INVESTIGATION INTO THE PROTEIN MODIFICATION OF NUTRITIONAL POWDER

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Abstract

Infant milk formula (IF) is a manufactured food that is fortified with nutrients to meet the composition of human mother's milk at the first 0-6 months postpartum. The common ingredients include bovine milk and/or whey as protein sources, lactose, vegetable oils, vitamins, and minerals. Unlike human milk, infant formula uses high- heat treatments to ensure microbiological safety and stability of the final product. These heat treatments are potentially detrimental to the nutrients by lowering the nutritional quality of the IF and can be seen as unnatural. Of particular interest is the effect of heating on proteins that contain the essential amino acids which supports the infant growth. The complex reaction between the proteins and lactose called *glycation*, measured as Furosine, is known to impair nutritional quality and protein digestibility. Hence, some infant formula manufacturers consider less heat treatment as a marker of naturalness.

Furosine was used as chemical marker in this study because it is known to be abundant in infant formula. The objective was to investigate if an IF that used a liquid skim had less modification as shown by furosine concentration, than an IF that used a Skimmed Milk Powder. Skimmed Milk Powder is produced by heating and dehydration and is usually rehydrated before it proceeds to the IF manufacture. Therefore, it experiences a double heat-treatment potentially resulting in a greater protein modification. The study showed that the commercial IFs with fresh Skimmed Milk had significantly lower modified proteins than an IF with Skimmed Milk Powder. The results suggested that the cumulative extent of modification found in the IF was greatest during spray drying. Additionally, it was shown that whey ingredients were responsible for the significant protein modification found in the batch tank. It was likely that the batch-to-batch variations of furosine observed in the commercial IFs were dominated by variations in the whey ingredients. The milk quality and the rigors of the subsequent whey protein production will be variable between factories, particularly those that use liquid whey. Therefore, the use of both liquid skim and liquid whey will minimize the contribution of the ingredients to the final IF.

The glycation and other parameters (denaturation and aggregation and lactulose) measured in this study gave insights to the possible differences of heat treatment and ingredients used between manufacturers. Lactulose content was analysed along with the total whey protein and β-lactoglobulin (β-LG) to gain information about the heat treatment history of the infant formula.

The outcome of this work can be used to guide manufacturers to decrease the protein modification in the commercial infant formula.

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Chapter 1. Introduction to thesis

1.1 Background

The World Health Organisation (WHO) and UNICEF recommend that infants must be exclusively breastfed for the first six months of life to achieve optimum growth, development, and health. Thereafter, infants should receive adequate nutrition to meet their evolving nutritional requirements from other food sources while breastfeeding. Dairy based infant formula products and formulated supplementary foods for young children are great substitute feed for infants who are unable to be breastfed. In New Zealand, exclusive breastfeeding at discharge remained steady between 2017-2019; 79.90% in 2017, 79.91% in 2018 and 79.19% in 2019 (New Zealand Breastfeeding Alliance, 2019). In 2020, the national exclusive breastfeeding rate dropped by 1.79% since and was sitting at 77.48% in 2020 (New Zealand Breastfeeding Alliance, 2020). This decline was due to the unprecedented barriers brought by the global pandemic, SARS-CoV-2. A recent study by Snyder and Worlton (2021) concluded the negative impact on a mother's ability to breastfeed especially for first-time mothers due to their inability to engage with health carers in-person and significant reduction to childcare access. However, Sakalidis et al. (2021) showed continued high breastfeeding rates in Australia and New Zealand. They highlighted the contribution of mothers spending more time at home with their child and the reported shortage of baby formula due to panic buying (Breathnach, 2020; Guynn, 2020; Juneau, 2020). Infant formula is forecasted to be worth USD 33.12 billion per annum between 2020-2024, progressing at over 7% compound annual growth rate (CAGR) in the forecasted period (Businesswire, 2020).

Milk is important in the human diet because it is a major source of high-quality protein. Specifically, the significant amount of essential amino acids it contains, and its higher bioavailability compared to plant proteins. Unlike human milk which has 60: 40 whey protein to casein ratio, bovine milk is casein dominant which constitutes ~ 80% and the whey protein only represents ~20% of the total protein (Masum, Chandrapala, Huppertz, Adhikari, & Zisu, 2021). With these differences in mind and the fact that the idea of having a dairy formula is to mimic human milk, it is apparent that the industrial considerations (e.g., processing and formulation) that must be taken into account to process the required final product are enormous and complicated.

The indispensable and conditionally indispensable amino acids in infant formula must be at least equal to that of breast milk under the Directive (European Union, 2013), refer to Table 1. Bovine milk contains amino acids in excess, but it is deficient in cysteine, methionine, and tryptophan. There are also differences between ratios of tyrosine/phenylalanine and methionine/cysteine in bovine and human milk which are considered important. Hence, the protein content of IF is

formulated higher (1.3-1.5g/100 g) than what is found in breast milk (0.8-1g/100g) to ensure that all AAs are adequately supplied.

Table 1: Levels of indispensable and conditionally indispensable AA in IF based on breast milk from European Union (2013)

	Per 100kJ ([1])	Per 100 kcal
Cysteine	9	39
Histidine	10	40
Isoleucine	22	90
Leucine	40	166
Lysine	27	113
Methionine	5	23
Phenylalanine	20	83
Threonine	18	27
Tryptophan	8	32
Tyrosine	18	76
Valine	21	88
([1])kJ = 0.239 kcal		

The effect of industrial processing on milk proteins is a well-studied area. Heat-treatment is one of them. This process is extensively used in milk processing for varying reasons but the most important is to destroy unwanted microbiological organisms or reduce to the limit allowed by regulations (Efigênia, Povoa, & Moraes-Santos, 1997). However, thermal treatment promotes denaturation of proteins, proteolysis, aggregation, and sometimes can cause undesirable chemical reactions that deteriorate the nutritional quality of the milk (such as the micronutrients and most importantly the bioavailability of essential amino acid such as lysine). To augment this in manufacturing IF, manufacturers introduce ingredients in the formulation which are normally in purer form. For example, vitamins, minerals, and sometimes amino acids that are commercially available. Whey protein concentrates (WPC), skim milk powders (SMP), whey protein hydrolysates (WPH), or demineralized whey (Demin whey) protein are used as protein sources to meet commercial scale and product requirements for IF and other paediatric nutritional products. The use of different protein sources in IF depends on the value proposition. For instance, WPH is used in infant formula with hypoallergenic claims because it has undergone a process called hydrolysis that reduces the amount of native protein that can cause allergenicity.

The cost of ingredients is another factor and the use of demineralized whey in the formulation is cheaper because it has high lactose content. As explained by Fenelon et al. (2019) about the formulation dynamics of infant formula, WPC (a more expensive ingredient) has higher protein (~80%) and will require 0.88g w/w to reach the target of 1.4% protein per 100 mL on reconstitution but 6.2g of lactose must be added to meet the typical 7.3g of lactose per 100 mL in infant formula upon reconstitution. In contrast, D90 (a cheaper ingredient) with 12.5% protein will require 5.6g to meet the protein content in 100 mL upon reconstitution but the quantity of lactose to be added is only 1.8g. Fresh milk is also used in IF manufacture to keep the natural nutritional benefits of milk. These differences in processing of ingredients have varying effects on the milk protein of the end-product including the overall bioavailability of the amino acid and their digestibility. Thus, this work focuses on determining a marker to measure the extent of these manufacturing differences on the milk proteins of the infant formula as the end-product which enables the manufacturer to control these changes if elimination is not possible.

1.2 Research aims and objectives

The main aim of the project is to provide understanding of the marker and the method suitable to measure the milk protein modification that occurs during the IF manufacture.

The proposed project will address the following key questions:

1. What are the suitable markers that can be used for milk protein modification determination?
2. What is the extent of protein modification occurring within each unit operation of IF manufacture?
3. What is the level of modification of each ingredient commonly used in NZ IF manufacture?
4. How does IF manufactured with Skimmed Milk compare to IF manufactured with Skimmed Milk Powder?
5. How does time since manufacture affect the protein in modification in the IF with Skimmed Milk and IF with Skimmed Milk Powder?
6. What strategy could reduce protein modification in the manufacture of infant formula?
7. How do different manufacturers compare?

Chapter 2. Literature Review

Paediatric formula is scientifically designed for complete and balanced nutrition which mimics human milk to support growth, development, increase immunity, and build a healthy appetite for infants and young children. Many infant formulas are based on dairy ingredients that undergo various processes including pH treatment, protein concentration and heat treatment that cause changes to dairy proteins. This section aims to identify from existing literature suitable protein modification markers that could measure the extent of changes and/or differences in the commercially available nutritional powders.

2.1 Milk Proteins

2.1.1 Whey

Whey protein (WP) is the soluble protein when casein is precipitated. The precipitation can be caused by lactic acid producing microorganisms such as making lactic acid whey, addition of acid in acid casein manufacture (acid whey) or by enzyme addition, used in rennet casein and cheese manufacture. Therefore, the whey from acid casein and cottage cheese manufacture is called an acid whey while the rest of whey produced is called sweet whey (A. Kilara & Vaghela, 2018). The liquid whey is a dilute stream and comprised of 94% water and 6% total solids (4.5% lactose, 0.8%protein and 0.7% minerals) (A. Kilara & Vaghela, 2004). The major WPs in bovine milk are β-Lactoglobulin (β-LG; approx. 50% of total WP), α-Lactalbumin (α-LA; approx. 20% of total WP), bovine serum albumin (BSA), immunoglobulin (Ig). In human milk, α-LA has the highest concentration among the WP with 20-25% (Lönnerdal, Forsum, Gebre-Medhin, & Hambraeus, 1976), therefore, there is a drive to fortifying IF with more α-LA (Murphy, 2015).

Structurally, whey proteins have globular shaped and well-defined tertiary and secondary structures. These structures can impair access of proteolytic enzymes to digest the proteins. Also, the structures make whey proteins prone to conformational changes during heat treatment. These changes affect the physical, structural, compositional properties of the proteins and drive protein-protein interaction, which changes the overall digestion kinetics of the protein in the infant stomach. Whey proteins provide a considerable amount of essential amino acids to support human growth (Wijayanti, Bansal, & Deeth, 2014) and emerging evidence from animal, in-vitro and limited human studies suggest that bioactivities can be linked to whey protein derivatives, e.g., concentrate, hydrolysates, isolates, peptides, and individual protein. For example, whey dominant or whey protein hydrolysate (WPH) based infant formula has shown to enhance immunity of the infant. Also, WPH is known to have less allergenicity, therefore the risk of infants developing allergy is low compared to the standard formula where whey proteins remain intact (Kelly, 2019).

2.1.2 Casein

Casein (CN) is precipitated during cheese making often referred as *curd*. In human milk, CN makes up 40% of the protein while in bovine milk CN makes up only approximately 20% of the protein. Furthermore, the major difference between the two is that human milk contains no α_{S2}-CN, while bovine milk contains casein types α_{S1}-CN, α_{S2}-CN, β-CN and K-CN (Packard, 2012). In bovine milk, α_{S1}-CN constitutes up to 40% of the total casein, α_{S2}-CN makes up to 10%, β-CN is up to 35% (the most hydrophobic of the CNs) and the remaining 15% is k-CN (Dziuba, Minkiewicz, & Darewicz, 2009). Caseins' key role is to carry calcium and phosphate from the mammary gland which are essential to neonates (Murphy, 2015). Furthermore, they can be divided into two groups: calcium binding (α_{S1}-CN, α_{S2}-CN, β-CN) and calcium non-binding (K-CN). In addition, caseins have varying phosphorylated groups which contribute to their high surface net charge.

In contrast to the whey protein, caseins exhibit a loose and very flexible structure and are associated with the supramolecular structures called micelles. They do not have tertiary structures and have less defined secondary structure and therefore do not undergo a *denaturation* as such (Pellegrino et al., 2011). This shows that in a raw milk system, native caseins can easily be digested in the stomach compared with native whey proteins. However, during the manufacturing process of milk where various heat treatment is involved caseins and whey proteins co-exist and undergo a series of complex reactions. The reaction products can be intensified further during IF processing. Thus, understanding these changes becomes important.

2.2 Common dairy protein sources in IF

2.2.1 Skim Milk (SM) and Skim Milk Powder (SMP)

Skim milk is produced by centrifugation of pasteurized raw milk; the other product is cream. Because it is in liquid form it has lower total solids (~9% solids; 0.1% fat and 8.9% solid non-fat) than skim milk powder, refer to Table 2 for composition proximate. Skim milk is used in IF as casein source.

SMP is also used as a source of casein and whey protein in IF and is obtained by removing water from pasteurized skim milk (Chandan, 2011; Murphy, 2015). The drying requires to reduce the moisture level down to 5% or less with a milkfat level of 1.5% and below, all in weight basis. The minimum protein content is 34% (weight basis) and this can be adjusted by a milk retentate or permeate. SMP can be classified into three heat treatments (low, medium, and high) depending on the whey protein nitrogen index (WPNI), a measure of undenatured whey protein nitrogen (WPN) which is inversely proportional to their heat treatment. Thus, the higher the thermal treatment the lower the WPNI as explained by Patel, Anema, Holroyd, Singh, and Creamer (2007). The application where the SMP is intended for often dictates the heat treatment it will have. For example, high-heat

SMP is commonly used in sweetened end-products such as confectioneries, UHT concentrated milk, caramel, milk chocolates, and in bakeries while medium heat is commonly used for beverages, yoghurts, and cultured products. Low heat SMP is suitable for fermented milk products, cheese milk and paediatric applications (New Zealand Milk Products, n.d.). Typical thermal treatments for low-heat SMP is 70-72°C/15s, 85°C/60s or 90-105°C/30s for medium heat and 120 °C for 1-2 mins or >120°C/ 4mins for high-heat SMP (Patel et al., 2007).

Table 2: Typical proximate of whole milk and skim milk from Chandan (2011)

	Water (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)
Skim Milk	90.9	0.1	3.3	5.0	0.7
Whole Milk	87.4	3.8	3.2	4.9	0.7

2.2.2 Demineralised whey (Demin whey)

Demin whey is manufactured by passing liquid whey through an ion exchanger and/or by electrodialysis. The demineralization level can be 70 or 90% (D90) which is suitable for 1st stage IF formulations, but 2nd stage, and follow-on formulas do not normally use demin whey because the 60:40 whey ratio is not required. Although demin whey is out of scope of the Whey Protein Concentrate (WPC) ingredient definition, the proportion of its lactose content (83%) relative to its protein level (13%) is a common ingredient in IF formulations. Figure 1 details the heat treatments used in a demin whey but excludes the temperatures used in the spray dryer because there are different variables that influence the drying conditions (e.g type and size of nozzle atomisers, rotation speed of the atomizer disc, air flow into the drying chambers, feeding flow to name a few). Table 3 summarizes the composition of different whey source in IF manufacture. There are no known standard spray drier conditions therefore, different manufacturers may use different conditions (Fenelon et al., 2019).

Table 3: Production specification of commercially traded whey protein products from Kelly (2019)

Composition (%)	WPC 35	WPC 80	WPI	D90
Protein (dry basis)	35	80	90	13
Fat	4	8	1	1
Ash	6	3.5	4	0.9
Lactose	60	3	1	83
Moisture	5	6	6	2.5

2.2.3 Whey Protein Concentrate (WPC)

The protein content of WPC used in IF ranges 35% to 80%. The production of this ingredient involves fat, lactose, and mineral removal to varying extents depending on the desired protein content. The processes include but are not limited to centrifugation, ultrafiltration, evaporation, and spray drying. WPCs with high protein levels (>65%) are often used in 1st stage IF and can replace demineralized whey in the formulation; but high-protein WPCs require more fortification of lactose. Furthermore, WPCs with low protein content (35 and 65%) are less expensive and can be incorporated in 1st stage IF; however, they can only be partially added because their mineral contents are high. Such restrictions are not applicable to 2nd stage and FOF. Therefore, low protein WPC can be used in 2nd stage and FOF formulations.

2.2.4 Whey Protein Hydrolysate (WPH)

WPH has been shown to improve the immune system of an infant (Holvoet et al., 2021; Kiewiet, Gros, Neerven, Faas, & Vos, 2015). Whey protein hydrolysate-based formula claims to be hypoallergenic (HA), less likely to cause allergies and less allergenic because in this form, milk proteins responsible for allergenic reactions are no longer present (Fenelon et al., 2019). Enzymatic hydrolysis of WP is a well-known process. The hydrolysis involves incubation of the substrate (e.g., WPC) with the desired enzymes at pH where the enzymes can achieve optimum activity in a reactor (e.g., a batch tank). The enzymes used gets inactivated using heat treatment and this could vary depending on the enzyme used (Guadix, Camacho, & Guadix, 2006). The inactivated hydrolyzed solution is dried back to powdered form.

2.2.5 Whey Protein Isolate (WPI)

WPI is manufactured using a microfiltration to remove fat, followed by ultrafiltration and diafiltration to increase protein content to at least 90% (Wang & Lucey, 2003). This whey protein can be further purified by ion-exchange chromatography to remove the glycomacropeptide (GMP) produced during the rennet step of cheesemaking. The purification results to an increase in concentration of individual whey proteins. Therefore, WPI provides a certain platform for development of new IF formulations with enriched bioactives (Fenelon et al., 2019).

Figure 1: General manufacturing process of demineralized and non-demineralized whey, whey protein concentrates and isolates. Heat map of the processes were included except the spray dryer temperatures from Fenelon, Hickey, Buggy, McCarthy, and Murphy (2019)

2.2.6 Whole Milk Powder (WMP)

WMP is often produced from standardized milk. Standardisation ensures that the desired fat content is achieved. The composition of whole milk is given in Table 2. The milk is then concentrated through evaporation and spray dried into a powder. Unlike SMP, whole milk powder is not categorized into different heat treatments, and it is pasteurized at 80-85^{o}C (Bylund, 1995). Whole cow's milk can be introduced to toddlers age 9 or 10 months (Larnkjær, Hoppe, Mølgaard, & Michaelsen, 2009).

A summary of dairy ingredients and their respective origin is depicted in Figure 2.

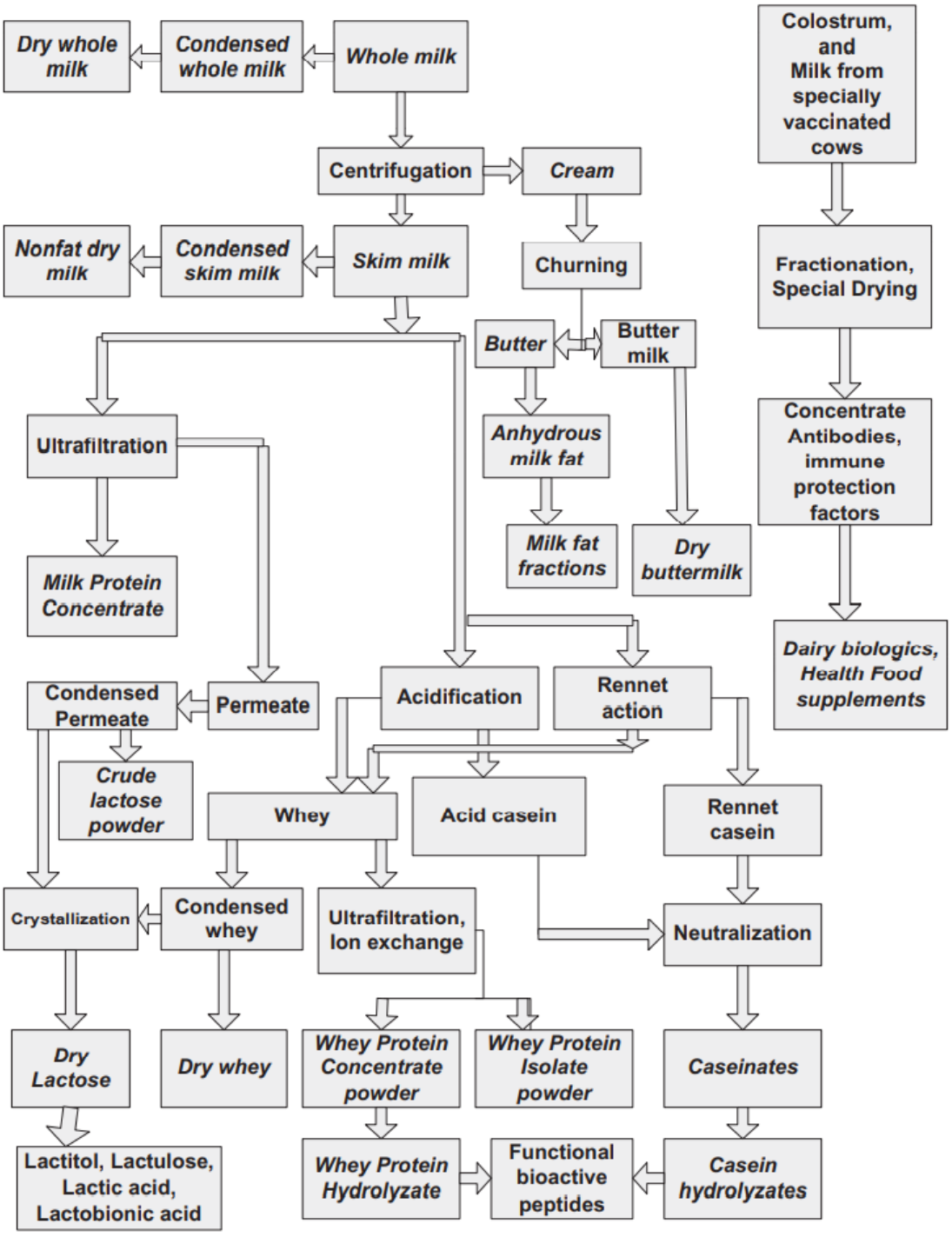


Figure 2: Dairy ingredients from De Wit (2003)

2.3 Nutritional Powders Processing

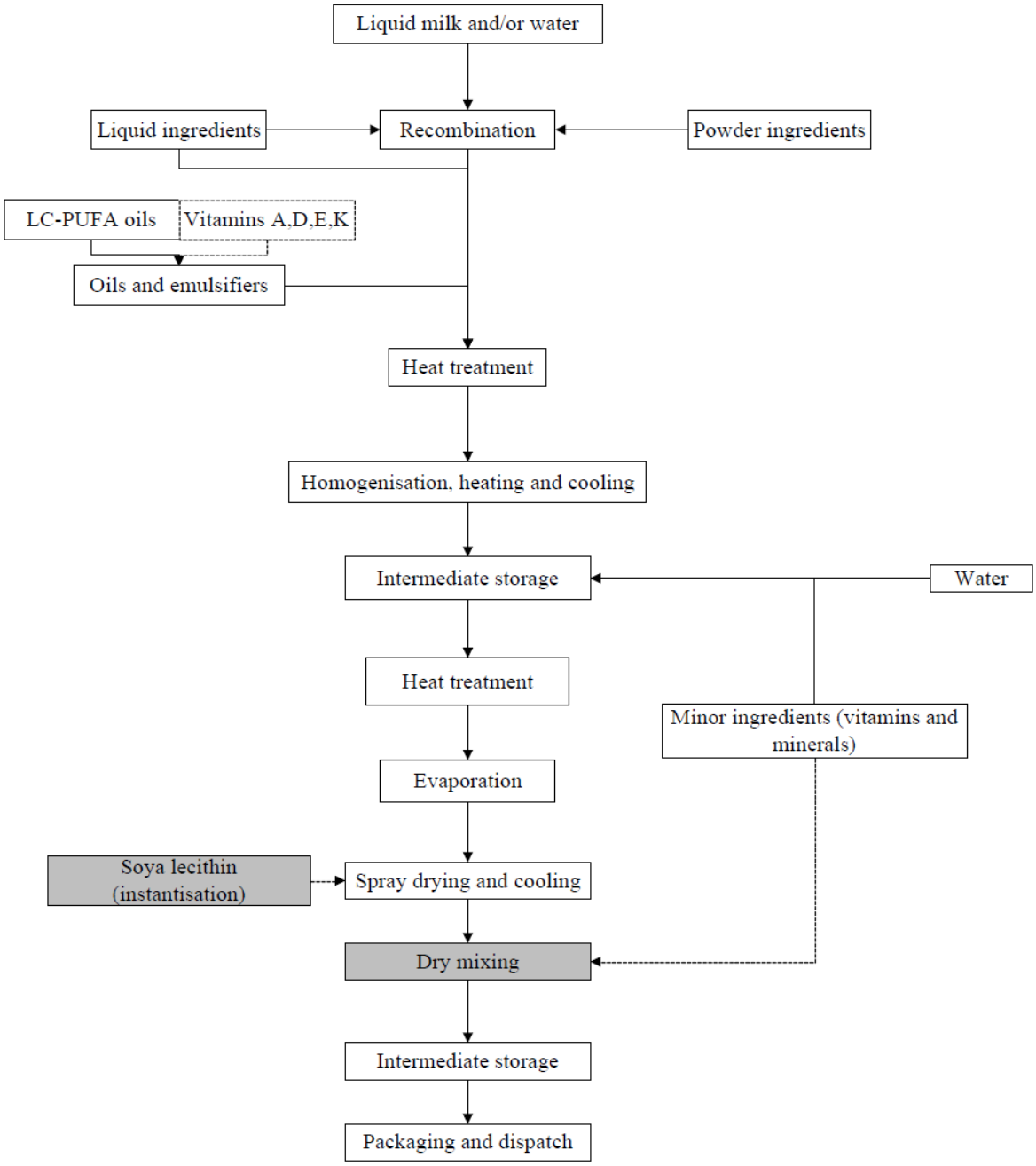


Figure 3: Generalized process diagram of IF and FOF manufacture (note: grey fill means process can be altered) from Murphy (2015)

Figure 3 depicts a general wet mix process of a commercial IF. Although the processes used by different manufacturers may vary, the entire process can be separated into three stages. The first stage involved hydration of powdered ingredients often involves the macro-ingredients (SMP or skim milk, WPC, sugar etc.), while oils are added once everything is in a wet mix. The second stage is when the wet-mix is homogenized to avoid fat separation and heat- treated thereafter to ensure

microbial safety. Finally, the emulsified and food safe mixture can be concentrated by evaporation into desired level of total solids and spray dried.

2.3.1 Effect of heat treatment

The dairy protein denaturation has been extensively studied and dates to Krueger, Ashworth, and Bendixen (1948); Rowland (1937) where both studied the effect of heat treatment and kinetics of milk protein denaturation. Recent advances around analytical methods and improved access to laboratory scale experiments that could mimic commercial scale manufacture allow precise results to be presented.

Heat-Treatment (pasteurization) prior to homogenization is often used to ensure microbial safety. The positioning of this heat-treatment also improves the emulsification as large aggregates of fat produced due to heat-treatment are disrupted by homogenization. There are two types of heat treatment commonly used in IF manufacture; 1. Indirect heating whereby heat is transferred indirectly to the product via a physical barrier e.g., tubular or plate heat exchangers, 2. Direct heating transfers to the product by direct contact e.g., steam infusion or steam injection. Direct heating provides a faster rate of heat transfer and therefore there is less exposure to higher temperatures than indirect. For example UHT temperatures can be achieved in as little as 1s for direct heating, while indirect heating requires an excess of 10s to achieve the same temperatures (Fenelon et al., 2019). As a result, denaturation of whey proteins such as β-LG and α-LA is lower in direct heat treatment than the indirect heat treatment. Boye and Alli (2000) observed that the conformational changes of β-LG started above 40^{o}C and unfolded at ~51^{o}C at pH 7. They also reported that the denaturation temperature (T_d) of β-LG in the absence of α-LA was at 71.9^{o}C and decreased by 2.8^{o}C (69.1^{o}C) in its presence. Conversely, the T_d of α-LA increased by 2.5^{o}C in the presence of β-LG which suggested that the latter can stabilize α-LA. All temperatures described were below the pasteurization temperature hence, it was expected that in IF manufacture, whey proteins are already partly denatured during heat-treatments before the evaporation process regardless of where they come from e.g., skim milk, SMP, and WPC. In conjunction, the kappa-caseins from heated skim milk form complexes with β-LG at pH <6.9 which stabilizes the casein micelle against precipitation (Singh & Fox, 1987). At 60:40, whey to casein ratio between pH 6.5-6.8 subjected to 140^{o}C for 80s heating, sediments of both whey and caseins were found, but at higher pH 6.9-7.1 only the β-LG was found in the supernatant after sedimentation (McSweeney, Mulvihill, & O'Callaghan, 2004).

Evaporation of IF wet-mix is used to remove water before spray drying, as illustrated in Figure 3. Removal of water by evaporation is preferred over spray drying due to lower energy costs and higher throughput. Evaporation is usually conducted under vacuum to reduce the evaporation

temperature to 50-70°C which minimizes damage to heat sensitive ingredients. The extent of concentration that can be achieved by evaporation is limited by the viscosity of the solution that increases with concentration. The most reported result on the effect of concentration on whey proteins during evaporation was β-LG denaturation decreases as the dry matter increases (Skelte G. Anema, 2000; Hillier, Lyster, & Cheeseman, 1979; McKenna & O'sullivan, 1971). Both lactose and dry matter did not to affect α-LA and since it is a monomeric species, the shift in molecular association was not expected (Skelte Anema, 2001). Nevertheless, the denaturation of whey proteins largely occurs in heat treatment. This means that if the protein ingredients were already in powdered form (e.g., SMP) that are used in IF manufacture, are anticipated to produce IF with higher proportion of denatured whey proteins than IF made from liquid ingredients such as skim milk.

Spray drying is the removal of water from the wet-mix by atomizing droplets into hot air. This process was found to have little effect on whey protein denaturation in skim milk systems (Guyomarc'h, Warin, Donald Muir, & Leaver, 2000; Oldfield, Taylor, & Singh, 2005) but it was found that the outlet air temperatures between 100-120°C can influence the denaturation of whey proteins (Murphy, 2015). The inlet air temperature has a minimal damage to milk proteins due to its evaporative cooling effect when water is removed rapidly and when particle is dried (Arun Kilara, 2011).

Table 4: Typical air temperatures used during spray drying of IF from Montagne, Van Dael, Skanderby, and Hugelshofer (2009)

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

2. 4 Modifications of milk protein due to processing

2.4.1 Physical Modification and measurement

Denaturation and Aggregation

Denaturation refers to the unfolding of proteins because of processing (Wijayanti et al., 2014). Thermal treatments and pH adjustments are known to drive conformational changes to dairy proteins. The extent of denaturation can either be *reversible* (partial unfolding), whereby whey proteins lose a helical structure or *irreversible* that leads to aggregation, such as formation of

disulphide linkages (Vasbinder & De Kruif, 2003) and intermolecular interactions such as electrostatic and hydrophobic interactions (SG Anema & Li, 2000; Hoffmann & van Mil, 1997; McMahon, Yousif, & Kalab, 1993). The irreversible denaturation dictates the functional properties of milk products (S. G. Anema, 2017). Thus, understanding the mechanism of these reactions is important because knowledge will help devise a way to improve the chemical and nutritional properties of dairy products.

Whey protein denaturation is one of the major modifications caused by heat treatment. In the study by S. G. Anema (2017), total whey protein in a reconstituted whole milk followed a second-order reaction. Using an Arrhenius plot, the total whey denaturation changed slope at 85^{o}C. The activation, enthalpy and entropy occurred in the temperature range for denaturation process which was at 70-85^{o}C, and these are pasteurization conditions. At 115^{o}C chemical reactions such as aggregation were observed. The kinetics of individual whey proteins from different dairy products were also successfully applied (Skelte G Anema & McKenna, 1996; Kessler & Beyer, 1991; Parris, Purcell, & Ptashkin, 1991). Hence, understanding the kinetics of denaturation principle is equally essential to predict the extent of denaturation after a defined heat treatment.

β-LG is often assumed to be the driver of WP denaturation due to its abundancy in milk compared to the other WPs. It is also known that denatured β-LG interacts with other WP (α-LA and BSA) and non-WPs such as κ-CN and an important milk protein change upon heating. This suggests a two-step mechanism when heat is applied; firstly, the β-LG unfolds and secondly, the unfolded structure could create protein complexes that are irreversible. In fact, Mulvihill and Donovan (1987) proposed a similar scheme. Their study showed that β-LG at room temperature 20^{o}C had a balance of monomeric and dimeric forms, but when temperature was increased by 10^{o}C and pH adjusted between 6 and 9, the dimer dissociated into monomeric forms. This observation was reversible at temperatures up to 70^{o}C. Loss of native tertiary and secondary structures were prominent, and the hidden disulphide bond of β-LG was exposed. Temperatures greater than 70^{o}C involved an irreversible protein complex both small (free thiol (-SH) oxidation and/or -SH/S-S (disulphide) linkages) and large (non-specific without -SH interaction) aggregates. However, Mulvihill and Donovan (1987) study was limited to isolated β-LG and α-LA from ultrafiltrate milk that was heated at 60 to 90^{o}C for 4 to 240 minutes (about 4 hours) and pH adjusted from 6 to 9. Other improved mechanisms were also proposed using a commercial WPC solution heated at 85^{o}C for 45 mins (Steventon, Gladden, & Fryer, 1991), a β-LG subjected into a lower heat treatment (60^{o}C to 70^{o}C) with a low ionic strength at neutral pH (Roefs & De Kruif, 1994), and a β-LG treated in a wider range of pH, temperature, ionic strength, and various time period between 0 to 5 hours (Verheul, Roefs, & de Kruif, 1998). Oldfield, Singh, Taylor, and Pearce (1998) studied the denaturation kinetics of whey

protein using a skim milk produced in a pilot-scale at ultra-high temperature (UHT) heated at 70^{o}C to 130^{o}C using a direct steam injection (DSI) system. Like Mulvihill and Donovan (1987), their model described the equilibrium existence of dimeric and monomeric form at room temperature and the former dissociate to monomeric forms once temperature increases and pH changes leading to formation of protein complexes. The addition lies in the presence of hydrophobic aggregates that convert into S-S linkages upon heating and the complexes are exacerbated by α-LA and denatured β-LG forming hydrophobic linkages at temperature >80^{o}C. Although there is no definite agreement on mechanism of physical and chemical interactions of whey proteins upon heat treatment and pH conditions, the common observation among studies about unfolding of proteins, exposure of protein components, association of proteins into both reversible and irreversible reactions, remain. This is an area still to be explored, especially with infant formula having a more complex system with other nutrients incorporated. Recent study by Halabi, Deglaire, Hamon, et al. (2020) demonstrated that the denaturation kinetics of α-LA, β-LG and LF were influenced by the composition of whey proteins in IF. They found that LF denaturation did not enhance in the presence of either α-LA and β-LG, but the denatured β-LG was modified in the presence of LF and α-LA caused by exposure of disulphide bonds which allows access to free thiols of β-LG. The amount of individual whey protein must also be considered as Surroca, Haverkamp, and Heck (2002) found that the maximum concentration of dimers, trimers and oligomers must reach maximum concentrations before polymerization occurs. Thus, the results show that the future focus in this area must be in understanding the interactions between the whey proteins in an infant formula.

2.4.2 Chemical Modification and measurement

Cross-linking

Non-enzymatic covalent cross-linkages due to heat treatment are formed from β-elimination of cystine/cysteine, serine or phosphoserine which results to dehydroalanine (DHA) is shown in Figure 4. Subsequent reaction of DHA with lysine, cystine and histidine yields to crosslinks lysinoalanine (LAL), lanthionine (LAN), and histidinoalanine (HAL), respectively. LAL has been the subject of interest among the β- elimination products because it exhibited renal lesion in rats after weeks of consuming LAL-rich diets, but this observation has not been seen in primates yet (De Groot, Slump, Feron, & Van Beek, 1976; Woodard & Short, 1977). According to De Groot et al. (1976), protein-bound LAL (up to 6000 ppm) does not induce renal activity in rats, but rather a peptide-bound and free LAL can at dietary levels of 100 ppm and above.

Figure 4: Protein cross-linkages via β- elimination from Pellegrino, Cattaneo, and De Noni (2011)

Friedman, Zahnley, and Masters (1981) studied the relationship between LAL of casein and in vitro digestibility. They found that LAL formation increased rapidly from pH 9 and formation of LAL reached 35% at pH 12.5. A dependency of LAL formation as function of temperature was also observed between 25 to 75^{o}C. The researchers ruled out that the nearly linear relationship between LAL formation and temperature was due to the inverse relationship of trypsin hydrolysis with temperature. This simply indicates an impairment of trypsin activity, which means a loss of susceptibility of enzyme cleavage causing a decline in digestibility. As demonstrated by Friedman et al. (1981), casein that was subjected to a 24-hour treatment time (pH 12.5 at 65^{o}C), had a noticeable

rapid increase in LAL formation up to 1-3hrs but the rates slowed thereafter. The result suggests that the changes in primary and secondary structures of protein induced by alkaline conditions promote hydrolysis in the first 3 hours and eventually reduces trypsin activity. This means that as the protein structural changes occur, it exposes specific amino acid residues required for the reaction and as the LAL formation (protein crosslinks) occurs and structural alteration continues, enzyme inhibition becomes possible. The essential AAs that go through this mechanism are rendered non-bioavailable. Thus, the extent of human body utilizing the heat induced LAL remains unclear and a subject to be explored. However, increase in LAL is not expected in infant formula manufacture as pH adjustments only reach to pH 6.8-7.

Deamidation

This post-translation modification is influenced by protein structure, temperature, and pH. Deamidation is undesirable because it can lead to structural changes of protein, stability, function, and immunogenicity. Deamidation refers to the hydrolysis of the amino side chain of asparagine (Asn) residue of protein to form an aspartic acid as seen in Figure 5 or glutamine (Glu) to form glutamic acid. *In vivo* deamidation is thought to act as a molecular timer in biological processes of aging. In pharmaceutical and other food industries, it relates to loss or changes in secondary, tertiary, and quaternary structure of protein which compromises its biological function or leads to an unwanted protein-protein aggregation (Gervais, 2016).

Deamidation can be facilitated by enzymatic or non-enzymatic reactions, the latter is most applicable to infant formula processing. Meltretter, Wüst, and Pischetsrieder (2014) concluded in their study that Asn deamidation is caused by heat and therefore, a good marker of rather intensive heat treatment. In addition, Ho, Zhu, Bansal, Boyce, and Le (2021) found that deamidation is pH dependent with Asn favouring the alkali condition (pH 10) and Glu in acidic condition (pH 3) and the latter, is also known to occur at slower rate (Bischoff & Kolbe, 1994). Hence, the level of deamidation in IF manufacture is expected to be very minimal because it uses a neutral pH (~6.8).

Also, the level of protein content is lower in IF (11-14% protein per 100g powder) unlike proteins used in biopharmaceuticals where they are usually purified, deamidation can have a deleterious impact on protein function. Certainly a case for recombinant human stem cell factor where haemoglobin deamidation was found to have lower oxygen affinity compared to non-deamidated variants as reviewed by Gervais (2016). There are also no link cases between poor digestion and deamidation of IF.

Figure 5: Non-enzymatic mechanism of asparagine deamidation (Gervais, 2016)

Dephosphorylation

Heat-induced dephosphorylation of phosphoserine undergoes hydrolysis which results to serine residue formation and can be involved in deamidation or proceed to form dehydroalanine (DHA) via β-elimination. DHA can further form lysinoalanine (LAL) by reacting with ε-amino group of lysine residues or with imidazole group attached to the histidine or thiols of cysteine which results to the formation of lanthionine (LAN) (Deeth & Lewis, 2016; Grewal, Huppertz, & Vasiljevic, 2018). For example, Meisel and Schlimme (1995) found dephosphorylation of phosphoserine residues in sterilized milk and a small extent of deamidation (van Boekel, 1999). In caseinate solution heated at 140℃ had very little dephosphorylation in the short term but increased by 50% after 30 minutes (van Boekel, 1999). Interestingly, Holland, Gupta, Alewood, and Deeth (2011) found no changes in deamidation levels in freshly prepared UHT milk and substantial concentrations were only found during storage at elevated temperatures. These inter and/ or intramolecular reactions that lead to undesirable cross-linkages are known to reduce digestibility (D'Agostina, Boschin, Rinaldi, & Arnoldi, 2003).

Furthermore, dephosphorylation of phosphoserine residue of casein due to heat treatment impairs the micelle's capacity to bind calcium and suppresses the production of caseinophosphopeptides which have been reported to play a role in the biological properties of casein (Pellegrino et al., 2011). Nonetheless, the fact remains that dephosphorylation of phosphoserine can proceed to form

crosslinks with other AA during heat treatment. Thus, one can only do a partial quantification of serine residues from dephosphorylation by measuring the DHA or the AA cross-linkages to use as a marker of this reaction. Also, the levels may remain minimal under IF manufacturing conditions.

Glycation

As milk contains high levels of lactose and protein, the abundance of Maillard Reaction Products (MRP) can be expected (Pischetsrieder & Henle, 2012). The most common degradation product of milk due to heat treatment is lactulosyllysine, an Amadori compound of lactose and the ε-amino group of lysine. This pathway is also illustrated in Figure 6. For extreme heat damage, this intermediate reaction proceeds to more reaction products called advanced glycation end products (AGEs). Thus, lactulosyllysine undergoes an oxidative degradation that yields to N^{ε} -carboxymethyllysine (CML) which is commonly associated with brown discoloration and flavour compounds (Mehta & Deeth, 2016). Other AGEs detected in processed milk N^{ε} -carboxyethyllysine (CEL), methylglyoxal-derived hydroimidazolone, oxalic acid monolysinylamide (OMA), pentoside and pyrraline. AGEs can also be derived from lactose degradation such as methylglyoxal reacting with an amino acid side chain of proteins. However, quantification of glycation is usually based on the Amadori product, lactulosyllysine, because lysine becomes biologically unavailable, which in turn reduces the protein and nutritional quality of milk products (Mehta & Deeth, 2016). Also, a recent study by Zenker, van Lieshout, van Gool, Bragt, and Hettinga (2020) that digestion of glycated casein can lead to formation of larger peptides that can survive gastrointestinal digestion.

Figure 6: Maillard Reaction products formation (Meltretter et al., 2014)

Unlike crosslinks of milk protein chains, lactosylation is a better marker of heat treatment in IF because of the natural abundance of lactose and lysine in milk. Most importantly, while lactose can

freely react with lysine, DHA can only react with an adjacent lysine. This partly explains why Pellegrino et al. (2011) reported far less LAL levels in some dairy products compared to furosine, a compound that is formed after the hydrolysis of lactulosyllysine in the powder. This will be discussed further later.

Raw bovine milk has been reported to contain 3-5mg furosine per 100g protein which increases over shelf life and estimated to increase by 7 mg per 100g every ten days at 23^{o}C. Pasteurised milk may contain up to 8 mg of furosine per 100g protein while ultra-high temperatures milk can range between 20- 250 mg per 100g protein (Sakkas, Moutafi, Moschopoulou, & Moatsou, 2014). Skimmed Milk Powder was reported to contain between 100-120 mg per 100g protein (Roland Van Renterghem & Jan De Block, 1996). Arena, Renzone, D'Ambrosio, Salzano, and Scaloni (2017) found that 50% of total protein lysine in Skimmed Milk Powder can be converted to lactulosyllysine.

Oxidation

Proteins can be subjected to oxidation modification via radical mediation, initiated usually by autoxidation of lipids and sugars or two-electron oxidation (Dean, Fu, Stocker, & Davies, 1997). The products include both stable and reactive species, and some are diagnostic of other damaging species. Other products can also arise from multiple pathways and are only used as generic markers of damage. Reactive radicals can also create major damage to the amino acid sidechains and backbone sites of the protein (Davies, 2016). Cysteine (Cys) and methionine (Met) residues are sulphur-containing AAs and can undergo direct oxidation. They were reported to be oxidized faster than protein peroxidation, and results to alteration of protein activity and function(Davies, 2016; Schöneich, 2017). Two Cys residues from heat treatment leads to disulphide (cystine) formation and are of particular importance as it yields to reversible intermolecular cross-linkages. In the presence(or absence) of oxidants these changes can be exacerbated and induces irreversible aggregation particularly the β-LG (Chen et al., 2019).

The aromatic amino acids histidine (His), tyrosine (Tyr) and tryptophan (Trp) are sensitive to oxidation because their radical reduction potentials are low, which give rise to oxidation species, 3-4-dihydroxyphenylalanine (DOPA) from Tyr, N-formylkynyrenine and kyunurenine from Trp, and crosslinked products such as di-Tyr and di-Trp. Aliphatic side chains namely arginine (Arg), lysine (Lys), proline (Pro) and threonine (Thr) can convert to unstable hydroperoxide (ROOH), alcohols and carbonyls via irreversible pathways, the reason alcohols and carbonyls are often used as markers of protein oxidation (Davies, 2016).

In the study conducted by Chen et al. (2019) showed that methionine sulphoxide (MetSO), a major methionine oxidation product, was significantly higher among all the protein oxidation markers they measured in four different IFs. Also, IF which used WPH had the highest MetSO level detected among the IF used in the experiment. This suggests that the additional processing employed in manufacturing the IF with protein hydrolysate contributed to the level found. The finding is unsurprising because side chains on hydrolyzed proteins are exposed and therefore more readily available to be oxidized compared to that of an intact protein. Therefore, this observation is not necessarily attributed by heat treatment but from hydrolysis that is part of hydrolysate manufacture. The MetSO levels the researchers presented must be also read with skepticism because the analysis presented in the paper did not include a correction factor for the hydrolysis undergone by IFs during the assay. This means that the reported results were probably higher than what were in the IF. Supplementary notes provided by the researchers did not clarify this. Also, MetSO can be converted back to methionine via renal MetSO reductase but there is always a small amount of it excreted in the urine as acetyl-MetSO with healthy patients' level at 0.02 nmol per mg creatinine (Zhao, Kim, & Levine, 2012). Thus, the use of this oxidation product for this work is not suitable.

Racemisation

This modification refers to conversion of L-amino acids (LAA) into D-amino acid (DAA) forms, known as the mirror image configuration. DAA is known to be indigestible because nature proteins are entirely built from LAA and most important AAs to human body exist in L-forms (Friedman & Levin, 2012). But it is known that free D-methionine (D-Met) is 100% bioavailable in rats, mice, poultry, and humans. Therefore, D-Met is extensively used as supplement in animal feeds to balance sulfur amino acids. In addition, D-tryptophan is also bioavailable to rats and a few other DAAs are partially available while the majority of them are not (Finot, 2005). For precautionary measures, only L- Met is intentionally added to foods for humans, hence the overall bioavailability of DAAs to humans whether they are consumed as free, or part of a protein remains unknown.

Racemization can be induced by factors such as oxidation reactions, high temperatures or changes in pH. The changes can lead to protein structure alterations, activities, properties and can increase toxicity (Chen et al., 2019). Finot (2005) mentioned that among the amino acids, aspartic acid is the most sensitive under severe heat treatment while in the alkaline conditions, the isomerization in a decreasing level follow this order; Ser, Cys, Asp, Thr, Phe, Met, Glu, Ala, Tyr, Lys, Leu, Ilu, and Val; branched AAs were the least converted. Schieber et al. (1997) reported that Sprague-Dawley rats did not show any pathological changes after feeding them 50mg D-Asp per kg weight orally, a known subacute toxicity level.

Comparison of Protein Modification

A comparison of different protein modification reactions is presented in Table 5 summarizing the markers, methods of quantification, whether the reactions are affected by heat treatments, pH adjustments and sources from the literature. Glycation and Aggregation and Denaturation were identified as primary markers to be used in this study because of their occurrence, abundancy, and accessibility of the methods.

Table 5: Known milk protein modifications in nutritional powders due to processing.

Protein Modification	Criteria						
	Markers	**Methods**	**Studies in commercial and model IF**	**Heat Treatment**	**Occurring at neutral pH**	**Nutritional Impact**	**Known levels in commercial IF**
Aggregation and Denaturation	*Cystine formation, k-CN/whey aggregates, whey-whey aggregation*	WPNI, SDSPAGE, RPHPLC, MWP, peptide profile,	Joyce, Brodkorb, Kelly, and O'Mahony (2017)	Yes	Yes	Slow stomach emptying	-
Cross-linking	*LAL, LAN, (DHA)*	RPHPLCMS, GCFID	D'Agostina et al. (2003)	Yes	Yes	Exhibits renal lesion in rat above 100ppm for both peptide bound and free LAL	2.0-8.0mg/100g protein
Deamidation	*Aspartic acid, iso-asparctic acid, glutamic acid, Ammonia,NPN*	LCQTRAPMS, FTIR, LCMS (MS)	-	Yes	Yes, *but increases more actively under alkaline condition*	Impairs protein function	-
Dephosphorylation	*Soluble inorganic phosphate*	Milk Alkaline Phosphatase	-	Yes	Yes	Heat-induced give serine residue,side-chain can give o-linked glycosylation, relates to diabetes (Grewal et al., 2018) and impairs calcium/iron binding(Pellegrino et al., 2011), improve digestibility of casein (Liu et al., 2016)	-
Glycation	*Furosine, CML*	HPLC-MS	Pellegrino et al. (2011),Martysiak-Zurowska and Stolyhwo (2007)	Yes	Yes	Impairs protein quality and protein digestion loss	779-1550.9mg/100gprotein
Oxidation	*Methionine Sulphoxide (MetSO), dityrosine*	HPLCQTOFMS	Chen et al. (2019)	Yes	Yes	MetSO can be converted back to Met	1.3mg/100g protein
Racemisation	*D-amino acids (e.g.Asp)*	HPLCQTOFMS	Chen et al. (2019)	Yes	Yes, *but increases more actively in both acidic and alkaline conditions*	Known to be non-digestible but D-amino acids but they can be converted back to L-forms via oxidative deamidation, therefore allow human utilization. D-Asp has no pathological impact in rats.	-

2.5 Storage

Milk powders are vastly more stable than liquid milk but maintaining quality and shelf life can be achieved by protecting milk from moisture, light, and heat. Milk powders can easily take up moisture from its surrounding (air) which causes a rapid deterioration of quality and post risk to food safety. Moisture promotes caking or lumping of the powder. Fat from milk creates off flavours when it reacts with oxygen from air. Microorganisms that are dormant, especially the oxygen-loving (aerobic) starts to grow exponentially when given the correct conditions. Also, during storage, the levels of amino acids can reduce to below the acceptable minimum limit for product specification due to protein modifications which were previously discussed. The dairy industry has continually aimed to improve the quality and nutritional benefit of IF by mimicking the composition of human milk. Thus, proper storage of the final product is equally important to achieving these.

Among the protein modifications discussed, it is the Maillard reaction that takes place easily during storage of IF due to its abundance in protein and carbohydrates (Jia, Chen, Qi, & Su, 2019). Lactose is the common carbohydrate in IF which represents about 35-50% of the bulk composition during IF manufacture (Masum et al., 2021). Other carbohydrates can also be used such as maltodextrin that can be added up to 30% of the IF total carbohydrate (C. A. Commission & Commission, 2007), but lactose must contribute to 18g per 100g of IF under the directive of the E. Commission (2006). Therefore, the lactosylation is inevitable in infant formula and studying the extent of it remains relevant to ensure that it is minimized.

There are several indicators of non-enzymatic browning to study thermal damage in protein during nutritional powders, but it is lactulosyllysine that will be used in this study. This compound is measured indirectly by furosine, a chemical compound that is formed during the assay. The principle is to conduct an acid hydrolysis in the powder, which converts lactulosyllysine into 40% lysine, 32% furosine and 28% pyridosine followed by analysis using a high- performance liquid chromatography (HPLC) (Bujard & Finot, 1978). It is a widely accepted method and well defined under International Organization for Standardization (2004). However, the major drawback of this technique is perhaps the fact that different concentrations of acid used lead to uncertainty of the conversion factor of lactosylysine to furosine (Aalaei, Rayner, & Sjöholm, 2019; Mehta & Deeth, 2016). Furthermore, the formation of lactulosyllysine is sensitive to temperature, moisture and water activity that allow close molecular proximity between lactose and lysine during storage (Pellegrino et al., 2011).

Table 6 shows the furosine levels in the final nutritional products gathered from literature.

Table 6: Reported furosine levels in nutritional products from several studies

Infant formula Final Product			
Author	**Production Scale**	**Furosine mg/100g Protein**	**%Blocked lysine (Total lysine)**
Martysiak-Zurowska and Stolyhwo (2007)	Commercial	1320.2- 1550.9	26.46- 33.94
Henle, Zehetner, and Klostermeyer (1995)	Commercial	930-1890	[1]not applicable
Contreras-Calderón, Guerra-Hernández, and García-Villanova (2009)	Commercial and Pilot plant	199- 1033	[1]not applicable
Contreras-Calderón, Guerra-Hernández, and García-Villanova (2008)	Commercial	354-1435	[1]not applicable
Birlouez-Aragon et al. (2004)	Commercial	[1]not applicable	30.4
Follow-On formula			
Martysiak-Zurowska and Stolyhwo (2007)	Commercial	931.9- 1156.7	19.57- 23.26
Liquid formula			
Henle et al. (1995)	Commercial	730-1250	[1]not applicable
Pellegrino et al. (2011)	[2]Unknown	204-444	8.1-15.9
Birlouez-Aragon et al. (2004)	Commercial	[1]not applicable	25.2

[1]not applicable: it was not presented in the study
[2]unknown: not indicated in the study

Moisture and water activity of IF powders play a role in the physicochemical properties such as lactose crystallization, glass transition (T_g), surface composition and morphology, caking, rehydration and Maillard browning. The moisture content of IF ranges between 2-4% and maintaining this level is important because higher moisture level leads to shorter shelf life (Masum et al., 2021). The amorphous lactose form of lactose is largely responsible for the moisture uptake of IF during storage due to its hygroscopic nature and susceptibility to warm and/or high relative humidity (RH). The amorphous lactose is formed during spray drying where rapid removal of water below saturation point of lactose solution takes place before it can crystallize (McCarthy et al., 2013). Structurally, the amorphous form is molecularly unordered, brittle, rigid and has high internal viscosity. During storage, the amorphous lactose can transform to a fluid state; the temperature where this transformation occurs is called glass transition, T_g. At temperatures above this T_g results to decrease in viscosity and increase molecular mobility that allows transformation of amorphous to a more molecularly ordered crystalline structure (Hogan, Famelart, O'Callaghan, & Schuck, 2010). These structural changes cause detrimental effect on the powder stability that lead to increase in free fat levels, off- flavors, powder stickiness and Maillard reaction products (Kim, Saltmarch, & Labuza, 1981). According to McCarthy et al. (2013), a powder with amorphous lactose and protein subjected to 54.4% RH for 48 hours had faster moisture uptake. But lower protein powders sorbed less water, therefore the onset of lactose crystallization began earlier. Conversely, the increase in protein led to increase in the sorbed moisture leading to a delay in lactose crystallization. Tham, Yeoh, and Zhou (2017) studied the effect of temperatures in IF which were sealed with metal cans and stored for 8 weeks. They measured the moisture every week and found that it did not significantly increase at both 25 and 45 ^oC but an increase was observed at 65^oC. The increase of moisture was possible at higher temperature because the initial stage of lactosylation or Maillard reaction involves a dehydration process (Brestenský et al., 2014). Cheng et al. (2017) also studied the effect of temperature on stability of IF stored at 25, 45, 55 and 70^oC. A weighed amount of two IFs (high lipid and high protein) were transferred into a clear aluminum press caps before storage. They found that the IFs stored at 25 and 40^oC for 25 and 102 days respectively had no significant change of the A_w. But for IFs stored at 55^oC, the A_w changed slightly whereas, the IFs stored at 70^oC had significant increase in A_w just after two days of storage. This suggests that higher temperatures lead to higher rate of vapor transmission and diffusion of moisture in powder. In addition, several studies had found non-enzymatic browning or crystallization were slower in open systems at constant RHs because water released during physical and chemical changes in a closed system are trapped that results to accelerated deterioration (Buera & Karel, 1995; Burin, Jouppila, Roos, Kansikas, & Buera, 2004; Kim et al., 1981; Roos & Karel, 1992).

2.6 Conclusion

Protein glycation is a concern in infant formula because the reactants, lactose and lysine are abundant. While it is known as a non-enzymatic reaction that results to browning and flavour compounds, it can also reduce the nutritional value of the infant formula. This modification may also affect digestibility of the infants because the enzyme cleaving sites can be blocked, which then prevents the digestive enzymes to cleave in these sites. Lactulosyllysine, measured as furosine is the first stable Amadori product which renders the lysine unavailable, and some infant formula can have up to 34% blockage.

In addition, the denaturation and aggregation of proteins are expected to occur in infant formula because of different heat treatments used to ensure microbial safety and product stability. Although the science behind heat-induced structural changes of proteins is well established, there is a limited information of it in the infant formula sold commercially. Other formulation conditions that may affect their behavior such as minerals, composition and levels of protein can also vary between manufacturers.

Overall, there is a lack of study on the physical and chemical effects of heat treatment in commercially available infant formula already in the market. Most studies to date have used an infant formula model (laboratory and pilot plant scale, with incomplete formulations), which does not represent the commercial conditions of IF, such as handling, transportation, and storage. Also, most glycation studies that used commercial infant formula in their experiments did not indicate the age and type of protein sources used. Hence, it remains unclear what are the causes of variations among the IFs available commercially.

Chapter 3. Experimental Design

3.1 Sample Preparation

For preliminary testing, IF Stage-1 samples were sourced from Palmerston North, New Zealand, and the Netherlands supermarkets. Samples were labelled as seen in Table 7. Samples were identified as IF with Skimmed Milk and IF with Skimmed Milk Powder samples based on the manufacturer's claim on the ingredients list, e.g., the use of *Skimmed Milk* and *milk solids* respectively. In addition, commercial knowledge was also used to support the differentiation between an IF with Skimmed Milk and IF with Skimmed Milk Powder.

Table 7: IF Stage-1 samples used in Preliminary Testing

Sample ID	Milk used	Manufacturing Process	Manufacturer (Brand)	Age upon testing (month)	Whey: Casein	Whey source as appeared on Ingredients list	Lactose content (g/100g IF)
S13	SM	IF with Skimmed Milk	A (1)	16	60:40	WPC,D90	54
S15	SM	IF with Skimmed Milk	A (2)	19	60:40	WPC,D90	56
S17	SM	IF with Skimmed Milk	A (2)	11	60:40	WPC,D90	56
S18	SM	IF with Skimmed Milk	A (2)	7	60:40	WPC,D90	56
S20	SMP	IF with Skimmed Milk Powder	B (1)	20	65:35	Whey Protein	57
S21	SMP	IF with Skimmed Milk Powder	B (2)	7	60:40	Milk Solids	57
S22	SMP	IF with Skimmed Milk Powder	B (3)	11	20:80	Not Applicable*	56
S24	SMP	IF with Skimmed Milk Powder	C (1)	5	60:40	Whey Protein	49
S27	SM	IF with Skimmed Milk	D (1)	9	60:40	D90 (milk)	55

Not Applicable*-this sample was an *IF from birth* that claimed to be casein dominant which used only SMP as source of proteins

Table 8 lists the InProcess samples of IF manufactured using commercial scale conditions. Sub-samples of each dairy protein ingredient used and its subsequent manufacturing processes, wherever it was safe to do so, were taken. The skim milk used was in liquid form (singly processed ingredient) whereas the main whey protein sources, WPC, and D90 were powders (doubly processed ingredients). The batch tank (recombination as seen in Figure 3) had all the rehydrated macro and micro ingredients at cooling temperature (4-8℃). The mix was then heat treated (heat treatment

before evaporation as seen in Figure 3) to ensure microbial safety and evaporated twice. The evaporated IF was then collected into a concentrate tank before it was spray dried.

Table 8: InProcess Samples

Sample ID	Manufacturing Process
Skimmed Milk	Dairy protein source 1
Whey Protein Concentrate (WPC)	Dairy protein source 2
Demineralised Whey (D90)	Dairy protein source 3
Batch Tank	InProcess
Evaporator 1	InProcess
Evaporator 2	InProcess
Concentrate Tank	InProcess
Dried Powder	Final Product

Table 9 lists the second set of infant formula bought from supermarkets in Palmerston North, New Zealand and the Netherlands were tested. This included different batches of the same brand from preliminary experiment to study the batch-to-batch variability. Additional samples from some batches in the preliminary experiment were also included to study the effect of age. IFs were stored in a temperature-controlled room at 20°C.

Table 9: IF Stage-1 samples used in Second Testing

Sample ID	Milk used	Manufacturing Process	Manufacturer (Brand)	Age upon testing (month)	Whey: Casein	Whey source as appeared on Ingredients' list	Lactose content (g/100g powder)
S23	SMP	IF with Skimmed Milk Powder	E (1)	9	60:40	Milk Solids	48
S26	SMP	IF with Skimmed Milk Powder	E (2)	8	60:40	Milk Solids	49
S31	SM	IF with Skimmed Milk	F (1)	17	60:40	D90,WPC,Whey Protein	54
S33	SMP	IF with Skimmed Milk Powder	C (1)	6	60:40	Whey Protein	49
S37	SMP	IF with Skimmed Milk Powder	B (2)	8	60:40	Milk Solids	57
S17	SM	IF with Skimmed Milk	A (2)	12	60:40	WPC,D90	56
S18	SM	IF with Skimmed Milk	A (2)	16	60:40	WPC,D90	56

3.2 Glycation

3.2.1 Furosine (FUR)

Infant formula samples were sent to an external laboratory for testing. The FUR method followed Acquistucci, Panfili, and Marconi (1996) with slight modifications. The milk powders were made 5%w/v. 2mL aliquot of this milk solution was treated with 6mL of 10.6 M HCl to make a final concentration of 8M HCl. The microwave hydrolysis used a stepwise ramp up to 175℃ which was held for 20 mins. The HPLC conditions followed the method of Troise, Fiore, Wiltafsky, and Fogliano (2015) but have been optimized for Ultra High-Performance Liquid Chromatography (UHPLC) and UV detection at 280 nm rather than an MS detection. The method was validated for infant formula and was fit for purpose.

The amount of FUR was used to calculate the %blocked lysine (%BL) based on Rufián-Henares, Delgado-Andrade, Jiménez-Pérez, and Morales (2007) method where 8M HCl was used. They reported that the acid hydrolysis conditions yielded to 40% of lysine, 36% FUR, and 24% pyridosine. Calculated available lysine (AL) was calculated from Amadori Products (AP) and Regenerated Lysine (RL) as expressed in the following equations.

Equation 1: Percentage Blocked Lysine (% of total lysine)

$$\%BL = \frac{2.78\ x\ \frac{\text{FUR}\ g}{100g\ protein}}{\left(\frac{\text{Total Lysine}\ g}{100g\ protein}\right)+\left(1.67x\left(\frac{\text{FUR}\ g}{100g\ protein}\right)\right)}\ x\ 100$$

Equation 2: Amadori Products (g 100g^{-} protein)

$$AP\ = \frac{FUR\ (\%)}{\left(\frac{36}{100}\right)}$$

RL which represented 40% of AP was calculated,

Equation 3: Regenerated Lysine (g 100g^{-} protein)

$$RL = \text{AP x}\ \frac{40}{100}$$

Therefore, AL was calculated

Equation 4: Calculated Available Lysine (g 100g^{-} protein)

$$AL = \text{total lysine} - \text{RL}$$

3.2.2 Total Lysine

This follows the AOAC 2018.06-2018 Total amino acids in Infant Formulas and Adult which hydrolyses the samples using 6 M HCl for 22h.

3.2.3 Lactulose

The infant formula samples were sent to an external laboratory for testing. The sugar component of the powdered samples was extracted by acid precipitation. The analytes were then separated by RPHPLC and detected by SRM targeted mass spectrometry.

3.2.4 Available Lysine (AL) by Homoarginine Method

The infant formula samples were sent to an external laboratory for testing. The method measured the lysine molecules that have not undergone structural changes and considered nutritionally available. This method uses a guanidation reaction whereby the reactive lysine (lysine which is not linked to lactose) reacts with o-methylsourea to convert to homoarginine before it is subjected to an acid protein hydrolysis. The amount of reactive lysine was determined based on the amount of homoarginine formed. Also, homoarginine is stable in acidic conditions. Therefore, subsequent amino acid hydrolysis allows the separation of two distinctive forms of lysine, one that is regenerated lysine and homoarginine (Brestenský et al., 2014).

The amount of homoarginine was used to calculate the available lysine by converting it to lysine by molar masses. For ease of calculations, results were converted from mg $100mg^{-1}$ of IF to g $100g^{-1}$ protein.

3.2.5 Moisture

This method follows the IDF Provisional Standard 26A: 1993 as cited by de Knegt and van den Brink (1998) with slight modification. As depicted in Figure 7, two grams of IF samples (W_1) were weighed into a pre-dried and pre-weighed flat-bottomed dish with well-fitted readily removal lids (W_2). Samples were left uncovered in the oven at controlled temperature 102+/-2°C for 3 hours. The samples were then covered and transferred into the desiccator and allowed to cool to temperature before re-weighing (W_3). The weight loss after heating is the moisture content and expressed in percentage (g $100g^{-1}$ IF).

Equation 5: Total Moisture (%w/w)

$$Total\ moisture = \frac{(W1+W2)-W3}{W1}\ x\ 100$$

Figure 7: Gravimetric determination of Moisture Content Set-up

3.2.6 Water activity (A_w)

Figure 8 shows the set-up of A_w testing. Infant formula powder was placed in the A_w dish up to the marked line. The A_w was measured by placing the dish inside the Aqualab 4TE Water Activity by Decagon Devices USA. The A_w value was recorded when the device stabilizes, prompted by a beep sound and the "running" sign was off. The device accuracy was verified before and after each use using verification standards (three lithium chlorides with 0.150, 0.250, and 0.500 A_w) and Milli-Q water with 1.000 Aw. The instrument was set at 25.0℃, with Temp Eq <Δ 4.0℃, sensor at dew point.

Figure 8: Water Activity Set-up

3.3 Whey Protein Denaturation and Aggregation

3.3.1 Acid precipitation of protein

The acid precipitation of protein follows the method of Joyce et al. (2017) with slight modification, detailed as follows. A solution was made up to 30 mL with 5.2% w/w protein of infant formula using Milli-Q water. The solution was labelled as *acid precipitate*. Acid precipitate solution was treated with 2.5mL of 10%w/v acetic acid and heated for 10 min at 40℃. Another 2.5mL of 1M sodium acetate was added into the heated mixture, and was further held at 40℃ for 10 min. The solution was cooled down to 22℃. When appropriate, the pH was adjusted to 4.6 using the 10%w/v acetic acid or 1M sodium acetate. Once the pH 4.6 was reached, the final weight of the solution was recorded and was centrifuged at 10000 g for 20 min at 22℃. The supernatant (milk serum) was transferred carefully into another centrifuge tube. This supernatant was labelled stock solution and was used for subsequent analysis. The *total soluble protein* of each sample was determined from the true total protein of the IF before acid precipitation (initial true total protein) and its corresponding supernatant using total nitrogen and non-protein nitrogen methods.

3.3.2 Total Nitrogen, TN

The total nitrogen of the infant formula and supernatant was determined by Kjeldahl method (Lynch & Barbano, 1999).

3.3.3 Non-Protein Nitrogen, NPN

This method follows ISO 8968-4:2016 (IDF 20-4:2016) of International Standard for Milk and Milk Products (*Milk and milk products : determination of nitrogen content*, 2016).

3.3.4 True Total Protein

TN by Kjeldahl method is the sum of all nitrogen in the sample including the NPN such as choline and urea (Donovan & Lönnerdal, 1989). Therefore, NPN was subtracted first from TN content before multiplying it to a protein factor of 6.25 (Maathuis, Havenaar, He, & Bellmann, 2017).

Equation 6: True Total Protein (g $100g^{-}$ IF)

$$\text{True Total Protein} = (\text{TN} - \text{NPN}) \times 6.25$$

To be consistent, other samples in this study also used this conversion factor.

3.3.5 Total Whey Protein by Reversed-phase high-performance liquid chromatography (RPHPLC)

The RPHPLC method follows Elgar et al. (2000) with slight modification.

3.3.5.1 RPHPLC System

It is consisted of a Waters e2695 Alliance Separation Module (Waters, Milford,MA, USA) accompanied with Waters 2489 UV/Vis detector and a Waters Empower®3 chromatographic data acquisition system. A 1 mL Resource™ RPC column supplied by Cytiva was used. Samples were queued under refrigeration condition at 5℃.

Solvent A was made of 0.1%v/v TFA in Milli-Q water while solvent B had 0.09% v/v TFA, 90% v/v acetonitrile in Milli-Q water. The column was operated at a flowrate of 1 mL/min under room temperature. It was equilibrated using 80% of solvent A for 1-min and after a sample injection it was followed by a 6-min isocratic period using 40% solvent B. A series of linear gradient up to 100% solvent B thereafter. The column was held at 100% solvent B for another minute and re-equilibrated by a 2-min linear gradient to 20% solvent B. Then the column was held at an isocratic period of 3 min. The total run time was 30 min with absorbance detection at 214 nm. See Table 10 for gradient summary.

Table 10: RPHPLC Gradient Summary

Time (mins)	Solvent A	Solvent B
0-1	80%	20%
1-6	60%	40%
6-16	55%	45%
16-19	50%	50%
19-20	50%	50%
20-23	30%	70%
23-24	0%	100%
24-25	0%	100%
25-27	80%	20%
27-30	80%	20%

3.3.5.2 Quantitation of Whey Protein

A commercially available bovine whey protein standards (alpha-lactalbumin (α-LA) , beta-lactoglobulin(βLG), bovine serum albumin (BSA), immunoglobulins (IgG) and proteose peptone (PP)) were used for calibration. A stock solution of each of these standards was made at 5-6mg/mL in Milli-Q water and stored at −20°C. A freshly prepared aliquot of each whey protein stock solution was mixed for calibration standards to give a working concentration. An 8-point standard curve from this mixed standard was generated by injecting volumes of 0.01-0.1 mL into the HPLC. For each IF sample, 1 mL of supernatant prepared in section 3.3.1 was carefully pipetted and diluted with 9 mL of milli-Q water in a separate 10 mL centrifuged tube. The diluted sample was centrifuged and filtered (0.4 µm) into HPLC vials. The final concentration was calculated to lie in the range of protein values within the linear portion of the standard curve as mentioned by Elgar et al. (2000). The total soluble whey protein was the concentration sum of all α-LA, βLG, BSA, IgG and PP5 including GMP

(glycomacropeptide) identified in the supernatant divided by the initial true total protein in the acid precipitation. Therefore, the total soluble whey protein of a sample was calculated as follows,

Equation 7: Total soluble whey protein (g/100g protein)

$$= \frac{Total\ whey\ protein\ concentration\ (\frac{g}{100g\ solution})}{Inital\ True\ total\ Protein\ (\frac{g}{100g\ solution})}\ x\ 100$$

3.3.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.6.1 (a) Acid Precipitation Supernatant (Non-Reduced Condition)

An SDS-stock solution was made from 1mL of supernatant and diluted with 4mL of milli-Q water, followed by a vortex into a 5mL centrifuge tube. A 1mL aliquot of this solution was pipetted into a separate 5mL centrifuge tube and made up to volume as a working solution using an SDS sample buffer (500mL milli-Q water, 125mL 0.5M Tris-HCl buffer at pH 6.8, 100mL glycerol, 200mL 10% w/v SDS and 25mL 0.4%w/v bromophenol blue solution) and vortexed. A 1.5mL aliquot was transferred into a 1.5mL Eppendorf tube and centrifuge at 13.5 rpm for 5 min. A 10µL of this centrifuged working solution was applied onto the gel supplied by Thermo Fisher Scientific (Invitrogen™ Bolt 12% Bis-Tris Plus 1.00 x 12 wells) using the running settings indicated on Table 11. A 30mL of 20x Bolt™ MES SDS Running Buffer by ThermoFisher Scientific was diluted with 570mL of Milli-Q water in a measuring cylinder, mixed thoroughly before transferring the full volume into the vertical electrophoresis cell.

Table 11: Running settings

Program	T/V-H
Volts	210V
Current	70mA
Power	6.5W (1gel), 13W(2gel)
Run dye front to edge	>1.3 for 1 gel

3.3.6.1 (b) Acid Precipitation Supernatant (Reduced Condition)

One mL of the working solution was pipetted into a separate microcentrifuge tube (e.g., 1.5mL Eppendorf tube). 20µL of beta-mercaptoethanol was used to reduce disulphide bonding. This mixture was mixed thoroughly using a vortex mixer before heating it using a heating block for 5 minutes at 98℃. The mixture was cooled down to room temperature before pipetting 10µL of the aliquot into the well.

3.3.6.2 Staining of Non-Reduced and Reduced Proteins

After electrophoresis, the gel was removed from the gel cassette and stained with 50mL Amido Black 10B Staining Solution (1.0g of Amino Black 10B dye in 250mL of milli-Q water, 100mL of glacial acetic acid and made it up to 1000mL solution with milli-Q water). The gel was left staining on the shaker for 1hour.

3.3.6.3 Destaining of Non-Reduced and Reduced Proteins

The gel was destained by draining the stain solution and replacing it with 50 mL destaining solution (10% v/v solution of glacial acetic acid in Milli-Q water) for an hour on the shaker. This was followed by re-draining the gel and replacing it with a fresh 100mL destain solution and put back shaking for further 19 hours.

3.3.6.4 Identification of Non-Reduced and Reduced proteins

The non- reduced and reduced proteins were quantified against a sample control (calci+ trim milk by Anchor™). Two 40µL aliquots of this milk was pipetted separately into a 1.5mL Eppendorf tubes and diluted with 1mL of sample buffer. The tubes were labelled as non-reduced and reduced controls, accordingly. The non-reduced control was vortexed well before 10µL of this was pipetted into the well with the rest of non-reduced samples. While the reduced control was added with 20µL of beta-mercaptoethanol and heated similarly like other reduced samples before 10µL was pipetted into the well.

3.4 Statistical Analysis

Minitab Statistical Software (x64) version 20.4.0 was used in statistical analyses. Two- sample t-test comparison with p-value set at 5% was used to test differences of the two processes followed by Pearson's correlation coefficient. One infant formula from each processing was duplicated in the preliminary investigation and average was taken for analysis.

Chapter 4. Investigation of Commercial Infant Formula

Introduction

This chapter reports if the protein modification markers selected were able to differentiate the IF that used skim milk from IF that used skim milk powder. The first marker, *glycation* was determined by measuring the FUR level in the IF while the second marker, *denaturation and aggregation* were characterized by the remaining soluble whey protein after pH 4.6 precipitation. The results from the first and second set of commercial IFs were combined and examined for ease of analysis. Moreover, the first set of IF samples were further characterized by available lysine using homoarginine method, lactulose, moisture content, A_w, and SDS-PAGE. In contrast, the second set of IFs were not tested for available lysine using homoarginine method, lactulose, and SDS-PAGE because such data were deemed unnecessary after the preliminary results came out. The analyses included the effect of storage time on the markers used. The percentage of blocked lysine(%BL) and available lysine (%AL) were calculated from FUR levels. They were then compared with available lysine measured by homoarginine method. The findings are discussed in relation to IF regulations.

As discussed in the literature review, glycation in the form of lactulosyllysine (LL) is a precursor of advanced glycation end products (AGEs) and gets converted into FUR during the acid hydrolysis. Lactulosyllysine is an advantageous protein modification marker to use because of the natural abundance of the reactants (lysine and lactose) in the product (Erbersdobler & Somoza, 2007). FUR is also a helpful indicator to use in IF shelf-life study because the reaction can proceed under minimal temperature and is sensitive to the rise in water activity and moisture (Pellegrino et al., 2011). The formation of this marker compound reduces the nutritional quality of the infant formula by impairing the essential amino acid lysine (Damjanovic Desic & Birlouez-Aragon, 2011).

There have been a limited number of studies that assessed protein modification using lactulosyllysine in commercial powdered infant formula (Akıllıoğlu & Lund, 2022; Birlouez-Aragon et al., 2004; Martysiak-Zurowska & Stolyhwo, 2007; Meltretter, Birlouez-Aragon, Becker, & Pischetsrieder, 2009; Milkovska-Stamenova & Hoffmann, 2017; Sabater et al., 2018). Quite recently Akıllıoğlu and Lund (2022) found 0.388g furosine per 100g protein in powdered infant formula. The study did not elaborate further about the infant formula used apart from they were commercially available. However, elevated levels of furosine were found by Martysiak-Zurowska and Stolyhwo (2007) in IF with Skimmed Milk Powder. They reported about 1.32 -1.55g furosine per 100g protein in powdered infant formula. Thus, these levels were used as point of reference to the likely concentrations in this study.

It is known that there are wide differences among infant formula in the market and the sensitivity to Maillard reactions could be different. In particular, the heat treatment during processing and forms

of proteins used in the formulation may influence the level of glycation in an infant formula (Aalaei, Sjöholm, Rayner, Teixeira, & Tareke, 2019). Lund, Bechshøft, Ray, and Lund (2022) studied the protein modification of two different forms of whey sources (liquid and powder) and found that liquid whey source contained significantly less FUR than dried whey.

The present study hypothesized that an IF derived from fresh skim has less FUR than an IF that has added SMP in the formulation. This is because the SMP has already been heated and spray dried then must be rehydrated and added as an ingredient, and spray dried as IF resulting in a double heat treatment of the protein. Therefore, the objective of this study was to evaluate the protein modification in commercial IFs and compare formulas produced using SMP with those produced using liquid skim milk. The effect of age on protein modification was also compared.

Results and Discussion

Table 12 shows the results of all commercial infant formula analysed in this study. IF with Skimmed Milk was found to be significantly different from IF with Skimmed Milk Powder in furosine (g $100g^{-1}$ protein), total lysine (g $100g^{-1}$ protein), %blocked lysine (g $100g^{-1}$ lysine), calculated available lysine and available lysine by Homoarginine (g $100g^{-1}$ protein) and total whey soluble protein (g $100g^{-1}$ protein). These observations will be discussed in the following sections.

Table 12: Individual results used to analyse the difference between an IF with Skimmed Milk and IF from Skimmed Milk Powder

IF with Skimmed Milk												
	g $100g^{-1}$ protein			g $100g^{-1}$ protein			mg $100g^{-1}$ lactose	g $100g^{-1}$ protein	g $100g^{-1}$ protein			
Sample Name	Furosine	Total Lysine	%Blocked lysine	Calculated Available Lysine	Available Lysine by Homoarginine	Total Initial Lysine	Lactulose	Lactose	β-LG	Total Whey Soluble Protein	%Moisture	Water Activity (A_w)
S13 (Skim)	0.94	9.95	22.63%	8.91	7.76	11.79	59	464	17.3%	42.2%	2.14%	0.161
S18 (Skim)	1.03	9.41	25.74%	8.27	6.41	11.43	49	487	15.7%	39.9%	2.01%	0.1408
S17 (Skim)	1.15	9.48	28.04%	8.20	8.19	11.74	54	485	17.7%	45.1%	1.89%	0.1477
S15 (Skim)	1.55	9.44	35.74%	7.72	7.21	12.46	48	493	15.1%	45.5%	2.21%	0.2063
S18 (Skim)	0.82	9.69	20.63%	8.78	n/a	11.30	n/a	490	18.2%	45.8%	2.05%	0.1378
S17 (Skim)	1.10	9.75	26.35%	8.53	n/a	11.90	n/a	483	19.2%	46.5%	1.89%	0.144
S27 (Skim)	0.80	9.39	20.74%	8.50	6.82	10.96	148	563	21.7%	45.2%	1.69%	0.1306
S31 (Skim)	1.03	9.36	25.80%	8.22	n/a	11.38	137	597	5.2%	16.0%	1.67%	0.1588
Mean ($STDEV$)	1.05 (0.235)	9.56 (0.214)	25.71 (0.049)	8.39 (0.374)	7.28 (0.128)	11.62 (0.398)	82.50 (46.5)	507.75 (46.5)	16.26 (0.049)	40.78 (0.103)	1.94 (0.002)	0.15 (0.024)
IF with Skimmed Milk Powder												
S20 (SMP)	1.44	8.91	35.45%	7.30	6.66	11.74	64	574	5.6%	30.1%	1.89%	0.1307
S21 (SMP)	2.01	9.13	44.79%	6.89	6.31	13.07	90	606	16.9%	36.2%	2.04%	0.144
S37 (SMP)	1.56	9.20	36.81%	7.46	n/a	12.27	n/a	590	17.6%	36.3%	2.03%	0.1434
S22 (SMP)	1.58	8.24	40.37%	6.48	6.40	11.34	99	514	3.6%	9.3%	1.81%	0.1251
S24 (SMP)	1.43	9.12	34.63%	7.52	6.30	11.93	912	503	2.5%	19.8%	2.42%	0.1893
S33 (SMP)	1.12	9.36	27.73%	8.11	n/a	11.55	n/a	525	2.2%	20.9%	2.66%	0.2134
S23 (SMP)	1.97	8.17	47.78%	5.98	n/a	12.03	n/a	520	0.0%	n/a*	2.42%	0.2021

S26 (SMP)	1.73	9.41	39.08%	7.49	n/a	12.79	n/a	512	9.9%	28.4%	2.42%	0.1546
Mean ($STDEV$)	1.61 (0.294)	8.94 (0.479)	38.33 (0.062)	7.15 (0.673)	6.42 (0.036)	12.09 (0.544)	219.25 (414)	543 (40.3)	7.29 (0.066)	25.86 (0.098)	2.21 (0.003)	0.16 (0.034)
p-value	0.001	0.005	0.000	0.000	0.016	0.218	0.243	0.129	0.20	0.012	0.056	0.529

Not applicable (n/a)- these samples were not tested because of testing laboratory issues, or the test has become irrelevant.
Not applicable (n/a*)- the method failed to extract the soluble whey protein because the IF used a thickener in the formulation.

Glycation of Milk proteins

Furosine. Storage time vs. FUR was plotted in Figure 9 for IF's from SMP and SM. There does not seem to be a relationship between age and FUR level. The correlation coefficient was -0.415 with R^2 of 17.24%. Nonetheless, Figure 9 shows that IF with SMP from ages 5 to 12 months had higher levels of FUR compared to IF with Skimmed Milk. This could imply that processing or the use of SMP in IF may have greater impact than age. However, the difference is not clear cut as Figure 9 shows that there is some overlap in FUR levels between IF produced with SMP and IF produced with Skimmed Milk. There is also variation between batches, some can be attributed to different manufacturing source or batch-to-batch variation within the same facility. For instance, samples S24 (1.43g FUR $100g^{-1}$protein) and S33 (1.12g FUR $100g^{-1}$protein) from manufacturer C which were of the same brand, but from different batches, and only a month old apart had a ~22% difference in FUR. Likewise, samples S21 (2.01g FUR $100g^{-1}$protein) and S37 (1.56g FUR $100g^{-1}$protein), from the same brand of manufacturer B but of different batches, and two months old apart had a ~22% difference in FUR level. In contrast, different batches of IF from manufacturer A(2) namely S18 (1.03g FUR $100g^{-1}$protein), S17(1.15g FUR $100g^{-1}$protein), and S15 (1.55g FUR $100g^{-1}$protein) had coincidentally increasing FUR level up to ~50% between 7 to 19 months of age. Thus, the observations were confounded by two factors: age and batch. To clarify the age effect further, more samples from S18 and S17 batches were studied. Both samples showed decreasing FUR level overtime by 20% and 11%, respectively. One can argue that these decreases were due to the formation of carboxymethyllysine (CML), an advanced glycation end-product (AGE) that proceeds after lactulosyllysine formation. However, Prosser, Carpenter, and Hodgkinson (2019) have studied that a spray-dried commercial IF stored in a tin can at ambient temperature was stable up to 80 months and had very little variation on CML. This conclusion concurs with Pellegrino et al. (2011) who did not observe formation of AGEs in high protein powdered infant formula stored at 25°C. Hence, the exact effect of age from a single batch of IF on FUR cannot be concluded at this point. A study using samples from the same batch and stored for different lengths of time would be required to clarify any effect of storage time on FUR levels.

Figure 10 shows that this difference between the use of Skimmed Milk and SMP in infant formula was significant (p-value=0.001). The boxplot shows that IF with Skimmed Milk generally have lower FUR compared to IF with Skimmed Milk Powder. However, overlapping FUR values between the two processes was again observed suggesting that a single value of FUR is not adequate to identify a fresh skim derived from SMP. There are possible causes of this overlap as mentioned before. It can be different ages of the IF which is now known to have lesser effect than processing. It can also be

the variation of the whey ingredients used between batches of IFs, the differences in whey sources (liquid or powder) used or heat treatment applied by different IF manufacturers.

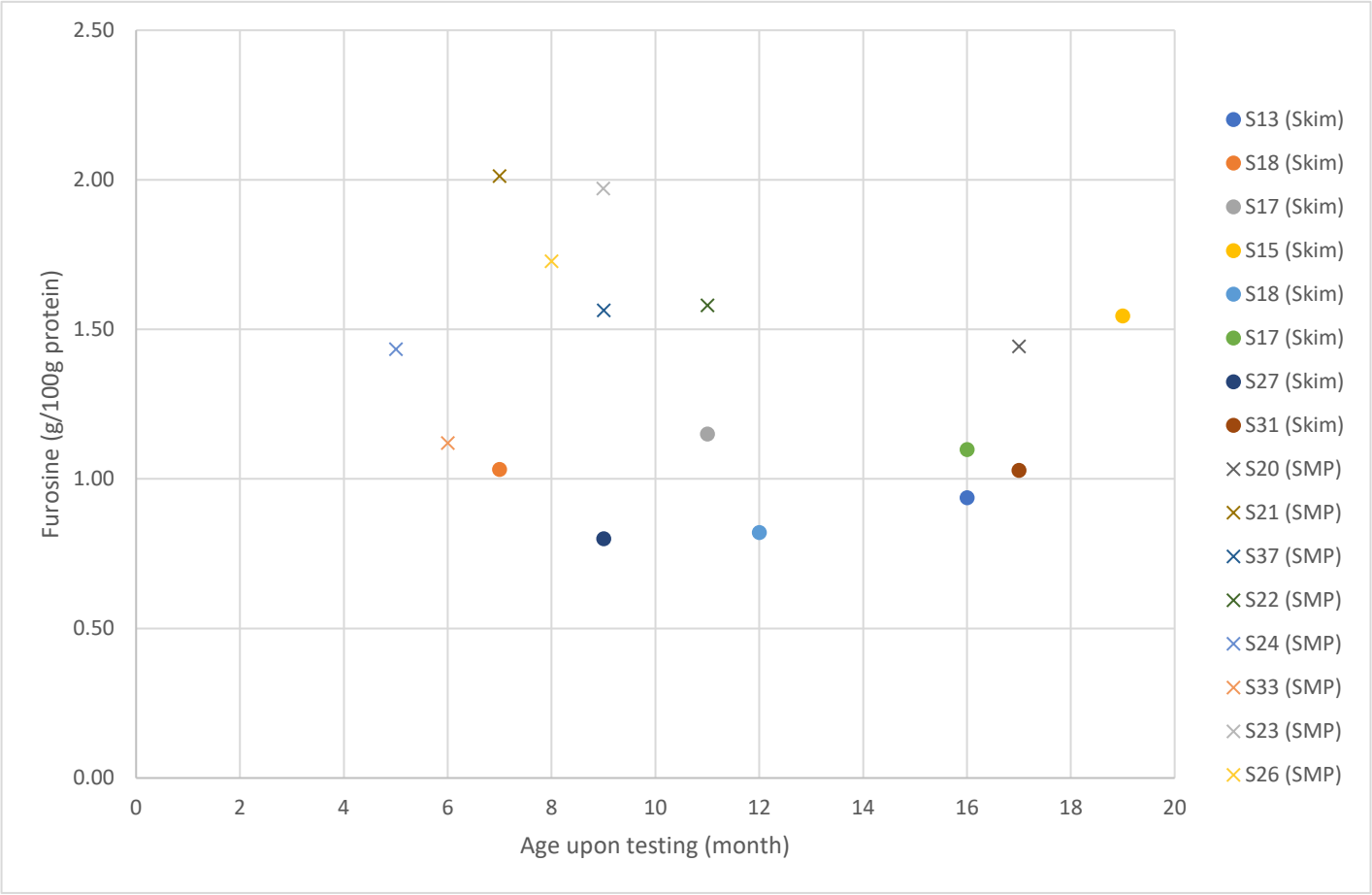


Figure 9: Scatterplot of Furosine (g $100g^{-1}$ protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder against time of testing

The batch-to- batch variation observed is undesirable because it decreases the possibility of effective and lean production line. The precision on shelf-life determination and recommendation can also be impaired. The heat load used during IF manufacture is not the only factor that can influence the FUR in IF, as briefly discussed in the earlier section. The recent study carried out by Lund, Bechshøft, et al. (2022) suggested that whey protein ingredients may also contribute to the furosine in the final product because it can also be introduced as a powder (doubly processed) or a liquid (singly processed). They found that a liquid serum protein concentrate (SPC) had significantly lower FUR than a powdered whey protein concentrate (WPC). The latter had ~0.32g FUR $100g^{-1}$ protein while SPC-IF contained about 0.26g FUR $100g^{-1}$ protein. This could explain the higher levels of furosine found in IF from manufacturer A, which claimed (based on ingredients list) that demineralized whey powder and WPC were used in samples S13, S15, S17 and S18, as seen in Table 12. Conversely, the

low furosine content of S27 from manufacturer D could be from the use of “demineralized milk”. It was the only whey source listed on the ingredients list of S27.

Other IFs with 60:40 whey to casein ratios in this study were not equally explicit with the whey source used in the IF, but it was either “milk solids” or “whey protein” that was on the ingredients list.

Sample S20 with higher 65:35 whey to casein ratio had strikingly lower FUR (1.44g FUR $100g^{-1}$protein) compared to some 60:40 IF with Skimmed Milk Powder. This is contradictory to the previous studies that indicated the whey-enriched infant formula to be more prone to the Maillard reaction (Birlouez-Aragon, Morales, Fogliano, & Pain, 2010; Contreras-Calderón et al., 2008). It could be explained by the fact that S20 was fortified with α-LA (25% of total whey). Previous work has shown that increasing the α-LA to β-LG ratio enhances the stability of the proteins (Crowley, Dowling, Caldeo, Kelly, & O’Mahony, 2016), which in turn, reduces the exposure of the reactive lysine to lactose. Sample S22 (1.58g FUR $100g^{-1}$protein) which used a 20:80 whey to casein ratio had comparable FUR content to S20. This possibly means that the effect of IF manufacturing conditions and the whey ingredients to FUR formation surpass not only the age but the whey to casein ratio as well.

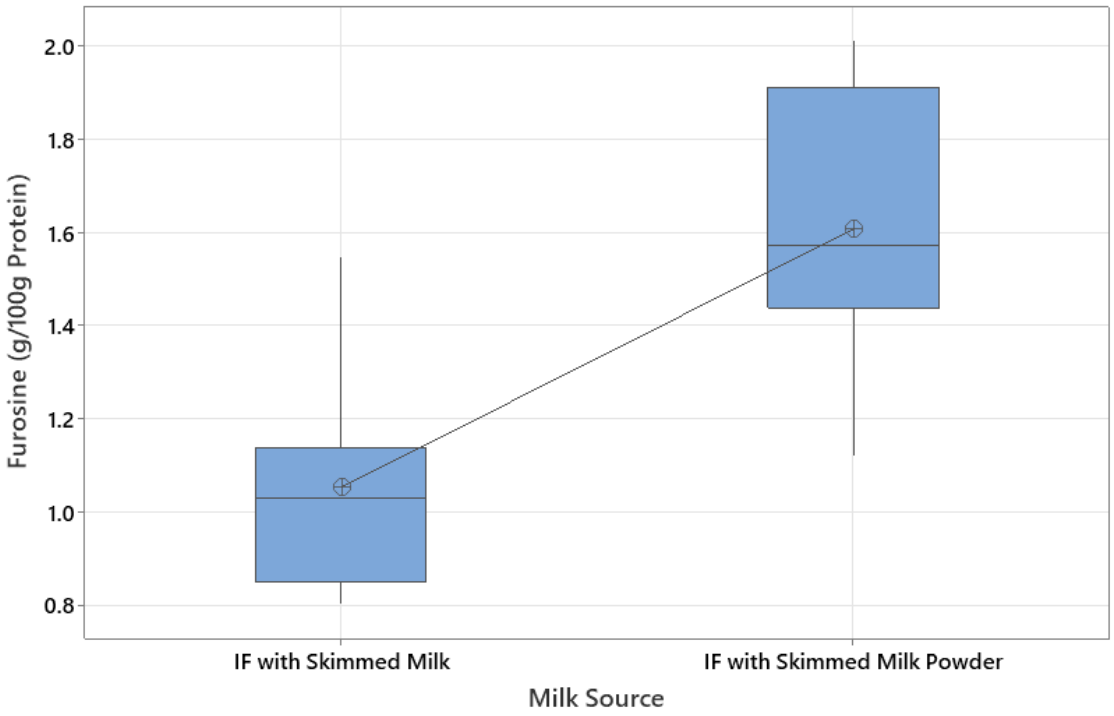


Figure 10: Boxplot of Furosine (g 100g-1 protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder

Hence, to challenge the batch-to-batch FUR variation of an IF and different FUR values across all IF brands further, the unit operations used in the IF manufacturing and the effect of the reactants must be understood. According to Lund, Mardal, Ray, and Lund (2022) the impact after pasteurization using DSI and evaporation on FUR level of an in-process milk caused it to increase by 3-fold (or a FUR increase of only 12% during processing) whilst spray drying caused a 19-fold increase from the non-heated mix. The mix prior to pasteurization had 0.0117g furosine $100g^{-1}$ protein and after spray drying it had 0.2180 g furosine $100g^{-1}$ protein. Furthermore, furosine and other Amadori products formed during heat processing are classified under second-order of reaction (Cantre, Garcia, Carpio, & Madamba, 2007). A critical review by van Boekel (2001) mentioned that conflicting results were reported in the literature and that a complex reaction such as MRP was hard to extrapolate beyond the conditions which the measurements were carried out. But this current study is designed to understand if the reactants had an obvious contribution to the observations made thus far.

Total Lysine. Figure 11 shows that the total lysine of the IF from the two processes were significantly different (p-value=0.005) with IF from Skimmed Milk Powder significantly lower than IF from fresh skim. This result is negatively correlated to FUR levels (Figure 10) which aligns with the theory that if more lysine is reacted with lactose to produce the Amadori product (lactulosyllysine) during the IF manufacture, the total lysine is expected to be less. Therefore, results from Figures 10 and 11 show agreement.

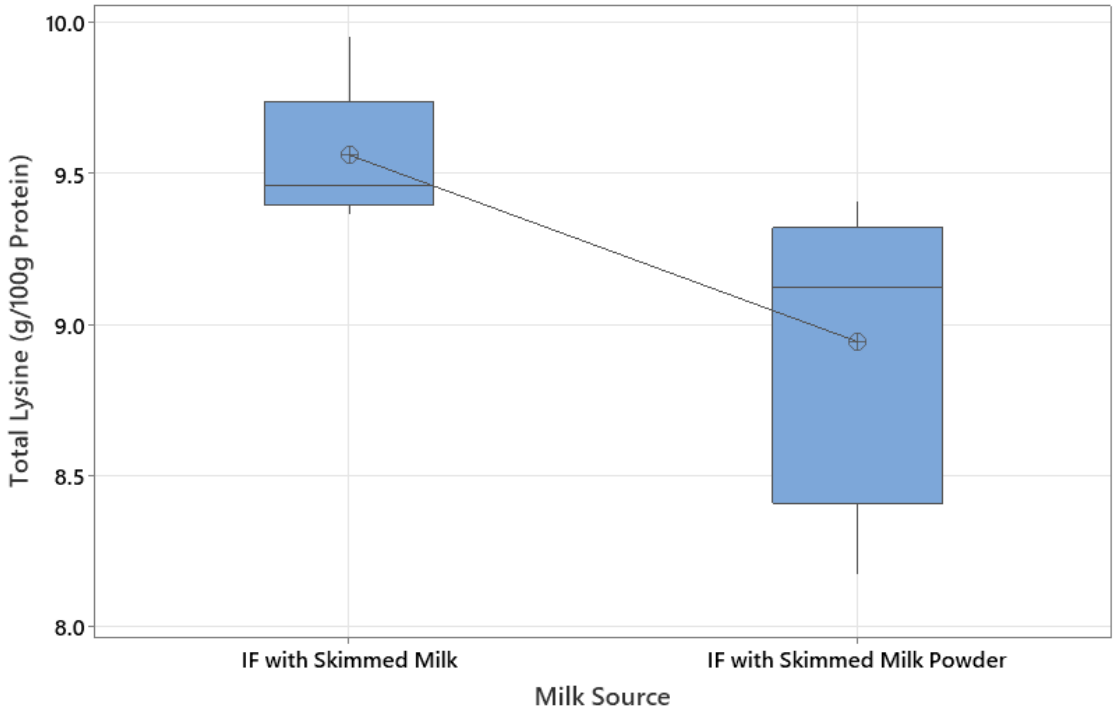


Figure 11: Boxplot of Total Lysine (g/100g protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder

In Figure 12, the total lysine plotted against FUR. The total lysine does show a negative correlation with FUR (r=-0.657). A simple linear regression showed a significant p-value of 0.006 but with low adjusted R^2 of 39.16%. Thus, it does not necessarily recommend total lysine to be the alternative of FUR or become the sole marker of protein modification. For example, the highest total lysine-containing sample in IF with Skimmed Milk was S13 (9.95g total lysine $100g^{-1}$ protein) and it had one of the lowest FUR contents (0.94g FUR $100g^{-1}$ protein), as also seen on Table 12. In IF with Skimmed Milk Powder, S23 (8.17g total lysine $100g^{-1}$ protein) had the least amount of total lysine and one of the highest FUR levels. However, this trend is not always true.

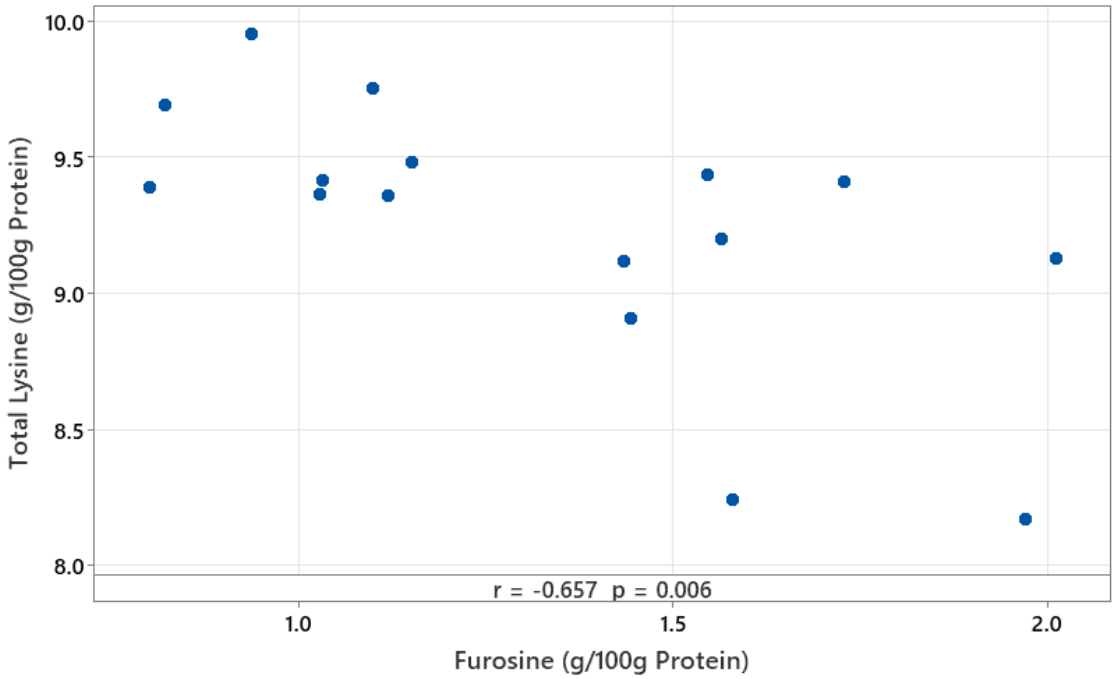


Figure 12. Pearson Correlation of Furosine and Total Lysine shows a significant negative correlation (r=-0.657, p-value= 0.006, r-sq(adj)= 39.16%)

Sample S27 had the least amount of FUR (0.80g FUR $100g^{-1}$ protein), at the same time had one of the lowest total lysine (9.39g total lysine $100g^{-1}$ protein) among the IF with Skimmed Milk. It can be argued that S27 had lower FUR because it had lower lysine added into the formulation to begin with. Hence, the initial lysine was also determined. The AL and AP as described in section 3.2.1 was added together to calculate the amount of initial lysine that went into the IF because it was impossible to measure. It was found that sample S27 had 10.72 g initial lysine $100g^{-1}$ protein, the lowest initial lysine among IF with Skimmed Milk and IF with Skimmed Milk Powder combined as indicated in Table 12. However, less FUR could also indicate more advanced glycation end products such as CML. Based on literature, the serum protein concentrate (SPC) contained a significant level of CML compared with WPC (Lund, Bechshøft, et al., 2022). This finding was surprising because heat treatment is known to promote CML formation. But when the SPC was used in an IF formulation, the CML result became insignificantly different from WPC-IF. Extra care should be taken when comparing with literature because of compounding factors. This conjecture regarding S27 cannot be proven with the current testing.

Overall, the calculated initial lysine between the two types IF presented in Figure 13 was found to be insignificant (p-value=0.218). This indicates that the FUR difference observed earlier was not due to the amount of lysine added into the formula.

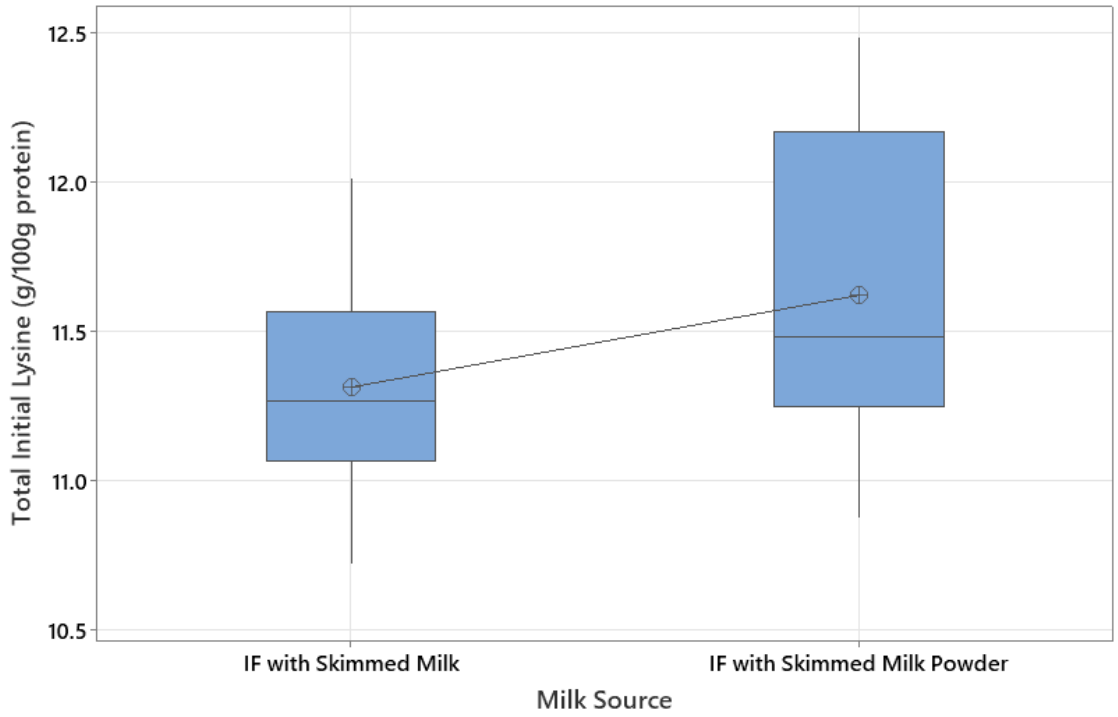


Figure 13: Boxplot of Total Initial Lysine (g/100g protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder

Lactose Content. It was observed that the total lysine of IF with SMP studied here had less than IF with fresh Skimmed Milk, but the total initial lysine added between the IFs was not significantly different. Hence, it is appropriate to investigate if the other reactant, lactose, had influenced the FUR formation of the IFs that resulted to this observation.

Figure 14 displays the difference between the lactose content obtained from the nutrition panel of each infant formula of the IF's produced with SMP and fresh Skimmed Milk. There was no significant difference between the two (p-value= 0.129). Lund, Bechshøft, et al. (2022) reported that increasing amount of lactose to whey ratios (0:100, 30:70 and 60:40) had significant FUR formation after spray drying but there were no significant differences between 60:40 and 90:10 ratios observed. The latter ratios suggest that lactose is not a limiting factor. Similarly, Birlouez-Aragon, Moreaux, Nicolas, and Ducauze (1997) has shown that increasing lactose levels promotes lysine blockage. As seen in Figure 14, all the IFs studied in this work had approximately used the same level of lactose in the formulation. Hence, the overlapping values of FUR between IF with Skimmed Milk and Skimmed Milk Powder could have not come from the reactants, leaving the heat treatments applied during IF manufacture and whey ingredients used to explain the observation.

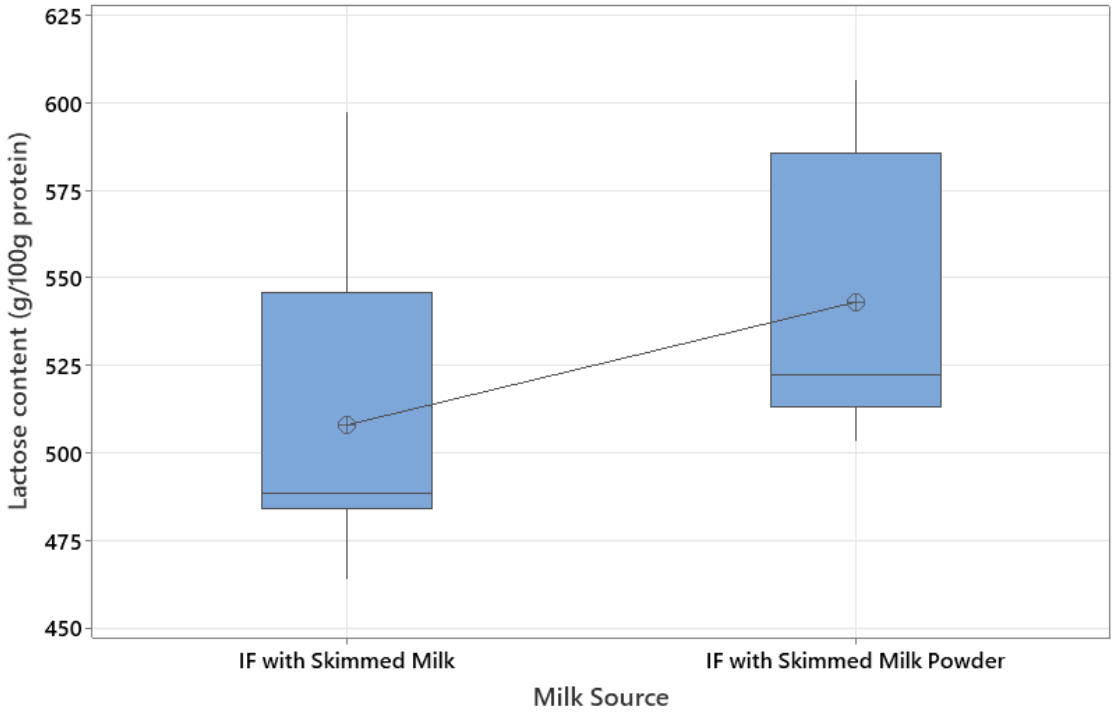


Figure 14: Boxplot of Lactose Content (g/100g protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder

Lactulose. This compound is an isomeric disaccharide and is not known to occur in milk naturally. It is from a lactose that had undergone the Lobry de Bruyn-Alberda van Eckenstein rearrangement during heat treatment (Pereyra Gonzáles et al., 2003). Hence, it is commonly used as a thermal indicator in heated dairy product. Figure 15 shows no significant difference in lactulose content between the two processes (p-value =0.243). However, sample S24 from manufacturer C, an IF manufactured with SMP was found to have ~11x more lactulose (912mg $100g^{-1}$ lactose or 4437mg kg^{-1} IF) compared to the average of IFs with Skimmed Milk Powder (84mg $100g^{-1}$ lactose). It is possible that sample S24 was treated with ultra-high temperature (UHT) during IF manufacture, but this cannot be confirmed. Leite, Croguennec, Halabi, and Costa Junior (2021) reported as high as 1365 mg lactulose L^{-1} in UHT- treated liquid infant formula. This result did not necessarily mean higher FUR content against other IF with Skimmed Milk Powder. For instance, the lactulose of S21 (90mg $100g^{-1}$ lactose) by manufacturer B was less than S24, yet its FUR content was higher, 2.01 g and 1.43g furosine $100g^{-1}$ protein, respectively. This proposes that lactulose and FUR formation due to thermalization of IF could be driven by two different processes during the IF manufacture. It may also mean that S24 used an ingredient that contained a high lactulose level to begin with compared to S21. Sample S21 indicated the use of "milk solids" while S24 used "whey protein" as sources of whey protein. Likewise, sample S27 by manufacturer D was found to contain the highest level of lactulose (148mg $100g^{-1}$ lactose) with lowest FUR level (0.80g $100g^{-1}$ IF) among IF with Skimmed Milk

and declared the use of “demineralized whey” as the only whey source. It remains unclear to what extent the contribution of the ingredient is to these markers at this point. Hence, quantifying these markers in the ingredients and through the IF manufacturing process will enhance our understanding.

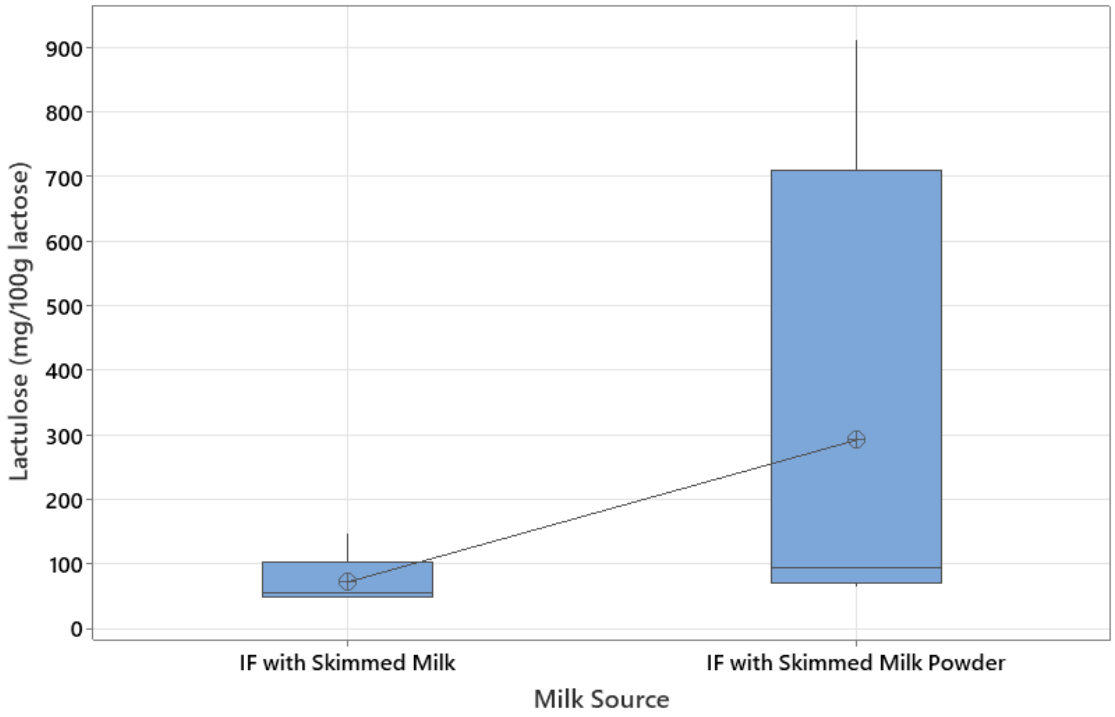


Figure 15: Boxplot of Lactulose (mg/100g lactose) of IF with Skimmed Milk and IF from Skimmed Milk Powder

As stated already, sample S27 indicated the use of demineralized milk as the main source of whey apart from Skimmed Milk. Demineralized whey has a lactose content of ~83g and 13g protein per 100g of powder (Paladii, Vrabie, Sprinchan, & Bologa, 2021). Therefore, it would not be surprising if the lactulose content of this ingredient is higher than WPC which lactose content can range between 4-21g per 100g powder (Paladii et al., 2021). Except for S24, S27, and S31, the average lactulose (66mg $100g^{-1}$ of lactose) found were comparable to the study carried out by Guerra-Hernández, García-Villanova, Leon, Romera, and Corzo (2002) on IF (361 mg kg^{-1} of IF ± 88.3) stored at 20°C. The three IFs (S24, S27, and S31) were excluded because of other measured parameters which could explain their lactulose levels. This will be discussed later.

IFs from manufacturer A had one-third lower lactulose content on average (50mg $100g^{-1}$ lactose) than S27 (148mg $100g^{-1}$ lactose) but the IFs had higher FUR (1.10g $100g^{-1}$ protein) than S27 (0.80g

$100g^{-1}$ protein). It was also suspected that S27 had used a liquid whey source because it had more intact β-LG among the IFs studied here, while S24 and S33 had hardly any left. This is an indication that the whey ingredient by manufacturers D and C may had the least and the worst heat treatment among the IF, respectively. This will also be discussed in detail later. For these reasons, the higher lactulose found in S27 could probably have come from the liquid demineralized milk used, that led to lower FUR content and higher intact β-LG.

Blocked Lysine. It can be seen in Figure 16 that the blocked lysine (% total lysine) between the two processes is significantly different (p-value= 0.000). This is unsurprising because the values were calculated from total lysine and furosine measurements. The percent blocked lysine for IF with Skimmed Milk ranges from ~21-36% while the IFs with Skimmed Milk Powder ranges between ~27-48%. Results are overlapping each other with the latter on the higher end.

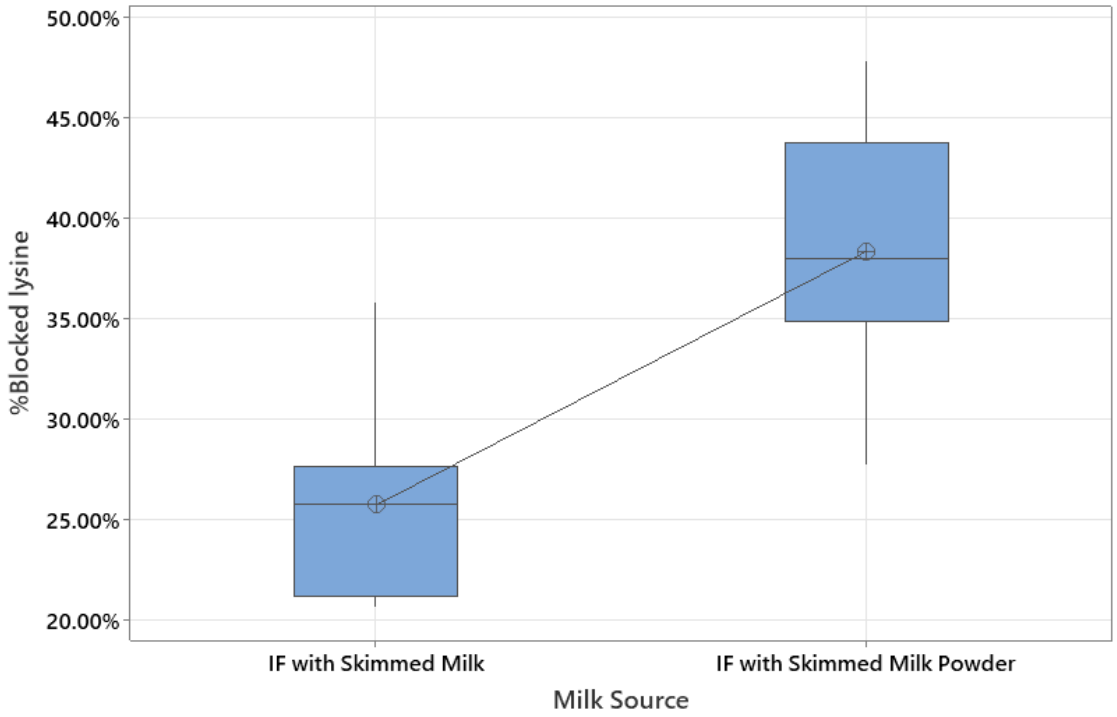


Figure 16: Boxplot of % Blocked Lysine of IF with skimmed Milk and IF from skimmed milk powder

Calculated Available Lysine. Figure 17 plots the calculated available lysine and shows that the two processes were significantly different (p-value= 0.000). This was again unsurprising because the values were calculated from FUR levels. The relevance of these results become apparent when they are converted to mg of lysine per 100 kcal of the IF, as seen in Figure 18. The difference between the two processes has remained significant (p-value=0.000) with IFs from Skimmed Milk Powder

serving lower available lysine per 100kcal. All the IFs tested in this study remained above the minimum limit of lysine set by EU and China regulators.

The available lysine in the present study for IF with Skimmed Milk was between 7.7- 8. g 100 g^{-1} protein. Ferrer, Alegría, Farré, Abellán, and Romero (2000) who reported a blocked lysine of 15.2-26.7% an available lysine of 8.71-11.15 g 100 g^{-1} protein. In contrast, Martysiak-Zurowska and Stolyhwo (2007) showed lower available lysine of 5.41-7.21% based on 100g protein with ~26-34% blocked lysine per 100g protein. The authors did not specify the dairy proteins used in the IFs examined. On the other hand, the IF with Skimmed Milk Powder in the present study had a percentage blockage of 27%- 48% and available lysine of 6%- 8% based on 100g protein. This agreed with Aalaei, Sjöholm, et al. (2019) who reported a range from 27-37% or 7.74-9.75 g available lysine 100 g^{-1} protein of IF. The authors indicated the use of SMP in the IFs studied. Evangelisti, Calcagno, and Zunin (1994) did not report the available lysine. What can be understood from the past and present studies is that IFs are vulnerable to glycation and if not controlled leads to the huge variation. Also, reporting the percentage of blocked lysine seems to be more appropriate rather than calculated available lysine (g/100g protein) when looking for differences between the two groups of IF.

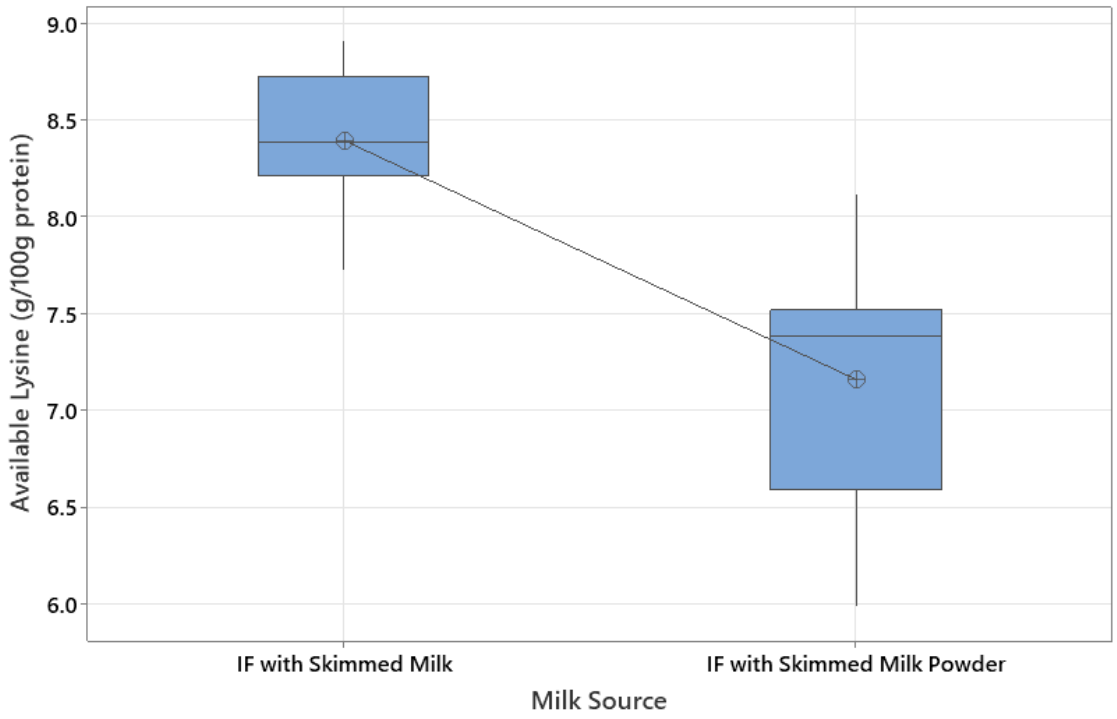


Figure 17: Boxplot of calculated Available Lysine (g/100g protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder

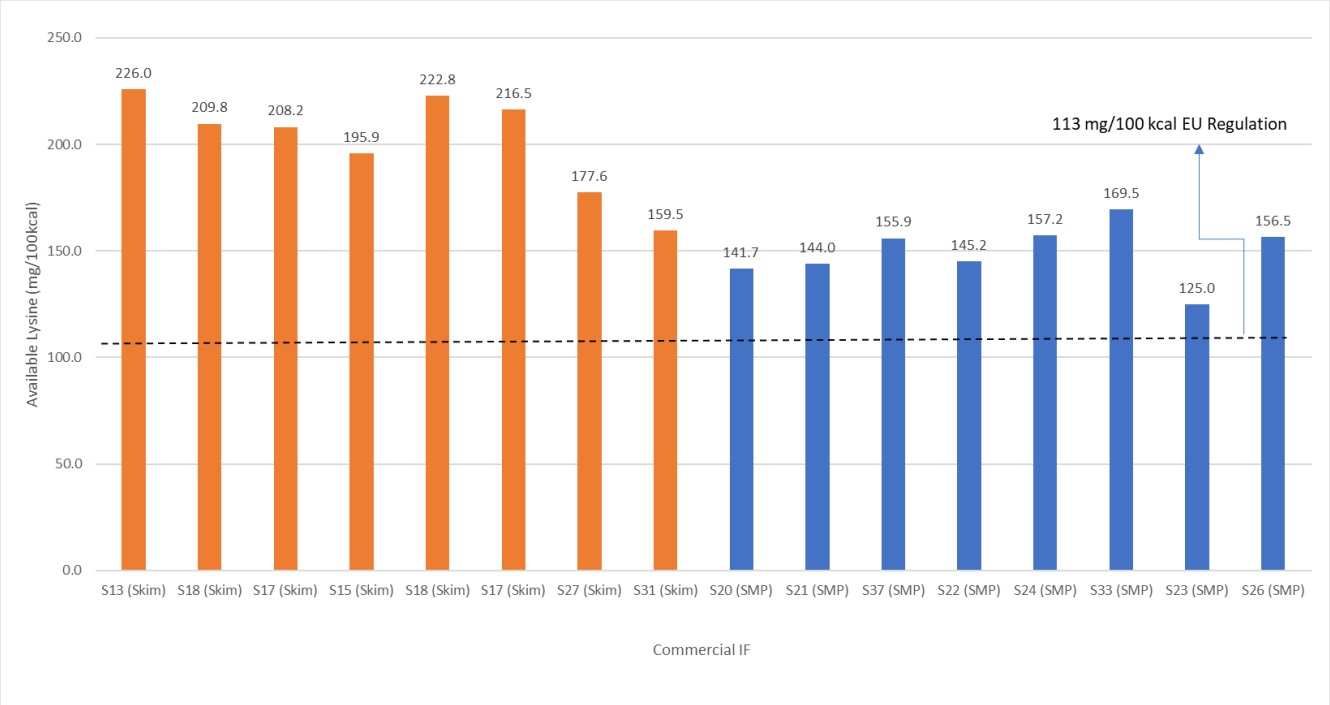


Figure 18: Column graph of Available Lysine (mg/100kcal) of IF with Skimmed Milk and IF from Skimmed Milk Powder based on Indispensable AA in IF based from China and EU regulations (114 and 113 mg/100kcal, respectively)

Available lysine by Homoarginine. The results shown in Figure 19 were only from the first set of IF because the global pandemic COVID-19 has caused disruption in the laboratory's workflow resulting to massive delays. Despite this, the results demonstrated a significant difference between the two processes (p-value = 0.029) with IF from Skimmed Milk on the higher side. This is consistent with FUR results and therefore the use of available lysine by Homoarginine was not considered necessary.

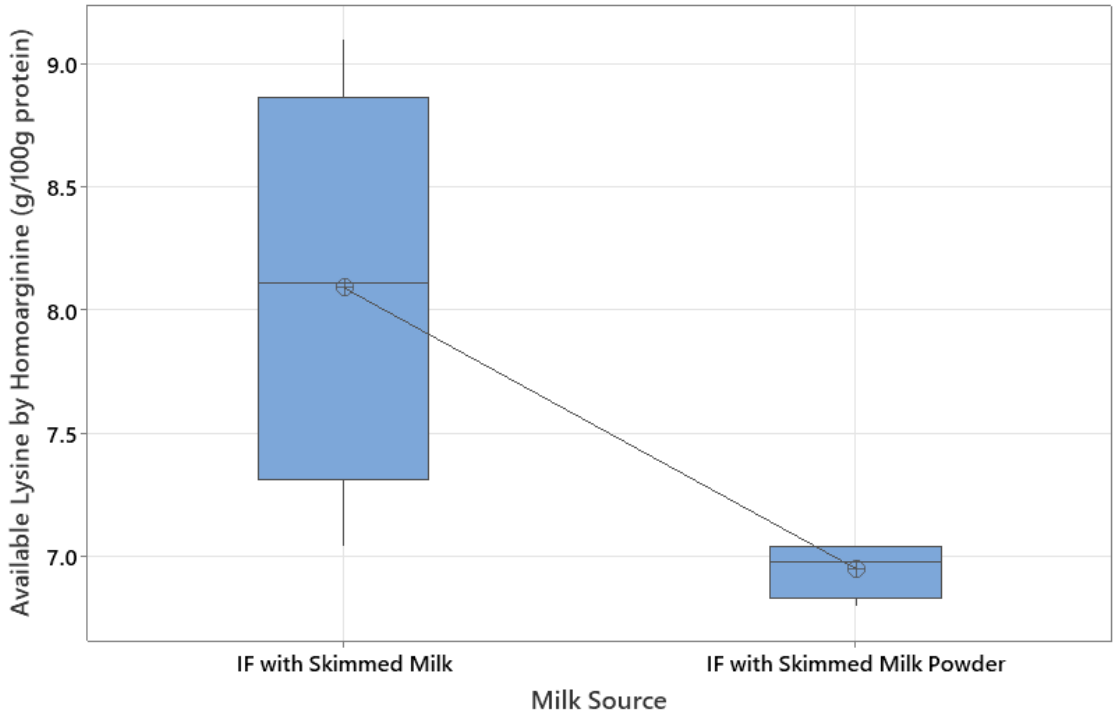


Figure 19: Boxplot of Available Lysine (g/100gprotein) by Homoarginine of IF with Skimmed Milk and IF from Skimmed Milk Powder

Moisture and A_w. The water content in IF is a crucial factor in progression of the Maillard reaction impacting its glass transition temperature. Glass transition is a phase where lactose is converted from a glassy and stable state to a rubbery state. The reaction rate is at the highest at an intermediate A_w 0.5-0.7 (Labuza & Saltmarch, 1982). During storage, the water content may increase with the progression of Maillard reaction which in turn results to an increase in the A_w of the product (Brestenský et al., 2014). However, Tham et al. (2017) reported that IF in sealed metal cans stored at 25℃ and 45℃ were stable throughout the ageing conditions they were subjected (i.e. 56days). Figures 20 and 21 present the moisture and Aw content of IFs studied in this work. The difference in the moisture and A_w between the IF with Skimmed Milk and IF with Skimmed Milk Powder was found to be insignificant with p-value >0.05. The observed values were comparable to Tham et al. (2017) who reported an average moisture of ~2% w/w and ~0.19 A_w across three commercial IFs.

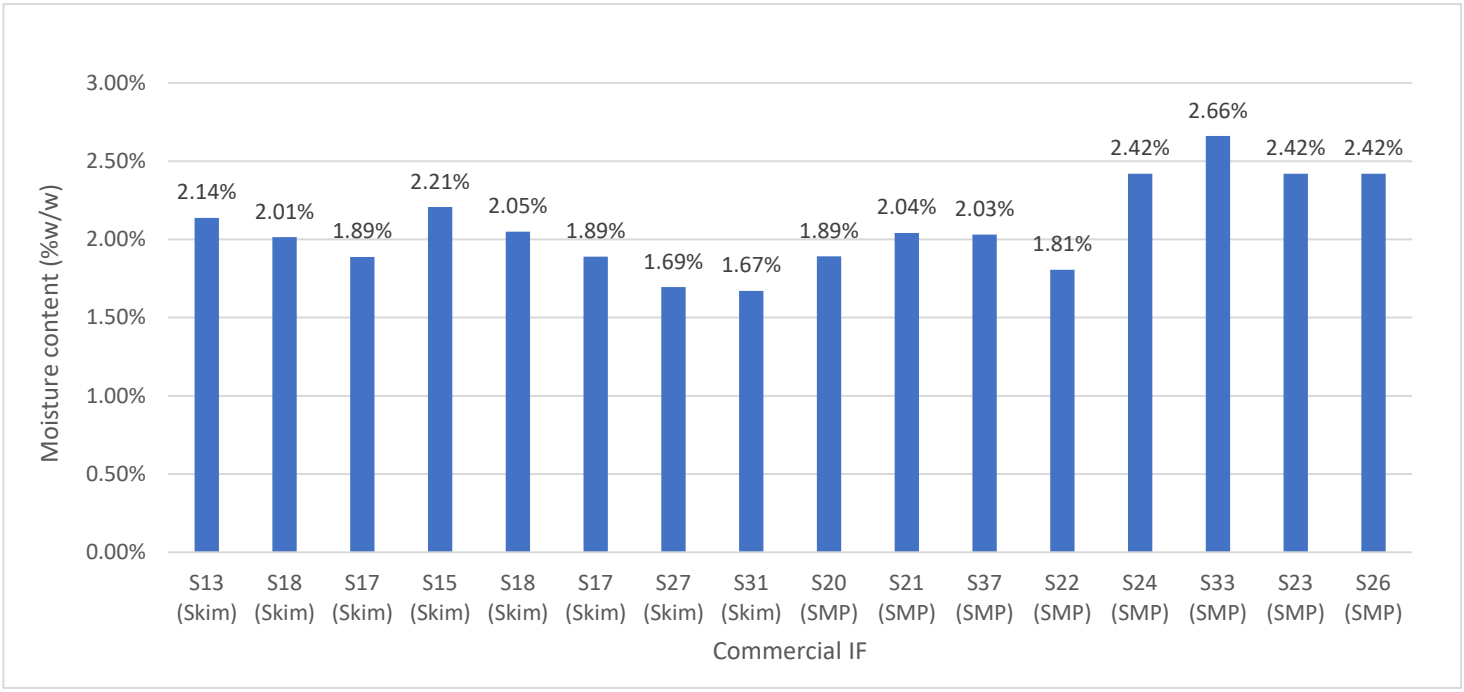


Figure 20: Column graph of moisture content (%w/w) of IF with Skimmed Milk and IF from Skimmed Milk Powder (p-value= 0.056)

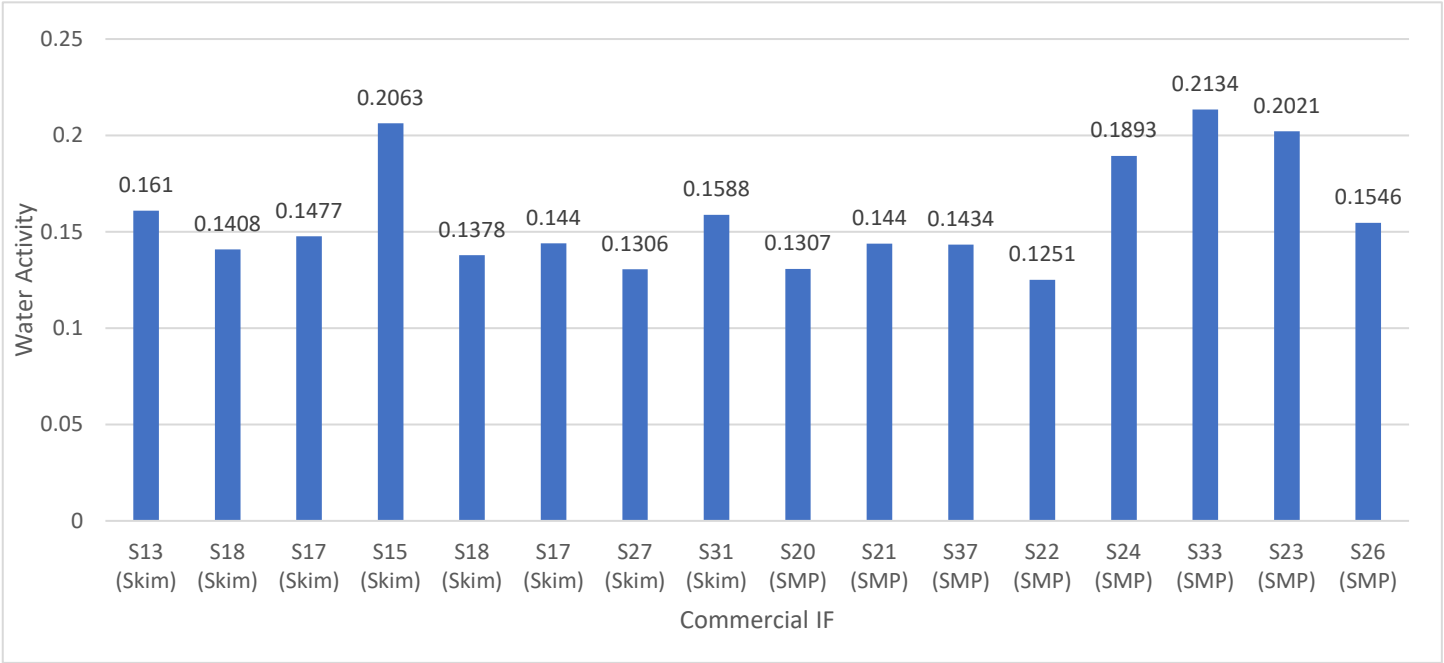


Figure 21: Column graph of water activities of IF with Skimmed Milk and IF from Skimmed Milk Powder (p-value= 0.891)

Denaturation and Aggregation of Milk proteins

In infant formula, the denaturation and aggregation of proteins involve the interaction between the free-SH group and S-S bond of cysteine-containing protein such as β-LG, α-LA, Κ-casein, and BSA via the -SH/S-S interchange reactions (Wijayanti et al., 2014). The reaction is usually driven by β-LG because of its higher concentration than other whey proteins in bovine milk. These protein complexes are consequences of the heat treatment conditions applied in milk (SG Anema & Li, 2000; McMahon et al., 1993; Vasbinder & De Kruif, 2003). Thus, this section has given emphasis to the total whey protein and β-LG remained in the soluble phase after the IFs were treated with acid to adjust its pH to 4.6.

Total soluble protein by RPHPLC. A comparison of total amount of soluble protein of IF for manufactured with SM and SMP is shown in Figure 22. These results were from chromatograms and plotted for ease of comparison. Figure 23 shows that significant amounts of glycomacropeptide (GMP), which ranges between 2.3-14.9g per 100g protein were present in the IFs. GMP is a glycosylated peptide formed during cheese making by rennet, were present in the IFs. GMP constitutes about 20-23% of the total protein in whey products (Farias, Martinez, & Pilosof, 2010). Hence for simplicity purposes, the calculations used in this section accounted for this soluble fraction. The principle of this method is that the more heat treatment the protein undergoes, the higher the denatured and aggregated casein-whey protein. These modified proteins are then precipitated leaving the soluble protein (whey) in the supernatant (Damjanovic Desic & Birlouez-Aragon, 2011; Yen, Lin, & Tu, 2015). Hence, if the 60:40 whey to casein ratio IF was to remain unaffected by the heat treatment, a maximum of 60g of total whey soluble protein is expected or 60% of 100g total protein. In this analysis, the combined data has shown that IF with Skimmed Milk had significantly more soluble whey protein compared to IF with Skimmed Milk Powder (p-value= 0.012). The former had soluble whey protein content ranging from 40-47g per 100g total protein, suggesting that 13-20g of the 60g total whey protein had been denatured and aggregated. However, S31 was an outlier and only retained ~16g of the total soluble whey or ~44g of the 60g total soluble whey protein precipitated out despite being manufactured with SM. As discussed previously, the lactulose level of S31 (137mg $100g^{-1}$ lactose) was comparable to sample S27 (148mg $100g^{-1}$ lactose) but the latter had retained ~45.2g of its total soluble whey protein. This suggests that S27 had far less heat treatment compared with S31. Although the heat treatment employed damaged the whey proteins of S31, it did not translate to an increase of FUR content suggesting that denaturation and aggregation of proteins are not driven by the spray dryer but the heat treatment that preceded it e.g., pasteurization or sterilisation. Lin, Kelly, O'Mahony, and Guinee (2018) studied the heat treatment effect during SMP manufacture which was mostly pronounced with increasing

severity of whey protein denaturation from 72℃ for 15s to 120 ℃ for 120s. A more recent study conducted by Halabi, Deglaire, Hennetier, et al. (2020) on heat-induced protein aggregates in model IF suggested that the soluble β-LG found were lower when the IF was heated at 80℃ than those heated at 67.5℃. At a fundamental level, the major proteins, β-LG and α-LA gets denatured significant upon heating the milk at temperatures above 70℃ (Skelte G. Anema, 2020). Hence, β-LG is a useful marker to study the extent of heat treatment.

Among IF with Skimmed Milk Powder at 60:40 whey to casein ratio, samples S24 and S33 from manufacturer C had the least total whey soluble protein averaging ~20.4g per 100g of total protein. Along with the high lactulose content of S24 (912 mg $100g^{-1}$ lactose) found earlier and lower FUR compared to IF with Skimmed Milk Powder, it was suspected that sample S24 had undergone a hasher thermal treatment prior to spray dryer than other IFs measured in this study. Sample S22 (9.3g soluble whey per 100g of total protein) was expected to contain a maximum of 20g total whey soluble protein because it was a casein dominant formula, which used a 20:80 whey to casein ratio.

It is worthy to note here that the data from the first set of IFs had shown insignificant difference (p-value= 0.051). Even though further data improved the p-value to 0.012, the test is not a suitable replacement for FUR. Batch-to-batch variation in the combined analysis was also noticeably less compared to the FUR results.

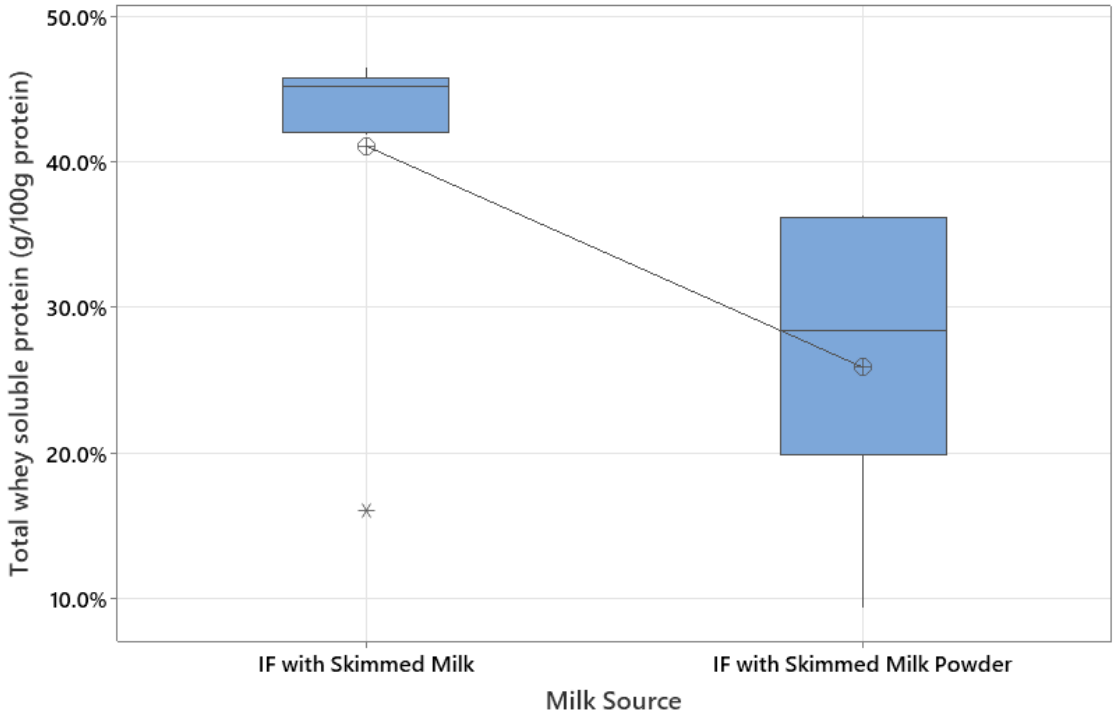


Figure 22: Boxplot of total whey soluble protein (g/100g protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder

Figure 23 illustrates the individual major whey proteins (β-LG, alac, pp5, bsa, and igg) and GMP. S27 from manufacturer D had the most soluble β-LG in IF with Skimmed Milk at 21.7g $100g^{-1}$ total protein. Sample S21(16.9g $100g^{-1}$ total protein) and S37(17.6g $100g^{-1}$ total protein) from manufacturer B of the same brand had the highest soluble β-LG in IFs with Skimmed Milk Powder. Low level of β-LG in S24 and S33 from manufacturer D (2.5 and 2.2 g 100 g^{-1} total protein, respectively) were found that suggested a harsher heat treatment compared to the rest of IFs studied here. Lin et al. (2018) reported that increasing the heat treatment from 72℃ for 15s to 120 ℃ for 120s of SMP prior to evaporation led to a significant increase in whey denaturation and reductions of whey soluble proteins. They also found that evaporation and spray drying did not induce whey denaturation.

As mentioned before, S22 was expected to contain less whey protein because it was a casein dominant IF. S22 which had less total whey soluble protein due to its 20:80 whey to casein ratio was surprisingly had more soluble β-LG (3.6g β-LG $100g^{-1}$ total protein) than samples S24 and S33. This supports further the use of higher heat treatment used by manufacturer D. The total soluble whey protein variation between batches was consistent which could mean that the heat treatment conditions used were constant. Hence, the batch-to-batch variation of FUR is derived elsewhere.

The soluble β-LG of S31 was also suspiciously low (5.8g per 100g protein) compared to the rest of the IF with Skimmed Milk formula that ranges ~15.1-21.7% β-LG. It is therefore speculated that this sample was also heated harshly in comparison to other IF with Skimmed Milk because of the lower total soluble whey protein found (16g per 100g total protein).

Also, S20 had expectedly high amount of total soluble whey because it used a 65:35 whey to casein ratio of which, ~26% of the total whey protein was α-LA. As presented in Figure 23, there was a reduction of ~3.4% of α-LA found in the soluble phase. Halabi, Deglaire, Hamon, et al. (2020) demonstrated that the heat-denaturation of α-LA was enhanced in the presence of β-LG. Their pasteurization simulations also revealed that the whey protein denaturation and aggregation was a function of the whey composition present in the IF and heat treatment intensity.

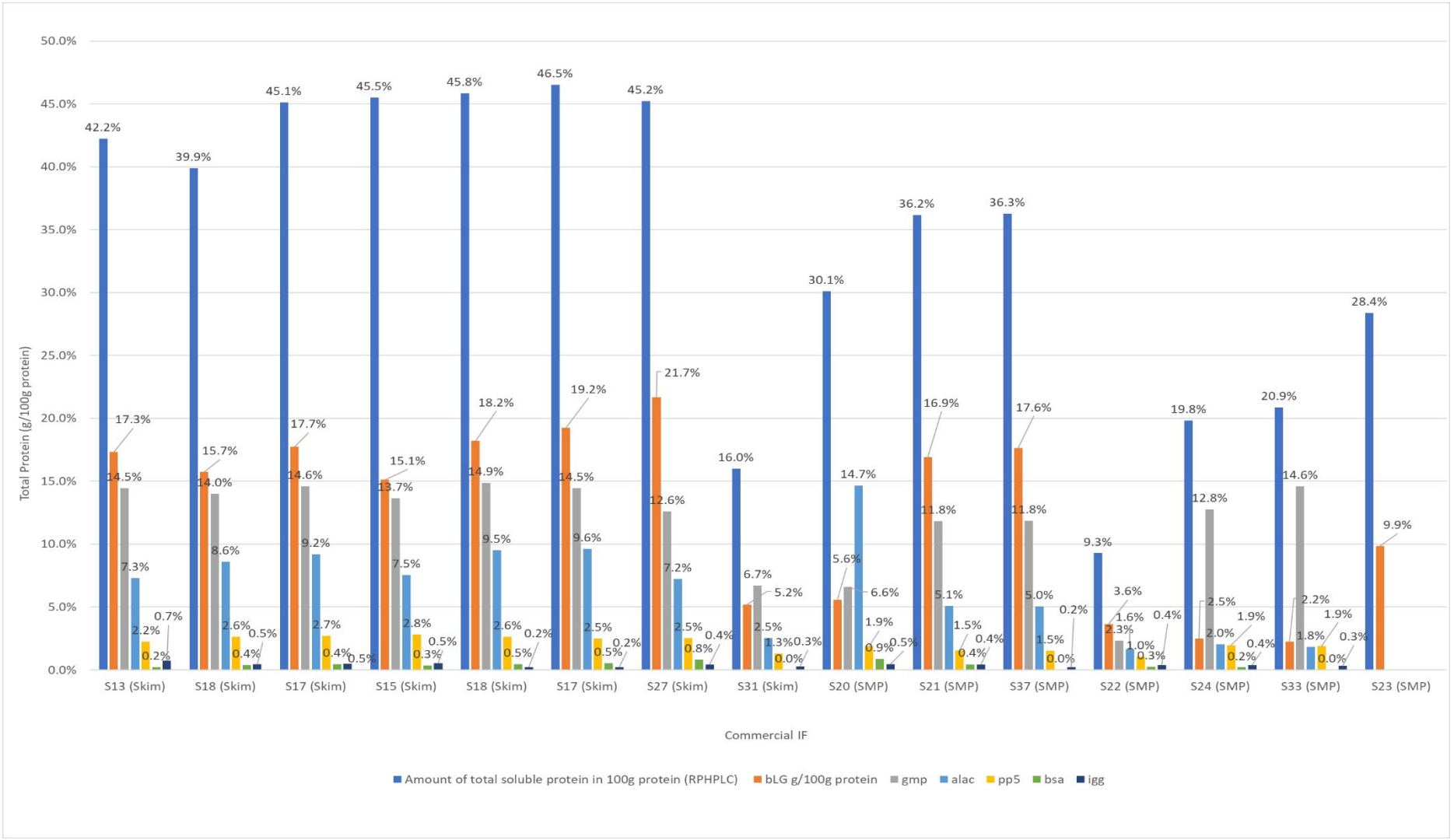


Figure 23: Column graph of total whey soluble protein (g/100g protein) and individual whey soluble protein (g/100g protein) of IF with Skimmed Milk and IF from Skimmed Milk Powder.

Most samples have 60:40 whey to casein ratio except S20 and S22 with 65:35 (α-LA- enriched) and 80:20 whey to casein ratios, respectively.

As observed in Table 12 and Figure 23, batches within manufacturers A(2), B(2), and C(1) have shown proportional relationship between lactulose, total soluble whey protein, and β-LG. The results could indicate:

1. these parameters occur at the same place before the spray dryer,

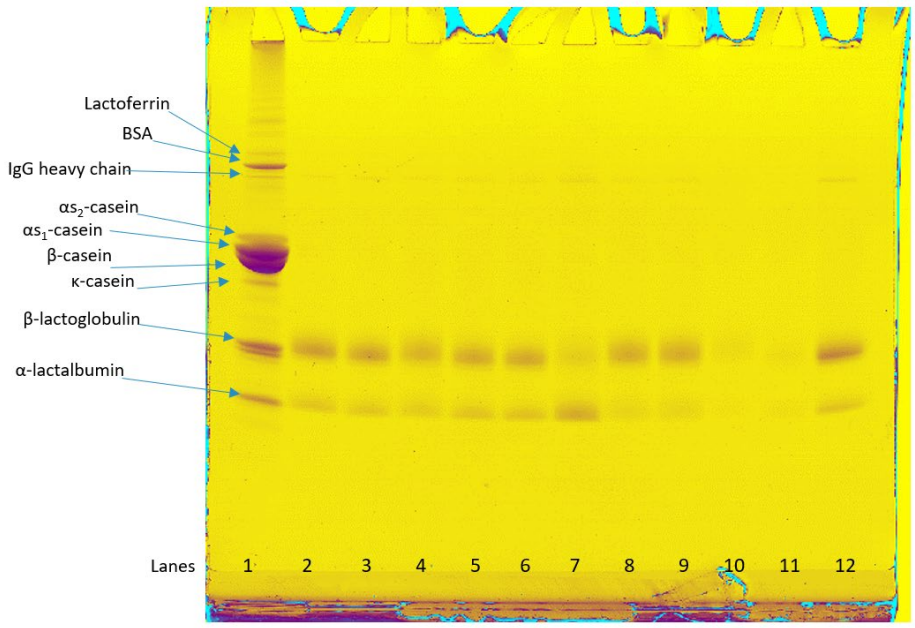
2. the batch-to-batch results using the total whey soluble protein and β-LG have shown consistency which may mean consistency in the process for the respective IF manufacturers,

3. total whey soluble protein and β-LG may be less sensitive to the ingredient contribution than FUR as a marker.

Hence, the batch-to-batch variations of FUR could likely be attributed by the batch-to-batch variation of the ingredients themselves, particularly, the other powdered whey ingredients such as WPC and D90.

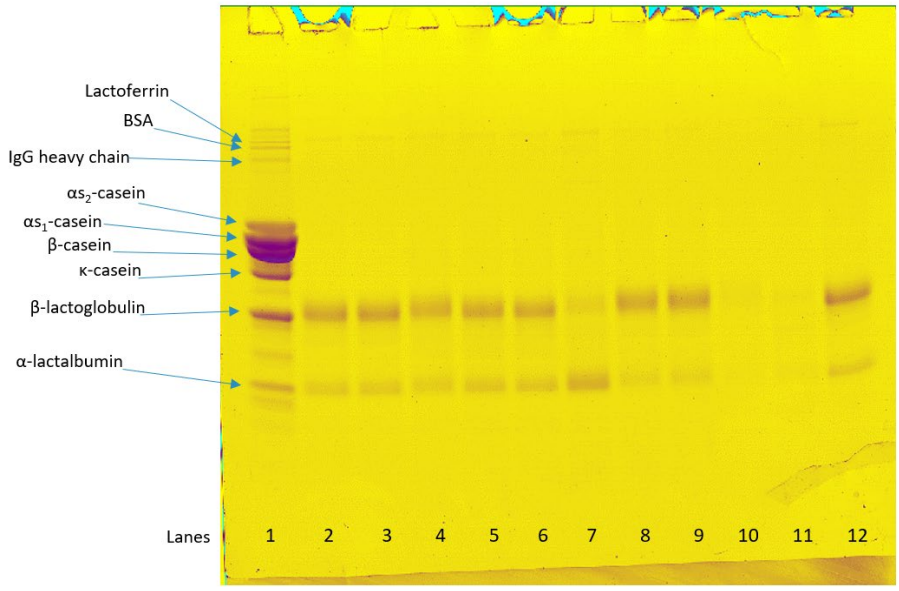
SDS-PAGE. The supernatants of the first set of IFs were further analysed using SDS-PAGE. Unlike RPHPLC, this method is not selective to whey. Therefore, it was used if there were any denatured and aggregated whey-casein protein that remained in the soluble phase after acid precipitation. The results from non-reduced SDS-PAGE (refer to Figure 24) found that casein for both IF with Skimmed Milk and IF with Skimmed Milk Powder were successfully precipitated. Among the whey proteins, only β-LG, α-LA, and minute levels of IGG were retained in the soluble phase. S20 had more α-LA because this was an α-LA enriched IF. S22 had stronger band on β-LG suggesting greater amount than S24. These results are consistent with the previous results. It is also noticeable that unlike the RPHPLC method, Figure 24 does not contain a GMP band. This is because GMP is very acidic and does not form a dye complex with standard SDS staining. Recently, Sharma, Sharma, Rajput, Mann, and Gandhi (2021) developed a method to improve this issue.

Under the reduced conditions, any aggregated with disulphide bridges will be released. Figure 25 shows that the amount of soluble whey proteins did not change. This means that soluble whey proteins from the non-reduced condition were not aggregated and denatured. The overall result from this analysis was the same as the RPHPLC, hence this method was deemed unnecessary to use in the second set of IFs.



Non- Reduced Infant Formula	
Lane 1	Trim Milk (control)
Lane 2	S13 (skim milk)
Lane 3	S13 dup (skim milk)
Lane 4	S15(skim milk)
Lane 5	S17 (skim milk)
Lane 6	S18 (skim milk)
Lane 7	S20 (SMP)
Lane 8	S21 (SMP)
Lane 9	S21 dup (SMP)
Lane 10	S22 (SMP)
Lane 11	S24 (SMP)
Lane 12	S27 (skim milk)

Figure 24: SDS-PAGE of the supernatant of IF from Acid Precipitation (Non-Reduced)



Reduced Infant Formula	
Lane 1	Trim Milk (control)
Lane 2	S13 (skim milk)
Lane 3	S13 dup (skim milk)
Lane 4	S15(skim milk)
Lane 5	S17 (skim milk)
Lane 6	S18 (skim milk)
Lane 7	S20 (SMP)
Lane 8	S21 (SMP)
Lane 9	S21 dup (SMP)
Lane 10	S22 (SMP)
Lane 11	S24 (SMP)
Lane 12	S27 (skim milk)

Figure 25: SDS-PAGE of the supernatant of IF from Acid Precipitation (Reduced)

Conclusion

Furosine was a good protein modification indicator to use. It was able to significantly differentiate between an IF with Skimmed Milk from those that used SMP. However, the data showed that some IF with Skimmed Milk had overlapping values with IF from Skimmed Milk Powder. The likely variations may be due to the heat treatment details used by IF manufacturers and the whey protein sources used. The latter was suspected of being the principal contributor because like the casein sources of IF they can be introduced as liquid or powdered as well. Age of the formula was not seen to be a factor to predict Furosine levels, but a shelf-life study on batches of samples is needed to confirm this

Lactulose did not significantly differentiate the IF with Skimmed Milk from those that used SMP. Along with total soluble whey protein and β-LG, they helped to decipher the heat treatment and whey sources used by different IF manufacturers.

RP-HPLC was a valuable tool in determining the denaturation and aggregation of soluble protein. SDS-PAGE confirmed other results but added no further useful information.

Overall, the exact contribution of both the commercial manufacturing unit operations and ingredients used to make the final product are something that could be the focus of future studies.

Chapter 5: Commercial Manufacturing process of IF

Introduction

This chapter reports on the effect of commercial IF manufacturing process on the protein modification. The previous investigation showed that there were significantly lower levels of FUR in IF that used Skimmed Milk versus those made with SMP. However, it also showed variability between batches and between IF manufacturers that caused the FUR data to overlap. This means that a single value of IF cannot differentiate if fresh skim was used or not. The previous chapter also showed that age did not appear to have an impact on these variations but proposed that the whey ingredients and manufacturing processes did. It showed that lactulose can be used as an indicator to the extent of heat treatment used by IF manufacturers prior to spray dryer along with the total soluble whey protein and β-LG contents. Even though the FUR was used as a marker to differentiate the fresh skim-IF from SMP-IF, the previous chapter also revealed that FUR found in the IFs examined may be driven by spray drying process.

This section hopes to unravel if the interpretation of observations made previously were true by studying the ingredients and the unit operations of an IF with Skimmed Milk under commercial conditions as described in section 3.1, Table 8. The other whey sources used here were in powdered form.

Results and Discussion

Furosine. Figure 26 shows the level of FUR of the different protein ingredients and the influence of the unit operations used in a commercial IF manufacture. Whey Protein Concentrate (WPC) and demineralized whey (D90) were added as powders whereas the skim milk was a liquid. The WPC had the highest level of FUR content among all the protein sources followed by D90 and the skim milk. Batch tank was measured at 0.734g FUR $100g^{-1}$ of protein. The contribution of individual dairy protein sources to the total FUR measured in the batch tank was then calculated. It was found that the WPC contributed to about ~74.2% of the total FUR in the batch tank while D90 and skim milk were responsible for ~23.4% and ~2.4%, respectively. This suggests that the initial FUR content of the individual ingredient and the amount of each ingredient used in the formulation mattered the most in predicting whether the FUR of the final product would be high or not. This also means that a variation in the WPC source will likely reflect in the final product and can potentially lead to a batch-to-batch variation.

The quality of WPC depends on the process history (Caric, 1994; Huffman & James Harper, 1999). Assuming one brand of IF uses the same type of WPC each time, the main variable comes from the breed of the dairy cattle (Mehra, O'Brien, Connolly, & Harrington, 1999). The composition of the

bovine milk affects protein levels, which in turn affect protein quality and its subsequent response to heat treatments (Onwulata, Konstance, & Tomasula, 2004; Walstra, 1999). Nonetheless, the reduction of FUR can possibly be achieved by using a liquid whey (Lund, Bechshøft, et al., 2022) or an acid whey where lactose has already been fermented before drying the WPC (Wherry, Barbano, & Drake, 2019).

Furthermore, the heat treatment from the batch tank to the concentrate tank increased the FUR by 19% (from 7.34g $100g^{-1}$ to 8.72g $100g^{-1}$) of which the first 12% occurred between the batch tank and the first evaporator. The cumulative heat treatment from the batch tank to the spray dryer caused the FUR content to double. Although previous studies reported different FUR values, the conclusions of the past and present studies remained the same, that is the FUR formation or loss of nutritional lysine are driven greatly by spray drying rather than pasteurization and sterilisation (Ferrer et al., 2000; Lund, Mardal, et al., 2022)

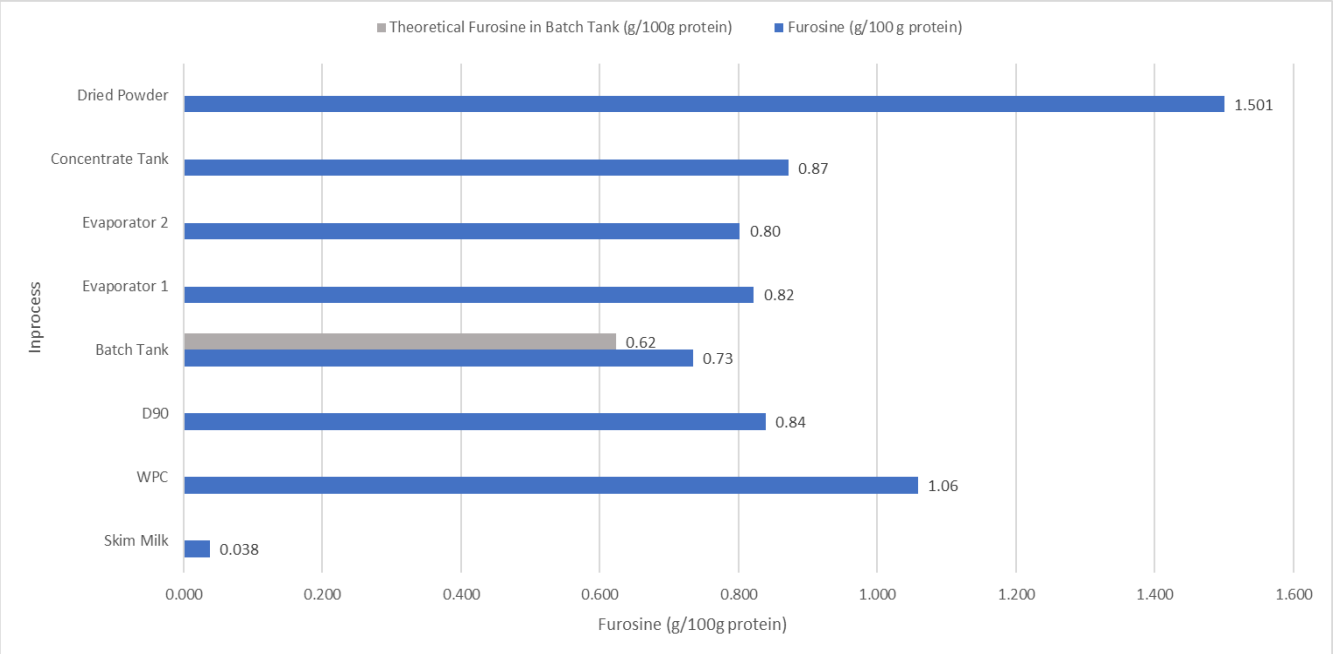


Figure 26: Bar graph of Furosine content (g/100g protein) of InProcess sample from a Commercial IF manufacture

This study also found that low heat SMP contained an average of 0.265g FUR $100g^{-1}$ protein. If used in infant formula, it could increase the contribution to the furosine in the batch tank by 14.5% or ~0.84 g FUR $100g^{-1}$ protein from 0.73g FUR $100g^{-1}$. R. Van Renterghem and J. De Block (1996) studied three classes SMPs namely, extra low-heat, low-heat, and medium-heat. They were freshly packed from a local dairy factory and used for storage experiments. The results showed comparable FUR concentrations (0.100-0.120g $100g^{-1}$ protein) at the start of the storage and significant increases

were observed for all the powders when stored at 37℃ with 65% relative humidity. In addition, the study also included the effects of heat treatment and holding times before evaporation and spray drying during an SMP manufacture. The researchers found that the heat treatment conditions <105℃ before evaporation had FUR content of the powders ranging between 0.170-0.300 g $100g^{-1}$ protein. They indicated no significant heat treatment and holding times effect apart from extreme conditions (temperature at >105℃ and holding times for 60 and 180s). Hence, they concluded that that the furosine formation observed was mainly due to the drying process and storage. These results demonstrate that the spray drying conditions between SMP manufacturers and the storage conditions of SMP used in the commercial IF with Skimmed Milk Powder may have contributed to the FUR variation observed.

Lactulose. Among the dairy protein sources, it was D90 that contained the highest level of lactulose (55mg $100g^{-1}$ total solids), as shown Figure 27. To remove the dilution differences between samples and ease of comparison, the values were expressed as mg lactulose per 100g total solids. It was followed by WPC (12mg $100g^{-1}$ total solids) and skim milk* had the least level of lactulose (<11mg $100g^{-1}$ total solids). Skim milk had barely detectable lactulose but was reported as <10mg L^{-1} because the detection limit of lactulose was <10mg L^{-1} of sample. Hence, to enable to compare the lactulose content of skim milk to the rest of the samples, 10mg L^{-1} was used in the calculation. This finding was in agreement with Morales, Romero, and Jiménez-Pérez (2000) but in contrast with other authors (Martínez-Castro, Calvo, & Olano, 1987) that reported a lactulose content of 5.02mg L^{-1} in milk that was heated at 63℃ for 30 minutes. The current study assumed that Skimmed Milk had 10mg L^{-1} to enable to calculate the theoretical lactulose content in the batch tank. The theoretical value was 15.77mg lactulose $100g^{-1}$ total solids. The InProcess samples in Figure 27 also suggests that lactulose was greatly influenced by the heat treatment prior evaporation (as seen on Figure 3) while the processes that proceeded it did not affect it.

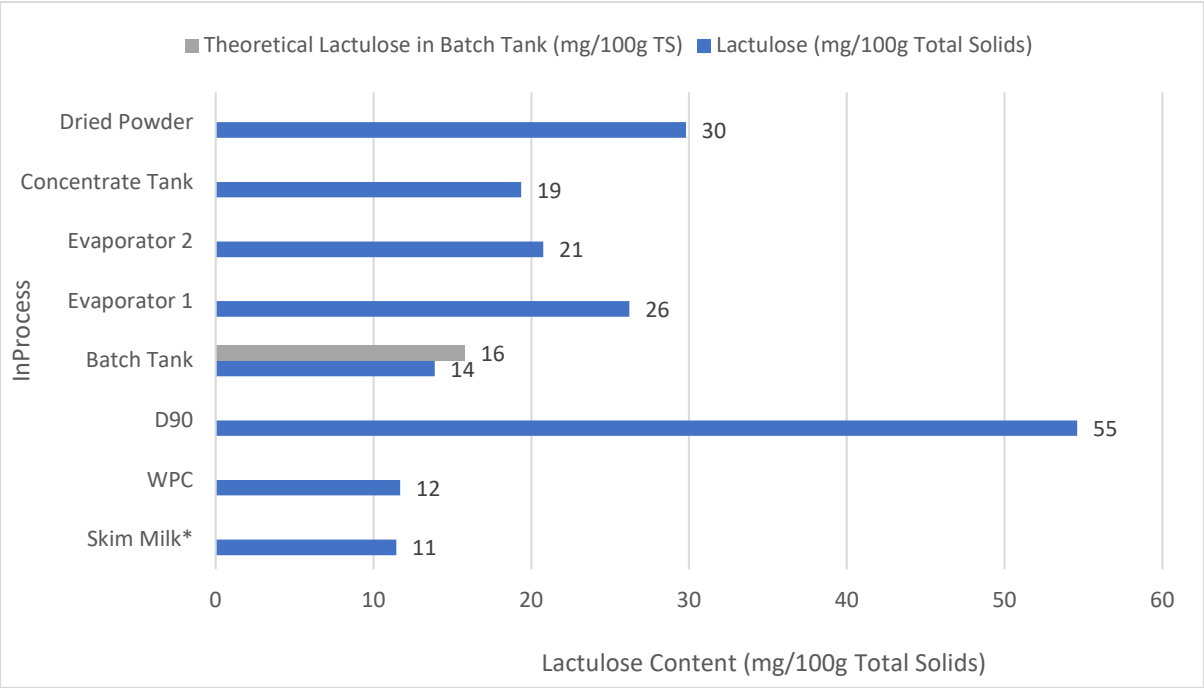


Figure 27: Bar graph of Lactulose content (mg/100g total solids) of InProcess sample from a Commercial IF manufacture. Note that Skim Milk* contains <11mg lactulose per 100g of total solids.

Total Soluble Protein by KJ and NPN. Total soluble protein refers to the remaining total protein found in the supernatant after acid precipitation. Figure 28 shows that that WPC had the highest total soluble protein amount among the ingredients and contributed to about ~67% found in the batch tank. It was followed by D90 which accounts to ~19% and skim milk at ~14% of the total soluble whey protein. It was also found that the heat treatment before evaporation (as seen in Figure 3) removed ~24% of the total soluble protein from the batch tank. The processes that followed did not create any significant further denaturation and aggregation.

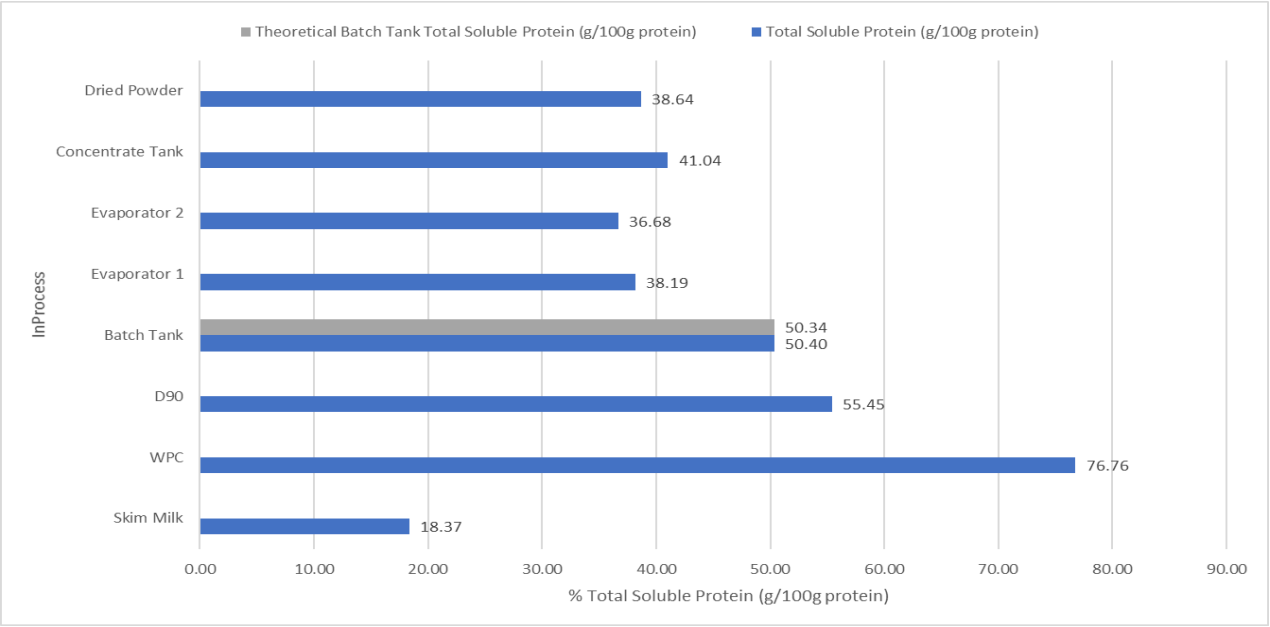


Figure 28: Total Soluble Protein content (g/100g protein) of InProcess sample from a Commercial IF manufacture

Total Soluble Whey Protein by RPHPLC. Total soluble whey protein refers to the remaining total whey protein found in the supernatant after acid precipitation. Theoretically, only proteins from whey will remain in the soluble phase. Hence, the values between the total soluble protein and total whey soluble methods were expected to be the same. Although the trend remained similar, the observed values seen in Figure 29 were higher compared to Figure 28. This could be from the systematic error between the two methods. Despite this, the contribution of WPC towards the total whey soluble found in the batch tank was ~67% while the D90 and skim milk were ~20% and ~13%, respectively. The results also found a ~19% decrease in total whey protein soluble after pasteurization (i.e., between batch tank and evaporator). It was also observed that ~27% of β-LG was lost after pasteurization. There were no significant decreases for both total soluble whey and β-

LG in the processes that followed. The total soluble whey protein and β-LG of the dried powder were comparable to commercial IF as plotted in Figure 23.

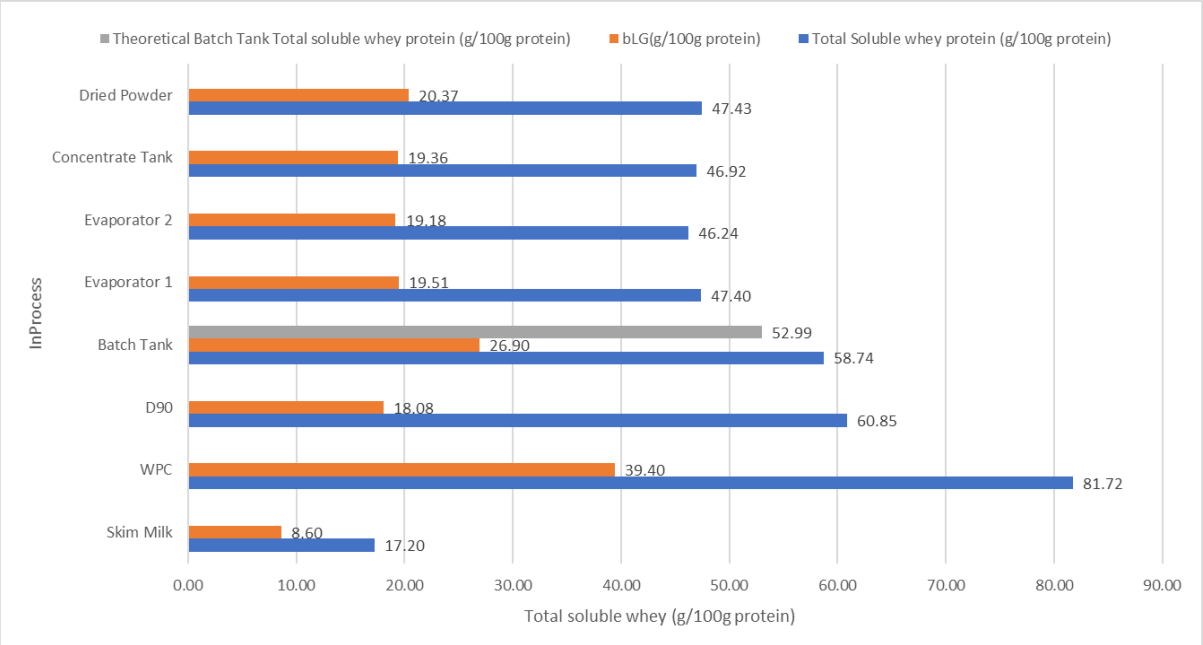
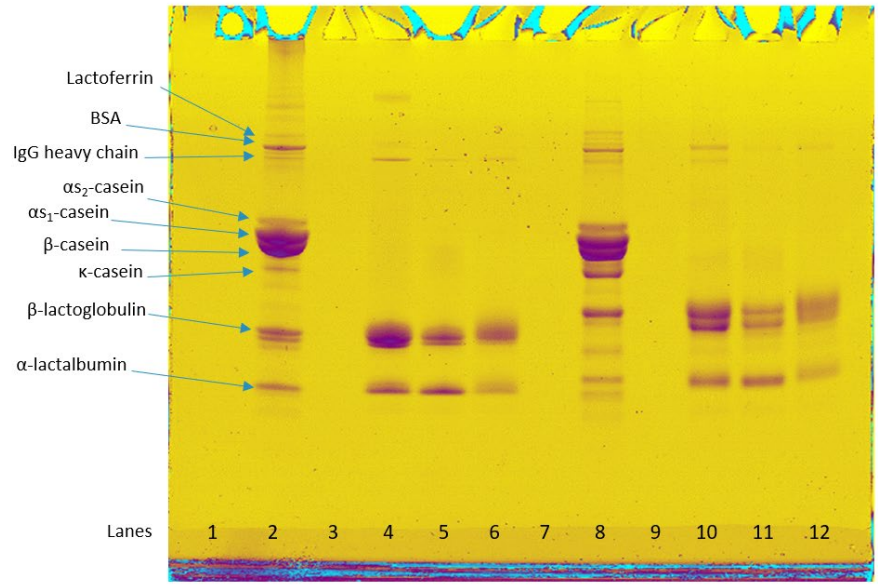


Figure 29: Total Soluble Whey Protein content (g/100g protein) of InProcess samples from a Commercial IF manufacture

Lactulose and total whey soluble protein showed the greatest change between the batch tank and Evaporator 1, i.e., during the heat treatment prior to the first evaporation step. Hence, these findings support the hypothesis that sample S24 with the highest lactulose and nearly totally denatured β-LG used a harsher heat treatment than any other commercial IF examined in this work. On the other hand, the high lactulose level found in S27 cannot possibly be attributed by heat treatment because its β-LG content and total soluble whey were the highest among the commercial IFs with Skimmed Milk. From Figure 27, D90 was found to contain the highest lactulose content among the ingredients. By taking all these observations into account and the ingredients list that claimed the use of "demineralized whey (milk)", it was possible that S27 did not only use an IF with Skimmed Milk but could have likely used a liquid D90 as well.

SDSPAGE. Figure 30 shows non reduced and reduced conditions soluble protein of the ingredients after acid precipitation. Skim milk has shown presence of BSA and IgG heavy chain under both conditions. Figures 31 and 32 show the in-process soluble protein under non-reduced and reduced conditions, respectively. Both figures show IgG heavy chain residues. It is not fully understood the extra band in the β-LG region in the figures. However, this could be an artifact from incomplete denaturation and disulphide bonds. Zhang, Wang, and Li (2019) achieved better result by combining

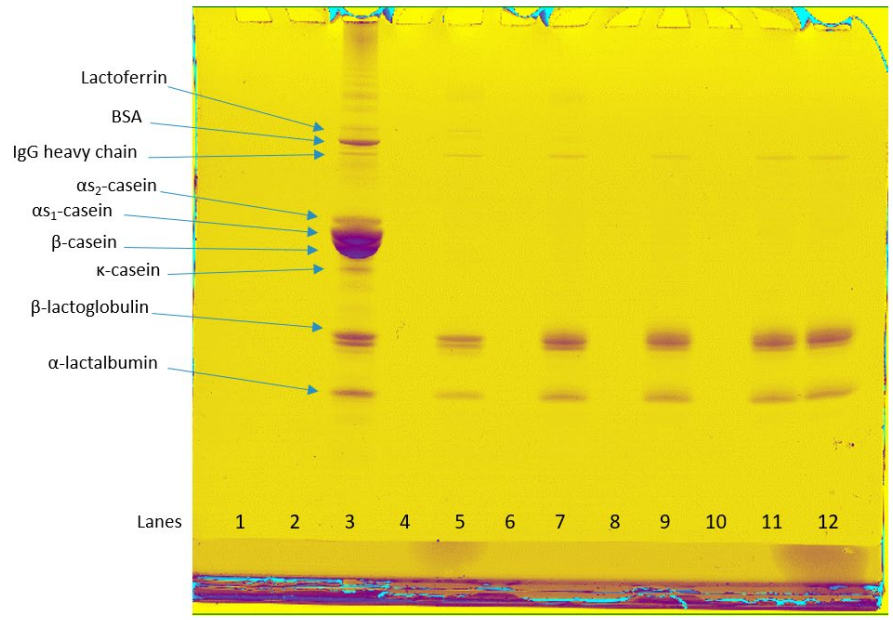
heating with iodoacetimide (IAM). The researchers also successfully minimized the artifacts by replacing the heat step with 8M urea.



Non- Reduced InProcess	
Lane 1	Blank
Lane 2	Trim Milk (control)
Lane 3	Blank
Lane 4	Skim Milk
Lane 5	WPC
Lane 6	D90

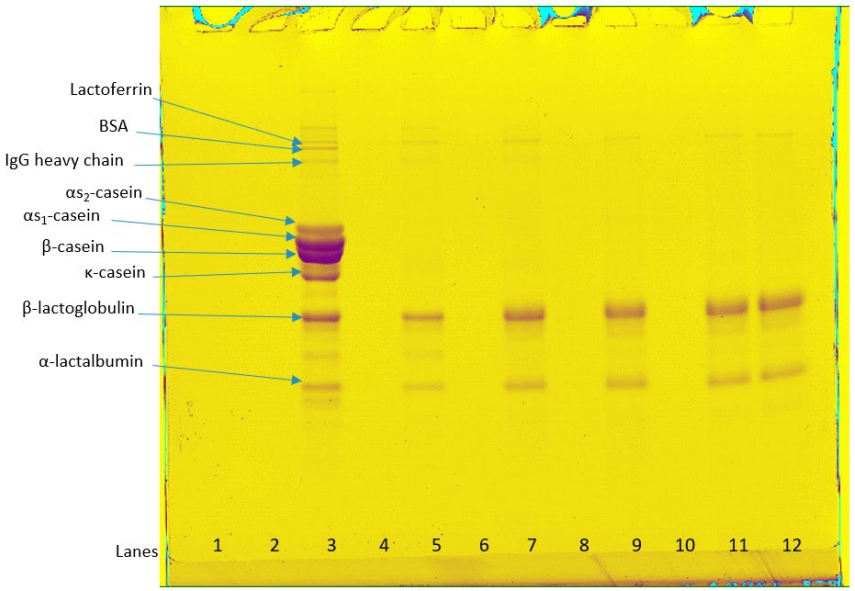
Reduced InProcess	
Lane 7	Blank
Lane 8	Trim Milk (control)
Lane 9	Blank
Lane 10	Skim Milk
Lane 11	WPC
Lane 12	D90

Figure 30: Non-Reduced and Reduced SDS-PAGE of Ingredients



Non-Reduced InProcess	
Lane 1	Blank
Lane 2	Blank
Lane 3	Trim Milk (control)
Lane 4	Blank
Lane 5	Batch Tank
Lane 6	Blank
Lane 7	Evaporator 1
Lane 8	Blank
Lane 9	Evaporator 2
Lane 10	Blank
Lane 11	Concentrated Tank
Lane 12	Final Product

Figure 31: Non- Reduced SDS-PAGE of InProcess



Reduced InProcess	
Lane 1	Blank
Lane 2	Blank
Lane 3	Trim Milk (control)
Lane 4	Blank
Lane 5	Batch Tank
Lane 6	Blank
Lane 7	Evaporator 1
Lane 8	Blank
Lane 9	Evaporator 2
Lane 10	Blank
Lane 11	Concentrated Tank
Lane 12	Final Product

Figure 32: Reduced SDS-PAGE of InProcess

Conclusion

Overall, the InProcess samples gave an insight to the potential cause of FUR variations between the batches of infant formula and between manufacturers. The measurement of lactulose, total soluble whey protein and β-LG were useful to understand the heat treatment variations between IF manufacturers. These markers are highly sensitive in the heat treatment (pasteurization) that comes before the evaporator whereas furosine is affected mostly by the spray drier. Among the ingredients, it was the powdered WPC and powdered demineralized whey (D90) that had the greatest contribution of FUR found in the batch tank. Hence, to ultimately keep the protein modification to a minimum and achieve consistency between batches, the use of both liquid skim and liquid whey can be considered.

Chapter 6. Overall Conclusion

Glycation was the most useful marker for measuring and comparing the heat-induced protein modification in infant formula. Lactulosyllysine, measured as furosine was a substantial indicator to differentiate the group of infant formula that used a Skimmed Milk and Skimmed Milk Powder. Collectively, the IF with Skimmed Milk Powder contained elevated furosine compared to the IF with Skimmed Milk. Nevertheless, the furosine value cannot always be used as a definitive indicator because of high batch-to-batch variation and different processing conditions between manufacturers. Interpretation was confused by variations in ingredients.

The InProcess samples suggested that the whey ingredient such as powdered WPC had the biggest contribution of furosine found in the batch tank, followed by powdered demineralized whey (D90). Among the IF manufacturing steps, the spray dryer had the greatest influence on furosine. Therefore, it is possible that the furosine variations observed between batches and IF manufacturers studied in this work were attributed by the differences in WPC and demineralized whey used in the IFs. While it is impossible to eliminate furosine, it is expected that the use of Skimmed Milk and liquid whey ingredients in an infant formula can minimize furosine and achieve a more consistent product.

On the other hand, the denaturation and aggregation can be used as a heat treatment marker. The InProcess results of lactulose, total soluble whey protein, and β-LG revealed that they are sensitive to the pasteurization step that comes before the evaporator. This information was vital in explaining the heat treatment history of the commercial IFs. Although reflective of heat treatment of the IF, they were not predictive of the observed furosine.

Age effect was difficult to study because samples of commercial IFs were unavailable at the time of manufacture. Preferably, a single batch of IF studied over time can determine the effects of aging.

Chapter 7. Recommendations

The use of liquid whey stream is an alternative to powdered whey ingredients to reduce the batch-to-batch variation and concentration of FUR in the infant formula. The use of acid whey can also be explored. A storage trial is recommended to monitor if the advantages of using a liquid skim in an IF are retained at the end of its shelf-life. Lastly, the future work can also include an in-vitro infant digestion model to understand if the FUR concentrations found in the current study affect the gastrointestinal digestion of infants.

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