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2 **Generating phytosterol nanoparticles in nanoporous bioaerogels via supercritical**
3 **carbon dioxide impregnation: Effect of impregnation conditions**

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Abstract

Phytosterols are known for promoting human health and wellness; however, their water-insolubility limits their bioaccessibility and consequently bioavailability. In this study, a novel method based on supercritical carbon dioxide (SC-CO₂) impregnation of phytosterols into nanoporous starch aerogels (NSAs) was optimized to form phytosterol nanoparticles with reduced crystallinity in order to improve their bioaccessibility. Impregnation conditions, namely temperature and cooling rates, were optimized for the highest impregnation capacity, smallest particle size, and improved phytosterol distribution. The highest impregnation capacity was 99 mg phytosterol/g NSA, and the size of the impregnated phytosterols ranged between 59 and 87 nm. More isolated phytosterol nanoparticles were generated via fast cooling which drastically decreased the solubility of phytosterols in SC-CO₂. The crystallinity of the impregnated phytosterols was reduced compared to the original phytosterol. The proposed method is a novel method to generate food grade phytosterol nanoparticles with reduced crystallinity which improves the water solubility.

Keywords: Phytosterol; supercritical; nanoparticles; starch; aerogel.

Abbreviations: Supercritical carbon dioxide (SC-CO₂), nanoporous starch aerogel (NSA), rapid expansion of supercritical solution into air (RESS), rapid expansion of supercritical solution into aqueous solution (RESSAS), depressurization of an expanded liquid organic solution (DELOS), sudden precipitation of supercritical solutions (SPSS), Brunauer–Emmett–Teller (BET), Barrett-Joyner-Halenda (BJH), scanning electron microscopy (SEM).

1. Introduction

Interest in preparing foods to improve the overall human health and wellness is growing with the trending food for health concept. One approach is to improve the nutritional value of the foods and beverages by incorporating bioactive compounds. Among bioactive compounds, phytosterols have received much attention in managing hypercholesterolemia, or abnormally high blood cholesterol, and recently inflammation (Navarro et al., 2001) and inflammatory bowel disease (IBD), a chronic inflammatory condition of the gastrointestinal tract (Cheon et al., 2006; Kang et al., 2013; Somani et al., 2015). Typical western diet is poor in terms of phytosterols and only ~5% of the dietary phytosterol is absorbed in the human body. Therefore, enriching foods with phytosterols is required, but incorporating phytosterols into foods is challenging because phytosterols are high melting point crystalline compounds that are insoluble in water and poorly soluble in fats and oils, which results in a gritty texture that affects the sensory and quality of the product negatively. Moreover, bioavailability of phytosterols is very low (1.5-5%) (Nguyen, 1999) due to poor water solubility and high crystallinity (Law, 2000).

Decreasing the size of bioactives is known to increase the bioaccessibility and in turn the bioavailability (Acosta, 2009; Kesisoglou et al., 2007). In recent years, there has been many efforts to decrease the size of phytosterols. These include emulsion formation (Chen et al., 2016; Ribeiro et al., 2016; Rozner et al., 2009), microencapsulation by spray drying (Di Battista et al., 2015), formation of inclusion complexes of phytosterols with hydroxypropyl β -cyclodextrin (Meng et al., 2012), and precipitation from organic solvents (Christiansen et al., 2003; Kawachi et al., 2006; Rossi et al., 2010). However, most of the current methods generate liquid products that are difficult to incorporate into all types of foods, have undesirable flavor profile due to the presence of surfactants, and their application is limited due to the use of organic solvents and/or

non-food grade surfactants. A recent study investigated the preparation of functional foods by co-crystallization of phytosterols and triacylglycerols; however, this approach is not applicable for many low fat foods which have an increasing consumer demand (Acevedo and Franchetti, 2016).

Particle formation using SC-CO₂ is a new and exciting area that offers alternative approaches to form various micro- and nanoparticles. CO₂ is non-toxic, non-flammable, environmentally friendly, inexpensive, abundant, and has mild critical temperature (31 °C) and pressure (7.4 MPa). So far, rapid expansion of supercritical solution into aqueous solution (RESSAS) (Türk and Lietzow, 2004), rapid expansion of supercritical solution into air (RESS) (Türk et al., 2002; Türk and Lietzow, 2008), and depressurization of an expanded liquid organic solution (DELOS) (Moreno-Calvo et al., 2014) processes have been implemented for micronization of phytosterols. However, agglomeration of the particles and use of organic solvents/surfactants are the disadvantages of those SC-CO₂-based techniques.

We have recently reported a novel approach to improving water solubility of the phytosterols using SC-CO₂ and a novel NSAs impregnation method (Ubeyitogullari and Ciftci, 2016b). In that approach, NSAs with extraordinary properties (59 m²/g surface area and 20 nm pore size) were used as a mold to generate phytosterol nanoparticles. Starch is cheap, abundant, biodegradable, biocompatible and food grade polysaccharide which can form nanoporous gel network without any chemical cross-linker. Therefore, starch is a great candidate to form NSAs for food applications (Ubeyitogullari and Ciftci, 2016a). In our previous study (Ubeyitogullari and Ciftci, 2016b) phytosterol nanoparticles were formed at a single impregnation condition (70 °C and 45 MPa), and the phytosterol impregnation capacity was relatively low (55 mg phytosterol/g NSA). It was also observed that the phytosterol nanoparticles form a very thin

plate-like structure. For an improved bioaccessibility, higher amount of isolated phytosterol nanoparticles are required. Hence, there is a critical need for detailed investigation of the impregnation conditions to improve the impregnation capacity and form isolated phytosterol nanoparticles.

Therefore, the objectives of this study were to (a) form NSAs (b) impregnate NSAs with phytosterols to form phytosterol nanoparticles using a novel SC-CO₂ impregnation method (c) investigate the effects of impregnation conditions, namely, temperature, pressure, and cooling rate, for the impregnation capacity, and phytosterol particle size and distribution.

2. Materials and methods

2.1. Materials

Wheat starch was kindly supplied by Manildra Milling Corporation (IA, USA). Crude phytosterols were obtained from MP Biomedicals (OH, USA) and their composition (52.2±0.7% β-sitosterol, 23.3±0.6% stigmasterol, and 24.5±0.1% campesterol) was determined using gas chromatography-mass spectrometry (GC-MS). Pyridine was purchased from EMD Chemicals, Inc. (NJ, USA). Sylon BFT [*N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA):trimethylchlorosilane (TMCS), 99:1] was obtained from Supelco Inc. (PA, USA) and 5α-cholestane (>98%) was purchased from Acros Organics (NJ, USA). Liquid CO₂ (purity> 99.99%) was supplied by Matheson Tri-Gas, Inc. (PA, USA), while ethanol (100%) was obtained from Decon Laboratories, Inc. (PA, USA).

2.2. Preparation of nanoporous starch aerogels (NSAs)

NSAs were produced from wheat starch according to the method that was optimized previously (Ubeyitogullari and Ciftci, 2016a). Briefly, starch was gelatinized in a closed high pressure reactor (4520 Bench Top Reactor, Parr Instrument Company, IL, USA) at 120 °C for 20

min with a mixing rate of 600 rpm to obtain the hydrogels, followed by a retrogradation step at 4 °C for 48 h. Subsequently, the water in the hydrogel was replaced with ethanol with a five-step solvent exchange (30, 50, 70, and 100% (v/v) ethanol for 1 h residence time then 100% ethanol for 24 h) to obtain an alcogel. Finally, the alcogels were converted to aerogels by SC-CO₂ drying of the alcogels at 40 °C and 10 MPa for 4h with a CO₂ flow rate of 0.5 L/min (measured at ambient conditions). NSAs were stored at room temperature (21 °C) until characterized (Ubeyitogullari and Ciftci, 2016a) and impregnated. The properties of the formed NSAs are presented in Table 1.

2.3. Impregnation of the NSAs with phytosterols

Phytosterols were impregnated into the NSAs using a custom-made laboratory scale SC-CO₂ impregnation system (Fig. 1). Impregnation vessel was divided into two compartments with a sintered filter. NSAs (1 g) and phytosterols (0.5 g) were separately wrapped in a Whatman #41 filter paper (NJ, USA). Wrapped phytosterols were placed at the bottom compartments, and the NSA was placed into the top compartment. The vessel temperature (70, 90 and 120 °C) was set prior to the impregnation and the temperature of the micrometering valve was set at 80 °C to prevent freezing due to Joule Thompson effect during depressurization. The system was pressurized with CO₂ to 45 MPa using the double head high pressure syringe pump (Model 260D, Teledyne Isco Inc., NE, USA) and kept at constant set pressure and temperature. Impregnations were carried out at semi-dynamic mode for 3 h. The shut off valve was opened every 10 min for 1 min and the CO₂ flow rate was adjusted to 1 L/min (measured at ambient conditions) using the micrometering valve. At the end of 3 h, the system was cooled down to 25 °C at different cooling rates with either natural cooling or fast cooling by blowing CO₂ to the impregnation vessel from a compressed CO₂ cylinder at 6 MPa. Table 2 shows the details of the

impregnation conditions. Then, the system was depressurized to atmospheric pressure at a CO₂ flow rate of 1 L/min (measured at ambient conditions). Finally, phytosterol-impregnated NSAs were collected from the vessel and stored at room temperature (21 °C) until characterized.

Phytosterols were also processed at the same impregnation conditions shown in Table 2 and collected by sudden precipitation of supercritical solutions (SPSS) without using NSAs. SPSS-phytosterols were recrystallized from phytosterol-SC-CO₂ solvato complex on the Whatman #41 filter paper (NJ, USA) in the top compartment of the vessel (Fig. 1).

2.4. Determination of the phytosterol impregnation capacity

Approximately 0.15 g of impregnated NSA was dispersed in 5 mL of chloroform in a 40-mL glass vial and heated at 50 °C for 1 h with occasional vortexing. Then, the mixture was filtered using a 0.45 µm pore-size filter. Filter cake and the vial were washed with 5 mL of chloroform three times and filtered. Finally, the chloroform in the combined filtrate was evaporated to dryness by blowing nitrogen using a Reacti-Vap evaporation unit (Model TS-18825, ThermoFisher Scientific, PA, USA) at room temperature (21 °C). The amount of phytosterols in the dry residue was determined using a GC as described in Section 2.5. Impregnation capacity was reported as mg phytosterol/g NSA. All experiments were carried out in duplicates.

2.5. Phytosterol analysis

Phytosterols in the dry residues were quantified by a GC (Hewlett-Packard (HP) 6890 GC) equipped with a flame ionization detector. Helium was used as the carrier gas with a flow rate of 0.5 mL/min. Phytosterols were derivatized prior to the injection into GC according to Ciftci et al. (2012a). Briefly, 50 µL of internal standard (5 α -cholestane, 2.25 mg/mL) was added onto the phytosterol residue and dried by blowing nitrogen at room temperature. Then, dry residues were dissolved in 0.3 mL pyridine and silylated with 1 mL of Sylon BFT for 30 min at 50 °C. The

silylated sterols were injected onto a GC column (DB-35MS, 25 x 0.20 mm x 0.33 μ m; J&W, Agilent Technologies, CA, USA) at splitless mode with a HP G1512A autosampler controller and a HP 6890 Series auto-injector. Data was recorded by using Agilent Chemstation software Rev. A. 10.02 [1757]. The oven temperature was programmed with an initial hold for 5 min at 100 $^{\circ}$ C, then raised to 250 $^{\circ}$ C at a heating rate of 25 $^{\circ}$ C/min and hold for 1 min at 250 $^{\circ}$ C, then increased to 290 $^{\circ}$ C at a rate of 3 $^{\circ}$ C/min and finally maintained at 290 $^{\circ}$ C for 40 min. The injector and detector temperature were set to 270 and 300 $^{\circ}$ C, respectively.

2.6. Morphology

The morphology of the phytosterols impregnated into the NSAs was analyzed and imaged by field emission scanning electron microscope (S4700 FE-SEM, Hitachi, Tokyo, Japan) operating at an accelerating voltage of 5 kV and 15 mA under low vacuum mode. The specimens were mounted on circular aluminum stubs with double-side conductive carbon tape and then sputter-coated with a chromium layer under vacuum (Desk V HP TSC, Denton Vacuum LLC, NJ, USA) prior to analysis.

2.7. Particle size analysis

Particle size measurements of the phytosterols impregnated into the NSAs were performed using ImageJ v. 1.50i software (public domain, National Institutes of Health, USA) (Kenar et al., 2014). Fifty particles were randomly selected from SEM images for particle size measurements and the results were reported as mean $\pm$ standard deviation.

2.8. Crystallinity

X-ray patterns of the samples were generated using a PANalytical Empyrean Diffractometer (Empyrean, PANalytical B.V., Almelo, Netherlands) according to Ubeyitogullari and Ciftci (2016b). The instrument was equipped with PIXcel^{3D} detector which was operated with 1D

detection. The x-ray diffraction analyses were performed at 45 kV and 40 mA. The powdered samples (mesh # 20) were scanned from 2° to 40° (2 θ) with a scanning speed of 0.927 °/min and sampling interval of 0.05°. The samples were spun continuously at the rate of 3.75 rpm throughout the analysis.

2.9. Statistical analysis

Statistical analysis of the obtained data was conducted using Minitab® 16.1.1 software (Minitab Inc., State Collage, PA, USA). The results were presented as the mean value $\pm$ standard deviation. Multiple comparison of the mean values was carried out by Tukey's test. The differences among treatments were considered statistically significant at a significance level of $p < 0.05$.

3. Results and discussion

NSAs were produced from wheat starch by converting the starch hydrogel into an alcogel, and then converting the alcogel to aerogel by SC-CO₂ drying. During gelatinization, the water is absorbed by starch granules and swollen granule sacs form porous hydrogel upon cooling (Ratnayake and Jackson, 2008). The water in the hydrogel was replaced with ethanol because removing ethanol with SC-CO₂ generates nanoporous and high surface area aerogels due to higher solubility of ethanol in SC-CO₂ (García-González et al., 2011). Our previous studies have shown that air drying to remove the water from the hydrogel does not generate starch aerogels (Ubeyitogullari and Ciftci, 2016a). SC-CO₂ eliminates the surface tension of the solvent in the pores (capillary forces in the pore walls) during drying and consequently preserves the structure of the gel (Pierre and Pajonk, 2002). However, other drying methods like air drying have high capillary forces which are capable to collapse all the pore volume during drying and end up with a nonporous material (Ubeyitogullari and Ciftci, 2016a). Consequently, NSAs were successfully

produced using SC-CO₂ drying with superior properties, such as surface area of 62 m²/g, pore size of 18 nm, pore volume of 0.27 cm³/g, and density of 0.11 g/cm³ with 93% porosity (Table 1). NSAs were used as a novel food grade template to form phytosterol nanoparticles with a novel simple physical nanomanufacturing method.

This novel nanomanufacturing method is based on recrystallization of the phytosterols from a phytosterol and SC-CO₂ solvato complex in the nanopores of the NSA via SC-CO₂ impregnation. Impregnation mechanism was based on the solubility of phytosterols in SC-CO₂. The pressure of impregnation was selected as 45 MPa based on the high solubility of phytosterols at that SC-CO₂ pressure (Ciftci et al., 2012a). This pressure is above the crossover pressure where the solubility increases with an increase in the temperature, because the increase in solubility due to an increase in vapor pressure of phytosterols is more dominant than the decrease in solubility due to a decrease in SC-CO₂ density (Güçlü-Üstündag and Temelli, 2004; Wong and Johnston, 1986). Therefore, the impregnation temperatures were selected as 70, 90, and 120 °C where higher phytosterol solubility in SC-CO₂ is expected. Static impregnation mode, where there was no CO₂ flow, resulted in lower impregnation capacities due to poor diffusion of the phytosterol-SC-CO₂ solvato complex into the NSA (data not shown), therefore, a semi-static impregnation was employed to improve the diffusion of phytosterol-SC-CO₂ solvato complex into NSA.

During SC-CO₂ impregnation, first phytosterols were dissolved in the SC-CO₂ and formed a solvato complex, and then the solvato complex diffused into the nanopores of the NSA. Then, the phytosterols were recrystallized in the nanopores by a sudden decrease in the temperature which results in a sudden decrease in the solubility of the phytosterols in the SC-CO₂ (Ubeyitogullari and Ciftci, 2016b). NSA played a critical role in the formation of phytosterol

nanoparticles because it acted as a mold which prevented the formation of large phytosterol crystals during recrystallization. The phytosterols were recrystallized in the SC-CO₂ by two different cooling mechanisms; natural and fast cooling. Figure 2 presents a representative pathway of the phase transitions of the CO₂ during natural and fast cooling at 90 °C. CO₂ followed a similar pathway at all studied temperatures. First, the temperature was decreased from 90 °C to 25 °C where the solubility of phytosterols in CO₂ is almost zero (Güçlü-Üstündag and Temelli, 2004). Decreasing the temperature from 90 °C to 25 °C decreased the pressure from 45 MPa to ca. 7.5 MPa due to contraction of CO₂. Then, the system was depressurized to atmospheric pressure (0.1 MPa) (Fig. 2). All the impregnations were carried out at the supercritical region where the solubility is higher than 5 mg sitosterol/g CO₂ (Stahl et al., 1988). However, depressurization of the system from supercritical phase may lead to loss of impregnated phytosterols from the NSA matrix due to dissolution of phytosterols in SC-CO₂. Therefore, temperature was decreased to 25 °C where CO₂ is liquefied due to contraction (Fig. 2). The advantage of depressurization of the system from the liquid CO₂ is that phytosterols have very limited solubility (less than 0.3 mg sitosterol/g CO₂) (Stahl et al., 1988) in liquid CO₂ which prevents the loss of phytosterols from the NSA matrix during depressurization.

This study introduces a novel mechanism to produce phytosterol nanoparticles in a simple and clean way using SC-CO₂ technology. Previously, phytosterol particles were produced using SC-CO₂ technology by RESSAS, RESS, and DELOS (Moreno-Calvo et al., 2014; Türk et al., 2002; Türk and Lietzow, 2004). RESSAS process dissolves phytosterols in SC-CO₂ from a packed column, then SC-CO₂ mixture expands into surfactant solutions to form phytosterol particles in the aqueous surfactant solutions. Similarly, RESS process utilizes the same fundamental principle but the SC-CO₂ mixture expands through a capillary nozzle into air to form particles. In

addition, an organic solvent (ethanol) is involved in DELOS process and the phytosterol-ethanol-SC-CO₂ mixture is depressurized to atmospheric pressure to precipitate particles. However, the agglomeration of the particles and the use of organic solvents are still potential concerns in those studies. As an alternative method, aerogels have been used as a mold to form phytosterol nanoparticles by our group for the first time (Ubeyitogullari and Ciftci, 2016b). In this study, the impregnation conditions were further optimized to improve the impregnation capacity and to have a better control over the size and distribution of the phytosterol particles.

3.1. Impregnation capacity

Figure 3 shows the phytosterol impregnation capacities at different impregnation conditions. The highest impregnation capacity (99 mg phytosterol/g NSA) was obtained at 90 °C and 45 MPa with three successive fast cooling. At every cycle of impregnation (heating-pressurization-cooling-depressurization), more phytosterols were impregnated into the nanopores of the aerogels due to improved diffusion of the phytosterol-NSA solvato complex. In the semi-dynamic mode, phytosterols are dissolved in the SC-CO₂ in the 10 min static mode, and then diffused into the NSA during the 1 min flow. Considering the small volume the aerogel occupies, and the amount of phytosterols dissolved in that volume of SC-CO₂, it is may be expected to have low loading capacities because phytosterol-NSA solvato complex enters and exits the NSA without recrystallization. However, in the three successive impregnation at fast cooling, phytosterols were deposited into the NSA at each depressurization step of the impregnation. Hence, significantly higher impregnation capacities were obtained with the three successive impregnation mode ($p < 0.05$). The impregnation capacity significantly increased from 61 to 73 with fast cooling at 90 °C; however, it decreased significantly at 70 °C (Fig. 3) ($p < 0.05$). On the

other hand, cooling rate did not affect the impregnation capacity at higher temperature (120 °C) ($p>0.05$).

Although the solubility of phytosterols in SC-CO₂ is expected to increase with temperature, there was no obvious effect of temperature on the impregnation capacity. Impregnation conditions of 1, 4, 5, and 6 had similar impregnation capacities (Fig. 3). Therefore, the limiting factor was not only the solubility of phytosterols in SC-CO₂ but also the diffusion into NSA matrix. A study by Díez-Municio et al. (2011) on impregnation of lactulose on chitosan using SC-CO₂ concluded that the highest impregnation capacity is not always obtained at the highest solubility of the solute in SC-CO₂.

Surface area of the aerogel has a critical role in impregnation capacity, which increases with an increasing surface area (García-González et al., 2015). To illustrate, corn starch aerogel with a surface area of 127 m²/g had drug impregnations capacities of 12.8 and 21.5 wt.% for ketoprofen and benzoic acid, respectively (García-González et al., 2015). Mehling et al. (2009) obtained 10 wt.% of impregnation capacity for both ibuprofen and paracetamol impregnations into potato starch aerogel (surface area of 72 m²/g) which is comparable with the impregnation capacities obtained in this study.

The impregnation capacity of phytosterols was improved from 55 mg phytosterol/g NSA, which was obtained in our previous study (Ubeyitogullari and Ciftci, 2016b), to 99 mg phytosterol/g NSA. To compare with other phytosterol carrier systems, microencapsulation of phytosterols by spray drying was studied by Di Battista et al. (2015) where surfactants (sodium lauryl sulfate or polysorbate Tween 20), arabic gum, and maltodextrin were used to prepare the liquid feeds. Phytosterol concentration of 6.7% (w/v) was used to prepare the feed liquid and the highest encapsulation efficiency was less than 10 g encapsulated phytosterol/ 100 g powder

collected with addition of 2% polysorbate Tween 20. In another study, the maximum encapsulation efficiency of phytosterols in colloidal dispersions (emulsions) was lower than 2 wt.%, where Tween 20 was used as emulsifier (Ribeiro et al., 2016).

3.2. Morphology and particle size

SEM images of crude phytosterol, SPSS-phytosterols and the phytosterol impregnated into the NSAs are presented in Figure 4. The size of crude phytosterol (as supplied from the manufacturer) was around 120 μm (Fig. 4a). Cooling rate was effective on the morphology of the SPSS-phytosterols obtained at different temperatures (Fig. 4b, b1). SPSS-phytosterols obtained with natural cooling had very thin plate-like (approximate thickness: 300 nm; lengths: 40-120 μm ; widths: 4-13 μm) well-ordered phytosterol crystals. Fast cooling resulted in similar thin layer-by-layer structure (thickness: 300 nm) but with small leaf plates (lengths or widths: 2-4 μm) entrapped between the large plates (lengths: 15-50 μm ; widths: 2-20 μm) (Fig. 4b1). Phytosterols impregnated into NSAs were all in spherical shape nanoparticles (Fig. 4c-e1). Fast cooling (Fig. 4c1, d1, e1) generated more isolated phytosterol nanoparticles than that of natural cooling (Fig. 4c, d, e).

During fast cooling, the drastic change in solubility of phytosterols in SC-CO₂ by fast cooling favored the nucleation rather than the crystal growth (López-Periago et al., 2008; Moreno-Calvo et al., 2014), and there was no enough time for the particles to come together to form aggregates of phytosterol particles. Three successive fast cooling resulted in aggregates of phytosterol nanoparticles due to the cumulative effect during impregnation (Fig. 4d2). Phytosterol particles have higher tendency to form aggregates with each other rather than being isolated in the second and third cycle impregnations, which is supported by the absence of interaction between the phytosterol nanoparticles and the NSA matrix (Ubeyitogullari and Ciftci, 2016b). It is expected

to have phytosterol nanoparticle aggregates at increased loading capacities because there will be more nanoparticles per unit area in the NSA. However, it should be noted that, it is more important to prevent formation of bigger phytosterol particles than obtaining nanoparticle aggregates. Behavior of the nanoparticle aggregates during digestion will determine the bioavailability, which requires a separate detailed study using *in vitro* or *in vivo* digestion models. Some studies have shown that aggregated nanoparticles, similar to the ones observed for three successive fast cooling, did not affect their function negatively (Liang et al., 2017; Dong and Hadinoto, 2017; Hari et al., 2016).

Previously, other SC-CO₂-based systems like RESS and RESSAS were used to produce phytosterol particles (Türk et al., 2002; Türk and Lietzow, 2004, 2008). RESS generated agglomerated phytosterol particles in the range of 166-219 nm by expansion of the supercritical mixture through a capillary nozzle (Türk et al., 2002). The same research group generated submicron phytosterol agglomerates by RESSAS where supercritical mixture was rapidly expanded into aqueous surfactant solution (Türk and Lietzow, 2004, 2008). However, the particles in those studies had very high tendency to aggregate (Valo et al., 2013). The aggregation of phytosterol nanoparticles are prevented by porous structure of the NSAs in this study. In another SC-CO₂-based study where the liquid organic solution of phytosterols was sprayed through a nozzle, micron size plate-like phytosterol crystals with a thickness ranging between 150 and 450 nm were generated (Moreno-Calvo et al., 2014). Rossi et al. (2010) obtained rod shaped colloidal phytosterol particles (length: 500-700 nm; diameter: 80-250 nm) by anti-solvent precipitation method where ethanol was used to dissolve phytosterols and then phytosterols were precipitated by mixing with water. Similarly, crystallization of β -sitosterol from other organic solvent (hexane) into water resulted in plate-like phytosterol crystals (length:

50-120 μm) (Kawachi et al., 2006). However, the use of non-food grade solvents and the difficulty in the controlling the particle size of phytosterols make those techniques not applicable in the food industry.

In emulsion-based approaches, needle like phytosterol crystals were obtained with different sizes; specifically larger than 100 μm (Chen et al., 2016) and larger than 10 μm (Rozner et al., 2009). Recently, Ribeiro et al. (2016) investigated oil-in-water emulsions using myritol and trilaurin as lipid matrix systems. Phytosterols were dissolved in those lipids at 100 °C before homogenization. Particle size of the prepared emulsions were larger than nano (>100 nm). Although most of the particles were entrapped in the oil droplets at low concentration (1 wt.% of phytosterol), there were micron size plate-like phytosterol crystals in the emulsions prepared with myritol at a concentration of 2 wt.% phytosterol which is much lower than the impregnation capacity (9.0 wt.%) obtained in this study. In another study, spray drying of the phytosterols resulted in very large crystals around 70 μm in size and spray drying of the emulsions (composed of arabic gum, maltodextrin, surfactants (sodium lauryl sulfate or polysorbate Tween 20), and phytosterols) generated particles in the range of 5.5-8.9 μm . One major drawback of liquid emulsion formulations is the difficulty in using them into real-world food applications. Moreover, the use of many chemicals (i.e. surfactants) throughout the process affects the consumer acceptability and final food product properties. The stability, handling, and storage of the emulsions are also disadvantageous against dry formulations.

3.3. Crystallinity

The crystallinity of the crude phytosterols, physical mixture of NSA with crude phytosterol (99 mg crude phytosterol/g empty NSA), phytosterol impregnated NSAs, and empty NSAs are presented in Figure 6. Crude phytosterols had characteristic diffraction peaks at $2\theta = 4.7^\circ$, 12.0° ,

15.0°, 18.5°, and 21.9°, respectively. Similar diffraction peaks were obtained in other studies for crude phytosterol powders with 75-80% β -sitosterol (Rossi et al., 2010) and 75.6% β -sitosterol (Moreno-Calvo et al., 2014). Empty NSA had one broad peak which is an indication of a complete gelatinization and disruption of semi-crystalline structure of starch (Ratnayake and Jackson, 2008). The intensity of the characteristic peaks in the phytosterol impregnated NSAs were much lower than that of the physical mixture of the NSA with crude phytosterols at the same mass ratio (Fig. 6), which indicates the reduced crystallinity of the phytosterol nanoparticles impregnated in the NSAs. The reduction in crystallinity improves the water solubility and in vitro dissolution rates due to higher lattice free energy (Babu and Nangia, 2011; Brittain et al., 2009; Rossi et al., 2010). Previously, DELOS process (Moreno-Calvo et al., 2014) generated micron size phytosterol particles but the crystallinity of the phytosterol particles increased.

4. Conclusions

Phytosterol nanoparticles were generated using a novel SC-CO₂ impregnation method that utilizes nanoporous aerogels made of starch. The highest impregnation capacity of 99 mg phytosterol/g NSA was achieved with impregnation temperature of 90 °C and three successive fast cooling. The size of average phytosterol nanoparticles ranged between 58.6 and 87.0 nm. Although the impregnation capacity and size of the phytosterol nanoparticles were not affected by the cooling rate, fast cooling resulted in more isolated phytosterol nanoparticles. The crystallinity of the phytosterols decreased after SC-CO₂ impregnation into NSAs which improves their water solubility and in turn their bioaccessibility. Dry formulation of phytosterol impregnated NSAs enables them to be added to numerous food products including low-fat products and beverages to prepare functional foods. This novel process is a simple

nanomanufacturing method to produce phytosterol nanoparticles with reduced crystallinity while eliminating the use of organic solvents and emulsifiers.

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Figure 1. Schematic diagram of SC-CO₂ drying of starch aerogels. Tags: (1) CO₂ cylinder; (2) needle valve; (3) high pressure pump; (4) preheater; (5) rupture disc; (6) pressure gauge; (7) high pressure vessel; (8) wrapped phytosterol; (9) glass wool sintered filter disc; (10) wrapped aerogel; (11) temperature controller; (12) micrometering valve; (13) cold trap; (14) sample vial; (15) flow meter.

Figure 2. SC-CO₂ impregnation, cooling, and depressurization pathways on the phase diagram of CO₂.

Figure 3. Phytosterol impregnation capacities of the NSAs generated at different SC-CO₂ impregnation conditions. Numbers from 1 to 7 refer to the operating conditions of the phytosterol impregnation into the NSAs given in the Table 2.

Figure 4. SEM micrographs of crude phytosterol (a), SPSS-phytosterols obtained at 70 °C with natural (b) and fast (b1) cooling, and phytosterol impregnated aerogels at 70 °C (c and c1), 90 °C (d, d1 and d2 (successive fast)) and 120 °C (e and e1) with natural and fast cooling, respectively.

Figure 5. Average particle size of the phytosterol nanoparticles obtained by varying impregnation conditions. Numbers from 1 to 7 refer to the operating conditions of the phytosterol impregnation into the NSAs given in the Table 2.

Figure 6. XRD patterns of crude phytosterols, physical mixture of NSA with crude phytosterol (99 mg crude phytosterol/g empty NSA), NSAs impregnated with phytosterols (impregnation condition: 7), and empty NSAs.

Table 1. The textural properties of the NSAs.

BET surface area (m ² /g)	61.6±1.4
BJH pore size (nm)	17.8±0.4
Pore volume (cm ³ /g)	0.27±0.01
Density (g/cm ³)	0.11±0.00
Porosity (%)	92.78±0.00

Table 2. Operating conditions of the phytosterol impregnation into the NSAs.

Impregnation conditions	Pressure (MPa)	Temperature (°C)	Cooling type	Cooling rate (°C/min)
1	45	70	Natural	0.6
2	45	70	Fast	10.0
3	45	90	Natural	0.7
4	45	90	Fast	10.3
5	45	120	Natural	0.8
6	45	120	Fast	12.5
7	45	90	Successive fast*	10.3

*Successive fast represents three fast cooling within the same run.