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SCHOOL OF FOOD AND NUTRITIONAL SCIENCES

DESIGN OF HIGH SOLIDS EMULSIONS FOR DEHYDRATION AND CAROTENOIDS STABILISATION

Thesis presented by

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For the degree of

Doctor of Philosophy

(Ph.D. in Food Science and Technology)

Under the supervision of

Professor Yrjö H. Roos

March 2017

Acknowledgments

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Aaron Lim

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Abstract

Microencapsulation is commonly accomplished using spray drying in the food and pharmaceutical industries. Concentrated solid feeds allow economic manufacturing of formulated foods in powder form. The application of layered interface in emulsion can improve microencapsulation, protection, and delivery of oil-soluble bioactive compounds in food materials. The present study investigated (i) flocculation in layer-by-layer (LBL) emulsion systems with high total solids content and deflocculation at various pH conditions, and the effects of whey protein isolate (WPI) concentration and total solids content on the stability of LBL emulsions; (ii) stability and loss kinetics of carotenoids in dehydrated emulsions to compare the ability of freeze-dried single-layer (SL) and LBL emulsions with trehalose as wall material to protect the carotenoids; (iii) spray drying of high total solids content SL and LBL emulsions with trehalose-low DE maltodextrin (DE 9-12), carbohydrate (trehalose) and non-carbohydrate (WPI), or trehalose-high DE maltodextrin (23-27) as wall materials; and (iv) the ability of the SL and LBL spray dried structures to protect encapsulated lutein and all-trans-β-carotene in entrapped oil upon storage in the vicinity of the glass transition temperature (T_g) of the glass forming wall components.

The ζ-potential measurements showed charge reversal upon addition of gum Arabic solution into SL emulsion with WPI as primary layer and confirmed the formation of LBL interface. Depletion flocculation of the oil particles occurred in LBL systems. Nonetheless, deflocculation into individual particles with increasing pH was possible in systems with sufficient primary layer as found from a decreased ζ-average diameter and visually under microscope. However, coalescence was observed in systems with lower amount of primary layer as confirmed by the ζ-average diameter and the presence of large bright particles under microscope. Viscosity of the systems was significantly ($P < 0.05$) increased with higher total solids contents. Accelerated destabilization test showed that systems with higher amount of emulsifier as primary layer and total solids contents exhibited the highest stability against creaming.

SL and LBL emulsions with trehalose as wall material were freeze-dried and humidified at 33% RH. On the other hand, high total solids (45-50%) SL and LBL emulsions with mixtures of trehalose-maltodextrin (M100), trehalose-maltodextrin (M250), and trehalose-WPI were successfully spray dried to give free flowing powders which showed little visual differences between the powders. There were differences in critical powder characteristics such as particle density, occluded air, and surface oil between the powders as the result of LBL interfacial structuring. Samples of all systems were hermetically sealed in glass vials, vacuum packed, and stored in the vicinity of the T_g. Degradation of the encapsulated lutein and all-trans-β-carotene was analysed using HPLC with C30 column and photodiode-array detector. Carotenoids degradation followed first order kinetics and increased with storage temperature but was less rapid in dehydrated LBL emulsions. Two-step degradation kinetics displaying rapid initial decrease followed by a second slower degradation was observed in spray dried systems. There was non-Arrhenian increase in carotenoids degradation with increasing storage temperature during the rapid initial degradation in all spray dried emulsions and in freeze-dried emulsions. LBL systems gave better protection towards carotenoid degradation during storage as carotenoids retention was higher in LBL systems upon long term storage. The application of LBL interfacial structure reduced carotenoids degradation and can be applied in formulated materials to control the stability of oil-soluble bioactives in the food and pharmaceutical industries.

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Introduction

Food product fortification can be done using bioactive compounds such as carotenoids to prevent degenerative and chronic diseases in humans (van Dokkum *et al.,* 2008). Highly lipophilic carotene, β-carotene, has the highest vitamin A conversion rate and activity among the pro-vitamin A carotenoids. β-carotene shows anticancer and antioxidant properties, and may prevent heart diseases (Bendich and Olson, 1989; Omenn *et al.,* 1996; Albanes, 1999; Grune *et al.,* 2010). Lutein on the other hand is categorized as xanthophyll due to the presence of two hydroxyl groups and is capable to reduce light-induced skin damage, cataract, and macular degeneration (Khachik *et al.,* 1997; Ribaya-Mercado and Blumberg, 2004). Carotenoids present in crystalline form or within protein complexes of fruits and vegetables have reduced gastrointestinal tract absorption during digestion that reduced bioavailability (Williams *et al.,* 1998). Nevertheless, bioavailability of carotenoids can be improved in the presence of oil as oil enhances carotenoids absorption (van het Hof, 2000). Carotenoids can be added into the lipid phase of an oil-in-water (O/W) emulsion to improve its bioavailability followed by emulsification with an emulsifier, addition of hydrophilic glass forming component, and emulsion dehydration to obtain a continuous phase of the dehydrated formulation (Ramoneda *et al.,* 2011; Mehanna *et al.,* 2015).

Surface active materials such as proteins, polyelectrolytes, small molecular surfactants, and phospholipids are commonly used as emulsifiers to assemble at the interface of oil surfaces during homogenization (McClements, 1999). Superior emulsifiers possess the ability to significantly and rapidly reduce surface tension at the interface between two immiscible liquids (Dickinson, 2003). Milk proteins have amphiphilic properties and are widely used in the preparation of food emulsions. Many studies have found that the layer-by-layer (LBL) interfacial structuring can improve stability of emulsions towards environmental stresses such as heat treatment, variations in pH, ageing, freeze-thaw cycles, lipid oxidation, and ionic strength (Moreau *et al.,* 2003; Ogawa *et al.,* 2003a; Aoki *et al.,* 2005; Güzey &

McClements, 2006a; Gharsallaoui *et al.,* 2010). LBL interfacial structuring is formed through electrostatic attraction between a charged primary layer with an oppositely charged secondary layer present in the continuous phase (Moreau *et al.*, 2003; Guzey & McClements, 2006b). The thicker and denser interfacial layer on the oil particles, lower van der Waals attraction, and higher steric repulsion may improve the stability of LBL systems (McClements, 1999; Moreau *et al.,* 2003; Gu *et al.,* 2005; Harnsilawat *et al.,* 2006; Benjamin *et al.,* 2012). Nonetheless, the ratio between the primary layer and secondary layer has an impact on the stability of the LBL emulsions as the presence of the secondary layer can lead to depletion or bridging flocculation (Dickinson, 2003; Guzey and McClements, 2006b). The close proximity of the oil particles in a system with depletion or bridging flocculation promotes creaming and coalescence (Chanamai and Mcclements, 2001; Gu *et al.,* 2005; Benjamin *et al.,* 2012).

Microencapsulation utilizes wall materials to entrap and protect core materials from environmental stresses for extended shelf life as well as enables controlled release of the core materials (Shahidi and Han, 1993). Both spray drying and freeze-drying are dehydration methods commonly utilized for microencapsulation (Desobry *et al.,* 1997; Ré, 1998; Harnkarnsujarit *et al.,* 2012b). Spray drying is often used as a dehydration method in the food industry as it is straightforward and economical giving powders of high quality and long shelf-life. Materials such as trehalose, maltodextrin, corn syrups, modified starches, and milk protein are frequently used as wall materials in spray drying (Desobry *et al.,* 1997; Hogan *et al.,* 2001a; Drusch *et al.,* 2006; Shaw *et al.,* 2007; Liang *et al.,* 2013; Mehanna *et al.,* 2015). Crystallization of the glass forming materials in powders results in the release of the encapsulated lipid that causes rapid degradation of the encapsulated materials (Buera *et al.,* 2005). Studies have found that mixtures of amorphous sugars with high molecular weight carbohydrates or protein can delay the crystallization rate of the amorphous sugar (Jouppila and Roos, 1994; Gabarra and Hartel, 1998; Haque and Roos, 2004; Potes *et al.,* 2012). Most study on spray dried LBL systems was done using systems of 20-30% total solids (%, mass) (Shaw *et al.,* 2007; Gharsallaoui *et al.,* 2010; Jiménez-Martín *et al.,* 2015; Kwamman and Klinkesorn, 2015). The most

concentrated system was found to have approximately 40% total solids (%, mass) (Carvalho *et al.*, 2014). Our study utilized high total solids LBL and single layer (SL) systems of 45-50% total solids (%, mass) for spray drying.

The objectives of studies covered by this thesis were:

i. To determine effects of emulsifier primary layer concentration and total solids content on the stability of the LBL emulsions.

ii. To investigate the characteristics of flocculated emulsified oil particles in systems with high total solids contents and their deflocculation.

iii. To determine carotenoids degradation and compare the ability of freeze-dried single layer (SL) and LBL emulsions with trehalose as the glass former to protect carotenoids upon storage in the vicinity of the T_g.

iv. To spray dry SL and LBL emulsions with high total solids content having different wall material mixtures.

v. To compare the ability of spray dried SL and LBL emulsions with trehalose and maltodextrin with low DE (9-12) mixture as wall material in preventing degradation of carotenoids upon storage in the vicinity of the T_g.

vi. To determine the ability of SL and LBL powders having trehalose and WPI mixture as wall material to protect carotenoids upon storage in the vicinity of the T_g of the carbohydrate.

vii. To investigate the ability of spray dried SL and LBL emulsions with trehalose and maltodextrin with high DE (23-27) contents as glass formers to protect encapsulated carotenoids upon storage at various temperatures.

viii. To compare the ability of spray dried and freeze-dried systems in preventing carotenoids degradation.

ix. To determine the ability of the powders having carbohydrate-carbohydrate and non-carbohydrate-carbohydrate mixtures as wall materials to protect carotenoids upon storage.

Chapter I

Literature Review

1.1 Emulsion Design

The knowledge of emulsions is commonly applied in the food industry to produce a range of our daily food products. Such products include mayonnaise, butter, beverages, milk, coffee creamer, and sauces, as well as in the pharmaceutical industry various oral products, lotions, and creams. The food and pharmaceutical industries commonly use conventional oil-in-water (O/W) and water-in-oil (W/O) emulsions in the production of emulsions. The use of O/W emulsion also allows utilization of the lipid phase to encapsulate lipophilic bioactives such as carotenoids (β-carotene, lutein, lycopene) (Ribeiro *et al.*, 2006; Cornacchia and Roos, 2011a; Frede *et al.*, 2014), polyunsaturated fatty acids (tuna oil, docosahexaenoic acid (DHA)) (Klinkesorn *et al.*, 2005; Aberkane *et al.*, 2014), vitamins (α-tocopherol) (Zhou and Roos, 2012), phytosterols (β-sitosterol, campesterol) (Engel and Schubert, 2005), and volatile organic compounds (1-butanol, octanol, ethyl hexanoate, trans-2-hexenal, α-ionone, 3-furaldehyde, 2-decanone, D-limonene) (Soottitantawat *et al.*, 2004; Benjamin *et al.*, 2012) that can then be added with glass former solutions and dehydrated using spray drying or freeze-drying to give a solid continuous phase (Drusch *et al.*, 2006; Ramoneda *et al.*, 2011). Nonetheless, there has been increasing interest in the study of emulsions with more complex structures such as multilayer or layer-by-layer (LBL) emulsions (Ogawa *et al.*, 2003a; Güzey & McClements, 2006; Gharsallaoui *et al.*, 2012), multiple emulsions (water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O)) (Su *et al.*, 2008; Hemar *et al.*, 2010), solid lipid particles (Cornacchia and Roos, 2011b) and nanoemulsions (Yuan *et al.*, 2008; Liang *et al.*, 2013).

Formation and Properties

In brief, emulsions are formed by homogenising two immiscible liquids such as water and oil in the presence of emulsifiers to give the dispersed phase and continuous phase (Dalgleish, 2004). The dispersed phase is defined as the tiny droplets that are distributed within the emulsions. The dispersed phase is also known as the discontinuous phase while the continuous phase is also known as the

dispersing phase which is the solution that surrounds the dispersed phase. The use of emulsifiers will prevent the breakdown of emulsions and increase stability of the emulsion by providing protection against aggregation during formation (Walstra, 1993). Proteins, small molecules surfactants, polysaccharides, and phospholipids are the common food-grade emulsifier used in the formation of emulsions (McClements, 1999). Emulsifiers are surface-active molecules that will readily adsorb at the surface of the dispersed phase during homogenization. The most important characteristic of emulsifiers is their ability to reduce interfacial tension significantly and quickly at the interface of the two immiscible liquids (Dickinson, 2003). Milk proteins with amphiphilic properties are commonly used in the preparation of food emulsions. Conformational changes occur in protein during adsorption and are controlled by the amino acid side chains and hydrophobic interactions between the surfaces (Haynes and Norde, 1995). The hydrophilic side of protein will protrude towards the hydrophilic phase while the hydrophobic portion will tend to assemble at the oil surface in an ideal situation (Dalgleish, 1997).

There are differences in the nature of casein, usually present as sodium caseinate (NaCas), and whey protein isolate (WPI) assembling at the oil-water interface that give variations to thickness, stability, and encapsulation properties (Dalgleish, 1997; Hu *et al.*, 2003; Cornacchia and Roos, 2011a). The main protein components present in casein are the mixture of α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein (Swaisgood, 2003) while WPI is comprised of β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), and immunoglobulins (de Wit, 1981). Casein will quickly spread and assemble at the oil-water interface to form a monolayer as cysteine and free thiol groups are not present in the main components of casein (α_{s1} and β) and cross linking of proteins may not occur (Fox and McSweeney, 1998). The arrangement of casein at the oil-water interface depends on the ratio of surface area to the amount of casein as an increase in the amount of casein in the system will lead to an increased packing of casein that pushes it away from the oil-water interface and more into the aqueous phase (Fang and Dalgleish, 1993). Interfacial layer formed by casein is about 10 nm in thickness while WPI formes thinner interfacial layers of about 2 nm and will form multilayers upon saturation of the monolayer (Dalgleish, 2004). WPI appeared to be

stiffer and had lower capability to stabilize the dispersed phase (Hunt and Dalgleish, 1994). Globular WPI will partially unfold and undergo macromolecular reorganization upon its assembly at the oil-water interface (Dickinson and Matsumura, 1994). Consequently, the thiol and disulphide as well as non-polar groups will be exposed causing thiol-disulfide exchange reactions or oxidation that give higher protein-protein hydrophobic interactions and cross-linking (Kinsella, 1982) resulting in the formation of WPI multilayers as observed in the increase of the lipid particle surface protein coverage with increasing amount of WPI (Cornacchia and Roos, 2011b).

An emulsion itself is a complex system and can be described by many properties and characteristics such as the droplet size, interfacial properties, droplet charge, droplet concentration, optical properties, and rheology. Emulsions can be further classified into macroemulsions, nanoemulsions, and microemulsions based on droplet diameter dominating the dispersed phase. A macroemulsion generally has a particle size between 100 nm – 100 μm, nanoemulsions between 20 nm – 100 nm, and microemulsions between 5 nm – 50 nm (McClements, 2010). The size of the dispersed phase that is close to the wavelength of visible light (400-700 nm) in macroemulsion will scatter light strongly giving the turbid or opaque appearance while nanoemulsion and microemulsion appear as transparent as diameter of the dispersed phase is smaller than the wavelength of light giving weak light scattering (McClements, 2010). Emulsion, generally referring to macroemulsion, is thermodynamically unstable and may undergo physical changes such as creaming, flocculation, coalescence, Ostwald ripening, and phase separation that are detrimental to the quality of the emulsion (Dickinson, 1992; McClements, 1999). Nonetheless, microemulsion is considered to be thermodynamically stable as the configurational entropy overcomes the interfacial free energy of emulsion with very small interfacial tension (Hunter, 1989).

1.1.1 Conventional O/W Emulsion

Conventional O/W emulsion is used widely in the food and pharmaceutical industries as it is fairly simple and cheap to produce compared to emulsions with more complex formation. Formation of conventional O/W emulsion requires energy in the form of mechanical agitation such as homogenization to disrupt and mix the immiscible continuous phase (aqueous phase) with the dispersed phase (lipid phase) in the presence of an emulsifier (Dalgleish, 2004). The energy supplied will result in the lipid phase covered with a thin interfacial layer of emulsifier and dispersed within the aqueous phase. The increase in energy supplied during homogenization produced smaller droplets of the dispersed phase as long as the emulsifier present is adequate to cover the surface of the droplets (McClements, 1999). Conventional O/W emulsion is classified as macroemulsion as the dispersed phase is generally present in between 100 nm – 100 μm giving the turbid or opaque appearance of this emulsion. Wall material components such as milk proteins (Hogan *et al.,* 2001a), trehalose (Drusch *et al.,* 2006), corn syrup (Shaw *et al.* 2007), maltodextrin (Ramoneda *et al.,* 2011), or modified starch (Carvalho *et al.,* 2014) can be added into the emulsion and dehydrated by the means of spray drying or freeze-drying to produce a continuous phase of the dehydrated formulation. The presence of an emulsifier at the oil-water interface has beneficial effects towards the stability of O/W emulsion by contributing to the reduced van der Waals attraction, increased steric stabilization, and the presence of electrostatic repulsion (Dalgleish, 1997; McClements, 1999).

All molecules regardless of their nature (polar, non-polar, or ionic) are subjected to van der Waals interactions (Israelachvili, 1992). Therefore, van der Waals interactions also act on macroscopic objects with high amount of molecules for example the dispersed phase in emulsions (Hiemenz 1986) and the interaction is always attractive in systems with similar molecules in a homogenous continuous phase (Hamaker, 1937). The overall van der Waals interaction is strongly affected by the presence of any molecules at the surface of the particles and the presence of emulsifier at the surface of the oil significantly affects interaction between the

dispersed phase particles particularly in close proximity (Vold, 1961; Israelachvili, 1992). Reduction in van der Waals attraction of up to approximately 20% was possible by having molecules absorbed to the oil surface and significant reduction can be achieved by using smaller oil particles or increasing interfacial layer thickness at the surface of the oil particle (Vold, 1961). The presence of emulsifier also improves the stability of conventional O/W emulsions as the result of steric stabilization since the hydrophilic portion of emulsifier can protrude into the aqueous continuous phase that inhibits the emulsifier coated oil particles by the means of mechanical and thermodynamic effects from being in close proximity (Dalgleish, 1999). Factors that improve steric stabilization includes (i) the use of emulsifiers with hydrophobic side groups that attach strongly on the oil surface and hydrophilic side groups that protrude significantly into the continuous phase, (ii) continuous phase that functions as a good solvent for the protruding hydrophilic side groups, (iii) ability of the repulsive forces to function over a distance close to the van der Waals attraction range, and (iv) the presence of thick layer of emulsifier at the oil surface (Dickinson, 1994; McClements, 1999). Increasing the volume fraction of the dispersed phase (φ) to above 0.56 was found to increase steric repulsion as the distance between the oil droplets became smaller and the hydrophilic portion was compacted or/and inter-penetrated (Tadros, 1994). The presence of electrostatic charges on the surface of the emulsifier-covered oil particles also contributed to improvement of the stability of the emulsion by electrostatic repulsion. Positively charged arginine, histidine, lysine, proline, and terminal amino group or negatively charged aspartic acid, glutamic acid, and terminal carboxyl group produced from the ionization of the amino group presents in protein will determine the overall net charge of the protein covered oil particles (Lehninger *et al.* 1993, Damodaran 1996). A bigger value of electrostatic charge will give a stronger electrostatic repulsion between the oil droplets producing emulsions with improved stability. Protein in principle is negatively charged at pH above the isoelectric point (pI) and positively charged at pH below the pI. Therefore, pH has an important role in controlling the stability of emulsion as the protein covered oil particles will possess no electrostatic charge at pH=pI leading to substantial aggregation of the oil droplets (Gu *et al.*, 2004; Kulmyrzaev & Schubert, 2004; Benjamin *et al.*, 2012).

Instability of O/W Emulsion

Nonetheless, conventional O/W emulsion is thermodynamically unstable and will break down with storage as the consequence of creaming, flocculation, coalescence, Ostwald ripening, and phase separation (Dickinson 1992; McClements 1999) particularly upon exposure to environmental stresses that include heating, changes in pH, freezing, dehydration, and ionic strength (McClements, 2010). The main physical instability mechanisms of O/W emulsions are creaming, flocculation, and coalescence. Creaming occurred as gravitational force acted on the emulsion that resulted in the dispersed phase to move upwards as the dispersed phase has lower density compared to the aqueous continuous phase (McClements, 1999). The creaming rate, *v*, of the oil droplets can be determined using Stokes' Law (1) where g is the gravitational acceleration, r is radius of oil droplets, ρ_2 is density of the dispersed phase, ρ_1 is density of the continuous phase, and η_1 is viscosity of the continuous phase (Tan, 2004). Therefore, stability of emulsion towards creaming can be controlled by reducing the size of the oil droplets, matching densities of the dispersed phase and continuous phase, and increasing viscosity of the continuous phase (Ogawa *et al.,* 2004; Mert, 2012). However, the oil droplets remained individual in creaming due to the presence of emulsifier on the oil droplet that allowed the oil particles to be redistributed by mild agitation as long as coalescence has not taken place (Damodaran, 2005). Nonetheless, creaming forces the oil particles to be in close proximity that can lead to flocculation, coalescence, and eventually phase separation (McClements, 1999).

$$v = \frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1} \qquad (1)$$

Gravity forces, thermal energy, and mechanical agitation caused oil droplets in an emulsion to be in constant movement and collide with each other that resulted in the aggregation of the oil particles (Lips *et al.,* 1993, Dukhin and Sjoblom, 1996). Flocculation and coalescence are the two main consequences of aggregation of oil droplets in emulsion (Dickinson and Stainsby, 1982; Dickinson, 1992). Flocculation

occurs as the result of non-covalent van der Waals attractive forces dominating and acting on the oil droplets at the distance of between 0.2 to more than 10 nm (Damodaran, 2005). Aggregation of the oil droplets in flocculation increased creaming rate due to the higher overall particle size and the packing of the oil particles in flocculation also increased the chance of coalescence (Borwankar *et al.*, 1992). Coalescence is said to happen when the interfacial layers of two or more oil droplet particles in close proximity are disrupted to form a single larger oil droplet particle and progress towards a more thermodynamically stable state as the result of reduced contact area between the lipid phase and aqueous phase (McClements, 1999). Amount of emulsifier is critical in the stability of O/W emulsion as insufficient emulsifier to cover all surface area of the oil droplets in emulsion causes rapid coalescence in order to reduce the total surface area for emulsifier coverage (Fang and Dalgleish, 1993). Coalescence of the oil droplets will lead to separation of the emulsion to eventually give a distinct layer of lipid phase on top of the aqueous phase (van Aken, 2004).

1.1.2 Layer-by-Layer (LBL) Interfacial Structure

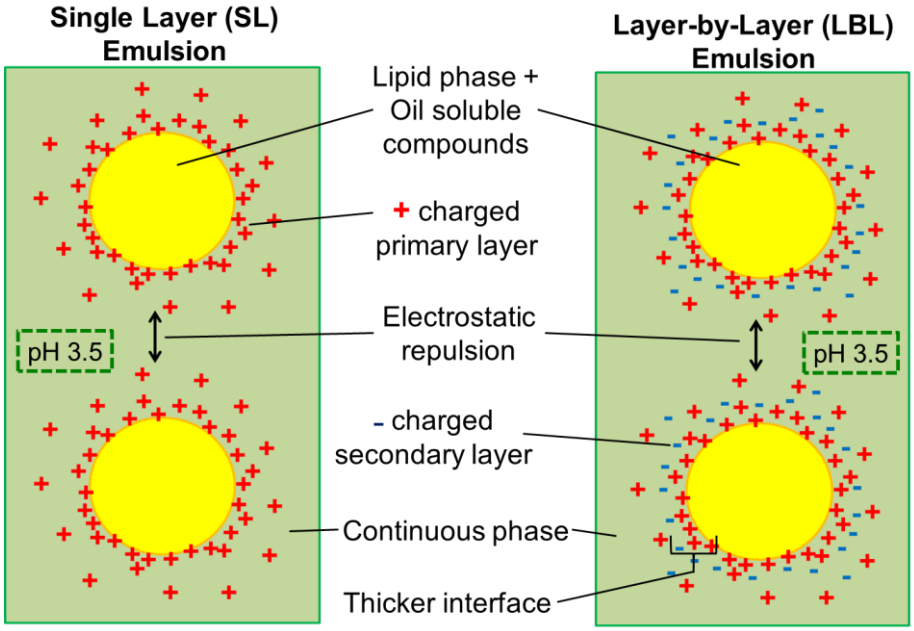

Figure 1.1. Schematic diagram showing the components and the difference in interfacial layer in single layer (SL) and layer-by-layer (LBL) emulsions.

The stability of an O/W emulsion can be further improved using the application of layer-by-layer (LBL) interfacial structure at the surface of the primary layer. LBL or multilayer emulsion is a conventional O/W emulsion with multiple layers of coatings on the oil droplets namely an emulsifier primary layer with a polyelectrolyte secondary layer (McClements, 2010). Figure 1.1 shows the structure of SL and LBL emulsions and the differences of the interfacial layers between SL and LBL systems. Single layer (SL) emulsions are conventional O/W emulsion with a single layer of emulsifier around the oil droplets. The formation of the LBL interfacial structure is obtained through electrostatic attraction between the primary and secondary layers (Moreau *et al.,* 2003; Guzey and McClements, 2006b). The addition of oppositely charged secondary layer will therefore result in the adsorption of the secondary layer around the primary layer covered oil droplets (McClements, 2010). Electrostatic attraction between the primary and secondary layers resulted in the thicker interfacial layer at the interface of the oil droplets (Ogawa *et al.,* 2003b; Klinkesorn *et al.,* 2005; Gharsallaoui *et al.,* 2012). Additional layers can be added to the layers present as long as the outer layer and the layers added were oppositely charged and the most outer layer generally determined the net charge of the system (Guzey and McClements, 2006b). Studies had found that the LBL system was more stable towards external stresses such as heat treatment, changes in pH, freeze-thaw cycles, a_w, ionic strength, ageing, and lipid oxidation (Aoki *et al.,* 2005; Gu *et al.,* 2005; Güzey and McClements, 2006a; Gharsallaoui *et al.,* 2010; Zhou and Roos, 2013; Jiménez-Martín *et al.,* 2015). The presence of a secondary layer at the oil-protein interface will further improve the steric repulsion, reduce the van der Waals interactions, and provide a thicker interfacial layer at the interface of the oil droplets (Moreau *et al.,* 2003; Gu *et al.,* 2005; Harnsilawat *et al.,* 2006; Benjamin *et al.,* 2012). The thicker interfacial layer in LBL system also reduces disruptions of the oil droplets during processing (McClements, 1999; Güzey and McClements, 2006a) such as atomization in spray drying that can provide improved stability and microencapsulation efficiency of the LBL system in the encapsulation of oil-soluble bioactives.

Table 1.1. List of primary layer and secondary layers used in the formation of layer-by-layer (LBL) emulsions.

Primary Layer	Secondary Layer	Citations
β-lactoglobulin	Alginate	Harnsilawat *et al.* (2006)
β-lactoglobulin	Pectin	Güzey and McClements (2006a)
β-lactoglobulin	Pectin	Benjamin *et al.* (2012)
β-lactoglobulin	ι-Carrageenan	Gu *et al.* (2005)
Lecithin	Chitosan	Jiménez-Martín *et al.* (2015)
Lecithin	Chitosan	Ogawa *et al.* (2003a)
Pea protein isolate	Pectin	Gharsallaoui *et al.* (2010)
Pea protein isolate	Pectin	Aberkane *et al.* (2014)
Sodium caseinate	Dextran sulfate	Jourdain *et al.* (2009)
Soy protein isolate	ι-Carrageenan	Zhou and Roos (2013)
Whey protein isolate	ι-Carrageenan	Zhou and Roos (2013)

Methods involved in the preparation of LBL emulsions has been described extensively in the literature (Moreau *et al.*, 2003; Güzey and McClements, 2006a; Klinkesorn *et al.*, 2006; Hou *et al.*, 2010; Gharsallaoui *et al.*, 2012; Aberkane *et al.*, 2014). Generally, preparation of LBL emulsions involves homogenizing the oil phase with an emulsifier containing aqueous phase. This is followed by addition of aqueous phase with the oppositely charged secondary layer components. Primary and secondary layers used in the production of LBL emulsion are shown in Table 1.1 with protein commonly selected as the primary layer. Protein is widely used in the production of LBL emulsions as protein has the ability to be positively or negatively charged with pH of the aqueous phase thus allowing better control of the electrostatic attraction between the primary layer and secondary layer (Guzey and McClements, 2006b). Limited studies done using gum Arabic as the secondary layer in LBL system were reported. Nonetheless, gum Arabic is widely used in the study on formation of complex coacervates (Weinbreck *et al.*, 2003; Jun-xia *et al.*, 2011; de Conto *et al.*, 2013; Butstraen and Salaün, 2014). The formation of LBL interfacial structure can be done using one-step mixing or two-step mixing (McClements, 2010). In one-step mixing, the primary and secondary layers are oppositely charged

at the time of mixing at a predetermined pH. On the other hand, the primary layer and secondary layers have similar charge during mixing followed by the adjustment of pH to give oppositely charged primary layer and secondary layer in two-step mixing.

Therefore, pH of the system has an important role in ensuring that the primary layer and secondary layer are oppositely charged for successful formation of the LBL interfacial structure through electrostatic attraction. Electrostatic attraction between primary layer on the oil droplets and the secondary layer in LBL system is affected by the change in pH that modifies the integrity, packing, and thickness of the LBL interfacial structure (Guzey and McClements, 2006b). The primary layer will have similar charge as the secondary layer at certain pH (depending on the pI or pK_a of the layers) resulting in the detachment of the secondary layer due to electrostatic repulsion between the primary layer and secondary layer, thus the loss of the LBL interfacial structure (Güzey and McClements, 2006a; Harnsilawat *et al.,* 2006; Klinkesorn *et al.,* 2006; Zeeb *et al.,* 2012). The detachment of the secondary layer results in the formation of conventional SL O/W emulsion with lower stability towards environmental stresses. However, the ability of the secondary layer to detach in LBL system enables the LBL interfacial structure to be designed and act as delivery system that allows for the controlled release of encapsulated materials using pH as the trigger (Gudipati *et al.,* 2010; Benjamin *et al.,* 2012).

Flocculation in LBL System

Although improved stability towards environmental stresses was observed in LBL systems, the presence of secondary layer can lead to flocculation of LBL systems, namely bridging flocculation and depletion flocculation. Bridging flocculation occurs if the polyelectrolyte present is insufficient to cover the entire interfaces of the oil droplets while depletion flocculation takes place due to the presence of excess polyelectrolyte in the aqueous phase (Dickinson, 2003; Guzey and McClements, 2006b). Figure 1.2 shows the differences of bridging flocculation and depletion flocculation in LBL systems. The close proximity of the oil droplets in bridging

flocculation and depletion flocculation increased the overall particle size that enhanced creaming rate and in long periods increased the possibility of coalescence in polyelectrolyte containing systems (Dickinson and Pawlowsky, 1997; McClements, 1999; Ogawa *et al.*, 2003a; Güzey and McClements, 2006a; Surh *et al*, 2006; Benjamin *et al.*, 2012). Therefore, it is vital to control the ratio of the secondary layer to primary layer to prevent bridging or depletion flocculation. The polyelectrolyte used as the secondary layer must be sufficient to cover all of the interfaces of the oil droplets, adsorb faster at the interfaces than droplet-droplet collisions, not in excess amount, and possess strong repulsive forces when adsorbed at oil droplets interfaces (Guzey and McClements, 2006b).

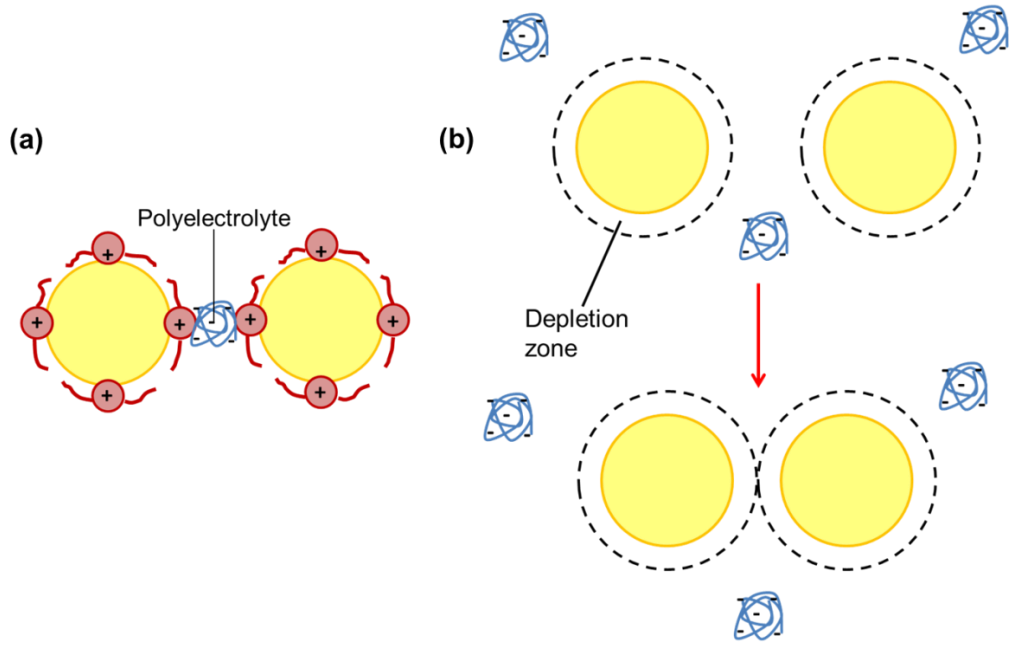

Figure 1.2. Bridging flocculation (a) showing a single polyelectrolyte between the interfaces of two oil droplets and depletion flocculation (b) due to the presence of excess polyelectrolytes occurring in layer-by-layer (LBL) system. (McClements, 1999).

Maximum bridging flocculation occurs as the polyelectrolytes present cover half of the oil droplets interface (Healy and La Mer, 1964). Bridging flocculation is the result of polyelectrolyte attaching to one or more of the interfaces of the oil droplets and connecting the tiny droplets into one big droplet (Guzey and McClements,

2006b). Electrostatic attraction between the polyelectrolyte and two or more oil droplets' interfaces produced attractive forces that were inflexible, strong, and irreversible in bridging flocculation (Blijdenstein *et al.,* 2004). On the other hand, excess polyelectrolyte present in the bulk aqueous phase while no polyelectrolyte present in the small region around the oil droplets, also known as the "depletion zone" that reaches out to a distance of about the radius of the polyelectrolyte from the oil droplets, gave an osmotic potential differences resulting in depletion flocculation (Figure 1.2) (Smith and Williams, 1995; Jenkins and Snowden, 1996; McClements, 2000). Therefore, aqueous phase within the "depletion zone" migrates into the bulk aqueous phase to decrease the concentration gradient resulting in the reduced "depletion zone" and increased aggregation of the oil droplets (McClements, 2000). Magnitude of the attractive forces of the aggregates is controlled by the concentration of polyelectrolytes while the range is controlled by the polyelectrolyte's radius of gyration giving flexible, weak, and reversible attractive forces (Blijdenstein *et al.,* 2004). Depletion attraction between the oil droplets increased with the increased non-sphericity of the secondary phase and the stiff, extended backbones of polyelectrolyte are efficient in promoting depletion flocculation (Piech and Walz, 2000).

1.1.3 Oil-soluble Bioactives

LBL O/W emulsion with improved stability towards environmental stresses (Moreau *et al.,* 2003; Ogawa *et al.,* 2003a; Güzey & McClements, 2006a; Gharsallaoui *et al.,* 2010) presents a great opportunity to incorporate oil-soluble bioactives into food products. Oil-soluble bioactives such as phytosterols (Engel and Schubert, 2005), carotenoids (Cornacchia and Roos 2011b), α-tocopherol (Zhou and Roos, 2012), and polyunsaturated fatty acids (Aberkane *et al.,* 2014) have been incorporated into O/W emulsion. Consuming food products consisting of bioactives has a significant role in preventing degenerative and chronic diseases in humans (van Dokkum *et al.,* 2008). However, the low bioaccessability of carotenoids when present as crystals or within protein complexes of vegetables and fruits decreased absorption during digestion in the gastrointestinal tract (Williams *et al.,* 1998). Oil-soluble bioactives such as

carotenoids are absorbed primarily in the duodenum through the mucosa of the small intestine (Chow, 2000; Noy, 2000; Alves-Rodrigues and Shao, 2004). Bioaccessability of carotenoids can be enhanced with oil as oil improved carotenoids absorption (van het Hof, 2000). Therefore, carotenoids can be dissolved and incorporated into the lipid phase of O/W emulsions to improve its bioaccessability and at the same time the emulsion will encapsulate and act as a delivery system for the carotenoids (McClements *et al.,* 2007; Yuan *et al.,* 2008; McClements, 2010; Cornacchia and Roos 2011a; Frede *et al.,* 2014). A good delivery system should have high loading capacity and retention of the bioactives, improve the bioaccessability of the encapsulated bioactives, and increase stability of the encapsulated bioactives towards chemical degradation (McClements *et al.,* 2007). The oil phase can then be homogenized with an emulsifier containing aqueous phase as in the usual steps to prepare O/W emulsion. Carotenoids, commonly present in the crystalline form need to be added at amounts below the saturation concentration in the lipid phase or by heating the lipid phase to ensure complete dissolution of the crystalline carotenoids (McClements and Li, 2010). The carotenoids containing emulsion can be blended with glass-forming hydrophilic components and dehydrated to obtain a solid continuous phase of the dried formulation (Drusch *et al.,* 2006; Klinkesorn *et al.,* 2006; Gharsallaoui, *et al.,* 2010; Ramoneda *et al.,* 2011).

Carotenoids

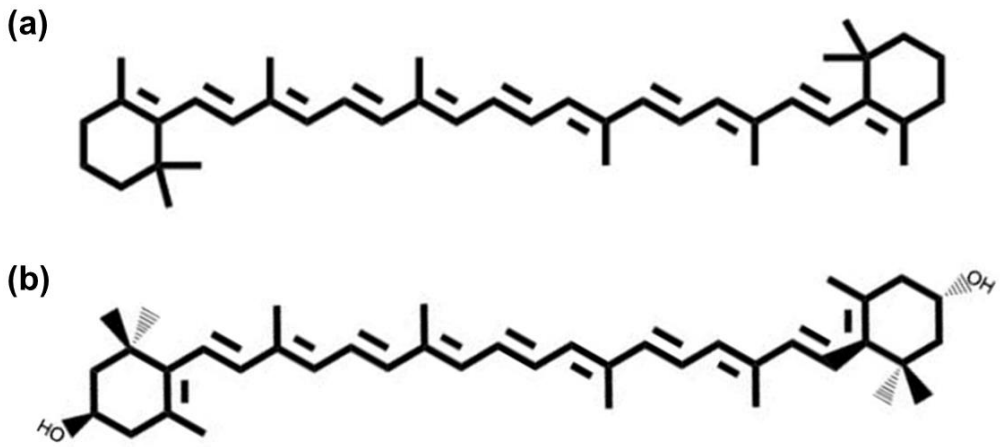

Figure 1.3. Structures of (a) all-trans-β-carotene (b) and lutein (Boon *et al.*, 2010).

There have been increasing interests in producing carotenoids containing foods as carotenoids have potential health benefits by preventing diseases (Boon *et al.*, 2010). Consumption of carotenoids was found to reduce the likelihood of cardiovascular diseases, and macular degeneration (Krinsky, 1993). Carotenoids are very hydrophobic red, yellow, and orange natural pigments, which contain 40-carbon molecules, identified by the conjugated double bonds with delocalized π-electrons and are predominantly found in fruits and vegetables (Britton, 1995; Failla *et al.*, 2007; Boon *et al.*, 2010). Plants are capable to synthesize carotenoids and they are often confined in the subcellular organelles such as chromoplasts and chloroplasts (Khoo *et al.*, 2011). Carotenoids may become linked with protein and act in photosynthesis as an accessory pigment in the chloroplasts. Carotenoids are also stored as crystalline or non-crystalline compounds in the chromoplasts (Bartley *et al.*, 1995). Carotenoids in plants occur naturally as all-trans isomers with tiny amounts of cis-isomers but when released or extracted for example in food processing isomerization to cis-isomers may take place (Schieber and Carle, 2005; Khoo *et al.*, 2011). Cis-isomers are often more polar, more stable towards crystallization, and they have better solubility in hydrocarbon solvents and oils (Castenmiller and West, 1998). Carotenoids can be further classified into carotenoids without oxygen, also known as carotenes (β-carotene), and carotenoids with oxygen, xanthophylls (lutein) (McClements *et al.*, 2007). The differences in all-trans-β-carotene and lutein structures are shown in Figure 1.3 showing the presence of two hydroxyl groups (-OH) in lutein.

Carotenes can be described using the general formula of $C_{40}H_{56}$ with the major forms of carotenes are α-carotene and β-carotene (Khoo *et al.*, 2011). All-trans-β-carotene has the highest vitamin A activity and vitamin A conversion rate among the provitamin A carotenoids (Grune *et al.*, 2010). Carotenoids with potential vitamin A precursors have at least one unsubstituted β-ionone ring with a polyene side chain with no less than 11 carbon atoms (Castenmiller and West, 1998). It was also found that β-carotene possess anticancer properties and antioxidant activities, and may provide protection against heart disease (Bendich and Olson, 1989; Omenn *et al.*, 1996; Albanes, 1999). The main carotenoid found in human diet is β-carotene

(Johnson, 2002). β-carotene is found mainly in vegetables and fruits with red, orange, and yellow colours (mangos, carrots) and also in green leafy vegetables (spinach, lettuce) (Holden *et al,* 1999). β-carotene with 11 double bonds found naturally is orange in colour (Rao and Rao, 2007). The lipophilic β-carotene found in vegetables and fruits may exist in crystalline form for example in tomatoes and carrots or non-crystalline form in lipid droplets in mangoes (Harris and Spurr, 1969; Vásquez-Caicedo *et al.,* 2006). Typically, β-carotene exists as all-trans-β-carotene in nature with tiny amounts of cis-β-carotene. Common forms of β-carotene occur in the order of all-trans-β-carotene > 9-cis-β-carotene > 13-cis-β-carotene > 15-cis-β-carotene (Guo *et al.,* 2008). The various isomers of β-carotene gave different bioconversion of the carotenoids to retinol with 9-cis-β-carotene and 13-cis-β-carotene having approximately half of the bioconversion of all-trans-β-carotene (Zechmeister, 1949; Bauernfeind, 1972). There is also a difference in intestinal absorption of carotenoids between all-trans and cis-β-carotene where all-trans-β-carotene was found to have significantly higher postprandial concentration than 9-cis-β-carotene (Johnson *et al.,* 1996).

Xanthophylls on the other hand are oxygenated carotenoids of general formula of $C_{40}H_{56}O_2$ with lutein carrying two hydroxyl groups; one at each end of the molecule (Figure 1.3) (Matsuno *et al.,* 1986; Alves-Rodrigues and Shao, 2004). Lutein has no provitamin A activity as it is not a substrate for the enzyme 15, 15'-monooxygenese to cleave into vitamin A (Alves-Rodrigues and Shao, 2004). It was shown that lutein has the ability to reduce cataracts, macular degeneration and light-induced skin damage (Khachik *et al.,* 1997; Ribaya-Mercado and Blumberg, 2004). Lutein absorbs blue light and therefore functions as blue-light filter to provide tissues protection against phototoxic damages (Gerster, 1991; Schalch and Weber, 1994). Besides, there have been indications that lutein is able to enhance skin's health, as well as to prevent stroke, coronary heart disease, and breast cancer (Johnson, 2000; Alves-Rodrigues and Shao, 2004). Lutein can be found in vegetables and fruits such as persimmon, kale, corn and spinach as well as egg yolk (Matsuno *et al.,* 1986; Holden *et al.,* 1999). Lutein available commercially is obtained from marigold flowers (Sowbhagya *et al.,* 2004). The ability of lutein to absorb blue light results in

the yellow colour of lutein (Khoo *et al.,* 2011). Bioavailability of lutein is higher than that of all-trans-β-carotene as the more polar lutein tends to appear at the outer layer lipid micelles in the gastrointestinal tract that allows easier absorption by the enterocyte membranes resulting in about five times higher uptake than is found for all-trans-β-carotene (van het Hof *et al.,* 1999; Yeum and Russell, 2002).

1.2 Microencapsulation and Dehydration

It is known that dehydration process reduces packaging materials, storage space, and shipping cost (Landström *et al.,* 2000). Above all, dehydration reduces powder stickiness and lipid oxidation while maintaining the initial emulsion structure (Millqvist-Fureby, 2003). The resulting carotenoids containing O/W emulsion with wall materials or glass formers can be dehydrated to give a continuous matrix that encapsulates the lipid phase, also known as microencapsulation. Microencapsulation is a method using homogeneous or heterogeneous matrix to entrap tiny droplets giving capsules with different properties acting as a physical barrier between the encapsulated materials and the environment that minimize contact and reaction with environmental oxidizing agents for example metal ions, light, and heat (Gharsallaoui *et al.*, 2007; Ye *et al.,* 2009). Microencapsulation is frequently used in the food industry to reduce concentration of the encapsulated materials when used in small quantity, to cover unwanted taste of the encapsulated materials, to enable controlled release of the encapsulated materials, to allow easier handling, to reduce migration of the encapsulated materials towards outside of the matrix, and to decrease reaction between the encapsulated materials and the environment (Shahidi and Han, 1993). Methods such as spray drying, freeze-drying, extrusion, fluidized-bed coating, coacervation, and spray chilling are commonly used in the microencapsulation of food systems (Desai and Park, 2005). Glass formation and the glassy structure are the foundation of matrices formed in spray drying and freeze-drying (Roos, 2009a). Microencapsulation can be used in combination with the LBL interfacial structure to provide the encapsulated bioactives with better protection against environmental stresses.

Microencapsulation

Microcapsules are defined as tiny spheres having one or more glass former mixtures around the encapsulated materials consisting of oil droplets, crystalline materials, or solids with the size of 200 nm up to 5000 μm (Ré, 1998; Gharsallaoui *et al.*, 2007). Microencapsulation provides food industry with the opportunity to add and encapsulate bioactives into food products, provide protection to the encapsulated bioactives during storage, produce new nutritional products, act as a mechanism for controlled release, and increase desirability for the end product (Balassa and Fanger, 1971). Glass formers or wall materials such as carbohydrates (maltodextrins, sucrose), hydrocolloids (gum Arabic, agar), and protein (whey protein, casein) have been used as a single ingredient or as mixtures for microencapsulation in the food industry (Shahidi and Han, 1993). These glass formers or wall materials are commonly added upon emulsification of the dispersed phase especially oil droplets with an emulsifier present in the aqueous phase (Heinzelmann and Franke, 1999; Klinkesorn *et al.*, 2006; Shaw *et al.*, 2007; Zhou and Roos, 2013). Table 1.2 provides reported data of the glass formers or wall materials and dehydration methods that are used for microencapsulation of O/W emulsion. Carbohydrates as glass formers and spray drying as dehydration method are frequently used for microencapsulation. Superior glass formers for microencapsulation should possess properties such as high solubility, low viscosity, non-reactive towards the encapsulated materials, the capability to maintain the encapsulated materials during storage, and provide the encapsulated materials with maximal protection against environmental stresses, economical, and food-grade quality (Shahidi and Han, 1993). Different types of microcapsules may be obtained such as basic spheres, irregular, tiny droplets of encapsulated materials dispersed within the glass formers, a few well defined encapsulated material droplets within the glass formers, and multi-walled depending on the microencapsulation method, glass formers mixtures, and physico-chemical properties of encapsulated materials (Gharsallaoui *et al.*, 2007).

Table 1.2. Glass formers or wall materials and dehydration methods commonly used in microencapsulation of oil-in-water (O/W) systems.

Glass formers/ Wall materials	Dehydration Method	Citations
Corn syrup solids	Spray drying	Klinkesorn *et al.* (2006)
Corn syrup solids	Spray drying	Shaw *et al.* (2007)
Glucose syrup	Spray drying	Drusch *et al.* (2006)
Gum Arabic	Spray drying	McNamee *et al.* (1998)
Lactose	Freeze-drying	Heinzelmann and Franke (1999)
Lactose-Milk protein isolate	Freeze-drying	Zhou and Roos (2012)
Lactose-Soy protein isolate	Freeze-drying	Zhou and Roos (2012)
Maltodextrin	Freeze-drying	Heinzelmann and Franke (1999)
Maltodextrin	Spray drying	Jiménez-Martín *et al.* (2015)
Sodium caseinate-Corn syrup solids	Spray drying	Hogan *et al.* (2001b)
Sodium caseinate-Maltodextrin	Spray drying	Hogan *et al.* (2001b)
Trehalose	Freeze-drying	Zhou and Roos (2013)
Trehalose	Spray drying	Drusch *et al.* (2006)
Whey protein isolate	Spray drying	Moreau and Rosenberg (1998)
Whey protein isolate	Spray drying	Rosenberg and Sheu (1996)
Whey protein isolate-Lactose	Spray drying	Rosenberg and Sheu (1996)

Carbohydrates such as maltodextrins, corn syrup solids, lactose, and trehalose have been used as glass formers in microencapsulation (Heinzelmann and Franke, 1999; Shaw *et al.,* 2007; Ramoneda *et al.,* 2011; Zhou and Roos, 2013; Jiménez-Martín *et al.,* 2015). Carbohydrates are considered excellent glass formers as they have low viscosities and great solubility even at high concentrations that enhance workability although carbohydrates do lack interfacial properties (Gharsallaoui *et al.,* 2007). Trehalose has been used successfully for the microencapsulation of bioactives (Elizalde *et al.,* 2002; Drusch *et al.,* 2007; Zhou and Roos, 2013). Trehalose, a disaccharide, is a non-reducing sugar made up of two glucose molecules linked by α, α-1, 1-glycosidic linkage and it is conformationally stable (Richards and Dexter,

2011). Commercial production of high purity crystalline trehalose is done using starch as the raw material in the presence of several enzymes such as (i) isoamylase to de-branch starch giving amylose, (ii) maltooligosyl-trehalose synthase to form the α, α-1, 1 bonds from α-1, 4 bonds at the reducing end of glucose giving amylosyl-trehalose, and (iii) maltooligosyl-trehalose trehalohydrolase to hydrolyze the α-1, 4 bonds between trehalose and amylosyl-moiety giving trehalose (Nakada *et al.,* 1995a, Nakada *et al.,* 1995b; Sugimoto, 1995). As trehalose is a non-reducing sugar, it will not contribute to Maillard reaction and therefore trehalose can be used in food products where browning is not desirable. The high glass transition temperature (T_g) of trehalose of around 115°C (Zhou and Roos, 2011) supports its use in microencapsulation. The T_g of trehalose is higher than that of many other disaccharides (Green and Angell, 1989). Trehalose forms dihydrate crystals with the chemical formula $C_{12}H_{22}O_{11}\cdot 2H_2O$ and molecular weight of 378.33 (Richards and Dexter, 2011). Water migrates to trehalose to form trehalose dihydrate thus giving desiccant effect to the amorphous phase that can give an increased T_g of the system (Aldous *et al.,* 1995; Schebor *et al.,* 2010).

However, caking, structural collapse, and crystallization of the amorphous sugar may occur during storage after microencapsulation (Gharsallaoui *et al.,* 2007). Crystallization of the amorphous sugar results in the release of the encapsulated bioactives containing lipid and rapid degradation of the encapsulated bioactives as they become unprotected within the matrix (Buera *et al.,* 2005). However, mixing of amorphous sugars with other carbohydrates may retard crystallization of the amorphous sugar (Mazzobre *et al.,* 1997; Gabarra and Hartel, 1998; Kouassi and Roos, 2001; Potes *et al.,* 2012). Maltodextrin may be used as a non-sweet polysaccharides made up of α-(1→4)-linked D-glucose obtained from the hydrolysis of starch. Maltodextrins have a dextrose equivalent (DE) < 20 (maltodextrin with low DE) while corn syrup solids have DE > 20 (maltodextrin with high DE) (Shahidi and Han, 1993; Chronakis, 1998). Maltodextrins or corn syrup solids are commonly used for microencapsulation as they are of low cost, effective, low viscosity at high total solids, and obtainable of a wide range of DE (DE 4-42) with decreased average molecular weight with increasing DE (Desobry *et al.,* 1997). Maltodextrins with low

DE (DE 9-12, ~160°C, anhydrous) have a higher T_g than maltodextrins with high DE (DE 23-27, ~140°C, anhydrous) (Potes *et al.,* 2012). Nonetheless, the T_g of amorphous sugar-maltodextrin mix was less significant than the presence of a high DE maltodextrin having smaller molecular size (Gabarra and Hartel, 1998). Maltodextrins with high DE are better in inhibiting crystallization as the smaller molecular size was more efficient in interrupting nucleation and crystal growth of the amorphous sugar (Potes *et al.,* 2012). Systems with a higher DE maltodextrin were also found to give better microencapsulation efficiency and retention of the encapsulated bioactives upon storage than those containing lower DE (Anandaraman and Reineccius, 1986; Wagner and Warthesen, 1995; Hogan *et al.,* 2001b). The presence of long chain saccharides in higher quantities in maltodextrins with low DE gave less dense and less flexible glassy matrix that increased oxygen permeability (Wagner and Warthesen, 1995).

Proteins, especially milk proteins such as whey protein and casein too have been used as wall materials for microencapsulation due to their exceptional surface active and colloid-stabilizing properties (Young *et al.,* 1993a; Dickinson, 1997; Moreau and Rosenberg, 1998; Hogan *et al.,* 2001; Gharsallaoui *et al.,* 2007). However, the use of protein alone as wall material is not very efficient in protecting the encapsulated materials against degradation but can be improved with the addition of carbohydrate as glass former (Kagami *et al.,* 2003; Bae and Lee, 2008). This was due to the lower particle density obtained in systems having protein as wall material compared to those having only carbohydrate as glass formers (Desobry *et al.,* 1997; Moreau and Rosenberg, 1999; Drusch *et al.,* 2006). The lower particle density and higher wall material porosity of systems with protein as wall material (Moreau and Rosenberg, 1998) allows easier migration of oxygen and metal ions towards the encapsulated materials. Nonetheless, addition of carbohydrates can increase density and reduce porosity of the wall material (Moreau and Rosenberg, 1999). Mixture of protein (whey protein isolate; whey protein concentrate; sodium caseinate) and carbohydrate (maltodextrin; corn syrup solid; lactose; highly branched cyclic dextrin) has been used successfully for microencapsulation (Young *et al.,* 1993a; Young *et al.,* 1993b; Moreau and Rosenberg, 1999; Kagami *et al.,* 2003; Sliwinski *et*

al., 2003; Bae and Lee, 2008). However, the highly surface active protein tends to migrate to the particle surface (air-water interface) in protein-carbohydrate mixtures giving a protein-rich layer that predominates at the air-water interface upon dehydration (Fäldt and Bergenståhl, 1994; Adhikari *et al.,* 2009; Wang *et al.,* 2013). This results in the discontinuity between the protein phase and carbohydrate phase used for microencapsulation in protein-carbohydrate systems and the encapsulation of the bioactives containing lipid phase within two separate phases giving differences in the stability of encapsulated bioactives. The presence of protein at the interface also resulted in surface indentations in the powders obtained from spray drying (Fäldt and Bergenståhl, 1994; Wang *et al.,* 2013). The increased flexibility of the particle surface due to the presence of protein inhibits skin rupturing during drying resulting in increased surface roughness and wrinkles and is a norm in spray dried milk and milk containing products (Wang and Langrish, 2010). Microencapsulation efficiency was also found to improve in systems with protein-carbohydrate mixtures as glass formers (Young *et al.,* 1993a; Young *et al.,* 1993b). Besides, mixtures of protein and carbohydrates were capable in retarding crystallization of the amorphous sugar components (Jouppila and Roos, 1994; Haque and Roos, 2004).

1.2.1 Spray Drying

Spray drying is the most common microencapsulation method used in the food industry with many applications in the microencapsulation of lipophilic bioactives (Drusch *et al.,* 2006; Shaw *et al.,* 2007; Aberkane *et al.,* 2014; Mahfoudhi and Hamdi, 2014). Among the advantages of spray drying is the availability of many types of spray dryers for various applications. Spray drying principle is a simple drying operation that allows continuous, full automatic control, and consistent powder flow obtained at constant drying conditions (Vega-Mercado *et al.,* 2001). The main steps involved in spray drying are atomization, hot air-droplets contact, water evaporation from droplets, and separation of dry particles from humid air (Murugesan and Orsat, 2012). The function of atomization is to increase the surface area of the feed for maximum heat transfer between the droplets and hot air

(Gharsallaoui *et al.,* 2007). Common atomizers include centrifugal disk, pressure nozzle, and pneumatic atomizer (Masters, 1968). Centrifugal disk operates between 4000 to 20000 rpm in pilot scale by fast release of uniform tiny droplets of the feed from the rapidly rotating disk's perimeter (Balassa and Fanger, 1971). Hot air in a spray dryer can be supplied using a co-current setup where the feed and hot air flow in the same direction or counter current setup where the feed and hot air flow in opposite directions (Gharsallaoui *et al.,* 2007). Spray drying with a co-current setup is widely used in food industry compared to other setups such as counter current operation (Zbicinski *et al.,* 2002). An inlet air temperature of 150°C to 220°C is common in the co-current setup allowing instantaneous evaporation of water to take place (Fleming, 1921). Typical inlet temperatures of 160-180°C and outlet temperatures of 70-90°C were commonly reported for microencapsulation (Rosenberg and Sheu, 1996; Drusch *et al.*, 2006; Bae and Lee, 2008; Aberkane *et al.,* 2014; Jiménez-Martín *et al.*, 2015).

Water evaporation from droplets in spray drying is the consequence of mass and heat transfer where heat from the hot air reaches droplet surfaces by convection and the heat provides the latent heat for water evaporation from the droplets (Murugesan and Orsat, 2012). Three consecutive steps can be identified in drying of the droplets: (i) namely increasing temperature of the droplets to a steady temperature known as the wet bulb temperature; (ii) water evaporation from the droplets at constant vapour partial pressure and temperature; and (iii) crust formation with the rapid reduction in drying rate that relies upon the rate of water diffusion through the crust (Gharsallaoui *et al.,* 2007). Crust formation or solidification of the droplet surface during drying yields free-flowing powders and prevents caking or stickiness of the powders within the spray dryer (Roos, 2010). Rapid instantaneous water evaporation of the droplets occurring in the dryer keeps the droplets temperature low until the dry state is achieved as heat is consumed by vaporizing the water (Gharsallaoui *et al.,* 2007); an effect also known as "evaporative cooling". Drying of droplets in a spray dryer usually occurs over 5-100 s although a well-designed dryer will have droplets residence time of 15-30 s in the drying zone (Fogler and Kleinschmidt, 1938; Corrigan, 1995). The drying particles achieve their final shapes as all water

evaporation has taken place (Ré, 1998). Powders obtained from the spray drying process are collected at the base of the drying chamber or guided to flow out with the outgoing humid air for separation in a cyclone or bag filter (Murugesan and Orsat, 2012). The design of a spray dryer is often based on trial-and-error, experience, and pilot scale studies due to the complexity of the steps involved in spray drying (Gharsallaoui *et al.,* 2007).

Spray Dried Materials

Properties of the powders obtained from spray drying can be manipulated by the change in the feed rate, powder temperature, feed's total solids content, atomization method, and air-flow configuration displaying the versatility of spray drying (Vega-Mercado *et al.,* 2001). Spray dried particles generally acquire a spherical shape with the dispersed phase trapped within the continuous phase as they fall through the dryer (Dziezak, 1988). Microcapsules having only carbohydrate as a glass former tend to have smooth spherical surfaces with some cracks due to brittleness of the carbohydrate while the addition of surface-active protein as a wall material results in microcapsules with wrinkled and folded surfaces, less spherical shape, and less cracks present (Fäldt and Bergenståhl, 1994; Drusch *et al.,* 2006; Wang and Langrish, 2010; Wang *et al.,* 2013). The dispersed phase is generally trapped within the continuous phase in the presence of air vacuoles as shown in the schematic diagram of a spray dried particle in Figure 1.4. Formation of air vacuoles in spray dried particles is frequently observed (Aguilera and Stanley, 1999; Keogh *et al.,* 2001; Soottitantawat *et al.,* 2005; Klinkesorn *et al.,* 2006; Bae and Lee, 2008). Air vacuoles are formed as a result of thermal expansion of trapped air bubbles in the liquid feed and air bubbles formed during atomization in spray drying (Duffie *et al.,* 1953; Verhey, 1972). Trapped oxygen within the spray dried particles is capable to accelerate degradation of encapsulated bioactives. Such reactions are commonly observed as a rapid initial degradation of spray dried materials (Desobry *et al.,* 1997; Desobry *et al.,* 1999; Jimenez *et al.,* 2004). Powders obtained from spray drying can be agglomerated to increase the overall particle size. Agglomerates also host channels between the bonded particles for water uptake giving better rehydration

during reconstitution of the powders (Roos, 2010). Controlled plasticization of the powder particles' surfaces results in agglomeration through adhesion of powder particles, interparticle liquid bridges developments, and finally agglomeration of the powders (Peleg and Mannheim 1977; Roos 1995; Palzer 2005).

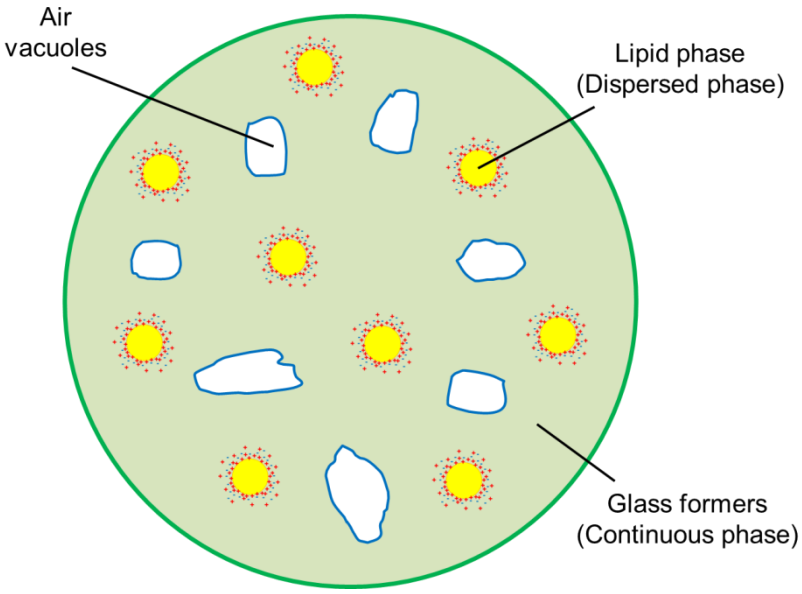

Figure 1.4. Schematic diagram of a spray dried particle showing the dispersed phase and trapped air vacuoles within the continuous phase.

1.2.2 Freeze-Drying

Freeze-drying is a superior dehydration method for the microencapsulation of heat-sensitive materials as drying occurs at a low temperature and pressure (Shahidi and Han, 1993). Microencapsulation of bioactives using freeze-drying has been successfully achieved (Heinzelmann *et al.*, 2000; Ramoneda *et al.*, 2010; Zhou and Roos, 2013; Mahfoudhi and Hamdi, 2015). Freeze-drying produced an amorphous, glassy structure through freezing of the water phase followed by sublimation of the ice crystals and elimination of unfrozen water from the freeze-concentrated materials (Roos, 1997). The three main phases in a standard freeze-drying process are freezing, primary drying, and secondary drying (Tang and Pikal, 2004). Water forms ice and becomes separated from the solutes to give a freeze-concentrated dispersed phase in freezing, ice sublimation occurs in primary drying, and lastly residual water

or unfrozen water is desorbed from the freeze-concentrated solids in which is referred to as secondary drying (Tang and Pikal, 2004). Sublimation of the ice crystals formed in freezing leaves pores within the adjoined solid structure network to give a porous structure (Harnkarnsujarit *et al.*, 2012a). Freeze-drying requires a temperature below the triple point of water for sublimation to occur and is commonly achieved by bringing the pressure to below that of the triple point which will be adjusted accordingly to regulate the sublimation temperature (Roos, 1997). Glass formation stops the formation of ice resulting in the maximally freeze-concentrated amorphous structure at low enough freezing temperatures (Slade and Levine, 1988; Roos and Karel, 1991a). The maximally freeze-concentrated structure is achieved at a temperature between the glass transition temperature of the maximally freeze-concentrated structure (T_g') and onset for ice-melting temperature of the maximally freeze-concentrated structure (T_m') (Roos, 1997). Maximally freeze-concentrated carbohydrate systems are generally made up of approximately 80% (w/w) noncrystalline solids and 20% (w/w) unfrozen water with T_g' considerably higher than the T_g of pure water (Roos, 1993). It is crucial in obtaining the maximally freeze-concentrated amorphous structure to ensure the ability of the unfrozen phase to support itself during sublimation (Pehkonen and Roos, 2008). Nonetheless, freeze-drying is less appealing for microencapsulation as it costs 30-50 times more than spray drying and is a time consuming process (Barbosa-Cánovas and Vega-Mercado, 1996; Desobry *et al.*, 1997).

Freeze-Dried Materials

Final product obtained from freeze-drying readily rehydrates, is highly porous, hygroscopic, and brittle (Roos, 1997). Figure 1.5 shows the dispersed and continuous phases in the porous structure of a freeze-dried material due to the presence of pores from ice sublimation. Pores size of freeze-dried materials is affected by the freezing temperature. Freezing at higher temperatures results in bigger pores as the lower cooling rate results in a slower nucleation rate, shorter time for crystal growth, and bigger ice crystals while smaller pores were obtained at lower freezing temperatures due to the higher cooling and nucleation rates (Harnkarnsujarit *et al.*, 2012a). The

pores obtained at higher freezing temperatures were heterogeneous whereas more homogeneous pores were found at lower freezing temperatures (Kang *et al.*, 1999). The bigger and heterogeneous pores also gave thicker pore membranes in systems with higher freezing temperatures in contrast with the thinner pore membranes in systems with lower freezing temperatures (Harnkarnsujarit *et al.*, 2012a). Freeze-drying should be completed at temperatures below the T_m' to maintain the volume of the freeze-concentrated solids and facilitate ice crystals removal as temperatures above the T_m' increase the amount of unfrozen water (Roos, 1997). The more porous structure obtained from freeze-drying has an effect on the stability of the encapsulated bioactives as degradation of bioactives was more rapid in freeze-dried systems than in spray dried materials (Mahfoudhi and Hamdi, 2014; Mahfoudhi and Hamdi, 2015).

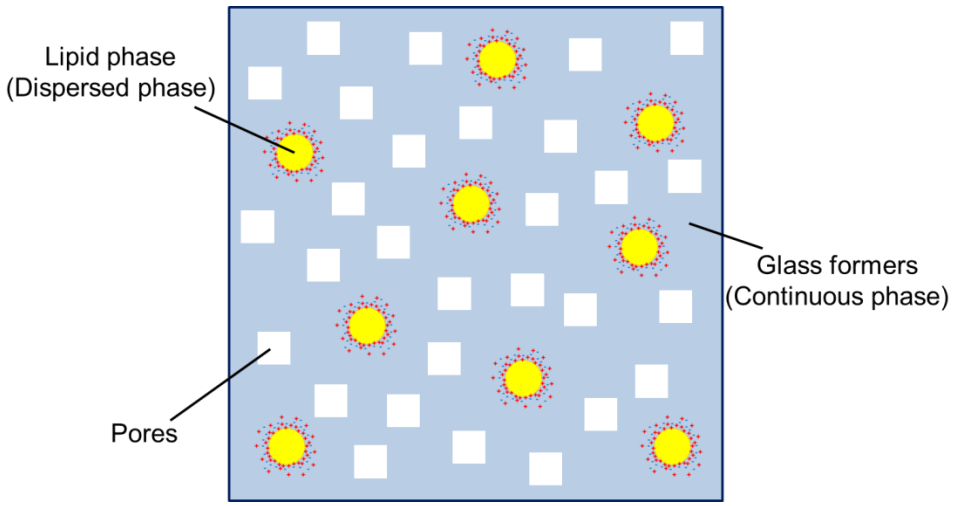

Figure 1.5. Schematic diagram of the dispersed phase and high amount of pores present within the continuous phase in freeze-drying giving a more porous structure.

1.3 Stability of Dehydrated Materials

Table 1.3. Differences in the physical properties of amorphous and crystalline solids (Bhandari and Roos, 2012).

Physical Properties	Amorphous Solid	Crystalline Solid
Softening Temperature	Low (T_g)	High (Melting)
Chemical reactivity	High	Low
Solvent Interaction	Fast	Slow
Compressibility	Good	Poor
Density	Low	High
Porosity	High	Low
Mechanical Strength	Brittle	Strong
Hygroscopicity	High	Low
Heat of Solution	Exothermic	Endothermic

Solid food materials may exist as amorphous or crystalline solids as well as partially crystalline solids. Amorphous solids are obtained from the rapid cooling of a liquid melt or rapid dehydration of solutions where the molecules present will have insufficient time to rearrange and, therefore, maintain their original orientation to give solids with liquid-like structures (Roos, 2002; Liu *et al.*, 2006). Food processing steps such as spray drying and freeze-drying generally produce amorphous solids (Drusch *et al.,* 2006; Ramoneda *et al.,* 2011). Amorphous solids are known to possess random and disordered molecular structures as the exact position of molecules at any given time cannot be established (Roos, 2010). The presence of the random, disordered, and high free volume state of molecules in amorphous structures enhance external interactions such as sorption of water (Bhandari and Roos, 2012). The amorphous solids can be classified as glassy or rubbery materials. The glass transition from glassy to rubbery state or vice versa is reversible and occurs over a glass transition temperature range, where T_g is taken from the onset or midpoint of temperature of the transition (Nelson and Labuza, 1994). The amorphous solids will be glassy at temperatures below the T_g and become rubbery at temperatures above the T_g. Viscosity of materials in glassy states is very high with values of exceeding

10^{12} Pa s. The viscosity decreases significantly over and above glass transition (Roos, 1995). The motion of molecules in glassy materials below the T_g are restricted to only rotations and vibrations giving solid-like characteristics while the increase of temperature to above the T_g results also in translational mobility of the molecules and molecules exist in supercooled liquid-like state with viscous flow characteristics (Sperling, 2006). Nonetheless, the metastability of the amorphous solids often results in crystallization in the rubbery state to form the thermodynamically equilibrium and stable crystalline solids (Roos, 1995). Table 1.3 shows the main differences in physical properties commonly associated with the amorphous and crystalline solids. Molecules in crystalline solids are arranged in a regular lattice with symmetrical long-range translational orientation (Liu *et al.,* 2006).

1.3.1 Glass Transition

Glass transition is characteristic of amorphous solids although glass transition has been poorly defined with constantly debated origins (Kauzmann, 1948; Angell, 2002). Glass transition is thermodynamically regarded as a second order phase transition as the transition shows a step change in heat capacity and it occurs without latent heat (Liu *et al,* 2006). Second-order transitions are described by the discontinuity in the second derivative of Gibbs energy or chemical potential, a step change in the heat capacity, and a step change in thermal expansion coefficient at the transition temperature (Roos, 1995). Glass transition is known as a kinetic and relaxation process related with α-relaxation that correlates to the translational motion of the molecules (Liu *et al,* 2006). The measurement of glass transition using a differential scanning calorimetry (DSC) shows an endothermic step change parallel with the change in heat capacity (ΔC_p) over the T_g range (Roos, 2010). Glass transition has a non-equilibrium nature and takes place over a temperature range with the possibility of showing time-dependent properties (Roos, 2012). Therefore, materials showing glass transition also require “time” as an extra parameter on top of thermodynamic conditions due to the non-equilibrium state of the materials (Roos, 1995; Roos, 2010).

The three principal theories of glass transition are the free-volume theory, kinetic theory, and thermodynamic theory (Roos, 2010). The free volume theory is based on the assumption that molecular motion relies on the existence of holes or free volume between molecules to allow molecular movement and rearrangement (Roos, 1995). Molecules present will move into the holes and switch places with one another that appear as the molecular movement (Sperling, 2006). The free volume of systems in glassy state is predicted to be between 2% to 11.3% of the overall volume and free volume may increase significantly at the T_g with the substantial increase in thermal expansion coefficient (Ferry, 1980). The size of the molecules also affects molecular movement as larger molecules require larger holes or higher free volume for diffusion (Nelson and Labuza, 1994). The free volume and viscosity relationship can be applied to demonstrate the relevance of Williams-Landel-Ferry (WLF) relationship fitting to relaxation time and viscosity data above glass transition (Williams *et al.,* 1955; Slade and Levine, 1991; Sperling, 2006; Roos, 2010). On the other hand, glass transition is defined as the temperature where the relaxation time of segmental motions of polymer chains approaches the experimental time scale (Roos, 1995). In kinetic theory, the change in free volume of a material around glass transition is taken into consideration and it was proposed that reduction in experimental frequency results in glass transition at a lower temperature (Sperling, 2006). Lastly, thermodynamic theory acknowledges the presence of a real second-order transition by employing the idea of equilibrium and thermodynamic requirements (Roos, 1995). However, the real thermodynamic state change happens at infinitely long time scales despite the fact that glass transitions are time-dependent (Gibbs and DiMarzio, 1958). The thermodynamic theory allows the prediction of changes in T_g with the change in variables such as molecular weight and diluent content (Sperling, 2006).

Glass Transition Temperature

The T_g can be described as the onset temperature for long-range, coordinated molecular motion that is not atypical of temperatures below the T_g as the molecules are “frozen” in the glassy state (Sperling, 2006). The mechanical and dielectric

relaxation times are taken as 100s at the onset of T_g but these are reduced significantly over the glass transition and therefore the onset of the T_g range is commonly used as the T_g (Roos, 2012). The T_g of most systems occurs about 100°C to 150°C below the equilibrium melting temperature of the crystals of the pure substance (Sperling, 2006). Thermal history and experimental conditions may give slight variation to the material specific T_g (Tant and Wilkes, 1981; Wunderlich, 1981). Molecular mass affects the T_g and low molecular weight molecules such as sucrose and glucose have low T_g while higher T_g was found for molecules with longer chain structures such as carbohydrate polymers (Bhandari and Howes, 1999). T_g increased exponentially with increasing molecular mass up to 25000 and there was a linear relationship between the reciprocal of average molecular weight and T_g (Fox and Flory, 1950). However, T_g of proteins and starches with very high molecular mass may not be attainable because of decomposition (Bhandari and Howes, 1999).

Water is capable of suppressing the T_g of amorphous carbohydrates as well as more complex food materials (Roos, 1987; Desobry *et al.*, 1999; Silalai and Roos, 2010; Ramoneda *et al.*, 2011; Potes *et al.*, 2012; Zhou and Roos, 2012). Water is a small molecular mass solvent and a strong plasticizer causing a significant decrease of the T_g (Slade and Levine, 1991). The T_g of amorphous water is found around -135°C with several values for the change in heat capacity (Sugisaki *et al.*, 1968; Hallbrucker *et al.*, 1989; Johari *et al.*, 1990; Angell, 2002). The small molecular size and low molecular mass of water caused a significant increase in molecular mobility showed by a high mobility, increased free volume, and decreased local viscosity (Ferry, 1980). The Gordon-Taylor relationship (2) often fits to plasticization data of water in amorphous polymer-solvent systems and it can be used to determine the effect of increasing water content on the reduction of T_g (Gordon and Taylor, 1952; Roos, 2009b). Besides water, sugars with very low T_g such as sucrose and fructose have reduced T_g of carbohydrate mixtures (Bhandari and Howes, 1999). However, mixing of carbohydrates (maltodextrin) with high T_g to form sugar matrixes can increase the overall T_g of the blend (Roos and Karel, 1991b; Gabarra and Hartel, 1998). The T_g of a binary system does not only depend on the properties of the

components in the mixtures but also on the miscibility of the components (Roos, 2008). Nonetheless, no significant increase in the T_g was observed after mixing high T_g carbohydrates at concentrations below 50% and a bigger increase in T_g was only observed at concentrations of 50% or higher (Roos and Karel, 1991b; Gabarra and Hartel, 1998; Potes *et al.,* 2012).

$$T_g = \frac{w_1 T_{g1} + k_{w2} T_{g2}}{w_1 + k w_2} \quad (2)$$

1.3.2 Structural Changes

It is crucial to maintain the physico-chemical state of the amorphous food systems to ensure quality of the products during storage. Changes in the physico-chemical properties has negative impacts such as structural changes, increased chemical reaction rates, and changes in microbial quality (Bhandari and Howes, 1999). The high hygroscopicity of dehydrated food materials can lead to water uptake and plasticization that changes the protective capability of the initially glassy matrix (Tant and Wilkes, 1981; Slade *et al.,* 1989). Storage of food materials above the T_g caused time-dependent physical changes and structural changes such as structural collapse, stickiness, caking, and crystallization (Özkan *et al.,* 2002; Prado *et al.,* 2006, Fan and Roos, 2016). These physical and structural changes occur at temperatures above the T_g at rates that are defined by the $T - T_g$ (Slade and Levine, 1991; Levi and Karel, 1995; Roos, 2009b). Structural collapse, stickiness, caking, and crystallization may also have an adverse effect on the handling properties of dehydrated food materials (Drusch *et al.,* 2006). The increase in free volume and molecular mobility as well as reduction in viscosity above the T_g (Roos and Karel, 1991c) results in structural collapse and crystallization that gave can give detrimental consequences to the quality of the food products and encapsulated materials such as bioactives, flavours, and volatiles. It was also likely that the chemical reaction rates were slower or reactions did not happen in the glassy state below the T_g due to the glassy state properties. However, reaction rates will increase significantly above the T_g in the rubbery state (Nelson and Labuza, 1994).

1.3.3 Degradation of Bioactives

The two main factors that affect reaction rates in food systems are temperature and water. Understanding the reaction kinetics is crucial to estimate stability and quality of food materials (Nelson and Labuza, 1994). Water activity was used to describe the impact of water on reaction rates. Water activity is a well-accepted approach in the prediction and control of food stability (Labuza, 1975). Glass transition was found to have an effect on the reaction rates of food systems (Le Meste *et al.,* 2002) and the link between water and reaction rates can be defined using the glass transition approach (Nelson and Labuza, 1994). Storage of food below glass transition provides longer shelf-life as lower diffusion rates in glassy state reduced reaction rates (Roos, 2009). Nonetheless, the consequence of glass transition on reaction rates is not well established in food processing (Roos, 2009). Carotenoids are highly vulnerable to degradation due to the presence of conjugated polyene chain (Boon *et al.,* 2010). Dissolving carotenoids in organic solution such as edible oil makes it less stable than naturally occurring carotenoids in tissues and these instability issues need to be addressed for successful utilization of carotenoids as functional food ingredients (Britton, 1995; Polyakov and Leshina, 2006; Boon *et al.,* 2010).

Carotenoids Loss

Changes that occur in food materials generally follow either zero or first order kinetics (Labuza and Riboh, 1982). The degradation of carotenoids is a typical first order reaction (Henry *et al.,* 1998; Desobry *et al.,* 1999; Hidalgo and Brandolini, 2008; Achir *et al.,* 2010; Harnkarnsujarit *et al.,* 2012b; Mahfoudhi and Hamdi, 2014) which shows increased rates with increasing storage temperature (Desobry *et al.,* 1997; Spada *et al.,* 2012). The first order reaction is described by equation (3) and the integrated equation of (3) displays a linear relationship in a plot of ln *C* against *t* (4) (Roos, 1995). The change in concentration takes place exponentially with time in a first order reaction and the rate constant, *k,* is defined by the slope of ln *C* against *t* plot (Roos, 1995). Temperature dependence of chemical reactions is commonly described with Arrhenius relationship (Glasstone, 1946). The Arrhenius relationship

is shown as equation (5) and can be re-written into the straight line form (6) where a plot of k against $1/T$ produces a straight line with a slope of E_a/R (Roos, 1995). It was established that the Arrhenius relationship is suitable to determine temperature dependence of reactions within the glassy state and 100°C above the T_g. Arrhenius kinetics, however, are not suitable to be used within the rubbery state (Slade *et al.*, 1989). Even though the WLF equation was deemed to be more applicable in determining temperature dependence of systems within the rubbery state, Arrhenius relationship is capable to predict changes in reaction rates within the rubbery state over a small temperature range (Nelson and Labuza, 1994). Arrhenius plot with data below and above the T_g of up to 40°C was found to be quite linear with r^2 of up to 0.999 (Karmas *et al.*, 1992; Lievonen *et al.*, 1998).

$$-\frac{dC}{dt} = kC \tag{3}$$

$$\ln C = \ln C_0 - kt \tag{4}$$

$$k = k_0 e^{-\frac{E_a}{RT}} \tag{5}$$

$$\ln k = \ln k_0 - \frac{E_a}{RT} \tag{6}$$

Various factors such as temperature, type of carotenoids, physical state, reaction medium, and environmental conditions may have an effect on the degradation kinetics of carotenoids (Pesek and Warthesen, 1988; Minguez-Mosquera and Jaren-Galan, 1995). Free radicals can be found in food materials as the result of reactions such as lipid oxidation (Choe and Min, 2006) that can cause carotenoids degration through electron transfer, hydrogen transfer, and formation of carotenoids-radical adducts (Britton, 1995; Young and Lowe, 2001; Mortensen, 2002). Carotenoids when exposed to heat in the presence of oxygen show degradation and form volatile and larger non-volatile compounds (Bonnie and Choo, 1999). Exposure of carotenoids to heat and light causes isomerization of the unstable all-trans β-carotene to cis-isomers (Khoo *et al.*, 2011). Carotenoids degradation is usually the

consequence of isomerization instead of decomposition (Chandler and Schwartz, 1988). The relocation of the single or double bonds to form different configurations of carotenoids requires isomerization energy (Kuki *et al.,* 1991). Electron transfer from neutral carotenoids to radicals or metal ions produced carotenoids radical cations ($Car^{\bullet+}$) or carotenoids radical dications (Car^{2+}) capable of isomerizing to different *cis*-species. Electron transfer plays a role in carotenoids isomerization to produce cations with lower configural transformation energy barriers than neutral carotenoids (Gao *et al,* 1996; Wei *et al.,* 1997). Heating of all-trans-β-carotene results in isomerization forming 13-cis-β-carotene, 15-cis-β-carotene, and 9-cis-β-carotene with 13-cis-β-carotene formed in the largest quantity (Chandler and Schwartz, 1988; Chen *et al.,* 1994; Henry *et al.,* 1998). The increase in 13-cis-β-carotene occurred simultaneously with the decrease in all-trans-β-carotene (Henry *et al.,* 1998). There can be increased difficulty in a single-step chromatographic separation of lutein (Rivas, 1989) as xanthophyll such as lutein is also capable to undergo esterification with fatty acids present to form lutein ester with lower polarity than the free lutein (Pérez-Gálves and Minguez-Mosquera, 2005). Carotenoids radicals can react with other radicals to form non-radical products in low oxygen condition (Burton and Ingold, 1984; Beutner *et al.,* 2001; Foti and Amorati, 2009).

1.4 Conclusions

O/W emulsions can be designed to act as delivery systems for oil soluble bioactives such as carotenoids. The application of LBL interfacial structure in O/W emulsions may further improve stability of the encapsulated bioactives. Nonetheless, LBL systems were commonly associated with flocculation, namely depletion flocculation and bridging flocculation due to the presence of the secondary layer. LBL technique has been known to produce emulsions with better stability towards environmental stresses but few studies have reported the use of high total solids LBL systems and dehydration of such systems. Dehydration of the LBL system by either freeze-drying or spray drying provides microencapsulation of the bioactives within glassy matrix for improved protection. The possibility to spray dry high total solid systems that gives high quality powders would be attractive to the food industry as spray drying

of high total solid systems greatly reduces production costs. Structural changes associated with glass transition such as structural collapse also change the properties of the continuous phase that affects the degradation of the encapsulated bioactives.

Chapter II

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Stability of flocculated particles in concentrated and high hydrophilic solids layer-by-layer (LBL) emulsions formed using whey proteins and gum Arabic

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Abstract

The objective of the present study was to investigate flocculation in layer-by-layer (LBL) emulsion systems with high total solids content and deflocculation at various pH conditions, and the effects of whey protein isolate (WPI) concentration and total solids content on the stability of LBL emulsions. WPI (1.96%, (1WPI), or 10.71%, (10WPI), w/w in water) was prepared in water and high-pressure homogenized with sunflower oil (10%, w/w, of total emulsion). Gum Arabic (0.15%, w/w, in total emulsion) was added to assemble electrostatically on WPI at oil particle interfaces at pH 3.5 using aqueous citric acid (10% w/w) forming LBL emulsion. The ζ-potential measurements showed charge reversal upon addition of gum Arabic solution into single layer (SL) emulsion confirming the formation of LBL interface. Trehalose:maltodextrin mixture (1:1, w/w, total emulsion, 28.57%, (28) or 57.14%, (57), w/w, in water) were used in the continuous phase. The high total solids content of the system result in depletion flocculation of the particles leading to bridging flocculation without coalescence as deflocculation into individual particles occurred with increasing pH from pH 3.5 to pH 6.5 in 10WPI systems. Deflocculation was evident in 10WPI-28 and 10WPI-57 as found from a decreased ζ-average diameter and visually under microscope. Coalescence was observed in 1WPI systems. Viscosity of the systems was significantly ($P < 0.05$) increased with higher total solids content. Accelerated destabilization test showed that systems at higher WPI and total solids contents exhibited the highest stability against creaming. Deflocculation in LBL systems can be controlled by pH while high solids in the aqueous phase provide stability against creaming.

Keywords: layer-by-layer emulsion, flocculation, deflocculation, high hydrophilic solids, WPI, gum Arabic

2.1 Introduction

Emulsifiers play an important role in reducing the interfacial tension between the oil and water phase resulting in smaller droplet size during homogenization and they

reduce flocculation throughout processing, storage and utilization (McClements, 1999). Common emulsifiers are surface active molecules such as phospholipids, proteins and polysaccharides that assembly at the interface around the oil droplets during homogenisation (Ogawa *et al.,* 2004; Gu *et al.,* 2005). Whey protein isolate (WPI) was used as emulsifier in this study. WPI contains β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and immunoglobulins (de Wit, 1981). Proteins, as emulsifier, generally are capable of favouring the formation of small oil droplets with poor stability towards environmental stresses. Contrarily, polyelectrolytes can provide good stability towards environmental stresses but are inferior in generating small droplets in emulsions or excess quantities may be required to stabilize small droplets (McClements, 2003).

Physiochemical processes such as flocculation followed by Ostwald ripening and coalescence will result in gravitational separation and destabilization of emulsions (McClements, 1999). LBL emulsions have better stability over a greater range of environmental stresses due to the strong electrostatic and steric repulsive forces of the thicker interfacial layer of LBL emulsion particles compared to a single emulsifier interface (Moreau *et al.,* 2003; Gu *et al.,* 2005; Harnsilawat *et al.,* 2006; Bouyer, *et al.,* 2011; Benjamin *et al.,* 2012). The layered interface could provide the particles with a higher resistance towards disruptions (McClements, 1999). The application of LBL technology with protein coated oil particles can enhance emulsion stability towards changes in pH, ionic strength, heat in thermal processing and drying, ageing, freeze thaw cycling and lipid oxidation (Ogawa *et al.,* 2003; Aoki *et al.* 2005; Gu *et al.,* 2005; Gharsallaoui *et al.,* 2010; Lim *et al.,* 2014). LBL emulsions are produced by the electrostatic attraction in the presence of the charged protein interface with an oppositely charged polyelectrolyte in the continuous phase (Moreau *et al.,* 2003; Gu *et al.,* 2005; Guzey and McClements, 2006b). Gum Arabic has a pK_a value of approximately 2.2 (Weinbreck *et al.,* 2004). Therefore, gum Arabic will be negatively charged at pH above 2.2. The ability of protein to become positively charged below its relatively low pH of the isoelectric point (pI) and negatively charged at pH above its pI justifies the use of protein as the primary layer in an edible emulsion (McClements, 1999; Gu *et al.,* 2004; Guzey *et al.,* 2004; Klein

et al., 2010). Beta-lactoglobulin has an isoelectric point (pI) of 5.2 (Bryant and McClements, 1998) while α-lactalbumin has pI of 4.1 (Weinbreck *et al.,* 2003).

The ratio between proteins and polyelectrolytes plays an important role in the stability of LBL emulsions as polyelectrolytes can contribute to bridging or depletion flocculation. Non-absorbed polyelectrolytes in the continuous phase enhanced depletion flocculation which promoted coalescence and creaming (Chanamai and McClements, 2001; Gu *et al.,* 2005; Guzey and McClements, 2006b; Benjamin *et al.,* 2012). The difference in osmotic pressure caused the solvent to migrate from depleted zone towards the surrounding polyelectrolytes-rich continuous phase in order to reduce the concentration gradient (McClements, 2000). Depletion flocculation occurred when non-absorbed polyelectrolytes produced an attractive osmotic force surpassing the repulsive forces present (Guzey and McClements, 2006b). Depletion flocculation occurred rapidly but was weak and reversible (Dickinson and Golding, 1997). On the other hand, bridging flocculation caused strong and irreversible bonds (Dickinson *et al.,* 1997; Blijdenstein *et al.,* 2004).

Our earlier studies showed that utilization of dehydrated LBL emulsion with trehalose as wall material as well as in extrudates reduced the loss rate of carotenoids (Lim *et al.,* 2014; Caliskan *et al.,* 2015). This finding supports the use of LBL emulsion in dehydration to improve the physicochemical stability and shelf life of formulated food systems, such as infant formula and nutritional supplements. In the present study, LBL emulsions were prepared using maltodextrin, trehalose, WPI and gum Arabic in the continuous phase at pH 3.5. Sunflower oil was emulsified with WPI dispersion prior to the addition of the polyelectrolyte and carbohydrates. Emulsions with high total solids contents were used in this study; as such systems are desired in spray drying and food processing. The objectives of the present study were to investigate the characteristics of flocculation of emulsified oil particles in systems with high total solids contents and their deflocculation at various pH conditions, and the effects of WPI concentration and total solids content on the stability of the LBL emulsions.

2.2 Materials and Methods

2.2.1 Materials

Trehalose (crystalline dehydrate) (Hayashibara Shoji Inc., Japan), maltodextrin (MD 150, DE 15, Grain Processing Corp, IA, USA), whey protein isolate (WPI) (Carbery Food Ingredients, WPI Isolac, Ballineen, Ireland) and gum Arabic (Sigma Aldrich Co. G9752, St Louis, MO, USA) were used. Sunflower oil was purchased from a local supplier (Mediterani, Pan Euro Foods, Dublin 15, Ireland). Citric acid and deionised water were obtained from KB Scientific Ltd. (Cork, Ireland). Potassium hydroxide was obtained from Merck (Darmstadt, Germany).

2.2.2 Primary Emulsion Preparation

Table 2.1. Amount of materials and water used for the preparation of layer-by-layer (LBL) emulsions (1WPI-28; 1WPI-57; 10WPI-28; 10WPI-57).

Materials	To prepare 1000g LBL emulsion			
	1WPI-28	**1WPI-57**	**10WPI-28**	**10WPI-57**
	Amount of materials (% w/w, in final emulsion)	**Amount of materials (% w/w, in final emulsion)**	**Amount of materials (% w/w, in final emulsion)**	**Amount of materials (% w/w, in final emulsion)**
WPI	0.2	0.2	1.2	1.2
GA	0.15	0.15	0.15	0.15
Trehalose:Maltodextrin	10+10	20+20	10+10	20+20
Sunflower oil	10	10	10	10

[a]pH of the systems were adjusted to pH 3.5 using citric acid solution (10% w/w) bringing the total amount to 100%.

[b]WPI: whey protein isolate; GA: gum Arabic

WPI (1.96% or 10.71%, w/w, in the aqueous phase of the primary emulsion) was hydrated in deionised water for 1 hour under stirring at room temperature (Table 2.1). The pH of the dispersion was adjusted to pH 3.5 with aqueous citric acid solution (10%, w/w) and hydration was continued for an additional 30 min. Sunflower oil (49.51% or 47.17%, w/w, in primary emulsions) was added into the WPI dispersion and blended with a high speed blender (T25 Digital, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 10000 rpm for 1 min to form a pre-emulsion. Primary emulsions were prepared from the pre-emulsions by homogenization with a two stage high pressure homogenizer (APV-1000 High Pressure Homogenizer, Wilmington, MA, USA) at 25 MPa (20 MPa for 1st stage and 5 MPa for 2nd stage) for three passes.

2.2.3 LBL and SL Emulsion Preparation

Gum Arabic (2.91% w/w, in solution; 0.15%, w/w, in final emulsion) was used as the polyelectrolyte of the LBL emulsion (Table 2.1). Gum Arabic was dissolved in deionised water under magnetic stirring for 1 h at room temperature. A mixture of trehalose and maltodextrin at a ratio of 1:1 (28.57% or 57.14%, w/w, in water; 20% or 40%, w/w, in final emulsion) was dissolved in deionised water at 70 °C under stirring with a magnetic stirrer. Maltodextrin was added initially and trehalose was dissolved to the clear maltodextrin solution. Aqueous gum Arabic and trehalose:maltodextrin solutions were adjusted to pH 3.5 with the citric acid solution (10%, w/w). The aqueous gum Arabic was combined with the primary emulsion to obtain the LBL structure through electrostatic attraction (Aoki *et al.,* 2005; Harnsilawat *et al.,* 2006; Zhou and Roos, 2013; Lim *et al.,* 2014) and stirring was continued for 30 min with magnetic stirrer. The primary emulsions and trehalose:maltodextrin solution were combined under continued stirring of 30 min. The citric acid solution and deionised water gave the LBL emulsions the final composition of 20% or 40% (w/w, total emulsion, 28.57% or 57.14%, w/w, in water) trehalose:maltodextrin solids, 10% (w/w, total emulsion) sunflower oil, 0.2% or 1.2% (w/w, total emulsion) WPI and 0.15% (w/w, total emulsion) gum Arabic. Four systems were used in this study: (i) 1.96% WPI and 28.7% trehalose:maltodextrin

solids in aqueous phase (1WPI-28); (ii) 1.96% WPI and 57.14% trehalose:maltodextrin solids in aqueous phase (1WPI-57); (iii) 10.71% WPI and 28.7% trehalose:maltodextrin solids in aqueous phase (10WPI-28); and (iv) 10.71% WPI and 57.14% trehalose:maltodextrin solids in aqueous phase (10WPI-57). The LBL emulsions were stored overnight at 4°C prior to experiments. Single layer (SL) emulsions were obtained using the same composition as LBL emulsions i, ii, iii, and iv without the addition of gum Arabic. Steps involved in the preparation of SL and LBL systems are shown in Figure 2.1.

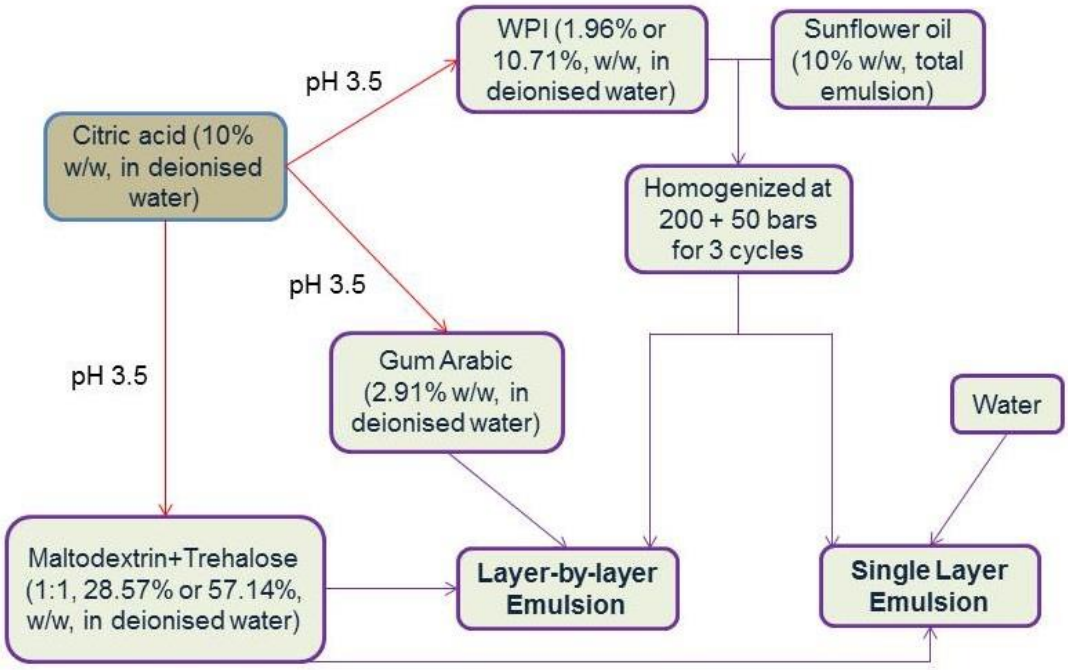

Figure 2.1. Flow diagram on the steps and materials involved in the preparation of single layer (SL) and layer-by-layer (LBL) systems.

2.2.4 Emulsion Characterisation

Particle size [ζ-average diameter and polydispersity index (PDI), using dynamic light scattering, measurement range: 0.3 nm - 10μm] and charge (ζ-potential, using electrophoretic light scattering) of the LBL and SL emulsions was determined with a Zetasizer (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK). A series of

solutions with different pHs were prepared at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5 in screw cap vials using 0.1M KOH for pH control. LBL emulsions were diluted in these solutions at ratio of 1:400 for the measurements of ζ-average diameter and ζ-potential. The diluted samples were then vortexed for 30 s to ensure homogeneity. Plastic cuvettes (Square cuvettes, PS, 10mmX10mmX45mm, SARSTEDT AG & Co, Nümbrecht, Germany) were filled with 1ml of the diluted emulsions and used to determine the particle size while plastic capillary cells (Folded capillary cells, DTS 1061, Malvern Instruments Ltd.) were used to determine the charge. Measurements were done at room temperature. Diluted emulsions in the vials were shaken before being transferred into cuvettes or capillary cells.

2.2.5 Optical Microscope

Three ml of LBL emulsions were transferred into glass vials with screw caps. The emulsions were diluted with 3 ml of aqueous citric acid at pH 3.5 to enhance particle separation and the pH of the emulsions was adjusted to pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 using 0.1M KOH and vortexed for 30 s to ensure homogeneity. The emulsions were allowed to stand overnight at room temperature for possible separation. A drop of the separated emulsions of all systems containing flocculated particles was transferred onto a glass slide and images were captured using optical microscope at 100X magnification (Olympus BX51) connected to a camera (PixeLink A662, Ottawa, ON) to record the effect of flocculation on the particles .

2.2.6 Separation Velocity

Emulsion stability was determined with LUMiSizer dispersion analyser (L.U.M. GmbH, Berlin, Germany). Sample cells were filled with 0.4 ml of emulsion. The measurements were completed at 20°C and 1500 rpm for 60 min. LUMiSizer is a centrifugal sedimentation method to determine emulsion stability enabling simultaneous measurement of the intensity of transmitted light as a function of time and position over the separation interface across the entire sample length. The

integral transmission (%) as a stability parameter for the emulsions was determined against time (s).

2.2.7 Apparent Viscosity Measurement

Apparent viscosity of the emulsions and the trehalose:maltodextrin solution (28.57% or 57.14%, w/w, in aqueous phase) as the aqueous phase was determined using Brookfield Viscometer (DV-1-RV viscometer; Brookfield Engineering Labs Inc., Stoughton, MA, USA) at 25°C. Measurements were made using concentric cylinder and cone spindle CC-45 at constant shear rate of 150 s^{-1} for 90 s. The average viscosity was recorded.

2.2.8 Statistical Analysis

Measurements of ζ-average diameter, ζ-potential and apparent viscosity were done in triplicate and the results were expressed as average ± SD. One way analysis of variance (ANOVA) was carried out to find significant differences using Tukey test at 95% confidence level. The SPSS Statistical Package (SPSS ver. 16, SPSS Inc., Chicago, IL, USA) was used.

2.3 Results and Discussion

2.3.1 Particle Size and Charge

2.3.1.1 ζ-potential

The ζ-potentials of all systems are given in Table 2.2. The ζ-potential for the layer-by-layer (LBL) emulsions was negative at all pH values regardless of the whey protein isolate (WPI) concentration and total solids content. The ζ-potential of all systems became increasingly negative with increasing pH. On the other hand, single layer (SL) emulsions of all systems had positive ζ-potential value as the pH of the emulsions (pH 3.5) was well below the isoelectric point (pI) of WPI of pH 5.2

(Bryant and McClements, 1998; Moschakis *et al.,* 2010). Gum Arabic in primary emulsion showed electrostatic attraction towards the positively charged protein (Figure 2.2) which led to formation of the LBL emulsion. Charge reversal is typical of the formation of the LBL interface and the potential of charged polyelectrolytes to cause charge reversal in emulsions is well-known (Chodanowski and Stoll, 2001; Moreau *et al.,* 2003; Guzey *et al.,* 2004; Surh *et al.,* 2006; Zhou and Roos, 2013; Lim *et al.,* 2014). Charge reversal may occur due to an excess of a polyelectrolyte as only small amounts of polyelectrolyte is required to neutralize the oppositely charged patches on protein particles.

Table 2.2. Changes in zeta-potential of layer-by-layer (LBL) emulsions (1WPI-28; 1WPI-57; 10WPI-28; 10WPI-57) with increasing pH and single layer (SL) emulsion at pH 3.5.

pH	Z-Potential (mV)			
	1WPI-28	**1WPI-57**	**10WPI-28**	**10WPI-57**
3.5	-11.2±1.2	-14.3±0.8	-1.3±0.2	-1.9±0.1
4.0	-27.3±0.2	-24.6±2.3	-2.4±0.2	-11.3±1.3
4.5	-30.6±0.7	-34.2±1.0	-13.7±0.8	-26.0±3.1
5.0	-36.8±0.9	-42.1±0.9	-23.9±0.7	-32.1±0.7
5.5	-42.7±1.4	-46.1±0.3	-39.1±1.0	-33.1±0.3
6.0	-53.5±0.9	-50.9±0.7	-52.3±1.0	-44.6±1.0
6.5	-64.0±1.1	-56.7±2.0	-57.1±1.2	-61.4±0.2
3.5 (SL)	17.3±0.1	18.7±0.4	23.1±0.6	27.3±0.9

[a]Values are means (n=3) ± SD

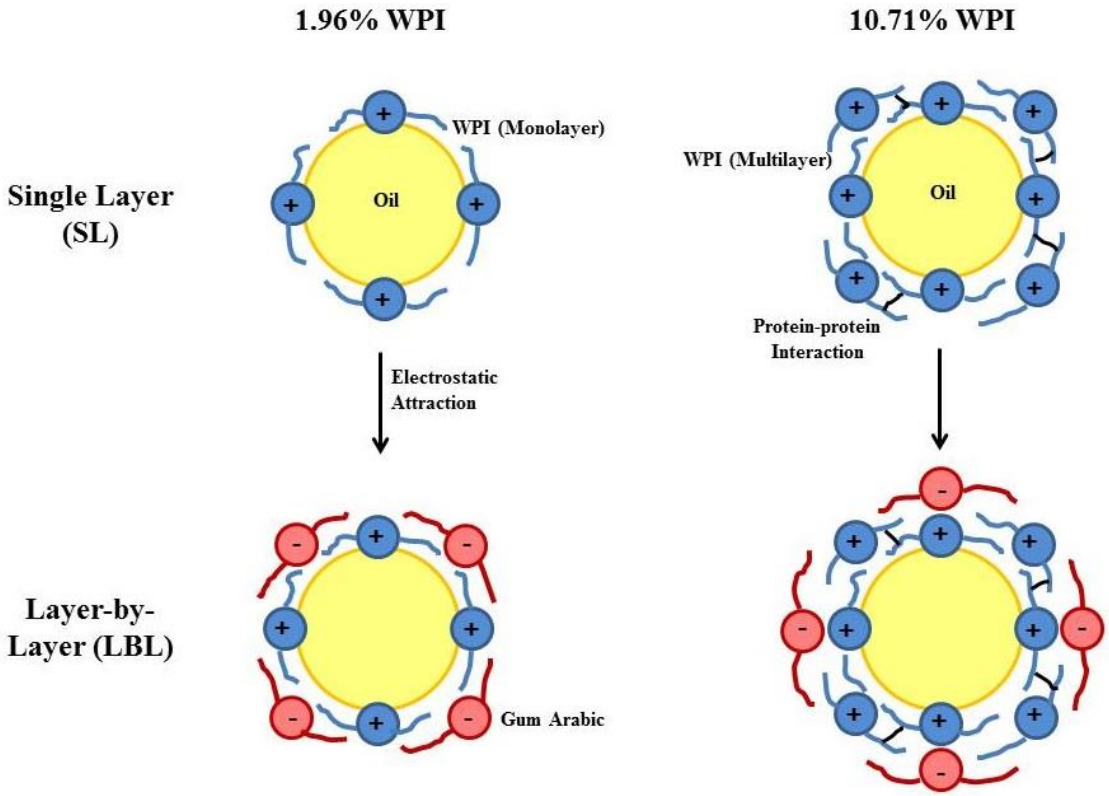

Figure 2.2. Schematic diagram of single layer (SL) and layer-by-layer (LBL) systems stabilized by whey protein isolate (WPI) as the primary layer and gum Arabic as the secondary layer.

Systems with the higher WPI concentration showed higher ζ-potential values at pH 3.5 as the higher WPI content increased positively charged patches at pH 3.5 for electrostatic attraction. As the amount of gum Arabic was kept constant, increasing the amount of WPI will reduce the ratio of gum Arabic to WPI resulting in the lower overall decrease of ζ-potential. Therefore, more anionic gum Arabic will be required to saturate positively charged patches. The negative charge around the pI of the protein was due to the anionic gum Arabic that was present. At pH above the pI of the protein, the negatively charged WPI and gum Arabic became electrostatically repellent (Guzey *et al.*, 2004; Gu *et al.*, 2005). The high electrostatic repulsion between the particles at a high pH above the pI of the protein could reduce flocculation of the oil particles in emulsion (Moreau *et al.*, 2003) as seen in the reduction of ζ-average diameter of all systems above the pI of WPI. The total solids content of the system had little effect on the ζ-potential as observed in the similar ζ-

potential between 1WPI-28 and 1WPI-57 as well as in 10WPI-28 and 10WPI-57. However, at the higher experimental pH, all systems had comparable negative ζ-potential values. The comparable negative ζ-potential values could be accounted for the absence of positively charged patches at pH above the pI of the protein as well as due to the presence of anionic gum Arabic.

2.3.1.2 ζ-average Diameter

Table 2.3. Changes in zeta-average and polydispersity index (PDI) of layer-by-layer (LBL) emulsions (1WPI-28; 1WPI-57; 10WPI-28; 10WPI-57) with increasing pH and single layer emulsion at pH 3.5.

pH	Z-Average (nm) (PDI)			
	1WPI-28	**1WPI-57**	**10WPI-28**	**10WPI-57**
3.5	3007±468^{c} (0.974^{c})	3586±332^{c} (0.641^{a})	1260±348^{ab} (0.950^{a})	972±203^{bc} (0.948^{b})
4.0	3612±277^{bc} (0.792^{bc})	2743±1070^{bc} (0.762^{a})	1979±882^{c} (0.697^{a})	1144±150^{cd} (0.959^{b})
4.5	2509±796^{abc} (0.599^{abc})	2405±867^{abc} (0.386^{a})	1968±92^{c} (0.681^{a})	1519±324^{cd} (1.000^{b})
5.0	1498±290^{abc} (0.461^{abc})	1386±551^{ab} (0.648^{a})	1149±68^{ab} (0.737^{a})	1598±283^{d} (1.000^{b})
5.5	1336±295^{a} (0.197^{a})	976±189^{a} (0.903^{a})	824±36^{a} (0.351^{a})	485±26^{ab} (0.238^{a})
6.0	1075±197^{ab} (0.335^{ab})	1050±133^{a} (0.462^{a})	462±26^{a} (0.304^{a})	413±13^{ab} (0.062^{a})
6.5	1116±91^{a} (0.219^{a})	1141±202^{a} (0.539^{a})	441±46^{a} (0.343^{a})	357 ±8^{a} (0.104^{a})
3.5 (Single Layer)	1394±591^{ab} (0.371^{ab})	1224±329^{ab} (0.750^{a})	517±92^{a} (0.474^{a})	204±20^{a} (0.271^{a})

[a]Values are means (n=3)±SD

[b]Values with different superscript are significantly different at $p<0.05$

ζ-average diameter and polydispersity index (PDI) of oil particles in LBL emulsions are given in Table 2.3. The ζ-average diameter decreased with increasing pH showing increasing repulsion and deflocculation of particles. The ζ-average diameter in systems with 10.71% WPI (10WPI-28 and 10WPI-57) was smaller than in system with 1.96% WPI (1WPI-28 and 1WPI-57) with increasing pH. The larger ζ-average diameter of 1WPI-28 and 1WPI-57 with increasing pH could be the result of coalescence occurring in these systems. An assumption that the oil particles were 200 nm in radius gave a surface area of $5.03x10^5$ nm^2 and an estimate of 100:1.92 ratio of oil to WPI as sufficient to form a WPI monolayer around the oil particles. Although the amount of WPI used in this study was in excess at 100:2 ratio of oil to WPI, such quantity was insufficient to form an adequately dense interface around dispersed oil particles to prevent coalescence as only a fraction of the WPI was likely to migrate onto the oil surface. The large ζ-average diameter and PDI at pH 3.5 could be due to flocculation in systems with 10.71% WPI and coalescence in systems with 1.96% WPI as a consequence of the high total solids of all systems resulting in the oil particles to be in close proximity. The oil particles being in close proximity could further lead to bridging flocculation in systems with 10.71% WPI as individual gum Arabic molecules could become electrostatically attracted to several WPI covered oil particles.

The largest ζ-average diameter of the flocculated particles ($p<0.05$) for systems with 10.71% WPI was found to be 1979 nm for 10WPI-28 at pH 4.0 and 1598 nm for 10WPI-57 at pH 5.0 which was near to the isoelectric point (pI) of WPI. The electrostatic forces between the oil particles at pI of WPI were unable to overcome the attractive interactions resulting in extensive flocculation (McClements, 1999). The large ζ-average diameter around pH 4.0 to pH 5.0 was in agreement with studies of Moreau *et al.* (2003), Gu *et al.* (2005), and Benjamin *et al.* (2012) who used β-lactoglobulin as the primary interface layer. For systems with 1.97% WPI (1WPI-28 and 1WPI-57), changes in ζ-average diameter were non-systematic as coalescence of the oil particles could occur randomly. Generally, high PDI values were measured for pH over the range from pH 3.5 to 5.0 showing that the LBL particles were strongly flocculated at such pH conditions. A study by Guzey *et al.*

(2004) of an emulsion stabilized by β-lactoglobulin and pectin showed that oil particles were highly flocculated at low pH values over the range of pH 3.5 to pH 5.0. Above pH 5.0, the PDI values were reduced indicating that the particles became less polydispersed and individual particles were reproduced. The ζ-average diameter of all systems at pH 6.5 was comparable to that of WPI-stabilized SL emulsions at pH 3.5 indicating that individual particles were released from flocculates. The large ζ-average diameter of SL systems with 1.97% WPI indicated that coalescence had occurred. Coalescence occurred in SL systems as well based on the ζ-average diameter and the coalescence could not result from flocculation as no polyelectrolyte was present which emphasized the importance of the amount of WPI towards emulsion stability preventing coalescence. Stability towards coalescence was affected by thickness of particle membranes and the thicker membranes of LBL systems can reduce the possibility of particle surfaces to become ruptured and provided better steric repulsion between particles (McClements, 1999).

The increased negative charge of WPI above its isoelectric point from pH 5.5 to pH 6.5 resulted in electrostatic repulsion of WPI-covered particles and gum Arabic in agreement with studies of Ogawa *et al.* (2003), Gu *et al.* (2005), and Guzey and McClements (2006b) leading to smaller particle size in systems with 10.71% WPI (10WPI-28 and 10WPI-57). Our results demonstrated that highly flocculated particles can remain individually stable and individual particles can be re-obtained upon deflocculation without coalescence based on the results from ζ-average diameter. However, the amount of the protein played an important role. Coalescence could occur if the primary emulsifier quantity was insufficient to encapsulate the oil particles as was found for 1WPI-28 and 1WPI-57 as small individual particles were not obtained upon deflocculation. Besides, the large ζ-average diameter of the SL system with 1.97% WPI further confirmed the occurrence of coalescence in the absence of the polyelectrolyte. Total solids content played little role in the ζ-average diameter of the systems as shown by the present study.

2.3.2 Optical Microscopy

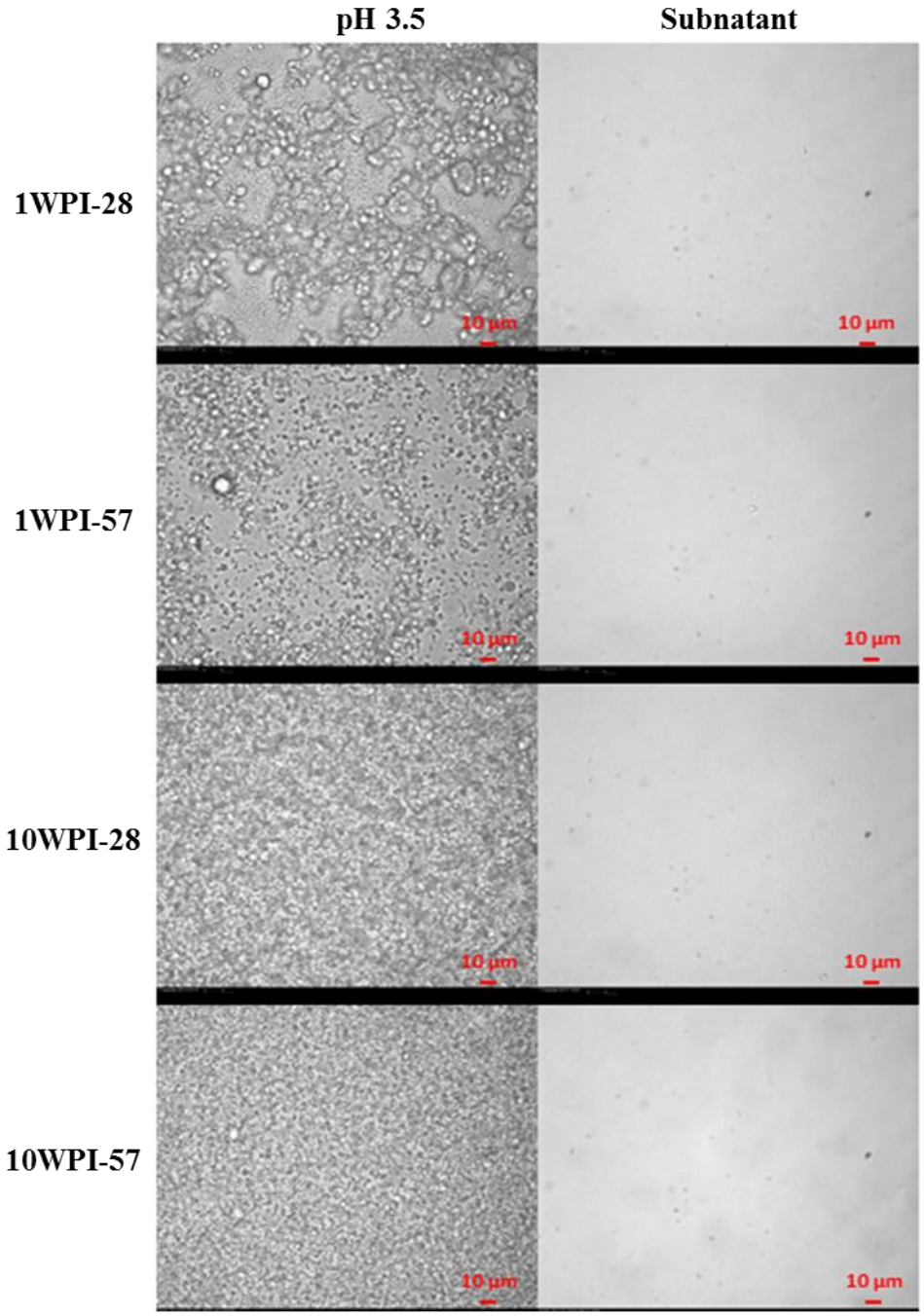

Figure 2.3. Particles of layer-by-layer (LBL) emulsions (1WPI-28; 1WPI-57; 10WPI-28; 10WPI-57) at pH 3.5 and their subnatant serum observed under optical microscope at 100x magnifications.

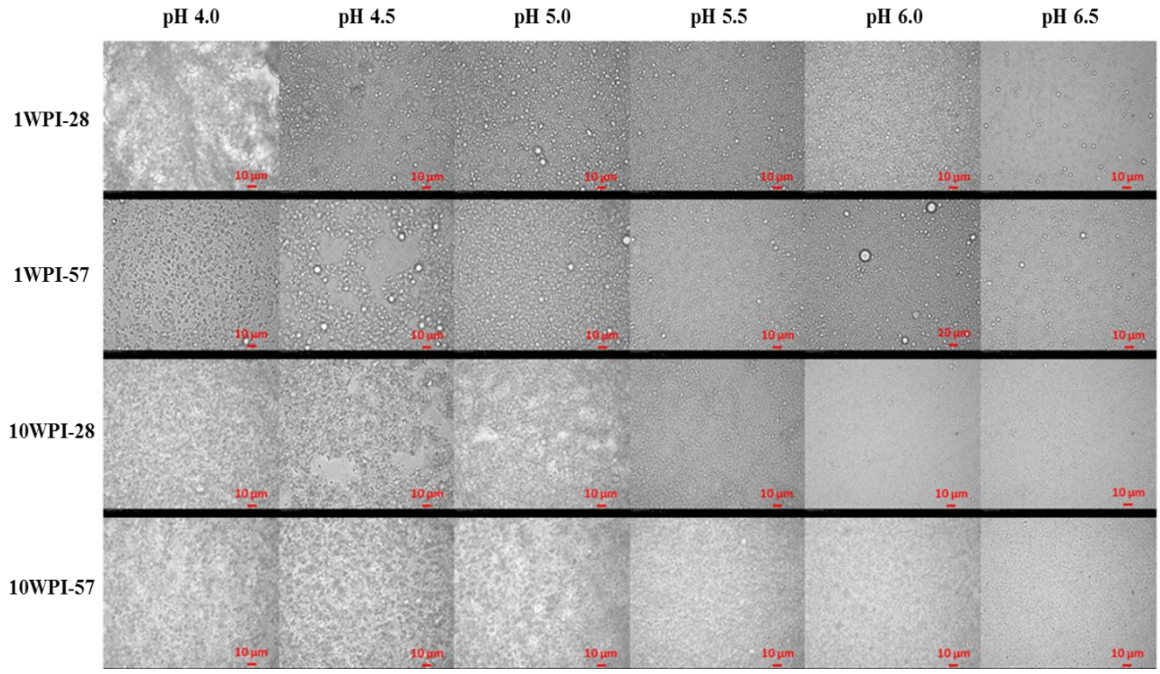

Figure 2.4. Effect of pH, WPI concentration and total solids content towards flocculated particles of layer-by-layer (LBL) emulsions (1WPI-28; 1WPI-57; 10WPI-28; 10WPI-57) observed under optical microscope at 100x magnifications.

Images of systems adjusted to pH 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5 as well as systems at their original pH 3.5 were taken under an optical microscope at 100X magnification. Figure 2.3 showed the particles at pH 3.5 and the separated bottom layer serum at pH 3.5 in all diluted systems. On the other hand, Figure 2.4 showed particles of all systems at pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. The particles of all systems were tightly packed at pH 3.5 as they underwent flocculation. The high total solids content of all systems could result in the particles to be in close proximity. Optical images confirmed that particles of all systems were highly flocculated over the range of pH 4.5 to pH 5.0 as the pH was around the pI of whey protein isolate (WPI). Studies by Moreau *et al.* (2003), Ogawa *et al.* (2003), Guzey *et al.* (2004), and Ogawa *et al.* (2004) reported that flocculation occurred in (LBL) emulsions with high polyelectrolytes concentrations at intermediate pH values of pH 4 to 6. Individual particles were regenerated above the pI of WPI at pH 5.5, 6.0 and 6.5 in all systems further confirming that flocculation was reversible. The high ζ-potential values above the pI of WPI in all systems lead to the negatively charged WPI covered

particles and anionic gum Arabic caused electrostatic repellence giving individual particles as shown in Figure 2.4 (Guzey *et al.* 2004; Gu *et al.,* 2005).

Coalescence could be confirmed in systems with 1.97% WPI (1WPI-28 and 1WPI-57) at all pH. The presence of large bright particles in Figure 2.3 and 2.4 indicated coalescence. Flocculation could enhance coalescence as particles became into close proximity with one another (Surh *et al.*, 2006). This observation was in agreement with the results measured for the ζ-average diameter which showed large ζ-average diameter values for 1WPI-28 and 1WPI-57 as the pH was increased. Surh *et al.* (2006) reported that flocculation and coalescence were visible in sodium caseinate-pectin LBL emulsions (oil to sodium caseinate ratio, 100:9) at pH 6 and 7. A study by Ye *et al.* (2004) showed that coalescence rate increased with increasing xanthan concentration due to a higher osmotic potential. Many small droplets were flocculated around large droplets with their interfaces broken causing coalescence to occur. At pH above the pI of WPI, gum Arabic was detached from the WPI's surface increasing osmotic pressure that can result in coalescence of the oil particles present. That phenomenon too formed a SL emulsion with reduced stability towards environmental stresses such as changes in pH. No particles were visible in the clear bottom layer of the continuous phase that was separated from the emulsion at pH 3.5 in all systems. This suggested that the subnatant consisted only when dissolved carbohydrates and the particles in the LBL emulsion were fully separated from the continuous phase. Total solids content had little role on the stability of particles present in the systems.

2.3.3 Separation Velocity and Apparent Viscosity

Table 2.4 gives the apparent viscosity of all systems and the aqueous phase containing trehalose:maltodextrin mixture (28.57% or 57.14%, w/w, in water) at pH 3.5. The apparent viscosity of the systems was mainly influenced by the hydrophilic total solids content as viscosity of systems with the higher total solids content was significantly higher ($P < 0.05$) than that of systems with lower total solids contents. It was noted that systems with higher amounts of whey protein isolate (WPI)

exhibited significantly higher ($P < 0.05$) apparent viscosity. The apparent viscosity of the systems increased significantly ($P < 0.05$) in the following order: 1WPI-28 < 10WPI-28 < 1WPI-57 < 10WPI-57. According to Stokes' Law, particles settling velocity increases with increasing diameter of particles while increasing viscosity reduces particles settling velocity. Data from ζ-average diameter provided information on the average diameter of the oil particles while viscometer on the apparent viscosity of the systems. These values were fitted into Stokes' Law to obtain velocity of particle's settling where higher velocity of particle's settling translated into system with lower stability.

Table 2.4. Apparent viscosity of layer-by-layer (LBL) emulsions (1WPI-28; 1WPI-57; 10WPI-28; 10WPI-57) and trehalose-maltodextrin mixture solution (28.57% or 57.14%, w/w, in water) at 25°C.

	Apparent Viscosity (mPa.s)
1WPI-28	3.93 ± 0.2^{b}
1WPI-57	33.01 ± 0.4^{d}
10WPI-28	7.70 ± 0.1^{c}
10WPI-57	42.88 ± 0.2^{e}
Trehalose:MD (28.57%, w/w)	2.33 ± 0.1^{a}
Trehalose:MD (57.14%, w/w)	59.90 ± 0.3^{f}

[a]Values are means (n=3)±SD

[b]Values with different superscript are significantly different at $p<0.05$

The effect of WPI concentration and total solids content on stability of the systems was determined with the accelerated destabilization test. The measurement used the intensity of the parallel transmitted NIR light over the entire sample length at various time intervals. The larger changes in integral transmission (%) with centrifugation indicated emulsion with lower stability (Lei *et al.*, 2014). Integral transmission (%) against time (s) for all systems is shown in Figure 2.5. The integral transmission (%) showed the length of aqueous phase in sample cells during centrifugation. Stability of emulsions can be enhanced with increased continuous phase viscosity and smaller

particle size (Mert, 2012). It can be seen from Figure 2.5 that 1WPI-28 and 1WPI-57 creamed more readily as compared to systems with 10.71% WPI (10WPI-28 and 10WPI-57). The higher creaming rate of 1WPI-28 and 1WPI-57 could be attributed to the fact that coalescence due to flocculation occurred in these systems resulting in particles with larger sizes. Although viscosity of 10WPI-28 was significantly lower than that of 1WPI-57, 10WPI-28 creamed at a slower rate further confirming that coalescence increased the creaming rate of systems with 1.96% WPI in agreement with Stokes' Law. The increase in diameters of oil particles in systems with 1.96% WPI greatly increases the velocity of particle's settling. Studies by Ogawa *et al.* (2004), and Guzey and McClements (2006b) also showed that emulsions with smaller particle size were more stable towards creaming. The higher amount of WPI in 10WPI-28 and 10WPI-57 could result in a WPI multilayer formation around the oil particles as a result of protein-protein interactions (Cornacchia *et al.*, 2011a) (Figure 2.2). Such WPI multilayer could form in 10WPI-28 and 10WPI-57 and give particles with a higher overall density in comparison to 1WPI-28 and 1WPI-57. The reduced density difference between the oil particles and aqueous phase reduces the driving force for creaming and effectively reduced the creaming rate (McClements, 1999). The lower density difference between the oil particles and aqueous phase will lower the velocity of particle's settling in accordance with the Stokes' Law. Mao *et al.* (2014) suggested that density matching the oil particles covered with WPI-pectin mixture and the aqueous phase contributed to slower creaming.

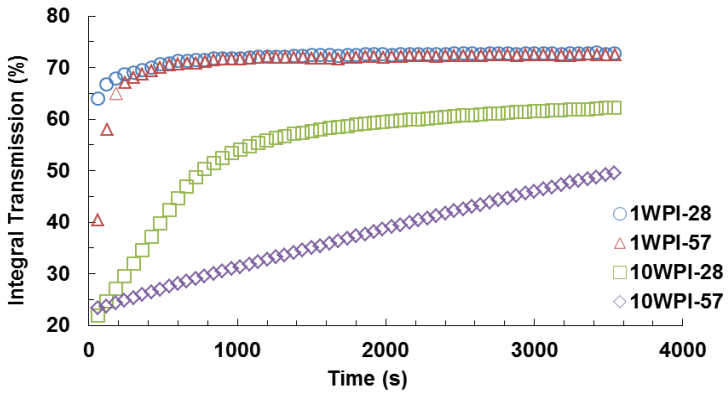

Figure 2.5. Influence of WPI concentration and total solids content on the stability of layer-by-layer (LBL) emulsions (1WPI-28; 1WPI-57; 10WPI-28; 10WPI-57) at pH 3.5 based on integral transmissions (%).

Systems with higher total solids content were more stable towards creaming as higher total solids content increased viscosity of the systems. The change in integral transmission (%) against time of 10WPI-57 was slower than in 10WPI-28 and 1WPI-57 was slower than 1WPI-28. Studies by Chanamai and McClements (2001) and Gu *et al.* (2005) stated that increased viscosity of aqueous phase reduced the creaming rate due to the slower movement of particles. Therefore, the higher total solids content in these systems may have delayed creaming due to the higher viscosity. Overall, our system with lower WPI concentration and lower total solids content (1WPI-28) had the poorest emulsion stability. Coalescence that increased the diameter of oil particles and the lower viscosity of the continuous phase in the system contributed to the faster creaming rate. On the other hand, systems with higher WPI concentrations and higher total solids contents (10WPI-57) had the highest emulsion stability. The higher WPI concentration prevented coalescence while the higher total solids content increased the viscosity of the continuous phase.

2.4 Conclusions

High hydrophilic solids layer-by-layer (LBL) emulsions were successfully obtained using whey protein isolate (WPI) as the primary layer and gum Arabic as the secondary layer at pH 3.5. The formation of LBL interface was confirmed from ζ-potential measurements which showed charge reversal upon addition of gum Arabic solution into single layer (SL) emulsion. The LBL systems had negative ζ-potential at all pH and the negative ζ-potential increased with increasing pH. Flocculated particles remained stable and deflocculated into separate individual particles at higher the pH. Such behaviour would be of significant importance in food and pharmaceutical systems as flocculation can occur at low pH conditions in stomach while deflocculation for nutrient or active component delivery can be expected to enhance release of components from the oil phase at the higher pH of the intestine. However, the amount of WPI is vital for the stability of the particles. Insufficient WPI will lead to coalescence as observed in results obtained from ζ-average diameter (particle size) and optical microscope in 1WPI-28 and 1WPI-57. No coalescence was found in 10WPI-28 and 10WPI-57. Particles observed under light

microscope confirmed a reduction in particles flocculation with increasing pH. Total solids content play a role in retarding creaming as higher total solids content increased viscosity of the aqueous phase. However, total solids content has lesser effect on the stability of particles in LBL emulsion. The system with the higher amount of WPI and higher total solids content had the highest stability towards creaming.

Chapter III

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Stability and Loss Kinetics of Lutein and β-Carotene Encapsulated in Freeze-Dried Emulsions with Layered Interface and Trehalose as Glass Former

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Abstract

Dehydration and subsequent storage of food materials may result in physicochemical changes and losses of bioactive substances. The purpose of the present study was to determine stability and loss kinetics of carotenoids in dehydrated emulsions, and to compare the ability of freeze dried single-layer (SL) and layer-by-layer (LBL) emulsions with trehalose as wall material to protect the carotenoids. WPI (2%, w/w, in oil) was used as emulsifier and homogenized with sunflower oil containing β-carotene (0.05%, w/w) and lutein (0.05%, w/w). Gum Arabic was added to obtain LBL emulsion. Trehalose (20%, w/w) was used as the wall material. Citric acid solution (10%, w/w) was used to adjust pH of emulsions to pH 3.5. The emulsions were then freeze-dried, humidified at 33% RH, stored at low oxygen (25°C, 37°C and 45°C) in closed containers, and analysed using HPLC with C30 column and photodiode-array detector. The loss of lutein and all-trans-β-carotene followed first order kinetics and was more rapid in SL system compared to LBL system. Carotenoids losses increased with increasing storage temperatures especially in SL systems. Activation energy of all-trans-β-carotene in SL was 62.1 kJ/mol compared to 43.9 kJ/mol in LBL and 58.9 kJ/mol in SL compared to LBL with 45.9 kJ/mol for lutein. The total β-carotene retention upon storage for 70 days was higher in LBL at all storage temperatures. However, the utilisation of LBL increased isomerization. LBL gave better protection towards carotenoids losses during storage and is highly applicable to formulated materials to control stability of oil soluble bioactive components.

Keywords: layer-by-layer emulsion, trehalose, β-carotene, lutein, HPLC

3.1 Introduction

β-carotene is a highly lipophilic hydrocarbon (terpenoid) which may occur in foods in noncrystalline and crystalline forms. β-carotene has received much interest due to its high provitamin A activity as well as antioxidant capacity. Carotenoids have low bioavailability as crystals or within protein complexes in fruits and vegetables

making them difficult to become absorbed in the gastrointestinal tract during digestion (Williams *et al.,* 1998). For optimal absorption to occur, the food matrix has to be properly digested to release carotenoids. This is followed by lipid micelles development in the small intestine and absorption of carotenoids by intestinal mucosal cells. Lastly, the carotenoids or their metabolic products will be transferred to the lymphatic and/or portal circulation (Erdman *et al.,* 1993). Yuan *et al.* (2008) showed that β-carotene protect against such diseases as lung cancer, heart disease and colorectal adenomas. Brennan *et al.* (2011) concluded that bioactive compounds, such as β-carotene, in diet could play a major role in preventing the development of chronic and degenerative diseases in humans. β-carotene degradation is typically a result of isomerization (Sweeney and Marsh, 1971) and oxidation (Simpson, 1985). On the other hand, lutein is classified as a xanthophyll due to the presence of hydroxyl groups (oxygen). Lutein occurs in green vegetables and leaves and is capable of preventing macular degeneration and cataracts (Khachik *et al.,* 1997). Xanthophylls are more heat sensitive than carotenes (hydrocarbons) and often show higher degradation (Dhuique-Mayer *et al.,* 2007).

Incorporation of β-carotene in the lipid phase of an oil-in-water (O/W) emulsion can dissolve β-carotene which enhances bioavailability (Yuan *et al.,* 2008). The inclusion of carotenoids in a food ingredient in a powder form or in formulated foods, such as infant formula, is often carried out by emulsifying an oil phase to a hydrophilic phase. The emulsion is subsequently dehydrated which requires the presence of a glass-forming hydrophilic component to form a continuous solid phase of the dried formulation (Roos, 1995; Drusch *et al.,* 2006). In such formulations, whey proteins may be used as edible emulsifiers as they exhibit amphiphilic characteristics and assembly on the oil/water interphase during homogenisation (Rahali *et al.,* 2000). Whey proteins show a positive charge at pH conditions below their isoelectric point of 5.2 (Bryant and McClements, 1998). Several studies have reported improved characteristics of emulsions with layered interface structures (Moreau *et al.,* 2003; Ogawa *et al.,* 2003). Layer-by-layer (LBL) emulsions can be produced by the electrostatic attraction between oppositely charged polyelectrolyte and the charged protein interface (Guzey and McClements, 2006b). Whey proteins at

a low pH can be covered by negatively charged polyelectrolytes, including pectins and gum Arabic (Moreau *et al.,* 2003; Güzey and McClements, 2006a). Gu *et al.* (2004) reported that LBL systems gave improved resistance of emulsions against freeze thaw cycles, lipid oxidation and high salt concentrations. Resistance of LBL emulsions towards changes in pH, ionic strength, heat in thermal processing and drying have also been reported (Gharsallaoui *et al.,* 2010). The pKa value of gum Arabic is approximately 2.2 (Moschakis *et al.,* 2010) and it may form LBL interface structures with whey proteins at higher pH. LBL emulsion particles with thicker interfacial layer compared to a single emulsifier layer (SL) increased the steric repulsion between particles (Harnsilawat *et al.,* 2006) as well as gave a better resistance towards disruptions (Güzey and McClements, 2006a). The stronger electrostatic and repulsive forces of the two layered molecular species in LBL emulsions compared to SL in general increased the stability of LBL emulsions towards environmental stresses (Benjamin *et al.,* 2012).

Degradation of β-carotene in dry emulsions can occur as a consequence of crystallization of the glass-forming sugar causing the release of the lipid phase, and occurrence of oxidation (Buera *et al.,* 2005). The use of low molecular weight carbohydrates can also lead to caking, structural collapse and crystallization (Drusch *et al.,* 2006). Therefore, a suitable wall material has to be selected to support the glass-formation and stability of the continuous phase in dehydrated materials. Trehalose is an excellent choice as it showed unique combination of water replacement properties, a high glass transition temperature and chemical inertness (Schebor *et al.,* 2010). The aim of the present study was to determine isomerisation and loss of carotenoids, and compare the ability of freeze-dried SL and LBL emulsions with trehalose as the glass-forming wall material to protect bioactive carotenoids upon storage in the vicinity of the glass transition. Study was done on systems with no oxygen. Trehalose, a well characterized glass formers was used to protect carotenoids against non-oxidative degradation. We report data on carotenoids loss kinetics and LBL ability in preventing losses of encapsulated carotenoids in freeze-dried systems during storage in closed containers. Such data are of high importance to food and pharmaceutical ingredients and formulations.

3.2 Materials and Methods

3.2.1 Materials

Whey protein isolate (WPI, Isolac, Carbery Food Ingredients, Ballineen, Ireland) was used as an emulsifier (the primary layer). Trehalose (crystalline dihydrate) (Hayashibara Shoji Inc., Japan) was used as the wall material. Gum Arabic (Sigma Aldrich G9752 Stenheim, Germany) was used as a polyelectrolyte (the secondary layer). Sunflower oil A (Musgrave Excellence^{TM}, Spain) or sunflower oil B (Pan Euro Foods, Ireland) was used as the lipid phase and the solvent for the lipophilic all-trans-β-carotene (crystalline Type I, synthetic, $\geq$ 93% (UV), Sigma-Aldrich, U.S.A.) and lutein (Marigold) (Shaanxi Sciphar Biotechnology Co. Ltd., China). Carotenoids were used as indicator to compare the systems in preventing bioactive compound losses. All other chemicals were purchased from Sigma-Aldrich, Inc. (Dublin, Ireland).

3.2.2 Emulsion Preparation

The emulsions were prepared in replicate in two batches using sunflower oil A and B. WPI (2%, w/w, in oil, 0.5%, w/w, in water) was dispersed in deionized water and stirred for 2 hours to enhance hydration of the proteins. pH was adjusted to pH 3.5 by citric acid solution (10% w/w). The oil phase was prepared by dispersing β-carotene (0.05%, w/w) and lutein (0.05%, w/w) in sunflower oil at 50°C by mixing with magnetic stirrer in a beaker covered with aluminium foil until a homogeneous dispersion was obtained. The oil phase and water phase were mixed and pre-homogenised using Ultra-Turrax (T25 Digital, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 10,000 rpm for 30s. 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). Trehalose (20% w/w total solids) was dissolved in water by stirring using a magnetic rod at 50°C for 2 hours and the pH was adjusted to pH 3.5 by citric acid solution (10% w/w). The emulsion was mixed

to an equal amount of trehalose solution (1:1 w/w) under magnetic stirring for 30 min to obtain the SL emulsion. To obtain the LBL emulsion, gum Arabic (0.5% w/w) was dispersed in deionized water and stirred 2 hours. The solution was then adjusted to pH 3.5 with citric acid solution (10% w/w). The primary emulsion was mixed with 1ml of gum Arabic solution for every 100g emulsion (0.005% w/w) at room temperature for 30 min to form the LBL system. Then, the emulsion (with gum Arabic) was mixed with equal amount of the trehalose solution (1:1 w/w) under magnetic stirring for 30 min. The zeta-average of the emulsion was determined to be 189.3±10 nm for SL and 220.7±17 nm for LBL. One the other hand, value of 13.43±0.5 mV was obtained for zeta-potential of SL and -4.08±0.2 for LBL.

3.2.3 Freeze-Drying

For freeze-drying (Lyovac GT2, Steris, Hurth, Germany), 4 ml aliquots of emulsions (SL and LBL) were transferred into 10 mL clear glass vials (Schott, Müllheim, Germany). Prior to freeze-drying, emulsions were frozen in vials at -20°C overnight (HLLF-240, Heto, Jouan Nordic A/S, Allerød, Denmark) and then tempered at -80ºC in a deep freezer (Icebird/Mini Freeze 80, Heto, Jouan Nordic A/S, Allerød, Denmark) for 3 hours. Freeze-drying was carried out under vacuum at pressure, p <0.1mbar (Lyovac GT 2, Steris®, Hürth, Germany) for at least 72 hours.

3.2.4 Water activity

All vials with samples were humidified at 33% RH in vacuum desiccators at room temperature. After equilibration, the sample vials were 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 and stored at 25°C, 37°C and 45°C for analysis. The experiment was carried out in replicate batches and samples were stored up to 70 days (sunflower oil A and sunflower oil B). The vacuum in the packages served as an indicator of isolation of the samples from the surrounding atmosphere as well as for leakage and possible loss of water during storage.

Water activity, a_w, 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 samples prepared using sunflower oil B after storage at 25°C, 37°C and 45°C was measured over 12 time points up to 72 days of storage.

3.2.5 Colourimetry

The colour was determined using a pre-calibrated colourimeter (Minolta, CR300 meter) for samples prepared using sunflower oil B after storage at 25°C, 37°C and 45°C at intervals for up to 72 days of storage. Colour of samples was measured directly from glass vials. Colour was expressed by the CIE L*a*b colour space which is an international standard for colour measurement adopted by the Commission Internationale de l'Eclairage (CIE) in 1976 (Quek e*t al.,* 2007). The L* value corresponded to the luminance or lightness component and ranged from 0-100, a* measured green to red values and b* from blue to yellow as the two chromatic components which ranged from -120 to 120 (Quek e*t al.,* 2007). L*a*b* values were measured in duplicate for both SL and LBL systems at each storage temperature over the 12 time points.

3.2.6 Extraction Method and HPLC Analysis

The study of carotenoids stability was carried out in replicate up to 70 days of storage using samples with sunflower oil A and sunflower oil B. Samples with sunflower oil A and sunflower oil B were studied at different time points. Duplicate samples of the freeze-dried emulsions (0.5 grams) were reconstituted in 2mL of deionized water (KB Scientific, Ireland) by vortexing (Scientific Industries Inc., G-560E, NY, USA) at room temperature for 30 s. In order to destabilize the emulsion, 3.9 mL methanol:ethlyacetate (1:1 v/v), serving also as organic solvents for the carotenoids, containing 0.2% butylated hydroxyl toluene (BHT) as an antioxidant, were added and vortexed for 30 s. BHT was added into the solvent to prevent loss of carotenoids during extraction process. The oil in the samples was saponified by

adding 1 mL of saturated potassium hydroxide in methanol (2M) and the mix was vortexed for 30 s to separate the lipid carrier (saponized fraction) from the β-carotene and lutein (unsaponised). In order to completely dissolve the β-carotene and lutein in an organic phase, 1mL dichlorometane was added and the samples were vortexed for 30s. Lastly, 3.8mL of hexane was added and the samples were vortexed for 30s to enhance separation of the organic phase. The extracts were left to stand for 30 min. The supernatant was removed with a pipette, filtered (Minisart RC 15, Sartorius Stedim Biotech GmbH, Goettingen, Germany), and transferred to HPLC vials (Dionex No: 055427, 1.5ml w/ Slit Septum , Unas, USA). A 10 µL injection of each sample was used in HPLC analysis of the β-carotene and lutein. A 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. The HPLC column was a 250 mm × 4.6 mm i.d., 5 µm, reversed-phase Acclaim C30 analytical column with a 4 mm × 4 mm i.d. guard column of the same material (Dionex, Sunnyvale, CA, U.S.A.). An eluent system was composed of acetonitrile (A), methanol:ethyl acetate (1:1) (B) and 0.5% of 200 mmol acetic acid in water (C) using a 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. Carotenoids were detected from absorbance at 450 nm. The relative amounts of β-carotene and lutein were derived from absorption peak areas. β-carotene and lutein degradation data were fitted to first-order kinetics: $\ln A/A_0 = -kt$, and the rate constants (k) were derived from the slopes of linear regressions. A first order kinetics was selected as plot of $\ln A/A_0$ against time (storage days) gave a straight line similar to those found in other studies (Hidalgo and Brandolini, 2008; Achir *et al.,* 2010; Ramoneda *et al.,* 2011). Activation energy was obtained using the relationship $k = Ae^{-Ea/(RT)}$ and plots of $\ln k$ against T^{-1}.

3.2.7 Statistical Analysis

The carotenoids stability study was carried out in replicate using sunflower A and B. Colour and water activity were obtained from duplicated measurements. One-way

analysis of variance (ANOVA) was used to determine the significant differences for multiple comparisons which was completed using Tukey test at $\alpha = 0.05$ (SPSS statistical package, ver.20.0).

3.3 Results and Discussions

3.3.1 Water Activity and Colourimetry

The water activity of both SL and LBL systems at 0 day and after 72 days of storage at 25°C, 37°C and 45°C are shown in Figure 3.1. There was a slight increase with significant difference in water activity during storage at 25°C. At 37°C and 45°C water activity decreased with time with significant difference in water activity of the SL and LBL systems at all storage temperatures. The retained vacuum in the sample packages during storage showed that the variation in a_w was a result of physicochemical changes as exchange of water with the surroundings was excluded and the samples were stored in closed containers. Potes *et al.*, (2013) stated that lipid oxidation produced reactive oxygen groups and free radicals that could lower a_w in sunflower oil based systems at 40°C. The decrease in a_w could result from hydration of double bonds of the unsaturated fatty acids at the higher temperatures. Also a decrease in a_w could occur as a result of hydration-related conformational changes of whey proteins that occurred at 35-40°C (Potes *et al.,* 2013). A study by Cardona *et al.* (1997) stated that the availability of water plays an important role in crystallisation of trehalose. Trehalose typically formed dihydrate crystals above the glass transition and water could migrate to the hydrated structure to form crystals with a consequent desiccant effect (Cerdeira *et al.,* 2005; Schebor *et al.,* 2010). Although such crystallization could explain the reduction in water activity at 37°C and 45°C, this hypothesis was not supported by the development of a hard, noncrystalline, poorly soluble structure of the materials at the end of storage.

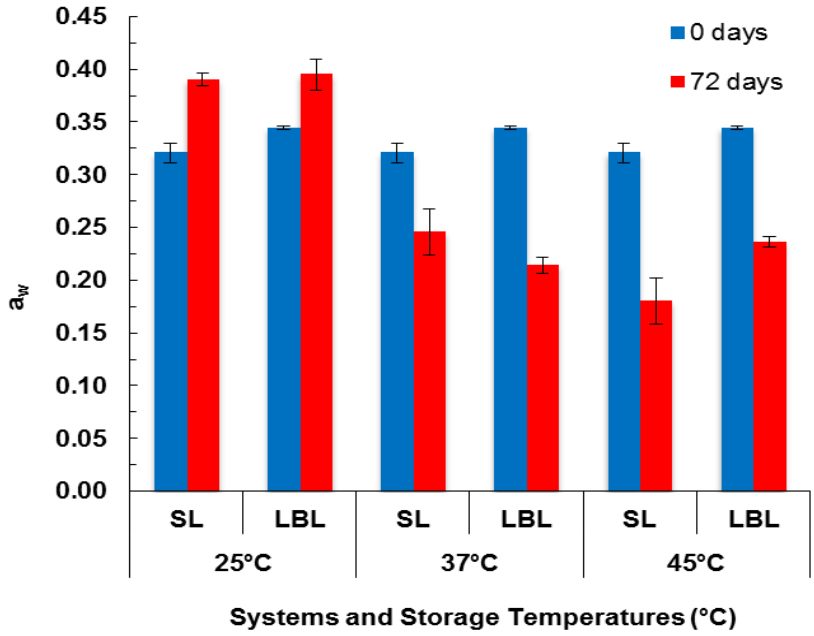

Figure 3.1. Water activity (a_w) of SL and LBL systems before and after storage for 72 days at 25°C, 37°C and 45°C.

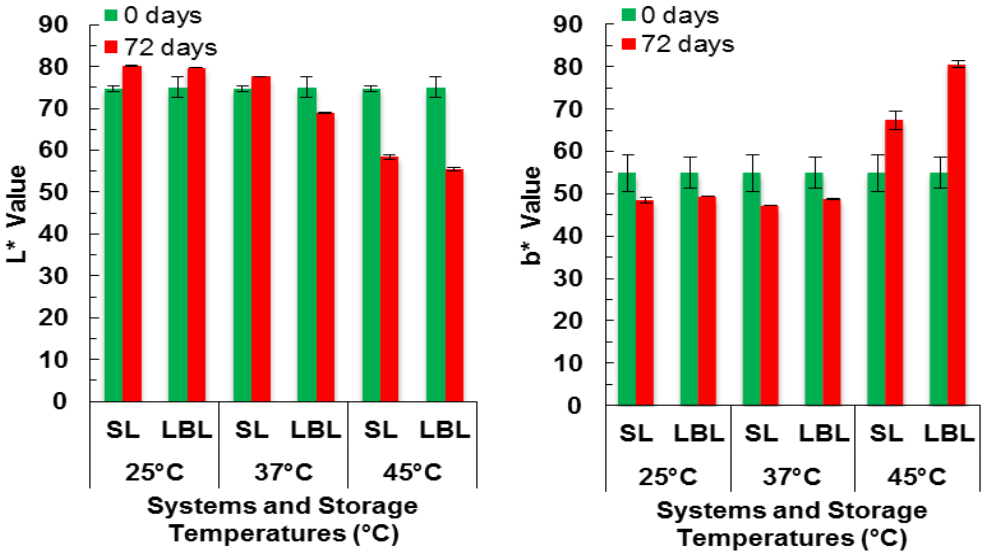

Figure 3.2. L* and b* values of SL and LBL systems before and after storage for 72 days at 25°C, 37°C and 45°C.

The differences in the colour values (L* and b*) of SL and LBL systems at 0 and after 72 days of storage at 25°C, 37°C and 45°C are shown in Figure 3.2. Minor variations were found for the a* values. The L* (lightness) values increased, and b* (yellowness) values decreased with significant difference after 72 days of storage.

More pronounced differences were found in the L* and b* values during storage at 45°C. The L* values decreased from 74.7 to 58.4 in SL and from 75.0 to 55.5 in LBL systems while the b* values increased from 54.9 to 67.4 in SL and from 55.1 to 80.6 in LBL systems. A study by Drusch *et al.* (2006) showed that the release of oil from microcapsules led to an increase in b* value. Free oil was found in the samples stored at 45°C in this study. Results of the study by Drusch *et al.* (2006) showed that when the materials were stored at 54% RH with trehalose as wall material, the L* values decreased and b* values increased in agreement with the results for samples stored at 45°C in the present study. The structural collapse during storage at 45°C caused bigger effect on colour values as compared to storage at 25°C and 37°C. Storage of samples above T_g led to a dynamic process of structural collapse following the rate controlled by the $T-T_g$ (Levi and Karel, 1995). Structural collapse allowed β-carotene containing oil to become released from the freeze-dried structure (Roos, 2002) and such free oil was present in the vials containing collapsed samples. The structural relaxations altered the appearance of the material. The changes in colour values were not related to non-enzymatic browning as trehalose is a non-reducing sugar (Drusch *et al.*, 2006) although lipid oxidation may also lead to browning (Venolia and Tappel, 1958; Zirlin and Karel, 1969; Pokorný *et al.*, 1974).

3.3.2 HPLC Analysis

There was a significant loss of lutein and all-trans β-carotene upon humidification over 5 days at 33% RH corresponding to approximately 43% and 23%, respectively. The water content in the humidified materials was approximately 7.42±0.07g/100g solids non-fat (SNF) for SL system and 7.44±0.08g/ 100g SNF for LBL. The glass transition temperature of the systems was found at 37°C. Study by Cardona *et al.* (1997) showed that the T_g of trehalose at 33% RH was approximately 35°C. The HPLC chromatograms (Figure 3.3) showed peaks identified as those of lutein with retention time of 5.7±0.3 min, 15-cis-β-carotene (20.8±0.6 min), 13-cis-β-carotene (24.9±0.4 min) and all-trans-β-carotene (29.5±0.9 min). All-trans-β-carotene possessed the highest provitamin A activity but exposure to heat during storage and processing may cause isomerization (Chandler and Schwartz, 1988).

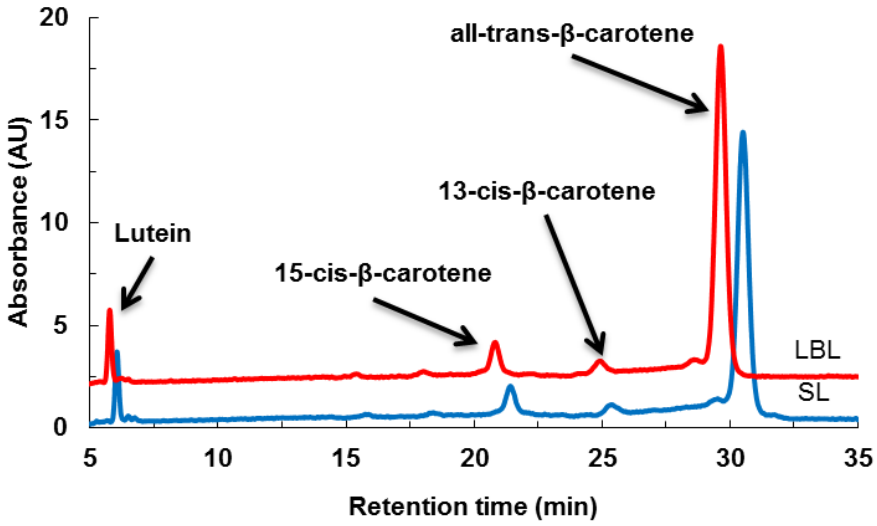

Figure 3.3. Peaks identified in chromatograms of humidified freeze dried SL and LBL emulsions (33% RH, 0 Day).

The data of peak areas for various carotenoids were used to derive quantitative values for isomerisation and retention, and first order kinetics were fitted to the data to obtain the first order degradation rate constants for β-carotene and lutein in SL and LBL systems. A first order kinetics was selected as the plot of ln A/A_0 against time (storage days) gave a straight line. Studies showed that carotenoids degradation is a typical first order reaction (Desobry *et al.,* 1997; Elizalde *et al.,* 2002; Zepka *et al.,* 2009; Achir *et al.,* 2010; Ramoneda *et al.,* 2011). The first order kinetic plot used replicate data obtained from sunflower oil A and sunflower oil B systems. Degradation of both all-trans-β-carotene and lutein increased with increasing storage temperature. Similar trend was found by Desorby *et al.* (1997), Dhuique-Mayer *et al.* (2007), Hidalgo and Brandolini (2008), and Qian *et al.* (2012). The first order rate constants of all-trans-β-carotene degradation were generally higher than those for lutein indicating a higher loss of all-trans-β-carotene during storage. The presence of two hydroxyl groups in lutein makes lutein more polar and hydrophilic than all-trans-β-carotene (Farombi and Britton, 1999; Updike and Schwartz, 2003; Alves-Rodrigues and Shao, 2004; Achir *et al.,* 2010). As a result, lutein molecules tend to assembly at the oil-WPI interface while all-trans-β-carotene may exist in the bulk oil. The presence of lutein at the oil-WPI interface may result in the lower rate of lutein degradation during storage. Citric acid that was added into the continuous phase

during emulsion preparation can provide lutein with protection against degradation. A study by Hraš *et al.* (2000) showed that citric acid provide small antioxidative effect. However, most of the lutein losses, 43%, occurred during humidification which could be due to the presence of lutein at the oil-WPI interface while only 23% of all-trans-β-carotene loss was accounted for humidification. Due to its molecular properties, lutein assembled at the WPI-oil interface could become exposed to the trapped oxygen within the matrix and therefore shows a more rapid degradation. Lutein may also interact with free fatty acids and the protein present at the oil interface. Lutein as a xanthophyll can undergo esterification with fatty acids present in sunflower oil. The carboxyl groups present in free fatty acids may assembly itself towards the oil-WPI interface as it is more polar. The presence of lutein and free fatty acids at the oil-WPI interface are likely to increase esterification of lutein and the proteins can increase losses in extraction of lutein (Peŕez-Gálvez and Mínguez-Mosquera, 2005). The highly oxygenated lutein could show a faster degradation as well (Dhuique-Mayer *et al.*, 2007). The polarity of the esterified lutein is lower and lutein esters can become concentrated in the core of the oil particles (Peŕez-Gálvez and Mínguez-Mosquera, 2005). The difference in polarity between lutein and lutein esters gives them different retention times in an HPLC analysis and effectively reduce the peak of lutein in HPLC chromatograms. The large difference in polarity between lutein and its esters increases the difficulty of their separation and analysis in a single step chromatographic method (Rivas, 1989). Our results also revealed that the rate constant of both lutein and all-trans-β-carotene losses were higher in SL systems than in LBL systems.

Oxidation plays a major role in the degradation of carotenoids (Achir *et al.*, 2010). The trapped oxygen within the glassy matrix during humidification in this study could contribute to oxidation of the carotenoids present in the systems and the initially rapid loss. The first order rate constants from each storage temperature were used in Arrhenius plots to obtain the activation energy for all-trans-β-carotene and lutein in SL and LBL systems. Arrhenius plots of all-trans-β-carotene and lutein are shown in Figure 3.4. The activation energy of SL system was higher than that of the LBL system for both all-trans-β-carotene and lutein. The higher activation energy for

the SL systems indicated that the carotenoid losses in SL were more temperature dependent. Activation energy of all-trans-β-carotene in SL was 62.1 kJ/mol compared to 43.9 kJ/mol in LBL and 58.9 kJ/mol in SL compared to LBL with 45.9 kJ/mol for lutein. A study by Ahmed *et al.* (2002) stated that carotenoids loss in papaya puree was heat sensitive and the activation energy was 20.6 kJ/mol. This value was lower than those obtained in this study. Henry *et al.* (1998) obtained activation energy of 109.6 kJ/mol for all-trans-β-carotene and 104.2 kJ/mol for lutein in oil model system stored at 75°C, 85°C and 95°C.

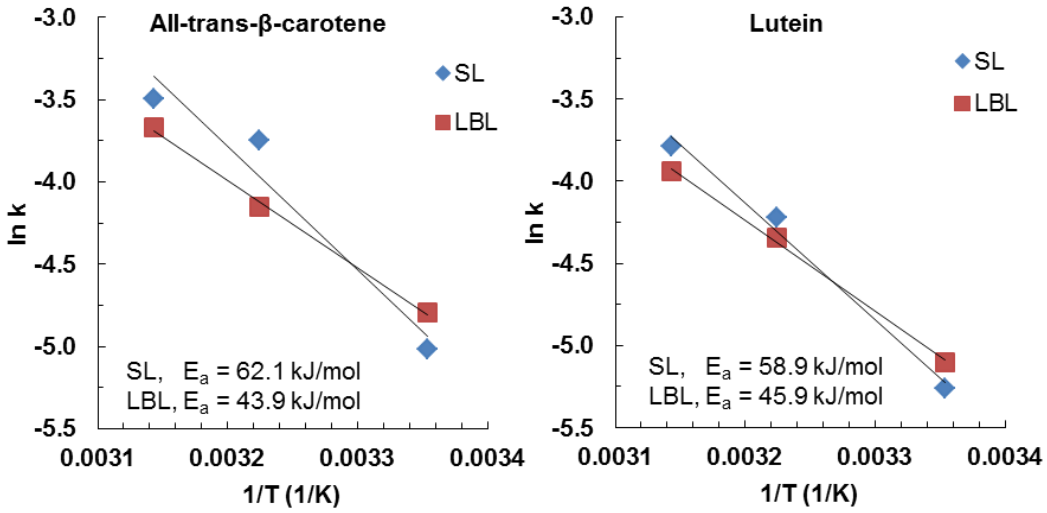

Figure 3.4. Arrhenius plot of all-trans-β-carotene and lutein in SL and LBL systems stored at 25°C, 37°C, and 45°C.

It was noted that structural collapsed occurred in all of the samples stored at 45°C after 24 hours storage. Structural collapse occurred randomly in SL and LBL after storage for 1 week at 37°C. Storage around and above the T_g could lead to collapse. Glass transition may cause the encapsulated β-carotene containing lipid to be released (Shimada *et al.*, 1991). Such release will increase isomerisation and loss of encapsulated carotenoids as they are not protected within the matrix. Structural collapse also caused increased insolubility as the samples became less water soluble. Therefore, collapsed samples were vortexed for a longer period to ensure complete carotenoids extraction. A study by Harnkarnsujarit *et al.* (2012b) concluded that β-carotene losses were reduced in freeze-dried solids with structural collapse.

Although all of the samples stored at 45°C undergo structural collapse, the rate constants of all-trans-β-carotene and lutein losses in both SL and LBL were higher than in samples stored at 25°C and 37°C. Ramoneda *et al.* (2011) reported that high β-carotene loss was associated with structural collapse. However, structural collapse in samples stored at 45°C suggested delayed carotenoids losses based on the rate constants obtained. The rate constant of carotenoids losses above the T_g of the systems increased at a smaller rate compared to those from 25°C to 37°C. It was noted that the structural changes above the T_g delayed degradation, especially in SL, but the glass transition of the solid encapsulant as such did not affect loss of the carotenoids.

Although oxidation is the main cause of carotenoid degradation, isomerisation may play a significant role in carotenoid stability during processing (Qiu *et al.*, 2009). Light exposure and temperature are the major causes of isomerization in carotenoids (Liu *et al.,* 2009; Khoo *et al.,* 2011). Both 15-cis-β-carotene and 13-cis-β-carotene were present in SL and LBL at all temperatures showed that isomerization occurred in both systems. The occurrence of both isomers increased with increasing storage temperature. Studies by and Chandler and Schwartz (1988), and Achir *et al.* (2010) found that heating decreased all-trans-β-carotene but at the same time increased isomerization. Isomers were formed in larger amounts in LBL systems compared to SL. The exposure to heat during storage caused isomerization in the samples. However, LBL systems may have prevented the total loss of all-trans-β-carotene and therefore larger amount of isomers were obtained compared to SL. Amount of 15-cis-β-carotene produced was higher than 13-cis-β-carotene. The amounts of both 15-cis-β-carotene and 13-cis-β-carotene generally fluctuated with storage. Although isomerization increased in LBL, the total loss of β-carotene (all-trans-β-carotene, 15-cis-β-carotene and 13-cis-β-carotene) in LBL was reduced (Figure 3.5) compared to SL systems. The retention of total β-carotene was higher in LBL at all storage temperatures. The higher retention in LBL system could be due to its higher physical stability towards environmental stresses. Studies by Moreau *et al.* (2003), Ogawa *et al.* (2003), Gu *et al.* (2005) and Harnsilawat *et al.* (2006) concluded LBL systems have better stability compared to SL systems. The retention of total β-carotene in SL

(48.1%) and LBL (50.7%) stored at 25°C were comparable upon storage for 70 days but higher retention occurred in LBL system stored at 37°C and 45°C. The retention of total β-carotene in SL stored at 37°C was 36.1% and 39.7% in LBL. Corresponding retention was found for 45°C where the retention was 12.1% in SL and 24.7% in LBL. These data showed that LBL reduced the loss of β-carotene with no significant difference especially at higher storage temperatures. The application of LBL technology with protein coated oil particles can enhance emulsion stability towards environmental stresses (Gharsallaoui *et al.*, 2010). The thick interfacial layer increased particles resistance towards disruptions (Güzey and McClements, 2006a). The increased stability of LBL system can prevent disruption of the oil particles that result in in the release of encapsulated carotenoids containing oil towards the matrix surface during storage.

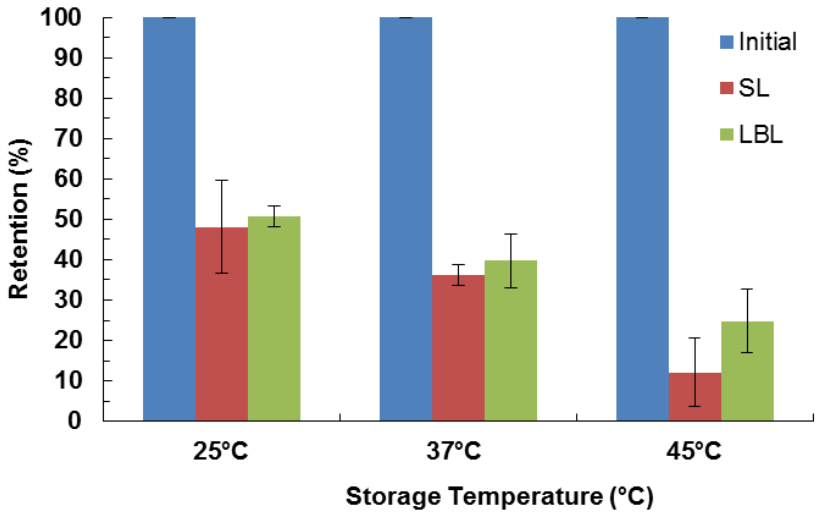

Figure 3.5. The difference in retention (%) of total β-carotene between SL and LBL systems stored at 25°C, 37°C, and 45°C for 70 days.

3.4 Conclusions

The use of LBL emulsion gave a better protection towards the loss of carotenoids in storage of freeze-dried emulsions with trehalose as wall material. Rate constants of all-trans-β-carotene degradation were higher than those of lutein. The retention of

total β-carotene upon storage for 70 days was higher in LBL at all storage temperatures. However, the application of LBL increased isomerization of all-trans-β-carotene. The activation energy of lutein and all-trans-β-carotene loss was higher in SL systems than in LBL. Structural collapse of the systems may have delayed the loss of carotenoids based on the rate constants obtained. The findings are highly applicable to formulated materials to control stability of oil soluble bioactive components, such as vitamins in infant formulas, nutritional supplements and medical foods.

Chapter IV

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Spray Drying of High Hydrophilic Solids Emulsions with Layered Interface and Trehalose-Maltodextrin as Glass Formers for Carotenoids Stabilization

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Abstract

Objectives of the present study were to spray dry high total solids content single layer (SL) and layer-by-layer (LBL) emulsions, and to compare the ability of the SL and LBL powders in preventing the loss of carotenoids upon storage in the vicinity of the glass transition temperature (T_g). Carotenoids stability in humidified spray dried (HSD), non-humidified freeze-dried (NHFD), and humidified freeze-dried (HFD) systems were determined as well. The loss of carotenoids followed first order loss kinetics. An initial rapid followed by a second slower first order loss kinetics was observed in the non-humidified spray dried (NHSD) systems. Storage of systems above the T_g delayed carotenoids losses based on the rate constants. The loss of carotenoids in LBL systems was more heat sensitive as the activation energies were generally higher. Activation energy decreased above T_g indicating that the loss of carotenoids became less temperature dependent. The application of LBL interface structure reduced the rate of carotenoids losses and can be applied to prevent loss of oil soluble bioactives in formulated materials.

Keywords: layer-by-layer; β-carotene; lutein; spray drying; glass transition; loss kinetics

4.1 Introduction

Bioactive compounds in food materials play an important role in the prevention of chronic and degenerative diseases in humans (van Dokkum *et al.*, 2008). Carotenoids are generally present in fruits and vegetables. β-carotene is a highly lipophilic hydrocarbon that is present in its non-crystalline or crystalline form in foods. Among the provitamin A carotenoids, β-carotene possessed the highest vitamin A activity as well as the highest rate of conversion to vitamin A (Grune *et al.*, 2010). Besides that, β-carotene also showed antioxidant activity and anticancer properties, and it may protect against heart disease (Bendich and Olson, 1989; Omenn *et al.*, 1996; Albanes, 1999). However, isomerisation (Sweeney and Marsh, 1971) and oxidation (Simpson, 1985) may lead to the degradation of β-carotene. On the other hand, lutein

is known as a xanthophyll due to the presence of hydroxyl groups in its molecular structure. Lutein was found to be able to prevent macular degeneration and cataracts (Khachik et *al.*, 1997) as well as play a role in minimizing light-induced skin damage (Ribaya-Mercado and Blumberg, 2004). Dhuique-Mayer *et al.* (2007) found that xanthophyll had faster degradation rates than carotenes and lower heat stability. However, carotenoids have low bioavailability when present as crystals or within protein complexes in fruits and vegetables. This causes difficulty in the adsorption of carotenoids during digestion in the gastrointestinal tract (Williams, 1998). Bioavailability of carotenoids can be enhanced by using delivery with edible oil as oil improves carotenoids adsorption (van Het Hof *et al.*, 2000).

Carotenoids can be dissolved and incorporated into the lipid phase of an oil-in-water (O/W) emulsion to improve its bioavailability. This will be followed by emulsifying of the oil phase with an aqueous phase containing an emulsifier. The emulsion can be dehydrated to obtain a continuous phase of the dried formulation using a glass-forming hydrophilic component (Drusch *et al.*, 2006; Ramoneda *et al.*, 2011; Spada *et al.*, 2012). Several studies have shown that layer-by-layer (LBL) emulsion with layered interface structures have a higher stability towards environmental stresses such as heat treatment, variations in pH, freeze-thaw cycles, lipid oxidation and ionic strength (Ogawa *et al.*, 2003; Aoki *et al.*, 2005; Güzey and McClements, 2006a; Gharsallaoui *et al.*, 2010; Lim *et al.*, 2014) which will result in an improved protection against degradation of the encapsulated bioactive compounds. The thicker interfacial layer too can increase the oxidative stability of LBL system as well as providing the particles with a higher stability towards disruptions (McClements, 1999; Klinkesorn *et al.*, 2005). LBL emulsion can be obtained via electrostatic attraction between a charged surface and oppositely charged polyelectrolyte present in the aqueous phase. Protein is commonly used as the primary layer to obtain LBL emulsion (Gu *et al.*, 2004; Gharsallaoui *et al.*, 2010; Klein *et al.*, 2010; Lim and Roos, 2015) as its charge can be modified by changing the pH of the aqueous phase. The isoelectric point (pI) of α-lactalbumin is 4.1 (Weinbreck *et al.*, 2003) while β-lactoglobulin has pI of 5.2 (Bryant and McClements, 1998). On the other hand, gum

Arabic has a pKa value of approximately 2.2 and will be negatively charged at above pH 2.2 (Weinbreck *et al.*, 2004).

Spray drying is a dehydration method commonly utilized in the food industry as it is economical and straightforward producing high quality dry powders with long shelf-life. Besides freeze drying (Desobry *et al.*, 1997; Harnkarnsujarit *et al.*, 2012b), spray drying is also used in the food industry for microencapsulation (Ré, 1998). Microencapsulation is a method where wall materials are used to entrap a core material to protect it from environmental stresses and to extend shelf life as well as provide controlled release of the core material (Shahidi and Han, 1993). Wall materials that have been used for spray drying include maltodextrins, trehalose, milk proteins, corn syrup and modified starch (Desobry *et al.*, 1997; Hogan *et al.*, 2001a; Drusch *et al.*, 2006; Shaw *et al.*, 2007; Liang *et al.*, 2013). However, crystallization of the glass formers; materials that are capable to form glassy structures in dried emulsions causes the release of the carotenoids containing lipid phase that will result in degradation of the carotenoids as a consequence of oxidation (Buera *et al.*, 2005). Nonetheless, studies have shown that mixtures of amorphous sugars with high molecular weight carbohydrates can delay crystallization of the amorphous sugar (Mazzobre *et al.*, 1997; Gabarra and Hartel, 1998; Kouassi and Roos, 2001; Potes *et al.*, 2012). Our previous study showed that freeze-dried LBL emulsion with trehalose as the wall material gave a better protection towards the loss of carotenoids as compared to SL system (Lim *et al.*, 2014). The objectives of the present study were to spray dry SL and LBL emulsions with high hydrophilic solids content, and to compare the ability of spray dried SL and LBL emulsions with mixture of maltodextrin and trehalose as wall materials in preventing the loss of carotenoids upon storage in the vicinity of the glass transition temperature of the encapsulant. The ability of humidified spray dried (HSD), non-humidified freeze-dried (NHFD), and humidified freeze-dried (HFD) systems in preventing the loss of carotenoids was determined as well. Data on carotenoid loss kinetics and the ability of SL and LBL systems to prevent losses of encapsulated carotenoids in spray dried systems during storage in closed containers were reported. These data are useful for formulated materials in food and pharmaceutical industries controlling the stability of oil soluble

bioactive compounds. The study will provide information on the effects of glass transition on stability of encapsulated bioactives and the ability of SL and LBL systems in protecting bioactives. The use of high total solids emulsions for spray drying too will be beneficial for the industry as there are few reports on the use of such concentrated systems available.

4.2 Materials and Methods

4.2.1 Materials

Whey protein isolate (WPI, Isolac) was obtained from Carbery Food Ingredients (Ballineen, Ireland), trehalose (crystalline dihydrate) from Hayashibara Shoji Inc. (Japan), maltodextrin (M100, DE 9-12) from Grain Processing Corporation (IA, U.S.A.), gum Arabic (Sigma Aldrich G9752) from Sigma Aldrich (Stenheim, Germany), sunflower oil from Musgrave Excellence^{TM} (Spain), lutein (Marigold) from Shaanxi Sciphar Biotechnology Co. Ltd. (China) and all-trans-β-carotene (crystalline Type I, synthetic, > 93% (UV)) from Sigma-Aldrich (U.S.A.) All other chemicals were purchased from Sigma-Aldrich, Inc. (Dublin, Ireland).

4.2.2 Emulsion Preparation

Deionized water was used to disperse WPI (10.71%, w/w, in water) and the dispersion was allowed to hydrate for 2 hours to enhance hydration of the proteins. Citric acid solution (10% w/w) was used to adjust pH of the dispersion to pH 3.5. Sunflower oil containing β-carotene (0.05%, w/w, of oil) and lutein (0.05%, w/w, of oil) was prepared and mixed with a Silverson mixer (Model AXR, Silverson Machines Ltd., Chesham, UK) at 50°C until a homogeneous dispersion was obtained. Pre-emulsion was obtained by mixing the oil phase and water phase using Silverson mixer at minimum speed for 60s. The pre-emulsions were subsequently homogenized for 3 cycles at 240 bar (200 bars for the first stage and 40 bars for the second) at room temperature using a two-stage valve homogenizer (APV-1000, APV Homogenizer Group, Wilmington, MA, USA). Trehalose and maltodextrin (1:1,

57.14%, w/w, in water) were dissolved in water at 65°C using a Silverson mixer. Maltodextrin was dissolved initially followed by trehalose and the pH was adjusted to pH 3.5 by citric acid solution (10% w/w). SL emulsion was obtained by mixing the emulsion to trehalose and maltodextrin solution for 30 min. Gum Arabic (2.91%, w/w, in water) was dispersed in deionized water and stirred for 2 hours. Citric acid solution (10% w/w) was used to adjust pH of the dispersion to pH 3.5. To obtain LBL emulsion, the gum Arabic solution was mixed with the emulsion at room temperature for 30 min. The emulsion with gum Arabic as secondary layer was then mixed with trehalose and maltodextrin solution for 30 min.

4.2.3 Spray Drying

The emulsions were dehydrated using a single stage Niro 25 spray dryer (GEA Niro, Soborg, Denmark) with rotating disc atomizer at 18000 rpm. The inlet and outlet temperatures were 185°C and 85°C, respectively. The powders 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 powders (2g) were then transferred into 10 mL clear glass vials (Schott, Müllheim, Germany) for further study.

4.2.4 Freeze-Drying

Freeze-dried SL and LBL emulsions were prepared using a freeze-drier (Lyovac GT 2, Steris®, Hürth, Germany) by transferring 2.5 ml of aliquots of emulsions into 10 mL clear glass vials (Schott, Müllheim, Germany). The emulsions were diluted with 2.5 ml of water adjusted to pH 3.5 using citric acid solution to allow proper drying in the freeze-drier. The emulsions were frozen in vials at −20 °C overnight (HLLF-240, Heto, Jouan Nordic A/S, Allerød, Denmark) and then tempered at −80 °C in a deep freezer (Icebird/Mini Freeze 80, Heto, Jouan Nordic A/S, Allerød, Denmark) for 3 h before freeze-drying. Freeze-drying was done for at least 72 h under vacuum at pressure, $p < 0.1$ mbar. Samples obtained from freeze drying were used for HPLC analysis for up to 8 days (7 time points).

4.2.5 Samples Packaging

A batch of spray dried and freeze-dried emulsions (SL and LBL) were humidified in vials over saturated solution of $MgCl_2$ (Sigma Chemical Co., St. Louise, MO, U.S.A.) at $0.33a_w$ in vacuum desiccators at room temperature. The humidified ($0.33a_w$) and non-humidified (as it is for spray dried and 0 a_w for freeze-dried) samples in vials were then hermetically sealed with vacuum using a freeze-drier. The samples were then 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 and stored at 25 °C, 45 °C, and 65 °C for non-humidified systems and 25 °C, 35 °C, and 45 °C for humidified systems prior to analyses. The vacuum in the packages represents isolation of the systems from surrounding atmosphere and as indicator for leakage that causes possible loss of water during storage. The glass transition temperatures (T_g) of the spray dried emulsions with mixture of trehalose and maltodextrin (DE 9-12, 1:1) as wall materials were determined using differential scanning calorimetry (DSC) (Mettler Toledo 821e, Schwerzenbach, Switzerland) with liquid N_2 cooling. The powders were prepared into DSC aluminium pans (40 µl; Mettler Toledo, Schwerzenbach, Switzerland), hermetically sealed, and analyzed. The thermograms were analyzed using STARe thermal analysis software, version 6.0 (Mettler Toledo, Schwerzenbach, Switzerland). The T_g of the systems were found to be 48 ± 1.2°C for SL non-humidified powders, 51± 1.2°C for LBL non-humidified powders, 39 ± 0.2°C for SL humidified powders and 40 ± 0.5°C for LBL humidified powders.

4.2.6 Water Activity

Water activity (a_w) of non-humidified spray dried (NHSD) systems stored at 25°C, 45°C and 65°C and humidified spray dried (HSD) systems stored at 25°C, 35°C, and 45°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 92 days of storage over 14 time points.

4.2.7 Colourimetry

The colour values (L*, a*, and b*) for both SL and LBL spray dried systems were determined using a pre-calibrated colourimeter (Minolta, CR300 meter, Osaka, Japan). The colour values were measured directly from glass vials after storage at intervals for up to 92 days of storage (14 time points). The L* value ranged from 0-100 and corresponded to the luminance or lightness component, while a* and b* ranged from -120 to 120 measuring the chromatic components of green to red values and blue to yellow values respectively.

4.2.8 Extraction Method and HPLC Analysis

Spray dried emulsions (1g) or freeze-dried emulsions (1g) were reconstituted in 2mL of deionized water (KB Scientific, Ireland) and vortexed (Scientific Industries Inc., G-560E, NY, USA) at room temperature for 30s. The emulsion was destabilized using 3.9 mL methanol:ethlyacetate (1:1 v/v) containing 0.2% butylated hydroxyl toluene (BHT) as an antioxidant to prevent loss of carotenoids during extraction process and vortexed for 30 s. Methanol:ethlyacetate also acted as organic solvents for the carotenoids. Oil present in the samples was saponified with 1 mL of saturated potassium hydroxide in methanol (2M) and the mix was vortexed for 30s to obtain the saponised fraction (lipid carrier) and unsaponised fraction (β-carotene and lutein). Then, 1 mL of dichloromethane was added and the samples were vortexed for 30s to ensure complete dissolution of β-carotene and lutein in the organic phase. Finally, to enhance separation of the organic phase, 3.8mL of hexane was added into the samples and vortexed for 30s. The samples were then left to stand in the dark for 30 min. A glass pipette was used to remove the organic phase, 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). A 10 μL injection of each sample was injected into the HPLC system. The β-carotene and lutein contents were quantified using 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.). The HPLC column used was a 250 mm × 4.6 mm i.d., 5 μm, reversed-phase Acclaim C30 analytical column with a 4 mm × 4 mm i.d. guard column of the same material (Dionex, Sunnyvale, CA, U.S.A.). An eluent system was composed of acetonitrile (A), methanol:ethyl acetate (1:1) (B) and 0.5% of 200 mmol acetic acid in water (C) using a 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. Detection of carotenoids was performed at 450 nm and relative amounts of β-carotene and lutein were derived from absorption peak areas. The study of carotenoids stability was carried out for up to 92 days of storage for spray dried samples and 8 days for freeze dried samples. Data of β-carotene and lutein 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. A first order kinetics was selected as plot of $\ln A/A_0$ against time (storage days) gave a straight line as carotenoids degradation is a typical first order reaction (Desorby *et al.*, 1997; Hidalgo and Brandolini, 2008; Harnkarnsujarit *et al.*, 2012b; Lim *et al.*, 2014). The Arrhenius relationship $k = Ae^{-Ea/(RT)}$ and plots of ln k against T^{-1} were used to obtain activation energy.

4.2.9 Optical Microscope

The SL and LBL powders from spray drying were dispersed in sunflower oil on a glass slide and images were captured using optical microscope at 100X magnification (Olympus BX51, Tokyo, Japan) connected to a video camera (PixeLink A662, Ottawa, ON, Canada).

4.2.10 Particle Size, Particle Density, Occluded Air, Interstitial Air, and Bulk Density

The particle size distribution of the spray dried emulsions was measured by laser diffraction using Malvern Mastersizer MSS with powder feeder unit (Malvern, Worcestershire, UK). Particle density was determined with helium gas Multivolume Pycnometer 1350 (Micromeritics, Georgia, USA). The occluded air and interstitial

air of the powders were determined according to the method of GEA Niro (2006). The tapped bulk density (100 taps) was determined using a tap volumeter (Copley, J. Engelsmann A.G., Ludwigshafen, Germany).

4.2.11 Powder Flow Testing

Flowability and bulk density of the powders obtained from spray drying was analyzed with Brookfield Powder Flow Tester (PFT) (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). The axial and torsional speed used was 1.0 mm/s and 1 rev/h, respectively. The powders were filled into an aluminium trough with perforated sheet of the annular shear cell and the powder surface was leveled using a curved profiled shaping blades. The weight of powders was recorded before testing. A vane profiled lids together with a simulated 2B finish for the flat lid were attached then to compression plate. Flowability of the powders was measured using an instantaneous flow function test. Five uniaxial normal stresses of between 0.2 and 4.8 kPa were applied and three over consolidation stresses at each normal stress.

4.2.12 Statistical Analysis

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

4.3 Results and Discussions

4.3.1 Powder Characteristics

Powders were successfully obtained from the spray drier with no visual differences between the powders using single layer (SL) and layer-by-layer (LBL) emulsions with high hydrophilic solids. The water content, particle density, occluded air, interstitial air, bulk density, tapped bulk density and particle size of the SL and LBL

powders are given in Table 4.1. Generally, there were significant differences between the characteristics of SL and LBL powders except for the bulk density. The amount of both occluded air and interstitial air were significantly higher in SL powders and the higher amount of occluded air resulted in the lower particle density of SL powders as compared to LBL powders. The application of layered interface on LBL system results in gum Arabic attaching electrostatically on the oil-protein surface increasing the overall density of the particles. The particles densities of both powders were comparable to powders obtained by Drusch *et al.* (2006) using trehalose or glucose syrups as wall materials. The low occluded air in the powders was due to the high total solids content emulsions used for spray drying. Mistry (2002) stated that powders obtained from low solids concentrates usually have low bulk density and high amount of occluded air. This can explain the higher amount of occluded air present in SL powders than in LBL powders as the application of LBL interface increases the particle density. An excess of gum Arabic too may increase the viscosity of the LBL emulsion prior to spray drying resulting in a slightly larger particle size. This account for the larger d [4,3], average volume-surface diameter obtained for LBL powders than in SL powders. The higher tapped density of LBL powders was the result of lower amount of interstitial air.

Table 4.1. Water content, particle density, occluded air, interstitial air, bulk density, tapped bulk density (100 times), and particle size of single layer (SL) and layer-by-layer (LBL) powders obtained by spray drying.

	SL	**LBL**
Water content (g/100g SNF)	6.15 ± 0.02^{a}	5.84 ± 0.03^{b}
Particle density (g/cm^3)	1.21 ± 0.01^{a}	1.26 ± 0.01^{b}
Occluded air (cm^3/100g)	7.74 ± 0.69^{a}	4.06 ± 0.36^{b}
Interstitial air (cm^3/100g)	88.91 ± 2.03^{a}	66.26 ± 5.16^{b}
Bulk density (g/cm^3)	0.45 ± 0.01^{a}	0.47 ± 0.01^{a}
Tapped density (g/cm^3)	0.58 ± 0.01^{a}	0.69 ± 0.02^{b}
D [4,3] (µm)	92.27 ± 0.64^{a}	107.30 ± 0.40^{b}

Values are means (n=3) ± SD.

Values followed by different superscripts are significantly different.

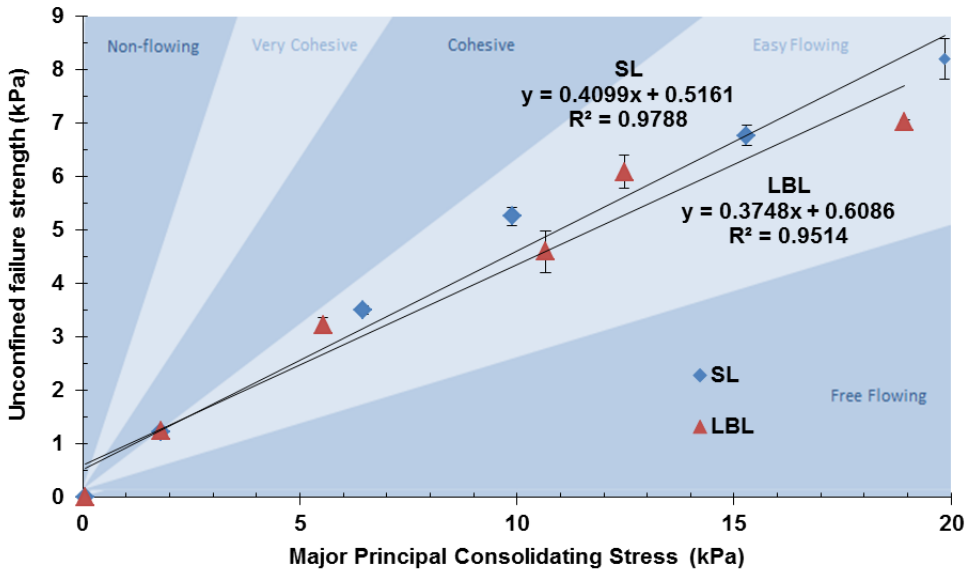

Figure 4.1. Flowability showing unconfined failure strength (kPa) as a function of major principal consolidation stress (kPa) of single layer (SL) and layer-by-layer (LBL) spray dried emulsions.

Figure 4.1 shows that SL and LBL powders were 'easy flowing' across the major principal consolidating stress (kPa). Flowability of powders is generally reduced with increasing water content or reducing particle size (Fitzpatrick *et al.*, 2004; Opaliński *et al.*, 2012). The lower water content and bigger particle size of LBL powders may lead LBL powders to be significantly more 'easy flowing' than SL powders. The smaller particle size of SL powders results in an enhanced total surface area that can increase affinity for water resulting in higher water content. The stress applied determines the flowability of powders and is crucial in processing and powder handling operations (Teunou *et al.*, 1999). The bulk density (Figure 4.2) of LBL powders was significantly higher than that of SL powders at all major principal consolidating stresses. This was in agreement with the higher interstitial air present in SL powders as higher interstitial air resulted in lower bulk density (Tamine, 2009). Images of the SL and LBL powders dispersed in sunflower oil and observed under optical microscope (100x magnifications) are shown in Figure 4.3. Both SL and LBL powders appeared similar under the microscope having spherical shapes. Dark spots were observed in both powders as well which indicated the presence of air vacuoles. High total solids emulsions were produced in our earlier study (Lim *et*

al. 2015) and these high solids emulsions were fed directly into spray drier in this study to obtain powders. The use of high solids feed is highly favourable in the industry and the results shown in Table 4.1, Figure 4.1, Figure 4.2, and Figure 4.3 showed that the physico-chemical properties of the powders obtain from high solids emulsions were satisfactory.

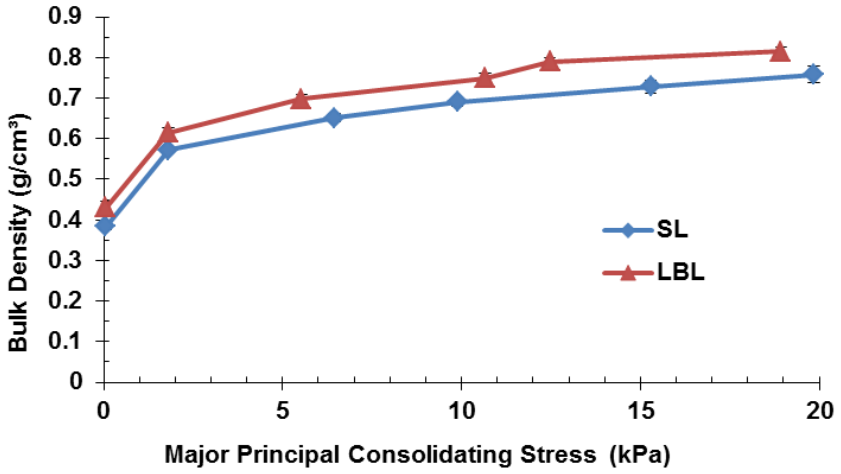

Figure 4.2. Bulk density of single layer (SL) and layer-by-layer (LBL) spray dried emulsions as a function of major principal consolidation stress (kPa).

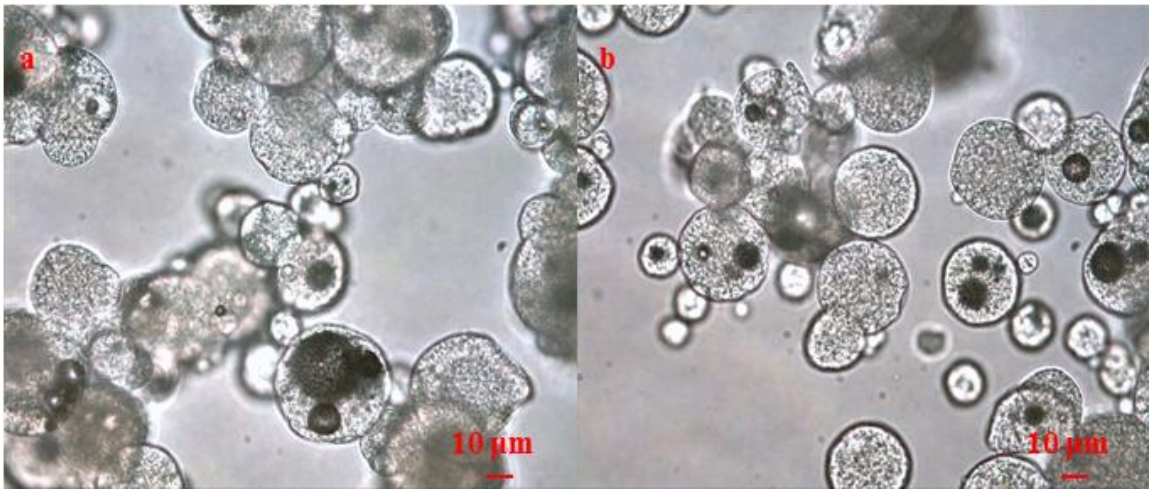

Figure 4.3. Single layer (SL) (a) and layer-by-layer (LBL) (b) powders dispersed in sunflower oil and observed under light microscope at 100x magnification.

4.3.2 Water Activity and Colour Measurements

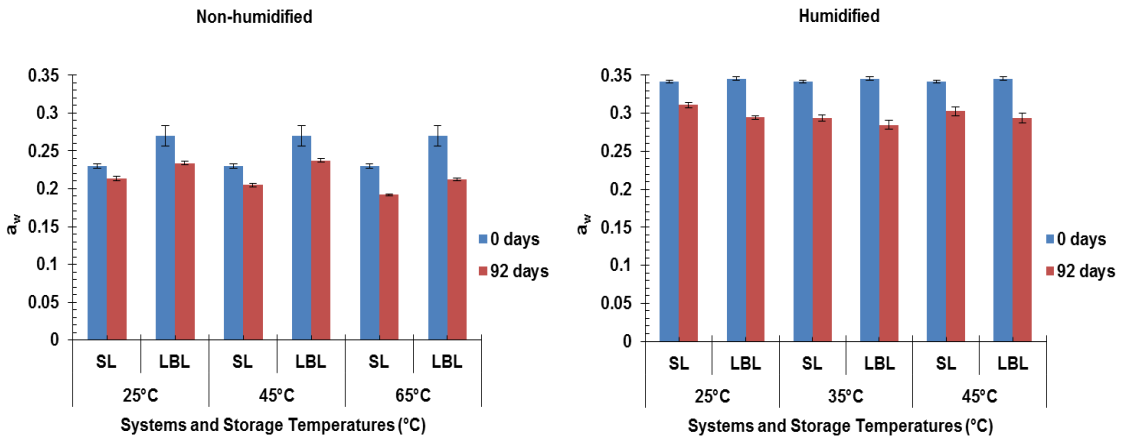

Figure 4.4. Water activity (a_w) of non-humidified and humidified single layer (SL) and layer-by-layer (LBL) spray dried emulsions before and after storage for 92 days at 25°C, 45°C, and 65°C and 25°C, 35°C, and 45°C, respectively.

Figure 4.4 shows the water activity (a_w) of non-humidified and humidified SL and LBL powders at 0 day and upon storage for 92 days at 25°C, 45°C, and 65°C and 25°C, 35°C, and 45°C, respectively. The a_w of non-humidified SL and LBL powders at day 0 represents the a_w of powders as it is after spray drying. It was noted that the a_w of non-humidified LBL powders were slightly higher than that of SL powders at day 0. The a_w of non-humidified SL ($0.23 \pm 0.01a_w$) and LBL ($0.27 \pm 0.01a_w$) powders which were obtained as it is from the spray drier were similar to powders commonly obtained from industrial spray drying of about $0.2a_w$ (Adhikari *et al.*, 2009). The a_w of both non-humidified and humidified systems decreased significantly upon storage at all storage temperatures. Nonetheless, the reductions of a_w in all systems were very small. The sample packages and vials maintained vacuum throughout the duration of the study indicating that no leakages occurred. This suggested that the decrease in a_w could be a result of physicochemical changes as the samples were stored in closed vials preventing changes of water with the environment. Hydration-related conformational changes in whey protein around 35-40°C can lead to reduction in a_w (Potes *et al.*, 2014). Potes *et al.* (2014) also stated that lipid oxidation resulting in reactive oxygen groups and free radicals, and

hydration of unsaturated fatty acids at higher temperatures can lead to a decrease in a_w.

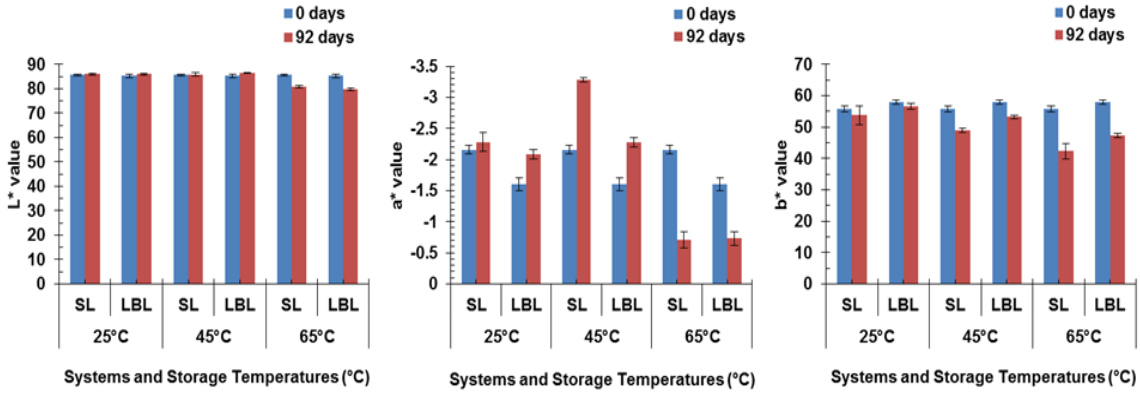

Figure 4.5. L*, a*, and b* values of non-humidified single layer (SL) and layer-by-layer (LBL) spray dried emulsions before and after storage for 92 days at 25°C, 45°C, and 65°C.

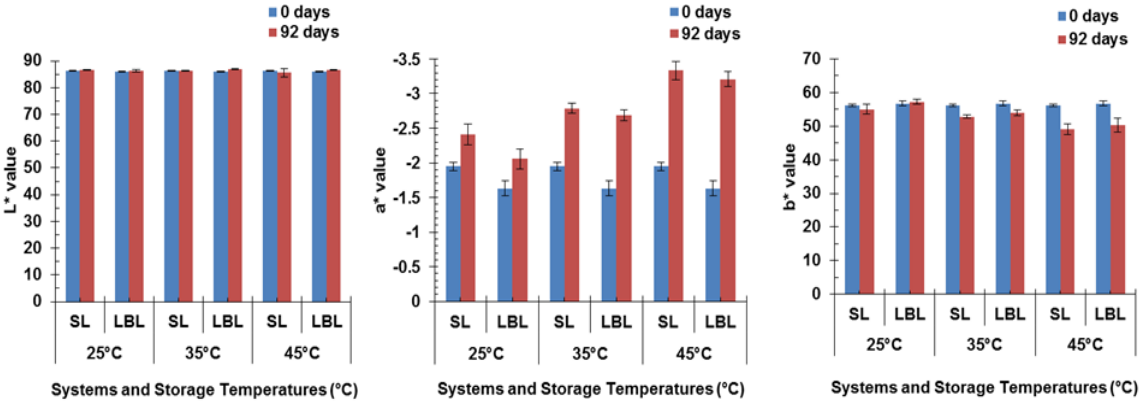

Figure 4.6. L*, a*, and b* values of humidified ($0.33a_w$) single layer (SL) and layer-by-layer (LBL) spray dried emulsions before and after storage for 92 days at 25°C, 35°C, and 45°C.

Figures 4.5 and 4.6 show the colour values (L*, a*, and b* values) of non-humidified and humidified SL and LBL spray dried systems at 0 and after 92 days of storage at 25°C, 45°C, and 65°C and 25°C, 35°C, and 45°C, respectively. Minor variations were found in L* (lightness) and a* (redness) values while bigger variations were observed in b* (yellowness) values. It was noted that the SL and LBL powders generally have similar colour values at 0 day for both non-humidified

and humidified systems. There were no significant differences in the changes of L* values except for non-humidified SL and LBL powders stored at 65°C where the L* values decreased significantly from 85.66 to 80.90 in SL system and from 85.24 to 79.81 in LBL system. This can be the result of non-enzymatic browning (Maillard reaction) due to the presence of protein amino groups (WPI), reducing sugars (maltodextrin), and heat. Lipid oxidation too can result in browning causing changes in the colour values (Venolia and Tappel, 1958; Zirlin and Karel, 1969). The a* values of all systems decreased significantly upon storage for 92 days except for non-humidified samples stored at 65°C where the a* values increased significantly. This corresponded to the L* values where non-humidified samples stored at 65°C showed a different trend compared to samples stored at 25°C and 45°C. Non-enzymatic browning in combination with lipid oxidation could result in the increased a* values of non-humidified samples stored at 65°C. The reduction of a* values upon storage at 25°C and 45°C agreed with findings of other authors using carotenoids containing systems as the consequences of carotenoids losses (Desobry *et al.,* 1997; Elizalde *et al.*, 2002; Mahfoudhi and Hamdi, 2014). The b* values of all systems generally decreased upon storage at 25°C, 45°C, and 65°C and 25°C, 35°C, and 45°C, respectively.

4.3.3 Carotenoids Stability

Figures 4.7 and 4.8 show the Arrhenius plots and activation energies for all-trans-β-carotene and lutein losses in non-humidified spray dried (NHSD) SL and LBL systems stored at 25°C, 45°C, and 65°C, and humidified ($0.33a_w$) spray dried (HSD) SL and LBL systems stored at 25°C, 35°C, and 45°C. Arrhenius plots and activation energies of the carotenoids loss in non-humidified freeze dried (NHFD) SL and LBL systems stored at 25°C, 45°C, and 65°C, and humidified ($0.33a_w$) freeze dried (HFD) SL and LBL systems stored at 25°C, 35°C, and 45°C are shown in Figures 4.9 and 4.10. The glass transition temperatures (T_g) of the systems were shown in the figures as well. T_g was not present in Figure 4.9 as the NHFD SL and LBL systems were stored below their respective T_g. The peak areas of carotenoids were used to obtain quantitative values for retention of carotenoids. These data were then fitted into first

order kinetics to get first order degradation kinetics for both all-trans-β-carotene and lutein in SL and LBL systems. The plots of ln A/A_0 against time (storage days) produced straight lines which justified the use of first order kinetics. Nonetheless, several authors also showed that carotenoids degradation was a typical first order reaction (Henry *et al.*, 1998; Hidalgo and Brandolini, 2008; Ramoneda *et al.*, 2011; Mahfoudhi and Hamdi, 2014; Mahfoudhi and Hamdi, 2015; Caliskan *et al.*, 2015). The retention time of lutein from the HPLC chromatograms was found to be 6.6 ± 0.2 min and all-trans-β-carotene 32.0 ± 0.6 min.

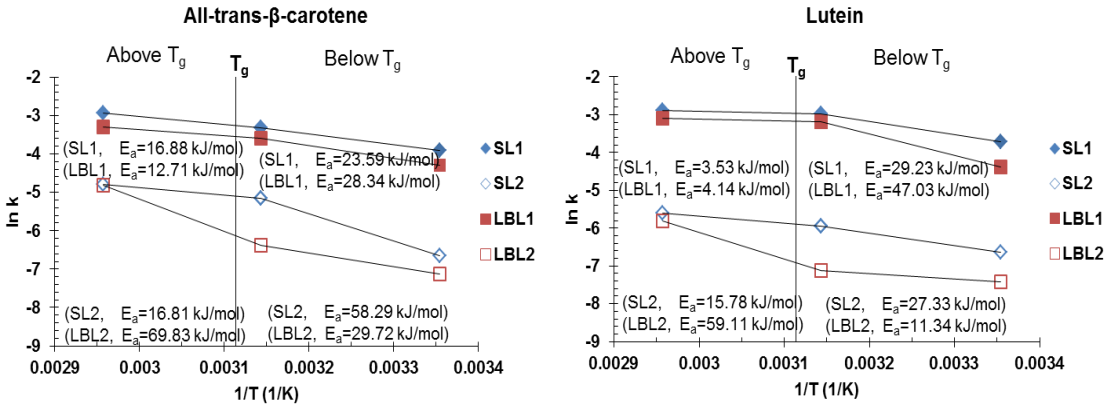

Figure 4.7. Glass transition temperature (T_g) as well as activation energies (E_a) and Arrhenius plots of all-trans-β-carotene and lutein in non-humidified single layer (SL) and layer-by-layer (LBL) powders stored for 92 days at 25°C, 45°C, and 65°C showing a rapid initial first order loss kinetic (SL1 & LBL1) followed by a second slower first order loss kinetic (SL2 & LBL2).

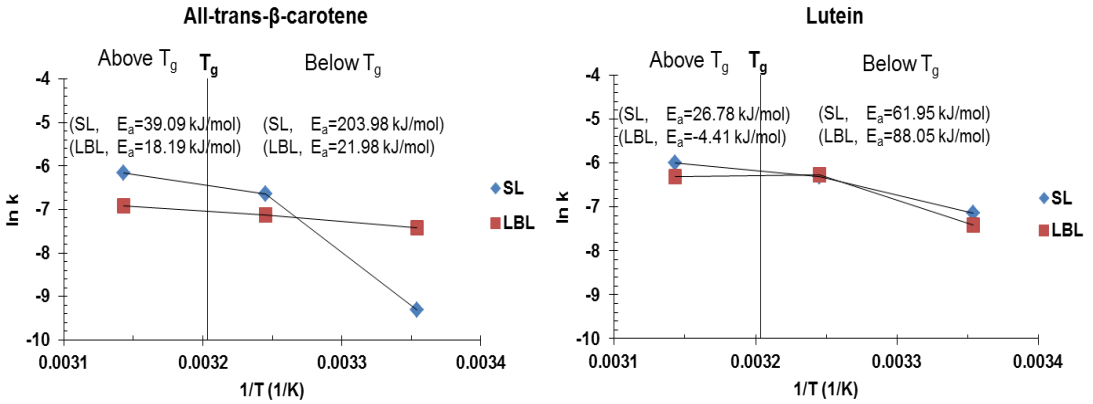

Figure 4.8. Glass transition temperature (T_g) as well as activation energies (E_a) and Arrhenius plots of all-trans-β-carotene and lutein in humidified ($0.33a_w$) single layer (SL) and layer-by-layer (LBL) powders stored for 92 days at 25°C, 35°C, and 45°C.

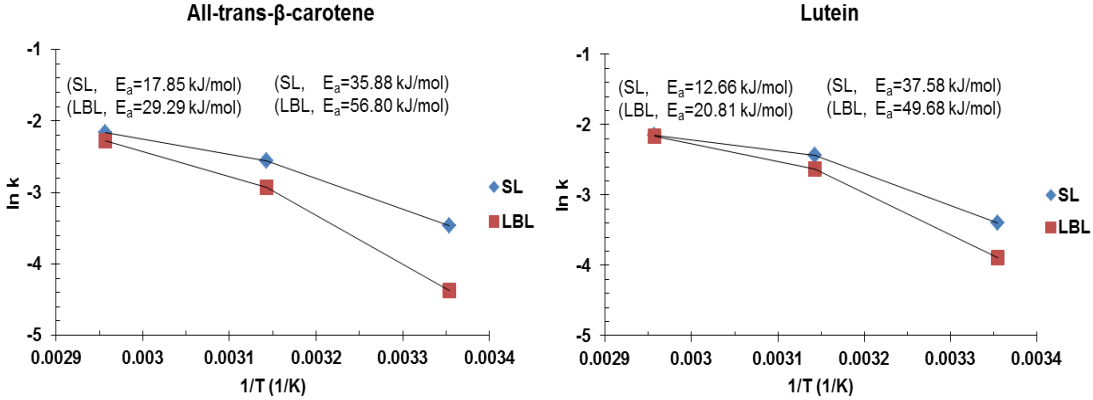

Figure 4.9. Activation energies (E_a) and Arrhenius plots of all-trans-β-carotene and lutein in non-humidified single layer (SL) and layer-by-layer (LBL) freeze dried systems stored for 8 days at 25°C, 45°C, and 65°C.

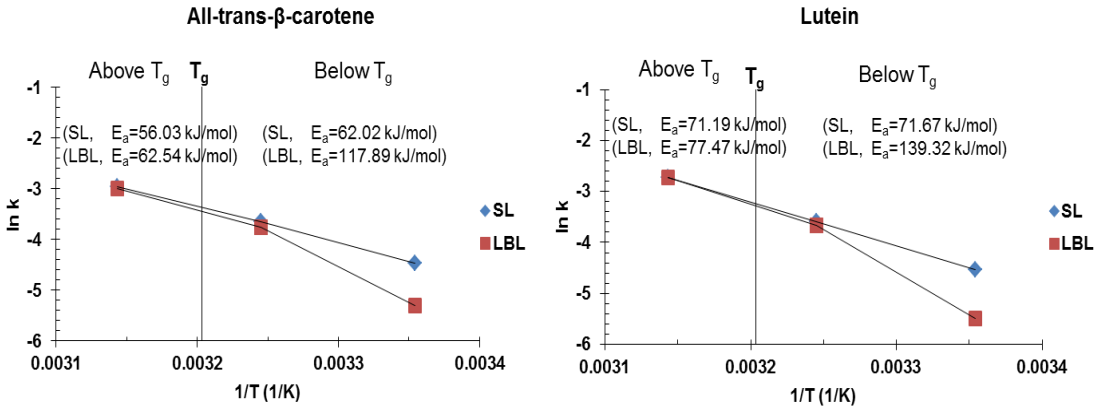

Figure 4.10. Glass transition temperature (T_g) as well as activation energies (E_a) and Arrhenius plots of all-trans-β-carotene and lutein in humidified ($0.33a_w$) single layer (SL) and layer-by-layer (LBL) freeze dried systems stored for 8 days at 25°C, 35°C, and 45°C.

A rapid initial first order loss kinetic followed by a second slower first order loss kinetic was observed in SL and LBL NHSD systems. Similar observations were made in other studies using spray dried carotenoids containing systems (Desobry *et al.*, 1997; Desobry *et al.*, 1999). Drusch *et al.* (2006) also found rapid increase of hydroperoxides within the first week of storage followed by moderate increase over the next eight weeks in fish oil containing spray dried emulsion. Oxygen may be present in vacuoles formed after hardening of the outer layer of the continuous phase followed by thermal expansion of the trapped air bubbles in droplets during spray drying (Aguilera 1990; Tewa-Tagne *et al.* 2007). Entrapped oxygen within the glassy matrix of spray dried emulsions will make contact with carotenoids present in the oil leading to rapid loss as oxidation is the leading cause of carotenoids degradation (Achir *et al.*, 2010). The higher amount of occluded air present within the SL NHSD solids results in a more rapid loss of carotenoids during the first 8 days of storage as compared to the LBL NHSD powder. As the oxygen present within the vacuoles were used up during storage, the loss of carotenoids become slower resulting in a second slower first order loss kinetic. The rapid initial first order loss kinetic was not observed in HSD systems, NHFD systems, and HFD systems. The rapid initial first order loss kinetic was not observed in HSD systems as the rapid initial loss could occur during the humidification process. As freeze drying

was done in vacuum, no oxygen was entrapped within the matrix that justifies the absence of the rapid initial first order loss kinetic in both NHFD and HFD systems. In addition, all vials were vacuumed sealed before further study ruling out the possibility of oxidation due to oxygen that may be present within the vials.

The carotenoids loss of all systems in this study was found to increase with increasing storage temperatures. This is a common occurrence and was well reported in other studies using carotenoids (Dhuique-Mayer *et al.,* 2007; Spada *et al.,* 2012; Qian *et al.,* 2012; Caliskan *et al.,* 2015). However, it was observed that storage of systems above the respective T_g or at higher storage temperature in NHFD systems have reduced carotenoids loss based on the rate constants obtained. Levi and Karel (1995) stated that a dynamic process of structural collapse occur in systems stored above its T_g governed by the rate defined by $T - T_g$. Although no obvious structural transformation was observed in the systems, the increased molecular mobility and decreased viscosity of systems stored above the T_g (Roos and Karel, 1991c) creates a thicker membrane around the powder surface in spray dried system or less porous structure in freeze-dried system resulting in denser and harder system. The denser and harder systems were capable of reducing heat transfer from the surroundings towards the encapsulated carotenoids containing oil particles reducing losses. Besides that, the solubility of oxygen in water decreased with increasing temperature up to 100°C (Hildebrand, 1956; Wilhelm *et al.* 1976). This translates to lower amount of oxygen present in the surface water of the powders with increasing storage temperature. As a result, diffusion of oxygen towards the encapsulated oil was reduced, reducing the loss of carotenoids. There was a reduced carotenoids loss with increasing temperature in NHFD system ($0a_w$) having no surface water showed that the formation of a denser and harder system with increasing temperature plays a major role in reducing the loss. This also shows that the reduced solubility of oxygen with increasing temperature played role in reducing the loss of carotenoids. The storage of these systems at higher storage temperatures too causes Maillard reactions that produce melanoidins. Melanoidins possesses antioxidant capacity (Wang *et al.,* 2011) providing the encapsulated carotenoids protection against radicals. However, it was noted that there was a large increase of carotenoids loss in LBL NHSD system

above the T_g during long term storage (second slower first order loss kinetic) based on the rate constants. Glass transition results in the release of carotenoids containing oil particles (Shimada *et al.,* 1991) increasing isomerisation and loss of carotenoids as these oil particles are not protected within the matrix. Isomerisation is a major contributor towards the loss of carotenoids (Qiu *et al.,* 2009) and is generally caused by exposure to heat and light.

Generally, the activation energies of LBL systems were higher than SL systems for all-trans-β-carotene and lutein. The higher activation energy of the LBL systems revealed that the loss of carotenoids in LBL systems were more temperature dependent. This is due to the difference in particle density between SL and LBL systems. At low storage temperature, the higher overall particle density of LBL system reduced heat transfer from surroundings towards the encapsulated carotenoids containing oil resulting in minimum loss of carotenoids. However, as the temperature increases, the ability of the LBL system to minimize heat transfer will reach a maximum limit, followed by an increased loss of carotenoids. Besides, gum Arabic at the WPI-oil interface changes the structure of the system and increasing local T_g and reducing collapse or flow as the temperature increased. This causes the LBL system to have less dense and hard continuous phase compared to SL systems resulting in the higher temperature dependence of carotenoids loss in LBL system. There was a change in the activation energy for the systems that were stored above T_g or with increasing storage temperature in NHFD systems. The activation energy of the systems generally decreased indicating that the loss of carotenoids in the systems became less temperature dependent. This was the result of better heat protection from the denser and harder continuous phase from the increased molecular mobility and reduced viscosity (Roos and Karel, 1991) allowing less heat transfer. However, the activation energy increased in LBL NHSD system above the T_g in the second slower first order loss kinetics and was in agreement with the large increase of carotenoids loss based on the rate constants.

In addition, the loss of all-trans-β-carotene and lutein was more rapid in freeze-dried systems than in spray dried systems. Freeze-drying produced a more porous matrix

than spray drying that increases the rate of heat transfer towards the encapsulated carotenoids containing oil particles resulting in higher loss of carotenoids. Spray drying on the other hand produces a denser and less porous structure resulting in reduced oxygen permeability and heat transfer. Two separate studies by the same authors showed that β-carotene degradation was more rapid in freeze-dried systems as compared to spray dried systems (Mahfoudhi and Hamdi, 2014; Mahfoudhi and Hamdi, 2015). It was also noted that the loss of carotenoids was faster in NHSD ($0.23a_w$ in SL and $0.27a_w$ in LBL) and freeze-dried ($0a_w$) systems than in both HSD and HFD systems ($0.33a_w$). This was attributed to the inhibitory effect of water towards oxidation due to hydrogen bonding with hydroperoxides produced during oxidation preventing further reactions, and inactivation by hydrating the trace metal presents reducing its catalytic effect (Karel *et al.*, 1967). Studies by Quast and Karel (1972) found that the oxidation rate of potato chips decreased as the water activity increased from $0.01a_w$ to $0.4a_w$. Similar observation was also made by Lavelli *et al.* (2007) in degradation of carotenoids in carrots. However, reaction rates in those studies increased as the water activity of the systems approaches $0.6a_w$.

It was noted that the degradation of lutein was generally more rapid than the degradation of all-trans-β-carotene in all systems based on the rate constants obtained. Lutein has two hydroxyl groups which results in lutein being more hydrophilic and polar than all-trans-β-carotene (Farombi and Britton, 1999; Updike and Schwartz, 2003). Due to the presence of hydroxyl groups, lutein will tend to concentrate at the oil-WPI interface while all-trans-β-carotene will be present within the bulk oil. Oxygen was trapped within the matrix of the wall materials upon spray drying of the emulsions. The presence of lutein molecules at the oil-WPI interface allows them to become easily exposed to the trapped oxygen as well as to the heat from the surroundings leading to faster degradation. Dhuique-Mayer *et al.* (2007) stated that xanthophylls degrades more rapidly and have lower heat stability than carotenes. Free fatty acids present in sunflower oil may concentrate at the oil-WPI interface as carboxyl group present in free fatty acids is more polar. This can result in esterification of lutein with the free fatty acids and presents increasing losses in lutein extraction. As lutein ester is less polar than lutein, lutein esters will tend to

concentrate within the core of the oil particles (Peŕez-Gálvez and Mínguez-Mosquera, 2005). Due to the difference in polarity between lutein and lutein ester, it will be more difficult to separate and analyze lutein in a single step chromatographic method (Rivas, 1989). The formation of lutein ester will reduce the lutein peak in HPLC chromatograms during study as the difference in polarity results in different retention time.

The degradation of both all-trans-β-carotene and lutein were generally more rapid in all SL systems than in LBL systems. This was in agreement with the results obtained in our earlier study using freeze-dried systems with trehalose as wall material (Lim *et al.,* 2014). The slower degradation of LBL systems also showed that the application of layered interface in LBL emulsion increased the stability of LBL systems towards environmental stresses such as heat reducing carotenoids loss. The thicker interfacial layer of LBL systems too will reduce heat transfer towards the encapsulated carotenoids containing oil. Many authors have concluded that the application of layered interface increased the stability of LBL system compared to SL systems as a result of thicker and denser interfacial layer of the particles, increased steric repulsion, and reduced strength of van der Walls attractions (Moreau *et al.* 2003; Gu *et al.,* 2005; Harnsilawat *et al.,* 2006; Benjamin *et al.,* 2012). The main isomers observed in the HPLC chromatograms were 15-cis-β-carotene and 13-cis-β-carotene. Exposure to heat during storage may lead to isomerisation in all systems and isomerisation increased with increasing storage temperature. A study by Chandler and Schwartz (1988) found that the loss of all-trans-β-carotene during heating correlates to the increase of isomerisation. Nonetheless, no observable trend was found in the amount of 15-cis-β-carotene and 13-cis-β-carotene as they fluctuated with storage.

4.4 Conclusions

Powders were obtained successfully using high hydrophilic solids emulsions having mixture of trehalose and maltodextrin as wall materials with a spray drier. All-trans-β-carotene and lutein were dissolved in the lipid phase of the emulsions. The loss of

all-trans-β-carotene and lutein followed first order loss kinetics. LBL systems gave a better protection towards carotenoids losses compared to SL systems upon storage for 92 days for spray dried systems and 8 days for freeze dried systems. The rate constants obtained showed that application of LBL technology on protein layered oil particles delayed the degradation of all-trans-β-carotene and lutein upon storage. A two first order loss kinetics of all-trans-β-carotene and lutein were observed in the non-humidified spray dried (NHSD) systems showing a rapid initial first order loss kinetic followed by a second slower first order loss kinetic. Carotenoids degradation was also more rapid in the more porous freeze-dried systems than in spray dried systems. It was noted that storage above the glass transition temperature (T_g) of the systems or with increasing storage temperature in non-humidified freeze-dried (NHFD) systems may have delayed carotenoids losses. The activation energies of LBL systems were generally higher than SL systems showing that the loss of carotenoids in these systems was more heat sensitive. There was a decrease in activation energy in systems stored above its T_g or with increasing storage temperature in NHFD systems showing that the loss of carotenoids in the systems became less temperature dependent. Water activity (a_w) values decreased with storage while minor variations were observed in L* and a* values, and bigger variations in b* values. Data from this study can be applied to formulated materials in food and pharmaceutical industries controlling the stability of oil soluble bioactive compounds.

Chapter V

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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 solid emulsions with carbohydrate (trehalose) and non-carbohydrate (WPI) solids to stabilize carotenoids upon storage at 35°C, 50°C, and 65°C. Carotenoid loss 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 carotenoid 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. Carotenoid 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

5.1 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 *et al.,* 2003a; Aoki *et al.,* 2005; Güzey and McClements, 2006a; Gharsallaoui *et al.,* 2010; Lim *et al.,* 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 Walls attraction strength (Moreau *et al.,* 2003; Gu *et al.,* 2005; Harnsilawat *et al.,* 2006; Benjamin *et al.,* 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, 2006a). 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 *et al.*, 2004; Gharsallaoui *et al.*, 2010; Klein *et al.*, 2010). The isoelectric point (pI) of β-lactoglobulin is 5.2 (Bryant and McClements, 1998) while α-lactalbumin is 4.1 (Weinbreck *et al.*, 2003). As gum Arabic has a pKa value of approximately 2.2, gum Arabic will be negatively charged above pH 2.2 (Weinbreck *et al.*, 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 *et al.*, 2010). β-carotene is also capable of showing antioxidant and anticancer properties, and may prevent heart diseases (Bendich and Olson, 1989; Omenn *et al.*, 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 and Blumberg, 2004) as well as prevents cataracts and macular degeneration (Khachik *et al.*, 1997). Chronic and degenerative diseases in humans can be reduced with the consumption of food products consisting of bioactive compounds (van Dokkum *et al.*, 2008). The low bioavailability of carotenoids as crystals or within protein complexes in fruits and vegetables reduces adsorption in the gastrointestinal tract during digestion (Williams *et al.*, 1998). Nonetheless, the use of carotenoids with oil can improve the bioavailability of carotenoids as oil

increases carotenoids adsorption (van Het Hof *et al.,* 2000). Therefore, the incorporation of carotenoids in the lipid phase of an oil-in-water (O/W) emulsion can enhance its bioavailability. The lipid phase can be emulsified with an emulsifier containing aqueous phase followed by addition of glass forming hydrophilic component and dehydration of the emulsion to produce a continuous phase of the dried formulation (Drusch *et al.,* 2006; Ramoneda *et al.,* 2011; Spada *et al.,* 2012; Mehanna *et al.,* 2015).

Microencapsulation utilizes wall materials to entrap core materials protecting it from environmental stresses to provide longer shelf life and enable controlled release of the core materials (Shahidi and Han, 1993). Microencapsulation is commonly achieved through spray drying in the food industry (Ré, 1998). Spray drying is also straightforward and economical giving high quality powders with a long shelf life. Maltodextrins, trehalose, milk proteins, corn syrup and modified starch are frequently used as wall materials in spray drying (Desobry *et al.,* 1997; Hogan *et al.,* 2001a; Drusch *et al.,* 2006; Shaw *et al.,* 2007; Liang *et al.,* 2013; Mehanna *et al.,* 2015). Crystallization of the wall materials in powders can lead to degradation of released carotenoids containing lipid phase as a result of direct exposure to environmental stresses (Buera *et al.,* 2005). Nevertheless, studies have shown that mixtures of sugars and proteins can delay the crystallization rate of the sugar component (Jouppila and Roos, 1994; Haque and Roos, 2004). Our earlier studies also showed that the application of LBL interfacial structures with trehalose, and mixture of trehalose and maltrodextrin (DE 10) as wall materials reduced the loss of carotenoids upon storage (Lim *et al.,* 2014; Lim and 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.

5.2 Materials and Methods

5.2.1 Materials

Sunflower oil was from Musgrave Excellence^{TM} (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.

5.2.2 Emulsion Preparation

The emulsions were prepared using methods modified from our earlier study (Lim and Roos, 2015; Lim and Roos, 2016). The emulsions prior to spray drying were SL and LBL which consisted of 18.2% sunflower oil and carotenoids, 18.18% trehalose, 9.09 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 (1.33 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 ± 1°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.

5.2.3 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 39.79% oil with carotenoids, 39.75% trehalose and 19.87% 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 5.1) and water activities were $0.14\pm0.01a_w$ for SL and $0.14\pm0.01a_w$ 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 N_2 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.

5.2.4 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.

5.2.5 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.

5.2.6 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:ethlyacetate (1:1, v/v) mixture acting as organic solvents for the carotenoids and vortexed for 30s. The methanol:ethlyacetate 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 μL 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 × 4.6 mm i.d., 5 μm, reversed-phase Acclaim C30 analytical column alongside a 4 mm × 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 and Brandolini, 2008; Lim *et al.,* 2014; Mahfoudhi and Hamdi, 2014). Activation energy was determined using Arrhenius relationship $k = Ae^{-Ea/(RT)}$ and plots of ln k against T^{-1}.

5.2.7 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 (PixeLink A662, Ottawa, ON).

5.2.8 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 2µm 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 nm^2 and integration time of 0.15s. Post processing and visualization was completed in Project Plus software (WITec).

5.2.9 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 ± 1°C and transferred into an oven set at 50 ± 1°C until constant weight was obtained.

5.2.10 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.

5.2.11 Emulsion Characterisation

Particle size ((ζ-average diameter), using dynamic light scattering, measurement range: 0.3nm – 10μm) 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.

5.2.12 Statistical Analysis

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

5.3 Results and Discussions

5.3.1 Powder Characteristics

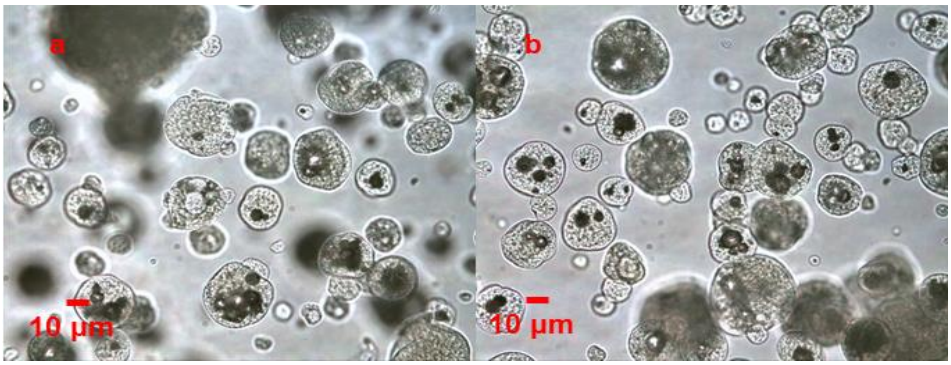

Figure 5.1. Images from light microscope at 100x magnification of single layer (SL) (a) and layer-by-layer (LBL) (b) powders dispersed in sunflower oil.

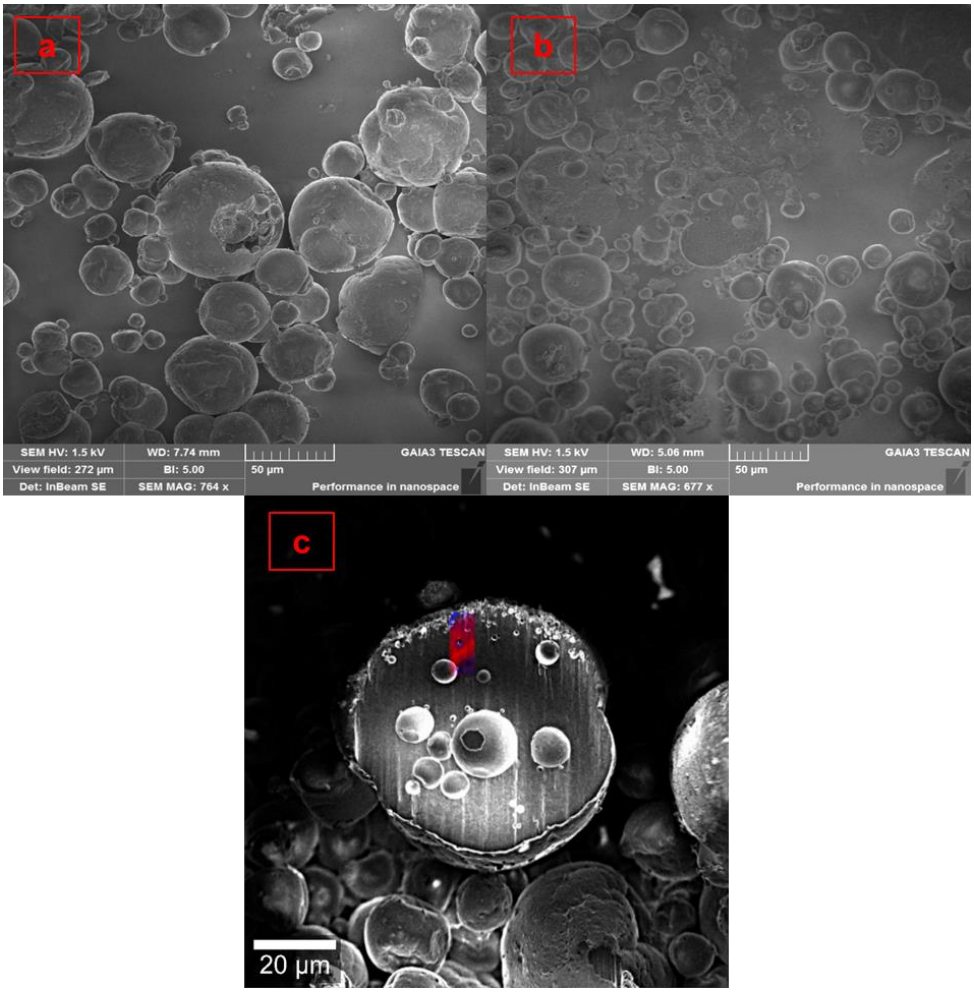

Figure 5.2. Raman-FIB-SEM images showing single layer (SL) powder particles (a), layer-by-layer (LBL) powder particles (b), and a cut powder particle with the presence of lutein (red) in large quantities at interfaces and all-trans-β-carotene (blue) dissolved in encapsulated oil and which appeared only in minor quantities at the cut surfaces (c).

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 5.1 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 5.2) 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 5.2. 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 5.1. 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 and 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 and McClements, 2006a) 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 5.3. 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).

Table 5.1. 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/cm^3)	**0.98±0.02**	**0.99±0.03**
Occluded air (cm^3/100g)	**20.67±1.29**	**18.51±2.74**
Interstitial air (cm^3/100g)	**101.63±1.29**	**98.79±2.74**
Bulk density (g/cm^3)	**0.33±0.01**	**0.35±0.01**
Tapped density (g/cm^3)	**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

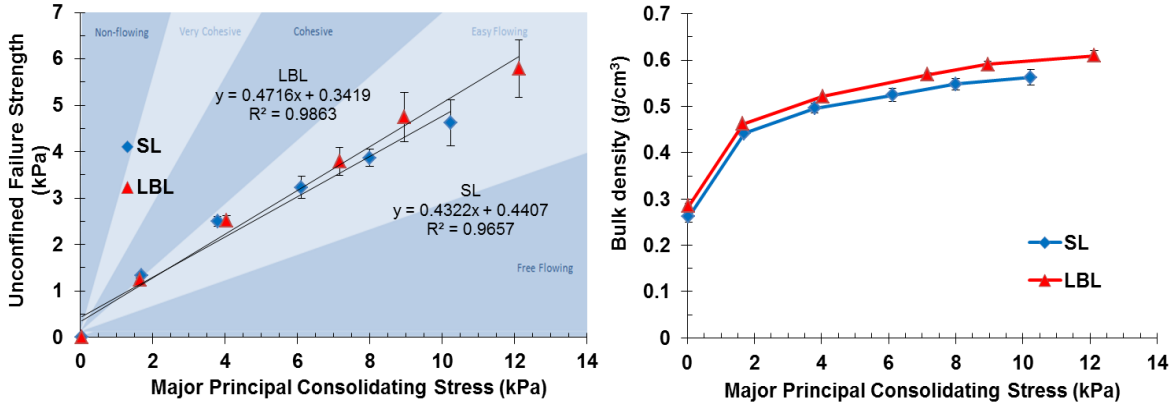

Figure 5.3. Flowability showing unconfined failure strength (kPa) and bulk density as a function of major principal consolidation stress (kPa) of single layer (SL) and layer-by-layer (LBL) powders.

5.3.2 Colour Measurements, Water Activity, and ζ-average Diameter

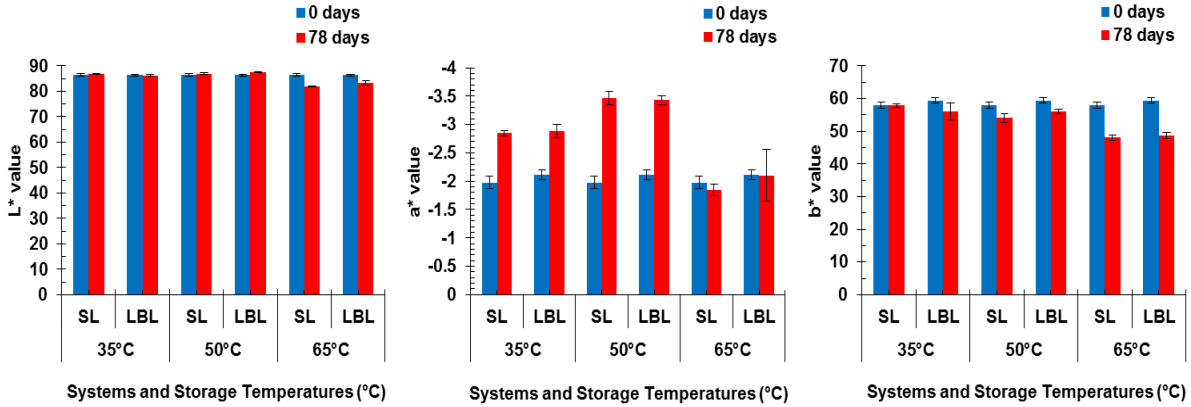

Figure 5.4. L* (lightness), a* (redness), and b* (yellowness) values of spray dried single layer (SL) and layer-by-layer (LBL) emulsions before and after storage for 78 days at 35°C, 50°C, and 65°C.

Figure 5.4 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 *et al.*, 2012; Mahfoudhi and 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 and Karel, 1969;

Pokorný *et al.,* 1974; Potes *et al.,* 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 *et al.,* 2002; Prado *et al.,* 2006; Spada *et al.,* 2012; Mahfoudhi and 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 5.5. The a_w of SL powders (0.14 ± 0.01a_w) and LBL powders (0.14 ± 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 *et al.,* 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 5.6. 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 *et al.* (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).

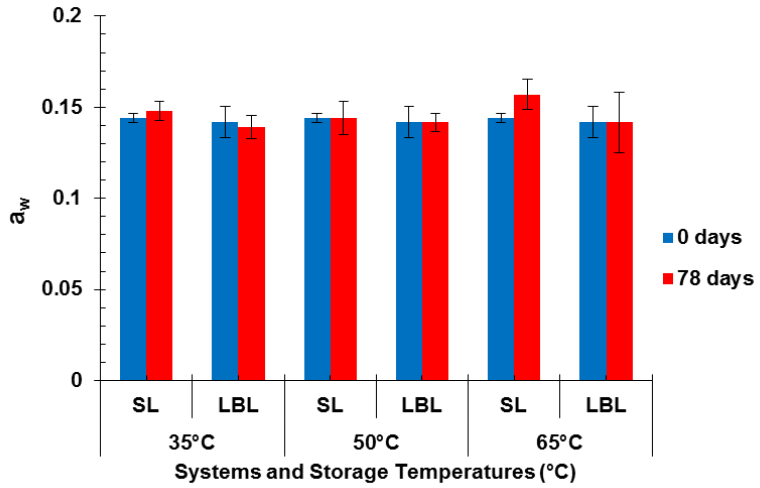

Figure 5.5. Water activity (a_w) of single layer (SL) and layer-by-layer (LBL) powders obtained by spray drying before and after storage at 35°C, 50°C, and 65°C for 78 days.

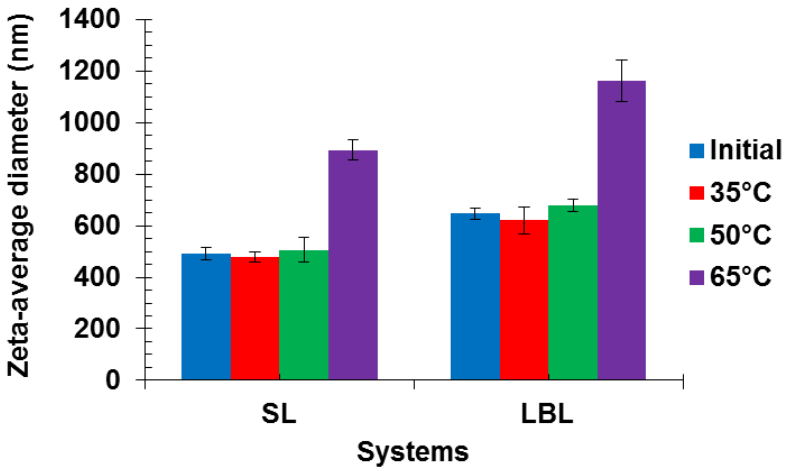

Figure 5.6. Zeta-average diameter (nm) of oil particles in reconstituted single layer (SL) and layer-by-layer (LBL) powders before and after storage at 35°C, 50°C, and 65°C for 92 days.

5.3.3 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 *et al.*, 1998; Desobry *et al.*, 1999; Achir *et al.*, 2010; Mahfoudhi and 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 5.7. 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 and 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.

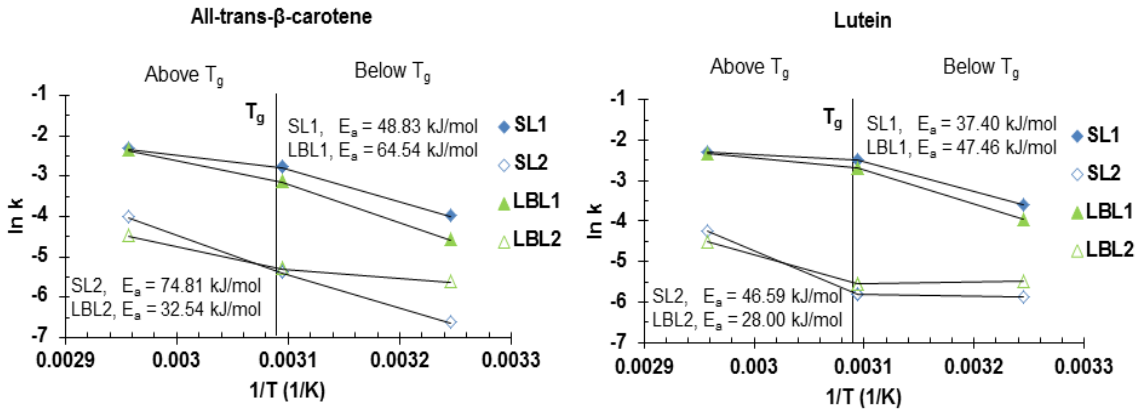

Figure 5.7. Arrhenius plots of all-trans-β-carotene and lutein in single layer (SL) and layer-by-layer (LBL) powders stored for 78 days at 35°C, 50°C, and 65°C having a rapid initial first order loss kinetic (SL1 & LBL1) followed by a second slower first order loss kinetic (SL2 & LBL2) and showing the activation energies and glass transition temperature (T_g).

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 and 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 *et al.,* 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 *et al.,* 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 and 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 *et al.,* 2007; Hidalgo and Brandolini, 2008; Spada *et al.,* 2012; Caliskan *et al.,* 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 *et al.,* 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 and 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 and 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 *et al.,* 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 *et al.* (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 and Roos, 2016) with mixture of trehalose and maltodextrin (DE 10) as glass formers based on the rate

constants. The mixture of carbohydrate (trehalose) and non-carbohydrate (WPI) as glass formers resulted in the higher porosity and lower density of the powders obtained. Density of spray dried powders was found to increase while the porosity decreased with increasing amount of lactose in a WPI-lactose wall matrix system (Moreau and Rosenberg, 1999). A study by Carneiro *et al.* (2013) also found that powders having mixture of maltodextrin and whey protein concentrate as wall materials has the lowest bulk density indicating higher amount of occluded air. The higher porosity and lower density of the powders in this study allows greater heat permeability towards the encapsulated carotenoids that increased carotenoids loss. WPI preferably migrates to the particle surface (air-water interface) due to its high surface activity forming a protein rich layer (Adhikari *et al.*, 2009; Wang *et al.*, 2013). Fäldt and Bergenståhl (1994) found that the surface-active protein predominates at the surface of spray dried carbohydrate-protein system. Discontinuity between carbohydrate and protein used as wall materials occurred in this present study due to the presence of surface-active WPI. As a result, the carotenoids containing oil will be encapsulated within the glass former and non-glass former during spray drying. The lesser amount of carotenoids containing oil trapped and protected within the denser glass former increased the loss of carotenoids. Besides, the high amount of protein present in the continuous phase of the emulsion results in surface indentations of the powders obtained (Fäldt and Bergenståhl, 1994; Wang *et al.*, 2013). This presence of WPI at particle surface increased the skin flexibility of the particles preventing skin rupturing during drying resulting in more wrinkles and surface roughness as observed in Figure 5.2 and is commonly observed in spray dried milk and milk containing products (Wang and Langrish, 2010). This increases the effective total surface 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 and Britton, 1999; Updike and 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 5.2 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.

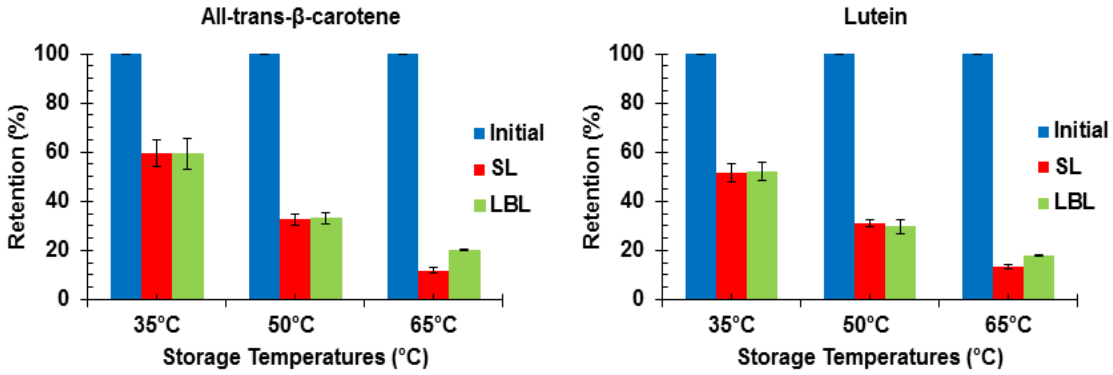

Figure 5.8. Retention (%) of all-trans-β-carotene and lutein in spray dried single layer (SL) and layer-by-layer (LBL) emulsions before and after storage at 35°C, 50°C, and 65°C for 78 days.

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 Walls 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 *et al.*, 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 5.8) 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 *et al.*, 2003b; Güzey and McClements, 2006a; Gharsallaoui *et al.*, 2010; Iwata *et al.*, 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.

5.4 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.

Chapter VI

Carotenoids Stability in Spray Dried High Solids Emulsions using Layer-by-Layer (LBL) Interfacial Structure and Trehalose-High DE Maltodextrin as Glass Former

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Abstract

The ability of powders obtained from spray drying of high hydrophilic solids emulsions with single layer (SL) or layer-by-layer (LBL) interface and trehalose-high DE maltodextrin (23-27) mixture as glass former to protect encapsulated lutein and all-trans-β-carotene in emulsified oil upon storage (35°C, 50°C, and 65°C) was investigated. Carotenoids degradation followed first order kinetics and increased with storage temperature but was less rapid in LBL system. Two-step degradation kinetics showing rapid initial decrease followed by a second slower degradation was observed. There was non-Arrhenian increase in carotenoids degradation during the initial step. Carotenoids degradation in LBL system was generally found to be more temperature dependent. Carotenoids retention was higher in LBL system with significant difference at 65°C. The main β-carotene isomers identified in this study were all-trans-β-carotene, 15-cis-β-carotene and 13-cis-β-carotene. LBL interfacial layer improved carotenoids stability and such design may enhance stability of dissolved or noncrystalline lipophilic bioactive compounds in formulated food and other biological materials.

Keywords: storage study; lutein; β-carotene; spray drying; layer-by-layer

6.1 Introduction

Carotenoids within fruits and vegetables as protein complexes or as crystals may not show bioaccessibility and bioavailability during digestion (Williams *et al.,* 1998; Carbonell-Capella *et al*., 2014). The lipid phase with carotenoids in manufacturing of formulated foods can be dispersed in water containing an emulsifier to produce an oil-in-water (O/W) emulsion. Fortification of the oil phase with dissolved carotenoids increases their bioavailability as oil enhances carotenoids absorption (van het Hof *et al.,* 2000). Water soluble glass forming hydrophilic components may be added and the emulsion dehydrated to encapsulate the lipid phase and produce shelf stable powders (Gharsallaoui, *et al.,* 2010; Lim *et al.,* 2014). Furthermore, interphase engineering using electrostatic attraction between charged primary

interphase layer and a subsequently introduced secondary layer with a charge difference is often used to produce layer-by-layer (LBL) emulsions (Ogawa *et al.*, 2003a; Benjamin *et al.*, 2012; Lim and Roos, 2015). In the present study whey protein isolate (WPI) and gum Arabic were used as the primary and secondary layers, respectively, as these polymers were oppositely charged at pH 3.5.

Many authors have reported the higher stability of LBL emulsion systems towards environmental stresses such as heat treatment, variations in pH, ageing, freeze-thaw cycles, lipid oxidation, and ionic strength (Moreau *et al.* 2003; Ogawa *et al.*, 2003a; Gharsallaoui *et al.*, 2010; Lim *et al.*, 2014). The thicker interfacial layer around particles, lower van der Waals attraction strength, and higher steric inter-particle repulsion increase the stability of LBL emulsions (Moreau *et al.* 2003; Gu *et al.*, 2005; Harnsilawat *et al.*, 2006; Benjamin *et al.*, 2012). The thicker interfacial layer in LBL system improves the stability of the oil particles against disruption to provide the encapsulated materials with improved protection (McClements, 1999). Microencapsulation of dispersed particles by dehydration of emulsions provides stability in powders and may facilitate controlled release of the entrapped core materials (Shahidi and Han, 1993). Spray drying is commonly utilized for microencapsulation in the food industry as spray drying is economic means to produce powders of high quality with long shelf-life. Spray drying often uses trehalose, maltodextrins, corn syrup, milk proteins, and modified starch as wall materials (Desobry *et al.*, 1997; Adhikari *et al.*, 2009; Spada *et al.*, 2012; Lim and Roos, 2016; Lim *et al.*, 2016). Nonetheless, crystallization of the glass-forming components may facilitate degradation of bioactive components (Buera *et al*., 2005). Crystallization of the amorphous sugar may therefore be decreased by the use of miscible carbohydrates with crystallizing amorphous sugars (Roos and Karel, 1991c; Potes *et al.*, 2012).

Our previous study showed that LBL systems with trehalose and maltrodextrin with low DE (9-12) or trehalose and WPI as wall materials provided better protection towards carotenoids loss (Lim and Roos, 2016; Lim *et al.*, 2016). Several other studies have shown that systems with higher DE maltodextrin gave better retention

of encapsulated bioactives upon storage than those of lower DE (Anandaraman & Reineccius, 1986; Wagner and Warthesen, 1995). The present study investigated the ability of dehydrated SL and LBL emulsions obtained from spray drying of concentrated hydrophilic solids emulsions of high trehalose and maltodextrin with high DE (23-27) contents as glass formers to protect encapsulated carotenoids upon storage at various temperatures (35°C, 50°C, and 65°C). The study provided data on loss kinetics and carotenoids retention in SL and LBL systems. The use of spray drying of emulsions of high total solids is rarely reported and our study will provide valuable information to the food and pharmaceutical industries.

6.2 Materials and Methods

6.2.1 Materials

Trehalose (crystalline dihydrate) was purchased from Hayashibara Shoji Inc. (Japan), maltodextrin (M250, DE 23-27) from Grain Processing Corporation (IA, U.S.A.), sunflower oil from Musgrave Excellence^{TM} (Spain), whey protein isolate (WPI, Isolac) was obtained from Carbery Food Ingredients (Ballineen, Ireland), gum Arabic (Sigma Aldrich G9752) from Sigma Aldrich (Stenheim, Germany), lutein (Marigold) from Shaanxi Sciphar Biotechnology Co. Ltd. (China), and all-trans-β-carotene (crystalline Type I, synthetic, > 93% (UV)) from Sigma-Aldrich (U.S.A.). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich, Inc. (Dublin, Ireland).

6.2.2 Emulsion Preparation

Emulsion was prepared with slight modification as described in Lim *et al.* (2014) and Lim and Roos (2016). Table 6.1 shows the amount, preparation, final emulsion composition (%), and powder composition (%) of whey protein isolate (WPI), sunflower oil and carotenoids, trehalose, maltodextrin (MD250), gum Arabic, water, and citric acid solution used to prepare single layer (SL) and layer-by-layer (LBL) emulsions. Deionized water was used to disperse WPI (12.7%, of mass, in water)

and the dispersion was left to hydrate for 2h to improve hydration of the proteins followed by pH adjustment of the dispersion using citric acid solution (10%, of mass, in water) to pH 3.5. Sunflower oil (20%, of mass, of total emulsion), used as lipid phase, was the solvent for all-trans-β-carotene (0.05%, of mass, of oil) and lutein (0.05%, of mass, of oil) at 50°C. The carotenoids were mixed with the oil phase with a Silverson mixer (Model AXR, Silverson Machines Ltd., Chesham, UK). The water phase and oil phase (at the ratio of 0.825 water used for dispersion: 1 sunflower oil) was then mixed for 60s with a Silverson mixer at minimum speed to produce pre-emulsion. Homogenization of the pre-emulsion was done at 240 bar (200 bars for the first stage and 40 bars for the second) at room temperature using a two- stage valve homogenizer (APV-1000, APV Homogenizer Group, Wilmington, MA, USA). Pre-emulsion was used in the preparation of the SL emulsion. For preparation of the LBL emulsion, deionized water was used to dissolve gum Arabic (13.04 %, of mass, in water) and the solution was stirred for 2 h. The gum Arabic solution (adjusted to pH 3.5 with citric acid solution) was mixed with the emulsion for 30 min at the ratio of 1:8.86 to obtain LBL emulsion.

Trehalose and maltodextrin mixture (1:1, 56.6% total solids, of mass, in water) was used as wall material. Maltodextrin was dissolved in deionized water initially at 55°C using a Silverson mixer followed by the addition of trehalose upon the dissolution of maltodextrin. The pH of the solution was adjusted to pH 3.5 using citric acid solution. The trehalose-maltodextrin solution was then added into the SL and LBL pre-emulsions at the ratio of 1.34:1 and 1.2:1, respectively, to obtain the final emulsions and mixed for 30 min using a Silverson mixer. Similar amount of water instead of gum Arabic solution was added into the SL emulsion to obtain corresponding final mass for both SL and LBL emulsions. The final total solids content of the SL and LBL emulsions was >50% of total mass. The emulsions comprised of 20.02% (mass) carotenoids containing sunflower oil, 15.0% (mass) trehalose, 15.0% (mass) maltodextrin, 2.4% (mass) WPI, and water (47.58%, mass, for SL, and 46.98%, mass, for LBL, modified to pH 3.5 with citric acid) with and without gum Arabic at 0.6%, respectively. Apparent viscosity (Rheometer ARES-G2, TA Instruments®, USA using aluminum “plane-plane”, 40 mm) of the SL and

LBL emulsions was determined at 25°C and constant shear rate of $150s^{-1}$ for 300s. The apparent viscosity of the SL emulsion was 36.64±1.4 mPa·s and that of the LBL emulsion was 103.69±1.4 mPa·s.

Table 6.1. Amount, preparation, final emulsion composition (%), and powder composition (%) of whey protein isolate (WPI), sunflower oil and carotenoids, trehalose, maltodextrin (MD250), gum Arabic, water, and citric acid solution used in the preparation of single layer (SL) and layer-by-layer (LBL) emulsions.

Component	Amount (g/1000g)		Preparation	Final Emulsion Composition (%)		Powder Composition (%)	
	SL	LBL		SL	LBL	SL	LBL
WPI	24	24	Dispersed in 165g of water at room temperature	2.4	2.4	4.6	4.5
Sunflower oil +Carotenoids	200.2	200.2	Carotenoids was dissolved in sunflower oil at 50°C	20.02	20.02	38.2	37.8
Trehalose	150	150	Dissolved in 230g of water at 55°C by addition of maltodextrin followed by trehalose	15	15	28.6	28.3
Maltodextrin (MD 250)	150	150		15	15	28.6	28.3
Gum Arabic	0	6	Dissolved in 40g of water at room temperature	0	0.6	-	1.1
Water	447.8	438.8	-	44.78	43.88	-	-
Citric Acid (10%, mass)	28	31	Used to adjust pH of the emulsions to pH 3.5	2.8	3.1	-	-

6.2.3 Spray Drying

Spray drying was done as reported by Lim and Roos (2016) with slight modifications. Single stage Niro 25 spray dryer (GEA Niro Production Minor,

Søborg, Denmark) with inlet and outlet temperatures of 183°C and 85°C, respectively and rotating disc atomizer at 24,000 rpm was used to spray dry the emulsions. The powder was immediately cooled to room temperature, sealed in plastic packages (PA/PE 90, Fispak Ltd., Dublin, Ireland), and stored in the dark at room temperature to reduce physico-chemical changes and water uptake prior to analyses. Solids of SL powders consisted of 38.2% (mass) carotenoids containing oil, 28.6% (mass) trehalose, 28.6% (mass) maltodextrin, and 4.6% (mass) WPI. Solids of LBL powders consisted of 37.8% (mass) carotenoids containing oil, 28.3% (mass) trehalose, 28.3% (mass) maltodextrin, 4.5% (mass) WPI, and 1.1% (mass) gum Arabic.

Two-gram samples of the powders were transferred into 10 mL clear glass vials (Schott, Müllheim, Germany) and hermetically sealed with septums under vacuum using a sealing assembly of a freeze-drier (Lyovac GT 2, Steris®, Hürth, Germany). The sealed vials were packaged in plastic laminate pouches (PA/PE 90, Fispar, Leamore Warehouse, Dublin, Ireland) in duplicates with a vacuum sealing system (Polar 80, Henkelman Vacuum Systems, Hentogenbosch, Netherlands). Vacuum present in the packages isolated the vials with samples from surrounding atmosphere and any loss of vacuum was an indicator of leakage. The samples were stored in incubators at 35°C, 50°C, and 65°C for further analyses of carotenoids stability at intervals. The powders at "as is" water content from the spray drier with the trehalose-maltodextrin (DE 23-27) as glass former showed onset glass transition temperature (T_g) of 62 ± 3°C for SL powders and 66 ± 2°C for LBL powders as measured using differential scanning calorimetry (DSC, Mettler Toledo 821e, Schwerzenbach, Switzerland).

6.2.4 Colourimetry

Colour of the powders was measured as reported in Lim *et al.* (2014). A pre-calibrated colourimeter (Minolta, CR300 meter) was used to measure the L*, a*, and b* values of SL and LBL systems. Measurements were completed directly from the glass vials upon storage at 35°C, 50°C, and 65°C for up to 99 days of storage over

14 time points. L* value ranged from 0-100 and was associated to the lightness or luminance component while the a* and b* values ranged from -120 to 120 measuring the chromatic components of green to red values and blue to yellow values, respectively.

6.2.5 Water activity

Water activity (a_w) of the powders was determined as described in Lim *et al.* (2014). AquaLab Water Activity Meter CX-2 with internal temperature control (Decagon Devices, Inc., Pullman WA, U.S.A.) was used to determine the a_w of SL and LBL systems stored at 35°C, 50°C, and 65°C. The a_w of the systems for up to 99 days of storage (14 time points) was determined in plastic cups (Disposable Sample Cups, AquaLab, Decagon Devices, Inc., Pullman WA, U.S.A.) at room temperature.

6.2.6 Extraction Method and HPLC Analysis

Extraction of carotenoids and determination of carotenoids loss using HPLC was done as reported by Lim *et al.* (2014) and Lim and Roos (2016) with slight modifications. Deionized water (KB Scientific, Ireland) (2 mL) was used to reconstitute 0.5g aliquots of the powders followed by vortexing (Scientific Industries Inc., G-560E, NY, USA) at room temperature for 30s. The reconstituted emulsion was destabilized with 3.9 mL methanol:ethlyacetate (1:1, v/v) mixture acting as organic solvents for the carotenoids and vortexed for 30s. To reduce oxidation of carotenoids during the extraction process, 0.2% butylated hydroxyl toluene (BHT) was added into the methanol:ethlyacetate mixture as antioxidant. Saponification of the oil particles was done using 1 mL of saturated potassium hydroxide in methanol (2M) followed by vortexing for 30s to separate the lipid carrier (saponised fraction) from the all-trans-β-carotene and lutein (unsaponised fraction). Dichloromethane (1 mL) was added into the sample and vortexed for 30s to ensure dissolution high level of extraction of the carotenoids present in the organic phase. Lastly, hexane (3.8 mL) was added into the sample followed by vortexing for 30s to enhance the organic phase separation. The extract was then allowed to stand in the dark for 30 min. Glass

pipette was used to remove the organic phase followed by filtering (Minisart RC 15, Sartorius Stedim Biotech GmbH, Goettingen, Germany) into HPLC vials (Dionex No: 055427, 1.5ml w/ Slit Septum, Unas, USA). Injection was done with 10 µL of sample into the HPLC system (Dionex ICS3000, Sunnyvale, CA, U.S.A.) equipped with autosampler (AS-1, Dionex, Sunnyvale, CA, U.S.A.), dual pump (DP-1, Dionex, Sunnyvale, CA, U.S.A.), and photodiode-array detector (PDA ICS Series, Dionex, Sunnyvale, CA, U.S.A.) to quantify the amount of β-carotene and lutein. A reversed-phase Acclaim C30 analytical column (250 mm × 4.6 mm i.d., 5 µm) equipped with a guard column (4 mm × 4 mm i.d.) of the same material (Dionex, Sunnyvale, CA, U.S.A.) was used. Acetonitrile (A), methanol:ethyl acetate (1:1) (B), and 0.5% of 200 mmol acetic acid in water (C) eluent system 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 was used. Absorption peak areas at 450 nm were used to derive the relative amounts of lutein and all-trans-β-carotene in the samples. First-order kinetics ($\ln A/A_0 = -kt$) was fitted to the data of carotenoids degradation and slopes of linear regressions were used to obtain the rate constants (k). A linear relationship obtained from the plot of $\ln A/A_0$ against time (storage days) supported the use of first order kinetics which was typical of carotenoids degradation (Wagner and Warthesen, 1995; Desobry *et al.*, 1997; Elizalde *et al.*, 2002; Mahfoudhi and Hamdi, 2014). Arrhenius relationship $k=Ae^{-Ea/(RT)}$ and plots of ln k against T^{-1} were used to determine activation energies. The study on carotenoids stability was carried out for up to 99 days of storage (14 time points).

6.2.7 Interstitial Air, Occluded Air, Particle Density, Bulk Density, Surface Oil, and Particle Size

Powder properties were determined as described in Lim *et al.* (2015) and Lim *et al.* (2016). The interstitial air and occluded air of the powders were determined as per GEA Niro (2006). Particle density was determined using a Multivolume Pycnometer 1350 (Micromeritics, Georgia, USA) equipped with helium gas. A tap volumeter (Copley, J. Engelsmann A.G., Ludwigshafen, Germany) was used to determine the

tapped bulk density (100 taps) of the powders. Hexane washing was done to determine surface oil of the powders. Hexane was used to wash the powders (10g) for four washes (50ml per wash). The beaker containing extracted surface fat and hexane was placed in water bath at 50°C to allow evaporation of hexane and moved into an oven at 50°C until constant weight was achieved. Malvern Mastersizer 3000 with powder feeder unit (Malvern, Worcestershire, UK) was used to determine the particle size distribution of SL and LBL powders by laser diffraction.

6.2.8 Statistical Analysis

The results were expressed as mean ± standard deviation and the analyses were completed in triplicates. Duplicated injections in replicates were carried out for the study of carotenoids stability.

6.3 Results and Discussions

6.3.1 Powder Properties

Table 6.2. Water content, interstitial air, occluded air, particle density, bulk density, tapped density (100 times), surface oil, and d [4,3] (average volume-surface diameter) of single layer (SL) and layer-by-layer (LBL) spray dried high hydrophilic solids emulsions.

	SL	LBL
Water content (g/100g SNF)	2.67±0.03	2.73±0.05
Interstitial air (cm^3/100g)	99.58±2.39	95.02±0.65
Occluded air (cm^3/100g)	7.73±2.06	3.08±0.65
Particle density (g/cm^3)	1.10±0.03	1.16±0.01
Bulk density (g/cm^3)	0.34±0.01	0.35±0.01
Tapped density (g/cm^3)	0.52±0.01	0.55±0.01
Surface oil (g/100g)	5.02±0.01	4.22±0.22
D [4,3] (µm)	67.73±0.09	72.37±0.25

Values are means (n=3) ± SD

High quality powders were successfully produced by spray drying of high total solids single layer (SL) and layer-by-layer (LBL) emulsions. The SL and LBL emulsions were visually similar in the powders obtained. Their respective water content, interstitial air, occluded air, particle density, bulk density, tapped density, surface oil, and particle size as d [4,3] are given in Table 6.2. There were no significant differences in the water contents and bulk densities of the powders obtained. SL emulsion powders had significantly higher interstitial air and occluded air contents than LBL emulsion powders. There can be non-adsorbed WPI present within the continuous phase of SL system that results in the mixture of carbohydrate and protein in the continuous phase. The presence of non-adsorbed WPI in the continuous phase results in the higher porosity of the wall materials upon spray drying that increased occluded air as observed in the higher occluded air in spray dried systems with trehalose-WPI as wall materials than trehalose-maltodextrin (M100) systems (Lim and Roos, 2016; Lim *et al.*, 2016). Nonetheless, the non-adsorbed WPI can be electrostatically attached on the added gum Arabic that effectively removes the non-adsorbed WPI from the continuous phase to the interface of the oil droplets (Figure 6.1). The presence of only carbohydrates as the wall materials therefore gave a denser and less porous powder translating to lower occluded air. Study by Moreau and Rosenberg (1999) also found that increasing amount of lactose in WPI-lactose spray dried systems increases the density and reduces the porosity of the powders obtained. The particle density of LBL emulsion powders was significantly higher as was shown by the lower occluded air. The electrostatic packing of gum Arabic at the ratio of 1:4 to WPI on the WPI-oil interface in LBL system increased the molecular density at the interface of the oil particles to be higher than that of SL system. The higher interstitial air and occluded air in SL emulsion powders also explained the significantly lower tapped density (100 taps). LBL interfacial structure increased stability of the oil particles towards disruption (Güzey and McClements, 2006a) during the atomization process to give LBL emulsion powders with significantly lower surface oil. Significant difference was observed in the powder particle size measured by d [4,3] (average volume-surface diameter) of SL and LBL spray dried emulsions with LBL powders having larger d [4,3] (72.37 ± 0.25μm) than SL powders (67.73 ± 0.09μm). The increase in

viscosity of LBL system due to the presence of gum Arabic may affect particle formation during atomization in the spray dryer as the higher viscosity of the emulsion increased the resistance of the LBL emulsion to flow out of the atomizer that gave larger atomized droplets and subsequently larger d [4,3] of the powders obtained. Generally, flowability of the SL and LBL emulsion powders were found to be 'easy flowing' as suggested by the results of powder flow tester (data not shown).

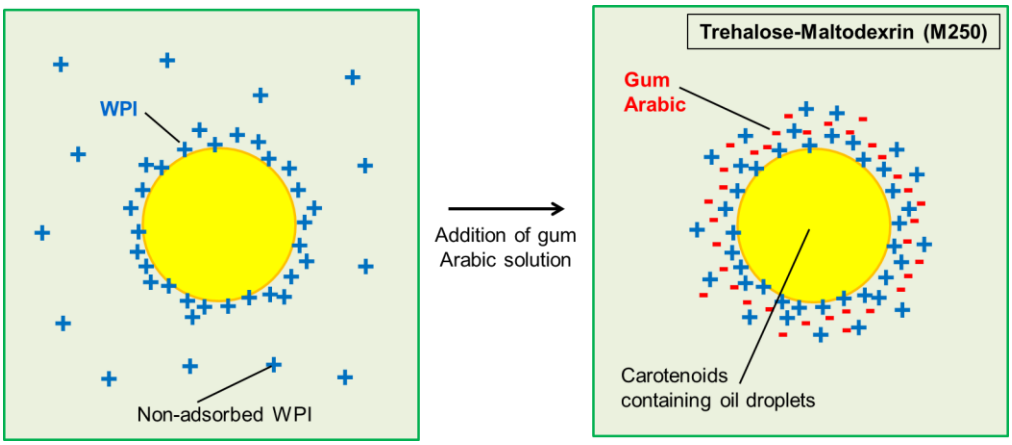

Figure 6.1. Images showing the presence of non-adsorbed whey protein isolate (WPI) within the trehalose-maltodextrin (M250) containing continuous phase in SL system and the migration of non-adsorbed WPI towards gum Arabic upon addition of gum Arabic solution in LBL system.

6.3.2 Water Activity and Colour

Figure 6.2 shows water activity (a_w) of SL and LBL emulsion powders before (day 0) and after storage for 99 days at 35°C, 50°C, and 65°C. The a_w of the powders at day 0 was the a_w measured after spray drying of the emulsions and cooling of the powders. There was no significant difference in the a_w between SL ($0.16 \pm 0.01 a_w$) and LBL ($0.17 \pm 0.01 a_w$) emulsion powders at day 0. The a_w of SL and LBL emulsion powders were below 0.20 a_w which is typical of spray drying in the food industry (Adhikari *et al.*, 2009). According to the stability map of foods as a function of a_w, reduction of non-enzymatic browning as well as inhibition of mold, yeast, and bacteria growth occurs in systems having a_w around 0.16 a_w (Labuza, 1971; Labuza and Dungan, 1971). There was no loss of vacuum in the packages used to store the

samples indicating the samples were isolated from the surroundings atmosphere that could affect the a_w during storage. The variations in the a_w of both systems upon 99 days storage were generally small and insignificant except at 50°C where the a_w reduced significantly although these changes were also small of only approximately 0.02 a_w.

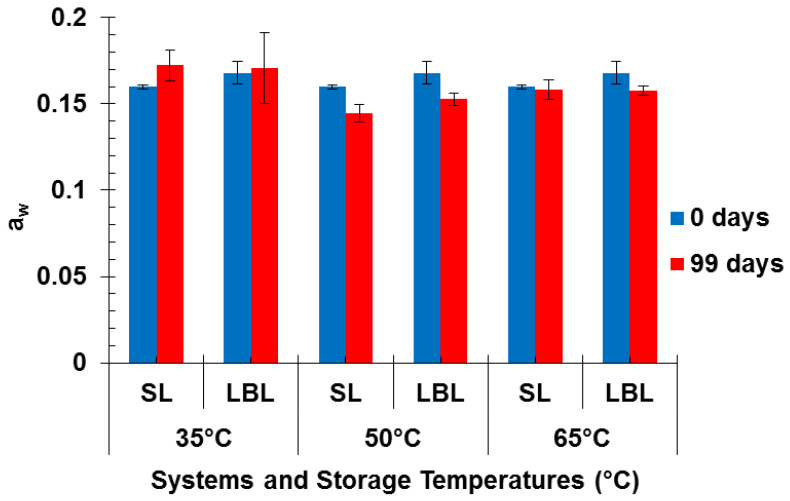

Figure 6.2. Water activity (a_w at room T) of single layer (SL) and layer-by-layer (LBL) spray dried high hydrophilic solids emulsions before and after 99 days storage at 35°C, 50°C, and 65°C.

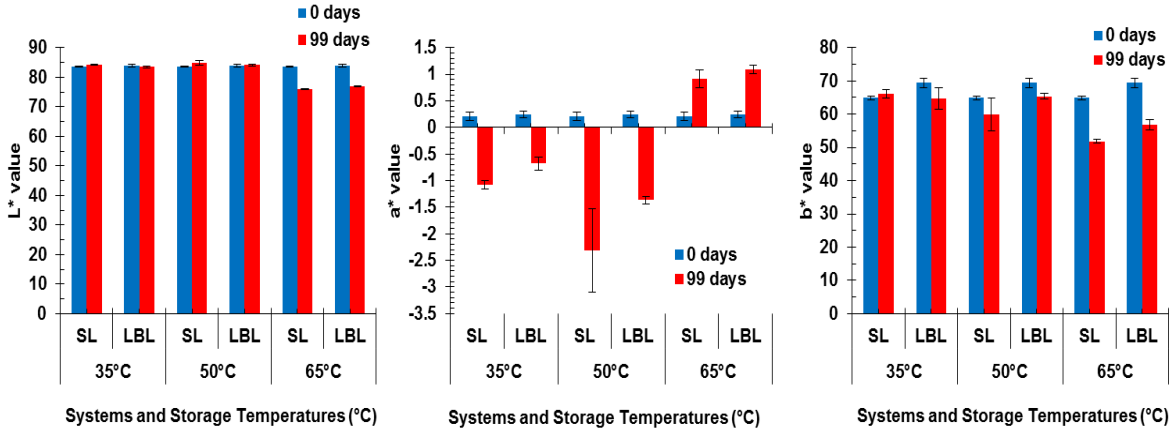

Figure 6.3. Colour values (L*, a*, and b* values) of single layer (SL) and layer-by-layer (LBL) powders obtained from spray drying at day 0 and upon storage at 35°C, 50°C, and 65°C for 99 days.

Colour measurement parameter values, L* (lightness), a* (redness), and b* (blueness), of SL and LBL emulsion powders before (day 0) and after storage at 35°C, 50°C, and 65°C for 99 days are shown in Figure 6.2. There were no significant differences in the L* and a* values between SL and LBL emulsion powders at day 0. Minor but insignificant changes were found for the L* values of both systems stored at 35°C and 50°C. However, the L* values in both SL and LBL systems decreased significantly during storage at 65°C for 99 days. Maillard reaction (non-enzymatic browning) occurred in emulsion powders as reactants, reducing sugars (maltodextrin components) and protein amino groups (WPI) were present that resulted in the lower L* values of systems stored at 65°C. Reactive oxygen species and free radicals from lipid oxidation too are capable of attacking the protein and amino groups in the continuous phase of the systems that causes browning (Venolia and Tappel, 1958; Potes *et al.*, 2014). Browning in oil and protein containing systems occurs due to the formation of lipid peroxides, followed by the formation of brown pigment precursors due to the interaction between peroxides or products of carbonylic peroxide decomposition with protein's active groups, and lastly the formation of brown pigments from the lightly-coloured or colourless precursors (Pokorný *et al.*, 1974). The a* values reduced significantly during storage for 99 days at 35°C and 50°C but increased significantly upon storage at 65°C. The a* values after storage for 99 days at the highest storage temperature (65°C) agreed with the respective L* values where a* values of systems stored at 65°C showed a different trend from systems stored at 35°C and 50°C. Therefore, the increase in a* values of systems stored at 65°C can be attributed to Maillard reaction and browning due to attack of amino acids by lipid oxidation (Zirlin and Karel, 1969; Pokorný *et al.*, 1974). On the other hand, the decrease in a* values upon storage at 35°C and 50°C was the result of degradation of carotenoids in the systems in agreement with the findings of other authors (Elizalde *et al.*, 2002; Qian *et al.*, 2012; Mahfoudhi and Hamdi, 2014). Similar trend in the changes of L* and a* values upon storage was observed in our earlier studies (Lim and Roos, 2016; Lim *et al.*, 2016). Reduction in b* values was generally observed in both systems at all storage temperatures upon storage with significance observed at 65°C. Browning that occurred at 65°C as observed from L* and a* values may result in the shift from yellowness to the darker hue of blueness that reduced the b* values.

Nonetheless, changes in b* values was not dominant in carotenoids containing systems as reported by other authors (Desobry *et al.*, 1997; Elizalde *et al.*, 2002; Mahfoudhi and Hamdi, 2014).

6.3.3 Loss Kinetics of Carotenoids

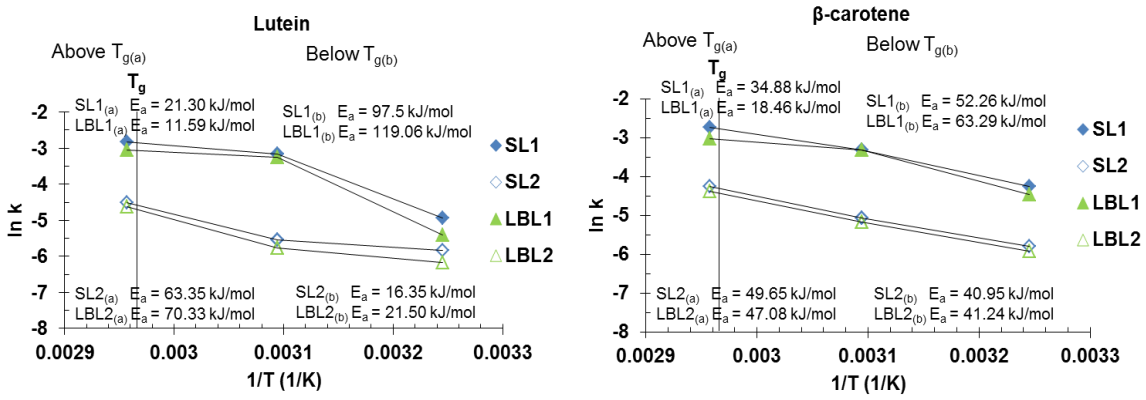

Figure 6.4. Glass transition temperature (T_g) and Arrhenius plots displaying the activation energies of lutein and all-trans-β-carotene in single layer (SL) and layer-by-layer (LBL) spray dried high hydrophilic solids emulsions stored at 35°C, 50°C, and 65°C for 99 days showing a rapid initial first order degradation kinetic (SL1 and LBL1) followed by a second less rapid first order degradation step (SL2 and LBL2).

The retention time of lutein was 5.6 ± 0.1 min and 28.5 ± 0.1 min for all-trans-β-carotene in the HPLC chromatograms. The loss of both lutein and all-trans-β-carotene was found to be less rapid in LBL systems. Figure 6.4 shows the Arrhenius plots for lutein and all-trans-β-carotene in SL and LBL spray dried emulsions stored at 35°C, 50°C, and 65°C for 99 days. Activation energies and glass transition temperatures (T_g) of the systems are also shown in the figure. Studies by other authors claimed that LBL interfacial layer properties decreased reaction rate as the thicker interfacial layer gave a better physical barrier (Klinkesorn *et al.*, 2005; Shaw *et al.*, 2007) which can provide an improved protection to the lipid phase. Oxygen may be trapped within the glassy matrix of the powders as observed by the presence of occluded air. Oxygen present can be the cause of carotenoids degradation as stated

by Achir *et al.* (2010). Water present in the powders too has dissolved oxygen (Wilhelm *et al.,* 1977) that is capable of causing loss of carotenoids. On the other hand, metal ions present in WPI (Carunchia Whetstine *et al.,* 2005) may diffuse in the presence of water and can catalyze oxidation of carotenoids. Metal ions results in carotenoids loss through electron transfer while adduct formation, hydrogen abstraction, and electron transfer occurs in the presence of oxygen to cause carotenoids degradation (Britton, 1995; Young and Lowe, 2001; Mortensen, 2002; Boon *et al.,* 2010). The higher amount of carotenoids containing surface oil on SL emulsion powder particles was likely to increase the loss rates of lutein and all-trans-β-carotene as surface oil was not protected by the glassy matrix and it was directly exposed to the surroundings. SL emulsion powder particles also possessed significantly higher amount of occluded air containing oxygen that contributed to higher carotenoids degradation rates.

The oxidation of carotenoids is a typical first order reaction (Wagner and Warthesen, 1995; Elizalde *et al.,* 2002; Mahfoudhi and Hamdi, 2014) and first order kinetics fitted to our carotenoids degradation data. Two-step first order degradation kinetics, however, was found for lutein and all-trans-β-carotene in both SL and LBL emulsion powders. Carotenoids showed rapid initial degradation kinetics (< 9 days) followed by a second less rapid step. Residual air present within the glassy matrix of the powders could increase oxidation of the entrapped carotenoids resulting in the rapid initial degradation. Vacuoles containing oxygen were formed within the glass matrix, as observed by the presence of occluded air (Table 6.2) as well as dark spots under optical microscopes in our earlier studies (Lim and Roos, 2016; Lim *et al.,* 2016), due to thermal expansion of air bubbles incorporated into the emulsion during emulsion preparation and air bubbles produced in atomization during the spray drying process (Duffie *et al.,* 1953; Verhey, 1972). Besides, the degradation of carotenoids in surface oil that was not protected within the glassy matrix also contributed to the rapid initial reduction. The higher occluded air in combination with higher surface oil in SL emulsion powder could explain the more rapid initial degradation of carotenoids. A study by Desobry *et al.* (1997) and our earlier studies (Lim and Roos, 2016, Lim *et al.,* 2016) also showed a two-step first order kinetics of

carotenoids degradation in spray dried solids. The second less rapid degradation step took place upon storage as the entrapped air was reduced and the degradation of carotenoids in the surface oil had already occurred. This confirmed less rapid degradation of carotenoids in oil droplets encapsulated within the continuous phase formed primarily by the glass forming carbohydrates. Boon *et al.* (2010) stated that carotenoids radicals formed by oxidation may undergo further chain reactions reacting among themselves and with other radicals present resulting in the further oxidation of carotenoids. Such mechanism contributed towards the second less rapid step of carotenoids degradation. Carotenoids radicals may also undergo isomerization from trans-isomers to cis-isomers which also as reduced quantities of original carotenoids isomers (Gao *et al.*, 1996; Gao and Kispert, 2003).

The degradation of lutein and all-trans-β-carotene increased in both systems with increasing storage temperature in agreement with several studies (Desobry *et al.*, 1997; Qian *et al.*, 2012; Lim *et al.*, 2014). Nonetheless, there was non-Arrhenian increase in the carotenoids loss rate with increasing storage temperature in the initial rapid degradation. Melanoidins produced through Maillard reactions have antioxidant capacity (Wang *et al.*, 2011) that is capable of providing protection to the encapsulated carotenoids from radicals that reduces carotenoids loss. A higher storage temperature could also reduce oxygen solubility in the water content at temperatures up to 100°C (Hildebrand, 1952; Wilhelm *et al.*, 1977). The lesser amount of oxygen present in the small quantity of water of the powders stored at higher storage temperature reduced oxygen diffusion towards the encapsulated carotenoids that effectively reduced carotenoids degradation. Storage of the systems in the vicinity of the T_g resulted also in structural collapse of the glass formers at the rate determined by $T\text{-}T_g$ (Levi and Karel, 1995). However, there was an increase in the rate of carotenoids degradation in emulsion powders stored at 65°C during long term storage. Long term exposure to the higher temperature resulted not only in decomposition of carotenoids but also isomerization as carotenoids were prone to isomerization that contributed to the decreasing quantity of all-trans-β-carotene (Chandler & Schwartz, 1988). The emulsion powders of a_w of approximately 0.16 a_w

also showed increased carotenoids degradation as systems with a_w below 0.2 or above 0.4 showed an increase in the rate of lipid oxidation (Labuza *et al.*, 1971).

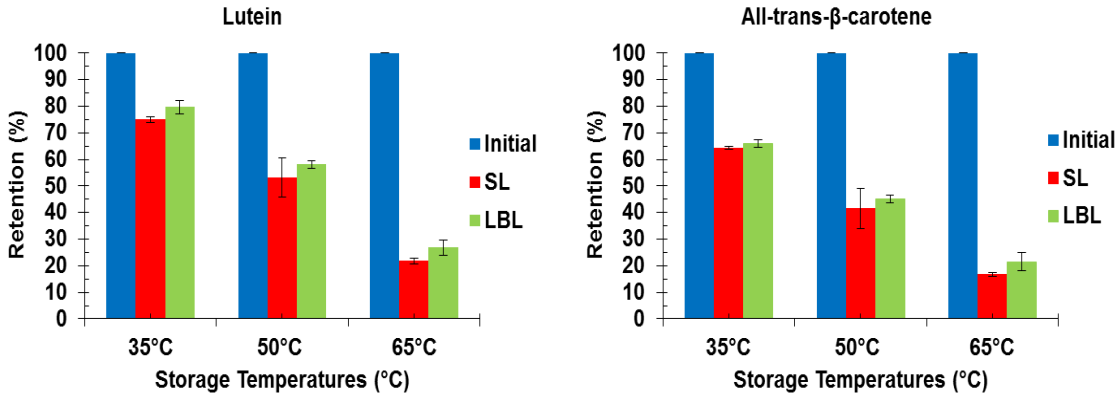

Figure 6.5. Retention (%) of lutein and all-trans-β-carotene upon storage at 35°C, 50°C, and 65°C for 99 days in single layer (SL) and layer-by-layer (LBL) powders.

There was a shift in the activation energies of carotenoids degradation with increasing storage temperature which was also reflected in the non-Arrhenian increase in the loss of carotenoids with increasing storage temperature. Lutein and all-trans-β-carotene contents in both SL and LBL emulsion powders upon long term storage for 99 days at 35°C, 50°C, and 65°C are shown in Figure 6.5. The retention of both lutein and all-trans-β-carotene was higher in LBL emulsion powder at all storage temperatures with significantly higher retention found for LBL emulsion powder stored at 65°C. This showed that the application of the LBL interfacial structure was able to significantly reduce degradation of carotenoids at higher storage temperatures. The improved protection by LBL systems towards environmental stresses such as heat treatment, ionic strength, ageing, lipid oxidation, freeze-thaw cycles and changes in pH was also reported by several other authors (Moreau *et al.*, 2003; Ogawa *et al.*, 2003a; Gharsallaoui *et al.*, 2010). The thicker interfacial layer, lower van der Walls attraction, and higher steric repulsion contributed towards the higher stability of LBL emulsions (Gu *et al.*, 2005; Güzey and McClements, 2006a; Harnsilawat *et al.*, 2006; Gharsallaoui *et al.*, 2010).

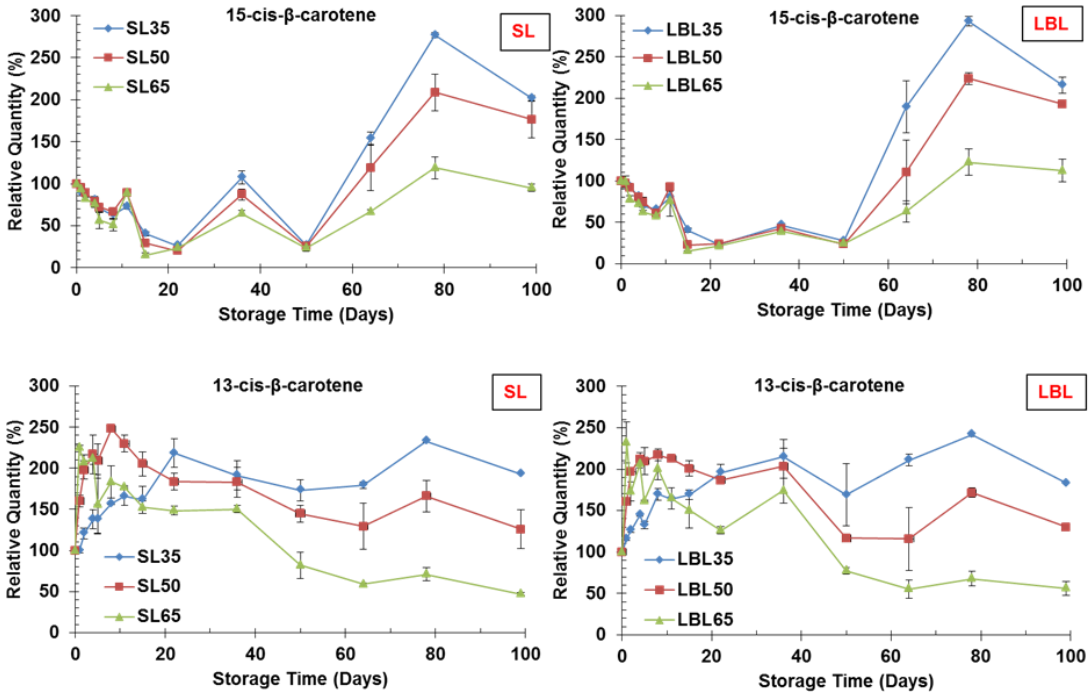

Figure 6.6. Variations in the relative quantity (%) of β-carotene isomers (15-cis-β-carotene and 13-cis- β-carotene) with storage time (days) in single layer (SL) and layer-by-layer (LBL) systems stored at 35°C, 50°C, and 65°C.

Isomerization of all-trans-β-carotene results in the formation of 15-cis-β-carotene and 13-cis-β-carotene as observed in the HPLC chromatograms. The exposure of the carotenoids containing systems to thermal treatment resulted in isomerization (Sweeney and Marsh, 1971). Isomerization could occur as carotenoids radicals formed such as cation radical (trans-carotenoids$^{\bullet+}$) and dication species (trans-carotenoids^{2+}) from all-trans-carotenoids isomerized to different cis species (Gao *et al.*, 1996). Heating resulted in the reduction of all-trans-β-carotene but at the same time increased the amount of β-carotene isomers (Chandler and Schwartz, 1988). Figure 6.6 shows relative quantities (%) of 15-cis-β-carotene and 13-cis-β-carotene in SL and LBL emulsion powders during storage at 35°C, 50°C, and 65°C for 99 days. The retention time in the HPLC chromatograms for 15-cis-β-carotene was 20.1 ± 0.1 min and 24.1 ± 0.1 min for 13-cis-β-carotene. Concentrations of 15-cis-β-carotene and 13-cis-β-carotene increased with increasing storage temperature initially with higher amount of isomers found in systems stored at higher storage temperatures. However, concentrations of these isomers were higher in emulsion

powders stored at 35°C during a long term storage indicating a higher total loss of β-carotene in emulsion powders stored at the higher storage temperatures. Chandler and Schwartz (1988) stated that all-trans-β-carotene readily converted to cis-isomers during thermal treatments rather than decreased in quantity as a result of thermal degradation. Generally, isomer concentrations fluctuated during storage but LBL emulsion powders showed slightly higher amounts of 15-cis-β-carotene and 13-cis-β-carotene than SL emulsion powders at all storage temperatures. The higher amount of the β-carotene isomers in LBL system suggested that the application of LBL interfacial structure reduced the amount of total degradation of β-carotene.

6.4 Conclusions

SL and LBL emulsions at high hydrophilic solids concentrations were successfully spray dried to give powders of corresponding visual and other powder characteristics as there were no significant differences in the water content, bulk density, water activity (a_w), L* values, and a* values of the fresh powders. The application of LBL interfacial structure was successful in reducing degradation of lutein and all-trans-β-carotene. Higher amounts of carotenoids were retained in LBL system upon long term storage of 99 days with significance observed at the higher storage temperature (65°C). There was non-Arrhenian relationship with increasing storage temperature for the degradation of carotenoids as storage in the vicinity of the T_g reduced degradation. It is crucial to identify factors that degrade oil-soluble bioactives during storage and methods to overcome degradation such as by the application of the LBL interfacial structure. Our results suggested that the LBL interfacial structure was able to reduce the loss of carotenoids and can be applied in the food and pharmaceutical industries to improve the stability of oil-soluble bioactives in formulated materials.

Chapter VII

General Discussion

7.1 Introduction

The use of concentrated solid feeds enables economic manufacturing of formulated foods in powder form. The present study emphasised on the production of high total solid emulsions stabilised with layer-by-layer (LBL) interfacial structuring as feed for spray drying. The LBL interfacial structure was acquired using gum Arabic as a polyelectrolyte to assemble at the whey protein isolate (WPI) surface through electrostatic attraction at pH 3.5. The lipid phase of an oil-in-water (O/W) emulsion offered a great opportunity for the encapsulation of oil-soluble bioactives. Oil-soluble bioactives such as carotenoids could be dissolved in the lipid phase prior to homogenization with emulsifier. These emulsions were designed with different wall material composites to provide protection to the encapsulated lutein and all-trans-β-carotene upon storage of the powders. The degradation kinetics of the encapsulated in powders upon storage at temperatures in the vicinity of the glass transition temperature (T_g) of the wall materials carbohydrate components was studied. Carotenoids degradation kinetics of LBL powders was compared to those of single layer (SL) interfaced spray dried emulsions.

7.2 LBL Emulsion

Conventional O/W or SL emulsion was thermodynamically unstable and could break down with storage (Dickinson, 1992; McClements, 1999) especially when exposed to environmental stresses such as heating, changes in pH, freezing, dehydration, and ionic strength (McClements, 2010). On the other hand, many studies found that LBL emulsions have higher stability towards external stresses such as heat treatment, variations in pH, freeze-thaw cycles, lipid oxidation, and ionic strength (Ogawa *et al.,* 2003a; Aoki *et al.,* 2005; Güzey and McClements, 2006a; Gharsallaoui *et al.,* 2010). LBL emulsion was formed through electrostatic attraction between oppositely charged primary layer and secondary layer which has been widely described in the literature (Moreau *et al.,* 2003; Grigoriev *et al.,* 2008; Gharsallaoui *et al.,* 2010; Benjamin *et al.,* 2012; Lim *et al.,* 2014; Jiménez-Martín *et al.,* 2015). Therefore,

LBL interfacial structuring was used in encapsulating carotenoids to further improve the stability of the encapsulated carotenoids.

7.2.1 Primary Layer

Nonetheless, the amount of primary layer or emulsifier played an important role in the stability of the LBL system. It was found in our study (Lim and Roos, 2015) that materials with lower amount of WPI primary layer (1:50 WPI to sunflower oil ratio) had lower oil droplets stability. Coalescence occurred in these systems and coalescence was irreversible as was observed from the large ζ-average diameter and the presence of large bright droplets in optical microscope images (Lim and Roos, 2015). Although depletion flocculation occurred in all LBL systems, individual oil droplets were obtained again in systems with higher amount of WPI as primary layer (6:50 WPI to sunflower oil ratio) upon deflocculation by increasing the pH of the aqueous phase. That result showed that sufficient primary layer prevented coalescence even though the oil droplets were in close proximity due to flocculation and this maintained the protection provided by the system to the encapsulated bioactives. Coalescence may lead to phase separation and the loss of protection provided to the encapsulated bioactives. Study by Cornacchia *et al.* (2011a) found that the concentration of emulsifier as primary layer had an impact on the emulsion particle size and stability of the encapsulated carotenoids. The low amount of emulsifier gave bimodal particle size distribution with visual oil separation. The stability of the emulsion affected carotenoids degradation rate where degradation was highest in system with insufficient amount of emulsifier (0.5:50 and 1:50 emulsifier to lipid phase ratio). The thickness of WPI as emulsifier at the surface of oil particles was found to increase with increasing concentration of WPI caused by protein-protein interaction (Cornacchia *et al.*, 2011a). Globular WPI at the surface of the O/W interface could undergo minor macromolecular rearrangement and partial denaturation (Dickinson and Matsumura, 1994). Partial unfolding of protein revealed the non-polar, thiol, and disulfide groups that enhanced protein-protein interactions and cross-linking due to thiol-disulfide exchange reaction or oxidation that can lead to protein polymerization (Kinsella, 1982). Therefore, the higher amount of WPI as

primary layer in our study (Lim and Roos, 2015) can form thicker layer around the oil droplets capable of preventing coalescence and providing better protection to the encapsulated biaoctives.

Protein is commonly used as the primary layer in the formation of LBL emulsions. Proteins such as sodium caseinate, WPI, β-lactoglobulin, pea protein isolate, and soy protein isolate have been used as the primary layer in other studies (Moreau *et al.*, 2003; Güzey and McClements, 2006a; Jourdain *et al.*, 2009; Gharsallaoui *et al.*, 2012; Zhou and Roos, 2013). The possibility to regulate the magnitude and charge of protein to be positive at pH below and negative at pH above the isoelectric point (pI) of the protein (Guzey and McClements, 2006b) is underpinning the common use of protein as the primary layer. Our study in the formation of LBL emulsion utilized WPI as the primary layer at pH 3.5. The main proteins in WPI, namely β-lactoglobulin and α-lactalbumin possessed pI of 5.2 and 4.1, respectively (Bryant and McClements, 1998; Weinbreck *et al.*, 2003). Therefore, the SL emulsions in our study were expected to be positively charged and this was confirmed by the results from ζ-potential measurements. There was also a difference in the magnitude of the ζ-potential based on the amount of the primary layer where the ζ-potential was higher in systems with higher amount of WPI. This suggested that WPI was capable to form a multilayer at the oil-water interface at high concentrations and was in agreement with the study by Cornacchia *et al.* (2011a). The amount of total hydrophilic solids was found to have minor effects on the ζ-potential as well as the ζ-average diameter of the SL emulsion droplets with similar amount of primary layer. The ζ-potential was generally related to the protein primary layer present while the ζ-average diameter was affected by the size of the oil droplets. However, the amount of the total hydrophilic solids was found to significantly affect the apparent viscosity of the aqueous phase. The apparent viscosity of the aqueous phase with lower amount of total hydrophilic solids (28.57%, mass) was found to be 2.33 ± 0.1 mPa.s but was 59.90 ± 0.3 mPa.s in aqueous phase with higher amount of total hydrophilic solids (57.14%, mass).

7.2.2 Secondary Layer and Emulsion Stability

Gum Arabic was used as the secondary layer in LBL interfacial structuring in our studies. The pKa of gum Arabic is known to be approximately 2.2 (Weinbreck *et al.*, 2004). Therefore, gum Arabic was negatively charged at pH 3.5 and it was electrostatically attracted to the positively charged WPI at the oil-water interface. The ability of the oppositely charged primary layer and secondary layer to be electrostatically attached to form the LBL interfacial structure has been widely reported (Moreau *et al.,* 2003; Ogawa *et al.,* 2003a; Gharsallaoui *et al.,* 2012; Zhou and Roos, 2013; Jiménez-Martín *et al.,* 2015). Alginate, pectin, ι-carrageenan, dextran sulfate and chitosan have been used as the secondary layer in the formation of the LBL interfacial structure (Gu *et al.,* 2005; Klinkesorn *et al.,* 2005; Harnsilawat *et al.,* 2006; Jourdain *et al.,* 2009; Aberkane *et al.,* 2014). The use of gum Arabic solution caused charge reversal in the ζ-potential from positively charged to be negatively charged as observed in our study (Lim and Roos, 2015). This was due to the negatively charged gum Arabic attaching to the WPI primary layer at the oil-water interface to provide the overall ζ-potential. Charge reversal is typical of the formation of LBL interfacial structure and is well-recognized (Surh *et al.,* 2006; Jones *et al.,* 2010; Lim *et al.,* 2014). Guzey and McClements (2006b) also stated that the net charge of a system is dictated by the charge of the most outer layer. The magnitude of the negative charge was found to be higher in emulsions with lower amount of WPI as ζ-potential was the sum of all the molecules with charges that were present and therefore the lower amount of WPI primary layer present gave lower positive charge. The amount of the total hydrophilic solids present in the LBL emulsions was found to have minor effects on the ζ-potential as carbohydrate does not contribute to the ζ-potential. Nonetheless, it was found that LBL systems with similar amount of primary layer but higher total hydrophilic solids had significantly higher apparent viscosity. The significantly higher apparent viscosity contributed towards the higher stability of the LBL emulsions with higher total hydrophilic solids towards gravitational separation as was observed using LUMiSizer accelerated destabilization test. This was in agreement with Stoke's Law where the particles separation velocity was inversely proportional to the viscosity of the system. The

increase in viscosity of the continuous phase minimized particle movement that decreased creaming rate (Chanamai and McClements, 2001; Gu *et al.,* 2005).

Ratio between the primary layer and secondary layer plays an important role in the stability of the LBL system as the addition of polyelectrolyte as secondary layer is known to cause depletion or bridging flocculation. It was reported that depletion flocculation or bridging flocculation occurred in LBL emulsions in studies by Ogawa *et al.* (2003a), Guzey *et al.* (2004), Mun *et al.* (2005), and Benjamin *et al.* (2012). Our study also found that LBL emulsions underwent depletion flocculation at pH 3.5 which contributed to the larger ζ-average diameter observed in all systems at pH 3.5. However, deflocculation was achieved in systems with higher amount of primary layer by increasing the pH of the systems with individual oil particles still intact as confirmed by the ζ-average diameter as the sufficient amount of primary layer prevented coalescence. The ζ-average diameter at pH 6.5 was comparable to those of SL emulsions at pH 3.5 which showed that the individual oil droplets were deflocculated. At pH above the pI of the primary layer, the primary layer and secondary layer will have similar electrostatic charge and this result in electrostatic repulsion between the primary and secondary layers (Guzey *et al.,* 2004; Gu *et al.,* 2005). As a consequence, the gum Arabic secondary layer may detach from the WPI primary layer to give SL emulsion. The ability of the secondary layer in LBL emulsion to detach with the change in pH allows the design of LBL interfacial structure as delivery system that enables controlled release of the encapsulated bioactives utilizing pH as the trigger (Gudipate *et al.,* 2010; Benjamin *et al.,* 2012). Nonetheless, systems with lower amount of primary layer were not deflocculated into individual oil droplets as coalescence occurred in these systems as observed in the ζ-average diameter. Coalescence occurring in LBL systems with lower amount of primary layer showed that the higher stability of LBL systems towards environmental stresses cannot be achieved if the SL system itself was not stable. Coalescence could also be observed under optical microscope where the presence of large bright particles indicated coalesced oil droplets (Lim and Roos, 2015). The close proximity of the oil droplets in flocculated emulsions promoted further flocculation (Surh *et al.,* 2006). Coalescence leads to systems with lower stability

against gravitational separation. Studies by Ogawa *et al.* (2004) and Guzey and McClements (2006b) found that systems with smaller oil droplets size had higher stability towards creaming. The lower stability of emulsion against gravitational separation was reflected in our study by the faster gravitational separation observed in LUMiSizer accelerated destabilization test in emulsions with lower amount of primary layer as the result of coalescence. This was in agreement with Stoke's Law where the particle size was directly proportional to the velocity of particles separation. Therefore, emulsion stability can be improved by increasing viscosity of the continuous phase and by having smaller particle size (Mert, 2012).

7.3 Dehydration of Emulsion and Powder Properties

The lipid phase of an O/W emulsion can be used for the encapsulation of oil–soluble bioactives. Our studies utilized lutein and all-trans-β-carotene as the oil–soluble bioactives for microencapsulation. The SL and LBL emulsions were mixed with various wall materials or wall material mixtures for dehydration. Our studies utilized trehalose, trehalose-maltodextrin (M100) (1:1), trehalose-maltodextrin (M250) (1:1), and trehalose-WPI (21:8) mixtures as wall materials in dehydration. We produced emulsions with high total solids of 50% (mass) when trehalose-maltodextrin (M100) and trehalose-maltodextrin (M250) were used and 45% total solids (mass) when trehalose-WPI was used for spray drying. Most studies of spray dried LBL systems were of more diluted systems with 40% or lesser total solids (Shaw *et al.,* 2007; Gharsallaoui *et al.,* 2010; Carvalho *et al.,* 2014; Jiménez-Martín *et al.,* 2015; Kwamman and Klinkesorn, 2015). We were also able to freeze-dry SL and LBL emulsions with trehalose and trehalose-maltodextrin (M100) mixtures as glass formers with encapsulated oil-soluble carotenoids.

7.3.1 Critical Characteristics of Powders

Free flowing SL and LBL powders with trehalose-maltodextrin (M100), trehalose-maltodextrin (M250), and trehalose-WPI mixtures as wall materials were obtained from the spray dryer. The SL and LBL powders obtained were visually similar,

spherical in shape under optical microscope observation, and were ‘easy flowing’ as determined by the powder flow tester. Flowability of powders was affected by the stress applied and it was critical during powder processing and handling (Teunou *et al.,* 1999). ‘Easy flowing’ powders obtained in our study would therefore enable easier post-drying handling of the powders. The properties of the powders such as surface oil, water content, particle density, occluded air, interstitial air, bulk density, tapped density, and particle size (d [4, 3]) were also determined. Generally, there was a significant difference in the critical powder characteristics between SL and LBL powders: namely the surface oil, particle density, occluded air, and interstitial air which may have an impact on the stability of the encapsulated carotenoids. Powders with less surface oil, occluded air, interstitial air, and higher particle density were expected to give better stability to the encapsulated carotenoids. Surface oil in LBL powders was found to be significantly lower than in SL powders. The application of LBL interfacial layer improved the stability of the oil droplets towards disruptions (Güzey and McClements, 2006a) during processing such as in the atomization of LBL emulsion in spray drying that reduced surface oil and improved microencapsulation efficiency. The lower amount of surface oil in LBL system reduces the amount of unprotected carotenoids containing lipid phase that can reduce carotenoids degradation.

The LBL powders also had significantly higher particle density, and significantly lower occluded air and interstitial air. The presence of gum Arabic at the interfaces of oil droplets may increase the overall particle density. Air vacuoles indicating the presence of occluded air can be observed under optical microscope reflected by the presence of dark spots in both SL and LBL powders. The higher amount of trapped oxygen showed by the higher amount of occluded air can result in the higher carotenoids degradation rate. On the other hand, the higher particle density can delay oxygen and metal ions migration towards the carotenoids containing oil phase that may reduce degradation of carotenoids. The presence of gum Arabic as secondary layer was found to increase the viscosity of LBL emulsion. The higher viscosity of the LBL system influenced particle formation during atomization as the increased viscosity could increase the resistance of the LBL emulsion to flow out of the

atomizer resulting in larger atomized droplets. Accordingly, the higher viscosity of LBL emulsions resulted in significantly larger d [4, 3] of the LBL powder particles. The use of carbohydrate-carbohydrate or carbohydrate-protein mixtures as wall materials also affected the critical properties of the powders. The particle density was significantly lower and occluded air significantly higher in powders produced using carbohydrate-protein mixtures as wall materials. The utilization of the more porous proteins as wall materials contributed to the differences in particle density and occluded air that may reduce the stability of the encapsulated carotenoids in system with carbohydrate-protein mixtures as wall materials. The wall materials used also affected particle size of the powders and carbohydrate-carbohydrate powder particles were significantly larger d [4, 3].

7.3.2 Water Activity and Colour

All of the powders obtained from spray drying were of between 0.14-0.25 a_w and the a_w of SL and LBL powders was generally similar initially. Corresponding powders produced in the industry have a_w of approximately 0.2 a_w (Adhikari *et al.,* 2009). Free flowing powders with a_w similar to those obtained from commercial spray drying were able to be produced from high total solids LBL emulsions in our studies. Overall shelf-life of the powders having a_w of approximately 0.2 a_w can also be improved. The powders will have better stability towards lipid oxidation and non-enzymatic browning as well as should not support mold, yeast, and bacteria growth as described by the stability map of food (Labuza, 1971; Labuza and Dungan, 1971). The a_w of both SL and LBL systems before and upon long term storage of up to 99 days were determined. It was found that a_w of the spray dried materials generally decreased upon storage although the a_w decreases were generally small and less than 0.05 a_w. However, there was significant decrease in the a_w of the freeze-dried material with trehalose as glass former upon storage at 37°C and 45°C. No leakages were observed in the vacuum bags used to store the powders indicating that the powders were isolated from the environment throughout storage that could affect the a_w. Therefore, the changes in a_w of the freeze-dried system with trehalose as glass former can be the result of physicochemical changes. Whey protein at 35-40°C can

undergo hydration-related conformational changes which may reduce a_w (Potes *et al.,* 2014). Thus, the freeze-dried system with trehalose as glass former and WPI as primary layer upon storage at 37°C and 45°C can undergo hydration-related conformational changes. Besides, the decrease in a_w can be a result of lipid oxidation and hydration of unsaturated fatty acids at elevated temperatures (Potes *et al.,* 2014) of the sunflower oil used as the dispersed phase.

Colour values (L*, a*, and b*) changed in both SL and LBL powders upon long term storage of up to 99 days. The initial (day 0) L*, a*, and b* values of the powders with different wall material mixtures were fairly similar. SL and LBL gave similar L*, a*, and b* values as well initially with no significant difference between the powders. There were no significant difference in the change of L* values in all systems regardless of the interfacial structure or wall materials upon storage except at the highest storage temperature (65°C) where the L* values reduced significantly. On the other hand, a* values found for the highest temperature generally increased upon storage while a* values of materials stored at the other two lower temperatures generally decreased. Reduction in a* values upon storage is commonly reported for carotenoids as the consequence of carotenoids degradation (Desobry *et al.,* 1997; Elizalde *et al.,* 2002; Qian *et al.,* 2012; Mahfoudhi and Hamdi, 2014). L* and a* values of both SL and LBL spray dried materials stored at the highest storage temperature showed a different trend in the colour change upon storage. The presence of reducing sugars (maltodextrin; residual lactose in WPI), amino groups (WPI), and heat (65°C) was capable of causing non-enzymatic browning (Maillard reaction) in these systems that gave the different trend in colour change. Free radicals and reactive oxygen species produced by lipid oxidation too have the ability to attack the protein amino groups which results in browning (Venolia and Tappel, 1958; Zirlin and Karel, 1969; Potes *et al.,* 2014). Browning in materials with oil and protein occurred due to the formation of lipid peroxides, followed by brown pigment precursors formation as the result of interaction between peroxides or products of carbonylic peroxide decomposition with protein's active groups, and finally brown pigments formation from the lightly-coloured or colourless precursors (Pokorný *et al.,* 1974). The b* values generally decreased with storage except for powders stored

at the lowest storage temperatures (25°C or 35°C). However, many authors have claimed that b* values are poor indicators as yellowness to blueness are not dominant in carotenoids containing systems (Desobry *et al.,* 1997; Elizalde *et al.,* 2002; Prado *et al.,* 2006; Spada *et al.,* 2012).

7.4 Loss Kinetics of Carotenoids

Bioactives such as carotenoids can undergo degradation during storage especially when exposed to elevated temperatures. The degradation of both lutein and all-trans-β-carotene in all systems in our studies obtained either by spray drying or freeze-drying increased with increasing storage temperature and similar findings have been widely reported (Desobry *et al.,* 1997; Dhuique-Mayer *et al.,* 2007; Koca *et al.,* 2007; Qian *et al.,* 2012; Spada *et al.,* 2012; Caliskan *et al.,* 2015). The degradation of carotenoids in all powders as well as freeze-dried systems in our studies was also found to be a first-order reaction as plots of ln A/A_o against storage time (days) produced straight lines (Wagner and Warthesen, 1995; Henry *et al.,* 1998; Elizalde *et al.,* 2002; Hidalgo and Brandolini, 2008; Achir *et al.,* 2010; Ramoneda *et al.,* 2011; Harnkarnsujarit *et al.,* 2012b; Lim *et al.,* 2014; Lim and Roos, 2016; Lim *et al.,* 2016).

7.4.1 SL *vs.* LBL Emulsion Powders

The degradation of lutein and all-trans-β-carotene was found to be less rapid in all LBL systems than in SL systems based on the rate constants. The less rapid degradation rate of carotenoids in LBL systems was observed in both freeze-dried and spray dried materials and showed that the application of LBL interfacial layer was able to reduce the degradation rate of the encapsulated carotenoids. Properties of the LBL interfacial layer reduced reaction rate as the thicker interfacial layer provided a better physical barrier to give an improved protection to the encapsulated lipid phase (Klinkesorn *et al.,* 2005; Shaw *et al.,* 2007). The thicker interfacial layer at the oil-water interface was assumed to be more efficient in minimizing diffusion of transition metal ions towards the oil phase (Aberkane *et al.,* 2014) that can cause

the degradation of the encapsulated carotenoids. Studies also found that lipid oxidation of the encapsulated lipids were reduced in spray dried systems with LBL interfacial layer (Shaw *et al.*, 2007; Aberkane *et al.*, 2014; Jiménez-Martín *et al.*, 2015) and freeze-dried (Klinkesorn *et al.*, 2005). The lower lipid oxidation rate can also reduce the degradation of carotenoids in LBL systems. Surface oil was found to be lower in LBL powders as the thicker LBL interfacial layer could prevent oil droplet disruption during atomization. The lower surface oil also contributed to the less rapid carotenoids degradation rate as less carotenoids containing oil phase was unprotected and exposed to the surrounding atmosphere. There was also significant difference in the occluded air content between SL and LBL systems. Occluded air was significantly higher in SL powders indicating higher amount of oxygen present that could contribute to the higher degradation rate of carotenoids. The total retention of both lutein and β-carotene was also higher in all LBL systems upon long term storage of spray dried (78-99 days) and freeze-dried emulsions (70 days). Significant difference in the total retention of carotenoids was generally observed upon storage at the highest storage temperature (65°C) in the spray dried systems.

There were differences between the degradation of carotenoids in humidified (0.33 a_w) spray dried and freeze-dried, and non-humidified (as is, 0.25 a_w for spray dried, 0 a_w for freeze-dried) materials. It was noted that carotenoids degradation was less rapid in the humidified system. Lipid oxidation was reduced in powders with a_w of approximately 0.3 a_w but oxidation increased below and above 0.3 a_w in agreement with the stability map of foods (Labuza, 1971; Labuza *et al.*, 1972). Labuza and Dungan (1971) proposed that the protective effect of water was due to water interaction with metal catalyst reducing their effectiveness as the result of transformation of the coordination sphere as well as bonding of water hydrogen with hydrogen peroxides to prevent initiation of decomposition reactions. Reduction in lipid oxidation of food systems was observed around a_w of the water monolayer, usually between 0.2-0.4 a_w in studies of Karel *et al.* (1967) and Labuza (1968). Velasco *et al.* (2003) attributed the role of a_w to the reduction of transition metals' prooxidant activity, higher free radicals and singlet oxygen quenching, as well as the delay in hydroperoxide decomposition. The reduction in lipid oxidation can translate

to the reduced degradation of carotenoids in the oil phase of the humidified (0.33 a_w) spray dried and freeze-dried systems that gives the lower degradation rate. Besides, the initial rapid degradation of carotenoids may have already occurred during the humidification process to obtain systems with a_w of 0.33 a_w. The initial degradation of carotenoids can result in the less rapid overall degradation rate of carotenoids in the humidified systems. The other main β-carotene isomers observed in the HPLC chromatograms were of 15-cis-β-carotene and 13-cis-β-carotene and similar peaks were observed in both SL and LBL systems. Heating reduced the amount of all-trans-β-carotene but simultaneously resulted in the increase of β-carotene isomers (Chandler and Schwartz, 1988). Carotenoids radicals formed namely the cation radical (trans-carotenoids$^{\bullet+}$) and dication species (trans-carotenoids^{2+}) from all-trans-carotenoids isomerized to different cis species in isomerization (Gao *et al.*, 1996). It was found that the relative amount (%) of 15-cis-β-carotene and 13-cis-β-carotene formed from the isomerization of all-trans-β-carotene was slightly higher in LBL systems at all storage temperatures suggesting that the application of LBL interfacial structure too prevented the total degradation of β-carotene.

7.4.2 Freeze-dried *vs.* Spray Dried Emulsions

It was found that the degradation of lutein and all-trans-β-carotene was more rapid in freeze-dried emulsions than in spray dried emulsions. The ln *k* value of the humidified (0.33 a_w) freeze-dried emulsions with trehalose-maltodextrin (M100) at the highest storage temperature (65°C) was about -3 day^{-1} while the ln *k* value of spray dried emulsion with similar a_w, glass formers, and storage temperature were much lower of about -6 day^{-1}. Our study using freeze-dried emulsion at 0.33 a_w with trehalose as glass former stored at 45°C also showed high ln *k* value of approximately -3.5 day^{-1}. Freeze drying gave a more porous and less dense structure than spray drying which may allow rapid diffusion of metal ions or oxygen towards the encapsulated carotenoids that can cause faster degradation of the carotenoids. Degradation of β-carotene was also found to be more rapid in freeze-dried system than in spray dried system in two separate studies by the same authors (Mahfoudhi and Hamdi, 2014; Mahfoudhi and Hamdi, 2015). It was noted that there was a two-

step first order carotenoids degradation kinetics showing a rapid initial degradation followed by a second less rapid degradation in all spray dried emulsions that were studied 'as is' from the spray dryer. Studies by Desobry *et al.* (1997) and Desobry *et al.* (1999) also made similar observation on the degradation of β-carotene in spray dried materials. This was associated to the vacuoles containing oxygen developed within the powder due to thermal expansion of the trapped air bubbles during the formation of the glassy matrix (Aguilera, 1990; Tewa-Tagne *et al.,* 2007). The trapped oxygen results in the degradation of the encapsulated carotenoids as oxidation is the leading cause of carotenoids degradation (Qiu *et al.,* 2009). Besides, the presence of surface oil can also contribute to the rapid initial degradation as these carotenoids containing oil phase are not protected within the matrix. Nonetheless, two-step first order carotenoids degradation kinetics was not observed in the freeze-dried emulsions. No oxygen was entrapped within the matrix of freeze-dried materials as freeze drying was done in vacuum. The absence of air in freeze-drying explains the absence of the rapid initial degradation. The rapid initial degradation of carotenoids was also not observed in the humidified spray dried emulsions as the rapid initial degradation could have already occurred during the humidification process.

7.4.3 Carbohydrate-Carbohydrate *vs.* Carbohydrate-Protein Wall Materials

The degradation rate of carotenoids in spray dried emulsions with carbohydrate-carbohydrate (trehalose-maltodextrin (M100) and trehalose-maltodextrin (M250)) as wall materials was found to be less rapid than in emulsions with carbohydrate-protein (trehalose-WPI) as wall material. The less rapid degradation rate of carotenoids with carbohydrate-carbohydrate wall materials was especially true in the rapid initial degradation and at the highest storage temperature. The significantly higher occluded air in the trehalose-WPI system (20.67±1.29 cm^3/100g in SL and 18.51±2.74 cm^3/100g in LBL) than in trehalose-maltodextrin (M100) (7.74±0.69 cm^3/100g in SL and 4.06±0.36 cm^3/100g in LBL) and trehalose-maltodextrin (M250) (7.73±2.06 cm^3/100g in SL and 3.08±0.65 cm^3/100g in LBL) contributed towards the higher degradation rate of carotenoids in carbohydrate-protein emulsions. The

use of carbohydrate and protein as wall materials can result in the discontinuity of the wall materials due to the presence of the surface-active WPI. Study by Fäldt and Bergenståhl (1994) showed that the surface-active protein predominated on the surface of carbohydrate–protein particles. The surface-active WPI tends to migrate to the surface (air-water interface) of the droplets during spray drying due to its high surface activity to give a protein rich layer (Adhikari *et al.,* 2009; Wang *et al.,* 2013). The presence of a thin layer of WPI at the hydrophilic-hydrophobic interfaces namely at the surface of the powder particle and around the air vacuoles in spray dried emulsion with trehalose-WPI mixtures as wall materials was observed in Figure 7.1 using Raman-Focused ion beam (FIB)-Scanning electron microscope (SEM) analysis. Therefore, the carotenoids containing oil droplets were encapsulated within the glass former (trehalose) and non-glass former (WPI) in carbohydrate-protein system. There was a lesser amount of the denser carbohydrate glass formers to encapsulate the carotenoids in the carbohydrate-protein system that could increase the degradation rate of carotenoids. More porous powders as observed in the higher amount of occluded air were obtained in carbohydrate-protein system due to the presence of protein as wall material compared to carbohydrate-carbohydrate systems. The higher amount of occluded air represents a system with higher amount of trapped oxygen that can cause increased oxidation of the carotenoids that result in the faster degradation rate.

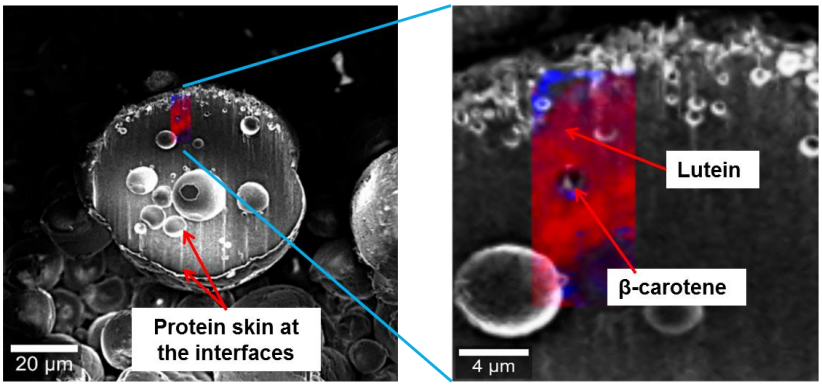

Figure 7.1. Raman-Focused ion beam (FIB)-Scanning electron microscope (SEM) images of a cut powder particle with trehalose-whey protein isolate (WPI) mixtures as wall materials showing the presence of a thin protein skin at the interfaces of the powder particle and air vacuoles as well as the differences in the location of lutein and all-trans-β-carotene within the particle structure. (Modified from Lim *et al.*, 2016)

The wall materials used also affected particle density of the powders obtained as particle density was significantly higher in powders with carbohydrate-carbohydrate as wall materials than with carbohydrate-protein as wall materials. Increasing the amount of lactose (carbohydrate component) in WPI-lactose spray dried systems was found to increase density and reduce porosity of powders (Moreau and Rosenberg, 1999). The differences of wall materials used also resulted in differences in carotenoids retention upon long term storage. Retention of lutein and all-trans-β-carotene was higher in spray dried emulsions with carbohydrate-carbohydrate wall materials than carbohydrate-protein wall materials at all storage temperatures. The higher amount of the denser carbohydrate glass former in carbohydrate-carbohydrate systems was also reflected by the higher particle density which provided improved encapsulation of the carotenoids. The higher density of the carbohydrate-carbohydrate wall materials can delay migration of oxygen and metal ions towards the encapsulated carotenoids that reduces degradation rate of carotenoids. It was noted that there was a non-Arrhenian increase in the degradation of carotenoids with increasing storage temperature in the initial rapid degradation kinetics. The carbohydrate glass formers used as wall materials can undergo dynamic process of structural collapse governed by the rate of T - T_g at storage temperatures above the

T_g (Levi and Karel, 1995). Storage of materials above T_g will decrease viscosity and increase molecular mobility within the solids (Roos and Karel, 1991). Structural collapse results in the harder and denser glass that can improve protection of the encapsulated carotenoids. The reduced degradation of encapsulated bioactives in collapsed systems was also observed by Selim *et al.* (2000) and Harnkarnsujarit and Charoenrein (2011). Prado *et al.* (2006) and Harnkarnsujarit *et al.* (2012b) stated that the improved stability of encapsulated bioactives in collapsed systems was due to the reduction in oxygen permeability across the collapsed matrix with significantly reduced micropores. Besides, the presence of reducing sugar (maltodextrin, residual lactose in WPI), protein amino groups (WPI), and heat in the systems can result in Maillard reaction. Maillard reaction generates melanoidins that possesses antioxidant capacity (Wang *et al.*, 2011). Melanoidins generated thus can give protection to the carotenoids present against attack from oxygen or metal ion present that reduced degradation of the carotenoids. Storage of the powders at high temperature can decrease solubility of oxygen in the water content of the system at temperatures up to 100°C (Hildebrand, 1952; Wilhelm *et al.*, 1977). The tiny quantity of water of the powders stored at higher storage temperature will therefore have smaller amount of oxygen. The lesser amount of oxygen can reduce oxygen diffusion towards the carotenoids containing oil phase that effectively reduced carotenoids degradation.

7.4.4 Lutein *vs.* All-trans-β-carotene

Degradation of lutein was predominantly more rapid than degradation of all-trans-β-carotene in the rapid initial degradation step of non-humidified spray dried emulsions, humidified spray dried emulsions, and in the non-humidified and humidified freeze-dried emulsions with trehalose-maltodextrin (M100) as wall materials. Lutein, an oxygenated carotenoid is classified as xanthophylls. The presence of two hydroxyl groups results in increased hydrophilicity and polarity of lutein (Farombi & Britton, 1999; Updike & Schwartz, 2003). Therefore, lutein tends to assemble at the interface of the oil droplets while all-trans-β-carotene is likely to remain in the bulk oil. Lutein at the interface of the oil droplets can act as antioxidant to the diffusing oxygen trapped within the wall materials resulting in the more rapid

degradation of lutein. Our study (Lim *et al.*, 2016) using the combination of Raman-FIB-SEM microscopy techniques confirmed the presence of lutein in large quantities at the interface that may have acted as antioxidant while all-trans-β-carotene was dissolved within the oil and only found in small quantities at the surface of a cut spray dried particle (Figure 7.1). The more rapid degradation of lutein was not observed in the second slower degradation kinetics in the non-humidified spray dried emulsion as well as in the degradation kinetics of freeze-dried system with trehalose as wall material. The degradation of lutein at the interface may have already occurred thoroughly during the rapid initial degradation in the non-humidified spray dried systems and during humidification process in the freeze-dried system with trehalose as wall material. However, retention of lutein was higher than retention of all-trans-β-carotene upon long term storage. This was observed in all non-humidified spray dried systems as well as in freeze-dried system with trehalose as wall material. The higher retention of lutein was attributed to the isomerization of all-trans-β-carotene to 15-cis-β-carotene and 13-cis-β-carotene as was observed in the HPLC chromatograms. All-trans-β-carotene was found to readily convert into cis-isomers during thermal treatment rather than undergoing decomposition that result in total degradation of the carotenoids (Chandler and Schwartz, 1988). The relative amount (%) of 15-cis-β-carotene and 13-cis-β-carotene generally fluctuated with storage but was found to be somewhat higher in the trehalose-maltodextrin (M250) LBL powder at all storage temperatures implying that reduced total degradation of β-carotene resulted from the application of LBL interfacial structure. Our study (Lim *et al.*, 2014) on freeze-dried system with trehalose as wall material also found that the retention (%) of total β-carotene (all-trans-β-carotene, 15-cis-β-carotene, and 13-cis-β-carotene) was higher in LBL system at all storage temperatures upon long term storage of 70 days.

7.5 Overall Conclusions and Application

Primary layer of O/W emulsion plays a crucial role in the stability of the emulsion as insufficient primary layer lead to coalescence that can lead to oil separation. The higher stability of LBL system towards environmental stresses cannot be achieved if

coalescence occurs as the bioactives containing lipid phase may separate out and will not be encapsulated. Besides, coalescence will increase the difficulty to spray dry such systems. The formation of LBL interfacial structure was confirmed by the charge reversal observed in the ζ-potential. Total solids content was found to have small effects on the ζ-potential and ζ-average diameter of systems with similar amount of primary layer. Flocculation occurred in LBL systems although deflocculation of LBL system with sufficient primary layer was possible with individual particles still intact. The ability of the secondary layer in LBL system to detach at pH > pI of the protein primary layer provides a great opportunity to use the LBL system as a delivery system that utilizes pH as a trigger for controlled release. LBL system will therefore be stable during storage and in the acidic condition of the stomach but the secondary layer of the LBL system can be detached in the alkaline condition of small intestine. The detachment of the secondary layer gave SL emulsion with lower stability towards environmental stresses that increased the release of the bioactives containing lipids from the emulsion for absorption. The possibility to spray dry high total solids LBL emulsions (45-50% mass) with wall material mixtures of trehalose-maltodextrin (M100), trehalose-maltodextrin (M250), and trehalose-WPI giving free flowing powders would be feasible to the food industry as it greatly reduces production costs. There were differences in the critical powder characteristics such as occluded air, particle density, and surface oil between SL and LBL powders as well as between powders with carbohydrate-carbohydrate and carbohydrate-protein mixtures as wall materials that may have an impact on the stability of the encapsulated carotenoids. The higher particle density and lower occluded air in LBL system and in systems with carbohydrate-carbohydrate mixtures as wall materials can reduce the degradation of the encapsulated carotenoids.

The a_w of the powders from spray drying were similar to those from commercial spray drying indicating the success in spray drying of the high total solids SL and LBL systems. Therefore, spray drying of high total solids LBL emulsion can be applied in the industry to provide the encapsulated oil-soluble bioactives with a better shelf-life. Small decrease in the a_w of no more than 0.05 a_w was observed in the powders upon storage. Non-enzymatic browning occurring in systems stored at

the highest storage temperature affected the L* and a* values showing different trends from powders stored at lower temperatures. The application of LBL interfacial structure was found to provide better protection to the encapsulated carotenoids upon storage compared to the conventional SL system based on the rate constants regardless of the dehydration method (spray drying or freeze-drying). Retention of carotenoids was also found to be higher in LBL systems upon long term storage with significance generally observed at the highest storage temperature. The higher retention of carotenoids upon long term storage allows the use of LBL systems to encapsulate various types of oil-soluble bioactives in the food and pharmaceutical industries for better control of their stability as encapsulated bioactives. Spray drying was found to be more effective in encapsulating carotenoids upon storage than freeze-drying based on the degradation kinetics as more porous structure was formed in freeze-drying. Therefore, the advantages and disadvantages of each dehydration method must be considered in the encapsulation of bioactives. Spray drying was capable of providing better protection to the encapsulated bioactives for products that will be exposed to heat during transportation and storage. Carotenoids degradation rate in spray dried emulsions having carbohydrate-carbohydrate mixture as wall materials was found to be less rapid than in carbohydrate-protein mixture. The slower degradation rate of systems with carbohydrate-carbohydrate mixture as wall materials was also reflected in the higher retention of carotenoids in spray dried emulsions with carbohydrate-carbohydrate mixture as wall materials upon long term storage. The higher amount of carbohydrate glass formers can provide better encapsulation of the bioactives suggesting that the use of carbohydrates as wall materials will be beneficial towards the protection of the encapsulated bioactives. Therefore, the use of LBL interfacial structure in combination with carbohydrate wall material can give improved shelf-life of the encapsulated oil-soluble bioactives. Overall data obtained from our studies are vastly applicable in the food and pharmaceutical industries to improve the stability of the oil-soluble bioactives.

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Appendix