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School of Food and Nutritional Sciences

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PHYSICOCHEMICAL CHARACTERISATION OF PROTEIN INGREDIENTS PREPARED FROM MILK BY ULTRAFILTRATION OR MICROFILTRATION FOR APPLICATION IN FORMULATED NUTRITIONAL PRODUCTS

Thesis presented by

Shane V. Crowley, B.Sc.

for the degree of

Doctor of Philosophy

in

Food Science and Technology

September, 2016

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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:** _____

Shane V. Crowley

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Abstract

Formulated food systems are becoming more sophisticated as demand grows for the design of structural and nutritional profiles targeted at increasingly specific demographics. Milk protein is an important bio- and techno-functional component of such formulations, which include infant formula, sports supplements, clinical beverages and elderly nutrition products. This thesis outlines research into ingredients that are key to the development of these products, namely milk protein concentrate (MPC), milk protein isolate (MPI), micellar casein concentrate (MCC), β-casein concentrate (BCC) and serum protein concentrate (SPC). MPC powders ranging from 37 to 90% protein (solids basis) were studied for properties of relevance to handling and storage of powders, powder solubilisation and thermal processing of reconstituted MPCs. MPC powders with ≥80% protein were found to have very poor flowability and high compressibility; in addition, these high-protein MPCs exhibited poor wetting and dispersion characteristics during rehydration in water. Heat stability studies on unconcentrated (3.5%, 140°C) and concentrated (8.5%, 120°C) MPC suspensions, showed that suspensions prepared from high-protein MPCs coagulated much more rapidly than lower protein MPCs. β-Casein ingredients were developed using membrane processing. Enrichment of β-casein from skim milk was performed at laboratory-scale using 'cold' microfiltration (MF) at <4°C with either 1000 kDa molecular weight cut-off or 0.1 µm pore-size membranes. At pilot-scale, a second 'warm' MF step at 26°C was incorporated for selective purification of micellised β-casein from whey proteins; using this approach, BCCs with β-casein purity of up to 80% (protein basis) were prepared, with the whey protein purity of the SPC co-product reaching ~90%. The BCC ingredient could prevent supersaturated solutions of calcium phosphate (CaP) from precipitating, although the amorphous CaP formed created large micelles that were less thermo-reversible than those in CaP-free systems. Another co-product of BCC manufacture, MCC powder, was shown to have superior rehydration characteristics compared to traditional MCCs. The findings presented in this thesis constitute a significant advance in the research of milk protein ingredients, in terms of optimising their preparation by membrane filtration, preventing their destabilisation during processing and facilitating their effective incorporation into nutritional formulations designed for consumers of a specific age, lifestyle or health status.

List of publications and conference contributions

The following papers arise from work undertaken during my PhD studies (2012-2016), and most are included in this thesis for examination purposes. At the time of writing most of the material presented in Part B (Chapters 6-11) was being prepared for submission to international peer-reviewed journals. It should be noted that some research articles and reviews listed (i.e., those marked with *), have not been included for consideration during the examination as they do not fit the core themes of the thesis, but research papers are included in Appendix 2 for information where published versions are available.

Peer-reviewed papers

***Crowley, S. V.**, Dowling, A. P., Caldeo, V., Kelly, A. L., and O'Mahony, J. A. (2016). Impact of α-lactalbumin:β-lactoglobulin ratio on the heat stability of model infant milk formula protein systems. *Food Chemistry*, 194, 184-190.

*Vos, B., **Crowley, S. V.**, O'Sullivan, J., Evans-Hurson, J., McSweeney, S., Krüse, J., Ahmed, M. R., Fitzpatrick, D., and O'Mahony, J. A. (2016). New insights into the mechanism of rehydration of milk protein concentrate powders determined by Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS). *Food Hydrocolloids*, 61, 933-945.

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Crowley, S. V., Jeantet, R., Schuck, P., Kelly, A. L., and O'Mahony, J. A. (2016). Rehydration and solubility characteristics of high-protein dairy powders, in, *Advanced Dairy Chemistry: Volume 1: Proteins, Part B, Applied Aspects*, 4th edn., P. L. H. McSweeney and J. A. O'Mahony (eds.), New York: Springer, pp. 99-131.

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***Crowley, S. V.**, and O'Mahony, J. A. (2016). Drying: Effect on Nutrients, Composition and Health, in, *Encyclopedia of Food and Health*, *Volume 2*, 1st ed., B. Caballero, P. M. Finglas, F. Toldrá (eds.), Oxford: Elsevier, pp. 439-445.

***Crowley, S. V.**, O'Mahony, J. A., and Fox, P. F., (2016). The proteins of milk, in, *Achieving Sustainable Production of Cow's Milk, Volume 1: Safety and Quality*, N. van Belzen (ed.). [in press]

Oral presentations

Crowley, S. V. (2016). Next generation milk protein ingredient isolation and functionality. IDF Parallel Symposia: Concentrated and Dried Milk Products, Dublin, Ireland, April 11-13th, 2016.

Crowley, S. V., Vos, B., O'Sullivan, J., Evans-Hurson, J., McSweeney, S., Krüse, J., Ahmed, M. R., Fitzpatrick, D., and O'Mahony, J. A. (2016). New insights into the mechanism of rehydration of milk protein concentrate powders determined by Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS). IDF Parallel Symposia: Concentrated and Dried Milk Products, Dublin, Ireland, April 11-13th, 2016.

Jeantet, R., **Crowley, S. V.,** and Auty, M. (2016). The rehydration dilemma: Panel discussion. IDF Parallel Symposia: Concentrated and Dried Milk Products, Dublin, Ireland, April 11-13th, 2016.

Crowley, S. V. (2014). Infant formula manufacture and novel ingredients. CDR Industry Team Research Forum, Madison, WI, USA, November 20th, 2014.

Poster presentations

Crowley, S. V., Molitor, M., Etzel, M. R., Kalscheuer, R., Lu, Y., Kelly, A. L., O'Mahony, J. A., and Lucey, J. A. (2016). Optimising the production of β-casein and co-products during membrane fractionation. 9th NIZO Dairy Conference, Papendal, the Netherlands, July 30th-August 2nd, 2015.

Crowley, S. V., Molitor, M., Kalscheuer, R., Lu, Y., Kelly, A. L., O'Mahony, J. A., and Lucey, J. A. (2016). Use of a liquid-solid hydrocyclone in the recovery of milk minerals from acid whey. IDF Parallel Symposia: Concentrated and Dried Milk Products, Dublin, Ireland, April 11-13th, 2016.

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Crowley, S. V., Gazi, I., Kelly, A. L., Huppertz, T., and O'Mahony, J. A. (2013). Influence of protein concentration factor on the physical characteristics of milk protein concentrate powders. Poster presentation: 42nd Annual Food Research Conference, Ashtown, Dublin, June 27-28th, 2013.

Part A

Characterisation of milk protein concentrates prepared at a range of protein concentration factors

Introduction and research objectives

Milk contains ~3.2% protein, 78% of which is casein, with the remaining 22% being whey protein (O'Mahony and Fox, 2013). These two major fractions can be separated from milk in (or close to) their native form using microfiltration (Saboya and Maubois, 2000). A rapidly growing area of application for these ingredients is protein-based beverages. In general, both casein and whey proteins are considered to have good nutritional quality, as they contain the full complement of indispensable amino acids, are highly digestible and contribute dietary bioactives in the form of proteins and peptides (Pellegrino *et al*., 2016). Casein, in its native, micellar form is used in sports nutrition applications as a 'slow-release' protein. Whey protein is an even more prevalent ingredient in sports nutrition, where it is marketed as a 'fast-release' protein, and is also used extensively in infant formulae. The slow/fast concept is related to rates of gastric emptying; Boirie *et al*. (1997) proposed that because casein forms a clot in the stomach the release of amino acids to the gut is slowed, while the non-clotting whey proteins result in a higher rate of release of amino acids. However, the relationship between protein type and postprandial digestion/absorption may be more complex and some researchers have proposed that nitrogen utilisation may benefit from a synergistic relationship between casein and whey proteins (Lacroix *et al*., 2006). Recent work has demonstrated that thermal treatment (90°C, 20 min) can alter the digestion characteristics of milk proteins, by incorporating whey proteins into the clot and altering the clot porosity, which in turn affects kinetics of protein hydrolysis (Ye *et al*., 2015).

In addition to differences in digestion and absorption properties, the two major milk proteins have many other unique characteristics. Due to their light-scattering properties, casein micelles are turbid in suspension, which is the primary reason for the characteristic whiteness that so distinguishes milk (Walstra *et al*., 1999). Casein micelles, and casein-derived phosphopeptides, bind a large quantity of calcium phosphate in a stable and highly bioavailable amorphous form (Cross *et al*., 2005). Casein is also a rich source of bioactive peptides, including those with opioid and ACE-inhibitory activities (Nongonierma *et al*., 2016). Whey proteins are particularly rich in branched-chain amino acids, which function as precursors to amino acids and proteins involved in muscle synthesis, promote the synthesis of

glutamine required for cell division and donate amino acids for the synthesis of the neurotransmitter, glutamate (Pellegrino *et al*., 2016). Many whey proteins are purported to have specific bio-functional roles in the body, including lactoferrin, which is thought to contribute anti-viral and anti-bacterial activity, stimulate the expression of growth factors and aid in the delivery of iron (Lönnerdal, 2014), and β-lactoglobulin, believed to have a role in the delivery of fat-soluble vitamins and the modification of digestive enzyme activity (O'Mahony and Fox, 2013). Casein and whey proteins have valuable nutritional characteristics and complementary roles in the human diet, as well as unique techno-functional characteristics. Ingredients that contain both of the major milk proteins in the native structures and ratio found naturally in milk therefore present exciting opportunities for the formulation of nutritional products.

Milk protein concentrate (MPC) is a term used to define an ingredient manufactured from skim milk in which the two major protein families (casein and whey protein) have both been concentrated by a membrane filtration process. This concentration is achieved through ultrafiltration (UF) of the skim milk, with diafiltration (DF) using water being applied to achieve greater depletion of non-protein components (Gésan-Guiziou, 2013). MPCs are classified according to their protein content, with MPC50 being an example of a low-protein MPC (~50% protein) and MPC80 representing a high-protein MPC (~80% protein). Milk protein isolate (MPI) and MPC90 are equivalent terms for MPC powders with ≥90% protein (solids basis). The use of a membrane process largely maintains the two major protein families in their native states (i.e., undenatured structure, low levels of process-induced covalently-bonded aggregates) and at their natural ratio (78:22 casein:whey protein). MPCs have a more favourable sensory profile compared to other casein-dominant ingredients, such as caseinates, acid casein and rennet casein (Smith *et al*., 2016). High-protein MPCs/MPIs are a key component of several nutritional beverages, including meal-replacers to prevent malnutrition in clinical environments, therapeutic drinks for the control of ageing-related diseases such as sarcopenia and lactose-free infant milk formulae. In these applications, MPCs are typically mixed with carbohydrates and oil, along with vitamins, minerals and emulsifiers, before being homogenised and thermally processed by in-container

sterilisation or ultra-high temperature (UHT) treatment (Liang *et al*., 2013, 2014) to produce liquid product formats or spray-dried into powders.

Much is known about the physical properties, solubility and heat stability of traditional dairy ingredients, such as skim milk powder (SMP) and whole milk powder (WMP), and methods to improve the functionality of these ingredients (Sharma *et al*., 2012; Kelly and Fox, 2016). The development of agglomeration technology facilitated the production of skim milk powders with good flowability and more 'instant' solubility characteristics; in addition, the adoption of lecithination resolved issues relating to the poor wettability of WMPs. Ingredients derived from whey, which were developed later and include whey protein concentrate (WPC) and whey protein isolate (WPI), benefited from this existing knowledge base. The routine application of both instantisation-enabling technologies to high-protein whey ingredients (WPC80, WPC85 and WPI) is now common, prompted by the dramatic growth in demand for fast-dissolving protein supplements for sports nutrition applications (Price, 2013). Factors influencing the destabilisation of milk during heating have also been extensively characterised, with variations in the levels of serum-phase constituents (e.g., ionic Ca, urea, phosphates) known to have a significant impact thereon (Huppertz, 2016). Comparatively little is known about the interrelationships between the process, composition and functionality of MPCs. Issues associated with poor solubility, which have been extensively studied and largely resolved for most dairy powders, have required the development of new analytical techniques (Fang *et al*., 2007) and solubility-enhancing technologies (Carr and Golding, 2016) designed specifically for MPCs. The studies detailed in this section involved the physicochemical characterisation of a range of MPCs before (physical properties, flowability), during (wetting, dispersion) and after (heat stability) rehydration. Results from these studies can inform strategies for the processing and application of MPCs in nutritional products and facilitate development of MPCs with unique/tailored functionality.

A key defect associated with high-protein MPCs is their slow rehydration rates, which is the subject of a review article in **Chapter 1**. The review details the current understanding of the causes of this defect, analytical methods to characterise the rehydration of MPCs and related ingredients, and strategies which are available to improve their solubility. Following the review article is a paper (**Chapter 2**) that

describes a study on the physical properties and flowability of a range of MPC powders, in which links are established between the composition of the liquid concentrates before spray-drying and their tendency to behave cohesively under an applied stress; this was one of the first published studies on food powders that used the recently developed Brookfield Powder Flow Tester. In **Chapter 3** a study is presented on the rehydration characteristics of the same set of MPC powders, focusing on the wetting and dispersion properties of the powders, studied using a range of experimental techniques developed by our lab group.

MPCs are used as ingredients in many different beverages, from low-protein lactose-free infant formulae to high-protein drinks designed for clinical applications. Heat treatments are essential during the manufacture of these products to produce a safe and shelf-stable product. There are no general guidelines for ensuring that MPCs do not become destabilised during heating, as they represent a very broad ingredient category with compositions that vary widely. Components which change in concentration (relative to protein) depending on the concentration factor achieved during UF/DF, i.e., lactose, non-protein nitrogen and minerals, have all been demonstrated to influence the heat stability of milk (Huppertz, 2016), and thus the heat stability of MPCs should also be expected to vary accordingly. This aspect of MPC technology was the subject of the two final papers that are presented in this section, which involved the characterisation of the heat stability profiles of MPCs at a protein content close to that of milk (3.5%) under lab-scale conditions representative of UHT processing (**Chapter 4**) and at protein levels close to those found in many commercial MPC-containing beverages (8.5%) when exposed to in-container sterilisation (**Chapter 5**) Findings from the various studies in Part A are discussed alongside those in Part B in **Chapter 12**.

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CHAPTER 1

Rehydration and solubility characteristics of high-protein dairy powders

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. PS and RJ contributed key ideas during its preparation. The chapter is included in its final published form (author's copy).

Rehydration and Solubility Characteristics of High-Protein Dairy Powders

4

S.V. Crowley, A.L. Kelly, P. Schuck, R. Jeantet, and J.A. O'Mahony

Abstract

Dairy powders derived from membrane filtration processes, such as milk protein concentrate (MPC) and phosphocaseinate (PC) powders, have considerable potential as functional ingredients due to their high protein content and quality. However, the use of these powders is sometimes limited or impaired by their poor rehydration characteristics in aqueous media, which has been linked with the formation of an inter-linked network of casein micelles at particle surfaces during processing and storage. Analytical tools are now available which can monitor the rehydration of dairy powders dynamically. This is a considerable development, as the rate-limiting stages of rehydration for individual powders (e.g., wetting, dispersion) can now be identified, quantified and targeted in attempts to improve rehydration properties. In addition, these technologies allow the negative effects of sub-optimal processing or storage conditions on powder rehydration and solubility characteristics to be measured, which allows preventative strategies against loss of solubility to be developed. Moreover, it is foreseeable that some of these technologies could be useful for in-line analysis and process control at an industrial scale. This review provides a detailed description of the underlying principles, data outputs and industrial relevance of current methods to monitor dairy powder rehydration. The technologies discussed in this review include viscometry and rheometry, turbidimetry, static light-scattering, focused beam reflectance

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measurement (FBRM), image analysis, nuclear magnetic resonance (NMR) relaxometry, thermochemistry, conductimetry and sound-based technologies. The contribution that these technologies have made to the current understanding of rehydration phenomena, with a particular emphasis on high-protein dairy powders (≥80 % protein), is discussed throughout. In addition, a comprehensive overview of rehydration and solubility characteristics, and the effects of process-, storage-, and additive-induced changes thereon, is given for high-protein dairy powders.

Keywords

Rehydration stages • Solubility • Instant properties • High-protein dairy powders • Rehydration properties • Characterisation of powder rehydration

Abbreviations

ADMI	American Dry Milk Institute
CaCas	Calcium caseinate
CN	Casein
CPMG	Carr-Purcell-Meiboom-Gill
DF	Diafiltration
FBRM	Focused beam reflectance measurement
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
IDF	International dairy federation
MDP	Maltodextrin powder
MF	Microfiltration
MPC	Milk protein concentrate
MR	Maillard reaction
NaCas	Sodium caseinate
NMR	Nuclear magnetic resonance
PC	Phosphocaseinate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLS	Static light-scattering
SMP	Skim milk powder
UF	Ultrafiltration
WMP	Whole milk powder
WP	Whey protein
WPI	Whey protein isolate
XPS	X-ray photoelectron spectroscopy

4.1 Introduction

Drying (e.g., roller-, spray-, freeze-drying) is commonly applied to dairy-based systems to enhance shelf-life, minimise transit costs and increase convenience levels for the end-user (Walstra *et al.*, 1999; Henning *et al.*, 2006; Schuck *et al.*, 2007). The ability of dehydrated ingredients to rehydrate readily in aqueous media is essential if their underlying functionality is to be exploited (Gaiani *et al.*, 2007; Mimouni *et al.*, 2009, 2010a; Fang *et al.*, 2011). Ease of rehydration is not solely an issue of consumer convenience, but is also crucial at an industrial level in relation to unit operations involving powders (Schober and Fitzpatrick, 2005; Hellborg *et al.*, 2010). Powder rehydration properties may be affected by raw material selection, pre-treatments, processing methods and storage conditions (Walstra *et al.*, 1999). In addition, intrinsic powder properties, such as surface and bulk composition, particle structure (e.g., morphology, presence of pores and capillaries) and rehydration conditions (e.g., stirring rate, temperature, solids content), can influence rehydration behaviour (Schuck *et al.*, 2012).

Developments in membrane filtration technologies have facilitated the fractionation of bovine milk to give liquid retentates with high

concentrations of protein (Kelly *et al*., 2000; Mistry, 2002). Retentates derived from membrane processes such as ultrafiltration (UF) and diafiltration (DF) are commonly dried into powders with high proportions of native-state proteins. Milk protein concentrates (MPCs) are produced by heat-treating skim milk and concentrating both whey protein (WP) and casein (CN) fractions in the retentate using UF and sometimes DF (Mulvihill and Ennis, 2003; Udabage *et al*., 2012). Phosphocaseinates (PCs; also termed native phosphocaseinates, native micellar CNs or micellar CN concentrates) are comprised primarily of the CN fraction, and associated minerals, retained following microfiltration (MF) or a combined MF/DF process (Fauquant *et al*., 1988; Pierre *et al*., 1992; Hurt and Barbano, 2010). On the other hand, WP isolates (WPIs) are obtained from UF and perhaps DF of whey or milk microfiltrate (Maubois and Olivier, 1997) or from whey using ion-exchange chromatography (Fox and McSweeney, 1998). After spray drying, which is sometimes preceded by an evaporation step, these dairy products have considerable value due to their innately high protein content and quality (Davenel *et al*., 2002; Mimouni *et al*., 2009); however, high-protein CN-dominant products such as PC powders, and to a lesser extent MPC powders, exhibit poor rehydration characteristics when introduced to aqueous media, due to low concentrations of lactose (Anema *et al*., 2006; Baldwin, 2010; Richard *et al*., 2013) and the poor dispersibility of powder particles with surfaces rich in inter-linked CN micelles (Havea, 2006; Gaiani *et al*., 2007; Mimouni *et al*., 2010a; Haque *et al*., 2012).

Standard methods (e.g., insolubility index) for measuring properties relating to powder solubility have the advantage of being easy to perform and interpret; however, results do not represent the entirety of the rehydration process, can have poor reproducibility and may not be applicable to more recently developed dairy powder ingredients, as discussed by Gaiani *et al*. (2005), Schuck *et al*. (2007) and Fang *et al*. (2008). In addition, these approaches for studying powder solubility do not provide information about the multiple stages of powder rehydration. Furthermore, the poor rehydration characteristics of high-protein dairy powders are probably due to inhibited water transfer during the period of analysis rather than insolubility *per se* (Schuck *et al*., 2007).

Recent research has focused on the dynamic monitoring of powder rehydration *in-situ*, which can allow the identification and quantification of the rate-limiting stage of rehydration for a given powder. Depending on powder type, the rate-limiting step can be wetting, the initial absorption of water by powder particles, or dispersion, the fragmentation of powder particles which have absorbed water. For example, it has been reported that the rate-limiting stage depends on whether the powder is CN- (dispersion-limiting) or WP- (wetting-limiting) dominant (Gaiani *et al*., 2007; Schuck *et al*., 2007; Hussain *et al*., 2011b). This information can be used as a basis to develop processes to improve powder solubility by reducing the duration of the rate-limiting stage of rehydration. In addition, such technologies could be useful for in-line process analysis and control at industrial scale.

This review includes an overview of dairy powder rehydration, the factors (composition-, process-, and storage-related) which influence rehydration behaviour and a discussion of the techniques which have been used to monitor powder rehydration phenomena dynamically. Particular focus has been placed on studies into high-protein dairy powders (≥80 % protein), as this is currently, and will remain for some time, a very active area of research.

4.2 Dairy Powder Rehydration

A general mechanism for the rehydration of an agglomerated, high-protein dairy powder is shown in Fig. 4.1. It is important to note that two or more of these stages may occur concurrently during rehydration. For this reason, stages such as wetting and swelling, or dispersion and dissolution, are often combined and considered as one. However, owing to the complexity of the powder rehydration process, all stages will be considered individually for the purpose of this review. In addition, some stages may not occur for certain

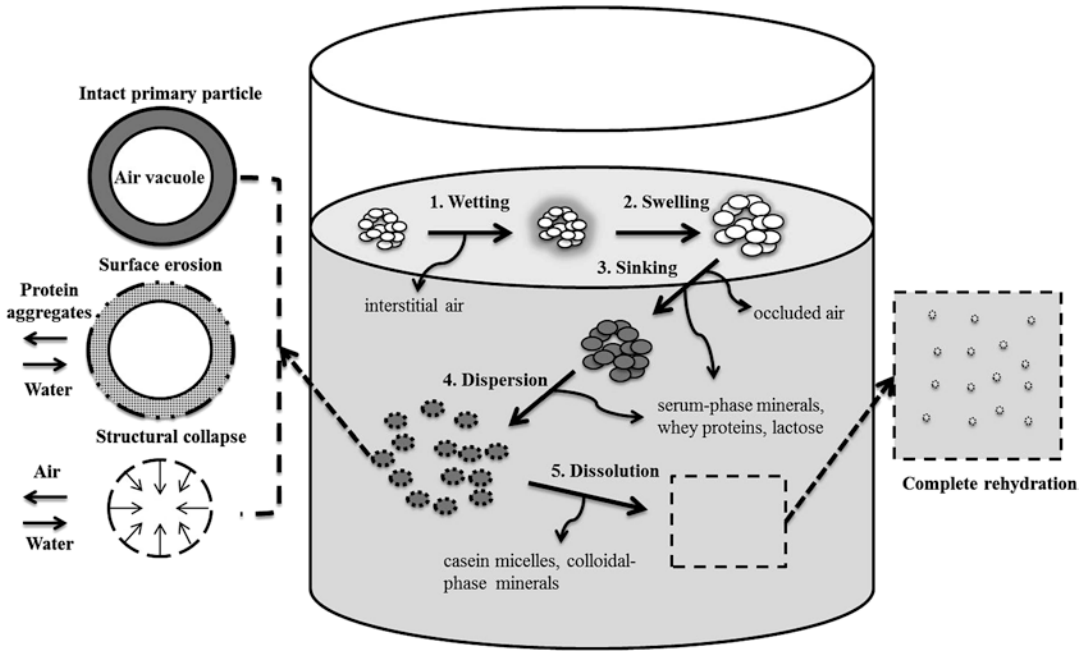

Fig. 4.1 Schematic representation of rehydration profile for an agglomerated high-protein dairy powder, showing *1* Wetting, *2* Swelling, *3* Sinking, *4* Dispersion and *5* Dissolution

powders (e.g., dispersion of agglomerated particles in non-agglomerated powders, swelling of particles and delayed surface erosion in WP-dominant powders).

4.2.1 Wetting

The wetting stage of powder rehydration refers to the initial absorption of water by introduced powder particles, the subsequent immersion of wetted powder particles, and their ultimate disappearance from the free surface of the liquid. Wetting was one of the first powder rehydration stages to be recognised and wettability is considered an important feature of instant powder products (Skanderby *et al*., 2009; Schuck *et al*., 2012). Particles of a skim milk powder (SMP) which has not been agglomerated make contact with the aqueous surface, wet rapidly and create a surface barrier which impedes subsequent wetting of other particles (Masters, 1985). Agglomeration minimises the specific surface area of powder particles and inhibits the formation of a surface film during rehydration, with a concomitant increase in the rate of wetting (Skanderby *et al*., 2009). Wetting of agglomerated powder particles involves the replacement of interstitial air at the solid surface with liquid, followed by inward diffusion of the liquid through the capillary network of the agglomerated powder particle (Galet *et al*., 2010; Forny *et al*., 2011). Wettability of powders may be measured using the International Dairy Federation (IDF) standard test for wettability (IDF, 1979). In this method, a glass plate holding a given quantity of powder is withdrawn over a defined period, the powder falls into the beaker and the time for all particles to become visibly wetted is recorded (Fig. 4.2).

Surface composition and contact angle (θ) have a marked influence on the wettability of powder surfaces (Fig. 4.3). Walstra *et al*. (1999) emphasised the importance of θ in a multi-phase system (solid, liquid and gas), stating that, if θ is <90°, wetting tends to be complete. Values of θ are typically derived from the Young equation (Forny *et al*., 2011):

$$\gamma_{SG=}\gamma_{LS+}\gamma_{LG}.\cos\theta \qquad (4.1)$$

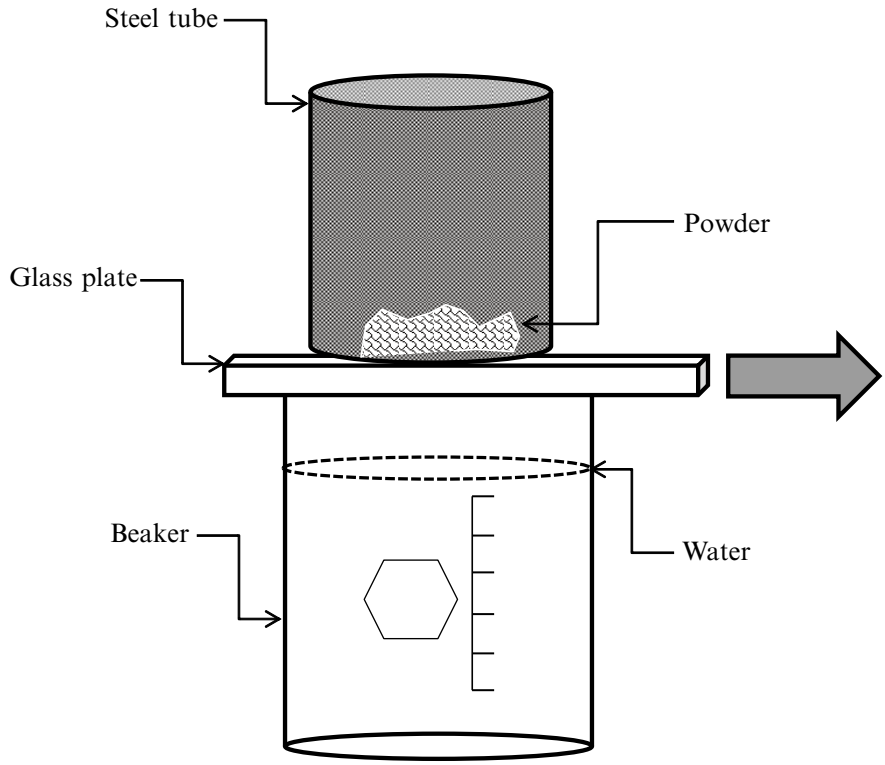

Fig. 4.2 Typical procedure used to determine the wettability of dairy powders

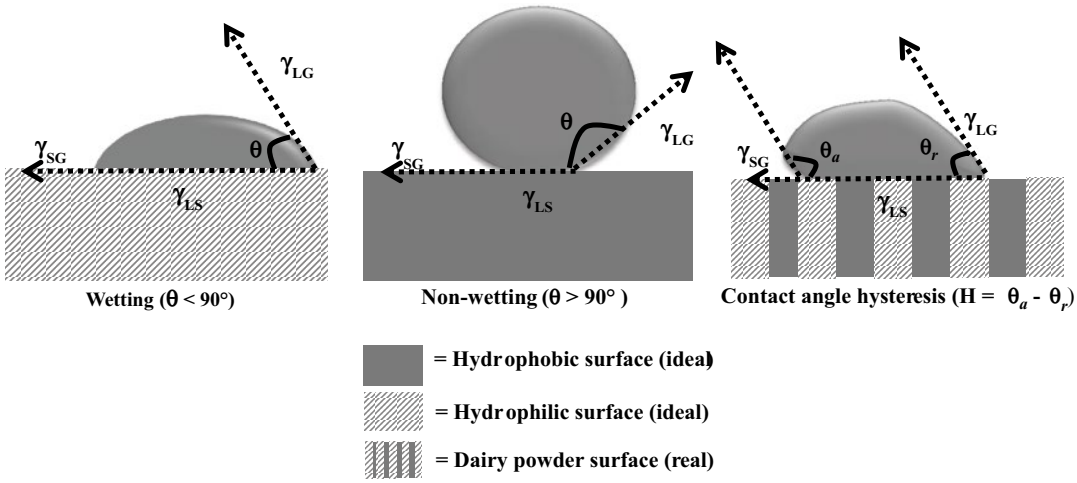

Fig. 4.3 The effect of surface composition on the contact angle (Θ) formed between a liquid drop and a solid surface. Interfacial tensions between solid-gas (γ_{SG}), liquid-solid (γ_{LS}) and liquid-gas (γ_{LG}) phases are illustrated along with Θ, as per Young's equation (4.1). Values of Θ_a and Θ_r represent the advancing and receding Θ, respectively, during contact angle hysteresis

where the interfacial tensions between the solid and gaseous phase, the liquid and solid phase, and the liquid and gaseous phase are represented by γ_{SG}, γ_{LS}, and γ_{LG}, respectively (Fig. 4.3). While it is difficult to obtain a true static Θ value for dairy powders, the change in Θ after a drop of water is deposited on a prepared bed of powder can give a useful indication of wetting properties across different samples. Using dynamic measurement of Θ, Gianfrancesco *et al.* (2011) reported that the presence of heat-denatured β-lactoglobulin or dissociated CN in powders yielded poor wetting properties compared to powders where these proteins were present in unmodified form. In addition, the decrease in Θ over 5 s after a drop of water was deposited on a 35 % protein MPC powder was >40 % compared to <10 % for a 86 % protein MPC powder, indicating that high-protein MPC powders imbibe water less readily (Crowley *et al.*, 2015). The results of Crowley *et al.* (2015) supported those of Fyfe *et al.* (2011), who reported that skins of inter-linked CN micelles at the surface of particles in MPC80 increased both non-polar bonding at dry particle surfaces and the attractive forces between reconstituted MPC80 and a hydrophobic surface, measured using X-ray photoelectron spectroscopy (XPS) and atomic force microscopy, respectively. Methods to measure Θ based on a combination of the Young equation with heat of immersion values, derived from calorimetric measurements, have also been proposed (Marabi *et al.*, 2008).

Young's equation pertains to ideal solid surfaces and yields the intrinsic Θ; however, uneven surface topography and chemical heterogeneity may yield larger apparent Θ values (Kwok and Neumann, 1999; Forny *et al.*, 2011). Deviations from ideality, as in the case of dairy powders, can result in multiple possible values of Θ at a given solid surface (Fig. 4.3). This phenomenon, known as contact angle hysteresis, arises from the difference between Θ values derived from advancing (Θ_a) and receding (Θ_r) liquids and results in discrepancies between experimentally derived and real values for Θ (Kwok and Neumann, 1999). However, despite the inherent difficulty in measuring Θ, the value provides a useful theoretical basis for framing discussions on the effects of surface composition on the wetting of dairy powders.

Hydrophobic materials, such as lipids, increase Θ and affect wetting negatively (Kim *et al.*, 2002) (Fig. 4.3). Conversely, high concentrations of lactose at the surface of dairy powders reduce wetting times, due to the hydrophilic nature of lactose (Gaiani *et al.*, 2006a). However, storage of dairy powders above the glass transition temperature (T_g) for lactose can result in its crystallisation, which has a negative effect on rehydration properties (Vega and Roos, 2006; Marabi *et al.*, 2007).

Gaiani *et al.* (2006a) referred to studies on the surface composition of powders based on scanning electron microscopy and stated that they tended to yield largely qualitative results. In addition, attempts to analyse quantitatively the levels of surface fat using solvent extraction can be compromised by simultaneous extraction of bulk lipids (Vega and Roos, 2006). In recent years, XPS has been a key technique for the characterisation of changes to surface composition in dairy powders. Murrieta-Pazos *et al.* (2012) outlined the principle of XPS in detail: to summarise briefly; irradiation of a sample surface by an X-ray source with known energy results in a total transition from photon energy to atomic electrons; levels of individual components at the powder surface are then calculated based on the elemental composition of the powder surface. Measured ratios of carbon, nitrogen and oxygen are then converted using a matrix formula into surface ratios of protein, lactose and fat.

XPS-based studies have demonstrated how the surface composition of dairy powders can change as a result of processing and storage, with obvious implications for powder wettability. Gaiani *et al.* (2006a) observed an over-representation of fat, relative to bulk composition, on the surfaces of high-protein PC powders. Nijdam and Langrish (2006) reported that higher inlet temperatures during the spray drying of milk concentrates, prepared by mixing reconstituted skim and full cream milk powders to fat in dry-matter contents in the range 1.1–29.8 %, w/w, promoted the transfer of lipid material to

particle surfaces and that lower inlet temperatures favoured the presence of protein at particle surfaces. Gaiani *et al*. (2009b) correlated reduced wettability with the migration of lipids to particle surfaces in a high-protein PC powder over storage. Vignolles *et al*. (2009) reported that, apart from surface fat, wettability of high-fat dairy powders was also affected negatively by factors such as the levels of amorphous lactose at particle surfaces, as well as the size and porosity of particles. Over-representation of fat, relative to bulk powder composition, has also been observed for model infant milk formula powders (McCarthy *et al*., 2013a).

Kim *et al*. (2009) reported that higher feed solids contents reduced surface lipid content while increasing surface protein and lactose content for both SMP and whole milk powder (WMP). Contrary to results reported by Nijdam and Langrish (2006), Kim *et al*. (2009) reported that higher drying temperatures promoted the presence of lactose at powder surfaces, a result which was confirmed by Vignolles *et al*. (2010). Kim *et al*. (2009) also observed that increasing the number of homogenisation passes resulted in lower lipid levels on particle surfaces in WMP samples, which was particularly apparent at low drying temperatures. Hanley *et al*. (2011) reported reduced wettability of infant formula powders with increased air velocity during pneumatic conveying; this coincided with increased bulk density and free fat levels (measured by solvent extraction), due to increased instances of attrition at higher air velocities.

Wetting is often considered to be the rate-limiting step during the rehydration of most dairy powders (Kim *et al*., 2002; Vega and Roos, 2006). Gaiani *et al*. (2007) demonstrated that agglomeration of a WPI powder accelerated wetting; however, the opposite effect was observed for PC powder. WPI powders have been shown to be as poorly-wettable as WMPs, despite the considerably lower fat content of the former (Gaiani *et al*., 2011). Hussain *et al*. (2011b) observed protracted wetting stages for WPI and high-protein PC powders when rehydrated in NaCl or $CaCl_2$ solutions, compared to rehydration in water, with $CaCl_2$ having a more marked influence due to Ca^{2+} having a greater ability than Na^+ to screen charge. Thus, the ionic environment influenced the wettability of both CN- and WP-dominant powders. Numerous studies have demonstrated that wettability is the rate-limiting stage of WP-dominant powders, and that agglomeration is of greater benefit to the rehydration of WP-dominant powders, while being potentially detrimental to the rehydration of CN-based powders (Schuck *et al*., 2007).

4.2.2 Sinking

After a powder has become sufficiently wetted, some occluded air is released from primary particles and replaced by liquid solvent, creating denser particles which descend through the solution (Walstra *et al*., 1999; Kelly *et al*., 2003; Richard *et al*., 2013) (Fig. 4.1). The rate at which this process occurs determines the sinkability of a powder. For sinking to occur, the wetted particle must be denser than the liquid in which it is suspended (Masters, 1985). According to Masters (1985), sinking is promoted by low levels of occluded air and high particle density. Powders with low bulk density will have a greater tendency to float on the surface when added to a liquid.

Determination of the sinkability of dairy powders is traditionally performed in static systems (Bullock and Winder, 1960; Tamsma *et al*., 1967); thus, as most dairy powders are added to liquids under agitation (Schober and Fitzpatrick, 2005; Jeantet *et al*., 2010; Richard *et al*., 2013), sinkability measurements have become largely redundant. Písecký (1997) stated that sinkability was once considered an integral part of the powder rehydration process; nevertheless, its non-decisive role, the difficulty inherent in its measurement and its redundancy as applied to industrial powder rehydration systems have resulted in sinking being regarded increasingly as a minor step during wetting. Hence, few studies have been carried out in relation to sinkability in recent years and it is unlikely to become the focus of studies on dairy powder rehydration in the future.

4.2.3 Swelling

Swelling of powder particles is being recognised increasingly as a distinct stage in the rehydration of CN-dominant powders. In rheological-based studies (Sect. 3.1.1), swelling of particles is observed as a peak in viscosity after initial particle wetting (Gaiani *et al.*, 2006b), while turbidimetry (Sect. 3.1.2) and static light-scattering (Sect. 3.1.3) measurements have identified swelling as a minimum in turbidity and a peak in particle size, respectively (Gaiani *et al.*, 2006b, 2007). Gaiani *et al.* (2006b) reported increases in PC powder particle size by as much as 35 % due to swelling during rehydration. It is conceivable that swelling may have an impact on subsequent dissolution of solid bridges and dispersion of powder particles; however, this may not be the case for WPI powders, where no clear swelling stage has been observed (Gaiani *et al.*, 2007; Hussain *et al.*, 2011b). Thus, WP-dominant powders may undergo rehydration as shown in Fig. 4.1, but without a swelling stage. Rehydrating CN-dominant powders at higher temperatures can reduce the duration of the swelling stage (Gaiani *et al.*, 2006b), which indicates that the beneficial effect of rehydrating powders at higher temperatures is due in part to a decreased swelling time. Currently, no standard method exists for the measurement of swelling time.

4.2.4 Dispersion

Dispersion involves the fragmentation of wetted powder particles and is closely associated with the instant properties of a powder (Singh and Newstead, 1992). The dispersion of the powder into component agglomerates, fragmentation of agglomerates into primary particles, and erosion of primary particles may all occur during this stage (Mimouni *et al.*, 2009; Skanderby *et al.*, 2009; Fang *et al.*, 2011) (Fig. 4.1).

An IDF standard method is available for measuring powder dispersibility (IDF, 1979). As shown in Fig. 4.4, the method proceeds as per the wettability test (Fig. 4.2), except that the moisture content of the powder is first determined and a spatula is used to prevent adherence of unwetted particles to beaker walls and to promote dispersion. Dispersibility (%) is defined as the ability of the powder to disintegrate into particles small enough to permeate a 150 µm sieve and is determined on a dry solids basis (Schuck *et al.*, 2012).

High-protein CN-dominant dairy powders contain particles with surfaces rich in inter-linked CN micelles, which undergo wetting, sinking, and possibly swelling, but do not become sufficiently dispersed to allow complete rehydration after a reasonable period of time (Anema *et al.*, 2006; Havea, 2006; Baldwin, 2010; Fang *et al.*,

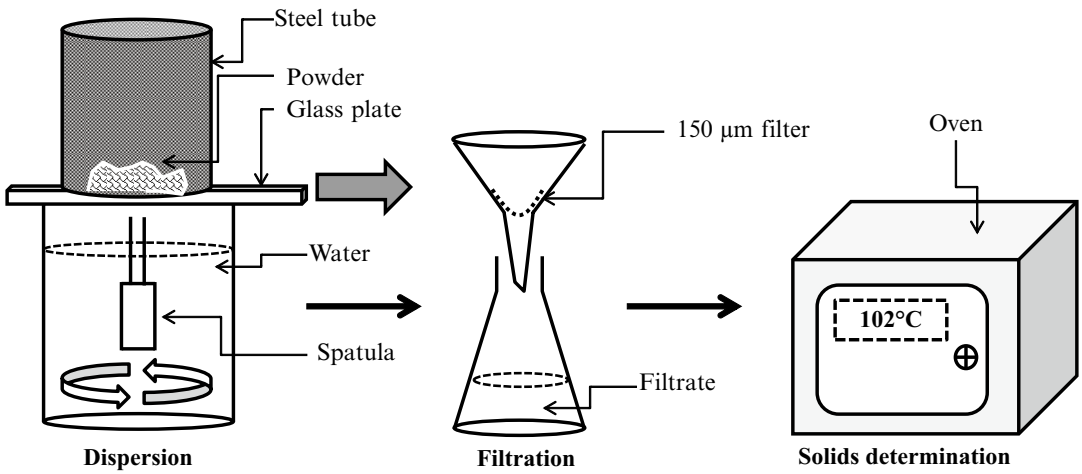

Fig. 4.4 Typical procedure used to determine the dispersibility of dairy powders

2012; Haque *et al*., 2012) results. Mimouni *et al*. (2009) hypothesised that complete rehydration of a high-protein MPC powder does not occur until the CN-rich skins of the primary particles become sufficiently eroded to induce structural collapse and release of all micellar components (such a process is shown during the dispersion stage in Fig. 4.1). This is supported by the continued presence of large primary particle-sized material in MPCs and PCs after extended periods of reconstitution using conventional mixing, even if solubility tests indicate rehydration is complete or near-complete (Chandrapala *et al*., 2014a; Crowley *et al*., 2015).

Mimouni *et al*. (2010a) identified two sets of components in a high-protein MPC powder, fast- and slow-dissolving, and reported that the former group was comprised of lactose, WP and monovalent ions, while the latter group consisted primarily of CN and associated colloidal minerals. As water penetration was sufficient to promote the complete release of fast-dissolving components from powder particles, the authors concluded that wettability could not be the rate-limiting step during rehydration of the MPC powder. Images from scanning electron microscopy seemed to confirm that primary particles of an MPC powder were porous enough to allow rapid inward diffusion of water, with concomitant release of fast-dissolving components, while the surface skin of inter-linked CN micelles prevented effective dispersion of particles, which limited release of micellar material (Mimouni *et al*., 2010b). As will be discussed in Sect. 3.2.5, sound-based technologies have revealed that water penetration can actually be delayed considerably in high-protein powders; moreover, the extrusion-porosification studies of Bouvier *et al*. (2013) demonstrated that increasing the size and number of pores in particles of these powders can markedly improve rehydration properties. Gaiani *et al*. (2011) reported that both PC and WPI powders (although still easy to rehydrate; see Sect. 3.1.2) were poorly-dispersible when compared with skim, semi-skimmed and whole milk powders. The authors attributed poor dispersibility of the powders to high and low concentrations of protein and hygroscopic compounds, respectively.

Augustin *et al*. (2012) produced MPCs through UF, UF and DF, or UF and evaporation, before the MPCs were spray-dried at high and low inlet/outlet temperatures (190/90 or 175/75 °C, respectively) into MPC powders (~82 %, w/w, protein in dry-matter); the authors reported that MPCs which were evaporated and/or spray-dried at high temperatures contained more insoluble material. Fang *et al*. (2012) analysed insoluble material in high-protein MPC powders which were rehydrated and subjected to a second spray drying step using a range of inlet temperatures (77–178 °C); there was an inverse relationship between inlet temperature and the concentration of soluble proteins, as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fang *et al*. (2012) reported that the CNs, in addition to α-lactalbumin, was the fraction most directly affected by higher drying temperatures, while β-lactoglobulin levels remained relatively constant. It is clear that the processing parameters used during the concentration and drying steps in the manufacture of high-protein CN-dominant powder impacts greatly their dispersion properties during subsequent rehydration.

Anema *et al*. (2006) characterised the insoluble material in an MPC powder (84.8 %, w/w, protein) using SDS-PAGE and high-performance liquid chromatography (HPLC); the authors identified the insoluble material as primarily CN-based, with minor WPs also present, and observed that insoluble material increased in quantity with the temperature (20–50 °C) and duration (0–60 days) of powder storage. In addition, Anema *et al*. (2006) demonstrated using mass spectrometry that lactosylation of the MPC powder occurred during storage, which suggested that the development of Maillard reaction (MR) products could contribute to the poor dispersibility of high-protein MPC powders. Le *et al*. (2011a) measured the development of early (lactulosyllysine—measured as furosine, itself a product of lactulosyllysine degradation), intermediate (hydroxymethylfurfural, HMF) and late (melanoidins—measured using colorimetry) MR products in an MPC powder (>80 %, w/w, protein) during storage (1–12 weeks) under a range

of relative humidities (44–84 %) and temperatures (25–40 °C); the authors observed that solubility decreased for the MPC powder with increasing storage time at all conditions studied, while the levels of all MR products increased, and that increased storage temperature and/or relative humidity increased the development of insoluble material and MR products.

Le *et al*. (2011b) compared changes in insolubility and MR product levels in four powders (SMP, WMP, MPC and WPC with 32, 24, 81 and 80 %, w/w, protein, respectively) over storage at 30 °C and a range of relative humidities (44–84 %); the authors reported that the development of furosine, HMF and melanoidins during storage was lower in MPC or WPC powders compared to WMP or SMP, which was attributed to the higher lactose:protein ratios in the latter powders, and that WPC was the only powder which did not become more insoluble over storage, presumably due to its lack of micellar CN. Indeed, Udabage *et al*. (2012) reported that a 95 %, w/w, protein CN-dominant powder without micellar casein (manufactured by spray drying of recombined concentrates of sodium caseinate (NaCas) and WPI to casein:whey ratios of 10:1 or 1:10) maintained 100 % solubility after high pressure treatment and/or 1 month storage at 40 °C; thus, the presence of micellar CN has a significant influence on the initial presence of insoluble material after manufacture and its development over storage. Le *et al*. (2011b) also reported that increased relative humidity during storage affected the solubility of SMP and MPC powders negatively but had no effect on WMP and WPC powder solubility; higher relative humidities promoted the development of furosine, HMF and melanoidins in SMP and WMP, while only furosine and melanoidin levels were affected in WPC and MPC powders. Elsewhere, Semagotoa *et al*. (2014) exposed an MPC70 powder to UV light during storage, which resulted in photoxidation-induced changes including lactosylation of casein and the formation of covalently-linked high molecular weight aggregates, with concomitant loss of solubility; conversely, MPC70 not exposed to UV light retained its solubility, unless the powder had been exposed to high temperature and relative humidity.

The studies of Anema *et al*. (2006) and Le *et al*. (2011a, b) revealed a possible relationship between the development of MR products and the poor dispersion properties of high-protein CN-dominant powders. Le *et al*. (2013) found that the insoluble material which developed over storage of an MPC powder (81 %, w/w protein) was primarily composed of α_{S1}-CN and subsequently investigated the effects of dephosphorylation, addition of lactose or addition of methylglyoxal (an advanced MR product) on the cross-linking behaviour of pure α_{S1}-CN on heating at 90 °C; the authors reported that the addition of lactose or methylglyoxal increased the development of cross-links during heating of α_{S1}-CN, while dephosphorylation had no effect. The results of Le *et al*. (2013) suggested that higher contents of lactose may increase the rate of development of MR products (e.g., methylglyoxal), with a concomitant increase in protein-protein cross-linking *via* amino acid residues such as lysine; conversely, a non-MR product, dehydroalanine (produced by heat- or alkaline-induced β-elimination of cysteine or phosphoserine residues), could not have participated in these protein-protein interactions as removal of phosphate from the phosphoserine residues of α_{S1}-CN did not affect subsequent cross-linking reactions on heating. Thus, it is possible that the development of MR products contributes to the poor dispersion characteristics of high-protein CN-dominant powders; indeed, Ennis and Mulvihill (1999b) also reported impaired solubility of rennet CN powders with increased furosine levels.

Anema *et al*. (2006) suggested that lower levels of "spacer" material, such as lactose, in high-protein MPC powders reduces the proximal distance between proteins, thereby promoting associations between CN micelles. Baldwin (2010) and Richard *et al*. (2013) suggested that lactose improves water penetration into powder particles and that a low level of lactose in high-protein dairy powders has a negative effect on solubility. Dissociation of CNs from the micellar phase of retentates during the manufacture of MPCs, is believed to be due to depletion of serum-phase minerals during UF and DF, which

creates a driving force for re-equilibration of calcium and inorganic phosphate with concomitant loss of micellar calcium phosphate. These effects have been attributed with an increased potential for hydrophobic interactions between CN micelles during processing, which may be responsible for the development of poorlydispersible inter-linked CN micelles in resultant MPC powders (Mimouni *et al.*, 2010b; Udabage *et al.*, 2012).

Udabage *et al.* (2012) applied high pressure treatment to MPCs before spray drying in the production of an 85 %, w/w, protein MPC powder and reported marked reductions in the insolubility of MPC powders pre-treated with high pressures. The application of 200 MPa of pressure at 40 °C resulted in a 19 % increase in solubility; the authors attributed the increased solubility of high pressure-treated MPC powders to increases in serum-based CN which reduced the proximity between CN micelles and thereby prevented their aggregation at particle surfaces during drying. Indeed, high pressure treatment of >100 MPa can cause extensive dissociation of CNs from micelles due to solubilisation of calcium phosphate from the micellar phase and increased ionisation under pressurised conditions (Huppertz *et al.*, 2006). Udabage *et al.* (2012) attributed increased solubility of powders manufactured from high pressure-treated MPCs to reduced levels of micellar CN; however, it is unclear whether increases in non-sedimentable CN caused by high pressure treatment are due to fragmentation of CN micelles into smaller micellar structures or the release of individual CNs from the colloidal phase (Huppertz *et al.*, 2006).

A number of studies on high-protein dairy powders using Fourier transform infrared spectroscopy have found only weak correlations between protein conformational changes and solubility in water (Kher *et al.*, 2007; Haque *et al.*, 2010; Sikand *et al.*, 2011). However, Hussain *et al.* (2011b) reported modifications to the secondary structure of proteins in PC and WPI powders when dissolved in NaCl or $CaCl_2$ solutions. Haque *et al.* (2012) reported enthalpy relaxations and thermal annealing in MPC powders during storage; the authors concluded that structural alterations over time created an increased potential for protein-protein interactions and the formation of a network of interlinked CN micelles at the powder surface. It was proposed that this network impaired the dispersion stage of MPC powder rehydration.

In recent years, numerous attempts have been made to improve the rehydration properties of high-protein CN-dominant powders through modifications to processing and rehydration conditions. Energy (thermal and/or kinetic) may be introduced to enhance powder dispersion (Jeantet *et al.*, 2010; Mimouni *et al.*, 2010a). Heating and stirring enhance dispersion by increasing the breakdown of solid bridges between particles but the energy inputs required depend on the physical structures and state of inter-particle bridges in the powder (Forny *et al.*, 2011). Increasing the stirring rate (Jeantet *et al.*, 2010) or using impeller designs that promote turbulence (Richard *et al.*, 2013) accelerates the rehydration of CN-dominant powders due to disruption of poorly-dispersible particles with surfaces rich in inter-linked CN micelles (Mimouni *et al.*, 2010b). The application of ultrasonication during the rehydration of a high-protein MPC powder was shown to improve its solubility dramatically compared to conventional mixing with an overhead stirrer at 50 °C (McCarthy *et al.*, 2013b); however, ultrasonication-induced cavitation resulted in increases in temperature to >70 °C, which, if not controlled by thermal dissipation, reduced the solubility of the MPC powder due to denaturation of WP. Chandrapala *et al.* (2014a) reported that ultrasonication, rotor-stator mixing or high-pressure homegenisation were all effective technologies for increasing the fragmentation of poorly-dispersible particles in high-protein MPC and PC powders.

Higher temperatures are commonly used to dissolve poorly-soluble powders but, in the case of dairy powders, care must be taken to avoid protein denaturation and aggregation, which could affect solubility negatively (El-Samragy *et al.*, 1993; Gianfrancesco *et al.*, 2011). Indeed, Fang *et al.* (2010) demonstrated that high temperatures could have a positive or negative effect on the rehydration of MPC powders

depending on powder composition, as well as their thermal and storage histories. Furthermore, high-protein dairy powders which develop considerable insolubility (i.e., after prolonged storage) may not undergo effective rehydration even if higher temperatures are used (Udabage *et al.*, 2012). Richard *et al.* (2013) reported that temperatures of ≥37 °C were sufficient to affect negatively the rehydration of a PC/WPI powder, a result attributed to aggregation, the opposite of effective dispersion.

An MPC produced by the ion-exchange method of Bhaskar *et al.* (2001), in which ~30 % of Ca^{2+} was replaced by Na^{+}, yielded low levels of insoluble material in the resultant MPC powder, presumably due to the removal of Ca^{2+} as an electrostatic bridging medium between CN micelles (Havea, 2006). Crowley *et al.* (2014) reported that reconstituted high-protein MPCs had a markedly higher Ca-ion activity than lower protein MPCs, due to greater loss of serum-phase minerals during UF/DF, with associated decreases in ionic strength and possibly a reduction in the influence of calcium binding salts (e.g., citrates and phosphates). Indeed, rehydration in phosphate or citrate solutions has been shown to improve the solubility of high-protein PC powders (Davenel *et al.*, 2002; Schuck *et al.*, 2002), indicating that disruption of inter-linked CN-micelles can be achieved using calcium binding agents.

The mineral environment into which a powder is added greatly impacts the rehydration process. Using milk as a medium for the rehydration of an MPC powder (85 %, w/w, protein), Udabage *et al.* (2012) reported marked increases in solubility compared to powders rehydrated in water; this effect was attributed to the higher total mineral content of milk, which may have induced greater changes to mineral equilbria and promoted the release of CN micelles from powder particle surfaces. Mao *et al.* (2012) manufactured MPCs with a diafiltration step that introduced a range of concentrations of NaCl; increasing the level of NaCl was found to increase MPC powder solubility, presumably due to ion-exchange effects and disruption of micellar integrity (Bhaskar *et al.*, 2001; Havea, 2006); in addition, β-mercaptoethanol and SDS also reduced aggregate formation, indicating that electrostatic, hydrophobic and disulphide linkages between proteins may all contribute to the poor dispersion properties of high-protein MPC powders. It has also been observed that the positive influence of rehydrating high-protein MPCs in high-ionic strength media (e.g., 80 mM KCl or milk permeate) is more pronounced at elevated temperatures (Crowley *et al.*, 2015).

Augustin *et al.* (2012) used ultrasonication (24 kHz, 600 W), two-stage homogenisation (350/100 bar) or microfluidisation (800 bar) of MPCs at ~50 °C before drying to investigate the effects of retentate pre-treatments with high shear technologies on the solubility of MPC powders (~82 %, w/w, protein in dry-matter) manufactured at pilot-scale; all high shear treatments increased powder solubility but microfluidised samples showed the most marked improvement in solubility after manufacture and after 8 months storage at ~22 °C. When microfluidisation was applied to commercial MPCs before drying into powders (~91 %, w/w, protein in dry-matter), Augustin *et al.* (2012) reported little improvement in powder solubility after manufacture compared to non-microfluidised samples; however, microfluidised samples displayed improved solubility compared to the control after both were stored for 6 months. Yanjun *et al.* (2014) pre-treated MPCs at ≤50 °C with ultrasonication (20 kHz, 600 W) before spray drying into an MPC powder (80 %, w/w, protein); these researchers reported reduced particle size and increased surface hydrophobicity in ultrasonicated samples, effects which were attributed to cavitation, turbulence and microstreaming induced by ultrasonication, with concomitant improvements in functional properties (e.g., solubility, emulsification, and gelation). Chandrapala *et al.* (2014b) ultrasonicated (20 kHz, 450 W) reconstituted MPC, WPC and CaCas powders before subjecting them to a second spray drying step; the sonicated MPC retained its solubility better over storage (60 days) than an unsonicated MPC at low or high relative humidity, with no differences observed for WPC and CaCas powders.

Gaiani *et al.* (2005) reported increased rehydration times for high-protein PC powders following agglomeration, which were correlated with reduced dispersibility values; hence, it is apparent that, where optimum solubility is required, agglomeration should be avoided during the manufacture of spray-dried CN-dominant powders, as it exacerbates their poor dispersion characteristics. Bouvier *et al.* (2013) reported that extrusion-porosification yielded MPC powders with considerably enhanced rehydration properties compared to spray-dried MPC powders due to the presence of micron-sized pores and nano-sized capillaries in the particles of the extrusion-porosified powders.

From the above studies, it is evident that dispersion is the rate-limiting stage during the rehydration of high-protein CN-dominant powders. This is due to the presence of poorly-dispersible particles with surfaces rich in inter-linked CN micelles, which can predominate because of multiple factors including an increased proximity between CN micelles due to high protein concentration factors during membrane processing (Anema *et al.*, 2006; Udabage *et al.*, 2012), changes to micellar structure caused by alterations to the concentration of minerals and mineral:protein ratios during membrane processing (Mimouni *et al.*, 2010b), increased denaturation and aggregation during evaporation and/or spray drying at high temperatures (Augustin *et al.*, 2012; Fang *et al.*, 2012), creation of additional poorly-dispersible material through agglomeration of CN-dominant powders (Gaiani *et al.*, 2005; Schuck *et al.*, 2007), and the development of MR products during prolonged storage, particularly at high temperature and/or relative humidity (Anema *et al.*, 2006; Le *et al.*, 2011a, b, 2013).

A number of strategies are available to reduce levels of poorly-dispersible material in high-protein CN-dominant powders. Introduction of monovalent ions using NaCl or KCl, either in the concentrate before drying (Bhaskar *et al.*, 2001; Mao *et al.*, 2012) or the dispersant before rehydration (Schuck *et al.*, 2002; Gaiani *et al.*, 2007; Crowley *et al.*, 2015) can improve dissociation of poorly-dispersible particles and improve solubility. In addition, Davenel *et al.* (2002) and Schuck *et al.* (2002) showed that rehydration in phosphate or citrate solutions improved the solubility of high-protein PC powders, while Udabage *et al.* (2012) demonstrated that milk had better dispersing characteristics than water for high-protein MPC powders. It should be noted, however, that strategies which involve modifying the mineral environment are often undesirable as mineral content and protein structure can be altered in the final product. Thus, physical interventions which improve powder solubility may be more appropriate in a broader range of applications, examples of which include increased stirring rate or temperature during rehydration (Jeantet *et al.*, 2010), use of turbulence-promoting impeller designs during powder rehydration (Richard *et al.*, 2013), application of ultrasonication during powder rehydration (McCarthy *et al.*, 2013b; Chandrapala *et al.*, 2014a), pre-treatment of concentrates before drying with ultrasonication (Augustin *et al.*, 2012; Chandrapala *et al.*, 2014b; Yanjun *et al.*, 2014), microfluidisation (Augustin *et al.*, 2012) or high pressure processing (Udabage *et al.*, 2012), and the use of alternative manufacturing processes to conventional spray drying, such as extrusion-porosification (Bouvier *et al.*, 2013). However, the benefits of some of these additive-free technologies may be off-set by the higher capital and running (e.g., energy) costs associated with their application.

4.2.5 Dissolution

Dissolution is the final stage of rehydration of dairy powders and coincides with the complete disappearance of granular structures (i.e., agglomerates, primary particles) and the release of constituent molecules (i.e., proteins, carbohydrates, minerals etc.) (Gaiani *et al.*, 2007) (Fig. 4.1). Purported functional properties of a powder will be affected negatively if dissolution is inhibited (Kher *et al.*, 2007). If a powder has successfully wetted, sunk, swelled and dispersed, the powder is said to have dissolved; thus, dissolution could be considered the end-point of rehydration rather than a stage in itself. Mimouni *et al.* (2010a) demonstrated that WP, monovalent

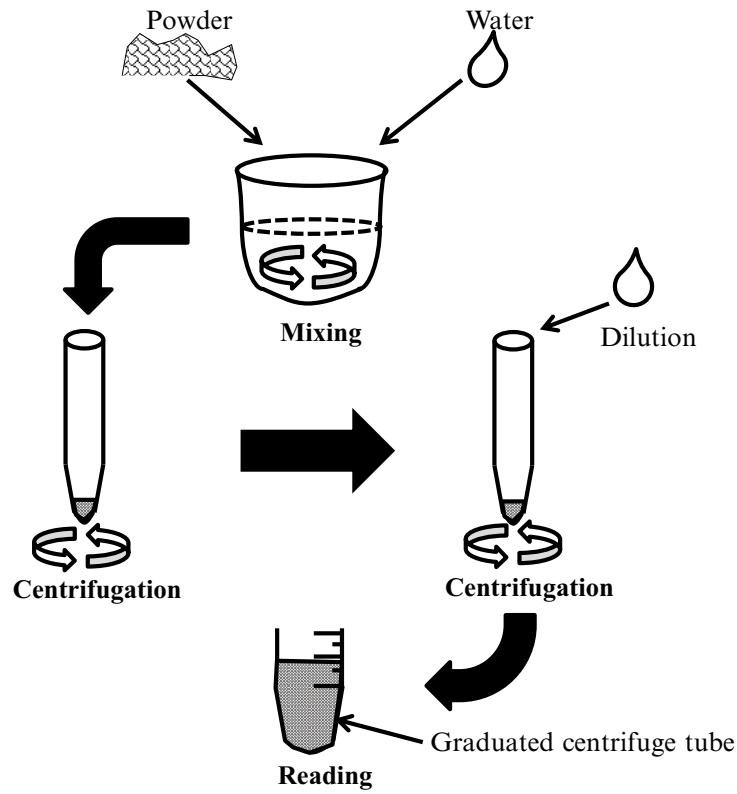

Fig. 4.5 Typical procedure used to determine the insolubility index of dairy powders (redrawn from Skanderby *et al*., 2009)

ions and lactose dissolve rapidly during rehydration and that CN micelles and associated minerals dissolve slowly. Thus, dissolution occurs throughout powder rehydration but is delayed by the poor dispersion of particles containing interlinked CN micelles and inhibited water transfer evident in high-protein CN-dominant powders.

Standard methods for the determination of powder solubility typically involve rehydration of a powder in water under controlled conditions, followed by centrifugation of the resultant mixture, and measurement of the amount of sedimentable or "insoluble" material (Kelly *et al*., 2003). Baldwin and Truong (2007) described the method outlined by the American Dry Milk Institute (ADMI, 1971) which was known as the *solubility index*. The ADMI method was updated in 1988 by the IDF, who entitled the resultant value the *insolubility index* (IDF, 1988; Písecký, 1997). The sediment can be quantified using centrifuge tubes with conically graduated bases or on a dry solids basis (Walstra *et al*., 1999; Skanderby *et al*., 2009). A typical experimental procedure for the measurement of insolubility index is outlined in Fig. 4.5. The insolubility index (ISI, in %) described by the IDF standard (IDF, 1988; Schuck *et al*., 2012) for skim milk is the volume of sediment (for 50 mL) after rehydration (10 g of powder in 100 mL of distilled water, at 25 °C), mixing (at 4000 rev min^{-1} for 90 s) and centrifugation (for 300 s at $160 \times g$). With this method, the quantity of insoluble material can be determined. However, it must be noted that the presence of sedimentable material may not be due to true insolubility, as in the case of denatured protein, but may instead be due to low water transfer during rehydration (Schuck *et al*., 2002).

Once granulate particles have disappeared after dispersion, rehydration is said to be complete; subsequently, numerous intrinsic and extrinsic factors influence the extent to which water becomes associated with proteins, with the extent of these protein-water interactions

influencing the functional properties of the dried ingredient (Kinsella and Fox, 1987). de la Fuente and Alais (1975) reported that solvation of CN micelles in milk decreased with acidification and increased with alkalisation. The authors also reported increasing solvation with heating (72–90 °C). Conversely, Snoeren *et al.* (1984) observed reduced micellar solvation with increasing temperature between 4 and 30 °C. The presence of charged and polar groups plays a central role in protein-water interactions (Hardy *et al.*, 2002). The extent to which bulk solvent can access these groups without steric hindrance is also important (Kinsella and Fox, 1987). The poor rehydration properties of high-moisture powders have been linked with depletion of potential hydrogen-bonding sites due to the presence of bound water (Marabi *et al.*, 2007; Syll *et al.*, 2012).

Heat-induced denaturation and aggregation of WP has been shown to have a negative effect on the solubility of skim milk retentate powders (El-Samragy *et al.*, 1993). However, the presence of denatured WP can have a positive impact on the functional properties of powders, relating to water-holding, viscosity and foaming (Henning *et al.*, 2006). Calcium binding agents are used during the rehydration of rennet CN powders to disrupt protein-protein cross-links and improve solvent access to proteins (Ennis *et al.*, 1998; Ennis and Mulvihill, 1999a, b, 2001). Addition of phosphates and citrates has been shown to increase protein-water interactions and reduce rehydration times for high-protein PC powders (Davenel *et al.*, 2002; Schuck *et al.*, 2002).

Shearing conditions may influence protein hydration positively by disrupting particle structure and making proteins more accessible to solvent, and/or by exposing hydrophilic groups and increasing the affinity of solvent for the proteins; however, shearing has potentially negative consequences if hydrophobic groups become exposed, with concomitant loss of solubility and/or aggregation (Ennis *et al.*, 1998). Ultrasonication of WPI or WP hydrolysate solutions (10 %, w/w, protein) caused an increase in conductivity, which was likely due to exposure of charged groups from within the globular structure of WPs, which resulted in greater solvent access to WPs with a concomitant improvement in solubility (Jambrak *et al.*, 2008); however, ultrasonication had little effect on the solubility of WPC solutions due to their higher content of lactose, which, it was proposed, had a protective effect against structural changes to proteins induced by ultrasonication.

de la Fuente and Alais (1975) reported decreased solvation of CN micelles on addition of $CaCl_2$, which was attributed to the associated pH decrease and clustering of the CN micelles; conversely, the researchers reported increased solvation of CN micelles, coinciding with increased pH, in calcium oxide-supplemented samples. Snoeren *et al.* (1984) also reported reduced solvation of CN micelles on supplementation of milk with $CaCl_2$. Exchange of H^+ for Ca^{2+} at the hydrophobic core of CN micelles has been linked with decreased micellar solvation on addition of $CaCl_2$ (Canabady-Rochelle *et al.*, 2009). Conversely, the introduction of Na^+ has been shown to increase micellar solvation (Le Ray *et al.*, 1998), although the addition of NaCl has a less pronounced effect on protein-water interactions than $CaCl_2$ (Davenel *et al.*, 2002; Schuck *et al.*, 2002), due to the ability of divalent cations to contribute to cross bridging, as well as screening of charge (Hussain *et al.*, 2012).

The rehydration profile of NaCas powder, monitored by turbidimetry (see Sect. 3.1.2), has been shown to be more similar to that of WPI powder than PC powder (Gaiani *et al.*, 2009a), presumably due to the absence of micellar structures in both NaCas and WPI powders. Indeed, protein-water interactions tend to be more pronounced for random-coil (e.g., CN) than globular (e.g., WP) proteins (Kinsella and Fox, 1987; Hussain *et al.*, 2011b). Hussain *et al.* (2011b) reported that higher quantities of NaCl than $CaCl_2$ were required to induce changes in the rehydration profiles of both PC and WPI powders. Structure loss in CN micelles due to Na^+/Ca^{2+} exchange has been linked with improved water transfer in PC powders (Hussain *et al.*, 2011a). Moreover, the rehydration properties of PC powders are affected differently by added salts, depending on the powder production mode

and the co-localisation of both protein and salts in the particle structure: Schuck *et al*. (2002) and Gaiani *et al*. (2007) showed that co-drying of PC and NaCl (i.e., mixing salt into the concentrate prior to spray drying) significantly improved solubility, while bi-drying (simultaneous drying of PC and NaCl through different nozzles) and dry-mixing (mixing of PC and NaCl powders) resulted in no or limited improvement.

4.3 Monitoring Powder Rehydration

Methods available to monitor the rehydration of powders can be divided into those which (1) discriminate between multiple rehydration stages (Sect. 3.1) and (2) solely measure total rehydration time and/or yield information on specific phenomena related to powder rehydration, e.g., mineral release, heat release, water penetration (Sect. 3.2). These differences in rehydration monitoring capability are illustrated in Fig. 4.6, along with information pertaining to any in-line capabilities for industrial powder rehydration processes. In addition, schematic representations of data outputs for high-protein CN-dominant powders using these techniques are shown in Fig. 4.7.

4.3.1 Multi-Stage Characterisation of Powder Rehydration

4.3.1.1 Rheology-Based Approaches

Rheology has been used to monitor dairy powder rehydration dynamically *in situ* and to identify individual stages of rehydration based on changes in viscosity. Ennis *et al*. (1998) monitored the rehydration of rennet CN powders in solutions of disodium orthophosphate, a calcium binding agent, at 55 °C under constant shear conditions (600 rpm). Peaks in the viscosity index were related to changes in particle size, inter-particle interactions and protein-water interactions, with specific stages in the rehydration process being identified by the researchers: (a) an initial increase in viscosity associated with wetting and swelling of particles, (b) a minor viscosity increase due to inter-particle interactions with the creation of small clusters, (c) network formation due to particle clumping and swelling, leading to a further viscosity increase, (d) a maximum viscosity index value related to absorption of solvent by particles, and (e) progressive viscosity decreases with shear, culminating in (f) a steady low viscosity reading indicative of complete rehydration.

Ennis *et al*. (1998) reported that concentrations of disodium orthophosphate up to 0.5 %,

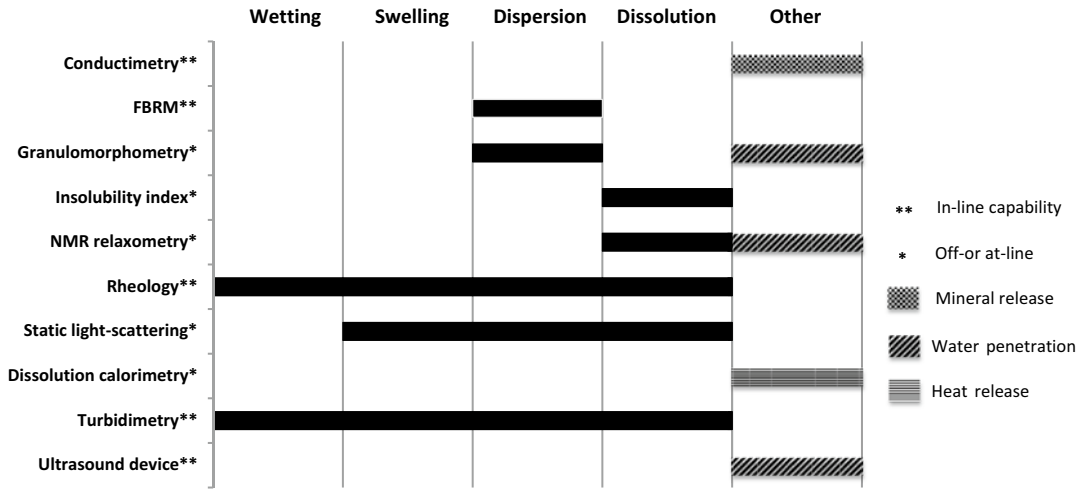

Fig. 4.6 Quantifiable rehydration stages, other data outputs and in- or off-line nature of methods for measuring the rehydration of dairy powders. *FBRM* focused beam reflectance measurement, *NMR* nuclear magnetic resonance

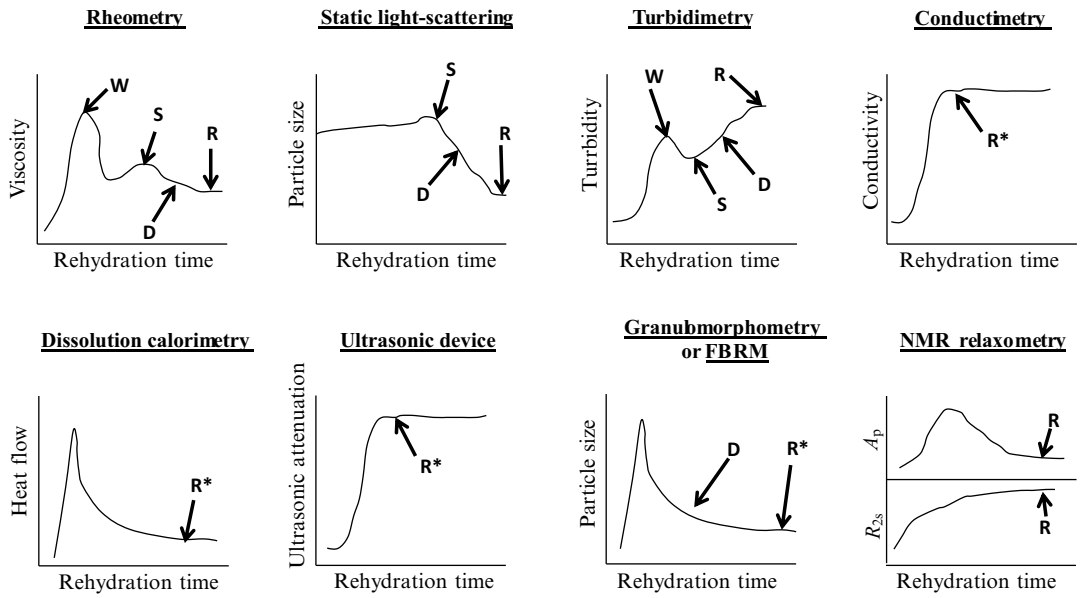

Fig. 4.7 Summary of typical rehydration profiles obtained for dairy powders using different measurement techniques. *W* wetting, *S* swelling, *D* dispersion, *R* time required for total rehydration, *FBRM* focused beam reflectance measurement, *NMR* nuclear magnetic resonance, *R** rehydration time may not indicate complete rehydration of powder in all cases

w/w, disrupted Ca^{2+}-mediated inter-particle cross bridging sufficiently to increase protein hydration, with a concomitant increase in the viscosity index; the authors also reported an increase in both the time required for particles to swell and for maximum viscosity index to be reached. At intermediate addition levels (0.35–0.7 %, w/w, disodium orthophosphate), viscosity decreased on continuing shear after reaching the maximum value, a phenomenon attributed to inhibited protein mobility, with resultant loss of water-holding capacity and reduced inter-protein interactions.

Ennis and Mulvihill (1999a) reported that monitoring of rehydration using viscometry was useful as a predictor of the performance of rennet CN powder during pilot-scale analogue cheese manufacture. After measuring rehydration using the viscometer method and furosine levels by HPLC, Ennis and Mulvihill (1999b) reported that differences in rehydration profiles of rennet CN powders were due, at least in part, to differences in levels of MR products in the rennet CN powders. Ennis and Mulvihill (2001) also observed reduced maximum viscosity index and time taken to reach maximum viscosity index in rennet CN powders manufactured from early- and late-lactation milk, which suggested that milk production season can influence the rehydration properties of rennet CN powders.

Gaiani *et al.* (2006b) used a rheometer to study the rehydration of a high-protein PC powder in water under constant shear rate (100 s^{-1}) at different concentrations, temperatures and rehydration times. This approach, when combined with particle size analysis, allowed the identification and quantification of the individual stages of the rehydration process (Fig. 4.7). Gaiani *et al.* (2006b) reported reduced swelling and rehydration times with increasing temperature from 5 to 45 °C. In efforts to improve rehydration properties, enhancement of water transfer into powder particles is thought to be crucial (Murrieta-Pazos *et al.*, 2011) and increased temperatures during rehydration are known to achieve this (Davenel *et al.*, 1997). The results of Gaiani *et al.* (2006b) suggested that the positive effect of high temperatures (40–50 °C) on the rehydration of CN-dominant powders may be linked to a reduced duration of the swelling stage.

Both Ennis *et al*. (1998) and Gaiani *et al*. (2006b) reported using "custom-built" paddle geometries for their viscometer- and rheometer-based studies, respectively. Ennis *et al*. (1998) used a paddle stirrer constructed from two flat stainless steel plates, both perforated twice, which were arranged at 90° to each other and positioned vertically in series along the rotating shaft, while the geometry used by Gaiani *et al*. (2006b) comprised four unperforated blades, also arranged at right angles relative to each other. The most noticeable geometrical variation between the paddle stirrers used by the two research groups was the presence or absence of plate perforations. The presence of narrow orifices in the design adopted by Ennis *et al*. (1998) would have increased mechanical agitation through the promotion of more turbulent mixing and thereby aided the dispersion of aggregated material; conversely, the configuration of Gaiani *et al*. (2006b), operated in the laminar regime, would have had a reduced energy input due to its lack of plate perforations. Indeed, Richard *et al*. (2013) demonstrated, using dynamic image analysis, that differences in impeller design can influence particle fragmentation and powder rehydration in the turbulent regime, which the authors attributed to associated variations in energy dissipation, suction phenomena and particle circulation.

The studies by Ennis *et al*. (1998) and Gaiani *et al*. (2006b) involved the analysis of different sample materials, thus negating any potential assessment of the influence of paddle geometry, stirring rate and flow regime; however, in future studies, with common sample bases, the effect of these factors on rehydration profiles should be taken into consideration. Indeed, it is often favourable to operate in the turbulent regime due to increased energy dissipation and promotion of powder rehydration. Thus, increased focus should be placed on the rehydration performance of dairy powders during turbulent flow, as it is probably of more direct relevance to industrial mixing operations, where shear rates in the range 10–500 s^{-1} and 10^2–10^4 s^{-1} are common during mixing and dispersion processes, respectively. According to Schuck *et al*. (2007), constant stirring rates are commonly used in industrial processes. Despite this, Gaiani *et al*. (2009a) considered that the constant speed stirring used in the studies of Ennis *et al*. (1998) and Gaiani *et al*. (2006b) deviated considerably from industrial stirring rates; if this is so, then adjusting the rate of shear to replicate these stirring rates more closely would seem a logical progression. It is also important to note that as Ennis *et al*. (1998) measured the rehydration of a CN-dominant powder in a solution of a calcium binding agent, which would cause a certain degree of *para*-caseinate dissociation with concomitant thickening, it is difficult to compare their results with those of Gaiani *et al*. (2006b); in addition, it is unclear if the viscometer method would be as sensitive to the comparatively lower viscosity changes measured by the latter researchers using rheometry for PC powders dispersed in water.

Rheological devices are available for direct in-line process stream analysis (Cullen *et al*., 2000) (Fig. 4.6), making rheology an industrially relevant technique for monitoring dairy powder rehydration without requiring sampling for off-line analysis. In addition, indirect methods of monitoring viscosity based on following the powder consumption (amperage) of the stirrer could be useful for in-line measurements of powder rehydration during processing; indeed, Schuck *et al*. (2005) found a strong correlation between the amperage of a concentrate vacuum pump and the viscosity of dairy concentrates.

4.3.1.2 Turbidimetry

The optical phenomenon of turbidity may be visualised as a haze or cloudiness in an otherwise transparent sample. Herri *et al*. (1999) outlined the physical principle of turbidity as follows: on passage through a fluid medium, a light beam of defined wavelength (λ) may encounter suspended particles with concomitant scattering of the incident beam. The resultant global extinction phenomenon yields an extinction coefficient of light-scattering, defined as turbidity (τ_λ) (see 4.2):

$$\tau_\lambda = \frac{\frac{1}{L}\log I_0}{I_L} \tag{4.2}$$

where I_0 equates to the intensity of the incident beam and I_L represents the intensity of the transmitted beam following passage through an optical path of length L. Thus, as the intensity of the transmitted beam decreases, turbidity is observed to increase.

Utilising light in the near-infrared region (860 nm), turbidity probes direct an incident beam into a sample of interest; any suspended particles which pass the incident beam reflect it back at a 180° angle into an electronic receptor located within the instrument. Data is reported in nephelometric turbidity units as a function of time, allowing continuous monitoring of dynamic processes such as dairy powder rehydration (Gaiani *et al*., 2005, 2009a).

Using turbidimetry and particle size analysis, Gaiani *et al*. (2005) studied the rehydration properties of both agglomerated and non-agglomerated high-protein PC powders, along with PC/ultrafiltrate powders. Four stages of rehydration for PC powder were observed: wetting, swelling, dispersion, and dissolution (Figs. 4.6, 4.7). Agglomeration had a negative effect on the rehydration properties of PC powders, which was correlated with reduced dispersibility values in agglomerated powders. It was also demonstrated that mixing with ultrafiltrate by co-drying yielded rehydration times 14 and 20 times faster for agglomerated and non-agglomerated PC powders, respectively, when compared to PC powders which were not co-dried with ultrafiltrate. Gaiani *et al*. (2007) demonstrated that the rate-limiting stage of PC powder rehydration was dispersion, that agglomeration had a positive impact on the rehydration of WP-dominant powders, and that adding WP to CN-dominant powders before spray drying can improve their rehydration properties.

Gaiani *et al*. (2009a) also used turbidimetry in a study of the rehydration of high-protein PC, NaCas, and WPI powders. During rehydration, turbidity increased steadily for PC powder but remained relatively low for WPI powder; as NaCas displayed a similar rehydration profile to WPI, the lack of micellar structures in both powders presumably caused reduced light-scattering effects. PC powders underwent rapid wetting compared to WPI powders; however, the total rehydration time for PC was 48,000 s compared to 239 s for WPI, due to a protracted dispersion stage for the former. A swelling stage was not observed for WPI, possibly due to a noisy turbidity signal. Hussain *et al*. (2011b) attributed the noisy turbidity signal of WPI powders during rehydration to the formation of lumps. Hussain *et al*. (2011b) speculated that reduced water binding by globular WPs, compared to random-coil CNs, explained the lack of a swelling stage during rehydration of the WPI powders.

Hussain *et al*. (2011b) reported that rehydration in NaCl solutions (0.75–3 %, w/v) instead of water delayed the stabilisation of turbidity for a high-protein PC powder; conversely, higher NaCl concentrations (6–12 %, w/v) eliminated the swelling stage and caused more rapid stabilisation of turbidity. In agreement with Gaiani *et al*. (2009a), these authors observed no distinct swelling stage, a longer wetting time and rapid turbidity stabilisation for a WPI powder rehydrated in water compared to a PC powder. Rehydration in 9–12 %, w/v, NaCl or 2.25–12 %, w/v, $CaCl_2$ solutions caused marked elongation of total rehydration time for WPI powder.

Turbidimetry is a useful tool to characterise dairy powder rehydration *in-situ*. In combination with particle size analysis, it gives particularly detailed information about the rehydration of CN-dominant powders (Gaiani *et al*., 2005; Schuck *et al*., 2007). Available as probes which can be inserted directly into a sample or process stream, turbidimetry has good potential as an in-line method for process analysis (Fig. 4.6). In addition, it is a versatile technique, which has been used to study both CN- and WP-dominant powders, rehydrated in water (Gaiani *et al*., 2009a) and in variable ionic environments (Hussain *et al*., 2011a, b).

4.3.1.3 Static Light-Scattering

Techniques based on light-scattering are commonly used to determine particle size and particle size distributions in both wet and dry samples. Scattering is a composite of light-matter interactions (reflection, refraction, diffraction) and as such is not a singular phenomenon (Webb, 2000).

Extinction of incident light is associated with the combined effects of both light scattering and absorption (Mori, 2007). Static light-scattering (SLS), also known as laser diffractometry or small-angle SLS, is an analytical technique which estimates particle size based on the specific angle at which particles scatter light; put simply, small particles scatter at large angles and large particles scatter at small angles (Keck and Müller, 2008; Mimouni *et al*., 2009).

In SLS, patterns of scattering are derived from intensity characteristics of the entire population of particles, with a particle size distribution and a mean particle diameter being determined (Moughal *et al.,* 2000; Gaiani *et al*., 2006b). The scattering pattern depends on the ratio between the particle size and the wavelength of incident light; thus, if the value for either parameter is kept constant whilst the other is modified, the resultant scattering pattern will be altered. Depending on the ratio between particle size and incident wavelength, Fraunhofer, Rayleigh or Mie scattering patterns may be observed (Keck and Müller, 2008). Fraunhofer patterns occur in systems wherein the particle size is much greater than the incident wavelength, whilst the inverse is true for Rayleigh scattering; Mie scattering occupies the intermediate region between these two patterns (Mori, 2007).

Particle size data from SLS instruments are typically derived from calculations based on Mie theory (also known as Lorenz-Mie theory; see Mie, 1908), which requires that some theoretical assumptions are made regarding the particles themselves (Webb, 2000; Mori, 2007; Keck and Müller, 2008). In essence, Mie theory considers the scattering and absorption of an incident beam of monochromatic light composed of plane waves by spherical particles which are isotropic in nature (Webb, 2000). As such, the data output of SLS relates to the equivalent spherical diameter of particles (Moughal *et al.*, 2000).

Moughal *et al*. (2000) used SLS technology to study particle size changes during the dissolution of calcium caseinate (CaCas) powders. Following an initial 180 min period of rehydration in water, the sample was introduced to the dispersing unit of the SLS system and analysed for changes in volume distribution and obscuration over 100 min. During analysis, the peak representing particles of 0.1–2.0 μm in size was observed to increase as the 2–80 μm peak decreased; this coincided with a steady decrease in obscuration (optical concentration) values, indicating the dissolution of particles which would otherwise contribute to light-scattering phenomena. The authors reported that rehydration times of up to 6 h were required for some CaCas powders to become rehydrated and that, as such, standard tests using rehydration times of ~1 h were inadequate.

In a study on the rehydration of high-protein PC powder, Gaiani *et al*. (2005) also used SLS technology, observing that, as rehydration time elapsed, particle size initially increased, before a substantial reduction in particle size, followed by a continuous reading for small particles (Fig. 4.7). Mimouni *et al*. (2009) studied the rehydration of a high-protein MPC powder at different temperatures using SLS. Aliquots (4 mL) of solution were taken and analysed for the change in size and volume concentration of particles. The rate at which the relative volume concentration of agglomerated particles decreased was more rapid than that of primary particles. The latter was thus identified as the rate-limiting step during the rehydration of MPC powder; the researchers found that the duration of this stage was reduced on increasing temperature (24–35 °C). Based on their results, Mimouni *et al*. (2009) developed a model for the rehydration of an agglomerated high-protein MPC powder which encompassed disruption of CN agglomerates, dispersion of primary particles, hydration of air vacuoles and erosion of the proteinaceous outer surface skin of particles, with concomitant release of CN micelles and associated minerals into solution.

Harper *et al*. (1963) hypothesised that high stirring rates increased powder solubilisation by reducing the concentration of solids in regions where particles are dissolving; to investigate this, the authors increased the spatial distance between powder particles by mixing them with sand before rehydration and found that minimising the

local concentration of solids around dissolving particles improved solubility. Indeed, mechanical agitation is commonly used to increase the rate of rehydration (Schober and Fitzpatrick, 2005; Jeantet *et al*., 2010). Jeantet *et al*. (2010) monitored the rehydration of a high-protein PC powder under different hydrodynamic conditions based on changes in particle size over time, as derived from SLS measurements. Increasing temperature from 26–30 °C had the same impact as a two-fold increase in stirring rate. The number of impeller rotations required for complete rehydration increased at higher solids content, but was independent of stirring rate. Thus, it was proposed that strategies to improve PC rehydration should proceed in order of most to least effective, i.e., temperature > stirring rate > solids content. Richard *et al*. (2013) showed that these findings could be extended to a range of other dairy powders (PC, mixes of PC and WPI, mixes of PC and lactose) and impeller designs.

SLS has been used to validate and supplement data from more recently developed methods of measuring dairy powder rehydration. Gaiani *et al*. (2005, 2006b) used SLS as a reference method for identifying different rehydration stages when studying the rehydration of a high-protein PC powder with turbidimetry and rheometry, respectively (see Sects. 3.1.1 and 3.1.2). However, SLS does not provide data pertaining to powder wetting (Fig. 4.6), which is a disadvantage when studying powders where it is the rate-limiting stage, such as WP-dominant powders (Gaiani *et al*., 2007; Schuck *et al*., 2007; Hussain *et al*., 2011b). In addition, it has also been noted that the SLS methods reported in the literature involved off-line sampling and dilution of samples prior to analysis (Fang *et al*., 2010), calling into question the extent to which the SLS-derived data represents the true nature of the powder rehydration process. It should be noted, however, that in-line SLS technology has recently been developed. If such technology can be verified for use in the monitoring of powder rehydration processes, the aforementioned issues associated with SLS measurements in these systems could be resolved.

4.3.1.4 Image Analysis

Gaiani *et al*. (2009a) used a phase contrast microscope with video camera attachment to study the rehydration of a high-protein PC powder; the microscopy images clearly displayed swelling of a PC powder particle from approximately 250–400 μm, with dispersion and disintegration of the particle as time elapsed. Mimouni *et al*. (2009) used a light microscope with video camera attachment to study morphological changes to MPC powder particles after exposure to a drop of water; this allowed the identification of air vacuoles inside primary particles (also observed as indentations using scanning electron microscopy) caused by spray drying. The authors hypothesised that erosion of the surrounding proteinaceous skin of these vacuoles would be required for structural collapse and concomitant solubilisation. Also using light microscopy, Fang *et al*. (2010) captured images of a poorly-soluble MPC powder, as well as a highly-soluble MPC powder, after rehydration; the images showed that larger particles persist in the former. Scanning electron microscopy images of an MPC powder after rehydration seemed to show that particles in a poorly-soluble MPC powder, which exhibited a surface skin consisting primarily of inter-linked CN micelles, was porous enough to imbibe water readily but that release of CN micelles was restricted (Mimouni *et al*., 2010b).

A granulomorphometer was used by Richard *et al*. (2013) to capture images of dairy powders during rehydration, and to measure particle counts, size distributions, inner diameter and mean particle diameter of powder particles during dissolution (Fig. 4.8). As the method required dilution of samples prior to analysis, it was not sensitive to the early stages of powder rehydration; in addition, due to limitations in the optics of the instrument, it did not allow a value for total rehydration time to be obtained (Fig. 4.6); however, the technique was applied successfully to visualise the impact of different impeller designs on fragmentation of particles and imbibing of liquid into particles. In addition, Richard *et al*. (2013) used granulomorphometry to investigate the interaction of water or ethanol with powder

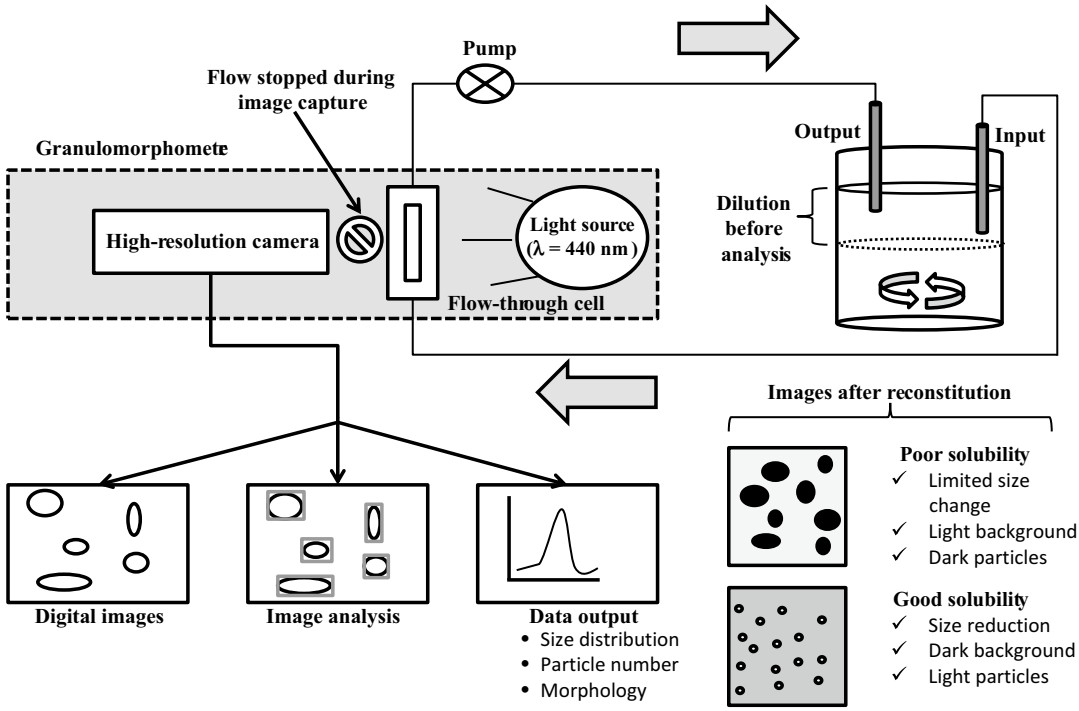

Fig. 4.8 Schematic representation of the principle of operation of the granulomorphometer (redrawn from Richard *et al.*, 2013)

particles; lightening of particle cores and darkening of image background were observed with water as the dispersant but not with ethanol; thus, these effects were reported as being due to water penetration into air vacuoles within primary particles and the advanced stages of powder dissolution, respectively.

As the above examples demonstrate, visual assessment of powder particles in solubilisation studies are commonly performed using off-line microscopy; these methods, however, require intensive sample preparation, are time-consuming, and are prone to yielding results with a high degree of error, as discussed by Gaiani *et al.* (2011). Thus, it is beneficial to use methods, such as granulomorphometry, which can monitor the rehydration of powders dynamically. However, there are a number of inherent limitations to granulomorphometry, as applied to studies into powder rehydration, namely, its requirement for sample dilution, its poor discrimination of fine particles and its off-line nature.

4.3.2 Single-Stage Characterisation of Powder Rehydration

4.3.2.1 Focused Beam Reflectance Measurement

Techniques based on laser light back-scattering, such as focused beam reflectance measurement (FBRM), are used in the analysis of particle size, particularly in the study of active pharmaceutical ingredients, where it is used to track dynamic phenomena such as crystallisation (Kail *et al.*, 2009).

The instrument (Fig. 4.9) houses its optics in a probe which can be immersed into a reaction vessel or process stream of interest. From within the probe, a laser ($\lambda = 780$ nm) is directed through an optics module, which focuses the beam at a narrow point near a sapphire window at the probe's base (Fang *et al.*, 2010). In a unique configuration, controlled speed rotation (2 m s^{-1}) of the optics directs the laser away from the middle axis of the probe, causing the beam to scan passing

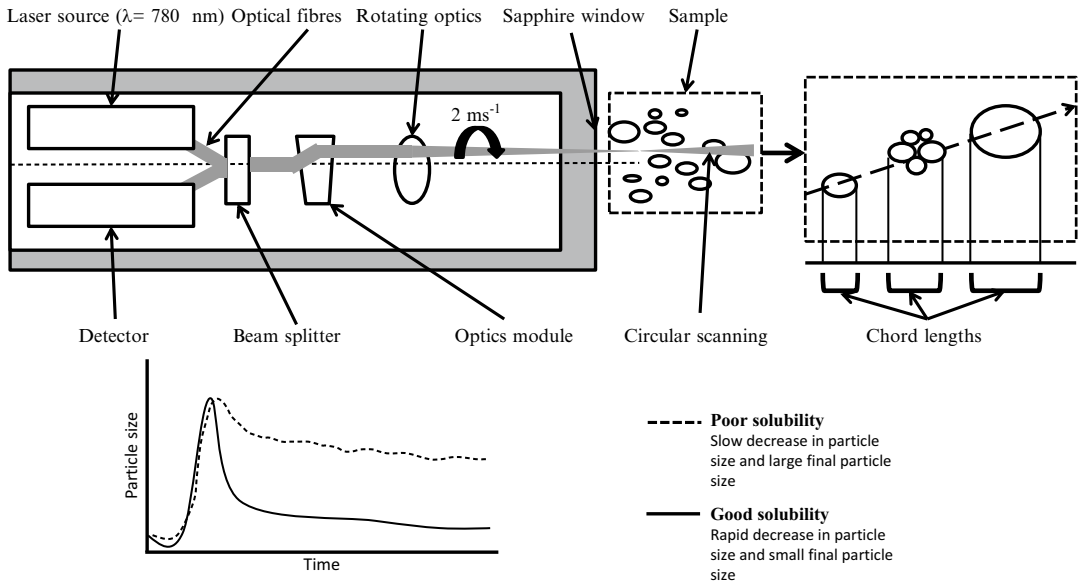

Fig. 4.9 Schematic representation of the principle of focused beam reflectance measurement (FBRM) (redrawn from Fang *et al.*, 2010)

particles in a circular manner (Dowding *et al.*, 2001). The rotating mechanism increases the spatial expanse covered by the laser and provides a more accurate representation of the particle size distribution (Singh, 2009). During analysis, laser light is first back-scattered (scattering angle of >90°) from particles; this light is then coupled by a beam splitter to an optical fiber and finally conducted onto a detector (Kail *et al.*, 2009). The duration of the resultant pulse depends on the time taken to scan completely a particle from one edge to another, which is computed by a special discrimination circuit (Dowding *et al.*, 2001). The pulse duration value is multiplied by the scan speed to yield the chord length. Thousands of chord lengths are typically recorded, which results in a chord length distribution, which can be re-calculated as either a number- or volume-weighted particle size distribution (Dowding *et al.*, 2001; Fang *et al.*, 2011). A chord length can be defined as a straight line stretching between two edges at opposite sides of a particle; in this sense, aggregates and agglomerates are analysed as single particles (Fig. 4.9).

Fang *et al.* (2010) used FBRM in their analysis of the rehydration properties of six MPC powders with varying protein contents, thermal and storage histories. For the six powders tested, a variable and rapid increase in chord length, coinciding with a low particle count, was attributed to wetting and sinking of particles. During rehydration, some MPC powders displayed reduced solubility with increasing temperature and others increased solubility with increasing temperature; these opposing effects were attributed to the differing compositions as well as storage and thermal histories of the samples. Thus, the most appropriate temperature for powder rehydration may be influenced considerably by the extent of storage- and process-induced changes.

Using FBRM, Fang *et al.* (2011) observed that the rehydration of agglomerated MPC powders was affected negatively by long storage durations (2 months compared to 2 weeks). Chord length decreased more rapidly for samples rehydrated at 50 °C (equilibrium particle size reading after 200 s) compared to 20 °C (equilibrium particle size reading after 30 min). The authors proposed a model of the rehydration of agglomerated MPC powders based on two stages: (1) dispersion of agglomerates (initial dissolution) and (2) dispersion of primary particles (equilibrium

dissolution); of course, the application of this model is restricted to agglomerated powders. Counts for different particle populations by FBRM showed that the number of large particles (150–300 μm) decreased rapidly over a period of ~300 s, while the number of small particles (1–10 μm) increased consistently during the 1800 s of rehydration. These data suggested that stages (1) and (2) initially occur in parallel but, as the former concludes, the latter continues for a protracted period; as such, stage (2) was considered to be the rate-limiting stage of rehydration for the agglomerated MPC powders studied.

Fang *et al.* (2012) rehydrated MPC powders and spray-dried them again at a range of inlet temperatures (77–178 °C), before analysing them for rehydration performance using FBRM. MPC powders subjected to a second spray drying step at low inlet temperatures (77 or 107 °C) maintained the same level of solubility as the original powder (made with one spray drying step), with both plateauing at a particle size of ~80 μm after 200 s. This effect was not observed for MPC powders dried at higher inlet temperatures (155 or 178 °C), which did not fully disperse after 30 min. The comparatively slow decrease in particle size for the control (one drying step), was attributed to the presence of greater amounts of agglomerated material, which necessitated additional dispersion; indeed, the presence of agglomerated material has been shown to affect negatively the rehydration of CN-dominant powders (Schuck *et al.*, 2007).

FBRM is a technique which allows the monitoring of dynamic processes *in situ* without the need for sample preparation or dilution (Dowding *et al.*, 2001) and it has been demonstrated to be a useful technique for studying the rehydration of dairy powders, particularly the dispersion stage of agglomerated CN-dominant powders (Fang *et al.*, 2011; see Figs. 4.6, 4.7). As such, FBRM has significant potential for use in the study of dairy powder rehydration in-line in a processing environment. However, in FBRM studies on the rehydration of dairy powders, powders were considered fully rehydrated when relatively large particle sizes (~50–100 μm) were still being measured; although particles of this size can remain in suspension in MPCs after prolonged rehydration (Mimouni *et al.*, 2009; Chandrapala *et al.*, 2014a; Crowley *et al.*, 2015), there is a question as to the sensitivity of FBRM to smaller particle sizes (e.g., CN micelles) and the time taken to reach total rehydration of powders (Fig. 4.7). In addition, the technology has yet to be evaluated for its suitability in the analysis of WP-dominant powders, or, indeed, dairy powders which are known to exhibit good rehydration properties.

4.3.2.2 Nuclear Magnetic Resonance Relaxometry

A nuclear magnetic resonance (NMR) relaxometry method, designed by Davenel *et al.* (1997), can be used to study dairy powder rehydration and has been shown to be highly sensitive to protein-water interactions. Davenel *et al.* (2002) and Schuck *et al.* (2002) both used a Minispec Bruker PC 10 NMR spectrometer (Bruker, Wissembourg, France) operated at a 10 MHz resonance frequency. During rehydration, decay curves were retrieved by recording a maximum of 845 spin echoes every 20 s from a Carr-Purcell-Meiboom-Gill (CPMG) sequence. Two exponential curves were summed in order to attain the CPMG curve, yielding the following equation:

$$S(t) = A_p \exp\left(-t.R_{2p}\right) + A_s \exp\left(-t.R_{2s}\right) \quad (4.3)$$

A_p and R_{2p} comprised the fast decay component, representing the amount of protons and relaxation rate, respectively, derived from exchangeable protons and water protons in the non-rehydrated solution. A_s and R_{2s} comprised the slow decay component, representing the amount of protons and relaxation rate, respectively, derived from exchangeable protons and water protons in the fully rehydrated solution. The relaxation rate value attributed to the slow component, R_{2s}, increased steadily as mixing progressed, whilst the number of protons associated with the fast component, A_p, decreased steadily as rehydration time elapsed; complete rehydration was observed as a constant reading for R_{2s} and a reduction to zero of protons associated with the fast component, A_p (Fig. 4.7).

Using the NMR method, Schuck *et al*. (2002), monitored the rehydration of a high-protein PC powder with minerals incorporated through different technological approaches (co-drying, bi-drying, dry-mixing; see Sect. 2.5), and observed absorption of water by particles (increased A_p) and solubilisation of particles (evolution of R_{2s}), in agreement with Davenel *et al*. (2002) (Fig. 4.7); thus, the NMR method was particularly sensitive to protein-water interactions associated with the advanced stages of rehydration (Fig. 4.6). The authors concluded that no loss of CN micelle integrity occurred when mineral salts were added by dry-mixing or bi-drying, but that structural alteration may occur if minerals are co-dried with PC. NaCl (for all methods of incorporation) elevated the concentration of free Ca^{2+} ions by 8 % compared with the control, reducing both the insolubility index and rehydration time for the PC, with little change in R_{2s} values. Davenel *et al*. (2002) also reported reduced rehydration times for PC, with no apparent change in R_{2s}, when NaCl was added before drying. These results indicated that minimal alterations to micellar properties occurred with NaCl addition and that improved rehydration was probably due to the innate hygroscopicity of the salt.

Addition of $CaCl_2$ resulted in a more pronounced impact on micellar integrity, with a large decrease in R_{2s} due to inhibited water transfer as a result of protein precipitation, with concomitant increase in insolubility and time to rehydration (Davenel *et al*., 2002; Schuck *et al*., 2002). Two calcium binding agents, sodium phosphate and citrate, were also tested by Schuck *et al*. (2002), with the greater reduction in R_{2s} values and rehydration times induced by the latter suggested a more marked effect on micellar integrity. Davenel *et al*. (2002) also observed greater reductions in rehydration time and relaxation rates for high-protein PC powders with the addition of citrates compared to phosphates. PC powder which had been freeze-dried showed delayed rehydration times compared to spray-dried PC powder; in addition, enrichment with WP or carbohydrate before drying improved rehydration of PC powder, as monitored by NMR relaxometry (Davenel *et al*., 2002). This was in agreement with the results of Mimouni *et al*. (2010a), who demonstrated that WP and carbohydrates readily dissolve during the rehydration of a poorly-soluble MPC powder.

NMR relaxometry is a highly sensitive method for studying changes to protein structure, relaxation rates and proton populations, making it a valuable tool for dairy powder rehydration studies (Schuck *et al*., 2007). Gaiani *et al*. (2009a) noted that, although the NMR method facilitated the study of certain aspects of powder rehydration, it did not allow characterisation of the wetting stage; moreover, it has little potential as an in-line method of analysis. However, the NMR technique facilitated the determination of rehydration rate and rehydration time; incomplete rehydration, due to the presence of insoluble material, could also be detected using the NMR method. Thus, NMR relaxometry is a highly sensitive method for studying the more advanced stages of powder rehydration (Fig. 4.6).

4.3.2.3 Thermochemistry

Marabi *et al*. (2007) used dissolution calorimetry to monitor thermodynamically the dissolution of SMP and maltodextrin powder (MDP). The microcalorimeter used (Calvet calorimeter, C80 Setaram, Caluire, France) compartmentalised the powder and the liquid prior to mixing. Following thermal equilibration, the powder was brought into contact with the dispersing medium to initiate wetting. Exothermic (negative enthalpy) and endothermic (positive enthalpy) responses during powder rehydration were then measured against a reference sample (water with no powder). High moisture contents and the presence of crystallised lactose were observed to yield reduced exothermic responses, which was in agreement with the negative effects of these compounds on powder solubility observed using image analysis. It was speculated that high-moisture powders had fewer hydrogen bonding sites due to the presence of bound water, and that the dissolution of crystallised material may have produced a positive enthalpic response which could have dampened the overall exothermic effect. Syll *et al*. (2012) reported an approximately linear correlation

between the water activity of powders and dissolution enthalpy values; this reduced exothermic response was again speculated to be a result of decreased availability of hydrogen bonding sites.

Also using dissolution calorimetry, Marabi *et al*. (2008) observed decreased heat release from lyophilised skim milk- or maltodextrin-based powders as fat content was increased. In addition, the authors investigated the mixing of pure fat with water, which yielded an endothermic response. It was concluded that increasing the fat level in powder systems has the effect of producing a higher endothermic response and that this would have a negative effect on powder rehydration by lowering the dissolution of enthalpy. The negative effect of a reduced heat of dissolution was correlated with longer rehydration times (determined by conductimetry; see Sect. 3.2.4). These data highlighted the fact that the negative effect of fat on powder solubilisation, widely attributed to increased hydrophobicity, may also be a result of reduced enthalpy of dissolution due to an increased endothermic response. However, the increase in rehydration time (113 s) for a powder with 45.0 % fat compared to 35.7 % fat coincided with only a minor decrease in the exothermic response; this led the authors to propose that the heat of dissolution was not capable of overcoming the rate-limiting step (wetting) for the 45 % fat powder.

Forny *et al*. (2011) highlighted the crucial role that thermodynamic approaches, such as dissolution calorimetry, could play in future studies of powder rehydration. The authors postulated that poorly-soluble powders could be characterised by their low exothermic response or an endothermic reading; furthermore, the authors proposed that powders composed of both amorphous and crystalline material could constitute a "thermodynamic microenvironment", in which poor solubility could be amended by a localised supply of heat flow from the amorphous to the crystalline phase during dissolution.

Citing the example of materials such as sodium chlorite, which is highly-soluble despite yielding an endothermic response of dissolution, Syll *et al*. (2012) stated that localised heat release as a method of improving powder rehydration was not feasible. In contrast to reports by Marabi *et al*. (2007, 2008) and Forny *et al*. (2011), these authors could find no reliable correlation between calorimetric response and dissolution time, determined by isothermal calorimetry and SLS, respectively, for various dairy powders. Moreover, Syll *et al*. (2012) reported rapid rehydration (measured using SLS) in powders which exhibited low exothermic responses. These results did not support the conclusions of Forny *et al*. (2011); thus, Syll *et al*. (2012) cautioned against using dissolution enthalpy values to investigate powder rehydration phenomena. They proposed that, used in conjunction with relaxation enthalpy measurements from differential scanning calorimetry, heat release data from isothermal calorimetry were most suitable for studying process- and storage-induced changes in amorphous powders, such as those occurring during ageing.

4.3.2.4 Conductimetry

Conductimetry measures the ability of a given solution to conduct an electric current (St-Gelais *et al*., 1995). The primary contributors to conductivity in milk systems are ions, amino acids (basic and acidic) and proteins; conversely, lactose and lipids are non-conductors (Therdthai and Zhou, 2001). Zhuang *et al.* (1997) discussed how the presence of lipids can reduce conductivity in milk by inhibiting the movement of ions. Mucchetti *et al*. (1995) demonstrated that skim milk ultrafiltrate had a similar conductivity to milk, while the retentate from diafiltration of skim milk had a significantly lower conductivity. Thus, it is minerals in the serum phase and not the colloidal phase which have the dominant influence on conductivity readings in milk (Zhuang *et al*., 1997). St-Gelais *et al*. (1995) reported that increasing temperature or acidification increased the conductivity of milk, presumably due to modifications in mineral equilibria between the serum and colloidal phases (Mucchetti *et al*., 1995).

Using a conductivity probe, inserted into a jacketed vessel, Marabi *et al*. (2008) monitored the dissolution of model food powders (SMP and MDP with different fat contents). Conductivity

values increased initially as powder was rehydrated (Fig. 4.7), presumably due to the release of charged species. An equilibrium conductivity reading was interpreted by the researchers as indicating complete rehydration. Increased fat content was reported to delay equilibrium conductivity readings (reduce solubility), with 45 % fat resulting in the longest dissolution time (~120 s). However, this effect could have been due to the inhibiting role of fat on any change in conductivity (Zhuang *et al*., 1997), rather than fat affecting directly the rehydration of other constituents in the powder.

Marabi *et al*. (2008) demonstrated that conductimetry was capable of monitoring the rehydration of both highly and poorly-soluble powders over time. Available as probes which can be inserted into samples or process streams, conductimetry also has significant potential as a method for in-line analysis. However, Marabi *et al*. (2008) conceded that conductivity data are not sufficient for physical modelling of the dissolution process, while Syll *et al*. (2012) questioned whether conductivity probes were capable of accurately identifying the end of the rehydration process. Indeed, in powders where particles containing poorly-dispersible surface skins of CN micelles remain after a reasonable period of rehydration (Mimouni *et al*., 2010a), an equilibrium conductivity reading may still be observed; thus, it would only indicate the rehydration of those constituents which are innately soluble within the time-course of the analysis.

Yanjun *et al*. (2014) reported reduced levels of insoluble material when MPC powder (80 %, w/w, protein) was ultrasonicated; despite this, conductivity levels did not change, indicating that conductimetry may not be able to detect changes in solubility. However, Gianfrancesco *et al*. (2011) used conductimetry to measure differences in the rehydration profiles of protein-based powders resulting from changes in the physicochemical state of the proteins in the powders; these researchers observed that heat-denatured β-lactoglobulin powder or NaCas powder, with or without lactose, exhibited considerably slower rehydration times than native β-lactoglobulin powder or PC powder, respectively; these results suggested that conductimetry was sufficiently sensitive to detect changes to rehydration behaviour induced by structural modifications to proteins. Indeed, Jambrak *et al*. (2008) reported increases in conductivity and solubility in ultrasonicated WPI and WP hydrolysate solutions, which was likely due to exposure of charged groups from within WPs with a concomitant increase in solvent access to proteins. Conductimetry is a useful method to measure the ionic strength of dairy systems (Crowley *et al*., 2014); thus, in studies on the rehydration properties of high-protein CN-dominant powders, it may be best applied to measure ionic strength for the determination of appropriate addition levels of salts such as NaCl, which are known to improve their solubility (Schuck *et al*., 2002).

4.3.2.5 Sound-Based Technologies

Ultrasonic spectroscopy has been used to characterise the rehydration of NaCas powder (Povey *et al*., 1999) and instant milk powder (Meyer *et al*., 2006); however, these studies were based on the properties of powders which had already been fully rehydrated. Richard *et al*. (2012) were the first to use ultrasonic tests *in situ* to monitor the rehydration of dairy powders dynamically. A schematic representation of the device is shown in Fig. 4.10. Instant extinction of the acoustic signal was observed as a high-protein PC powder was added to the stirred vessel (Fig. 4.7). As the powder dissolved and attenuation decreased, the acoustic signal recovered to an equilibrium value after 42 min; however, relaxation of the ultrasound signal did not coincide with complete dissolution, with particle sizes of between 20 and 30 μm measured by SLS after an equilibrium ultrasound reading; thus, ultrasonic relaxation measurements may not be suitable for assessing the total rehydration times of powders (Fig. 4.6). Crucially, the results of the study suggested that the release of occluded air is not as rapid as has been suggested previously by Mimouni *et al*. (2010a, b) for high-protein MPC powders and that, in fact, water penetration may be inhibited considerably by the surface skin of inter-linked CN micelles present at the surfaces of particles in high-protein CN-dominant powders.

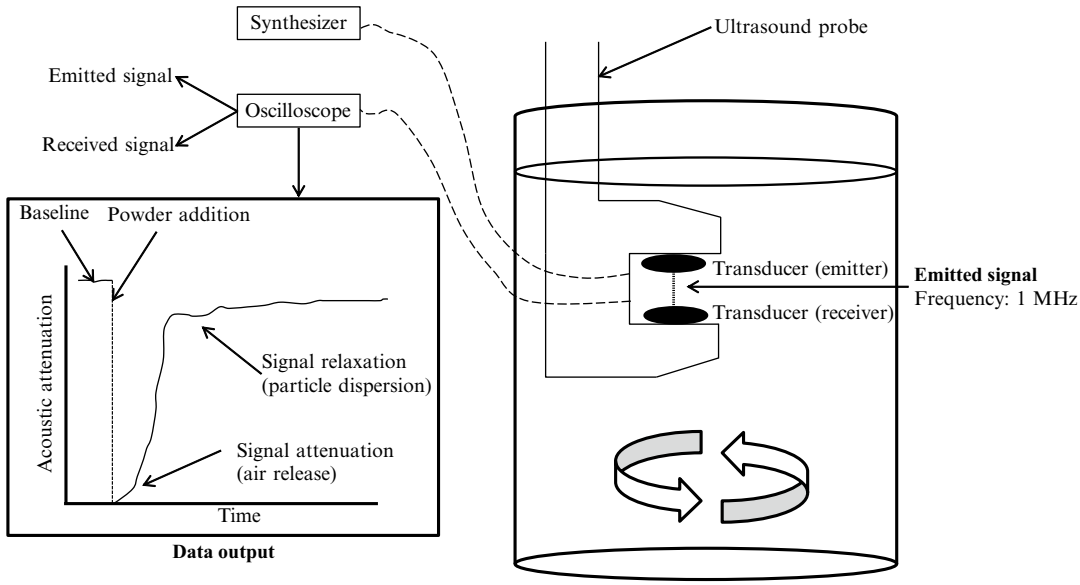

Fig. 4.10 Schematic representation of the principle and data outputs for an ultrasound device used for measuring the rehydration of dairy powders (redrawn from Richard *et al.*, 2012)

Release of occluded air from primary particles and its replacement with water is known to occur during the rehydration of dairy powders (Walstra *et al.*, 1999; Kelly *et al.*, 2003). Richard *et al.* (2012) determined that air release, as a result of solvent penetration with concomitant release of air occluded in the primary particles, was primarily responsible for ultrasound signal attenuation. The observation that attenuation levels did not recover completely following ultrasound relaxation suggested that air release did not occur during the later stages of rehydration. Thus, the technique was shown to be useful as a method to monitor the early to intermediate stages of powder rehydration. Moreover, it was shown that the ultrasound relaxation times were highly correlated with the overall rehydration times for different dairy powders (WPI, mix of PC and WPI, mix of PC and lactose). These results suggested that penetration of water into powder particles, with concomitant release of occluded air, is a key step during the rehydration of powders, as it precedes the fragmentation of primary particles and thus markedly impacts the time required to achieve complete rehydration. However, it is possible that primary particles of CN-dominant powders may imbibe water readily while retaining a surface rich in inter-linked CN micelles (Mimouni *et al.*, 2010a, b); in this case, air release would not necessarily precede fragmentation of the primary particles, but rather their sedimentation due to increased particle density (Masters, 1985).

It is clear from the aforementioned studies that further research is required to confirm whether delayed water penetration inhibits the effective dispersion of particles in high-protein dairy powders. Sound-based technologies will be important in this respect, as they are sensitive to the release of air, which can be used to account for changes in the penetration of water into particles between different powders. Other sound-based technologies also have potential applications in studies of dairy powder rehydration. For example, broadband acoustic resonance spectroscopy (BARDS), developed by Fitzpatrick (2011), has been used to study the rehydration of chemical compounds and mixtures but, until recently, had not been used in the analysis of complex, multi-component systems such as dairy powders. As the acoustic resonance measured by BARDS is associated with the release of gas from dissolving compounds, it is a useful method for monitoring the

stages of powder rehydration associated with water penetration and air release. Indeed, results from BARDS analysis indicated that air release took ~10 min for an MPC35 powder compared to ~50 min for an MPC90 powder (Vos *et al*., 2015); both powders were rehydrated to 0.2 %, w/w, in water, with significant increases in air release times expected at the higher target concentrations that these powders would typically be rehydrated to.

4.4 Conclusions

Rehydration should be considered the first and most essential attribute of high-protein dairy powders, as their use as ingredients in the food industry and other industries commonly requires them to rehydrate easily in aqueous media. A wide range of technologies are now available to study powder rehydration. Certain technologies, such as turbidimetry, conductimetry and FBRM, could find applications in industrial processes due to their in-line capabilities. Others, such as NMR-, thermochemical-, and sound-based technologies, will continue to yield new insights into the complex interactions which occur between powder components and water during rehydration.

Schuck *et al*. (2007) compared results from NMR, turbidimetry and rheometry studies into the rehydration of dairy powders. There were wide variations between the rehydration times obtained from the different methods and little agreement with insolubility index results in certain cases. The mechanisms behind rehydration, particularly for novel high-protein powders, remains poorly understood. There is a multitude of technologies available, but each varies considerably in terms of their underlying principles and data-outputs, and no method, used in isolation, would appear to be capable of fully characterising the rehydration process. Thus, future studies would benefit from an integrated approach, where two or more of these technologies are combined, to study high-protein CN-dominant powders such as PC, MPC and milk protein isolate powders. Moreover, measuring the rehydration properties of dairy powders is one of the best methods of determining the degree to which ageing-related changes have occurred during storage (Anema *et al*., 2006; Mimouni *et al*., 2009). Thus, the evolution of structure in dairy powders during storage should be a central focus in future research, as it will help explain resultant rehydration behaviour and make it possible to control this important property better.

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CHAPTER 2

Influence of protein concentration on the physical characteristics and flow properties of milk protein concentrate powders

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. SVC co-designed the study, measured the particle size and specific surface area of powders, determined flowability, wall friction and dynamic bulk density characteristics, analysed the data and performed statistical analysis. IG and TH provided the powders, powder composition and pycnometry data. The chapter is included in its final published form.

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Influence of protein concentration on the physical characteristics and flow properties of milk protein concentrate powders

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ABSTRACT

This study investigated the physical characteristics and flow properties of seven milk protein concentrate (MPC) powders, ranging from 36.6 (MPC35) to 89.6 (MPC90) % (w/w) protein in dry-matter. MPC80, MPC85 and MPC90 had the smallest particle sizes ($P < 0.05$) and significantly higher ($P < 0.05$) specific surface areas, as measured using nitrogen adsorption. The bulk density of MPC powders decreased with increasing protein content, due to increased ($P < 0.05$) levels of interstitial and occluded air. Flow testing indicated that high protein MPC powders were more compressible ($P < 0.05$), had lower flow index values ($P < 0.05$) and required larger ($P < 0.05$) outlet diameters for optimal flow in mass-flow hoppers compared to lower protein MPC powders.

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1. Introduction

Fractionated and concentrated milk protein-based ingredients are being increasingly dried into high protein powders (Gésan-Guiziou, 2013; Mistry, 2002), e.g., milk protein concentrate (MPC), milk protein isolate (MPI), micellar casein isolate (MCI), whey protein concentrate (WPC) and whey protein isolate (WPI) powders. These products are concentrated in protein and depleted in serum-phase constituents such as lactose, minerals and non-protein nitrogen using membrane-based separation technologies such as ultrafiltration (UF) or microfiltration, either of which may be combined with diafiltration (DF) to achieve higher protein concentrations (Gésan-Guiziou, 2013; Mulvihill and Ennis, 2003).

Food powders are commonly stored in bulk silos before packaging, dry-mixing with other powders or rehydration. The flowability of a powder during discharge from a silo depends to a large extent on the composition and physical properties of the powder, which are affected by its processing and storage history (Janjatović et al., 2012). High moisture levels affect flowability negatively, due to increased liquid bridging and capillary interactions between particles. Moisture levels can increase over storage, particularly if lactose is present in the amorphous state, with increased water sorption resulting in reduced flowability (Fitzpatrick, 2007). Migration of fat to particle surfaces, which occurs during spray drying (Nijdam and Langrish, 2006) and storage (Gaiani et al., 2009), also reduces flowability by increasing liquid bridging between particles (Kim et al., 2005).

A large particle size improves flowability due to a decreased specific surface area (SSA), with a concomitant reduction in inter-particle interactions (Fitzpatrick et al., 2004a); conversely, a small particle size increases SSA, which creates greater cohesive forces between particles (Reisner and Eisenhart Rothe, 1971). However, although particle size is considered one of the major factors influencing the flowability of powders, it is often weakly correlated with flowability (Fitzpatrick, 2007; Fitzpatrick et al., 2004a).

As described by Chen et al. (2012), the flow of a powder out of a silo is typically classified into two patterns, namely, mass-flow and core-flow, with the occurrence of either being dictated by the dimensions of the converging section of the silo, referred to as the hopper (Fig. 1). Most powder handling issues are caused by core-flow, where the powder particles initially form a funnel which does not collapse until the mobile powder particles flow through it; however, powder particles may remain at the internal walls and not flow from the hopper, a phenomenon known as rat-holing (Fitzpatrick et al., 2004a). It is possible to determine the minimum outlet diameter to prevent rat-holing (D_{RH}; see Fig. 1) during core-flow; however, many powder flow issues may be resolved by simply transforming the flow pattern from core-flow to mass-flow. For

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Nomenclature

BET	Brunauer–Emmett–Teller
CI	Compressibility index (%)
d_{10}	Particle size below which 10% of material volume exists (μm)
d_{50}	Particle size below which 50% of material volume exists – median (μm)
d_{90}	Particle size below which 90% of material volume exists (μm)
$D_{arching}$	Arching diameter (m)
D_{RH}	Rat-hole diameter (m)
ff	Flow factor (–)
FF	Flow function (–)
i	Jenike flow index (–)
MPCS	Major principal consolidating stress (kPa)
SSA	Specific surface area ($m^2\ g^{-1}$)
UFS	Unconfined failure strength (kPa)
V_{ia}	Volume of interstitial air (ml $100\ g^{-1}$)
V_{oa}	Volume of occluded air (ml $100\ g^{-1}$)
WYL	Wall yield locus
δ_c	Critical angle of internal friction (°)
δ_e	Effective angle of internal friction (°)
θ	Angle of hopper wall to vertical for mass flow (°)
p_b	Bulk density ($g\ cm^{-3}$)
p_c	Critical bulk density ($kg\ m^{-3}$)
$p_{compressed}$	Compressed bulk density ($g\ cm^{-3}$)
p_p	Particle density ($g\ cm^{-3}$)
p_{poured}	Poured bulk density ($g\ cm^{-3}$)
p_t	True density ($g\ cm^{-3}$)
σ_c	Critical stress (kPa)
ϕ_c	Critical angle of wall friction (°)
ϕ_w	Angle of wall friction (°)

a cohesive powder, a stable arch may still form at the hopper outlet during mass-flow; this effect, known as arching, creates a no-flow situation and the immobilised powder will require dislodgement for processing to continue. Hence, it is important to determine the minimum hopper outlet diameter and the minimum hopper half-angle to prevent arching during mass-flow of a given powder (Iqbal and Fitzpatrick, 2006) (Fig. 1).

The flow properties of skim milk powder (SMP), whole milk powder (WMP) and WPC have been well characterised (Fitzpatrick, 2007; Kim et al., 2005; Rennie et al., 1999; Teunou et al., 1999), and those of sodium caseinate and rennet casein have also been studied (Fitzpatrick et al., 2007). Comparatively little is known about the behaviour of ingredients enriched in micellar casein, such as MPC powders. MPCs are manufactured using UF, and sometimes DF, which concentrates both caseins and whey proteins in the retentate. UF/DF retentates may be spray-dried into powders containing between 35% and 90% (w/w) protein on a dry-matter basis (MPC35, MPC70, MPC90 etc.), depending on the protein concentration factor achieved during membrane processing (Mulvihill and Ennis, 2003). MPC products have found application as ingredients in many food systems, including cheese, yoghurt, ice cream and nutritional beverages (Gésan-Guiziou, 2013). For application as food ingredients, MPC powders are likely to be stored in silos prior to use; thus, it is important to determine how they may behave during storage and handling.

This study included seven MPC powders, with a diverse range of compositions, from MPC35, a product close in gross composition to a typical SMP, to MPC90, effectively a MPI. MPC powders were assessed for particle size distribution, SSA, particle and bulk density, interstitial and occluded air, flowability, internal and wall friction angles, and compressibility. In addition, flow, bulk density and wall friction data for the MPC powders were used to determine design parameters for appropriate mass-flow hoppers.

2. Materials and methods

2.1. Manufacture and composition of milk protein concentrate powders

MPCs were produced from pasteurised (72 °C for 15 s) skimmed bovine milk at NIZO food research (Ede, The Netherlands) as

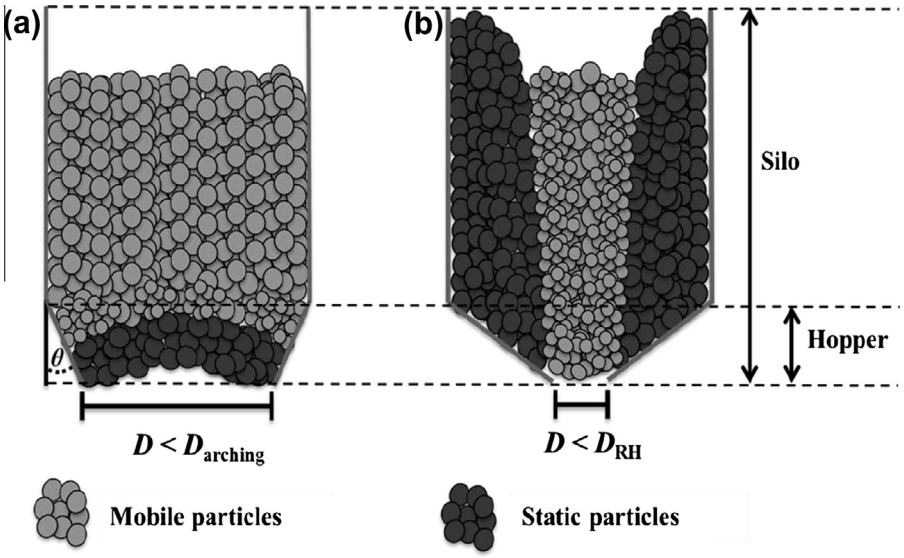

Fig. 1. Schematic diagram of flow problems, namely arching and rat-holing, encountered in hoppers during (a) mass-flow and (b) core-flow, respectively, where D = outlet diameter, $D_{arching}$ = minimum arching diameter, D_{RH} = minimum rat-hole diameter and θ = hopper half angle.

Table 1
Parameters related to manufacture of milk protein concentrates (MPCs). Solids contents are the means of data from duplicate analysis, while viscosity values are from a single analysis.

MPC	Volume concentration factor		Total solids content (%, w/w)	Viscosity at 50 °C (mPa s)
	Ultrafiltration	Diafiltration		
35	–	–	35.5	6
50	2.0	–	36.9	18
60	3.5	–	36.3	45
70	4.0	0.5	32.8	42
80	5.0	3.5	15.7	3
85	5.0	6.5	14.7	2
90	6.0	10.0	16.4	3

Table 2
Composition of milk protein concentrate (MPC) powders. Values are means of data from duplicate analysis, except for lactose content which was the result of a single analysis.

MPC	Protein (%, w/w)	Lactose (%, w/w)	Water (%, w/w)	Ash (%, w/w)
35	35.4	49.6	3.4	8.1
50	49.9	35.8	3.8	7.8
60	60.8	24.5	4.0	7.7
70	68.3	18.0	3.6	8.0
80	79.1	6.4	4.6	7.7
85	84.0	1.8	4.8	7.5
90	85.9	0.4	4.2	7.6

described by Crowley et al. (2014). Details of the volume concentration factors used during ultrafiltration (UF) and diafiltration (DF), use of evaporation (where applicable), and the solids content and viscosity of liquid concentrates before spray drying are given in Table 1. The MPC powders were non-agglomerated, which is of significance as recent research has indicated that agglomeration may affect the rehydration properties of casein-dominant powders negatively (Schuck et al., 2007).

Total solids content of samples was determined by oven drying (IDF, 1987). The viscosity of retentates and liquid concentrates was measured with an AR2000 rheometer (TA instruments, New Castle, DE, USA) at a shear rate of 500 s^{-1} at 50 °C using a double concentric cylinder geometry. Measurement of protein, lactose, moisture and ash in the MPC powders was performed as reported by Crowley et al. (2014). Fat levels were estimated to be between 0.5% and 1.7% (w/w). The composition of the MPC powders is shown in Table 2.

2.2. Particle size distribution and specific surface area

The particle size distribution of MPC powders was determined using a RODOS laser diffraction system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) using an R5 lens for particles between 0.5 and 875 μm and a dry feeder attachment comprising a hopper and vibrating plate (Sympatec VIBRI). At the beginning of the analysis, the hopper was raised automatically by ~1.0–3.3 mm and the powder was transferred to the dry dispersion unit by the vibrating plate (70%). Powders were then pumped with pressurised air (1.0 bar) into the path of the laser (λ = 632.8 nm) until an optical concentration of >5% was achieved. Results were calculated using the Fraunhofer approximation and presented as d_{10}, d_{50} and d_{90}, indicating the particle size below which 10%, 50% and 90% of material volume exists, respectively.

A Gemini VI Surface Area Analyzer (Micromeritics, Norcross, GA, USA) was used to determine the SSA of MPC powders, with nitrogen gas as the adsorbate and an operating pressure of 1.03–1.24 bar. Degassing was performed before each analysis using a FlowPrep™ 060 degassing unit (Micromeritics, Norcross, GA, USA). Values of SSA were calculated from a nine-point sorption isotherm (temperature maintained at −196 °C with liquid nitrogen) using the Brunauer–Emmett–Teller (BET) equation, as described by Brunauer et al. (1938).

2.3. Bulk density, particle density, occluded air and interstitial air

The tapped (100 taps) bulk density (ρ_{tapped}) of the MPC powders was measured as per GEA Niro (2006a), using a STAV 2003 Stampfvolumeter (J. Engelsmann Apparatebau, Ludwigshafen, Germany). Particle density (ρ_p) was measured as per GEA Niro (2006b), using a Beckman air comparison pycnometer model 930 (Beckman Instruments Inc., Brea, CA, USA). Values of true density (ρ_t) for each MPC powder were calculated based on the levels and densities of individual components in the powders, with density values of milk components taken from Walstra et al. (2006b). The volume of interstitial air (V_{ia}) and the volume of occluded air (V_{oa}) were determined as per GEA Niro (2006b).

2.4. Powder flow testing

A Powder Flow Tester (PFT) from Brookfield (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) was used in the analysis of flowability, wall friction and bulk density (ρ_b) of MPC powders. Axial and torsional speeds for the PFT were 1.0 mm s^{-1} and 1 rev h^{-1}, respectively. Samples were filled into the aluminium trough (230 cm^3, 15.2 cm internal diameter) of the annular shear cell at ~20 °C. The base of the trough was fitted with a perforated screen to prevent powder at the base of the cell from moving during shear. Curved- or flat-profiled shaping blades were used to level the powder surface in the trough for flow- or wall friction-testing, respectively. The mass of the powder was recorded before testing, with the axial distance between the lid and the powder used to calculate changes in the volume of powder during testing. Vane- or flat-profiled lids (15.2 cm external diameter), both made of 304 stainless steel, with a simulated 2B finish for the flat lid, were attached to the compression plate of the PFT for flow- or wall friction-testing, respectively.

2.4.1. Flowability

Flowability of the MPC powders was measured using an instantaneous flow function (FF) test. This involved the application of five uniaxial normal stresses (between 0.2 and 4.8 kPa) and three overconsolidation stresses at each normal stress. Under known normal stress, powders were first critically consolidated, and then the shear stress needed to cause the powder to fail (i.e., lose sufficient strength to flow under an applied stress) while subject to four normal stresses less than the consolidating stress, as well as at the consolidating stress itself, were measured. The shear stress at failure was then plotted against normal stress at each consolidating stress to construct a best-fit yield locus. This procedure was repeated five times to create five yield loci for each consolidating stress. The steady-state point, consolidation end point and multiple overconsolidation points comprised each yield locus (Fig. 2).

Unconfined failure strength (UFS) and major principal consolidating stress (MPCS) were calculated from two specific Mohr circles from each yield locus (Fig. 2). The effective angle of internal friction (δ_e) was calculated as the angle formed between a line, which began at the origin and was tangent to the consolidation Mohr circle, and the normal stress axis. Flow factor (ff) values for each powder were determined from a Jenike design chart for a conical hopper using values of δ_e and wall friction angle (ϕ_w; see Section 2.4.2) (Jenike, 1964). The intersection of the plot of FF and a straight line of ff drawn from the origin yielded the critical stress (σ_c). The FF curve was plotted from UFS and MPCS values

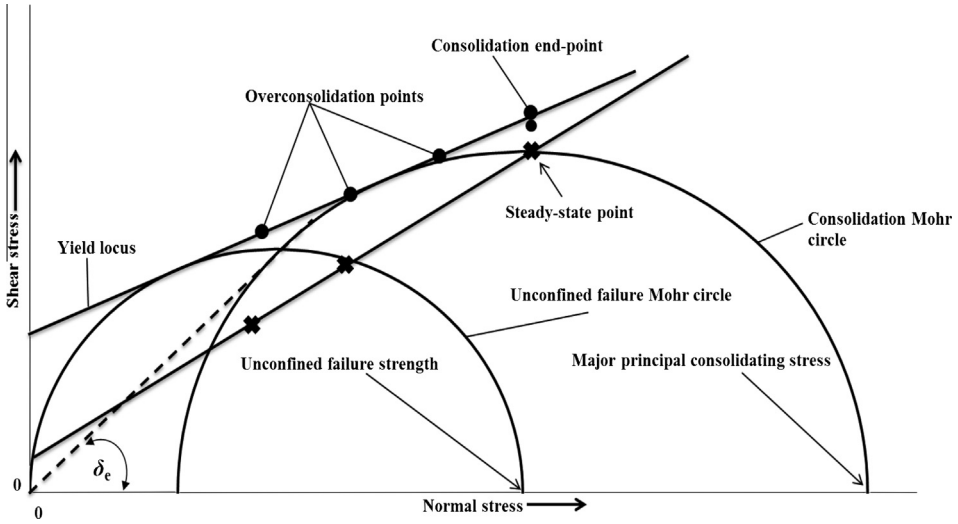

Fig. 2. Schematic representation of stress data from flow testing showing a yield locus and Mohr circles, as well as the extraction of data relating to powder flow properties.

calculated for each yield locus. Flow index (i) values were calculated from the inverse slope of a straight line fitted through the FF data points (Teunou et al., 1999). Values for ρ_b were also measured at each yield locus and plotted against their corresponding MPCS. Poured bulk density (p_{poured}) and compressed bulk density ($p_{compressed}$) were determined at minimum and maximum MPCS, respectively; compressibility index (CI) was calculated based on the % change in ρ_b between p_{poured} and $p_{compressed}$.

2.4.2. Wall friction angle

The shear stresses required to move MPC powders continuously (steady-state flow) across a stainless steel surface under three normal stresses, 4.8, 3.2 and 1.6 kPa, applied in order of decreasing normal stress, were measured in the calculation of ϕ_w values. A wall yield locus (WYL) was constructed for each MPC powder based on the maximum shear stress developed between the bulk powder and the wall material under the three normal stresses before steady-state flow occurred. The maximum WYL was constructed from the highest shear stress of any WYL at its corresponding normal stress (Fig. 3). The coefficient of wall friction (μ) was calculated from the slope of a straight line plotted from the origin and intersecting the maximum WYL at a normal stress value of 4.8 kPa, with values for ϕ_w calculated as follows:

$$\phi_w = \arctan(\mu) \quad (1)$$

2.4.3. Hopper design for mass-flow

Values for σ_c and critical ρ_b (ρ_c) were derived from FF and ρ_b plots, respectively, with $D_{arching}$ (see Fig. 1) calculated as follows:

$$D_{arching} = \frac{2 \times \sigma_c \times 1000}{p_c \times g} \quad (2)$$

where g = acceleration due to gravity (m s^{-2}).

Values for δ_e and ϕ_w were used to calculate minimum hopper half angle (θ) based on the following equation for a conical hopper:

$$\theta = \left[90 - \frac{1}{2}\arccos\left(\frac{1-\sin\delta_c}{2\sin\delta_c}\right)\right] - \frac{1}{2}\left[\phi_w + \arcsin\left(\frac{\sin\phi_w}{\sin\delta_c}\right)\right] \quad (3)$$

where δ_c = critical angle of internal friction (°).

Both FF and ϕ_w data were used in the calculation of θ, with the value of ϕ_w used in Eq. (3) derived from the point at which the WYL intersected the consolidation Mohr circle.

2.5. Statistical analysis

Measurements of powder composition, bulk and particle density, and occluded and interstitial air were performed in duplicate, with all other analyses performed in triplicate. Analysis of variance (ANOVA; Tukey's HSD) was performed using Minitab (version 16.2.2, Minitab Ltd., Coventry, UK) statistical analysis package. The level of statistical significance was determined at $P < 0.05$.

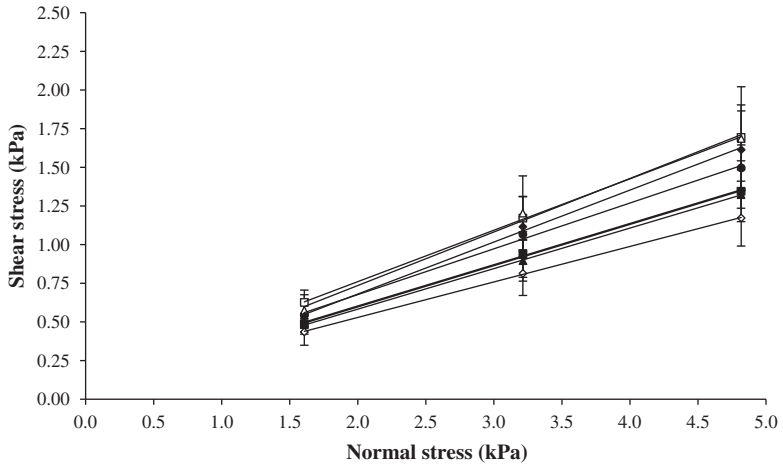

Fig. 3. Wall yield loci for milk protein concentrate (MPC) 35 (■), MPC50 (◇), MPC60 (▲), MPC70 (●), MPC80 (□), MPC85 (Δ) and MPC90 (♦). Values are means ± standard deviations of data from triplicate analysis.

3. Results and discussion

As outlined previously, the flowability of a powder is influenced primarily by its bulk and surface composition, as well as its physical properties. It is clear that increased protein concentration factor during membrane processing (Table 1) resulted in marked differences in the gross composition of MPC powders (Table 2). Protein content increased due to retention of casein and whey by the 10 kDa ultrafiltration (UF) membrane, whereas lactose and minerals were depleted considerably due to their loss in the permeate; other than the influence of lactose on water sorption during storage, very little is known about the contribution of these components to flowability. In addition, levels of moisture and fat, which are the components believed to have the most significant influence on flowability, did not differ markedly between the MPC powders. Hence, differences in gross composition were considered to have had a negligible direct effect on the flow properties of the MPC powders. Surface composition is also an important contributor to flow properties but was outside the scope of the present study. Thus, differences in physical properties between the MPC powders, as affected by the processing conditions used and characteristics of the MPCs before drying, were considered to be the primary factors influencing the flow behaviour of the MPC powders. This relationship will be the focus of the following sections.

3.1. Physical properties

3.1.1. Particle size distribution and specific surface area

The largest median particle size (d_{50}) was observed for MPC60 ($P < 0.05$), while MPC80, MPC85 and MPC90 had significantly ($P < 0.05$) lower d_{50} values compared to all other MPC powders (Table 3). Differences in d_{50} values can be explained by the properties of the retentates and concentrates which entered the spray drier. MPC60, which had the largest d_{50} value, was produced from a liquid concentrate with the highest viscosity (Table 1). The retentates of MPC80, MPC85 and MPC90 had substantially lower viscosity values, due to their low solids contents. Feed materials with high solids contents yield larger particle sizes in spray-dried powders (Keogh et al., 2003), due to impaired atomisation caused by high viscosity of the concentrates (Walstra et al., 2006a). In addition, samples with less water to be removed, due to a high solids content of the feed material, typically display decreased droplet shrinkage, with a concomitant increase in particle size (Fu et al., 2013). Hence, increased atomisation efficiency and droplet shrinkage were probably responsible for the smaller particle sizes observed in MPC80, MPC85 and MPC90.

Specific surface area (SSA) values are typically inferred from particle size data, and are thus often representative only of the SSA of equivalent spheres. Conversely, methods based on nitrogen adsorption do not include an assumption of sphericity. Values of SSA, measured using nitrogen adsorption, were between 1.7 and 2.4 times higher ($P < 0.05$) for MPC80, MPC85 and MPC90 compared to lower protein powders (Table 3). Although significant differences in d_{50} values were measured between MPC35, MPC50, MPC60 and MPC70, differences in SSA were minimal; this indicated that the high SSA values observed for MPC80, MPC85 and MPC90 may not have been due to differences in particle size alone, suggesting that alterations to surface morphology may have been a contributing factor.

3.1.2. Bulk density, particle density, occluded air and interstitial air

MPC80, MPC85 and MPC90 had significantly lower ($P < 0.05$) particle density (ρ_p) values than the other MPC powders, due to increases ($P < 0.05$) in the volume of occluded air (V_{oa}) in these powders (Table 3). Lower ($P < 0.05$) ρ_{tapped} values for MPC80, MPC85 and MPC90 compared to lower protein MPC powders were due to higher volumes of interstitial air (V_{ia}) and higher V_{oa} values ($P < 0.05$). Low feed solids contents in spray drying typically results in powder particles with high V_{oa} and low bulk density (ρ_b) values (Mistry, 2002; Mistry and Pulgar, 1996). The low viscosity of the retentates for MPC80, MPC85 and MPC90 probably increased the incorporation of air into the feed, with a concomitant increase in V_{oa}, while the greater presence of small particles in these powders would be expected to increase V_{ia} (Skanderby et al., 2009).

3.2. Flow properties

3.2.1. Flowability, internal friction angle, compressibility and wall friction angle

Results from flow function (FF) testing indicated that different MPC powders were free-flowing, easy-flowing or cohesive depending on the major principal consolidating stress (MPCS) applied (Fig. 4). The flowability of powders is often stress-dependant (Chen et al., 2012; Teunou et al., 1999), which has important implications for the flow behaviour in different regions of the hopper and at different fill heights. The consolidating stress transmitted through a powder by its own self-weight increases in proportion to the distance from the apex of the hopper (Schulze, 2004); hence, MPC70, which was easy-flowing at MPCS values of >7.0 kPa but was cohesive at a MPCS of 2.0 and 4.4 kPa, may behave more cohesively at the hopper outlet, increasing the potential for arching (Fig. 1). Moreover, as the fill level of a hopper decreases during discharge of a powder, the consolidating stress due to self-weight of the powder would also decrease, which may cause a powder such as MPC70 to behave more cohesively below a certain fill height.

Fig. 4 clearly illustrates that MPC80, MPC85 and MPC90, and to a lesser extent MPC70, exhibit drastically impaired flow properties. Flow index (i) values (Table 4) for the MPC powders were calculated from FF plots (Fig. 4) and assessed based on Jenike's classification for flowability (Jenike, 1964; Teunou et al., 1999). MPC35 and MPC50 were free-flowing, while MPC60 and MPC70 were easy-flowing. Results for MPC35, MPC50, MPC60 and MPC70 were

Table 3
Values (mean ± standard deviation) relating to the physical properties of milk protein concentrate (MPC) powders. d_{10} = particle size below which 10% of material volume exists ($n = 3$), d_{50} = particle size below which 50% of material volume exists ($n = 3$), particle size below which 90% of material volume exists ($n = 3$), SSA = specific surface area ($n = 3$), ρ_p = particle density ($n = 2$), ρ_{tapped} = tapped bulk density ($n = 2$), V_{ia} = volume of interstitial air ($n = 2$), V_{oa} = volume of occluded air ($n = 2$).

MPC	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)	SSA ($m^2\ g^{-1}$)	ρ_p ($g\ cm^3$)	ρ_{tapped} ($g\ cm^3$)	V_{ia} (ml 100 g^{-1})	V_{oa} (ml 100 g^{-1})
35	$12.9^{c} \pm 0.2$	$35.3^{d} \pm 0.6$	$64.6^{bc} \pm 1.7$	$0.34^{bc} \pm 0.01$	$1.23^{a} \pm 0.01$	$0.56^{b} \pm 0.00$	$98^{d} \pm 0$	$18.1^{d} \pm 0.7$
50	$15.3^{b} \pm 0.2$	$43.0^{b} \pm 0.2$	$72.1^{b} \pm 0.5$	$0.44^{b} \pm 0.07$	$1.25^{a} \pm 0.00$	$0.59^{a} \pm 0.00$	$88^{e} \pm 1$	$14.1^{e} \pm 0.2$
60	$18.1^{a} \pm 0.2$	$48.9^{a} \pm 0.5$	$90.6^{a} \pm 0.6$	$0.32^{c} \pm 0.02$	$1.12^{b} \pm 0.00$	$0.54^{c} \pm 0.00$	$95^{d} \pm 0$	$21.4^{c} \pm 0.1$
70	$14.9^{b} \pm 0.2$	$39.6^{c} \pm 0.4$	$72.7^{b} \pm 0.2$	$0.38^{bc} \pm 0.01$	$1.09^{c} \pm 0.00$	$0.49^{d} \pm 0.00$	$111^{c} \pm 1$	$23.0^{c} \pm 0.2$
80	$10.8^{d} \pm 0.5$	$27.9^{e} \pm 0.8$	$58.0^{cd} \pm 7.5$	$0.76^{a} \pm 0.04$	$0.80^{e} \pm 0.01$	$0.30^{e} \pm 0.00$	$206^{b} \pm 1$	$53.7^{a} \pm 1.3$
85	$9.6^{e} \pm 0.0$	$26.1^{f} \pm 0.2$	$51.9^{d} \pm 0.9$	$0.74^{a} \pm 0.06$	$0.84^{d} \pm 0.01$	$0.29^{f} \pm 0.00$	$229^{a} \pm 3$	$47.2^{b} \pm 0.8$
90	$10.7^{d} \pm 0.2$	$26.8^{ef} \pm 0.1$	$52.5^{d} \pm 0.3$	$0.77^{a} \pm 0.03$	$0.85^{d} \pm 0.00$	$0.29^{f} \pm 0.00$	$230^{a} \pm 2$	$46.2^{b} \pm 0.2$

[a–f]Values within columns with different superscripts are significantly different ($P < 0.05$).

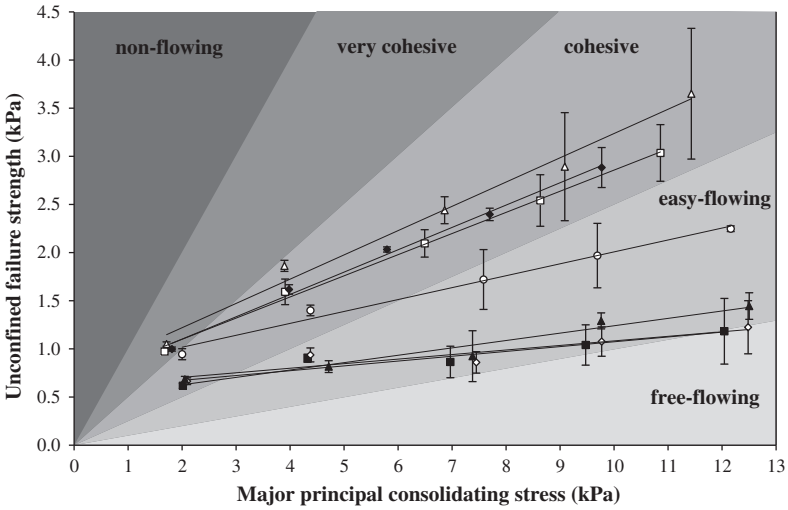

Fig. 4. Flow function curves showing unconfined failure strength as a function of major principal consolidating stress for milk protein concentrate (MPC) 35 (■), MPC50 (◇), MPC60 (▲), MPC70 (●), MPC80 (□), MPC85 (△) and MPC90 (♦). Values are means ± standard deviations of data from triplicate analysis.

similar to those reported by Teunou et al. (1999) and Fitzpatrick et al. (2004b, 2007) for SMP, which has a similar composition to MPC35 and is known to exhibit good flowability. MPC80, MPC85 and MPC90 were cohesive, indicating that they deviated considerably from the other MPC powders or what would be expected for a typical SMP. Although the flowability of powders enriched in micellar casein has not been characterised previously, Fitzpatrick et al. (2007) reported that rennet casein and sodium caseinate powders were also cohesive. The poor flowability of MPC80, MPC85 and MPC90 was probably due to their small particle size and high SSA values (Table 3), which would be expected to increase particle–particle interactions. Indeed, effective angle of internal friction (δ_e) values increased with increasing protein content of the MPC powders (Table 4), indicating that more cohesive interactions occurred between particles in high protein MPC powders.

All MPC powders became compressed on the application of increasing MPCS, with concomitant increases in ρ_b (Fig. 5). Significantly higher ($P < 0.05$) compressibility index (CI) values were observed for MPC80, MPC85 and MPC90 compared to the lower protein MPC powders (Table 4). The void-filling action of the small particles in high protein MPC powders, combined with their high V_{ia} values (Table 3), probably allowed increased evacuation of air during compression, with a concomitant reduction in proximal distance between particles. High CI values indicated that MPC80, MPC85 and MPC90 may compress under self-weight during storage in hoppers, which may result in altered handling properties and ρ_b values which deviate from specifications.

The angle of wall friction (ϕ_w) is an essential parameter in the design of appropriate mass-flow hoppers. High ϕ_w values indicate that low normal loads will be transmitted to the walls of the hopper, which can result in deposition or segregation of powder (Iqbal and Fitzpatrick, 2006). Wall yield loci for the MPC powders (Fig. 3) were used to derive ϕ_w values at 4.8 kPa. Values of ϕ_w increased with increasing protein content of the MPC powders, which was probably due to increasing SSA (Table 3), with a concomitant increase in particle–wall interactions. Values of ϕ_w were between 15.9° and 21.7° for the MPC powders (Table 4), which was within the range 11.8–27.3° reported by Fitzpatrick et al. (2004a) for various food powders. MPC35 had a ϕ_w of 16.8°, which was similar to the value of 15.4° reported by Fitzpatrick et al. (2004b) for SMP. Increases in ϕ_w with protein content indicated that high protein MPC powders may have a greater tendency to undergo deposition or segregation in a hopper.

3.2.2. Hopper design for mass-flow

Differences in ϕ_w are the dominant factor influencing the minimum hopper half angle (θ) required for ensuring a mass-flow pattern during powder discharge from a hopper (Iqbal and Fitzpatrick, 2006). Due to their higher ϕ_w values, high protein MPC powders had lower θ values (steeper hopper walls) compared to lower protein MPC powders (Table 4). The θ for MPC35 was 24°, which was lower than the value of 32° reported by Teunou et al. (1999) for an agglomerated SMP. Results from the present study showed that MPC powders with higher protein contents will require steeper hopper walls to achieve mass-flow; indeed, MPC80, MPC85 and MPC90 had θ values which were <20° (even without the subtraction of the commonly used 2–3° safety margin) which is commonly used as a 'rule-of-thumb' for achieving mass-flow (Chen et al., 2012; Fitzpatrick et al., 2004a).

Table 4
Values (mean ± standard deviation; $n = 3$) relating to flow properties and hopper design, derived from shear cell testing, for milk protein concentrate (MPC) powders. δ_e = effective angle of internal friction, ϕ_w = angle of wall friction, σ_c = critical stress, CI = compressibility index, $D_{arching}$ = minimum outlet diameter to prevent arching, θ = minimum hopper half-angle, ff = flow factor, i = flow index.

MPC	δ_e (°)	ϕ_w (°)	σ_c (kPa)	CI (%)	ff (–)	i (–)	$D_{arching}$ (m)	θ(°)
35	$40.2^{c} \pm 1.1$	$16.8^{bc} \pm 1.9$	$0.62^{a} \pm 0.09$	$47.3^{b} \pm 3.5$	$1.49^{a} \pm 0.05$	$13.4^{a} \pm 5.1$	$0.24^{c} \pm 0.03$	$24^{a} \pm 3$
50	$41.1^{bc} \pm 0.4$	$15.9^{c} \pm 1.0$	$0.66^{a} \pm 0.05$	$45.0^{b} \pm 1.9$	$1.48^{ab} \pm 0.01$	$13.0^{a} \pm 3.0$	$0.25^{c} \pm 0.01$	$24^{a} \pm 6$
60	$41.8^{abc} \pm 0.1$	$15.9^{c} \pm 0.9$	$0.54^{a} \pm 0.09$	$46.7^{b} \pm 8.7$	$1.46^{abc} \pm 0.02$	$9.9^{ab} \pm 0.8$	$0.24^{c} \pm 0.04$	$21^{a} \pm 5$
70	$42.0^{abc} \pm 0.6$	$18.0^{abc} \pm 1.1$	$0.94^{b} \pm 0.14$	$50.5^{b} \pm 4.7$	$1.46^{abc} \pm 0.02$	$6.0^{bc} \pm 0.4$	$0.42^{b} \pm 0.06$	$24^{a} \pm 1$
80	$43.0^{ab} \pm 0.7$	$21.7^{a} \pm 1.4$	$0.96^{b} \pm 0.05$	$78.6^{a} \pm 0.8$	$1.40^{bc} \pm 0.03$	$3.9^{bc} \pm 0.1$	$0.69^{a} \pm 0.04$	$6^{b} \pm 4$
85	$43.9^{a} \pm 1.2$	$20.8^{ab} \pm 2.0$	$1.10^{b} \pm 0.14$	$90.6^{a} \pm 7.7$	$1.39^{c} \pm 0.04$	$3.5^{c} \pm 0.4$	$0.78^{a} \pm 0.05$	$18^{ab} \pm 5$
90	$41.8^{abc} \pm 0.8$	$19.6^{abc} \pm 2.7$	$0.95^{b} \pm 0.07$	$79.2^{a} \pm 9.1$	$1.44^{abc} \pm 0.03$	$3.6^{bc} \pm 0.4$	$0.72^{a} \pm 0.03$	$18^{ab} \pm 8$

[a–c]Values within columns with different superscripts are significantly different ($P < 0.05$).

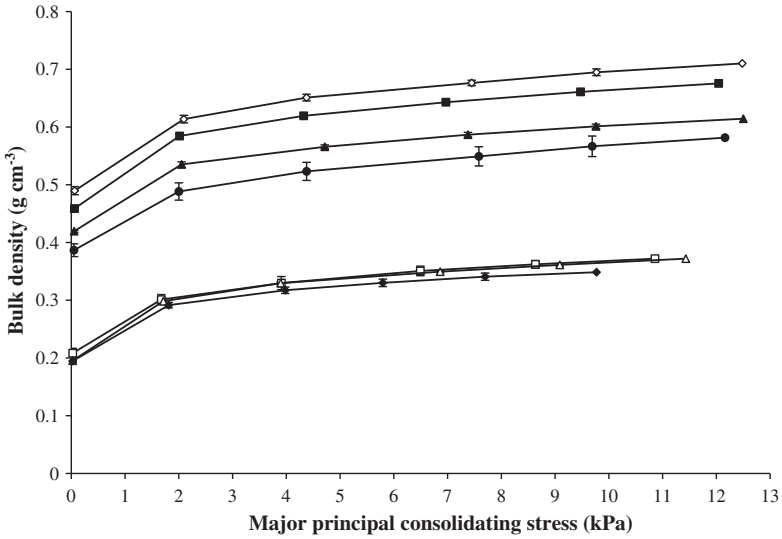

Fig. 5. Bulk density as a function of major principal consolidating stress for milk protein concentrate (MPC) 35 (■), MPC50 (◇), MPC60 (▲), MPC70 (●), MPC80 (□), MPC85 (△) and MPC90 (♦). Values are means ± standard deviations of data from triplicate analysis.

MPC70, MPC80, MPC85 and MPC90 had significantly higher ($P < 0.05$) critical stress (σ_c) values than lower protein MPC powders (Table 4), indicating that they have a tendency to develop cohesive arches which require greater stresses to collapse (Reisner and Eisenhart, 1971; Schulze, 2004). Indeed, $D_{arching}$ values were significantly higher ($P < 0.05$) for MPC70, MPC80, MPC85 and MPC90 compared to lower protein MPC powders. MPC70 occupied an intermediate $D_{arching}$ region between low and high protein MPC powders, being significantly different ($P < 0.05$) from both groups. It is clear from these results that a single hopper design will not facilitate effective handling of MPC powders when substantial variation in physical characteristics and flow properties exist between different powders.

4. Conclusions

This study demonstrated that differences related to the composition and processing of MPCs across a range of protein concentrations resulted in powders with altered physical characteristics, which, in turn, affected their flow properties negatively; as a result, steeper hopper walls and larger outlet diameters were calculated in the design of mass-flow hoppers for high protein MPC powders. High specific surface areas were probably responsible for the markedly reduced flowability of high protein MPC powders. In addition, increased volumes of interstitial air and the void-filling action of small particles resulted in high degrees of compressibility in high protein MPC powders, suggesting that they may be susceptible to compression under their own self-weight during storage in a hopper.

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CHAPTER 3

Rehydration characteristics of milk protein concentrate powders

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. SVC co-designed the study, provided analytical training/support to BD and analysed the data. SVC and BD carried out analytical centrifugation, light-scattering and conductimetry experiments. BD assisted with contact angle experiments. IG and TH provided the powders, powder composition data, pycnometry data and light microscopy images. The chapter is included in its final published form. Supplementary data for this chapter are included in Appendix 1.

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Rehydration characteristics of milk protein concentrate powders

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ABSTRACT

Low-(MPC35, MPC50), medium-(MPC60, MPC70) and high-(MPC80, MPC85, MPC90) protein content milk protein concentrate (MPC) powders, manufactured at pilot-scale, were evaluated for their rehydration characteristics. Optical tensiometry confirmed that water droplets were imbibed more slowly as protein content of the MPCs increased, indicating impaired wetting. Casein micelles comprised only <2% of the particle population by volume in MPC70, MPC80, MPC85 or MPC90 after 90 min of rehydration at 25 °C, as primary particles which had not dispersed fully remained in suspension. The quantity of sediment, measured using analytical centrifugation, increased in the order MPC70 < MPC80 < MPC85 < MPC90 after 90 min of rehydration at 25 °C, with lower protein MPCs forming no sediment. No sediment formation was observed in any of the MPCs after 24 h of rehydration at 25 °C, despite the predominance of primary particles in suspensions of high-protein MPCs. Increasing the temperature of reconstitution from 25 to 50 °C during 90 min of rehydration caused a 41.4% decrease in sediment height for MPC90 in water; however, reductions in sediment height of 89.9% and 99.5% were achieved when MPC90 was rehydrated in milk permeate or 80 mM KCl, respectively. It is evident that low ionic strength (confirmed using conductimetry) has a strong negative effect on the rehydration properties of high-protein MPCs, and that the synergistic effect of increasing ionic strength and temperature can substantially accelerate rehydration.

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1. Introduction

Complete rehydration is normally a prerequisite for effective expression of a dried protein ingredient's underlying functionality. Dairy powders which are both high in protein and casein-dominant are difficult to reconstitute fully, even after extended periods of rehydration, due to inhibited transfer of water into powder particles (Schuck et al., 2007) and slow dispersion of a "skin" of casein micelles present at particle surfaces (Mimouni et al., 2009). Milk protein concentrates (MPCs) are one such category of dairy ingredient, the poor reconstitution properties of which require end-users to modify existing unit operations or product formulations so that powder rehydration may be accelerated.

Anema et al. (2006) identified caseins as the primary components in the poorly-dispersible particle population in MPC85. Mimouni et al. (2009) proposed that the rate-limiting step during the rehydration of MPC85 was the dispersion of inter-linked casein micelles predominating at the surface of primary particles, which preceded the subsequent release of colloidal material (i.e., casein micelles and associated minerals). Numerous studies have confirmed that slow dispersion of primary particles is responsible for the extended rehydration times of casein-dominant powders (Gaiani et al., 2005; Fang et al., 2011; Richard et al., 2013), an effect which, perhaps counter intuitively, becomes more pronounced when these powders are agglomerated (Gaiani et al., 2005; Schuck et al., 2007).

According to Mimouni et al. (2010b), the surface of slowly-dispersing primary particles in MPC85 was sufficiently porous to allow water to be imbibed rapidly, with a subsequent fast release of whey proteins, serum-phase minerals and lactose, while the release of casein micelles and associated minerals was retarded. In support of this, the addition of "fast-dissolving" components, such as NaCl (Schuck et al., 2002) or whey proteins (Gaiani et al., 2007), has been shown to improve the rehydration properties of micellar casein isolate (MCI) powders. However, increasing the size and number of pores in MPC powder particles through extrusion-porosification also markedly enhanced rehydration properties (Bouvier et al., 2013). The latter result suggests that, while not being the rate-limiting stage in itself, slow penetration of water into primary particles may contribute strongly to the poor rehydration properties of casein-dominant powders; ultrasound attenuation measurements support this, with Richard et al. (2012) reporting delayed air release from vacuoles within primary particles in MCI powder.

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Table 1
Compositional and physical properties of milk protein concentrate (MPC) powders. All data are presented as the means of duplicate analysis, except for lactose, which was the result of a single analysis.[a]

	Composition				Physical properties	
	Protein (%, w/w)	Lactose (%, w/w)	Ash (%, w/w)	Fat (%, w/w)	d_{50} [b] (μm)	Tapped density (g cm^{-3})
MPC35	35.4	49.6	8.06	0.5	35.3	0.56
MPC50	49.9	35.8	7.75	0.8	43.0	0.59
MPC60	60.8	24.5	7.74	1.5	48.9	0.54
MPC70	68.3	18.0	7.99	1.2	39.6	0.49
MPC80	79.1	6.36	7.69	1.7	27.9	0.30
MPC85	84.0	1.81	7.54	1.2	26.1	0.29
MPC90	85.9	0.37	7.59	1.6	26.8	0.29

[a] Adapted from Crowley et al. (2014a).
[b] Particle size below which 50% of material volume exists – median.

Previous studies have confirmed that a poorly-dispersible casein fraction is responsible for the slow rehydration of MPC powders containing ⩾80% protein, and that this phenomenon is exacerbated during storage, particularly at elevated temperature and/or relative humidity (Mimouni et al., 2010a). MPCs, however, comprise a broad product category, spanning low-, intermediate- and high-protein powders, the entire breadth of which has not been researched extensively. In addition, classical definitions or determinants of solubility, relevant to skim or whole milk powders and whey powders, do not account completely for the rehydration-state of MPCs; thus, there is a need to expand the criteria against which the performances of MPCs during reconstitution are assessed. This study utilised an integrated experimental approach, including measurement of changes in contact angle, conductivity and particle size distribution over time, in addition to measuring sedimentation behaviour using analytical centrifugation, to characterise the rehydration properties (i.e., wetting, mineral release, dispersion) of MPC powders across a range of protein concentrations (from MPC35, close in composition to skim milk powder, to MPC90, effectively a milk protein isolate). The effect of temperature and additives (deionised water, milk permeate, calcium-binder, non-ionic surfactant or monovalent ions, at 25 or 50 °C) on the sedimentation behaviour of MPC90 was also investigated, to develop an understanding of the mechanism dictating its dispersion characteristics and potential strategies to ameliorate same.

2. Materials and methods

2.1. Milk protein concentrate powders: processing, composition and physical properties

The manufacture and composition of the MPC powders used in this study were described in detail by Crowley et al. (2014a, b). Briefly, fresh bovine milk was skimmed and pasteurised followed by ultrafiltration (MPC50, MPC60) or combined ultrafiltration and diafiltration (MPC70, MPC80, MPC85, MPC90) of milk to different protein concentration factors at 50 °C with 10 kDa molecular weight cut-off membranes; MPC35 was not subjected to membrane filtration. MPC35, MPC50, MPC60 and MPC70 were evaporated to increase total solids content of the retentates prior to spray drying, while MPC80, MPC85 and MPC90 were not evaporated. All MPCs were spray-dried in a single-stage drier with nozzle atomisation, an air inlet temperature of 185–190 °C, and an outlet temperature of 85–90 °C. Selected compositional and physical characteristics (based on relevance to rehydration properties) of each MPC powder were analysed as per Crowley et al. (2014a, b) and are shown in adapted form in Table 1. Light microscopy images of particles in MPC powders were also captured at 40× magnification with a light microscope after dispersing powders in paraffin oil.

2.2. Wetting: optical tensiometry

Contact angle (θ) was measured using an optical tensiometer (PocketGoniometer PGX®, Plasmatreat Ltd., Bicester, UK) using the Sessile drop technique and the dynamic measurement option at ~22 °C, with a measurement time of 5 s and a deionised water droplet volume of 5.26 ± 0.43 μL. Measurement of θ was carried out on discs of MPC powder with a diameter of 13 mm, prepared by a Specac® hydraulic compressor (Perkin Elmer, Buckinghamshire, UK), using a fill height of 1 mm and a compression force of 8000 kg. Values of θ were collected every second for 5 s, and images were extracted from the PGX software at 0, 3 and 5 s intervals.

2.3. Powder rehydration

Powders were added to 300 mL of deionised water in 400 mL beakers to attain 1.5% (w/v) protein suspensions. In some analytical centrifugation experiments (see Section 2.6), deionised water was replaced with milk permeate (Carbery, Ballineen, Co. Cork, Ireland), or solutions of KCl, tri-sodium citrate, or polysorbate 80 (Sigma Chemical Co., St Louis, Missouri, MO, USA), all with a theoretical ionic strength of 80 mM. For dynamic analysis of conductivity over 90 min, MPC suspensions were stirred at controlled temperature (25 °C) using an overhead stirrer, with a four-impeller design, and an impeller length of 2 cm, operated at 2025 rpm with no vortex formation in the turbulent regime (Re > 10,000). MPC suspensions rehydrated for 90 min for single-point measurement of particle size distribution (PSD) and sedimentation were stirred magnetically under conditions of controlled temperature (25 °C); the magnetic stirring bar had a length of 2.5 cm, and was operated at 500 rpm with no vortex formation in the turbulent regime (Re > 10,000). MPC suspensions rehydrated over 24 h were first stirred magnetically for 8 h at 25 °C, after which samples were stirred at ambient temperature (22 °C) for the remaining 16 h. Reported stirring rates were selected to achieve a turbulent regime (non-limiting hydrodynamics) without vortex formation (to prevent excessive aeration). Wetted powder adhering to the internal walls of the beaker, when observed, was removed by gentle washing using a Pasteur pipette filled with the solution being studied. Rehydration experiments for each MPC powder were conducted at least in duplicate.

2.4. Mineral release/ionic strength: conductivity

Conductivity was measured using a Titrando autotitrator, equipped with a five-ring conductivity measuring cell and accompanying Tiamo v2.3 software (Metrohm Ireland Ltd, Athy Road, Co. Carlow, Ireland). The probe was calibrated at 25 °C with a KCl solution of known conductivity (12.9 mS cm^{-1}). Sufficient time (1 min)

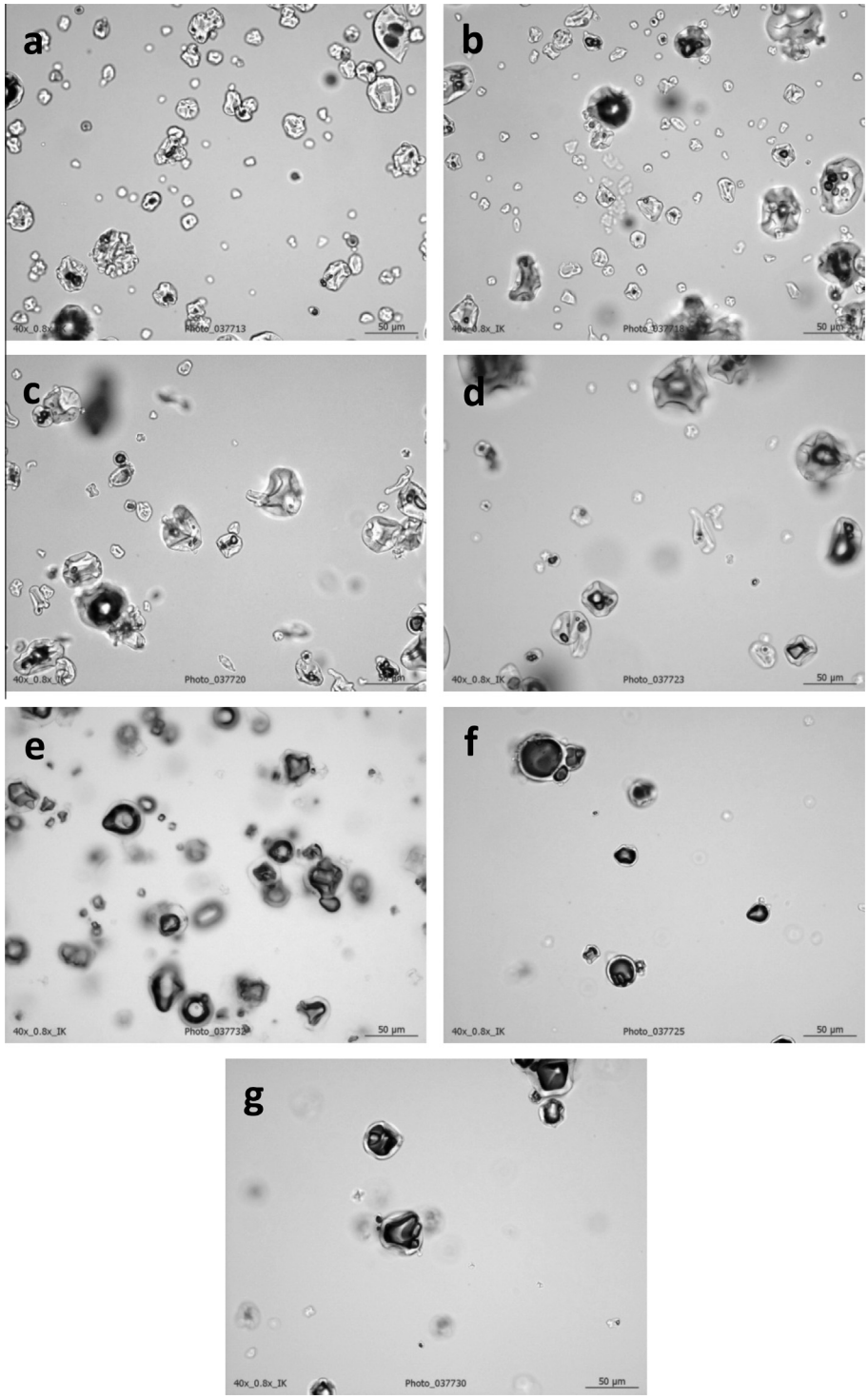

Fig. 1. Light microscopy images at 40× magnification of milk protein concentrate (MPC) 35 (a), MPC50 (b), MPC60 (c), MPC70 (d), MPC80 (e), MPC85 (f) and MPC90 (g). Powders were dispersed in paraffin oil on glass slides.

was allowed to elapse prior to powder addition to establish a baseline reading for conductivity, with powder then being added over a period of 1 min.

2.5. Dispersion: particle size distribution

A Malvern Mastersizer 2000 S (Malvern Instruments Ltd., Malvern, UK), a single lens static light-scattering system, with a helium–neon laser with 2 mW power and a dual-wavelength of 466 and 633 nm, was used to measure PSD in MPC suspensions after rehydration for 90 min and 24 h. Analysis of PSD was performed using the generalised polydisperse model with the 3NHD presentation using the following parameters: particle refractive index of 1.46, absorption of 0.1 and dispersant refractive index of 1.33, based on the recommendations of the manufacturer. MPC suspensions were introduced into the dispersing unit (deionized water as dispersant) of the instrument until a laser obscuration of 14 ± 1% was achieved. Data were presented as volume-based

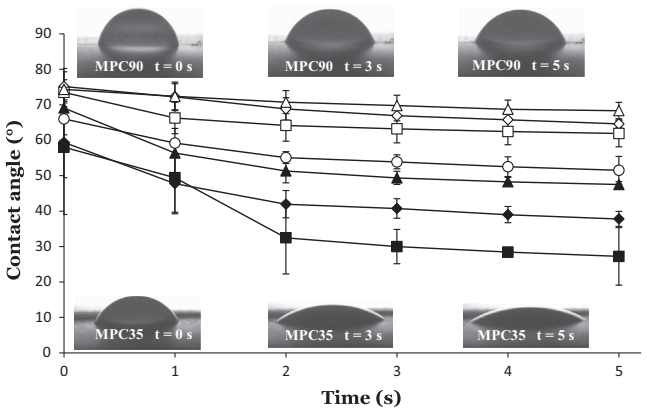

Fig. 2. Contact angle developed between a deposited drop of deionised water and the surface of a compressed disc of milk protein concentrate (MPC) 35 (■), MPC50 (♦), MPC60 (▲), MPC70 (○), MPC80 (◇), MPC85 (□) and MPC90 (Δ) powder over time. Values are presented as mean ± standard deviation of data from three independent trials. Representative images of droplets on MPC35 and MPC90 after 0, 3 or 5 s of analysis are shown in inset.

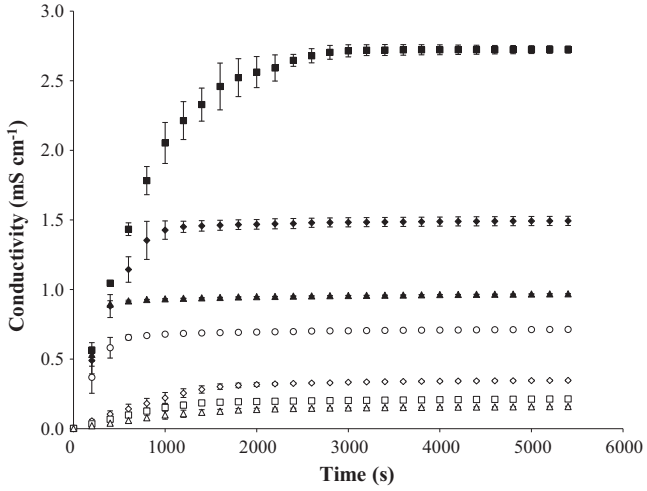

Fig. 3. Conductivity of suspension during the rehydration of milk protein concentrate (MPC) 35 (■), MPC50 (♦), MPC60 (▲), MPC70 (○), MPC80 (◇), MPC85 (□) and MPC90 (Δ) in deionised water measured dynamically over 90 min at 25 °C. Values are presented as mean ± standard deviation of data from three independent trials. Every 100th profile is shown.

PSDs. For each MPC, a peak <1 μm was observed and was deemed to represent casein micelles, and a second peak at >10 μm was deemed to represent primary powder particles (i.e., non-agglomerated particles formed from atomized droplets during spray drying).

2.6. Dispersion: sedimentation and sediment compression

An analytical centrifuge (LUMiSizer®, L.U.M. GmbH, Berlin, Germany), which measures the intensity of transmitted NIR light as a function of time and position over the length of a cell held horizontally over the light path, was used to measure sedimentation behaviour in MPC suspensions rehydrated for 90 min and 24 h. The measuring principle of the instrument was described in detail elsewhere (Crowley et al., 2014c). Polycarbonate cells (2 mm light path) were filled with 400 μL of MPC suspension with a wide-bore (16.5 gauge) needle. Measurements were performed using two centrifugal steps, 36*g* for 10 min followed by 168*g* for 10 min. The temperature during centrifugation was maintained at 25 °C. The transmission profiles after 10 s, and 5, 10, 15 and 20 min centrifugation are presented. Sediment height was calculated by first plotting the movement of the phase boundary (sediment-aqueous phases) against time and then subtracting the steady-state value from the position of the cell bottom (129 mm, determined based on sedimentation profile of deionised water).

3. Results and discussion

The composition and physical properties of the powders relevant to the current rehydration study are shown in Table 1. MPC powders were characterised by increasing levels of protein and marked depletion of lactose and minerals as increasing protein concentration factors were applied using membrane filtration during manufacture (Crowley et al., 2014d). There was a trend of increasing occluded and interstitial air content as protein content increased, resulting in reduced bulk density (Table 1); in addition, particle size increased with protein content for MPC35, MPC50 and MPC60, and decreased thereafter for higher protein MPCs, due to differences in the viscosity of the retentates and liquid concentrates which entered the spray drier (Crowley et al., 2014a). Images from light microscopy (Fig. 1) showed that, as protein content increased for the MPC powders, dark regions became more prominent within the particles; these dark regions, surrounded by distinct white regions, indicated the presence of air vacuoles and, by inference, protein-rich "skins" at the surface of the particles. The large air vacuoles observed for MPC80, MPC85 and MPC90 have also been observed in commercial high-protein MPC powders (Mimouni et al., 2009).

3.1. Wetting behaviour

Contact angle (θ) is a commonly used index of wetting behaviour in a diverse number of subject areas, including food-, soil-, plant-, nano-, and pharmaceutical-sciences. On initial deposition of a water droplet on the surface of powder discs, values of θ between 55° and 75° were measured (Fig. 2). High-protein MPC powders (MPC80, MPC85, MPC90) displayed θ values at $t = 0$ of ~75°, which is close to the value of 90° reported typically for non-wetting materials, such as waxes and lipids (Kwok and Neumann, 1999). However, it must be noted that the non-uniform surface topography and chemical heterogeneity of these powders may have resulted in higher measured θ values than real θ values (Forny et al., 2011); thus, it is more useful to consider the change in θ values over time (Gianfrancesco et al., 2011).

It can be seen clearly from Fig. 2 that high-protein MPC powders did not imbibe the deposited water droplet to any great extent during the period of analysis (5 s); this is in contrast to the low-protein powders (MPC35, MPC50), in particular, and also intermediate-protein (MPC60, MPC70) powders, which underwent marked reductions in θ values over 5 s. The higher levels of lactose in the low- and intermediate-protein MPC powders are likely to have increased water transfer into these powders due to increased hydrophilicity (Gaiani et al., 2006). The protein-rich skins observed in high-protein MPCs (Fig. 1) may have also increased the hydrophobicity of the powders; indeed, Fyfe et al. (2011) reported that the development of these skins, or "crusts", during storage of an MPC80 resulted in increased non-polar bonding at dry particle surfaces (measured using X-ray photoelectron spectroscopy) and greater attractive forces between rehydrated MPC80 and a hydrophobic surface (measured using atomic force microscopy).

Results of measurement of θ are most relevant to the initial stages of rehydration, in which powder particles make contact with water prior to subsequent sinking, swelling, dispersion and dissolution (Crowley et al., 2014b). Although extraction of a true static value for θ is not possible, owing to the complex surface

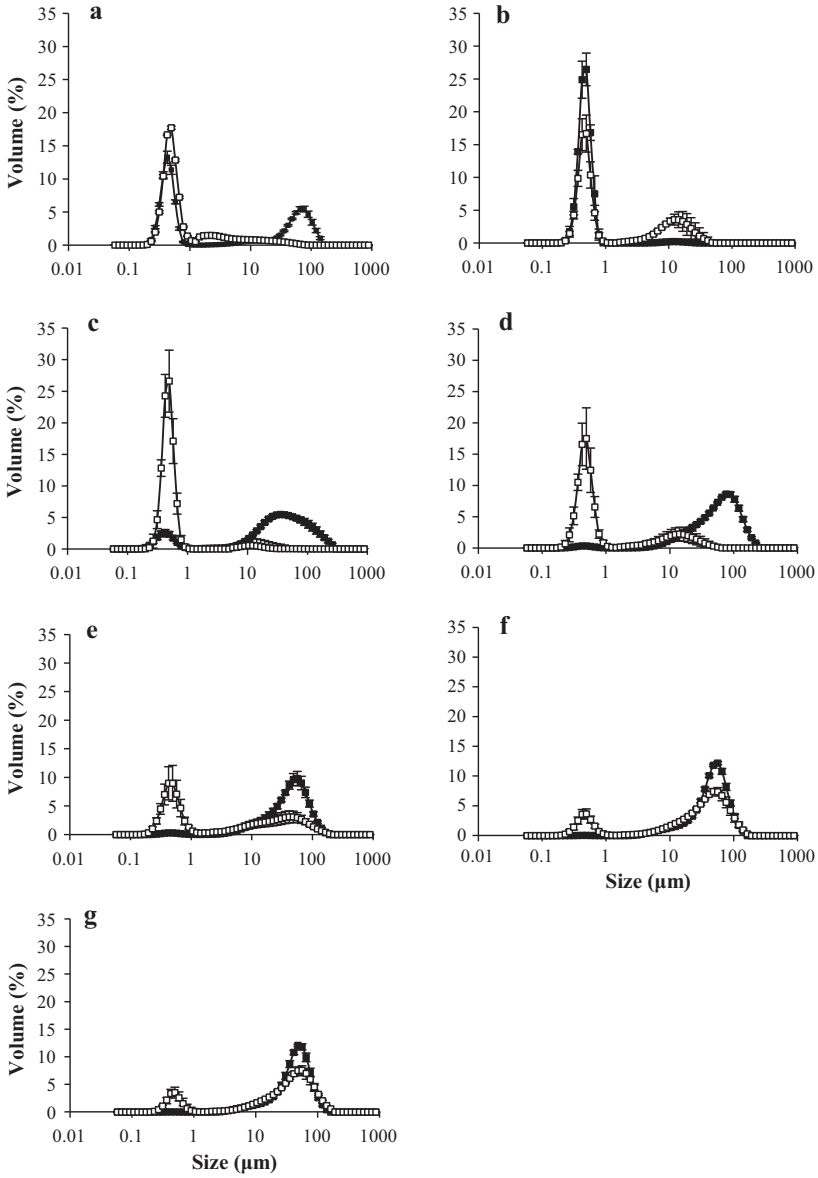

Fig. 4. Particle size distribution (PSD) data for milk protein concentrate (MPC) 35 (a), MPC50 (b), MPC60 (c), MPC70 (d), MPC80 (e), MPC85 (f) and MPC90 (g) after 90 min (■) and 24 h rehydration (□) in deionised water at 25 °C. Values are presented as mean ± standard deviation of data from three independent trials.

chemistry and topology of the MPC powders, it is clear that low-, intermediate- and high-protein powders could be classified into three distinct groups, according to their wetting behaviour (Fig. 2), with increasing protein content negatively affecting wetting. Thus, although wetting is known not to be the rate-limiting stage of rehydration for such casein-dominant powders (Schuck et al., 2007), wetting times are still likely to increase with increasing protein content, which, hence, negatively affects rehydration.

3.2. Mineral release/ionic strength

Measurement of conductivity is a useful technique for the investigation of the release of minerals during the rehydration of powders and the ionic strength of reconstituted powders. In conductivity measurements, the contribution of proteins, colloidal minerals and lactose do not need to be considered (Therdthai and Zhou, 2001), while the contribution of fat, which can reduce conductivity through inhibition of ion mobility (Zhuang et al., 1997), can be ignored in this case due to the very low levels found in the MPC powders (Table 1). Thus, free cations and anions in the serum phase of rehydrating MPC suspensions can be considered as the primary species contributing to conductivity profiles.

Although Marabi et al. (2008) considered steady-state conductivity to indicate complete rehydration of their model food powder systems, this is not appropriate for MPC powders, where primary particles with poor water transfer and dispersion properties persist after extended periods of rehydration (see Section 3.3), irrespective of the dissolution state of other components. The data presented in Fig. 3 indicate that the release of minerals was complete for all MPC powders within the 90 min period of rehydration, in agreement with the findings of Mimouni et al. (2010a), who stated that non-micellar minerals comprised the fast-release fraction, along with lactose and whey proteins, during the rehydration of MPC85. Normalisation of the conductivity data indicated that there were no distinct trends with regard to differences in the rate at which minerals were released during rehydration of the powders (see Fig. S1).

It is known that high-protein casein-dominant powders, which are otherwise poorly-dispersible, rehydrate readily in milk

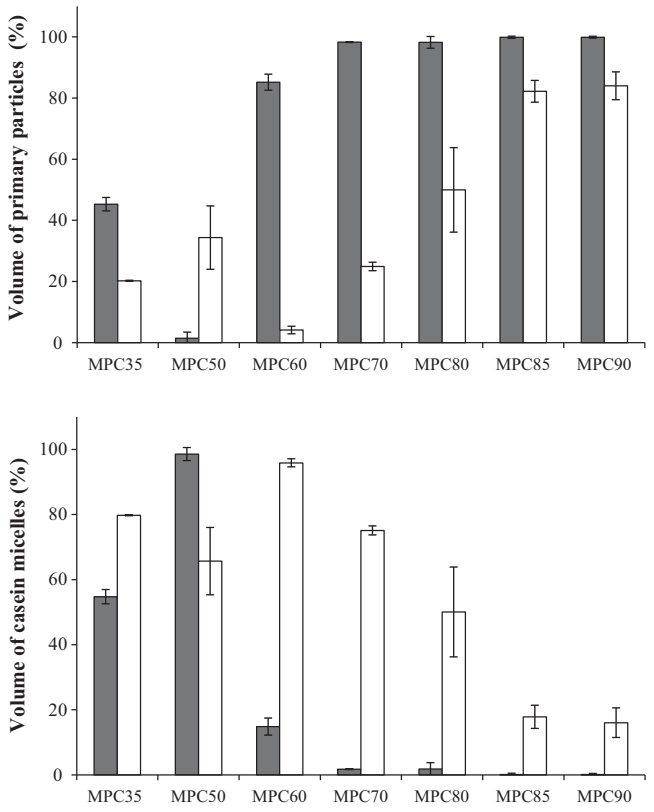

Fig. 5. Volume (% of total particle population) of primary particles and casein micelles after 90 min rehydration (grey bar) or 24 h rehydration (white bar) in deionised water at 25 °C. Values are presented as mean ± standard deviation of data from three independent trials.

(Udabage et al., 2012) or other high-ionic strength media such as NaCl solutions (Schuck et al., 2002; Gaiani et al., 2007; Hussain et al., 2011). It is clear from the conductivity profiles for the MPC suspensions during rehydration (Fig. 3) that steady-state values of conductivity decreased markedly with increasing protein content. This indicates a progressively lower ionic strength as protein content increased, due to depletion of minerals from the serum phase of skim milk when higher protein concentration factors were applied during the ultrafiltration/diafiltration process, which itself contributes to the markedly higher Ca-ion activity of high-protein MPCs reported previously (Crowley et al., 2014d). During the concentration and/or drying of MPCs, this high Ca-ion activity may facilitate enhanced charge-screening between casein micelles, with concomitant formation of poorly-dispersible aggregates *via* hydrophobic interactions (Havea, 2006). Indeed, replacement of Ca^{2+} in MPCs with Na^{+} using cation-exchange improves the rehydration properties of the resultant powders (Bhaskar et al., 2001; Havea, 2006). From the results in Fig. 3, it is clear that sufficient quantities of ions (e.g., sodium, phosphate, citrate) do not become available during the reconstitution of high-protein MPCs to counteract the potentially negative effects of high Ca-ion activity.

3.3. Dispersion characteristics

Analysis of PSD, after 90 min and 24 h rehydration, confirmed the results of numerous previous studies which reported the presence of large poorly-dispersible particles in high-protein MPCs after reasonable periods of rehydration (Fang et al., 2010, 2011; Mimouni et al., 2009, 2010b). In addition, it is clear that this phenomenon is not restricted to powders with >80% protein, with MPC60 and MPC70 also displaying similar behaviour. Two peaks were visible in the particle size profiles, representing (1) casein micelles at <1 μm and (2) primary powder particles at >10 μm (Fig. 4). After 90 min of rehydration, most MPC suspensions exhibited bimodal distributions, indicating the presence of both primary particles and casein micelles. At most, only ~2% of the particles in MPC70, MPC80, MPC85 or MPC90 consisted of casein micelles after 90 min of rehydration (Fig. 5). After 24 h of rehydration, the proportion of casein micelles increased, likely due to their release from the poorly-dispersible skin at the surface of primary particles, the proportion of which decreased simultaneously (Figs. 4 and 5). The one powder which deviated from this trend was MPC50, which had a greater proportion of larger particles when rehydration time was increased. Of the MPCs which were subjected to membrane filtration, MPC50 underwent the least extensive concentration. Thus, the resulting slight alterations in protein content and mineral balance may have created favourable conditions for protein–protein interactions, which resulted in aggregation during extended rehydration times; however, further research is required to investigate this possibility. In general, the results in Figs. 4 and 5 suggest that a greater quantity of primary powder particles remained undispersed as the protein content of MPC powders increased.

Analytical centrifugation was used to obtain information regarding the optical properties of the MPC suspensions, the sedimentation behaviour of primary particles, and the compressibility of the resultant sediment. Three representative sedimentation profiles for low- (MPC35), intermediate-(MPC70) and high-protein (MPC90) powders, after rehydration for 90 min, are shown in Fig. 6; other powders within these protein classes displayed very similar behaviour to the MPC powders selected. Depending on the powder, different regions can be identified in the sample cell as centrifugation progressed: stable dispersion, i.e., casein micelles in colloidal suspension; primary particles, i.e., primary powder particles concentrated towards the base of the cell, initially, but which sediment over time; initial sediment, i.e., formed from primary powder particles during low-speed centrifugation; and compressed sediment, i.e., a sediment layer with reduced height due to compaction with increased centrifugation speed.

After 90 min of rehydration, MPC35 did not undergo any sedimentation, despite the fact that >45% of its particle population was comprised of primary particles (Fig. 5). Conversely, the primary particles in MPC70 and MPC90 formed a distinct sediment layer during centrifugation, characterised by an optically dense region at the base of the cell (Fig. 6). For MPC70, this material was concentrated close to the base of the cell prior to the formation of a sediment layer, while for MPC90 it was dispersed throughout a larger region within the sample cell, resulting in far lower transmission readings than the region characterised by colloidal stability. Sediment height after 90 min of rehydration increased with protein content from MPC70 to MPC90 (Fig. 7), despite similar PSDs (Figs. 4 and 5). The sediment layers formed from these samples underwent compression when a higher centrifugal force was applied, with this effect being more pronounced for high-protein powders than MPC70 (Fig. 7). Lower transmission values above the region of sediment were observed as protein content increased; as protein content was equal in all suspensions, this effect was most likely due to entrapment of casein micelles (the dominant light-scattering population in skim milk) within the skin at the surface of sedimentable primary particles.

The sedimentation profiles for all samples after 24 h of rehydration indicated complete suspension stability, as per the profile of MPC35 in Fig. 6, despite the fact that primary particle-sized material remained in the suspensions (Figs. 4 and 5). Irrespective of rehydration time, the median diameter of primary particles which were present in high-protein MPC powders was ~50 μm (Fig. 4). Conversely, sedimentation behaviour in high-protein MPC powders was strongly dependent on rehydration time, with the

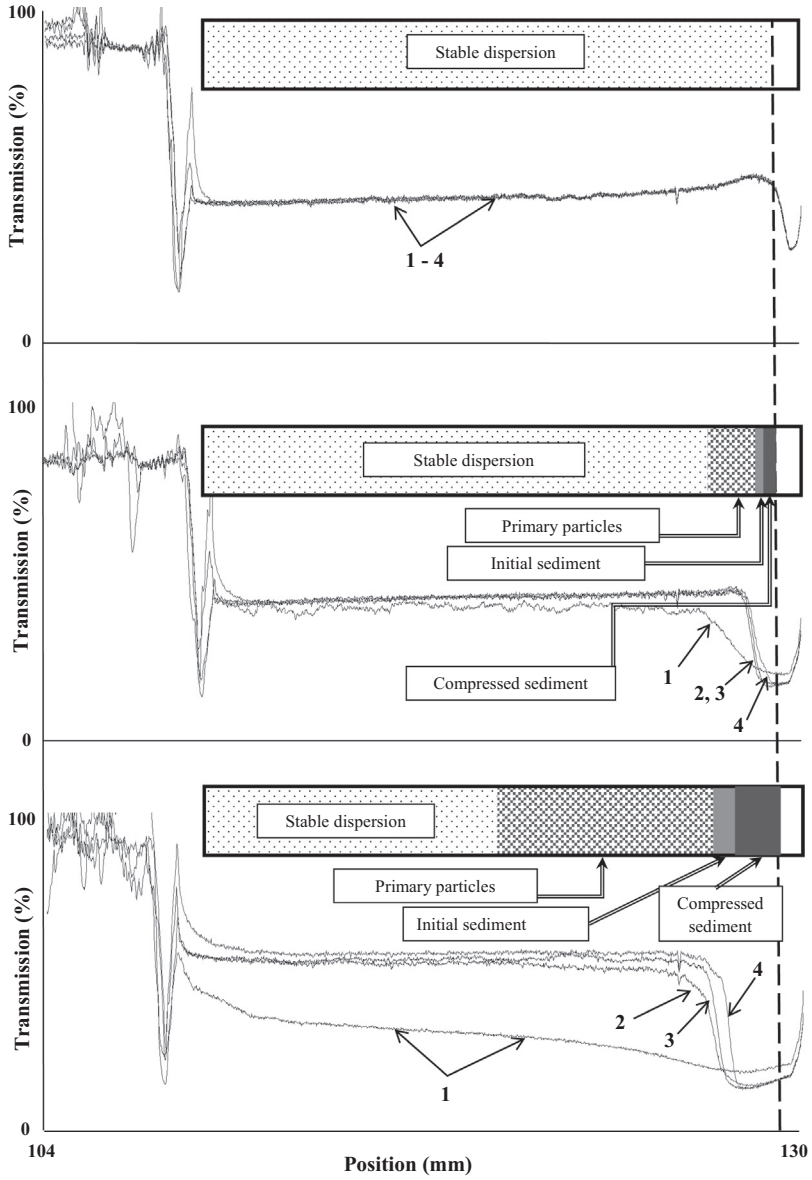

Fig. 6. Representative sedimentation profiles for milk protein concentrate (MPC)35 (top), MPC70 (middle) and MPC90 (bottom) after rehydration for 90 min in deionised water at 25 °C, showing transmission of NIR light through sample cells as a function of time (1 = 10 s, 2 = 5 min, 3 = 10 min, 4 = 20 min) and position in the sample cell. Profiles were captured as samples were centrifuged at 36*g* for 10 min followed by 168*g* for 10 min. A schematic illustration explaining the distinct regions which developed within the sample cell during centrifugation is shown in inset. The broken line indicates the position of the base of the sample cell, from which the height of the sediment, if present, can be calculated. In MPC35, the sample is dominated by casein micelles, which are stable in suspension and do not sediment, resulting in no change in transmission over time. Conversely, for MPC90, there are primary particles present throughout the cell initially, which cause very low transmission after 10 s; during centrifugation, a sediment forms from these particles, resulting in low transmission towards the base of the cell and high transmission elsewhere; this sediment then becomes compressed at increased centrifugation speed (giving higher optical density and reduced sediment height).

primary particles sedimenting after 90 min but not 24 h of rehydration, despite having the same effective particle size. Analysis of the PSD of MPC90 after 24 h rehydration with the addition of octanol in the dispersing line of the light-scattering instrument was used to assess if air incorporation was responsible for the larger peak. The results (see Fig. S2) showed the same PSD with or without octanol, indicating that the peak representing large particles in rehydrated MPCs was not due to air bubbles. Thus, these results appear to support the hypothesis of Mimouni et al. (2009) that, where extensive erosion of the protein-rich surface skin has occurred, primary particles in high-protein MPC powders may still retain the same effective size if sufficient quantities of material have not been removed to induce total structural collapse. However, while particle sizes may indeed be similar, the density of eroded primary particles would be expected to be much lower, and the viscosity of the continuous phase slightly higher, resulting in the observed stability to centrifugation of MPC suspensions containing poorly-dispersible primary particles.

3.4. Effect of temperature and additives on MPC90 solubility

To measure the effect of temperature and additives on the dispersion characteristics of MPC90, solutions were prepared using KCl, trisodium citrate (TSC) or polysorbate80 (PS80). The dispersants had a theoretical ionic strength of 80 mM (equivalent to 0 mM for PS80, as it is a non-ionic surfactant), to match that of the milk UF permeate (UFP), which was also tested. MPC90 was then rehydrated at 25 or 50 °C for 90 min and sediment height was assessed after centrifugation at 36*g* for 10 min to avoid

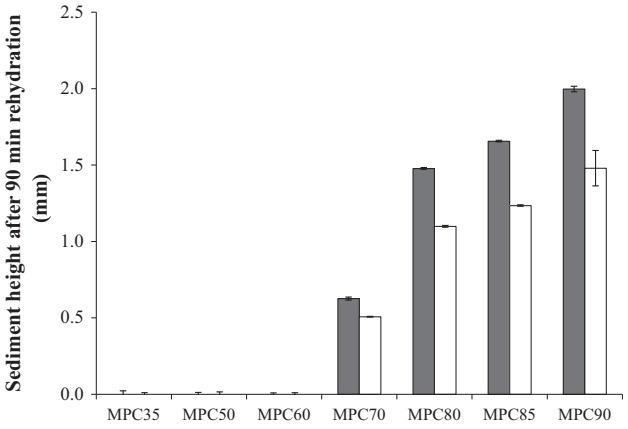

Fig. 7. Height of sediment formed for milk protein concentrate (MPC) powders, which had been rehydrated for 90 min, after centrifugation at 36*g* for 10 min (grey bar) followed by 168*g* for a further 10 min (white bar) at 25 °C. Values are presented as mean ± standard deviation of data from three independent trials.

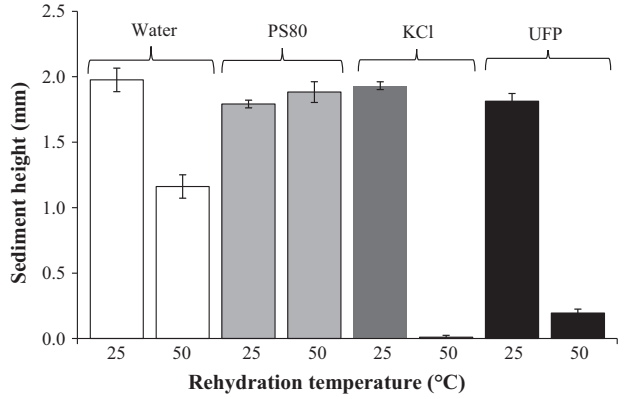

Fig. 8. Height of sediment formed for milk protein concentrate (MPC)90 powder, which had been rehydrated for 90 min at 25 or 50 °C in water, polysorbate 80 (PS80), KCl, or ultrafiltration permeate from milk (UFP), after centrifugation at 36*g* for 10 min. Values are presented as mean ± standard deviation of data from two independent trials.

compression of the sediment at higher centrifugal forces (see Fig. 7), with results shown in Fig. 8.

Increasing the temperature of reconstitution to ~50 °C is common industrial practice when a dairy powder is difficult to reconstitute; however, the correlation between increased temperature and improved rehydration is often weak (Fang et al., 2010; Richard et al., 2013), as it depends on the composition, process- and storage-history of the powder. A higher temperature of reconstitution resulted in a strong decrease in sediment height for MPC90 rehydrated in water (Fig. 8); however, a considerable amount of sedimentable material remained. Thus, a greater input of thermal energy was not sufficient to eliminate sedimentation of proteins after rehydration of MPC90 in the time-course studied.

Irrespective of temperature, rehydration of MPC90 in TSC caused extensive dissociation of casein micelles (NIR light transmission >80%; see Fig. S3). A few mm-sized wetted lumps of powder remained floating on the surface of MPC90/TSC suspensions after 90 min (disappeared by ~110 min) which could not be filled into the LUMiSizer sample cells; thus, no sediment could be detected for MPC90/TSC. TSC is a strong calcium-binder and, when added to milk, causes a shift in calcium and phosphorus from the micellar- to the serum-phase, with concomitant dissociation of casein micelles (Gaucheron, 2005). Although TSC has been reported to improve the solubility of high-protein casein-dominant powders (Schuck et al., 2002), it is clear that it also extensively alters the properties of the micellar phase as a secondary effect, which may be undesirable in many applications where maintenance of micellar structure is considered important.

PS80 is a non-ionic surfactant commonly used as an ingredient in ice-cream, where it contributes to shape retention and melting properties (Muse and Hartel, 2004). In addition, Lallbeeharry et al. (2014) reported that whole milk powder was more wettable when manufactured by co-drying of milk with PS80 compared to milk co-dried with lecithin, an ionic surfactant. In this study, PS80 caused only a slight decrease in sediment height at 25 °C, while the otherwise positive effect of increased rehydration temperature on the solubility of MPC90 was eliminated (Fig. 8). These results suggest that the presence of PS80 does not improve the solubility of high-protein MPCs and that, in addition, where elevated reconstitution temperatures are used, PS80 may actively prevent effective dispersion of poorly-dispersible material. The concentration of PS80 used in this study (80 mM) was considerably higher than the critical micelle concentration (~0.01 mM). As these micelles were uncharged, electrostatic interactions with proteins can be discounted. However, at elevated temperatures, hydrophobic interactions between micellar PS80 and casein would have been promoted. As a result of hydrophobic interactions with PS80 micelles, the dispersion of poorly-dispersible casein particles may have been inhibited further.

The greatest reduction in sediment height was found when MPC90 was reconstituted in KCl or UFP, but only at a higher temperature of reconstitution (Fig. 8). KCl had a slightly more positive effect than UFP, suggesting that the presence of lactose and a more complex salt system in the latter did not improve MPC90 rehydration. These results indicate that ionic strength is a key factor during the rehydration of MPCs. However, even after rehydrating MPC90 in KCl or UFP at 50 °C, primary-sized particles were still measured (see Fig. S4), indicating again that dispersion was not complete. It should also be noted that the pH values of MPC90/KCl and MPC90/UFP suspensions were 7.2 ± 0.02 and 6.54 ± 0.02, respectively, suggesting a possible role of pH in determining the rehydration properties of MPCs. A more acidic pH would increase Ca-ion activity further (Crowley et al., 2014d), resulting in even greater screening of charge between casein micelles, which could exacerbate the tendency for casein micelles to form, and remain as, large aggregates. The pH of MPC90/water was somewhat alkaline at 7.05 ± 0.06 and yet yielded a larger sediment than MPC90/UFP, which seems to contradict this principle; however, the former system had a far lower ionic strength (Fig. 3), which would also contribute to an increased Ca-ion activity. The individual roles of pH, ionic strength and ionic composition in determining the solubility of MPCs are poorly understood, and further study is warranted to separate their respective effects.

4. Conclusion

High-protein MPC powders (e.g., MPC80, MPC85, MPC90) exhibited the poorest rehydration properties, but the rehydration properties of intermediate-protein powders (e.g., MPC70), which are less well characterised, were also observed to be poor. Low ionic strength seems to limit the dispersion of primary particles during the rehydration of intermediate- and high-protein MPCs. This effect is likely due to a combination of high Ca-ion activity and poor solvent quality, which promotes non-covalent interactions between casein micelles and stabilises the resultant aggregates. Restoration of the ionic strength of milk using KCl or UFP, when combined with an elevated temperature of reconstitution, is an effective method of attaining a stable suspension after a short rehydration time (90 min). However, although stable suspensions of MPCs can be attained after extended periods of rehydration, or

using appropriate combinations of elevated temperature and additives, primary particle-sized material may still remain, with potentially negative implications for MPC functionality (e.g., gelling, emulsification).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfoodeng.2014.09.033.

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CHAPTER 4

Heat stability of reconstituted milk protein concentrate powders

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. SVC co-designed the study, provided analytical support/training to MM and analysed the data. MM assisted with heat coagulation time experiments. IG and TH provided the powders, powder composition data, calcium-ion activity data and protein profile data. The chapter is included in its final published form.

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Heat stability of reconstituted milk protein concentrate powders

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ABSTRACT

Milk protein concentrates (MPCs) were manufactured from skim milk at pilot scale by ultrafiltration, or ultrafiltration and diafiltration, to different protein concentration factors. MPCs were then spray-dried into seven powders ranging from 36.6% (w/w; MPC35) to 89.6% (w/w; MPC90) protein in dry matter. Powders were reconstituted to 3.5% (w/w) protein and the heat stability of MPC suspensions measured at 140 °C in the pH range 6.3–7.3. At pH < 6.8, the heat stability of MPC suspensions decreased with increasing protein content of the MPC powders, due to high Ca-ion activity. At pH > 6.8, the destabilising influence of increased Ca-ion activity with increasing protein content of the MPC powders was countered partially by reduced heat-induced κ-casein dissociation. The heat stability of MPC80 was restored by re-establishment of the serum composition of skim milk; fortification with lactose or urea only affected heat stability outside the pH region where rapid Ca-induced coagulation occurred.

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1. Introduction

Milk is subjected regularly to thermal treatments, the intensity of which varies widely, and while milk typically withstands most of these treatments, instability to heating can occur in extreme conditions. Such instability manifests itself in the form of heat-induced coagulation of milk. Compositional factors influence greatly the heat-induced destabilisation of milk and other dairy protein-based systems (Horne & Muir, 1990). The formation of complexes between β-lactoglobulin and κ-casein, in the serum or colloidal phases, is associated with regions of minimum and maximum heat stability in milk, respectively (Oldfield, Singh, Taylor, & Pearce, 2000; Rose, 1961). The production of organic acids (mainly formic) through heat-induced degradation of lactose (Fox, 1981; O'Brien, 2009) and high Ca-ion activity (Philippe, Gaucheron, Le Graët, Michel, & Garem, 2003; Sievanen, Huppertz, Kelly, & Fox, 2008) have negative effects on heat stability. Urea, the principal component of the non-protein nitrogen (NPN) fraction of milk, has a stabilising influence during the heating of milk, which is attributed primarily to the buffering effect of its degradation products (Metwalli & van Boekel, 1996). Reduced levels of phosphate cause a shift in regions of heat stability to more alkaline pH values (O'Connell & Fox, 2003; Rose, 1961). In addition, phosphates, as well as other Ca-binding salts, are sometimes added to milk to improve heat stability, due to their ability to increase buffering capacity and reduce Ca-ion activity (De Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2012).

Milk protein concentrates (MPCs) are produced by concentrating pasteurised skim milk using ultrafiltration (UF), which is sometimes followed by diafiltration (DF) and/or evaporation. MPCs may then be spray-dried into powders with protein concentrations ranging between 35% (w/w; MPC35) and 90% (w/w; MPC90) protein in dry matter, depending on the protein concentration factor achieved during membrane processing (Mulvihill & Ennis, 2003). MPC powders have been studied as ingredients in numerous food products including ice cream (Alvarez, Wolters, Vodovotz, & Ji, 2005), high protein bars (Loveday, Hindmarsh, Creamer, & Singh, 2009), cheese (Francolino, Locci, Ghiglietti, Iezzi, & Muccheti, 2010) and dairy beverages (Giroux, Houde, & Britten, 2010). The solubility characteristics of MPC powders have also been the focus of a number of studies (Anema, Pinder, Hunter, & Hemar, 2006; Fang, Selomulya, & Chen, 2010; Havea, 2006). However, the heat stability of reconstituted MPC powders has yet to be established. In various applications, MPC powders are likely to be reconstituted and subjected to heat treatments such as ultra-high temperature processing or retort sterilisation; thus, it is important to determine the heat stability of reconstituted MPC powders to prevent process variability or losses in product quality due to heat-induced destabilisation.

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The objective of this study was to determine the heat stability at 140 °C of a range of MPC powders after reconstitution to 3.5% (w/w) protein MPC suspensions. Ca-ion activity and the distribution of proteins in unheated and heated samples were measured to develop mechanistic understanding for differences observed in pH-heat coagulation time (pH-HCT) profiles. In addition, the serum phase of MPC80 was modified by dialysis against skim milk or the reintroduction of various serum phase constituents (i.e., lactose, minerals, and urea) to determine the influence of these constituents on heat stability.

2. Materials and methods

2.1. Materials and manufacture of MPC powders

Low-heat skim milk powder (SMP) was supplied by the Irish Dairy Board (Fermoy, Ireland). Dialysis tubing (12–14 kDa cut-off) was obtained from Medicell (London, UK). Urea was supplied by Merck Group (Darmstadt, Germany). The maltodextrin used was Maldex 170 (Tereos Syral, Marckolsheim, France), with a dextrose equivalent of 17, which was supplied by Corcoran Chemicals Ltd. (Dublin, Ireland). All other chemicals and reagents were acquired from Sigma Chemical Co. (St Louis, MO, USA).

MPCs were produced from two batches of pasteurised (72 °C for 15 s) skimmed bovine milk of identical composition at NIZO food research (Ede, The Netherlands). For each MPC produced, ~700 kg of milk were used. UF and DF were performed at 50 °C using 10 kDa cut-off membranes (Microdyn-Nadir GmbH, Wiesbaden, Germany) with a total membrane surface area of 36 m^2. Following membrane processing, the retentates for MPC35, MPC50, MPC60 and MPC70 were concentrated on a 4-stage falling film evaporator. For MPC80, MPC85 and MPC90, no evaporation step was applied. All samples were dried using a single-stage Niro 25 spray drier (GEA Niro, Soborg, Denmark) with nozzle atomisation and air inlet and outlet temperatures of 185–190 °C and 85–90 °C, respectively. MPC powders were packaged in cans immediately after manufacture and stored at −25 °C.

2.2. Compositional analysis

Total solids content was determined by oven drying (IDF, 1987). Lactose content was determined by high-performance liquid chromatography (HPLC) using the method of Koops and Olieman (1985). Total nitrogen, non-protein nitrogen, and true protein were determined as outlined in (ISO, 2001a, 2001b, 2001c) using the Kjeldahl method and a nitrogen-to-milk protein conversion factor of 6.38. Ash content was determined after heating samples at 550 °C until a white ash was obtained, as outlined by NEN (1998). The ash was analysed subsequently by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) to determine the concentration of total Ca. Quantification of inorganic phosphorus (P_i) was performed on the filtrate of the 12% trichloroacetic acid-soluble fraction of 3.5% protein MPC suspensions according to IDF (1990). The composition of the MPC powders after reconstitution to 3.5% protein (see Section 2.3) was calculated from the measured MPC powder composition data and is summarised in Table 1.

2.3. Preparation of MPC suspensions for heat stability testing

For heat stability testing at 140 °C, MPC powders were reconstituted to 3.5% protein in deionised water. MPC suspensions were stirred magnetically at ~20 °C for a minimum of 3 h and then held at 4 °C in sealed beakers for ~18 h to achieve complete rehydration. In addition, MPC80 was reconstituted in simulated milk ultrafiltrate (SMUF; Jenness & Koops, 1962) or in solutions of 0, 100, 200, 300 or 400 mg L^{-1} urea, 0, 0.72, 1.72, 2.72 or 4.62% (w/w) lactose or 4.62% (w/w) maltodextrin. Finally, reconstituted MPC80 (pH adjusted to 6.8) was also dialysed (12–14 kDa cut-off) against 2 × 16.7 volumes of reconstituted SMP (3.5% protein) at 4 °C. After dialysis, deionised water was added to counteract osmosis-mediated concentration of the MPC80 suspension.

2.4. Heat stability testing

The heat coagulation time (HCT) of the MPC suspensions was determined at 140 °C as a function of pH (6.3–7.3) using the method of Davies and White (1966). Samples (2.5 mL) were filled into glass tubes (length, 130 mm; external diameter, 10 mm; wall thickness, 2 mm) and placed in an oil bath, with HCT determined as the time that elapsed between placing samples in the oil bath and the first visible onset of coagulation. Samples were rocked at a rate of ~8 min^{-1} and the heating up time for samples to reach 140 °C was ~2 min.

2.5. Protein distribution in unheated and heated MPC suspensions

MPC powders were reconstituted to 3.5% protein, with adjustment of pH to 6.5, 6.8 or 7.1 using 1 M NaOH or 1 M HCl. Subsamples were heated at 90 °C for 30 min, and then equilibrated at 20 °C for 1 h. Control MPC suspensions were prepared in a similar manner, without heat treatment. The equilibrated MPC suspensions were ultracentrifuged at 100,000 × *g* and 20 °C for 1 h. Whole samples and supernatants were analysed for the concentration of κ-, α_{S1}- and β-casein, and β-lactoglobulin and α-lactalbumin by reversed-phase HPLC as outlined by Hinz, Huppertz, and Kelly (2012). The percentage of sedimentable and non-sedimentable protein was calculated based on the peak areas for each of the five proteins in the whole sample and the supernatant.

2.6. Ca-ion activity in MPC suspensions

MPC powders were reconstituted to 3.5% (w/w) protein and adjusted to pH 6.3, 6.5, 6.8 or 7.1 using 1 M NaOH or 1 M HCl. Standard solutions were prepared with KCl and $CaCl_2$ to an ionic strength of 10, 20, 40, 60 or 80 mM and a Ca concentration of 0.00, 0.25, 0.50, 1.00, 2.00 or 3.00 mM at each ionic strength. All samples

Table 1
Composition of milk protein concentrate (MPC) suspensions calculated from measured values for MPC powders. Values are the means of data from duplicate analysis, with the exception of lactose and inorganic phosphorus values, each of which are from a single analysis.

MPC	Protein (%, w/w)	Non-protein nitrogen (%, w/w)	Lactose (%, w/w)	Ash (%, w/w)	Solids (%, w/w)	Calcium (mg g^{-1})	Inorganic phosphorus (mg g^{-1})
MPC35	3.50	0.032	4.90	0.80	9.55	1.35	0.84
MPC50	3.50	0.016	2.51	0.54	6.75	1.21	0.63
MPC60	3.50	0.010	1.41	0.45	5.53	1.14	0.53
MPC70	3.50	0.008	0.92	0.41	4.94	1.12	0.50
MPC80	3.50	0.004	0.28	0.34	4.22	1.06	0.41
MPC85	3.50	0.003	0.08	0.31	3.97	1.06	0.36
MPC90	3.50	0.002	0.02	0.31	3.91	1.06	0.36

were equilibrated overnight at 20 °C. The pH of the milk samples was measured again after equilibration and re-adjusted if necessary.

Measurements on all samples and standards were performed using a Ca-ion-specific electrode and a conductivity meter. A linear relationship was established between conductivity (κ, in mS cm^{-1}) and the estimated ionic strength (I, in mM) according to:

$$\kappa = 0.1181 \times I - 0.0354 \quad (1)$$

Equation (1) was used to estimate the ionic strength of the MPC samples based on their measured conductivity. It should be noted here that the true ionic strength and conductivity of the serum phase of samples is the sum of the contributions of each individual cation and anion; however, given the dominant contribution of K^+ and Cl^- to the ionic strength and conductivity of milk (Walstra, Wouters, & Geurts, 2006), KCl was considered to provide an accurate representation of differences in conductivity and ionic strength among samples.

In addition, the following relationship was established between the measured Ca electrode potential (E_{Ca}, in mV) and the free Ca-ion concentration (c_{Ca}, in mM) present in the standard solutions:

$$E_{Ca} = a \times \log(c_{Ca}) + b \quad (2)$$

where a and b are constants dependent on ionic strength. The ionic strength dependencies of the constants a and b were found to be:

$$a = -0.236 \times \ln(I) + 13.573 \quad (3)$$

and

$$b = -3.543 \times \ln(I) - 32.199 \quad (4)$$

Therefore, with the aid of equations (1)–(4), the concentration of free Ca^{2+} ions could be calculated in the MPC suspensions.

The Ca-ion activity (γ_{Ca}) coefficient was calculated from the Debye–Hückel limiting law:

$$\log(\gamma_{Ca}) = \frac{-0.5 \times (Z)^2 \times \sqrt{I}}{1 + \sqrt{I}} \quad (5)$$

where Z is the valence number of Ca^{2+} (=2). Finally, the Ca-ion activity (a_{Ca}, in mM), was calculated as follows:

$$a_{Ca} = \gamma_{Ca} \times c_{Ca} \quad (6)$$

3. Results

3.1. Ca-ion activity in MPC suspensions

Ca-ion activity in MPC suspensions increased with increasing protein content of the MPC powders used at all pH values studied (Table 2). These increases can be attributed to two factors, i.e., small increases in Ca-ion concentration with increasing protein content of the MPC powders and strong increases in the Ca-ion activity coefficient due to the decreases in ionic strength of the serum phase of suspensions of high protein MPC powders (results not shown). In addition, Ca-ion activity decreased with increasing pH for suspensions reconstituted from all MPC powders (Table 2), presumably due to complexation of Ca with inorganic or organic phosphate as a result of the reduced solubility of Ca phosphates with increasing pH (Vaia, Smiddy, Kelly, & Huppertz, 2006). The increase in Ca-ion concentration in suspensions of high protein MPC powders is probably related to the fact that, on reconstitution of the samples, the serum phase is initially not saturated with respect to Ca phosphate; as a result, some micellar Ca and phosphate will dissolve until saturation is reached. In the serum phase of milk, Ca and phosphate are present at near-equimolar concentrations, most (~70%) of the serum Ca is in the form of the Ca citrate anion, and only ~20% of serum Ca is in the form of ionic Ca. In contrast, most phosphate is in the form of the mono- and di-hydrogen phosphate anions, resulting in a 1:4 molar ratio for calcium:(mono- + di-hydrogen phosphate) in milk serum (Walstra et al., 2006). However, solubilisation of micellar Ca phosphate from casein micelles, in a serum phase which can be considered to be essentially water, will result in a Ca:phosphate ratio which is much closer to unity and, hence, a higher concentration of ionic Ca, despite the lower solubility of Ca phosphate in the low ionic strength medium.

Table 2
Influence of pH on Ca-ion activity in suspensions of milk protein concentrates (MPCs) containing 3.5% (w/w) protein. Values are the means of data from duplicate analysis.

MPC	pH	Ca-ion activity (mM)
MPC35	6.30	1.30
	6.49	0.99
	6.81	0.63
	7.09	0.43
MPC60	6.31	1.74
	6.50	1.33
	6.80	0.87
	7.07	0.59
MPC80	6.30	2.20
	6.50	1.72
	6.79	1.14
	7.09	0.73
MPC90	6.31	2.68
	6.51	2.03
	6.80	1.49
	7.11	0.98

3.2. Protein distribution in heated and unheated MPC suspensions

Little or no denaturation of β-lactoglobulin or α-lactalbumin occurred during MPC production (data not shown), which is in agreement with the high level of non-sedimentable whey proteins in all unheated MPC suspensions (Fig. 1). Levels of non-sedimentable whey proteins were considerably lower in heated suspensions, which can be attributed to heat-induced denaturation of whey proteins and their aggregation and association with casein micelles (Oldfield et al., 2000). Virtually complete denaturation was observed for both α-lactalbumin and β-lactoglobulin in all heated samples (data not shown). Levels of non-sedimentable denatured whey protein increased with increasing pH at which the heat treatment was performed and decreased with increasing protein content of the MPC used.

Low levels of non-sedimentable α_{s1}- and β-casein were found in unheated samples. These levels increased slightly with increasing pH and, in most cases, decreased slightly as a result of heat treatment (Fig. 1). The protein content of the MPC powders used did not have a strong effect on these phenomena. Levels of non-sedimentable κ-casein also increased slightly with increasing pH in unheated MPC suspensions (Fig. 1). After heat treatment (30 min at 90 °C) at pH 6.5, the level of non-sedimentable κ-casein was lower in all samples than in their unheated counterparts, whereas at pH 6.8, heat treatment increased the level of non-sedimentable κ-casein in suspensions of MPC35, MPC50 and MPC60, but reduced it in suspensions of MPC80, MPC85 and MPC90. After heat treatment at pH 7.1, the level of non-sedimentable κ-casein increased in all samples, except for reconstituted MPC90. Overall, the extent of heat-induced dissociation of κ-casein in MPC

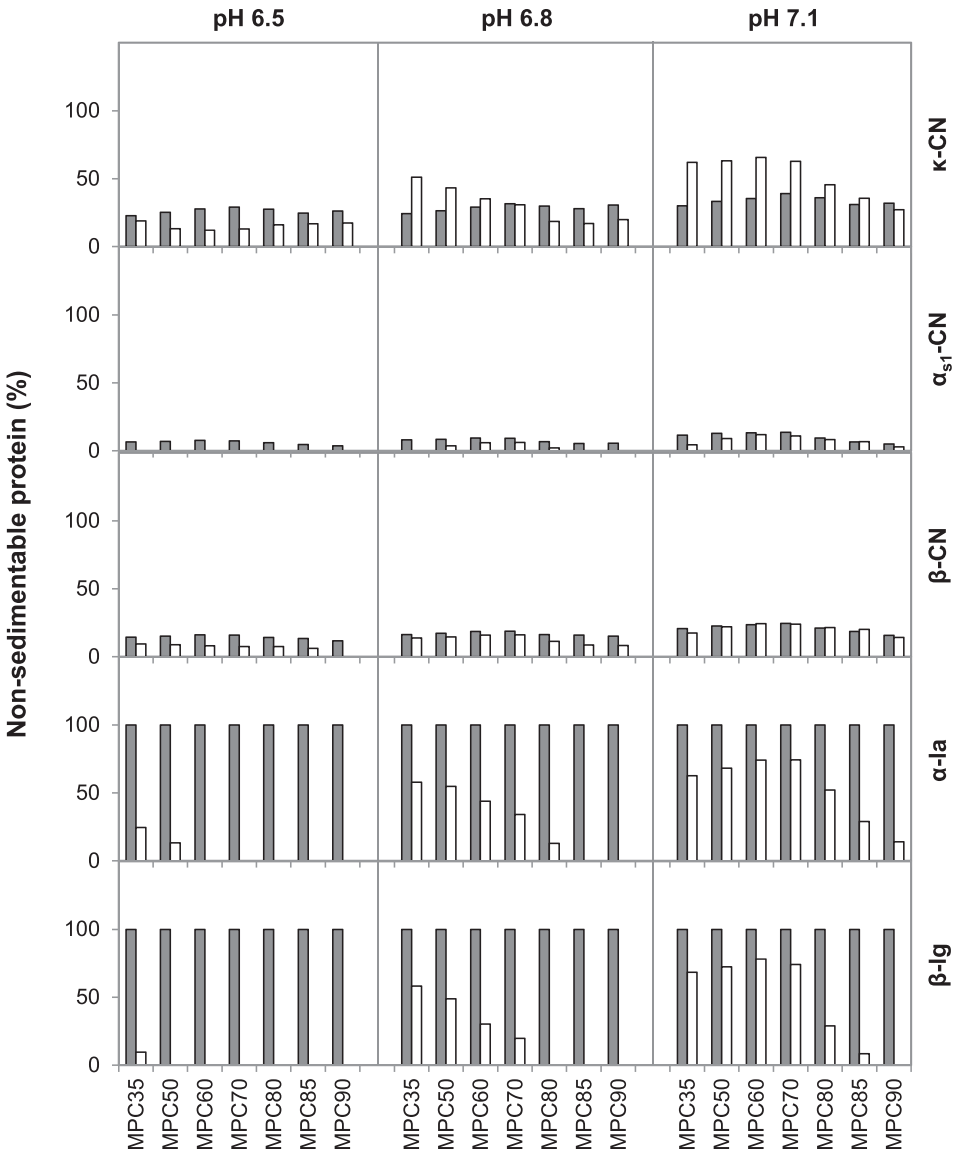

Fig. 1. Influence of pH (6.5, 6.8 or 7.1) on the percentage of κ-casein (κ-CN), α_{s1}-casein (α_{s1}-CN), β-casein (β-CN), α-lactalbumin (α-la) and β-lactoglobulin (β-lg) in the ultracentrifugal (100,000 × *g* for 60 min at 20 °C) supernatant of unheated (■) and heated (□; 30 min at 90 °C) suspensions of milk protein concentrate (MPC)35, MPC50, MPC60, MPC70, MPC80, MPC85 and MPC90 containing 3.5% (w/w) protein. Values are the means of data from duplicate analysis.

suspensions decreased with increasing protein content of the MPC powder used and increased with increasing pH at which the heat treatment was carried out (Fig. 1). Increased heat-induced dissociation of κ-casein with increasing pH is in agreement with numerous previous studies, as reviewed by Anema (2009). In addition, the reduced extent of heat-induced dissociation of κ-casein with increasing protein content of the MPC powders is in agreement with previous findings by Anema, Singh, and Creamer (1993) that more extensive heat-induced dissociation of κ-casein occurred in suspensions of SMP in SMUF than in water, and that the extent of heat-induced dissociation of κ-casein increased when increasing levels of NaCl were added to milk (Huppertz & Fox, 2006).

3.3. Heat stability of MPC suspensions

Suspensions of MPC35 exhibited a pH-HCT profile similar to that observed typically for bulk skim milk, i.e., with a maximum at pH ~6.7 and a minimum at pH ~7.0 (Fig. 2). For MPC50, a profile with a maximum and a minimum was also observed, but these occurred at a higher pH (7.0 and 7.2, respectively), while the HCT at the maximum and minimum were also higher than for MPC35. For suspensions of MPC60-MPC90, pH-HCT profiles did not exhibit a maximum or minimum. Instead, these MPC suspensions had pH-HCT profiles with very low HCT values at the acidic side of the spectrum. Above a certain pH, which increased with increasing protein content of the MPC powder used, HCT increased with increasing pH. For the suspension of MPC90, HCT was <2 min throughout the entire pH range studied (Fig. 2).

Added urea (0–400 mg L^{-1}) did not affect the HCT of suspensions of MPC80 at pH ≤ 6.8; however, at pH ≥ 6.9, HCT increased with increasing levels of added urea (Fig. 3). Likewise, the addition of lactose or maltodextrin did not affect the HCT of suspensions of MPC80 at ≤ 6.8 whereas, at pH ≥ 6.9, HCT decreased with increasing levels of added lactose (Fig. 4). The addition of maltodextrin also reduced HCT in this pH region, but to a considerably lesser extent than that observed for a similar quantity of lactose (Fig. 4).

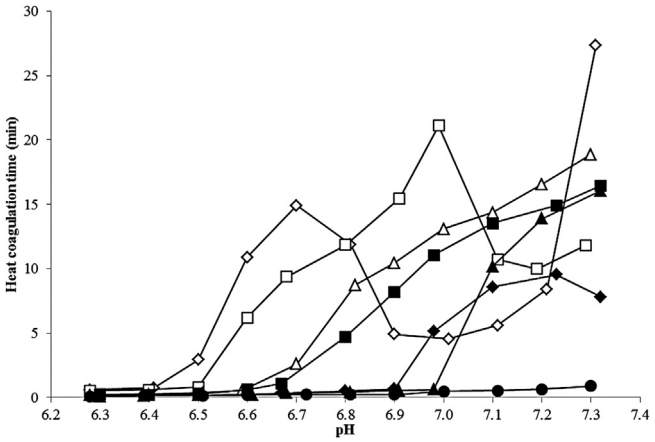

Fig. 2. pH-heat coagulation time (pH-HCT) profiles at 140 °C for milk protein concentrate (MPC)35 (◇), MPC50 (□), MPC60 (△), MPC70 (■), MPC80 (◆), MPC85 (▲) and MPC90 (●) reconstituted to 3.5% (w/w) protein. Values are the means of data from duplicate analysis.

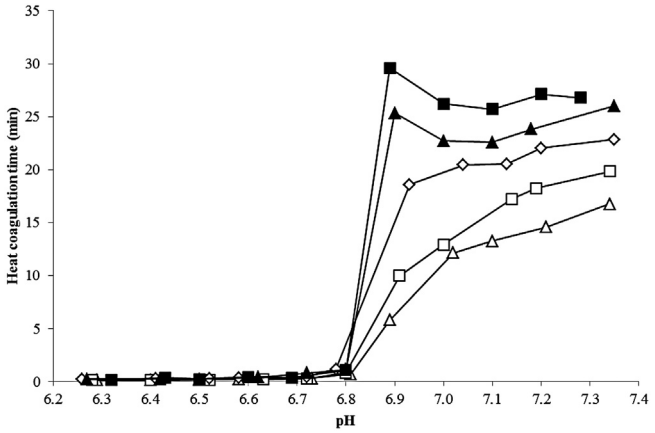

Fig. 3. pH-heat coagulation time (pH-HCT) profiles at 140 °C for a 3.5% (w/w) protein suspension of milk protein concentrate (MPC)80, with urea added at levels of 0 (△), 100 (□), 200 (◇), 300 (▲) or 400 (■) mg L^{-1}. Values are the means of data from duplicate analysis.

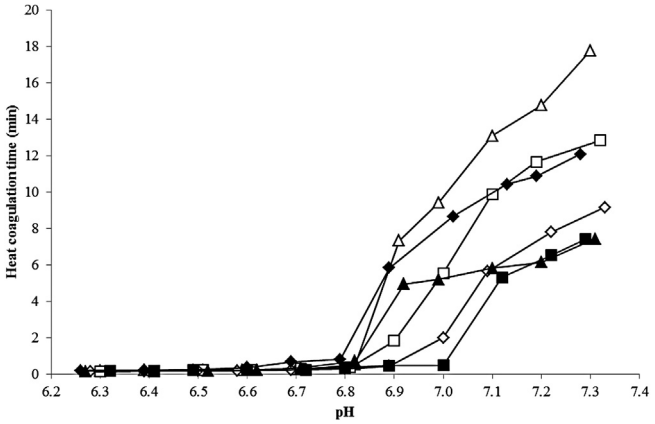

Fig. 4. pH-heat coagulation time (pH-HCT) profiles at 140 °C for a 3.5% (w/w) protein suspension of milk protein concentrate (MPC)80 with added lactose concentrations (%, w/w) of 0.00 (△), 0.72 (□), 1.72 (◇), 2.72 (■) or 4.62 (▲) or with 4.62% (w/w) added maltodextrin (◆). Values are the means of data from duplicate analysis.

When MPC80 was reconstituted in SMUF, or SMUF fortified with lactose and urea, rather than in water, HCT at pH ≤ 6.9 increased markedly and a maximum in the pH-HCT profile occurred at pH 6.7–6.8 (Fig. 5). In addition, a very broad HCT minimum, centred at pH ~7.1, was observed in suspensions of MPC80 in SMUF or SMUF fortified with lactose and urea. A more defined minimum, as well as a maximum, was observed in the pH-HCT profile of suspensions of MPC80 dialysed against reconstituted SMP (Fig. 5). The pH and HCT at the maximum and minimum for MPC80 dialysed against reconstituted SMP (Fig. 5) were comparable to that of MPC50 (Fig. 2).

4. Discussion

Fig. 2 illustrates clearly that the pH-HCT profiles of MPC suspensions are affected strongly by the protein content of the MPC powders from which they are reconstituted; this, in turn, also affects strongly the composition of the suspensions (Table 1). At equal protein content (3.5%), suspensions of MPC powders with increasing protein content were characterised by progressively lower total solids, lactose, NPN, ash, P_i and Ca levels. When considering the heat stability of milk and related systems, a number of parameters related to the serum phase are key contributors, most notably the destabilising influence of ionic Ca, the stabilising role of urea and the role of lactose, which may be destabilising (through heat-induced acidification) or stabilising (through Maillard reactions). In addition, heat-induced dissociation of κ-casein influences heat stability strongly, and particularly the occurrence and position of a minimum in the pH-HCT profile (O'Connell & Fox, 2003; Singh, 2004; Van Boekel, Nieuwenhuijse, & Walstra, 1989).

The heat-induced destabilisation of milk at pH ≤ 6.7 is attributed typically to a high Ca-ion activity in this pH region, combined with heat-induced acidification arising from degradation of lactose and heat-induced precipitation of tertiary Ca phosphate. The heat-induced association of denatured whey proteins with casein micelles is believed to have a stabilising action in this pH region (O'Connell & Fox, 2003; Singh, 2004; Van Boekel et al., 1989). Urea may have a stabilising role through the buffering capacity of one of its degradation products, ammonia. Another degradation product of urea, isothiocyanate, can provide a stabilising effect through the formation of homocitrulline after interactions with lysine (Metwalli & van Boekel, 1996). Heat-induced dissociation of κ-casein does not occur at pH < 6.7 (Anema, 2009; Singh & Fox, 1986, 1987) and is therefore not believed to be a major contributor to heat-induced

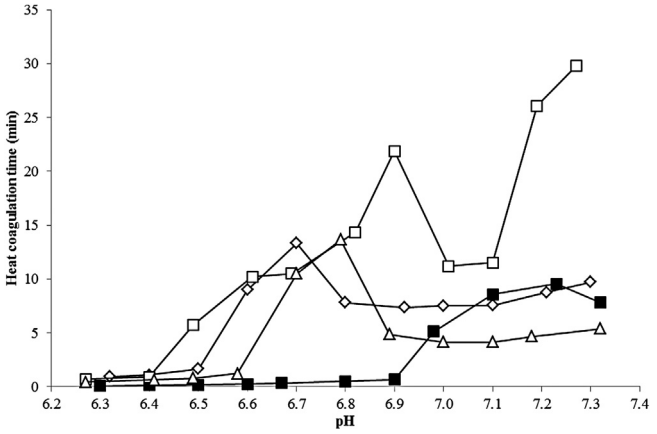

Fig. 5. pH-heat coagulation time (pH-HCT) profiles at 140 °C for milk protein concentrate (MPC)80 reconstituted to 3.5% (w/w) protein in water (■), simulated milk ultrafiltrate (SMUF) (◇), SMUF fortified with 300 mg L^{-1} urea and 4.62% (w/w) lactose (△), or MPC80 dialysed against reconstituted skim milk powder (□). Values are the means of data from duplicate analysis.

coagulation in this pH region (O'Connell & Fox, 2003; Singh, 2004; Van Boekel et al., 1989).

When considering the various factors outlined above in relation to the markedly reduced heat stability of MPC suspensions with increasing protein content of the MPC powder used in the pH range 6.3–6.7 (Fig. 2), increased Ca-ion activity is identified readily as a primary contributor to reduced heat stability. The negative effect of increased Ca-ion activity on the heat stability of milk has been reported by numerous researchers (De Kort et al., 2012; Gaucheron, 2005; Philippe et al., 2003; Sievanen et al., 2008). The lower levels of urea in suspensions of high protein MPC powders can be ruled out as the primary destabilising mechanism, as the addition of 400 mg urea L^{-1}, which is even slightly higher than levels typically found in milk, did not increase the heat stability of suspensions of MPC80 at pH $\leq$ 6.7 (Fig. 3). Likewise, stabilisation through lactose-mediated Maillard reactions did not increase heat stability in this pH region (Fig. 4). With respect to stabilisation to heat-induced coagulation through interactions of whey proteins with casein micelles, the results in Fig. 1 actually show increased association of denatured whey protein with casein micelles in heated MPC suspensions with increasing protein content of the MPC powder used, which would be expected to provide stabilisation, rather than the destabilisation which was observed.

A final contributor, heat-induced acidification, is also unlikely to be enhanced in suspensions of high protein MPC powders because the lactose content is lower in these samples, as are total Ca and P_i (Table 1) and serum Ca and P_i (data not shown). Hence, the low heat stability of suspensions of high protein MPC powders can be attributed primarily to the high Ca-ion activity in these MPC suspensions (Table 2). This is supported further by the fact that restoring the serum phase to a composition similar to that of skim milk, and hence of lower Ca-ion activity, increased HCT markedly between pH 6.3–6.7 (Fig. 5).

At pH values > 6.7, the HCT of skim milk first decreases to a minimum with increasing pH while, on the alkaline side of the minimum, HCT increases again strongly with pH, as observed for the suspension of MPC35 (Fig. 2). The initial reduction in HCT with increasing pH > 6.7 has been linked with the fact that heat-induced dissociation of κ-casein becomes more pronounced in this pH range and increases strongly with increasing pH. The specific occurrence of the minimum may be attributable to extensive heat-induced dissociation of κ-casein, combined with a sufficiently high Ca-ion activity to cause rapid heat-induced coagulation of κ-casein-depleted casein micelles (O'Connell & Fox, 2003; Singh, 2004; Van Boekel et al., 1989). At higher pH values, HCT increases again, presumably due to reductions in Ca-ion activity. As outlined before, the presence of urea can have a stabilising action in this region, whereas lactose may have a stabilising or destabilising action (O'Connell & Fox, 2003; Singh, 2004; Van Boekel et al., 1989).

When considering the heat stability of MPC suspensions in this pH range (6.7–7.3), and factors contributing to this, the increased Ca-ion activity (Table 2) and reduced extent of heat-induced dissociation of κ-casein (Fig. 1) can be identified as the main contributors to the effects observed, with the former destabilising and the latter stabilising the system. Hence, the very rapid heat-induced coagulation of reconstituted high protein MPC powders, which occurred over a much wider pH region than is typically observed for skim milk (Fig. 2), can be attributed to a combination of high Ca-ion activity and sufficient heat-induced dissociation of κ-casein. Re-establishment of the serum composition by dialysis against skim milk or reconstitution in SMUF largely reversed these changes in the pH-HCT profile (Fig. 5).

The fact that the pH region in which HCT remains < 2 min extends with increasing protein content of the MPC powder, and extends over the entire pH region studied for reconstituted MPC90 (Fig. 2), suggests that Ca-ion activity has a dominant effect, despite marked reductions in heat-induced dissociation of κ-casein. It should be noted here that the extent of heat-induced dissociation of κ-casein under the conditions used in this study (90 °C for 30 min) is expected to be lower than that observed at 140 °C; however, nonetheless, trends with respect to differences between samples will remain (Anema, 2009). The very low heat stability of some samples did not allow measurement of heat-induced dissociation of κ-casein at 140 °C, as a result of which the conditions of 90 °C for 30 min, at which all samples remained relatively stable, were selected. Above the pH where HCT values are < 2 min, HCT increases with pH (Fig. 2) because Ca-ion activity is too low, and the extent of heat-induced dissociation of κ-casein is not sufficiently high, to result in near-instantaneous coagulation. At this point, the acidification of milk as a result of heat-induced degradation of lactose becomes an important factor; hence, the buffering action of the degradation products of urea can stabilise MPC suspensions against heat-induced coagulation (Fig. 3) and the presence of increasing levels of lactose destabilise milk through more extensive heat-induced acidification (Fig. 4). Compared with lactose, maltodextrin had a less pronounced impact on heat stability (Fig. 4), which was likely due to a less rapid degradation process: maltodextrin must first be degraded into mono- and di-saccharides, and these mono- and di-saccharides can subsequently be degraded into organic acids through Maillard reactions. This is a much less rapid process than the isomerisation and subsequent degradation of lactose described earlier, which may have limited the influence of maltodextrin on the heat stability of MPC80.

5. Conclusion

Increased protein content of MPC powders had a strong effect on the heat stability of 3.5% protein MPC suspensions. At pH < 6.8, strong destabilisation was observed with increasing protein content of the MPC powders used due to an increased Ca-ion activity in MPC suspensions. At pH > 6.8, the increased Ca-ion activity also had a destabilising effect, but this was countered partially by reduced heat-induced dissociation of κ-casein. As a result, both increases and decreases in heat stability were observed with increasing protein content of the MPC powders in this pH region. The prominent role of the serum mineral content in determining the heat stability of MPC suspensions was highlighted further by the reversal of the pH-HCT profile of MPC80 towards a shape more typical of skim milk when the serum composition of skim milk was re-established. The addition of urea or lactose to suspensions of MPC80 affected HCT only in pH regions where rapid Ca-induced coagulation of casein micelles did not occur and, thus, only modulated heat stability in pH regions where reasonably high heat stability was observed.

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CHAPTER 5

Stability of milk protein concentrate suspensions to in-container sterilisation heating conditions

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. SVC co-designed the study, provided analytical support/training to MB and analysed the data. MB assisted wth heat coagulation time experiments. BC helped with serum-phase protein gelation experiments. IG and TH provided the powders, powder composition data, calcium-ion activity data and protein profile data. The chapter is included in its final published form.

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Stability of milk protein concentrate suspensions to in-container sterilisation heating conditions

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ABSTRACT

Milk protein concentrate (MPC) powders, ranging from 35 (MPC35) to 87 (MPC90)% protein, were reconstituted to 8.5% protein and assessed for heat stability at 120 °C, Ca-ion activity, heat-induced dissociation of κ-casein, and heat-induced gelation of serum-phase proteins in ultracentrifugal supernatants of unheated MPC suspensions. Heat stability of MPC suspensions depended on the protein content of the powder from which the suspensions were prepared. MPC70 had excellent heat stability compared with MPC35; however, MPC80, MPC85 and MPC90 were highly unstable to heating. Ca-ion activity increased with increasing protein content of the MPCs, whereas the extent of heat-induced dissociation of κ-casein and gelation of serum-phase proteins decreased. Increased heat stability with increasing protein content from MPC35 to MPC70 was attributed to decreased κ-casein dissociation and reduced gelation of serum-phase proteins. Despite these stabilising factors, excessively high Ca-ion activity caused MPC80, MPC85 and MPC90 to have very poor heat stability at pH 6.3–6.8, 6.3–7.1 and 6.3–7.3, respectively.

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1. Introduction

The heat stability of milk protein concentrates (MPCs) reconstituted to 3.5% protein was previously investigated by Carr (1999) for MPC85 at 120 °C and Crowley et al. (2014) for MPC35 (i.e., skim milk powder), MPC50, MPC60, MPC70, MPC80, MPC85 and MPC90 at 140 °C. Carr (1999) demonstrated that increasing the level of whey protein denaturation up to 86% in MPC85 had a negligible effect on heat stability, while further increases to ≥90% had a negative effect. Crowley et al. (2014) reported that MPC suspensions became less heat-stable when prepared from MPC powders with high protein contents, due primarily to increased Ca-ion activity. The results of these studies are useful for predicting and controlling the heat stability of unconcentrated MPC suspensions during thermal processing (e.g., in-container sterilisation, UHT treatment).

To further expand knowledge pertaining to the stability of MPCs during thermal processing, the present study investigated the stability of concentrated MPC suspensions under heating conditions resembling those of in-container sterilisation. In-container sterilisation involves the application of a lower temperature (typically ~120 °C) and a longer treatment time (typically 10–20 min) than UHT, and is widely applied to concentrated milk (Singh, 2004). Sterilisation is also commonly used in the manufacture of ready-to-drink beverages with high protein contents (~5–10% protein), which is a product category in which high-protein MPCs (i.e., MPC80, MPC85, MPC90) are increasingly being used as ingredients.

It is well known that the heat stability of milk protein systems is concentration-dependent. Concentrated milk has a lower heat stability than unconcentrated milk, which can be improved considerably by pre-heating the milk or by the addition of Ca-binding agents (De Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2012; Rose, 1961; Singh, 2004). The highest heat stability in concentrated milk tends to occur at a lower pH (6.4–6.6) than in unconcentrated milk (6.6–6.8) and heat-induced changes generally proceed to a less advanced degree (Singh, 2004). This study evaluated the stability of a range of MPC powders when reconstituted to 8.5% protein and heated at 120 °C. Measurement of protein distributions, Ca-ion activity and serum-phase protein

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gelation were used to develop a mechanistic understanding of observed pH-dependence of heat stability of the concentrated MPCs. The heat stability profiles of two MPC80s with differing degrees of whey protein denaturation were also compared, to investigate if pre-heating influences the heat stability of concentrated MPCs.

2. Materials and methods

2.1. Production and composition of milk protein concentrates

MPC powders of different protein contents used in the present study were the same as described by Crowley et al. (2014), with the additional inclusion of a medium-heat MPC80 (MH-MPC80), produced from milk heated at 95 °C for 45 s, as described by Gazi, Vilalva, and Huppertz (2014), instead of 72 °C for 15 s. Composition of the 8.5% protein MPC suspensions (Table 1) was estimated from measured values of the powders (Crowley et al., 2014). MPC suspensions were prepared from powders as described by Crowley et al. (2014).

2.2. Heat coagulation time

Heat coagulation time (HCT) of the MPC suspensions was determined for three independently prepared suspensions of each MPC at 120 °C as a function of pH (6.3–7.3) using the method of Davies and White (1966), as described by Crowley et al. (2014).

2.3. Heat-induced gelation of serum-phase proteins

Ultracentrifugal supernatants (1 h at 100,000 × *g* at 20 °C) from two independently-prepared 8.5% protein suspensions of MPC35, MPC60, MPC80 and MPC90 at pH 6.8 were heated in an oil bath at 120 °C, for the time equivalent to the HCT of the whole MPC suspension at pH 6.8 (see Section 2.2). In unheated MPC suspensions, ultracentrifugation separates casein micelles from the proteins in the serum phase, with the latter being comprised primarily of whey proteins and minor levels of casein. The resultant supernatant was considered a system representative of the serum-phase of the complete MPC suspension. To investigate the gelation of serum-phase proteins, the supernatants were assessed for visual indicators of heat-induced changes (i.e., coagulation, particulation/flecking and thickening). In addition, the particle size distribution (PSD) of the heated supernatants was determined with a Malvern Mastersizer S (Malvern Instruments, Malvern, UK). PSD was calculated using a generalised polydisperse model using a particle and dispersant refractive index of 1.46 and 1.33, respectively, and an absorption coefficient of 0.1.

2.4. Ca-ion activity and heat-induced κ-casein dissociation in MPC suspensions

Ca-ion activity of unheated MPC suspensions was determined in duplicate as a function of pH using a Ca-ion selective electrode as described by Crowley et al. (2014). Protein profiles of ultracentrifugal supernatants of heated (30 min at 90 °C) suspensions (8.5% protein) of MPC35, MPC60, MPC80 and MPC90 at pH 6.5, 6.8 or 7.1 were determined in duplicate using reversed-phase HPLC, as described by Hinz, Huppertz, and Kelly (2012).

3. Results

3.1. Heat stability of MPCs

Suspensions of MPC35, MPC50, MPC60 and MPC70 showed distinct maxima in their pH–HCT profiles (Fig. 1), as is typically observed for concentrated skim milk (Singh, 2004). Both the pH at which the maximum occurred (from 6.6 to 6.9) as well as the HCT at the maximum (from 13 to 74 min) increased with increasing protein content of the MPC. For MPC80, MPC85 and MPC90, HCT increased with increasing pH. For these samples, HCT was <2 min (i.e., within the heating period to reach 120 °C) at pH values ≤ 6.7, 7.1 and 7.2, respectively; notable heat stability was only observed at higher pH values (Fig. 1). The heat stability of MPC90 was exceptionally low across the entire pH range, with MPC85 having a HCT which was nearly 10-fold higher than that of MPC90 at pH 7.3. Due to a more intense pasteurisation treatment (95 °C for 45 s), MH-

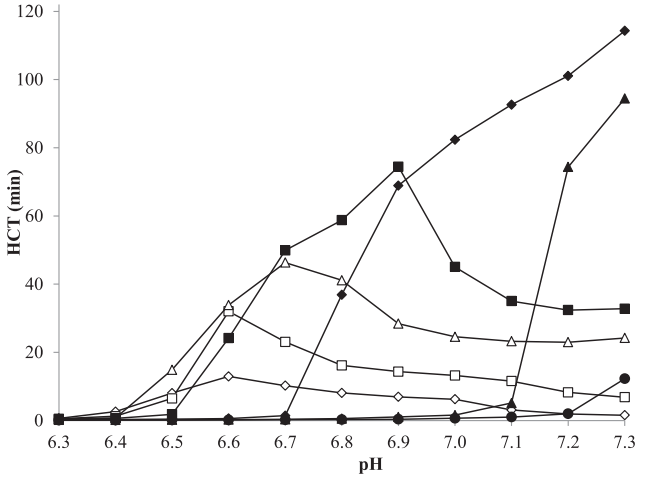

Fig. 1. pH-heat coagulation time (pH–HCT) profiles at 120 °C for MPC35 (◇), MPC50 (□), MPC60 (△), MPC70 (■), MPC80 (◆), MPC85 (▲) and MPC90 (●) reconstituted to 8.5% (w/w) protein. Values are the means of duplicate data from three independent trials.

Table 1
Composition of reconstituted milk protein concentrate (MPC) powders calculated from measured values for MPC powders.[a]

MPC	Non-protein nitrogen (%, w/w)	Lactose (%, w/w)	Ash (%, w/w)	Solids (%, w/w)	Calcium (mg g^{-1})	Inorganic phosphorus (mg g^{-1})
MPC35	0.08	11.9	1.94	23.2	3.28	2.04
MPC50	0.04	6.10	1.31	16.4	2.94	1.53
MPC60	0.02	3.42	1.09	13.4	2.77	1.29
MPC70	0.02	2.23	1.00	12.0	2.72	1.21
MPC80	0.01	0.68	0.83	10.2	2.57	1.00
MPC85	0.01	0.19	0.75	9.64	2.57	0.87
MPC90	0.00	0.05	0.75	9.50	2.57	0.87

[a] All powders were reconstituted to 8.5% (w/w) protein; data were calculated based on the values reported by Crowley et al. (2014).

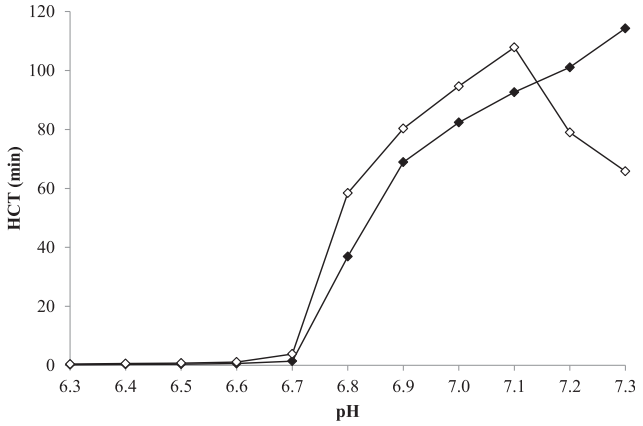

Fig. 2. pH-heat coagulation time (pH–HCT) profiles at 120 °C for low-heat (◆) and medium-heat (◇) MPC80 reconstituted to 8.5% (w/w) protein in water. Values are the means of duplicate data from three independent trials.

MPC80 contained more denatured whey protein (~25% of α-lactalbumin and ~65% of β-lactoglobulin denatured) than the MPC80 made from milk pasteurised at 72 °C for 15 s (<5% of α-lactalbumin and β-lactoglobulin denatured) (Gazi & Huppertz, 2015). The HCT of MH-MPC80 was slightly but consistently higher than that of MPC80 at pH 6.3–7.1, with both samples having the same overall pH–HCT profile in this pH region; however, MH-MPC80 was less heat-stable at pH 7.2 and 7.3 (Fig. 2).

3.2. Ca-ion activity of MPCs

Ca-ion activity increased with increasing protein content of the MPC powders and decreased with increasing pH (Table 2), as also reported for 3.5% protein MPC suspensions (Crowley et al., 2014). For MPC35 and MPC60, Ca-ion activities were lower for the 8.5% protein suspensions than those reported for the corresponding 3.5% protein suspensions by Crowley et al. (2014). However, for MPC80, Ca-ion activity was the same for the 3.5 and 8.5% protein suspensions and, for MPC90, Ca-ion activity was higher for the 8.5% protein suspension compared with the 3.5% protein suspensions.

Table 2
Influence of pH on Ca-ion activity in suspensions of MPC35, MPC60, MPC80 and MPC90 containing 8.5% (w/w) protein. Values are the means of data from duplicate analysis.

MPC	pH	Ca-ion activity (mM)
MPC35	6.28	0.83
	6.49	0.58
	6.79	0.37
	7.10	0.25
MPC60	6.29	1.50
	6.49	1.06
	6.80	0.71
	7.10	0.46
MPC80	6.30	2.19
	6.52	1.62
	6.80	1.13
	7.12	0.80
MPC90	6.29	3.52
	6.52	2.71
	6.82	1.93
	7.11	1.52

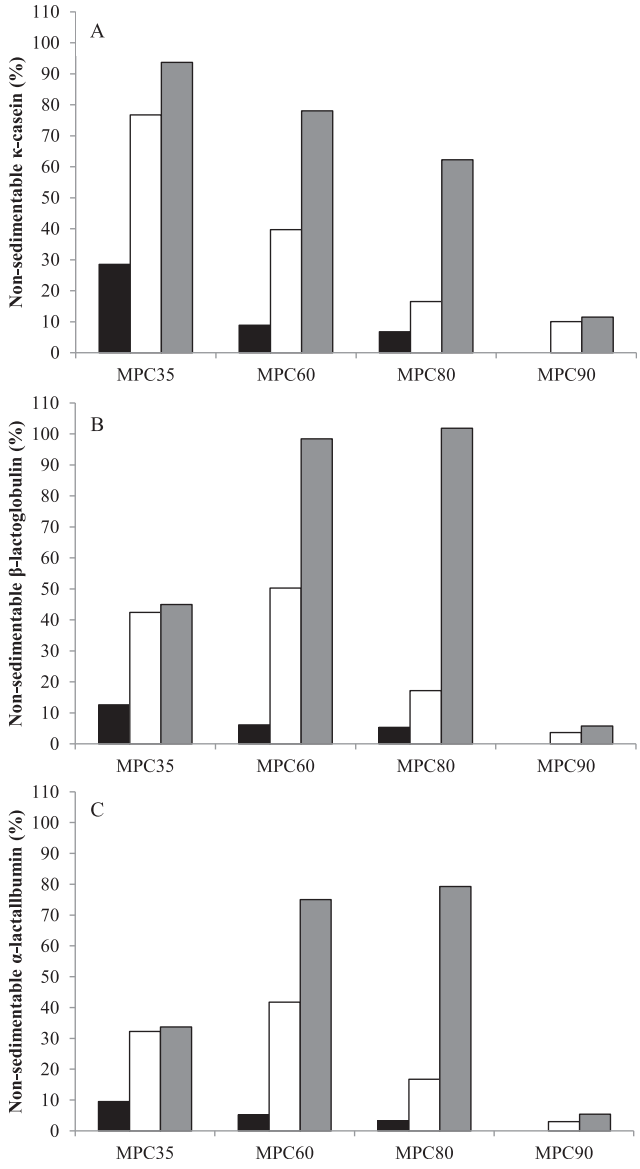

Fig. 3. Influence of pH (■, 6.5; □, 6.8; ▩, 7.1) on the percentage of (A) κ-casein, (B) β-lactoglobulin and (C) α-lactalbumin present in the ultracentrifugal (100,000 × *g* for 60 min at 20 °C) supernatant of heated (30 min at 90 °C) suspensions of milk protein concentrates MPC35, MPC60, MPC80, and MPC90 containing 8.5% (w/w) protein. Values are the means of data from duplicate analysis. MPC90 at pH 6.5 showed visible coagulation after heat treatment and was not analysed further.

3.3. Protein distribution in heated MPCs

The distributions of κ-casein, β-lactoglobulin and α-lactalbumin between the sedimentable and non-sedimentable phase of heated (30 min at 90 °C) 8.5% protein suspensions of MPC35, MPC60, MPC80 and MPC90 at pH 6.5, 6.8 or 7.1 are shown in Fig. 3. For the MPC90 suspension at pH 6.5, visible coagulation occurred during heating so this sample was not analysed further. Levels of non-sedimentable κ-casein in heated MPC suspensions increased with pH and decreased with the protein content of the MPC (Fig. 3). Levels of non-sedimentable β-lactoglobulin and α-lactalbumin decreased with increasing protein content of the MPC at pH 6.5, whereas at pH 6.8 and 7.1, the highest level of non-sedimentable β-lactoglobulin and α-lactalbumin was observed for MPC60 and MPC80, respectively. For both pH values, levels of non-

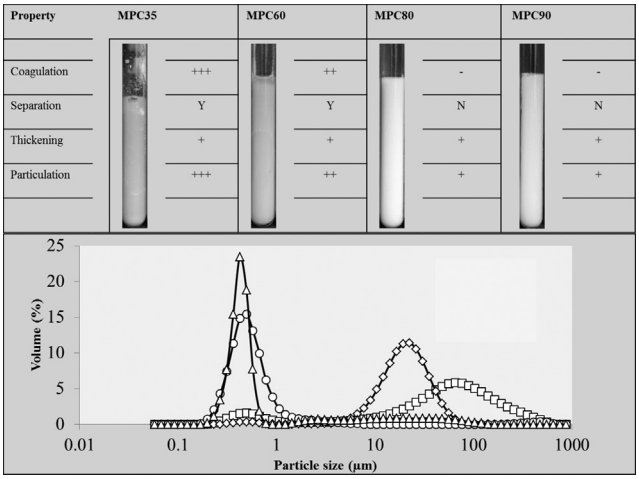

Fig. 4. Degree of serum-phase protein gelation determined based on visual observation and particle size distribution analysis of ultracentrifugal (100,000 × *g* for 60 min at 20 °C) supernatants of 8.5% protein milk protein concentrates (MPCs) after heating (□, MPC35; ◇, MPC60; ○, MPC80; △, MPC90). Supernatants were heated at pH 6.8 and 120 °C until the experimentally determined heat coagulation time of whole MPC suspensions was reached. Inset images show samples after heating. +++ = extensive, + = minimal, − = negligible, Y = yes, N = no.

sedimentable β-lactoglobulin and α-lactalbumin were considerably lower for MPC90 than for any of the other MPCs (Fig. 3).

3.4. Gelation of serum-phase proteins

MPC suspensions in the present study contained approximately 1.7% (w/w) whey protein. At these concentrations, heat-induced aggregation and gelation of proteins in the serum phase of the sample can be an important contributor to heat-induced coagulation (Singh, 2004). The effects of heat treatment on the serum phases of MPC suspensions at pH 6.8 are shown in Fig. 4. The heating time (8, 41, 73 or 0.3 min for MPC35, MPC60, MPC80 and MPC90, respectively) was selected based on the HCT of the MPC suspension at pH 6.8 (Fig. 1), to establish if gelation of proteins in the serum phase could be a contributor to the observed heat-coagulation of the MPC suspensions.

Before heating, all supernatants were clear and no turbidity was evident (results not shown). After heating, MPC35 and MPC60 exhibited coagulation and separation (Fig. 4); however, these effects were not observed for MPC80 and MPC90, but turbidity did increase on heating for these MPCs, with the formation of some fine particles also evident. PSD analysis revealed that primarily submicron-sized particles were present in heated supernatants of MPC80 and MPC90, whereas considerably larger particles were found in the heated supernatants of MPC35 and MPC60 (Fig. 4). While these studies were carried out in systems devoid of micellar casein, the absence of heat-induced flocculation or gelation of serum phase proteins for MPC80 and MPC90 under conditions of pH, temperature and time where the complete samples destabilised, strongly indicates that coagulation of these MPC suspensions is primarily driven by aggregation of casein micelles and not by serum protein gelation. However, for MPC35 and MPC60, the results shown in Fig. 4 suggest that serum-phase protein gelation may be a noteworthy contributor to the observed heat-induced coagulation.

4. Discussion

Trends observed in pH–HCT profiles for 8.5% protein MPC suspensions (Fig. 1) can be attributed to the combined effect of differences in Ca-ion activity, heat-induced κ-casein dissociation and heat-induced gelation of serum-phase proteins. The heat stability of MPC80 was improved slightly at pH 6.3–7.1 by increasing the level of denatured whey proteins in the MPC (Fig. 2). However, increased whey protein denaturation resulted in the MH-MPC80 being less stable at pH 7.2 and 7.3 (Fig. 2), suggesting that the additional whey protein at the surface of casein micelles may have rendered them more susceptible to destabilising factors such as the precipitation of tertiary Ca phosphate. Carr (1999) studied the influence of denatured whey protein on the heat stability of unconcentrated MPC85 (3.5% protein) and reported a negligible effect at denaturation levels of up to 86% and a negative effect at levels up to 90%. The results of the present study indicate that the influence of pre-heating on heat stability is largely positive when applied to a concentrated MPC system.

Ca-ion activity increased both with increasing protein content of the MPC powder and with decreasing pH (Table 2), similar to effects previously reported for 3.5% protein MPC suspensions (Crowley et al., 2014). Ca-ion activity values for the 8.5% protein suspensions were lower (MPC35, MPC60), equivalent (MPC80) or higher (MPC90) than those of the 3.5% protein systems. The main determinants of Ca-ion activity are the total concentration of Ca-ions and the Ca-ion activity coefficient. The latter is reduced by increasing ionic strength, thereby explaining the lower Ca-ion activity for 8.5% protein suspensions of MPC35 and MPC60 (Table 2) compared with their 3.5% protein counterparts (Crowley et al., 2014). However, for MPC90, higher Ca-ion activity was observed for the 8.5% protein suspension compared with the 3.5% protein suspension. Due to extensive diafiltration during the manufacture of MPC90, most soluble salts had been removed and the residual soluble salts were primarily counter-ions of the negatively-charged proteins. In addition, the Ca-ion content in MPC suspensions is subject to changes during the establishment of mineral equilibria between casein micelles and the serum phase. When high-protein MPC powders are hydrated in water, there is an entropic drive for the release of Ca and phosphate into the serum phase to reach saturation. Ca is associated with casein micelles via (1) calcium phosphate nanoclusters, (2) serine phosphate groups, and (3) glutamic acid and aspartic acid residues (Holt, 1997), with these forms of micellar Ca being expected to dissolve in the order (3) > (2) > (1). As the concentration of (3) was low in the 3.5% protein systems of Crowley et al. (2014), its dissolution was unlikely to have achieved saturation by itself; hence, (2) and (1) would also need to dissolve. In 8.5% protein suspensions there was a much greater concentration of (3), meaning that less of (2) and (1) were required to reach saturation. Thus, when MPC90 was reconstituted to 8.5% protein, the serum phase still ultimately became saturated, but at a higher Ca:phosphate ratio than in the 3.5% protein systems, and thus a higher Ca-ion activity (Table 2). A similar shift in serum-phase mineral composition and Ca-ion activity was observed on addition of calcium chloride to concentrated milk (Sievanen, Huppertz, Kelly, & Fox, 2008).

Heat-induced dissociation of κ-casein was found to decrease with increasing protein content of the MPCs, and increase with increasing pH (Fig. 3), as reported by Crowley et al. (2014) for 3.5% protein MPC systems. However, α-lactalbumin and β-lactoglobulin did not follow this trend and the highest levels of non-sedimentable whey proteins were seen for MPC60 at pH 6.8 and MPC80 at pH 7.1. Since no native whey protein remained after heat treatment at 90 °C for 30 min (data not shown) the non-sedimentable whey protein is denatured, either in the form of whey protein aggregates or whey protein–κ-casein aggregates. The sedimentable whey protein is either associated with casein micelles or in whey protein aggregates sufficiently large to sediment on ultracentrifugation. The latter may have formed on heating

suspensions of MPC35 at pH 6.8 and 7.1, under which conditions MPC35 contained high levels of non-sedimentable k-casein but considerably lower levels of non-sedimentable whey proteins (Fig. 3). In contrast, suspensions of MPC60 and MPC80 heated at these pH values showed higher levels of non-sedimentable whey proteins than κ-casein, indicating that some whey protein was present in the form of non-sedimentable aggregates, which is also in line with observations on heating of the serum phase (Fig. 4). For MPC90, virtually all κ-casein, β-lactoglobulin and α-lactalbumin were sedimentable.

Fig. 4 demonstrates that interactions between serum-phase proteins in micelle-depleted systems were sufficient to induce network formation only in MPC35 and, to a lesser extent, MPC60. Serum-phase protein gelation did not occur in MPC80 and MPC90, but serum-phase protein aggregates were formed (Fig. 4). Indeed, stable suspensions of aggregated whey proteins can be generated when whey proteins are heated in $CaCl_2$ solutions at neutral pH (Phan-Xuan et al., 2014). The higher turbidity of the heated supernatant of MPC80 compared with that of MPC90 (Fig. 4) was likely due to more extensive aggregation of serum-phase proteins caused by the considerably longer heating time (37 min versus 0.3 min, respectively).

The poor heat stability of all 8.5% protein MPC suspensions at pH < 6.5 (Figs. 1 and 2) is probably due to extensive gelation of serum-phase proteins, with concomitant formation of a casein-whey protein network (Huppertz, 2014; Singh, 2004); this is supported by the predominance of sedimentable whey proteins in these systems after heating (Fig. 3). Increased heat stability with increasing protein content from MPC35 to MPC70 and shifts in the HCT maximum to more alkaline values (Figs. 1 and 2) can be related to a reduced extent of heat-induced protein gelation in the serum-phase (Fig. 4) and a reduced extent of heat-induced dissociation of κ-casein (Fig. 3). However, increased Ca-ion activity counters these stabilising mechanisms and becomes the dominant influence for high-protein MPCs, in which strong reductions in heat stability were observed. For these MPCs, reasonable heat stability was only observed at higher pH values, where Ca-ion activity was lower (Table 2).

5. Conclusion

Although there are a growing number of commercial applications in which MPCs feature as the primary protein source, e.g., high-protein ready-to-drink beverages, little is known about the heat stability of such systems. To address this research gap, the stability of MPCs to heating at 120 °C after reconstitution to 8.5% protein was investigated and found to be strongly influenced by the protein content of the original MPC powder. On comparing skim milk powder (i.e., MPC35) with MPC50, MPC60 and MPC70, heat stability was improved in the latter, due to reduced dissociation of κ-casein and decreased gelation of serum-phase proteins. It can thus be predicted that MPC powders containing up to 70% protein will not present significant heat stability issues during thermal processes developed for other concentrated dairy systems. On the other hand, concentrated suspensions prepared from high-protein powders (MPC80, MPC85 and MPC90) coagulated more quickly during heating, due to their high Ca-ion activity, irrespective of reduced dissociation of κ-casein and decreased gelation of serum-phase proteins. For thermal processing of concentrated high-protein MPCs without significant destabilisation, strategies may need to be developed to improve their heat stability, which could include the application of pre-heating, demonstrated in this study to increase the heat stability of MPC80.

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Part B.

Development and scale-up of microfiltration-based methods for producing β-casein ingredients

Introduction and research objectives

Milk is a colloidal system, in which both casein micelles and fat globules scatter light, resulting in a liquid with a white appearance. On removal of fat, milk remains opaque, due to the strong light-scattering properties of the micellar phase. Casein micelles, with an average hydrodynamic diameter of ~150-200 nm, have specific physicochemical properties which facilitate their ability to remain in a stable suspension (O'Mahony and Fox, 2013). However, deliberate destabilisation of the micellar phase, resulting in the separation of casein from whey, has long been practised. The earliest example of such a process is probably cheese-making, whereby κ-casein at the micellar surface is hydrolysed to remove it as a steric stabiliser against coagulation in the presence of calcium ions.

The manufacture of casein ingredients in the dairy industry has mostly relied on procedures based on micellar destabilisation (Carr and Golding, 2016). Casein ingredients obtained from precipitation-based processes are insoluble, unless subjected to additional chemical treatments. Solubilisation of rennet casein necessitates the use of calcium chelators and high mixing temperatures (Ennis *et al*., 1998), while conversion of acid casein into soluble caseinates requires alkalisation treatments using sodium or calcium hydroxide, for example, to generate sodium and calcium caseinate, respectively (O'Mahony and Fox, 2013). These types of casein ingredients are primarily valued for their techno-functional properties in specific food products. For example, rennet casein is a key ingredient in cheese analogues, were it provides water-binding and textural properties (Ennis *et al*., 1998), while sodium caseinate is an important ingredient in cream liqueurs, where it can emulsify fat in an ethanol-rich solvent phase (Mezdour *et al*., 2006)

Over the last few decades, there has been a marked shift in the dairy industry away from precipitation-based approaches to prepare protein ingredients towards methods based on membrane filtration technology. This has facilitated the manufacture of milk protein concentrates (MPCs), discussed in Part A of this thesis, and also micellar casein concentrates (MCCs), casein-based ingredients which contain casein in a form very close to that found in milk (Gésan-Guiziou, 2013; O'Mahony and Tuohy, 2013). The adoption of MPCs by manufacturers has so far outpaced that of MCCs, but MCCs are increasingly used in commercial applications

including sports supplements, cheese and Greek-style yoghurt; however, the rehydration characteristics of MCCs are even more challenging than those of MPCs (Jeantet *et al*., 2010; Crowley *et al*., 2016). Enrichment and purification of individual caseins can also be achieved by combining these membrane technologies with (reversible) modifications in the association state of caseins. It has been demonstrated, for example, that 'cold' (e.g., <5°C) microfiltration of milk enriches β-casein in the whey stream, while subsequent warming of the demineralised β-casein-enriched whey can be used to fractionate β-casein micelles from whey proteins by 'warm' (e.g., 26°C) microfiltration (O'Mahony *et al*., 2014).

Commercial availability of β-casein is limited, due to the challenges inherent in its manufacture by membrane processing, including low permeate flux, destabilisation during processing, relatively low yield and purity values, and uncertainty surrounding the use of co-products (which include modified MCC). Nonetheless, interest in the ingredient remains high, due to its known ability to function in emulsion- and foam-stabilising roles (Le Meste *et al*., 1990; Coppola *et al*., 2014), and also its potential use in nutritional and medicinal applications in food and pharmaceuticals (Shapira *et al*., 2010; Danino *et al*., 2014). As the predominant casein in human milk is β-casein, these type of ingredients also open up new possibilities in the area of humanising the casein fraction of infant formula. However, much is unknown about how β-casein ingredients extracted from bovine milk compare to native human micelles. Although human casein contains ~80% β-casein, there is also κ-casein present to act as a colloidal stabiliser; furthermore, human milk contains multiple phosphoforms of β-casein, with varying calcium-sensitivities and dissociation characteristics (Sood *et al*., 1997; McCarthy *et al*., 2013), while bovine milk contains β-casein with one predominant degree of phosphorylation. Thus, there are many questions surrounding β-casein in the infant formula context, including its comparative ability to stabilise calcium phosphate and form stable micellar structures. Many of these factors are also relevant to applications of β-casein that have been proposed for encapsulation of vitamins (Danino *et al*., 2014) and drugs (Shapira *et al*., 2010), with the potential implications of cross-linking of β-casein by calcium phosphate in these systems yet to be established.

Enriched and purified fractions of β-casein are some of the most recently developed casein-based ingredients. Production of these fractions involves membrane processes, in which unique challenges are encountered, as previously outlined, but the final ingredient exhibits potential in areas ranging from functional to nutritional and medicinal applications. In this section of the thesis, research concerning the preparation and characterisation of β-casein is described. Firstly, aspects related to the formulation and process requirements for infant formula products, progress in the humanisation of infant formulae (e.g., enrichment with α-lactalbumin, β-casein) and factors influencing the stability of these products during processing and storage are reviewed in **Chapter 6**. There are reports that some types of non-bovine milk are better tolerated by infants who find bovine milk difficult to digest, which in some cases has been linked to their β-casein content; indeed, in certain respects, milk from goats, horses and camels are more similar to human milk than bovine milk. In **Chapter 7**, the compositional properties of human milk and bovine milk are compared, along with a range of other mammalian milk types, in order to evaluate the potential of alternative milk types as sources of ingredients (including β-casein) for infant formula.

Processes for the preparation of β-casein ingredients involve the integration of a number of membrane filtration steps, with the success of each fractionation step depending on a complex interplay between protein association-state (as determined by pre-treatments and processing parameters) and membrane pore-size. Results of an investigation of the influence of membrane pore-size and separation polymer material on a process for the laboratory-scale enrichment of β-casein during cold microfiltration of milk are presented in **Chapter 8**. While β-casein-enriched whey fractions may be sufficient in many applications, or ideal in the case of first-age infant formula, the development of more purified fractions will afford greater flexibility for the use of β-casein in a broader range of applications. A study concerning the development and optimisation of novel pilot-scale processes for the purification of β-casein from such an enriched whey stream is detailed in **Chapter 9**. In addition to questions surrounding the optimal conditions for controlling β-casein association-state during its preparation, much remains unknown about the functionality of the final β-casein ingredient, which differs markedly from that of analytical-grade materials studied most frequently by previous researchers. Findings

related to the self-association of a β-casein ingredient prepared using one of the integrated membrane filtration processes are discussed in **Chapter 10**. A major concern for potential adopters of the aforementioned membrane technology to manufacture β-casein commercially is the utility of the numerous co-products which are generated, one of which is MCC. It is presently unclear to what extent cold microfiltration modifies the composition and functionality of this material compared to that made using a traditional warm process. **Chapter 11** includes a detailed characterisation of the influence of cold microfiltration on the rehydration characteristics of a resultant MCC powder. Findings from the various studies in Part B are discussed alongside those in Part A in **Chapter 12**.

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CHAPTER 6

Studies on the formulation, processing and storage of infant milk formulae

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Declaration

This chapter was written by author SVC and reviewed by his co-authors.

Abstract

Infant milk formulae (IMFs) are some of the most complex nutritional products in the food industry. An IMF comprises a blend of multiple ingredients, which needs to meet different process- and storage-stability criteria depending on the product format to be manufactured (e.g., powdered, ready-to-feed). Heat-induced destabilisation, creaming of fat, oxidation of fat and non-enyzymatic browning cause stability issues in IMFs. The number of ingredients used in IMFs is continually expanding, and now include novel protein ingredients which are intended to 'humanise' their protein profile. Active research into the enrichment and isolation of individual dairy proteins from bovine milk (e.g., α-lactalbumin, β-casein) for humanisation purposes, and the implications of such formulation changes for the techno-functionality of IMFs, will be critical to the successful development of next-generation IMFs. The field of study concerned with the techno-functional properties of IMF systems is growing steadily and there is a need to review the recent advances which have been made; this chapter provides an overview of the latest research in this area.

1. Introduction

Research into infant formula is intensifying, as evidenced by the rising number of peer-reviewed research papers and review articles in recent years (Fig. 1). Infant milk formula (IMF) is a human milk substitute manufactured mainly using bovine milk as its base ingredient. There has been some growth in the market for IMFs derived from non-bovine milk, which is discussed in Chapter 7, but will not be considered further here. The use of plant-based ingredient sources, most notably soy, is important in the production of formulae for infants intolerant of milk protein-based IMFs (Bhatia *et al*., 2008), but is outside of the scope of this review. First-age IMFs are those products formulated specifically for the first 6 months of the human neonate's life. In recent times, second- and third-age IMFs have also been developed which are marketed as "follow-on" and "toddler" milks for infants from 6-12 and over 12 months old, respectively.

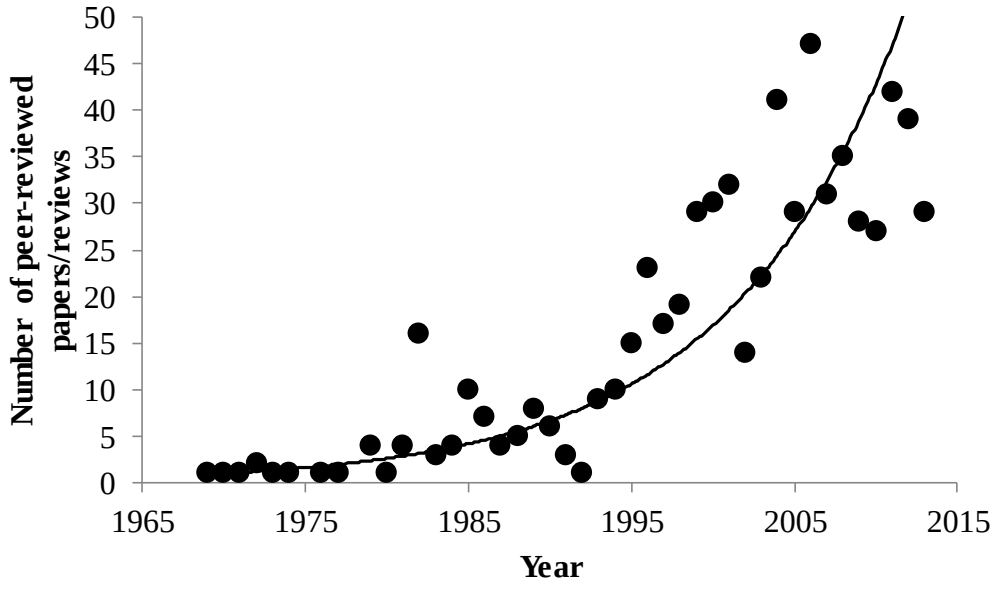

Fig. 1. Number of peer-reviewed papers and review articles captured under search query "infant formula" in food, agriculture and chemistry journals between 1969 and 2015 (source: Scopus).

Differences in the gross composition of human milk, bovine milk and age-specific IMFs are shown in Table 1.

Table 1. Typical gross composition of bovine and human milks, and age-specific infant milk formulae.

	Mammalian milks[a]		**Infant milk formulae**[b]		
	Bovine	Human	First-age	Second-age	Third-age
Protein (%)	3.4	0.9	1.3	1.4	1.5
WP:CN (-)	~20:80	~60:40	~60:40	~20:80	~20:80
Carbohydrate (%)	4.8	7.0	7.3	7.5	8.2
Fat (%)	3.7	3.8	3.5	3.0	3.0

[a] taken from Uniacke-Lowe *et al*. (2010)

[b] mean values from survey of ingredient listings of four commercial formulae for each age-category

WP = whey protein, CN = casein

Human milk has a lower protein content and a higher carbohydrate content than bovine milk; in addition, the protein system of the former is whey protein-dominant, while the latter is casein-dominant. To achieve the same whey protein:casein ratio as human milk, manufacturers of IMFs mix together sufficient quantities of skim milk and whey protein ingredients (e.g., demineralised whey, whey protein concentrate/isolate) (de Wit, 1998) (Fig. 2). For later stage age-specific IMFs, the contribution of whey protein ingredients to the protein profile is generally reduced, to yield casein-dominant formulae (Table 1).

Innate minerals in skim milk, both those associated with proteins (e.g., micellar calcium phosphate) and those which exist in the serum phase, contribute a substantial proportion of the final mineral content in IMFs (Fig. 3). However, to meet regulatory requirements, additional fortification with minerals, such as calcium salts, is necessary (Bass and Chen, 2006). Other major macronutrients (lipids and lactose), in addition to processing aids (e.g., emulsifiers, stabilisers) (Zou and Akoh, 2013) and micro-nutrients (e.g., vitamins, nucleotides, probiotics) (Thompkinson and Karb, 2007), are also added to different degrees depending on the manufacturer.

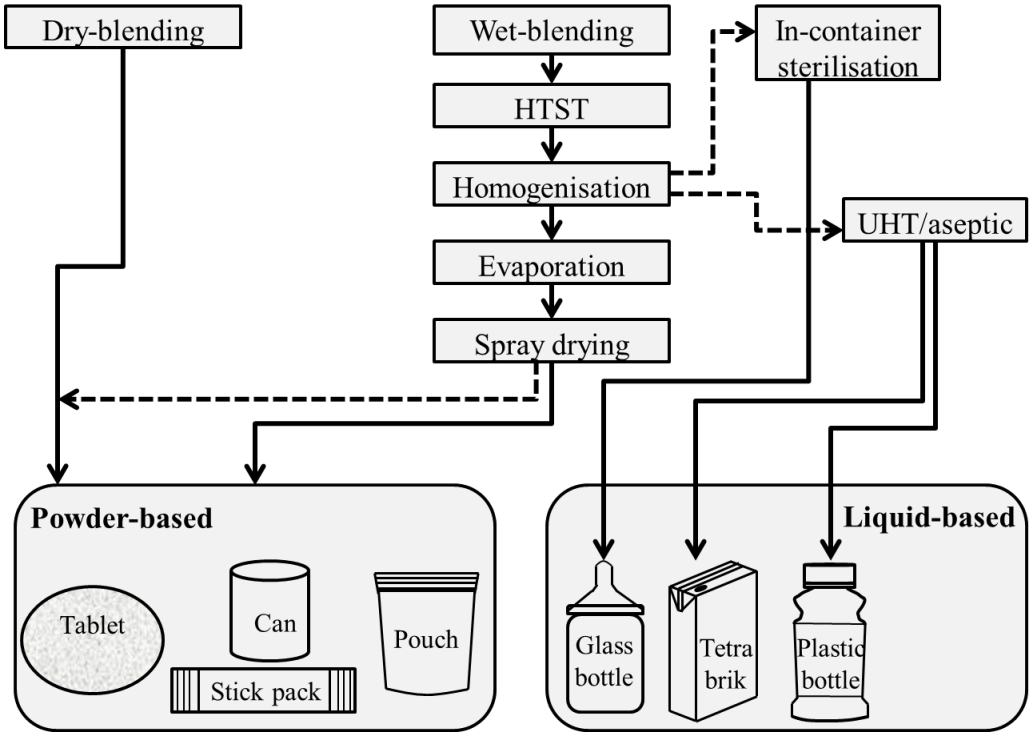

Fig. 2. Process flow diagram for manufacture of infant milk formula in different product formats.

The multi-component IMF system is then subjected to a processing procedure involving multiple heating steps, which may include some combination of heat treatment (HTST, ultra-high temperature (UHT) treatment, in-container sterilisation), evaporation and spray drying (Guerra-Hernandez *et al*., 2002; Sarriá *et al*., 2001) (Fig. 2). Unless carefully controlled, this thermal load can result in extensive denaturation and possible destabilisation of proteins (McSweeney *et al*., 2004). Other product-specific problems may also arise such as, for example, creaming in liquid "ready-to-feed" products (McCarthy *et al*., 2012; Zou and Akoh, 2013), or crystallisation of amorphous lactose in IMF powders (Nasirpour *et al*., 2006; Schmitz-Schug *et al*., 2013)

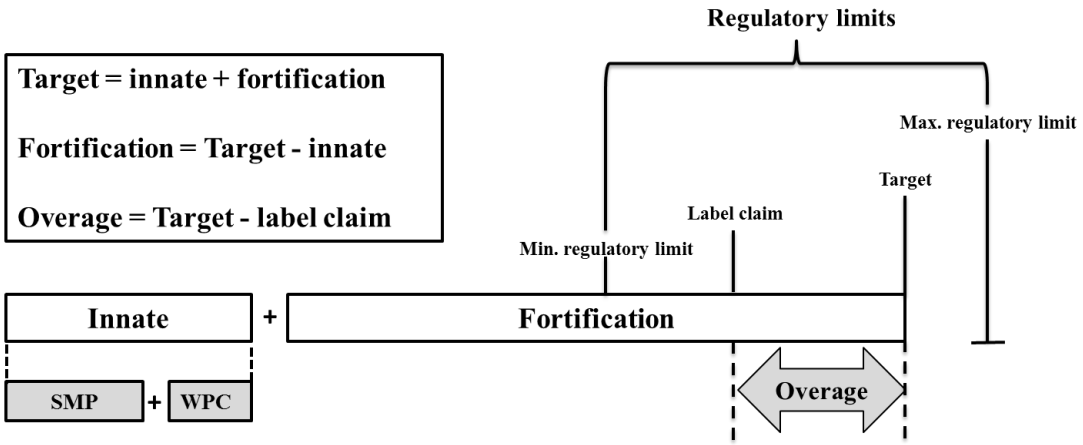

Fig. 3. Schematic showing the formulation and regulatory considerations which determine the mineral profile of infant milk formulae.

Formulation practices in the IMF industry continue to grow in complexity, as illustrated by the ingredient web shown in Figure 4. Currently, IMFs are formulated to a protein content which is higher than that of human milk (Table 1). The purpose of this excess of proteins is to ensure that essential amino acids are supplied to the infant in the required quantities and proportions (European Commission Directive 2006/141/EC). Ongoing research aims to reduce the levels of proteins such as β-lactoglobulin, which is absent from human milk, in IMFs, and increase the levels of proteins such as α-lactalbumin (Pearce, 1995; Crowley *et al*., 2016) and β-casein (McCarthy *et al*., 2013b, 2014) which are present at higher levels in human milk. Modification of the protein profile of IMFs in this way could decrease the risk of allergenic response to milk proteins in infants, and may also allow manufacturers to reduce the protein content of IMFs to levels closer to that of human milk while still satisfying amino acid requirements (Kuhlman *et al*., 2003). However, IMFs with humanised protein profiles may behave differently to traditional formulae during processing, becoming more or less stable depending on the type of proteins used and their interactions with other ingredients (McCarthy *et al*., 2013b; Crowley *et al*., 2016).

This review provides a comprehensive overview of recent technological developments in the formulation and manufacture of IMFs. In Section 2, focus is placed on recent research into the stability of IMF systems during formulation (e.g.,

calcium fortification), processing (e.g., heat treatment, pneumatic conveying) and storage (e.g., Maillard browning, lipid oxidation, crystallisation of lactose). In Section 3, recent advances in the manufacture and use of novel protein ingredients for IMF applications is given particular attention, as these ingredients are likely to become an important focal point of research into the humanisation of IMFs in future years.

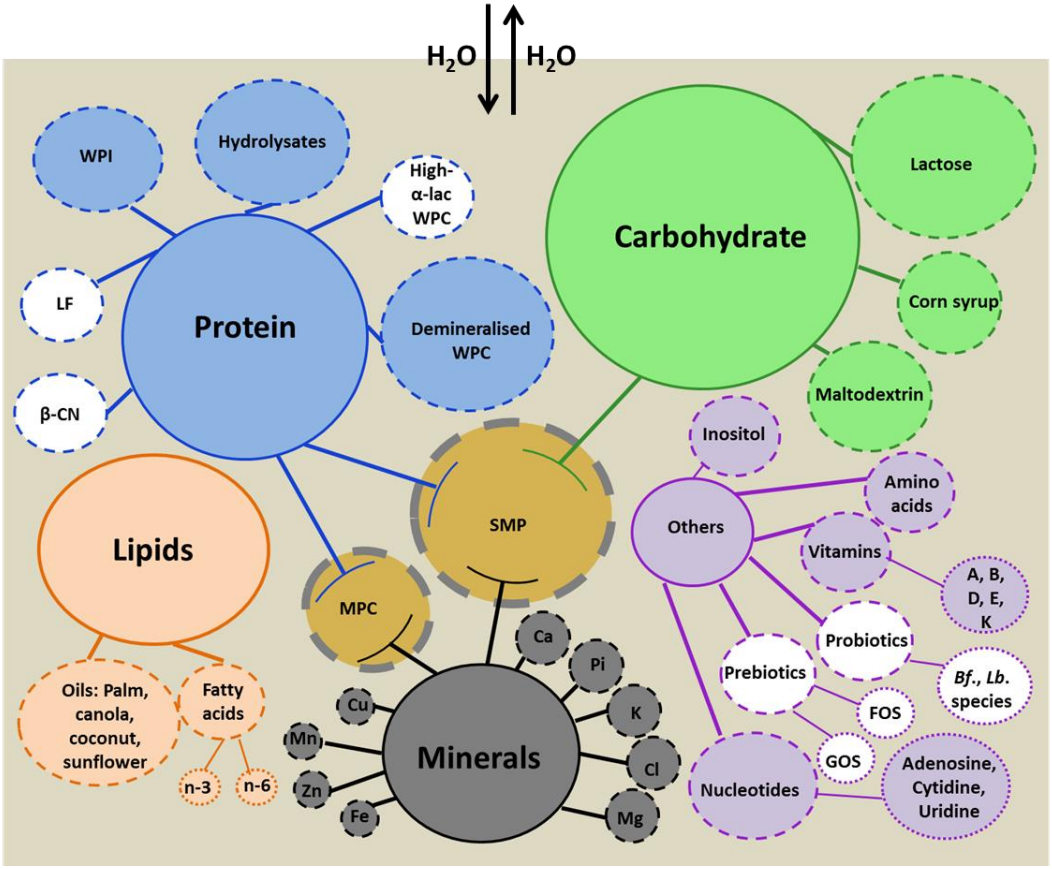

Fig. 4. Ingredient web showing the primary ingredients (broken circles) which contribute to the major nutrient classes (solid circles) in infant milk formulae. The size of circles roughly approximates their relative contribution. White circles indicate that an ingredient is currently used in niche or premium applications. Gold circles indicate blend-type ingredients, which contribute to two or more major nutrient classes. Water may be added (dilution, mixing) or removed (evaporation, drying) during processing to achieve correct proportions. Abbreviations: β-CN – β-casein; LF – lactoferrin; α-lac – α-lactalbumin; WPI – whey protein isolate; WPC – whey protein concentrate; SMP – skim milk powder; MPC; milk protein concentrate; FOS – fructooligosaccharides; GOS – galactooligosaccharides.

2. Factors affecting the stability of infant milk formulae

In the following sections, various aspects of IMF stability are discussed. The sections are divided into those dealing with changes during processing (2.1) and storage (2.2); in addition, as browning can occur during both processing and storage, this is housed under a separate section (2.3). In the sub-headers of each sub-section, the letter(s) in parentheses indicates whether the phenomenon being discussed is relevant to IMF mixes/products in the liquid (L) and/or powder (P) form.

2.1. Process-induced changes

2.1.1. Destabilisation due to calcium fortification (L)

Like many other nutritional beverages, IMFs are fortified with minerals. Although much of the minerals in IMFs are supplied by the reconstituted skim milk powder, and whey-based ingredients to a lesser degree, further fortification is required to meet regulatory limits (Fig. 3). The addition of minerals, and calcium in particular, can impair the stability of IMF formulations during processing and subsequent storage.

While the negative effects of calcium fortification on systems such as skim milk have been well characterised (Deeth and Lewis, 2014), there has been surprisingly little research on these effects in IMFs. The nature of the destabilisation caused by fortification of dairy protein-based systems with calcium depends on whether the salt is soluble or insoluble (Omoarukhe *et al*., 2010; Crowley *et al*., 2014b). Insoluble calcium salts (e.g., salts of phosphate, carbonate or citrate) can sediment during processing of liquids or after reconstitution of fortified powders (Williams *et al*., 2005), and are known to impart undesirable sensory qualities (Singh *et al*., 2007). To improve the physical stability of insoluble calcium salts, formulators can use micronized forms of calcium and/or thickening agents such as carrageenan (Gerhart and Schottenheimer, 2013). Sedimentation, or "fall-out", of insoluble calcium salts in IMFs often necessitates an overage, where an excess of calcium is added to ensure the label claim is met post-processing and that quality assurance limits are satisfied over shelf-life (Fig. 3).

Although insoluble calcium salts are typically considered inert, recent studies have demonstrated that addition of hydroxyapatite to milk protein solutions can result in adsorption of proteins to the insoluble calcium salt (Tercinier *et al*., 2013, 2014b); protein adsorption, studied in sodium caseinate and whey protein solutions, was found in these studies to be much more pronounced for caseins than for whey proteins. Wen *et al*. (2016) studied additional calcium salts in adsorption studies of sodium caseinate, finding that the adsorption characteristics of hydroxyapatite and tri-calcium phosphate were similar, but that far less casein adsorbed to calcium carbonate; the much lower specific surface area of the calcium carbonate compared to the other salts was linked by the researchers to its low protein adsorption, due to a reduction in potential adsorption sites. Tercinier *et al*. (2014a) added hydroxyapatite to solutions containing native casein micelles (milk or micellar casein solutions) and found evidence that its addition caused changes in mineral equilibria, which in turn caused increases in serum casein that subsequently bound to the hydroxyapatite.

Soluble calcium salts (e.g., lactate, chloride, gluconate or hydroxide) increase levels of ionic calcium, with a concomitant increase in heat-induced destabilisation due to increased charge-screening between proteins (Vyas and Tong, 2004; Singh *et al*., 2007; Omoarukhe *et al*., 2010; On-Nom *et al*., 2012; Crowley *et al*., 2014b) or protein-stabilised fat globules (McCarthy *et al*., 2014). One method proposed for maintaining the heat stability in calcium-fortified systems is to mix sterile calcium solutions with the product after it has been heat-treated (Harada *et al*., 1989). Similarly, the use of dry-blending systems can prevent some of these destabilisation reactions (Fig. 2). Alternatively, the calcium-ion activity of dairy protein-based systems can be reduced by the addition of calcium-binding agents (de Kort *et al*., 2012). Factors related to the heat stability of IMFs are covered in greater detail in Section 2.1.2. Approximately two-thirds of the calcium in milk is associated with the micellar phase, mostly in the form of amorphous calcium phosphate; on addition of soluble calcium, a considerable proportion becomes complexes with casein micelles as calcium phosphate, which is associated with a pH drop, an increase in sedimentable protein and a partial desolvation of micelles (Philippe *et al*., 2003; Crowley *et al*., 2014b). Future strategies to increase the stability of fortified calcium could involve the use of amorphous calcium phosphate that has been pre-complexed with casein. Holt *et al*. (1998) demonstrated that

hydrophilic phosphopeptides derived from β-casein can stabilise supersaturated solutions of calcium phosphate; it has been proposed that stable beverages containing up to 30-times the calcium content of milk can be prepared using this approach (Holt, 2009). Thachepan *et al*. (2010) achieved a similar stabilising effect with solutions of intact (non-hydrolysed) β-casein.

Some general characteristics of common calcium salts used for fortification purposes are shown in Table 2. It is clear that these calcium salts have very different physicochemical effects on a dairy protein system. It is also worth noting the marked differences in the contribution of calcium to the total mass of the salts (i.e., potency), which is rarely addressed in the literature; calcium gluconate, for example, is expensive on a mass basis, which is exacerbated by the high fortification levels required to achieve a given target increase in total calcium and the quantity of alkali which must be added to offset the acidification caused by its addition. However, what is most unique about calcium fortification practices in the IMF industry is that anything from one to four salts may be added, depending on the manufacturer and the product type (powder or liquid), with different combinations of soluble and insoluble calcium salts used by different manufacturers (Bass and Chen, 2006). This 'calcium cocktail' approach is due to the need to balance the need to increase calcium levels with the technological implications of adding the salt and the regulatory constraints surrounding the addition of the counterion (e.g., chloride, phosphate). In the model IMFs described by Michel *et al*. (1993), 38% of total calcium was comprised of calcium chloride/citrate or calcium chloride/hydroxide combinations for sterilised and spray-dried formulae, respectively; although the researchers commented that the combinations of calcium salts were selected for optimal heat stability, there was no detailed discussion of the relative merits of each combination or why different salts were used for the different processes.

It is notable that fortification practices can differ depending on whether the product is liquid or powder, which is related to different physicochemical properties of these ingredients (Table 2). At the typical pH of IMFs (pH 6.4-7.5; Chávez-Servín *et al*., 2015), insoluble calcium salts in liquid products will have a tendency to sediment during processing or storage.

Table. 2. Physicochemical properties of calcium salts and their effects when added to milk[a].

Ca salt	Formula	Potency (%)	Solubility	ΔpH[b]	$[Ca^{2+}]$[b]	HCT
Carbonate	$CaCO_3$	40	Insoluble	~	~	~
Chloride	$CaCl_2.2H_2O$	27	Soluble	- - -	+++	- - -
Citrate	$Ca_3(C_6H_5O_7)_2.4H_2O$	21	Insoluble	~	~	-
Gluconate	$C_{12}H_{22}CaO_{14}.H_2O$	8.9	Soluble	- - -	+++	- - -
Hydroxide	$Ca(OH)_2$	54	Soluble	+++	++	- -
Phosphate	$CaHPO_4.2H_2O$	23	Insoluble	~	~	-

[a] adapted from Crowley *et al*. (2014b)

[b] symbols indicate nature and extent of change: +++ most severe increase, - - - most severe decrease, ~ no measurable change.

ΔpH = change in pH on addition to milk

$[Ca^{2+}]$ = ionic calcium level of milk after fortification

HCT = heat coagulation time, after addition to milk

Liquid IMFs could potentially be stored for up to two years, which is more than sufficient time for the sedimentation of suspended particles; conversely, powdered IMFs are consumed ≤24 h after reconstitution, within which time sedimentation would be less severe. Suspension stability issues occurring during processing related to the use of insoluble calcium salts can be offset somewhat by the adoption of dry-mixing processes, as shown in Figure 2. Calcium hydroxide is an ingredient in several commercial IMFs, but research into its effects on the stability of dairy protein-based systems to processing has, to these authors' knowledge, so far been limited to the study of Crowley *et al*. (2014b). In that study, it was observed that calcium hydroxide had slightly lower destabilising effect on skim milk during heating compared to other calcium salts; however, an increase in casein micelle size associated with its addition resulted in micelles which underwent the most rapid sedimentation during accelerated physical stability testing. Much remains unclear about the influence of fortification with different combinations of calcium salts on the stability of liquid and powder IMFs. Even the effects of commonly used salts, such as calcium hydroxide, have been infrequently studied in isolation, and never in a whey protein-dominant system.

2.1.2. Destabilisation during thermal processing (L)

The manufacture of IMFs can involve multiple heating steps: recombination of mixed dry ingredients at elevated temperatures (~50-60°C) with water, oil and liquid concentrate ingredients; pasteurisation of the liquid mix; heating by UHT, in-container sterilisation or high-temperature short-time heating (HTST) treatments; and evaporation and spray drying (Fig. 2) for powder manufacture. Particularly heat-labile ingredients (e.g., vitamins) may be added to the IMF after spray drying; alternatively, dry-blending of some or all ingredients may be used to limit degradation of ingredients (Fig. 2).

The two major protein fractions in milk, casein and whey proteins, can be generally classified as heat-stable and heat-labile, respectively (O'Mahony and Fox, 2013). As IMFs are whey protein-dominant products, they are more susceptible to heat-induced denaturation, aggregation and coagulation than typical milk products (Rattray and Jelen, 1997; Singh *et al*., 2014). IMFs are also high-fat systems; as the fat globules in IMFs are coated at least partially by negatively-charged proteins, they can undergo similar heat-induced destabilisation to those reported for proteins during heating (McCarthy *et al*., 2014). Furthermore, the presence of high levels of lactose and added calcium in IMFs could also negatively affect their heat stability, due to increased heat-induced acidification and increased ionic-calcium activity, respectively (Crowley *et al*., 2014b).

Michel *et al*. (1993) reported that holding liquid mixes at 101°C caused a marked reduction in levels of soluble calcium and zinc during the production of sterilised and spray-dried infant formulae. These results were supported by the work of Guo *et al*. (1996), who reported extensive association of casein and casein-bound calcium, zinc, phosphorus and magnesium, in addition to whey proteins, with the lipid fraction in five commercial IMFs. From electron microscopy and SDS-PAGE, Guo *et al*. (1996) concluded that casein micelles adsorbed to the lipid-aqueous phase interface, which was attributed to homogenisation; in addition, heat-induced interactions between denatured β-lactoglobulin and κ-casein at the surface of casein micelles seemed to have contributed to the high levels of whey protein at the interface. These results were subsequently confirmed by Guo *et al*. (1998), who suggested that HTST heating and homogenisation had the most significant influence

on the incorporation of proteins and minerals into the lipid phase of IMFs prepared at pilot scale, while evaporation and spray drying had little effect.

In a comprehensive evaluation of various model IMF systems, McSweeney *et al*. (2004) studied the effects of modifying the pH, whey protein:casein ratio, total solids concentration, and lipid, mineral and lactose content of blends of reconstituted skim milk and electrodialysed whey powders on heat stability. Regions of high and low heat stability were pH-dependant, and associated with the presence of β-lactoglobulin/κ-casein complexes at the surface of casein micelles or in the serum phase, respectively, a well-known phenomenon in studies relating to the heat stability of milk (Singh, 2004). As whey protein:casein ratio increased, the regions of high heat stability became limited to narrower pH limits. The addition of calcium or lipids shifted regions of high heat stability to more alkaline pH values, while lactose only had a negative effect in regions of maximal heat stability. As the pH values of commercial IMFs were shown by Chávez-Servín *et al*. (2015) to vary quite widely, the pH-dependency of the heat-induced destabilisation of IMFs is an important consideration during the design of appropriate thermal processes. Mulcahy *et al*. (2016) demonstrated that conjugation of whey protein hydrolysate with maltodextrin can markedly reduce the extent of heat-induced turbidity development and gelation.

α-Lactalbumin-enriched IMFs are now available commercially. The availability of these IMFs is due to advances in the manufacture of α-lactalbumin-based ingredients (see Section 3.1). This is a critical development in the humanisation of IMFs, as α-lactalbumin is one of the primary whey proteins in human milk (Table 3). However, the possible technological implications of modulating the whey protein profile of IMF systems in this way have not been researched extensively. Crowley *et al*. (2016) investigated the influence of different α-lactalbumin:β-lactoglobulin ratios in model whey protein-dominant IMF protein systems prepared by reconstituting milk protein concentrate (80% protein) and purified fractions of α-lactalbumin- or β-lactoglobulin-enriched whey protein ingredients in simulated milk ultrafiltrate (SMUF). Heat stability at 140°C increased markedly as α-lactalbumin:β-lactoglobulin ratio increased, due to the limited ability of α-lactalbumin to participate in destabilising reactions, in comparison to the more

heat-labile β-lactoglobulin (Rattray and Jelen, 1997; Wijayanti *et al.*, 2014). Similarly, in an investigation of model IMFs with partially or selectively hydrolysed whey proteins, Murphy *et al*. (2015) observed markedly reduced post-heating viscosity in IMFs which contained extensively hydrolysed β-lactoglobulin. The authors highlighted the implications of reduced post-heating viscosity during the manufacture of IMFs; lower viscosity affords greater opportunities to maintain a turbulent flow regime during thermal processing, which would reduce residence time at heat-exchanger walls, thereby reducing fouling. In addition, as excessive thickening limits the ability of manufacturers to dry IMF concentrates at high solids contents, a lower feed viscosity may allow high-solids drying, which is more cost-effective.

Due to the high fat content of IMFs, ingredients with surfactant properties are commonly added to improved emulsion stability. McSweeney *et al*. (2008) investigated the effect of the addition of emulsifiers (lecithin or monoglycerides) on the heat stability of fat-containing model whey protein-dominant IMFs, and observed that addition of lecithin did not change the zeta-potential of the emulsions but did increase their heat stability, while monoglycerides decreased both the zeta-potential and the heat stability of the emulsions. The researchers therefore hypothesised that lecithin, a negatively-charged phospholipid, replaced or interacted with negatively-charged proteins at the surface of fat globules in such a way that repulsion due to steric and electrostatic forces was enhanced; on the other hand, the negative effect of monoglycerides on heat stability was considered to be due to their displacement of proteins at the surface of fat globules, with a concomitant reduction in steric and electrostatic repulsive forces. The properties of surfactants at and around the lipid-aqueous phase interface have important implications for the development of next-generation IMFs. Currently, β-casein and lactoferrin are two of the most interesting candidates for further humanisation of IMFs, due to their greater contribution to the protein profile of human milk compared to bovine milk (Table 3). In addition to their importance in the improvement of the nutritional quality of IMFs, they may also have important implications for heat stability during processing. Dephosphorylation of purified β-casein using potato acid phosphatase resulted in β-casein-stabilised emulsions which were less susceptible to calcium-induced aggregation (McCarthy *et al*., 2013b), which suggests that such an approach

may be a useful way of limiting the destabilising influence of ionic calcium (Table 2) during the heating of IMFs. The use of dephosphorylated β-casein is also relevant to efforts which aim to humanise IMFs, as the majority of β-casein forms in human milk (β-casein exists in multiple degrees of phosphorylation in human milk) have less phosphate groups than the equivalent β-casein in bovine milk (Sood and Slattery, 1997).

Using low-amplitude oscillatory rheology, McCarthy *et al*. (2014) measured the increase in storage modulus (*G'*) as an index of heat-induced coagulation of emulsions (pH 7.0) stabilised by β-casein, lactoferrin or 1:1 mixtures of both during heating to 90°C, holding, and cooling to 45°C. The researchers reported that the negatively charged β-casein (isoelectric point of 4.6-4.8) was heat-stable unless subject to charge-screening by additional calcium ions while, conversely, the heat-labile lactoferrin (isoelectric point of 8.0-8.5) became more stable to heating when the level of calcium chloride was increased to 5 mM; this was proposed to be due to binding of ionic calcium by carboxyl groups of sialic acid present in the glycan chains of lactoferrin, with a concomitant increase in attractive forces between the glycan chains and amino acids of lactoferrin, which could have inhibited heat-induced unfolding of the protein. These researchers found that a 1:1 mixture of both proteins yielded a heat-stable calcium-containing emulsion, with calcium improving the heat stability of lactoferrin, and lactoferrin, in turn, improving the heat stability of β-casein in the presence of calcium.

Future studies on the heat stability of model IMF systems may benefit from the development of appropriate dispersants for dry protein ingredients. Crowley *et al*. (2015b) reported that a model whey protein-dominant IMF was more stable to heating at 140°C when reconstituted in ultrafiltration permeate from a commercial IMF, compared to SMUF or skim milk ultrafiltrate; the result was attributed to the lower calcium-ion concentration in the IMF permeate. Further research is required in this area, however. Differences in the mineral fortification strategies between manufacturers, in addition to the different solids levels encountered during various IMF processes, mean that, in reality, IMFs are exposed to a range of mineral environments, which need to be further characterised if more appropriate methods to characterise the heat stability of model IMFs are to be developed.

Table. 3. Protein profiles of bovine milk, human milk and first-age infant milk formula. Values reported for mammalian milks are for term milk. ND indicates that the level of that protein was not determined. α-Lac = α-lactalbumin, β-lg = β-lactoglobulin, Ig = immunoglobulins, LF = lactoferrin, LPO, lactoperoxidase, OPN = osteopontin, TGF-β, transforming growth factor β, MFGM-E8 = milk fat globule membrane E-8.

Fraction	Protein	MW (kDa)	Bovine milk	Human milk (g/L)	Formula
Total casein			**30**	**3.5-4.4**	**4.8**
	α_{s1}	23	12-15	0.6	1.74
	α_{s2}	25	3-4	0.0	0.48
	β	24	9-11	2.7	1.62
	κ	19	3-4	0.9	0.6
	γ	12-21	1-2	0.0	0.36
Total whey protein			**5.0**	**5.3-6.6**	**7.2**
	α-lac	14	1-1.5	2-3	1.4-2.3
	β-lg	18	3-4	0.0	3.5
	Ig	75-95	0.6-1.0	1.2	ND
	LF	80	0.01-0.1	1-2	0.05
	LPO	80	0.01-0.03	0.005	ND
	OPN	60	0.018	0.138	0.01
Other proteins					
	TGF-β	25	1.3×10^{-5}	0.001	ND
	MFGM-E8	47/50	28.2-48.4	0.05-0.10	ND

[a] modified from Chatterton *et al*. (2013)

2.1.3. Breakage of agglomerates during powder transport (P)

The majority of commercial IMF powders are agglomerated during manufacture. Agglomeration improves many of the functional properties of IMFs, such as flowability and rehydration, due to increases in particle size and altered surface reactivity (Cuq *et al*., 2013). However, the large size and irregular morphology of agglomerates, in comparison to primary particles, makes them more susceptible to breakage when exposed to external mechanical forces (Barrett and Peleg, 1995). One practical implication of the relative mechanical instability of agglomerated particles is that the measurement of particle size distribution using static light-scattering can be compromised if dry dispersion (i.e., using pressurised air as a continuous phase) is applied; Kwak *et al*. (2009) demonstrated that the particle size of an agglomerated IMF powder decreased consistently with increasing air pressures in the range 0.1-2.0 bar.

It is common industrial practice to transport agglomerated IMF powders using air in lean- or dense-phase format to various locations in the processing plant (e.g., hoppers, packing areas) through a network of steel pipes (Fig. 2). This process, known as pneumatic conveying, brings agglomerated particles into contact with each other and also the walls of the piping; it has been suggested that both particle-particle interactions (Richardson *et al*., 2009) and particle-wall interactions (Hanley *et al*., 2013) are the dominant factors influencing particle breakage in such scenarios. The potential for the breakage of agglomerates is a considerable concern for a number of reasons. At a practical level, it has been reported frequently that small particles and a high specific surface area are primary contributors to poor flowability (Fitzpatrick *et al*., 2004; Crowley *et al*., 2014a); hence, after an IMF has been pneumatically conveyed to a hopper, it may fail to flow through the outlet as predicted. Mor seriously, however, the fracturing of small particles from agglomerates is likely to alter bulk density of the powder; as IMFs are dispensed by the user on a volume basis (i.e., using scoops) to attain a specific composition, any change in bulk density may result in inappropriate nutrient delivery (Hanley *et al*., 2011a; Crowley and O'Mahony, 2016).

Hanley *et al*. (2011a, b) used a custom-built pneumatic conveying rig at pilot scale to assess the effect of pneumatic conveying parameters and composition of

IMF powders, respectively, on the physical and chemical properties of conveyed IMFs. Seven conveying parameters were varied by Hanley *et al*. (2011a) to determine the significance of their impact on these properties. Operation mode (lean- versus dense-phase) was the only parameter to significantly affect particle size and wettability, while operation mode, air velocity and conveying passes all significantly affected bulk density; however, air velocity was only significant during dilute-phase conveying, which is rarely used in the transport of IMF powders. The researchers modified the pneumatic conveying parameters based on these results and reported that changes in particle size and bulk density were negligible; the principal feature of the optimised conveying process was operation in dense-phase flow at a low air velocity.

Pneumatic conveying in dilute-phase mode at high air velocity, and to a lesser extent in dense-phase mode at low air velocity, were identified again by Hanley *et al*. (2011b) as having a marked negative effect on the physical and chemical properties of IMF powders due to agglomerate breakage. Results from this study also suggested that differences in the fat:protein ratio of the different IMFs studied had a strong influence on the mechanical stability of the agglomerates. Uniaxial compression testing was used to measure the normal force at failure and stiffness of individual agglomerated particles, with both values generally increasing with increasing protein:fat ratio. IMF powders which displayed high force at failure and stiffness tended to undergo the least significant changes in bulk density during pneumatic conveying.

Hanley *et al*. (2012) used discrete element modelling to gain further insights into the principal mechanisms governing failure during uniaxial compression of individual agglomerates in IMF powder, with data inputs from the compression experiments of Hanley *et al*. (2011b), in addition to microscopic analysis of the powders. Under compression, the agglomerates, simulated as a polydisperse network of interacting rigid spheres, separated into distinct sub-agglomerates due to breakage of inter-particle bonds; the researchers proposed that the initial shape of a given agglomerate dictated the extent to which it fragmented into sub-agglomerates, while the sub-agglomerates were stable structures which resisted further fragmentation.

2.2. Storage-induced changes

2.2.1. Creaming of fat globules (L)

It has long been known that the amphiphilic nature of milk proteins, the addition of surfactants or thickeners, and the application of homogenisation can all be exploited in various combinations to improve the emulsion stability of IMFs. Such formulation strategies result in fat globules that are much smaller than their equivalents in human milk (Michalski *et al*., 2005), but they are nonetheless a prerequisite for the consistent quality which is expected of liquid IMFs over an extended shelf-life.

Based on the results of ultracentrifugal separation experiments with radio-labelled casein and whey proteins, Rowley and Richardson (1985) discussed how the presence of insufficient protein at the lipid-aqueous phase interface could promote coalescence and creaming, while too much protein at the interface could result in gelation; therefore, an intermediate level of protein-lipid interactions was apparently required for optimal emulsion stability. In hypoallergenic IMFs, in which proteins are hydrolysed, the hydrolysates may not have the same ability to stabilise the emulsion as the intact protein (Sawatski *et al*., 1994); however, it has been proposed that, in partially hydrolysed ingredients, peptides may interact with proteins in a synergistic manner, creating a weak gel-like network which inhibits coalescence of fat globules in the same manner as a thickener such as carrageenan (Lajoie *et al*., 2001). Murphy *et al*. (2015) reported that IMFs containing partially-hydrolysed whey proteins had significantly larger fat globules than the non-hydrolysed control before and after heating/homogenisation; however, a selectively-hydrolysed IMF containing no intact β-lactoglobulin had the same fat globule size as the control. The authors attributed the unchanged emulsion characteristics of the selectively-hydrolysed IMF to the inability of α-lactalbumin to undergo extensive heat-induced denaturation/aggregation in the absence of sufficient levels of intact β-lactoglobulin (Wijayanti *et al*., 2014). It has been demonstrated that the stability of whey protein hydrolysate-based IMF emulsions can be improved by addition of lecithin (Drapala *et al*., 2015) or conjugation of the hydrolysate with maltodextrin (Drapala *et al*., 2016a, b).

Fligner *et al*. (1990) measured the effect of altering carrageenan and lecithin levels, in addition to whey protein:casein ratio, on the emulsion stability of a sterilised concentrated IMF using gravitational creaming, accelerated creaming (ultracentrifugal separation) and fat globule size distribution analyses. The researchers reported that higher whey protein:casein ratios yielded the most stable emulsions, possibly due to the greater emulsifying ability of denatured whey proteins or an increased viscosity; in addition, the presence of denatured whey protein-casein micelle complexes at the surface of fat globules (Michel *et al*., 1993; Guo *et al*., 1996, 1998) may have resulted in fat globules of greater density and/or increased steric repulsion between fat globules (Fligner *et al*., 1991). Fligner *et al*. (1990) identified an optimal lecithin addition level of 0.5% as being required to improve emulsion stability without incurring the negative effects of excessive protein displacement from the lipid-aqueous phase interface. The effect of emulsifiers (lecithin or monoglycerol) or thickeners (locust bean gum or carrageenan) on the emulsion stability of IMFs were measured by Zou and Akoh (2013), with emulsifiers having a more pronounced stabilising effect at the levels studied.

The casein and whey proteins present in the dairy ingredients used to manufacture an IMF act as natural emulsifiers. As the protein profile of IMFs becomes more humanised, the protein content could be lowered while the amino acid requirements are still satisfied (Kuhlman *et al*., 2003). Such a formulation change may have implications for the techno-functional properties of the IMFs, due to a concurrent reduction in the emulsifying capacity of the system. For example, lowering the protein:fat ratio to different ratios between 0.43 and 0.21 resulted in progressive increases in fat globule size, with a concomitant increase in creaming, as measured using analytical centrifugation (McCarthy *et al*., 2012). The emulsifying capacity of β-casein, which is highly surface-active and likely to be used as an ingredient in humanised IMFs, may help offset some of these issues in next-generation IMFs. McCarthy *et al*. (2013b) compared the ability of control phosphorylated β-casein and an enzymatically dephosphorylated form to stabilise emulsions. The former yielded smaller fat globules than the latter, which suggests that a high degree of phosphorylation improves the stabilising effect of β-casein; however, when heated in the presence of calcium, the dephosphorylated form was markedly more stable.

Other improvements in the emulsion stability of IMFs may be achieved by adopting new processing technologies. Murphy *et al*. (2011a) used high-velocity steam injection to heat-treat a high-solids (60%) model IMF, which had been homogenised using a colloid mill; the researchers reported increased emulsion stability, measured using analytical centrifugation, after steam injection due to reduced fat globule size and increased viscosity. In another study, Murphy *et al*. (2011b) compared a control IMF subjected to a typical HTST treatment at low solids (30%) to an IMF heat-treated with high-solids steam injection; despite a lower viscosity and larger fat globule size in the latter, the stability of both IMFs to creaming was similar.

2.2.2. Oxidation of lipids (L, P)

Human milk provides infants with an abundant supply of long-chain polyunsaturated fatty acids (LCPFAs), which have important functions in biological membranes, and are believed to influence cognitive and retinal development (de la Presa-Owens *et al*., 1995; Sawatski *et al*., 1994). For many years, researchers have attempted to fortify IMFs with LCPFAs to achieve a closer match to the lipid profile of human milk. Sawatski *et al*. (1994) discussed how two commonly used sources of LCPFAs, i.e., fat from the organs of animals and fish oils, could be used in combination to attain balanced levels of n-3 and n-6 LCPFAs; however, these sources of LCPFAs were said to have the disadvantages of a risk of toxicity and a tendency to undergo oxidation, respectively.

Oxidation has been one of the major obstacles to the incorporation of LCPFAs into IMFs. As an alternative, Sawatski *et al*. (1994) proposed the inclusion of lipids from the eggs of chickens which had underwent a controlled feeding regime; the egg lipids, when added along with a typical oil blend for IMFs (milk fat, in addition to coconut/palm kernel, palm, sunflower, soybean, and evening primrose oils), were said to yield LCPFA profiles close to human milk without increasing the susceptibility of IMFs to oxidation over storage. Oxidation of lipids in IMFs is undesirable due to resultant rancidity, which may render the sensory properties of the product unacceptable; in addition, the release of free radicals that can scavenge

electrons from cell membranes could have negative health consequences. de la Presa-Owens *et al*. (1995) demonstrated that the addition of 2% microencapsulated fish oil into a commercial IMF powder reduced its shelf-life by approximately 4 months, with markedly higher peroxide values and rancidity being measured after 18 months.

IMFs are rich in metals, such as iron and copper, which act as pro-oxidants that catalyse lipid oxidation reactions during storage. As ingredients such as iron need to be incorporated into IMFs to satisfy regulatory requirements (Thompkinson and Karb, 2007), there is interest in the functionality of other ingredients which may inhibit the action of pro-oxidants. An iron-binding protein, lactoferrin, has already attracted interest as an ingredient in IMF due to its high levels in human milk (Table 3) and antimicrobial activity. In addition, Satué-Gracia *et al*. (2000) reported that lactoferrin was a powerful antioxidant when added to whey protein- or casein-dominant liquid IMFs fortified with lactoferrin at levels up to 220 µM. Bovine serum albumin was also tested as a control, but exhibited no antioxidant activity. Interestingly, the casein-dominant IMF was reported as being more susceptible to oxidation, which was postulated to be due (1) to the presence of soya lecithin, which may have become oxidised itself or facilitated the reaction of metals and oxygen through its emulsifying activity, and/or (2) the low levels of disulphide links and absence of free thiol groups, which can scavenge free radicals. Other ingredients which may reduce lipid oxidation as a secondary function are lecithin and carrageenan, which are sometimes added as emulsifiers and thickeners, respectively. Zou and Akoh (2013) reported significant negative correlations between carrageenan or lecithin content and the extent of lipid oxidation in model liquid IMFs.

Romeu-Nadal *et al*. (2007) investigated the effect of storage temperature (25 or 37°C) and time (up to 15 months storage) on the oxidative stability of IMF powders with a low or high level of supplementation with n-3 and n-6 LCPFAs; the researchers reported that higher LCPFA content, elevated temperature and prolonged storage time all contributed to increased oxidation of lipids and rancidity. Carmen García-Martinez *et al*. (2010) characterised both total lipids and the free oil fraction of IMF powders stored for 3 months at 25, 30 or 37°C for the products of lipid oxidation and the degradation of tocopherols. Despite being identified as rancid by a

sensory panel, IMF powders stored at the highest temperature exhibited no differences in the level of oxidation compounds in their total lipids fraction; however, when the free oil fraction was analysed, it was found to be highly oxidised and low in tocopherols. Thus, the authors of the study considered analysis of the free oil fraction to be crucial when lipid oxidation tests are being performed on IMF products.

In a later study, García-Martinez *et al*. (2012) studied the effect of in-container sterilisation (120°C, 15 min) on lipid oxidation and degradation of tocopherols in whey protein- and casein-dominant liquid IMFs. While the researchers observed no changes in lipid oxidation, the thermal treatment caused substantial degradation of tocopherols. As tocopherols function as antioxidants in IMF systems, it was proposed that in-container sterilisation may indirectly increase oxidation over extended storage; however, the researchers reported that protein, particularly casein, had a protective effect against thermal degradation of tocopherol, with the casein-dominant IMF displaying the lowest losses of the antioxidant. Alternative antioxidants to tocopherol have also been successfully used in IMF systems; Zou and Akoh (2015) reported that an annatto tocotrienol-rich fraction, considered as a 'natural' antioxidant, was a more effective antioxidant than tocopherol in an IMF system.

2.2.3. Crystallisation of amorphous lactose (P)

Lactose often exists in an amorphous state in fresh dairy powders (Roos, 2002). As lactose in the amorphous state is highly hygroscopic, it has a tendency to absorb moisture present as vapour in the surrounding air, particularly under conditions of high relative humidity and/or temperature. Subsequently, the plasticising effect of sorbed water depresses the glass transition temperature and increases molecular mobility, with concomitant transition to the rubbery state, thereby facilitating the formation of crystalline lactose (Roos, 2002). Crystallisation of lactose is the cause of a wide range of defects, including reductions in flowability, solubility and sensory acceptability, and is an important consideration in the development of IMFs, in which the carbohydrate content is high (Table 1).

Chuy and Labuza (1994) investigated caking and stickiness phenomena related to the crystallisation of lactose in three commercial IMFs. The researchers reported that an IMF in which anhydrous lactose contributed 100% of total carbohydrate was more prone to lactose crystallisation and associated defects than an IMF in which a 1:1 mixture of lactose/maltodextrin comprised the carbohydrate fraction. Indeed, high-molecular weight carbohydrates such as maltodextrin are often added to IMFs and other products to enhance powder stability by elevating glass transition temperature. Proteins have also been reported to increase glass transition temperature, with Nasirpour *et al*. (2006) reporting that increasing β-lactoglobulin content increased the minimum relative humidity required to induce crystallisation of lactose in freeze-dried model IMF powders. These results were confirmed by Nasirpour *et al*. (2007), who also observed reduced lactose crystal size in IMFs with increased levels of β-lactoglobulin.

As stated previously, advances in ingredient development may facilitate the formulation of lower protein IMFs which still satisfy amino acid requirements. However, the role of protein in inhibiting lactose crystallisation may be diminished in this scenario. To investigate this, McCarthy *et al*. (2013a) studied moisture sorption, lactose crystallisation and fat release in model IMFs with five different protein:fat ratios between 0.21 and 0.43. Reduced moisture sorption and increased rates of lactose crystallisation were measured as protein:fat ratio was decreased, with migration of fat to particle surfaces (where fat was over-represented relative to bulk composition) as lactose crystallised. The researchers concluded that protein:fat ratio could be decreased to 0.32 without significant negative implications for the stability of IMF powders. Further decreases in protein:fat ratio, without destabilisation, may be possible through the partial replacement of lactose with a carbohydrate like maltodextrin (Chuy and Labuza, 1994); however, significant reductions in innate levels of lactose may require partial replacement of skim milk powder with milk protein concentrate.

Little is currently known about the nature of the crystals which are formed in IMF powders and if, for example, there are certain crystal types which may have less deleterious implications for product stability than others. Schmitz-Schug *et al*. (2013) measured changes in molecular mobility associated with changes in the

physical state of lactose in model IMF powders; they proposed that crystallisation conditions (i.e., water activity, temperature) influence the nature of the crystals formed, based primarily on the observation that changes in these factors affect the transversal relaxation times in the crystalline state, as measured using nuclear magnetic resonance spectroscopy. Such analytical developments present an opportunity to gain a better understanding of the crystalline phase of aged IMF powders, which may facilitate more controlled crystallisation for enhanced stability over shelf-life.

2.3. Changes occurring during processing and storage

2.3.1. Non-enzymatic browning (P, L)

Non-enzymatic browning, *via* the Maillard reaction, can occur during processing and storage of IMFs. In addition to the undesirable sensory characteristics associated with excessive browning, the development of Maillard reaction products can have a negative effect on nutritional properties, primarily due to lysine loss, in addition to altered bioavailability of minerals. High levels of lactose and lysine, together with the extensive thermal treatments and storage times which can be encountered, make IMFs particularly susceptible to such non-enzymatic browning. Ferrer *et al*. (2000) studied process- and storage-induced changes in levels of available lysine and furfural in first- and second-age IMFs. The researchers reported that spray drying resulted in greater lysine loss than pasteurisation of milk or sterilisation of the liquid concentrate for both IMFs. Subsequently, browning reactions continued for the second-age formula during storage of the powder, while the first-age IMF was relatively stable; the second-age IMF contained more protein, and casein comprised a greater proportion of this, and, in addition, maltodextrin comprised 40% of the total carbohydrate (lactose was the only carbohydrate in the first-age formula), suggesting that these compositional differences were responsible for observed differences in browning during storage.

Sarriá *et al*. (2001) assessed the extent of the Maillard reaction in sterilised and spray-dried IMFs, and its relationship with calcium bioavailability. The researchers found that the Maillard reaction was more advanced in IMFs which had

been subjected to in-container sterilisation compared to spray-dried IMFs; consequently, *in vivo* (rat) models indicated that absorption of calcium from the sterilised IMF was higher, but calcium utilisation was decreased. Instead of in-container sterilisation or spray drying, UHT treatment is sometimes applied to IMFs during processing (Fig. 2). Guerra-Hernandez *et al*. (2002) measured the development of the Maillard reaction in two liquid IMFs, one of which included a UHT treatment step during processing, over storage; the researchers observed a greater extent of browning in the IMF which had been subjected to UHT treatment. The rapid progression of the Maillard reaction in IMFs subjected to UHT treatment was confirmed by Roux *et al*. (2009), who simulated the process using ohmic heating at lab-scale.

The extent of browning in IMFs increases with increasing storage/heating time and temperature and, in addition, the changes are more pronounced in nitrogen-rich than in oxygen-rich gaseous environments (Guerra-Hernandez *et al*., 2002a, b). In general, the generation of Maillard reaction products increases further when the seal of the IMF container is broken, due to exposure to UV light and air (Chávez-Servín *et al*., 2015). Increased moisture sorption at high relative humidity, with a concomitant increase in water activity, also results in increased generation of Maillard reaction products during storage of IMFs containing amorphous lactose; however, when lactose is partially replaced with a non-reducing sugar, such as starch, the extent of non-enzymatic browning is reduced (Nasirpour *et al*., 2006). Enrichment of IMFs with microencapsulated fish oil (to increase levels of long-chain polyunsaturated fatty acids; see Section 2.2.2) by dry-blending has been shown to have no influence on the extent of non-enzymatic browning in IMF powders (Chávez-Servín *et al*., 2006). Schmitz-Schug *et al*. (2013) observed that the extent of lysine loss over time during heating of IMFs at 90°C decreased as the lactose:protein ratio increased. The researchers also observed that lactose in IMFs with low lactose:protein ratios tended to remain in the rubbery state above the glass transition temperature and, hence, had a higher molecular mobility than IMFs with higher lactose:protein ratios, in which more lactose was crystalline. Thus, it was hypothesized that the presence of crystalline lactose inhibits the progression of the Maillard reaction in IMF powders.

3. Progress in the development of protein ingredients for infant milk formulae

3.1. Whey protein ingredients with increased α-lactalbumin:β-lactoglobulin ratios

β-Lactoglobulin is the principal whey protein in bovine milk but is absent from human milk (Table 3), and is therefore thought to stimulate an allergenic response in susceptible infants. Hypoallergenic formulae are based primarily on the enzymatic hydrolysis of proteins; however, extensively hydrolysed proteins often have inferior functional and sensory characteristics (Bu *et al*., 2013). Thus, much research in recent years has focused on reducing the potential allergenicity of IMFs by depleting β-lactoglobulin from raw ingredients, such as whey protein concentrates. On the other hand, α-lactalbumin is the principal whey protein in human milk and a small segment of the IMF market now consists of products fortified with α-lactalbumin. Although there are α-lactalbumin isolates available, it is more common for levels to be increased using whey protein concentrates with high α-lactalbumin:β-lactoglobulin ratios.

Bottomly (1991) reported that ultrafiltration/diafiltration of whey protein concentrate at 50°C using a 100 kDa membrane could be used to generate permeates which had α-lactalbumin:β-lactoglobulin ratios up to 3-fold higher than that of the starting whey. Most researchers have used pre-treatment (i.e., high heat and/or acidification) of whey to selectively precipitate either α-lactalbumin or β-lactoglobulin and enhance the fractionation of these whey proteins, which otherwise have similar molecular weights (Table 3). Pearce (1995) described the following method for the generation of enriched fractions of α-lactalbumin and β-lactoglobulin using a combination of acidification and heating to selectively aggregate α-lactalbumin: reduction in density and ionic strength by diafiltration of whey; pH-adjustment to 4.3 with agitation; heating of acidified whey at 63°C for 10 min; cooling to 50°C and holding for 10 min; and centrifugal separation or microfiltration of the liquid into a β-lactoglobulin-enriched supernatant/permeate and α-lactalbumin-enriched pellet/retentate for further concentration and drying. α-Lactalbumin only comprised 2.5% of protein in the fraction in enriched in β-lactoglobulin (71% of total protein), although the purity of the α-lactalbumin fraction was not reported.

de Wit and Bronts (1995) exposed whey to a calcium-binding ion-exchange resin to remove bound calcium from α-lactalbumin, thereby transforming it into its *apo* form, which is more susceptible to aggregation (Brew, 2003). The whey was separated from the resin, acidified to pH 4.6, and incubated at <50°C to selectively aggregate and precipitate the *apo*-α-lactalbumin. The precipitate was then separated by centrifugation or microfiltration to yield a pellet/retentate enriched in α-lactalbumin (>60% of protein) and a supernatant/permeate enriched in β-lactoglobulin (>80% of protein). Restoration of the native state of α-lactalbumin may be important for the functionality of the final ingredient (Lam and Nickerson, 2015); Lucena *et al*. (2006) proposed that *apo*-α-lactalbumin, prepared from whey protein concentrate using a similar method to de Wit and Bronts (1995), could be transformed into *holo*-α-lactalbumin by solubilisation of the precipitate in a calcium solution at pH 7.5. Wu (2001) reported that, when whey was acidified to pH 3.5, α-lactalbumin could be concentrated in the retentate during processing with 20 kDa ultrafiltration membranes, due to an apparently marked increase in its molecular weight; the researchers reported that this method could be used to prepare an ingredient in which α-lactalbumin comprised 65% of protein. Uchida *et al*. (1996) selectively aggregated β-lactoglobulin using an acid/heat treatment of: pH adjustment (if necessary) of whey to pH ~6.0; UHT treatment of pH-adjusted whey followed by cooling to 50°C; and ultrafiltration (150 kDa) or microfiltration (0.15 μm) to yield a β-lactoglobulin-enriched retentate and α-lactalbumin-enriched permeate. α-Lactalbumin:β-lactoglobulin ratios in the permeates were 9-14 fold higher than the starting whey.

Etzel (1999) highlighted the main disadvantage of precipitation- and/or membrane-based enrichment of the two major whey proteins, stating that they require large volumes of feed material to achieve sufficiently high yields. Instead, the author proposed separation by ion-exchange to produce two enriched fractions based on differences in the isolelectric points of β-lactoglobulin and α-lactalbumin: pH-adjustment of whey to 4.5; processing of pH-adjusted whey at >40°C through a cation-exchange resin which reversibly binds both β-lactoglobulin and α-lactalbumin; and finally flushing with elution buffer at 4.9 and 6.5 for elution of β-lactoglobulin and α-lactalbumin, respectively. Unlike earlier ion-exchange methods, which used different NaCl concentrations in combination with pH-adjustment for the

fractionation of whey proteins (Ahmed *et al*., 1998), the method of Etzel (1999) was based entirely on pH, and therefore did not require intensive washing and conditioning steps between elutions. Lozano *et al*. (2008) reported the preparation of β-lactoglobulin by selective enrichment from other whey proteins using precipitation with different concentrations of ammonium sulphate, followed by isolation using cation-exchange chromatography.

Due to its slightly lower molecular weight, native α-lactalbumin tends to permeate wide-pore ultrafiltration membranes to a greater degree than β-lactoglobulin, which can be exploited in the fractionation of the two proteins. A two-stage ultrafiltration and diafiltration process (300 kDa) was used by Muller *et al*. (1999) to obtain an enriched α-lactalbumin fraction (~50% pure) from acid casein whey. Marella *et al*. (2011) processed pre-microfiltered cheese whey with wide-pore (50, 100 or 300 kDa) membranes, and reported that α-lactalbumin purities of >50% could be attained with 50 and 100 kDa membranes; these researchers reported that high trans-membrane pressures were required to achieve this degree of purity with the latter membrane. It is difficult to achieve high purity values when separating β-lactoglobulin and α-lactalbumin based on molecular weight alone, as they are similar in this regard (Table 3). More recently, positively-charged ultrafiltration membranes, which exploit differences in the isoelectric points as well as the molecular weight of proteins, have been successfully applied in the fractionation of β-lactoglobulin and α-lactalbumin at much higher purities (80-87%) than has been achieved with uncharged wide-pore membranes (Arunkumar and Etzel, 2013, 2014).

Kuhlman *et al*. (2003) used a whey protein concentrate which had been enriched in α-lactalbumin in the formulation of an IMF. The presence of higher quantities of α-lactalbumin allowed the researchers to reduce total protein content from 1.5% to 1.4%, to more closely resemble human milk (Table 1), while still satisfying amino acid requirements set forth in regulations without fortification with amino acids. This more humanised product was demonstrated to be more acceptable to infants, with more efficient protein utilisation also being reported.

3.2. Infant milk formulae with increased levels of β-casein and lactoferrin

Progress in the humanisation of the casein fraction of IMFs is less advanced than that of the whey protein fraction, presumably because the latter accounts for a greater proportion of total protein in most IMFs (Table 3). The primary casein in human milk is β-casein (Table 3). To these authors' knowledge, there are no β-casein-enriched-IMFs manufactured in the Americas, Europe or Australasia, although there are some reports of these IMFs being available in Asia. IMFs manufactured with milk containing a specific genetic variant (A2) of β-casein (note: they are not enriched in β-casein relative to the other caseins) are being manufactured and marketed in Australia and New Zealand based on a hypothesis centered on a premise that the more common form (A1) is a risk factor in illnesses including diabetes, heart disease, schizophrenia and autism; however, the claims surrounding 'A2 milk' have been strongly refuted (Truswell, 2005; Chin-Dusting *et al*., 2006). There are few studies on differences in the physicochemical properties of the two genetic variants of β-casein; however, Raynes *et al*. (2015) recently reported that solutions of the A2 variant formed larger β-casein micelles and contained more β-casein monomers than the A1 variant, differences which were deemed responsible for the greater ability of the former to inhibit the aggregation of lysozyme or α-lactalbumin during heating.

The ability of β-casein ingredients to form micelles, bind calcium and phosphorus, and inhibit aggregation of proteins (i.e., chaperone-like activity) will be important considerations in the development of β-casein-enriched IMFs. In human milk at 37°C, β-casein is present in micelles which are digested differently to bovine casein (Li-Chan and Nakai, 1988); as in bovine milk, these micelles are stabilised primarily by a combination of hydrophobic and electrostatic interactions (Sood *et al*., 1997). The size and stability of human β-casein micelles is affected by β-casein concentration, ionic strength, calcium-ion content and temperature (Sood and Slattery, 2001); this is also true for bovine β-casein (O'Connell *et al*., 2003), although differences in the degree of phosphorylation between the two mammalian β-caseins means they have different temperature-dependant association behaviour (Sood and Slattery, 1997, 2001; McCarthy *et al*., 2013b). A significant replacement of the whole casein present in IMFs with β-casein would likely result in pronounced

differences in optical, rheological and process-stability characteristics. Furthermore, it has been demonstrated that β-casein can inhibit the aggregation of β-lactoglobulin (Kehoe and Foegeding, 2014) and α-lactalbumin (Raynes *et al*., 2015) through its aforementioned chaperone-like activity, indicating that heat stability of IMFs may also be affected by such a formulation change. The possible implications of β-casein enrichment for the stability of IMFs need to be investigated in greater detail.

There remains a pronounced disparity between the levels of casein profile of human milk and IMFs, and there is a need to develop ingredients which can be used to address this. Multiple methods to manufacture β-casein, primarily based on the dissociation of β-casein from the micellar phase into the serum phase at cold temperatures and subsequent separation of the phases using membrane filtration, have been developed over the last 50 years; while outside the scope of the present review, these methods are the basis of a comprehensive review being prepared (Crowley *et al*., 2017) on the enrichment and purification of β-casein from milk.

The large-scale manufacture of lactoferrin, one of the primary whey proteins in human milk (Table 3) using cation-exchange chromatography began in earnest over 30 years ago, and lactoferrin-supplemented IMFs are commercially available in Asia (Tomita *et al*., 2009). However, very large quantities of whey are needed to attain practical yields of lactoferrin. Etzel (2004) reported that 10,000 L of whey is required to generate just 1 kg of the protein. Thus, lactoferrin is considered a prohibitively expensive ingredient by the majority of IMF manufacturers. For these reasons, most commercial IMFs contain lactoferrin at levels below the 100-300 mg $100g^{-1}$ found in human milk, with concentrations of 50-75 mg 100 g^{-1} being measured in 12 commercial IMFs by Zhang *et al*. (2014).

3.3. Cheese whey-derived protein ingredients in infant milk formula manufacture

Annatto is frequently used to imbue cheese with a yellow/orange colour. This is particularly important where cheese milk is derived from non-pasture fed cows, which results in less carry-over of naturally occurring carotenoids. Residual annatto in whey protein ingredients derived from cheese whey is undesirable in many food applications (Kang *et al*., 2010). For this reason, bleaching of cheese whey is often

performed during the manufacture of whey protein powders (Listiyani *et al*., 2011). There are strict regulatory limits on the level of norbixin, the principal carotenoid in annatto, which can be present in whey protein ingredients intended for use in IMF applications (Campbell *et al*., 2014). In addition, the use of bleaching agents, such as benzoyl peroxide or hydrogen peroxide, in the production of IMF ingredients, is either prohibited or heavily regulated; for example, benzoyl peroxide, when reacted with annatto, forms benzoic acid, the presence of which in whey ingredients is severely restricted in the USA and Europe, and banned in China (Kang *et al*., 2010).

Some recent research has focused on the production of cheese-whey-derived ingredients which are low in norbixin. These products could satisfy regulatory requirements for norbixin while limiting the need to bleach. In whey, norbixin is thought to exist as micelles which are associated with the milk fat globule membrane (MFGM) fraction (Zhu and Damodaran, 2012). When complexes formed between MFGM and chitosan were removed from cheese whey by microfiltration, the resultant whey protein concentrate had a high degree of clarity (Lucey *et al*., 2010). Bixin, a non-polar colourant, was shown to have a greater affinity for the lipid phase retained in the curd during cheese-making (Smith *et al*., 2014). The use of β-carotene, which is naturally present in milk, has also been proposed as an effective alternative colourant with a performance comparable to that of annatto (Moeller *et al*., 2014).

Stronger regulations on the quality of cheese whey-derived protein ingredients intended for use in IMFs will necessitate the adoption of technologies which reduce carry-over of colourant and/or bleach residues. If these technologies are not successfully applied then alternative sources of whey proteins may need to be considered by IMF manufacturers. One such source is the whey stream generated during the microfiltration of milk, which can be further concentrated by ultrafiltration/diafiltration into a ‘native whey’ or ‘serum protein’ concentrate/isolate.

3.4. Cheese whey-derived protein ingredients depleted in glycomacropeptide

Glycomacropeptide (GMP) is the hydrophilic fragment released from the micellar phase during rennet-induced proteolysis of κ-casein during cheese

manufacture (Sharma Neelima *et al*., 2013). GMP is present in whey protein concentrates but not in human milk; however, GMP is ultimately liberated by pepsin during digestion of casein (Sandström *et al*., 2008). GMP is a rich source of threonine, which has been linked with instances of hyperthreoninemia in infants fed whey protein-dominant IMFs (Rigo *et al*., 2001), although later study failed to support this hypothesis (Sandström *et al*., 2008). Low-threonine GMP isolates have recently been developed (McMahon *et al*., 2014).

In itself, GMP is a highly valuable ingredient, particularly as a dietary source for sufferers of phenylketonuria (van Calcar *et al*., 2009). Large-scale purification of GMP from cheese whey can be achieved, with the most successful methods involving ion-exchange (Etzel, 1999; McMahon *et al*., 2014). The by-product of these ion-exchange processes is a sweet whey-derived protein ingredient substantially depleted in GMP. Although ingredients depleted in GMP have received interest for application in humanised IMFs, so has GMP itself, despite its absence from human milk. Human milk contains much higher levels of sialic acid than both bovine milk and commercial IMFs (Wang *et al*., 2001), and interest in the fortification of IMFs with GMP is primarily due to its high sialic acid content (McMahon *et al*., 2006). Although believed to contribute an antipathogenic role in infants, sialic acid has been primarily linked with enhancements in cognitive development (Wang and Brand-Miller, 2003). Sialic acid supplementation was shown to increase performance in memory and learning tasks in piglet models (Wang *et al*., 2007). Casein-dominant IMFs were shown to contain less sialic acid than whey-protein-dominant IMFs (Wang *et al*., 2001).

Sialic acid is localised within different macroconstituents in human milk and bovine milk/IMFs. In the former sialic acid is mainly bound to oligosaccharides, while in the latter it is bound to glycoproteins (Wang *et al*., 2001). Research into the use of oligosaccharides (e.g., galactooligosaccharides, fructooligosaccharides, inulin) as pre-biotics for improving the bifidogenic properties of IMFs is ongoing (Vandenplas, 2002); it is possible that such pre-biotics may have a secondary benefit of offsetting the sialic acid deficit in IMFs. Moreover, increasing levels of other glycoproteins, such as lactoferrin, in IMFs may allow further increases in levels of sialic acid.

3.5. Formulae for improved dietary, clinical and developmental outcomes

Although much of the present review has focused on bovine milk-based first-age IMFs, the most widely used IMF product, this is only one product in an increasingly broad product range. There are also different age-categories, which have already been mentioned, as well as IMFs designed to meet specific dietary requirements. It is important to note again at this point that (1) formulae based on other mammalian milks (e.g., caprine, equine) are sometimes considered as useful alternatives for infants who are intolerant or allergic to bovine milk-based IMFs and that (2) interest in the use of ingredients from non-mammalian sources (e.g., soy, rice) has intensified in recent years (El-Agamy, 2007). However, as (1) and (2) are outside the scope of this review, emphasis in this section will continue to be placed on bovine-based formulations. This section closes with a brief discussion of some additional efforts in formulation advances for the improvement in health outcomes for formula-fed infants.

3.5.1. Cow milk protein allergy

Cow milk protein allergy (CMPA) is an allergenic response to bovine milk proteins with a prevalence which can approach 10% of a given country's populations (El-Agamy, 2007). When the compositions of human and bovine milks are compared, certain discrepancies in protein profile can be identified as potentially influencing the allergenicity of IMFs. In particular, the absence of α_{s1}-casein and β-lactoglobulin in human milk renders their presence in IMFs conspicuous where allergenicity is concerned (Table 3).

In Section 3.1 the research which has been carried out to develop β-lactoglobulin-depleted whey protein ingredients was discussed. IMFs containing trace levels or no β-lactoglobulin have yet to be developed, however. More common is the use of proteolytic enzymes to produce extensively-hydrolysed IMFs, which are considered more palatable than 'elemental' amino acid-based IMFs (Clemente, 2000). IMFs containing hydrolysed proteins are marketed as 'hypoallergenic', as they are almost completely devoid of the intact proteins which normally provoke an allergenic response in sufferers of CMPA (El-Agamy, 2007). Hypoallergenic IMFs

have been available for many years but have certain undesirable properties, such as a high degree of bitterness (Clemente, 2000). It is desirable to hydrolyse those proteins primarily linked with CMPA (e.g., α_{s1}-casein and β-lactoglobulin), but hydrolysis of proteins in the production of hypoallergenic IMFs is not specific; thus, techno- and bio-functionality may be negatively affected in the absence of these other proteins.

Recent research has shown that selective hydrolysis of whey proteins is possible when pH, in particular, is controlled. Lieske and Konrad (1996) observed that the proteolytic enzyme, papain, preferentially hydrolysed α-lactalbumin and β-lactoglobulin at acidic and alkaline pH values, respectively, as also reported in later studies (Cheison *et al*., 2011, 2012). The pH-dependant selectivity of proteolytic enzymes for the two major whey proteins is due to conformational changes in protein structure. At pH >7.5, the internal structure of β-lactoglobulin becomes more exposed to the aqueous phase (Tanford *et al*., 1959), increasing its susceptibility to enzymatic hydrolysis, while the globular structure of α-lactalbumin is relatively unchanged; conversely, protonation of Asp residues at pH <5 results in the inability of the *apo* form of α-lactalbumin to bind calcium (Brew, 2003; Chatterton *et al*., 2006), transforming it into a form which is more easily hydrolysed, whereas β-lactoglobulin is highly stable to proteolysis under these conditions (Lieske and Konrad, 1996; Cheison *et al*., 2011, 2012). The findings of these researchers support the development of selectively-hydrolysed IMFs, which may have better functionality and palatability, while still being hypoallergenic. A commercial prototype of a selectively-hydrolysed whey protein ingredient was successfully used by Murphy *et al*. (2015) to prepare model IMFs with hydrolysed β-lactoglobulin and intact α-lactalbumin.

Research related to the conjugation of whey proteins with different sugars and polysaccharides has intensified in recent years (Lucey *et al*., 2009; Corzo-Martinez *et al*., 2010; Böttger *et al*., 2012). It is hypothesised that the presence of sugar groups in these conjugates could restrict binding of IgE to the protein moiety, thereby reducing allergenicity (Lucey *et al*., 2009). Conjugated β-lactoglobulin has been shown to have altered digestibility (Corzo-Martinez *et al*., 2010; Böttger *et al*., 2012) and techno-functional properties (Lillard *et al*., 2009). Reports as to the success of conjugating whey protein in reducing allergenicity have been mixed

(Corzo-Martinez *et al*., 2010; Zhong *et al*., 2014). Further research is required to verify if conjugation is useful for reducing the allergenicity of whey proteins, and if the process can be optimised for large-scale manufacture.

3.5.2. Digestive discomfort

IMFs are high in lactose (Table 1), which is problematic for those who are intolerant to this sugar. Skim milk powder is a key ingredient in IMFs, being the main contributor of casein, innate minerals and additional whey protein; the use of skim milk powder is unsuitable in lactose-free IMFs, and high-protein milk protein concentrates typically replace skim milk powder in these applications. Available literature on lactose-free IMFs is limited. Where the use of milk protein concentrates is concerned, caution is warranted in terms of stability of the IMF during processing. Compared to skim milk, milk protein concentrates (MPCs) may present manufacturers with stability problems during reconstitution of the MPC powder (Crowley *et al*., 2015a) and thermal processing (Crowley *et al*., 2014c). It is unclear how prominent these issues may be in an IMF system, in which MPC is only one component in a complex system. IMFs for the alleviation of symptoms associated with gastroenteritis mainly fall into two categories, i.e., acidified and casein-dominant; the hypoallergenic IMFs discussed in Section 3.6.1 are sometimes recommended in these cases also. As IMF research has primarily focused on whey protein-dominant IMFs at pH ~7.0 in recent years, little is known about formulation and processing considerations for these products. Similar recommendations are sometimes made in instances of colic or sleep-disturbance, but supporting evidence for such recommendations is limited.

It has been known for many years that human milk clots differently during digestion compared to bovine milk. Nakai and Li-Chan (1987) showed that increasing the β-casein content of bovine milk rendered its clotting and proteolytic properties more similar to human milk. More recently, Liu *et al*. (2016) reported that dephosphorylation of the casein fraction modified the digestibility of milk protein concentrates during infant *in vitro* gastrointestinal digestion. Prakash *et al*. (2014) found that regular, lactose-free, soy-based and thickened (anti-reflux) first-age IMFs

all decreased in viscosity during simulated gastrointestinal digestion, but that the anti-reflux IMF maintained a higher viscosity throughout. Homogenisation and pasteurisation were shown to affect the digestion of lipids and proteins in IMFs (Bourlieu *et al*., 2015b) due to alterations in fat globule size and interfacial composition; indeed, marked changes in the lipid fraction of milk caused by pasteurisation, homogenisation and spray drying have been measured during the manufacture of milk powder (Yao *et al*., 2015). Potential influences of lipid source, structure and interfacial composition on digestibility and the infant microbiome were reviewed by (Bourlieu *et al*., 2015a). An excellent overview of studies on the in vitro digestion of IMFs was written by Nguyen *et al*. (2015).

3.5.3. Proteins for improved clinical and developmental outcomes in formula-fed infants

The application of other, low-abundance bovine milk proteins in IMFs has emerged as an area of interest in recent years owing to an increased understanding of the human milk proteome. These proteins are present in higher quantities in human milk compared to bovine milk and IMFs (Table 3) and include immunoglobulins, MFGM, osteopontin and TGF-β (Chatterton *et al*., 2013). These proteins are considered to be highly bioactive, with roles in physiological process such as immunofunction, bone maintenance and cognitive development. Some, such as osteopontin, are produced at a commercial scale and are marketed specifically for IMF applications; however, to these authors' knowledge, none have been included in commercial IMFs. The main factor limiting the use of these ingredients is presumably the high cost associated with their isolation from bovine milk.

3.6. Development of liquid base ingredients for infant milk formula manufacture

Protein ingredients used in IMFs are typically added as powders. These powders, which already have an extensive thermal history (e.g., pasteurisation, evaporation, spray drying), must be reconstituted and mixed together at elevated temperatures prior to another sequence of heat treatments, which are often followed

by drying (Fig. 2). This high thermal load is undesirable due to the potential for destabilisation (Singh, 2004) and loss of nutritional quality due to protein denaturation/aggregation, degradation of vitamins, fatty acids and lysine (Crowley and O'Mahony, 2016). Moreover, the transport of the dried ingredients to the IMF manufacturer and the multiple thermal treatments applied after they have been mixed amounts to a considerable environmental cost. Thus, improvements in the IMF manufacturing process are being sought in the form of integrated membrane systems for the production of humanised, liquid base ingredients from which IMFs can be formulated. Tobin *et al*. (2013) described such a process, wherein fresh milk is subjected to several microfiltration and ultrafiltration steps, in addition to nanofiltration, to create an IMF with a macronutrient and mineral composition which closely matches that which would typically be the target for an IMF manufacturer using skim milk powder and whey protein concentrates/isolates.

4. Conclusions and future perspectives

While they are already complex systems, the formulation and processing of IMFs will become even more sophisticated in the coming years. Stability issues, such as creaming, browning and heat-induced destabilisation, will need to be closely monitored as novel ingredients are incorporated. Growth in the market for second- and third-age IMFs may also inform developments in formulation. Ingredients such as milk protein concentrates and isolates may be particularly applicable for casein-dominant systems, as they will allow fortification with protein along with associated calcium and phosphorus without the introduction of substantial quantities of other components (e.g., sodium, lactose, non-protein nitrogen).

Advances in the separation of proteins from bovine milk using cross-flow membrane technology are likely to drive further developments in the area of humanisation. For economic reasons, enrichment of proteins will probably be more important than isolation of individual fractions. Co-enrichment of valuable fractions (i.e., α-lactalbumin, β-casein) for the creation of protein base ingredients for IMF applications will be a key objective. The change in protein profile which these ingredients will incur should be beneficial for the nutritional status of the infant;

however, performance of these modified IMFs during processing and storage will need to be carefully monitored to ensure that the final product is stable.

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CHAPTER 7

Potential applications of non-bovine mammalian milk in infant nutrition

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Declaration

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1. Introduction

Several animals have been domesticated over the centuries for dairying purposes, but the milk of cows is by far the most abundant type of milk produced in the world today (Faye and Konuspayeva, 2012; Claeys *et al*., 2014). Consumption of milk from non-bovine species is primarily a feature of developing countries, where other lactating animals have been found to serve the local population with adequate supplies of milk within the prevailing terrain, climate and culture (Faye and Konuspayeva, 2012; Medhammar *et al*., 2012).

The most important non-bovine milk in human society is, of course, the milk produced by a mother for her infant; generally capable of completely satisfying the nutritional requirements of the infant, this complex biological fluid undergoes dynamic changes during lactation to keep pace with the infant's growth and development (Lönnerdal, 2003; Morgan, 2006). Although a mother's milk is the most ideal and universally recommended nutrient source for human infants, an alternative is often sought where effective breast-feeding is not possible or practiced. Most commonly, the chosen alternative is an infant milk formula (IMF) based on bovine milk. However, some infants are allergic or intolerant of bovine milk, in addition to the milk of other species, and are therefore fed milk protein hydrolysate-based or plant-based products (Klemola *et al*., 2002; El-Agamy, 2007).

Certain types of non-bovine mammalian milk have also attracted interest for their potential in infant nutrition, due to some characteristics that they share with human milk. This interest can probably be traced back to anecdotal reports of babies tolerating a certain milk source (e.g., caprine) better than the prevailing type (e.g., bovine) available to a given community (Haenlein, 2004); more recently, IMF products made from non-bovine sources such as caprine milk have been manufactured commercially and have been reported as being comparable to bovine milk-based IMFs in terms of infant development (Grant *et al.*, 2005; Han *et al.*, 2011; Zhou *et al.*, 2013). Differences in the technological characteristics of dairy products derived from non-bovine milk types were also recognised, with, for example, caprine milk yielding softer curds than bovine milk upon rennet coagulation (Park *et al*., 2007). The curds formed during digestion of the milk of humans, horses and camels are also softer than those of bovine or buffalo milk,

which may contribute to the degree to which the milk of different species can be tolerated by human infants (Park, 2007; Uniacke-Lowe *et al*., 2010; Barłowska *et al*., 2011; Claeys *et al*., 2014). Presently, comprehensive data is available on the numerous inter-species differences in compositional, physicochemical and biochemical characteristics of milk (Barłowska *et al*., 2011; Claeys *et al*., 2014), which allows some key characteristics of relevance to infant nutrition to be identified. In general, a comparison of the milk of different species from the perspective of infant nutrition is far more complex than considering a single criterion, such as curd-formation, and extends to such factors as total protein content, whey protein:casein ratio, casein microheterogeneity, micellar characteristics, fatty acid profile, and oligosaccharide content, among many others.

The consensus among health-care professionals and experts in nutrition is that infants should exclusively consume human breast milk after birth for a period of ~6 months (World Health Organisation, 2001). When circumstances dictate that an infant cannot consume human milk, IMF products are considered a suitable alternative. On the other hand, feeding infants with bovine milk has been strongly discouraged for many years, as it is too dissimilar from human milk in its unmodified form. However, bovine milk remains the prevalent base from which IMFs are produced, owing to its large production volumes, established distribution networks and the proven functionality of its components. However, detailed analysis of the milk of other mammalian species, such as those of the goat, horse and camel, indicate a high degree of compositional similarity to human milk compared with bovine milk (Barłowska *et al*., 2011; Medhammar *et al*., 2012; Claeys *et al*., 2014), which may make them promising candidates as human milk replacers or bases for IMF manufacture.

The current standard practice for IMF manufacture is to use bovine milk-derived ingredients as bases for the manufacture of a *humanised* product. The term humanised refers to the fact that the blend of ingredients is intended to yield an IMF which more closely resembles human milk than bovine milk alone. Advances in the area of humanisation typically occur when novel ingredients are developed which allow the further humanisation of IMFs, one early example being the development of demineralised whey protein ingredients (Smithers, 2008). Demineralised whey may be blended with skim milk to allow alteration of the casein:whey protein ratio from

80:20 (bovine milk) to 40:60 (human milk) (de Wit, 1998). More recently, processes for the production of individual casein (e.g., β-casein) fractions, in addition to whey protein (e.g., α-lactalbumin, lactoferrin, osteopontin) and milk fat globule membrane (MFGM) fractions (Chatterton *et al*., 2004; O'Mahony and Fox, 2013; Smithers, 2015), have been developed, offering new possibilities for humanising the protein profile of IMFs. In addition, while matching the chemical composition and profile of human milk is an important element of humanisation, so too is the need to match the physiological and health-related outcomes associated with its consumption by infants; an example of this is the use of galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin, which, although not present in human milk, are being added to mimic the functionality of prebiotics which are native to human milk (Vandenplas, 2002; Bode, 2009; Barille and Rastall, 2013).

Some non-bovine mammalian milk types can be considered as *innately humanised* when compared to current-generation IMFs, in that they have certain features common to human milk (i.e., equivalent casein:whey protein ratio, absence of β-lactoglobulin, or high β-casein:α_s-casein ratio), which are challenging to achieve, particularly in combination, during the formulation of IMFs. The purpose of this review is to highlight these common features, so that the potential application of certain non-bovine milk types in infant nutrition can be evaluated. Possibilities for the development of novel IMF ingredients from non-bovine milk sources will be considered, which may further aid the development of humanised (e.g., β-lactoglobulin-free) formulae in the future.

At the outset of this chapter, an overview of infant formula is given. Every advance in humanisation-led formulation results in IMFs moving closer to human milk and further from bovine milk; however, this transition continues and may never be complete due to the complexity of human milk, which is biologically customised for each individual infant. It is the purpose of this chapter to identify mammalian milk types which are closer in composition to human milk than bovine milk. This may afford greater options for the manufacture of humanised IMFs, a concept which is illustrated in Figure 1.

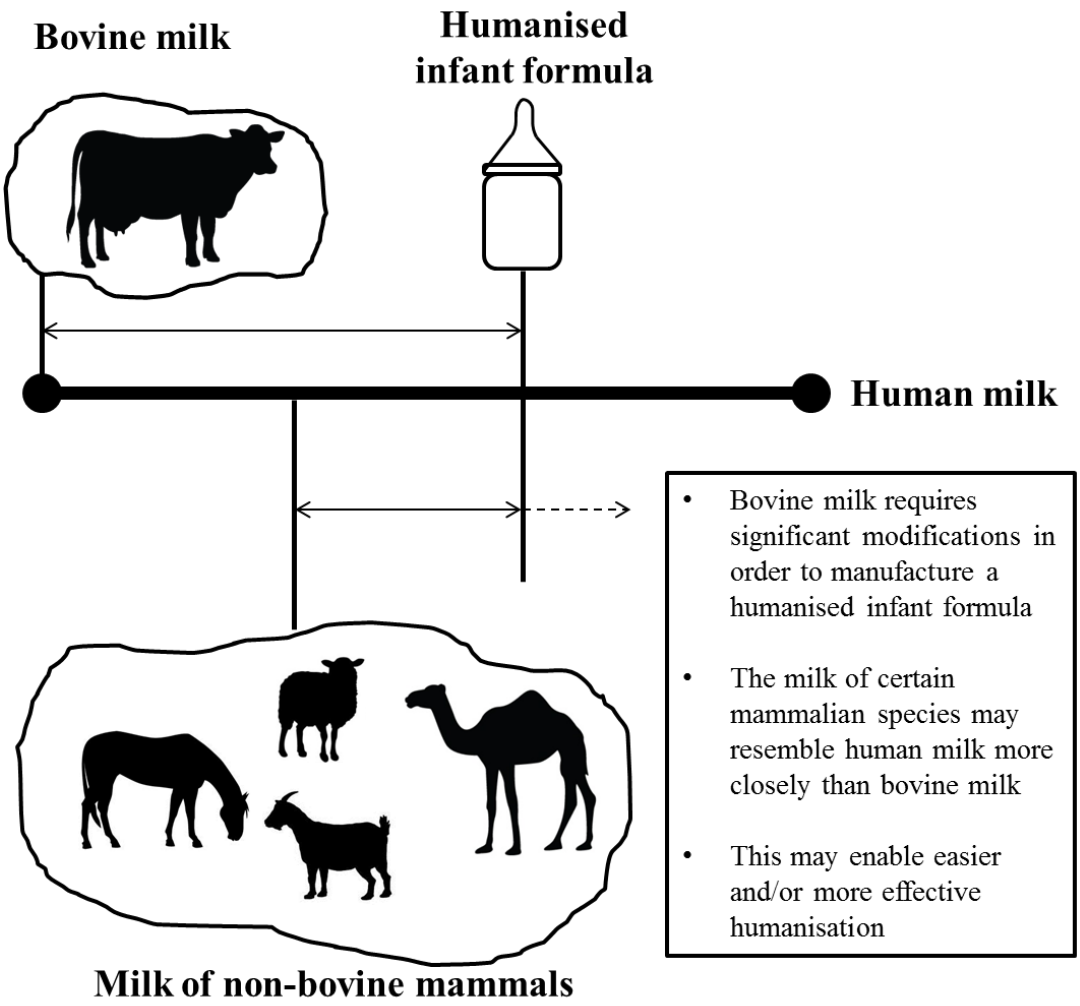


Fig. 1. Conceptual illustration of the potential application of milk of different non-bovine mammalian species in humanised infant formula products.

2. Overview of infant formula

Infant formula is a broad term for a large range of commercial products used as nutrient delivery media in infant nutrition. Some of the general characteristics of a 1st age (see Section 2.1.1) IMF product are that it is:

- Intended for consumption by human infants as a primary nutrient source;
- Formulated to mimic human milk as closely as possible, subject to scientific knowledge, technological capabilities, and cost considerations;
- Considered a safe alternative to human milk, where the latter is not available in sufficient quantity or quality;
- Subject to ongoing humanisation-led reformulation based on

scientific advances in the knowledge of human milk composition, the recommendations of expert panels, and developments in technology platforms.

Beyond these core characteristics, there are many differences in product types, ingredient sources and manufacturing processes, which will be discussed briefly in this section.

2.1 Product types

2.1.1. Age categories

Formulae can be categorised based on the age range of the infant for whom they are formulated. For example, those intended for consumption by infants of 0-6 months are termed '1st age', while those for 6-12 months are termed '2nd age' or 'follow-on' formulae, and those for 1+ years are termed '3rd, 4th. . . ', and so forth. The 1st age IMFs are intended to replace human milk as the sole source of nutrition for the infant, and are formulated to mimic human milk as closely as possible. Follow-on/toddler formulae, however, are usually casein-dominant, and are typically higher in levels of total protein and certain micronutrients such as iron; in addition, unlike 1st-age IMFs, which are intended to be used as the sole nutrient source in the absence of human milk, follow-on IMFs are supposed to be just one component of a balanced infant diet (Morgan, 2006). In subsequent sections of this chapter, the focus will be placed on 1st age IMFs, as these products are the subject of greatest scientific inquiry and exhibit a high degree of nutritional complexity.

2.1.2. Specialised formulae

Areas of new product development in infant formulae have continued to expand and diversify in recent years, with innovation in follow-on and toddler formulae being particularly active. In addition, IMFs which can be consumed by those with specific intolerances (e.g., lactose) or digestion-related difficulties (e.g., regurgitation) have been developed. In the formulation of lactose-free products, skim milk is replaced by milk protein isolate and products of corn starch hydrolysis, such as maltodextrin, are used as an alternative carbohydrate source to lactose. Easy-to-digest and/or hypoallergenic formulae manufactured from hydrolysed proteins are readily available (El-Agamy, 2007; Murphy *et al.*, 2015). As well as hydrolysed

IMFs, those based on plant (e.g., soy, rice) proteins can be used in cow milk protein allergy (CMPA) cases, but it is possible for infants to have allergenic reactions to both hydrolysates and plant proteins (El-Agamy, 2007; Uniacke-Lowe *et al*., 2010; Klemola *et al*., 2002;); furthermore, soy proteins are considered nutritionally inferior to dairy proteins, and, for example, contain phytate which inhibits calcium absorption (Bass and Chen, 2006), while hydrolysates are known to have poor sensory properties including bitterness. Replacement of human milk/bovine milk-based IMFs either directly with non-bovine milk (e.g., caprine milk) or with a non-bovine milk-based IMF (e.g., caprine milk formula) is sometimes practiced but, again, infants susceptible to CMPA may also be allergic to proteins in these milk systems (El-Agamy, 2007).

As determined by Armaforte *et al*. (2010), pre-term human milk (milk expressed by lactating female for an infant born *before* 37 weeks of gestation) has a number of biochemical differences to term human milk (milk expressed by a lactating female for an infant born *after* 37 weeks of gestation), with formulations designed for pre-term and low-birth-weight infants being developed (Georgi *et al*., 2006) and available commercially. Still other IMFs are claimed to be easier to digest, promote sleep, reduce regurgitation and decrease symptoms of colic, though evidence supporting such claims is currently limited. The design of IMFs in which the protein profile has been humanised to a greater-than-normal extent (e.g., with modified levels of α-lactalbumin, lactoferrin or β-casein) is an area of high research activity (Pearce, 1995; Kuhlman *et al*., 2003; Chatterton *et al*., 2004; McCarthy *et al*., 2013, 2014; Crowley *et al*., 2016). Currently, most efforts aimed at humanising IMFs are focused on modifications to protein profile, and, as such, this will be a central theme of the present chapter.

2.2. Formulation considerations

The aim in formulating IMF products is to blend bovine milk-derived ingredients and other ingredients (e.g., vegetable oils, vitamins) to mimic the composition of human milk, which differs compositionally from bovine milk in a multitude of ways (Table 1). The final IMF typically has a protein content of ~1.2-1.8%, which is much lower than that of bovine milk; in addition, the fat and ash contents are also significantly lower, while the lactose content is markedly higher (Table 1). Furthermore, the whey protein:casein ratio is different and, to invert the

ratio from casein-dominant (bovine) to whey-protein dominant (human), appropriate quantities of demineralised whey or whey protein isolate are added to skim milk (de Wit, 1998). Human milk also contains higher proportions of certain proteins, such as tryptophan-rich α-lactalbumin, and it is for this reason that IMFs are typically manufactured to contain higher protein contents than human milk, so that requirements for essential amino acids are satisfied (de Wit 1998; European Commission Directive 2006/141/EC).

2.3. Manufacture

IMFs are available commercially in various formats (Fig. 2). In the manufacture of all product formats, IMFs are subjected to a processes designed to ensure a final product which is safe to consume, contains appropriate levels of essential nutrients, and is stable over its stated shelf-life. The various formats are exposed to processing conditions which, to different degrees, may influence their nutritional profile and quality. The ready-to-feed products, for example, are exposed to thermal treatments which may be sufficient to cause protein denaturation/aggregation if appropriate controls are not implemented (McSweeney *et al*., 2004; Crowley *et al*., 2016), and heat-induced changes could affect, for example, protein digestibility (Ye *et al*., 2016). IMF systems are particularly susceptible to heat-induced destabilisation due to their high proportion of the more heat-labile whey proteins (Rattray and Jelen, 1997; McSweeney *et al*., 2004). Furthermore, chemical reactions during processing and storage of IMFs can lead to generation of Maillard reaction products (Ferrer *et al*., 2000; Chávez-Servín *et al*., 2006, 2015; Nasirpour *et al*., 2006), in addition to oxidation of fatty acids (de la Presa-Owens *et al*., 1995; Satué-Gracia *et al*., 2000; Romeu-Nadal *et al*., 2007) and degradation of vitamins, particularly heat-labile variants such as vitamin C (Chávez-Servín *et al*., 2008a, b).

Steps in the manufacturing process which are most likely to result in destabilisation or nutrient loss in IMFs are those involving the application of high temperatures, which can be summarised as follows:

- Reconstitution of powders at temperatures ~50-60°C with water, oil, minerals and liquid concentrate ingredients;
- Ultra-high temperature (>135°C × 1-2 s), in-container sterilisation (~110-

120°C × 10-20 min) or high-temperature short-time heating (~72-75°C × 15-20 s) of a liquid ingredient blend;

- Evaporation and spray drying for powder manufacture.

Heat-labile micronutrients, such as vitamins, can sometimes be added post-heating. In addition, strategies based on dry-blending of powder ingredients can be employed to offset degradative changes otherwise occurring during heating of liquid mixes. Micronutrient loss due to thermally-induced degradation can also be combatted by use of appropriate overages, which refers to the use of higher levels of a given nutrient, intended to compensate for losses incurred during processing. Appropriate overages are calculated based on a program which involves the collection of both predictive and real-time data for the degradation of different nutrients during processing and storage (Crowley and O'Mahony, 2016); however, protein denaturation/aggregation is often irreversible when it happens and there are increasing efforts to minimise its occurrence in the first place.

It should be stated at the outset that any prospective alternatives to bovine milk-based IMFs, whether involving the use of milk of other mammalian species as direct replacements or as bases for IMF products, are likely to require a similarly intensive thermal treatment to ensure safety for human consumption. In general, the milk of different species responds to heat treatments in a broadly similar manner to bovine milk as far as micronutrient degradation is concerned (Claeys *et al*., 2014). However, different degrees of process stability may be encountered in different non-bovine milk types. For example, caprine milk exhibits poor heat stability due to its high calcium-ion activity (Park *et al*., 2007), but this instability can be decreased through the addition of calcium-binding agents (Chen *et al*., 2012).

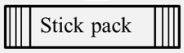
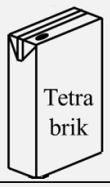
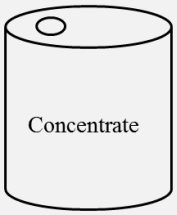
Format	Physical State	Features of the format
	Powder	• Free-flowing bulk powder in multi-use can • Consumer scoops designated quantity for correct nutrient density • Dissolved in sterilised water/container prior to consumption
	Powder	• Compressed powder in single-serve tablet • Correct nutrient density contained within tablet • Dissolved in sterilised water/container prior to consumption
	Powder	• Free-flowing powder in single-serve sachet • Correct nutrient density contained within sachet • Dissolved in sterilised water/container prior to consumption
	Liquid	• Ready-to-drink/pre-sterilised • Re-sealable • Multi-use (within 48 h after opening)
	Liquid	• Ready-to-drink/pre-sterilised • Single-use (within 48 h after opening)
	Liquid	• Ready-to-drink/pre-sterilised • Single-use (within 48 h after opening)
	Powder	• Free-flowing bulk powder in plastic pouch • Used to refill cleanable, reusable tubs
	Liquid	• Concentrated liquid infant formula • Consumer mixes with designated quantity of sterilised water for correct nutrient density • Ease-of-preparation is intermediate between can format (intensive) and ready-to-feed formats (easy)

Fig. 2. Different commercial product formats for infant formula products

Camel milk is highly unstable to heating, even though its whey protein fraction is more heat stable than that of bovine or buffalo milk (Farah, 1986; El-Agamy, 2000; Alhaj *et al*., 2011), with whey cheese products reportedly being difficult to produce from camel milk (Ramet, 2001). The low κ-casein content and absence of a β-lactoglobulin-like protein in camel milk (Hinz *et al*., 2012; Claeys *et al*., 2014) probably reduces its heat stability by limiting the formation of stabilising complexes between the two proteins at the surface of micelles (Singh, 2004); on the

other hand, the lack of β-lactoglobulin likely increases the stability of the whey protein fraction as the free thiol group of this protein would otherwise facilitate more extensive aggregation of denatured whey proteins (Wijayanti *et al*., 2014). The heat stability of camel milk has been increased by the addition of κ-casein or EDTA (Alhaj *et al*., 2011).

Although equine milk might be expected to be highly susceptible to heat-induced destabilisation, as high whey protein:casein ratios are normally associated with greater instability in milk systems (Rattray and Jelen, 1997; McSweeney *et al*., 2004), the low total protein level and low proportion of β-lactoglobulin, combined with the absence of a free thiol group in equine β-lactoglobulin, are said to contribute to a generally higher heat stability compared to bovine milk (Uniacke-Lowe *et al*., 2010). Indeed, the heat stability of bovine milk-based IMFs can be improved by increasing the α-lactalbumin:β-lactoglobulin ratio (Crowley *et al*., 2016). Fat, which is emulsified by protein in milk systems, can also participate in reactions culminating in heat-induced destabilisation. Whole human milk was less stable to heating at 130°C than bovine milk, despite the lower protein content of the former, but was more heat stable after the fat was removed by skimming (Burns, 2013).

3. Comparison of bovine, non-bovine and human milk

All mammalian milk types show wide compositional variation due to a combination of interacting factors such as maternal diet and health status, age of the infant mammal and the gestational period of delivery (e.g., pre-term, full-term). Taken individually, the influence of these factors on the milk of each species would require an intensive survey of published research in their own right. As this is beyond the scope of the current chapter, these factors will be excluded from the ensuing discussion. Instead, the general composition of full-term (or mature) milk will be compared for inter-species differences. In addition, human milk is known to be rich in a wide range of bioactives, such as hormones, immune-regulatory proteins (e.g., cytokines), growth factors and enzymes (Morgan, 2006; Garofalo, 2010; Petherick, 2010), which are thought to make important contributions to infant development (Lönnerdal, 2003). This will be a crucial area in future advancements in infant nutrition products but, again, is outside the scope of this review. Where comparisons are made to IMFs, the products considered will be 1st-age, which are those designed for feeding of infants between 0 and 6 months (See Section 2).

Table 1. General composition of the milk of different mammalian species and infant formula[a].

	Infant formula*	Human	Bovine	Caprine	Ovine	Buffalo	Equine	Camel	Yak	Reindeer
Solids (%)	12.5	10.7-12.9	11.8-13.0	11.9-16.3	18.1-20.0	15.7-17.2	9.3-11.6	11.9-15.0	13.5-18.4	20.1-27.1
Protein (%)	1.3-1.8	0.9-1.9	3.0-3.9	3.0-5.2	4.5-7.0	2.7-4.7	1.4-3.2	2.4-4.2	4.2-5.9	7.5-13.0
Lactose (%)	7.0-7.5	6.3-7.0	4.4-5.6	3.2-5.0	4.1-5.9	3.2-4.9	6.3-7.0	3.5-5.1	3.3-6.2	1.2-2.7
Fat (%)	3.0-3.5	2.1-4.0	3.3-5.4	3.0-7.2	5.0-9.0	5.3-9.0	0.3-4.2	2.0-6.0	5.3-9.5	10.2-21.5
Ash (%)	0.2-0.4	0.2-0.3	0.7-0.8	0.7-0.9	0.9-1.0	0.8-0.9	0.3-0.5	0.69-0.9	0.4-1.0	1.2-2.7
NPN[#] (g/L)	n.d.	0.45	0.27-0.38	0.40-0.61	n.d.	n.d.	0.38	0.68	0.4-0.5	2.2
OGS (g/L)[+]	n.d.	8	0.03-0.06	0.25-0.30	0.02-0.04	n.d.	n.d.	n.d.	n.d.	n.d.
Energy (kJ/L)	2500-2950	2843	2709-2843	2802-2894	4038-4439	4244-4779	1936-2050	2410-3286	3811-4295	5541-8436

[a] modified from Claeys *et al*. (2014)

* based on typical levels reported for commercial products

+ values taken from Oliveira *et al*. (2015

NPN – non-protein nitrogen

OGS - Oligosaccharides

3.1. Gross composition: General comments

The gross composition of infant formula, bovine milk and a range of other mammalian milk types are shown in Table 1. Bovine milk deviates from human milk in a number of ways, with the former having much higher protein and ash contents, while lactose levels are conversely lower; these differences also exist between human milk and that of many other ruminant species. In terms of gross composition, the milk of the only monogastric species shown, the horse, bears several notable similarities to human milk, being somewhat similar in its contents of protein, lactose and ash in particular; these similarities are also characteristic of the milk of other monogastric species, such as the donkey (Claeys *et al*., 2014). The milk of some other ruminants, such as sheep and reindeer, differ markedly from human milk in several respects, with fat and protein contents being particularly high (Table 1).

3.2. Protein profile

3.2.1 General characteristics

When considering protein profile in relation to infant formulae, it is useful to begin with the casein:whey protein ratio. One of the major innovations in the humanisation of infant formula was their transformation from casein- to whey protein-dominant products (de Wit, 1998). Although the individual proteins within these two protein classes exhibit many inter-species differences, the general properties of both are the same (Table 2). Owing to these physicochemical differences, casein and whey proteins are digested differently by the body, which affects the rate at which they release amino acids and other nutrients. Compared to casein, whey proteins have higher levels of essential and branched-chain amino acids, which are released quickly during digestion, while casein forms a clot from which calcium phosphate and amino acids are released by slow acid-solubilisation and proteolysis, respectively (Holt *et al*., 2013; Pellegrino *et al*., 2013; Ye *et al*., 2016).

Table 2. General properties of the two major protein fractions in milk, based on information presented in O'Mahony and Fox (2013).

Property	**Casein***	**Whey proteins***	**Notable inter-species differences**
Size	Large (<600 nm)	Small (<10 nm)	Size of casein micelles (Claeys *et al.*, 2014)
Physical state	Micelles	Monomers or small quaternary structures	Predominance of monomeric or dimeric β-lactoglobulin (Sawyer, 2013)
Phosphorylation	High	None	Degree of phosphorylation of caseins (Martin *et al.*, 2013)
Glycosylation	κ-casein is glycosylated	Lactoferrin is glycosylated	Human κ-casein is more glycosylated (Martin *et al.*, 2013)
Mineralisation	Caseins in micellar form bind large quantities of calcium and phosphate, and much lower levels of magnesium, citrate, sodium and potassium	α-lactalbumin binds one calcium ion per mol. Lactoferrin binds iron and osteopontin binds calcium.	A different balance between micellar and soluble minerals can exist in the milk of various species (Park *et al.*, 2007)
Solubility at pH 4.6	Insoluble	Soluble	The casein of some species requires a lower pH to precipitate (O'Mahony and Fox, 2013)
Calcium sensitivity	Can be precipitated by calcium	Calcium promotes aggregation of whey proteins	Calcium sensitivity varies depending on the different degrees of phosphorylation
Heat stability	Highly stable due to rheomorphic structure of caseins	Prone to denaturation at temperatures >65°C	Some milk does not contain β-lactoglobulin, or contains β-lactoglobulin without a free thiol group (Uniacke-Lowe *et al.*, 2010), which may alter denaturation/aggregation behaviour (Claeys *et al.*, 2014)
Physical stability	Sedimentable, except for a small proportion of monomeric casein	Non-sedimentable, unless associated with casein micelles or present as large aggregates	Certain milk (e.g., caprine) has a high proportion of soluble casein which is non-sedimentable (Park *et al.*, 2007)
Cold-dissociation	Progressive, but limited, dissociation of caseins (primarily β-casein) as temperature is reduced to <20°C.	None	Milk with higher levels of β-casein undergoes more extensive cold-induced dissociation (Lamothe *et al.*, 2007)

High levels of casein are believed to contribute to infants' inability to digest bovine milk comfortably; therefore, 1st-age IMFs are formulated to be whey protein-dominant, as in human milk (Table 3). On the other hand, formulae intended for infants over the age of 6 months are formulated to be casein-dominant. Of the mammalian milk types shown in Table 3, the casein:whey protein ratio of equine milk (approximately 50:50) is the closest to that of human milk. The milk of the ruminant species are all casein-dominant, being similar to bovine milk in this regard. Although it might therefore be predicted that the casein-dominant milk of ruminant species is uniformly unsuitable to infants in terms of digestibility, this may not be true, as certain milk, such as caprine milk, yield casein curds which are markedly less firm than those of bovine milk (Park *et al*., 2007). In addition, it has been reported that camel milk does not form a cohesive curd during rennet- or acid-gelation, but rather exhibits limited floc formation (Ramet, 2001; Barłowska *et al*., 2011).

The nature of the casein curds formed from different milk is a function of the complex interplay between pH, total casein content, casein micelle size distributions, relative proportions of individual caseins, micellar calcium phosphate concentration, and processing (Park *et al*., 2007). This is an area requiring more attention, particularly where digestibility is concerned. Ye *et al*. (2016) demonstrated that heat treatment (90°C × 20 min) could significantly alter the structure of the protein clot formed from bovine milk during simulated gastric digestion; heated milk formed a less rigid clot with larger pores which was subject to more rapid proteolysis. Digestion of proteins is also influenced by their localisation within different phases in IMF systems, with proteins adsorbed at lipid-water interfaces undergoing more rapid proteolysis than bulk-phase proteins (Bourlieu *et al*., 2015). The digestibility of the casein of different species is likely a function of the unique casein profile, micellar properties and interfacial composition of each, with these factors perhaps also being influenced to different degrees by processing depending on the species of milk.

Table 3. Protein profile (g L^{-1}) of the milk of different species [a].

	Infant formula*	Human	Bovine	Caprine	Ovine	Buffalo	Equine	Camel	Yak	Reindeer
Total casein	4.8	2.4-4.2	24.6-28	23.3-46.3	41.8-46	32-40	9.4-13.6	22.1-26.0	34.3-45.8	70-80
α_{s1}-casein	1.74	0.77	8.0-10.7	0.0-13.0	15.4-22.1	8.9	2.4	n.d.	9.3-13.1	n.d.
α_{s2}-casein	0.48	-	2.8-3.4	2.3-11.6	n.d.	5.1	0.2	n.d.	3.6-6.5	n.d.
β-casein	1.62	3.87	8.6-9.3	0.0-29.6	15.6-17.6	12.6-20.9	10.66	n.d.	15.0-20.6	n.d.
κ-casein	0.6	0.14	2.3-3.3	2.8-13.4	3.2-4.3	4.1-5.4	0.24	-	4.9-8.5	n.d.
γ-casein	0.36	-	0.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total whey protein	7.2	6.2-8.3	5.5-7.0	3.7-7.0	10.2-11	6	7.4-9.1	5.9-8.1	n.d.	13.4
β-lactoglobulin	3.5	-	3.2-3.3	1.5-5.0	6.5-8.5	3.9	2.55	-	3.4-10.1	n.d.
α-lactalbumin	1.4-2.3	1.9-3.4	1.2-1.3	0.7-2.3	1.0-1.9	1.4	2.37	0.8-3.5	0.2-1.7	n.d.
Serum albumin	n.d.	0.4-0.5	0.3-0.4	n.d.	0.4-0.6	0.29	0.37	7-11.9	0.2-3.1	n.d.
Proteose peptone	n.d.		0.8-1.2	n.d.	n.d.	3.31	n.d.	n.d.	n.d.	n.d.
Lactoferrin	0.05	1.5-2.0	0.02-0.5	0.02-0.2	0.8	0.03-3.4	0.1-2.0	0.02-7.28	n.d.	n.d.
Lysozyme	n.d.	0.1-0.9	(70-600) x 10^{-6}	250 x 10^{-6}	100 x 10^{-6}	(120-152) x 10^{-6}	0.5-1.33	(60-1350) x 10^{-6}	n.d.	n.d.
Osteopontin	0.01	0.138*	0.018*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Immunoglobulins	n.d.	1.0-1.3	0.5-1.0	n.d.	0.7	10.66	1.63	n.d.	n.d.	n.d.
IgG	n.d.	0.03	0.15-0.8	0.1-0.4	n.d.	0.37-1.34	0.38	n.d.	n.d.	n.d.
IgA	n.d.	0.96	0.05-0.14	0.03-0.08	n.d.	0.01-0.04	0.47	n.d.	n.d.	n.d.
IgM	n.d.	0.02	0.04-0.1	0.01-0.04	n.d.	0.04-1.91	0.03	n.d.	n.d.	n.d.
CN:WP ratio	40:60	29:71-33:67	82:18	78:22	76:24	82:18	52:48	73:27-76-24	82:18	80:20-83:17

n.d. = no data

[a] modified from Claeys *et al*. (2014)

* values from Chatterton *et al*. (2013)

3.2.2. Heterogeneity of proteins

Both the casein and whey protein fractions in mammalian milk are heterogeneous. Differences in the levels of individual proteins within each fraction in bovine milk and human milk are an important aspect of current humanisation efforts. El-Agamy (2007) described CMPA as an immunogenic response to bovine milk proteins which has a prevalence approaching 10% of infants within a given country (incidence significantly decreases with increasing age). When the protein profiles of human milk and bovine milk are compared (Table 3), key differences in the proportions of certain proteins can be readily highlighted as potential contributors to CMPA. Most notably, β-lactoglobulin is the primary whey protein in the milk of many mammalian species, but is absent from human milk.

Potential methods to humanise bovine milk and improve its suitability for sufferers of CMPA include increasing the α-lactalbumin:β-lactoglobulin ratio in IMFs using α-lactalbumin-rich ingredients (Pearce, 1995; Stack *et al*., 1999; Kuhlman *et al*., 2003; Lucena *et al*., 2006; Davis *et al*., 2007), removal of intact β-lactoglobulin in whey protein ingredients through its selective hydrolysis (Murphy *et al*., 2015), conjugation of β-lactoglobulin with carbohydrates to reduce potentially negative immune reactions (Taheri-Kafrani *et al*., 2009; Böttger *et al*., 2013), and genetic modification of lactating cows to produce β-lactoglobulin-free milk (Jabed *et al*., 2012). In light of these intensive and diverse efforts, it is interesting to note that camel milk, like human milk, reportedly does not contain β-lactoglobulin (Table 3); other minor species milk types which also do not reportedly contain this protein include those of rodents, lagomorphs and chimpanzees (Sawyer, 2013). While it does contain β-lactoglobulin, equine milk has a much higher α-lactalbumin:β-lactoglobulin ratio than bovine milk (Table 3); it is important to note here that most IMF manufacturers utilise ingredients consisting of enriched, rather than purified fractions, and a complete absence of β-lactoglobulin is therefore not a prerequisite for the commercial uptake of an ingredient. Indeed, current practice involves the use of α-lactalbumin-enriched fractions to alter the α-lactalbumin:β-lactoglobulin ratio in IMFs (Kuhlman *et al*., 2003). These practices are targeted at improving the amino acid profile, protein content and digestibility of IMFs rather than alleviate symptoms of CMPA. Camel milk reportedly contains no β-lactoglobulin (Hinz *et al*., 2012; Claeys *et al*., 2014) and may be an effective alternative to formulae based on plant

proteins or hydrolysates for treatment of CMPA. It has been reported that there is no cross-reactivity of camel milk proteins with allergenic bovine milk proteins while other non-bovine species do exhibit cross-reactivity (El-Agamy, 2007, 2009). In one study of 28 CMPA-afflicted infants, 80% had negative skin-prick tests to camel milk and those who tested negative all tolerated camel milk during subsequent feeding (Ehlayel, 2011).

The high protein content of current-generation IMFs, compared to human milk (Table 1), has been linked with adverse physiological consequences such as increased bodyweight in IMF-fed infants (Koletzko *et al*., 2009). Including ingredients with higher α-lactalbumin:β-lactoglobulin ratios in IMFs allows a reduction in protein content from current levels to levels closer to those found in human milk, as amino acid requirements (particularly those of tryptophan and cysteine) will be satisfied to a greater extent (Kuhlman *et al*., 2003). However, the manufacture of α-lactalbumin, and to a greater extent lactoferrin, at purities and volumes sufficient to supply the IMF industry at affordable prices is extremely challenging. Manufacturing processes for lactoferrin involving cation-exchange chromatography are well established, and premium IMF products which are enriched in lactoferrin are available commercially in Asia (Tomita *et al*., 2009). However, 10,000 L of whey is required to generate a mere 1 kg of lactoferrin (Etzel, 2004), with these low yields resulting in the protein being an overly expensive for most IMF manufacturers (reported price of US$300 per kg). Thus, IMFs typically contain lactoferrin levels which are below the 100-300 mg 100 g^{-1} found in human milk, with Zhang *et al*. (2014) measuring concentrations of 50-75 mg 100 g^{-1} in a range of commercial IMF samples. Although there is some variation in the data, high levels of lactoferrin have been found in buffalo, camel and equine milk (Table 3). Non-bovine milk types that are already enriched in key proteins (i.e, α-lactalbumin, lactoferrin) may be a viable alternative to, or source of ingredients for, bovine milk-based IMFs which may otherwise require humanisation with expensive ingredients from bovine milk.

The casein fraction of IMFs has been subject to much less intensive humanisation efforts. Compared to bovine milk, the casein fraction of human milk is characterised by a relatively high β-casein:α_s-casein ratio (α_{s2}-casein is also notably absent from human milk), which is also the case for caprine, buffalo, equine and yak

milk (Table 2); for this reason, the manufacture of casein isolates from the milk of some of these species is an interesting prospect. Although commercial β-casein ingredients are available to a limited extent, fortification of commercial IMFs with this protein does not yet appear to be practiced to the authors' knowledge. In milk low in α_s-caseins, such as that of the goat and human, the curds which are formed are much less rigid than those in high α_s-casein systems such as bovine and buffalo milk (Claeys *et al*., 2014), which may have important implications for the digestibility of caseins (El-Agamy, 2007; Park *et al*., 2007; Ye *et al*., 2016). Compared to its whey protein profile, which boasts some interesting similarities with human milk, less detailed information on the casein profile of camel milk is available.

The major whey protein in bovine milk, β-lactoglobulin, is commonly considered a key allergen in cases of CMPA because it is not present in human milk; however, the low degree of similarity between the caseins of different species with their human homologues may also contribute to the allergenicity of these proteins to infants (Jenkins *et al*., 2007). The α_s-casein proteins of the non-human species have a very low degree of homology with the human forms, while the inter-species homology of β-casein is higher (Table 4). This may partially explain why milk types with low α_s-casein:β-casein ratios (e.g., caprine, equine) are less likely to provoke an allergic response in infants (Uniacke-Lowe *et al*., 2010). In comparison to the caseins, there is generally a high degree of inter-species homology between the principal whey proteins (Brew, 2013; Sawyer, 2013). The less similar a protein is to its human homologue the more likely it is to provoke an allergenic response when consumed by an infant; for this reason, reducing the α_s-casein:β-casein ratio will be an important objective for the humanisation of IMFs in the coming years. To achieve this, casein fractions generated from milk with a low α_s-casein:β-casein ratio, such as caprine or equine milk, could be used in IMFs; alternatively, β-casein-enriched ingredients could be produced from milk and used for fortification of IMFs.

Table 4. Casein microheterogeneity of the milk of different species[a].

	Human	Bovine	Caprine	Ovine	Equine	Camel
α_{s1}-casein						
Genetic variants	-	7	13	5	-	-
Phosphorylation sites*	6/0-8	9/9	11/11	10/10	10/?	6/?
Glycosylation	No	No	no	no	no	no
Identity to closest human homologue [b]	100	29	29	28	44	36
α_{s2}-casein						
Genetic variants	-	4	6	3	-	-
Phosphorylation sites*	-	17/?	16/?	17/13	-	9/?
Glycosylation	No	No	no	no	no	no
Identity to closest human homologue [b]	100	16	17	17	-	11
β-casein						
Genetic variants	-	9	3	2	-	-
Phosphorylation sites*	6/6	6/5	6/6	6/6	9/7	4/?
Glycosylation	No	No	no	no	no	no
Identity to closest human homologue [b]	100	53	54	54	58	58
κ-casein						
Genetic variants	-	-	-	-	-	-
Phosphorylation sites*	5/?	5/3	6/3	5/3	2S/6T/?	?/?
Glycosylation	yes	Yes	yes	yes	yes	yes

[a] modified from Martin *et al*. (2013)

[b] taken from Jenkins *et al*. (2007)

***** putative/effective

Several methods for the separation and enrichment of β-casein from bovine milk, largely based on microfiltration at cold temperatures, have been described in the literature (Woychik *et al*., 1992; O'Mahony *et al*., 2014; Crowley *et al*., 2015a); however, industrial uptake of such processes has been low, which some have attributed to limitations in yield and temperature control (Lamothe *et al*., 2007). The milk of non-bovine species may have beneficial properties which aid the development of such protein ingredients. Lamothe *et al*. (2007) described the markedly enhanced separation of β-casein from caprine milk compared to bovine milk when rennet curds of both were held at cold temperatures (0, 4, 8, 16°C), according to the method of Huppertz *et al*. (2006). Caprine milk contains a higher total level of β-casein than bovine milk (Table 3), in addition to a greater ratio of soluble to micellar casein (Park *et al*., 2007), which make it a promising raw material for the manufacture of β-casein for IMF applications; furthermore, the relatively weak curds it forms during renneting may facilitate optimal diffusion of β-casein from caprine casein curds incubated at low temperatures compared to bovine casein curds (Lamothe *et al*., 2007). The disadvantage of processes for the separation of β-casein from casein curds is that the value of the curd material is low; it is therefore preferable to not have to form a casein curd in the first place. Methods for producing β-casein from bovine milk based on membrane separation yield a micellar casein concentrate as a co-product (O'Mahony *et al*., 2014; Crowley *et al*., 2015a); hence, membrane separation may be a better approach to preserve the value of all streams during the separation of β-casein from caprine milk.

3.2.3. Microheterogeneity of caseins

Two important properties of caseins are phosphorylation and glycosylation. Individual bovine caseins are phosphorylated in the order $\alpha_{s2} > \alpha_{s1} > \beta > \kappa$ (O'Mahony and Fox, 2013); the higher the degree of phosphorylation, the more calcium-sensitive the protein. Casein phosphorylation exhibits a high degree of inter-species variability (Table 4); for example, β-casein in bovine milk occurs primarily in a single, quintuply-phosphorylated phosphoform, while human β-casein is phosphorylated at six different levels from 0-5 phosphates per mol and mainly occurs in the quadruply-phosphorylated form (Sood *et al*., 1996). A range of phosphoforms have also been found in ovine milk, while camel β-casein primarily occurs in a triply-phosphorylated state (Martin *et al*., 2013). The multi-

phosphorylated β-casein in human milk is known to have unique association characteristics which differ to that of the β-casein in bovine milk (Sood *et al*., 1996; Sood and Slattery 1997, 2001), and which may be an important factor influencing differences in micellar properties between the two species. In the future, efforts to humanise the casein profile of IMFs may extend beyond adjustments to the levels of individual proteins. For example, dephosphorylation of bovine β-casein resulted in a β-casein which was more similar to the prinicpla form in human milk (in terms of phosphorylation, charge, gelation) and β-casein-stabilised emulsions which had larger fat globules sizes and greater calcium-stability (McCarthy *et al*., 2013). Identification of non-bovine caseins with phosphorylation patterns similar to those in human casein may allow modifications to the micellar and lipid components of IMFs to achieve greater agreement with human milk.

The only casein which is glycosylated in milk is κ-casein (Table 4), but the degree to which this protein is glycosylated is also species-specific. Glycomacropeptide (GMP) is a hydrophilic fragment of κ-casein liberated from casein micelles on renneting. GMP consists of 5-10% and 55% carbohydrate (by weight) in bovine milk and human milk, respectively (van Halbeek *et al*., 1985; Sood *et al*., 2003), while ovine and caprine GMP are known to be even less glycosylated than the bovine form (Park *et al*., 2007). IMFs prepared using cheese or rennet casein-derived whey protein ingredients contain GMP. As GMP is rich in threonine, its presence in IMFs has been associated with cases of hyperthreoninemia in IMF-fed infants (Rigo *et al*., 2001), although a later study failed to support this hypothesis (Sandström *et al*., 2008). Due to these concerns, strategies for the removal of GMP from sweet whey have been developed and low-threonine GMP isolates have been produced (Georgi *et al*., 1999; McMahon *et al*., 2014).

IMF manufacturers are interested in the potential use of GMP as an ingredient because it is a source of sialic acid (McMahon *et al*., 2014). The sialic acid content of human milk is significantly higher than that of bovine milk and bovine-milk-based IMFs (Wang et al., 2001), and sialic acid is believed to play important roles in the development of cognition and defence against pathogens (Wang and Brand-Miller, 2003). Dietary intake of sialic acid increased the performance of piglets in memory and learning tasks (Wang *et al*., 2007). In human milk, sialic acid is mainly bound to free oligosaccharides, while in bovine milk-based IMFs it is mainly bound to the GMP region of κ-casein, which is likely to

significantly affect the manner in which the body utilises sialic acid from these two systems (Wang *et al*., 2001). There is limited information available on the sialic acid profiles of the milk of other species.

3.2.4. Micellar characteristics

Caseins are predominately present in the form of micelles in the milk of all mammalian species, although the composition (Table 3) and size (Table 5) of these micelles varies considerably between species. The casein micelles of bovine milk are by far the most studied of the mammalian species. Extensive studies of bovine micelles, and some detailed characterisation of micelles of other species (mostly human), allow some general statements to be made regarding inter-species differences:

- Micelle size is inversely correlated with the concentration of κ-casein (Horne, 2006; Barłowska *et al*., 2011; Martin *et al*., 2013);
- Micellar solvation in different species is inversely correlated with the concentration of micellar calcium phosphate (Park *et al*., 2007);
- Higher β-casein:α_s-casein ratios result in greater quantities of serum-phase casein and more extensive dissociation of micelles when temperature is reduced to <20°C (Sood *et al*., 1996; Lamothe *et al*., 2007);
- In micelles of different phosphoforms of β-casein, the extent of the release of β-casein into the serum phase on addition of EDTA is lowest for non- and singly-phosphorylated forms (Sood *et al*., 1996), as other forces (e.g., hydrophobic) are likely to play a more prominent role in maintaining their association with micelles;
- High levels of non-micellar ionic calcium are associated with greater destabilisation of casein during heating, which can be improved through the addition of calcium-binding agents (Alhaj *et al*., 2011; Chen *et al*., 2012), a strategy which is often used to increase the heat stability of sterilised milk;
- The flocculation of casein micelles which precedes rennet coagulation, and the firmness of the resultant coagulum, varies widely between species, which may be due to a number of factors, including total casein content, differences in the ratio of β-casein:α_s-casein, degree of casein phosphorylation, levels of

micellar calcium phosphate, casein micelle size distribution, and/or the specific enzymes used to induce coagulation (Kapeller *et al*., 2006; Park *et al*., 2007; Uniacke-Lowe *et al*., 2010; Barłowska *et al*., 2011; Claeys *et al*., 2014).

Table 5. Mean sizes of the two major light-scattering particle populations of the milk of different species [a].

	Casein micelles (nm)	Fat globules (μm)
Infant formula	~150-182*	<1+
Human	64-80	4.0
Bovine	150-182	2.8-4.6
Caprine	260	2.6-3.5
Ovine	180-210	3.0-4.6
Buffalo	180	4.1-8.7
Equine	255	2-3
Camel	380	3.0
Yak	-	4.4

[a] modified from Claeys *et al*. (2014)

[*] considered equivalent to that of bovine milk

[+] taken from Bourlieu *et al*. (2015)

The properties of casein micelles have important implications for mineral binding and delivery, protein digestibility and physical stability during IMF processing (Table 2), yet, little research has focused on this aspect of inter-species comparison. β-Casein is the primary casein in human milk; at 37°C, human casein exists as micelles which are smaller (Table 4) and more easily digestible (Nakai and Li-Chan, 1987; Li-Chan and Nakai, 1988) than those formed by bovine casein. The association of human β-casein into micelles is influenced by β-casein concentration, ionic strength, calcium-ion content and temperature (Sood and Slattery, 2001), which is also the case for bovine β-casein (O'Connell *et al*., 2003), although differences in the degree of phosphorylation between these two mammalian β-caseins means they have their own unique temperature-dependant association behaviour (Sood and Slattery, 1997, 2001; McCarthy *et al*., 2013). There is insufficient data available on such properties of casein micelles other than those available for bovine and human milk.

3.3. Lipids

3.3.1. Physical state

Fat in milk occurs as polydisperse, spherical globules stabilised by a milk fat globule membrane (MFGM) comprised of lipids, phospholipids, cerebrosides, cholesterol and proteins; populations of these fat globules can span sizes between 0.1 and 18 μm across the milk of different species (Barłowska *et al*., 2011). The average milk fat globule diameter is inversely correlated with the specific surface area of the dispersed lipid phase. It is thought that milk types in which smaller fat globules are present exhibit more rapid lipid digestion, as more area at the surfaces of lipids is available for lipolytic enzymes to act (Bourlieu *et al*., 2015). Ovine milk and to a much greater extent camel milk and caprine milk have smaller fat globules than bovine milk; on the other hand, the average fat globule size of buffalo milk is almost double that of bovine milk (El-Zeini, 2006).

The average fat globule size in human milk (full term) generally approximates that of ovine, caprine and bovine milk; however, the average fat globule size in all of these milk types is about 10 times higher than in commercial IMFs (Table 5). IMFs are subjected to homogenisation, which physically disrupts fat globules and reduces their size significantly; subsequent process steps, such as evaporation and spray drying, can result in slight increases and decreases in size, respectively, but fat globules remain far smaller than in regular milk and are typically in the sub-micron range (McCarthy *et al*., 2012; Drapala *et al*., 2015). The process of homogenisation also alters the surface composition of fat globules, with increased adsorption of both casein and whey proteins at the lipid-water interface (McCarthy *et al*., 2012; Bourlieu *et al*., 2015). In contrast to human milk, in which MFGM emulsifies the fat, milk proteins act as the primary emulsifiers in IMFs, with a minor contribution from MFGM. Bourlieu *et al*. (2015) compared model IMFs containing native milk fat globules (~4 μm, MFGM coated) or homogenised milk fat globules (≤ 1 μm, protein coated); the presence of smaller fat globules resulted in more rapid lipolysis, while the presence of protein at the interface of these small globules increased the rate of proteolysis. Efforts to modify the composition of the lipid-water interface in IMFs to more closely resemble the interfacial composition in human milk though the addition of bovine milk phospholipids during processing have been successful (Gallier *et al*., 2015). Stabilising IMF emulsions through processing while ensuring that interfacial composition and fat globule size are close

to those found in human milk will be a key challenge in future years.

3.3.2. Composition of lipid component

Lipid content is a major determinant of the energy density of mammalian milk. As can be seen in Table 6, milk of certain mammals (ovine, buffalo and reindeer) are energy-dense, due to their high fat levels, while others, equine milk in particular, have very low fat and energy levels. The total fat content of human milk is generally in line with the types of ruminant milk considered for comparison in Table 6; however, the lipid profile of the milk of different species deviate considerably from each other. Milk of ruminants generally has higher saturated fatty acid (SFA) contents than human milk, while their monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) levels are lower (Table 6). The fatty acid profiles of milk from equine and camel sources are most similar to that of human milk, with camel milk having the highest SFA content. Contents of conjugated linoleic acid (CLA) and cholesterol in human milk are broadly similar to those of ruminant milk, with equine milk being lower in both, and camel and yak milk being much higher in cholesterol. Cholesterol levels are higher in human milk compared to IMFs; indeed, concentrations of cholesterol in the blood sera of human milk-fed infants are higher than those fed IMFs in which the lipid component is comprised of blends of vegetable oils (Morgan, 2006). In general, the fatty acid profile of human milk is characterised by very low levels of C4:0, C6:0, C8:0 and C10:0, with camel milk being similar to human milk in this regard. In addition, levels of C18:1 and C18:2 are particularly high in human milk (Barłowska *et al*., 2011).

Table 6. Fatty acid profile (% of total fatty acids), cholesterol content (mg 100 mL milk^{-1}) and proportion of C16:0 esterified at the *sn-2* position (%) of the milk of different species [a].

	SFA (%)	MUFA (%)	PUFA (%)	n-6:n-3 (-)	CLA (%)	Cholesterol (mg 100 mL^{-1})	*sn*-2 C16:0* (%)
Human	39.4-45	33.2-45.1	8.1-19.1	7.4-8.1	0.2-1.1	14-20	74
Bovine	55.7-72.8	22.7-30.3	2.4-6.3	2.1-3.7	0.2-2.4	13.1-31.4	38
Caprine	59.9-73.7	21.8-35.9	2.6-5.6	4	0.3-1.2	10.7-18.1	36
Ovine	57.5-74.6	23.0-39.1	2.5-7.3	1.0-3.8	0.6-1.1	14-29.0	29
Buffalo	62.1-74	24.0-29.4	2.3-3.9	n.d.	0.4-1	4-18.0	37
Equine	37.5-55.8	18.9-36.2	12.8-51.3	0.3-3.5	0.02-0.1	5.0-8.8	n.d.
Camel	47-69.9	28.1-31.1	1.8-11.1	n.d.	0.4-1	31.3-37.1	n.d.
Yak	60-65	3.8-18	2-6.2	n.d.	0.2	22	n.d.
Reindeer	84	20	4	n.d.	n.d.	n.d.	n.d.

n.d. = no data

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acid

[a] modified from Claeys *et al*. (2014)

* taken from Freeman *et al*. (1965)

3.3.3. Stereo-specific triglycerol structures

For many years, the lipid component of IMFs was comprised of bovine milk fat, which differs significantly from human milk fat (Table 6). In modern IMFs, blends of vegetable oils are more commonly used, with a concomitant increase in the PUFA levels of these products. Another important aspect of inter-species differences in milk fat is the stereo-specific positioning of fatty acids in the central (*sn*-2) and terminal (*sn*-1,3) positions of the glycerol backbone. In human milk, the *sn*-1,3 positions are primarily occupied by oleic acid (Bar-Yoseph *et al*., 2013), with high levels (~70%) of palmitic acid positioned at the *sn*-2 position of the glycerol backbone. This level of palmitic acid is approximately double that found in the *sn*-2 position in bovine, caprine, ovine and buffalo milk (Table 5), while *sn*-2 palmitic acid levels in donkey milk (54%) have been reported to be closer to those of human milk (Blasi *et al*., 2008). Straarup *et al*. (2006) surveyed a range of IMF products and reported that the proportion of palmitic acid positioned at the *sn*-2 position of the glycerol backbone ranged widely from 3.4 to 21% in non-specialised 1st-age products. The stereo-specific positioning of fatty acids on the glycerol backbone is believed to affect fatty acid absorption, with the fatty acids in human milk displaying superior absorption properties to infant formula despite similar fatty acid composition (Thompkinson and Karb, 2007). In addition, the high level of *sn*-2 palmitic acid in human milk has been linked with improved calcium absorption and softer stools in infants (Bar-Yoseph *et al*., 2013). Structured triglycerides have been developed in which vegetable oils are enzymatically modified to contain more *sn*-2 palmitic acid, with oleic acid positioned at *sn*-1,3, as in human milk fat; in addition to contributing to improvements in fatty acid absorption, calcium absorption and stool properties, these structured triglycerides have been linked with better bone development, altered intestinal microflora, reduced intestinal inflammation, and decreased instances of colic (Bar-Yoseph *et al*., 2013).

IMFs are often supplemented with polyunsaturated fatty acids (PUFAs), as long-chain PUFAs are present at high levels in human milk, in which they are thought to contribute to cognitive and visual development (de la Presa-Owens *et al*., 1995). Equine milk is high in PUFAs compared to the other milk types (Table 6), which is particularly notable given that the protein content of this milk (Table 1) means that minimal dilution would be required to manufacture an IMF. PUFAs can be oxidised under adverse conditions during processing, and, in particular, storage

(Romeu-Nadal *et al*., 2007), a phenomenon aided by the presence of pro-oxidants such as iron in IMFs (Thompkinson and Karb, 2007). Lactoferrin, which binds iron and comprises a much higher proportion of whey protein in human milk than in the milk of other species, with camel milk perhaps having the most comparable levels (Table 3), can counteract oxidation when used to supplement IMFs (Satué-Gracia et al., 2000). Regardless of the type of milk used, the final PUFA content of IMFs is a function of a complex interplay between lactoferrin and prooxidant levels, in addition to PUFA supplementation and storage conditions. Encapsulation-based technologies are also being studied for the protection of PUFAs (de la Presa-Owens *et al*., 1995; Chávez-Servín *et al*., 2006; Crowley and O'Mahony, 2016) and are used commercially.

3.4. Carbohydrate profile

3.4.1. Major components

Lactose is the principal carbohydrate in milk, and levels of this disaccharide are broadly similar across the milk of various ruminants (Table 1). Equine milk has higher levels of lactose than ruminant milk and has levels closest to those found in human milk. Pure lactose is widely available and the presence of low levels in milk is easy to rectify. Levels of glucose, fructose and sucrose are negligible in milk, but these sugars are sometimes added to IMFs at low levels for sensory purposes (Nguyen *et al*., 2015). Maltodextrins, corn syrup solids and starches are also sometimes added to IMFs, either as a carbohydrate source in lactose-free IMFs or, in the case of starch, as a means to increase viscosity in thickened, anti-regurgitation formulae.

3.4.2. Oligosaccharides

Recommended levels for oligosaccharides in IMFs have not been defined; however, oligosaccharides are present in human milk at much higher concentrations than the milk of other mammalian species (Table 1). In excess of 200 different oligosaccharides have been identified thus far in human milk (Wu *et al*., 2010, 2011). Oligosaccharides are the subject of much interest due to their apparent biofunctional role as prebiotics (i.e., non-digestible carbohydrates which promote the growth of probiotic bacteria). Levels of oligosaccharides are several-fold higher in human milk compared to the milk of other species (Claeys *et al*., 2014). Among the

ruminants, caprine milk has one of the highest levels of oligosaccharides, which makes it an attractive raw material for their separation and purification by membrane technology. Oligosaccharide-enriched fractions have been obtained from caprine cheese whey using ultrafiltration with 1 kDa membranes (Martinez-Ferez *et al*., 2006; Oliveira *et al*., 2012a) or ultrafiltration with 10 kDa membranes combined with enzymatic hydrolysis of lactose and solid-phase extraction to remove glucose, galactose and non-hydrolysed lactose (Thum *et al*., 2015).

Ingredients, such as GOS, FOS and inulin, which are purported to simulate some of the health benefits of oligosaccharides in human milk, have been developed and are used in the manufacture of IMF products (Vandenplas, 2002; Bode, 2009; Barille and Rastall, 2013). Limitations in this approach include the comparatively simple structures of these oligosaccharides compared to those present in human milk, with a lack of sialic acid and fucose groups in particular; as such, the search for alternative sources continues (Bode, 2009). Despite these limitations, early indicators suggest that prebiotic supplementation of IMFs may be effective. Bifidobacteria are often the dominant species in the gastrointestinal tract of infants fed human milk, and promotion of this species in the microbiota of infants fed prebiotic-supplemented IMFs has been linked with several positive health effects including improved stool characteristics, reduced risk of infectious diseases, and lower incidences of atopic dermatitis (Janthscher-Krenn and Bode, 2012).

3.5. Micronutrients

The milk of any species is a plentiful dietary source of a diverse range of micronutrients; however, there are many differences in the micronutrient profiles of various species. Highly pure forms of minerals and vitamins are readily available, making micronutrient fortification, in principal, a simpler task than modifying the protein profile of IMFs, for example; however, there remain significant challenges in the fortification of IMFs with micronutrients. In particular, addition of calcium salts can cause significant destabilisation (Omoarukhe *et al*., 2010), while vitamins can undergo degradation during storage (Chávez-Servín *et al*., 2008a, b).

3.5.1. Minerals

The milk of ruminants contains significantly higher levels of most minerals compared with human milk (Table 7).

Table 7. Levels of individual minerals (mg 100 mL^{-1}) for the milk of different species[a] and the degree to which adequate intake (AI) is satisfied.

	AI*	Human		Bovine		Caprine		Ovine		Buffalo		Equine		Camel		Yak		Reindeer	
		min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max
Calcium	200	28	34	112	123	85	198	159	242	112	220	50	135	105	157	119	221	163	320
% AI		109	133	437	480	332	772	620	944	437	858	195	527	410	612	464	862	636	1248
Phosphorus	100	14	43	59	119	79	153	124	175	85	293	20	121	58	104	92	163	64	270
% AI		109	335	460	928	616	1193	967	1365	663	2285	156	944	452	811	718	1271	499	2106
Potassium	400	53	62	106	163	140	242	94	162	92	182	25	87	124	179	75	179	80	156
% AI		103	121	207	318	273	472	183	316	179	355	49	170	242	349	146	349	156	304
Magnesium	30	3	4	7	12	10	36	16	25	2	39	3	12	8	16	11	19	11	22
% AI		78	104	182	312	260	936	416	650	52	1014	78	312	208	416	286	494	286	572
Sodium	120	10	18	58	58	28	59	30	75	35	95	8	58	36	73	19	41	25	50
% AI		65	117	377	377	182	384	195	488	228	618	52	377	234	475	124	267	163	325
Citrate	n.d.	n.d.	n.d.	160	177	70	180	104	205	144	224	n.d.	n.d.	128	185	n.d.	n.d.	n.d.	n.d.
% AI		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chloride	180	60	63	100	119	104	209	99	160	57	75	19	19	132	132	28	144	68	80
% AI		260	273	433	516	451	906	429	693	247	325	82	82	572	572	121	624	295	347
Iron	0.27	0.04	0.20	0.03	0.10	0.05	0.10	0.08	0.10	0.04	0.20	0.02	0.15	0.07	0.37	0.07	0.07	0.065	0.07
% AI		116	578	87	289	144	289	231	289	121	578	58	433	202	1069	202	202	188	188
Zinc	2	0.20	0.40	0.30	0.55	0.40	0.60	0.40	0.60	0.15	0.73	0.09	0.64	0.19	0.60	0.26	0.71	1.00	1.30
% AI		78	156	117	215	156	234	156	234	59	285	35	250	74	234	101	277	390	507
Copper	0.2	0.02	0.06	0.01	0.08	0.02	0.05	0.02	0.05	0.01	0.04	0.02	0.11	0.01	0.19	0.01	0.01	nd	Nd
% AI		78	234	39	312	78	195	78	195	27	156	78	429	39	741	39	39	nd	Nd

[a] adapted from Claeys *et al*. (2014)

* AI (mg day^{-1}) for an infant (0-6 mo) was taken from IOM (2006, 2011); calculation of % AI satisfied was based on consumption of 780 mL of milk, which is the average volume of human milk consumed by infants aged 0-6 mo (van Guodoever, 2015). n.d. = no data.

Levels of calcium and phosphorus are particularly low in human milk, likely due to its lower casein content (Table 3). Equine milk has a mineral profile which most closely resembles that of human milk, particularly with regard to non-micellar minerals, although chloride content is comparatively low (Table 7). The types of milk which are most promising in relation to the formulation of IMFs, in terms of protein profile (caprine, camel and equine milk), all contain relatively high ash levels (Table 1). One of the reasons that bovine milk is unsuitable for infant consumption is its high concentration of protein and minerals relative to human milk. The high protein and mineral levels in bovine milk result in it having a dangerously high potential renal solute load (PRSL; a function of the dietary intake of protein, sodium, potassium, chloride and phosphorus) for infants, who have a limited ability to concentrate and dilute urine, which could put the infant at risk of developing hypertonic dehydration (Morgan, 2006). For use in IMF applications, bovine milk is diluted with water and blended with demineralised whey protein ingredients, thereby reducing its PRSL considerably; such an approach would also be required for other milk types, with many (e.g., ovine, buffalo, reindeer) containing even higher levels of the solutes which contribute to PRSL than bovine milk (Table 1, 7).

In general, adequate intake (AI) values of individual minerals for an infant of 0-6 months are attained (~100% of AI) by the consumption of 780 mL of human milk (average daily intake for an infant; van Guodoever, 2015), while consumption of the same volume of most of the other milk types results in the AI of a large range of minerals being over-supplied to a very large degree (Table 7). The implications of the high mineral levels of various non-bovine milk types (Table 7) for the prospective manufacture of an IMF depends on other compositional factors also. Caprine milk, for example, would need to be diluted at least two-fold to achieve an appropriate protein content (Table 1), and may require the addition of demineralised whey protein to achieve the correct casein:whey protein ratio (Table 3), which would bring the mineral profile closer to that of human milk than in its unmodified form; on the other hand, the milk of the horse, and particularly the camel, contain excessively high levels of various minerals for infant nutrition applications (Table 7), but could not be diluted to a great extent as they are already close to the protein content of human milk (Table 1); in addition, they would require less demineralised whey protein than a bovine-milk based IMF as they have lower casein:whey protein ratios (Table 3). Thus, alternative approaches, based on the use of

concentrated/demineralised protein fractions and the implementation of mineral fortification strategies may be required to use such non-bovine mammalian milks in IMF applications.

Most ions in milk, such as sodium, potassium and chloride, exist in ionic form in the serum phase, while others, such as calcium, phosphorus and iron, are distributed either as free ions or associated with various macromolecules. Iron stores in the liver are sufficient for infants of <6 months, and the iron present in human milk is mostly bound to the MFGM and lactoferrin. Iron levels in many types of milk are comparable to that of human milk (Table 7). However, the iron present in human milk is known to be absorbed very efficiently compared to bovine milk (Morgan, 2006). Of the calcium in bovine milk, ~67% is associated with casein micelles and 33% is present in the serum phase, with only ~1% located in the lipid fraction, and even less (0.15%) associated with α-lactalbumin; the calcium in human milk is distributed differently, with 6% present in the micellar phase and 40% soluble in the serum phase, 29% associated with whey proteins (1% with α-lactalbumin) and up to 26% associated with milk fat (Flynn and Cashman, 1992). Further deviations in mineral distribution are observed in the milk of other species. Attia *et al*. (2000) reported that the distribution of calcium between the micellar and soluble phases in camel milk is similar to that of bovine milk, although the micelles contained 1.5 times more total minerals; in particular, the proportions of magnesium (67%), phosphorus (67%) and citrate (33%) in the micellar phase were notably higher than in bovine milk (40%, 60% and 10%, respectively). Casein micelles in caprine milk and ovine milk are more highly mineralised than those in bovine milk (Park *et al*., 2007). Ultrafiltration of camel (Mehaia, 1996), buffalo (Patel and Mistry, 1997) and caprine (Moreno-Montoro *et al*., 2015) milk results in concentration of protein, as well as a number of minerals, particularly the calcium and phosphorus associated with the micellar phase, which also occurs during ultrafiltration of bovine milk (Lin *et al*., 2015).

IMF products can be considered to be broadly comprised of two classes of minerals, innate minerals (introduced in dairy ingredients such as skim milk) and fortified minerals (added as pure salts). It is preferable to maximise the levels of the former, as they exist in a more stable equilibrium with proteins and are less likely to contribute to instability during processing and storage. Fortification with salts promotes instability in IMFs if not carefully controlled. This is particularly true of

calcium, which can destabilise milk systems in a number of ways, such as the poor heat stability which develops when soluble salts are added and the mineral 'fall-out'/sedimentation which occur when insoluble salts are used (Omoarukhe *et al*., 2010). In addition to process instability, fortification with calcium derived from soluble salts has been linked with poor nutritional outcomes, such as reduced calcium retention and fat absorption in infants, due to what is presumed to be modifications to fat globule structure (Bass and Chen, 2006). The high micellar calcium contents of caprine and ovine milk may allow a reduction in the levels of fortified calcium in IMF products, with a greater incorporation of innate minerals; however, high-calcium milk systems (e.g., caprine) are also high in free, ionic calcium, presumably due to insufficient citrate for complexation (see Table 7), which makes them prone to heat-induced destabilisation, and may necessitate the use of stabilising citrate or phosphate salts (Chen *et al*., 2012). Calcium absorption from mammalian milk is higher than from plant sources, as phytate present in the latter forms complexes with calcium which limit absorption; for this reason, soy-based IMFs require >30% more total calcium than bovine milk-based IMFs, while calcium absorption from bovine milk-based IMFs and human milk are similar at ~60% (Bass and Chen, 2006).

3.5.2. Vitamins

Monogastric mammals, such as the horse and donkey, produce milk which contain lower levels of vitamins generally, with the same also being true for the milk of certain ruminants (i.e., the camel), although levels of vitamin C in camel milk are notably high (Barłowska *et al*., 2011; Claeys *et al*., 2014). Caprine, ovine and bovine milk contain lower levels of vitamin C than human milk, while the vitamin A contents of human milk and caprine milk are similar, with ovine milk, and, in particular, bovine milk, being lower in vitamin A than human milk (Barłowska *et al*., 2011; Claeys *et al*., 2014).

It is easier to adjust the vitamin profile of a milk or IMF which is too low in a given vitamin, rather than one which is too high, with appropriate supplementation. Many vitamins in caprine, ovine and bovine milk, such as thiamine (all), riboflavin (all), niacin (caprine, ovine), pantothenic acid (all), vitamin B6 (all), biotin (all), vitamin B12 (all) are present at much higher levels than in human milk (Park *et al*., 2007), even when milk standardised to the protein levels of IMFs is considered; as a

result, in some cases the milk of these species may not meet the strict regulatory limits for IMF products and there may be a risk that infants could consume these vitamins at overly high levels during feeding; however, when the combined effects of dilution to an appropriate protein content and losses from thermal processing and storage are considered, vitamin levels in IMFs manufactured from these milk types may fall below regulatory limits. On the other hand, 'goat milk anaemia' in adults is associated with consumption of caprine milk, due to the low levels of vitamin B12 and folic acid present relative to bovine milk (Park *et al*., 2007). If a caprine milk diluted ~2-fold to a protein content closer to that of human milk is considered, the vitamin B12 content is sufficient, but the folic acid content is much too low; however, this latter issue could be easily addressed with appropriate supplementation.

4. Non-bovine mammalian milk in infant nutrition: the case of caprine milk

Although bovine milk is the most extensively used base from which IMFs are manufactured, IMFs based on the milk of other species have also been developed and are available commercially. A notable example is caprine milk-based IMFs, which are now available in a number of countries, including New Zealand and the UK. There have been several controversial or contentious reports and claims concerning the benefits of feeding 'raw goat milk' or 'homemade goat milk infant formula' to infants, practices which can have severe health implications for infants (Basnet *et al*., 2010). Suitably-modified caprine milk-based IMFs are, however, suitable for infant consumption. Caprine milk is not recommended for infants in unmodified form, therefore it must be converted into IMF through appropriate dilution, blending and fortification, in much the same way as bovine milk; these modifications negate many of the principal differences which exist between caprine and bovine milk (Silanikove *et al*., 2010). Despite conflicting evidence regarding the cross-reactivity of bovine and caprine milk proteins (Haenlein, 2004; El-Agamy, 2007), caprine products are not regarded as suitable for infants with bovine milk protein allergy (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012). An interesting aspect of caprine milk-based IMFs is that they do not seem to require an inversion of the naturally occurring whey protein:casein ratio (~20:80), as caprine milk proteins satisfy amino acid requirements in the native casein-dominant

form (Zhou *et al*., 2014); however, whey protein-dominant bovine milk-based IMFs and casein-dominant caprine milk-based IMFs have been reported to result in similar developmental outcomes when fed to infants (Grant *et al*., 2005; Han *et al*., 2011; Zhou *et al*., 2013).

It is unclear how caprine milk-based IMFs compare to the more advanced IMF products which have been developed, such as those enriched in α-lactalbumin; as far as the authors are aware, such a comparison has not been made. Caprine IMFs, like bovine IMFs, still deviate from human milk in innumerable respects, and manufacturers of the former may need to develop humanisation strategies to keep pace with an IMF industry which is continuously attempting to 'close the gap' with human milk, not just in terms of chemical composition, but also in infant growth and developmental outcomes. The humanisation challenge is perhaps even greater for manufacturers of caprine IMFs; although ingredients such as α-lactalbumin, lactoferrin, β-casein and osteopontin are available commercially, they are bovine milk-derived, which may limit their application in products marketed as being 'goat milk infant formula' (note: mixed-species IMFs are reportedly in development, with one company in New Zealand planning to manufacture a product termed 'shegoa', a blend of ovine and caprine milk, for IMFs). Thus, these protein fractions may need to be manufactured from caprine milk at commercial scale to allow next-generation, humanised caprine IMFs to be developed. Such fractionation technologies have been developed at a small scale (see, for example, Lamothe *et al*., 2008), but an industry for such caprine milk protein fractions does not currently exist. Despite these factors, caprine IMFs are an example of how a non-bovine mammalian milk can be successfully developed into a commercial infant nutrition product.

5. Outlook and future perspectives

In this chapter, the properties of milk of non-bovine mammalian species were compared with those of human milk, with a view to assessing their relative merits as potential human milk replacers or raw materials for IMF manufacture compared to bovine milk. Owing to the unparalleled suitability of breast milk for infants, the ability of any non-human mammalian milk to act safely and effectively as a direct replacement in all cases is minimal. The diversity of macro- and micro-nutrient

compositions across the milk of different species should continue to be investigated for any potential nutritional and health benefits they may confer when consumed whole by *human adults*. It must be reiterated, however, that no non-human milk is suitable as a direct replacement for breast milk. As such, although caprine, horse and camel milk have certain characteristics which make them preferable to other milk types for application in infant nutrition, they should still be considered primarily as potential raw materials for the production of IMFs, which may allow the composition of IMFs to be further humanised (Fig. 1). Thus, although the use of camel milk as an alternative to human milk is attractive due to its reported lack of β-lactoglobulin (Table 3), this milk is still casein-dominant (Table 3) and too high in ash (Table 1), for example, both of which may need to be modified as per current practice with bovine milk-based IMFs. However, where a direct replacement of human milk with another whole milk is the only option, the milk of monogastric species and camels seem to be far more appropriate alternatives compared with ruminant milk, with the compositional profiles of the ruminants generally deviating much more significantly from that of human milk (Table 1, 3, 6, 7). The relative advantages and disadvantages of the most promising milk for infant nutrition applications from select species are summarised in Table 8.

An area that requires more attention is the potential for non-bovine mammalian milk to function as rich sources of ingredients for humanised IMF products. Equine milk (and donkey milk) have several notable similarities to human milk, including total protein, lactose and ash contents (Table 1), whey protein:casein ratio and β-casein:α_s-casein ratio (Table 3); in addition, the α-lactalbumin:β-lactoglobulin ratio in equine milk compares favourably to that of bovine milk (Table 3). Research involving the concentration of equine milk using ultrafiltration for use in model IMF systems should be considered, as this may provide a human milk-like protein fraction to which other IMF ingredients (e.g., oils, minerals) could be added in correct proportions. Caprine milk does not compare with human milk as closely as equine milk, but it has certain features in common with human milk, such as its β-casein:α_s-casein ratio (Table 3), and should be considered favourably in light of evidence supporting it as a tolerable protein source in some cases of CMA in infants (El-Agamy, 2007; Uniacke-Lowe *et al*., 2010) and its use in the manufacture of IMFs deemed to have comparable developmental outcomes to bovine milk-based IMFs for infants (Grant *et al*., 2005; Han *et al*., 2011; Zhou *et al*., 2013).

Table. 8. Assessment of the relative advantages and disadvantages relating to some general properties of the milk types identified as potentially useful for infant nutrition applications. This information is a rough approximation based on data shown elsewhere in this chapter, and should be used as a rough guide only. The number of ✓ (similar) or × (dissimilar) symbols is in proportion to the similarity of a given milk to human milk in that particular property.

Property	Bovine	Caprine	Equine	Camel
Total protein	×××	×××	✓✓	✓
Whey protein:casein ratio	×××	×××	✓✓	××
Casein profile	×××	✓	✓✓	n.a.
Whey protein profile	×××	×××	✓	✓✓
Lactose	✓	✓	✓✓✓	✓
Total fat	✓✓✓	✓✓✓	✓✓✓	✓✓✓
Ash	×××	×××	✓✓	×××
Oligosaccharides	×××	✓	n.a.	n.a..

n.a.. = not applicable (due to insufficient data)

Many types of milk, such as caprine, buffalo, equine and yak milk, contain higher β-casein:α_s-casein ratios to bovine milk, and could serve as β-casein or casein concentrate sources for IMF production. Indeed, successful generation of β-casein-enriched fractions from caprine milk has already been achieved (Lamothe *et al*., 2007). Caprine milk has also been demonstrated to be an excellent source of oligosaccharides, which can be enriched using membrane processing (Martinez-Ferez *et al*., 2006; Oliveira *et al*., 2012a; Thum *et al*., 2015) for possible inclusion in IMF products due to their functionality as prebiotics (Oliveira *et al*., 2012b). The development of such caprine milk fractions may be particularly important for the humanisation of current caprine milk-based IMFs.

Camel milk, like human milk, reportedly does not contain β-lactoglobulin, and could be used to manufacture whey protein ingredients for use in IMFs; appropriate demineralisation would eliminate the high ash content of camel milk, which is not compatible with safe infant nutrition. To the authors' knowledge, the only studies involving the membrane processing of camel milk have involved concentration by ultrafiltration (Mehaia, 1996); further studies in which microfiltration is applied in the fractionation of casein and whey protein fractions could be interesting for ingredient development purposes.

A major limitation to the development of dairy protein ingredients from non-bovine sources is the low volumes of milk which are available for enrichment/purification processes such as membrane separation. Two of the more interesting milk types from an infant nutrition perspective, equine and camel, are often reported to have poor gelation/cheese-making characteristics, and therefore do not provide a dependable source of high volumes of whey, while milk which consistently yields whey streams (e.g., buffalo, caprine, ovine) do not have a whey protein profile comparable to human milk and, hence, are of less interest. Thus, the use of caprine and ovine whey, for example, will probably be of more interest due to any unique techno-functional properties that they may have (Sanmartín *et al*., 2014) or simply to reduce wastage (Macedo *et al*., 2015).

Improving the cheese-making characteristics of non-bovine milk, such as that of the camel, may provide the secondary benefit of an increased supply of a whey source with unique potential in the realm of infant nutrition. Indeed, it is now known that recombinant camel chymosin results in very effective clotting of camel milk, which increases the cheese-making potential of camel milk and, in turn, may increase the volumes of camel cheese whey which are available for further processing (Kapeller *et al*., 2006). The development of such materials from different non-bovine milk sources should first be pursued with a view to eventually assessing if the inclusion of such ingredients in IMFs may impart beneficial nutritional characteristics to these products.

6. Concluding remarks

If the stated goal of infant formula manufacturers is to produce products which resemble human milk as closely as possible, then notice must be taken of the striking similarities which exist between human milk and the milk of certain non-bovine species. While a search for a universally safe, direct replacement for human milk is ill-advised, due to the complete nutritional profile of human milk and the high likelihood that a certain proportion of the population will exhibit intolerances or adverse reactions to the alternatives, these types of milk should be viewed as useful potential sources of raw materials for infant formulae. While there are significant limitations on the volume of some non-bovine mammalian milk available for large-scale processing, particularly where purification of individual components (e.g., by membrane separation) to supply a global market is concerned, this area should be

pursued, even if it is initially of only "academic interest". However, increased production volumes of certain types of non-bovine milk may need to be achieved if they are to be considered a realistic option by dairy ingredient and IMF manufacturers, particularly if their use outside of niche applications is envisaged. Given the relative merits of the milk of different species, the future development of specific mixed-species milk products (e.g., camel whey protein/caprine casein blend, humanised bovine IMF with caprine oligosaccharidees) should be considered.

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CHAPTER 8

Processing and protein-fractionation characteristics of different polymeric membranes during filtration of skim milk at refrigeration temperatures

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. The chapter has been published as: Crowley, S. V., Caldeo, V., McCarthy, N. A., Fenelon, M. A., Kelly, A. L., and O'Mahony, J. A. (2015). Processing and protein-fractionation characteristics of different polymeric membranes during filtration of skim milk at refrigeration temperatures. *International Dairy Journal*, 48, 23-30. SVC co-designed the study, configured the bench-top membrane filtration unit, determined composition and physicochemical properties, performed electrophoresis, carried out analytical centrifugation tests and analysed the data. VC provided assistance with HPLC analysis.

Abstract

Serum protein concentrates (SPCs) were generated from reconstituted skim milk (3.2% protein) using lab-scale membrane filtration at 3-4°C. The influence of membrane type on process performance (e.g., permeate flux) and protein-enrichment (e.g., protein profile) was assessed with polyvinylidene-difluoride membranes (0.1 μm and 0.45 μm pore-size), and a polyethersulfone membrane (1000 kDa cut-off). The 1000 kDa membrane exhibited the highest starting flux (6.7 L m^{-2} h^{-1}), followed by the 0.1 μm (5.4 L m^{-2} h^{-1}) and 0.45 μm (4.8 L m^{-2} h^{-1}) membranes. Flux decreased by >40% during filtration with the 1000 kDa and 0.1 μm membranes, while the decrease was lower (<20%) with the 0.45 μm membrane. β-Casein comprised >97% of casein in SPCs from the 0.1 μm and 1000 kDa membranes. SPCs from the 0.45 μm membrane had higher β-casein:α_s-casein ratios than the feed and higher levels of minor whey proteins (e.g., lactoferrin) relative to the other SPCs.

1. Introduction

In the manufacture of bovine milk-based first age infant milk formula (IMF) products, the protein component is formulated by reconstituting skim milk and whey powders in combination to yield a casein:whey ratio of approximately 40:60, which is equivalent to that of human milk (de Wit, 1998), and markedly different to that of bovine milk (80:20). Efforts to extend the humanisation of IMF typically involve increasing the levels of β-casein, α-lactalbumin and lactoferrin, while reducing levels of α_s-casein and β-lactoglobulin (Guo, 2014). Ingredients such as α-lactalbumin-enriched whey protein concentrates are already being incorporated into IMFs (Kuhlman *et al*., 2005). Similarly, the application of β-casein-enriched ingredients is expected to increase as manufacturers attempt to further bridge the gap between the protein profile of IMFs and human milk. However, industrial processes for the production of β-casein-enriched ingredients remain in the early stages of development commercially.

One of the most promising processes for the manufacture of β-casein-enriched serum protein concentrates (SPCs) is pressure-driven membrane filtration. Traditionally, filtration processes (e.g., ultrafiltration, microfiltration), are performed at high temperatures of 45-50°C, where there is the advantage of a high flux (short processing time for a given volume of product) and limited growth of mesophilic bacteria (Zulewska *et al*., 2009; Hurt *et al*., 2010; Gésan-Guiziou, 2013; O'Mahony and Tuohy, 2013). For the enrichment of β-casein, filtration is performed at cold (typically 4-10°C) temperatures (Woychik *et al*., 1992; Le Berre and Daufin, 1994; O'Mahony *et al*., 2014; Holland *et al*., 2011; Christensen and Holst, 2014; Coppola *et al*., 2014; Seibel *et al*., 2014), under which conditions β-casein exists in its monomeric state in the serum phase (Payens and van Markwijk, 1963; Rose, 1968). In the dairy industry, the term "cold", as applied to membrane separation processes, currently encompasses a relatively broad temperature range and can include any filtration process temperatures <20°C. As low filtration temperatures may have additional benefits distinct from β-casein separation (e.g., reduction in the denaturation of whey proteins, reduced fouling of membranes by calcium phosphate and reduced growth of thermophiles), many manufacturers are currently transitioning from traditional "warm" (~40-50°C) processes to cold processes (Lawrence *et al*., 2008). However, the lower temperatures reduce diffusivity, and therefore the mass

transfer coefficient, with a concomitant marked decrease in permeate flux (Le Berre and Daufin, 1994). This issue is exacerbated when polymeric membranes (e.g., polyvinylidene-difluoride, PVDF, and polyethersulfone, PES) are used instead of ceramic membranes, as the latter generally have considerably higher flux values (Zulewska *et al*., 2009; Beckman *et al*., 2010). For many years, ceramic membranes were the most widely used material for membrane separation processes in the dairy industry, due to their excellent pH-, temperature- and cleaning-tolerance, in addition to high flux performance; however, in recent years, polymeric membranes have become increasingly popular due to their cost-effectiveness (O'Mahony and Tuohy, 2013).

The materials and conditions which have been described for the enrichment of β-casein using membrane filtration vary widely, and few researchers have provided a detailed analysis of processing performance. Woychik *et al*. (1992) reported filtration of milk at temperatures of 2-8°C using microfiltration membranes with pore sizes of 0.1 and 0.2 µm or an ultrafiltration membrane with a molecular weight cut-off (MWCO) of 100 kDa in a plate-and-frame configuration. Le Berre and Daufin (1994) separated β-casein from sodium caseinate at 4°C using tubular ceramic membranes (ZrO_2 filtering layer on carbon supporting layer) with pore sizes ranging between 0.02 and 0.08 µm. Microfiltration of skim milk at 2-5°C using 0.55 µm spiral-wound polymeric (PVDF) membranes has also been reported (O'Mahony *et al*., 2014). Holland *et al*. (2011) removed β-casein from skim milk using 80 kDa MWCO polymeric ultrafiltration (PES) membranes in a plate-and-frame configuration at 7°C. More recently, Glas *et al*. (2013) used different membranes with pore sizes of between 0.3 and 0.5 µm in a spiral-wound configuration for enrichment of β-casein from skim milk at much higher temperatures (10-20°C), while Christensen and Holst (2014) reported using a 800 kDa MWCO membrane in a spiral-wound configuration to generate a β-casein-enriched permeate from micellar casein isolate. Most of these researchers noted that the separation of β-casein had potential application in infant formula products.

As the above examples illustrate, studies on the enrichment of β-casein have involved the use of a multitude of membrane materials, pore sizes and MWCOs, configurations and processing temperatures. Presently, the optimal materials and conditions for the filtration of skim milk at refrigeration temperatures are unclear. Le Berre and Daufin (1994) performed a thorough analysis of processing characteristics

during their β-casein enrichment experiments; however, the researchers used a 1% (w/w) sodium caseinate solution as the feed and ceramic membranes for the separation. In industrial applications for the development of infant formula ingredients, skim milk is more likely to be used as the feed material for β-casein enrichment, as it facilitates co-enrichment with whey proteins and the generation of a separate "native" functional retentate stream enriched in casein micelles (O'Mahony *et al*., 2014; Seibel *et al*., 2014).

In this study, membrane filtration at refrigeration temperatures (≤4°C) was performed using polymeric microfiltration membranes (0.1 μm and 0.45 μm pore-size PVDF) and a polymeric ultrafiltration membrane (1000 kDa MWCO PES), with detailed analysis undertaken of the filtration process itself, in addition to the composition, physicochemical properties and protein profile of the process streams generated, with a view to developing a protein base for IMF manufacture.

2. Materials and methods

2.1. Materials

Low-heat skim milk powder (SMP) was supplied by the Irish Dairy Board (Fermoy, Ireland). Reconstituted SMP was prepared in deionised water under constant magnetic stirring at 22°C to attain a 3.2 % (w/w) true protein suspension. Reconstituted SMP was then stored at 4°C for ~23 h to ensure complete rehydration.

2.2. Membrane filtration: processing parameters, process analysis and cleaning

Filtration experiments were performed at lab-scale using a pressure-driven, membrane filtration device (Pellicon 2 mini-holder; Merck-Millipore, Tullagreen, Carrigtwohill, Ireland), as described by Crowley *et al*. (2015), who reported using the unit for ultrafiltration of reconstituted SMP and IMF, with the following modifications: in the present study, both PES (Biomax, Merck-Millipore) and PVDF (Durapore, Merck-Millipore) membranes were used in individual trials (Table 1); the heat-exchanger (plate and frame) was used to control temperature at between 3-4°C; an equilibration time of at least 30 min was allowed to ensure stable conditions (e.g., temperature and flux). Reconstituted SMP was concentrated to a volume concentration factor (VCF) of 3 by removing permeate, with the retentate being continuously recirculated back to the feed. Samples of the retentate and permeate were taken for analysis when VCF = 3, while the feed was sampled before

concentration (VCF = 1). Critical flux was determined as described by Crowley *et al*. (2015).

Table 1. Geometric and hydrodynamic properties of membranes used for filtration of reconstituted skim milk.

Membrane code	**PES1000**	**PVDF0.1**	**PVDF0.45**
Molecular weight cut-off (kDa)[a]	1000	n.a.	n.a.
Pore-size (μm)[a]	n.a.	0.1	0.45
Spacer present[a]	Yes	Yes	Yes
Channel length (m)[a]	0.16	0.16	0.16
Channel height (m)[a]	0.001	0.001	0.001
Channel width (m)[a]	0.04	0.04	0.04
Total active membrane width (m)[a]	0.625	0.625	0.625
Membrane area (m^2)[a]	0.1	0.1	0.1
Number of feed channels[a]	12	12	12
Number of permeate channels[a]	13	13	13
Total hydraulic diameter (m)[b]	0.021	0.021	0.021
Water permeability (L m^{-2} min^{-1} bar^{-1})[b]	1666	2057	2006
Volumetric flow rate (L m^{-2} min^{-1})[b]	3	3	3
Cross-flow velocity (m s^{-1})[b]	0.14	0.14	0.14

[a] values taken from documentation received from, or personal communication with, Merck-Millipore technical representatives

[b] values calculated as described by Crowley *et al*. (2015)

n.a. = not applicable

The filtration system was cleaned fully after each concentration process and critical flux determination using flushing and recirculation steps with water, sodium hypochlorite and phosphoric acid for PVDF membranes and water and NaOH for the PES membrane. The contribution of the membrane, reversible fouling and irreversible fouling to hydraulic resistance were determined for each membrane by resistance-in-series modelling of water flux data at 25°C on clean and fouled membranes, according to Darcy's law (Beckman and Barbano, 2013): viscosity of water was taken as 8.91×10^{-4} Pa.s, water flux was determined at a TMP of 0.1 bar, and a cross-flow velocity of 0.16 m s^{-1} was used. The degree of fouling was determined as described by Beckman and Barbano (2010).

2.3. Composition and physicochemical analyses

Total nitrogen (TN) and non-protein nitrogen (NPN) were determined as per ISO 8968-IDF 20 (Barbano, 2001, parts 1, 4 and 5), using the Kjeldahl method and a nitrogen-to-milk protein conversion factor of 6.38 for calculation of crude protein (CP) and true protein (TP). A HB43-S Halogen rapid moisture analyser (Mettler Toledo GmbH, Schwerzenbach, Switzerland) was used to measure total solids (TS) content. Conductivity was measured at 5°C with a five-ring conductivity measuring cell (Metrohm Ireland Ltd., Carlow, Ireland). Apparent viscosity was measured with a Thermo Scientific HAAKE RotoVisco 1 viscometer (Karlsruhe, Germany) at a shear rate of 300 s^{-1}, using a cup and bob geometry cooled with water at 5°C. A portable refractometer (N1-α, 0-32% Brix, Atago USA Inc., Bellevue, WA, USA) was used to measure the Brix value of the retentate during concentration, in order to estimate changes in solids content.

2.4. Electrophoresis

Qualitative assessment of the protein profile of feed, retentate and permeate streams was performed using electrophoresis. Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970), with minor modifications. Prepared samples were run on pre-cast 4-20% acrylamide tris-glycine gels (Pierce Protein Research Products, Scoresby, Australia) using an AcquaTank mini gel unit (Acquascience, Bellbrook Industrial Estate, Uckfield, UK). SDS-PAGE gels were scanned using a desktop scanner (HP Scanjet G4010, HP, Leixlip, Ireland). All gels were Coomassie-stained.

2.5. Reversed-phase high-performance liquid chromatography

The protein profile of feed, retentate and permeate streams was measured quantitatively using a Varian high-performance liquid chromatography (RP-HPLC) system (Varian Associates Inc., Walnut Creek, CA, USA) comprising an autosampler, a ProStar solvent delivery system with three pumps, and a ProStar programmable multi-wavelength spectrophotometer. The RP-HPLC system was interfaced with a PC with Varian Star Workstation v.6 software package for system control and data acquisition

A Phenomenex Widepore XB-C18 reversed-phase column with 3.6 μm particle size and 250 × 4.6 mm SecurityGuard Ultra UPLC Widepore guard column

for 4.6 mm ID (Phenomenex, Macclesfield, UK) was used for protein separation. The column temperature was maintained at 45°C. Elution was monitored at 214 nm at a flow rate of 1 mL min^{-1} and a mobile phase of two solvents, A, containing 10% acetonitrile (HPLC far UV grade; Labscan Ltd, Blackrock, Ireland) and 0.1% trifluoroacetic acid (TFA, sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Ireland) in deionised water and B, containing 90% HPLC-grade acetonitrile, 9.93% deionised water and 0.07% TFA. The gradient was as follows: linear gradient from 5% to 25% B in 5 min, increasing from 25% to 45% B in 25 min, increasing from 45% to 95% B in 1 min, holding at 95% B for 9 min, and reducing from from 95% B to 5% B in 1 min, followed by 5% B for 40 min.

A solution (pH 7) containing 0.1 M BisTris buffer, 6 M guanidine hydrochloride, 5.37 mM sodium citrate, and 19.5 mM di-dithiothreitol was added to samples (volume such as to contain 10 mg protein) in a 1:1 ratio (v:v) at room temperature. Each sample was incubated for 1 h at room temperature, and diluted 1:1 (v:v) with solvent A. Before injection, samples were filtered through a 0.20 µm filter (MINIsart RC-15, Sartorius AG, Göttingen, Germany) and 40 µL was injected. α_{s2}-Casein was omitted from HPLC analysis due to previously reported difficulties associated with its quantification (Hinz *et al*., 2012).

2.6. Statistical analysis

Results reported are the means of data from three independent trials on separate days, unless stated otherwise. Analysis of variance (one-way ANOVA) of protein profile data from protein peak areas identified by RP-HPLC were conducted using SPSS Version 20.0 for Mac OS X (SPSS Inc., Chicago, IL, USA). When differences were significant ($P < 0.05$), the means were analysed using Tukey's HSD test.

3. Results

3.1. Membrane characteristics and process design

Key geometrical parameters (e.g., membrane area and hydraulic diameter) were identical for the three membranes used, with the membranes differing only in their material of construction and pore-size/MWCO (Table 1). A low cross-flow velocity (0.14 m s^{-1}) was selected to allow operation at minimum TMP to limit the effects of membrane and/or deposit layer compaction (Persson *et al*., 1995), which could alter membrane selectivity.

Measurement of the impact of TMP on permeate flux (Fig. 1) during recirculation of skim milk at <4°C at a cross-flow velocity of 0.14 m s^{-1} showed that PES1000 had the highest critical flux (7.8 L m^{-2} h^{-1} at a TMP of 0.027 bar), followed by PVDF0.1 (5.2 L m^{-2} h^{-1} at a TMP of 0.031 bar) and PVDF0.45 (3.5 L m^{-2} h^{-1} at a TMP of 0.032 bar).

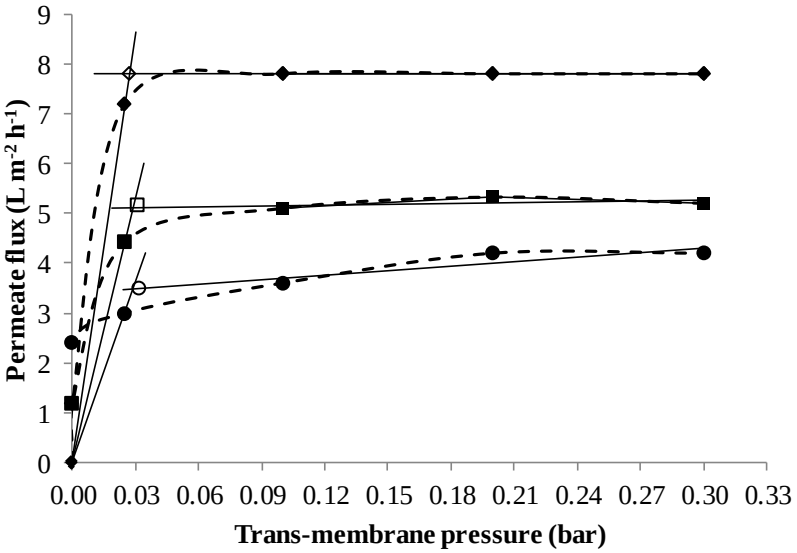

Fig. 1. Permeate flux as a function of trans-membrane pressure during the filtration of reconstituted skim milk at 3.4 ± 0.3°C in full recirculation mode with a 0.1 µm polyvinylidene-difluoride (■), 0.45 µm polyvinylidene-difluoride (●) or 1000 kDa polyethersulfone (◆) membrane. Critical flux values are indicated by open symbols. Results are from a single trial for each membrane.

Critical flux values for all membranes, particularly the PVDF membranes, were substantially lower than the starting flux values which have been reported for microfiltration at 10 (17 L m^{-2} h^{-1}; Lawrence *et al*., 2008;), 21 (14 L m^{-2} h^{-1}; O'Mahony *et al*., 2014) or 50°C (14 L m^{-2} h^{-1}; Beckman and Barbano, 2010) using polymeric membranes, and particularly for microfiltration of skim milk at 50°C

using ceramic membranes (54 L m^{-2} h^{-1}; Hurt *et al*., 2010, 64-72 L m^{-2} h^{-1}; Zulewska *et al*., 2009).

Operation at sub-critical flux was selected for subsequent generation of MF permeates, as this is known to limit the effects of fouling (Bacchin *et al*., 2006). Critical flux data for the membranes did not correlate well with results for water permeability measurements (Table 1), with the lowest water permeability value measured for the PES1000 membrane. This membrane exhibited the highest flux with skim milk as the feed, but exerted the greatest hydraulic resistance against a fluid which did not contain foulants (water). This result suggested that reduced interactions between proteins with the PES1000 membrane compared to the PVDF membranes (possibly due to a lower hydrophobicity of the former) were responsible for the higher flux; indeed, adhesion of proteins to membrane surfaces is known to affect polymeric membranes more than ceramic membranes (O'Mahony and Tuohy, 2013).

3.2. Process performance of membranes during filtration

Across the individual trials with the three membranes, temperature was maintained at 3.4 ± 0.3°C during concentration, while pH was 6.68 ± 0.05. Temperature decreased by 0.4 ± 0.2°C when the process was switched from full recirculation to concentration mode, due to improved efficiency of the heat-exchanger in cooling the reduced volume of feed/retentate on removal of permeate.

Permeate flux declined during concentration with all three membranes (Fig. 2a), which is likely due to a combination of rapid concentration polarisation and gradual fouling (Beckman and Barbano, 2013). This decrease in flux was approximately linear, with R^2 values of 0.92, 0.98 and 0.81 for PES1000, PVDF0.1 and PVDF0.45, respectively. Flux decline (expressed as a percentage of the starting flux) was >40% for PES1000 and PVDF0.1, but <20% for PVDF0.45 membranes.

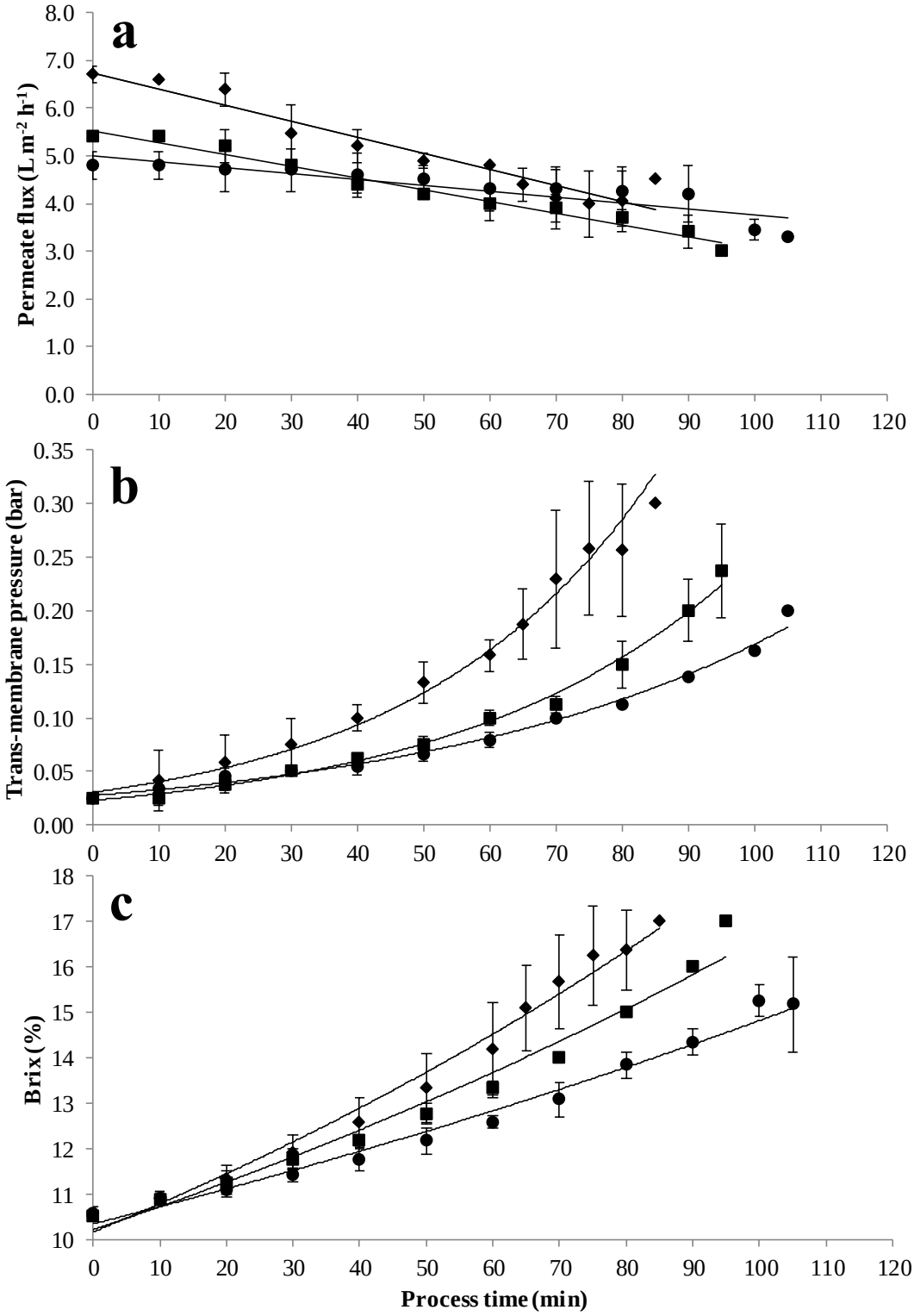

Fig. 2. Permeate flux, trans-membrane pressure and brix as a function of time during the filtration of reconstituted skim milk at 3.4 ± 0.3°C with a 0.1 µm polyvinylidene-difluoride (■), 0.45 µm polyvinylidene-difluoride (●) or 1000 kDa polyethersulfone (◆) membrane. Permeate was continuously removed during filtration to achieve a volume concentration factor of 3. Results are the means ± standard deviations of data from three independent trials.

The decrease in flux for the PES1000 and PVDF0.1 membranes was comparable to values reported by O'Mahony *et al*. (2014), who reported decreases of 40-60% during microfiltration/diafiltration of skim milk with 0.55 µm spiral-wound PVDF membranes. In addition, strong exponential increases in TMP ($R^2 \geq 0.99$; Fig. 2b) and brix values ($R^2 \geq 0.98$; Fig. 2c) were recorded during processing with all three membranes.

The higher starting flux obtained with the PES1000 membrane resulted in a processing time of 80 ± 5 min to achieve a VCF of 3, which was more rapid than both the PVDF0.1 (95 ± 0 min) and PVDF0.45 (98 ± 8 min) membranes. Resistance-in-series modelling (Table 2) confirmed that the formation of a reversible fouling layer (i.e., which could be removed with cleaning agents but not water) was the largest single contributor to overall resistance, in agreement with the results of Le Berre and Daufin (1994). Resistance due to the membrane itself and irreversible fouling (i.e., that not removable by cleaning agents) were comparatively minimal, and their influence decreased further as reversible fouling increased during the latter stages of processing (Table 2). Measurement of degree of fouling from differences in clean and fouled water permeability for two independent trials, where 100% indicates total blockage, indicated that the fouling decreased in the order PVDF0.1 (40.7 ± 9.4%), PES1000 (19.7 ± 0.4%) and PVDF0.45 (3.5 ± 2.7 %). Despite such large differences in fouling, cleaning of the PES and PVDF membranes was effective in maintaining the water permeability at ±20% of the original value of the membrane at first use in all cases.

Table 2. Resistance-in-series modelling of fouling of membranes at 0 and 60 min processing time during filtration of reconstituted skim milk at refrigeration temperatures. Results are the means of data from three independent trials. R_t = total resistance; R_m = membrane resistance; R_r = resistance due to reversible fouling; R_i = resistance due to irreversible fouling.

t ~ 0	R_t (10^{12} Pa)	R_m (10^{11} Pa)	R_r (10^{12} Pa)	R_i (10^{10} Pa)	R_t/R_m -	R_m/R_t (%)	R_i/R_t (%)	R_r/R_t (%)
PVDF0.1	9.7	2.0	9.4	9.8	49.5	2.04	0.99	97.0
PES1000	7.4	2.4	7.1	7.5	30.4	3.35	1.03	95.6
PVDF0.45	8.5	2.0	8.3	0.2	41.5	2.45	0.03	97.5
t = 60	R_t (10^{13} Pa)	R_m (10^{11} Pa)	R_r (10^{13} Pa)	R_i (10^{10} Pa)	R_t/R_m -	R_m/R_t (%)	R_i/R_t (%)	R_r/R_t (%)
PVDF0.1	4.5	2.0	4.4	9.8	228	0.45	0.21	99.3
PES1000	5.8	2.4	5.8	7.5	239	0.42	0.13	99.4
PVDF0.45	2.7	2.0	2.7	0.2	133	0.76	0.01	99.2

3.3. Gross composition and physicochemical properties of process streams

Membrane filtration of skim milk at refrigeration temperatures to a VCF of 3 resulted in increased levels of true protein and solids in retentate fractions and the permeation of a limited quantity of true protein into the permeate (Table 3). As the PVDF0.45 membrane was more porous than the PES1000 and PVDF0.1 membranes, it allowed the permeation of additional protein into the permeate. More than 20% of the total nitrogen in the permeate from the PES1000 and PVDF0.1 membranes was comprised of NPN, while the value was 13% for the higher protein permeate of the PVDF0.45.

After processing, the pH of retentate and permeate fractions were slightly higher than that of the feed (Table 3). Conductivity values of all retentates decreased relative to the feed, while the conductivity of the permeate increased compared to that of the feed, due to depletion and enrichment of minerals in the serum phase of skim milk, respectively. The retentate of the PVDF0.45 membrane had a lower viscosity than the other retentates, due to loss of micellar casein and a lower solids content (Table 3). The presence of micellar material was evidenced by the high turbidity of the PVDF0.45 permeate, as assessed both visually and using analytical centrifugation (results not shown); conversely, the permeates from the PES1000 and PVDF0.1 membranes were clear.

Table 3. Composition and physicochemical properties of individual process streams after filtration of reconstituted skim milk (feed) at refrigeration temperatures using different polymeric membranes. Physicochemical properties were measured at ~5°C. Results are the means ± standard deviations of data from three independent trials.[a]

		Composition			Physicochemical properties		
Membrane	Stream	True protein (%, w/w)	Non-protein nitrogen (%, w/w)	Total solids (%, w/w)	pH (-)	Conductivity (mS cm^{-1})	Viscosity (mPa.s)
All	Feed	3.21 ± 0.04	0.027 ± 0.000	11.5 ± 0.25	6.65 ± 0.01	3.05 ± 0.02	3.20 ± 0.31
PVDF0.1	Retentate	7.76 ± 0.22	0.027 ± 0.001	17.7 ± 0.19	6.71 ± 0.04	2.54 ± 0.01	11.5 ± 0.75
PVDF0.1	Permeate	0.56 ± 0.05	0.026 ± 0.001	7.30 ± 0.15	6.71 ± 0.03	3.14 ± 0.02	1.95 ± 0.16
PVDF0.45	Retentate	6.36 ± 0.86	0.026 ± 0.001	15.9 ± 1.34	6.70 ± 0.00	2.67 ± 0.07	9.00 ± 2.19
PVDF0.45	Permeate	1.22 ± 0.42	0.026 ± 0.001	8.35 ± 0.61	6.65 ± 0.06	3.13 ± 0.04	2.57 ± 0.38
PES1000	Retentate	7.56 ± 0.06	0.026 ± 0.001	18.0 ± 0.33	6.70 ± 0.00	2.52 ± 0.06	13.3 ± 0.81
PES1000	Permeate	0.56 ± 0.01	0.025 ± 0.001	7.28 ± 0.29	6.70 ± 0.04	3.12 ± 0.06	2.08 ± 0.19

3.4. Protein profile of process streams

SDS-PAGE analysis of the process streams showed that β-lactoglobulin and α-lactalbumin, in addition to β-casein, were enriched in the permeate of the PVDF0.1 and PES1000 membranes (Fig. 3). RP-HPLC was also used to quantify individual casein and whey proteins in the membrane streams (Fig. 4); β-casein comprised >97% of the casein protein fraction in the permeates from the PVDF0.1 and PES1000 membranes (Table 4). In comparison, β-casein was reported to have accounted for ~50% of the casein fraction in the permeates generated by Woychik *et al*. (1992) using milk at temperatures between 2-8°C and microfiltration membrane pore-sizes of 0.1-0.2 μm.

Compared to permeates from PVDF0.1 and PES1000 membranes, significantly ($P < 0.05$) higher levels of α_{s1}- and κ-caseins were identified in the permeates of the PVDF0.45 membrane (Table 4), due to permeation of small casein micelles; however, separation using the PVDF0.45 membrane still enriched for β-casein relative to the other caseins (Table 4), due to the high levels of serum-phase β-casein at ≤4°C. Based on true protein (Table 3) and protein profile (Table 4) data, it was calculated that the β-casein content of permeate from the PVDF0.1, PVDF0.45 and PES1000 membranes was 37, 31 and 35 g 100 g^{-1} total protein, respectively. The permeates from the PVDF0.1 and PES1000 membranes were β-casein-enriched SPCs, with markedly higher whey protein:casein ratios than the feed (Table 4). The permeate from the PVDF0.45 membrane also had a higher whey protein:casein ratio than the feed, but the increase was less marked than in the permeates from the other membranes. The generation of a more whey-dominant system is important for first-age IMF applications, where the final product should have a high whey protein:casein ratio similar to that of human milk (Guo, 2014).

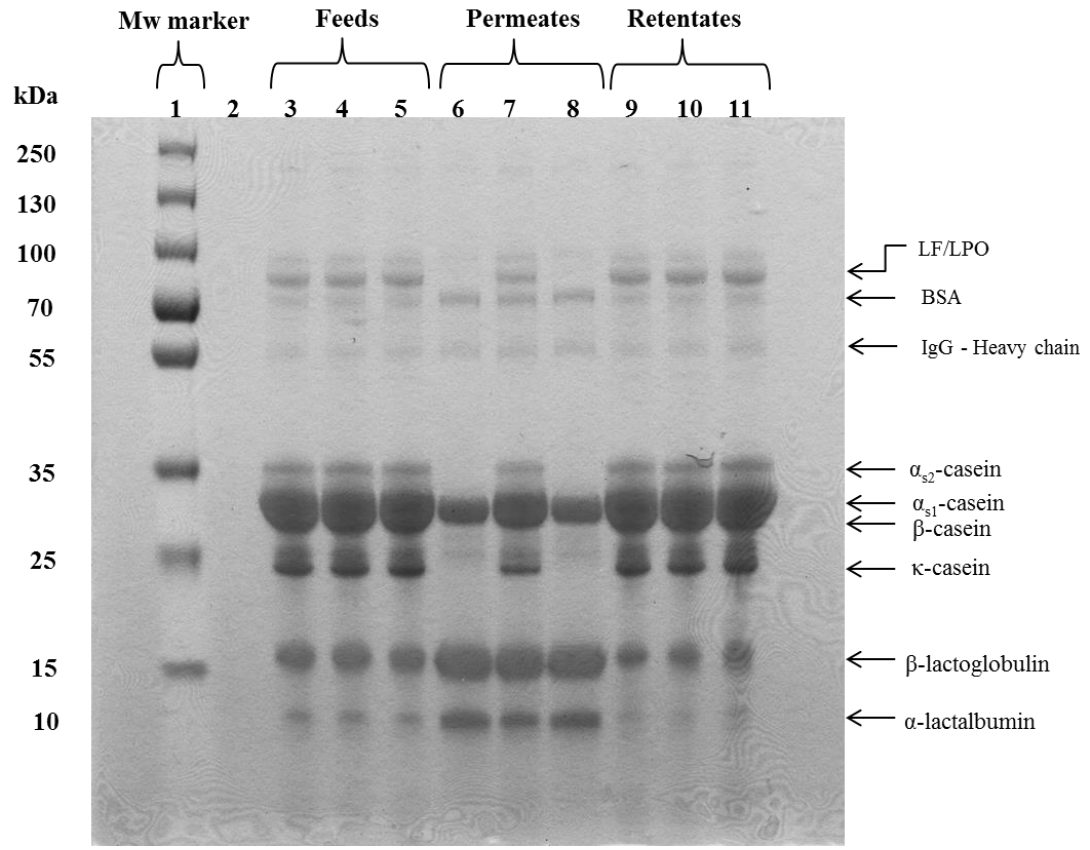

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoretogram of molecular weight marker (lane 1), and feeds (lanes 3, 4, 5), permeates (lanes 6, 7, 8) and retentates (lanes 9, 10, 11) from filtration of reconstituted skim milk at 3.4 ± 0.3°C with 0.1 μm polyvinylidene-difluoride, 0.45 μm polyvinylidene-difluoride or 1000 kDa polyethersulfone membrane (samples from membranes are shown in this order from left to right for individual streams).

The loss of minor whey protein (with MW of ~80 kDa) was observed in permeates generated from the PVDF0.1 and PES1000 membranes, but not for the PVDF0.45 membrane, in which the minor whey protein was enriched relative to the feed (Fig. 3). Based on data from Anema (2009) and the use of internal standards in SDS-PAGE (data not shown), the main high-MW whey proteins were identified as the heavy chain of IgG, BSA and lactoferrin (LF) in order of increasing MW. The results in Figure 3 indicated that LF may be present in substantial quantities in the permeate of the PVDF0.45 membrane, the larger pore size of which facilitated its permeation during membrane filtration, while it was absent or present at only trace levels in permeates from the PES1000 and PVDF0.1 membranes; however, differentiation between LF and lactoperoxidase (LPO) using SDS-PAGE was not possible due to their approximately equivalent MWs (Veith and Reynolds, 2004).

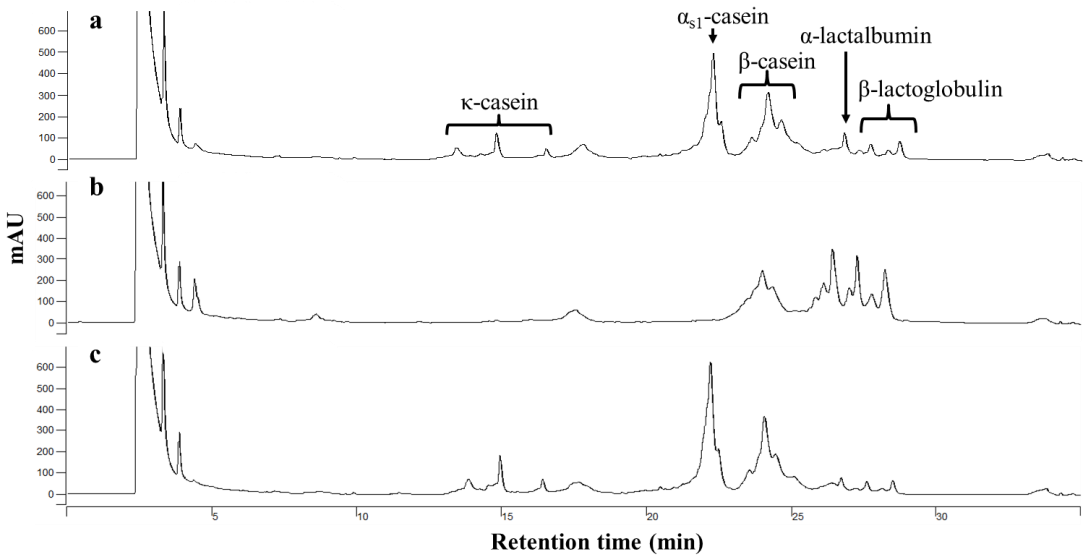

Fig. 4. Chromatograms from reversed-phase high-performance liquid chromatography for (a) feed and (b) permeate and (c) retentate streams from membrane separation of skim milk at 3.4 ± 0.3°C using a 0.1 µm pore-size polyvinylidene-difluoride membrane, showing the identification of peaks associated with proteins of interest.

4. Discussion

Selection of an appropriate membrane is one of the key considerations when designing separation processes for the enrichment of milk proteins, including β-casein. Both PVDF and PES membranes have been used in studies on the enrichment of β-casein using cold membrane filtration, but their performance in terms of both processing and protein-enrichment characteristics has not been compared in substantial detail previously.

In terms of the composition and physicochemical properties (Table 3), and protein profile (Fig. 3, 4; Table 4) of the process streams, the PES1000 and PVDF0.1 membranes are essentially equivalent; however, where these membranes diverge is primarily in their influence on the filtration process itself. The higher flux afforded by the PES1000 membrane facilitated a more rapid separation process (Fig. 1, 2). The former factor cannot be underestimated, as the low flux afforded by cold filtration processes is one of the single biggest factors preventing their uptake at commercial level. Moreover, the PES1000 membrane had the additional benefit of being easier to clean, with most of the clean water permeability being restored by a simple clean with warm water. As the selectivity of the PES1000 and PVDF0.1 was

essentially equivalent, the differences in process performance may be due to factors such as differences in membrane hydrophobicity (O'Mahony and Tuohy, 2013).

Both the PES1000 and PVDF0.1 membranes were suitable for the manufacture of β-casein-enriched SPCs, where 97-100% of total casein consisted of β-casein. Approximately 20% of the total nitrogen in these permeates was comprised of NPN (Table 3), which is similar to the proportions found in human milk; in addition, the permeates had increased whey protein:casein ratios compared to the feed (Table 4). However, β-lactoglobulin was present in all of the SPCs, although it is absent from human milk (Guo, 2014). Thus, levels of β-lactoglobulin would need to be reduced in sufficient quantities in the SPCs produced using the PES1000 and PVDF0.1 membranes for them to be promising ingredients in humanised IMFs (Woychik, 1992). More generally, these SPCs could find use in other food application as, for example, increasing levels of β-casein in whey protein-based ingredients has been shown to improve functional properties such as foaming (Coppola *et al*., 2014).

In contrast to the other membranes studied, the PVDF0.45 membrane allowed some micellar casein to pass into the permeate (Table 4), resulting in a casein fraction in which β-casein comprised only 53%. Interestingly, this membrane displayed certain desirable processing characteristics, such as low flux decline (Fig. 2a) and minimal fouling. The good processing characteristics of this membrane may be due to the loss of small casein micelles into the permeate, as evidenced by high turbidity (results not shown), higher protein and solids content (Table 3) and the presence of high levels of α_s- and κ-caseins (Fig. 3, 4, Table 4); permeates from the tighter membranes only became turbid and sedimentable when incubated at elevated temperatures (i.e., 50°C), due to thermally-induced micellisation of β-casein (results not shown). Reduced numbers of small micelles would result in a reduced polydispersity of the casein micelle population in the retentate, with a concomitant decrease in the propensity for tight packing of micellar material at the membrane surface. Pre-treatment of feeds to lower levels of small casein micelles may be a useful way of reducing fouling and improving membrane performance.

Table 4. Protein profile of individual process streams after filtration of reconstituted skim milk (feed) at refrigeration temperatures using different polymeric membranes determined using reverse-phase high-performance liquid chromatography (RP-HPLC). Note: total whey protein was considered to consist of only β-lactoglobulin and α-lactalbumin. Results are the means ± standard deviations of data from three independent trials.

Membrane	Stream	% of total casein peak area			% of total whey protein peak area		% of total peak area	
		α_{S1}-casein	β-casein	κ-casein	β-lactoglobulin	α-lactalbumin	Casein	Whey protein
All	Feed	46.3	41.4	12.3	67.6	32.4	90.2	9.8
PVDF0.1	Permeate	0.00^{a}	100^{b}	0.00^{a}	69.6^{a}	30.4^{a}	49.2^{a}	50.8^{b}
PVDF0.45	Permeate	36.6^{b}	53.2^{a}	10.2^{b}	70.5^{a}	29.5^{a}	77.7^{b}	22.3^{a}
PES1000	Permeate	0.72^{a}	96.5^{b}	2.74^{a}	68.0^{a}	32.0^{a}	48.3^{a}	51.7^{b}
PVDF0.1	Retentate	48.9^{x}	38.0^{x}	13.1^{x}	69.0^{x}	31.0^{x}	94.2^{x}	5.82^{x}
PVDF0.45	Retentate	48.0^{x}	39.0^{x}	13.0^{x}	66.9^{x}	33.1^{x}	94.1^{x}	5.86^{x}
PES1000	Retentate	47.5^{x}	37.4^{x}	15.1^{x}	71.4^{x}	28.6^{x}	94.4^{x}	5.64^{x}

$^{a-b}$ values for permeates within a column with different superscripts were significantly different ($P < 0.05$).

^{x} values for retentates within a column with different superscripts were significantly different ($P < 0.05$).

For the first time, the effect of membrane processing on the minor, high MW whey proteins during the generation of β-casein-enriched SPCs was also investigated. PVDF0.45 was the only membrane studied in which LF/LPO appeared to be present in substantial quantities in the permeate after filtration of skim milk at refrigeration temperatures, with markedly higher levels compared to permeates from the other two membranes studied (Fig. 3). As previously stated, LF and LPO cannot be differentiated based on MW by SDS-PAGE; however, as levels of LF are several fold higher than that of LPO in bovine milk (de Wit, 1998; Korhonen, 2011; Mills *et al*., 2011), much of the intensity of the LF/LPO band in Figure 3 can be attributed to LF. The effect of membrane type on LF levels during the enrichment of β-casein may have important implications for infant formulae ingredient development. An iron-binding protein, LF is considered to be highly bioactive, with purported antimicrobial, antioxidative, and immunomodulatory functions among others (Korhonen, 2011; Mills *et al*., 2011); in addition, LF is considered an important constituent of the protein profile of human milk, where it is present at much higher levels than bovine milk (Guo, 2014). These factors have led to increasing interest in the use of LF as a fortificant in IMFs (Korhonen, 2011; McCarthy *et al*., 2014). Despite an associated decrease in the purity of β-casein in the casein fraction (Table 4), the finding that LF levels may not be depleted relative to the feed (Fig. 3) supports the potential application of more porous membranes such as PVDF0.45 in the development of β-casein-enriched ingredients for infant formulae. Future studies on SPCs generated using membrane filtration would benefit from additional analysis of the minor whey protein fraction using other powerful techniques for protein profiling (i.e., mass spectrometry).

5. Conclusion

The selection of different membrane materials can have a marked influence on filtration performance at refrigeration temperatures during the enrichment of β-casein from skim milk. A PES membrane with a 1000 kDa MWCO and a PVDF membrane with a 0.1 μm pore-size were both used successfully to enrich β-casein at high casein purity, producing SPCs of equivalent composition, physicochemical properties and protein profile. However, although the selectivity of these membranes was essentially identical, the PES membrane displayed improved flux and low fouling. The 0.45 μm PVDF membrane was not as selective for β-casein, but it

yielded a permeate with a unique protein profile (i.e., increased β-casein:α_s-casein ratio, LF not depleted); in addition, it was the least susceptible to fouling of all the membranes tested, which was hypothesised to be due to a reduced packing ability of the fouling layer caused by loss of small casein micelles to the permeate. This study will aid in the selection of parameters for refrigerated filtration processes for the development of β-casein-enriched ingredients for nutritional applications such as infant formulae.

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CHAPTER 9

Preparation of β-casein concentrates using integrated membrane filtration processes

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. SVC co-designed the pilot plant trials and laboratory experiments, participated in processing, data collection and sampling during the pilot plant trials, conducted lab-scale charged-membrane experiments, performed turbidimetry, conductivity and light-scattering experiments, and analysed all data generated. MM and RK contributed to the design and completion of pilot plant trials. ME and AA provided resources, guidance and assistance with charged-membrane trials. YL analysed process streams and powders for composition.

Abstract

For many decades, processors have separated milk into its two major protein families, casein (CN) and serum (or whey) proteins (SPs). Traditionally, this separation was achieved by precipitating the CN but, in recent years, the use of cross-flow microfiltration (MF) has become more common. A more recent development is the fractionation of an individual CN, β-CN, from milk using MF, although this is at an early stage of development compared to the fractionation of individual SPs (e.g., lactoferrin, α-lactalbumin). To build knowledge in this area, β-CN concentrates (BCCs) and SP concentrates (SPCs) were manufactured from skim milk using two different MF procedures, both of which exploit temperature-induced changes in the association state of β-CN, and can be described briefly as follows: (1) 'warm-then-cold' (WTC) processing, in which skim milk was fractionated into CNs and SPs by warm MF (>25°C), with the CN stream used to enrich β-CN in the cold MF (<5°C) permeate; (2) 'cold-then-warm' (CTW) processing, in which skim milk was processed with cold MF to generate a β-CN-enriched permeate, which was fractionated into β-CN and SPs by warm MF (>25°C). WTC processing generated a BCC with good purity (70% β-CN, protein-basis) and SPCs with very low levels of β-CN contamination (<4%). An optimised CTW process yielded a BCC of the highest purity (~80%) of the processes studied and an SPC with slightly higher β-CN contamination (7%); to achieve these high purity values during the CTW process, ionic strength needed to be carefully controlled during warm MF, at a level sufficient to promote aggregation of β-CN without causing destabilisation.

1. Introduction

Milk is a biological fluid containing protein (~3.2%), which consists of two major fractions, caseins (CNs, ~78% of total protein) and serum proteins (SPs, ~22% of total protein), in addition to fat (~4.5%), lactose (~4.1%) and minerals (ash content of ~0.8%) among many other minor constituents (O'Mahony and Fox, 2013). SPs primarily consist of β-lactoglobulin (β-lg, ~50% of SP) and α-lactalbumin (α-la, ~20% of SP), which are present in soluble form primarily as small monomers and dimers at the natural pH of milk (i.e., pH ~ 6.7), while CNs exist as large colloidal structures known as 'casein micelles'; these two protein fractions can be separated from each other by microfiltration (MF), typically using membranes with pore-sizes of 0.1-0.2 μm (Pierre *et al*., 1992; Saboya and Maubois, 2000; Jimenez-Lopez *et al*., 2008). MF is an alternative approach to traditional methods involving acid-induced (isoelectric) or rennet-induced (proteolytic) precipitation of CN and has the benefit of maintaining CN and SP streams closer to the 'native' states in which they are found in milk; on the other hand, precipitation methods yield insoluble CN fractions and SP streams which are compositionally different to native SP streams, such as the mineral-rich 'acid whey' or glycomacropeptide-containing 'rennet whey' (Carr and Golding, 2016).

Of the four principal CNs in milk, β-CN has attracted the most interest as a potential ingredient. The relative ease with which β-CN can be extracted from milk compared to the other CNs has facilitated efforts to prepare enriched and purified fractions of the protein. β-CN is also known to have excellent techno-functional properties. β-CN has a high interfacial activity, which makes it a good stabiliser of emulsions and foams (Murphy and Fox, 1991, Dauphas *et al*., 2005; Coppola *et al*., 2014; Li *et al*., 2016), while its ability to self-associate has been exploited in the encapsulation of hydrophobic drugs (Shapira *et al*., 2010) and vitamins (Barenholz and Danino, 2014). Development of β-CN ingredients is also a requirement of the infant formula industry, as there is a need to humanise the casein profile of 1st-age formulae (for 0-6 mo infants) by increasing the level of β-CN, which is the major CN in human milk (McCarthy *et al*., 2013). The β-CN-rich micelles of human milk form much softer curds in the stomach compared to those of bovine milk, and it has been demonstrated that increasing the β-CN content of bovine milk results in curd-

forming properties that more closely resemble human milk (Nakai and Li-Chan, 1987).

While just conventional membrane technology is needed, extraction of β-CN from milk using cross-flow filtration is a unique and complex separation process, requiring temperature-induced changes in the association state of β-CN to facilitate its selective fractionation from milk (Christensen and Holst, 2014; O'Mahony *et al*., 2014). Furthermore, it is typically preferred that any changes in the association state of β-CN are reversible, so that its functionality as an ingredient is maintained, which necessitates careful control of factors such as temperature, ionic strength and protein concentration. Other non-membrane approaches based on the extraction of β-CN from rennet-coagulated CN have also been developed (Le Magnen and Maugas, 1992; Huppertz *et al*., 2006), but these methods have several disadvantages, including the generation of an insoluble co-product and proteolysis of β-CN due to residual enzyme activity.

CN micelles contain α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN, in the approximate ratio 4:1:4:1. Two of the major stabilising factors which maintain the structure of CN micelles in milk are hydrophobic interactions between apolar amino acid residues of the CNs and electrostatic bridging between the phosphoseryl residues of CNs mediated primarily by amorphous calcium phosphate nanoclusters (Holt *et al*., 2013). When the temperature of milk is decreased from ambient (20-25°C) the influence of the hydrophobic effect is reduced and the solubility of calcium phosphate is increased, which reduces the strength of these stabilising factors; this effect becomes notable at ~15°C, but becomes progressively more pronounced the closer the temperature is to ≤5°C (Downey and Murphy, 1970; Creamer *et al*., 1977). β-CN, the CN with the greatest number of hydrophobic amino acids and second lowest degree of phosphorylation (O'Mahony and Fox, 2013), is most susceptible to release from the micellar phase when temperature is reduced, which is a key enabler for its enrichment by cold MF (Woychik, 1992; Christensen and Holst, 2014; Glas *et al*., 2014; O'Mahony *et al*., 2014; Crowley *et al*., 2015; Tobin and Verdurmen, 2015). At low temperatures (0-15°C), native CN micelles will be retained by a 0.1-0.5 µm MF membrane, while any β-CN in the serum phase will permeate. In the absence of CN micelles, and at concentrations above its critical micelle

concentration (CMC), β-CN will form its own micelles/aggregates at temperatures sufficient to facilitate hydrophobic interactions (Leclerc and Calmettes, 1997; O'Connell *et al*., 2003; Dauphas *et al*., 2005). The micellisation of β-CN is influenced by temperature and ionic strength. Portnaya *et al*. (2006) reported CMC values measured at 17-28°C for the micellisation of β-CN dissolved in phosphate buffer with 0.1 mM ionic strength (CMC ≈ 0.05-0.25%, w/v) or pure water (CMC ≈ 0.14-0.31%, w/v), with CMC decreasing as temperature increased. In a manner similar to native CN micelles, it has been demonstrated that β-CN micelles can be concentrated using 0.5 μm MF membranes (O'Mahony *et al*., 2014). Increased ionic strength or ionic calcium is known to promote the self-association of β-CN (Dauphas *et al*., 2005; Portnaya *et al*., 2006), but this has not been tested as a method to enhance retention of the protein during MF.

β-CN ingredients can be prepared as enriched or purified fractions. For the purposes of this article the following terminology is used: 'β-CN-enriched' refers to a whey stream with SPs as the dominant protein component but in which the levels of β-CN have been increased relative to the *ca.* 5% β-CN (protein-basis) typical of native whey; 'purified β-CN' refers to a stream in which β-CN comprises the dominant protein fraction, after SPs have been partially depleted from the system. As shown in Figure 1, two main strategies are currently available to obtain purified β-CN using MF: (1) 'warm-then-cold' MF (WTC-MF), in which warm MF (i.e., >25°C) of skim milk is performed to separate micellar CN (retentate) from SPs (permeate) before cold (i.e., <5°C) MF of the micellar CN to create a β-CN-depleted micellar CN stream (retentate) and purified β-CN (permeate); and (2) 'cold then warm' MF (CTW-MF), in which skim milk is subjected to cold MF to generate a β-CN-enriched SP stream (permeate) and a β-CN-depleted micellar CN stream (retentate), followed by warm MF of the permeate to separate β-CN micelles (retentate) from SPs (permeate). In either case, after appropriate down-stream concentration by some combination of MF and ultrafiltration (UF)/diafiltration (DF) and spray drying, several co-products are manufactured: a β-CN concentrate (BCC), a micellar CN concentrate (MCC) and a SP concentrate (SPC).

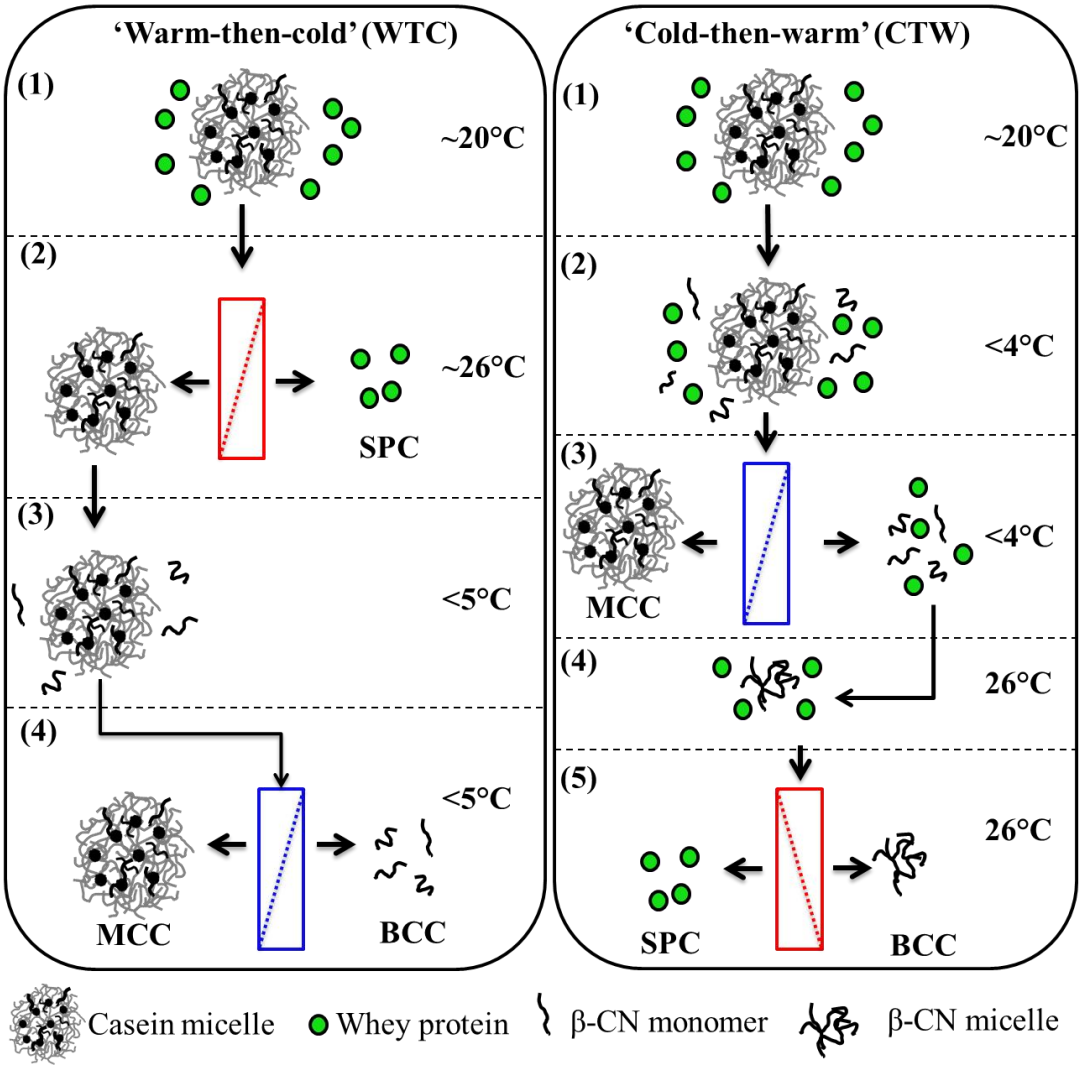

Fig. 1. Schematic representation of the molecular basis underpinning β-casein enrichment and purification from milk using microfiltration. MCC = micellar casein concentrate. BCC = β-casein concentrate.

In either of the approaches outlined in Figure 1, a β-CN-depleted MCC is generated; this type of MCC powder is soluble and has been reported to have modified functionality compared to regular MCCs (O'Mahony *et al*., 2008, 2009; Seibel *et al*., 2014). The properties of these MCC powders will not be the focus of this study. Instead, the primary objective was to compare the two processes based on the purity of the final BCC and SPC materials. MF-based processing of milk has been described in recent studies as generating products with a range of β-CN purities. Enrichment of β-CN using cold MF yielded a product with ~24% β-CN on a protein-basis (Coppola *et al*., 2014), while the incorporation of a warm MF step after cold

MF (i.e., CTW process) resulted in higher purities of ~40% (O'Mahony *et al*., 2014). A version of the WTC process was reported to yield a product which was ~75% β-CN (Christensen *et al*., 2014). The authors are not aware of any studies which have investigated process modifications to increase the purity of both the BCC and SPC fractions prepared from skim milk using two-step MF processes.

There is significant interest in β-CN-enriched SPCs, particularly in relation to applications in infant formula (McCarthy *et al*., 2013; Crowley *et al*., 2015), but more pure BCC-type ingredients are likely to find broader use in emulsification, foaming and encapsulation applications. On the other hand, the behaviour of SPCs during processing and their nutritional profile would be altered by 'contamination' with β-CN, which may be a concern for manufacturers. Previously, Kelly *et al*. (2000) reported on aspects related to the development of integrated membrane processes (MF, UF, DF, and electrodialysis) for the production of whey protein concentrates and MCCs at pilot-scale. There is currently a lack of similar literature on practical approaches to improve the preparation of next-generation dairy ingredients, such as BCCs and their co-products, in kg quantities. In the present study, results from process optimisation experiments for the manufacture of BCCs, SPCs and MCCs at pilot-scale using integrated membrane processes (WTC-MF, CTW-MF, DF, UF, and charged UF) are reported. As a part of this study, novel approaches were developed in attempts to improve the separation of β-CN from SPs, including negatively-charged UF membranes for charge-based fractionation and a DF medium consisting of reduced-strength milk UF permeate to promote β-CN micellisation for enhanced size-based separation by MF.

2. Materials and methods

In all cases in the descriptions below, MF membranes were made from polyvinylidenedifluoride (PVDF). In the majority of cases, the material of the UF membranes was polyethersulfone (PES), with the exception of charged UF membranes, which are described in more detail below. The configuration of the pilot-scale membranes was spiral-wound, while lab-scale membrane filtration experiments were conducted using plate-and-frame technology. In the dilution or DF of filtration streams reverse osmosis (RO) water was typically used, but deionised

(DI) water was used when generation of sufficient volumes of RO water was not feasible. Drying of membrane streams was in all cases carried out with a single-stage spray-drier with nozzle atomisation (PSD 55; APV, Copenhagen, Denmark).

2.1. Influence of membrane pore-size and temperature on protein transmission dynamics

Pasteurised skimmed milk (227 L) was processed with a pilot-scale MF system equipped with two spiral-wound MF membrane elements arranged in parallel, one with 0.08 µm pore-size and the other with 0.20 µm pore-size (Synder Filtration, CA, USA). Each element had 7.06 m^2 of membrane area. The experimental process comprised five distinct stages: (1) *start-up*, in which the system was operated in total recirculation mode (i.e., permeates and retentate returned to feed tank) at 3.5±1.8°C for 89 min; (2) *warming*, in which the system was heated to 26.9°C from 2.7°C at a rate of 0.5°C min^{-1} in total recirculation mode; (3) *fractionation*, in which permeate was removed at 27.1±0.1°C for 120 min with continuous DF using milk UF permeate; (4) *cooling*, in which the system was returned to total recirculation mode and cooled from 27.2°C to 3.2°C at a rate of 0.3°C min^{-1} over 81 min; and (5) *β-CN removal*, in which permeate was removed at 3.5±0.9°C for 198 min with continuous DF using milk UF permeate. Trans-membrane pressure (*TMP*), calculated from feed (P_f), retentate (P_r) and permeate (P_p) pressures according to equation 1, was maintained at 0.65±0.13 bar through appropriate control of feed pump, recirculation pump and a throttling valve on the retentate side.

$$TMP = \left(\frac{P_f + P_r}{2}\right) - P_p \qquad \text{(eq. 1)}$$

During the experiment, permeate flux (J) was recorded and turbidity visually assessed for both permeates using individual rotameters for each membrane element. Intermittent samples of each permeate were taken for analysis of true protein content using mid-infrared (MIR) spectroscopy (Eurofins, Mount View, MN, USA). Select

samples were also analysed for basic composition (i.e., solids, protein) and protein profile by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), as described in Section 2.5. In sections 2.2-2.4, MF denotes the use of the 0.08 µm PVDF membrane.

2.2. Influence of temperature and time on protein transmission during microfiltration of β-casein-rich stream

The cold MF permeate (β-CN-enriched) generated in stage 5 (Section 2.1) was concentrated using UF at 10.5±0.8°C with a single 10 kDa UF element (Synder Filtration, CA, USA) with 7.06 m^2 membrane area. DF of the concentrate was performed with RO water. *TMP* was 3.2±0.4 bar and *J* was 26.1±4.1 L m^{-2} h^{-1} during UF/DF. The UF/DF concentrated solution (38 L) was then subjected to MF under conditions of total recirculation (~1% feed solids) at a *TMP* of 0.67 bar for 104 min, with temperature increasing from 10 to 26°C during the first 72 min, followed by a holding period of 25 min, with permeate samples being taken for protein analysis by MIR spectroscopy. Selected samples were also analysed for composition and protein profile, the latter by SDS-PAGE (see Section 2.5). The purpose of the experiment was to determine the time required to cause a theoretical reduction in the transmission of β-CN to 0%.

2.3. Manufacture of β-casein concentrate using warm-then-cold MF

2.3.1. Trial 1: Initial process

The principle of the WTC-MF process is illustrated schematically in Figure 1 and a process flow diagram is shown in Figure 2.

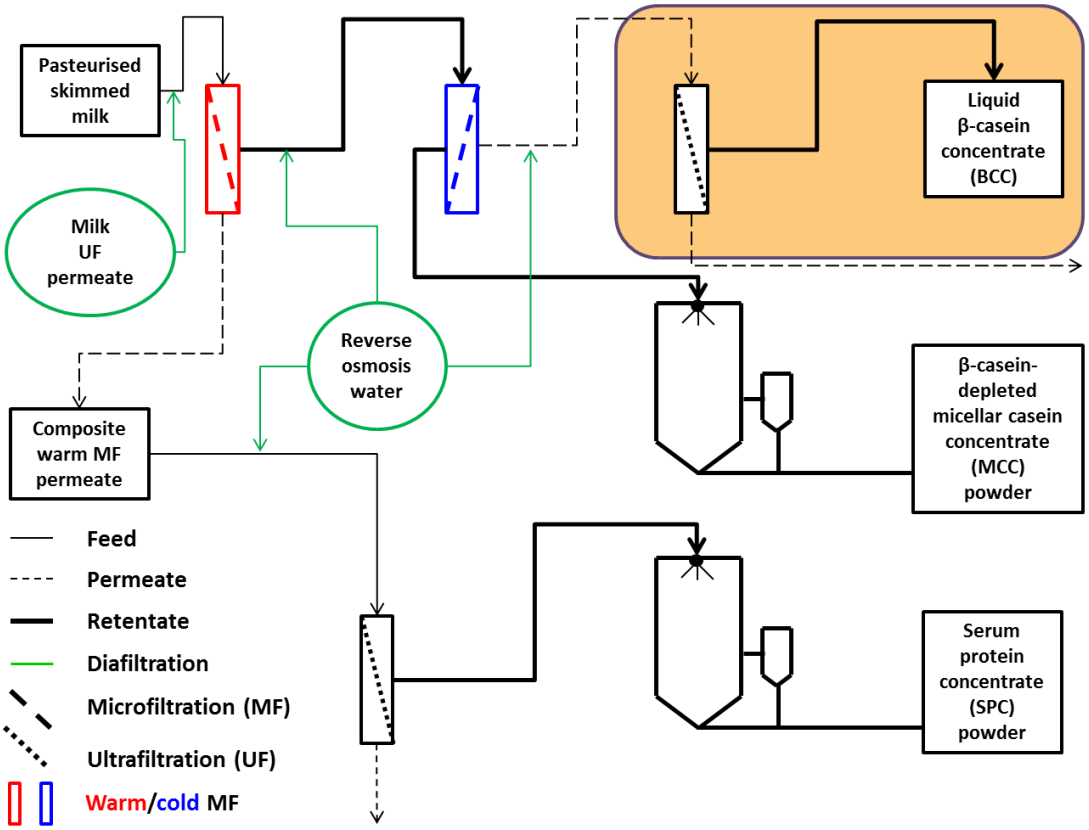

Fig. 2. Process flow diagram for the manufacture of β-casein concentrate using 'warm-then-cold' microfiltration. The orange area indicates where the process was optimised by maintaining cold temperatures to prevent precipitation and incorporating a negatively-charged ultrafiltration membrane step to increase protein purity.

Skim milk (1196 L) was subjected to MF at 26.3±0.3°C with two MF elements arranged in parallel (47.9 m^2 total membrane area) to generate micellar CN (retentate) and SP (permeate) fractions; a 2× VCF was achieved and DF of the MF retentate was performed with milk UF permeate. *TMP* and *J* values were 0.69±0.1 bar and 18.3±0.3 L m^{-2} h^{-1}, respectively, during MF/DF. The micellar CN fraction was processed with MF/DF at 1.1±0.4°C to yield a β-CN-enriched permeate stream. The MF/DF permeate containing purified β-CN stream (38 L) was then processed by UF/DF (using 114 L of RO water) at 13.8±2.9°C to generate a BCC for spray drying. The UF was operated with two 10 kDa membrane elements arranged in series for a total membrane area of 14.1 m^2 and an average *TMP* of 3.75±0.03 bar. During UF of the BCC, temperature was increased from 4 to 20°C to compensate for prohibitively low *J*, but this resulted in precipitation and consequently the BCC could not be spray-dried. An SPC was produced from the SP fraction (the permeate from warm

MF/DF of milk) by UF/DF with RO water at 26±0.4°C using six 10 kDa UF elements with a total membrane area of 49.05 m^2 (*TMP* = 2.69±0.2 bar, *J* = 18.0±1.4 L m^{-2} h^{-1}). Approximately 38 L each of MCC (16% solids) and SPC (18% solids) were both spray-dried with inlet and outlet temperatures of 182-186°C and 80-82°C, respectively. Drying yielded 1.5 kg of MCC powder and 6.4 kg of SPC powder.

2.3.2. Lab-scale testing: fractionation of β-casein/serum protein mixture using charged ultrafiltration membranes

To investigate the potential of charged membranes for the production of purified BCC, a β-CN/SP mixture generated using cold MF of skim milk (see Section 2.4.1) was adjusted to pH 5.5 or 6.0 with 1 N HCl and processed with 100 or 300 kDa molecular weight cut-off (MWCO) viscous (V)-screen Pellicon 2 membrane cartridges (EMD Millipore, Billerica, MA) comprising flat-sheet membranes (0.1 m^2 total membrane area) of composite regenerated cellulose (Ultracel™ PLC) which were chemically modified as described by Arunkumar and Etzel (2015) to have a negative charge. The conductivity of the feed was close to that of milk or milk permeate (8-9 mS cm^{-1}) giving an ionic strength of 80 mM. Fractionation was performed in total recirculation mode using 500 mL of feed maintained at 15°C by holding of the feed vessel in ice. *J* was allowed to reach steady state (24 L m^{-2} h^{-1} for 100 kDa, 36 L m^{-2} h^{-1} for 300 kDa), with feed, retentate and permeate streams being sampled. Each stream was then analysed quantitatively for β-lg and α-la levels using SDS-PAGE using internal standards of those proteins according to the method of Arunkumar and Etzel (2013); sieving coefficient (S_o) values were then calculated for each protein according to their concentration in the permeate (C_p) and retentate (C_r):

$$S_o = \frac{C_p}{C_r} \qquad \text{(eq. 2)}$$

An additional experiment was performed at pH 5.2 using the 300 kDa membrane; however, the very low *J* (6 L m^{-2} h^{-1}) indicated extensive fouling of the membrane,

presumably caused by destabilisation and precipitation of β-CN, so the experiment was not performed with the 100 kDa membrane. Cleaning was performed after each total recirculation experiment according to the procedure described by Arunkumar and Etzel (2013).

2.3.3. Trial 2: Optimised process (incorporation of low-temperature concentration and charged ultrafiltration step)

The process as outlined in Figure 2 was repeated in modified form based on results of trials described in Section 2.3.1 and 2.3.2: Firstly, skim milk (1,662 L) was concentrated to a VCF of 2× before DF with milk UF permeate (MF conditions: temperature = 25.8±0.6°C, *TMP* = 0.69±0.03 bar, total membrane area = 48 m^2) to generate a micellar CN stream depleted in SPs. The micellar CN (332 L) was then used as feed for a cold MF process (MF conditions: temperature = 3.13±0.47°C, *TMP* = 0.61±0.03 bar, total membrane area = 48 m^2), with continuous DF using 265 L of milk UF permeate.

The retentate from the cold MF process was concentrated from 5.9% to 7.6% total solids using UF operated at 4.65±0.66°C (to prevent protein destabilisation) and continuous DF with milk UF permeate (to maintain constant ionic strength). UF was carried out with two 10 kDa membrane elements arranged in series for a total membrane area of 14.1 m^2 and an average *TMP* of 2.69±0.68 bar. The retentate from the UF process (i.e., liquid BCC) was then adjusted to pH 5.4±0.1 (to promote SP removal during charged membrane filtration based on results of experiments described in Section 2.3.2) by slow addition of 0.27 kg 35-38% HCl. The acidified liquid BCC was then processed using a 100 kDa regenerated cellulose UF membrane (Microdyn-Nadir GmBH, Wiesbaden, Germany), with a total membrane area of 7.0 m^2, modified to have a net negative charge as described by Arunkumar and Etzel (2015). *TMP* and *J* during charged UF were 1.04±0.35 bar and 12.9±0.5 L m^{-2} h^{-1}, respectively, while the filtration temperature was 5.62±2.30°C. Permeate was removed from the charged UF membrane for ~60 min before continuous DF commenced with RO water until permeate solids reached <0.5%. The resultant liquid BCC (13 L) was spray-dried at 8.5% solids (inlet temperatures 189-191°C, outlet temperatures 79-80°C) to yield 1.09 kg of BCC.

2.4. Manufacture of β-casein concentrate using cold-then-warm microfiltration

2.4.1. Trial 1: Initial process (warm microfiltration at low ionic strength)

The process used for the preparation of β-CN from skim milk was essentially as described by O'Mahony *et al*. (2014), according to the concepts illustrated in Figure 1 and the process flow diagram shown in Figure 3.

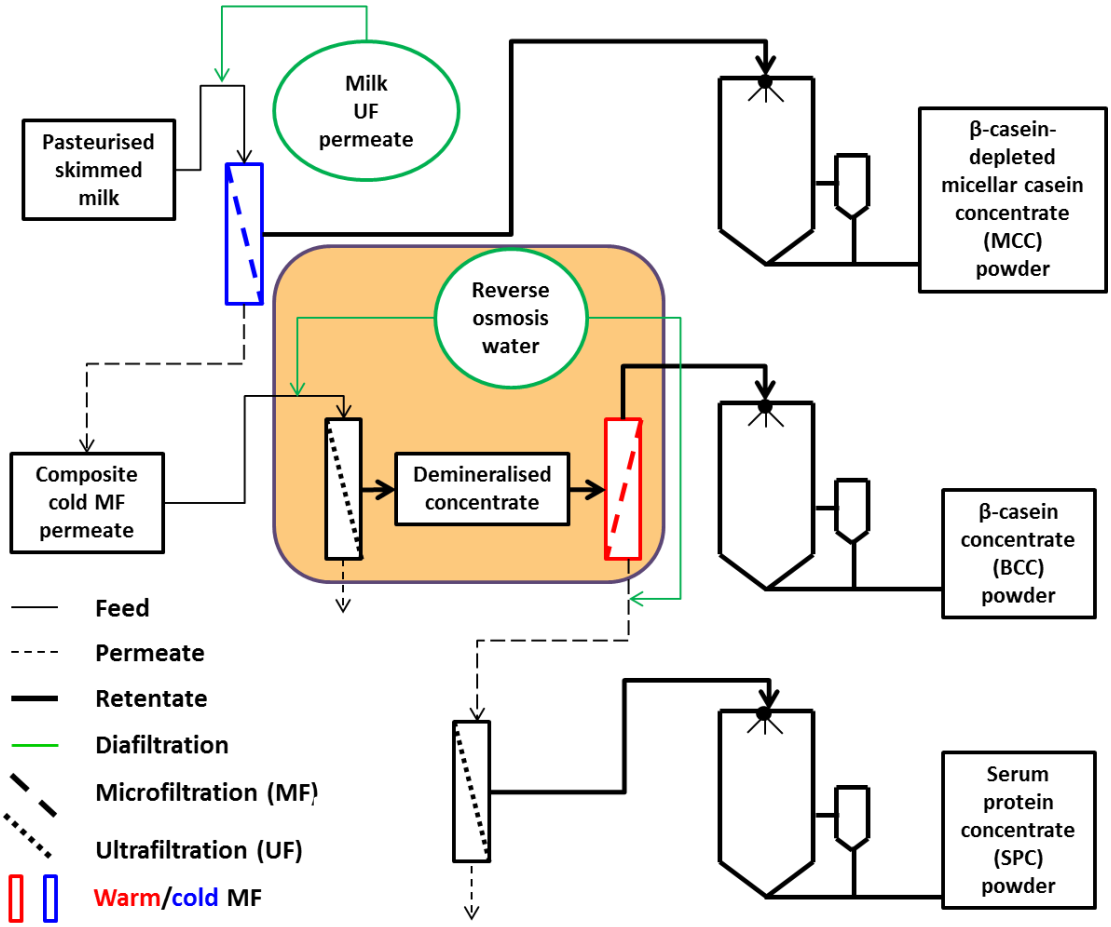

Fig. 3. Process flow diagram for manufacture of β-casein concentrate using 'cold-then-warm' microfiltration. The orange area indicates where the process was optimised through fractionation of β-casein from serum proteins using warm MF (without concentration) at a controlled ionic strength of ~32 mM.

A pore-size of 0.08 µm was selected both for 'cold MF' and 'warm MF', which was much smaller than the 0.5 µm pore-size used in the previous study. In this study, MF was performed with two MF elements arranged in parallel for 47.9 m^2 total membrane area. Cold MF was carried out at 1.5 ± 0.2°C, 0.69 ± 0.02 bar and a *J* of 8.6 ± 2.4 L m^{-2} h^{-1}), with skim milk (2362 L) concentrated to a 2× VCF and then subject to DF with milk UF permeate (1942 L) to generate a β-CN/SP mixture (3471 L composite permeate).

The cold MF permeate was processed using four UF elements with a 10 kDa MWCO and a total membrane area of 32.7 m^2. UF was carried out at 5.9 ± 0.27°C (2.43 ± 0.98 bar, *J* of 13.2 ± 2.4 L m^{-2} h^{-1}) until retentate solids had increased from 7 to 12%, after which UF/DF was performed at 19.1 ± 0.8 using 659 L of RO water as the DF medium until permeate solids approached 0.5%. At this point, a sub-sample of the β-CN/SP mixture (~2 L) was taken after UF (but before DF) and sodium azide (0.02%) was added before storage at ~4°C for the lab-scale charged membrane experiments described in Section 2.3.2 and β-CN micellisation/aggregation experiments described in Section 2.4.2. The UF retentate was then diluted using 114 L with RO water from 9.8 to 4.5% total solids, giving a solution estimated to have <5% the ionic strength of milk UF permeate, at which point sub-samples could be heated indefinitely at 90-100°C without any visual indication of precipitation (Coppola *et al*., 2014).

After holding at 26°C for ~90 min, the diluted UF concentrate (363 L) was subjected to warm MF (temperature = 25.9 ± 1.6°C, *TMP* = 0.6 ± 0.0 bar, *J* = 33.2 ± 2.9 L m^{-2} h^{-1}) to purify β-CN by depleting SPs. Warm MF was stopped when the solids content of the permeate decreased from 2.5% to 0.0%, which indicated that SPs were no longer being removed in substantial quantities. The BCC fraction (warm MF retentate) was spray-dried at 11.6% solids (inlet temperatures 177-183°C, outlet temperatures 82-83°C) without further processing; as it was being generated, the warm MF permeate was continuously supplied to a UF process (7.0 m^2 membrane area, temperature = 30°C ± 3.0°C, *TMP* = 3.2 ± 0.1 bar, *J* = 36.8 ± 11.0 L m^{-2} h^{-1}) for concentration to 21.8% solids prior to drying (inlet temperatures 184-187°C, outlet temperatures 80-81°C). The MCC generated during cold MF was also spray-dried at 10.3% solids (inlet temperature 183-186°C, outlet temperatures 81-82°C). During the

drying process, 10.8 kg of MCC, 6.3 kg of SPC and 3.2 kg of BCC were produced from 129 L, 34 L and 30 L of liquid feed, respectively.

2.4.2. Lab-scale testing: influence of ionic strength on the aggregation of β-casein

The UF-concentrated β-CN/SP mixture generated in Section 2.4.1 was diluted to 1.2% protein using different ratios of milk UF permeate and DI water (0:100, 20:80, 40:60, 60:40, 80:20, 100:0) to attain different ionic strengths. The 1.2% protein systems (all at pH 6.8) were then transferred in 30 mL volumes to 50 mL plastic centrifuge tubes and incubated at 26±0.5°C for 30 min; subsequently, the incubated samples were removed from the water bath and transferred to 30 mL glass tubes for analysis of turbidity using a nephelometer (Hach, model 2100N). The samples were then stored at 4°C and analysed for any irreversible destabilisation after ~18 h. Based on these experiments, the optimal ionic strength of the DF medium for stable aggregation of β-CN during its fractionation by warm MF was estimated.

2.4.3. Trial 2: Optimised process: Warm microfiltration at controlled ionic strength

The process (Fig. 3) described in Section 2.4.1 was repeated with some major modifications, particularly in relation to the warm MF step. Cold MF and warm MF involved the use of two 0.08 µm MF membrane elements arranged in parallel with 47.9 m^2 total membrane area. A β-CN-enriched SP stream was generated as the permeate during cold MF of skim milk (1181 L) to a VCF of 3×, at which point DF with RO water (568 L) was performed until permeate solids reached ~3.0%. MF/DF was performed at a temperature of 2.8°C ± 0.3°C, a *TMP* of 30.7 ± 0.0 bar and a *J* of 7.3 ± 1.8 L m^{-2} h^{-1}. The MF/DF permeate was fed to a UF process consisting of two membrane elements with a total membrane area of 14.1 m^2 (*TMP* = 2.2±0.5 bar, *J* = 24.0±5.3 L m^{-2} h^{-1}), which proceeded at 6.8 ± 1.3°C until total solids in the permeate reached 2.5% solids. This permeate was estimated to have ~40% of the ionic strength of milk UF permeate and was used as the DF medium during subsequent warm MF/DF.

The UF-concentrated cold MF permeate was warmed at 26°C for 90 min prior to warm MF/DF, in which the 40%-strength milk UF permeate was used as a continuous DF medium. The MF was conducted at 26.8 ± 0.7°C, a *TMP* of 0.60±0.04 bar and a *J* of 48.1±2.4 L m^{-2} h^{-1}) using a single element with a membrane area of 7.0 m^2. During MF, feed solids did not increase due to the sufficiently high rate of DF, which reduced the risk of β-casein precipitation caused by over-concentration. Warm MF/DF was continued until the total solids of the MF permeate stabilised at a value of 2.8%, which was close to the UF permeate solids (2.5%), indicating that protein was no longer being removed in substantial quantities. During warm MF/DF, the permeate being generated was fed to a UF process, and the UF permeate (also 2.5% solids) was recycled back into the warm MF/DF feed; after warm MF/DF, the UF feed was subjected to DF with DI water until permeate solids decreased from 2.5% to 0.2%, before it was concentration to >20% solids for spray drying into an SPC powder. The retentate from the warm MF/DF process was also fed to a UF process, in which DF was carried out to reduce permeate solids to 0.5%, before solids were increased to 7.0% for spray drying into a BCC powder. UF of SPC and BCC streams was performed with a single UF element with a membrane area of 7.0 m^2. Regular sampling of the warm MF/DF feed for turbidity (using nephelometry) and particle size (using dynamic light-scattering (Malvern Zetasizer nano ZSP, Malvern Instruments, Worcestershire, UK) analysis was performed to investigate the association state of β-CN and its influence on fractionation performance. From 26.5 L of liquid BCC, 2.3 kg of powder was spray-dried, while 2.9 kg of powder was spray-dried from 15 L of liquid SPC.

2.5. Analysis of composition and physicochemical properties

Analysis of crude and true protein, total solids and total CN were as per standard methods (AOAC, 2003). SDS-PAGE was carried out according to Shapiro *et al*. (1967). Tabulated protein values represent crude protein unless otherwise indicated; this value over-represents protein in systems with high non-protein nitrogen levels (e.g., skim milk, MF permeate of milk), but was found to be in close agreement with true protein values in concentrated or purified fractions. The SDS-PAGE described in Section 2.3 is a modified technique for quantitative

determination using laser densitometry, and has been described in detail elsewhere (Arunkumar and Etzel, 2013, 2015).

In-process true protein values were analysed using MIR spectroscopy by an external laboratory (Eurofins, Mount View, MN, USA). The MIR method is specific for protein (does not measure NPN) and is considered independent of the amino acid composition of the milk protein (Dupont *et al*., 2013); in addition, the ratio of MIR protein values to Kjeldahl protein values for SPC and BCC samples of ~80% purity approached unity in both cases (data not shown). Quantification of individual milk proteins was performed with reversed phase-high-performance liquid chromatography (RP-HPLC); samples were prepared according to Bobe *et al*. (1998) with RP-HPLC analysis carried out as per the conditions described by Bonfatti *et al*. (2008).

Turbidity was analysed in 30 mL glass tubes using a nephelometer (Hach, model 2100N) with a tungsten light-source and a detector placed at 90° to the incident light. Particle size was measured in diluted (1:10) samples after filtering (0.22 µm) by dynamic light-scattering using a Malvern Zetasizer nano ZSP (Malvern Instruments, Worcestershire, UK). Data collection and analyses were performed using DTS (Nano) software (Version 5.02; Malvern Instruments). Analysis of composition and physicochemical properties was performed in at least duplicate with mean values reported.

2.6. Calculation of contamination, purity and yield values

The mass of β-CN in BCC and SPC powders was calculated from CN nitrogen and HPLC data according to the following equation:

$$Mass\ of\ \beta - CN\ (g) = Total\ CN\ in\ powder\ (g) \times \frac{\beta - CN\ (\%\ of\ CN\ peak\ area\ )}{100} \quad \text{(eq. 3)}$$

These values were used to calculate contamination of the SPC, purity of the BCC and yield of β-CN in the BCC powders. For the purpose of this study, β-CN was

considered a 'contaminant' of the SPC stream (i.e., SPs defined the purity of that ingredient); contamination was therefore calculated as:

$$Contamination\ (\%) = \frac{Mass\ of\ \beta-CN\ in\ SPC\ (g)}{Mass\ of\ protein\ in\ SPC\ (g)} \times 100 \quad \text{(eq. 4)}$$

On the other hand, β-CN content determined the purity of the BCC:

$$Purity\ (\%) = \frac{Mass\ of\ \beta-CN\ in\ BCC\ (g)}{Mass\ of\ protein\ in\ BCC\ (g)} \times 100 \quad \text{(eq. 5)}$$

Yield was calculated on a β-CN basis, with only the BCC powders considered:

$$Yield\ (\%) = \frac{Mass\ of\ \beta-CN\ in\ BCC\ (g)}{Mass\ of\ \beta-CN\ in\ skim\ milk\ (g)} \times 100 \quad \text{(eq. 6)}$$

For the yield calculations, β-CN was considered to account for 35% of the CN in the skim milk (O'Mahony and Fox, 2013) used to manufacture a given BCC.

The amount of milk required to produce 1 kg of β-CN (as a proportion of a given BCC) was also calculated:

$$Milk\ needed\ for\ 1\ kg\ \beta-CN\ (L) = \frac{Volume\ of\ milk\ processed\ (L)}{Mass\ of\ \beta-CN\ recovered\ in\ BCC\ (kg)} \times 1 \quad \text{(eq. 7)}$$

3. Results and discussion

The results of screening experiments to determine the influence of membrane pore-size and β-CN dissociation/association dynamics are discussed first in this section, before the two principal processes (WTC and CTW) are described separately. In their respective sections, results of an initial trial (WTC1, CTW1) are

reported for both processes, followed by lab-scale experiments aimed at establishing a method to optimise the process and finally the results of the optimised pilot-scale process (WTC2, CTW2). Compositional data of select liquid streams and all powders generated during all pilot-scale trials are shown in Table 1 for comparison.

Certain key membrane steps were repeated in different trials which allowed assessment of their reproducibility. For example, cold MF of skim milk had a standard deviation for J which was <7% of the mean (n = 2), while warm MF of skim milk had a standard deviation for J which was <1% of mean J (n = 2), indicating both processing steps were highly reproducible. In the remainder of the text, values are derived from single experiments, deemed representative of their respective processes.

Table 1. Composition of skim milk (n = 2), and microfiltration retentates (MFRs) and microfiltration permeates (MFPs) generated at <4 °C during the manufacture of β-casein concentrate (BCC), micellar casein concentrate (MCC) and serum protein concentrate (SPC) using 'warm-then-cold' (WTC) or 'cold-then-warm' (CTW) MF processes in different trials (1, 2). MFR/MFPs were produced directly from skim milk or from microfiltered milk during CTW and WTC processing, respectively.

	Solids (%, w/w)	**Protein** (%, w/w)	**Protein** (%, solids)	**Casein** (%, protein)	**β-CN** (%, protein)
Skim milk	8.81	3.24	36.3	77.5	27.1 *
WTC1					
Cold MFR	9.61	4.39	45.7	90.9	-
Cold MFP	5.76	0.38	6.60	42.1 (74.8)	-
MCC (powder)	96.4	57.8	60.0	93.1 (93.8)	-
SPC (powder)	95.3	57.7	60.5	4.02 (4.10)	< 4
BCC (liquid) ~	2.61	0.68	26.1	57.4 (60.5)	-
WTC2					
Cold MFR	10.5	5.29	50.4	93.6	-
Cold MFP	6.68	0.89	13.3	56.2 (70.1)	-
MCC (powder)	Not manufactured during this trial				
SPC (liquid)	0.53	0.18	34.0	5.56 (4.71)	-
BCC (powder)	93.9	89.4	95.2	74.4 (74.5)	70.4
CTW1					
Cold MFR	10.3	4.76	46.2	87.2	-
Cold MFP +	10.1	4.23	41.9	36.9	-
MCC (powder)	96.7	49.6	51.3	90.5	-
SPC (powder)	95.1	93.0	97.8	19.1	14.3
BCC (powder)	95.5	92.4	96.8	77.1	73.3
CTW2					
Cold MFR	7.95	5.55	69.8	96.6	-
Cold MFP	3.05	0.84	27.5	42.8	-
MCC (powder)	Not manufactured during this trial				
SPC (powder)	94.8	90.5	92.0	10.8	6.91
BCC (powder)	94.5	87.1	90.0	83.0	80.2

Note: casein values in parentheses were calculated on a true protein basis

* values estimated based on established mean values for milk

~ precipitated during concentration; data representative of solution after precipitate removal.

+ permeate was concentrated by ultrafiltration

3.1. Screening of microfiltration membranes and measurement of temperature-dependent protein transmission dynamics

Values for *Cp* and *J* during total recirculation/permeate removal experiments with 0.08 and 0.20 µm pore-sizes are shown in Figure 4. The experimental results can be divided into five main stages as follows:

I: *Total recirculation*; β-CN and SPs were present in the serum phase of skim milk at <5°C, and both types of protein permeated the membrane;

II: *Warming*; transmission of protein through the membranes decreased due to thermally-induced association of β-CN with the micellar phase. *J* increased due to increased diffusion of solutes at the higher temperature;

III: *CN-SP fractionation*; marked decreases in protein levels in the permeate occurred over time, as large quantities of SPs were removed to the composite permeate, while CN micelles were retained. *J* began to decrease due to fouling;

IV: *Cooling*; recovery of protein levels in the permeate was measured as levels of transmittable β-CN (i.e., present in the serum phase of the feed) increased due to the reduced strength of hydrophobic interactions. *J* decreased further due to reduced diffusion of proteins at the low temperature;

V: *Micellar CN-β-CN fractionation*; hysteresis of *J* compared to start-up (i.e., <100 min) occurred due to fouling of the membrane during the experiment. Fluctuating levels of β-CN in permeate are noted, probably due to cyclical re-equilibration of β-CN between micellar and serum phases to replenish serum-phase β-CN removed in the permeate according to the following relationship:

$$\beta - \text{CN release from micelle} \leftrightharpoons \beta - \text{CN removal in permeate}$$

Figure 4 demonstrates that more protein permeated the 0.20 µm membrane than the 0.08 µm membrane during MF of skim milk, with a higher turbidity also

visually observed (Fig. 4, rotameter images). Kjeldahl nitrogen determination revealed that the 0.20 µm permeate contained 33% CN (true protein-basis), which was higher than the 32% measured for the 0.08 µm permeate. As micellar CN is the dominant light-scattering component in milk protein systems, the increased CN content and turbidity of the 0.20 µm permeate strongly indicated loss of CN micelles through the membrane (Jimenez-Lopez *et al*., 2008). Differences in protein transmission between the membranes became less obvious in the later stages (Fig. 4), likely due to the formation of a fouling layer which began to influence the separation process; indeed, fouling was evidenced by hysteresis of *J*. Based on these data, the 0.08 µm membrane was selected for further trials; the reasons for this selection were two-fold: (1) to minimise micellar contamination of MF permeate streams generated from skim milk or micellar CN (during both WTC and CTW processes) and (2) to achieve maximum retention of β-CN micelles during warm MF (during the CTW process).

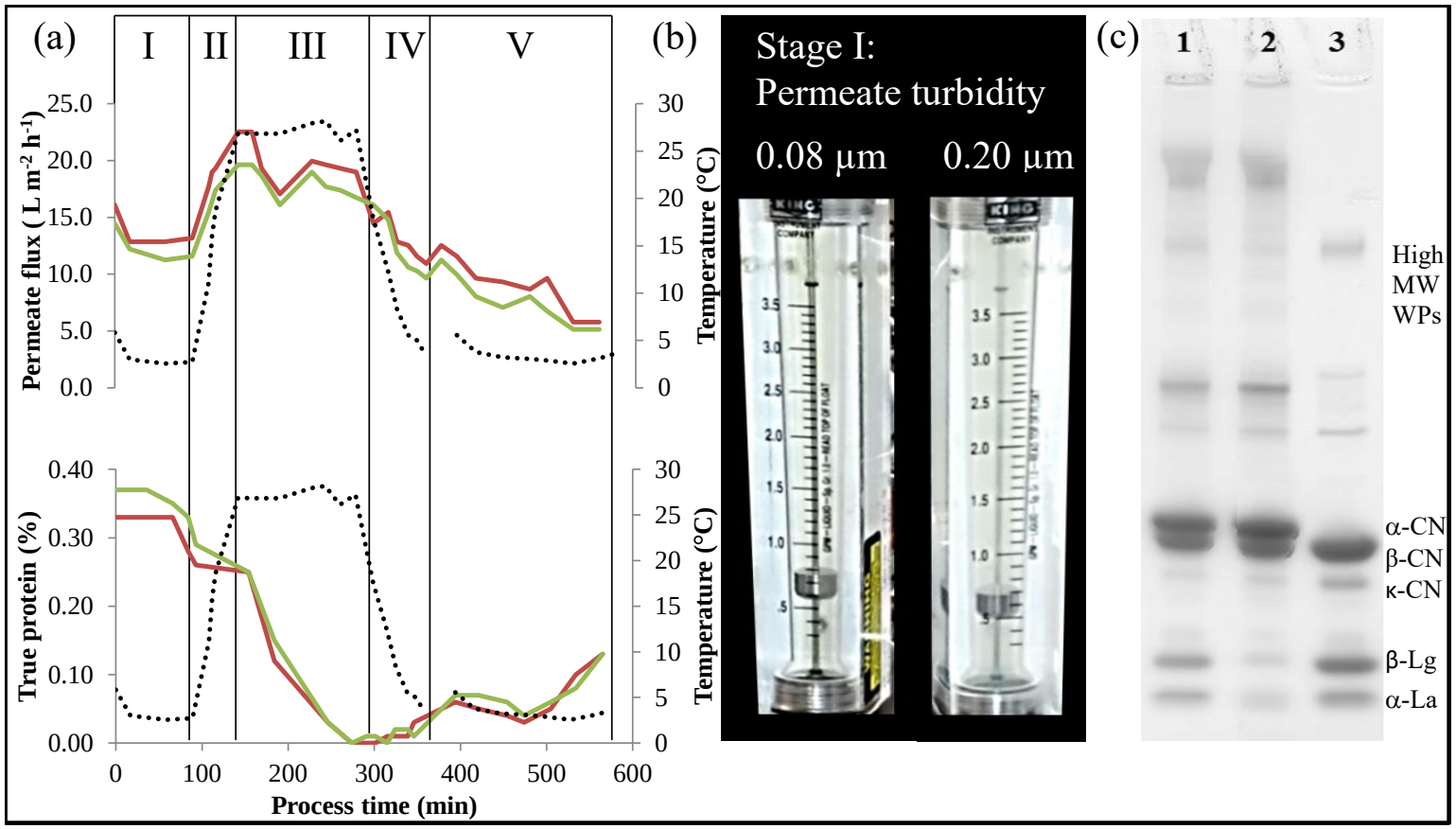

Fig. 4. (a) True protein and permeate flux during experiments using microfiltration (MF) membranes (0.08 µm, —, and 0.20 µm, —) under warm (>25°C) and cold (<5°C) conditions. Depending on the stage, the MF process was operated in total recirculation or permeate removal mode. Temperature is indicated by a broken black line. Images of cold MF permeate during stage I are also pictured (b); obscuration of the float in the rotameter indicates higher turbidity in the permeate of the 0.20 µm membrane. (c) SDS-PAGE gel showing: 1, skim milk used as feed in stage I; 2, MF retentate generated in stage III; 3, β-casein concentrate generated in stage V.

Figure 5 shows the relationship between *Cp* with temperature for a 0.82% protein BCC solution produced through UF of the permeate collected from the 0.08 and 0.02 µm membranes during stage V, followed by exhaustive DF with RO water to <0.2% total solids in the permeate. In this experiment, the BCC solution was recirculated only through the 0.08 µm MF element. *Cp* decreased during warming from 10 to 20°C, decreasing more slowly thereafter during heating to and holding at 26°C. O'Connell *et al*. (2013) demonstrated that self-association of β-CN with an increase in the size and number of micelles was dictated by the increase in temperature within the range 10-25°C. Given that SPs do not undergo aggregation in the temperature range, the reduction in *Cp* seen in Figure 5 must be due to β-CN micellisation. As 50% of the protein in the BCC solution was comprised of CN, >90% of which was β-CN (SDS-PAGE; data not shown), the decrease in the level of transmittable protein by 50% (assuming 100% transmission of monomers and dimers at ≤5°C) was taken to indicate maximal retention of β-CN micelles by the membrane. These data suggest that holding times of 60-90 min at 26°C are sufficient to attain a high degree of β-CN retention during warm MF.

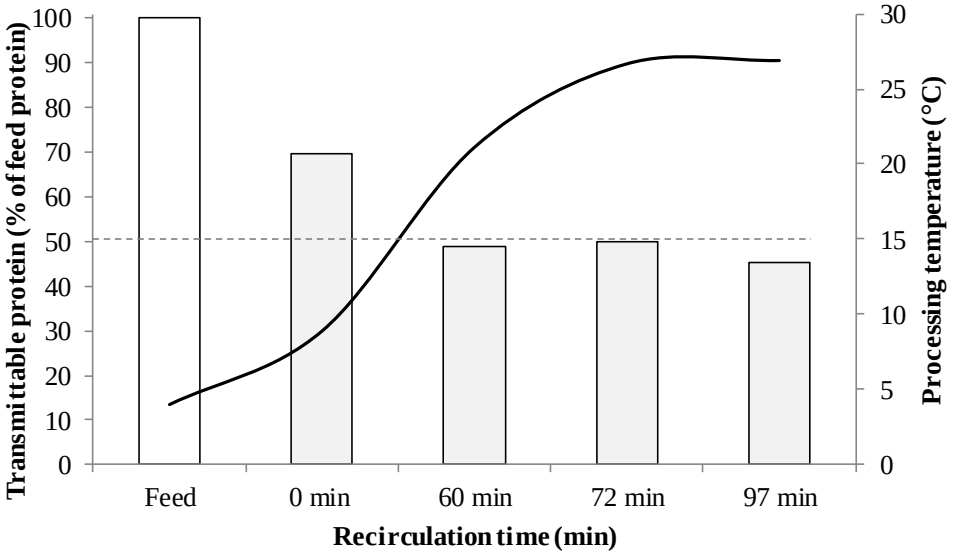

Fig. 5. True protein content of permeates (bars) during total recirculation experiments where a liquid β-casein concentrate solution, containing 0.82% total protein (0.41% total casein), white bar, was subject to a temperature ramp from 5 to 26°C (curve). The protein content of the starting feed (white bar) was compared to that of permeate samples (grey bars) to determine the influence of β-casein aggregation on changes in transmittable protein. The broken line is included to guide the eye, with a reduction in transmittable protein of ~50% considered to coincided with maximal retention of β-casein during microfiltration.

3.2. Manufacture of β-casein concentrate using warm-then-cold microfiltration process

In the first WTC trial (WTC1), the aim was to extensively deplete SPs from skim milk using warm MF prior to fractionation of β-CN from the micellar phase using cold MF. The cold MF permeate was highly enriched in CN (Table 1), giving a β-CN purity of ~71.2% based on SDS-PAGE (data not shown). During the concentration of this stream by UF, a temperature increase to ~20°C caused precipitation of β-CN, resulting in a decrease in *J* from 21 to 13 L m^{-2} h^{-1} and a further decrease to <10 L m^{-2} h^{-1} when temperature was reduced to 15°C in an attempt to prevent further precipitation. Approximately 19% of the CN was lost as precipitate (Table 1) and the BCC was not spray-dried as a result. The SP stream generated by warm MF of milk was concentrated and spray-dried into an SPC with a very low degree of contamination by β-CN (<4%, Table 1).

To investigate potential methods of increasing the purity of β-CN in BCCs manufactured using the WTC process, lab-scale fractionation of a β-CN/SP mixture using negatively-charged UF membranes was performed. In the pH range studied (5.2-6.0), all three proteins were negatively charged; however, due to differences in their respective isoelectric points, the net negative charge varied (α-la > β-CN > β-lg), while MW of the proteins decreased in the order β-CN > β-lg > α-lac. Measured S_o values (Table 2) indicated that protein fractionation was dominated by MW; this is supported by data showing that increasing the MWCO of the membrane from 100 to 300 kDa resulted in greater permeation of SPs (Table 2).

In all experiments, β-CN could not be detected in any of the permeate samples, and was thus assumed to have been fully retained (S_o = 0), whether processed at 4 or 15°C, while the SPs permeated; moreover, no isoelectric precipitation was visually observed in permeates adjusted to pH 4.6 using 1 N HCl, indicating an absence of CN.

Table 2. Sieving coefficient (S_o) values for major protein fractions during fractionation with negatively-charged UF membranes with MWCOs of 100 and 300 kDa at lab-scale. Experiments conducted at 15°C used freshly generated β-casein-enriched serum protein concentrate as the feed solution, while those conducted at 5°C used thawed β-casein-enriched serum protein concentrate to which 3 g L^{-1} whey protein isolate was added.

pH (-)	**Temperature** (°C)	**100 kDa S_o values** (-)			**300 kDa S_o values** (-)		
		β-CN	α-La	β-Lg	β-CN	α-La	β-Lg
5.2	15	-	-	-	0	0.33	0.07
5.5	5	0	0.10	0.04	-	-	-
5.5	15	0	0.21	0.03	0	0.20	0.12
6.0	5	0	0.06	0.02	-	-	-
6.0	15	0	0.21	0.03	0	0.22	0.10

In the second WTC trial (WTC2), based on S_o data generated at lab-scale (Table 2), a charged UF step was incorporated to increase the purity of β-CN in the enriched stream. During this process, the concentration of proteins in the cold MF permeate by UF was maintained at low temperatures to prevent precipitation of β-CN. After processing with charged UF (also performed cold) a BCC was dried that had a higher CN purity (75%) than to the cold MF permeate (70%). This result indicated that removal of SPs during charged UF increased the purity of the resultant BCC, although the purity value was close to that of the liquid BCC in the WTC1 trial: as determined by HPLC, the BCC had a β-CN purity of 70.4% (Table 1), which was also close to the values reported by Christensen and Holst (2014), who used a similar WTC-based process. Although an SPC was not dried in this trial, the liquid SPC again had a very low degree of CN contamination (<5%, Table 1).

3.3. Manufacture of β-casein concentrate using cold-then-warm microfiltration process

In Section 3.2, processes were described in which micellar CN was first fractionated from skim milk, with the micellar fraction then used as a starting material from which β-CN could be purified by cold MF. In this section, processes are described in which skim milk was first processed with cold MF to generate a β-

CN-enriched SP stream, which was then fractionated into SPC and BCC streams using warm MF, as first proposed by O'Mahony *et al*. (2014).

In Table 1, the results of the first CTW process (CTW1) trial are shown. Cold MF of skim milk created a β-CN-enriched SP stream. After exhaustive DF, followed by dilution, with RO water to an ionic strength estimated to be <5 mM, this material was adjusted to 26°C for fractionation using warm MF. Warm MF under these conditions yielded a BCC powder with high β-CN purity (~73%) after drying, which is slightly higher than the purities obtained with the WTC process and also represents a considerable improvement on the value of ~40% reported by O'Mahony *et al*. (2014). The higher BCC purity compared to the value reported by O'Mahony *et al*. (2014) was probably due to a combination of factors, including higher β-CN enrichment during cold MF due to the lower temperatures (2-3°C compared to 3-6°C) and greater β-CN retention during warm MF owing to the tighter membranes used (0.1 µm compared to 0.5 µm). Micellar β-CN has a particle size of ~30 nm (O'Connell *et al*., 2013), which is much smaller than the 150-200 nm characteristic of native micellar CN (O'Mahony and Fox, 2013); thus, the required pore-size for retention of β-CN micelles is smaller.

The SPC from the CTW1 process was contaminated with 14% β-CN (Table 1), which indicates that micellisation of β-CN under these conditions (i.e., low ionic strength) was sub-optimal for achieving purity values for the SPC fraction close to those obtained with the WTC process. To determine the optimal ionic strength to promote micellisation of β-CN, the β-CN/SP mixture generated during cold MF and concentrated by UF in the CTW1 trial was incubated at 26°C after dilution in different ratios of milk permeate and deionised water. As expected, a strong linear relationship existed between conductivity and the concentration of milk permeate (Fig. 6, inset). In Figure 6 (main), it can be seen that increased ionic strength resulted in higher turbidity caused by some combination of an increase in the size, number and/or density of β-CN micelles. When conductivity was between 1 and 4 mS cm^{-1}, changes in turbidity were completely reversed by refrigeration, indicating that the micellisation was thermo-reversible (Fig. 6).

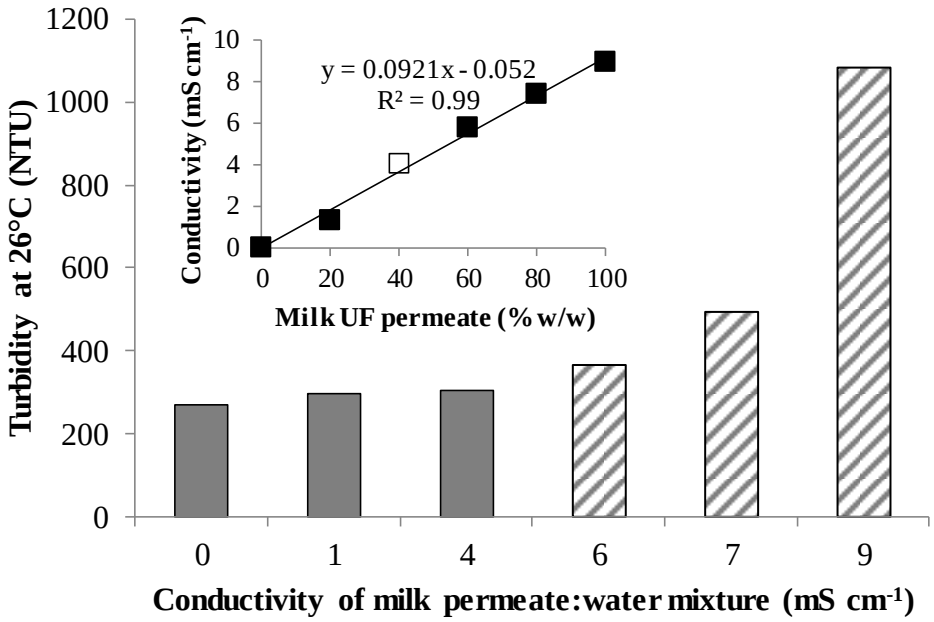

Fig. 6. Turbidity/stability of a β-casein-enriched serum protein concentrate diluted to different ionic strengths (as measured by conductimetry) using different ratios of milk ultrafiltration permeate and deionised water. Solid grey bars indicate that no irreversible destabilisation occurred, while bars with white lines indicate that samples were observed to have precipitated after holding at 4°C for 16 h. Inset shows the relationship between conductivity (representative of ionic strength) and concentration of milk permeate in permeate/water mixtures; the open box is the optimal ionic strength for promoting micellisation without destabilisation.

At high ionic strengths, where milk permeate exceeded 40% strength, the solutions formed a sediment after cooling, indicating that a precipitation process had been initiated during warming which could not be reversed by cooling (Fig. 6). Based on the results in Figure 6, the CTW process was modified to incorporate a warm MF step (again at 26°C) in which ionic strength was maintained at 40% of that of milk permeate (~32 mM), and protein levels were maintained at ~1.2% in an attempt to prevent precipitation due to concentration effects. In the optimised process (CTW2), the purity values of both the BCC and the SPC were increased (Table 1), due to the increased retention of β-CN by the MF membrane. Before warm MF, β-CN existed as monomers of <10 nm in diameter at <5°C, which resulted in minimal turbidity (Fig. 7); at 26°C, particles with a mean diameter of ~30 nm (characteristic of β-CN micelles; see O'Connell *et al*., 2013) were formed, which remained stable at a constant size during the entirety of the warm MF run.

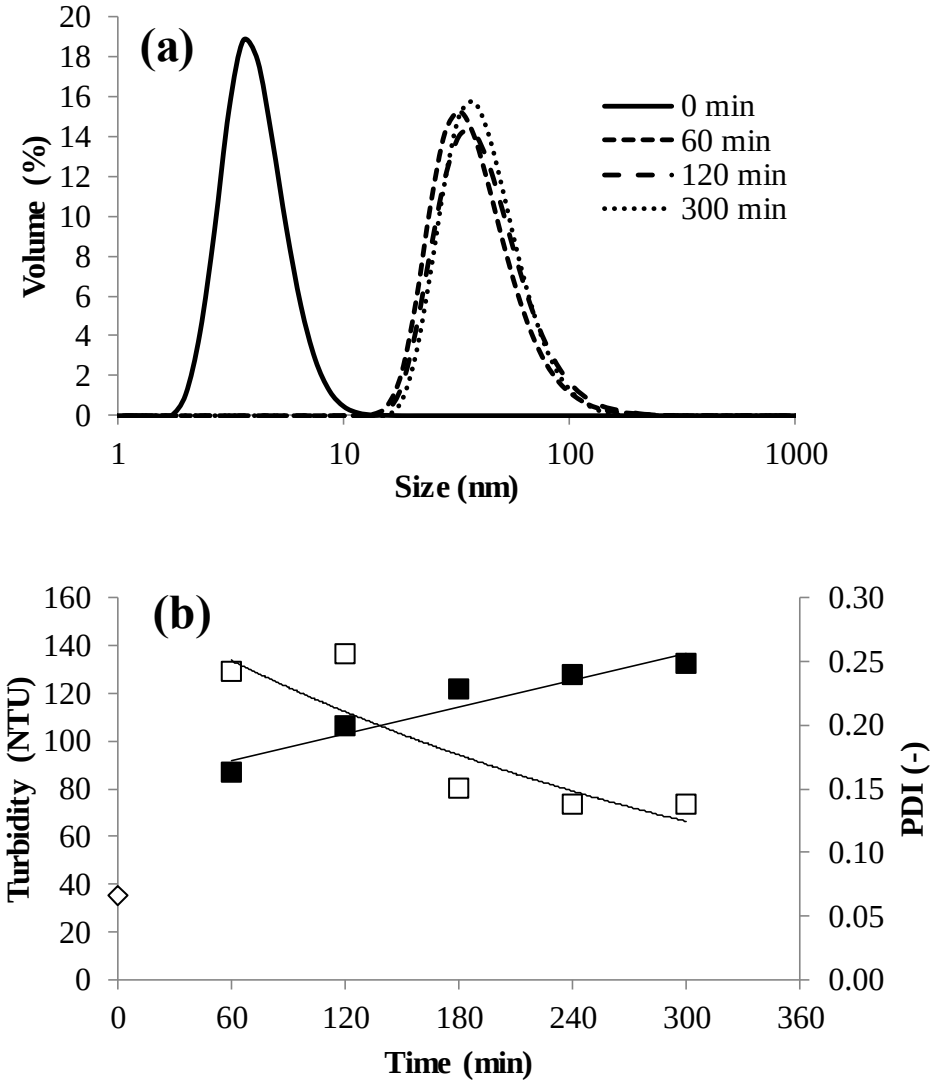

Fig. 7. (a) Particle size distribution and (b) turbidity values (closed boxes) and polydispersity index (PDI) values (open boxes) of β-casein monomers at <10°C (0 min) and β-casein micelles during microfiltration at 26°C. Time-points are omitted for particle size data for clarity of presentation, but the results shown are representative of the overall trend.

Interestingly, a trend of increasing turbidity in the feed during warm MF was observed; as SP was being removed and mean particle size was largely unchanged during the process (Fig. 7), the increased turbidity was likely due to concentration of β-CN micelles during warm MF, which would explain the decreasing PDI of the particle population (Fig. 7), i.e., the micellar phase began to dominate the particle population.

As predicted by lab-scale experiments, β-CN remained stable during warm MF at a controlled ionic strength (~32 mM). Ionic strength was held constant

through continuous DF with 40%-strength milk UF permeate. The feed for the warm MF process consisted of 1.26% total protein with 0.46% total CN, in close agreement with the 1.20% protein and 0.44% casein system studied at lab-scale. However, as SPs were being depleted continuously during warm MF, and total solids decreased slightly from 4.4% to 3.9% during the run, potential fluctuation in the levels of β-CN in the feed were possible. As the solids content of the serum-phase during warm MF can be considered to be equivalent to the DF medium (i.e., 2.5% total solids), the proportion of solids comprised of protein in the feed during warm MF could be estimated as follows:

$$Total\ protein\ in\ MF\ concentrate\ (\%) = Total\ feed\ solids - DF\ solids \quad \text{(eq. 8)}$$

Based on eq. 8, the total protein at the end (202 min) of warm MF was estimated at 1.40%, a marginal increase from the 1.26% protein in the feed.

The β-CN purity (80.2%) of the BCC powder (Table 1) could be used to calculate the β-CN solids in the concentrate at the end of the warm MF run, as the UF-concentration step which was applied to the MF concentrate before drying would not be expected to alter the protein profile:

$$\beta - \text{CN in MF concentrate}\ (\%) = 1.4 \times 0.802 \quad \text{(eq. 9)}$$

This calculation indicates that levels of β-CN increased from 0.46% to 1.12% during the warm MF run, with this increase likely being responsible for the increased turbidity and reduced PDI (Fig. 7). That the concentration of β-CN more than doubled during warm MF without causing destabilization at the ionic strength studied suggests that warm MF with higher concentrations of β-CN in the feed may be worth investigating as an approach to increase BCC/SPC purity further.

3.4. Comparison of permeate flux, protein transmission and yield of the processes studied

Values for *J* and *Cp* during the cold and warm MF steps for both processes are compared in Figure 8. The major factors influencing *J* during filtration of different milk protein systems with a common membrane type are temperature, protein type and fouling layer characteristics. Native CN micelles are the largest protein particles in milk and are highly hydrated (becoming more so at lower temperatures), making them the dominant contributor to viscosity and fouling when present in significant quantities (Gésan-Guiziou, 2013). The WTC process (Fig. 8a, b) is characterised by two low-J MF steps, the first (warm MF of milk) because micellar CN comprises 78-95% of the protein and the second (cold MF of MCC) because micellar CN comprises ~95% of the protein and the temperature is <5°C. The CTW process (Fig. 8c, d) is characterised by only one low-J step (cold MF of milk), caused again by the predominance of micellar CN and the low temperature; on the other hand, the fractionation of β-CN from SPs is a high-J step, due to the absence of native micellar CN, the dilute nature of the system and the high temperature.

In both BCC processes, two principal co-products are generated, MCC and SPC. Comparing protein transmission during warm MF and cold MF of milk in Figure 8, it is evident that SP removal is markedly more efficient in the former, which is responsible for the slightly higher CN purity in the MCC produced using the WTC process compared to the CTW process (Table 1). Despite this efficient removal of SP during warm MF of milk, the BCC produced using the WTC process had the lowest purity (70%), indicating that appreciable levels of SP were still being removed during cold MF. The SPC generated during the WTC process was, however, characterized by a lower level of contamination than the CTW process (Table 1). Yield and purity data are both shown in Figure 9. To produce an equivalent quantity of β-CN, the optimised CTW process was estimated to require 56% of the skim milk needed for the CTW1 process and 28% of the skim milk needed for the WTC2 process (Fig. 9). The optimization sequences for the two BCC manufacturing processes (i.e., WTC and CTW) are summarised in Figure 10.

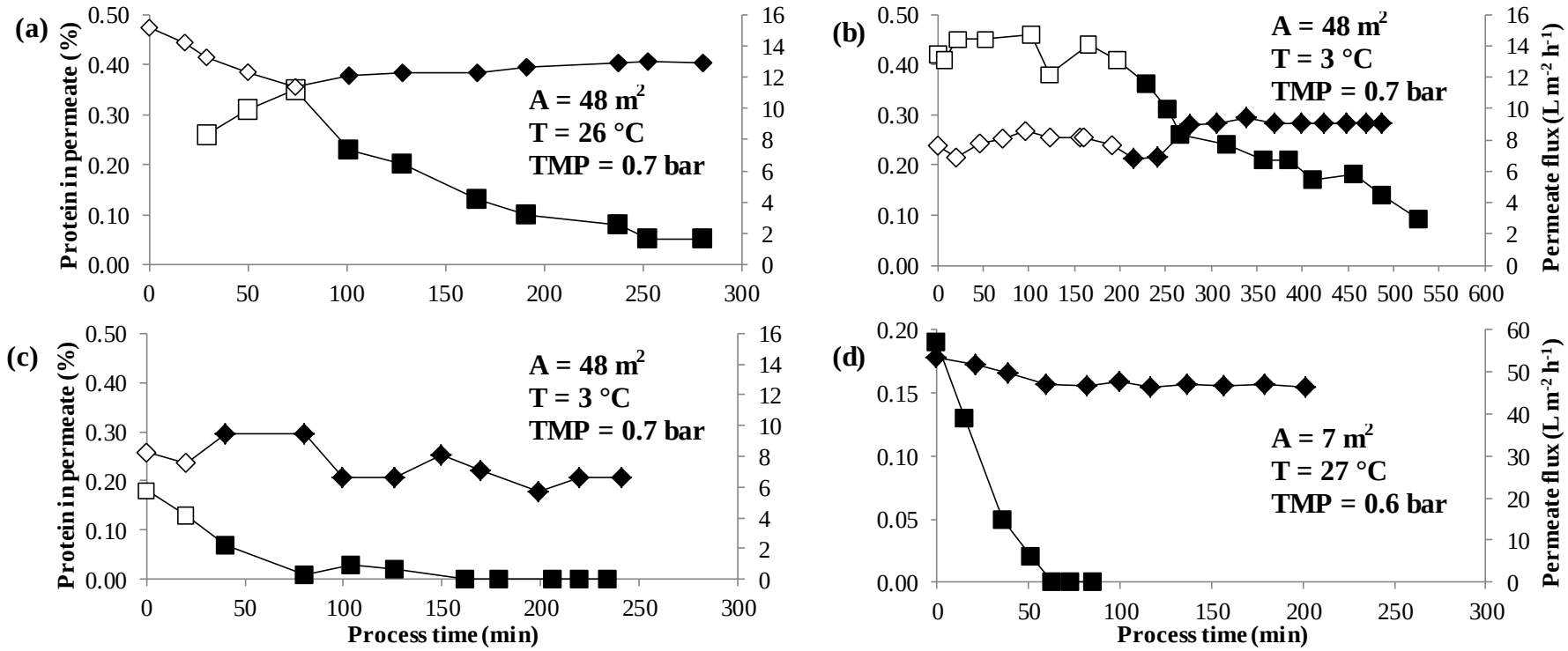

Fig. 8. Protein content of permeates (squares) and permeate flux values (diamonds) during steps in the 'warm then cold' process; (a) warm MF of skim milk, (b) cold MF of skim milk, and the 'cold then warm' process; (c) cold MF of micellar casein concentrate and (d) warm MF of cold MF permeate from skim milk. Where present, open symbols indicate the concentration phase, in which permeate was removed until a volume concentration factor of 2 was achieved. Closed symbols indicate DF, in which milk UF permeate was continuously added during permeate removal. PVDF membranes of 0.08 µm pore-size were used in all cases. A = membrane area, T = feed temperature, TMP = trans-membrane pressure.

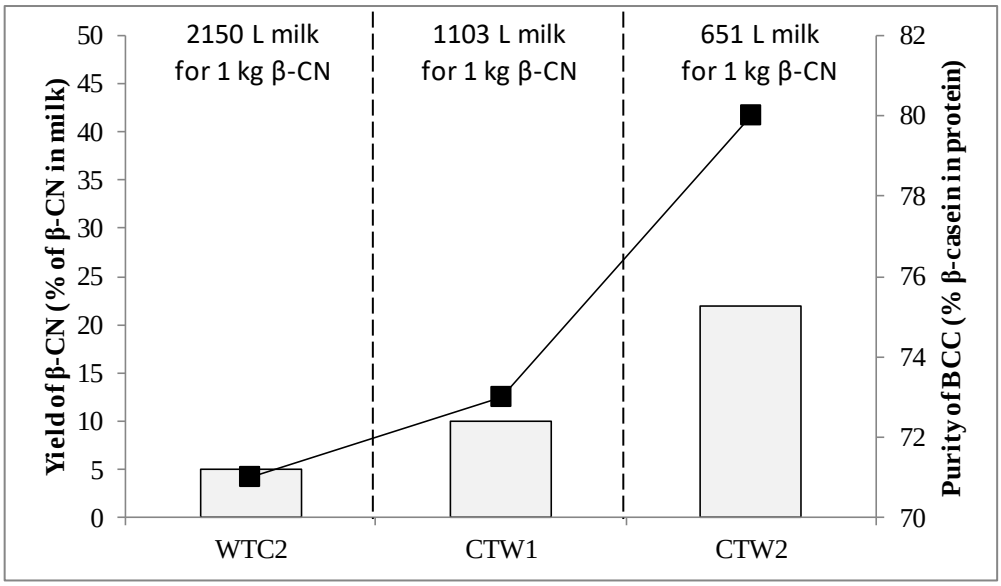

Fig. 9. Protein yield (bars) and purity (line) data for the three trials in which BCC powders were manufactured. These data were used to calculate the amount of skim milk required to produce 1 kg of β-casein (inset).

4. Conclusions

Two processes were described for BCC manufacture: (1) WTC; in which skim milk was fractionated into micellar CN and SPC streams, with the CN fraction used to generate BCC by cold MF, and (2) CTW; in which skim milk was processed with cold MF, with the resultant β-CN-enriched permeate being used to generate BCC and SPC by warm MF. Use of the micellar CN feed in the WTC process has the advantage of generating an SPC co-product which is low in CN (i.e., close in composition to a traditional whey protein concentrate); however, improving the purity of β-CN by this process is difficult, as β-CN monomers permeate the MF membrane along with residual SPs. Negatively-charged UF membranes showed promise as a method to increase the purity of BCCs manufactured by this approach without requiring controlled aggregation of β-CN. The CTW process affords greater flexibility for controlling the purity of BCC and SPC streams. By using 40% strength milk permeate as the DF medium during warm MF, β-CN formed stable aggregates which could be fractionated to yield and ingredient with 80% purity if ionic strength was maintained at a sufficiently high level; in addition, the CN levels in the SPC co-product were reduced substantially compared to an equivalent process in which warm MF was performed at low ionic strength. To the knowledge of the authors, the

purity of this BCC is the highest reported for a pilot-scale membrane-based process for fractionating milk. The BCC in question is a unique ingredient, analogous to a milk protein isolate in that it retains the CN:SP ratio of milk, but with β-CN replacing native micellar CN.

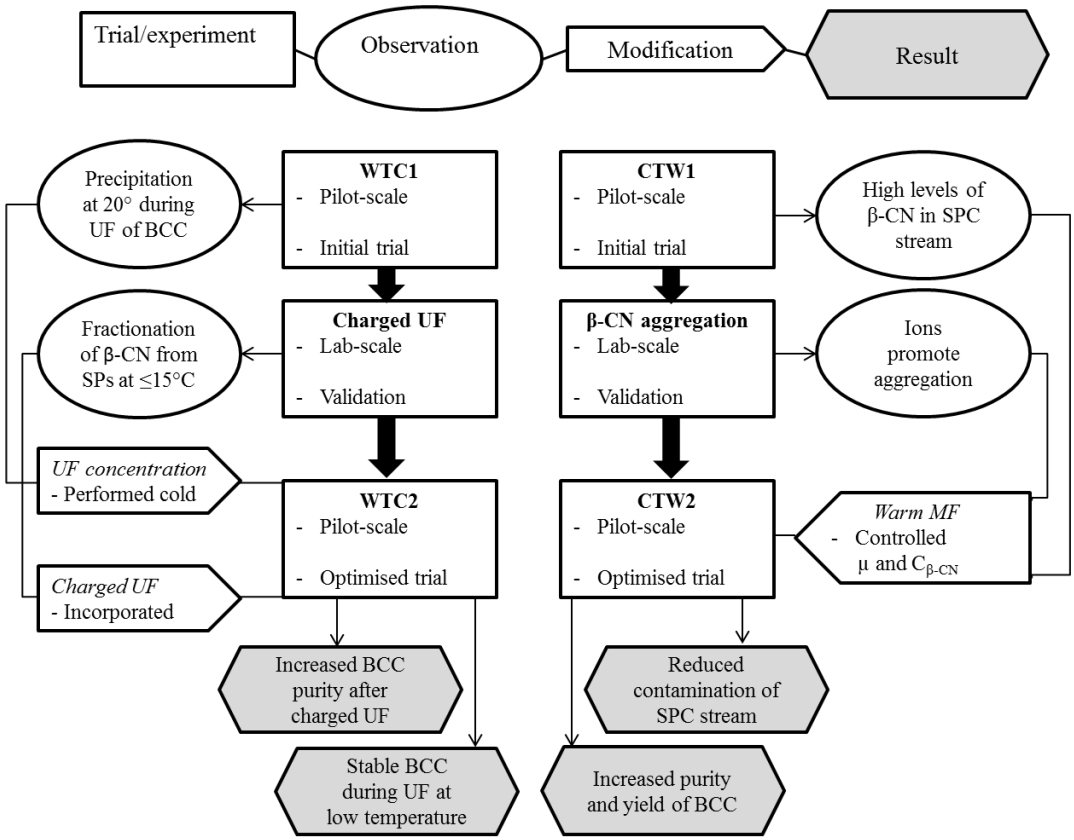

Fig. 10. Optimisation procedure for warm-then-cold (WTC) and cold-then-warm (CTW) processes for β-casein concentrate (BCC) and serum protein concentrate (SPC) manufacture. UF = ultrafiltration, μ = ionic strength, $C_{β-CN}$ = concentration of β-casein.

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CHAPTER 10

Turbidity, thermo-reversibility and physical stability of heat-induced complexes in β-casein concentrate solutions

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. SVC co-designed the study and generated all data included in this chapter, with the exception of composition and protein profile data, which were determined during the study described in Chapter 9 and are presented here in modified form.

Abstract

Monomeric bovine β-casein self-associates into micelles under appropriate conditions of protein concentration, serum composition and temperature. The present study investigated self-association characteristics of a β-casein concentrate (BCC) manufactured at pilot-scale using membrane filtration of milk. The BCC had a casein:whey protein ratio of 77:23, where ~95% of casein consisted of β-casein, with the remainder mostly consisting of κ-CN. BCC was reconstituted to 1.2% protein in various liquid media at pH 6.8 and incubated at different temperatures from 4-63°C for 30 min. Self-association of β-casein was thermo-reversible in deionised water, lactose (4, 6 or 8%) or calcium (9 mM) solutions. In most serum-phases, BCC became highly opaque after incubation at 63°C, but clarified rapidly during cooling to 25°C. However, in simulated milk ultrafiltrate (SMUF), which has a high ionic strength and is supersaturated in calcium phosphate (CaP), BCC remained opaque during cooling to 25°C, and retained residual turbidity after 15 h holding at 4°C; if SMUF was prepared without phosphate then turbidity development in BCC solutions was markedly reduced. The complexes responsible for this turbidity development could be dissociated with 50 mM trisodium citrate. Results from pH-drift and light microscopy experiments indicate that BCC stabilises CaP in the amorphous form, and that CaP in turn serves an integrating role for the β-casein-rich micelles which are protected by a surface layer of κ-CN. A model is proposed to explain the formation, protein profile and destabilisation of complexes in BCC systems.

1. Introduction

The caseins (CNs) of bovine milk are a heterogeneous group of four phosphoproteins (α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN). In bovine milk, the CNs self-assemble into a polydisperse population (size range of ~ 50-500 nm, mean ≈ 150-180 nm) of colloids termed 'casein micelles', which are stable in the conditions of high ionic strength (80 mM) and soluble calcium (9 mM) which prevail in the system (O'Mahony and Fox, 2013). The CNs associate into micelles associate primarily through a combination of hydrophobic and electrostatic interactions, the former occurring directly between hydrophobic regions of the CNs and the latter mediated by 'nanoclusters' of calcium phosphate (CaP) (Horne, 1998). The nanoclusters, often referred to as colloidal calcium phosphate (CCP), are sequestered in the amorphous form at clusters of phosphorylation sites located at the hydrophilic tails of CNs; this process prevents precipitation of CaP, which is supersaturated in milk, and facilitates delivery of significant dietary CaP without pathological precipitation in the mammary gland during milk secretion (Horne, 2006; Dalgleish, 2011; Holt and Carver, 2012; Holt, 2016).

Among the CNs, κ-CN has the lowest degree of phosphorylation (1 residue), giving it a low Ca-sensitivity and eliminating its potential as a nucleation site for CCP; hence, κ-CN is thought to terminate a polymerisation process that would otherwise continue indefinitely, and is generally agreed to occupy a position at the micellar surface where it stabilises micelles electrostatically and sterically (O'Mahony and Fox, 2013). The more Ca-sensitive CNs, β-CN and the α_s-CNs, contain the prerequisite number of phosphorylated residues (≥3) for sequesterisation of CCP and form much of the internal structure of the CN micelle (Dalgleish, 2011; Holt, 2016).

Removal of significant quantities of CCP through, for example, addition of calcium-binding agents, can result in non-selective dissociation of all CNs from the micellar framework (Griffin *et al*., 1988). The casein micelles of milk are responsible for its white colour, and removal of CCP with concomitant dissociation of the micellar structure causes a major decrease in the turbidity of milk, where both function as major light-scattering species (Smiddy *et al*., 2006). On the other hand, if milk is cooled to <5°C, the influence of hydrophobic interactions is minimised while

partial dissolution of CCP occurs, resulting in 'cold-induced dissociation' of a limited quantity of monomeric β-CN into the serum phase, while the integrity of the micellar framework is maintained (Rose, 1968; Creamer *et al*., 1977; Dalgleish, 2011). β-CN has the most hydrophobic amino acids of the CNs in milk, and this strongly affects its own ability to self-associate when in solution. In the absence of native CN micelles, β-CN forms micelles with a mean diameter of 20-30 nm when above its critical micelle concentration (CMC) and at temperatures >4°C (Leclerc and Calmettes, 1997; O'Connell *et al*., 2003; Portnaya *et al*., 2006). These two phenomena, (1) cold-induced dissociation of β-CN from native casein micelles and (2) self-association of β-CN in systems devoid of native micelles, can both be exploited in the separation of β-CN from the other CNs and the separation of β-CN from whey proteins, respectively, using membrane filtration technology (Coppola *et al*., 2014; O'Mahony *et al*., 2014; Crowley *et al*., 2016b).

As an ingredient, β-CN has many potential applications, including some, such as foaming and emulsification, which exploit its very high surface activity (Le Meste *et al*., 1990; Coppola *et al*., 2014). More recently, a growing body of evidence supports the application of β-CN as an encapsulating agent for hydrophobic compounds of nutritional and clinical relevance (Shapira *et al*., 2010a, 2010b, 2012; Esmaili *et al*., 2011; Bachar *et al*., 2012; Semenova *et al*., 2012; Razmi *et al*., 2013; Moeiniafshari *et al*., 2015; Turovsky *et al*., 2012). Furthermore, unlike bovine milk, β-CN is the dominant CN in human milk, which makes β-CN ingredients promising candidates for humanising the CN fraction of next-generation infant milk formulae (McCarthy *et al*., 2013). In several respects, CN micelles in human milk are markedly different from those in bovine milk, being smaller, more hydrated and more prone to cold-induced dissociation as a few examples (Sood *et al*., 1997), and it is unclear to what degree β-CN ingredients can be used to replicate these properties. It is important to develop humanised micellar systems for infant formulae, as the CN of human milk have altered clotting properties, which could influence their digestion (Nakai and Li-Chan, 1987).

Certain factors affecting the self-association of β-CN, such as pH and temperature, or the presence of urea, sugars, denatured whey protein, or plant proteins (napin), have been studied by various researchers (Yong and Foegeding, 2008; O'Connell *et al*., 2013; Schwartz *et al*., 2015; Setter and Livney, 2015). Setter

and Livney (2015) demonstrated that sugars affect the self-association of β-CN, although the influence of lactose, the primary carbohydrate in most infant formula systems, and a common excipient in pharmaceuticals, has yet to be studied. Holt *et al*. (1996, 1998) demonstrated that hydrophilic fragments of β-CN could stabilise supersaturated solutions of CaP, while Van Kemendae and De Bruyn (1989) and Thachepan *et al*. (2010) have reported similar findings for highly pure intact β-CN; however, other than these studies, little research has been carried out into the self-association of β-CN in supersaturated solutions of CaP, despite the significant role of CCP in influencing the structure and physicochemical properties of native CN micelles (Holt and Horne, 1996; Horne, 1998; Horne, 2006; Holt and Carver, 2012). The results of previous studies have suggested that intact β-CN (Van Kemendae and De Bruyn, 1989; Thachepan *et al*., 2010) and β-CN phosphopeptide (Holt *et al*., 1996, 1998) have the ability to stabilise amorphous CaP, as CN micelles do in milk. No self-association studies have yet included β-CN materials generated using membrane filtration, one of the most viable processes for the manufacture of β-CN commercially, which tend to yield quite heterogeneous CN profiles.

The purpose of this study was to explore the effects of serum-phase composition on the self-association and stability of a membrane process-derived β-CN concentrate (BCC), with a particular focus on the behaviour of BCC in simulated milk ultrafiltrate (SMUF), a mineral solution which replicates the complex salt system of milk serum. BCC generated at pilot scale according to a process described by Crowley *et al*. (2016b) was used. The process involved no precipitation steps, relying mainly on thermo-reversible changes in β-CN's association-state, which preserves the solubility of β-CN and maintains the protein its native form. BCCs produced in this manner contain significant levels of κ-CN (~5% of total CN), which could markedly alter their self-association characteristics. Association properties in BCC were investigated in a range of serum-phase compositions and a range of temperatures (4-63°C). A protein content of 1.2% was studied, which is much higher than most previous work on β-CN self-association but closer to levels found in human milk and infant formula. A model was developed based on experimental data on BCC self-association, dissociation and destabilisation when dissolved in SMUF, which could inform the use of BCCs in infant formula and other applications.

2. Materials and methods

2.1. Materials

BCC was manufactured according to the 'cold-then-warm 1 (CTW1)' process described by Crowley *et al*. (2016b), using pressure-driven separation with spiral-wound polymeric membranes. Briefly, pasteurised and skimmed milk was microfiltered and diafiltered (with milk ultrafiltration permeate) using a 0.08 µm pore-size membrane at 1.5°C to separate β-CN and whey proteins from casein micelles; the β-CN/whey protein mixture was concentrated with a 10 kDa ultrafiltration membrane at <10°C, followed by exhaustive demineralisation through diafiltration with reverse osmosis (RO) water at 19°C; the demineralised concentrate was warmed to 26°C to form micelles of β-CN which could then be separated from whey proteins using microfiltration (0.08 µm); the liquid BCC was further concentrated/demineralised by microfiltration/diafiltration prior to drying with a single-stage spray-drier with nozzle atomisation (PSD 55; APV, Copenhagen, Denmark). BCC powder was stored in air-tight bags at 4°C immediately after manufacture.

Lactose monohydrate (99.5%) and $Na_3C_6H_5O_7.2H_2O$ were provided by Fisher Scientific (Fair Lawn, NJ, USA). The following salts were used to prepare simulated milk ultrafiltrate (SMUF) according to the method of Jenness and Koops (1962): $CaCl_2.2H_2O$, K_2SO_4, KCl, KH_2PO_4 (Fisher Scientific, Fair Lawn, NJ, USA) and $K_3C_6H_5O_7.H_2O$, K_2CO_4 and $Mg_3(C_6H_5O_7)_2.9H_2O$ (Sigma-Aldrich, St. Louis, MO, USA). SMUF was prepared fresh for each experiment by adding salts individually to deionised water. The SMUF was then left to stir at 22°C for 60 min. After this period, >35% HCl was added drop-wise until a pH ~ 3.0 was reached. The solution was stirred for a further 60 min to ensure complete solubilisation of salts, particularly CaP. The solution was then restored to pH 6.8 by drop-wise addition of 2 and 10 N KOH. After bringing to volume the SMUF was inverted several times in a stoppered flask and used as a dispersant for BCC powder within 1-2 h.

2.2. Compositional analysis and protein profiling

BCC was analysed for total solids, protein and casein using standard procedures (AOAC, 2003). Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was performed to determine the levels of individual CNs (Bobe *et al*., 1998; Bonfatti *et al*., 2008).

2.3. Preparation of β-casein concentrate (BCC) solutions

BCC was reconstituted in a number of different liquid media, which were deionised water, lactose (4, 6 or 8%), SMUF, SMUF containing 4, 6 and 8% lactose, SMUF with a 3-fold increase in calcium (SMUF-HC), and SMUF containing no phosphate (SMUF-NP). SMUF is a complex mineral solution which approximates the salts system of milk serum and consists of 9 mM L^{-1} Ca, 12 mM L^{-1} P, 3.2 mM L^{-1} Mg, 18 mM L^{-1} Na, 39 mM L^{-1} K, 32 mM L^{-1} Cl (Jenness and Koops, 1962). In all cases, BCC was added slowly to the dispersant over the course of 5-6 h under gentle mixing to prevent caking of the powder at the liquid surface. Once all of the powder had been added, the system was let rehydrate for a further 2 h, at which point the solution was sealed and stored at ~4°C overnight to ensure complete rehydration of the protein. The rehydration temperature used was ~22°C in most cases; however, BCC was rehydrated in SMUF at ≤15, unless stated otherwise, as rehydration temperature was observed to influence self-association. A completely clear BCC solution could be formed at ≤15°C; however, a temperature of 22°C resulted in irreversible turbidity development, which caused greater turbidity development during subsequent incubation experiments. The BCC solutions had a native pH of 6.7-6.9, and were adjusted to pH 6.8 if necessarym with drop-wise addition of 0.1 N HCl or 0.1 N NaOH under constant magnetic stirring.

2.4. Self-association in β-CN concentrate (BCC) solutions

2.4.1. Effect of serum composition and incubation temperature

Refrigerated BCC solutions (30 mL at pH 6.8) were transferred to 50 mL plastic graduated centrifuge tubes and equilibrated to a solution temperature of 20°C.

The centrifuge tubes were then submerged in a water bath and incubated at 26, 37 or 63°C (±0.5°C) for 30 min, unless otherwise indicated. At the designated time, samples were quickly removed from the water bath and transferred to glass, screw-capped tubes (30 mL capacity) and immediately analysed for turbidity within 30-60 s using a nephelometer (Hach, model 2100N) with a tungsten light-source and a detector placed at 90° to the incident light. No dilution was performed prior to turbidity analysis to maintain the protein content, association state and serum environment; the exception to this was dissociation experiments, which are discussed in Section 2.4.2.

Immediately after incubation, BCC solutions were held in the glass tubes and turbidity was monitored during cooling to 45, 37 and 26°C. BCC solutions were also analysed for turbidity at 4°C before incubation and at 4°C after 15-16 h refrigeration post-incubation; comparison of these two values gave an indication of the thermo-reversibility of the aggregation process.

2.4.2. *Dissociation of complexes in β-CN concentrate (BCC) solutions*

BCC was rehydrated in SMUF 22°C, which yielded a more turbid BCC/SMUF system. These solutions were then analysed for turbidity immediately after being incubated at 37°C for 30 min or after post-incubation holding at 4°C for 15 h. Dissociation of complexes was studied through 1:1 dilution with deionised water, SMUF or tri-sodium citrate (TSC, 100 mM) added at the same temperature as the sample to be analysed (4 or 37°C).

2.5. Size of complexes in β-CN concentrate (BCC) solutions

The particle size distribution of BCC solutions prepared in deionised water or SMUF were compared using dynamic light-scattering (DLS); BCC/SMUF was prepared at 15°C to ensure that no turbidity development occurred prior to incubation. BCC/SMUF was incubated at 4, 26, 37 or 63°C as described previously, with an additional incubation at 20°C, and samples were then diluted 1:10 in SMUF before being syringe-filtered (0.22 µm pore-size). The filtered solution was then analysed at 4, 20, 25, 35 or 60°C by DLS with a back-scattering angle of 173°C

using a Malvern Zetasizer nano ZSP (Malvern Instruments, Worcestershire, UK); BCC/water solutions incubated at 4 and 25°C were also analysed. Three scans were performed in each measurement. Data collection and analyses were performed using DTS (Nano) software (Version 5.02; Malvern Instruments), with the temperature-dependence of solvent viscosity being factored into size calculations. Data was transformed from intensity-weighted distributions to volume-weighted distributions using protein and solvent refractive indices of 1.45 and 1.33, respectively.

2.5. *Sedimentation behaviour of complexes in BCC solutions*

An analytical centrifuge (LUMISizer®, L.U.M. GmbH, Berlin, Germany) was used to study the sedimentation of β-CN complexes in select samples. Two main experiments were performed; *constant incubation time*, in which the sedimentation of BCC/water and BCC/SMUF were compared with milk protein concentrate 35 (MPC35, also prepared in SMUF) after incubation for 30 min at different temperatures, and *constant incubation temperature* in which BCC/SMUF was analysed after incubation at 37°C for 90, 150 or 210 min. The principle of the instrument has been described in detail previously (Crowley *et al*., 2014). A cycle of two centrifuge speeds was applied at a controlled temperature of 37°C, the first at 36 g for 10 min followed by the second at 2300 g for 60 min. The lower centrifuge speed allowed the measurement of light transmission before protein sedimentation (T_{start}). Protein sedimentation occurred at the higher speed, and could be calculated by fitting a straight line as follows:

$$CR = \frac{T}{t+c} \qquad \text{(eq. 1)}$$

where CR = clarification rate (%T h^{-1}), T = transmission (%) and t = time (h).

The final transmission value after 60 min at 2300 g was denoted as T_{end} and compared to T_{start} to determine the extent of sedimentation during the measurement.

2.6. *Formation of calcium phosphate during incubation*

SMUF and BCC/SMUF solutions were supersaturated with respect to CaP. At pH 6.8, an elevation of temperature should increase the degree of supersaturation and result in the formation of calcium phosphate (CaP) particles. In milk systems this process can be represented as follows (Lewis, 2010):

$$3Ca^{2+} + 2HPO_4^{2-} \leftrightarrow Ca_3PO_4 \downarrow + 2H^+$$

As seen by this reaction, the formation of CaP on incubation should be accompanied by a pH drop, meaning that acidification can be used to track the reaction, as demonstrated previously by Spanos *et al*. (2007) for SMUF at 60°C. Acidification in SMUF and BCC/SMUF solutions were monitored continuously during incubation at ~60°C. First, a probe was submerged in 10 mL of solution, with pH and temperature being measured at room temperature for 30 s; this was followed by heating to and holding at 60°C, followed by cooling back to room temperature for 10 min. A holding time of 40 min was used for BCC/SMUF, but this was extended to 50 min for SMUF to investigate if steady-state would be reached. Straight lines were fitted to key stages to determine acidification rates. Hysteresis of pH (ΔpH) was also measured, to determine the reversibility of the acidification, as follows:

$$\Delta pH = pH_{start} - pH_{cooled} \text{ @ } 25°C \qquad \text{(eq. 2)}$$

with pH_{start} and pH_{cooled} indicating the pH measured before incubation and after cooling, respectively.

A BX51 light microscope (Olympus, Tokyo, Japan) was used to assess if crystallisation of CaP occurred during heating these solutions. Drops of SMUF or BCC/SMUF were transferred to glass slides, covered with glass slips, heated to and held at 60°C on a controlled-temperature stage. The conditions were designed to closely mimic those used in the pH-drift experiments. Images of the solutions were

taken continuously during the experiment using a light-microscope with a 40× objective lens.

2.7. *Turbidity and protein loss on extended incubation*

Turbidity of BCC/SMUF solutions was determined as described in Section 2.4, except that 14 solutions (seven time-points, n = 2) were incubated at 37°C for up to 210 min and removed at 30 min intervals for immediate analysis of turbidity. A separate experiment was performed where BCC/SMUF incubated at 90, 150 and 210 min were incubated under the same conditions before storage at 4°C for 15 h in sealed containers; these solutions were then centrifuged at 10,000 *g* for 30 min at 10°C. The supernatant, after separation from the pellet, was diluted 1:20 and absorbance measured at 280 nm using a spectrophotometer. The absorbance values were then compared with an unincubated BCC/SMUF solution to determine protein loss caused by incubation.

2.8. Levels of water, inorganic and organic matter in centrifugal pellets

The centrifugal pellets obtained as described in Section 2.7 were drained of supernatant upside-down for ~30 min and stored in sealed containers at 4°C for ~48 h. The pellets were kept intact as a solid mass and frozen at -20°C after removing any remaining drops of supernatant with filter paper. After ~48 h, the pellets were thawed at 4°C and weighed before being segmented into mm-sized chunks with a spatula. Individual pellet segments were then analysed using thermo-gravimetric analysis (TGA) with a TGA 500 (TA Instruments Ltd., UK). Segments of ~ 2 mg on platinum pans were transferred to an inert nitrogen-flushed atmosphere and heated (10°C min^{-1}) from ambient temperature to 550°C with weight loss from the sample being continuously monitored. Weight loss was characterised by two major stages: vaporisation of water up to 200°C and decomposition of organic matter up to 550°C. In this way, the content of water and ash could be measured for the pellets, with organic matter calculated as follows:

$$Organic\ matter\ (\%) = 100 - [water\ (\%) + ash\ (\%)] \quad \text{(eq. 3)}$$

As there was no organic material in the SMUF and 97% of dry-matter in the BCC powder was protein, the organic material was taken to consist of protein derived from the BCC. The BCC/SMUF solution was measured to have a total solids content of 1.75 ± 0.06%, BCC contributing 1.24% and SMUF contributing 0.51%. Measured ash was therefore attributed to the SMUF, assuming a negligible concentration in the BCC powder. With these assumptions in mind, the yield of insoluble solids, protein and ash were calculated as follows:

$$Yield\ of\ insoluble\ component\ (\%) = \frac{Quantity\ in\ pellet\ (mg)}{Quantitiy\ in\ 30\ mL\ solution\ (mg)} \times 100 \quad \text{(eq. 4)}$$

3. Results

3.1. Protein profile of β-casein concentrate

The BCC studied consisted of 97% protein, the profile of which is shown in Table 1. BCC had a CN:whey protein ratio (77:23) which approximated that of milk and milk protein concentrates.

Table 1. Protein and casein profile of BCC before and after reconstitution. Data are for the powder are from the means of duplicate experiments; the solution values are estimated from these for a 1.2% solution.

Component	**BCC powder** (w/w)	**BCC solution** (w/v)
Total protein (% dry-matter)	96.8	1.20
Whey protein (% protein)	22.9	0.275
Total casein (% protein)	77.1	0.925
β-Casein (% protein)	73.3	0.880
κ-Casein (% protein)	3.33	0.040
α_s-Casein (% protein)	0.42	0.005

RP-HPLC indicated that >95% of the CN fraction was comprised of β-CN, with the remainder of the CN being primarily composed of κ-CN (4.3%). Trace levels of α_s-CN were detected (0.5%), which represented <0.01% of reconstituted BCC solution on a w/v basis. At the protein content that the BCC was reconstituted to (1.2%), β-CN was at least an order of magnitude higher than its CMC at >4°C.

3.2. Effect of temperature and serum-phase composition on turbidity development

At all serum compositions, turbidity of BCC increased with increasing temperature after incubation for 30 min (Fig. 1), with no visible precipitation or sedimentation observed. The contribution of whey proteins to turbidity development was considered negligible as whey protein isolate solution (0.3%, w/w, protein, i.e., total whey protein level in reconstituted BCCs) prepared in either water or milk ultrafiltrate had turbidity values of <10 NTU after incubating at temperatures between 25-63°C for 30 min (data not shown); indeed, temperatures of >70°C are typically required to cause extensive denaturation/aggregation of whey proteins (Wijayanti *et al*., 2014). Turbidity development in the temperature range studied can therefore mainly be attributed to self-association of the CNs.

Compared to BCC/water systems, increases in turbidity were far more pronounced in BCC/SMUF (Fig. 1b), as the latter has a much higher ionic strength (~80 mM) and contains 9 mM calcium, which promote β-CN self-association (Dauphas *et al*., 2005); SMUF is also supersaturated in CaP, which increases at elevated temperatures (Spanos *et al*., 2007), and could have contributed to increased light-scattering if complexed with CN complexes as nanoclusters (Smiddy *et al*., 2006). In BCC/SMUF samples, an increasing level of lactose from 0 to 4% resulted in increased turbidity at 37°C, but this effect was not observed for 6 and 8%, which seemed to slightly inhibit turbidity development. Adjustments in the mineral profile of SMUF were made to distinguish between the influence of ionic Ca and CaP formation. In Figure 1c it can be seen that increasing Ca content to ~27 mM caused turbidity development to double in BCC/SMUF-HC compared to BCC/SMUF, while when BCC/SMUF was prepared with no phosphate (BCC/SMUF-NP), turbidity development was greatly minimised.

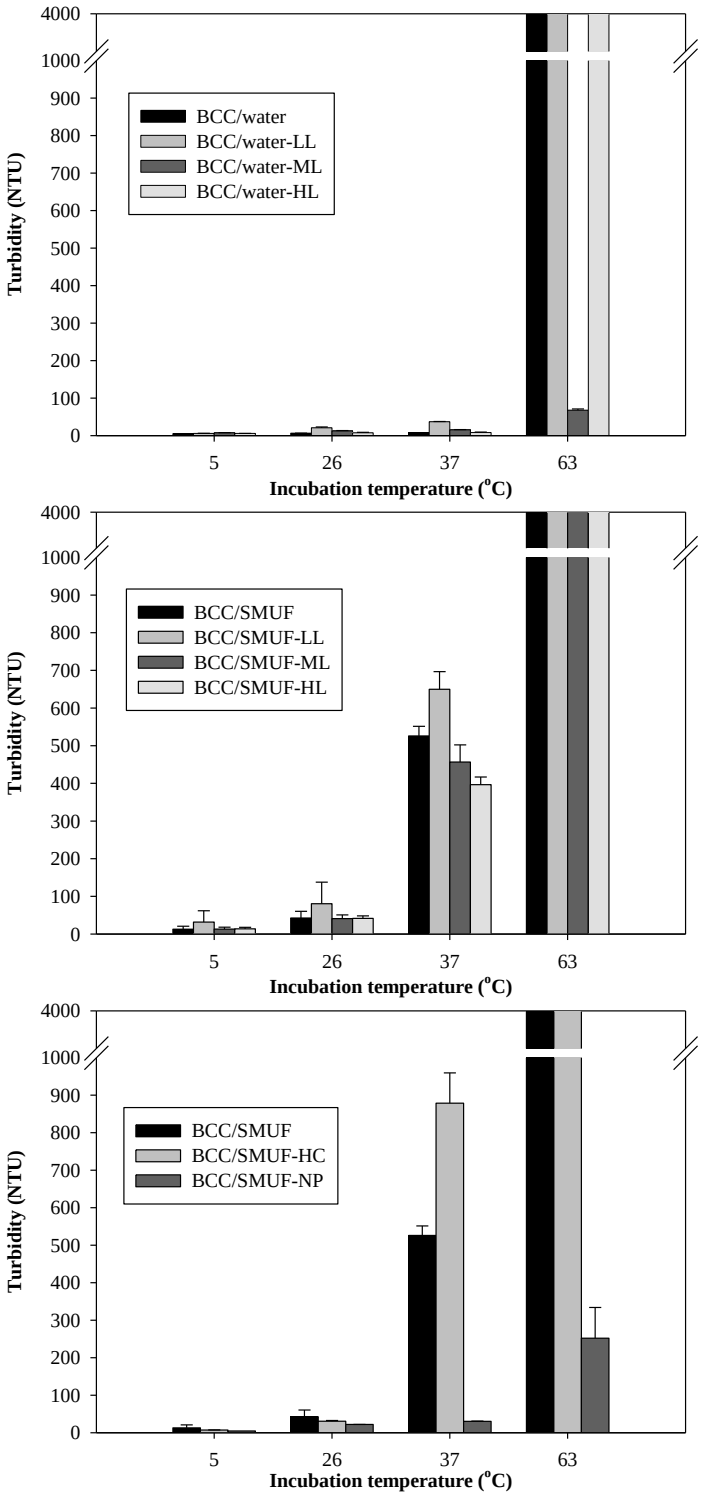

Fig. 1. Turbidity of β-casein concentrate (BCC) solutions prepared in water or simulated milk ultrafiltrate (SMUF) with 4% (LL), 6% (ML) or 8% (HL) lactose, and incubated at specific temperatures for 30 min. SMUF was also prepared with 27 mM Ca (SMUF-HC) and with no phosphate (SMUF-NP) and compared to standard SMUF with 9 mm Ca and in the presence of phosphate. Data are the means (± standard deviations) of experiments on solutions prepared, incubated and analysed on at least two separate occasions. Detection limit = 4000 NTU.

3.4. Influence of temperature on the size of protein complexes

The particle size distributions of BCC/SMUF solutions after incubation at for 30 min at various temperatures are shown in Figure 2.

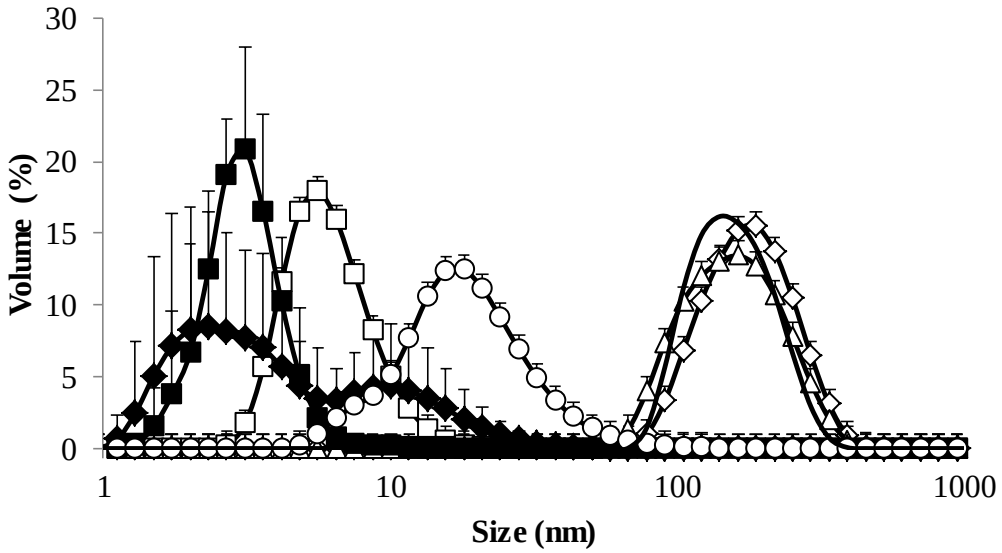

Fig. 2. Particle size distributions of β-casein concentrate (BCC) solutions prepared in simulated milk ultrafiltrate (SMUF), and incubated at 4, 20, 26 and 37°C for 30 min analysis at 4°C (□), 20°C (○), 25°C (◊), 35°C (Δ) or 63°C (—), respectively. Solutions were diluted (1:10) and filtered (0.22 μm) prior to analysis. Results for BCC/water solutions at 4°C (■) and 25°C (♦) are included for comparison. Data are the means (± standard deviations) of solutions subjected to two separate incubation experiments, prepared and analysed for size in duplicate. The 63°C data was the result of a single analysis on pooled filtrate due to low sample volume.

At 4°C, BCC/SMUF samples primarily contained particles of <10 nm, with a mean value of ~6 nm, indicative of monomeric β-CN (O'Connell *et al*., 2003). BCC/water at this temperature contained particles in a similar size range, albeit slightly smaller. At 20°C, a shift to larger sizes of 10-60 nm (mean ≈ 20 nm) is evident in BCC/SMUF, indicative of the formation of micellar β-CN (O'Connell *et al*., 2003); a slight shoulder in the peak at ≤10 nm suggests that monomeric species remained at this temperatures. Unlike BCC/water, in which a monomer/micelle mixture predominated, neither monomers nor micelles were detected in BCC/SMUF at 25°C; instead, a broad population of much larger particles (~50-400 nm) was observed, consistent with a polydisperse population of colloidal aggregates similar in size to native CN micelles in bovine milk (~50-500 nm). At 25, 37 or 63°C the overall

shape of the particle size distribution in BCC/SMUF was largely unchanged, with average sizes (~140-190 nm) that are much larger than those of β-CN micelles; however, there was a notable trend of decreasing size with increasing temperature in this range (Fig. 2), indicating a possible elevation of cohesive forces within these particles at higher temperatures (Beliciu and Moraru, 2009).

3.5. Influence of temperature on the physical stability of complexes

Clarification profiles for BCC/water and BCC/SMUF solutions obtained from analytical centrifugation experiments are shown in Figure 3. MPC35/SMUF was included to compare the separation of β-CN micelles with native CN micelles. The results indicate negligible light-scattering in BCC/water solutions at both temperatures, with no evidence of a sedimenting population of particles. For solutions incubated at 37°C, BCC/SMUF had a reduced transmission compared to BCC/water at the early stages of centrifugation; this effect was especially pronounced after incubation at 63°C, in agreement with turbidity data (Fig. 1). The change in transmission with centrifugation time for the BCC/SMUF sample incubated at 37°C was relatively constant and slightly uneven (Fig. 3A1); this unusual profile was due to masking of the sedimentation phenomenon (top half of cell) with continued self-association of β-CN (bottom half of cell), as can be seen in Figure 3A2. This effect was not observed for BCC/SMUF incubated at 63°C, for which a very low T_{start} (~10%) was detected, and which displayed almost complete sedimentation of particles within 2000 s of centrifugation (Fig. 3B1). In contrast, the sedimentation behaviour of MPC35 was unaffected by incubation temperature, as native CN micelles are not subject to such dynamic and dramatic shifts in association-state under these conditions.

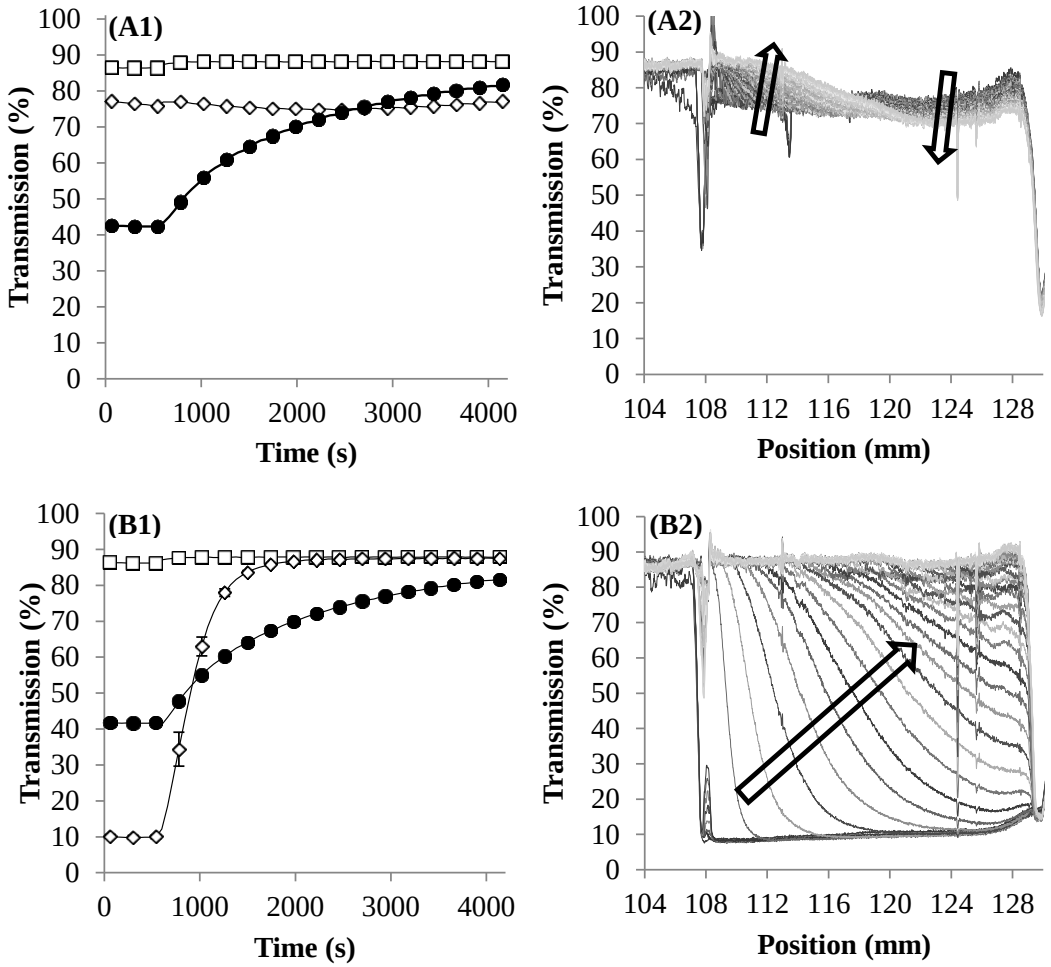

Fig. 3. Transmission of near-infrared light during analytical centrifugation of solutions of β-casein concentrate (BCC) prepared in water or simulated milk ultrafiltrate (SMUF) and milk protein concentrate 35 (MPC35) prepared in SMUF. Solutions were either incubated at (A) 37°C or (B) 60°C for 30 min before centrifugation, with (1) showing transmission as a function of time derived from (2) time- and space-resolved transmission profiles. Data in (1) are the means (± standard deviations) of solutions subjected to two separate incubation experiments, while data in (2) are from a single analysis representative of replicate profiles.

It is interesting to compare the sedimentation behaviour of MPC35 and BCC, with SMUF as dispersant and incubation at 63°C, as both had equivalent protein contents and CN:whey protein ratios but different casein profiles. The BCC/SMUF exhibited a more rapid and extensive sedimentation than MPC35/SMUF, which is seemingly inconsistent with its smaller particle size (mean of ~140 nm for BCC, compared to ~170 nm for MPC35 – Thom Huppertz, personal communication). This may be due to an underestimation of size caused by the removal of large particles during the filtration step applied before DLS analysis; however, the ~30% lower transmission measured for BCC/SMUF compared to MPC35/SMUF during low-speed centrifugation (Fig. 3B1, 0-600 s) is consistent with the BCC/SMUF having greater light-scattering due to the presence of smaller aggregates with a higher specific surface area (Madadlou *et al*., 2009). Another explanation, however, could be an increased influence of hindered settling in MPC35. A consistently sharper phase boundary was detected for BCC/SMUF compared to MPC35 (not shown), which is indicative of a more polydisperse sedimentation; this was confirmed by particle velocity distribution analysis, with a median particle velocity of 7.2 μm s^{-1} and span of 1.2 calculated for BCC/SMUF, compared to 4.6 μm s^{-1} and 1.6 for MPC35/SMUF. Thus, it is possible that the more polydisperse BCC/SMUF had a lower overall sedimentation rate due to the greater presence of slow-sedimenting particles which hindered the settling of the fast-sedimenting population.

3.6. Thermo-reversibility of turbidity development

Samples which had been incubated at 63°C were subjected to cooling at 22°C to investigate the degree to which turbidity development during incubation was thermo-reversible (Fig. 4). For samples incubated at 63°C, it took 5, 15 and 40 min to reach 45, 37, and 26°C, respectively. During this time, all BCC/SMUF systems remained visually opaque (milk-like) and were above the detection limit of the nephelometer (Fig. 4). Conversely, in most cases, the turbidity of BCC/water systems began to decrease instantaneously and was completely reversed.

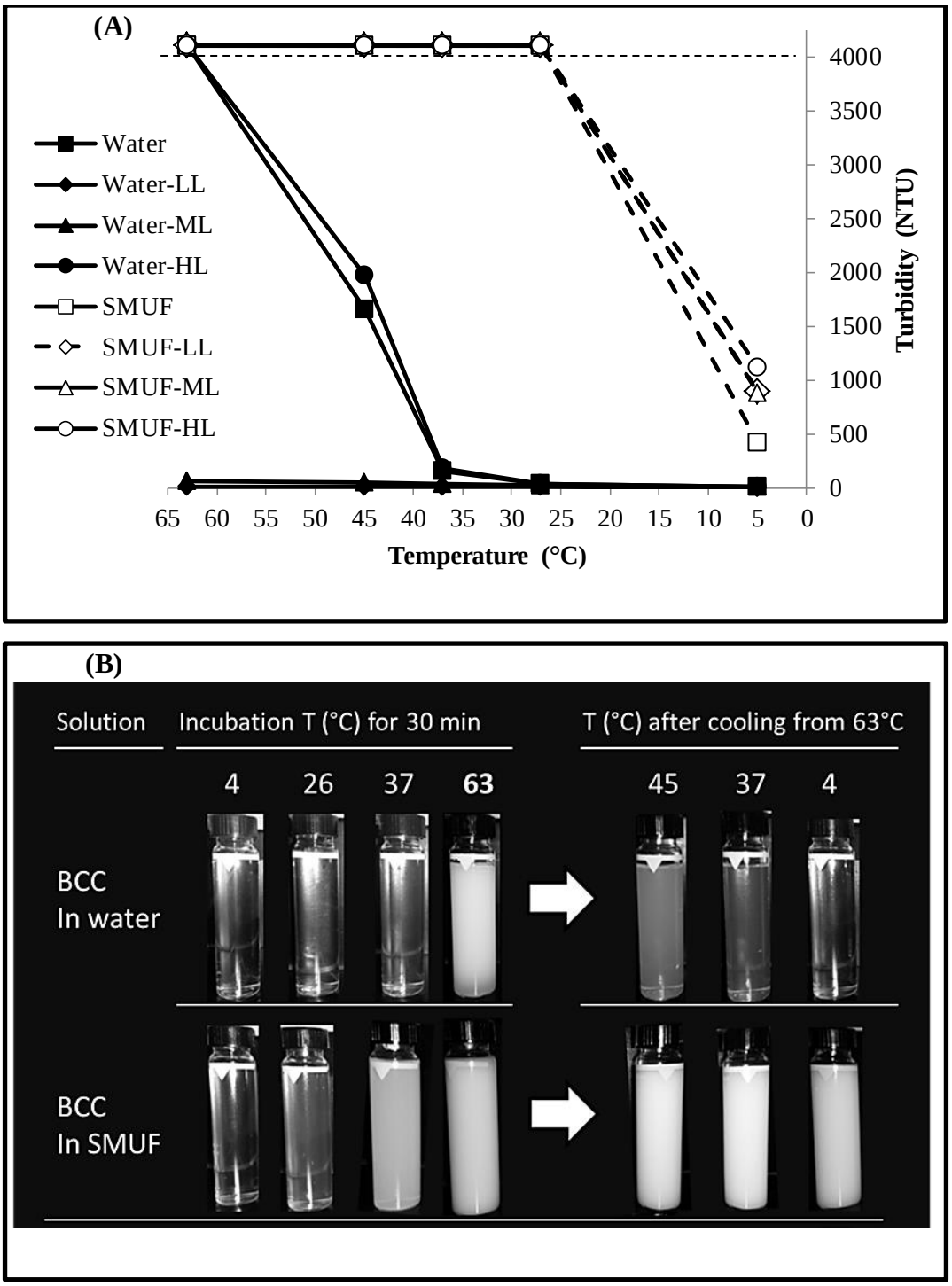

Fig. 4. (a) Turbidity of β-casein concentrate (BCC) solutions during cooling from 63°C and (b) photographs of representative samples. LL – 4% lactose, ML = 6% lactose, HL = 8% lactose. Data are the means of experiments on solutions prepared, incubated and analysed on at least two separate occasions.

Also shown in Figure 4 is the turbidity of the systems after 15 h of cooling at 4°C; notably, all BCC/SMUF systems retained a degree of residual turbidity (~400-1100 NTU), while all BCC/water systems had NTU values <10. Similar, if less pronounced and consistent, trends were observed for samples incubated at 37°C (data not shown). The results of cooling experiments indicated that there is a population of complexes in BCC/SMUF solutions incubated at ≥37°C which are slowly- or non-thermo-reversible, suggesting that non-hydrophobic interactions, presumably CCP-based, are also involved as integrating forces.

3.6. Dilution-induced dissociation of complexes in BCC solutions

The results in Section 3.5, which describe the change in turbidity when incubated BCC systems were cooled, indicate the degree to which the complexes formed during heating dissociate when hydrophobic attractions are reduced. The finding that the thermo-reversal of turbidity development in BCC/SMUF systems is remarkably slow, and ultimately incomplete, indicates that the integrity of the complexes formed cannot be attributed to hydrophobic attractions alone. To investigate this further, a BCC/SMUF system was prepared at 22°C and incubated at 37°C. Samples were then analysed for turbidity at 37°C undiluted, or after 1:1 dilutions in water, SMUF or TSC. The same experiment was performed after first cooling the incubated sample at 4°C for 15 h, with samples being analysed either undiluted or in the aforementioned diluents.

The results of warm and cold dissociation experiments are shown in Figure 5. In BCC/SMUF systems at 37°C, dilution with water resulted in an 82% decrease in turbidity. Dilution in SMUF resulted in an increase in turbidity, which placed the sample outside the limit of detection for the instrument; the solution was already very close to this limit, and the increase in turbidity was probably due to an effective doubling of incubation time with SMUF as diluent and an equilibration time of 30 min following dilution. Dilution in TSC resulted in a reversal of turbidity to values close to those observed for unincubated BCC/SMUF systems at 4°C (~13 NTU). TSC has a limited impact on the refractive index of the solvent (Smiddy *et al*., 2006), and additional losses in turbidity caused by TSC can therefore be attributed to its dissociating action (i.e., sequestering of Ca). After incubation, cooling of undiluted

BCC/SMUF to 4°C resulted in a 93% reduction in turbidity, although turbidity was still quite high (252 NTU). At this temperature, dilution with water or SMUF achieved a decrease in turbidity of ~50%. The presence of TSC at 4°C caused more extensive dissociation than at 37°C, resulting in an NTU of 2.6.

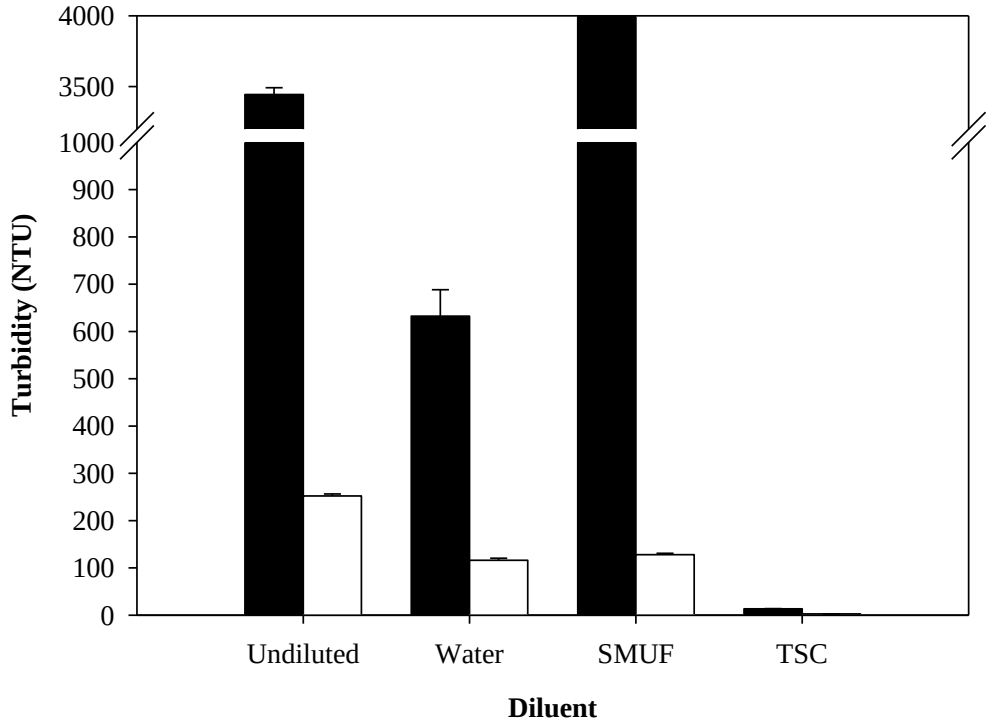

Fig. 5. Turbidity of β-casein concentrate (BCC) solutions prepared in simulated milk ultrafiltrate (SMUF) at 22°C and incubated at 37°C for 30 min followed by immediate dilution in different solvents (grey bar) or dilution after holding at 4°C for 15 h (white bar). Dilutions were 1:1 in all cases. Data are the means (± standard deviations) of solutions subjected to two separate incubation/dilution/cooling experiments. Detection limit = 4000 NTU.

3.7. Formation of calcium phosphate during incubation

It was observed that SMUF incubated at 37 or 63°C for 30 min precipitated (not shown) but that no precipitation occurred under these conditions in BCC/SMUF solutions. These data suggested that the crystallisation of CaP was being prevented by proteins in the BCC. CaP formation was tracked dynamically using pH-drift experiments conducted during heating to and holding at 60°C (Fig. 6).

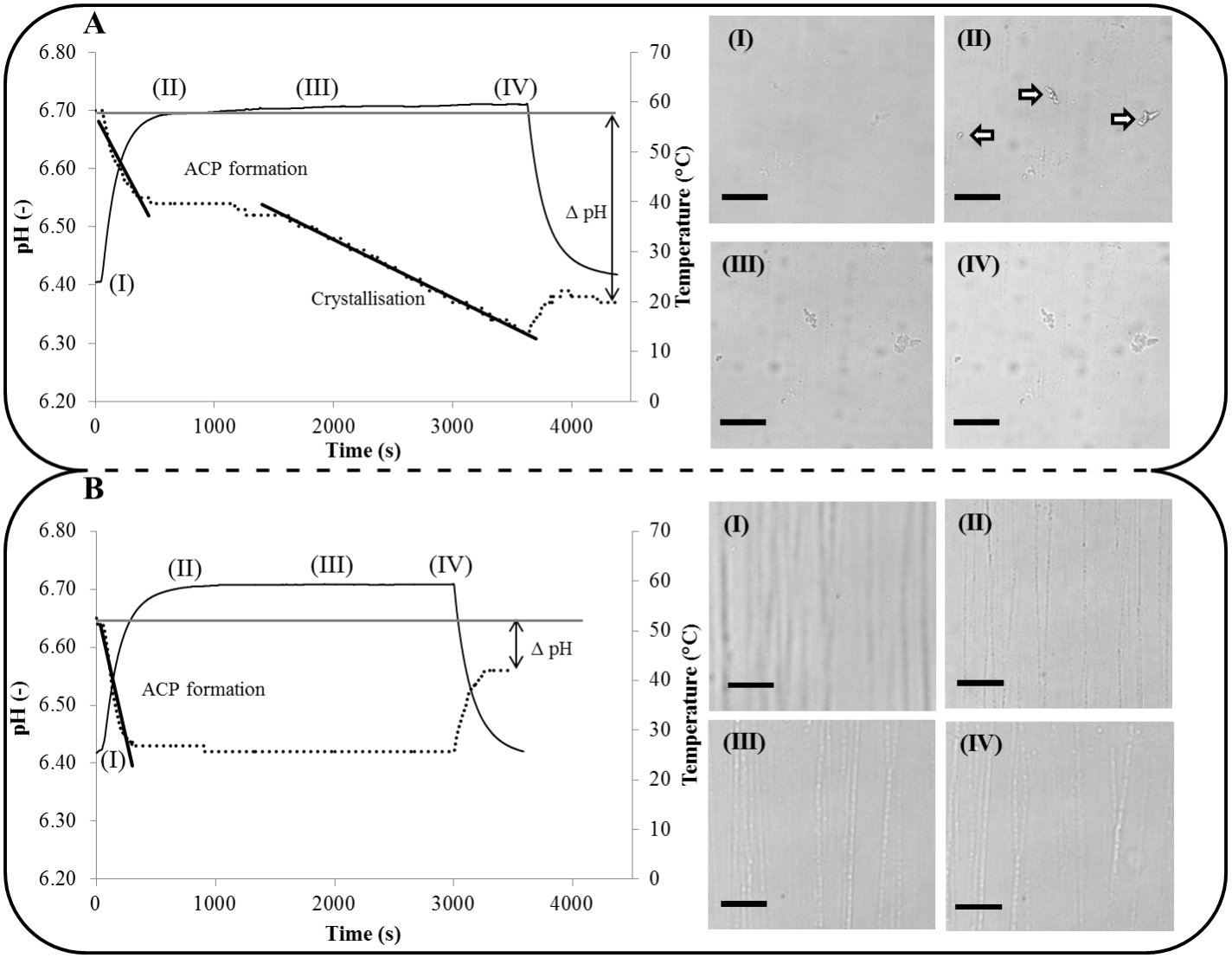

Fig. 6. Changes in pH during incubation of (A) simulated milk ultrafiltrate (SMUF) and (B) β-casein concentrate (BCC) in SMUF at ~60°C. Images taken by a light microscope during an equivalent heating cycle at corresponding stages (I-IV) are shown. Temperature is shown as a solid curve and pH as a broken curve. A grey horizontal line is shown extending from starting pH to guide the eye for pH hysteresis (ΔpH) calculation. Linear fits are fitted to pH curves to estimate the rate of amorphous calcium phosphate (ACP) formation and crystallisation Scale bars represent a size of 50 µm. Examples of crystals are indicated by an arrow. Data are from a single analysis but are representative of replicate profiles.

Data for SMUF are shown in Figure 6A. An initial decrease from pH 6.8 to 6.5 occurred during the first 500 s of heating from 25°C, followed by a lag phase lasting ~1000 s and a second acidification stage which continued until the temperature was reduced. When subject to cooling the original pH was partially restored. For BCC/SMUF (Fig. 6B), the initial stage of acidification was similar to that observed for SMUF. After this point, however, the two samples diverged. No second acidification stage was detected for BCC/SMUF; instead, an extended lag phase at pH 6.4 was measured. A partial restoration of pH was, again, measured after cooling.

Results of modelling of pH-drift data are shown in Table 2.The two-stage acidification that was a feature of SMUF was classified as consisting of stage A, which was rapid, and stage B, which was slow. Only stage A was detected in BCC/SMUF, the rate of this acidification was double that of SMUF (Table 2).

Table 2. Results of modelling of pH-drift data during heating of simulated milk ultrafiltrate (SMUF) and β-casein concentrate (BCC) dissolved in SMUF (BCC/SMUF) at 60°C. Data are the means (± standard deviation) of duplicate experiments, with the exceptions of those marked * and +, which are explained in footnotes.

Calculated values	**SMUF**	**BCC/SMUF**
Stage A rate (pH unit s^{-1}, $\times 10^{-4}$)	-4 ± 0	-8 ± 1
Stage B rate (pH unit s^{-1}, $\times 10^{-4}$)	-1 ± 0	n.d.
pH hysteresis (pH unit)	0.33 ± 0.01	0.10 ± 0.01
Optical properties	Precipitated	Opaque suspension
Crystal size range at 60°C (μm)*	20-33	n.d.
Crystal growth at 60°C (μm)+	4 ± 1	n.d.

n.d. = not detected

* range of sizes for three crystals between 0-50 min holding at 60°C

+ average change in the size of three crystals at 0 min compared to 50 min (at 60°C)

After cooling for 10 min, a ΔpH of 0.3 was measured for SMUF, while the value for BCC/SMUF was only 0.1. Light microscopy images of SMUF during heating and holding indicated the presence of crystals (Fig. 6A), while no crystals were observed

in BCC/SMUF (Fig. 6B). The lengths of the three prominent crystals in the images in Figure 6 were measured and found to increase on average by ~17% during the duration of holding (Table 2). Holt and Carver (2012) described how CaP precipitation in supersaturated solutions commences with the formation of an amorphous state that serves as a precursor phase to lower energy crystalline states (i.e., octocalcium phosphate, hydroxyapatite); in the presence of phosphoproteins, CaP formation is arrested at the amorphous stage. Thachepan *et al*. (2010) found that β-CN could stabilised CaP in the amorphous state during incubation >35°C. Results from pH-drift and light microscopy images for SMUF and BCC/SMUF are consistent with the reports of these previous authors and can be used to draw the following conclusions:

- The initial (rapid) acidification (stage A) is due to the formation of amorphous CaP;
- A subsequent (slow) acidification (stage B) is due to the formation of crystalline CaP;
- Amorphous CaP is a precursor to an energetically-favourable crystal phase in SMUF;
- Crystallisation and precipitation of CaP are suppressed by phosphoproteins in BCC;
- Phosphoserine sites of β-CN serve as a nucleation site for amorphous CaP, resulting in a faster rate of stage A acidification (i.e., faster formation of the amorphous phase);
- Values for ΔpH are higher in SMUF than in BCC/SMUF due to the persistence of the more thermodynamically stable CaP crystals during cooling.

3.8. Turbidity development and colloidal destabilisation during extended incubation

When BCC/SMUF was incubated at 37°C, turbidity increased consistently over time (Fig. 7a). Irreversible destabilisation occurred for incubation periods of ≥60 min, with precipitates observed after subsequent cooling (~15 h at 4°C). When the destabilised BCC/SMUF systems were centrifuged, the supernatants were measured to have an almost 10% decrease in absorbance of light at 280 nm, indicating protein loss compared to the unincubated control (Fig. 7b).

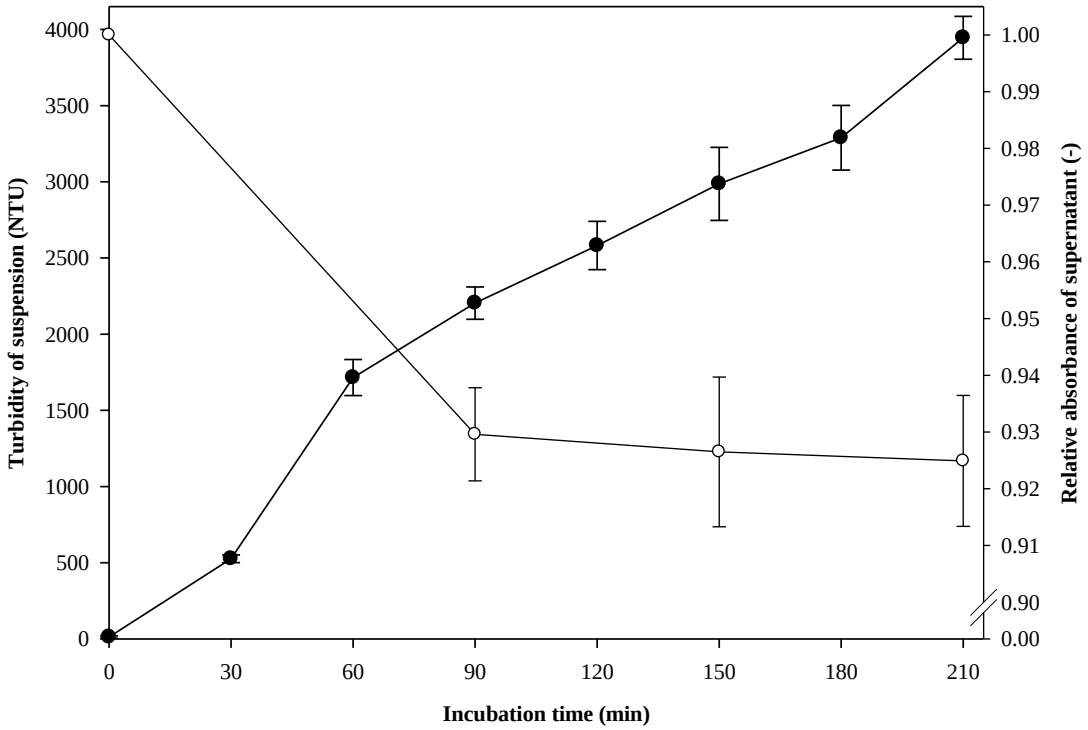

Fig. 7. Turbidity of β-casein concentrate (BCC)/simulated milk ultrafiltrate (SMUF) solutions during incubation at 37°C at different times (open symbols) and the absorbance at 280 nm of soluble protein in the supernatant after refrigeration (~15 h) and centrifugal removal of precipitated material. Data are the means (± standard deviations) of solutions subjected to two separate incubation experiments, with supernatants generated in duplicate and prepared for analysis separately.

The results in Figure 7 indicate that, when BCC was incubated in SMUF for >30 min, an aggregation process was initiated which ultimately led to the irreversible destabilisation of a limited quantity of protein. This phenomenon was very likely to be heat-induced rather than cooling-induced, as demonstrated by data from analytical centrifugation experiments performed immediately after incubation at 37°C for 30,

90, 150 or 210 min (Table 3). As expected, there was no measurable sedimentation in the 30 min sample. There was a strong, positive linear relationship between incubation time and clarification rate, *CR* (R^2 = 0.99). Evaluation of T_{start} and T_{end}, indicates that longer incubation times resulted in an increase in the number of sedimentable complexes (low T_{start}) and a greater loss of complexes due to sedimentation (high T_{end}).

Table 3. Results of analytical centrifugation experiments for β-casein concentrate dissolved in simulated milk ultrafiltrate (BCC/SMUF) incubated for different times at 37°C. Data shown is the starting transmission reading at the earliest point of centrifugation (T_{start}), the last transmission reading at the end of centrifugation (T_{end}), the rate of change in transmission over time (clarification rate, CR) and the goodness of fit for the clarification rate (R^2). Data are the means (± standard deviation) of duplicate experiments

Incubation time (min)	T_{start} (%)	T_{end} (%)	*CR* (% h^{-1})	R^2
30	77.2 ± 0.0	77.2 ± 0.2	0.31 ± 0.1	0.96-0.97
90	69.6 ± 0.5	78.5 ± 0.4	11.3 ± 0.3	≥0.99
150	66.6 ± 0.3	80.4 ± 0.1	18.2 ± 0.3	≥0.99
210	61.5 ± 0.7	82.2 ± 0.5	26.6 ± 1.0	≥0.99

Pellets obtained from centrifugation of BCC/SMUF incubated for 90, 150 and 210 min were analysed by TGA to determine levels of water, organic and inorganic constituents. Representative TGA profiles are shown in Figure 8a. Weight loss due to water vaporisation is seen up to 200°C, followed by decomposition of organic material up to 550°C; the residue at the end of the heating ramp is inorganic material. Although TGA facilitates discrimination between free and bound water, the sum of both were considered for the purposes of this study. Organic and inorganic material were considered to be composed of protein (from BCC) and ash (from SMUF), respectively. Increasing incubation time resulted in increases in the protein and ash levels of pellets, with a notable decrease in water content between the 150 and 210 min incubation times compared to pellet from 90 min incubation (Fig. 8B). Trend in the yield of insoluble protein were generally consistent with abs. 280 nm data, with

both sets of data suggesting only a limited degree of protein destabilisation (7-13%), reaching a maximum after ~150 min incubation.

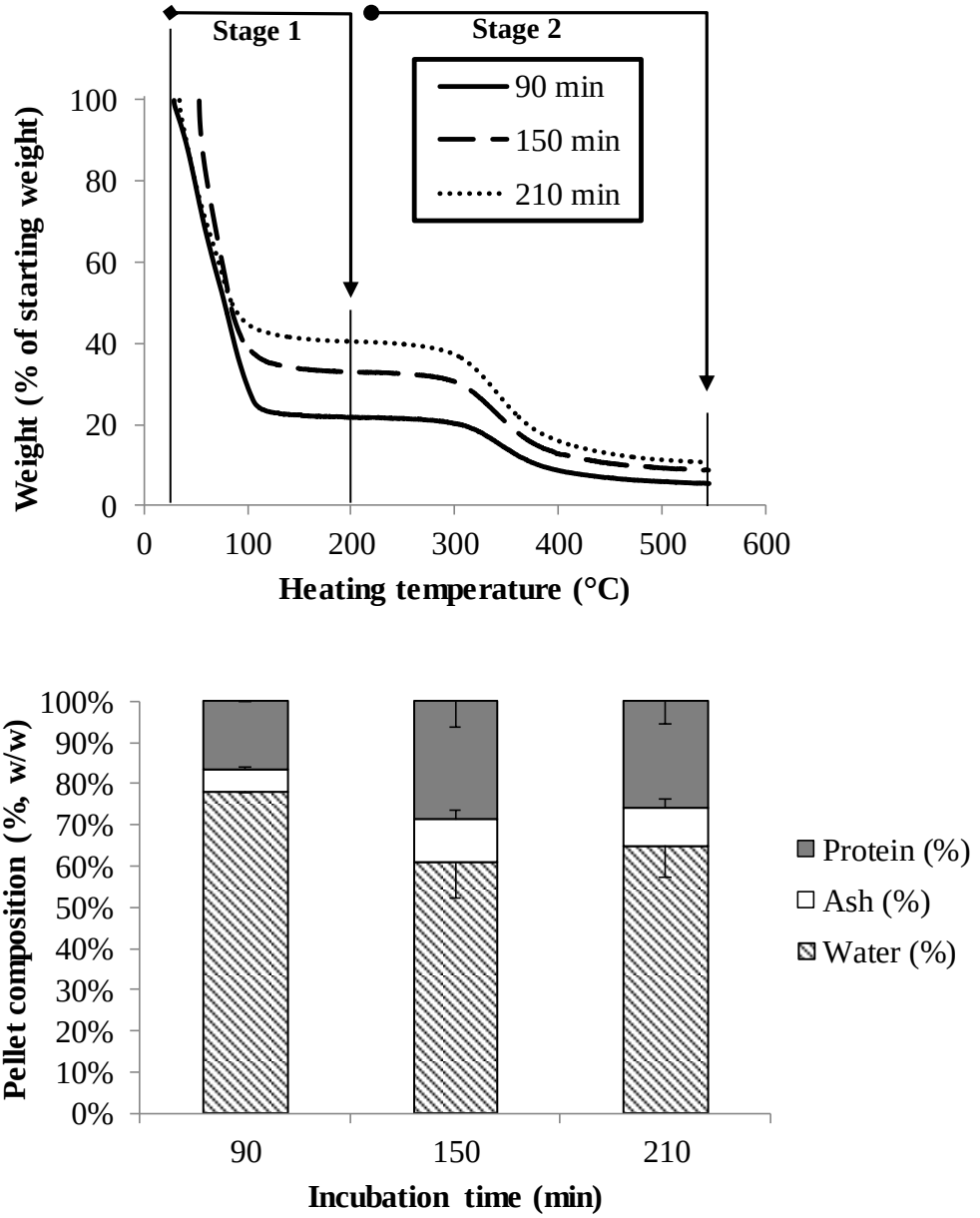

Fig. 8. Results of thermo-gravimetric analysis on 2.0-2.5 mg sub-samples of pellets obtained from β-casein concentrate (BCC)/simulated milk ultrafiltrate (SMUF) solutions after incubation at 37°C for 90, 150 or 210 min, refrigeration for 15 h, centrifugation and removal of supernatant. Weight loss curves during heating (top) and compositional data derived from measurement of evaporative loss of water (Stage 1) and decomposition of organic material (Stage 2). Data are the means (± standard deviations) of solutions subjected to two separate incubation experiments, with pellets generated in duplicate and prepared for analysis separately.

The quantity of pellet obtained from BCC/SMUF increased with incubation time, with a two-fold increase in the yield of both insoluble protein and insoluble ash when incubation time exceeded 90 min (Fig. 9a).

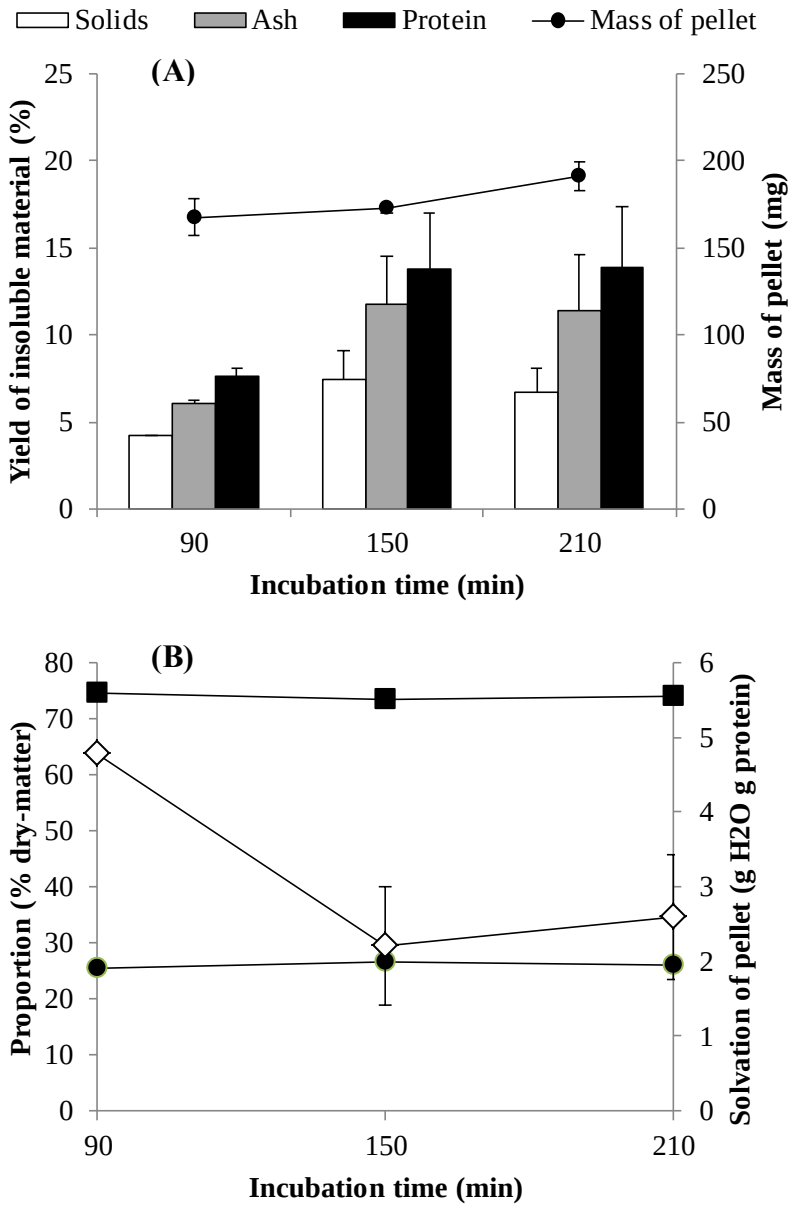

Fig. 9. (a) Yield of pellet (scatter-plot) and individual dry-matter components in the pellet (bars) after centrifugation of incubated solutions of β-casein concentrate (BCC)/simulated milk ultrafiltrate (SMUF) and (b) the proportion of protein (■) and ash (●) in the dry-matter of the pellet, and the solvation of protein in the pellet (◊). Data are the means (± standard deviations) of solutions subjected to two separate incubation experiments, with pellets generated in duplicate and prepared for analysis separately.

Despite the differences in the yield of protein and ash, the protein:ash ratio was constant across all of the pellets; however, the solvation of the protein (g H_2O g protein) decreased markedly between 90 and 150 min incubation (Fig. 9b). As the protein:ash ratio was constant between the pellets, dehydration due to different degrees of mineralisation (De La Fuente and Alais, 1975; Crowley *et al*., 2014) can be ruled out. In addition, changes in moisture due to crystallisation of CaP is unlikely, as it was determined by pH-drift experiments at a starting pH of 6.69 and temperature of 38°C (not shown) that the average pH between these three different incubation times was 6.49 ± 0.02. The change in solvation may therefore be due to a structural arrangement of the protein complexes involving hydrophobic interactions.

4. Discussion

The BCC investigated in this study exhibited self-association behaviour which deviated from that of the β-CN samples investigated by previous researchers. Self-association phenomena were most distinctive for the BCC when dispersed in a medium that was supersaturated in CaP, SMUF, which has been used infrequently in studies of β-CN self-association. Highly opaque solutions were formed at temperatures ≥25°C (Fig. 1), due to the formation of protein assemblies that were larger than typical β-CN micelles (Fig. 2) and sedimented more rapidly than native CN micelles (Fig. 3). The high RI of CCP likely contributed to the marked opacity of BCC/SMUF solutions compared to BCC/water. Unlike the findings of other researchers, these β-CN complexes, when formed at ≥37°C, were not fully thermo-reversible, or at least not within the 15 h of cooling studied (see Fig. 4), indicating that hydrophobic interactions were supplemented by CCP as an integrating factor; this contention was supported by the extensive dissociation of these complexes caused by TSC (Fig. 5). The slow and incomplete thermo-reversal of this polymerisation process can be attributed to the slow rate of dissolution of CCP cross-links compared to the relatively fast decrease in the strength of hydrophobic interactions; a decrease in the Ca-binding ability of caseins at low temperatures may also have contributed to observed dissociation phenomena (Horne and Lucey, 2014). Cold-dissociation of micelles in BCC/SMUF is far more extensive than that observed for native bovine CN micelles, and more closely resembles the behaviour

of human CN micelles, which dissociated by ≥80% during cold storage (Sood *et al*., 1997).

The integrating role of CCP and the heterogeneous casein profile of the BCC (Table 1) largely explains the findings from incubation experiments on BCC/SMUF. On increasing temperature from 4-20°C, BCC/SMUF underwent a classic monomer-micelle transition, not unlike that observed for BCC/water at 4-25°C (Fig. 2) and in agreement with the results of previous researchers (Leclerc and Calmettes, 1997; O'Connell *et al*., 2003). In BCC/SMUF at 20°C the average micelle was ~ 20 nm, the largest being ~68 nm, with monomers of <10 nm also present; this distribution is consistent with the limited hydrophobic self-association of β-CN monomers into small micelles. However, at ≥25°C the particles formed were too large to be consistent with this form of β-CN micelle (i.e., hydrophobic core with a stabilising hydrophilic shell). Instead, the 5 phosphoseryl residues of the β-CN molecule likely served as nucleation sites for CCP, which explains how BCC inhibits crystallisation of CaP (Fig. 6). The CCP, in turn, would have facilitated the cross-bridging of the hydrophilic N-terminal of β-CN at the phosphorylated region forming core-shell structures in closer compliance with the model proposed by Holt *et al*. (1998), who studied the stabilisation of CaP nanoclusters by the N-terminal of β-CN, isolate using tryptic digestion; the major difference between the BCC studied here and the phosphopeptide of Holt *et al*. (1998) was that the hydrophobic region was not removed, and would therefore have extended into the solvent as the surrounding shell. A continuous polymerisation process could therefore be initiated, as represented in (1), where a polymerisation pathway a single molecule wide is shown radiating in one direction from a single CCP nanocluster, with binding based on CCP cross-linking of phosphorylation sites (P) and direct protein-protein interactions *via* hydrophobic regions (H):

(1) [CCP] + [(P)β-CN(H)] + [(H)β-CN(P)] + [CCP] + [(P)β-CN(H)] . . .

In three dimensions, each CCP nanocluster would bind ~4 β-CN molecules. During short periods of incubation (i.e., ≤30 min) the polymerisation process (1) was

probably terminated by κ-CN. Although the proportion of κ-CN in the casein fraction of BCC appears low (Table 1), it is within the range reported for the milk of certain mammalian species, such as equine and human milk (Crowley *et al*., 2016a), and is therefore capable of terminating polymerisation according to (2), with its negatively-charged C-terminal (C) stabilising the structure at the surface:

(2) [CCP] + [(P)β-CN(H)] + [(H)κ-CN(C)].

Consumption of free κ-CN by (2) may have resulted in a gradual shift to a continuous process (1), due to an absence of sufficient polymerisation blocker. Thus, at incubation times of >30 min, the co-precipitation of organic (protein) and inorganic (minerals) materials which occurred (Fig. 7, 8) may have been due to the presence of β-CN/CCP complexes which were not shielded from inter-micelle hydrophobic interactions.

The example in (2) can be used to roughly approximate the radius of the smallest theoretical micelle allowed by this model. Assuming that each dimeric/polymeric arrangement of β-CN molecules associated end-to-end, and binds two complementary CCP units, and that a single κ-CN terminates polymerisation in a given direction (see (2)), with multiplication by 2 for conversion from radii values, the approximate diameter of a stable micelle ($d_{micelle}$) in a BCC system can be calculated according to:

$$d_{micelle} = \left(n\left[d_{\mathrm{CCP}} + 2d_{\beta-\mathrm{CN}}\right] + [\,\mathrm{d}_{\kappa-\mathrm{CN}}]\right) \times 2 - (\,2d_{\beta-\mathrm{CN}} + d_{\mathrm{CCP}}\,) \qquad \text{(eq. 5)}$$

where n = the number of CCP/β-CN complexes.

Inserting diameter (d) values of 6 nm for β-CN monomers (from Fig. 2, BCC/SMUF at 4°C), 5 nm for the exposed portion of κ-CN at the micelle surface (Holt and Horne, 1996), and 4.6 nm for the CCP nanocluster (Holt *et al*., 1998) into eq. 5, $d_{micelle}$ values of 27 nm (n=1, minimum size), 60 nm (n=2), 93 nm (n=3), 126 nm

(n=4) and so forth can be calculated by varying *n*, with each CCP/β-CN complex increasing $d_{micelle}$ by 33 nm. The micelle sizes of BCC/SMUF at 37-60°C ranged from ~60-400 nm, corresponding to *n* values of 2-12.

Based on eq. 5, *d* in BCC systems was dictated by the number of CCP/β-CN complexes within a micelle, while stability depended on the availability of κ-CN. In Figure 3, it can be seen that sedimentation rates for BCC particles increased with increasing temperature, suggesting that continuous polymerisation may have resulted in the creation of larger particles which sedimented more quickly. However, results from pH-drift experiments indicated that CCP formation was complete after only ~5 min of incubation (Fig. 6), meaning that *n* values for κ-CN-terminated micelles were likely unchanged after an initial period of nucleation and polymerisation. Furthermore, particle size actually decreased with increasing temperature from 25 to 60°C (Fig. 2), similar to the results of Beliciu and Moraru (2009) for native CN micelles. Sedimentation rate also increased with increasing incubation time at 37°C (Table 2). Incubation times of >30 min yielded insoluble material with a constant protein:ash ratio, but solvation of the protein decreased markedly on increasing incubation time from 90 min to 150 min. A model for the self-association of CN in BCC/SMUF based on the data discussed in this section is described below.

Proposed model for self-association behaviour in BCC systems

From the results of this study, the principal reactions underpinning protein self-association in BCC can be postulated as depicted in Figure 10, which aids in the explanation of some of the observed physicochemical properties of these complexes. This model borrows generously from key ideas presented by both David Horne and Carl Holt for the structure of native casein micelles in milk. The nature of the interactions between CCP and hydrophilic regions of the caseins was adapted from Holt (1992, 1998), while the formation of the internal structure of the micelle by hydrophobic interactions was based on Horne (1998). However, the model in Figure 10 is used to describe the BCC solutions, which has a very different casein profile compared to bovine milk, as discussed earlier.

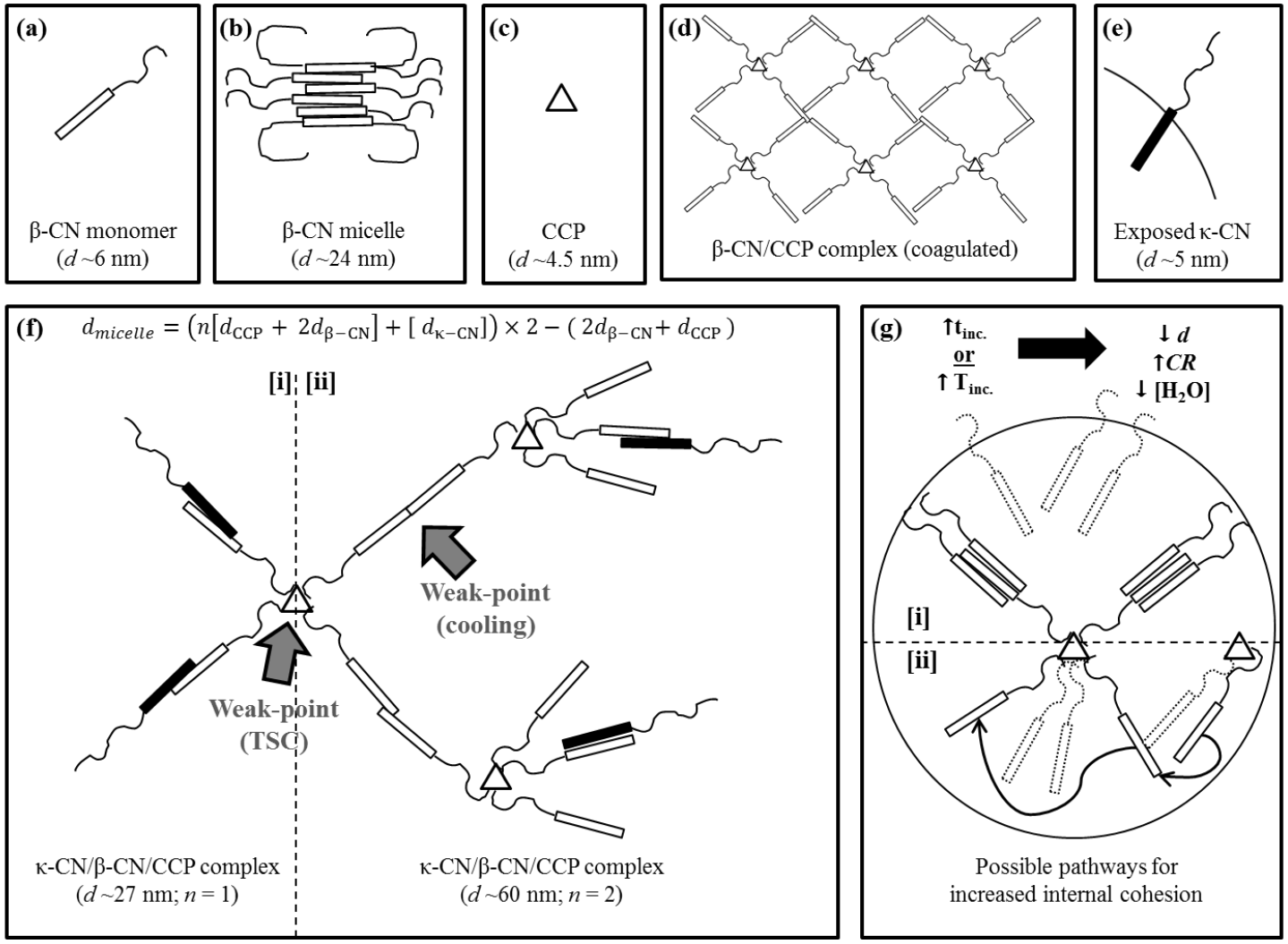

Fig. 10. Proposed model for the self-association of casein (CN) in β-casein concentrate (BCC) solutions dissolved in simulated milk ultrafiltrate (SMUF). CCP = colloidal calcium phosphate, d = diameter, n = number of size-determining CCP-β-CN-β-CN complexes, $t_{inc.}$ = incubation time, TSC = tri-sodium citrate, $T_{inc.}$ = incubation temperature, CR = clarification rate, [H_2O] = protein solvation.

At low temperature (<5°C) monomeric β-CN (Fig. 10a) predominates due to negligible hydrophobic interactions and low CaP supersaturation. In the absence of CaP, β-CN micelles form with a hydrophobic core, hydrophilic shell and overall crab-like shape (Fig. 10b). In supersaturated CaP solutions (i.e., SMUF >20°C), the phosphoserine residues at the of β-CN monomers act as a nucleation site for CCP (Fig. 10c); in a system devoid of free κ-CN, the solvent-facing orientation of β-CN's hydrophobic region would cause continuous hydrophobically-driven polymerisation culminating in coagulation (Fig. 10d). However, in the BCC system studied there was sufficient κ-CN to act as a polymerisation blocker, resulting in the formation of stable micellar complexes as illustrated in Figure 10(f).

The size of the stable complexes was determined by Eq. 5, as shown in regions [i] and [ii] of the micelle bisected in the vertical plane (Fig. 10f). The extensive micellar dissociation measured when incubated BCC/SMUF was cooled to <5°C was due to [(P)β-CN(H)] + [(H)β-CN(P)] regions, which were vulnerable to dispersion when hydrophobic interactions were low, while the integrating strength of CCP was less weakened by cooling but was eliminated by TSC (Fig. 10f); therefore, the residual turbidity detected in cooled BCC/SMUF solutions incubated at ≥37°C was likely due to the persistence of micelles with an *n* value of 1.

Destabilisation of BCC/SMUF systems occurred on extended incubation and coincided with an increasing quantity of insoluble material; this effect was likely due to the coagulation of κ-CN-free complexes, as depicted in panel (d) of Figure 10, when κ-CN became mostly confined to the (stable) micellar phase shown in panel (f). The loss of protein at incubation times of 90 min-210 min was broadly similar, reaching a maximum of 7-13% based on both abs. 280 nm and TGA data; this suggests that the proportion of κ-CN in BCC, although much lower than in bovine milk, is sufficient to stabilise the majority of the β-CN in the system even after extended incubation.

The decrease in micelle size between 25 and 63°C after 30 min can be explained by the strengthening of internal hydrophobic interactions. The micelle shown bisected in the horizontal plane in Figure 10 (g) shows how this may be caused by incorporation of additional β-CN into the micellar phase [i] or through increased cohesion between hydrophobic groups within the micelles [ii]. These

effects may have had a role in the increased turbidity and decreased physical stability at increasing temperatures, due to increased micellar density; this effect may also have occurred on extended incubation, which would explain the desolvation of sedimentable protein on increasing incubation time from 90 to 150/210 min, potentially caused by expulsion of water from the micellar framework as it contracted. The model (Fig. 10) is based on the experimental data generated during the present study. Further work will be required to verify if it holds when exposed to certain conditions, such as renneting, which would allow the proposed stabilising role of κ-casein to be tested.

5. Conclusion

β-CN ingredients prepared using recently developed membrane separation processes have self-association behaviour which deviates from analytical-grade β-CN due to their unique CN profile. In this study, such an ingredient, termed 'BCC', was assessed for its association and dissociation behaviour. Both the extent and reversibility of self-association was confirmed to be influenced by reconstitution temperature, serum-phase composition, incubation temperature and time. A key finding was that β-CN complexes could stabilise a supersaturated solution of CaP, such as exists in milk; in turn, CaP promoted the formation of β-CN complexes which were more resistant to dissociation. Irreversible, albeit limited, coagulation occurred on extended incubation, which was attributed to a continuous polymerisation process mediated by hydrophobic interactions. The nature of the association and dissociation characteristics of protein-mineral complexes in BCCs could have important implications for their use in certain applications, such as the humanisation of infant formulae, encapsulation of drugs or bioactives and the stabilisation of emulsions and foams. Future work will seek to verify the proposed model for self-association in BCCs and establish potential implications for the techno-functionality of BCC-type ingredients.

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Chapter 11

Micellar casein concentrate powders made using cold microfiltration of skim milk have improved rehydration characteristics

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Declaration

This chapter was written by author SVC and reviewed by his co-authors. SVC co-designed the study, provided analytical training/support to EB and analysed the data. EB assisted with contact angle, conductivity, particle size and analytical centrifugation experiments. JVCS carried out confocal laser scanning microscopy and helped with calcium-ion analysis. NAM, HBW and MAF provided the powders, which were prepared at pilot scale in Teagasc, powder composition data and colloidal data on the powders after reconstitution.

Abstract

Microfiltration (MF) of skim milk is used to fractionate casein micelles from whey proteins and non-protein constituents. When combined with diafiltration (DF), this allows the manufacture of liquid micellar casein concentrate (MCC), which can be spray-dried into high-protein (≥80% protein, dry-basis) powders. MCC powders rehydrate very slowly, which is considered a defect in most industrial handling operations. This study investigated if cold (<10°C) or warm (50°C) MF/DF altered the rehydration characteristics of the powders (MCC_{cold} and MCC_{warm}, respectively). The wetting properties of the MCC powders were equivalent, but pronounced differences in dispersion characteristics were measured. After 90 min rehydration at 50°C, casein micelles accounted for only 7.5% of particle volume in MCC_{warm} compared to 48% in MCC_{cold}. As a result of its superior dispersion characteristics, MCC_{cold} yielded 50-60% less sediment during analytical centrifugation experiments. These results indicate that cold MF/DF is a viable option to improve the solubility of MCCs.

1. Introduction

Of the 35 g L^{-1} protein in bovine milk, approximately 28 g L^{-1} consists of caseins (mainly present as casein micelles), with the remainder being whey proteins (O'Mahony and Fox, 2013). Casein micelles are large (mean hydrodynamic diameter ≈ 150 nm) assemblies of four individual phosphoproteins (α_{s1}-, α_{s2}-, β-, and κ-casein), which bind ~ 67% of calcium and ~ 50% of inorganic phosphate in milk as colloidal calcium phosphate (CCP). Casein has long been fractionated from milk and converted into powders for use in both non-food, e.g., paint, glue (Audic *et al*., 2003) and food-based, e.g., analogue cheese, cream liqueur (O'Mahony and Fox, 2013) applications. Two traditional methods of purifying casein are isoelectric precipitation through acidification ('acid casein') or enzymatic hydrolysis with rennet ('rennet casein'). Both of these approaches yield a solid curd, which can be mechanically disrupted and dried into powders that are insoluble. Additional processing steps are required to generate a soluble material for drying; for example, acid casein can be converted into sodium caseinate through alkalisation (Carr and Golding, 2016).

A more recent technology to manufacture soluble casein in its 'native' (micellar) form is microfiltration (MF), a pressure-driven separation process incorporating semi-permeable membranes with a pore-size of ~0.1 µm (Pierre *et al*., 1992; Saboya and Maubois, 2000). Diafiltration (DF) with deionised water during MF facilitates the production of MCCs in which the protein fraction is usually 85-95% casein. MCCs are often used as ingredients in protein supplements and clinical nutrition products. As these applications can require reconstitution of MCCs by the manufacturer (i.e., wet mixing of dry ingredients) and/or the consumer (i.e., reconstitution of dried blended powders for consumption), it is preferable that the powders rehydrate quickly and completely. For milk powders, the rehydration process is characterised by multiple stages, including wetting of the powder when added to the liquid and subsequent dispersion of the powder particles; the duration of these stages, wetting and dispersion, have been reported to be protracted for high-protein powders (Crowley *et al*., 2016). For MCCs the wetting stage, and in particular the dispersion stage, are slow. Poor dispersion characteristics can negatively affect powder handling in manufacturing facilities, due to blockages in process lines, and impair consumer acceptability of final products, due to the presence of lumps or sediment (Mitchell *et al*., 2015). Although MCC powders are

soluble, they exhibit exceptionally long rehydration times in comparison to other milk-derived powders (e.g., skim milk powder, whey protein concentrate, sodium caseinate); milk protein concentrate powders (MPCs) have a similar challenge, although it is less pronounced than for MCCs (Crowley *et al*., 2016) due to the higher proportion of the more soluble whey proteins in the former (Mimouni *et al*., 2010).

Various strategies, typically targeted towards improving dispersion characteristics, have been developed to improve the rehydration of MCCs and MPCs. Many successful approaches have been based on pre-treatments applied to concentrated protein fractions from milk prior to drying, such as that of Bhaskar *et al*. (2001), in which calcium was removed from ultrafiltered milk by ion-exchange before mixing with untreated concentrate and drying into an MPCs powders. Incorporation of sodium caseinate into the concentrate before the drying of MCC increased its solubility (Schokker *et al*., 2011), while the application of high-pressure treatments to the concentrate before drying of MPCs also resulted in solubility enhancement (Udabage *et al*., 2012). Bouvier *et al*. (2013) investigated an alternative drying technology, extrusion-porosification, which was found to produce a more soluble powder than spray drying. Others have developed strategies which can be applied during powder reconstitution itself, with various researchers reporting that high-shear and ultrasonication technologies were effective when applied during reconstitution of MCCs and MPCs (Augustin *et al*., 2012; McCarthy *et al*., 2013; Chandrapala *et al*., 2014a). Increasing either temperature or the number of stirrer revolutions during rehydration improved the rehydration properties of an MCC (Jeantet *et al*., 2010). An elevated reconstitution temperature, combined with an increased level of monovalent salts (KCl), was more effective in promoting the dispersion of MPCs than either method alone (Crowley *et al*., 2015).

There is a lack of options available for processors who wish to improve the rehydration performance of MCCs without potentially: (I) incurring significant capital expenditures (to procure solubility-enhancing equipment); (II) modifying ingredient techno-functionality (by replacing or dissociating micellar casein); or (III) altering ingredient listings (through the use of additives). It is perhaps surprising therefore that the temperature at which MF is carried out during MCC manufacture has not received more attention. MF in the dairy industry have traditionally been

performed at ~50°C, which is optimal for high permeate flux and efficient removal of whey proteins (Hurt *et al*., 2015); however, MF (and also ultrafiltration) at temperatures <15°C is becoming more common in the dairy industry (Lawrence *et al*., 2008), and studies have shown that this may facilitate operation with lower fouling (Luo *et al*., 2015), and enrichment of β-casein in the whey protein stream (Coppola *et al*., 2014; O'Mahony *et al*., 2014), in addition to modifications of the gelation (O'Mahony *et al*., 2009) and emulsifying (Luo *et al*., 2015) properties of the MCC fraction. However, no studies have reported the influence of MF temperature on the rehydration characteristics of resultant MCC powders. In the present study, two MCCs were manufactured, one using traditional warm MF (50°C, MCC_{warm}) and another using cold MF (<10°C, MCC_{cold}) before drying. The rehydration characteristics of these powders were then compared using a range of analytical techniques to understand the impact of MF temperature on rehydration characteristics of MCC powders.

2. Materials and methods

2.1 Manufacture of micellar casein concentrate powders

The two MCC powders were manufactured as described by McCarthy *et al*. (2016). The processes can be briefly summarised as follows; skim milk was batch-diafiltered 1:2 with reverse osmosis (RO) water and held overnight at ~4°C, before holding at <10°C or 50°C prior to membrane filtration. MF/DF of the milk was performed with 0.14 µm Tami Isoflux® ceramic membranes (Tami Industries, Nyons Cedex, France) on a GEA Model F filtration unit (GEA Process Engineering A/S, Skanderbog, Denmark) operated in retentate recirculation mode. The temperature throughout processing was maintained at <10°C or 50°C using an in-line heat exchanger. MF was performed until the volume of the milk/RO water was reduced by a factor of 9. Liquid MCCs were then evaporated using a Tetra Scheffers® falling-film single-stage evaporator (Tetra Pak, Gorredijk, the Netherlands). Spray drying of the MCCs was carried out using a pilot scale Anhydro Lab 3 spray dryer (SPX Flow Technology A/S, Soeborg, Denmark) with a wheel atomizer operating with inlet and outlet temperature of 178°C and 88°C, respectively. All subsequent analyses and experiments (Sections 2.2-2.6) on the two MCC powders were performed in at least

duplicate, with results presented as the means of at least two independent measurements on freshly prepared samples.

2.2. Composition of powders and colloidal properties of reconstitutes solutions

Protein content of the MCC powders was measured by the Kjeldahl method using a nitrogen-to-protein conversion factor of 6.38 (IDF, 2001). Fat and moisture were determined by the Rose-Gotlieb method (IDF, 2008) and oven drying (IDF, 2004), respectively. Mineral profiling was carried out using inductively-coupled plasma mass spectrometry (Herwig *et al.*, 2011). The size and charge of casein micelles in reconstituted solutions was assessed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) according to McCarthy *et al*. (2014). Protein profile was determined by reversed phase-high performance liquid chromatography (RP-HPLC), as detailed by McCarthy *et al*. (2016).

2.3. Distribution of protein and fat in powder particles

The distribution of protein and fat in MCC powder particles was determined using a Leica TCS SP5 confocal laser scanning microscope (CLSM; Leica Microsystems CMS GmbH, Wetzlar, Germany). Dual labelling using Nile Red (0.1% w/v in propanediol) and Fast Green FCF (0.01% w/v in water) was carried out to visualise the protein and fat phases, respectively, in the powder particles. The dye solutions were mixed in a ratio that allowed diffusion of the dyes into the powder particles whilst preventing their solubilisation, as proposed by Maher *et al*. (2015). The observations were performed using 63× oil immersion objective (numerical aperture = 1.4) at excitation wavelengths of 488 nm and 633 nm provided by Ar and He/Ne lasers. Images of 512 × 512 pixels in size were acquired using zoom factor of 3. At least three specimens of each sample were examined to obtain representative images.

2.4. Wetting behaviour: Optical Tensiometry

Measurements of contact angle were carried out according to the Sessile drop method using a Theta Optical Tensiometer (Attension, Biolin Scientific Ltd., Espoo, Finland). Discs (d ≈ 13 mm, h ≈ 1.5 mm) of MCC powders were prepared by compression of powders with a Specac® manual hydraulic press (Perkin Elmer, Buckinghamshire, UK), which applied a load of 8,000 kg. A droplet of deionised water (5 µL) was placed on the disc and contact angles were measured over time at 20°C.

2.5. Ion release: Conductimetry and calcium-ion concentration

Monitoring of ion release during the rehydration of MCC powders was carried out using a Titrando autotitrator and accompanying Tiamo v2.3 software equipped with either a five-ring conductivity measuring cell or a calcium (Ca)-ion-selective electrode (Metrohm Ireland Ltd, Athy Road, Co. Carlow, Ireland). The probes were calibrated at 25°C or 50°C (depending on the rehydration experiment temperature) with buffer solutions of known conductivity or Ca-ion concentration. A period of 1 min was allowed to elapse for establishment of a baseline before the powder was added, over a period of 2 min, with continuous measurement throughout. Ca-ion standards were prepared to have an ionic strength equivalent to that of milk or milk serum (80 mM). MCC suspensions have a much lower ionic strength than milk, particularly at the early stages of rehydration, so the Ca-ion concentration data reported are not *true* values, and, in fact, are likely to be underestimated due to the higher ion activity coefficient of Ca at low ionic strength; however, these data provide a means to discriminate between differences in the Ca-ion release behaviour of the two MCC powders.

2.6. Dispersion: Particle size distribution and analytical centrifugation

A Malvern Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK) was used to measure the particle size distribution (PSD) in MCC suspensions after rehydration for 90 min. Analysis of PSD was performed using a particle refractive index of 1.46, absorption of 0.1 and dispersant refractive index of 1.33. MCC

suspensions were introduced into the dispersing unit of the instrument with deionised water as dispersant until a laser obscuration of 12.5 ± 1% was achieved. Data are presented as volume-based PSDs.

To measure the sedimentation behaviour in MCC suspensions rehydrated for 90 min, an analytical centrifuge (LUMISizer®, L.U.M. GmbH, Berlin, Germany) was used according to the method of Crowley *et al*. (2015), in which the intensity of transmitted NIR light (880 nm) was measured as a function of time and position over the length of a polycarbonate cell held horizontally over the light path during centrifugation. The height of initial sediments formed after centrifugation at 36g for 10 min, and the compressed sediments formed during subsequent centrifugation at 168g for 10 min, were measured by subtracting the position of the supernatant/sediment boundary from the position of the cell bottom. Mean transmission values were also calculated for the region above the (compressed) sediment and below the meniscus (114-124 mm, common for all experimental runs). Transmission values can be considered the inverse of turbidity, with low values indicating a high degree of light-scattering. As the light source was in the NIR region, scattering can be considered to dominate relative to absorption (Omar and Matjafri, 2009).

3. Results

3.1. Composition and physicochemical properties of powders

Operation of the MF/DF process at <10°C or 50°C resulted in several differences between the MCC_{cold} and MCC_{warm} powders. The proportion of whey protein was higher in the former, the β-casein:α_s-casein ratio was lower, and there was a decrease in the level of both calcium and phosphorus (Table 1). In addition, there was a slightly higher level of fat in the MCC_{warm} powder. Levels of the monovalent ions measured were, however, relatively unchanged. The influence of processing temperature on colloidal properties of reconstituted MCCs was also comparatively minor, but the size and net negative charge of the casein micelles was slightly higher for the MCC_{cold} (Table 1).

Table 1. Composition and colloidal properties of micellar casein concentrate (MCC) powders manufacture using microfiltration at < 10 °C (cold) or 50 °C (warm).

Property	MCC	
	Warm	Cold
Protein		
Total (%, w/w)	75.5	74.5
Total (% solids)	79.0	77.8
Whey (% protein)	9.02	13.8
β-CN:α_s-CN ratio	1.0:1.0	1.0:1.1
Total fat (%, w/w)	1.4	1.1
Minerals (mg g^{-1})		
Sodium	2.33	2.13
Potassium	8.17	8.71
Calcium	28.8	25.0
Phosphorus	19.1	17.7
Micellar phase		
Size (nm)	147	153
Zeta potential (mV)	-25.1	-26.9

3.2. Component distribution in MCC powder particles

Representative CLSM images of the MCC powders are shown in Figure 1. The particles in both MCC powders were characterised by large protein-dense regions interspersed with a minor proportion of fat globules, which surrounded internal air vacuoles (black regions). The main difference observed between the two MCC powders appeared to be the greater number and size of the fat globules in the MCC_{warm} powder compared to the MCC_{cold} powder.

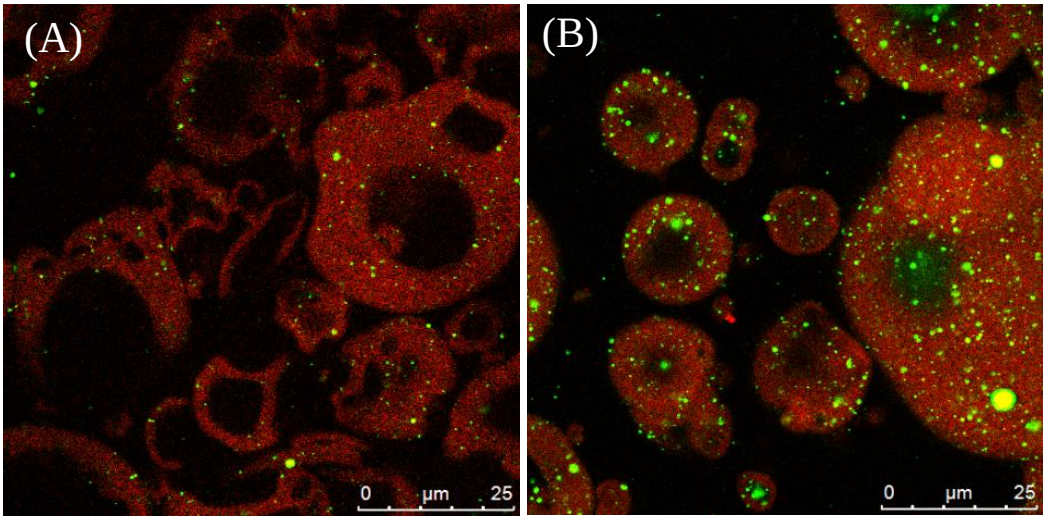

Fig. 1. Representative confocal laser scanning microscopy images of micellar casein concentrate (MCC) powders manufactured by microfiltration at <10°C (A) or 50°C (B), followed by evaporation and spray drying. Green indicates fat and red indicates protein, while black regions within particles are air vacuoles.

3.3. Wetting behaviour of MCC powders

Wetting behaviour was analysed by measuring the contact angle formed between a droplet of water and a compressed disc of each MCC powder. Assuming that interference from topological differences was negligible, a high value for contact angle indicates that a powder is less wettable (i.e., more hydrophobic), while a reduction in contact angle over time is caused by spreading at the surface (Mitchell *et al*., 2015). The data from these experiments showed that there were no apparent differences in the initial wetting behaviour of the powders on initial contact with the droplet or over time (Fig. 2).

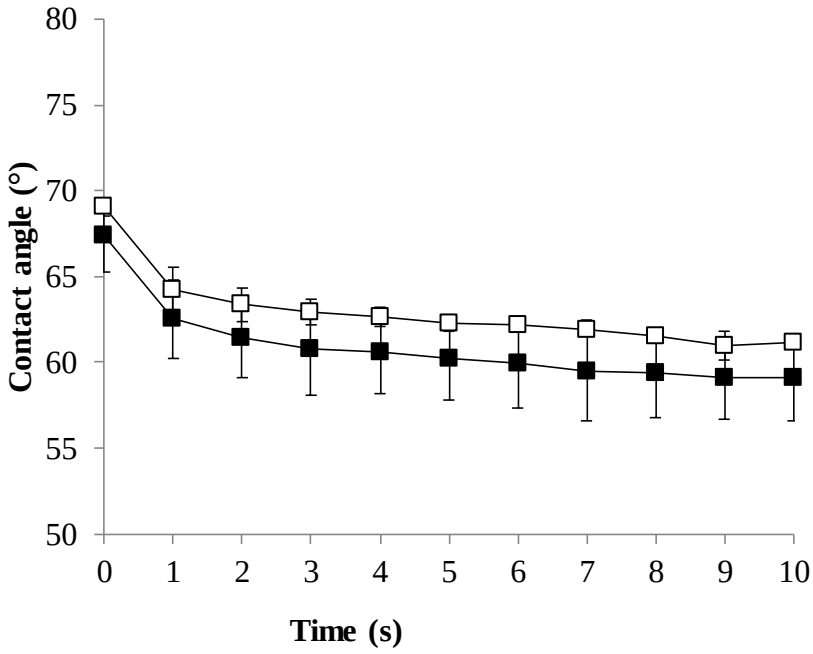

Fig. 2. Contact angle values over time for a droplet of deionised water deposited on compressed discs of micellar casein concentrate (MCC) powder manufactured by microfiltration at <10°C (□) or 50°C (■) followed by evaporation and spray drying. Data points are the mean ± standard deviation of replicate experiments (n = 3).

3.4. Ion release from MCC powders during rehydration

Conductivity was measured continuously during the rehydration of the MCC powders. There was an initial sharp increase in conductivity as ions were released from the powder on introduction to water and an eventual steady-state condition as the release of ions was completed (Fig. 3A). For both powders, rehydrated to 1.5% protein, the time to reach steady-state was ~3000 s (Fig. 3A). Rehydration at 50°C resulted in a higher conductivity reading throughout the experiment compared to rehydration at 25°C, due to increased diffusion coefficients for the ions at the higher temperature; however, no trends for the effect of rehydration temperature on the time to reach steady-state conductivity were observed.

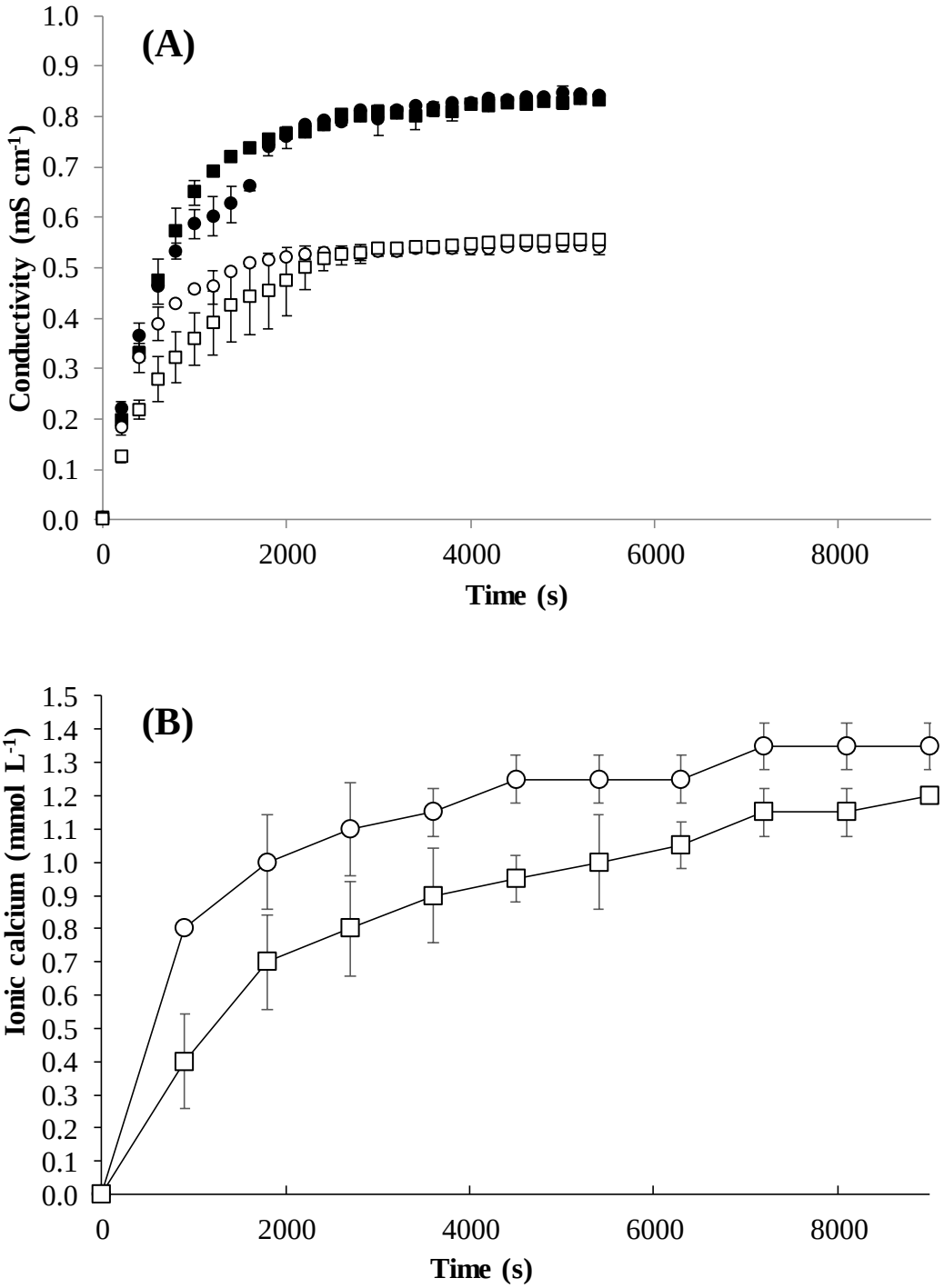

Fig. 3. Conductivity (A) and calcium-ion concentration (B) over time during the rehydration of micellar casein concentrate (MCC) manufactured by microfiltration at <10°C (○, ●) or 50°C (□, ■); open and closed symbols represent powders rehydrated at 25 and 50°C, respectively. Data points are the mean ± standard deviation of replicate experiments (n =2).

The final conductivity was directly proportional to the amount of powder added to the water (data not shown). More pronounced differences in ion release were detected when ionic Ca was measured in isolation (Fig. 3B). The MCC_{cold} powder exhibited a faster release of Ca, a quicker return to steady-state, and a higher total Ca level throughout.

3.5. Dispersion behaviour of MCC powders

The progression of dispersion for a casein-dominant powder such as MCC can be tracked by measuring the PSD after a period of rehydration (Crowley *et al*., 2015). The dispersion process of a MCC powder can be considered as comprising primarily of the disappearance of micron-sized primary powder particles (after wetting and submersion) and the release of nanometer-sized casein micelles; when this process is complete, the powder can be considered dissolved. In Figure 4A, it can be seen that primary particles dominate the PSD after 90 min rehydration at 25°C; this does not necessarily mean that casein micelles have not been released, but only that they are contributing little to the overall particle volume. Under these conditions, the particles in the MCC_{warm} were notably larger, indicating that dispersion was less advanced. When dispersion was promoted by increasing temperature of reconstitution to 50°C a casein micelle population was apparent for both powders (Fig. 4B). The MCC_{cold}, however, contained a much higher proportion of casein micelles after 90 min rehydration at 50°C compared to the MCC_{warm}, indicating that the former powder had far better dispersion characteristics; from Table 2, it can be seen that these rehydration conditions led to almost 50% of the particle volume in the MCC_{cold} being comprised of casein micelles (<1 μm), while this proportion was only <10% for the MCC_{warm}.

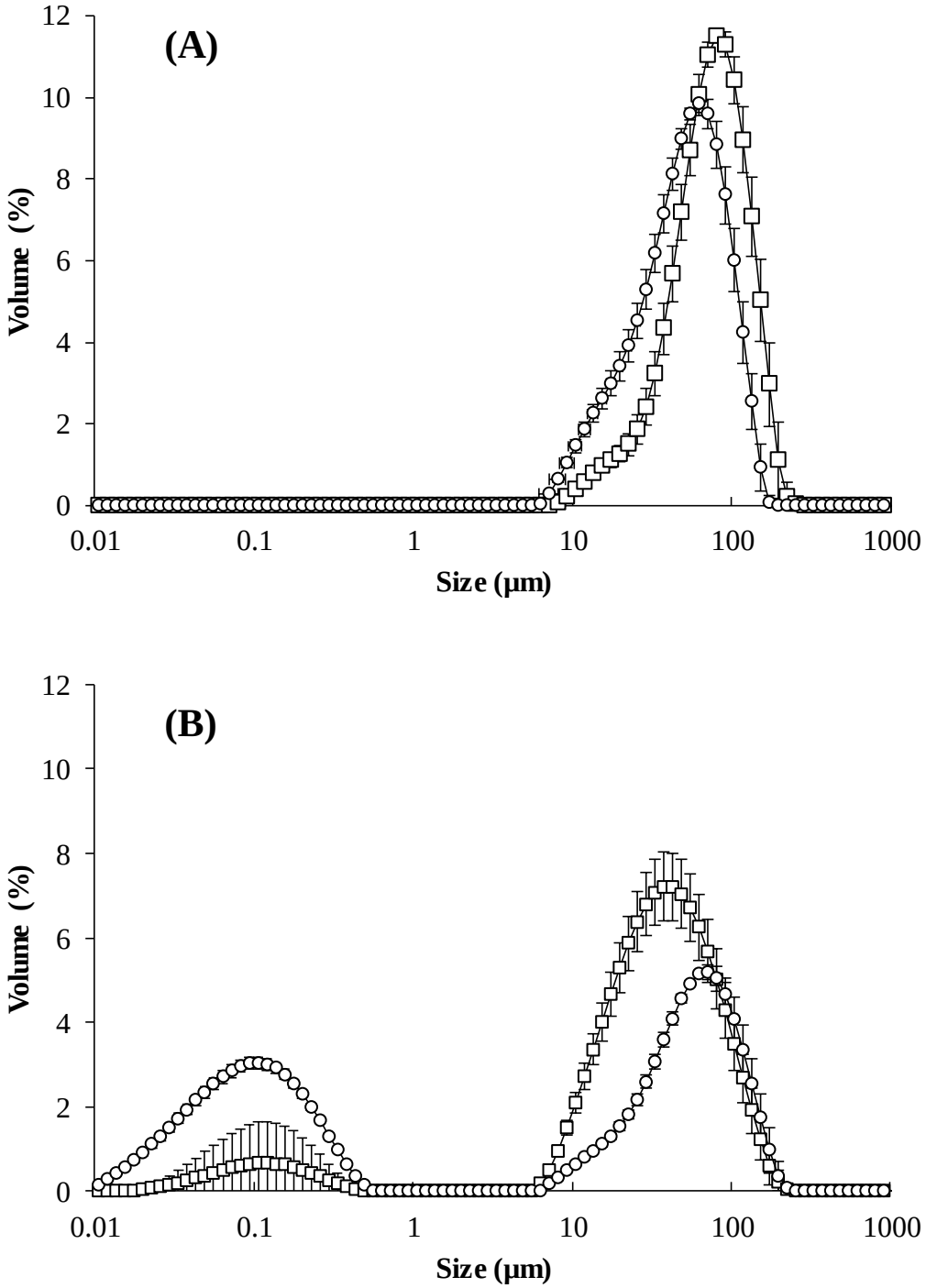

Fig. 4. Particle size distributions for micellar casein concentrate (MCC) powder manufactured by microfiltration at <10°C (○) or 50°C (□), followed by evaporation and spray drying, and rehydrated at 25 °C (A) or 50 °C (B) for 90 min. Data points are the mean ± standard deviation of replicate experiments (n = 3).

Table 2. Proportion of particles in specific size classes after rehydration of micellar casein concentrate (MCC) powders at 25 or 50 °C for 90 min.

	Rehydration temperature			
	25°C		**50°C**	
Size class (μm)	MCC_{warm}	MCC_{cold}	MCC_{warm}	MCC_{cold}
	% of total particle volume			
0-1	0.00	0.00	7.50	48.2
1-10	0.24	0.07	2.50	0.91
10-50	26.1	49.8	58.3	25.5
50-100	43.8	38.5	23.3	22.7
100-1000	29.8	11.7	8.33	2.73

To investigate the influence of these differences in dispersion state (Fig. 4, Table 2) on the sedimentation behaviour of the MCC suspensions on rehydration, an analytical centrifuge was used. In Figure 5, representative sedimentation profiles are shown for each MCC after rehydration at 25 or 50°C, indicating the transmission (T%) through the length of the sample cell during centrifugation. Reading from left to right, these profiles represent an increasing distance from the rotor of the centrifuge, and can be characterised by an initial high T% region (air), a boundary region (meniscus), a long low T% region (suspension) leading to a sharp reduction in T% (sediment). The depressed T% in the region above the meniscus for MCCs rehydrated at 50°C, which was also observed in our previous study on MPCs (Crowley *et al*., 2015), is noted, and is likely due to condensation. In addition, it was observed that T% profiles were noisier for the earlier profiles, which was attributed to the presence of an inhomogeneous population of wetted powder particles in suspension prior to their sedimentation.

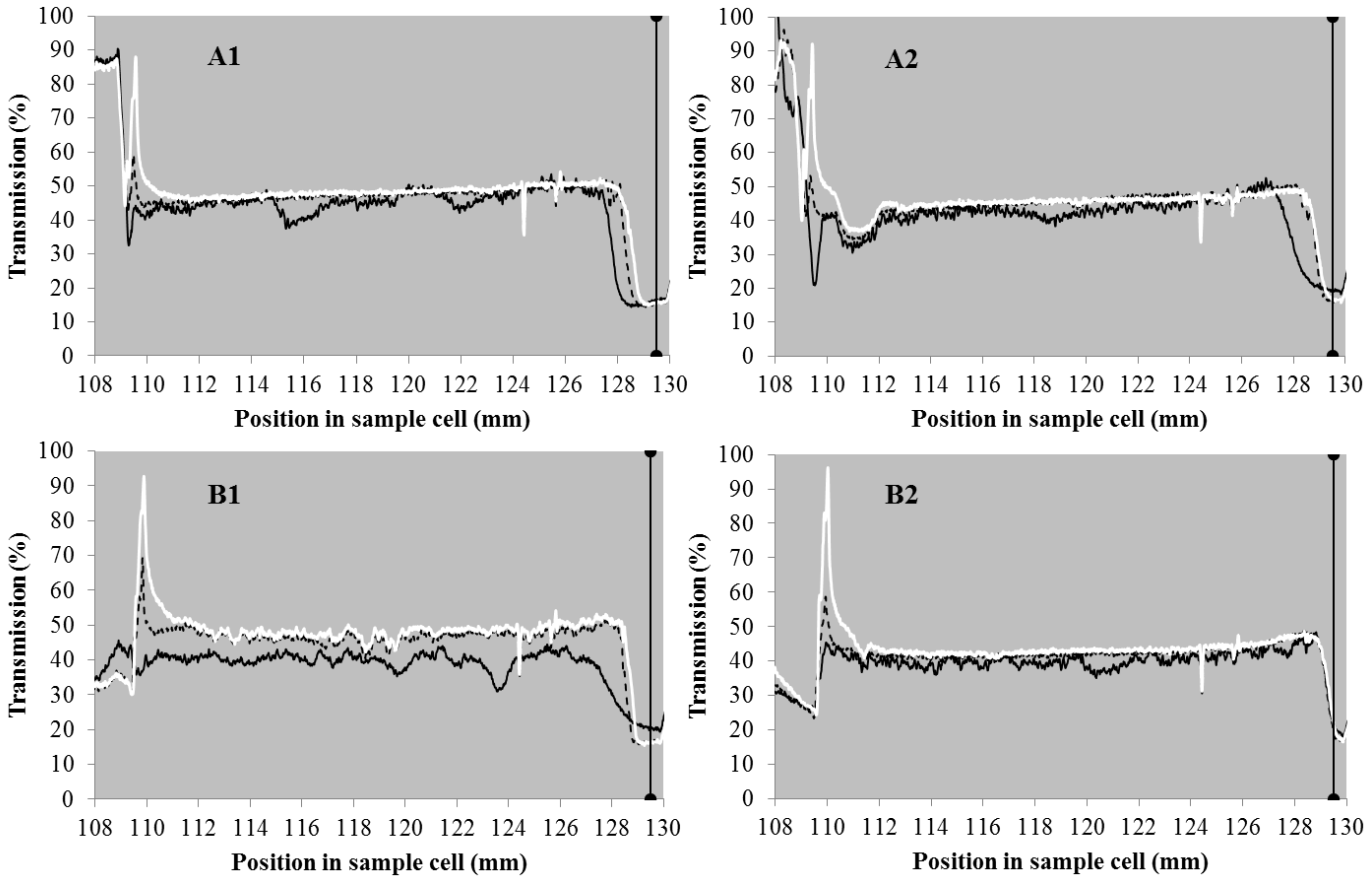

Fig. 5. Sediment formation and compression during the centrifugation of suspensions of micellar casein concentrate (MCC) powders manufactured by microfiltration at <10°C (MCC_{cold}) or 50°C (MCC_{warm}), followed by evaporation and spray drying, and rehydrated at different temperatures for 90 min. (A1) MCC_{warm} rehydrated at 25°C, (A2) MCC_{cold} rehydrated at 25°C, (B1) MCC_{warm} rehydrated at 50°C and (B2) MCC_{cold} rehydrated at 50°C. Three profiles are indicated for each sample: the first detected profile (black line), the profile after the first centrifugation step of 36g for 10 min (broken black line) and the profile after the second centrifugation step of 168g for 10 min (white line). The cell bottom (129.5 mm) is indicated by a vertical black line to guide the eye.

A larger sediment was observed for the MCC_{cold} compared to the MCC_{warm} on rehydration at 25°C, both of which became compressed at the second (higher) centrifugation speed. Increasing rehydration temperature to 50°C appeared to reduce the amount of sediment formed (Fig. 5). These profiles were used to calculate sediment heights and mean T% values for the MCC powders rehydrated at different temperatures (Fig. 6).

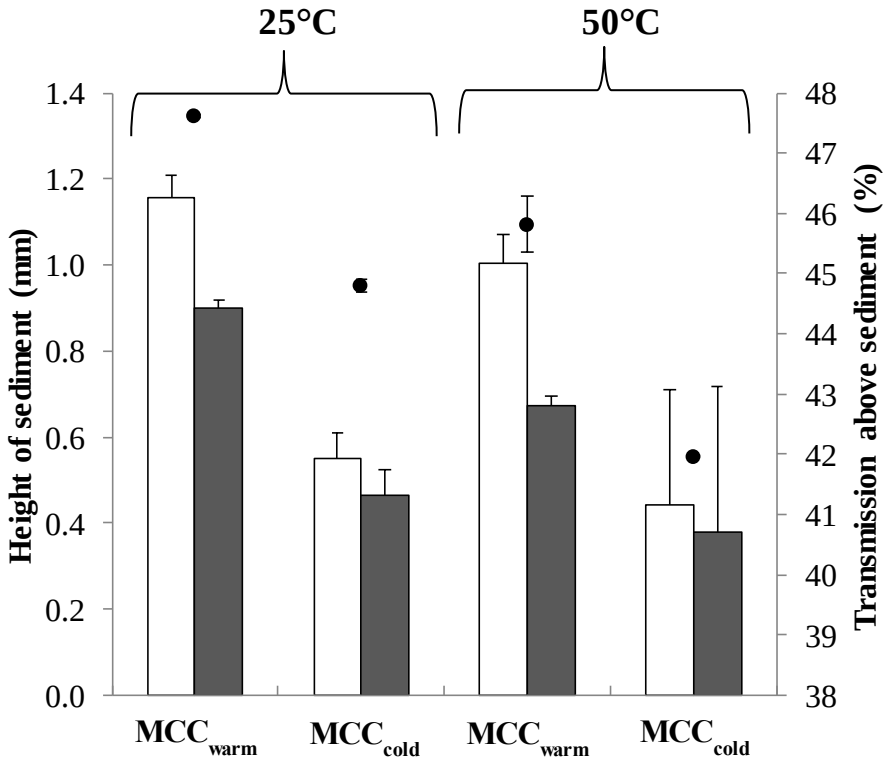

Fig. 6. Height of sediment (bars) and transmission of near-infrared light above the sediment (scatter-plot) during analytical centrifugation experiments of micellar casein concentrate (MCC) manufactured by microfiltration at <10°C (MCC_{cold}) or 50°C (MCC_{warm}). Rehydration experiments were performed at 25 or 50°C. White bars indicate the initial height of sediment after 10 min at 36g and grey bars represent compressed sediments after an additional 10 min at 168g. Transmission values (●) were taken for each MCC at both rehydration temperatures after the full 20 min centrifugation cycle. Data points are the mean ± standard deviation of replicate experiments (n=2).

After 10 min at 36g, the height of sediments formed from MCC_{cold} suspensions were 53-56% smaller than sediments from the MCC_{warm}, The dispersion of both MCCs was promoted by the higher rehydration temperature (i.e., reduced

sediment height), although the reduction in sediment height associated with an increase in rehydration temperature from 25 to 50°C was greater for the MCC_{warm} (20% reduction) than the MCC_{cold} (14% reduction). The larger sediments formed in the MCC_{warm} were also more susceptible to compression when subjected to a second centrifugation step at 168g for 10 min. Based on the data in Figure 6, MCC_{warm} sediments compressed by 22 and 33% after rehydration at 25 and 50°C, respectively, while the equivalent values for MCC_{cold} sediments were 16 and 14%.

4. Discussion

Milk-derived powders which contain >70% protein, of which 80-95% is micellar casein, are known to have poor rehydration properties; in particular, the release of discrete casein micelles from powder particles in MCCs and MPCs is slow due to the poor dispersion characteristics of the powder particles (Gaiani *et al*., 2007; Crowley *et al*., 2015), which has been linked with inhibited transfer of water into the powder (Richard *et al*., 2013; Vos *et al*., 2016). In this study, the influence of MF temperature on the rehydration performance of MCC powders was investigated. Cold MF was found to have a positive impact on the rehydration characteristics of MCC, due to modifications in the composition, and, perhaps, the colloidal properties of the powder (Table 1).

Some of the changes in MCC composition caused by the lower MF temperature (<10°C) compared to the higher MF temperature (50°C), including a higher proportion of whey proteins and a reduction in Ca content (Table 1), have been demonstrated by previous researchers to improve the rehydration of casein-dominant powders. Indeed, Richard *et al*. (2013) showed that increasing the level of whey proteins in MCC powders improved their dispersibility, while Bhaskar *et al*. (2001) developed a method of improving the dispersion of MPCs based on removal of Ca. The lower casein:whey protein ratio in the MCC_{cold} was due to a reduced efficiency of whey protein removal at lower filtration temperatures (Karasu *et al*., 2010; O'Mahony *et al*., 2014), while the lower Ca level was caused by dissolution of calcium phosphate from the micellar phase at the low temperature (Luo *et al*., 2015). It is possible that other modifications to the casein fraction caused by the lower MF temperature, such as the reduced β:α_s-casein ratio and the increased zeta-

potential of the casein micelles (Table 1), may have influenced the rehydration characteristics of the MCCs. β-Casein is the most hydrophobic of the caseins, and its depletion may make the MCC_{cold} better at absorbing water; however, this is not supported by contact angle data (Fig. 2). Alternatively, the tendency for casein micelles to become inter-linked, resulting in the formation of a poorly-dispersible 'skin' (Crowley *et al*., 2016), may be inhibited by alterations in micellar structure (due to decreased β-casein:α_s-casein ratio) or increased electrostatic repulsion (caused by increased zeta-potential). It is, however, difficult to ascertain the influence of these factors compared to differences in the levels of calcium and whey protein, the importance of which to milk protein powder rehydration have been more widely reported.

Gaiani *et al*. (2009) has previously shown that fat migration to the surface of powder particles during storage is an important factor influencing the rehydration behaviour of MCCs, in particular the wetting behaviour. In this study, there seemed to be a greater number and size of fat globules in the MCC_{warm} (Fig. 1), probably due to the higher fat level in this powder (Table 1), but this did not influence the wetting behaviour of the MCCs, which were equivalent (Fig. 2). Trends in conductivity during the rehydration of the MCC powders were also essentially the same for the two MCC powders (Fig. 3A), likely due to domination of conductivity changes by ions such as Na, Cl and K which are released quickly during rehydration (Mimouni *et al*., 2010). However, the release of ionic Ca was faster and progressed to a greater degree during the rehydration of MCC_{cold} compared to MCC_{warm} (Fig. 3B). As a large proportion of Ca is associated with casein micelles in casein-dominant powders, a delay in its ionisation may be due to a slow release of micelles during rehydration, which would reduce the rate at which Ca is re-equilibrated from the micellar to the serum phase (Mimouni *et al*., 2010).

Measurement of particle size after 90 min rehydration confirmed that the dispersibility of the two MCC powders was different (Fig. 4). Dispersion of primary powder particles was far more advanced in the MCC_{cold} powder after this period of rehydration, which resulted in a greater proportion of discrete casein micelles being released, particularly after rehydration at 50°C (Table 2). Increasing rehydration temperature above ambient is commonly used to promote the dispersion of these

powders (Jeantet *et al*., 2010), and these results indicate that MCC_{cold} is more susceptible to the positive influence of this approach compared to MCC_{warm}.

As a result of the lower levels of primary powder particles in suspension after rehydration (Fig. 4), MCC_{cold} yielded approximately half of the sediment that MCC_{warm} produced during centrifugation (Fig. 5 and 6). The turbidity of the supernatant after sedimentation was higher for MCC_{cold} (Fig. 6) as more casein micelles had been released into a stable suspension and were capable of scattering light. The highest turbidity and lowest sediment were measured in the MCC_{cold} rehydrated at 50°C, indicating it had the fastest and most complete dispersion properties. In addition to a greater degree of sedimentation, the sediment yielded during centrifugation of MCC_{warm} was more compressible (Fig. 6). Although the higher rehydration temperature (50°C) reduced the sediment generated by both MCC suspensions, the compressibility of the MCC_{warm} sediments was higher compared to those formed at 25°C. This higher compressibility of sediment may be due to a greater degree of water transfer into the powder particles at the higher temperature, which was not sufficient to disperse the sedimentable particles, but resulted in a material which was more mechanically pliable. Thus, the strategy of increasing mixing temperature to promote dispersion of these powders may create a sedimentable phase in MCC_{warm} that is subject to consolidation during storage, which may in turn make it more difficult to re-suspend this material by actions such as shaking and stirring.

5. Conclusions

It is important to develop methods to improve the rehydration properties of MCCs, a challenge which needs to be addressed to fulfil their potential as ingredients in techno-functional and/or nutritional roles. This study demonstrated that the dispersion characteristics of MCCs could be improved by simply reducing the temperature of the MF/DF step during their manufacture. There is a growing trend towards cold membrane filtration for other reasons (e.g., lower fouling, microbial control, β-casein enrichment), and increased MCC solubility could be an additional/secondary benefit in these cases. In others, cold MF/DF may present an

alternative to methods for solubility-enhancement based on the use of extra equipment or additives.

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Chapter 12.

General Discussion and Future Perspectives

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Declaration

This chapter was written by author SVC.

12.1. Milk protein concentrates

12.1.1. Background and outlook

In classical approaches for the manufacture of casein-dominant powders, high-protein ingredients are generated by default, i.e., an insoluble protein fraction is prepared and the majority of the soluble components are removed. Thus, individual products such as rennet casein, acid casein or co-precipitates generally tend to have protein levels ≥85%. A benefit of processes based on membrane filtration is the relative flexibility afforded for controlling the composition of the final product (Carr and Golding, 2016). Milk protein concentrate (MPC) is not a single ingredient, but, rather, a category of ingredients, with each individual MPC having a composition determined, in part, by the parameters of the membrane process used. During the membrane filtration of milk, the concentration factor achieved during ultrafiltration (UF) and the extent of diafiltration (DF) greatly influence the protein, lactose, non-protein nitrogen and mineral levels of the MPC. The serum phase is significantly altered as the extent of UF/DF increases, while the proximity of the casein micelles progressively increases. These compositional and physicochemical factors can result in MPC powders having very different properties before, during and after rehydration, which was the subject of the investigations included in Part A of this thesis.

Previous research has mostly focused on single MPC powders, most typically MPC80- or MPC85-type ingredients. There is a good reason for this: high-protein MPCs are the most widely-used in food applications and there have been reports of issues with their processing performance (i.e., difficult handling properties, long rehydration times and poor heat stability). For food formulators, it is important to have options when choosing an ingredient for a product to be developed. Currently, many different MPCs are manufactured using membrane processing, which would seem to present an array of options, yet the choices manufacturers make are largely based on the gross composition of powders, due to a lack of information concerning trade-offs between protein purity and ingredient stability or functionality. It is crucial therefore to establish a link between protein concentration factors achieved during MPC production and the physicochemical and properties and process stability of the resultant powders. Results from our studies

may help inform processors during their selection and use of MPCs for particular applications, while providing fundamental information to underpin future research into strategies to improve the processability of MPCs

Any issue encountered during the use of an MPC, be it a tendency to cake in a hopper, form a sediment after rehydration, or coagulate during heating, is largely determined by changes occurring during the manufacturing process. A range of strategies are the subject of research efforts that aim to prevent or inhibit these process-induced changes and thereby limit destabilisation of MPCs in applications in which they are used. However, it is uncertain whether these efforts will be successful or, if they are, how widely they will be adopted. It may therefore be considered that there is an urgent need to provide manufacturers with strategies that allow them to minimise destabilisation of current-generation MPCs. As high-protein MPCs have been reported as having some of the least desirable characteristics from a processing point of view, they are the most likely to present manufacturers and consumers with issues related to their storage, handling and use. The results of the studies reported in this thesis point to some potential methods for improving the functionality of these high-protein MPCs.

12.1.2. Overview of findings

The most immediate set of characteristics which define a food powder is its behaviour as a bulk solid; even the most sophisticated of designs for liquid processing will be rendered obsolete by an immobilised column of powder in a silo. Studies on the flowability of dairy powders have been infrequent, and have generally been the domain of chemical engineers rather than food scientists. The availability of analytical tools such as the Powder Flow Tester (PFT) from Brookfield and the FT4 Powder Rheometer System from Freeman Technology are important developments in the field of food science, as they provide easy-to-use and rapid methods to generate a multitude of data related to the flowability of powders. This should facilitate a more in-depth exploration of the relationships between processing of milk and the properties of the milk powders. In Crowley *et al*. (2014a), the PFT device was used to characterise different MPC powders, which were found to span a range of flow behaviours from easy-flowing to cohesive

(**Chapter 2**). As discussed by Fitzpatrick (2007), particle size, although somewhat associated with flow behaviour, was not a strong predictor of MPC flowability. Specific surface area was better in this regard, and was strongly correlated with flow index values. Differences in specific surface area could be linked back to the viscosity of the liquid concentrates which were dried into the MPC powders. Particle size generally increased with protein content for MPC35, MPC50 and MPC60, which might be expected to improve flowability, but a relatively constant specific surface area and flow index values were measured; MPC70 had a similar specific surface area to these powders but had poorer flowability. These data indicate that specific surface area is a better predictor of flowability than particle size, and that specific surface area is influenced by factors other than particle size, such as particle shape. The trend of decreasing flowability with increasing protein content was not necessarily confined to physical properties, with the aforementioned poor flow behaviour of MPC70 occuring despite a specific surface area that was similar to that of the easy-flowing powders. Kelly *et al*. (2015) demonstrated that these MPC powders have markedly different particle surface compositions, with a marked increase in protein at the surface and slight increase in surface fat with protein content; it is known that factors such as free fat can influence inter-particle interactions in powders (Fitzpatrick, 2007), although very little is known about the influence of protein. Thus, although physical properties such as specific surface area are strong predictors of flow behaviour, surface composition analysis can play an important role in establishing a potential link between surface chemistry and bulk solids properties, and may help explain the poor flowability of MPC70-type powders.

The term ‘good solubility’, as applied to dairy powders, can be defined generally as:

> *the quality of a powder that allows its constituents to be returned quickly to their original undried state through reconstitution in water without the need for major process interventions.*

Many MPCs fulfil the criteria set out in this definition. In our study (**Chapter 3**), MPC35, MPC50 and MPC60 can be said to have good solubility. This quality, however, cannot be attributed to MPC70 and, in particular, the high-protein powders

MPC80, MPC85 and MPC90. For these powders, the rehydration process can be represented by a series of slow stages that cumulatively contribute to an exceptionally long rehydration time; for the purpose of this discussion, the following stages are considered based on our review of the literature (Crowley *et al*., 2016) in **Chapter 1**, the results of our study on the solubility of MPCs (Crowley *et al*., 2015b) in **Chapter 3** and findings from a recent study on the water transfer properties of the same MPC powders (see Appendix 2; '*New insights into the mechanism of rehydration of milk protein concentrate powders determined by Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS)*'):

1. Interaction of powder with water and immersion of particles (wetting);
2. Imbibition of water through particle surfaces into internal vacuoles (water transfer);
3. Decrease in particle density rendering the system non-sedimentable by classical centrifugal conditions for solubility testing (particle erosion);
4. Disappearance of primary (powder) particle-sized material (dispersion, dissolution).

The acceleration of (1) is normally achieved through agglomeration, but Ji *et al*. (2016) showed that this negatively affects (3) and (4) for MPC powders. Measurement of contact angle provides a rapid (5-60 s) method of measuring wetting properties of non-agglomerated and agglomerated MPC powders (Crowley *et al*., 2015b; Ji *et al*., 2016). Most research has focused on increasing the rate of (3) and (4), which typically requires high temperatures, high shear, extended reconstitution times, and the use of additives (e.g., chelating salts) or unit operations (e.g., ultrasound, high pressure) that promote protein dissociation (Crowley *et al*., 2016). A combination of sedimentation and particle size analysis is very effective for monitoring the progress of (3) and (4), respectively (Crowley *et al*., 2015b). The analytical centrifugation approach to dispersion analysis developed in our lab has several advantages over the traditional (in)solubility index-type methods:

- Only 400 µL of dispersion is required;
- Twelve suspensions can be analysed simultaneously;

- The high sensitivity to optical density eliminates the need for washing/re-centrifugation step;
- A cycle of centrifuge speeds can be applied for compressibility analysis;
- Additional analysis (e.g., turbidity changes, sedimentation rates) is possible.

Alterations in ionic composition were demonstrated by Crowley *et al*. (2015b) to have good potential in improving the dispersion characteristics of a high-protein MPC, when combined with an elevated rehydration temperature; this is consistent with the observation that reconstituting MPCs in milk at elevated temperatures reduces the so-called 'cheese nugget' defect associated with their application in cheese (Bhaskar *et al*., 2007); cheese nuggets were characterised by Carr and Golding (2016) as 'small fat-free translucent protein lumps'. The influence of (2) has largely been overlooked until the recent development of analytical tools capable of monitoring air release during rehydration (for examples see Richard *et al*., 2012 and the paper in Appendix 2 titled; '*New insights into the mechanism of rehydration of milk protein concentrate powders determined by Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS)*').

The measures manufacturers are willing to take to address solubility issues with MPCs will depend on their own criteria for success and the resources they are willing to allocate to achieve it. The fast wetting of an agglomerated MPC may give an initial impression of good solubility, but this may not prevent the formation of a sediment (Ji *et al*., 2016). The adoption of reconstitution protocols involving high temperature and/or high ionic strength have been shown to eliminate sediment, but micron-sized particles may remain in suspension (Crowley *et al*., 2015b), which could cause sensory or functionality defects. Ca-binding technology that dissociates casein micelles will convert the system to one with 'good solubility', but functionality of the MPC might be irreversibly altered and may more closely resemble a caseinate if extensive (Carr and Golding, 2016). The acceptability of incurring additional energy and/or ingredient costs will be key a consideration for any solubility-enhancing technique. As such, the poor solubility of high-protein MPCs is a problem for which the solutions are very much application-specific.

Perhaps the most unusual feature of MPC powders is that they contain primary particles which remain undispersed and seem to retain much of their size and integrity after extended periods of rehydration. This property was a surprise initially, when, for example, attempts in our laboratory to measure particle size dynamically (using both focused-beam reflectance and light-scattering) during the dissolution of MPC90 yielded results that indicated only minor changes in particle size after over an hour of mixing (unpublished, preliminary data). However, this result was found consistently for high-protein MPCs, is in agreement with reports in the literature (Fang *et al*., 2011) and can be reasonably expected to be related to defects such as the presence of 'cheese nuggets' when MPCs are use in cheese milk, which is the subject of a series of patents describing methods to eliminate their presence (including Bhaskar *et al*., 2007 and Carr *et al*., 2004). The persistence of large particles during the rehydration of MPCs has been attributed to the presence of inter-linked casein micelles present at the surface of the particles (Mimouni *et al*., 2009, 2010). After a relatively short rehydration time (e.g., ~90 min) these particles tended to sediment readily, but, with increasing rehydration time (e.g., ~24 h), the suspension stability improved and a sediment was no longer detected (Crowley *et al*., 2015); this suggests that the so-called 'skin' might be comprised of multiple layers of inter-linked micelles surrounding the internal vacuole, like a hollow onion, with the erosion of the outermost layers perhaps being responsible for the gradual reductions in particle density. Dry MPC80, MPC85 and MPC90 all had particle density values which were 0.8-0.9 g cm^{-3}. Fitzpatrick *et al*. (2016) recently measured very similar values (0.8 g cm^{-3}) for a commercial MPC90-type ingredient (referred to as an 'MPI'); the authors considered that this density value should result in flotation in water and, indeed, detected a cream-type layer after centrifugation, which they attributed to particle flotation, while also observing sedimentation. For a particle of density (ρ) 0.8 g cm^{-3}, it can be predicted that differences in $\Delta\rho$ ($\rho_{solvent}$ - $\rho_{particle}$) might affect buoyancy as follows:

a) A non-interacting, aerated particle will float in the solvent (high $+\Delta\rho$);
b) A non-dissolving particle that imbibes water will sink (high $-\Delta\rho$);
c) Slow imbibition and partial erosion will lead to a low $\Delta\rho$ value;
d) Complete imbibition and extensive erosion may lead to negligible $\Delta\rho$.

The main factors influencing the suspension stability of MPCs during rehydration can therefore be considered the air:water ratio in the internal vacuoles of particles and the mass of solid matter at the particle surface; with imbibition primarily reducing flotation and erosion of particle surfaces being mainly responsible for decreasing sedimentation. The ideal conditions outlined in (a) and (b) are not met by during the rehydration of a high-protein MPC powder; instead, the rehydration process is likely to involve a transition from (c) to (d), resulting in the presence of particles comprised of a large volume of solvent (i.e., the serum phase of MPC suspension) surrounded by a thin (potentially nm-thick, at the lower end) layer of casein micelles. Assuming that the total volume of such a particle primarily consists of material of the same density as the dispersing phase, these particles are unlikely to sediment under the conditions used most commonly to measure solubility (<1000 *g*, <30 min); furthermore, a very large number of casein micelles may be released even when much larger particles are the predominant population by volume, as detected by light-scattering (it would take ~1×10^6 casein micelles with a diameter of 0.1 µm to occupy the same volume as a single 10 µm primary particle) and there may therefore simply not be enough of these large particles to generate a measurable sediment. This unique population of large particles can be considered as emulsified droplets in a monophasic system (i.e., a porous particle with a surface of casein micelles and an interior composition equivalent to the surrounding solvent); it is unclear whether there is a critical density at which deleterious effects on MPC functionality may become negligible. Currently, researchers can measure solubility values of ~80-100% (based on sediment) and a mean particle size of 10-50 µm (by light-scattering) for a casein-dominant MPC (with an expected micelle size of 150-200 nm). If the large non-sedimentable particles are found to influence MPC functionality, then they may need to be considered an important criterion within an expanded definition of 'good solubility'.

The heat stability of milk has been a central topic of dairy research for decades and there have been several important developments in the area (Singh, 2004). The influence of individual milk components, such as urea (Muir and Sweetsur, 1976) and ionic Ca (On-Nom *et al*., 2012; Crowley *et al*., 2014) on stability during heating have been determined. The mechanisms responsible for destabilisation at various pH values have been characterised (Singh and Fox, 1985;

Donato and Guyomarc'H, 2009) and the heat stability profiles of concentrated and unconcentrated milk are well understood (Singh, 2004). Methods to increase heat stability by using, for example, pre-heating or addition of Ca-binders (de Kort *et al*., 2012; Huppertz, 2016), are well established. It has long been recognised that casein micelles are remarkably heat-stable, withstanding temperatures above boiling for far longer than whey proteins. This point is well illustrated by the fact that a product has been developed specifically for the purpose of cooking/boiling in the kitchen, which consists of a whey protein-depleted micellar casein fraction (Heino and Huumonen, 2015). Certain casein-based ingredients, such as sodium caseinate, are known to be even more heat-stable than native casein micelles. Despite the common use of MPCs in UHT-treated and in-container sterilised beverages, there is virtually no information available on how the heat stability of different MPCs compares, and how heat-induced destabilisation varies for MPCs during different heating regimes. In addition, it is unclear which existing strategies to address stability issues (e.g., pre-heating, Ca-binders) in milk are appropriate for combatting the destabilisation issues encountered with MPCs.

The heat stability studies on unconcentrated MPCs (Crowley *et al*., 2014c) in **Chapter 4** and concentrated MPCs (Crowley *et al*., 2015a) in **Chapter 5** show a set of MPC ingredients with a complex range of heat stability profiles. Heat stability generally decreased with increasing protein content of the MPC powder; in particular, high-protein MPCs (i.e., MPC80, MPC85 and MPC90) had poor heat stability. This poor heat stability can be mostly explained by their high ionic-Ca activity, despite the role of other factors (i.e., reduced lactose level and κ-casein dissociation) that would otherwise favour stability; for concentrated systems, it was found that whey proteins had an important influence. Although the pH-dependent behaviour of each MPC was complex in its own right, and further complexities became apparent when concentrated and unconcentrated MPCs were compared, it can be said that the poor stability of high-protein MPCs was consistently found. Among the other MPCs, an increase in protein:lactose ratio was, in some cases, seen to have a positive effect on heat stability; this was particularly true in concentrated systems, with MPC70, for example, having excellent heat stability. Therefore, a fair response to the question 'Are MPCs heat-stable?' could be 'Which one?', followed by '. . . and for what thermal process???'. To incorporate high-protein MPCs into

beverages that will be exposed to intense thermal treatments, it should be considered firstly if a high-protein MPC is absolutely necessary for the intended application; if not, then MPCs with intermediate protein:lactose ratios may be a less challenging option. In the event that a high-protein MPC is to be used then stabilisers may be required. The results of our studies point in one direction in this regard: salts, such as phosphates and citrates, which function by binding ionic Ca. It was shown that heat stability of an unconcentrated MPC system could be improved greatly by restoring the ionic composition of milk; although this may not be achievable in a practical sense in many beverage applications, it does suggest that methods to reduce Ca-ion activity (e.g., use of Ca-binders, increasing ionic strength) could be successful. In addition, pre-heat treatment (95°C, 45 s) of skim milk seemed to have some slight beneficial effects on the heat stability of MPC80 in concentrated systems.

12.1.3. Recommendations for future research

Assessment of flowability of milk protein concentrates dried at constant viscosity:

A key limitation of the experimental study of MPC flowability described in Crowley *et al*., 2014a) was that the high-protein MPCs were spray-dried at a much lower total solids than the lower protein powders. This resulted in the high-protein MPCs having much smaller particle sizes, due to the lower viscosity of the feed entering the atomiser. Thus, the influence of protein concentration factor was confounded with differences in physical properties (mainly particle size and specific surface area). Specific surface area was a good predictor of flowability, and the high values for the cohesive MPCs were likely influenced by their small particle sizes, along with the fact that casein-dominant suspensions tend to adopt buckled, uneven surface morphologies during drying (Sadek *et al*., 2016). A powder such as MPC70, which, despite not having a significantly higher specific surface area, was more cohesive than the lower protein MPCs, is suggestive of a potentially important role for particle surface composition on flowability. A valuable follow-up study would be one where the liquid concentrates were dried at similar viscosities to generate particles of broadly equivalent size distributions, which would facilitate closer evaluation of the influence of surface composition and particle morphology. The

recently developed single-droplet drying approaches adopted by other researchers (see Fu *et al*., 2012 and Sadek *et al*., 2016) could be useful for such experiments, when combined with the low-volume sample cell feature of the PFT.

Study of the potential impact of time-consolidation on flowability and solubility:

Given that the high-protein MPCs studied here were prone to consolidation, it is likely that these powders would become compressed if exposed to prolonged stress (i.e., in a stack of powder sacks) over time. As these powders have also been demonstrated to be poorly-dispersible during rehydration, it is worth investigating if periods of defined consolidation exacerbate their poor flowability and their slow dispersion rate.

Investigation of the tableting properties of high-protein milk protein concentrates:

One of the main logistical advantages of food powders is their low bulk volume, which allows nutrient-dense product formats to be shipped around the world. There is an increasing interest in the potential to tablet these powders, as the compression involved in tabletting would further reduce shipping costs. Furthermore, tablets could afford greater control of nutrient delivery in powdered infant milk formula and meal replacers, for example, by eliminating the need for consumers to weigh out powders accurately. Given the high protein and calcium phosphate content of MPI/MPC90-type products, they may be attractive as supplements in tablet form; in addition, this type of ingredient is commonly the main protein source in clinical nutrition products, and the tabletting properties of blends of MPI/MPC90, carbohydrate and vitamins, etc., may also be of interest. That the high-protein MPCs investigated in our study are highly compressible indicates that they may have good tabletting properties (e.g., ability to retain shape and integrity), which could form the basis of tablet designs for oral consumption either directly or after mixing with water; in the case of the latter, methods to enhance dispersion would need to be investigated, such as the design of effervescent milk protein tablets. Patents have already been filed for tablets in which high-protein MPCs comprise up to 18-28% of the tablet weight, for the treatment of oropharyngeal candidiasis in immune-

compromised patients (Attali, 2009) and orofacial herpes (Attali and Constantini, 2011); in these formulations, the MPC (an MPC85) is claimed to have 'mucoadhesive' properties, in which the tablet sticks to mucous membranes in an area such as the gum for controlled release of the active ingredient. Studies on the process-composition-physical-chemical-tabletting properties of different MPCs could contribute to such developments.

Assessment of whether persistent micron-size particles influence functional and sensory properties:

Even after long periods of rehydration, particles as large as those found in the dry powder itself can be detected in suspensions of high-protein MPCs. These particles are wetted and submerged powder particles which have not fully dispersed due to the strong interacting forces between casein micelles at their surface. These particles may be responsible for the 'cheese nugget' defect associated with cheese manufactured using MPCs and could have other negative impacts on MPC functionality. However, although these particles comprise a significant proportion of the total particle volume in these suspensions, they may not necessarily sediment and be detected as insoluble material in a centrifugation experiment. It is important to determine if these particles have any measurable impact on functional properties (e.g., gelation, emulsification and foaming); if they do, then standard solubility tests may need to be revised to take account of their presence.

Incorporation of calcium-binding agents to increase heat stability:

It was conclusively demonstrated in both heat stability studies that one of the major factors influencing the heat stability of MPCs was their Ca-ion activity. One approach to improve their heat stability might be to bind a proportion of this free Ca using citrate or phosphate salts, although care would need to be taken to avoid excessive dissociation of the micellar phase.

Exploitation of high heat stability of MPC70-type ingredients in beverage systems:

Manufacturers who wish to use MPC ingredients should also consider the entire range of MPCs when selecting one for their application; MPC50, MPC60 and MPC70, for example, may facilitate the fortification of beverages with good quality milk protein without incurring the challenges associated with higher protein varieties. It cannot be said, based on our results, that the heat stability of MPCs uniformly decreases with increasing protein:lactose ratio. Indeed, powders such as MPC70 compared very favourably to skim milk in terms of heat stability, particularly in concentrated systems. If the potential challenges of processing certain high-protein MPCs must be considered, then so should the potential opportunities with heat-stable ingredients such as MPC70. MPC70-type ingredients could be examined in model formulations of products in which MPI/MPC90s are more common (e.g., clinical nutrition beverages), to assess whether protein purity can be sacrificed to improve heat stability in certain applications.

12.2. β-Casein ingredients and co-products

12.2.1. Background and outlook

For many years, there has been interest in the use of β-casein as a techno- and bio-functional material in food and pharmaceutical applications. Despite this, commercial production of β-casein-enriched ingredients is in its infancy, particularly when compared to the rate of innovation in the fractionation of individual whey proteins (Smithers, 2008, 2015). Membrane filtration is one of the best technology platforms for the manufacture of industrial-scale quantities of enriched or purified β-casein from milk (Christensen *et al*., 2014; O'Mahony *et al*., 2014). To date, however, there have been few studies investigating how the extraction of β-casein from milk can be optimised. When one considers the innate complexity of a typical β-casein enrichment/purification process (e.g., integration of various filtration steps, use of multiple diafiltration media, control of protein association-state), along with its apparent limitations (low flow rates, risk of coagulation, low yield and purity), the lack of supporting research for the development of these processes is likely to have hindered progress in this area.

As discussed in the introduction to Part B of this thesis, use of membrane filtration has led to a shift away from classical approaches in which the principal milk proteins were isolated using precipitation. The production of purified β-casein from milk involves the combined application of membrane filtration and reversible changes in protein structure, an approach that has also been used successfully to develop α-lactalbumin ingredients (Lucena *et al*., 2006). Separation processes for protein ingredient development in the dairy industry can be generally described as having a two-way split in terms of the major streams generated: cheese and sweet whey, acid casein and acid whey, sweet whey and whey permeate, milk protein concentrate and milk permeate, micellar casein and native whey, and so forth. Essentially, these processes involve the concentration of naturally larger molecules (stream 1) by passing the feed over a semi-permeable membrane that allows the passage of smaller molecules (stream 2); β-casein fractionation, on the other hand, does not fit within this, admittedly, simple definition. Taking the protocol of O'Mahony *et al*. (2014) for preparation of β-Casein as an example, the first separation phenomenon occurring during β-casein manufacture does not involve any membrane, but rather the redistribution of β-casein into the serum-phase at <5°C while α_s- and κ-caseins mostly remain in the micellar phase. After this component distribution, the large casein micelles (stream 1) can be fractionated from the smaller β-casein and whey proteins (stream 2). The latter stream is comprised of two protein classes which are too similar in size to be separated by conventional membranes; hence, the β-casein is subject to controlled self-association which allows it to be concentrated (stream 3) while the whey proteins permeate (stream 4). Thus, the β-casein process can be said to involve a convergence of several important concepts which are emerging and driving advances in the separation of milk proteins. We consider these concepts to be:

1. The structure of proteins must not be irreversibly altered;
2. Ideally, the value of all co-products should be maintained or even enhanced;
3. Reversible changes in protein association-state can enhance separation.

Much of the commercial interest in β-casein arises from a desire among manufacturers to humanise the casein profile of infant formula, yet the ability of β-casein to mimic the functionality of native casein micelle in milk is unclear. Many of the studies on the self-association of β-casein have been performed on analytical-grade preparations of the protein, which may not be adequate predictors of the behaviour of β-casein prepared using membrane processing. The casein micelles in milk have several key characteristics which are important during general processing and application of milk-based products:

- Casein micelles can withstand temperatures of >100 °C for extended periods;
- Systems supersaturated in calcium phosphate are stabilised by casein micelles;
- Milk opacity does not change perceptibly with the fluctuations in temperature encountered during storage and warming/cooling for consumption.

The many developments in the processing of infant formula, including the use of demineralised whey, modification of the casein:whey protein ratio, and increasing α-lactalbumin:β-lactoglobulin ratio (see **Chapter 6**) have largely evolved around a casein profile which has remained constant. The partial substitution, and potentially the complete replacement, of native casein micelles with β-casein ingredients therefore poses a significant challenge to the stability of infant formula within the existing processing framework; however, this challenge must be considered in the light of exciting opportunities for the manufacture of infant formula with micellar phases more closely resembling those in human milk. Human micelles are smaller than bovine micelles (Ruegg and Blanc, 1982), more prone to dissociation (Sood *et al*., 1997) and tend to form a less rigid clot in the stomach (Nakai and Li Chan, 1987); some of these features are shared with the milk of non-bovine species (see **Chapter 7**) and have been attributed to the predominance of β-casein in human milk, which suggests that β-casein ingredients may allow their properties to be replicated.

12.2.2. Overview of findings

All of the microfiltration processes studied in **Chapter 8** exhibited low permeate flux, which is a feature of cold filtration processes (Gésan-Guiziou, 2013). For these low-flux processes, any increase in flux can have an economic advantage to a processor. A polyethersulfone (PES) membrane (with comparative β-casein enrichment performance) yielded a flux >20% higher than that of a polyvinylidenedifluoride (PVDF) membrane, the latter being the most commonly supplied polymeric membrane industrially (Pearce, 2007). The PES membrane also displayed a much lower degree of fouling, which suggests it may be a more amenable to extended operation.

Protein fractionation processes in the dairy industry which incorporate microfiltration membranes typically demand an efficient separation of casein and whey proteins. Therefore, casein in whey streams is normally considered a contaminant, particularly when the increasing use of whey protein in clear, acidic beverages is considered (Etzel, 2004). The 1000 kDa PES and 0.1 µm PVDF membranes completely retained micellar casein, with only β-casein permeating along with the whey in significant quantities. This separation profile is desirable if, for example, the β-casein-enriched material is intended to be further fractionated into a more purified β-casein; in this context, the 0.45 µm PVDF membrane would be unsuitable. However, this membrane generated an ingredient of great novelty in certain respects, as it contained native casein micelles but was also enriched in β-casein. The micelles which permeated would be expected to be smaller than the average micelle size in the feed, as reported recently by Jørgensen *et al*. (2016) for the micellar fraction permeating a 0.22 µm ceramic membrane on microfiltration of skim milk. Other notable properties of the permeate from the 0.45 µm membrane were a reduced protein content (~1.2%), a more whey protein-dominant protein profile than the feed (skim milk) and a minor whey protein component which was relatively unchanged compared to permeates generated using the tighter membranes; interestingly, these characteristics coincide with many of the formulation targets associated with the manufacture of first-age infant formula (see **Chapter 6**). In the short-term, β-casein ingredients are unlikely to be included in formulations as a 1:1 replacement for skim milk (in regular infant formula) or milk protein

concentrate/isolate (in lactose-free formulae), due to a number of technical, logistical and economic considerations. The first generation of β-casein-enriched infant formulae (some of which are reportedly on the market) are more likely to comprise a mixture of native micellar casein and fortified β-casein, diluted to ~1.2% protein in the case of ready-to-feed systems, much like the microfiltration permeate generated using the 0.45 µm membrane.

For the manufacture of purified β-casein, as described in **Chapter 9**, the enrichment step needed to be completed with minimal contamination of the whey stream with micellar casein. Secondly, the pore size of the membrane needed to be sufficiently small to allow the fractionation of β-casein micelles from whey proteins, particularly considering the smaller size of these micelles compared to native casein micelles in milk; these conditions were satisfied with a 0.08 µm membrane. The most successful approach to purification was that in which a β-casein-enriched whey stream was subjected to conditions of controlled ionic strength, protein concentration and temperature designed to promote retention of β-casein micelles during microfiltration. This novel approach yielded an ingredient with an 80% β-casein purity - one of the highest reported for such a process. However, the potential application of this technology is not limited to purification of β-casein from milk. The adoption of low-temperature filtration across the dairy industry has led to an increase in β-casein levels in many native whey streams; the presence of β-casein in these streams can cause complications with down-stream processing and/or product applications, and so controlled self-association of β-casein could be used as a clean-up step in these situations.

Studies on the self-association of β-casein have revealed some consistent findings: the micelles are small (~20-30 nm); micelle formation is a thermo-reversible process, although β-casein is irreversibly destabilised in the presence of ~10 mM calcium. In **Chapter 10**, the β-casein concentrate (BCC) studied differed from the analytical-grade proteins used in most previous studies, with whey protein comprising >20% of total protein and, most importantly, κ-casein contributing a non-negligible 5% of total casein in the BCC. In addition, the investigation differed from many recent studies in focusing on self-association behaviour in a supersaturated calcium phosphate solution (SMUF). Given that, arguably, the most

widely studied and debated feature of casein micelle structure in the last half-century is the role of calcium phosphate, it is surprising that its influence on the behaviour of β-casein in solution has not received more attention. This knowledge gap becomes more glaring still when one considers that one of the most immediate technical challenges associated with humanising the casein profile of infant formula will be design of a stable micellar structure which binds amorphous calcium phosphate.

In 1998, Carl Holt reported that a hydrophilic peptide of β-casein could stabilise supersaturated solutions of calcium phosphate for months or even years; this technology is the basis of phosphopeptide-amorphous calcium phosphate materials proposed for use as additives in enamel-remineralising toothpastes (Reynolds, 2004; Holt, 2009). In the study of Holt (1998), the hydrophobic terminus of β-casein was removed using enzymatic hydrolysis to prevent hydrophobic interactions from negatively affecting β-casein stability. Results from **Chapter 10** show that prolonged incubation of our β-casein ingredients (with intact hydrophobic groups) in SMUF did lead to precipitation of protein, albeit to a minor extent. However, the ingredient was still highly effective at stabilising calcium phosphate at 37-63°C, with pH-drift analysis, light microscopy and visual assessment confirming that calcium phosphate crystallisation/precipitation was prevented. That aggregation of β-casein continued despite an absence of a marked pH drop, and that precipitation could be prevented if solutions were cooled after a short incubation, indicated that the destabilisation process was hydrophobically driven. Dissociation experiments at low temperature revealed that the β-casein ingredient displayed similar behaviour to human milk micelles, with extensive but incomplete dissociation of the micellar phase (Sood *et al*., 1997), likely due to the stabilising role of calcium phosphate. These data are consistent with the core-shell model proposed by Holt (1998) for calcium phosphate (core) and phosphopeptide (shell) co-assemblies. Sood and Slattery (2002) reported that ~95% of human β-casein with phosphorylation levels of 4-5 could be stabilised if there was one κ-casein molecule for every 4-6 β-casein molecules, which is close to the ratio found in human milk; the researchers also showed that the human β-CN, with phosphorylation levels of 0-3, required even less κ-casein. That ~85-95% of the protein in the β-casein solutions investigated in our study remained stable at elevated temperature in a high ionic strength environment strongly indicates that the κ-casein present largely terminated the polymerisation

process. These findings indicate that membrane process-derived β-casein may have promise as a micellar carrier of calcium phosphate.

During the enrichment/purification of β-casein, MCC is produced in the largest volume of any single stream, which makes the utilisation of this ingredient a major challenge for those seeking to adopt this approach to manufacturing β-casein. Despite the poor rehydration characteristics of MCCs, which are known to be even worse than those of high-protein MPCs (Crowley *et al*., 2016), very little is known about the influence of microfiltration temperature on MCC solubility. It was found in the studies reported in **Chapter 11** that there were marked compositional differences between two MCC powders, manufactured by cold and warm processes, most notably in terms of their protein and mineral profile. The MCC prepared using cold microfiltration had two properties which likely had a major impact on its improved rehydration performance, i.e., (1) a greater proportion of whey protein and (2) decreased levels of calcium and phosphorus.

It is interesting to note that the improvement in rehydration performance associated with cold filtration was not noted by either Seibel *et al*. (2015) or Luo *et al*. (2015), who measured the solubility of MCCs and MPCs, respectively, manufactured using both cold and warm temperatures. The MPC powders studied by Luo *et al*. (2015) were <60% protein and were freeze-dried rather than spray-dried; in addition, due to the ultrafiltration process used, they did not have different proportions of whey proteins regardless of the temperature of the membrane process. Issues relating to poor solubility are generally experienced with casein-dominant powders until the protein content exceeds ~70%, and these issues are not encountered to the same extent with freeze-dried powders; furthermore, the powders were rehydrated for an extended period, i.e., two hours at room temperature followed by overnight rehydration in the fridge. Although Seibel *et al*. (2015) reported using the same rehydration time (i.e., 90 min), 10 min of the rehydration time consisted of high-shear mixing, which can readily break particles into smaller fragments that are less likely to settle (and therefore may not be detected as insoluble). The methodology used by these researchers make it difficult to compare our results with theirs, which otherwise showed similar trends in factors (mineral and protein profile) that are known to be important contributors to MCC/MPC solubility. It should also be mentioned that insolubility was measured by these

researchers using a single-point measurement of the level of sedimentable solids after centrifugation. These MCC and MPC powders are defined by a slow dispersion of particles rather than an single absolute mass of insoluble material; hence, large undispersed particles can be present while not necessarily being sedimentable under the centrifugal conditions used (Crowley *et al*., 2015). It is for this reason that a range of experimental techniques (Fang *et al*., 2007) have been, and continue to be, developed for measuring their rehydration characteristics. Cold microfiltration was demonstrated to have a positive effect on the solubility of MCC powder (**Chapter 11**), suggesting that modification of filtration temperature could provide an effective, easy to implement, low capital cost and additive-free approach to modulating the rehydration characteristics of these poorly soluble powders.

12.2.3. Recommendations for future research

The fate of minor whey proteins during β-casein production:

In **Chapter 8**, it was demonstrated that enrichment of β-casein using membranes which completely blocked passage of micellar casein also resulted in depletion of minor whey proteins. The mechanism for this phenomenon is not clear, but it is likely that the positively-charged whey proteins, lactoferrin and lactoperoxidase, are electrostatically attracted to the negatively-charged surface of casein micelles. As a result, the complete retention of casein micelles results in simultaneous concentration of these minor whey proteins in the microfiltration retentate. To investigate this effect further, more quantitative techniques (capillary electrophoresis, mass spectrometry) than were used in our study could be used. As factors such as temperature, ionic strength and pH influence the strength of electrostatic attractions between oppositely-charged proteins, it should be possible to counteract this effect. An interesting study therefore would be to measure the effects of these factors on transmission of the minor whey proteins through the membrane during a β-casein enrichment process. If the effective transmission of proteins such as lactoferrin could be achieved, then the resultant β-casein ingredient would more closely resemble the protein profile of human milk.

Preparation of enriched β-casein fractions with small native micelles:

It was shown that use of a sufficiently wide-pore membrane for cold microfiltration causes a proportion of micelles to permeate the membrane along with the β-casein. Although this material is not suitable for the production of purified β-casein, it does constitute a novel milk protein ingredient. This material could be investigated as a model infant milk formula at the natural protein content (1.2%) of the permeate after a 3× concentration, given that it is enriched in β-casein, does not appear to be depleted in minor whey proteins, and likely contains micelles which are smaller than those in bovine milk (and perhaps closer to those in human milk). In addition, the permeate should be taken and concentrated by ultrafiltration, with diafiltration as needed, to generate model high-protein beverages of 8-12% protein for comparison with standard MPCs with the same protein content. Differences in the protein profile and size distribution of the micellar fraction may possibly have favourable properties for such beverages, including reduced viscosity and mouth-coating.

Modulation of β-casein:κ-casein ratio in β-casein-rich ingredients:

Although the focus of our work was on the enrichment of β-casein from milk, there are potential benefits of purposeful co-enrichment with other individual caseins. For example, dissociation of greater quantities of κ-casein may bring the β-casein:κ-casein ratio closer to that found in human milk. That κ-casein only accounts for 5% of the casein in the materials we generated limits its potential for playing a strong stabilising role during the aggregation of β-casein. In human milk, there is a greater proportion of κ-casein, but human β-casein is also multi-phosphorylated, and forms of β-casein with few or no phosphoseryl residues have also been linked with a stabilising role (Sood *et al*., 1997). Therefore, investigations into strategies to promote the dissociation of additional κ-casein are warranted. In certain respects, one might expect κ-casein to be more susceptible to cold-induced dissociation than β-casein, given that it occupies a position largely on the surface of casein micelles and is therefore readily accessible to the solvent; in addition, it is less phosphorylated than β-casein, and hence in theory relies more on hydrophobic attractions to integrate it into the micellar framework, given that it has few nucleation sites for binding of CCP.

Laboratory-scale optimisation of β-casein fractionation from whey proteins:

The β-casein purification process we describe was carried out at pilot scale. This was a particularly useful exercise in demonstrating how kg-quantities of β-casein could be produced, but also highlighted how the process could be modified based on laboratory testing to increase purity and yield. However, there is a need to study the fractionation of β-casein from whey proteins in greater depth such as is possible at lab-scale. The flexibility afforded by this scale would allow the influence of the full range of factors (e.g., protein content, ionic strength, pH, membrane fouling, membrane pore-size, charged *vs.* uncharged membranes) to be investigated more thoroughly, to inform larger-scale processes by availability of more complete data.

Plasmin activity and plasmin-mediated proteolysis in β-casein ingredients:

It is well known that the majority of plasmin in milk is associated with the micellar casein fraction (Ismail and Nielson, 2010). For this reason, concentration of micellar casein by ultrafiltration or microfiltration typically results in an increase in plasmin activity (Gazi *et al*., 2014). As plasmin is associated with proteolysis of casein and destabilisation of milk during storage, it is critical to determine the influence of processing conditions on plasmin activity. It is surprising that plasmin activity in enriched and purified β-casein fractions has not been investigated. It would be expected, that because plasmin is associated with the casein micelles, it should be retained during microfiltration and therefore be present in negligible quantities in the β-casein fractions. However, preliminary data from our laboratory indicates that there is a significant level of plasmin activity in purified β-casein ingredients prepared by microfiltration of milk. The presence of plasmin in these ingredients is an important finding, given that β-casein is the main substrate for plasmin in milk (O'Mahony and Fox, 2013). Indeed, we have seen that β-casein was readily hydrolysed over 14 d storage, with the plasmin activity, and thus proteolysis, being influenced by the different processing conditions described in **Chapter 9**. Plasmin-mediated hydrolysis of β-casein could impact the functionality of these ingredients, and its ability to affect these properties and methods to mitigate its activity need to be investigated.

Co-assembly of β-casein and calcium phosphate:

In the study in **Chapter 10**, the self-association of β-casein was strongly impacted by the presence of calcium phosphate. There are surprisingly few studies in this area and many opportunities for future work. In our study, we investigated one protein (1.2% protein) and one degree of calcium phosphate supersaturation. Future studies should be developed to investigate the full range of protein levels that an ingredient such as β-casein might encounter. If the BCCs we discussed are considered as potential emulsifiers/foamers (due to their high surface activity) but in another sense are analogous to MPCs (i.e., casein-dominant ingredient with micellar phase and whey protein) then this range could vary from, for example, 0.1% protein (use as an emulsifier) to 12% protein (clinical beverage applications). When an MPC is introduced into a formulation at increasing concentrations, there is a simultaneous increase in calcium and phosphorus, which is a unique feature of the ingredient and is considered nutritionally favourable in many applications. Thresholds need to be established for β-casein levels, in terms of what protein:calcium phosphate ratio yields a stable system with no precipitation of protein or minerals. As a part of such an investigation, the inclusion of BCC/MPC mixtures should be studied, to assess if small proportions of native micellar casein can promote stability in β-casein-rich solutions.

Heat stability of β-casein ingredients:

β-Casein was shown to exhibit highly dynamic shifts in association state with temperature (**Chapter 10**). However, in commercial liquid processing, such an ingredient is likely to encounter much higher temperatures than those investigated in this thesis. As one example, everything from high temperature-short time pasteurisation to in-container sterilisation and ultra-high temperature treatment may be encountered depending on the product format in infant formula manufacture. Predicting the influence of the fortification of infant formula with β-casein on its heat stability is difficult; on one hand, β-casein has been shown to display chaperone-like activity in preventing the aggregation of denatured whey protein (Kehoe and Foegeding, 2011), which could be beneficial in whey protein-dominant formulae. However, it is unclear if the very low levels of κ-casein in β-casein

ingredients can facilitate the formation of micellar structures which withstand coagulation at temperatures of >100°C, a characteristic of casein micelles which facilitates much of modern thermal processing of dairy products.

Encapsulation properties of β-casein ingredients:

The tendency for β-casein, in low ionic strength systems, to self-associate into micelles, with a hydrophobic core and hydrophilic shell, has led to some exciting research on the ability of β-casein to encapsulate fat-soluble vitamins (Barenholz and Danino, 2014) and hydrophobic drugs which target stomach tumours (Shapira *et al*., 2010). The formation of co-assemblies of β-casein and amorphous calcium phosphate may have potential implications for such studies, raising questions such as:

- Does the presence of calcium phosphate alter the release of hydrophobic substances in the stomach, due to associated changes in structure and buffering capacity?
- Do the structural changes caused by the presence of amorphous calcium phosphate alter the encapsulation properties of β-casein micelles?
- Does the ability of calcium phosphate to modulate the thermo-reversibility of heat-induced β-casein aggregates have potential applications in, for example, refrigerated beverages containing encapsulated substances?
- Could characteristics of native micelles, such as acid and enzymatic coagulability, be expected to be relevant also for a micellar β-casein system, and could this be used to develop protein gels containing materials encapsulated in the micellar phase?
- Will the temperature-dependent changes in micellisation result in undesirable and dramatic shifts in opacity as the consumer purchases (~25°C), warms (~37°C) and refrigerates (~4°C) β-casein-enriched infant milk formulae?

Rehydration of β-casein ingredients:

β-Casein ingredients present unique challenges during rehydration in mineral solutions. When rehydrated at warm temperatures (≥20°C), β-casein will immediately begin a polymerisation process, which will, at a minimum, influence subsequent aggregation when exposed to higher temperatures, and at worst could result in destabilisation during rehydration. The BCC powders we studied were highly aerated, as they were dried at low total solids level. Although the poor wetting properties of the powders were probably influenced by the high hydrophobicity of the protein, the high level of aeration likely exacerbated this effect. It will be important to study the spray drying of β-casein concentrates at higher feed solids and assess the influence of this on the wetting properties of resultant powders. However, this is unlikely to fix the issue with premature self-association in mineral solutions. This property may be particularly problematic in infant formula, where ingredients are mixed together at temperatures as high as 63°C. For the adoption of β-casein ingredients by this sector, the order of ingredient addition and the degree to which β-casein replaces casein from skim milk will be key considerations influencing stability. Several questions can be raised:

- Should β-casein be rehydrated in water before the other ingredients are added?
- If β-casein is adsorbed to the O/W interface in an emulsion system, will it be less susceptible to aggregation?
- Do either calcium or phosphate need to be added in insoluble form, or do citrates need to be added, to prevent destabilisation of β-casein?
- What quantity of native micellar casein is required to stabilise the ingredients at the liquid mixing stage?

Rehydration characteristics during powder ageing:

The micellar casein material manufactured as a co-product during β-casein enrichment was shown to have improved dispersion characteristics relative to micellar casein made by traditional warm microfiltration (**Chapter 11**). However,

this study was conducted on fresh powders analysed soon after their manufacture. Although fresh MCC and MPC powders are known to have solubility problems immediately post-drying, these issues typically increase during storage, particularly when exposed to adverse conditions of temperature and/or humidity. It will be important, therefore, to determine if the benefits of cold microfiltration on the rehydration properties of MCC powders persist throughout the storage conditions typically encountered during storage and transport of milk powders. In such a study, the application of novel measurement techniques such as BARDS (see Appendix 2) will further aid understanding of the differences in dispersibility of these high-protein milk powders.

Thermo-reversible sol-gel transitions in high-protein suspensions:

There have been only a limited number of studies on characteristics of MCC powders after rehydration as affected by microfiltration temperature used in their manufacture, and such studies were mostly related to acid and rennet gelation properties. Preliminary work from our laboratory on MCCs prepared by cold or warm microfiltration indicates that, when the MCC powders (from cold and warm microfiltration processes) are reconstituted to ~18% protein, and refrigerated for 30 h, both form gels; when subsequently incubated at 37°C, both gels reverted to a liquid-like state but required different times to do so: the MCC_{cold} took 15 min while the MCC_{warm} took 34 min. The thermo-reversible gels formed when high-protein suspensions of casein micelles are cooled have recently shown promise as long shelf-life (i.e., several weeks) alternatives to spray-dried powders (Amelia and Barbano, 2013). Preliminary findings indicate that the temperature of microfiltration could be used to control the rate of the gel-sol transition; this could allow, for example, reduction of the warming time required before gelled MCC can be mixed with other powder or liquid ingredients in formulated food products.

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Appendix 1

Supplementary data for Chapter 3

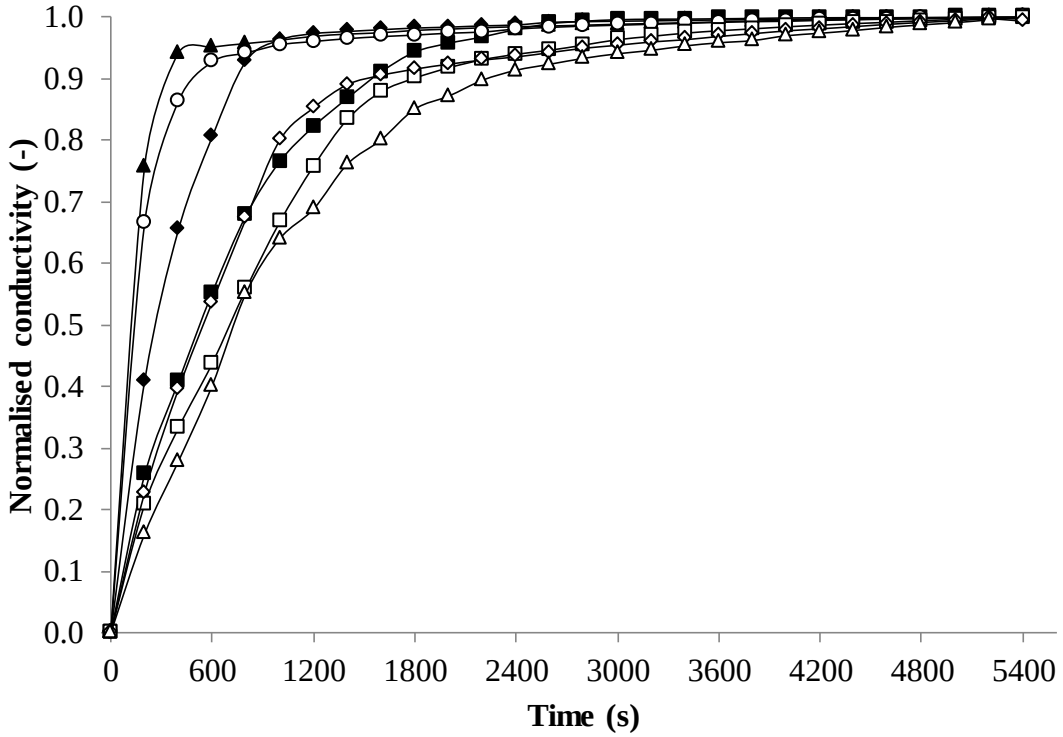

Fig. S1. Normalised conductivity during the rehydration of milk protein concentrate (MPC) 35 (■), MPC50 (♦), MPC60 (▲), MPC70 (○), MPC80 (◊), MPC85 (□) and MPC90 (Δ) in deionised water measured dynamically over 90 min at 25°C. Every 100th profile is shown.

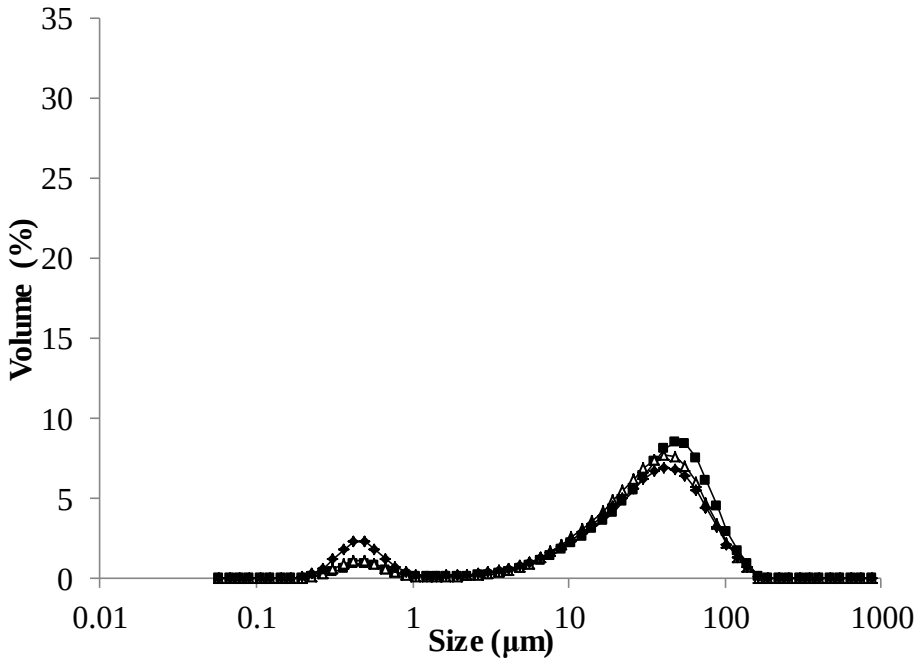

Fig. S2. Particle size distribution (PSD) data for milk protein concentrate (MPC)90 after 24 h rehydration in deionised water with (■) or without (Δ) octanol or in 80 mM KCl (♦) at 25°C. The PSD of MPC90 rehydrated in water was measured with and without octanol in the dispersing line of the Mastersizer to assess the potential influence of entrained gas bubbles on light scattering. Note: ranges of values shown in axes match those of PSD profiles in main manuscript.

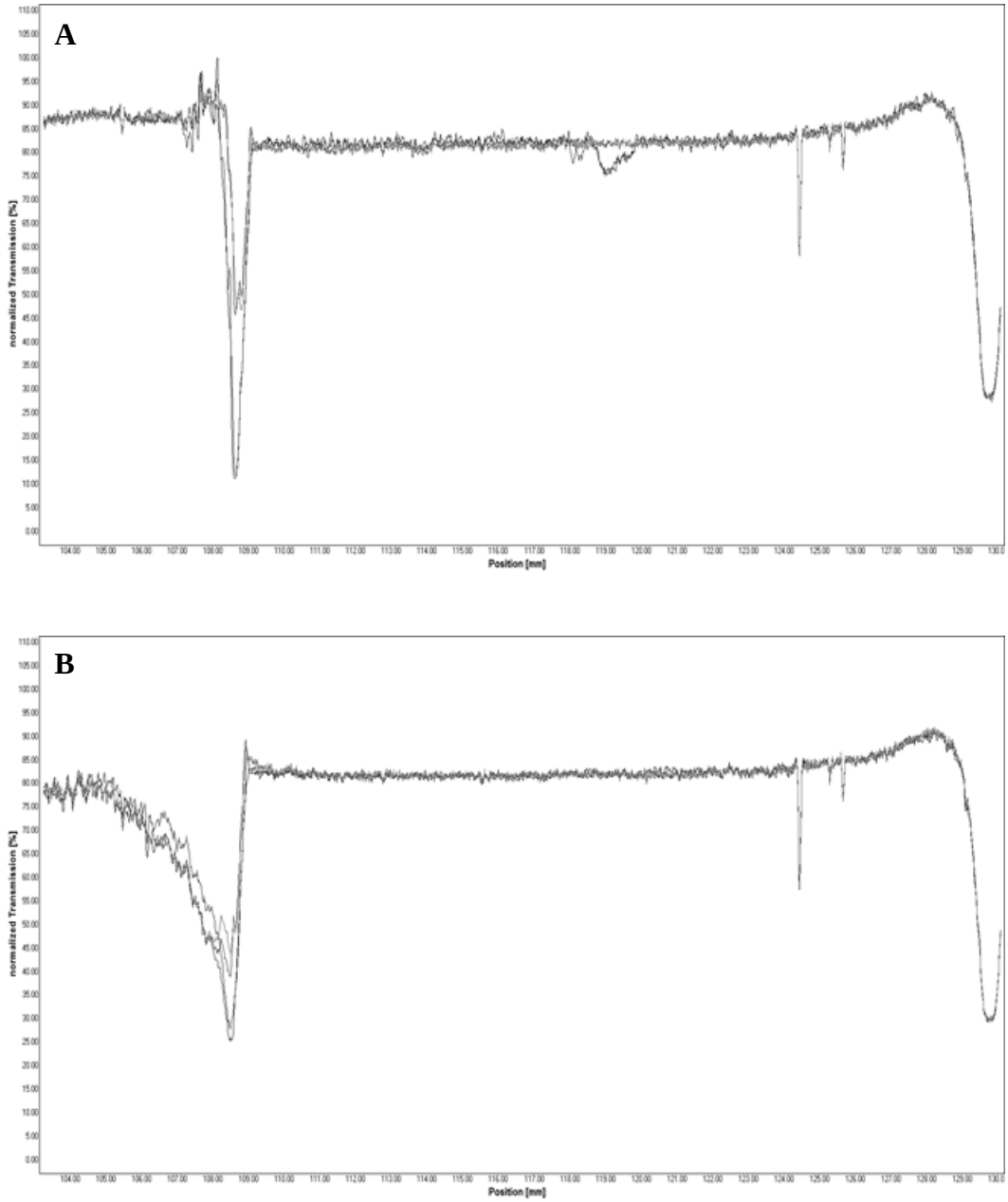

Fig. S3. Profiles showing change in transmission of NIR light through sample suspensions as a function of time and position in the sample cell during centrifugation at 36 × *g* for 10 min followed by 168 × *g* for 10 min of a milk protein concentrate (MPC) powder (MPC90) rehydrated in 80 mM trisodium citrate for 90 min at 25°C (A) or 50°C (B).

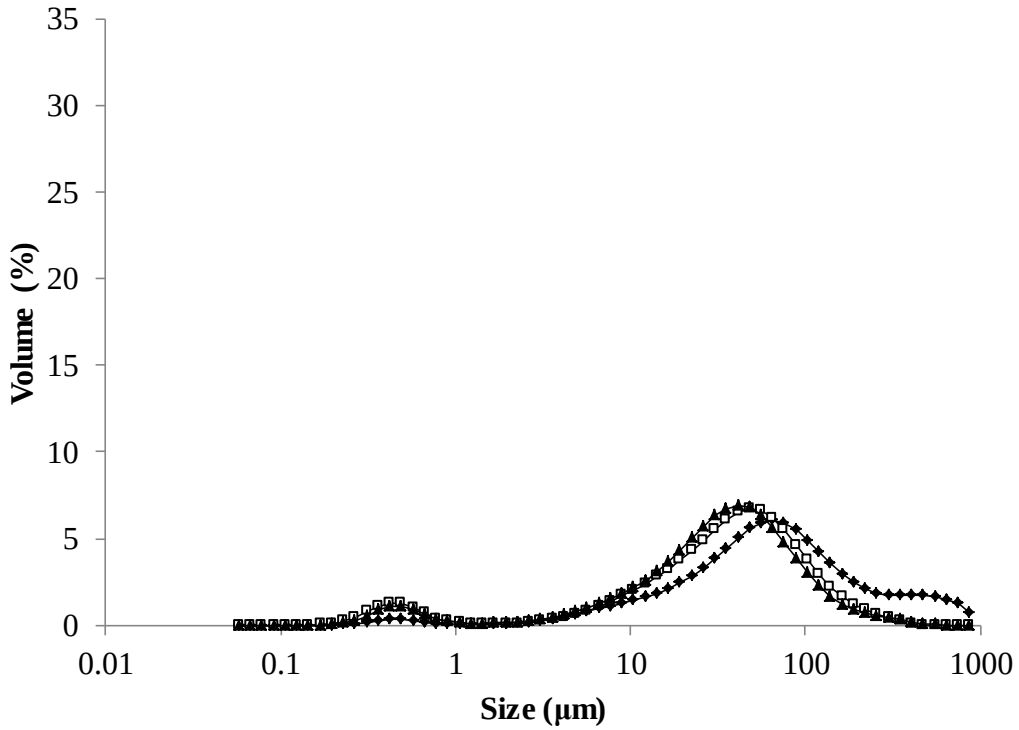

Fig. S4. Particle size distribution (PSD) data for milk protein concentrate (MPC)90 after 90 min rehydration in deionised water (♦), 80 mM KCl (□) or milk permeate (▲) at 50°C. Note: ranges of values shown in axes match those of PSD profiles in main manuscript.

Appendix 2

Additional research outputs

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Use of ultrafiltration to prepare a novel permeate for application in the functionality testing of infant formula ingredients

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ABSTRACT

Ultrafiltration (UF) permeates produced from reconstituted infant milk formula powder (IMF; 1.3%, w/w, protein) and reconstituted skim milk powder (SMP; 3.2% protein) were compared with simulated milk ultrafiltrate (SMUF) in terms of composition, physicochemical properties and impact, as dispersants, on the heat stability of model infant formula systems. Permeates from IMF and SMP were generated at 15 °C using a lab-scale UF unit with a 10 kDa cut-off polyethersulfone membrane. Operation at optimal cross-flow velocity and sub-critical flux allowed 1 L of IMF to be concentrated by a volume concentration factor (VCF) of 3 in 20 ± 2 min, with minimal flux decline and constant trans-membrane pressure (*TMP*); conversely, UF took 33 ± 4 min for SMP, with a decrease in flux and increase in *TMP* over that time. Permeate from IMF (IMF_p) had a markedly different mineral profile to SMP permeate (SMP_p), with the former having considerably lower levels of the major ions (e.g., calcium, phosphorus and sodium). IMF_p, SMP_p, SMUF or deionised water was used to reconstitute milk protein concentrate (MPC)80 and whey protein isolate (WPI) powders in combination to give 5.5% total protein and a 60:40 ratio of whey protein:casein. These model IMFs were assessed for heat stability at pH 6.8 and 140 °C; the type of dispersant used influenced heat stability strongly, with heat stability decreasing in the order water > IMF_p > SMP_p > SMUF. Calcium-ion concentrations of 0.01, 0.71, 1.51 and 1.77 mM L^{-1} were measured for water, IMF_p, SMP_p and SMUF, respectively, indicating that increased heat stability of proteins dispersed in IMF_p, compared to SMP_p or SMUF, may have been due to lower calcium-ion concentration. This study highlights the influence of serum phase composition on the heat-induced destabilisation of infant formula ingredients and outlines a novel approach for the generation of IMF_p, which is of importance in the development of ingredients which remain stable during the processing of IMF products.

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1. Introduction

Research into infant formula is undergoing a period of substantial growth, owing to a number of converging factors, including increased understanding of compositional differences between human and bovine milk [1], diversification of infant formula product ranges and types [24], increase in the demand for infant formulae due to economic growth of nations in Asia, the Middle East, North Africa and South America [3], and a limited literature base related to changes in infant formulae during formulation, processing and storage.

Laboratory-based studies on infant formulae typically necessitate the reconstitution of dried ingredients (e.g., casein and whey protein powders, and lactose) before analysis, to create a model infant milk formula [22,5,21]. During this reconstitution step, water or buffer solutions may be used as dispersants for the powders; in addition, water or buffers may be used as diluents for liquid infant milk formulae in the analysis of certain physicochemical properties (e.g., protein particle size measurement by dynamic light-scattering).

Jenness and Koops [19] formulated simulated milk ultrafiltrate (SMUF) based on the composition of the ultrafiltration (UF) permeate of milk, and SMUF is used in a range of applications in dairy science laboratories. Some of these applications include, *inter alia*, dispersion of milk protein powders [5,8], dilution of milk protein solutions for particle size and zeta potential measurement [14], and modelling of ion equilibria in milk [10]. A buffer simulating the aqueous phase of cheese, synthetic cheddar cheese aqueous phase (SCCAP) has also been developed, based on analysis of the liquid expressed when cheese was compressed in a hydraulic press [4]. Both SMUF and SCCAP are prepared using readily available lab-grade minerals, and the underlying "recipe" can be customised by the end-user for specific applications. For example, O'Mahony

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Nomenclature

CP	concentration polarisation
HCT	heat coagulation time
IMF	reconstituted infant milk formula powder
IMF_p	infant milk formula permeate
J	permeate flux
J_{crit}	critical flux
J_{lim}	limiting flux
J_w	water flux
L_p	hydraulic permeability
Q	volumetric feed flow rate
R_m	hydraulic resistance
SMP	reconstituted skim milk powder
SMP_p	skim milk permeate
SMUF	simulated milk ultrafiltrate
TMP	trans-membrane pressure
V	cross-flow velocity
VCF	volume concentration factor

et al. [25] modified SCCAP with varying levels of calcium chloride to facilitate direct measurement of the effect of colloidal calcium phosphate levels associated with the casein network on cheese textural and rheological properties. In many instances, where it is important to replicate the full complexity of the serum phase of a system such as milk (e.g., lactose, non-protein nitrogen, in addition to minerals), centrifugation, microfiltration or ultrafiltration may be performed to obtain a casein micelle-depleted serum phase [11]; however, guidelines for those wishing to generate these materials at lab-scale are limited.

To the authors' knowledge, a method for preparing a permeate by membrane filtration which replicates the serum phase of an infant milk formula has not been described. The development of such a permeate presents an opportunity to make studies on the techno-functional properties of model infant milk formulae more representative of real systems. The present study involved the preparation of UF permeates from reconstituted skim milk and infant formula powders, and comparison of these with SMUF. Optimal process conditions were determined for a rapid and sustainable UF process. The composition and physicochemical properties of the UF permeates and SMUF were measured. These materials, in addition to deionised water, were then used as dispersants for protein powders to elucidate the influence of serum phase composition on the heat stability of a model whey-dominant infant formula protein system.

2. Experimental

2.1. Materials

Low-heat skim milk powder was provided by the Irish Dairy Board (Fermoy, Co. Cork, Ireland). A commercial first-age infant milk formula was purchased from a local supermarket. Reconstituted skim milk powder (SMP) was prepared by slow addition of powder to deionised water under constant magnetic stirring at 50 °C to obtain a 3.21 ± 0.06% (w/w) crude protein suspension with a native pH of 6.7 ± 0.0. Reconstituted infant milk formula (IMF) was prepared under the same conditions to 1.28 ± 0.03% (w/w) crude protein with a native pH of 7.4 ± 0.1. The pH of the IMF was within the range of values (6.3–7.5) measured for non-acidified commercial infant formulae by Chávez-Servín et al. [6]. Once all powder had been added, solutions were left stirring for ~2 h and then stored overnight at 4 °C. SMUF at pH 6.7 ± 0.0 was prepared as described by Jenness and Koops [19], using analytical grade salts (obtained from Sigma–Aldrich, St Louis, Missouri, MO, USA) dissolved in deionised water.

2.2. Ultrafiltration rig

Lab-scale ultrafiltration (UF) experiments were performed using a pressure-driven, cross-flow filtration device, consisting of a membrane cartridge (Millipore Biomax: 10 kDa molecular weight cut-off, 1000 cm^2 total membrane area, V (viscous)-screen) enclosed within a stainless steel membrane holder (Pellicon 2 mini-holder), all supplied by Merck-Millipore (Tullagreen, Carrigtwohill, Co. Cork, Ireland). Additional information on the geometric and hydrodynamic properties of the membrane cartridge is provided in Table 1.

The feed (1 L) was subjected to continuous magnetic stirring in a glass beaker. A Watson-Marlow 520 s variable speed tri-lobed peristaltic pump (Lennox Pump and Process, John F. Kennedy Drive, Naas Road, Co. Dublin, Ireland) delivered the feed into the feed port of the membrane unit; the volumetric feed flow rate (Q) was controlled by adjusting the rpm of the pump, and values of Q were converted to cross-flow velocity (V) according to Table 1. Braided tubing was installed between the pump and the feed port to minimise oscillatory flow fluctuations caused by pulsation. The retentate passed through a plate heat-exchanger, with counter-flow coolant in alternate channels supplied by a water bath set at 15 °C. The retentate was recirculated to the feed vessel throughout processing, thereby maintaining feed temperature at 15 °C. Two pressure gauges were installed close to the feed and retentate ports, with a valve located on the retentate side for control of trans-membrane pressure (TMP). Permeate was collected in a 1 L graduated cylinder during processing, with all feed materials being concentrated by a volume concentration factor (VCF) of 3. This VCF corresponded to final protein in dry-matter values of 59.5% and 16.5% for retentates of SMP and IMF, respectively. Permeate flux (J) was recorded in duplicate during processing by measuring the volume of permeate generated in a graduated cylinder over 30 s.

2.2.1. Determination of operational parameters

Critical (J_{crit}) and limiting (J_{lim}) flux for SMP and IMF were determined in duplicate in one freshly prepared sample of each at different values of V in constant-TMP mode [27,2] at a constant VCF of 1 using the configuration described earlier, with the exception that both permeate and retentate lines were returned to the feed vessel (i.e., full recirculation mode). TMP was increased step-wise in 0.2 bar increments by partially closing the valve on the retentate side. J was measured in duplicate after an equilibration time of 3–4 min at each TMP. Step-wise increases in TMP were continued until the initially linear TMP–J relationship (pressure-dependant region) reached a plateau (pressure-independent region). Values of J_{crit} and J_{lim} were determined as described by Youravong et al. [29], who performed both constant-J and constant-TMP experiments on skim milk and reported that both methods yielded the same results. Starting values of J for permeate generation were selected for operation in the sub-J_{crit} region. The slope (t) of a linear fit of log J_{lim} as a function of log V was used to assess the influence of turbulence on J for SMP and IMF (Table 1). Hysteresis, due to irreversible fouling [2], was assessed by returning TMP to 0.2 bar and repeating the measurement of J. To determine if the feed

Table 1
Geometric and hydrodynamic properties of polyethersulfone membrane cassette, with 1000 cm^2 surface area and 10 kDa molecular weight cut-off, used during ultrafiltration experiments. J_w = water flux (L m^{-2} h^{-1}); J = permeate flux (L m^{-2} h^{-1}), J_{lim} = limiting flux (L m^{-2} h^{-1}); $\mu_w^{15°C}$ = dynamic viscosity of water at 15 °C (Pa·s); *TMP* = trans-membrane pressure (Pa); c = y-intercept of linear regression; Q = volumetric flow rate (L s^{-1}) of feed; IMF = reconstituted infant milk formula, SMP = reconstituted skim milk.

Geometrical properties	Symbol	Value	Source
Spacer present	–	Yes	Merck-Millipore technical representative
Channel height (cm)	h	0.09	
Channel width (cm)	w	4.0	
Number of feed channels (–)	n	12	
Hydrodynamic properties			*Calculation*
Hydraulic diameter (cm)	d_H	2.1	$d_H = \left(4 \times \frac{w \times h}{2(w+h)}\right) \times n$
Hydraulic permeability (L m^{-2} h^{-1}/Pa)	L_p	99	$J_w = L_p(TMP)$
Hydraulic resistance (m^{-1}) at 2.0×10^4 Pa *TMP*	R_m	1.9×10^{13}	$R_m = \frac{TMP}{\mu_w^{15°C}} \times J_w$
Hydraulic resistance (m^{-1}) at 1.2×10^5 Pa *TMP*		2.9×10^{13}	
Cross-flow velocity of feed (m s^{-1})	V	0.09–0.36	$V = \frac{Q}{w \times h}$
Influence of hydraulic turbulence on J (SMP)	t	0.82	$logJ_{lim} = t(logV) + c$
Influence of hydraulic turbulence on J (IMF)		0.62	

materials exhibited the weak- or strong-form of J_{crit}, the *TMP*–J profiles were compared to those established at each V for deionised water, as described by Youravong et al. [29]; the slope of the plot of water flux (J_w) against *TMP* was used to calculate the hydraulic permeability (L_p) and resistance (R_m) of the membrane (Table 1).

2.2.2. Cleaning procedure

The UF system was cleaned fully after each concentration process and determination of J_{crit}. First, the feed line was removed from the feed vessel to allow air to force residual feed material out of the unit. Subsequently, the system was flushed with distilled water until liquid expressed through the permeate and retentate lines was clear. The system was then flushed with ~2.5 L m^{-2} of 0.1 N NaOH at a *TMP* of 0.0 bar and another ~2.5 L m^{-2} at a *TMP* of 0.5 bar. Recirculation of 0.1 N NaOH was then carried out for 45–60 min. The membrane cartridge was subsequently removed and placed in a sealed container of 0.1 N NaOH and stored at 4 °C. On start-up, approximately 100–150 L m^{-2} of distilled water was flushed through the unit until the pH of the feed, retentate and permeate lines were within 0.1–0.2 pH units of each other. The feed vessel was then flushed with deionised water, before recirculation of same. Cleaning efficiency was assessed by measuring water permeability after cleaning. If water permeability exceeded a difference of ±20% from the previous run, cleaning was repeated. For cleaning of the heat exchanger, the peristaltic pump was disconnected from the membrane unit and connected directly to the heat exchanger. The cleaning cycle for the heat exchanger was as follows: 2 L of water followed by 1 L of 1.0 N NaOH, followed by 2 L of water, followed by 1 L of 1.0 N HNO_3, and a final flush with 2 L water. All cleaning steps, for both the membrane unit and the heat exchanger, were performed at a controlled temperature of 50 °C.

2.3. Composition and physicochemical analyses of process streams

Ash content was determined after heating samples at 550 °C until a white ash was obtained [23]. Inductively-coupled plasma mass spectrometry (ICP-MS) was used to measure mineral profile, as described by Herwig et al. [16]. Crude protein, true protein and non-protein nitrogen (NPN) were determined as per ISO [18], using the Kjeldahl method and nitrogen-to-milk protein conversion factors of 6.38 for SMP/SMP_p and 6.25 for IMF/IMF_p. Solids content was determined with a HB43-S Halogen rapid moisture analyser equipped with a halogen bulb and balance (Mettler Toledo GmbH, Schwerzenbach, Switzerland). Lactose content was determined by iodometric titration [17]. Calcium (Ca)-ion concentration was determined with a Ca-ion selective electrode, as described by Crowley et al. [7], except that the calibration curve was constructed from standard solutions containing 2, 4, 6, 8 and 10 mM L^{-1} of Ca. Conductivity was determined at 25 °C with a five-ring conductivity measuring cell (Metrohm Ireland Ltd., Athy Road, Co. Carlow, Ireland). Viscosity was measured with a Thermo Scientific HAAKE RotoVisco 1 viscometer (Dieselstrasse 4, D-76227, Karlsruhe, Germany) at 50 °C and a shear rate of 300 s^{-1}, using a cup and bob geometry. Heat stability was determined at 140 °C as per the method of Davies and White [9], except that heat coagulation time (HCT) was determined at a single pH (6.8) as described by Vyas and Tong [28]; the pH of individual samples post-coagulation was measured after samples were held at 20 °C for 7 min (to allow safe removal of tubes from rack), held in iced-water for a further 3 min, and pooled (due to limitations in quantity of sample). Results for composition, physicochemical properties and heat stability are the means of data from the analysis of three independently prepared samples, except for the mineral profiles of SMUF (n = 2) and the feeds (n = 1).

3. Results and discussion

3.1. Operational parameters and hydrodynamics of ultrafiltration

For both feed materials (SMP and IMF), J_{crit} values were below J_{lim} and increased with increasing V (Fig. 1). In complex systems such as milk, J is influenced strongly by the balance between the rates of solute deposition and the rate of solute removal at the membrane surface. By increasing V, turbulence is promoted, which increases the forces applied tangentially to the membrane (i.e., wall shear stress; see [12]) and thereby the rate at which solutes are removed from the membrane surface; this has the effect of reducing the hydraulic resistance created by deposited solutes, resulting in increased J values [15].

SMP exhibited lower J_{crit} values than IMF, for an equivalent value of V (Fig. 1). During cold UF with polymeric membranes, such as the 10 kDa PES membrane used in the present study, the dominant foulants are proteins, rather than minerals [29,13]; thus, the lower J values measured for SMP were likely due to it having a higher protein content than IMF, which resulted in increased deposition of solutes on the membrane surface. Log–log plots of J_{lim} against V yielded linear relationships for SMP (R^2 = 0.97) and IMF (R^2 = 0.99), the slopes of which demonstrated that the positive effect of V on J was more pronounced for SMP than IMF (Table 1), indicating the occurrence of greater solute deposition and, therefore, the greater positive influence of turbulence on processing of the former. It should be noted that, in general, no trends were observed in terms of J hysteresis (Fig. 1); thus, the

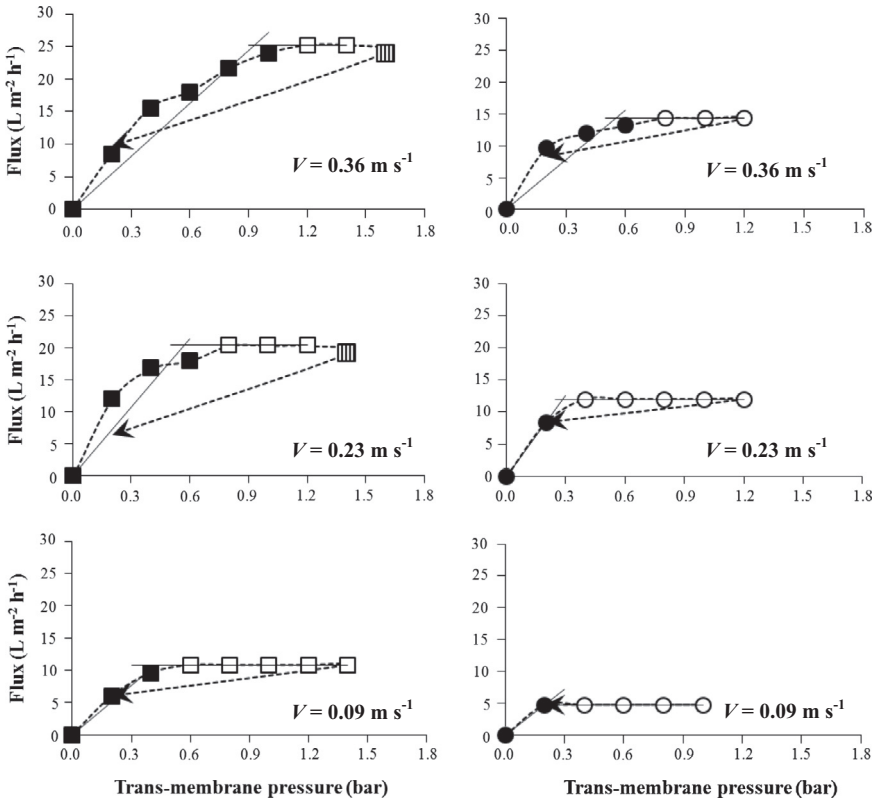

Fig. 1. Permeate flux as a function of trans-membrane pressure (*TMP*) and cross-flow velocity (*V*) at 15 °C for reconstituted skim milk powder (SMP; circles) and reconstituted infant milk formula powder (IMF; squares). Regions where flux (*J*) is pressure-dependant and pressure-independent are denoted by closed and open symbols, respectively; linear fits were applied to both of these regions, and critical flux was determined as being the last point in the pressure-dependant region. The broken line is a plot through all the data points, where the arrow indicates whether hysteresis of *J* occurred due to membrane fouling when *TMP* was returned to 0.2 bar. Symbols with vertical lines inset indicate that limiting flux has been exceeded.

majority of *TMP–J* profiles were deemed not to be influenced greatly by extensive pore-blocking and other irreversible fouling mechanisms [2]. However, when J_{lim} was exceeded at a *V* of 0.23 m s^{-1} for IMF, hysteresis of *J* occurred with a decrease in *J* at 0.2 bar *TMP* of ~50% (Fig. 1); this effect was not observed when J_{lim} was exceeded at a higher *V* of 0.36 m s^{-1}, highlighting further the positive role of turbulence in the inhibition of fouling. In contrast to results for both SMP and IMF, the *TMP–J* relationship for deionised water was linear at all *V* values (Fig. 2), i.e., as no fouling material was present, hydraulic resistance was due solely to the membrane itself, with higher *V* values exerting no apparent influence on its innate permeability. Hydraulic resistance of the membrane (R_m) increased at increasing *TMP* (Table 1) due to physical compression of the membrane [26]. As the initially linear regions of the *TMP–J* profiles for SMP and IMF both had much lower slopes than those for water, the J_{crit} values obtained can be classified as weak-form in both cases [2], as reported by Youravong et al. [29] for skim milk.

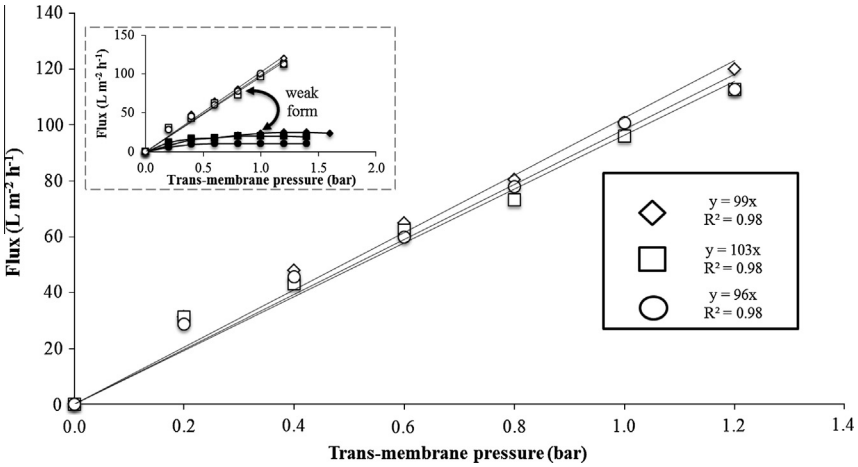

Fig. 2. Main: Permeate flux (*J*) as a function of trans-membrane pressure (*TMP*) during ultrafiltration of deionised water at a cross flow velocity (*V*) of 0.09 (○), 0.23 (□) or 0.36 (◇) m s^{-1}, and a temperature of 15 °C. The inset figure shows a comparison of the *TMP–J* relationship for deionised water and reconstituted infant milk formula powder (IMF), indicating weak-form critical flux; data for IMF at *V* values of 0.09 (●), 0.23 (■), and 0.36 (♦) m s^{-1} are presented.

3.2. Process performance during ultrafiltration

Based on the results shown in Fig. 1, the UF of SMP and IMF was performed at sub-J_{crit} (SMP: <15 L m^{-2} h^{-1}; IMF: <25 L m^{-2} h^{-1}) using the highest V value measured (0.36 m s^{-1}). Sub-J_{crit} operations are optimal for dairy systems in terms of process efficiency and sustainability, despite the slower process they afford compared to operation at J_{lim} [29] and the associated requirement for larger membrane area [20]. In addition, in this study, it was important to generate permeates which had not been depleted of any components due to excessive fouling. It is sometimes undesirable to operate at high V, even when increased J can be attained (resulting in reduced process times), due to the requirement for higher capacity pumps and increased energy inputs during scale-up (Merck-Millipore technical representative; personal communication); however, as this study was focused on the development of a dispersant for the evaluation of model infant milk formula systems at lab-scale, the highest V value was selected for subsequent concentration experiments.

During processing, J was much higher for IMF than SMP (Fig. 3), resulting in a considerably reduced total run time for IMF (20 ± 2 min) compared to SMP (33 ± 4 min). Interestingly, after ~16 min processing, J and *TMP* were found to decrease and increase, respectively, for SMP, while no similar trends were observed for IMF. These results suggested that a concentration polarisation (CP) layer was formed immediately on start-up for both feeds [20], as indicated by the weak form J_{crit} shown in Fig. 2, which then remained stable during UF of IMF.

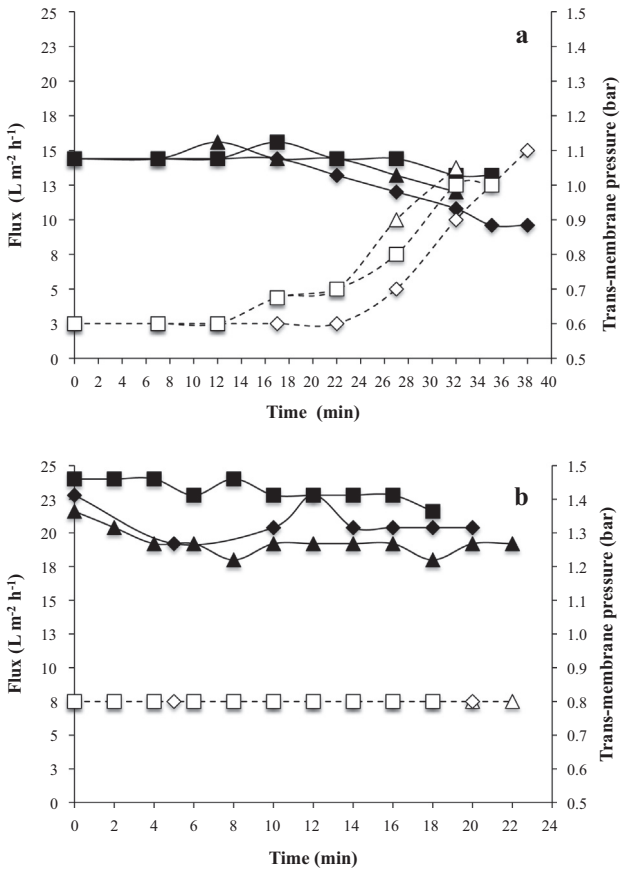

Fig. 3. Permeate flux (solid lines, closed symbols) and trans-membrane pressure (broken lines, open symbols) during ultrafiltration of reconstituted skim milk powder (SMP; a) and reconstituted infant milk formula powder (IMF; b) at 15 °C. Data from three independent trials are presented where diamonds indicate trial 1, triangles indicate trial 2 and squares indicate trial 3.

For SMP, flux began to decline steadily after ~16 min, indicating that, in addition to CP, minor fouling, which can occur during sub-J_{crit} UF of skim milk [29], may have contributed to increased hydraulic resistance [20]. Viscosity of SMP would have increased markedly as concentration progressed, resulting in reduced turbulence and wall shear stress, and, subsequently, a sufficient decrease in the rate of solute removal from the membrane surface to allow more extensive fouling. Indeed, the final retentate viscosity values for SMP and IMF were 28.4 ± 3.8 and 7.0 ± 0.3 mPa s, respectively; the fact that viscosity was fourfold higher for the former suggests strongly that it played a prominent role in the observed decrease in J, while the low viscosity of IMF retentate permitted a sufficiently consistent rate of solute removal from the membrane surface to allow steady J and *TMP* during processing. Increased concentration during the UF of SMP also resulted in an increase in *TMP* (Fig. 3), which may have further exacerbated the issue of decreased J by compression of the fouling layer and/or the membrane itself (see Table 1 and [26]).

3.3. Composition of ultrafiltration permeates and SMUF

As expected, SMUF and SMP_p displayed very similar composition and physicochemical properties (Table 2), indicating that the UF process used in this study generated a permeate which was an accurate representation of the serum phase of milk. IMF_p was characterised as having a higher lactose (and thereby, solids) content, and slightly lower NPN, than SMP_p (Table 2), due to innately different levels of these components in the feed materials. True protein values for the permeates indicated negligible transmission of protein through the membrane. Lower ash levels, conductivity values and Ca-ion concentration for IMF_p showed that IMF contained a markedly different serum phase compared to SMP_p and SMUF. The lower Ca-ion concentration in IMF_p, compared with SMP_p or SMUF, may have been caused by the higher pH of the IMF (which may have resulted in formation of insoluble tertiary Ca phosphate), the use of insoluble Ca salts (i.e., salts of carbonate or phosphate) during fortification, or the presence of higher levels of Ca-binding ions (e.g., citrate) in the IMF.

Low ash levels and conductivity values for IMF_p compared to SMP_p were consistent with the mineral profile results, with considerably lower levels of major minerals measured in the former (Table 3). Transmission from the feed to the permeate of monovalent ions (sodium and potassium), which exist predominately in the serum phase of milk, was equivalent for both feeds (Table 3); conversely, only ~25% of calcium and ~50% of magnesium (again equivalent for both feed materials) were transmitted during UF of SMP and IMF (Table 3), as approximately 70% and 35%, respectively, of these divalent cations are associated with casein micelles in milk [11]. The feeds differed substantially in terms of transmission of phosphorus (which is also distributed between casein micelles and the serum phase) into the permeate, with values of 35% and 22% for SMP and IMF, respectively. The level of phosphorus was ~4 times higher in SMP than in IMF, but ~6 times higher in SMP_p compared to IMF_p, due to the reduced transmission of phosphorus during UF of IMF (Table 3). It is unclear why transmission of phosphorus into the permeate was lower for IMF than SMP, but increased association of phosphate with caseins at the higher pH of the IMF feed may have been responsible [11]. SMP generally had 2–3 times the levels of sodium, potassium, calcium and magnesium than IMF in both feeds and permeates.

3.4. Effect of dispersant type on heat stability of model IMF

When considering the HCT values shown in Fig. 4 for model IMF systems prepared with water, IMF_p, SMP_p or SMUF, as dispersant, it is important to consider the composition and physicochemical

Table 2
Composition and physicochemical properties of the ultrafiltration permeates of reconstituted skim milk powder (SMP_p) and reconstituted infant formula powder (IMF_p), in addition to simulated milk ultrafiltrate (SMUF).

Compositional properties						Physicochemical properties		
Sample	Solids	TP [a]	Lactose % (w/w)	NPN [b]	Ash	Viscosity mPa s	Conductivity mS cm^{-1}	Ionic Ca[c] mM L^{-1}
SMP_p	5.71 ± 0.01	0.02 ± 0.01	3.39 ± 0.18	0.02 ± 0.00	0.49 ± 0.03	1.62 ± 0.19	5.69 ± 0.55	1.51 ± 0.08
IMF_p	7.37 ± 0.02	0.00 ± 0.00	4.54 ± 0.11	0.01 ± 0.00	0.18 ± 0.01	1.67 ± 0.22	2.25 ± 0.13	0.71 ± 0.03
SMUF	0.76 ± 0.03	N/A	N/A	N/A	0.44 ± 0.01	1.68 ± 0.01	6.59 ± 0.09	1.77 ± 0.03

[a] TP = true protein i.e., (total nitrogen − non-protein nitrogen) × nitrogen-to-protein conversion factor.
[b] NPN = non-protein nitrogen.
[c] Ca = calcium.

Table 3
Mineral profile of reconstituted skim milk powder (SMP), ultrafiltration permeate from SMP (SMP_p), reconstituted infant milk formula powder (IMF), ultrafiltration permeate from IMF (IMF_p), and simulated milk ultrafiltrate (SMUF).

Ion	SMP	IMF	SMP_p mg kg^{-1}	IMF_p	SMUF	SMP ⇒ SMP_p Transmission (%)[a]	IMF ⇒ IMF_p Transmission (%)[a]
Sodium	375	168	307 ± 22	137 ± 7	413 ± 8	81.9	81.5
Potassium	1771	673	1400 ± 97	529 ± 37	1522 ± 28	79.1	78.6
Calcium	1206	353	299 ± 22	87.6 ± 6.4	280 ± 1	24.8	24.8
Phosphorus	1030	256	358 ± 24	55.7 ± 4.2	318 ± 4	34.8	21.8
Magnesium	112	55.9	59.4 ± 4.6	28.5 ± 1.9	72.4 ± 0.7	53.0	51.0

[a] Transmission was calculated as $\frac{Level\ of\ ion\ in\ permeate}{Level\ of\ ion\ in\ feed} \times 100$.

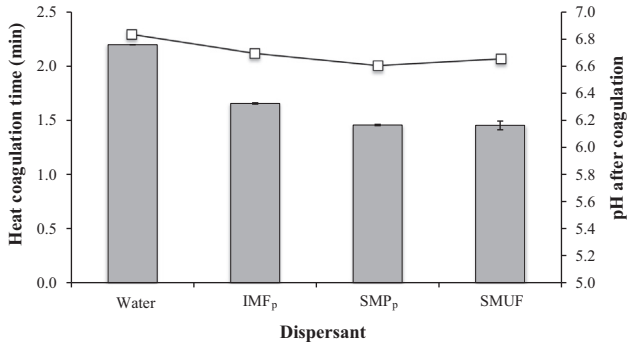

Fig. 4. Heat coagulation time (bars) at 140 °C and pH after coagulation (□) as a function of dispersant type for model whey-dominant infant milk formula protein system. SMP_p = ultrafiltration permeate from reconstituted skim milk powder; IMF_p = ultrafiltration permeate from reconstituted infant milk formula; SMUF = simulated milk ultrafiltrate.

properties of each dispersant. Certain components of NPN (e.g., urea) can stabilise milk to heat-induced coagulation, while lactose can either destabilise (through heat-induced acidification) or stabilise (through Maillard reactions) milk proteins during heating [8]. However, in this study, the contribution of heat-induced acidification can be safely ignored, as the HCT times were too short (<2 min) for a sufficient decrease in pH to occur (Fig. 4); in addition, the low HCT values and pH of 6.8 meant that Maillard reactions were unlikely to have had a marked influence on heat stability [8]. Thus, Ca-ion-mediated charge-screening with concomitant interactions between negatively-charged proteins was probably the primary destabilising mechanism [28,7,8]). SMUF and SMP_p were the least stable to heating (Fig. 4), likely due to high Ca-ion concentration (Table 2); conversely, the low Ca-ion concentration of IMF_p, and the very low levels of ionic Ca in deionised water (0.01 mM L^{-1}), may have been responsible for the highest HCT values.

4. Conclusion

A method for the generation of IMF permeate (IMF_p) using lab-scale UF was developed. IMF_p, when used as a dispersant for protein powders, increased the heat stability of a model IMF protein system compared with SMP_p or SMUF, due mainly to lower levels of minerals, and in particular free Ca ions, in the IMF_p. The process described herein generates a dispersant/diluent which could be used to evaluate the functionality of infant formula ingredients in a serum phase which is more representative of the fully formulated system. In this way, the predictive power of analytical tests to determine the destabilisation of ingredients during the processing of infant formulae (e.g., coagulation, fouling) may be enhanced. As the equipment described in this study for generating the novel permeate may not be commonly available, the development of simulated infant milk ultrafiltrate (SIM-UF), composed of readily available lab-grade mineral salts, based on the protocol described herein for the generation and analysis of IMF permeates, which would serve as models for the SIM-UF, warrants further research and development.

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Impact of α-lactalbumin:β-lactoglobulin ratio on the heat stability of model infant milk formula protein systems

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ABSTRACT

Model infant milk formula systems (5.5% protein) were formulated to contain α-lactalbumin:β-lactoglobulin ratios of 0.1, 0.5, 1.3, 2.1 or 4.6 and assessed for heat stability and heat-induced changes. 'Humanising' the model formulas by increasing α-lactalbumin:β-lactoglobulin enhanced heat stability at 140 °C in the pH range 6.6–6.9. The model formulas were analysed after lab-scale high-temperature short-time heating at pH 6.8. Gel electrophoresis indicated that increased heat stability in high α-lactalbumin:β-lactoglobulin samples was due to decreased covalent interactions between proteins. In low α-lactalbumin:β-lactoglobulin formulas, protein–protein interactions caused marked increases in protein particle size and viscosity of the heated systems; conversely, covalent interactions between proteins were minimal in high α-lactalbumin:β-lactoglobulin formulas. Reduced protein–protein interactions with increasing α-lactalbumin:β-lactoglobulin has important implications for subsequent processing; for example, lower viscosity post-heating may affect bulk density in spray-dried products or physical stability in ready-to-feed products.

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1. Introduction

One of the key compositional differences between human and bovine milks is protein, in terms of both quantity and quality. For several decades, first-age infant milk formula products (IMFs) have been formulated to be whey protein-dominant (i.e., containing a whey protein:casein ratio of ~60:40 in the final product). Demineralised whey or whey protein concentrate/isolate, when added to skim milk in the correct proportions, can be used to attain the desired whey protein:casein ratio (De Wit, 1998; Fomon, 2001). This protein base, to which other ingredients (e.g., lipids, carbohydrates, minerals and vitamins) are added, is standardised to give the required protein content (typically 1.2–1.8% protein) in the final product. This protein level is higher than that of human milk (~0.9–1.1%), which is an intentional compensation to ensure the provision of sufficient quantities of essential amino acids, such as tryptophan, tyrosine and cysteine, which are present in higher concentrations in human milk than in bovine milk (De Wit, 1998; European Commission 2006/141/EC).

The whey protein fraction in bovine milk is dominated by β-lactoglobulin (β-lg), which accounts for ~50% of total whey protein, while α-lactalbumin (α-lac) comprises 20% of total whey protein. However, β-lg is absent from human milk and α-lac predominates (Armaforte et al., 2010). In fact, the presence of β-lg in IMFs is believed to be one of the primary stimulators of allergenic responses in IMF-fed infants, which has resulted in the development of hydrolysed or "hypoallergenic" IMFs (Murphy et al., 2015). Another approach to reducing allergenicity involves the conjugation of β-lg with sugars to decrease its allergenicity to susceptible individuals (Böttger, Etzel, & Lucey, 2013; Taheri-Kafrani et al., 2009). In addition, methods to deplete β-lg from whey to produce α-lac-enriched ingredients for use in applications such as IMF formulation are well established (Lucena, Alvarez, Menendéz, & Alvarez, 2006; Pearce, 1995; Stack, Hennessy, Mulvihill, & O'Kennedy, 1999). Formulation of IMFs to contain higher α-lac:β-lg ratios can be achieved through fortification with these α-lac-enriched whey protein ingredients to yield a more humanised product, with scientifically-validated physiological benefits for the infant (Davis, Harris, Lien, Pramuk, & Trabulsi, 2007; Kuhlman, Lien, Weaber, & O'Callaghan, 2003; Sandström, Lonnerdal, Graverholt, & Hernell, 2008). Furthermore, enrichment with α-lac may allow a reduction in the protein content of IMFs to values closer to those found in human milk, as the amino acid requirements set forth in international regulations (European Commission 2006/141/EC) for IMFs would be satisfied to a greater degree (Kuhlman et al., 2003).

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It is important to investigate the implications of increasing α-lac:β-lg ratio on heat stability and heat-induced changes of IMFs, as IMFs are commonly subjected to several heating steps during their manufacture, which can include combinations of high-temperature short-time (HTST) heating, evaporation, ultra-high temperature (UHT) treatment and in-container sterilisation (Jiang & Guo, 2014). As whey protein, in particular β-lg, is one of the dominant contributors to heat-induced changes in milk (Rattray & Jelen, 1997; Singh, 2004), any alteration to α-lac:β-lg ratio in IMFs is likely to alter their behaviour during thermal processing, which, in turn, may affect the quality of IMF products (Murphy, Fenelon, Roos, & Hogan, 2014).

In this study, model IMFs with a protein content of 5.5% (typical for IMFs after wet-mixing of ingredients and at the point of heat treatment), a constant whey protein:casein ratio of 60:40, and varying ratios of α-lac:β-lg were formulated. The role of α-lac:β-lg ratio in determining the extent of heat-induced changes in model IMFs was then determined in a series of experiments. Stability to heating at 140 °C and viscosity changes during lab-scale high-temperature short-time (HTST) were studied. Samples before and after HTST heating were the subject of further analysis, including particle size distribution (PSD) using dynamic-light scattering, physical stability using analytical centrifugation, and protein profile using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2. Materials and methods

2.1. Materials

Milk protein concentrate 80 (MPC80) with a protein content of 82.8% (w/w) was supplied by NIZO food research (Zb Ede, The Netherlands). Whey protein isolates comprising α-lac (90% purity, 93.5% protein) and β-lg (90% purity, 88.7% protein) were obtained from Davisco Foods International (Le Sueur, MN, USA).

2.2. Preparation of model infant milk formula protein systems

Model IMF protein systems were formulated to contain 5.5% total protein, with MPC80 contributing 50% of protein, and the remaining 50% comprised of five different blends of α-lac and β-lg. All samples were reconstituted in simulated milk ultrafiltrate (SMUF; Jenness & Koops, 1962). Powders were reconstituted by magnetic stirring at 22 °C for 3 h, with whey protein powder being reconstituted first. Each sample was then adjusted to the desired pH using 0.5 M HCl or 0.5 M NaOH and stored at 4 °C for 18 h to facilitate complete rehydration. Before analysis, samples were stirred magnetically at 22 °C for 1 h and pH was re-adjusted if necessary.

The protein profiles of all model IMFs were determined by reversed-phase high-performance liquid chromatography (RP-HPLC) using the method of Crowley et al. (2015), and are shown in Fig. 1; for the remainder of this paper, the model IMFs will be referred to by their α-lac:β-lg ratios (Fig. 1). The whey protein:casein ratio, where total whey protein was considered to comprise α-lac and β-lg, was measured as 59 ± 3:41 ± 3 for the model IMFs.

2.3. Compositional analysis

Total solids content was measured by oven drying (IDF, 1987). Total nitrogen was determined using the macro-Kjeldahl method (IDF, 1986), and a nitrogen–protein conversion factor of 6.38 was used to calculate total protein. Fat content was determined using the Gerber method (IDF, 1991). Lactose content was measured by HPLC as described by Indyck, Edwards, and Woollard (1996), except that the protein precipitation step was performed using tungstic acid. Mineral profiles were determined by inductively-coupled plasma mass spectrometry (ICP-MS) according to the method of Herwig, Stephan, Panne, Pritzkow, and Vogl (2011). Calcium (Ca)-ion concentration of model IMFs at 20 °C was measured using a polymer membrane Ca-ion-selective electrode (Metrohm Ltd., Ireland), as described by Crowley, Kelly, and O'Mahony (2014), except that the standard curve was prepared using standards containing 2, 4, 6, 8 and 10 mM L^{-1} of Ca. Results showing the complete compositional profile of the model IMFs are presented in Table S1.

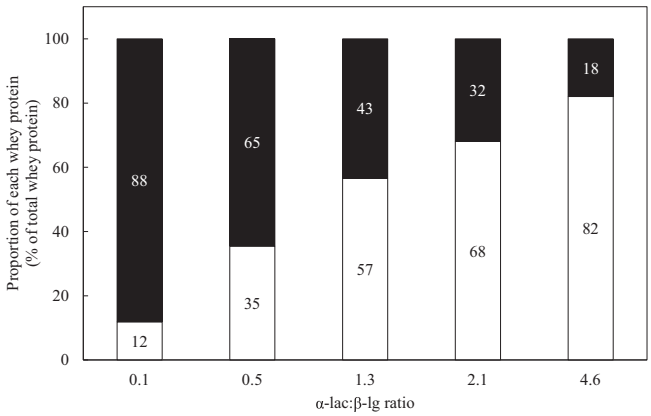

Fig. 1. Relative proportions of β-lactoglobulin, β-lg (■) and α-lactalbumin, α-lac (□) in model infant milk formula (IMF) samples with different α-lac:β-lg ratios. Data are from reversed-phase high-performance liquid chromatography analysis of at least two freshly prepared samples of each model IMF. "Total whey protein" refers to α-lac + β-lg.

2.4. Heat stability at 140 °C as a function of pH

Samples were prepared as outlined in Section 2.2 and divided into 11 aliquots before adjustment of pH at 0.1 pH unit intervals in the range 6.2–7.2 using 0.5 M HCl or 0.5 M NaOH. Heat stability was measured at 140 °C by the method of Davies and White (1966) under the conditions specified by Crowley et al. (2014). In commercial UHT processing, the duration of the thermal treatment is much shorter than that in a typical heat stability test; however, tests in which heat coagulation time is measured are a very commonly used index of heat stability in milk protein systems, and are regarded as a reliable method of measuring inter-sample differences and trends in heat stability (Singh, 2004).

2.5. Simulated HTST treatment

The change in viscosity of model IMFs at pH 6.8 during a heating, holding and cooling cycle designed to simulate HTST treatment was measured using a TA Instruments AR-G2 controlled-stress rheometer (Crawley, West Sussex, UK) with an aluminium parallel plate (60 mm diameter) and TRIOS v8.32 software, as described by Crowley et al. (2014), except that samples were heated to 85 °C before cooling to 5 °C.

To increase the quantity of heated sample available for further analysis, the rheometer was equipped with a starch pasting cell geometry with 28 mL sample capacity. The same heating–holding–cooling regime detailed above for the parallel plate was applied, and the contents of the starch pasting cell were subjected to a constant shear rate of 15 s^{-1}. Before and after heat-treatment, samples were analysed as described in Sections 2.6–2.8.

2.6. Protein particle size distribution

Unheated and heated model IMFs were diluted 1:200 in SMUF and equilibrated for at least 1 h at 22 °C. Protein particle diameter and polydispersity index were measured at 25 °C, after 120 s of temperature equilibration, using dynamic light-scattering (Zetasizer Nano series HT, Malvern Instruments Ltd., Worcestershire, United Kingdom) and accompanying Malvern Zetasizer software v.7.02. The apparatus was equipped with a He–Ne laser emitting at 633 nm. A solvent viscosity of 0.8872 mPa s and solvent refractive index of 1.33 were used in particle size calculations. Intensity-weighted Z-average particle diameter and polydispersity index values are reported. A total of five measurements were taken for each individual replicate using a back-scattering configuration with a scattering angle of 173°. The term *protein particle size* is used in this manuscript as a general term which encompasses the size of native casein micelles, micelles with which whey proteins have complexed, and serum protein aggregates. Changes in protein particle size after heating were considered to have occurred due to a combination of whey protein–casein and whey protein–whey protein interactions.

2.7. Accelerated physical stability

The physical stability of unheated and heated model IMFs at 20 °C was measured using an analytical centrifuge (LUMiSizer, L.U.M. GmbH, Berlin, Germany) as described by Crowley et al. (2014), except that a shorter centrifugation time of 1 h was used. For calculating the change in transmission over time, integration limits were set at 109 and 127 mm in order to exclude the meniscus and sediment regions. A total of 60 profiles were collected over 1 h.

2.8. Protein profile analysis by SDS–PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under reducing and non-reducing conditions as described by Laemmli (1970) on the model IMFs before and after heating. An AcquaTank mini gel unit (Acquascience, Bellbrook Industrial Estate, Uckfield, UK) was used for running the gels, which were pre-cast 4–20% acrylamide tris–glycine gels (Pierce Protein Research Products, Australia). The staining solution used was Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories, Inc., USA) and gels were scanned using a desktop scanner (HP Scanjet G4010).

2.9. Statistical data analysis

One-way analysis of variance (ANOVA) with Tukey's HSD on selected data from at least two independent trials on freshly prepared model IMFs was carried out using MINITAB® v.16.2.4 (Minitab Ltd, Coventry, UK) statistical analysis package. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Composition

There were no significant differences ($P > 0.05$) in the composition of the model IMF protein systems in terms of total solids, crude protein, lactose or mineral profile (Table S1), with fat levels <0.20% in all samples. Altered α-lac:β-lg ratios in model IMFs formulated with different proportions of α-lac- or β-lg-enriched WPIs were confirmed by HPLC (Fig. 1).

3.2. Heat stability at 140 °C as a function of pH

In model IMFs with α-lac:β-lg ratios of 0.1 and 0.5, samples showed very poor heat stability (HCT < 2 min) across the entire pH range (Fig. 2), with a very slight increase in stability as pH increased. In model IMFs with α-lac:β-lg ratios of 1.3, 2.1 and 4.6, broad areas of increased heat stability were observed at pH 6.6–6.9 (Fig. 2). Maximum HCT for these samples increased with increasing α-lac:β-lg and shifted to slightly lower pH values. Heat stability at pH < 6.6 and >7.1 remained very low for all samples irrespective of α-lac:β-lg ratio (Fig. 2). Overall, increasing α-lac:β-lg ratio improved the heat stability of model IMF protein systems.

3.3. Viscosity changes during lab-scale HTST heating

No significant differences ($P > 0.05$) in viscosity were measured between the model IMFs during initial holding at 20 °C (Fig. 3). After HTST treatment and during holding at 5 °C, model IMFs with α-lac:β-lg ratios of 1.3 or 4.6 were significantly ($P < 0.05$) less viscous than samples with α-lac:β-lg ratios of 0.1 or 0.5 (Fig. 3). These results demonstrated that heat-induced increases in viscosity were reduced as α-lac:β-lg ratio increased.

3.4. Protein particle size before and after HTST treatment

Unheated model IMFs had mean protein particle diameters between 181 and 187 nm (Table 1), which is typical of values reported for casein micelles (Anema, Lowe, & Li, 2004; Crowley et al., 2014; Nair, Dalgleish, & Corredig, 2013). Heated samples with α-lac:β-lg ratios of 0.1, 0.5 or 1.3 exhibited significant ($P < 0.05$) increases in protein particle diameter compared to the samples before heating (Table 1) whereas, at higher α-lac:β-lg ratios (2.1 or 4.6), no significant changes in protein particle size were detected after heating. There was also a trend towards decreasing polydispersity after heating as α-lac:β-lg increased (Table 1). The PSD of the model IMF with the highest α-lac:β-lg (4.6) was monomodal, irrespective of whether it was heated or not, but displayed a shift to slightly smaller protein particle sizes after heating (Table 1; Fig. S1). A second population of small protein particles with diameters ranging from 40 to 100 nm was

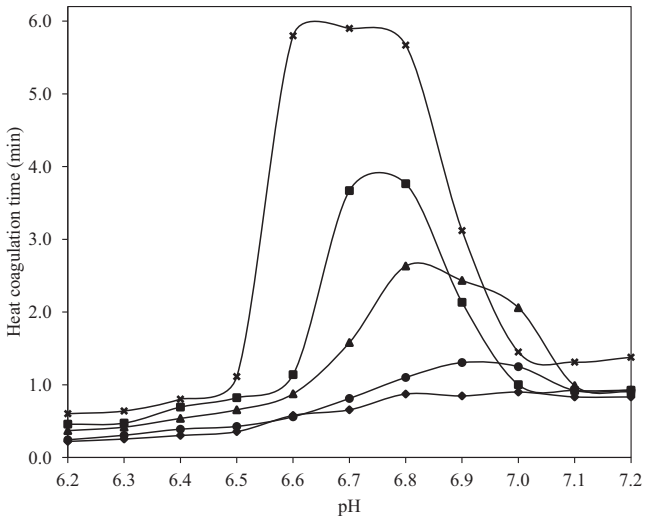

Fig. 2. pH-heat coagulation time (HCT) profiles for model infant milk formula samples with α-lactalbumin (α-lac): β-lactoglobulin (β-lg) ratios of 0.1 (♦), 0.5 (●), 1.3 (▲), 2.1 (■) or 4.6 (×) in the pH range 6.2–7.2. Results are the means of data from at least three independent trials on freshly prepared samples.

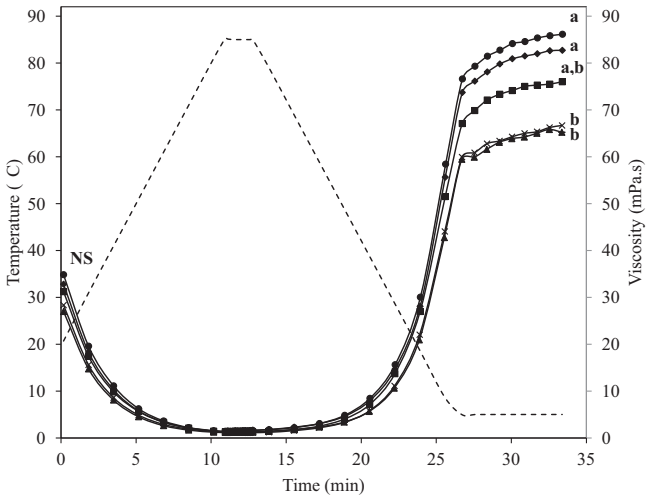

Fig. 3. Temperature (broken line) and viscosity (symbols) during lab-scale high-temperature short-time (HTST) treatment of model infant milk formula samples with α-lactalbumin (α-lac): β-lactoglobulin (β-lg) ratios of 0.1 (♦), 0.5 (●), 1.3 (▲), 2.1 (■) or 4.6 (×) at pH 6.8. Results are the means of data from at least three independent trials on freshly prepared samples. Different letters after the curve indicate that samples were significantly different ($P < 0.05$) at 5 °C; NS indicates no significant difference between samples at 20 °C.

Table 1
Protein particle size and polydispersity index values of model infant milk formula samples with different α-lactalbumin (α-lac):β-lg (β-lactoglobulin) ratios, before and after application of lab-scale high-temperature short-time heating. Results are the means ± standard deviations of data from three independent trials on freshly prepared samples.

α-lac:β-lg ratio	Protein particle size (nm)		Polydispersity index (–)	
	Unheated	Heated	Unheated	Heated
0.1	187 ± 2.07[a]	319 ± 80.2[b]	0.19 ± 0.02[a]	0.34 ± 0.09[b]
0.5	182 ± 2.08[a]	249 ± 35.9[b]	0.17 ± 0.01[a]	0.26 ± 0.07[a]
1.3	181 ± 4.88[a]	252 ± 41.2[b]	0.17 ± 0.01[a]	0.22 ± 0.05[a]
2.1	184 ± 3.62[a]	233 ± 33.7[a]	0.18 ± 0.03[a]	0.17 ± 0.04[a]
4.6	186 ± 4.73[a]	165 ± 2.00[a]	0.19 ± 0.02[a]	0.10 ± 0.01[b]

[a,b]Means within rows are significantly ($P < 0.05$) different after treatment if they carry different superscript letters.

apparent in model IMFs with α-lac:β-lg ratios of 0.1 and 0.5 after heating, indicating the presence of serum protein aggregates (Donato and Guyomarc'h, 2009); in addition, micron-sized particles were formed in the sample with α-lac:β-lg of 0.1 (Fig. S1), suggesting that particularly extensive aggregation had occurred in this sample.

3.5. Physical stability before and after HTST treatment

Physical stability was measured using an analytical centrifuge. The transmission of near-infrared light through the sample cell at the beginning and end of centrifugation was ~30% and 65%, respectively, for all unheated model IMF samples (Fig. 4). Under these conditions, this increase in transmission during centrifugation can be attributed to the sedimentation of large particles such as casein micelles (Crowley et al., 2014; Tobin, Fitzsimons, Kelly, & Fenelon, 2011) and presumably large whey protein aggregates. All samples were more physically stable after heating. For heated systems with α-lac:β-lg ratios of 0.1–2.1, the transmission at the beginning of centrifugation decreased to between 10% and 15% compared to unheated samples (Fig. 4), due to the increased light-scattering by the large protein particles which were formed (Table 1), while a much smaller decrease in transmission was observed for the 4.1 α-lac:β-lg sample due to negligible protein aggregation (Table 1). Fig. 4 shows that the value for transmission after 60 min of centrifugation increased with increasing α-lac:β-lg ratio, indicating decreased physical stability. The model IMF with an α-lac:β-lg ratio of 0.1 was the most stable to protein sedimentation on centrifugation after heating (Fig. 4). This result seems counterintuitive, as this sample had the largest average protein particle diameter and may, thus, have been expected to sediment more rapidly (Guyomarc'h, Nono, Nicolai, & Durand, 2009). It is not clear why samples with larger protein particles were more physically stable, but the markedly increased polydispersity of these systems (Table 1) indicates that populations of particles in these systems were very complex, likely comprising both casein micelle-whey protein complexes and serum protein aggregates (Fig. S1). Possible explanations for reduced sedimentation in these systems include: (1) that the formation of a large range of aggregate sizes, including small serum protein aggregates, resulted in increased inter-aggregate proximity with a concomitant increase in stabilising interactions between aggregates during centrifugation; (2) that a significant portion of the particle population was comprised of aggregates with higher hydration or lower density after heating, with, for example, Guyomarc'h et al. (2009) demonstrating that complexes formed between whey proteins were more dense than complexes formed between κ-casein and whey proteins.

3.6. Protein profile before and after HTST treatment

After heating, the intensity of both β-lg and α-lac bands decreased strongly under non-reducing conditions at α-lac:β-lg ratios between 0.1 and 1.3 (Fig. 5a). A substantial proportion of α-lac did not form covalent associations with other proteins during heating in samples with α-lac:β-lg ratios of 2.1 and 4.6, as indicated by the markedly higher band intensity in non-reducing SDS–PAGE gels (Fig. 5a). These results indicate that both β-lg and α-lac readily formed covalently-linked complexes with casein and/or themselves at low α-lac concentrations; the dissociation of these complexes under reducing conditions, indicated by the presence of distinct, intense bands (Fig. 5b), suggests that the protein–protein interactions were predominately covalent in nature (i.e., linked by disulphide bonds).

An increase in the levels of high molecular weight (MW) material remaining in the wells of the non-reducing gel as α-lac:β-lg ratio increased was also observed (Fig. 5a); in addition, there was noticeable streaking in the region between the BSA band and the loading well in samples with α-lac:β-lg ratios of 1.3, 2.1 and 4.6. The high MW material was also covalently linked, as evidenced by its dissociation under reducing conditions (Fig. 5b). The sample with the highest α-lac:β-lg ratio (4.6) had the greatest quantity of material remaining in the loading wells of the non-reducing SDS–PAGE gel (Fig. 5a); however, the same sample underwent the least significant changes in particle size distribution (Table 1) and viscosity (Fig. 3) during HTST treatment, and exhibited the highest heat stability at 140 °C (Fig. 2). The intensity of the BSA band decreased in heated IMFs as α-lac:β-lg increased, particularly after the high MW complexes had been dissociated (Fig. 5b). The high MW BSA has a free thiol group and, hence, can form complexes with α-lac (Wijayanti, Bansal, & Deeth, 2014). Thus, it is possible that, as α-lac:β-lg ratio is increased, BSA begins to play a more prominent role in influencing protein–protein interactions while, under normal conditions, β-lg is present in sufficient quantities to mask the influence of BSA. Although the level of covalently-linked high MW material remaining in wells was observed to increase in non-reducing SDS–PAGE gels as α-lac:β-lg ratio increased (Fig. 5a), this appeared to be insufficient

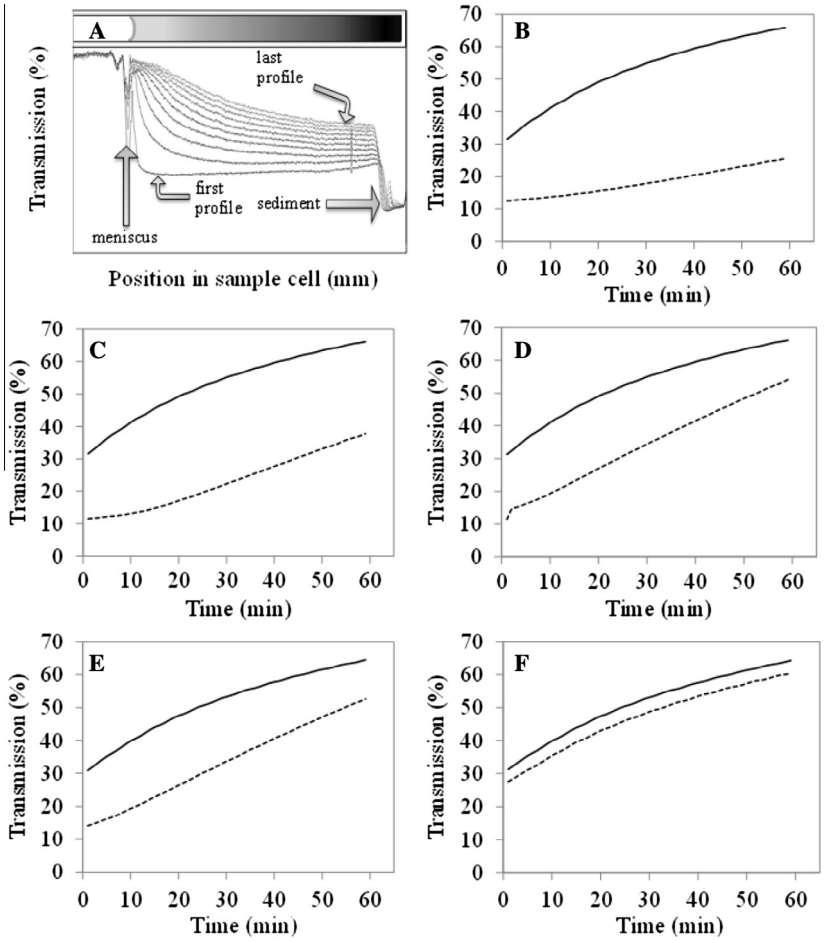

Fig. 4. Clarification of model infant milk formula samples during centrifugation (2300*g*; 20 °C). Increasing transmission was used as an index of clarification due to protein sedimentation, and was calculated by integrating raw profiles showing the transmission of near-infrared light across the length of a sample during centrifugation. An example raw profile is shown in (A); integration limits were set between the meniscus and sediment (region of clarification). Changes in transmission are shown for model infant milk formula samples with α-lactalbumin (α-lac):β-lactoglobulin (β-lg) ratios of (B) 0.1, (C) 0.5, (D) 1.3, (E), 2.1 or (F) 4.6 before (—) and after (- - -) heating. Results are the means of data from at least three independent trials on freshly prepared samples.

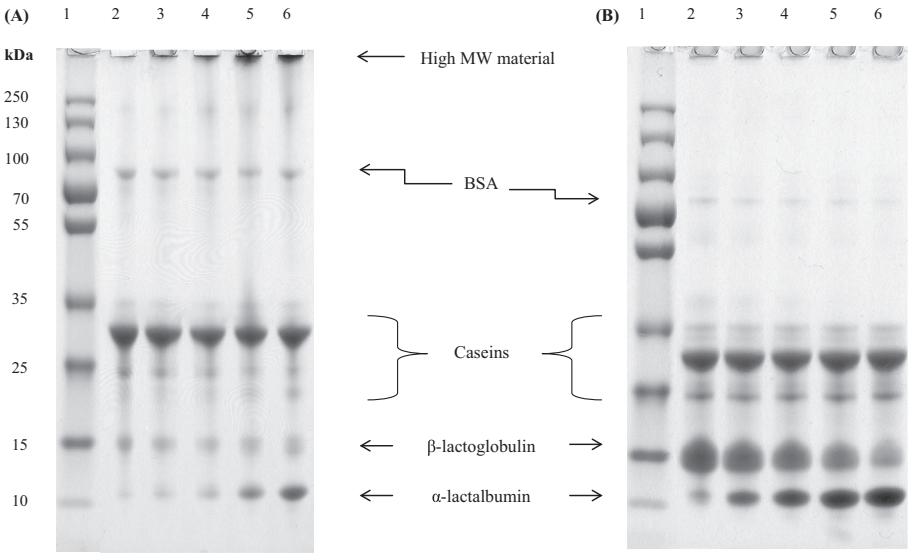

Fig. 5. Sodium dodecyl sulphate–polyacrylamide gel electrophoretograms (SDS–PAGE) under (A) non-reducing and (B) reducing conditions for heated model infant milk formula (IMF) samples. Wells were loaded with molecular weight marker (lane 1), and heated model IMF samples with α-lactalbumin (α-lac):β-lactoglobulin (β-lg) ratios of 0.1 (lane 2), 0.5 (lane 3), 1.3 (lane 4), 2.1 (lane 5) or 4.6 (lane 6).

to induce significant physicochemical changes of relevance to processing.

4. Discussion

This study conclusively demonstrated that modulating the α-lac:β-lg ratio of model IMF protein systems significantly affects their heat stability. Differences in the heat stability of these model IMFs can be primarily attributed to β-lg being more heat-labile than α-lac; hence, when α-lac:β-lg ratio was increased the extent of protein–protein interactions decreased, which resulted in increased heat stability. In addition to protein–protein interactions, multiple other compositional factors can influence the heat stability of milk (e.g., lactose content and Ca-ion activity); however, in this study, these factors were constant across the model IMFs (Table S1), and their contribution to inter-sample differences were therefore considered to be negligible. Model IMFs with high α-lac:β-lg ratios were the most resistant to heat-induced coagulation (Fig. 2). In high α-lac:β-lg ratio systems, protein–protein interactions were less pronounced, as evidenced by the less extensive increases in protein particle size (Table 1), viscosity (Fig. 3) and covalent interactions between proteins (Fig. 5) after heating. These data demonstrate that increasing α-lac:β-lg ratio decrease the protein–protein interactions that negatively affect heat stability. This is consistent with the report of Rattray and Jelen (1997), that blends (3.4% protein) of β-lg/skim milk were generally much less heat-stable than those of α-lac/skim milk at various casein:-whey protein ratios and pH values. It is clear that, in the pH range 6.6–6.9, increasing α-lac:β-lg ratio increased the heat stability of model IMFs at 140 °C (Fig. 2). Outside of this pH range, a higher Ca-ion activity at pH < 6.6 and increased dissociation of κ-casein at pH > 6.9 (Crowley et al., 2014; Singh, 2004) may have limited the otherwise strong stabilising effect of increased α-lac:β-lg.

Protein–protein interactions occurring in milk during heating can have both stabilising and destabilising influences on milk proteins. When milk is heated, the formation of complexes between whey proteins themselves and between whey proteins and casein can occur. Where reported, increases in protein particle size shown in Table 1 for model IMFs are likely due to the formation of both types of protein complexes. Whey proteins, and in particular β-lg, have a strong influence on the pH-dependant stability of milk to heating, and contribute to high heat stability in unconcentrated milk when complexed with casein micelles (Singh, 2004). In concentrated milks, substantial dissociation of κ-casein occurs throughout the pH-HCT profile, which is promoted by interactions with β-lg (Pearce, 1979; Singh & Creamer, 1991). In systems containing no casein, the β-lg is far more prone to heat-induced coagulation than α-lac; hence, increasing the level of β-lg in milk systems considerably reduces maxima values for heat stability, while increasing the levels of α-lac has a negligible or even positive effect on heat stability (Rattray & Jelen, 1997). In mixed systems containing both casein and whey proteins, association of whey proteins with casein micelles occurs during heating, but α-la only participates in these reactions if in the presence of sufficient quantities of β-lg and to a much more limited extent compared to β-lg (Oldfield, Singh, & Taylor, 1998; Oldfield, Singh, Taylor, & Pearce, 2000; Vasbinder & de Kruif, 2003). Thus, of the principal whey proteins, β-lg has the dominant influence on heat stability, whether positive or negative. The results of the present study indicate that the destabilising effects of β-lg (i.e., increased whey protein aggregation and/or increased κ-casein dissociation) had a stronger influence on the heat stability of model IMFs than the stabilising effects (i.e., formation of complexes at micellar surfaces), and that this factor was responsible for the positive effect of increased α-lac:β-lg ratio on heat stability. Thus, altering α-lac:β-lg ratio in the production of humanised IMFs may affect performance during thermal processes such as UHT.

In the manufacture of an IMF by a "wet-mixing" approach, the liquid IMF at ~4–7% total protein and pH 6.6–7.0 is typically subjected to HTST treatment followed by refrigerated storage, during which refrigerated storage heat-labile ingredients are often added before further processing. In this study, such a HTST-cooling cycle was simulated at lab-scale. Heating milk at 75–100 °C increases its viscosity, with the extent of this increase commonly linked with the size and volume fraction of casein micelles, which are increased through their interactions with denatured whey proteins (Anema et al., 2004). Analysis of the model IMFs post-HTST heating generally indicated that the extent of the increase in viscosity decreased with increasing α-lac:β-lg (Fig. 3), due to less extensive protein–protein interactions (Table 1, Fig. 5). It is evident from the results of simulated HTST treatment that increasing α-lac:β-lg ratio alters the physicochemical properties of IMFs (e.g., reduces viscosity) after heating. This may have important implications for manufacturers of IMFs in powder and liquid formats. For example, in IMF powder manufacture, differences in feed solution viscosity after HTST (Fig. 3) and vacuum evaporation would be expected to change certain physical properties of spray-dried IMFs, such as bulk density (Keogh, Murray, & O'Kennedy, 2003; Murphy et al., 2014). In addition, analytical centrifugation experiments indicated that physical stability after HTST heating decreased with increasing α-lac:β-lg (Fig. 4). In liquid IMF manufacture, where more intense heat treatments such as UHT are applied, stabiliser addition may be required to limit the more rapid sedimentation of protein particles in high α-lac:β-lg samples during storage (Tobin et al., 2011).

5. Conclusion

Increasing α-lac:β-lg ratio conferred improved heat stability on model IMF protein systems by limiting the extent of whey protein–casein interactions. Protein particle size in high α-lac:β-lg IMFs did not increase to the same extent as that in samples with low α-lac:β-lg ratio during HTST-heating, resulting in less extensive heat-induced viscosity increases in the former. Observed differences in heat stability and post-heating viscosity between IMFs with different α-lac:β-lg ratios may have practical implications for the manufacture of humanised IMF liquids and powders, in terms of destabilisation during thermal treatment or storage, and the physical properties of IMF powders, respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.07.077.

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New insights into the mechanism of rehydration of milk protein concentrate powders determined by Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS)

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ABSTRACT

This study investigated the transfer of water into milk protein concentrate (MPC) powder particles using Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS) as a detection method for the first time. BARDS analysis is based on an acoustic phenomenon which occurs during powder rehydration. Release of air from the powder into the solvent during rehydration leads to outgassing in the solvent, which results in changes in solvent compressibility that are monitored through accompanying changes in induced resonance frequencies in the dissolution vessel. BARDS confirmed that water transfer into MPC particles became increasingly inhibited as protein content of the powder increased. The reproducibility of the data indicates that air release from internal vacuoles within powder particles in high-protein MPCs is a highly ordered process, occurring over a protracted time scale. Kinetic modelling of gas volume data from BARDS confirmed that the release of occluded air caused the changes in solvent compressibility during rehydration. The physicochemical properties of solubilised protein had a slight inhibitory effect on escape of bubbles from the solvent, but the primary factor limiting gas release from high-protein MPCs was water transfer into powder particles and the concomitant release of occluded air into the solvent. In agreement with many previous studies, cryo-SEM analysis showed that particles in high-protein MPCs were slow to disperse; the current study, in addition, highlights inhibited water transfer into particles as another factor which may contribute to their poor rehydration properties. A potential link between inhibited water transfer and poor dispersibility is proposed.

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1. Introduction

Milk protein concentrate (MPC) powders are recently developed ingredients which contain the two major protein fractions of bovine milk at the ratio that they occur naturally in milk (80:20 casein:whey protein). MPCs are typically manufactured using pressure-driven membrane separation processes, where ultrafiltration (UF) alone, or a combined UF and diafiltration (DF) process, is used to concentrate protein while removing smaller molecules including lactose, salts, and non-protein nitrogen (Carr & Golding, 2016). After membrane processing, MPCs are usually spray-dried into powders. Currently, the most widely used MPC powders in commercial applications are the high-protein varieties (i.e., those containing ≥79% protein). High-protein MPCs are exploited for their functional attributes (e.g., viscosity, emulsification, curd-forming ability) and nutritional features (e.g., high protein, low lactose) in a range of commercial applications (Agarwal, Beausire, Patel, & Patel, 2015).

When milk protein concentrate (MPC) powders are manufactured to a final protein content ≥70%, solubility is often impaired (Crowley et al., 2015), with the rate of liberation of casein micelles from powder particles during rehydration decreasing with

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increasing protein content of the powders (Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010b). As casein is the predominant component in high-protein MPCs, and the majority of caseins exist in the micellar state, the persistence of these poorly-dispersible particles for extended periods after wetting and submersion of the powder can result in suspensions with an unacceptably high quantity of sedimentable solids (Havea, 2006; Sikand, Tong, Roy, Rodriguez-Saona, & Murray, 2011). Furthermore, after extended rehydration, these particles may not be of sufficient density to sediment, but may still remain suspended as large, highly-hydrated particles (Crowley et al., 2015; Fang, Selomulya, Ainsworth, Palmer, & Chen, 2011). Incomplete rehydration of MPCs is an issue which is encountered both during mixing of dried ingredients by processors and reconstitution of dried products by consumers, and can have a negative influence on the functional and sensory properties of the final product (Carr & Golding, 2016).

Micellar casein is primarily responsible for the solubility issues encountered during the rehydration of MPCs (Anema, Pinder, Hunter, & Hemar, 2006; Gazi & Huppertz, 2014; Havea, 2006; McKenna, 2000; Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010a). The particular solubility issues associated with MPCs are not found in whey protein-dominant powders (i.e., whey protein concentrates/isolates), casein-dominant powders which are relatively low in protein (i.e, skim milk/nonfat dry milk powders) or high-protein casein-dominant powders which do not contain micellar casein (e.g., sodium caseinate). Micellar casein concentrates (MCCs), powders with higher casein:whey protein ratios than MPCs, are known to have similarly poor, and even more challenging, rehydration performance (Crowley, Jeantet, Schuck, Kelly, & O'Mahony, 2016); when levels of lactose or whey proteins are increased in MCCs they dissolve more quickly, due to a concurrent decrease in the level of micellar casein and possible improvements in the water transfer properties of the powder due to the presence of the more soluble components (Richard et al., 2012).

In high-protein MPC powders, casein micelles are considered to be the molecular building blocks of a 'skin' at the surface of primary powder particles, which may prevent the release of casein micelles during rehydration (Crowley et al., 2016; Fyfe et al., 2011; Ji et al., 2016; McKenna, 2000; Mimouni et al., 2010b). The solubility of MPCs deteriorates further during storage under adverse conditions (Anema et al., 2006; Fyfe et al., 2011; Gazi & Huppertz, 2014), due to the decreased solubility of micellar casein, while the solubility of whey proteins is largely retained unless they have been denatured to a significant degree during processing (Gazi & Huppertz, 2014). The central role of micellar casein in the development of insolubility issues in MPCs is further supported by some of the techniques which have been used to improve their solubility, including high pressure treatment (Udabage, Puvanenthiran, Yoo, Versteeg, & Augustin, 2012), ion-exchange (Bhaskar, Singh, & Blazey, 2001) and CO_2 injection (Marella, Salunke, Biswas, Kommineni, & Metzger, 2012), all of which are primarily based on structural modification of casein micelles (Carr & Golding, 2016).

There is a need to develop *in-situ* techniques for the dynamic monitoring of powder rehydration phenomena, as this will allow the identification of the stages (i.e., wetting, water transfer, or dispersion) which are responsible for prolonged rehydration times (Crowley et al., 2016; Fang, Selomulya, & Chen, 2008). Dynamic studies of MPC powder rehydration have primarily focused on advanced stages of rehydration, such as dispersion, which has been identified as the rate-limiting step in the rehydration process for MPCs in experiments where the changes in particle size over time were measured (Fang et al., 2011; Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2009). However, other than the study of Hauser and Amamcharla (2016) on commercial MPC80, there are limited studies in the literature in which water transfer into particles during the rehydration of MPC powders has been investigated. Water transfer was studied in MCCs by Schuck et al. (2002) and Richard et al. (2012) using nuclear magnetic resonance relaxometry and ultrasound attenuation measurements, respectively, with both studies demonstrating that water transfer can be markedly inhibited in MCCs. Bouvier, Collado, Gardiner, Scott, and Schuck (2013) demonstrated that increasing the size and number of pores in particles can improve the rehydration properties of MCC powders, supporting the concept that enhancing water transfer can improve the dispersibility of these powders.

Generating data on water transfer phenomena in MPCs could potentially inform strategies to modify particle structure (i.e., during or after spray drying) in order to improve their rehydration characteristics. Thus, in this study, a new form of acoustic spectroscopy, Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS), was used to monitor water transfer related phenomena in a range of MPC powders for the first time. BARDS is an analytical platform technology with multiple applications, such as blend uniformity analysis, discrimination of polymorphs and drug loading on sugar spheres for controlled release formulations (Fitzpatrick et al., 2012a, 2012b, 2014). The technique is based on real-time changes in the compressibility of a solvent as a solute dissolves, which can be monitored acoustically via changes in induced resonant frequencies of the dissolution vessel. In this study, the rehydration behaviour of MPCs with varying protein contents was assessed over time using BARDS. Experiments were devised to isolate the influence of water transfer on changes in gas volume during rehydration. A novel kinetic approach was used to confirm the role of occluded air release in determining BARDS spectra. The influence of serum-phase composition (i.e., soluble protein) on the escape of gas from the solvent was considered for the first time in a study of dairy powder rehydration based on changes in gas volume. Cryo-SEM micrographs were also collected during the rehydration of selected powders to establish a potential link between water transfer and particle dispersion-state.

2. Experimental

2.1. Materials

The MPC powders used in the current study were prepared at pilot-scale by NIZO food research (Ede, the Netherlands) as described by Crowley, Gazi, Kelly, Huppertz, and O'Mahony (2014), Crowley, Megemont, Gazi, Kelly, Huppertz, and O'Mahony, (2014). In brief, pasteurised skim milk was subject to UF (MPC50, MPC60) or UF and DF (MPC70, MPC80, MPC85, MPC90) to different protein concentration factors at 50 °C with 10 kDa molecular weight cut-off membranes. MPC35 did not undergo any membrane filtration, and is essentially skim milk powder. MPC35, MPC50, MPC60 and MPC70 were evaporated before being spray-dried, while MPC80, MPC85 and MPC90 were not subjected to evaporation. Spray drying involved nozzle atomisation, an air inlet temperature of 185–190 °C and an outlet temperature of 85–90 °C. The composition and selected physical properties (measured as described by Crowley, Gazi et al. 2014; Crowley, Megemont et al., 2014) of the MPC powders are shown in Table 1. Analar grade KCl was purchased from Sigma Aldrich. The solvent used for rehydration experiments was deionised water unless otherwise indicated.

2.2. Instrumentation

The BARDS spectrometer consists of a closed chamber with a dissolution vessel (soda lime glass), microphone (Sony ECM-CS10, range 100 Hz–16 kHz), a magnetic stirrer and follower. A

Table 1
Composition and physical properties of milk protein concentrates (MPCs). Data presented are the means of duplicate analysis, with the exception of lactose, which was the result of a single analysis.[a]

	Composition				Physical properties		
	Protein	Lactose	Ash	Fat	d_{50}[b]	Interstitial air	Occluded air
	(%, w/w)				(μm)	(ml 100 g^{-1})	
MPC35	35.4	49.6	8.06	0.5	35.3	98	18.1
MPC50	49.9	35.8	7.75	0.8	43.0	88	14.1
MPC60	60.8	24.5	7.74	1.5	48.9	95	21.4
MPC70	68.3	18.0	7.99	1.2	39.6	111	23.0
MPC80	79.1	6.36	7.69	1.7	27.9	206	53.7
MPC85	84.0	1.81	7.54	1.2	26.1	229	47.2
MPC90	85.9	0.37	7.59	1.6	26.8	230	46.2

[a] Taken from Crowley et al. (2014a).
[b] Particle size below which 50% of material volume exists – median.

schematic diagram is shown in Fig. 1, demonstrating the principle of BARDS as applied in the current study and the basic components of the apparatus. There is access at the front of the chamber for the dissolution vessel and at the top in order to place a sample in a weighing boat on an automated tipper motor for introduction of the powder. The microphone is positioned above the top of the glass within the housing for these studies. The glass, containing 25 mL of deionised water, is placed on the stirrer plate. The stirrer motor underneath is positioned so as to allow the magnetic follower to gently tap the inner vessel wall. In this way, the follower acts as a source of broadband acoustic excitation, thereby inducing various acoustic resonances in the glass, the liquid and the air column above the liquid. The audio is sampled at a rate of 44.1 kHz. A fast Fourier transform is applied to the signal, resulting in a typical BARDS frequency response. The resonances of the liquid vessel are recorded in a frequency band of 0–20 kHz. The frequency response was measured during the rehydration of 0.04–0.20% (w/v) suspensions of MPCs in 25 mL water.

2.2.1. Theoretical background of BARDS

The BARDS response results from changes in the compressibility of a solvent during the dissolution of a compound, in which compressible gas bubbles are introduced or generated. Changes in compressibility alter the speed of sound resulting in frequency changes of induced acoustic resonances within the solvent. The principles underlying the BARDS response are as follows. The sound velocity (v) in a medium (m s^{-1}), whether air or liquid phase, is determined by Equation (1).

$$v_{(sound)} = \sqrt{\frac{1}{K.\rho}} \tag{1}$$

where ρ is the density (kg m^{-3}) and K is the compressibility (inverse of the bulk modulus) of the medium (Pa^{-1}). Generation of micro bubbles in a liquid decreases the density in a negligible way in comparison to a large increase in compressibility. The net effect is a significant reduction of the sound velocity in the liquid. The following relationship between the fractional bubble volume and the sound velocity in water was derived by Frank S. Crawford, as given in equation (2) (Crawford, 1982):

$$\frac{v_w}{v} = \sqrt{(1 + 1.49 \times 10^4 . f_a)} \tag{2}$$

where v_w and v are the velocities of sound (m s^{-1}) in pure and bubble-filled water, respectively, and f_a is the fractional volume occupied by air bubbles. Equation (2) is based on an approximation presented originally by Wood (1930).

BARDS analysis of an induced acoustic excitation of the vessel containing the fluid is focused on the lowest variable frequency time-course, i.e., the fundamental resonance mode of the liquid. The fundamental resonant frequency is determined by the sound velocity in the liquid and the approximate but fixed height of the liquid level, which corresponds to one quarter of its wavelength.

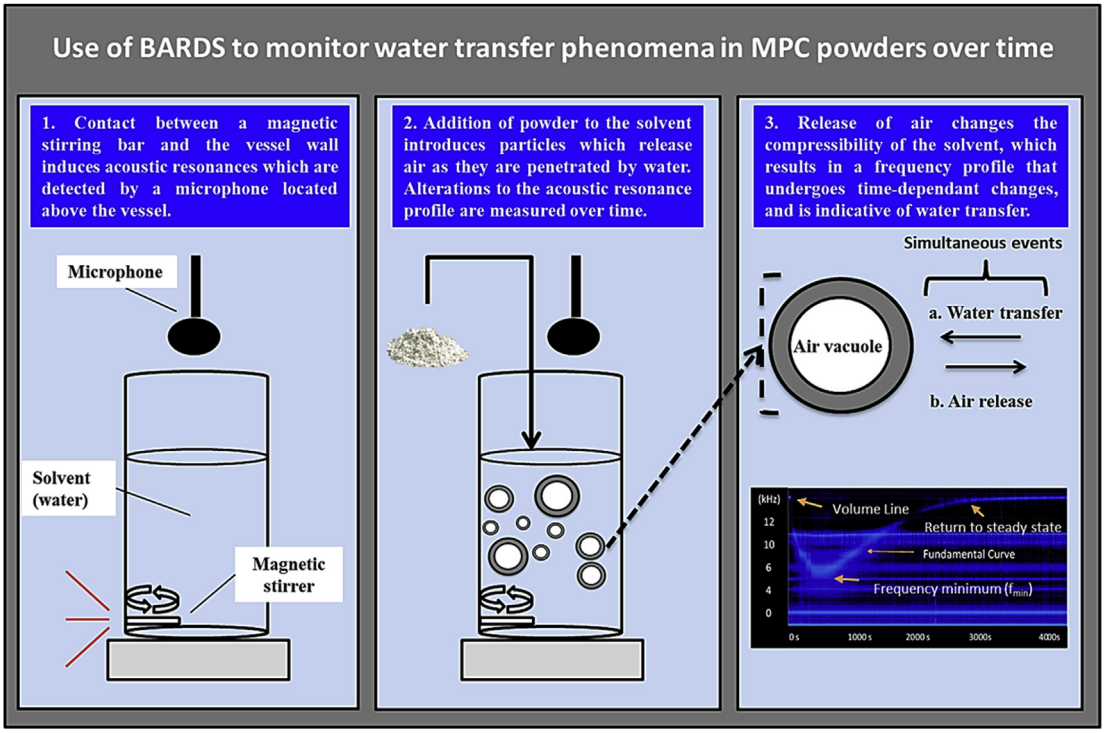

Fig. 1. Principle of BARDS analysis as applied to MPC rehydration: Panel 1, Schematic of the BARDS Instrument; Panel 2, Addition of MPC powder to the BARDS dissolution vessel; Panel 3, Water/Air transfer of an MPC particle during rehydration and raw BARDS spectra of the rehydration of 50 mg of MPC90 in 25 mL of deionised water at 22 °C.

The frequency response is described as:

$$freq = \frac{freq_w}{\sqrt{1 + 1.49 \times 10^4 . f_a}} \quad (3)$$

where $freq_w$ and $freq$ are the resonance frequencies (kHz) of the fundamental resonance modes in pure and bubble-filled water, respectively. A comprehensive outline of the principles and underlying processes involved in BARDS analysis is given by Fitzpatrick et al. (2012a).

2.3. Experimental procedure for BARDS experiments

In a typical experiment, the spectrometer records the steady-state resonances of the system as a reference for 30 s after the stirrer has been set in motion (Fig. 1, panel 1). The pitch of the resonance modes in the suspension change significantly when the powder is added (Fig. 1, panel 2), before gradually returning to steady-state over several minutes (Fig. 1, spectrum in panel 3). The amounts used are expressed as solid/liquid concentration (w/v) in all figures and throughout the text. Gas oversaturation of water prior to introduction of powders was removed through agitation by shaking vigorously for 60 s and then resting for 10 min. Otherwise, remaining gas oversaturation may lead to an over-response (Fitzpatrick et al. 2013).

The frequency-time response of the fundamental resonance is presented as manually extracted data from the total acoustic response. The steady-state frequency before addition of the powder is designated as the 'volume line', so called as it varies depending on the liquid volume in the vessel. Spectra were recorded for 3000–4000 s depending on the rate of return of the BARDS response to steady state. All experiments were performed in duplicate at ambient temperature (~22 °C) and atmospheric pressure. Average readings with error bars representing the standard deviation are presented.

2.4. Characterisation of the microstructure of MPC powders in their dry state

Scanning electron microscopy (SEM; Philips XL30 FEG ESEM) was used to characterise the microstructure of MPC35, MPC70 and MPC90 to assess any morphological differences. MPC powder samples were placed upon double-sided adhesive conductive carbon tape, sputter-coated with gold and scanned at 10 kV.

2.5. Characterisation of the microstructure of MPC powders during rehydration

Cryogenic scanning electron microscopy (Cryo-SEM; Philips XL30 FEG ESEM), with a Gatan low temperature preparation system, was used to visualise the microstructure of MPC35 and MPC90 at rehydration times of 100, 1000 and 3000 s. Cryo-SEM analysis was performed to assess differences in particle dispersion between the two powders, to supplement water transfer data generated using BARDS. One drop of liquid was frozen to approximately −180 °C in liquid nitrogen slush. Samples were then fractured and etched for 1 min at a temperature of −95 °C inside the preparation chamber. Afterwards, samples were sputter coated with gold and scanned at 3 kV, during which the temperature was maintained below −160 °C by addition of liquid nitrogen to the system.

3. Results

3.1. Composition and physical properties of the MPC powders

Data related to the composition and physical properties of the MPCs are shown in Table 1. Reductions in lactose and mineral levels were measured in the MPC powders, corresponding with increasing protein concentration. Particle size increased with increasing protein content for MPC35, MPC50 and MPC60, and decreased thereafter as protein content increased further. There were no apparent trends in the volume of interstitial and occluded air when MPC35, MPC50, MPC60 and MPC70 were compared; however, MPC80, MPC85 and MPC90 were 2–3 times more aerated than the former powders, although there were only minor differences in aeration within this class of high-protein MPC powders.

3.2. Interpretation of BARDS profiles

Fig. 1, panel 3, shows a typical BARDS spectrum during the rehydration of MPC90. The acoustic frequency profile of interest is called the fundamental curve. The frequency minimum (f_{min}) represents an equilibrium between the rate of introduction of gas as bubbles into solution and the rate of elimination of these bubbles at the surface of the solution. In BARDS analysis, the fundamental curve is used to make comparisons between individual experiments. The acoustic frequencies of the vessel remained steady for the first 30 s until the addition of MPC90; thereafter, the resonant frequency at 14 kHz decreased to 6 kHz and gradually returned to steady-state. The constant frequency at 11 kHz is just one of many resonant frequencies of the vessel that is not dependent on the liquid compressibility and therefore remained unchanged as gas volume levels fluctuated.

Figs. 2 and 3 show the acoustic profiles for all seven MPCs with a concentration of 0.2%. There is a gradual increase in the deflection to f_{min} with increasing protein content. Powders with higher protein concentrations exhibited a distinct change in the rate of gas release into the solvent (reduced down-slope) compared to lower protein powders, as indicated by the increased amount of time required to reach f_{min}. The disappearance of gas from the solvent after f_{min} also proceeded more slowly (reduced up-slope) as protein content of the MPC powder increased, which resulted in considerably extended times to reach steady-state. Most notable was the time required (~3000 s) to reach steady-state for MPC90.

The acoustic profiles (Fig. 2) strongly indicate differences in the volume of gas generated, the rate of gas release from the powders,

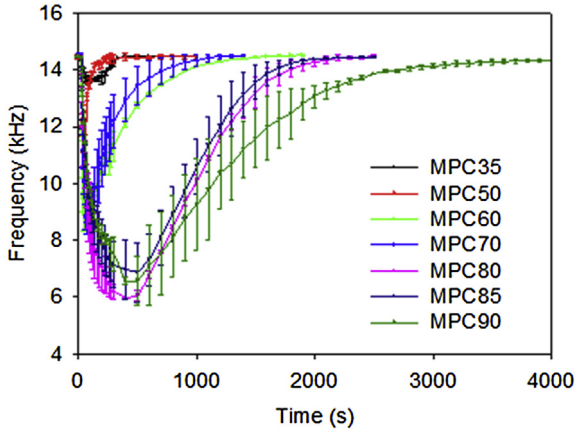

Fig. 2. Comparison of BARDS spectra of all MPC powders dissolved in 25 mL deionised water at 22 °C with a consistent sample concentration of 0.2% (w/v).

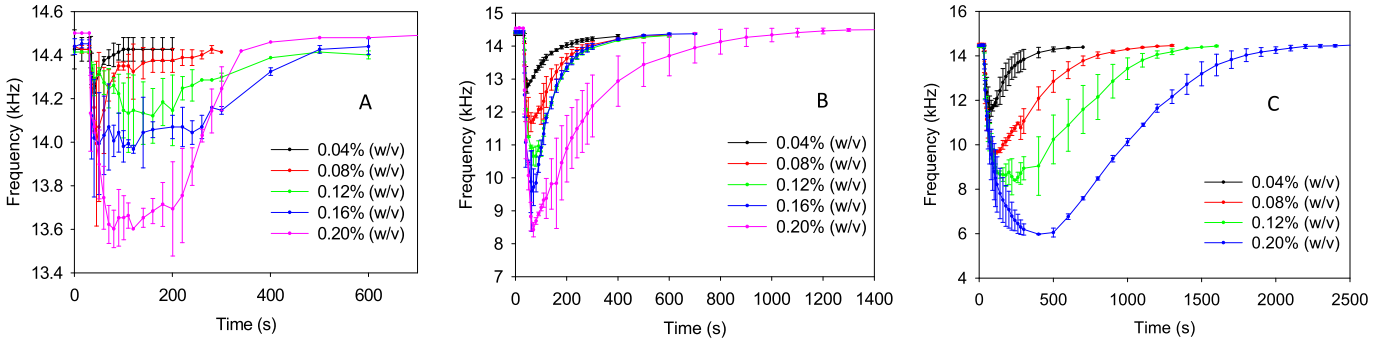

Fig. 3. Concentration-dependence of BARDS frequency response during rehydration of MPC in deionised water at 22 °C: (A) MPC35; (B) MPC70 and (C) MPC80.

and the rate of gas disappearance from the solvent during the rehydration of different MPCs. These factors were investigated more closely by tracking changes in gas volume in the following sections.

3.3. Changes in compressible gas volume during rehydration of MPC powders

Equation (3) was applied to the BARDS frequency data from the MPC experiments (Fig. 2) to generate data relating to the fractional gas volume (f_a) occupied by compressible gas during the dissolution of 0.2% of each of the MPCs. The gas volume plots presented in Fig. 4(A) and (B) concern absolute volumes ($f_a \times V_{solution}$). The initial up-slope indicates the rate at which gas was released from the powder. The data suggests that a significant change in rehydration behaviour occurred when the protein content exceeded 79% in the MPC powders. MPC35 generated a negligible gas volume during rehydration (see higher resolution data in Fig. 4B). MPC50 and MPC60 exhibited a very rapid release of a limited quantity of gas, the disappearance of which from the solvent began immediately and proceeded rapidly. Conversely, for MPC80, MPC85 and MPC90, there was a very gradual increase in the gas volume to a high maximum, after which point gas volume remained constant in the system for ~200 s, due to a balance of gas release and disappearance, before gas disappearance from the liquid surface became dominant. The steady increase in the compressibility of the solvent for these powders during ~500 s of rehydration indicates that the immersed particles themselves, containing occluded air prior to significant water transfer, are non-compressible and that, as such, the release of gas from the particles contributes to changes in the compressibility of the suspension. If the particles themselves were compressible, an immediate and marked increase in gas volume would be expected to occur as soon as the powder submerged.

3.4. Kinetic analysis of changes in compressible gas volume during rehydration of the MPCs

When the gas volume data (Fig. 4A and B) is plotted using a logarithmic scale, as shown in Fig. 4(C), the disappearance rate constant (k) for compressible gas in the suspension is given by the descending slope (assuming a first-order process). Table 2 presents the results of this gas disappearance analysis, with values for k and the time range from which the descending slope was calculated. For the MPC suspensions at the highest concentration studied (0.2%), a gradual decrease in gas disappearance rate is observed with increasing protein content of the powder. The k value of the lowest protein powder (MPC35) was five times that of the highest protein powder (MPC90), indicating a profound shift in water transfer behaviour.

Based on visual assessment of wetting behaviour, kinetic data in Table 2 and gas volume-time plots (Fig. 4), it is possible to distinguish four categories of MPC based on data for 0.2% systems:

Fast wetting/fast water transfer/fast gas disappearance: MPC35 and MPC50

These powders wetted rapidly at the water surface and underwent fast sinking. The volume response-time curves for these samples show a subsequent fast release of compressible gas from the powder into the solvent, indicating that liquid transfer into particles was rapid after sinking. In contrast to the MPC50, the MPC35 response seems to indicate a relatively slower rate of water

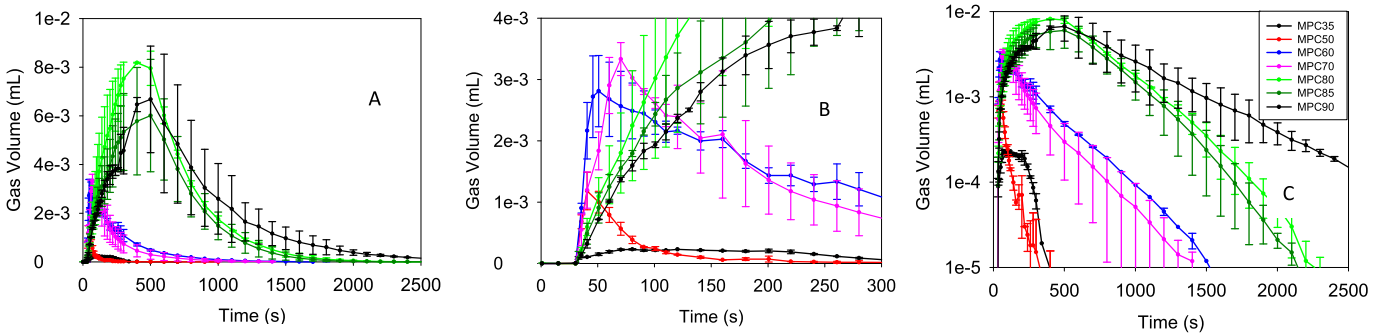

Fig. 4. Gas volume-time plots derived from BARDS frequency data: (A) Gas volume plots of all MPCs tested during rehydration of 50 mg MPC in 25 mL water at 22 °C; (B) Magnification of gas volume profile during the initial phase shown in (A); (C) A log plot of the gas volumes in (A), the slopes of which are used to calculate the BARDS first-order rate constants (k).

transfer, despite having a lower protein content. Both powders exhibited high gas disappearance rates ($k \approx 1–3 \times 10^{-2}$ s^{-1}) compared to the other MPCs. These results are generally in line with previous studies demonstrating that low-protein MPCs have good solubility characteristics (Crowley et al., 2015; Sikand et al., 2011).

Fast wetting/fast water transfer/intermediate gas disappearance: MPC60 and MPC70

These samples exhibited rapid wetting at the powder surface and gas generation in the solvent, suggesting that water transfer into particles in these powders was not severely inhibited compared to MPC35 and MPC50. Like MPC50, these powders exhibited a more rapid rate of water transfer than MPC35. However, both powders exhibit slower gas disappearance rates ($k \approx 3.7–6.6 \times 10^{-3}$ s^{-1}) than MPC35 and MPC50, indicating that escape of bubbles from the solvent was inhibited compared to the lower protein powders, likely due to the increasing influence of solubilised protein. For example, a 0.2% suspension of MPC70 will have approximately double the protein content of MPC35. Particles in this set of MPCs with <80% protein disperse relatively quickly (Crowley et al., 2015), and therefore increasing the protein content from MPC35/MPC50 to MPC60/MPC70 may have increased the levels of soluble protein to a degree sufficient to inhibit bubble escape (Ybert & di Meglio, 1998).

Slow wetting/slow water transfer/intermediate gas disappearance: MPC80 and MPC85

The initial part of the response was likely influenced by slow wetting, with MPC80 and MPC85 observed to require ~200 s to fully disappear from the liquid surface; however, this cannot account for the 500 s of gas generation which elapsed prior to the initiation of the gas disappearance phase, which was strongly indicative of inhibited water transfer into powder particles. As with MPC60 and MPC70, intermediate gas disappearance rates ($k \approx 3.4–3.9 \times 10^{-3}$ s^{-1}) were measured, which suggests that this period of inhibited water transfer did not continue into the gas disappearance phase and influence derived k values. Thus, the effect of soluble proteins might be considered to dominate gas disappearance, as per the lower protein MPCs.

Slow wetting/slow water transfer/slow gas disappearance: MPC90

Similarly to MPC80 and MPC85, slow wetting was observed for MPC90 (~200 s). The initial part of the response of MPC90 is also similar to that of MPC80 and MPC85, and strongly indicates inhibited water transfer into the submerged particles. The gas disappearance rate for MPC90 was the lowest of all the powders ($k \approx 1.9 \times 10^{-3}$ s^{-1}), and suggested that water transfer into particles may have continued during the gas disappearance phase.

The slow gas generation for high-protein MPCs strongly indicates inhibition of water transfer into powder particles. However, it is not clear, especially for MPC90, which of the two primary phenomena (water transfer into particles and gas elimination from the solvent) are rate-determining for the observed trends in gas disappearance based on the kinetic data for 0.2% systems alone. Further analysis of the concentration-dependency of the BARDS response for different MPCs was performed to obtain more reliable mechanistic and kinetic information.

3.5. Analysis of the concentration-dependency of the BARDS response for MPCs

The concentration-dependency of the BARDS response for four of the seven MPCs (MPC35, MPC70, MPC80 and MPC90), spanning the four aforementioned categories, is shown in Fig. 4. The comparative kinetic analysis of the BARDS data is based on the related gas volume data and is presented in Fig. 5 using a logarithmic scale.

The results for individual powders can be summarised as follows:

MPC35 (Fig. 5A):

An immediate, very rapid gas disappearance was observed for 0.04 and 0.08% systems ($k \approx 5 \times 10^{-2}$ s^{-1}). A short time period (~200 s) of constant gas volume was observed at the higher concentrations of 0.12% (~100 s) and 0.16 and 0.20% (~200 s) before the gas volume started to decrease. The periods of constant volume may be attributed to slower powder wetting and uptake into the solvent, which was observed with higher quantities of added MPC35. In the gas disappearance phase, a k value of ~1×10^{-2} s^{-1} was calculated for concentrations of 0.08–0.20%.

MPC70 (Fig. 5B):

The gas disappearance rate for the concentrations 0.04–0.16% decreased gradually with time. Therefore, the curves have been characterized by two gas disappearance rate constants, an initial fast release and a subsequent slow release: the values are $k \approx 1.2–1.7 \times 10^{-2}$ s^{-1} for the initial part of each curve and

Table 2
Results from kinetic analysis of log gas volume-time plots taken from BARDS measurement of different MPC powders during their rehydration in water at different concentrations (0.04–0.20%, w/v). The compressible gas volume disappearance rate constant (k, s^{-1}) and the time range (s) of the linear fit applied are presented. If the rate of gas disappearance changed over time two values for k were calculated by separate linear fits, one for the initial stage (unshaded rows) and another for the terminal stage (grey shaded rows).

% (w/v)	MPC35		MPC50		MPC60		MPC70		MPC80		MPC85		MPC90	
	k (s^{-1})	Range (s)	k (s^{-1})	Range (s)	k (s^{-1})	Range (s)	k (s^{-1})	Range (s)	k (s^{-1})	Range (s)	k (s^{-1})	Range (s)	k (s^{-1})	Range (s)
0.20			2.5E-02	40–160			6.6E-03	70–300						
	1.0E-02	220–500	9.5E-03	160–300	3.70E-03	240–1500	3.7E-03	400–1400	3.4E-03	500–2000	3.9E-03	800–1800	1.9E-03	500–2500
0.16							1.3E-02	120–200						
	1.0E-02	220–500					7.9E-03	220–500					1.8E-03	400–2700
0.12							1.3E-02	120–200						
	1.0E-02	220–500					7.9E-03	220–500	4.3E-03	400–1300			1.8E-03	400–2100
0.08	4.4E-02	60–90					1.6E-02	120–220						
	1.0E-02	120–260					7.0E-03	240–500	4.9E-03	400–1200			2.1E-03	700–2400
0.04							1.1E-02	60–180	9.8E-03	100–220				
	5.3E-02	40–90					7.1E-03	260–400	6.7E-03	300–600			2.8E-03	600–1500

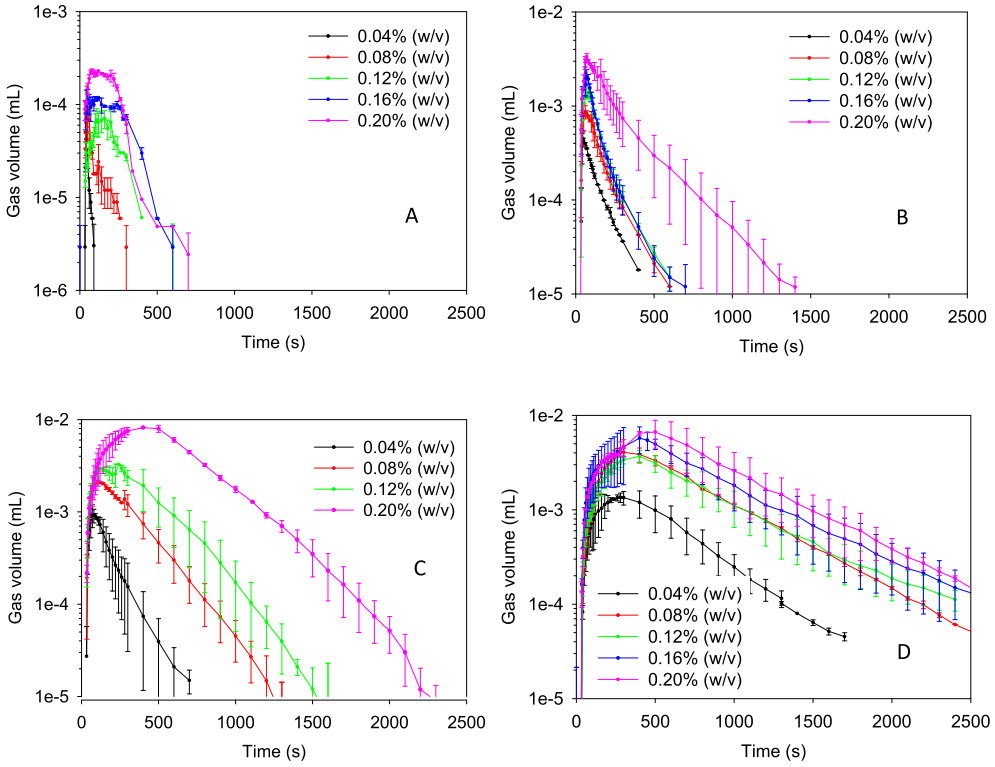

Fig. 5. Gas volume plots for (A) MPC35, (B) MPC70, (C) MPC80 and (D) MPC90, using a logarithmic scale for the gas volume.

$k \approx 7–8 \times 10^{-3}$ s^{-1} for the terminal parts. The gas disappearance for the 0.2% system was much slower than these lower concentrations with $k \approx 6.6 \times 10^{-3}$ s^{-1} for the initial part and $k \approx 3.7 \times 10^{-3}$ s^{-1} for the terminal part of the curve. The overall decrease in disappearance rate with rehydration time and with increase of MPC70 concentration, may suggest an increasing influence of solvent properties (e.g., increasing viscosity or 'protein drag force' effects) on the release of bubbles from the solvent as protein is solubilised.

MPC80 (Fig. 5C):

The time taken for the gas volume to reach its maximum value increased with increasing concentration, due to the influence of increasingly longer wetting times (200 s at the highest mass added). The gas disappearance rate constants decreased with increasing concentration from $k \approx 6.7 \times 10^{-3}$ s^{-1} at 0.04% to $k \approx 3.4 \times 10^{-3}$ s^{-1} at 0.2%.

MPC90 (Fig. 5D):

Compared to MPC80, the concentration-dependency of the time for the gas volume to reach its maximum seemed less prominent with MPC90, despite this powder having similar wetting times. In addition, the gas disappearance rate appears to be relatively independent of concentration ($k \approx 2.0–3.0 \times 10^{-3}$ s^{-1}) compared to the other MPCs.

The rate-limiting stage for the descending slope (representing gas disappearance) seems to be gas elimination from the solvent for MPC70. Values for k decrease with increasing concentration, indicating that increasing levels of solubilised components may have retarded bubble escape to a greater degree. On the other hand, for MPC90, gas release from MPC particles appears to be rate-limiting, as gas disappearance was effectively independent of concentration. A slow and ordered process of water transfer into MPC90 particles would explain this observation.

The MPC80 data indicate a concentration-dependent transition between the processes described which determine the gas disappearance rate for MPC70 and MPC90. Like MPC90, there is evidence that this powder has poor water transfer properties, due to the extended duration of its gas generation phase, but its gas disappearance behaviour is broadly similar to MPC70. It is proposed that the factor which extends the gas disappearance phase of MPC90 compared to MPC80 is a slower water transfer process.

3.6. Validation of water transfer as key stage influencing BARDS spectra for MPC90

The data presented in previous sections indicate that inhibited water transfer into particles in high-protein MPCs strongly influences the BARDS spectra, with powders such as MPC90 containing particles which require longer water transfer times. An experiment was designed to investigate whether the slow decrease in the compressible gas volume for high-protein MPC samples (especially MPC90) is due to steady transfer of gas out of the MPC powder particles (during water transfer) or due to other processes which affect the loss of gas at the liquid surface - for instance, an increase in viscosity, surface tension or drag forces acting on ascending bubbles (Ybert & di Meglio, 1998). To this end, KCl was used as a monitoring compound, to investigate whether the physicochemical properties of rehydrated MPC90 (post steady-state) inhibited the ability of gas to escape from the liquid. Fig. 6(A) shows the BARDS responses during the dissolution of 0.5 M KCl in water and also the dissolution of 0.2% MPC90 in water. KCl exhibits immediate release of gas and a fast return to steady state within 200 s.

A second experiment was performed whereby the same amount of KCl was added to a solution of 0.2% MPC90 which had been

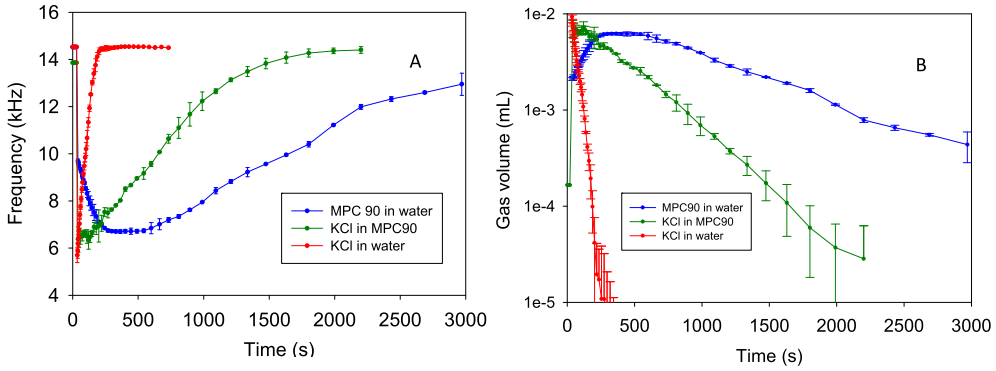

Fig. 6. Investigation of influence of solvent properties on gas disappearance: (A) Frequency-time plot of MPC dissolved in water, KCl dissolved in water or KCl dissolved in MPC90 solution; (B) log plot of gas volume data derived from (A).

rehydrated until a steady-state BARDS response was achieved (Fig. 6A). Again, there was an immediate generation of gas observed, but the return to steady-state took ~10 times longer due to replacement of water with MPC90 suspension. This result shows that the presence of soluble proteins impedes gas disappearance. Despite the slower escape of gas from the liquid, it took a significantly shorter time for gas from KCl to disappear from MPC suspension compared to the disappearance of gas during the rehydration of MPC90. The first-order k values can be derived from the descending slopes in Fig. 6(B), and were found to be 2.82×10^{-3} s^{-1} for KCl dissolved in MPC90 solution compared to the lower k value of 1.54×10^{-3} s^{-1} for MPC90 on its own. This strongly indicates that for MPC90 the terminal gas disappearance rate is determined by the process of continued water transfer into particles which generates compressible gas from MPC90 during rehydration.

In pharmacokinetics, an analogous process to that observed for MPC90 rehydration can be described in which the terminal stage of the concentration time-course of a drug in the blood reflects the drug absorption process instead of the elimination process as a 'flip-flop' system (Boxenbaum, 1998). In this study, generation of compressible gas bubbles through water transfer into particles can replace absorption, in the pharmacokinetic sense, for the kinetic analysis of MPC90 rehydration presented in the following section.

3.7. Verification that occluded air accounted for total gas volume using flip-flop kinetics

The gas volume time-course of MPC90 (see Fig. 6B) was used to establish the total amount of compressible gas that was produced during the rehydration experiment. An approach was followed similar to that used in pharmacokinetic studies, in which one distinguishes the absorption of a drug into the body, its distribution and its subsequent elimination. The concentration-time profile is determined in the central compartment (blood/plasma). The area under the concentration/time curve (AUC), combined with the drug distribution volume (V_d) and its first-order elimination rate constant (k_{el}, s^{-1}) are used to calculate the dose (D) that has entered the central compartment using Equation (4).

$$D = AUC \times V_d \times k_{el} \quad (4)$$

In an adjusted approach used for the gas volume analysis, the absorption is replaced by the generation of compressible gas into the solution (the central compartment) following addition of MPC90 to the solvent. The dose administered in pharmacokinetics becomes the total amount of compressible gas produced. In contrast to pharmacokinetics, the distribution volume (V_d) is now simply the volume of the solution ($V_{solution}$) (Rowland & Tozer, 1989). The total amount of compressible gas produced during dissolution (D_{gas}) can then be calculated using Equation (5).

$$D_{gas} = AUV \times k_{el} \quad (5)$$

where D_{gas} (mL) is the total amount of compressible gas produced during the rehydration of MPC90. AUV (mL.s) is the total area under the gas volume/time curve (the volume of compressible gas is calculated as $f_a \times V_{solution}$) and k_{el} (s^{-1}) is the rate constant of the first-order gas elimination process. In the calculations, the rate constant determined for KCl in MPC90 ($k_{el} = 2.82 \times 10^{-3}$ s^{-1}) is used for the elimination process in the MPC90 experiment. The AUV was calculated to be 7.87 mL s. The total amount of gas generated (D_{gas}) was 2.22×10^{-2} mL, calculated as $AUV \times k_{el}$. The total amount of occluded gas in the MPC90 sample used in the experiment was 2.31×10^{-2} mL. This was estimated for a 0.2% MPC90 system in 25 mL of water from the occluded air value in Table 1. The total amount of gas generated during powder rehydration (estimated from BARDS data) is in close agreement with the occluded air content of the powder.

The first-order k value derived from the descending slope of MPC90 in Fig. 6(B), was found to be 1.54×10^{-3} s^{-1}, and is assumed to describe the gas generation process (k_{gen}). Note that $k_{el} > k_{gen}$, implying flip-flop characteristics of gas production and elimination.

The values obtained above for k_{el}, k_{gen} and D_{gas} were used to simulate the gas volume-time of MPC90. The result is shown in Fig. 7(A) together with the data earlier presented in Fig. 6(B). The red profile represents 0.2% MPC90 data and the black profile is the simulation.

A similar set of experiments with rehydration of MPC90 in water and KCl in rehydrated MPC90, but performed under slightly altered conditions (i.e., using a glass vessel with different dimensions and therefore slightly different solution mixing dynamics) yielded different gas elimination rate constants. The results of the 0.2% MPC90 experiment are shown in Fig. 7(B) as the red profile. The value for k_{el} of 5.17×10^{-3} s^{-1} was derived from the KCl gas elimination rate in MPC90 solution. The AUV was 4.36 mL s and the total amount of gas generated ($AUV \times k_{el}$) was 2.25×10^{-2} mL, which is again in close agreement with the total amount of occluded gas in the MPC90 sample used. The calculations demonstrate that the two experiments are in good agreement in terms of the total amount of gas generated. The value is therefore independent of the differences in response observed between the two experiments and the different rate constants used in the calculations.

A value of 1.99×10^{-3} s^{-1} for k_{gen} was derived from MPC90 terminal gas disappearance rate. The black profile in Fig. 7(B) shows the simulation of the gas volume-time profile, based on the values found for k_{el}, k_{gen} and D_{gas}.

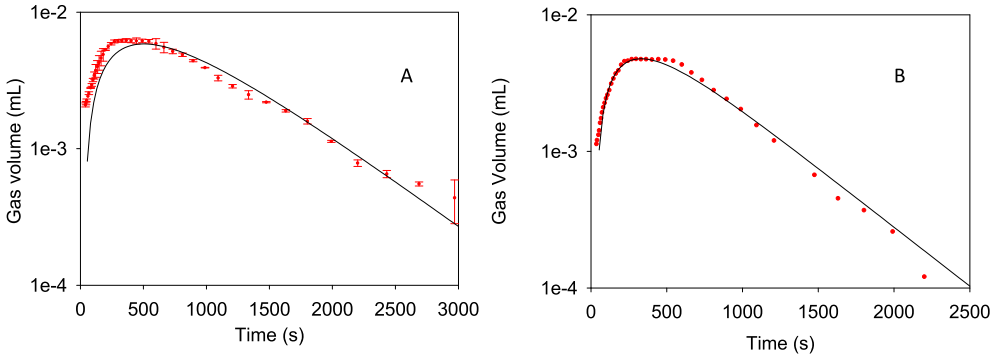

Fig. 7. Modelling of the BARDS data of 0.2% MPC90 using flip-flop kinetics: (A) Log Simulation of the gas volume data as presented in Fig. 6(B). (B) Simulation of 0.2% MPC90 gas volume data obtained under slightly different conditions.

Crucially, the values calculated using flip-flop kinetics for the amount of gas generated during the rehydration of MPC90 indicate that the gas detected by BARDS originates exclusively from the occluded gas fraction of the powder. Thus, when considering the BARDS spectra for the MPC90, the influence of interstitial air can be considered negligible, which allows isolation the gas generation phenomenon as one of water transfer into powder particles.

3.8. Microstructure of dry MPC powders

SEM micrographs of representative low- (MPC35), intermediate- (MPC70) and high-protein (MPC90) powders are shown in Fig. 8. In agreement with particle size data (Table 1), there was a greater quantity of small particles in the MPC90, while the MPC35 and MPC70 were similar in this respect. Increasing protein content was associated with two distinct morphological changes, the smoothening of particle surfaces and partial deflation of the surface towards the particle interior. Smooth particle surfaces may be attributable to differences in compositional homogeneity of the particle surfaces in the MPC powders. Kelly et al. (2015) determined that protein constituted 63, 79 and 93% of the surface of MPC35, MPC70 and MPC90 (same sample set), respectively; the surface of MPC35 contained a large quantity of lactose (31%), while the surface of MPC90 contained <1% lactose. The deflation effect is characteristic of casein-dominant dairy powders, such as MPCs and MCCs, and is not observed for whey protein-dominant powders (Sadek et al., 2016). It is generally associated with powders containing high levels of occluded air, which is the case for MPC70 and, in particular, MPC90 (Table 1), where distinct internal air vacuoles and external protein rich layers are present. Recent studies suggest that highly concentrated casein suspensions undergo a form of gelation during drying, and that this surface gel has distinct mechanical properties which result in this final deflated or buckled powder particle shape (Sadek et al., 2016).

3.9. Microstructure of MPC powders during rehydration

To investigate the dispersion state of powder particles during rehydration, cryo-SEM was used to visualise powders with fast (MPC35) and slow (MPC90) water transfer characteristics. Cryo-SEM micrographs of MPC35 and MPC90 powders during rehydration are shown in Fig. 9. The three different time points represent pre-steady-state for both powders (100 s), steady state for MPC35 and not MPC90 (1000 s), and post-steady-state for both powders

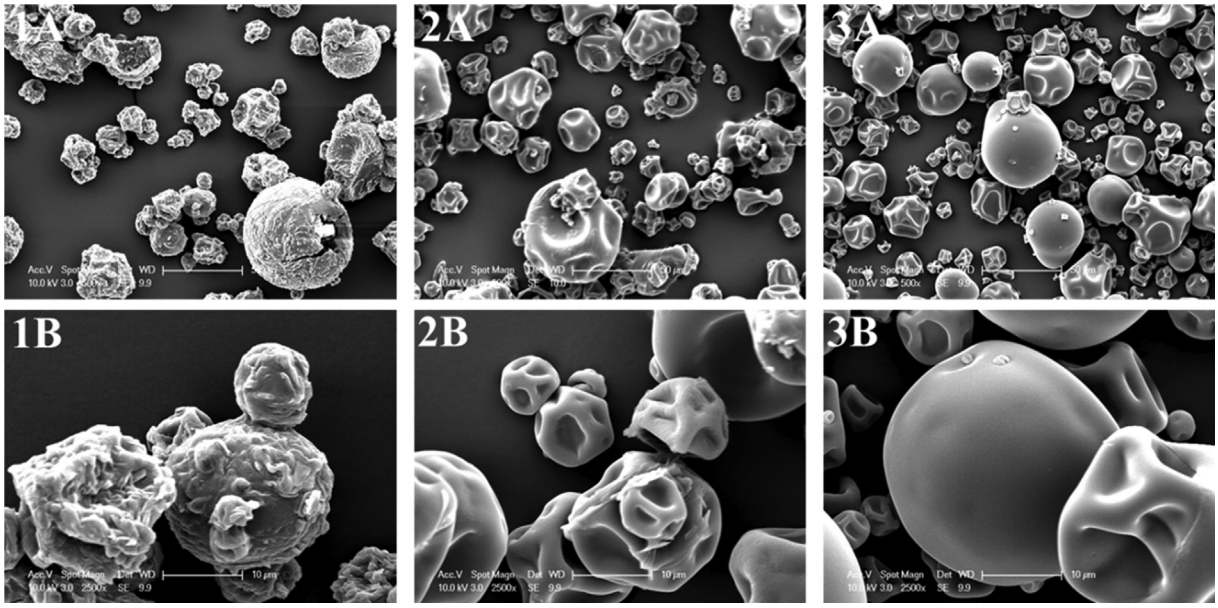

Fig. 8. Cryo-SEM micrographs of dry (1) MPC35, (2) MPC70 and (3) MPC90 at magnifications of (A) 500× and (B) 2500×, with scale bars of 50 and 10 μm, respectively, for the magnifications.

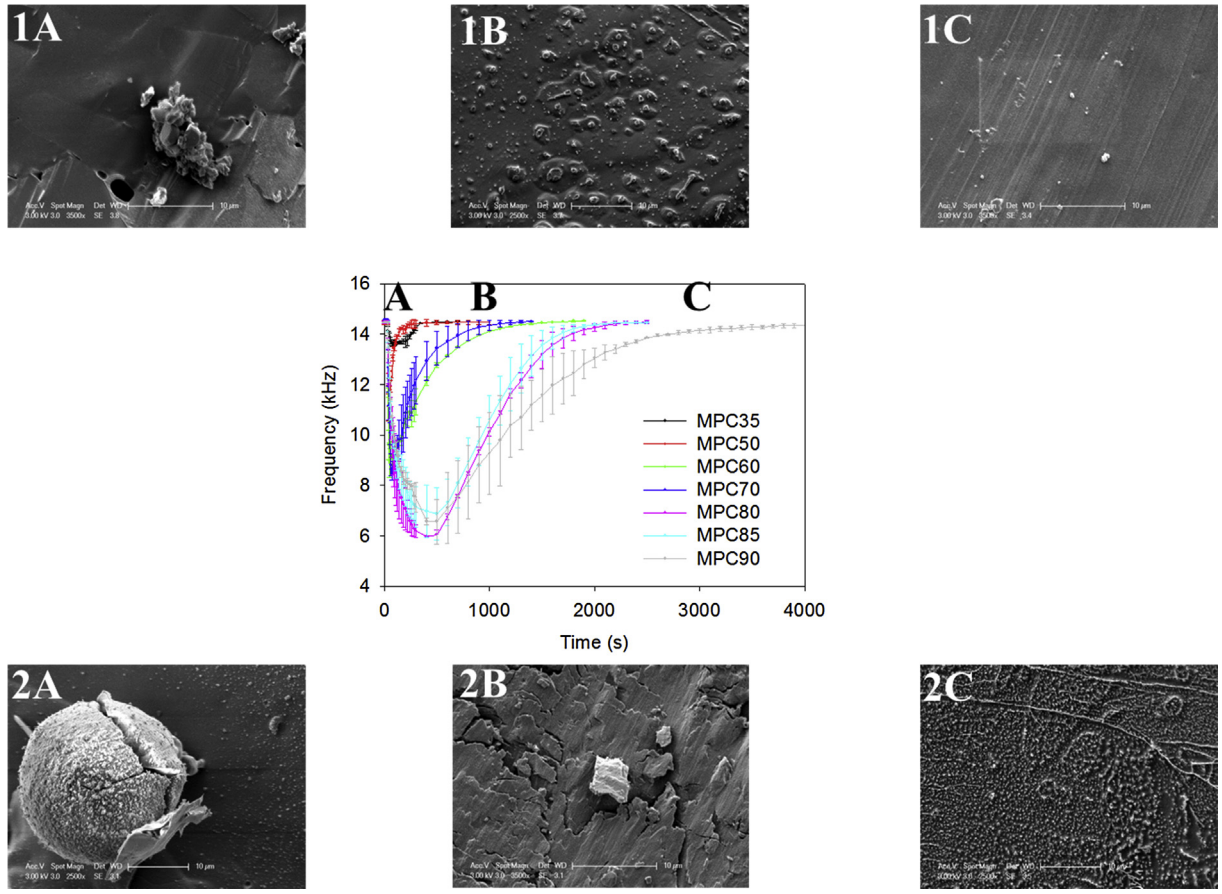

Fig. 9. BARDS spectra of all MPCs added at 0.2% w/v and cryo-SEM micrographs of (1) MPC35 and (2) MPC90 after rehydration for (A) 100 s, (B) 1000 s, and (C) 3000 s. Corresponding rehydration times in the BARDS spectra and micrographs are indicated for comparison.

(3000 s), as determined by BARDS.

After a short period of 100 s, partially-dispersed or fragmented particles were present in both MPC35 and MPC90; however, the latter also contained intact powder particles, similar in size (~20 μm) to the particles observed in the corresponding micrographs for dry powders (Fig. 8). When the powders were rehydrated for 1000 s, numerous small, distinct particles predominated in MPC35 which were ~1 μm in size, while several larger particles (~5 μm) remained in MPC90 with few distinct particles in general being visible. After 3000 s of rehydration, the majority of particles in MPC35 were <1 μm, with a minor distribution of micron-sized particles, while, in contrast, MPC90 was still populated largely by particles >1 μm; in addition, ring-link structures can be seen in Fig. 9 (1C), consistent with the possible presence of hydrated but undispersed powder particles, as suggested previously (Crowley et al., 2015; Mimouni et al., 2009). Fig. 9 also shows the BARDS frequency-time profiles for all seven MPCs with a sample mass of 50 mg (0.2%). The BARDS measurement times corresponding to the rehydration times where the micrographs were captured are indicated. It can be seen in Fig. 9 that for an equivalent stage of water transfer, such as the steady-state of all MPCs at 3000 s, different dispersion states can exist. This is because BARDS is a technique that detects the completion of water transfer into particles but not necessarily the disappearance of granular particle structures. However, both water transfer and dispersion occur simultaneously, indicating that a possible relationship between the two phenomena exists; this is expanded in Section 4.

4. Discussion

This is the first study reporting on the gas release/water transfer properties of a full range of MPCs, ranging from low to high protein. Results from BARDS analysis indicated that water transfer into MPC powder particles became inhibited as the protein content of the MPC powders increased (Figs. 2 and 3, Table 1). For example, rehydration of MPC35 yielded a minimal BARDS response and a rapid return to steady-state (<400 s), while the time (60 min) required to reach steady-state for MPC90 (0.2%) (Fig. 2) is unprecedented in its length compared to previous BARDS studies (Fitzpatrick et al., 2012a,b, 2013, 2014). Release of gas from powders can be used to indirectly investigate water transfer in dairy powders (Hauser & Amamcharla, 2016; Richard et al., 2012). Gas in powders consists of interstitial (between particles) and occluded (within particles) air. In the samples studied, greater quantities of both were present in high-protein powders such as MPC90 (Table 1), but flip-flop kinetic analysis of MPC90 (0.2%) indicated that only occluded air was detected by BARDS (see Section 3.7); thus, the higher levels of occluded air in high-protein MPCs were responsible for the greater total volume of gas which was released during their rehydration (Fig. 3). The volume of compressible gas generated was, as would be expected, in proportion to the mass of powder added to the water (Fig. 5).

Fig. 4B demonstrates that at concentrations of 0.2% gas was released much more slowly into the solvent during the rehydration of high-protein MPCs (MPC80, MPC85 and MPC90) compared to the lower protein MPCs. Gas generation in these high-protein MPCs was partially delayed by slow wetting, due to their high air content

and consequent poor sinkability (Table 1) but also the high hydrophobicity indicated by the large contact angle formed between the powders and water (Crowley et al., 2015). However, although wetting lasted 200 s for these powders, gas generation was still dominating over gas elimination until 500 s in the BARDS spectra of 0.2% MPC80, MPC85 and MPC90, confirming that water transfer into particles continued after wetting. For MPC80 and MPC85, Fig. 3C and Table 2 show that the gas disappearance phase was intermediate among the powders and similar to MPCs which did not exhibit inhibited water transfer (MPC60, MPC70). For this reason, inhibited water transfer was not considered to strongly affect the gas disappearance behaviour in MPC60, MPC70, MPC80, MPC85; instead, the impeding influence of solubilised protein on bubble escape (Ybert & di Meglio, 1998) was considered to define gas elimination in these systems. The more rapid gas disappearance for MPC35 and MPC50, which displayed similarly fast water transfer to MPC60 and MPC70, was likely due to the lower levels of protein available to impede bubble escape. Indeed, it was demonstrated in this study that the properties of a protein suspension can influence bubble escape (Fig. 6) during the rehydration of a solute, which is an important consideration when conducting rehydration assessments using BARDS and other sound-based methods.

The rate of gas disappearance for MPC90 was the slowest of all the powders, which suggested that water transfer into powder particles was still influential during the gas disappearance phase; however, the influence of water transfer and solvent properties needed to be differentiated. Although certain physicochemical properties of a 0.2% MPC90 suspension (which had undergone complete water transfer) affected gas bubble elimination (Fig. 6), slow water transfer into the powder particles was determined to be the major factor limiting gas disappearance from the solvent. The influence of the markedly slow process of water transfer into particles therefore persisted throughout the early, intermediate and late stages in the BARDS spectra for MPC90. A study of concentration-dependency, however, revealed differences between MPC80 and MPC90, which has also been observed previously for their dispersion characteristics (Crowley et al., 2015). For MPC80 (gas elimination-limiting), the rate of gas disappearance was affected by concentration effects such as soluble protein, but this was not the case for MPC90 (gas generation-limiting), as the process of water transfer was not influenced by concentration (Fig. 6).

Cryo-SEM micrographs indicated that the dispersion of particles on the transition from a dry powder (Fig. 8) into a rehydrated suspension (Fig. 9) was slower and less complete for MPC90 compared to MPC35, due to the poor dispersibility of particles in high-protein MPCs (Crowley et al., 2015; Fang et al., 2011; Ji et al., 2016; Mimouni et al., 2009). The cryo-SEM micrographs can be compared to the BARDS spectra in Fig. 10. At 100 s of rehydration, MPC35 had a limited BARDS frequency deflection due to the quick dispersion of particles capable of releasing the minor levels of occluded air present (Table 1). As large structures capable of entrapping air were no longer present due to effective dispersion, MPC35 rapidly reached steady state before 1000 s had elapsed; conversely, water transfer into MPC90 particles was slow, and the particles themselves underwent more limited dispersion, resulting in continued air release from the particles. When particles in both MPCs had undergone significant dispersion into smaller fragments and dissolution into component molecules, neither powder exhibited any air release (~3000 s). However, at this point, the rehydration state of both powders cannot be considered equivalent, as it is clear that much larger particle structures remained in the MPC90. The MPC35 primarily consisted of particles <1 μm, which would be expected for the nanoscale proteins present in milk. On the other hand, MPC90 contained a substantial proportion of micron-sized particles, which were presumably undispersed powder particle fragments.

BARDS data indicating inhibited water transfer during the rehydration of high-protein MPCs, particularly MPC90, must be considered in the context of a growing body of evidence supporting the presence of a 'skin' of inter-linked casein micelles at the surface of high-protein MPC particles, which has been linked with the poor rehydration characteristics of these powders (Crowley et al., 2016; Fyfe et al., 2011; Ji et al., 2016; McKenna, 2000; Mimouni et al., 2010b). The results of the present study suggest that this skin of

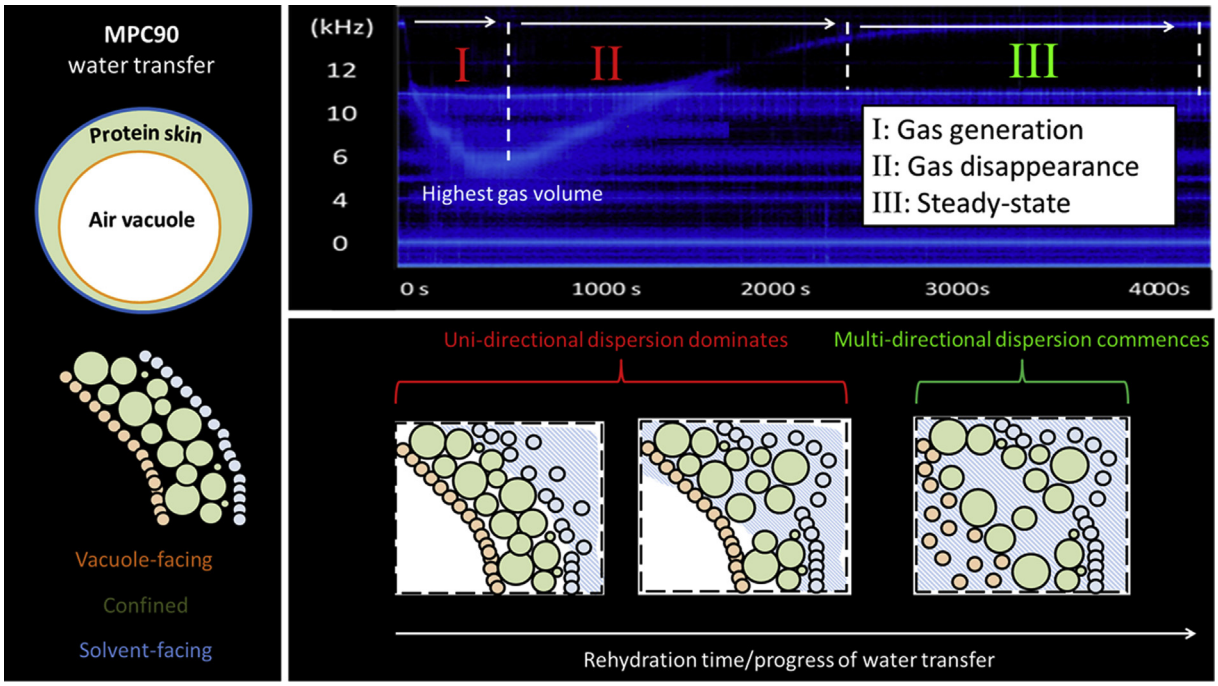

Fig. 10. Schematic representation of protein 'skin' at the surface of a primary powder particle in a high-protein MPCs and the hypothesised relationship between inhibited water transfer and the poor dispersion of these particles. A BARDS profile for MPC90 is shown.

inter-packed casein micelles may act as a barrier which reduces the rate of water transfer into particles during the rehydration of high-protein MPCs. The protein:lactose ratio at the surface of the MPC particles studied here decreased significantly as the protein content of the powders increased (Kelly et al., 2015). This altered surface composition may have removed lactose as a hydrophilic channel for effective water transfer into the particle resulting in a relatively homogenous and hydrophobic particle surface (Crowley et al., 2015; Fyfe et al., 2011). The absence of lactose as a physical 'spacer' may also have promoted the tendency for proteins-protein interactions resulting in cohesive protein skin (Anema et al., 2006; Havea, 2006). The BARDS data and cryo-SEM micrographs in Figs. 8–10 strongly support that both water transfer and dispersibility are impaired in high-protein MPCs. This has also been found for MCCs, where a link between rapid water transfer and effective dispersion has been proposed (Richard et al., 2012) and demonstrated (Bouvier et al., 2013).

The nature of the relationship between water transfer and dispersion has yet to be established, although it is evident from the present study that MPCs with poor water transfer properties also have poor dispersion characteristics. One possibility is that incomplete water transfer results in regions of the particles remaining effectively 'dry', thereby limiting their ability to attain the molecular mobility necessary to disperse effectively. This concept is illustrated in Fig. 10 with corresponding BARDS frequency profile for MPC90, which shows how the presence of dry regions near the internal air vacuole of the particle could result in the predominance of uni-directional (towards the bulk solvent) dispersion, where components in immediate contact with the solvent are released first by an erosion-like process. Transfer of water through the protein skin during rehydration could eventually expose dry regions to a second solvent-front located in the interior of the particle. The presence of these two solvent-fronts would then promote collapse of the particle through multi-directional (towards the particle interior and the bulk solvent) dispersion.

5. Conclusion

BARDS was demonstrated to be an effective method for discriminating between MPC powders with different rehydration characteristics. The BARDS experiments only required 25 mL of water and 10–50 mg of each MPC (0.04–0.20% for suspensions), which minimises greatly the quantities of powder required for comparable tests. An additional advantage is that BARDS is non-invasive, as acoustic responses are derived from a non-contact microphone rather than a submerged probe. MPC35, MPC50, MPC60 and MPC70 exhibited similar water transfer properties, and differences in their BARDS spectra were primarily caused by their different air contents and the effect of increasing protein content on bubble escape. High-protein MPC powders (MPC80, MPC85 and MPC90) exhibited a characteristic BARDS response during rehydration involving a prolonged period of gas generation to reach a maximum solvent compressibility, due mainly to inhibited water transfer into the powder particles. The period of gas generation during the rehydration of high-protein MPCs was followed by a prolonged return to steady-state equilibrium; the disappearance of gas from the solvent during this phase was influenced by the impeding effect of soluble protein on bubble escape; however, for MPC90, inhibited water transfer was still dominant during gas disappearance. The water transfer properties of high-protein MPCs were poor, but they were exceptionally poor for MPC90. BARDS is one of the few techniques currently available which facilitates the dynamic monitoring of water transfer during powder rehydration. Further BARDS studies will focus on the effect of varying solvent composition and temperature of rehydration on water transfer properties. BARDS may also be an attractive option for identifying defects in the rehydration characteristics of high-protein dairy powders caused by process- or storage-induced degradative changes.

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