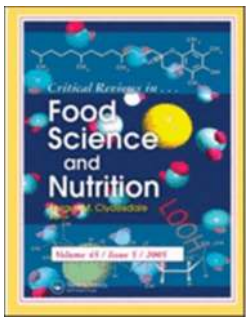

A COMPREHENSIVE OVERVIEW ON THE MICRO- AND NANO-TECHNOLOGICAL ADVANCES FOR ENHANCING THE CHEMICAL STABILITY AND BIOAVAILABILITY OF CAROTENOIDS

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A COMPREHENSIVE OVERVIEW ON THE MICRO- AND NANO-TECHNOLOGICAL ENCAPSULATION ADVANCES FOR ENHANCING THE CHEMICAL STABILITY AND BIOAVAILABILITY OF CAROTENOIDS

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ABSTRACT

Carotenoids are lipophilic secondary plant compounds, and their consumption within fruits and vegetables has been positively correlated with a decreased risk of developing several chronic diseases. However, their bioavailability is often compromised due to incomplete release from the food matrix, poor solubility and potential degradation during digestion. In addition, carotenoids in food products are prone to oxidative degradation, not only lowering the nutritional value of the product but also triggering other quality deteriorative changes such as formation of lipid pro-oxidants (free radicals), development of discolorations or off-flavor defects. Encapsulation refers to a physicochemical process aiming to entrap an active substance in structurally engineered micro- or nano-systems in order to develop an effective thermodynamical and physical barrier against deteriorative environmental conditions, such as water vapor, oxygen, light, enzymes or pH. In this context, encapsulation of carotenoids has shown to be a very effective strategy to improve their chemical stability under common processing conditions including storage. In addition, encapsulation may also enhance bioavailability (via influencing bioaccessibility and absorption) of lipophilic bioactives, via modulating their release kinetics from the carrier system, solubility and interfacial properties. In the present paper, it is aimed to present the state of the art of carotenoid microencapsulation in order to enhance storability and bioavailability alike.

Keywords: Bioactive compounds; bioaccessibility; controlled release; storage stability; functional food; structural design

INTRODUCTION

Carotenoids are natural organic pigments comprising eight isoprene units joined to a skeleton of 40 carbon atoms. Carotenoids can be generally classified into *oxygen containing,* i.e. xanthophylls (e.g. lutein, cryptoxanthin, astaxanthin) and *hydrocarbon* carotenoids, i.e. carotenes (e.g. *α-, β-* carotene, lycopene, phytoene). Carotenoids are synthesized by all plants and many microorganisms (bacteria and fungi), but not by animals including humans. On many occasions, carotenoids are responsible for the red, yellow and orange color of fruit, vegetables, flowers etc. (Britton, 1996).

The presence of a conjugated, delocalized π-electron system (polyene chain) is strictly associated with the particular physicochemical properties of carotenoids. They are comparatively lipophilic compounds with octanol-water coefficients of ca. 10-18 (Cooper et al., 1997). They also easily isomerize and are oxidized; they absorb light, imparting their color features as well as exerting significant free radical scavenging ability, acting as singlet oxygen quenchers or electron donators/acceptors (Krinsky and Johnson, 2005).

At least in part due to their antioxidant properties, adoption of a carotenoid-rich diet has been suggested to confer important health benefits to humans by retaining main bodily functions and to prevent inflammation related disease such as cardiovascular, ophthalmological, pulmonary and neurodegenerative disorders as well as some types of cancer (Krinsky and Johnson, 2005; Stahl and Sies, 2005). This may be related to the carotenoids' impact on cellular signaling cascades such as various transcription factors, influencing the expression of genes associated with antioxidant defense, anti-inflammatory or anti-cancer properties (Kaulmann and Bohn, 2014; Stahl and Sies, 2005). Unfortunately, due to their required emulsification in form of mixed micelles in the gut prior to their uptake (Bohn, 2008), bioavailability from fruits and vegetables, their main dietary sources, is low, around 10-20% for carotenes, and up to 40% for xanthophylls (Bohn, 2008; Salar-Behzadi et al., 2013).

In food industry, carotenoids are used as natural pigments to impart color or to recover color losses, following severe processing and prolonged storage of e.g. meat and dairy products, ice cream, beverages, or confectionaries (Britton and Khachik, 2009). Moreover, carotenoids are considered as natural antioxidants, preventing lipid oxidation in o/w and w/o emulsions such as in salad dressings, mayonnaises, dairy and fat spreads etc. (Dimakou and Oreopoulou, 2012; Lee and Choe, 2013; Yanishlieva et al., 2001). Delivering carotenoids to humans via functional food carriers can improve their bioavailability through improving matrix release kinetics (Qian et al., 2012c), solubility (Ribeiro et al., 2010), and may reduce their degradation during digestion (Wang and Bohn, 2012). Such carriers may include dehydrated matrices produced by physical methods (e.g. spray-, freeze- or fluidized bed-drying), micro- or nano-emulsions, hydrogel-based microbeads, self-assembled biopolymer matrices, nanostructured particles and others. Moreover, specific food processing such as thermal processing, size reduction or emulsification may promote carotenoid bioavailability. On the other hand, abusive processing including prolonged storage time, exposure to oxygen, severe heat treatment etc. can damage carotenoids due to instability against heat and light (Wang and Bohn, 2012). Thus, maintaining carotenoid biological activity, particularly for processed food, is a challenge for the food industry. In the present paper, we review recent advances in carotenoid micro- and nano-encapsulation aiming to retain their stability under typical food processing and storage conditions (Fig. 1) and to ensure maximal bioavailability.

THE CONCEPT OF ENCAPSULATION

Encapsulation refers to a physicochemical process aiming to entrap active compounds, e.g. phytochemicals, living cells, essential oils etc. as core materials in a micro-environment of a composite compound (wall material), and generally fall into two main categories: *reservoir* and *matrix* type. The former is characterized by a shell-like structure with the active compound residing in the interior of the capsule. In the matrix type, the bioactive compound

is uniformly distributed over the formed biopolymer carrier system, i.e. the active compound can be found also on the encapsulate surface. Regardless of the encapsulation technique, the carrier material should provide a thermodynamical and physico-chemical barrier against external environmental or processing conditions, such as water vapor, heat, oxygen, light, enzymes or pH (Augustin and Sanguansri, 2008). Furthermore, the carrier material should be inert, consisting of natural food components (or such generally recognized as safe (GRAS)), inexpensive, technologically (good film forming, gelling, water binding and emulsifying capacity, low viscosity) and organoleptically (neutral taste and odor, colorless) versatile and also, on specific occasions, should convey controlled release (Augustin and Sanguansri, 2008; Gonnet et al., 2010; Zuidam and Shimoni, 2010). With respect to carotenoids, the design of the carrier system should consider also the solubility, crystallinity, and droplet particle size changes during incorporation in the selected structure, as these parameters are directly associated with release kinetics and bioaccessibility and therefore carotenoid bioavailability (Ribeiro et al., 2010). For lipophilic compounds, the following various types of microcarriers may be distinguished (McClements, 2010):

a) Conventional emulsions: Consisting of a lipophilic emulsified particle and the emulsifying agent;
b) Nanoemulsions: as conventional emulsions, but of mean droplet size < ca. 250 nm;
c) Liposomes: a lipid bilayer encapsulating an aqueous solution inside this hydrophobic shield;
d) Hydrogel particles: network of hydrophilic biopolymers trapping solvent molecules;
e) Solid-lipid particles: solid (e.g. crystalline lipid) dispersed in oil.

Typical ingredients for encapsulation include amorphous sugars (sucrose, lactose, trehalose), polyols (sorbitol, mannitol) and non-crystalline polymeric materials such as chemically modified starch, maltodextrins, polysaccharides (arabic, mesquite or gellan gum, alginates,

pectins) and proteins (gelatin, whey proteins, sodium caseinate, soy protein). Physical state transitions (e.g. glassy to rubbery) in biopolymer matrices inducing their structural collapse can also adversely impact the stability of the encapsulated material. As a consequence, the release of lipophilic bioactive core material and the increased permeability and diffusivity to gasses (water vapor, oxygen) of the encapsulating matrix may trigger degradation, e.g. lipid oxidation, discoloration, off-flavor development etc. It has been reported that carotenoids exert high stability at low (a_W : 0.11-0.3, glassy state) to intermediate water activities (glassy state controlled by Williams-Landel-Ferry (WLF) kinetics), but low stability upon full matrix plasticization (Desobry et al., 1997; Selim et al., 2000; Sutter et al., 2007).

Selecting the appropriate encapsulation strategy of bioactive compounds is challenging, as core and wall material properties, capsule-matrix characteristics (e.g. ease of dispersion, particle size, morphology, structural aspects, solubility), the stability against ageing (e.g. matrix collapse), and release mechanisms/kinetics during gastrointestinal (GI) digestion should be considered. Finally, the encapsulation technique should be technically and economically feasible, up-scalable from the laboratory to the industrial level and sustainable.

MICROENCAPSULATION TECHNOLOGIES

1. Spray drying

General aspects

Spray drying is a unit operation where a solution, emulsion or suspension comprising the capsule's wall and core material is atomized by passing through a nozzle and mixed with a stream of hot air, usually around 140-180°C, in a drying chamber (Gharsallaoui et al., 2007). Spray drying is a cost-effective and high throughput/handling capacity process resulting in controlled particle size formation, good technological powder characteristics and short residence time in the drying chamber (1-30 s) (Gharsallaoui et al., 2007; Gouin, 2004).

For lipophilic compounds such as for carotenoids, o/w emulsion formation is required prior to atomization for high encapsulation efficiency and narrow particle size distribution (Ribeiro et al., 2010). Ingredients with good emulsifying and film forming capacity, high solubility and low viscosity are predominantly used (Gharsallaoui et al., 2007). Spray drying conditions, e.g. air and carrier solution flow rates, inlet/outlet temperature, cyclone separator pressure, and nozzle geometry can affect encapsulation efficiency, the particle size and morphology as well as carotenoid degradation due to heat induced (cis-) isomerization, oxidation (Goula and Adamopoulos, 2012; Nunes and Mercadante, 2007; Ribeiro et al., 2010; Shu et al., 2006), epoxide (Perez-Galvez et al., 2005) and apo-carotenal formation (Boon et al., 2010).

As a general rule, increasing the core to wall material ratio, lower feeding temperature and inlet temperature of spray drying is associated with enhanced carotenoid encapsulation efficiency (Goula and Adamopoulos, 2012; Mestry et al., 2011; Shu et al., 2006; Wang et al., 2012b). In most of the cases, the retention of carotenoids in the spray dried matrices can be as high as 90-94% (Table 1), with the remainder ending up uncoated, rather than oxidized. In addition, carotenoid encapsulation efficiency can be further improved by reducing the mean lipid droplet size due to faster water evaporation rates and the shorter film formation time (Shen and Quek, 2014; Shu et al., 2006).

Effectiveness of encapsulation depending on core:wall ratio and materials employed

Sufficient encapsulation requires a certain amount of wall material. Adverse effects on both encapsulation efficiency and storage stability were detected when increasing the core:wall material ratio from 1:19 to 3:17 for a broad range of wall materials including gelatin, gum arabic, gellan gum, mesquite gum, and soluble soybean polysaccharide (Hojjati et al., 2011; Rodríguez-Huezo et al., 2004; Shu et al., 2006).

Starch derivatives such as maltodextrins and modified starches have also been successfully employed to develop carotenoid loaded microcapsules (Loksuwan, 2007). The presence of

surface active functional groups (e.g. 1-octenyl succinate), the amount of free soluble amylose, the thickening capacity of starch, are known to impact carotenoid encapsulation efficiency. Acid modification of native tapioca starch enhanced β-carotene retention and chemical stability upon storage and reduced its surface deposited amount. This was attributed to the ability of amylose to form a continuous network via hydrogen bonds, immobilizing carotene and increasing film forming capacity, creating a protective physical barrier against heat and oxygen. Similarly, lycopene encapsulation by hydrophobically modified waxy maize starch (Capsul®) allowed for efficient encapsulation and increased storage stability at 10 and 20°C, compared to its free form (Rocha et al., 2012).

Protein ingredients such as whey protein concentrate, skim milk powder and sodium caseinate have also been used to produce carotenoid loaded dried microspheres (Pu et al., 2011; Shen and Quek, 2014). The formation of spray dried microcapsules via casein micelle dissociation – re-assembly mechanisms was an efficient strategy to encapsulate and stabilize bixin, a natural apocarotenoid and a food colorant (Zhang and Zhong, 2013). The microcapsules exerted very good dispersibility over a broad pH range (2-10), while their instability close to the casein isoelectric point was minimized by adding soluble soy polysaccharides (Table 1), adsorbing onto casein particles under acidic conditions. Upon rehydration, the spray dried microcapsules underwent structural reformation, resulting in bixin core/casein shell particles of very small mean size (90 nm) and of high stability against thermal/light induced carotenoid degradation.

In addition to the wall materials, the purity of the carotenoid extract has been reported to impact encapsulation efficiency, with a minimum purity being required to achieve adequately high retention in the spray dried matrix (Shu et al., 2006). Investigating lycopene oleoresin it was found that a purity of 52% was required to maximize encapsulation efficiency to approx. 80% in gelatin/sucrose based microcapsules, while further increases of oleoresin purity

conferred only minor improvement of the encapsulation. The reasons remain speculative, but it was suggested that additional compounds present at lower purity, e.g. fatty acids, glycerides, and unsaponifiable compounds hindered a proper wall formation.

Storage stability of encapsulated carotenoids and influence of water activity

Storage stability of carotenoids encapsulated in spray dried food matrices is affected by many parameters, such as the thermal history, the composition, physical state and structural aspects of the microparticles, the storage conditions such as relative humidity, temperature, presence of light (Zhang and Zhong, 2013), vacuum, oxygen, inert gases etc., and the packaging material properties, e.g. permeability to water vapor and oxygen, and light absorbance (Table 1). Matrix compositional elements govern carotenoid degradation either via their free radical scavenging activity or their wall barrier properties.

Using surface active ingredients such as milk proteins (whey protein concentrate, sodium caseinate, β-lactoglobulin), chemically modified starches, gelatine, mesquite and arabic gum have shown to enhance carotenoid storage stability (Barbosa et al., 2005; Liang et al., 2013b; Loksuwan, 2007; Rocha et al., 2012; Shu et al., 2006; Wang et al., 2013) by up to 5 times. Their encapsulating potential relies primarily on their ability to interact with lipophilic compounds via hydrogen bonds or van der Waals forces, diminishing the amount of carotenoids deposited on the microcapsule surface (readily degradable) and interacting in the air/liquid surface upon dehydration, creating protective films. Using surface active compounds as primary (sole) or secondary (with other) encapsulating agents can drastically (0.5 to 5-fold) enhance carotenoid stability over time compared to non-encapsulated ones. In addition, carotenoid photostability can be influenced by the wall materials' ability to alter the powder's reconstitution properties. For instance, it was demonstrated that bixin degradation in solutions exposed to light, prepared by dispersing carriers containing Tween 80 or gum

Arabic, was reduced by 3-4 times (compared to control solutions with maltodextrins) due to their lower water solubility and higher turbidity (Barbosa et al., 2005).

Maintaining low temperature and low water activity, protecting the native carrier system from structural collapse or physical ageing are pre-requisites to ensure maximum stability of the core material. A direct relation between water activity of spray dried matrices and carotenoid degradation upon storage has been shown. A linear correlation between β-carotene degradation and glass transition temperature was observed for a critical range of a_w (i.e. 0.11-0.75), while further increasing residual water abruptly reduced β-carotene degradation, due to complete structural collapse of the carrier, lowering diffusion rates of oxygen (Liang et al., 2013b; Prado et al., 2005; Serris and Biliaderis, 2001). Transitions from the glassy to the rubbery state and matrix collapse increase solute molecular mobility and translational energy of macromolecules and result in a shift from monolayer to multilayer water sorption states. Liang et al. (2013b) demonstrated that degradation kinetics of β-carotene in the amorphous state are driven by water vapor, oxygen diffusion and mobility constraints related to the systems' microstructure and porosity. However, structural collapse may raise technological concerns such impaired flowability and dispersibility, caking, microbial spoilage etc. and thus production of high moisture carriers are rather undesired.

Spray drying: Monodisperse and combined with other microencapsulation technologies

Single droplet (monodisperse) spray drying has been also implemented for microencapsulating carotenoids promoting better control of microparticle size distribution, allowing powder production under the same thermal conditions, and offering consistency of biological activity (Dai et al., 2013; Schutyser et al., 2012). Microencapsulation of astaxanthin with lactose and sodium caseinate by monodisperse droplet spray drying allowed producing narrowly sized microparticles that exhibited good technological features with respect to flowability, agglomeration, residual moisture, and oil surface coverage. Despite the

high temperature (105°C) in the drying chamber, the method did not significantly influence the radical scavenging activity of astaxanthin. The authors further confirmed the controlled release of astaxanthin in a buffer solution at neutral pH and at room temperature. However, the method is much less cost effective compared to conventional spray drying, due to lack of atomizing techniques able to handle large production capacities (Dai et al., 2013).

Spray drying can easily be combined with other microencapsulation techniques, such as inclusion complexation. Supercritical fluid and self-assembled protein carriers such as casein have been also successfully implemented (Jarunglumlert and Nakagawa, 2013). In their study, protein aggregation under acidic conditions (pH = 5.5) improved β-carotene loading to the matrix, due to the formation of cluster aggregates. Furthermore, aggregation at slightly higher pH (6.0) reduced β-carotene deposition on microparticle surfaces, mainly due to the structural rearrangement of the protein cluster aggregates, reducing the risk of heat induced degradation of β-carotene throughout the drying as well as its release rate into water.

2. Freeze drying

General aspects

Freeze drying is widely applied for heat labile bioactive compounds and living cells. The method is based on freezing aqueous samples (solution, suspension or o/w emulsion) at very low temperature (–80 to –40 °C) and their successive drying by water sublimation at low pressure (< ca. 5 mbar). Although the method has found many applications particularly in the case of anhydrobiotics and sensitive encapsulates including liposomes (Meng et al., 2008), several disadvantages such as high operational costs (energy and time intensive), restricted barrier properties (high porosity) of the capsules (Krokida and Maroulis, 1997) and often the need for size reduction (e.g. grinding, pulverization) to facilitate rapid drying exist.

For carotenoids, freeze drying provides a considerable protection against oxidation and isomerization occurring in other dehydration processes involving hot air streams (spray

drying, fluidized bed drying, air drying etc.). Contrary to convective heat processing, freeze drying, due to the low temperature and pressure, has rather a low impact on the encapsulation efficiency and carotenoid degradation, not triggering water phase reactions or oxidation. Desobry and others (1997) compared spray, freeze and drum drying, finding that freeze drying had only a minor impact on β-carotene encapsulation efficiency and microparticle surface coverage compared to spray drying (8 vs 11% and 35 vs 38%, respectively), while drum drying resulted in higher carotenoid degradation. On the other hand, the comparably large mean size (up to 100 μm and high porosity) together with the high surface β-carotene load of the freeze-dried particles negatively affected β-carotene stability throughout storage under controlled temperature and relative humidity compared to e.g. drum-drying.

Interestingly, degradation of β-carotene encapsulated in amorphous matrices such as maltodextrins exhibited a transition from first to second order kinetics, resulting in decreased β-carotene oxidation, ca. 20% (Desobry et al., 1997). Similarly, freeze- drying for developing carotenoid (lutein, zeaxanthin, and canthaxanthin) rich egg yolk powders improved the retention rates of xanthophylls compared to spray-dried samples by ca. 25%, though no difference in encapsulation efficiency was detected (Wenzel et al., 2010).

Structural collapse occurring during freeze drying might also lead to enhanced carotenoid retention during storage (Harnkarnsujarit et al., 2012a). Investigating the storage stability of β-carotene entrapped in freeze dried maltodextrin systems revealed that the structural collapse upon freeze drying at temperatures below Tg′ and Tm′ was associated with improved β-carotene retention. This was mainly attributed to the reduced oxygen and water vapor diffusivity of the matrices, due to the formation of a low porosity crust. On the other hand, operating freeze dryers at high temperature leads to more porous matrices, with the mean pore diameter and thickness govern carotenoid stability, with smaller and thinner pores

in conjunction with lower thickness known to cause highest degradation of β-carotene due to increased oxygen exposure of the larger surface (Harnkarnsujarit et al., 2012a).

Wall material for freeze drying encapsulation and additives

Amorphous sugars and polyols, gelatine, modified starch, maltodextrins, gum arabic, pullulan, polyvinylpyrrolidone, sodium caseinate and β-cyclodextrins (CDs) have been used for carotenoid encapsulating employing freeze drying (Table 1). For sugars and polyols, physical state transitions e.g. from the amorphous to the fully crystalline, occurring due to freeze-concentration may lead to poor encapsulation efficiency and storage stability. This may be avoided by adding crystallization inhibitors such as amino acids, phosphate anions or divalent cations, which enhance the ability of the sugar matrix to retain higher amounts of carotenoids. Sutter et al. (2007) demonstrated that divalent cations (Zn^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+}) and glycine, at concentrations of 20 mM, can improve β-carotene encapsulation efficiency of phosphate buffered mannitol systems up to 3 fold, via cation-hydrogen bonding via hydroxyl groups, forming a glycine physical barrier on the liquid-solid interfaces.

Chemically modified starch and maltodextrins for carotenoid encapsulation were investigated in a series of studies (Grattard et al., 2002; Ramoneda et al., 2011; Sousdaleff et al., 2012; Spada et al., 2012a; Spada et al., 2012b). Their good filming and emulsifying properties promote the formation of a physical barrier against oxidation. For example, in a study by Sousdaleff et al. (2012) curcumin (a lipophilic polyphenol) encapsulated by freeze-drying, using maltodextrin DE20 based matrices improved its stability against degradation in the absence or presence of light and oxygen (air), resulting in a retention of 71% over 30 d.

Similarly, freeze-dried matrices made of native pinhão starch exerted a poor retention of β-carotene during storage, due poor interfacial properties (Spada et al., 2012b). Chemical modification of native pinhão starch enhanced β-carotene binding and retention, attributed to the increased flexibility and lower oxygen permeability of the dextrin-based matrices, as well

as to the presence of free soluble amylose. The latter can form gel-like structures at liquid-air interfaces, creating a physical barrier under harsh conditions. Moreover, it can effectively entrap β-carotene via hydrogen bond formation (Spada and others 2012b). The hydrolytic breakdown of starch, has been reported to improve the encapsulation efficacy and retention of carotenoids (Spada et al., 2012b; Wagner, 1995) as well as blending maltodextrins with other biopolymers such as gelatin or gum arabic (Ramoneda et al., 2011).

Spada et al. (2012a) showed that gelatin as a co-encapsulating agent does not enhance the encapsulation efficiency of native and hydrolyzed starch based matrices, though a ca. 5 fold reduction of surface deposited β-carotene was achieved. Furthermore, gelatin reduced the matrices' solubility in cold water, indicating reduced diffusivity and permeability of the freeze-dried matrices, possibly due to formation of an external coating layer.

Using disaccharide – macromolecule blends to entrap carotenoids in freeze-dried matrices can alternatively be employed to control phase transitions, reducing glass transition temperature, enhancing carotenoid storage stability (Elizalde et al., 2002; Harnkarnsujarit et al., 2012a; Tang and Chen, 2000). Studies have shown that biopolymers such as starch and starch hydrolyzates, whey proteins or gelatin significantly hinder recrystallization even under high RH (up to 84%). Alternatively, adding electrolytes has been suggested to control physical state transitions in dehydrated food matrices, due to their ability to influence the water phase and therefore the physicochemical interplay of the present biomolecules (Buera et al., 2005). The retention of labile compounds (enzymes, bioactives, living cells) can also be affected by the type of cations used to control recrystallization of amorphous sugar based dry matrices. For example, Mg^{2+} and K^{+} in trehalose-salt based matrices lowered trehalose recrystallization and improved the ability of the matrix to retain β-galactosidase. However, adding Mg^{2+} to enhance β-carotene retention in freeze-dried gelatin–trehalose matrices via attempting to delay sugar crystallization did not confer any beneficial effects in terms of

storage stability (Elizalde et al. (2002). This implies that benefits from electrolytes also depend on the physicochemical profile of the wall material and its interaction with the lipophilic components (e.g. changes in gelatin conformation at the lipid droplet interface induced by $MgCl_2$) may also affect crystallization phenomena (Elizalde et al. 2002).

Modifying the sugar composition of maltodextrin-based freeze dried matrices can dramatically change β-carotene stability during storage, due to their impact on α-relaxation time (solute molecular mobility) and water sorption and phase transition phenomena. Harnkarnsujarit et al. (2012) demonstrated that β-carotene degraded according to the order Fru/Glu/Suc<Glu<Fru<Suc, whilst all systems performed better compared to pure maltodextrin one. This was explained by the sugars' ability to exert higher fluidity due to the increased $T-T_g$ (Harnkarnsujarit et al., 2012a; Harnkarnsujarit et al., 2012b), resulting in a partial flow and protective coating, reducing porosity and oxygen diffusion.

3. Coacervation

Coacervation refers to salting out or phase separation of lyophilic colloids into liquid microdroplets rather than aggregates (Mellema, 2003). The phase with a higher concentration of colloid components is the coacervate, while the biopolymer depleted phase is the equilibrium solution (de Kruif et al., 2004). Simple and complex coacervation mainly refers to single and at least binary biopolymer systems, respectively. Complex coacervation occurs due to the electrostatic interaction of cationic (gelatin, chitosan, plant proteins) and anionic (pectin, alginate, arabic gum, carboxymethylcellulose) biopolymers (de Kruif et al., 2004; Ducel et al., 2004). At pH conditions at which the polyelectrolytes are oppositely charged the biopolymer solution separates into a coacervated phase in form of microdroplets and a dilute phase. The resulting system is characterized by highly viscous random-coil macromolecules that allow water retention between the interspaces (Strauss and Gibson, 2004). Gelation of the coacervate microdroplets can be achieved in different ways, such as by pH or temperature

change, as well as by adding inorganic electrolytes (Mellema, 2003), producing gelled microparticles (de Kruif et al., 2004). Hardening the surrounding hydrogel-based shell of the formed microparticles is usually carried out by chemical or enzymatic cross-linking means to enhance barrier properties and to achieve sustained release (Gouin, 2004).

Compared to other microencapsulation methods such as emulsion templating, extrusion and inclusion complexation, coacervation is more competitive in terms of bioactive throughput (up to 99%), and the microcapsules' release properties under mechanical stress, temperature, and GI conditions, e.g. pH, bile salts, enzymatic activity (de Kruif et al., 2004; Gouin, 2004). However, coacervation is rather expensive and complex, using cross-linking agents that may raise food safety issues, e.g. glutaraldehyde. Operational costs of coacervation can be reduced by implementing inexpensive and straightforward techniques such as spray drying for dehydrating the obtained microcapsules. On the other hand, conventional non-food grade cross-linking agents can be sufficiently substituted by plant phenolics, enzyme cross-linkers such as transglutaminase, or heat-induced molecular complexation (Jones et al., 2009; Lv et al., 2014; Strauss and Gibson, 2004). Strauss and Gibson (2004) demonstrated that polyphenols (from coffee or grape juice) formed cross-links in proteins (gelatin) under oxidizing conditions. The cross-linked gelatin coacervates exerted high mechanical strength and reduced swelling due to the presence of a dense polymer network. Also, the cross-linked microparticles were more lipophilic and heat stable, suggesting an improved ability to uphold lipophilic bioactive compounds without coalescencing or disintegration.

To date, most of coacervation food applications refer to lipophilic compounds such as flavors (Leclercq et al., 2009; Yang et al., 2014b), essential oils (Gan et al., 2008; Lv et al., 2014; Prata et al., 2008; Sutaphanit and Chitprasert, 2014), oleoresins (Liu et al., 2007a; Zuanon et al., 2013) and carotenoids (Qv et al., 2011; Rocha-Selmi et al., 2013b; SILVA et al., 2012;

Sun et al., 1995). Novel applications for encapsulating sweeteners (Rocha-Selmi et al., 2013c; Rocha-Selmi et al., 2013a) and living cells (e.g. probiotics) have also been realized. Microencapsulating carotenoids by coacervation is based on their emulsification in an aqueous solution of the two biopolymer phases. Adjustment of pH of the colloid system leads to associative phase separation which triggers the deposition of the coacervate phase around the emulsified lipophilic core material. Carotenoid encapsulation efficiency and stability is influenced by several parameters related to the coacervation process, e.g. pH (due to changes of ζ-potential) (SILVA et al., 2012), temperature and type of biopolymers and cross-linking agents, the drying process (Qv et al., 2011; SILVA et al., 2012) and storage conditions (Qv et al., 2011; Rocha-Selmi et al., 2013b; SILVA et al., 2012; Xiao et al., 2014).

Gelatin/gum arabic comprises the most common biopolymer blend for coarcervation, mainly due to the good interfacial properties of arabic gum (emulsifying the lipid core material) and gelatin due to its acceptable melting behavior, flexibility and mechanical strength along with its high resistance against the proteolytic activity of gastric juice (de Kruif et al., 2004; Mellema, 2003). Encapsulating lutein in freeze dried gelatin/gum arabic coacervates improved its stability under light, high temperature and high RH (Qv and others 2011). It was demonstrated that vitrification significantly affected the structural collapse of the microcapsules and therefore their ability to retain lutein upon storage.

The structural collapse and formation of structural imperfections during the drying has also been reported to influence carotenoids encapsulated in gelatine based coarcervates. Spray drying appeared more efficient to retain the spherical/structural integrity of the coarcevates while freeze drying resulted in surface imperfections such as cracks, creases or fissures (Qv et al., 2011; Rocha-Selmi et al., 2013b; SILVA et al., 2012). On the other hand, freezing can generate carotenoid loaded coarcervates via electrostatic interactions of oppositely charged biopolymers in the cryo-concentrated aqueous phase (Nakagawa and Nagao, 2012).

Nakagawa & Nagao (2012) reported that freezing β-carotene containing emulsions (stabilized by a blend of gelatin and gum acacia), resulted in core-shell type nanoparticulates enclosing β-carotene without affecting the structure of the surrounding membranes, whereas the freezing rate impacted both encapsulation yield and release. On the other hand, freezing of emulsions pre-adjusted to a pH where coarcervation is induced may led to partial destruction of the polymeric membranes of the microparticles by penetrating ice crystals.

Plant proteins have also been used successfully to develop complex coarcervates for encapsulating carotenoids (Xiao et al., 2014). Using chitosan and soy protein isolate to form coacervates containing capsanthin, it was shown that the emulsification conditions (homogenization speed, temperature), the core:wall ratio and wall material concentrations influence microencapsulation yield and efficiency, as well as the microparticulate morphological characteristics. Chitosan led to a noticeable improvement of the polymeric membrane barrier, reducing water vapor and oxygen diffusion while reducing light penetration. However, the hygroscopicity of proteins may be a restrictive factor for stabilizing carotenoids, particularly at high RH (Qv et al., 2011; Xiao et al., 2014).

4. Liposomes

Liposomes consist of a molecular lipid bilayer that separates the inner aqueous phase from the external continuous water phase. Liposomes are formed via hydrophilic-hydrophobic interactions between an amphipilic agent, e.g. phospholipids, and water molecules. They play an important role for delivering hydrophobic drugs (Gonnet et al., 2010; Gouin, 2004). For microencapsulation, the bioactive compound is entrapped either in the inner aqueous phase (low loading capacity) or within the membrane (higher loading capacity), the size of carriers varying from 30 nm to a few μm (Zuidam and Shimoni, 2010). Due to the limited chemical and physical stability of liposomes (they easily undergo aggregation, coalescence, phospholipid hydrolysis and oxidation) and low encapsulation efficiency, the formation of

large unilamellar vesicles is preferred for food ingredients (Gouin, 2004), allowing for higher encapsulation efficiency, smaller capsule size, and higher cost effectiveness.

In general, liposomes are natural, biodegradable and non-toxic, exert good stability against high a_w, allow sustained release of bioactive compounds under specific temperature, i.e. above the melting point of phospholipids (ca.180-200°C) (Gouin, 2004). Up-scaling liposome production in their dry state (proliposomes) is particularly relevant for the food industry: combining liposome formation with drying methods (spray drying, freeze drying, supercritical fluid precipitation) can be a cost-effective and sustainable alternative for encapsulation (Alves and Santana, 2004; Moraes et al., 2013; Xia et al., 2012).

Moraes et al. (2013) reported that proliposome technology can be used to develop vehicles for entrapping β-carotene, with very good technological properties (density, solubility, and hygroscopicity) and high encapsulation capacity (up to 100%). Degradation of β-carotene in proliposomes stored under vacuum remained as low as 8% during 60 days of storage, while degradation in powders stored under air was 30%. Thermal degradation during spray drying process, the formation of pre-oxidation products (triggering carotenoid auto-oxidation), the oxygen permeability and the physical state (e.g. glassy or rubbery) of the proliposome matrix have been reported to influence carotenoid storage stability (Moraes et al., 2013). Also reconstituting proliposomes in water, e.g. containing xanthan gum (a liposome stabilizer), is associated with acceptable colloidal stability (in terms of average hydrodynamic volume, polydispersity index and zeta potential) and carotenoid losses of 25% were reported after 100 days of storing liposome aqueous suspension at room temperature (Moraes et al., 2013).

5. Inclusion complexation

CDs (α-, β- or γ-), are cyclic oligosaccharides which can serve as carriers for incorporated small lipophilic bioactive compounds (Blanch et al., 2007; Kim et al., 2010; Pinho et al., 2014). The encapsulation of carotenoids in CDs is taking place on a supramolecular basis, i.e.

the enthalpy rich water molecules within the CDs are substituted by hydrophobic molecules, without cleavage or formation of covalent bonds between the CD and the inclusion compound (IC) (Pinho et al., 2014). The inclusion complexed carotenoids remain in the interior of the CDs central cavity via hydrophobic forces, van der Waals interactions or hydrogen bonds, rendering the inclusion complex thermodynamically stable (Blanch et al., 2007; Mourtzinos et al., 2008; Pinho et al., 2014). CDs are inexpensive and non-toxic, exert very low hygroscopicity and high thermal stability (up to 100°C), they permit taste modification and mask undesired off-flavors, are not absorbed in the upper GI tract and they are completely metabolized by the colon microflora (Szente and Szejtli, 2004).

Encapsulating carotenoids by CDs can enhance their water solubility, reduce oxidation, light- and heat-induced degradation, or control their release, improving bioavailability (Polyakov et al., 2004; Szente and Szejtli, 2004). The inclusion of α- and β-carotene, lycopene and lutein in a carotenoid-rich canola oil in α- and β-CD complexes (molar ratio of CDs : carotenoids 1:0.5) enhanced carotene stability in the presence of air (Basu and Vecchio, 2001), and astaxanthin encapsulated β-CD complexes showed 7-9 fold improved stability against heat (65 to 100°C), UV-light, and oxygen (Kim et al., 2010).

Factors such as stability and release may be fine-tuned by altering CD structure, e.g. ring size (α-, β- and γ-CDs with 6, 7, and 8 membered sugar-rings, respectively) or hydroxypropylation/methylation (Basu and Vecchio, 2001; Blanch et al., 2007; Huang et al., 2002; Kim et al., 2010; Mele et al., 1998; Pfitzner et al., 2000; Yuan et al., 2013). According to Blanch et al. (2007), the van der Waals surface area of lycopene was associated with the encapsulation efficiency of CDs, with β-CD favoring complexation with *all-trans*-lycopene. Similarly, the inclusion-complexation of CDs with astaxanthin was facilitated for β-CD, decreasing complex mean size compared to native CDs, while no altered structure was found for α- and γ-CD (Blanch et al., 2007; Kim et al., 2010). In addition to enhancing solubility,

Pfitzner et al., (2000) showed that methyl-β-CD complexes possess a high stability against *cis-trans* isomerization, with all-*trans* isomers being of higher thermodynamic stability than the *cis*-ones. Though additional chemical modification of CDs was reported to favor the formation of ICs, it led to dark colored powder products, indicating poor encapsulating.

A method for including bixin rich plant extracts (1:1 ratio) with curcumin in β-CD (1:2 ratio) was investigated (Marcolino et al., 2011). Co-precipitation for complexing curcumin and bixin resulted in the most stable ICs (confirmed by TGA/DSC). Curcumin and bixin complexes exerted high decomposition temperatures and low water evaporation enthalpies, suggesting that the interaction between CDs and carotenoid-rich extracts is energetically favorable, facilitating displacing water molecules from the central cavity of β-CD. Kneading also exhibited a similar behavior to that of co-precipitating β-CD, while simple mixing did not improve stability of the microencapsulated carotenoids.

6. Extrusion

Fabricating microspheres by single or co-extrusion technology comprises entrapping bioactive substance by a biopolymer cross-linked by ionotropic gelation. Though microsphere formation via covalent cross-linking of reactive biopolymers (e.g. alginates, pectins, chitosan or κ-carrageenan) is similar for single or co-extrusion, the techniques are yielding microcapsules of different structure, e.g. matrix in the case of single extrusion and core-shell in the case of co-extrusion. Modifications related to the technical set-up of extrusion devices have also been reported, mainly focusing on the nozzle conformation (i.e. single vs. dual feed) and atomization principle (i.e. stationary, rotating or vibrating) (Zuidam and Shimoni 2010). Both techniques are suitable to produce spherical microcapsules (>160 μm to few mm), depending on nozzle geometry, atomization process (jet cutter, vibration nozzle, atomizing disc, electrostatic potential), biopolymer solution viscosity, feed rate, ionic strength of gelling bath etc. (Zuidam and Shimoni 2010). To date, extrusion has proven to be

one of the most feasible techniques to encapsulate probiotics (Burgain et al., 2011), though other bioactive compounds have been encapsulated such as hydrophilic (Lupo et al., 2014) and lipophilic polyphenols (Waterhouse et al., 2014), and vitamins (Wichchukit et al., 2013). Finally, extrusion has been successfully implemented for covalently gelled microbeads (Donhowe et al., 2014; Kittikaiwan et al., 2007; Laos et al., 2007; Leach et al., 1998).

Sodium alginate is the most frequently used biopolymer for carotenoid loaded microbead fabrication, while other biopolymers exerting ionotropic gelation such as furcellaran (a co-polymer of β- and κ-carregeenan) have also been tested. Among the first applications, Leach and others (1998) tested sodium alginate based microbeads for β-carotene encapsulatuion. Increasing sodium alginate concentration (0.33, 0.47 and 0.73% w/v) decreased β-carotene encapsulation efficiency during the complexation of Ca^{2+} with sodium alginate (from 45.5 to 19.1 g/kg from wet beads) and improved its chemical stability during fluidized bed drying (losses declined from 21 to 13%). However, β-carotene chemical stability under detrimental (light/oxygen) and non-detrimental (dark/nitrogen) conditions ameliorated for microbeads comprising 0.33% w/v sodium alginate, indicating lower gas diffusion and light transmission. Moreover, though fluidized bed drying resulted in high β-carotene isomerization (*9-cis*-isomers increased from 36.5 to 50%), the obtained dry microbeads were quite stable against isomerization during storage, depending on alginate concentration (Leach et al., 1998).

Recently, spray drying vs. extrusion for β-carotene encapsulation efficiency and release kinetics under simulated digestion was compared (Donhowe et al., 2014). Although spray drying (with maltodextrin as a carrier) resulted in smaller particles than extrusion (chitosan coated alginate microbeads), i.e. 11 vs. 942 μm, extrusion decreased surface deposition (0.0004 vs. 39.5%) and increased encapsulation efficiency (54.7 vs. 37.7%), possibly due to poorer β-carotene emulsification of the spray dried product. High β-carotene encapsulation efficiency (ca. 97%) was also reported for microbeads comprised of covalently cross-linked

furcellaran with Ca^{2+} or K^{+} (Laos et al., 2007). Notably, parameters such as the ionic strength of the cross-linker, the cation type and the carotenoid:biopolymer ratio should be considered upon microbead formation, as they may impact the mechanical strength of the end-product. Finally, coating microbeads using film forming biopolymers such as chitosan has been reported to slow down carotenoid degradation during storage (Kittikaiwan et al., 2007). A five-fold coating of astaxanthin loaded beads using a 1.5% w/v chitosan solution in glacial acetic acid followed by an ionic setting using a $Na_5P_3O_{10}$ solution led to the formation of a 100 μm external film layer, improving the chemical stability of astaxanthin under abusive storage conditions. The coated pellets were characterized by higher astaxanthin retention (after exposure to light and oxygen) compared to the uncoated systems. Both systems showed notable antioxidant activity (ABTS method), though chitosan coated beads had lower IC_{50} after 24 weeks of storage (-18 and 30˚C), indicating superior antioxidant capacity.

NANOENCAPSULATION TECHNOLOGIES

1. Nano-liquid lipid carriers (LLCs)

Incorporating carotenoids into lipid droplets stabilized by an interfacial layer of surface active compounds is an efficient encapsulation strategy. Though microemulsions have a remarkable ability to retain lipophilic compounds, nanoemulsions as delivery carriers exert advantages such as high optical clarity, enhanced physical stability (gravitational separation, droplet coalescence), processability and bioavailability (Silva et al., 2012). Nanoemulsion generally refers to colloidal systems of lipid droplets (mean size 10-100 nm) dispersed into a continuous aqueous phase, stabilized by a surrounding interfacial layer of at least one emulsifier. Nanoemulsions loaded with carotenoids can be fabricated by their dissolution into the bulk lipid phase and emulsification into the water phase by high-energy (high pressure homogenization, microfluidization, sonication, high shearing homogenization) or low-energy (phase inversion temperature (PIT), solvent displacement, spontaneous emulsification)

techniques (Ezhilarasi et al., 2013; Silva et al., 2012). The chemical stability of carotenoids during emulsification and storage depends on compositional (carotenoid type, type/ concentration of the lipid phase, type/content of surfactants, presence of oxygen scavengers etc.), colloidal (particles size, interfacial layer composition), intrinsic (pH, ionic strength, droplet surface charge) and extrinsic (light, oxygen, temperature) conditions (Table 3).

Effect of carrier lipid phase

Interfacial engineering of o/w emulsions, e.g. tailoring of the emulsion phase domains (lipid, aqueous and interfacial layers) promote chemical stability of the active substance. Focusing on the lipid phase, it was shown that the oxidation/degradation patterns of carotenoids present in the bulk (non-emulsified) or emulsified state are considerably different. Carotenoid oxidation (in the bulk phase) is governed by oxygen transport in the air/oil interface, while emulsified carotenoids are less prone to oxidation as oxygen transport follows a more complicated pathway including oxygen dissolution in the continuous aqueous phase, partitioning in the oil/water interface and diffusion to the lipid droplet surface. Thus, parameters that control mass transfer (pressure, temperature, aqueous phase microviscosity) or contribute to the formation of colloidal barriers on the lipid droplet surface (thickness and density of the interfacial layers) such as the bulk oil and surface active compounds, do influence carotenoid stability throughout storage.

The type of carrier lipid phase is inextricably associated with the chemical stability of carotenoids, for two main reasons. First, the physicochemical properties of the oil interplays with the colloidal aspects of the formed droplets and thus with the partitioning of pro-oxidative components between the water and the oil phase. Second, the oil phase may carry functional groups that are prone to oxidation or minor chemical compounds exerting antioxidant activity, e.g. tocopherols. Boon and others (2008) studied the impact of the fatty acid composition of oils and their antioxidants on lycopene oxidation encapsulated in 5%

(w/w) o/w corn oil based emulsions. Lycopene was rapidly degraded in stripped (purified) corn oil emulsions, followed by lycopene dissolved in corn oil and hexadecane. Presumably, the naturally inherent antioxidants in the oil provided protection of carotenoids against degradation. However, at the end of the storage trials, losses of ca. 80% of the initial carotenoid amount were observed. On the other hand, the reduced formation of secondary oxidation products such as hexanal (observed in the emulsified corn oil systems compared to the stripped or bulk corn oil), suggested that both the emulsification and the chemical composition of the oil carrier can enhance the ability of lycopene to quench alkoxy radicals, inhibiting β-scission reaction producing hexanal.

Szterk and others (2013) affirmed that β-carotene stability emulsified in refined palm olein, linseed oil or rapeseed oil strongly depended on the susceptibility of the bulk lipid phase (oil + carotenoids) to autoxidation. Highest β-carotene stability (12 wks, 2˚C) was found with emulsified in palm olein, and lowest in linseed oil (higher PUFA content). A negative correlation between the amount retained β-carotene and formed hydro-peroxides (PV) was detected, underlining carotenoid antioxidant capacity.

Effect of surfactant type and content

Testing the effect of various polysorbates (Tweens 20, 40, 60 and 80) on the colloidal and chemical stability of nanoemulsions containing β-carotene (Yuan and others (2008), Tween 20 induced the greatest size reduction, resulting in droplets of 132 to 173 nm, with finer particles being obtained at highest surfactant amounts (12% w/w). Milk proteins (sodium caseinate and whey proteins) were successfully adopted to produce physically and chemically stable nanoemulsions containing carotenoids. Qian and others (2012a) used β-lactoglobulin (2% w/w) as the primary surfactant in orange oil based o/w nanoemulsions (10% w/w oil) containing β-carotene. Storage (2 wks at 5, 20, 37 and 55˚C) of the emulsions showed that β-carotene content decreased by 43 to 100% with increasing temperature. Particle size analysis

affirmed that higher temperature reduced their physical stability, primarily associated with the ability of the globular proteins to undergo conformational transitions, favoring hydrophobic interactions between the lipid droplets, leading to their flocculation. In the same study, β-lactoglobulin exerted improved carotenoid retention compared to Tween 20, perhaps due to the free radical scavenging ability of β-lactoglobulin at the oil-water interface (via cysteine residues, disulfide bonds and thiol groups), its ability to form complexes with carotenoids, and to improve the barrier properties of the interfacial layers surrounding the lipid droplets (Qian et al., 2012b). In a successive study, WPI (0.5% w/w) was used to stabilize 5% w/w MCT based o/w emulsions containing β-carotene (Xu et al., 2013b). Storing the emulsions (55˚C, 7 days) induced a pH and ionic strength dependent degradation of β-carotene. Some amino acids, e.g. cysteine, tryptophan, and methionine, present in WPI, may act as free radical scavenging and metal-chelating agents and WPI antioxidant ability is controlled by the reactivity of free amino groups with carotenoid peroxyl radicals.

Yi and others (2014) investigated sodium caseinate as a physical and chemical stabilizing agent in o/w (10% w/w) emulsions containing β-carotene homogenized at different pressures (10-103 MPa). Beta-carotene degradation exhibited a linear pattern over storage time (30 days, 25˚C) with highly pressurized systems being the most unstable. Caseins are known to control lipid oxidation by reducing the accessibility of metal ions or other pro-oxidants to unsaturated fatty acids and carotenoids, and by binding ferric ions, shifting the equilibrium from the hydroperoxide forming ferrous to ferric iron (Díaz et al., 2003; Yi et al., 2014). However, producing very fine lipid droplets triggers carotenoid oxidation due to increased surface area, minimizing the protective role of absorbed caseins (Yi et al., 2014).

Hydrophobically modified starch or maltodextrins and surface active polysaccharides (e.g. arabic gum, pectins) can also be used as primary emulsifiers in nanoemulsions containing lipophilic substances. Liang et al. (2013a) investigated the impact of sodium octenyl succinic

acid (OSA) modified starches on β-carotene stability. The starches' molecular properties (molecular weight (MW), radius of gyration and dispersed molecular density) interfered with β-carotene degradation only at ambient storage (i.e. 25˚C), light and oxygen. Starches of high MW and dispersed molecular density were associated with higher β-carotene retention during 30 days of storage, attributed to the starches' ability to form thicker and denser interfacial layers surrounding the lipid droplets, providing a physical barrier against oxidation. However, no significant differences in β-carotene retention were detected with OSA-modified starch stabilized nanoemulsions stored at 4˚C in the absence of light and oxygen.

Impact of pH and ionic strength

Processing LLC containing carotenoids (blending with acidic formulations, salt or sugars) may modify pH and ionic strength, directly influencing the physical stability of emulsions (inducing droplet flocculation, phase separation or creaming), and indirectly the chemical stability of carotenoids (Qian et al., 2012b; Xu et al., 2013a; Xu et al., 2013b; Yi et al., 2014). For example, acidic conditions may produce ion-pairs that dissociate, forming carotenoid carbocations undergoing cis-trans isomerization and degradation (Boon et al., 2010). Qian et al. (2012a) reported that exposing emulsified β-carotene to pH 3-7 altered its degradation kinetics at 25˚C, with acidic pH causing highest degradation, corroborating observations by Boon et al., (2009) using hexadecane (5% w/w) based emulsions containing lycopene. Losses at pH 7 and 3 during incubation (94 h, 15˚C) amounted to ca. 50% and 98%, respectively. Carotenoid instability at acidic pH was attributed to formed ion-pairs promoting carotenoid isomerization, increasing iron solubility, the binding of iron to the surface of emulsion droplets, and metal-lipid interactions and oxidation reactions (Boon et al., 2009).

Interfacial coating modification of the lipid droplets can improve the stability of emulsified carotenoids (Xu and others 2013a,b). Whey proteins on the lipid droplet surface slightly improved the stability of β-carotene containing nanoemulsions stored for 7 days at 55˚C. In

general, β-carotene degradation was higher at pH<pI for whey proteins, attributed to pH-dependent changes of colloidal aspects of the emulsions. Emulsions adjusted to a pH of 6-7 exerted higher β-carotene retention (29-34%) vs. systems with a pH of 3-4 (21-12%). Though the high stability of carotenoids at pH>pI appears to be associated with the binding of soluble iron in the water-oil interface, the increased stability at low pH was attributed to their higher cationic charge density, facilitating repulsive interactions with pro-oxidant metals (Xu and others 2013b). Similarly, forming complex interfaces on lipid droplets, e.g. beet pectin-whey protein isolate conjugates, enhanced β-carotene retention (Xu and others 2013a). However, the stabilizing effect of protein-pectin conjugates was superior at pH 7 than under acidic conditions. For a certain pH, no significant differences in the surface charge of lipid droplets were detected, suggesting that the antioxidant role of protein-polysaccharide conjugates emanates from their ability to create a dense and cohesive interfacial layer, preventing the penetration of pro-oxidants (iron ions, free-radicals) present in the aqueous phase (Xu and others 2013a). Thus, though ionic strength plays is important for reducing electrostatic repulsions between lipid droplets, favoring particle aggregation via van der Waals and hydrophobic attraction, increased salt concentration may improve the stability of carotenoids in nanoemulsions. Qian et al. (2012a) reported enhanced β-carotene retention in nanoemulsions (stored at 55˚C for 7 days) when sodium chloride (400-500 mM) was added.

Presence of pro-oxidant scavengers

Carotenoid oxidation in nanoemulsions can be triggered by many factors such as heat, light, singlet oxygen, transition metals and free radicals. Carotenoid autoxidation results in the formation of reaction products such as epoxides, endoperoxides, apocarotenals and apocarotenones (Krinsky and Yeum, 2003), with altered or decreased biological activity. Two groups of antioxidants are used to control carotenoid autoxidation: singlet oxygen or free radical scavengers and transition metal chelators. Depending on processing conditions,

transition metals can be found either in aqueous phases or in oil-water interfaces. Emulsions containing 200 and 500 mM of desferoxamine (an iron chelator) enhanced β-carotene content after storage by 25% and 37% (7 days, 55˚C) compared to nanoemulsions without chelator. EDTA, even at low levels (1 mM), pronouncedly influenced the color stability of β-carotene containing emulsions (Qian et al., 2012b). EDTA may prevent metal redox cycling, the formation of insoluble metal complexes, the occupation of metal coordination sites, and sterically hinder the interaction between metals and lipid substrates (Decker 1998). A similar pro-oxidant scavenging activity for EDTA was reported for other carotenoids, e.g. for lycopene (Boon et al., 2009). Contrarily, Bou et al. (2011) demonstrated that the effectiveness of metal chelators not only depended on their amount, but also on the surface charge of lipid droplets and pH, as well as the acid dissociation constant (pKa) of the metal chelating agents. Using Tween 20, Bou et al. (2011) showed that lycopene retention in 5% w/w emulsions was superior in systems without metal chelators compared to those containing EDTA, citric acid or tripolyphosphate. The poor effectiveness of EDTA was attributed to its ability to increase the solubility of ferric iron, favoring its interaction with lycopene, inducing autoxidation.

Qian et al. (2012b) investigated the impact of water (ascorbic acid) and oil soluble (α-tocopherol acetate, coenzyme Q10) antioxidants on the degradation of β-carotene encapsulated in 10% corn oil (w/w) – β-lactoglobulin stabilized nanoemulsions. Though ascorbic acid and coenzyme Q10 controlled β-carotene stability throughout storage (55˚C, 15 days), different prooxidant-scavenging mechanisms were observed. While polar compounds can act as prooxidant-scavengers via their ability to absorb to oil-water interfaces (where they come into contact with peroxyl radicals) or by chelating transition metals in the water phase, lipophilic compounds mainly act as free radicals scavengers at the lipid droplet surface.

Research on α-tocopherol pro-oxidant scavenging activity in nanoemulsions containing carotenoids resulted in contradictory findings. According to Bou et al. (2011), α-tocopherol

exerted the most pronounced carotenoid retention compared to more polar antioxidants (gallic acid, propyl gallate). Contrarily, Qian et al., (2012b) reported that α-tocopherol conferred only minor scavenging activity compared to ascorbic acid and coenzyme Q10. Interestingly, Xu et al. (2013a) demonstrated that the antioxidant ability of α-tocopherol (200 vs. 500 mg/kg) in nanoemulsions containing β-carotene stabilized by protein-polysaccharide conjugates strongly depended on the pH, with free radical scavenging action increasing with pH, with acidic conditions (pH 4) not showing improved carotenoid retention.

2. Nanoencapsulation using supercritical fluids

A supercritical fluid (SCF) is a dense liquid with the physicochemical properties of gases. Close to its critical point the fluids' solubility, viscosity, diffusivity, and thermal conductivity change radically (Taleb 2010). Employing SFCs is an emerging method to extract and purify heat labile compounds, as well as for the structural design of particles (Jung and Perrut, 2001; Mattea et al., 2009b). SFC particle design is advantageous over conventional precipitation as it promotes good particle size and morphology control, reduces isomerization and thermal degradation of heat labile compounds, improves encapsulation and precipitation yield and minimizes harmful chemical residues (Santos and Meireles, 2010). CO_2 is predominantly used as an anti-solvent for food applications, exerting a low critical point temperature (T_c = 31.1 °C, P_c = 7.38 MPa), is non-toxic, inflammable, inexpensive, relatively inert, has adjustable solvent power/selectivity and can easily be eliminated from the food matrix (Gutiérrez et al., 2013; Santos and Meireles, 2010) by evaporation.

Supercritical fluid precipitation techniques for carotenoid nanoencapsulation can be divided into five main categories (Jung and Perrut, 2001; Mattea et al., 2009a): rapid expansion of supercritical fluids (RESS), gas anti-solvent (GAS), supercritical anti-solvent (SAS/SEDS), particles from gas-saturated solutions (PGSS), and supercritical extraction from an emulsion (SFEE). Several factors can influence the physical state, morphology, mean particle size of

encapsulates, and micro-encapsulation efficiency, including pressure and temperature of the supercritical fluid, the geometry of the precipitation vessel and the atomization nozzle, the anti-solvent flow rate, type (e.g. emulsion, suspension, solution) and flow rate of the carrier aliquot system, the carotenoid type and the carotenoid: wall material ratio (Table 2).

RESS assisted encapsulation uses the solvation power of supercritical fluids by adjusting pressure and temperature. The bioactive compound and the polymeric coating material are co-dissolved in supercritical CO_2 and then co-precipitated via rapid depressurization of the obtained organic solution through a nozzle (Cocero et al., 2009; Jung and Perrut, 2001). The poor solubility of many active substances and coating materials in supercritical CO_2 is the major drawback of RESS (Cocero et al., 2009). In addition, the morphology and particle size and thereby the bioactive payload are difficult to control, as co-precipitation is a rapid process. Though RESS is scarcely used to encapsulate food bioactives, its feasibility for micronization and encapsulation of carotenoids (astaxanthin) and organic pigments (rutin, anthocyanins and quercetin) has been demonstrated (QUAN et al., 2009; Santos and Meireles, 2013). Quan et al. (2009) showed that RESS assisted micronization of astaxanthin produced small sphere-like particles (mean particle size 0.5 μm), being almost 10 times smaller than microparticles obtained by solvent (n-hexane) assisted crystallization. Low pressure and high temperature pre-expansion facilitated the formation of tiny particles, possibly due to decreased critical nuclei size and increased concentration during atomization. Studying rutin, it was demonstrated that the bioactive substance solubility in the supercritical solvent (CO_2 with ethanol) determines the encapsulation mechanism and efficiency, particle mean size and morphology. For bioactives with low solubility in the supercritical solvent, the core material is not dissolved but suspended in the continuous phase. Upon expansion, encapsulation is completed by depositing coating material on the suspended particles, creating a polymeric layer on the particles' surface (Santos and Meireles, 2013).

The PGSS process consists of saturating a solution with the bioactive compound, using a supercritical fluid. The gas-saturated solution is expanded under atmospheric pressure through an atomization nozzle, the resulting vaporization of the dissolved gas induces cooling of the solution (Joule-Thomson effect), promoting nucleation and precipitation (Martín and Weidner, 2010). An advantage of the PGSS is that it allows particle formation from aqueous solutions, and the combination with conventional drying methods, e.g. spray drying. Due to the efficient atomization and the remarkably lower temperatures implemented in the spray tower, PGSS is useful for carriers with low melting points (close to room temperature) or heat sensitive bioactives (de Paz et al., 2012b; de Paz et al., 2012a; Martín and Weidner, 2010). However, the mean size of the obtained particles is generally larger than those obtained by other supercritical fluid based techniques, ranging from a few to several hundred microns.

Recently, encapsulating carotenoids by PGSS has been realized using soybean lecithin and poly-ε-caprolactones (de Paz et al., 2012b; de Paz et al., 2012a). Using poly-ε-caprolactones of low (4000 g mol^{-1}) and high MW (10000 g mol^{-1}) for β-carotene encapsulation was challenging as apart from the MW, a thorough control of the operating conditions in the precipitation vessel (homogenization time, pressure and temperature) and the molar ratio of β-carotene:polymeric carrier is required (de Paz et al., 2012b). Low MW polymers can drastically improve encapsulation parameters (process yield, particle mean size, agglomeration phenomena, β-carotene payload). This has mainly been attributed to the lower viscosity (1690 vs. 9300 mPa*s) and higher solubility in supercritical CO_2 (2.7 vs. 5.4% w/w). Thus, atomization is facilitated and the higher amount of solubilized supercritical gas favors smaller droplets/smaller particle formation during the expansion in the nozzle.

However, compared to SFEE, PGSS appears inferior regarding encapsulation efficiency and technological/morphological properties of the produced particles. De Paz et al. (2012a) studied the effectiveness of PGSS combined with liposome technology to produce pro-

liposomes loaded with β-carotene. The process yield was higher (20-50%) compared to that of the PGSS-poly-ε-caprolactone method (5-44%), whereas encapsulation efficiency ranged from 40-60%. Particle size and morphology were influenced by the pre-expansion pressure and temperature in the homogenization vessel, with high pressure and low temperature reducing droplet size during atomization. It was also demonstrated that increased gas to product and lecithin to β-carotene ratios can hinder atomization, due to increased viscosity of the gas saturated solution. However, the increased lecithin content in the solution improved the encapsulation efficiency, by reducing interfacial tension of the gas saturated solution, facilitating the interplay at the oil-water interfaces, forming a core surrounding shell that can be soundly maintained even during the expansion step. The reconstituted pro-liposomes in water produced liposomes of 1-5 μm mean size and of multilamellar structure, with β-carotene being entrapped in the hydrophobic region (internal space of the lipid bilayer).

SAS/SEDS are based on mixing a supercritical fluid stream of a desired temperature and pressure conditions with a solution to induce recrystallization due to supersaturation (Cocero et al., 2009; Mattea et al., 2009a). SAS/SEDS was successfully applied (Fig. 2) to carotenoid extraction/micronization and encapsulation (Table 2) (Davarnejad et al., 2008; Franceschi et al., 2009; Miguel et al., 2006; Santos and Meireles, 2013). The encapsulation efficiency of SAS/SEDS as well as the particles' size and morphological characteristics depend on several parameters, e.g. the affinity between the core/coating compounds, the core to coating material ratio, the pre-expansion pressure and temperature as well as the anti-solvent flow rate (Franceschi et al., 2008; Martín et al., 2007). Co-precipitation with β-carotene and lutein revealed that the encapsulation efficiency was affected by the type of carotenoid, CO_2 flow rate and core to coating ratio. Though in both cases prismatic/needle-like carotenoid particles were covered by amorphous spherical PEG particles, lutein encapsulation efficiency increased with high CO_2 flow rates and high polymer:carotenoid ratios.

Franceschi et al. (2008) investigated the impact of pressure, solution, and anti-solvent flow rate on the encapsulation efficiency and morphology of β-carotene – PHBV (poly-3-hydroxybutyrate-*co*-hydroxyvalerate) SEDS co-precipitates. The formation of small particles with good β-carotene coverage improved at high pressure and low solution flow rates, which was attributed low viscosity of the solution and enhanced mass transfer and diffusion rates of CO_2 to and from the droplets during atomization/depressurization. Corroborating previous studies, the reduced β-carotene:PHBV ratio also reduced encapsulation efficiency and increased surface deposited carotenoids due to the faster precipitation of the polymeric material (Franceschi et al., 2008; Priamo et al., 2010). Successive studies on β-carotene- and astaxanthin-PHBV SEDS produced nanoparticulates (Machado Jr et al., 2014; Priamo et al., 2011; Priamo et al., 2010) confirmed that the encapsulation efficiency and the percentage of carotenoids entrapped in the polymeric matrix were maximal at highest pressure (20 and 10 MPa, respectively) and carotenoid concentration in the organic solution (~1:1 and 1:2 β-carotene and astaxanthin to PHBV ratio). β-Carotene release in the organic phase depended on the used solvent type (being faster for ethyl acetate and n-hexane than for ethanol) and the loaded bioactive amount (Priamo et al., 2011).

Pre-expansion temperature plays an important role for co-precipitating active substances with biopolymers. Operating precipitation vessels at high temperature (above the glass transition temperature of the coating material) can result in obstructed particle formation, development of fibrous, film-like or highly agglomerated particles (Nalawade et al., 2006). Mattea et al. (2008) studied the effect of temperature on the morphology of β-carotene loaded PEG microparticles produced by SEDS. Fabricating microparticles was arduous above 288K, due to strong agglomeration and film forming phenomena, due to the apparition of a liquid-liquid phase instead of a supercritical phase, thus supersaturation and precipitation kinetics were considerably slowed down. Experiments at a constant temperature of 288K (threshold for

particle precipitation) and increasing the PEG : β-carotene ratio improved coverage of the precipitated carotenoid particles and transition from the initial irregular structure to hollow spheres and finally to concavity-absent microspheres (Mattea et al., 2008).

Proteins can also constitute carotenoid co-precipitants as they facilitate absorption on their interfaces, and due to their good sustained released properties. Zein constituted a primary substrate for lutein encapsulation by SEDS (Hu et al., 2012). Lutein encapsulation efficiency increased with higher pressure, temperature, and decreased lutein:zein ratio. Adopting these conditions, morphological (from large spherical particles with less agglomeration to uniform nanospheres) and particle size changes (198 to 355 nm) were also observed. The lutein-zein blend and their SEDS co-precipitate (10MPa, 45˚C, 1:18) showed a glass transition region (50-80˚C) in both systems, though a clear lutein melting peak (Tm 180-190˚C) was observed only for the zein-lutein blend, indicating that zein with lutein was embedded in the amorphous state, as well as good thermodynamical compatibility of both materials (lutein-zein interactions via intermolecular hydrogen bonds). Finally, the SEDS zein-lutein co-precipitate exhibited slower release in PBS with no detectable burst point (near zero order kinetics), contrarily to pure lutein or lutein-zein blends, revealing that the release of the SEDS zein-lutein precipitate was associated with water molecule diffusion to the matrix, increasing zein polymeric network interspaces, facilitating diffusion of lutein to the bulk aqueous phase.

SAS/SEDS techniques have also been successfully combined with other microencapsulation technologies such as liposome or inclusion-complexation (Lesoin et al., 2011; Nerome et al., 2013; Xia et al., 2012). SAS implementation to produce pro-liposomes may bring advantages compared to conventional organic solvent methods (Bangham method), as it is more efficient, environmental-friendly, is carried out under mild temperature, and reduces the amount of residual organic solvent in the final dry product (Lesoin et al., (2011). However, no

significant differences were noticed between SAS and the Bangham process in terms of encapsulation efficiency (approx. 20%), liposome storage stability and mean particle size.

Xia et al. (2012) presented a SEDS assisted method to produce lutein loaded pro-liposomes. Lutein loading of the carrier substrate was pressure – temperature dependent; operating conditions of 35°C and 8 MPa provided highest encapsulation efficiency (90%). The SEDS obtained microparticles were free flowing microspheres of 100–300 nm diameter, due to the ability of CO_2 to form a single phase, increasing nucleation rates. Nerome et al. (2013) introduced an SEDS assisted method to produce lycopene/β-CD inclusion complexes in dry form, resulting in nanoparticles with a mean size of 40-150 μm, depending on phase equilibria conditions (pressure, temperature) and organic solution and anti-solvent flow rates. High pressure, temperature and solution flow rate resulted in smaller, uniform and spherical particles due to enhanced lycopene solubility in the solvent phase (DMF), and a turbulent flow in the jet edge region, promoting supersaturation and droplet break-up.

SFEE is an alternative to emulsion templating/precipitation methods where the bioactives incorporated in o/w emulsions are recrystallized, implementing the supercritical anti-solvent method. SFEE is considered advantageous over conventional emulsion templating techniques due to reduced amounts of organic solvents needed during the precipitation process. Moreover, SFEE overcomes the challenges related to SAS, that is, the difficulty to produce small (nanoscale) particles and the tendency of the obtained powders to agglomerate (Mattea et al., 2009a). The presence of surface active compounds in the emulsion promotes nanoparticle formation without agglomeration, while their growth is restricted due to high nucleation rates (Mattea et al., 2009a).

Santos et al. (2012) produced nanoparticles containing β-carotene or lycopene by SFEE. An increased emulsion flow (from 2.5 to 4.5 mL/min) was found to be associated with improved encapsulation efficiency (61 to 89% and 34 to 71% for β-carotene and lycopene, respectively)

though isomerization also increased slightly. The lower encapsulation efficiency of lycopene was mainly attributed to its higher solubility in supercritical CO_2 and better partitioning in the antisolvent phase), but differences in the nucleation rate, particle specific surface area, and interactions between the carrier and carotenoids were also stated.

However, carotenoid isomerization during SFEE processing seems to occur mainly during solvent extraction, i.e. after particle formation (Mattea et al., 2009b), as increased emulsion flow rates lead to prolonged solvent extraction and higher isomerization (Santos et al., 2012). Using SFEE, pressure and temperature remarkably effected both encapsulation efficiency and isomerization of carotenoids (β-carotene, lycopene, astaxanthin) (Mezzomo et al., 2012; Santos et al., 2012). An optimum temperature and pressure needs to be found to maximize encapsulation efficiency, as they influence thermodynamic properties of carotenoids and coating material (Mezzomo et al., 2012). While low pressure reduces carotenoid solubility in the antisolvent phase (de la Fuente et al., 2006), higher pressure may prevent film formation of the polymeric coating material (Mezzomo et al., 2012). It should also be emphasized that the encapsulation carrier and carotenoid strongly determine the impact of pressure on the encapsulation efficiency, using SFEE. Depending on the carotenoid, pressure may influence carotenoid trans-cis-isomerization, though literature data are contradictory. While Santos et al. (2012) reported that a pressure increase from 7 to 13 MPa reduced lycopene isomerization (from 2 to 0.02%), an increase of cis-isomers was reported at higher pressure operating conditions, i.e. 20 to 40 MPa (Yi et al., 2009). Though it is unclear how pressure influences isomerization, the increased density of the antisolvent phase (increased pressure in the precipitation vessel) may be the key point (Santos et al., 2012).

SFEE appears also efficient to maintain carotenoid storage stability (de Paz et al., 2013; Mezzomo et al., 2012; Santos et al., 2012). Mezzomo and others (2012) compared the stability of astaxanthin in systems prepared by reconstituting SFEE or SAS co-precipitates,

reporting that the SFEE obtained systems exhibited the highest stability (7 days of storage), whereas the stability of SAS derived systems strictly depended on supercritical co-precipitation conditions and astaxanthin extract concentration. In addition to high encapsulation efficiency, small particle size and enhanced morphological features, the carrier type may also influence the storage stability of carotenoid nanoparticulates produced by SFEE. de Paz et al. (2013) revealed that β-carotene retention in SFEE received freeze-dried nanoparticles improved with high MW/high melting point poly-ε-caprolactones compared to lower MW/melting point analogues.

3. Protein self-assembled nanoparticles

The broad range of techno-functional and physicochemical properties of proteins renders them as natural vehicles for the encapsulation and sustained release of bioactive compounds. Ion binding, binding of hydrophobic compounds, surface active properties, ability to form molecular self- or co-assemblies, complexation with oppositely charged polysaccharides, acid or heat induced gelation, pH triggered gel swelling, adsorption at the air/water or oil/water interfaces, formation of covalent conjugates via Maillard reaction, UV light absorbance, and enhanced bioavailability by controlling the access of digestive enzymes are the most important properties of proteins for constituting effective carriers to deliver bioactive compounds and living cells (e.g. probiotic bacteria) to the human host (Livney, 2010).

For instance, milk proteins (casein micelles, β-lactoglobulin) are well known for their excellent self-assembly/co-assembly properties (Fig. 4). The formed protein nano-vehicles can easily be loaded with lipophilic bioactive compounds and provide an effective physical barrier against harsh environmental conditions (Livney, 2010; Zimet et al., 2011; Zimet and Livney, 2009). Proteins are further widely available, inexpensive, have high nutritional value and satisfactory sensory characteristics and are generally recognized as safe (GRAS).

Casein micelles are clusters of α-, β- and κ-caseins, stabilized by hydrophobic interactions and serine-calcium phosphate bridges. The micelles are further electrostatically and sterically stabilized by κ-casein "hairy" layers on the micelle surface. Encapsulating lipophilic compounds in casein nanoparticles can be achieved via two main strategies: a) ethanol and heat assisted dissociation of casein micelles, binding the bioactive ligand to soluble caseins (α-, β-, κ-CN) and micelle formation via reconstitution of the native mineral milk composition, and b) incorporating lipophilic compounds into casein micelles by ethanol assisted high pressure homogenization, followed by solvent evaporation or emulsification-evaporation (Esmaili et al., 2011; Mantzouridou et al., 2012; Pan et al., 2013; Trejo and Harte, 2010). Encapsulating lipophilic compounds in self-assembled casein nanoparticles facilitates the incorporation of bioactive compounds into the food matrix. In addition, casein nanoparticles are of higher stability compared to microemulsions or liposomes, are cost efficient and environmentally friendly (Gutiérrez et al., 2013).

Casein self-assembled nanoparticles have been successfully used to encapsulate many lipophilic bioactive compounds including curcumin (Esmaili et al., 2011; Pan et al., 2013) and β-carotene (Agüeros Bazo et al., 2013; Danino et al., 2011; Sáiz-Abajo et al., 2013). According to Sáiz-Abajo and others (2013), β-carotene encapsulation in self-assembled casein nanoparticles allowed its complete entrapment in the interior of the matrices, though β-carotene degradation due to heat, light and oxygen was approx. 40%. However, casein nanoparticles improved the stability of β-carotene under heat (80 °C, 8 h) and high hydrostatic pressure processing, compared to its free form. Incorporating β-carotene loaded CN nanoparticles into cookie dough and baking (180°C) resulted in a 42% β-carotene degradation, compared to 67% for free β-carotene.

Elucidating protective mechanisms of r-CN on β-carotene, Sáiz-Abajo and others (2013) reported that β-carotene was linked via hydrophobic interactions to the hydrophobic domain

of CN micelles (κ-casein) following heat/ethanol assisted dissociation (Fig. 4). During the CN re-assembly, β-carotene remained inside the hydrophobic core of the micelles, protected from external conditions. Using purified β-CN to develop nanoparticles has been reported an alternative efficient strategy for encapsulating carotenoids (Danino et al., 2011; Esmaili et al., 2011; Pan et al., 2013). Nanoparticles from self-assembled β-CN formed under acidic conditions (i.e. pH< pI) showed high stability between pH 2.4-4.2 and 1-45°C. The narrow particle size distribution provided satisfactory organoleptic aspects when incorporated into food matrices, including clear beverages (Danino et al., 2011; Semo et al., 2007).

Bovine β-lactoglobulin (β-LG) is the main whey protein in cow milk, characterized by cold, hot or acid induced gelation, interaction at the air/liquid interfaces, foam and emulsion stabilizing capacity, film forming ability, and water and oil binding capacity (Nicolai et al., 2011). The central cavity of β-LG provides a ligand-binding site for hydrophobic molecules, including carotenoids (Mensi et al., 2013a), and opening its hydrophobic pocket (by increasing pH and ionic strength) could enhance binding affinity (Zhang et al., 2013b). Investigating the binding affinity of several carotenoids to variants A, B of milk derived β-LGs, β-carotene exhibited a better binding affinity to β-LG compared to α-carotene and β-cryptoxanthin, while no significant differences in their binding affinities as a function of β-LG variants were observed (Mensi et al., 2013a). The potential of β-LG as an encapsulating agent is associated with its ability to form aggregates under controlled temperature, pH and ionic strength or by high pressure via hydrophobic attraction and disulphide/sulfhydryl (S-S/SH) bridges (Mensi et al., 2013b; Nicolai et al., 2011). For example, encapsulation efficiency of α-tocopherol in nanoparticles formed by Zn^{2+} induced β-LG cold gelation improved by increasing the α-TOC/β-LG molar fraction, while low amounts of Ca^{2+} conferred benefits on the binding affinity of the lipophilic compound, due to lowering electrostatic repulsions between β-LG molecules (Somchue et al., 2009).

Implementing heat induced aggregation-encapsulation, an increased encapsulation efficiency of β-LG aggregates with both the amount protein and the β-LG/EGCG molar ratio was reported (Sphigelman et al., 2012). The authors suggested a cooperative effect between ligands and β-LG, as protein molecules complexed together more easily entrap a larger amount of (stacked) ligands. Binding ligands to β-LG aggregates primarily takes place via hydrophobic interactions/hydrogen bonding, and the negligible changes in the electrophoretic mobility of the nanoparticles on the modification of the ligand : β-LG ratio omit the possibility of electrostatic interactions (Shpigelman et al., 2012). Of note, the colloidal stability of globular milk protein assembled nanoparticles as flocculation phenomena are constrained due to electrostatic repulsions and Brownian motion, whereas freeze drying and reconstituting the nanoparticles had only minor effects on their colloidal aspects.

High hydrostatic treatment of β-LG can be adopted as a minimal processing and sustainable alternative to develop self-assembly nanovehicles. Mensi et al. (2013b) investigated the impact of high pressure processing (pressurization conditions, β-LG amount, pH and ionic strength adjustment) on the efficiency of nanoassemblies to load and retain β-carotene during storage and under simulated GI conditions. The encapsulation efficiency was high (e.g. 89-99%), depending on ionic strength and pH (low pH and salt concentration facilitated the aggregates' loading with carotenoids). High pressure formed nanocomplexes of enhanced storage photostability at 4°C, compared to β-carotene bound to native β-LG or in its free form, with all-*trans* β-carotene retention being 75-100, 62 and 17%, respectively.

The natural (cold, heat, pressure or acid) self-assembly capacity of β-LG can be further improved via its ability to interact with other biopolymers through covalent conjugation or electrostatic complexation (Nicolai et al., 2011). Maillard reaction-formed conjugates between proteins and polysaccharides can self-assemble or absorb to oil-water interfaces creating a 'hairy layer' with the polysaccharide block. This layer can stabilize the colloidal

emulsion by steric exclusion or by increased microviscosity of the continuous water phase around the droplets (Nicolai et al., 2011). Maillard reaction conjugates have been successfully used to encapsulate β-carotene (Kosaraju et al., 2009; Pan et al., 2007). Protein-dextran copolymers formed by Amadori rearrangement to encapsulate β-carotene exhibited adequate encapsulation efficiency (up to 54%), stability against $FeCl_3$ induced oxidation and promoted stability against pepsin/trypsin (Pan et al., 2007).

Electrostatic complex formation of oppositely charged biopolymers is an established method to encapsulate bioactive compounds, including living cells. For lipophilic molecules, layer-by-layer deposition can be applied to nano-coated emulsion droplets (containing the bioactives), via attractive molecular forces forming core-shell type nanoparticles. Proteins such as β-LG and caseins can be used as they interact with anionic or cationic (below or above their pI, respectively) compounds, stabilizing emulsions (Nicolai et al., 2011). Though molecular complexation can also occur above the pI of proteins, a reduced pH favors stable precipitates via electrostatic interactions between the anionic groups of the polysaccharide (e.g. pectin, chitosan etc.) and the cationic patches of globular protein surfaces (Jones et al., 2009). Ionic strength, pH, temperature, molecular characteristics (charge density and distribution) and the molar ratio of the biopolymers govern the nature of the complexes (Jones et al., 2009; Matalanis et al., 2011).

4. Solid lipid nanoparticles (SLNs)

Solidifying the lipid phase (fully or partially crystalline fat droplets) of liquid-liquid nanoparticles (LLNs) such as o/w nano-emulsions and liposomes, results in novel delivery systems known as solid liquid nanoparticles (SLNs), which owe certain advantages over LLNs. SLNs are commonly fabricated using a lipid phase with a narrow melting range, e.g. pure TAGs or hydrocarbons in order to produce a highly ordered crystalline structure, enabling the physical entrapment of lipophilic bioactive compounds. There is great interest of

SLNs for food industry applications, as they combine the high bioaccessibility/ bioavailability of emulsions, the membrane permeability of liposomes and the ability of biopolymer nanoparticles for controlled release. SLN technology also reduces the disadvantages of conventional delivery systems (organic solvents), i.e. low bioactive compound payload, low chemical stability, bio-toxicity of the carrier, and challenges of LLN up-scaling. The major drawback of SLN delivery rests in the expulsion of bioactive compounds due to polymorphic transitions of the crystalline lipid structures (i.e. α→β´→β-crystal) during storage. Polymorphic transitions result in conformational modification of the lipid nanoparticles (from spherical to platelet-like), increasing their surface area and inducing particle aggregation due to hydrophobic interactions, decreasing stability. Using lipid ingredients with a broad melting range or blending lipids of different melting profile can be strategies to increase the structural disorder of the lipid crystal lattices and to alter the physical stability of SLNs towards particle aggregation or gelation. For food applications, delivery systems should employ food grade lipid sources with acceptable mouth-melting properties with have neutral effects on blood lipids. Biopolymers for stabilizing fat crystalline structures must be edible, biodegradable and have sufficient barrier properties against water vapor and oxygen, without hindering the release of the bioactive compounds.

A growing number of studies have investigated the efficacy of SLN based carotenoid delivery systems in terms of encapsulation/loading efficiency, physical/colloidal stability, carotenoid stability, stability of the lipid phase against primary/secondary oxidation as well as sustained release (Cornacchia and Roos, 2011; De Lara Gomes et al., 2013; Eltayeb et al., 2013; Helgason et al., 2009; Hentschel et al., 2008; Lacatusu et al., 2013; Liu and Wu, 2010; Nik et al., 2012; Qian et al., 2013). The physicochemical properties of the lipid phase (e.g. melting profile, crystalline state, TAG composition) and physicochemical (melting point) and molecular characteristics (head and tail groups) of the surfactants have been reported to be

crucial for the physical stability of the nanostructured lipid carriers (NLCs) and the chemical stability of the encapsulated carotenoids (Helgason et al., 2009; Qian et al., 2013).

Lucatusu et al. (2013), using a lipid carrier (carnauba wax), glycerol stearate and fish oil fortified with lutein reported an encapsulation efficiency of 56-89% with 200 nm or smaller particles, with lower amounts of fish oil decreasing encapsulation efficiency. Improved oxygen scavenging ability was detected for the antioxidant activity of lutein entrapped in SLNs vs. free lutein, though this did not depend on lutein content. The colloidal features of the lutein loaded SLNs, though significantly affected by the amount of oil carrier and lutein (both with increasing amounts reducing the hydrodynamic volume of the nanovehicles), exerted good physical stability against particle aggregation at 25°C. Contrarily, Qian and others (2013), Helgason and others (2009) and Cornacchia and Roos (2011) found that entrapped β-carotene SLNs reduced chemical stability, postulating that the restricted stability of β-carotene in SLNs was related to its expulsion from the fat crystals, undergoing aggregative (through non-polar patches of the particles or partial fat coalescence) and polymorphic changes. Helgason et al. (2009) investigated the impact of several emulsifiers (differing in their molecular conformation and melting points), emphasizing that β-carotene degradation during SLNs storage was associated with lipid phase structural modification upon crystallization. When nucleation was initiated on the fat droplet surface, a solid was formed that protected against pro-oxidants in the aqueous phase. In contrast, when nucleation starts in the interior of the fat droplets, β-carotene may be expelled to the water/oil interface, exposing it to pro-oxidants (Helgason et al., 2009). Similarly, Hentschel et al. (2008) reported that β-carotene degradation in propylene glycol monostearate (PGMS) based SLNs started shortly after storage at 20°C, though no polymorphic transitions were detected. It was also demonstrated that co-encapsulating of β-carotene with antioxidants, including α-tocopherol, protected against degradation, even when NLCs were dispersed in water.

5. Nanostructured lipid carriers (NLCs)

The challenges of SLNs, e.g. low payload, high water content and tendency to form aggregates that expulse the bioactives, have led to development of a second generation of SNLs known as nanostructured lipid carriers (NLCs) (Müller et al., 2002; Tamjidi et al., 2013). NLCs are considered as modified SLNs in which the lipid phase comprises both solid (fat) and liquid (oil) lipids at room or body temperature. Regarding the solid lipid phase, fat is found either in a partially crystalline or amorphous state, increasing the structural disorder of the nanoparticulates, thus minimizing the expulsion of the bioactive due to fat polymorphism (Tamjidi et al., 2013). The major advantage of NLCs is their higher payload as imperfections within the fat structure can accommodate more efficiently the bioactives. Three conformation types of NLCs exist: the imperfect, the amorphous and the multiple (Fig. 5). In the imperfect case, the "tightly packed" fat structure of SLNs is disrupted by creating internal voids, using lipid components exerting high spatial incompatibility (large enough distances between the fatty acids of the glycerides). This can be achieved by blending solid lipids with liquid lipids of different chemical composition, e.g. mixing tristearin with MCT (Yang et al., 2014a). The amorphous type is based on controlling the fat crystallization to produce crystalline conformations prone to polymorphic transitions, i.e. structureless solid amorphous matrices. This can be realized by blending specific lipids (e.g. hydroxyl-octacosanylhydroxystearate with isopropylmyristate), where lack of crystallinity leads to enhanced stability against expulsion of the bioactive. Finally, for multiple systems, a complex oil-in-fat-in-water emulsion comprising tiny liquid oil nanocompartments is produced, with the bioactive being dissolved in the oil phase (Müller et al., 2002; Tamjidi et al., 2013).

Depending on the carrier specification (heat stability, hydrophobicity of the bioactive compounds, emulsifier cloud point etc.), NLCs can be produced by hot or cold homogenization. In the former, the bioactive compound is dissolved in the molten fat/oil

emulsifier system. After high pressure homogenization or ultrasonication, the nanoemulsion is cooled to initiate fat crystallization and precipitation of the nanoparticles. In the second case, the emulsion is quenched by liquid nitrogen and the resulting solid matrix is cold-milled to obtain fine particulates. Though the latter method appears to be advantageous for heat sensitive compounds, most NLC applications for carotenoids rely on hot homogenization, due to sufficient heat stability of carotenoids (retention >80-90%) (Helgason et al., 2009; Hentschel et al., 2008). In addition, problems related to uncontrollable fat crystallization can be overcome by proper selection of the emulsifying system (Gutiérrez et al., 2013).

Recently, solvent diffusion (dissolution of lipid phase into ethanol followed by blending with a hydrophilic emulsifier in water) has been successfully applied for β-carotene loaded NLCs (Hejri et al., 2013). The method provided good β-carotene stability (up to 98%), though degradation depended on the amount of lipid phase, surfactant concentration, and dissolution temperature. PIT was also successfully used to produce NLCs at low energy, cost efficiency and minor equipment demand (Zhang et al., 2013a).

To date, NLCs are predominantly employed for drug delivery, though there is an increasing number of applications for delivering dietary derived compounds, including hydrophobic polyphenols (Aditya et al., 2013) and carotenoids (Hejri et al., 2013; Hentschel et al., 2008; Hung et al., 2011; Nik et al., 2012; Quan et al., 2013; Zhang et al., 2013a) in food items. Homogenization conditions (Hung et al., 2011; Liu and Wu, 2010), melting profile of the lipid ingredients (Yang et al., 2014a; Zhang et al., 2013a), surfactants (Hejri et al., 2013), concentration of the lipid phase (Hejri et al., 2013), fat crystallization, and ionic strength of the emulsion (Zhang et al., 2013a) all influence the encapsulation efficiency and the stability of carotenoids during storage. Liu and Wu (2010), studying ultrasonication effecting lutein encapsulation by NLCs, found a decreased NLC mean size (from 228 to 110 nm) with increasing ultrasonication time (from 2 to 10 min). No re-aggregation was detected with

excess energy input as previously stated (Kentish et al., 2008), though excessive ultrasonication did not enhance lutein loading efficiency.

Surfactant type and amount directly impact carotenoid payload in NLCs and their stability during storage. This is generally associated with the surfactants' ability to reduce the surface area of the lipid particles, affecting fat polymorphic transitions, controlling mass transfer phenomena, e.g. oxygen diffusion (Hejri et al., 2013; Helgason et al., 2009). In the latter study, β-carotene stability improved with increasing surfactant amount until a maximum; further increase significantly impaired stability against oxidation, likely due to the formation of a rigid shell protecting carotenoids. Zheng et al. (2013) investigated several hydrophilic surfactants (Tween 20, arabic gum, sucrose esters, and polyglycerol fatty acid esters) for colloidal aspects, crystallinity and physical stability of NLCs. The loading efficiency and storage stability of α-lipoic acid improved by surfactants with small and flexible hydrophilic head groups such as Tween 20 or PGFE. Contrarily to sucrose esters and arabic gum, these systems were stable towards flocculation and fat polymorphic transitions.

Ionic strength can also affect the effectiveness of surfactants. Depending on surfactant to ionic strength ratio, the formation of gels or phase separation may take place. Zhang et al. (2013a), implementing the PIT method to fabricate NLCs loaded with β-carotene reported that increasing NaCl concentration (from 0 to 1.0 M) decreased PIT and facilitated NLC formation at lower temperature. Moreover, adjusting ionic strength during NLC production enhances their physical stability (absence of coalescence or Ostwald ripening phenomena) and also remarkably reduces β-carotene degradation during storage.

It was shown that NLC nanoparticles can be used to fabricate the core of gelatine-arabic gum coacervates containing fucoxanthin (Quan et al., 2013). The physicochemical properties of the lipid core components (cetyl palmitate vs. canola oil) significantly affected the morphological, payload and release properties of the capsules. Increasing the fat crystalline

fraction in the lipid core increased the irregularity of the obtained microspheres and lowered the efficiency to uphold fucoxanthin due to the build-up of a "brick-like" structure devoid of internal pockets for taking up fucoxanthin. However, the presence of compact lipid core structures (due to the increase of the solid to liquid fraction) lowered disintegration and slowed the release after incubation in gastric/intestinal fluids, and enhanced storage retention at various RH. The ability of high melting point lipid components to control mass transport phenomena (oxygen and GI-fluids) even under conditions where structural collapse of the hydrogel shell structure was noticed, was made responsible (Quan et al., 2013).

6. Solvent displacement

Solvent displacement or anti-solvent precipitation is generally based on altering the "quality" of the solvent around the biopolymer molecules, to induce a spontaneous self-assembly when a critical solvent concentration is attained (Joye and McClements, 2013; Matalanis et al., 2011). This can be achieved by adding a miscible anti-solvent to a biopolymer solution where the active compound comprises a co-solute of the initial biopolymer solution. Nanoparticle formation takes place by rapid diffusion of the organic solvent in the aqueous phase and the deposition of the active substance in the solvent – anti-solvent interface (Chu et al., 2007a). The polymer molecules (in the solvent phase) diffuse together with the solvent and self-assemble at the interface, incorporating the active substance (Fig. 6). The surfactant in the anti-solvent phase stabilizes the formed nanoparticles, preventing aggregation. The solvent can then be eliminated by evaporation. As for supercritical fluid induced precipitation (e.g. SAS/SEDS), supersaturation is the driving force of encapsulation effectiveness, determining mean size, crystallinity, purity and morphology of the formed particles.

In general, high supersaturation and low surface tension enable fast nucleation and small crystal growth (controlled by secondary crystallization and Ostwald ripening) and particle formation with narrow size distribution (Joye and McClements, 2013; Liu et al., 2007b).

Nanoparticles formed by anti-solvent precipitation show low polydispersity and high encapsulation efficiency/stability. This is achievable either thermodynamically (by stabilizers/media controlling colloidal interactions between nanoparticles) or kinetically (energy input to compensate excess surface energy due to the particles' high surface area) (Thorat and Dalvi, 2012). Parameters such as anti-solvent to solvent ratio, the speed of anti-solvent to solvent addition or vice versa, active substance concentration, precipitation temperature, the physicochemical profiles of the anti-solvent, surfactant and biopolymers (e.g. polarity, surface charge, aggregation ability, interfacial properties) impact the size, crystallinity and morphology of the nanoparticles (Joye and McClements, 2013).

With respect to food industry, nanoprecipitates produced by solvent displacement mainly aim to incorporate active substances that degrade under common processing conditions (heat, pH, light and mechanical stress), enhancing bioactive payload compared to conventional encapsulation, and to achieve customized release under GI digestion conditions. Solvent displacement nanoprecipitation is regarded as a very efficient method to entrap and stabilize lipophilic substances including carotenoids or hydrophobic polyphenols (curcumin), in food matrices, as well as to improve bioavailability (Chu et al., 2007a; Faisal et al., 2013; Kakran et al., 2012; Lobato et al., 2013; Mitri et al., 2011; Panagiotou and Fisher, 2013; Patel et al., 2010; Ribeiro et al., 2008; Tachaprutinun et al., 2009; Yin et al., 2009).

Though solvent displacement appears to be advantageous for up-scaling, proper selection of nanoparticle components (e.g. polymers, surfactants, water miscible organic solvents) may be demanding (Joye and McClements, 2013). The most commonly employed organic solvents are ethanol and acetone, while a great number of surface active compounds have been successfully used for nanoprecipitation, including milk proteins (whey protein isolates/ concentrates, sodium caseinate), plant proteins (zein), polysorbates, and sucrose esters (Chu et al., 2007a; Ribeiro et al., 2008; Yin et al., 2009). Polymers can also be used as supportive

coating materials, particularly when sustained release of the bioactive substances is required. For most applications, polymeric materials exerting fair interfacial properties (gelatin, poly-D,L-lactide (PLA), poly-D,L-lactide-co-glycolide (PLGA), poly-ε-caprolactone (PCL), arabic gum) are used for anti-solvent nanoprecipation (Lobato et al., 2013; Ribeiro et al., 2008), though in many cases polymers as co-stabilizer are not required.

Chu et al. (2007) and Ribeiro et al. (2008) used solvent displacement to investigate colloidal and stability characteristics of nanoparticles loaded with β-carotene and stabilized either by different types of milk proteins or by a blend of surfactants (Tween 20) and surface active polymers (gelatin, PLA and PGLA). Though nanoparticle formation mechanisms were similar in both cases (movement of the lower surface tension phase to the aqueous one and creating a solvent boundary layer where supersaturation, nucleation and β-carotene crystal formation took place), the formed particles were stabilized either only by the surfactants or by synergistic actions of the surfactants and polymeric material. In the latter case, the surfactants rapidly adsorbed on the oil-water interfaces, preventing droplet coalescence due to electrostatic repulsion, while the polymer material deposited on the stabilized particles, creating a rigid and thick outer layer, hindering particle growth due to Ostwald ripening.

It was also shown that the surfactant type was the main factor affecting mean particle size, whereas surfactant concentration, the emulsification process and amount of β-carotene loaded may interplay with the colloidal aspects of the obtained particles. Among different types of milk proteins, sodium caseinate was optimum to stabilize β-carotene nanoparticles, as casein submicelles (primarily) and micelles (secondarily) undergo conformational changes upon solvent displacement, interacting with β-carotene via hydrophobic binding (Chu et al., 2007). Whey protein concentrates were the least effective stabilizing agents, increasing particle polydispersity (due to their lower protein purity, MW homogeneity, and structural flexibility compared to SC). In the same study, it was shown that a minimum amount of SC (0.5% wt)

was required to ensure an adequate number of casein submicelles to stabilize the precipitated β-carotene via self-assembled caseins. Further increasing the SC concentration did not significantly change β-carotene uptake. Fabricating particularly small nanoparticles may increase β-carotene susceptibility to degradation during storage. Yin et al., (2009), studying the influence of several surfactant systems on colloidal aspects and stability of β-carotene loaded nanoparticles revealed that larger particles improved biological activity due to better protection by the thicker crust/envelop of the surface nanodeposited emulsifiers.

Recently, bixin was successfully encapsulated in PCL-sorbitan monostearate stabilized nanoparticles by anti-solvent (1:8 ethanol : acetone ratio) precipitation (Lobato et al., 2013). The approach allowed a very high encapsulation efficiency (>98.6%), probably due to the capric/caprylic TAG based core, promoting bixin solubilization. The loaded nanoparticles exerted good stability against Ostwald ripening and coalescence during storage (119 days, room temperature), though bixin retention declined to 46%.

7. Emulsification evaporation

This method comprises dissolving the active substance in an organic solvent and emulsifying the apolar phase using an aqueous phase containing the surfactant. The formed o/w emulsion is then converted to a nanodispersion by solvent evaporation (Fig. 7). The particles' morphology rests usually in the thermodynamically stable crystal structure, as solid formation takes place by evaporation crystallization at low supersaturation (Chu et al., 2007b). The morphological characteristics and polydispersity of the nanoparticles are influenced by many parameters, i.e. the surfactant, the homogenization process and conditions (pressure, number of passes), the organic : aqueous phase ratio, the organic solvent and amount, evaporation temperature etc. (Anarjan et al., 2011b; Anarjan and Tan, 2013b; Cheong and Tan, 2010; Chu et al., 2007b; Tan and Nakajima, 2005). Together with storage conditions, these parameters may also indirectly impact the stability of the encapsulate (Anarjan and Tan, 2013c).

Emulsifiers are the driving force for nanoparticle formation, reducing interfacial tension between immiscible phases, reducing the energy required to disrupt the droplets during emulsification. Surfactants absorb on the oil-water interfaces, forming a protective layer surrounding the droplets, preventing coalescence (Chu et al., 2007b). Chu and others (2007b) investigated milk protein emulsifiers (sodium caseinate, whey protein concentrate (WPC) and isolate (WPI) and hydrolyzate (WPH)) with respect to properties of β-carotene containing particles. Factors such as interfacial tension reduction, molecular flexibility and structural packing of the protein, MW, protein purity as well as exposure of hydrophobic areas affected nanoparticle characteristics. SC was the most efficient surfactant, favoring formation of monomodally distributed fine nanoparticles, followed by WPH, WPI and WPC. Anarjan and Tan (2013a) studied polysorbates (Tween 20, 40, 60 and 80) and sucrose ester emulsifier functionality, reporting that Tweens more efficiently reduced the mean size of astaxanthin loaded nanodispersions than sucrose esters, due to their higher HLB and shorter fatty acid chain lengths. On the other hand, the polydispersity of nanoparticles was lower for sucrose esters; perhaps due to their higher entrapment efficiency or critical micelle concentration than polysorbates, facilitating the formation of astaxanthin nanodispersions.

Using co-surfactants can remarkably modify mean nanoparticle diameter as well as their polydispersity index (Anarjan and Tan, 2013b). Blending non-adsorbing emulsifiers such as sodium caseinate or arabic gum with Tween 20 to create binary surfactant systems significantly increased the mean size of astaxanthin loaded nanodispersions. Several mechanisms were proposed for these adverse effects, such as competitive adsorption (restricting the non-adsorbing emulsifiers in a small area, leading to a thicker interfacial layer), depletion flocculation (increased attractive forces between the droplets leading to droplet coalescence) and bridging flocculation (droplet collision due to electrostatic interactions between charged domains of the biopolymers). However, using ternary systems

(Tween 20 and two non-adsorbing emulsifiers) significantly reduced nanoparticle mean size; emphasizing the synergy of the surfactants in terms of stabilizing and emulsifying ability, forming a closely-packed interfacial layer of the intercalating surfactant molecules.

Microfluidization is the most common homogenization technique for nanoparticles production by solvent-diffusion. In several studies (Anarjan et al., 2011b; Chu et al., 2007b; Tan and Nakajima, 2005), increased homogenization pressure and cycles was generally associated with finer nanoparticles, as higher shear forces and turbulence was achieved. However, parameters such as the evaporation temperature and the organic to aqueous ratio can also effect the homogenization to control fine particle formation. Anarjan et al. (2011) reported that a minimum critical temperature was required for fine nanoparticle fabrication. In addition, increasing the amount of organic solvent produces larger particles, mainly due to increased microviscosity at the polar-apolar interfaces, reducing disruptive forces due to cavitation (Tan and Nakajima, 2005). An antagonistic action on astaxanthin nanodispersion particle size and polydispersity index was observed with water immiscible (dichloromethane) and water miscible (acetone) solvents. Tuning the ratio of water miscible to immiscible solvents proved effective to control nanoprecipitation. Adjusting solvent proportions controls solvent diffusion in the aqueous phase, the active substance solubility and stabilizer as well as the interfacial properties of the system. Therefore, a critical apolar : polar solvent ratio exists to obtain the finest particles with narrow particle size distribution. This critical point is generally associated with rapid nucleation rates, controlled particle growth, hindered particle collision phenomena, and adequately low surface tension (Anarjan et al., 2011a).

In general, emulsification-evaporation (due to relatively mild processing conditions) allows rather high encapsulation yields (up to 97%). Homogenization conditions (pressure, number of cycles), the organic solvent and stabilizer type as well as the evaporation temperature are the most crucial parameters for carotenoid degradation during the nanoprecipitation process.

Tan and Nakajima (2005) reported a 1-8 % β-carotene loss after the emulsification-evaporation process, which was mainly attributed to the temperature raise during microfluidization and the presence of heat, light and oxygen during the evaporation. Anarjan et al. (2011b) demonstrated that astaxanthin losses (4-45%) during nanoprecipitation were predominantly influenced by increasing cycle numbers and homogenization pressure, while evaporation temperature conferred only a minor effect. Anarjan and Tan (2013a) investigated the impact of surfactant type on astaxanthin losses during nanoprecipitation, revealing that emulsifiers carrying unsaturated fatty acids (e.g. Tween 80 or oleic acid sucrose esters) increased oxidation. Astaxanthin losses were also lower for nanodispersions with larger particle mean size (e.g. sucrose ester based), due to larger interfacial layers providing protection of the lipophilic active substance. In a successive study, Anarjan and Tan (2013b), using a ternary emulsifying system (Tween 20, arabic gum and sodium caseinate), reported that the emulsifier amount as well as the emulsifier to organic phase ratio can influence astaxanthin losses during emulsification-evaporation. Generally, a high emulsifier and organic phase concentration enhanced astaxanthin stability, primarily due to their impact on the oil-water interfaces and limited exposure of carotenoids to external conditions. Using binary solvent systems (water immiscible and water soluble solvents) has also been highlighted as an alternative to control carotenoid losses during nanoprecipitation. Anarjan and others (2011a) showed that trans-cis- isomerization of astaxanthin was higher in nanodispersions fabricated using pure dichloromethane (24%) compared to using acetone (13%). The authors reported that astaxanthin cis-isomer concentration was minimized with a dicholoromethane to acetone ratio at 3:7 (for 9-cis) and 5:5 (for 13-cis).

Incorporating antioxidants (α-tocopherol, ascorbic acid) into astaxanthin loaded nanodispersions can improve autoxidation induced losses of carotenoids during emulsification-evaporation (Anarjan et al., 2013). Lipophilic antioxidants (α-tocopherol)

were more effective against carotenoid oxidation, due to their ability to react with free radical intermediates of carotenoid peroxidation and peroxides as well as due to their ability to act as reducing agents for transition metals. Controlling carotenoid stability in nanodispersions is challenging, as it is not only affected by storage conditions but also by other factors such as carotenoid oxidation induced during processing, the morphological and compositional profile of the nanoparticles, the presence of antioxidants as well as the targeted food matrix (Anarjan and Tan, 2013a; Anarjan and Tan, 2013c). In general, storing carotenoid loaded nanodispersions at high temperature and light/oxygen exposure accelerate their oxidation. For example, increasing storage temperature by 10˚C resulted in 7- and 24-fold increased astaxanthin oxidation in nanodispersions stabilized by Tween 20 and sodium caseinate, respectively. Similarly, astaxanthin containing nanodispersion exposure to fluorescent/UV light resulted in a respective 3/5-fold increased oxidation for Tween 20 based systems and a 5/ 9-fold increase for sodium caseinate based ones (Anarjan and Tan, 2013c).

Severe homogenization conditions triggered β-carotene autoxidation (retention decreased from 56 to 36%) when homogenization pressure increased (from 60 to 140 MPa) during nanodispersion storage (4 °C, 12 weeks). Moreover, the increased ratio of the organic to water phase from 1:9 to 2:8 significantly improved β-carotene storage. The formation of free radicals during homogenization and the impact of the solvent phase and stabilizing agents on the specific surface area of the particles and the colloidal aspects of the protective interfacial layers surrounding the formed nanoparticles are the main explanations for these results (Tan and Nakajima 2005). Scavenging free radicals produced during homogenization by adding antioxidants appears to be effective to extend the shelf-life of astaxanthin loaded nanodispersions. Using ascorbic acid (40 mg/L) and α-tocopherol (60 mg/L) as additives in astaxanthin nanodispersions produced very low degradation rates (<0.00001/ week), while no significant differences in astaxanthin content within the first month (at 5 °C) were observed

(Anarjan et al., 2013). Anarjan and Tan (2013b) also scrutinized the effect of the food system (deionized water, skimmed milk or orange juice) on the stability of astaxanthin encapsulated in nanodispersions stabilized by a ternary surfactant system (Tween 20, sodium caseinate, arabic gum). Astaxanthin stability was higher in skimmed milk and orange juice compared to deionized water, whereas orange juice was the best matrix to incorporate astaxanthin, which was attributed to the high ascorbic acid content of orange juice and low pH, retarding free radical induced carotenoid autoxidation (Anarjan and Tan 2013b).

ENCAPSULATION TO IMPROVE BIOACCESSIBILITY AND BIOAVAILABILITY OF CAROTENOIDS

Bioavailability of a compound is generally defined as the fraction that can be absorbed and used for physiological functions and/or storage. The bioavailable fraction (F) of lipophilic bioactives can be expressed as (McClements et al., 2008; McClements and Li, 2010):

$$F = F_B \times F_C \times F_M \quad (1)$$

where F_B is the bioaccessibility, F_C equals transport (the fraction of the released bioactive compound that is transported across the intestinal epithelium) and F_M represents metabolism (here: the fraction that ends up in circulatory system via the lymph), though also further biodistribution and excretion (half-life in tissues) would play a role.

Due to their limited solubility in lipids and aqueous solutions including saliva and intestinal fluids, carotenoids exhibit low bioavailability, that is, up to 20% for carotenes and up to ca. 40% for xanthophylls (Bohn, 2008). Emulsification of carotenoids in the bulk lipid phase by surface active lipids (phospholipids, FFAs, MAGs or DAGs) can enhance their solubility in the intestine (Borel et al., 1996; Huo et al., 2007). In addition, lipase hydrolytic activity promoting micellization is generally higher in the case of emulsified TAGs, indicating that liquid lipid carriers (emulsions) are natural systems promoting carotenoids bioavailability

(Huo et al., 2007). Taking into account the structural, colloidal and physicochemical diversity of LLCs, a large number of factors can influence carotenoid bioavailability (Fig. 8):

a) the molecular conformation of the lipid carrier, e.g. type and position of the fatty acid chains of the glycerol backbone;

b) the colloidal and interfacial properties of the lipid phase components as well as the presence of surface active compounds in the food matrix or in intestinal fluids;

c) the physical state of the lipid phase, e.g. presence and amount of crystalline fat, polymorphic state of crystalline fat;

d) the structure of the food matrix and its disintegration during digestion; and

e) the direct/ indirect interaction of food matrix with the lipid phase, modulating digestion (prevention of lipid droplet disruption, reduction of lipase activity, binding of bile salts) and absorption (modulation of mass transfer, e.g. enzyme or bile salt diffusion, micelle hydrodynamic volume) (Bohn, 2008; McClements et al., 2008).

Thus, encapsulation of carotenoids adopting interfacial and structural engineering practices can also promote the bioavailability of carotenoids, via: i) improved stability against isomerization and degradation, such as being the case for epoxycarotenoids (undergoing epoxide-furanoid transition due to the acid gastric pH); ii) enhanced solubility and increased bioaccessibility in the gut; iii) target release kinetics e.g. for a precise delivery or release patterns over time in the small intestine (main organ for carotenoid absorption), likewise minimizing potential negative effects of other matrix constituents such as dietary fiber.

LLCs encapsulated carotenoids

Most studies scrutinizing the impact of encapsulation on carrier physicochemical and colloidal characteristics have been conducted on o/w micro- or nano-emulsions. Carotenoid type and amount of added to delivery systems are known to influence their bioaccessibility. In general, increasing the carotenoid amount is related with a higher recovered fraction at the

end of the digestion process (Wang et al., 2012a). However, higher doses tend to lower bioavailability, possibly due to saturation processes during absorption (Bohn, 2008).

The molecular structure of the lipid carrier is critical for carotenoid bioaccessibility; their bioacessibility is enhanced by long chain TAGs compared to medium or small chain TAGs (Qian et al., 2012a; Tyssandier et al., 2001), in part as the latter are taken up via the portal vein and contribute less to micelle formation. The bioaccessible β-carotene fraction in in-vitro digested nanoemulsions prepared with long-chain (corn oil), medium chain triglycerides (MCT) or orange oil was drastically reduced by decreasing TAG chain length (Qian et al., 2012a), (Table 3), possibly due to the ability of LCT to form mixed micelles with a large hydrophobic core, adequately accommodating β-carotene. In addition, orange oil was very poorly digested. Similarly, β-carotene bioaccessibility emulsified either in corn oil or lemon oil was lower in the non-digestible oil system, as carotenoids may become trapped in the oil droplets (Rao et al., 2013). A linear relationship between β-carotene bioaccessibility and the amount of FFAs released during lipolysis was also detected. It is also noticeable that TAG lipolysis is more crucial for carotenes than for xanthophylls (Tyssandier et al., 2001). Xanthophylls are situated mainly in the oil/water interface rather than in the hydrophobic core of lipid droplets, facilitating their transport to aqueous medium during the micellization process (Tyssandier et al., 2001) and their release for subsequent enterocyte uptake.

Synergism between TAG composition (LCT vs MCT) and oil concentration regarding β-carotene bioaccessibility in low (1% w/w) and high (4% w/w) fat nanoemulsions was recently studied. Though oil digestibility was linearly reduced in the high fat and high LCT content systems, the amount of oil in the lipid phase modifying β-carotene bioaccessibility was MCT to LCT ratio dependent (Salvia-Trujillo et al., 2013a). For low fat systems, a negative linear correlation between FFAs released during digestion and β-carotene bioaccessibility was observed, with a higher LCT:MCT ratio favoring β-carotene

bioaccessibility. For high fat systems, β-carotene bioaccessibility decreased when the LCT concentration in the oil phase increased from 0 to 50%, whereas a further increase of the LCT percentage (from 50 to 100%) enhanced bioacessibility. The reduced bioaccessibility at high to intermediate MCT:LCT ratios was associated with an increase of non-digested TAGs, leading to higher retention of β-carotene in the core of the non-digested lipid droplets.

Mean lipid droplet size is another factor that may modify carotenoid bioaccessibility. For example, reducing lipid droplet size in microfluidized LLCs improved the bioaccessibility of zeaxanthin (3 to 33%), and β-carotene (2 to 18%) (Cha et al., 2011). Though lipid droplet size can dramatically be affected by physicochemical phenomena (droplet coalescence, disruption, dissolution) during digestion, the presence of small lipid droplets may enhance carotenoid bioaccessibility, due to the increased surface area of the lipid droplets exposed to lipolytic enzymes and surface active compounds, facilitating micellization (Yonekura and Nagao, 2007). Salvia-Trujillo et al. (2013b) showed that reducing lipid droplet size from 23 to 0.23 μm (4% corn oil based o/w emulsions containing 0.5% (w/w) β-carotene, 1.5% Tween 20) increased β-carotene bioaccessibility from 34 to 59%. A positive correlation of carotenoid bioaccessibility and the completeness of lipid digestion was also detected.

Droplet size is also associated with carotenoid bioaccessibility due to the effect of bile salts and lipase. (Wang et al., 2012a) demonstrated that bile and lipase exert a governing role on micellar carotenoid recovery in the presence of large lipid droplets (e.g. 0.68 to 18 μm) while no influence on carotenoid solubilization and micelle formation was detected when nanodispersions with particles <100 nm were tested. However, the latter systems improved β-carotene bioaccessibility (48 to 61%). This suggests the existence of a lower particle size threshold where the disruption of the lipid droplets via the action of bile and pancreatic lipase can be achieved, whereas a further size reduction can enhance carotenoid bioaccessibility only via facilitating transport of the carotenoid molecules to mixed micelles.

The composition and colloidal properties of the interfacial layers surrounding the lipid droplets may also influence carotenoid availability. This may be attributed to the: a) increased stability of lipid droplets against coalescence or disruption during GI passage, b) modulation of lipid droplet exposure to intestinal enzymes and bile salts, and c) control of the transfer of carotenoids from the lipid droplets to the mixed micelles, via competitive absorption of surface active compounds to the lipid droplets during digestion.

Parameters such as electrostatic charge, thickness, permeability, microviscosity, and environmental response of the lipid droplets are known to impact the transfer of lipophilic compounds to mixed micelles (McClements et al., 2008; Troncoso et al., 2012). The bioaccessibility of carotenoids loaded into interfacial engineered lipid droplets via surface active compounds such as milk proteins (sodium caseinate, whey proteins), soy protein isolates, sucrose esters, polysorbates or polyglycerol esters of fatty acids has been extensively studied (Table 3). Liu et al., (2012) investigated the impact of the interfacial layer composition (whey protein isolate, decaglycerol monostearate and soybean soluble polysaccharides) of lipid droplets of LCCs loaded with β-carotene on their bioaccessibility. The authors reported that the surfactant type pronouncedly affected the stability of lipid particles during in vitro digestion. Whey protein stabilized interfacial layers exerted the lowest stability (droplet size increased from 0.6 to 18 μm) in the gastric phase, probably due to their susceptibility to pepsin, disintegrating the interfacial layers, promoting droplet flocculation (Liu et al., 2012; Nik et al., 2011). Emulsion exposure to duodenal and intestinal fluids remarkably reduced particle size, with the WPI- and SSPS-stabilized ones showing the strongest response. Whey proteins in the interfacial layers surrounding the lipid droplets were of highest β-carotene bioaccessibility. This was primarily attributed to the ability of the whey proteins absorbed to the oil/water interface to unfold and covalently cross-link, enhancing the lipase action at the interfaces and therefore the extent of emulsified β-carotene. The lower

biaccessibility of the SSPS stabilized lipid droplets was attributed to soluble dietary fiber, suppressing β-carotene transport to the aqueous phase (Yonekura and Nagao, 2009).

Soluble dietary fiber present in the continuous phase of LLCs has been identified to adversely impact carotenoid bioaccessibility (Yonekura and Nagao, 2009). Several mechanisms have been suggested, including a) reduced enzyme activity (pancreatic lipase); b) adsorption of fiber to the interfacial layers surrounding the lipid droplet, reducing the accessibility to lipolytic enzymes; c) increased bulk and viscosity of the aqueous phase, reducing the mass transfer (diffusion) rates of molecules (e.g. bile salts, enzymes, micelles) to and from the lipid droplet surface; d) binding bile salts, hindering their emulsifying activity.

Yonekura and Nagao (2009) investigated the effect of several soluble dietary fibers (medium- and high-viscosity sodium alginate, apple and citrus pectin, methyl- and carboxymethyl-cellulose) on the micellization and (Caco-2) cellular uptake of β-carotene/ lutein emulsified in soybean oil droplets. Though all fiber-containing systems reduced carotenoid bioaccessibility, the mechanisms depended on the hydrocolloid type. Sodium alginate appeared to reduce carotenoid bioacccessibilty mainly by increasing the macroviscosity of the bulk aqueous phase and formating hydrogel aggregates, entrapping lipophilic matter in the gastric phase, while pectins exerted a more complex activity that did not solely rely on their viscosifying action but presumably on their bile salt binding activity as well.

Recently, the impact of pectin concentration and degree of methyl-esterification (DE) on the bioaccessibility of β-carotene loaded LLCs was studied (Verrijssen et al., 2014). Increasing pectin concentrations did not significantly change colloidal structures and bioaccessibility of β-carotene during GI passage (Table 3). However, DE affected both the colloidal state of the lipid droplets and microviscosity of the bulk aqueous phase throughout GI transit, and consequently bioaccessible β-carotene. Most bioaccessible β-carotene (ca. 60%) was detected in digested emulsions comprising high DE pectin (66 or 99%), whereas digesta containing

low DE pectin (14%) bioaccessible β-carotene was lower (ca 35%). This was attributed to the formation of gel-like aggregates (via ionotropic interactions of galacturonic acid units with Ca^{2+}), entrapping β-carotene, reducing lipase activity due to sterical hindrance associated with constrained mass transport (controlled by viscosity) or reduced droplet surface area, limiting enzymatic attack. Furthermore, the low DE pectins were associated with limited lipid digestibility and adsorption of lipophilic compounds to the droplet interfaces.

Bioaccesibility of carotenoids encapsulated in solid micro- and nano-particulates

Solid encapsulation matrices include dry particulates, hydrogel microbeads, molecular assemblies, solid lipid carriers etc. To date, a rather limited number of studies have been conducted on the impact of structural aspects of solid-based encapsulation vehicles, their disintegration, and the colloidal changes of their particular components on the bioavailability of encapsulated bioactive compounds. Donhowe et al., (2014) evaluated the release and bioaccessibility of β-carotene encapsulated either in maltodextrin spray dried microcapsules or alginate-chitosan microbeads (prepared by ionotropic gelation), incorporated in two different fat-free food products (tapioca pudding, yogurt). Highest β-carotene bioaccessibility was achieved in the spray dried matrices (13 and 1% for pudding and yogurt, respectively), while the amount of bioaccessible β-carotene in the gelled microbeads was as low as 0.2 %, regardless the food matrix. This was ascribed to the restricted mass transport of pancreatic lipases and bile to and from the lipid droplets interfaces embedded in the hydrogel matrix and limited micellization due to soluble dietary fiber present at the intestinal phase.

Encapsulation of β-carotene in WPI-alginate-chitosan microbeads was assessed as a strategy to enhance the carotenoid content of almond butter and to improve its bioaccessibility (Roman et al., 2012). The authors reported that the bioaccessible fraction of both free and encapsulated β-carotene was below 1%. It was deduced that especially the binding of carotenoids (via the emulsification of lipid phase) by whey protein and the hindrance of the

micellization process (due to soluble fiber) were responsible for these results. On the other hand, nanoparticles based on low MW chitosan cross-linking initiated by tripolyphosphate resulted in 28% higher lutein bioaccessibility compared to artificial mixed micelles (Arunkumar et al., 2013). It was believed that the restricted hydrophobic interaction between chitosan and lutein was the major parameter enhancing carotenoid micellization.

NLCs also showed good release in vitro compared to conventional LLCs. Lutein release from encapsulated NLCs (glycerol stearate, fish oil and carnauba wax in buffered saline media) increased by ca. 30% compared to conventional nanoemulsions (no fish oil, Lacatusu et al. (2013)). Fish oil was crucial for lutein release, probably as it modulated carotenoid hydrophobic binding in the emulsified lipid phase. A similar lutein release was found using diffusion cells with dialysis membranes (Liu and Wu 2010). However, lutein encapsulated in lyophilized NLCs of solid lipids (glyceryl palmitostearate, palmitic acid MAGs, C:12-C:16 FA,), surfactants (polysorbates, Pluronic F68) and corn oil was of slower release (in media) compared to LLCs (based on caprylic/capric TAGs, identical to NLCs surfactants) after 24h. Lutein released in simulated gastric/intestinal fluids followed the order of SIF>SIF (no pancreatin)>GF(no pepsin)>GF. Only for SIF there was an increased lutein release rate during the first 6h, indicating disintegration of the lipid structures due to pancreatic lipase. In the other systems, a gradual increase of the released lutein during the first 24h was observed, reaching eventually a plateau, suggesting good resistance of the NLCs to gastric juice.

In vitro release (in PBS-SDS medium) of lutein encapsulated in nanoparticules prepared by supercritical fluids exhibited differences compared to pure lutein and a lutein-zein mixture (Hu et al., 2012). A near zero-order release was detected for the latter, without an initial release burst as for pure lutein/the zein-lutein mixture. The slow release was attributed to the restricted diffusion of the solute molecules from the bulk aqueous phase into the solid matrix due to the zein polymeric network. However, the amount of lutein released after 300 min was

equal for all test systems, indicating that temporal water upholding leads to the relaxation of polymeric entanglements, facilitating the transfer of lutein to the bulk aqueous phase.

Cellular uptake of encapsulated carotenoids

Carotenoid absorption is a complex dynamic process involving different physicochemical and biochemical phenomena, including their release from the food matrix, solubilization into mixed micelles in the gut, uptake by enterocytes, incorporation into chylomicrons and secretion into the lymph (Harrison, 2012). Caroteoid absorption efficacy as well as their tissue concentrations are known to depend on the molecular structure and physiochemical profile, e.g. hydrophobicity (logP, logD) (Sy et al., 2012). Though human studies are still perceived as the "gold standard" to assess carotenoid bioavailability, restrictions such as the need to administer high amounts of carotenoids in order to observe significant variations in plasma, and ethical and cost constraints pose limits (Biehler and Bohn, 2010). In vitro testing of carotenoids using cells mimicking the human intestinal epithelium (e.g. Caco-2) can be a convenient, inexpensive and reproducible tool to evaluate the digestive stability, micellization, uptake and transport as well as metabolism. Parameters such as dissolution rate, hydrophilicity, particle size and surface area, cis-trans-isomerization, lipid polymorphism, surfactants (e.g. lysophosphatidylcholine, FFA), presence of free vs. esterified protein bound complexes, and interactions with dietary compounds (lipids, fiber, phytosterols etc.) have been reported to affect carotenoid cellular uptake (Failla et al., 2008; Kotake-Nara and Nagao, 2012; Yonekura and Nagao, 2009).

Ribeiro et al. (2006) assessed the Caco-2 and HT-29 cellular uptake of astaxanthin and lycopene in o/w emulsions prepared by different emulsifiers, namely Tween 20, sucrose laurate, whey protein isolate (BiPro®), and hydrolyzed whey protein isolate (BioZate®), both containing Tween 20). The presence of whey proteins in the encapsulation carrier system resulted in a significant increase of astaxanthin and lycopene cellular uptake (ca. 7- and 11-

fold, respectively). The enhanced carotenoid uptake in the presence of β-lactoglobulin was attributed to its binding capacity through hydrophobic bonds as well as to its molecular conformation, facilitating carotenoid emulsification. Takaishi et al., (2012) examined the Caco-2 cellular uptake of β-cryptoxanthin in Satsuma mandarin fruit pulp, juice and LLCs (5% mandarin extract, 5% triglycerides, 76% glycerol, 9% polyglyceryl fatty acid esters and 5% water). Emulsified β-cryptoxanthin was incorporated 5-times higher than mandarin juice, whereas its esterified : free form ratio decreased by approx. 23% compared to mandarin juice. This was explained by the preferential absorption of free β-cryptoxanthin and conversion of the esterified to free form during Caco-2 cellular uptake.

Soluble dietary fiber as a main or co-encapsulation component may compromise carotenoid absorption. Caco-2 cell uptake of β-carotene/lutein from artificial micelles declined in the presence of high viscosity sodium alginate (60% and 51% for β-carotene and lutein respectively), apple (66%, 58%) and citrus (78%, 72%) pectins (Yonekura and Nagao (2009). In high viscosity aqueous environments, cellular uptake is constrained by slow diffusion.

Anarjan et al., (2011a) investigated the cellular uptake (HT-29) of astaxanthin loaded in nanodispersions prepared with different organic-phases, e.g. DCM, acetone and their mixtures. Astaxanthin cellular uptake was maximized with a blend of 31% DCM and 69% acetone. Factors such as particle mean size, PDI and concentration of 9-*cis* and 13-*cis* isomers significantly influenced astaxanthin uptake, which was higher from fine particulates, exerting a high level of polydispersity and low *trans*- to *cis*- isomerization.

Encapsulation of carotenoids via molecular inclusion has been associated with reasonable cellular uptake. Pfitzner et al. (2000) investigated several carotenoids in methyl-CD based vehicles, using human skin fibroblasts. Beta-carotene within methyl-β-CD complexes had a 40% higher cellular uptake compared to free carotenoids in organic solvent, suggesting good bioavailability due to the high solubility and continuous release of the bioactive. However, GI

digestion was not studied. Lancrajan et al. (2001), assessing the impact of liposomes vs. ICs based on β-CDs on the incorporation of β-carotene, lutein and canthaxanthin into natural membrane fractions (mitochondrial, microsomal and plasma) found that carotenoid lipophilicity and membrane characteristics (cholesterol to phospholipid ratio) affected absorption into pig liver cells and retinal epithelial cells. While β-CD-lutein complexes exerted highest plasma membrane uptake of ICs vs. liposomes (explained by less competition with cholesterol), β-carotene showed similar availability for both liposome and β-CD ICs. Fernández-García et al. (2010) studied the uptake of β-carotene, lutein and lycopene encapsulated in ICs with β-CD by brush border membrane vesicles (BBMVs). Carotenes exerted a higher uptake compared to lutein both in their free (carotenoids + Tween 20) and microencapsulated (ICs with β-CD) form. Due to the increased β-CD concentration in the donor solution, a higher amount of carotenoids was available for later assimilation due to the shift of the dissociation equilibrium toward the free forms of the inclusion complexes. Interestingly, substances hindering receptors involved in carotenoid absorption such as lipoproteins did not adversely impact the assimilation process. On the contrary, for both carotenes and xanthophylls, a synergistic interaction between β-CDs and lipoproteins occurred, promoting the dissociation and release of carotenoids and therefore, their bioavailabilty. Similarly, (Madhavi and Kagan, 2008) disclosed that Caco-2 cellular uptake of lutein in CD inclusion complexes was remarkably influenced by parameters such as the drying method (spray vs. freeze drying), the CD type (α-, β-, γ- or hydroxypropyl-β-CD) as well as the excipient to dissolute the dry CD-carotenoid complexes. Lutein uptake into Caco-2 cells was higher for the freeze dried α- and γ-CD complexes dispersed in vegetable oil containing lecithin. These formulations exerted also a better bioavailability in-vivo, affirmed in a clinical trial with six healthy subjects following a modest carotenoid rich diet. Plasma

lutein levels after 24h were 60% higher in subjects who ingested the lutein-CD-vegetable oil-lecithin formulation and only 20% when consuming the lutein-vegetable oil.

Liposomal encapsulation of astaxanthin and uptake into hepatic (Hep3B, HepG2) cells was studied by Peng and others (2010). Liposomes were fabricated using phosphatidyl- choline (4% w/v/DMSO), cholesterol (1% w/v/DMSO), PLGA (1% w/v/DMSO) added into a binary blend of chloroform/methanol, sonicated, solvent evaporated and vacuum dried. The obtained liposomes (due to their fine particle size and excellent dispersion) displayed a higher cellular uptake than free astaxanthin (in THF or DMSO). After 18h of incubation, astaxanthin concentration was 2.4, 1.2 and 0.7 μg/dish (Hep3B), and 2.0, 0.9 and 0.5 μg/dish (HepG2) for liposomes, DMSO and THF. Antioxidant activity of intracellular enzymes such as superoxide dismutase, catalase and glutathione S-transferase was also 3-fold higher in cells treated with the liposome encapsulated astaxanthin compared to its free form.

Encapsulation of β-carotene in protein (sodium caseinate, whey protein isolate or soybean protein isolate) nano-assemblies via emulsification-solvent displacement slightly enhanced its antioxidant properties (in Caco-2 cells) compared to its crystalline form. The authors deduced that the larger particles' surface area and enhanced β-carotene solubility in the aqueous medium due to its amorphous state and the intermolecular hydrophobic interactions with proteins were associated with this observation. Similarly, carotenoid bioavailability from β-CN microparticles was improved due to their distribution over a very large surface area of the caseins, promoting digestion, mixed micelles incorporation and absorption. In addition, the open tertiary molecular structure of the re-assembled β-CN facilitates access to proteases and the sustained release of carotenoids (Danino et al., 2011). Finally, in-vitro studies have suggested that the cytoxicity of lipophilic bioactive compounds, e.g. curcumin can be reduced upon their enclosure in β-CN assembled matrices contrarily to other encapsulating agents

such as chemically modified starches or CDs (Esmaili et al., 2011; Pan et al., 2013), though results may depend on cell lines and resulting uptake and metabolism capabilities.

In vivo studies related to bioavailability of encapsulated carotenoids

Though in vitro studies can provide insights into the colloidal, physicochemical and structural principles governing the bioavailability of nutrients, in most cases (due to the complexity of the human digestive system), in vivo studies with animals or humans are necessary to obtain a more reliable perception of the processes of digestion and absorption. With respect to carotenoids, only few studies have been conducted using encapsulated forms, in contrast to studies with raw or processed plant food matrices, e.g. fruits and vegetables. With respect to animal studies, one of the main parameters to be considered is the cleavage efficacy of provitamin A carotenoids (Biehler and Bohn, 2010; Borel, 2012), with ferrets, preruminant calves, and gerbils being regarded as best models to study carotenoid absorption.

Faisal et al., (2013) created a self-emulsifying drug delivery system (SEDDS) comprised of 40% LCT (olive oil), 20% surfactant (Cremophor RH), and co-surfactant (Tween 85), a solid dispersion (SD) based on 100% gelucire, as well as a lipid based solid dispersion (LBSD) for oral lycopene administration. Following dosing to fasted pigs, lycopene encapsulated in SEDDS was not quantifiable, while for SD, after an initial lag phase, lycopene was detected in the plasma of pigs, reaching a maximum concentration (5.5 ng/mL) at 10h. Comparing the bioavailability of micellar amorphous and LBSD encapsulated lycopene, a 2.4-fold increase in plasma was detected compared to the more delayed peaking of the micellar form (t_{peak} = 10.8h). It was deduced that the formulation solubililzation and the ability of the excipients to stimulate lymphatic carotenoid transport were bioavailability determinants.

Absorption of lutein encapsulated in self-emulsifying suspensions (SEPS) by rats and beagle dogs was studied by Shanmugam et al., (2011). A lutein suspension (25 mg, 40% in sunflower oil) was emulsified in a blend of MCT phospholipids (0, 250, 500 mg), Tween 80

(75 mg), glycerol (30 mg), α-tocopherol (10 mg) and propylene glycol caprylate (50 mg). The emulsions were filled into hard gelatine capsules. Lutein emulsified by phospholipids increased plasma concentrations 4-11 fold compared to those without phospholipids or the commercial suspension (CF) of (non-emulsified) lutein. Similarly, relative bioavailability of the emulsion systems containing 250 and 500 mg of MCT phospholipids was 180 and 470% higher compared to that of LLCs without phospholipids. The ability of phospholipids to facilitate the transition of lutein from the lipid phase to mixed micelles and therefore its enterocyte uptake can explain these observations. In the same study, lutein amounts in eye tissues of rats fed with SEPS delivered lutein was 16 and 4 times higher compared to placebo (vegetable oil without lutein) and CF. High amounts of phospholipids may promote HDL formation, known lutein transfer vehicles to extrahepatic tissues including the eye.

Taha and others (2007) studied the vitamin A bioavailability enhancing capacity of a SEDDS system composed of vitamin A acetate and palmitate, emulsified into a lipid phase containing soybean oil, polyoxyl 35 castor oil, MC mono- and di-glycerides, either filled into HPMC hard capsules or mixed with microcrystalline cellulose and compressed into tablets. Vitamin A bioavailability of SEDDS in form of capsules and tablets was 207 and 144% higher than that of soybean oil-vitamin A suspensions. The authors deduced that dissolution is the rate-limiting step in absorption and bioavailability of vitamin A and therefore the presence of surfactant/co-surfactant facilitates the incorporation of retinol into mixed micelles.

Arunkumar et al., (2013) determined the appearance of lutein in plasma, eye or liver tissue of mice fed with either a chitosan encapsulated form or with micellar lutein prepared by organic solvent dissolution and evaporation. The encapsulation of lutein in chitosan carriers was associated with higher lutein appearance in plasma (55%), liver (54%) and eye tissues (63%). It was assumed that the fine particle size and the prolonged plasma half-life of the nanocapsules increased lutein tissue levels.

CONCLUSIONS

A variety of strategies have been sought to encapsulate labile nutritious and health beneficial compounds such as carotenoids to reap their full benefits as bioactive functional food ingredients, including spray drying, freeze-drying, liposome formation, coacervation, extrusion, or via inclusion complexes. Strategies to enhance carotenoid stability against detrimental environmental conditions during processing and/or storage such as light, temperature, oxygen, and undesired reactions with other food compounds such as reactive oxygen species or metal ions have focused on micro-and nanoencapsulation, including particles generated by LLCs, SLNs, NLCs, SCF or self-assembly. Promising materials to generate emulsions include proteins (e.g. caseins), Tween, maltodextrins or other polysaccharides such as pectins, and inclusion complexes such as by CDs could constitute promising candidates. Many strategies have highlighted the technological feasibility to produce carotenoid encapsulated materials that are sufficiently stable, with a high loading capacity, and being of sufficiently small size to be integratable into a variety of products without causing detrimental effects on quality attributes such as sensorial ones.

Another, less studied aspect is the potential positive effect that encapsulated carotenoids could pose on bioavailability. Especially emulsions with particles of small size and being digestible GI, such as gelatins, have shown promising results in terms of bioaccessibility and in part in bioavailability, by facilitating matrix release, enhancing micelle formation and promoting rapid transfer to the enterocytes, allowing for targeted GI delivery. Future studies on food grade formulations will have to enhance the body of evidence that such formulations will not only be beneficial with respect to product stability, but can also enhance the bioactivity of these promising compounds via improving aspects of bioavailabilty.

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FIGURE CAPTIONS:

FIG. 1: Cause and effect diagram illustrating parameters associated with processing conditions and matrix compositional and structural characteristics influencing the chemical stability of carotenoids

FIG. 2: Visualization of the microstructural aspects of micronized (A: crystalline structure), and microencapsulated (B: amorphous structure) β-carotene implementing supercritical fluid technology. In both cases, the nanoprecipitates were obtained using a pressure of 120 bar, and a temperature of 313K; flow rates of organic solution and antisolvent were 1 mL min^{-1} and 40 mL min^{-1} and β-carotene content was set at 4 mg mL^{-1} (image adopted by Priamo et al., (2010) and reproduced with the permission of Elsevier).

FIG. 3: Formation of carotenoids loaded into casein self-assemblies.

FIG. 4: Mechanistic overview of the formation of Maillard reaction derived protein-carbohydrate conjugates for encapsulating carotenoids.

FIG. 5: Illustration of different strategies for the formation of solid liquid nanoparticles (SLNs) or nanostructured lipid carriers (NLCs). In general, the NLCs exert a better stability against polymorphic transitions occurring during processing and storage and therefore enhanced controlled release properties and chemical stability of carotenoids.

FIG. 6: Schematic representation of the fabrication of carotenoid loaded nanoparticles using the solvent displacement method (modified from Ribeiro et al., (2008)).

FIG 7: Illustration of the production of encapsulated carotenoids into nanoparticles using the emulsification – solvent evaporation method.

FIG. 8: Schematic overview of structural and colloidal transformations of encapsulation carrier systems taking place during oral, gastric and duodenal/small intestine phase (illustration of the human digestive system reproduced from Wikipedia as a free- licensed material).

TABLE 1: Effects of wall material, drying and storage conditions on the encapsulation efficiency, particle mean size and morphology, and carotenoid stability in dried microparticulates.

Ingredient	Drying method and conditions	Carotenoid type	Encapsulation efficiency (%)	Surface coverage (%)	Particle mean size (µm)	Morphology of microcapsules	Retention rate throughout storage (%, days, conditions)	Reference
Native tapioca starch	Spray drying 170°C, 95°C	β-carotene	68.4	104	106-250	Spherical, inner hollow	nd	(Loksuwan, 2007)
Acid modified tapioca starch	Spray drying 170°C, 95°C	β-carotene	82.2	19.6	75-150	Spherical, inner hollow	nd	(Loksuwan, 2007)
Chemically modified maize starch (Capsul®)	Spray drying 180°C, 98°C, 0.6L/h	lycopene	21-30	nd	nd	Spherical, inner hollow, surface dents	78-83% (73 d,10°C, air) 58-67% (73 d, 25°C, air)	(Rocha et al., 2012)
Maltodextrin DE24	Spray drying 170°C, 95°C	β-carotene	46.7	73	106-250	Spherical, inner hollow	nd	(Loksuwan, 2007)
Maltodextrin DE25	Spray drying 170°C, 95°C	β-carotene	nd	nd	nd	nd	nd	(Loksuwan 2007
Gum arabic – sucrose (4:1)	Spray drying 170°C, 113°C	lycopene	94-96	nd	nd	Sperical, surface dents, absence of cracks	nd	(Nunes and Mercadante, 2007)
Gum arabic – sucrose (19:1) Maltodextrin DE20 Maltodextrin DE20 – sucrose (4:1) Maltodextrin DE20 – Tween 80 (499:1)	Spray drying	nd	nd	nd	nd	nd	nd	(Barbosa et al., 2005)
Sodium caseinate – lactose (1:1)	Spray drying 180°C, 83°C, 433kg/h	astaxanthin	86.1	0.04	6-100	Spherical, surface dents	74, 60 and 47% (26 d, 5,25 and 40°C, air)	(Pu et al., 2011)
Whey protein isolate – maltodextrin DE20	Spray drying 160-180°C, 70-80°C, 2L/h	astaxanthin	63-93	nd	2-25	Spherical, surface dents	45-54% (10 d, 33%RH, 45°C, air or nitrogen)	(Shen and Quek, 2014)
Soybean polysaccharide (SSPS)	Spray drying 170°C, 95°C, 0.3L/h	canthaxanthin	78.5 – 90.1	10.3-14.3	0.02-0.2	Spherical, smooth surface	44-57% (16 wks, 25°C, air) 63-75% (16 wks, 25°C, dark)	(Hojjati et al., 2011)
Gelatine – sucrose (3:7)	Spray drying	lycopene	12-82 (6-90% extract purity)	nd	2-15	Spherical, bee-net like inner structure	80% (28 days, 0°C, air)	(Shu et al., 2006)
Gellan gum-maltodextrin DE10 Arabic gum – maltodextrin DE10 Mesquite gum – maltodextrin DE10	Spray drying 170°C , 80°C, 1.2L/h	nd	nd	nd	nd	Spherical, inner hollow, surface dents, porous shell	nd	(Rodríguez-Huezo et al., 2004)
Arabic gum-pequi fruit	Spray drying	total	8-52	nd	1-500	Spherical, smooth	nd	(Santana et

pulp	152-200°C, 108-149°C,	carotenoids				surface		al., 2013)
Microalgal paste	Spray drying 180°C, 70°C	fucoxanthin, diadinoxanthin	75	nd	nd	nd	negligible (35 d, -20, 5 and 20°C vacuum, dark)	(Ryckebosch et al., 2011)
Tomato pulp	Spray drying	lycopene	nd	nd	nd	Nd	nd	(Goula et al. 2012)
Paprika oleoresin	Spray drying 160-200°C, 110°C	total carotenoids	nd	nd	nd	nd	(vacuum, P)	(Rascón et al., 2011)
Casein plus SSPS (0.3% w/v)	Spray drying	bixin	90	nd	0.09	nd	nd	Zhang and Zhong (2013)
Maltodextrin DE25	Freeze drying -35°C	β-carotene	92%	35%	80	nd	50% (105 d, 25°C, 32% RH, air, light exposure)	(Desobry et al., 1997)
Pullulan	Freeze drying -18°C,	saffron carotenoids	nd	nd	nd	nd	nd	(Selim et al. 2000)
Egg yolk solids	Freeze drying -	lutein zeaxanthin canthaxanthin	nd	nd	nd	nd	nd	(Wenzel et al. 2010)
Gelatine – trehalose	Freeze drying -110°C, 4×10^{-1} mbar	β-carotene	nd	nd	nd	nd	(156 d, 25°C, 11,44 and 75% RH, air)	(Elizalde et al., 2002)
β-cyclodextrin carotenoid to β-CD molar ratios 1:1 and 1:4	Freeze drying -18°C	lycopene	50% for 1:4 no complex formation for 1:1	nd	nd	Irregular structures of varying size Presence of small CD fragments	nd	(Nunes and Mercadante, 2007)

Nd: not determined; CD: cyclodextrin, DE: dextrose equivalent, RH: relative humidity.

TABLE 2: Overview of methods for encapsulating carotenoids by supercritical fluid techniques, including parameters characterizing the performance of the employed methods.

Method	Carotenoid and coating type	Pressure (MPa)	Temperature (˚C)	CO_2 flow rate (mL/min)	Solution flow rate (mL/min)	Core to coating ratio	Encapsulation efficiency (EE) or variation coefficient (CV), (%)	Particle mean size (µm)	Particle morphology	Reference
RESS	Rutin, PEG	20	40	nd	nd	1:2, 1:10	EE: 22.6 for 1:2; EE: 44.2 for 1:10	42.9	Amorphous, flaky agglomerates	(Santos and Meireles, 2013)
SAS	Bixin extract, PEG	10	40	0.6, 1.5	1	1:2, 1:10	EE: 62	33	Flaky agglomerates	(Santos and Meireles, 2013)
SAS	β-carotene, lutein, PEG	8 for β-carotene 8-10 for lutein	15	2.5, 3.5	1.7 kg/h	1:10, 3:40 for β-carotene 1:4-1:12 for lutein	nd	~1 for β-carotene ~50 for lutein	Spherical PEG particles deposited on β-carotene Spherical and amorphous PEG particles	(Martín et al., 2007)
SEDS	β-carotene, PHBV	8-12	40	20-40	1	1:10-2:5	VC: 21-31	0.28 – 0.57	Quasi spherical interconnected particles	(Franceschi et al., 2008)
SEDS	β-carotene, PHBV	8-20	40	40	1	1:15-1:1	EE: 1.8-55.5	<1.5	Quasi spherical interconnected particles	(Priamo et al., 2010)
SEDS	astaxanthin, PHBV	8, 10	35	20	1	1:4-1:2	EE: 21.5-48.3	0.13-0.27	nd	(Machado Jr et al., 2014)
SEDS	lutein, zein	10-15	32-45		0.5 to 1	1:24-1:12	nd	nd	nd	(Hu et al., 2012)
SEDS	lycopene, β-CD	10-14	40-50	15-25	0.25-0.75		nd	0.04-0.15	Spherical particles of varied level of agglomeration	(Nerome et al., 2013)
SEDS	β-carotene, PHBV	8	40	39	1	1:40-2:5	EE: 16.7-34.7	nd	Quasi spherical particles of varied porosity; evidence of β-carotene deposited on surface at low PHBV amounts	(Franceschi et al., 2010)
PGSS	β-carotene, soybean lecithin	8.1-10.2	102-132	n/a	GPR: 21-32	1:169 - 1:221	EE: 29-59	13.4-448	Collided spherical particles exerting high agglomeration	(de Paz et al., 2012a)
PGSS	β-carotene, poly-ε-caprolactones (CAPA) (MW 4000 & 10000)	11, 15	50, 70	n/a	n/a	1:8-1:4	EE:	111-652	Flat or sphere like particles attached and agglomerated by long polymer filaments (MW 10000); no agglomeration for CAPA MW 4000	(de Paz et al., 2012b)

SFEE	β-Carotene, lycopene, OSA-modified starch	7-13	80	n/a	2.5-5.5	n/a	EE: 34-89	345-366	Spherical shaped particles of low porosity (spray dried material obtained from the SF treated nanoemulsions)	(Santos et al., 2012)
SFEE	Astaxanthin, Pluronic F127	8-12	35-45	1-5	1-5	1:5-5:6	EE:19.7-93.1	nd	Flat, flaky particles (co-precipitates); Spherical particles with evidence of very low agglomeration (spray dried material)	(Mezzomo et al., 2012)

CD: cyclodextrin; GAS: gas anti-solvent; GPR: gas to product ratio, n/a: not applied; MW: molecular weight; nd: not determined; OSA: octenyl succinic acid; PEG: polyethylenglycol; PGSS: particles from gas-saturated solutions; PHBV: copolymer of 3-hydroxybutanoic acid and 3-hydroxypentanoic acid; RESS: rapid expansion of supercritical fluids; SAS: supercritical anti-solvent; SEDS: solution enhanced dispersion by supercritical fluids; SF: supercritical fluid; SFEE: supercritical extraction from an emulsion.

TABLE 3: Compositional, physicochemical and bioavailability aspects of carotenoids encapsulated in liquid lipid carriers (LLCs).

Carotenoid, concen-tration	Lipid phase composition	Surfactants	Lipid droplet size (μm)	Carotenoid retention during storage	Bioavailability aspects				Reference
					Digestion conditions at 37°C	Extent of lipolysis	Bioaccessible fraction	Cellular uptake	
β-carotene (0.15% w/w)	MCT (10% w/w)	Decaglycerol monolaurate (ML750); WPI; SSPS (4% w/w)	0.2 (ML750), 0.58 (WPI) and 0.75 (SSPS)	nd	Gastric (pH 2), duodenal and small intestine fluids	nd	64 (ML750), 69 (WPI) and 48% (SSPS)	nd	(Liu et al., 2012)
β-carotene (0.05% w/w)	Soybean oil (10% w/w)	SPI (1.5% w/w)	0.31	nd	Gastric (SFGs, pH 2, 1h,), duodenal (PC, COL, PL, BS, pH 6.5, 2h)	80%	32% (without PL) 80% (PL presence)	nd	(Nik et al., 2011)
β-carotene (0.05% w/w)	Soybean oil (10% w/w)	SPI (1.5% w/w)	0.23	nd	Gastric (SFGs, pH 2, 1h,), duodenal (BS,BS-COL, BS-PL, BS-PL-COL, BS-PL-COL-LPL, pH 6.5, 2h)	51 to 79%	34 to 80%	nd	(Malaki Nik et al., 2011)
β-carotene (0.01% w/w)	Corn oil (10% w/w)	Sodium caseinate (2% w/w)	0.12-37 (fine emulsions); 10.7 (coarse emulsion)	nd	Gastric (SFGs, pH 2, 1h,), duodenal (pH 7, 2h)	66 to 82% (fine emulsions); 60% (coarse emulsion)	50 to 73% for fine emulsions	nd	(Yi et al., 2014)
β-carotene (0.01% w/w)	Soybean oil (10% w/w)	WPI (0.5% w/w)	0.18	nd	Gastric (SFGs, pH 2, 1h,), duodenal (BS,BS-COL, BS-PL, BS-PL-COL, BS-PL-COL-LPL, pH 6.5, 2h)	47 (BS), 44 (BS-PL & BS-PL-COL), 64 (BS-COL), 72% (BS-PL-COL-LPL)	31 (BS & BS-PL), 29 (BS-PL-COL), 42 (BS-COL), 49% (BS-PL-COL-LPL)	nd	(Nik et al., 2010)
β-carotene (n/a)	Olive oil (5% w/w)	Citrus pectin of varying esterification level e.g. 14, 66 & 99% (1 & 2% w/w)	8.1 and 4.5 for CP99; 5.8 and 4.8 for CP66 3.6; 4.5 for CP14	nd	Gastric (pH 1.3, 2h) and small intestine fluids (urea, pancreatin, lipase, bile extract, pH 7, 2h)	nd	57 to 62% for CP99; 56 to 60% for CP66; 33 to 37% for CP14	nd	(Verrijssen et al., 2014)
β-carotene (0.025% w/w)	Soybean oil (10% w/w)	ML750 (0.9% w/w)	0.68- 1.8 for HP (10, 50, 140 MPa) homogenized systems; 18.3 (coarse emulsion)	nd	Gastric (pH 2, 1h), small intestine fluids (pancreatin, bile extract, pH 7.5, 2h)	nd	7.2 to 10% for sub-micron LLCs 5.6 to 6.2% for coarse emulsions	nd	(Wang et al., 2012a)
β-carotene	Orange oil, 4% oil phase,	1.5% Tween 20	0.2 (initial); 0.8 (intestine)	nd	Oral (pH 6.8, 10min), gastric (pH 2.5, 2h), small intestine fluids (pancreatin, bile extract,	nd	66% for LCT and 2% for MCT	nd	Qian et al., 2012a)

					pH 7, 2h				
β-carotene (0.025% w/w)	Orange oil (10% w/w)	β-lactoglobulin (2% w/w)	0.078	0, 7, 44 and 57% (5, 20, 37 and 55˚C, 14d, dark, ambient air)	nd	nd	nd	nd	(Qian et al., 2012b)
β-carotene (0.075% w/w)	MCT (5% w/w)	WPI (0.5% w/w)	0.2 to 0.5	21, 12, 40, 44 % (pH 3, 4, 6, & 7, 55˚C, 7d, dark, ambient air). 82% (pH 7, 200μm α-tocopherol) (55˚C, 7d, dark, ambient air)	nd	nd	nd	nd	(Xu et al., 2013b)
β-carotene (0.02% w/w)	Corn oil + MCT at 0, 25, 50, 75, 100%; 1 & 4% w/w	Tween 20 (1.5% w/w)	0.15 to 0.42	nd	Small intestine fluids (pancreatin, bile extract, pH 7.0, 2h)	85 to 124% at 1% w/w LLCs 32 to 88% at 4% w/w LLCs	14 to 88% at 1% w/w 20 to 91% at 4% w/w	nd	(Salvia-Trujillo et al., 2013a)
β-carotene (0.1% w/w)	Linseed oil, rapeseed oil refined palm olein (5% w/w)	Arabic gum (12% w/w)	0.34, 0.36 & 0.43 for LO, RO and RPO	73, 82 & 84% for LO, RO and RPO (2˚C 12 weeks)	nd	nd	nd	nd	(Szterk et al., 2013)
β-carotene (0.02% w/w)	Corn oil blended with lemon oil at varying ratios (0, 33, 50, 66 & 100%)	Sucrose monopalmitate (0.4% w/w) Lysolecithin (0.1% w/w)	0.13-0.24 lemon oil presence led to higher lipid hydrodynamic volumes	nd	Oral (mucin, pH 6.8, 10min), gastric (SFG, pH 2.5, 2h), intestinal phase (pancreatin, bile, pH 7, 2h)	0-0.57 mmol/L FFA released Pure lemon oil did not induce lipolysis	5-75%; pure lemon oil systems exerted the lowest β-carotene bioaccessibility	nd	(Rao et al., 2013)
β-carotene (0.09% w/w)	MCT (30% w/w)	Sodium octenyl succinate starches (HI-CAP 100, CAPSUL, CAPSUL-TA) (21% w/w)	ca. 0.14 for CAPSUL and CAPSUL-TA ca. 0.16 for HI-CAP 100	64-78% for HI-CAP 100; 51-76% for CAPSUL; 56-77% for CAPSUL-TA; LLCs stored at 4 or 25˚C under dark or light and with or without N_2	Gastric (SGF, pH 2.0, 1h) and small intestine phase (pancreatin, bile extract, pH 7.5, 2h)	120% for HI-CAP 100 & CAPSUL 117% for CAPSUL-TA	19% (HI-CAP 100), 25% (CAPSUL-TA), 35% (CAPSUL) 3% in pure MCT	nd	(Liang et al., 2013a)
lycopene astaxanthin (n/a)	Fractionated palm oil (10% w/w)	Tween 20, sucrose laurate in their binary blends with WPI or hydrolyzed WPI (n/a)	0.2 to 0.35 for lycopene, 0.15 to 0.35 for astaxanthin containing LLCs	100% in WPI systems with/without α-tocopherol; 68-75% (lycopene); 74-84% (astaxanthin), systems with/without α-tocopherol	n/a	nd	nd	HT-29: 50-270 lycopene) 130-270 (astaxan-thin) pmol/g protein. Lower in Tween 20, WPI systems	(Ribeiro et al., 2006)

SFGs: simulated gastric fluids, PC: pancreatin, COL: colipase, BS: bile salts, PL: phospholipase, LPL: lyso-phospholipase, nd: not determined, n/a: not available, MCT: medium-chain triglycerides; WPI: whey protein isolate; FFA: free fatty acids; SPI: soybean protein isolate; PC: phosphatidylcholine; BS: bile salts; LO: linseed oil; RO: rapeseed oil; RPO: refined palm olein; SSPS: soy soluble polysaccharides; SFG: small fat globules.

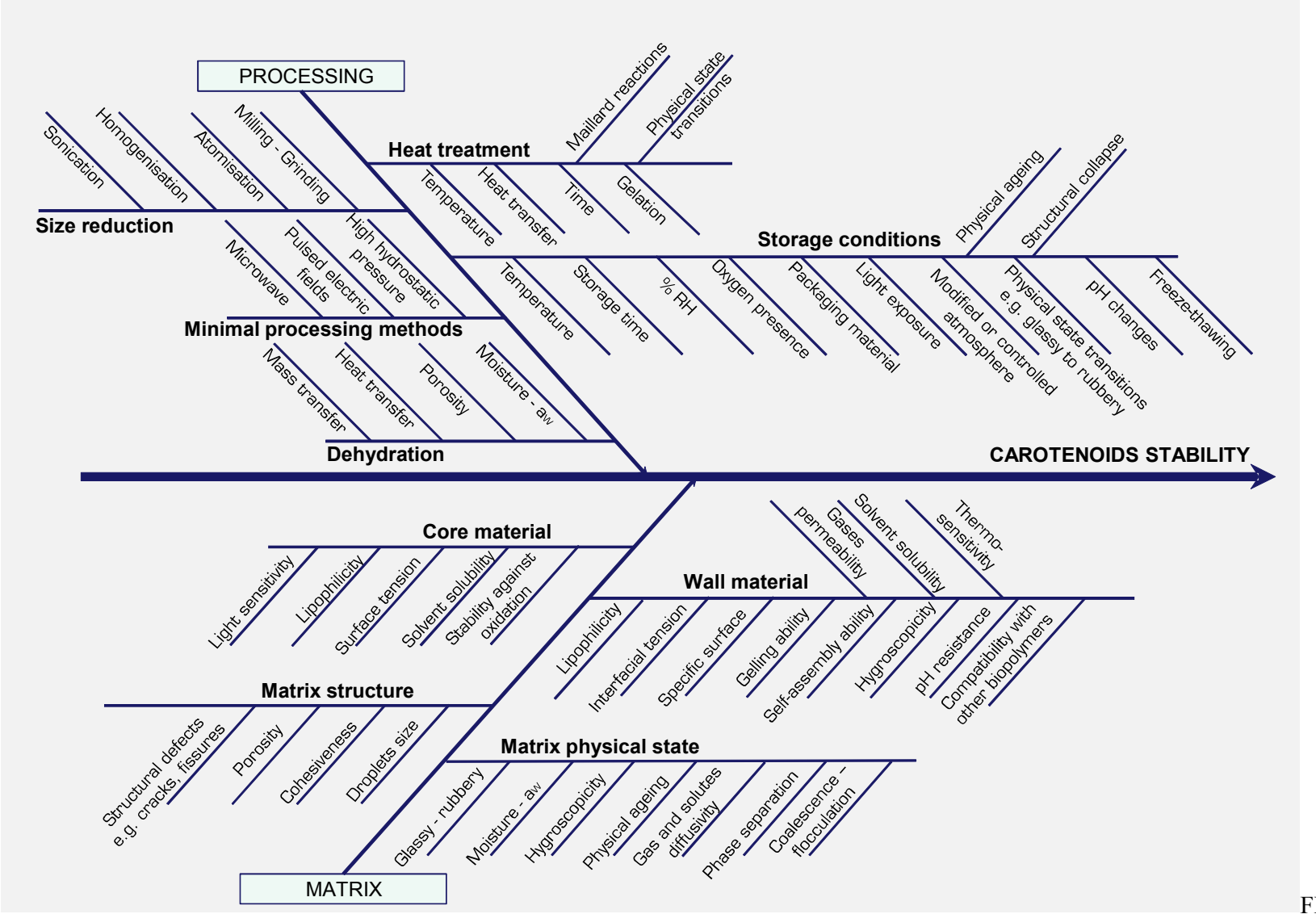
PROCESSING
Sonication
Homogenisation
Atomisation
Milling - Grinding
Size reduction
Microwave
Pulsed electric fields
High hydrostatic pressure
Minimal processing methods
Heat treatment
Maillard reactions
Physical state transitions
Temperature
Heat transfer
Time
Gelation
Storage conditions
Physical ageing
Structural collapse
Temperature
Storage time
% RH
Oxygen presence
Packaging material
Light exposure
Modified or controlled atmosphere
Physical state transitions e.g. glassy to rubbery
pH changes
Freeze-thawing
Mass transfer
Heat transfer
Porosity
Moisture - aw
Dehydration
CAROTENOIDS STABILITY
Core material
Light sensitivity
Lipophilicity
Surface tension
Solvent solubility
Stability against oxidation
Wall material
Gases permeability
Solvent solubility
Thermo-sensitivity
Lipophilicity
Interfacial tension
Specific surface
Gelling ability
Self-assembly ability
Hygroscopicity
pH resistance
Compatibility with other biopolymers
Matrix structure
Structural defects e.g. cracks, fissures
Porosity
Cohesiveness
Droplets size
Matrix physical state
Glassy - rubbery
Moisture - aw
Hygroscopicity
Physical ageing
Gas and solutes diffusivity
Phase separation
Coalescence - flocculation
MATRIX

FIGURE 1

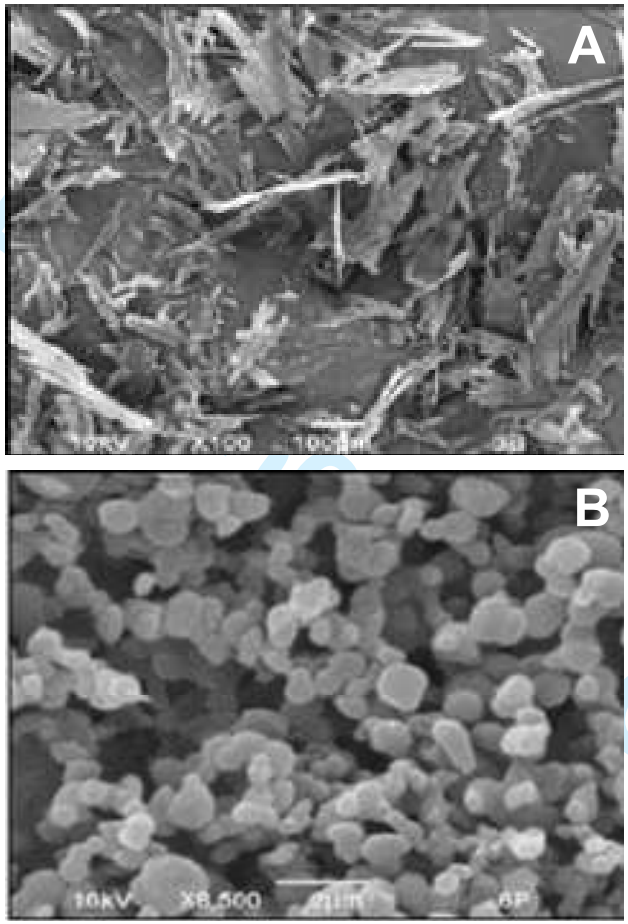

FIGURE 2

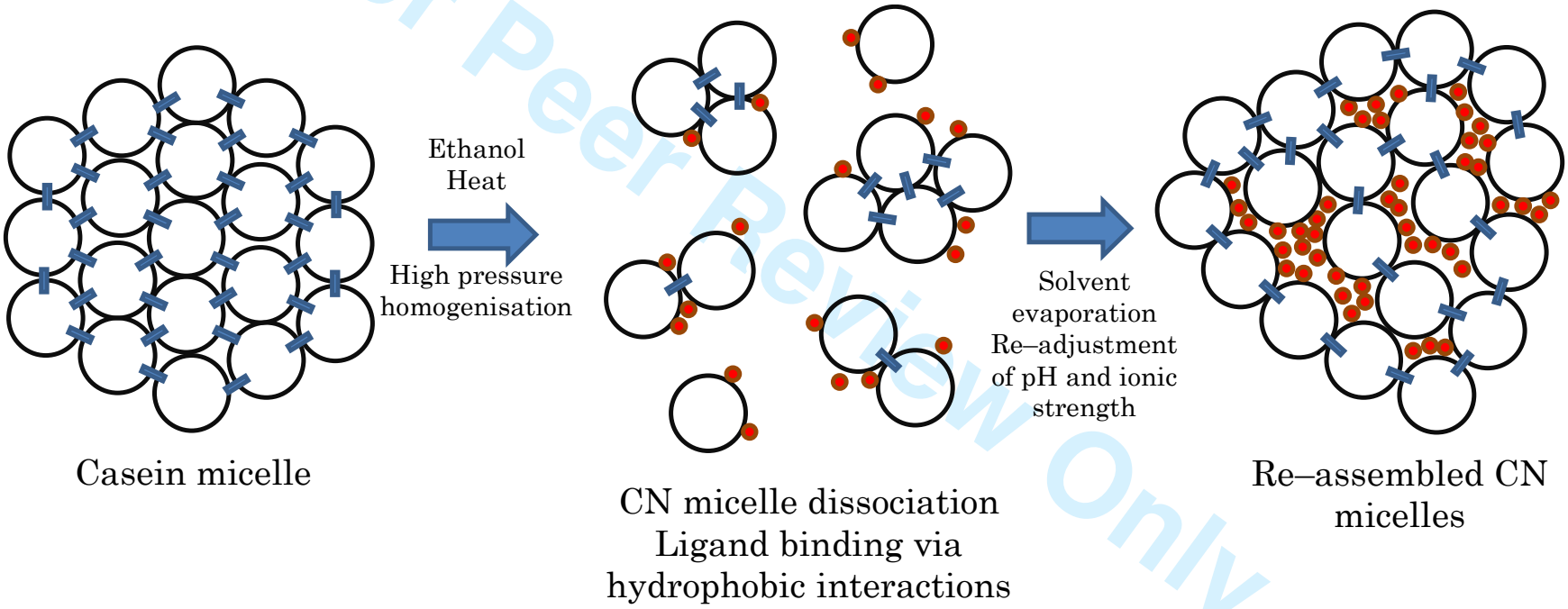

FIGURE 3

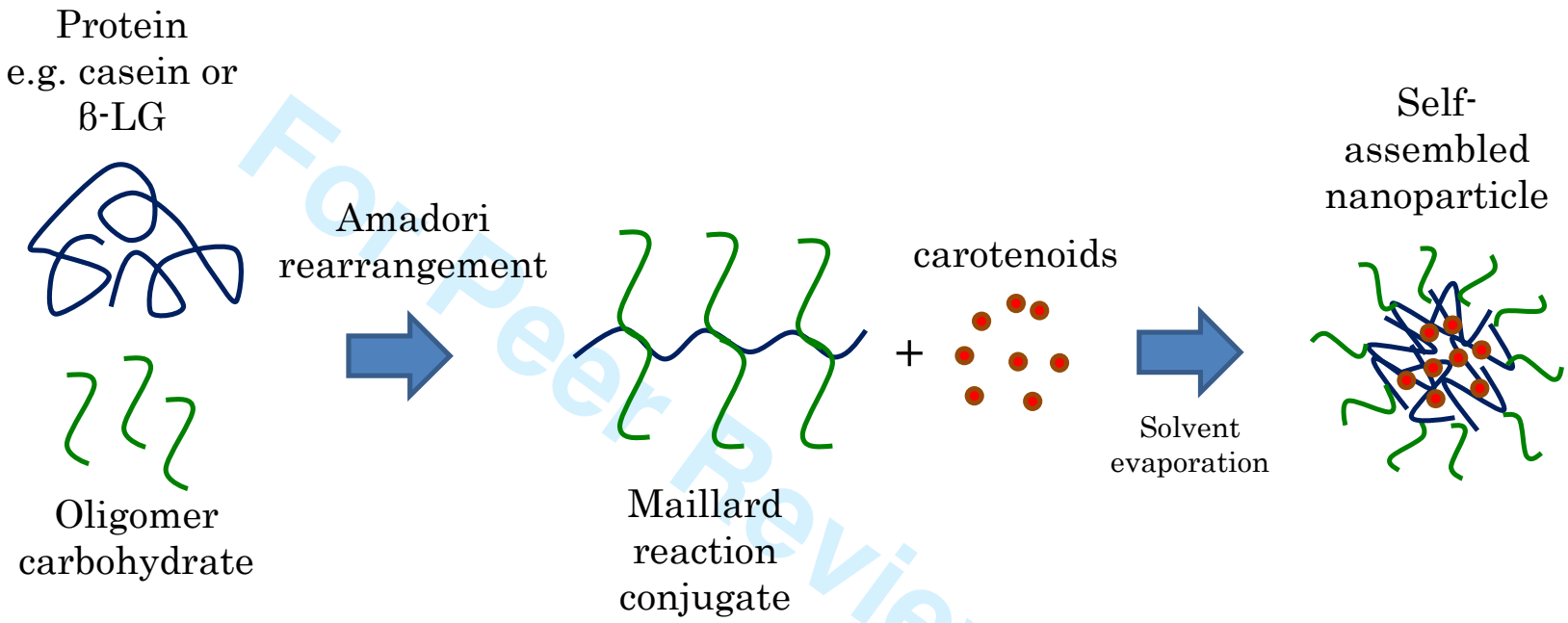

FIGURE 4

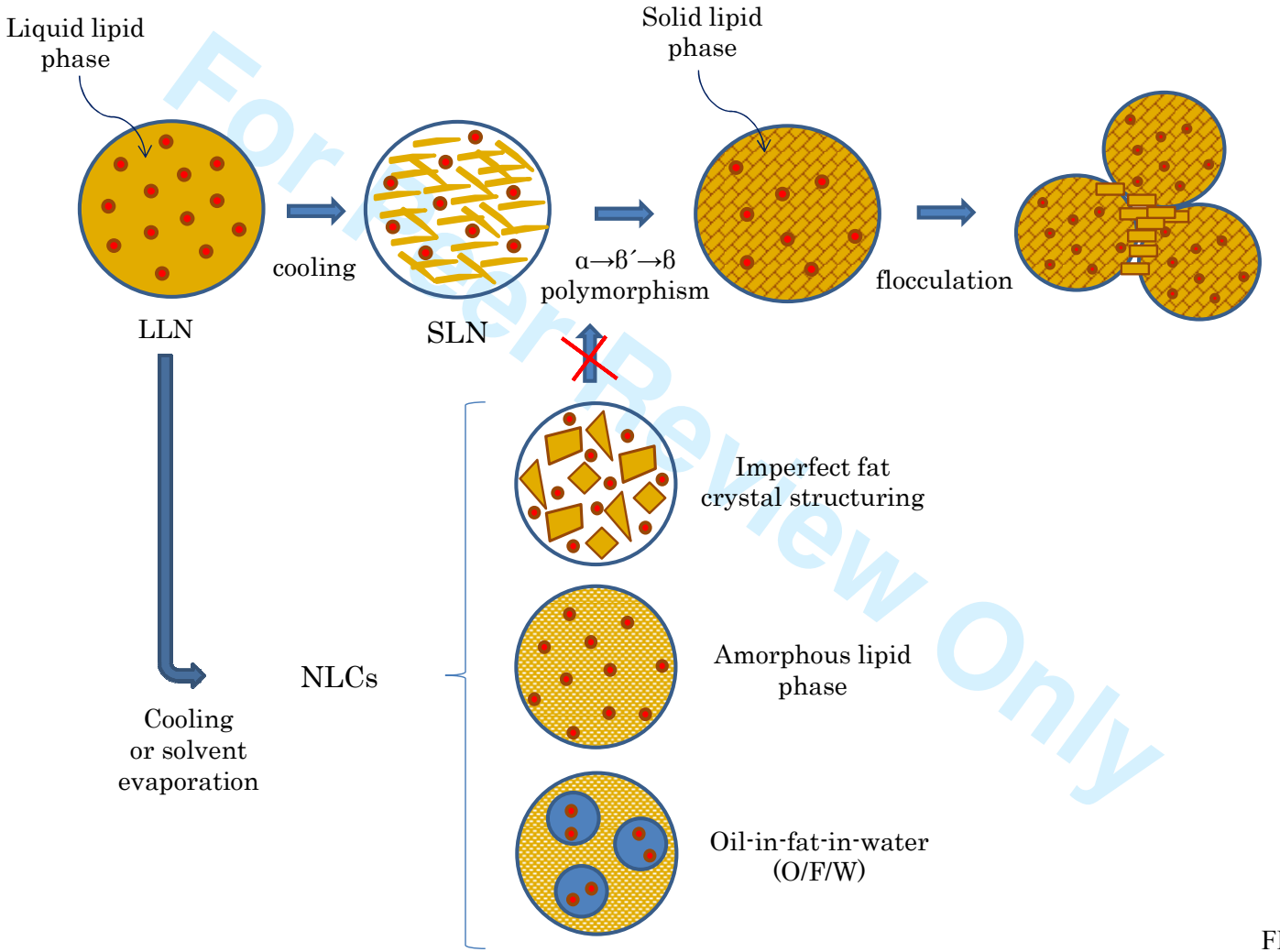

FIGURE 5

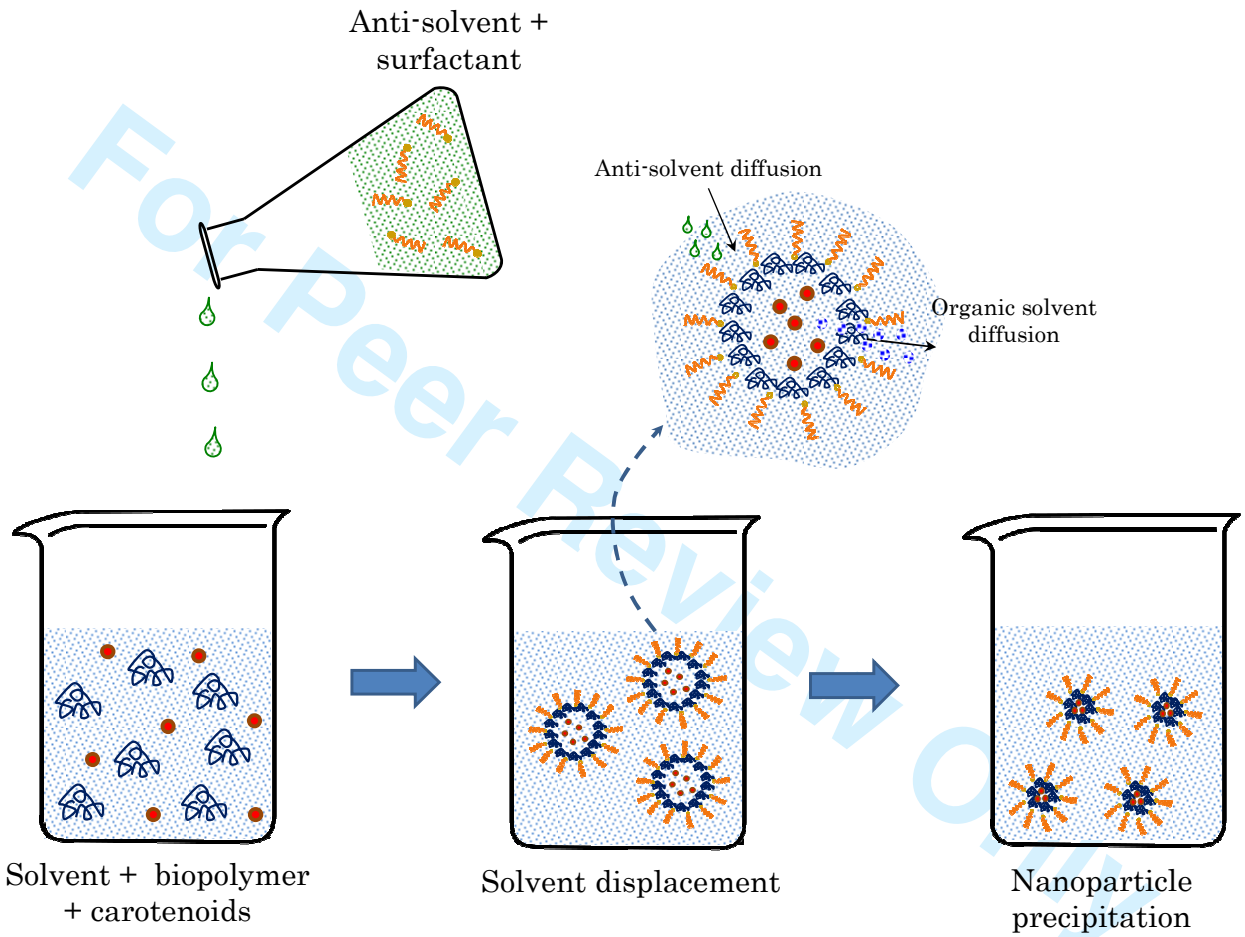

FIGURE 6

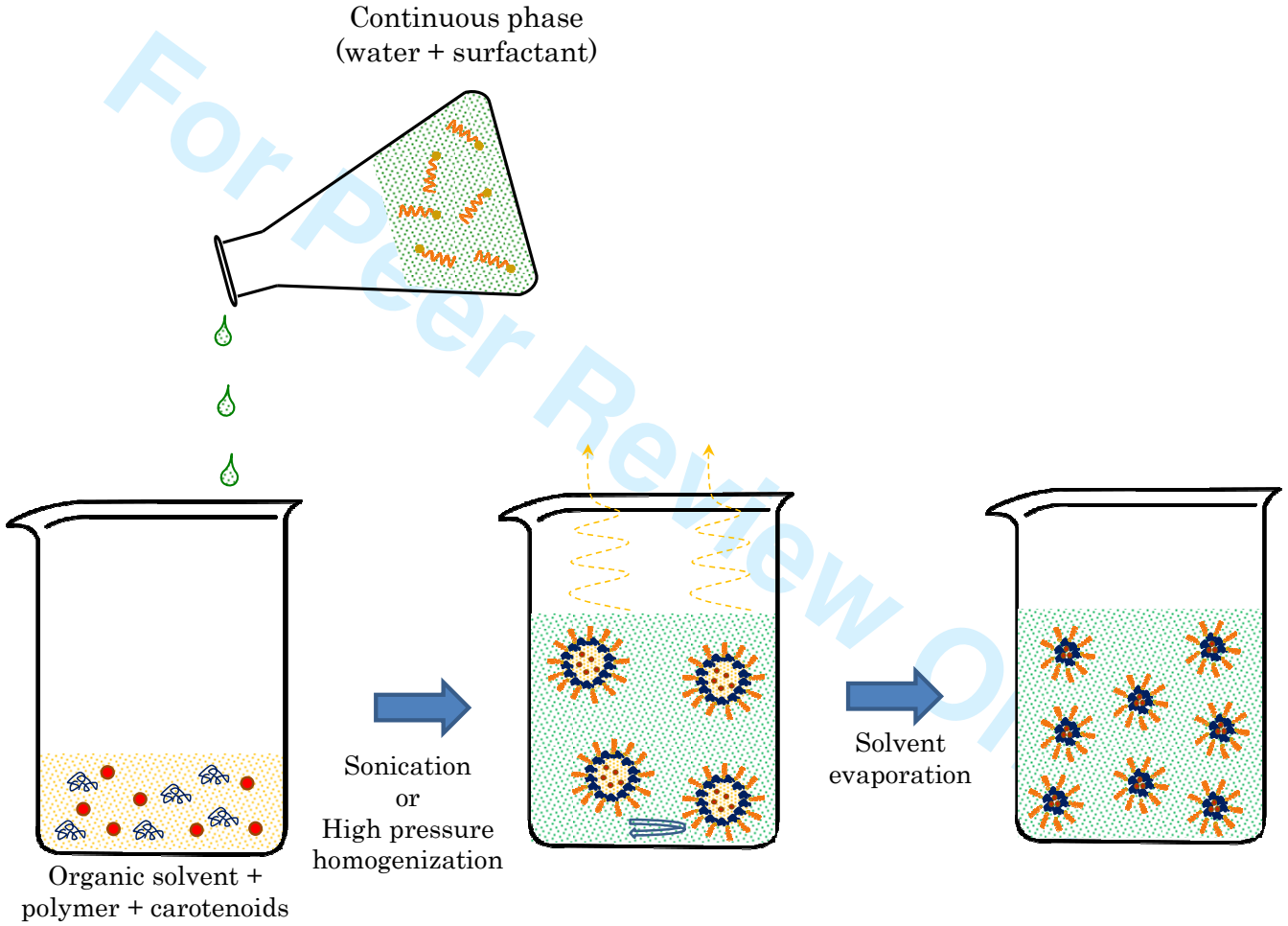

FIGURE 7

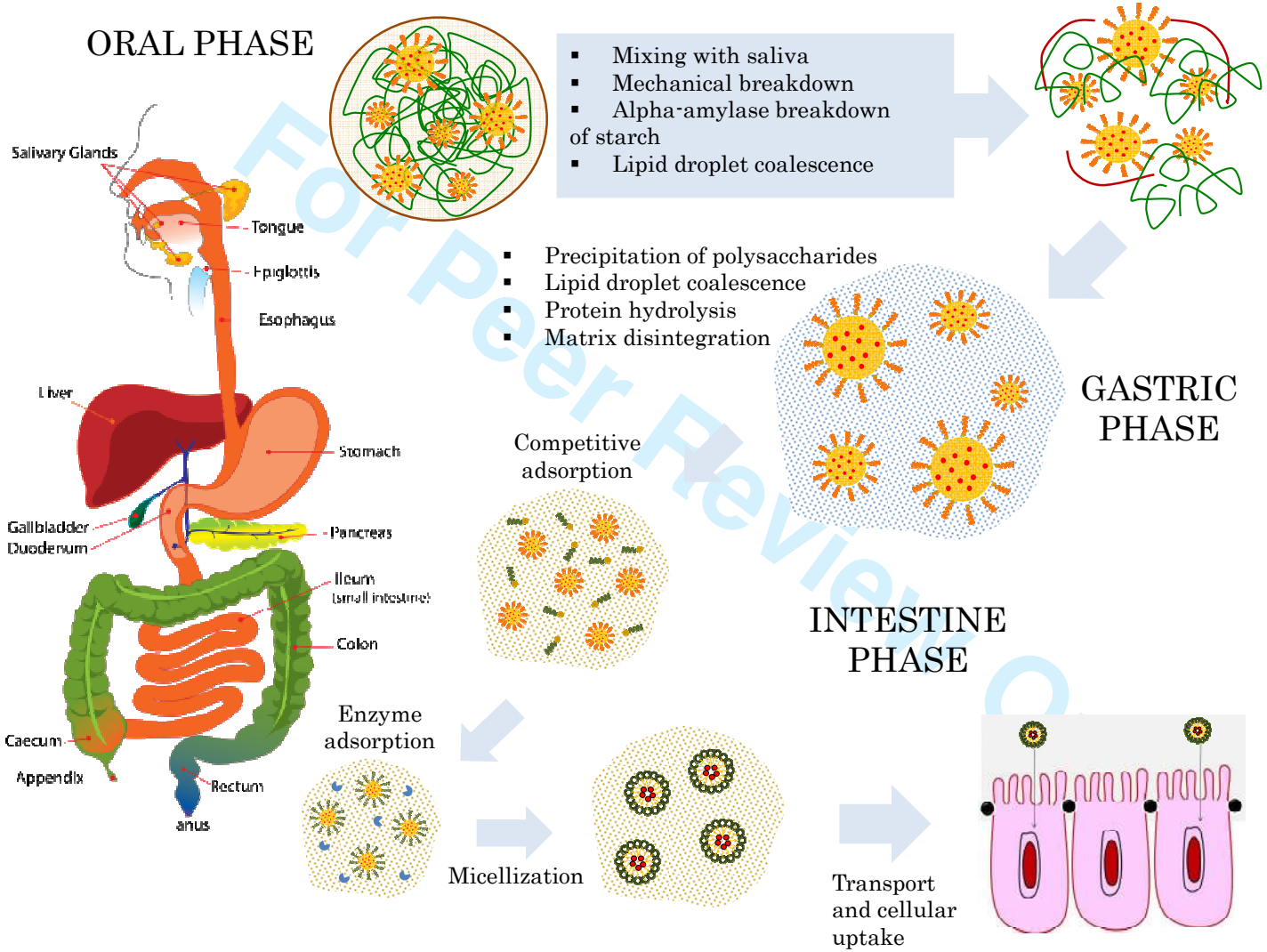
ORAL PHASE
Mixing with saliva
Mechanical breakdown
Alpha-amylase breakdown of starch
Lipid droplet coalescence
Salivary Glands
Tongue
Epiglottis
Esophagus
Liver
Stomach
Gallbladder
Duodenum
Pancreas
Ileum
(small intestine)
Colon
Caecum
Appendix
Rectum
anus
Precipitation of polysaccharides
Lipid droplet coalescence
Protein hydrolysis
Matrix disintegration
GASTRIC PHASE
Competitive adsorption
INTESTINE PHASE
Enzyme adsorption
Micellization
Transport and cellular uptake

FIGURE 8