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Teagasc Food Research Centre
Moorepark, Fermoy, Co. Cork, Ireland



University College Cork
Ireland

**Infant Milk Formula Manufacture: Process and Compositional
Interactions in High Dry Matter Wet-Mixes**

A thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

Eoin Gerard Murphy, B.Eng. (Hons.)

School of Food and Nutritional Sciences

University College Cork

April 2015

Supervisors:

Dr Mark A. Fenelon, Dip. Proc. Chem. Eng., B.Sc, PhD

Prof Yrjö H. Roos, M.Sc, PhD

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Declaration

I hereby declare that the work submitted in this thesis 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: _____

Eoin Gerard Murphy

Dedication

For:
Jim and Mary Murphy


Abstract

Infant milk formula (IMF) is fortified milk with composition based on the nutrient content in human mother's milk, 0 to 6 months postpartum. Primary ingredients include bovine milk and/or whey, lactose, vegetable oils and vitamin/mineral premixes. Extensive medical and clinical research has led to advances in the nutritional quality of infant formula, bringing it closer to human milk with claims of improved gut health, immunological response, cognitive health etc., however, relatively few studies have focused on interactions between nutrients and the manufacturing process.

The objective of this research was to investigate the impact of composition and processing parameters on the physical behaviour of high dry matter (DM) IMF systems with a view to designing more sustainable manufacturing processes. The study showed that commercial IMF, with similar compositions, manufactured by different processes, had markedly different physical properties in dehydrated or reconstituted state. Commercial products made with hydrolysed protein were more heat stable compared to products made with intact protein, however, emulsion quality was compromised. Heat-induced denaturation of whey proteins resulted in increased viscosity of wet-mixes, an effect that was dependant on both whey concentration and interactions with lactose and caseins. Expanding on fundamental laboratory studies, a novel high velocity steam injection process was developed whereby high DM (60%) wet-mixes with lower denaturation/viscosity compared to conventional processes could be achieved; powders produced using this process were of similar quality to those manufactured conventionally. Hydrolysed proteins were also shown to be an effective way of reducing viscosity in heat-treated high DM wet-mixes. In particular, using a whey protein concentrate whereby β -Lactoglobulin was selectively hydrolysed, i.e., α -Lactalbumin remained intact, reduced the viscosity of wet-mixes during processing while still providing good emulsification.

The thesis provides new insights into interactions between nutrients and/or processing which influence physical stability of IMF both in concentrated liquid and in powdered form. The outcomes of the work have applications in such areas as; increasing the DM content of spray drier feeds in order to save energy, and, controlling final powder quality.

Publications



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The effect of high velocity steam injection on the colloidal stability of concentrated emulsions for the manufacture of infant formulations

Eoin G. Murphy^a, John T. Tobin^a, Yrjö H. Roos^b, Mark A. Fenelon^{a*}

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Eoin G. Murphy · John T. Tobin · Yrjö H. Roos ·
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Decoupling Macronutrient Interactions during Heating of Model Infant Milk Formulas

Eoin G. Murphy,[†] Mark A. Fenelon,^{*,†} Yrjö H. Roos,[§] and Sean A. Hogan[†]

[†]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland

[§]School of Food and Nutritional Sciences, University College, Cork, Ireland

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Physical stability of infant milk formula made with selectively hydrolysed whey proteins



Eoin G. Murphy^a, Yrjö H. Roos^b, Sean A. Hogan^a, Patrick G. Maher^a, Cal G. Flynn^c,
Mark A. Fenelon^{a,*}

^aTeagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

^bSchool of Food and Nutritional Sciences, University College Cork, Ireland

^cKerry Ingredients and Flavours, Tralee Road, Listowel, Co. Kerry, Ireland

Abbreviations

α-La	α -Lactalbumin
ANOVA	Analysis of variance
ANS	1-Anilinonaphthalene-8-Sulfonic Acid
ATR	Attenuated Total Reflectance
β-Lg	β -Lactoglobulin
BSA	Bovine Serum Albumin
C	Casein
Ca⁽²⁺⁾	Calcium (or Calcium ion)
CaCl₂.2H₂O	Calcium Chloride Dihydrate
cm	Centimetre
CMP	Casein-Macropptide
Cp	Heat Capacity
Cys	Cysteine
D	Hydraulic Diameter
D(v,0.1)	Diameter below which 10% (by volume) of the a particle size distribution exist
D(v,0.9)	Diameter below which 90% (by volume) of the a particle size distribution exist
D[3,2]	Sauter mean diameter
D[4,3]	Volume mean diameter
Da	Dalton
DE	Dextrose Equivalent
DH	Degree of Hydrolysis
DM	Dry Matter
DSC	Differential Scanning Calorimeter
DWP	Demineralised Whey Powder
F_d	Drum Flowability
FO	Follow On
FOS	Fructo-Oligo-Saccharide
F_R	Relative Fluorescence
FTIR	Fourier Transform Infra-Red (spectroscopy)

g	Gram
g	Gravitational force
G	Mass Velocity
GOS	Galacto-Oligo-Saccharide
h	hour
HSSI	High Solids Steam Injection
HT	Heat Treatment
HTST	High Temperature Short Time (pasteurisation)
h_x	Heat Transfer Coefficient of x
i	Jenike Flow Index
IMF	Infant Milk Formula
k	Thermal Conductivity
K₂CO₃	Potassium Carbonate
K₂SO₄	Potassium Sulphate
K₃citrate.H₂O	Potassium Citrate Monohydrate
kcal	Kilocalorie
KCl	Potassium Chloride
kDa	Kilodalton
kg	Kilogram
KH₂PO₄	Potassium Phosphate Monobasic
kJ	Kilojoule
KOH	Potassium Hydroxide
L	Litre
L	Lactose
LC-PUFA	Long-Chain Poly-Unsaturated Fatty Acids
m	Metre
μ	Apparent viscosity
m	Slope
M	Molar
mg	Milligram
Mg⁽²⁺⁾	Magnesium (or Magnesium ion)
MgCl₂.6H₂O	Magnesium Chloride Hexahydrate
min	Minute

mL	Millilitre
μL	Microlitre
mm	Millimetre
μm	Micrometre
μm	Micrometre
mM	Millimolar
mPa	Millipascal
MPa	Megapascal
MPC	Milk Protein Concentrate
ms	Milliseconds
Na₃citrate.5H₂O	Sodium Citrate Pentahydrate
NaOH	Sodium Hydroxide
N_c	Number of Channels (heat exchanger)
NH	Non-Hydrolysed
nm	nanometre
No.	Number
PAGE	Polyacrylamide Gel Electrophoresis
PCN	Phospho-Casein
PH	Partially Hydrolysed
PHE	Plate Heat Exchanger
PI	Pressure Indicator
PP	Proteose Peptone
Pr	Prandtl Number
Q	Heat Transfer Rate
r	Pearson's correlation coefficient
ρ	Density
Re	Reynolds Number
RH	Relative Humidity
RI	Refractive Index
RO	Reverse Osmosis
RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
rpm	revolutions per minute
s	Second

SDS	Sodium Dodecyl Sulphate
SE	Standard Error
SH	Selectively Hydrolysed
SMP	Skim Milk Powder
SMUF	Simulated Milk Ultrafiltrate
Sn	Stereoisomeric number
SO	Sunflower Oil
t	time
T	Temperature
T_d	Denaturation Temperature
T_g	Glass Transition Temperature
TI	Temperature Indicator
T_v	Temperature of onset of viscosity increase
U	Overall Heat Transfer Coefficient
UHT	Ultra High Temperature (heat treatment)
USFDA	United States Food and Drug Administration
v/v	Volume/Volume
W	Whey
w/w	Weight/Weight
WPC	Whey Protein Concentrate
WPI	Whey Protein Isolate
WPNI	Whey Protein Nitrogen Index
%	Percentage
°C	Degree Celsius

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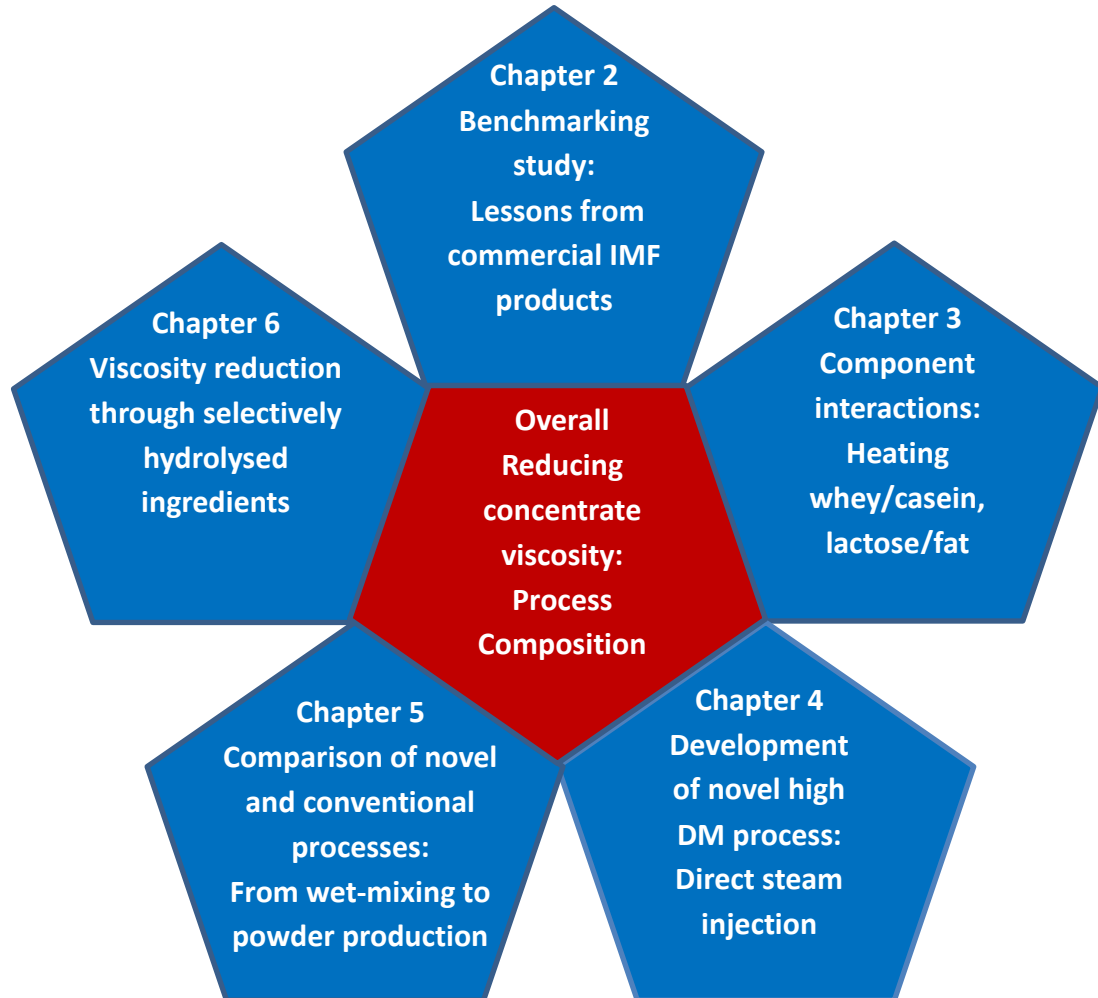
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Objectives

The relationships between the objectives of individual research chapters and the overall objectives of this study are presented in the figure below:



Chapter 1: Literature review – processing and compositional considerations in infant milk formula manufacture

1.1 Infant Milk Formula

Infant milk formulae (IMF) are tailored milks for infants based on the nutritional profile of human milk. IMF products are consumed by infants from birth and must satisfy the nutritional requirements of new-borns until they can be introduced to appropriate complimentary food (European Commission, 2006; Codex Alimentarius, 2007). Associated follow-on (FO-IMF) products are also common, intended for babies and children from 6 months through to 7 years of age.

Bovine milk is not a suitable source of nutrition for neonates. Despite this, bovine milk derivatives are the most common source of non-fat constituents in IMF (Nasripour et al., 2006). The differences between human milk, bovine milk and IMF are shown in Table 1.1 When bovine skim milk, for example, is used as an ingredient in IMF, protein and mineral content must be reduced. This is achieved by addition of lactose and/or demineralised whey powder (DWP; > 80% w/w lactose). Addition of DWP and/or whey protein concentrates (WPC) changes casein to whey ratio from that of bovine milk (approximately 80:20) to that of human milk (most commonly cited as 40:60 but subject to change depending on stage of lactation; Lonnerdal, 2003). Casein to whey ratio is not controlled in Europe, however, it is often changed to include more whey protein; Chinese regulation requires the protein in IMF to contain a minimum of 60% whey protein (P.R. China, 2010). Special blends of vegetable oils are added to the formulation to simulate the nutritional qualities of human milk fat (Bar-Yoseph et al., 2013).

Table 1.1. Composition of IMF, human and bovine milk

	IMF	Human	Bovine
		per 100 mL	
Energy (kcal)	60 - 70	71	69
Total Protein (g)	1.2 - 2.0*	0.9	3.3
Casein	N.S	0.3	2.6
Whey	N.S	0.6	0.7
Fat (g)	2.9 - 3.9*	3.8	3.7
Carbohydrate (g)	5.9 - 9.1*	7	4.8

Data: Gurr, 1981; Thompkinson and Kharb, 2007

*Calculated from European Commission, 2006 for energy content = 65 kcal 100 mL⁻¹

IMF made with bovine milk ingredients is not suitable for all infants and several alternatives exist. For example, IMF made with hydrolysed proteins is available for

infants with an allergy or intolerance to bovine milk protein (Brew, 2003). IMF intended for low birth weight infants, or infants born prematurely, have higher calorific value and contain greater amounts of proteins, vitamins and minerals. When infants are introduced to solid food, follow-on (FO-IMF) formulae are commonly used as a complimentary food source. Formulations generally decrease in fat and increase in protein as the child progresses in age (McSweeney, 2008)

Physical characteristics are affected by composition during manufacture of IMF and FO-IMF. McCarthy et al. (2012) found that decreasing protein-to-fat ratio significantly decreased stability of IMF emulsions. Interactions caused by compositional variation and manufacturing process type will also affect physical characteristics. For example, the main whey protein in bovine milk, β -Lactoglobulin (β -Lg) denatures at temperatures above 65 °C which can lead to increases in viscosity, gelation, and fat globule aggregation (Walstra and Jenness, 1984; Euston et al., 2000; Singh and Havea, 2003). The extent of denaturation, however, may be reduced by direct heat treatments; such as direct steam injection, which can also reduce the size of fat globules in the IMF emulsion (Zadow, 1969; van Boekel and Folkerts, 1991; Ye et al., 2005). Dry matter (DM) content of IMF wet-mixes is also a key consideration, as the processing and compositional factors listed above are likely to be affected by the concentration of components relative to water. The purpose of this review is to compile the existing literature documenting compositional and processing interactions pertinent to IMF manufacture.

1.2 Composition

1.2.1 Protein

IMF must contain at least the same quantity of essential and semi-essential amino acids (see Table 1.2) as human milk (Koletzko et al., 2005; Thompkinson and Kharb, 2007). While bovine milk contains an excess of some amino acids, for infant nutrition, it is deficient in methionine, cystine and tryptophan. The minimum crude protein content of IMF manufactured with bovine milk proteins is 1.8 g 100 kcal⁻¹ (European Commission, 2006; Codex Alimentarius, 2007; P.R. China, 2010). IMF manufactured with hydrolysed bovine proteins and soy proteins should contain a minimum protein content of 2.25 g 100 kcal⁻¹ (European Commission, 2006; Codex Alimentarius, 2007; P.R. China, 2010). The

ratio of whey to casein is often changed from 20:80 (as in bovine milk) to 60:40 (as in human milk) by addition of whey protein ingredients. Simulating the whey to casein ratio found in human milk may help to produce a softer curd which is easier for digestion (Thompkinson and Kharb, 2007).

Table 1.2. The minimum levels of essential and semi-essential amino acids in IMF

Amino acid	mg 100 kJ⁻¹
Arginine	16
Cystine	6
Histidine	11
Isoleucine	17
Leucine	37
Lysine	29
Methionine	7
Phenylalanine	15
Threonine	19
Tryptophan	7
Tyrosine	14
Valine	19

Source: European Commission 2006

1.2.2 Whey proteins

Whey proteins are proteins remaining in solution after removal of casein from milk (Mulvihill and Donovan, 1987). The major whey proteins in bovine milk are, in order of concentration, β -Lactoglobulin (β -Lg; ~50% of total whey protein), α -Lactalbumin (α -La; ~20%) and bovine serum albumin (BSA; 5-10%) (Fox and McSweeney, 1998b; Thompkinson and Kharb, 2007). α -La is the most abundant whey protein in human milk (Thompkinson and Kharb, 2007; Shi et al., 2011). Modern IMF may be enriched in α -La as a result of advances in dairy technology allowing for the production of α -La-enriched whey concentrates (Holt et al., 1999).

Whey proteins, in their native form, are compact globular structures with non-polar groups, such as hydrophobic amino acids, arranged in the centre of folded peptide chains. Whey proteins possess intricate secondary and tertiary structures (Singh, 2011). Feng and Baugh (2013) measured whey protein content of 12 IMF products and found whey protein content varied from 18 to 63% of total protein. No details were provided on

the age categories of the products, however, it is likely that high whey protein contents measured corresponded to IMF products with lower whey protein contents corresponding to FO-IMF. Protein content of IMF sold in China must consist of at least 60% whey proteins (P.R. China, 2010). The susceptibility of whey proteins to denature at elevated temperatures is of particular importance to dairy processing and IMF manufacture given that whey proteins can be present in high levels (see Section 1.3.3). Denaturation by heat, which causes unfolding of whey protein structure, can lead to association of whey proteins with themselves (Simmons et al., 2007) and also with caseins (Corredig and Dalgleish, 1999; Nguyen et al., 2012).

IMF may contain a large proportion of Casein Macropeptide (CMP) depending on the source whey proteins used in formulation. CMP is a by-product of cheese and/or rennet casein manufacture, resulting from the enzymatic cleavage of κ -casein during renneting. Cleavage occurs at the position 106 in the amino acid sequence of κ -casein, forming a water soluble macropeptide (residues 106-169) (Fox and McSweeney, 1998a). The CMP released into the whey stream constitutes a significant portion of the nitrogenous material; a study characterising 8 whey protein isolates/concentrates has shown that upwards of 25% of nitrogenous material can be CMP (Holt et al., 1999). Therefore, depending on the whey protein content of IMF, CMP could constitute upwards of 15% of protein.

Proteose-peptone (PP) is a group of heat stable proteins found in bovine milk and its derivatives. PPs are defined as being a heat stable group i.e. the TCA-insoluble proteins in acid whey (pH 4.6) prepared from milk heated at 90 °C for 30 min (Rowland, 1938). PP content of milk ranges between 0.5 and 3 g L⁻¹ in (Andrews, 1979; Pâquet, 1998). PP may be present in significant quantities in IMF formulations; apart from its presence in milk it has also been shown to constitute between 17 and 19% of the total whey protein in WPC (Innocente et al., 2011). The PP fraction in milk is quite heterogeneous but can be characterised into two broad groups - 1) those derived by proteolysis of caseins and 2) minor indigenous milk proteins such as osteopontin and PP3, also known as lactophorin (Fox, 2003). PPs derived from proteolysis of whey protein during storage have also been found in WPC (Innocente et al., 2011).

1.2.3 Caseins

A large proportion of the caseins (α , β and κ) in bovine milk exist in complexes with small, inorganic salts, called casein micelles. Casein micelles are highly hydrated spheres with up to 4 g water per gram of protein chemically bound or physically entrained within the micelle (Morris et al., 2000). Individually, the caseins are relatively small molecules (20 to 25 kDa), but casein micelles have molecular weights in the range of 10^6 to 10^9 Da (Fox and McSweeney, 1998a). Casein micelles have been shown to have an average diameter of approximately 200 nm (De Kruif, 1998), however, the exact structure of the casein micelle is still not fully understood. Recently, the nanocluster model has gained interest, where α and β -casein bind to colloidal calcium phosphate, fixing these calcium sensitive caseins in the structure of the micelle which is stabilised by a diffuse hairy layer of κ -casein at the micellar surface (Holt, 1992; De Kruif, 1999). κ -casein is unique among the casein in that it contains a carbohydrate moiety which confers hydrophilicity to the individual casein and also the casein micelle, by virtue of the hairy layer which extends from the surface of the micelle into the aqueous phase (Fox and McSweeney, 1998a; De Kruif, 1999). The κ -casein layer also provides electrostatic and steric repulsion between individual micelles (De Kruif and Zhulina, 1996).

1.2.4 Non protein nitrogen

The non-protein nitrogen content of human milk is much higher than that of bovine milk - ~20% compared to ~5% (Fox and McSweeney, 1998a). IMF is commonly fortified with taurine, which is the second most abundant free amino acid in human milk, but is absent from mature bovine milk (Thompkinson and Kharb, 2007). A study on the free amino acid composition of 12 commercially available IMF showed that, similar to human milk, free glutamic acid plus glutamine were the most abundant free amino acids, however, the levels present were much lower than human milk (Agostoni et al., 2000). Choline is often added to infant formula at levels of between 7 and 30 mg 100 kcal⁻¹ (Koletzko et al., 2005).

1.2.5 Carbohydrates

Lactose, as the predominant carbohydrate in bovine (and human) milk, is the main carbohydrate present in IMF. The European Commission (2006) directive states that at

least 4.5 g 100⁻¹ kcal of IMF must consist of lactose. After meeting this requirement, the remaining digestible carbohydrate may consist of:

- maltodextrin
- glucose syrups
- maltose
- pre-cooked starch
- gelatinised starch
- sucrose
- glucose

Glucose polymers such as maltodextrin and glucose syrup are, along with lactose, the recommended carbohydrate source for IMF (Codex Alimentarius, 2007). Pre-cooked and gelatinised starch must not exceed 30% of total carbohydrate and be naturally free of gluten (European Commission, 2006; Codex Alimentarius, 2007). Sucrose and glucose may be added to IMF produced with hydrolysed proteins in order to mask bitter flavours. Fructose is not suitable for new-borns (Koletzko et al., 2005; Code Alimentarius, 2007).

Maltodextrin and glucose syrup consist of D-glucose units connected in chains of variable length. They are derived from partial hydrolysis of starch the extent of which determines the length of the connecting chains, which is termed the dextrose equivalent value (DE value). The higher the DE value, the shorter the glucose chains in the maltodextrin/glucose syrup. The distinction between these starch derivatives is stated in the European Union's Combined Nomenclature code – at DE values greater than 20 the derivative is known as glucose syrup (European Commission, 2013).

Non-digestible carbohydrates such as galacto-oligo-saccharide (GOS) and/or fructo-oligo-saccharide (FOS) are commonly added to IMF to promote the growth of bifidobacteria (O'Callaghan et al., 2011). GOS is manufactured from lactose which has been transgalactosylated by β -galactosidase to produce a mixture of GOS polymers (tri-, tetra- and penta-GOS). Commercial oligosaccharides are available in both powder and syrup forms. Syrups can contain up to 75% (w/v) solids, of which up to 60% can be oligosaccharides (Playne and Crittendon, 2009). An oligosaccharide content of approximately 0.5% (GOS:FOS = 9:1) on a dry basis is suggested by the European Commission's Scientific Committee on Food (Scientific Committee for Food, 2001).

1.2.6 Fat content

The fat content of IMF should account for between 40 and 55% of the total energy (European Commission, 2006). Fat in infant formula usually consists of a blend of vegetable oils; milk fat is occasionally used, but at low levels (Montagne et al., 2009). Oils such as soya, sunflower, coconut, palm etc. are blended to emulate human breast milk in fatty acid composition, degree of saturation and the content of trans fatty acids (Nasripour et al., 2006). The purpose of this is to ensure efficient fat and mineral absorption by the infant; for example, high saturated fat content, as in bovine milk fat, may result in reduced calcium absorption (Williams et al., 1970).

Essential fatty acids (linoleic and α -linolenic) contents are regulated due to their role in synthesising long-chain poly unsaturated fatty acids (LC-PUFA). Although LC-PUFAs can be synthesised from linoleic acid and α -linolenic acid, IMF is often enriched with LC-PUFA due to beneficial effects on visual and cognitive development (Emmet and Rogers, 1997). Oil blends supplemented with LC-PUFAs are particularly susceptible to oxidation and as a result oil is usually stored under an inert gas (Montagne et al., 2009).

The distribution of fatty acids on the glycerol backbone of triglycerides in human milk is different to that of vegetable oils. The three positions on the glycerol backbone are numbered Sn-1 to Sn-3; in human milk, the saturated fatty acid, palmitic acid, is preferentially located at the central Sn-2 position. In contrast, vegetable oils contain a high proportion of palmitic acid at the outer Sn-1 and Sn-3 positions (Bar-Yoseph et al., 2013). Studies have shown that the Sn-2 position of palmitic acid in human milk promotes the absorption of fat and calcium in infants (López-López et al., 2001; Ramírez et al., 2001). As a result, vegetable oils with enhanced levels of palmitic acid in the Sn-2 position have become available, known commonly as structured lipids. The various effects of one commercially available structured lipid have been reviewed by Bar-Yoseph (2013).

1.2.7 Minerals and Vitamins

It is necessary to fortify bovine derived IMF with minerals. Addition of demineralised whey, ultrafiltered whey concentrates and lactose to skim milk dilutes the concentration of minerals present. Table 1.3 shows the acceptable range of minerals and

vitamins allowed in IMF. Commonly used minerals include: Calcium carbonate, calcium phosphates, dibasic magnesium phosphate, potassium citrate, magnesium chloride etc. IMF must also be fortified with vitamins, which can be divided into two groups – fat soluble vitamins (A,D,E and K) and water soluble vitamins.

1.2.8 Functional Ingredients

Use of emulsifiers in IMF has been reviewed in detail by McSweeney (2008). While dairy proteins are capable of emulsifying fat in IMF to an acceptable degree (McCarthy et al., 2012) it is permitted to add non-protein emulsifiers to aid emulsification. Non-protein emulsifiers are low weight molecular weight surfactants which are classified as food additives and their use in IMF is regulated. Addition of lecithin and mono-/di-glycerides is permitted in the manufacture of IMF manufactured from intact dairy proteins. In IMF for special purposes, emulsification of fat may be more challenging due to, for example, extensive hydrolysis of protein; a more extensive list of surfactants are allowed in these products (McSweeney, 2008)

Table 1.3. Regulatory limits for minerals and vitamins in IMF

	unit	Minimum	Maximum
Calcium	mg 100 ⁻¹ mL	12	33
Phosphorous	mg 100 ⁻¹ mL	6	22
Magnesium	mg 100 ⁻¹ mL	1.2	2.6
Sodium	mg 100 ⁻¹ mL	5	14
Chloride	mg 100 ⁻¹ mL	12	38
Potassium	mg 100 ⁻¹ mL	15	38
Iron	mg 100 ⁻¹ mL	0.07	0.3
Copper	µg 100 ⁻¹ mL	8.4	25
Zinc	mg 100 ⁻¹ mL	0.12	0.36
Manganese	µg 100 ⁻¹ mL	0.25	25
Fluoride	µg 100 ⁻¹ mL	0	25
Iodine	µg 100 ⁻¹ mL	2.5	12
Selenium	µg 100 ⁻¹ mL	0.25	2.2
Vitamin A	µg 100 ⁻¹ mL	14	43
Vitamin D	µg 100 ⁻¹ mL	0.25	0.65
Vitamin E	mg 100 ⁻¹ mL	**	1.2
Vitamin K	µg 100 ⁻¹ mL	1	6
Vitamin B ₁	µg 100 ⁻¹ mL	14	72
Vitamin B ₂	µg 100 ⁻¹ mL	19	95
Vitamin B ₃	µg 100 ⁻¹ mL	72	375
Vitamin B ₅	µg 100 ⁻¹ mL	94	475
Vitamin B ₆	µg 100 ⁻¹ mL	9	42
Vitamin B ₁₂	µg 100 ⁻¹ mL	0.025	0.12
Vitamin C	mg 100 ⁻¹ mL	2.5	7.5
Folic acid	µg 100 ⁻¹ mL	2.5	12
Biotin	µg 100 ⁻¹ mL	0.4	1.8

Source: European Commission 2006; ** 0.5 mg g⁻¹ of PUFA expressed as linoleic acid

1.2.9 Infant formula for special purposes

IMF made with intact proteins derived from bovine milk is not suitable for every infant. Bovine milk proteins account for 10-40% of all food allergies (Miciński et al., 2013). The major ways of reducing the allergenic effects of bovine milk protein are:

- Hydrolysis – In hydrolysed IMF, allergenic proteins are converted into peptides of lower molecular weight by enzymatic action. The hypoallergenic effects

depend on degree of hydrolysis. IMF can be extensively hydrolysed or partially hydrolysed. Extensively hydrolysed formulae contain peptides derived from hydrolysis of molecular weight below 5000 Da. Partially hydrolysed formulae contain peptides of 8000 to 20000 Da as well as intact proteins. Extensively hydrolysed formulae have been shown to be not quite hypoallergenic due residual levels of antigenicity. Partially hydrolysed formulae are less effective, claiming a reduced allergenic affect (Maldonado et al., 1998).

- Substitution – Soy protein is the only alternative source of protein in IMF mentioned in regulations (European Commission, 2006; Codex Alimentarius, 2007). Regulation stipulates that the minimum protein content of soy-based formulae should be higher ($2.25 \text{ g } 100 \text{ kcal}^{-1}$) than that of bovine milk-based formulae ($1.8 \text{ g } 100 \text{ kcal}^{-1}$); in addition, it is necessary to supplement soy based formulae with methionine, carnitine, copper, zinc, iron and calcium (Maldonado et al., 1998). IMF made with other protein sources, such as rice protein, can be considered (Piacentini et al., 2003) but only on a case-by-case basis, as published data on the use of these protein sources is limited (Koletzko et al., 2005).

Lactose-free formulae are also available; IMF containing a mixture of corn-syrup and sucrose as the carbohydrate source was described by Heubi et al. (2000). IMF thickened using pre-gelatinised cornstarch has been shown to be effective in treatment of reflux (Xinias et al., 2005). Regulation limits the concentration of pre-gelatinised or cooked starch to below $2 \text{ g } 100^{-1} \text{ mL}$ or 30% of the total carbohydrate (European Commission, 2006).

1.3 IMF manufacture overview

Powdered IMF, in general, is produced by three types of manufacturing processes (McSweeney, 2008): (1) wet-mixing, (2) dry blending or (3) a combination of wet processing and dry-blending. In wet-mixing, ingredients are hydrated in water to the desired composition and are subsequently spray dried, whereas dry blending involves mixing powdered ingredients.

IMF is prone to microbiological spoilage and therefore the primary objective in any manufacturing process is to ensure the microbial safety of the final product (D'Agostina et al., 2003; Mullane et al., 2007). *Cronobacter sakazakii* (previously *Enterobacter sakazakii*) and *Salmonella* are dangerous microorganisms which must be

absent from IMF. *C. sakazakii* is associated with meningitis, necrotising enterocolitis and bacteraemia in infants (Mullane et al., 2006; Mullane et al., 2007). A disadvantage of the dry blending process is that, as there is no heat treatment step, the microbiological quality of the final product will be dependent on raw material quality. In wet-blending processes, heat treatment is applied to formulations. Heat treatment is a critical control point in ensuring microbiological safety of IMF. Other advantages and disadvantages of wet and dry processing are summarised in Table 1.4.

Table 1.4. Advantages and disadvantages of wet and dry processing

	Advantages	Disadvantages
Wet processing	<ul style="list-style-type: none"> - Microbial quality of final product can be controlled by heat treatment - Oil can be incorporated - High degree of control over physical quality of final product (i.e. wettability, solubility) - Homogenous product 	<ul style="list-style-type: none"> - Higher cost - More maintenance - Both wet and dry areas exists in one manufacturing facility
Dry processing	<ul style="list-style-type: none"> - No water in processing area easier to control bacterial growth - Lower energy - Less equipment 	<ul style="list-style-type: none"> - Microbial quality of final product dependant on raw materials - Physical quality of final product dependant on raw materials - Oil cannot be incorporated (must be pre-encapsulated) - De-blending of ingredients during transport

Sources: US Food and Drug Administration, 2003; Montagne et al, 2009

Figure 1.1 shows a generalised wet process for the manufacture of powdered IMF. The specific processes used in the manufacture of IMF vary between manufacturers; however, the process can be broken into non-specific stages. To begin with, powdered ingredients are hydrated in skim milk or water to give the desired macro-composition (see Section 1.3.1). Heat sensitive micro-ingredients, such as minerals and vitamins, may be added at a later stage to avoid thermal processing. Oils are incorporated into the wet-mix which is then homogenised to prevent subsequent separation of oil (see Section 1.3.2). Wet-mixes are heat treated to ensure microbial stability (see Section 1.3.3). Finally, it is

sometimes required to concentrate the wet-mix by evaporation (see Section 1.3.4) before powder is produced by spray drying (see Section 1.3.5).

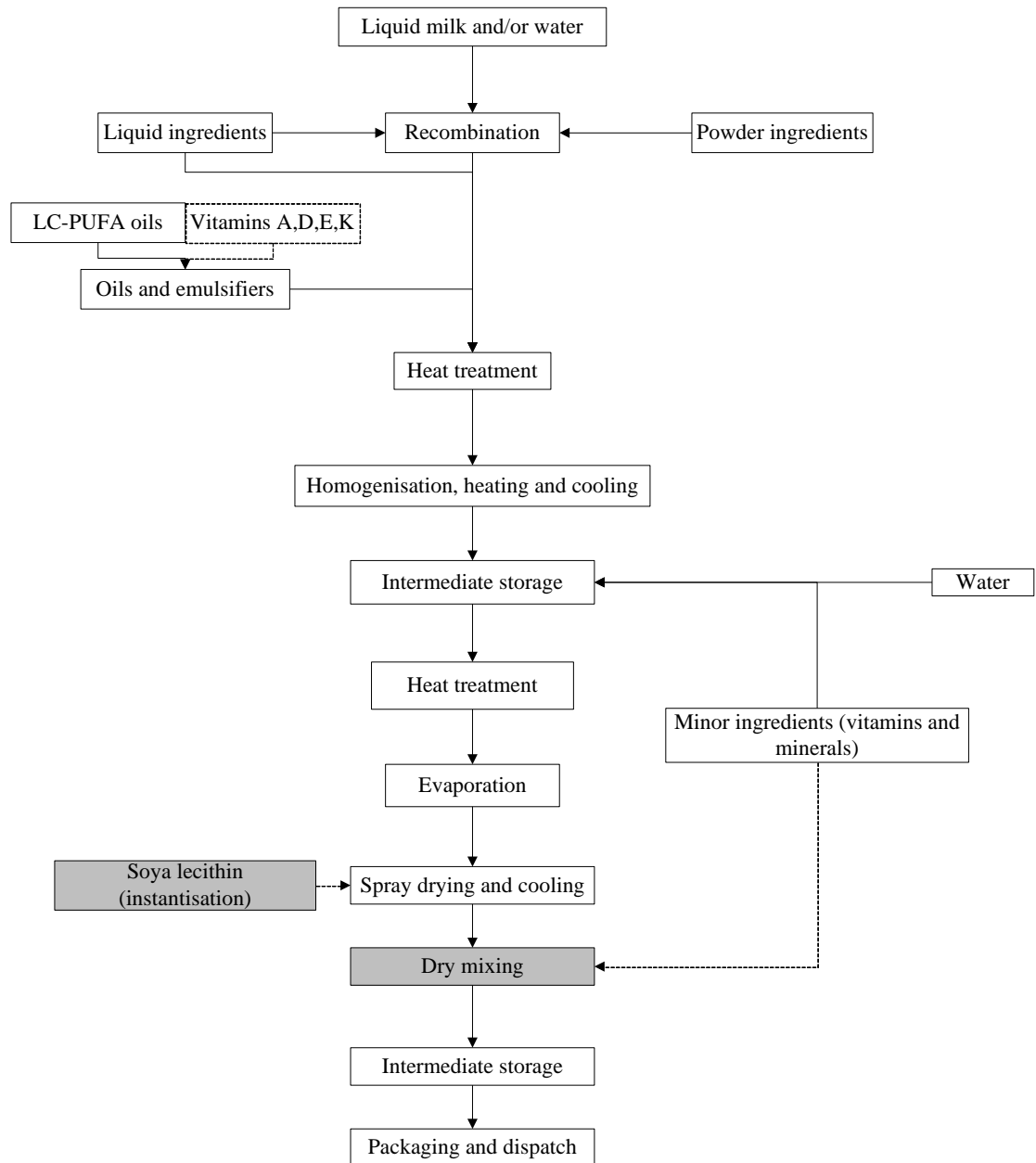


Figure 1.1. Generalised powdered IMF and FO-IMF manufacturing process. Note: grey fill indicates alternative processing (Sorensen et al., 1992; Montagne et al., 2009)

1.3.1 Rehydration of raw materials

Raw materials

Table 1.5 shows the raw materials commonly used in IMF. The quality and pre-treatment of ingredients will influence the physical properties of IMF wet-mixes. Skim milk powder (SMP) is available in three qualities: low, medium and high heat skim. The classification is dependent on the severity of heat treatment undergone during manufacture (Martin et al., 2007). The classification is also descriptive of the extent of denaturation undergone by whey proteins during manufacture. Functional properties of low, medium and high heat SMP are shown in Table 1.6. For fresh skim milk heat treatment has been found to result in higher viscosities after evaporation compared to non-heat treated fresh skim milk (Bienvenue et al., 2003).

Table 1.5. Typical raw material for IMF

Casein Source	Skim milk powder (SMP), evaporated skim milk, acid casein, sodium/calcium/potassium caseinate, milk protein concentrate (MPC*)
Whey Source	Demineralised whey powder (DWP), whey protein concentrates (WPC*), whey protein isolates (WPI), hydrolysed whey ingredients
Alternative protein source	Soy milk, soy protein isolate, locust bean seed protein, amino acids
Oils	Soy, corn, safflower, sunflower, colza, palm, copra, structured lipids
Carbohydrates	Lactose, starch, sucrose, corn syrup, corn syrup solids
Major minerals	Calcium carbonate, calcium phosphates, dibasic magnesium phosphate, potassium citrate, magnesium chloride
Minor minerals	Potassium iodide, ferrous sulphate, manganese sulphate, copper sulphate, zinc sulphate
Vitamins	A, D, E, K, B1, B2, B6, B12, niacin, folic acid, pantothenic acid, biotine, choline, inositol
Functional Ingredients	Soy lecithin, mono- and di-glycerides

*MPC and WPC can have varying protein content e.g. MPC 80 = 80% protein; WPC35 = 35% protein

Processing of whey protein products during production affects their functional properties. Native whey protein content in commercially available WPC has been shown to vary between 35 and 91% (de Wit et al., 1983). Solubility of whey products (which affects emulsification and gelation properties) is proportional to native whey content (Morr, 1979; de Wit, 1984). Acid whey products made by precipitation of casein under acidic conditions do not contain CMP, and could have different functionality to CMP-containing cheese-whey products; CMP is much more heat stable than α -La and β -Lg (O'Loughlin et al., 2012).

Table 1.6. Influence of heat treatment on functional properties of skim milk powders

Classification	WPNI	Typical heat treatment	Functional properties
Low heat	> 6.0	70 °C for 15 s	Solubility, lack of cooked flavour
Medium heat	4.51-5.99	85 °C for 60 s or 90-105 °C for 30s	Emulsification, foaming, water absorption, viscosity
High heat	<1.5	90 °C for 5 min or 120 °C for 1-2 min	Heat stability, water binding, gelation, water absorption

Source: Kelly et al., 2003; WPNI: Whey protein nitrogen index (g undenatured WPN·g of powder⁻¹)

Hydration of raw materials

The hydration of powders is typically considered to comprise of several stages: wetting, sinking, dispersion and dissolution (Simatos et al., 2009). High shear devices can be used to disperse raw materials to reduce hydration time (McCarthy et al., 2012; McCarthy et al., 2013). Temperature of dispersion is also critical to ensuring adequate hydration at a commercial scale; wettability increases as temperature is increased from 10 °C to 50 °C (Bylund, 1995a). Lactose content of raw materials also plays an important role in their hydration. It has been demonstrated that lactose plays an important role in preserving the native structure of proteins during drying (Allison et al., 1999; Baldwin, 2010). In both casein dominant powders (skim milk with adulterated lactose content) and whey dominant powders (whey and WPC35) increased lactose content has a positive effect on the hydration of powders (Baldwin and Woodhams, 1974; Lamiot et al., 1998). Therefore, high lactose content powders such as SMP and DWP, in contrast to MPC80 and WPC80, can give favourable hydration properties during wet-mixing of IMF.

Lactose is a relatively insoluble sugar; at 25 °C its solubility is 21.6 g lactose 100 g⁻¹ water in comparison to approximately 200 g sucrose 100 g⁻¹ water. As a result, during wet-mixing of IMF there exists a critical dry matter concentration at which lactose is no longer soluble. Figure 1.2 shows, that at 40% (w/w) dry matter, an IMF consisting of 59% (w/w) lactose on a dry basis, the temperature required for solubilising lactose is greater than 45 °C.

Viscosity of wet mix

The viscosity of the wet-mix prior to heat treatment and homogenisation depends largely on DM content and composition; Snoeren et al.(1982) showed apparent viscosity of concentrated skim milk was dependant on the volume fraction of dispersed solid material. Casein was experimentally determined to be the more voluminous than native whey protein, $3.57 \times 10^{-3} \text{ m}^3 \text{ kg}^{-1}$ compared to $1.07 \times 10^{-3} \text{ m}^3 \text{ kg}^{-1}$. Denatured whey proteins, however, were determined to have a voluminosity of $3.09 \times 10^{-3} \text{ m}^3 \text{ kg}^{-1}$; therefore, the pre-treatment of whey ingredients will also have an impact on the viscosity of infant formulae wet-mixes. For IMF containing glucose polymers, DE value could affect viscosity of wet-mixes; Dokic et al. (1998) found that solution viscosity decreased with increasing DE value.

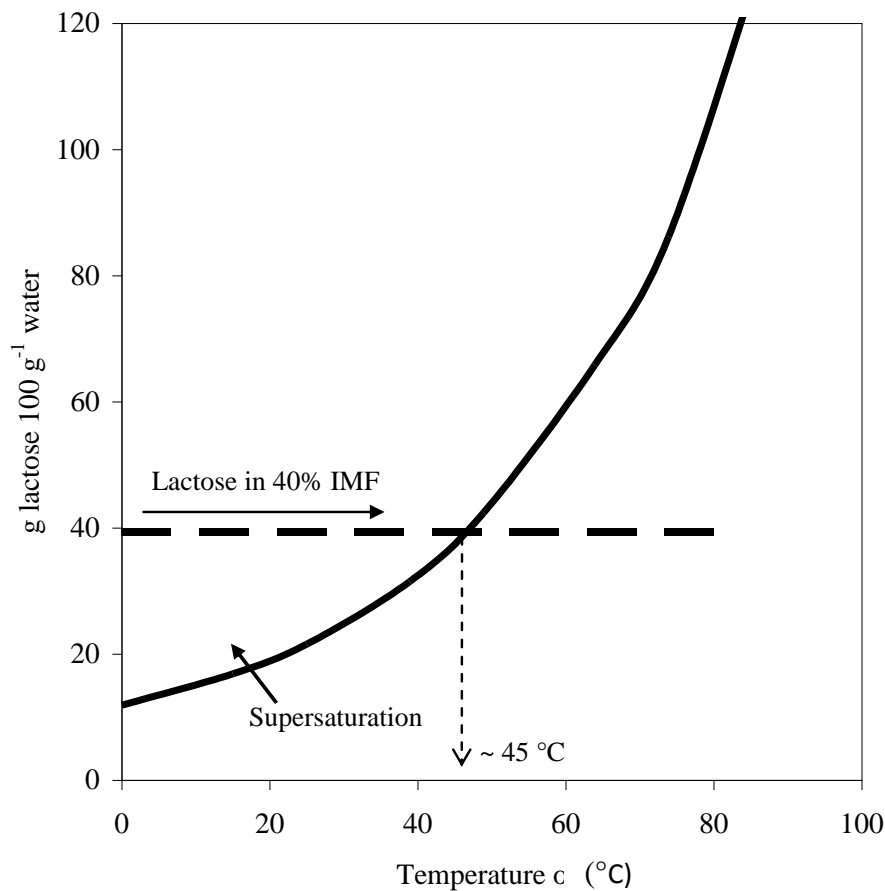


Figure 1.2. Solubility of lactose in a 40% DM IMF wet mix. The solid trace represents lactose solubility. The dashed trace represents the concentration of lactose in a 40% DM IMF

1.3.2 Homogenisation and emulsification

Stabilisation of fat against separation is an essential element during wet-mixing of IMF. Proteins are amphiphilic molecules containing both hydrophobic and hydrophilic regions and are largely responsible for emulsification in IMF. During homogenisation, fat is disrupted into small globules and is stabilised by proteins which absorb to at the fat and water interface. Absorbed proteins have hydrophobic regions oriented towards the fat phase and hydrophilic regions oriented towards the aqueous phase. In addition IMF, particularly IMF made with hydrolysed proteins, may also contain low weight molecular weight surfactants which act as emulsifying aids. Similar to proteins, surfactants contain hydrophobic regions which absorb to the interface between fat and water and hydrophilic regions which are in contact with the aqueous phase. Emulsification of fat by milk

proteins is reviewed Singh (2011); emulsification in IMF products is reviewed in detail by McSweeney (2008)

Homogenisation

Homogenisation of dairy products is generally achieved by high-pressure valve-type homogenisation (Bylund, 1995b). This unit operation consists of a high pressure pump which forces product through a valve. The homogenising pressure is the pressure required to open the valve (Caric, 1994). In many cases, homogenisation is carried out in two stages; pressure is higher in the first stage (e.g. 14 MPa) followed by a lower second stage pressure (e.g. 3 MPa) to prevent and disrupt formation of fat globule clusters (McCarthy et al., 2012). The main process factors effecting homogenisation are; pressure, temperature, flow rate and fat content (Phipps, 1985). Greater homogenisation pressure results in smaller fat globules. Goulden and Phipps (1964) found that for bovine milk and creams, average fat globule diameters increased with fat content; at higher fat contents (> 12% w/w fat) elevated temperatures and flow rates reduced fat globule size.

Compositional factors affecting emulsification

The ratio of whey proteins to caseins (W: C) present in a wet-mix plays an important role in emulsion formation and stability. Sourdet et al. (2002) found that emulsions (9% w/w palm kernel oil; 3% w/w protein) prepared with SMP (W: C = 20: 80) and SMP/WPI mixtures (W: C = 60: 40) had more protein absorbed at the oil-water interface compared to similar emulsions prepared with WPI (W: C = 100: 0). In emulsions made with SMP/WPI mixtures casein was absorbed preferentially at the oil water interface; a similar observation was reported by McCarthy et al. (2012) for reconstituted IMF emulsions (W: C = 60: 40). Emulsions made with WPI alone were more flocculated than casein containing emulsions. These flocculated fat droplets were disrupted by addition of SDS suggesting hydrophobic interactions were responsible for flocculation. It was postulated the presence of casein at the oil-water interface increased steric repulsion between fat globules and prevented flocculation. Granger et al. (2005) also reported that emulsions (8% w/w fat) made with SMP underwent far less hydrophobic flocculation compared to emulsions made with a mixture of whey proteins and caseins (W: C = 60: 40). In emulsions made with varying ratios of unheated WPI and sodium caseinate (30% w/w soya oil; 1% w/w protein) protein absorbed at the oil-water

interface was also higher at higher casein contents. Creaming over a storage period of 4 months was greatest in emulsions made from sodium caseinate alone (Britten and Giroux, 1991).

Different sources and types of whey proteins and caseins are available for IMF manufacture (see Table 1.5); the processes employed to isolate different components from bovine milk can influence functional properties, including emulsification. The extent of whey protein denaturation, for example, can affect emulsification. The effect of denaturation on emulsifying properties of whey proteins reported in the literature is variable. Millqvist-Fureby et al. (2001) found emulsification, as indicated by fat globule size (3% w/w rapeseed oil; 3% w/w protein) was reduced as the level of denaturation in whey proteins increased. Voutsinas et al. (1983) showed that emulsification of whey proteins was not substantially changed by heating. However, in the same study, the emulsification activity of a β -Lg isolate was found to decrease after heating. The source of whey protein could also have an affect; whey obtained from cheese manufacture can contain significant quantities of CMP which has lesser emulsifying properties than whey proteins (Martin-Diana et al., 2005). To increase similarities between IMF and human milk, α -La enriched protein ingredients are used in IMF; this may reduce the emulsification efficiency as α -La is less surface active than β -Lg (Yamauchi et al., 1980; McSweeney, 2008). Similarly, casein type can also effect emulsification; sodium caseinate has been shown to be more surface active and produce finer emulsions compared to casein micelles (Courthaudon et al., 1999)

The emulsifying properties of milk protein ingredients become altered by hydrolysis. Contradictory reports exist in the literature regarding the effect hydrolysis has on emulsifying properties. Chobert et al. (1988) found that emulsifying properties of whey proteins were enhanced by a limited degree of tryptic hydrolysis. In contrast, Agboola and Dalgleish (1996) found that fat globule size of emulsions stabilised with hydrolysed whey proteins increased with degree of hydrolysis. Similarly, for casein, some researchers have found positive effects of hydrolysis on emulsifying properties (Haque and Mozaffar, 1992) while others have found negative effects (Chobert et al., 1988; Agboola and Dalgleish, 1996). Differences in degree of hydrolysis (DH), molecular weight of peptides and peptide amphiphilicity could account for these contradictory findings. The effect of hydrolysis on emulsification is reviewed in more detail by McSweeney (McSweeney, 2008).

1.3.3 Heat treatment

Heat treatment is a critical control point in ensuring microbiological quality of IMF. The positioning of heat treatment within the process is flexible, as indicated in Figure 1.1. It may be beneficial to position the heat treatment prior to homogenisation in order to produce a more stable emulsion (Varnam and Sutherland, 1994) as aggregates of fat globules produced during heat treatment (McSweeney et al., 2004) could be disrupted by homogenisation. Heat treatment was carried prior to homogenisation in the work of McCarthy et al. (2012; 2013).

Direct vs. indirect heat treatment

Two general types of heat treatment exist in the dairy industry; indirect – heat is transferred across a physical barrier which separates product from heating medium (e.g. plate or tubular heat exchangers) and; direct – product and heating medium come into direct contact (e.g. steam injection or infusion). Direct heat treatments have a lower associated heat load than indirect treatments (Lewis and Deeth, 2009). Direct heat treatments can achieve ultra-high temperature treatments in as little as 1 s, compared to in excess of 10 s for indirect treatments. As a result, denaturation of β -Lg and α -La resulting from direct UHT is lower than from indirect UHT (Tran et al., 2008). Direct steam injection has an homogenising affect (Zadow, 1969; Datta et al., 2002) which could disrupt formation of aggregated fat globules during heat treatment (McSweeney et al., 2004). Adamopoulos and Petropakis (1999) described direct steam injectors where a two-phase mixture of steam and liquid is accelerated to supersonic velocity as a result of the injector's geometry; this produces cavitation and sharp changes in shear rate within the injector which result in homogenisation of fat particles within the mixture.

Heat stability of whey proteins

β -Lg, as the main whey protein in bovine milk, can play a particularly important role in determining physical characteristics of IMF during heating. β -Lg's sensitivity to heat is due, largely, to the presence of a free sulphydryl (thiol) group (Papiz et al., 1986; Brownlow et al., 1997). During heat treatment, denatured and unfolded β -Lg can participate in intermolecular reactions via thiol-disulphide interchanges which can lead to aggregation of β -Lg molecules with adjacent β -Lg molecules (Manderson et al., 1998;

Galani and Apenten, 1999), other whey proteins (Schokker et al., 2000) and casein micelles (Fox and Morrissey, 1977). However, it should be noted that not all aggregation of β -Lg is caused by thiol-disulphide interchanges; hydrophobic interactions also contribute to aggregation (Manderson et al., 1998; Galani and Apenten, 1999).

α -La is stabilised by four disulphide bonds and association with Ca^{2+} . α -La does not contain a free thiol group to serve as a starting point for covalent aggregation (Brew, 2003). As a result, denaturation of pure α -La has been observed to be reversible, and gel formation upon heating is far reduced in comparison to β -Lg (Boye et al., 1997). In systems containing β -Lg and α -La, aggregates are formed both by thiol-disulphide interchanges and by hydrophobic interactions (Dalglish et al., 1997; Schokker et al., 2000).

CMP consists of a hydrophilic region of the κ -casein which is separated from the intact protein during manufacture of cheese or rennet casein. CMP contains no sulphhydryl groups (Fox and McSweeney, 1998a). As a result CMP does not undergo physical changes due to heat treatment. O'Loughlin et al. (2012) found the CMP fraction of WPI to be resistant to heat treatments of up to 80 °C for 10 min, especially when compared to the β -Lg A, β -Lg B and α -La fractions which were reduced by 77, 65 and 64%, respectively. At neutral pH, gelation does not occur in CMP solutions at concentrations of up to 40% (w/w) (Martinez et al., 2010).

Heat stability of casein - interactions with whey proteins

Caseins have relatively little higher structure and are heat-stable in comparison to whey proteins (Donella-Deana et al., 1985; Swaisgood, 2003). Sodium caseinate can be held at 140 °C for more than 1 h without visible changes in physicochemical properties (Fox and McSweeney, 1998b). Caseins, however, do interact with whey proteins during heat-treatment. The heat stability of skim milk, for example, is linked to cysteine residues in both β -Lg, the most abundant whey protein, and κ -casein, located at the surface of the casein micelles. When mixtures of β -Lg and κ -casein are heated they aggregate through the formation disulphide bonds and/or hydrophobic interactions (Guyomarc'h et al., 2009). Jeurink and De Kruif (1993) found that unfolding of β -Lg and subsequent association with casein micelles was responsible for increasing the viscosity of skim milk after heating at 85 °C. When skim milk is heated, depending on the pH, the formation of

complexes of β -Lg and κ -casein can either have a favourable or detrimental effect on heat stability (Tessier and Rose, 1964). At $\text{pH} < 6.9$, β -Lg complexes with κ -casein attached to the casein micelle, stabilising the micelle against precipitation. At $\text{pH} > 6.9$, κ -casein dissociates from the casein micelle to form the complex in the serum phase, resulting in destabilisation of the casein micelle (Singh and Fox, 1987). Similarly, interactions between casein and whey in IMF systems have been shown to be pH dependant. At $\text{pH} 6.5 - 6.8$, β -Lg in a dispersion of SMP and electro dialysed whey (whey 60%; casein 40%) was found to co-sediment with caseins after heating ($140\text{ }^{\circ}\text{C}$ for 80 s); at $\text{pH} 6.9 - 7.1$ β -Lg was found in the supernatant after sedimentation (McSweeney et al., 2004).

Interactions of proteins in emulsified systems

Heat treatment of IMF emulsions has been shown to result in an increase in fat globule size. When heated at $140\text{ }^{\circ}\text{C}$ for 80 s the fat globule size of a $\text{pH} 6.8$ IMF emulsion increased from below $1\text{ }\mu\text{m}$ before heating to $1-10\text{ }\mu\text{m}$ after heating (McSweeney et al., 2004). Despite containing 40% casein (of total protein) the mechanism attributed to the increase was postulated to be the same as presented by Euston et al. (2000) for increases in fat globule size after heating of whey-stabilised emulsions (See Figure 1.3). When protein-stabilised emulsions are heated, interactions occur between proteins adsorbed at the fat-liquid interface and serum proteins, resulting in the formation fat globule aggregates. Flocculation of fat globules is undesirable as it has a detrimental effect on the creaming stability of emulsions (Keowmaneechai and McClements, 2006).

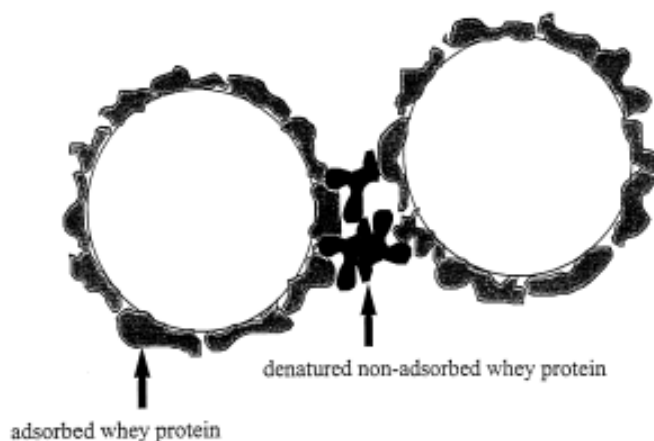


Figure 1.3. Proposed mechanism for aggregation of fat globules during heat treatment of whey stabilised emulsions (Euston et al., 2000). The mechanism was also proposed for IMF systems (whey 60%; casein 40%) (McSweeney et al., 2004)

Solvent affects – minerals and lactose

The continuous phase in which proteins and fat globules are dispersed affects heat stability. Dissolved minerals have a big effect on the stability of dispersions and emulsions. Increasing the ionic strength of the continuous phase reduces electrostatic repulsion between dispersed particles (Bryant and McClements, 1998). Casein and denatured whey proteins can be precipitated from dispersion by any of several salts (Fox and McSweeney, 1998a). Divalent cations Ca^{2+} and Mg^{2+} decrease denaturation temperature of β -Lg and increase extent of denaturation and aggregation in whey proteins heated at neutral pH (Varunatian et al., 1983). Divalent cations are often chelated with citrate and/or added in insoluble form to avoid formation of coagulants during heat treatment of IMF (Montagne et al., 2009).

Sugars have an inhibitory effect on whey protein denaturation. Sucrose and lactose, have been shown to increase whey protein denaturation temperature and thermal coagulation time of whey proteins (Garrett et al., 1986; Kulmyrzaev et al., 2000). Interactions between sugars (and some other co-solvents) and the backbone of whey proteins are thermodynamically unfavourable (Liu and Bolen, 1995). As a result sugars are preferentially excluded from the vicinity of the protein; equivalently, proteins in these systems are termed as preferentially hydrated (Timasheff, 1993). Thus, when heat is applied to a preferentially hydrated protein, any potential interaction with a preferentially excluded co-solvent is unfavourable and will result in the protein retaining its compact native structure resulting in an increase T_d i.e. a state where the contact surface area is

minimised will prevail to higher temperatures. In contrast, lactose was found to have a negative effect on the heat stability of model IMF emulsions heated at 140 °C, presumably as a result of enhanced acid formation caused by degradation of lactose which reduced the time taken for coagulation (McSweeney et al., 2004). It should be noted that excessive acid formation is not expected in IMF under less severe heat treatments.

Effect of increasing concentration during heat treatment

Studies documenting the effect of increasing dry matter content on the denaturation of whey protein are summarised in Table 1.7. The main systems studied have been skim milk and cheese whey, with considerable variation in findings, perhaps dependant on the system and or conditions of heating. The most commonly reported finding was that whey protein, or β -Lg, denaturation decreased with increasing concentration (McKenna and O'Sullivan, 1971; Hillier et al., 1979; Anema, 2000). Anema (2000), however, reported the effect of concentration was reduced as temperature increased and was negligible at 100 °C. It was proposed that the thermodynamic favourability of reducing interaction between whey protein and lactose could explain these observations. The increased presence of lactose at high DM content may have prevented the transition of β -Lg from dimeric to monomeric form, which was suggested to be rate determining at < 90 °C. At >90 °C, aggregation is the rate determining step of β -Lg denaturation in milk; lactose was less effective in stabilising against these interactions, and hence, increased dry matter content was not effective in preventing denaturation at elevated temperatures. The above explanation was consistent with the observation that increased lactose and dry matter content was found not to effect α -La denaturation (Anema, 2001); α -La is a monomeric species, therefore no rate-determining shift in molecular association occurs. Oldfield (1996) found that increased dry matter content resulted in increased denaturation of whey proteins at 110 °C, suggesting that at temperatures approaching UHT, increased dry matter content has the opposite effect compared to < 90 °C. This is in contrast with Hillier et al. (1979) who found that increasing dry matter content reduced β -Lg denaturation and increased α -La denaturation at > 100 °C. The methods of heat treatment between the studies were quite different, the former employing a dynamic direct contact heat treatment on skim milk, the latter

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employing a static treatment of unknown duration on cheese whey. These differences likely account for the contrasting results; the same may be said for the older studies mentioned in Table 1.7 i.e., Hartland et al. (1952) and Guy et al. (1967).

Table 1.7. Summary of studies on effect of increasing dry matter content in skim milks and cheese whey

Study	Year	Protein/systems	Conc.	Heating methods and temperatures	Findings
Harland et al.	1952	Whey in skim milk	9 - 36 (% w/w)	Not available. Data from Anema (2000)	Concentration had little effect on whey protein denaturation
Guy et al.	1967	Whey in cottage cheese whey	5 - 40 (% w/w)	Heated at 87, 84.5 and 74 °C in water bath for up to 30 min	Whey protein denaturation was at a minimum at 20% w/w
McKenna and O' Sullivan	1971	Whey in skim milk	9 - 44 (% w/w)	Heated to 75 and 80 °C in test tubes for from 5 to 20 min	Whey protein denaturation decreased with concentration
Hillier et al.	1979	α -La and β -Lg in cheese whey	1.9 - 11.4 (mg protein/mL)	Small volumes sealed in capillaries and heated from 70 to 130 °C. No heating times mentioned	Denaturation of α -Lac increased with concentration; Denaturation of β -Lg decreased with concentration
Oldfield	1996	α -La and β -Lg in skim milk	6 - 13 (% w/w)	Heated to 110 °C in a direct steam injection plant	Whey protein denaturation increased with concentration
Anema	2000	β -Lg in skim milk	9.6 - 38.4 (% w/w)	75 to 100 °C in sealed plastic tubes in water bath for up to 15 min	β -Lg denaturation decreased with increasing concentration; effect of concentration decreased with increased heating temperatures - at 100 °C there was no affect
Anema	2001	α -La in skim milk	9.6 - 38.4 (% w/w)	75 to 100 °C in sealed plastic tubes in water bath for up to 15 min	α -La denaturation was not affected by concentration

Effect of increasing concentration on thermal properties

Increasing DM content also changes the thermal properties of milk which will affect energy required for heat treatment. The specific heat capacity (C_p ; $\text{kJ kg}^{-1} \text{K}^{-1}$) will increase with increasing concentration due to relatively larger amount of energy required to break hydrogen bonds in water (Edsall and Wyman, 1962; Fernandez-Martin, 1971).

Thermal conductivity (k ; $\text{W m}^{-1} \text{K}^{-1}$) of milk also decreases with increasing dry matter content (More and Prasad, 1988). These thermal parameters affect heat transfer coefficients in forced convection such as found in tubular and plate heat exchangers and are thus very important to heat exchanger design (Coulson and Richardson, 1963).

1.3.4 Evaporation

Evaporation of IMF wet-mixes is often employed to remove water prior to spray drying (Figure 1.1). Water can be removed by evaporation with lower energy costs compared to spray drying (Fox et al., 2010). Falling film evaporators are commonly employed by the dairy industry. During falling film evaporation, the wet-mix flows by gravity through a number of tubes, forming a film on the inside of each tube as it flows downwards. Live steam is applied to the outside of the tubes causing evaporation of water from the wet-mix. Evaporation is carried out under vacuum (~ 50 to 70 °C) which allows for the evaporation of heat sensitive wet-mixes. The extent of concentration achievable is determined by viscosity increase; in order for efficient drying to subsequently take place, post-evaporation viscosity of whole milk should not exceed $60 - 100 \text{ mPa s}$ (Westergaard, 2004). In addition to changes in viscosity, evaporation can affect the physical state of wet-mix constituents. Liu et al. (2012) reported that concentration of skim milk affected micellar hydration, aggregation and the amount of calcium associated with the micelle. Transfer of calcium phosphate from soluble to colloidal micellar state reduces pH during evaporation (Singh, 2007). McCarthy et al. (2012) observed an increase in fat globule size of IMF emulsions during evaporation.

1.3.5 Spray drying

IMF powders are produced by spray drying. Hot air is used to remove water from wet-mixes which have been atomised into fine droplets ($10-400 \mu\text{m}$) to increase the area of contact with the hot air (Westergaard, 2004). IMF powders are generally produced using two or three stage spray dryers. Two or three stage spray drying consists of a large drying chamber (stage 1) in which the bulk of water is removed, followed by supplementary drying using an internal fluidised bed (stage 2, located at the bottom of the drying chamber) and/or external fluidised bed (stage 3). Table 1.8 shows the range of temperatures employed in spray drying IMF. Agglomeration of powder particles is

employed to enhance wettability and flowability of IMF. Spontaneous agglomeration of powder particles will occur from random collisions of atomised particles during drying. Forced primary agglomeration consists of positioning two or more atomising devices so that the atomised particles from each overlap. In forced secondary agglomeration fine particles which exit in the exhaust air of the dryer and external fluid bed are re-introduced to the drying chamber. More information on atomisation, agglomeration and spray drying of dairy and IMF systems can be found in the reviews of Kelly et al. (2003), Skanderby et al. (2009) and Montagne et al. (2009).

Table 1.8. Typical air temperatures used during spray drying of IMF

Location	Temperature (°C)
Drying chamber inlet	180-200
Internal fluidised bed	50-60
External fluidised bed	20-30
Exhaust air	80-100

Source: (Montagne et al., 2009)

Protein changes during spray drying

The viscosity of pre-spray drying wet-mixes is a function of the composition of the wet mix and the various processing steps to which it has been subjected. Higher viscosity wet-mixes result in an increase in the size of droplets produced by atomisation (Hogan et al., 2001). Larger droplets result in slower drying (Vignolles et al., 2007) which can decrease the solubility of the resulting powders (Skanderby et al., 2009; Schuck et al., 2012a). Development of insolubility in milk powders during drying is generally associated with high powder particle temperature at moisture contents between 10 and 30% (Straatsma et al., 1999; Sharma et al., 2012). Some authors report that casein micelles are the main constituent of insoluble material in bovine milk powders (Baldwin and Truong, 2007; Baldwin, 2010). Casein micelles are highly hydrated (see Section 1.3.4), and during dehydration, micelles reconfigure. The re-ordering of the micelle structure increases hydrophobicity and leads to insolubility upon rehydration, however, the original structure of the micelle will be restored over time (Baldwin, 2010). Other authors suggest that association of β -Lg with casein plays a role in formation of insoluble particles (Straatsma et al., 1999; Sharma et al., 2012).

Spray drying has been found to have little effect on the denaturation of whey proteins in skim milk systems (Guyomarc'h et al., 2000; Oldfield et al., 2005). For much of the spray drying process, atomised droplet temperatures are lower than that of the inlet air, due to evaporative cooling, and may not exceed denaturation temperature until towards the end of the drying process. Towards the end of drying, the dried particle will approach the dryer outlet air temperature, but should not exceed 70 °C until almost all the water has been removed (Singh and Newstead, 1992). Whey content of IMF is much higher than bovine milk, so it follows that whey protein denaturation and aggregation could be more significant in the spray drying of IMF. Anandharamakrishnan et al. (2008) found that denaturation of whey proteins during the spray drying of WPC was significantly higher at air outlet temperatures in the region of 100-120 °C compared to 60-80 °C. Bernard et al. (2011) found insoluble aggregates in reconstituted spray-dried WPC (1% w/w solution; insoluble aggregates removed by centrifugation – 15,000 x g for 15 min) increased by ~8% (w/w) after spray drying with an inlet temperature of 260 °C. Whey protein denaturation and aggregation during spray drying can also affect emulsion properties. Sliwinski et al. (2003) studied the interfacial protein in spray dried emulsions (20% w/w soybean oil; 2.4% w/w protein) prepared from mixtures of sodium caseinate and WPI. Interfacial proteins in casein dominant emulsions were not affected by spray drying, however, in emulsions containing greater than 60% WPI (of total protein) caseins at the oil-water interface were displaced by whey proteins. Furthermore, when WPI content of emulsions exceeded 70% (of total protein) reconstituted fat globule sizes were significantly increased compared to casein dominant emulsions.

Free fat and powder surface composition

During spray drying, fat is liberated from protein stabilised fat droplets. Free fat has a large effect on physical and reconstitution properties of powders (see Section 1.4) and has a greater susceptibility to oxidation. Fat may become liberated due to collisions of fat droplets during water removal as suggested by Vignolles et al. (2010); it is also possible that displacement of caseins at the oil-water interface by whey proteins could liberate some free fat in IMF systems (Sliwinski et al., 2003). Emulsion quality prior to spray drying has a large influence on the free fat; un-homogenised emulsions have been found to have higher free fat content than corresponding homogenised emulsions (De Vilder et al., 1979; Vignolles et al., 2009). In WPI-stabilised emulsions, free fat was

observed to increase with fat and protein content but decrease with lactose content (Keogh and O'Kennedy, 1999).

In recent years, distribution of fat (and other constituents) within dairy powders has been studied in detail (Kim et al., 2005a; Gaiani et al., 2006; Vignolles et al., 2009; Vignolles et al., 2010). Fat supramolecular structure is defined as the location and state (free or globular) of fat within the powder particle (Vignolles et al., 2010). Fat (both globular and free) has been observed to be overrepresented (compared to bulk composition) at the surface of spray dried dairy powders (Kim et al., 2005a; Vignolles et al., 2009; Vignolles et al., 2010). Kim et al (2005a) showed flowability of dairy powders was increased after surface free fat was removed by a brief wash with petroleum ether. This indicates the importance of surface composition, and in particular surface free fat, in determining the physical properties of dairy powders. Other authors have studied effects of surface composition on powder properties such as wettability, and stability to oxidation (Keogh et al., 2001; Gaiani et al., 2006).

In addition to fat, protein has also been found to be overrepresented at dairy powder surfaces (Gaiani et al., 2006; Vignolles et al., 2010). The high surface composition of fat and protein was shown by Kim et al. (2003) to be related to solute/solvent segregation during drying. As water (the solvent) diffuses to the surface of the droplet to evaporate, solutes (lactose, minerals, protein, fat droplets, free fat) diffuse towards the centre of the particle. The diffusion of lactose and minerals towards the centre of the particle occurs at a quicker rate compared to protein and fat, thus accounting for overrepresentation of the latter constituents at surface. Formation and effect of free fat in dairy ingredients is reviewed in detail by Vignolles et al. (2007).

Importance of lactose and other carbohydrates during spray drying

Carbohydrate-protein mixtures are good encapsulating materials for fat and are commonly used in the dairy industry. Prior to drying, the emulsifying properties of proteins stabilise fat; during drying, carbohydrates form an amorphous glass which has reduced molecular mobility and restricts the movement of the encapsulated fat (Zhou and Roos, 2011). In addition, different sugars have been shown to protect protein from conformational changes during spray drying by forming hydrogen bonds with the protein, thus acting as water substitutes (Carpenter et al., 1993; Allison et al., 1999; Baldwin and Truong, 2007). Sugars also have a protective effect on denaturation and could reduce

aggregation of whey protein during drying. As a result it is likely that the presence of sugar leads to a more stable interface during spray drying and it has been shown that free fat in dairy powders is decreased at higher lactose: protein ratios (Young et al., 1993; Keogh and O'Kennedy, 1999).

As mentioned above, an advantage of amorphous glass matrices in spray dried particles is the limited mobility of encapsulated material through the matrix. However, under certain conditions (high temperature and/or moisture contents) amorphous glasses undergo a transition to a rubbery state where molecules are much more mobile. The temperature at which this transition occurs is called “glass transition temperature” (T_g). Subjecting powders to conditions where they are in excess of T_g is not desirable in terms of encapsulation, but can also cause stickiness during spray drying and crystallisation during storage of powders. Stickiness in powders occurs at a certain temperature above glass transition temperature ($T - T_g$) and is particularly important during spray drying, where sticky powders can reduce yield and in extreme cases block the spray dryer (Hogan and O'Callaghan, 2010; Mounsey et al., 2012). Crystallisation of lactose during storage of powder has been well documented and has detrimental effects on powder flow properties due to formation of solid bridges of crystalline material forming between powder particles (Fitzpatrick et al., 2004). Lactose crystal formation can also disrupt fat globules and cause free fat (McCarthy et al., 2013). Of the mono- and di-saccharides used in IMF, lactose has the highest T_g (101 °C) and fructose has the lowest (5 °C); sucrose has an intermediate T_g of 62 °C (Bhandari and Howes, 1999). Therefore, lactose would seem to be the best choice of mono- or disaccharide for ease-of-drying and limiting crystallisation during storage. For carbohydrates derived from hydrolysis of starch, T_g decreases with extent of hydrolysis i.e. as DE number increases (Bhandari and Howes, 1999).

1.4 Powder properties

Powder size and morphology

Powder particle size and morphology play an important role in determining functional properties of IMF such as flowability and rehydration properties. Nikolova et al. (2014) studied the effects of outlet air temperature and feed DM content on SMP size and morphology. It was found, in keeping with Hogan et al (2001) that median particle size increased with increasing DM content of feed to the spray dryer. Two complementary mechanisms are likely responsible for the increase; as DM content

increases less moisture is removed from the particle during drying – therefore less particle shrinkage occurs, and; feed viscosity is higher at increased DM contents leading to formation of coarser droplets during atomisation. It was also observed that when feed concentration was lower, particles were more spherical with smoother surfaces.

Agglomeration achieved by returning fine powder particles to the drying chamber increases powder particle size. The effectiveness of agglomeration is related to T_g of powder particles as increased stickiness will promote adhesion of particles (Turchiuli et al., 2011). Agglomeration has a large influence on the shape of particles; aggregation of roughly spherical primary particles results in more structured powder. Based on their appearance, agglomerates are descriptively termed “onion” “raspberry” or “grape” (Skanderby et al., 2009).

Pneumatic conveying is commonly used to transport IMF powders within manufacturing facilities. During transportation attrition as a result of contact-at-speed between agglomerates and transport lines can cause breakage of powder with detrimental effects on powder functionality. Resistance of particle to breakage by attrition was found to be related to protein content, with higher protein content powders being more resistant (Hanley, 2011).

Flowability

Flowability is a measure of how powder particles move with respect to each other. Powder flow properties are important in the manufacture, handling and packing of powders (Sharma et al., 2012). Powders with larger particles generally have better flow properties as the specific surface area of contact between particles is lower than for smaller particles (Ortega-Rivas, 2009). However, other factors can affect flowability which can sometimes lead to a weak correlation between flowability and particle size (Fitzpatrick et al., 2004). Surface composition of powder particles (see Section 1.3.5) can be especially important and high quantities of free fat at the powder surface can reduce flowability (Kim et al., 2005a); Yazdanpanah and Langrish (2011) reported that SMP particles with crystalline surfaces and amorphous cores had better flowability than skim milk with fully amorphous particles. Powder particle shape also plays a role in determining flow properties with more spherical particles resulting in better flow properties (Fu et al., 2012).

Rehydration

IMF powders should dissolve rapidly upon addition to water. Sharma et al. (2012) reviewed parameters important to rapid rehydration. Wettability, measures the ability of a powder to penetrate the surface of non-agitated water. Sinkability, measures the ability of powder to sink into the water. Dispersibility, measures the ability of a powder to break apart into individual particles when added to water under gentle agitation. Solubility, measures the amount of powder brought into solution. Agglomeration is generally believed to increase wettability and dispersibility; Neff et al. (1968) and Freudig et al. (1999) reported that agglomeration to a particle size of 200 and 400 nm, respectively, led to optimum rehydration properties in SMP. Recently, the work of Gaiani et al. has studied the factors affecting the various stages of rehydration (Gaiani et al., 2005; Gaiani et al., 2006). Gaiani et al. (2007) found that agglomeration reduced rehydration time of whey protein powder but increased rehydration time of casein powder. This finding was attributed to the different rate controlling steps i.e., wettability for whey protein and dispersibility for casein powders. Solubility of SMP decreased with increasing particles size (Straatsma et al., 1999); this was attributed to quicker drying in smaller particles and hence less conformational changes to protein as particles dry (see section 1.3.5). Similarly, Buma (1971) found that smaller whole milk powder particles had better solubility than larger particles, attributed to the greater area of contact between water and fine particles.

Surface composition of powder particles affects rehydration. Surface free fat reduces wettability by increasing the hydrophobicity of the surface and increasing the contact angle with the water (Vignolles et al., 2007). Kim et al (2002) found that wettability, in 3 out of 4 powders, greatly increased after surface free fat was removed by a brief wash with petroleum ether; wettability of WPC was not affected and this was attributed to the high surface protein content (> 90%) after washing. Several other studies have reported similar, negative effects of surface free fat on wettability. Kim et al. (2005b) suggested that the extent of this negative effect on wettability was related to the melting point of the surface free fat, which may be different to that of total fat. Buma (1971) studied the effect of free fat in whole milk powder on dispersibility and solubility. While some correlations were observed it was not possible to determine definite relationships. For example, fine particles collected from the pilot spray dryer cyclone

were much higher in free fat and smaller in size compared to main portion of whole milk powder collected. Solubility was higher in fine particles, most likely due to small particle size, not high free fat.

1.5 Conclusion

Interaction between process and composition of dairy systems has a large effect in determining the physical characteristics of concentrates and resulting powders. The literature reviewed in this chapter deals mostly with unmodified milk systems or isolated ingredients. However, the physical properties of globular whey proteins and micellar casein, in combination with the processing, can be expected to greatly influence the physical state of IMF and FO-IMF wet mixes. The upcoming chapters study the effects of processing and composition in systems pertinent to IMF manufacture.



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Chapter 3: Decoupling macronutrient interactions during heating of model infant milk formulae

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Decoupling Macronutrient Interactions during Heating of Model Infant Milk Formulas

Eoin G. Murphy,[†] Mark A. Fenelon,^{*†} Yrjö H. Roos,[§] and Sean A. Hogan[†]

[†]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland

[§]School of Food and Nutritional Sciences, University College, Cork, Ireland

Abstract

Understanding macronutrient interactions during heating is important for controlling viscosity during infant milk formula (IMF) manufacture. Thermal behaviour of macronutrients (casein, whey, lactose, fat) was studied, in isolation and combination, over a range of concentrations. Addition of phospho-casein to whey protein solutions elevated denaturation temperature (T_d) of β -Lg and the temperature at which viscosity started to increase upon heating (T_v). Secondary structural changes in whey proteins occurred at higher temperatures in dispersions containing phospho-casein; the final extent of viscosity increase was similar to that of whey protein alone. Addition of lactose to whey protein solutions, delayed secondary structural changes, increased T_d and T_v , and reduced post-heat treatment viscosity. This study demonstrated that heat induced changes in IMF associated with whey protein (denaturation, viscosity) are not only a function of concentration but are also dependent on interactions between macronutrients.

3.1 Introduction

Infant milk formula (IMF) must provide sufficient balance of proteins, fats, carbohydrates, vitamins and minerals required for the development of infants. Standard, non-specialised, formulations are based typically on bovine skim milk solids mixed with whey protein, lactose and vegetable oils in ratios representative of human milk. The ingredients used in formulations have different forms – for example, sources of casein may come from liquid, fresh skim milk, or powdered solids, such as skim milk powder or caseinates (Montagne et al., 2009). Ingredient suppliers also provide whey proteins in different forms, such as demineralised whey powder with over 80% lactose, or, whey protein concentrates, which contain up to 80% protein (Nasripour et al., 2006). Consequently, manufacturers adopt a number of manufacturing procedures based on availability or preference for particular ingredients.

Manufacturers of powdered IMF may use (1) full wet-mixing, (2) full dry-blending, or (3) a combination of wet-mixing and dry-blending.(USFDA, 2003) In wet-mixing, ingredients are rehydrated in water, or liquid skim milk, to obtain desired blend composition and spray dried to yield a final, powdered product (McCarthy et al., 2012). Heat treatment is a critical control point of such wet-mixing process as it ensures the microbial safety of the finished powder. Wet-mixing and heat treatment are often carried out at relatively low dry matter (DM) contents (20-40% DM) before concentration by falling film evaporation (~ 55% DM) and spray drying. (McCarthy et al., 2012; McCarthy et al., 2013) In dry-blending, all ingredients are mixed together in their powdered form. In one example of a combined process, powders containing protein and fat ingredients are produced by spray drying; lactose, minerals and vitamins are then dry-blended, as solids, into the final product (Mullane et al., 2007). For wet-mixing and combined processes, essential heat treatment operations can cause physical changes to the wet-mix, such as increased viscosity. These changes must be understood and controlled to ensure powder quality, as elevated viscosity can lead to problems such as insoluble particles in the finished powder (Fox et al., 2010).

Whey proteins are sensitive to denaturation by heat. As a result the behaviour of IMF during heat treatment is highly influenced by its whey protein content, in particular β -lactoglobulin (β -Lg), which is the most abundant whey protein in bovine milk and highly reactive in its denatured state (Fox and McSweeney, 1998a; Thompkinson and Kharb, 2007). Heating β -Lg at temperatures greater than 60 °C can cause almost complete

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loss of α -helical secondary structure (Qi et al., 1997). Thiol groups exposed as a result of structural changes can lead to thiol-disulphide linkages of β -Lg with itself and other proteins (Papiz et al., 1986; Brownlow et al., 1997). These interactions, along with hydrophobic interactions, can cause gelation if the concentration of protein is sufficiently high. Caseins, in contrast are relatively heat stable; sodium caseinate can be held at 140 °C for more than 1 h without noticeable change in physicochemical functionality (Fox and McSweeney, 1998b). In skim milk, solvent-dependent, whey protein-casein interactions can have significant effects on the volume fraction of the dispersed micelle, and hence, viscosity (Anema and Li, 2003a). Lactose and other sugars have an inhibitory effect on heat-induced changes in whey proteins with the effect of increasing denaturation temperature and decreasing gel forming properties (Garrett et al., 1986; Kulmyrzaev et al., 2000; Rich and Foegeding, 2000).

Most studies on heat-induced changes of IMF constituents have been carried out on bovine milk systems or isolated whey ingredients. The aim of this study was to elucidate protein-protein and protein-carbohydrate interactions at ratios relevant to IMF manufacture i.e. at DM contents up to 58% (w/w). Differential scanning calorimetry (DSC) and Fourier transform infra-red spectroscopy (FTIR) were used to investigate, at a fundamental level, heat induced changes of protein in the presence or absence of lactose. These findings were then related to heat-induced viscosity measurements (with the addition of fat) in model IMF systems over a range of DM content.

3.2 Materials and Methods

3.2.1 Materials

Whey protein Isolate (WPI; Isolac; 90% protein) was obtained from Carbery (Cork, Ireland). Phospho-casein (PCN; 80% protein) was obtained from Sachsenmilch (Leppersdorf, Germany). Edible lactose (L) was obtained from Glanbia (Kilkenny, Ireland). Sunflower oil (SO; Solesta, Warwickshire, UK) was obtained from a local supermarket. Mineral salts (> 98% purity) were obtained from Sigma Aldrich (Wicklow, Ireland).

3.2.2 Basis

Heat induced changes in dispersions of IMF constituents (protein, lactose and fat) were studied for individual components and in combinations. Dispersions were prepared to correspond to an IMF intended for new-born infant feeding. The ratios of total protein to lactose and whey to casein were 1.4 to 7.2 and 6.0 to 4.0, respectively. For each combination of ingredients (WPI, PCN and lactose) four concentration levels were prepared in simulated milk ultrafiltrate (SMUF), which approximated DM contents at various processing stages during IMF manufacture (i.e. 25.8 to 58.2% DM; Table 3.1).

Table 3.1. IMF constituents in dispersion/emulsions

Concentration Level	Whey g	Casein g	Fat ¹ g	Lactose g	SMUF g	DM %
1	2.4	1.6	10.3	20.6	100.0	25.8
2	4.8	3.2	20.6	41.1	100.0	41.1
3	7.2	4.8	30.9	61.7	100.0	51.1
4	9.6	6.4	41.1	82.3	100.0	58.2

¹added to heat treatment experiments only

For example, at the lowest concentration studied, WPI solutions contained 2.4 g whey protein per 100 g SMUF, while WPI-PCN contained 2.4 g whey protein and 1.6 g casein per 100 g SMUF. Table 3.2 shows the whey protein concentration of whey-containing solutions/dispersions as a function of overall weight of formulations (% w/w). For viscosity measurements, SO was also used in the systems at a protein to oil ratio of 1.4 to 3.6 (see section 3.2.7). All dispersions and emulsions were prepared and analysed in triplicate.

Table 3.2. Whey protein as a percentage of total matter in whey containing non-fat systems

Concentration Level	WPI	WPI-L	WPI-PCN	WPI-PCN-L
	% whey protein (w/w)			
1	2.3	2.0	2.3	1.9
2	4.6	3.3	4.4	3.2
3	6.7	4.3	6.4	4.1
4	8.8	5.0	8.3	4.8

3.2.3 Preparation of dispersions

SMUF was prepared using deionised water according to the method of Jenness and Koops (1962). Proteins, at levels stated in Table 3.1, were allowed to hydrate in SMUF overnight before use. The composition of SMUF was as follows: KH_2PO_4 – 11.61 mM; $\text{K}_3\text{citrate}\cdot\text{H}_2\text{O}$ – 3.70 mM; $\text{Na}_3\text{citrate}\cdot 5\text{H}_2\text{O}$ – 6.09 mM; K_2SO_4 – 1.03 mM; K_2CO_3 – 2.17 mM; KCl – 8.05 mM; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ – 8.98 mM; $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ – 3.21 mM. Sodium azide (0.03% w/w) was added as a preservative. Dispersions and emulsions were standardised to pH 6.7 by addition of 0.5 M NaOH. Where necessary, lactose was added to dispersions at 25 °C and agitated for 2 h before use.

3.2.4 Denaturation temperature

Differential scanning calorimetry (DSC) was carried out using a Q2000 DSC (TA Instruments, New Castle, DE, USA). Samples (ca. 20 mg) were heated from 40 °C to 100 °C at a rate of 5 °C min⁻¹ in a nitrogen-purged environment. Heat flow to the sample was compared against an empty reference pan. Denaturation temperature (T_d) of β -Lg was obtained using Universal Analysis 2000 software (TA Instruments) where T_d was the temperature corresponding to the peak minimum of the denaturation endotherm.

3.2.5 Fourier transform infra-red spectroscopy

Fourier transform infra-red spectroscopy (FTIR) spectroscopy (Bruker Tensor 27, Bruker Optik GmbH, Germany) provided qualitative information on the physical state of proteins in dispersion. Attenuated total reflectance (ATR), mid-IR spectra were obtained using a thermally controlled BioATR cell II TM. Spectra used were an average of 300

scans at 4 cm^{-1} . Samples were measured, step-wise from 50 to 90 °C at 10 °C intervals using fresh dispersions at each temperature. Background readings were taken against SMUF at each measurement temperature and subtracted automatically. Atmospheric corrections (H_2O and CO_2 compensations), vector normalisation and Fourier self-deconvolution (Lorentzian shape with band-width and noise reduction factors of 20.31 and 0.36, respectively) of the Amide I region (1700 to 1600 cm^{-1}) were carried out using OPUS 5.5 software (Hogan et al., 2012). FTIR measurements were carried out in triplicate at the highest whey protein concentration ($9.6\text{ g }100\text{ g}^{-1}$ SMUF). In lactose-containing dispersions, the concentration of lactose ($82.3\text{ g }100\text{ g}^{-1}$ SMUF) was present at levels greater than its theoretical solubility limit in water – $42.4\text{ g }100\text{ g}^{-1}$ water at 49 °C (Hunziker and Nissen, 1926). Samples were centrifuged at $1000 \times g$ for ca. 1 min in order to sediment crystalline lactose, and the supernatant used for analyses.

3.2.6 Viscosity

Viscosity of dispersions/emulsions was monitored throughout heating using a starch pasting cell in a rheometer (AR 2000 rheometer, TA Instruments, New Castle, DE, USA). All samples (28 g) were heated from 40 °C to 95 °C at a rate of $22\text{ }^\circ\text{C min}^{-1}$, held at 95 °C for 5 min before cooling to 40 °C at a rate of $18\text{ }^\circ\text{C min}^{-1}$. During the measurement samples were sheared with the starch pasting cell impeller at a rate of 16.8 s^{-1} (Kett et al, 2013). For samples containing lactose, a solubilisation step (60 °C for 30 min) was included prior to the heating ramp described above.

3.2.7 Homogenisation

Emulsions were prepared (for viscosity experiments) by homogenising mixtures of sunflower oil with dispersions (WPI, WPI-L, PCN, PCN-L, WPI-PCN, WPI-PCN-L at each concentration level) prepared above. Homogenisation was carried out at 65 °C using a two-stage, valve-type homogeniser, Model NS2006H (Niro Soavi, Parma, Italy), employing a first-stage pressure of 15 MPa and a second-stage pressure of 5 MPa.

3.2.8 Statistical analysis

Analysis of variance (ANOVA) was carried out using the Minitab 15 (Minitab Ltd, Coventry, UK, 2007) statistical software. The significance of formulation type on heat induced viscosity increase was estimated ($P < 0.05$).

3. Decoupling macronutrient interactions during heating of model infant milk formulae

Where the slope (m) of a regression line is reported, the 95% confidence limits associated with the slope are also specified ($m \pm t SE_m$). The standard error of the estimated slope (SE_m) were calculated using Microsoft Excel's LINEST function. The confidence interval was then calculated as the product of SE_m and t , the 2-tailed t value associated with $P = 0.05$ and $n - 2$ degrees of freedom.

3.3 Results and discussion

3.3.1 Denaturation temperature

Denaturation temperature (T_d) of β -Lg was measured by DSC in the various dispersions, starting with WPI in deionised water, followed by the stepwise addition of the other constituents; mineral salts (SMUF), phospho-casein (PCN) and lactose (L). The effect of concentration was also studied; solids content of dispersions corresponded to concentrations found typically in IMF systems (Table 3.1). Figure 3.1 shows a representative trace of denaturation in a WPI solution. The shape of the thermograms is similar to those reported previously with the peak minimum corresponding to T_d of β -Lg (Boye and Alli, 2000; Fitzsimons et al., 2007). T_d of α -Lactalbumin was identified as a small shoulder occurring 10 to 15 °C lower than T_d β -Lg on DSC thermograms but was detectable only at whey protein concentrations greater than 8% (w/w).

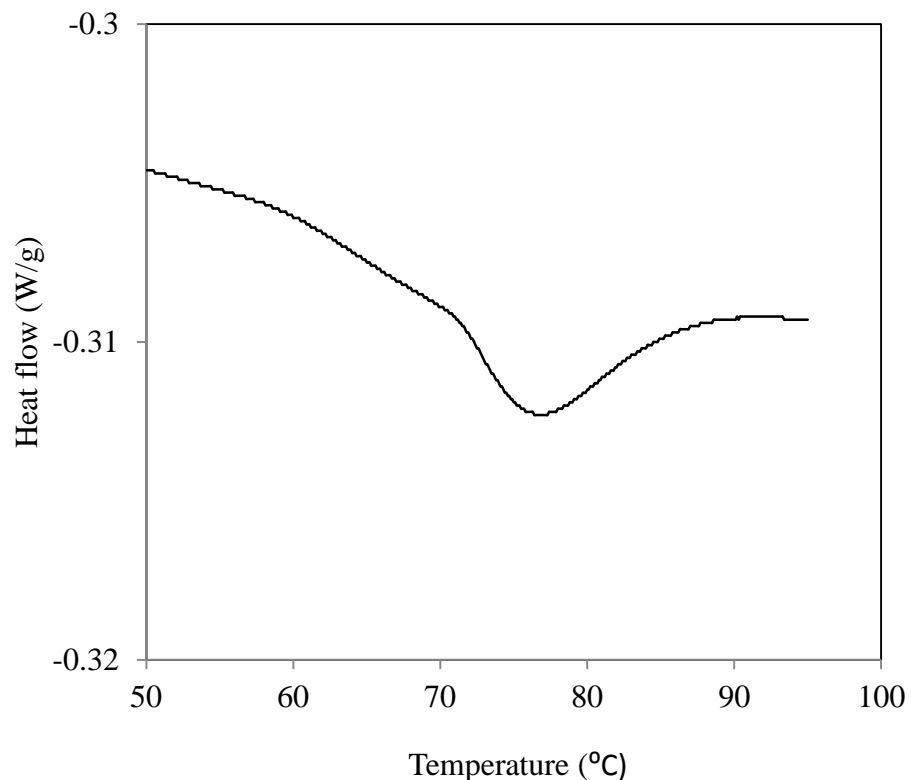


Figure 3.1. DSC thermogram of WPI solution (8.8% whey protein w/w in SMUF)

T_d of β -Lg in WPI hydrated in deionised water and SMUF was within the range reported by previous studies i.e. 75 to 85 °C depending on concentration, genetic variant

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of β -Lg, scanning rate etc. (Imafidon et al., 1991; Qi et al., 1995). In general, T_d decreased with increasing protein concentration (Figure 3.2). Qi et al. (1995) reported that increasing the concentration of purified β -Lg increased T_d . T_d values reported across a number of studies suggest that increasing protein concentration in non-purified systems tends to decrease β -Lg denaturation temperature (Imafidon et al., 1991; Boye and Alli, 2000; Haug et al., 2009).

Hydrating WPI in SMUF, prior to DSC analysis, had the effect of increasing T_d of β -Lg compared to that of WPI in deionised water (Figure 3.2) The effect of salts on denaturation temperature of proteins can be related to the Hoffmeister lyotropic series, according to which, ions are classified as chaotropic or kosmotropic, depending, respectively, on their destabilising or stabilising effects (Kendrick et al., 2002). Chaotropes disrupt hydrophobic aggregation and promote unfolding of proteins and thereby reduce T_d . In contrast, kosmotropic agents promote hydrophobic interactions and lead to an increase in T_d (Tobias and Hemminger, 2008). T_d of β -Lg in WPC has been shown to increase in the presence of NaCl and decrease in the presence of $MgCl_2$ or $CaCl_2$ (Varunsatian et al., 1983; Kunz et al., 2004). It appears from the present study that the net effect of the milk serum salts in SMUF is kosmotropic in nature i.e. the presence of mineral salts had the effect of increasing T_d of β -Lg. These results show a clear effect of SMUF on T_d , therefore, as SMUF plays an important role in stabilising rehydrated PCN, all subsequent dispersions/solutions were hydrated in SMUF; this allowed comparisons to be made more easily between the various systems.

Addition of PCN to systems containing WPI increased T_d of β -Lg (Figure 3.2). Casein micelles require a significant amount of water for hydration; extrapolating from previously reported data, casein micelles, at temperatures of 70 to 80 °C have a water content of approximately 2.2 g water g^{-1} casein (Sood et al., 1976). It could be argued that PCN increased the effective concentration of whey protein in the continuous phase due to the large voluminosity of casein micelles, rendering a significant proportion of solvation water unavailable to whey proteins. At the highest casein content of the present study (6.4 g $100 g^{-1}$ SMUF) the propensity of casein micelles to hydrate and compete for water could decrease available water by ca. 14.1 g. On this basis, it might be expected that addition of PCN should decrease T_d , given that increasing concentration of WPI dispersions resulted in a decrease in T_d . However, increased T_d of β -Lg in WPI-PCN dispersions suggests PCN had a stabilising effect on whey protein denaturation. In

previous studies, the effects of individual caseins (α -, β -, κ -) on T_d were inconsistent (Blanc et al., 1977; Paulsson and Dejmek, 1990; Imafidon et al., 1991); Imafidon et al. (1991) found that κ -casein variants AB and BB decreased T_d while variant AA increased T_d . Inconsistencies may be due to differences in buffer systems source, genetic variants and pre-processing of proteins used. Furthermore, these studies did not examine the effect of intact casein micelles on T_d of β -Lg.

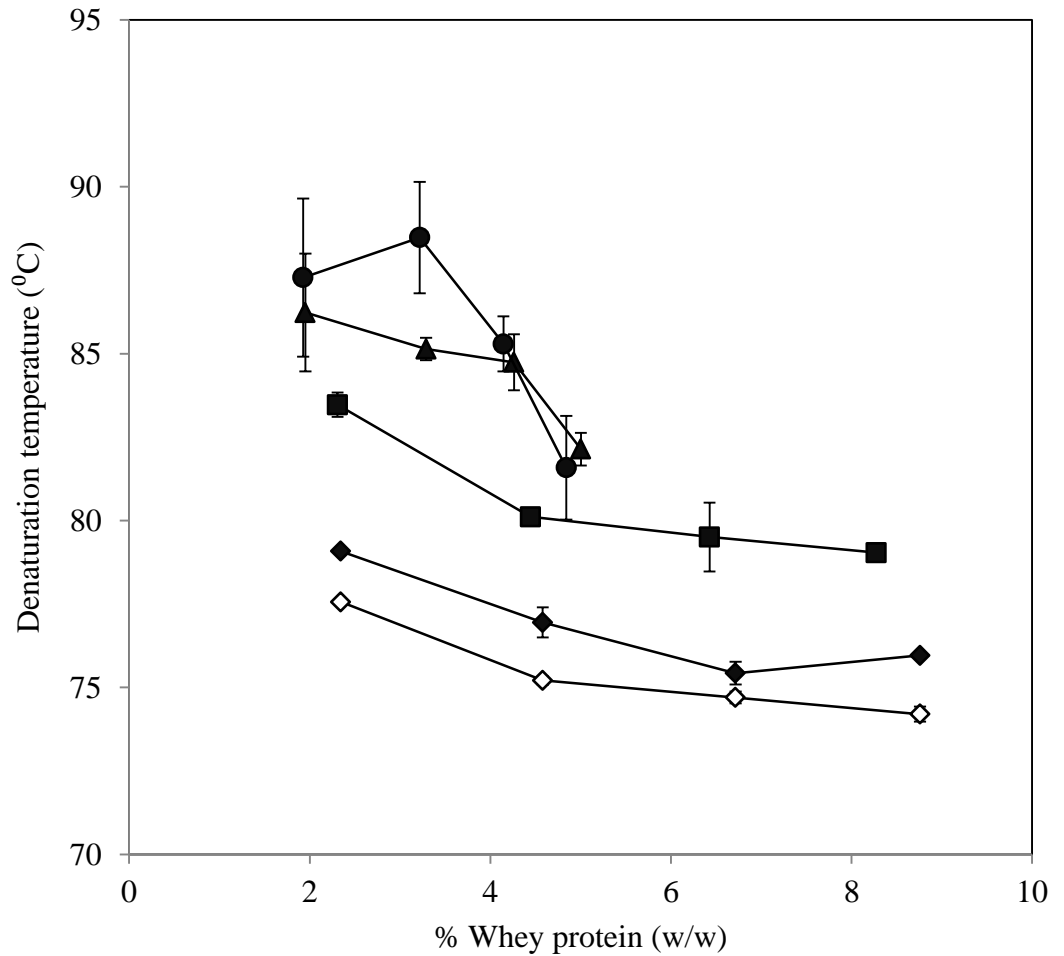


Figure 3.2. Denaturation temperature (T_d) in SMUF (solid fill) and deionised water (no fill); WPI (\diamond, \blacklozenge), WPI-PCN (\blacksquare), WPI-L (\blacktriangle) and WPI-PCN-L (\bullet) at increasing protein concentrations. Error bars represent the standard deviation of three replicate trials.

Addition of lactose to systems containing WPI resulted in an increase in T_d of β -Lg compared to lactose-free systems at equivalent protein concentrations (Figure 3.2). The stabilising effect of sugars, including lactose, against whey protein denaturation has been well documented (Itoh et al., 1976; Bull and Breese, 1978; De Wit and Klarenbeek, 1981; Boye and Alli, 2000). Interactions between sugars and the hydrophobic backbone of whey proteins are thermodynamically unfavourable (Liu and Bolen, 1995). As a result,

sugars become preferentially excluded from the vicinity of the protein in order to minimise protein-carbohydrate contact (Timasheff, 1993). Similarly, when heat is applied to whey protein, any potential interaction with a co-solvent sugar is unfavourable, which results in retention of the compact, native structure of the protein to higher temperatures. The stabilising effect of lactose was least apparent at high protein levels; possibly due to low lactose solubility at high DM concentrations and particularly so in the presence of PCN.

3.3.2 Fourier transform infra-red spectroscopy

The amide I region ($1700 - 1600 \text{ cm}^{-1}$) of FTIR spectra was examined in formulations equivalent to 58.2% DM (see Table 3.1). This region is associated with C=O and C–N stretching vibrations and is indicative of protein secondary structure (Hogan et al., 2012). Changes in amide I spectra, with respect to temperature, are summarised in Table 3.3. As expected, little effect of temperature was observed for PCN dispersions, due to a relative lack of secondary structure in casein proteins. In the case of WPI, however, increasing temperature from 60 °C to 90 °C resulted in significant changes in secondary structure, particularly decreases in intramolecular β -sheet ($1630\text{-}1627 \text{ cm}^{-1}$) and α -helix (1654 cm^{-1}) conformations.

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Table 3.3. Summary of secondary structural changes with respect to temperature

		1684 cm ⁻¹	1672 cm ⁻¹	1654/1645 cm ⁻¹	1630-1627 cm ⁻¹	1616/1617 cm ⁻¹
	°C	Intermolecular sheet	β- Turns	α-helix/random	Intramolecular β-sheet	Intermolecular β-sheet
WPI	50	O	O	O	O	O
	60	-	-	-	O	+
	70	-	-	--	--	+++
	80	-	-	-	-	+
	90	-	-	-	O	+
WPI-L	50	O	O	O ^a	O	O
	60	-	-	--	O	+++
	70	+	+	-	O	O
	80	+	+	-	--	+
	90	+	+	-	-	+
WPI-PCN	50	O	O	O	O	O
	60	+	+	+	+	+
	70	+	+	O	-	O
	80	O	O	--	---	+
	90	O	+	-	-	-
WPI-PCN-L	50	O	O	O ^a	O	O
	60	O	+	-	O	+++
	70	+	+	O	O	O
	80	+	++	O	--	+
	90	O	+	O	--	+

(Jackson and Mantsch, 1992; Boye et al., 1996; Qi et al., 1997; Barth and Zscherp, 2002; Kehoe et al., 2008)

(o) no change from previous temperature

(+/++/+++)
(-/-/---)

small/intermediate/large increase

small/intermediate/large decrease

Analysis based on three replicate trials

^a Increased absorbance compared to lactose-free dispersion

3. Decoupling macronutrient interactions during heating of model infant milk formulae

A concomitant increase in the absorbance spectra in the region of 1617 cm^{-1} indicated the formation of aggregates with intermolecular β -sheet structures (Jackson and Mantsch, 1992; Kehoe et al., 2008). The greatest changes in secondary structure and intermolecular aggregate formation occurred between 60 and 70 °C. Qi et al (1997) also reported a significant reduction in α -helix conformation over these temperatures, which, it was suggested, plays an important role in the exposure of the free thiol group, Cys-121. Exposure of this group is of particular significance to IMF manufacture as it is associated with potentially large increases in viscosity, due to whey protein aggregation following intermolecular thiol-disulphide interchange reactions.

At 50 °C, secondary structure of WPI-PCN dispersions was similar to that of WPI. The presence of PCN delayed temperature-induced losses of β -sheet structure. A major decrease in intramolecular β -sheet structure was not observed until $> 70\text{ °C}$, compared to $> 60\text{ °C}$ for WPI in the absence of PCN. Delayed loss of secondary structure correlated well with DSC analysis, where PCN caused an increase in T_d . Surprisingly, between 80 and 90 °C, where intramolecular β -sheet structure decreased, intermolecular β -sheet structure also decreased. Increases in turns/random coil structure ($\sim 1672\text{ cm}^{-1}$) were also observed as temperature increased. Overall, it is evident that PCN altered the effects of temperature on secondary structural changes in whey proteins.

Addition of lactose to SMUF had negligible effect on absorbance of SMUF buffers in the Amide I region. Lactose, however, did influence the spectra of WPI solutions in the Amide I region (Figure 3.3a). A notable difference between the spectra of WPI and WPI-L is the relatively large absorbance peak in the region $1670\text{ to }1640\text{ cm}^{-1}$ in lactose-containing samples. Peaks in this region ($1654\text{ and }1645\text{ cm}^{-1}$) have been associated previously with α -helical and random coil conformations.(Qi et al., 1997; Kehoe et al., 2008) However, it is also possible that the presence of lactose altered the local structure of water resulting in preferential hydration of proteins (Huang et al., 1995).

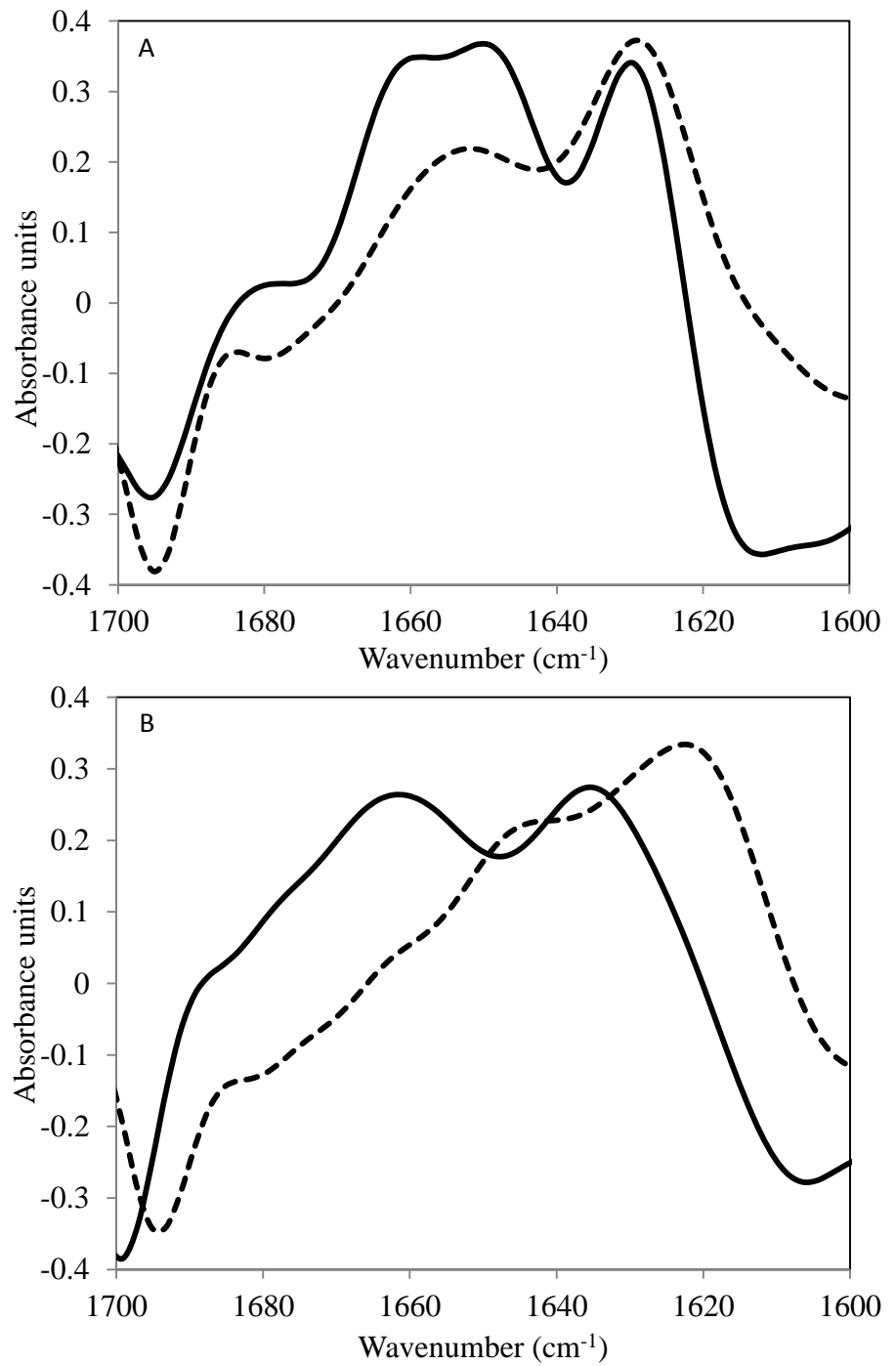


Figure 3.3. FTIR spectra of WPI (dashed) and WPI-L (solid) as 50 °C (A) and 80 °C (B)

Another notable difference occurred at $\sim 1617\text{ cm}^{-1}$, an area associated with intermolecular aggregates, where WPI-L systems had a lower absorbance in comparison to WPI (Figure 3.3a). It is also possible that spectral changes of WPI in the presence of lactose were due to the Maillard reaction. Czerwenka et al. (2006) reported that lactosylation of β -Lg (over 120 h at 60 °C) resulted in approximately 1.6 molecules of lactose bound to each β -Lg molecule. It is suggested that the extent of the Maillard reaction was limited over the timeframe of FT-IR measurements reported here (approximately 6 min per analysis). Schiff base intermediates have been reported to absorb at 1647 cm^{-1} (Wnorowski and Yaylayan, 2003); in the current study, intensity at 1647 cm^{-1} diminished with increasing temperature. Based on the above, it appears unlikely that possible spectral interferences due to lactosylation were a confounding factor.

Heating from 50 to 60 °C increased the aggregation band (1617 cm^{-1}) of WPI-L (Table 3.3). An accompanying reduction in the 1670 to 1640 cm^{-1} region, indicated that some of the additional secondary structure, due to the presence of lactose, was lost upon heating. Intramolecular β -sheet structure was preserved to a higher temperature in WPI-L solutions (Table 3.3), where no loss was observed until $> 70\text{ °C}$, compared to $> 60\text{ °C}$ in the absence of lactose. Spectra of WPI-L solutions, heated from 50 to 80 °C did not change in the same manner as WPI alone (compare Figure 3.3a and 3.3b), in which, the main peak shifted from intramolecular (1630 cm^{-1}) towards intermolecular β -sheet structure (1617 cm^{-1}). Delayed loss of structure and aggregation were most likely due to the stabilisation effect of lactose, which has previously been shown to delay aggregation in β -Lg solutions, albeit to a lesser extent than other sugars (Boye et al., 1996). The findings are consistent with DSC results reported above, which showed that T_d of WPI in the presence of lactose was higher than in its absence. Increased absorbance at 1672 cm^{-1} indicated an increase in turns/random coil structure upon heating, which was similar to the effect of PCN upon WPI.

Lactose in WPI-PCN dispersions had a similar effect to lactose in WPI solutions i.e. a relatively large peak, compared to lactose-free dispersions, in the range of 1640 to 1670 cm^{-1} was observed. As with WPI-L dispersions, a decrease was observed in α -helix/random conformation between 50 and 60 °C, and, intramolecular β -sheet structure remained intact until the temperature exceeded 70 °C (Table 3.3).

3.3.3 Heat treatment experiments

The effect of heat treatment on viscosity of IMF formulations was determined by heat treatment at 95 °C for 5 min. Samples were heated from 40 to 95 °C at a rate of 22 °C min⁻¹. During this temperature ramp, viscosity first decreased, before a critical temperature was exceeded (T_v) and viscosity increased exponentially (Figure 3.4). Measurement of T_v provides practical information relevant to existing, industrial heat treatment scenarios, given that it was carried out under shear conditions and the rate of temperature increase was high (22 °C min⁻¹) compared to T_d measurements. As observed by DSC and FTIR data reported above, β -Lg in WPI is sensitive to heat denaturation and is highly reactive when unfolded due to the exposure of a free sulfhydryl (thiol) group (Papiz et al., 1986; Brownlow et al., 1997). During heat treatment, denatured and unfolded β -Lg forms aggregates through self-association, and association with other milk proteins (Fox and Morrissey, 1977; Manderson et al., 1998; Galani and Apenten, 1999; Schokker et al., 2000). Denatured whey proteins and aggregates have a greater hydrated volume than native proteins which leads to significant increases in viscosity (Snoeren et al., 1982; Ndoye et al., 2013). Therefore, it is not surprising that all WPI-containing systems increased in viscosity as temperature approached T_d . The trends observed for T_v were similar to those observed for T_d (Figure 3.2) i.e. addition of lactose and/or PCN increased T_v . In some cases it was observed that T_v occurred prior to T_d , especially at higher whey protein concentrations. This could be explained by differences in heating and shearing rates between the rheometer and DSC methods. In the case of PCN and PCN-L dispersions viscosity did not increase due to the absence of whey protein.

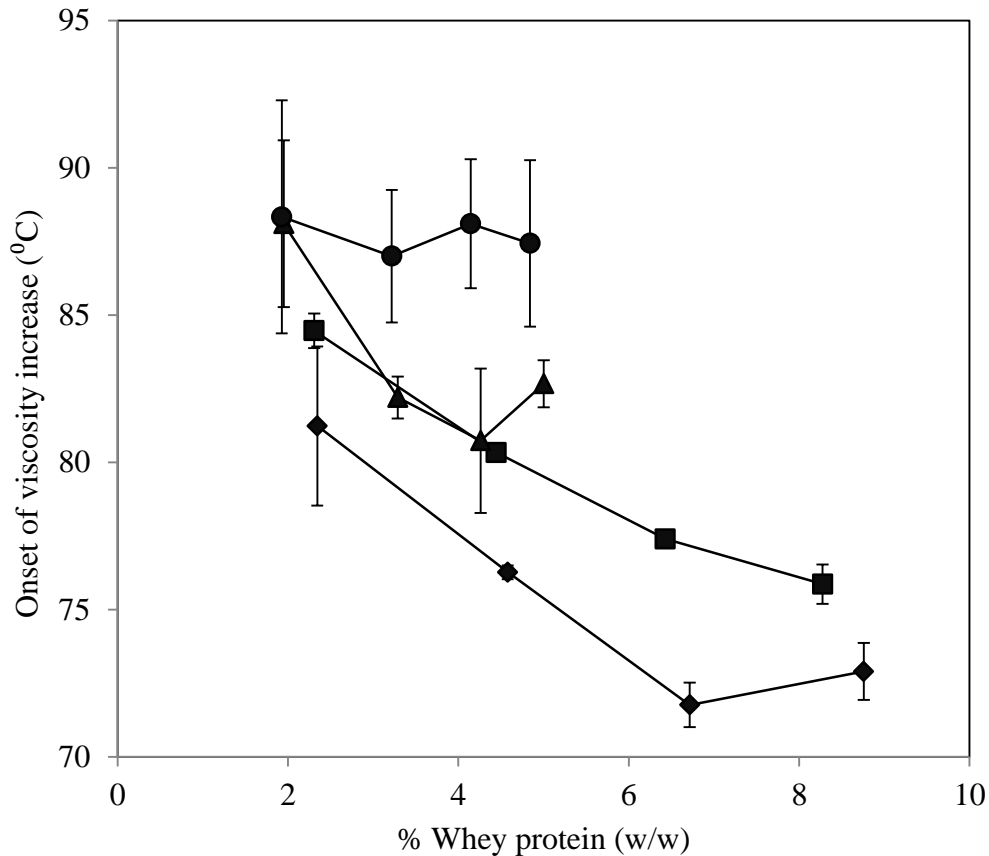


Figure 3.4. Onset of viscosity increase during heating; WPI (◆), WPI-PCN (■), WPI-L (▲) and WPI-PCN-L (●) at increasing protein concentrations. Error bars represent the standard deviation of three replicate trials.

The extent of viscosity change during heat treatment was calculated as the ratio of apparent viscosities (at 40 °C) after and before heating at 95 °C for 5 min (Figure 3.5). Heating for 5 min represents a more severe heat treatment than that generally applied during manufacture (McCarthy et al., 2013; Murphy et al., 2013). As a result, aggregates were observed in concentrated WPI-containing systems which would not be expected in an industrial process. The shearing action of the starch pasting cell ensured aggregates remained in suspension. Despite the severity of the treatment, it has the benefit of showing the potential for viscosity increase in a given system while providing indicative trends relevant to manufacture. Dispersions of PCN, had lower viscosities following heat treatment, post heat treatment viscosity, at 40 °C, was 95 to 99% of the pre heat treatment viscosity. Viscosity in milk protein dispersions is related to the hydrated volume of the micelle (Horne, 1998). At higher temperatures the hydrophobic effect, responsible for much of the stability of the casein micelle, is greater, resulting in a “tightening” or deswelling of the micelle and a decrease in micellar hydration (Horne, 1998). The lower

viscosity of PCN dispersions after cooling is postulated to have been caused by high-temperature association of calcium phosphate with the micelles, which subsequently prevented re-swelling of micelles upon cooling (Gaucheron, 2005).

Figure 3.5 shows the extent of viscosity increase in WPI containing dispersions/solutions as a result of heating. The large viscosity increases observed in systems containing WPI following heat treatment were related to the extensive water holding capacity of denatured whey proteins and aggregates. An effect supported by the findings of O'Loughlin et al (2012). The extent of viscosity increase was a linear function of whey protein content (Figure 3.5). The 95% confidence intervals of both WPI and WPI-PCN dispersions overlap, suggesting there was no difference in extent of viscosity increase with whey protein concentration for these two systems. This was despite T_d and T_v occurring at higher temperatures in the presence of PCN; however, whey protein-casein interactions may also contribute to heat-induced increases in viscosity, depending on heating and solvent conditions and could explain the similar degree of viscosity increase (Anema and Li, 2003a).

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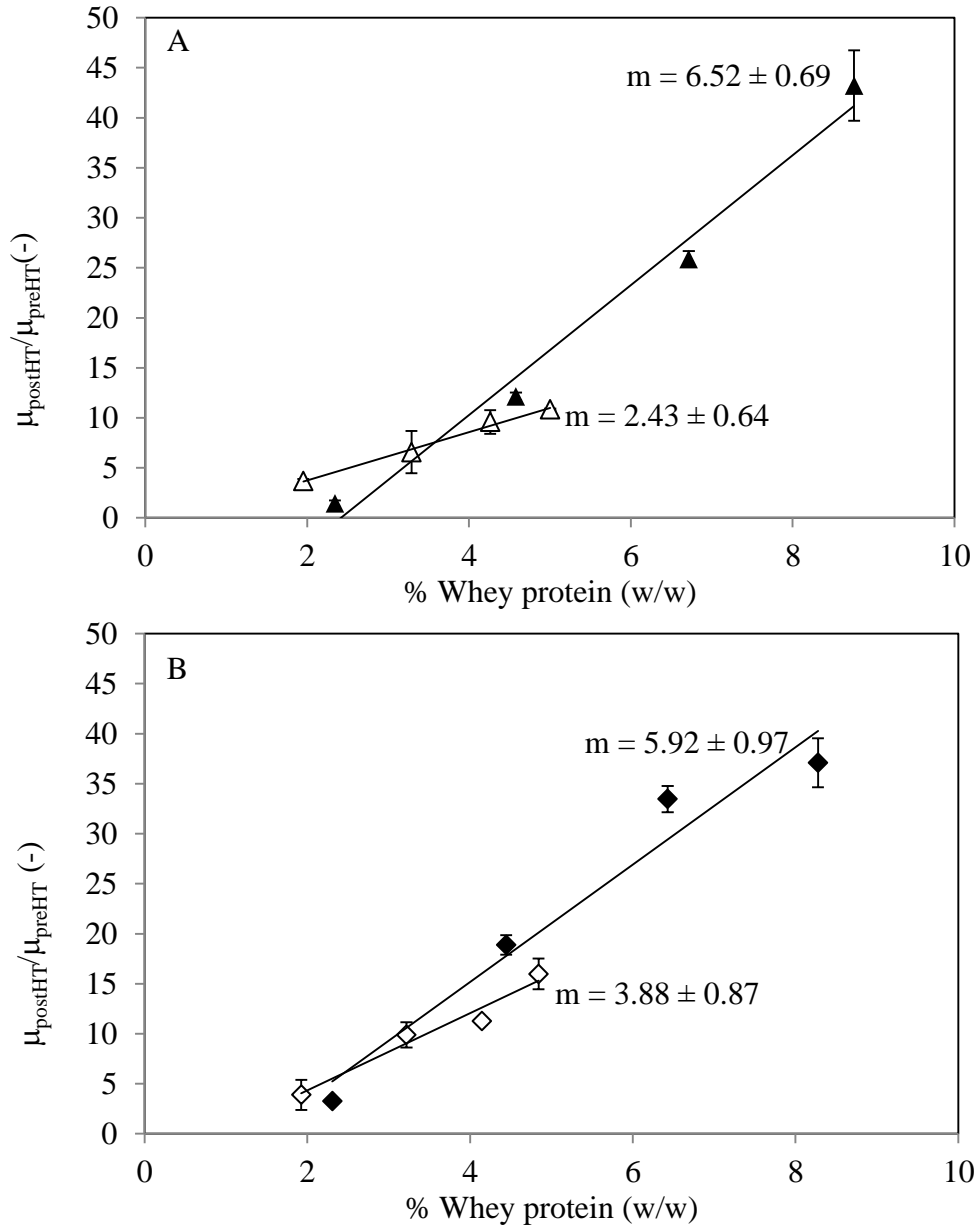


Figure 3.5. Ratio of apparent viscosity ($40\text{ }^{\circ}\text{C}$; 16.8 s^{-1}) post (μ_{postHT}) and pre (μ_{preHT}) heat treatment ($95\text{ }^{\circ}\text{C}$ for 5 mins) in WPI containing systems. A: WPI (\blacktriangle), WPI-L (\triangle). B: WPI-PCN (\blacklozenge), WPI-PCN-L (\diamond). Error bars represent the standard deviation of three replicate trials. Slopes (m) are given \pm 95% confidence intervals.

As fat is a vital component in IMF, emulsions were also prepared and heat treated. In all cases addition of fat to formulations resulted in higher viscosities, post-heat treatment, compared to corresponding oil-free systems. During homogenisation protein adsorbs to the oil-water interface and protects fat droplets from coalescence. Therefore, protein in emulsified systems can be divided into two classes: adsorbed and non-adsorbed. Despite this difference, the general trends observed upon heating of fat-free systems were reflected in emulsified systems. Major increases in viscosity were observed

only in WPI-containing systems; PCN stabilised emulsions were remarkably stable to heat treatment over the whole range of DM content studied. Lactose increased the heat stability of emulsions. Figure 3.6 shows the viscosity after heat treatment of the different formulations as a function of total DM content. In emulsified systems, a major increase in viscosity was observed between 25 and 35% DM (w/w) for lactose-free emulsions; similar viscosity increases were not observed before DM content reached 50% (w/w) in emulsions containing lactose. This is particularly relevant for ingredient/IMF manufacturers that produce lactose-depleted base powders with the intention of adding crystalline lactose at a later stage (Mullane et al., 2007). Maximising DM content prior to heat treatment of lactose-depleted base mixtures could have significant impact on the viscosity of concentrates, which in extreme cases causes problems such as inefficient atomisation during spray drying (Fox et al., 2010).

Lactose increased heat stability. The rate of change of viscosity increase per unit change in whey protein concentration (as indicated by the slope of regression lines in Figure 3.5) was lower for systems containing lactose; the 95% confidence intervals of the slopes did not overlap with those of lactose-free systems, suggesting lactose had a significant effect. Addition of lactose resulted in lower post heat-treatment viscosity at higher DM content. For example, the most concentrated WPI solution had a DM content of 8.8% (w/w) compared to 47.9% (w/w) for the most concentrated WPI-L solution, yet the apparent viscosity of the former, post-heat treatment, was twice that of the latter. Rich and Foegedding (2000) found that the onset of WPI gelation was delayed in the presence of lactose and that subsequent gel strength was reduced.

The extensive increases in viscosity following heat-treatment of WPI-containing emulsions suggests that, during heat treatment, protein-stabilised oil droplets associated with non-absorbed denatured whey proteins, and/or each other, to form a transient network of flocculated oil droplets, with an associated increase in volume fraction (Euston et al., 2000). Such changes were sufficient to alter the viscoelastic properties of emulsions and confer a macrostructure with more 'solid-like' properties.

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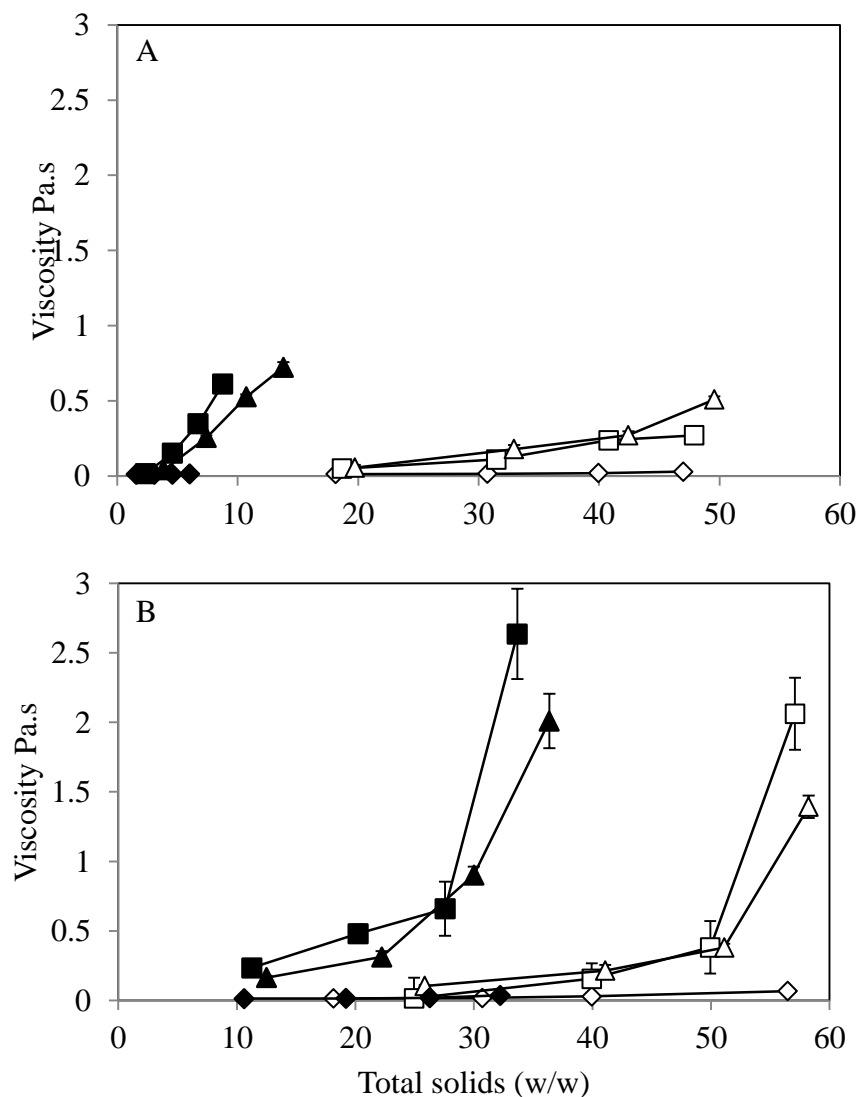


Figure 3.6. Apparent viscosities ($40\text{ }^{\circ}\text{C}$; 16.8 s^{-1}) of oil-free (A) and homogenised oil-containing (B) IMF model systems after heat treatment ($95\text{ }^{\circ}\text{C}$ for 5 mins). Closed symbols denote lactose-free dispersions/emulsions; PCN (◆), WPI (■), WPI-PCN (▲). Open symbols denote lactose-containing dispersions/emulsions; PCN-L (◇), WPI-L (□), WPI-PCN-L (△). Error bars represent the standard deviation of three replicate trials.

3.4 Conclusion

The extent of whey protein structural changes and their influence on the viscosity of IMF dispersion/emulsions were significantly affected by their interactions with other macronutrient components. Lactose content, in particular, played an important protective role during the heat treatment of IMF, retarding and reducing viscosity increases in dispersions and emulsions alike. This is in keeping with the theory of preferential hydration of proteins in the presence of carbohydrates resulting in stabilisation of the protein. Lactose content of wet-mixes should be a key consideration when designing IMF manufacturing processes. PCN (micellar casein) also had a protective effect on β -Lg denaturation. Despite increasing the denaturation temperature and delaying the onset of viscosity increase in WPI, in a manner similar (albeit diminished) to lactose, it seems that PCN interacted with WPI, during heating, in ways that lead to similar levels of viscosity increase to that observed in WPI alone. Overall, this study provided insight into the relationships that exist between macronutrients as a function of concentration and temperature and should provide information of direct relevance to manufacturers of IMF.

Chapter 4: Increasing dry matter content in infant milk formula manufacture – development of novel processing path

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The effect of high velocity steam injection on the colloidal stability of concentrated emulsions for the manufacture of infant formulations

Eoin G. Murphy^a, John T. Tobin^a, Yrjo H. Roos^b, Mark A. Fenelon^{a*}

^aTeagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

^bUniversity College Cork, School of Food and Nutritional Sciences, Cork City, Ireland

Abstract

Dry matter (DM) content is an important consideration in the manufacture of infant milk formulations (IMF). This study looked at the physical properties of IMF formulations at various DM contents and proposed a novel process for heat treatment of high DM content formulations. Increasing DM content of IMF formulations from 20 to 60% (w/w) resulted in an exponential increase in apparent viscosity ($\mu = 0.0009e^{0.044DM}$). pH was a linear function ($R^2 = 0.93$) of both DM content and temperature ($pH = 7.19 - 0.01DM - 6.84 \times 10^{-3} \text{ Temperature}$). The extent of size reduction of fat globules during rotor-stator emulsification was found to increase concomitantly with DM content. Based on physical characteristics measured, the calculated heat transfer in a plate heat exchanger was lower at high DM contents. The ratio of convective to conductive heat transfer (Nusselt number) was reduced by approximately 25%, largely due to reduced turbulence caused by higher viscosity. The Reynolds number decreased from approximately ~1000 to ~150 as DM content increased from 20 to 60% (w/w). When heated at 80 °C, β -Lactoglobulin denaturation decreased with increasing DM content and no changes in viscosity were observed. Heating at 95 °C resulted in large viscosity increases for high DM content formulations and the rate of denaturation of β -Lg was not affected by DM content. A high DM content (60% w/w) novel process utilising rotor-stator emulsification and direct steam injection (heat treatment = 120 °C x 3 s) was developed which produced stable emulsions with significantly ($P < 0.05$) more native whey protein and lower viscosity compared to indirectly heated formulations. The study illustrated the difficulty of heating high DM content at > 90 °C due to viscosity increases, and, proposed a novel process which was capable of limiting viscosity increases and denaturation at these conditions.

4.1 Introduction

Powdered infant milk formula (IMF) manufacturers may employ combinations of various processing steps/unit operations to ensure the microbial and physical stability of final products. Bovine milk derivatives are the most commonly used raw materials in the production of IMF. Commonly, raw materials (proteins, carbohydrates) are mixed in water and combined with vegetable fat (and water in the case of powdered ingredients) to give the desired composition of constituents (Nasripour et al., 2006). Processing of these formulations includes heat treatment to ensure microbial stability and, homogenisation to ensure stability to separation (USFDA, 2003). In many cases, there is flexibility within the process in terms of choosing the dry matter (DM) content at which processing is carried out.

Increasing DM content of milk-based wet-mixes will affect physical properties. Several researchers have found that increasing DM content can exponentially increase viscosity of bovine milk-based systems, due to an increase in the volume fraction of dispersed constituents such as casein, whey and fat (Fernandez-Martin, 1972; Snoeren et al., 1982; Snoeren et al., 1984; Trinh et al., 2007). Density of formulations can also change as a result of increases in proteins and lactose, both of which are more dense than water (Walstra et al., 1984). Specific heat capacity and thermal conductivity decrease with increasing DM content (Fernandez-Martin, 1971; Gavrilla et al., 2005). Convective heat transfer is affected by all these parameters. For example, the efficiency of convective heat transfer is proportional to turbulence, which can be seen as a ratio of internal to viscous forces in a flowing fluid, therefore, increases in viscosity can reduce turbulence and, thus, heat transfer (Coulson and Richardson, 1963).

DM content could also influence fouling of heat transfer surfaces resulting in a reduction in heat transfer. It has been shown that β -Lactoglobulin (the most abundant whey protein in bovine milk; β -Lg) denaturation can play an important role in the fouling mechanism (De Jong et al., 1992; Changani et al., 1997; De Jong, 1997). Anema (2000) reported that β -Lg denaturation in skim milk was reduced at higher DM contents when heated at < 90 °C; however when heated at > 90 °C, DM content had little effect on the rate of denaturation. This was attributed to a change in the rate limiting step of denaturation from monomer unfolding (at < 90 °C) to aggregate formation (at > 90 °C). Oldfield (1996) found that increased DM content resulted in increased denaturation of whey proteins at 110 °C. Heating high DM content formulations at temperatures > 90 °C

may present problems of heat induced viscosity increases due to aggregation of β -Lg, with associated fouling.

Direct heat treatments have high heating rates, allowing the desired heating temperature to be reached quickly (Lewis and Deeth, 2009). Tran et al. (2008) found in a survey of time-temperature profiles of 22 Australian UHT plants that both α -Lactalbumin (α -La) and β -Lg denaturation was lower in direct plants than in indirect. Therefore, direct heat treatment processes could be applied to limit the extent of β -Lg denaturation and/or viscosity increases in sensitive formulations. The objectives of this study were to:

1. Understand the effect of increasing DM on processing IMF
2. Develop a novel high DM content (60% w/w) process allowing for the heat treatment and homogenisation of infant formulations of suitable physical stability and concentration for spray drying.

4.2 Materials and methods

4.2.1 Materials

Medium heat skim milk powder (SMP) and demineralised whey powder (DWP) were supplied by Dairygold Food Ingredients (Mitchelstown, Co. Cork, Ireland). Lactose was supplied by Friesland Foods Domo, (The Netherlands), under the product name Lactopure. Sunflower oil (SO) was supplied by Trilby Trading (Drogheda, Co Louth, Ireland). Potassium hydroxide (KOH) was supplied by BDH Laboratory Supplies (Poole, England). Compositions of the ingredients are shown in Table 4.1.

Table 4.1. Compositional analysis of ingredients used

Ingredient	Protein (% w/w)	Casein (% w/w)	Whey Protein (% w/w)	Fat (% w/w)	Lactose (% w/w)	Moisture (% w/w)
SMP	34.0	27.2	6.8	1.3	52.0	3.7
DWP	12.0	0.0	12.0	0.0	83.0	3.0
Lactose	0.0	0.0	0.0	0.0	95.0	5.0
Sunflower oil	0.0	0.0	0.0	100.0	0.0	0.0

4.2.2 Lab scale experiments

4.2.2.1 Batch formulation

Model first stage infant milk formulae (IMF) were prepared as outlined in Table 4.2. Emulsions (500 g) of increasing dry matter (DM) content (20-60% w/w) were prepared at 65 °C using an Ultra-Turrax rotating at (24000 rpm) for 5 min (IKA-Werke GmbH & Co. KG, Staufen, Germany). Larger batches (5 kg) were prepared using a Silverson overhead mixer (Silverson, UK). Reynolds (Re) number was estimated for the mixing operation using:

$$\text{Re} = \frac{D^2 N \rho}{\mu} \quad (\text{eq 4.1})$$

where D is the diameter of the impeller (0.015 m), N is the rotational speed (revolutions s⁻¹), ρ is density kg/m³ and μ is viscosity (Pa s). The above relationship was used for illustrative purposes. It is likely that other factors relating to the geometry of the rotor-stator mixer could influence Reynolds number.

Table 4.2. Batch recipes at increasing DM contents

DM content (% w/w)	SMP (% w/w)	DWP (% w/w)	Lactose (% w/w)	SO (% w/w)	Water (% w/w)
20	3.2	9.4	2.0	5.7	79.6
30	4.9	14.2	3.1	8.5	69.4
40	6.5	18.9	4.1	11.4	59.2
50	8.1	23.6	5.1	14.2	48.9
60	9.7	28.3	6.1	17.1	38.7

4.2.2.2 Heat treatment

Formulations were heated by either of two ways:

1. Formulations (28 g) were heated using the starch pasting cell attachment of an AR 2000 rheometer (TA Instruments, Crawley, UK). Samples were heated from 40 °C to 80 or 95 °C at a rate of 22 °C min⁻¹, held at 80 or 95 °C for 5 min before cooling to 40 °C at a rate of 18 °C min⁻¹.
2. Formulations (5kg) were pre-heated to 65 °C before heating to 120 °C for 3 s using a MicroThermics tubular heat exchanger (MicroThermics, North Carolina, U.S.A.).

4.2.3 Pilot scale experiments

4.2.3.1 Batch Formulation

Model first stage IMFs (200 kg) were prepared at a target DM content (~ 60% w/w) using the ingredients outlined in Table 4.2. A portion of each batch (50 kg) was diluted to 20% (w/w) prior to heat treatment. Trials were carried out in triplicate.

4.2.3.2 Ingredient hydration and emulsion formation

Rehydration of powdered ingredients and subsequent emulsification of oil was carried out using a YTRON ZC powder induction unit (YTRON Process Technology GmbH, Germany), equipped with a rotor-stator dispersion head (3 mm tooling). Suction created by the dispersion head pulled ingredients from the induction hopper into the liquid stream, thus wetting and dispersing the powders. This unit was connected to a jacketed tank (300 kg capacity), RO water (80 kg) was added to the tank and circulated through the ZC (operating at 100% capacity), while applying steam to the jacket. The contents of the tank were constantly agitated at approximately 500rpm by means of an

impeller. At approximately 50 °C (in tank) the lactose (11.97 kg) and a portion of the oil (11 kg at 20 °C) were added to the tank, to minimise foaming during the induction of demineralised whey protein and skim milk powder. The tank was allowed to reach 74 °C (to aid solubilisation of lactose) before induction through the high shear powder induction unit commenced. Ingredient induction through the YTRON ZC was carried out in the following order for each trial, DWP (54.80 kg), SMP (19.20 kg) and SO (22.70 kg). Induction of ingredients lasted between 18-21 min followed by 10 min of recirculation through the dispersion head and back into the jacketed tank below the level of fluid in the tank. The pH was adjusted to 6.85 with 4 M KOH prior to heat treatment.

4.2.3.3 Heat treatment

Heat treatment (120 °C for 3 s) was carried out in a custom built heat treatment unit, incorporating a Maklad high velocity steam injector (Maklad Innovative Fluid & Systemtechnik GmbH, Wien, Austria). This heat treatment unit consisted of a pressure sensing multistage pump CRNE3-19 (Grundfoss Ltd, Dublin, Ireland), a Maklad direct steam injector, holding tube (approx. volume 1.75 L) and plate heat exchanger. Two batches 60% and 20% (w/w) DM content (150 kg) were heat treated for each trial. The high velocity steam injector was stabilised on water before switching to the product. A process flow diagram is shown in Figure 4.1.

4.2.4 Physical characterisation of formulations

4.2.4.1 Particle size distribution and dry matter content

The particle size distribution of formulations was measured using a Mastersizer 2000S (Malvern Instruments Ltd., UK). The optical parameters selected were a sample refractive index of 1.46, a particle absorbance of 0.001 and a dispersant refractive index of 1.33 (water). Volume mean diameter (D[4,3]) and D(v,0.9) (equivalent diameter which 90% of particles are smaller than) were used to characterise emulsions.

DM contents were measured using a Smart System 5, Smart Trac System (CEM Corporation, NC, USA).

4.2.4.2 Apparent viscosity (μ)

Viscosity measurements were carried out on an AR G2 Rheometer (TA Instruments, Crawley, UK). For lab scale formulations, the effect of DM content (20 to

60% w/w) and temperature (45 to 90 °C) on viscosity was measured using concentric cylinder geometry. For pilot scale formulations samples were standardised to a DM content of 12.5% (w/w) to eliminate concentration differences and viscosity was measured at 20 °C using a 60mm diameter parallel plate. In both cases, samples were pre-sheared at 300 s⁻¹ for 1 min, equilibrated for 1 min and then ramped from 1 to 300 s⁻¹, while operating under conditions of steady state flow.

Viscosity was also modelled using Eiler's equation, as specified by Singh et al. (1997):

$$\mu = \mu_0 \left(1 + \frac{1.25 \sum \phi_i}{1 - \sum \phi_i / \phi_{\max}}\right)^2 \quad (\text{eq 4.2})$$

where μ is the viscosity of a milk product, μ_0 is the viscosity of a the portion of the product consisting of water and components smaller than lactose, ϕ_i is the volume fraction of an individual component (fat, casein, whey, lactose), ϕ_{\max} is an assumed value corresponding to the maximum packing of all particles (0.9 for fluid milk products).

Volume fraction is given by:

$$\phi_i = V_i c_{v,i} \quad (\text{eq 4.3})$$

where V_i is the voluminosity of each component (m³/kg) and $c_{v,i}$ is the concentration of each component in the product (kg/m³). Values of V for milk components were taken from Singh et al. (1997):

Fat globules ~ 1.1 x 10⁻³ m³/kg

Casein ~ 3.9 x 10⁻³ m³/kg

Whey proteins ~ 1.5 x 10⁻³ m³/kg

Lactose ~ 1 x 10⁻³ m³/kg

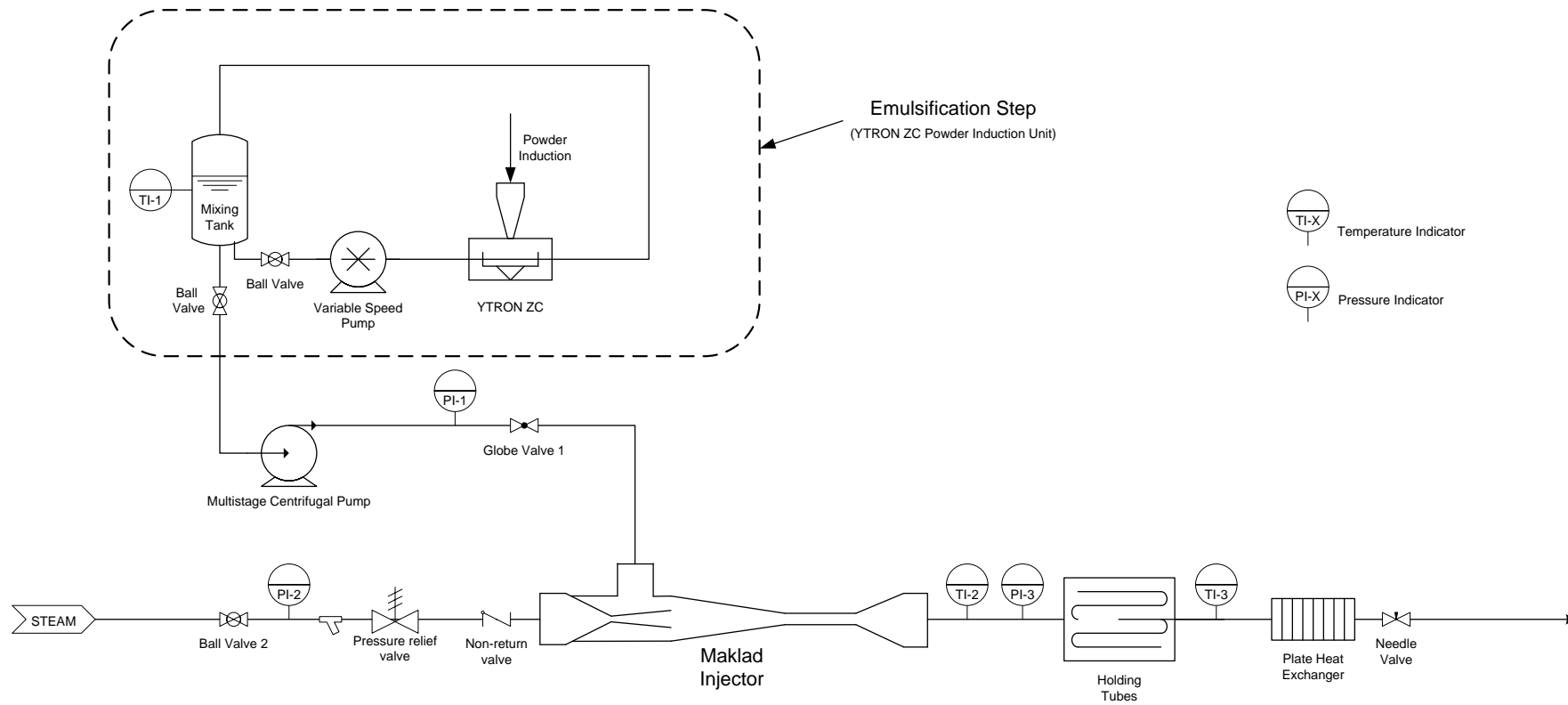


Figure 4.1. Process flow diagram for novel process, including emulsification step.

4.2.4.3 pH and density (ρ)

The pH of formulations was measured using a SenTix 41 electrode with automatic temperature compensation (WTW, Germany). Density was measured using a 100 mL glass density bottle. A correction factor was applied to allow for the expansion of glass with temperature

4.2.4.4 Emulsion stability

Stability pilot scale formulations to separation was measured using a LumiFuge 116 stability analyser (L.U.M GmbH, Berlin, Germany) as described by McCarthy et al. (2012). Samples were centrifuged at 1500 rpm (221 – 287 g, dependent on the distance from the rotor within the length of the measurement cell) for 7.5 h, simulating approximately 3 months ageing under conditions of normal gravity.

4.2.4.5 Specific heat capacity (C_p) and thermal conductivity (k)

Specific heat capacity of 20-60% (w/w) formulations was measured using a Q2000 differential scanning calorimeter (TA Instruments, Crawley, UK) which was calibrated using Indium standards. Samples were heated from 60 to 120 °C at 20 °C min⁻¹. Analysis was performed on 20-30 mg of sample hermetically sealed in aluminium pans. Thermal conductivity, as a function of DM content and temperature (T), was estimated using the following equation (Gavrilla et al., 2005):

$$k = 10^{-4}(0.1(100 - DM) + 1)(T) + 0.006(100 - DM) - 0.0186 \quad (\text{eq. 4.4})$$

4.2.4.6 High performance liquid chromatography separation of native whey protein

Separation of whey proteins was performed by reverse phase high performance liquid chromatography (RP-HPLC) at 28 °C with an Agilent Technologies 1200 Series system (Santa Clara, CA, USA). The column used was a 4.6 x 150 mm, XBridge BEH300 C4 with a particle size of 3.5 μm (Waters, Milford, MA, USA). The solvents used were – (A) Aqueous Phase: 0.1% trifluoroacetic acid (TFA) v/v%, and (B) Organic Phase: 90% acetonitrile (ACN), 0.1% TFA. The gradient used was as follows: 30% Organic Phase for 2.5 min, increasing to 50% Organic Phase over 12.5 min. Samples were diluted to approximately 0.125% w/v whey protein, adjusted to pH 4.6 before further dilution to 0.1% w/v (native and non-native) whey protein. Samples were filtered

through 0.2 µm Supor syringe filter (Pall Corporation, East Hills, NY, USA), and 10 µL of each was injected onto the column.

The orders of the denaturation reactions for α-La and β-Lg were determined as specified by Anema (2000). For $n = 1$, $\ln(C_t/C_0)$ is a linear function of time. For $n = 1.5$, $(C_t/C_0)^{-0.5}$ is a linear function of time. Where, n is order of reaction, C_t is concentration at time = t , and C_0 is concentration at time = 0.

4.2.5 Heat transfer properties

For illustrative purposes, the effect of DM content on heat transfer properties in a gasket plate heat exchanger was calculated using physical properties measured above. The methodology used was adapted from Naik and Matawala (2013). The gasket plate heat exchanger had a total heat transfer area of 61 m², with 30 channels (N_c) on the formulation side and a plate thickness (t) of 0.001 m. The channels were assumed to have a circular cross sectional area (CSA), therefore the characteristic length equal to $(4 \times \text{CSA})/\text{wetted perimeter}$ was the diameter, D , of the channels. The plates were constructed of stainless steel ($k_{\text{plate}} = 16.5 \text{ W}\cdot\text{m}^{-2}\cdot\text{K}$). Mass velocity of formulation per channel (G) was $296 \text{ kg}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The chevron angle (β) in the corrugated plates was 60°. Formulations were heated from 20 to 80 °C using water ($G_{\text{water}} = 400 \text{ kg}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Convective heat transfer was a function of Reynolds (Re) and Prandtl (Pr) numbers given by the following equations:

$$\text{Re} = \frac{GD}{\mu} \quad (\text{eq. 4.5})$$

$$\text{Pr} = \frac{C_p \mu}{k} \quad (\text{eq. 4.6})$$

The heat transfer coefficients of formulations (h_f) were calculated using the following relationship between Nusselt (Nu), Pr and Re numbers (Naik and Matawala, 2013):

$$\text{Nu} = \frac{h_f D}{k} = (0.335 - 0.105 \sin[3.8(\beta - 41)]) \text{Re}^{0.6} \text{Pr}^{0.333} \quad (\text{eq. 4.7})$$

The above equation is operational in the ranges of $50 < \text{Re} < 10000$ and $3 < \text{Pr} < 75$.

Thus, the overall clean heat transfer coefficient (U_{clean}) and heat transfer rate (Q) were calculated:

$$\frac{1}{U_{\text{clean}}} = \frac{1}{h_f} + \frac{1}{h_{\text{water}}} + \frac{t}{k_{\text{plate}}} \quad (\text{eq. 4.8})$$

$$Q = U_{clean} A \frac{(T_{water,hot} - T_{f,cold}) - (T_{water,cold} - T_{f,hot})}{\ln \left(\frac{T_{water,hot} - T_{f,cold}}{T_{water,cold} - T_{f,hot}} \right)} \quad (\text{eq. 4.9})$$

4.2.6 Statistical analysis

For pilot scale experiments, unless specified, differences between formulations before and after heat treatment were tested for significance using ANOVA (Minitab Ltd, Coventry, UK).

4.3 Results and Discussion

4.3.1 Effect of DM content on physical properties of IMF emulsions

Lab scale formulations were prepared at various DM contents using a rotor-stator mixer. Figure 4.2 shows that as DM content increased the particle size of emulsions was reduced. Rotor-stator homogenisation is not generally used for homogenisation of milk (Phipps, 1985); however, it would seem that as DM content increases so does the emulsification efficiency of rotor-stator mixers. The results indicate that rotor-stator mixers could be a viable alternative to conventional valve-type homogenisation at high DM content. However, for process optimisation and scale up, parameters governing droplet formation should be understood. The hydrodynamic conditions during mixing will affect the mechanism of oil droplet formation. In laminar flow, viscous shear forces (τ_v) deform fluid particles, leading to breakup and deformation. This is opposed by surface tension stresses (τ_s). The ratio of τ_v/τ_s is termed the Capillary (Ca) number, and, when Ca exceeds a critical value, droplet formation will occur. In turbulent flow, breakup is independent of viscous shear forces and caused by pressure fluctuations at droplet surfaces (Lemenand et al., 2013). Therefore, viscosity of fluid is a key consideration during rotor-stator homogenisation for determining the flow regime of mixing and the mechanism of subsequent droplet formation.

Apparent viscosity of IMF formulations was found to increase with DM content (Figure 4.3). Similar relationships have been observed in skim milk (Snoeren et al., 1984) and whole milk systems (Fernandez-Martin, 1972). Snoeren et al (1982) found that the viscosity could be modelled as a function of voluminosity of dispersed constituents. Casein was experimentally determined to be the more voluminous than native whey protein, $3.57 \times 10^{-3} \text{ m}^3 \cdot \text{kg}^{-1}$ compared to $1.07 \times 10^{-3} \text{ m}^3 \cdot \text{kg}^{-1}$. Compared to whole milk, the emulsions in the current study had lower total protein and casein contents which resulted in lower viscosity. Viscosity as a function of DM content started to increase significantly between 40 and 50% DM (w/w) compared to between 30 and 40% DM (w/w) in the whole milk system of Trinh et al. (2007). The relationship proceeded as predicted by Eiler's equation (eq 4.2) until DM content exceed 50% (w/w); at 60% (w/w) calculated viscosity was higher than experimental viscosity. This could be due to changes occurring in voluminosity of components as DM increases or because of some lactose sedimentation during viscosity analysis.

Re of the mixing operation was reduced from 30369 to 5562 as DM increased from 20 to 60% (w/w). In general, fully turbulent flows can be expected for $Re > 10^4$, therefore, it is likely that as DM content increased, the flow regime changed from turbulent to transitional (Coulson and Richardson, 1996). Transitional flow is complex, however, it is possible that the lack of fully turbulent flow at high DM allowed increased viscous forces to influence droplet disruption to a greater extent compared to the fully turbulent flows at low DM content, where viscosity has negligible effect on droplet formation.

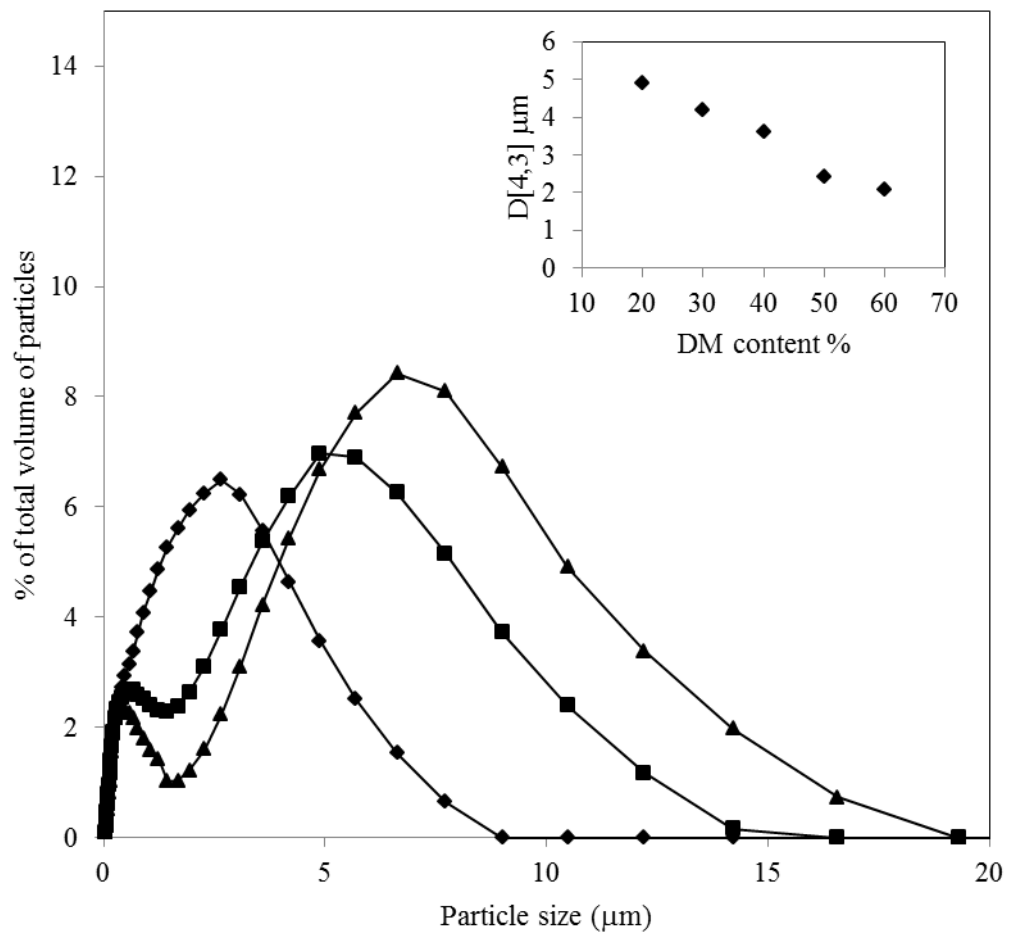


Figure 4.2: Effect of dry matter content on particle size distribution after rotor-stator mixing. The main figure shows particle size distribution of 60 (♦), 40 (■) and 20% (w/w) (▲) model IMF wet-mixes. The inset figure shows the effect of DM content on the volume mean diameter, $D[4,3]$, obtained.

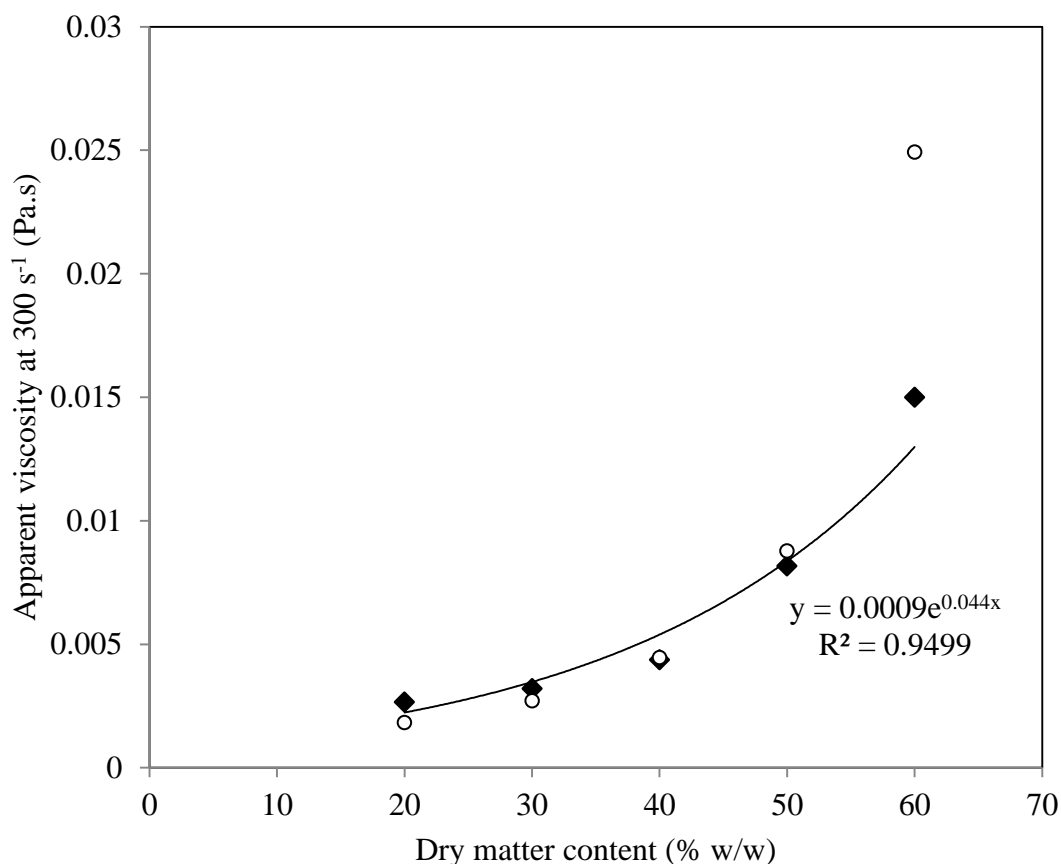


Figure 4.3. Effect of solids content on viscosity of IMF formulations at 60 °C; experimental values and associated exponential model (◆); values estimated using Euler's equation (○)

The effect of temperature on apparent viscosity of formulations is shown in Figure 4.4. At lower DM contents, apparent viscosity was reduced by temperature, however, at > 50% DM (w/w), apparent viscosity increased at 90 °C. This heating likely caused an increase in voluminosity of dispersed constituents as a result of whey protein denaturation, and, subsequent aggregation reactions both with itself and casein micelles (Anema and Li, 2003b). Heat exchangers may become fouled at high DM contents, owing to a combination of high viscosity, pH and interactions with mineral salts (Deeth and Haranto, 2009). Consequently, the relationship between concentration, temperature and viscosity increase is of extreme importance to IMF processing.

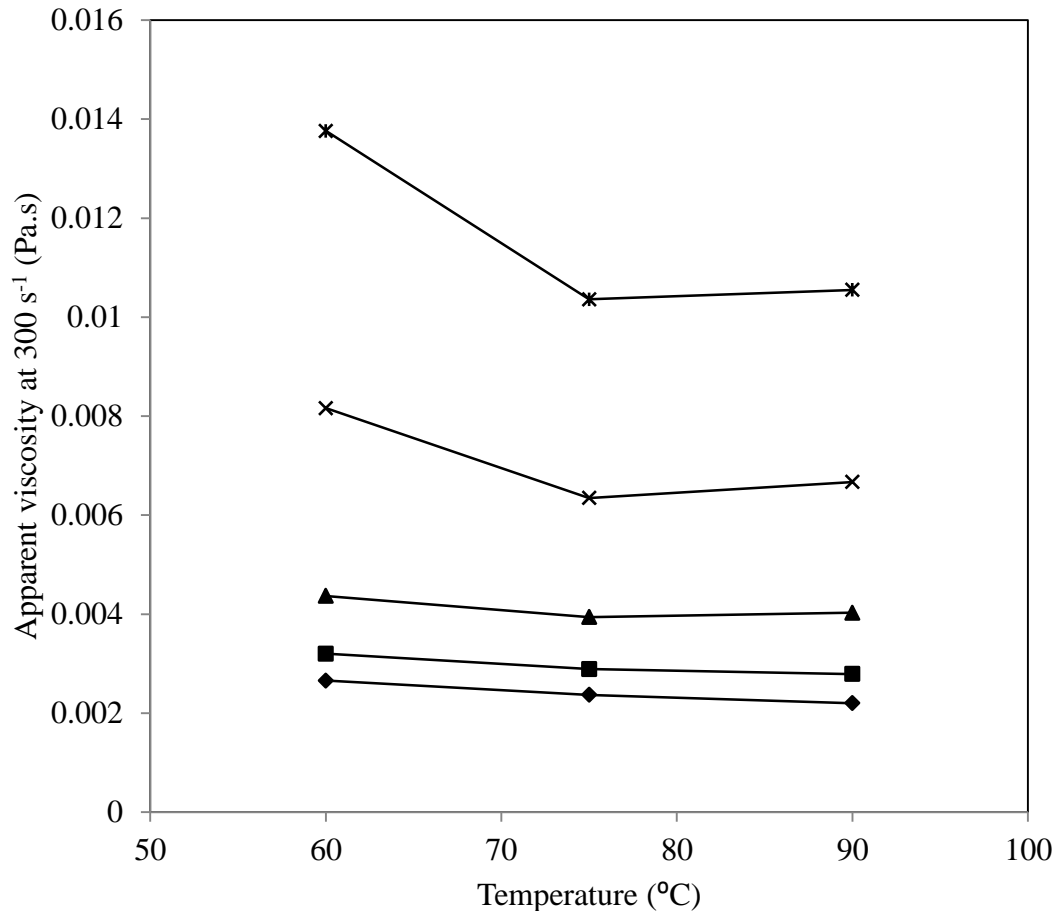


Figure 4.4. Effect of temperature on apparent viscosity of formulations of various DM contents. (♦) 20% (w/w); (■) 30% (w/w); (▲) 40% (w/w); (x) 50% (w/w); (*) 60% (w/w)

Figure 4.5 shows the pH of formulations decreased with increasing temperature and/or DM content due to calcium phosphate precipitation. Solubility of calcium phosphates decreases with increasing temperature and the release of $[H]^+$ ions during precipitation which has been suggested to account for approximately 60% of the temperature induced acidification of milk (Chaplin and Lyster, 1988). The pH may also drop as a result of temperature induced changes between sensing and reference electrodes used in pH measurement (Barron et al., 2006). Similarly, when DM content is increased, various forms of calcium phosphate can become saturated in solution and precipitate, releasing $[H]^+$ ions which can reduce pH. A linear model was applied to the data, resulting in the following equation ($R^2 = 0.93$):

$$\text{pH} = 7.19 - 0.011\text{DM} - 6.84 \times 10^{-3}\text{Temperature}$$

While the linear model is broadly correct, the lines in Figure 4.5 do not fit exactly with the experimental data. For a more accurate model, the interaction between the effect of

DM and Temperature should be considered i.e., it appears that the effect of temperature was greater at higher DM content.

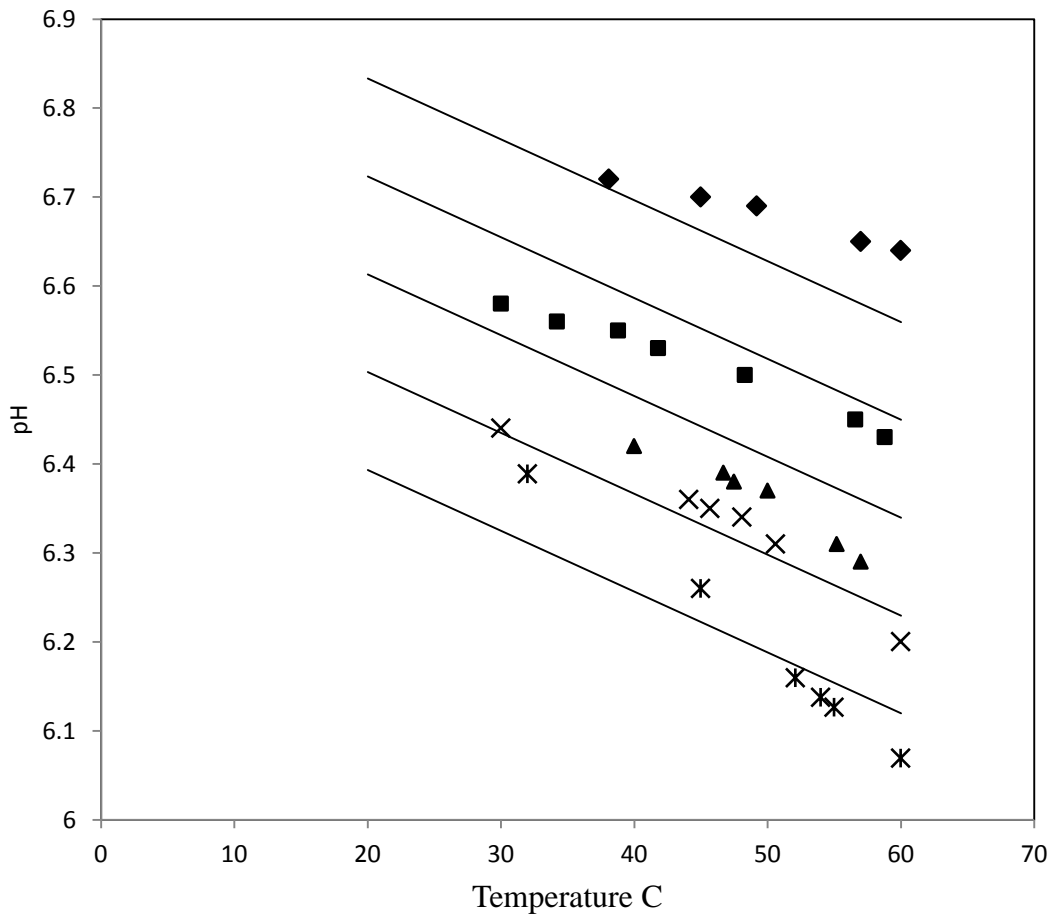


Figure 4.5. Effect of DM content and temperature on pH of formulations (♦) 20% (w/w); (■) 30% (w/w); (▲) 40% (w/w); (x) 50% (w/w); (*) 60% (w/w). Straight lines illustrate the linear model fitted.

Density of formulations increased with increasing DM content and decreased with increasing temperature (Figure 4.6). The effect of DM content was due to the relatively higher densities of non-aqueous components such as lactose and protein, 1780 kg and 1400 m⁻³ respectively (Walstra et al., 1984). Sunflower oil has a lower density than water, however, the quantities of lactose and proteins in formulations were enough to counteract the tendency of sunflower oil to reduce density. The decrease in density with temperature was a result of the thermal expansion of the components, most significantly, water (McCarthy and Singh, 2009).

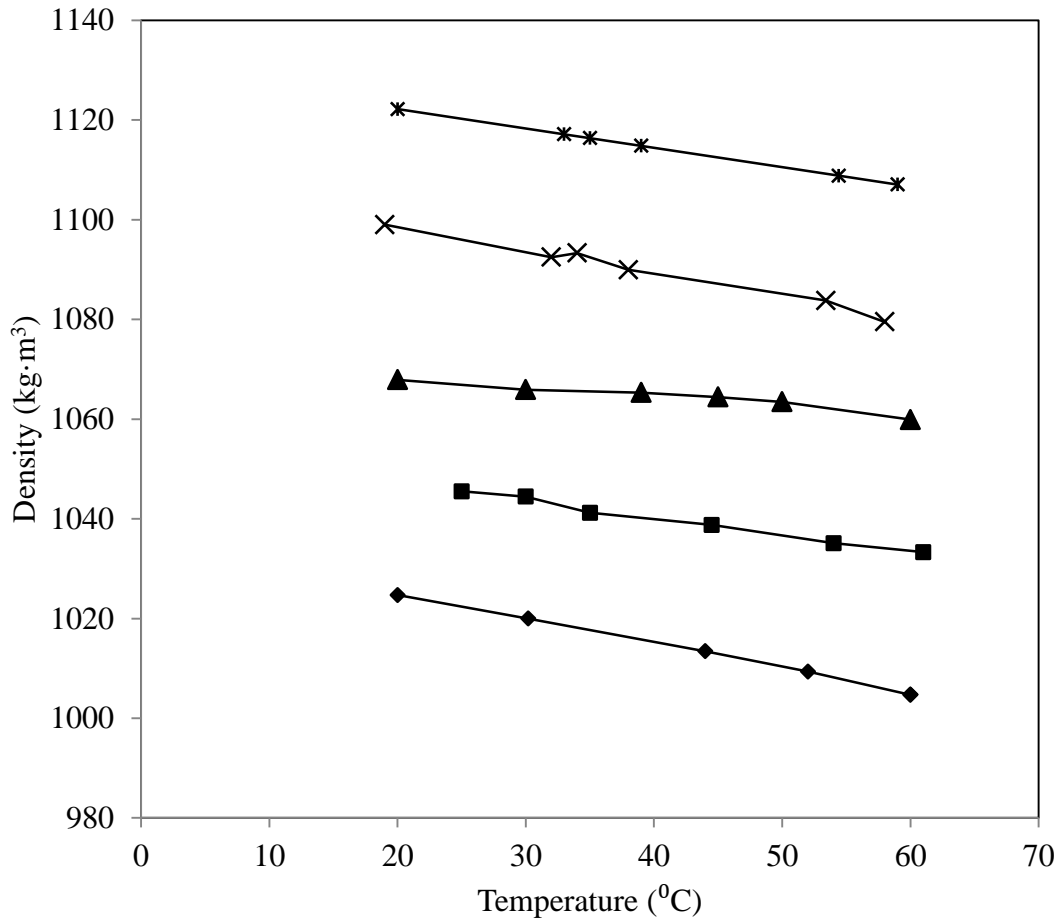


Figure 4.6. Effect of temperature on density of formulations (◆) 20% (w/w); (■) 30% (w/w); (▲) 40% (w/w); (x) 50% (w/w); (*) 60% (w/w)

4.3.2 Effect of DM content on heat induced changes

Formulations were heated in a starch pasting cell at temperatures of 80 °C and 95 °C for 5 min (Figure 4.7). Large viscosity increases were observed when high DM content formulations were heated at 95 °C; 50 and 60% (w/w) formulations were visibly aggregated after heating (Figure 4.7a). When heated at 80 °C, high DM content formulations were stable to heat-induced viscosity increase, in fact, viscosity of formulations was reduced after heat treatment (e.g. 56.7 mPa s to 50.6 mPa s; not visible in Figure 4.7b), possibly as a result of solubilisation of lactose, which was not fully solubilised at 40 °C for 60% (w/w) formulations.

The large viscosity increases in high DM content formulations heated at 95 °C was correlated to greater losses in native whey content during heating (Figure 4.8) – after 5 min heating at 95 °C, 60% (w/w) formulations lost approximately 3 times more native β -Lg compared to heating for the same time at 80 °C. At 95 °C, the observed rate of

denaturation of β -Lg was similar at 20% and 60% (w/w); however, at 80 °C, the rate of denaturation of β -Lg was retarded at 60% (w/w). These findings are consistent with the work of Anema (2000); at 80 °C, the rate-determining step in β -Lg denaturation is thought to be the unfolding of monomeric β -Lg, however, it has been suggested that the presence of lactose results in a shift towards a dimeric form as this decreases thermodynamically undesirable contact between protein and lactose (Timasheff, 1993). The net effect is a reduction of the reactive monomeric form and thus a retardation of denaturation at higher lactose/DM contents. At higher temperatures the rate determining step is aggregation of unfolded β -Lg (Dannenberg and Kessler, 1988), therefore, denaturation of β -Lg was less effected by DM content when heated at 95 °C. Denaturation of α -La was not dependent on DM content, in accordance with Anema (2001). In keeping with previous studies, the order of reaction for β -Lg denaturation was 1.5, compared to 1 for α -La denaturation (Anema 2000, 2001)

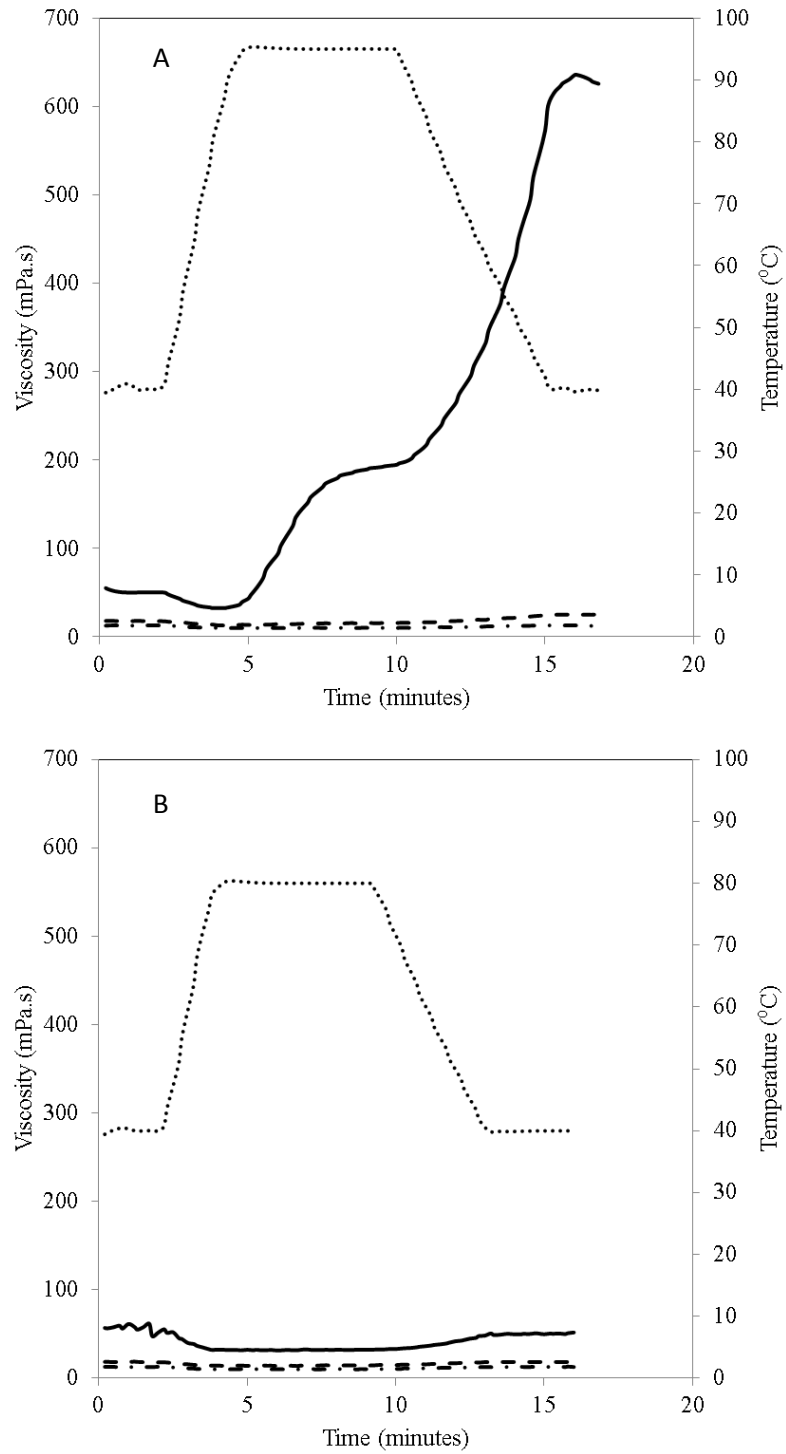


Figure 4.7. Effect of DM content and heat on viscosity of formulations. Samples heated for 5 min at: A – 95 °C; B – 80 °C. 60% (w/w) (solid trace); 40% (w/w) (dashed trace); 20% (w/w) (dash-dot trace); Temperature (dotted trace)

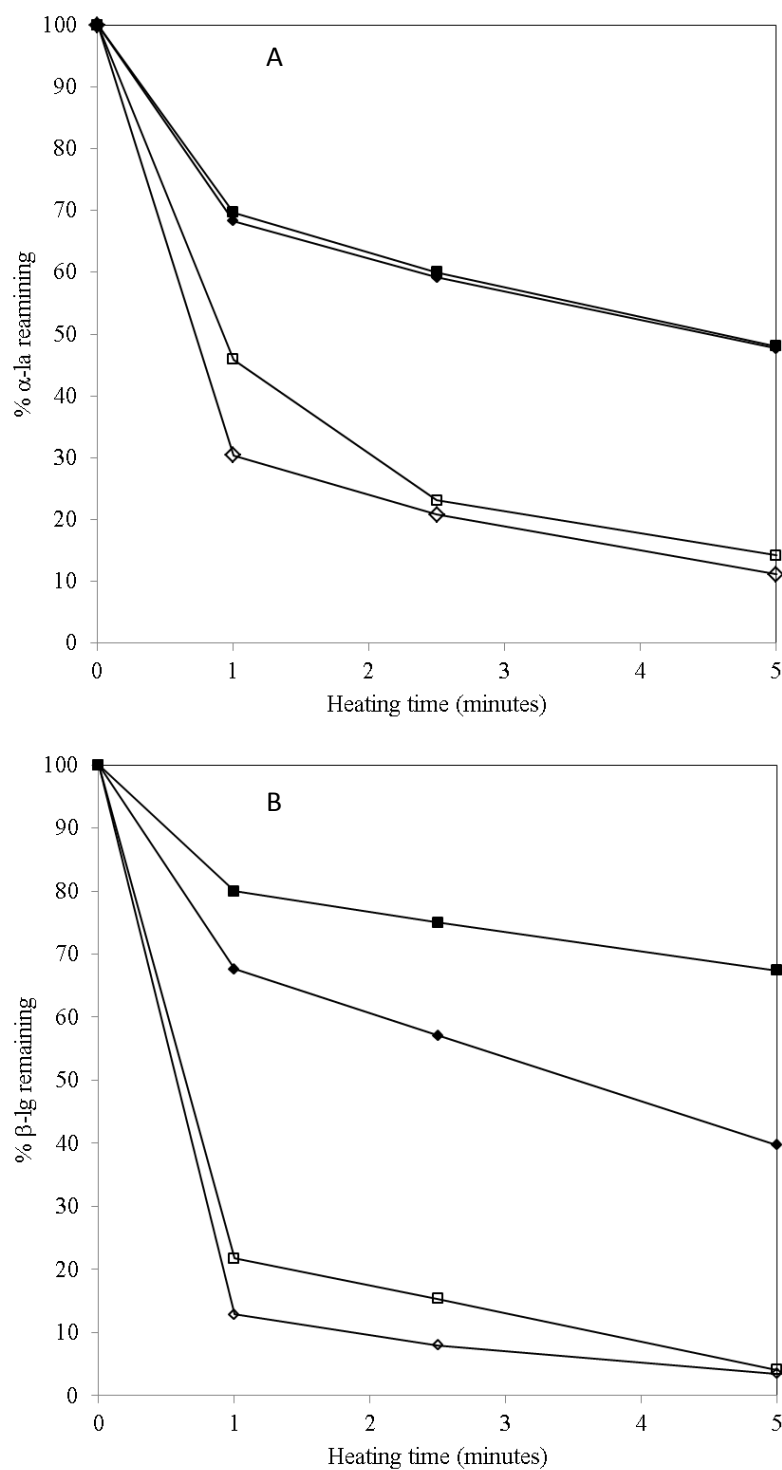


Figure 4.8. Effect of DM content on loss of native α -La (A) and β -Lg (B) at 80 °C (60% (w/w) [■]; 20% (w/w) [◆]) and 95 °C (60% (w/w) [□]; 20% (w/w) [◇]).

4.3.3 Effect of DM content on heat transfer properties

Figure 4.9 shows, in accordance with Fernandez-Martin (1971), heat capacity of formulations decreased as a function of DM content. Heat capacity measures the amount of energy taken up by a substance's molecules as the temperature rises; if more energy is absorbed by, for example, breaking of intermolecular bonds, the heat capacity will increase. When water is heated, energy is taken up due to the breaking of hydrogen bonds, resulting in a high heat capacity (Edsall and Wyman, 1962). Therefore as the water content of the formulations was reduced the amount of energy required to raise the temperature decreased.

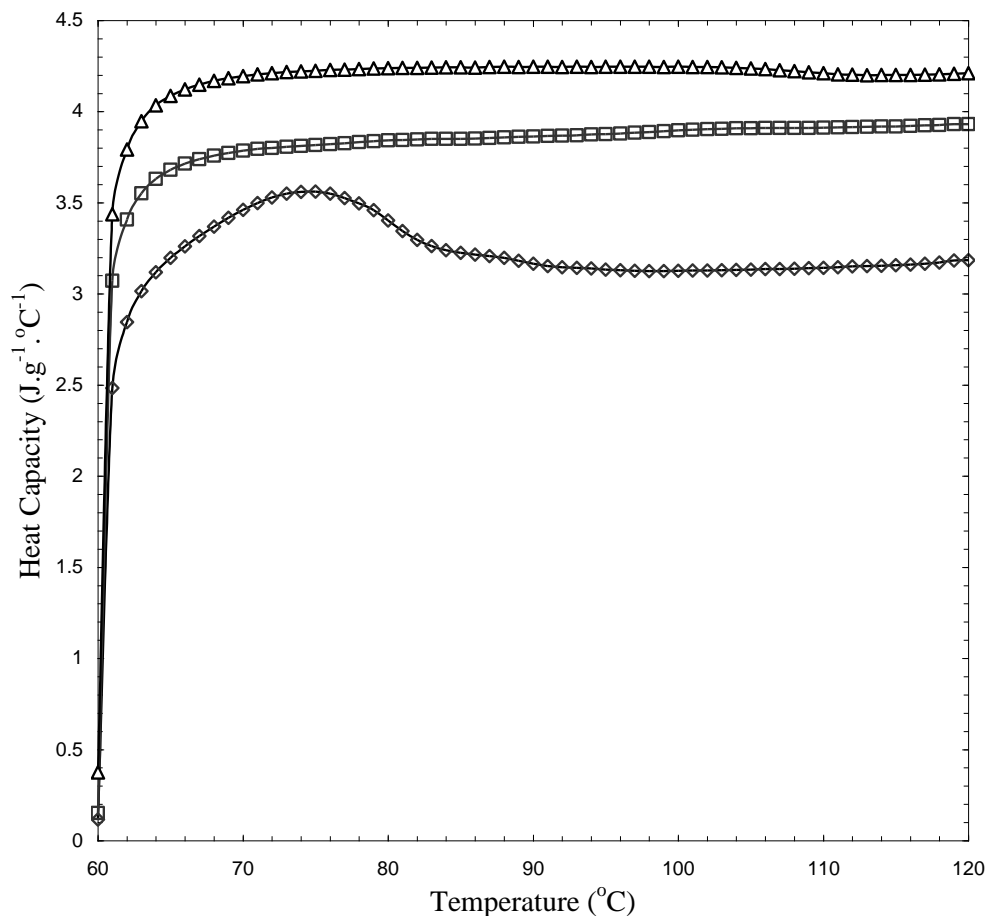


Figure 4.9. Heat capacity plotted against temperature for 60% (w/w) (◇), 20% (w/w) (□) and water (Δ).

50 (not shown in Figure 4.9) and 60% (w/w) formulations exhibited heat capacity peaks in the range of 60-95 °C, as shown in Figure 4.9 (for 60% w/w). These formulations were visibly gelled after DSC analysis. Based on the temperature range over which the peak appeared and its endothermic nature it is likely that it shows denaturation

of whey proteins. The aggregation involved in gelation is exothermic; however when gelation is observed using conventional DSC, its peak is dwarfed by the denaturation reaction and, hence, was not observed (Fitzsimons et al., 2007).

The reduction in specific heat capacity with increasing DM content will be accompanied by a reduction in thermal conductivity. The changes in physical properties outlined in previous sections were used to calculate the overall effect of increasing DM content on heat transfer. Figure 4.10 shows the effect of increasing DM content on Reynolds (Re.) number and Prandtl (Pr.) number in the plate heat exchanger (PHE) described in section 4.2.5. The flowrate through the PHE was 22 kg s^{-1} of formulation, which was to be heated from 20 to 80 °C. A temperature of 80 °C was chosen because it is close to HTST pasteurisation temperature and also because the physical properties were more stable at this temperature than at 90+ °C (see Figure 4.7). The change in Reynolds number was greater in magnitude than the change in Prandtl number, which is particularly significant in calculating the heat transfer coefficient of the formulation. As shown in equation 4.4 the exponent on Reynolds number is 0.6, compared to 0.33 for Prandtl number. As a result, the formulation heat transfer coefficient, and thus, the overall heat transfer coefficient (U_{clean}) decreased with increasing DM content. The overall effect of this was that at higher DM contents the required rate of heat transfer was not achievable with the current PHE – a larger heat transfer area would be required to carry out the required heating (Figure 4.11). Note: it should be stated that at 60% (w/w) Pr was approximately 3 times in excess of the maximum value for which eq 4.7 was tested. However, it would still be expected that as the turbulence decreases the overall heat transfer coefficient would be reduced resulting in insufficient heat transfer.

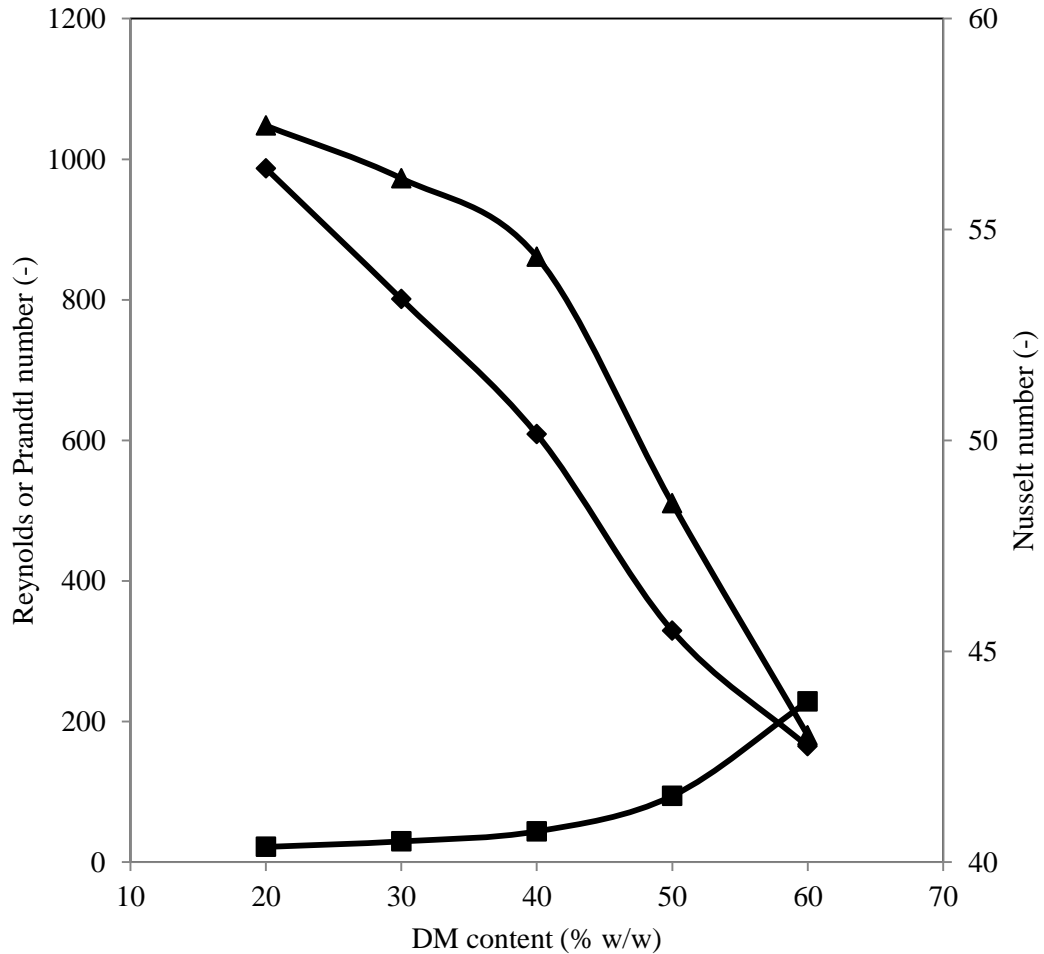


Figure 4.10. Effect of increasing DM content on Reynolds (◆) Prandtl (■) and Nusselt (▲) numbers

The above example illustrates the dependence of heat transfer on $Re.$, which at constant mass flowrate is a function of viscosity only (see equation 4.2). Figures 4.4 and 4.7 show that viscosity of formulations is likely to remain constant at the temperature used in calculations, however, at > 90 °C large viscosity increases during heat treatment at high DM content could further reduce heat transfer properties. Furthermore, these viscosity increases are the result of denaturation/aggregation of whey, particularly β -Lg. It has been reported that unfolded and/or aggregated β -Lg adhering to walls in processing plants is a major factor in fouling (De Jong et al., 1992; Changani et al., 1997; De Jong, 1997). Fouling results in a lower overall heat transfer coefficient ($U_{dirty} < U_{clean}$), therefore, at high DM contents a process where denaturation of whey proteins is reduced is desirable.

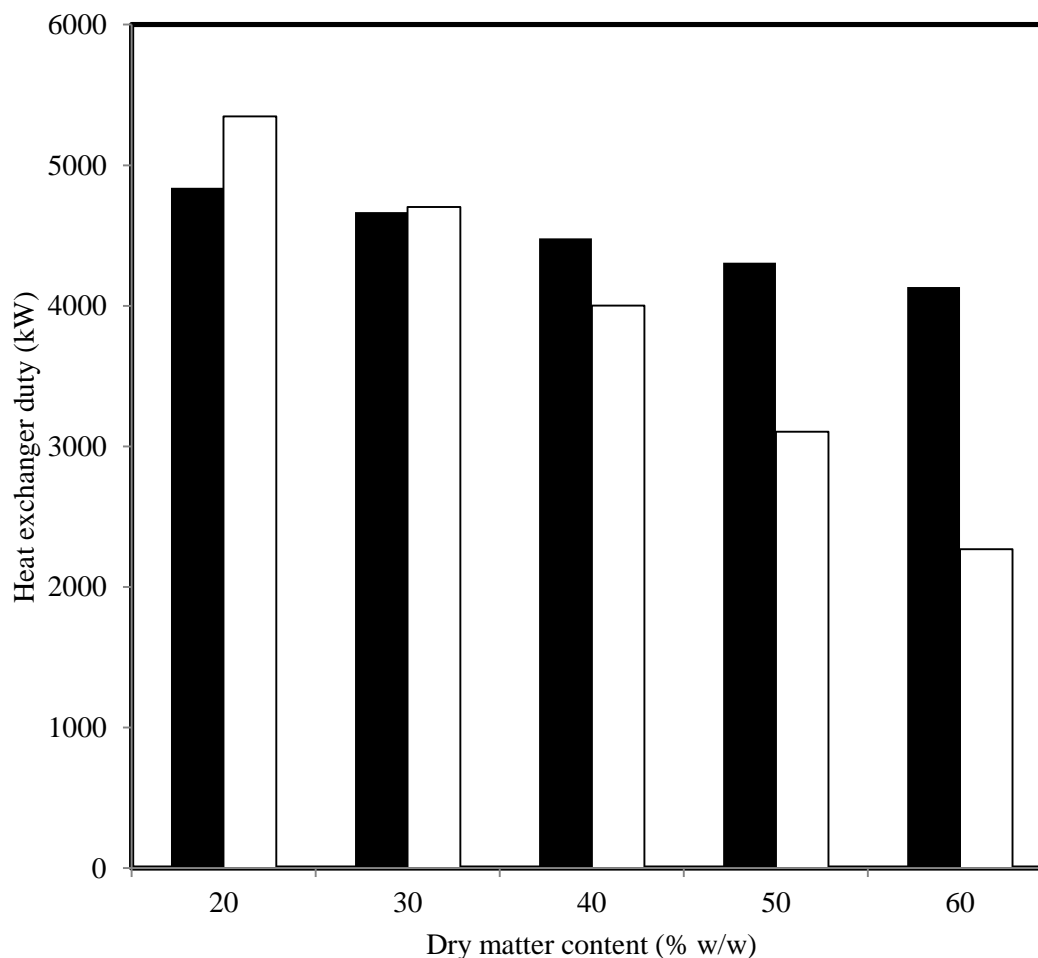


Figure 4.11. Effect of DM content on the performance of a plate heat exchanger. Required heat transfer rate (black columns); achievable heat transfer rate (white columns)

4.3.4 Novel processing path for high DM formulations – direct steam injection

20% and 60% (w/w) formulations were prepared by a novel processing path which utilised rotor-stator homogenisation in combination with direct steam injection (Figure 4.1). Table 4.3 shows the physical properties of formulations pre and post-heat treatment. Similar to lab scale trials, rotor-stator homogenisation produced a stable emulsion at 60% (w/w). For 20% (w/w) formulations, rotor-stator homogenisation was carried out at 60% (w/w) followed by dilution, due to insufficient emulsification at 20% (w/w). This is in keeping with the observations made from Figure 4.2 that rotor-stator homogenisation is more efficient at high DM contents. As result of the condensation of steam during the direct heat treatment, the formulations were diluted post heat treatment by approximately 4 and 2% (w/w) for 60 and 20% (w/w) formulations, respectively.

Table 4.3. Properties of formulations pre and post-heat treatment¹

Stage	Particle size D[4,3]	DM % (w/w)	Viscosity ² @300 s ⁻¹ mPa.s
Pre HT - 60% Trials	1.28 ± 0.03	59.09 ± 0.13	1.93 ± 0.06
Post HT - 60% Trials	1.10 ± 0.03	54.76 ± 0.49	2.08 ± 0.10
Pre HT - 20% Trials	1.57 ± 0.43	20.00	1.84 ± 0.04
Post HT - 20% Trials	1.14 ± 0.12	17.80 ± 0.10	1.85 ± 0.15

¹Analysed using two-way ANOVA (factors: DM content and heat treatment)

²Standardised to 12.5% (w/w)

Values reported are the means of three replicate trials ± standard deviations

A homogenisation effect was found to occur within the injector, heat treatment was found to significantly ($P < 0.05$) reduce D[4,3] of formulations. This was most likely due to shear created within the injector. As shown in Adamopoulos and Petropakis (1999) cavitation occurs at various points along an injector system including the point of steam condensation in the narrow section of the injector. A flowing fluid (in this case a two-phase mixture of steam and formulation) has both a dynamic and static pressure; when dynamic pressure exceeds static pressure cavities of gas or vapour are formed in the flow which, when subjected to rapid increases in pressure implode producing localised high pressures and temperatures i.e. cavitation. The degree of cavitation has been reported to play an important role in fat globule size reduction (Kessler, 2002).

DM content of formulations was standardised to 12.5% (w/w) for comparative purposes. Table 4.3 shows that heat treatment of 60% (w/w) formulations resulted in the highest viscosity. In contrast, heat treatment of 20% (w/w) formulations did not increase viscosity. This was in keeping with observations made in Figure 4.7 i.e., viscosity of 60% (w/w) formulations increased markedly during heating at 95 °C for 5 min compared to 20% (w/w) formulations where viscosity did not increase over the course of heating. However, no significant ($P = 0.922$) increase in the 20% (w/w) formulations was observed. Viscosity increase during heating by direct steam injection was correlated with whey protein denaturation. Figure 4.12 shows that 60% (w/w) formulations lost significantly ($P < 0.05$) more β -Lg during heat treatment than 20% (w/w) formulations. Oldfield (1996) also found β -Lg denaturation increased with DM content for skim milk heated at 110 °C. As aggregation reactions are thought to be rate limiting at > 100 °C this

suggests that, in the current study, denaturation of whey protein increased with DM content due to an increase in aggregation reactions (Dannenberg and Kessler, 1988).

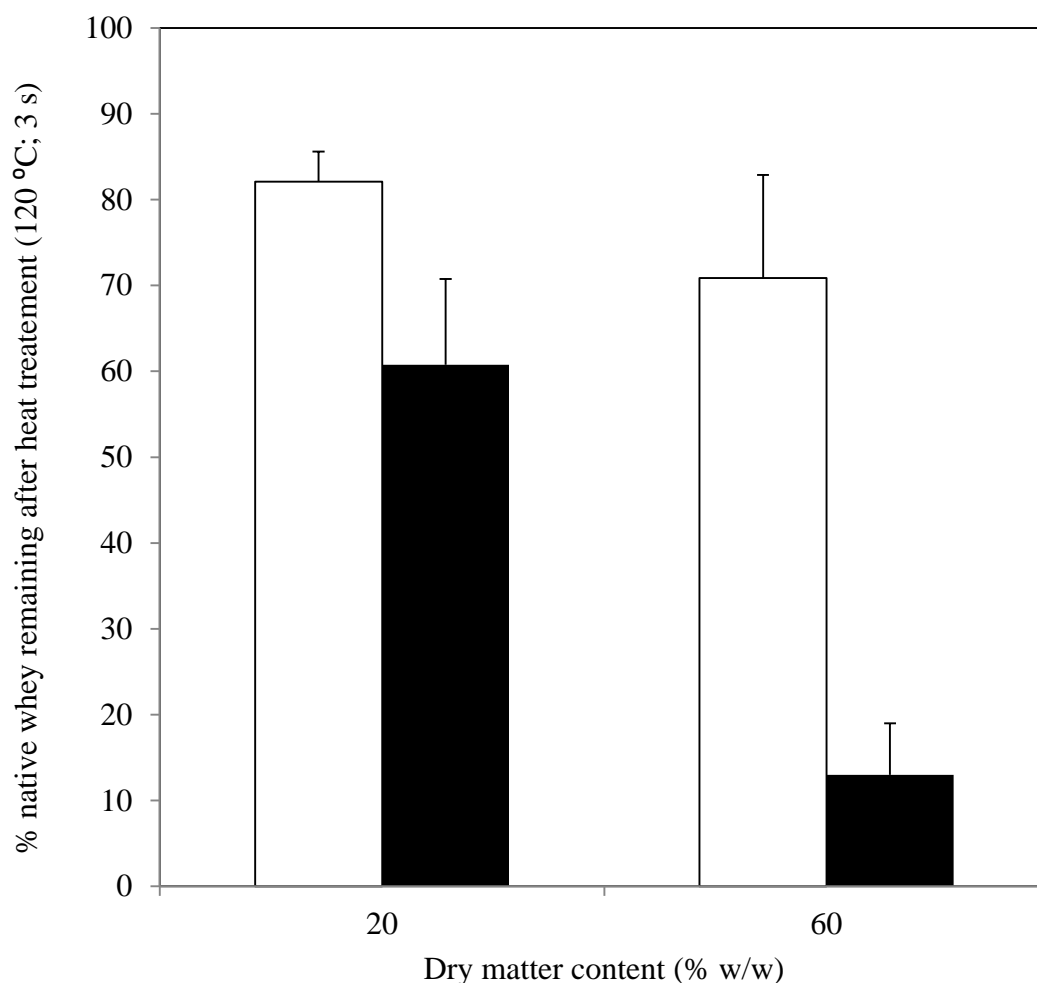


Figure 4.12. Effect of DM content on the loss of whey protein during heat treatment by direct steam injection (α -La – white columns; β -Lg – black columns). Values reported are the means of three replicate trials \pm standard deviations

Figure 4.13 shows heat treated 60% (w/w) formulations had the lowest creaming rate, due to the homogenisation effect and viscosity increase incurred by steam injection. The creaming rate, $0.32 \pm 0.05 \text{ mm} \cdot \text{day}^{-1}$, was similar to reported values for reconstituted (12% w/w) model IMF manufactured using a conventional process, consisting of heat-treatment and valve-type homogenisation at 42% (w/w) followed by evaporation and spray drying (McCarthy et al., 2012). This demonstrated that the novel processing path was capable of producing a stable emulsion without using conventional valve-type homogenisation.

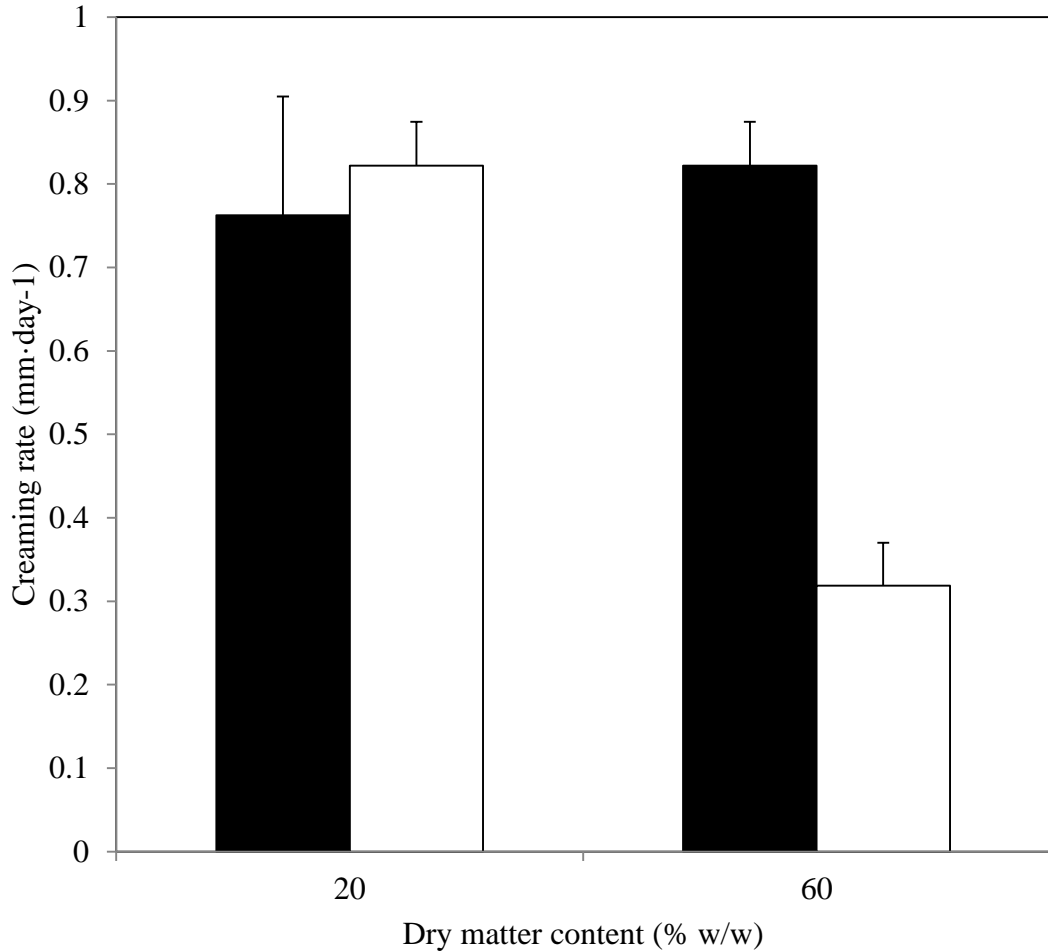


Figure 4.13. Creaming rates of 20% and 60% (w/w) formulations pre (black columns) and post (white columns) heat treatment by direct steam injection (DM standardised to 12.5% w/w). Values reported are the means of three replicate trials \pm standard deviations.

Formulations heated by novel processing path were compared to similar formulations heated by an indirect tubular heat exchanger. The apparent viscosity (at a standardised DM content of 12.5%) of indirectly heated 60% (w/w) formulations was significantly ($P < 0.05$) higher (2.37 ± 0.06) than formulations heated using direct steam injection (2.08 ± 0.10). This was due to lower losses in native α -La and β -Lg arising from steam injection, $50.0 \pm 1.1\%$, compared to $70.5 \pm 1.4\%$ for indirect heat treatment. Therefore, direct heat treatments should be utilised at high DM content to reduce denaturation and viscosity of concentrates.

4.4 Conclusion

Increasing DM content of model IMF formulations had a significant effect on physical properties. Viscosity of formulations increased exponentially with increased DM content, resulting in a decrease in heat transfer efficiency by forced convection. Rotor-stator homogenisation efficiency was increased at higher DM contents.

When heated at < 90 °C increasing DM resulted in less denaturation of β -Lg and viscosity of formulations were not sensitive to heat treatment. At > 90 °C, increasing DM content did not reduce β -Lg denaturation; 50 and 60% (w/w) formulations were sensitive to heat-induced viscosity increases as a result of denaturation/aggregation of whey proteins.

A novel processing path was designed for production of high DM content formulations using rotor-stator homogenisation and direct steam injection (120 °C for 3 s). The novel path produced physically stable formulations, and, while viscosity increases and denaturation were greater at higher DM contents, the extent of these heat-induced changes were significantly ($P < 0.05$) lower than in indirectly heated formulations of equivalent DM content. Therefore, the processing path provided a feasible means of producing lower viscosity high DM content formulations and could reduce fouling associated with high viscosity and denaturation of whey proteins.

Chapter 5: A high-solids steam-injection process for the manufacture of powdered infant milk formula

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ORIGINAL PAPER

A high-solids steam injection process for the manufacture of powdered infant milk formula

**Eoin G. Murphy · John T. Tobin · Yrjo H. Roos ·
Mark A. Fenelon**

Abstract

Introduction of more energy efficient processing practices, such as increasing the initial solids content from which powder is manufactured, are of interest to the infant formula industry. This study evaluated the use of an inline rotor-stator mixer followed by direct steam-injection to disperse and heat-treat (110 °C, 3 s) high-solids (60% w/w) formulations, for the production of powdered infant milk formula. As a control, 30% (w/w) infant milk formulations were subjected to a typical process i.e. heat-treatment in a tubular heat exchanger, valve-type homogenisation, evaporation (to 55% w/w solids content) and spray drying. Both formulations were dried using a 3-stage dryer with two-fluid nozzle atomisation at inlet and outlet temperatures of 187 and 85 °C respectively. Formulations subjected to the steam-injection process had significantly ($P < 0.05$) lower viscosity compared to control formulations at equivalent solids contents (55% w/w). This was partly attributed to lower levels of whey protein denaturation ($76.2 \pm 0.09\%$) compared to indirect heat-treatment in the control process ($87.0 \pm 0.5\%$) as measured by high-performance-liquid-chromatography. Prior to spray drying, volume-mean particle size of both processes was not significantly different ($P > 0.05$) – 1.42 ± 0.46 and 1.29 ± 0.01 μm for the control and high-solids steam-injection processes respectively. Powders produced by both processes had statistically similar ($P > 0.05$) surface free fat content, wettability and dispersibility. The study showed that it is possible to produce quality model infant milk formula powders from a high-solids concentrate while considerably reducing process complexity.

5.1 Introduction

Companies which manufacture powdered infant milk formula (IMF) must comply with strict microbiological regulations while ensuring good powder reconstitution properties. Current manufacturing processes are often adapted from well-established unit operations within the dairy industry, which can vary between manufacturers (Pisecky, 1997). It is, however, in the interest of IMF manufacturers to explore alternative processes, which offer improvements in efficiency and nutritional quality while meeting the requirements outlined above.

The behaviour of ingredients during processing is an important consideration when investigating an alternative process or unit operation. Bovine milk derivatives are the principle ingredients in the manufacture of IMF (Nasripour et al., 2006); however, the distribution of macronutrients in bovine milk means that it is not ideal for consumption by infants, especially between the ages of 0 - 6 months. To comply with nutritional regulations, protein-to-carbohydrate ratio and casein-to-whey ratio of bovine milk must be altered by addition of whey and lactose ingredients (Nasripour et al., 2006). In formulations where the level of whey protein is relatively high, the method of heat-treatment is a key consideration as β -Lactoglobulin (β -Lg), the most abundant whey protein in bovine milk, denatures at heat-treatment temperatures (generally above 70 °C) leading to increases in viscosity, and gelation above a critical protein concentration (Walstra and Jenness, 1984; Singh and Havea, 2003). Denatured whey proteins have also been linked to emulsion instability, acting as a bridging mechanism between fat globules (Euston et al., 2000). The behaviour of whey proteins during heat-treatment of IMF can significantly influence viscosity and particle size which may also affect subsequent powder properties.

A common heat-treatment method used in the dairy industry is indirect heat-treatment, which involves heat transfer through an interface (typically stainless steel) into the product. Alternatively, direct heat-treatment can also be used; in this scenario, the product comes into direct contact with the heating medium, for example, steam-injection and steam-infusion (Bylund, 1995c). Zadow (1969) showed that for whole bovine milk indirect heat-treatment resulted in higher levels of whey protein denaturation than direct heat-treatment. The reduced thermal load of direct heat-treatment is due to the almost instantaneous achievement of heat-treatment temperature associated with these treatments (Lewis and Deeth, 2009). In addition, direct heat treatments have also been shown by

several studies to disrupt fat globules and reduce their size (Zadow, 1969; van Boekel and Folkerts, 1991; Ye et al., 2005).

In the manufacture of powdered IMF, formulations are often evaporated prior to spray drying. Ye et al. (2004) showed that evaporation disrupted the fat globules of pasteurised whole fat milk resulting in a decrease in fat globule size and an increase in adsorption of caseins and whey to the fat globule surface. In contrast, evaporation of a homogenised whey dominant IMF has been shown to increase fat globule size (McCarthy et al. 2012).

In some circumstances, dependent on logistic or seasonal influences, skim milk powder (SMP) is used as the casein source for IMF, rather than liquid or concentrated skim milk. In this scenario, formulations can be prepared over a wide range of concentrations, depending on the capabilities of processing equipment. Higher concentration formulations require less water removal during powder manufacture, and, under certain conditions it may be possible to proceed to spray drying without an evaporation step. However, the extent of whey protein denaturation during heat-treatment, viscosity increase, and associated fouling, is dependent on concentration of whey protein (Dannenberg and Kessler, 1988; Fryer, 1989). Therefore, in order to avoid excessive viscosity increases and fouling at high-solids contents, the thermal load associated with heat-treatment has to be minimised, while ensuring compliance with microbiological regulations.

In Chapter 4, a process was developed utilising a rotor-stator dispersion step in combination with direct steam-injection to heat-treat model IMF at high-solids concentrations (60% w/w). The steam-injector used was a patented (Maklad Fluid GmbH) injector/ejector system similar to those described by Adamopoulos and Petropakis (1999). Within the injector, a shockwave front is formed as a result of steam condensation, providing a homogenisation effect capable of reducing the fat globule size, resulting in colloidally stable high-solids content formulations. These formulations, however, were not spray dried to produce powdered IMF.

The aim of this study was to manufacture model IMF powders utilising the high-solids (60% w/w) steam-injection (HSSI) approach developed in Chapter 4. This novel process, which did not include an evaporation step, and the powders produced were compared to a typical industrial process, consisting of indirect heat-treatment at 30% (w/w) solids, followed by homogenisation, evaporation and spray drying.

5.2. Materials and Methods

5.2.1 Materials

Medium heat skim milk powder (SMP) and lactose were supplied by Glanbia Food Ingredients (Ballyragget, Co. Kilkenny, Ireland). Demineralised whey powder (DWP) was supplied by Dairygold Food Ingredients (Mitchelstown, Co. Cork, Ireland). Sunflower oil was supplied by Trilby Trading (Drogheda, Co. Louth, Ireland). Potassium hydroxide (KOH) was supplied by Sigma Aldrich Ireland (Arklow, Co. Wicklow, Ireland).

5.2.2 Batch Formulation

Batches (150 kg) of model IMF were formulated to a solids content of 60% (w/w) (HSSI process) and 30% (w/w) (control process) using the ingredients and quantities outlined in Table 5.1. Reverse osmosis (RO) water was heated (to 55 °C for the control process and 70 °C for the HSSI process) in order to aid solubilisation of ingredients. The order of ingredient addition during batch make-up was lactose, DWP, sunflower oil followed by SMP. The target composition (on a moisture-free basis) of the powders manufactured from both processes was 11.3, 29.1 and 58.1% (w/w) for protein, fat and lactose contents respectively. The target whey to casein ratio was 60:40.

A YTRON ZC powder induction unit (YTRON Process Technology GmbH, Bad Endorf, Germany), consisting of a high shear rotor-stator mixer connected to a recirculation pump, was used for ingredient induction. For both HSSI and control processes, powders and sunflower oil were inducted under vacuum into the rotor-stator mixer which was connected to a mixing tank via a closed circulation loop. After ingredient induction, formulations were recirculated through the rotor-stator mixer at approximately 2 kg s^{-1} for a further 10 min to disperse the ingredients. This corresponded to 8 passes through the mixer. The pH of HSSI (60% w/w) formulations was adjusted to 6.6 (at 60 °C) using $4 \text{ mol}\cdot\text{L}^{-1}$ KOH; an equivalent amount of KOH (i.e. the ratio of KOH to IMF solids was the same) was added to the 30% (w/w) formulations.

Table 5.1. Ingredient composition and quantities used in processing

	SMP	DWP	Lactose	SO	Water
	Ingredient composition (kg·kg ⁻¹)				
Casein	0.28	0.00	0.00	0.00	0.00
Whey	0.07	0.13	0.00	0.00	0.00
Lactose	0.52	0.83	0.99	0.00	0.00
Fat	0.01	0.01	0.00	1.00	0.00
Moisture	0.04	0.03	0.01	0.00	1.00
Ash	0.08	0.01	0.00	0.00	0.00
	Quantities used in processing (kg)				
Control (30% w/w)	7.29	20.40	5.54	12.82	103.95
HSSI (60% w/w)	14.57	40.80	11.08	25.64	57.90

SMP skim milk powder, *DWP* demineralised whey powder, *SO* Sunflower oil, *HSSI* high-solids steam-injection

5.2.3 Heat-treatment

HSSI formulations (60% w/w) were heat-treated using a shockwave steam-injector (Maklad Innovative Fluid & Systemtechnik GmbH, Wien, Austria) at 110 °C for 3 s. The injector was part of a custom built pilot line consisting of a pressure sensing multistage pump CRNE3-19 (Grundfoss Ltd, Dublin, Ireland) which delivered the formulation, at constant pressure (0.70 MPa), to the steam-injector. Steam at approximately 0.65 MPa was injected into the formulation at high velocity. After steam-injection the formulation was passed through a holding tube of approximately 1.75 L before being cooled to ~ 55 °C in a plate heat exchanger prior to spray drying. Control, 30% (w/w) formulations were heat-treated at 110 °C for 3 s using a MicroThermics tubular heat exchanger (MicroThermics, North Carolina, U.S.A.).

5.2.4 Homogenisation

Control formulations (30% w/w) were homogenised using two-stage valve-type homogeniser, Model NS2006H (Niro Soavi, Parma, Italy) employing a first-stage pressure of 13.8 MPa and a second-stage pressure of 3.5 MPa. Homogenisation was carried out downstream of the heat-treatment step. Control formulations were held overnight at 4 °C prior to evaporation and drying.

5.2.5 Evaporation and Spray Drying

Control formulations were evaporated to a solids content of 55% (w/w) using a single-stage falling film evaporator operating at 65 °C. Control and HSSI formulations were spray dried using an Anhydro 3-stage dryer with fines return (SPX Flow Technology, Soeberg, Denmark) equipped with a two-fluid nozzle atomiser. Formulations were maintained at approximately 60 °C in the dryer balance tank. The inlet temperature and outlet temperatures were 187 and 85 °C respectively. Note: HSSI formulations were spray dried directly after heat-treatment using identical conditions to the control formulations.

5.2.6 Particle size distributions

Particle size distributions of control and HSSI formulations were measured using a Mastersizer S (Malvern Instruments Ltd., UK). For analyses of emulsions taken from the wet process and reconstituted formulations, samples were diluted in water and the following optical parameters were used: RI of dispersed phase = 1.46, absorption index of dispersed phase = 0.001, RI of water = 1.33. For analysis of powder particle size, the following optical parameters were used: RI of dispersed phase = 1.45, absorption index of dispersed phase = 0.1, RI of air = 1. Volume mean diameter, $D[4,3]$ was used to characterise particle size.

5.2.7 Viscosity and solids content

Viscosity was measured using an AR G2 rheometer (TA Instruments, Crawley, UK). For both control and HSSI processes, samples taken during wet processing were analysed using concentric cylinder geometry at 55 °C. The analysis of reconstituted powders (12.5% w/w at 20 °C) was performed using parallel plate geometry. The procedure for both geometries was: samples were pre-sheared at 500 s⁻¹ for 1 min followed by equilibration at 0 s⁻¹ for 1 min. The shear rate was then increased from 5 to 500 s⁻¹ over 2 min, held at 500 s⁻¹ for 1 min, then decreased from 500 to 5 s⁻¹ over 2 min. Solids content of formulations was measured using a Smart System 5, Smart Trac (CEM Corporation, North Carolina, USA).

5.2.8 Native whey protein content of formulations

The level of native whey protein in ingredients was quantified using the Whey Protein Nitrogen Index (WPNI) method (GEA-Niro, 2012). Whey protein denatured during processing was measured by reverse-phase high-performance liquid-chromatography (RP-HPLC). Control and HSSI formulations were sampled after each unit operation step (dispersion, heat-treatment, evaporation and spray drying) and diluted to a constant protein content of $2.5 \text{ g}\cdot\text{L}^{-1}$ protein in MilliQ water. The pH was reduced to 4.6 (using 1 N HCl) followed by centrifugation at 20,000 g for 15 min to remove denatured whey proteins and casein. The supernatant was then filtered through $0.22 \mu\text{m}$ PES filters (Pall Corporation) for HPLC analysis. The HPLC system used was an Agilent 1200 series (Santa Clara, CA, USA) equipped with a SourceTM 5RPC, 4.6 x 150 mm column (GE Healthcare Life Sciences, Buckinghamshire, UK). The solvents used were – (A) aqueous phase: 0.1% w/v trifluoroacetic acid (TFA) in MilliQ water and (B) organic phase: 90% w/v acetonitrile and 0.1% w/v TFA, in MilliQ water. The sample (20 μL) was injected onto the column and eluted using the following gradient - 30% B for 2.5 min, which increased to 50% B over 12.5 min, followed by an increase to 70% B over 3 min. Absorbance was measured at 214 nm. The level of whey protein denaturation was determined by differences in peak areas between samples taken before and after individual unit operations.

5.2.9 Powder physical properties

Free moisture content was measured using a HR83 Halogen Moisture Analyser (Mettler Toledo, Columbus OH, USA). Surface free fat, dispersibility, wettability and bulk volume were determined using GEA Niro analytical methods (GEA-Niro, 2012). Bulk density was calculated from the bulk volume. Water sorption behaviour was measured between 0 and 70% relative humidity (RH) using an SPS11-10 μ dynamic vapour sorption (DVS) unit. The nitrogen content of powders was determined by the Kjeldahl method (IDF, 2001). The protein content was calculated using the conversion – Nitrogen x 6.38 (Merrill and Watt, 1973).

5.2.10 Reconstituted properties of formulations

Powders were reconstituted to a solids content of 12.5% (w/w) by adding the powder to 40 °C water in a plastic container and shaking for 10 s. Stability of formulations

to both sedimentation and flotation (creaming) was then measured immediately, using a LumiFuge 116 stability analyser (L.U.M GmbH, Berlin, Germany) as described by (Tobin et al., 2011). Samples were centrifuged at 300 rpm (9.3 – 11.5 g, dependent on the distance from the rotor within the length of the measurement cell) for 10 min, simulating two hours storage under conditions of normal gravity. Two hours was chosen to represent the normal time within which IMF is consumed after reconstitution. Separation behaviour was analysed using Sepview 4.1 (L.U.M GmbH, Berlin, Germany) software.

5.2.11 Statistical analysis

All trials were carried out in triplicate and the subsequent data sets were subjected to analysis using MINITAB® 15 (Minitab Ltd, Coventry, UK) statistical analysis package. The statistical significance of differences between the means of variables within control and HSSI processes was analysed by means of an un-paired T-test (confidence interval of 0.95). To determine the effect of spray drying on particle size, a paired T-test was (confidence interval of 0.95).

5.3. Results

5.3.1 Wet processing properties

Figure 5.1 shows the particle size distribution of formulations during processing. The effectiveness of emulsification achieved by rotor-stator dispersion was found to be concentration dependant. After 10 min recirculation through the rotor-stator mixer the volume-mean particle size ($D[4,3]$) of HSSI (60% w/w) formulations was $1.53 \pm 0.11 \mu\text{m}$, compared to $2.88 \pm 0.25 \mu\text{m}$ for control (30% w/w) formulations, subjected to the same recirculation time. The $D[4,3]$ of the HSSI formulations was reduced from 1.53 ± 0.11 to $1.29 \pm 0.01 \mu\text{m}$ after steam-injection. After dispersion using the rotor-stator, control formulations were heated in a tubular heat exchanger followed by conventional valve type homogenisation, which resulted in a $D[4,3]$ reduction from 2.88 ± 0.25 to $0.59 \pm 0.06 \mu\text{m}$. However, after evaporation to a solids content of $55.2 \pm 0.5\%$ (w/w) the $D[4,3]$ increased to $1.42 \pm 0.46 \mu\text{m}$, thus there was no significant difference ($P > 0.05$) in $D[4,3]$ between control and HSSI formulations, prior to spray drying. The use of steam as a heating medium in HSSI formulations reduced the solids content from approximately 60 to $55.5 \pm 0.4\%$ (w/w) due to steam condensation, consequently there was no significant difference ($P > 0.05$) in solids content between the control ($55.2 \pm 0.5\%$ w/w) and HSSI formulations prior to drying.

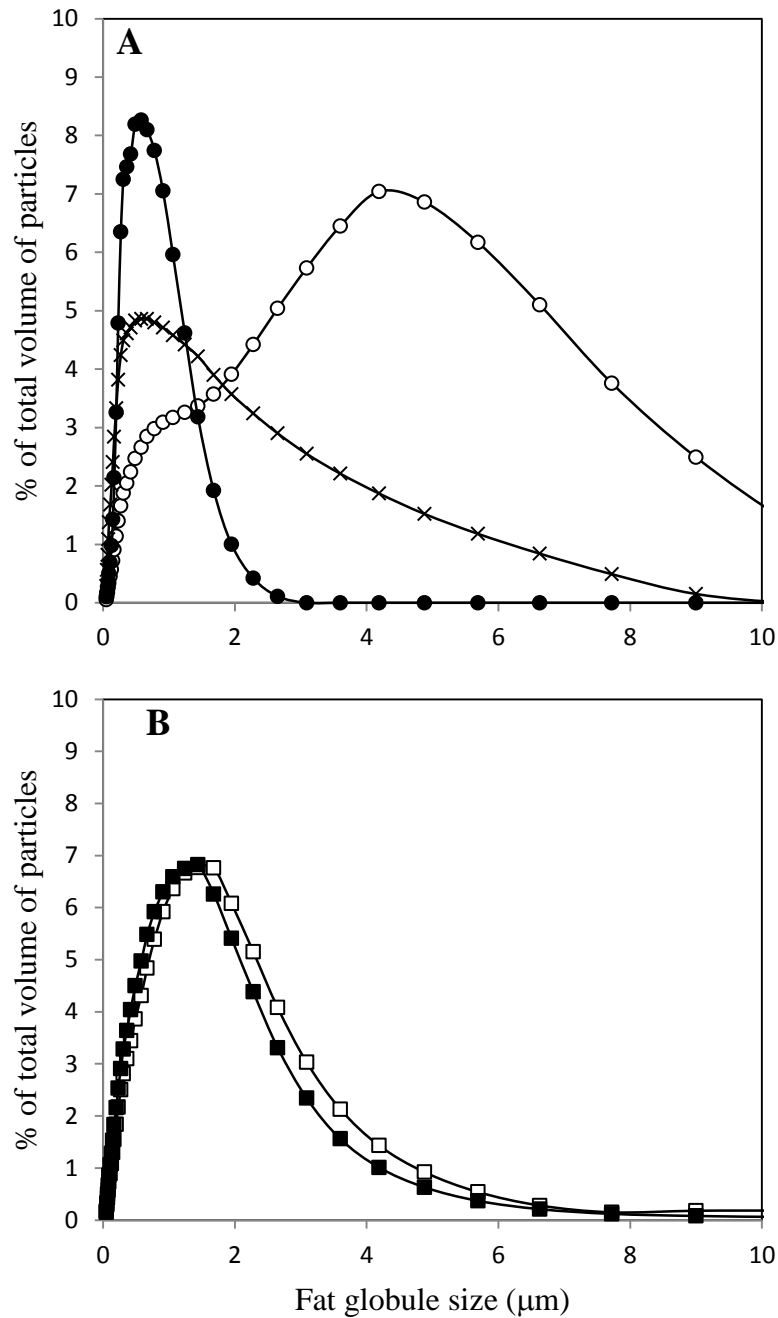


Figure 5.1 Particle size distributions: a – Control formulations: post dispersion (○), post heat-treatment and homogenisation (●) post evaporation (×), b – HSSI treated formulations: post dispersion (□) and post heat-treatment (■)

Figure 5.2 shows the change in viscosity of control and HSSI formulations throughout processing. For both formulations, heat-treatment did not significantly ($P > 0.05$) affect viscosity; however, the solids content of the HSSI formulations was reduced by approximately 5% (w/w). This indicates that the dilution caused by steam-injection offset any increases in viscosity during heating. After evaporation to approximately 55% (w/w) solids content, viscosity (at 500 s^{-1}) of control formulations was approximately 2.5

times that of the HSSI treated formulation (43.0 ± 3.5 and 18.9 ± 0.7 mPa s respectively) at equivalent solids content. A power law equation was applied to the rheological data of both control and HSSI formulations at 55% (w/w). The rate index of control formulations was 0.815 ± 0.031 in comparison to 0.976 ± 0.003 for HSSI formulations.

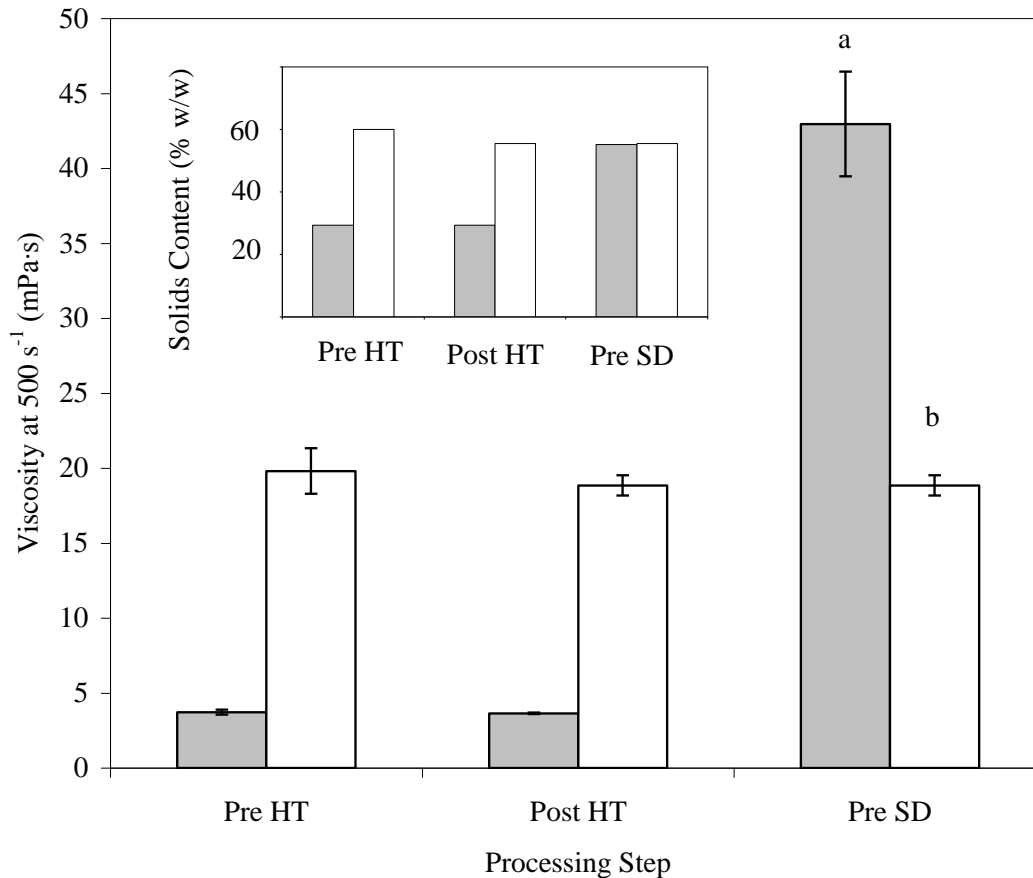


Figure 5.2 Apparent viscosity at various processing stages, pre and post heat-treatment (HT) and pre spray drying (SD). The control process is represented by grey columns and the HSSI process by white. Columns annotated with the same letter (a-b) did not differ significantly ($P < 0.05$). Also included (inset) is the solids content at corresponding processing stage.

The native whey content of control and HSSI formulations throughout processing is shown in Figure 5.3. The native whey content of control and HSSI formulations prior to heat treatment was found to be $44.4 \pm 0.3\%$ by WPNI. Direct heat treatment of control formulations resulted in the largest decrease in native whey content, from $44.4 \pm 0.3\%$ before processing to $13.0 \pm 0.5\%$ after heat treatment. In contrast, the direct treatment received by HSSI formulations resulted in significantly lower denaturation, from $44.4 \pm 0.3\%$ to $23.8 \pm 0.9\%$. Subsequent processing steps, evaporation in the case of control

formulations, and spray drying, were found to have relatively little effect on the level of native whey in formulations.

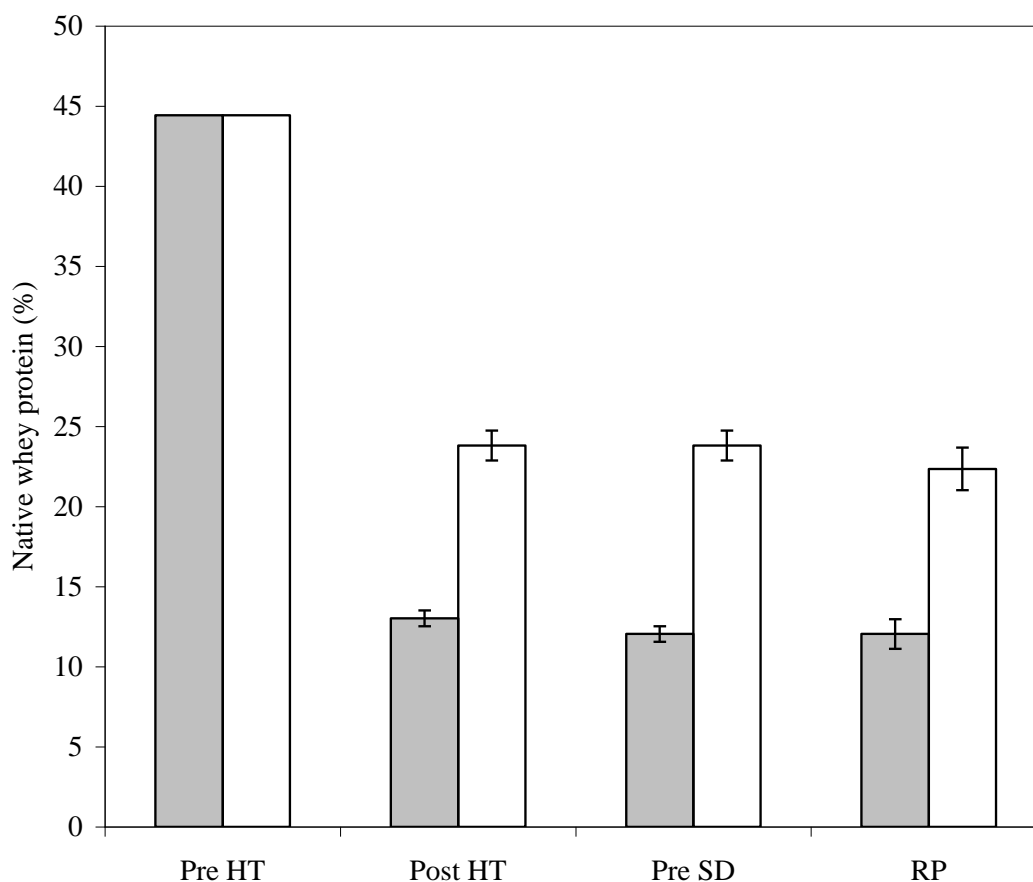


Figure 5.3 Native whey protein content of control and HSSI formulations pre heat-treatment (HT), post heat-treatment, pre spray drying (SD) and in the reconstituted powders (RP). The control process is represented by grey columns and the HSSI process by white.

5.3.2 Powder and reconstituted properties

Spray drying of HSSI formulations resulted in powders with lower moisture content and lower volume mean diameter $D[4,3]$ than control powders (Table 5.2). Both control and HSSI powders were instantly wettable (approximately 7 s) with dispersibilities of approximately 95%, while surface free fat was less than 1% (w/w). The protein contents of powders manufactured by both control and HSSI processes did not vary significantly ($P > 0.05$) and the target composition of 11.3% (w/w) was achieved (Table 5.2). Despite the differences in powder particle size no difference in water sorption behaviour was observed.

Reconstituted control formulations (12.5% w/w) exhibited slightly higher viscosity than reconstituted HSSI formulations at 2.10 ± 0.04 mPa·s compared to 1.95 ± 0.02 mPa·s respectively. The D[4,3] of reconstituted HSSI formulations was slightly larger than reconstituted control formulations at 1.31 ± 0.31 and 1.03 ± 0.38 μm respectively. Higher levels of denaturation were observed in reconstituted control and HSSI powders compared to values before spray drying; however, when compared to the levels of denaturation occurring during heat-treatment the increase was small (Figure 5.3).

Both reconstituted control and reconstituted HSSI formulations showed a slight separation tendency during simulated storage. However, the separation rate was statistically similar ($P > 0.05$) for control and HSSI formulations at $0.9 \pm 0.4\%$ h^{-1} and $1.2 \pm 0.4\%$ h^{-1} respectively.

Table 5.2. Physical characteristics of powders produced from control and High-Solids Steam-Injection (HSSI) formulations

Process	Moisture Content*	D[4,3]*	Bulk Density	Surface Free Fat	Wettability	Dispersibility	Protein Content
	% (w/w)	µm	g mL ⁻¹	% (w/w)	s	% (w/w)	% (w/w)
Control	1.64 ± 0.09	192.5 ± 18.5	0.51 ± 0.01	0.6 ± 0.2	7.6 ± 0.5	95.1 ± 3.0	11.4 ± 0.1
HSSI	1.36 ± 0.02	138.3 ± 9.4	0.51 ± 0.01	0.9 ± 0.1	7.5 ± 0.6	95.1 ± 3.3	11.4 ± 0.1

* indicates parameter differed significantly ($P < 0.05$) between control and HSSI processes

5.4. Discussion

5.4.1 Wet processing properties

The shear effect of the rotor-stator dispersion head successfully created a monomodal emulsion which was stable during steam-injection. At equivalent solids content (~55% w/w) the D[4,3] of control (post evaporation) and HSSI formulations was not significantly different ($P > 0.05$). Thus, the use of a dispersion head as the emulsification step before heat-treatment of high-solids content formulations was a suitable alternative to the more conventional practice of homogenisation followed by evaporation. However, emulsification efficiency within the dispersion head was dependent on the solids content of the formulation; thus, at lower solids contents (i.e. control formulations) a broader particle size was observed. At higher solids contents (60% w/w) more shear forces are generated due to the close proximity of the solids during recirculation through the rotor-stator dispersion head resulting in more efficient emulsification of the oil. Typically homogenisation is carried out downstream of heat-treatment, to disrupt any aggregated protein or flocculated fat globules occurring during heat-treatment (Hillbrick et al., 1999). The findings demonstrate that emulsification, as in the HSSI experiments, can be carried out before heat-treatment; the rapid change in shear rate caused by steam condensation within the steam-injector results in a further particle size reduction (Adamopoulos and Petropakis, 1999; Murphy et al., 2011). This removes the need for downstream homogenisation within the process, thereby reducing process complexity and associated costs. In the control process, there was a 2.5 fold increase in particle size during evaporation which was attributed to shearing effects coupled with interaction between denatured whey proteins positioned at the oil-water interface, causing flocculation of fat globules. An alternative mechanism for this particle size increase may be compression and distortion of fat globules during water removal, leading to rupture and subsequent coalescence (Aranberri et al., 2004).

In Chapter 4, the extent of whey protein denaturation after heating at 120 °C was found to increase with increasing DM content; therefore, high levels of whey protein denaturation could be expected in the HSSI formulations. However, HSSI formulations had significantly lower levels of whey protein denaturation than control formulations, even though heating was carried out at 60% (w/w) in comparison to 30% (w/w) for the control. The results indicate that, in agreement with Zadow (1969), formulations treated

by the HSSI process received a lower thermal load (at the same time/temperature combination, 110 °C for 3 s) than the control process, in which a tubular heat-exchanger was used. This is most likely due to the rapid rate of change of product temperature associated with direct heat-treatments and resulted in lower levels of denaturation (Lewis and Deeth, 2009). Subsequent evaporation (in the case of the control formulations) and spray drying was found to have a comparatively small effect on the level of denaturation, due to the relatively low temperatures received by the formulations during these operations. Thus, the level of native whey protein in the final powders was largely determined by the heat-treatment step, indicative of the importance of this step in the processing of whey-rich formulations.

Viscosity is another indicator of the intensity of thermal treatment received by a formulation. The results indicated that HSSI formulations received lower heat loads than control formulations. Despite the majority of whey protein denaturation taking place during the heat-treatment step (Figure 5.3), no significant increase in viscosity was observed in control formulations after indirect heat-treatment, due in part to the relatively low solids content of the formulation (30% w/w). However, after evaporation, the viscosity of control formulations was significantly ($P < 0.05$) higher than HSSI formulations at equivalent solids contents (55% w/w). As evaporation had little effect on whey protein denaturation, the relatively higher viscosity of control formulations can be attributed to a combined effect of higher levels of whey protein denaturation during indirect heat-treatment coupled with the effect of evaporation. The higher voluminosity of unfolded whey proteins (Snoeren et al., 1982) and closer proximity of reactive groups, during evaporation, leads to interaction between whey proteins, increasing viscosity. Another possibility is association of denatured whey protein with casein micelles, particularly during water removal. Martin et al. (2007) found that casein micelle size increased substantially after evaporation of skim milk, and proposed that it was as a result of denatured whey proteins adhering to casein micelles. Furthermore, in fat containing systems, both caseins and whey have been found to adsorb to fat globules during evaporation (Ye et al., 2004). Therefore, it is likely that the increase in particle size found post-evaporation of control formulations was as a result of flocculation of fat globules caused by protein interactions. Control formulations at 55% (w/w) were found to exhibit shear thinning behaviour (rate index of power law < 1) indicating flocculation of fat globules (Demetriades et al., 1997a, 1997b). The rate index of HSSI formulations at 55%

was close to 1 (Newtonian), indicating that little flocculation had occurred. Flocculation of fat globules results in emulsions with higher viscosity than unflocculated emulsions, due to the larger volume fraction of the flocculated material (Keowmaneechai and McClements, 2002). Therefore, a higher amount of denatured whey protein in combination with a larger degree of fat globule flocculation resulted in control formulations with higher viscosity than HSSI formulations prior to spray drying.

Formulation viscosity can be a limiting factor in spray drying. Thus, the lower viscosity of HSSI formulations, in combination with a particle size within the range of control formulations post evaporation, suggests that higher concentration (> 55% w/w) formulations could be successfully spray dried, lowering water removal requirements, with associated cost benefits.

5.4.2 Powder and reconstituted properties

Wettability and dispersibility are important parameters in the production of instant powders. Both control and HSSI powders can be considered wettable as their wetting times were less than 120 s (Schuck et al., 2012a). Surface free fat reduces the wettability of powder particles as it increases the hydrophobicity at the particle surface (Faldt, 1995) and is therefore, also an important parameter in the production of instant powders. The surface free fat (< 1% w/w) and wettability (approximately 7 s) of both the control and HSSI powders correlated with the four typical IMF powders characterised by Hanley et al. (2011), which were in the range of 0.3 - 0.8% (w/w) and 7 – 23 s respectively. The dispersibility of control and HSSI powders (~ 95%) was higher than the target for whole milk powder (85%) as reported by Tuohy (1989). The high dispersibility and low wetting time (i.e. less than 20 s) of control and HSSI powders indicates that they may be considered instant powders (Schuck et al., 2012a). The protein content did not vary significantly ($P > 0.05$) between control and HSSI powders, suggesting that concentration and temperature-induced instability and associated fouling of process surfaces, did not occur for HSSI formulations, an observation which correlates with the low levels of whey protein denaturation in the HSSI process (Fryer, 1989). However, moisture content and powder particle size differed significantly ($P > 0.05$) between control and HSSI powders. Both these parameters can be linked to solution viscosity immediately prior to spray drying, which effects droplet formation during two-fluid nozzle-atomisation, resulting in a reduction in droplet diameter as viscosity decreases (Masters, 2002a). Thus, in the lower viscosity HSSI treated formulations the smaller droplets produced during

atomisation had a larger surface area compared to control formulations, which increased the area of contact between the surface of the droplets and the drying air, resulting in lower moisture powders (Masters, 2002b).

The stability to separation in both reconstituted control and HSSI powders did not vary significantly. Stokes' law predicts that the separation rate of a particle in suspension is directly proportional to its particle size and inversely proportional to its viscosity (Robins, 2000), and therefore, predicts a lower stability to separation in the reconstituted HSSI powders as a result of their higher particle size and lower viscosity compared to the reconstituted control powders. However, this was not observed, indicating that reconstituted HSSI formulations were as stable to separation as reconstituted control formulations over the maximum shelf life of a reconstituted infant milk formula (approximately 2 h).

5.5 Conclusion

High-solids steam-injection (HSSI) is a feasible alternative for the production of powdered IMF from concentrated formulations. The reconstitution properties of IMF powders produced by the combination of rotor-stator dispersion followed by direct steam-injection compared favourably with those of powders produced in a conventional (control) process. Furthermore, the HSSI process caused less thermal denaturation of whey proteins than the control process, even though heat-treatment was carried out at twice the concentration (60% w/w). The lower denaturation level also resulted in lower viscosity prior to spray drying, suggesting that the HSSI process could be suitable for the production of IMF at even higher concentrations (> 60% w/w), which would further reduce the energy requirements associated with water removal in powder production. Finally, as the HSSI process does not require a homogeniser downstream of heat-treatment or evaporation prior to spray drying, process complexity and associated costs are reduced.

Chapter 6: Physical stability of infant milk formula made with selectively hydrolysed whey proteins

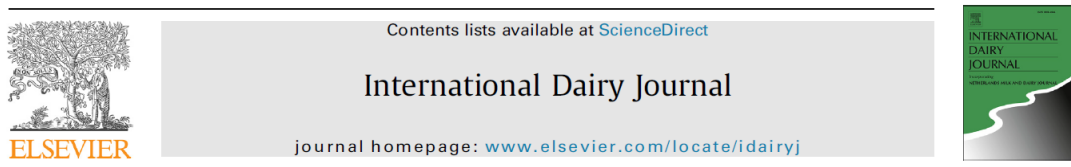
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Physical stability of infant milk formula made with selectively hydrolysed whey proteins



Eoin G. Murphy ^a, Yrjö H. Roos ^b, Sean A. Hogan ^a, Patrick G. Maher ^a, Cal G. Flynn ^c, Mark A. Fenelon ^{a,*}

^a Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

^b School of Food and Nutritional Sciences, University College Cork, Ireland

^c Kerry Ingredients and Flavours, Tralee Road, Listowel, Co. Kerry, Ireland

Abstract

A whey protein ingredient, in which β -Lactoglobulin was selectively hydrolysed, was evaluated during manufacturing of infant formulae. Three model infant milk formula (IMF) powders were produced containing: non-hydrolysed (NH) proteins (60:40; whey proteins: caseins); partially hydrolysed (PH) caseins and whey proteins; and selectively hydrolysed (SH) whey proteins. After homogenisation, particle size (D[4,3]) of the SH formulae was similar ($P > 0.05$) to NH formulae and was significantly ($P < 0.05$) smaller than PH formulae. Prior to spray drying (~55% w/w), the viscosity of SH formulae (14.8 ± 0.3 mPa s) was significantly lower ($P < 0.05$) than that of the NH (48.6 ± 0.8 mPa s) or PH formulae (27.6 ± 1.5 mPa s). Surface free fat, wettability and glass transition temperature of powders were not significantly ($P > 0.05$) different. IMF manufactured with SH ingredients have applications in high dry matter processes with potential for reduced energy costs in spray drying.

6.1. Introduction

Infant milk formula (IMF) powders are comprised of macronutrients (proteins, lipids, carbohydrates) and micronutrients (vitamins, minerals). The level of each nutrient is set to simulate the composition of human milk and is governed by various regulations, e.g., European Commission (2006) and Codex Standard (2007). While primarily intact proteins from bovine milk are used as the nutritional base for IMF (Asselin et al., 1988), hydrolysed proteins are used in comfort or speciality formulations; i.e., for easy digestion and/or reduced allergenicity. Hydrolysed proteins can impart health benefits without changing the nutritional value of the formulation (Boza et al., 1994; Alles et al., 2004).

During the manufacture of IMF, proteins play an important role in determining the physical characteristics of formulations, in particular, stabilisation of the oil phase into an oil-in-water emulsion (McDermott, 1987). In whey-dominant first-age IMF, whey proteins, which constitute typically 60% of total protein, can also play an important role in heat stability of formulations. β -Lactoglobulin (β -Lg), the most abundant whey protein in bovine milk-derived IMF, is a heat sensitive protein and unfolds to interact with itself, other whey proteins and casein micelles at temperatures above 65 °C (McDermott, 1987). The interactive nature of β -Lg is due, predominantly, to the presence of a free thiol group, which leads to reorganisation of disulphide linkages from intra- to inter-molecular form (Sawyer, 1968; Shimada and Cheftel, 1989). In many instances, β -Lg plays a central role in fouling on the surfaces of heat exchangers; for example, milk, which contains approximately 2.5% (w/w) β -Lg on a dry matter basis, can form a deposit which contains approximately 50% protein, the majority of which is β -Lg, when heated at temperatures up to 110 °C (Fryer, 1989). Milk salts are also a major contributor to fouling deposits. Fouling deposits are decreased by turbulent flow, which is inversely proportional to viscosity; as a result dry matter content can be a limiting factor during heat treatment of IMF (Fryer, 1989).

α -Lactalbumin (α -La) is the most abundant whey protein in human milk and α -La-enriched IMF is widely available. α -La does not contain the free thiol group required to initiate covalent aggregation (Brew, 2003) and is less likely to cause fouling during heating. Aggregation of commercially available α -La during heating (95 °C for 1 h) can be between 20 and 70% reversible, depending on source (McGuffey et al., 2007) and heat-induced gel formation of α -La is less extensive compared to β -Lg (Boye and Alli,

2000). Addition of β -Lg to α -La systems has been shown to reduce denaturation reversibility and alter the aggregation process (McGuffey et al., 2007). It has been suggested that β -Lg could react via thiol disulphide interchange with one of the four disulphide bonds in α -La producing β -Lg/ α -La aggregates with a free thiol group. This dimer would further react with α -La or β -Lg producing mixed aggregates of α -La and β -Lg (Schokker et al., 2000).

Hydrolysed proteins have relatively little secondary structure and altered functional properties compared to intact proteins (Chobert et al., 1988; Kilara and Panyam, 2003). Good solubility, emulsification capacity and thermal stability are important attributes of a protein ingredient and determine rheological / structural behaviour within a formulation. This ultimately determines the processing limits on parameters such as the heat treatment used during manufacture. Hydrolysis of protein can decrease emulsion stability (Nakai and Li-Chan, 1989) with proteolytic enzyme type, substrate and degree of hydrolysis, reported to impact on emulsifying properties (Mahmoud et al., 1992; Darewicz et al., 2000; Tuncturk and Zorba, 2006). Chobert et al. (1988) suggested that in order to maintain good emulsification properties peptides derived from whey should not be lower than 5 kDa. Singh and Dalgleish (1998) found that peptides derived from whey with a molecular weight of 0.52 kDa were sufficient to produce a mono-disperse emulsion with a $D[4,3] < 1 \mu\text{m}$. The majority of studies on hydrolysed proteins in IMF have focused on nutritional and allergenic aspects, rather than functional characteristics such as emulsification and viscosity. The significance of protein content on IMF emulsion stability has recently been reported (McCarthy et al., 2012, 2013). Production of a stable emulsion during manufacture of IMF is required to minimise surface free fat and protect against creaming in reconstituted IMF powders. In addition, lower viscosity systems allow spray-drying of emulsions with higher dry matter contents, thereby reducing cost of the unit operation.

The objective of the present study was to investigate the use of a selectively hydrolysed whey protein ingredient for manufacture of IMF powders. Model IMF powders were produced containing intact proteins from skim milk and demineralised whey, partially hydrolysed proteins (hydrolysed skim milk and demineralised whey), or a whey protein ingredient whereby only β -Lg had been selectively hydrolysed, leaving α -La intact. The rationale was to optimise the functionality of the protein system by removing the primary heat-labile protein, β -Lg, while maintaining good emulsification

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through remaining unhydrolysed α -La. The study demonstrated a novel approach to manufacture of infant formula with reduced allergenicity and improved thermal stability throughout processing. The functional benefits arising from inclusion of hydrolysed proteins and removal of β -Lg, such as lower viscosity and thermal stability, should provide a means of manufacturing IMF products at high dry matter contents ($\geq 55\%$ w/w) without detrimental impact on final product quality.

6.2. Materials and methods

6.2.1 Materials

Medium heat skim milk powder (SMP), demineralised whey powder (DWP), hydrolysed skim milk preparation (Hyprol™ 9092, HSMP), hydrolysed demineralised whey powder (Hyprol™ 9093, HDWP) and a selectively hydrolysed prototype whey protein powder (Hyprol™, SH-WP) were all donated by Kerry Ingredients and Flavours (Listowel, Ireland). Edible lactose was sourced locally. High oleic sunflower oil, palm oil, soya oil and coconut oil were supplied by Trilby Trading (Drogheda, Ireland). U.S. Pharmacopeia and British Pharmacopeia grade mineral salts were purchased from Sigma Aldrich Ireland (Arklow, Ireland). Compositions of the powdered ingredients used are given in Table 6.1.

Table 6.1. Composition (g kg⁻¹) of ingredients used in study.

Composition	SMP	DWP	HSMP	HDWP	SH-WP	Lactose
Caseins	266	0	251	0	0	0
Whey proteins	66	139	80	147	731	4
Fat	8	11	10	14	8	1
Moisture	42	18	23	15	41	5

6.2.2 Experimental overview

Three different formulations were manufactured. An ‘intact’ (NH) formulation containing non-hydrolysed proteins (60% whey proteins and 40% caseins (w/w), of total protein) was made using SM and DW as protein sources. A ‘partially’ hydrolysed protein (PH) formulation was made using HSM and HDW (60% whey derived peptides/protein; 40% casein derived peptides/protein). A ‘selectively’ hydrolysed protein (SH) formulation was made containing as the exclusive protein source, a whey protein ingredient (SH-WP) in which β -Lg had been hydrolysed.

The target composition of final powders was 11.5% (w/w), 29.5% (w/w) and 59.0% (w/w) of proteins, fat and lactose, respectively. Each formulation was manufactured in triplicate. Each replicate was made on a separate day.

6.2.3 Molecular weight distribution

Molecular weight distribution of protein ingredients was determined using a modification of the method of O'Loughlin et al. (2012). Size-exclusion chromatography was carried out using TSK Gel G2000SW_{XL} and G3000SW_{XL} (7.8 mm x 300 mm) columns, operated in series (TosoHaas Bioscience GmbH, Stuttgart, Germany), under isocratic conditions (20 mM sodium phosphate buffer at pH 7) over 60 min and at a flow rate of 0.5 mL min⁻¹. Samples were made up to 2.5 g L⁻¹ protein in 20 mM phosphate buffer and applied in 20 µL volumes to the column following filtration using 0.45 µm low protein binding membrane filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The column was calibrated using the following molecular weight standards: α-La, β-Lg A and B, bovine serum albumin, lactoferrin, caseino-macropeptide (Sigma-Aldrich, Dublin, Ireland), Ribonuclease A, cytochrome c, aprotinin, bacitracin, His-Pro-Arg-Trp, Leu-Trp-Met-Arg, bradykinin, Leu-Phe, and Tyr-Glu (Bachem AG, Bubendorf, Switzerland).

6.2.4 Surface hydrophobicity

Surface hydrophobicity of protein ingredients was determined using a modification of the method of Chaudhuri et al. (1993). Hydrophobicity was measured by ligand binding of an aromatic fluorescence probe, 1-Anilinonaphthalene-8-Sulfonic Acid (ANS) to protein dispersions at concentrations from 0 to 0.5 g L⁻¹, using a Cary Eclipse Fluorescence Photometer (Agilent Technologies, Santa Clara, CA, USA). Protein dispersions (2 mL) were prepared in 20 mM sodium phosphate buffer (pH 7) before addition of 0.1 mL of 0.945 mM ANS. Excitation and emission wavelengths were 370 and 470 nm, respectively, with emission band passes of 5 nm. Surface hydrophobicity was determined by the slope of the line obtained by plotting protein concentration against relative fluorescence (F_R), defined as: $F_R = (F - F_0)/F_0$, where, F is the fluorescence intensity of the protein-ANS conjugate and F_0 that of ANS alone.

6.2.5 Wet-mix preparation

For each formulation, 60 kg wet-mixes were prepared at 55% (w/w) dry matter in a jacketed tank containing reverse osmosis (RO) purified water at approximately 60 °C. The order of ingredient addition was as follows: lactose, dehydrated whey protein source

(DWP, HDWP or SH-WP), fat blend followed by dehydrated casein/whey source (SM or HSM).

Dehydrated ingredients were hydrated, and the oil blend dispersed, using a high-shear overhead mixer, during which, minerals were also added. The whole sequence of batch preparation took 10 min, during which, the temperature was maintained at 60 °C. Minerals added were: calcium carbonate, calcium hydroxide, ferrous sulphate (heptahydrate), magnesium chloride (hexahydrate), potassium bicarbonate, potassium chloride, potassium hydrogen phosphate, potassium hydroxide and trisodium citrate (dihydrate). The pH of each wet-mix was adjusted to 6.8 (at 60 °C) using potassium hydroxide.

The oil blend used contained vegetable oils (sunflower, soyabean, palm and coconut) mixed at a ratio designed to simulate the fatty acid profile of human milk. The ratio of linoleic acid to α -linolenic acid was 15:1 in accordance with the limits set by Codex Standard 72 (2007).

6.2.6 Heat treatment, homogenisation and spray drying

Wet-mixes were heat treated at 100 °C for 30 s using a MicroThermics tubular heat exchanger (MicroThermics, Raleigh, NC, U.S.A.) and subsequently homogenised (at 65 °C) in an in-line, two-stage, valve-type homogeniser (Model NS2006H, Niro Soavi, Parma, Italy) with first-, and second-stage pressures of 13.8 MPa and 3.5 MPa, respectively. Immediately after heat treatment, formulations were transferred to a balance tank, where they were held at approximately 60 °C before spray drying using an Anhydro 3-stage drier with fines return to the top of the drier (SPX Flow Technology, Soeborg, Denmark). Inlet and outlet air temperatures were 187 and 85 °C, respectively.

6.2.7 Emulsion particle and powder particle size distribution

Emulsion particle size distribution of wet-mixes was measured before and after heat-treatment and homogenisation, and in reconstituted powders using a Mastersizer S (Malvern Instruments Ltd., Malvern, UK). The optical parameters used were: refractive indices of 1.46 and 1.33 for sample and dispersant (water), respectively, and particle absorbance of 0.001. Volume mean diameter, $D[4,3]$ was used to characterise emulsion, reconstituted powder and powder (MS64 Dry Powder Feeder unit) particle size distributions.

6.2.8 Viscosity and dry matter content

Viscosity for each formulation, before and after heat-treatment, was measured using an AR G2 Rheometer (TA Instruments, Crawley, UK) with concentric cylinder geometry at 55 °C. Analysis of reconstituted powders (12.5% w/w at 20 °C) was performed using the same geometry. In both cases, samples were pre-sheared at 500 s⁻¹ for 1 min followed by equilibration at 0 s⁻¹ for 1 min. The shear rate was then increased from 5 to 500 s⁻¹ over 2 min, held at 500 s⁻¹ for 1 min, then decreased from 500 to 5 s⁻¹ over 2 min. Dry matter content of formulations was measured using a Smart System 5, Smart Trac (CEM Corporation, Matthews, NC, USA).

6.2.9 Electrophoresis

Proteins in ingredients were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions, using the method of Laemmli (1970).. Powders for analysis were dissolved (4 g protein L⁻¹) in 0.5 mol L⁻¹ Tris-HCl buffer (pH 6.8), containing 0.01% (v/v) bromophenol blue and 2% (w/v) SDS, and heated to 95 °C for 5 min in the presence of 2-mercaptoethanol. Electrophoresis was carried out using a mini-protean 3 cell electrophoresis unit and a 20% acrylamide gel (Bio-rad Laboratories LTd, Dublin, Ireland). Running conditions were 160 V for 60 min. Gels were stained with Coomassie Brilliant Blue R-250 in an acetic acid:isopropanol:water mixture (10:25:65, v/v). Gels were destained in an acetic acid:isopropanol:water mixture (10:10:80, v/v). Protein bands were compared to a molecular weight standard – PAGERuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA)

6.2.10 Powder physical properties

Moisture content was measured using a HR83 Halogen Moisture Analyser (Mettler Toledo, Columbus, OH, USA). Surface free fat, dispersibility, wettability and bulk density were determined using GEA Niro analytical methods (GEA-Niro, 2012).

6.2.11 Differential scanning calorimetry

Thermal transitions in powders were measured using a Q2000 differential scanning calorimeter (DSC; TA Instruments, Crawley, UK) calibrated using Indium standards. Hermetically sealed pans, containing approximately 10 mg powder, were

heated, in a nitrogen purged environment, from 0 to 100 °C at 5 °C min⁻¹, cooled at 10 °C min⁻¹ to 0 °C, before final heating to 100 °C at 5 °C min⁻¹. Heat flow to the sample was compared against an empty reference pan.

6.2.12 Emulsion stability of reconstituted formulations

Emulsions were reconstituted from powders to a dry matter content of 12.5% (w/w) by adding the powder to water in a 500 mL screw-cap plastic container at 40 °C and shaking for 10 s. Stability of emulsions to both sedimentation and flotation (creaming) was measured immediately, using a LumiFuge 116 stability analyser (L.U.M GmbH, Berlin, Germany) as described by (Tobin et al., 2011). Samples were centrifuged at 9.3 – 11.5 g, for 10 min, which is equivalent to 2 h storage under conditions of normal gravity and considered representative of the time within which IMF is typically consumed after reconstitution. Separation behaviour was analysed using Sepview 4.1 (L.U.M GmbH) software.

6.2.13 Statistical analysis

All formulation types were processed in triplicate and subsequent data sets subjected to one-way ANOVA using MINITAB[®] 15 (Minitab Ltd, Coventry, UK) statistical analysis package. Means with significant differences were compared using Fisher's individual error rate with significance at $P < 0.05$. A paired t-test was used to determine significance ($P < 0.05$) of treatments within each type of formulation (e.g. before and after spray drying).

6.3. Results and Discussion

6.3.1 Protein ingredient properties

Figure 6.1 (lanes 1-3) shows reducing SDS-PAGE of non-hydrolysed (NH), partially hydrolysed (PH) and selectively hydrolysed (SH) protein ingredients, respectively. SDS-PAGE under reducing conditions disrupts both hydrophobic and disulphide interactions between proteins, giving an indication of the gross constituents of a protein mixture. NH protein ingredients (lane 1) contained both intact caseins and whey proteins (α -La, β -Lg), as to be expected. PH protein ingredients (lane 2) may have contained small amounts of caseins, β -Lg, and α -La, as indicated by faint traces on the SDS-PAGE, which may be expected for partially hydrolysed proteins from skim milk and whey, respectively. However, it is also possible that the band over the range of 10 – 20 kDa for PH protein ingredients could be that of hydrolysed casein (Chobert et al., 1988). Lane 3 shows the SH protein ingredients; the absence of β -Lg and presence of α -La is indicative of the selectively hydrolysed nature of the SH protein ingredients, however, peptides expected to be present were not observed under the specific conditions of SDS-PAGE used in this study. The presence of lower molecular weight material was therefore studied by size-exclusion HPLC.

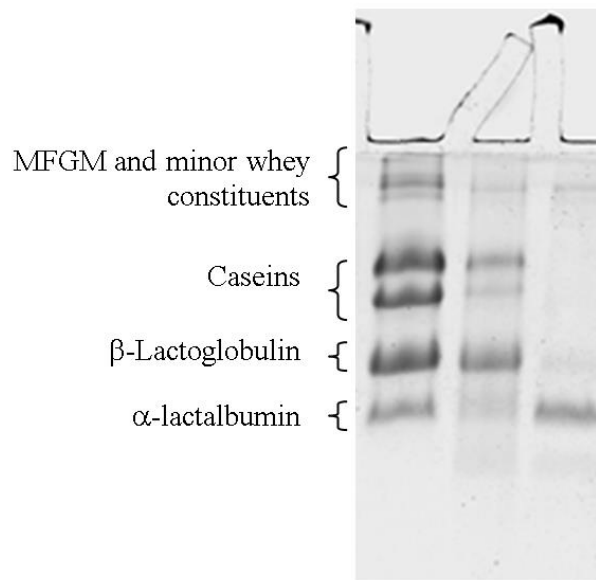


Figure 6.1. SDS-PAGE under reducing conditions (20% acrylamide gel). Lanes 1 – 3 show total protein (caseins and whey proteins) composition of non-hydrolysed, partially hydrolysed and selectively hydrolysed protein ingredients, respectively. Protein concentration on gels was 2 g L^{-1} .

Figures 6.2 and 6.3 show molecular mass profiles of ingredients measured by size-exclusion HPLC. As NH protein ingredients were not subjected to hydrolysis, there was little material present below 10 kDa (2.3%). There were, however, large amounts of aggregates greater than 40 kDa present (71.2%), an indication of both the casein micelles present and the propensity for intact whey proteins to aggregate during processing i.e., self-aggregation and/or aggregation with caseins during heat-treatment prior to spray drying (Anema and Li, 2003a). PH protein ingredients contained more hydrolysed material (approximately 32% of the material which passed through the size-exclusion column was lower than 10 kDa). SH protein ingredients were the most extensively hydrolysed and least aggregated with approximately 45% of material lower than 5 kDa, and less than 5% of material greater than 40 kDa. In addition, 25% of material present was in the range of 10 -20 kDa, another indication of the high α -La content ($MW_{\alpha-La} = 14.2$ kDa; Barbana and Perez (2011)). The chromatograph area of PH protein ingredients was lower than NH and SH protein ingredients indicative of a loss of material, most likely aggregates, during sample preparation and/or due to accumulation at the entrance to the swollen pores of the guard or size exclusion column (Mahler et al., 2008; Zolls et al., 2011). It is possible that partial hydrolysis of caseins and whey proteins led to the formation of aggregates through peptide-peptide interactions and/or interactions with some of the remaining intact proteins (Liu et al., 2007; Creusot and Gruppen, 2008).

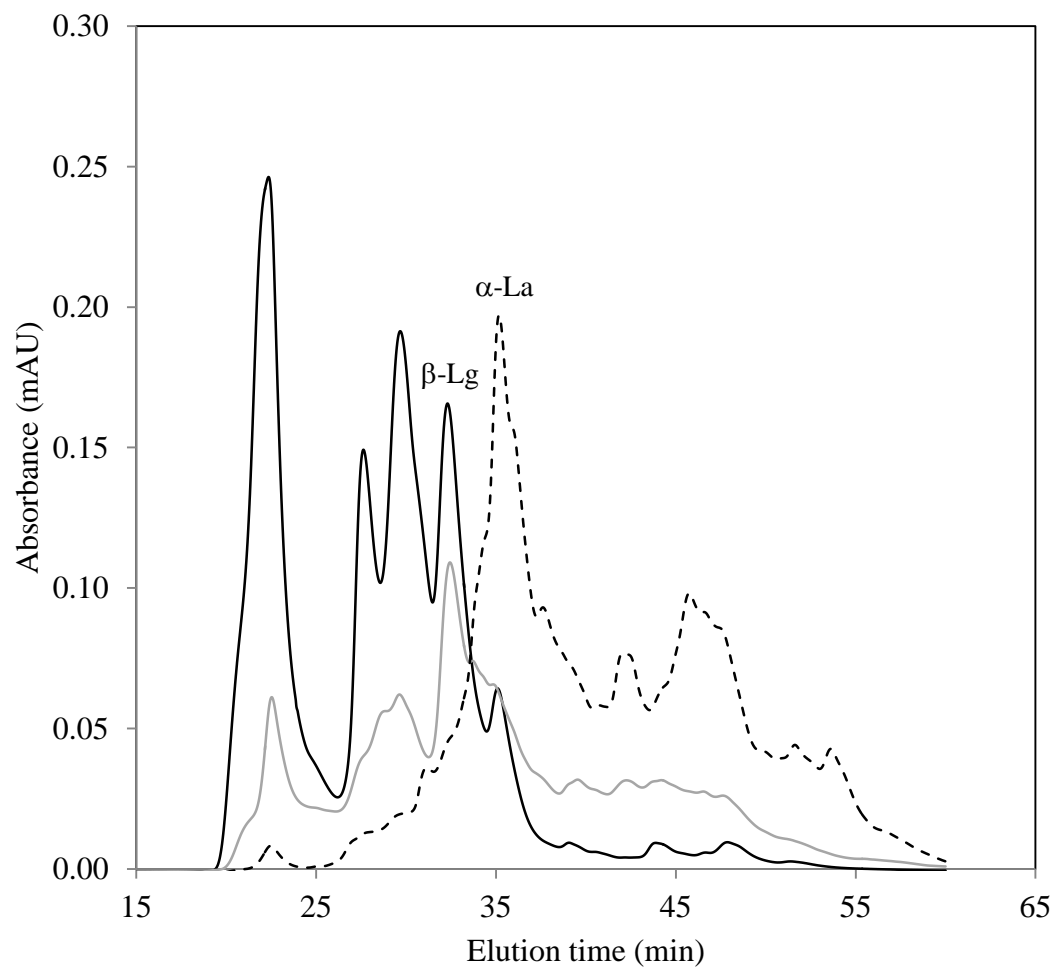


Figure 6.2. Size-exclusion high-performance liquid-chromatography profiles of non-hydrolysed (black), partially hydrolysed (grey) and selectively hydrolysed (dashed) protein ingredients. Peaks corresponding to β -Lactoglobulin (β -Lg) and α -Lactalbumin (α -La) are marked. Samples were prepared at 2.5 g L^{-1} protein.

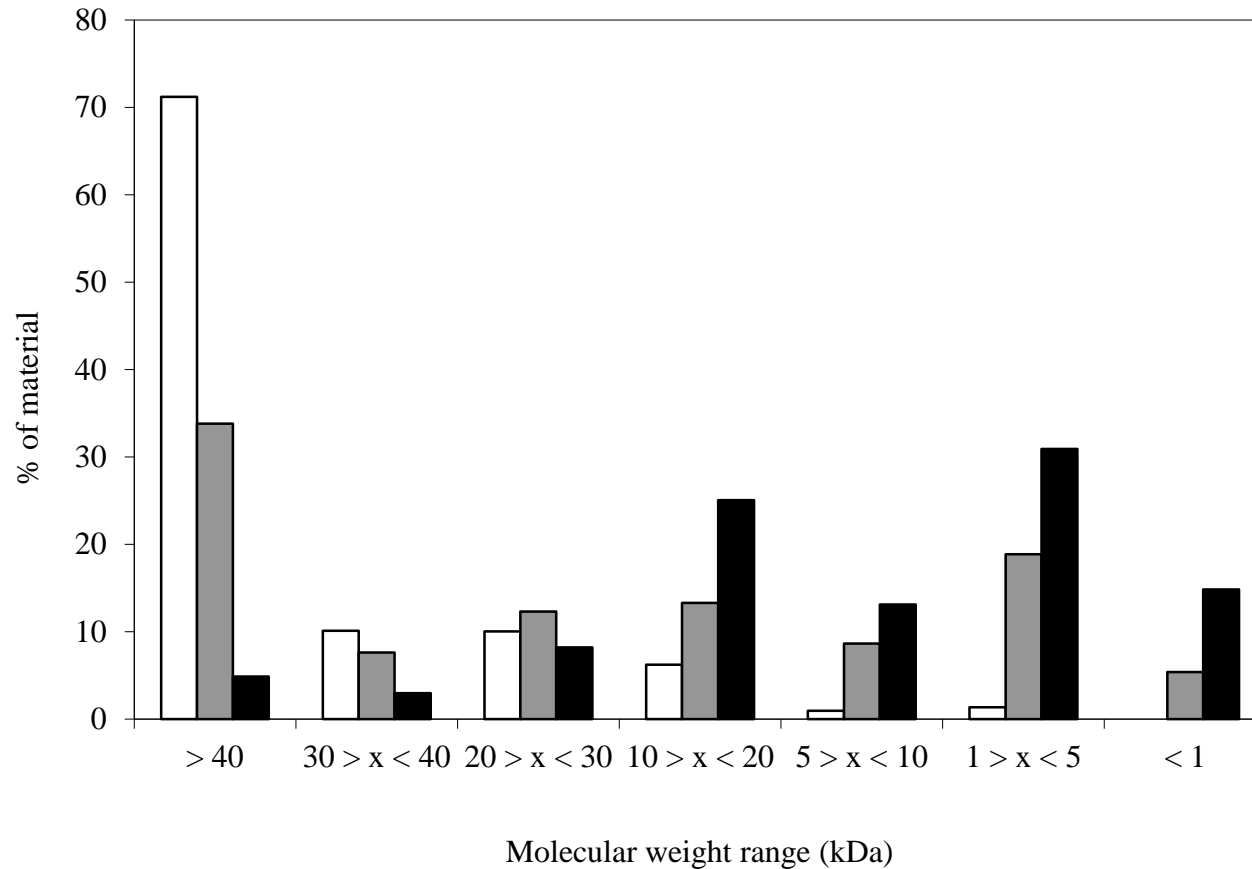


Figure 6.3. Molecular weight distribution of formulations, as measured by size-exclusion high-performance liquid-chromatography. Columns represent: Non-hydrolysed (no fill), partially hydrolysed (grey fill) and selectively hydrolysed (black fill) protein ingredients. Samples were prepared at 2.5 g L⁻¹ protein.

Figure 6.4 shows the surface hydrophobicity of the NH, PH and SH protein ingredients. Several authors have reported positive correlations between surface hydrophobicity and emulsifying capacity (Kato and Nakai, 1980; Mahmoud et al., 1992; Moro et al., 2001). NH protein ingredients had the highest surface hydrophobicity, as indicated by the slope in Figure 6. 4, followed by PH, with SH having the lowest surface hydrophobicity. Hydrolysed caseins, which have been shown to have reduced emulsification capacity in comparison to intact caseins, may have contributed to the lower surface hydrophobicity of the PH protein ingredients (Chobert et al., 1988; Mahmoud et al., 1992). Similarly, the large amount of low MW material in SH protein ingredients may have contributed to its lower surface hydrophobicity (Chobert et al., 1988; Turgeon et al., 1992a, b; Singh and Dalgleish, 1998).

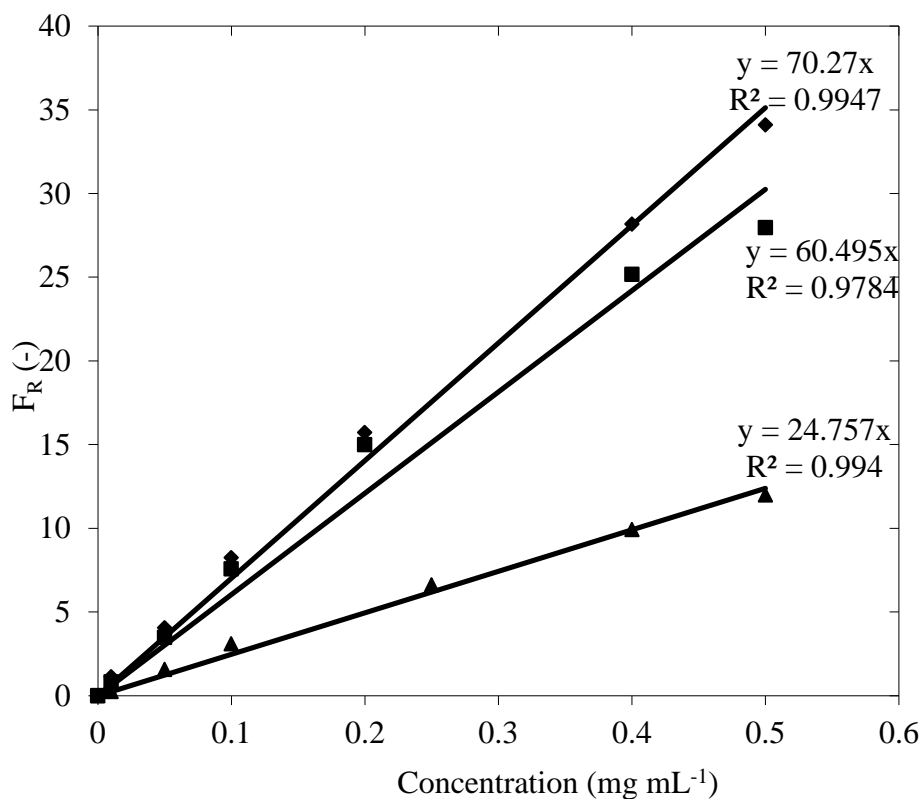


Figure 6.4. Relative fluorescence (F_R), determined by ANS (1-Anilinonaphthalene-8-Sulfonic Acid) probe method, as a function of protein concentration for non-hydrolysed (♦), partially hydrolysed (■), and selectively hydrolysed protein (▲) ingredients.

6.3.2 Wet-processing of formulations

6.3.2.1 Emulsion particle size distribution

Emulsion particle size distribution in each wet-mix was monitored throughout processing (Table 6.2). A high shear mixer was used to make a homogenous mixture of proteins, fat, carbohydrate and minerals in the form of a coarse emulsion (prior to heat treatment and homogenisation). Coarse emulsions containing intact proteins had significantly ($P < 0.05$) lower volume mean particle size (D[4,3] i.e., $1.61 \pm 0.32 \mu\text{m}$; Table 6.2) compared to coarse emulsions containing partially ($3.88 \pm 0.21 \mu\text{m}$) or selectively hydrolysed proteins ($3.49 \pm 0.22 \mu\text{m}$), i.e., PH or SH respectively.

Table 6.2. Volume mean diameter D[4,3] pre- and post-heat treatment and homogenisation (HT&H) of wet mixes

Wet-mix	Pre HT&H (μm)	Post HT&H (μm)
Non-hydrolysed	$1.61 \pm 0.32^{\text{a}}$	$0.64 \pm 0.20^{\text{a}}$
Partially hydrolysed	$3.88 \pm 0.21^{\text{b}}$	$2.01 \pm 0.46^{\text{b}}$
Selectively hydrolysed	$3.49 \pm 0.22^{\text{b}}$	$0.52 \pm 0.04^{\text{a}}$

Values presented are the means of 3 replicates \pm standard deviation. Fisher's individual error rate was used to calculate significance

^{a,b} Values within a column not sharing a common superscript differ significantly, $P < 0.05$

After heat treatment and homogenisation, both NH and SH wet-mixes had significantly ($P < 0.05$) lower D[4,3] compared to PH wet-mixes. SH wet-mixes, which contained intact α -La, had the lowest D[4,3] of $0.52 \pm 0.04 \mu\text{m}$ after heat treatment and homogenisation. The findings demonstrate that in hydrolysed ingredients where proteins were indiscriminately hydrolysed, as in the PH wet-mixes, homogenisation was reduced compared to those where selective proteins remained intact i.e. as in the SH wet-mixes where the α -La was not hydrolysed. While SDS-PAGE analysis indicated the presence of some intact β -Lg in PH ingredients (Figure 6.1) it appears not to have been enough to emulsify the fat to the same extent as SH protein ingredients. The findings are interesting as the SH protein ingredients had the lowest surface hydrophobicity (Figure 6.4) and contained the highest amount of peptide material less than $< 5 \text{ kDa}$, i.e., 46% compared to 24% in the PH protein ingredients (Figure 6.3). It is possible that other factors such as viscosity of wet-mixes and/or heat induced changes (wet-mixes were heat-treated before

homogenisation) could influence homogenisation. For example, heat treatment of β -Lg (present in both NH and PH wet-mixes) has been found to reduce surface activity and emulsification capacity through hydrophobic interactions (Voutsinas et al., 1983). α -La, in contrast, exhibits much more reversible behaviour during heat treatment especially in the absence of β -Lg as was the case in SH wet-mixes (McGuffey et al., 2007). Therefore, α -La in SH wet-mixes may have retained its hydrophobicity after heat treatment, thus maintaining its ability to emulsify.

6.3.2.2 Viscosity

Figure 6.5 shows viscosity of wet-mixes before and after heat treatment and homogenisation. Apparent viscosity (at 500 s^{-1}) of SH wet-mixes was significantly lower ($P < 0.05$) prior to heat treatment and homogenisation compared to NH and PH wet-mixes (i.e. after ingredient addition and formation of coarse emulsion). This suggested lower voluminosity in hydrolysed proteins compared to intact proteins. During heat treatment, both PH and SH wet-mixes increased in viscosity to a lesser extent than NH wet-mixes (Figure 6.5). Intact β -Lg appears to have played an important role in viscosity increase during heat treatment. Whey proteins in NH wet-mixes contained a greater proportion of intact β -Lg (Figure 6.1.), which, due to the presence of a free thiol group may have caused large increases in viscosity by increasing voluminosity of dispersed proteins and fat globules as result of thiol-disulphide interchanges and hydrophobic interactions (Snoeren et al., 1982; Dalgleish et al., 1997; Schokker et al., 2000; Anema and Li, 2003a; Guyomarc'h et al., 2009). PH wet-mixes which contained some residual β -Lg underwent a lower extent of heat-induced viscosity increase compared to NH formulations. The absence of β -Lg from SH wet-mixes would appear to account for their lower viscosity (Figure 6.1.). It is possible that α -La denaturation in SH wet-mixes during heat treatment was reversible due to the lack of a free thiol group (found in β -Lg) to initiate irreversible heat induced changes and viscosity increase (Schokker et al., 2000; McGuffey et al., 2007).

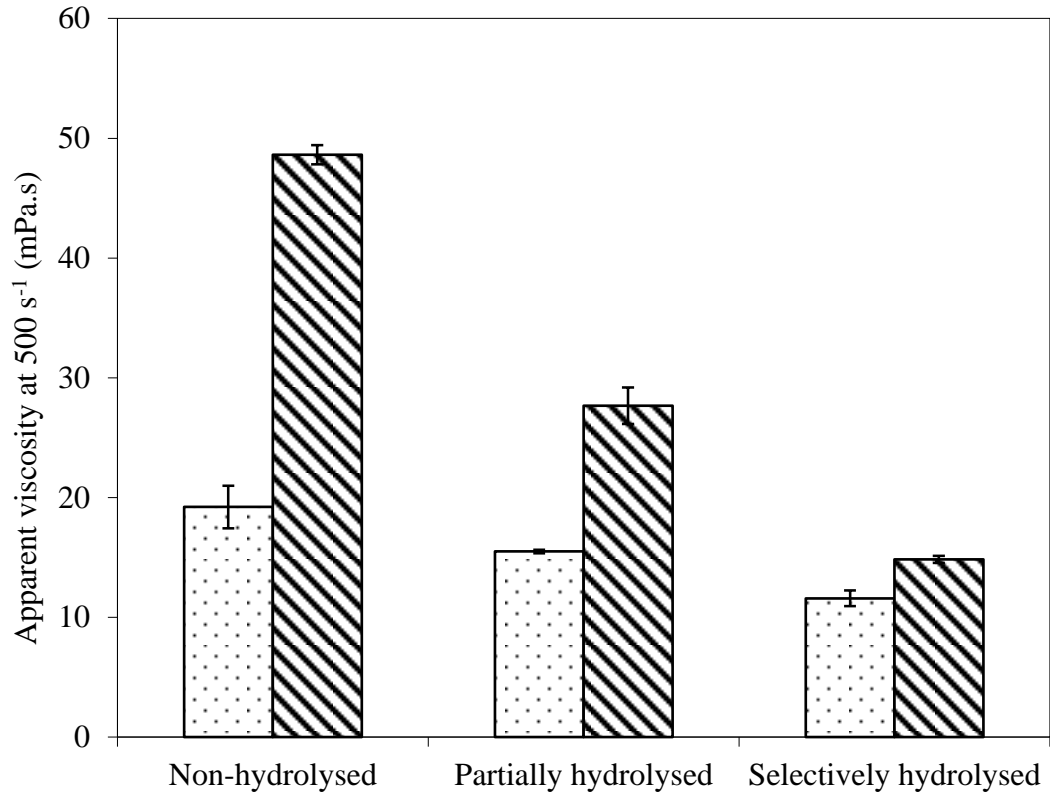


Figure 6.5. Apparent viscosity (500 s^{-1} ; $55 \text{ }^\circ\text{C}$) of wet-mixes before (dotted fill) and after (shaded fill) heat treatment and homogenisation. Error bars represent standard deviation of three replicate trials.

All minerals were in specification, with the exception of sodium in SH wet-mixes, owing to differences in the innate sodium contents of the SH protein ingredient (Codex Alimentarius, 2007). The sodium content in reconstituted SH powders (12.5% w/w) was $80.5 \pm 0.2 \text{ mg } 100 \text{ g}^{-1}$ compared to 20.2 ± 3.6 and $21.8 \pm 1.8 \text{ mg } 100 \text{ g}^{-1}$ for reconstituted NH and PH powders respectively. Higher sodium content has been found to increase protein aggregation and reduce gelation time in whey protein systems as a result of shielding of negative charges on protein molecules by Na^+ ions (Mulvihill and Kinsella, 1988; Tang et al., 1993; Verheul et al., 1995; Puyol et al., 2001; Caussin et al., 2003; Fitzsimons et al., 2007). In the specific case of β -Lg, which was selectively hydrolysed in SH formulations, it has been found that addition of NaCl resulted in the formation of more voluminous aggregates upon heating (Durand et al., 2002). However, in the current study the SH formula had lower viscosity, suggesting that hydrolysis of the β -Lg negated

any effect of the Na⁺ ions; thus, selective hydrolysis of β -Lg was most likely responsible for the low viscosity in the SH formulations shown in Figure 6.5.

Low viscosity in combination with good emulsification properties and the possible reduced tendency to fouling make SH protein ingredients suitable for high-dry matter (> 55% w/w) manufacturing of IMF. Dry matter content, and thus viscosity of wet-mixes, can be a limiting factor during spray drying; above a critical limit, viscosity of concentrates during atomisation can produce droplets too large to be dried efficiently. It is suggested that optimal viscosity for whole milk powder, for example, should not exceed between 60 and 100 mPa s for efficient atomisation (Westergaard, 2004). Inefficient drying may lead to technological problems such as increase in the insoluble fraction of resulting powders (Fox et al., 2010). A major benefit of increasing dry matter content of spray-dryer feeds is that it simply reduces the amount of water to be removed during drying. Energy costs required for spray drying are extremely high - as much as 4500 MJ per tonne of water removed (Fox et al., 2010). Increasing the dry matter content of a 55% (w/w) concentrate by 1% reduces the amount of water to be removed by some 22 kg (if 55% (w/w) concentrate contains 1 tonne water) leading to a decrease in energy requirements for drying by approximately 100 MJ. The findings in this study suggest that by using SH protein ingredient in formulations, IMF manufacturers can develop concentrates with good physical stability throughout thermal and spray drying processes.

6.3.3 Powder properties

The volume mean diameter, D[4,3], and moisture content of powders were correlated to the viscosity of feed formulations (Table 6.3). NH powders had significantly ($P < 0.05$) higher powder D[4,3] and moisture content compared to SH and PH powders. It is possible that during the drying process atomisation of lower viscosity hydrolysed wet-mixes produced droplets with greater specific surface area and increased contact with the drying air, thus, improving efficiency of drying. This would result in smaller powder particles of lower moisture content. Surface free fat, dispersibility and wettability were not affected by the proteins (NH, PH or SH) used and were comparable to those found in commercially available infant formula powders (Hanley et al., 2011).

It has been suggested that protein hydrolysates may decrease glass transition temperature (T_g) in food powders (Netto et al., 1998). Mounsey et al. (2012) found that hydrolysed proteins lowered T_g in sodium caseinate-lactose powders. However, T_g did not

differ significantly between powders with various levels of hydrolysis (NH, PH or SH). It would appear that, at the protein/peptide to lactose ratio examined, it is lactose, rather than proteins, that determines the glass transition behaviour (Bhandari and Howes, 1999).

6.3.4 Powder reconstitution properties

The D[4,3] of powders reconstituted in water (12.5% w/w) was lower than that of feed dispersions prior to spray drying. This indicates that a further homogenisation effect occurred during atomisation (Table 6.4). Viscosities of reconstituted powders reflected those of wet-mixes prior to drying i.e. reconstituted NH powders exhibited the highest viscosity (3.80 ± 0.07 mPa s), SH powders exhibited the lowest (3.36 ± 0.03 mPa s).

No differences were observed in the separation stability (as measured by analytical centrifugation) of reconstituted powders during accelerated storage over 2 h. No free fat or particulate matter was observed in any of the reconstituted powders. Such findings suggest that IMF powders, with satisfactory reconstitution properties, were produced in each case and that inclusion of hydrolysed proteins had no negative impact on the creaming stability of reconstituted IMF powders.

Table 6.4. Properties of powders reconstituted in water (12.5% w/w) at 20 °C

Reconstituted powder	Viscosity ¹ (mPa s)	Volume mean diameter (D[4,3]) (µm)	Clarification* (% h ⁻¹)
Non-hydrolysed	3.80 ± 0.07^a	0.52 ± 0.11^a	0.41 ± 0.39^a
Partially hydrolysed	3.53 ± 0.02^b	1.61 ± 0.39^b	0.71 ± 0.78^a
Selectively hydrolysed	3.36 ± 0.03^c	0.48 ± 0.06^a	0.49 ± 0.51^a

¹ Apparent viscosity at 500 s⁻¹

Values presented are the means of 3 replicates ± standard deviation. Fisher's individual error rate was used to calculate significance

^{a,b,c} Values within a column not sharing a common superscript differ significantly, $P < 0.05$

*Change in transmission of light through reconstituted powder over simulated 2 h storage

6. Physical stability of infant milk formula made with selectively hydrolysed whey proteins

Table 6.3. Physical characteristics of manufactured infant milk formula (IMF) powders

IMF powder	Protein content (% w/w)	Volume mean diameter (D[4,3]) (μm)	Moisture content (% w/w)	Surface free fat (% w/w of powder)	Dispersibility (%)	Bulk density (g mL^{-1})	Wettability (s)	Glass transition temperature (T_g) ($^{\circ}\text{C}$)
Non-hydrolysed	11.82 \pm 0.21 ^a	155.43 \pm 5.74 ^a	1.95 \pm 0.11 ^a	0.68 \pm 0.14 ^a	95.61 \pm 1.82 ^a	0.43 \pm 0.01 ^a	24.88 \pm 0.52 ^a	53.04 \pm 2.92 ^a
Partially hydrolysed	11.61 \pm 0.30 ^a	118.28 \pm 6.70 ^b	1.82 \pm 0.12 ^{a,b}	0.63 \pm 0.08 ^a	96.73 \pm 0.14 ^a	0.52 \pm 0.01 ^b	20.27 \pm 3.94 ^b	55.82 \pm 1.12 ^a
Selectively hydrolysed	11.89 \pm 0.17 ^a	103.00 \pm 3.03 ^c	1.68 \pm 0.08 ^b	0.56 \pm 0.05 ^a	98.05 \pm 1.26 ^a	0.45 \pm 0.01 ^a	24.13 \pm 0.54 ^a	53.94 \pm 3.78 ^a

Values presented are the means of 3 replicates \pm standard deviation. Fisher's individual error rate was used to calculate significance.

^{a,b,c} Values within a column not sharing a common superscript differ significantly, $P < 0.05$

6.4 Conclusions

This study has shown that hydrolysed milk proteins may be successfully used to produce IMF powders with good physical characteristics. Heat-treated and homogenised wet-mixes containing hydrolysed ingredients had significantly ($P < 0.05$) lower viscosities than formulations containing non-hydrolysed ingredients. Ingredients in which β -Lg was selectively hydrolysed resulted in wet-mixes with the lowest viscosity. Emulsification, drying and reconstitution properties of selectively hydrolysed wet-mixes were comparable or better than standard, non-hydrolysed wet-mixes.

Production of low viscosity wet-mixes, using selectively hydrolysed whey proteins provides a potential mechanism for drying IMF at high dry matter content while maintaining good concentrate atomisation and ensuing powder functionality (flowability, wettability). Maximisation of concentrate dry matter content, whilst avoiding associated processing problems, provides the opportunity for significant cost savings during large-scale production of IMF powders.

Chapter 7: Overall discussion and conclusions

7.1 IMF manufacture

Infant and follow-on nutritional formulations (liquid or powder) are a diverse range of fortified foods designed to meet the nutritional requirements of neonates and toddlers. Infant milk formula (IMF) is manufactured worldwide by a diverse range of companies with food, nutritional and/or medical backgrounds. Consequently, methods for manufacture may vary between companies. The present study focused on IMF powder manufacture by wet-mixing, whereby, protein and carbohydrate ingredients are hydrated in water, recombined with vegetable oils, and, spray dried. Most standard IMF is manufactured from bovine milk constituents. However, the macro-composition (protein; fat; lactose) of IMF is variable and quite different from bovine milk (European Commission, 2006). In addition, specialised IMF products are also available, e.g., utilising, hydrolysed proteins to reduce allergenicity or increase digestibility (Maldonado et al., 1998).

Compositional and process interactions can have large effect on the physical characteristics of wet-mixes. For example, wet-mixes may contain relatively high quantities of whey protein and thus β -Lactoglobulin (β -Lg), as it is the most abundant whey protein in bovine milk. β -Lg is particularly heat sensitive and will aggregate with adjacent β -Lg molecules and other proteins (both whey proteins and caseins) when heated at temperatures greater than 65 °C (Walstra and Jenness, 1984; Anema and Li, 2003a; Singh and Havea, 2003). Furthermore, in emulsified systems, thermal denaturation of whey protein can cause aggregation of fat globules (Euston et al, 2000). In wet-mixes containing hydrolysed protein, physical behaviour during heat treatment may be significantly different to those containing intact non-enzymatically modified proteins; Britten et al. (1994) reported aggregate formation during heat treatment of whey proteins was dependent on both degree of hydrolysis and pH. The type of heat treatment, direct or indirect, may also have an effect on physical characteristic. Direct heat treatment is relatively less severe, compared to indirect, and may result in reduced heat induced changes, such as whey protein denaturation (Datta et al., 2002).

The physical characteristics of wet-mixes of bovine milk proteins prior to spray drying have a significant effect on ensuing powder quality (De Vilder et al., 1976, 1979). Viscosity of wet-mixes may be a limiting factor during evaporation and spray drying. Evaporation is more energy efficient than spray drying, however, the extent of concentration by evaporation is restrained by the viscosity of the resultant concentrate

which should not be too high in order to ensure efficient spray drying (Westergaard, 2004). Therefore, dry matter (DM) content of spray drying feeds is limited by viscosity; thus, the economic implications of concentrate viscosity are important as it effectively determines the amount of water to be removed during the energy-intensive spray drying operation. The empirical investigations discussed hereafter explored the effect of compositional and processing on viscosity during IMF manufacture.

7.2 Modularisation of IMF manufacture

The microstructure of commercially available powders was investigated in Chapter 2; presence of large lactose tomahawk crystals in approximately 50% of powders was indicative of processes in which lactose was dry blended into a spray dried base powder. This represents a “modularisation” of manufacture where powders containing certain constituents (e.g. proteins and fat) could be manufactured and dry blended with powders containing other constituents (e.g. lactose and/or proteins) to produce powders of the required nutritional specification (such as described by Mullane et al., 2007). Powders containing lactose crystals did not exhibit inferior flow characteristics compared to lactose crystal-free powders, despite having properties reported to contribute to poor flowability e.g. small particle size and lower sphericity (Fitzpatrick et al., 2004; Ortega-Rivas, 2009; Fu et al., 2012). It was postulated that crystalline lactose may have better flow properties compared to amorphous surfaces of milk powder particles (as observed by Yazdanpanah and Langrish (2011) for SMP particles) and/or lactose crystals could act as barriers between milk powder particles, thereby reducing contact and increasing flowability. When rehydrated to 55% (w/w) and heated, powders containing lactose crystals behaved differently to rehydrated lactose crystal-free powders, which could have been a result of process and compositional interactions in the manufacturing of lactose-depleted base powders.

Implications of the observation made in Chapter 2, i.e., that some IMF products may be made from combining lactose-depleted base powders with crystalline lactose, were seen in Chapter 3. In accordance with previous studies, lactose played an important role in stabilising whey proteins during heat treatment; in the presence of lactose, heat induced changes to β -Lg occurred at higher temperatures, and heat induced viscosity increase was lower, compared to lactose free systems. (Garrett et al., 1986; Kulmyrzaev et al., 2000; Rich and Foegeding, 2000). Additionally, casein micelles were found to increase the temperature at which whey protein started to denature and lose secondary

structure. Previous work in this area had focused on the effect of individual casein molecules rather than casein micelles and results vary (Blanc et al., 1977; Paulsson and Dejmek, 1990; Imafidon et al., 1991). Despite having a stabilising effect against denaturation, casein micelles did not significantly reduce the extent of heat-induced viscosity increase, which was likely influenced by aggregation of unfolded whey proteins with casein micelles (Anema and Li, 2003).

7.3 High dry matter content IMF: development of a novel process

Effects of increasing DM content on physical and heat transfer properties of IMF wet-mixes were investigated in Chapter 4. Figure 4.3 shows the extent of increase in viscosity of IMF wet-mixes between 20 and 60% (w/w). Increased viscosity of high DM wet-mixes may be a limiting factor during heat treatment, especially when indirect heating methods are employed. Fouling may be increased by the lack of turbulence associated with high viscosity wet-mixes. High DM content wet-mixes would require increased heat transfer areas due to lower heat transfer coefficients (see Figure 4.11). Fouling could be further exasperated by heat-induced viscosity increases when wet-mixes > 50% (w/w) are heat treated > 90 °C. When 50 and 60% (w/w) wet-mixes were heated at 95 °C, extensive viscosity increase due to aggregation of whey proteins was observed. Therefore, fouling during heat treatment (>90 °C) of high DM wet-mixes may be intensified by unfolded and/or aggregated β -Lg adhering to walls in processing plants (De Jong et al., 1992; Changani et al., 1997; De Jong, 1997).

A novel high-solids steam-injection (HSSI) process was developed with the aim of limiting heat induced denaturation of whey proteins and viscosity increases in high DM wet-mixes (see Figure 4.1). Rotor-stator homogenisation was applied to 60% (w/w) wet-mixes, prior to heat-treatment using direct steam injection. The HSSI process resulted in physically stable heat treated wet-mixes, with lower levels of denaturation and lower viscosity compared to 60% (w/w) wet-mixes heat treated using a tubular heat exchanger. This was most likely due to the lower severity of direct heat treatments compared to indirect treatments (Lewis and Deeth, 2009). In accordance with previous studies of direct heat treatment, an accompanying homogenising effect was observed within the homogeniser which further contributed to increasing physical stability (Zadow, 1969; van Boekel and Folkerts, 1991; Ye et al., 2005). Homogenisation effects were likely caused by shockwave fronts created during condensation of steam (Adamopoulos and Petropakis, 1999).

In Chapter 5, the direct heating employed in the HSSI process produced spray-drying feeds (55% w/w) of significantly reduced viscosity and higher native whey protein content compared to a process employing indirect heat treatment at 35% (w/w) followed by evaporation to 55% (w/w). Powders produced from both processes were of similar quality, indicating the feasibility of the HSSI process. The reduced viscosity associated with HSSI has interesting implications for processing of IMF at > 60% (w/w); production of more concentrated spray-drying feeds using the HSSI process could reduce water removal costs while limiting problems associated with atomisation of high viscosity fluids (Westergaard, 2004). In addition, production of acceptable quality powders from a process utilising rotor-stator homogenisation is noteworthy, given that the alternative, valve-type homogenisation, is ubiquitous within the infant formula industry and dairy science literature.

7.4 Hydrolysed protein ingredients

In Chapter 2, it was observed that reconstituted commercial powders made with hydrolysed proteins (n=3) were less stable to separation compared to reconstituted powders made with intact proteins (n=12). This was partially due to the presence of starch in two of the three hydrolysed powders which resulted in sedimentation, however, the poor stability to creaming observed in the remaining reconstituted powder was likely due to poor stabilisation of fat by the hydrolysed protein ingredient. Altered emulsification properties of hydrolysates compared to intact proteins can be expected and are dependent on degree of hydrolysis (Euston et al., 2001). Similarly, reaction of hydrolysed whey proteins to heat treatment is related to degree of hydrolysis, which may explain the low viscosity increase observed during heating of the starch-free, reconstituted hydrolysed IMF powder (see Table 2.1; 55% w/w) compared to the majority of reconstituted powders made with intact proteins (Britten, 1994).

In Chapter 6, the effect of hydrolysed ingredients on viscosity and emulsification was investigated during manufacture of IMF powders. Three protein systems were employed: 1) non-hydrolysed (NH) whey proteins and caseins, 2) partially hydrolysed (PH) whey proteins and caseins, and 3) selectively hydrolysed (SH) whey proteins, where β -Lactoglobulin (β -Lg) was selectively hydrolysed. Viscosities (at 55% w/w) of heat-treated wet-mixes made with SH (14.8 ± 0.3 mPa s) and PH (27.6 ± 1.5 mPa s) proteins were lower than wet-mixes made with NH (48.6 ± 0.8 mPa s) proteins. Lower viscosity of heat-treated wet-mixes made with PH proteins compared to NH proteins may have

been related to degree of hydrolysis (Britten 1994). The low viscosity of heat treated SH wet-mixes was probably more related to absence of β -Lg (see Figure 6.1) than degree of hydrolysis. Denaturation of α -Lactalbumin (α -La) in SH wet-mixes during heat treatment could have been largely reversible, due to the lack of a free thiol group (found in β -Lg) which plays a key role in irreversible heat induced changes such as viscosity increase (Schokker et al., 2000; McGuffey et al., 2007). Despite observations made in Chapter 2 that emulsification in hydrolysed commercial IMF powder was inferior to non-hydrolysed powders, emulsion quality of wet-mixes made with SH protein ingredients was similar to NH protein ingredients. This was hypothesised to have been a result of α -La present in SH protein ingredients, which may be largely absent from indiscriminately hydrolysed protein ingredients. Powders produced from SH protein ingredients had similar physical qualities to powders made from NH protein ingredients, which indicated the feasibility of the approach. As in Chapter 5, low viscosity prior to spray drying associated with SH protein ingredients indicates the potential to further increase DM content (> 60% w/w).

7.5 Overall conclusions

Overall, the research presented in this thesis explored various aspects of IMF manufacture, particularly viscosity development during heat treatment of high DM content wet-mixes. Reduction of viscosity was explored using three approaches: 1) Compositional – study of the interaction between IMF components, 2) Process – development of novel HSSI processing path, and 3) Enzymatic – utilisation of a novel enzymatically modified protein ingredient.

Micro-structural analysis of commercially available IMF powders indicated different methods of manufacture are applied across the industry. Results suggest that some commercial IMF powders are produced by blending dried ingredients (base powders) together, rather than by spray drying of wet-mixes containing 100% of constituents. Composition of base powders is likely to be quite variable and many combinations of different types of powder are possible to reach the final nutritional specification after dry-blending. Heat induced changes in wet-mixes during manufacture of IMF and base powders associated with whey protein (denaturation, viscosity) are not only a function of concentration but are also dependent on interactions between macronutrients. Therefore, in designing processes for manufacture of IMF and base powders it is essential to consider compositional interactions in relation to heat treatment.

Direct heat treatment is an effective way of reducing whey protein denaturation during heat-treatment of IMF compared to indirect heat treatment. The relatively lower severity of direct heat treatment can also limit the extent of viscosity increase. Similarly, alteration of the thermally reactive whey protein, β -Lg, through selective hydrolysis is an effective manner of reducing viscosity in high DM content wet-mixes, while maintaining good emulsification properties compared to partially hydrolysed proteins. The combination of low viscosity at high DM content associated with both direct heat-treatment and enzymatic approach would reduce costs associated with water removal by spray drying, while limiting deleterious effects associated with drying of high viscosity fluids.

In conclusion, this research has provided theoretical and practical insights relevant to viscosity reduction in IMF manufacture. The research is particularly relevant to IMF manufacturers seeking methods to reduce production costs. Many aspects of this study, including a confidential process, have been transferred to industry. Suitable follow-up studies to the work presented in this thesis are:

- Assessment of fouling associated with the HSSI process in comparison to conventional processes over extended timeframes.
- Determination of maximum DM content which can be feasibly heat-treated using HSSI processing.
- Study the effect of utilising selectively hydrolysed ingredients in the HSSI process.
- Apply HSSI processes and shockwave fronts in manufacture of other dairy ingredients

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Appendix: Published literature