Carotenoids Stability in High Total Solids Spray Dried Emulsions with gum Arabic Layered Interface and Trehalose-WPI Composites as Wall Materials

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Abstract

The present study investigated the ability of spray dried single layer (SL) and layer-by-layer (LBL) high total solids emulsions with carbohydrate (trehalose) and non-carbohydrate (WPI) solids to stabilize carotenoids upon storage at 35°C, 50°C, and 65°C. Carotenoids loss was determined using HPLC with C30 column and photodiode-array detector followed first order loss kinetics, and increased with increasing storage temperature. Rapid initial first order loss followed by a second, less rapid first order loss was observed. Storage of the systems above the T_g reduced carotenoids loss in the initial first order loss. The loss of carotenoids in LBL system was more temperature dependent initially but SL system was more temperature dependent in the second first order loss step. LBL system showed slower loss rate of carotenoids in the initial first order loss step and at 65°C in the second step. Carotenoids retention was significantly higher in LBL system upon storage at 65°C. The LBL interfacial structure gave better protection to the encapsulated carotenoids over storage and it was highly applicable to formulated materials to protect oil soluble bioactives.

Keywords: high hydrophilic solids emulsion; layer-by-layer; lutein; β -carotene; spray drying; storage study

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1. Introduction

Studies have found that the application of layer-by-layer (LBL) interfacial structures in LBL emulsions can result in emulsions with higher stability towards environmental stresses such as heat treatment, variations in pH, freeze-thaw cycles, lipid oxidation and ionic strength (Ogawa, Decker, & McClements, 2003a; Aoki, Decker, & McClements 2005; Güzey & McClements, 2006; Gharsallaoui, Saurel, Chambin, Cases, Voilley, & Cayot, 2010; Lim, Griffin, & Roos, 2014). The higher stability of LBL system is due to the thicker and denser interfacial layer of the particles, higher steric repulsion, as well as lower van der Waals attraction strength (Moreau, Kim, Decker, & McClements, 2003; Gu, Decker, & McClements, 2005; Harnsilawat, Pongsawatmanit, & McClements, 2006; Benjamin, Silcock, Leus, & Everett, 2012). The thicker interfacial layer of LBL systems can increase the stability of the oil particles towards disruptions providing better protection towards the encapsulated materials (McClements, 1999; Güzey and McClements, 2006). LBL emulsion can be produced via electrostatic attraction between charged primary layer with oppositely charged secondary layer present in the continuous phase (Moreau *et al.*, 2003; Lim and Roos, 2015). Whey protein isolate (WPI) was used as primary layer and

gum Arabic as secondary layer in this study. Due to its ability to be positively or negatively charged by changing the pH of the aqueous phase, protein is widely used as the primary layer in LBL systems (Gu, Decker, & McClements, 2004; Gharsallaoui *et al.*, 2010; Klein, Aserin, Ishai, & Garti, 2010). The isoelectric point (pI) of β -lactoglobulin is 5.2 (Bryant and McClements, 1998) while α -lactalbumin is 4.1 (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003). As gum Arabic has a pKa value of approximately 2.2, gum Arabic will be negatively charged above pH 2.2 (Weinbreck, Tromp, & de Kruif, 2004). Generally, the use of proteins as emulsifiers results in small oil droplets in emulsions with poor stability towards environmental stresses. On the other hand, polyelectrolytes produced oil droplets with better stability towards environmental stresses but were incapable to produce small oil droplets unless used in excess quantities (McClements, 2003).

Carotenoids are commonly found in fruits and vegetables with β -carotene, a highly lipophilic carotene having the highest vitamin A activity and rate of conversion to vitamin A among the provitamin A carotenoids (Grune, Lietz, Palou, Ross, Stahl, Tang, Thurnham, Yin, & Biesalski, 2010). β -carotene is also capable of showing antioxidant and anticancer properties, and may prevent heart diseases (Bendich & Olson, 1989; Omenn, Goodman, Thornquist, Balmes, Cullen, Glass, Keogh, Meyskens, Valanis, Williams, Barnhart, & Hammar, 1996; Albanes, 1999). Degradation of β -carotene occurs mainly through oxidation (Simpson, 1985) and isomerisation (Sweeney & Marsh, 1971). On the contrary, lutein, due to the presence of hydroxyl groups in its molecular structure is categorized as xanthophyll. Lutein is commonly found in green vegetables and can minimize light-induced skin damage (Ribaya-Mercado & Blumberg, 2004) as well as prevents cataracts and macular degeneration (Kachik, Bernstein, & Garland, *et*

70 *al.*, 1997). Chronic and degenerative diseases in humans can be reduced with the consumption of
71 food products consisting of bioactive compounds (van Dokkum, Frølich, Saltmarsh, Gee, 2008).
72 The low bioavailability of carotenoids as crystals or within protein complexes in fruits and
73 vegetables reduces adsorption in the gastrointestinal tract during digestion (Williams, Boileau, &
74 Erdman, 1998). Nonetheless, the use of carotenoids with oil can improve the bioavailability of
75 carotenoids as oil increases carotenoids adsorption (van Het Hof, West, Weststrate, & Hautvast,
76 2000). Therefore, the incorporation of carotenoids in the lipid phase of an oil-in-water (O/W)
77 emulsion can enhance its bioavailability. The lipid phase can be emulsified with an emulsifier
78 containing aqueous phase followed by addition of glass forming hydrophilic component and
79 dehydration of the emulsion to produce a continuous phase of the dried formulation (Drusch,
80 Serfet, Van Den Heuvel, & Schwarz, 2006; Ramoneda, Ponce-Cevallos, Buera, & Elizalde,
81 2011; Spada, Noreña, Marczak, & Tessaro, 2012; Mehanna, Alwattar, & Elmaradny, 2015).

82
83 Microencapsulation utilizes wall materials to entrap core materials protecting it from
84 environmental stresses to provide longer shelf life and enable controlled release of the core
85 materials (Shahidi & Han, 1993). Microencapsulation is commonly achieved through spray
86 drying in the food industry (Ré, 1998). Spray drying is also straightforward and economical
87 giving high quality powders with a long shelf life. Maltodextrins, trehalose, milk proteins, corn
88 syrup and modified starch are frequently used as wall materials in spray drying (Desobry, Netto,
89 & Labuza, 1997; Hogan, McNamee, O’Riordan, & O’Sullivan, 2001; Drusch *et al.*, 2006; Shaw,
90 McClements, & Decker, 2007; Liang, Huang, Ma, Shoemaker, & Zhong, 2013; Mehanna *et al.*,
91 2015). Crystallization of the wall materials in powders can lead to degradation of released
92 carotenoids containing lipid phase as a result of direct exposure to environmental stresses (Buera,

Schebor, & Elizalde, 2005). Nevertheless, studies have shown that mixtures of sugars and proteins can delay the crystallization rate of the sugar component (Jouppila & Roos, 1994; Haque & Roos, 2004). Our earlier studies also showed that the application of LBL interfacial structures with trehalose, and mixture of trehalose and maltodextrin (DE 10) as wall materials reduced the loss of carotenoids upon storage (Lim *et al.*, 2014; Lim & Roos, 2016). The objectives of the present study were to obtain single layer (SL) and LBL powders by spray drying emulsions with high total solids content as well as to determine the ability of the SL and LBL powders having carbohydrate (trehalose) and non-carbohydrate (WPI) mixture as wall materials to protect carotenoids upon storage in the vicinity of the glass transition temperature (T_g) of the carbohydrate. Data on carotenoid loss kinetics and the ability of SL and LBL systems in preventing the loss of encapsulated carotenoids in spray dried systems stored in closed containers were reported providing important information to food and pharmaceutical ingredients and formulations. The application of concentrated systems having carbohydrate and non-carbohydrate mixture as wall materials for spray drying will be beneficial for the industries as there are few reports on the use of such system.

2. Materials and Methods

2.1 Materials

Sunflower oil was Musgrave ExcellenceTM (Spain), whey protein isolate (WPI, Isolac) was from Carbery Food Ingredients (Ballineen, Ireland), gum Arabic (Sigma Aldrich G9752) from Sigma Aldrich (Stenheim, Germany), trehalose (crystalline dehydrate) from Hayashibara Shoji Inc.

(Japan), all-trans- β -carotene (crystalline Type I, synthetic, >93% (UV)) from Sigma-Aldrich (U.S.A.), and lutein (Marigold) from Shaanxi Sciphar Biotechnology Co. Ltd. (China). All other chemicals were purchased from Sigma-Aldrich, Inc. (Dublin, Ireland) and they were of analytical grade.

2.2 Emulsion Preparation

The emulsions were prepared using methods modified from our earlier study (Lim & Roos, 2015; Lim & Roos, 2016). The emulsions prior to spray drying were SL and LBL which consisted of 18.2% sunflower oil and carotenoids, 18.18% trehalose, 8.11% WPI, and water (adjusted to pH3.5 using citric acid) and with and without gum Arabic (0.27%), respectively. WPI was dispersed in deionized water (19.36%, w/w, in water) and the dispersion was allowed to hydrate for 2 hours to enhance its hydration. The dispersion was adjusted to pH 3.5 using citric acid solution (10% w/w). β -carotene (0.05%, w/w, of oil) and lutein (0.05%, w/w, of oil) was dissolved in sunflower oil and mixed with a Silverson mixer (Model AXR, Silverson Machines Ltd., Chesham, UK) at 50°C. A Silverson mixer was used to mix the oil phase (2 part of sunflower oil) and water phase (1 part of water used initially for the dispersion of WPI) at minimum speed for 60s to obtain pre-emulsion. The pre-emulsions were subsequently homogenized at room temperature using a two-stage valve homogenizer (APV-1000, APV Homogenizer Group, Wilmington, MA, USA) for 3 cycles at 240 bar (200 bars for the first stage and 40 bars for the second). A mixture of trehalose and WPI (ratio of 21:8) was used as wall material. Trehalose (46.41%, w/w) was dissolved in deionized water and citric acid solution mixture (40.21% water and 59.79% citric acid solution) using a Silverson mixer at $50 \pm 1^\circ\text{C}$.

WPI (30.77%, w/w) was dispersed in deionized water, stirred with a rod, and allowed to hydrate for 2 hours to ensure complete hydration. The trehalose solution and WPI dispersion were then mixed together using a Silverson mixer and the pH was adjusted to pH 3.5 using citric acid solution. To obtain SL emulsion, the emulsion was mixed with the trehalose and WPI mixture at the ratio of 1:2.14 for 30 min. Gum Arabic (5.66%, w/w, in water) was dissolved in deionized water and stirred for 2 hours. The gum Arabic solution was adjusted to pH 3.5 with citric acid solution. LBL emulsion was obtained by mixing the gum Arabic solution with the emulsion at the ratio of 1:6 at room temperature for 30 min using a Silverson mixer. The emulsion with gum Arabic as secondary layer was then mixed with the trehalose and WPI mixture at the ratio of 1:1.83 for 30 min. The SL emulsion was added with similar amount of water instead of gum Arabic solution to achieve the same final weight as LBL emulsion.

2.3 Spray Drying and Samples Packaging

The emulsions were dehydrated using a single stage Niro 25 spray dryer (GEA Niro Production Minor, Soborg, Denmark) with inlet and outlet temperatures set at 185°C and 85°C, respectively equipped with a rotating disc atomizer at 24000 rpm. The powder solids consisted of 40.66% oil with carotenoids, 40.62% trehalose and 18.12% WPI as their main components and they were rapidly cooled to room temperature, sealed in plastic bags, and stored at room temperature to prevent water uptake and physico-chemical changes prior to analysis. The water contents after spray drying were less than 3% (Table 1) and water activities were $0.14 \pm 0.01a_w$ for SL and $0.14 \pm 0.01a_w$ for LBL powders, respectively. The powders (2g) were then transferred into 10 mL clear glass vials (Schott, Müllheim, Germany). The samples in vials were hermetically sealed

with vacuum using a freeze-drier (Lyovac GT 2, Steris[®], Hürth, Germany) and packaged in plastic pouches (PA/PE 90, Fispar, Leamore warehouse, Dublin, Ireland) using a vacuum sealing system (Polar 80, Henkelman vacuum systems, Hentogenbosch, Netherlands) in duplicates. The vacuum in the packages represents isolation of the systems from surrounding atmosphere and served as an indicator for leakage that could have led to possible loss or uptake of water during storage. Differential scanning calorimetry (DSC) (Mettler Toledo 821e, Schwerzenbach, Switzerland) with liquid N₂ cooling was used to determine the glass transition temperature (T_g) of the powder solids. Samples of powders were prepared in DSC aluminium pans (40 µl; Mettler Toledo, Schwerzenbach, Switzerland), hermetically sealed, and analyzed. STARE thermal analysis software, version 6.0 (Mettler Toledo, Schwerzenbach, Switzerland) was used to analyze the thermograms. The T_g for the powder solids with mixture of trehalose and WPI as wall materials was found to be 50 ± 0.2°C for SL powders and 51 ± 0.2°C for LBL powders. Samples of powders (2 g) in vials were stored in the vicinity of the T_g in incubators (TS 8136, Termaks, Bergen, Norway) set at 35 ± 1°C, 50 ± 1°C, and 65 ± 1°C.

2.4 Colourimetry

The colour values (L*, a*, and b*) of SL and LBL systems were measured directly from glass vials upon storage at 35°C, 50°C, and 65°C for up to 78 days of storage (13 time points) using a pre-calibrated colourimeter (Minolta, CR300 meter). The a* and b* ranged from -120 to 120 measuring the chromatic components of green to red values and blue to yellow values, respectively while the L* value ranged from 0-100 and corresponded to the luminance or lightness component.

2.5 Water activity

Water activity (a_w) of SL and LBL systems stored at 35°C, 50°C, and 65°C was determined at room temperature using an AquaLab Water Activity Meter CX-2 with internal temperature control (Decagon Devices, Inc., Pullman WA., U.S.A.). The a_w of the systems was determined in plastic cups for up to 78 days of storage over 13 time points.

2.6 Extraction Method and HPLC Analysis

SL and LBL powders (0.5g) were reconstituted in 2mL of deionized water (KB Scientific, Ireland) and vortexed (Scientific Industries Inc., G-560E, NY, USA) at room temperature for 30s. This was followed by destabilization of the emulsion using 3.9 mL methanol:ethylacetate (1:1, v/v) mixture acting as organic solvents for the carotenoids and vortexed for 30s. The methanol:ethylacetate mixture contained 0.2% butylated hydroxyl toluene (BHT) as antioxidant to prevent the loss of carotenoids during extraction process. The oil particles within the samples was saponified using 1 mL of saturated potassium hydroxide in methanol (2M) and was vortexed for 30s separating the mix into the saponised fraction (lipid carrier) and unsaponised fraction (β -carotene and lutein). To ensure complete dissolution of the carotenoids present in the organic phase, 1 mL of dichloromethane was added and the samples were vortexed for 30s. Finally, 3.8mL of hexane was added into the samples and vortexed for 30s to improve separation of the organic phase. The samples were then left to stand for 30 min in the dark. The organic phase was removed with a glass pipette, filtered (Minisart RC 15, Sartorius Stedim Biotech GmbH,

Goettingen, Germany), and transferred into HPLC vials (Dionex No: 055427, 1.5ml w/ Slit Septum, Unas, USA). The samples were injected at a volume of 10 μ L into the HPLC system. Dionex ICS3000 (Sunnyvale, CA, U.S.A.) with a dual pump (DP-1, Dionex, Sunnyvale, CA, U.S.A.), autosampler (AS-1, Dionex, Sunnyvale, CA, U.S.A.), and photodiode-array detector (PDA ICS Series, Dionex, Sunnyvale, CA, U.S.A.) was used to quantify the amount of β -carotene and lutein. Column used in the study was a 250 mm $\times$ 4.6 mm i.d., 5 μ m, reversed-phase Acclaim C30 analytical column alongside a 4 mm $\times$ 4 mm i.d. guard column of the same material (Dionex, Sunnyvale, CA, U.S.A.). An eluent system composing of acetonitrile (A), methanol:ethyl acetate (1:1) (B) and 0.5% of 200 mmol acetic acid in water (C) was used with gradient profile of 84.5% A, 15% B, 0.5% C at -5 to 2 min, gradient of A and B from 2 to 12 min to 64.5% A, 35% B, 0.5% C, and return from 34 to 40 min to 84.5% A, 15% B, 0.5% C. Relative amounts of β -carotene and lutein were derived from absorption peak areas performed at 450 nm. The study on the stability of β -carotene and lutein was carried out for up to 78 days of storage (13 time points). Data of carotenoids degradation were fitted to first-order kinetics ($\ln A/A_0 = -kt$) and the rate constants (k) were derived from the slopes of linear regressions. Plot of $\ln A/A_0$ against time (storage days) gave a straight line which justified the use of first order kinetics as carotenoids degradation is a typical first order reaction (Desobry *et al.*, 1997; Hidalgo & Brandolini, 2008; Lim *et al.*, 2014; Mahfoudhi & Hamdi, 2014). Activation energy was determined using Arrhenius relationship $k = Ae^{-E_a/(RT)}$ and plots of $\ln k$ against T^{-1} .

2.7 Optical Microscope

The spray dried emulsions were dispersed in sunflower oil on a glass slide and images were captured using optical microscope at 100X magnification (Olympus BX51) attached to a video camera (PikeLink A662, Ottawa, ON).

2.8 Raman-FIB-SEM analysis

Raman-FIB-SEM analysis was done using RISE microscope; a unique integration of high resolution Confocal Raman Microscope (CRM) (WITec) with field-emission ultra-high resolution Scanning Electron Microscope (SEM) and Focused Ion Beam (FIB;TESCAN GAIA3 GMU) allowing the combination of all devices into one chamber. The precision of about 2 μ m of movement from FIB-SEM to the Raman position allowed straightforward identification of the exact place which was visualized under CRM and cut under the FIB-SEM. The powder was first visualized in SEM to locate a suitable position for FIB milling. Rough milling (750 pA) was performed in order to reduce the cutting time followed by the polishing procedure (approx. 100 pA). Then the powder was tilted to the position where the revealed surface was horizontal. After the SEM image was captured, the powder was moved to the Raman position (approx. movement of 180 mm). Under CRM, the position for Raman imaging was selected on the base of the white light image. Raman mapping was performed with 532 nm green laser with pixel size of 100x100 nm² and integration time of 0.15s. Post processing and visualization was completed in Project Plus software (WITec).

2.9 Particle size, particle density, occluded air, interstitial air, bulk density, and surface oil

The particle size distribution of SL and LBL powders was determined by laser diffraction using Malvern Mastersizer 3000 with powder feeder unit (Malvern, Worcestershire, UK). A helium gas Multivolume Pycnometer 1350 (Micromeritics, Georgia, USA) was used to determine particle density. The occluded air and interstitial air of the spray dried emulsions were determined as per GEA Niro (2006). Tapped bulk density (100 taps) of the powders was determined with a tap volumeter (Copley, J. Engelsmann A.G., Ludwigshafen, Germany). Surface oil of the powders was determined by hexane washing. The powders (10g) were washed for four times using 50ml of hexane per wash. The hexane containing extracted fat residue was allowed to evaporate in water bath set at $50 \pm 1^{\circ}\text{C}$ and transferred into an oven set at $50 \pm 1^{\circ}\text{C}$ until constant weight was obtained.

2.10 Powder flow testing

Brookfield Powder Flow Tester (PFT) (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) was used to analyze flowability and bulk density of the powders. Axial and torsional speed of 1.0 mm/s and 1 rev/h, respectively was used. An aluminium trough with perforated sheet of the annular shear cell was filled with powders, leveled using a curved profiled shaping blade, and weight of powders was recorded before testing. Then, vane profiled lids with simulated 2B finish for the flat lid was attached to compression plate. Instantaneous flow function test was used to measure flowability of the powders. Five uniaxial normal stresses of between 0.2 and 4.8 kPa were applied and three over consolidation stresses at each normal stress.

2.11 Emulsion Characterisation

Particle size (ζ -average diameter), using dynamic light scattering, measurement range: 0.3nm – 10 μ m) of the reconstituted powders before and after 92 days of storage at 35°C, 50°C, and 65°C was determined using Zetasizer (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK). The powders were reconstituted to initial concentration (45% total solids content) using deionized water adjusted to pH3.5 with citric acid solution. The emulsions were then diluted with deionized water (pH 3.5) at ratio of 1:500 for the measurements of ζ -average diameter. Lastly, the diluted samples were vortexed for 30s to ensure homogeneity. Plastic cuvettes (Square cuvettes, PS, 10mmX10mmX45mm, SARSTEDT AG & Co, Nümbrecht, Germany) were used to determine the particle size with 1ml of diluted emulsions. Measurements were completed at room temperature.

2.12 Statistical Analysis

The analyses were completed in triplicates and results were expressed as mean $\pm$ standard deviation. The carotenoids stability study was carried out in replicates with duplicated injections.

3. Results and Discussions

3.1 Powder Characteristics

High total solids single layer (SL) and layer by layer (LBL) emulsions were successfully spray dried producing high quality powders with no visual differences between the powders. The

powders obtained were dispersed in sunflower oil and images were captured under light microscope at 100x magnifications. Figure 1 showed that the SL and LBL powders obtained were similar in shape and contained dark spots indicating the presence of air vacuoles. Images from the Raman-FIB-SEM (Figure 2) also showed that both SL and LBL powder particles had spherical shapes with slight wrinkles and surface roughness. The surface of a cut particle is shown in Figure 2. The water content, particle density, occluded air, interstitial air, bulk density, tapped bulk density, surface oil, and particle size of the SL and LBL powders obtained were shown in Table 1. The powders have very similar characteristics as there were no significant differences in the particle density, occluded air, interstitial air, bulk density and tapped bulk density. Although there were no significant differences in the particle density, occluded air, and interstitial air between the powders, the higher particle density in LBL powder while higher occluded air and interstitial air in SL powder showed similar trend to the powders obtained in our earlier study (Lim & Roos, 2016). Gum Arabic attached electrostatically on the charged WPI-oil interface in LBL layered interface resulting in higher overall particle density. Overall particle density of the powders obtained was lower than those having only carbohydrates as wall materials (Desobry *et al.*, 1997; Drusch *et al.*, 2006) as the presence of protein reduced particle density (Moreau & Rosenberg, 1999). The water content in SL powder was significantly higher than in LBL powder. This was due to the smaller particle size of SL powder that gave a higher total surface area for water sorption from the surroundings during cooling of the powders to room temperature. The occluded air was fairly low in both powders as high total solids emulsions were used for spray drying. High occluded air and low bulk density of powders are the consequences of low total solids formulations used for drying (Mistry, 2002). The surface oil of the powders was found to be significantly higher in SL system than in LBL system. The

application of LBL interfacial structure increases the stability of the oil particles towards disruption (Güzey & McClements, 2006) occurring during atomization increasing microencapsulation efficiency of the carotenoids containing oil. There was a significant difference in the $d_{[4,3]}$, average volume-surface diameter between SL and LBL powders with LBL powders having bigger particle size. The presence of gum Arabic in the continuous phase of the LBL emulsion increased its viscosity which affected particle formation during atomization in the spray drier producing larger particles. The flowability and bulk density as a function of major principal consolidation stress (kPa) of the SL and LBL powders are shown in Figure 3. Both the SL and LBL powders were generally ‘easy flowing’ with no significant differences in their flowability across the major principal consolidation stress (kPa). On the other hand, LBL powders have significantly higher bulk density across the major principal consolidation stress (kPa).

3.2 Colour measurements, water activity, and ζ -average diameter

Figure 4 shows the colour values (L^* , a^* , and b^* values) of SL and LBL spray dried systems at 0 and after 78 days of storage at 35°C, 50°C, and 65°C. There was no significant difference in the colour values between SL and LBL systems at day 0. Generally, minor changes occurred in the L^* (lightness) and a^* (redness) while bigger changes was observed in b^* (yellowness) values. The changes in L^* values were not significant except at 65°C where L^* values of both SL (from 86.39 to 81.88) and LBL (from 86.29 to 83.18) powders decreased significantly. On the other hand, the a^* values of systems stored at 35°C and 50°C decreased significantly upon storage. This was in agreement with results obtained in other studies using

systems with carotenoids upon storage due to carotenoids loss (Desobry *et al.*, 1997; Qian, Decker, Xiao, & McClements, 2012; Mahfoudhi & Hamdi, 2014). However, minor changes with no significant differences in a^* values were observed in systems stored at 65°C that corresponds to the L^* values where systems stored at 65°C showed a different trend compared to systems stored at 35°C and 50°C. Residual lactose present in WPI were capable of causing Maillard reaction (non-enzymatic browning) that resulted in the changes of L^* and a^* values stored at 65°C. Besides, lipid oxidation generates free radicals and reactive oxygen groups capable of attacking proteins and amino groups present in the continuous phase that results in browning (Zirlin & Karel, 1969; Pokorný, El-Zeany, Kolakowska, & Janíček, 1974; Potes, Kerry, & Roos, 2014). Generally, the b^* values reduced upon storage with significant difference observed in systems stored at 50°C and 65°C. However, several authors claimed that b^* value was not a good indicator for surface colour changes in systems with β -carotene as the colour range b^* value characterizes were not dominant (Elizalde, Herrera, & Buera, 2002; Prado, Buera, & Elizalde, 2006; Spada *et al.*, 2012; Mahfoudhi & Hamdi, 2014).

Water activity (a_w) of the SL and LBL systems before and after storage at 35°C, 50°C, and 65°C for 78 days is shown in Figure 5. The a_w of SL powders ($0.14 \pm 0.01a_w$) and LBL powders ($0.14 \pm 0.01a_w$) were similar to the a_w of powders frequently obtained from spray drying in the industry of around 0.2 a_w (Adhikari, Howes, Bhandari, & Langrish, 2009). Generally, there was no significant difference in the water activity before and after storage for 78 days at all temperatures in both SL and LBL systems. Vacuum was preserved in the sample packages and vials during the duration of the study and showed that no leakages occurred that could have affected the a_w . The effect of storage at 35°C, 50°C, and 65°C for 92 days on ζ -average diameter

in reconstituted SL and LBL powders is shown in Figure 6. It can be seen initially at day 0 that the ζ -average diameter of SL oil particles was significantly smaller than those of LBL. The application of LBL interfacial structure increased the ζ -average diameter due to the presence of both WPI and gum Arabic at the oil interface. There was no significant difference in the changes of ζ -average diameter of SL and LBL systems upon storage at 35°C and 50°C for 92 days. However, storage at 65°C resulted in significant increase of the ζ -average diameter in both SL and LBL systems. Kim, Decker, & McClements (2002) suggested that the denaturation of β -lactoglobulin resulted in widespread flocculation of oil droplets in O/W emulsions because of the increased surface hydrophobicity that increases hydrophobic interaction between oil particles. Besides, protein sulfhydryl groups will be exposed due to protein denaturation increasing disulfide bond between proteins covered oil particles. β -lactoglobulin and α -lactalbumin on the surface of oil particles will unfold upon heating above 65°C as observed in DSC (Dalgleish, 1996).

3.3 Carotenoids Stability

Quantitative values of carotenoids retention were determined using the peak areas of all-trans- β -carotene and lutein. First order kinetics was used to fit the data producing first order degradation kinetics of carotenoids in both SL and LBL systems. Straight lines obtained in the plots of $\ln A/A_0$ against storage days (time) supported the application of first order kinetics. Many other authors also found that the degradation of carotenoids is a typical first order reaction (Henry, Catignani, & Schwartz, 1998; Desobry, Netto, & Labuza, 1999; Achir, Randrianatoandro, Bohuon, Laffargue, & Avallone, 2010; Mahfoudhi & Hamdi, 2014).

Arrhenius plots and activation energies of all-trans- β -carotene and lutein for SL and LBL spray dried systems stored at 35°C, 50°C, and 65°C for 78 days is shown in Figure 7. Glass transition temperature (T_g) of the systems too was presented in the figure. The HPLC chromatograms showed that the retention time of all-trans- β -carotene was 32.5 ± 0.1 min and 6.8 ± 0.1 min for lutein. The increase in isomers corresponds to the loss of all-trans- β -carotene during heat treatment (Chandler & Schwartz, 1988). The main isomers observed in this study were the 15-cis- β -carotene and 13-cis- β -carotene. However, no observable trends can be obtained from the isomers as they fluctuate with time.

The presence of a two-step loss kinetics was noted in the loss of both all-trans- β -carotene and lutein showing a fast initial first order loss kinetic followed by a second less rapid first order loss kinetic. This was in agreement with the observation made on the non-humidified spray dried system used in our earlier study (Lim & Roos, 2016). Similar observation was also made by Desobry *et al.* (1997) and Desobry *et al.* (1999) on the loss of β -carotene in spray dried system. Nonetheless, study by Drush *et al.* (2006) too found rapid initial increase of hydroperoxides followed by a second period of slower increase of hydroperoxides. The presence of surface oil on the powder surfaces increases the exposure of the carotenoids containing oil to the heat from surrounding as well as residual oxygen and oxygen present on the surface water. These exposures on the unprotected carotenoids containing oil led to the rapid initial loss of carotenoids. The higher amount of surface oil on SL powders led to the higher degradation rate of both all-trans- β -carotene and lutein in SL system during the rapid initial loss. Besides, oxygen trapped within the glassy matrix of the powders is capable of causing oxidation leading to rapid loss of carotenoids as oxidation is the main cause of carotenoids degradation (Qiu, Chen, & Li,

2009). Oxygen containing vacuoles were formed within the powders as a result of thermal expansion of trapped air bubbles upon hardening of the glass formers (Aguilera, 1990; Tewa-Tagne, Briançon, & Fessi, 2007). The activation energies of LBL system was higher than SL system in the initial first order loss indicating that the loss of carotenoids in LBL system was more temperature dependent. This was attributed to the presence of gum Arabic at the oil-WPI interface that reduced heat transfer towards the encapsulated carotenoids and effectively reducing carotenoids loss. Nonetheless, the ability of the LBL system in reducing heat transfer will reach a maximum limit with increasing storage temperature resulting in higher carotenoids loss. Likewise, the local T_g at the oil-WPI interface was increased by the presence of gum Arabic reducing flow or structural collapse at higher temperature. Therefore, LBL powders have lower density or hardness at the vicinity of the oil particles increasing the temperature dependence on the loss of carotenoids. However, in the second first order loss kinetic, the loss of carotenoids in SL system was more temperature dependent than LBL system. This showed that the SL system was less stable towards environmental stresses such as heat during long term storage. As structural collapse occurred at the rate of $T - T_g$ (Levi & Karel, 1995), the presence of gum Arabic at oil-WPI interface result in a harder and denser area around the oil particles over long term storage reducing the temperature dependence of carotenoids loss in LBL system.

The loss of both all-trans- β -carotene and lutein in this study increased with increasing storage temperature and is a phenomena commonly observed in other carotenoids containing studies (Desobry *et al.*, 1997; Koca, Burdurlu, & Karadeniz, 2007; Hidalgo & Brandolini, 2008; Spada *et al.*, 2012; Caliskan, Lim, & Roos, 2015). Nonetheless, it was noted that the loss of carotenoids above the T_g was reduced based on the rate constants acquired in the initial rapid first

order loss kinetics. This can be attributed to the lower solubility of oxygen with rising temperature of up to 100°C (Hildebrand, 1952; Wilhelm, Battino, & Wilcock, 1977). The lower amount of oxygen dissolved in the surface water of the systems with higher storage temperatures led to the lesser amount of amount of oxygen available to diffuse towards the carotenoids containing oil particle. This effectively reduced the loss of carotenoids in both SL and LBL powders. Besides, storing the systems above the T_g caused a dynamic process of structural collapse at the rate of $T - T_g$ (Levi & Karel, 1995). Even though no physical collapse of the powders was observed, the increased molecular mobility and reduced viscosity above the T_g of the systems (Roos & Karel, 1991) lead to a thicker membrane on the powder particles. The resulting harder and denser powder particles effectively reduced carotenoids loss by reducing heat transfer from the surrounding towards the encapsulated carotenoids containing oil. However, during long term storage, a higher increase in the loss of all-trans- β -carotene and lutein was observed above the T_g as seen in the second slower first order loss kinetics. The loss of carotenoids and increased isomerisation gave higher loss rate constants above the T_g during long term storage as glass transition lead to the release of the encapsulated oil (Shimada, Roos, & Karel, 1991). The main factor for carotenoids loss is isomerisation (Qiu *et al.*, 2009) and is largely due to light and heat exposure. Free radicals generated from lipid oxidation over storage as well as reactive oxygen species are capable of reacting with the carotenoids present increasing the loss of carotenoids. Labuza, Heidelbaugh, Silver, & Karel, (1971) stated that the rate of lipid oxidation increased in systems with a_w below 0.2 or above 0.5.

The loss of carotenoids in this present study was more rapid than those of the non-humidified spray dried powders from earlier study (Lim & Roos, 2016) with mixture of trehalose

460 and maltodextrin (DE 10) as glass formers based on the rate constants. The mixture of
461 carbohydrate (trehalose) and non-carbohydrate (WPI) as glass formers resulted in the higher
462 porosity and lower density of the powders obtained. Density of spray dried powders was found to
463 increase while the porosity decreased with increasing amount of lactose in a WPI-lactose wall
464 matrix system (Moreau & Rosenberg, 1999). A study by Carneiro, Tonon, Grosso, & Hubinger,
465 (2013) also found that powders having mixture of maltodextrin and whey protein concentrate as
466 wall materials has the lowest bulk density indicating higher amount of occluded air. The higher
467 porosity and lower density of the powders in this study allows greater heat permeability towards
468 the encapsulated carotenoids that increased carotenoids loss. WPI preferably migrates to the
469 particle surface (air-water interface) due to its high surface activity forming a protein rich layer
470 (Adhikari *et al.*, 2009; Wang, Jiang, & Zhou, 2013). Fäldt & Bergenståhl (1994) found that the
471 surface-active protein predominates at the surface of spray dried carbohydrate-protein system.
472 Discontinuity between carbohydrate and protein used as wall materials occurred in this present
473 study due to the presence of surface-active WPI. As a result, the carotenoids containing oil will
474 be encapsulated within the glass former and non-glass former during spray drying. The lesser
475 amount of carotenoids containing oil trapped and protected within the denser glass former
476 increased the loss of carotenoids. Besides, the high amount of protein present in the continuous
477 phase of the emulsion results in surface indentations of the powders obtained (Fäldt &
478 Bergenståhl, 1994; Wang *et al.*, 2013). This presence of WPI at particle surface increased the
479 skin flexibility of the particles preventing skin rupturing during drying resulting in more wrinkles
480 and surface roughness as observed in Figure 2 and is commonly observed in spray dried milk and
481 milk containing products (Wang & Langrish, 2010). This increases the effective total surface
482 area of the powder particles allowing more surface area for heat transfer resulting in the higher

loss of all-trans- β -carotene and lutein. It was also noted that the loss of lutein was slightly more rapid than all-trans- β -carotene based on the rate constant. The presence of two hydroxyl groups increased the polarity and hydrophilicity of lutein (Farombi & Britton, 1999; Updike & Schwartz, 2003). As a result, lutein will be more likely to assemble at WPI-oil interface while all-trans- β -carotene will be present within the bulk oil. It can be seen from Figure 2 that lutein was present at the interfaces within a cut particle and appeared in large quantities on surfaces while all-trans- β -carotene was dissolved within the encapsulated oil and was detectable only in small quantities on the SEM images. The location of lutein in oil particles allows lutein to be more susceptible to heat from surroundings as well as trapped oxygen within the powder particles resulting in the faster degradation rate.

It can be observed that the degradation rate of all-trans- β -carotene and lutein was faster in SL system than in LBL system in the initial rapid first order loss kinetic, and at higher storage temperature above the T_g in the second less rapid first order loss kinetic. The denser and thicker interfacial layer of the LBL particles, lower van der Waals attractive forces, and higher steric repulsions contributed to the higher stability of LBL systems than SL systems (Moreau *et al.* 2003; Gu *et al.*, 2005; Harnsilawat *et al.*, 2006; Bouyer, Mekhloufi, Le Potier, De Kerdaniel, Grossiord, Rosilio, & Agnely, 2011). This can be attributed to the higher stability of LBL system towards heat from surrounding due to the application of LBL layered interface. The LBL layered interface reduced heat transfer from the surrounding towards the encapsulated carotenoids reducing loss. On the other hand, the higher amount of surface oil on SL powders resulted in the higher loss of carotenoids in the SL system during the initial rapid first order loss. The significantly smaller particle size ($d [4,3]$) of SL powders too results in higher carotenoids loss in

SL system as the increased effective total surface area of the powders permits higher amount of heat transfer. The total retention of all-trans- β -carotene and lutein in SL and LBL systems upon storage for 78 days (Figure 8) also showed that the retention of carotenoids was significantly higher in LBL powders at higher storage temperature (65°C). This indicated that the application of LBL layered interface was capable in providing the encapsulated carotenoids containing oil in LBL system with significantly better protection against the loss of carotenoids at high storage temperature. The higher stability of LBL system towards environmental stresses such as heat, changes in pH, ionic strength, lipid oxidation, and freeze-thaw cycles too have been reported in several other studies (Ogawa *et al.*, 2003a; Ogawa, Decker, & McClements, 2003b; Güzey & McClements, 2006; Gharsallaoui *et al.*, 2010; Iwata, Neves, Watanabe, Sato, & Ichikawa, 2014). However, no significant difference was observed in the total retention of carotenoids between SL and LBL systems stored below and in the vicinity of the T_g .

4. Conclusions

Spray drying of high total solids single layer (SL) and layer-by-layer (LBL) emulsions with mixture of trehalose and WPI as wall materials produced high quality powders. The powders appeared similar visually as well as under light microscope observation. There was also no significant difference in the particle density, occluded air, interstitial air, bulk density and tapped bulk density between SL and LBL powders. The degradation of all-trans- β -carotene and lutein increased with increasing storage temperatures and followed first order loss kinetics. The presence of two step loss kinetics of all-trans- β -carotene and lutein showing a fast initial first order loss kinetic followed by a second less rapid first order loss kinetic was observed. There

was reduced loss of carotenoids above the glass transition temperature (T_g) based on the rate constants in the initial rapid first order loss kinetics. The loss of carotenoids in LBL system was more temperature dependent as LBL system has higher activation energies than SL system. Nonetheless there was a shift in the activation energies in the second first order loss kinetic as the loss of carotenoids in SL system became more temperature dependent than LBL system. Overall, the application of LBL interfacial structure on the oil particles reduced the loss of carotenoids in the initial rapid first order loss kinetic, and at higher storage temperature above the T_g in the second less rapid first order loss kinetic. The retention of carotenoids were also significantly higher in LBL powders at higher storage temperature (65°C) upon storage for 78 days indicating that the application of LBL interfacial structure provided the encapsulated carotenoids containing oil in LBL system with significantly better protection towards the high storage temperature. Minor changes occurred in the L^* (lightness) and a^* (redness) while bigger changes occurred in b^* (yellowness) values upon storage. No significant difference was observed in the water activity (a_w) of both SL and LBL systems before and after storage at all storage temperatures. Data obtained from this current study is highly applicable in the food and pharmaceutical industries to increase stability of oil soluble bioactives.

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FIGURES

Figure 1. Images from light microscope at 100x magnification of single layer (SL) (a) and layer-by-layer (LBL) (b) powders dispersed in sunflower oil.

Figure 2. Raman-FIB-SEM images showing single layer (SL) powder particles (a), layer-by-layer (LBL) powder particles (b), and a cut powder particle with the presence of lutein (red) in large quantities at interfaces and all-trans- β -carotene (blue) dissolved in encapsulated oil and which appeared only in minor quantities at the cut surfaces (c).

Figure 3. Flowability showing unconfined failure strength (kPa) and bulk density as a function of major principal consolidation stress (kPa) of single layer (SL) and layer-by-layer (LBL) powders.

Figure 4. L* (lightness), a* (redness), and b* (yellowness) values of spray dried single layer (SL) and layer-by-layer (LBL) emulsions before and after storage for 78 days at 35°C, 50°C, and 65°C.

Figure 5. Water activity (a_w) of single layer (SL) and layer-by-layer (LBL) powders obtained by spray drying before and after storage at 35°C, 50°C, and 65°C for 78 days.

Figure 6. Zeta-average diameter (nm) of oil particles in reconstituted single layer (SL) and layer-by-layer (LBL) powders before and after storage at 35°C, 50°C, and 65°C for 92 days.

Figure 7. Arrhenius plots of all-trans-β-carotene and lutein in single layer (SL) and layer-by-layer (LBL) powders stored for 78 days at 35°C, 50°C, and 65°C having a rapid initial first order loss kinetic (SL1 & LBL1) followed by a second slower first order loss kinetic (SL2 & LBL2) and showing the activation energies and glass transition temperature (T_g).

Figure 8. Retention (%) of all-trans-β-carotene and lutein in spray dried single layer (SL) and layer-by-layer (LBL) emulsions before and after storage at 35°C, 50°C, and 65°C for 78 days.

TABLE

Table 1. Water content, particle density, occluded air, interstitial air, bulk density, tapped bulk density (100 times), surface oil, and average volume-surface diameter (d [4,3]) of single layer (SL) and layer-by-layer (LBL) spray dried emulsions with trehalose and WPI mixture as wall materials.

	SL	LBL
Water content (g/100g SNF)	2.74±0.03	2.89±0.02
Particle density (g/cm³)	0.98±0.02	0.99±0.03
Occluded air (cm³/100g)	20.67±1.29	18.51±2.74
Interstitial air (cm³/100g)	101.63±1.29	98.79±2.74
Bulk density (g/cm³)	0.33±0.01	0.35±0.01
Tapped density (g/cm³)	0.49±0.01	0.50±0.01
Surface oil (g/100g)	6.90±0.14	3.90±0.04
D [4,3] (μm)	46.77±0.17	48.23±0.13

Values are means (n=3) ± SD