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Processing and Stability of Infant Formula-Based Emulsions as Affected by Emulsifier Type

Thesis presented by

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Declaration

I hereby declare that the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

_____ Date: _____

Kamil P. Drapala

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Summary

Infant formulae (IF), which are emulsion-based nutritional products available commercially in either liquid or powder format, are prone to destabilisation during processing and on storage due to their inherent thermodynamic instability. Ongoing efforts to further the humanisation of IF products drive modifications to the composition and structural organisation of oil globule interfaces in IF products, aimed at matching the biofunctionality of such IF products to that of the fat globule membranes in human milk. Similarly, changes to the protein component of IF (e.g., source, profile, peptide chain length) to cater for specific nutritional requirements, or the presence of non-protein emulsifiers (e.g., low molecular weight, L_{Mw}, polar lipids) to improve stability of these products, significantly affect the composition and structure of oil globule interfaces in IF products. This thesis represents new and innovative research on the properties and performance of selected innate and added emulsifiers in IF products (i.e., whey protein isolate, WPI; whey protein hydrolysate, WPH; phospholipids and citric acid esters of glycerides) and more novel emulsifiers formed by Maillard-induced conjugation of WPH with maltodextrin (MD). This research focused predominantly on the surface activity of emulsifiers, their ability to form emulsions and to subsequently stabilise them against shear-, heat- and storage-induced changes. Conditions used to test the stabilisation properties included refrigerated storage (14 d at 4°C), thermal processing (75-100°C × 15 min), accelerated shelf-life testing and spray drying. Studies on the stability of model IF emulsions (protein = 15.5 g L^{-1}, fat = 35.0 g L^{-1}, carbohydrate = 70.0 g L^{-1}; pH 6.8) to thermal processing (100°C × 15 min) and to accelerated shelf-life testing showed exceptionally high stability of emulsions prepared using the novel WPH-MD conjugate. The stability of the WPH-MD-stabilised emulsions conjugate was due to the strong steric hindrance effect provided by the conjugate, which prevented interactions between adjacent oil globules. Conversely, extensive aggregation of oil globules on heating (~72°C), observed for WPH-based emulsions was induced by the high levels of exposed reactive sites (e.g., free -SH) resulting from hydrolysis of the whey protein molecules in the parent WPI ingredient. Inclusion of L_{Mw} emulsifiers (0-9 g L^{-1}) in the WPH-based emulsions improved

their heat stability during thermal processing (95°C × 15 min); however, it resulted in poor shelf life stability due to the competitive displacement of proteins/peptides from the oil-water interface by L_{Mw} emulsifiers. Study of the spray drying properties of emulsion-based IF liquid concentrates (32% TS) demonstrated superior functionality of the WPH-MD conjugate, as evidenced by the greatest emulsion quality on rehydration of the IF powders stabilised by the conjugate, compared to the powders stabilised by the other emulsifier systems (WPI, WPH and WPH+L_{Mw}). The findings presented in this thesis constitute a significant advancement in research on mechanisms of stabilisation of IF emulsions provided by a potent steric barrier to oil globules using glycated proteins/peptides; this work adds to the rapidly expanding research on interfacial composition and structural organisation of oil globules in IF products and opens up new possibilities for designing novel emulsion-based nutritional products.

List of publications and conference contributions

Peer-reviewed scientific articles:

Drapala, K.P., Auty, M.A.E., Mulvihill, D.M., and O'Mahony, J.A. (2015). Influence of lecithin on the processing stability of model whey protein hydrolysate-based infant formula emulsions. *International Journal of Dairy Technology*, 68, 322–333.

Drapala, K.P., Auty, M.A.E., Mulvihill, D.M., and O'Mahony, J.A. (2016). Improving thermal stability of hydrolysed whey protein-based infant formula emulsions by protein-carbohydrate conjugation. *Food Research International*, 88, 42–51.

Drapala, K.P., Auty, M.A.E., Mulvihill, D.M., and O'Mahony, J.A. (2016). Performance of whey protein hydrolysate-maltodextrin conjugates as emulsifiers in model infant formula emulsions. *International Dairy Journal*, 62, 76–83.

Drapala, K.P., Auty, M.A.E., Mulvihill, D.M., and O'Mahony, J.A. (2017). Influence of emulsifier type on the spray-drying properties of model infant formula emulsions. *Food Hydrocolloids*, 69, 56–66.

O'Mahony, J.A., Drapala, K.P., Mulcahy, E.M., and Mulvihill, D.M. (2017). Controlled glycation of milk proteins and peptides: Functional properties. *International Dairy Journal*, 67, 16–34.

Oral conference contributions:

Drapala, K.P., Mulvihill, D.M., and O'Mahony, J.A. (2014). Improving heat stability of model hydrolysed infant formula emulsions by protein-carbohydrate conjugation. Presented at 43rd Annual Food Research Conference, Dublin, Ireland (10-11 December 2014).

Drapala, K.P., Mulvihill, D.M., and O'Mahony, J.A. (2015). Engineering of infant formula emulsions to enhance protein thermal stability through Maillard conjugation. Presented at ADSA® –ASAS Joint Annual Meeting 2015 as a part of the *Dairy Foods Graduate Student Oral Competition*, Orlando, FL, USA (12-16 July 2015).

Drapala, K.P., Mulvihill, D.M., and O'Mahony, J.A. (2015). Engineering of infant formula emulsions to enhance protein thermal stability through Maillard conjugation. Presented at Delivery of Functionality 6th International Symposium, Paris, France (14-17 July 2015).

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Drapala, K.P., Auty, M.A.E., Mulvihill, D.M., and O'Mahony, J.A. (2015). Performance of whey protein hydrolysate-maltodextrin conjugates as emulsifiers in model infant formula emulsions. Presented at IDF Parallel Symposia, Dublin, Ireland (11-13 April 2016).

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Literature Review

Chapter 1

Infant formula: An overview of product categories, ingredients, formulation and processing

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Abstract

Human milk is recognised as complete and the best source of nutrients for infants; however, it is not always possible to supply new-borns with mother's milk. In such cases, infant and follow-on formulae provide an alternative to human milk and are tailored to meet the nutritional requirements of a child at different stages of its development. To design an appropriate formula, the differences in composition between its main ingredient source (i.e., generally bovine milk) and human milk need to be considered. Typically, fractionation processes are applied to give a desired protein profile, combined with the inclusion of carbohydrates, oil blends, minerals and vitamins in the formulation of these nutritional products. This review provides a context and background to such nutritional products, with a focus on regulatory requirements, selection of ingredients and manufacturing processes typical of these products. Current trends in commercially relevant research into humanisation of infant and follow-on formulae are also reviewed, with an emphasis on the interfacial composition of oil globules and the influence of these interfaces on metabolism of lipids from infant nutritional products.

1.1. Introduction

It is well established that breast milk is the best source of nutrition for newborn infants and breastfeeding is recommended for the first 2 years of a child's life by the World Health Organisation. Breastfeeding during the first 6 months after birth has been shown to have significant beneficial effects on the development of an infant's immunological, digestive and cognitive systems (Andreas, Kampmann, and Mehring Le-Doare, 2015; Eidelman et al., 2012; Neville et al., 1984). However, it is not always possible to provide an infant with its mother's milk due to factors including poor nutritional status of the mother or health-related and socio-cultural reasons, resulting in a need for another form of early-stage nutrition.

Infant formula (IF) and follow-on milk products are designed to provide for the nutritional needs of infants and young children during the early stages of their life (i.e., from birth to 3-4 years) by matching the composition of these products to that of human milk (particularly for the former) at different stages of lactation (Jardí Piñana, Aranda Pons, Bedmar Carretero, and Arija Val, 2014; Maldonado, Gil, Narbona, and Molina, 1998; Pehrsson, Patterson, and Khan, 2014; Tudehope, Page, and Gilroy, 2012). Bovine milk is the primary ingredient base used for formulating IF products; however, its composition is significantly different to that of human milk. Differences in levels of key components (e.g., protein, lipid, carbohydrate, vitamins and minerals), as well as the protein and fatty acid profiles, have to be addressed when developing age-appropriate infant and follow-on formulae products. Similarly, the structural assembly (i.e., localisation of individual components within the food matrix) of the relevant components in the (emulsion-based) IF products needs to be carefully designed to best match the digestive behaviour of IF products to that of human milk (Lopez, Cauty, and Guyomarc'h, 2015). In particular, the interfacial composition and structural arrangement of oil globules in emulsion-based systems has been shown to have a significant effect on digestion of protein and lipids (Bourlieu et al., 2015; Oosting et al., 2014).

The majority of IF products are manufactured by wet mixing (i.e., reconstituting and blending) of dehydrated ingredients and liquid

concentrates, followed by homogenisation and a range of heat treatments, depending on the product, manufacturing process, format and the desired shelf life. These emulsions are further converted into stable final products in either powder or liquid (i.e., ready to feed, RTF) formats by means of spray drying or sterilisation (ultra high temperature or in-container sterilisation), respectively. The various unit operations involved in the manufacture of these products involve high stress (e.g., temperature, shear forces), which can give rise to challenges with the stability of emulsion systems within such formulations. In addition, challenges can also be experienced during storage of IF products due to their composition (e.g., lipid oxidation of unsaturated fatty acids) (Zunin, Boggia, Turrini, and Leardi, 2015). Therefore, it is important to carefully consider and maintain a balance between interfacial composition and structure, stability to processing and stability to extended storage (i.e., shelf life), digestibility when designing IF-based emulsions in a range of physical formats of finished products.

1.2. Categories of infant nutritional products

The nutritional requirements of a child change as it grows and develops, which is reflected by the natural changes in the composition of human milk during the lactation cycle (Ballard and Morrow, 2013; Csapo and Salamon, 2009). These changes are also addressed by the manufacturers of IF and follow-on products by supplying a range of specifically formulated product categories with nutrient profiles to meet the needs of a growing child at different stages of its development (Fig. 1.1). Commercially available formulae are generally categorised based on the stage of child nutrition, as determined by age, into first (0-6 months), second (6-12 months), third (1-3 years) and fourth (3+ years) stages, while Codex Alimentarius (2007), groups these products into two age categories, namely infant formula (from birth to 12 months) and follow-on formula (from 12-36 months). The regulatory guidelines for composition of these two categories of children's nutritional products are presented in Table 1.1. IF products can also be categorised based on the source and profile of their protein component; formulae based on cow's milk can be either whey- or casein-dominant (typically whey:casein ratio of 60:40 to match that of human milk) (Dupont, 2003), while soy and rice are typical

Table 1.1. Regulatory limits for first stage and follow-on children nutrition formulae.

Nutrient	Units per 100 kcal	Infant formula				Follow-on formula			
		CODEX[1]		EU[2]		CODEX		EU	
		Min	*Max*	*Min*	*Max*	*Min*	*Max*	*Min*	*Max*
Protein	g	1.80	3.00	1.80	3.00	3.00	5.50	1.80	3.50
Lipid	g	4.40	6.00	4.40	6.00	3.00	6.00	4.00	6.00
Linoleic acid	mg	300	-	300	1200	300	300	1200	-
Linolenic acid	mg	50.0	-	50.0	-	-	50.0	-	-
Carbohydrate	g	9.00	14.0	9.00	14.0	-	9.00	14.0	-
Vitamin A	IU	200	600	200	600	250	750	200	600
Vitamin D	IU	40.0	100	40.0	100	40.0	120	40.0	120
Vitamin E	IU	0.50	-	0.50	5.00	0.70	-	0.50	5.00
Vitamin K	µg	4.00	-	4.00	25.0	4.00	-	4.00	25.0
Vitamin B_1	µg	60.0	-	60.0	300	40.0	-	60.0	300
Vitamin B_2	µg	80.0	-	80.0	400	60.0	-	80.0	400
Vitamin B_6	µg	35.0	-	35.0	175	45.0	-	35.0	175
Vitamin B_{12}	µg	0.10	-	0.10	0.50	0.15	-	0.10	0.50
Niacin	µg	300	-	300	1500	250	-	300	1500
Folic Acid	µg	10.0	-	10.0	50.0	4.00	-	10.0	50.0
Panthotenic acid	µg	400	-	400	2000	300	-	400	2000
Biotin	µg	1.50	-	1.50	7.50	1.50	-	1.50	7.50
Vitamin C	mg	10.0	-	10.0	30.0	8.00	-	10.0	30.0
Choline	mg	7.00	-	7.00	50.0	-	-	-	-
Inositol	mg	4.00	-	4.00	40.0	-	-	-	-
Calcium	mg	50.0	-	50.0	140	90.0	-	50.0	140
Phosphorus	mg	25.0	-	25.0	90.0	60.0	-	25.0	90.0
Magnesium	mg	5.00	-	5.00	15.0	6.00	-	5.00	15.0
Iron	mg	0.45	-	0.30	1.30	1.00	2.00	0.60	2.00
Zinc	mg	0.50	-	0.50	1.50	0.50	-	0.50	1.50
Manganese	µg	1.00	-	1.00	100	-	-	1.00	100
Copper	µg	35.0	-	35.0	100	-	-	35.0	100
Iodine	µg	10.0	-	10.0	50.0	5.00	-	10.0	50.0
Sodium	mg	20.0	60.0	20.0	60.0	20.0	85.0	20.0	60.0
Potassium	mg	60.0	180	60.0	160	80.0	-	60.0	160
Chloride	mg	50.0	160	50.0	160	55.0	-	50.0	160
Selenium	µg	1.00	-	1.00	9.00	-	-	1.00	9.00
L-Carnitine	mg	1.20	-	1.20	-	-	-	-	-
Taurine	mg	-	12.0	-	12.0	-	-	-	12.0
Nucleotides	mg	-	-	-	5.00	-	-	-	5.00

[1] FAO/WHO Codex Alimentarius Commission Standard for infant formulae (Stan 72-1981) and for follow-on formulae (Stan 156-1987), including revisions and amendments.

[2] Commission of the European Communities Directive 2006/141/EC on infant formulae and follow-on formulae, Official Journal of the European Communities, 2006, amending Directive 1999/21/EC.

Source: O'Callaghan, O'Mahony, Ramanujam, and Burgher (2011).

protein sources for the plant protein-based formulae category (O'Callaghan, O'Mahony, Ramanujam, and Burgher, 2011). Other categories of IF products include hydrolysed infant formulae, pre-term formulae, low-birthweight formulae, post-discharge formulae and foods for special medical purposes (e.g., lactose free formulae, antiregurgitation formulae, and low phenylalanine formulae; Fig. 1.1) (O'Callaghan et al., 2011). The hydrolysed infant formulae can be further classified into 'easy-to-digest' (partially hydrolysed formulae) and clinical nutrition products (extensively hydrolysed and amino acid-based formulae), depending on the degree of hydrolysis.

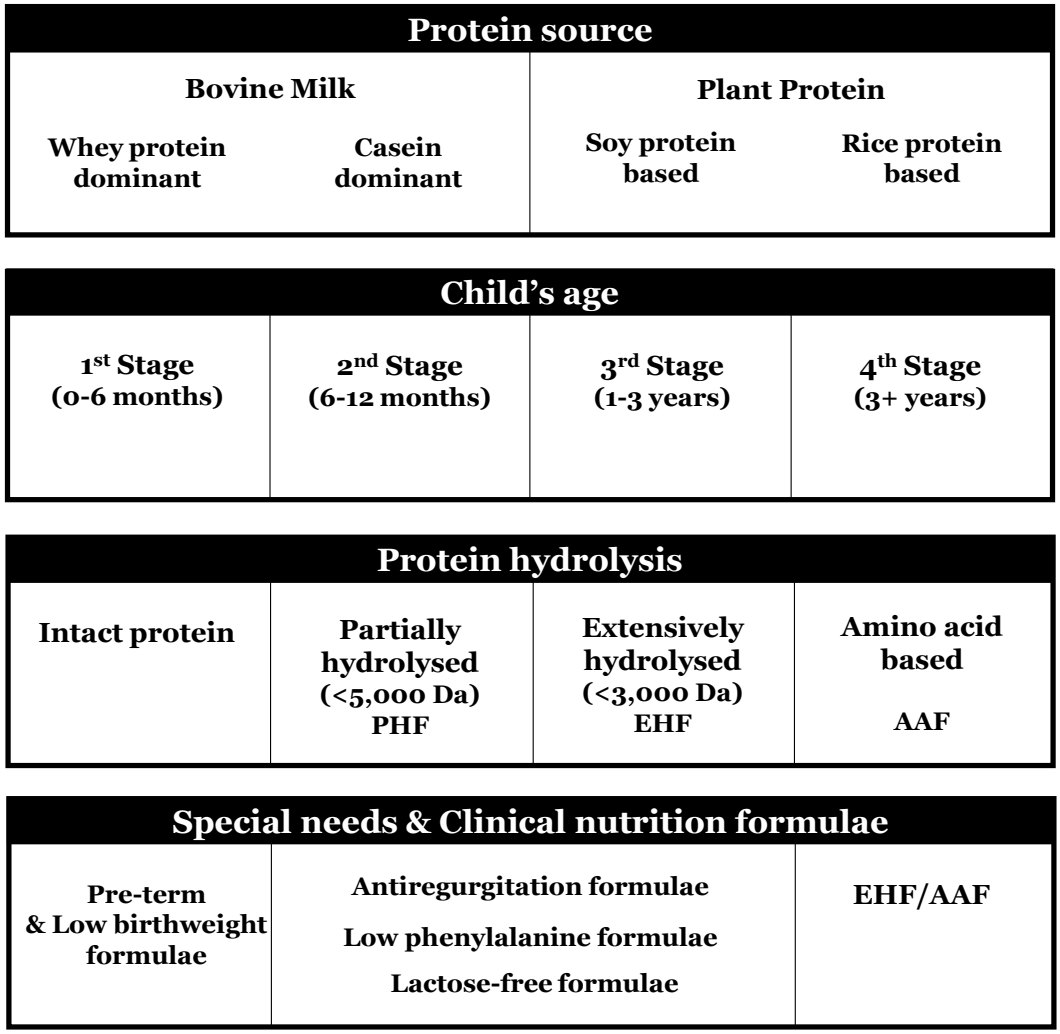

Figure 1.1. Schematic representation of the different categories of commercially available nutritional products intended for infants and young children.

1.2.1. Whey protein hydrolysate-based infant formulae

Hydrolysed protein ingredient-based IF products are very often based exclusively on whey protein hydrolysate (WPH) ingredients; these products can be further divided into partially hydrolysed, extensively hydrolysed and amino acid-based formulae (PHF, EHF and AAF, respectively), depending on the extent of protein hydrolysis (Fig. 1.1). In the AAF the proteins/peptides are hydrolysed to their constituent amino acids; EHF contain oligopeptides with molecular weight less than 3,000 Da, while PHF contain oligopeptides with molecular weight less than 5,000 Da (Exl, 2001; Greer, Sicherer, and Burks, 2008; Lowe et al., 2011). While AAF and EHF products are mainly intended for therapeutic purposes in infants suffering from, or with a high risk of cow's milk allergy (CMA), infant nutrition products from the PHF group cannot be used for therapeutic purposes but are recommended for infants at risk of CMA as they have been shown to have a preventive effect thereon (Chandra, 1997; Exl, 2001; von Berg et al., 2008). PHF products, also referred to as 'easy to digest', have been shown to have benefits including increased digestibility, more rapid amino acid release and to increase the gastric emptying rate (Fig. 1.2) (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, and Recio, 2014).

Owing to the nature of hydrolysed whey protein (i.e., altered molecular weight distribution and functional properties), manufacture of hydrolysed protein-based IF products can often be more challenging compared with the manufacture of intact protein-based counterparts. While reduction in the molecular weight typically improves surface activity and emulsion capacity of proteins/peptides, their emulsion stabilising properties can be impaired due to a thinner and less structured interfacial layer, thus promoting coalescence of oil globules post homogenisation (Agboola and Dalgleish, 1996; Agboola, Singh, Munro, Dalgleish, and Singh, 1998; Singh and Dalgleish, 1998). Protein hydrolysis has also been shown to negatively affect thermal stability of emulsions due to the increased number of reactive sites (i.e., free thiol groups) at the interface of oil globules and in the emulsion bulk phase, effectively increasing protein-mediated aggregation of oil globules (Drapala, Auty, Mulvihill, and O'Mahony, 2016; Ye and Singh, 2006). This is a particular

challenge for infant formula manufacturers as formulations typically undergo several heating steps (i.e., pasteurisation, evaporation, spray drying) and impaired heat stability can result in product defects such as formation of visible aggregated material (i.e., white flecks) (Regost, 2016) or coalescence and breaking of oil globules and the presence of free fat in the RTF formula or in the reconstituted powder and in-process challenges with fouling and poor heat transfer.

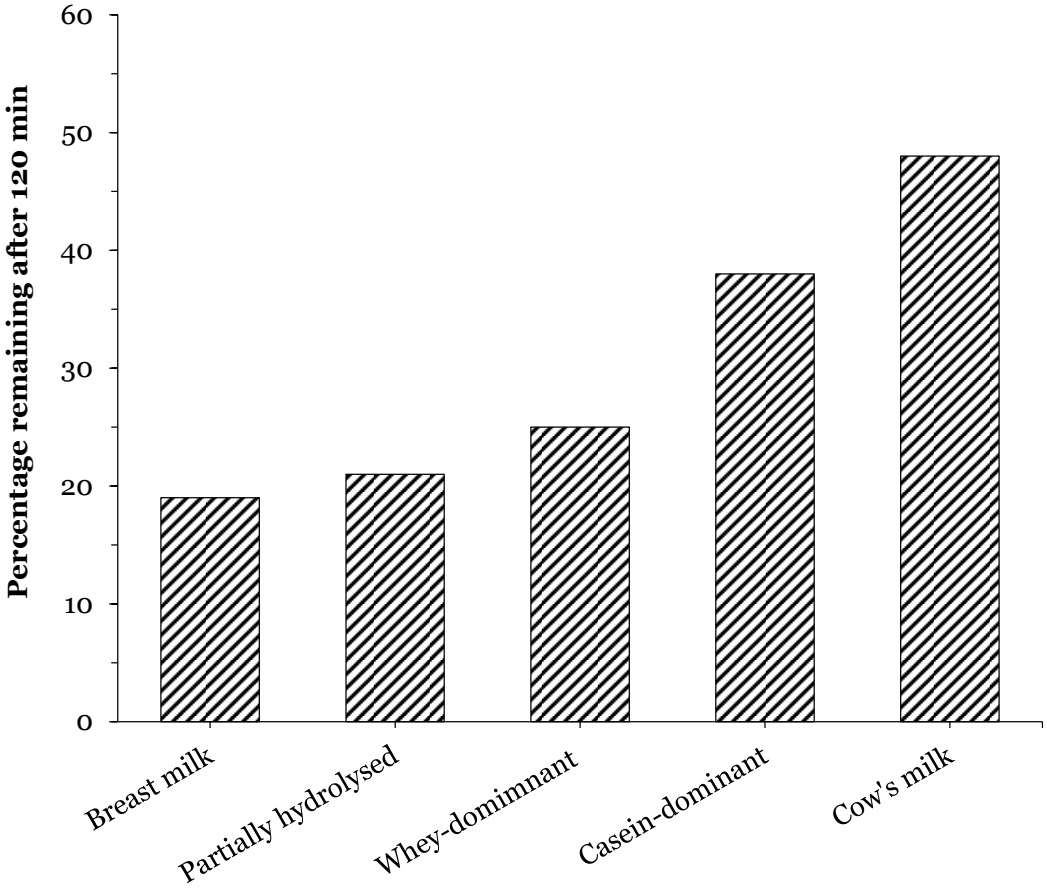

Figure 1.2. Gastric emptying rate in infants as affected by type of milk.

Source: www.nestlenutrition-institute.org.

Improving thermal stability of WPH-based emulsions has been the focus of a small number of studies; the strategies investigated typically involved the addition of low molecular weight surfactants (i.e., lecithin and/or citric acid esters of mono- and di-glycerides, CITREM) (McSweeney, 2008) and

hydrolysis of the starch component to reduce the influence of depletion flocculation (Tirok, Scherze, and Muschiolik, 2001). In parallel, other studies have reported on improvements in the thermal stability of intact whey protein-based emulsions on conjugation of the protein with carbohydrates through the Maillard reaction (Kasran, Cui, and Goff, 2013; Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, and Dewettinck, 2004; Setiowati, Saeedi, Wijaya, and Van der Meeren, 2016); this approach may also be beneficial in improving the emulsion stability of hydrolysed whey protein-based IF systems.

1.3. Ingredients used in infant formulae

Bovine milk is the primary source of ingredients for IF products (e.g., protein and lactose); however, its composition is significantly different to that of human milk, thus fractionation and selective enrichment is required to make it suitable for use in infant nutrition. Some of the greatest differences between bovine and human milk are in their protein and fat components, hence, the majority of research and product development efforts have been traditionally focused on these macro-nutrients.

Matching the detailed macro- (i.e., protein, lipid and carbohydrate) and micro-components (e.g., vitamins, minerals) of human milk requires careful selection of bovine milk-based (and other) ingredients to achieve an IF product with a nutritional profile as close as possible to that of human milk. Fractionation of bovine milk into individual constituents (i.e., lactose, different protein-enriched fractions and milk fat) followed by blending of selected constituents with other components from sources other than cow's milk (e.g., blends of vegetable and fish oils and maltodextrin) is central to development of IF products.

1.3.1. Protein-based ingredients

The protein content of human milk is lower than that of bovine milk; its protein profile is also considerably different, as evidenced by the whey:casein ratio, but also by the different profile of the whey protein component (Table 1.2). The whey protein:casein ratio in human milk is known to change throughout the lactation cycle, typically being 80:20 in early lactation, 60:40

in mid lactation and 50:50 in late lactation, while for bovine milk it is much less variable throughout lactation normally averaging 20:80 (Lönnerdal, 2003). The major whey proteins in human milk are α-lactalbumin and lactoferrin, making up 29% and 24% of the total protein, respectively; these proteins only represent 3.7% and 0.6% of the total protein, respectively, in bovine milk (Lien, 2003). α-Lactalbumin is recognised as playing a significant role in regulating an infant's physiological processes, antioxidant systems and in brain development, due to its high content of tryptophan, cysteine and lysine (Lien, 2003). Similarly, lactoferrin is important for its antimicrobial and immunotropic properties, and it promotes the growth of beneficial lactic acid bacteria, suppresses growth of pathogenic bacteria and facilitates iron adsorption (Artym and Zimecki, 2005). In addition, β-lactoglobulin, the dominant whey protein fraction in bovine milk constituting approximately 50% of total whey protein, is absent from human milk (Marshall, 2004). In the casein protein fraction of human milk, β-casein is the major constituent, which owing to its extensive phosphorylation, is capable of complexing with calcium, zinc and other divalent ions, effectively facilitating their adsorption (Chen et al., 2016; Lönnerdal, 2003). Differences in the ratios of individual proteins between bovine and human milk effectively lead to different concentrations of essential amino acids, in different milks. As a result of these differences in the amino acid profile, the protein content of IF products is typically higher than that in human milk (i.e., 13-15 *vs* 9-11 g L^{-1}, respectively) (Lien, Davis, and Euler, 2004); enrichment of IF products with selected whey protein and casein fractions such as α-lactalbumin, lactoferrin and β-casein is also sometimes practiced (Aly, Ros, and Frontela, 2013; Buggy, McManus, Brodkorb, McCarthy, and Fenelon, 2017; Crowley, Dowling, Caldeo, Kelly, and O'Mahony, 2016; Crowley et al., 2015; Lien, 2003; O'Mahony, Smith, and Lucey, 2007; Rueda et al., 2008; Sadler and Smith, 2013; Wernimont, Northington, Kullen, Yao, and Bettler, 2015). Traditionally, cheese or casein manufacturing processes are the main sources of whey for the recovery of whey protein; however, owing to the nature of these processes, resultant wheys typically contain unwanted residual components, including colouring agent (e.g., annatto, not approved as food additive for IF in Europe, US and China), starter cultures, glycomacropeptide and high mineral levels. To comply with

regulations, certain components may need to be (at least partially) removed (e.g., by filtration and ion exchange demineralisation processes) to make the resultant whey protein products suitable for use in IF products (EU Commission, 2016; McSweeney, 2008; Smithers, 2015).

Table 1.2. Composition of human and bovine milk.

Component	**Human milk**	**Bovine milk**
	g 100 mL^{-1}	
Protein	**1.03**	**3.30**
Whey protein	0.61	0.68
α-Lactalbumin	0.29	0.12
β-Lactoglobulin	0.00	0.33
Lactoferrin	0.25	0.02
Casein	0.42	2.62
Lipid	**4.40**	**3.70**
Triglycerides	4.31	3.63
Saturated	2.07	2.40
Monounsaturated	1.72	1.10
Polyunsaturated	0.52	0.15
Mono-/di-glycerides and polar lipids	0.09	0.07
Carbohydrate	**7.20**	**4.70**
Lactose	6.70	4.70
Oligosacharides	0.50-2.00	~0.00
Ash	**0.20**	**0.70**
Water	**87.5**	**87.8**

Sources: Packard (2012); www.nestlenutrition-institute.org.

1.3.2. Lipid-based ingredients

The fatty acid composition of bovine milk is considerably different to that of human milk; these differences are mostly due to the lower levels of short chain fatty acids and higher proportion of polyunsaturated fatty acids in human milk compared to bovine milk (Packard, 2012). The ratio of saturated to unsaturated fatty acids is generally 50:50 in human milk, while it is 65:35 in bovine milk; palmitic acid is the major saturated fatty acid in both human and bovine milk (Packard, 2012); the breakdown of fatty acids into saturated, mono- and poly-unsaturated classes for human and bovine milks is presented in Table 1.2. The lipid component of commercial IF products is typically comprised of a blend of various vegetable oils (e.g., soybean, sunflower, high-oleic safflower, coconut oil, palm oil, and low-erucic rapeseed oils) to achieve the specific fatty acid levels required by the infant (EU Regulation, 2013; Codex Alimentarius, 2007). The regulations contain a general requirement for maximum caloric intake of the lipid component (i.e., 4.4-6.0 g 100 $kcal^{-1}$), where the α-linoleic acid is to constitute 5-20% of the total lipid and the α-linoleic:linolenic ratio is to be between 5 and 15 to ensure adequate balance between the ω3 and ω6 metabolic pathways (Zunin et al., 2015). However, the triglyceride configuration of the vegetable oils is different to that observed in human milk due to the preferential location of the palmitic acid at the *Sn*-1 and *Sn*-3 positions of the triglyceride in vegetable oils, in contrast to the *Sn*-2 preferential location for human milk fat (O'Callaghan et al., 2011). Transesterification is sometimes applied to vegetable oils used in the manufacture of IF products in order to enrich the finished product with palmitic acid at the *Sn-2* position (Bourlieu et al., 2015; López-López, López-Sabater, Campoy-Folgoso, Rivero-Urgell, and Castellote-Bargalló, 2002).

Another important group of lipids present in human milk (and all mammalian milks) are polar lipids (i.e., phospholipids and lipoproteins), which are predominantly present at the surface of fat globules, where they assemble as components of the milk fat globule membrane (MFGM). These polar lipids form a tri-layer, interrupted by patches of protein and glycoprotein units (Fig. 1.3); this heterogeneous interface surrounds the triglyceride core, protecting and facilitating delivery of bioactive compounds and nutrients to

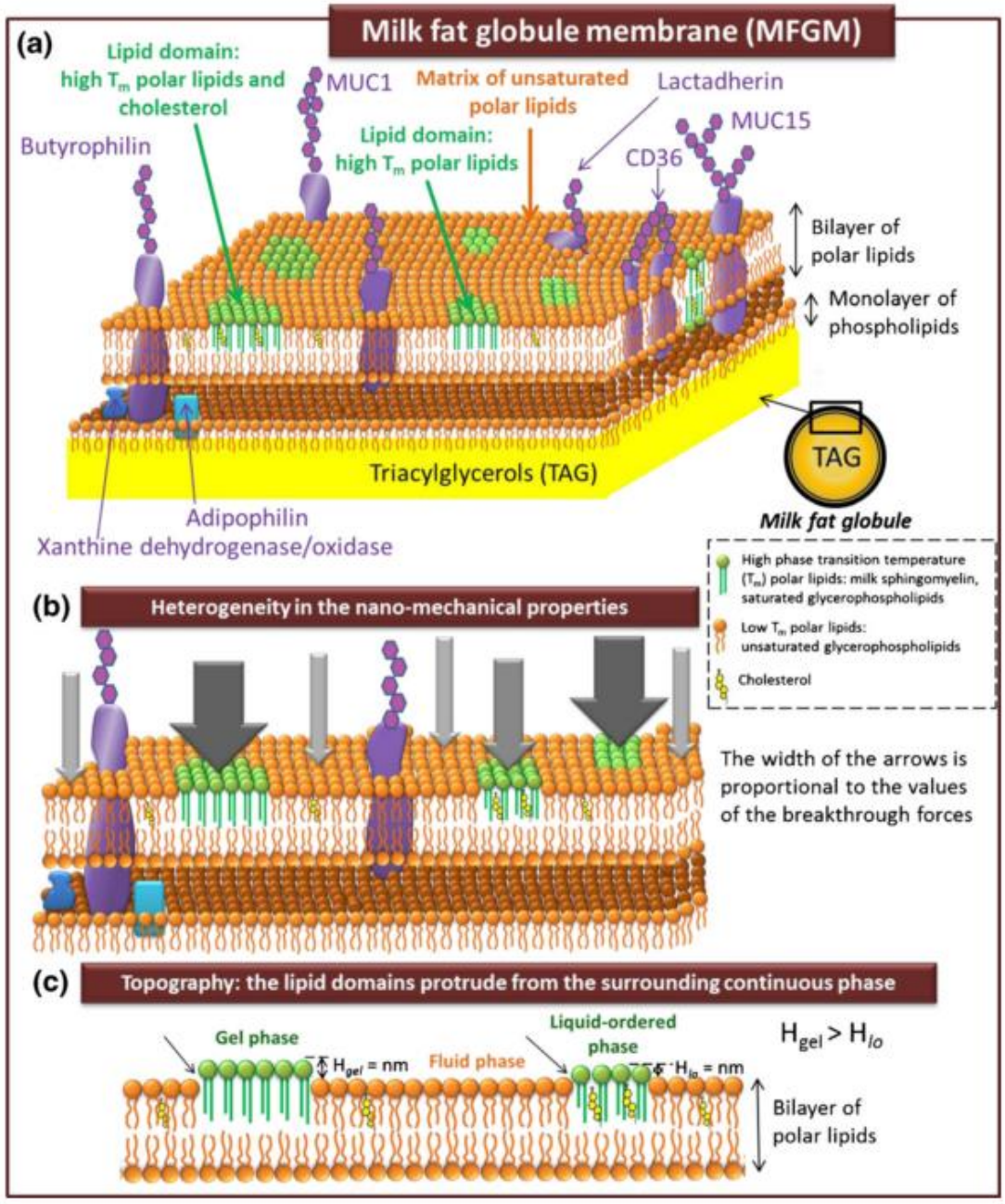

Figure 1.3. Model of the organization of the biological membrane surrounding fat globules in milk, the milk fat globule membrane (MFGM). (**a**) 3-Dimensional representation of the trilayered membrane, with the heterogeneous distribution of proteins and the phase coexistence of lipids: lateral segregation of high T_m polar lipids in domains. (**b**) Heterogeneities in the nano-mechanical properties of the outer bilayer of the MFGM. The width of the arrows is proportional to the mean values of the breakthrough forces determined by atomic force mapping. (**c**) Schematic representation showing that the MFGM is not flat. H_{gel} and H_{lo} are the average height difference between the two lipid phases. *Source: Lopez, Cauty, and Guyomarc'h (2015).*

the infant in an efficient manner (Lopez et al., 2015). The structure of the MFGM is considered unique compared to any other biological lipid transport system (Spertino et al., 2012) and it is believed to play an important role in lipid metabolism (Singh and Gallier, 2016). However, similar to other formulated dairy products, the oil globules in IF are mostly coated and stabilised by casein and/or whey proteins due to the preferential adsorption of these components during homogenisation. The efforts to characterise and understand the role of MFGM in the metabolism of human milk, as well as engineering formulae with human milk-like fat globule membranes (FGM) at the surface of oil globules have been particularly active areas of research over the last 4-5 years (Contarini and Povolo, 2013; Liao, Alvarado, Phinney, and Lonnerdal, 2011; Lopez et al., 2015; Lopez and Ménard, 2011). Gallier et al. (2015) studied a prototype IF product (Nuturis®, Danone), where large oil globules (~4 μm) partially coated by phospholipids were produced in an effort to mimic the oil globule structures of human milk. The focus of the study was to characterise the structure of the fat droplets of the concept IF and compare it to the structure of fat globules in human milk and in control IF with the aid of microscopic techniques (confocal laser scanning microscopy and transmission electron microscopy). An example of a commercially available product with MFGM-like structures is Enfamil Enspire® from Mead Johnson Nutrition.

1.3.3. Carbohydrate-based ingredients

Similar to bovine milk, lactose is the main carbohydrate component in human milk being present at ~6.7%, compared to ~4.7% in bovine milk; its structure is the same in all mammalian milks (Packard, 2012). Infant formula products based on bovine milk are typically produced from skim milk powder or skim milk concentrate, much of the former containing ~52% lactose and ~34% protein. To reach the required carbohydrate content in the formulation, further addition of lactose or products of starch hydrolysis (e.g., maltodextrin or corn syrup solids) is required. Typically, healthy full-term infants do not exhibit difficulties with digesting lactose, and for those suffering from problems with lactose metabolism, part of the lactose (typically ~30%) is replaced with simple sugars (e.g., glucose) (Packard, 2012).

In addition to lactose, human milk contains a significant proportion of oligosaccharides (5-20 g L^{-1} in mature milk), which are believed to play an important role in influencing and regulating the intestinal microbiota (i.e., as prebiotics) and providing potent antimicrobial protection, as well as being involved in brain and cognition development (Andreas et al., 2015; Bode, 2012; Vandenplas, 2002). Supplementation of IF products with oligosaccharides has been shown to have beneficial effects on the gut microbiota, metabolic activity, stool consistency and development of the immunological system (Goehring et al., 2016; Seppo, Autran, Bode, and Järvinen, 2016; Vandenplas, Zakharova, and Dmitrieva, 2015).

1.4. Processes used in infant formulae manufacture

Infant formula products are typically available in two physical states, as a spray dried powder or as a ready to feed (RTF) liquid emulsion. Preparation of powdered formula can be achieved by wet mixing (i.e., reconstituting the ingredients followed by spray-drying), by dry blending (i.e., mixing of dehydrated components to constitute a uniform blended product) or by a combination of the two (i.e., production of a base powder by wet mixing and spray drying processes followed by dry blending with carbohydrate, minerals and/or vitamins) (McSweeney, 2008). The powder and RTF formats of IF generally undergo similar unit operations in the initial stages of processing (unless the powdered formula is dry blended; Fig. 1.4); these typically involve blending of liquid and powdered ingredients at a desired ratio, heat treatment and standardisation of the wet mix to the desired nutritional profile. In the blending process, proper hydration of protein can be facilitated by using high shear mixing devices (O'Sullivan, Schmidmeier, Drapala, O'Mahony, Kelly, 2016) or increased mixing temperatures (i.e., ≤ 50°C; Bylund, 1995). Heat treatment (i.e., pasteurisation, ~72-75°C × 15-20 s) typically follows the wet mixing stage to ensure the subsequent microbial quality of the product. Subsequent homogenisation allows homogenous distribution of the oil phase in the liquid phase; although less common, the homogenisation can also be carried out prior to pasteurisation (Buggy et al., 2017). Heat labile ingredients (e.g., vitamins) can be added after pasteurisation; microbial quality of these ingredients is critical as their point of addition is after the main heat treatment

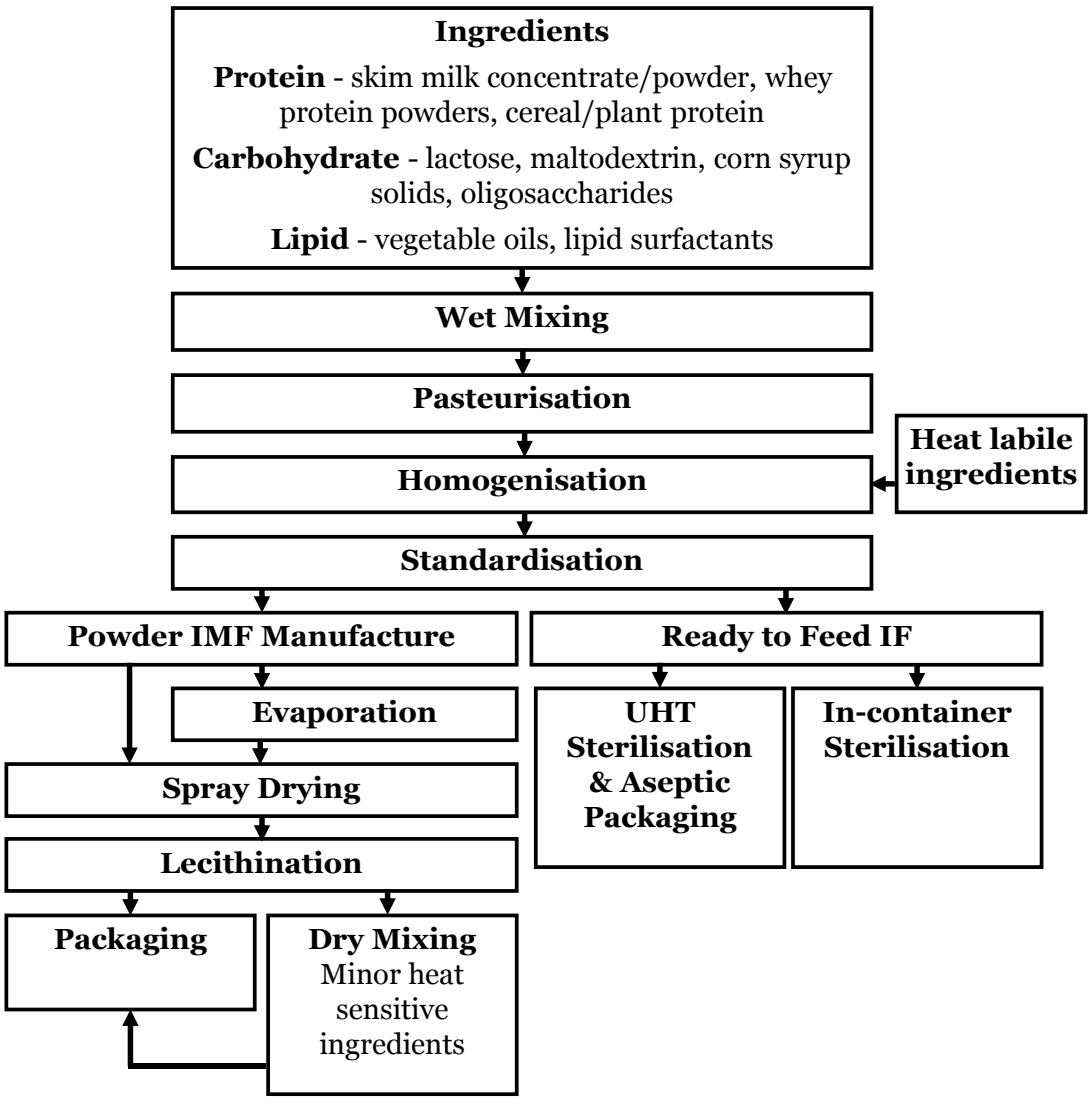

Figure 1.4. Flow diagram showing the different stages involved in the typical manufacture processes for infant formulae and follow-on formulae in both powder and liquid (RTF) formats.

step. Generally, formation of small oil globules (< 1 μm) is desirable during homogenisation of IF to ensure stability of the emulsion against separation (McCarthy et al., 2012; McSweeney 2008). However, recent studies have shown that IF products with larger oil globules, compared to traditional IF products, may offer metabolic and digestive properties that are closer to those observed for human milk (Bourlieu et al., 2015; Gallier et al., 2015; van Aken, 2010). Following pasteurisation and homogenisation, the product destined for powdered formula is evaporated to the target total solids concentration of ~50-

55%, unless already formulated at final solids content (Fig. 1.4), and spray dried, while the product destined for RTF formula (at ~12% solids content) is either sterilised by a UHT process (135-140°C × 2-3 s) followed by aseptic packaging or by in-container sterilisation (120°C × 10-20 min; glass jars, plastic bottles, laminate pouches or tinplate cans) (Fig. 1.4). The heating stages of the IF process are critical control points that ensure the microbial quality of the final product; however, due to the conditions employed and the heat-sensitive nature of the product, they present challenges in relation to heat stability of the product. Efforts have been made to understand the nature of, and to minimise the undesirable, heat-induced changes to the systems during processing; these changes include fouling of heat exchanger surfaces, denaturation and aggregation of protein and interactions between protein and oil globules (e.g., flocculation, coalescence, flecking) (McCarthy, Kelly, O'Mahony, and Fenelon, 2014; McSweeney, Healy, and Mulvihill, 2008; McSweeney, Mulvihill, and O'Callaghan, 2004; Regost, 2016).

The continuous improvement efforts to increase the resemblance of early nutrition products to that of human milk in regards to composition, structure and bio-functionality are steadily moving such formulated products even closer to human milk. However, a significant gap still exists between commercial IF products and breast milk and a small number of research groups worldwide are working towards closing that gap. The more promising areas of this research include redesigning the interfacial structure of oil globules, addressing the function of (human) milk oligosaccharides and their enrichment in IF, and efficient and sustainable fractionation of bovine milk for enrichment of IF with α-lactalbumin, lactoferrin, β-casein and other bio-functional proteins and peptides.

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Chapter 2

A review of the analytical approaches used for studying the properties and stability of nutritional beverage emulsions

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Table of Contents

Abstract

Emulsions contain water and oil, stabilised by surface active agents, and are amongst the most widely present, diverse and complex food components. These products are susceptible to changes induced by manufacturing processes and on storage, resulting in challenges with their stability, quality and shelf life. An understanding of the relationship between structure and stability of an emulsion is essential to designing and competently formulating food products with the desired nutritional functionality and sensory properties, while achieving the required shelf life. This article critically reviews a broad range of commonly-used analytical approaches focused on emulsion formation dynamics, emulsion structure, techno-functional properties and stability to intrinsic and environmental factors. A brief overview of the fundamentals of the stability of emulsions to separation based on their measurable physical parameters (i.e., oil globule size, phase density, viscosity) is presented, as well as a discussion on the rheological properties of an emulsion as affected by interactions between its components. The effectiveness of oil globule stabilisation by electrostatic and steric barriers under various environmental conditions is reviewed, with particular focus on the strategies used to influence and measure these stabilisation phenomena. Finally, the assembly of the interfacial layers in infant formula emulsions is discussed, with a focus on the current trends therein for this high value food sector.

2.1. Introduction

Many food products are emulsion-based systems (e.g., protein-based beverages, ready to feed infant formulae, ice cream and mayonnaise); in some food products the emulsion is immobilised by subsequent processing (e.g., spray drying, gelation or freezing). Owing to the immiscible nature of the two major components of an emulsion (i.e., water and oil), these systems are inherently thermodynamically unstable, hence, efforts to improve their stability are an ongoing focus of food research. Stability of a food emulsion can be divided into stability against time-induced changes resulting from the differences in the density of the two immiscible components of the emulsion (i.e., floatation and phase separation; Fig. 2.1 B) as described by Stokes' law and stability of the system against undesirable interactions between its individual components (i.e., flocculation, aggregation and coalescence of oil globules) (Fig. 2.1 C and D).

Based on Stokes' law, a relationship between the parameters of an emulsion (i.e., particle size, phase density and viscosity) and the velocity of separation (i.e., creaming) can be established (Equation 2.1). This relationship helps to explain how changes to the emulsion parameters affect its stability to gravity-governed changes under laminar flow:

$$v_{creaming} = \frac{2r^2(\rho_{oil\ globule} - \rho_{serum})g}{9\eta} \quad (2.1)$$

Where $v_{creaming}$ describes the velocity of upward movement (i.e., creaming) of an oil globule, r is the radius of the oil globule, ρ is the density of the corresponding dispersed phase (i.e., the oil globule) and the dispersant (i.e., serum phase), g is the acceleration due to gravity, and η refers to the viscosity of the continuous phase. Conversely, when the dispersed phase has a higher density compared to the continuous phase, a downwards movement is observed for the dispersed particles (i.e., sedimentation).

It should be noted that Stokes equation assumes that the separating particles are spherical, which is not always true for oil globules with structured

interfacial layers. In addition, the equation does not take into account charge-based interactions between particles (i.e., the electroviscous effects) nor the hydrodynamic interactions between the particles and the solvent (McClements, 1999).

Processing stability and shelf life stability are other terms commonly used when discussing emulsion stability; this terminology is especially relevant from an industrial perspective as it covers the stability of a formulation (i.e., mixture of ingredients prepared according to a formula) during different stages of product manufacture and stability of the final product during its intended storage time. Processing stability refers to the ability of emulsions to resist changes incurred in response to adverse processing conditions during a relatively short time (i.e., heat treatment, high shear forces, turbulent flow and short time storage); these generally include changes due to the interactions between the components of the system as detailed in Fig. 2.1 C. These undesirable changes include flocculation and coalescence of oil globules, aggregation of protein and formation of complexes between different component classes (i.e., bridging flocculation, surfactant/protein/ polysaccharide complexes) (Antipova, Semenova, Belyakova, and Il'in, 2001; Drapala, Auty, Mulvihill, and O'Mahony, 2015; Drapala, Auty, Mulvihill, and O`Mahony, 2016a,b; Regost, 2016; Ye and Singh, 2006). On the other hand, shelf life stability represents a holistic view on the stability of these systems and it addresses changes that happen gradually over a relatively long time (i.e., weeks-months); these incorporate both changes in the system based on Stokes' law (i.e., creaming, sedimentation; Fig. 2.1 B), changes due to undesirable interactions between components (Fig. 2.1 D) as well as chemical reactions that can take place in a food emulsion (e.g., lipid oxidation).

Numerous approaches have been developed and employed to test emulsion stability, studying the prevalence of the undesirable changes during processing (Guzey and McClements, 2006; Liu, Sun, Xue, and Gao, 2016; McCarthy et al., 2012; McSweeney, Healy, and Mulvihill, 2008; Mustapha, Ruttarattanamongkol, and Rizvi, 2012) and during shelf life (Pan, Tikekar, and Nitin, 2013; Sarkar, Arfsten, Golay, Acquistapace, and Heinrich, 2016; Tcholakova, Denkov, Ivanov, and Campbell, 2006; Xu, Wang, Jiang, Yuan,

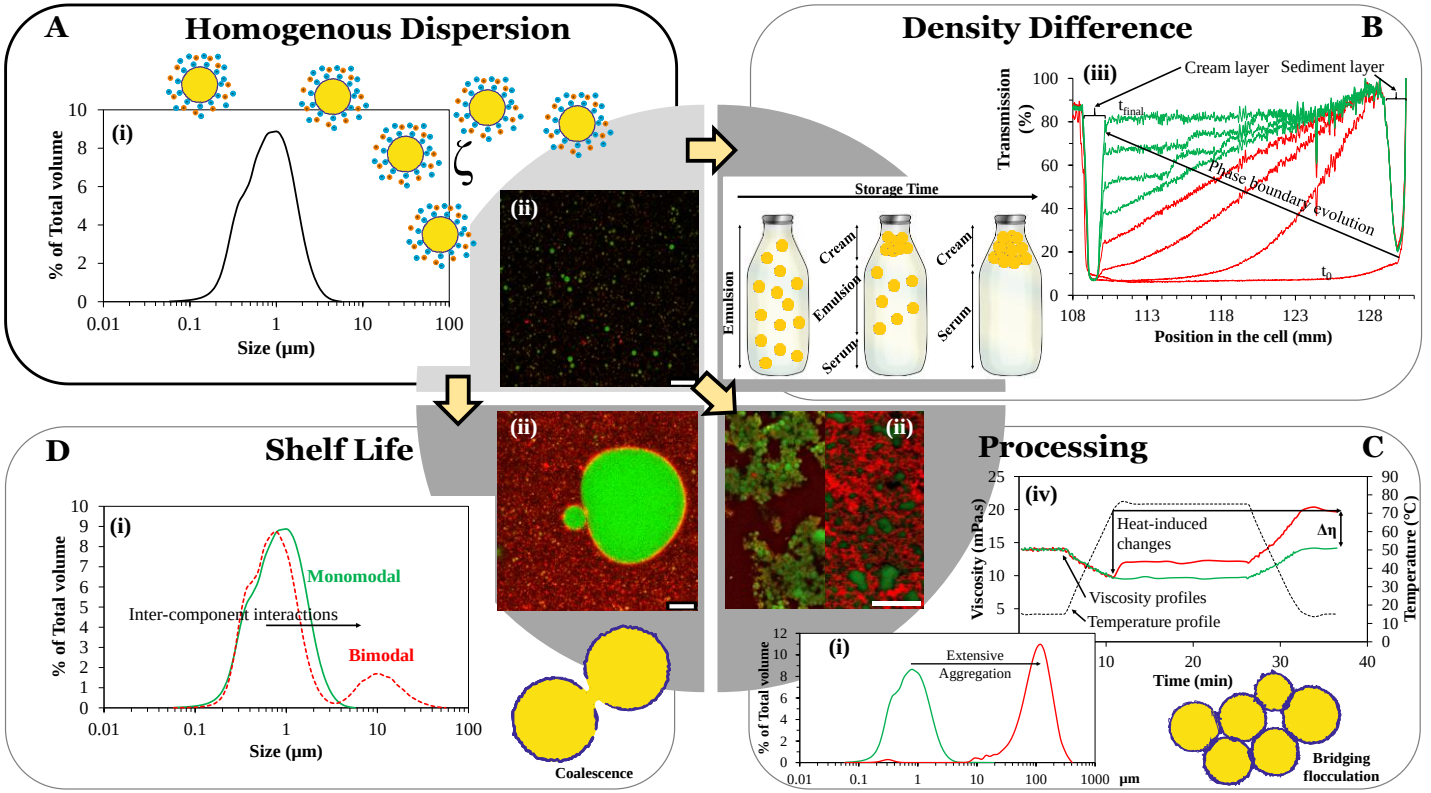

Figure 2.1. Schematic representation of changes that can affect (A) a typical IF emulsion as a result of (B) density difference (creaming of oil globules), (C) thermal processing (protein mediated aggregation of oil globules) and (D) long term storage (coalescence of oil globules) as evidenced by various analytical techniques typically used to characterise emulsions: (i) particle size analysis measured using laser diffraction; (ii) confocal laser scanning microscopy (CLSM) images showing the distribution of protein (red) and neutral lipids (green) in emulsions; (iii) accelerated creaming stability analysis with an analytical centrifuge; and (iv) simulated heat treatment with dynamic sample viscosity information obtained using a rheometer, equipped with a starch pasting cell.

and Gao, 2012). Due to the impact the emulsion parameters, as described by Stokes law, have on its physical stability, many of the analytical approaches have been directed at measuring and controlling fat globule size distribution, viscosity, flow behaviour and phase separation. Selected methods will be discussed in detail in this review. Likewise, approaches used to study the prevalence of undesirable interactions between components of emulsions during processing and shelf life will also be discussed to provide a detailed overview of the current methodology used in understanding, controlling and predicting emulsion stability. In this review, selected analytical approaches, used for studying the formation, assembly and properties of emulsions and the role of interfaces of oil globules on mechanisms of emulsion stabilisation and destabilisation will be discussed.

2.2. Formation of emulsion interfaces

Surface active compounds (e.g., emulsifiers) are essential to provide a reasonable stability and homogeneity to a system composed of two (or more) immiscible phases (i.e., oil and water in food emulsions) due to their affinity for both hydrophobic and hydrophilic phases (McClements, 1999). These compounds are added to an oil and water mixture prior to a mechanical process, such as homogenisation or high shear mixing, which are designed to disperse one phase in another by breaking the dispersed phase into a large number of small subunits (i.e., oil globules for oil-in-water emulsions). The increase in the contact area between the two phases is accompanied by an increase in the surface free energy, resulting in increased thermodynamic instability with a strong drive to minimise the contact area between the two phases (i.e., coalescence of oil globules and phase separation). However, with the presence of emulsifiers, their rapid migration to, and adsorption at, the oil/water (O/W) interface reduces the surface free energy (i.e., interfacial tension) and effectively impedes the drive towards coalescence of oil globules. In addition, a physical barrier is formed by the adsorbed compounds at these interfaces, giving further protection against coalescence.

The term surface active ingredient covers a broad range of components, the effectiveness of which for formation and stabilisation of an emulsion system

depends on their amphiphilic balance, molecular size and structure. Proteins are common, naturally occurring emulsifiers, widely used in a broad range of food applications; however, other compounds such as lipid-based low molecular weight surfactants (e.g., phospholipids, mono- and di-glycerides, esterified glycerides) are also commonly used (McSweeney, 2008). In general, smaller emulsifiers display higher mobility, compared to larger emulsifiers; smaller emulsifiers tend to dominate the interface in a shorter time. In addition, these small emulsifiers can display higher packing density at the interface (i.e., have a more densely populated interface), compared to large, more structured, surfactants (e.g., protein), resulting in lower interfacial tension (Pugnaloni, Ettelaie, and Dickinson, 2005).

To assess the ability of an emulsifier to stabilise an interface, analysis of interfacial tension is typically performed; such analysis provides information on the emulsifier adsorption rates as well as on the extent of reduction in interfacial tension. A comprehensive review of different approaches used to study surface and interfacial tension has been published by Drelich, Fang, and White (2002) and the main principles of these approaches, with some examples of their applications, are summarised briefly in the current review. The most commonly used methods for the analysis of interfacial tension can be divided into 2 categories, based on the measurement principle employed: (1) direct measurement of the repulsive force between two immiscible phases and (2) analysis of the shape of a droplet as affected by interfacial tension. Both of these approaches are based on quantification of the force resisting an increase in the surface area (i.e., surface tension) promoted by forces acting in the opposing direction (e.g., pull, gravitational or centrifugal forces; Fig. 2.2).

The repulsive forces acting at the interface between two immiscible phases can be quantified using a microbalance or capillary pressure approaches. For the former approach, a probe (typically Wilhelmy plate or Du Noüy ring), connected to the microbalance, is placed directly above the surface/interface and is brought into contact with it. Resultant wetting of the probe by the liquid, due to the capillary forces (Fig. 2.2 a), causes an increase in the surface area; at the same time the inherent drive of the system to reduce the surface area is acting in the opposite direction. The net effect is that a pull force acts on the

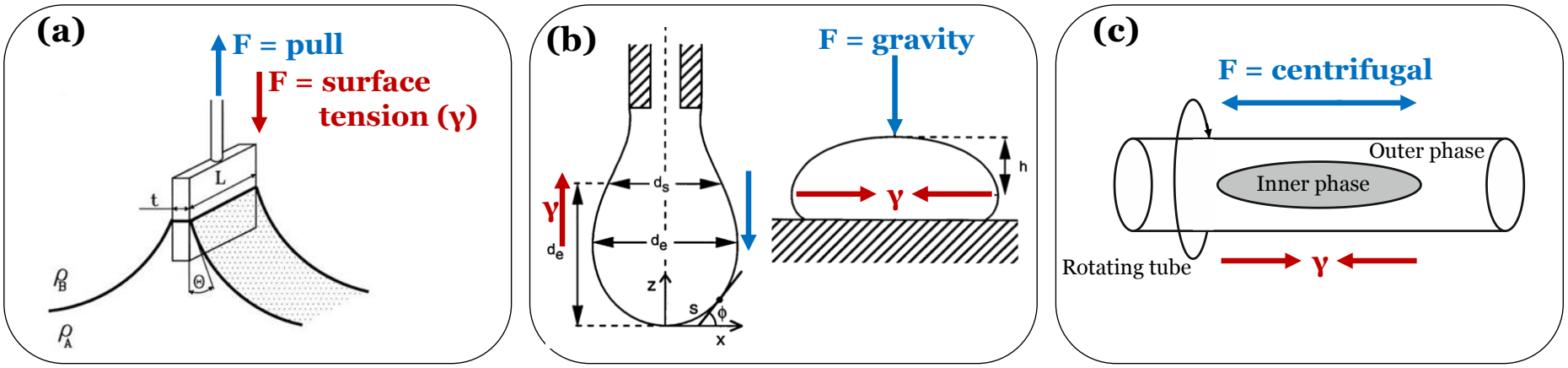

Figure 2.2. Schematic representations of principles of typical analytical techniques used for determination of interfacial tension (γ): (a) microbalance technique with the Wilhelmy plate geometry; (b) pendant (left) and sessile (right) drop techniques using image analysis software; (c) spinning drop method using image analysis software.

Adapted from: Drelich, Fang and White (2002); Leick, Henning, Degen, Suter and Rehage (2010); Thiessen and Man (2000).

probe, which is quantified by the microbalance and used to calculate the surface/interfacial tension (Equation 2.2). The presence of a surface active compound results in a decrease of that pull force, owing to the reduced surface free energy upon its adsorption at the interface.

$$\gamma = \frac{F}{L\,cos\theta} \tag{2.2}$$

Where γ is the surface/interfacial tension (mN m^{-1}), F is the pull force (mN) acting on the plate, L is the wetted length (i.e., 2 × plate width; mm) and θ is the contact angle between liquid meniscus and the plate.

Using the Du Noüy ring attachment allows only a single point measurement, where the force required to detach the ring from the lamella is related to the γ. On the other hand, the Wilhelmy plate attachment allows study of a dynamic γ by its continuous measurement without the detachment step, making it a geometry of choice for measuring surface properties of emulsifiers.

Kim, Cornec, and Narsimhan (2005) used the Wilhelmy plate approach to study the effect of heat-induced changes in the secondary structure of β-lactoglobulin on its interfacial properties at the planar soybean oil-water interface. Those authors correlated the conformational changes to the protein with the interfacial area populated by the native or denatured/aggregated protein and with the flexibility of the protein at the interface (i.e., increase in flexibility due to protein denaturation), linking the improved flexibility to better emulsifying functionality of the ingredient. Using a similar approach, Jara, Carrera Sánchez, Rodríguez Patino, and Pilosof (2014) studied surface pressure isotherms for individual whey proteins (i.e., β-lactoglobulin, β-lg; α-lactalbumin, α-la; bovine serum albumin, BSA) as affected by protein conformation at different pH values. The authors reported different interfacial behaviour for these proteins, where after initial adsorption at the interface, native α-la displayed limited unfolding (i.e., rigid structure) at pH 6 and a flexible structure at pH 3. Similarly, BSA displayed increased surface activity at pH 3 compared to pH 6; conversely, the native β-lg, was shown to display higher surface activity at pH 6 compared to that at pH 3. Modification of surface properties of protein by hydrolysis was studied using the Wilhelmy

plate method by Turgeon, Gauthier, Molle, and Leonii (1992). In that study the authors identified peptide fractions, from the hydrolysis of β-lg, with improved interfacial properties; the molecular structure of these peptides was further assessed using reversed phase high-performance liquid chromatography and analysis of surface hydrophobicity. Their research concluded that the best surface activity for hydrolysed β-lg was observed at low M_w (i.e., ~2000 kDa) combined with clustering in distinct polypeptide regions of hydrophilic and hydrophobic residues. Effectively, those authors showed that surface tension analysis, combined with the other methods, allowed a holistic evaluation of hydrolysed protein fractions for improving surface activity. Yang et al. (2013) used the Wilhelmy plate method to measure the surface activity of low molecular weight (L_{Mw}) surfactants (i.e., phospholipids and non-ionic polymeric surfactants) and correlated it with their emulsion capacity, as determined by measurement of fat globule size distribution post homogenisation.

Surface tension analysis is often used for studying the compatibility between different surface active components in a system, where it can help with assessing the shelf life stability of emulsions. Cai and Ikeda (2016) studied the competitive displacement of native or conjugated whey protein, by L_{Mw} surfactant, Tween 20, at an air-water (A/W) surface. Those authors reported that surface tension analysis allowed tracking of protein displacement from the surface upon introduction of the L_{Mw} surfactant. In that study, the authors showed that conjugation of protein with the network-forming polysaccharide, gellan, provided a resistance to protein displacement from the interface. Competitive displacement behaviour and its effect on the shelf life of an emulsion-based product can also be characterised using a surface rheology approach (Dickinson, 2001); this approach typically uses modified surface tension methods and will be discussed later in this section.

With the capillary pressure approach, a pressure difference across a curved interface is measured. Based on the Young-Laplace equation, the pressure is higher on the concave side and lower on the convex side of a curved interface. By measuring the pressure required to force a gas bubble through the capillary into the liquid, the tension associated with that interface can be calculated.

Tamm, Sauer, Scampicchio, and Drusch (2012) used the capillary approach to measure the changes in the pressure difference across the interface to monitor dynamic protein adsorption at the air/water interface and correlated that information with foam capacity and foam stability. Similarly, Marinova et al. (2009) studied the adsorption kinetics for caseins and whey proteins using a Langmuir trough, where the differences in surface properties were correlated with the differences in foamability of the proteins.

The second category of methods employed to measure surface/interfacial tension is based on the shape of a liquid droplet. In this approach, the interfacial tension is calculated from the drop dimension and the volume of the liquid used to form the droplet. The shape of a liquid droplet is resultant from the two opposing forces acting on it; surface tension, which pulls the liquid upwards towards a spherical drop, and the force of gravity, which pulls the liquid downwards causing an elongation of a pendant drop or widening of a sessile drop (Equations 2.3 and 2.4, respectively; Fig. 2.2 b) (Drelich, Fang, and White, 2002).

$$\gamma = \frac{\Delta\rho g D^2}{H} \tag{2.3}$$

$$\gamma = \frac{\Delta\rho g z_e^2}{2} \tag{2.4}$$

Where γ is the surface tension; $\Delta\rho$ is the difference in density of the two phases; g is the force of gravity acting on the droplet; D is the equatorial diameter; H is the shape dependent parameter, which can be obtained from shape factor tables available in the literature (Rusanov and Prokhorov, 1996; Staufer, 1965), and z_e is the height from the top of the drop to its equator for the sessile drop (Drelich et al., 2002).

It is worth noting that the main limitation for using pendant and sessile drop methods is their low sensitivity for low surface tension systems (Sagis and Scholten, 2014). It is often possible to combine the surface tension measurement, using the pendant drop approach, with surface rheology analysis, whereby the sample dosing system allows inducing deformations to

the interfacial area by fluctuating the volume of the drop at controlled amplitude and frequency – the resultant response to the sinusoidal compression and expansion of the interface is related to the elasticity and flexibility of the interface. Kaltsa, Paximada, Mandala, and Scholten (2014) used the pendant drop method to study the evolution of interfacial pressure at O/W interfaces in systems containing WPI, Tween 20 and a 1:1 mixture of the surfactants. Using this method, those authors were able to record faster rate of adsorption and lower final interfacial tension for the L_{Mw} surfactant, compared to the protein; the authors were also able to observe reorganisation of molecules at the interface, where protein were displaced by the L_{Mw} surfactant. Dombrowski, Johler, Warncke, and Kulozik (2016) used the pendant drop method to study the surface tension and the dynamics of surface adsorption for soluble aggregates of β-lg, prepared under different ionic strength and pH conditions, at the A/W interface. Those authors measured the interfacial dilatational properties of films formed by β-lg aggregates by drop oscillation. Effectively, the information on surface behaviour of these aggregates generated using the pendant drop approach was correlated to their foam forming and stabilising properties.

Drop detachment is a more traditional variant of the pendant drop method, where the force of gravity is utilised to measure the surface/interfacial tension; in this approach the weight or volume of the liquid required to detach the drop from the needle is measured. This information is then calculated to give surface tension; the force required to detach the droplet is directly proportional to its surface tension (i.e., higher γ will require the drop to be bigger/heavier) (Dunkhin, Kretzschmar, and Miller, 1995). This method was traditionally used to study the surface and interfacial tension before more advanced, camera-based devices became available. The spinning drop method is a more advanced variant of the liquid drop shape analysis, used for a low interfacial tension (γ_I) range; in this method drop deformations are measured as a result of radial pressure gradients acting on the drop in a rapidly spinning tube (Fig. 2.2 c). The spinning drop method is used for studying the interfacial tension between two liquids, where the continuous heavy phase is placed in the measurement cell (i.e., transparent horizontal tube) and a drop of the

lower density phase is injected into the tube as it is spinning (Thiessen and Man, 2000). The extent of drop deformation (i.e., elongation) is inversely proportional to the γ_I; the higher the γ_I the more spherical the shape of the spinning drop. Jeirani et al. (2013) used the spinning drop method to measure the γ_I of model triglyceride microemulsion systems consisting of palm oil and de-ionised water. That approach was used to screen co-surfactants (Tween 20, Tween 80, glycerol, sorbitan monooleate, glyceryl monooleate and saponin) for their ability to achieve ultra-low γ_I values (i.e., <0.01 mN m^{-1}). Those authors were able to measure very low γ_I values, where the lowest γ_I, 0.0002 mN m^{-1}, was reported for the microemulsion system stabilised by glyceryl monooleate.

Analysis of interfacial rheology typically involves a modification of one of the approaches discussed above; it allows the generation of dynamic information on the adsorbed layer, its flexibility and strength (i.e., viscoelastic properties) by measuring the deformation of the interface as a function of force and time (Bos and van Vliet, 2001; Karbaschi et al., 2014). The equipment and methodology used to study interfacial rheology is, generally, based on the drop volume/shape approaches used for studying surface/interfacial tension, with modifications to allow the exertion of a deformation force on the interface and measuring the responses of the interface to such a force. Studying interfacial rheology can provide valuable information on different surfactants and mixed surfactant systems, especially for applications in emulsion processing stability (i.e., rigidity of the interfacial layer and stability against coalescence on high impact collisions) or in foam stability, where the voluminous and relatively heavy food structure is supported by a network of interfacial films. The influence of food emulsifiers on the rheological properties of interfaces in food systems have been reviewed by Murray (2002) and Karbaschi et al. (2014). Tamm and Drusch (2017) measured the rheological properties of O/W interfaces in systems containing β-lg with different extents of hydrolysis (3 and 6%) and pectins with different extents of methoxylation (32, 35 and 64%). In that study, the authors reported differences in the rheological properties of the samples as affected by interfacial layer composition; native β-lg/pectin complexes displayed higher dilatational elastic moduli compared to

hydrolysed β-lg/pectin; however, the hydrolysed β-lg/pectin complexes exhibited greater strength of the interfacial layer on application of shear (i.e., time and amplitude sweeps), compared to the native β-lg/pectin systems. The authors also reported interactions between pectins and proteins/peptides as evidenced by measured differences in their adsorption rate. Hong and Fischer (2016) used the interfacial rheology approach to study the differences in the properties of interfacial layers stabilised by hydrophobic and hydrophilic colloidal particles (clays) and related these properties to stability of canola O/W emulsions. Those authors found that stabilisation of emulsions by a mixture of both hydrophilic and hydrophobic colloids resulted in strong association between the two and, effectively, significantly increased interfacial modulus improving stability to coalescence of the emulsions.

Analysis of the interfacial properties of emulsions can provide valuable information like emulsifier adsorption rates, compatibility between emulsifiers in a multi-emulsifier system or the strength of the interfacial layer, for producing a homogenous and stable emulsion system. However, it must be noted that these analyses have different limitations (e.g., unsuitability for low γ values, only model interfaces analysed) and they also measure changes at the interface (e.g., adsorption and displacement) over a relatively short period of time and do not necessarily relate to changes that can take place in these systems over storage and shelf life. Hence, it is advised that this approach is combined with other analyses that measure changes in the system during processing or storage.

2.3. Emulsion quality – homogeneity and size distribution

The size of oil globules in an emulsion is a major factor governing the quality of the system and its stability to gravity-induced separation as described by Stokes law. Emulsion quality and shelf life stability is strongly related to its homogeneity, specifically the size and distribution of oil globules in the emulsion system. Thus, formation of an emulsion with sufficiently small oil droplets is a key initial requirement for stability of such systems; typically, emulsions with mean oil globule diameter < 1 μm, that display a monomodal distribution, are considered to be stable to phase separation (McClements,

1999). The size distribution of oil globules in food emulsions is also important for their sensory properties; parameters such as mouthfeel, flavour release and colour are related to the fat globule size distribution (FGSD) of the system (Benjamins, Vingerhoeds, Zoet, de Hoog, and van Aken, 2009; van Aken, 2010).

It is essential to be able to measure and/or predict the quality of an emulsion after homogenisation, it is also important to monitor changes that can take place in the system during processing (e.g., heating, pumping and short-term storage) and during the desired product shelf life. The main approach to track these changes is by monitoring changes in the FGSD using static light scattering (i.e., laser diffraction), where particles dispersed in a liquid medium (typically water) scatter laser light at different angles, depending on their size. Generally, large particles scatter light at narrow angles, and small particles scatter light at wide angles; the intensity of detected scattered light is then calculated to provide information on the particle size distribution. Laser diffraction instruments (i.e., Mastersizer, Malvern Instruments Ltd., Malvern, UK), used for measuring the FGSD, operate within the size range between 10 nm to 3500 μm, which encompasses the typical oil globule size range for food emulsions. The presentation of FGSD results obtained with laser diffraction instruments typically includes a size distribution profile (Fig. 2.1 i), values for particle size at the 10, 50 and 90% quantiles of the distribution ($D_{v,0.1}$, $D_{v,0.5}$ and $D_{v,0.9}$, respectively), particle mean diameters (volume-based, $D_{4,3}$ and surface area-based, $D_{3,2}$), specific surface area and distribution span. The size distribution profile gives a visual representation of the distribution of oil globule sizes in the emulsion samples based on the volume rather than the number of particles, meaning that the presence of a small number of large oil globules is not obscured by the presence of a large number of small oil globules. The use of distribution profiles for presentation of the FGSD results is useful for assessing the homogeneity of the system and tracking changes in the system caused by interactions between its components (i.e., monomodal *vs* bi- or multi-modal distribution). Van der Ven, Gruppen, de Bont, and Voragen (2001) used size distribution profiles to compare the emulsifying properties of different casein and whey protein hydrolysates, where depending

on the degree of hydrolysis and the hydrolysis conditions, different profile modes (i.e., mono- and bi-modal) and the width of distributions of oil globules in resultant emulsions were observed. It may sometimes be useful to construct a cumulative distribution plot, to show the population size at a given percentile value; such representation of the FGSD data allows comparison of the range (i.e., span) of size distribution between samples, which is especially useful in systems displaying a monomodal size distribution. The cumulative distribution profiles can also be used to track changes in the FGSD in response to adverse environmental conditions or prolonged storage. Kasran, Cui, and Goff (2013) used this approach of data presentation to discuss stability of emulsions made using non conjugated and conjugated (with fenugreek gum) soy whey protein during 28 d of storage; similarly, in the work of Shimoni, Shani Levi, Levi Tal, and Lesmes (2013), cumulative distribution profiles were used to study stability of emulsions containing lactoferrin nano-particles to gastric digestion. Calculating the span of the distribution is another way to show the range of the size distribution of particles in a sample; it is given by the following relationship between the three quantile parameters (Equation 2.5).

$$Span = \frac{D_{v,0.9} - D_{v,0.1}}{D_{v,0.5}} \tag{2.5}$$

The information on the particle size at the extremities of the distribution can be extracted by studying the $D_{v,0.1}$ and $D_{v,0.9}$ parameters; these parameters are important when monitoring changes in sample FGSD (e.g., after certain treatments or during shelf life stability testing). Since the $D_{v,0.1}$ parameter is sensitive to the fraction of small particles, its increase can indicate an assembly and formation of particles from small components of the sample, that were not detectable or included in the initial profile (i.e., before the treatment or at the initial time point of storage). Alba, Ritzoulis, Georgiadis, and Kontogiorgos (2013) showed that an increase in $D_{v,0.1}$ for model Okra-based acidic (pH 3) emulsions during storage can be used as an indicator of Ostwald ripening, where a certain population of oil globules grows in size at the expense of other,

smaller globules in the system. Conversely, an increase in the $D_{v,0.9}$, with little changes to the other quantile parameters would typically indicate formation of a small number of large particles in the sample. In an emulsion system, this normally indicates flocculation or coalescence of oil globules (Drapala, Auty, Mulvihill, and O'Mahony, 2015; Łuczak and Fryźlewicz-Kozak, 2013; McClements, 2015). When the $D_{v,0.9}$, increases, the choice of the dispersing media when using the FGSD analysis allows one to identify which of the two undesirable processes (i.e., coalescence or flocculation) is taking place in an emulsion system; dissociating media like sodium dodecyl sulphate (SDS) has been used to discriminate between reversible and irreversible interactions between oil globules (Bazmi, Duquenoy, and Relkin, 2007; Liang et al., 2014). If the increase in FGSD is reversible upon the use of dissociating agent, the interactions are due to flocculation of oil globules; conversely, an irreversible increase in the FGSD would typically indicate coalescence.

The two different approaches for calculating the mean particle (i.e., oil globule) diameter are based on the particle volume ($D_{4,3}$, de Brouckere mean diameter) or on the particle surface area ($D_{3,2}$, Sauter mean diameter). $D_{4,3}$, is the most commonly used parameter when discussing FGSD results as it shows where the mass/volume fraction of the system lies; the $D_{4,3}$ is sensitive to changes at the extremes of the distribution (i.e., especially the large particles with large volume) as well as the span and the homogeneity of the distribution. The $D_{4,3}$ parameter is suitable for screening of emulsifiers for their ability to form small oil globules during homogenisation and to prevent interactions between oil globules on processing and storage; small changes in the size distribution can be easily detected with this volume-based mean parameter (Martinet et al., 2005; Mwangi, Ho, Tey, and Chan, 2016; Ye, Hemar, and Singh, 2004). Conversely, $D_{3,2}$ is more sensitive to the small particles in the distribution (i.e., those with greatest surface area to volume ratio) and it is less often used in describing FGSD of emulsions. One of the examples where the use of the $D_{3,2}$ parameter is useful is in work from Van der Meeren, El-Bakry, Neirynck, and Noppe (2005), where the authors measured particle size distribution of coffee cream soybean oil-based emulsions containing protein and lecithin before and after heat treatment; two separate techniques were

used by those authors, photon correlation spectroscopy and static laser light diffraction, to obtain harmonic intensity-weighted average hydrodynamic diameter and $D_{3,2}$, respectively. In that work, the authors reported increases in the intensity-weighted diameter and no change in the $D_{3,2}$, from which they concluded deposition of protein on the surface of oil globules in these emulsions as an effect of heat treatment (i.e., in-container sterilisation; >12 min at 119°C, 45 min total heating time). Similarly, McCarthy et al. (2012) used the $D_{3,2}$ parameter for correlation with the protein load at oil globule interfaces in model infant formula emulsions. Those authors reported an increase in the $D_{3,2}$ with decreasing protein:fat ratio and parallel decrease in the specific surface area of oil globules; however, no differences in the protein load were reported for those systems. Specific surface area measured by laser diffraction can provide indirect information on changes in the particle size within the population, i.e., large surface area would indicate large number of small particles and the opposite for small surface area. However, measurement of surface area using laser diffraction does not take into account the porosity and topography of the surface and other methods (e.g., Brunauer–Emmett–Teller relationship or mercury porositometry methods used for solid particles) might be more appropriate if the surface area is the principal focus (Alghunaim, Kirdponpattara, and Newby, 2016; Arvaniti et al., 2014; Williams, 2007).

A significant volume of information can be generated using the laser diffraction technique for an emulsion system; it is often possible to discriminate between emulsion-related destabilisation pathways such as coalescence, flocculation and Ostwald ripening as well as provide information on the performance of protein-based emulsifiers in these systems (e.g., heat-induced aggregation, protein-mediated bridging flocculation and changes in the interfacial protein load). Euston, Finnigan, and Hirst (2001) measured the rate of apparent aggregation of emulsion globules by monitoring the change in the ratio between initial number of emulsion droplets and the number of emulsion droplets after heating; they established that the aggregation of oil globules in emulsions stabilised by whey protein follows the same order of kinetics (i.e., 1.5) as observed for heat-induced aggregation of β-lg. Liang et al. (2014) studied the influence of different sugars (i.e., glucose, maltose,

trehalose and maltodextrin) on emulsification and heat stability of milk protein concentrate (MPC)-based O/W emulsions. Those authors used FGSD analysis to track the evolution in particle size distribution in those emulsions as a function of heating time (0-20 min at 140°C); the authors reported changes to the casein micelle and oil globule populations as a result of the heat treatments, as evidenced by a shift from bimodal to multimodal size distribution and a progressive shift of the peaks towards the large particle region of the size distribution profile as the holding time increased. Chevallier et al. (2016) studied the influence of whey protein microgel (WPM) particles on the heat stability of emulsions containing whey proteins; the authors found that the presence of whey proteins (WPM or native) at the surface of oil globules resulted in decreased heat stability compared to emulsions where oil globules were stabilised by caseins, as evidenced by heat-induced increases in particle size and changes in the microstructure of emulsions. However, the presence of WPM in the serum phase of casein-stabilised emulsions allowed formation of heat-stable whey protein rich emulsions. Laser diffraction is often used in combination with other analytical techniques, (e.g., microscopy, creaming velocity, protein load analysis or zeta potential analysis) to provide more holistic information about the system and changes that take place within. Some of these other approaches will also be discussed in this review.

2.4. Phase separation and emulsion shelf life

Emulsions display an inherent tendency to destabilise by phase separation owing to the differences in the density between their main components (lipid and water). According to Stokes law (see Equation 2.1 in Section 2.1), the rate of separation is influenced by four parameters (density, viscosity, particle size and acceleration due to gravity). From these four parameters, three are directly related to the emulsion system itself and can be, to some extent, controlled by the formulation and the processes used (phase densities, oil globule size and viscosity of the dispersed phase). Since most emulsions are produced using either innate or added emulsifiers in the formulation and high pressure homogenisation (typically), the initial emulsion quality is generally good and it is difficult to determine its creaming rate, and effectively shelf life, under ambient storage conditions. Hence, approaches utilising exaggeration of

external conditions (i.e., centrifugation, increased temperature) are used to facilitate generation of information on emulsion stability in a reasonable time frame.

In this review, the approach for measuring emulsion stability to creaming under accelerated conditions will be discussed. A range of commercially available analytical centrifuges (L.U.M. GmbH, Berlin, Germany) allow for acceleration of the phase separation process by controlling the centrifugal speed and the temperature while live monitoring movement of the particles (e.g., oil globules and protein particles) through the measurement cell. The principle of this method has been comprehensively detailed by Lerche and Sobisch (2007; 2011); in summary, a liquid sample is introduced to a transparent measurement cell and placed in the centrifuge. Transmission of light through the cell is measured as a function of time and movement of the particles towards the top (creaming) or bottom (sedimentation) of the cell can be observed. Collected data is typically presented as STEP profiles (space- and time-resolved extinction profiles), detailing the changes in the light transmission through the cell during the analysis, integral transmission (cumulative changes in the transmission through the cell) and front tracking (movement of the phase boundary) profiles, as shown in Fig. 2.1 iii. Creaming velocity of the sample can be calculated from the front tracking profile and recalculated to a corresponding creaming velocity under ambient gravity forces (Drapala et al., 2016b); similarly the evolution of the thickness of the cream layer can be calculated from the front tracking profiles. It should be noted that the accelerated separation approach speeds up the separation of components in the system due only to size of particles in the dispersed phase, viscosity of the continuous phase and the difference in density between the dispersed and continuous phases. In addition, progressive creaming and sedimentation can result in changes in viscosity during the course of the analysis.

Application of the analytical centrifuge approach for emulsion systems can be useful when screening effectiveness of emulsifiers or when reformulating existing products, where it can provide information on the effects of the new ingredients on the creaming rates of the system, compared to the original

formulation. Liu et al. (2016) studied the stability of walnut oil emulsions to various unit operations, changes in composition and storage conditions such as freeze-thaw cycles and pH fluctuation; these authors used the integral transmission data to investigate the effects these conditions had on the creaming behaviour of emulsions as a function of the emulsifier used. Shimoni et al. (2013) reported on Pickering stabilisation of olive oil-based emulsions by lactoferrin nanoparticles; those authors used the STEP profiles and calculated creaming velocities to describe differences in the stability of those emulsions after homogenisation and after *in vitro* acid digestion. In that study, the authors used the separation velocity data to calculate the harmonic mean droplet size, as detailed previously by Detloff, Sobisch, and Lerche, (2006) and Lerche and Sobisch (2011), and correlated the size of oil globules with their creaming rates and zeta potential. Those authors reported that the nanoparticles were more successful in stabilising coarse (mean oil globule size ~ 65-85 μm) emulsions than fine (mean oil globule size ~ 4-6 μm) emulsions; it was also reported that addition of carrageenan significantly increased the zeta potential (ζ) of oil globules and reduced the creaming rates in fine emulsions stabilised by the nanoparticles, however, no viscosity data was provided for those systems. Similarly, Lei, Liu, Yuan, and Gao, (2014) used the analytical centrifuge approach to study the effect of emulsifier type on the physicochemical properties of β-carotene emulsions; the authors correlated changes in the system viscosity and oil globule size with the differences in their creaming rates. In another study, Meshulam, Slavuter, and Lesmes (2014) investigated destabilisation of inulin-stabilised emulsions by saliva additon and they showed that the creaming velocity of emulsions were not affected by differences in the ζ of oil globules achieved by changing the system pH (i.e., pH 2-10). This finding is not surprising as accelerated conditions used in the analytical centrifuge approach cause movement of the system components in accordance with Stokes law (unless the movement takes place in an electric field). Hence, the changes typically observed under quiescent conditions, such as interactions of oil globules due to low electrostatic repulsion, are excluded from the analysis due to its time-scale being too short for their role to be important in separation. On the other hand, modification of the steric stabilisation system for oil globules can potentially yield different stability to

separation under accelerated conditions owing to increased thickness of the interfacial layer (i.e., increased surface load of high density components), effectively slowing down the separation due to lowered density difference between the dispersed and continuous phases (van Lent, Le, Vanlerberghe, and Van der Meeren, 2008; Klein et al., 2010). Extensive protrusion of the steric layer on the surface of oil globules into the aqueous phase can potentially affect the separation rate by retarding the upward motion of oil globules in the emulsion. Stability of an emulsion to creaming can be modified by controlling the density of its constituent oil droplets, which can be achieved by selection and/or modification of the oil component to allow effective interactions of its carbonyl groups with polar functional groups of emulsifiers (e.g., amine, amide, carboxyl and hydroxyl groups) (Klein, Aserin, Svitov, and Garti, 2010). Another strategy for controlling the oil globule density was shown by Ruiz-Rodriguez, Meshulam, and Lesmes (2014), who studied the effect of incorporation of silica nanoparticles into oil globules on emulsion creaming rates. These authors showed that by increasing the density of the dispersed phase they were able to decelerate creaming and even achieve sedimentation of oil globules in an aqueous phase at high levels of inclusion (≥ 1%, w/w) of silica nanoparticles. In an analogous manner, an increase of the protein load at the emulsion interface will give rise to increased density of emulsified oil globules and, effectively lower difference in the densities between the two phases, resulting in slower creaming rates (van Lent et al., 2008).

Based on the same principle, analytical centrifugation can provide information on the rate and extent of sedimentation in an emulsion system, where deposition of material at the bottom of the product container is, generally, undesirable. This can often be the case in nutritional beverage formulations (e.g., infant, sports nutrition or elderly nutritional products), often produced from dried protein base ingredients, where the protein ingredient can display poor solubility and undergo changes in hydration during beverage storage (Crowley et al., 2015; De Wit, 1990; Pelegrine and Gaspareto, 2005). Crowley, Kelly, and O'Mahony (2014) discussed sedimentation rates for casein micelles in reconstituted skim milk powder, which were found to increase with calcium fortification, as measured with the analytical centrifugation approach.

Sedimentation is often a considerable challenge for plant protein-based formulations and since such products have been gaining significant commercial interest, it is of relevance to measure and predict both creaming and sedimentation behaviour in such emulsion-based products. In a study by Makinen, Uniacke-Lowe, O'Mahony, and Arendt, (2015) separation rates for bovine milk and plant-based milk substitutes were compared using integral transmission profiles. The authors reported that creaming was the main mechanism of separation for the bovine milk, while creaming and sedimentation were observed for the plant-based systems. The accelerated stability approach has also been used to study the reconstitution properties of model infant formula powders, where the measured sedimentation and creaming rates provided information on the quality of the product (Murphy et al., 2015).

Another interesting approach was shown by Iritani, Katagiri, Aoki, Shimamoto, and Yoo (2007), who studied the kinetics of floatation of oil globules in an emulsion using a correlation between creaming rates and surface area and porosity of interfaces of oil globules. It should be pointed out that, despite the fact that the accelerated separation technique is often used to investigate shelf life stability of emulsions, it can only reflect separation based on the current-state properties of the system, without considering changes that take place in complex multi-component systems (e.g., coalescence, flocculation, aggregation, competitive displacement) over time and which are influenced by storage conditions (e.g., temperature and time). Thus, when the creaming behaviour of the product during its shelf life is the focus, single point testing is not enough to provide sufficient information and sample incubation needs to be built into the experimental design. Storage of emulsion samples at one or more elevated temperatures (compared to normal storage conditions for the product) can be coupled with testing samples at different storage times for creaming velocity in order to construct a stability map as a function of time (and storage temperature) (Lerche and Sobisch, 2011). This approach allows data collection for the evolution of creaming rates in the product stored and tested under accelerated conditions, to give a predictive shelf life stability model for a given system. Effectively, a correlation can be developed to

estimate product shelf life under its typical storage conditions (Lerche and Sobisch, 2011).

2.5. Interactions between emulsion components – rheological characterisation

Rheological properties of liquid food systems describe their flow behaviour and viscosity characteristics and these strongly dictate the processing behaviour and shelf life stability of liquid foods, as well as appearance, texture, mouthfeel and flavour release (Fischer and Windhab, 2011). Studying the rheological properties of an emulsion can provide indirect information on the size and shape of its components as well as on the interactions between these components (e.g., oil globules, protein aggregates, hydrocolloids). A typical approach for measuring the rheological properties of a liquid is to measure the resistance of the system to applied stress (e.g., rotational or oscillatory stress), where the stress causes deformation of the system; extensive literature is available on the fundamental principles of the approach (Chung and McClements, 2014; Erni, Fischer, and Windhab, 2007; McClements, Monahan, and Kinsella, 1993; Norton, Spyropoulos, and Cox, 2010) and only selected relevant concepts will be considered in this review. The greater the force resisting deformation, the higher the viscosity of the system and the linearity of the system deformation depends on the interactions of its components. Lack of such interactions results in a linear response (i.e., typical for water; Newtonian flow, where the response of apparent viscosity to shear rate is linear), while a nonlinear response indicates disruption (shear thinning) or formation (shear thickening) of inter-component interactions on application of the stress. Shear thinning behaviour is typical for food protein systems, owing to associative interactions between proteins, forming weak structures that are interrupted/broken when the sample is sheared (Williams, 2007). In general, the magnitude of shear thinning behaviour increases with increasing protein concentration. While dilute emulsions (Sünder, Scherze, and Muschiolik, 2001), typically display near-Newtonian flow behaviour; a deviation from Newtonian behaviour to shear-thinning is usual for concentrated emulsions (Liang, Patel, Matia-Merino, Ye, and Golding, 2013). Effectively, increasing the concentration of dispersed phase particles (e.g., oil

globules, protein and other constituents), changes the rheological properties of the system due to increasing potential for interactions between the dispersed particles (Fischer, Pollard, Erni, Marti, and Padar, 2009). The viscosity of emulsions increases with increasing concentration of the dispersed phase (i.e., volume fraction, ϕ), where increasing ϕ can result in a change in the flow regime from turbulent to laminar flow (Tadros, 2013), until a critical ϕ is reached (~0.63). At ϕ greater than the critical ϕ the emulsion cannot flow easily due to the high packing density of globules (Piorkowski and McClements, 2014). Different geometries can be used on viscometers and rheometers, depending on the sample (e.g., parallel plate, concentric cylinder, cup and bob with single and double gap design), where the sample is sheared between the stationary and moving components. The rotational approach is generally used for studying the viscosity and flow behaviour, while the oscillatory approach is sometimes used to provide information on the viscoelastic properties of the system without applying rotational shear; the latter is often used for concentrated, high volume fraction systems (Tadros, 2013).

The rheological properties of emulsions are dictated by the concentration, shape, and size of constituents of the dispersed phase as well as their ability to participate in various interactions (e.g., hydrophobic interactions, covalent bonds, electrostatic and steric interactions) with other components. Such interactions are inherent to food-based emulsions in liquid format, which are generally comprised of a heterogeneous mixture of biopolymers. In particular, food emulsions contain a variety of particles with a broad range of sizes and structures; oil globules, proteins (e.g., casein micelles, globular protein, denatured and aggregated protein), carbohydrates, L_{Mw} emulsifiers and other smaller components (ions, vitamins) that can influence the flow behaviour and viscosity of emulsions. In O/W emulsions, oil globules are, typically, the largest physical components; theoretically, a spherical shaped particle has minimal effect on flow behaviour as it does not cause high disturbance in flow, compared to irregularly shaped particles that tend to rotate before assuming an optimal orientation in regards to direction of the flow (Mueller, Llewellin, and Mader, 2010; Sandler and Wilson, 2010) (Fig. 2.3). In addition, the

spherical conformation would, in theory, indicate a minimal extent of steady state interactions, as opposed to components with a linear or branched conformation (i.e., polypeptide chain of protein or polysaccharide chain of carbohydrates) (Neelima, Sharma, Rajput, and Mann, 2013; Nobel, Weidendorfer, and Hinrichs, 2012). However, the interfaces of oil globules are seldom smooth and inert since they are populated by surface-active species (e.g., proteins, peptides, L_{Mw} surfactants), often making their contribution to the rheological properties of the system quite significant. Oil globules can often participate in interactions with other globules (e.g., flocculation, aggregation, complexation) or with components of the serum phase (e.g., aggregation, segregative separation and complexation), or a combination of both (Chevallier et al., 2016; Drapala et al., 2016a, 2016b; Liang et al., 2013). These interactions are particularly important during unit operations applied in the preparation of food emulsions (e.g., heating, pumping, short-time storage and spray drying). Thermal processing typical for these systems (thermisation, pasteurisation, UHT) causes an increase in protein-mediated interactions due to protein denaturation and aggregation (Chevallier et al., 2016; Liang et al., 2013); the increase in the temperature also provides higher mobility of solutes in the liquid, thus accelerating the rate of physicochemical changes in the system (interactions at the surface, surfactant exchange) (Ryu and Free, 2003). Buggy, McManus, Brodkorb, McCarthy and Fenelon (2017) studied physical stability of model infant formulae emulsions enriched with α-lactalbumin by measuring viscosity of emulsions as affected by the process configuration, where heat treatment (65°C × 30 s) was carried out either before or after homogenisation (2-stage, 21 MPa total pressure). Those authors reported that a process where heat treatment was carried out post-homogenisation, resulted in significantly higher viscosity of emulsions, compared to that where heating was carried out prior to homogenisation; these differences were also reflected by the presence of significantly bigger protein/lipid particles (as measured by laser diffraction and electrophoresis) in the former systems. Liang et al. (2013) used steady-state flow measurements (shear rate ramp from 0.001-500 s^{-1} using cup and bob geometry) to assess the ability to limit interactions between emulsion components on heating by heat-induced pre-aggregation of whey proteins in

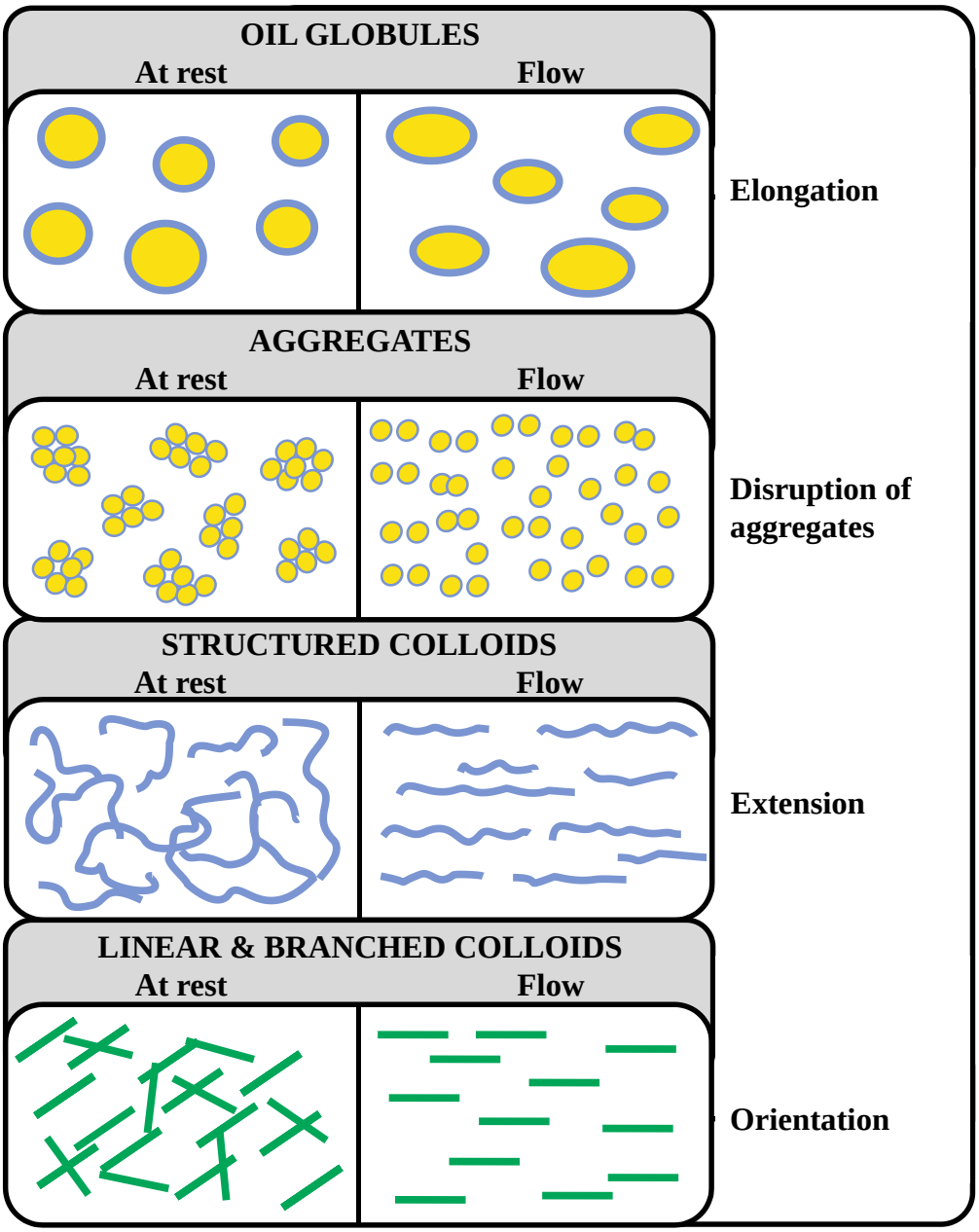

Figure 2.3. Schematic representation of re-arrangement of macrocomponents in food emulsions (e.g., oil globules, proteins/peptides, carbohydrates) as affected by application of shear (i.e., horizontal flow). Flow behaviour of food emulsions is affected by the changes in the orientation/structures of its macrocomponents: deformation (i.e., elongation) of oil globules, disruption of aggregates, extension of structured colloids (e.g., proteins/peptides) and orientation of linear/branched carbohydrates.

solution for MPC-stabilised emulsions. Those authors used viscosity to monitor protein-protein interactions in both the protein solutions and in the final emulsions, reporting lower apparent viscosity in emulsions stabilised by pre-aggregated protein. They were also able to identify reversible changes in these emulsions on heating, as exhibited by a shear thinning behaviour, which they associated with disruption of heat-induced oil globule clusters on shearing; confocal laser scanning microscopy was also used to support these observations. In another study, Liang et al. (2014) compared the effects of addition of different sugars (glucose, maltose, trehalose, sucrose) on the heat stability of MPC-stabilised emulsions. A range of rheological measurements was used by the authors (shear rate ramp from 0.001-500 s^{-1}; flow behaviour analysis, shear rate ramp 1-1000 s^{-1}; and viscosity index, constant shear rate of 1 s^{-1}, using cone and plate geometry) to study the steady-state flow properties, the effect of viscosity ratio between the dispersed and continuous phases on the droplet break-up in emulsions and the attractiveness between the system components for unheated and heated samples. Integration of these rheological approaches with particle size distribution and microstructural analyses allowed the authors to identify aggregation of casein micelles, oil globules and mixtures of both and determine if these interactions were reversible or not by using a dissociating agent (1 mM Tween 20 and 12.8 mM EDTA at pH 10). In a study by Lei et al. (2014), the authors used rheological flow behaviour analysis for β-carotene-containing emulsions stabilised by chitosan, physical complexes of chitosan and epigallocatechin-3-gallate (EGCG) and chitosan-EGCG conjugates to provide information on the shear and yield stress, consistency index and flow behaviour index of these systems. The authors related the observed differences in the creaming stability of these emulsions to their different rheological behaviour in accordance with Stokes law, reporting slower emulsion separation rates for systems stabilised by the chitosan-EGCG complexes, which displayed non-Newtonian flow behaviour and had higher viscosity. The authors also used the viscoelastic response of the emulsions to predict the extent of flocculation of oil globules in the emulsions.

Shear-thinning behaviour of dilute emulsions has been associated with disruption of weak interactions between the components of the continuous

and dispersed phases in an emulsion system (Ruiz-Rodriguez et al., 2014); it can be used to discriminate between reversible and irreversible (i.e., bridging flocculation) associations between oil globules in an emulsion (Dickinson, 2001). A different approach to study the rheological properties of emulsions was reported by Degrand, Michon and Bosc (2016), where they used multi-speckle diffusing-wave spectroscopy (MS-DWS) technology to characterise evolution of stability in rapeseed oil O/W emulsions stabilised by Tween 20 or proteins from whey protein concentrate (WPC). These authors used the elasticity index as a parameter for describing the rheological behaviour of emulsions, which facilitated tracking local organisation of oil globules in the emulsion. Effectively, that study used MS-DWS to successfully monitor flocculation of oil globules (bridging flocculation or depletion-flocculation) and protein network rearrangement. The conclusion of that study was that the MS-DWS approach was useful for studying depletion-flocculation, destabilisation kinetics and network packing in emulsion systems.

2.6. Modes of oil globule stabilisation

Electrostatic repulsion is one of the primary mechanisms of stabilisation of milk protein-based emulsions owing to the charge on the protein adsorbed at the interfaces of oil globules. In an environment where the pH is away from the isoelectric point (pI) of proteins, they possess a charge (i.e., a net negative charge at pH > the typical pI range of 4.6-5.2 for dairy proteins). Such charged interfaces provide repulsion between oil globules and effectively prevent their coalescence and globule-globule interactions post-homogenisation (Damodaran, 2005). The electrostatic charge (zeta potential, ζ) is highest at a pH far from the pI of the emulsifiers and is also influenced by the type and concentration of salts (i.e., ionic strength of the system); hence, it is important to characterise the potential of an emulsifier to exert an electrostatic barrier under desired environmental conditions. Zeta potential is typically determined by measuring the velocity of particles in an electric field, hence the devices available for ζ analysis normally combine light scattering and electrostatic field approaches. During a ζ measurement, a dilute liquid sample is placed in a sealed cell containing two electrodes, and as current is passed through the sample the movement (i.e., electrophoretic mobility) of particles

is measured and expressed as ζ. A strong relationship between ζ of oil globules and system pH in nanoemulsions stabilised by L_{Mw} surfactants (lecithin and saponin) has been shown by Ozturk, Argin, Ozilgen, and McClements (2014); those authors showed a significant decrease in the ζ, from -60 to -5 mV on reducing pH from pH 8 to pH 2, with a concomitant decrease in stability of the emulsions. Aggregation of oil globules at high salt concentrations (i.e., >100 mM NaCl) in those nanoemulsions, as a result of electrostatic screening, was also reported. The electrostatic screening effect describes accumulation of monovalent ions of an opposite charge at the surface of the oil globules, neutralising the charge on the globules and effectively disabling the barrier protecting the emulsion from globule-globule interactions. On the other hand, the presence of divalent ions in an emulsion can facilitate flocculation of oil globules by formation of crosslinks, mediated by such ions (Dickinson, 2001). The electrostatic barrier is sensitive to the composition and properties of the emulsion interfacial layer, which is prone to changes during processing (e.g., heat-induced protein deposition) or storage (e.g., surfactant displacement). Modification of the emulsifier or the interfacial layer to control the ζ of oil globules is a commonly-used strategy; in work by Lei et al. (2014), cationic L_{Mw} chitosan was modified by physical complexation and covalent conjugation with a polyphenol, and it was shown that emulsions formed with those complexes and conjugates did not vary in ζ from emulsions stabilised by native chitosan; they displayed greater storage stability compared to the native chitosan-stabilised emulsions, owing to steric stabilisation.

Nowadays, consumers seek new sensory experiences, which often extend beyond the traditional, neutral pH dairy-based beverages; these applications pose challenges for emulsion stability due to lower electrostatic repulsion between protein-stabilised oil globules, which possess little charge close to their pI. In an effort to meet that demand, food manufacturers need to look for science-based approaches in order to address the challenges encountered with stability of such products. Designing the emulsion interfaces to control its charge, in order to confer electrostatic stabilisation during processing, typically involves the measurement of ζ of the oil globules. Similarly, emulsion stability to acidic environment is often needed when using the oil globules as

delivery vessels for sensitive bio-components, where they have to be stable under the acidic environment of the stomach. Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, and Dewettinck (2004) studied the electrophoretic mobility (i.e., ζ) of conjugated WPI and anionic pectin near the pI of β-lg (pH 5.5) to determine their ability to form and stabilise O/W emulsions at these adverse conditions. Improvement in stability of emulsions formed with the conjugated WPI at the acidic pH, was reported, owing to the greater negative charge of oil globules in that environment compared to emulsions stabilised by WPI alone. Manipulation of the electrostatic charge of oil globules in an emulsion can also be achieved using multilayer deposition of charged species at the emulsion interface, as shown by Zhao, Wei, Wei, Yuan, and Gao (2015), where stabilised O/W emulsions with the cationic protein lactoferrin and formed a secondary interfacial layer by electrostatic adsorption of anionic polysaccharides (from pectin or soybean). They reported changes in the ζ of oil globules as a function of lactoferrin and polysaccharide concentrations, which ranged from +40 to -40 mV. A similar approach was investigated by Liu, Wang, Sun, McClements and Gao (2016), where layer-by-layer electrostatic deposition of lactoferrin (cationic) and polyphenolics (anionic) was performed for β-carotene emulsions. A number of variants of the interface with different deposition sequences were tested by the authors and these interfacial configurations were discussed in the context of electrostatic and steric repulsion and resultant stability to heating and storage of the emulsions. Shimoni et al. (2013) showed that increasing the ζ of oil globules by complexation of lactoferrin nanoparticles with carrageenan yielded good stability of those systems to gastric conditions; the study specifically identified the benefits of synergies between electrostatic and steric stabilisation for emulsion stability in an acidic environment. Phoon, San Martin-Gonzalez, and Narsimhan (2014) investigated differences in the oxidative stability of Menhaden fish oil-in-water emulsions stabilised by hydrolysates of soy β-conglycinin under a wide pH range (pH 3.0 – 12.5) as affected by the ζ of proteins/peptides and conformation of the proteins/peptides at oil-mimicking, functionalized silver surfaces. These authors reported that the modification of the surface charge of the oil globules from non-polar to anionic led to changes in protein/peptide conformation at the interface (i.e., increased

formation β-sheet); however, they reported no improvement in the oxidative stability of emulsions with increasing the ζ of oil globules. Adjonu, Doran, Torley, and Agboola (2014) compared ζ of oil globules in emulsions stabilised by intact and hydrolysed (degree of hydrolysis 10-11%) WPI and reported higher electrostatic stabilisation of the intact WPI-based emulsions, which gave the emulsions enhanced stability during storage compared to emulsions formed with the hydrolysed WPI.

2.7. Emulsion microstructure

Visualisation of emulsion microstructure provides valuable information on the structural assembly of components of the system and allows a better understanding of the nature of changes taking place in the system. Several microscopic approaches based on different technologies are available (e.g., optical microscopy, confocal laser scanning microscopy, CLSM; confocal raman microscopy, CRM; scanning electron microscopy, SEM; atomic force microscopy, AFM; or transmission electron microscopy, TEM techniques) and choosing the right technique for a given sample depends on the physical format of the sample, the information required on the sample and the desired level of detail. Confocal laser scanning microscopy (CLSM) is the technique of choice for studying food emulsion systems as it allows gathering ample system information by individually labelling and localising its individual components (e.g., protein and lipid); hence this technique is the main focus of this review. The first publications using CLSM for studying a food system were focused on O/W emulsions, where the displacement of sodium caseinate by monoacylglycerols from an O/W interface was the focus (Heertje, Nedelof, Kendrickx, and Lucassen-Reynders, 1990; Heertje, Van Aalst, Blonk, Nederlof, and Lucassesn-Reynders, 1996). Analysis of a sample using CLSM requires application of a fluorescent dye, which possesses affinity for a specific component of the sample – the dye has particular excitation and emission spectra and emits fluorescent light upon laser-induced excitation and the resultant emission at a specific wavelength indicates the localisation of the stained component within the sample matrix. Several components of the sample can be visualised at the same time, given that the emission wavelengths spectra do not overlap. Comprehensive information on the principles of CLSM

analysis as well as important considerations for its use in various food matrices are available elsewhere (Auty, Morris, and Groves, 2013; Auty, Twomey, Guinee, and Mulvihill, 2001; Everett and Auty, 2008).

Labelling the protein and lipid components of an emulsion can provide important information on the microstructure of emulsion-based systems and more elaborate approaches can be used to label other components, such as polar lipids and glycoproteins (Bourlieu et al., 2015; Lopez, Madec, and Jimenez-Flores, 2010; Lopez and Ménard, 2011) or a specific protein class (Sørensen et al., 2007). Visualisation of the locations of components within the system matrix can aid understanding of the structure of native food matrices (e.g., human or bovine milk fat globule membranes) to allow effective engineering of food systems to replicate naturally-occuring structures. Lopez, Cauty, and Guyomarc'h (2015) used CLSM to investigate differences in the microstructure of oil globules in native human and bovine milks and in commercial IF products. They identified that, apart from differences in size of oil globules, the differences between human/bovine and IF milks were due to differences in the composition and structural organisation of the emulsion interfacial layers. Localisation of a specific component in a food matrix can also be helpful to study the pathways for rearrangement of the system during its processing. Munoz-Ibanez et al. (2016) used a combination of microscopic techniques (i.e., optical microscopy, SEM and CRM) to study the microstructure and localisation of components (i.e., gum arabic, sunflower oil and maltodextrin) in spray-dried emulsions and reported the over-representation of the surface-active gum arabic at the surface of powder particles, indicating its rapid adsorption at the surface of atomised droplets during the spray drying process.

The CLSM technique is often used to study changes taking place in food systems as influenced by processing conditions, storage time (i.e., shelf life stability), or conditions during ingestion and digestion; application of CLSM can significantly aid interpretation of data obtained using other analytical techniques. Processing conditions can affect the structure and properties of a food system, for example, powder-based emulsion-containing systems undergo several processing steps (e.g., homogenisation, heat treatments,

concentration and spray drying) before they are packaged as a final product. The effect of homogenisation on the composition and organisation of the O/W interfacial layer in bovine milk was reported by Lopez et al. (2015); selective staining of polar lipids and protein allowed to observe a homogenisation-driven shift from polar lipid- to protein-dominant emulsion interfaces. Differences in the thickness of interfacial layers in emulsions stabilised by β-casein and WPI were reported by Li, Auty, O'Mahony, Kelly and Brodkorb (2016); with the aid of image analysis of CLSM micrographs, they observed thicker interfaces for β-casein-stabilised emulsions compared to those stabilised with whey protein. Sørensen et al. (2007) used CLSM to study surface behaviour of caseins and whey proteins in milk under different homogenisation conditions. They reported that modification of homogenisation temperatures (50 and 72 °C) and pressures (5.0, 15.0 and 22.5 MPa) resulted in different interfacial composition of oil globules in fish oil-enriched milk emulsions, as evidenced by regions of co-localisation of lipid and protein (casein or lactoferrin) and protein aggregation. Differences in the interfacial composition of emulsions observed by the authors were linked with different oxidative stability of these systems during storage.

The changes in microstructure of emulsions during *in vitro* digestion of infant formula were studied by Bourlieu et al. (2015) with the aid of CLSM, where fluorescent labelling of protein, neutral and polar lipids allowed to track changes in the system during simulated digestion stages. Reorganisation of the system components and aggregation of protein and oil globules in model IF emulsions during their digestion, demonstrated by the micrographs, were used to study the kinetics of digestion of these systems as affected by their processing (i.e., homogenisation and pasteurisation conditions). Differences in emulsion interface composition were linked to their potential effect on the infant digestive physiology and lipid metabolism of infant formula (IF) products. Displacement of emulsifiers from the interface and changes in the microstructure of the system during processing, and under model digestion conditions, were also reported by Yang et al. (2013). Qiu, Zhao, Decker and McClements (2015) presented CLSM micrographs of fish oil-based O/W emulsions stabilised with different proteins at different stages of an *in vitro*

digestive track (i.e., before digestion, in mouth, in stomach and in the intestine). Visualisation of disruption of the emulsions at these 4 stages of digestion allowed the authors to determine the role of interfacial composition on emulsion stability and fat release during digestion. In recent work, Gallier et al. (2015) used CLSM and TEM techniques to evaluate the interfacial composition of oil globules in a concept IF product and compared it with those of traditional IF products and human milk. The concept IF emulsion was designed to mimic the polar lipid-dominant interfacial layer known to exist in human milk; using these microscopic techniques, provided guidance for the microstructural design of interfaces of oil globules in the concept IF product. Gallier et al. (2015) reported a thin and heterogeneous interface for the concept product composed of a mixture of phospholipids, proteins, lipoproteins and fragments of milk fat globule membranes. Microstructural approaches can be powerful in engineering food structures for specific biological functions through the control of digestive and metabolic properties of food-based emulsions. Understanding the relationship between food structure and its sensory properties can be aided by the application of CLSM approaches, which can effectively enable design of new formulation/structure strategies for reducing the fat content of food products. Abhyankar, Mulvihill and Auty (2014) used this combined analytical approach to study the deformation and effective break-up of oil globules in emulsion-filled gels.

CLSM can be used to identify the mechanisms of emulsion destabilisation such as coalescence, flocculation or protein aggregation (Fig. 2.1 ii); this technique can also be used for studying the arrangement and properties of the emulsion interfacial layer, its thickness, density and continuity, which can be observed from the cross-sectional analysis of oil globules. Thermal stability of model IF emulsions was studied with the aid of CLSM by Drapala et al. (2016a), whereby the microscopic technique facilitated tracking of heat-induced protein deposition on the oil globule surfaces and protein-mediated bridging flocculation of oil globules during heating. Formation of buoyant white flecks in whey protein-based formulae was reported, which upon visualisation with CLSM, were shown to be oil globules entrapped within a protein network. Similarly, visualisation, using CLSM, of associative interactions between

components in IF products induced by processing steps in their manufacture (i.e., heat treatments performed for the microbial safety of these products) have been discussed by Lopez et al. (2015), where aggregation of protein and interactions between protein and oil globules to form lipoprotein complexes were reported. Drapala, Auty, Mulvihill, and O'Mahony (2017) used CLSM to show differences in the physical stability to spray drying of model IF emulsions stabilised by different emulsifier systems. In their work, the authors fluorescently-labelled the protein and neutral lipids in the dried powder particles to visualise the distribution of oil globules within the powder matrices. They were able to link the inhomogeneous distribution of oil globules in samples to poor thermal stability of emulsions and competitive adsorption mechanisms between surfactants used in the formulations.

Time-related changes of an emulsion system govern its shelf life stability; generally, the displacement of emulsifiers at the interfaces of oil globules can lead to coalescence and, effectively, phase separation. Labelling of protein components in an emulsion system containing other, non-protein emulsifiers, and monitoring the changes in the continuity of the protein layer at the oil globule surfaces has been shown to give information related to protein displacement from the interface by L_{Mw} polar lipid surfactants (e.g., lecithin) (Drapala et al., 2015). Those authors showed regions of discontinuity in the protein interfacial layer, linked to its competitive displacement by lecithin and such discontinuity resulted in poor stability of these emulsions caused by coalescence. Ye et al. (2004) used CLSM to study storage stability of highly-hydrolysed (27% DH) WPH-based O/W emulsions containing different levels of xanthan and reported different extents of coalescence of these systems depending on the addition level of the polysaccharide.

The shelf life of food emulsions is often increased by water removal through spray drying, effectively immobilising the oil globules and other components of the continuous phase (Vega and Roos, 2006); stability of such 'immobilised' emulsions to storage can be assessed with the aid of microscopic techniques (Lim, Burdikova, Sheehan, and Roos, 2016; Lim, Griffin, and Roos, 2014; Lim and Roos, 2016). McCarthy et al. (2013) studied the changes in oil distribution within the matrix of emulsion-based powders during storage. They reported

changes in the size of oil globules and the amount of surface free oil during storage and correlated these changes to lactose crystallisation in the powders during storage.

2.8. Other considerations

A number of other analytical techniques may also be applied when studying emulsion systems; these are aimed at assessing the performance of emulsifiers and the physicochemical properties and stability of the emulsions. These techniques can include, but are not restricted to, measurement of surface hydrophobicity and reactive groups of an emulsifier, measurement of the interfacial protein load and interfacial composition, heat coagulation time of an emulsion or the oxidative stability of an emulsion.

Measurement and modification of the hydrophilic-hydrophobic balance of an emulsifier can provide control over its surface activity and influence its emulsion capacity and activity properties (Hamada and Swanson 1994; Lei, Zhao, Selomulya, and Xiong, 2015; Morand, Dekkari, Guyomarc'h, and Famelart, 2012). This approach typically involve spectrofluorometric analysis of surface hydrophobicity, where the hydrophobic groups of an emulsifier are quantified based on their interactions with the fluorescent probe anilino naphthalene sulfonic acid (ANS) (Bonomi, Iametti, Pagliarini, and Peri, 1988).

Food emulsions stabilised by whey protein often display poor stability to thermal processing due to high reactivity of the free thiol groups of β-lg (Simmons, Jayaraman, and Fryer, 2007; Wijayanti, Bansal, and Deeth, 2014) as evidenced by formation of buoyant lipoprotein complexes (i.e., white flecks) (Drapala et al., 2016a). The level of free thiol groups in a protein sample can be measured by the spectrophotometric method of Hoffmann and van Mil (1997) and Alting, Hamer, De Kruif, Paques and Visschers (2003). This approach can be useful when focusing on improvement of heat stability of emulsions by controlling the reactivity of its protein component. The protein load (i.e., mg protein per m^2 fat surface area) and composition of the emulsion interfacial layer can also be determined; this approach typically involves separation of the oil globules from the serum phase of the emulsion by means of centrifugation, followed by analysis of protein content by standard

analytical methods (e.g., Lowry method, Kjeldahl method, protein assay kits) (Alba et al., 2013; Sünder et al., 2001). However, this approach for determination of the surface composition of oil globules in emulsions has its limitations due to its invasive nature, leading to changes to the emulsion interfacial layer (e.g., coalescence of oil globules during centrifugation, displacement and rearrangement protein at the interface during the washing steps) and it may not provide representative information on the system studied (Holzmüller, Müller, Himbert, and Kulozik, 2016). It is also possible to measure the thickness of the interfacial layer and formation of multilayer structures at the O/W interface using a range of techniques, including spectroscopy, light scattering, microscopy, gravimetric and reflectivity techniques, as detailed by Guzey and McClements (2006).

Heat stability of emulsions is often assessed using various modifications of a method by Miller and Sommer (1940), where a liquid sample is placed in an oil bath at high temperatures (120-140°C) until onset of coagulation is observed (O'Connell and Fox, 2000). The incubation time of the sample until coagulation is noted and it represents the heat coagulation time (HCT) and HCT typically decreases as the pH of the sample approaches the pI of the protein stabilising the emulsion. This method is commonly used to assess the stability of emulsions to heat treatment during processing, especially for formulations destined for UHT treatment (Sievanen, Huppertz, Kelly, and Fox, 2008).

Oxidative stability is an essential consideration when designing emulsion-based food systems; its role is especially significant in high added value nutritional formulations that typically contain unsaturated fatty acids which are prone to oxidation. A range of assays and techniques are generally used to monitor the progression of lipid oxidation in emulsions under accelerated storage conditions, these are typically targeted at monitoring the levels of oxidation products. Spectrophotometric assays (peroxide value; *para*-anisidine value; and thiobarbituric acid reactive substances, TBARS assays) are typically used to quantify the levels of primary or secondary oxidation products (i.e., hydroperoxides or aldehydes and ketones, respectively) (Djordjevic, McClements, and Decker, 2004; Mei, McClements, Wu, and

Decker, 1998; Qiu et al., 2015). Gas chromatography and mass spectrometry methods are also commonly used to monitor oxygen consumption (Lethuaut, Métro, and Genot, 2002) or production of volatile oxidation products (e.g., headspace propanal or hexanal) (Cho, McClements, and Decker, 2002; Lee and Decker, 2011).

Food emulsions are complex and dynamic systems and numerous techniques directed at the assessment of their physicochemical properties, the prediction of their stability and digestive fate have been detailed in the scientific literature. Generally, studies focused on emulsion systems involve a combination of different analytical approaches to provide comprehensive meaningful information. These approaches cover a broad spectrum of emulsion evaluations, from characterisation of emulsion building blocks, the dynamics of emulsion formation and system microstructure, to the identification and control of the interactions between components of emulsions. Designing a study focused on emulsion stability should incorporate techniques capable of measuring thermodynamic-driven changes (based on Stokes law) affecting the system, as well as those that can take place under environmental conditions (e.g., physical stress, temperature extremes, high ion concentrations and low pH) representative of processing and storage. Good initial quality of an emulsion may not necessarily confer good emulsion stability due to interactions between its components promoted by the processing conditions or by changes that affect the emulsion during its shelf life. The use of food emulsions for nutrient delivery, where the oil globules play the role of delivery vessels, requires a detailed understanding of the system formation and stability to ensure effective protection and targeted, predictable release of the transported bioactive component. Microstructural approaches have shown to be particularly useful for such studies as they enable detailed profiling of the interfacial layers and allow study of the evolution of microstructure at different stages during the digestion process. Analytical approaches used for assessment of emulsion properties and stability range from simple assays to sophisticated techniques that require access to expensive analytical equipment. Choosing the most suitable approach will ultimately depend on the specific focus of the study and the facilities available.

A combination of several complementary techniques is typically required to provide comprehensive information on the emulsion system and to facilitate engineering of a bio-functional and stable food product.

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Chapter 3

A review of the impact of conjugation on functional properties of milk proteins and peptides

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Table of Contents

Abstract

Glycation of milk proteins and peptides can be achieved by Maillard-induced conjugation of reducing carbohydrates with the available amino groups of proteins/peptides during the early stages of the Maillard reaction. This conjugation can be achieved under wet or dry heating conditions, with the choice of heating mode influencing the rate and extent of conjugation, in addition to the functionality of the conjugated protein/peptides. Conjugation has been shown to modify the technological and nutritional properties of a range of milk protein/peptide-based ingredients. This review focuses mainly on modifications to physicochemical properties and technological functionality (i.e., solubility, heat stability, emulsification, foaming and gelation properties) of milk proteins and peptides by conjugation. Particular emphasis is placed on understanding of the relationships between changes in protein/peptide molecular structure/conformation, physicochemical properties and technological functionality, as influenced by glycation.

3.1. Introduction

A conjugated protein is defined as a protein to which another chemical group (e.g., carbohydrate) is attached by either covalent bonding or other interactions (Wong, 1991). Milk proteins and peptides, in the presence of reducing carbohydrates, can undergo a series of complex chemical changes during heating, known as the Maillard reaction. Conjugation occurs naturally during the early stages of the Maillard reaction when a covalent bond forms between the protein and carbohydrate components, resulting in the release of water (i.e., condensation reaction). The resulting covalently-linked Schiff base product can undergo irreversible Amadori rearrangement, leading to the formation of Amadori products (Ames, 1992; Liu, Ru, and Ding, 2012; Zhu, Damodaran and Lucey, 2008). Conjugation of food proteins with carbohydrates *via* the Maillard reaction (i.e., glycation) is a growing area of interest, with many studies completed, particularly over the last 10-15 years, on the use of conjugation to modify physicochemical and functional properties of proteins and peptides. Section 3.2 provides an overview of the Maillard reaction and the various factors affecting the reaction, respectively.

Milk protein ingredients are utilised in the formulation of a wide range of food, clinical and pharmaceutical products, due to their unique functional and nutritional attributes (Smithers, 2015). In the food industry, the principal technological hurdles limiting the use of milk (especially whey) protein ingredients in the formulation of value-added beverages and powders are, (1) poor solubility of intact proteins in high acid ready-to-drink beverages, resulting in the development of turbidity and phase separation (Akhtar and Dickinson, 2007), (2) poor emulsification properties of hydrolysed proteins (Singh and Dalgleish, 1998; Agboola, Singh, Munro, Dalgleish, and Singh, 1998a, b), causing challenges with emulsion formation, stabilisation and spray drying (e.g., powder stickiness and high free fat) during the manufacture of powdered nutritional products, and (3) physical instability such as aggregation, sedimentation and creaming during processing and shelf life in high ionic strength environments and during thermal processing (Yadav, Parris, Johnston, Onwulata, and Hicks, 2010). Conjugation has been shown to be successful in modifying the functional properties of a range of milk

protein/peptide-based ingredients. Section 3.3 provides an overview of how the key compositional, structural and physicochemical properties of protein/peptide and carbohydrate substrates influence the progression of conjugation and the functionality of the resulting conjugated proteins/peptides. A detailed comparison of the differences between the two main modes of achieving conjugation (i.e., dry and wet heating) is provided in Section 3.4, while Sections 3.5-3.9 of this review provide detailed information on the effects of conjugation on solubility, heat stability, emulsification, foaming and gelation properties of the principal milk proteins/peptide ingredients used in the food industry. Section 3.10 provides an overview of approaches developed for enriching and purifying conjugates.

3.2. Principles and factors affecting the Maillard reaction

The Maillard reaction (Maillard, 1912) describes a complex series of reaction pathways, many of which proceed concurrently during heating and/or storage of protein/carbohydrate mixtures. The chemistry of the Maillard reaction can be divided into three stages - the early, intermediate and advanced stages (Fig. 3.1) (Hodge, 1953).The early stage of the Maillard reaction involves a series of individual reactions that are initiated when the ε-amino groups of lysine, or to a lesser extent, the imidazole and indole groups from histidine and tryptophan, respectively, and the α-amino groups of terminal amino acids in proteins/peptides condense with the carbonyl groups of reducing carbohydrates, to form a Schiff base, with the release of a molecule of water (Ames, 1992). The Schiff base is thermodynamically unstable and undergoes spontaneous rearrangement to form either an Amadori (in the case of aldoses) or Heyn's (in the case of ketoses) product (Wrodnigg and Eder, 2001). The intermediate stage of the Maillard reaction involves the degradation of the Amadori and/or Heyn's rearrangement products by a number of different reactions, including cyclisation, dehydration, retro-aldolisation, isomerisation and further condensation, which causes degradation of amino acids and carbohydrates (Ames, 1998). The advanced stages are complex and variable, depend on the reaction conditions, and involve dehydration and decomposition of the early reaction products, resulting in the production of many advanced Maillard reaction products (AMP) and coloured nitrogenous

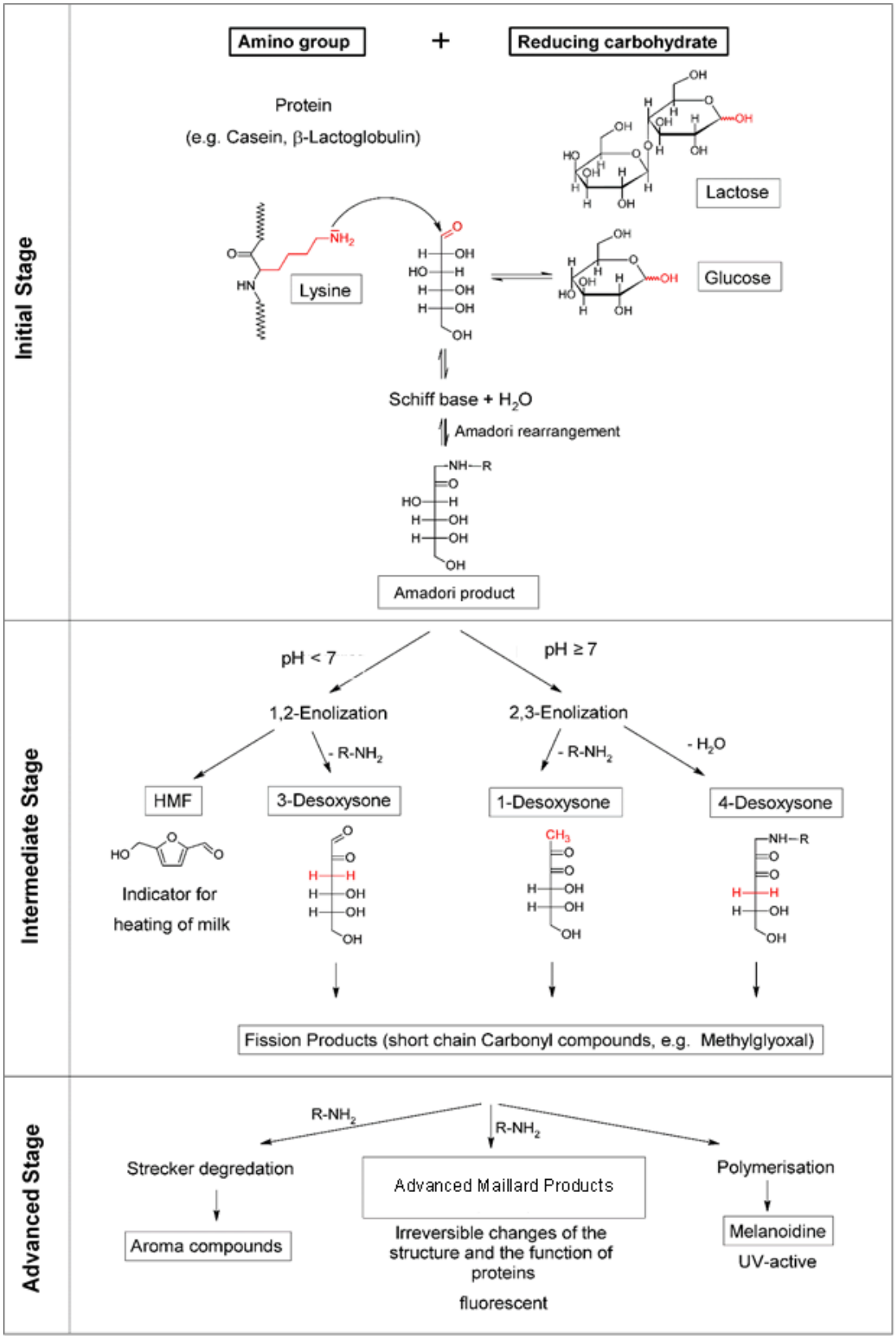

Figure 3.1. Simplified overview of the Maillard reaction in milk and milk products (based on Hodge, 1953; Ames 1998).

polymers and co-polymers, known collectively as melanoidins (Ames, 1998; Hodge, 1953). While, from a functionality perspective, it is desirable to achieve conjugation in the early stages of the Maillard reaction, it is normally desirable to limit the progression of the Maillard reaction to advanced stages, as AMP's are largely responsible for some of the less desirable consequences of the Maillard reaction, e.g., generation of off-flavours, loss of nutritional value, protein crosslinking and generation of potentially toxic compounds (Uribarri et al., 2005).

The progression of the Maillard reaction and, effectively production of protein/peptide-carbohydrate conjugates is affected by a number of intrinsic and extrinsic factors, and these typically include the nature of the reactants, properties of the systems or the environmental conditions (Ames, 1990; de Oliveira, Coimbra, de Oliveira, Zuñiga, and Rojas, 2016; Liu et al., 2012; Oliver, Melton and Stanley, 2006a; Van Boekel, 2001). The physicochemical properties (i.e., molecular weight, M_w; structure/conformation and surface charge) of the amino and carbonyl compounds, and their molar ratios, all govern the rate and extent of the Maillard reaction, and consequently, the physicochemical properties of the conjugated proteins/peptides. Reactivity of compounds tend to decrease with increasing M_w, due to the greater contribution of steric hindrance with increasing M_w; as an example, mono-saccharides are more reactive with proteins than di- or oligo-saccharides under conditions which favour conjugation. For protein hydrolysates, the degree of hydrolysis, M_w profile and charge of the peptides are important in determining their reactivity during Maillard-induced conjugation (Drapala, Auty, Mulvihill, and O'Mahony, 2016a, b; Mulcahy, Park, Drake, Mulvihill, and O'Mahony, 2016b; Van Lancker, Adams, and De Kimpe, 2011).

Temperature has a significant impact on the rate of the Maillard reaction, which increases with increasing temperature and duration of heating (Maillard, 1912). Higher temperatures cause opening up of the protein structure, providing greater accessibility to reactive protein/peptide functional groups; increasing temperature also affects the reactivity of sugars as the proportion of reducing sugar molecules present in the open-chain form (i.e., the more reactive form) increases (Van Boekel, 2001), due in part to the

faster rate of mutarotation of the sugar molecules. It should be noted that heat-induced structural/conformational changes (e.g., denaturation and aggregation) of milk proteins/peptides may result in amino groups becoming less available for participation in the Maillard reaction (Chevalier, Chobert, Popineau, Nicolas, and Haertlé, 2001; Jiang and Brodkorb, 2012; Mehta and Deeth, 2016).

The reactivity of proteins and carbohydrates in the Maillard reaction is greatly influenced by the pH of the system; a basic environment can catalyse the initial stages of the Maillard reaction by deprotonating the amino groups, which in turn increases reactivity with carbonyl groups of reducing carbohydrates. The open chain form of the carbohydrate and the un-protonated form of the amino group, which are considered to be the most reactive forms, are usually favoured at higher pH, up to a maximum of pH ~9-10 (Martins, Jongen, and Van Boekel, 2000). The pH of protein/peptide-carbohydrate mixtures can decrease (depending on the buffering capacity) as the Maillard reaction progresses due to the formation of acids (e.g., formic and acetic acids), the consumption of basic amino groups (e.g., lysine) or the loss of carboxyl groups during Strecker degradation, resulting in the production of carbon dioxide (Nursten, 2005). Furthermore, products derived from the intermediate and advanced stages of the Maillard reaction are degraded by different reaction pathways depending on the pH of the system. Increasing water activity (a_w) of protein/peptide-carbohydrate mixtures generally increases the rate and extent of conjugation, due to the increased diffusion and mobility of reactants; however, high water concentrations/a_w can negatively influence progression of the Maillard reaction (Morgan, Léonil, Mollé, and Bouhallab, 1999b).

Factors, other than those outlined above, can impact the Maillard reaction, including the presence of sulphur dioxide in food systems which has been shown to delay the development of brown colour (Ames, 1990) and the presence of metal ions, which can accelerate or inhibit the Maillard reaction, depending on their concentration (Ramonaitytė, Keršienė, Adams, Tehrani and De Kimpe, 2009). In model systems, the presence of tertiary amine salts, acetic acid and free radicals have been shown to promote the Maillard reaction; however, these factors may often, in practice, be of minor

significance relative to the nature of the reactants, temperature, time and moisture content (O'Brien, 1997). Non-thermal energy sources (e.g., ionizing radiation, UV irradiation and ultrasound treatment) have also been shown to produce Maillard reaction products, including brown pigments and volatile flavour compounds (O'Brien, 1997).

3.3. Substrates for Maillard reaction

The conjugation of milk proteins/peptides has been studied using many categories of milk protein-based ingredients as substrates, including, but not limited to, whey protein concentrates (WPC) and isolates (WPI), individual whey protein fractions (in particular β-lactoglobulin, β-lg; α-lactalbumin, α-lac; and bovine serum albumin, BSA), sodium caseinate, casein fractions (β-casein) and hydrolysates of whey proteins (WPH) and caseins (e.g., hydrolysed sodium caseinate). For the reasons outlined in Section 3.2, it is desirable that the proteins/peptides used are soluble under the conditions of conjugation; hence only soluble forms of casein (e.g., sodium caseinate) have been studied. It is also desirable that the proteins used for conjugation are present in a conformation which ensures a high degree of accessibility of carbonyl groups to amino groups, which is one of the main reasons why, in the study of casein-based conjugates, sodium caseinate, with an open/flexible structure, and extremely low levels of non-protein components, has been extensively used as the casein protein substrate; the authors are not aware of any studies performed using micellar casein for production of casein-based conjugates.

In addition, whey proteins are more susceptible than caseins to heat-induced aggregation under the conditions used for conjugation (particularly under wet heating conditions), which would be expected to restrict accessibility of carbonyl groups to amino groups on the protein/peptide molecules. β-Lg typically represents ~50-60% of total protein in WPC, WPI and WPH ingredients and has two disulphide bonds and one free thiol group, which are deemed responsible for the irreversible thermal aggregation and gelling properties of this protein (Brodkorb, Croguennec, Bouhallab, and Kehoe, 2016). In contrast, α-lac has a single polypeptide chain, containing four disulphide bonds, and no free sulphydryl group (Permyakov and Berliner,

2000), making it less susceptible to heat-induced denaturation/aggregation under the conditions used in conjugation of whey protein (Enomoto et al., 2009). Furthermore, Nieuwenhuizen et al. (2003) reported that the availability of the lysine groups in α-lac is modified by the binding of calcium; five lysine residues were available for reaction in apo-α-lac compared to four available lysine residues in holo-α-lac.

It is desirable to have low levels of non-protein components (e.g., lactose, minerals and lipid) in the protein-containing ingredients used as substrates for conjugation, as lactose contributes strongly to brown colour and flavour compound formation (Lillard, Clare, and Daubert, 2009), minerals promote aggregation of whey proteins (Brodkorb et al., 2016), lipid material can contribute to off-flavour formation (Liu and Zhong, 2014; Lloyd, Hess, and Drake, 2009) and lactose can also compete with other carbohydrates for conjugation to the protein substrate during heating under conditions required to achieve conjugation. Therefore, high protein content WPC and WPI, or pure protein fraction ingredients are most commonly used for conjugation purposes.

The whey proteins generally have slightly higher normalised levels of lysine residues than the caseins. Hydrolysis of casein and whey protein molecules increases the number of free amino groups available to react with carbonyl groups during conjugation and can also lead to increased exposure and accessibility to previously-buried lysine residues. Protein hydrolysates are generally characterized by their degree of hydrolysis (DH), which expresses the number of peptide bonds cleaved as a percentage of the total number of peptide bonds available (Foegeding, Davis, Doucet, and McGuffey, 2002). Hydrolysis of whey proteins, due to reduction of average M_w and levels of secondary structure, enhances their stability to heat-induced aggregation, which can facilitate enhanced retention of amino groups in a form accessible for conjugation during heating. For example, Ju, Otte, Madsen and Qvist (1995) reported that limited hydrolysis of WPI (DH 2-7%), using trypsin, prevented heat-induced gelation of a WPI solution (12%, w/v, protein) on heating at 80°C for 30 min at pH 3 and 7. Mulcahy et al. (2016b) reported that WPH with a low degree of hydrolysis (DH 9.3%) had 55.4% higher levels of

available amino groups compared with an intact WPI counterpart, which contributed to more rapid and extensive conjugation of maltodextrin (MD) with the WPH than with the WPI.

The conjugation of milk proteins/peptides has been studied using many different types of carbohydrate ingredients, including, but not limited to, lactose, MD, corn syrup solids (CSS), dextrans, glucose, maltose, ribose, guar gum, pectin, fenugreek gum, oligosaccharides and glucosamine. From the point of view of their ability to participate in Maillard-induced conjugation of milk proteins/peptides, and the functionality of the resultant conjugates, the key differences between these carbohydrates are chain length, structure (i.e., linear *vs* branched and ketoses *vs* aldoses) and charge (neutral *vs* charged). In general, the shorter the chain length of the carbohydrate component, the faster the rate, and the greater the extent of conjugation. On conjugation of whey protein with MD or CSS, having dextrose equivalent (DE) values in the range 6-38, at an initial pH 8.2, at 90°C for up to 24 h, the extent of conjugation increased with increasing DE value of the MD and CSS ingredients (Mulcahy et al., 2016a). Delahaije, Gruppen, van Nieuwenhuijzen, Giuseppin and Wierenga (2013) studied the stability of emulsions of patatin conjugated to the same extent with different mono- and oligosaccharides (xylose, glucose, maltotriose and maltopentaose) and reported that attachment of monosaccharides did not affect the flocculation behaviour of the emulsion; however, the attachment of maltotriose and maltopentaose (M_w > 0.5 kDa) provided stability against flocculation of the emulsions at pH 5, due to increased steric stabilization contributed by the higher M_w carbohydrates. Brands and van Boekel (2001) reported that ketoses degraded during heating, whereas aldoses were involved in formation of the covalent bond between proteins and carbohydrates during the Amadori stage of the Maillard reaction.

3.4. Mode of conjugation

The main variables that can be controlled during conjugation of milk proteins/peptides are temperature, time, pH, moisture content, relative humidity (RH) and/or a_w. These variables can be grouped to give 2 distinct approaches for achieving conjugation – (1) wet heating and (2) dry heating.

The wet heating approach normally involves incubation of an aqueous solution of protein/peptide and carbohydrate reactants, commonly pre-adjusted to a target pH (normally pH 6.0-11.0), for a pre-determined time (min-d) at a set temperature (typically in the range 60-95^{o}C). The conjugation reaction is normally stopped (or slowed considerably) by cooling and further processing (e.g., freeze or spray drying) of the conjugated protein/peptide solution. The dry heating approach normally involves incubation of a co-dried mixture (commonly pre-adjusted to a target pH) of the protein/peptide and carbohydrate ingredients for a pre-determined time (min-d) at a set temperature (typically in the range 60-130^{o}C) at a set RH (typically 60-80%).

Both approaches have been used extensively for conjugation of milk proteins/peptides and both have their advantages and limitations. The mobility of reactants is higher with the wet heating than the dry heating approach and higher temperatures (for shorter times) are generally used with the former than with the latter; however, some recent studies have used considerably higher temperature (130^{o}C) and shorter times (<30 min) than previous studies to achieve conjugation of WPI with lactose or MD under dry heating conditions at 79% RH (Liu and Zhong, 2014). Similarly, Guo and Xiong (2013) reported that WPI was successfully conjugated with lactose or MD (DE18) at 130°C for 20 min and 79% RH, with both systems having less colour development that WPI-lactose/MD conjugated at 80°C for 2 h.

To achieve maximum reactivity between the protein/peptide and carbohydrate components using dry heating, it is necessary to prepare a solution of the two components, which is dried before being conjugated by dry heating, and the conjugated powder typically requires down-stream drying due to release of water during the early stages of the Maillard reaction. This latter issue can also lead to localised browning of the powdered reaction mixture during conjugation due to sugar crystallisation (Lievonen, Laaksonen, and Roos, 1998) using the dry heating approach, which is not an issue with the wet heating approach. While most of the research published to date using the wet heating approach has been conducted at temperature ranging from 60-95°C for time periods of minutes-days (Chevalier et al., 2001; Darewicz, and Dziuba, 2001; Drapala et al., 2016a, b; Morgan et al., 1998; Mulcahy et al.,

2016a,b; Zhang et al., 2012; Zhu et al., 2008), some studies have reported the use of higher temperatures (i.e., 100-130°C) for shorter times (≤6 h) to induce conjugation using wet heating; for example, Chen et al. (2013b) reported that phosvitin and dextran were conjugated by heating in an aqueous solution at 100°C for 6 h.

In addition to the differences in energy costs and efficiency between wet and dry heating approaches, the use of dry heating at lower temperatures (<70°C) has been shown to result in greater preservation of the native 3-dimensional structure of whey proteins, compared with wet heating approaches, which has important implications for selected functional properties, such as solubility and interfacial properties (Gauthier, Bouhallab, and Renault, 2001; Morgan et al., 1998; Morgan et al., 1999a,b). The use of macromolecular crowding to effectively restrict denaturation and, in particular, aggregation of whey proteins has also shown promise on conjugation of WPI with dextran (Ellis, 2001; Perusko, Al-Hanish, Velickovic, and Stanic-Vucinic, 2015; Zhu, et al., 2008).

3.5. Solubility

Milk proteins used in food products are generally required to have high levels of solubility in order to facilitate expression of the desired functional properties such as gelation, aeration, water-binding, foaming and emulsification (de Wit, 1989; O'Regan, Ennis and Mulvihill, 2009). Solubility of milk proteins is influenced by many physicochemical properties of the protein molecules themselves, i.e., M_w, conformation (e.g., as affected by denaturation/aggregation), amino acid composition, physical state, exposure of selected functional groups, surface hydrophobicity, and environmental factors, i.e., pH, temperature, ionic strength and nature of the solvent (De Wit and Klarenbeek, 1984; Hayakawa and Nakai, 1985; Vojdani, 1996).

Protein-carbohydrate conjugation *via* the Maillard reaction has been shown to be an effective means of increasing the solubility of milk proteins. Native whey protein molecules are globular in structure and are susceptible to heat-induced changes (>70°C) such as denaturation and aggregation (Wijayanti, Bansal, and Deeth, 2014a), while caseins are non-globular proteins, with more open,

flexible structures and can be heated at 140°C, at pH 6.7, for at least 40 min before coagulation occurs (Fox and Hoynes, 1975). Sodium caseinate has very different functionality to whey proteins (i.e., high viscosity at low concentrations and poor solubility at pH ~4.6) and is used as an emulsifier, texturizer and stabilizer in food products such as cured meats, processed cheese, coffee whiteners, high fat powders, bakery and confectionary products (Carr and Golding, 2016; O'Regan and Mulvihill, 2011; Swaisgood, 1993).

Improvements in the solubility of sodium caseinate at its isoelectric point would be expected to help broaden its application in food products; O'Regan and Mulvihill (2009) reported that sodium caseinate conjugated with MD, with DE values of 4 or 10, had improved protein solubility (~5-90%) in the pH range 4.0-5.5, compared to sodium caseinate, particularly around the isoelectric point (~pH 4.6) of the protein. This increase in protein solubility on conjugation was attributed to an increase in the hydration of the protein due to the covalent attachment to the protein molecules of hydrophilic MD glucose polymer side chains, and modification of the net charge of the protein, contributing to greater repulsion between the protein molecules. The increase in the net negative charge of the protein on conjugation with carbohydrate may be attributed to the consumption of charged amino acids, such as the basic amino acid lysine during the Maillard reaction (Ames, 1998; Brands and van Boekel, 2002; Lertittikul, Benjakul, and Tanaka, 2007; Wang and Zhong, 2014). Interestingly, it was noted that, at similar extents of conjugation, the conjugated sodium caseinate-MD10 had higher protein solubility (~50-80% increase) across the pH range 4.0-4.5 than the conjugated sodium caseinate-MD4. Similar results were reported by Shepherd, Robertson and Ofman (2000) and Oliver, Melton and Stanley (2006b), with conjugation of sodium caseinate with MD under dry heating conditions, leading to increases in protein solubility, particularly at pH 4.0-4.6, which was again attributed to increased steric repulsion between conjugated protein molecules. Grigorovich et al. (2012) reported that sodium caseinate conjugated with MD with DE values of 2 or 10, under dry heating conditions at an initial pH of 7, at 60°C and 79% RH for 72 h, had improved solubility (~10-80% increase) across the pH range 3.5–5.0, compared with sodium caseinate alone. The authors

reported that the improvement in protein solubility of the sodium caseinate-MD conjugate solutions was determined mainly by the molar ratio of the protein:carbohydrate and the DE value of the MD used for conjugation. Similar to the study of O'Regan and Mulvihill (2009), the most pronounced increase in solubility was achieved using MD with the higher DE value (i.e., DE 10).

However, it should be noted that conjugation of milk proteins with carbohydrates does not always result in increased protein solubility as the type and extent of modification of the functional properties are very dependent on the nature of the reactants, reaction conditions and the pathways followed by the Maillard reaction (Hiller and Lorenzen, 2010). Corzo-Martínez, Carrera-Sanchez, Villamiel, Rodriguez-Patino, and Moreno (2012b) reported that dry heating of sodium caseinate and galactose, at an initial pH of 7.0, at 50-60°C, 67% RH, for 4 and 72 h, resulted in a 20% reduction in the solubility of the protein at pH 7.0, compared to the unheated sodium caseinate control. The authors attributed the decreased protein solubility on conjugation to an increase in the surface hydrophobicity of the protein on heating. However, at pH 5.0, Corzo-Martínez et al. (2012b) reported that conjugated sodium caseinate-galactose displayed an increase of ~10% in solubility, compared to the unheated and dry heated sodium caseinate controls due to the shift in the isoelectric point of the conjugated protein to a lower pH as a result of a moderate increase in its net negative charge following conjugation.

The dry heating approach has also been used extensively to conjugate whey proteins with carbohydrates as it is claimed to result in less heat-induced conformational changes to the whey protein molecules (Oliver et al., 2006b; Zhu et al., 2008) as lower temperatures are typically used (Li, Enomoto, Ohki, Ohtomo, and Aoki, 2005) than with wet heating. Wang and Ismail (2012) demonstrated that WPI conjugated with dextran by dry heating at 60°C and 49% RH, for 96 h, had enhanced protein solubility (85.7 and 89.0% increase) at pH 4.5 and 5.5, respectively, when they were subsequently heated to 80°C for 30 min, compared to the respective WPI control. The authors reported that the enhanced solubility of WPI on conjugation with dextran was attributed to suppressed intermolecular protein-protein interactions, along with

structural/physicochemical changes to the protein, including a shift in the isoelectric point of the protein to a more acidic pH, reduction in the surface hydrophobicity of the whey protein molecules and increased resistance to thermal denaturation, resulting in a reduced exposure of free sulfhydryl groups after conjugation of the protein with dextran. Similarly, other studies have shown that conjugation of casein or whey proteins with carbohydrates resulted in a shift in the isoelectric point of proteins towards more acidic pH due to the consumption of positively charged lysine residues during conjugation (Jiménez-Castaño, Villamiel, and López-Fandiño, 2007; Martinez-Alvarenga et al., 2014).

In a study by Wang, He, Labuza and Ismail (2013), the authors characterised the structural changes in whey protein molecules conjugated with dextran (at 60°C and 49% RH for 96 h) using surface-enhanced Raman spectroscopy. Those authors reported that the Raman spectra of the conjugated WPI-dextran samples had an additional peak at 983 cm^{-1}, which they attributed to the formation of a Schiff base, which was accompanied by deprotonation of carboxyl groups, contributing to higher net negative charge along with re-organisation of the sulphide linkages. These conformational changes in the whey protein molecules imparted structural rigidity to the conjugated WPI-dextran system, which in turn increased protein solubility on thermal treatment (75°C for 30 min) over a wide pH range (3.4-7.0), compared to previously unheated WPI. Wang et al. (2013) also reported that the β-sheet configuration of the whey protein molecules in the conjugated WPI-dextran had increased band intensity in the Raman spectra, compared to that of the unheated WPI control. Wang et al. (2013) and Damodaran (2008) reported that the β-sheet configuration is more thermally stable than the α-helix and other disordered structure configurations in whey protein molecules, thus an increase in the β-sheet configuration may explain the improvements in the thermal stability at pH 4.5 and 5.5 of the conjugated WPI-dextran.

A limited number of studies have reported modification of functional properties of whey proteins conjugated with carbohydrates using wet heating conditions. The likely reason for this is that heating of whey protein in an aqueous environment at ≥70°C can result in denaturation and aggregation,

which have been reported to reduce whey protein solubility (Liu et al., 2012; Pelegrine and Gasparetto, 2005; Zhu, Damodaran, and Lucey, 2010). However, Jiang and Brodkorb (2012), Lillard et al. (2009) and Liu and Zhong (2015) have investigated the use of high temperatures (95-130°C) to induce conjugation of whey proteins or isolated whey protein fractions with carbohydrates, and have reported improvements in the antioxidant activity, emulsification properties and heat stability, respectively, of whey protein-carbohydrate conjugates.

3.6. Heat Stability

Glansdorff, Prigogine and Hill (1973) defined thermal or heat stability as the ability of a substance to resist irreversible change in its chemical or physical structure, often by resisting polymerisation, under defined conditions (i.e., temperature, pH and ionic strength). Globular whey proteins are very susceptible to heat-induced (>70°C) changes such as denaturation and aggregation (Wijayanti et al., 2014a), therefore, this section will focus mainly on the heat stability of whey proteins and improvement thereof by conjugation. The thermal stability of whey proteins has been the subject of extensive research and there are many reports in the literature on the denaturation and aggregation of whey proteins under different solution and processing conditions (Brodkorb et al., 2016; Donovan and Mulvihill, 1987; Marangoni, Barbut, McGauley, Marcone, and Narine, 2000; Oldfield, Singh, and Taylor, 2005; Ryan, Zhong, and Foegeding, 2013; Sağlam, Venema, de Vries, and van der Linden, 2014).

Several approaches have been investigated to control aggregation of whey proteins, including the addition of hydrophobic/amphiphilic compounds prior to heating, such as molecular chaperones, alcohols, hydrolysed/hydroxylated lecithin, and saturated/unsaturated fatty acids, or removal of intermediate aggregates and modification of the ionic environment of the protein solution (Yong and Foegeding, 2008; Wijayanti, Bansal, Sharma and Deeth, 2014b). Protein-carbohydrate conjugation via the Maillard reaction has been shown to be an effective method to improve the thermal stability of milk proteins.

Zhu et al. (2010) conjugated WPI with dextran (M_w 440 kDa) by heating a solution of 10% WPI and 30% dextran, at an initial pH of 6.5 at 60°C for 48 h. The authors measured the thermal stability of the conjugated WPI-dextran solution (0.1%, w/v, protein) by heating at 80°C for 30 min and subsequently measuring the development of turbidity in the solutions (i.e., with increasing development of turbidity there was a higher absorbance at 500 nm; A_{500}), across the pH range 3.0-7.5. The absorbance of the conjugated WPI-dextran solution did not change on heating; however, there was a ~10 fold increase in A_{500} of the WPI solution that was heated at 80°C for 30 min in the pH range 4.5-5.5, which was attributed to the formation of large protein aggregates that scattered light. The authors reported that the unheated WPI had a typical differential scanning calorimetry (DSC) denaturation profile, with an endothermic peak at ~74°C attributed to the denaturation of β-lg, and a shoulder at ~66°C, attributed to the denaturation of α-lac; however, the conjugated WPI-dextran solution had a flat line profile suggesting that whey protein in the WPI had less secondary structure, due to the covalent attachment of the dextran which contributed to a higher denaturation temperature and improvements in thermal stability. Similar DSC profiles were reported by Hattori, Nagasawa, Ametani, Kaminogawa and Takahashi (1994), Liu and Zhong (2013) and Wang and Ismail (2012) who showed that the denaturation temperature of whey protein-carbohydrate conjugates was higher than that of the corresponding unconjugated whey proteins.

Chevalier et al. (2001) reported that β-lg conjugated with either ribose, arabinose, glucose, galactose, lactose or rhamnose, at pH 6.5 and 60°C for 72 h in an aqueous environment (0.4% protein, 0.4% carbohydrate), exhibited greater thermal stability at pH 5.0, when heated at 70–90°C for up to 1 h, than unheated and heated β-lg controls (i.e., without added carbohydrate). The improvement in thermal stability of the solution (0.2%, w/v, protein), as measured by the concentration of protein in the supernatant of the heated solutions after centrifugation (15 min at 15,000 g), was dependent on the carbohydrate as follows; ribose>arabinose>rhamnose>glucose=galactose >lactose. However, the choice of carbohydrate used in conjugation is known to alter the extent of protein-carbohydrate conjugation, making it difficult to

distinguish if the changes in the functional properties were due, directly, to compositional/structural differences between the carbohydrates or, indirectly, to their differing effects on the extent of conjugation (Chen, Liu, Labuza, and Zhou, 2013a; Li et al., 2009; Mulcahy et al., 2016a; ter Haar, Schols, and Gruppen, 2011).

Liu and Zhong (2013) conjugated WPI with either glucose, lactose or MD (M_w 1 kDa) by dry heating at an initial pH of 7.0 at 80°C and 80% RH for 2 h, in a mass ratio of 1:1, and evaluated heat stability by reconstituting samples to 7%, w/v, protein, adding 0-150 mM NaCl or $CaCl_2$, adjusting the solutions to pH between 3.0-7.0, and heating for 2 min at 88°C, simulating a hot-fill beverage process (Etzel, 2004). The authors assessed the thermal stability by visual observation of turbidity development after heating; the solutions prepared from the conjugated WPI-MD and WPI-lactose remained transparent under all conditions tested, while the unheated and dry heated WPI controls with added salt became turbid on heating at pH 6.0. Liu and Zhong (2013) reported that the conjugated WPI-MD had a higher denaturation temperature and a more negative net charge across the pH range 2.0-7.0 than the unheated WPI control, which may have contributed to the increased thermal stability of the former.

Several authors reported that improvements in heat stability of whey protein-carbohydrate conjugates can be related to the number and chain length of the carbohydrates attached to the whey protein molecules, along with the location at which they are attached on the protein molecules; the attachment of higher M_w carbohydrates has been shown to have a greater impact on improving the thermostability of whey proteins, due to increased steric repulsion, compared to conjugation with monosaccharides (Aoki et al., 1999; Corzo-Martinez et al., 2012b, c; Morris, Sims, Robertson, and Furneaux, 2004; Mulcahy et al., 2016a, Tuinier, Rolin, and De Kruif, 2002; Wong, Day, and Augustin, 2011; Wooster and Augustin, 2006).

WPHs have been reported to have impaired functional properties compared to their intact counterparts and have been shown to be more susceptible to destabilisation when heated, due to the exposure of buried hydrophobic

residues and/or release of specific peptides that promote peptide-peptide and peptide-protein aggregation (Adjonu, Doran, Torley, and Agboola, 2013; Creusot and Gruppen, 2007). Mulcahy et al. (2016b) reported that WPH (DH 9.3%) conjugated with MD (DE 17) under wet heating conditions at an initial pH of 8.2 and 90°C for 8 h, had superior thermal stability to further heating at 85°C for 10 min with 40 mM NaCl added, compared to those of the unheated or heated WPH control solutions. The unheated or heated WPH control solutions precipitated and phase separated on heating at 85°C for 10 min due to the formation of large protein aggregates (~10-50 μm), whereas, the conjugated WPH-MD solution (i.e., previously heated for 8 h at 90°C at an initial of pH 8.2) that was further heated with 40 mM added NaCl remained stable and the protein aggregates present remained small (<~1 μm).

The conditions used during the Maillard reaction impact the thermal stability of the resulting conjugates; Wang and Zhong (2014) dry heated WPI-MD in the mass ratio 1:1, at 80°C and 65% RH for 4 h, at different pHs (i.e., pH 4.0, 5.0, 6.0 and 7.0). The solutions prepared from the conjugated WPI-MD at pH 6.0 (5% protein, and 0-150 mM added NaCl) that was subsequently heated at 138°C for 1 min (to simulate UHT treatment), had improved thermal stability (i.e., remained transparent as evaluated by the visual assessment of turbidity) compared to the solution prepared from the WPI-MD conjugated at pH 4.0. The improvement in thermal stability was attributed to the greater extent of covalent attachment of MD molecules to the whey protein molecules at pH 6.0, resulting in reduced protein-protein interactions, lower surface hydrophobicity of the protein, a shift in the isoelectric point (from 4.63 to 4.07) of the protein to lower pH and a higher protein denaturation temperature compared to the WPI-MD conjugate prepared at pH 4.0.

3.7. Emulsification

Emulsifiers act by reducing the surface free energy at the interface between oil and aqueous phases, and thereby provide an effective interfacial barrier to help resist the thermodynamic tendency of emulsions to destabilise (McClements, 2015). Proteins are the most commonly used class of food emulsifiers, due to their excellent surface activity, diverse and desirable nutritional profile, wide

availability and positive consumer perception (Bos and van Vliet, 2001; Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, and Recio, 2014; Lam and Nickerson, 2013). In addition, surface and interfacial properties of proteins can be modified through controlled hydrolysis (i.e., increasing molecular mobility) (Panyam and Kilara 1996; Tamm, Sauer, Scampicchio, and Drusch, 2012; Turgeon, Gauthier, Molle, and Leonii, 1992), controlled denaturation (i.e., opening up of the protein structure) (Raikos 2010; Rullier, Novales, and Axelos, 2008), change in the charge (Hamada and Swanson, 1994) or by complexation with another component (i.e., polyphenols, carbohydrates) (Dickinson, 2010) to enable best matching of their functionality to specific product and process applications.

There has been considerable growth in interest in the area of modification of emulsification properties of proteins by their conjugation with various carbohydrates through the Maillard reaction (Drapala et al., 2016a, b; de Oliveira et al., 2016; Foegeding and Davis 2011; Lam and Nickerson 2013; Liu et al., 2012; Oliver et al., 2006a). Protein-carbohydrate conjugates consist of two composite moieties, where, in an emulsion system, the more surface-active component (i.e., protein) adsorbs at the oil/water (O/W) interface, while the more hydrophilic component (i.e., carbohydrate) extends into the bulk aqueous phase of the emulsion; the two components display two distinct, complimentary and synergistic roles in bringing about the action of conjugate-based emulsifiers.

Conjugation of proteins with carbohydrates can improve their emulsion formation properties indirectly by enhancing protein solubility (see Section 3.5), increasing their effective concentration and mobility in aqueous solution. Changes in conformation of proteins arising from conjugation (i.e., unfolding of the protein structure and exposure of hydrophobic and hydrophilic groups) result in a more flexible protein structure, enabling it to move faster towards, and adsorb at, the O/W interface, compared to unconjugated protein (Báez, Busti, Verdini, and Delorenzi, 2013; Corzo-Martínez et al., 2011; Gauthier et al., 2001). Improvements in emulsification properties of WPI on conjugation with dextran (Zhu et al., 2010) or of sodium caseinate conjugated with glucosamine (Jiang and Zhao, 2011), both under wet heating conditions, have

been reported. Protein type influences the effect of conjugation on the emulsion formation ability of proteins, where the emulsification properties of native globular proteins (e.g., whey proteins) can benefit more from conjugation than those of less-structured proteins, due to the unfolding of the compact globular structure, increasing molecular flexibility and surface hydrophobicity (Einhorn-Stoll, Ulbrich, Sever, and Kunzek, 2005; Evans, Ratcliffe, and Williams, 2013). In an analogous manner, it is reasonable to assume that the effect of conjugation on emulsification properties of hydrolysed proteins/peptides would largely depend on the degree of protein hydrolysis/conformation change and the M_w of the protein/peptide and carbohydrate components of the conjugates, while there appears to be no information available on this subject in the scientific literature.

Carbohydrate moieties covalently attached to protein on conjugation act like a tail, and are effectively towed by the protein as it migrates through the bulk aqueous phase towards the O/W interface, as the carbohydrate generally does not provide a driving force for this migration of the conjugated protein molecules. Despite its passive role in the formation of emulsions, the carbohydrate component of protein-carbohydrate conjugates generally does not impede the movement of the conjugated protein through the bulk phase, except when the size ratio between the protein and carbohydrate is disproportional. Matemu, Kayahara, Murasawa and Nakamura (2009) and Akhtar and Dickinson (2007) reported that increasing the M_w of the carbohydrate component reduced emulsifying activity index (EAI) of food protein-carbohydrate conjugates made therefrom, using tofu whey and bovine milk-derived WPI, respectively. The larger hydrodynamic radius of protein-carbohydrate conjugates, compared to the protein alone, can potentially result in a decreased rate of diffusion in the bulk phase and reduce the rate of adsorption of conjugates at the interface (Ganzevles, van Vliet, Stuart, and de Jongh, 2007). As an example, lower emulsion formation ability was reported for WPI conjugated with high M_w MD (DE 2; M_w 280 kDa), an effect which was not observed for low M_w MD (DE 19; M_w 8.7 kDa), compared to non-conjugated WPI (Akhtar and Dickinson, 2007).

The improved emulsion formation properties of milk protein-carbohydrate conjugates, compared to unconjugated protein, can also be attributed to their strong steric stabilisation properties; as the emulsifier adsorbs at the surface of newly-formed oil globules on homogenisation, it prevents their coalescence by means of steric repulsion (Liu, Ma, McClements, and Gao, 2016). The carbohydrate moiety anchored at the surface of an oil globule by the protein, protrudes into the aqueous phase of the emulsion and prevents coalescence on high impact collisions between individual oil globules during the dynamic homogenisation process (Corzo-Martínez et al., 2011). The emulsion formation properties of protein are highly dependent on the environmental conditions under which emulsification takes place; high salt concentration and acidic environment usually reduce protein solubility, due to their influence on electrostatic repulsion. A high salt content screens charges of protein molecules, while low pH reduces their charge due to proximity to the isoelectric point of the proteins (i.e., pH 4.6-5.3 for bovine milk protein) - effectively protein-protein interactions are promoted, resulting in decreased solubility and protein precipitation, and negatively impacting their surface/interfacial activity (Damodaran 2005; Bos and van Vliet 2001; Zhai, Day, Aguilar, and Wooster, 2013).

Conjugation of milk proteins with carbohydrates generally enhances their emulsion formation and stabilisation properties at high salt concentrations and under acidic conditions, due to improved protein solubility under such environmental conditions. Covalent attachment of MD or corn fibre gum to globular whey proteins (i.e., β-lg and proteins in WPI) by conjugation has been shown to enhance the emulsifying properties of the proteins across a broad pH range (3.2-5.5), by significantly increasing protein solubility (Akhtar and Dickinson 2007; Yadav et al., 2010). Similarly, enhanced emulsion formation properties, attributed to increased protein solubility, over a broad pH range (pH 2-11) have been reported for a range of milk protein ingredients (β-lg, α-lac, BSA and sodium caseinate) conjugated under dry and/or wet heating conditions with a number of different carbohydrates (glucosamine, galactose and dextran) (Corzo-Martínez et al., 2011; Jiang and Zhao 2011; Jimenez-Castano, Villamiel, and Lopez-Fandino, 2007). Conjugation of protein can

greatly improve its solubility at acidic pH and high ionic strength conditions due to the additional steric barrier provided by the conjugated carbohydrate component preventing protein aggregation and precipitation (see Section 3.5). Additionally, conjugation can shift the isoelectric point of protein to lower pH as reported for individual whey proteins (β-lg, α-lac and BSA) conjugated with MD (Jimenez-Castano et al., 2007). Such enhanced protein functionality under challenging environmental conditions offers significant potential for the development of novel emulsion-based food formulations.

Stability of an emulsion refers to its ability to withstand deteriorative changes (i.e., physical or chemical) during processing and/or storage. The main mechanism responsible for physical stability of protein-based emulsions is long-range electrostatic repulsion; proteins adsorbed at the O/W interface confer an electrostatic charge (i.e., a negative charge in the case of most milk protein-based emulsions at near neutral pH) to the oil globules, effectively preventing their flocculation and coalescence. In addition to electrostatic repulsion, emulsions formulated with conjugated proteins are also stabilised by the additional steric hindrance provided to the adsorbed conjugated protein molecules by the carbohydrate component. The carbohydrate component of the conjugate is anchored at the O/W interface by surface active protein and, due to its hydrophilicity, it extends into the aqueous phase and acts to physically hinder interactions between oil globules. Sterically-stabilised emulsions are, generally, more robust and resilient to changes to the system (i.e., temperature, concentration, pH and ionic strength), compared to emulsions stabilised solely by electrostatic repulsion, making them attractive for providing emulsion stability to formulation and manufacturing processes as well as during product storage (Fig. 3.2) (Evans et al., 2013; Liu et al., 2012).

Stability of an emulsion to processing can be described as the stability to high stress processes to which these systems can be subjected during manufacture, including thermal treatments, changes in ionic strength, high shear forces and freeze-thaw cycles (Guzey and McClements 2006; McClements, 2015). Heat treatment of protein-stabilised emulsions can often result in interactions (i.e., mediated by free sulphydryl groups and hydrophobic interactions) between proteins located at the interfacial layers of different globules, as well as with

un-adsorbed protein in the serum phase, leading to protein-mediated bridging flocculation of oil globules (Fig. 3.2) (Dickinson 2001; Piorkowski and McClements 2014; Tcholakova, Denkov, Ivanov, and Campbell, 2006). Such bridging flocculation can result in fouling of heat exchange surfaces, the generation of buoyant protein-lipid flecks, impaired emulsion shelf life or, in extreme cases, complete emulsion destabilisation (Prakash, Kravchuk, and Deeth, 2015; Petit, Six, Moreau, Ronse, and Delaplace, 2013; Drapala et al., 2016a, b). Drapala et al. (2016a, b) showed that model infant formula emulsions stabilised by WPH-MD conjugates, produced by the wet heating approach, were resistant to heat-induced bridging flocculation, compared to those stabilised by non-conjugated WPH. The authors reported that the conjugate-stabilised systems showed no changes in viscosity or particle size distribution after a high temperature-short time (HTST) treatment of between 75-100°C for 15 min, in contrast to emulsions stabilised by intact, hydrolysed or pre-heated hydrolysed whey protein. In addition, significant improvements in heat stability of O/W emulsions stabilised by WPI conjugated with low methoxyl-pectin under dry heating conditions (60°C at 74% RH for 16 d) have been reported by Setiowati, LienVermeir, Martins, De Meulenaer, and Van der Meeren (2016).

The good thermal stability of emulsions stabilised by conjugated protein is predominantly due to the physical restriction of access (by serum phase constituents such as un-adsorbed proteins) to the potentially reactive inner interfacial layer (i.e., protein) by the unreactive outer interfacial layer (i.e., carbohydrate). Strong steric hindrance and increased thickness of the interfacial layer in conjugate-stabilised, compared to protein-stabilised, O/W emulsions, can efficiently prevent flocculation of oil globules when electrostatic stabilisation is disabled (i.e., by charge screening or by proximity to the protein isoelectric point) (Fig. 3.2). The greater thickness of the interfacial layer in conjugate-stabilised emulsions can provide additional stability of oil globules to mechanical stress and high shear forces, commonly experienced during unit operations such as mixing, pumping, flow or atomisation (Sagis and Scholten, 2014). Wooster and Augustin (2006) reported that the thickness of the interfacial layer in O/W emulsions

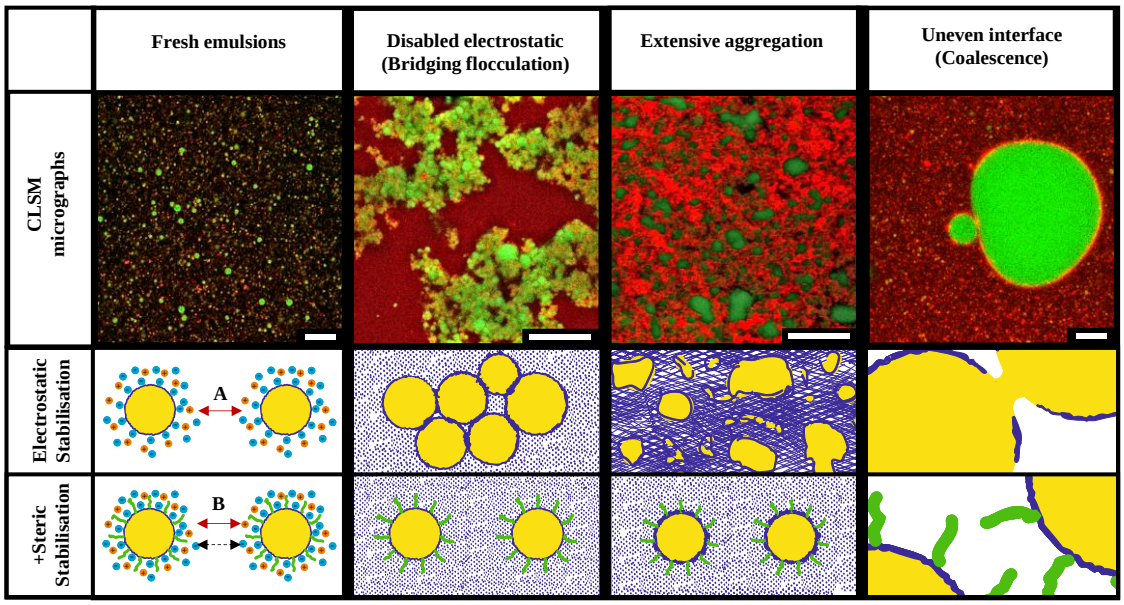

Figure 3.2. A schematic diagram illustrating differences between emulsions stabilised by either electrostatic repulsion (A; 2nd row) or a combination of electrostatic and steric repulsion mechanisms (B; 3rd row); oil droplets (yellow) coated by protein (blue) or conjugate (blue + green) and surrounded by a cloud of ions (anions – blue and cations - orange). Confocal laser scanning microscope (CLSM) images (1st row) show examples of corresponding detrimental changes taking place in such systems, as presented on a model nutritional emulsion formulation system, adapted from Drapala et al. (2015; 2016a). ***Fresh emulsions*** were globally the same for the two different models – homogenous, where small globules ($D_{4,3}$ <1 μm) followed a monomodal distribution; no differences in viscosity and particle size were observed between the conjugated and unconjugated systems. ***Bridging flocculation*** occurs when globules show attractive/cohesive interactions upon collisions/contact and attach; these can also be mediated by serum phase proteins displaying similar cohesive behaviour. ***Extensive aggregation*** occurs when protein-stabilised globules are exposed to prolonged adverse conditions (i.e., high temperature, pH near isoelectric point), oil pools entrapped within the protein matrix giving the aggregates buoyant nature (resemblance in behaviour to flecks as reported by Drapala et al., (2016a). ***Coalescence*** occurs when, upon contact, repulsion forces and strength of the interfacial layer are not sufficient to prevent lipid-lipid contact and resultant mass transfer Drapala et al. (2016b). *Scale bar = 10 μm. Scales for the confocal images vary to best show the relevant features. CLSM legend: green=oil; red=protein.*

stabilised by β-lg-dextran conjugates can be modified by using carbohydrates with different M_w. Fundamentally, increasing the M_w of the carbohydrate moiety yields increased thickness of the interfacial layer and confers greater steric stabilisation as a result (Akhtar and Dickinson, 2007). However, factors such as the kinetics of conjugation (see Section 3.2) and the rate of diffusion/adsorption of the conjugate (i.e., as discussed earlier in this section) can both be negatively impacted by increasing M_w of the carbohydrate and need to be considered when using higher M_w carbohydrates. Emulsion stability can be enhanced by conjugation of protein with charged carbohydrates; Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, and Dewettinck (2004) reported an improved stability of O/W emulsions due to strong electro-steric stabilisation functionality of WPI-pectin conjugates.

Stability of emulsions during storage can present challenges, in regards to deteriorative changes of either a physical nature, due to thermodynamic instability (i.e., coalescence, flocculation, gelation, creaming and oiling off) or a chemical nature (i.e., lipid oxidation) (Tcholakova et al., 2006; Piorkowski and McClements 2014; McClements, 2015; Dalgleish 1997; Chaiyasit, Silvestre, McClements, and Decker, 2000). Physical instability of emulsions can be separated into that governed directly by Stokes law (i.e., gravitational separation of components of different density) and that resulting from interactions between oil globules (i.e., coalescence, flocculation). Steric stabilisation of emulsions containing protein-carbohydrate conjugates can effectively prevent interactions between oil globules over prolonged storage, owing to the strong physical barrier provided by the interfacial layer (Fig. 3.2). O/W emulsions stabilised by conjugated milk proteins have shown no changes in the size of fat globules during storage (24 h-21 d at 22-40^{o}C under quiescent conditions), compared to the corresponding systems stabilised by non-conjugated proteins (Drapala et al., 2016b; Lesmes and McClements 2012; Liu et al., 2016; Medrano, Abirached, Moyna, Panizzolo, and Añón, 2012; O'Regan and Mulvihill, 2013).

Emulsions stabilised by conjugated milk proteins display greater oxidative stability than those stabilised by protein alone, possibly due to the increased thickness of the interfacial layer and the physical barrier that restricts the

access of pro-oxidant species to oxidation-sensitive components such as lipids and lipid-soluble compounds. A significant improvement in the oxidative stability of emulsions containing β-carotene, stabilised by lactoferrin conjugated with dextran, compared to emulsions stabilised by the protein alone, was reported recently by Liu et al. (2016), where the anti-oxidant effect was attributed to restriction of physical contact between pro-oxidants and lipids by the thick interfacial layer of the conjugate-stabilised emulsion. Furthermore, it has been shown that certain (especially late-stage) Maillard reaction products have anti-oxidant properties when incorporated into O/W emulsions (Markman and Livney 2012; O'Regan and Mulvihill, 2010); for example, conjugation of WPI, sodium caseinate and lactose-hydrolysed skim milk powder (SMP) with glucose, lactose, pectin or dextran under dry heating conditions at 70^{o}C and 65% RH for up to 240 h was reported to increase the anti-oxidant capacity of the systems due to production of late-stage Maillard reaction products with antioxidant activity (Hiller and Lorenzen, 2010).

Low ζ potential of oil globules near the isoelectric point of milk proteins, and screening of the electrostatic charge by excess ions, can promote flocculation of protein-coated oil globules, leading to breakage of the emulsion and phase separation (McClements, 2015; Piorkowski and McClements, 2014; Sarkar and Singh, 2016), whereas the presence of a strong steric barrier by protein-carbohydrate conjugates can oppose emulsion destabilisation under these environmental conditions. Lesmes and McClements (2012) demonstrated that conjugation of β-lg with dextran, under dry heating conditions (60^{o}C and 76% RH for 24 h), enhanced the formation and stability of O/W emulsions prepared at pH 7 using the conjugated protein on subsequent acidification to pH 5. The authors reported that the thick O/W interfacial layer formed with the high M_w dextran (i.e., $M_w \geq 40$ kDa) was responsible for the greater stability of the conjugate-based emulsions, compared to emulsions made using unconjugated protein.

Good stability to storage at high salt concentration (0.2 M citrate buffer) and under acidic conditions (pH 3.2) were reported for emulsions stabilised by conjugates of β-lg or WPI with corn fiber gum prepared using dry heating conditions at 75^{o}C and 79% RH for time periods ranging from 2 h to 7 d (Yadav

et al., 2010); in these systems, the branched nature of the corn fiber gum resulted in good emulsion stability, even at low levels of conjugation. Considerably improved resistance to flocculation for emulsions stabilised by conjugates of β-lg and dextran (M_w 27-200 kDa) at high salt (i.e., 0-20 mM $CaCl_2$) addition levels was also reported by Wooster and Augustin (2006). In that study, the authors reported that a significant increase (~12-fold) in particle size for emulsions stabilised by unconjugated protein was observed at ≥10 mM calcium (Ca) content, while no changes were observed at all added Ca levels for the conjugate-based systems. The superior stability of conjugate-stabilised emulsions was attributed to the thickness and steric stabilisation effects of the outer interfacial layer (i.e., dextran), which effectively offset the electrostatic screening effect of Ca addition. Similar findings were reported for lactoferrin-dextran conjugates, where strong steric stabilisation of oil globules resulted in emulsion stability at high ionic strength (Liu et al., 2016). Likewise, Akhtar and Dickinson (2007) reported that emulsions stabilised by WPI-MD conjugates (DE 19) and containing high levels of sodium lactate (5% w/w) did not show any changes in particle size distribution after 21 d of storage at 22°C, in contrast to a ~2-fold increase in mean volume diameter for emulsions stabilised by unconjugated protein or by gum arabic (a naturally-occurring protein-carbohydrate conjugate).

The unique functionality of milk protein-carbohydrate conjugate-based emulsifiers is particularly interesting for emulsion-based food products exposed to challenging environmental and processing conditions such as low pH, high ionic strength and severe thermal processes (e.g., fruit beverages, infant formula, clinical nutrition products and acidified milk drinks). Such products can pose challenges with processing and shelf life stability and, in some cases, hydrocolloids are added to retard phase separation. Milk protein/peptide-carbohydrate conjugate-based emulsifiers also offer a significant potential for applications in emulsion-based delivery systems, where their interfacial functionality can facilitate controlled release of sensitive bio-actives (e.g., vitamins) in the small intestine, avoiding acid-mediated emulsion destabilisation and loss of the encapsulated material in the stomach. Gumus, Davidov-Pardo, and McClements (2016) reported that, as

well as stability to acidic conditions, emulsions stabilised by casein-dextran conjugates, prepared under dry heating conditions (60^{o}C and 76% RH for 48 h), were additionally resistant to enzymatic digestion by pepsin, which prevented issues with premature release of encapsulated lutein in the stomach. Proteolysis of the interfacial layer was retarded by the thick outer carbohydrate layer, which restricted the pepsin from accessing the inner protein interfacial layer. In addition, the authors showed that the use of conjugate-based emulsifiers did not interfere with release of encapsulated material in the intestine, where bile salts displaced the emulsifier from the surface of oil globules. Similarly, in the study of Lesmes and McClements (2012), β-lg-dextran conjugate-stabilised emulsions displayed good stability to stomach-like environmental conditions, due to strong steric stabilisation and subsequent release of encapsulated fatty acids occurred in the intestinal stage, due to emulsifier displacement by bile salts.

3.8. Foaming

Somewhat like emulsions, foams are systems comprising of two phases (i.e., air and water), both with different densities; their formation requires an aeration process (i.e., whipping or sparging) and it is aided by the presence of surface active compounds (i.e., proteins, peptides, low M_w surfactants) which can adsorb at the air/water (A/W) interface and reduce its surface free energy, allowing retention of air within the foam matrix. The foaming properties of proteins/peptides are often assessed by their rate of adsorption at the A/W interface, as measured by the volume of air incorporated in the foam (i.e., foam capacity) and their ability to subsequently retain that volume of incorporated air (i.e., foam stability) (Foegeding, Luck, and Davis, 2006). Stability of foams is inherently lower than that of emulsions, due to the greater tendency to separation of the two phases, owing to the larger diameter and lower density of the dispersed phase particles (i.e., air bubbles and oil globules, respectively) in foams than in emulsion systems. In addition, solubility of the dispersed phase in the continuous phase and macroscopic processes such as liquid drainage, Ostwald ripening (i.e., disproportionation) and bubble coalescence also contribute to foam instability. Surface activity of a protein/peptide is the main factor affecting foam capacity, therefore, solubility and mobility of

proteins/peptides in the bulk phase strongly determine the initial foam volume. Conversely, foam stability is largely governed by the rheological properties of the interfacial layer. Despite slower interfacial adsorption rates, compared with lower M_w non-protein surfactants, proteins confer important functionality to foams, owing to their interfacial flexibility and viscoelastic properties (Damodaran, 2005). Modification of the structure/conformation of food proteins or, more specifically, milk proteins can offer important functional benefits in applications involving foam formation and stabilisation; alteration of the hydrophilic-hydrophobic balance (Hamada and Swanson 1994; Lei, Zhao, Selomulya, and Xiong, 2015; Morand, Dekkari, Guyomarc'h, and Famelart, 2012), controlled denaturation and aggregation (Dombrowski, Johler, Warncke, and Kulozik, 2016; Kim, Cornec, and Narsimhan, 2005) or formation of nanoparticles by electrostatic complexation of sodium caseinate and gum arabic (Ye, Flanagan, and Singh, 2006) or complexation and conjugation of different milk proteins with carbohydrates (Báez et al., 2013; Jian, He, Sun, and Pang, 2016; Jiang and Zhao, 2011; Turgeon, Schmitt, and Sanchez, 2007) have all been shown to improve surface activity and foaming properties of proteins.

Foam capacity is governed by the properties of the proteins/peptides present in a system; high surface activity, high mobility in the bulk phase and the presence of hydrophobic regions are the main factors affecting foam overrun (i.e., volume of air incorporated). Adsorption of hydrophobic regions of the protein molecule at the A/W interface is thermodynamically favourable, due to the hydrophobic nature of the interface (i.e., due to constant evaporation), and it is regarded as one of the main driving forces for protein adsorption and anchoring at the interface (Dickinson, 2010). The adsorption process in protein-based food systems can often be self-limiting, due to gradual development of a barrier acting to impede adsorption; physical restriction of space or electrostatic repulsion between protein molecules can promote formation of such a barrier (Wierenga, Meinders, Egmond, Voragen, and De Jongh, 2005). Dombrowski et al. (2016) reported that increasing hydrophobicity of β-lg, by controlled aggregation, increased the probability of its adsorption at the interface (as measured by changes in the surface

pressure), regardless of the barrier counteracting adsorption. The authors also reported that the surface adsorption of heat-induced aggregates of β-lg was not different to that observed for the native protein, demonstrating that diffusion kinetics of β-lg to the interface were unaltered by denaturation/aggregation, signifying that the effect of increased protein hydrophobicity was dominant over the changes in size brought about by aggregation of the protein molecules. In addition, foaming properties of protein tend to be optimal at pH near their isoelectric point, due to lowered electrostatic repulsion forces between proteins adsorbing at the interface, allowing for higher density interfacial packing (Marinova et al., 2009). A similar net effect can be achieved with the addition of salts/ions due to screening of the electrostatic charges and reducing the intensity of charge-based repulsion forces (Foegeding et al., 2006). Moreover, decreasing the electrostatic repulsion forces, by pH adjustment or salt addition, can promote interactions between individual proteins/peptides, resulting in conformational rearrangement and increased hydrophobicity, thus intensifying the driving force for adsorption.

Changes to structure/conformation of proteins, resulting from their conjugation with carbohydrates, generally contribute to increased protein solubility, higher protein mobility and, effectively, faster adsorption at A/W interfaces (see Section 3.7). Improvement in foam capacity for BSA conjugated with glucose in a wet heating process (45°C for 2 h with continuous stirring), compared to BSA conjugated with mannose or unconjugated BSA, was reported by Jian et al. (2016). In this study, conjugation resulted in changes in protein conformation, yielding a more flexible and loosened structure which, effectively, increased the rate of protein adsorption at the A/W interface; however, a decrease in surface hydrophobicity and decreased foam stability was reported for conjugated BSA. Similar findings were reported for foams stabilised by β-lg-glucose conjugates (dry heating; 50°C at 65% RH for 96 h) (Báez et al., 2013), where improved foam capacity, compared to using unconjugated β-lg, was explained by heat-induced conformational changes in the structure of the whey protein molecules, conferring more open and flexible structures, thus allowing more rapid formation of the interfacial layer. A

combination of increased hydrophobicity and changes in the conformation of protein can offer increased foam overrun as reported for supramolecular α-lac-glycomacropeptide complexes (i.e., stabilised by non-covalent interactions) by Diniz et al. (2014).

Foams are thermodynamically unstable systems that can undergo rapid destabilisation (i.e., bubble coarsening, coalescence, film drainage, film rupture and foam collapse) (Considine, 2012; Foegeding et al., 2006). The key component conferring stability to foams is the interfacial layer, occupied by surface active compounds (i.e., proteins and peptides), providing a viscoelastic structure to the system, serving to stabilise it against the forces of gravity acting on it and limiting mass transfer between air bubbles (Drenckhan and Saint-Jalmes, 2015; Murray, 2002; Stuart, Norde, Kleijn, and van Aken, 2005). The rheological properties of the interfacial layer govern the stability of the foam; in theory, a thermodynamically-stable foam can be achieved when its interfacial layer has purely elastic behaviour (Wijnen and Prins, 1995). However, this is rarely the case in food systems; hence, one of the objectives in engineering stable foams is to develop a greater level of elastic behaviour in the rheological properties of the interfacial layer (Báez et al., 2013; Drenckhan and Saint-Jalmes, 2015; Jiang and Zhao, 2011). The most common strategies applied to ensure desirable viscoelastic properties of interfacial films of foams involve decreasing of the long-range electrostatic repulsion between protein molecules to increase their packing density at the A/W interface (Dickinson, 2010). This is generally done by either decreasing the pH towards the isoelectric point of the protein, by increasing the ionic strength to screen the electrostatic repulsion forces, or by a combination of both (Wierenga and Gruppen, 2010). However, such environmental conditions also coincide with a strong tendency for protein to aggregate and precipitate, due to diminished electrostatic repulsion, dramatically reducing their functionality (Lucey, 2016). Conjugation of protein with carbohydrates allows the avoidance of extensive protein aggregation when the electrostatic repulsion forces are disabled (i.e., at acidic pH or high ionic strength). In effect, denser protein packing without extensive aggregation can be achieved using conjugated protein (Rade-Kukic, Schmitt, and Rawel, 2011).

Jimenez-Castano et al. (2007) reported that conjugation of milk proteins (β-lg, α-lac and BSA) with dextran in a dry-heating process (55°C at 0.44 a_w for up to 96 h) resulted in a reduction in isoelectric point of each protein and improved their solubility and heat stability around the isoelectric point of the protein. Such modified functionality (i.e., good heat stability at low pH) can offer potential in protein-based foam applications, allowing considerable protein unfolding when heated at low pH, without extensive protein aggregation or precipitation. Controlled aggregation under these conditions, combined with flexible unfolded protein structures, and low electrostatic repulsion, offer significant potential for stabilisation of foam systems. Other approaches directed at improving foam stability involve increasing the thickness and elasticity of the interfacial film by increasing the size of its building blocks (i.e., controlled protein aggregation) (Báez et al., 2013; Dombrowski et al., 2016; Foegeding et al., 2002; Rullier et al., 2008; Tamm et al., 2012) or by conformational changes to the protein structure (i.e., partial unfolding of globular protein) (Dissanayake and Vasiljevic, 2009; Dombrowski et al., 2016; Morales, Martínez, Pizones Ruiz-Henestrosa, and Pilosof, 2015). These approaches closely match the changes to protein structure/conformation and functionality offered by protein conjugation; increased size of interfacial building blocks, controlled protein aggregation on conjugation and opening up of the protein structure have been shown to improve stability of foams formed with conjugated milk proteins (Corzo-Martínez et al., 2012b; Hiller and Lorenzen, 2010).

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In using protein/peptide-carbohydrate conjugates to stabilise foams, the thickness of the interfacial layer and therefore, effectiveness of steric stabilisation, can be controlled using carbohydrates with different M_w (Wooster and Augustin, 2006). Hiller and Lorenzen (2010) reported increased stability of foams prepared with a range of protein (WPI, sodium caseinate and lactose-hydrolysed skim milk) and carbohydrate (glucose, lactose, pectin and dextran) conjugates (produced by dry heating at 70°C and 65% RH for up to 240 h) due to formation of thick and viscoelastic interfacial films that prevented disproportionation of gas bubbles. Increasing the thickness of the interfacial film can effectively improve its rheological properties in addition to providing an effective steric barrier with good dilatational properties (Dombrowski et al., 2016). Similarly, Kim, Cornec, and Narsimhan (2005) reported that denaturation and unfolding of β-lg resulted in increased shear elasticity and viscosity of the interfacial layer due to increased flexibility of the partially-denatured globular protein.

The viscoelastic properties of protein-stabilised foams are strongly dependent on the structure/conformation of the protein; globular proteins (e.g., whey proteins) tend to give interfacial films with greater viscoelasticity, due to higher packing density, compared to less ordered proteins (e.g., caseins) (Bos and van Vliet, 2001). Conjugation of less-ordered proteins offers good potential for improvement of their foam stabilising properties due to increases in the thickness of the interfacial layer and, effectively, better dilatational properties of the A/W interface (Dombrowski et al., 2016). Jiang and Zhao (2011) reported that modification of casein (sodium caseinate) by its cross-linking using transglutaminase and/or conjugation with glucosamine (using a wet heating approach at 37°C for up to 5 h with continuous agitation) significantly increased apparent viscosity of the caseinate solutions. The authors showed that a combination of cross-linking and conjugation increased both storage and loss moduli of casein suspensions and that the elastic properties of the cross-linked and conjugated casein solutions were more dominant, indicating solid-like response to dynamic deformation, in contrast to unmodified and cross-linked casein suspensions. Modification of the viscoelastic properties of the interfacial layer of foams by conjugation of casein with glucosamine conferred enhanced stability against bubble coalescence (increased by 20.8% compared to unconjugated casein) (Jiang and Zhao, 2011). Facilitating dense packing and interactions between protein-based building blocks are effective means of improving viscoelastic properties of the interfacial layer of a foam (Mackie and Wilde, 2005). In a similar way, facilitating interactions between carbohydrate components of protein-carbohydrate conjugates adsorbed at A/W interfaces of foams can provide not only a strong steric barrier but also improved viscoelastic properties of the interface. Cai and Ikeda (2016) reported increased resistance against surfactant-induced displacement of protein from the A/W interface in foams stabilised with WPI-gellan conjugates prepared by dry heating at 80°C and 79% RH for 2 h, compared to systems containing unconjugated WPI and the surfactant Tween 20. The authors attributed the greater resistance to displacement of protein in the conjugate-based foam system to the ability of the gellan moiety, covalently attached to the whey protein molecules, to form

a carbohydrate network at the interface, effectively immobilising the conjugate-covered interface.

Conjugation of protein with carbohydrates alters the hydrophobic-hydrophilic balance of protein and conformational changes to the protein structure caused by conjugation increase its surface hydrophobicity, generally resulting in improved emulsion formation properties of conjugated proteins (see Section 3.7). On the other hand, hydrophilicity of the resulting ingredient is increased by the attachment of the hydrophilic carbohydrate moieties (see Section 3.7). Conversely, greater hydrophilicity can yield better foam stability due to improved water holding capacity by the conjugate located at the interfacial layer, and effectively restrict liquid drainage in the foam (Báez et al., 2013). The hydrophilic nature of the carbohydrate anchored at the A/W interface by the protein, viscoelastic properties of the interface, and higher viscosity for conjugated protein-carbohydrate systems (WPI, SMP, sodium caseinate, glucose, lactose, pectin, dextran), compared to native protein, have been shown by Hiller and Lorenzen (2010) to be the main factors responsible for increased foam stability. In contrast, another study by Jian, He, Sun, and Pang (2016) have claimed that the increased hydrophilicity of BSA resulting from its conjugation with glucose or mannose decreased foam stability. Jiang and Zhao (2011) elucidated that a shift in the amphiphilic nature of casein towards more hydrophilic behaviour, following conjugation with glucosamine, reduced ability of foam to retain the incorporated air. It is important to consider that both of these, apparently contradictory, findings can hold true, with the precise impact of conjugation being very much dependent on differences in protein structure (globular, ordered, unordered etc), nature of the carbohydrate (chain length, charge etc) and conditions employed for conjugation and foam formation.

3.9. Gelation and textural properties

Whey protein gels are three-dimensional, self-supporting, networks, within which the aqueous solution and any dispersed elements (e.g., fat) are entrapped. Gelation of whey proteins involves a controlled increase in protein-protein interactions, while carefully maintaining a balance with protein-

solvent interactions (Brodkorb et al., 2016). During gelation, the number and combined strength of protein-protein interactions (e.g., disulphide, hydrophobic and electrostatic interactions) determine the mechanical and rheological properties of the resultant gel network. High whey protein content ingredients (i.e., WPC and WPI) are commonly used in food applications which require gelation of the protein for the expression of functionality (e.g., recombined meat products, desserts, yoghurts, puddings, mousses). Many compositional and environmental factors affect the formation and rheological properties of whey protein gels, including protein concentration, pre-denaturation and aggregation of protein, salts, temperature and pH (Foegeding, Bowland, and Hardin, 1995; Langton and Hermansson, 1992; Mulvihill and Kinsella, 1987).

It is known for over 20 years that heating solutions of globular milk proteins (e.g., lysozyme and BSA) and reducing sugars (e.g., lactose, ribose and xylose), at temperatures of 90-121^{o}C, results in the formation of gels with higher firmness and elasticity than gels made using the proteins alone in solution (Armstrong, Hill, Schrooyen, and Mitchell, 1994; Easa, Hill, Mitchell, and Taylor 1996). The increased strength of these protein-carbohydrate gels is due to Maillard reaction-mediated reduction in pH and by cross-linking of the protein molecules (e.g., *via* lysinoalanine). The gel strength (but also colour development) increases with decreasing M_w of the sugars (Hill, Mitchell, and Armstrong, 1992), while the pH required to achieve gelation decreases with increasing sugar concentration and reactivity. In combination, these effects of sugar incorporation on gelation properties of globular protein on heating, means that it is possible to reduce the amount of protein required for gel formation (Azhar, 1996; Oliver et al., 2006b).

More recent work has focused on studying the gelation properties of milk proteins (especially whey proteins) conjugated with higher M_w carbohydrates under dry heating conditions, due to the challenges associated with denaturation and aggregation of whey proteins under wet heating conditions (Gauthier et al., 2001; Morgan et al., 1999a). Conjugation of whey proteins in WPI with dextran has been shown to influence the rheological properties of heat-induced gels made therefrom (Spotti et al., 2013a, b; 2014a, b; Sun et al.,

2011). Conjugation of WPI with dextran of M_w 6, 40 and 70 kDa, under dry heating conditions at 60°C for 2-9 d at 63% RH was shown to result in whey protein-conjugate gels with lower fracture stress and Young's Modulus as measured by uniaxial compression testing (Spotti et al., 2013a; 2013b) and lower gel firmness (i.e., storage modulus) as measured by dynamic low amplitude oscillatory shear rheology (Spotti et al., 2014a; 2014b), compared with WPI alone or unconjugated WPI-dextran mixtures. Similar results were reported by Sun et al. (2011) for WPI conjugated with dextran (average M_w 150 kDa) at 60°C for 7 d at 79% RH.

The lower strength of heat-set WPI-based gels made from whey protein conjugated with dextran, compared with unconjugated whey protein or mixtures of whey protein and carbohydrates is attributed to several factors, with the relative contribution of the individual factors dependent on the system composition and conditions of conjugation. Under the heating conditions typically required to achieve conjugation (see Section 3.4), denaturation and aggregation of whey proteins can occur, serving to alter exposure and reactivity of functional groups (e.g., free sulphydryl and hydrophobic groups) and the surface charge of protein molecules, all of which influence protein-protein and protein-water interactions (Brodkorb et al., 2016). Covalent attachment of the carbohydrate molecules also increases the hydrophilicity and steric barrier properties of the conjugated proteins, both of which result in decreased protein-protein interactions and increased protein-water interactions.

3.10. Enrichment and purification of conjugates

It is desirable to enrich the protein-carbohydrate conjugates from the reaction mixtures in which they are produced in order to remove unreacted carbohydrate, unreacted protein and possibly soluble Maillard reaction products, while increasing conjugated protein concentration. Such processes need to be food-grade, efficient, economical and have acceptable yield – enrichment, as opposed to purification, of the protein-carbohydrate conjugate is normally sufficient.

There has been limited work published to date on the enrichment/purification of milk protein-carbohydrate conjugates, and the studies that have been reported (Bund, Allelein, Arunkumar, Lucey, and Etzel, 2012; Etzel and Bund, 2011) are very much informed by approaches used in the pharmaceutical industry for purification of various therapeutic proteins conjugated with polyethylene glycol (i.e., PEGylated proteins), with separation being achieved largely based on differences in hydrophobicity (i.e., using hydrophobic interaction chromatography; Mayolo-Deloisa, González-Valdez, and Rito-Palomares, 2016) and charge density (Abe, Akbarzaderaleh, Hamachi, Yoshimoto, and Yamamoto, 2010) between conjugated and unconjugated proteins.

An initial study by Etzel and Bund (2011) involved laboratory-scale, analytical separation and enrichment of whey protein-dextran conjugates from mixtures of unreacted dextran and whey protein using cation exchange column chromatography with traditional chromatographic beads or porous polymethacrylate monolithic media and sodium lactate/sodium chloride-containing elution buffers. Using such an approach, unreacted dextran eluted first, followed by the conjugated protein and finally the unreacted protein; a portion of the unreacted whey protein was isoelectrically precipitated from the feed stream at pH 5.0, before chromatographic separation. The monolith media resulted in a similar dynamic binding capacity as the traditional beaded support (4-6 g L^{-1}) but with 42-fold higher mass productivity and 48-fold higher flow rate, while yielding a conjugate-enriched stream with lower purity. The use of cation exchange chromatography, as originally proposed by Etzel and Bund (2011) has been successfully scaled up to a preparative scale (i.e., 160 fold up-scaling from 5 mL to 800 mL columns) by Bund et al. (2012). On scale up, the upfront partial removal of unreacted whey protein by isoelectric precipitation was shown to be effective in reducing the buffer volumes required, purification time and the number of chromatography cycles required for purification of the conjugates. The yield of conjugated protein was ~18% on a protein basis, with the losses mainly associated with incomplete conversion of unconjugated to conjugated whey protein during the conjugate production process. Opportunities for increasing this conversion rate should

be evaluated in future studies, with integration of conjugation and fractionation steps to reintroduce unreacted dextran and protein or the use of on-column conjugation having being suggested by Fee and Van Alstine (2006) and Bund et al. (2012). In addition, progressively increasing the salt concentration during elution for the enrichment/purification of milk protein-carbohydrate conjugates would be expected to facilitate separation of conjugates based on differences in their degree of glycosylation, as is the case with PEGylated lysozyme and BSA (Abe et al., 2010).

The vast majority of the studies to date on functional properties of conjugated milk proteins/peptides have been completed on mixtures of conjugated and unconjugated proteins/peptides (i.e., without removal of unconjugated protein/peptide and carbohydrate material). Further development of approaches for enrichment and purification of conjugated proteins/peptides from unconjugated proteins/peptides and carbohydrates will allow more systematic and deeper understanding of the role of residual unconjugated protein/peptide and carbohydrate material in determining the overall functionality of conjugated mixtures.

Modification of the techno-functional properties of proteins by their conjugation with carbohydrates has been shown to facilitate formation of complex food matrices (e.g., emulsions, foams and gels) with improved stability and/or textural characteristics. Research reviewed in this chapter indicates the significant potential of conjugated proteins as ingredients for applications in a broad range of food systems.

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Experimental Chapters

Objectives

The principal objective of the research reported on in this thesis was to develop new fundamental knowledge on the effects of selected emulsifiers on the formation and stability of model infant formulae (IF) products. A particular focus was placed on determining, for the first time, the functionality of Maillard-induced whey protein hydrolysate-maltodextrin (WPH-MD) conjugates as emulsifiers in IF products. Another aim of the research was to demonstrate how the mode of stabilisation of oil globules (i.e., electrostatic *vs* steric) in model IF emulsions affects stability of these systems to a range of industrial manufacturing processes (i.e., short time storage, heat treatment, spray drying and powder rehydration) and to prolonged storage (accelerated shelf life testing, accelerated creaming analysis and lipid oxidation analysis).

The specific aims of the research were as follows:

- To investigate the surface activity of proteins/peptides and low molecular weight (L_{Mw}) emulsifiers (i.e., phospholipids from soybean lecithin) at air-water and oil-water interfaces, and to correlate these functional properties of ingredients with their ability to form an emulsion.

- To characterise destabilisation processes typically experienced for different types of IF emulsions based on intact and hydrolysed whey protein and to elucidate the mechanisms responsible for these processes.

- To investigate competitive displacement and destabilisation mechanisms in mixed emulsifier-based IF emulsions (proteins/peptides + L_{Mw} emulsifiers) and to relate these properties to the stability of IF emulsions to processing and to prolonged storage.

- To evaluate the effectiveness of steric stabilisation of oil globules in model IF emulsions, formed with WPH-MD conjugate ingredient, in conferring stability of emulsions to heat treatment, spray drying and prolonged storage.

Chapter 4

Influence of lecithin on the processing stability of model whey protein hydrolysate-based infant formula emulsions

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Abstract

Whey protein hydrolysate (WPH)-based oil-in-water (O/W) emulsions containing lecithin (0–5%, w/w, oil) were produced and stored at 4 °C for 14 days. Surface tension and interfacial tension of these systems were measured for formulation development. Fat globule size distribution (FGSD) analysis and confocal laser scanning microscopy (CLSM) were used to assess the physical stability of emulsions during storage and identify mechanisms of instability. Lecithin decreased interfacial tension between oil and aqueous phases of model emulsions and allowed formation of smaller oil droplets on homogenisation. However, low-intermediate levels (1–3%) of lecithin caused coalescence and shift to bimodal FGSD during storage of emulsions.

4.1. Introduction

Bovine milk is widely used as a base material for the manufacture of infant nutritional products; however, its composition differs considerably from that of human milk. Differences in the protein content (i.e., 33 and 9-11 g L^{-1} in bovine and human milk, respectively), ratio of casein:whey protein (i.e., 80:20 and 40:60 in bovine and human milk, respectively), and amino acid composition need to be considered during ingredient selection and formulation development in the manufacture of infant formula (IF) products. Other ingredients used in the formulation of IF products are lactose, maltodextrins and corn syrup solids as sources of carbohydrates, blends of vegetable and fish oils (to mimic the fatty acid composition of human milk), minerals, vitamins and emulsifiers such as lecithins or mono- and di-glycerides (Alles et al., 2004; MacLean et al., 2010).

Cow's milk allergy (CMA) is a condition observed in early childhood and on average 2.2% of children below the age of two years are affected (Natale et al., 2004; Tammineedi et al., 2013). Partial or limited enzymatic hydrolysis of protein can help in reducing CMA-related issues by offering 'pre-digested' formula for infants. Manufacturers of infant nutritional ingredients/products employ enzymatic hydrolysis to produce formulas which are easier to digest; these products are generally modified cow's milk-based formulae, often based exclusively on whey protein, and are suggested as being suitable for infants experiencing feeding discomfort and digestion-related issues (O'Mahony et al., 2011).

Considerable challenges encountered in the manufacture of partially hydrolysed whey protein-based IF emulsions are related to poor heat stability during processing, coalescence and creaming and lipid oxidation on storage. Several studies have focused on improving the stability of these types of IF systems (Tirok et al., 2001; Christiansen et al., 2004; Ye et al., 2004; Ye and Singh, 2006). Emulsion stability and fat globule size distribution (FGSD) of model IF are known to be influenced by protein content (McCarthy et al., 2012). Improvement of heat stability (McSweeney et al., 2004, 2008) and oxidative stability (Zou and Akoh, 2013) of IF systems with increasing lecithin

content has also been reported. However, intact dairy protein was used as the protein source in the aforementioned studies, and there is currently a lack of detailed information on the processing stability of hydrolysed whey protein-based IF type emulsions. Studies involving hydrolysed whey protein and their properties have shown that the degree of hydrolysis (DH) is generally a good indicator of protein functionality (Foegeding et al., 2002) and low levels of hydrolysis (i.e., DH ≤10-20%) are beneficial (Singh and Dalgleish, 1998; Caessens et al., 1999; Van der Ven et al., 2001; Luck et al., 2002; Ruiter and Voragen, 2002) while more extensively hydrolysed (i.e., DH >20%) proteins increasingly lose their structure and display decreased techno-functionality (Agboola and Dalgleish, 1996a, 1996b; Scherze and Muschiolik, 2001).

The incorporation of low molecular weight emulsifiers, such as phospholipids (commonly from lecithin) or mono- and di-glycerides, generally increases the physical stability of hydrolysed protein-based emulsions due to their ability to adsorb at the oil/water interface and effectively decrease the interfacial tension (Tirok et al., 2001; Ruiter and Voragen, 2002). Arising from decreased interfacial tension, smaller fat globules can be formed during homogenisation (Van Aken et al., 2003; Diftis and Kiosseoglou, 2004; O'Brien, 2009) and the rapid action of low molecular weight surfactants adsorbing at the interface prevents rapid coalescence (Dickinson et al., 1989). Production of emulsions with narrow FGSD, where volume-surface average diameter is <1.0 μm (i.e., typically ~0.5 μm) (Buchheim and Dejmek, 1997) is desirable in the manufacture of infant formula emulsions and, when achieved, usually indicates good emulsifying properties of the ingredients (McCarthy et al., 2012).

Lecithin is used in the manufacture of IF products to enhance emulsifying properties or storage stability by forming a physical barrier in the form of a cohesive film around oil globules (McClements, 2004; Ghosh and Rousseau, 2010). Lecithin is also known to enhance stability to heating of emulsions (Agboola et al., 1998a; Van der Meeren et al., 2005; Tran Le et al., 2007; McSweeney et al., 2008) which is especially important in the manufacturing processes of IF emulsions based on hydrolysed protein. Additionally, the amphipathic nature of lecithin gives it good functionality as a wetting agent,

therefore it can also be used to aid instant properties of milk/infant formula powders (O'Mahony et al., 2011; Sharma et al., 2012; Hammes et al., 2015).

The use of hydrolysed whey proteins in the formulation of IF products is of growing interest; however, limited formulation research has been carried out in this area. In this study, the effects of lecithin on the processing and physical stability of model IF emulsions prepared with hydrolysed whey protein were studied. The effect of different levels of addition of lecithin on the interfacial tension in O/W systems and the consequences thereof for the manufacture and stability of model hydrolysed whey protein-based IF emulsions during storage were investigated.

4.2. Materials and Methods

4.2.1. Materials

Whey protein hydrolysate (WPH), Hyprol®, was obtained from Kerry Group (Listowel, Co. Kerry, Ireland). Maltodextrin (Maldex 170 with dextrose equivalent value of 17) and de-oiled powdered soybean lecithin (Ultralec® P) were obtained from Syral Belgium N.V. (Aalst, Belgium) and ADM (Decatur, IL, USA), respectively. Soybean oil (Organic Soya Oil, Clearspring Ltd., London, UK) was purchased from a local commercial outlet. All other chemicals, reagents and minerals used in the study were purchased from Sigma Aldrich (St. Louis, MO, USA).

4.2.2. Characterisation of whey protein hydrolysate

Protein, ash, moisture and fat contents of WPH were determined by Kjeldahl (IDF Standard 20-1, 2014), ashing at 500°C for 5 h (IDF Standard 90, 1979), oven drying at 103°C for 5 h (IDF Standard 26, 2004) and Rose Gottlieb method (AOAC, 1995), respectively; lactose content was determined by difference. Degree of hydrolysis of the WPH ingredient was determined by the trinitrobenzenesulfonic acid method as described by Adler-Nissen (1979).

Size distribution of peptides in the WPH was determined by size exclusion chromatography (SEC) using a TSK G2000SW, 600 × 7.5 mm column (10 µm, Sigma-Aldrich, Dublin); elution was with an isocratic gradient of 30%

acetonitrile containing 0.1% TFA (v/v) at 1.0 mL/min. The samples were diluted in water or running buffer and 20 μL of 1 g/L protein/peptide solutions were injected onto the column and the eluate was monitored with UV absorbance at 214 nm. Commercial β-lactoglobulin A, bovine serum albumin and caseinomacropeptide (CMP) (Sigma-Aldrich, Dublin, Ireland) were used as standards with Ribonuclease A, Cytochrome C, Aprotinin, Bacitracin, His-Pro-Arg-Tyr, Leu-Tyr-Met-Arg, Bradykinin, Leu-Phe, Tyr-Glu (Bachem AG, Bubendorf, Switzerland) used as molecular weight standards. Standards were pre-filtered through 0.22 μm low protein binding membrane filters (Sartorius Stedim, Surrey, UK) and centrifuged at 10,000 × g for 20 min prior to application to the column. All solvents were filtered under vacuum through a 0.45 μm high velocity filter (Millipore Ltd., Durham, UK).

4.2.3. Preparation of emulsions

Model infant formula emulsions containing 1.55, 3.50 and 7.00 g/100 mL of protein, oil and carbohydrate, respectively, were prepared as follows: WPH and maltodextrin (MD) were solubilised in ultrapure water preheated to 75°C with continuous mixing using an overhead stirrer at 500 rpm for 1 h. Solutions (1 mL) containing individually iron sulphate heptahydrate, zinc sulphate heptahydrate, manganese sulphate monohydrate or copper sulphate (to give final added iron, zinc, manganese and copper concentrations of 800, 600, 33 and 5 μg/100 mL, respectively) were then added to the protein/carbohydrate solution.

Stock lecithin-containing soybean oil was prepared by adding lecithin to preheated (55°C) oil during continuous mixing with magnetic stirring on a hotplate (55°C) for 60 min. The stock solution was then added to soybean oil (55°C) to give five different lecithin concentrations (1-5%, w/w, oil). Aqueous and oil phases were subsequently mixed and maintained at 55°C until homogenization. A control was prepared with soybean oil alone (i.e., no added lecithin). Emulsions were formed by pre-homogenisation with an Ultra-Turrax at 10,000 rpm for 2 min followed by two stage homogenisation (double pass) at 10 and 2 MPa, using a valve homogeniser (APV GEA Niro-Soavi S.p.A., Italy) at 50°C. Following homogenisation, the pH of each emulsion was

adjusted to 6.8 with 0.1 N HCl and/or 0.1 N NaOH and sodium azide (0.05%, w/v) was added to prevent microbial growth during storage.

4.2.4. Compositional analysis of emulsions

Kjeldahl (IDF Standard 20-1, 2014) and Gerber (IDF Standard 105, 2008) methods were used for determination of protein and fat levels of emulsions, respectively. Moisture content was measured by oven drying at 103°C for 5 h (IDF Standard 21, 2010). Ash was measured using muffle furnace heating at 500°C for 5 h (AOAC, 2002). Carbohydrate content was calculated by difference.

4.2.5. Surface and interfacial tension analysis

4.2.5.1. Dynamic surface tension

Surface tension (γ_S) measurements were performed at 55°C (to best replicate emulsion preparation conditions) under atmospheric pressure with a Krüss K12 Tensiometer (Krüss GmbH, Hamburg, Germany) equipped with a Wilhelmy plate. γ_S was measured over 60 min after formation of the surface in ultrapure water, WPH solution (1.55%, w/v) and soybean oil containing different levels of soybean lecithin (0-5%, w/w). Samples containing lecithin were prepared by adding the lecithin to preheated (55°C) oil and allowing it to mix fully at 55°C with continuous intermediate-speed stirring for 60 min. The protein solution was prepared as described earlier with only protein added; ultrapure water was used as a control. Aliquots (25 mL) were placed in the sample vessel and air bubbles (if present) were removed with a Pasteur pipette. Before each measurement, the plate attachment and the sample vessel were washed with acetone and ultrapure water followed by annealing over a flame to ensure removal of all organic matter. Glassware used in the analysis were subjected to an acid wash, i.e., after thorough washing with detergent and water, glassware were filled to overflow with 1 N nitric acid, left overnight and rinsed 3 times with ultrapure water before drying.

4.2.5.2. Dynamic interfacial tension

Measurements of interfacial tension (γ_I) at the soybean oil/lecithin (0-5%, w/w) interface with ultrapure water or protein solution (1.55%, w/v; protein from WPH) were also carried out with a Krüss K12 Tensiometer using the Wilhelmy plate method. Samples were prepared as detailed earlier for dynamic surface tension and measured at 55°C over 60 min; 25 mL of heavy phase (water or protein solution) and 25 mL of light phase (oil or oil containing lecithin) were used. γ_I was recorded continuously from 0 to 5 min and at 10, 15, 30 and 60 min after forming the interface. The measurement program was set to record a maximum of 80 readings per given time point at 1 s intervals, unless the standard deviation was ≤0.01 over ten consecutive readings, in which case the measurement would stop for the given time point. The sample vessel and the Wilhelmy plate were cleaned and annealed before each measurement and all glassware was acid washed as described earlier.

4.2.6. Measurement of fat globule size distribution

Fat globule size distribution of the emulsions was measured using a laser light-diffraction unit (Mastersizer S, Malvern Instruments Ltd., Worcestershire, UK) equipped with a 300 RF (reverse fourier) lens and He-Ne laser (λ of 633 nm). A polydisperse model with 3NAD presentation and a particle and dispersant refractive index of 1.46 and 1.33 were selected for data analysis as described by McCarthy et al. (2012). Sample was introduced to the mixing chamber and dispersed in ultrapure water until a laser obscuration of 14% (± 0.5%) was reached. Measurements were made on emulsions immediately after homogenisation (day 0) and after 4, 7, 11 and 14 d of storage at 4°C.

4.2.7. Confocal laser scanning microscopy analysis

The microstructural analysis of emulsions was performed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany). Protein and lipid were fluorescently labelled with Nile blue dye (Sigma-Aldrich, Wicklow, Ireland); 50 µL of the dye solution was added to 1 mL of emulsion followed by vortex mixing for 5 s. Visualisation of oil and protein in emulsions (10 µL) was carried out using an

Ar laser operating at an excitation wavelength of 488 nm with emission detected between 500 and 530 nm and a He-Ne laser operating at an excitation wavelength of 633 nm with emission detected between 650 and 700 nm for oil and protein, respectively (Auty et al., 2001). The observations were performed using 20× and 63× oil immersion objectives. At least three specimens of each sample were observed to obtain representative micrographs of samples.

4.2.8. Statistical data analysis

Analysis of variance (ANOVA) was carried out using Minitab® 16 (Minitab Ltd, Coventry, UK, 2010) statistical analysis package. The Tukey method was used to obtain grouping information on the treatment means. The level of significance was determined at $P < 0.05$.

4.3. Results and Discussion

4.3.1. Characterisation of whey protein hydrolysate

The composition, degree of hydrolysis and peptide size distribution data of the WPH used in the preparation of emulsions are shown in Table 4.1. Lactose levels (i.e., innate carbohydrate component of formulation) were taken into consideration when preparing the emulsions.

4.3.2. Composition of emulsions

Compositional analysis of emulsions showed that measured levels (Table 4.2) were satisfactorily near target levels. Ash levels were found to be statistically different ($P < 0.05$) and an increase in the level of ash was found to follow the level of lecithin addition to the emulsions, suggesting that the contribution of ash present in the lecithin (9.6%) led to the differences in ash levels between formed emulsions.

4.3.3. Surface and interfacial tension data

4.3.3.1. Dynamic surface tension

Upon formation of a new surface in the soybean oil sample with no lecithin added the initial surface tension (γ_S) was 30.8 mN m^{-1} (Table 4.3, Fig. 4.1); this

Table 4.1. Composition, degree of hydrolysis (DH) and peptide size distribution of the whey protein hydrolysate (WPH) used in the preparation of emulsions.

Compositional parameter	**%**
Protein	77.7
Lactose	11.6
Ash	4.92
Moisture	4.83
Fat	0.99
Degree of hydrolysis	10.7
Peptide distribution (based on molecular weight)	**% of total protein**
>20 kDa	4.68
10-20 kDa	3.85
5-10 kDa	5.65
2-5 kDa	21.2
1-2 kDa	24.5
0.5-1 kDa	22.5
<0.5 kDa	17.6

decreased to 30.3 once equilibrium surface tension (γ_{SEq}) was reached after 1 h (Table 4.3, Fig. 4.1). This initial γ_S value (30.8 mN m^{-1}) of the control oil (i.e., no lecithin added) was assumed to be representative of a clean surface (i.e., at point of surface formation), and was used as the initial value in all measured systems (Fig. 4.1). A rapid decrease in the γ_S was observed in all oil samples containing lecithin as the surface aged (i.e., time after formation of a new surface). The majority of the decrease was observed to take place within the first 5 min of surface ageing and the rate and extent of decrease in γ_S increased with increasing lecithin content (Fig. 4.1). Values presented in Table 4.3 show that addition of 1% lecithin resulted in γ_{SEq} of 29.4 mN m^{-1}, a reduction of 1.4 mN m^{-1} compared to the control. When the lecithin content

Table 4.2. Composition of model infant formula emulsions containing different levels of lecithin (0-5%, w/w, oil).

Emulsion (g lecithin 100 g^{-1} oil)	**Fat** (g 100 mL^{-1})	**Protein**	**Carbohydrate**	**Moisture**	**Ash**
			(g 100 g^{-1})		
0.00	3.48 ± 0.09[a]	1.67 ± 0.02[a]	7.78 ± 0.48[a]	87.0 ± 0.55[a]	0.10 ± 0.00[a]
1.00	3.44 ± 0.04[a]	1.68 ± 0.03[a]	8.02 ± 0.80[a]	86.7 ± 0.77[a]	0.10 ± 0.01[a]
2.00	3.43 ± 0.02[a]	1.69 ± 0.01[a]	8.10 ± 0.56[a]	86.7 ± 0.54[a]	0.11 ± 0.00[b]
3.00	3.44 ± 0.01[a]	1.69 ± 0.06[a]	8.23 ± 1.03[a]	86.5 ± 1.03[a]	0.11 ± 0.01[b]
4.00	3.47 ± 0.01[a]	1.70 ± 0.02[a]	8.12 ± 0.63[a]	86.6 ± 0.63[a]	0.12 ± 0.00[c]
5.00	3.51 ± 0.01[a]	1.70 ± 0.03[a]	8.29 ± 0.67[a]	86.4 ± 0.69[a]	0.12 ± 0.01[c]

[(a-c)] *Values within a column not sharing a common superscript differed significantly ($P < 0.05$)*

was increased to 2% the γ_{SEq} was further reduced by 1.0 mN m^{-1} (i.e., γ_{SEq} of 28.4 mN m^{-1}). Higher levels of addition of lecithin (i.e., 3-5 g 100 g^{-1} oil) did not contribute to any further decrease in γ_S (no significant differences in γ_{SEq} between these samples) and γ_{SEq} values for samples containing 2-5% lecithin were found to be within a narrow range (i.e., 28.2 – 28.7 mN m^{-1}). A similar trend was reported by McSweeney et al. (2008) where addition of lecithin up to a level of 2 g L^{-1} facilitated formation of small oil globules in model IF emulsions where average globule diameter decreased with increasing lecithin level; however, lecithin levels greater than 2 g L^{-1} did not contribute to further reduction in oil globule sizes.

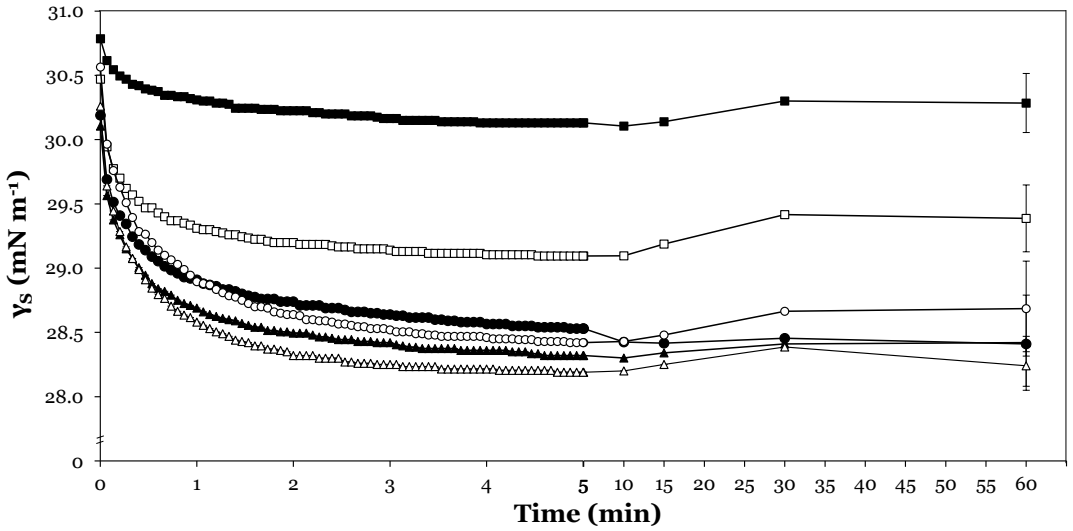

Figure 4.1. Dynamic surface tension of soybean oil samples containing different levels of lecithin (%, w/w); no lecithin (■), 1% (□), 2% (●), 3% (○), 4% (▲) and 5% (Δ) lecithin (w/w).

4.3.3.2. Dynamic interfacial tension

Interfacial tension (γ_I) between oil and aqueous phases displayed a similar pattern as observed for surface tension, where initial γ_I decreased rapidly following formation of the interface. Initial γ_I recorded at the interface between soybean oil (SBO) and ultrapure water (i.e., control system) of

Table 4.3. Surface and interfacial tension of soybean oil-protein systems containing different levels of lecithin (w/w, oil) measured at 55°C.

Sample	**Surface tension**		**Interfacial tension**		
	γ_S	$\Delta\gamma_S$[5]	Interface[2]	γ_I	$\Delta\gamma_I$[5]
	Initial γ_S/γ_I[3]		(mN m^{-1})		
SBO[1]	30.8 ± 0.1	-	O/W	9.4 ± 0.3	-
			O/P	4.3 ± 0.1	-
	Equilibrium $\gamma_{SEq}/\gamma_{IEq}$[4]		(mN m^{-1})		
SBO	30.3 ± 0.2[a]	0.5	O/W	3.2±0.1[a]	6.2
			O/P	1.1 ± 0.2[b]	8.3
SBO and 1% lecithin	29.4 ± 0.3[ab]	1.4	O/P	<1.0[6]	>8.4
SBO and 2% lecithin	28.4 ± 0.1[b]	2.4	O/P	<1.0	>8.4
SBO and 3% lecithin	28.7 ± 0.4[b]	2.1	O/P	<1.0	>8.4
SBO and 4% lecithin	28.4 ± 0.4[b]	2.4	O/P	<1.0	>8.4
SBO and 5% lecithin	28.2 ± 0.2[b]	2.5	O/P	<1.0	>8.4

[1] SBO represents soybean oil

[2] Interface: O/W represents an interface between the oil and filtered deionized water; O/P represents an interface between oil and the protein solution (1.55%, w/v)

[3] Initial surface or interfacial tension recorded immediately upon formation of the surface/interface

[4] Equilibrium surface and interfacial tension (γ_{SEq} and γ_{IEq}, respectively) recorded at 1 h of surface/interface age

[5] The total decrease in γ from the formation of clean surface/interface until reaching the equilibrium is presented as Δγ

[6] The sensitivity limit of the instrument was 1 mN m^{-1}, thus samples showing γ values lower that this limit are presented as <1.0

[(a-b)] Values within a column not sharing a common superscript differed significantly ($P < 0.05$)

9.4 mN m^{-1} was used as an initial γ_I for all measured systems (Fig. 4.2) as it represented a clean interface (i.e., an interface with no surfactants present). Equilibrium interfacial tension (γ_{IEq}) of the control system, recorded after 1 h was 3.2 mN m^{-1} (Table 4.3). The majority of the decrease in γ_I was achieved within 15 min of the interface formation.

Measured γ_{IEq} between soybean oil and protein solution was 1.1 mN m^{-1}. The further reduction in γ_{IEq} of 2.1 mN m^{-1} (i.e., from 3.2 to 1.1 mN m^{-1}) observed in the soybean oil and aqueous phase system when hydrolysed protein was introduced indicated the effectiveness of hydrolysed whey protein (DH 10.7%) in decreasing the γ_I. The rate at which γ_I decreased upon interface formation was markedly higher in the protein containing system (i.e., majority of the decrease was observed within 5 min of formation of the interface; Fig. 4.2). This shows the high mobility and effectiveness of peptides in rapidly reducing γ_I (Chobert et al., 1988; Turgeon et al., 1992; Singh and Dalgleish, 1998; Kong et al., 2007). The effectiveness of partially hydrolysed protein in reducing the interfacial tension is due to the presence of low-intermediate molecular weight peptides (Table 4.1) and their flexible structure with both hydrophobic and hydrophilic sites localized along the peptide chain, unfolding (i.e., bigger peptides) and aligning upon adsorption at the interface, thereby forming a viscoelastic film (Lam and Nickerson, 2013) and lowering the interfacial tension between the two phases.

Samples where lecithin was added (1-5%, w/w) to the oil phase displayed rapid reduction in the γ_I reaching values lower than 1.0 mN m^{-1} (sensitivity limit) immediately after formation of the interface (Fig. 4.2). This can be explained by the behaviour of small surface active agents such as those present in lecithin (i.e., phospholipids) which migrate rapidly through the dispersant and adsorb at the interface allowing the γ_{IEq} to be reached in a very short time (Mezdour et al., 2008). Such a rapid decrease in γ_I by phospholipids was also reported by Kabalnov (1995) where the γ_{IEq} was reached in <1 s. Low molecular weight phospholipids display higher mobility and maneuverability compared to proteins (and large peptides); thus, they can displace larger surfactants (i.e., such as protein and peptides) from the surface/interface (Van Aken et al.,

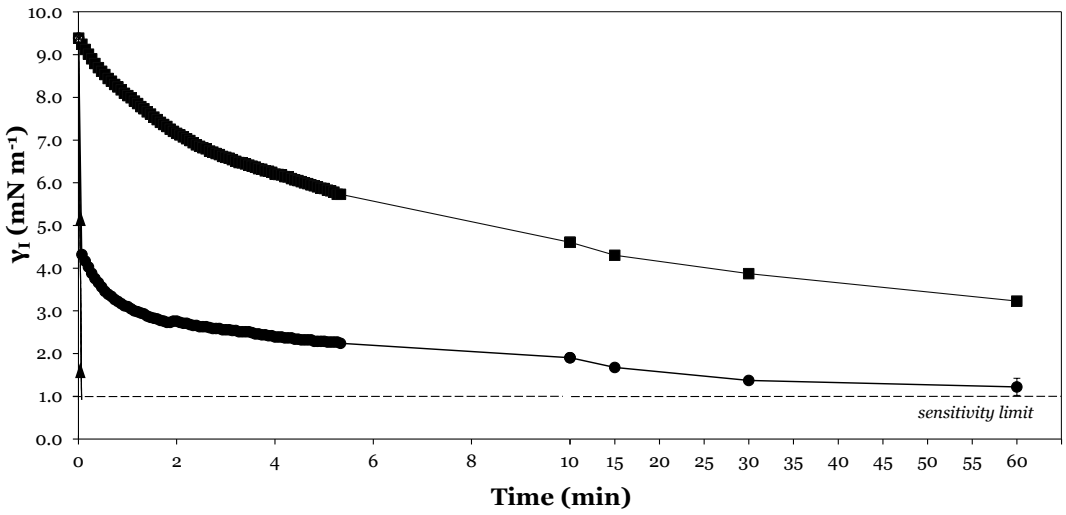

Figure 4.2. Dynamic interfacial tension in samples composed of soybean oil/water (■) and soybean oil/protein (1.55%, w/v, protein) (●). Vertical line(s) (▲) represent the γ_I of lecithin-containing systems. Horizontal dashed line represents the sensitivity limit (1 mN m^{-1}) of the K12 Processor Tensiometer. Any readings below this limit are not shown on the diagram.

2003; Diftis and Kiosseoglou, 2004; Lam and Nickerson, 2013) resulting in rapid reduction of γ_I.

4.3.4. Fat globule size distribution in emulsions

Fat globule size distribution (FGSD) of oil globules in emulsions after homogenization (0 d) showed that all samples, irrespective of level of addition of lecithin, were able to form good quality emulsions with narrow size distribution (Table 4.4). The mean volume diameter ($D_{4,3}$) of a control emulsion (i.e., emulsion prepared without lecithin) was 0.97 μm and increasing lecithin level generally resulted in lower values of $D_{4,3}$ with the smallest $D_{4,3}$ value of 0.86 μm found in the 5% (w/w, oil) lecithin-containing emulsion. Size distributions of oil globules of all formed emulsions were very similar with a general trend of smaller globules formed in emulsions with higher level of addition of lecithin which was also reported by McSweeney et al. (2008) for intact milk protein-based model IF emulsions.

Changes in FGSD of lecithin-containing emulsions were observed during storage at 4°C, while no changes were observed in the control emulsion (Fig. 4.3). $D_{4,3}$ increased in all emulsions formed with lecithin on storage at 4°C and this effect was most pronounced for samples containing 1-3% (w/w, oil) lecithin (Fig. 4.4). Emulsions prepared with 3% (w/w, oil) lecithin showed greatest increase in particle size (Table 4.4); $D_{4,3}$ increased from 0.91 μm (0 d) to 2.33 μm (14 d). Development of large oil globules on storage decreased at higher (4-5%, w/w, oil) lecithin addition levels giving $D_{4,3}$ of 1.63 and 1.30 μm for 4 and 5% (w/w, oil) lecithin-containing emulsions at 14 d, respectively. In the emulsions that displayed increases in particle size during storage, this was represented by a shift of FGSD distribution from monomodal to bimodal (Fig. 4.3) with a second peak evident at ~10 μm within 4 d of storage at 4°C. FGSD profiles showed a decrease in the number of larger oil globules (i.e., flattening of the second peak) as the lecithin level in emulsions was increased, particularly to 4 and 5% lecithin. The total percentage of large oil globules (i.e., the area under second peak) was approximately 2- and 3-fold lower in emulsions with lecithin content of 4 and 5%, respectively, as compared to that of the 3% lecithin-containing emulsion. FGSD results (Table 4.4) also showed that changes in particle size were only detected in the 90% quantile of the size distribution (i.e., $D_{v,\,0.9}$). After 14 d of storage at 4°C, emulsions with 1, 2 and 3% (w/w, oil) lecithin showed an increase in $D_{v,\,0.9}$ of 5.51 (i.e., from 1.69 to 7.20 μm), 6.00 (i.e., from 1.68 to 7.68 μm) and 6.06 (i.e., from 1.67 to 7.73 μm) μm, respectively, while it increased by 0.46 (i.e., from 1.60 to 2.06 μm) and 0.11 (i.e., from 1.59 to 1.70 μm) μm in 4 and 5% lecithin-containing emulsions, respectively. No increase in $D_{v,\,0.9}$ was observed in the control emulsion during storage. The destabilising effect of lecithin on oil globules in emulsions was also observed by Zou and Akoh (2013) who showed that the presence of lecithin (0.4 g L^{-1}) resulted in larger particle sizes after storage (28 d at room temperature) in intact milk protein model IF emulsions.

Results from the current study indicated that the presence of soybean lecithin in WPH-based emulsion systems can promote interactions between oil globules and result in their coalescence. Studies by Cruijisen (1996) and Agboola et al. (1998a) showed similar trends where the presence of

Table 4.4. Fat globule size distribution of model infant formula emulsions prepared with different levels (0-5%, w/w, oil) of lecithin during storage at 4°C at 0, 4 and 14 d post homogenisation.

Lecithin addition	**Storage time**	**Fat Globule Size Parameter[1]**				
(%, w/w, oil)	**(days)**	**$D_{4,3}$**	**$D_{3,2}$**	**$D_{v,0.1}$**	**$D_{v,0.5}$**	**$D_{v,0.9}$**
0.00 (Control)	0	0.97 ± 0.03^{a}	0.63 ± 0.02^{a}	0.32 ± 0.01^{a}	0.82 ± 0.02^{a}	1.80 ± 0.08^{a}
	4	0.96 ± 0.02^{a}	0.61 ± 0.02^{a}	0.32 ± 0.02^{a}	0.81 ± 0.03^{a}	1.81 ± 0.09^{a}
	14	0.97 ± 0.03^{c}	0.63 ± 0.02^{ab}	0.32 ± 0.01^{a}	0.82 ± 0.02^{a}	1.79 ± 0.05^{a}
1.00	0	0.93 ± 0.01^{ab}	0.62 ± 0.02^{a}	0.32 ± 0.01^{a}	0.79 ± 0.01^{ab}	1.69 ± 0.01^{a}
	4	1.49 ± 0.24^{a}	0.61 ± 0.03^{a}	0.32 ± 0.02^{a}	0.77 ± 0.03^{ab}	1.90 ± 0.20^{ab}
	14	2.26 ± 0.26^{ab}	0.66 ± 0.01^{a}	0.33 ± 0.01^{a}	0.77 ± 0.01^{b}	7.20 ± 1.04^{b}
2.00	0	0.89 ± 0.00^{bc}	0.60 ± 0.01^{a}	0.31 ± 0.00^{a}	0.77 ± 0.01^{bc}	1.68 ± 0.04^{ab}
	4	1.31 ± 0.31^{a}	0.60 ± 0.04^{a}	0.31 ± 0.01^{a}	0.75 ± 0.03^{ab}	1.78 ± 0.09^{a}
	14	2.29 ± 0.37^{ab}	0.64 ± 0.03^{ab}	0.31 ± 0.01^{a}	0.74 ± 0.02^{b}	7.68 ± 1.81^{b}
3.00	0	0.91 ± 0.02^{bc}	0.60 ± 0.02^{a}	0.31 ± 0.01^{a}	0.76 ± 0.02^{bc}	1.67 ± 0.03^{b}
	4	1.60 ± 0.27^{a}	0.60 ± 0.03^{a}	0.31 ± 0.02^{a}	0.75 ± 0.03^{ab}	1.99 ± 0.43^{a}
	14	2.33 ± 0.33^{a}	0.63 ± 0.01^{ab}	0.31 ± 0.01^{a}	0.74 ± 0.01^{b}	7.73 ± 1.44^{b}
4.00	0	0.87 ± 0.01^{bc}	0.59 ± 0.02^{a}	0.31 ± 0.01^{a}	0.75 ± 0.02^{bc}	1.60 ± 0.03^{b}
	4	1.17 ± 0.22^{a}	0.59 ± 0.03^{a}	0.31 ± 0.01^{a}	0.73 ± 0.03^{ab}	1.69 ± 0.11^{a}
	14	1.63 ± 0.01^{bc}	0.61 ± 0.01^{ab}	0.31 ± 0.01^{a}	0.73 ± 0.02^{b}	2.06 ± 0.16^{a}
5.00	0	0.86 ± 0.03^{c}	0.58 ± 0.03^{a}	0.31 ± 0.02^{a}	0.74 ± 0.02^{c}	1.59 ± 0.01^{b}
	4	1.13 ± 0.27^{a}	0.58 ± 0.02^{a}	0.31 ± 0.01^{a}	0.72 ± 0.02^{b}	1.66 ± 0.13^{a}
	14	1.30 ± 0.21^{c}	0.60 ± 0.02^{b}	0.31 ± 0.01^{a}	0.72 ± 0.02^{b}	1.70 ± 0.18^{a}

[1] Fat globule size distribution parameters for emulsions: $D_{4,3}$ represents volume mean diameter; $D_{3,2}$ represents Sauter mean diameter; $D_{v,0.1}$, $D_{v,0.5}$ and $D_{v,0.9}$ represent fat globule size in the 10, 50 and 90% quantiles of the distribution, respectively.

(a-c) Values within a column not sharing a common superscript differed significantly ($P<0.05$)

unmodified soybean lecithin (1.5 and 1.0 – 2.5 g L^{-1}, respectively) promoted coalescence of oil globules in caseinate- and WPH-based (DH 27%) O/W emulsions, respectively. A study by Van der Meeren et al. (1995) showed that low values of interfacial tension due to the presence of lecithin at the O/W interface had a negative effect on stability of emulsions against flocculation. Decreased physical stability of emulsions containing WPH and lecithin was also observed by Tirok et al. (2001) using more extensively hydrolysed protein (DH 23-29%) and lecithin (4.8 g L^{-1}). In the current study, a shift in FGSD from monomodal to bimodal was observed in lecithin-containing emulsions on storage at 4°C. However, only a small proportion of the total population of oil globules was affected by the size increase and distribution shift as changes in oil globule size in all lecithin-containing emulsions were only found for the 90% size distribution quantile (i.e., $D_{v,\,0.9}$) while $D_{v,\,0.1}$ or $D_{v,\,0.5}$ did not display any concurrent increase in size. Interestingly, the stability to coalescence in emulsions containing lecithin improved with its higher levels of addition; formation of large oil globules was found to be significantly lower in 4% and lower still in 5% lecithin-containing emulsions. It is worthwhile to note that, even with the development of a second peak, FGSD remained narrow in the majority of the population (i.e., first peak) of all lecithin-containing emulsions.

A preliminary experiment (Fig. 4.3 g – h) was carried out, where stored (10 d at 4°C) emulsions were treated with a dissociating agent, sodium dodecyl sulphate (SDS) and their FGSD was subsequently measured using an approach similar to that used by Tomas et al. (1994), Agboola et al. (1998b) and Tirok et al. (2001). A bimodal distribution and large particle size (as represented by the 90% quantile) continued to be detected following addition of dissociating agent which suggested that coalescence (as opposed to flocculation) of oil globules was the main mechanism of emulsion instability in the samples. This is in agreement with work carried out by Agboola et al. (1998b) and Ye and Singh (2006) who reported a similar destabilisation mechanisms in WPH-based emulsions.

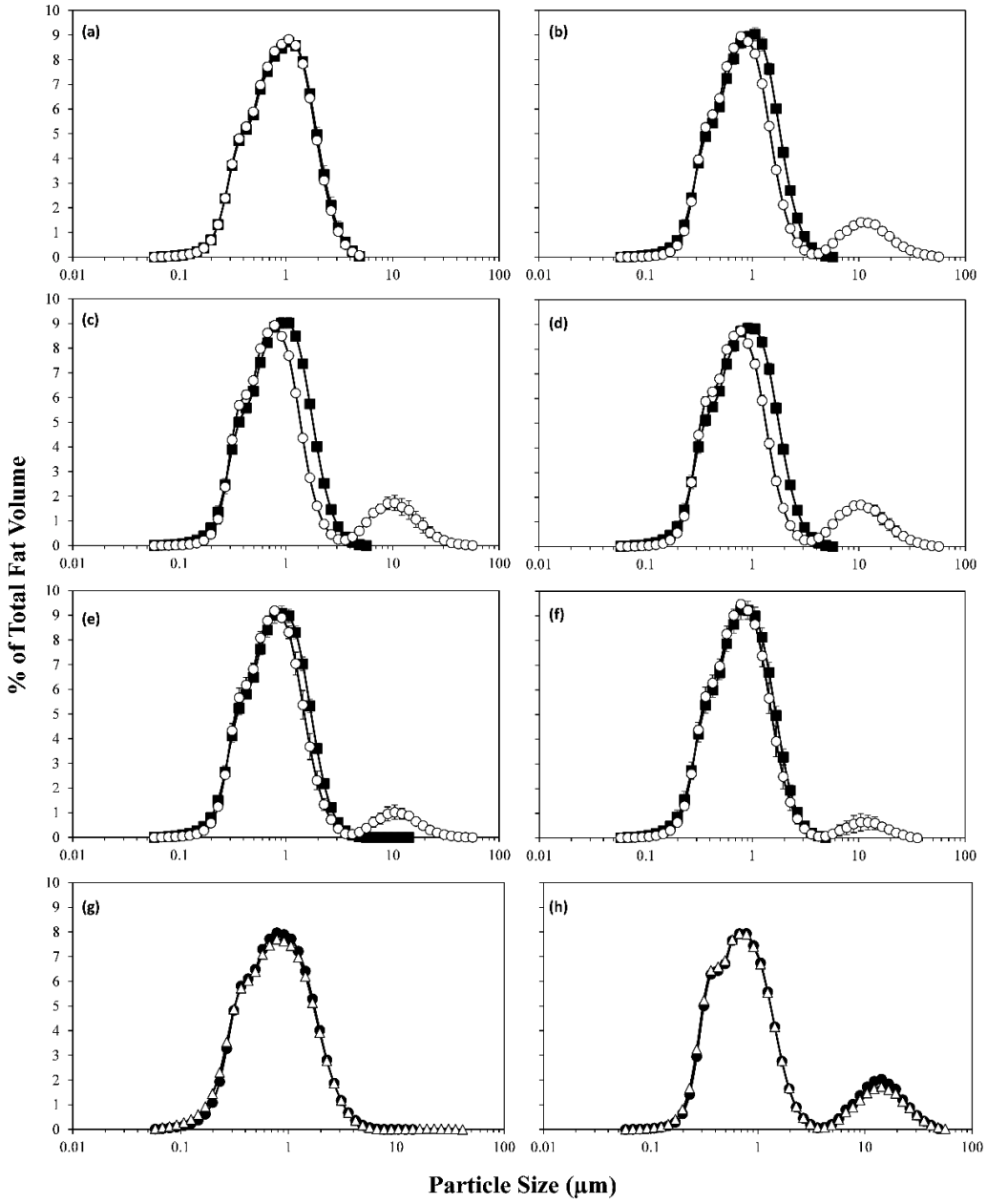

Figure 4.3. Fat globule size distribution profiles of emulsions prepared with lecithin at levels of 0% (a), 1% (b), 2% (c), 3% (d), 4% (e) or 5% (f) (w/w, oil) post homogenisation (■) and after 14 d storage at 4°C (○). Size distribution of oil globules in emulsions containing 0% (g) and 1% (h) lecithin (w/w, oil) after 10 d storage at 4°C with water (●) or 0.2%, w/v, SDS (□) used as the dispersant.

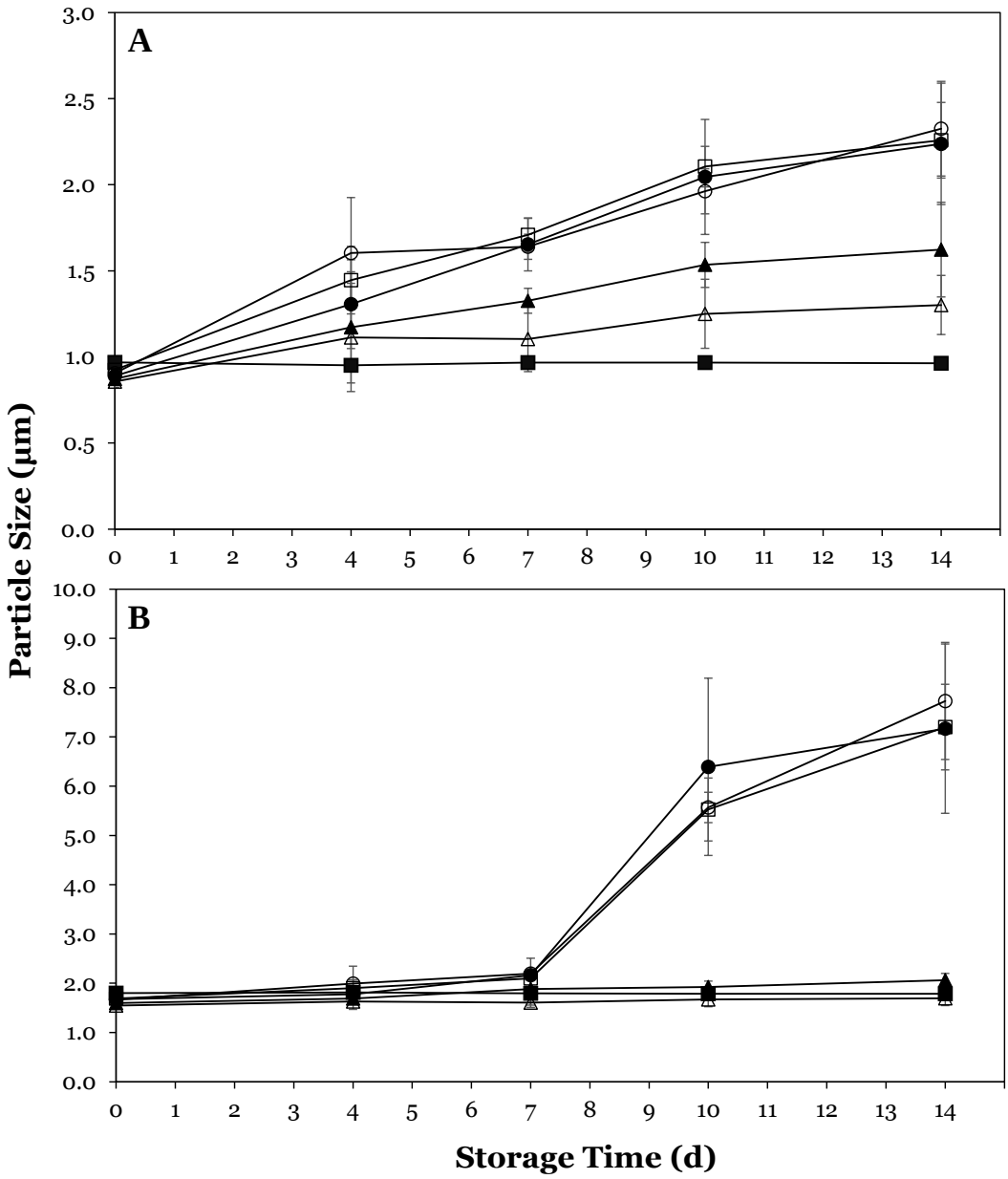

Figure 4.4. Fat globule size distribution parameters: (A) mean volume diameter ($D_{4,3}$) and (B) 90% quantile size distribution ($D_{v,0.9}$) for oil globules in emulsions prepared with lecithin at levels of 0% (■), 1% (□), 2% (●), 3% (○), 4% (▲) and 5% (Δ) (w/w, oil) stored at 4 °C over 14 days.

4.3.5. Confocal laser scanning imaging of emulsions

Confocal laser scanning microscopy (CLSM) showed that freshly-prepared (i.e., 1 d after homogenisation) emulsions, had fine and uniformly distributed oil globules (Fig. 4.5). There were no visible differences between the control

(i.e., 0% lecithin), 1 and 5% lecithin (w/w, oil) emulsions, supporting the FGSD results. Development of a small number of larger oil globules (10-15 μm) was observed in emulsions containing 1% lecithin (w/w, oil) after 7 d of storage at 4°C. No changes in the size of oil globules were observed for control and 5% lecithin (w/w, oil) emulsions at that time point. CLSM micrographs showed increased numbers of large (10-30 μm) oil globules in emulsions containing lower lecithin levels (i.e., 1-3%) after 14 d of storage at 4°C. In emulsions containing 4-5% lecithin some bigger oil globules (5-10 μm) were formed after 14 d of storage at 4°C. These globules, however, were smaller, considerably less numerous and formed at a slower rate (i.e., changes were not observed until 14 d of storage) as compared to emulsions with lower lecithin levels (1-3%). No visual differences in size distribution of oil globules after 14 d of storage at 4°C were observed in the control sample. Large oil globules were also evident in emulsions containing >1% lecithin (w/w, oil) (data not shown). WPH-based emulsions formed with these lecithin levels formed large oil globules during short term refrigerated storage with significant changes observed after 7 d. CLSM micrographs confirmed that lecithin (when added at levels of 1-3%, w/w, oil) in model WPH-based IF emulsions promoted coalescence of oil globules during storage (14 d) at 4°C. Micrographs showed large and uniform oil globules in the emulsion containing 1% lecithin (w/w, oil) after 14 d storage. This supported earlier findings suggesting that coalescence was the main mechanism responsible for emulsion instability in lecithin-containing WPH-based emulsions.

Differences in the interface of larger (> 3 μm) fat globules were seen at higher magnification and an example of this is given in Fig. 4.6, which shows an uneven thickness of protein at the oil globule interface (Fig. 4.6 b, arrows). This uneven protein thickness was observed for many of the larger oil globules in all samples containing lecithin, but was not observed in globules of the WPH control (0 % lecithin) sample (Fig. 4.6 a). This suggests a possible partial displacement and aggregation of interfacial whey protein by the lecithin over time, which may also help explain the coalescence of oil globules containing lecithin. More study is needed to characterise the precise nature of the interfacial material, for example using fluorescently-labelled phospholipids.

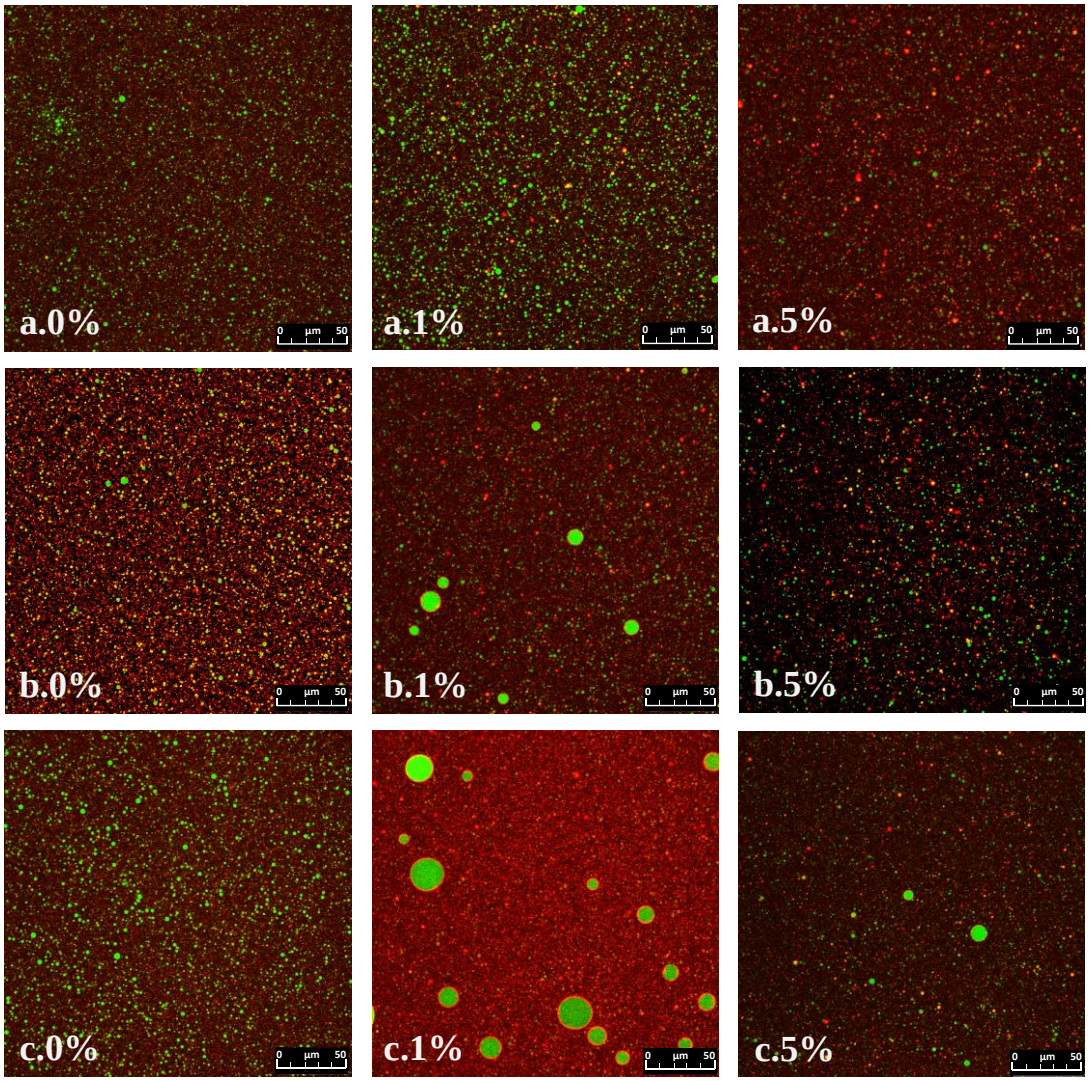

Figure 4.5. Confocal laser scanning microscopy images of model infant formula emulsions containing 0, 1 or 5% lecithin (w/w, oil) after 1 (a), 7 (b) and 14 (c) days of storage at 4°C. Micrographs present overall size distribution of oil globules (green) in different emulsions over time. Scale bar (bottom right) = 50 µm.

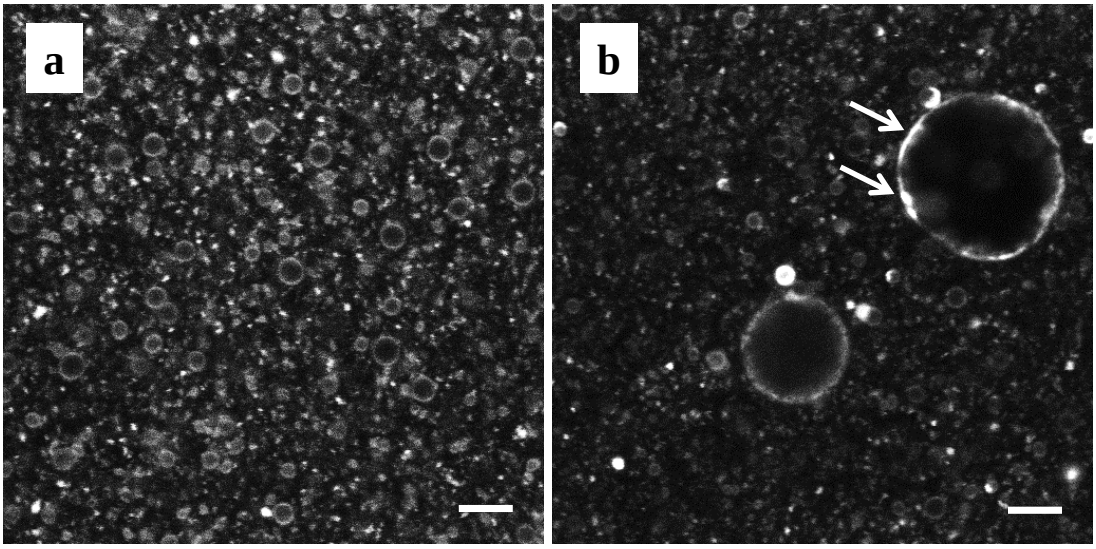

Figure 4.6. High magnification confocal micrographs of emulsions after storage at 4°C for 14 days showing the protein-labelled channel. a) 0 % lecithin; b) 1 % lecithin (w/w, oil). Arrows indicate variable thickness of protein at the oil globule interface. Scale bar = 5 μm.

4.4. Conclusions

This study shows that an effective decrease in the interfacial tension between the oil and aqueous phase in the manufacture of model infant formula emulsions produced with hydrolysed whey protein can be achieved by incorporation of low levels of lecithin (i.e., 1%, w/w, oil). Emulsions formed with hydrolysed whey protein displayed narrow size distribution of oil globules which was further reduced by incorporation of lecithin. It was, however, shown that low-to-intermediate levels (1-3%) of lecithin decreased stability of emulsions during storage at 4°C by promoting coalescence of oil globules. Confocal microscopy proved to be a helpful tool for studying coalescence in emulsions and it complemented light scattering work in the current study.

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Chapter 5

The effect of lecithin on the oxidative stability of model infant formula emulsions produced with hydrolysed whey protein

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Abstract

Model infant formula emulsions containing 1.55, 3.50 and 7.00 g per 100 mL protein (whey protein hydrolysate, WPH; degree of hydrolysis 10.7), soybean oil and maltodextrin (MD; dextrose equivalent 17), respectively, were prepared. Emulsions contained 0-5 g soybean lecithin per 100 g oil. Emulsions were stored for 14 d at 40°C with constant agitation to promote lipid oxidation. All emulsions had mono-modal oil globule size distributions immediately post-homogenisation and after the 14 d storage; a limited increase in the mean size of oil globules was observed for the stored emulsions (i.e., <1 μm post-homogenisation and <2 μm after the 14 d storage). Differences in the level of peroxides (primary lipid oxidation products) and thiobarbituric acid reactive substances (TBARS; secondary lipid oxidation products) were observed for the emulsions during storage. Lecithin addition of 1 g per 100 g oil, reduced the rate of primary and secondary lipid oxidation; no additional antioxidative effect was observed for higher lecithin addition levels (2-5 g 100 g^{-1}). The oxidative stability of bulk soybean oil containing different levels of lecithin (0-5 g 100 g^{-1}) was measured at 120°C using Rancimat. Lecithin addition had a strong antioxidative effect on the oil stability, evidenced by decreasing the oxidation induction time ($T_{I\text{-}ox}$). The rate of lipid oxidation in the soybean oil was largely dependent on the lecithin addition level, where $T_{I\text{-}ox}$ increased with increasing lecithin content. The antioxidative effect of the lecithin, in both emulsion and in bulk oil systems, was linked to its surface activity and its ability to bind free radicals and pro-oxidants.

5.1. Introduction

Infant nutrition is one of the most important aspects of life-stage nutrition as all the nutrients required for proper growth and development of an infant, for at least the first few weeks, come from a single food source – milk. It is indisputable that breastfeeding is the best option for infant nutrition (Anatolitou, 2012); however, it is not always possible to provide every infant with its mother's milk, for reasons such as breastfeeding difficulties, cultural and social incompatibilities, or poor health and nutritional status of the mother. Thus, infant formula (IF) products are designed to closely match the nutritional requirements of an infant and offer it the best possible alternative to breast milk (i.e., specific composition and bio-functionality).

Numerous studies have focused on characterisation of the individual components of human milk (i.e., relative levels, structures and bio-functionality) (Andreas, Kampmann, and Mehring Le-Doare, 2015; Dupont, 2003; Guerra et al., 2016; Kreissl et al., 2015; Sundekilde et al., 2016; Wada and Lönnerdal, 2014), and consequently, on the development of formulations for infant nutrition (Alles, Scholtens, and Bindels, 2004; Bourlieu et al., 2015; Crowley, Dowling, Caldeo, Kelly, and O'Mahony, 2016; Gallier et al., 2015; Joyce, Brodkorb, Kelly, and O'Mahony, 2017; McSweeney, 2008; Nguyen, Bhandari, Cichero, and Prakash, 2015). Formulae based primarily on whey protein (60:40 whey protein:casein ratio, WP:CN, respectively) are the main category of infant nutrition products. Mild or extensive hydrolysis of proteins in the ingredients can also be used to improve digestibility and nutrient absorption (O'Callaghan, O'Mahony, Ramanujan, and Burgher, 2011) as well as reduce allergenicity (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, and Recio, 2014), respectively. IF products also contain carbohydrates (e.g., lactose and maltodextrin), vegetable oils, minerals, vitamins and other constituents (e.g., choline) required for proper development of an infant (Koletzko et al., 2005; Koletzko, Shamir, and Ashwell, 2012).

In addition to changes in protein bio-availability, hydrolysis of protein also affects protein techno-functionality; moderate hydrolysis (<20% degree of

hydrolysis, DH) generally improves protein solubility, surface activity and emulsification properties (Agboola and Dalgleish, 1996a, 1996b; Banach, Lin, and Lamsal, 2013; Foegeding and Davis, 2011; Panyam and Kilara, 1996). However, thermal stability of emulsions containing whey protein hydrolysate (WPH) is often impaired, compared to intact protein-based emulsions, due to the lower steric stabilisation properties and higher number of reactive sites with the former (Drapala, Auty, Mulvihill, and O'Mahony 2016a; Ye and Singh, 2006). Low molecular weight (L_{MW}) surfactants, including lecithin, mono- and di-glycerides of fatty acids and their esters (CITREM) are permitted for use in hydrolysed IF (Codex Alimentarius, 2011) to aid with emulsification. Lecithin is a collective term used to describe a mixture of phospholipids (i.e., phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid) originating from a variety of food sources (e.g., soybean, sunflower, rapeseed, egg); it is most commonly extracted from crude vegetable oils by degumming followed by thin layer evaporation (Ceci, Constenla, and Crapiste, 2008; Scholfield, 1981). In addition to its physicochemical functionality, lecithin is sometimes added to IF products to help meet required choline levels (MacLean et al., 2010).

Ready to feed (RTF) formats of IF are emulsions, sterilised (i.e., ultra-high temperature followed by aseptic filling or in-container sterilisation) to ensure microbial safety, packaged in single use containers (i.e., plastic, laminate or glass) and stored at room temperature for several weeks to months. To meet a desired shelf life stability of such products, detrimental changes that can take place during storage due to the physical (e.g., phase separation, component interactions) or chemical (e.g., lipid oxidation, formation of complexes) reactions must be limited. Lipid oxidation is a group of deteriorative reactions causing formation and decomposition of lipid hydroperoxides (ROOH) into peroxyl (ROO) and alkoxyl (RO) radicals (i.e., primary and secondary oxidation, respectively) (Frankel, 1998). Lipid oxidation causes changes to the food's organoleptic properties (i.e., rancid smell and taste), nutritional profile (loss of essential fatty acids) and involves production of potentially toxic components (McClements and Decker, 2000). The rate of lipid oxidation in oil-in-water (O/W) emulsions can be limited by modifying the emulsion

interfacial layer through changing the surfactant system. Generally, surfactants present at the interfaces of oil globules decrease the surface free energy, reducing thermodynamic instability of the system; these compounds can also provide a physical barrier that limits undesirable globule-globule interactions, such as coalescence and flocculation. It has been shown that a number of surface active compounds additionally carry an antioxidative potential; reports on reduced lipid oxidation rate in emulsions stabilised by sodium caseinate (Nielsen, Horn, and Jacobsen, 2013; O'Dwyer, O'Beirne, Eidhin, and O'Kennedy, 2013; Qiu, Zhao, Decker, and McClements, 2015), whey protein (Tong, Sasaki, McClements, 2000) or by L_{Mw} surfactants (including lecithin) (Mancuso, McClements, and Decker, 1999; Pan, Tikekar, and Nitin, 2013; Zou and Akoh, 2013) indicate that in conjunction with physical stabilisation, these compounds can additionally provide a chemical/physical antioxidative barrier.

Reports on antioxidant properties of lecithin in liquid (Kargar, Spyropoulos, and Norton, 2011) and spray dried (Klinkesorn, Sophanodora, Chinachoti, McClements, and Decker, 2005) emulsions have shown its benefits for use in these systems. Conversely, the presence of lecithin in emulsions has been associated with adverse effects on their physical stability (Drapala, Auty, Mulvihill, and O'Mahony, 2015, 2016b; Ozturk, Argin, Ozilgen, and McClements, 2014; Tirok, Scherze, and Muschiolik, 2001), depending on the lecithin state (i.e., native or hydrolysed) and its addition level. Currently, the knowledge regarding the oxidative stability of WPH-based infant nutrition products is limited and this study aims to develop much-needed understanding of this area. The objective of the current study was to investigate the effects of changing the composition and, effectively, the properties of the emulsion interfacial layer (i.e., by using different lecithin inclusion levels) on the oxidative stability of model WPH-based RTF IF systems.

5.2. Materials and methods

5.2.1. Materials

Whey protein hydrolysate (WPH; degree of hydrolysis, DH, 10.7%) was obtained from Kerry Group (Listowel, Co. Kerry, Ireland). The composition and molecular weight profile of the WPH are as detailed by Drapala et al. (2015). Maltodextrin (MD, dextrose equivalent, DE, 17), and de-oiled powdered soybean lecithin (hydrophilic-lipophilic balance, HLB, 7) were obtained from Syral Belgium N.V. (Aalst, Belgium) and ADM (Decatur, IL, USA), respectively. Soybean oil (Organic Soya Oil, Clearspring Ltd., London, UK) was purchased from a local commercial outlet. All other chemicals, reagents and minerals used in the study were purchased from Sigma Aldrich (Dublin, Ireland).

5.2.2. Analysis of fatty acid composition of soybean oil

The fatty acid composition of the soybean oil was analysed by gas liquid chromatography (GLC); sample preparation, GLC instrument, column details and parameters of the analysis were as detailed by O'Dwyer et al. (2013).

5.2.3. Emulsion formulation and compositional analysis

Model infant formula emulsions containing 1.55, 3.50 and 7.00 g/100 mL of protein, oil and carbohydrate, respectively, and 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 g lecithin per 100 g oil were prepared as detailed by Drapala et al. (2015) (Chapter 4, pp. 153-154). Solutions of iron, zinc, manganese and copper salts were added to the aqueous phases prior to homogenisation to meet the typical IF levels of 800, 600, 33 and 5 μg 100 mL^{-1}, respectively, as detailed by Drapala et al. (2015). Aqueous and oil phases were blended at 55°C, pre-homogenised and homogenised (double pass) using a 2-stage valve homogeniser with 10 and 2 MPa valve pressures as detailed by Drapala et al. (2015) (Chapter 4, pp. 153-154). The resulting emulsions were adjusted to pH 6.8 (with 0.1 N HCl and/or 0.1 N NaOH) and sodium azide was added (0.05 g 100 mL^{-1}) as a preservative. Aliquots (60 mL) of each emulsion were placed in loosely-capped baffled flasks (Thomas Scientific, Swedesboro, NJ, USA) to promote emulsion aeration and

placed in a shaking incubator (Orbital Incubator SI 50, Bibby Scientific Limited, Staffordshire, UK) at 40°C for 14 d with a low agitation speed of 30 rpm. The composition of the emulsions was measured according to IDF standard methods as detailed by Drapala et al. (2015).

5.2.4. Measurement of fat globule size distribution

Fat globule size distribution (FGSD) of the emulsions was measured using a static laser light-diffraction instrument (Mastersizer S, Malvern Instruments Ltd., Worcestershire, UK) equipped with a 300 RF (reverse fourier) lens and He-Ne laser (λ of 633 nm). A polydisperse model with 3NAD presentation and particle and dispersant refractive indices of 1.46 and 1.33, respectively, were selected for data analysis as described by McCarthy et al. (2012). Sample was introduced to the small-volume mixing chamber and dispersed in d.H_2O until a laser obscuration of 14.0% (± 0.5%) was reached. Measurements were made on emulsions immediately after homogenisation and after 14 d of accelerated storage at 40°C.

5.2.5. Oxidative stability of bulk oil

The oxidative stability of soybean oil containing 0.0-5.0 g 100 g^{-1} lecithin was measured using a rapid oxidation stability analyser (Rancimat 743, Metrohm Ireland Ltd., Dublin, Ireland). The instrument operates on the principle of the Active Oxygen Method of Gray (1985), where oil samples are heated and the progress of oxidation is monitored by measuring the increase in production of volatile products of oxidation through changes in conductivity (Läubli and Bruttel, 1986). Soybean oil with different levels of added lecithin (0.0-5.0 g 100 g^{-1} oil) were prepared as described in Section 5.2.3. Samples (3 g) were placed in reaction tubes, heated to and maintained at 120°C during analysis (i.e., up to 30 h, depending on the sample); air was passed through the samples at a steady flow rate (20 L h^{-1}) and subsequently fed into the corresponding measurement vessels filled with ultrapure water and equipped with a conductivity probe (2 steel electrodes, 1 kHz, 1.7 V). Production of volatile organic compounds (secondary products of lipid oxidation) was measured based on changes in the conductivity recorded in the contents of the measurement vessel; the increase in conductivity and the oxidation induction

times ($T_{I\text{-}ox}$) (i.e., time taken to achieve onset of an exponential increase in conductivity) were recorded and used as an index of oxidative stability of samples. All samples were tested in triplicate.

5.2.6. Oxidative stability of model IF emulsions

5.2.6.1. Primary lipid oxidation of emulsions

Primary lipid oxidation in the emulsions was determined by measuring peroxide levels at 5 time points, immediately after homogenisation and after 3, 7, 11 and 14 d of storage at 40°C as follows: aliquots (0.5 ml) of each emulsion were placed in rubber-sealed plastic tubes to which 5 mL of iso-octane/2-propanol (3:1) was added; the tubes were vortexed 3 times for 10 s each, centrifuged at 1780 g for 4 min and the resulting supernatants were used for analysis. Supernatant (0.05 mL), 2.95 mL of methanol/1-butanol (2:1), 15 µL ammonium thiocyanate (3.94 M) and 15 µL ferrous iron solution (prepared with $BaCl_2$ and $FeSO_4$ to give final concentrations of 0.132 and 0.144 M, respectively) were placed into tubes, vortexed, transferred to capped cuvettes and stored in the dark for 20 min. Sample blank and solvent blank containing 0.05 mL of d.H_2O and 0.05 mL of iso-octane/2-propanol, respectively, instead of the supernatant, were prepared following the same procedure as detailed for the samples. The absorbance at 510 nm of all samples was measured using a Cary 300 Bio UV-visible spectrophotometer (Varian Inc., CA, USA). The concentration of peroxides in samples was calculated from a standard curve (0-30 mM hydrogen peroxide, H_2O_2) prepared with a 30% H_2O_2 solution.

5.2.6.2. Secondary lipid oxidation in emulsions

The progress of secondary lipid oxidation during accelerated storage at 40°C for 14 d of the emulsions was measured at the same time points as detailed for measurement of primary oxidation (see Section 5.2.6.1) by measuring levels of thiobarbituric acid reactive substances (TBARS) using a modified method of Siu and Draper (1978) as follows: emulsions (5.00 ± 0.05 g) were placed in plastic tubes to which 5 mL of d.H_2O and 5 mL of trichloroacetic acid (TCA; 10 g 100 mL^{-1}) were added; the tubes were shaken vigorously for 30 s and the contents were filtered through Whatman No 1 filter paper. Resulting filtrates

(4 mL) were placed in glass tubes (10 mL), had 1 mL of thiobarbituric acid (TBA) reagent (i.e., 2-thiobarbituric acid in d.H_2O; 6 mM) added, and were sealed, vortexed and placed in a water bath at 70°C for 1 h. Samples were prepared in duplicate from each of the emulsions. The blank was prepared in the same way as detailed for the samples, except that d.H_2O (2 mL) and TCA (2 mL; 10 g 100 mL^{-1}) were used in place of the sample filtrate. After incubation at 70°C for 1 h, samples were left to cool under ambient conditions and their absorbance was measured at 532 nm using a Cary 300 Bio UV-visible spectrophotometer (Varian Inc., CA, USA). Each sample was measured in duplicate, giving in total 4 readings per emulsion. Malondialdehyde (MDA) levels (i.e., mg MDA per kg of sample) were calculated using molar extinction coefficient (1.56×10^5 M^{-1} cm^{-1}) and expressed as TBARS number.

5.2.7. Statistical analysis

All emulsions were prepared in three independent trials and all measurements were carried out in at least duplicate. Analysis of variance (ANOVA) was carried out using the Minitab® 16 (Minitab Ltd., Coventry, UK, 2010) statistical analysis package. The Tukey method was used to obtain grouping information. The level of significance was determined at $P < 0.05$.

5.3. Results

5.3.1. Composition of soybean oil and emulsions

The fatty acid composition of the soybean oil is shown in Table 5.1; this shows the largely unsaturated nature of fatty acids in the oil, as evidenced by the high content of linoleic (51%; C18:2), oleic (25%; C18:1) and linolenic (7%; C18:3) fatty acids. The composition of the emulsions used in the current study are as reported by Drapala et al. (2015). The composition of the emulsions was satisfactorily near the target levels; the ranges of protein, fat, carbohydrate, total solids and ash in all emulsions were 1.67-1.70 g 100 g^{-1}, 3.43-3.51 g 100 mL^{-1}, 7.78-8.29 g 100 g^{-1}, 13.0-13.6 g 100 g^{-1} and 0.10-0.12 g 100 g^{-1}, respectively. No significant differences were observed in the protein, fat, carbohydrate and total solids content of the emulsions; however, significant differences were observed in the ash content, which increased with increasing

Table 5.1. Fatty acid composition of soybean oil used as the bulk oil in the Rancimat method and as the oil component in model infant formula emulsions.

Fatty acid	Fatty acid number	% of Total fatty acids
Palmitic	C16:0	9.08 ± 0.06
Stearic	C18:0	4.25 ± 0.29
Oleic	C18:1	25.3 ± 0.26
Vaccenic	C18:1	1.25 ± 0.02
Linoleic	C18:2	51.2 ± 0.37
α-Linoleic	C18:3	n.d.
Linolenic	C18:3	6.88 ± 0.04
Arachidic	C20:0	0.43 ± 0.01
Gadoleic	C20:1	n.d.
Behenic	C22:0	0.43 ± 0.01

n.d. – not detected

lecithin addition level. The differences in the ash content are likely due to the contribution of ash present in the lecithin component of the formulations (i.e., 9.60 g 100 g^{-1}).

5.3.2. Fat globule size distribution in emulsions

Fat globule size distribution (FGSD) data for the model IF emulsions showed that all emulsions had very similar size distributions of oil globules immediately post homogenisation, and the size distribution did not change significantly during accelerated storage for 14 d at 40°C (Fig. 5.1, Table 5.2). Although not statistically significant, an increase in the mean volume diameter ($D_{4,3}$) was observed for all emulsions after 14 d of accelerated storage compared to the corresponding values post homogenisation (Table 5.2). The increase in $D_{4,3}$, with no obvious changes in the other FGSD parameters, or in the size distribution profiles, most likely indicated formation of a small number of large particles, to which the volume-based $D_{4,3}$ parameter is

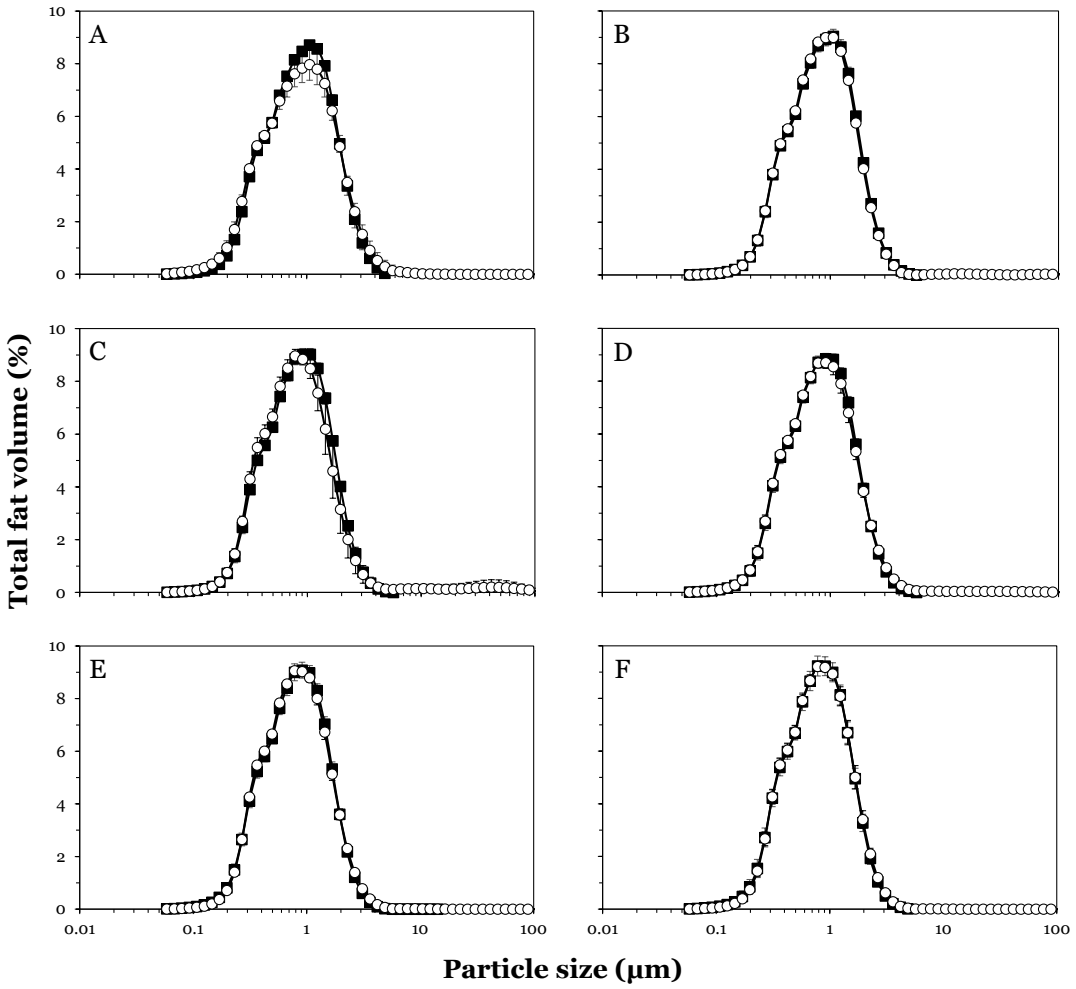

Figure 5.1. Fat globule size distribution profiles for whey protein hydrolysate-based model infant formula emulsions containing lecithin at different addition levels, 0.0 (A), 1.0 (B), 2.0 (C), 3.0 (D), 4.0 (E) and 5.0 (F), g 100 g^{-1} oil, (■) post-homogenisation and (○) after 14 d storage at 40°C.

particularly sensitive (McClements, 2015). Such formation of large oil globules is common in emulsions containing proteins/peptides and L_{Mw} emulsifiers(e.g., lecithin) (Drapala et al. 2016a, b; Kaltsa, Paximada, Mandala, and Scholten, 2014; Wilde, Mackie, Husband, Gunning, and Morris, 2004). Despite the limited changes in the size distributions of oil globules in the emulsion samples, all samples displayed good physical stability during accelerated storage and there were no visual or otherwise noteworthy differences in FGSD between the emulsions after 14 d of storage at 40°C (Table 5.2).

Table 5.2. Fat globule size distribution of model infant formula emulsions containing different lecithin addition levels (0.0–5.0 g 100 g^{-1} oil) immediately post-homogenisation and after 14 d of storage at 40°C.

Emulsions (g lecithin 100 g^{-1} oil)	**Measurement stage**	**Fat globule size distribution parameter**[1]				
		$D_{4,3}$	$D_{3,2}$	$D_{v,\ 0.1}$	$D_{v,\ 0.5}$	$D_{v,\ 0.9}$
0.0	Post-homogenisation	0.97 ± 0.042^{aA}	0.63 ± 0.025^{aA}	0.32 ± 0.010^{aA}	0.82 ± 0.023^{aA}	1.80 ± 0.095^{aA}
	14 d storage	1.32 ± 0.620^{aA}	0.60 ± 0.024^{aA}	0.30 ± 0.017^{aA}	0.81 ± 0.018^{aA}	1.95 ± 0.226^{aA}
1.0	Post-homogenisation	0.93 ± 0.005^{aAB}	0.62 ± 0.017^{aA}	0.32 ± 0.010^{aA}	0.79 ± 0.009^{aAB}	1.69 ± 0.008^{aAB}
	14 d storage	1.64 ± 1.272^{aA}	0.62 ± 0.000^{aA}	0.32 ± 0.003^{aA}	0.78 ± 0.003^{aAB}	1.69 ± 0.038^{aAB}
2.0	Post-homogenisation	0.89 ± 0.000^{aBC}	0.60 ± 0.000^{aA}	0.31 ± 0.000^{aA}	0.77 ± 0.009^{aBC}	1.68 ± 0.043^{aAB}
	14 d storage	1.78 ± 1.484^{aA}	0.60 ± 0.020^{aA}	0.31 ± 0.010^{aA}	0.74 ± 0.030^{aB}	1.78 ± 0.173^{aAB}
3.0	Post-homogenisation	0.91 ± 0.020^{aBC}	0.60 ± 0.024^{aA}	0.31 ± 0.013^{aA}	0.76 ± 0.023^{aBC}	1.67 ± 0.028^{aAB}
	14 d storage	0.87 ± 0.018^{aBC}	0.59 ± 0.028^{aA}	0.31 ± 0.013^{aA}	0.75 ± 0.024^{aAB}	1.71 ± 0.013^{aAB}
4.0	Post-homogenisation	1.08 ± 0.277^{aA}	0.59 ± 0.022^{aA}	0.31 ± 0.013^{aA}	0.75 ± 0.018^{aBC}	1.60 ± 0.025^{aB}
	14 d storage	0.89 ± 0.004^{aA}	0.59 ± 0.021^{aA}	0.31 ± 0.011^{aA}	0.74 ± 0.018^{aAB}	1.61 ± 0.011^{aAB}
5.0	Post-homogenisation	0.87 ± 0.018^{aC}	0.58 ± 0.033^{aA}	0.31 ± 0.018^{aA}	0.74 ± 0.020^{aC}	1.59 ± 0.013^{aB}
	14 d storage	1.37 ± 0.900^{aA}	0.59 ± 0.026^{aA}	0.31 ± 0.013^{aA}	0.73 ± 0.030^{aB}	1.56 ± 0.060^{aB}

[1] Fat globule size distribution parameters are: $D_{4,3}$, volume mean diameter; $D_{3,2}$, Sauter mean diameter; $D_{v,0.1}$, fat globule size in the 10% quantile of the distribution; $D_{v,0.5}$, fat globule size in the 50% quantile of the distribution; $D_{v,0.9}$, fat globule size in the 90% quantile of the distribution.

(a-b) Values for a given emulsion and given FGSD parameter at the two different measurement stages (i.e., post-homogenisation and after 14 d of accelerated storage) not sharing a common superscript differed significantly ($P < 0.05$).

(A-C) Values for a given measurement stage for all lecithin addition levels not sharing a common superscript differed significantly ($P < 0.05$).

5.3.3. Oxidative stability of bulk soybean oil

Differences in the oxidative stability of bulk soybean oil containing different levels of lecithin were evidenced by an increase in the oxidation induction time with increasing lecithin content of the oil (Fig. 5.2). The $T_{I\text{-}ox}$ for oils with added lecithin were significantly higher compared to the control (i.e., oil with no added lecithin) and there was a direct relationship between the lecithin content and the onset of secondary oxidation in the bulk oil samples. The oil with the highest lecithin content (i.e., 5.0 g 100 g^{-1}) had the longest $T_{I\text{-}ox}$, which was >10 fold higher than that of the control sample. Similar findings were reported by Judde, Villeneuveb, Rossignol-Castera, and Le Guillou (2003) where lecithin was found to lower the rate of lipid oxidation for a range of bulk oils (i.e., palm, rapeseed, soybean, sunflower, walnut, fish oils and lard). Those authors attributed the reported results to a synergistic effect between phospholipids and tocopherols present in most of these oils as well as to the radical and pro-oxidant chelating properties of phospholipids.

5.3.4. Oxidative stability of model IF emulsions

5.3.4.1. Primary lipid oxidation in emulsions

The progress of primary lipid oxidation in model IF emulsions during accelerated storage for 14 d at 40°C indicated a strong antioxidative effect of lecithin (Fig. 5.3A); all lecithin addition levels resulted in a reduced oxidation rate, as evidenced by the production of peroxides, compared to the control (i.e., emulsion with no lecithin added). No statistically significant differences were observed in the levels of peroxides between emulsions at different time points of storage; however, a trend was observed, where after 10 d of storage, the control sample had higher levels of primary oxidation products (i.e., 6.91 mM H_2O_2) compared to the emulsions containing lecithin (i.e., 2.76, 3.08, 3.00, 3.05 and 3.04 mM H_2O_2 for 1.0, 2.0, 3.0, 4.0 and 5.0 g lecithin 100 g^{-1} oil, respectively). These differences were more pronounced after 14 d of storage (Fig. 5.3A, Table 5.3). Although the differences in the progress of primary lipid oxidation between the emulsions were not significant due to the large standard errors observed for these systems (Table 5.3), a clear trend was

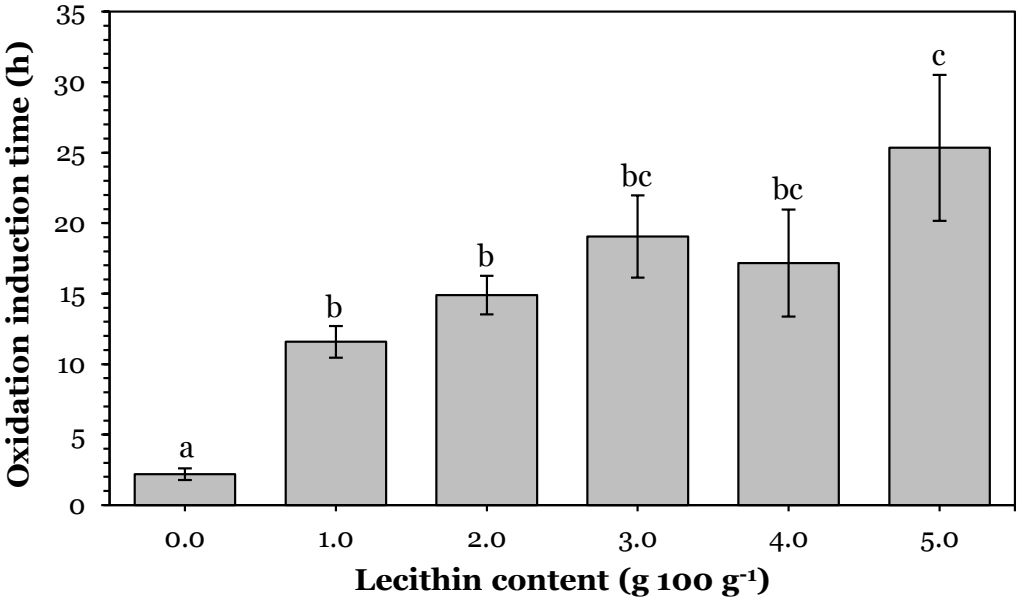

Figure 5.2. Oxidative stability of soybean oil containing different levels (0.0-5.0 g 100 g^{-1}) of soybean lecithin measured with the Rancimat. Induction time describes the time needed to detect an exponential increase in the formation of volatile compounds (i.e., secondary oxidation products). Lower case letters (a-c) show sample grouping information according to statistical differences ($P < 0.05$).

observed, where lecithin addition considerably reduced the progress of primary oxidation. Large variability between trials is often a challenge in lipid oxidation studies (Mancuso et al., 1999; Osborn and Akoh, 2004) due to numerous factors affecting these reactions (i.e., complexity of the food matrix or the method of oil recovery) and due to the instability of peroxides (Clark, 2001). All 3 trials performed in the current study displayed the same general trends. The progress of primary lipid oxidation was greatly reduced by addition of lecithin; however, increasing the lecithin addition level from 1.0 to 5.0, g 100 g^{-1} oil, did not show any additional antioxidative effect.

5.3.4.2. Secondary lipid oxidation in emulsions

The progress of formation of secondary lipid oxidation products in model IF emulsions during accelerated storage for 14 d at 40°C also showed that lecithin

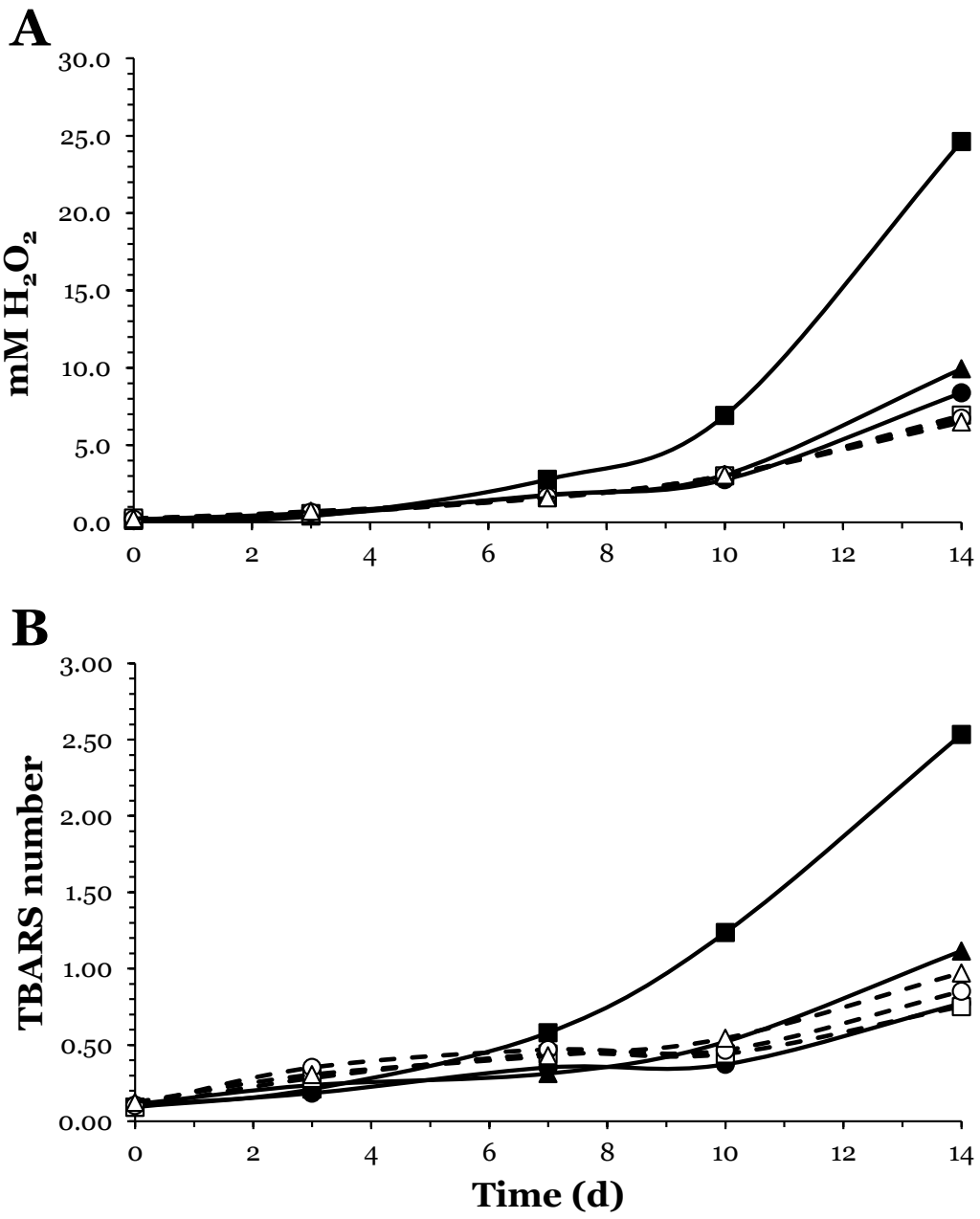

Figure 5.3. Progress of (A) primary and (B) secondary lipid oxidation, measured by the production of peroxides and malondialdehyde, respectively, in whey protein hydrolysate-based model infant formula emulsions with (■) no lecithin addition and (●) 1.0, (▲) 2.0, (□) 3.0, (○) 4.0 and (Δ) 5.0, g 100 g^{-1} oil, lecithin addition during accelerated storage for 14 d at 40°C. For the information regarding variability in the progress of primary and secondary lipid oxidation in the emulsions please refer to Table 5.3.

Table 5.3. The progress of primary and secondary lipid oxidation, measured by the production of peroxides and malondialdehyde, respectively, in whey protein hydrolysate-based model infant formula emulsions with different levels of lecithin addition (0.0–5.0 g 100 g^{-1} oil) during accelerated storage for 14 d at 40°C.

Incubation time (d)	**Emulsions** (g lecithin 100 g^{-1} oil)					
	0.0	1.0	2.0	3.0	4.0	5.0
	Primary lipid oxidation (mM H_2O_2)					
0	0.13 ± 0.16^{aA}	0.23 ± 0.32^{aA}	0.19 ± 0.24^{aA}	0.27 ± 0.27^{aA}	0.22 ± 0.20^{aA}	0.23 ± 0.22^{aA}
3	0.40 ± 0.19^{aA}	0.46 ± 0.01^{aAB}	0.63 ± 0.14^{aAB}	0.55 ± 0.05^{aAB}	0.65 ± 0.02^{aAB}	0.71 ± 0.07^{abB}
7	2.78 ± 1.11^{abA}	1.79 ± 0.46^{aA}	1.74 ± 0.47^{aA}	1.62 ± 0.40^{abA}	1.64 ± 0.40^{bA}	1.57 ± 0.19^{bA}
10	6.91 ± 6.96^{abA}	2.76 ± 0.23^{aA}	3.08 ± 0.02^{aA}	3.00 ± 0.12^{bA}	3.05 ± 0.10^{cA}	3.04 ± 0.29^{cA}
14	24.6 ± 17.4^{bA}	8.37 ± 2.42^{bA}	9.93 ± 3.65^{bA}	6.93 ± 1.06^{cA}	6.74 ± 0.53^{dA}	6.48 ± 0.85^{dA}
	Secondary lipid oxidation (TBARS number)					
0	0.09 ± 0.00^{aA}	0.11 ± 0.01^{aA}	0.11 ± 0.02^{aA}	0.09 ± 0.00^{aA}	0.10 ± 0.01^{aA}	0.12 ± 0.04^{aA}
3	0.21 ± 0.03^{aAB}	0.18 ± 0.02^{aA}	0.24 ± 0.04^{aABC}	0.28 ± 0.03^{abBCD}	0.35 ± 0.02^{aD}	0.31 ± 0.04^{abCD}
7	0.58 ± 0.37^{aA}	0.35 ± 0.11^{aA}	0.31 ± 0.03^{aA}	0.44 ± 0.06^{bA}	0.47 ± 0.23^{abA}	0.43 ± 0.16^{abA}
10	1.24 ± 1.48^{aA}	0.37 ± 0.04^{aA}	0.52 ± 0.21^{aA}	0.44 ± 0.04^{bA}	0.47 ± 0.11^{abA}	0.54 ± 0.04^{abA}
14	2.53 ± 2.39^{aA}	0.77 ± 0.23^{bA}	1.12 ± 0.85^{aA}	0.75 ± 0.20^{cA}	0.85 ± 0.25^{bA}	0.97 ± 0.55^{bA}

(a-d) Oxidation values for a given emulsion within a column not sharing a common superscript differed significantly ($P < 0.05$)

(A-D) Oxidation values for a given time point during accelerated storage within a row not sharing a common superscript differed significantly ($P < 0.05$)

addition greatly slowed the oxidation rate (Fig. 5.3B). Similar to the primary lipid oxidation results, large standard errors (Table 5.3) found for the levels of secondary lipid oxidation products resulted in no statistically significant differences in the TBARS numbers between the samples during accelerated storage. The trends observed for secondary oxidation were consistent between all 3 trials and showed that after the 14 d of storage the control emulsion sample (i.e., no lecithin addition) displayed the greatest extent of oxidation (TBARS=2.53), compared to the lecithin-containing emulsions (i.e., TBARS=0.75-1.12; Table 5.3, Fig. 5.3B). The first obvious divergence in the extent of secondary oxidation between emulsions were observed at 10 d of accelerated storage, where the TBARS number for the control emulsion was at least double the TBARS numbers measured for the lecithin-containing emulsions (Fig. 5.3B, Table 5.3).

5.4. Discussion

Results presented in the current study demonstrated that incorporation of soybean lecithin in a simple food matrix (i.e., bulk soybean oil), as well as in a more complex multicomponent model food formulation (i.e., model IF), improved oxidative stability of both systems. Effectively, when lecithin was added to the bulk oil or included in the formulation of model IF emulsions, lipid oxidation, as evidenced by the levels of primary (hydroperoxides) and secondary (alcohols, carbonyl compounds and carboxylic acids) oxidation products was retarded.

In the model IF emulsions, adding lecithin at 1.0 g per 100 g oil, greatly reduced lipid oxidation, whereas any further increases in the lecithin content to 5.0 g per 100 g oil, had no measured additional effect on lipid oxidation. The antioxidative properties of lecithin in both bulk oil and emulsion systems are related to its surface activity, as well as its ability to interact with reactive oxygen species, thus limiting their availability for interactions with lipids in the system. In the Rancimat method for oxidative stability analysis of bulk oils, air bubbles are constantly being created and the oxygen reacts with lipids as it passes through the sample. Surface-active lecithin (i.e., a mixture of phospholipids) very quickly migrates towards and adsorbs at the newly-

formed air-water interface (Drapala et al., 2015) and effectively limits the extent of interactions between oxygen and the lipids (1) by forming a physical barrier and (2) by interacting chemically with the pro-oxidant species (i.e., binding metal ions) (Pan et al., 2013). Lecithin can arrange into micelles when present at a concentration sufficient to do so (critical micelle concentration for lecithin ~1.5 g 100 mL^{-1}) and these structures have been reported to provide an antioxidative effect in oil-in-water emulsions by interacting with ions (e.g., iron) and with hydroperoxides, solubilising them and effectively limiting their contact with lipids (Cho, McClements, and Decker, 2002; Huang et al., 2001; Nuchi, Hernandez, McClements, and Decker, 2002; Waraho, McClements, and Decker, 2011; Zou and Akoh, 2013). Similarly, in an emulsion system, the antioxidative effect of lecithin can be separated into its effects in both the serum and oil phases of the emulsion (i.e., present in both phases due to its amphipathic nature) as well as physical antioxidative effect at the interface of the emulsion. Thus lecithin, adsorbed at the interface of oil globules, provides both physical (i.e., through a physical restriction) and chemical (i.e., through radical-scavenging properties) barrier properties.

The effect of the properties of the interfacial layer of an emulsion on its oxidative stability have been studied by Kargar et al. (2011), who found that the oil phase fraction, emulsifier type and concentration and size distribution of oil globules in the emulsion had strong influences on the oxidative stability of emulsions. Pan et al. (2013) reported that unmodified lecithin reduced the permeation of free radicals into the oil phase of an emulsion and significantly reduced the rate of lipid oxidation. Thickness of the interfacial layer also plays a role in influencing lipid oxidation, where it limits the access of the radicals and pro-oxidants to the lipid globules. Generally, oil globules stabilised by intact or moderately-hydrolysed (i.e., <20% DH) proteins confer a thicker interfacial layer compared to L_{Mw} surfactants (e.g., lecithin) due to the greater sizes and more complex structures of the former (Drapala et al., 2016b; Scherze and Muschiolik, 2001). A thinner and non-continuous interfacial layer have been reported for WPH-based model IF emulsions containing lecithin (1.0-5.0, g 100 g^{-1} oil) (Drapala et al., 2015). Despite the presence of thinner and non-continuous interfacial layer in the emulsions stabilised by both WPH

and the lecithin, compared to the emulsions stabilised by the WPH alone, the oxidation was greatly reduced in the former. Based on these observations, it is concluded that it is mainly the chemical antioxidative effect of the L_{Mw} emulsifier (i.e., lecithin) that governed the rate of lipid oxidation in these model WPH-based emulsion systems, and that the thicker O/W interfaces formed with the WPH provide only a limited antioxidative barrier.

5.5. Conclusions

The results presented in this study show a strong antioxidative effect of soybean lecithin, when present in bulk soybean oil and in a model WPH-based IF emulsions. In the bulk oil, lecithin addition at 1 g 100 g^{-1} dramatically reduced lipid oxidation and increasing its addition level increased the antioxidative effect, as evidenced by a concomitant increase in the oxidation induction time. Similarly, in model IF-based emulsion systems lecithin addition reduced the progress of primary and secondary lipid oxidation; however, the effect of increasing the addition level of lecithin >1 g 100 g^{-1} was not observed. The antioxidative effect of the soybean lecithin is mainly attributed to a combination of its surface active properties (i.e., presence of the surfactant at the oil/air and oil/water interfaces in the bulk oil and in emulsion systems, respectively) and its ability to bind free radicals and pro-oxidants (i.e., making them unavailable for propagation of lipid oxidation).

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Chapter 6

Improving thermal stability of hydrolysed whey protein-based infant formula emulsions by protein-carbohydrate conjugation

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Abstract

Whey protein hydrolysate (WPH) ingredients are commonly used in the manufacture of partially-hydrolysed infant formulae. The heat stability of these emulsion-based formulae is often poor, compared with those made using intact whey protein. The objective of this study was to improve the heat stability of WPH-based emulsions by conjugation of WPH with maltodextrin (MD) through wet heating. Emulsions stabilised by different protein ingredients, whey protein isolate (WPI_E), whey protein hydrolysate (WPH_E), heated WPH (WPH-H_E), and WPH conjugated with MD (WPH-C_E) were prepared and heat treated at 75°C, 95°C or 100°C for 15 min. Changes in viscosity, fat globule size distribution (FGSD) and microstructure, evaluated using confocal laser scanning microscopy (CLSM), were used to monitor the effects of hydrolysis, pre-heating and conjugation on the heat stability of the emulsions. Heat stability increased in the order WPH_E < WPI_E << WPH-H_E <<< WPH-C_E; emulsions WPH_E, WPI_E and WPH-H_E destabilised on heating at 75°C, 95°C or 100°C, respectively. Flocculation and coalescence of oil droplets were mediated by protein aggregation (as evidenced by CLSM) on heat treatment of WPH-H_E emulsion at 100 °C, while no changes in FGSD or microstructure were observed in WPH-C_E emulsion on heat treatment at 100°C, demonstrating the excellent thermal stability of emulsions prepared with the conjugated WPH ingredient, due principally to increased steric stabilisation as a result of conjugation.

6.1. Introduction

Human milk is widely accepted as the best source of nutrients required for proper short- and long-term development of infants. The composition of mother's milk is compatible with the infant's digestive system and is known to minimise the risk of gastrointestinal and respiratory infections (Alles, Scholtens and Bindels, 2004; Exl, 2001; O'Mahony, Ramanujam, Burgher and O'Callaghan, 2011). However, it is not always possible to provide the infant with mother's milk. Efforts to develop humanised formulae for infant nutrition are focused on many aspects of formula composition and functionality including matching protein content and profile (i.e., whey-dominant protein profile and α-lactalbumin enrichment) (Chatterton, Rasmussen, Heegaard, Sørensen and Petersen, 2004; Crowley et al., 2015; Hambraeus, 1977; Ogra and Greene, 1982; O'Mahony et al., 2011), fatty acid profile (Berger, Fleith and Crozier, 2000), carbohydrate, vitamin and mineral levels to those present in human milk (Pehrsson, Patterson and Khan, 2014).

Formulae manufactured using whey protein hydrolysate (WPH) ingredients can be categorised based on the degree of hydrolysis of the protein; the main categories are amino acid-based formulae (AAF), where proteins/peptides are hydrolysed to their constituent amino acids; extensively hydrolysed formulae (EHF) containing oligopeptides with molecular weight below 3,000 Da and partially hydrolysed formulae (PHF) containing oligopeptides ranging in molecular weight up to 20,000 Da (Exl, 2001; Lowe et al., 2011). While AAF and EHF products are mainly intended for therapeutic purposes in infants suffering from, or with a high risk of cow's milk allergy (CMA), infant nutrition products from the PHF group cannot be used for therapeutic purposes but are recommended for infants at risk of CMA as they have been shown to provide a preventive effect thereon (Chandra, 1997; Exl, 2001; von Berg et al., 2008). Partially hydrolysed formulae are often also referred to as 'pre-digested' formulae based on their improved digestibility and absorption in the gut, helping to reduce gastrointestinal discomfort issues (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo and Recio, 2014).

Hydrolysis causes alteration to the functional properties of proteins and hydrolysate functionality is ultimately dependent on a number of factors including enzyme type and specificity, hydrolysis conditions and method of enzyme inactivation (Panyam and Kilara, 1996; Tavano, 2013). Generally, moderate hydrolysis improves surface activity of proteins/peptides as the hydrolysate fractions migrate rapidly to surfaces/interfaces which can give rise to improved functional properties such as foaming and emulsification (Agboola and Dalgleish, 1996a, 1996b; Banach, Lin and Lamsal, 2013; Foegeding and Davis, 2011; Kilara and Panyam, 2003). Moderate hydrolysis of globular proteins (i.e., whey proteins) improves their heat stability as a result of the diminished secondary structure; however, this improvement does not always translate directly to more complex systems such as emulsions made using hydrolysed whey protein, where heat stability has been shown to be negatively affected by hydrolysis of whey protein (Singh and Dalgleish, 1998; Ye and Singh, 2006). Responsibility for poor heat stability of hydrolysed whey protein-based emulsions is related to reduced steric hindrance (Ye, Hemar and Singh, 2004) and increased number of available (i.e., exposed) reactive sites on protein/peptide molecules at the oil globule surface and in the serum phase of the emulsion (Euston, Finnigan and Hirst, 2000; Hunt and Dalgleish, 1995).

Conjugation of proteins with carbohydrates using the Maillard reaction has been shown to be effective in modifying protein functionality (Liu, Ru and Ding, 2012; O'Regan and Mulvihill, 2010a, 2010b; Oliver, Melton and Stanley, 2006). Extensive research documenting the beneficial effects of protein modification through conjugation is available in the scientific literature; improved functional properties of proteins including solubility, emulsification, encapsulation and emulsion stability (Akhtar and Dickinson, 2003; Kasran, Cui and Goff, 2013a, 2013b; Lei, Wang, Liang, Yuan and Gao, 2014), thermal stability (Jimenez-Castano, Lopez-Fandino, Olano and Villamiel, 2005; Kato, Aoki, Kato, Nakamura and Matsuda 1995; Liu et al., 2012; O'Regan and Mulvihill, 2010a; Wang and Zhong, 2014) or foaming and gelation properties (Campbell, Raikos and Euston, 2003; Martínez and Pilosof, 2013) as a result of conjugation are well documented. However,

published scientific reports on the properties and functionality of hydrolysed whey protein ingredients modified by Maillard conjugation appear to be limited; the authors are not aware of any published studies reporting on the performance of such ingredients in oil-in-water emulsion systems, particularly in infant formula (IF) systems. The current study aims to investigate and report on the performance of ingredients produced by conjugation of hydrolysed whey protein with maltodextrin in comparison to that of intact whey protein in production and stabilisation of model IF emulsions.

6.2. Materials and methods

6.2.1. Materials

Whey protein isolate (WPI) and whey protein hydrolysate (WPH; 8% degree of hydrolysis; DH) were obtained from Carbery Food Ingredients Ltd (Ballineen, Co. Cork, Ireland). Composition of WPI and WPH ingredients was determined using standard International Dairy Federation (IDF) methods and molecular weight profile of the protein ingredients was determined using size exclusion chromatography as detailed by Drapala, Auty, Mulvihill and O'Mahony (2015). The composition, DH and molecular weight profile of the WPI and WPH ingredients are shown in Table 6.1. Maltodextrin (MD) was obtained from Corcoran Chemicals Ltd. (Dublin, Ireland) and had moisture and ash contents of <5% and <0.2%, respectively. Soybean oil was obtained from Frylite Group Ltd (Strabane, Co. Tyrone, Northern Ireland). All other chemicals and reagents used in the study were of analytical grade and sourced from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland).

6.2.2. Conjugate and stock protein solutions

Two unheated stock solutions (5%, w/v, protein) were prepared from WPI and WPH and allowed to hydrate for 18 h at 4°C and pH was adjusted to 6.8 before being used for emulsion formulation. The protein-carbohydrate conjugate solution was prepared by solubilising required quantities of WPH and MD in ultrapure water for 2 h at 20°C using a magnetic stirrer to give 5% (w/v) protein and 5% (w/v) carbohydrate. The solution was adjusted to pH 8.2 with 0.5 N potassium hydroxide (KOH) and allowed to hydrate for 18 h at 4°C,

before being readjusted to pH 8.2 with 0.5 N KOH at 20°C. Aliquots (250 mL) of this solution were placed in 500 mL screw-capped, glass conical flasks and heated at 90°C for 8 h. After heating for 8 h, the solutions were cooled immediately to 4°C and stored at that temperature overnight. A control for the heat treatment was prepared in exactly the same way as outlined above with

Table 6.1. Composition, degree of hydrolysis and molecular weight profile of the whey protein isolate (WPI) and whey protein hydrolysate (WPH) ingredients used in the preparation of emulsions.

Composition	**WPI**	**WPH**
	% w/w	
Protein	87.2 ± 0.9	83.7 ± 0.5
Fat	0.72 ± 0.1	0.67 ± 0.1
Carbohydrate[1]	4.21	7.80
Ash	2.76 ± 0.1	2.92 ± 0.1
Moisture	5.11 ± 0.0	4.91 ± 0.1
Degree of hydrolysis	NA[2]	8.00
Molecular weight profile	**% of total protein**	
Insoluble	0.00	2.00 ± 0.6
>20 kDa	28.0 ± 3.4	12.0 ± 1.6
10-20 kDa	50.5 ± 3.7	24.2 ± 8.8
5-10 kDa	3.90 ± 0.2	9.49 ± 1.9
2-5 kDa	15.6 ± 0.3	11.9 ± 1.6
1-2 kDa	0.92 ± 0.1	9.30 ± 2.0
0.5-1 kDa	0.29 ± 0.0	11.0 ± 1.9
<0.5 kDa	0.83 ± 0.7	20.2 ± 3.0

[1]*Carbohydrate content determined by difference.*

[2]*NA = not applicable.*

the exception that no MD was added to the WPH. In summary, four stock protein or protein-carbohydrate solutions were prepared and were subsequently used to formulate emulsions that are referred to as whey protein isolate emulsion (WPI_E), whey protein hydrolysate emulsion (WPH_E), heated whey protein hydrolysate emulsion (WPH-H_E) and conjugated whey protein hydrolysate emulsion (WPH-C_E), respectively.

6.2.3. Measurement of free thiol groups

The level of free thiol groups in the stock protein solutions was determined following an assay described by Hoffmann and van Mil (1997) with the exception that a Bis-Tris/HCl buffer (pH 6.8) was used in place of the Tris-HCl buffer (as performed by Alting, Hamer, De Kruif, Paques and Visschers, 2003). Aliquots (0.05 mL) of stock protein solutions (5% w/v) were added to 2.70 mL of 0.05 M Bis-Tris/HCl buffer (pH 6.8) before adding 0.25 mL of Ellman's reagent (107.5 mg/100 g of the buffer) (Ellman, 1959). Solutions were vortexed and absorbance was measured using a dual beam UV-visible spectrophotometer (Varian Cary 300, Varian Ltd., Walton-on-Thames, UK) at a wavelength of 412 nm. Measurements were completed in triplicate and the level of thiol groups was calculated using a molar extinction coefficient for 2-nitro-5-mercapto-benzoic acid (i.e., Ellman's reagent) of 13,600 M^{-1} cm^{-1}.

6.2.4. Preparation of emulsions

Model infant formula emulsions containing 1.55, 3.50 and 7.00 g/100 mL of protein, oil and carbohydrate, respectively, were prepared as follows: stock protein or protein-carbohydrate solutions (see Section 6.2.2) were diluted with ultrapure water to the appropriate concentration followed by addition of MD as required with continuous mixing using a magnetic stirrer at intermediate speed for 1 h at 22°C to prepare the aqueous phases of the emulsions. Innate levels of lactose present in the protein powders were taken into account when calculating the requirement for added carbohydrate (i.e., MD). Emulsions were prepared as described by Drapala et al. (2015) except that higher 1st and 2nd stage homogeniser pressures of 15 and 3 MPa, respectively, were used.

6.2.5. Composition and colour analysis of emulsions

Protein, fat, moisture, ash and carbohydrate content of emulsions were determined using standard IDF methods as detailed by Drapala et al. (2015). The colour of the emulsions was measured using a pre-calibrated colorimeter (Minolta Chroma Meter CR-400, Minolta Ltd., Milton Keynes, U.K.) The emulsions were loaded into a glass cell (CM-A98, optical path length: 10 mm) held in position by means of a transmittance specimen holder (CM-A96) and positioned with a white plate behind the glass cell. Colour was expressed using the Commission Internationale de l'Eclairage (CIE) colour chromaticity L* a* b* scale (L = dark/light, a = red/green, b = yellow/blue).

6.2.6. Measurement of fat globule size distribution and zeta potential

Fat globule size distribution (FGSD) of the emulsions was measured using a laser light-diffraction unit (Mastersizer S, Malvern Instruments Ltd., Worcestershire, UK) equipped with a 300 RF (reverse fourier) lens and He-Ne laser (λ of 633 nm). A polydisperse model with 3NAD presentation was used for unheated and heated emulsions as described by McCarthy et al. (2012). The 3NHD presentation was also used for heated emulsions to estimate the size of protein particles/aggregates present as described by Ciron, Gee, Kelly and Auty (2010). The samples were introduced to the mixing chamber and dispersed in ultrapure water to reach an obscuration of 14% (± 0.5%). Measurements of FGSD were made on emulsions on the day of homogenisation and immediately after heat treatment. The zeta potential (ζ) of oil globules in emulsions was measured using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) as detailed by Joshi et al. (2012). Each emulsion was diluted 1:100 with ultrapure water, adjusted to pH 6.8 with KOH or HCl and allowed to equilibrate at 25°C for 120 s in the cuvette prior to analysis. The measurement was performed on the day of homogenisation using an automatic voltage selection and ζ was calculated using the Smoluchowski model (Kirby and Hasselbrink 2004).

6.2.7. Measurement of emulsion viscosity on heating

Emulsions (28 g) were heated in an AR-G2 controlled stress rheometer (TA Instruments, Crawley, West Sussex, UK) equipped with a starch pasting cell (SPC) geometry. The heating program was chosen to allow sample equilibration for 2 min at 15°C with no shearing followed by holding for 5 min at 15°C, heating at 10°C/min to reach the required target temperature (75°C or 95°C), peak temperature hold for 15 min, cooling at 10°C/min to reach 15°C and holding at 15°C for 5 min while constantly shearing at a rate of 15 s^{-1} throughout analysis. Apparent viscosity (η) data was recorded at 1 s intervals during the heating program. An oil bath was used to heat treat more stable emulsions at 100°C; samples (2.5 mL) were placed in glass tubes, stoppered and immersed in an oil bath for 15 min at 100°C with constant mixing of tube contents by gently rocking at approx. 8 min^{-1}, giving a constant, gentle, flow of the liquid in the tube. Emulsion samples were recovered after all heat treatments (i.e., from SPC and oil bath tubes) and used for further analysis (FGSD and microstructural analysis).

6.2.8. Confocal laser scanning microscopy analysis

Microstructural analysis of emulsions was performed using a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems, Heideberg GmbH, Mannheim, Germany) as detailed by Drapala et al. (2015). Protein and lipid were fluorescently labelled with Nile Blue dye (Sigma-Aldrich, Dublin, Ireland). Visualisation of oil and protein in emulsions (10 μL) was carried out using an Ar laser (excitation = 488 nm, emission = 500-530 nm) and a He-Ne laser (excitation = 633 nm, emission = 650-700 nm) for oil and protein, respectively (Auty, Twomey, Guinee and Mulvihill, 2001). The observations were performed using 20x and 63x oil immersion objectives. At least three specimens of each sample were observed to obtain representative micrographs of samples.

6.2.9. Statistical data analysis

Analysis of variance (ANOVA) was carried out using the Minitab® 16 (Minitab Ltd, Coventry, UK, 2010) statistical analysis package. The Tukey method was

used to obtain grouping information. The level of significance was determined at $P < 0.05$.

6.3. Results

6.3.1. Composition and colour of emulsions

Compositional analysis of emulsions showed that measured levels (Table 6.2) were in line with target levels for all samples (i.e., 1.39 – 1.45% protein, 3.35 – 3.42% fat and 6.47 – 6.52% carbohydrate) and no significant differences in composition were found between samples. Emulsions stabilised by heated/conjugated proteins (i.e., WPH-H$_E$ and WPH-C$_E$) differed slightly but significantly in lightness (L* values; Table 6.2) from emulsions stabilised by unheated WPI or WPH; lowest L* value was observed for WPH-C$_E$ (82.6) followed by WPH-H$_E$ (83.2), WPH$_E$ (83.7) and WPI$_E$ (84.0). A similar trend was observed in the intensity of the yellow colour of the emulsions where all samples were statistically different from each other and the highest b* value was observed for WPH-C$_E$ (4.48) followed by WPH-H$_E$ (2.24), WPH$_E$ (0.78) and WPI$_E$ (0.30). Lower L* and higher b* values in emulsions WPH-C$_E$ and WPH-H$_E$, as compared to emulsions WPH$_E$ and WPI$_E$, can be directly related to production of coloured compounds such as melanoidins during the later stages of the Maillard reaction (Oliver et al., 2006). Although no MD was added prior to heating of the WPH solution, innate lactose (a reducing sugar) present in the WPH powder (Table 6.1) would have contributed to some Maillard-induced browning during heating. As shown by Liu and Zhong (2015) lactose is more reactive than maltodextrin and therefore has greater propensity for Maillard-induced colour development as it contains more reducing groups per unit weight as compared to MD.

6.3.2. Fat globule size distribution and ζ-potential

Narrow and monomodal size distributions of oil globules were observed in all four emulsions post-homogenisation (Fig. 6.1) with the samples having mean volume diameters ($D_{4,3}$; Table 6.3) of 0.85, 0.83, 0.80 and 0.79 μm for WPI$_E$, WPH$_E$, WPH-H$_E$ and WPH-C$_E$ emulsions, respectively. The size distribution data showed that all of the protein ingredients had good emulsifying properties

Table 6.2. Composition and colour of model infant formula emulsions stabilised by the different whey protein ingredients.

Emulsion	Protein (%)	Fat	Carbohydrate	Ash	Total Solids	Tristimulus coordinates		
						L*	a*	b*
WPI_E	1.45 ± 0.04^a	3.35 ± 0.03^a	6.52 ± 0.11^a	0.06 ± 0.03^a	11.4 ± 0.05^a	84.0 ± 0.11^a	-0.87 ± 0.01^a	0.30 ± 0.03^a
WPH_E	1.43 ± 0.04^a	3.39 ± 0.01^a	6.47 ± 0.04^a	0.10 ± 0.01^a	11.4 ± 0.06^a	83.7 ± 0.12^a	-1.08 ± 0.06^b	0.78 ± 0.06^b
$WPH\text{-}H_E$	1.39 ± 0.01^a	3.39 ± 0.05^a	6.50 ± 0.10^a	0.21 ± 0.15^a	11.5 ± 0.08^a	83.2 ± 0.26^b	-1.07 ± 0.05^b	2.24 ± 0.04^c
$WPH\text{-}C_E$	1.42 ± 0.02^a	3.42 ± 0.01^a	6.52 ± 0.18^a	0.13 ± 0.05^a	11.5 ± 0.11^a	82.6 ± 0.16^c	-1.01 ± 0.04^b	4.48 ± 0.02^d

$^{(a\text{-}c)}$ *Values within a column not sharing a common superscript differed significantly ($P<0.05$).*

as indicated by $D_{4,3}$ values of all emulsions less than 1 μm which was consistent with results of McCarthy et al. (2012) and Drapala et al. (2015). There were no significant differences ($P<0.05$) in FGSD between emulsions formed with the different ingredients immediately post homogenisation.

The net negative charge (i.e., ζ potential) of oil globules in emulsions was lowest in WPI_E (-48.0 mV; Table 6.3) followed by WPH_E (-49.6 mV), WPH-H_E (-53.1 mV) and WPH-C_E (-55.0 mV). Although ζ was slightly higher for emulsion globules stabilised by heated WPH than for those stabilised by WPI and WPH, and was slightly higher still for emulsion globules stabilised by conjugated WPH, no significant differences were observed in ζ between globules in the 3 emulsions prepared using hydrolysed whey protein. The positively charged amino acid lysine is predominantly involved in covalent attachment with reducing sugars during the Maillard reaction; thus, by its interaction during heating/conjugation the net negative charge on the protein, and as a result, on the surface of the oil globules in the emulsions increased, which was consistent with the results of Acedo-Carrillo et al. (2006), Liu et al. (2012) and Wang and Zhong (2014). Additionally, as a consequence of thermal denaturation of proteins, charged groups buried within the native structure of globular proteins are exposed, and this may also have contributed to the change in net protein charge (Tcholakova, Denkov, Ivanov and Campbell, 2006).

After heating at 75°C for 15 min in the starch pasting cell (SPC), the particle size distribution of WPI_E emulsion showed a limited broadening of the profile (Fig. 6.1 a); however, only a minor difference was found in the $D_{4,3}$ before (0.85 μm) and after (0.87 μm) heat treatment of the emulsion under these conditions (Table 6.3). The WPH_E emulsion destabilised during heat treatment at 75°C as evidenced by the presence of large particles ($D_{4,3}$ of 120 μm) in the sample after heat treatment (Fig. 6.1 b; Table 6.3). Results obtained using both the 3NAD (i.e., selective for oil) and 3NHD (i.e., selective for protein) presentations displayed essentially the same size distribution profiles (Fig. 6.1 b). Size distribution data and visual observation (i.e., phase separation with large particles buoyant in the semi-transparent serum phase and no free oil; Fig. 6.2 a) of WPH_E emulsion heat treated at 75°C suggested that the

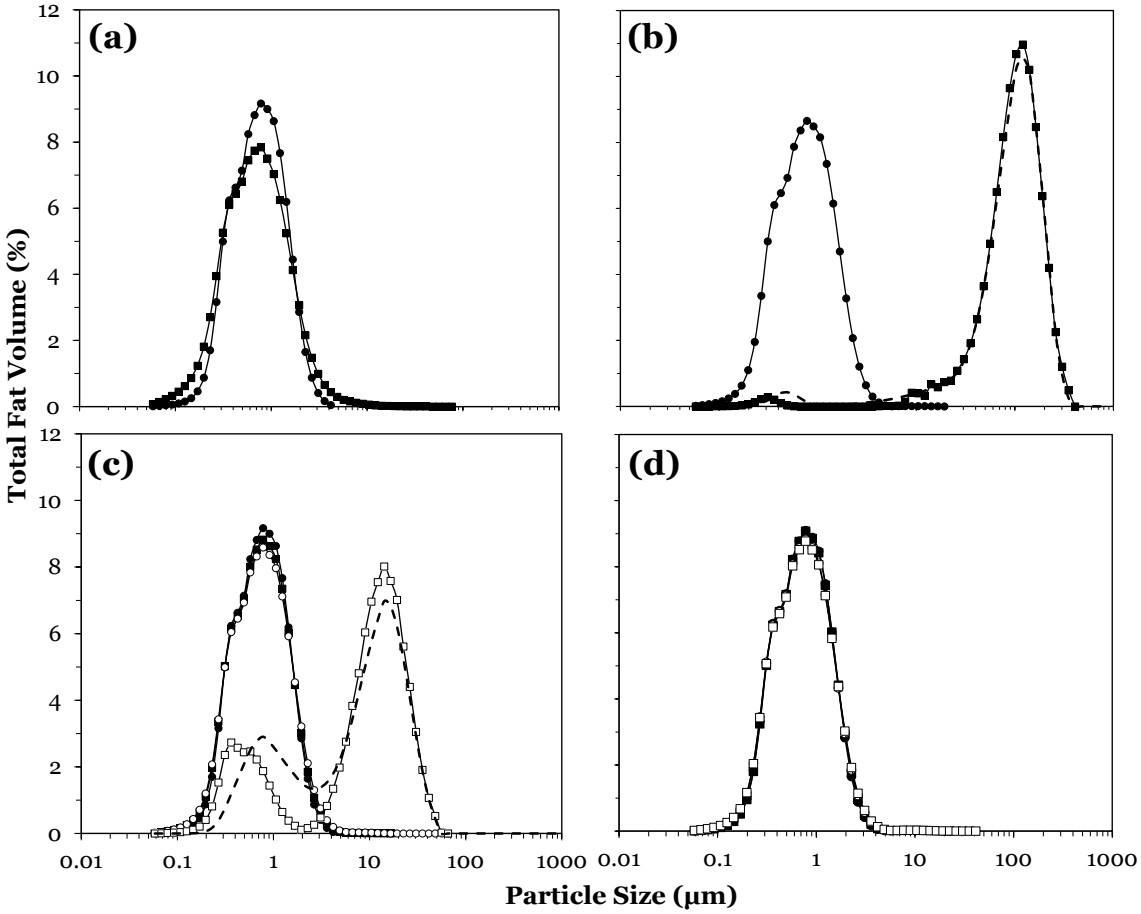

Figure 6.1. Fat globule size distribution in WPI_E (**a**), WPH_E (**b**), WPH-H_E (**c**) and WPH-C_E (**d**) emulsions post-homogenisation (●) and after 15 min of heat treatment at 75°C (■), 95°C (○) and 100°C (□). Dashed line (---) represents the 3NHD presentation profiles for destabilised emulsions formed with hydrolysed protein (**b**, **c**).

emulsion destabilised through aggregation of globules via protein/peptides on the surface of oil globules and possibly aggregation of globules via interaction with non-adsorbed protein/peptides present in the serum resulting in entrapment of oil in the aggregated protein network. Previous studies have indicated that heat-induced destabilisation of protein-based oil-in-water (O/W) emulsions is often mediated by non-adsorbed serum proteins/peptides that, upon heating, interact with each other and with adsorbed proteins/peptides, thus causing formation of protein/oil complexes (Euston et al., 2000; Hunt and Dalgleish, 1995). In contrast to the WPH_E emulsion, no

changes in particle size distribution were observed for WPH-H_E or WPH-C_E emulsions on heating at 75°C for 15 min (Fig. 6.1 c, d; Table 6.3).

Emulsions that displayed good thermal stability during heat treatment at 75°C for 15 min were subjected to a more severe treatment of 95°C for 15 min in the SPC. The WPI_E emulsion destabilised during heat treatment at 95°C. Visual inspection of the sample after heat treatment indicated formation of a separated coarse protein network and serum phase (Fig. 6.2 b); hence, particle size distribution data for the WPI_E emulsion after the heat treatment at 95°C could not be determined. The WPH-H_E and WPH-C_E emulsions displayed good thermal stability to heating at 95°C for 15 min as evidenced by no significant increase in $D_{4,3}$ (<0.05 μm) on heating (Table 6.3; Fig. 6.1 c, d) and the visual appearance of these samples after heating (Fig. 6.2 c, d). Differences in thermal stability of WPH-H_E and WPH-C_E emulsions were observed when the emulsions were heated at 100°C for 15 min in an oil bath; visual assessment post heating indicated formation of large particles (i.e., aggregates) in the WPH-H_E emulsion (Fig. 6.2 e). This was confirmed by particle size analysis

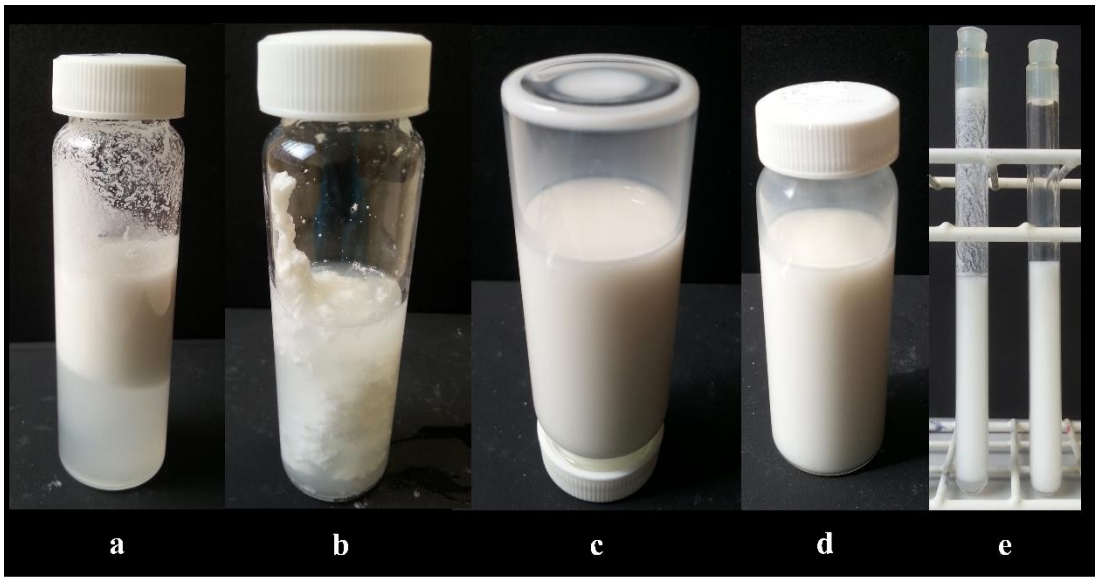

Figure 6.2. Photographs of emulsions post-heat treatment at 75°C x 15 min (**a**: WPH_E) and at 95°C x 15 min (**b**: WPI_E; **c**: WPH-H_E; **d**: WPH-C_E). Emulsions WPH-H_E and WPH-C_E were also heated at 100°C x 15 min in an oil bath (**e**: left=WPH-H_E; right=WPH-C_E).

Table 6.3. Fat globule size distribution (FGSD) and zeta potential (ζ) of oil globules in model infant formula emulsions WPI_E, WPH_E, WPH-H_E and WPH-C_E post homogenisation and post heating at 75, 95 or 100°C for 15 min.

Emulsion	*Heat treatment*	Fat Globule Size Parameter[1] (μm)					ζ Potential (mV)
		$D_{4,3}$	$D_{3,2}$	$D_{v,0.1}$	$D_{v,0.5}$	$D_{v,0.9}$	
WPI_E	*unheated*	0.85 ± 0.0[a]	0.56 ± 0.0[a]	0.30 ± 0.0[a]	0.70 ± 0.0[a]	1.52 ± 0.0[a]	-48.0 ± 2.6[a]
	75°C x 15 min	0.87 ± 0.1[a]	0.48 ± 0.0[a]	0.24 ± 0.0[a]	0.64 ± 0.0[a]	1.69 ± 0.3[a]	n.d.[2]
	95°C x 15 min	n.d.[2]	n.d.	n.d.	n.d.	n.d.	n.d.
	100°C x 15 min	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
WPH_E	*unheated*	0.83 ± 0.1[a]	0.54 ± 0.0[a]	0.29 ± 0.0[a]	0.69 ± 0.1[a]	1.54 ± 0.1[a]	-49.6 ± 3.0[ab]
	75°C x 15 min	120 ± 30[b]	50.9 ± 37[b]	38.3 ± 3.8[b]	112 ± 29[b]	212 ± 59[b]	n.d.
	95°C x 15 min	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	100°C x 15 min	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
WPH-H_E	*unheated*	0.80 ± 0.0[a]	0.55 ± 0.0[a]	0.30 ± 0.0[a]	0.68 ± 0.0[a]	1.46 ± 0.0[a]	-53.1 ± 1.2[ab]
	75°C x 15 min	0.81 ± 0.0[a]	0.53 ± 0.0[a]	0.29 ± 0.0[a]	0.68 ± 0.0[a]	1.51 ± 0.1[a]	n.d.
	95°C x 15 min	0.85 ± 0.0[a]	0.53 ± 0.0[a]	0.28 ± 0.0[a]	0.68 ± 0.0[a]	1.58 ± 0.1[a]	n.d.
	100°C x 15 min	38.8 ± 29[b]	3.72 ± 2.3[b]	13.5 ± 19[a]	37.2 ± 39[a]	66.4 ± 61[b]	n.d.
WPH-C_E	*unheated*	0.79 ± 0.0[a]	0.54 ± 0.0[a]	0.29 ± 0.0[a]	0.67 ± 0.0[a]	1.45 ± 0.0[a]	-55.0 ± 3.1[b]
	75°C x 15 min	0.80 ± 0.0[a]	0.54 ± 0.0[a]	0.29 ± 0.0[a]	0.68 ± 0.0[a]	1.47 ± 0.1[a]	n.d.
	95°C x 15 min	0.81 ± 0.0[a]	0.53 ± 0.0[a]	0.28 ± 0.0[a]	0.67 ± 0.0[a]	1.50 ± 0.1[a]	n.d.
	100°C x 15 min	0.83 ± 0.0[a]	0.53 ± 0.0[a]	0.28 ± 0.0[a]	0.67 ± 0.0[a]	1.52 ± 0.1[a]	n.d.

[1] *Fat globule size distribution parameters $D_{4,3}$ and $D_{3,2}$ represent volume mean diameter and Sauter mean diameter, respectively. $D_{v,0.1}$, $D_{v,0.5}$ and $D_{v,0.9}$ represent fat globule size in the 10, 50 and 90% quantiles of the distribution, respectively.*

[2] *n.d. = not determined. ζ potential measured in emulsions post homogenisation only.*

whereby the FGSD profile of WPH-H_E showed a shift from monomodal to bimodal (Fig. 6.1 c). The emulsion stabilised by WPH-C was stable to heat treatment at 100°C for 15 min; no significant differences in $D_{4,3}$ (<0.05 μm) or in FGSD profiles were observed after heat treatment as compared to post-homogenisation (Table 6.3, Fig. 6.1 d) and no visual evidence of destabilisation (Fig. 6.2 e) was observed in the emulsion after heating.

6.3.3. Apparent viscosity of emulsions on heating

Apparent viscosity of all emulsions before heating (i.e., at 15°C) was similar with no significant ($P<0.05$) differences were found between samples (Table 6.4). On increasing temperature from 15˚C to 75˚C, apparent viscosity of all emulsion samples decreased (Fig. 6.3 a). Decreasing viscosity with increasing temperature is commonly observed in protein solutions; however, the decrease in viscosity normally continues until a protein-specific temperature is reached, at which point physical changes to the protein affect its structure (i.e., unfolding of polypeptide/peptide chain, disruption of hydrophobic interactions and aggregation by covalent and non-covalent bonding), generally causing an increase in viscosity (Considine, Patel, Anema, Singh and Creamer, 2007; Goetz and Koehler, 2005). Hence, the onset of structural changes and interactions (eventually leading to destabilisation) in protein-based emulsions can be identified by tracking changes in their apparent viscosity during heat treatment. Although final viscosity of the WPI_E emulsion (i.e., after cooling to 15°C) was slightly higher compared to the initial viscosity (i.e., at 15°C before heating) of the sample, no significant differences were found between viscosity of the WPI_E emulsion before and after heating at 75°C for 15 min (Table 6.4). An increase in viscosity from 9.6 to 12.7 mPa.s (Fig. 6.3 a) before reaching the peak hold temperature (75°C) was observed for WPH_E. Final viscosity of the WPH_E emulsion (i.e., after cooling to 15°C) was higher by 8.5 mPa.s compared to the initial viscosity of the sample at 15°C (Table 6.4) and visual assessment of the sample after heating indicated extensive destabilisation of the emulsion (Fig. 6.2 a). After heat treatment, apparent viscosity (at 15°C) of WPH-H_E and WPH-C_E emulsions was no different to that measured before heating of these emulsions but was significantly lower than the viscosity of the WPH_E emulsion after the same heat treatment (Fig. 6.3 a; Table 6.4).

During more severe thermal treatment at 95°C for 15 min of the WPI_E emulsion, a sharp increase in viscosity was observed during the heating phase on reaching 81°C (Fig. 6.3 b); formation of distinct separated coarse protein network and serum phases was observed on visual assessment of the heated WPI_E emulsion (Fig. 6.2 b). No significant ($P<0.05$) differences in viscosity of WPH-H_E and WPH-C_E emulsions were observed after heating at 95°C for 15 min compared to unheated emulsions (Fig. 6.3 b; Table 6.4); visual inspection of samples after heating indicated that the WPH-H_E and WPH-C_E emulsions were stable to the heat treatment (Fig. 6.2 c, d). No viscosity data was recorded during heat treatment at 100°C as this heat treatment was performed in an oil bath.

6.3.4. Free thiol groups and thermal stability of emulsions

The level of free thiol (-SH) groups was significantly different in the stock protein solutions used to prepare emulsions and increased in the order WPI<<<WPH-H<<WPH-C<WPH (i.e., 2.49, 7.89, 10.9 and 11.5 μmol -SH/g protein, respectively). WPI consists of intact whey protein where most of the reactive -SH groups are buried within its globular structure, while WPH has more -SH groups exposed due to enzymatic hydrolysis of the compact globular structure (Panyam and Kilara, 1996). Significantly ($P<0.05$) lower levels of free -SH groups measured in the WPH-H solution as compared to the WPH solution indicated their reduction on heating due to the involvement of -SH groups in formation of di-sulphide bridges (-S-S-) (Adjonu, Doran, Torley and Agboola, 2013; Singh, 2011). Significantly ($P<0.05$) higher levels of free -SH groups were measured in the WPH-C solution compared to the WPH-H solution, although the two solutions were subjected to the same heating conditions (i.e., 8 h at 90°C). The difference in the levels of free -SH groups observed between WPH-H and WPH-C may be associated with two factors: (1) a macromolecular crowding effect (Zhu, Damodaran, and Lucey, 2008, 2010) caused by the higher number of macromolecules (i.e., MD also present in the WPH-C sample) limiting mobility and interactions between proteins/peptides in the solution during heat treatment; and (2) the access to free -SH groups may be restricted by steric hindrance of already conjugated protein/peptides, thus limiting the formation of -S-S- bonds.

Table 6.4. Apparent viscosity of WPI_E, WPH_E, WPH-H_E or WPH-C_E emulsions at different stages of heat treatment using a starch pasting cell at 75°C and 95°C for 15 min.

Holding Temperature	Measurement Stage	Viscosity (mPa.s)			
		WPI_E	**WPH_E**	**WPH-H_E**	**WPH-C_E**
75°C	*Pre-heating*	14.3 ± 0.2^{aA}	13.5 ± 0.8^{aA}	14.0 ± 0.0^{aA}	14.0 ± 0.1^{aA}
	Reaching peak temperature	10.6 ± 0.3^{aB}	12.7 ± 1.0^{bA}	9.69 ± 0.1^{aB}	9.40 ± 0.2^{aB}
	Peak hold	10.4 ± 0.1^{abB}	13.1 ± 1.7^{aA}	9.69 ± 0.0^{bB}	9.68 ± 0.0^{bB}
	Post-heating	15.2 ± 0.3^{aA}	22.0 ± 3.2^{bB}	14.1 ± 0.0^{aA}	14.1 ± 0.1^{aA}
95°C	*Pre-heating*	14.6 ± 0.6^{aA}	n.d.[1]	14.1 ± 0.1^{aB}	14.1 ± 0.1^{aA}
	Reaching peak temperature	176 ± 201^{aA}	n.d.	8.71 ± 0.0^{aA}	8.99 ± 0.2^{aB}
	Peak hold	457 ± 357^{bA}	n.d.	9.09 ± 0.0^{aA}	9.05 ± 0.0^{aB}
	Post-heating	98.6 ± 71^{bA}	n.d.	15.0 ± 0.4^{aC}	14.8 ± 0.6^{aA}

[1] *n.d. = not determined as sample destabilised during less severe heat treatment (i.e., 75°C for 15 min).*

(a-b) Values within a row (horizontal) not sharing a common superscript differed significantly (P<0.05).

(A-B) Values within a column (vertical) for each of the heat treatments (i.e., 75 or 95°C) not sharing a common superscript differed significantly (P<0.05).

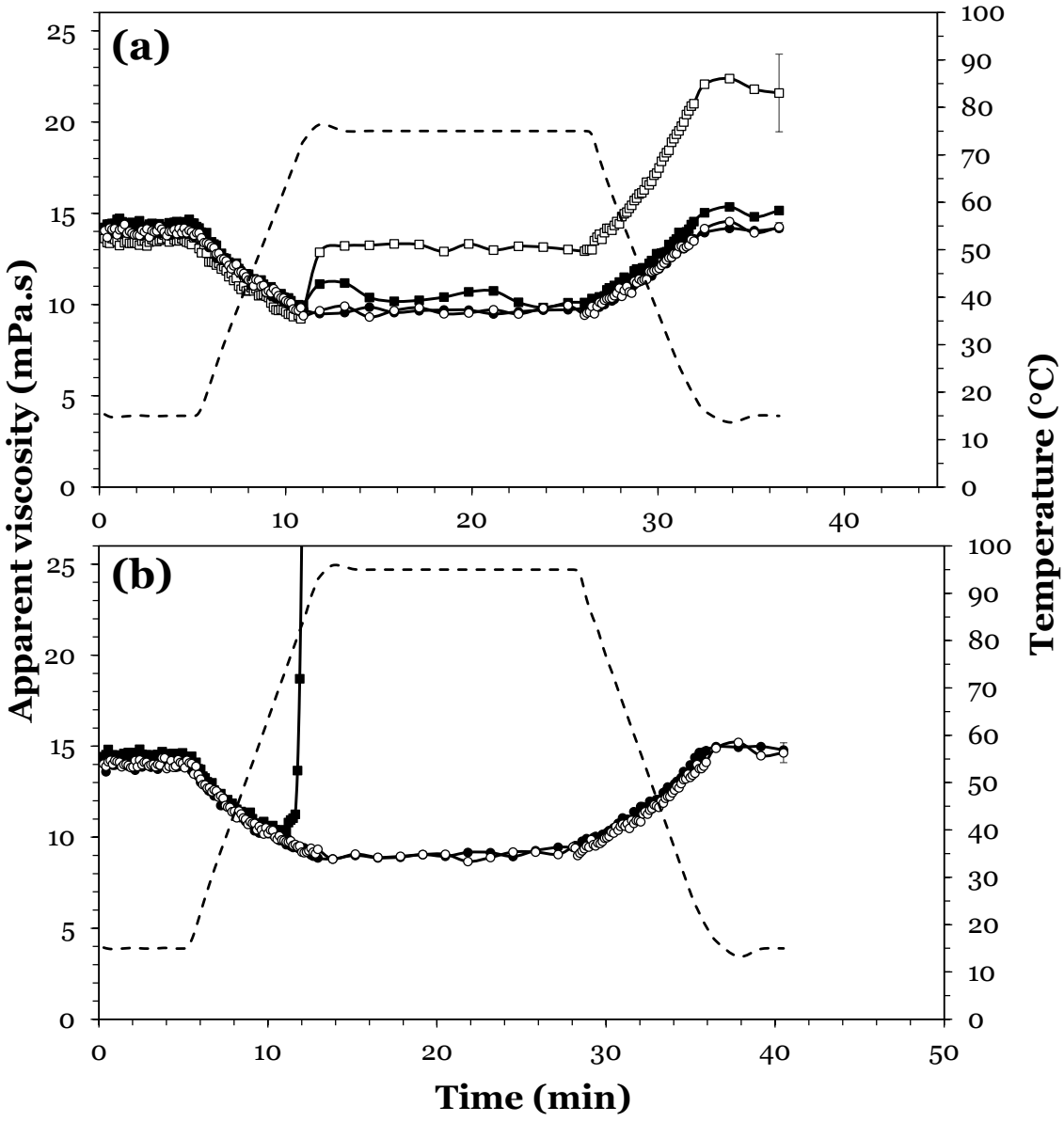

Figure 6.3. Apparent viscosity profiles of WPI_E (■), WPH_E (□), WPH-H_E (●) and WPH-C_E (○) emulsions during starch pasting cell (SPC) heat treatments with peak hold at 75°C (a) and 95°C (b). Dashed line represents the temperature profile.

6.3.5. Confocal laser scanning microscopy

Microstructural analysis of WPH-HE and WPH-CE emulsions showed small, uniform and homogenously distributed fat globules in both samples post homogenisation with no differences between the samples (Fig. 6.4 a1, b1). However, major differences were observed between the two emulsions after heat treatment at 100°C for 15 min, supporting the FGSD data (Table 6.3). No changes were observed in the WPH-CE emulsion after heating at 100°C for 15 min. Conversely, the WPH-HE emulsion displayed a heterogeneous microstructure with a number of mechanisms involved in the emulsion destabilisation being identified. Bridging flocculation of oil globules was

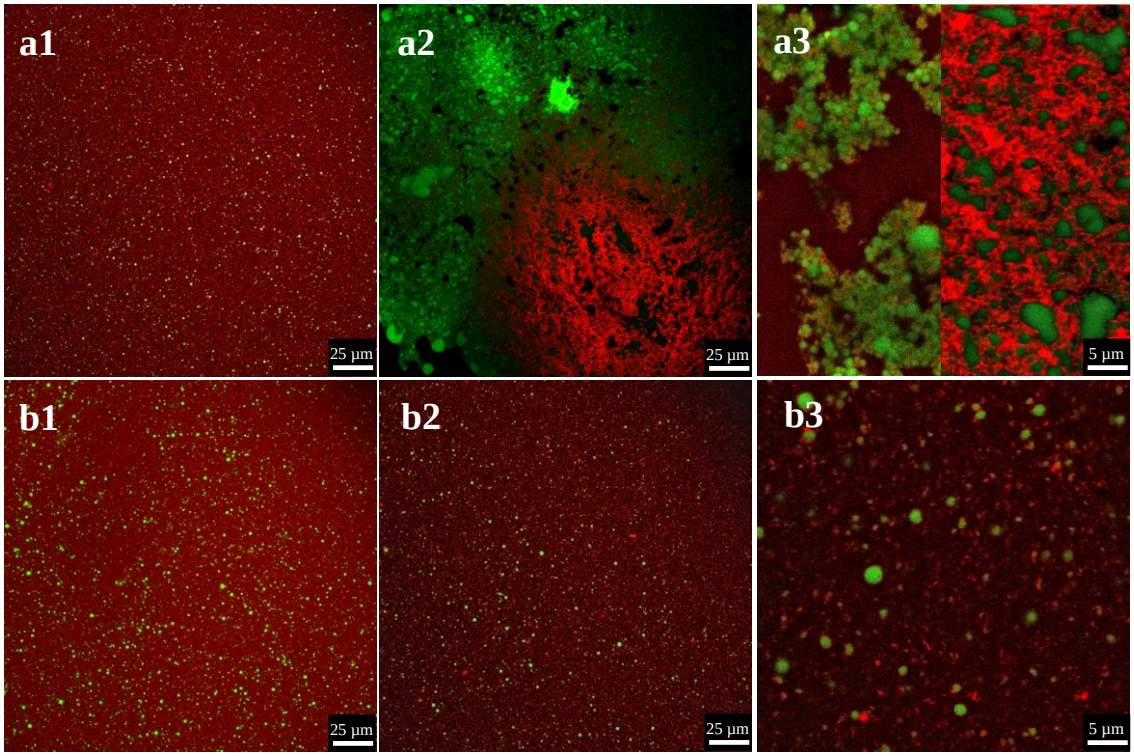

Figure 6.4. Confocal laser scanning micrographs of WPH-HE (**a**) and WPH-CE (**b**) emulsions before (**1**) and after (**2**, **3**) heat treatment in oil bath at 100°C for 15 min. Protein=RED; Oil=GREEN. Scale bar (bottom right) is 25 µm (**1**, **2**) and 5 µm (**3**).

Note: Figure a3 is a combination of 2 micrographs (i.e., left and right) to give more comprehensive representation of the heterogeneous structure observed in the WPH-H emulsion after heat treatment.

observed for the heat treated WPH-H_E emulsion, where a distinct, dense protein layer surrounded the individual oil globules (Fig. 6.4 a3-left). This mechanism of destabilisation is common for whey protein-based O/W emulsions where proteins adsorbed at the interface of different oil globules react with each other on heating through formation of disulphide bonds (Dickinson, 2001). Coalescence and formation of larger oil globules was also observed in the WPH-H_E emulsion and this mechanism often occurs in conjunction with flocculation (Raikos, 2010; Tcholakova et al., 2006), where the interfacial film between oil globules ruptures and the globules merge to form larger oil globules (Tcholakova et al., 2006; Ye et al., 2004). Formation of a dense protein network with pools of oil trapped within it, visible in the heat treated WPH-H_E emulsion, is a consecutive step in the thermal destabilisation process that follows bridging flocculation and coalescence (Lam and Nickerson, 2013). With prolonged exposure to high temperature, interactions between proteins/peptides at interfaces of different oil globules and between proteins/peptides at interfaces and serum proteins/peptides grow stronger forming a cohesive protein network (as seen in Fig. 6.4 a3-right). No changes in the microstructure of the WPH-C_E emulsion after heat treatment at 100°C for 15 min (Fig. 6.4 b2, b3) indicated that the emulsion was stable to the heating process.

6.4. Discussion

The stability of the emulsions to thermal processing under controlled conditions (i.e., temperature, heating/cooling rate and shear rate) was found to be markedly different for emulsions stabilised by the different protein ingredients; the observed order of the heat stability (least-to-most stable) was WPH_E<WPI_E<<WPH-H_E<<<WPH-C_E. Results presented in this work (1) identified differences in destabilisation mechanisms between emulsions formed with intact and hydrolysed whey proteins and (2) demonstrated that modification of hydrolysed whey protein/peptides by conjugation with MD gave an ingredient with superior thermal stability in infant formula-based O/W emulsion systems. It was also shown that pre-heating of the hydrolysed whey protein ingredient improved thermal stability of emulsions formed therefrom.

Clear differences in destabilisation behaviour were observed for emulsions prepared using intact (WPI_E) and hydrolysed (WPH_E) whey protein as evidenced by the magnitude of viscosity increase of emulsions upon destabilisation (Fig. 6.3), final viscosity (Table 6.4) and protein particle/aggregate size and physical appearance (Fig. 6.2) of destabilised WPI_E and WPH_E emulsions. These results demonstrate that formation of a coarse protein network or formation of a large number of relatively small (10 to 400 μm) protein aggregates/oil particles are the final stages of thermal destabilisation of intact (WPI_E) and hydrolysed (WPH_E) whey protein-based emulsions, respectively. Denaturation and aggregation of intact whey protein involve a number of sequential stages such as unfolding, association (non-covalent followed by covalent bonding), propagation (i.e., formation of polymers) and termination (Mulvihill and Donovan, 1987; Oldfield, Singh, Taylor and Pearce, 1998). The differences outlined above in the nature of thermally-induced destabilisation observed in WPI_E and WPH_E emulsions indicated that the propagation stage is limited and formation of a large number of small aggregate complexes is favoured, over extensive protein network formation (as observed in intact protein systems) in the WPH-stabilised emulsions. According to a study by Surroca, Haverkamp and Heck (2002), during the thermal denaturation and aggregation of intact whey protein, aggregates need to reach their maximum concentrations before the polymerisation stage can occur. However, in a system containing hydrolysed protein, the termination stage can take place before polymerisation due to blocking of thiol groups (-SH) by peptides in the surrounding serum phase and on the interfaces of nearby oil globules. Successful efforts to limit aggregation of whey proteins by blocking –SH groups have been documented (Sakai, Sakurai, Sakai, Hoshino and Goto, 2000; Wijayanti, Bansal, and Deeth, 2014).

The results of this study have shown that the role of serum proteins in mediating aggregation and destabilisation of WPH-stabilised emulsions can be diminished by preheating of the protein ingredient prior to emulsion formation. This is clear from the current study where improved thermal stability of emulsions stabilised by hydrolysed whey protein was evident where the level of free thiol groups had been reduced through pre-heating. Similarly,

previous studies have shown that (1) serum (non-adsorbed) proteins play a major role in aggregation and destabilisation of whey protein-stabilised emulsions (Euston et al., 2000; Hunt and Dalgleish, 1995), (2) blocking potential reactive sites on proteins/peptides (i.e., hydrophobic or thiol groups) allows improvement of heat stability of protein-based systems (Baier and McClements, 2001; Rich and Foegeding, 2000; Smulders and Somers, 2012) and (3) pre-heating of protein reduces the number of reactive groups – mainly free thiol groups (Liang, Patel, Matia-Merino, Ye, and Golding, 2013; Livney, Corredig, and Dalgleish, 2003; Wijayanti et al., 2014).

The results of this study have demonstrated that the emulsion stabilised with the WPH-C displayed superior stability to thermal processing, where oil globule-globule interactions, observed in WPI_E, WPH_E and $WPH\text{-}H_E$ emulsions, were prevented. It is proposed that primarily steric stabilisation and, to a lesser extent, increased ζ potential, provided by the protein-carbohydrate conjugate, limited interactions (i.e., coming in contact and subsequent aggregation) between proteins/peptides adsorbed at the interfaces of different oil globules in the $WPH\text{-}C_E$ emulsion conferring superior stability compared with WPI or WPH ingredients. Limiting interactions between oil globules in emulsions is one of the main strategies to improve stability of these systems. An increase in steric stabilisation through adsorption/attachment of flexible, hydrophilic macromolecules to the emulsion globules effectively limits close contact and subsequent interactions between oil globules (Dalgleish 1997). Stabilisation of emulsions with conjugated protein/maltodextrin provides the O/W interfacial layer with increased thickness and effectively produces a better steric barrier to the oil globules. In keeping with this, Wong, Day and Augustin (2011) reported that increased steric stabilisation resulting from the thicker interfacial layer of conjugate (composed of wheat protein/dextran) gave better emulsion stability. Additionally, conjugation has been reported to improve stability of emulsions in cases where these systems were subjected to stressed or unfavourable conditions; improved heating and freeze-thaw stability of emulsions formed with casein/maltodextrin conjugates was reported by O'Regan and Mulvihill (2010a) and improvement in long term stability of emulsions stabilised with

WPI/dextran conjugates with low ζ potential (i.e., <30 mV) was reported by Akhtar and Dickinson (2003). In the current study, combining pre-heating with attachment of hydrophilic polysaccharide groups to hydrolysed whey protein by Maillard conjugation resulted in a protein-based emulsifier characterised by its ability to confer improved thermal stability to infant formula type emulsions.

6.5. Conclusions

This study showed that heat stability of model infant formula emulsions based on hydrolysed whey protein ingredients can be markedly improved by modification of the protein ingredient through conjugation with carbohydrate. Covalent bonding between proteins/peptides in hydrolysed whey protein and maltodextrin produced an ingredient with enhanced performance during thermal processing of the model infant formula emulsion where, due to increased steric and electrostatic repulsion, interactions between and subsequent destabilisation of oil globules during heat treatment were supressed. It was also shown that pre-heating of hydrolysed whey protein prior to its use in emulsion preparation resulted in enhanced heat stability of the emulsion, as a result of a reduction in the level of reactive sites (i.e., free thiol groups) through protein-protein interactions. Incorporation of protein-carbohydrate conjugates in the formulation of nutritional products could potentially allow for the displacement (at least partial) of non-protein emulsifiers without compromising stability or quality of the product and offers potential for application in other nutritional products naturally containing hydrolysed whey protein and maltodextrin, such as clinical nutrition products.

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Chapter 7

Performance of whey protein hydrolysate-maltodextrin conjugates as emulsifiers in model infant formula emulsions

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Abstract

Model infant formula emulsions containing 15.5, 35.0 and 70.0 g L^{-1} protein, soybean oil and maltodextrin (MD), respectively, were prepared. Emulsions were stabilised by whey protein hydrolysate (WPH) + CITREM (9 g L^{-1}), WPH + lecithin (9 g L^{-1}) or WPH conjugated with MD (WPH-MD). All emulsions had mono-modal oil droplet size distributions post-homogenisation with mean oil droplet diameters ($D_{4,3}$) of <1.0 μm. No changes in the $D_{4,3}$ were observed after heat treatment (95°C, 15 min) of the emulsions. Accelerated storage (40°C, 10 d) of unheated emulsions resulted in an increase in $D_{4,3}$ for CITREM (2.86 μm) and lecithin (5.36 μm) containing emulsions. Heated emulsions displayed better stability to accelerated storage with no increase in $D_{4,3}$ for CITREM and an increase in $D_{4,3}$ for lecithin (2.71 μm) containing emulsions. No increase in $D_{4,3}$ over storage was observed for unheated or heated WPH-MD emulsion, indicating its superior stability.

7.1. Introduction

The incorporation of whey protein hydrolysates (WPH) with a moderate degree of hydrolysis into nutritional formulations tailored for athletes, the elderly or infants is increasing due to growing demand for products which contain amino acids in a rapidly digestible form. The enhanced gut absorption and efficient metabolisation of hydrolysates (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, and Recio, 2014) make these ingredients particularly useful for consumers seeking to increase the rate of muscle synthesis or limit ageing-related muscle loss (Jonker, Deutz, Erbland, Anderson, and Engelen, 2014; Pimenta, Abecia-Soria, Auler, and Amaya-Farfan, 2006). Infant formulae containing moderately hydrolysed WPH are not intended for medical purposes in infants suffering from cows' milk allergy; however, these formulae can improve comfort in infants that suffer from difficulty digesting intact proteins (Bourlieu et al., 2015; Nguyen, Bhandari, Cichero, and Prakash, 2015).

A common challenge encountered during the preparation of emulsions containing hydrolysates is their diminished processing stability (i.e., short term storage of unheated emulsions and thermal stability) and shelf life stability (i.e., long term storage of heat treated emulsions) compared with emulsions containing intact whey protein (Drapala, Mulvihill and O'Mahony, 2015, 2016; Singh and Dalgleish, 1998; Ye and Singh, 2006). Poor thermal stability of WPH-based emulsions is related to the reduced steric hindrance between oil globules provided by peptides compared to intact protein (Ye, Hemar and Singh, 2004). This reduced steric hindrance increases the interactions which occur between oil globules during heating and storage of emulsions. In addition, a high number of exposed reactive sites (such as free -SH groups) at both the oil/water interface and in the serum phase of WPH-based emulsions promotes protein/peptide-protein/peptide interactions (i.e., mainly through formation of disulphide bridges, -S-S-) resulting in flocculation of oil globules (Adjonu, Doran, Torley and Agboola, 2013, 2014; Drapala et al., 2016; Singh, 2011; Panyam and Kilara, 1996).

Non-protein emulsifiers, such as CITREM (i.e., citric acid esters of monoglycerides) or lecithin, are often included in the formulation of emulsions to facilitate the formation of small oil globules on homogenisation, improve stability of emulsions to thermal processing and reduce creaming during storage. Lecithin or CITREM are routinely used in the manufacture of infant formulae (IF) which contain hydrolysed milk proteins, where they are used at up to 5 and 9 g L^{-1}, respectively (Codex Alimentarius Commission, 1981; McSweeney, 2008). These low molecular weight emulsifiers adsorb rapidly at the oil/water interface during homogenisation allowing the formation of small oil globules. They also interact with proteins adsorbed at the interface and in the serum phase reducing the availability of thiol groups at the oil/water interface and in the serum phase and limiting interactions between oil globules during heating (Euston, Finnigan, and Hirst, 2001; McCrae, 1999; McSweeney, Healy, and Mulvihill, 2008). CITREM and lecithin contain charged domains (anionic and zwitterionic, respectively) within their structures and they confer a charge to the surface of oil globules upon adsorption. Charged molecules promote electrostatic stabilisation of emulsions and the impact of globule charge on thermal stability of IF type emulsions have been well documented (Kasinos et al., 2013; McCarthy, Kelly, O'Mahony, and Fenelon, 2014).

Modification of protein by conjugation with carbohydrate (i.e., maltodextrin, dextran or pectin) by exploiting the early/intermediate stages of the Maillard reaction has been shown to improve the functional properties of different proteins. Conjugation of casein with maltodextrin has been reported to improve solubility, foaming and emulsification properties (Jiang and Zhao, 2011), freeze-thaw stability (O'Regan and Mulvihill, 2010a), encapsulation efficiency (O'Regan and Mulvihill, 2010b) and emulsion stability at acidic pH (Shepherd, Robertson, and Ofman, 2000). Conjugation of whey proteins with pectin improved emulsifying properties at neutral pH (Xu, Wang, Jiang, Yuan, and Gao, 2012) and acidic pH (Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, and Dewettinck, 2004); moreover, protection of sensitive, oil soluble compounds against oxidation was enhanced using whey protein-pectin conjugates (Xu et al., 2012). Modification of protein/peptides present in WPH

by conjugation can help alleviate issues encountered with stability in emulsions stabilised by hydrolysates; indeed, improved stability of model hydrolysed IF emulsions to thermal processing on conjugation has already been reported (Drapala et al., 2016).

The objective of this study was to identify addition levels of CITREM and lecithin required to produce emulsions with thermal stability similar to that measured for a WPH-maltodextrin (WPH-MD) stabilised emulsion. The performance of WPH-MD conjugates as emulsifiers in IF type emulsions will be compared with the performance of WPH plus added non-protein emulsifiers (i.e., CITREM and lecithin; added at the predetermined levels) used commercially in such products to determine if such conjugates can replace (at least partially) non-protein emulsifiers traditionally used in these products.

7.2. Materials and Methods

7.2.1. Materials

Whey protein hydrolysate (WPH; 8.0% degree of hydrolysis, DH) was obtained from Carbery Food Ingredients Ltd (Ballineen, Ireland) and had 86.3% protein (IDF Standard 20-1, 2014), 5.0% moisture (IDF Standard 26, 2004), 2.8% ash (IDF Standard 90, 1979), 0.7% fat (IDF Standard 9C, 1987) and 5.2% lactose (determined by difference). Maltodextrin (MD; Maldex 120 with a dextrose equivalent value of 12) was obtained from Corcoran Chemicals Ltd. (Dublin, Ireland) and had moisture and ash contents of <5.0% and <0.2%, respectively. The majority of the MD population had an average molecular weight of 5.9 kDa as determined by multiangle laser light scattering with size exclusion chromatography (Lucey, Srinivasan, Singh, and Munro, 2000). Soybean oil was obtained from Frylite Group Ltd (Strabane, UK). CITREM (Grindsted® CITREM N12) was obtained from Dupont Nutrition Biosciences ApS (Braband, Denmark) and de-oiled powdered soybean lecithin (Ultralec® P) was obtained from ADM (Decatur, IL, USA). All other chemicals and reagents used in the study were of analytical grade and sourced from Sigma-Aldrich (Dublin, Ireland).

7.2.2. Stock protein solutions and conjugation

A stock protein solution (50.0 g L^{-1}, protein; pH 6.8) was prepared with WPH as detailed by Drapala et al. (2016). In brief, the WPH-MD conjugate solution was prepared by heating a WPH-maltodextrin solution (50.0 g L^{-1} protein, 50.0 g L^{-1} maltodextrin; pH 8.2) at 90°C for 8 h (Mulcahy, Mulvihill, and O'Mahony, 2015; Mulcahy, Park, Drake, Mulvihill, and O'Mahony, 2016). The stock protein solution (non-conjugated) was subsequently used to formulate emulsions containing different levels (0-9 g L^{-1}) of either CITREM or lecithin and the stock conjugate solution was used to formulate WPH-MD conjugate-based emulsions.

7.2.3. Preparation of emulsions

Model infant formula emulsions containing 15.5, 35.0 and 70.0 g L^{-1} protein, oil and total maltodextrin, respectively, were prepared from stock WPH or stock WPH-MD conjugate solutions essentially as detailed by Drapala et al. (2015, 2016). Non-protein emulsifiers (CITREM or lecithin) were added to emulsions prepared from the stock WPH solution. For emulsions containing CITREM, the CITREM (0-9 g L^{-1}; dissolved in ultrapure water at 65°C) was added to the aqueous phase prior to mixing with the oil phase. For emulsions containing lecithin, the lecithin (0-9 g L^{-1}; dissolved in soybean oil at 65°C) was added to the oil phase prior to mixing with the aqueous phase. For emulsions containing WPH-MD conjugate, all protein was provided by the stock conjugate solution and the MD was added to reach the target concentration (i.e., 70 g L^{-1}). Aqueous and oil phases of emulsions were mixed together at 50°C and then pre-homogenised with an Ultra-Turrax (T25, IKA-Werke GMBH and Co. KG, Staufen, Germany) at 10,000 rpm for 2 min followed by two stage homogenisation (double pass) at 15 and 3 MPa, using a valve homogeniser (APV GEA Niro-Soavi S.p.A., Parma, Italy) at 50°C. Following homogenisation, the pH of emulsions was measured and, if needed, readjusted to pH 6.8 with 0.1 N HCl or 0.1 N KOH. Emulsion aliquots used for accelerated stability testing had sodium azide (0.50 g L^{-1}) added to prevent microbial growth.

7.2.4. Fat globule size distribution and zeta potential

Fat globule size distribution (FGSD) of the emulsions was measured using a laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK) equipped with a 300 RF (reverse fourier) lens, an LED light source (λ of 470 nm) and a He-Ne laser (λ of 633 nm). A polydisperse model with particle and dispersant refractive index of 1.46 and 1.33, respectively, were selected for data analysis (McCarthy et al., 2012). Samples were introduced to the mixing chamber and dispersed in ultrapure water until a laser obscuration of 5-8% was reached and three readings were taken for each sample. FGSD was measured within 1 h post homogenisation (d 0), immediately post heating and after 3, 6, 8 and 10 d of accelerated storage at 40°C. The zeta potential (ζ) of oil globules in emulsions was measured using a Zetasizer Nano-ZS (Malvern Instruments), as detailed by Drapala et al. (2016).

7.2.5. Screening of thermal stability of emulsions

Model IF emulsions stabilised by WPH-MD conjugate, WPH + CITREM (0-9 g L^{-1}) or WPH + lecithin (0-9 g L^{-1}) were heat treated at 95°C for 15 min. Thermal stability of these emulsions was assessed by changes in FGSD of emulsions after heat treatment as compared to the FGSD measured immediately post homogenisation. The heat treatment (95°C for 15 min using an oil bath) was used to initially screen the thermal stability of emulsions containing lower levels of non-protein emulsifiers (0-5 g L^{-1}; CITREM or lecithin) in order to identify very unstable samples. Emulsions stabilised by WPH + CITREM or WPH + lecithin (5-9 g L^{-1}) were heated (fresh aliquots) under controlled conditions with an AR-G2 controlled stress rheometer (TA Instruments, Crawley, UK) equipped with a starch pasting cell (SPC). The thermal treatment applied to the samples was as detailed by Drapala et al. (2016) and involved heating to 95°C, holding for 15 min at peak temperature and cooling to 15°C with constant shear-rate (15 s^{-1}). Apparent viscosity was recorded at 1 s intervals during heating, holding and cooling. Emulsion samples were recovered after all heat treatments (i.e., from oil bath tubes and from the SPC) and their thermal stability was assessed by visual observation and analysis of FGSD, as described in Section 7.2.4. Addition levels of CITREM

and lecithin required to obtain thermal stability equivalent to that measured for the WPH-MD sample were identified. The three emulsion systems evaluated in the remainder of this study are referred to as conjugate-based emulsion (CON_e), CITREM containing emulsion (CIT_e) and lecithin containing emulsion (LEC_e).

7.2.6. Assessment of properties of emulsions

7.2.6.1. Determination of composition of emulsions

The chemical composition (i.e., total solids, protein, ash and carbohydrate content) of the CON_e, CIT_e, and LEC_e emulsions was determined using the methods detailed for the WPH ingredients in Section 7.2.1. The fat content of emulsions was determined using the Gerber method (IDF Standard 105, 2008). The carbohydrate content of emulsions was calculated by difference.

7.2.6.2. Determination of apparent viscosity of emulsions

Viscosity of CON_e, CIT_e and LEC_e emulsions post homogenisation and post heating at 95°C for 15 min was measured using a rotational viscometer (Haake RotoVisco 1 Rotational Viscometer, Thermo Fisher Scientific, MA, USA) equipped with a cylindrical double gap cup and rotor (DG43, Thermo Fisher Scientific, MA, USA) as described by Mulcahy et al. (2015). The average apparent viscosity at 300 s^{-1} of each emulsion was determined at 20°C (± 0.1°C).

7.2.6.3. Accelerated storage stability testing of emulsions

To determine stability of unheated CON_e, CIT_e and LEC_e emulsions to accelerated storage, aliquots (50 mL) were transferred to plastic containers, sealed and incubated at 40°C. FGSD of the emulsions was measured after 3, 6, 8 and 10 d storage. A parallel experiment was carried out to determine the stability of heated (95°C for 15 min) CON_e, CIT_e and LEC_e emulsions to accelerated storage (10 d at 40°C).

7.2.6.4. Accelerated creaming stability testing of emulsions

Creaming velocities of unheated and heated CON_e, CIT_e and LEC_e emulsions were measured using an analytical centrifuge (LUMiSizer, L.U.M. GmbH, Berlin, Germany). The principle of analysis by LUMiSizer has been detailed by Lerche and Sobisch (2011). Stability of emulsions to creaming was determined at 23°C and 563 g for 8.5 h as detailed by Shimoni, Shani Levi, Levi Tal and Lesmes (2013). Creaming velocity was calculated from front tracking profiles as detailed by Lerche and Sobisch (2011).

7.2.6.5. Microstructural analysis of emulsions

The microstructural analysis of emulsions was performed using a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems, Heideberg GmbH, Mannheim, Germany) as detailed by Drapala et al. (2015). In brief, protein and lipid were fluorescently labelled with Nile Blue dye and visualisation in emulsions was carried out using He-Ne (633 nm) and Ar (488 nm) lasers for protein and lipid, respectively. The observations were performed using a 63× oil immersion objective. At least three specimens of each emulsion were observed to obtain representative micrographs of samples.

7.2.7. Statistical analysis

All emulsions were prepared in three independent trials and all measurements were carried out in at least duplicate. Analysis of variance (ANOVA) was carried out using the Minitab® 16 (Minitab Ltd, Coventry, UK, 2010) statistical analysis package. The Tukey HSD test was used to obtain grouping information. The level of significance was determined at $P < 0.05$.

7.3. Results

7.3.1. Influence of emulsifier type and concentration on thermal stability of emulsions

Thermal stability results for emulsions stabilised by WPH and different levels (0-9 g L^{-1}) of CITREM or lecithin and by the WPH-MD conjugate are shown in

Table 7.1. Emulsions containing low to intermediate levels of lecithin (1-5 g L^{-1}) displayed poor stability to thermal processing at 95°C for 15 min. Extensive heat-induced coagulation was observed in the emulsion containing 1 g L^{-1} lecithin, where protein/peptide aggregates and a distinct serum phase were observed in the sample after heat treatment. No coagulation was observed at lecithin addition levels > 1 g L^{-1}; however, destabilisation of emulsions containing low to intermediate levels (2-5 g L^{-1}) of lecithin was observed, as evidenced by the presence of relatively large aggregates in the emulsions after heating. Increasing the lecithin concentration in emulsions improved their thermal stability and, at ≥ 5 g L^{-1} lecithin, no extensive destabilisation was observed. Emulsions containing ≥ 5 g L^{-1} lecithin were heat treated at 95°C for 15 min in the SPC. Similar to the emulsions heated in the oil bath, the destabilising effects of thermal processing decreased as the level of lecithin increased. The presence of aggregates in the heated emulsions was observed at up to 7 g L^{-1} lecithin addition. Emulsions containing ≥ 8 g L^{-1} lecithin had mean volume diameter of oil globules < 1 μm, which is generally an indicator of a physically stable emulsion (McCarthy et al., 2012; Drapala et al., 2015). Lecithin-containing emulsions that were heated in the SPC displayed a tendency to foul the cell (i.e., deposit a protein/oil layer on the metal surface they were in contact with during thermal processing). Fouling decreased with increasing lecithin level; however, it was still observed (although to a limited degree) even at the highest level (i.e., 9 g L^{-1}) of lecithin addition (Table 7.1). A lecithin addition level of 9 g L^{-1} was identified as being able to provide best thermal stability to the WPH-based emulsion, essentially equivalent to that of an emulsion stabilised by the WPH-MD conjugate.

Emulsions containing low (1-4 g L^{-1}) levels of CITREM displayed poor stability to heating at 95°C for 15 min and coagulation was observed in emulsions containing 1 and 2 g L^{-1} CITREM. The presence of large aggregates without coagulation was observed in emulsions containing 3 and 4 g L^{-1} CITREM and the $D_{4,3}$ of particles decreased by ~50% on increasing the level from 3 to 4 g L^{-1} (Table 7.1). Similar to lecithin-containing emulsions, samples containing 5-9 g L^{-1} CITREM were heated in the SPC. Formation of aggregates was observed in emulsions containing up to 7 g L^{-1} CITREM; however, unlike

Table 7.1. Influence of lecithin or CITREM addition level on stability of model whey protein hydrolysate (WPH)-based infant formula (IF) emulsions, pH 6.8 containing 1.55, 3.50 and 7.00% protein, fat and carbohydrate, respectively, on heat treatment at 95°C for 15 min using an oil bath or a rheometer compared to the stability of a model IF emulsion stabilised by WPH-maltodextrin (WPH-MD) conjugate.

Mapping parameter	**WPH + Lecithin (g L^{-1})**									**WPH + CITREM (g L^{-1})**									**WPH-MD conjugate**
	1[a]	2[a]	3[a]	4[a]	5[ab]	6[b]	7[b]	8[b]	9[b]	1[a]	2[a]	3[a]	4[a]	5[ab]	6[b]	7[b]	8[b]	9[b]	
Coagulation	+[2]	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Aggregation	NA[3]	+++	++	++	++	+	+	-	-	NA	NA	+++	++	+	+	+	-	-	-
Fouling	NA	NA	NA	NA	+++	+++	++	++	+	NA	NA	NA	NA	-	-	-	-	-	-
$D_{4,3}$[1] (µm)	NA	117	96.7	73.7	17.5	3.42	1.22	0.96	0.90	NA	NA	119	58.8	3.99	0.98	0.81	0.70	0.62	0.79

[a-b] Heat treatment method: a) oil bath, b) rheometer equipped with a starch pasting cell (SPC)

[1] $D_{4,3}$; mean volume diameter of particles as measured by laser diffraction

[2] Symbols: '+' and '-' describe either presence or absence, respectively, of the corresponding mapping parameters in an emulsion sample after the heat treatment; higher number of '+' refers to higher magnitude of the corresponding parameter

[3] NA = not applicable

lecithin-containing emulsions, no fouling was observed in the SPC at any CITREM addition level. The emulsion containing 9 g L^{-1} CITREM displayed the highest thermal stability, the stability being similar to that observed for the emulsion stabilised by the WPH-MD, hence this level of the emulsifier was selected for subsequent analyses.

Model infant formula emulsions stabilised by the WPH-MD conjugate, WPH + CITREM (9 g L^{-1}) or WPH + lecithin (9 g L^{-1}) displayed good thermal stability to heating at 95°C for 15 min in the SPC. The effects of the heat treatment on selected emulsion properties were assessed by determining FGSD, apparent viscosity, stability to creaming, ζ potential and microstructural properties. Emulsions stabilised by WPH-MD conjugate, WPH + CITREM (9 g L^{-1}) and WPH + lecithin (9 g L^{-1}) will subsequently be referred to as CON_e, CIT_e and LEC_e emulsions, respectively, in this study.

7.3.2. Properties of emulsions

7.3.2.1. Composition of emulsions

Compositional analysis of CON_e, CIT_e and LEC_e emulsions showed that measured/calculated levels of protein (15.0, 15.1 and 15.4 g L^{-1}, respectively), fat (36.8, 36.7 and 37.3 g L^{-1}, respectively) and carbohydrate (64.5, 70.9 and 71.1 g L^{-1}, respectively) were sufficiently near target levels. The total solids content of the CON_e emulsion (117.5 g L^{-1}) was lower than in the CIT_e and LEC_e emulsions (123.8 and 125.2 g L^{-1}, respectively), due to the presence of non-protein emulsifiers in addition to the target protein content in the CIT_e and LEC_e emulsions.

7.3.2.2. Size and charge of oil globules in emulsions

CON_e, CIT_e and LEC_e emulsions had narrow particle size distributions (Fig. 7.1) and mean volume diameter ($D_{4,3}$) of 0.79, 0.62 and 0.72 μm (Table 7.2), respectively, immediately post homogenisation. The CON_e and CIT_e emulsions displayed good stability to heat treatment at 95°C for 15 min as indicated by no significant changes in the $D_{4,3}$, $D_{3,2}$ (Sauter mean diameter), $D_{v,0.5}$ and $D_{v,0.9}$ (fat globule size in the 50% and 90% quantiles of the distribution, respectively)

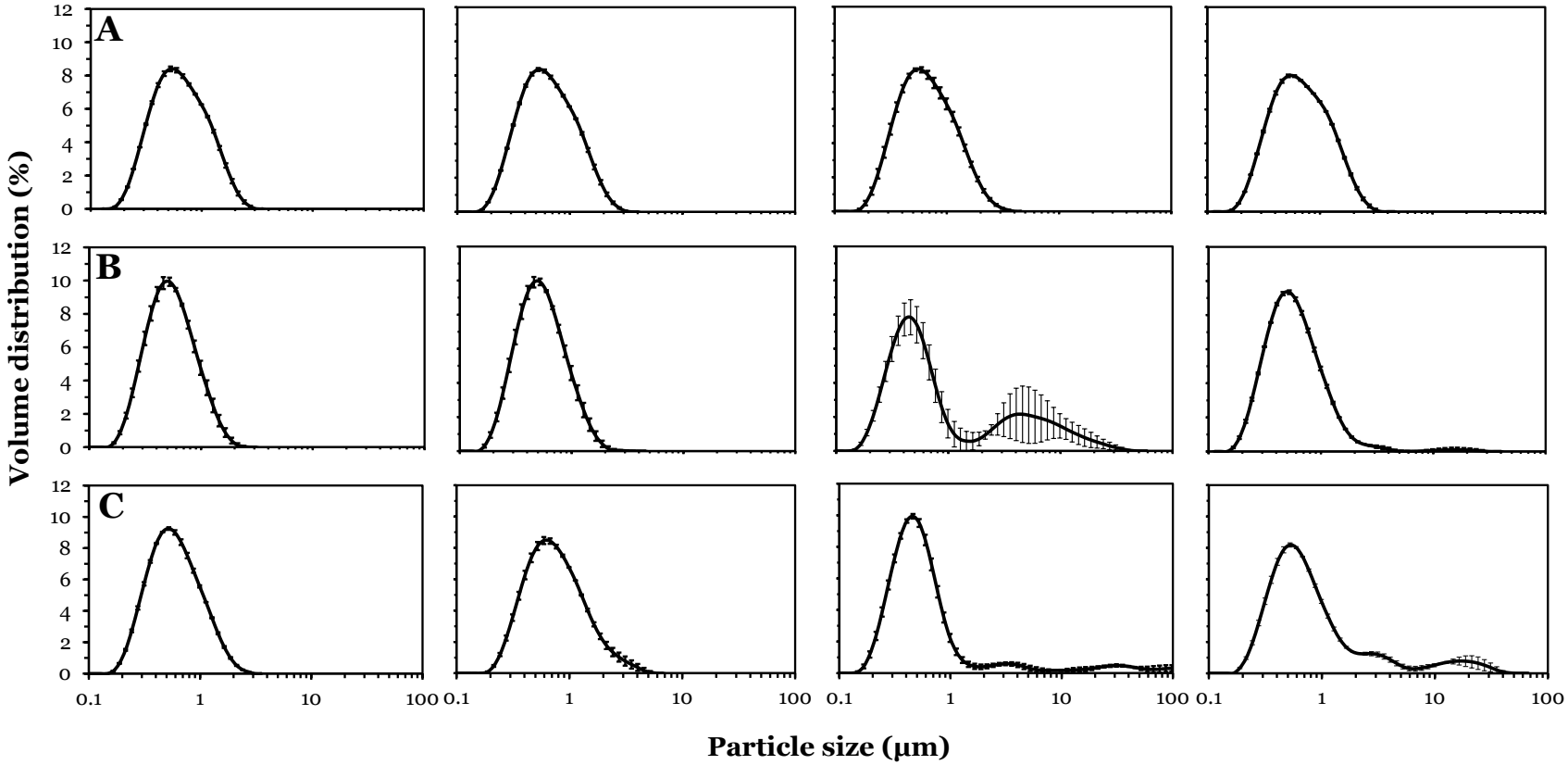

Figure 7.1. Fat globule size distribution profiles of model whey protein hydrolysate (WPH)-based infant formula emulsions stabilised by (A) WPH-maltodextrin conjugate and (B) WPH + CITREM at 9 g L^{-1} or (C) WPH + lecithin at 9 g L^{-1} (horizontally, left to right) post homogenisation, after heat treatment (95°C x 15 min), after 10 d of storage at 40°C of unheated emulsions and after 10 d of storage at 40°C of heated emulsions. Large error bars observed for the unheated CIT_e emulsion after 10 d of storage reflect a large variability in the extent of destabilisation of the emulsions, however, the same trend was observed for the sample for all 3 independent trials.

Table 7.2. Fat globule size distribution and zeta potential (ζ) of model whey protein hydrolysate (WPH)-based infant formula emulsions stabilised by WPH-maltodextrin conjugate (CON_e), WPH + CITREM (9 g L^{-1}; CIT_e) or WPH + lecithin (9 g L^{-1}; LEC_e) post homogenisation, post heating at 95°C for 15 min and after an accelerated storage at 40°C for 10 d post homogenisation of unheated and heated emulsions.

Emulsion	Measurement stage	Fat globule size parameter (µm)				ζ Potential (mV)	Apparent viscosity (mPa.s)	Creaming velocity (mm d^{-1})
		$D_{4,3}$ [1]	$D_{3,2}$ [2]	$D_{v,0.5}$ [3]	$D_{v,0.9}$ [4]			
CON_e	Post homogenisation	0.79 ± 0.02^{a}	0.57 ± 0.01^{a}	0.67 ± 0.02^{a}	1.43 ± 0.03^{a}	- 53.3 ± 0.54^{A}	1.86 ± 0.19^{A}	0.28 ± 0.03^{A}
	Heated at 95°C, 15 min	0.79 ± 0.01^{a}	0.57 ± 0.02^{a}	0.66 ± 0.01^{a}	1.44 ± 0.03^{a}	- 50.7 ± 0.69^{A}	1.90 ± 0.12^{A}	0.24 ± 0.03^{AB}
	Accelerated storage: unheated	0.82 ± 0.02^{a}	0.60 ± 0.02^{a}	0.67 ± 0.02^{a}	1.50 ± 0.04^{ab}	n.d.[5]	n.d.	n.d.
	Accelerated storage: heated	0.83 ± 0.02^{a}	0.62 ± 0.02^{a}	0.69 ± 0.01^{a}	1.55 ± 0.02^{b}	n.d.	n.d.	n.d.
CIT_e	Post homogenisation	0.62 ± 0.04^{a}	0.49 ± 0.04^{a}	0.54 ± 0.03^{a}	1.07 ± 0.08^{a}	- 57.7 ± 0.34^{B}	2.15 ± 0.26^{A}	0.18 ± 0.05^{B}
	Heated at 95°C, 15 min	0.62 ± 0.04^{a}	0.49 ± 0.03^{a}	0.54 ± 0.03^{a}	1.07 ± 0.08^{a}	- 53.1 ± 0.83^{A}	2.45 ± 0.05^{B}	0.06 ± 0.02^{C}
	Accelerated storage: unheated	2.86 ± 1.22^{b}	0.62 ± 0.11^{a}	0.67 ± 0.15^{a}	7.81 ± 4.84^{a}	n.d.	n.d.	n.d.
	Accelerated storage: heated	0.88 ± 0.14^{a}	0.55 ± 0.04^{a}	0.60 ± 0.03^{a}	1.31 ± 0.06^{a}	n.d.	n.d.	n.d.
LEC_e	Post homogenisation	0.72 ± 0.00^{a}	0.52 ± 0.04^{a}	0.58 ± 0.04^{a}	1.21 ± 0.11^{a}	- 52.3 ± 0.83^{A}	2.01 ± 0.21^{A}	0.22 ± 0.05^{AB}
	Heated at 95°C, 15 min	0.90 ± 0.11^{a}	0.62 ± 0.10^{a}	0.72 ± 0.07^{b}	1.65 ± 0.26^{a}	- 49.7 ± 2.13^{A}	2.20 ± 0.03^{AB}	0.17 ± 0.05^{B}
	Accelerated storage: unheated	5.36 ± 1.98^{b}	0.51 ± 0.01^{a}	0.53 ± 0.01^{a}	8.29 ± 9.22^{a}	n.d.	n.d.	n.d.
	Accelerated storage: heated	2.71 ± 0.75^{ab}	0.64 ± 0.03^{a}	0.72 ± 0.03^{b}	4.98 ± 1.46^{a}	n.d.	n.d.	n.d.

[1-4] Fat globule size distribution parameters: 1) $D_{4,3}$, volume mean diameter; 2) $D_{3,2}$, Sauter mean diameter; 3) $D_{v,0.5}$, fat globule size in the 50% quantile of the distribution; 4) $D_{v,0.9}$, fat globule size in the 90% quantile of the distribution.

[5] n.d. = not determined

[(a-b)] FGSD values for a given emulsion within a column not sharing a common superscript differed significantly ($P < 0.05$)

[(A-B)] ζ potential, apparent viscosity and creaming velocity values for a measurement stage for each emulsion within a column not sharing a common superscript differed significantly ($P < 0.05$)

and no change in the FGSD profiles post heating compared to post homogenisation (Table 7.2; Fig. 7.1). A limited increase in the $D_{v,0.5}$ and broadening of the size distribution profile was observed for the LEC_e emulsion post heating at 95°C for 15 min (Table 7.2; Fig. 7.1, C2). However, no significant changes were measured for all the other FGSD parameters and the $D_{4,3}$ of the LEC_e emulsion remained < 1 μm after heating, also indicating good heat stability. Zeta potential values showed that oil globules in freshly prepared CIT_e emulsion had significantly higher net negative charge (-57.7 mV) compared to the CON_e (-53.3 mV) and LEC_e (-52.3 mV) emulsions (Table 7.2). Heating at 95°C for 15 min reduced the ζ of oil globules in emulsions by 4.6, 2.6 and 2.6 mV for CIT_e, CON_e and LEC_e emulsions, respectively. No significant differences in the ζ were found between heated CIT_e, CON_e and LEC_e emulsions.

7.3.2.3. Apparent viscosity of emulsions

No significant differences in viscosity were observed for CON_e, CIT_e and LEC_e emulsions immediately post homogenisation (Table 7.2). Viscosity of all emulsions increased on heat treatment at 95°C for 15 min. Viscosity of the heated CIT_e emulsion was found to be significantly higher than the viscosity of heated CON_e emulsion; no significant differences were observed between heated CON_e and LEC_e and between heated CIT_e and LEC_e emulsions.

7.3.2.4. Accelerated storage stability of emulsions

The unheated CON_e emulsion displayed excellent stability over the 10 d accelerated storage at 40°C with no changes observed in the $D_{4,3}$, $D_{3,2}$ and $D_{v,0.5}$ or in the FGSD profiles (Fig. 7.1, A1 and A3; Table 7.2); a marginal increase (i.e., 0.07 μm) in the $D_{v,0.9}$ was observed for the unheated CON_e emulsion on accelerated storage. An increase in $D_{4,3}$ to 2.86 μm and a shift in the size distribution profile from monomodal to bimodal (Fig. 7.1, B1 and B3; Table 7.2) was observed for the unheated CIT_e emulsion after 10 d of accelerated storage. Particle size parameters $D_{3,2}$, $D_{v,0.5}$ and $D_{v,0.9}$ for the unheated CIT_e emulsion also increased after storage compared to post homogenisation; however, the increases were not found to be significant due to large standard errors observed for the stored emulsion (Table 7.2). The

unheated LEC_e emulsion displayed the least stability to accelerated storage; $D_{4,3}$ increased to 5.36 μm and the presence of a small number of large (~30 μm) oil globules was observed on the FGSD profile (Fig. 7.1, C3). An increase in $D_{v,0.9}$ was observed for the unheated LEC_e emulsion after the storage compared to post homogenisation; however, the difference was not found to be significant due to large standard error observed for the stored emulsion (Table 7.2). Additionally, a complete phase separation, evidenced by the presence of free oil floating on top of the emulsion in the container, was observed as early as 6 d into the storage of the unheated LEC_e emulsion. No phase separation was observed for either the unheated CON_e or CIT_e emulsions over the 10 d of storage at the accelerated conditions.

The stability of heated (95°C for 15 min) emulsions to accelerated storage (10 d at 40°C) was also determined and the results were similar to the unheated systems. A marginal increase in the $D_{4,3}$, $D_{3,2}$ $D_{v,0.5}$ and $D_{v,0.9}$ were measured for the heated CON_e and CIT_e emulsions after 10 d storage compared to FGSD values immediately after heat treatment (Table 7.2). No differences in the FGSD profiles were observed for the heated CON_e emulsion after the storage compared to post heating (Fig. 7.1, A2 and A4). A marginal broadening of the FGSD profile was observed for the heated CIT_e emulsion after 10 d storage compared to post heating (Fig. 7.1, B2 and B4). Following the trend observed for the unheated emulsions, the biggest changes on accelerated storage were observed for the heated LEC_e emulsion. Large (~20 μm) oil globules were present in the heated LEC_e emulsion after 10 d storage (Fig. 7.1, C4). The $D_{4,3}$ and $D_{v,0.5}$ for the heated LEC_e emulsion increased after the 10 d of storage compared to after heat treatment; however, this increase was not found to be significant due largely to variability in data observed for $D_{4,3}$ and $D_{v,0.9}$ (Table 7.2). No differences were found in the $D_{3,2}$ and $D_{v,0.5}$ after the accelerated storage compared to values measured immediately after the heat treatment. Interestingly no free oil was observed for the heated LEC_e emulsion after 10 d storage.

7.3.2.5. Accelerated creaming stability of emulsions

Creaming velocity measured immediately post homogenisation was highest for the CON_e emulsion followed by the LEC_e and CIT_e emulsions (Table 7.2). However, all emulsions displayed creaming velocity below 1 mm d^{-1}, which is considered an indicator of good stability to creaming in oil in water (O/W) emulsions (Dickinson 1992; McClements 1999). A limited decrease in the creaming velocity was observed for the CON_e and LEC_e emulsions as a result of the heat treatment (95°C for 15 min) (Table 7.2); a significant ($P < 0.05$) decrease was observed for the CIT_e emulsion after heating.

7.3.2.6. Microstructural analysis of emulsions

Microstructural analysis of the emulsions showed that all samples had fine and uniformly distributed oil globules immediately post homogenisation (Fig. 7.2). Similarly, a homogenous distribution of small (~ 1 μm) oil globules was observed in all emulsions after the heat treatment at 95°C for 15 min. Microstructural analysis showed that the heat treatment resulted in an increased number of protein aggregates/complexes in the serum phase of the emulsions; this observation was especially pronounced in the CIT_e emulsion (Fig. 7.2 B, panel 2).

Pronounced differences in the microstructure were observed between unheated CON_e, CIT_e and LEC_e emulsions after the 10 d storage at 40°C. No changes in the microstructure were observed for the unheated CON_e emulsion after 10 d storage compared to post homogenisation. Relatively large (i.e., 5-10 μm) oil globules were present in the unheated CIT_e emulsion and still larger globules (i.e., 10-20 μm) in the unheated LEC_e emulsion (Fig. 7.2 B, panel 3; and C, panel 3, respectively) after 10 d storage. Additionally, large (1-2 μm) protein complexes were observed in the unheated CIT_e emulsion after 10 d of storage at 40°C.

Results for the accelerated storage of the heated CON_e emulsion showed no evident increase in the size of oil globules and no obvious differences in the microstructure of the emulsion compared to post homogenisation (Fig. 7.2 A panel 4). The heated CIT_e emulsions also displayed a good stability to

accelerated storage and no evident changes resulting from the storage were observed in the heated CIT_e emulsion (Fig. 7.2 B, panel 4). Large (i.e., 5-15 µm) oil globules were observed for the heated LEC_e emulsion after 10 d storage at 40°C (Fig. 7.2 C, panel 4).

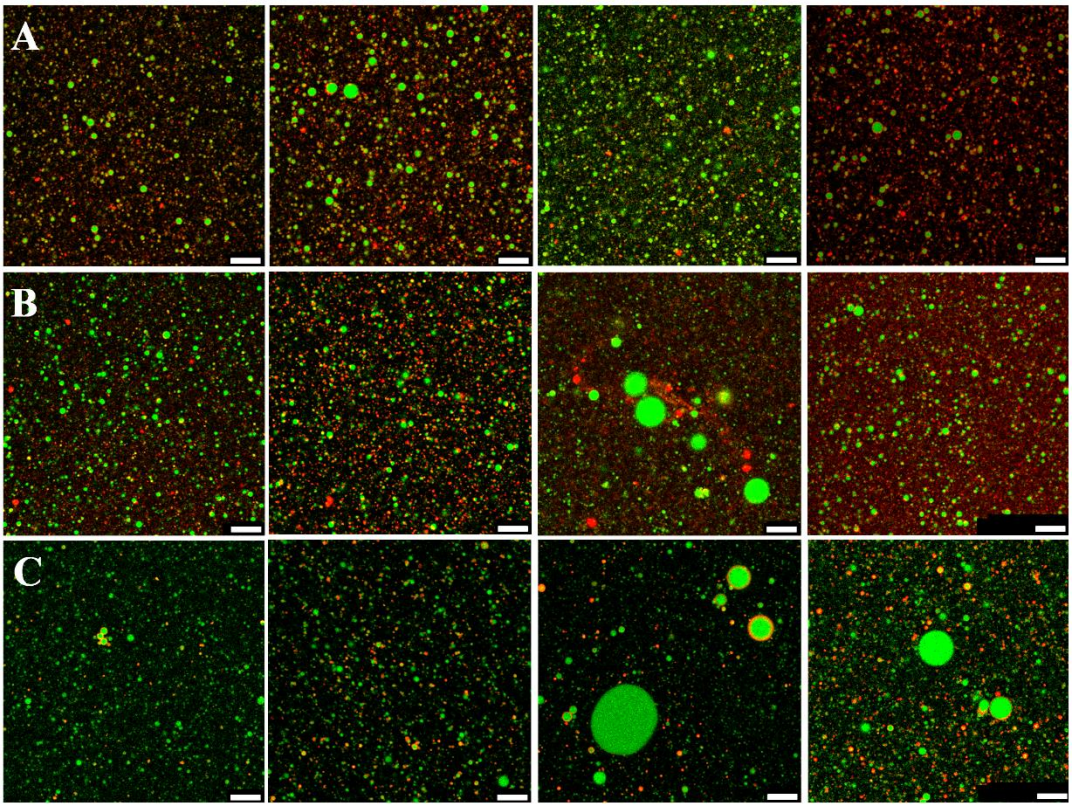

Figure 7.2. Confocal laser scanning microscopy images of model whey protein hydrolysate (WPH)-based infant formula emulsions stabilised by (A) WPH-maltodextrin conjugate and (B) WPH + CITREM at 9 g L^{-1} or (C) WPH + lecithin at 9 g L^{-1} (horizontally, left to right) post homogenisation, after heat treatment (95°C x 15 min), after 10 d of storage at 40°C of unheated emulsions and after 10 d of storage at 40°C of heated emulsions. Emulsions were labelled with Nile Blue and the micrographs show distribution of oil globules (green) and protein particles (red). Scale bar = 10 µm.

7.4. Discussion

The results presented in the current study show that the WPH-MD conjugate conferred excellent stability to IF type emulsion products containing hydrolysed whey protein. Emulsions stabilised by the WPH-MD conjugate displayed the greatest stability to thermal processing (95°C for 15 min) and

accelerated storage (40°C for 10 d), in both unheated and heated emulsions. There were marginal or no changes in the size distribution of oil globules in the WPH-MD conjugate stabilised emulsion, compared to emulsions stabilised by WPH + lecithin or WPH + CITREM (Fig. 7.1 and 7.2, Table 7.2). The superior stability of the CON_e emulsion can be attributed to the ability of the WPH-MD conjugate to enhance steric stabilisation of oil globules in an emulsion system. Upon adsorption of the surface active WPH-MD conjugate at the interface of oil globules during homogenisation, the carbohydrate, a hydrophilic component of the conjugate, protrudes into the serum phase of the emulsion, in effect, increasing thickness of the interfacial layer, conferring enhanced steric stabilisation and limiting interactions between oil globules (Kasran, Cui, and Goff, 2013; Wong, Day, and Augustin, 2011). Additionally, it is proposed that the covalent attachment of the MD to WPH reduces the potential of the interfacial protein/peptide-MD layer to interact with protein/peptides in the serum. This is due to a physical restriction of access to the interfacial protein/peptides that are in close proximity to the covalently attached carbohydrate (i.e., space interference). Such space interference can effectively improve thermal stability of WPH-based emulsions, where protein/peptide mediated bridging flocculation is a common processing challenge (Dickinson 2001; Drapala et al., 2016; McSweeney, Mulvihill, and O'Callaghan, 2004; Ye and Singh, 2006).

WPH-MD conjugates can be used as an alternative ingredient for stabilizing WPH-based emulsions where the addition of non-protein emulsifiers (i.e., low molecular weight surfactants like CITREM and lecithin) can be, at least partially, replaced. Thus, competitive destabilisation, often observed in systems containing protein and low molecular weight surfactants, could be avoided (Kaltsa, Paximada, Mandala and Scholten, 2014; Wilde, Mackie, Husband, Gunning and Morris, 2004). This destabilisation, which takes place during storage of emulsions containing protein/peptides and low molecular weight surfactants, can result in a non-continuous interfacial layer with certain regions dominated by protein/peptides and others by the surfactant (Drapala et al., 2015). Such unordered structure of the interface can in effect promote coalescence (Tirok, Scherze and Muschiolik, 2001) and, under particularly

adverse conditions, result in phase separation. Hofman and Stein (1991) and Mezdour, Lepine, Erazo-Majewicz, Ducept and Michon (2008) have reported a detrimental effect of lecithin on emulsion stability, which was linked to reduced interfacial tension and, effectively, and decreased rigidity and strength of the interfacial layer. Coalescence, evidenced by the presence of large oil globules, was observed in the unheated CIT_e and unheated and heated LEC_e emulsions, while both the unheated and heated CON_e emulsions displayed resistance to coalescence during accelerated storage.

The different extents of emulsion instability observed for unheated emulsions containing the low molecular weight surfactants CITREM or lecithin can be explained by the ability of the ionic surfactant CITREM to interact and form ternary complexes with polysaccharides and protein (Antipova, Semenova, Belyakova, Il'in, 2001; McSweeney 2008; Semenova, Myasoedova and Antipova, 2001) (Fig. 7.2 B). Formation of such complexes may have curtailed the mobility of the CITREM in the serum phase during storage and limited its role in the competitive destabilisation at the emulsion interfaces, thus, enhancing the stability of the CITREM containing emulsion compared to the lecithin containing emulsion. A positive effect of the ternary complexes formed by CITREM and polysaccharides-protein on the emulsion stability was observed when the emulsions were heated. It is proposed that on heating, formation of the complexes and limited protein/peptide aggregation, provide a synergistic stabilising effect; the presence of these combined complexes and aggregates at the surfaces of oil globules may have contributed to steric stabilisation. Additionally, the number of CITREM molecules in the serum phase, that would potentially be available to displace protein/peptides at the interfaces, is decreased.

Electrostatic repulsion also has a role to play in stabilisation of oil globules in O/W emulsions against undesirable globule-globule interactions. Modification of WPH by conjugation with MD affects the charge on proteins/peptides as positively charged ε-amino groups of the lysine residues are blocked by the covalently attached MD (Acedo-Carrillo et al., 2006; Liu, Ru, and Ding, 2012). It has been shown by Drapala et al. (2016) that oil globules in emulsions stabilised by a WPH-MD conjugate displayed greater

negative charge compared to those stabilised by WPH alone. The interactions between protein at the emulsion interfaces and protein in the emulsion serum phase have been widely reported and reviewed (Raikos, 2010); these interactions affect not only the structural arrangement at the interfaces but also impact on the charge of the oil globules. The results presented in the current study showed that the initial differences in the ζ between CON_e, CIT_e and LEC_e emulsions measured post homogenisation were diminished after heat treatment (Table 7.2). Reported reduction in the ζ of oil globules in all emulsions as a result of heat treatment can be explained by interactions between proteins/peptides at the surface of oil globules and proteins/peptides in the serum phase and by a rearrangement of emulsifiers at the interface. The CIT_e emulsions displayed a bigger reduction in ζ after the heat treatment, compared to the other emulsions. This indicates increased interactions (i.e., through combined complex formation and aggregation) at the oil-water interfaces and supports the concept of CITREM-based complexes playing a role in steric stabilisation. In the current study, the differences in the ζ of oil globules in the heated CON_e, CIT_e and LEC_e emulsions were insignificant, however, major differences in the storage behaviour were observed for these emulsions. The best shelf life stability displayed by the CON_e emulsion compared to the other emulsions was linked directly to the properties of the interfacial layer of oil globules in the CON_e emulsion with the most effective steric hindrance and absence of the competitive destabilisation observed for the CIT_e and LEC_e emulsions.

7.5. Conclusions

The results presented in the current study show that the performance of WPH-MD conjugates in stabilising model WPH-based IF emulsions was superior to that observed for emulsions stabilised by WPH + CITREM (9 g L^{-1}) or WPH + lecithin (9 g L^{-1}). The greater thermal and storage stability of the emulsion stabilised by the WPH-MD conjugate is attributed to enhanced steric stabilisation of oil globules in the emulsion as a result of conjugation. Undesirable interactions between oil globules during heat treatment and accelerated storage were markedly reduced in the emulsion stabilised by the WPH-MD conjugate compared to emulsions stabilised by WPH with CITREM

or with lecithin. The novel WPH-MD conjugate emulsifiers can provide a valuable and highly functional alternative to the inclusion of non-protein emulsifiers in nutritional formulations.

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Chapter 8

Influence of emulsifier type on the spray drying properties of model infant formula emulsions

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Abstract

The objective of this study was to compare the drying performance and physicochemical properties of model infant formula (IF) emulsions containing 43, 96 and 192 g L^{-1} protein, oil and maltodextrin (MD), respectively, prepared using different emulsifier systems. Emulsions were stabilised using either whey protein isolate (WPI), whey protein hydrolysate (WPH; DH 8%), WPH + CITREM (9 g L^{-1}), WPH + lecithin (5 g L^{-1}) or WPH conjugated with maltodextrin (DE 12) (WPH-MD). Homogenised emulsions had 32% solids content and oil globules with mean volume diameter <1 μm. Powders were produced by spray drying with inlet and outlet temperatures of 170 and 90°C, respectively, to an average final moisture content of 1.3%. The extent of powder build-up on the dryer wall increased in the order; WPH-MD<<WPH≤WPI<WPH+LEC≤WPH+CIT. The same trend was observed for the extent of spontaneous primary powder agglomeration, as confirmed by particle size distribution profiles and scanning electron micrographs, where the WPH-MD and WPH+CIT powders displayed the least and greatest extent of agglomeration, respectively. Analysis of elemental surface composition of the powders showed that surface fat, protein and carbohydrate decreased in the order: WPH+CIT>WPH+LEC>WPH>WPH-MD>WPI, WPI>WPH>WPH-MD>WPH+LEC>WPH+CIT and WPH-MD>WPI>WPH >WPH+LEC>WPH+CIT, respectively. Additionally, differences in wettability, surface topography and oil globule distribution within the powder matrix and in reconstituted powders were linked to the emulsifier system used. Inclusion of the WPH-MD conjugate in the formulation of IF powder significantly improved drying behaviour and physicochemical properties of the resultant powder, as evidenced by lowest powder build-up during drying and greatest emulsion quality on reconstitution, compared to the other model formula systems.

8.1. Introduction

Protein-based added-value nutritional formulations have been gaining a significant share of the global food market over the last decade, especially those tailored for athletes, the elderly and infants; the total global market for these product types is predicted to exceed 100 billion USD by 2020. Formulations for such products generally contain protein (e.g., whey protein), oils rich in unsaturated fatty acids (i.e., blends of vegetable oils) and carbohydrates (e.g., maltodextrin) as the main components. Whey protein hydrolysate (WPH) is often used as a protein source in such nutritional formulae due to its desirable amino acid composition, high digestibility and rapid absorption in the gut (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, and Recio, 2014).

Modification of proteins *via* hydrolysis has been extensively studied, with reports on improvement in protein functionality in the areas of solubility, surface activity, foaming and emulsifying properties available in the scientific literature (Agboola and Dalgleish, 1996a, b; Banach, Lin, and Lamsal, 2013; Foegeding and Davis, 2011; Kilara and Panyam, 2003). However, incorporation of WPH into nutritional formulations such as powdered formulae or ready to drink products is often associated with processing and shelf life challenges such as protein/peptide-mediated bridging flocculation and coalescence, due to reduced steric stabilisation and increased number of exposed reactive sites, compared to formulations based on intact whey protein (Drapala, Auty, Mulvihill, and O'Mahony, 2016a, b; Euston, Finnigan, and Hirst, 2000; Hunt and Dalgleish, 1995). Irrespective of the format of the final product (i.e., liquid or powder), the formulations for both physical formats have to undergo a number of thermal treatments (e.g., pasteurisation, sterilisation, spray drying) as a liquid. Therefore, additional non-protein surface active components are often included in the formulation of WPH-based emulsions in order to improve their processing and shelf life stability; these surfactants are usually lipid-based emulsifiers, including lecithin or citric acid esters of mono- and di-glycerides (CITREM).

Spray drying is one of the most common processes used in the manufacture of dairy ingredients and nutritional products; rapid water removal results in increased product shelf life, reduced shipping and storage costs and provides the consumer with a convenient and stable product. In this complex process, multiple factors such as feed characteristics (e.g., composition and rheological properties), process parameters (e.g., atomiser type and fines return) and external factors (e.g., air humidity, temperature) significantly impact the drying performance and the physicochemical properties of the final product. The composition (i.e., the type and content of protein, carbohydrate, fat and emulsifier, total solids content) and properties (i.e., flow behaviour and viscosity) of the emulsion destined for spray drying have a strong influence on its drying properties; extensive scientific reports and reviews focusing on the effects these factors have on the properties of the resulting powders have been published (Adhikari, Howes, Wood, and Bhandari, 2009; Jayasundera, Adhikari, Aldred, and Ghandi, 2009; Ji et al., 2016; Kim, Chen, and Pearce, 2009; Millqvist-Fureby, Elofsson, and Bergenståhl, 2001; Taneja, Ye, Jones, Archer, and Singh, 2013; Vega and Roos, 2006; Vignolles, Jeantet, Lopez, and Schuck, 2007).

It is well established that there is a strong relationship between the surface composition of powder particles and their drying performance in addition to the properties (e.g., cohesiveness, shelf life) of the final product (Kelly, O'Mahony, Kelly, and O'Callaghan, 2014; Nijdam and Langrish, 2006; Sadek et al., 2015). In the production of high-fat powders, high surface fat content can lead to powder stickiness, low powder recovery (i.e., yield) and production down-time (i.e., due to powder build-up on the dryer walls) as well as poor shelf life and undesirable properties of the final product (i.e., lipid oxidation, caking, low solubility and dispersibility) (Paterson, Zuo, Bronlund, and Chatterjee, 2007). Surface composition of an emulsion-based powder is governed considerably by the emulsifier system used; upon atomisation, a new air/liquid interface is created and surface active components (i.e., protein, peptides, low molecular weight surfactants) present in the emulsion, migrate rapidly towards, and adsorb at, the new interface, effectively reducing the surface free energy and enhancing the thermodynamic stability of the system

(Munoz-Ibanez et al., 2016). Effectively, surfactants are over-represented at the droplet/powder particle surface, affecting in-process and in-application behaviour of these products, as exhibited by interactions of particles with the dryer wall and with other droplets/powder particles. Thus, a better understanding of the emulsifier system and its modification to tailor it to a specific formulation has an important role in increasing drying efficiency in producing a powder with desired properties.

Conjugation of milk proteins with carbohydrates through the Maillard reaction has been reported to produce emulsifiers with exceptional functionality, especially with respect to stability of emulsion to unfavourable thermal and/or storage conditions (Akhtar and Dickinson, 2003; Drapala et al., 2016a, b; Kasran, Cui, and Goff, 2013a, 2013b; O'Regan and Mulvihill, 2010a 2010b; Wooster and Augustin, 2006). WPH-maltodextrin (WPH-MD) conjugates have been shown to confer strong steric stabilisation to oil droplets, effectively limiting globule-globule interactions and preventing emulsion destabilisation (i.e., flocculation and/or coalescence) (Corzo-Martínez et al., 2011; Liu, Ma, McClements, and Gao, 2016).

There is potential for these conjugates to affect surface properties of spray-dried emulsions, effectively influencing their behaviour during drying and properties of the final product. Good interfacial barrier properties and inherent ability of WPH-MD conjugates to adsorb at the newly formed air/water interface (O'Mahony, Drapala, Mulcahy, and Mulvihill, 2017) can offer an ingredient capable of deterring interactions between atomised emulsion droplets/powder particles. However, currently there are no published studies reporting on the use of WPH-based conjugates in spray-dried emulsions nor on the properties of the resultant powders. This study aims to directly compare the spray drying performance and powder physical properties of spray-dried emulsions stabilised with different emulsifier systems, namely, conjugated whey proteins/peptides (WPH-MD), not conjugated whey proteins/peptides (WPI, WPH) and not conjugated WPH with the addition of low molecular weight lipid-based surfactants (i.e., WPH + CITREM and WPH + lecithin).

8.2. Materials and methods

8.2.1. Materials

Whey protein isolate (WPI) and whey protein hydrolysate (WPH; 8% degree of hydrolysis; DH) were obtained from Carbery Food Ingredients Ltd. (Ballineen, Co. Cork, Ireland). The WPI and WPH ingredients had protein contents of 87.2 and 83.7%, respectively, and ash contents of 2.76 and 2.92%, respectively, as reported by Drapala et al. (2016a). Maltodextrin (MD) was obtained from Corcoran Chemicals Ltd. (Dublin, Ireland) and had moisture and ash contents of <5.0% and <0.2%, respectively. Soybean oil was obtained from Frylite Group Ltd. (Strabane, Co. Tyrone, Northern Ireland). CITREM (Grindsted® CITREM N12) was obtained from Dupont Nutrition Biosciences ApS (Brabrand, Denmark) and de-oiled powdered soybean lecithin (Ultralec® P) was obtained from ADM (Decatur, IL, USA). All other chemicals and reagents used in the study were of analytical grade and sourced from Sigma-Aldrich (Arklow, Co.Wicklow, Ireland).

8.2.2. Preparation of emulsions

Emulsions ($_e$) for model infant formula (IF) powders ($_p$) were prepared at pH 6.8 using protein, soybean oil and maltodextrin in the ratios 1.0:2.3:4.5, respectively. The protein component was either WPI, WPH or WPH conjugated with MD in a wet heating process as detailed by Drapala et al. (2016a). Additionally, non-protein emulsifiers, citric acid esters of mono- and di-glycerides (CITREM; 9 g L^{-1}) and soybean lecithin (5 g L^{-1}) were incorporated into the formulation of selected IF emulsions destined for subsequent spray drying. Emulsions were prepared by dissolving oil-soluble components, where applicable, in soybean oil and water-soluble components in ultrapure water, followed by two-stage homogenisation (double pass) at 15 and 3 MPa, using a valve homogeniser (APV GEA Niro-Soavi S.p.A., Parma, Italy) at 50°C. All emulsions were prepared to a total solids (TS) target of 32% as measured with a rapid moisture analyser (HB43-S, Mettler-Toledo LLC, Columbus, OH, USA). In total, five emulsions based on WPI, WPH, WPH +

CITREM (WPH+CIT), WPH + lecithin (WPH+LEC) and WPH conjugated with maltodextrin (WPH-MD) were produced in the current study.

8.2.3. Spray drying of emulsions

Powders were produced from emulsions using a bench-top spray dryer (B-191, BÜCHI Labortechnik AG, Flawil, Switzerland) with a maximum evaporation capacity of 1.5 L H_2O h^{-1}. Inlet temperature was set at 170°C and outlet temperature was maintained at 90-95°C by controlling the aspirator power (i.e., in the range of 40-60 m^3 h^{-1}) and the feed flow rate (i.e., in the range 1.2-1.4 L h^{-1}). Effectively, drying temperatures were kept within the industry relevant range typical for IF manufacture by using high feed flow rate (95-100%) and relatively low aspirator power (80-90%); however, this was achieved at the expense of product yield (Fig. 8.1). The powders were collected in the collection chamber as detailed in Fig. 8.1, and transferred to zip-sealed low density polyethylene bags (VWR International, Leuven, Belgium), followed by vacuum packing in heat-sealed polyamide/polyethylene bags (Fispak Ltd., Dublin, Ireland) with a moisture permeability of 2.6 g m^{-2}.d. The powders were stored in the dark at ambient conditions (i.e., ~20°C) until further analyses within 4 weeks of spray drying. Powder recovery was calculated on a TS basis (i.e., [final powder product TS/feed liquid TS] ×100) from the total amount of powder obtained in the collection chamber. Losses on drying were due to unrecoverable powder, which stuck to the wall of the dryer main chamber or fell and accumulated at the base of the main chamber during spray drying (Fig. 8.1). Powder stickiness was visually assessed based on the extent of wall coating by powder in the cyclone, in order to provide information on particle cohesion arising from surface characteristics (Fig. 8.1).

8.2.4. Particle size distribution

Particle size distribution (PSD) of the emulsions immediately after homogenisation and after powder reconstitution (i.e., 12%, w/v) was measured using a laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) equipped with a 300 RF (reverse fourier) lens, an LED light source (λ of 470 nm) and a He-Ne laser (λ of 633 nm) as detailed by Drapala et al. (2016b). The size distribution of the model

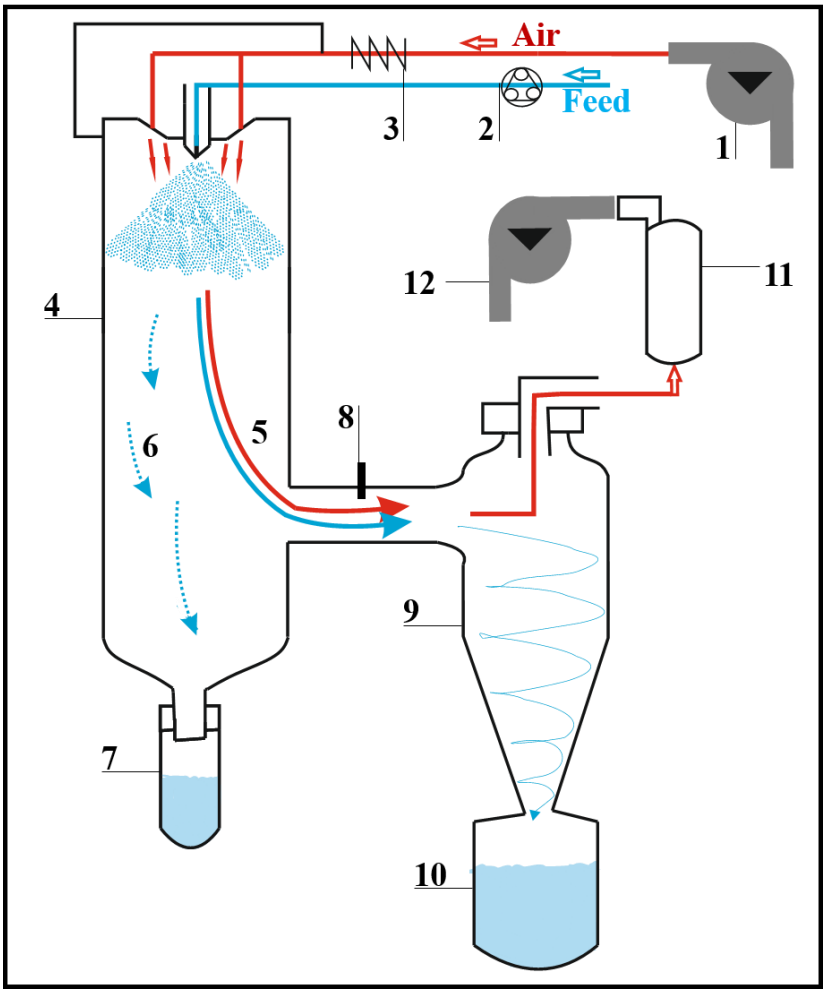

Figure 8.1. Schematic diagram showing the set-up and the principle of operation for the laboratory-scale BÜCHI B-191 spray drier. The inlet temperature is regulated directly by the power of the heater (3) and the outlet temperature (measured at 8) is regulated indirectly by controlling the feed flow rate (2) and the air flow (1). Feed is introduced into the main drying chamber (4) by a 2-fluid nozzle atomiser, where it is rapidly dried by heated air; dried particles are pulled into the cyclone (9) by means of an aspirator (12). Large and heavy particles (i.e., wet lumps and scorched particles, falling off the build-up around the nozzle and around hot air inlets, respectively) are separated from the powder by means of the air pull and gravity (5 and 6, respectively). By design, air pull is insufficient to move larger and heavier particles into the cyclone, making them fall into the waste collection container (7) at the bottom of the dryer main chamber. Dried powder particles are further separated from air in the cyclone and the final powder is collected in the powder collection container (10) at the bottom of the cyclone. The clarified air is exhausted at the top of the bag filter (11).

infant formula powders was measured using a Mastersizer 3000 equipped with a dry powder disperser cell (Aero S). Approximately 3.0 g of powder was placed in the feed hopper, containing a ball bearing to facilitate powder flow, with the feed pressure set at 1 bar, powder flow rate at 40-70% and the hopper height at 2 mm. All measurements were taken at 1-2% obscuration. The background and sample measurement duration was set at 20 s with the material refractive and absorption indexes of 1.46 and 0.01, respectively.

8.2.5. Rheological measurements

The apparent viscosity of emulsions was measured at 20°C using a rotational viscometer (Haake RotoVisco 1, Thermo Fisher Scientific, MA, USA) equipped with a cylindrical double gap cup and rotor (DG43, Thermo Fisher Scientific, MA, USA) as described by Mulcahy, Mulvihill and O'Mahony (2016). The shear rate was increased from 0 to 300 s^{-1} over 5 min, held at 300 s^{-1} for 2 min and decreased to 0 s^{-1} over 5 min; the average apparent viscosity was determined at 300 s^{-1} (η_{300}) for each emulsion. The power law applied to shear stress (τ) versus shear rate (γ) was used to obtain the flow behaviour parameters, consistency coefficient (K) and flow behaviour index (n) as detailed by Anema, Lowe, Lee, and Klostermeyer (2014). The flow behaviour index values are used to describe the flow behaviour of liquid samples where $n < 1$, $n > 1$ and $n = 1$ indicate shear-thinning, shear-thickening and Newtonian flow behaviour, respectively.

8.2.6. Composition and colour analyses of powders

The chemical composition of the model infant formula powders was determined using standard International Dairy Federation (IDF) methods as detailed by Drapala, Auty, Mulvihill, and O'Mahony (2015). Colour of the powders was measured using a pre-calibrated colorimeter (Minolta Chroma Meter CR-400, Minolta Ltd., Milton Keynes, U.K.) equipped with a granular materials attachment CR-A50. Colour was expressed using the Commission Internationale de l'Eclairage (CIE) colour chromaticity L* a* b* scale (L = dark/light, a = red/green, b = yellow/blue).

8.2.7. Powder wettability

The sessile drop goniometric method was used to determine the wettability of powders. All powders were compressed for 10 s at 78.4 MPa using a manual press (15 ton Manual Hydraulic Press, Specac Ltd., Orpington, UK) to form pellets (13 mm diameter); all pellets had a density of 1.08 (± 0.05) g cm^{-3}. Subsequently, the mean contact angle (θ) was determined directly using an optical tensiometer (Attension Theta, Biolin Scientific, Stockholm, Sweden); a drop (10 µl) of ultrapure water was formed and deposited on top of a powder pellet and the reduction in contact angle during the first 30 s was recorded using a high-resolution digital camera (15 frames per second) and processed using image analysis software (OneAttension, Biolin Scientific).

8.2.8. Surface composition of powders

The surface free fat content of powders was determined using the GEA Niro analytical method (GEA Niro, 2005) as described by McCarthy et al. (2013) with modified quantities of powder (5.0 g), petroleum ether (30 mL) and filtrate (15 mL) used. Elemental composition of powder surfaces was determined by X-ray photoelectron spectroscopy (XPS; Kratos Axis 165, Kratos Analytical, UK) as detailed by McCarthy et al. (2013). A matrix formula was used to calculate relative amounts of protein, fat and carbohydrate on the powder surface, as detailed by Fäldt, Bergenståhl, and Carlsson (1993).

8.2.9. Microstructure of powders

8.2.9.1. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) analysis of powder particles was performed using a confocal laser scanning microscope (TCS SP, Leica Microsystems CMS GmbH, Wetzlar, Germany). Powders were deposited onto a glass slide and excess sample was removed with compressed air. The powder samples were stained with a mixture (3:1) of Nile Red (0.10 g L^{-1} in polyethylene glycol) and Fast Green (0.01 g L^{-1} in water) fluorescent dyes (Sigma Aldrich, Wicklow, Ireland) to label the fat and protein components of the powders, respectively. Visualisation of oil and protein in the powders was

carried out using an Ar laser (excitation = 488 nm, emission = 500-530 nm) and He-Ne laser (excitation = 633 nm, emission = 650-700 nm), respectively. At least 3 representative images of each sample were taken using a 63× oil immersion objective.

8.2.9.2. Scanning electron microscopy

Scanning electron microscopy (SEM) analysis of powders was performed using a scanning electron microscope (JSM-5510, Jeol Ltd., Tokyo, Japan). Samples were mounted on double-sided carbon tape, attached to SEM stubs, and then sputter-coated with gold/palladium (10 nm; Emitech K550X, Ashford, UK). Representative micrographs were taken at 5 kV at 1000× (i.e., overview of powder population) and 3000× (i.e., shape and surface topography of powder particles) magnifications. At least three specimens of each sample were observed to obtain representative micrographs of samples.

8.2.10. Statistical data analysis

All powders were prepared in three independent trials and all measurements were carried out in at least duplicate. Analysis of variance (ANOVA) was carried out using the Minitab® 16 (Minitab Ltd., Coventry, UK, 2010) statistical analysis package. The Tukey method was used to obtain grouping information. The level of significance was determined at $P < 0.05$.

8.3. Results

8.3.1. Emulsion characteristics

The emulsions had TS levels ranging from 32.2 to 32.7% prior to spray drying (Table 8.1). Particle size analysis showed that all emulsions had oil globules with mean volume diameter ($D_{4,3}$) < 1 μm and no statistically-significant differences in $D_{4,3}$ were found between the emulsions (Table 8.1). Similarly, no significant differences in the apparent viscosity (η_{300}) were observed between WPI_e, WPH_e, $WPH+CIT_e$ and $WPH+LEC_e$ emulsions; however, the η_{300} for the $WPH-MD_e$ emulsion was significantly lower than that of the WPI_e, and $WPH+CIT_e$ emulsions (Table 8.1). Analysis of the flow behaviour showed no

Table 8.1. Characteristics of emulsions prepared using different emulsifiers; whey protein isolate (WPI_e), whey protein hydrolysate (WPH_e), WPH + CITREM ($WPH+CIT_e$), WPH + lecithin ($WPH+LEC_e$) and WPH-maltodextrin conjugate ($WPH\text{-}MD_e$), used to produce model infant formula powders.

		Emulsions				
Emulsion characteristics		**WPI_e**	**WPH_e**	**$WPH+CIT_e$**	**$WPH+LEC_e$**	**$WPH\text{-}MD_e$**
Solids content	(%, w/w)	32.6 ± 0.16[a]	32.2 ± 0.69 [a]	32.5 ± 0.10[a]	32.2 ± 0.04[a]	32.7 ± 0.18[a]
PSD[1] (μm)	$\mathbf{D_{4,3}}$	0.76 ± 0.05[a]	0.78 ± 0.14[a]	0.81 ± 0.21[a]	0.58 ± 0.06[a]	0.67 ± 0.05[a]
	$\mathbf{D_{v,0.1}}$	0.25 ± 0.07[a]	0.21 ± 0.04[a]	0.11 ± 0.07[a]	0.15 ± 0.01[a]	0.24 ± 0.05[a]
	$\mathbf{D_{v,0.5}}$	0.55 ± 0.06[a]	0.55 ± 0.01[a]	0.38 ± 0.08[a]	0.46 ± 0.12[a]	0.55 ± 0.03[a]
	$\mathbf{D_{v,0.9}}$	1.26 ± 0.10[a]	1.40 ± 0.12[a]	1.07 ± 0.07[a]	1.52 ± 0.85[a]	1.23 ± 0.04[a]
Flow behaviour[2]	$\boldsymbol{\eta_{300}}$ (mPa.s)	13.5 ± 0.55[a]	11.9 ± 1.27[ab]	13.0 ± 0.49[a]	11.9 ± 0.24[ab]	10.9 ± 0.31[b]
	$\boldsymbol{K}$ (Pa.s^n; ×10^2)	1.57 ± 0.19[a]	1.18 ± 0.22[a]	2.92 ± 0.87[a]	1.64 ± 1.25[a]	2.19 ± 0.50[a]
	$\boldsymbol{n}$	0.97 ± 0.02[a]	1.00 ± 0.02[a]	0.85 ± 0.06[a]	0.98 ± 0.16[a]	0.87 ± 0.05[a]

[1] Particle size distribution (PSD) parameters: $D_{4,3}$, volume mean diameter of oil globules; $D_{v,0.1}$, $D_{v,0.5}$, and $D_{v,0.9}$ representing particle size in the 10%, 50% and 90% quantiles of the distribution.

[2] Flow behaviour parameters: (η_{300}), apparent viscosity measured at 300 s^{-1}; (K), consistency coefficient; (n), flow behaviour index.

[(a-b)] Values for a given parameter (i.e., within each row) for all powders, not sharing a common superscript differed significantly ($P < 0.05$).

significant differences between emulsions, where most emulsions displayed a shear-thinning behaviour (i.e., $n < 1$) (Table 8.1). A reduction in the viscosity during shearing (i.e., shear-thinning) of protein solutions is, generally, a result of spatial rearrangement of protein molecules in the liquid and of disruptions in their steady-state interactions (Walstra, Wouters, and Geurts, 2006); in emulsions, shear-thinning can be associated with flocculation of oil droplets (Xu, Wang, Jiang, Yuan, and Gao, 2012). Additionally, in a concentrated emulsion system (i.e., TS = 32%), packing of oil globules is denser than in a dilute emulsion (i.e., TS ≤ 12%) and interactions between its constituents, as monitored by flow behaviour analysis, can also be related to physical contact between molecules located at the interfaces of oil globules (O'Mahony, et al., 2017). The formation of ternary complexes between unadsorbed protein/peptides, CITREM and maltodextrin (Drapala et al., 2016b; Semenova, Myasoedova, and Antipova, 2001) in the WPH+CIT$_e$ emulsion, or the presence of intact whey protein in the serum phase and at the interfaces of oil globules in the WPI$_e$ emulsion, are likely to have contributed to higher viscosity of these emulsions, compared to the other samples.

8.3.2. Drying performance

Fig. 8.2 illustrates differences in drying behaviour between liquid concentrates/powders as evidenced by different levels of wall-coating (i.e., multilayer particle cohesion) by fine powder particles in the cyclone of the spray dryer. The extent of this coating is assumed to be directly related to powder stickiness; the observed stickiness can be divided into 3 groups based on the level of coating, i.e., non-sticky (negligible coating), moderately sticky (partial coating) and very sticky (complete coating) (Fig. 8.2; Table 8.3). Using this classification, the WPI$_p$ and WPH$_p$ powders were moderately sticky, WPH+CIT$_p$ and WPH+LEC$_p$ powders were very sticky and the WPH-MD$_p$ powder was non-sticky.

Differences in the stickiness of powders had a direct impact on the powder recovery (i.e., product yield; Table 8.3); the recovery of product was lower for powders with higher levels of stickiness. Powders containing non-protein emulsifiers (WPH+LEC$_p$ and WPH+CIT$_p$) displayed the lowest powder

recovery (18.1 and 21.3%, respectively) followed by WPI_p (22.0%), WPH_p (26.1%) and $WPH\text{-}MD_p$ (55.3%). It should be noted that, in order to facilitate the use of industry-relevant drying temperatures (i.e., 170°C and 90-95°C for inlet and outlet, respectively) high feed flow rate (95-100%) and relatively low aspirator power (80-90%) conditions were used. These conditions caused deposition of higher-moisture particles at the periphery of the atomised feed jet on the inner wall of the main drying chamber (Fig. 8.1) and contributed to the low powder yield. Sticking of powders to the inner wall of a spray dryer is a common challenge in industry and it directly affects the product yield and drying efficiency (i.e., cleaning and down-time). In high-fat powders (e.g., infant formulae) stickiness is strongly related to the powder surface composition, while, in low-fat protein-dominant powders, it is generally related to the efficiency of water removal and glass transition properties of the system (Kelly et al., 2014). Generally, the more fat at the powder surface the greater the challenges with powder stickiness (Sharma, Jana, and Chavan, 2012; Paterson et al., 2007).

The highest levels of stickiness in this study were observed for powders containing lipid-based emulsifiers (CITREM and lecithin) while the powder containing the protein-based conjugate displayed the lowest stickiness. The physicochemical characteristics of CITREM and lecithin have directly affected cohesiveness (i.e., stickiness) of powders; their high mobility and surface activity facilitates rapid migration to the surface of emulsion droplets formed on atomisation and their relatively low melting temperatures (55-65°C) make them plastic and adhesive under the environmental conditions of spray drying. Similarly, the surface active WPH-MD conjugate can also rapidly move to and adsorb at the surface of atomised droplets (O'Mahony et al., 2017).

8.3.3. Powder analyses

8.3.3.1. Composition and colour of powders

Compositional analysis of powders showed that the measured levels (Table 8.2) were in line with the target levels for all samples (i.e., 12.1-12.7% protein, 26.9-29.0% fat and 56.1-58.8% carbohydrate). No significant differences were found in the fat, carbohydrate or moisture content between the powders. No

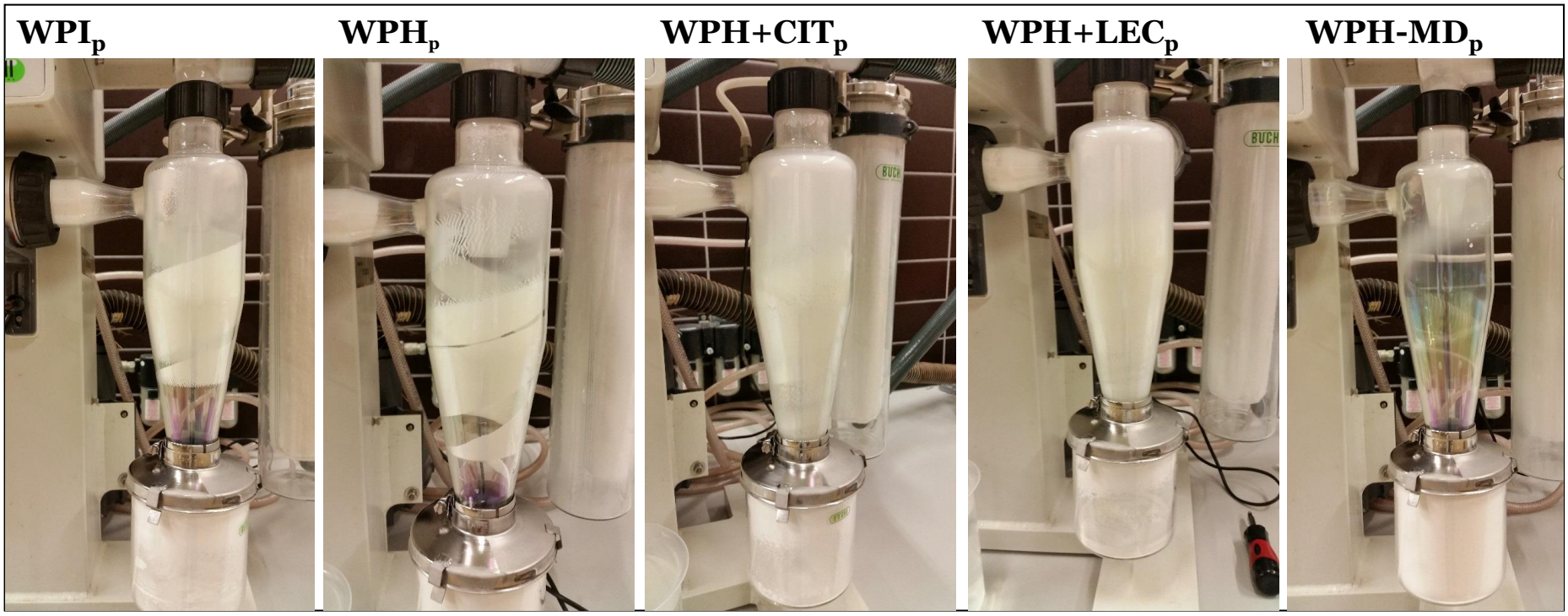

Figure 8.2. Differences in the build-up of fine powder on the wall of the cyclone during spray drying of powders ($_p$) containing different emulsifier systems: whey protein isolate (WPI$_p$), whey protein hydrolysate (WPH$_p$), WPH + CITREM (WPH+CIT$_p$), WPH + lecithin (WPH+LEC$_p$) and WPH-maltodextrin conjugate (WPH-MD$_p$). The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191). The photographs were taken ~30 min after starting the drying run for all powders.

significant differences in colour were found between WPI_p, WPH_p and $WPH+CIT_p$ powders; these powders had high L* and low b* values compared to the $WPH-MD_p$ and $WPH+LEC_p$ powders (Table 8.2). These differences were most likely due to the presence of melanoidins (conjugation products) and carotenoids (naturally present in lecithin) in the $WPH-MD_p$ and $WPH+LEC_p$ powders, respectively (Liu, Ru, and Ding, 2012; McSweeney, 2008; Scholfield, 1981) as previously reported by Drapala et al. (2016b).

8.3.3.2. Particle size distribution of powders

All powders had relatively small particles (i.e., $D_{4,3}$ of 14.2–41.1 μm; Table 8.3). The biggest particles were observed for the $WPH+LEC_p$, followed by the $WPH+CIT_p$, WPI_p, WPH_p and $WPH-MD_p$ powders (Table 8.3, Fig. 8.3B). In addition, powders containing lipid-based surfactants, $WPH+LEC_p$ and $WPH+CIT_p$, had a distinct shoulder on the higher end (i.e., at ~100 μm) of the size range, with a notable proportion of the particle population (i.e., 7.78 and 4.05%, respectively) in these powders having diameter >100 μm (Fig. 8.3B; Table 8.3). A much smaller shoulder was also present in the WPI_p and smaller still in the WPH_p powders (i.e., 2.93 and 2.26% of particle population were >100 μm, respectively). The $WPH-MD_p$ powder had a monomodal profile with the narrowest size distribution, where the majority (i.e., ~99%) of particles had diameters <40 μm (Fig. 8.3B); this sample also had the largest proportion of fine particles (i.e., 19.9% of total population had diameter <5 μm; Table 8.3). The greater proportion of small particles in the $WPH-MD_p$ powder, compared to the other powders is likely related to this liquid concentrate feed having the lowest viscosity of all samples (Pisecky, 2012). A relationship between feed viscosity and the size of particles in the resultant powder was also reported by Crowley, Gazi, Kelly, Huppertz, and O'Mahony (2014), where particle size increased with increasing feed viscosity.

8.3.3.3. Powder wettability

The results for contact angle (θ) analysis showed that the highest θ was observed for $WPH+CIT_p$, followed by WPI_p > $WPH+LEC_p$ > $WPH-MD_p$ > WPH_p (Table 8.3). Generally, the more hydrophobic the surface (i.e., surface of powder pellet), the lower is its affinity for interactions with water and,

Table 8.2. Composition and colour of model infant formula powders ($_p$) produced with different emulsifier systems: whey protein isolate (WPI_p), whey protein hydrolysate (WPH_p), WPH + CITREM ($WPH+CIT_p$), WPH + lecithin ($WPH+LEC_p$) and WPH-maltodextrin conjugate ($WPH-MD_p$). The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191).

Powder	Composition (%, w/w)					Colour coordinates		
	Protein	Fat	Carbohydrate	Ash	Moisture	L*	a*	b*
WPI_p	12.1 ± 0.21^{a}	28.4 ± 1.33^{a}	57.7 ± 0.99^{a}	0.52 ± 0.17^{a}	1.73 ± 0.35^{a}	96.1 ± 0.26^{a}	-1.26 ± 0.09^{b}	3.15 ± 0.24^{a}
WPH_p	12.6 ± 0.10^{b}	29.0 ± 1.58^{a}	56.1 ± 1.50^{a}	0.67 ± 0.10^{ab}	1.08 ± 0.66^{a}	96.3 ± 0.16^{a}	-1.30 ± 0.11^{b}	3.02 ± 0.15^{a}
$WPH+CIT_p$	12.3 ± 0.13^{ab}	28.8 ± 0.34^{a}	56.6 ± 0.43^{a}	0.87 ± 0.19^{ab}	1.36 ± 0.91^{a}	95.8 ± 0.49^{ab}	-1.26 ± 0.06^{b}	3.35 ± 0.26^{a}
$WPH+LEC_p$	12.7 ± 0.22^{b}	26.9 ± 2.44^{a}	58.2 ± 1.84^{a}	0.71 ± 0.13^{ab}	1.48 ± 0.34^{a}	93.8 ± 1.28^{c}	-1.96 ± 0.08^{a}	6.37 ± 0.25^{c}
$WPH-MD_p$	12.5 ± 0.09^{b}	26.9 ± 2.56^{a}	58.8 ± 3.17^{a}	0.97 ± 0.13^{b}	0.89 ± 0.34^{a}	94.1 ± 0.52^{bc}	-0.85 ± 0.07^{c}	4.77 ± 0.38^{b}

$^{(a-c)}$ *Values for a given parameter (i.e., within each column) for all powders, not sharing a common superscript differed significantly ($P < 0.05$).*

effectively, the higher the θ with the droplet of water placed on that surface. Thus, the contact angle analysis is often used to study the affinity of powders for water, providing information on powder wettability (i.e., lower θ = better wettability). The differences in wettability between the WPI_p and WPH_p powders, evidenced by different θ, were most likely directly related to differences in the physical state of protein (i.e., native *vs* hydrolysed, respectively). Solubility is generally enhanced by protein hydrolysis due to partial disruption of protein secondary and tertiary structure resulting in increased water access and faster hydration in hydrolysed, compared with intact, protein-based powders (Banach et al., 2013; Chobert, Bertrand-Harb, and Nicolas, 1988; Kelly, O'Mahony, Kelly, and O'Callaghan, 2016; Panyam and Kilara, 1996). Longer wettability times for model infant formula powders based on intact whey protein compared to partially hydrolysed whey protein were reported previously by Murphy et al. (2015). Wettability of the WPH-MD_p was similar to that observed for the WPH_p (Table 8.3). The better powder wettability observed for the WPH+LEC_p, compared to the WPH+CIT_p, was likely due to the differences in the nature of the two surfactants; CITREM and lecithin are anionic and zwitterionic (i.e., amphoteric) surfactants, respectively (McSweeney 2008). Lecithin is often coated onto the surface of dairy powders in a fluidised bed to facilitate improved wetting properties (i.e., instantisation) (Hammes, Englert, Zapata Norena, and Medeiros Cardozo, 2015).

8.3.3.4. Surface composition of powders

No significant differences were found in the free fat content for all powders due to large standard deviations, especially observed for the WPH+LEC_p powder (Table 8.3). A trend was observed, where free fat content was generally higher, for the WPH+CIT_p, WPH_p and WPH+LEC_p powders (i.e., 20.0, 22.9 and 25.4%, w/w, free fat, respectively), compared to the WPH-MD_p and WPI_p powders (i.e., 13.3 and 14.1%, w/w, free fat, respectively). Table 8.3 shows differences in the surface composition (i.e., as measured using XPS) between the spray-dried model IF powders prepared in this study. The level of protein at the surface was highest for the WPI_p powder followed by WPH_p, WPH-MD_p, WPH+LEC_p and WPH+CIT_p powders. The highest levels of surface fat were

Table 8.3. Properties of spray dried model infant formula powders ($_p$) prepared with different emulsifier systems: whey protein isolate (WPI_p), whey protein hydrolysate (WPH_p), WPH + CITREM ($WPH+CIT_p$), WPH + lecithin ($WPH+LEC_p$) and WPH-maltodextrin conjugate ($WPH-MD_p$). The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191).

Powder characteristics		**WPI_p**	**WPH_p**	**$WPH+CIT_p$**	**$WPH+LEC_p$**	**$WPH-MD_p$**
Drying performance[1]	Powder recovery (%)	22.0 ± 6.59^{a}	26.1 ± 3.27^{a}	21.3 ± 6.67^{a}	18.1 ± 2.56^{a}	55.3 ± 10.8^{b}
	Stickiness (relative)	+	+	++	++	-
PSD Powders[2] (µm)	$D_{4,3}$	26.5 ± 16.9^{ab}	25.4 ± 4.79^{ab}	30.8 ± 2.94^{ab}	41.1 ± 13.2^{a}	14.2 ± 4.79^{b}
	$D_{v,0.1}$	5.75 ± 0.56^{a}	5.85 ± 0.21^{a}	7.87 ± 0.54^{b}	9.52 ± 0.73^{c}	4.76 ± 0.27^{a}
	$D_{v,0.5}$	15.5 ± 2.29^{ab}	15.1 ± 0.33^{ab}	18.4 ± 1.64^{bc}	22.7 ± 2.41^{c}	12.2 ± 0.94^{a}
	$D_{v,0.9}$	59.5 ± 48.3^{a}	40.4 ± 3.22^{a}	56.0 ± 15.4^{a}	95.1 ± 43.6^{a}	26.6 ± 2.33^{a}
	% <5 µm	10.5 ± 2.16^{bc}	13.5 ± 0.71^{b}	6.33 ± 1.64^{cd}	2.84 ± 0.81^{d}	19.9 ± 2.71^{a}
	% >100 µm	2.93 ± 6.92^{a}	2.26 ± 1.13^{a}	4.05 ± 0.93^{a}	7.78 ± 5.29^{a}	0.00 ± 0.00^{a}
Contact angle (θ)		42.1 ± 0.08^{b}	36.9 ± 1.45^{d}	46.7 ± 1.00^{a}	40.5 ± 2.27^{bc}	37.2 ± 0.91^{cd}
Surface free fat (%)		14.1 ± 2.68^{a}	22.9 ± 4.85^{a}	20.0 ± 5.05^{a}	25.4 ± 17.9^{a}	13.3 ± 1.18^{a}
Surface composition (%)	Protein	50.7 ± 6.42^{a}	37.1 ± 6.22^{b}	27.0 ± 2.81^{b}	29.1 ± 4.03^{b}	32.3 ± 2.02^{b}
	Fat	34.1 ± 9.42^{a}	50.9 ± 6.47^{ab}	64.2 ± 6.22^{b}	61.8 ± 6.82^{b}	50.0 ± 3.23^{ab}
	Carbohydrate	15.2 ± 3.02^{ab}	12.0 ± 0.91^{ab}	8.85 ± 3.50^{b}	9.12 ± 3.17^{b}	17.7 ± 1.61^{a}
PSD Reconstituted[2] (µm)	$D_{4,3}$	2.42	5.72	5.00	1.47	0.84
	$D_{v,0.1}$	0.15	0.35	0.31	0.35	0.17
	$D_{v,0.5}$	0.57	4.68	1.10	1.18	0.51
	$D_{v,0.9}$	8.02	13.3	14.4	3.07	1.82

[1] Drying performance describing powder recovery (%, w/w total solids, TS; powder TS/feed TS) and powder stickiness; stickiness classification: -, non-sticky; +, moderately sticky; ++, very sticky.

[2] Particle size distribution parameters (PSD): $D_{4,3}$, volume mean diameter; $D_{v,0.1}$, $D_{v,0.5}$, and $D_{v,0.9}$ representing particle size in the 10%, 50% and 90% quantiles of the distribution. Particle size distribution analysis for reconstituted powders was carried out only on one trial.

[(a-d)] Values for a given parameter (i.e., within each row) for all powders, not sharing a common superscript differed significantly ($P < 0.05$).

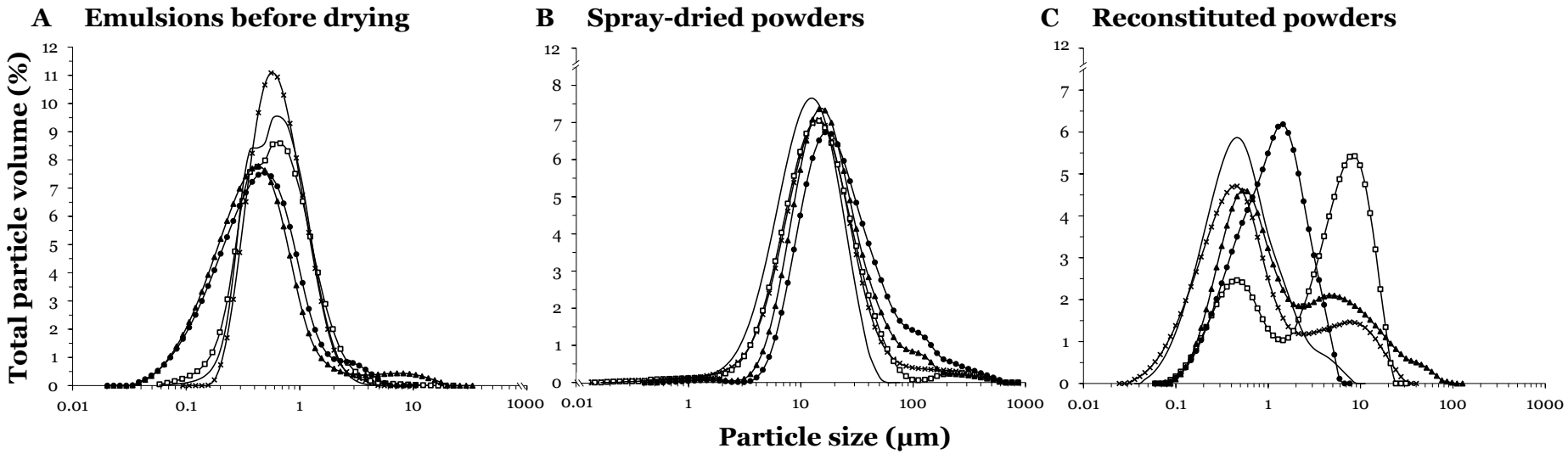

Figure 8.3. Particle size distribution for (A) homogenised emulsions (dryer feeds), model infant formula powders (B) after spray drying and (C) after powder reconstitution. The formulations contained different emulsifier systems: (×) whey protein isolate, (□) whey protein hydrolysate, (▲) WPH + CITREM, (●) WPH + lecithin and (–) WPH-maltodextrin conjugate. The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191).

found in the WPH+CIT$_p$ and WPH+LEC$_p$ powders. The amount of carbohydrate present at the surface was significantly higher for the WPH-MD$_p$ powder compared to the 2 powders containing lipid-based surfactants (i.e., WPH+LEC$_p$ and WPH+CIT$_p$).

The differences between the surface fat composition as measured by the solvent extraction and by the XPS methods can be explained by the different principles underpinning these methods. For the solvent extraction method, the results are presented as the weight of extractable fat as a % of the powder sample weight; conversely, in the XPS method, the results are presented as the % of surface area of the powder particle occupied by fat. For the XPS method, only a 10 nm depth of the surface of the powder particle is analysed (Kim, Chen, and Pearce, 2009). Conversely, the solvent extraction approach extracts fat present at the surface of the powder particle as well as fat present at other locations within its interior. According to a model proposed by Buma (1971), the solvent-extractable free fat for dairy powders consists of surface fat, outer layer fat from fat globules within the surface layer of the particle, capillary fat constituted by fat globules that can be reached by the solvent through capillary forces, and dissolution fat consisting of fat reached by solvent through holes left by already extracted fat. The review of solvent extraction-based methods for assessment of the amount of free or surface fat in spray-dried emulsions, reported in the scientific literature, was compiled by Roos and Vega (2006), where it was shown that these methods use different solvent types (petroleum ether, hexane, pentane and carbon tetrachloride), solvent-to-powder ratios (5:1 – 40:1), and powder-solvent contact times (30 s – 48 h). The solvent extraction method used in this study (GEA Niro, 2005) for quantification of the surface free fat in the milk powders, with an extraction time of 15 min, could have led to the extraction of lipid material in addition to surface fat alone (i.e., fat from the surface and from the interior of the powder particles).

8.3.3.5. Microstructure of powders

Scanning electron microscopy

Fig. 8.4 A and B illustrate the detailed morphology (shape and structure) of the spray-dried model IF powders. Differences between samples were mainly manifested by the extent of particle agglomeration (i.e., spontaneous agglomeration of primary particles) and the topography of the particle surfaces in the powders. Powders containing lipid-based emulsifiers, WPH+CIT$_p$ and WPH+LEC$_p$, displayed the greatest extent of particle agglomeration, followed by WPI$_p$, WPH$_p$ and WPH-MD$_p$ (Fig. 8.4A). Such agglomeration is generally caused by extensive particle cohesion (i.e., sticking) and is evidenced by the presence of 'bunch of grape'-type agglomerates (Pisecky, 2012), as observed in this study for the WPH+CIT$_p$, WPH+LEC$_p$ and, to a lesser extent, WPI$_p$ powders (Fig. 8.4A). These observations closely match the particle size distribution data discussed in Section 8.3.3.2. and indicate cohesive interactions between particles during spray drying (i.e., spontaneous agglomeration).

The surface topography was also different between the powders; smooth surfaces were observed for the WPI$_p$ and to a lesser extent for WPH-MD$_p$ while the powder particles in the WPH$_p$, WPH+CIT$_p$ and WPH+LEC$_p$ had an uneven surface with numerous bumps (WPH$_p$) or craters (WPH+CIT$_p$ and WPH+LEC$_p$) present on the surface (Fig. 8.4B). The presence of crater-like structures on the surface of spray-dried emulsions/powders has been associated with broken oil globules resulting in high levels of surface fat (Drusch and Berg, 2008). Additionally, WPH-MD$_p$ powder particles appeared to be partially collapsed (i.e., shrivelled) unlike particles in the other powders. Such shrivelled/buckled structures in spray-dried powders has been linked with temperature-dependent changes in the volume of occluded air (i.e., inflation followed by deflation of intra-particle air as the particle moves from hot toward the cooler regions of the dryer) (Walton and Mumford, 1999) and with the mechanical properties of the skin layer of the drying particles (Sadek et al., 2015, 2016).

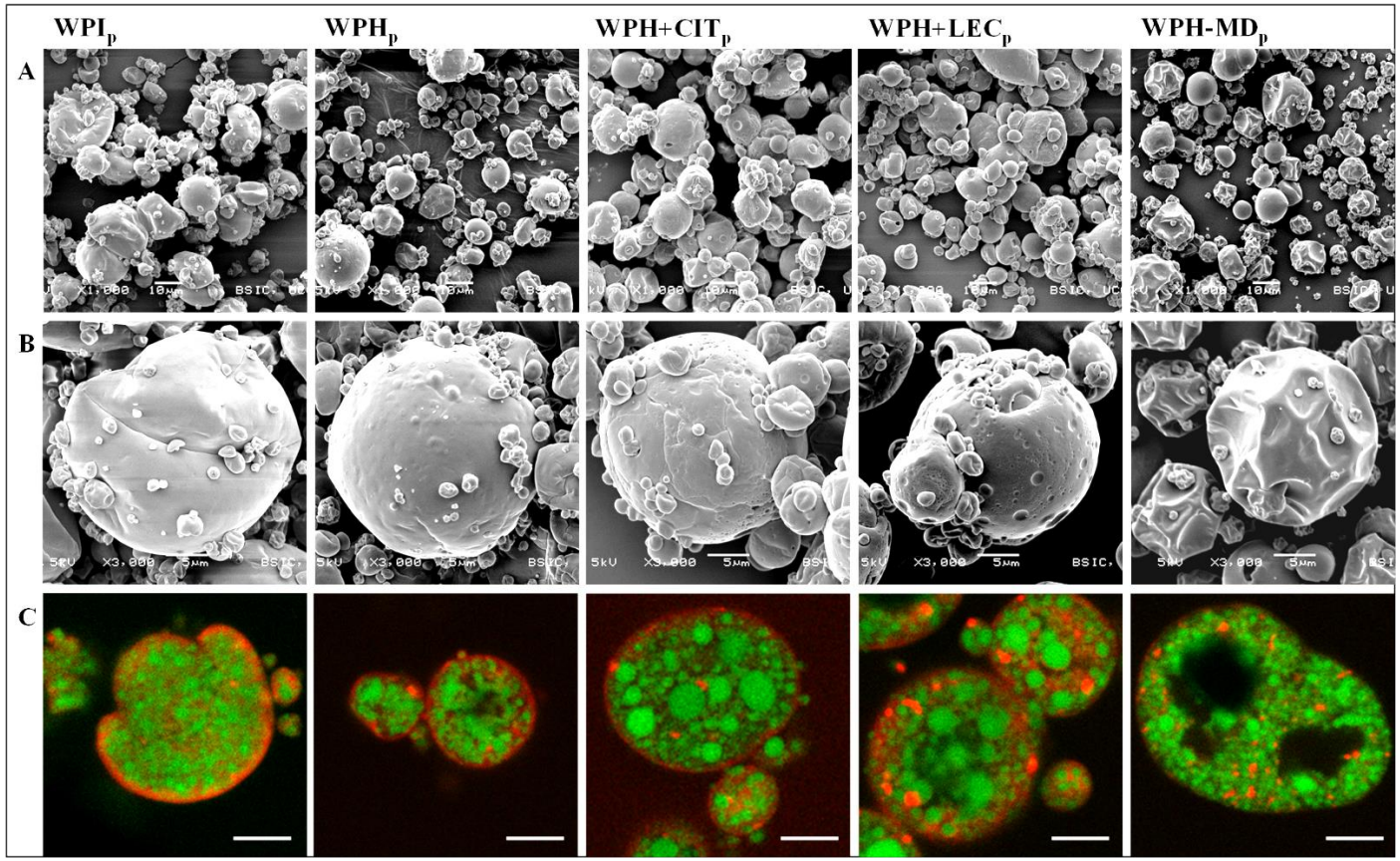

Figure 8.4. Scanning electron microscope (SEM; A and B) and confocal laser scanning microscope (CLSM; C) images of model infant formula powders ($_p$) containing different emulsifier systems: whey protein isolate (WPI$_p$), whey protein hydrolysate (WPH$_p$), WPH + CITREM (WPH+CIT$_p$), WPH + lecithin (WPH+LEC$_p$) and WPH-maltodextrin conjugate (WPH-MD$_p$). For the CLSM analysis powders were labelled with Nile Red:Fast Green (3:1) and the micrographs show distribution of oil droplets (green) and protein particles (red). Scale bar for the micrographs: A = 10 μm, B = 5 μm, and C = 5 μm. The powders were produced using a laboratory scale spray dryer (BÜCHI B-191).

Confocal laser scanning microscopy

Powders produced in the current study had generally similar particle structures, where individual oil droplets were homogenously distributed within a protein-carbohydrate network (Fig. 8.4C). The only exception was the WPH_p powder, where the oil phase appeared to be largely present as irregular and extensive oil pools. Differences in the size of oil droplets within the powder matrix were observed; powders containing lipid-based surfactants, $WPH+CIT_p$ and $WPH+LEC_p$ had markedly bigger (2-3 μm) oil droplets embedded in the powder structure, compared to apparently smaller (≤ 1 μm) oil droplets in the WPI_p and $WPH\text{-}MD_p$ powders. Pools of oil or large oil droplets observed in CLSM micrographs can be related to poor stability of these emulsions to processing. Additionally, 'empty' regions were observed in the centre of the $WPH\text{-}MD_p$ powder (Fig. 8.4C); these regions most likely indicate the presence of internal air pockets (i.e., vacuoles) in particles of this powder as discussed in Section 8.3.3.5. Formation of vacuoles and shrivelling of powder particles have been shown to take place concomitantly (Sadek et al., 2015) and is strongly linked to the surface composition of the droplet and, effectively, its drying kinetics (Nijdam and Langrish, 2006; Vignolles et al., 2007).

8.3.3.6. Particle size distribution after reconstitution of powders

Notable differences were observed in the PSD between the reconstituted IF powders (Table 8.3; Fig. 8.3C); the mean volume diameter ($D_{4,3}$) and the value for the 90% quantile of the size distribution ($D_{v,0.9}$) were higher for all reconstituted powders compared to the emulsions prior to spray drying (Tables 8.1 and 8.3; Fig. 8.3A and C). The observed increases in $D_{4,3}$ and $D_{v,0.9}$ were most pronounced for the WPH_p and $WPH+CIT_p$ powders (i.e., increases in $D_{4,3}$ and $D_{v,0.9}$ to ≥ 5 μm and >13 μm, respectively); only a limited increase was observed for the $WPH\text{-}MD_p$ powder (i.e., $D_{4,3}$ < 1 μm and $D_{v,0.9}$ < 2 μm) (Table 8.3). The $D_{4,3}$ and $D_{v,0.9}$ parameters are particularly sensitive to changes at the large particle periphery of the size distribution and their increase can be used as an indicator of associations between the larger components in a system (i.e., coalescence and/or flocculation of oil globules in this case).

These differences reflect different stabilities of the corresponding formulations to the spray drying conditions (i.e., stability of oil globules against coalescence in a concentrated emulsion system and stability to high heat and high shear stress within the atomiser and upon atomisation) and support the CLSM observations (see Section 8.3.3.5).

8.4. Discussion

The stability of emulsions to spray drying was different for the studied formulations, as illustrated by the size distribution of oil globules in the powder matrix and in the reconstituted emulsions. These differences can be explained by the properties of the emulsifier systems used in these formulations, and their effect on stabilising emulsions against globule coalescence or heat-induced aggregation of oil globules during processing. During spray drying, emulsion-based systems are subjected to considerable stresses which can cause protein aggregation, breaking and coalescence of oil globules; this can lead to high surface free fat content and, effectively, undesirable properties of the resultant powder. Emulsions stabilised by high molecular weight (M_w) surfactants (e.g., protein) usually have thick and elastic interfacial films and are more stable to stress, compared to those stabilised by low M_w surfactants (e.g., CITREM, lecithin), which are prone to coalescence when forced into close contact (Taneja et al., 2013). Formulations based on WPH often display poor thermal stability, due to exposure of reactive sites (e.g., free sulphydryl groups) at the surfaces of oil globules and in the bulk phase, often resulting in bridging flocculation of oil globules (Agboola, Singh, Munro, Dalgleish, and Singh, 1998; Drapala et al., 2016a). Such behaviour was also reported in the current study, where oil pools in the WPH_p powder matrix and large oil globules were present after reconstitution of this powder.

CITREM and lecithin are often added to improve thermal stability of WPH-based emulsions; however, their presence can lead to competitive destabilisation, where protein/peptide-based surfactants are displaced from the interfaces by smaller surfactants, promoting coalescence of oil globules (Drapala et al., 2016a; Kaltsa, Paximada, Mandala, and Scholten, 2014; Mackie, Gunning, Wilde, and Morris, 1999; Van Aken, 2003; Wilde, Mackie,

Husband, Gunning, and Morris, 2004). This was observed in the current study for CITREM- and lecithin-containing powders, where large oil globules were observed in the powder matrix and in the reconstituted emulsions (Fig. 8.4C, Table 8.3). In addition, topographical features observed for samples containing lipid-based emulsifiers (i.e., craters; Fig. 8.4B) indicated that coalescence of oil globules resulted in the presence of damaged oil globules at the powder surface (Drusch and Berg, 2008). It is generally accepted that strong steric stabilisation of oil globules, provided by protein-carbohydrate conjugates, can greatly limit these forms of destabilisation (O'Mahony et al., 2017; Oliver, Melton, and Stanley, 2006). The presence of WPH-MD conjugate in emulsions prevents interactions between individual oil globules and interactions with bulk protein/peptides, resulting in enhanced stability. Results presented in the current study show that superior stability of emulsions to spray drying was achieved when the WPH-MD conjugate was present in the formulation, compared to formulations containing CITREM or lecithin.

In an emulsion, surface active molecules (e.g., protein, peptides, lecithin, CITREM, conjugates) are adsorbed at the oil/water interface, where they stabilise oil globules; these compounds are, generally, also abundant in the emulsion bulk phase as they are present in excess of the concentration required for oil stabilisation. Upon atomisation, a new interface (water/air) is formed at the surface of the atomised droplets and, during very short time scales, surface active components move from the bulk to this new surface, adsorb and rearrange (Munoz-Ibanez et al., 2016). Smaller surfactants move and adsorb faster due to their higher mobility compared to large surfactants (Landstrom, Alsins, and Bergenstahl, 2000). Similar to the stabilisation of oil globules, the composition and structure of the interfacial layer of atomised droplets dictate their potential for interactions (i.e., stickiness, agglomeration) (Nijdam and Langrish, 2006); in effect, surface composition and physicochemical properties of the resulting powder are largely dependent on the surfactant system of the emulsion. The high surface fat level observed for the WPH+CIT_p and WPH+LEC_p powders and the high surface maltodextrin level observed for the WPH-MD_p powder, could indicate preferential

adsorption of lipid-based and conjugate-based emulsifiers, respectively, at the surfaces of atomised droplets in these powders. Owing to the different surface compositions, powders displayed different propensity for interactions between individual atomised droplets/particles (i.e., primary spontaneous agglomeration) and with the wall of the spray dryer (as measured by powder build-up in the cyclone). It is generally recognised that high levels of surface free fat cause challenges with cohesive interactions of powders (Jayasundera et al., 2009; Vega and Roos, 2006). Similarly, in the current study, the likely preferential presence of lipid-based emulsifiers on the surface of some of the powders may have contributed to greater cohesiveness and, effectively, could have promoted agglomeration and powder build-up, compared to the other powders.

Properties of the feed and drying kinetics generally govern the shape of powder particles (Walton and Mumford, 1999). Distinctive shrivelled particles observed for the WPH-MD_p powder were likely related to significantly lower viscosity of that emulsion, compared to the other emulsions (i.e., at the same TS content), effectively, impacting the rate of water removal. Additionally, the more hydrophilic nature of the surface of atomised droplets/powder particles for the WPH-MD_p system, resulting from higher surface maltodextrin content, compared to the other samples could have promoted faster water removal as evidenced by the lower moisture content of the resultant powder. According to a study by Sheu and Rosenberg (1998), surface indentation for whey protein-based powders was promoted by high drying rates, leading to wall solidification before the onset of particle inflation. With progressive water removal during drying of a dairy-based system, a skin layer is formed at the droplet surface and its properties further affect the kinetics of drying and the final shape of the dried particles. Sadek et al. (2015) presented a model for mechanical properties of skin layer of a droplet during drying, where, depending on protein type present at the surface (i.e., whey protein or micellar casein), the mechanical properties of the skin were different and affected the shape of the resultant dried particles. Those authors showed that in casein micelle-dominant skins, the elastic modulus increased faster and the protein skin reached the plasticity region earlier, producing shrivelled particles with

ductile and plastic skin, while it took longer for the whey protein-dominant skin to reach the plasticity region, giving round particles with brittle and plastic skins. Particle indentation for whey protein-based powders was reported to be linked to the ratio of protein to maltodextrin at the surface of powder particles (Rosenberg and Young, 1993; Sheu and Rosenberg, 1998), where surface indentation was inversely related to the proportion of whey protein in the particle skin. In the study by Sheu and Rosenberg (1998), the authors showed that increasing the maltodextrin proportion in the skin decreased its elasticity and, effectively led to the formation of shrivelled powder particles. Such shrivelled morphology was observed in this study for the WPH-MD_p powder particles. In addition, the presence of vacuoles observed in the WPH-MD_p powder sample supports its fit to the model proposed by Sadek et al. (2015), where vacuole formation and particle shrivelling were concomitant. With rapid water removal from the atomised droplets during spray drying, less latent heat energy is required due to lower moisture content, and the energy (i.e., temperature) acting on the non-water powder components is increased. This, effectively, can result in increased inflation of the droplet due to the expanding volume of air occluded within, followed by particle collapse (i.e., deflation) as the particles move away from the heat source, resulting in a shrivelled hollow powder particle (Hecht and King, 2000; Walton and Mumford, 1999). The use of different emulsifier systems resulted in different surface composition of the resultant powders as well as different quality of reconstituted emulsions. It was demonstrated that the differences in powder surface composition influenced the kinetics of drying for these formulations and governed the cohesive interactions between atomised droplets/powder particles. Effectively, the presence of lipid-based emulsifiers (i.e., CITREM or lecithin) in formulations greatly increased the cohesive interactions resulting in extensive spontaneous primary agglomeration and, effectively, reduced product yield. On the other hand, when the conjugate-based emulsifier was present in the formulation, these cohesive interactions were markedly reduced.

8.5. Conclusions

The current study demonstrated that using the WPH-MD conjugate in the formulation of emulsion-based model IF powder improved its processing stability and affected the surface composition of resultant powder. The use of the conjugate in the formulation gave powder with decreased surface fat and increased surface carbohydrate levels, compared to systems containing lipid-based emulsifiers (i.e., CITREM or lecithin). In effect, the conjugate-based powder displayed reduced cohesive behaviour, resulting in decreased agglomeration and markedly higher product yield; the opposite was observed for the powders containing lipid-based emulsifiers. This study showed that the surface composition of an emulsion-based powder and, effectively, its drying performance and final product characteristics were greatly improved by utilisation of interactions (i.e., conjugation) between the two components of the formulation (i.e., protein and carbohydrate).

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Chapter 9

General discussion

&

Recommendations for future research

9.1. General Discussion

9.1.1. Background and outlook

Many nutritional products are emulsion-based systems, where the innate (e.g., protein) or added (e.g., lecithin) surface-active components (i.e., emulsifiers) facilitate formation of homogenous aqueous-oil dispersions and govern the shelf life of what are inherently thermodynamically unstable systems. Emulsifiers adsorb at the oil-water (O/W) interface, decrease the surface free energy and form a protective barrier around oil globules, conferring globule stabilisation by means of electrostatic and/or physical (i.e., steric) repulsion mechanisms. Therefore, the composition, structure and physicochemical properties of emulsifiers and the resultant composition, structure and physicochemical properties of the interfacial layer of an O/W emulsion strongly influence the stability of the emulsion. Infant formula (IF) products are emulsion-based systems, commercially available in both liquid and powder formats. Such emulsions are typically stabilised mainly by bovine or plant (e.g., soy, rice) protein, often with the addition of low molecular weight (L_{Mw}) surfactants to improve their stability (McSweeney, 2008). Formulae based on partially hydrolysed whey protein constitute a significant share of the infant nutrition market owing to their 'easy-to-digest' profile (i.e., limited curd formation during acidification in the gastric conditions of an infant – pH 4.5), as evidenced by the current product portfolio of the major manufacturers of IF ingredients and products (e.g., SMA Comfort, Nestle; Aptamil Gold+ HA, Danone Nutricia; Enfamil Gentlease, Mead Johnson Nutrition; Hyvital, Friesland Campina). The aim of this thesis was to investigate the effects of different emulsifiers, including intact and hydrolysed whey protein, conjugated hydrolysed whey protein and L_{Mw} surfactants (lecithin and citric acid esters of mono- and di-glycerides, CITREM) on the mechanisms driving emulsion formation, processing performance and stability of emulsions in the context of model infant formula systems.

9.1.2. Overview of findings

The composition of the interfacial layer plays an important role in the stability of oil globules in an emulsion; this is particularly the case in emulsions

involving mixtures of emulsifiers, due to the competitiveness of the emulsifiers for the interfacial space and the rheological properties of the interfaces. The rates of adsorption at the O/W interface are significantly higher for L_{Mw} emulsifiers, compared to proteins/peptides from whey protein hydrolysate (WPH) due to the higher surface activity and higher mobility of the former (**Chapter 4**). Hence, the use of L_{Mw} emulsifiers generally allows formation of smaller oil globules during homogenisation, compared to emulsions prepared using whey protein isolate (WPI)/WPH alone (**Chapters 4**, **5**, 7, and **8**). Conversely, when WPH was conjugated with maltodextrin (MD), the conjugate retained its good interfacial properties as evidenced by no differences in the fat globule size distribution (FGSD) of oil globules post homogenisation, regardless of the higher molecular weight of the WPH-MD, compared to WPH (**Chapters 6** and **8**). All emulsifier systems studied in this thesis were able to form good emulsions under the homogenisation conditions used (i.e., 12-18 MPa, 2-stage, double pass at 50-55°C), where the mean volume diameter ($D_{4,3}$) of oil globules ranged from 0.58 to 0.97 μm (**Chapters 4-8**).

Despite the smaller FGSD achieved in the emulsions containing a mixture of WPH and L_{Mw} emulsifiers (i.e., lecithin and CITREM), these emulsions displayed poor stability on storage (i.e., at 4 and 40°C), compared to emulsions stabilised solely by the intact, hydrolysed or conjugated whey protein ingredients (**Chapters 4** and **6-8**); this inferior stability was linked to the irregular composition of the interfacial layer of oil globules (**Chapter 4**; Fig. 9.1). From the micrographs obtained using confocal scanning microscopy and FGSD analysis (**Chapter 4**), it was evident that the presence of lecithin and CITREM in emulsions resulted in irregular interfaces of oil globules, effectively leading to coalescence of the globules (i.e., competitive destabilisation mechanism; **Chapters 7** and **8**).

In a similar manner, the surface composition of spray-dried model infant formula products was shown to be largely dependent on the emulsifiers used to form the emulsion, owing to their rapid adsorption at newly created air/water interfaces of atomised emulsion droplets (**Chapter 8**). Differences in surface composition affected the drying performance (e.g., cohesive

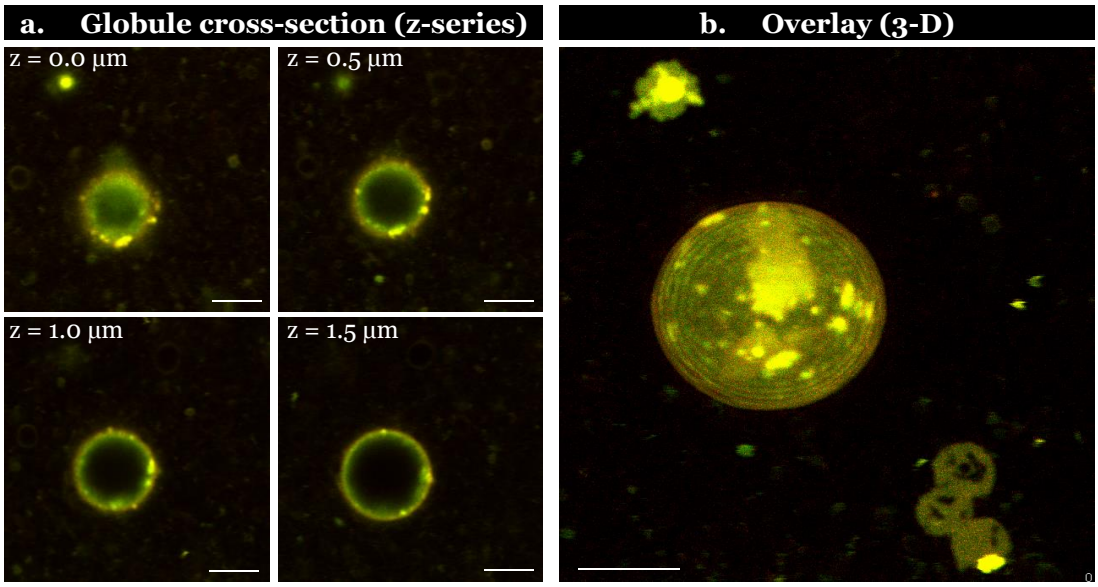

Figure 9.1. Interfacial location of phospholipids (bright yellow regions) on the surface of an oil globule in a model infant formula emulsion (15.5, 35.0 and 70.0 g L^{-1} of protein, lipid and carbohydrate, respectively; prepared as detailed in **Chapter 4**). The emulsion was stabilised by moderately hydrolysed whey protein (degree of hydrolysis = 10.7%) and soybean lecithin (3% of the total lipid weight). The lecithin (i.e., phospholipids) were stained with fluorescent dye Rhod-DOPE (Avanti Polar Lipids) according to the protocol of Lopez, Madec and Jimenez-Flores (2010); scale bar = 5 µm; distance between the cross-sections of the globule (i.e., z-distance) = 0.5 µm. Drapala, Auty, Mulvihill, and O'Mahony (2015; **Unpublished results**).

interactions) of emulsions and influenced the rheological properties of the wall of the drying droplets and affected the drying kinetics of these systems. Extensive cohesiveness of powder observed for systems containing L_{Mw} emulsifiers (i.e., CITREM and lecithin) was associated with the high levels of surface lipid in these powders, as measured by X-ray photoelectron spectroscopy (XPS); while significantly lower cohesiveness of the WPH-MD conjugate-based powder was associated with the low surface lipid content, and faster drying rates, owing to the lower viscosity of that formulation. The topographical features of powder particles were used to predict the rheological properties of the drying wall, in accordance with models available in the

scientific literature (Rosenberg and Young, 1993; Sadek et al., 2015, 2016; Sheu and Rosenberg, 1998); systems stabilised by the conjugated protein were associated with lower flexibility and faster solidification of the drying wall, compared to the powders based on protein/peptides with and without the inclusion of L_{Mw} emulsifiers.

The interfacial layer of oil globules in an emulsion generally provides stabilisation *via* mechanisms of repulsive colloidal interactions, which are based either on electrostatic repulsion forces (i.e., through the charge of the emulsifiers) or on steric hindrance (i.e., physical restriction of contact between individual globules by components of their interfaces). The novel scientific studies presented in this thesis demonstrated that steric stabilisation of oil globules in model IF emulsions provided by the WPH-MD conjugate was superior to the electrostatic stabilisation provided by the proteins/peptides alone or proteins/peptides in combination with L_{Mw} emulsifiers.

Regardless of the emulsifier system used to stabilise the emulsions (i.e., intact whey protein, WPI; WPH, WPH + L_{Mw} emulsifiers and WPH conjugated with MD), only limited differences were observed in the zeta potential (ζ) of oil globules in these emulsions (i.e., ζ ranged from -48.0 to -57.7 mV at pH 6.8; **Chapters 6** and **7**). Moderate hydrolysis of whey protein (i.e., 8% degree of hydrolysis, DH) had only a limited effect on ζ, with emulsions stabilised by intact and hydrolysed whey protein having ζ of -48.0 and -49.6 mV, respectively (**Chapter 6**). The higher ζ of the oil globules stabilised by the WPH conjugated with maltodextrin (i.e., -55.0 mV), compared to those stabilised by the unconjugated WPH (**Chapter 6**), was attributed to blocking of the positively-charged amino acid lysine on conjugation. Inclusion of the L_{Mw} emulsifiers, lecithin or CITREM, in the formulation of WPH-based emulsions also increased the ζ of oil globules, compared to emulsions stabilised by WPH alone (**Chapter 6** and **7**). Predominantly zwitterionic (i.e., possessing both positive and negative charge on the polar head) phospholipid components of lecithin contributed to a limited increase in ζ of oil globules (-52.3 mV), while the presence of negatively-charged organic acid groups of CITREM contributed to higher ζ (i.e., -57.7 mV), compared to the emulsions containing WPH alone as emulsifier. Heating of emulsions stabilised by WPH-

MD conjugate and WPH+L_{Mw} reduced the ζ of oil globules (by 2.6 to 4.6 mV), which was attributed to heat-induced structural changes to the proteins/peptides at the interfaces and limited interactions between serum and interfacial proteins/peptides (**Chapter 7**).

Electrostatic stabilisation has its limitations, such as the requirement for a specific pH range to allow the emulsifiers to exert a strong electrostatic charge (i.e., far from the isoelectric point, pI, of the emulsifier), and the electrostatic stabilisation is most effective under quiescent and ambient conditions, where the combination of Brownian motion and ζ keeps the small oil globules (<1 μm) homogenously dispersed throughout the emulsion. The studies presented in this thesis demonstrated that emulsions, in which electrostatic stabilisation was the dominant stabilisation mechanism showed inferior stability to long time storage (**Chapters 4 and 7**), thermal processing (**Chapters 6 and 7**) and spray drying (**Chapter 8**), compared to emulsions where the oil globules were stabilised by steric hindrance provided by the conjugated WPH. Steric hindrance allows physical stabilisation of oil globules in O/W emulsions by extending the hydrophilic moiety of an emulsifier into the serum phase of the emulsion, resulting in a physical restriction of access to oil globules, greatly limiting globule-globule interactions. Steric stabilisation of oil globules, provided by the WPH-MD conjugate yielded emulsions with superior stability, compared to emulsions stabilised predominately by means of electrostatic repulsion (i.e., WPI-, WPH-, WPH+L_{Mw}-based; **Chapters 6-8**), as evidenced by stability against coalescence of oil globules during extended storage (10 d × 40°C) and against globule aggregation during thermal processing (100°C × 15 min; shear rate 15 s^{-1}).

The results presented in **Chapters 4-8** showed that stability of model IF emulsions was largely affected by the nature of the emulsifier system used for their stabilisation; emulsions stabilised by moderately hydrolysed whey protein did not display any increase in FGSD during storage at 4°C for 14 d (**Chapter 4**); however, when L_{Mw} emulsifiers, CITREM or lecithin, were present in these emulsions, they promoted competitive destabilisation, as evidenced by extensive coalescence of oil globules (**Chapters 4** and **7**).

Similar to WPH-based emulsions, good storage stability was observed for the WPH-MD-based emulsions, which did not display any changes in FGSD during storage at 40°C (**Chapter 7**). However, stability of emulsions to thermal processing (75-100°C × 15 min) did not follow the same trend as observed for the storage stability. Poor heat stability of WPH-based emulsions was linked to the high number of free thiol groups (–SH), exposed by protein hydrolysis, which promoted protein-mediated aggregation of oil globules (i.e., bridging flocculation) during heating of the emulsions (**Chapter 6**). Destabilisation of the WPH-based emulsions through bridging flocculation was evidenced by formation of small (~100 μm) buoyant particles; these clusters of aggregated oil globules surrounded by a protein/peptide network closely match the description of white flecks, a common (yet poorly understood) product quality challenge in infant formula products (Regost, 2016). Reducing the number of free –SH groups, by preheating of the WPH prior to emulsion formation, was shown to be a successful strategy for improving thermal stability of the emulsions, due to limiting of interactions between serum and interfacial proteins and this approach increased the onset temperature for emulsion destabilisation (i.e., from ~72 to ~100°C; **Chapter 6**).

Another recent approach used to increase thermal stability of whey protein-rich emulsions was reported by Chevalier et al. (2016), where the protein-mediated aggregation of oil globules was limited by a combination of exclusive stabilisation of oil globules with caseins and subsequent (i.e., post-homogenisation) inclusion of whey protein-microgels, pre-associated to limit their interactions with emulsion interfaces on heating. In this thesis, providing a strong steric barrier to oil globules in whey protein/peptide-based emulsions, by their stabilisation with WPH-MD conjugates, was shown to successfully prevent globule-globule interactions over a range of heating temperatures (75-100°C), as evidenced by the absence of any heat-induced increases in FGSD (**Chapters 6** and **7**). Heat stability equivalent to that of non-conjugated WPH-based emulsions was achieved at high CITREM and lecithin inclusion levels (9 g L^{-1}); however, defects such as fouling of heat exchange surfaces during thermal treatment of the lecithin-containing

emulsions and inferior stability to storage of L_{Mw}-containing emulsions demonstrated the advantage of the conjugated WPH for overall emulsion stabilisation (**Chapter 7**). Stability of emulsions based on conjugated WPH-MD extended to unit operations other than thermal processing, as evidenced by distinctive powder microstructure (i.e., surface topography and distribution of oil globules within the powder matrix) and good emulsion quality upon reconstitution of model IF powders, compared to powders based on WPI, WPH and WPH+L_{Mw} emulsifiers (**Chapter 8**). Efficient steric stabilisation of emulsions, where the hydrophobic components of the interfacial layer physically restricts interactions between oil globules during unit operations, prolonged storage and/or under adverse environmental conditions (e.g., pH = ~pI of the emulsifier), offers significant potential for addressing stability challenges of existing formulations and for development of novel food products (i.e., acidic milk-type beverages, milk fat globule membrane-based systems).

9.2. Recommendations for future research

Imitating milk fat globule membranes

Harnessing the strong steric stabilisation potential of WPH-MD conjugates can provide significant potential for production of novel IF products, engineered to closer resemble interfacial structures present in human milk, where oil globules are predominately populated by polar lipids and sterically-stabilised by glycoproteins (i.e., milk fat globule membranes; Fig. 1.3., **Chapter 1**, page 25). It would be worth investigating the assembly of IF fat globule membranes (FGM) with WPH-MD conjugates incorporated in them to determine the optimum inclusion levels required to provide desired stability (i.e., against process- and storage-induced changes). It would also have to be considered that the levels of included conjugated protein should only constitute a small fraction of the total protein in the formulation to keep the nutrient profile, total protein and the amino acid levels within the regulatory guidelines. Relevant information on the structural engineering of model FGM interfaces could be obtained by investigating the effects of modification of the protein moiety (e.g., by hydrolysis) and resulting changes in the M_w, hydrophilic/hydrophobic balance and localisation of the hydrophilic and hydrophobic groups within the polypeptide chain on adsorption and preferential localisation of these emulsifiers at the interfaces of oil globules dominated by polar lipids. Such an investigation could be aided by the combination of size exclusion, spectrofluorimetry, surface/interfacial tension techniques and confocal microscopy (i.e., aimed at localisation of the polar lipids and protein/peptide-based components). Similarly, it would be worth investigating how different physicochemical properties of the hydrophilic moiety (e.g., molecular weight, linear *vs* branched structure and electrostatic charge) influence formation and stability of such advanced emulsion systems. Designing and investigating these novel interfaces for IF products could provide effective science-based solutions for the next generation of IF and nutritional beverages designed to allow improved nutrient delivery, where the oil globules can pass through the stomach and subsequently be disassembled by the bile salts in the small intestine (Singh and Gallier, 2016; van Aken, 2010). Broadening the scientific knowledge in the area of structural

engineering of FGM interfaces would complement the current efforts of the IF industry, focused on the development of next-generation IF products tailored to more closely resemble the structures and metabolic fate of human milk. The drive by the IF industry to develop such products can be evidenced by recent publications reporting on prototype children nutrition products containing MFGM components from companies like Danone (Gallier et al., 2015) and Lactalis (Veereman-Wauters et al., 2012), patents on formulae incorporating bovine MFGM (WO 2011/069987 A1, US 2012/0321600 A1) and new IF products containing MFGM components (Enfamil Enspire, Mead Johnson).

Surface composition of emulsion globules

The emulsions studied in **Chapters 4-8**, were stabilised by mixed emulsifier systems (i.e., proteins/peptides- and lipid-based emulsifiers) and by different ratios of these emulsifiers (i.e., CITREM and lecithin inclusion levels of 0-9 g L^{-1}, proteins/peptides at an inclusion level of 15.5 g L^{-1}), which significantly affected the stability of the emulsions. A significant reduction in the creaming velocity, without accompanying changes in the particle size distribution and only a limited increase in the viscosity of emulsions stabilised by WPH and CITREM (**Chapter 7**) was linked to the formation of complexes between CITREM, proteins/peptides and carbohydrates in the serum and at the interfaces of oil globules as previously reported by Semenova, Myasoedova and Antipova (2001). It would be worth investigating the interfacial composition of oil globules in such emulsions; however, current methodology used for providing that information (e.g., the washed cream method; Oortwijn and Walstra, 1979) has limitations due to the invasive nature of the method (centrifugation of the emulsion and washing steps), which can lead to coalescence of the oil globules, changes in the interfacial area, displacement and rearrangement of the surfactants at the O/W interface (Holzmüller, Müller, Himbert, and Kulozik, 2016). The next generation of confocal and spectroscopy-based raman microscopy (Leica TCS SP8 CLSM, Nikon C2 CLSM, WITec ALPHA300) are promising techniques, which may allow investigation of the interfacial components of emulsions in their native state

and environment, where non-invasive localisation of multicomponent structures could be achieved for emulsion globules at the nano-scale (Lim, Burdikova, Sheehan, and Roos, 2016).

Rheological properties of conjugate-stabilised emulsions

Rheological properties of emulsions are affected by the size and shape of their components (e.g., oil globules, proteins, carbohydrates and other hydrocolloids) and interactions between these components (e.g., aggregation, orientation and/or physical contact). The hairy layer of the oil globules, provided by the hydrophilic MD moiety extending into the aqueous phase in emulsions stabilised by the WPH-MD conjugate is likely to participate in non-attractive steady-state interactions with other components of the serum phase (i.e., protein, carbohydrates) and interfaces of other oil globules, especially in the concentrated system investigated in **Chapter 8** (i.e., 32% total solids content). In **Chapter 8**, emulsions stabilised by the WPH-MD conjugate displayed higher shear-thinning behaviour, compared to emulsions stabilised by the WPI or WPH (i.e., flow behaviour index = 0.97, 1.00 and 0.87 for WPI-, WPH- and WPH-MD-based emulsions, respectively). Shear-thinning behaviour in O/W emulsions is typically associated with flocculation of oil globules, disrupted on application of shear forces. However, owing to the low propensity to flocculation documented (**Chapters 6** and **7**) for the WPH-MD-based emulsions and to different interfacial structures of oil globules in these emulsions, compared to emulsions stabilised by non-conjugated proteins/peptides, the shear thinning behaviour was attributed to the steric interactions promoted by the emulsion interfacial layer. Further investigation of the effect of the hairy interfaces in emulsions stabilised by conjugated protein, on their flow properties would provide novel relevant rheological information on these systems and may allow formation of food matrices with unique texture and mouthfeel experiences.

Drying kinetics and interfacial rheology

Differences in the spray drying behaviour of model IF emulsion systems, evidenced by different topographies of powder particles and different extent of particle cohesion, were associated with the physicochemical properties of emulsifier systems used to stabilise these emulsions (**Chapter 8**). The composition of the interfacial films of oil globules in the emulsions were shown to impact on the rheological properties of these films (e.g., rigidity-flexibility balance) and, effectively influence the stability of emulsions against globule-globule interactions (**Chapters 4** and **8**). Topographical features of model IF powders reported in **Chapter 8** (i.e., round *vs* shrivelled surfaces, presence/absence of vacuoles) revealed a relationship between emulsifier systems used in the formulations and mechanical properties of skin/wall layer of an atomised droplet/powder particle during drying (i.e., ductile *vs* brittle wall, rate of wall solidification). Further investigation of the influence of emulsifiers on the drying kinetics, powder surface composition and physicochemical properties of powders may provide important insights into optimisation of the industrial conditions (e.g., inlet and outlet temperatures, atomiser selection) for drying dairy-based powders, depending on the emulsifiers present in the formulation. Scientific outcomes from such study may lead to greater control of the drying behaviour and physicochemical properties of resultant powders, (e.g., controlling particle size by promoting/limiting 'spontaneous' agglomeration; controlling powder flowability by influencing surface topography; controlling powder stickiness by manipulation of surface composition) by modification of the composition and functional properties of emulsifiers used (e.g., surface adsorption rates, hydrophobic-hydrophilic balance and M_w).

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Appendix:

Peer-reviewed scientific articles

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ORIGINAL RESEARCH

Influence of lecithin on the processing stability of model whey protein hydrolysate-based infant formula emulsions

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Whey protein hydrolysate (WPH)-based oil-in-water (O/W) emulsions containing lecithin (0–5%, w/w, oil) were produced and stored at 4 °C for 14 days. Surface tension and interfacial tension of these systems were measured for formulation development. Fat globule size distribution (FGSD) analysis and confocal laser scanning microscopy (CLSM) were used to assess the physical stability of emulsions during storage and identify mechanisms of instability. Lecithin decreased interfacial tension between oil and aqueous phases of model emulsions and allowed formation of smaller oil droplets on homogenisation. However, low-intermediate levels (1–3%) of lecithin caused coalescence and shift to bimodal FGSD during storage of emulsions.

Keywords Whey protein hydrolysate, Emulsion, Lecithin, Confocal laser scanning microscopy, Infant formula, Interfacial tension.

INTRODUCTION

Bovine milk is widely used as a base material for the manufacture of infant nutritional products; however, its composition differs considerably from that of human milk. Differences in the protein content (i.e. 33 g/L and 9–11 g/L in bovine and human milk, respectively), ratio of casein: whey protein (i.e. 80:20 and 40:60 in bovine and human milk, respectively) and amino acid composition need to be considered during ingredient selection and formulation development in the manufacture of infant formula (IF) products. Other ingredients used in the formulation of IF products are lactose, maltodextrins and corn syrup solids as sources of carbohydrates, blends of vegetable and fish oils (to mimic the fatty acid composition of human milk), minerals, vitamins and emulsifiers such as lecithins or mono- and diglycerides (Alles *et al.* 2004; MacLean *et al.* 2010).

Cow's milk allergy (CMA) is a condition observed in early childhood, and on average 2.2% of children below the age of 2 years are affected (Natale *et al.* 2004; Tammineedi *et al.* 2013). Partial or limited enzymatic hydrolysis of protein can help in reducing CMA-related issues by offering 'predigested' formula for infants. Manufacturers of infant nutritional ingredients/products employ enzymatic hydrolysis to produce formulas which are easier to digest; these products are generally modified cow's milk formula, often based exclusively on whey protein, and are suggested as being suitable for infants experiencing feeding discomfort and digestion-related issues (O'Mahony *et al.* 2011).

Considerable challenges encountered in the manufacture of partially hydrolysed whey protein-based IF emulsions are related to poor heat stability during processing, coalescence and creaming and lipid oxidation on storage. Several studies have focused on improving the stability of these types of IF systems (Tirok *et al.* 2001; Christiansen *et al.* 2004; Ye *et al.* 2004; Ye and Singh 2006). Emulsion stability and fat globule size distribution (FGSD) of model IF are known to be influenced by protein content (McCarthy *et al.* 2012). Improvement of heat stability (McSweeney *et al.* 2004, 2008) and oxidative stability (Zou and Akoh 2013) of IF systems with increasing lecithin content has also been

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reported. However, intact dairy protein was used as the protein source in the aforementioned studies, and there is currently a lack of detailed information on the processing stability of hydrolysed whey protein-based IF emulsions. Studies involving hydrolysed whey protein and their properties have shown that the degree of hydrolysis (DH) is generally a good indicator of protein functionality (Foegeding *et al.* 2002) and low levels of hydrolysis (i.e. DH ≤10–20%) are beneficial (Singh and Dalgleish 1998; Caessens *et al.* 1999; Van der Ven *et al.*, 2001; Luck *et al.* 2002; Ruiter and Voragen 2002) while more extensively hydrolysed (i.e. DH >20%) proteins increasingly lose their structure and display decreased techno-functionality (Agboola and Dalgleish 1996a,b; Scherze and Muschiolik 2001).

The incorporation of low molecular weight emulsifiers, such as phospholipids (commonly from lecithin) or mono- and diglycerides, generally increases the physical stability of hydrolysed protein-based emulsions due to their ability to adsorb at the oil/water interface and effectively decrease the interfacial tension (Dickinson 1998, 2001; Tirok *et al.* 2001; Ruiter and Voragen 2002). Arising from decreased interfacial tension, smaller fat globules can be formed during homogenisation (Van Aken *et al.* 2003; Diftis and Kiosseoglou 2004; O'Brien 2009) and the rapid action of low molecular weight surfactants adsorbing at the interface prevents rapid coalescence (Dickinson *et al.* 1989). Production of emulsions with narrow FGSD, where volume–surface average diameter is <1.0 μm (i.e. typically ~0.5 μm) (Buchheim and Dejmek 1997), is desirable in the manufacture of infant formula emulsions and, when achieved, usually indicates good emulsifying properties of the ingredients (McCarthy *et al.* 2012).

Lecithin is used in the manufacture of IF products to enhance emulsifying properties or storage stability by forming a physical barrier in the form of a cohesive film around oil droplets (McClements 2004; Ghosh and Rousseau 2010). Lecithin is also known to enhance stability of emulsions while heating (Agboola *et al.* 1998a; Van der Meeren *et al.* 2005; Le *et al.* 2007; McSweeney *et al.* 2008), which is especially important in the manufacturing processes of IF emulsions based on hydrolysed protein. Additionally, the amphiphilic nature of lecithin gives it good functionality as a wetting agent; therefore, it can also be used to aid instant properties of milk/infant formula powders (O'Mahony *et al.* 2011; Sharma *et al.* 2012; Hammes *et al.* 2015).

The use of hydrolysed whey proteins in the formulation of IF products is of growing interest; however, limited formulation research has been carried out in this area. In this study, the effects of lecithin on the processing and physical stability of model IF emulsions prepared with hydrolysed whey protein were studied. The effect of different levels of addition of lecithin on the interfacial tension in O/W systems and the consequences thereof for the manufacture and stability of model hydrolysed whey protein-based IF emulsions during storage were investigated.

MATERIALS AND METHODS

Materials

Whey protein hydrolysate (WPH), Hyprol®, was obtained from Kerry Group, plc. (Listowel, Co. Kerry, Ireland). Maltodextrin (Maldex 170 with dextrose equivalent value of 17) and de-oiled powdered soya bean lecithin (Ultralec® P) were obtained from Syral Belgium NV (Aalst, Belgium) and ADM (Decatur, IL, USA), respectively. Soya bean oil (Organic Soya Oil, Clearspring Ltd., London, UK) was purchased from a local commercial outlet. All other chemicals, reagents and minerals used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Characterisation of whey protein hydrolysate

Protein, ash, moisture and fat contents of WPH were determined by Kjeldahl (IDF Standard 20-1, 2014), ashing at 500 °C for 5 h (IDF Standard 90, 1979), oven-drying at 103 °C for 5 h (IDF Standard 26, 2004) and Rose-Gottlieb method (IDF Standard 9C, 1987), respectively; lactose content was determined by difference. Degree of hydrolysis of the WPH ingredient was determined by the trinitrobenzenesulphonic acid method as described by Adler-Nissen (1979).

Size distribution of peptides in the WPH was determined by size exclusion chromatography (SEC) using a TSK G2000SW, 600 × 7.5 mm column (10 μm, Sigma-Aldrich, Dublin, Ireland); elution was with an isocratic gradient of 30% acetonitrile containing 0.1% TFA (v/v) at 1.0 mL/min. The samples were diluted in water or running buffer, 20 μL of 1 g/L protein/peptide solutions was injected onto the column and the elution was monitored with UV absorbance at 214 nm. Commercial β-lactoglobulin A, bovine serum albumin and caseinomacropeptide (CMP) (Sigma-Aldrich, Dublin, Ireland) were used as standards with ribonuclease A, cytochrome C, aprotinin, bacitracin, His-Pro-Arg-Tyr, Leu-Tyr-Met-Arg, bradykinin, Leu-Phe and Tyr-Glu (Bachem AG, Bubendorf, Switzerland) used as molecular weight standards. Standards were prefiltered through 0.22-μm low-protein-binding membrane filters (Sartorius Stedim, Surrey, UK) or centrifuged at 10 000 × *g* for 20 min prior to application to the column. All solvents were filtered under vacuum through a 0.45-μm high-velocity filter (Millipore Ltd., Durham, UK).

Preparation of emulsions

Model infant formula emulsions containing 1.55, 3.50 and 7.00 g/100 mL of protein, oil and carbohydrate, respectively, were prepared as follows: WPH and maltodextrin (MD) were solubilised in de-ionised water (d.H_2O) and preheated to 75 °C with continuous mixing using an overhead stirrer at 500 rpm for 1 h. Solutions (1 mL) containing individually iron sulphate heptahydrate, zinc sulphate heptahydrate, manganese sulphate monohydrate or copper sulphate (to give final added iron, zinc, manganese and

copper concentrations of 800, 600, 33 and 5 μg/100 mL, respectively) were then added to the protein/carbohydrate solution.

Stock lecithin-containing soya bean oil was prepared by adding lecithin to preheated (55 °C) oil during continuous mixing with magnetic stirring on a hotplate (55 °C) for 60 min. The stock solution was then added to soya bean oil (55 °C) to give five different lecithin concentrations (1–5%, w/w, oil). Aqueous and oil phases were subsequently mixed and maintained at 55 °C until homogenisation. A control was prepared with soya bean oil alone (i.e. no added lecithin). Emulsions were formed by prehomogenisation with an Ultra-Turrax at 710 g for 2 min followed by two-stage homogenisation (double pass) at 10 and 2 MPa, using a valve homogeniser (APV GEA Niro-Soavi S.p.A., Italy) at 50 °C. Following homogenisation, the pH of each emulsion was adjusted to 6.8 with 0.1 N HCl and/or 0.1 N NaOH and sodium azide (0.05%, w/v) was added to prevent microbial growth during storage.

Table 1 Composition, degree of hydrolysis (DH) and peptide size distribution of the whey protein hydrolysate (WPH) used in the preparation of emulsions

Characteristics	*% (w/w)*
Protein	77.7
Lactose	11.6
Ash	4.92
Moisture	4.83
Fat	0.99
Degree of hydrolysis (%)	10.7
Peptide distribution (based on molecular weight)	% of total protein
>20 kDa	4.68
10–20 kDa	3.85
5–10 kDa	5.65
2–5 kDa	21.2
1–2 kDa	24.5
0.5–1 kDa	22.5
<0.5 kDa	17.6

Compositional analysis of emulsions

Kjeldahl (IDF Standard 20-1, 2014) and Gerber (IDF Standard 105, 2008) methods were used for the determination of protein and fat levels of emulsions, respectively. Moisture content was measured by oven-drying at 103 °C for 5 h (IDF Standard 21,2010). Ash was measured using muffle furnace heating at 500 °C for 5 h (AOAC, 2002). Carbohydrate content was calculated by difference.

Surface and interfacial tension analysis

Dynamic surface tension

Surface tension (γ_S) measurements were performed at 55 °C (to best replicate emulsion preparation) under atmospheric pressure with a Krüss K12 tensiometer (Krüss GmbH, Hamburg, Germany) equipped with a Wilhelmy plate. γ_S was measured over 60 min after formation of the surface in filtered d.H_2O (Milli-Q system), WPH solution (1.55%, w/v) and soya bean oil containing different levels of soya bean lecithin (0–5%, w/w). Samples containing lecithin were prepared by adding the lecithin to preheated (55 °C) oil and allowing it to mix fully at 55 °C with continuous intermediate-speed stirring for 60 min. The protein solution was prepared as described earlier with only protein added; filtered d.H_2O was used as a control. Aliquots (25 mL) were placed in the sample vessel, and air bubbles (if present) were removed with a Pasteur pipette. Before each measurement, the plate attachment and the sample vessel were washed with acetone and d.H_2O followed by annealing over a flame to ensure removal of all organic matter. Glassware used in the analysis was subjected to an acid wash; that is, after thorough washing with detergent and water, glassware was filled to overflow with 1 N nitric acid, left overnight and rinsed 3 times with d.H_2O before drying.

Dynamic interfacial tension

Measurements of interfacial tension (γ_I) at the soya bean oil/lecithin (0–5%, w/w) interface with d.H_2O or protein

Table 2 Composition of model infant formula emulsions containing different levels of lecithin (0–5%, w/w, oil)

Lecithin content (%, w/w)	*Protein (%, w/w)*	*Fat (%, w/v)*	*Carbohydrate (%, w/w)*	*Moisture (%, w/w)*	*Ash (%, w/w)*
0.00	1.67 ± 0.02[a]	3.48 ± 0.09[a]	7.78 ± 0.48[a]	87.0 ± 0.55[a]	0.10 ± 0.00[a]
1.00	1.68 ± 0.03[a]	3.44 ± 0.04[a]	8.02 ± 0.80[a]	86.7 ± 0.77[a]	0.10 ± 0.01[a]
2.00	1.69 ± 0.01[a]	3.43 ± 0.02[a]	8.10 ± 0.56[a]	86.7 ± 0.54[a]	0.11 ± 0.00[b]
3.00	1.69 ± 0.06[a]	3.44 ± 0.01[a]	8.23 ± 1.03[a]	86.5 ± 1.03[a]	0.11 ± 0.01[b]
4.00	1.70 ± 0.02[a]	3.47 ± 0.01[a]	8.12 ± 0.63[a]	86.6 ± 0.63[a]	0.12 ± 0.00[c]
5.00	1.70 ± 0.03[a]	3.51 ± 0.01[a]	8.29 ± 0.67[a]	86.4 ± 0.69[a]	0.12 ± 0.01[c]

[(a–c)]Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

solution (1.55%, w/v; WPH) were also carried out with a Krüss K12 tensiometer using the Wilhelmy plate method. Samples were prepared as detailed earlier for dynamic surface tension and measured at 55 °C over 60 min; 25 mL of heavy phase (water or protein solution) and 25 mL of light phase (oil or oil containing lecithin) were used. γ_I was recorded continuously from 0 to 5 min and at 10, 15, 30 and 60 min after forming the interface. The measurement program was set to record a maximum of 80 readings per given time point at 1sec intervals, unless the standard deviation was ≤0.01 in ten consecutive readings, in which case the measurement would stop for the given time point. The sample vessel and the Wilhelmy plate were cleaned and annealed before each measurement, and all glassware was acid-washed as described earlier.

Measurement of fat globule size distribution

Fat globule size distribution (FGSD) of the emulsions was measured using a laser light diffraction unit (Mastersizer S, Malvern Instruments Ltd., Worcestershire, UK) equipped with a 300 RF (reverse fourier) lens and He-Ne laser (λ of 633 nm). A polydisperse model with 3NAD presentation and a particle and dispersant refractive index of 1.46 and 1.33 were selected for data analysis as described by McCarthy *et al.* (2012). Sample was introduced to the mixing chamber and dispersed in d.H_2O until a laser obscuration of 14% (± 0.5%) was reached. Measurements were taken on emulsions immediately after homogenisation (day 0) and after 4, 7, 11 and 14 days of storage at 4 °C.

Confocal laser scanning microscopy analysis

The microstructural analysis of emulsions was performed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany). Protein and lipid were fluorescently labelled with Nile blue dye (Sigma-Aldrich, Wicklow, Ireland); 50 μL of the dye solution was added to 1 mL of emulsion followed by vortex mixing for 5 s. Visualisation of oil and protein in emulsions (10 μL) was carried out using an Ar laser operating at an excitation wavelength of 488 nm with emission detected between 500 and 530 nm and a He-Ne laser operating at an excitation wavelength of 633 nm with emission detected between 565 and 615 nm for oil and protein, respectively (Auty *et al.* 2001). The observations were performed using 20× and 63× oil immersion objectives. At least three specimens of each sample were observed to obtain representative micrographs of samples.

Statistical data analysis

Analysis of variance (ANOVA) was carried out using Minitab® 16 (Minitab Ltd, Coventry, UK, 2010) statistical analysis package. The Tukey method was used to obtain grouping information on the treatment means. The level of significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Characterisation of whey protein hydrolysate

The composition, degree of hydrolysis and peptide size distribution data of the WPH used in the preparation of emulsions are shown in Table 1. Lactose levels (i.e. innate carbohydrate component of formulation) were taken into consideration when preparing the emulsions.

Composition of emulsions

Compositional analysis of emulsions showed that measured levels (Table 2) were satisfactorily near target levels. Ash levels were found to be statistically different ($P < 0.05$), and an increase in its level was found to follow the increase in the levels of lecithin addition to the emulsions, suggesting that the contribution of ash present in the lecithin

Table 3 Surface and interfacial tension of soya bean oil systems containing different levels of lecithin (w/w, oil) measured at 55 °C

	Surface tension		*Interfacial tension*		
Sample	γ_S	$\Delta\gamma_S$[5]	*Interface*[2]	γ_I	$\Delta\gamma_I$[5]
	mN/m				
	Initial γ_S/γ_I[3]				
SBO[1]	30.8 ± 0.1		O/W	9.4 ± 0.3	–
			O/P	4.3 ± 0.1	–
	Equilibrium $\gamma_{SEq}/\gamma_{IEq}$[4]				
SBO	30.3 ± 0.2[a]	0.5	O/W	3.2 ± 0.1[a]	6.2
			O/P	1.1 ± 0.2[b]	8.3
SBO & 1% lecithin	29.4 ± 0.3[ab]	1.4	O/P	<1.0[6]	>8.4
SBO & 2% lecithin	28.4 ± 0.1[b]	2.4	O/P	<1.0	>8.4
SBO & 3% lecithin	28.7 ± 0.4[b]	2.1	O/P	<1.0	>8.4
SBO & 4% lecithin	28.4 ± 0.4[b]	2.4	O/P	<1.0	>8.4
SBO & 5% lecithin	28.2 ± 0.2[b]	2.5	O/P	<1.0	>8.4

[1]SBO represents soya bean oil.

[2]Interface: O/W represents an interface between the oil and filtered de-ionised water; O/P represents an interface between oil and the protein solution (1.55%, w/v).

[3]Initial surface or interfacial tension recorded immediately upon formation of the surface/interface.

[4]Equilibrium surface and interfacial tension (γ_{SEq} and γ_{IEq}, respectively) recorded at 1 h of surface/interface age.

[5]The total decrease in γ from the formation of clean$_{surface/interface\ until}$ reaching the equilibrium is presented as $\Delta\gamma$.

[6]The sensitivity limit of the instrument is 1 mN/m; thus, samples showing γ values lower than this limit are presented as <1.0.

[(a–b)]Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

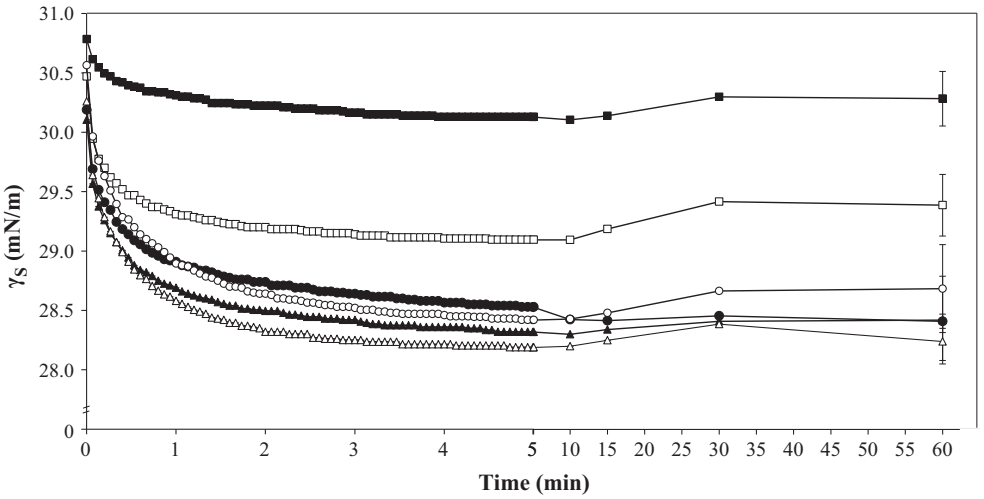

Figure 1 Dynamic surface tension of soya bean oil samples containing different levels of lecithin (%, w/w): no lecithin (■), 1% (□), 2% (●), 3% (○), 4% (▲) and 5% (△) lecithin (w/w).

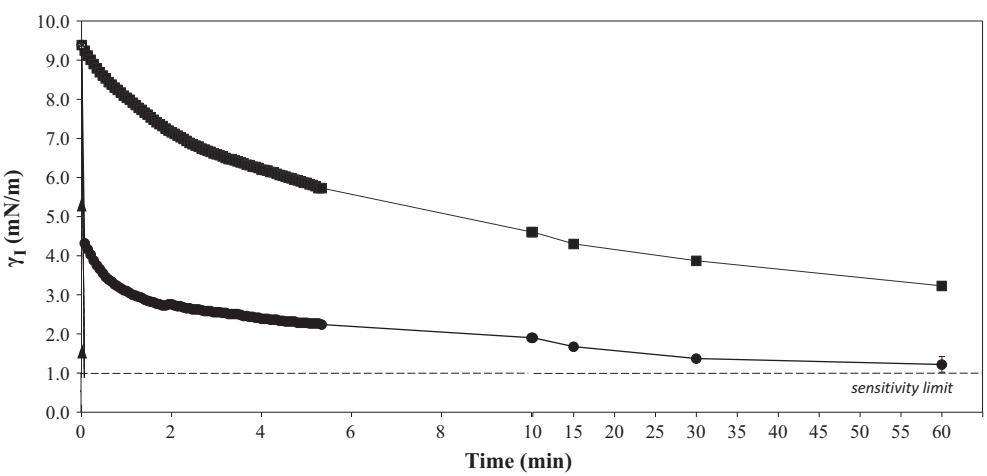

Figure 2 Dynamic interfacial tension in samples composed of soya bean oil/water (■) and soya bean oil/protein (1.55%, w/v, protein) (●). Vertical line(s) (▲) represent the γ_I of lecithin-containing systems. Horizontal dashed line represents the sensitivity limit (1 mN/m) of the K12 processor tensiometer. Any readings below this limit are not shown in the diagram.

(9.6%) led to the differences in ash levels between formed emulsions.

Surface and interfacial tension data

Dynamic surface tension

Upon formation of a new surface in the soya bean oil sample with no lecithin added, the initial surface tension (γ_S) was 30.8 mN/m (Table 3, Figure 1); this decreased to 30.3 once equilibrium surface tension (γ_{SEq}) was reached after 1 h (Table 3, Figure 1). This initial γ_S value (30.8 mN/m) of the control oil (i.e. no lecithin added) was assumed to be representative of a clean surface (i.e. at point of surface formation) and was used as the initial value in all measured systems (Figure 1). A rapid decrease in the γ_S was observed in all oil samples containing lecithin as the surface aged (i.e. time after formation of a new surface). The majority of the decrease was observed to take place within the first 5 min of surface ageing, and the rate and extent of decrease in γ_S increased with increasing lecithin content (Figure 1). Values presented in Table 3 show that addition of 1% lecithin resulted in γ_{SEq} of 29.4 mN/m, a reduction of 1.4 mN/m compared to the control. When the lecithin content was increased to 2%, the γ_{SEq} was further reduced by 1.0 mN/m (i.e. γ_{SEq} of 28.4 mN/m). Higher levels of addition of lecithin (i.e. 3–5%) did not contribute to any further decrease in γ_S (no significant differences in γ_{SEq} between these samples), and γ_{SEq} values for samples containing 2–5% lecithin

Table 4 Fat globule size distribution of model infant formula emulsions prepared with different levels (0–5%, w/w, oil) of lecithin during storage at 4 °C at 0, 4 and 14 days post homogenisation

		Fat Globule Size Parameter (μm)				
Lecithin addition (%, w/w, oil)	*Storage time (days)*	$D_{[4,3]}$ [1]	$D_{[3,2]}$ [2]	$D_{(v,0.1)}$ [3]	$D_{(v,0.5)}$ [4]	$D_{(v,0.9)}$ [5]
0.00 (Control)	0	0.97 ± 0.03^{a}	0.63 ± 0.02^{a}	0.32 ± 0.01^{a}	0.82 ± 0.02^{a}	1.80 ± 0.08^{a}
	4	0.96 ± 0.02^{a}	0.61 ± 0.02^{a}	0.32 ± 0.02^{a}	0.81 ± 0.03^{a}	1.81 ± 0.09^{a}
	14	0.97 ± 0.03^{c}	0.63 ± 0.02^{ab}	0.32 ± 0.01^{a}	0.82 ± 0.02^{a}	1.79 ± 0.05^{a}
1.00	0	0.93 ± 0.01^{ab}	0.62 ± 0.02^{a}	0.32 ± 0.01^{a}	0.79 ± 0.01^{ab}	1.69 ± 0.01^{a}
	4	1.49 ± 0.24^{a}	0.61 ± 0.03^{a}	0.32 ± 0.02^{a}	0.77 ± 0.03^{ab}	1.90 ± 0.20^{ab}
	14	2.26 ± 0.26^{ab}	0.66 ± 0.01^{a}	0.33 ± 0.01^{a}	0.77 ± 0.01^{b}	7.20 ± 1.04^{b}
2.00	0	0.89 ± 0.00^{bc}	0.60 ± 0.01^{a}	0.31 ± 0.00^{a}	0.77 ± 0.01^{bc}	1.68 ± 0.04^{ab}
	4	1.31 ± 0.31^{a}	0.60 ± 0.04^{a}	0.31 ± 0.01^{a}	0.75 ± 0.03^{ab}	1.78 ± 0.09^{a}
	14	2.29 ± 0.37^{ab}	0.64 ± 0.03^{ab}	0.31 ± 0.01^{a}	0.74 ± 0.02^{b}	7.68 ± 1.81^{b}
3.00	0	0.91 ± 0.02^{bc}	0.60 ± 0.02^{a}	0.31 ± 0.01^{a}	0.76 ± 0.02^{bc}	1.67 ± 0.03^{b}
	4	1.60 ± 0.27^{a}	0.60 ± 0.03^{a}	0.31 ± 0.02^{a}	0.75 ± 0.03^{ab}	1.99 ± 0.43^{a}
	14	2.33 ± 0.33^{a}	0.63 ± 0.01^{ab}	0.31 ± 0.01^{a}	0.74 ± 0.01^{b}	7.73 ± 1.44^{b}
4.00	0	0.87 ± 0.01^{bc}	0.59 ± 0.02^{a}	0.31 ± 0.01^{a}	0.75 ± 0.02^{bc}	1.60 ± 0.03^{b}
	4	1.17 ± 0.22^{a}	0.59 ± 0.03^{a}	0.31 ± 0.01^{a}	0.73 ± 0.03^{ab}	1.69 ± 0.11^{a}
	14	1.63 ± 0.01^{bc}	0.61 ± 0.01^{ab}	0.31 ± 0.01^{a}	0.73 ± 0.02^{b}	2.06 ± 0.16^{a}
5.00	0	0.86 ± 0.03^{c}	0.58 ± 0.03^{a}	0.31 ± 0.02^{a}	0.74 ± 0.02^{c}	1.59 ± 0.01^{b}
	4	1.13 ± 0.27^{a}	0.58 ± 0.02^{a}	0.31 ± 0.01^{a}	0.72 ± 0.02^{b}	1.66 ± 0.13^{a}
	14	1.30 ± 0.21^{c}	0.60 ± 0.02^{b}	0.31 ± 0.01^{a}	0.72 ± 0.02^{b}	1.70 ± 0.18^{a}

[1]$D_{[4,3]}$ represents volume mean diameter.

[2]$D_{[3,2]}$ represents Sauter mean diameter.

[3]$D_{(v,0.1)}$ represents fat droplet size in the 10% quantile of the distribution.

[4]$D_{(v,0.5)}$ represents fat droplet size in the 50% quantile of the distribution.

[5]$D_{(v,0.9)}$ represents fat droplet size in the 90% quantile of the distribution.

$^{(a–c)}$Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

were found to be within a narrow range (i.e. 28.2–28.7 mN/m). A similar trend was reported by McSweeney *et al.* (2008) where addition of lecithin up to a level of 2 g/L facilitated formation of small oil droplets in model IF emulsions where average droplet diameter decreased with increasing lecithin level; however, lecithin levels above 2 g/L did not contribute to further reduction in oil droplet sizes.

Dynamic interfacial tension

Interfacial tension (γ_I) between oil and aqueous phases displayed a similar pattern as observed for surface tension, where initial γ_I decreased rapidly following formation of the interface. Initial γ_I recorded at the interface between soya bean oil (SBO) and filtered d.H_2O (i.e. control system) of 9.4 mN/m was used as an initial γ_I for all measured systems (Figure 2) as it represented a clean interface (i.e. an interface with no surfactants present). Equilibrium interfacial tension (γ_{IEq}) of the control system, recorded after 1 h, was 3.2 mN/m (Table 3). The majority of the decrease in γ_I was achieved within 15 min of the interface formation.

Measured γ_{IEq} between soya bean oil and protein solution was 1.1 mN/m. The further reduction in γ_{IEq} of 2.1 mN/m (i.e. from 3.2 to 1.1 mN/m) observed in the soya bean oil and aqueous phase system when hydrolysed protein was introduced indicated the effectiveness of hydrolysed whey protein (DH 10.7%) in decreasing the γ_I. The rate at which γ_I decreased upon interface formation was markedly higher in the protein-containing system (i.e. majority of the decrease was observed within 5 min of formation of the interface; Figure 2). This shows the high mobility and effectiveness of peptides in rapidly reducing γ_I (Chobert *et al.* 1988; Turgeon *et al.* 1992; Singh and Dalgleish 1998; Kong *et al.* 2007 Seta *et al*. 2014). The effectiveness of partially hydrolysed protein in reducing the interfacial tension is due to the presence of low–intermediate molecular weight peptides (Table 1) and their flexible structure with both hydrophobic and hydrophilic sites localised along the peptide chain, unfolding (i.e. bigger peptides) and aligning upon adsorption at the interface, thereby forming a viscoelastic film (Lam and Nickerson 2013) and lowering the interfacial tension between the two phases.

Samples where lecithin was added (1–5%, w/w) to the oil phase displayed rapid reduction in the γ_I reaching values lower than 1.0 mN/m (sensitivity limit) immediately after

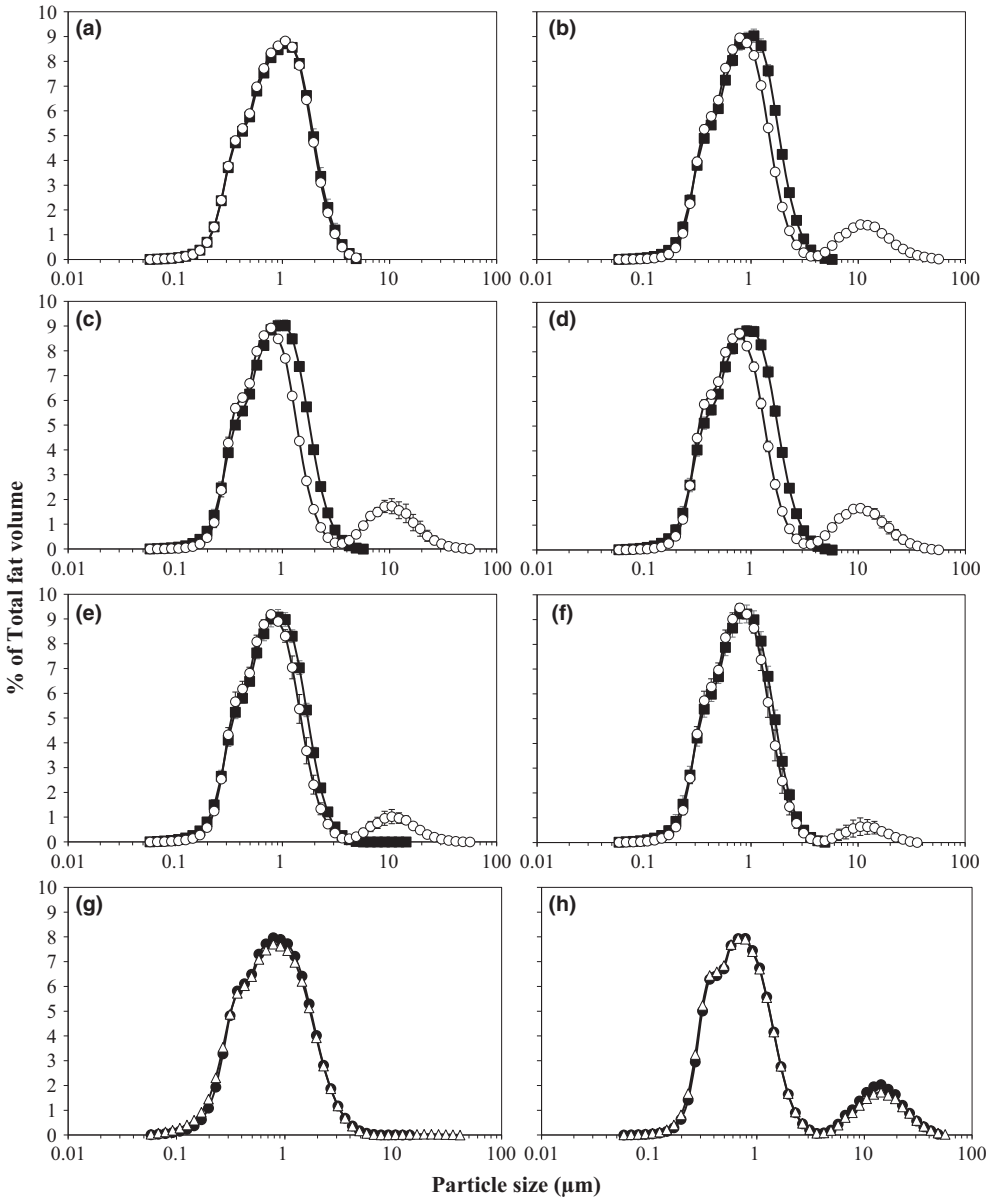

Figure 3 Fat globule size distribution profiles of emulsions prepared with lecithin at levels of 0% (a), 1% (b), 2% (c), 3% (d), 4% (e) or 5% (f) (w/w, oil) post-homogenisation (■) and after 14 days storage at 4 °C (○). Size distribution of oil droplets in emulsions containing 0% (g) and 1% (h) lecithin (w/w, oil) after 10 days of storage at 4 °C with water (●) or 0.2%, w/v, SDS (△) used as the dispersant.

formation of the interface (Figure 2). This can be explained by the behaviour of small surface active agents such as those present in lecithin (i.e. phospholipids) which migrate rapidly through the dispersant and adsorb at the interface allowing the γ_{IEq} to be reached in a very short time (Mezdour *et al.* 2008). Such a rapid decrease in γ_I by phospholipids was also reported by Kabalnov *et al.* (1995) where the γ_{IEq} was reached in <1 s. Low molecular weight phospholipids display higher mobility and manoeuvrability compared to proteins (and large peptides); thus, they can displace larger surfactants (i.e. such as protein and peptides) from the surface/interface (Van Aken *et al.* 2003; Diftis and Kiosseoglou 2004; Lam and Nickerson 2013) resulting in rapid reduction of γ_I.

Fat globule size distribution in emulsions

Fat globule size distribution (FGSD) of oil droplets in emulsions after homogenisation (0 day) showed that all samples, irrespective of level of addition of lecithin, were able to form good quality emulsions with narrow size distribution (Table 4). The mean volume diameter (D[4,3]) of a control emulsion (i.e. emulsion prepared without lecithin) was

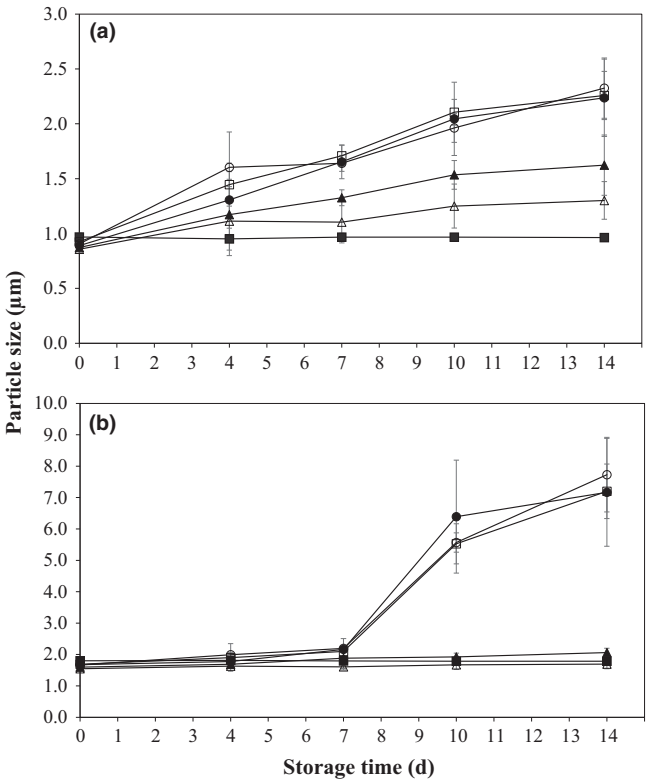

Figure 4 Fat globule size distribution parameters: (a) mean volume diameter ($D_{4,3}$) and (b) 90% quantile size distribution ($D_{(v,0.9)}$) for oil droplets in emulsions prepared with lecithin at levels of 0% (■), 1% (□), 2% (●), 3% (○), 4% (▲) and 5% (△) (w/w, oil) stored at 4 °C over 14 days.

0.97 µm, and increasing lecithin level generally resulted in lower values of D[4,3] with smallest D[4,3] value of 0.86 µm found in the 5% (w/w, oil) lecithin-containing emulsion. Size distributions of oil droplets of all formed emulsions were very similar with a general trend of smaller droplets formed in emulsions with higher level of addition of lecithin, which was also reported by McSweeney *et al.* (2008) in intact milk protein-based model IF emulsions.

Changes in FGSD of lecithin-containing emulsions were observed during storage at 4 °C, while no changes were observed in the control emulsion (Figure 3). D[4,3] increased in all emulsions formed with lecithin on storage at 4 °C, and this effect was most pronounced for samples containing 1–3% (w/w, oil) lecithin (Figure 4). Emulsions prepared with 3% (w/w, oil) lecithin showed greatest increase in particle size (Table 4); D[4,3] increased from 0.91 µm (0 day) to 2.33 µm (14 days). Development of large oil droplets on storage decreased at higher (4–5%, w/w, oil) lecithin addition levels giving D[4,3] of 1.63 and 1.30 µm for 4 and 5% (w/w, oil) lecithin-containing emulsions at 14 days, respectively. In the emulsions that displayed increases in particle size during storage, this was represented by a shift of FGSD distribution from monomodal to bimodal (Figure 3) with a second peak evident at ~10 µm within 4 days of storage at 4 °C. FGSD profiles showed a decrease in the number of larger oil droplets (i.e. flattening of the second peak) as the lecithin level in emulsions was increased, particularly to 4% and 5% lecithin. The total percentage of large oil droplets (i.e. the area under second peak) was approximately 2- and 3-fold lower in emulsions with lecithin content of 4% and 5%, respectively, as compared to that of the 3% lecithin-containing emulsion. FGSD results (Table 4) also showed that changes in particle size were only detected in the 90% quantile of the size distribution (i.e. D(v, 0.9)). After 14 days of storage at 4 °C, emulsions with 1, 2 and 3% (w/w, oil) lecithin showed an increase in D(v, 0.9) of 5.51 (i.e. from 1.69 to 7.20 µm), 6.00 (i.e. from 1.68 to 7.68 µm) and 6.06 µm (i.e. from 1.67 to 7.73 µm), respectively, while it increased by 0.46 (i.e. from 1.60 to 2.06 µm) and 0.11 µm (i.e. from 1.59 to 1.70 µm) in 4 and 5% lecithin-containing emulsions, respectively. No increase in D(v, 0.9) was observed in the control emulsion during storage. The destabilising effect of lecithin on oil droplets in emulsions was also observed by Zou and Akoh (2013) who showed that the presence of lecithin (0.4 g/L) resulted in larger particle sizes after storage (28 days at room temperature) in intact milk protein model IF emulsions.

Results from the current study indicated that the presence of soya bean lecithin in WPH-based emulsion systems can promote interactions between oil droplets and result in their coalescence. Studies by Cruijisen (1996) and Agboola *et al.* (1998a) showed similar trends where the presence of unmodified soya bean lecithin (1.5 and 1.0–2.5 g/L, respectively) promoted coalescence of oil globules in caseinate- and WPH-based (DH 27%) O/W emulsions, respectively. A study by Van der Meeren *et al.* (1995) showed that low values of interfacial tension due to the presence of lecithin at the O/W interface had a negative effect on the stability of emulsions against flocculation. Decreased physical stability of emulsions containing WPH and lecithin was also observed by Tirok *et al.* (2001) using more extensively hydrolysed protein (DH 23–29%) and lecithin (4.8 g/L). In the current study, a shift in FGSD from monomodal to bimodal was observed in lecithin-containing emulsions on storage at 4 °C. However, only a small proportion of the total population of oil droplets was affected by the size increase and distribution shift as changes in oil droplet size in all lecithin-containing emulsions were only found for the 90% size distribution quantile (i.e. D(v, 0.9)) while D(v, 0.1) or D(v, 0.5) did not display any concurrent increase in size. Interestingly, the stability to coalescence in emulsions containing lecithin improved with its higher levels of addition; formation of large oil droplets was found to be significantly lower in 4% and lower still in 5% lecithin-containing emulsions. It is worthwhile to note that, even with the development of a second peak, FGSD remained narrow in the majority of the population (i.e. first peak) of all lecithin-containing emulsions.

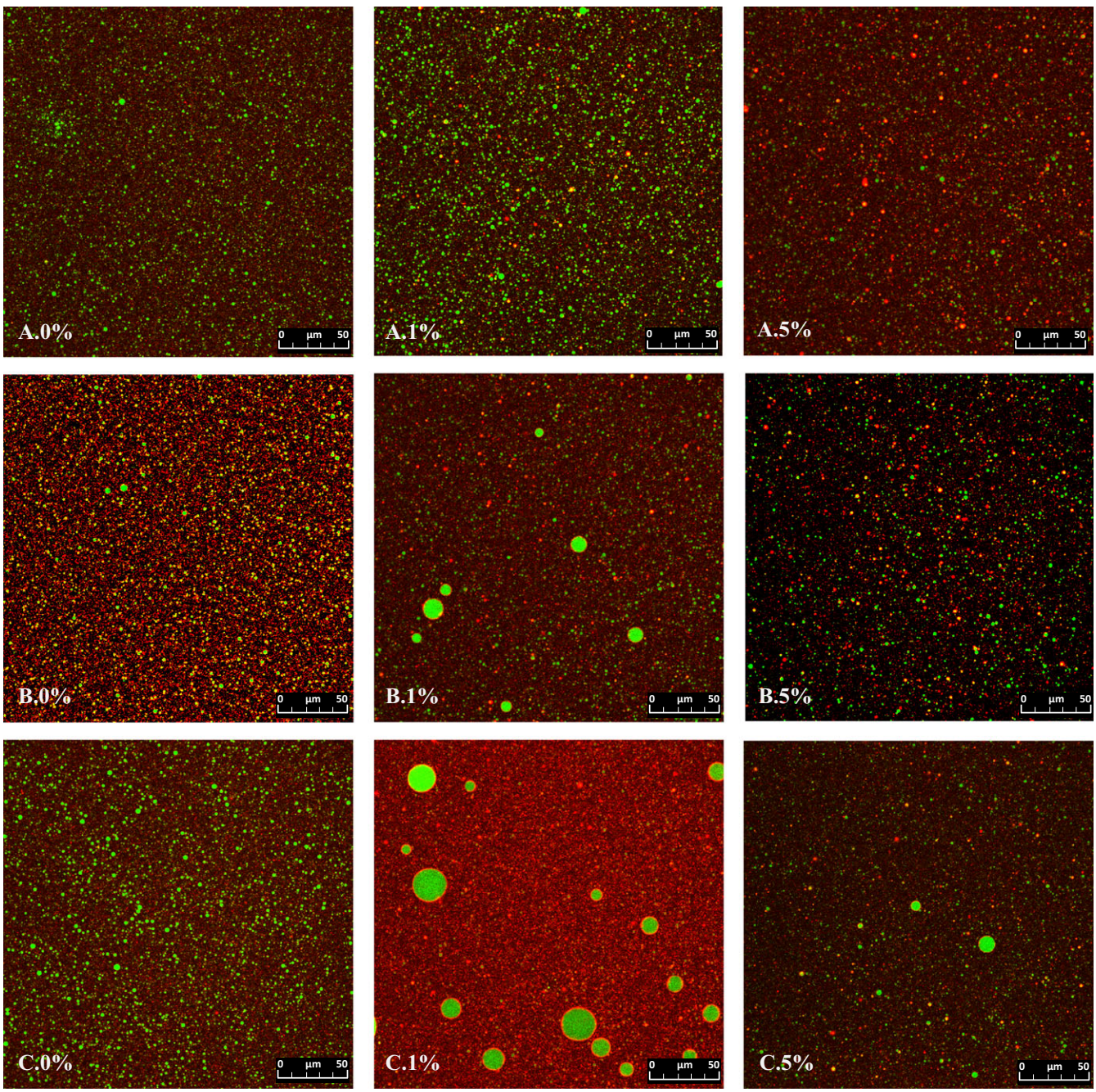

Figure 5 Confocal laser scanning microscopy images of model infant formula emulsions containing 0%, 1% and 5% lecithin (w/w, oil) after 1 (A), 7 (B) and 14 (C) days of storage at 4 °C. Micrographs present overall size distribution of oil droplets (green) in different emulsions over time. Scale bar (bottom right) = 50 µm.

A preliminary experiment (Figure 3g–h) was carried out where stored (10 days at 4 °C) emulsions were treated with the dissociating agent, sodium dodecyl sulphate (SDS), and FGSD was subsequently measured using an approach similar to that used by Tomas *et al.* (1994), Agboola *et al.* (1998b) and Tirok *et al.* (2001). A bimodal distribution and large particle size (as represented by the 90% quantile) continued to be detected following addition of dissociating agent which suggests that coalescence (as opposed to flocculation) of oil droplets was the main mechanism of emulsion instability in the samples. This is in agreement with the work carried out by Agboola *et al.* (1998b) and Ye and Singh (2006) who reported a similar destabilisation mechanisms in WPH-based emulsions.

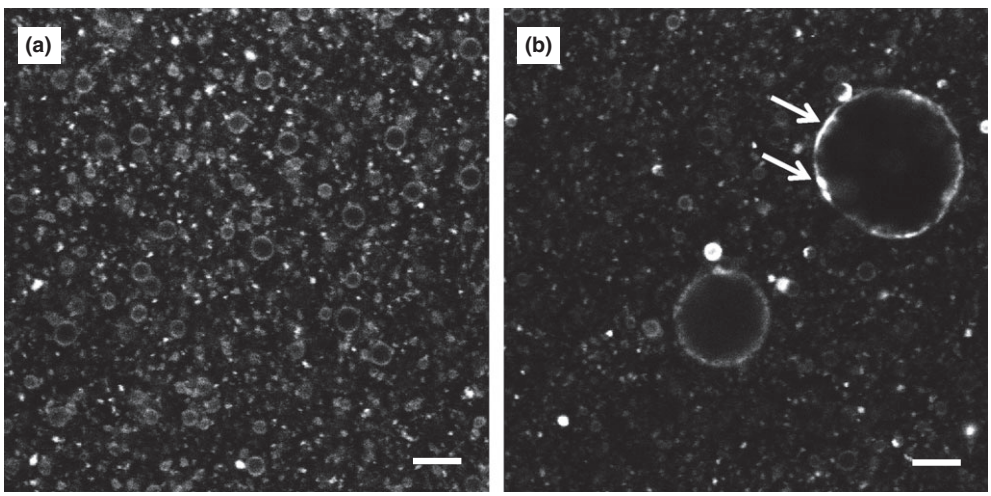

Figure 6 High-magnification confocal micrographs of emulsions after storage at 4 °C for 14 days showing the protein-labelled channel. (a) 0% lecithin; (b) 1% lecithin (w/w, oil). Arrows indicate variable thickness of protein at the oil droplet interface. Scale bar = 5 μm.

Confocal laser scanning imaging of emulsions

Confocal laser scanning microscopy (CLSM) showed that freshly prepared (i.e. 1 day after homogenisation) emulsions had fine and uniformly distributed oil droplets (Figure 5). There were no visible differences between the control (i.e. 0% lecithin), 1 and 5% lecithin (w/w, oil) emulsions, supporting the FGSD results. Development of a small number of larger oil droplets (10–15 μm) was observed in emulsions containing 1% lecithin (w/w, oil) after 7 days of storage at 4 °C. No changes in the size of oil droplets were observed for control and 5% lecithin (w/w, oil) emulsions at that time point. CLSM micrographs showed increased numbers of large (10–30 μm) oil droplets in emulsions containing lower lecithin levels (i.e. 1–3%) after 14 days of storage at 4 °C. In emulsions containing 4–5% lecithin, some bigger oil globules (5–10 μm) were formed after 14 days of storage at 4 °C. These droplets, however, were smaller, considerably less numerous and formed at a slower rate (i.e. changes were not observed until 14 days of storage) as compared to emulsions with lower lecithin levels (1–3%). No visual differences in size distribution of oil droplets after 14 days of storage at 4 °C were observed in the control sample. Large oil droplets were also evident in emulsions containing >1% lecithin (w/w, oil) (data not shown). WPH-based emulsions formed with these lecithin levels formed large oil droplets during short-term refrigerated storage with significant changes observed after 7 days. CLSM micrographs confirmed that lecithin (when added at levels of 1–3%, w/w, oil) in model WPH-based IF emulsions promoted coalescence of oil droplets during storage (14 days) at 4 °C. Micrographs showed large and uniform oil droplets in the emulsion containing 1% lecithin (w/w, oil) after 14 days storage. This supported earlier findings suggesting that coalescence was the main mechanism responsible for emulsion instability in lecithin-containing WPH-based emulsions.

Differences in the larger (>3 μm) fat globule interface were seen at higher magnification, and an example of this is given in Figure 6, which shows an uneven thickness of protein at the oil–droplet interface (Figure 6b, arrows). This uneven protein thickness was observed for many of the larger oil droplets in all samples containing lecithin, but was not observed in droplets of the WPH control (0% lecithin) sample (Figure 6a). This suggests a possible partial displacement and aggregation of interfacial whey protein by the lecithin over time, which may also help explain the coalescence of oil droplets containing lecithin. More study is needed to characterise the precise nature of the interfacial material, for example using fluorescently labelled phospholipids.

CONCLUSIONS

This study shows that an effective decrease in the interfacial tension between the oil and aqueous phase in the manufacture of model infant formula emulsions produced with hydrolysed whey protein can be achieved by the incorporation of low levels of lecithin (i.e. 1%, w/w, oil). Emulsions formed with hydrolysed whey protein displayed narrow size distribution of oil droplets which was further reduced by the incorporation of lecithin. It was, however, shown that low-to-intermediate levels (1–3%) of lecithin decreased the stability of emulsions during storage at 4 °C by promoting coalescence of oil droplets. Confocal microscopy proved to be a helpful tool for studying coalescence in emulsions, and it complemented light scattering work in the current study.

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Improving thermal stability of hydrolysed whey protein-based infant formula emulsions by protein–carbohydrate conjugation

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ABSTRACT

Whey protein hydrolysate (WPH) ingredients are commonly used in the manufacture of partially-hydrolysed infant formulae. The heat stability of these emulsion-based formulae is often poor, compared with those made using intact whey protein. The objective of this study was to improve the heat stability of WPH-based emulsions by conjugation of WPH with maltodextrin (MD) through wet heating. Emulsions stabilised by different protein ingredients, whey protein isolate (WPI_E), whey protein hydrolysate (WPH_E), heated WPH (WPH-H_E), and WPH conjugated with MD (WPH-C_E) were prepared and heat treated at 75 °C, 95 °C or 100 °C for 15 min. Changes in viscosity, fat globule size distribution (FGSD) and microstructure, evaluated using confocal laser scanning microscopy (CLSM), were used to monitor the effects of hydrolysis, pre-heating and conjugation on the heat stability of the emulsions. Heat stability increased in the order WPH_E < WPI_E << WPH-H_E <<< WPH-C_E; emulsions WPH_E, WPI_E and WPH-H_E destabilised on heating at 75 °C, 95 °C or 100 °C, respectively. Flocculation and coalescence of oil droplets were mediated by protein aggregation (as evidenced by CLSM) on heat treatment of WPH-H_E emulsion at 100 °C, while no changes in FGSD or microstructure were observed in WPH-C_E emulsion on heat treatment at 100 °C, demonstrating the excellent thermal stability of emulsions prepared with the conjugated WPH ingredient, due principally to increased steric stabilisation as a result of conjugation.

1. Introduction

Human milk is widely accepted as the best source of nutrients required for proper short- and long-term development of infants. The composition of mother's milk is compatible with the infant's digestive system and is known to minimise the risk of gastrointestinal and respiratory infections (Alles, Scholtens, & Bindels, 2004; Exl, 2001; O'Mahony, Ramanujam, Burgher, & O'Callaghan, 2011). However, it is not always possible to provide the infant with mother's milk. Efforts to develop humanised formulae for infant nutrition are focused on many aspects of formula composition and functionality including matching protein content and profile (i.e., whey-dominant protein profile and α-lactalbumin enrichment) (Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004; Crowley, Dowling, Caldeo, Kelly, & O'Mahony, 2016; Hambraeus, 1977; Ogra & Greene, 1982; O'Mahony et al., 2011), fatty acid profile (Berger, Fleith, & Crozier, 2000), carbohydrate, vitamin and mineral levels to those present in human milk (Pehrsson, Patterson, & Khan, 2014).

Formulae manufactured using whey protein hydrolysate (WPH) ingredients can be categorised based on the degree of hydrolysis of the protein; the main categories are amino acid-based formulae (AAF), where proteins/peptides are hydrolysed to their constituent amino acids; extensively hydrolysed formulae (EHF) containing oligopeptides with molecular weight below 3000 Da and partially hydrolysed formulae (PHF) containing oligopeptides ranging in molecular weight up to 20,000 Da (Exl, 2001; Lowe et al., 2011). While AAF and EHF products are mainly intended for therapeutic purposes in infants suffering from, or with a high risk of cow's milk allergy (CMA), infant nutrition products from the PHF group cannot be used for therapeutic purposes but are recommended for infants at risk of CMA as they have been shown to provide a preventive effect thereon (Chandra, 1997; Exl, 2001; von Berg et al., 2008). Partially hydrolysed formulae are often also referred to as 'pre-digested' formulae based on their improved digestibility and absorption in the gut, helping to reduce gastrointestinal discomfort issues (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014).

Hydrolysis causes alteration to the functional properties of proteins and hydrolysate functionality is ultimately dependent on a number of factors including enzyme type and specificity, hydrolysis conditions and method of enzyme inactivation (Panyam & Kilara, 1996; Tavano, 2013). Generally, moderate hydrolysis improves the surface activity of proteins/peptides as the hydrolysate fractions migrate rapidly to

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surfaces/interfaces which can give rise to improved functional properties such as foaming and emulsification (Agboola & Dalgleish, 1996a,b; Banach, Lin, & Lamsal, 2013; Foegeding & Davis, 2011; Kilara & Panyam, 2003). Moderate hydrolysis of globular proteins (i.e., whey proteins) improves their heat stability as a result of the diminished secondary structure; however, this improvement does not always translate directly to more complex systems such as emulsions made using hydrolysed whey protein, where heat stability has been shown to be negatively affected by hydrolysis of whey protein (Singh & Dalgleish, 1998; Ye & Singh, 2006). Responsibility for the poor heat stability of hydrolysed whey protein-based emulsions is related to reduced steric hindrance (Ye, Hemar, & Singh, 2004) and increased number of available (i.e., exposed) reactive sites on protein/peptide molecules at the oil globule surface and in the serum phase of the emulsion (Euston, Finnigan, & Hirst, 2000; Hunt & Dalgleish, 1995).

Conjugation of proteins with carbohydrates using the Maillard reaction has been shown to be effective in modifying protein functionality (Liu, Ru, & Ding, 2012; Oliver, Melton, & Stanley, 2006; O'Regan & Mulvihill, 2010a,b). Extensive research documenting the beneficial effects of protein modification through conjugation is available in the scientific literature; improved functional properties of proteins including solubility, emulsification, encapsulation and emulsion stability (Akhtar & Dickinson, 2003; Kasran, Cui, & Goff, 2013a,b; Lei, Wang, Liang, Yuan, & Gao, 2014), thermal stability (Jimenez-Castano, Lopez-Fandino, Olano, & Villamiel, 2005; Kato, Aoki, Kato, Nakamura, & Matsuda, 1995; Liu et al., 2012; O'Regan & Mulvihill, 2010a; Wang & Zhong, 2014) or foaming and gelation properties (Campbell, Raikos, & Euston, 2003; Martínez & Pilosof, 2013) as a result of conjugation are well documented. However, published scientific reports on the properties and functionality of hydrolysed whey protein ingredients modified by Maillard conjugation appear to be limited; the authors are not aware of any published studies reporting on the performance of such ingredients in oil-in-water emulsion systems, particularly in infant formula (IF) systems. The current study aims to investigate and report on the performance of ingredients produced by conjugation of hydrolysed whey protein with maltodextrin in comparison with that of intact whey protein in production and stabilisation of model IF emulsions.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) and whey protein hydrolysate (WPH; 8% degree of hydrolysis; DH) were obtained from Carbery Food Ingredients Ltd. (Ballineen, Co. Cork, Ireland). Composition of WPI and WPH ingredients was determined using standard International Dairy Federation (IDF) methods and molecular weight profile of the protein ingredients was determined using size exclusion chromatography as detailed by Drapala, Auty, Mulvihill, and O'Mahony (2015). The composition, DH and molecular weight profile of the WPI and WPH ingredients are shown in Table 1. Maltodextrin (MD) was obtained from Corcoran Chemicals Ltd. (Dublin, Ireland) and had moisture and ash contents of <5.0% and <0.2%, respectively. Soybean oil was obtained from Frylite Group Ltd. (Strabane, Co. Tyrone, Northern Ireland). All other chemicals and reagents used in the study were of analytical grade and sourced from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland).

2.2. Conjugate and stock protein solutions

Two unheated stock solutions (5.00 g/100 mL protein) were prepared from WPI and WPH and allowed to hydrate for 18 h at 4 °C and pH was adjusted to 6.8 before being used for emulsion formulation. The protein–carbohydrate conjugate solution was prepared by solubilising required quantities of WPH and MD in ultrapure water for 2 h at 20 °C using a magnetic stirrer to give 5.00 g/100 mL protein and 5.00 g/100 mL carbohydrate. The solution was adjusted to pH 8.2 with 0.5 N potassium hydroxide (KOH) and allowed to hydrate for 18 h at 4 °C, before being readjusted to pH 8.2 with 0.5 N KOH at 20 °C. Aliquots (250 mL) of this solution were placed in 500 mL screw-capped, glass conical flasks and heated at 90 °C for 8 h. After heating for 8 h, the solutions were cooled immediately to 4 °C and stored at that temperature overnight. A control for the heat treatment was prepared in exactly the same way as outlined above with the exception that no MD was added to the WPH. In summary, four stock protein or protein–carbohydrate solutions were prepared and were subsequently used to formulate emulsions that are referred to as whey protein isolate emulsion (WPI_E), whey protein hydrolysate emulsion (WPH_E), heated whey protein hydrolysate emulsion (WPH-H_E) and conjugated whey protein hydrolysate emulsion (WPH-C_E), respectively.

Table 1
Composition, degree of hydrolysis and molecular weight profile of the whey protein isolate (WPI) and whey protein hydrolysate (WPH) ingredients used in the preparation of emulsions.

Composition	WPI	WPH
	% *w*/*w*	
Protein	87.2 ± 0.9	83.7 ± 0.5
Fat	0.72 ± 0.1	0.67 ± 0.1
Carbohydrate[a]	4.21	7.80
Ash	2.76 ± 0.1	2.92 ± 0.1
Moisture	5.11 ± 0.0	4.91 ± 0.1
Degree of hydrolysis	NA[b]	8.00
Molecular weight profile	% of total protein	
Insoluble	0.00	2.00 ± 0.6
>20 kDa	28.0 ± 3.4	12.0 ± 1.6
10–20 kDa	50.5 ± 3.7	24.2 ± 8.8
5–10 kDa	3.90 ± 0.2	9.49 ± 1.9
2–5 kDa	15.6 ± 0.3	11.9 ± 1.6
1–2 kDa	0.92 ± 0.1	9.30 ± 2.0
0.5–1 kDa	0.29 ± 0.0	11.0 ± 1.9
<0.5 kDa	0.83 ± 0.7	20.2 ± 3.0

[a] Carbohydrate content determined by difference.
[b] NA = not applicable.

2.3. Measurement of free thiol groups

The level of free thiol groups in the stock protein solutions was determined following an assay described by Hoffmann and van Mil (1997) with the exception that a Bis-Tris/HCl buffer (pH 6.8) was used in place of the Tris–HCl buffer (as performed by Alting, Hamer, De Kruif, Paques, & Visschers, 2003). Aliquots (0.05 mL) of stock protein solutions (5.00 g/100 mL) were added to 2.70 mL of 0.05 M Bis-Tris/HCl buffer (pH 6.8) before adding 0.25 mL of Ellman's reagent (107.5 mg/100 g of the buffer) (Ellman, 1959). Solutions were vortexed and absorbance was measured using a dual beam UV–visible spectrophotometer (Varian Cary 300, Varian Ltd., Walton-on-Thames, UK) at a wavelength of 412 nm. Measurements were completed in triplicate and the level of thiol groups was calculated using a molar extinction coefficient for 2-nitro-5-mercapto-benzoic acid (i.e., Ellman's reagent) of 13,600 M^{-1} cm^{-1}.

2.4. Preparation of emulsions

Model infant formula emulsions containing 1.55, 3.50 and 7.00 g/100 mL of protein, oil and carbohydrate, respectively, were prepared as follows: stock protein or protein–carbohydrate solutions (see Section 2.2) were diluted with ultrapure water to the appropriate concentration followed by addition of MD as required with continuous mixing using a magnetic stirrer at intermediate speed for 1 h at 22 °C to prepare the aqueous phases of the emulsions. Innate levels of lactose present in the protein powders were taken into account when calculating the requirement for added carbohydrate (i.e., MD). Emulsions were prepared as described by Drapala et al. (2015) except that higher 1st and

2nd stage homogeniser pressures of 15 and 3 MPa, respectively, were used.

2.5. Composition and colour analysis of emulsions

Protein, fat, moisture, ash and carbohydrate content of emulsions were determined using standard IDF methods as detailed by Drapala et al. (2015). The colour of the emulsions was measured using a pre-calibrated colorimeter (Minolta Chroma Meter CR-400, Minolta Ltd., Milton Keynes, U.K.) The emulsions were loaded into a glass cell (CM-A98, optical path length: 10 mm) held in position by means of a transmittance specimen holder (CM-A96) and positioned with a white plate behind the glass cell. Colour was expressed using the Commission Internationale de l'Eclairage (CIE) colour chromaticity L* a* b* scale (L = dark/light, a = red/green, b = yellow/blue).

2.6. Measurement of fat globule size distribution and zeta potential

Fat globule size distribution (FGSD) of the emulsions was measured using a laser light-diffraction unit (Mastersizer S, Malvern Instruments Ltd., Worcestershire, UK) equipped with a 300 RF (reverse Fourier) lens and He–Ne laser (λ of 633 nm). A polydisperse model with 3NAD presentation was used for unheated and heated emulsions as described by McCarthy et al. (2012). The 3NHD presentation was also used for heated emulsions to estimate the size of protein particles/aggregates present as described by Ciron, Gee, Kelly, and Auty (2010). The samples were introduced to the mixing chamber and dispersed in ultrapure water to reach an obscuration of 14% (±0.5%). Measurements of FGSD were made on emulsions on the day of homogenisation and immediately after heat treatment. The zeta potential (ζ) of oil droplets in emulsions was measured using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) as detailed by Joshi et al. (2012). Each emulsion was diluted 1:100 with ultrapure water, adjusted to pH 6.8 with KOH or HCl and allowed to equilibrate at 25 °C for 120 s in the cuvette prior to analysis. The measurement was performed on the day of homogenisation using an automatic voltage selection and ζ was calculated using the Smoluchowski model (Kirby & Hasselbrink, 2004).

2.7. Measurement of emulsion viscosity on heating

Emulsions (28 g) were heated in an AR-G2 controlled stress rheometer (TA Instruments, Crawley, West Sussex, UK) equipped with a starch pasting cell (SPC) geometry. The heating programme was chosen to allow sample equilibration for 2 min at 15 °C with no shearing followed by holding for 5 min at 15 °C, heating at 10 °C/min to reach the required target temperature (75 °C or 95 °C), peak temperature hold for 15 min, cooling at 10 °C/min to reach 15 °C and holding at 15 °C for 5 min while constantly shearing at a rate of 15 s^{-1} throughout analysis. Apparent viscosity (η) data was recorded at 1 s intervals during the heating programme. An oil bath was used to heat treat more stable emulsions at 100 °C; samples (2.5 mL) were placed in glass tubes, stoppered and immersed in an oil bath for 15 min at 100 °C with constant mixing of tube contents by gently rocking at approx. 8 min^{-1}, giving a constant, gentle, flow of the liquid in the tube. Emulsion samples were recovered after all heat treatments (i.e., from SPC and oil bath tubes) and used for further analysis (FGSD and microstructural analysis).

2.8. Confocal laser scanning microscopy analysis

Microstructural analysis of emulsions was performed using a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) as detailed by Drapala et al. (2015). Protein and lipid were fluorescently labelled with Nile Blue dye (Sigma-Aldrich, Dublin, Ireland). Visualisation of oil and protein in emulsions (10 μL) was carried out using an Ar laser (excitation = 488 nm, emission = 500–530 nm) and a He–Ne laser (excitation = 633 nm, emission = 565–615 nm) for oil and protein, respectively (Auty, Twomey, Guinee, & Mulvihill, 2001). The observations were performed using 20× and 63× oil immersion objectives. At least three specimens of each sample were observed to obtain representative micrographs of samples.

2.9. Statistical data analysis

Analysis of variance (ANOVA) was carried out using the Minitab® 16 (Minitab Ltd., Coventry, UK, 2010) statistical analysis package. The Tukey method was used to obtain grouping information. The level of significance was determined at $P < 0.05$.

3. Results

3.1. Composition and colour of emulsions

Compositional analysis of emulsions showed that measured levels (Table 2) were in line with target levels for all samples (i.e., 1.39–1.45% protein, 3.35–3.42% fat and 6.47–6.52% carbohydrate) and no significant differences in composition were found between samples. Emulsions stabilised by heated/conjugated proteins (i.e., WPH-H_E and WPH-C_E) differed slightly but significantly in lightness (L* values; Table 2) from emulsions stabilised by unheated WPI or WPH; the lowest L* value was observed for WPH-C_E (82.6) followed by WPH-H_E (83.2), WPH_E (83.7) and WPI_E (84.0). A similar trend was observed in the intensity of the yellow colour of the emulsions where all samples were statistically different from each other and the highest b* value was observed for WPH-C_E (4.48) followed by WPH-H_E (2.24), WPH_E (0.78) and WPI_E (0.30). Lower L* and higher b* values in emulsions WPH-C_E and WPH-H_E, as compared to emulsions WPH_E and WPI_E, can be directly related to production of coloured compounds such as melanoidins during the later stages of the Maillard reaction (Oliver et al., 2006). Although no MD was added prior to heating of the WPH solution, innate lactose (a reducing sugar) present in the WPH powder (Table 1) would have contributed to some Maillard-induced browning during heating. As shown by Liu and Zhong (2015) lactose is more reactive than maltodextrin and therefore has greater propensity for Maillard-induced colour development as it contains more reducing groups per unit weight as compared to MD.

3.2. Fat globule size distribution and ζ-potential

Narrow and monomodal size distributions of oil droplets were observed in all four emulsions post-homogenisation (Fig. 1) with the samples having mean volume diameters ($D_{4,3}$; Table 3) of 0.85, 0.83, 0.80 and 0.79 μm for WPI_E, WPH_E, WPH-H_E and WPH-C_E emulsions, respectively. The size distribution data showed that all of the protein ingredients had good emulsifying properties as indicated by $D_{4,3}$ values of all emulsions less than 1 μm which was consistent with the results of McCarthy et al. (2012) and Drapala et al. (2015). There were no significant differences ($P < 0.05$) in FGSD between emulsions formed with the different ingredients immediately post homogenisation. The net negative charge (i.e., ζ potential) of oil droplets in emulsions was lowest in WPI_E (−48.0 mV; Table 3) followed by WPH_E (−49.6 mV), WPH-H_E (−53.1 mV) and WPH-C_E (−55.0 mV). Although ζ was slightly higher for emulsion droplets stabilised by heated WPH than for those stabilised by WPI and WPH and was slightly higher still for emulsion droplets stabilised by conjugated WPH, no significant differences were observed in ζ between droplets in the 3 emulsions prepared using hydrolysed whey protein. The positively charged amino acid lysine is predominantly involved in covalent attachment with reducing sugars during the Maillard reaction, thus by its interaction during heating/conjugation the net negative charge on the protein, and as a result, on the surface of the oil droplets in the emulsions increased, which was consistent with the results of Acedo-Carrillo et al. (2006); Liu et al.

Table 2
Composition and colour of model infant formula emulsions stabilised by the different whey protein ingredients.

Emulsion	Protein	Fat	Carbohydrate (%)	Ash	Total solids	Tristimulus coordinates		
						L*	a*	b*
WPI_E	1.45 ± 0.04^a	3.35 ± 0.03^a	6.52 ± 0.11^a	0.06 ± 0.03^a	11.4 ± 0.05^a	84.0 ± 0.11^a	-0.87 ± 0.01^a	0.30 ± 0.03^a
WPH_E	1.43 ± 0.04^a	3.39 ± 0.01^a	6.47 ± 0.04^a	0.10 ± 0.01^a	11.4 ± 0.06^a	83.7 ± 0.12^a	-1.08 ± 0.06^b	0.78 ± 0.06^b
WPH-H_E	1.39 ± 0.01^a	3.39 ± 0.05^a	6.50 ± 0.10^a	0.21 ± 0.15^a	11.5 ± 0.08^a	83.2 ± 0.26^b	-1.07 ± 0.05^b	2.24 ± 0.04^c
WPH-C_E	1.42 ± 0.02^a	3.42 ± 0.01^a	6.52 ± 0.18^a	0.13 ± 0.05^a	11.5 ± 0.11^a	82.6 ± 0.16^c	-1.01 ± 0.04^b	4.48 ± 0.02^d

$^{(a–c)}$Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

(2012) and Wang and Zhong (2014). Additionally, as a consequence of thermal denaturation of proteins, charged groups buried within the native structure of globular proteins, are exposed and this may also have contributed to the change in net protein charge (Tcholakova, Denkov, Ivanov, & Campbell, 2006).

After heating at 75 °C for 15 min in the starch pasting cell (SPC), the particle size distribution of WPI_E emulsion showed a limited broadening of the profile (Fig. 1a); however, only a minor difference was found in the $D_{4,3}$ before (0.85 μm) and after (0.87 μm) heat treatment of the emulsion under these conditions (Table 3). The WPH_E emulsion destabilised during heat treatment at 75 °C as evidenced by the presence of large particles ($D_{4,3}$ of 120 μm) in the sample after heat treatment (Fig. 1b; Table 3). Results obtained using both the 3NAD (i.e., selective for oil) and 3NHD (i.e., selective for protein) presentations displayed essentially the same size distribution profiles (Fig. 1b). Size distribution data and visual observation (i.e., phase separation with large particles buoyant in the semi-transparent serum phase and no free oil; Fig. 2a) of WPH_E emulsion heat treated at 75 °C suggested that the emulsion destabilised through aggregation of droplets via protein/peptides on the surface of oil droplets and possibly aggregation of droplets via interaction with non-adsorbed protein/peptides present in the serum resulting in entrapment of oil in the aggregated protein network. Previous studies have indicated that heat-induced destabilisation of protein-based oil-in-water (O/W) emulsions is often mediated by non-adsorbed serum proteins/peptides that, upon heating, interact with each other and with adsorbed proteins/peptides, thus causing formation of protein/oil complexes (Euston et al., 2000; Hunt & Dalgleish, 1995). In contrast to the WPH_E emulsion, no changes in particle size distribution were observed for WPH-H_E or WPH-C_E emulsions on heating at 75 °C for 15 min (Fig. 1c, d; Table 3).

Emulsions that displayed good thermal stability during heat treatment at 75 °C for 15 min were subjected to a more severe treatment of 95 °C for 15 min in the SPC. The WPI_E emulsion destabilised during heat treatment at 95 °C. Visual inspection of the sample after heat

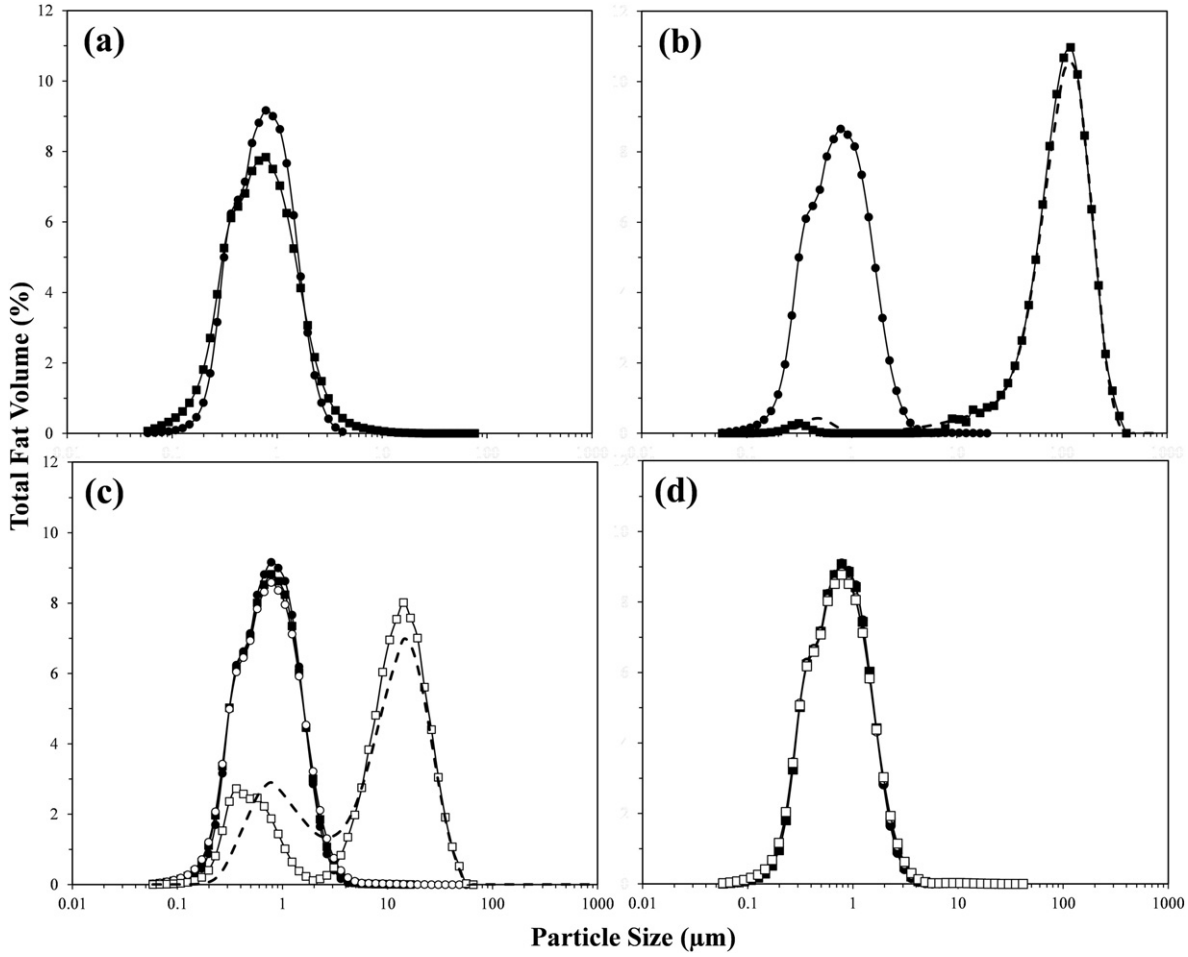

Fig. 1. Fat globule size distribution in WPI_E (a), WPH_E (b), WPH-H_E (c) and WPH-C_E (d) emulsions post-homogenisation (●) and after 15 min of heat treatment at 75 °C (■), 95 °C (○) and 100 °C (□). Dashed line (—) represents the 3NHD presentation profiles for destabilised emulsions formed with hydrolysed protein (b, c).

Table 3
Fat globule size distribution (FGSD) and zeta potential (ζ) of oil droplets in model infant formula emulsions WPI_E, WPH_E, WPH-H_E and WPH-C_E post homogenisation and post heating at 75, 95 or 100 °C for 15 min.

Emulsion	Heat treatment	Fat globule size parameter (μm)					ζ potential (mV)
		$D_{4,3}$[1]	$D_{3,2}$[2]	$D_{v,0.1}$[3]	$D_{v,0.5}$[4]	$D_{v,0.9}$[5]	
WPI_E	Unheated	0.85 ± 0.0[a]	0.56 ± 0.0[a]	0.30 ± 0.0[a]	0.70 ± 0.0[a]	1.52 ± 0.0[a]	−48.0 ± 2.6[a]
	75 °C × 15 min	0.87 ± 0.1[a]	0.48 ± 0.0[a]	0.24 ± 0.0[a]	0.64 ± 0.0[a]	1.69 ± 0.3[a]	n.d.[6]
	95 °C × 15 min	n.d.[7]	n.d.	n.d.	n.d.	n.d.	n.d.
	100 °C × 15 min	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
WPH_E	Unheated	0.83 ± 0.1[a]	0.54 ± 0.0[a]	0.29 ± 0.0[a]	0.69 ± 0.1[a]	1.54 ± 0.1[a]	−49.6 ± 3.0[ab]
	75 °C × 15 min	120 ± 30[b]	50.9 ± 37[b]	38.3 ± 3.8[b]	112 ± 29[b]	212 ± 59[b]	n.d.
	95 °C × 15 min	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	100 °C × 15 min	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
WPH-H_E	Unheated	0.80 ± 0.0[a]	0.55 ± 0.0[a]	0.30 ± 0.0[a]	0.68 ± 0.0[a]	1.46 ± 0.0[a]	−53.1 ± 1.2[ab]
	75 °C × 15 min	0.81 ± 0.0[a]	0.53 ± 0.0[a]	0.29 ± 0.0[a]	0.68 ± 0.0[a]	1.51 ± 0.1[a]	n.d.
	95 °C × 15 min	0.85 ± 0.0[a]	0.53 ± 0.0[a]	0.28 ± 0.0[a]	0.68 ± 0.0[a]	1.58 ± 0.1[a]	n.d.
	100 °C × 15 min	38.8 ± 29[b]	3.72 ± 2.3[b]	13.5 ± 19[a]	37.2 ± 39[a]	66.4 ± 61[b]	n.d.
WPH-C_E	Unheated	0.79 ± 0.0[a]	0.54 ± 0.0[a]	0.29 ± 0.0[a]	0.67 ± 0.0[a]	1.45 ± 0.0[a]	−55.0 ± 3.1[b]
	75 °C × 15 min	0.80 ± 0.0[a]	0.54 ± 0.0[a]	0.29 ± 0.0[a]	0.68 ± 0.0[a]	1.47 ± 0.1[a]	n.d.
	95 °C × 15 min	0.81 ± 0.0[a]	0.53 ± 0.0[a]	0.28 ± 0.0[a]	0.67 ± 0.0[a]	1.50 ± 0.1[a]	n.d.
	100 °C × 15 min	0.83 ± 0.0[a]	0.53 ± 0.0[a]	0.28 ± 0.0[a]	0.67 ± 0.0[a]	1.52 ± 0.1[a]	n.d.

[a–b] Values within a column, for individual heat treatments not sharing a common superscript differed significantly ($P < 0.05$).
[1] $D_{4,3}$ represents volume mean diameter.
[2] $D_{3,2}$ represents Sauter mean diameter.
[3] $D_{v,0.1}$ represents fat globule size in the 10% quantile of the distribution.
[4] $D_{v,0.5}$ represents fat globule size in the 50% quantile of the distribution.
[5] $D_{v,0.9}$ represents fat globule size in the 90% quantile of the distribution.
[6] n.d. = not determined. ζ potential measured in emulsions post homogenisation only.
[7] n.d. = not determined due to emulsion destabilisation and presence of large flocs of protein. In addition, samples that destabilised during a heat treatment were not subjected to more severe treatment.

treatment indicated formation of a separated coarse protein network and serum phase (Fig. 2b); hence, particle size distribution data for the WPI_E emulsion after the heat treatment at 95 °C could not be determined. The WPH-H_E and WPH-C_E emulsions displayed good thermal stability to heating at 95 °C for 15 min as evidenced by no significant increase in $D_{4,3}$ (<0.05 μm) on heating (Table 3; Fig. 1c, d) and the visual appearance of these samples after heating (Fig. 2c, d).

Differences in the thermal stability of WPH-H_E and WPH-C_E emulsions were observed when the emulsions were heated at 100 °C for 15 min in an oil bath; visual assessment post heating indicated formation of large particles (i.e., aggregates) in the WPH-H_E emulsion (Fig. 2e). This was confirmed by particle size analysis whereby the FGSD profile of WPH-H_E showed a shift from monomodal to bimodal (Fig. 1c). The emulsion stabilised by WPH-C was stable to heat treatment at 100 °C for 15 min; no significant differences in $D_{4,3}$ (<0.05 μm) or in FGSD profiles were observed after heat treatment as compared to post-homogenisation (Table 3, Fig. 1d) and no visual evidence of destabilisation (Fig. 2e) was observed in the emulsion after heating.

3.3. Apparent viscosity of emulsions on heating

Apparent viscosity of all emulsions before heating (i.e., at 15 °C) was similar with no significant ($P < 0.05$) differences found between samples (Table 4). On increasing temperature from 15 °C to 75 °C apparent

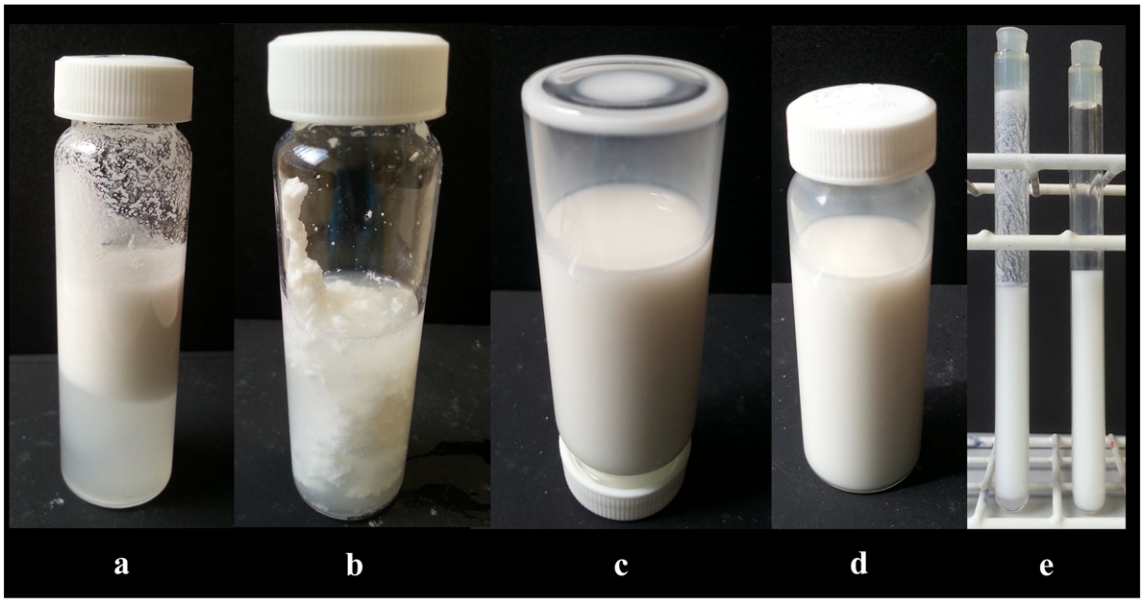

Fig. 2. Photographs of emulsions post heat treatment at 75 °C for 15 min (a: WPH_E) and at 95 °C for 15 min (b: WPI_E; c: WPH-H_E; d: WPH-C_E). Emulsions WPH-H_E and WPH-C_E were also heated at 100 °C for 15 min in an oil bath (e: left = WPH-H_E; right = WPH-C_E).

Table 4
Apparent viscosity of WPI_E, WPH_E, WPH-H_E or WPH-C_E emulsions at different stages of heat treatment using a starch pasting cell at 75 °C and 95 °C for 15 min.

Holding temperature (°C)	Measurement stage	Viscosity (mPa·s)			
		WPI_E	WPH_E	WPH-H_E	WPH-C_E
75	Pre-heating	14.3 ± 0.2^{aA}	13.5 ± 0.8^{aA}	14.0 ± 0.0^{aA}	14.0 ± 0.1^{aA}
	Reaching peak temperature	10.6 ± 0.3^{aB}	12.7 ± 1.0^{bA}	9.69 ± 0.1^{aB}	9.40 ± 0.2^{aB}
	Peak hold	10.4 ± 0.1^{abB}	13.1 ± 1.7^{aA}	9.69 ± 0.0^{bB}	9.68 ± 0.0^{bB}
	Post-heating	15.2 ± 0.3^{aA}	22.0 ± 3.2^{bB}	14.1 ± 0.0^{aA}	14.1 ± 0.1^{aA}
95	Pre-heating	14.6 ± 0.6^{aA}	n.d.[1]	14.1 ± 0.1^{aB}	14.1 ± 0.1^{aA}
	Reaching peak temperature	176 ± 201^{aA}	n.d.	8.71 ± 0.0^{aA}	8.99 ± 0.2^{aB}
	Peak hold	457 ± 357^{bA}	n.d.	9.09 ± 0.0^{aA}	9.05 ± 0.0^{aB}
	Post-heating	98.6 ± 71^{bA}	n.d.	15.0 ± 0.4^{aC}	14.8 ± 0.6^{aA}

[a–b]Values within a row (horizontal) not sharing a common superscript differed significantly ($P < 0.05$).
[A–B]Values within a column (vertical) for each of the heat treatments (i.e., 75 or 95 °C) not sharing a common superscript differed significantly ($P < 0.05$).
[1] n.d. = not determined as sample destabilised during less severe heat treatment (i.e., 75 °C for 15 min).

viscosity of all emulsion samples decreased (Fig. 3a). Decreasing viscosity with increasing temperature is commonly observed in protein solutions; however, the decrease in viscosity normally continues until a protein-specific temperature is reached at which point physical changes to the protein affect its structure (i.e., unfolding of polypeptide/peptide chain, disruption of hydrophobic interactions and aggregation by covalent and non-covalent bonding), generally causing an increase in viscosity (Considine, Patel, Anema, Singh, & Creamer, 2007; Goetz & Koehler, 2005). Hence, the onset of structural changes and interactions (eventually leading to destabilisation) in protein-based emulsions can be identified by tracking changes in their apparent viscosity during heat treatment. Although final viscosity of the WPI_E emulsion (i.e., after cooling to 15 °C) was slightly higher compared to the initial viscosity (i.e., at 15 °C before heating) of the sample, no significant differences were found between viscosity of the WPI_E emulsion before and after heating at 75 °C for 15 min (Table 4). An increase in viscosity

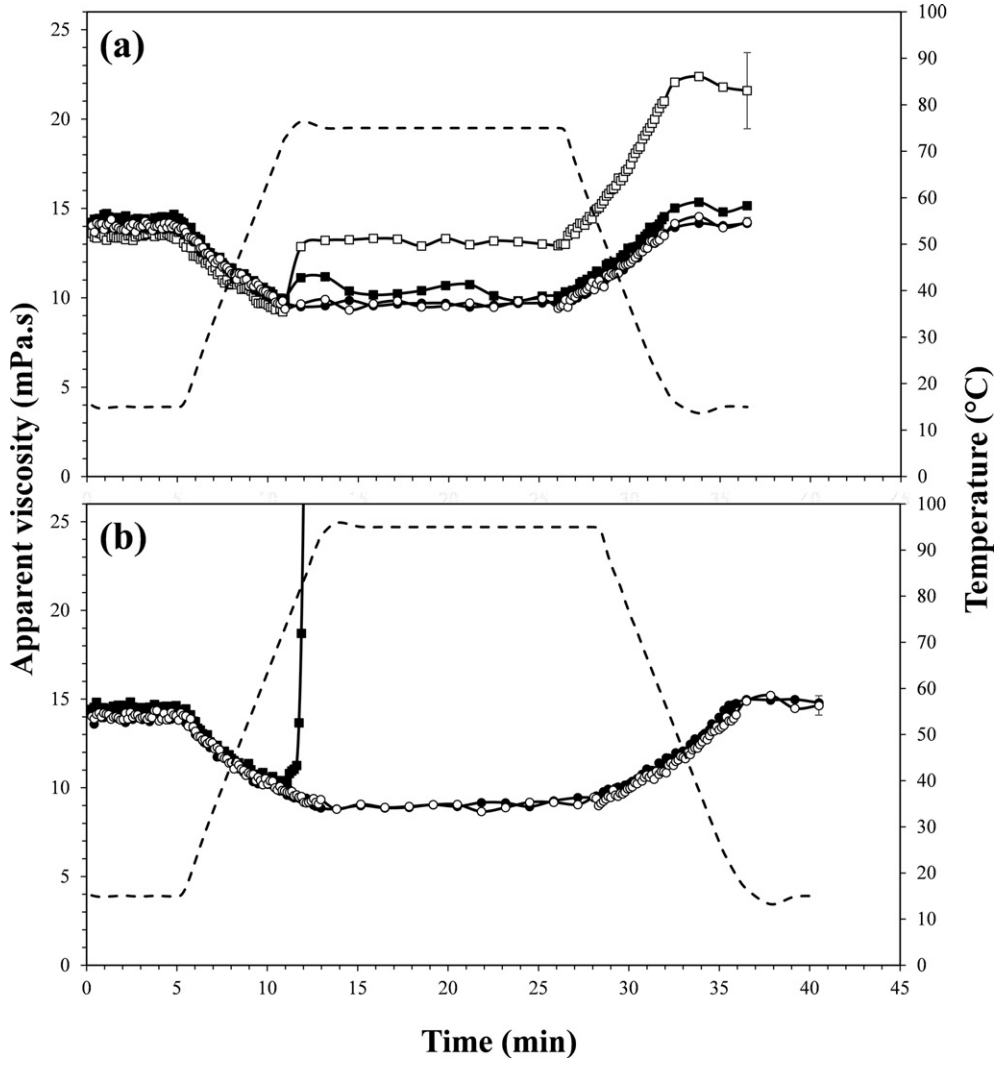

Fig. 3. Apparent viscosity profiles of WPI_E (■), WPH_E (□), WPH-H_E (●) and WPH-C_E (○) emulsions during heat treatments in a starch pasting cell (SPC) with peak temperature hold of 75 °C (a) and 95 °C (b). Dashed line represents the temperature profile.

from 9.6 to 12.7 mPa·s (Fig. 3a) before reaching the peak hold temperature (75 °C) was observed for WPH_E. Final viscosity of the WPH_E emulsion (i.e., after cooling to 15 °C) was higher by 8.5 mPa·s compared to the initial viscosity of the sample at 15 °C (Table 4) and visual assessment of the sample after heating indicated extensive destabilisation of the emulsion (Fig. 2a). After heat treatment, apparent viscosity (at 15 °C) of WPH-H_E and WPH-C_E emulsions was no different to that measured before heating of these emulsions but was significantly lower than the viscosity of the WPH_E emulsion after the same heat treatment (Fig. 3a; Table 4).

During more severe thermal treatment at 95 °C for 15 min of the WPI_E emulsion, a sharp increase in viscosity was observed during the heating phase on reaching 81 °C (Fig. 3b); formation of distinct separated coarse protein network and serum phases was observed on visual assessment of the heated WPI_E emulsion (Fig. 2b).

No significant ($P < 0.05$) differences in viscosity of WPH-H_E and WPH-C_E emulsions were observed after heating at 95 °C for 15 min compared to unheated emulsions (Fig. 3b; Table 4); visual inspection of samples after heating indicated that the WPH-H_E and WPH-C_E emulsions were stable to the heat treatment (Fig. 2c, d). No viscosity data was recorded during heat treatment at 100 °C as this heat treatment was performed in an oil bath.

3.4. Free thiol groups and thermal stability of emulsions

The level of free thiol (—SH) groups was significantly different in the stock protein solutions used to prepare emulsions and increased in the order WPI <<< WPH-H << WPH-C < WPH (i.e., 2.49, 7.89, 10.9 and 11.5 μmol —SH/g protein, respectively). WPI consists of intact whey protein where most of the reactive —SH groups are buried within its globular structure, while WPH has more —SH groups exposed due to enzymatic hydrolysis of the compact globular structure (Panyam & Kilara, 1996). Significantly ($P < 0.05$) lower levels of free —SH groups measured in the WPH-H solution as compared to the WPH solution indicated their reduction on heating due to the involvement of —SH groups in formation of di-sulphide bridges (—S—S—) (Adjonu, Doran, Torley, & Agboola, 2013; Singh, 2011). Significantly ($P < 0.05$) higher levels of free —SH groups were measured in the WPH-C solution compared to the WPH-H solution, although the two solutions were subjected to the same heating conditions (i.e., 8 h at 90 °C). The difference in the levels of free —SH groups observed between WPH-H and WPH-C may be associated with two factors: (1) a macromolecular crowding effect (Zhu, Damodaran, & Lucey, 2008, 2010) caused by the higher number of macromolecules (i.e., MD also present in the WPH-C sample) limiting mobility and interactions between proteins/peptides in the solution during heat treatment and (2) the access to free —SH groups may be restricted by steric hindrance of already conjugated protein/peptides, thus limiting the formation of —S—S— bonds.

3.5. Confocal laser scanning microscopy

Microstructural analysis of WPH-H_E and WPH-C_E emulsions showed small, uniform and homogenously distributed fat globules in both samples post homogenisation with no differences between the samples (Fig. 4a1, b1). However, major differences were observed between the two emulsions after heat treatment at 100 °C for 15 min, supporting the FGSD data (Table 3). No changes were observed in the WPH-C_E emulsion after heating at 100 °C for 15 min. Conversely, the WPH-H_E emulsion displayed a heterogeneous microstructure with a number of mechanisms involved in the emulsion destabilisation being identified. Bridging flocculation of oil droplets was observed for the heat treated WPH-H_E emulsion, where a distinct, dense protein layer surrounded the individual oil droplets (Fig. 4a3—left). This mechanism of destabilisation is common for whey protein-based O/W emulsions where proteins adsorbed at the interface of different oil droplets react with each other on heating through formation of disulphide bonds (Dickinson, 2001). Coalescence and formation of larger oil droplets was also observed in the WPH-H_E emulsion and this mechanism often occurs in conjunction with flocculation (Raikos, 2010; Tcholakova et al., 2006), where the interfacial film between oil droplets ruptures and the droplets merge to form larger oil droplets (Tcholakova et al., 2006; Ye et al., 2004). Formation of a dense protein network with pools of oil trapped within it, visible in the heat treated WPH-H_E emulsion, is a consecutive step in the thermal destabilisation process that follows bridging flocculation and coalescence (Lam & Nickerson, 2013). With prolonged exposure to high temperature, interactions between proteins/peptides at interfaces of different oil droplets and between proteins/peptides at interfaces and serum proteins/peptides grow stronger forming a cohesive protein network (as seen in Fig. 4a3—right). No changes in the microstructure of the WPH-C_E emulsion after heat treatment at 100 °C for 15 min (Fig. 4b2, b3) indicated that the emulsion was stable to the heating process.

4. Discussion

The stability of the emulsions to thermal processing under controlled conditions (i.e., temperature, heating/cooling rate and shear rate) was found to be markedly different for emulsions stabilised by the different protein ingredients; the observed order of the heat stability (least-to-most stable) was WPH_E < WPI_E << WPH-H_E <<< WPH-C_E. Results presented in this work identified (1) differences in destabilisation mechanisms between emulsions formed with intact and hydrolysed whey proteins and (2) demonstrated that modification of hydrolysed whey protein/peptides by conjugation with MD gave an ingredient with superior thermal stability in infant formula-based O/W emulsion systems. It was also shown that pre-heating of the hydrolysed whey protein ingredient improved the thermal stability of emulsions formed therefrom.

Clear differences in destabilisation behaviour were observed for emulsions prepared using intact (WPI_E) and hydrolysed (WPH_E) whey protein as evidenced by the magnitude of viscosity increase of emulsions upon destabilisation (Fig. 3), final viscosity (Table 4) and protein particle/aggregate size and physical appearance (Fig. 2) of destabilised WPI_E and WPH_E emulsions. These results demonstrate that formation of a coarse protein network or formation of a large number of relatively small (10 to 400 μm) protein aggregates/oil particles are the final stages of thermal destabilisation of intact (WPI_E) and hydrolysed (WPH_E) whey protein-based emulsions, respectively. Denaturation and aggregation of intact whey protein involve a number of sequential stages such as unfolding, association (non-covalent followed by covalent bonding), propagation (i.e., formation of polymers) and termination (Mulvihill & Donovan, 1987; Oldfield, Singh, Taylor, & Pearce, 1998). The differences outlined above in the nature of thermally-induced destabilisation observed in WPI_E and WPH_E emulsions, indicated that the propagation stage is limited and formation of a large number of small aggregate complexes is favoured, over extensive protein network formation (as observed in intact protein systems) in the WPH-stabilised emulsions. According to a study by Surroca, Haverkamp, and Heck (2002), during the thermal denaturation and aggregation of intact whey protein, aggregates need to reach their maximum concentrations before the polymerisation stage can occur. However, in a system containing hydrolysed protein, the termination stage can take place before polymerisation due to the blocking of thiol groups (—SH) by peptides in the surrounding serum phase and on the interfaces of nearby oil droplets. Successful efforts to limit aggregation of whey proteins by blocking —SH groups have been documented (Sakai, Sakurai, Sakai, Hoshino, & Goto, 2000; Wijayanti, Bansal, & Deeth, 2014).

The results of this study have shown that the role of serum proteins in mediating aggregation and destabilisation of WPH-stabilised emulsions can be diminished by preheating of the protein ingredient prior to emulsion formation. This is clear from the current study where the improved thermal stability of emulsions stabilised by hydrolysed

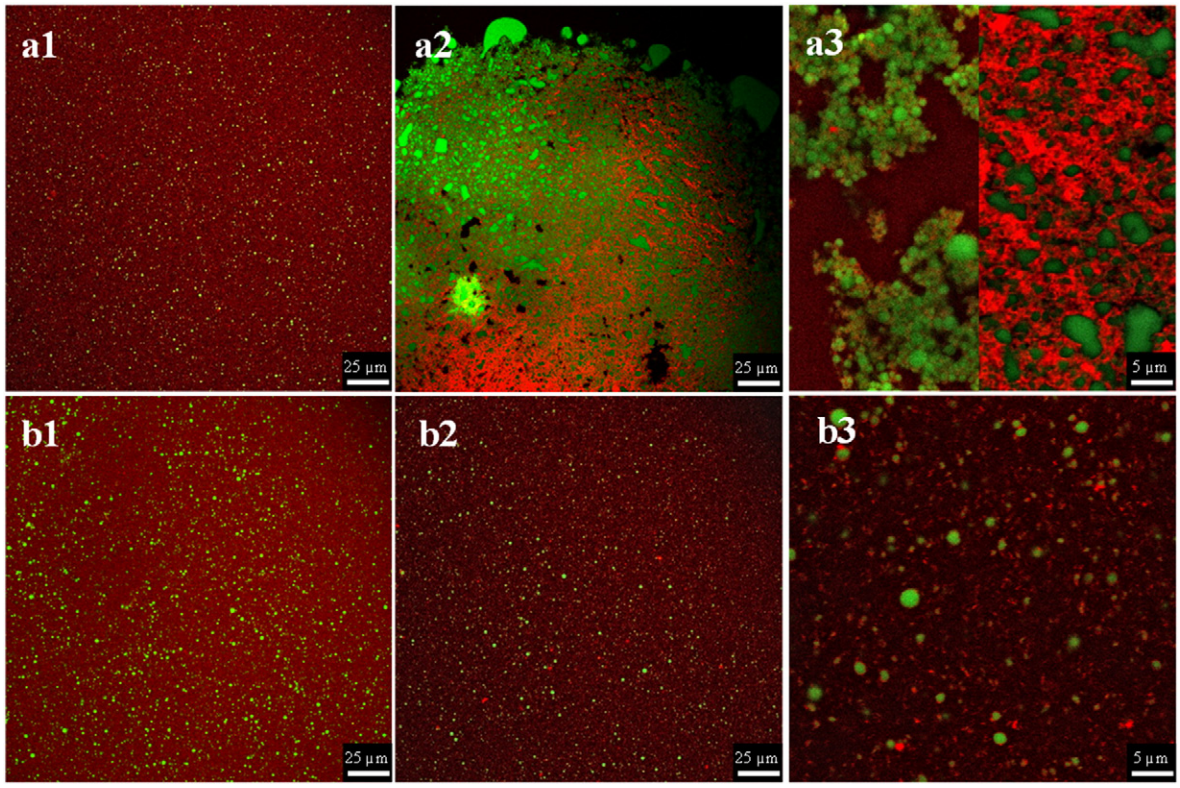

Fig. 4. Confocal laser scanning micrographs of WPH-H_E (a) and WPH-C_E (b) emulsions before (1) and after (2, 3) heat treatment in an oil bath at 100 °C for 15 min. Protein = red; oil = green. Scale bar (bottom right) is 25 μm (1, 2) and 5 μm (3). *Note*: Figure a3 is a combination of 2 micrographs (i.e., left and right) to give more comprehensive representation of the heterogeneous structure observed in the WPH-H emulsion after heat treatment.

whey protein was evident where the level of free thiol groups had been reduced through pre-heating. Similarly, previous studies have shown that (1) serum (non-adsorbed) proteins play a major role in aggregation and destabilisation of whey protein-stabilised emulsions (Euston et al., 2000; Hunt & Dalgleish, 1995), (2) blocking potential reactive sites on proteins/peptides (i.e., hydrophobic or thiol groups) allows improvement of the heat stability of protein-based systems (Baier & McClements, 2001; Rich & Foegeding, 2000; Smulders & Somers, 2012) and (3) pre-heating of protein reduces the number of reactive groups — mainly free thiol groups (Liang, Patel, Matia-Merino, Ye, & Golding, 2013; Livney, Corredig, & Dalgleish, 2003; Wijayanti et al., 2014).

The results of this study have demonstrated that the emulsion stabilised with the WPH-C displayed superior stability to thermal processing, where oil droplet–droplet interactions, observed in WPI_E, WPH_E and WPH-H_E emulsions, were prevented. It is proposed that, primarily steric stabilisation and, to a lesser extent, increased ζ potential, provided by the protein–carbohydrate conjugate, limited interactions (i.e., coming in contact and subsequent aggregation) between proteins/peptides adsorbed at the interfaces of different oil droplets in the WPH-C_E emulsion conferring superior stability compared with WPI or WPH ingredients. Limiting interactions between oil droplets in emulsions is one of the main strategies to improve the stability of these systems. An increase in steric stabilisation through adsorption/attachment of flexible, hydrophilic macromolecules to the emulsion droplets effectively limits close contact and subsequent interactions between oil droplets (Dalgleish, 1997). Stabilisation of emulsions with conjugated protein/maltodextrin provides the O/W interfacial layer with increased thickness and effectively produces a better steric barrier to the oil droplets. In keeping with this, Wong, Day, and Augustin (2011) reported that increased steric stabilisation resulting from the thicker interfacial layer of conjugate (composed of wheat protein/dextran) gave better emulsion stability. Additionally, conjugation has been reported to improve the stability of emulsions in cases where these systems were subjected to stressed or unfavourable conditions; the improved heating and freeze–thaw stability of emulsions formed with casein/maltodextrin conjugates was reported by O'Regan and Mulvihill (2010a) and an improvement in the long term stability of emulsions stabilised with WPI/dextran conjugates with low ζ potential (i.e., <30 mV) was reported by Akhtar and Dickinson (2003). In the current study combining pre-heating with the attachment of hydrophilic polysaccharide groups to hydrolysed whey protein by Maillard conjugation resulted in a protein-based emulsifier characterised by its ability to confer improved thermal stability to infant formula type emulsions.

5. Conclusions

This study shows that the heat stability of model infant formula emulsions based on hydrolysed whey protein ingredients can be markedly improved by modification of the protein ingredient through conjugation with carbohydrate. Covalent bonding between proteins/peptides in hydrolysed whey protein and maltodextrin produced an ingredient with enhanced performance during the thermal processing of the model infant formula emulsion where, due to increased steric and electrostatic repulsion, interactions between and subsequent destabilisation of oil droplets during heat treatment were suppressed. It was also shown that pre-heating of hydrolysed whey protein prior to its use in emulsion preparation resulted in the enhanced heat stability of the emulsion, as a result of a reduction in the level of reactive sites (i.e., free thiol groups) through protein–protein interactions. Incorporation of protein–carbohydrate conjugates in the formulation of nutritional products could potentially allow for the displacement (at least partial) of non-protein emulsifiers without compromising the stability or quality of the product and offers potential for application in other nutritional products naturally containing hydrolysed whey protein and maltodextrin, such as clinical nutrition products.

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Performance of whey protein hydrolysate–maltodextrin conjugates as emulsifiers in model infant formula emulsions

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ABSTRACT

Model infant formula emulsions containing 15.5, 35.0 and 70.0 g L^{-1} protein, soybean oil and maltodextrin (MD), respectively, were prepared. Emulsions were stabilised by whey protein hydrolysate (WPH) + CITREM (9 g L^{-1}), WPH + lecithin (9 g L^{-1}) or WPH conjugated with MD (WPH–MD). All emulsions had mono-modal oil droplet size distributions post-homogenisation with mean oil droplet diameters ($D_{4,3}$) of <1.0 μm. No changes in the $D_{4,3}$ were observed after heat treatment (95 °C, 15 min) of the emulsions. Accelerated storage (40 °C, 10 d) of unheated emulsions resulted in an increase in $D_{4,3}$ for CITREM (2.86 μm) and lecithin (5.36 μm) containing emulsions. Heated emulsions displayed better stability to accelerated storage with no increase in $D_{4,3}$ for CITREM and an increase in $D_{4,3}$ for lecithin (2.71 μm) containing emulsions. No increase in $D_{4,3}$ over storage was observed for unheated or heated WPH–MD emulsion, indicating its superior stability.

1. Introduction

The incorporation of whey protein hydrolysates (WPHs) with a moderate degree of hydrolysis into nutritional formulations tailored for athletes, the elderly or infants is increasing due to growing demand for products that contain amino acids in a rapidly digestible form. The enhanced gut absorption and efficient metabolism of hydrolysates (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014) make these ingredients particularly useful for consumers seeking to increase the rate of muscle synthesis or limit ageing related muscle loss (Jonker, Deutz, Erbland, Anderson, & Engelen, 2014; Pimenta, Abecia-Soria, Auler, & Amaya-Farfan, 2006). Infant formulae containing moderately hydrolysed WPH are not intended for medical purposes in infants suffering from cows' milk allergy, however, these formulae can improve comfort in infants that suffer from difficulty digesting intact proteins (Bourlieu et al., 2015; Nguyen, Bhandari, Cichero, & Prakash, 2015).

A common challenge encountered during the preparation of emulsions containing hydrolysates is their diminished processing stability (i.e., short term storage of unheated emulsions and thermal stability) and shelf life stability (i.e., long term storage of heat treated emulsions) compared with emulsions containing intact whey protein (Drapala, Auty, Mulvihill, & O'Mahony, 2015, in press; Singh & Dalgleish, 1998; Ye & Singh, 2006). Poor thermal stability of WPH based emulsions is related to the reduced steric hindrance between oil droplets provided by peptides compared with intact protein (Ye, Hemar, & Singh, 2004). This reduced steric hindrance increases the interactions that occur between oil droplets during heating and storage of emulsions. In addition, a high number of exposed reactive sites (such as free –SH groups) at both the oil/water interface and in the serum phase of WPH based emulsions promotes protein/peptide–protein/peptide interactions (i.e., mainly through formation of disulphide bridges, –S–S–) resulting in flocculation of oil droplets (Adjonu, Doran, Torley, & Agboola, 2013, 2014; Drapala et al., in press; Panyam & Kilara, 1996; Singh, 2011).

Non-protein emulsifiers, such as CITREM (i.e., citric acid esters of monoglycerides) or lecithin, are often included in the formulation of emulsions to facilitate the formation of small oil droplets on homogenisation, improve stability of emulsions to thermal processing and reduce creaming during storage. Lecithin and CITREM are routinely used in the manufacture of infant formulae (IF) that contain hydrolysed milk proteins, where they are used at up to 5 and 9 g L^{-1}, respectively (Codex Alimentarius Commission, 1981; McSweeney, 2008). These low molecular weight emulsifiers

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adsorb rapidly at the oil/water interface during homogenisation allowing the formation of small oil globules. They also interact with proteins adsorbed at the interface and in the serum phase reducing the availability of thiol groups at the oil/water interface and in the serum phase and limiting interactions between oil droplets during heating (Euston, Finnigan, & Hirst, 2001; McCrae, 1999; McSweeney, Healy, & Mulvihill, 2008). CITREM and lecithin contain charged domains (anionic and zwitterionic, respectively) within their structures and they confer a charge to the surface of oil droplets upon adsorption. Charged molecules promote electrostatic stabilisation of emulsions and the impact of droplet charge on thermal stability of IF type emulsions have been well documented (Kasinos et al., 2013; McCarthy, Kelly, O'Mahony, & Fenelon, 2014).

Modification of protein by conjugation with carbohydrate (i.e., maltodextrin, dextran or pectin) by exploiting the early/intermediate stages of the Maillard reaction has been shown to improve the functional properties of different proteins. Conjugation of casein with maltodextrin has been reported to improve solubility, foaming and emulsification properties (Jiang & Zhao, 2011), improve freeze–thaw stability (O'Regan & Mulvihill, 2010a), encapsulation efficiency (O'Regan & Mulvihill, 2010b) and emulsion stability at acidic pH (Shepherd, Robertson, & Ofman, 2000). Conjugation of whey proteins with pectin improved emulsifying properties at neutral pH (Xu, Wang, Jiang, Yuan, & Gao, 2012) and acidic pH (Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, & Dewettinck, 2004); moreover, protection of sensitive, oil soluble compounds against oxidation was enhanced using whey protein–pectin conjugates (Xu et al., 2012). Modification of protein/peptides present in WPH by conjugation can help alleviate issues encountered with stability in emulsions stabilised by hydrolysates; indeed, improved stability of model hydrolysed IF emulsions to thermal processing on conjugation has already been reported (Drapala et al., in press).

The objective of this study was to identify addition levels of CITREM and lecithin required to produce emulsions with thermal stability similar to that measured for a WPH–maltodextrin (WPH–MD) stabilised emulsion. The performance of WPH–MD conjugates as emulsifiers in IF type emulsions was compared with the performance of WPH plus added non-protein emulsifiers (i.e., CITREM and lecithin; added at the predetermined levels) used commercially in such products to determine if such conjugates can replace (at least partially) non-protein emulsifiers traditionally used in these products.

2. Materials and methods

2.1. Materials

Whey protein hydrolysate (WPH; 8.0% degree of hydrolysis, DH) was obtained from Carbery Food Ingredients Ltd (Ballineen, Ireland) and had 86.3% protein (Standard 20-1; IDF, 2014), 5.0% moisture (Standard 26; IDF, 2004), 2.8% ash (Standard 90; IDF, 1979), 0.7% fat (Standard 9C; IDF, 1987) and 5.2% lactose (determined by difference). Maltodextrin (MD; Maldex 120 with a dextrose equivalent value of 12) was obtained from Corcoran Chemicals Ltd. (Dublin, Ireland) and had moisture and ash contents of <5.0% and <0.2%, respectively. The majority of the MD population had an average molecular mass of 5.9 kDa as determined by multiangle laser light scattering with size exclusion chromatography (Lucey, Srinivasan, Singh, & Munro, 2000). Soybean oil was obtained from Frylite Group Ltd (Strabane, UK). CITREM (Grindsted® CITREM N12) was obtained from Dupont Nutrition Biosciences ApS (Brabrand, Denmark) and de-oiled powdered soybean lecithin (Ultralec® P) was obtained from ADM (Decatur, IL, USA). All other chemicals and reagents used in the study were of analytical grade and sourced from Sigma–Aldrich (Dublin, Ireland).

2.2. Stock protein solutions and conjugation

A stock protein solution (50.0 g L^{-1}, protein; pH 6.8) was prepared with WPH as detailed by Drapala et al. (in press). In brief, the WPH–MD conjugate solution was prepared by heating a WPH–maltodextrin solution (50.0 g L^{-1} protein, 50.0 g L^{-1} maltodextrin; pH 8.2) at 90 °C for 8 h (Mulcahy, Mulvihill, & O'Mahony, 2016; Mulcahy, Park, Drake, Mulvihill, & O'Mahony, 2016). The stock protein solution (non-conjugated) was subsequently used to formulate emulsions containing different levels (0–9 g L^{-1}) of either CITREM or lecithin and the stock conjugate solution was used to formulate WPH–MD conjugate based emulsions.

2.3. Preparation of emulsions

Model infant formula emulsions containing 15.5, 35.0 and 70.0 g L^{-1} protein, oil and total maltodextrin, respectively, were prepared from stock WPH or stock WPH–MD conjugate solutions essentially as detailed by Drapala et al. (2015, in press). Non-protein emulsifiers (CITREM or lecithin) were added to emulsions prepared from the stock WPH solution. For emulsions containing CITREM, the CITREM (0–9 g L^{-1}; dissolved in ultrapure water at 65 °C) was added to the aqueous phase prior to mixing with the oil phase. For emulsions containing lecithin, the lecithin (0–9 g L^{-1}; dissolved in soybean oil at 65 °C) was added to the oil phase prior to mixing with the aqueous phase. For emulsions containing WPH–MD conjugate all protein was provided by the stock conjugate solution and the MD was added to reach the target concentration (i.e., 7.0%). Aqueous and oil phases of emulsions were mixed together at 50 °C and then pre-homogenised with an Ultra-Turrax (T25, IKA-Werke GMBH & Co. KG, Staufen, Germany) at 10,000 rpm for 2 min followed by two stage homogenisation (double pass) at 15 and 3 MPa, using a valve homogeniser (APV GEA Niro-Soavi S.p.A., Parma, Italy) at 50 °C. Following homogenisation, the pH of emulsions was measured and, if needed, readjusted to pH 6.8 with 0.1 N HCl or 0.1 N KOH. Emulsion aliquots used for accelerated stability testing had sodium azide (0.50 g L^{-1}) added to prevent microbial growth.

2.4. Fat globule size distribution and zeta potential

Fat globule size distribution (FGSD) of the emulsions was measured using a laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK) equipped with a 300 RF (reverse Fourier) lens, an LED light source (λ of 470 nm) and a He–Ne laser (λ of 633 nm). A polydisperse model with particle and dispersant refractive index of 1.46 and 1.33, respectively, were selected for data analysis (McCarthy et al., 2012). Samples were introduced to the mixing chamber and dispersed in ultrapure water until a laser obscuration of 5–8% was reached and three readings were taken for each sample. FGSD was measured within 1 h post homogenisation (d 0), immediately post heating and after 3, 6, 8 and 10 d of accelerated storage at 40 °C. The zeta potential (ζ) of oil droplets in emulsions was measured using a Zetasizer Nano-ZS (Malvern Instruments), as detailed by Drapala et al. (in press).

2.5. Screening of thermal stability of emulsions

Model IF emulsions stabilised by WPH–MD conjugate, or WPH + CITREM (0–9 g L^{-1}) or WPH + lecithin (0–9 g L^{-1}) were heat treated at 95 °C for 15 min. Thermal stability of these emulsions was assessed by changes in FGSD of emulsions after heat treatment as compared to the FGSD measured immediately post homogenisation. The heat treatment (95 °C for 15 min using an oil bath) was used to initially screen the thermal stability of emulsions

containing lower levels of non-protein emulsifiers (0–5 g L^{-1}; CITREM or lecithin) to identify very unstable samples. Emulsions stabilised by WPH + CITREM or WPH + lecithin (5–9 g L^{-1}) were heated (fresh aliquots) under controlled conditions with an AR-G2 controlled stress rheometer (TA Instruments, Crawley, UK) equipped with a starch pasting cell (SPC). The thermal treatment applied to the samples was as detailed by Drapala et al. (in press) and involved heating to 95 °C, holding for 15 min at peak temperature and cooling to 15 °C with constant shear-rate (15 s^{-1}). Apparent viscosity was recorded at 1 s intervals during heating, holding and cooling. Emulsion samples were recovered after all heat treatments (i.e., from oil bath tubes and the SPC) and their thermal stability was assessed by visual observation and analysis of FGSD, as described in Section 2.4. Addition levels of CITREM and lecithin required to obtain thermal stability equivalent to that measured for the WPH–MD sample were identified. The three emulsion systems evaluated in the remainder of this study are referred to as conjugate based emulsion (CON_e), CITREM containing emulsion (CIT_e) and lecithin containing emulsion (LEC_e).

2.6. Assessment of properties of emulsions

2.6.1. Determination of composition of emulsions

The chemical composition (i.e., total solids, protein, ash and carbohydrate content) of the CON_e, CIT_e, and LEC_e emulsions was determined using the methods detailed for the WPH ingredients in Section 2.1. The fat content of emulsions was determined using the Gerber method (Standard 105; IDF, 2008). The carbohydrate content of emulsions was calculated by difference.

2.6.2. Determination of apparent viscosity of emulsions

Viscosity of CON_e, CIT_e and LEC_e emulsions post homogenisation and post heating at 95 °C for 15 min was measured using a rotational viscometer (Haake RotoVisco 1 Rotational Viscometer, Thermo Fisher Scientific, MA, USA) equipped with a cylindrical double gap cup and rotor (DG43, Thermo Fisher Scientific, MA, USA) as described by Mulcahy et al. (2015). The average apparent viscosity at 300 s^{-1} of each emulsion was determined at 20 °C (±0.1 °C).

2.6.3. Accelerated storage stability testing of emulsions

To determine stability of unheated CON_e, CIT_e and LEC_e emulsions to accelerated storage, aliquots (50 mL) were transferred to plastic containers, sealed and incubated at 40 °C. FGSD of the emulsions was measured after 3, 6, 8 and 10 d storage. A parallel experiment was carried out to determine the stability of heated (95 °C for 15 min) CON_e, CIT_e and LEC_e emulsions to accelerated storage (10 d at 40 °C).

2.6.4. Accelerated creaming stability testing of emulsions

Creaming velocities of unheated and heated CON_e, CIT_e and LEC_e emulsions were measured using an analytical centrifuge (LUMiSizer, L.U.M. GmbH, Berlin, Germany). The principle of analysis by LUMiSizer has been detailed by Lerche and Sobisch (2011). Stability of emulsions to creaming was determined at 23 °C and 563 g for 8.5 h as detailed by Shimoni, Shani Levi, Levi Tal, and Lesmes (2013). Creaming velocity was calculated from front tracking profiles as detailed by Lerche and Sobisch (2011).

2.6.5. Microstructural analysis of emulsions

The microstructural analysis of emulsions was performed using a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) as detailed by Drapala et al. (2015). In brief, protein and lipid were fluorescently labelled with Nile Blue dye and visualisation in emulsions was carried out using He–Ne (633 nm) and Ar (488 nm) lasers for protein and lipid, respectively. The observations were performed using a 63× oil immersion objective. At least three specimens of each emulsion were observed to obtain representative micrographs of samples.

2.7. Statistical analysis

All emulsions were prepared in three independent trials and all measurements were carried out in at least duplicate. Analysis of variance (ANOVA) was carried out using the Minitab® 16 (Minitab Ltd, Coventry, UK) statistical analysis package. The Tukey HSD test was used to obtain grouping information. The level of significance was determined at $P < 0.05$.

3. Results

3.1. Influence of emulsifier type and concentration on thermal stability of emulsions

Thermal stability results for emulsions stabilised by WPH and different levels (0–9 g L^{-1}) of CITREM or lecithin and by the WPH–MD conjugate are shown in Table 1. Emulsions containing low to intermediate levels of lecithin (1–5 g L^{-1}) displayed poor stability to thermal processing at 95 °C for 15 min. Extensive heat induced coagulation was observed in the emulsion containing 1 g L^{-1} lecithin, where protein/peptide aggregates and a distinct serum phase were observed in the sample after heat treatment. No coagulation was observed at lecithin addition levels >1 g L^{-1}, however, destabilisation of emulsions containing low to intermediate levels (2–5 g L^{-1}) of lecithin was observed, as evidenced by the presence of relatively large aggregates in the emulsions after heating. Increasing the lecithin concentration in emulsions improved their thermal stability and at ≥5 g L^{-1} lecithin no extensive destabilisation was observed. Emulsions containing ≥5 g L^{-1} lecithin were heat treated at 95 °C for 15 min in the SPC. Similar to the emulsions heated in the oil bath, the destabilising effects of thermal processing decreased as the level of lecithin increased. The presence of aggregates in the heated emulsions was observed at up to 7 g L^{-1} lecithin addition. Emulsions containing ≥8 g L^{-1} lecithin had mean volume diameter of oil droplets <1 μm, which is generally an indicator of a physically stable emulsion (Drapala et al., 2015; McCarthy et al., 2012). Lecithin containing emulsions that were heated in the SPC displayed a tendency to foul the cell (i.e., deposit a protein/oil layer on the metal surface they were in contact with during thermal processing). Fouling decreased with increasing lecithin level; however, it was still observed (although to a limited degree) even at the highest level (i.e., 9 g L^{-1}) of lecithin addition (Table 1). A lecithin addition level of 9 g L^{-1} was identified as being able to provide best thermal stability to the WPH based emulsion, essentially equivalent to that of an emulsion stabilised by the WPH–MD conjugate.

Emulsions containing low (1–4 g L^{-1}) levels of CITREM displayed poor stability to heating at 95 °C for 15 min and coagulation was observed in emulsions containing 1 and 2 g L^{-1} CITREM. The presence of large aggregates without coagulation was observed in emulsions containing 3 and 4 g L^{-1} CITREM and the $D_{4,3}$ of particles decreased by ~50% on increasing the level from 3 to 4 g L^{-1} (Table 1). Similar to lecithin containing emulsions, samples containing 5–9 g L^{-1} CITREM were heated in the SPC. Formation of aggregates was observed in emulsions containing up to 7 g L^{-1} CITREM; however, unlike lecithin containing emulsions, no fouling was observed in the SPC at any CITREM addition level. The emulsion containing 9 g L^{-1} CITREM displayed the highest thermal stability, the stability being similar to that observed for the

Table 1
Influence of lecithin or CITREM addition level on stability of model whey protein hydrolysate (WPH) based infant formula (IF) emulsions on heat treatment compared with the stability of a model IF emulsion stabilised by WPH–maltodextrin (WPH–MD) conjugate.[a]

Mapping parameter	WPH + lecithin (g L^{-1})									WPH + CITREM (g L^{-1})									WPH–MD conjugate
	1^{ob}	2^{ob}	3^{ob}	4^{ob}	$5^{ob,r}$	6^{r}	7^{r}	8^{r}	9^{r}	1^{ob}	2^{ob}	3^{ob}	4^{ob}	$5^{ob,r}$	6^{r}	7^{r}	8^{r}	9^{r}	
Coagulation	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Aggregation	NA	+++	++	++	++	+	+	−	−	NA	NA	+++	++	+	+	+	−	−	−
Fouling	NA	NA	NA	NA	+++	+++	++	++	+	NA	NA	NA	NA	−	−	−	−	−	−
$D_{4,3}$ (μm)	NA	117	96.7	73.7	17.5	3.42	1.22	0.96	0.90	NA	NA	119	58.8	3.99	0.98	0.81	0.70	0.62	0.79

[a] WPH based IF emulsions, pH 6.8, contained 1.55, 3.5 and 7.0% protein, fat and carbohydrate, respectively, were heat treated at 95 °C for 15 min using an oil bath or a rheometer equipped with a starch pasting cell (SPC); heat treatment method indicated by superscript letters: ^{ob}, oil bath; ^{r}, rheometer. $D_{4,3}$ is the mean volume diameter of particles as measured by laser diffraction. The symbols + and − describe either presence or absence, respectively, of the corresponding mapping parameters in an emulsion sample after the heat treatment; higher number of + refers to higher magnitude of the corresponding parameter; NA, not applicable.

emulsion stabilised by the WPH–MD, hence this level of the emulsifier was selected for subsequent analyses.

Model infant formula emulsions stabilised by the WPH–MD conjugate, WPH + CITREM (9 g L^{-1}) or WPH + lecithin (9 g L^{-1}) displayed good thermal stability to heating at 95 °C for 15 min in the SPC. The effects of the heat treatment on selected emulsion properties were assessed by determining FGSD, apparent viscosity, stability to creaming, ζ potential and microstructural properties. Emulsions stabilised by WPH–MD conjugate, WPH + CITREM (9 g L^{-1}) and WPH + lecithin (9 g L^{-1}) will subsequently be referred to as CON_e, CIT_e and LEC_e emulsions, respectively, in this study.

3.2. Properties of emulsions

3.2.1. Composition of emulsions

Compositional analysis of CON_e, CIT_e and LEC_e emulsions showed that measured/calculated levels of protein (15.0, 15.1 and 15.4 g L^{-1}, respectively), fat (36.8, 36.7 and 37.3 g L^{-1}, respectively) and carbohydrate (64.5, 70.9 and 71.1 g L^{-1}, respectively) were sufficiently near target levels. The total solids content of the CON_e emulsion (117.5 g L^{-1}) was lower than in the CIT_e and LEC_e emulsions (123.8 and 125.2 g L^{-1}, respectively), due to the presence of non-protein emulsifiers in addition to the target protein content in the CIT_e and LEC_e emulsions.

3.2.2. Size and charge of oil droplets in emulsions

CON_e, CIT_e and LEC_e emulsions had narrow particle size distributions (Fig. 1) and mean volume diameter ($D_{4,3}$) of 0.79, 0.62 and 0.72 μm (Table 2), respectively, immediately post homogenisation. The CON_e and CIT_e emulsions displayed good stability to heat treatment at 95 °C for 15 min as indicated by no significant changes in the $D_{4,3}$, $D_{3,2}$ (Sauter mean diameter), $D_{v,0.5}$ and $D_{v,0.9}$ (fat droplet size in the 50% and 90% quantiles of the distribution, respectively) and no change in the FGSD profiles post heating compared with post homogenisation (Table 2; Fig. 1). A limited increase in the $D_{v,0.5}$ and broadening of the size distribution profile was observed for the LEC_e emulsion post heating at 95 °C for 15 min (Table 2; Fig. 1, C2). However, no significant changes were measured for all the other FGSD parameters and the $D_{4,3}$ of the LEC_e emulsion remained <1 μm after heating also indicating good heat stability.

Zeta potential values showed that oil droplets in freshly prepared CIT_e emulsion had significantly higher net negative charge (−57.7 mV) compared with the CON_e (−53.3 mV) and LEC_e (−52.3 mV) emulsions (Table 2). Heating at 95 °C for 15 min reduced the ζ potential of oil droplets in emulsions by 4.6, 2.6 and 2.6 mV for CIT_e, CON_e and LEC_e emulsions, respectively. No significant differences in the ζ potential were found between heated CIT_e, CON_e and LEC_e emulsions.

3.2.3. Apparent viscosity of emulsions

No significant differences in viscosity were observed for CON_e, CIT_e and LEC_e emulsions immediately post homogenisation (Table 2). Viscosity of all emulsions increased on heat treatment at 95 °C for 15 min. Viscosity of the heated CIT_e emulsion was found to be significantly higher than the viscosity of heated CON_e emulsion; no significant differences were observed between heated CON_e and LEC_e and between heated CIT_e and LEC_e emulsions.

3.2.4. Accelerated storage stability of emulsions

The unheated CON_e emulsion displayed excellent stability over the 10 d accelerated storage at 40 °C with no changes observed in the $D_{4,3}$, $D_{3,2}$ and $D_{v,0.5}$ or in the FGSD profiles (Fig. 1, A1 and A3; Table 2); a marginal increase (i.e., 0.07 μm) in the $D_{v,0.9}$ was observed for the unheated CON_e emulsion on accelerated storage. An increase in $D_{4,3}$ to 2.86 μm and a shift in the size distribution profile from monomodal to bimodal (Fig. 1, B1 and B3; Table 2) was observed for the unheated CIT_e emulsion after 10 d of accelerated storage. Particle size parameters $D_{3,2}$, $D_{v,0.5}$ and $D_{v,0.9}$ for the unheated CIT_e emulsion also increased after storage compared to post homogenisation; however, the increases were not found to be significant due to large standard errors observed for the stored emulsion (Table 2). The unheated LEC_e emulsion displayed the least stability to accelerated storage; $D_{4,3}$ increased to 5.36 μm and the presence of a small number of large (~30 μm) oil droplets was observed on the FGSD profile (Fig. 1, C3). An increase in $D_{v,0.9}$ was observed for the unheated LEC_e emulsion after the storage compared with post homogenisation; however, the difference was not found to be significant due to large standard error observed for the stored emulsion (Table 2). Additionally, a complete phase separation, evidenced by the presence of free oil floating on top of the emulsion in the container, was observed as early as 6 d into the storage of the unheated LEC_e emulsion. No phase separation was observed for either the unheated CON_e or CIT_e emulsions over the 10 d of storage at the accelerated conditions.

The stability of heated (95 °C for 15 min) emulsions to accelerated storage (10 d at 40 °C) was also determined and the results were similar to the unheated systems. A marginal increase in the $D_{4,3}$, $D_{3,2}$, $D_{v,0.5}$ and $D_{v,0.9}$ were measured for the heated CON_e and CIT_e emulsions after 10 d storage compared with FGSD values immediately after heat treatment (Table 2). No differences in the FGSD profiles were observed for the heated CON_e emulsion after the storage compared to post heating (Fig. 1, A2 and A4). A marginal broadening of the FGSD profile was observed for the heated CIT_e emulsion after 10 d storage compared to post heating (Fig. 1, B2 and B4). Following the trend observed for the unheated emulsions, the biggest changes on accelerated storage were observed for the heated LEC_e emulsion. Large (~20 μm) oil droplets were present in the heated LEC_e emulsion after 10 d storage (Fig. 1, C4). The $D_{4,3}$ and $D_{v,0.5}$ for the heated LEC_e emulsion increased after the 10 d of

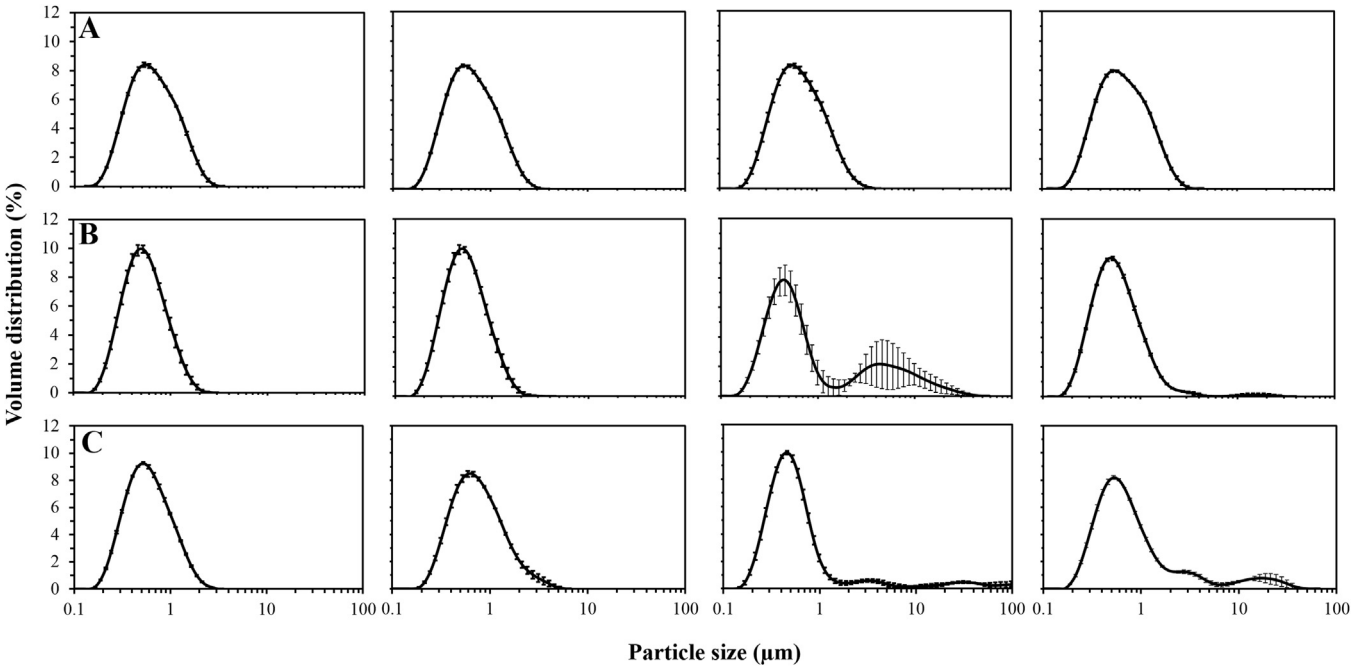

Fig. 1. Fat globule size distribution profiles of model whey protein hydrolysate (WPH) based infant formula emulsions stabilised by (A) WPH–maltodextrin conjugate and (B) WPH + CITREM at 9 g L^{-1} or (C) WPH + lecithin at 9 g L^{-1} (horizontally, left to right) post homogenisation, after heat treatment (95 °C × 15 min), after 10 d of storage at 40 °C of unheated emulsions and after 10 d of storage at 40 °C of heated emulsions. Large error bars observed for the unheated CIT_e emulsion after 10 d of storage reflect a large variability in the extent of destabilisation of the emulsions; however, the same trend was observed for the sample for all 3 independent trials.

Table 2
Fat globule size distribution and zeta potential (ζ) of model whey protein hydrolysate (WPH) based infant formula emulsions stabilised by WPH–maltodextrin conjugate (CON_e), WPH + CITREM (CIT_e) or WPH + lecithin (LEC_e) post homogenisation, post heating at 95 °C for 15 min and after an accelerated storage at 40 °C for 10 d post homogenisation of unheated and heated emulsions.[a]

Emulsion	Measurement stage	Fat globule size parameter (μm)				ζ potential (mV)	Apparent viscosity (mPa s)	Creaming velocity (mm d^{-1})
		$D_{4,3}$	$D_{3,2}$	$D_{v,0.5}$	$D_{v,0.9}$			
CON_e	Post homogenisation	0.79 ± 0.02^{a}	0.57 ± 0.01^{a}	0.67 ± 0.02^{a}	1.43 ± 0.03^{a}	−53.3 ± 0.54^{A}	1.86 ± 0.19^{A}	0.28 ± 0.03^{A}
	Heated 95 °C, 15 min	0.79 ± 0.01^{a}	0.57 ± 0.02^{a}	0.66 ± 0.01^{a}	1.44 ± 0.03^{a}	−50.7 ± 0.69^{A}	1.90 ± 0.12^{A}	0.24 ± 0.03^{AB}
	Accelerated storage: unheated	0.82 ± 0.02^{a}	0.60 ± 0.02^{a}	0.67 ± 0.02^{a}	1.50 ± 0.04^{ab}	n.d.	n.d.	n.d.
	Accelerated storage: heated	0.83 ± 0.02^{a}	0.62 ± 0.02^{a}	0.69 ± 0.01^{a}	1.55 ± 0.02^{b}	n.d.	n.d.	n.d.
CIT_e	Post homogenisation	0.62 ± 0.04^{a}	0.49 ± 0.04^{a}	0.54 ± 0.03^{a}	1.07 ± 0.08^{a}	−57.7 ± 0.34^{B}	2.15 ± 0.26^{A}	0.18 ± 0.05^{B}
	Heated 95 °C, 15 min	0.62 ± 0.04^{a}	0.49 ± 0.03^{a}	0.54 ± 0.03^{a}	1.07 ± 0.08^{a}	−53.1 ± 0.83^{A}	2.45 ± 0.05^{B}	0.06 ± 0.02^{C}
	Accelerated storage: unheated	2.86 ± 1.22^{b}	0.62 ± 0.11^{a}	0.67 ± 0.15^{a}	7.81 ± 4.84^{a}	n.d.	n.d.	n.d.
	Accelerated storage: heated	0.88 ± 0.14^{a}	0.55 ± 0.04^{a}	0.60 ± 0.03^{a}	1.31 ± 0.06^{a}	n.d.	n.d.	n.d.
LEC_e	Post homogenisation	0.72 ± 0.00^{a}	0.52 ± 0.04^{a}	0.58 ± 0.04^{a}	1.21 ± 0.11^{a}	−52.3 ± 0.83^{A}	2.01 ± 0.21^{A}	0.22 ± 0.05^{AB}
	Heated 95 °C, 15 min	0.90 ± 0.11^{a}	0.62 ± 0.10^{a}	0.72 ± 0.07^{b}	1.65 ± 0.26^{a}	−49.7 ± 2.13^{A}	2.20 ± 0.03^{AB}	0.17 ± 0.05^{B}
	Accelerated storage: unheated	5.36 ± 1.98^{b}	0.51 ± 0.01^{a}	0.53 ± 0.01^{a}	8.29 ± 9.22^{a}	n.d.	n.d.	n.d.
	Accelerated storage: heated	2.71 ± 0.75^{ab}	0.64 ± 0.03^{a}	0.72 ± 0.03^{b}	4.98 ± 1.46^{a}	n.d.	n.d.	n.d.

[a] CITREM and lecithin were used at 9 g L^{-1}. Fat globule size distribution parameters are: $D_{4,3}$, volume mean diameter; $D_{3,2}$, Sauter mean diameter; $D_{v,0.5}$, fat droplet size in the 50% quantile of the distribution; $D_{v,0.9}$, fat droplet size in the 90% quantile of the distribution; values for a given emulsion within a column not sharing a common lower case letter differed significantly ($p < 0.05$). ζ potential, apparent viscosity and creaming velocity values for a measurement stage for each emulsion within a column not sharing a common superscript uppercase letter differed significantly ($p < 0.05$); n.d., not determined.

storage compared with after heat treatment; however, this increase was not found to be significant due largely to variability in data observed for $D_{4,3}$ and $D_{v,0.9}$ (Table 2). No differences were found in the $D_{3,2}$ and $D_{v,0.5}$ after the accelerated storage compared with values measured immediately after the heat treatment. Interestingly no free oil was observed for the heated LEC_e emulsion after 10 d storage.

3.2.5. Accelerated creaming stability of emulsions

Creaming velocity measured immediately post homogenisation was highest for the CON_e emulsion followed by the LEC_e and CIT_e emulsions (Table 2). However, all emulsions displayed creaming velocity below 1 mm d^{-1}, which is considered an indicator of good stability to creaming in oil in water (O/W) emulsions (Dickinson, 1992; McClements, 1999). A limited decrease in the creaming velocity was observed for the CON_e and LEC_e emulsions as a result of the heat treatment (95 °C for 15 min) (Table 2); a significant ($P < 0.05$) decrease was observed for the CIT_e emulsion after heating.

3.2.6. Microstructural analysis of emulsions

Microstructural analysis of the emulsions showed that all samples had fine and uniformly distributed oil droplets immediately post homogenisation (Fig. 2). Similarly, a homogenous distribution

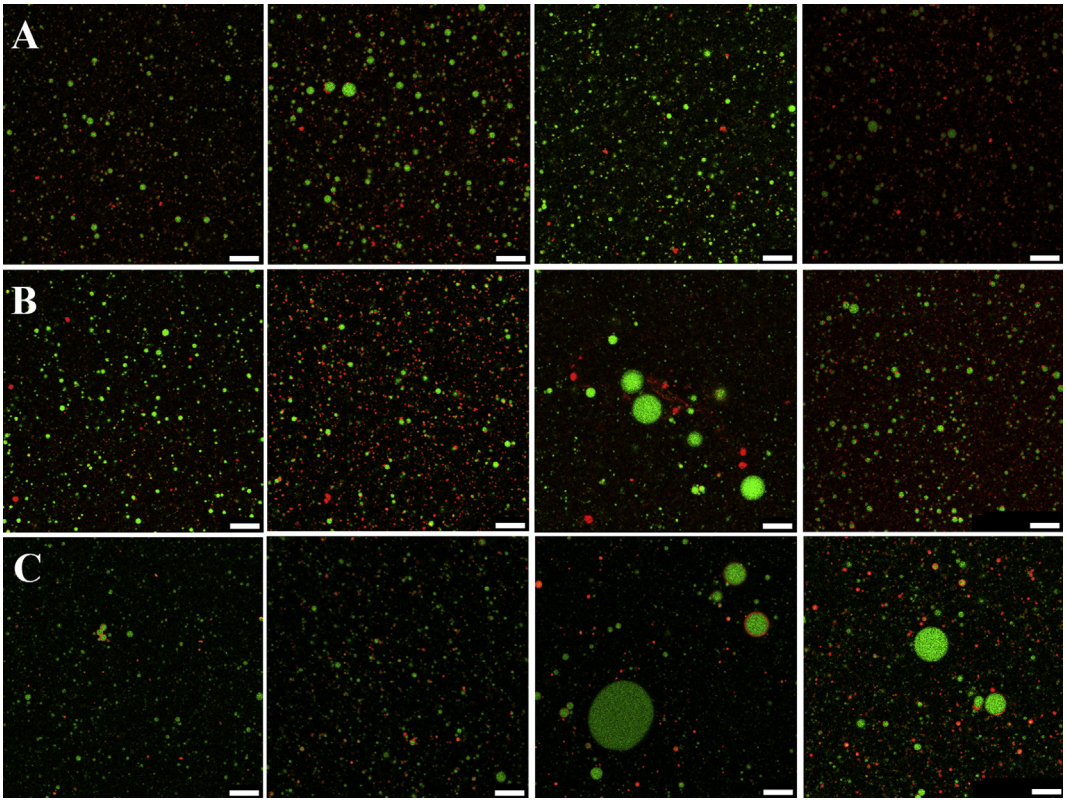

Fig. 2. Confocal laser scanning microscopy images of model whey protein hydrolysate (WPH) based infant formula emulsions stabilised by (A) WPH–maltodextrin conjugate and (B) WPH + CITREM at 9 g L^{-1} or (C) WPH + lecithin at 9 g L^{-1} (horizontally, left to right) post homogenisation, after heat treatment (95 °C × 15 min), after 10 d of storage at 40 °C of unheated emulsions and after 10 d of storage at 40 °C of heated emulsions. Emulsions were labelled with Nile Blue and the micrographs show distribution of oil droplets (green) and protein particles (red). Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of small (~1 μm) oil droplets was observed in all emulsions after the heat treatment at 95 °C for 15 min. Microstructural analysis showed that the heat treatment resulted in an increased number of protein aggregates/complexes in the serum phase of the emulsions; this observation was especially pronounced in the CIT_e emulsion (Fig. 2, B2).

Pronounced differences in the microstructure were observed between unheated CON_e, CIT_e and LEC_e emulsions after the 10 d storage at 40 °C. No changes in the microstructure were observed for the unheated CON_e emulsion after 10 d storage compared with post homogenisation. Relatively large (i.e., 5–10 μm) oil droplets were present in the unheated CIT_e emulsion and still larger droplets (i.e., 10–20 μm) in the unheated LEC_e emulsion (Fig. 2, B3 and C3, respectively) after 10 d storage. Additionally, large (1–2 μm) protein complexes were observed in the unheated CIT_e emulsion after 10 d of storage at 40 °C.

Results for the accelerated storage of the heated CON_e emulsion showed no evident increase in the size of oil droplets and no obvious differences in the microstructure of the emulsion compared with post homogenisation (Fig. 2, A4). The heated CIT_e emulsions also displayed a good stability to accelerated storage and no evident changes resulting from the storage were observed in the heated CIT_e emulsion (Fig. 2, B4). Large (i.e., 5–15 μm) oil droplets were observed for the heated LEC_e emulsion after 10 d storage at 40 °C (Fig. 2, C4).

4. Discussion

The results presented in the current study show that the WPH–MD conjugate conferred excellent stability to IF type emulsion products containing hydrolysed whey protein. Emulsions stabilised by the WPH–MD conjugate displayed the greatest stability to thermal processing (95 °C for 15 min) and accelerated storage (40 °C for 10 d), in both unheated and heated emulsions. There were marginal or no changes in the size distribution of oil droplets in the WPH–MD conjugate stabilised emulsion, compared with emulsions stabilised by WPH + lecithin or WPH + CITREM (Figs. 1 and 2, Table 2). The superior stability of the CON_e emulsion can be attributed to the ability of the WPH–MD conjugate to enhance steric stabilisation of oil droplets in an emulsion system. Upon adsorption of the surface active WPH–MD conjugate at the interface of oil droplets during homogenisation, the carbohydrate, a hydrophilic component of the conjugate, protrudes into the serum phase of the emulsion, in effect, increasing thickness of the interfacial layer, conferring enhanced steric stabilisation and limiting interactions between oil droplets (Kasran, Cui, & Goff, 2013; Wong, Day, & Augustin, 2011). Additionally, it is proposed that the covalent attachment of the MD to WPH reduces the potential of the interfacial protein/peptide-MD layer to interact with protein/peptides in the serum. This is due to a physical restriction of access to the interfacial protein/peptides that are in close proximity to the covalently attached carbohydrate (i.e., space interference). Such space interference can effectively improve thermal stability of WPH based emulsions, where protein/peptide mediated bridging flocculation is a common processing challenge (Dickinson, 2001; Drapala et al., in press; McSweeney, Mulvihill, & O'Callaghan, 2004; Ye & Singh, 2006).

WPH–MD conjugates can be used as an alternative ingredient for stabilizing WPH based emulsions where the addition of non-protein emulsifiers (i.e., low molecular weight surfactants like

CITREM and lecithin) can be, at least partially, replaced. Thus, competitive destabilisation, often observed in systems containing protein and low molecular weight surfactants, could be avoided (Kaltsa, Paximada, Mandala, & Scholten, 2014; Wilde, Mackie, Husband, Gunning, & Morris, 2004). This destabilisation, which takes place during storage of emulsions containing protein/peptides and low molecular weight surfactants, can result in a non-continuous interfacial layer with certain regions dominated by protein/peptides and others by the surfactant (Drapala et al., 2015). Such unordered structure of the interface can in effect promote coalescence (Tirok, Scherze, & Muschiolik, 2001) and, under particularly adverse conditions, result in phase separation. Hofman and Stein (1991) and Mezdour, Lepine, Erazo-Majewicz, Ducept, and Michon (2008) have reported a detrimental effect of lecithin on emulsion stability, which was linked to reduced interfacial tension and, effectively, decreased rigidity and strength of the interfacial layer. Coalescence, evidenced by the presence of large oil droplets, was observed in the unheated CIT_e and unheated and heated LEC_e emulsions, while both the unheated and heated CON_e emulsions displayed resistance to coalescence during accelerated storage.

The different extents of emulsion instability observed for unheated emulsions containing the low molecular weight surfactants CITREM or lecithin can be explained by the ability of the ionic surfactant CITREM to interact and form ternary complexes with polysaccharides and protein (Antipova, Semenova, Belyakova, & Il'in, 2001; McSweeney, 2008; Semenova, Myasoedova, & Antipova, 2001) (Fig. 2B). Formation of such complexes may have curtailed the mobility of the CITREM in the serum phase during storage and limited its role in the competitive destabilisation at the emulsion interfaces, thus, enhancing the stability of the CITREM containing emulsion compared with the lecithin containing emulsion. A positive effect of the ternary complexes formed by CITREM and polysaccharides–protein on the emulsion stability was observed when the emulsions were heated. It is proposed that on heating, formation of the complexes and limited protein/peptide aggregation, provide a synergistic stabilising effect; the presence of these combined complexes and aggregates at the surfaces of oil droplets may contribute to steric stabilisation. Additionally, the number of CITREM molecules in the serum phase, that would potentially be available to displace protein/peptides at the interfaces, is decreased.

Electrostatic repulsion also has a role to play in stabilisation of oil droplets in O/W emulsions against undesirable droplet–droplet interactions. Modification of WPH by conjugation with MD affects the charge on proteins/peptides as positively charged ε-amino groups of the lysine residues are blocked by the covalently attached MD (Acedo-Carrillo et al., 2006; Liu, Ru, & Ding, 2012). It has been shown by Drapala et al. (in press) that oil droplets in emulsions stabilised by a WPH–MD conjugate displayed greater negative charge compared with those stabilised by WPH alone. The interactions between protein at the emulsion interfaces and protein in the emulsion serum phase have been widely reported and reviewed (Raikos, 2010); these interactions affect not only the structural arrangement at the interfaces but also impact on the charge of the oil droplets. The results presented in the current study showed that the initial differences in the ζ potential between CON_e, CIT_e and LEC_e emulsions measured post homogenisation were diminished after heat treatment (Table 2). Reported reduction in the ζ potential of oil droplets in all emulsions as a result of heat treatment can be explained by interactions between proteins/peptides at the surface of oil droplets and proteins/peptides in the serum phase and by a rearrangement of emulsifiers at the interface. The CIT_e emulsions displayed a bigger reduction in ζ potential after the heat treatment, compared with the other emulsions. This indicates increased interactions (i.e., through combined complex formation and aggregation) at the oil–water interfaces and supports the concept of CITREM based complexes playing a role in steric stabilisation. In the current study the differences in the ζ potential of oil droplets in the heated CON_e, CIT_e and LEC_e emulsions were insignificant, however, major differences in the storage behaviour were observed for these emulsions. The best shelf life stability displayed by the CON_e emulsion compared with the other emulsions was linked directly to the properties of the interfacial layer of oil droplets in the CON_e emulsion with the most effective steric hindrance and absence of the competitive destabilisation observed for the CIT_e and LEC_e emulsions.

5. Conclusions

The results presented in the current study show that the performance of WPH–MD conjugates in stabilising model WPH based IF emulsions was superior to that observed for emulsions stabilised by WPH + CITREM (9 g L^{-1}) or WPH + lecithin (9 g L^{-1}). The greater thermal and storage stability of the emulsion stabilised by the WPH–MD conjugate is attributed to enhanced steric stabilisation of oil droplets in the emulsion as a result of conjugation. Undesirable interactions between oil droplets during heat treatment and accelerated storage were markedly reduced in the emulsion stabilised by the WPH–MD conjugate compared to emulsions stabilised by WPH with CITREM or lecithin. The novel WPH–MD conjugate emulsifiers can provide a valuable and highly functional alternative to the inclusion of non-protein emulsifiers in nutritional formulations.

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Influence of emulsifier type on the spray-drying properties of model infant formula emulsions

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ABSTRACT

The objective of this study was to compare the drying performance and physicochemical properties of model infant formula (IF) emulsions containing 43, 96 and 192 g L^{-1} protein, oil and maltodextrin (MD), respectively, prepared using different emulsifier systems. Emulsions were stabilised using either whey protein isolate (WPI), whey protein hydrolysate (WPH; DH 8%), WPH + CITREM (9 g L^{-1}), WPH + lecithin (5 g L^{-1}) or WPH conjugated with maltodextrin (DE 12) (WPH-MD). Homogenised emulsions had 32% solids content and oil globules with mean volume diameter <1 μm. Powders were produced by spray-drying with inlet and outlet temperatures of 170 and 90 °C, respectively, to an average final moisture content of 1.3%. The extent of powder build-up on the dryer wall increased in the order; WPH- MD << WPH ≤ WPI < WPH + LEC ≤ WPH + CIT. The same trend was observed for the extent of spontaneous primary powder agglomeration, as confirmed by particle size distribution profiles and scanning electron micrographs, where the WPH-MD and WPH + CIT powders displayed the least and greatest extent of agglomeration, respectively. Analysis of elemental surface composition of the powders showed that surface fat, protein and carbohydrate decreased in the order; WPH + CIT > WPH + LEC > WPH > WPH- MD > WPI, WPI > WPH > WPH- MD > WPH + LEC > WPH + CIT and WPH- MD > WPI > WPH > WPH + LEC > WPH + CIT, respectively. Additionally, differences in wettability, surface topography and oil globule distribution within the powder matrix and in reconstituted powders were linked to the emulsifier system used. Inclusion of the WPH-MD conjugate in the formulation of IF powder significantly improved drying behaviour and physicochemical properties of the resultant powder, as evidenced by lowest powder build-up during drying and greatest emulsion quality on reconstitution, compared to the other model formula systems.

1. Introduction

Protein-based added-value nutritional formulations have been gaining a significant share of the global food market over the last decade, especially those tailored for athletes, the elderly and infants; the total global market for these product types is predicted to exceed 100 billion USD by 2020. Formulations for such products generally contain protein (e.g., whey protein), oils rich in unsaturated fatty acids (i.e., blends of vegetable oils) and carbohydrates (e.g., maltodextrin) as the main components. Whey protein hydrolysate (WPH) is often used as a protein source in such nutritional formulae due to its desirable amino acid composition, high digestibility and rapid absorption in the gut (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014). Modification of protein *via* hydrolysis has been extensively studied, with reports on improvement in protein functionality in the areas of solubility, surface activity, foaming and emulsifying properties available in the scientific literature (Agboola & Dalgleish, 1996a,b; Banach, Lin, & Lamsal, 2013; Foegeding & Davis, 2011; Kilara & Panyam, 2003). However, incorporation of WPH into nutritional formulations such as powdered formulae or ready to drink products is often associated with processing and shelf life challenges such as protein/peptide-mediated bridging flocculation and coalescence, due to reduced steric stabilisation and increased number of exposed reactive sites, compared to formulations based on intact whey protein (Drapala, Auty, Mulvihill, & O'Mahony, 2016a,b; Euston, Finnigan, & Hirst, 2000; Hunt & Dalgleish, 1995). Irrespective of the format of the final product (i.e., liquid or powder), the formulations for both physical formats have to undergo a

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number of thermal treatments (e.g., pasteurisation, sterilisation, spray-drying) as a liquid. Therefore, additional non-protein surface active components are often included in the formulation of WPH-based emulsions in order to improve their processing and shelf-life stability; these surfactants are usually lipid-based emulsifiers, including lecithin or citric acid esters of mono- and di-glycerides (CITREM).

Spray-drying is one of the most common processes used in the manufacture of dairy ingredients and nutritional products; rapid water removal results in increased product shelf-life, reduced shipping and storage costs and provides the consumer with a convenient and stable product. In this complex process, multiple factors such as feed characteristics (e.g., composition and rheological properties), process parameters (e.g., atomiser type and fines return) and external factors (e.g., air humidity, temperature) significantly impact the drying performance and the physico-chemical properties of the final product. The composition (i.e., the type and content of protein, carbohydrate, fat and emulsifier, total solids content) and properties (i.e., flow behaviour and viscosity) of the emulsion destined for spray-drying have a strong influence on its drying properties; extensive scientific reports and reviews focusing on the effects these factors have on the properties of the resulting powders have been published (Adhikari, Howes, Wood, & Bhandari, 2009; Jayasundera, Adhikari, Aldred, & Ghandi, 2009; Ji et al., 2016; Kim, Chen, & Pearce, 2009; Millqvist-Fureby, Elofsson, & Bergenståhl, 2001; Taneja, Ye, Jones, Archer, & Singh, 2013; Vega & Roos, 2006; Vignolles, Jeantet, Lopez, & Schuck, 2007).

It is well established that there is a strong relationship between the surface composition of powder particles and their drying performance in addition to the properties (e.g., cohesiveness, shelf-life) of the final product (Kelly, O'Mahony, Kelly, & O'Callaghan, 2014; Nijdam & Langrish, 2006; Sadek et al., 2015). In the production of fat-rich powders, high surface fat content can lead to powder stickiness, low powder recovery (i.e., yield) and production down-time (i.e., due to powder build-up on the dryer walls) as well as poor shelf-life and undesirable properties of the final product (i.e., lipid oxidation, caking, low solubility and dispersibility) (Paterson, Zuo, Bronlund, & Chatterjee, 2007). Surface composition of an emulsion-based powder is governed mainly by the emulsifier system used; upon atomisation, a new air/liquid interface is created and surface active components (i.e., proteins, peptides, low molecular weight surfactants) present in the emulsion, migrate rapidly towards, and adsorb at, the new interface, effectively reducing the surface free energy and enhancing the thermodynamic stability of the system (Munoz-Ibanez et al., 2016). Effectively, surfactants are over-represented at the droplet/powder particle surface, affecting in-process and in-application behaviour of these products, as exhibited by interactions of particles with the dryer wall and with other droplets/powder particles. Thus, a better understanding of the emulsifier system and its modification to tailor it to a specific formulation has an important role in increasing drying efficiency in producing a powder with desired properties.

Conjugation of milk proteins with carbohydrates through the Maillard reaction has been reported to produce emulsifiers with exceptional functionality, especially with respect to stability of emulsion to unfavourable thermal and/or storage conditions (Akhtar & Dickinson, 2003; Drapala et al., 2016a,b; Kasran, Cui, & Goff, 2013a,b; O'Regan & Mulvihill, 2010a,b; Wooster & Augustin, 2006). WPH-maltodextrin (WPH-MD) conjugates have been shown to confer strong steric stabilisation to oil droplets, effectively limiting globule-globule interactions and preventing emulsion destabilisation (i.e., flocculation and/or coalescence) (Corzo-Martínez et al., 2011; Liu, Ma, McClements, & Gao, 2016).

There is potential for these conjugates to affect surface properties of spray dried emulsions, effectively influencing their behaviour during drying and properties of the final product. Good interfacial barrier properties and inherent ability of WPH-MD conjugates to adsorb at the newly formed air/water interface (O'Mahony, Drapala, Mulcahy, & Mulvihill, 2017) can offer an ingredient capable of deterring interactions between atomised emulsion droplets/powder particles. However, currently there are no published studies reporting on the use of WPH-based conjugates in spray dried emulsions nor on the properties of the resultant powders. This study aims to directly compare the spray drying performance and powder physical properties of spray dried emulsions stabilised with different emulsifier systems; namely, conjugated whey proteins/peptides (WPH-MD), not conjugated whey proteins/peptides (WPI, WPH) and not conjugated WPH with the addition of low molecular weight lipid-based surfactants (i.e., WPH + CITREM and WPH + lecithin).

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) and whey protein hydrolysate (WPH; 8% degree of hydrolysis; DH) were obtained from Carbery Food Ingredients Ltd. (Ballineen, Co. Cork, Ireland). The WPI and WPH ingredients had protein contents of 87.2 and 83.7%, respectively, and ash contents of 2.76 and 2.92%, respectively, as reported by Drapala et al. (2016a). Maltodextrin (MD) was obtained from Corcoran Chemicals Ltd. (Dublin, Ireland) and had moisture and ash contents of <5.0% and <0.2%, respectively. Soybean oil was obtained from Frylite Group Ltd. (Strabane, Co. Tyrone, Northern Ireland). CITREM (Grindsted® CITREM N12) was obtained from Dupont Nutrition Biosciences ApS (Brabrand, Denmark) and de-oiled powdered soybean lecithin (Ultralec® P) was obtained from ADM (Decatur, IL, USA). All other chemicals and reagents used in the study were of analytical grade and sourced from Sigma-Aldrich (Arklow, Co.Wicklow, Ireland).

2.2. Preparation of emulsions

Emulsions ($_e$) for model infant formula (IF) powders ($_p$) were prepared at pH 6.8 using protein, soybean oil and maltodextrin in the ratios 1.0:2.3:4.5, respectively. The protein component was either whey protein isolate (WPI), whey protein hydrolysate (WPH) or WPH conjugated with maltodextrin (MD) in a wet heating process as detailed by Drapala et al. (2016a). Additionally, non-protein emulsifiers, citric acid esters of mono- and di-glycerides (CITREM; 9 g L^{-1}) and soybean lecithin (5 g L^{-1}) were incorporated into the formulation of selected IF emulsions destined for subsequent spray-drying. Emulsions were prepared by dissolving oil soluble components, where applicable, in soybean oil and water soluble components in ultrapure water, followed by two stage homogenisation (double pass) at 15 and 3 MPa, using a valve homogeniser (APV GEA Niro-Soavi S.p.A., Parma, Italy) at 50 °C. All emulsions were prepared to a total solids (TS) target of 32% as measured with a rapid moisture analyser (HB43- S, Mettler- Toledo LLC, Columbus, OH, USA). In total, five emulsions based on WPI, WPH, WPH + CITREM (WPH + CIT), WPH + lecithin (WPH + LEC) and WPH conjugated with maltodextrin (WPH-MD) were produced in the current study.

2.3. Spray-drying of emulsions

Powders were produced from emulsions using a bench-top spray dryer (B-191, BÜCHI Labortechnik AG, Flawil, Switzerland) with a maximum evaporation capacity of 1.5 L H_2O h^{-1}. Inlet temperature was set at 170 °C and outlet temperature was

maintained at 90–95 °C by controlling the aspirator power (i.e., in the range of 40–60 $m^3 h^{-1}$) and the feed flow rate (i.e., in the range 1.2–1.4 L h^{-1}). Effectively, drying temperatures were kept within the industry relevant range typical for IF manufacture by using high feed flow rate (95–100%) and relatively low aspirator power (80–90%); however, this was achieved at the expense of product yield (Fig. 1). The powders were collected in the collection chamber as detailed in Fig. 1, transferred to zip-sealed low density polyethylene bags (VWR International, Leuven, Belgium), followed by vacuum packing in heat-sealed polyamide/polyethylene bags (Fispak Ltd., Dublin, Ireland) with a moisture permeability of 2.6 g m^{-2}.d. The powders were stored in the dark at ambient conditions (i.e., ~20 °C) until further analyses within 4 weeks of spray drying. Powder recovery was calculated on a TS basis (i.e., [Final powder product TS/feed liquid TS] × 100) from the total amount of powder obtained in the collection chamber. Losses on drying were due to unrecoverable powder, which stuck to the wall of the dryer main chamber or fell and accumulated at the base of the main chamber during spray-drying (Fig. 1). Powder stickiness was visually assessed based on the extent of wall coating by powder in the cyclone, in order to provide information on particle cohesion arising from surface characteristics (Fig. 1).

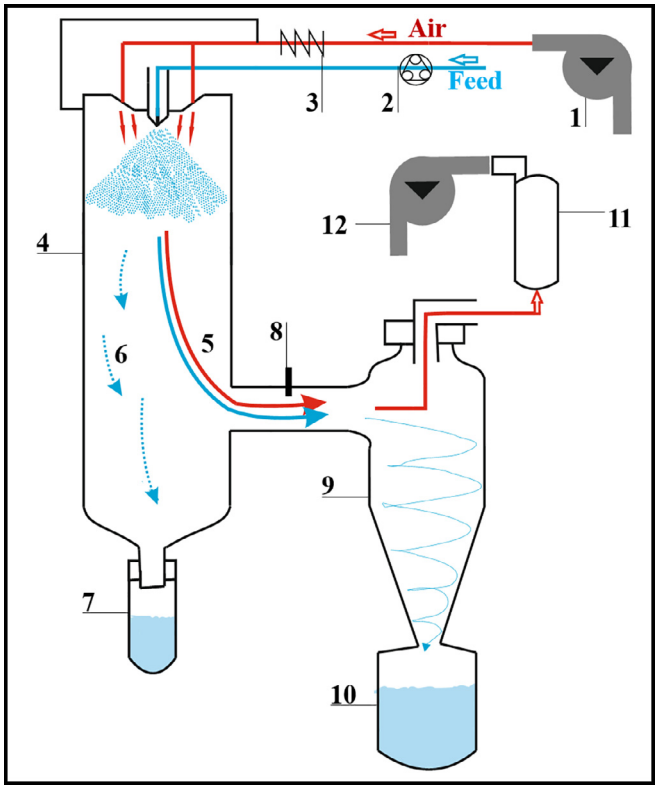

Fig. 1. Schematic diagram showing the set-up and the principle of operation for the laboratory-scale BÜCHI B-191 spray drier. The inlet temperature is regulated directly by the power of the heater (3) and the outlet temperature (measured at 8) is regulated indirectly by controlling the feed flow rate (2) and the air flow (1). Feed is introduced into the main drying chamber (4) by a 2-fluid nozzle atomiser, where it is rapidly dried by heated air; dried particles are pulled into the cyclone (9) by means of an aspirator (12). Large and heavy particles (i.e., wet lumps and scorched particles, falling off the build-up around the nozzle and around hot air inlets, respectively) are separated from the powder by means of the air pull and gravity (5 and 6, respectively). By design, air pull is insufficient to move larger and heavier particles into the cyclone, making them fall into the waste collection container (7) at the bottom of the dryer main chamber. Dried powder particles are further separated from air in the cyclone and the final powder is collected in the powder collection container (10) at the bottom of the cyclone. The clarified air is exhausted at the top of bag filter (11).

2.4. Particle size distribution

Particle size distribution (PSD) of the emulsions immediately after homogenisation and after powder reconstitution (i.e., 12%, w/v) was measured using a laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) equipped with a 300 RF (reverse fourier) lens, an LED light source (λ of 470 nm) and a He-Ne laser (λ of 633 nm) as detailed by Drapala et al. (2016b). The particle size distribution of the model infant formula powders was measured using a Mastersizer 3000 equipped with a dry powder disperser cell (Aero S). Approximately 3.0 g of powder was placed in the feed hopper, containing a ball bearing to facilitate powder flow, with the feed pressure set at 1 bar, powder flow rate at 40–70% and the hopper height at 2 mm. All measurements were taken at 1–2% obscuration. The background and sample measurement duration was set at 20 s with the material refractive and absorption indexes of 1.46 and 0.01, respectively.

2.5. Rheological measurements

The apparent viscosity of emulsions was measured at 20 °C using a rotational viscometer (Haake RotoVisco 1, Thermo Fisher Scientific, MA, USA) equipped with a cylindrical double gap cup and rotor (DG43, Thermo Fisher Scientific, MA, USA) as described by Mulcahy, Mulvihill, and O'Mahony (2016). The shear rate was increased from 0 to 300 s^{-1} over 5 min, held at 300 s^{-1} for 2 min and decreased to 0 s^{-1} over 5 min; the average apparent viscosity was determined at 300 s^{-1} (η_{300}) for each emulsion. The power law applied to shear stress (τ) *vs* shear rate (γ) was used to obtain the flow behaviour parameters, consistency coefficient (K) and flow behaviour index (n) as detailed by Anema, Lowe, Lee, and Klostermeyer (2014). The flow behaviour index values were used to describe the flow behaviour of liquid samples where $n < 1$, $n > 1$ and $n = 1$ indicate shear-thinning, shear-thickening and Newtonian flow behaviour, respectively.

2.6. Composition and colour analyses of powders

The chemical composition of the model infant formula powders was determined using standard International Dairy Federation (IDF) methods as detailed by Drapala, Auty, Mulvihill, and O'Mahony (2015). Colour of the powders was measured using a pre-calibrated colorimeter (Minolta Chroma Meter CR-400, Minolta Ltd., Milton Keynes, U.K.) equipped with a granular materials attachment CR-A50. Colour was expressed using the Commission Internationale de l'Eclairage (CIE) colour chromaticity L* a* b* scale (L = dark/light, a = red/green, b = yellow/blue).

2.7. Powder wettability

The sessile drop goniometric method was used to determine the wettability of powders. All powders were compressed for 10 s at 78.4 MPa using a manual press (15 ton Manual Hydraulic Press, Specac Ltd., Orpington, UK) to form pellets (13 mm diameter); all pellets had a density of 1.08 (±0.05) g cm^{-3}. Subsequently, the mean contact angle (θ) was determined directly using an optical tensiometer (Attension Theta, Biolin Scientific, Stockholm, Sweden); a drop (10 μl) of ultrapure water was formed and deposited on top of a powder pellet and the reduction in contact angle during the first 30 s was recorded using a high-resolution digital camera (15 frames per second) and processed using image analysis software (OneAttension, Biolin Scientific).

2.8. Surface composition of powders

The surface free fat content of powders was determined using the GEA Niro analytical method (GEA Niro, 2005) as described by McCarthy et al. (2013) with modified quantities of powder (5.0 g), petroleum ether (30 mL) and filtrate (15 mL) used. Elemental composition of powder surfaces was determined by X-ray photoelectron spectroscopy (XPS; Kratos Axis 165, Kratos Analytical, UK) as detailed by McCarthy et al. (2013). A matrix formula was used to calculate relative amounts of protein, fat and carbohydrate on the powder surface, as detailed by Faldt, Bergenstahl, and Carlsson (1993).

2.9. Microstructure of powders

2.9.1. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) analysis of powder particles was performed using a confocal laser scanning microscope (TCS SP, Leica Microsystems CMS GmbH, Wetzlar, Germany). Powders were deposited onto a glass slide and excess sample was removed with compressed air. The powder samples were stained with a mixture (3:1) of Nile Red (0.10 g L^{-1} in polyethylene glycol) and Fast Green (0.01 g L^{-1} in water) fluorescent dyes (Sigma Aldrich, Wicklow, Ireland) to label the fat and protein components of the powders, respectively. Visualisation of oil and protein in the powders was carried out using an Ar laser (excitation = 488 nm, emission = 500–530 nm) and He- Ne laser (excitation = 633 nm, emission = 650–700 nm), respectively. At least 3 representative images of each sample were taken using a 63× oil immersion objective.

2.9.2. Scanning electron microscopy

Scanning electron microscopy (SEM) analysis of powders was performed using a scanning electron microscope (JSM- 5510, Jeol Ltd., Tokyo, Japan). Samples were mounted on double-sided carbon tape, attached to SEM stubs, and then sputter-coated with gold/palladium (10 nm; Emitech K550X, Ashford, UK). Representative micrographs were taken at 5 kV at 1000× (i.e., overview of powder population) and 3000× (i.e., shape and surface topography of powder particles) magnifications. At least three specimens of each sample were observed to obtain representative micrographs of samples.

2.10. Statistical data analysis

All powders were prepared in three independent trials and all measurements were carried out in at least duplicate. Analysis of variance (ANOVA) was carried out using the Minitab® 16 (Minitab Ltd., Coventry, UK, 2010) statistical analysis package. The Tukey method was used to obtain grouping information. The level of significance was determined at $P < 0.05$.

3. Results

3.1. Emulsion characteristics

The emulsions had TS levels ranging from 32.2 to 32.7% prior to spray-drying (Table 1). Particle size analysis showed that all emulsions had oil globules with mean volume diameter ($D_{4,3}$) less than 1 μm and no statistically-significant differences in $D_{4,3}$ were found between the emulsions (Table 1). Similarly, no significant differences in the apparent viscosity (η_{300}) were observed between WPI_e, WPH_e, WPH + CIT_e and WPH + LEC_e emulsions; however, the η_{300} for the WPH- MD_e emulsion was significantly lower than that of the WPI_e, and WPH + CIT_e emulsions (Table 1). Analysis of the flow behaviour showed no significant differences between emulsions, where most emulsions displayed a shear-thinning behaviour (i.e., $n < 1$) (Table 1). A reduction in the viscosity during shearing (i.e., shear-thinning) of protein solutions is, generally, a result of spatial rearrangement of protein molecules in the liquid and of disruptions in their steady-state interactions (Walstra, Wouters, & Geurts, 2006); in emulsions, shear-thinning can be associated with flocculation of oil droplets (Xu, Wang, Jiang, Yuan, & Gao, 2012). Additionally, in a concentrated emulsion system (i.e., TS = 32%), packing of oil globules is denser than in a dilute emulsion (i.e., TS ≤ 12%) and interactions between its constituents, as monitored by flow behaviour analysis, can also be related to physical contact between molecules located at the interfaces of oil globules (O'Mahony et al., 2017). The formation of ternary complexes between unadsorbed protein/peptides, CITREM and maltodextrin (Drapala et al., 2016b; Semenova, Myasoedova, & Antipova, 2001) in the WPH + CIT_e emulsion, or the presence of intact whey protein in the serum phase and at the interfaces of oil globules in the WPI_e emulsion, are likely to have contributed to higher viscosity of these emulsions, compared to the other samples.

3.2. Drying performance

Fig. 2 illustrates differences in drying behaviour between liquid concentrates/powders as evidenced by different levels of wall-coating (i.e., multilayer particle cohesion) by fine powder particles in the cyclone of the spray dryer. The extent of this coating is assumed to be directly related to powder stickiness; the observed stickiness can be divided into 3 groups based on the level of coating, i.e., non-sticky (negligible coating), moderately sticky (partial coating) and very sticky (complete coating) (Fig. 2; Table 3). Using this classification, the WPI_p and WPH_p powders were moderately sticky, WPH + CIT_p and WPH + LEC_p powders were very sticky and the WPH-MD_p powder was non-sticky.

Differences in the stickiness of powders had a direct impact on the powder recovery (i.e., product yield; Table 3); the recovery of product was lower for powders with higher levels of stickiness. Powders containing non-protein emulsifiers (WPH + LEC_p and WPH + CIT_p) displayed the lowest powder recovery (18.1 and 21.3%, respectively) followed by WPI_p (22.0%), WPH_p (26.1%) and WPH-MD_p (55.3%). It should be noted that in order to facilitate the use of industry-relevant drying temperatures (i.e., 170 °C and 90–95 °C for inlet and outlet, respectively) high feed flow rate (95–100%) and relatively low aspirator power (80–90%) conditions were used. These conditions caused deposition of higher-moisture particles at the periphery of the atomised feed jet on the inner wall of the main drying chamber (Fig. 1) and contributed to the low powder yield. Sticking of powders to the inner wall of a spray dryer is a common challenge in industry and it directly affects the product yield and drying efficiency (i.e., cleaning and down-time). In high-fat powders (e.g., infant formulae) stickiness is strongly related to the powder surface composition, while in low-fat, protein-dominant powders, it is generally related to the efficiency of water removal and glass transition properties of the system (Kelly et al., 2014). Generally, the more fat at the powder surface the greater the challenges with powder stickiness (Paterson et al., 2007; Sharma, Jana, & Chavan, 2012).

The highest levels of stickiness in this study were observed for powders containing lipid-based emulsifiers (CITREM and lecithin) while the powder containing the protein-based conjugate displayed the lowest stickiness. The physicochemical characteristics of CITREM and lecithin have directly affected cohesiveness (i.e., stickiness) of powders; their high mobility and surface activity facilitates rapid migration to the surface of emulsion droplets formed on atomisation and their relatively low melting temperatures

Table 1
Characteristics of emulsions prepared using different emulsifiers; whey protein isolate (WPI_e), whey protein hydrolysate (WPH_e), WPH + CITREM ($WPH + CIT_e$), WPH + lecithin ($WPH + LEC_e$) and WPH-maltodextrin conjugate ($WPH\text{-}MD_e$), used to produce model infant formula powders.

Emulsion characteristics		Emulsions				
		WPI_e	WPH_e	$WPH + CIT_e$	$WPH + LEC_e$	$WPH\text{-}MD_e$
Total solids content	(%, w/w)	32.6 ± 0.16[a]	32.2 ± 0.69 [a]	32.5 ± 0.10[a]	32.2 ± 0.04[a]	32.7 ± 0.18[a]
PSD[1] (μm)	$D_{4,3}$	0.76 ± 0.05[a]	0.78 ± 0.14[a]	0.81 ± 0.21[a]	0.58 ± 0.06[a]	0.67 ± 0.05[a]
	$D_{v,0.1}$	0.25 ± 0.07[a]	0.21 ± 0.04[a]	0.11 ± 0.07[a]	0.15 ± 0.01[a]	0.24 ± 0.05[a]
	$D_{v,0.5}$	0.55 ± 0.06[a]	0.55 ± 0.01[a]	0.38 ± 0.08[a]	0.46 ± 0.12[a]	0.55 ± 0.03[a]
	$D_{v,0.9}$	1.26 ± 0.10[a]	1.40 ± 0.12[a]	1.07 ± 0.07[a]	1.52 ± 0.85[a]	1.23 ± 0.04[a]
Flow behaviour[2]	η_{300} (mPa.s)	13.5 ± 0.55[a]	11.9 ± 1.27[ab]	13.0 ± 0.49[a]	11.9 ± 0.24[ab]	10.9 ± 0.31[b]
	K ($Pa.s^n$; $x10^2$)	1.57 ± 0.19[a]	1.18 ± 0.22[a]	2.92 ± 0.87[a]	1.64 ± 1.25[a]	2.19 ± 0.50[a]
	n	0.97 ± 0.02[a]	1.00 ± 0.02[a]	0.85 ± 0.06[a]	0.98 ± 0.16[a]	0.87 ± 0.05[a]

[1] Particle size distribution parameters: $D_{4,3}$, volume mean diameter of oil globules; $D_{v,0.1}$, $D_{v,0.5}$, and $D_{v,0.9}$ represent particle size in the 10%, 50% and 90% quantiles of the distribution.
[2] Flow behaviour parameters; (η_{300}) apparent viscosity measured at 300 s^{-1}; (K) consistency coefficient; (n) flow behaviour index.
[(a-b)] Values for a given parameter (i.e., within each row) for all powders, not sharing a common superscript differed significantly ($P < 0.05$).

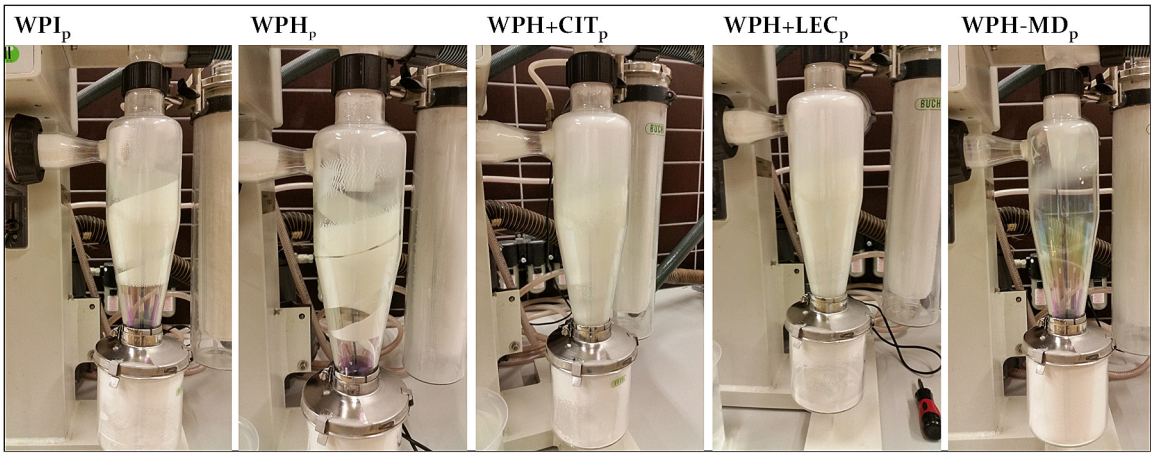

Fig. 2. Differences in the build-up of fine powder on the wall of the cyclone during spray-drying of powders ($_p$) containing different emulsifier systems: whey protein isolate (WPI_p), whey protein hydrolysate (WPH_p), WPH + CITREM ($WPH + CIT_p$), WPH + lecithin ($WPH + LEC_p$) and WPH-maltodextrin conjugate ($WPH\text{-}MD_p$). The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191). The photographs were taken ~30 min after starting the drying run for all powders.

(55–65 °C) make them plastic and adhesive under the environmental conditions of spray-drying. Similarly, the surface active WPH-MD conjugate can also rapidly move to and adsorb at the surface of atomised droplets (O'Mahony et al., 2017).

3.3. Powder analyses

3.3.1. Composition and colour of powders

Compositional analysis of powders showed that the measured levels (Table 2) were in line with the target levels for all samples (i.e., 12.1-12.7% protein, 26.9-29.0% fat and 56.1-58.8% carbohydrate). No significant differences were found in the fat, carbohydrate or moisture content between the powders. No significant differences in colour were found between WPI_p, WPH_p and $WPH + CIT_p$ powders; these powders had high L* and low b* values compared to the $WPH\text{-}MD_p$ and $WPH + LEC_p$ powders (Table 2). These differences were most likely due to the presence of melanoidins (conjugation products) and carotenoids (naturally present in lecithin) in the $WPH\text{-}MD_p$ and $WPH + LEC_p$ powders, respectively (Liu, Ru, & Ding, 2012; McSweeney, 2008; Scholfield, 1981) as previously reported by Drapala et al. (2016b).

3.3.2. Particle size distribution of powders

All powders had relatively small particles (i.e., $D_{4,3}$ of 14.2-

Table 2
Composition and colour of model infant formula powders ($_p$) produced with different emulsifier systems: whey protein isolate (WPI_p), whey protein hydrolysate (WPH_p), WPH + CITREM ($WPH + CIT_p$), WPH + lecithin ($WPH + LEC_p$) and WPH-maltodextrin conjugate ($WPH\text{-}MD_p$). The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191).

Powder	Composition (%, w/w)					Colour coordinates		
	Protein	Fat	Carbohydrate	Ash	Moisture	L*	a*	b*
WPI_p	12.1 ± 0.21[a]	28.4 ± 1.33[a]	57.7 ± 0.99[a]	0.52 ± 0.17[a]	1.73 ± 0.35[a]	96.1 ± 0.26[a]	−1.26 ± 0.09[b]	3.15 ± 0.24[a]
WPH_p	12.6 ± 0.10[b]	29.0 ± 1.58[a]	56.1 ± 1.50[a]	0.67 ± 0.10[ab]	1.08 ± 0.66[a]	96.3 ± 0.16[a]	−1.30 ± 0.11[b]	3.02 ± 0.15[a]
$WPH + CIT_p$	12.3 ± 0.13[ab]	28.8 ± 0.34[a]	56.6 ± 0.43[a]	0.87 ± 0.19[ab]	1.36 ± 0.91[a]	95.8 ± 0.49[ab]	−1.26 ± 0.06[b]	3.35 ± 0.26[a]
$WPH + LEC_p$	12.7 ± 0.22[b]	26.9 ± 2.44[a]	58.2 ± 1.84[a]	0.71 ± 0.13[ab]	1.48 ± 0.34[a]	93.8 ± 1.28[c]	−1.96 ± 0.08[a]	6.37 ± 0.25[c]
$WPH\text{-}MD_p$	12.5 ± 0.09[b]	26.9 ± 2.56[a]	58.8 ± 3.17[a]	0.97 ± 0.13[b]	0.89 ± 0.34[a]	94.1 ± 0.52[bc]	−0.85 ± 0.07[c]	4.77 ± 0.38[b]

[(a-c)] Values for a given parameter (i.e., within each column) for all powders, not sharing a common superscript differed significantly ($P < 0.05$).

41.1 μm; Table 3). The biggest particles were observed for the WPH + LEC_p, followed by the WPH + CIT_p, WPI_p, WPH_p and WPH-MD_p powders (Table 3, Fig. 3B). In addition, powders containing lipid-based surfactants, WPH + LEC_p and WPH + CIT_p, had a distinct shoulder on the higher end (i.e., at ~100 μm) of the size range, with a notable proportion of the particle population (i.e., 7.78 and 4.05%, respectively) in these powders having diameter >100 μm (Fig. 3B; Table 3). A much smaller shoulder was also present in the WPI_p and smaller still in the WPH_p powders (i.e., 2.93 and 2.26% of particle population were >100 μm, respectively). The WPH-MD_p powder had a monomodal profile with the narrowest size distribution, where the majority (i.e., ~99%) of particles had diameters <40 μm (Fig. 3B); this sample also had the largest proportion of fine particles (i.e., 19.9% of total population had diameter <5 μm; Table 3). The greater proportion of small particles in the WPH-MD_p powder, compared to the other powders is likely related to this liquid concentrate feed having the lowest viscosity of all samples (Pisecky, 2012). A relationship between feed viscosity and the size of particles in the resultant powder was also reported by Crowley, Gazi, Kelly, Huppertz, and O'Mahony (2014), when particle size increased with increasing feed viscosity.

3.3.3. Powder wettability

The results for contact angle (θ) analysis showed that the highest θ was observed for WPH + CIT_p, followed by WPI_p > WPH + LEC_p > WPH-MD_p > WPH_p (Table 3). Generally, the more hydrophobic the surface (i.e., surface of powder pellet), the lower is its affinity for interactions with water and, effectively, the higher the θ with the droplet of water placed on that surface. Thus, the contact angle analysis is often used to study the affinity of powders for water, providing information on powder wettability (i.e., lower θ = better wettability). The differences in wettability between the WPI_p and WPH_p powders, evidenced by different θ, were most likely directly related to differences in the physical state of protein (i.e., native *vs* hydrolysed, respectively). Solubility is generally enhanced by protein hydrolysis due to partial disruption of protein secondary and tertiary structure resulting in increased water access and faster hydration in hydrolysed, compared with intact, protein-based powders (Banach et al., 2013; Chobert, Bertrand-Harb, & Nicolas, 1988; Kelly, O'Mahony, Kelly, & O'Callaghan, 2016; Panyam & Kilara, 1996). Longer wettability times for model infant formula powders based on intact whey protein compared to partially hydrolysed whey protein were reported previously by Murphy et al. (2015). Wettability of the WPH-MD_p was similar to that observed for the WPH_p (Table 3). The better powder wettability observed for the WPH + LEC_p, compared to the WPH + CIT_p, was likely due to the differences in the nature of the two surfactants; CITREM and lecithin are anionic and zwitterionic (i.e., amphoteric) surfactants, respectively (McSweeney, 2008). Lecithin is often coated onto the surface of dairy powders in a fluidised bed to facilitate improved wetting properties (i.e., instantisation) (Hammes, Englert, Zapata Norena, & Medeiros Cardozo, 2015).

3.3.4. Surface composition of powders

No significant differences were found in the free fat content for all powders due to large standard deviations, especially observed for the WPH + LEC_p powder (Table 3). A trend was observed, where free fat content was generally higher, for the WPH + CIT_p, WPH_p and WPH + LEC_p powders (i.e., 20.0, 22.9 and 25.4%, w/w, free fat, respectively), compared to the WPH-MD_p and WPI_p powders (i.e., 13.3 and 14.1%, w/w, free fat, respectively).

Table 3 shows differences in the surface composition (i.e., as measured using XPS) between the spray-dried model IF powders prepared in this study. The level of protein at the surface was highest for the WPI_p powder followed by WPH_p, WPH-MD_p, WPH + LEC_p and WPH + CIT_p powders. The highest levels of surface fat were found in the WPH + CIT_p and WPH + LEC_p powders. The amount of carbohydrate present at the surface was significantly higher for the WPH-MD_p powder compared to the 2 powders containing lipid-based surfactants (i.e., WPH + LEC_p and WPH + CIT_p).

The differences between the surface fat composition as measured by the solvent extraction and by the XPS methods can be explained by the different principles underpinning these methods. For the solvent extraction method the results are presented as the weight of extractable fat as a % of the powder sample weight; conversely in the XPS method, the results are presented as the % of surface area of the powder particle occupied by fat. For the XPS method only a 10 nm depth of the surface of the powder particle is

Table 3
Properties of spray dried model infant formula powders ($_p$) prepared with different emulsifier systems: whey protein isolate (WPI_p), whey protein hydrolysate (WPH_p), WPH + CITREM (WPH + CIT_p), WPH + lecithin (WPH + LEC_p) and WPH-maltodextrin conjugate (WPH-MD_p). The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191).

Powder characteristics		WPI_p	WPH_p	WPH + CIT_p	WPH + LEC_p	WPH-MD_p
Drying performance[1]	Powder recovery (%)	22.0 ± 6.59^{a}	26.1 ± 3.27^{a}	21.3 ± 6.67^{a}	18.1 ± 2.56^{a}	55.3 ± 10.8^{b}
	Stickiness (relative)	+	+	++	++	−
PSD (μm) Powders[2]	$D_{4,3}$	26.5 ± 16.9^{ab}	25.4 ± 4.79^{ab}	30.8 ± 2.94^{ab}	41.1 ± 13.2^{a}	14.2 ± 4.79^{b}
	$D_{v,0.1}$	5.75 ± 0.56^{a}	5.85 ± 0.21^{a}	7.87 ± 0.54^{b}	9.52 ± 0.73^{c}	4.76 ± 0.27^{a}
	$D_{v,0.5}$	15.5 ± 2.29^{ab}	15.1 ± 0.33^{ab}	18.4 ± 1.64^{bc}	22.7 ± 2.41^{c}	12.2 ± 0.94^{a}
	$D_{v,0.9}$	59.5 ± 48.3^{a}	40.4 ± 3.22^{a}	56.0 ± 15.4^{a}	95.1 ± 43.6^{a}	26.6 ± 2.33^{a}
	% <5 μm	10.5 ± 2.16^{bc}	13.5 ± 0.71^{b}	6.33 ± 1.64^{cd}	2.84 ± 0.81^{d}	19.9 ± 2.71^{a}
	% >100 μm	2.93 ± 6.92^{a}	2.26 ± 1.13^{a}	4.05 ± 0.93^{a}	7.78 ± 5.29^{a}	0.00 ± 0.00^{a}
Contact angle (θ)		42.1 ± 0.08^{b}	36.9 ± 1.45^{d}	46.7 ± 1.00^{a}	40.5 ± 2.27^{bc}	37.2 ± 0.91^{cd}
Surface free fat (%)		14.1 ± 2.68^{a}	22.9 ± 4.85^{a}	20.0 ± 5.05^{a}	25.4 ± 17.9^{a}	13.3 ± 1.18^{a}
Surface composition (%)	Protein	50.7 ± 6.42^{a}	37.1 ± 6.22^{b}	27.0 ± 2.81^{b}	29.1 ± 4.03^{b}	32.3 ± 2.02^{b}
	Fat	34.1 ± 9.42^{a}	50.9 ± 6.47^{ab}	64.2 ± 6.22^{b}	61.8 ± 6.82^{b}	50.0 ± 3.23^{ab}
	Carbohydrate	15.2 ± 3.02^{ab}	12.0 ± 0.91^{ab}	8.85 ± 3.50^{b}	9.12 ± 3.17^{b}	17.7 ± 1.61^{a}
PSD (μm) Reconstituted[2]	$D_{4,3}$	2.42	5.72	5.00	1.47	0.84
	$D_{v,0.1}$	0.15	0.35	0.31	0.35	0.17
	$D_{v,0.5}$	0.57	4.68	1.10	1.18	0.51
	$D_{v,0.9}$	8.02	13.3	14.4	3.07	1.82

[1] Drying performance describing powder recovery (%, w/w total solids, TS; powder TS/feed TS); stickiness classification: -, non-sticky; +, moderately sticky; ++, very sticky.
[2] Particle size distribution (PSD) parameters: $D_{4,3}$, volume mean diameter; $D_{v,0.1}$, $D_{v,0.5}$, and $D_{v,0.9}$ representing particle size in the 10%, 50% and 90% quantiles of the distribution. Particle size distribution analysis for reconstituted powders was carried out only on one trial.
[(a-d)] Values for a given parameter (i.e., within each row) for all powders, not sharing a common superscript differed significantly ($P < 0.05$).

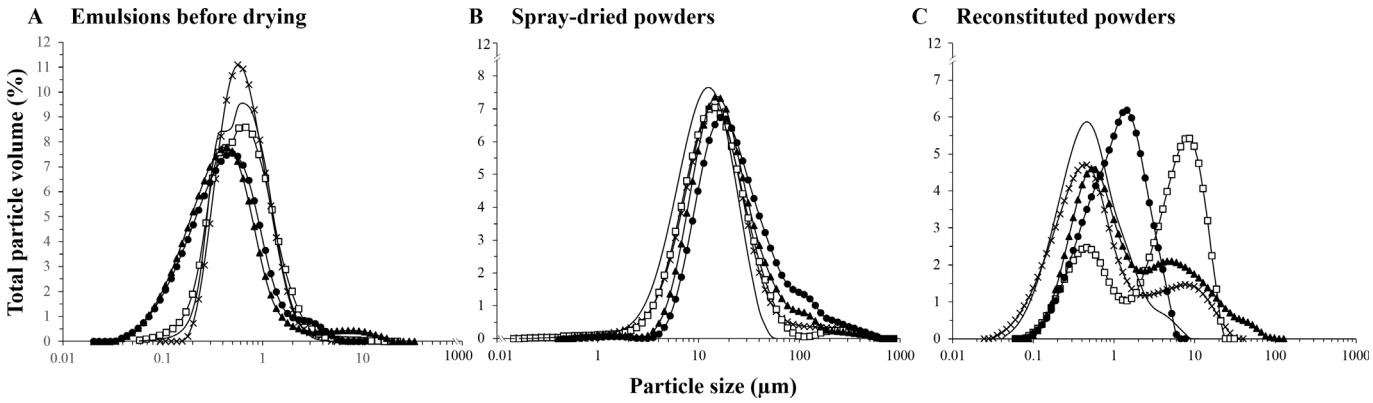

Fig. 3. Particle size distribution for (A) homogenised emulsions (dryer feeds), model infant formula powders (B) after spray-drying and (C) after powder reconstitution. The formulations contained different emulsifier systems: (×) whey protein isolate, (□) whey protein hydrolysate, (▲) WPH + CITREM, (●) WPH + lecithin and (–) WPH-maltodextrin conjugate. The powders were produced using a laboratory-scale spray dryer (BÜCHI B-191).

analysed (Kim et al., 2009). Conversely, the solvent extraction approach extracts fat present at the surface of the powder particle as well as fat present at other locations within its interior. According to a model proposed by Buma (1971) the solvent-extractable free fat for dairy powders consists of surface fat, outer layer fat from fat globules within the surface layer of the particle, capillary fat constituted by fat globules that can be reached by the solvent through capillary forces, and dissolution fat consisting of fat reached by solvent through holes left by already extracted fat. A review of solvent extraction-based methods for assessment of the amount of free or surface fat in spray-dried emulsions, reported in the scientific literature, was compiled by Vega and Roos (2006) and it was shown that these methods use different solvent types (petroleum ether, hexane, pentane and carbon tetrachloride), solvent-to-powder ratios (5:1–40:1), and powder-solvent contact times (30 s–48 h). The solvent extraction method used in this study (GEA Niro, 2005) for quantification of the surface free fat in the milk powders, with an extraction time of 15 min, could have led to the extraction of lipid material in addition to surface fat alone (i.e., fat from the surface and from the interior of the powder particles).

3.3.5. Microstructure of powders

3.3.5.1. Scanning electron microscopy. Fig. 4 A and B illustrate the detailed morphology (shape and structure) of the spray-dried model IF powders. Differences between samples were mainly manifested by the extent of particle agglomeration (i.e., spontaneous agglomeration of primary particles) and the topography of the particle surfaces in the powders. Powders containing lipid-based emulsifiers, WPH + CIT_p and WPH + LEC_p, displayed the greatest extent of particle agglomeration, followed by WPI_p, WPH_p and WPH-MD_p (Fig. 4A). Such agglomeration is generally caused by extensive particle cohesion (i.e., sticking) and is evidenced by the presence of 'bunch of grape'-type agglomerates (Pisecky, 2012), as observed in this study for the WPH + CIT_p, WPH + LEC_p and, to a lesser extent, WPI_p powders (Fig. 4A). These observations closely match the particle size distribution data discussed in Section 3.3.2 and indicate cohesive interactions between particles during spray-drying.

The surface topography was also different between the powders; smooth surfaces were observed for the WPI_p and to a lesser extent for WPH-MD_p while the powder particles in the WPH_p, WPH + CIT_p and WPH + LEC_p had an uneven surface with numerous bumps (WPH_p) or craters (WPH + CIT_p and WPH + LEC_p) present on the surface (Fig. 4B). The presence of crater-like structures on the surface of spray-dried emulsions/powders has been associated with broken oil globules resulting in high levels of surface fat (Drusch & Berg, 2008). Additionally, WPH- MD_p powder particles appeared to be partially collapsed (i.e., shrivelled) unlike particles in the other powders. Such shrivelled/buckled structures in spray-dried powders has been linked with temperature-dependent changes in the volume of occluded air (i.e., inflation followed by deflation of intra-particle air as the particle moves from hot toward the cooler regions of the dryer) (Walton & Mumford, 1999) and with the mechanical properties of the skin layer of the drying particles (Sadek et al., 2015, 2016).

3.3.5.2. Confocal laser scanning microscopy. Powders produced in the current study had generally similar particle structures, where individual oil droplets were homogenously distributed within a protein-carbohydrate network (Fig. 4C). The only exception was the WPH_p powder, where the oil phase appeared to be largely present as irregular and extensive oil pools. Differences in the size of oil droplets within the powder matrix were observed; powders containing lipid-based surfactants, WPH + CIT_p and WPH + LEC_p had markedly bigger (2–3 μm) oil droplets embedded in the powder structure, compared to apparently smaller (≤1 μm) oil droplets in the WPI_p and WPH-MD_p powders. Pools of oil or large oil droplets observed in CLSM micrographs can be related to poor stability of these emulsions to processing. Additionally, 'empty' regions were observed in the centre of the WPH-MD_p powder (Fig. 4C); these regions most likely indicate the presence of internal air pockets (i.e., vacuoles) in particles of this powder as discussed in Section 3.3.5.1. Formation of vacuoles and shrivelling of powder particles have been shown to take place concomitantly (Sadek et al., 2015) and is strongly linked to the surface composition of the droplet and, effectively, its drying kinetics (Nijdam & Langrish, 2006; Vignolles et al., 2007).

3.3.6. Particle size distribution after reconstitution of powders

Notable differences were observed in the PSD between the reconstituted IF powders (Table 3; Fig. 3C); the mean volume diameter ($D_{4,3}$) and the value for the 90% quantile of the size distribution ($D_{v,0.9}$) were higher for all reconstituted powders compared to the emulsions prior to spray drying (Tables 1 and 3; Fig. 3A and C). The observed increases in $D_{4,3}$ and $D_{v,0.9}$ were most pronounced for the WPH_p and WPH + CIT_p powders (i.e., increases in $D_{4,3}$ and $D_{v,0.9}$ to ≥ 5 μm and >13 μm, respectively); only a limited increase was observed for the WPH-MD_p powder (i.e., $D_{4,3}$ < 1 μm and $D_{v,0.9}$ < 2 μm) (Table 3). The $D_{4,3}$ and $D_{v,0.9}$ parameters are particularly sensitive to changes at the large particle periphery of

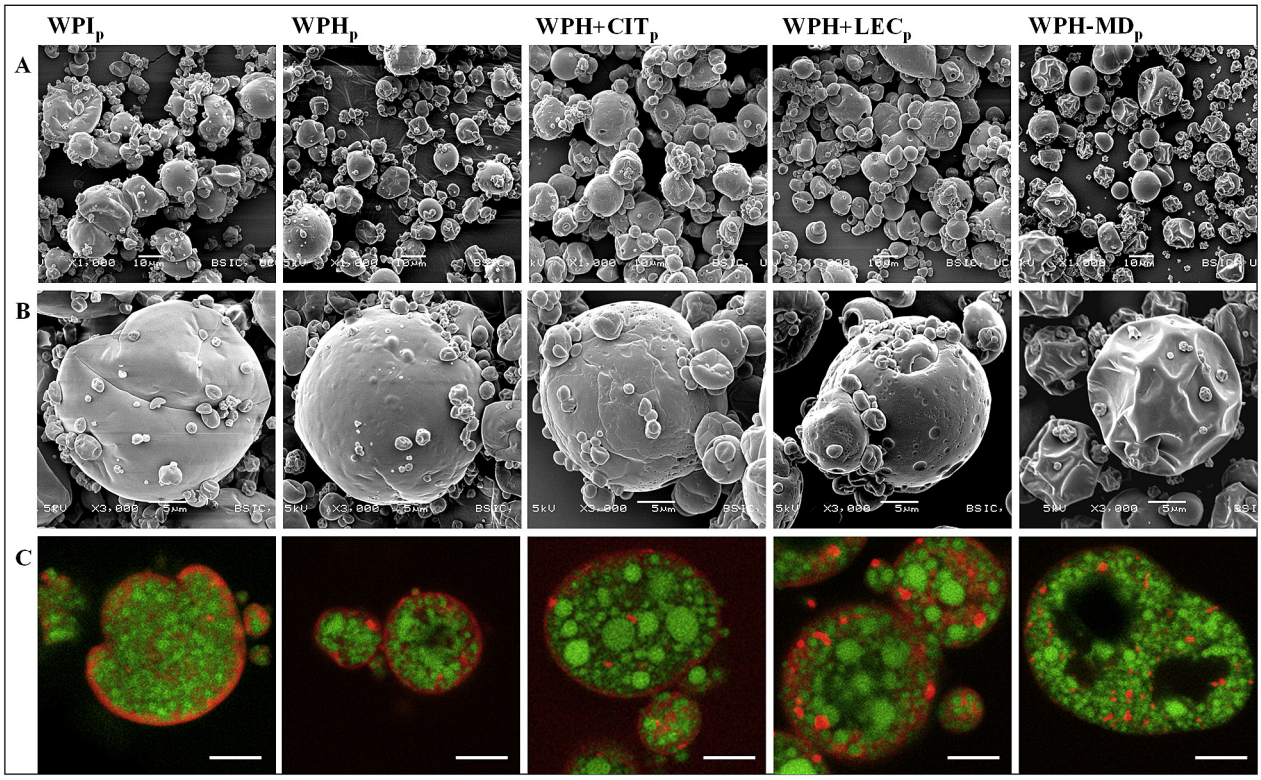

Fig. 4. Scanning electron microscope (SEM; A and B) and confocal laser scanning microscope (CLSM; C) images of model infant formula powders ($_p$) containing different emulsifier systems: whey protein isolate (WPI$_p$), whey protein hydrolysate (WPH$_p$), WPH + CITREM (WPH + CIT$_p$), WPH + lecithin (WPH + LEC$_p$) and WPH-maltodextrin conjugate (WPH-MD$_p$). For the CLSM analysis powders were labelled with Nile Red:Fast Green (3:1) and the micrographs show distribution of oil droplets (green) and protein particles (red). Scale bar for the CLSM micrographs = 5 μm. The powders were produced using a laboratory scale spray dryer (BÜCHI B-191). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the size distribution and their increase can be used as an indicator of associations between the larger components in a system (i.e., coalescence and/or flocculation of oil globules in this case).

These differences reflect different stabilities of the corresponding formulations to the spray-drying conditions (i.e., stability of oil globules against coalescence in a concentrated emulsion system and stability to high heat and high shear stress within the atomiser and upon atomisation) and support the CLSM observations (see Section 3.3.5.2).

4. Discussion

The stability of emulsions to spray-drying was different for the studied formulations, as illustrated by the size distribution of oil globules in the powder matrix and in the reconstituted emulsions. These differences can be explained by the properties of the emulsifier systems used in these formulations, and their effect on stabilising emulsions against globule coalescence or heat-induced flocculation during processing. During spray-drying, emulsion-based systems are subjected to considerable stresses which can cause protein aggregation, breaking and coalescence of oil globules; this can lead to high surface free fat content and, effectively, undesirable properties of the resultant powder. Emulsions stabilised by high molecular weight (M_W) surfactants (e.g., protein) usually have thick and elastic interfacial films and are more stable to stress, compared to those stabilised by low M_W surfactants (e.g., CITREM, lecithin), which are prone to coalescence when forced in close contact (Taneja et al., 2013). Formulations based on WPH often display poor thermal stability, due to exposure of reactive sites (e.g., free sulphydryl groups) at the surfaces of oil globules and in the bulk phase, often resulting in bridging flocculation of oil globules (Agboola, Singh, Munro, Dalgleish, & Singh, 1998; Drapala et al., 2016a). Such behaviour was also reported in the current study, where oil pools in the WPH$_p$ powder matrix and large oil globules were present after reconstitution of this powder.

CITREM and lecithin are often added to improve thermal stability of WPH-based emulsions; however, their presence can lead to competitive destabilisation, where protein/peptide-based surfactants are displaced from the interfaces by smaller surfactants, promoting coalescence of oil globules (Drapala et al., 2016b; Kaltsa, Paximada, Mandala, & Scholten, 2014; Mackie, Gunning, Wilde, & Morris, 1999; Van Aken, 2003; Wilde, Mackie, Husband, Gunning, & Morris, 2004). This was observed in the current study for CITREM- and lecithin-containing powders, where large oil globules were observed in the powder matrix and in the reconstituted emulsions (Fig. 4C, Table 3). In addition, topographical features observed for samples containing lipid-based emulsifiers (i.e., craters; Fig. 4B) indicated that coalescence of oil globules resulted in the presence of damaged oil globules at the powder surface (Drusch & Berg, 2008). It is generally accepted that strong steric stabilisation of oil globules, provided by protein-carbohydrate conjugates, can greatly limit these forms of destabilisation (Oliver, Melton, & Stanley, 2006; O'Mahony et al., 2017). The presence of WPH-MD conjugate in emulsions prevents interactions between individual oil globules and interactions with bulk protein/peptides, resulting in enhanced stability. Results presented in the current study show that superior stability of emulsions to spray-drying was achieved when the WPH-MD conjugate was present in the formulation, compared to formulations containing CITREM or lecithin.

In an emulsion, surface active molecules (e.g., protein, peptides,

lecithin, CITREM, conjugates) are adsorbed at the oil/water interface, where they stabilise oil globules; these compounds are, generally, also abundant in the emulsion bulk phase as they are present in excess of the concentration required for emulsion formation. Upon atomisation, a new interface (water/air) is formed at the surface of the atomised droplets and, during very short time scales, surface active components move from the bulk to this new surface, adsorb and rearrange (Munoz-Ibanez et al., 2016). Smaller surfactants move and adsorb faster due to their higher mobility compared to large surfactants (Landstrom, Alsins, & Bergenstahl, 2000). Similar to the stabilisation of oil globules, the composition and structure of the interfacial layer of atomised droplets dictate their potential for interactions (i.e., stickiness, agglomeration) (Nijdam & Langrish, 2006); in effect, surface composition and physicochemical properties of the resulting powder are largely dependent on the surfactant system of the emulsion. The high surface fat level observed for the WPH + CIT_p and WPH + LEC_p powders and the high surface maltodextrin level observed for the WPH-MD_p powder, could indicate preferential adsorption of lipid-based and conjugate-based emulsifiers, respectively, at the surfaces of atomised droplets in these powders. Owing to the different surface compositions, powders displayed different propensity for interactions between individual atomised droplets/particles (i.e., primary spontaneous agglomeration) and with the wall of the spray dryer (as measured by powder build-up in the cyclone). It is generally recognised that high levels of surface free fat cause challenges with cohesive interactions of powders (Jayasundera et al., 2009; Vega & Roos, 2006). Similarly, in the current study, the likely preferential presence of lipid-based emulsifiers on the surface of some of the powders may have contributed to greater cohesiveness and, effectively, could have promoted agglomeration and powder build-up, compared to the other powders.

Properties of the feed and drying kinetics generally govern the shape of powder particles (Walton & Mumford, 1999). Distinctive shrivelled particles observed for the WPH-MD_p powder were likely related to significantly lower viscosity of that emulsion, compared to the other emulsions (i.e., at the same TS content), effectively, impacting the rate of water removal. Additionally, the more hydrophilic nature of the surface of atomised droplets/powder particles for the WPH-MD_p system, resulting from higher surface maltodextrin content, compared to the other samples could have promoted faster water removal as evidenced by the lower moisture content of the resultant powder. According to a study by Sheu and Rosenberg (1998), surface indentation for whey protein-based powders was promoted by high drying rates, leading to wall solidification before the onset of particle inflation. With progressive water removal during drying of a dairy-based system, a skin layer is formed at the droplet surface and its properties further affect the kinetics of drying and the final shape of the dried particles. Sadek et al. (2015) presented a model for mechanical properties of skin layer of a droplet during drying, where, depending on protein type present at the surface (i.e., whey protein or micellar casein), the mechanical properties of the skin were different and affected the shape of the resultant dried particles. Those authors showed that in casein micelle-dominant skins, the elastic modulus increased faster and the protein skin reached the plasticity region earlier, producing shrivelled particles with ductile and plastic skin, while it took longer for the whey protein-dominant skin to reach the plasticity region, giving round particles with brittle and plastic skins. Particle indentation for whey protein-based powders was reported to be linked to the ratio of protein to maltodextrin at the surface of powder particles (Rosenberg & Young, 1993; Sheu & Rosenberg, 1998), where surface indentation was inversely related to the proportion of whey protein in the particle skin. In the study by Sheu and Rosenberg (1998), the authors showed that increasing the maltodextrin proportion in the skin decreased its elasticity and, effectively led to the formation of shrivelled powder particles. Such shrivelled morphology was observed in this study for the WPH-MD_p powder particles. In addition, the presence of vacuoles observed in the WPH-MD_p powder sample supports its fit to the model proposed by Sadek et al. (2015), where vacuole formation and particle shrivelling were concomitant. With rapid water removal from the atomised droplets during spray-drying, less latent heat energy is required due to lower moisture content, and the energy (i.e., temperature) acting on the non-water powder components is increased. This, effectively, can result in increased inflation of the droplet due to the expanding volume of air occluded within, followed by particle collapse (i.e., deflation) as the particles move away from the heat source, resulting in a shrivelled hollow powder particle (Hecht & King, 2000; Walton & Mumford, 1999). The use of different emulsifier systems resulted in different surface composition of the resultant powders as well as different quality of reconstituted emulsions. It was demonstrated that the differences in powder surface composition influenced the kinetics of drying for these formulations and governed the cohesive interactions between atomised droplets/powder particles. Effectively, the presence of lipid-based emulsifiers (i.e., CITREM or lecithin) in formulations greatly increased the cohesive interactions resulting in extensive spontaneous primary agglomeration and, effectively, reduced product yield. On the other hand, when the conjugate-based emulsifier was present in the formulation, these cohesive interactions were markedly reduced.

5. Conclusions

The current study demonstrated that using the WPH-MD conjugate in the formulation of emulsion-based model IF powder improved its processing stability and affected the surface composition of resultant powder. The use of the conjugate in the formulation gave powder with decreased surface fat and increased surface carbohydrate levels, compared to systems containing lipid-based emulsifiers (i.e., CITREM or lecithin). In effect, the conjugate-based powder displayed reduced cohesive behaviour, resulting in decreased agglomeration and markedly higher product yield; the opposite was observed for the powders containing lipid-based emulsifiers. This study showed that the surface composition of an emulsion-based powder and, effectively, its drying performance and final product characteristics were greatly improved by utilisation of interactions (i.e., conjugation) between the two components of the formulation (i.e., protein and carbohydrate).

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Review

Controlled glycation of milk proteins and peptides: Functional properties

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ABSTRACT

Glycation of milk proteins and peptides can be achieved by Maillard-induced conjugation of reducing carbohydrates with the available amino groups of proteins/peptides during the early stages of the Maillard reaction. This conjugation can be achieved under wet or dry heating conditions, with the choice of heating mode influencing the rate and extent of conjugation, in addition to the functionality of the conjugated protein/peptides. Conjugation has been shown to modify the technological and nutritional properties of a range of milk protein/peptide-based ingredients. This review focuses mainly on modifications to physicochemical properties and technological functionality (i.e., solubility, heat stability, emulsification, foaming and gelation properties) of milk proteins and peptides by conjugation. Particular emphasis is placed on understanding of the relationships between changes in protein/peptide molecular structure/conformation, physicochemical properties and technological functionality, as influenced by glycation.

Contents

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1. Introduction

A conjugated protein is defined as a protein to which another chemical group (e.g., carbohydrate) is attached by either covalent bonding or other interactions (Wong, 1991). Milk proteins and peptides, in the presence of reducing carbohydrates, can undergo a series of complex chemical changes during heating, known as the Maillard reaction. Conjugation occurs naturally during the early stages of the Maillard reaction when a covalent bond forms between the protein and carbohydrate components, resulting in the release of water (i.e., condensation reaction). The resulting covalently-linked Schiff base product can undergo irreversible Amadori rearrangement, leading to the formation of Amadori products (Ames, 1992; Liu, Ru, & Ding, 2012; Zhu, Damodaran, & Lucey, 2008). Conjugation of food proteins with carbohydrates *via* the Maillard reaction (i.e., glycation) is a growing area of interest, with many studies completed, particularly over the last 10–15 years, on the use of conjugation to modify physicochemical and functional properties of proteins and peptides. Sections 2 and 3 provide an overview of the Maillard reaction and the various factors affecting the reaction, respectively.

Milk protein ingredients are utilised in the formulation of a wide range of food, clinical and pharmaceutical products, due to their unique functional and nutritional attributes. In the food industry, the principal technological hurdles limiting the use of milk (especially whey) protein ingredients in the formulation of value-added beverages and powders are: (i) poor solubility of intact proteins in high-acid ready-to-drink beverages, resulting in the development of turbidity and phase separation (Akhtar & Dickinson, 2007), (ii) poor emulsification properties of hydrolysed proteins (Agboola, Singh, Munro, Dalgleish, & Singh, 1998a, 1998b; Singh & Dalgleish, 1998), causing challenges with emulsion formation, stabilisation and spray drying (e.g., powder stickiness and high free fat) during the manufacture of powdered nutritional products, and (iii) physical instability such as aggregation, sedimentation and creaming during processing and shelf-life in high ionic strength environments and during thermal processing (Yadav, Parris, Johnston, Onwulata, & Hicks, 2010).

Conjugation has been shown to be successful in modifying the functional properties of a range of milk protein/peptide-based ingredients. Sections 4 and 5 provide an overview of how the key compositional, structural and physicochemical properties of protein/peptide and carbohydrate substrates, respectively, influence the progression of conjugation and the functionality of the resulting conjugated proteins/peptides. A detailed comparison of the differences between the two main modes of achieving conjugation (i.e., dry and wet heating) is provided in Section 6, while Sections 7–11 of this review provide detailed information on the effects of conjugation on solubility, heat stability, emulsification, foaming and gelation properties of the principal milk protein/peptide ingredients used in the food industry. Section 12 provides an overview of approaches developed for enriching and purifying conjugates.

2. The Maillard reaction

The Maillard reaction (Maillard, 1912) encompasses a complex series of reaction pathways, many of which proceed concurrently during heating and/or storage of protein/carbohydrate mixtures. Understanding of the complexity of the Maillard reaction has been advancing steadily through the years, and a brief overview is provided here in the context of the Maillard reaction being the main mechanism by which milk proteins and peptides are glycated (i.e., conjugated with carbohydrate molecules). Hodge (1953) was the first to develop a simplified, integrated scheme for the Maillard reaction, which has been advanced further and refined by researchers from different fields over the years (Henle, Walter, & Klostermeyer, 1991; Van Boekel, 1998; Zhang & Zhang, 2007). In essence, Hodge (1953) divided the chemistry of the Maillard reaction into three stages - the early, intermediate and advanced stages (Fig. 1). The early stage of the Maillard reaction involves a series of individual reactions that are initiated when the ε-amino groups of lysine, or to a lesser extent, the imidazole and indole groups from histidine and tryptophan, respectively, and the α-amino groups of terminal amino acids in proteins/peptides condense with the carbonyl groups of reducing carbohydrates, to form a Schiff base, with the release of a molecule of water (Ames, 1992). The Schiff base is thermodynamically unstable and undergoes spontaneous rearrangement to form either an Amadori (in the case of aldoses) or Heyn's (in the case of ketoses) product (Wrodnigg & Eder, 2001).

The intermediate stage of the Maillard reaction involves the degradation of the Amadori and/or Heyn's rearrangement products by a number of different reactions, including cyclisations, dehydrations, retro-aldolisations, isomerisations and further condensations, which causes degradation of amino acids and carbohydrates (Ames, 1998). The advanced stages are complex and variable, depend on the reaction conditions, and involve dehydration and decomposition of the early reaction products, resulting in the production of many advanced Maillard reaction products (AMP) and coloured nitrogenous polymers and co-polymers, known collectively as melanoidins (Ames, 1998; Hodge, 1953). While, from a functionality perspective, it is desirable to achieve conjugation in the early stages of the Maillard reaction, it is normally desirable to limit the progression of the Maillard reaction to advanced stages, as AMPs are largely responsible for some of the less desirable consequences of the Maillard reaction, e.g., generation of off-flavours, loss of nutritional value, protein crosslinking and generation of potentially toxic compounds (Uribarri et al., 2005). There have been many analytical approaches researched and reported for monitoring the formation of Maillard reaction products and for determining the progression of the Maillard reaction through the early-, intermediate- and late-stages. An overview of the analytical approaches most commonly used in protein/peptide-carbohydrate conjugation studies is provided in Table 1.

3. Factors affecting Maillard-induced conjugation of proteins/peptides

In the production of protein/peptide-carbohydrate conjugates, the rate, extent and course of the Maillard reaction are influenced by several intrinsic and extrinsic factors, including, but not limited to, nature of the reactants, temperature, time, pH and water activity (a_w) (Ames, 1990; de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Liu et al., 2012; Oliver, Melton, & Stanley, 2006a; Van Boekel, 2001). Understanding and manipulation of these factors allow control of the yield, quality and functionality of conjugated proteins/peptides.

3.1. Nature of the reactants

The physicochemical properties (i.e., molecular weight, M_w; structure/conformation and surface charge) of the amino and carbonyl compounds, and their molar ratios, all govern the rate and extent of the Maillard reaction, and consequently, the physicochemical properties of the conjugated proteins/peptides. Reactivity of compounds tend to decrease with increasing M_w, due to the greater contribution of steric hindrance with increasing M_w; as an example, monosaccharides are more reactive with proteins than di- or oligosaccharides under conditions that favour conjugation. For protein hydrolysates, the degree of hydrolysis, M_w profile and

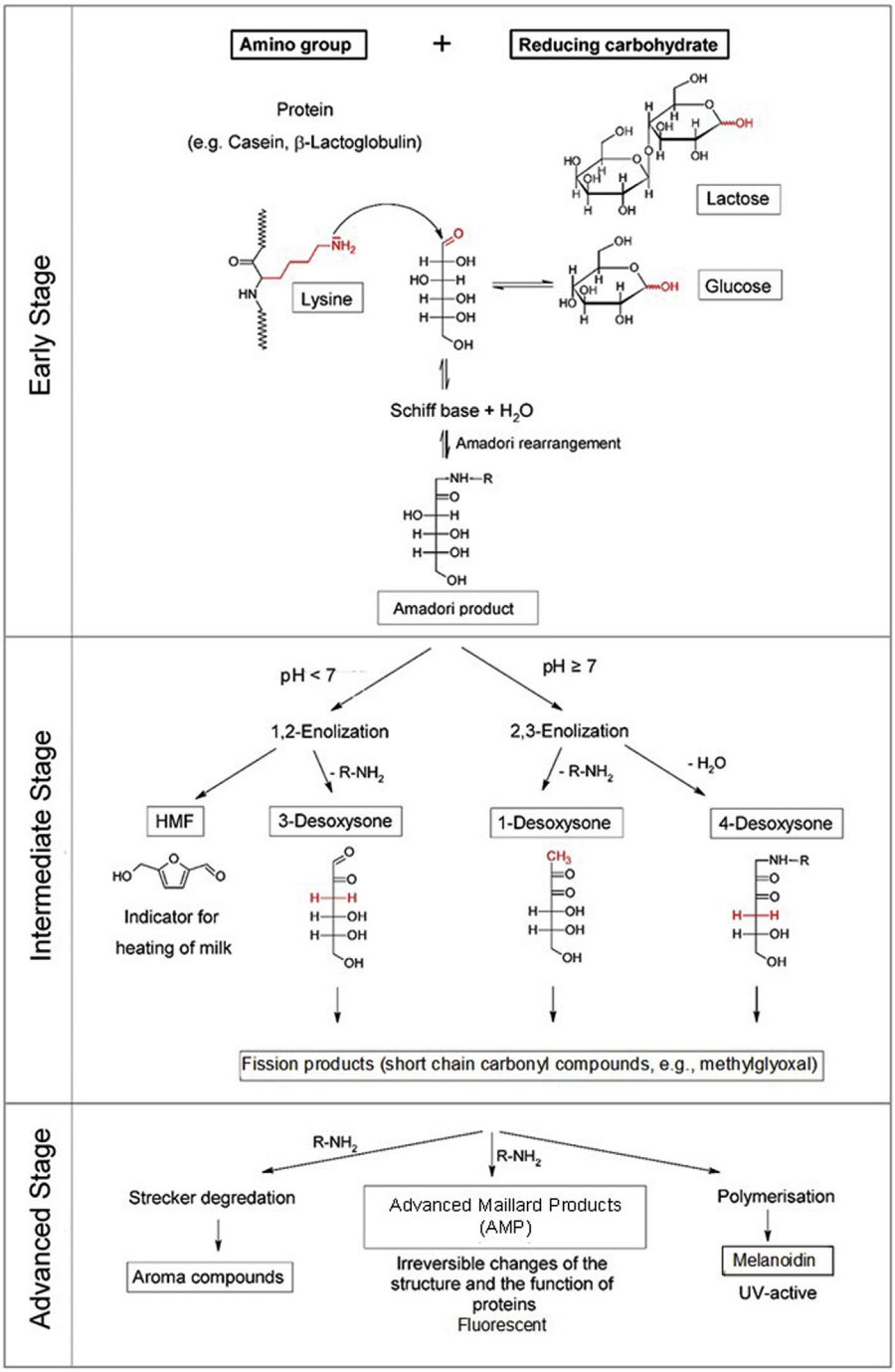

Fig. 1. Schematic overview of the Maillard reaction in milk and milk products (based on Hodge, 1953; Ames, 1998).

charge of the peptides are important in determining their reactivity during Maillard-induced conjugation (Drapala, Auty, Mulvihill, & O'Mahony, 2016a, 2016b; Mulcahy, Park, Drake, Mulvihill, & O'Mahony, 2016b; Van Lancker, Adams, & De Kimpe, 2011).

3.2. Temperature and time

Louis-Camille Maillard (Maillard, 1912) was the first to report that the rate of the Maillard reaction increased with increasing temperature and duration of heating. In addition, more recent research has shown that temperature also affects the nature (e.g., conformation and accessibility to reactive protein/peptide functional groups) of the reactants. The reactivity of sugars increases with increasing temperature as the proportion of reducing sugar molecules present in the open-chain form (i.e., the more reactive form) increases (Van Boekel, 2001), due in part to the faster rate of mutarotation of the sugar molecules. Heat-induced structural/conformational changes (e.g., denaturation and aggregation) of milk proteins/peptides may result in amino groups becoming less available for participation in the Maillard reaction (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001; Jiang & Brodkorb, 2012; Mehta & Deeth, 2016).

Table 1
An overview of the analytical approaches most commonly used in protein/peptide-carbohydrate conjugation studies (modified from O'Brien, 1997).

Measurement	Reference
Early stage	
Reduction in chemically reactive amino groups including 1-fluoro-2,4-dinitrobenzene (FDNB), trinitrobenzenesulfonic acid (TNBS), guanidination, sodium borohydride, *o*-phthaldialdehyde (OPA) and dye-binding methods	Mehta and Deeth (2016)
Formation of lactulosyllysine	Henle et al. (1991)
Absorbance of Schiff base	Zhu et al. (2008)
HPLC analysis of derived Amadori products (e.g., furosine, carboxymethyllysine)	Erbersdobler and Somoza (2007)
Amino acid analysis	Rutherfurd, Bains, and Moughan (2012)
Identification of conjugation site by electrospray ionisation or matrix assisted laser desorption/ionisation mass spectrometry	Oliver (2011)
Intermediate stage	
HPLC analysis of intermediate reaction products or their derivatives (e.g., hydroxymethylfurfural)	Morales, Romero, and Jiménez-Pérez (1995)
pH decrease	O'Brien (1997)
Advanced stage	
Colour development (absorbance or colorimeter)	Ames (1998)
Fluorescence of advanced Maillard products	Birlouez-Aragon et al. (1998)
Strecker degradation volatiles by gas chromatography mass spectrometry/flame ionisation	Jansson et al. (2014)
Enzyme-linked immunosorbent assay	Horiuchi, Araki, and Morino (1991)

3.3. pH

pH influences the reactivity of both the carbohydrate and protein components of mixtures during the Maillard reaction; a basic environment can catalyse the initial stages of the Maillard reaction by deprotonating the amino groups, which in turn increases reactivity with carbonyl groups of reducing carbohydrates. The open chain form of the carbohydrate and the un-protonated form of the amino group, which are considered to be the most reactive forms, are usually favoured at higher pH, up to a maximum of pH ~9–10 (Martins, Jongen, & Van Boekel, 2000). The use of buffers, such as phosphate, phthalate and acetate, to help minimise changes in pH on heating, have been reported to catalyse the Maillard reaction, as measured by loss of available amino groups and development of brown colour (Bell, 1997; Van Boekel, 2001). For example, at a constant pH between 5 and 7, the presence of phosphate buffer has been shown to increase the rate of the Maillard reaction by ~15 fold compared with a non-buffered system, as the phosphate acts as an acid-base catalyst during the Amadori rearrangement (Potman & Van Wijk, 1989). The pH of protein/peptide-carbohydrate mixtures can decrease (depending on the buffering capacity) as the Maillard reaction progresses due to the formation of acids (e.g., formic and acetic acids), the consumption of acidic amino groups (e.g., lysine) or the loss of carboxyl groups during Strecker degradation, resulting in the production of carbon dioxide (Nursten, 2005).

Furthermore, products derived from the intermediate and advanced stages of the Maillard reaction are degraded by different reaction pathways depending on the pH of the system. For example, degradation of the Amadori products at pH <7 takes place via the 1, 2-enolisation pathway that favours the formation of furfural or hydroxymethylfurfural (HMF), whereas, at pH ≥7, the degradation of Amadori products proceeds through the 2, 3-enolisation pathway, favouring the production of reductones and fragmentation products such as hydroxyacetone and 2, 3-butanedione, minimising the formation of HMF (Ames, 1998; Liu et al., 2012).

3.4. Water activity/relative humidity

Increasing water activity of protein/peptide-carbohydrate mixtures generally increases the rate and extent of conjugation, due to the increased diffusion and mobility of reactants; however, high water concentrations/a_w can negatively influence progression of the Maillard reaction. Morgan, Léonil, Mollé, and Bouhallab (1999a) reported that, when heated at the same temperature, the rate and extent of protein-carbohydrate conjugation in an aqueous solution was lower than when a dry heating approach was used, as the presence of water inhibits the initial Amadori condensation reaction between the available amino and carbonyl groups. The physicochemical state of the reactants in mixtures of proteins, peptides and carbohydrates can also influence the progression of Maillard-induced conjugation; when reactants (e.g., sugars) in the amorphous state are exposed to high humidity (a_w) they generally absorb water until the reactant molecules acquire sufficient mobility (generally at a_w ~0.6–0.7) and space to form a crystalline lattice. On crystallisation of sugars, water is released and may become trapped in a localised manner within protein/peptide-carbohydrate mixtures, and facilitate further interactions/conjugation between proteins/peptides and amorphous sugars (Lievonen, Laaksonen, & Roos, 1998, 2002; Roos, Jouppila, & Zielasko, 1996).

3.5. Other factors

Factors, other than those outlined above, can impact the Maillard reaction, including the presence of sulphur dioxide in food systems that has been shown to delay the development of brown colour (Ames, 1990) and the presence of metal ions, which can accelerate or inhibit the Maillard reaction, depending on their concentration (Ramonaitytė, Keršienė, Adams, Tehrani, & De Kimpe, 2009). In model systems, the presence of tertiary amine salts, acetic acid and free radicals have been shown to promote the Maillard reaction; however, these factors may often in practice be of minor significance relative to the nature of the reactants, temperature, time and moisture content (O'Brien, 1997). Non-thermal energy sources (e.g., ionising radiation, UV irradiation and ultrasound treatment) have also been shown to produce Maillard reaction products, including brown pigments and volatile flavour compounds (O'Brien, 1997).

4. Protein/peptide substrates used in conjugation

The conjugation of milk proteins/peptides has been studied using many categories of milk protein-based ingredients as substrates, including, but not limited to, whey protein concentrates (WPC) and isolates (WPI), individual whey protein fractions (in

particular β-lactoglobulin, β-lg; α-lactalbumin, α-lac; and bovine serum albumin, BSA), sodium caseinate, casein fractions (β-casein) and hydrolysates of whey proteins (WPH) and caseins (e.g., hydrolysed sodium caseinate). For the reasons outlined in Section 3, it is desirable that the proteins/peptides used are soluble under the conditions of conjugation; hence only soluble forms of casein (e.g., sodium caseinate) have been studied. It is also desirable that the proteins used for conjugation are present in a conformation which ensures a high degree of accessibility of carbonyl groups to amino groups, which is one of the main reasons why, in the study of casein-based conjugates, sodium caseinate, with an open/flexible structure, and extremely low levels of non-protein components, has been extensively used as the casein protein substrate; the authors are not aware of any studies performed using micellar casein for production of casein-based conjugates.

In addition, whey proteins are more susceptible than caseins to heat-induced aggregation under the conditions used for conjugation (particularly under wet heating conditions), which would be expected to restrict accessibility of carbonyl groups to amino groups on the protein/peptide molecules. β-Lactoglobulin typically represents ~50–60% of total protein in WPC, WPI and WPH ingredients and has two disulphide bonds and one free thiol group, which are deemed responsible for the irreversible thermal aggregation and gelling properties of this protein (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016). In contrast, α-lac has a single polypeptide chain, containing four disulphide bonds, and no free sulphydryl group (Permyakov & Berliner, 2000), making it less sensitive to heat-induced denaturation/aggregation under the conditions used in conjugation of whey protein (Enomoto et al., 2009). Furthermore, Nieuwenhuizen et al. (2003) reported that the availability of the lysine groups in α-lac are modified by the binding of calcium; five lysine residues were available for reaction in apo-α-lac compared with four available lysine residues in the halo-α-lac.

It is desirable to have low levels of non-protein components (e.g., lactose, minerals and lipid) in the protein-containing ingredients used as substrates for conjugation, as lactose contributes strongly to brown colour and flavour compound formation (Lillard, Clare, & Daubert, 2009), minerals promote aggregation of whey proteins (Brodkorb et al., 2016), lipid material can contribute to off-flavour formation (Liu & Zhong, 2014; Lloyd, Hess, & Drake, 2009) and lactose can compete with other carbohydrates for conjugation to the protein substrate during heating under conditions required to achieve conjugation. Therefore, high protein content WPC and WPI, or pure protein fraction ingredients are most commonly used for conjugation purposes.

The whey proteins generally have slightly higher normalised levels of lysine residues than the caseins. Hydrolysis of casein and whey protein molecules increases the number of free amino groups available to react with carbonyl groups during conjugation and can also lead to increased exposure and accessibility to previously-buried lysine residues. Protein hydrolysates are generally characterised by their degree of hydrolysis (DH), which expresses the number of peptide bonds cleaved as a percentage of the total number of peptide bonds available (Foegeding, Davis, Doucet, & McGuffey, 2002). Hydrolysis of whey proteins, due to reduction of average M_w and levels of secondary structure, enhances their stability to heat-induced aggregation, which can facilitate enhanced retention of amino groups in a form accessible for conjugation during heating. For example, Ju, Otte, Madsen, and Qvist (1995) reported that limited hydrolysis of WPI (DH 2–7%), using trypsin, prevented heat-induced gelation of a WPI solution (12%, w/v, protein) on heating at 80 °C for 30 min at pH 3 and 7. Mulcahy et al. (2016b) reported that WPH with a low degree of hydrolysis (DH 9.3%) had 55.4% higher levels of available amino groups compared with an intact WPI counterpart, which contributed to more rapid and extensive conjugation of maltodextrin (MD) with the WPH than with the WPI.

5. Carbohydrate substrates used in conjugation

The conjugation of milk proteins/peptides has been studied using many different types of carbohydrate ingredients, including, but not limited to, lactose, MD, corn syrup solids (CSS), dextrans, glucose, maltose, ribose, guar gum, pectin, fenugreek gum, oligosaccharides and glucosamine. From the point of view of their ability to participate in Maillard-induced conjugation of milk proteins/peptides, and the functionality of the resultant conjugates, the key differences between these carbohydrates are chain length, structure (i.e., linear versus branched and ketoses versus aldoses) and charge (neutral versus charged). In general, the shorter the chain length of the carbohydrate component, the faster the rate, and the greater the extent of conjugation. On conjugation of whey protein with MD or CSS, having dextrose equivalent (DE) values in the range 6–38, at an initial pH 8.2, at 90 °C for up to 24 h, the extent of conjugation increased with increasing DE value of the MD and CSS ingredients (Mulcahy, Mulvihill, & O'Mahony, 2016a). Delahaije, Gruppen, van Nieuwenhuijzen, Giuseppin, and Wierenga (2013) studied the stability of emulsions of patatin conjugated to the same extent with different mono- and oligosaccharides (xylose, glucose, maltotriose and maltopentaose) and reported that attachment of monosaccharides did not affect the flocculation behaviour of the emulsion; however, the attachment of maltotriose and maltopentaose (molecular mass > 0.5 kDa) provided stability against flocculation of the emulsions at pH 5, due to increased steric stabilisation contributed by the higher molecular mass carbohydrates. Brands and van Boekel (2001) reported that ketoses degraded during heating, whereas aldoses were involved in formation of the covalent bond between proteins and carbohydrates during the Amadori stage of the Maillard reaction.

6. Mode of conjugation

The main variables that can be controlled during conjugation of milk proteins/peptides are temperature, time, pH, moisture content, relative humidity (RH) and/or a_w. These variables can be grouped to give 2 distinct approaches for achieving conjugation – (1) wet heating and (2) dry heating. The wet heating approach normally involves incubation of an aqueous solution of protein/peptide and carbohydrate reactants, commonly pre-adjusted to a target pH (normally pH 6.0–11.0), for a pre-determined time (min-d) at a set temperature (typically in the range 60–95 °C). The conjugation reaction is normally stopped (or slowed considerably) by cooling and further processing (e.g., freeze or spray drying) of the conjugated protein/peptide solution. The dry heating approach normally involves incubation of a co-dried mixture (commonly pre-adjusted to a target pH) of the protein/peptide and carbohydrate ingredients for a pre-determined time (min-d) at a set temperature (typically in the range 60–130 °C) at a set RH (typically 60–80%).

Both approaches have been used extensively for conjugation of milk proteins/peptides and both have their advantages and limitations. The mobility of reactants is higher with the wet heating than the dry heating approach and higher temperatures (for shorter times) are generally used with the former than with the latter; however, some recent studies have used considerably higher temperature (130 °C) and shorter times (<30 min) than previous studies to achieve conjugation of WPI with lactose or MD under dry heating conditions at 79% RH (Liu & Zhong, 2014). Similarly, Guo and Xiong (2013) reported that WPI was successfully conjugated

with lactose or MD (DE18) at 130 °C for 20 min and 79% RH, with both systems having less colour development that WPI-lactose/MD conjugated at 80 °C for 2 h.

To achieve maximum reactivity between the protein/peptide and carbohydrate components using dry heating, it is necessary to prepare a solution of the two components, which is dried before being conjugated by dry heating, and the conjugated power typically requires down-stream drying due to release of water during the early stages of the Maillard reaction. This latter issue can also lead to localised browning of the powdered reaction mixture during conjugation due to sugar crystallisation (Lievonen et al., 1998) using the dry heating approach, which is not an issue with the wet heating approach. While most of the research published to date using the wet heating approach has been conducted at temperature ranging from 60 to 95 °C for time periods of min-d (Chevalier et al., 2001; Darewicz & Dziuba, 2001; Drapala et al., 2016a, 2016b; Morgan et al., 1998; Mulcahy et al., 2016a, 2016b; Zhang et al., 2012; Zhu et al., 2008), some studies have reported the use of higher temperatures (i.e., 100–130 °C) for shorter times (≤6 h) to induce conjugation using wet heating; for example, Chen et al. (2013b) reported that phosvitin and dextran were conjugated by heating in an aqueous solution at 100 °C for 6 h.

In addition to the differences in energy costs and efficiency between wet and dry heating approaches, the use of dry heating at lower temperatures (<70 °C) has been shown to result in greater preservation of the native 3-dimensional structure of whey proteins, compared with wet heating approaches, which has important implications for selected functional properties, such as solubility and interfacial properties (Gauthier, Bouhallab, & Renault, 2001; Morgan et al., 1998, 1999a, 1999b). The use of macromolecular crowding to effectively restrict denaturation and, in particular, aggregation of whey proteins has also shown promise on conjugation of WPI with dextran (Ellis, 2001; Perusko, Al-Hanish, Velickovic, & Stanic-Vucinic, 2015; Zhu et al., 2008).

7. Solubility

Milk proteins used in food products are generally required to have high levels of solubility to facilitate expression of the desired functional properties such as gelation, aeration, water-binding, foaming and emulsification (De Wit, 1989; O'Regan, Ennis, & Mulvihill, 2009). Solubility of milk proteins is influenced by many physicochemical properties of the protein molecules themselves, i.e., M_w, conformation (e.g., as affected by denaturation/aggregation), amino acid composition, physical state, exposure of selected functional groups, surface hydrophobicity, and environmental factors, such as pH, temperature, ionic strength and nature of the solvent (De Wit & Klarenbeek, 1984; Hayakawa & Nakai, 1985; Vojdani, 1996).

Protein-carbohydrate conjugation via the Maillard reaction has been shown to be an effective means of increasing the solubility of milk proteins. Native whey protein molecules are globular in structure and are susceptible to heat-induced changes (>70 °C) such as denaturation and aggregation (Wijayanti, Bansal, & Deeth, 2014a), while caseins are non-globular proteins, with more open, flexible structures and can be heated at 140 °C, at pH 6.7, for at least 40 min before coagulation occurs (Fox & Hoynes, 1975). Sodium caseinate has very different functionality to whey proteins (i.e., high viscosity at low concentrations and poor solubility at pH ~4.6) and is used as an emulsifier, texturiser and stabiliser in food products such as cured meats, processed cheese, coffee whiteners, high fat powders, bakery and confectionary products (Carr & Golding, 2016; O'Regan & Mulvihill, 2011; Swaisgood, 1993).

Improvements in the solubility of sodium caseinate at its isoelectric point would be expected to help broaden its application in food products; O'Regan and Mulvihill (2009) reported that sodium caseinate conjugated with MD, with DE values of 4 or 10, had improved protein solubility (~5–90%) in the pH range 4.0–5.5, compared with sodium caseinate, particularly around the isoelectric point (~pH 4.6) of the protein. This increase in protein solubility on conjugation was attributed to an increase in the hydration of the protein due to the covalent attachment to the protein molecules of hydrophilic MD glucose polymer side chains, and modification of the net charge of the protein, contributing to greater repulsion between the protein molecules. The increase in the net negative charge of the protein on conjugation with carbohydrate may be attributed to the consumption of charged amino acids, such as the basic amino acid lysine during the Maillard reaction (Ames, 1998; Brands & van Boekel, 2002; Lertittikul, Benjakul, & Tanaka, 2007; Wang & Zhong, 2014). Interestingly, it was noted that, at similar extents of conjugation, the conjugated sodium caseinate-MD10 had higher protein solubility (~50–80% increase) across the pH range 4.0–4.5 than the conjugated sodium caseinate-MD4. Similar results were reported by Shepherd, Robertson, and Ofman (2000) and Oliver, Melton, and Stanley (2006b), with conjugation of sodium caseinate with MD under dry heating conditions, leading to increases in protein solubility, particularly at pH 4.0–4.6, which was again attributed to increased steric repulsion between conjugated protein molecules. Grigorovich et al. (2012) reported that sodium caseinate conjugated with MD with DE values of 2 or 10, under dry heating conditions at an initial pH of 7, at 60 °C and 79% RH for 72 h, had improved solubility (~10–80% increase) across the pH range 3.5–5.0, compared with sodium caseinate alone. The authors reported that the improvement in protein solubility of the sodium caseinate-MD conjugate solutions was determined mainly by the molar ratio of protein:carbohydrate and the DE value of the MD used for conjugation. Similar to findings in the study of O'Regan and Mulvihill (2009), the most pronounced increase in solubility was achieved using MD with the higher DE value (i.e., DE 10).

The majority of studies on conjugation of casein-based ingredients have been completed using dry heating approaches (Corzo-Martínez, Carrera-Sánchez, Villamiel, Rodríguez-Patino, & Moreno, 2012b; Corzo-Martínez, Moreno, Villamiel, & Harte, 2010a; Corzo-Martínez, Soria, Belloque, Villamiel, & Moreno, 2010b; Markman & Livney, 2012; Morris, Sims, Robertson, & Furneaux, 2004) while a limited number of studies have been completed using wet heating approaches (e.g., Cardoso et al., 2011). Darewicz, Dziuba, and Mioduszewska (1998) showed that a β-casein-glucose solution, heated at an initial pH of 7.4, at 37 °C for 24 h under wet heating conditions to achieve conjugation, had a ~5–30% improvement in solubility across the pH range 2.0–8.0, compared with the unheated β-casein control, with the greatest improvement in solubility occurring around the isoelectric point of the protein (~pH 4.6). This improvement in solubility of β-casein was attributed to the covalent attachment of glucosyl residues to β-casein, resulting in an increased hydrophilicity and steric hindrance of the β-casein-glucose conjugates, compared with the unheated mixture. Similar results were also described by Groubet, Chobert, Haertlé, and Nicolas (1999) who prepared conjugated β-casein with either arabinose, lactose or ribose (molar ratio 1:100, protein:carbohydrate) by heating at 60 °C for 3 d in an anaerobic, aqueous environment and reported that all β-casein-sugar conjugates had increased solubility at pH 4.5–6.0.

However, it should be noted that conjugation of milk proteins with carbohydrates does not always result in increased protein solubility as the type and extent of modification of the functional properties are very dependent on the nature of the reactants, reaction conditions and the pathways followed by the Maillard

reaction (Hiller & Lorenzen, 2010). Corzo-Martínez et al. (2012b) reported that dry heating of sodium caseinate and galactose, at an initial pH of 7.0, at 50–60 °C, 67% RH, for 4 and 72 h, resulted in a 20% reduction in the solubility of the protein at pH 7.0, compared with the unheated sodium caseinate control. The authors attributed the decreased protein solubility on conjugation to an increase in the surface hydrophobicity of the protein on heating. However, at pH 5.0, Corzo-Martínez et al. (2012b) reported that conjugated sodium caseinate-galactose displayed an increase of ~10% in solubility, compared with the unheated and dry heated sodium caseinate controls due to the shift in the isoelectric point of the conjugated protein to a lower pH as a result of a moderate increase in its net negative charge following conjugation.

Heat treatment of proteins can lead to the formation of reactive intermediates (e.g., methylglyoxal or dehydroalanine), which can then react with the ε-amino group of lysine, resulting in the formation of protein crosslinks, leading to modification of functional properties and loss of nutritional value (Calabrese, Mamone, Caira, Ferranti, & Addeo, 2009; Le, Holland, Bhandari, Alewood, & Deeth, 2013; Pellegrino, Van Boekel, Gruppen, Resmini, & Pagani, 1999). In particular, the development of lysinoalanine (LAL) in protein solutions during heat treatment has been reported to be responsible for protein crosslinking (Gerrard, 2002). Mulcahy et al. (2016b) reported that a WPI solution heated for 8 h at 90 °C had a higher level of LAL (179 mg 100 g^{-1} protein) than the level of LAL in a solution of WPI conjugated with MD6 (58.8 mg 100 g^{-1} protein) under the same heating conditions; as the ε-amino groups of lysine are also consumed by the covalent attachment of carbohydrate to protein during the Maillard reaction, competition for the ε-amino reaction sites is likely to be responsible for the lower levels of LAL found in the conjugated protein-carbohydrate solution, compared with protein solution heated alone (Mulcahy et al., 2016a).

The dry heating approach has also been used extensively to conjugate whey proteins with carbohydrates as it is claimed to result in less heat-induced conformational changes to the whey protein molecules (Oliver et al., 2006b; Zhu et al., 2008) as lower temperatures are typically used (Li, Enomoto, Ohki, Ohtomo, & Aoki, 2005) than with wet heating. Wang and Ismail (2012) demonstrated that WPI conjugated with dextran by dry heating at 60 °C and 49% RH, for 96 h, had enhanced protein solubility (85.7 and 89.0% increase) at pH 4.5 and 5.5, respectively, when they were subsequently heated to 80 °C for 30 min, compared with the respective WPI control. The authors reported that the enhanced solubility of WPI on conjugation with dextran was attributed to suppressed intermolecular protein–protein interactions, along with structural/physicochemical changes to the protein, including a shift in the isoelectric point of the protein to a more acidic pH, reduction in the surface hydrophobicity of the whey protein molecules and increased resistance to thermal denaturation, resulting in a reduced exposure of free sulfhydryl groups after conjugation of the protein with dextran.

A further study by Wang, He, Labuza, and Ismail (2013) characterised the structural changes in whey protein molecules conjugated with dextran (at 60 °C and 49% RH for 96 h) using surface-enhanced Raman spectroscopy. The authors reported that the Raman spectra of the conjugated WPI-dextran samples had an additional peak at 983 cm^{-1}, which they attributed to the formation of a Schiff base, which was accompanied by deprotonation of carboxyl groups, contributing to higher net negative charge along with re-organisation of the sulphide linkages. These conformational changes in the whey protein molecules imparted structural rigidity to the conjugated WPI-dextran system, which in turn increased protein solubility on thermal treatment (75 °C for 30 min) over a wide pH range (3.4–7.0), compared with previously unheated WPI. Wang et al. (2013) also reported that the β-sheet configuration of the whey protein molecules in the conjugated WPI-dextran had increased band intensity in the Raman spectra, compared with that of the unheated WPI control. Wang et al. (2013) and Damodaran (2008) reported that the β-sheet configuration is more thermally stable than the α-helix and other disordered structure configurations in whey protein molecules, thus an increase in the β-sheet configuration may explain the improvements in the thermal stability at pH 4.5 and 5.5 of the conjugated WPI-dextran.

Martinez-Alvarenga et al. (2014) studied the effect of temperature, time, water activity and molar ratio of reactants on the functional properties of WPI conjugated with MD (molecular mass of 1.7 kDa); the WPI-MD with the lowest extent of conjugation (dry heated at 50 °C, 50% RH, for 24 h in the molar ratio of 1:1 protein:carbohydrate) had an increase in protein solubility of just 3% at pH 5.0, compared with the unheated control, due to the covalent attachment of the hydrophilic MD to the protein molecules. The authors reported that WPI-MD with the greatest extent of conjugation (achieved by heating at 50–60 °C, 80% RH, for 48 h in the molar ratio of 1:1 or 1:2 protein:carbohydrate) had a shift in the isoelectric point, from pH 5.0 for the unheated control, to pH 4.0–4.5, due to the consumption of positively charged lysine residues during conjugation.

Jimenez-Castano, Villamiel, and Lopez-Fandino (2007) conjugated individual whey protein fractions (β-lg; α-lac; BSA) with dextran (10 or 20 kDa) by dry heating at an initial pH of 7.0, at 60 °C and 44% RH for 24–72 h. The authors reported that the extent of conjugation, decreased in the following order; BSA>β-lg>α-lac and demonstrated that conjugation of β-lg with dextran (20 kDa), for either 36 or 60 h, improved its solubility by ~40% at pH 5.0. However, the solubility of the β-lg-dextran conjugate was ~20–30% lower at pH 4.0, compared with the unheated or heated β-lg control samples, which may be attributed to the consumption of positively charged amino groups (i.e., lysine) causing a shift in the isoelectric point to a more acidic pH. In contrast, Chevalier et al. (2001) reported that β-lg conjugated with galactose, glucose, lactose or rhamnose, by wet heating at an initial pH of 6.5, at 60 °C for 72 h, had increased solubility of ~25% at pH 4.5, compared with the respective heated β-lg control due to changes in the conformation and hydrophobicity of the protein molecules. Jimenez-Castano et al. (2007) also reported that α-lac-dextran conjugates exhibited a higher solubility (~5–50% increase), compared with the unheated control, in the pH range 3.0–5.0, with the greatest increase in solubility occurring at pH 4.0; similar trends were reported for BSA–dextran conjugates that had higher solubility around the isoelectric point (pH 4.7–4.9) than the unheated control.

A limited number of studies have reported modification of functional properties of whey proteins conjugated with carbohydrates using wet heating conditions. The likely reason for this is that heating of whey protein in an aqueous environment at ≥70 °C can result in denaturation and aggregation, which have been reported to reduce whey protein solubility (Liu et al., 2012; Pelegrine & Gasparetto, 2005; Zhu, Damodaran, & Lucey, 2010). However, Jiang and Brodkorb (2012), Lillard et al. (2009) and Liu and Zhong (2015) have investigated the use of high temperatures (95–130 °C) to induce conjugation of whey proteins or isolated whey protein fractions with carbohydrates, and have reported improvements in the antioxidant activity, emulsification properties and heat stability, respectively, of whey protein-carbohydrate conjugates.

Mulcahy et al. (2016b) reported that at pH 3.5, a conjugated WPI-MD solution, prepared by heating at an initial pH of 8.2, at 90 °C for 8 h, had higher protein solubility (50.7%) than the WPI solution heated without MD (26.7% solubility). The authors also reported that the protein solubility of a conjugated WPH-MD

solution, prepared by heating at 90 °C for 8 h, increased 80.9% at pH 4.0–4.5, compared with that of the heated WPH solution (75.3%). The increase in protein solubility of the conjugated WPI-MD and WPH-MD solutions, compared with solutions of heated whey protein (90 °C for 8 h) without MD, was attributed to enhanced hydration of the protein and increased steric hindrance between the protein molecules provided by the attachment of the bulky dextran molecules.

Some alternative approaches to achieving conjugation have also shown promise in increasing protein solubility; for example, Sun, Yu, Zeng, Yang, and Jia (2011b) reported that WPI-dextran conjugates, prepared by application of a pulsed electric field (15 and 30 kV cm^{-1}, flow rate ~30 mL min^{-1}) at an initial pH of 10 and at 30 °C for 7.35 ms, had higher solubility (10–30% increase) at pH 4.0–6.0 than the control WPI solution treated with pulsed electric field.

8. Heat stability

Glansdorff, Prigogine, and Hill (1973) defined thermal or heat stability as the ability of a substance to resist irreversible change in its chemical or physical structure, often by resisting polymerisation, under defined conditions (i.e., temperature, pH and ionic strength). Globular whey proteins are very susceptible to heat-induced (>70 °C) changes such as denaturation and aggregation (Wijayanti et al., 2014a), therefore, this section will focus mainly on the heat stability of whey proteins and improvement thereof by conjugation. The thermal stability of whey proteins has been the subject of extensive research and there are many reports in the literature on the denaturation and aggregation of whey proteins under different solution and processing conditions (Brodkorb et al., 2016; Donovan & Mulvihill, 1987; Marangoni, Barbut, McGauley, Marcone, & Narine, 2000; Oldfield, Singh, & Taylor, 2005; Ryan, Zhong, & Foegeding, 2013; Sağlam, Venema, de Vries, & van der Linden, 2014).

Several approaches have been investigated to control aggregation of whey proteins, including the addition of hydrophobic/amphiphilic compounds prior to heating, such as molecular chaperones, alcohols, hydrolysed/hydroxylated lecithin, and saturated/unsaturated fatty acids, removal of intermediate aggregates and modification of the ionic environment of the protein solution (Wijayanti, Bansal, Sharma, & Deeth, 2014b; Yong & Foegeding, 2008). Protein-carbohydrate conjugation via the Maillard reaction has been shown to be an effective method in improving the thermal stability of milk proteins.

Zhu et al. (2010) conjugated WPI with dextran (440 kDa) by heating a solution of 10% WPI and 30% dextran, at an initial pH of 6.5 at 60 °C for 48 h. The authors measured the thermal stability of the conjugated WPI-dextran solution (0.1%, w/v, protein) by heating at 80 °C for 30 min and subsequently measuring the development of turbidity in the solutions (i.e., with increasing development of turbidity there was a higher absorbance at 500 nm; Abs_{500}), across the pH range 3.0–7.5. The absorbance of the conjugated WPI-dextran solution did not change on heating; however, there was a ~10 fold increase in Abs_{500} of the WPI solution that was heated at 80 °C for 30 min in the pH range 4.5–5.5, which was attributed to the formation of large protein aggregates that scattered light. The authors reported that the unheated WPI had a typical differential scanning calorimetry (DSC) denaturation profile, with an endothermic peak at ~74 °C attributed to the denaturation of β-lg, and a shoulder at ~66 °C, attributed to the denaturation of α-lac; however, the conjugated WPI-dextran solution had a flat line profile suggesting that whey protein in the WPI had less secondary structure, due to the covalent attachment of the dextran which contributed to a higher denaturation temperature and improvements in thermal stability. Similar DSC profiles were reported by Hattori, Nagasawa, Ametani, Kaminogawa, and Takahashi (1994), Liu and Zhong (2013) and Wang and Ismail (2012) who showed that the denaturation temperature of whey protein-carbohydrate conjugates was higher than that of the corresponding unconjugated whey proteins.

Chevalier et al. (2001) reported that β-lg conjugated with either ribose, arabinose, glucose, galactose, lactose or rhamnose, at pH 6.5 and 60 °C for 72 h in an aqueous environment (0.4% protein, 0.4% carbohydrate), exhibited greater thermal stability at pH 5.0, when heated at 70–90 °C for up to 1 h, than unheated and heated β-lg controls (i.e., without added carbohydrate). The improvement in thermal stability of the solution (0.2%, w/v, protein), as measured by the concentration of protein in the supernatant of the heated solutions after centrifugation (15 min at 15,000 × *g*), was dependent on the carbohydrate type as follows; ribose > arabinose > rhamnose > glucose = galactose > lactose. However, the choice of carbohydrate used in conjugation is known to alter the extent of protein-carbohydrate conjugation, making it difficult to distinguish if the changes in the functional properties were due, directly, to compositional/structural differences between the carbohydrates or, indirectly, to their differing effects on the extent of conjugation (Chen, Liu, Labuza, & Zhou, 2013a; Li et al., 2009; Mulcahy et al., 2016a; ter Haar, Schols, & Gruppen, 2011).

Liu and Zhong (2013) conjugated WPI with either glucose, lactose or MD (M_w 1 kDa) by dry heating at an initial pH of 7.0 at 80 °C and 80% RH for 2 h, in a mass ratio of 1:1, and evaluated heat stability by reconstituting samples to 7%, w/v, protein, adding 0–150 mM NaCl or $CaCl_2$, adjusting the solutions to pH between 3.0 and 7.0, and heating for 2 min at 88 °C, simulating a hot-fill beverage process (Etzel, 2004). The authors assessed the thermal stability by visual observation of turbidity development after heating; the solutions prepared from the conjugated WPI-MD and WPI-lactose remained transparent under all conditions tested, while the unheated and dry heated WPI controls with added salt became turbid on heating at pH 6.0. Liu and Zhong (2013) reported that the conjugated WPI-MD had a higher denaturation temperature and a more negative net charge across the pH range 2.0–7.0 than the unheated WPI control, which may have contributed to the increased thermal stability of the former.

Several authors reported that improvements in heat stability of whey protein-carbohydrate conjugates can be related to the number and chain length of the carbohydrates attached to the whey protein molecules, along with the location at which they are attached on the protein molecules; the attachment of higher M_w carbohydrates has been shown to have a greater impact on improving the thermostability of whey proteins, due to increased steric repulsion, compared with conjugation with monosaccharides (Aoki et al., 1999; Corzo-Martínez, Sánchez, Moreno, Patino, & Villamiel, 2012c; Corzo-Martínez et al., 2012b; Morris et al., 2004; Mulcahy et al., 2016a; Tuinier, Rolin, & De Kruif, 2002; Wong, Day, & Augustin, 2011; Wooster & Augustin, 2006).

WPHs have been reported to have impaired functional properties compared with their intact counterparts and have been shown to be more susceptible to destabilisation when heated, due to the exposure of buried hydrophobic residues and/or release of specific peptides that promote peptide–peptide and peptide–protein aggregation (Adjonu, Doran, Torley, & Agboola, 2013; Creusot & Gruppen, 2007). Mulcahy et al. (2016b) reported that WPH (DH 9.3%) conjugated with MD (DE 17) under wet heating conditions at an initial pH of 8.2 and 90 °C for 8 h, had superior thermal stability to further heating at 85 °C for 10 min with 40 mM NaCl added, compared with those of the unheated or heated WPH control solutions. The unheated or heated WPH control solutions precipitated and phase separated on heating at 85 °C for 10 min due to the

formation of large protein aggregates (~10–50 μm), whereas, the conjugated WPH-MD solution (i.e., previously heated for 8 h at 90 °C at an initial of pH 8.2) that was further heated with 40 mM added NaCl remained stable and the protein aggregates present remained small (<~1 μm).

The conditions used during the Maillard reaction impact the thermal stability of the resulting conjugates; Wang and Zhong (2014) dry heated WPI-MD in the mass ratio 1:1, at 80 °C and 65% RH for 4 h, at different pHs (i.e., pH 4.0, 5.0, 6.0 and 7.0). The solutions prepared from the conjugated WPI-MD at pH 6.0 (5% protein, and 0–150 mM added NaCl) that was subsequently heated at 138 °C for 1 min (to simulate UHT treatment), had improved thermal stability (i.e., remained transparent as evaluated by the visual assessment of turbidity) compared with the solution prepared from the WPI-MD conjugated at pH 4.0. The improvement in thermal stability was attributed to the greater extent of covalent attachment of MD molecules to the whey protein molecules at pH 6.0, resulting in reduced protein–protein interactions, lower surface hydrophobicity of the protein, a shift in the isoelectric point (from 4.63 to 4.07) of the protein to lower pH and a higher protein denaturation temperature compared with the WPI-MD conjugate prepared at pH 4.0.

Protein-carbohydrate conjugates produced by alternative methods, such as sonication, have also been shown to have improved functionality; Perusko et al. (2015) conjugated a WPI-arabinose-polyethylene glycol (PEG) solution at an initial pH of 8.0, at ~5–10 °C by sonication (20 kHz frequency) for 60 min. The authors reported that the conjugated WPI-arabinose-PEG solution had a greater extent of conjugation (10% increase), due to the presence of PEG facilitating macromolecular crowding, compared with the sonicated solution of WPI-arabinose without PEG. The conjugated WPI-arabinose-PEG solution had higher protein solubility (~10–40% increase) when the solution was heated at 80–100 °C for 15 min compared with the sonicated WPI control solution and the conjugated WPI-arabinose solution without PEG. The authors attributed this increase in thermal stability of the conjugated WPI-arabinose-PEG solution to attachment of the arabinose units, which interfere with protein aggregation due to steric hindrance limiting protein–protein interactions.

The use of protein-carbohydrate conjugation has also been shown to enhance the functional properties of WPC; Liu and Zhong (2014) prepared a defatted WPC (34% protein) by adjusting a WPC solution to pH 4.0, centrifuging, and spray drying the resulting supernatant. The resulting defatted WPC was conjugated by heating under dry conditions, at 130 °C for either 20 or 30 min, or 60 °C for either 24 or 48 h, at 79% RH, which resulted in the whey proteins conjugating with the innate lactose (68.8%) present in the ingredient. The authors assessed the thermal stability of solutions (4%, w/v, protein) prepared from the conjugated WPC by adjusting the pH of the solution to 3.0–7.0 and heating the solutions at 88 °C for 2 min or at 138 °C for 1 min. The WPC conjugated at 130 °C for 30 min remained transparent once further heated at 138 °C for 1 min with 150 mM added NaCl; however, the authors did not suggest a mechanism for the apparent improvement in thermal stability.

Conjugation has also been shown to be beneficial in producing heat stable whey protein nanofibrils; Liu and Zhong (2013) produced protein nanofibrils (pH 2.0, heated at 85 °C for 24 h) from solutions of WPI and lactose which had previously been conjugated under dry heating conditions (80 °C and 70% RH for 2 h). The nanofibrils prepared from the conjugated WPI-lactose were highly dispersible and remained transparent after heating (88 °C for 2 min or 138 °C for 1 min) in the pH range 4.0–7.0, even with up to 150 mM NaCl added, compared with the nanofibrils formed from a WPI solution, which became turbid under all heating conditions tested. The greater thermal stability of the nanofibrils produced from the conjugated protein was attributed to the lactose on the nanofibril surface providing additional steric hindrance.

9. Emulsification

Emulsifiers act by reducing the surface free energy at the interface between oil and aqueous phases, and thereby provide an effective interfacial barrier to help resist the thermodynamic tendency of emulsions to destabilise (McClements, 2015). Proteins are the most commonly used class of food emulsifiers, due to their excellent surface activity, diverse and desirable nutritional profile, wide availability and positive consumer perception (Bos & van Vliet, 2001; Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Lam & Nickerson, 2013). In addition, surface and interfacial properties of proteins can be modified through controlled hydrolysis (i.e., increasing molecular mobility; Panyam & Kilara, 1996; Tamm, Sauer, Scampicchio, & Drusch, 2012; Turgeon, Gauthier, Molle, & Leonii, 1992), controlled denaturation (i.e., opening up of the protein structure; Raikos, 2010; Rullier, Novales, & Axelos, 2008), change in the charge (Hamada & Swanson, 1994) or by complexation with another component (i.e., polyphenols, carbohydrates; Dickinson, 2010) to enable best matching of their functionality to specific product and process applications.

There has been considerable growth in interest in the area of modification of emulsification properties of proteins by their conjugation with various carbohydrates through the Maillard reaction (Drapala et al., 2016a, 2016b; Foegeding & Davis, 2011; Lam & Nickerson, 2013; Liu et al., 2012; Oliver et al., 2006a; de Oliveira et al., 2016). Protein-carbohydrate conjugates consist of two composite blocks, where, in an emulsion system, the more surface-active component (i.e., protein) adsorbs at the oil/water (O/W) interface, while the more hydrophilic component (i.e., carbohydrate) extends into the bulk aqueous phase of the emulsion; the two components display two distinct, complimentary and synergistic roles in bringing about the action of conjugate-based emulsifiers.

Conjugation of proteins with carbohydrates can improve their emulsion formation properties indirectly by enhancing protein solubility (see Section 7), increasing their effective concentration and mobility in aqueous solution. Changes in conformation of proteins arising from conjugation (i.e., unfolding of the protein structure and exposure of hydrophobic and hydrophilic groups) result in a more flexible protein structure, enabling it to move faster towards and adsorb at the O/W interface, compared with unconjugated protein (Báez, Busti, Verdini, & Delorenzi, 2013; Corzo-Martínez et al., 2011; Gauthier et al., 2001). Improvements in emulsification properties of WPI on conjugation with dextran (Zhu et al., 2010) or of sodium caseinate conjugated with glucosamine (Jiang & Zhao, 2011), both under wet heating conditions, have been reported. Protein type influences the effect of conjugation on its emulsion formation abilities, where the emulsification properties of native globular proteins (e.g., whey proteins) can benefit more from conjugation than those of less-structured proteins, due to the unfolding of the compact globular structure, increasing molecular flexibility and surface hydrophobicity (Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005; Evans, Ratcliffe, & Williams, 2013). In an analogous manner, it is reasonable to assume that the effect of conjugation on emulsification properties of hydrolysed proteins/peptides would largely depend on the degree of protein hydrolysis/conformation change and the M_w of the protein/peptide and carbohydrate components of the conjugates, while there appears to be no information available on this subject in the scientific literature.

Carbohydrate moieties covalently attached to protein on conjugation act like a tail, and are effectively towed by the protein

as it migrates through the bulk aqueous phase towards the O/W interface, as the carbohydrate generally does not provide a driving force for this migration of the conjugated protein molecules. Despite its passive role in the formation of emulsions, the carbohydrate component of protein-carbohydrate conjugates generally does not impede the movement of the conjugated protein through the bulk phase, except when the size ratio between the protein and carbohydrate is disproportional. Matemu, Kayahara, Murasawa, and Nakamura (2009) and Akhtar and Dickinson (2007) reported that increasing the M_w of the carbohydrate component reduced emulsifying activity index (EAI) of food protein-carbohydrate conjugates made therefrom, using tofu whey and bovine milk-derived WPI, respectively. The larger hydrodynamic radius of protein-carbohydrate conjugates, compared with the protein alone, can potentially result in a decreased rate of diffusion in the bulk phase and reduce the rate of adsorption of conjugates at the interface (Ganzevles, van Vliet, Stuart, & de Jongh, 2007). As an example, lower emulsion formation ability was reported for WPI conjugated with high molecular mass MD (DE 2; 280 kDa), an effect that was not observed for medium molecular mass MD (DE 19; 8.7 kDa), compared with non-conjugated WPI (Akhtar & Dickinson, 2007).

The improved emulsion formation properties of milk protein-carbohydrate conjugates, compared with unconjugated protein, can be also attributed to their strong steric stabilisation properties; as the emulsifier adsorbs at the surface of newly-formed oil globules on homogenisation, it prevents their coalescence by means of steric repulsion (Liu, Ma, McClements, & Gao, 2016). The carbohydrate moiety anchored at the surface of an oil globule by the protein, protrudes into the aqueous phase of the emulsion and prevents coalescence on high impact collisions between individual oil globules during the dynamic homogenisation process (Corzo-Martínez et al., 2011). The emulsion formation properties of protein are highly dependent on the environmental conditions under which emulsification takes place; high salt concentration and acidic environment usually reduce protein solubility, due to their influence on electrostatic repulsion. A high salt content screens charges of protein molecules, while low pH reduces their charge due to proximity to the isoelectric point of the proteins (i.e., pH 4.6–5.3 for bovine milk protein) - effectively protein–protein interactions are promoted, resulting in decreased solubility and protein precipitation, negatively impacting their surface/interfacial activity (Bos & van Vliet, 2001; Damodaran, 2005; Zhai, Day, Aguilar, & Wooster, 2013).

Conjugation of milk proteins with carbohydrates generally enhances their emulsion formation and stabilisation properties at high salt concentrations and under acidic conditions, due to improved protein solubility under such environmental conditions. Covalent attachment of MD or corn fibre gum to globular whey proteins (i.e., β-lg and proteins in WPI) by conjugation has been shown to enhance the emulsifying properties of the proteins across a broad pH range (3.2–5.5), by significantly increasing protein solubility (Akhtar & Dickinson, 2007; Yadav et al., 2010). Similarly, enhanced emulsion formation properties, attributed to increased protein solubility, over a broad pH range (2–11) have been reported for a range of milk protein ingredients (β-lg, α-la, BSA and sodium caseinate) conjugated under dry and/or wet heating conditions with a number of different carbohydrates (glucosamine, galactose and dextran) (Corzo-Martínez et al., 2011; Jiang & Zhao, 2011; Jimenez-Castano, Villamiel, & Lopez-Fandino, 2007). Conjugation of protein can greatly improve its solubility at acidic pH and high ionic strength conditions due to the additional steric barrier provided by the conjugated carbohydrate component preventing protein aggregation and precipitation (see Section 7). Additionally, conjugation can shift the isoelectric point of protein to lower pH as reported for individual whey proteins (β-lg, α-lac and BSA) conjugated with MD (Jimenez-Castano et al., 2007). Such enhanced protein functionality under challenging environmental conditions offers significant potential for the development of novel emulsion-based food formulations.

Stability of an emulsion refers to its ability to withstand deteriorative changes (i.e., physical or chemical) during processing and/or storage. The main mechanism responsible for physical stability of protein-based emulsions is long-range electrostatic repulsion; proteins adsorbed at the O/W interface confer an electrostatic charge (i.e., a negative charge in the case of most milk protein-based emulsions at near neutral pH) to the oil globules, effectively preventing their flocculation and coalescence. In addition to electrostatic repulsion, emulsions formulated with conjugated proteins are also stabilised by the additional steric hindrance provided to the adsorbed conjugated protein molecules by the carbohydrate component. The carbohydrate component of the conjugate is anchored at the O/W interface by surface active protein and, due to its hydrophilicity, it extends into the aqueous phase and acts to physically hinder interactions between oil globules. Sterically-stabilised emulsions are, generally, more robust and resilient to changes to the system (i.e., temperature, concentration, pH and ionic strength), compared with emulsions stabilised solely by electrostatic repulsion, making them attractive for providing emulsion stability during formulation and processing as well as during product storage (Fig. 2) (Evans et al., 2013; Liu et al., 2012).

Stability of an emulsion to processing can be described as the stability to high stress processes to which these systems can be subjected during manufacture, including thermal treatments, changes in ionic strength, high shear forces and freeze-thaw cycles (Guzey & McClements, 2006; McClements, 2015). Heat treatment of protein-stabilised emulsions can often result in interactions (i.e., mediated by free sulphydryl groups and hydrophobic interactions) between proteins located at the interfacial layers of different globules, as well as with un-adsorbed protein in the serum phase, leading to protein-mediated bridging flocculation of oil globules (Fig. 2) (Dickinson, 2001; Piorkowski & McClements, 2014; Tcholakova, Denkov, Ivanov, & Campbell, 2006). Such bridging flocculation can result in fouling of heat exchange surfaces, the generation of buoyant protein-lipid flecks, impaired emulsion shelf-life or, in extreme cases, complete emulsion destabilisation (Drapala et al., 2016a, 2016b; Petit, Six, Moreau, Ronse, & Delaplace, 2013; Prakash, Kravchuk, & Deeth, 2015). Drapala et al. (2016a, 2016b) showed that model infant formula emulsions stabilised by WPH-MD conjugates, produced by a wet heating approach, were resistant to heat-induced bridging flocculation, compared with those stabilised by non-conjugated WPH. The authors reported that the conjugate-stabilised systems showed no changes in viscosity or particle size distribution after a high temperature-short time (HTST) treatment of between 75 and 100 °C for 15 min, in contrast to emulsions stabilised by intact, hydrolysed or pre-heated hydrolysed whey protein. In addition, significant improvements in heat stability of O/W emulsions stabilised by WPI conjugated with low methoxyl-pectin under dry heating conditions (60 °C at 74% RH for 16 d) have been reported by Setiowati, LienVermeir, Martins, De Meulenaer, and Van der Meeren (2016). The good thermal stability of emulsions stabilised by conjugated protein is predominantly due to the physical restriction of access (by serum phase constituents such as un-adsorbed proteins) to the potentially reactive inner interfacial layer (i.e., protein) by the unreactive outer interfacial layer (i.e., carbohydrate).

Strong steric hindrance and increased thickness of the interfacial layer in conjugate-stabilised, compared with protein-stabilised O/W emulsions, can efficiently prevent flocculation of oil globules when electrostatic stabilisation is disabled (i.e., by charge screening or by proximity to the protein isoelectric point) (Fig. 2). The greater

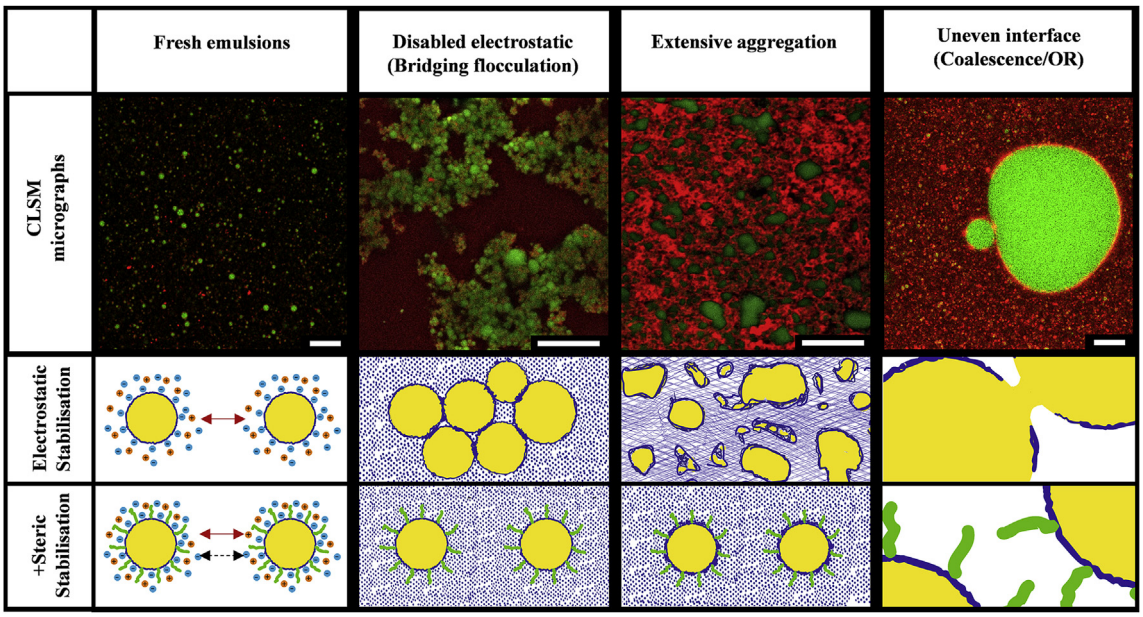

Fig. 2. Schematic representation of the differences in emulsions stabilised by either electrostatic repulsion (2nd row) or a combination of electrostatic and steric repulsion mechanisms (3rd row); oil droplets (yellow) coated by protein (blue) or conjugate (blue + green) and surrounded by a cloud of ions. Confocal laser scanning microscope (CLSM) images (1st row; scale bar equals 10 μm, but scales for the confocal images vary to best show the features being described. Green, oil; red, protein) show examples of corresponding destabilisation mechanisms taking place in such systems, as presented for model nutritional beverage emulsion systems, adapted from Drapala, Auty, Mulvihill, and O'Mahony (2015) and Drapala et al. (2016a). Fresh emulsions were essentially the same for the two different stabilisation mechanisms (i.e., electrostatic and steric) – homogenous, where small globules ($D_{4,3}$ < 1 μm) followed a monomodal distribution; no differences in viscosity and particle size were observed between the conjugated and unconjugated systems. Bridging flocculation occurs when globules show attractive/cohesive interactions upon collisions/contact and attach; these can also be mediated by serum phase proteins displaying similar cohesive behaviour. Extensive aggregation occurs when protein-stabilised globules are exposed to prolonged adverse conditions (i.e., high temperature, pH near isoelectric point), oil pools entrapped within the protein matrix giving the aggregates a buoyant nature, resembling the behaviour of flecks as reported by Drapala et al. (2016a). Coalescence/Ostwald ripening occurs when, upon contact, repulsion forces and strength of the interfacial layer are not sufficient to prevent lipid–lipid contact resulting in mass transfer (Drapala et al., 2016b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thickness of the interfacial layer in conjugate-stabilised emulsions can provide additional stability of oil globules to mechanical stress and high shear forces, commonly experienced during unit operations such as mixing, pumping, flow or atomisation (Sagis & Scholten, 2014). Wooster and Augustin (2006) reported that the thickness of the interfacial layer in O/W emulsions stabilised by β-lg-dextran conjugates can be modified by using carbohydrates with different M_w. Fundamentally, increasing the M_w of the carbohydrate moiety yields increased thickness of the interfacial layer and confers greater steric stabilisation as a result (Akhtar & Dickinson, 2007). However, factors such as the kinetics of conjugation (see Section 3) and the rate of diffusion/adsorption of the conjugate (i.e., as discussed earlier in this section) can both be negatively impacted by increasing M_w of the carbohydrate and need to be considered when using higher M_w carbohydrates. Emulsion stability can be enhanced by conjugation of protein with charged carbohydrates; Neirynck, Van der Meeren, Bayarri Gorbe, Dierckx, and Dewettinck (2004) reported improved stability of O/W emulsions due to strong electro-steric stabilisation functionality of WPI-pectin conjugates.

Stability of emulsions during storage can present challenges, in regards to deteriorative changes of either a physical nature, due to thermodynamic instability (i.e., coalescence, flocculation, gelation, creaming and oiling off) or a chemical nature (i.e., lipid oxidation) (Chaiyasit, Silvestre, McClements, & Decker, 2000; Dalgleish, 1997; McClements, 2015; Piorkowski & McClements, 2014; Tcholakova et al., 2006). Physical instability of emulsions can be separated into that governed directly by Stokes law (i.e., gravitational separation of components of different density) and that resulting from interactions between oil globules (i.e., coalescence, flocculation). Steric stabilisation of emulsions containing protein-carbohydrate conjugates can effectively prevent interactions between oil globules over prolonged storage, owing to the strong physical barrier provided by the interfacial layer (Fig. 2). O/W emulsions stabilised by conjugated milk proteins have shown no changes in the size of fat globules during storage (24 h–21 d at 22–40 °C under quiescent conditions), compared with the corresponding systems stabilised by non-conjugated proteins (Drapala, Auty, Mulvihill, & O'Mahony, 2016b; Lesmes & McClements, 2012; Liu et al., 2016; Medrano, Abirached, Moyna, Panizzolo, & Añón, 2012; O'Regan & Mulvihill, 2013).

Emulsions stabilised by conjugated milk proteins display greater oxidative stability than these stabilised by protein alone, possibly due to the increased thickness of the interfacial layer and the physical barrier that restricts the access of pro-oxidant species to oxidation-sensitive components such as lipids and lipid-soluble compounds. A significant improvement in the oxidative stability of emulsions containing β-carotene, stabilised by lactoferrin conjugated with dextran, compared with emulsions stabilised by the protein alone, was reported recently by Liu et al. (2016), where the anti-oxidative effect was attributed to restriction of physical contact between pro-oxidants and lipids by the thick interfacial layer of the conjugate-stabilised emulsion. Furthermore, it has been shown that certain (especially late-stage) Maillard reaction products have anti-oxidant properties when incorporated into O/W emulsions (Markman & Livney, 2012; O'Regan & Mulvihill, 2010); for example, conjugation of WPI, sodium caseinate and lactose-hydrolysed skim milk powder (SMP) with glucose, lactose, pectin or dextran under dry heating conditions at 70 °C and 65% RH for up to 240 h was reported to increase the anti-oxidant capacity of the systems due to production of late-stage Maillard reaction products with antioxidant activity (Hiller & Lorenzen, 2010).

Low ζ potential of oil globules near the isoelectric point of milk proteins, and screening of the electrostatic charge by excess ions, can promote flocculation of protein-coated oil globules, leading to breakage of the emulsion and phase separation (McClements, 2015;

Piorkowski & McClements, 2014; Sarkar & Singh, 2016), whereas the presence of a strong steric barrier provided by protein-carbohydrate conjugates can oppose emulsion destabilisation under these environmental conditions. Lesmes and McClements (2012) demonstrated that conjugation of β-lg with dextran, under dry heating conditions (60 °C and 76% RH for 24 h), enhanced the formation and stability of O/W emulsions prepared at pH 7 using the conjugated protein on subsequent acidification to pH 5. The authors reported that the thick O/W interfacial layer formed with the high molecular mass dextran (i.e., ≥40 kDa) was responsible for the greater stability of the conjugate-based emulsions, compared with emulsions made using unconjugated protein.

Good stability to storage at high salt concentration (0.2 M citrate buffer) and under acidic conditions (pH 3.2) were reported for emulsions stabilised by conjugates of β-lg or WPI with corn fibre gum prepared using dry heating conditions at 75 °C and 79% RH for time periods ranging from 2 h to 7 d (Yadav et al., 2010); in these systems, the branched nature of the corn fibre gum resulted in good emulsion stability, even at low levels of conjugation. Considerably improved resistance to flocculation for emulsions stabilised by conjugates of β-lg and dextran (27–200 kDa) at high salt (i.e., $CaCl_2$) addition levels was also reported by Wooster and Augustin (2006). In that study, the authors reported that a significant increase (~12-fold) in particle size for emulsions stabilised by unconjugated protein was observed at ≥10 mM calcium (Ca) content, while no changes were observed at all added Ca levels (0–20 mM) for the conjugate-based systems. The superior stability of conjugate-stabilised emulsions was attributed to the thickness and steric stabilisation effects of the outer interfacial layer (i.e., dextran), which effectively offset the electrostatic screening effect of Ca addition. Similar findings were reported for lactoferrin-dextran conjugates, where, strong steric stabilisation of oil globules resulted in emulsion stability at high ionic strength (Liu et al., 2016). Likewise, Akhtar and Dickinson (2007) reported that emulsions stabilised by WPI-MD conjugates (DE 19) and containing high levels of sodium lactate (5% w/w) did not show any changes in particle size distribution after 21 d of storage at 22 °C, in contrast to ~2-fold increase in mean volume diameter for emulsions stabilised by unconjugated protein or by gum arabic (a naturally-occurring protein-carbohydrate conjugate).

The unique functionality of milk protein-carbohydrate conjugate-based emulsifiers is particularly interesting for emulsion-based food products exposed to challenging environmental and processing conditions such as low pH, high ionic strength and severe thermal processes (e.g., fruit beverages, infant formula, clinical nutrition products and acidified milk drinks). Such products usually pose challenges with processing and shelf-life stability and, in some cases, hydrocolloids are added to retard phase separation. Milk protein/peptide-carbohydrate conjugate-based emulsifiers also offer significant potential for applications in emulsion-based delivery systems, where their interfacial functionality can facilitate controlled release of sensitive bio-actives (e.g., vitamins) in the small intestine, avoiding acid-mediated emulsion destabilisation and loss of the encapsulated material in the stomach. Gumus, Davidov-Pardo, and McClements (2016) reported that, as well as stability to acidic conditions, emulsions stabilised by casein-dextran conjugates, prepared under dry heating conditions (60 °C and 76% RH for 48 h), were additionally resistant to enzymatic digestion by pepsin, which prevented issues with premature release of encapsulated lutein in the stomach. Proteolysis of the interfacial layer was retarded by the thick outer carbohydrate layer, which restricted the pepsin from accessing the inner protein interfacial layer. In addition, the authors showed that the use of conjugate-based emulsifiers did not interfere with release of encapsulated material in the intestine, where bile salts displaced the emulsifier from the surface of oil globules. Similarly, in the study of Lesmes and McClements (2012), β-lg-dextran conjugate-stabilised emulsions displayed good stability to stomach-like environmental conditions, due to strong steric stabilisation and subsequent release of encapsulated fatty acids occurred in the intestinal stage, due to emulsifier displacement by bile salts.

10. Foaming

The formation and stabilisation of foams by milk proteins/peptides have been extensively detailed and reviewed in the scientific literature; for more information on the foaming functionality of milk proteins/peptides the reader is referred to publications by Damodaran (2005), Dickinson (2010), Foegeding, Luck, and Davis (2006), Huppertz (2010), and Lam and Nickerson (2013). The current review sets out to discuss foaming functionality of milk proteins/peptides as affected by conjugation with carbohydrates.

Changes to structure/conformation of proteins, resulting from their conjugation with carbohydrates, generally contribute to increased protein solubility, higher protein mobility and, effectively, faster adsorption at air/water (A/W) interfaces (see Section 7). Improvement in foam capacity for BSA conjugated with glucose in a wet heating process (45 °C for 2 h with continuous stirring), compared with BSA conjugated with mannose or unconjugated BSA, was reported by Jian, He, Sun, and Pang (2016). In this study, conjugation resulted in changes in protein conformation, yielding a more flexible and loosened structure that, effectively, increased the rate of protein adsorption at the A/W interface; however, a decrease in surface hydrophobicity and decreased foam stability was reported for conjugated BSA. Similar findings were reported for foams stabilised by β-lg-glucose conjugates (dry heating; 50 °C at 65% RH for 96 h) (Báez et al., 2013), where improved foam capacity, compared with using unconjugated β-lg, was explained by heat-induced conformational changes in the structure of the whey protein molecules, conferring more open and flexible structures, thus allowing more rapid formation of the interfacial layer. A combination of increased hydrophobicity and changes in the conformation of protein can offer increased foam overrun as reported for supramolecular α-lac-glycomacropeptide complexes (i.e., stabilised by non-covalent interactions) by Diniz et al. (2014).

Conjugation of protein with carbohydrates allows the avoidance of extensive protein aggregation when the electrostatic repulsion forces are disabled (i.e., at acidic pH or high ionic strength). In effect, denser protein packing without extensive aggregation can be achieved using conjugated protein (Rade-Kukic, Schmitt, & Rawel, 2011). Jimenez-Castano et al. (2007) reported that conjugation of milk proteins (β-lg, α-lac and BSA) with dextran in a dry-heating process (55 °C at 0.44 a_w for up to 96 h) resulted in a reduction in isoelectric point of each protein and improved their solubility and heat stability around the isoelectric point of the protein. Such modified functionality (i.e., good heat stability at low pH) can offer potential in protein-based foam applications, allowing considerable protein unfolding when heated at low pH, without extensive protein aggregation or precipitation. Controlled aggregation under these conditions, combined with flexible unfolded protein structures, and low electrostatic repulsion, offer significant potential for stabilisation of foam systems. Other approaches directed at improving foam stability involve increasing the thickness and elasticity of the interfacial film by increasing the size of its building blocks (i.e., controlled protein aggregation) (Báez et al., 2013; Dombrowski, Johler, Warncke, & Kulozik, 2016; Foegeding et al., 2002; Rullier et al., 2008; Tamm et al., 2012) or by conformational changes to the protein structure (i.e., partial unfolding of globular protein; Dissanayake & Vasiljevic, 2009; Dombrowski et al., 2016; Morales, Martínez, Pizones Ruiz-Henestrosa, & Pilosof, 2015). These approaches closely match the changes to protein structure/conformation and functionality offered

by protein conjugation; increased size of interfacial building blocks, controlled protein aggregation on conjugation and opening up of the protein structure have been shown to improve stability of foams formed with conjugated milk proteins (Corzo-Martínez et al., 2012b; Hiller & Lorenzen, 2010).

In using protein/peptide-carbohydrate conjugates to stabilise foams, the thickness of the interfacial layer and therefore, effectiveness of steric stabilisation, can be controlled using carbohydrates with different M_w (Wooster & Augustin, 2006). Hiller and Lorenzen (2010) reported increased stability of foams prepared with a range of protein (WPI, sodium caseinate and lactose-hydrolysed skim milk) and carbohydrate (glucose, lactose, pectin and dextran) conjugates (produced by dry heating at 70 °C and 65% RH for up to 240 h) due to formation of thick and viscoelastic interfacial films that prevented disproportionation of gas bubbles. Increasing the thickness of the interfacial film can effectively improve its rheological properties in addition to providing an effective steric barrier with good dilatational properties (Dombrowski et al., 2016). Similarly, Kim, Cornec, and Narsimhan (2005) reported that denaturation and unfolding of β-lg resulted in increased shear elasticity and viscosity of the interfacial layer due to increased flexibility of the partially-denatured globular protein.

The viscoelastic properties of protein-stabilised foams are strongly dependent on the structure/conformation of the protein; globular proteins (e.g., whey proteins) tend to give interfacial films with greater viscoelasticity, due to higher packing density, compared with less ordered proteins (e.g., caseins; Bos & van Vliet, 2001). Conjugation of less-ordered proteins offers good potential for improvement of their foam stabilising properties due to increases in the thickness of the interfacial layer and, effectively, better dilatational properties of the A/W interface (Dombrowski et al., 2016). Jiang and Zhao (2011) reported that modification of casein (sodium caseinate) by its cross-linking using transglutaminase and/or conjugation with glucosamine (using a wet heating approach at 37 °C for up to 5 h with continuous agitation) significantly increased apparent viscosity of the caseinate solutions. The authors showed that a combination of cross-linking and conjugation increased both storage and loss moduli of casein suspensions and that the elastic properties of the cross-linked and conjugated casein solutions were more dominant, indicating solid-like response to dynamic deformation, in contrast to unmodified and cross-linked casein suspensions. Modification of the viscoelastic properties of the interfacial layer of foams by conjugation of casein with glucosamine conferred enhanced stability against bubble coalescence (increased by 20.8% compared with unconjugated casein; Jiang & Zhao, 2011). Facilitating dense packing and interactions between protein-based building blocks are effective means of improving viscoelastic properties of the interfacial layer of a foam (Mackie & Wilde, 2005).

In a similar way, facilitating interactions between carbohydrate components of protein-carbohydrate conjugates adsorbed at A/W interfaces of foams can provide, not only a strong steric barrier, but also improved viscoelastic properties of the interface. Cai and Ikeda (2016) reported increased resistance against surfactant-induced displacement of protein from the A/W interface in foams stabilised with WPI-gellan conjugates prepared by dry heating at 80 °C and 79% RH for 2 h, compared with systems containing unconjugated WPI and the surfactant Tween 20. The authors attributed the greater resistance to displacement of protein in the conjugate-based foam system to the ability of the gellan moiety, covalently attached to the whey protein molecules, to form a carbohydrate network at the interface, effectively immobilising the conjugate-covered interface.

Conjugation of protein with carbohydrates alters the hydrophobic-hydrophilic balance of protein and conformational changes to the protein structure caused by conjugation increase its surface hydrophobicity, generally resulting in improved emulsion formation properties of conjugated proteins (see Section 9). On the other hand, hydrophilicity of the resulting ingredient is increased by the attachment of the hydrophilic carbohydrate moieties (see Section 9). Conversely, greater hydrophilicity can yield better foam stability due to improved water holding capacity by the conjugate located at the interfacial layer, and effectively restrict liquid drainage in the foam (Báez et al., 2013). The hydrophilic nature of the carbohydrate anchored at the A/W interface by the protein, viscoelastic properties of the interface and higher viscosity for conjugated protein-carbohydrate systems (WPI, SMP, sodium caseinate, glucose, lactose, pectin, dextran), compared with native protein, have been shown by Hiller and Lorenzen (2010) to be the main factors responsible for increased foam stability. In contrast, other authors have claimed that the increased hydrophilicity of BSA resulting from its conjugation with glucose or mannose decreased foam stability (Jian et al., 2016). Jiang and Zhao (2011) elucidated that a shift in the amphiphilic nature of casein towards more hydrophilic behaviour, upon conjugation with glucosamine, reduced ability of foam to retain the incorporated air. It is important to consider that both of these, apparently contradictory, findings can hold true, with the precise impact of conjugation being very much dependent on differences in protein structure (globular, ordered, unordered, etc.), nature of the carbohydrate (chain length, charge, etc.) and conditions employed for conjugation and foam formation.

11. Gelation and textural properties

Whey protein gels are three-dimensional, self-supporting, networks, within which the aqueous solution and any dispersed elements (e.g., fat) are entrapped. Gelation of whey proteins involves a controlled increase in protein–protein interactions, while carefully maintaining a balance with protein-solvent interactions (Brodkorb et al., 2016). During gelation, the number and combined strength of protein–protein interactions (e.g., disulphide, hydrophobic and electrostatic interactions) determine the mechanical and rheological properties of the resultant gel network. High whey protein content ingredients (i.e., WPC and WPI) are commonly used in food applications which require gelation of the protein for the expression of functionality (e.g., recombined meat products, desserts, puddings, mousses). Many compositional and environmental factors affect the formation and rheological properties of whey protein gels, including protein concentration, pre-denaturation and aggregation of protein, salts, temperature and pH (Foegeding, Bowland, & Hardin, 1995; Langton & Hermansson, 1992; Mulvihill & Kinsella, 1987).

It has been known for over 20 years that heating solutions of globular milk proteins (e.g., lysozyme and BSA) and reducing sugars (e.g., lactose, ribose and xylose), at temperatures of 90–121 °C, results in the formation of gels with higher firmness and elasticity than gels made using the proteins alone in solution (Armstrong, Hill, Schrooyen, & Mitchell, 1994; Easa, Hill, Mitchell, & Taylor, 1996). The increased strength of these protein-carbohydrate gels is due to Maillard reaction-mediated reduction in pH and by cross-linking of the protein molecules (e.g., via lysinoalanine). The gel strength (but also, undesirably, colour development) increases with decreasing M_w of the sugars (Hill, Mitchell, & Armstrong, 1992), while the pH required to achieve gelation decreases with increasing sugar concentration and reactivity. In combination, these effects of sugar incorporation on gelation properties of globular protein on heating, means that it is possible to reduce the amount of protein required for gel formation (Azhar, 1996; Oliver et al., 2006b).

More recent work has focused on studying the gelation properties of milk proteins (especially whey proteins) conjugated with higher M_w carbohydrates under dry heating conditions, due to the challenges associated with denaturation and aggregation of whey

proteins under wet heating conditions (Gauthier et al., 2001; Morgan et al., 1999a). Conjugation of whey proteins in WPI with dextran has been shown to influence the rheological properties of heat-induced gels made therefrom (Spotti et al., 2013a, 2013b, 2014a, 2014b; Sun et al., 2011a). Conjugation of WPI with dextran of 6, 40 and 70 kDa, under dry heating conditions at 60 °C for 2–9 d at 63% RH was shown to result in whey protein-conjugate gels with lower fracture stress and Young's Modulus as measured by uniaxial compression testing (Spotti et al., 2013a, 2013b) and lower gel firmness (i.e., storage modulus) as measured by dynamic low amplitude oscillatory shear rheology (Spotti et al., 2014a, 2014b), compared with WPI alone or unconjugated WPI-dextran mixtures. Similar results were reported by Sun et al. (2011a) for WPI conjugated with dextran (average molecular mass 150 kDa) at 60 °C for 7 d at 79% RH.

The lower strength of heat-set WPI-based gels made from whey protein conjugated with dextran, compared with unconjugated whey protein or mixtures of whey protein and carbohydrates is attributed to several factors, with the relative contribution of the individual factors dependent on the system composition and conditions of conjugation. Under the heating conditions typically required to achieve conjugation (see Section 6), denaturation and aggregation of whey proteins can occur, serving to alter exposure and reactivity of functional groups (e.g., free sulphydryl and hydrophobic groups) and the surface charge of protein molecules, all of which influence protein–protein and protein-water interactions (Brodkorb et al., 2016). Covalent attachment of the carbohydrate molecules also increases the hydrophilicity and steric barrier properties of the conjugated proteins, both of which result in decreased protein–protein interactions and increased protein-water interactions.

12. Enrichment and purification of conjugates

It is desirable to enrich the protein-carbohydrate conjugates from the reaction mixtures in which they are produced to remove unreacted carbohydrate, unreacted protein and possibly soluble Maillard reaction products, while increasing conjugated protein concentration. Such processes need to be food-grade, efficient, economical and have acceptable yield – enrichment, as opposed to purification, of the protein-carbohydrate conjugate is normally sufficient.

There has been limited work published to date on the enrichment/purification of milk protein-carbohydrate conjugates, and the studies that have been reported (Bund, Allelein, Arunkumar, Lucey, & Etzel, 2012; Etzel & Bund, 2011) are very much informed by approaches used in the pharmaceutical industry for purification of various therapeutic proteins conjugated with polyethylene glycol (i.e., PEGylated proteins), with separation being achieved largely based on differences in hydrophobicity (i.e., using hydrophobic interaction chromatography; Mayolo-Deloisa, González-Valdez, & Rito-Palomares, 2016) and charge density (Abe, Akbarzaderaleh, Hamachi, Yoshimoto, & Yamamoto, 2010) between conjugated and unconjugated proteins.

An initial study by Etzel and Bund (2011) involved laboratory-scale, analytical separation and enrichment of whey protein-dextran conjugates from mixtures of unreacted dextran and whey protein using cation exchange column chromatography with traditional chromatographic beads or porous polymethacrylate monolithic media and sodium lactate/sodium chloride-containing elution buffers. Using such an approach, unreacted dextran eluted first, followed by the conjugated protein and finally the unreacted protein; a portion of the unreacted whey protein was isoelectrically precipitated from the feed stream at pH 5.0, before chromatographic separation. The monolith media resulted in a similar dynamic binding capacity as the traditional beaded support (4–6 g L^{-1}) but with 42-fold higher mass productivity and 48-fold higher flow rate, while yielding a conjugate-enriched stream with lower purity. The use of cation exchange chromatography, as originally proposed by Etzel and Bund (2011) has been successfully scaled up to a preparative scale (i.e., 160 fold up-scaling from 5 mL to 800 mL columns) by Bund et al. (2012). On scale up, the upfront partial removal of unreacted whey protein by isoelectric precipitation was shown to be effective in reducing the buffer volumes required, purification time and the number of chromatography cycles required for purification of the conjugates. The yield of conjugated protein was ~18% on a protein basis, with the losses mainly associated with incomplete conversion of unconjugated to conjugated whey protein during the conjugate production process. Opportunities for increasing this conversion rate should be evaluated in future studies, with integration of conjugation and fractionation steps to reintroduce unreacted dextran and protein or the use of on-column conjugation having being suggested by Bund et al. (2012) and Fee and Van Alstine (2006). In addition, progressively increasing the salt concentration during elution for the enrichment/purification of milk protein-carbohydrate conjugates would be expected to facilitate separation of conjugates based on differences in their degree of glycosylation, as is the case with PEGylated lysozyme and BSA (Abe et al., 2010).

The vast majority of the studies to date on functional properties of conjugated milk proteins/peptides have been completed on mixtures of conjugated and unconjugated proteins/peptides (i.e., without removal of unconjugated protein/peptide and carbohydrate material). Further development of approaches for enrichment and purification of conjugated proteins/peptides from unconjugated proteins/peptides and carbohydrates will allow more systematic and deeper understanding of the role of residual unconjugated protein/peptide and carbohydrate material in determining the overall functionality of conjugated mixtures.

13. Conclusions and future perspectives

The proteins of milk are considered its most valued constituents from both techno-functional and nutritional perspectives (Augustin & Udabage, 2007) and many milk protein-based ingredients have been developed over the years which harness the unique functional properties of milk proteins (Smithers, 2008). As exemplified in this review, milk protein functionality continues to be an active area of research with Maillard-induced glycation of milk proteins/peptides offering considerable potential in the development of milk protein-based ingredients with enhanced heat stability, solubility, emulsification and foaming properties. However, some challenges with the use of conjugation to modify milk protein/peptide functionality exist and need to be addressed if the opportunities presented herein are to be fully realised and commercialised. One of the challenges experienced in interpreting data from studies completed to date on the influence of conjugation on functional properties of milk proteins/peptides is the ability to decouple the effects of protein denaturation/aggregation and conjugation on the functional property of interest. To facilitate this decoupling it is critically important to include appropriate control samples (i.e., unheated versus heated versus conjugated protein/peptide) in the design of such studies.

While it is desirable to achieve conjugation during the early stages of the Maillard reaction, it is also beneficial to limit the progression of the Maillard reaction to advanced stages, as the latter are largely responsible for some of the less desirable aspects of the Maillard reaction. In addition to bringing about glycation, the Maillard reaction (through the Strecker degradation pathway) contributes to both colour and flavour development under

conditions required to achieve conjugation (Van Boekel, 2006). Little information exists in the peer-reviewed literature on the sensorial properties of milk protein-carbohydrate conjugate ingredients and further research is required to develop a better understanding of sensory properties of milk protein-based conjugates. Bitterness in protein hydrolysates is associated with greater exposure of hydrophobic amino acid residues in peptides than in the intact proteins (FitzGerald & O'Cuinn, 2006; Newman, O'Riordan, Jacquier, & O'Sullivan, 2015); the covalent attachment of hydrophilic carbohydrate moieties to peptides during conjugation may present opportunities for reducing bitterness with conjugated milk protein hydrolysates. Maillard reaction products in conjugated milk protein-carbohydrate systems have been associated with high levels of antioxidant activity and milk protein/peptide-carbohydrate conjugates may be of interest as antioxidants in formulated food systems (Gu et al., 2009; Jiang & Brodkorb, 2012).

A number of authors have reported that conjugation of milk proteins with carbohydrates may alter the digestibility and immunogenicity of the proteins, to an extent dependent on the heat treatment applied, exact conformational changes to the proteins and the M_w of the carbohydrate moiety covalently attached (Böttger, Etzel, & Lucey, 2013; Corzo-Martínez et al., 2010b; Corzo-Martínez, Ávila, Moreno, Requena, & Villamiel, 2012a; Ikeda et al., 1996; Kobayashi et al., 2001). Further research on the nutritional and toxicological aspects of milk protein/peptide-carbohydrate conjugates and how conjugation impacts protein digestion and allergenicity would help in the development of these ingredients for hypoallergenic food applications.

Some information on the kinetics of adsorption and viscoelastic properties of interfacial (i.e., O/W and A/W) layers stabilised by milk protein-carbohydrate conjugates has been included in this review (see Sections 9 and 10); however, there are important questions remaining to be answered. For example, better understanding and control of the hydrophilic-hydrophobic balance of conjugated proteins, as affected by carbohydrate type and protein conformational changes, could assist in developing conjugates that confer greater foam capacity and stability. Similarly, studying the influence of M_w ratios and conformation of carbohydrate and protein moieties (i.e., as controlled by hydrolysis) would expand current knowledge in the area of interfacial packing and thickness of conjugates and assist in developing tailored solutions for improving protein functionality in areas such as emulsion stability, foam stability and encapsulation. Applying techniques used for studying the viscoelastic properties of interfaces, such as pendant drop tensiometry (Tamm et al., 2012), dilatational and shear rheology (Karbaschi et al., 2014), non-linear rheology (Sagis & Fischer, 2014) and surfactant diffusion kinetics (Dombrowski et al., 2016) would facilitate greater understanding of the interfacial properties of milk protein/peptide-carbohydrate conjugates.

In contrast to O/W emulsions stabilised by protein or low M_w surfactants, where the majority of the emulsifier is located at the interface, conjugate-based emulsifiers (i.e., mainly the hydrophilic carbohydrate moiety) can extend considerably into the serum phase. As a result of these differences, it is likely that the rheological properties of conjugate-stabilised O/W emulsions would be influenced by the interactions between the 'hairy' interfacial layers of individual oil globules and between these globules and other serum phase components (e.g., protein aggregates and hydrocolloids, if present) and is an area that warrants further investigation.

In using milk protein/peptide-carbohydrate conjugates as emulsifiers in powdered emulsion-based products, information is required in understanding how these ingredients may affect spray drying properties and stability of the resultant powders. It has been reported recently that on spray drying emulsions, the concentration of emulsifiers used is disproportionately higher on the surface, compared with the bulk phase of spray dried powder particles (Munoz-Ibanez et al., 2016) and it is also generally accepted that the surface composition of a powder strongly influences its functionality (e.g., solubility) and stability during storage (e.g., caking; Kelly et al., 2015; Vega & Roos, 2006; Vignolles, Jeantet, Lopez, & Schuck, 2007). It is also likely that conjugation of milk proteins/peptides with carbohydrates influences their spray drying properties, as during drying, the mechanical properties of the droplet skin layer are affected by the properties and structure of the protein/surfactant present at the A/W interface, which affects the kinetics of moisture removal and the overall kinetics of particle drying as shown with a single droplet drying model (Sadek et al., 2015).

In addition to the conventional dry and wet heating approaches, some alternative/non-thermal technologies and combinations thereof, such as sonication (Perusko et al., 2015; Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Velickovic, 2013; Yu, Seow, Ong, & Zhou, 2017), microfluidisation (Huang et al., 2013; Zhong et al., 2014), high hydrostatic pressure (Moreno, Molina, Olano, & López-Fandiño, 2003) and microwave treatment (Wang et al., 2013) are increasingly being studied for application in the pre-treatment of substrates prior to conjugation or directly in the production of conjugates and may offer promise for the production of milk protein/peptide conjugates with modified functionality.

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