**Encapsulation of fish oil into hollow solid lipid micro- and nanoparticles using carbon
dioxide**

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Abstract

Fish oil was encapsulated in hollow solid lipid micro- and nanoparticles formed from fully hydrogenated soybean oil (FHSO) using a novel green method based on atomization of supercritical carbon dioxide (SC-CO₂)-expanded lipid. The highest fish oil loading efficiency (97.5%, w/w) was achieved at 50%, w/w, initial fish oil concentration. All particles were spherical and in the dry free flowing form; however, less smooth surface with wrinkles was observed when the initial fish oil concentration was increased up to 50%. With increasing initial fish oil concentration, melting point of the fish oil-loaded particles shifted to lower onset melting temperatures, and major polymorphic form transformed from α to β and/or β' . Oxidative stability of the loaded fish oil was significantly increased compared to the free fish oil ($p < 0.05$). This innovative method forms free-flowing powder products that are easy-to-use solid fish oil formulation, which makes the handling and storage feasible and convenient.

Keywords: Fish oil; Microparticle; Nanoparticle; Oxidation; Encapsulation; Supercritical carbon dioxide.

1. Introduction

Growing demand for health and wellness improving foods has led the food industry to develop foods containing bioactives. Among bioactives, omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), received a lot of interest due to their unique health benefits (Chow, 2000). EPA and DHA are special fatty acids with health benefits such as prevention of cardiovascular disease (Din, Newby, & Flapan, 2004), some types of cancer (Larsson, Kumlin, Ingelman-Sundberg, & Wolk, 2004), and inflammatory neurodegenerative diseases such as rheumatoid arthritis, diabetes, and Alzheimer's (Yashodhara, Umakanth, Pappachan, Bhat, Kamath, & Choo, 2009; Valenzuela, Bascuñan, Valenzuela, & Chamorro, 2009). The role of DHA in neural and visual development in infants is well known (Arab-Tehrany, Jacquot, Gaiani, Imran, Desobry, & Linder, 2012; Lands, 2005). In order to get the benefits of EPA and DHA, the World Health Organization (WHO) and North Atlantic Treaty Organization (NATO) recommend consumption of 0.3-0.5 g of EPA and DHA per day (Kris-Etherton, Harris, & Appel, 2002); however, the average consumption is below the recommended amounts. Therefore, there is a growing interest in food industry to develop foods supplemented with EPA and DHA.

Fish oil is one of the main sources of EPA and DHA; however, incorporation of fish oil into foods is challenging because of the low solubility of fish oil in aqueous food systems and its high susceptibility to oxidation (Aghbashlo, Mobli, Madadlou, & Rafiee, 2013; Aghbashlo, Mobli, Madadlou, & Rafiee, 2012). Encapsulation has been used as the most common approach to protect fish oil from oxidation. Fish oil has been encapsulated in various matrices such as gelatin (Cho, Shim, & Park, 2003), β -cyclodextrin (Choi, Ruktanonchai, Min, Chun, & Soottitantawat, 2010), starch (Tan, Xu, Li, Liu, Song, & Wang, 2009), protein (Wang, Tian, &

Chen, 2011), and chitosan (Chatterjee & Judeh, 2015) using different techniques such as freeze drying (Chung, Sanguansri, & Augustin, 2011), inclusion complexation (Choi et al., 2010), emulsification (Nasrin & Anal, 2014), electrospinning (García-Moreno et al., 2016) and spray-drying (Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2016). Among those methods, spray drying has been used widely for encapsulation but it requires an aqueous feed solution which limits the wall material and the active compound to water-soluble compounds. Therefore, typically, an oil-in-water emulsion is prepared before spray drying for the encapsulation of fish oil, and emulsion formation step prior to spray drying requires high energy methods such as high pressure homogenization and microfluidization.

In this study, a single step “green” process is used to develop spherical fish oil-loaded hollow solid lipid micro- and nanoparticles. In this process, the lipid phase which contains a solid lipid as wall material and a liquid fish oil as core material is expanded by dissolving supercritical carbon dioxide (SC-CO₂) in the lipid phase, and then the SC-CO₂-expanded lipid phase is atomized through a nozzle (Yang & Ciftci, 2016a; Yang & Ciftci, 2016b). Fully hydrogenated soybean oil was selected as a model solid lipid because it is inexpensive, does not contain trans fatty acids, and stable against oxidation due to high stearic acid content (~86%) (Yang et al., 2016a). Unlike spray drying, this process does not require formation of emulsion prior to particle formation or any organic solvent to dissolve the lipid phase. Hollow solid lipid micro- and nanoparticles are alternatives to solid lipid nanoparticles, which are one of the latest generation of drug delivery systems. Nevertheless, solid lipid nanoparticles have some disadvantages such as expelling the loaded bioactive, limited loading capacity, consumption of high energy or use of solvents, and scale up issues (Mukherjee, Ray, & Thakur, 2009). Hollow solid lipid particles offer enhanced protection and biocompatibility as solid lipid nanoparticles

but with minimized or no bioactive expelling and improved loading capacity. Moreover, the reported process in this study generates dry free-flowing hollow solid lipid micro- and nanoparticles in powder form. The success of this process depends on better understanding of the behavior of each active ingredient during processing, because the behavior (e.g., melting, solubility, phase behavior) of each lipid and active ingredient differs at differing processing conditions. This is a promising novel green method for formation of dry micro- and nanoparticles; however, its potential for developing bioactive-carriers has not been fully explored yet. Utilization of inexpensive food grade lipids to develop fish oil-carrier systems using this process has the potential to develop novel fish oil-carrier systems in a simple and clean manner. To the best of our knowledge, there is no report on the encapsulation of fish oil in hollow solid lipid particles using the SC-CO₂-based particle formation process.

Therefore, the main objective of this study was to develop novel fish oil carrier hollow solid lipid micro- and nanoparticles using a simple and green process based on SC-CO₂. The specific objectives were to: i) load fish oil into the hollow solid lipid micro- and nanoparticles made of fully hydrogenated soybean oil (FHSO) via atomization of SC-CO₂-expanded lipid, ii) characterize the particles in terms of fish oil loading efficiency, particle morphology, particle size and size distribution, melting properties, and polymorphism, and iii) determine the oxidative stability of the fish oil loaded in the hollow solid lipid micro- and nanoparticles.

2. Materials and methods

2.1. Materials

FHSO was kindly provided by ConAgra Foods Inc. (Omaha, NE, USA). Liquid CO₂ (99.99% purity) was purchased from Matheson (Lincoln, NE, USA). Fish oil from menhaden

and cumene hydroperoxide (80% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). p-anisidine (99% purity) was purchased from ACROS Organics (Mumbai, India). All other chemical reagents were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA).

2.2. Production of the fish oil-loaded hollow solid lipid micro- and nanoparticles using CO₂

Fish oil-loaded hollow solid lipid micro- and nanoparticles were formed using a home-made SC-CO₂ particle formation system. Details of the particle formation system and its operation was reported previously (Yang et al., 2016a). Temperature of the expansion vessel was set to 57 °C, which is the lowest temperature where FHSO is in the liquid state at the expansion pressure of 120 bar and above (Yang et al., 2016a). Temperature of the depressurization valve and nozzle was set at 90 °C to prevent freezing due to Joule-Thomson effect during atomization. First, molten FHSO (80 °C) was manually injected into the expansion vessel through the sampling port, and then predetermined amounts of fish oil was manually injected into the vessel through the sampling port to obtain initial fish oil mass concentrations of 10%, 20%, 30%, 40% and 50% in the lipid mixture. Then, the sampling port was closed and the expansion vessel was pressurized with CO₂ to 200 bar using the syringe pump. Pressurized CO₂ in the supercritical state and the liquid lipid mixture was mixed for 1 h using the magnetic drive to obtain a SC-CO₂-expanded lipid mixture. Then, the magnetic drive was turned off and waited for 10 min to stabilize the SC-CO₂-expanded lipid mixture. The pressure of the syringe pump was set at 10 bar above the pressure of the expansion vessel, and CO₂ inlet valve was opened, then depressurization valve was immediately opened and the SC-CO₂-expanded lipid mixture was atomized through a 50 µm diameter nozzle. Solid lipid particles were collected in the sample

collection vessel. Particle formation conditions (200 bar expansion pressure, 57 °C, and 50 µm nozzle diameter) were based on previously optimized conditions that yielded the smallest spherical ($d_{50\%} = 278$ nm) hollow solid lipid micro- and nanoparticles (Yang et al., 2016a).

2.3. Determination of the fish oil loading efficiency

Approximately, 7 mg of fish oil-loaded solid lipid particles was methylated according to Belayneh, Wehling, Cahoon, & Ciftci (2015). The fatty acid methyl esters (FAME) were analyzed by a gas chromatograph (GC) (7890A GC systems, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame ionization detector. FAME were separated on an Agilent HP-INNOWAX capillary column (30 m × 0.25 mm × 0.25 µm). Oven temperature was held at 90 °C for 1 min, then heated to 235 °C at 10 °C/min, and then kept at 235 °C for 5 min. Hydrogen was used as a carrier gas. EPA and DHA were identified by comparing retention times with those of authentic standards. Loading efficiency was calculated according to Eq. (1):

$$LE (\%) = \frac{\text{Experimental loading capacity}}{\text{Theoretical loading capacity}} \times 100 \quad (1)$$

where, experimental loading capacity is the total mass of EPA+DHA in the solid lipid particles, and theoretical loading capacity is the total mass of EPA+DHA in the initial physical fish oil and FHSO mixture. Physical mixtures of the fish oil and FHSO was prepared by mixing fish oil and FHSO to obtain the initial fish oil concentrations used in the particle formation experiments.

2.4. Determination of the particle size and size distribution

Particle size and size distribution of the fish oil-loaded solid lipid particles were measured using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments

Ltd., Worcestershire, UK). Approximately 30 mg of samples were dispersed in 25 mL distilled water containing 0.4% (w/w) of polyoxyethylene sorbitan monooleate (Tween 80). Before each analysis, suspensions were sonicated in an ultrasonic water bath (3510 R-MTH, Branson Ultrasonics Corporation, Danbury, CT, USA) for 30 min. The refractive index (RI) was set as 1.46 with distilled water (RI = 1.33) as dispersant. The obscuration value was in the range of 5-7% for each analysis.

2.5. Scanning Electron Microscopy analysis

Samples were spread onto a sample mount with a double-sided carbon tape and sputter coated in argon atmosphere with chromium using HiPace 80 (Pfeiffer Vacuum, Germany). Then, morphology of the fish oil-loaded solid lipid particles was analyzed using a Field Emission-Scanning Electron Microscope (FE-SEM) (S4700, Hitachi High-Technologies Corporation, Japan).

2.6. Transmission Electron Microscopy analysis

Samples were dispersed in water and then filtered using a 450 nm pore size syringe filter. Few droplets of the filtrate were deposited on a carbon-coated copper grid (200 mesh), negatively stained with 2% uranyl acetate solution, and waited to dry before examination. Then, samples were analyzed with a Transmission Electron Microscope (TEM) (H7500, Hitachi High-Technologies Corporation, Japan).

2.7. Confocal fluorescence microscopy analysis

A confocal fluorescence microscope (A1, Nikon Instruments Inc., Japan) was used to

investigate the loading of fish oil in the solid lipid micro- and nanoparticles. Prior to analysis, fish oil-loaded solid lipid particles (3-5 mg) were mixed with 40 μ L Nile Red solution (0.125%, w/v) with propane-1,2-diol. Then, the mixture was vortexed for 10 seconds, and then stained for 40 min before image collection. The confocal images were recorded by a fluorescence microscope (90i, Nikon Instruments Inc., Japan) using z series scanning at each 1 μ m distance. Analyses were conducted at the excitation wavelengths of 561 nm and 640 nm and the emission wavelength of 600 nm for red fluorophores.

2.8. Determination of the melting profile of the fish oil-loaded hollow solid lipid micro- and nanoparticles

Melting profile of the samples were determined using a differential scanning calorimeter (DSC) (Diamond DSC, Perkin Elmer, Waltham, Massachusetts, USA). Samples (5-7 mg) were placed in an aluminum DSC pan and hermetically sealed. An identical empty sealed pan was used as the reference pan. The pans were placed in the DSC and stabilized at 25 $^{\circ}$ C for 1 min. Then, the samples were heated from 25 $^{\circ}$ C to 100 $^{\circ}$ C at 5 $^{\circ}$ C/min to obtain the melting peak.

2.9. Determination of the polymorphism of the fish oil-loaded hollow solid lipid micro- and nanoparticles

Polymorphism of the samples was determined using a PANalytical Empyrean Diffractometer (XRD) unit (Empyrean, PANalytical, Westborough, MA, USA) according to Yang et al. (2016a) by running the powder samples from 2 to 50 degrees under continuous scan at a step size of 0.026 with 2θ min^{-1} .

2.10. Oxidative stability of the loaded fish oil in hollow solid lipid micro- and nanoparticles

Oxidative stability of the fish oil loaded in the solid lipid particles was studied using a modified Schaal oven test (Rossell, 1986). Fish oil-loaded hollow solid lipid particles, as well as the free fish oil and empty hollow solid lipid particles, were placed in an open-lid container and stored at 40 °C in an oven. Samples were withdrawn everyday over three weeks of storage and analyzed for peroxide value to determine the primary oxidation products, and anisidine value for secondary oxidation products as described below.

2.10.1. Peroxide value

Peroxide value (PV) was determined according to the colorimetric method described by Bae and Lee (2008) with some modifications. Depending on the concentration of hydroperoxides, the fish oil-loaded particles and free fish oil (1-30 mg) was diluted with chloroform/methanol (7:3, v/v) so that the final absorbance of the reacted sample remained below 1.0 cm⁻¹. The mixture (50 µL) was then mixed with 8 mL chloroform/methanol (7:3, v/v) mixture in a glass tube, followed by the addition of 50 µL of ammonium thiocyanate solution and 50 µL of ferrous chloride solution. The final mixture was then vortexed and incubated for 20 min in a dark cabinet at ambient temperature. After incubation, the absorbance of the sample was measured with a UV/Vis spectrophotometer (Evolution 201, Thermo Fisher Scientific, Shanghai, P.R. China) at 505 nm. The concentration of the hydroperoxides was determined based on a standard curve of cumene hydroperoxide (0-25 µM). PV was expressed as milliequivalents (meq) hydroperoxide per kg of fish oil.

2.10.2. Anisidine value

Anisidine value (AV) was determined according to AOCS Official Method Cd 18-90 with some modifications. Depending on the concentration of the secondary oxidation products, samples (1-30 mg) were weighed into a glass test tube and dissolved with 8 mL iso-octane. The iso-octane was used as the reference (blank) solution. Absorbance of the mixture (A_1) was measured at 350 nm using a UV/Vis spectrophotometer (Evolution 201, Thermo Fisher Scientific, Shanghai, P.R. China). An aliquot of mixture or blank (2.5 mL) was then transferred to each of two test tubes, and 0.5 mL of p-anisidine solution (0.25%, w/v, solution in glacial acetic acid) was added to each test tube. The test tubes were then capped, vortexed, and allowed to react for 10 min in a dark cabinet at ambient temperature. The absorbance (A_2) was measured at 350 nm against the iso-octane blank containing p-anisidine. AV was calculated by the following formula:

$$AV = 25 \times (1.2A_2 - A_1) / \text{sample weight (g)} \quad (2)$$

2.11. Statistical analysis

Data are presented as mean $\pm$ standard deviation based on triplicate experiments and analyses. The data were analyzed by ANOVA using SAS (version 9.3) software package (SAS Institute Inc., NC, USA) at 95% confidence interval. Differences among the means were compared using Tukey's multiple range test.

3. Results and discussion

3.1. Particle morphology

SEM images of the fish oil-loaded hollow solid lipid micro- and nanoparticles are shown in Figure 1a-e, and Figure 1f presents the TEM images of the solid lipid nanoparticles that were separated from the microparticles after filtering the water dispersions of the particles with 50% initial fish oil concentration. Particles generated at all fish oil concentrations were spherical and in the form of free-flowing powder. However, the surface morphology of the particles depended on the initial fish oil concentration. Empty particles had a smooth surface, whereas presence of fish oil resulted in formation of a wrinkled surface, and increasing the initial fish oil concentration increased the wrinkles on the surface. When the SC-CO₂-expanded lipid mixture is atomized at the nozzle upon depressurization, a liquid droplet of fish oil, FHSO, and SC-CO₂ mixture was formed and then this droplet turned into a liquid lipid bubble due to expansion of the SC-CO₂ at atmospheric pressure. Depending on the pressure in the expansion vessel, force exerted on the liquid lipid bubble from inside during expansion varied; higher forces exerted at higher expansion pressures. At the same time, temperature of the atomized particles decreased suddenly due to Joule-Thomson effect (Yang et al., 2016a). Upon sudden cooling, liquid FHSO bubble solidified and loaded with the liquid fish oil.

No apparent cracks or breakages were observed on the particles. An intact particle morphology is important for better protection of the fish oil which oxidizes in the presence of oxygen. This process generated more spherical particles compared to spray drying which typically generates indented surfaces due to moisture loss during drying followed by cooling (Tonon, Brabet, & Hubinger, 2008). In this SC-CO₂-based atomization process, absence of water in the lipid mixture and the absence of drying resulted in the formation of true spherical particles. Solubility of the SC-CO₂ in the lipid and solidification rate of the liquid lipid bubble to solid lipid sphere after atomization were also important factors affecting the particle morphology. It

was found that SC-CO₂ has higher solubility in the lower melting point fats than the higher melting point ones which results in higher volumetric expansion (Ciftci & Temelli, 2014; Jenab & Temelli, 2012). Previously, it was reported that mass fraction of CO₂ in canola oil and fully hydrogenated canola oil blend (30% canola oil) at 70 °C was around 0.16 at 200 bar, whereas the same solubility was obtained at 80 bar at the same temperature for canola oil (Jenab et al., 2012). When the fish oil concentration in the lipid mixture was increased, more SC-CO₂ was dissolved in the lipid mixture and therefore the lipid droplet formed during atomization contained more SC-CO₂. Presence of more SC-CO₂ in the liquid lipid droplet resulted in more force exerted in the liquid lipid bubble. When the liquid lipid bubble was formed, particle solidified spontaneously due to sudden cooling. When the cooling rate was fast, lipid bubble did not have enough time to create irregular surface due to CO₂ expansion, but at slower cooling rates there was more time for non-smooth surface formation.

3.2. Fish oil loading efficiency

Figure 2 presents the loading efficiency of the fish oil in the hollow solid lipid micro- and nanoparticles via atomization of the SC-CO₂-expanded lipid mixture. Loading efficiency was 80.2% for the particles obtained at 10% initial fish oil concentration, whereas it significantly increased to 97.5% at 50% initial fish oil concentration ($p < 0.05$). Increasing the initial fish oil concentration from 10% to 20% increased the loading efficiency significantly from 80.2% to 93.2% ($p < 0.05$); however, there was no significant increase with a further increase in the initial fish oil concentration ($p > 0.05$). Loading efficiency in such particle formation system depends on the phase composition of the SC-CO₂-expanded lipid phase. In the expansion vessel, two phases are formed after pressurization with CO₂; bottom phase is the SC-CO₂-expanded lipid mixture

286 that contains fish oil and FHSO, and the top phase that contains mainly SC-CO₂ and small
287 amount of oil dissolved in the SC-CO₂. Particles are formed from the SC-CO₂-expanded lipid
288 mixture; therefore, there was more fish oil for loading at the increased initial fish oil
289 concentrations. Previously, it was reported that the encapsulation efficiency decreases with
290 increasing oil content for encapsulation of flaxseed oil (Tonon, Grosso, & Hubinger, 2011) and
291 fish oil (Tan, Chan, & Heng, 2005) by spray drying. In this study, loading efficiency did not take
292 the surface oil into account due to difficulty in the determination of the surface oil. Surface oil
293 determination for non-fat wall systems has been done by washing the oil on the surface of the
294 particles with a solvent such as hexane (Tan et al., 2005). In such washing, hexane extracts the
295 oil on the surface but does not extract the wall material. In this study, both the wall material and
296 the loaded material are oil which makes the “surface washing” with a solvent impossible,
297 because the solvent extracts both the surface oil and the wall material. To the best of our
298 knowledge, there is no reported study on the encapsulation of oils in solid fats using the process
299 reported in this study. In an effort to compare the amount of surface oil on the solid lipid spheres,
300 the particles were analyzed by confocal fluorescence microscopy. Confocal fluorescence
301 microscopy z-series scanning images revealed that the particles were hollow and the lipid shell
302 contained fish oil (Fig. 3). These images show the cross-section of the spherical particles. The
303 fish oil in the cavity of the particles was not visually obvious mainly due to the insufficient
304 penetration of the fluorescent dye into the cavity. Supplementary Figure 1 shows the surface
305 images of the particles without scanning the cross-section in order to see the surface oil. Fish oil
306 on the surface and in the shell increased significantly at 40% and 50% initial fish oil
307 concentration in the lipid mixture. Increased surface oil has been reported for encapsulation of
308 fish oil (Tan et al., 2005) and lemon myrtle oil (Huynh, Caffin, Dykes, & Bhandari, 2008) by

spray drying. Jafari, Assadpoor, He, and Bhandari (2008) attributed the lower encapsulation efficiency at higher oil loads to the presence of greater amount of core oil close to the drying surface which leads to shorter diffusion path length to the air-particle interface. In this SC-CO₂-based process, the particle formation mechanism, and therefore surface oil formation, is different than that of spray drying. At increased fish oil concentration, there was more fish oil in the lipid droplet interface. When the liquid lipid droplet solidified immediately due to Joule-Thomson effect, there was more fish oil on the surface of the solidified lipid particle.

3.3. Particle size and size distribution

Both empty particles and the fish oil-loaded particles presented a bimodal size distribution (Supplementary Figure 2). The mean particle size of the fish oil-loaded particles ranged from 5 to 18 μm . The mean particle size increased with initial fish oil concentration. Ten percent ($d_{10\%}$), fifty percent ($d_{50\%}$) and ninety percent ($d_{90\%}$) of the particles generated at 10% initial fish oil concentration were smaller than 0.08 μm , 6.3 μm , and 17.8 μm , respectively, whereas they were 0.07 μm , 17.7 μm , and 53.2 μm at 50% initial fish oil concentration, respectively. Based on the particles size analysis results, 10% of the particles were nanoparticles (Supplementary Figure 2). Even though particle size results presented increased particle size with increasing fish oil concentration, such an increase was not observed from SEM images. Increase in the particle size was mainly due to agglomeration of the particles due to increased surface oil. Particles were stickier at higher surface oil amounts and therefore agglomerated. Particle agglomerated with increasing fish oil concentration (Supplementary Figure 3). However, particles generated at 50% fish oil concentration were still free-flowing particles at room temperature. Previously, increase in the particle size with increasing fish oil loading was reported

for fish oil loaded β -cyclodextrin and polycaprolactone particles (Choi et al., 2010). The particle size analysis showed that $d_{10\%}$ of the particles ranged between 55.1 and 89.2 nm at varying initial fish oil concentrations, and the presence of the nanoparticles were confirmed by the TEM analysis (Fig. 1f). When the powder samples were dispersed in water and the microparticles were separated from the nanoparticles (<400 nm) (Yang et al., 2016a), a clear liquid was formed and the nanoparticles were not visible by naked eye.

3.4. Melting profile of the fish oil-loaded hollow solid lipid micro- and nanoparticles

Melting profile of the fish oil-loaded solid lipid particles is critical since one of the objectives of this study is to develop free-flowing dry powder formulations that can carry and protect fish oil. Free-flowing powder form is desirable for easy handling, storage, and consumption. Figure 4 presents the DSC melting curves of the FHSO, empty hollow solid lipid particles, and the fish oil-loaded solid lipid particles generated at varying initial fish oil concentrations. FHSO melted between 69.4 °C and 73.1 °C with a single endothermic peak at 71.7 °C. Tristearin accounted for 86.2% of the triacylglycerols in the FHSO; therefore, a relatively sharper single melting peak was observed. However, empty hollow solid lipid particles that were formed from 100% FHSO exhibited two major melting peaks at 55.5 °C and 65.9 °C and the onset temperature of the major melting peak decreased to 61.4 °C. This decrease in the major melting peak temperature was due to decreased particle size, hollow structure with a thin shell, and change in the polymorphic forms. The presence of lower melting point peak for the empty particles was attributed to the presence of nanoparticles (Yang et al., 2016a). Addition of liquid oil into a solid fat results in formation of less ordered crystals and leads to decreased melting point (Awad, Helgason, Weiss, Decker, & McClements, 2009), but interestingly, melting

point of the particles increased when the particles were loaded with 10% fish oil. A weak lower melting point peak at 53.6 °C was observed at 10% initial fish oil concentration but it disappeared above 10% initial fish oil concentration. This was attributed to the agglomeration of the nanoparticles due to increased surface oil at increased fish oil concentration. Above 10% initial fish oil concentration, the major melting peak of the solid lipid particles gradually shifted to a lower onset melting temperature with increasing initial fish oil concentration. The major melting peak of the solid lipid particles with 10% initial fish oil concentration was 69.5 °C, whereas it decreased to 65.1 °C at 50% initial fish oil concentration. When the very high and similar loading efficiencies at all initial fish oil concentrations were considered, the decrease in the melting point was not surprising. Fish oil is a liquid oil; therefore, increasing fish oil amount in the high melting point FHSO decreased the melting point of the particles.

3.5. Polymorphism of the fish oil-loaded hollow solid lipid micro- and nanoparticles

Polymorphism is another important parameter affecting the thermal properties of the solid lipid particles. Fats can crystallize in three main polymorphic forms, namely, α , β' , and β . α is the least stable polymorphic form, whereas β is the most stable polymorphic form, and β' is the metastable polymorphic form (Rousseau, Marangoni, & Jeffrey, 1998). Melting point of α is lower than that of β , due to the fact that the more stable the crystal structure, the more organized the triacylglycerol molecules packing within the lattice (Lawler & Dimick, 2008; Yang & Ciftci, 2016a). Figure 5 reveals that the initial fish oil concentrations affected the polymorphism of the fish oil-loaded solid lipid particles. The short spacings (d) of 0.46 nm, 0.37 nm, and 0.38 nm indicated that predominate polymorphic forms in the FHSO were β and β' , whereas it was α (d = 0.41 nm) for empty hollow solid lipid particles (Yang et al., 2016a). However, polymorphic form

α became less pronounced and β became more pronounced with increasing fish oil concentration. This was due to more ordered packing of the triacylglycerol molecules during solidification in the presence of fish oil due to slower solidification rate during particle formation. As mentioned before, solidification rate was lower at increased fish oil concentrations due to significantly lower melting point of fish oil compared to FHSO; therefore, lipid mixture had more time to pack, and, in turn, to form a more ordered packing during slower solidification.

Polymorphic transitions also play role in the changes in the polymorphic form during storage. Accelerated transition of α to β was reported previously for the presence of 25% fish oil within the tripalmitin lipid droplets (Awad et al., 2009). Aggregation of the particles at increased fish oil concentration also suggested that there could be polymorphic transitions in the presence of fish oil. It has been reported that extensive particle aggregation in solid lipid nanoparticles often occurs when the polymorphic form transform from α to β (Helgason, Awad, Kristbergsson, McClements, & Weiss, 2008).

3.6. Oxidative stability of the fish oil in the hollow solid lipid micro- and nanoparticles

Oxidative stability of the fish oil loaded in the hollow solid lipid micro- and nanoparticles was evaluated by following peroxide value and anisidine value over three weeks with an accelerated storage test at 40 °C (Fig. 6). No peroxide formation was observed for empty hollow solid lipid micro- and nanoparticles over three weeks. Empty particles were formed of FHSO, which consisted of 86.2% stearic acid; therefore, it is oxidatively stable. No oxidation of the FHSO shows that all peroxide formation is from fish oil. First significant peroxide formation in the free fish oil was observed on day 4 (813 meq peroxide/kg fish oil), and reached a maximum level of 1761 meq peroxide/kg fish oil on the day 10 ($p < 0.05$). A sharp increase in the peroxide

value was observed for hollow solid lipid micro- and nanoparticles with 50% initial fish oil concentration on day 6 (2658 meq peroxide/kg fish oil), whereas a similar sharp increase was observed for the particles with 40% initial fish oil concentration on day 8 (2973 meq peroxide/kg fish oil). Particles with 30% initial fish oil concentration presented a gradual oxidation starting from day 6 until reaching its maximum (3170 meq peroxide/kg fish oil) on day 13. Particles with 20% fish oil had a similar oxidation trend reaching its maximum on day 17 (2803 meq peroxide/kg fish oil). The slowest oxidation was observed for the particles with 10% fish oil where the highest peroxide value was observed on day 23 (2623 meq peroxide/kg fish oil). Peroxide value of all fish oil-loaded particles decreased after reaching their maximum peroxide values (Fig. 6a), whereas anisidine values increased with decreasing peroxide value (Fig. 6b). Peroxide value is an indicator of the formation of primary oxidation products (hydroperoxides), whereas anisidine value indicates the formation of secondary oxidation products such as aldehydes, ketones, and alcohols that are formed by degradation of the primary oxidation products. Trend in anisidine value for each sample was similar to that of peroxide value. The highest anisidine values were observed for free fish oil (1285) on day 12 and particles with 50% fish oil (1550) on day 9. The slowest anisidine formation was observed for particles with 10% fish oil on day 23 (1768), and this was the same anisidine value for the particles with 20% fish oil but on day 15. Anisidine value for the particles with 30% fish oil started to increase on day 9 and reached its maximum on day 15 (2840).

Formation of peroxides in all particles were mainly due to oxidation of the fish oil on the surface of the particles. As mentioned before, particles with 40% and 50% fish oil concentration had higher amount of surface oil compared to other concentrations; therefore, a quick oxidation was observed for those particles. Even though oxidation time for the free fish oil and the

particles with 50% fish oil was similar, the amount of peroxides formed was higher in the particles. This was attributed to the higher surface area of the fish oil on the surface of the micro- and nanoparticles. Surface fish oil formed a thin layer with a high surface area on the particles; therefore, it was easier for oxygen to diffuse into the fish oil per unit time. The fish oil-loaded particles obtained at 10%, 20% and 30% initial fish oil concentration had higher oxidative stability due to lower amount of surface oil. When oxidative stabilities are evaluated, it should be noted that an accelerated oxidation test was used in this study; therefore, real life storage times will be longer than the reported accelerated storage times. Another important point to consider, when compared with other studies, is the storage of the samples in closed or open containers. In this study samples were stored in open containers at elevated temperatures; therefore, oxidation times are shorter due to continuous exposure to oxygen as opposed to samples stored in closed containers.

3.7. Comparison with other methods

Previously, solid lipid particles containing glyceryl monostearate and silanized TiO₂ were formed using a process similar to our process, called particles from gas saturated solutions (PGSS) at 13 MPa and 72 °C to encapsulate caffeine, glutathione, and ketoprofen (García-González, Argemí, Sampaio de Sousa, Duarte, Saurina, and Domingo et al., 2010). They reported loading efficiencies of 4.2, 5.6, and 16.1% for caffeine, glutathione, and ketoprofen, respectively. Their SEM images showed that the particles had sponge-like morphology and their size was greater than 10 µm. PGSS was also used to form caffeine-carrier glyceryl monostearate particles (Sampaio de Sousa, Simplício, de Sousa, & Duarte, 2007). They generated nonspherical porous needle aggregates greater than 50 µm. Caffeine content of the particles was 140 mg/g,

which was 20% less than the initial caffeine content used for loading. In another study, PGSS was used to form solid lipid microparticles made of tristearin, phosphatidylcholine and polyethylene glycol (PEG) to carry a protein (PEGylated Robonuclease A, RNase) ((Vezzù, Borin, Bertucco, Bersani, Salmaso, & Caliceti, 2010). In their process, the micronization was promoted by air and nitrogen streams, and particles were formed at pressures between 100 and 180 bar and temperatures between 45 and 64 °C using nozzle diameters between 180 and 440 µm. Protein encapsulation efficiency was 82% for the PEGylated RNase, and RNase microparticles had an average size of 5 µm. In a recent study on PGSS process, PEG was used to encapsulate mackerel oil enriched with EPA and DHA via an enzymatic reaction (Haque & Chun, 2016). Similar to our findings, they did not find a significant change in the concentration of the encapsulated EPA and DHA compared to the initial oil. Even though the original PEG was spherical, the morphology was denatured after particle formation leading to formation of irregular shape aggregates or fibrous structures. Those results also suggested the all oil was not encapsulated. In that study, effect of process on the stability was reported but no shelf life study was conducted.

In one of the studies where fish oil was encapsulated using methods other than supercritical fluid technology, Wang et al. (2011) encapsulated fish oil in solid microcapsules made of barley protein with homogenization of pre-emulsified barley followed by microfluidization, and then spray drying at 150 °C. They reported loading efficiencies of 92.9-100.2%. Particles were spherical with nonsmooth surface, internal morphology was honeycomb-like, and the size of the particles ranged between 1 and 5 µm based on the SEM images. Barley protein particles were able to protect the fish oil up to 3-4 weeks reaching peroxide values of 45-76 meq peroxide/kg oil at 40 °C storage. Those authors also attributed the oxidation to the

surface oil. However, as opposed to our oxidative stability test, they stored their samples in closed containers, which exposed the particles to less amount of oxygen.

In another spray drying process, fish oil was encapsulated in different matrices, namely, soybean soluble polysaccharides, maltodextrin, hydroxypropyl betacyclodextrin, and modified starch using spray granulation, spray drying, and freeze drying (Anwar & Kunz, 2011). They reported that spray granulation produces “multiple encapsulations” granules which provide maximum protection to the encapsulated fish oil. They reported the highest encapsulation efficiency for spray granulation (96%), followed by spray drying (83.6%) and freeze drying (<50%). Size of the spray-dried particles were in the range of 50-150 μm , whereas it was 500-600 μm for spray granulation. Surface oil content was highest for freeze drying (11.8%), followed by spray drying (2.6%), and spray granulation (0.3%). Spray drying generated more spherical-like particles, but other methods generated more irregular-shape particles. Except for spray granulation, peroxides were formed in the fish oil encapsulated with spray drying and freeze drying in 3 days during storage at room temperature.

In another study, fish oil was encapsulated in chitosan using ultrasonic atomizer and three processing steps, namely, emulsification, ultrasonic atomization, and freeze drying (Klaypradit & Huang, 2008). Spherical particles of encapsulated powder made from the chitosan-maltodextrin and chitosan whey protein isolate mixtures had a particle size of 8.4 and 10.2, respectively, and encapsulation efficiencies of 83.5 and 79.3%, respectively.

When compared with other methods, most of them need an emulsion formation method before micronization process. However, in this study, there is no emulsion formation step; particles are directly formed from mixtures of solid lipid and fish oil. Used temperatures are also relatively milder in this study as spray drying uses higher temperatures. Moisture content in the

final product is a concern in the above mentioned methods but it is not a concern in this process as there is no water used in the process. In the above-mentioned processes, droplet size of the fish oil in the emulsion is controlled but in this study, particle size is mostly dictated by nozzle diameter and expansion pressure.

4. Conclusions

This study reports formation of fish oil-loaded hollow solid lipid micro- and nanoparticles from FHSO using a novel green process based on the atomization of the SC-CO₂-expanded lipid mixture. The highest loading efficiency of 97.5% was achieved at 50% initial fish oil concentration. The process was able to form spherical intact fish oil-loaded particles at varying initial fish oil concentrations. Thermal analysis showed that the melting point shifted to lower temperatures with increasing initial fish oil concentrations in comparison with FHSO. Major polymorphic form of the particles transformed from α to β' and/or β when the initial fish oil concentration was increased. Moreover, the fish oil-loaded spherical hollow solid lipid particles obtained at 10%, 20% and 30% initial fish oil concentrations could significantly improve the oxidative stability of the loaded fish oil compared to the free fish oil. Considering the higher amount of loaded fish oil and lowest degree of oxidation, 30% initial fish oil concentration is suggested as the optimum initial fish oil concentration.

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Notes

The authors declare no conflict of interest.

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Figure captions

Figure 1. SEM images of the fish oil-loaded hollow solid lipid microparticles obtained at varying initial fish oil concentrations: (a) 10%, (b) 20%, (c) 30%, (d) 40%, and (e) 50%; (f) TEM image of the fish oil-loaded hollow lipid nanoparticles obtained at 50% initial fish oil concentration after filtration using 450 nm syringe filter.

Figure 2. Fish oil loading efficiency at different initial fish oil concentrations in the lipid mixture.

Figure 3. Confocal fluorescence microscopy z-series scanning images showing the fish oil-loaded hollow solid lipid micro- and nanoparticles obtained at varying initial fish oil concentrations: (a) 0%, (b) 10%, (c) 20%, (d) 30%, (e) 40%, and (f) 50%. **Black color shows the FHSO, red color on the particles shows the fish oil on the surface of the particles, and the dark red color in the particles in figures b-f shows the loaded fish oil.**

Figure 4. DSC melting curves of the fish oil-loaded hollow solid lipid micro- and nanoparticles obtained at varying initial fish oil concentrations.

Figure 5. XRD patterns showing the polymorphism of the fish oil-loaded hollow solid lipid micro- and nanoparticles obtained at varying initial fish oil concentrations.

Figure 6. Change in the peroxide value (PV) (top) and anisidine value (AV) (bottom) of the fish oil-loaded hollow solid lipid micro- and nanoparticles during accelerated storage test at 40 °C over three weeks.

Figure S1. Confocal fluorescence microscopy z-series scanning images showing the surface of the fish oil-loaded hollow solid lipid micro- and nanoparticles obtained at varying initial fish oil concentrations: (a) 10%, (b) 20%, (c) 30%, (d) 40%, and (e) 50%.

Figure S2. Particle size and size distribution of the fish oil-loaded hollow solid lipid micro- and nanoparticles obtained at varying initial fish oil concentrations: (a) 10%, (b) 20%, (c) 30%, (d) 40%, and (e) 50%.

Figure S3. SEM images of the fish oil-loaded hollow solid lipid micro- and nanoparticles obtained at varying initial fish oil concentrations: (a) 10%, (b) 20%, (c) 30%, (d) 40%, and (e) 50%.

Figure 1

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698 **Figure 2**

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