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# Modified milk protein concentrates in high-protein nutrition bars

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**Modified milk protein concentrates in high-protein nutrition bars**

by

**Justin Charles Banach**

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

Major: Food Science and Technology

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Ames, Iowa

2016

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## CHAPTER 1. GENERAL INTRODUCTION

### 1.1 Research Problem

Milk protein concentrates (MPCs) (42-85% protein w/w) are powder protein ingredients that are produced by concentrating and drying the proteins in bovine skim milk. Common food protein functional properties (e.g., solubility, gelation, heat-stability) are well covered in the literature for MPCs (Agarwal and others 2015). High-protein ( $\geq 80\%$  protein w/w) MPCs (i.e., MPC80, MPC85) have poor solubility that worsens during storage. This limits its usable shelf life and improving its solubility has been a primary focus. Our approach focused on using high-protein MPCs in intermediate moisture food (IMF) systems where complete protein dissolution is not a prerequisite for performance and in some cases could be texturally detrimental (Cho 2010).

High-protein nutrition (HPN) bars (20-50% protein w/w) are a target application for high-protein MPCs. Nutritionally, MPCs had the highest digestible indispensable amino acid score (DIAAS; 1.18) when compared with soy protein isolate (SPI; 0.91), whey protein concentrate (WPC; 1.10), and several other commercial protein ingredients (Rutherford and others 2015). Unlike protein hydrolysates, a mainstay in HPN bars, MPCs are not bitter and have clean, milky flavor. However, when MPCs are added into a HPN bar formulation they cause the system to harden and lose cohesion during storage. Consumers do not desire a hard, crumbly HPN bar and this drastically limits the product's textural shelf life. This has limited the inclusion of high-protein MPCs in HPN bars.

### 1.2 Overall Goal and Study Hypotheses

The overall goal of this work is to improve the performance of MPCs in HPN bars by modifying their functional properties using food processing techniques such that they

increase stability and cohesion of the final product. This was accomplished in a series of study as presented in Chapter 3 through 7. In study 1 (Chapter 3), the microstructural changes in HPN bars formulated with extruded or toasted MPC80 were studied as these modifications slowed or accelerated texture change, respectively (Banach and others 2014). The hypothesis was that toasting increased and extruding decreased the free sulfhydryl content of MPC80 and this increased and decreased their ability to participate in texture altering disulfide bond formation during HPN bar storage. In study 2 (Chapter 4), instrumental and sensory texture attributes of HPN bars formulated with extruded MPC80 were evaluated and correlated. The hypothesis was that the two techniques were correlated and that extruded MPC80 affects several HPN bar texture attributes other than hardness. In study 3 (Chapter 5), transglutaminase crosslinked milk protein ingredients and a reduced-calcium MPC (RCMPC) were texturally evaluated in HPN bars. The hypothesis was that crosslinked proteins would be less able to participate in Maillard-induced aggregations during HPN bar storage and that RCMPC would keep stable texture by slowing internal moisture migration. In study 4 (Chapter 6), extrusion was used to modify MPC80 functionality with the hypothesis that physical property alteration influences its chemical reactivity within HPN bars and can be used to explain textural change during storage. In study 5 (Chapter 7), MPC85 was jet-milled with the hypothesis that powder particle size reduction will alter powder characteristics and allow for better hydration during HPN bar production and that will help the product maintain better cohesion and texture stability.

### 1.3 Significance

Increased utilization of MPCs in HPN bars would allow the US dairy industry to gain recognition in a category not recognized as being dairy and one that is growing. Growth will continue as consumers demand convenient sources of higher quality protein. Modified MPCs have potential use in HPN bars and would be able to substitute imported (e.g., caseinates) and non-dairy (e.g., soy) protein ingredients used in commercial HPN bars.

### 1.4 Dissertation Organization

This work is presented in eight chapters. Chapter 2 provides background information on both MPC and HPN bars that is pertinent to all the following chapters. Chapters 3 through 7 are manuscript chapters that have been written, submitted, or accepted as journal articles. Chapter 8 is a general conclusion about all the works presented in this dissertation. Formatting follows the author guidelines set forth by the *Journal of Food Science*.

### 1.5 References

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## CHAPTER 2. MILK PROTEIN CONCENTRATE AND HIGH-PROTEIN NUTRITION BARS

### 2.1 Abstract

This literature review provides background information about milk protein concentrate (MPC) powders and high-protein nutrition (HPN) bars. The production of MPC from fluid milk and its generalized functional properties are briefly described. It is well known that MPCs produce HPN bars that rapidly undergo texture changes during storage. The texture change mechanisms for MPC-formulated HPN bars have not yet been fully elucidated, but they are likely related to those used to explain texture changes in other model HPN bars as discussed in this review. The functional properties of MPC ingredients are reviewed and these properties are discussed in terms of their potential to affect performance in HPN bars. Finally, potential techniques to modify MPC functionality to produce HPN bars with improved texture and enhanced textural stability are discussed.

### 2.2 Milk Protein Concentrate Powders

#### 2.2.1 Milk: A Precursor to Milk Protein Ingredients

Milk is defined as the lacteal secretion, practically free from colostrum, obtained from the complete milking of one or more healthy cows (Milk 2015). Packaged fluid milk for beverage consumption must be pasteurized or ultra-pasteurized and contain at least 8.25% (w/w) milk solids not fat (SNF) and not less than 3.25% (w/w) milkfat (Milk 2015). Table 2-1 provides the proximate, mineral, vitamin, lipid, and amino acid content of producer fluid milk with 3.7% milkfat (USDA 2015c). Annual worldwide milk production is expected to surpass 500 million metric tons (MMT) in 2016 and 96.3 MMT

of that will be produced in United States (US) (USDA 2015b). However, only 28% of the fluid milk produced in the US is used as fluid milk. A majority of the excess fluid milk is processed into dairy powders by water removal processes (e.g., concentration, drying) that extends the shelf life of this once perishable product and allows for global trade (Lagrange and others 2015; Cessna and Kuberka 2015). The most basic dairy powders include skim milk powder (SMP) (Codex Standard for Milk...1999), nonfat dry milk (NFDM) (Nonfat Dry Milk 2015), and whole milk powder (WMP) (Dry Whole Milk 2015). SMP, NFDM, and WMP each contain a high concentration of  $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucose (i.e., lactose, milk sugar) (Table 2-2), which causes gastrointestinal issues upon consumption in lactose intolerant individuals (Deng and others 2015). Despite this, milk powders are one of the oldest industrial ingredients used by the food industry (Lagrange and others 2015).

**Table 2-1 Composition<sup>1</sup> of milk (producer, fluid, 3.7% milkfat) per 100 g**

<b><u>Proximates</u></b>		<b><u>Lipids</u></b>	
Water	87.69 g	Saturated	2.278 g
Energy	64 kcal	4:0	0.119 g
Protein	3.28 g	6:0	0.07 g
Total fat	3.66 g	8:0	0.041 g
Ash	0.72 g	10:0	0.092 g
Carbohydrate	4.65 g	12:0	0.103 g
Fiber	0 g	14:0	0.368 g
		16:0	0.963 g
		18:0	0.444 g
		Monounsaturated	1.057 g
<b><u>Amino Acids</u></b>		16:1	0.082 g
Tryptophan	0.046 g	18:1	0.921 g
Threonine	0.148 g	20:1	0 g
Isoleucine	0.198 g	22:1	0 g
Leucine	0.321 g	Polyunsaturated	0.136 g
Lysine	0.26 g	18:2	0.083 g
Methionine	0.082 g	18:3	0.053 g
Cystine	0.03 g	18:4	0 g
Phenylalanine	0.158 g	20:4	0 g
Tyrosine	0.158 g	20:5 n-3 (EPA)	0 g
Valine	0.22 g	22:5 n-3 (DPA)	0 g
Arginine	0.119 g	22:6 n-3 (DHA)	0 g
Histidine	0.089 g	Cholesterol	14 mg
Alanine	0.113 g	Phytosterols	0 mg
Aspartic acid	0.249 g	<b><u>Vitamins</u></b>	
Glutamic acid	0.687 g	Vitamin C	1.5 mg
Glycine	0.069 g	Thiamin	0.038 mg
Proline	0.318 g	Riboflavin	0.161 mg
Serine	0.178 g	Niacin	0.084 mg
<b><u>Minerals</u></b>		Pantothenic acid	0.313 mg
Calcium, Ca	119 mg	Vitamin B-6	0.042 mg
Iron, Fe	0.05 mg	Folate, total	5 µg
Magnesium, Mg	13 mg	Folic acid	0 µg
Phosphorus, P	93 mg	Folate, food	5 µg
Potassium, K	151 mg	Folate, DFE	5 µg
Sodium, Na	49 mg	Vitamin B-12	0.36 µg
Zinc, Zn	0.38 mg	Vitamin A, RAE	33 µg
Copper, Cu	0.01 mg	Retinol	31 µg
Manganese, Mn	0.004 mg	Vitamin A	138 IU
Selenium, Se	2 µg		

<sup>1</sup> Compositional data obtained from the National Nutrient Database for Standard Reference (USDA 2015c).

**Table 2-2 Composition<sup>1</sup> (g per 100 g) of skim milk powder (SMP), nonfat dry milk (NFDM), and whole milk powder (WMP)**

Proximates	SMP	NFDM	WMP
Water	3.8	3.2	2.5
Energy (kcal)	363	362	496
Protein	33	36	26
Fat	0.8	0.8	27
Carbohydrate	56	52	38
Fiber	0	0	0
Ash	7.9	8.4	5.8

<sup>1</sup> Compositional data obtained from the National Nutrient Database for Standard Reference (USDA 2015d, 2015e) and Lagrange and others (2015).

Adding milk powder to foods improves their protein quality and mineral content, and does not introduce anti-nutritional factors found in some plant proteins. These attributes make fortification with milk powders nutritionally beneficial for the world's malnourished population; especially those who suffer from severe protein deficiency (i.e., kwashiorkor) (Hoppe and others 2008). The world's population is expected to grow to 9.7 billion by 2050 and 11.2 billion by 2100 (United Nations 2015). Urbanization and the growth of the middle class will increase the demand for protein; especially those derived from animal sources such as meat and milk. Animal proteins are complete proteins whereas those derived from other sources (e.g., grains, seeds) are typically deficient in at least one essential amino acid. Literature suggests that consuming 25-30 g protein per meal, particularly those rich in branched chain amino acids (e.g., leucine), can promote muscle growth and reduce sarcopenia in aging adults (Paddon-Jones and Rasmussen 2009). A high-protein diet can help maintain a healthy body weight and provides a satiating effect (Westerterp-Plantenga and others 2012; Veldhorst and others 2012). Science-based media reports ([www.ift.org](http://www.ift.org)) have bolstered the importance of dietary protein and consumers have responded by increasing their consumption. As a

result, the food industry has developed quick and convenient foods, such as beverages, bars, and snacks, with more protein at reasonable prices (Layman 2014).

The recommended daily allowance (RDA), which is the amount required per day to prevent deficiency related complications, for protein is set at 0.8 g/kg body mass. For athletes, 1.2 to 1.4 g protein per kg body mass has been suggested, although the recommended increase was not fully substantiated (Lamont 2012). The US set the daily reference value (DRV) for protein at 50 g (Nutrition Labeling of Food 2015). If world's 7.3 billion people in 2015 (United Nations 2015) consumed protein at the DRV for a year, it would require 133 MMT protein (7.3 billion people  $\times$  18.25 kg protein/yr-person). Assuming that all fluid milk contains 3.4% protein, 17 MMT of milk protein will be produced in 2016 with 3.3 MMT of that being produced in the US. Dairy proteins can only partially meet the world's demand for protein and they must be combined with other sources, especially since milk protein allergy limits consumption by a fraction of the population (Pereira 2014).

### *2.2.2 Membrane usage in Milk Protein Powder Processing*

While fluid milk, SMP, NFDM, and WMP powder are good sources of dairy protein, their practical functionality in processed foods is limited. Typical uses of these ingredients include recombined fluid milk, cheese, sweetened condensed milk, ice cream, confections, baked products, evaporated milks, and other beverages (Oldfield and Singh 2005). Milk powder addition to formulations to standardize, increase, or improve the protein content is limited by the introduction of excessive lactose. Microbial metabolism of excess lactose may cause unwanted or secondary fermentations during cheese and yogurt production (Sankarlal and others 2015; Wolf and others 2015). Lactose may



crystallize during ice cream storage, which causes unwanted “sandiness” to develop (Patel and others 2006). Moreover, lactose imparts very few physiochemical properties in foods, as its simple disaccharide structure does not allow for it. Lactose has been the subject of further processing to enhance its value in food and nonfood applications (Seki and Saito 2012).

In the early 1960s, the dairy industry began to utilize membrane technology for concentrations (i.e., water removal) and solid/liquid or liquid/liquid separations (Pouliot 2008). As this technology became more economically and technically advanced, so did the production of whey protein based ingredients, including whey protein concentrates and isolates (i.e., WPCs and WPIs, respectively) (Smithers 2008). Instead of disposing cheese whey, which had environmental and economic cost, it was membrane-filtered to remove lactose and other low molecular weight compounds (i.e., permeate) whereas the retained protein-rich fraction (i.e., retentate) was spray dried to produce highly functional and nutritional ingredients with extended shelf life. Diafiltration and electro dialysis are unit operations that are used to increase protein content of the final product by more complete removal of low molecular weight soluble compounds and minerals, respectively, prior to drying. Microfiltration (MF;  $> 0.1 \mu\text{m}$ ), UF (1-500 nm), nanofiltration (NF; 0.1-1 nm), and reverse osmosis (RO;  $< 0.1 \text{ nm}$ ) membranes are now commonly used in the dairy processing to manufacture new protein ingredients, and concentrated and extended shelf life milks (Pouliot 2008).

### *2.2.3 Production of Membrane Concentrated Milk Protein Powders*

WPCs (34-89% protein d.b.) and WPIs ( $\geq 90\%$  protein d.b.) paved the way for the production of other dry dairy protein ingredients (Table 2-3). MF or UF of milk can be

used to produce micellar casein concentrates (MCCs) or low lactose MCCs, respectively, that have sensory properties superior to dried casein and caseinates whereas the serum protein fraction that permeates has optimal solubility and clarity for use in protein beverages (Hurt and Barbano 2015). UF of skim milk followed by concentration and spray drying can be used to produce a total milk protein concentrate (MPC) with casein-to-whey protein ratio (80:20) the same as typical bovine milk (Singh 2007). MCCs and MPCs are produced with different final protein content, which is identified by the number directly following MPC (i.e., MPC80 has 80% protein d.b.). MPCs with protein content greater than or equal to 90% (d.b.) are more commonly referred to as milk protein isolates (MPIs). Specialized dairy protein ingredients, such as enzyme hydrolyzed MPCs, can be produced using membrane technology (Ewert and others 2015).

**Table 2-3 Composition<sup>1</sup> (g per 100 g) of several dairy protein ingredients: Milk protein concentrate (MPC), micellar casein concentrate (MCC), and whey protein concentrate (WPC)**

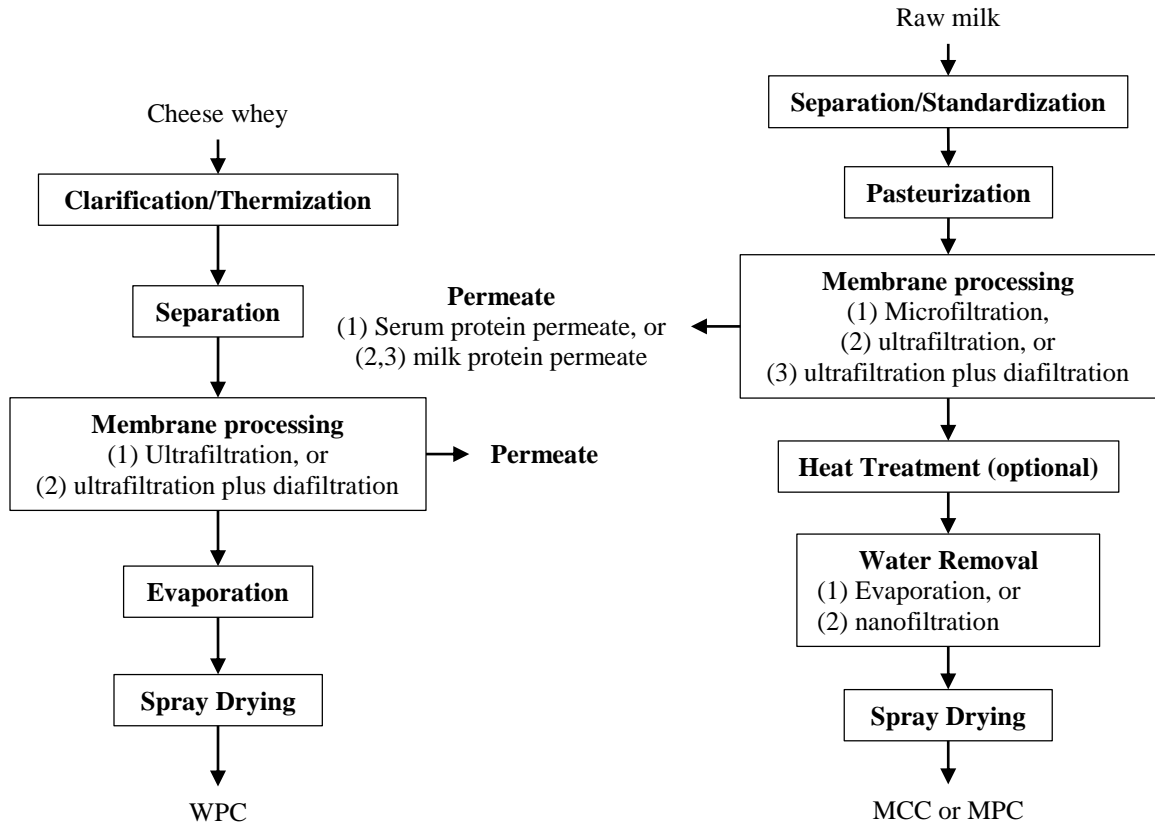
Ingredient	Product	Protein <sup>2</sup>	Fat <sup>3</sup>	Lactose	Ash	Moisture
Milk protein concentrate	MPC42	41.5	1.25	51.0	10.0	5.0
	MPC70	69.5	2.50	20.0	10.0	5.0
	MPC80	79.5	2.50	9.0	8.0	6.0
	MPC90/MPI	89.5	2.50	5.0	8.0	6.0
Micellar casein concentrate	MCC42	41.5	1.25	51.0	6.0	5.0
	MCC70	69.5	2.50	16.0	8.0	5.0
	MCC80	79.5	3.00	10.0	8.0	6.0
	MCC90	89.5	3.00	1.0	8.0	7.0
Whey protein concentrate	WPC34	34.0	4.5	52.0	8.0	4.5
	WPC80	79.5	8.0	8.0	4.0	4.5
	WPI	89.5	1.0	1.0	3.0	4.5

<sup>1</sup> All compositional data obtained from Dairy Management Inc. (2015b, 2015c).

<sup>2</sup> Protein content is specified as a minimum value and is reported on a dry basis for products labeled 80 and above.

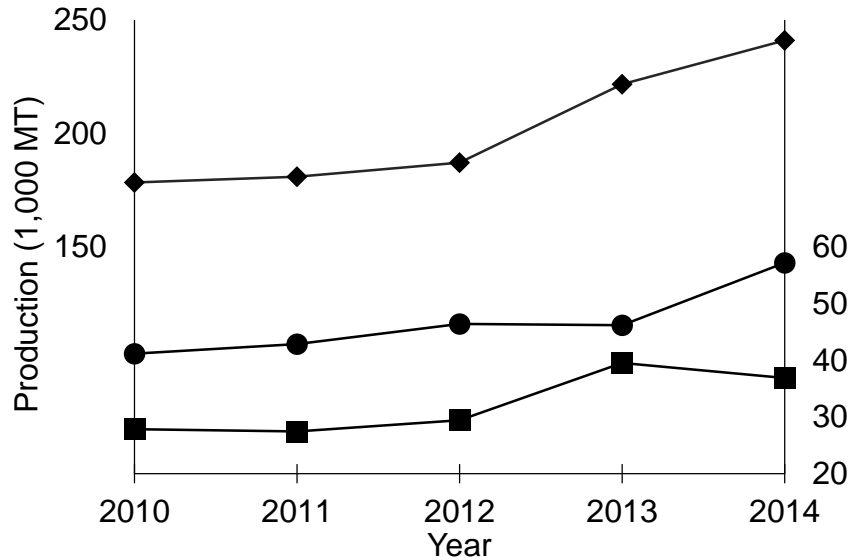
<sup>3</sup> Maximum values on an as-is basis are specified for fat, lactose, ash, and moisture.

Production of WPC/WPI, MCC, and MPC/MPI is summarized in Figure 2-1. Processing parameters vary by production system and product and are summarized elsewhere (Marella and others 2015; da Silva and others 2015; Hurt and Barbano 2010). It should be noted that MPCs, MPIs, and MCCs do not possess a standard of identity. The terminology MCC is used to describe a casein-rich product produced by membrane processing with membranes such that its micellar structure is maintained. Precipitation of casein from milk by acid or rennet followed by washing and drying produces casein powder (Codex Standard for Edible...1995). Neutralization of dissolved casein powder with sodium, potassium, or calcium hydroxide followed by drying produces sodium, potassium, or calcium caseinate, respectively, which have improved functionality and are commonly used in industrial applications. MCC possess superior functional properties, compared to casein powder and caseinates, and while it has been used in some nutritional products, its domestic and foreign production numbers are unknown (Lagrange and others 2015). MPCs can also be produced by co-precipitating the casein and whey proteins in skim milk followed by drying or by dry blending dairy powders together such that the casein-to-whey protein ratio is approximately 80:20 (Kelly 2011). Compared with NFDM, MCCs and MPCs are relatively new protein ingredients and slowly they are starting to replace traditional dairy based ingredients in processed foods.



**Figure 2-1 Generalized production of whey protein concentrate (WPC), micellar casein concentrate (MCC), and milk protein concentrate (MPC).**

The US produced, on average, 703 thousand MT of NFDM per year inclusively between 2010 and 2014 (Figure 2-2) (USDA 2015a, 2014, 2013, 2012, 2011). Annual production of human-grade WPC was between 178 and 241 thousand MT tons during the same period. MPC production during the same period was lower, but it increased from 41 thousand MT in 2010 to 57 thousand MT in 2014. WPI production was lower than WPC and MPC production. The USDA reports total production and protein content differences between the ingredients are unaccounted. A protein powder with higher protein content is more valuable and potentially more functional than a low protein counterpart. NFDM has limited functionality yet continually out produces more functional protein concentrates and isolates due to economic factors, which are well beyond the scope of this review.



**Figure 2-2 Production of milk protein concentrate (MPC; ●), whey protein concentrate (WPC; ◆), and whey protein isolate (WPI; ■) in the United States between 2010 and 2014.** MPC protein content ranges from 40.0 to 89.9%. WPC includes powders produced for humans with protein content ranging from 25.0 to 89.9%. WPI protein content is greater than or equal to 90%. Data obtained from the USDA (2011, 2012, 2013, 2014, 2015a).

#### 2.2.4 Structure-function of Milk Proteins

Proteins are macromolecules assembled by linking amino acids together through peptide bonds. Food proteins provide nutrition to the consumer, and add structure and functionality to the food product. After consuming protein, it is digested to oligopeptides and amino acids, which are absorbed through the gastrointestinal tract. Amino acids are used for anabolic synthesis of proteins, co-enzymes, pigments, and nucleic acids.

Oligopeptides are bioactive, and benefit the consumer in some way greater than its simple amino acid composition, or in some instances they initiate an allergic response (Picariello and others 2013). The nutritional, bioactive, and allergenic properties of dairy proteins have been studied and the results are often conflicting due to methodological differences. Proteins serve as the primary structural building blocks in foods such as meat (e.g., steak), cheese, and yogurt (Foegeding and Davis 2011). Concentrated and isolated protein powders can be used to build and stabilize food structures by imparting their

functional properties. These functional or physicochemical properties include solubility, gelation, emulsification, foam stability, heat stability, water binding, and many others.

Food protein ingredients are not purified, rather are mixtures of several different proteins (Table 2-4) each with different structure and function. In foods, proteins interact with themselves and with other components (e.g., carbohydrates, lipids, etc.). Food processing can cause protein unfolding or denaturation, which does not necessarily mean loss of function, but rather possession of new functionality. Protein denaturation causes changes in secondary (e.g.,  $\alpha$ -helix,  $\beta$ -sheet) and tertiary structure elements (e.g., overall structure, epitopes), but food protein structure-function studies broadly focus on changes in surface topology (Foegeding and Davis 2011). The functional behavior of protein in foods is mostly observed on a macroscopic level and is then related to microstructural properties, but discussions rarely proceed to molecular comparisons.

**Table 2-4 Major proteins (% protein-basis) found in milk protein concentrate (MPC), micellar casein concentrate (MCC), whey protein concentrate (WPC), and whey protein isolate (WPI)**

Protein	MPC <sup>1</sup>	MCC <sup>2</sup>	WPC <sup>3</sup>	WPI <sup>3</sup>
<i>Caseins</i>	80	92	0	0
$\alpha_{s1}$	34	39		
$\alpha_{s2}$	8	9		
$\beta$	25	29		
$\kappa$	9	10		
$\gamma$	4	5		
<i>Whey</i> <sup>4</sup>	20	8	100	100
$\beta$ -Ig	9	4	50-60	44-69
$\alpha$ -la	4	2	12-16	14-15
P-p	4	2		
BSA	1	< 1	3-5	1-3
Ig	2	< 1	5-8	2-3
GMP			15-21	2-20
LF			< 1	NR

<sup>1</sup> Protein composition was the same as fluid milk (Swaisgood 2008).

<sup>2</sup> Casein-to-whey protein ratio was set at 92:8 (Dairy Management Inc. 2015a).

<sup>3</sup> Residual caseins are present (Burrington 2012).

<sup>4</sup>  $\beta$ -Ig, beta-lactoglobulin.  $\alpha$ -la, alpha-lactalbumin. P-p, proteose-peptones. Ig, immunoglobulins. GMP, glycomacropeptide. LF, lactoferrin.

Despite the complexity of foods, food scientists continually measure the functionality of concentrated or isolated protein ingredients. New protein sources, such as insect (Yi and others 2013) and algae (Pelofske 2015), protein extraction processes, and modifications are continually tested for food usage. Researchers utilize many different methodologies to test the same properties without accounting for its interactions with other constituents in the final product (Foegeding and Davis 2011). Nevertheless, researchers typically measure a protein's in-solution functional properties (Sun-Waterhouse and others 2014). Protein powders are hydrated at different concentrations, pHs, and ionic strengths, and then functionality, such as emulsification, foaming, heat stability, water holding, gelation, and other related properties are measured. While it is possible to measure in-solution functionalities, it is difficult to relate these properties to performance in processed foods, especially those that are solid-like. Protein ingredients are modified to impart different functionality when used in a food and can be used to create new and improved foods (Sun-Waterhouse and others 2014).

Another approach for determining protein structure-function properties is to create a model system comprised of the main components of the food product being studied (Harper 2009). The main components should be selected through either preliminary experiments, or by surveying commercial products and previously reported literature models. Eliminating minor components, for example minerals (e.g., sodium chloride), might seem harmless since they contribute little functionality alone, but when used in the presence of proteins or other hydrocolloids, such low molecular weight, minor compounds can alter macromolecule structure and functionality. A solid-type model food also allows for testing at a realistic level of protein incorporation and hydration as

opposed to in-solution tests that require dilute solutions and fully hydrated proteins (Harper 2009). Moreover, a model system allows the production processes (e.g., heat, shear, pH) to be simulated and, although scale-up poses a challenge for the future, knowledge about processability is generated in addition to data obtained from evaluating the model system (Harper 2009). Models generate empirical data in that researchers easily find out what happened, but more in-depth tests are required to figure out the mechanism(s) as to why or how it happened. It is easier to explain a protein's performance in a food based on its performance using a model system. Similarly, a protein's functional properties are often better suited to retroactively describe performance in a food or model system rather than predict its behavior (de Wit 1998). Model food systems are useful in understanding how reactions, such as oxidation and Maillard browning, proceed in complex systems and are used to establish shelf life parameters. They are also helpful in determining the feasibility of using a new protein ingredient developed or modified for a specific food application. While traditional functional property evaluation may be useful for some simpler food systems, such as beverages and salad dressings, protein structure-function in more complex, solid-type foods is better evaluated using a model system.

#### *2.2.5 Functionality and Applications of Milk Protein Powders*

The functional properties of WPC/WPI, MPC/MPI, acid casein, calcium caseinate, sodium caseinate, MCC, and hydrolysates were qualitatively summarized and example food applications where those functionalities are useful are provided (Table 2-5). The functionalities listed in Table 2-5 were adapted to better compare the protein powder ingredients listed. For example, one research institute report stated that MPCs



have high solubility (Dairy Management Inc. 2015a) whereas it is known that solubility of MPC powders decreases with increasing storage time (Haque and others 2010). The mechanisms for decreasing MPC solubility have been reviewed (Fan and others 2014) and processing modifications (Cao and others 2015b; Augustin and others 2012; Carr 2002) and optimal dissolution conditions (Li and others 2015; McCarthy and others 2014) have been explored. Alternatively, WPC/WPI are very soluble by comparison, and while MPCs do dissolve they do not hydrate as easily as WPC/WPI, and therefore are not noted for solubility in Table 2-5. The functionality of a protein powder ingredient depends on its processing conditions and its final composition. For example, low-protein MPCs (e.g., MPC35, MPC50) dissolved and wetted more readily than those with intermediate (e.g., MPC60, MPC70) and high-protein (e.g., MPC80, MPC85, MPI) (Crowley and others 2015b). If functional properties vary by protein content so do the food applications where they can be applied.

**Table 2-5 Milk protein powder functional properties and example food applications<sup>1</sup>**

<b>Functionality</b>	<b>WPC/WPI</b>	<b>MPC/MPI</b>	<b>Acid Casein</b>	<b>Ca-Caseinate</b>	<b>Na-Caseinate</b>	<b>MCC</b>	<b>Hydrolysate</b>	<b>Food Application</b>
Thickening/Viscosity		✓		✓	✓	✓		confectionary, meat, bakery, soups, sauces
Wettability	✓			✓			✓	beverages
Dispersibility	✓			✓	✓		✓	beverages
Solubility	✓			✓	✓		✓	beverages
Emulsification	✓	✓			✓			coffee whitener, meat, soups, sauces
Heat stability		✓		✓	✓	✓	✓	soups, sauces, recombined evaporated milk
Heat gelation	✓							meringues, cakes, egg white substitutes
Foaming/Whipping	✓	✓		✓	✓	✓	✓	ice cream, desserts, whipped topping
Opacity		✓		✓	✓	✓		protein shakes, nutritional beverages
Clarity	✓						✓	fruit flavored beverages, protein waters
Clean flavor		✓				✓		nutritional products, yogurt
Protein fortification	✓	✓	✓	✓	✓	✓	✓	performance nutrition, dietary supplements
Acid stability	✓		✓				✓	acidified and fermented beverages

<sup>1</sup> Milk protein powder functionalities and food applications were adapted from multiple sources (Dairy Management Inc. 2015a; Baldwin and Pearce 2005; Oldfield and Singh 2005).

The potential food applications listed in Table 2-5 are limited in scope and in some instances are irrelevant for individuals seeking to substantially boost protein intake and for the dairy industry seeking to increase protein sales. For example, the emulsifying properties of MPCs were evaluated in 3% (w/w) solution (Dybowska 2008), and a product requiring such emulsification (e.g., salad dressing) would not be heavily consumed per meal. Emulsifiers, other than proteins, are also readily available to

stabilize emulsions at lower addition levels and more affordably. While soups and sauces can be thickened with dairy proteins, such thickening is more cheaply accomplished using starches. Some “innovative” applications for MPCs include yogurts, processed cheeses, NFDM replacements, protein standardization, nutritional bar applications, and other underutilized yet technically feasible applications (e.g., soups, sauces, etc.) (Agarwal and others 2015). Processed cheeses are the largest user of MPCs, but in the US powdered MPCs are prohibited in cheeses with a standard of identity (Lagrange and others 2015). Low-protein MPCs are starting to replace NFDM in some applications.

## **2.3 High-protein Nutrition Bars and Texture Changes during Storage**

### *2.3.1 The Rise of High-protein Nutrition bars*

Nutrition and HPN bar sales grew 71% from 2006 to 2011, and topped \$1.7 billion in sales during the latter year with future growth expected (Mintel 2012). Approximately 226 products were on the market in 2005 and in 2015 that number grew to 1,012 different nutritional bars (Dizik 2015). Nutritional bars that highlight protein and convenience sell better than products marketed on fiber content, weight loss claims, and those featuring cereals (e.g., granola bars) (Dizik 2015). Many different nutritional bars, once available only in specialty shops, are now readily available at grocery and convenience stores. These nutritional bars are broadly categorized as high-protein (i.e., HPN bars), balanced nutrition (40%/30%/30% carbohydrates/non-trans fat/protein caloric-basis), carb conscious, and carbohydrate-rich (Table 2-6).

**Table 2-6 Example nutritional bars by category**

Name	Example flavor	Protein (g)	Serving Size (g)	Protein Wt. (%)	Protein Blend <sup>1</sup>
<i>High-protein</i>					
Clif® Builder's	Chocolate mint	20	68	29	SPI, SPC
PowerBar® Protein Plus™	Chocolate brownie	30	90	33	SPI, WPI, Ca-CN
QuestBar® Protein Bar	Chocolate chip cookie dough	21	60	35	MPI, WPI
BNRG® Power Crunch®	French vanilla crème	14	40	35	H-WP, WPI, MPI
Met-RX® Big 100	Chocolate chip cookie dough	28	100	28	WPC, MPC, Ca-CN, Na-CN, WPI, EW
<i>Energy</i>					
Clif® Energy	Cool mint chocolate	10	68	15	SPI
Snickers® Marathon® Energy	Chewy chocolatey peanut	13	55	24	SPI, PF, MPC
PowerBar® Performance Energy	Peanut butter	9	65	14	SPI
Tiger's® Milk	Peanut butter crunch	6	35	17	SPI, Ca-CN
<i>Balanced Nutrition</i>					
Balance Bar®	Cookie dough	15	50	30	SPI, WPI, H-MPI, CN, Ca-CN
Zone Perfect®	Chocolate chip cookie dough	10	45	22	Na-CN, SPI, WPI, WEP
<i>Carb Conscious</i>					
Detour® Lower Sugar	Chocolate chip caramel	15	43	35	WPC, WPI, H-WP, Ca-CN, SPI
Carb Conscious Supreme Protein®	Caramel nut chocolate	30	96	31	WPI, WPC, MPI, SPI, PF, H-C
AdvantEdge® Carb Control™	Chocolate peanut butter crisp	17	60	28	SPI, MPC, Ca-CN, WPC, PF

<sup>1</sup> Only protein powders used in the soft-textured portion of the bar are included. Proteins included in coatings or other layers, as specified on the product label, were not listed here. SPI and SPC, soy protein isolate and soy protein concentrate, respectively. WPI, whey protein isolate. Ca-CN and Na-CN, calcium and sodium caseinate, respectively. MPI, milk protein isolate. EW, egg white protein. PF, peanut flour. H-MPI and H-WP, hydrolyzed milk protein isolate and hydrolyzed whey protein, respectively. CN, casein. WEP, whole egg powder. H-C, hydrolyzed collagen.

There is no standard of identity stipulated for HPN bars. Their name suggests that protein content should be “high” and thus they should provide at least 20% DRV (50 g) for protein (Nutrient Content Claims...2015). At a minimum, a HPN bar shall provide 10 g protein per reference amount customarily consumed per eating occasion (Reference Amounts...2015) to be labeled as a high-protein product. The example HPN bars listed in Table 2-6 provide 14-30 g protein per 40-100 g serving and they can be formulated to contain up to 50% protein (w/w) (Imtiaz and others 2012), thus, can potentially utilize a large amount of milk protein powders. These high-protein systems are preferably cold processed, that is, formed (e.g., rolled, pressed), shaped (e.g., low-pressure extrusion), cut, and packaged without cooking, because the protein component tightly binds water which prevents its release during baking (Burrington 2007).

### *2.3.2 Generalized High-protein Nutrition Bar Composition with Emphasis on the Protein Component*

HPN bars are comprised of 20-50% protein, 10-50% carbohydrates, and 10-15% fats on a weight-basis (Imtiaz and others 2012; Zhu and Labuza 2010). The HPN bars listed in Table 2-6 contain 28-35% protein (w/w). A formulation constraint in soft-textured, non-baked HPN bars is water activity ( $a_w$ ), which must be kept less than 0.65 to ensure microbial safety (Loveday and others 2009). Some literature based model formulations exclude free water all together and instead rely on sugar syrups (e.g., HFCS), polyols (e.g., glycerol), and sugar alcohols (e.g., sorbitol) to bind the system together while maintaining the low  $a_w$ . Flavorings, vitamin/mineral premixes, coatings, and other textural elements are added to HPN bars.

Dairy powders, namely, calcium caseinates and whey protein hydrolysates, and soy protein powders, such as concentrates and isolates, are usually blended as protein sources in commercial HPN bar formulations (Imtiaz and others 2012). Careful protein ingredient selection is required as when proteins become highly concentrated in HPN bars, they adversely affect texture and accelerate undesirable texture changes, namely hardening, during storage. MPCs/MPIs are avoided in HPN bars since their products quickly harden and lose cohesiveness during storage (Imtiaz and others 2012; Loveday and others 2009; Li and others 2008; Baldwin and Pearce 2005), especially in comparison to other protein powders, such WPCs/WPIs. MPCs and MPIs, which are nutritionally superior to soy protein isolates (SPIs) and several other common protein ingredients (Table 2-7) (Rutherford and others 2015), have slowly penetrated HPN bar formulations as they negatively impact textural quality. MetRX®, a nutritional company, first used MPCs/MPIs in their products, including HPN bars, during the early 1990s, but since then few products have incorporated these high-quality proteins at a substantial level (Agarwal and others 2015). HPN bar texture literature has focused on whey proteins (Rao and others 2013a; Zhu and Labuza 2010; McMahon and others 2009) although other proteins, including egg white, SPI, calcium caseinate, and MPI, in similar systems have also been studied (Rao and others 2013b; Li and others 2008). Even though many different protein powders have been evaluated in model HPN bar systems, it remains unclear what functionality they should possess to a balance firmness and cohesiveness while maintaining textural stability during storage.

**Table 2-7 Protein digestibility-corrected amino acid scores (PDCAAS) and digestible indispensable amino acid scores (DIAAS) of some common protein ingredients<sup>1</sup>**

<b>Protein Ingredient</b>	<b>DIAAS</b>	<b>PDCAAS</b>
Milk protein concentrate (MPC 4850, Fonterra)	1.18	1.00
Whey protein isolate (WPI 8855, Fonterra)	1.09	1.00
Whey protein concentrate (WPC 392, Fonterra)	0.973	1.00
Soy protein isolate (Supro 670, Solae)	0.906	1.00
Soy protein isolate (Supro XF, Solae)	0.898	0.979
Pea protein concentrate (Nutralys S85, Roquette)	0.822	0.893
Wheat bran (local)	0.411	0.525
Rice protein concentrate (Oryzatein 90, Axiom Foods)	0.371	0.419

<sup>1</sup> PDCAAS and DIAAS were determined by Rutherford and others (2015).

### 2.3.3 Model High-protein Nutrition Bars in Texture Evaluation

A number of different models have been used to study intermediate moisture food (IMF), a category that includes HPN bars, texture, stability, and the mechanisms for texture change during storage (Table 2-8). These models test the effect of protein source, mainly dairy derived whey proteins to align with commercial utilization, and ingredient type (e.g., isolate, concentrate, hydrolysate) on HPN bar texture change. Protein ingredients evaluated include SPI, soy protein concentrate (SPC), WPI, WPC, caseinates, egg white, MPC, MPI, and any of their hydrolysates. WPC, WPI, and/or their hydrolysates are predominantly used in the model HPN bars. Egg white protein, MPC, and MPI are used sparingly in the soft-textured HPN bars, although the latter two components listed may be present in a coating or layer within the HPN bar.

**Table 2-8 Model high-protein nutrition bar formulations used in the literature**

Protein				Other Constituents	
Source	Ingredient <sup>1</sup>	Ingredient Wt. (%)	Content (%)	Ingredient (Formula Wt. %)	Reference
Whey	WPI/H-WPI	38.0	32.7	HFCS or sorbitol syrup (42.7), vegetable shortening (19.3)	McMahon and others (2009)
Dairy	MPC80	37.4	30.0	Glycerol (21.5), palm kernel stearin (18.5), maltitol syrup (12.0), HFCS (10.0), water (0.6)	Banach and others (2014)
Dairy	MPC80, WPI, or Ca-CN	20.0	16.3, 18.2, or 18.4	Glucose (40.0), glycerol (15.0), water (15.0), cocoa Butter (10.0)	Loveday and others (2009) Loveday and others (2010)
Dairy	MPC/WPC	35.5-37.0	30.0	Glucose syrup (34.9), glycerol (17.4), maltodextrin (3-3.5), palm kernel oil (5.7-6.7), lecithin (0.5), water (0.3-1.6)	Imtiaz and others (2012)
Dairy	WPI, WPI/CN, or WPI/H-CN	60.0	58.1	Water or phosphate buffer (40)	Rao and others (2016) Zhou and others (2008a)
Soy	SPI	34.2	30.0	Corn syrup (26.1), HFCS (21.4), rice syrup solids (7.9), cocoa powder (5.1), glycerol (4.0), vitamin/mineral premix (0.7), chocolate flavor (0.5), vanilla flavor (0.1), salt (0.1)	Cho (2010)
Whey	WPI	45	43.6	Fructose or sorbitol (25), glycerol (17.5), water (12.5)	Zhou and others (2013)
Egg	EW/HEW	75	54.3	Water (25)	Rao and others (2013b)

<sup>1</sup> WPI and WPC, whey protein isolate and concentrate, respectively. H-WPI, hydrolyzed whey protein isolate. MPC, milk protein concentrate. MPC80, milk protein concentrate with 80% protein. SPI, soy protein isolate. CN, Ca-CN, and H-CN, caseinate, calcium caseinate, and hydrolyzed caseinate, respectively. EW, egg white powder. HEW, hydrolyzed egg white powder.



The simplest HPN bar model combines protein powder with water or pH stabilized phosphate buffer (Rao and others 2016; Zhou and others 2008a). Simplified models allow for a mechanistic approach, however, their results might not transfer when the protein ingredient is used in a more complex, market-ready HPN bar formulation. Complex models include flavorings and added vitamins/minerals, and while these constituents are not expected to contribute texturally to the dough system, they may influence textural stability (Cho 2010). Texture changes in a complex model are not attributable to any one cause.

A consistency between all models is that total protein content (16-60% protein w/w) is clearly stated or readily estimated based on the formulation. Some literature models are protein ingredient dependent and this makes it difficult to simply substitute a new protein ingredient for evaluation. A model formulation set up into bar form when MPC80 and caseinate were utilized at 20% (w/w), but the one prepared with WPI at the same level of inclusion never solidified and hence was not a HPN bar (Loveday and others 2010, 2009).

#### *2.3.4 High-protein Nutrition Bar Texture Change and Quantitation*

After model HPN bars are prepared, they are stored at room (~22°C) or elevated temperatures for accelerated storage, and texture is measured using instrumental and, only in some instances, trained sensory panel analysis. Instrumental puncture testing is the most utilized technique to generate sample hardness data. Essentially a small diameter (2-5 mm) cylindrical probe is used to puncture hand-pressed HPN bar or IMF dough to predefined strain (25-50%) at constant speed (1 mm/s) while compressive force and time or distance are recorded by a texture analyzer (Rao and others 2016, 2013b;

Zhou and others 2013, 2008a; Zhu and Labuza 2010; Liu and others 2009; Li and others 2008). Hardness is reported as the maximum force obtained during compression or the force at specified strain. Puncture analysis does not allow for quantitation of any other texture attributes and thus it is unclear how this technique adequately describes a complete chewing experience. McMahon and others (2009) used a 45° chisel blade to shear samples 3 cm wide and 1 cm thick to 85% strain and reported hardness as the maximum compressive load. Loveday and others (2010, 2009) uniaxially compressed cylindrical (dia. = 16 mm; h = 20 mm) HPN bars and reported fracture stress as a measure of hardness. These instrumental methods only characterized hardness related texture attributes whereas other relevant HPN bar texture attributes, such as those obtained by texture profile analysis (TPA) or quantitative descriptive analysis (QDA), are missed using this convenient method.

Puncture methodologies are mainly used to measure hardness of models formulated with whey proteins, and while hardening is the major textural concern in these systems, it is not known how the other attributes change. Li and others (2008) punctured HPN bars prepared by blending WPI, SPI, and MPI, and supplemented instrumental hardness with “bar integrity” and stickiness data acquired by a small (n = 5) trained panel. Bar integrity combined crumbliness (i.e., 1 = crumbly and low bar integrity) and fluidity (i.e., 10 = high fluidity and low bar integrity), and highlighted the importance of other HPN bar textural attributes and the fact that overall texture depends on the protein ingredient type and not just the total protein content.

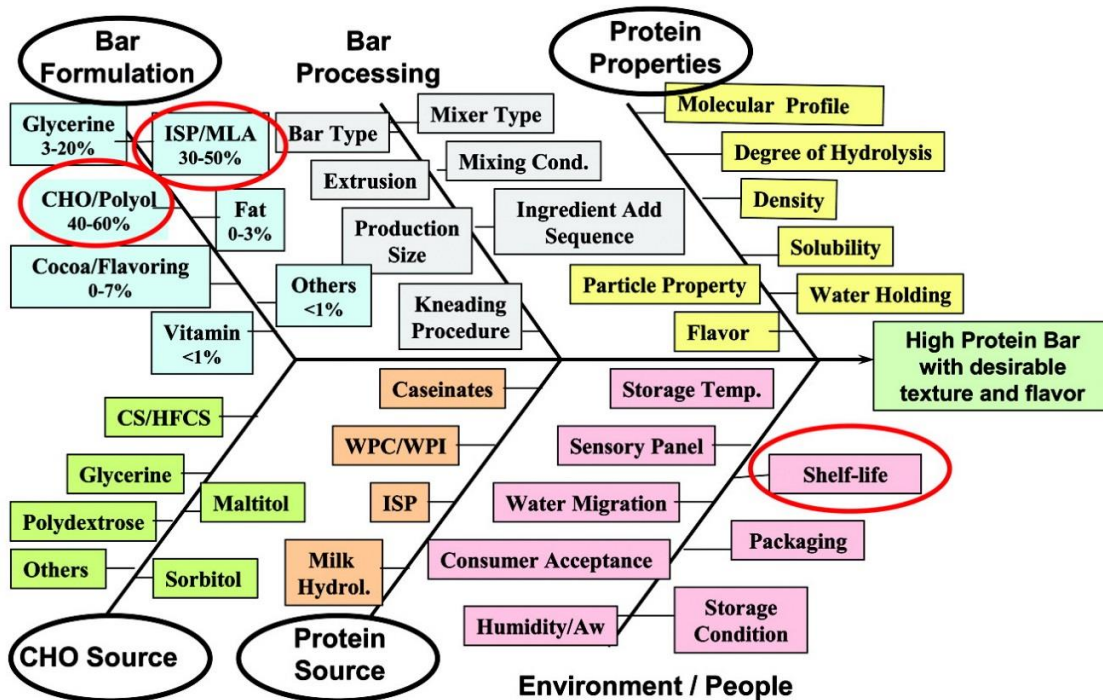
WPC and MPC were blended and made into model HPN bars that were instrumentally punctured throughout yearlong storage at room temperature (Imtiaz and

others 2012). A trained sensory panel evaluated in-hand firmness, crumbliness, smoothness, and stickiness, and in-mouth hardness, fracturability, chewiness, dissolvability, cohesiveness of mass, powderiness, and tooth packing only after 1 month storage. Instrumental analysis, including TPA's standardized definitions (Gunasekaran and Ak 2003), could not measure HPN bar smoothness, dissolvability, powderiness, and tooth packing. Sensorial firmness was correlated with puncture peak force and this added validity to preceding works that were working under this assumption. Another key finding was that absolute peak force registered during probe withdrawal correlated with sensory panel perceived crumbliness/cohesiveness. The texture of HPN bars, especially those formulated with high-protein MPCs, cannot be described by hardness data alone and they should be supplemented with crumbliness/cohesiveness and other relevant data (Imtiaz and others 2012).

### *2.3.5 Postulated Mechanisms of High-protein Nutrition Bar Texture Change*

Literature has predominantly focused on the role of protein during HPN bar texture change, which has been overly simplified to instrumental puncture hardening. Other HPN bar components, such as free sulfhydryl blocking compounds (i.e., N-ethylmaleimide), reducing agents (i.e., cysteine), sugar alcohols (i.e., maltitol, sorbitol), and other polyols (i.e., propylene glycol, glycerol) also affect HPN bar texture and its stability during storage (Zhu and Labuza 2010; Liu and others 2009). Therefore, HPN bar texture work is somewhat empirical, with results dependent on the model system utilized. For example, protein powder/water systems harden with time without added sugars, carbohydrates, and lipids, and despite those components being present in commercial systems, hardening of that model must involve the protein component. Other

factors that influence HPN bar texture and its change during storage were broadly classified into six categories (Cho 2010): (1) bar formulation, (2) bar processing (e.g., mixing time, order of ingredient addition, pressing force), (3) protein properties, (4) carbohydrate source, (5) protein source, and (6) environment/people (Figure 2-3). Based on these categories and other works, several mechanisms of HPN bar hardening have been suggested and are detailed below:



**Figure 2-3 Factors that influence high-protein nutrition (HPN) bar texture and its change during storage.** ISP, isolated soy protein or soy protein isolate. CHO, carbohydrate component. CS, corn syrup. HFCS, high-fructose corn syrup. A<sub>w</sub>, water activity. Reprinted from Cho (2010) with permission. Copyright© 2010 American Chemical Society.

### 2.3.5.1 Glass Transition Temperature

One of the most important aspects pertaining to HPN bar texture is the protein glass transition temperature ( $T_g$ ) or the analogous protein powder glass rubber transition temperature ( $T_{gr}$ ) (Kelly and others 2015; Rao and others 2013b).  $T_g$  is interpreted thermodynamically by differential scanning calorimetry whereas  $T_{gr}$  is determined by thermomechanical rheology (Hogan and others 2016). Spray dried protein powders exist

in the glassy state when kept at temperatures less than  $T_g$ . A higher protein  $T_g$  indicates lower molecular mobility when the powder is stored at room temperature and thus has greater resistance to physical and chemical change. Protein powder  $T_g$  is a function of its moisture content, average molecular weight, and protein content.  $T_g$  decreases as average molecular weight decreases and as moisture content increases (Zhou and others 2014).  $T_{gr}$  for protein powders, specifically MPC, increased as the protein content increased (Kelly and others 2015). During HPN bar production, if the temperature of the dough exceeds the protein powder  $T_{gr}$ , the particles collapse, that is, they lose their structure and plasticize the final system in the rubbery state (Hogan and others 2016). Whey protein hydrolysates, which have lower average molecular weight and thus lower  $T_g$  than their intact parent proteins, maintain the HPN bar in the pliable rubbery state throughout storage at a temperature greater than  $T_g$  (Rao and others 2013a). Elevated HPN bar viscosity in the chemically more reactive rubbery state slows the progression of any texture degrading reactions (Rao and others 2013a) or perhaps time-dependent texture change is not driven by chemical changes and texture remains soft solely due to the system maintaining the rubbery state. Proteins with higher  $T_g$  are expected to produce HPN bars that exist in the texturally hard, glassy state or one that hardens as the system returns to the glassy state. Humectants such as glycerol ( $T_g=-93^\circ\text{C}$ ), sorbitol ( $T_g=-2^\circ\text{C}$ ), and maltitol ( $T_g=44^\circ\text{C}$ ) can lower overall HPN bar  $T_g$  keeping the protein plasticized, the system soft, and protein aggregation to a minimum during storage (Liu and others 2009).

### 2.3.5.2 Solidification and Particle Size

If protein powder  $T_{gr}$  is too high, particle collapse will not occur and its structure will show through in the HPN bars, as was the case when formulated with MPC80 at

20% (w/w) (Loveday and others 2009). Incomplete particle collapse during HPN bar production will likely produce a non-plasticized, crumbly system. Additionally, the protein volume fraction required for system solidification, the point when the HPN bar lipid/polyol blend changes from liquid to solid while slowly adding powder, depends mainly on the volume-volume constraints dictated by the protein powder particle size (Hogan and others 2016). When volume-volume constraints form the basis of initial HPN bar texture, higher-volume fractions are obtained using protein powders with smaller size and/or bi-modal distributions such that smaller particles interject themselves in the voids formed between larger particles (Huppertz and Hogan 2015). This may give a HPN bar with more fluid texture whereas solidity obtained at a low volume fraction with large particles may be less cohesive and may fracture under lower stresses. If the protein powder particles remain suspended, that is solidification does not occur, the system is metastable and does not age texturally (Hogan and others 2016). Solidification is required in order for the final product to be a HPN bar. The protein volume fraction where solidification occurs depends on particle size and particle collapse in the lipid/polyol blend used in HPN bar formulation. When formulating HPN bars at a fixed protein content, a common practice in literature (Banach and others 2014; Imtiaz and others 2012), initial texture likely depends on the protein powder fraction required for solidification and the amount of powder needed to obtain the desired protein level on a weight-basis.

#### 2.3.5.3 *Moisture Migration*

Moisture migration between HPN bar constituents can lead to texture changes, but there are conflicting reports about the direction water moves. Theoretically, water should

migrate from constituents with relatively higher  $a_w$  (e.g., sugar syrups) to those with lower  $a_w$  (e.g., protein powder) (Hazen 2010; Book 2008; Li and others 2008; Gautam and others 2006). Carbohydrate syrup dehydration may lead to sugar crystallization, which can impart a sandy texture and cause the system to harden by increased crystallinity (Hutchinson 2009). As non-plasticized protein powder particles sorb water from other constituents, they swell while the system maintains a fixed geometry and hardens by increased packing density (Huppertz and Hogan 2015). However, some NMR analyses revealed that low molecular weight constituents pulled water away from the protein using osmotic pressure (Loveday and others 2010, 2009). Water activity increased during HPN bar storage, which suggested that water did not become more associated with the protein, rather it became freer in the system, and this occurred with matrix hardening (McMahon and others 2009). Moisture migration away from a plasticized or partially plasticized protein powder causes its  $T_{gr}$  to increase, after which the particles lose plasticity as they enter the glassy state causing texture changes. Added minerals (e.g.,  $Na^+$ ,  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ) may alter protein conformation and may increase internal moisture migration, both of which may contribute to textural changes (Book 2008). Moisture migration may or may not occur within moisture-limited ( $a_w \leq 0.65$ ) HPN bars, but keeping all components adequately hydrated and minimizing change are likely keys to stabilizing texture.

#### *2.3.5.4 Disulfide Bond Formations and Protein Aggregations*

Soft and pliable HPN bars exist in the rubbery state and are prone to chemical and physical changes despite having high viscosity (Zhou and others 2014; Rao and others 2013a). Protein/water systems hardened as disulfide-linked whey proteins aggregated

into a more complete network (Zhou and others 2008a, 2008b). Blocking protein free sulfhydryl groups with N-ethylmaleimide extended the model's textural shelf life by 135 days (Zhu and Labuza 2010). Cysteine, a reducing agent, addition to the same model at molar ratios of 0.05 and 0.25, extended (+15 days) and shortened (-11 days) the shelf life, respectively (Zhu and Labuza 2010). Cysteine delayed or accelerated HPN bar hardening by affecting sulfhydryl-disulfide exchange and the formation of disulfide-linked protein aggregates. Maillard-induced protein aggregations were also related to HPN bar hardening and when reducing sugars were replaced with non-reducing sugar alcohols, texture changes were slowed by inhibition of the reaction (Liu and others 2009). However, Maillard browning cannot be the only cause of texture change as models formulated with sorbitol instead of fructose still hardened during storage (McMahon and others 2009).

#### *2.3.5.5 Macro-constituent Phase Separations*

Another suggested mechanism for HPN bar texture change is macro-constituent, specifically protein and sugar/polyol syrups, separation during storage (McMahon and others 2009). Partitioning the co-solvent away from the local protein domain allows for protein aggregations (e.g., disulfide bond, Maillard-induced), which increases the  $T_g$  and contributes to hardening as previously discussed (McMahon and others 2009). Phase separations were limited in HPN bars formulated with WPI hydrolysates, which were more hydrophilic and were better able to associate with the sugar/polyol syrup, and this limited hardening during storage. When selecting a protein ingredient for a HPN bar formulation, its interaction with glycerol, sugar syrups, and other polyols such as sugar alcohols need to be considered to ensure compatibility. The importance of the protein



powder's  $T_{gr}$  becomes clear while discussing particle size and solidification, moisture migration, protein aggregation, and macronutrient phase separations as mechanisms of HPN bar hardening. Other HPN bar texture attributes that change during storage (e.g., crumbliness) have not been detailed in the literature and have not had their mechanism elucidated, but are likely related to those previously discussed for hardening.

### *2.3.6 Protein Ingredient Functionality for Better Performance in High-protein Nutrition Bars*

In addition to the protein ingredient utilized, formulation, processing, carbohydrate source, and environmental conditions potentially influence initial HPN bar texture as well as its change during storage (Figure 2-3). While many different protein ingredients have been tested in model HPN bars (Hogan and others 2012; Li and others 2008), the specific properties a protein ingredient should possess to impart softness, cohesiveness, and other desirable textural attributes remains unclear. Molecular profile and degree of hydrolysis, powder density, solubility, and water holding capacity are important protein properties to consider during HPN bar production. Cho (2010) identified protein powder solubility and degree of hydrolysis as primary factors that influence texture and stability. Bulk density had a secondary effect when these primary factors were held constant, and particle size influenced texture when these primary and secondary factors fell within a similar range (Cho 2010). A key finding was that higher protein ingredient solubility imparts hardness whereas lower solubility imparted crumbliness (Cho 2010). The effects of degree of hydrolysis, bulk density, and particle size on the texture of two different model HPN bars are provided in Table 2-9. Other protein ingredient parameters, such as particle density, may also be relevant to

performance, but these and those described by Cho (2010) are not discussed in most HPN bar reports.

**Table 2-9 Protein powder properties and their effect on high-protein nutrition (HPN) bar texture in two different formulations<sup>1</sup>**

Property <sup>3</sup>	HPN Bar Formulations <sup>2</sup>	
	30% protein (w/w) SPI and HFCS	35-50% protein (w/w) SPI/Dairy and low-carbohydrate
Solubility	Higher: hard, chewy Lower: hard, crumbly Optimum: $30 \leq \text{SSI} (\%) \leq 40$	Higher: soft, chewy, and sticky
DH (%)	Higher: hard, chewy, and sticky	Higher: soft, sticky, and bitter
Bulk Density	Higher: soft and easy to process	Higher: soft and easy to process
Particle Size	Larger: soft	Larger: soft

<sup>1</sup> Adapted from Cho (2010) with permission. Copyright© 2010 American Chemical Society.

<sup>2</sup> SPI, soy protein isolate. HFCS, high-fructose corn syrup.

<sup>3</sup> SSI, soluble solids index. DH, degree of hydrolysis.

## 2.4 Functionality of Milk Protein Concentrate with Emphasis on High-protein Nutrition Bars

### 2.4.1 Introduction

HPN bars prepared with high-protein MPCs or MCCs are powdery, crumbly, and hard, and these unfavorable attributes worsen as the product ages (Hogan and others 2012; Imtiaz and others 2012). While it is possible that poor performance is due to the elevated  $T_g$  of these proteins (Rao and others 2013a), this would not necessarily be the case if the proteins were not fully plasticized during HPN bar production. Although MPCs and MCCs are relatively new dairy protein ingredients, with respect to those derived from whey (Smithers 2008), their functional properties in solution are well characterized. It is challenging, if not impossible, to relate in-solution functionalities, especially those measured at dilute concentrations, to the behaviors and performance in solid-type, IMFs such as HPN bars. However, based on the work of Cho (2010), some relatability between HPN bar texture and the protein ingredient's functional properties

can be expected (Table 2-9). The key benefits of MPCs in HPN bars were identified as nutritional protein, water binding, foaming, and whipping (Agarwal and others 2015). It is not immediately clear how the latter three functionalities, especially foaming and whipping, present themselves in HPN bars. The following section briefly reviews the most current literature about MPCs' functional properties and discusses their textural importance in HPN bars.

#### *2.4.2 Primary Protein Structure*

Molecular weight and its influence on  $T_g$ , and amino acid composition become relevant if HPN bar texture change is in fact influenced by disulfide bond and Maillard-induced protein aggregations. Table 2-10 provides the amino acid composition and molecular weight of the major bovine milk proteins. Beta-lactoglobulin ( $\beta$ -lg) contains 1 (Cys121) free sulfhydryl group whereas  $\alpha_{s2}$ -casein,  $\kappa$ -casein, and alpha-lactalbumin ( $\alpha$ -la) contain 1, 1, and 4 disulfide bonds, respectively. Cys121 is more prone to disulfide bond formation and oxidation during HPN bar storage than cysteines in disulfide bond form, although those too are reactive through sulfhydryl-disulfide exchange. Maillard browning requires a free amine compound, such as lysine, in order to proceed. Each major bovine milk protein contains anywhere from 9 to 24 lysine residues. Disulfide bond formation may be less pronounced in HPN bars formulated with MPCs due to lower  $\beta$ -lg and free sulfhydryl concentration. Maillard browning is expected to proceed in MPC formulated HPN bars just as it does in those formulated with whey protein as it contains an adequate amount of lysine.

**Table 2-10 Major bovine milk protein<sup>1</sup> amino acid composition and molecular weight<sup>2</sup>**

AA	$\alpha_{s1}$ -CN	$\alpha_{s2}$ -CN	$\beta$ -CN	$\kappa$ -CN	$\beta$ -lg	$\alpha$ -la
Asp	7	4	4	4	11	9
Asn	8	14	5	7	5	12
Thr	5	15	9	14	8	7
Ser	8	6	11	12	7	7
SerP	8	11	5	1	0	0
Glu	24	25	18	12	16	8
Gln	15	15	21	14	9	5
Pro	17	10	35	20	8	2
Gly	9	2	5	2	3	6
Ala	9	8	5	15	14	3
½ Cys	0	2	0	2	5	8
Val	11	14	19	11	10	6
Met	5	4	6	2	4	1
His	11	11	10	13	10	8
Leu	17	13	22	8	22	13
Tyr	10	12	4	9	4	4
Phe	8	6	9	4	4	4
Trp	2	2	1	1	2	4
Lys	14	24	11	9	15	12
His	5	3	5	3	2	3
Arg	6	6	4	5	3	1
PyroGlu	0	0	0	1	0	0
Residues	199	207	209	169	162	123
MW (kDa)	23,612	25,228	23,980	19,005	18,362	14,174

<sup>1</sup> CN, casein.  $\beta$ -lg,  $\beta$ -lactoglobulin.  $\alpha$ -la,  $\alpha$ -lactalbumin.

<sup>2</sup> Amino acid (AA) and molecular weight (MW) values obtained from O'Mahony and Fox (2013).

### 2.4.3 Solubility

MPCs are criticized for their poor rehydration characteristics, which worsen with time stored under ideal (e.g., refrigerated, low relative humidity) and adverse (e.g., high temperature, high relative humidity) conditions. MPCs produced by different manufacturers will have different solubility and mineral profile due to production differences (Mao and others 2012; Sikand and others 2011). High-protein MPCs are more prone to solubility losses during storage; MPC90's solubility decreased to 50% soluble solids in fewer than 30 days while it took 120 days for MPC60 to see a similar

reduction (Gazi and Huppertz 2015). The development of insoluble material has been associated with casein micelle-micelle interactions during drying and powder storage which leads to the formation of a moisture impermeable skin that does not involve non-micellar caseins and whey proteins (Gazi and Huppertz 2015; Haque and others 2015; Havea 2006). MPCs possess good solubility immediately after manufacture, but it is the decrease during storage that limits their usage.

Attempts have been made to improve MPCs' rehydration characteristics. MPCs produced with sodium chloride or potassium chloride in the diafiltration water (150 mM) were always more soluble than the control after storage (Sikand and others 2016). Retentate concentration by nano-filtration instead of heat-based evaporation produced MPC60 with improved solubility and storage stability (Cao and others 2015a, 2015b). Retentate acidification during processing produced a MPC with lower solubility at its native pH (i.e., approaching casein's isoelectric point), but after neutralization protein solubility was greater than the non-acidified control (Luo and others 2016). Carbon dioxide acidified retentate produced a MPC powder that was less prone to decreasing solubility during storage (Marella and others 2015). Difficulties in rehydrating powder MPCs for liquid applications have spurred interest in highly concentrated liquid proteins (Lu and others 2015).

HPN bars formulated with high-protein MPCs are crumbly and lack cohesion (Banach and others 2014; Imtiaz and others 2012). This is due to low solvation of MPCs in the formulation and higher solubility is likely required to improve their ability to impart cohesion. SPI's optimal SSI range for balancing HPN bar firmness and cohesiveness was 30% to 40% (Cho 2010), but the optimal range for high-protein MPCs

has not yet been determined. Toasted MPC80 had lower solubility than the control and produced HPN bars that were the most susceptible to hardening (Banach and others 2014, 2013a). Protein insolubility may decrease chemical reactivity in HPN bars, but it will also decrease system cohesiveness. MPC solubility, and thus the powder's shelf life, need to be considered when formulating HPN bars.

#### *2.4.4 Water Holding Capacity and Water Sorption*

Water holding or hydration capacity (WHC) is the water mass that a powder can take up during specified hydration conditions and hold onto under defined centrifugal force. In most instances excess water is added to a protein powder, which is then agitated for hydration and centrifuged to expel un-held water. After decanting the supernatant, the amount of water occluded in the pellet, including the native powder moisture, is used to calculate WHC (water (g)/dry solids (g)). An improved method only utilizes enough water to saturate the material such that soluble solids are not lost during the decanting step (Quinn and Paton 1979). Water sorption isotherms are used to measure the equilibrium moisture content of a protein powder at specified relative humidity. Protein powder WHC and equilibrium moisture content may influence the extent of moisture migration in HPN bars. Higher WHC may cause water to be pulled to the protein powder during HPN bar production and storage, and may impart shorter, less cohesive, and moderately hard texture (Cho 2010). High-protein MPCs (i.e., MPC85, MPC90) sorbed less water over a full sorption cycle (0 to 90% RH) and had higher monolayer moisture binding, a necessary minimum for chemical reactivity, than their low protein counterparts (Kelly and others 2015). This indicated that high-protein MPCs are stable during storage, but also that they might not fully plasticize during HPN bar production and hence fail to

produce a cohesive product. Minimizing moisture migration within a HPN bar during storage will like increase its textural stability and shelf life.

#### *2.4.5 Heat Stability and Gelation*

Protein heat stability and gelation are likely not all that important for HPN bar textural stability. Soft-textured HPN bars are not typically baked due to their tendency to retain moisture, and thus no chemical, enzymatic, or temperature induced mechanism of gelation exists. Heat stability of MPC35 through MPC70 increased with protein content, however, it decreased with increasing protein content from MPC80 through MPC90 due to high calcium ion activity. Reconstituted high-protein MPCs had lower heat coagulation time than low-protein MPCs when prepared at fixed protein content and heat stability was further improved at alkaline pH (Crowley and others 2015a, 2015b). Gels prepared by acidifying protein solutions (7.5% protein w/w) with glucono-delta-lactone (GDL) were more porous and held less water when prepared from higher protein MPCs (Meletharayil and others 2015). However, a MPC80 solution (3.5% protein w/w) did not form a gel after  $\kappa$ -casein cleavage by rennet unless supplemented with 2 to 3 mM calcium chloride to restore serum calcium, after which it gelled similarly as raw milk and confirmed that micelle structure was maintained during MPC manufacture (Martin and others 2010).

#### *2.4.6 Surface Hydrophobicity*

Hydrophobic amino acid exposure allows proteins to interact with hydrophobic components, such as oil droplets in an emulsion (Damodaran 1997), and their exposure can also be used as way to measure protein unfolding or denaturation. Molecular probes, such as 8-anilino-1-naphthalene-sulphonic acid (ANS), are used measure surface

hydrophobicity by fluorescence when they bind hydrophobic protein patches (Haskard and Li-Chan 1998). However, ANS depends on electrostatic interaction to bind the protein's surface and modifications that increase negative charge (e.g., succinylation) may interfere with its ability to bind any newly exposed hydrophobic residues. Intrinsic tryptophan reflectance and red shifts in peak emission wavelength have also been used to indicate protein unfolding (Qi and Onwulata 2011). Both ANS measurement and tryptophan reflectance requires that the protein be in solution at relatively dilute concentrations since both require transparency and a response measurable by a fluorimeter. This poses a problem for native MPCs since they are difficult to rehydrate at native pH and although soluble at pH extremes, this might cause unrelated unfolding. Modified MPCs may be even more insoluble and only the most hydrophilic fraction would persist in solution (Banach and others 2013a). Denatured proteins may function as inert structural elements in HPN bars and those with greater hydrophobic exposure may help prevent internal moisture migration.

#### *2.4.7 Powder Properties*

Food powder properties are influenced by the general properties of the material as well as processing and drying conditions (Schuck and others 2012). General properties include overall composition (e.g., protein, carbohydrate, fat, minerals),  $a_w$ ,  $T_g$ , microbiological properties, and organoleptic properties (Schuck and others 2012). The production process influences many bulk properties including, but not limited to, loose, tapped, and true densities, occluded and interstitial air volumes, rehydratability, wettability, caking, flowability, floodability, friability, color, and hygroscopicity (Schuck and others 2012; Murrieta-Pazos and others 2012). Particle size influences many of these



properties, and while its distribution is measured in bulk, it begins to decipher differences in the individual particles that make up the powder. Particles possess different shapes (e.g., circularity, elongation, convexity) and surface features (e.g., microstructure, porosity). Particle surfaces are characterized physically (e.g., atomic force microscopy), microstructurally (e.g., scanning electron microscopy), molecularly (e.g., dynamic vapor sorption, inverse gas chromatography), and atomically (e.g., x-ray photoelectron spectroscopy). The functionality of a powder depends on these surface attributes (Murrieta-Pazos and others 2012); especially when particle structure is maintained in MPC formulated HPN bars.

MPC particle size varies with manufacturer and while measurements are made to track powder dissolution (Fyfe and others 2011), particle size distributions (PSDs) of the powder itself are less commonly reported. Particle diameters, including  $D_{10}$ ,  $D_{50}$ ,  $D_{90}$ , and  $D_{4,3}$ , increased with protein content of MPC35 through MPC60, decreased slightly for MPC70, and those of MPC80, MPC85, and MPC90 were less than those reported for MPC35 ( Kelly and others 2015; Crowley and others 2014). MPC bulk, tapped, and particle densities decreased with increasing protein content whereas interstitial and occluded air increased (Crowley and others 2014). These powder properties are dependent on the solids content and viscosity at atomization; high viscosity leads to poor atomization, less water removal, and inhibition of droplet shrinkage (Crowley and others 2014). MPC80 and above are diafiltered during production and are not subjected to further concentration prior to spray drying. Thus smaller high-protein MPC droplets are atomized, which allows for more water removal, particle shrinkage, and air entrapment during spray drying (Kelly and others 2015; Crowley and others 2014).

Particle parameters are not typically reported in HPN bar studies, especially when formulated with whey proteins. Smaller MPC particles or powders with bimodal PSDs may offer improved textural performance in HPN bars by increasing the volume fraction at solidity by allowing smaller particles to position themselves between larger ones (Huppertz and Hogan 2015). Smaller MPC particles have increased adhesion for each other, and with increased surface area they will hydrate better than larger particles during HPN bar production. If particle structure is maintained during HPN bar production, the smaller particles will adhere better to each other and will improve HPN bar cohesiveness. If hydration sufficiently causes particle collapse, then the system becomes adequately plasticized in the rubbery state and the resultant HPN bar will have decreased crumbliness. Particle collapse or prior size reduction would decrease occluded air and increase the denseness of HPN bars prepared with high-protein MPCs.

High-protein MPC particles are not likely to collapse during HPN bar manufacture and thus their surface properties are relevant. X-ray photoelectron spectroscopy (XPS) showed that protein and fat orientate themselves at the particle surface whereas lactose was progressively located in bulk as MPC protein content increased (Kelly and others 2015). Care must be taken when analyzing XPS results since components measured on the particle surface are often overestimated and not correlated with the components measured in bulk (Murrieta-Pazos and others 2012). Surface fat would likely contribute to increased particle surface hydrophobicity, which would inhibit rehydration during HPN bar production. XPS and AFM analysis showed that MPC80 powder particle surface hydrophobicity increased as it aged (Fyfe and others 2011). High-protein MPCs were more hydrophobic than low-protein MPCs as determined by the

sessile drop technique (Alghunaim and others 2016; Crowley and others 2015b). Higher hydrophobicity may prevent moisture migration to the protein, but MPC's inability to hydrate rapidly during HPN bar production may be detrimental to its texture and stability.

## **2.5 Modification of Milk Protein Concentrate for Enhanced Performance in High-protein Nutrition Bars**

### *2.5.1 Introduction*

It is well known that protein ingredient functionality can be modified using chemical, enzymatic, and physical methods to improve their performance in specific food applications (Phillips and others 1994). Each will be elaborated on in the following sections with pertinence to HPN bar applications. However, very few protein modifications are carried out for improved performance in high-protein, solid IMFs such as HPN bars. Thus, a majority of the discussion provided is hypothetical in nature and based off what is known about initial HPN bar texture and its stability during storage. Despite being complex, multi-component systems, the protein source (i.e., soy vs. whey) used in a HPN bar influences the texture of the final product (Childs and others 2007). Another study showed that MPC modification, although proprietary, can be used to alter the resultant firmness and cohesiveness of HPN bars formulated with blended dairy proteins (Imtiaz and others 2012). Protein ingredient modification for use in HPN bars is understudied, and modifications that can be used to soften and improve cohesion as well as improve textural stability are not inherently clear.

### *2.5.2 Chemical Modification*

Well-known protein chemical modification techniques, such as alkylation, acylation, phosphorylation, amidation, esterification, glycosylation, sulfitolysis, cysteinylolation, and glutathiolation can be used to alter protein structure and resultant

physicochemical properties (Damodaran 2008). Care must be utilized during chemical modifications not only because the consumer becomes wary of anything labeled as chemical, but also because there is concern that some of the reactants required to carry out the modification are toxic. None of these chemical modifications have been used directly to improve protein performance in HPN bars. However, many of these modify structure by blockage of reactive amino acid side chains, such as lysine and cysteine, which have been previously implicated in HPN bar hardening. Enzymatic hydrolysis and Maillard-induced protein glycation are the two main ways food proteins are modified and they are each discussed in their own sections.

### *2.5.3 Enzymatic Hydrolysis and Crosslinking*

Enzymes are the most common way to hydrolyze proteins for use in food since acid and alkaline hydrolysis are less specific and degrade its nutritional quality. Other than our work (Banach and others 2014, 2013a), and the proprietary modifications conducted by Imtiaz and others (2012), hydrolysis has been the only proposed protein modification technique to improve the texture and stability of HPN bars. The function of protein hydrolysates in HPN bars has been previously discussed and is well characterized (Rao and others 2013a). Casein hydrolysates blended into WPI at 10% (w/w) produced a softer dough system that hardened slower during storage (Rao and others 2016). Model HPN bars formulated with a higher weight percent of a hydrolyzed WPI remained softer and exhibited greater microstructural stability under accelerated storage conditions (McMahon and others 2009).

MPC80 was enzymatically hydrolyzed (Banach and others 2013b) and while it would likely improve its performance in HPN bars, such evaluation was not conducted.

Enzymatically hydrolyzing MPC retentate, the liquid protein concentrate produced during ultrafiltration, to produce a hydrolyzed MPC powder after spray drying is feasible. One processing consideration would be whether the hydrolysis conditions are suitable for both casein and whey proteins in highly concentrated solutions. For example, enzyme activity might be favored at a pH that causes casein precipitation or  $\kappa$ -casein hydrolysis from the micelle, which may lead to protein aggregation and difficulties with downstream processing. Another unique and often highlighted aspect of MPCs is that their casein micelle structure is maintained during processing and since hydrolysis would disrupt this structure, evaluation would be required to see if there was a functional or economic benefit of hydrolyzing MPC retentate versus utilizing an alternative protein.

After separating the casein/whey and casein proteins from skim milk in the form of MPC and MCC retentates, respectively, Salunke (2013) used transglutaminase (Tgase) to enzymatically crosslink the caseins prior to spray drying. These crosslinked protein ingredients were evaluated in a number of different processed cheese and yogurt applications (Salunke and others 2013a, 2013b, 2013c, 2012a, 2012b, 2012c). Tgase crosslinked proteins are known to add structure and body to solid food systems and were evaluated in HPN bars as part of the following study. Since they have increased net molecular weight and hence lower molecular mobility, these protein ingredients might produce HPN bars that are less prone to chemical changes such as Maillard-induced protein aggregations and may also improve textural stability during storage.

#### *2.5.4 Dry Heat Toasting and Glycation*

Heating protein powders in the dry-state can cause partial protein denaturation and aggregate formation, and this alters its surface dependent functional properties.

Heating WPI and egg white protein in the dry state improved foam stability and maintained foamability (Nicorescu and others 2011). Glycation may occur during heating since these proteins contain residual sugars such as lactose and glucose, respectively. Co-dissolving protein powders with a carbohydrate component, such as dextran or maltodextrin, followed by drying and heating to initiate glycation has improved protein heat stability, solubility, and emulsion stability (O'Regan and Mulvihill 2013; Wang and Ismail 2012). MPC80 was previously dry heat treated at either 75°C or 110°C for 4 h to alter its functional properties (Banach and others 2013a). When evaluated in a model HPN bar, the MPC toasted at 75°C did not differ texturally from the control whereas the one formulated with MPC80 toasted at 110°C performed much worse (Banach and others 2014). Based on these results, dry heat toasting without adding any other constituents would not be good option for improving the performance of MPC80 in HPN bars. Protein-maltodextrin conjugation may improve textural performance by enhancing protein powder ability to interact with the other constituents and thus minimize macronutrient phase separations within the system.

#### *2.5.5 Mineral Substitution and Reduction*

Recent MPC modification has focused on alteration of their mineral profile during production. Divalent calcium ions can be replaced by diafiltering with sodium chloride or potassium chloride (150 mM) (Sikand and others 2013; Mao and others 2012), or by cation exchange to improve rehydration (Bhaskar and others 2007; Dybing and others 2007). MPC retentate acidification by carbon dioxide injection allowed for the dissociation of calcium and phosphate from the micelle and thus the spray dried MPC contained less ash and calcium, and had lower net negative charge (Marella and others

2015). Calcium reduction also improved solubility (Marella and others 2015), and while protein solubility is not a prerequisite for performance in HPN bars, mineral content and surface charge can influence internal moisture migration. Preventing moisture migration during storage may improve the storage stability of the HPN bars. The textural performance of a reduced-calcium MPC was evaluated in a model HPN bar as part of the following study.

#### *2.5.6 Extrusion*

Extrusion is simply forcing a pumpable product through a die. Pumping is accomplished by pistons, rollers, or screws, with the latter being most common in food processing. Single or twin screws rotating in an enclosed circular or figure-eight shaped barrel convey material from the infeed through a die. As material moves, it is subjected to shear force, frictional and applied heat, and high-pressure at the die-end. A myriad of feed (e.g., composition, moisture, particle size), equipment (e.g., twin or single screw, screw profile, length to diameter ratio), and processing (e.g., material feed rate, screw speed, barrel temperature) conditions exist and this makes extrusion a versatile and economic food processing unit operation. The food industry uses extrusion to manufacture pastas, ready-to-eat cereals, puffed snacks, pet foods, candies, and meat analogs (Heldman and Hartel, 1997).

Proteins are also commonly extruded to produce crisps (Tremaine and Schoenfuss 2012) and meat analogs (Lin and others 2002). The frictional heat, shearing forces, and high pressures exerted on protein during extrusion cause protein denaturation, which brings out new functionality. Melt temperature, a function of both applied and frictional heat, has a profound effect on denaturation and it works with shear to alter a protein's

native structure (Qi and Onwulata 2011). Notable protein changes during extrusion are surface exposure of once buried hydrophobic residues, the formation of covalent bonds (e.g., disulfide), and increased non-covalent interactions (e.g., hydrogen bonds, hydrophobic interactions), all of which ultimately reduce protein solubility. Many commonly reported functional properties (e.g., gelation, emulsification, water holding capacity) are highly dependent on solubility and thus extrusion processing has a negative effect if these properties are desired. MPC (Banach and others 2013a), pea protein isolate (Osen and others 2015), WPC (Nor Afizah and Rizvi 2014), and SPI (Fang and others 2014) each has had its functionality modified with extrusion processing.

Starchy materials are easily extruded to produce puffed snacks with low nutritional quality. Adding native protein to the mix for improved nutritional quality often limits extrudate expansion and hinders textural appeal. Most literature focuses on protein-starch interaction during extrusion and its effect on the resultant functional properties without any regard for potential application other than the obvious puffed snacks (Zhang and others 2016). Onwulata (2010) included pre-extruded WPI in puffed corn meal that resulted in higher expansion index and crispness than the one extruded with native WPI. Extruded and coarsely milled MPC80 produced softer, more stable HPN bars (30% protein w/w) than the control (Banach and others 2014) and these textural differences were discussed in terms MPC80's extrusion-modified functionality, such as reduced solubility, WHC, and in-solution surface hydrophobicity (Banach and others 2013a). Cho (2010) formulated HPN bars with finely ( $\sim 100\% < 150 \mu\text{m}$ ) and coarsely ( $\sim 80\% < 150 \mu\text{m}$ ) milled soy protein crisps, which were essentially milled extrudates, but textural comparisons were made only to analyze the effect of particle size



for which the larger protein particles produced a softer, more stable product.

Microstructural changes including protein aggregations, free sulfhydryl change, free amine change, and macronutrient separations are looked at in HPN bars formulated with extruded MPC80 in the following study.

## 2.6 Conclusions

MPCs are a relatively new protein powder ingredient with respect to WPC and NFDM. The functional properties of MPCs are well characterized in solution, but it is still unclear why they produce HPN bars that harden rapidly and lose cohesiveness during storage. While potentially due to some of the previously suggested mechanisms of HPN bar texture change, it may also be due to the unique properties that MPC powders display and such properties are not commonly reported for proteins to be processed into model HPN bars. The performance of MPCs in HPN bars can be improved by physical modification, such as extrusion and particle size reduction, and these are the main subjects of the following study.

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### CHAPTER 3. MICROSTRUCTURAL CHANGES IN HIGH-PROTEIN NUTRITION BARS FORMULATED WITH EXTRUDED OR TOASTED MILK PROTEIN CONCENTRATE

Modified from a paper published in the *Journal of Food Science*<sup>1</sup>

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#### 3.1 Abstract

Milk protein concentrates with more than 80% protein (i.e., MPC80) are underutilized as the primary protein source in high-protein nutrition bars as they impart crumbliness and cause hardening during storage. High-protein nutrition bar texture changes are often associated with internal protein aggregations and macronutrient phase separation. These changes were investigated in model high-protein nutrition bars formulated with MPC80 and physically modified MPC80s. High-protein nutrition bars formulated with extruded MPC80s hardened slower than those formulated with toasted or unmodified MPC80. Extruded MPC80 had reduced free sulfhydryl group exposure, whereas measurable increases were seen in the toasted MPC80. High-protein nutrition bar textural performance may be related to the number of exposed free sulfhydryl groups in MPC80. Protein aggregations resulting from ingredient modification and high-protein nutrition bar storage were studied with sodium dodecyl sulfate polyacrylamide gel electrophoresis. Disulfide-based protein aggregations and changes in free sulfhydryl concentration were not consistently relatable to high-protein nutrition bar texture change. However, the high-protein nutrition bars formulated with extruded MPC80 were less

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prone to phase separations, as depicted by confocal laser scanning microscopy, and underwent less texture change during storage than those formulated with toasted or unmodified MPC80.

### **3.2 Practical Application**

High-protein nutrition bars formulated with extruded MPC80 underwent fewer microstructural changes during storage. Disulfide crosslink formation and free sulfhydryl content changes were not always indicative of texture changes in high-protein nutrition bars. Texture change in high-protein nutrition bars formulated with MPC80 was, thus, only partly due to these aggregations. Pre-extruded MPC80 may produce high-protein nutrition bars with an extended textural shelf life compared to those produced with unmodified MPC80.

### **3.3 Introduction**

Powder milk protein concentrates (MPCs), particularly those with more than 80 g protein per 100 g product (i.e., MPC80), possess poor rehydration and solubility characteristics that worsen during storage (Havea 2006; Anema and others 2006; Haque and others 2010). High-protein nutrition (HPN) bars, which contain 20-50% protein (w/w), are intermediate moisture systems that do not require complete protein solubility and are a potential application for MPCs (Cho 2010). However, when utilized in HPN bars, MPCs present challenges in balancing cohesiveness (e.g., too crumbly), firmness (e.g., too hard), and texture change over the product's shelf life (Baldwin and Pearce 2005; Imtiaz and others 2012; Li and others 2008; Loveday and others 2009). Texture change of HPN bars during storage is likely due to a combination of different phenomena, for example, moisture migration between constituents, macronutrient phase

separations, and disulfide bond- and Maillard-induced protein aggregations (Zhou and others 2008a; Loveday and others 2009; McMahon and others 2009; Zhou and others 2013).

In addition to protein, HPN bars are comprised of 10-50 g carbohydrate and 10-15 g fat per 100 g (Zhu and Labuza 2010). Free water is minimized and water activity is kept less than 0.65 to ensure microbial shelf stability (Loveday and others 2009). While other ingredients (e.g., sugar alcohols) and other factors (e.g., storage conditions) can influence HPN bar texture, protein source (e.g., dairy, soy) and type (e.g., concentrate, hydrolysate, crisp) have direct impact (Childs and others 2007; McMahon and others 2009; Imtiaz and others 2012). The physicochemical properties of MPC can be tailored for HPN bars using physical, chemical, or enzymatic modifications (Imtiaz and others 2012). The texture of HPN bars formulated at 30% protein (w/w) with physically modified MPC80 was evaluated over 42 days storage at 22°C, 32°C, and 42°C (Banach and others 2014). HPN bars produced with extruded MPC80 hardened slower than those made with toasted or unmodified MPC80. MPC80 toasted at 75°C or 110°C for 4 h produced HPN bars that had minimal texture change or increased fracture force, respectively, when compared to those formulated with control MPC80. Extruded MPC80s had reduced protein solubility and, based on the rate of free amine reduction during HPN bar storage, were less chemically reactive (Banach and others 2014, 2013).

Free amine reduction was one chemical change that occurred during storage of HPN bars, but it insufficiently explains texture change (Rao and others 2013; McMahon and others 2009; Baier and others 2007; Banach and others 2014). Protein aggregations, including those from disulfide crosslink formations and Maillard reactions, during

storage have also been implicated in texture change (Zhou and others 2013; Zhou and others 2008a, 2008b). N-ethylmaleimide prevented disulfide bond formation and extended textural shelf life of a model intermediate moisture food (IMF) 6-times the control (Zhu and Labuza 2010). Free sulfhydryl interactions were texturally relevant in the same IMF, as molecular cysteine slowed or accelerated hardening when added at low or high levels, respectively (Zhu and Labuza 2010). The objective of the present study was to determine the effect extrusion and toasting had on the free sulfhydryl content of MPC80 and to verify the occurrence of disulfide crosslinking within HPN bars formulated with those modified protein ingredients. Additionally, confocal laser scanning microscopy (CLSM) was used to study macronutrient phase separations in these HPN bars. Instrumental texture properties were presented in detail elsewhere (Banach and others 2014); however, they are related to the microstructural changes presented in this study.

### **3.4 Materials and Methods**

#### *3.4.1 Materials and Reagents*

MPC80 (79.9% protein, 4.6% moisture) was purchased from Idaho Milk Products (Jerome, ID). Glycerol, boric acid, sodium chloride, ethylenediaminetetraacetic acid (EDTA), urea, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Pierce™ BCA protein assay, and Nile red (MP Biomedicals, LLC) were purchased from Fisher Scientific (Waltham, MA). L-cysteine hydrochloride monohydrate, sodium tetraborate decahydrate, and fluorescein isothiocyanate (FITC) isomer 1 were purchased from Sigma-Aldrich (St. Louis, MO). The reducing agent compatible bicinchoninic acid (BCA) protein assay was purchased from G-Biosciences® (St. Louis, MO). The 2x Laemmli Sample Buffer,



precast 4-20% gradient Mini-Protean® TGX™ gels, Bio-Safe™ Coomassie Stain, and Precision Plus Protein™ Standards were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

### *3.4.2 Milk Protein Concentrate Modification and High-protein Nutrition Bar Production*

MPC80 was modified with extrusion or dry-heat toasting. MPC80 moisture content was adjusted to 38% and extruded at die-temperature of 65°C or 120°C using a low-shear screw profile. The extrudate was dried, milled, and sieved through a 250 µm mesh, as detailed elsewhere (Banach and others 2014, 2013). For dry-heat toasting, MPC80 was put in a laboratory oven at 75°C or 110°C for 4 h and passed through the same screen. These modified proteins are referred to as E65 (78.4% protein, 7.3% moisture), E120 (79.5% protein, 5.8% moisture), T75 (80.6% protein, 4.1% moisture), and T110 (81.7% protein, 3.0% moisture), respectively.

HPN bars, with protein and moisture content indicated, were prepared by Banach and others (2014) using control MPC80 (31.4% protein, 14.4% moisture), E65 (31.7% protein, 14.2% moisture), E120 (31.6% protein, 13.6% moisture), T75 (31.6% protein, 13.4% moisture), and T110 (31.5% protein, 13.5% moisture). After 0, 6, 13, 22, or 42 days storage at 32°C, the HPN bars were frozen in liquid nitrogen, ground with a laboratory blender, and kept at -80°C until free sulfhydryl measurement and SDS-PAGE in the present study.

### *3.4.3 Free Sulfhydryl Measurement*

The free sulfhydryl content of each protein ingredient and HPN bar was determined by Ellman's assay with modifications (Beveridge and others 1974). Free sulfhydryl extraction buffer (pH 8.5) contained 8 mol urea plus 4.1 mmol EDTA per L

and was prepared in borate buffer (100 mmol boric acid, 75 mmol sodium chloride, and 25 mmol sodium tetraborate decahydrate per L). Protein ingredients (0.75 g) were mixed with degassed extraction buffer (11.25 g) for 2 h in 15-mL centrifuge tubes. HPN bars (2.04 g) and degassed extraction buffer (9.96 g) were mixed in 25-mL Erlenmeyer flasks for the same time. For the HPN bars prepared with T110, 2.55 g was mixed with 12.45 g extraction buffer. Protein ingredient and HPN bar dispersions were centrifuged for 15 min at 12,000 g and 15,000 g, respectively.

Sample supernatants (0.5 mL) or cysteine standards (0.5 mL) were vortexed with 50  $\mu$ L of 10 mmol DTNB/L and 2.5 mL extraction buffer, which was held at room temperature for 15 min and absorbance read at 412 nm. Sample and standard blanks were prepared by substituting DTNB with extraction buffer. Standard net absorbance was plotted against seven free sulfhydryl concentrations (25 to 493  $\mu$ mole/L) and was fitted with a linear ( $R^2 \geq 0.995$ ) curve (not shown) used to determine sample free sulfhydryl concentration. These values were divided by the BCA assay determined soluble protein (g/L) and free sulfhydryl content was reported as  $\mu$ mole per g protein.

#### *3.4.4 Non-reduced and Reduced SDS-PAGE*

Sample supernatants from the free sulfhydryl assay (above) were used for non-reduced SDS-PAGE. Reduced extraction followed the same procedures except the extraction buffer contained 50 mL  $\beta$ -mercaptoethanol/L. Soluble protein was diluted to 4 mg/mL and was verified using the appropriate BCA assay. Non-reduced dilutions contained 3.7-4.4 mg protein/mL whereas the reduced dilutions contained 3.8-5.6 mg protein/mL. The non-reduced samples were diluted 1:2 with both reducing and non-reducing 2x Laemmli Sample Buffer. The reduced samples were only diluted 1:2 with

reducing 2x Laemmli Sample Buffer. The protein standard and samples were loaded onto the gel at equal volume (10  $\mu$ L) and were electrophoresed at 150 V for 50 min using standard SDS-PAGE running buffer (250 mmol tris, 1.92 mol glycine, and 10 g SDS per L). The gels were fixed in methanol/acetic acid/Millipore water (40/10/50) for 30 min, stained for 1 h, and de-stained with Millipore water.

#### *3.4.5 Confocal Laser Scanning Microscopy of the High-protein Nutrition Bars*

CLSM methodologies were adapted from literature to detect possible macronutrient phase separations within the HPN bars during storage (McMahon and others 2009). A separate 50 g batch of each HPN bar was prepared with the same lot of ingredients. In addition to the protein ingredients described above, each model contained 21.5 g glycerol (99.8% glycerol, 0.1% water), 18.4 g palm kernel stearin, 12.0 g maltitol syrup (Lycasin®80/55, 51.7% D-maltitol, 3.0% D-sorbitol, 24.5% water, Roquette America, Keokuk, IA), 10.0 g high-fructose corn syrup (CornSweet®55, 55% fructose, 41% dextrose, 4% higher saccharides, 23% water, Archer Daniels Midland, Decatur, IL), and distilled water to standardize protein ingredient moisture content per 100 g. A mechanical stand mixer was used to combine the ingredients, according to Banach and others (2014), and a small portion was leveled into a press-to-seal silicone isolator (13 mm diam.  $\times$  2 mm depth, Grace™ Bio-Labs, Bend, OR) mounted on a glass microscope slide. One drop of FITC-acetone solution (0.2 g FITC/kg) and one drop of Nile red-acetone solution (0.2 g Nile red/kg) were applied to the HPN bar surface with a glass Pasteur pipette. A glass coverslip was placed over the sample and, along with the base of the push-to-seal isolator, was sealed into place with silicone. The freshly prepared slides

were kept at room temperature (~22°C) overnight and day 0 images were acquired the following day.

CLSM micrographs were acquired with a SP5 X MPC confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL) using the 10x objective lens with 2x digital zoom. Three representative images (775  $\mu\text{m} \times 775 \mu\text{m}$ , 1024 px  $\times$  1024 px) of each HPN bar were acquired using filters to capture FITC (i.e., protein) and Nile red (i.e., lipid) fluorescence. The fluorescence signals were auto-contrasted and overlaid in Leica LAS AF Lite software. The same slides were imaged after 6, 22, and 42 days at 32°C after equilibrating to room temperature.

#### *3.4.6 Statistical Analyses*

A mixed linear model was used to discern free sulfhydryl content differences between the protein ingredients. Independent variables were protein ingredient and ingredient preparation, and their interaction was the random term. HPN bar free sulfhydryl content was also modeled using the mixed linear method. The independent variables were protein ingredient, storage time, and their interaction. Protein ingredient and storage time slicing factors were applied separately to analyze changes within each HPN bar throughout storage and between HPN bars at fixed time, respectively. In each model, Satterthwaite's method was used to compute denominator degrees of freedom and means were compared using Tukey's adjusted p-value. All statistical analyses were performed with SAS® software (version 9.3, SAS Institute Inc., Cary, NC).

### 3.5 Results and Discussion

#### 3.5.1 Free Sulphydryl Content of Modified MPC80 Ingredients

We have hypothesized that the textural performance of MPC80 protein ingredients in HPN bars is related to their initial free sulphydryl content. Protein modifications that increase free sulphydryl concentration or increase exposure by way of protein unfolding could accelerate disulfide bond formation during HPN bar storage. Free sulphydryl content of the protein ingredients and their corresponding HPN bars after storage at 32°C is shown in Table 3-1. Control MPC80 in the present study had 4 µmole free sulphydryl per g soluble protein. Mao and others (2012) reported that MPC80 had approximately 9.5 µmole free sulphydryl per g soluble protein, while MPC with 62% protein (w/w) had 4.8 µmole free sulphydryl per g soluble protein (Cao and others 2015). While on the same order of magnitude, free sulphydryl differences can be attributed to production scale, storage time and conditions, and modifications made to Ellman's assay.

**Table 3-1 Free sulphydryl (SH) content (µmole/g protein ± SD) of the protein ingredients and high-protein nutrition (HPN) bars after storage at 32°C**

Protein <sup>1</sup>	Ingredient SH	HPN Bar SH after Storage				
		day 0	day 6	day 13	day 22	day 42
MPC80	4.0±0.3 <sup>bc</sup>	5.3±1.3 <sup>a,z</sup>	5.3±1.4 <sup>ab,z</sup>	5.4±1.2 <sup>a,z</sup>	4.9±0.9 <sup>a,z</sup>	5.0±1.8 <sup>b,z</sup>
T75	4.5±0.1 <sup>bc</sup>	5.3±0.9 <sup>a,z</sup>	5.5±1.4 <sup>a,z</sup>	5.2±0.9 <sup>a,z</sup>	4.7±1.0 <sup>a,z</sup>	4.5±0.7 <sup>b,z</sup>
T110	5.6±0.7 <sup>c</sup>	4.0±0.9 <sup>a,y</sup>	5.5±0.8 <sup>a,yz</sup>	5.6±0.9 <sup>a,yz</sup>	6.0±0.9 <sup>a,yz</sup>	7.1±1.2 <sup>a,z</sup>
E65	3.0±0.2 <sup>b</sup>	3.7±0.8 <sup>a,z</sup>	3.4±0.9 <sup>b,yz</sup>	1.5±0.3 <sup>b,y</sup>	1.7±1.3 <sup>b,y</sup>	1.8±0.7 <sup>c,yz</sup>
E120	0.7±0.3 <sup>a</sup>	0.6±0.7 <sup>b,z</sup>	0.7±0.4 <sup>c,z</sup>	0.6±0.5 <sup>b,z</sup>	0.5±0.7 <sup>b,z</sup>	0.2±0.5 <sup>c,z</sup>

<sup>1</sup> MPC80, unmodified MPC80. T75 and T110, MPC80 toasted for 4 h at 75°C and 110°C, respectively.

E65 and E120, MPC80 extruded at die temperatures of 65°C and 120°C, respectively.

<sup>a-c</sup> Means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>y,z</sup> Means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

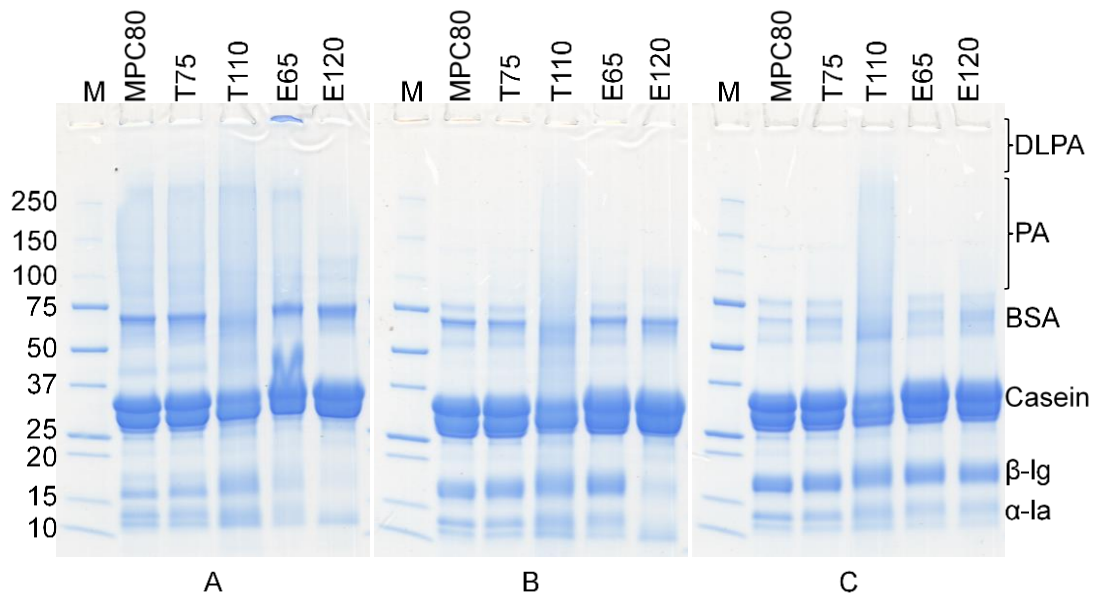
Extrusion reduced the free sulphydryl content of MPC80 by imparting both heat and shear force (Table 3-1); E65 and E120 had 3.0 and 0.7 µmole per g soluble protein, respectively. Higher extrusion temperatures reportedly caused greater free sulphydryl

loss in texturized whey protein concentrate (WPC) and whey protein isolate (WPI) (Qi and Onwulata 2011a; Qi and Onwulata 2011b; Manoi and Rizvi 2009; Nor Afizah and Rizvi 2014). The die-end melt temperature of E120 was greater than that of E65 and it was this temperature difference that significantly reduced E120's free sulfhydryl content ( $P < 0.05$ ).

T75 and T110 had 4.5 and 5.6  $\mu$ mole free sulfhydryl per g soluble protein, respectively (Table 3-1). Dry heating beta-lactoglobulin ( $\beta$ -lg) and WPI caused partial protein unfolding and increased free sulfhydryl accessibility to DTNB in the absence of SDS (Gulzar and others 2011a, 2011b). When the assay buffer included SDS, which increased DTNB access to the protein's buried free sulfhydryl groups via denaturation, the measured free sulfhydryl content of the same proteins decreased, which was the result of disulfide bond formation and free sulfhydryl oxidation (Gulzar and others 2011a, 2011b). Although urea denatures proteins differently than SDS, it should have sufficiently solubilized and unmasked the buried free sulfhydryl groups found within the toasted MPC80. Increased free sulfhydryl content in the toasted MPC80 did not align with previous results (Gulzar and others 2011a, 2011b). Sulfhydryl-disulfide and free sulfhydryl oxidations occurred minimally in toasted MPC80s since free sulfhydryl content increased in the presence of urea and greater exposure occurred at the higher toasting temperature. Reduced free sulfhydryl content, as was the case with extruded MPC80, produced softer and more texturally stable HPN bars than those formulated with T75 and T110, which had relatively unaltered and increased free sulfhydryl content, respectively (Banach and others 2014).

### 3.5.2 SDS-PAGE Protein Profiles of the Modified MPC80 Ingredients

SDS-PAGE protein profiles of toasted, extruded, and unmodified MPC80 were used to explain their measured free sulfhydryl content (Figure 3-1). The protein ingredients were solubilized in either non-reducing (Figure 3-1A, B) or reducing (Figure 3-1C) extraction buffer, without (3-1A) or with  $\beta$ -mercaptoethanol (3-1B and 3-1C) added to the SDS-PAGE sample buffer. The profiles of T75 matched those found in unmodified MPC80 under the same set of running conditions. Therefore, the fact that these two protein ingredients had statistically equivalent free sulfhydryl content (Table 3-1) and that they produced HPN bars with similar textural properties was not surprising (Banach and others 2014). More noticeable differences were visualized for T110, E65, and E120, and are discussed below.



**Figure 3-1 Non-reduced (A) and reduced (B, C) SDS-PAGE protein profiles for MPC80, T75, T110, E65, and E120 extracted with non-reducing (A, B) or reducing (C) buffer.** MPC80, unmodified MPC80. T75 and T110, MPC80 toasted 4 h at 75°C and 110°C, respectively. E65 and E120, MPC80 extruded at die temperature of 65°C and 120°C, respectively. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include:  $\alpha_{S2}$ ,  $\alpha_{S1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -Ig, beta-lactoglobulin.  $\alpha$ -Ia, alpha-lactalbumin.

Measured free sulfhydryl interpretation was the primary purpose for SDS-PAGE comparison and hence discussion will focus on the free sulfhydryl-containing proteins in MPC, including bovine serum albumin (BSA) (Cys34) and  $\beta$ -lg (Cys121), which have the potential to form disulfide bonds during HPN bar storage. Protein disulfide bond formations can be visualized on SDS-PAGE gels by disappearance or reappearance of bands when a reducing agent is excluded or included (Onwulata and others 2010). BSA (66 kDa) remained soluble in each modified MPC80 and, with the exception of T110, its appearance remained the same with fixed SDS-PAGE conditions. BSA contains 17 disulfide bonds and so partial reduction, as indicated by fading band intensity, occurred on the gels that included  $\beta$ -mercaptoethanol (Figure 3-1B, C). Disulfide bond formation involving BSA as a participant in T110 was unlikely, as solubility was not regained with reduced extraction (Figure 3-1C).

Under non-reduced conditions, the soluble  $\beta$ -lg in E65 was limited and it was almost nonexistent in E120 when compared with MPC80 (Figure 3-1A). Extrusion of MPC80 at a die temperature of 120°C made  $\beta$ -lg insoluble, which corroborates its low, yet detectable, free sulfhydryl content (Table 3-1). Soluble disulfide linked protein aggregates (DLPA) too large to enter the gel were noted in E65, but were absent in E120 (Figure 3-1A).  $\beta$ -mercaptoethanol reduced the DLPA found in E65 and helped identify the participating proteins (Figure 3-1B).  $\beta$ -lg band intensity in E65 was regained, resembling that found in MPC80, and confirmed its involvement in the DLPA that resulted from extrusion at 65°C (Figure 3-1B). DLPA are also found in the region labeled simply as protein aggregates (PA) for E65 and E120 as protein band smearing occurred vertically in these lanes (Figure 3-1A) and clarity was regained with reducing



agent addition (Figure 3-1B, C). Intensity in the region labeled PA was greater in E65 than in E120. However, the figure was labeled with PA versus DLPA, as some aggregates remain in this region for some of the proteins (i.e., T110) after reduction. The  $\beta$ -lg band was still absent in E120 after reducing agent addition to the SDS-PAGE sample buffer, thus, did not participate as heavily in the formation of soluble DLPA (Figure 3-1B).

The casein proteins, including the  $\alpha_{s2}$ ,  $\alpha_{s1}$ ,  $\beta$ , and  $\kappa$  units, found between 37 kDa and 25 kDa, were altered more by toasting at 110°C than the other treatments. Casein in T110 was less soluble, as indicated by reduced band intensity, than in MPC80 under the same conditions. The casein proteins do not contain any free sulfhydryl groups, but as solubility decreased under strictly non-reduced conditions, the  $\beta$ -lg in T110 became more concentrated when compared with the visual band intensity of  $\beta$ -lg in MPC80 (Figure 3-1A). PA in T110 remained after reduction (Figure 3-1B, C), which suggested resultant aggregation involved Maillard-type aggregations that involved the casein proteins more than the whey proteins. Although T110's free sulfhydryl content was not significantly greater than MPC80's (Table 3-1), its elevated magnitude likely resulted from increased  $\beta$ -lg and less casein in solution.

Dissolution of E65, E120, and T75 in reducing buffer produced protein profiles almost identical to unmodified MPC80 (Figure 3-1C).  $\beta$ -lg in E120 solubilized under these conditions, which indicated that insolubility under non-reduced conditions was from disulfide cross-linked aggregations that formed during extrusion. Unlike the soluble DLPA in E65, those found in E120 were mostly insoluble under non-reduced conditions, which was attributed to the higher extrusion temperature. The  $\beta$ -lg bands for E65, E120,

and T110 on this gel are broader and shifted upwards, and their  $\alpha$ -la bands lacked definition compared with MPC80 (Figure 3-1C). T110 still had a vertically smeared SDS-PAGE protein profile, which indicated that non-reducible Maillard induced PA formed during modification.

### 3.5.3 Free Sulfhydryl Content of the High-protein Nutrition Bars during Storage

Changes in protein solubility during storage might influence HPN bar free sulfhydryl measurements. Soluble protein extractable from the HPN bars was significantly influenced by protein ingredient and storage time. Soluble protein ranged from 40-45, 32-37, 44-46, 29-39, and 42-50 mg/mL for the HPN bars formulated with E65, E120, T75, T110, and MPC80, respectively, during 42 d storage. Measured protein solubility was the lowest on day 42 for the HPN bars prepared with T75, T110, E65, and MPC80. However, protein solubility in the E120 formulated HPN bars tended to increase with storage time, a trend that made the interaction term significant ( $P < 0.05$ ). When day 0 protein solubility was compared with day 42 protein solubility, only the T110 formulated HPN bar had significantly lower solubility on day 42. While the T110 formulated HPN bars produced less supernatant overall, the soluble protein concentration was only significantly lower than all other samples on day 42. Soluble protein extractable from an IMF reportedly decreased during storage and was related to matrix hardening (Zhou and others 2008a). In the present study, a significant reduction in protein solubility was not observed for all HPN bars during storage even though they all underwent significant texture change during the same time (Banach and others 2014).

Only the second preparation of the HPN bars made by Banach and others (2014) was used to evaluate free sulfhydryl change during storage (Table 3-1), which was

satisfactory since protein ingredient preparation ( $n = 2$ ) did not influence free sulfhydryl content ( $P > 0.05$ ). No difference between the measured free sulfhydryl content of a protein ingredient and its respective HPN bar was expected on day 0. While differences were observed in the extruded MPC80s, larger deviations were found between the protein ingredient and the HPN bar free sulfhydryl content when prepared with toasted and unmodified MPC80. Initially, the HPN bar formulated with T110 had lower free sulfhydryl content than the HPN bars formulated with MPC80 and T75, a trend that was reversed within the protein ingredient category. While the HPN bar was more complex than the protein ingredient, any background noise from the extra constituents was subtracted from the sample prior to calculating free sulfhydryl content with the standard curve.

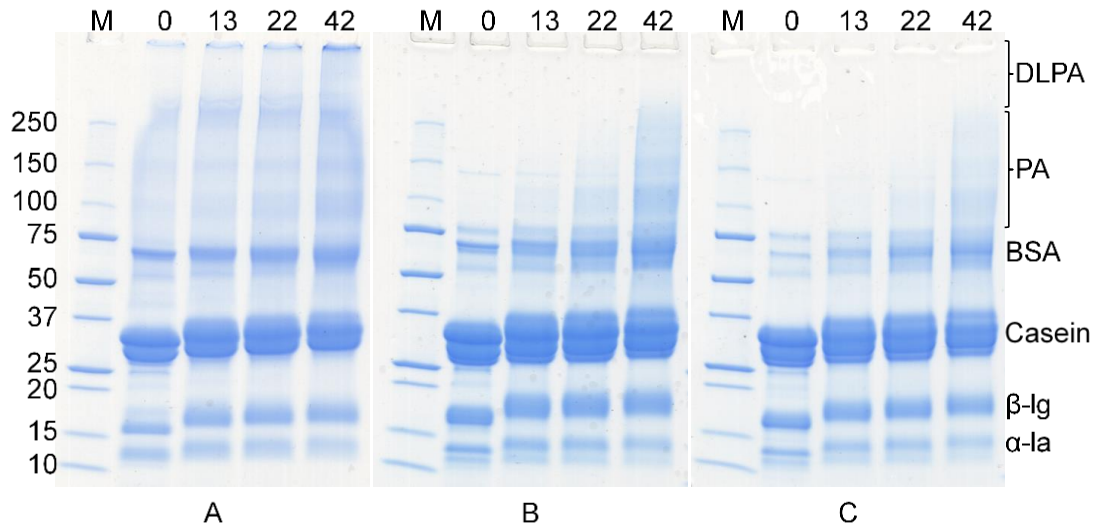
Free sulfhydryl content in HPN bar was significantly affected by the protein ingredient used and its interaction with storage time ( $P < 0.05$ ), but storage time alone did not have a significant effect ( $P > 0.05$ ). No initial differences were detected between the HPN bars formulated with MPC80, T75, T110, and E65 ( $P > 0.05$ ), whereas the E120 formulated HPN bars had significantly lower free sulfhydryl content. Although the numbers trended towards reduction, significant free sulfhydryl change was not detected during HPN bar storage when formulated with MPC80, T75, or E120 (Table 3-1). Free sulfhydryl content in E65 formulated HPN bars decreased significantly ( $P < 0.05$ ) after 13 days and did not differ from the one formulated with E120 for the remainder of the study. The free sulfhydryl concentration in T110 formulated HPN bars increased ( $P < 0.05$ ) with storage and was significantly greater than the other HPN bars on day 42 (Table 3-1).

Decreasing free sulfhydryl concentration during storage would indicate free sulfhydryl oxidation or the formation of disulfide bonds and that the HPN bar texture changes observed by Banach and others (2014) followed the protein aggregation mechanism previously reported (Zhou and others 2008a, 2008b). While all the HPN bars analyzed by Banach and others (2014) hardened, the HPN bar formulated with E65 was the softest and hardened the slowest. Yet, the present study revealed a significant free sulfhydryl content decrease in this sample within the same storage period. On the other hand, the T110 formulated HPN bars performed poorly from a texture standpoint and had increased free sulfhydryl concentration during storage. The insignificant free sulfhydryl decrease observed in the HPN bars formulated with MPC80 and T75, which behaved similarly from a texture standpoint, may or may not be sufficient to induce textural change. However, the significant interaction between protein ingredient and storage time disproves disulfide bond formation as the main mechanism of HPN bar texture change when formulated with MPC80.

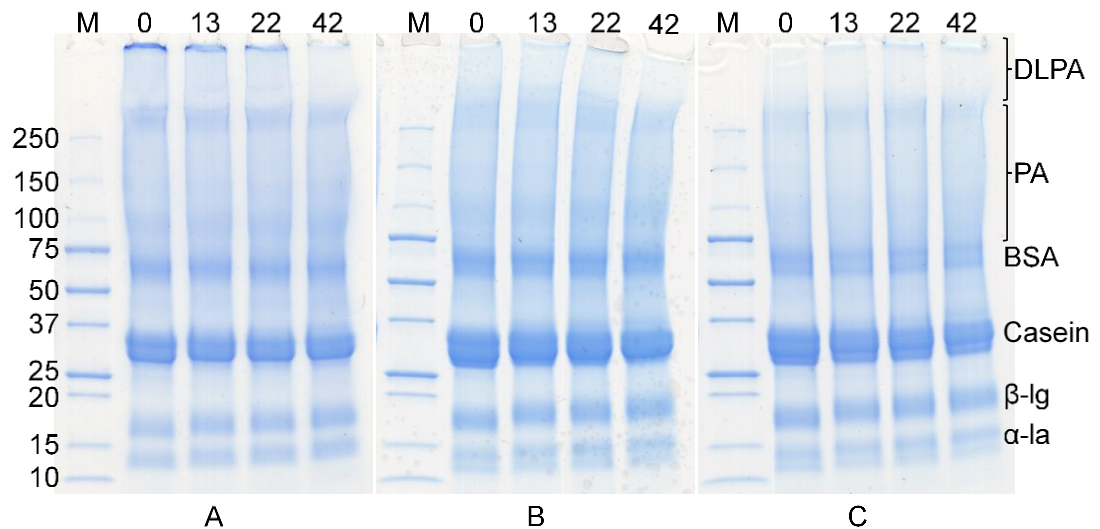
#### *3.5.4 SDS-PAGE Protein Profiles of the High-protein Nutrition Bars during Storage*

Reduced and non-reduced SDS-PAGE protein profiles for the HPN bars formulated with unmodified (Figure 3-2), toasted (Figure 3-3), and extruded (Figure 3-4 and Figure 3-5) MPC80 were used to verify disulfide bond formation during storage. In Figure 3-2 to Figure 3-5, images A and B show the proteins extractable under non-reduced conditions whereas C shows the proteins soluble in a reducing buffer. Gel A was run without  $\beta$ -mercaptoethanol, but it was included in the SDS-PAGE sample buffer for gels B and C. Under the same SDS-PAGE conditions, the protein profiles of the HPN

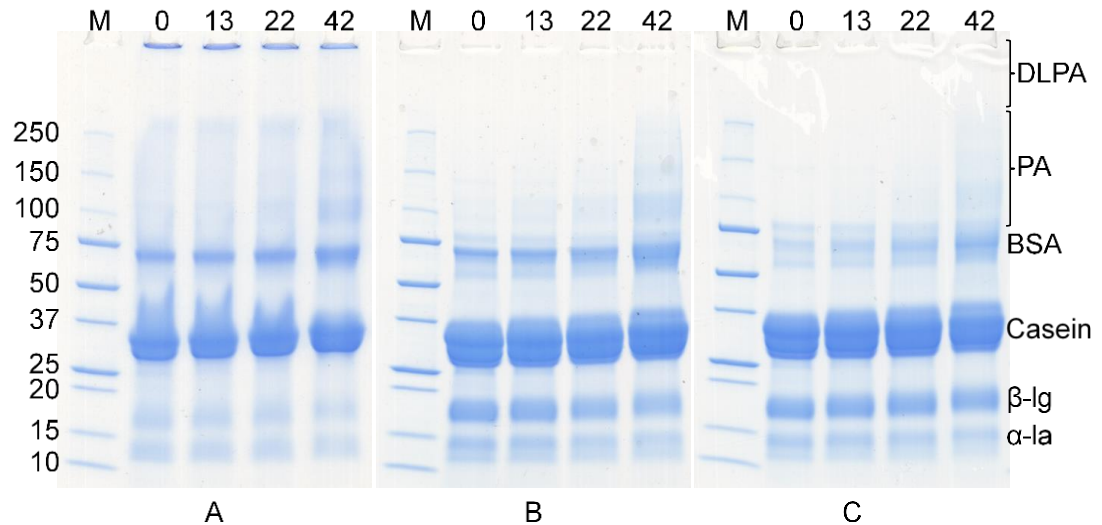
bars prepared with T75 matched those prepared with the control MPC80 and thus are not shown.



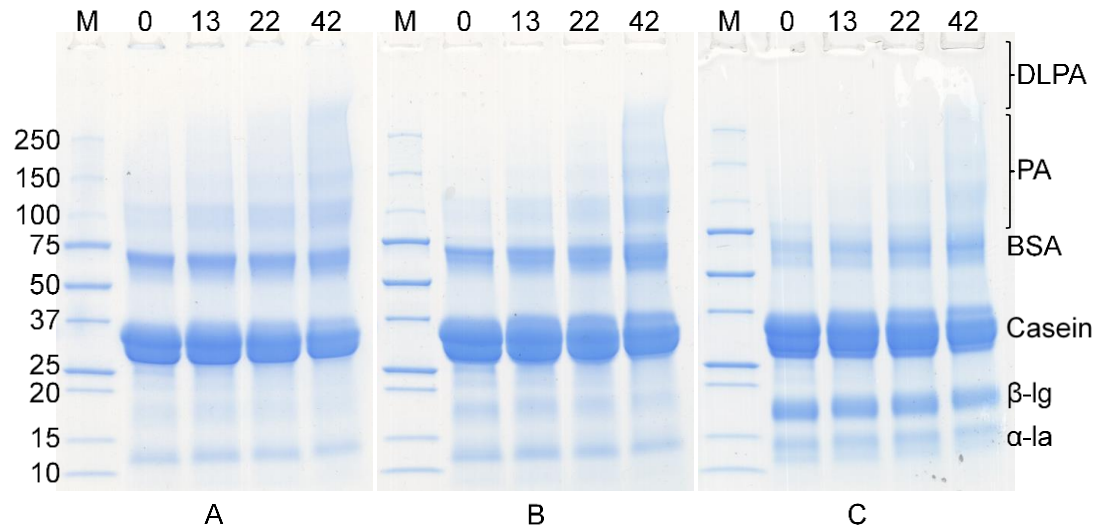
**Figure 3-2 Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with unmodified MPC80 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. Caseins, from high to low molecular weight, include:  $\alpha_{S2}$ ,  $\alpha_{S1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -Ig, beta-lactoglobulin.  $\alpha$ -la, alpha-lactalbumin.**



**Figure 3-3 Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with T110 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel. T110, MPC80 toasted at 110°C for 4 h. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include:  $\alpha_{S2}$ ,  $\alpha_{S1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -Ig, beta-lactoglobulin.  $\alpha$ -la, alpha-lactalbumin.**



**Figure 3-4 Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with E65 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel.** E65, MPC80 extruded at a die temperature of 65°C. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include:  $\alpha_{S2}$ ,  $\alpha_{S1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -Ig, beta-lactoglobulin.  $\alpha$ -Ia, alpha-lactalbumin.



**Figure 3-5 Non-reduced (A) and reduced (B, C) SDS-PAGE of the proteins extractable from the high-protein nutrition (HPN) bar formulated with E120 using non-reducing (A, B) or reducing (C) buffer after storage at 32°C for the days indicated at the top of each gel.** E120, MPC80 extruded at a die temperature of 120°C. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include:  $\alpha_{S2}$ ,  $\alpha_{S1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -Ig, beta-lactoglobulin.  $\alpha$ -Ia, alpha-lactalbumin.

DLPA accumulated just below the loading well for the HPN bars formulated with MPC80, T75, T110, and E65 (Figures 3-2A, 3-3A, and 3-4A). In the HPN bars formulated with MPC80 or T75, the formation of soluble DLPA increased throughout

storage period, as indicated by band intensity (Figure 3-2A). However, the same protein aggregations decreased during storage in the T110 formulated HPN bars (Figure 3-3A). The DLPA in E65 were of higher molecular weight, as the band was highly concentrated at the top of the gel and DLPA migration into the gel was virtually nonexistent (Figure 3-4A). In this case, the DLPA remained nearly constant and thus these aggregations did not change during storage as they did in the HPN bars formulated with toasted and unmodified MPC80. These DLPA, especially those that did not enter the gels, were inferred due to disulfide crosslink formation, as a reducing agent in the sample buffer allowed the proteins involved to enter the gel (Figures 3-2B, 3-3B, and 3-4B). The HPN bars formulated with E120, in line with the protein ingredient, did not show any soluble DLPA initially nor were any formed during storage (Figure 3-5A).

Directly below the DLPA region, a strip labeled PA, which consists of both disulfide crosslinked aggregates as well as those due to Maillard-induced protein aggregations, was identified (Figure 3-2 to Figure 3-5). Vertical band smearing on each storage day became less intense when a reducing agent was added to the SDS-PAGE sample buffer or both the SDS-PAGE sample and extraction buffers. Disruption of these PA was from reduction of disulfide bonds that were present initially (i.e., Day 0) in each HPN bar from protein ingredient modification or natively found in MPC80. Disulfide linked aggregates were less common in the PA region for the T110 formulated HPN bars, as reducing agent addition did not decrease vertical band smearing and thus was inferred to be from non-reducible, Maillard-induced PA formed during initial protein modification (Figure 3-1). However, on the gels with a reducing agent, vertical band smearing within the lanes increased with the storage time when formulated with extruded (Figure 3-4 and

Figure 3-5B or C) or unmodified MPC80 (Figure 3-2B or C) and remained constant when formulated with the heavily pre-aggregated T110 (Figure 3-3B or C). The development of non-reducible, Maillard-induced PA with storage may have contributed to HPN bar texture change as previously reported (Banach and others 2014; Zhou and others 2013), even though this was suggested not to be a mechanism of texture change by McMahon and others (2009).

Individual protein bands (e.g., casein,  $\beta$ -lg) on the non-reduced gels were slightly smeared; however, their resolution improved with reducing agent addition to the SDS-PAGE sample buffer alone or to both extraction and SDS-PAGE sample buffers (Figure 3-2 to Figure 3-5). The casein proteins, including  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -, and  $\kappa$ -casein, separated at lower resolution on the non-reduced gels when compared to the reduced gels, especially as storage time increased. Decreased casein mobility after day 0 on the non-reduced SDS-PAGE gels for the HPN bars formulated with MPC80 (Figure 3-2A) and T75 (not shown) was due to increased molecular weight from protein glycation that occurred during storage (Loveday and others 2009; Zhou and others 2013). With longer storage, the caseins in the HPN bars formulated with MPC80 (Figure 3-2), T75 (not shown), and to a lesser extent, those with extruded MPC80 (Figure 3-4 and Figure 3-5) had improved resolution on the reduced SDS-PAGE gels. The caseins, which account for 80% protein in any membrane concentrated MPC, do not contain any free sulfhydryl groups, but the  $\alpha_{s2}$ -casein (Cys36–Cys40) and the  $\kappa$ -casein (Cys11–Cys88) each have a disulfide bond (Bouguyon and others 2006; Rasmussen and others 1992). Since improved casein separation occurred only when a reducing agent was added, it might involve sulfhydryl-disulfide interchange amongst cysteine-containing  $\beta$ -lg,  $\kappa$ -casein,  $\alpha_{s2}$ -casein, and  $\alpha$ -la.



However, the small change in molecular weight that improved casein separation may have been from glycation of the protein.

The observed  $\beta$ -lg, which contains one free sulfhydryl group, on the non-reduced SDS-PAGE gels, was relatable to the free sulfhydryl content of the HPN bars on each respective storage day.  $\beta$ -lg band intensity from the HPN bars formulated with MPC80 (Figure 3-2A) or T75 (not shown) remained fairly constant throughout storage, as did the measured free sulfhydryl concentration (Table 3-1).  $\beta$ -lg solubility decreased with storage for the HPN bar formulated with E65 (Figure 3-4A) and was absent in the samples prepared with E120 (Figure 3-5A). The extractable  $\beta$ -lg content increased with storage for the HPN bars formulated with T110 (Figure 3-3A). The decreasing, missing, and increasing  $\beta$ -lg within the HPN bars formulated with E65, E120, and T110, respectively, corresponded with free sulfhydryl content (Table 3-1). While disulfide bond formation occurred during HPN bar storage, the differences in the SDS-PAGE protein profiles and free sulfhydryl contents show that it cannot be the only source of texture change. The non-reducible PA, represented by band smearing on the SDS-PAGE gels, and especially prevalent in the HPN bars formulated with T110, also played a role in both initial texture and change during storage.

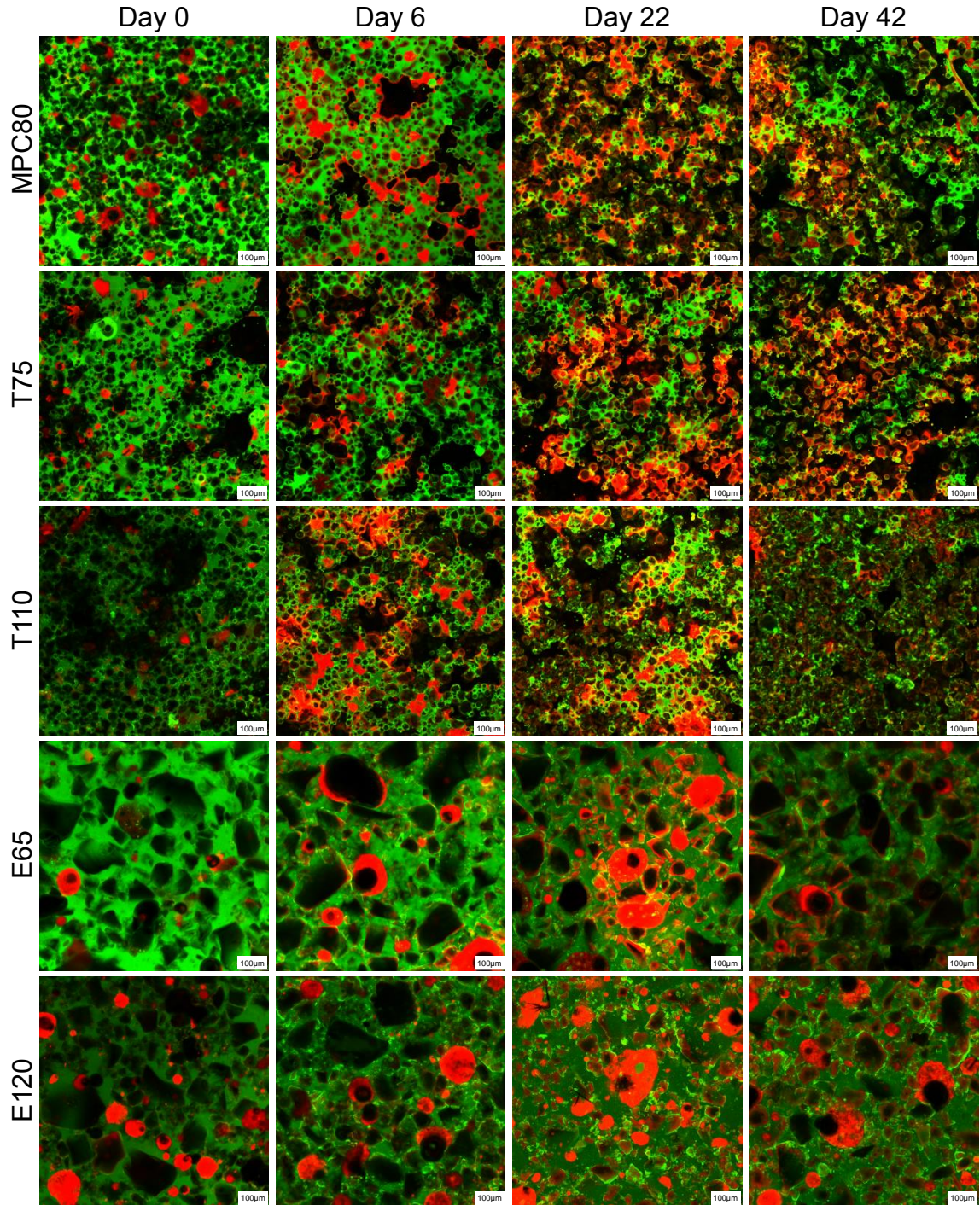
### *3.5.5 Confocal Micrographs of the High-protein Nutrition Bars during Storage*

Initial differences in HPN bar microstructure were more apparent when formulated with extruded MPC80 versus toasted MPC80 and compared with unmodified MPC80 (Figure 3-6). Similar to published CLSM images of HPN bars formulated with MPC80 (Loveday and others 2009), a green proteinaceous continuous phase was observed on day 0. The intense FITC background staining may have hindered the

appearance of Nile red. Its intensity decreased with storage, which allowed for lipid depiction (Loveday and others 2010).

The larger black regions present on the micrographs of the HPN bars formulated with control MPC80, T75, or T110 are non-fluorescing components (McMahon and others 2009). The smaller unstained regions with circular or concave shape might be undissolved, unmodified or toasted MPC80 powder since there was not enough free water in this formulation for complete protein hydration (McMahon and others 2009; Loveday and others 2009). The slightly larger unstained regions with concave shape on the micrographs for the HPN bars formulated with extruded MPC80 are likely undissolved protein particles with limited FITC uptake. Although all protein ingredients were passed through a 250  $\mu\text{m}$  mesh, the extruded MPC80 had a larger size distribution and average diameter when compared with control MPC80. The particles in the control MPC80 were no larger than 100  $\mu\text{m}$  (Crowley and others 2014). Extruded MPC80, which was milled using centrifugal mill equipped with a 500  $\mu\text{m}$  mesh, had approximate  $d_{80}$  of 250  $\mu\text{m}$  (Vargo 2014). The larger protein particles served as inert structural elements, or structure breakers, that physically disrupted the HPN bar matrix and with limited solubility were less likely to participate in chemical reactions during storage (Purwanti and others 2010). Larger particle size and decreased surface area was one factor that slowed free amine reduction in the HPN bars formulated with extruded MPC80 (Banach and others 2014). The larger sized particles found in E65 did not slow free sulfhydryl content reduction between day 6 and day 13 in the HPN bar formulated with that protein ingredient (Table 3-1).

Limited microstructural changes were observed in the HPN bars formulated with extruded MPC80 through the 42 day storage period (Figure 3-6). The green, protein-based continuous phase remained prominent in the HPN bars formulated with E65 or E120. On day 22 and day 42, larger lipid droplets and what appeared to be lipid coated protein particles were seen for these HPN bars. McMahon and others (2009) saw more lipid coalescence in HPN bars that contained more WPI hydrolysate versus native WPI, and those samples remained softer during storage. Additionally, the HPN bars formulated with lower weight percentages of hydrolyzed WPI hardened quicker and the CLSM images showed the development of protein-rich and carbohydrate-rich regions (McMahon and others 2009). The HPN bars formulated with extruded MPC80 maintained an unvarying protein-rich phase throughout storage and HPN bar hardening was slowed by preventing macronutrient (i.e., protein, carbohydrate, fat) phase separation.



**Figure 3-6 Confocal micrographs (775  $\mu\text{m}$  x 775  $\mu\text{m}$ ) of high-protein nutrition (HPN) bars formulated with unmodified (MPC80), toasted (T75 and T110), or extruded (E65 and E120) MPC80.** HPN bars (30% protein (w/w)) were stored for 0, 13, 22, or 42 days at 32°C. MPC80, unmodified MPC80. T75 and T110, MPC80 toasted 4 h at 75°C and 110°C, respectively. E65 and E120, MPC80 extruded at die temperature of 65°C and 120°C, respectively. Fluorescein isothiocyanate (FITC) stained the protein component green and Nile red stained the lipid component red. The length of the white bar on each micrograph represents 100  $\mu\text{m}$ .

CLSM also revealed that microstructural changes were more conspicuous in HPN bars formulated with unmodified or toasted MPC80, which were less texturally stable (Banach and others 2014). During storage, the continuous protein-rich phase on day 0 was penetrated by Nile red stained lipids and blackened, particle-clustered regions. Loveday and others (2010, 2009) also reported decreased protein solubility and increased particle clustering during storage of HPN bars formulated with MPC80 or calcium caseinate as their pourable HPN bar formulation set into a firm matrix within a day of manufacture. Although particle clustering was not apparent in WPI formulated HPN bars, unstained regions did develop in those that hardened more rapidly, which were suggested to be carbohydrate-rich regions (McMahon and others 2009). The MPC80 particle surfaces were hydrated during protein bar production, but this surface layer hydration was lost as water molecules moved to associate with polyhydroxy compounds used in the model (Loveday and others 2009). Inadequate protein particle surface hydration in the present study potentially limited fluorescence in the HPN bars formulated with unmodified or toasted MPC80. If water molecules continued to disassociate from the particle surface, it partially explains why more unstained regions appeared during storage.

The water activity of the HPN bars formulated with unmodified or toasted MPC80 increased quickly during the first 4 days at 32°C and then remained fairly constant (Banach and others 2014). Increased water activity would support the notion of water molecule movement to the bulk phase and concurrently less association with the protein. The water activity of the HPN bars formulated with extruded MPC80 did not increase rapidly during the first 4 days of storage, rather it increased slowly and

approached the plateau value obtained for the other HPN bars (Banach and others 2014). Water activity measurement employed lacked sensitivity and even though it plateaued early on for the HPN bars formulated with unmodified or toasted MPC80, a slow yet continual shift of water molecules to the bulk phase might be one reason for the disappearance of the continuous green background on the micrographs during storage (Figure 3-6). On the contrary, CLSM images for the HPN bars formulated extruded MPC80, especially those formulated with E120 and stored 22 and 42 days, had small regions with high levels of FITC fluorescence, which confirmed that these regions were not becoming moisture depleted. Therefore, extruded MPC80 was better able to utilize water molecules as a plasticizer in their intermediately bound state, which helped maintain the soluble protein network and improved textural stability during HPN bar storage (McMahon and others 2009; Li and others 2008).

### 3.6 Conclusions

Extrusion decreased and toasting increased the free sulfhydryl content of MPC80. The HPN bars produced with extruded or toasted MPC80 were less and more prone, respectively, to texture change when compared to each other and the control MPC80. The free sulfhydryl content during HPN bar storage increased when formulated with T110, decreased when formulated with E65, and did not change significantly when formulated with T75, E120, or unmodified MPC80. During HPN bar storage, soluble DLPA increased for MPC80 and T75, decreased for T110, remained constant for E65, and were absent in E120. The formation of soluble DLPA and free sulfhydryl change during storage were not consistently relatable to HPN bar texture change. Microstructurally and texturally, the HPN bars formulated with extruded MPC80

exhibited greater stability, and use of this modified protein in HPN bars may be useful in extending textural shelf life.

### 3.7 Acknowledgement

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## CHAPTER 4. INSTRUMENTAL AND SENSORY TEXTURE ATTRIBUTES OF HIGH-PROTEIN NUTRITION BARS FORMULATED WITH EXTRUDED MILK PROTEIN CONCENTRATE

Modified from a paper published in the *Journal of Food Science*<sup>1</sup>

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### 4.1 Abstract

Previous instrumental study of high-protein nutrition (HPN) bars formulated with extruded milk protein concentrate (MPC) indicated slower hardening compared to bars formulated with unmodified MPC. However, hardness, and its change during storage, insufficiently characterizes high-protein nutrition bar texture. In this study, MPC80 was extruded at two different conditions and model HPN bars were prepared. A trained sensory panel and instrumental techniques were used to measure HPN bar firmness, crumbliness, fracturability, hardness, cohesiveness, and other attributes to characterize texture change during storage. Extrusion modification, storage temperature, and storage time significantly affected the instrumental and sensory panel measured texture attributes. The HPN bars became firmer and less cohesive during storage. When evaluated at the same storage conditions, the texture attributes of the HPN bars formulated with the different extrudates did not differ significantly from each other. However, textural differences were noted most of the time between the control and the HPN bars formulated with extruded MPC80. An adapted HPN bar crumbliness measurement technique produced results that were correlated with sensory panel measured crumbliness ( $r = 0.85$ )

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and cohesiveness ( $r = -0.84$ ). Overall, the HPN bars formulated with extruded MPC80 were significantly softer, less crumbly, and more cohesive than the control during storage.

#### 4.2 Practical Application

Extruding milk protein concentrate with 80% protein produced a functional ingredient that, when incorporated in high-protein nutrition bars, resulted in favorable texture attributes, e.g., reduced firmness and improved cohesiveness, when compared to the unmodified control. Instrumental texture attributes were correlated with their respective sensory attributes. High-protein nutrition bar crumbliness measurement by sieve analysis promises to be a useful tool for quantifying crumbliness and cohesiveness as results were strongly correlated with the sensory panel.

#### 4.3 Introduction

It is well known that high-protein nutrition (HPN) bars (20-50% protein w/w) and other shelf-stable, intermediate moisture foods (IMFs; 10-40% moisture;  $0.55 \leq a_w \leq 0.90$ ) problematically harden to unpalatable levels during storage (Rao and others 2013a; Imtiaz and others 2012; Loveday and others 2009; Banach and others 2014). Reducing the average molecular weight of a protein by hydrolysis can soften HPN bars and slow their hardening by suppressing the system's glass transition temperature ( $T_g$ ) (Rao and others 2013a; McMahon and others 2009). While enzyme hydrolysates have improved digestibility (Potier and Tome 2008) and reduced allergenicity (Verhoeckx and others 2015), they cost more to produce and taste bitter. Encapsulated casein hydrolysate added at 3% (w/w) to protein bars did not impart bitterness, but encapsulation also increased hydrolysate  $T_g$ , and since texture was not measured, it is unknown if the hydrolysate

retained its desirable texture-softening functionality (Rocha and others 2009). While other protein modification techniques, including physical (Osen and others 2015; Banach and others 2013) and chemical (Zhang and others 2015) for improved functionality are available, their focus has been on altering a protein's solubility-dependent properties, such as gelation, emulsification, and foaming, which are unrelated to performance in IMFs or HPN bars that are more solid than fluid.

Milk protein concentrates (MPCs), particularly those with high protein (i.e.,  $\geq 80\%$ ;  $\geq$  MPC80), are not preferentially utilized in HPN bars (Baldwin and Pearce 2005). HPN bars formulated with MPC harden during storage (Banach and others 2014). Hardness and hardening rate alone inadequately characterizes these systems, which also suffer from decreased cohesiveness and increased crumbliness during storage (Imtiaz and others 2012; Loveday and others 2009; Li and others 2008). In our previous study, texture profile analysis (TPA) and shear testing demonstrated that HPN bars formulated with extruded MPC80 remained softer than unmodified controls during storage (Banach and others 2014). TPA is an instrumental texture technique where two successive sample compressions are used to roughly simulate two bites by a consumer with output that has been used to describe the texture of many different foods (Gunasekaran and Ak 2003). TPA has the potential to describe the texture attributes of HPN bars better than the puncture test favored in IMF-based literature, but their correlation with sensory panel perceived attributes remains unknown.

Trained sensory panels can also be used to describe the texture of HPN bars, but such evaluation is more time-consuming and costly and less utilized when describing these systems. A sensory-based texture study most pertinent to the current work involved

two proprietarily modified MPCs and a non-hydrolyzed whey protein concentrate (WPC) (Imtiaz and others 2012). These were blended to make HPN bars with different protein composition at fixed protein content (30% protein w/w) that had altered cohesiveness/crumbliness. The same study found correlation between the results of instrumental puncture with a 5 mm cylindrical probe and select sensory attributes measured by a trained panel. Another sensory-based study found that the predominant protein source (i.e., whey vs. soy) influenced sensory texture in a more realistic HPN bar formulation (Childs and others 2007). Literature has focused on the role of protein in texture change and determined that functionalization prior to HPN bar production can impart textural stability.

Multiple factors affect complete HPN bar texture change during storage. Commercially produced HPN bars are complex systems of blended proteins mixed with carbohydrates (e.g., maltodextrins), lipids (e.g., palm oil), plasticizers (e.g., glycerol, sugar alcohols), and other components (e.g., minerals) that can alter the system's stability during storage. Storage conditions and other added constituents, such as polyols and free sulfhydryl-containing compounds, are also known to affect the rate of hardening (Liu and others 2009; Zhu and Labuza 2010). Simplified models have been used to mechanistically describe texture change, namely hardening, that occurs during storage, but the results might not translate to commercial HPN bars. Simple models are key for mechanistic studies, but their scope is more limited than those using a more realistic HPN bar formulation, like the one used in the present study, that have not been reported in abundance (Hogan and others 2012).

The following study was designed to thoroughly characterize the texture attributes of HPN bars formulated with ground extruded MPC80. Commonly reported instrumental TPA attributes were correlated with those measured by the sensory panel. Since increased crumbliness and decreased cohesiveness have been previously reported and observed in MPC-containing HPN bars (Imtiaz and others 2012; Banach and others 2014), a sieve analysis after TPA was employed to better characterize these properties.

#### **4.4 Materials and Methods**

##### *4.4.1 Milk Protein Concentrate Extrusion*

MPC80 (80% protein w/w dry-basis, Milk Specialties Global, Eden Prairie, MN) was fed (25 kg/h) into a co-rotating twin-screw extruder (DN DL 44, Bühler AG, Uzwil, Switzerland) at the Joseph J. Warthesen Food Processing Center (University of Minnesota, St. Paul, MN) using systems previously described (Tremaine and Schoenfuss 2014). Screw speed (350 rpm), MPC80 feed rate, and set barrel temperature (50°C) were fixed. Water addition was lowered from 11 kg/h to 10 kg/h to produce extrudates with circular die (3 mm) melt temperature of ~105°C (i.e., E105) and ~116°C (i.e., E116), respectively. Extrudates were pelletized and dried partially on a fluidized bed dryer (OTW 05TRR2, Bühler AG, Braunschweig, Germany). Drying continued at 40°C in a forced draft oven for 26 h. The protein pellets were coarsely ground as described (Banach and others 2014) and the resultant powder was jet-milled.

##### *4.4.2 Protein Powder Particle Size Measurement*

Particle size distributions (PSD) were measured (n = 2) by laser diffraction (Mastersizer 2000, Malvern Inc., Worcestershire, United Kingdom) (Gazi and Huppertz 2015). 450 mL isopropanol (Fisher Scientific, Waltham, MA) in a 600 mL beaker was

stirred at 2,000 rpm by the wet dispersion accessory (Hydro 2000MU, Malvern Inc., Worcestershire, United Kingdom). Powder was added to the dispersant such that obscuration was 10-20% and triplicate measurements were taken automatically. Isopropanol's refractive index and sensor threshold were 1.39 and 64, respectively. MPC's refractive index and absorption value were 1.46 and 0.1, respectively (Crowley and others 2015).

#### *4.4.3 High-protein Nutrition Bar Preparation*

Protein ingredient moisture content was determined after drying 16 h at 102°C and protein was measured by Dumas nitrogen combustion (AOAC 1998). HPN bars were prepared (n = 2) at 30% protein (w/w) using either control MPC80 (76.8% protein, 5.2% moisture), E105 (74.3% protein, 7.5% moisture), or E116 (74.4% protein, 7.4% moisture). 1.21 kg MPC80, 1.25 kg E105, and 1.25 kg E116 were each dry-blended with 155 g maltodextrin (Maltrin®180, 16.5-19.9 dextrose equivalent, 6% moisture, Grain Processing Corporation, Muscatine, IA). 175 g high-fructose corn syrup (CornSweet®55, 55% fructose, 41% dextrose, 4% higher saccharides, 23% water, Archer Daniels Midland, Decatur, IL), 647 g glycerol (99.7% glycerol, USP Grade, US Glycerin, Jackson, MI), 321 g maltitol syrup (Lycasin®80/55, 51.7% D-maltitol, 3.0% D-sorbitol, 24.5% water, Roquette America, Keokuk, IA), and 111, 69, or 71 g distilled water were combined and heated to 60° for the HPN bars to be prepared with MPC80, E105, or E116, respectively. 465 g non-hydrogenated, trans-free palm oil (SansTrans®39, IOI Loders Crocklaan, Channahon, IL) was melted with 15.5 g low-viscosity liquid lecithin (Beakin®LV1, 0.8% moisture, Archer Daniels Midland, Decatur, IL). The wet ingredients were first combined and then the dry ingredient blend was slowly added over

the course of 4.5 min mixing with the paddle attachment on speed 1 (A200, Hobart Corporation, Troy, OH).

HPN bar dough was transferred and pressed into two parchment paper-lined cookie sheets (22.9 cm x 33 cm x 1.6 cm). A rolling pin was used to press the HPN bar dough flush with the upper edge of the pan, removing or adding more sample as needed to ensure a uniform height. Each pan was wrapped with lightly oiled plastic wrap and remaining HPN bar dough was pressed into water activity ( $a_w$ ) cups as described previously (Banach and others 2014). Samples were kept at room temperature ( $\sim 22^\circ\text{C}$ ) overnight.

A circular cutter (ID = 1.91 cm) punched samples from each HPN bar sheet. The samples were expelled directly onto heavy-duty waxed plates, which were then heat-sealed in metallized bags (S-16891, Uline, Pleasant Prairie, WI). Samples formulated with E105 and E116 were refrigerated ( $4^\circ\text{C}$ ) for 1 h prior to cutting. Samples were assigned to room temperature ( $\sim 22^\circ\text{C}$ ) or incubated storage ( $32^\circ\text{C}$ ) the following day.

#### *4.4.4 Panelist Recruitment and Training*

This study was approved for human subjects by the Office of Responsible Research at Iowa State University (Institutional Review Board # 14-166). Eight female panelists were trained to evaluate the textural attributes of HPN bars for a minimum of 7 h over the course of 8 1-hour training sessions. Panelists measured firmness and crumbliness using their hands and fracturability, hardness, cohesiveness, and mouth coating in their mouths using anchored 15-cm lines (Table 4-1).



**Table 4-1 High-protein nutrition (HPN) bar texture attributes and sensory panel anchors<sup>1</sup>**

Attribute	Definition	Anchors
Firmness	Force required to compress a sample between thumb and index finger	0 cm - Sara Lee® White Bread 7 cm - DiLusso's Wisconsin American Cheese 15 cm - Baby Carrot
Crumbliness	Extent to which pieces break from a sample after one in-hand compression	0-2 cm - DiLusso's Wisconsin American Cheese 7 cm - HyVee® Chocolate Chip Granola Bar 13-14 cm - Nabisco® Grahams Original
Fracturability	Force required for the sample to break between one's incisors	0-1 cm - Philadelphia® Neufchatel Cheese 6 cm - Nabisco® Grahams Original 14 cm - Old London® Melba Toast
Hardness	Force required to bite through the sample with one's molars	0-1 cm - Philadelphia® Neufchatel Cheese 4-5 cm - DiLusso's Wisconsin American Cheese 12-13 cm - Baby Carrot
Cohesiveness	Degree to which the sample holds together in a mass after three chews	0-2 cm - Baby carrot 7-8 cm - DiLusso's Wisconsin American Cheese 13-14 cm - Little Debbie® Cosmic Brownie

<sup>1</sup> Attributes, definitions, and anchors adapted from Childs and others (2007), Imtiaz and others (2012), and Meilgaard and others (2007).

HPN bar texture was evaluated immediately after being cut (i.e., week 0) and then weekly for up to 6 weeks. Since the samples were not placed into storage until day 1 and they were removed from storage 4.5 h prior to each evaluation session for temperature equilibration, the storage time at 32°C was less than each identified week (i.e., 1 wk = 5.7 d, 2 wk = 12.7 d, etc.). With 2 HPN bar preparations, there were two evaluation sessions each week and 6 HPN bars (i.e., 3 proteins × 2 storage temperatures) were evaluated per session. Panelists were randomly presented 3 cut HPN bar samples identified only by a 3 digit code on a white paper plate. One sample was used for in-hand evaluation and the other two were for in-mouth tests. Panelists were provided water, unsalted crackers, and unscented wet wipes to cleanse their palate and hands between HPN bars.

#### *4.4.5 Instrumental Texture Evaluation*

HPN bars for instrumental texture evaluation were removed from incubated storage concurrent those for sensory evaluation and were evaluated the following day. HPN bar samples (n = 3) were compressed with a flat plate (TA-30) at 2 mm/s to 60% strain using the TPA test format while force (N) versus time (s) data were recorded (TA-XT2, Texture Technologies, Scarsdale, NY) (Banach and others 2014). Other HPN bar samples (n = 3) were sheared across their circular cross-section with a 45° chisel blade (TA-42) at 1 mm/s (Banach and others 2014). Max force (N) during the first TPA compression and shear force (N) were used to report HPN bar hardness. Adhesiveness (J) was taken as the absolute area under the force versus time curve during probe withdrawal after the first compression. Cohesiveness (%) was the ratio of area under the second compression curve to the area under first compression curve.

A sieve analysis was used to measure HPN bar crumbliness by modifying a method used to measure the same parameter of Queso Fresco cheese (Hwang and Gunasekaran 2001). After TPA, the sample was transferred to a stack of 3" sieves with descending aperture (i.e., 5.6, 4.0, 2.8, 2.0, 1.4, 1.0, and 0.5 mm). The stack was placed into a custom-made 8" to 3" adapter and was shaken for 30 s on speed 3 (Shaker #18480, CSC Scientific Sieve, Fairfax, VA). Mass percent finer than the top sieve (No. 3.5) was reported as crumbliness.

#### *4.4.6 Color Water Activity, pH, Moisture, and Protein Measurement*

HPN bar color and  $a_w$  were measured ( $n = 3$ ) as described elsewhere (Banach and others 2014). 20% HPN bar dispersions were prepared in Millipore water, mixed for 16 h, and pH was measured ( $n = 2$ ). HPN bar moisture content was measured ( $n = 3$ ) by difference after drying 1 g samples at 102°C for 26 h. HPN bars were frozen in liquid nitrogen on the day of manufacture and after 29 weeks storage, kept at -80°C, and were used to determine average HPN bar protein content by Dumas nitrogen combustion (AOAC 1998).

#### *4.4.7 Statistical Analyses*

Instrumental measurements were averaged by protein ingredient, storage temperature, storage time, and preparation ( $n = 2$ ). Sensory panel responses were not averaged prior to statistical analysis. The dependent variables were modeled using the mixed procedure with protein (i.e., MPC80, E105, and E116), time (i.e., weeks), and temperature (i.e., 22°C and 32°C) set as the independent variables. Panelist and preparation of each HPN bar were set as the random effects; only the latter applied to instrumental analysis. Slicing factors were applied to analyze between proteins at fixed

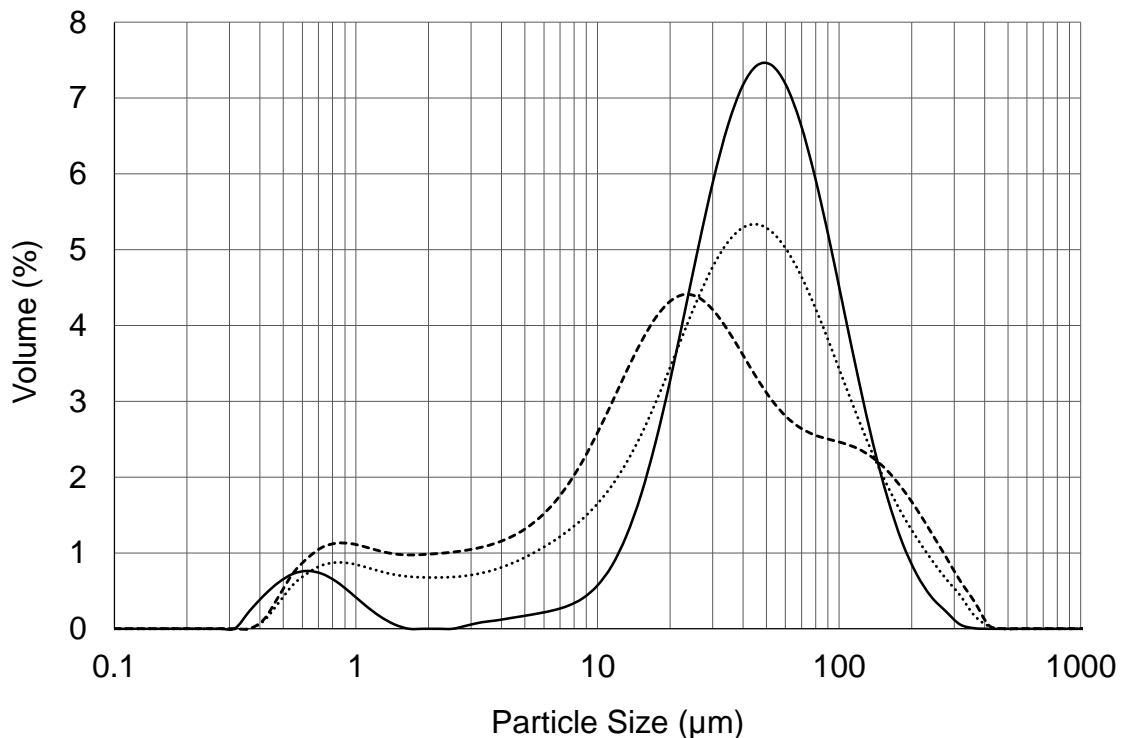
time and also within each HPN bar over storage. The Tukey-Kramer adjusted  $P$ -value ( $\alpha = 0.05$ ) was used to determine differences between the least squares means. For correlation analysis, sensory panel responses were averaged by protein ingredient, storage temperature, storage time, and preparation ( $n = 2$ ). Pearson correlation coefficients ( $r$ ) were calculated between sensory and instrumental responses. All statistical analyses were performed with SAS® software (version 9.4, SAS Institute Inc., Cary, NC).

## 4.5 Results and Discussion

### 4.5.1 Protein Powder Particle Size and its Influence on High-protein Nutrition Bar Production

$D_{90}$ ,  $D_{50}$ , and  $D_{10}$  of MPC80 were set as the processing targets for jet-milling such that any HPN bar texture differences were attributable to the extrusion modification rather than a confounded PSD effect. Protein powder volume mean diameters ( $D_{4,3}$ ) were measured ( $\pm$  SD) at 53 ( $\pm 0.1$ ), 57 ( $\pm 0.8$ ), and 61 ( $\pm 0.8$ )  $\mu\text{m}$  for E105, E116, and MPC80, respectively. Although  $D_{4,3}$  ranged only 8  $\mu\text{m}$ , particle size span (i.e.,  $(D_{90}-D_{10})/D_{50}$ ) for E105 (5.7), E116 (3.3), and MPC80 (2.1) indicated that the jet-milled powders had broader PSD than the more uniform spray-dried MPC80 (Figure 4-1). On average ( $\pm$  SD), 1162 ( $\pm 7$ ), 1372 ( $\pm 9$ ), and 1365 g ( $\pm 5$ ) of HPN bar dough prepared with MPC80, E105, and E116, respectively, was required to fill each production pan (1209  $\text{cm}^3$ ). The control HPN bar (0.96  $\text{g cm}^{-3}$ ) was less dense than those prepared with E105 (1.13  $\text{g cm}^{-3}$ ) and E116 (1.13  $\text{g cm}^{-3}$ ). The finer protein particles, which were more common in the milled extrudates, positioned themselves between the larger powder particles. E105 had the largest span, smallest  $D_{4,3}$ , and produced the densest HPN bar. The control protein powder, with more uniform PSD, could not accomplish this level of particle packing due to volume constraints within the HPN bar. Excess pressure was unable to add more mass

to the control HPN bar, and if applied, would cause textural differences from production rather than protein modification. Uniform sample geometry was important for texture analysis and, despite density differences, the HPN bar dough was pressed to a uniform height.



**Figure 4-1 Particle size distributions (PSD) for control and extruded MPC80 powders.** MPC80 (—), spray-dried control milk protein concentrate with 80% protein. E105 (---) and E116 (···), jet-milled MPC80 that was extruded at die-end melt temperature of 105°C and 116°C, respectively.

HPN bar doughs prepared with extruded MPC80 had higher fluidity than the control during manufacture and were pourable whereas the control required force to take shape. This fluidity made it difficult to remove cut samples from the sheeted HPN bars prepared with extruded MPC80 and prompted chilling prior to cutting. The control HPN bar was rigid and samples were easily cut at room temperature. The samples prepared with extruded MPC80 (14.3 mm ± 0.5) were about 1.5 mm shorter than those prepared with control MPC80 (15.8 mm ± 0.0), but all samples maintained their cylindrical shape

during storage. Height differences were attributed to the incompressibility of unmodified MPC80 and potential settling within the HPN bars formulated with extruded MPC80.

The less viscous HPN bar dough formulated with extruded MPC80 may be more difficult to process into and hold bar form.

Particle size parameters of protein powder, including diameter, uniformity, span, and PSD should not be ignored while discussing HPN bar texture. These parameters will affect the volume fraction required to obtain HPN bar solidity and texture change during storage (Hogan and others 2016; Thomar and others 2012). Protein powders that form a suspension in a particular HPN bar formulation rather than a jammed or solid product are not expected to change texturally during storage (Hogan and others 2016). In our previous study involving extruded MPC80 in HPN bars, E65, E120, and the control had  $D_{4,3}$  ( $\pm$  SD) of 119 ( $\pm$  12), 88 ( $\pm$  15), and 73  $\mu\text{m}$   $D_{4,3}$  ( $\pm$  2), respectively (unpublished data). These extruded MPC80s had larger average particle size than the control and produced HPN bars that were softer and less prone to hardening (Banach and others 2014). This result aligned with the work of Cho (2010), who found that coarsely ground ( $\sim 84\% < 150 \mu\text{m}$ ) soy protein crisps, or extruded and milled soy protein concentrate, produced HPN bars that were softer and less prone to hardening than those produced using the finely ground ( $\sim 100\% < 150 \mu\text{m}$ ) fraction. In the present study, E105 and E116 were milled slightly finer than the control MPC80, and if repeatable textural results are obtained, it can be partially attributed to the extrusion modification, despite there being an incompletely accounted for PSD effect. More in-depth particle size and density discussions will serve as topic of interest in future studies, but its effect on texture change are beyond the scope of this study.

#### 4.5.2 High-protein Nutrition Bar Protein, Moisture, Water Activity, Color, and pH

HPN bar protein (%  $\pm$  SD) was  $32.2 \pm 0.9$ ,  $32.6 \pm 0.5$ , and  $32.5 \pm 0.7$  when formulated with MPC80, E105, and E116, respectively. Changes in as-is protein during storage were not expected, but might have occurred from measurable moisture content change ( $P < 0.05$ ) (Table 4-2). Initial HPN bar moisture (%  $\pm$  SD) was  $17.9 \pm 0.9$ ,  $14.7 \pm 0.2$ , and  $14.9 \pm 0.1$  when formulated with MPC80, E105, and E116, respectively. Any increase during storage was due to more water in the bulk phase as verified by increasing  $a_w$  (Table 4-3). HPN bar  $a_w$  increased slightly during storage ( $P < 0.05$ ), but after day 3 no significant change was detected. HPN bars prepared with extruded MPC80 maintained lower  $a_w$  than the control when stored at 22°C. Increasing HPN bar  $a_w$  during storage was observed in other samples formulated with extruded MPC80 and was explained on a microstructural basis (Banach and others 2016, 2014). HPN bar color (Figure 4-2) change during storage was dependent on protein, time, and temperature ( $P < 0.05$ ). The HPN bars formulated with extruded MPC80 did not undergo significant total color change ( $\Delta E$ ) while stored at 22°C for 6 weeks (Table 4-3). Extrusion can destroy lysine, which limits its ability to participate in Maillard browning during HPN bar storage (Banach and others 2014). Sample pH was measured to determine if browning was possibly affected by differences in initial pH (Table 4-2). However, protein ingredient did not have an effect on pH ( $P > 0.05$ ), and although it decreased slightly during storage and was influenced by storage temperature, no trend with  $\Delta E$  was observed.

**Table 4-2 Moisture Content (%) and pH of the high-protein nutrition (HPN) bars after 0, 6, and 29 weeks at 22°C or 32°C**

Property	°C	Protein <sup>1</sup>	Week 0	Week 6	Week 29
Moisture (%)	22	MPC80	17.9 <sup>a,y</sup>	20.2 <sup>a,z</sup>	21.6 <sup>a,z</sup>
		E105	14.7 <sup>b,y</sup>	18.4 <sup>a,z</sup>	20.3 <sup>a,z</sup>
		E116	14.9 <sup>b,y</sup>	19.1 <sup>a,z</sup>	19.9 <sup>a,z</sup>
	32	MPC80	-	19.3 <sup>a,z</sup>	21.6 <sup>a,z</sup>
		E105	-	18.7 <sup>a,z</sup>	19.5 <sup>b,z</sup>
		E116	-	19.0 <sup>a,z</sup>	19.5 <sup>b,z</sup>
pH	22	MPC80	6.77 <sup>a,z</sup>	6.53 <sup>a,y</sup>	6.47 <sup>a,y</sup>
		E105	6.78 <sup>a,z</sup>	6.49 <sup>a,y</sup>	6.42 <sup>a,y</sup>
		E116	6.72 <sup>a,z</sup>	6.52 <sup>a,y</sup>	6.24 <sup>b,x</sup>
	32	MPC80	-	6.53 <sup>a,z</sup>	6.09 <sup>a,y</sup>
		E105	-	6.46 <sup>a,z</sup>	6.10 <sup>a,y</sup>
		E116	-	6.50 <sup>a,z</sup>	6.08 <sup>a,y</sup>

<sup>1</sup> MPC80, unmodified milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.

<sup>a,b</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column for each property at fixed temperature.

<sup>x,z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row for each property at fixed temperature.

**Table 4-3 Water activity ( $a_w$ ) and total color change ( $\Delta E$ ) of the high-protein nutrition (HPN) bars during 6 weeks storage at 22°C or 32°C**

Property	°C	Protein <sup>1</sup>	Week 0	Week 3 <sup>2</sup>	Week 1	Week 3	Week 6
$a_w$	22	MPC80	0.48 <sup>a,y</sup>	0.51 <sup>a,z</sup>	0.51 <sup>a,z</sup>	0.50 <sup>a,z</sup>	0.50 <sup>a,z</sup>
		E105	0.44 <sup>b,y</sup>	0.47 <sup>b,z</sup>	0.48 <sup>b,z</sup>	0.48 <sup>b,z</sup>	0.48 <sup>b,z</sup>
		E116	0.44 <sup>b,y</sup>	0.47 <sup>b,z</sup>	0.48 <sup>b,z</sup>	0.48 <sup>b,z</sup>	0.49 <sup>ab,z</sup>
	32	MPC80	-	-	0.51 <sup>a,z</sup>	0.50 <sup>a,z</sup>	0.50 <sup>a,z</sup>
		E105	-	-	0.49 <sup>b,z</sup>	0.50 <sup>a,z</sup>	0.50 <sup>a,z</sup>
		E116	-	-	0.49 <sup>b,z</sup>	0.50 <sup>a,z</sup>	0.49 <sup>a,z</sup>
$\Delta E$	22	MPC80	0.0 <sup>a,y</sup>	2.8 <sup>a,z</sup>	3.5 <sup>a,z</sup>	3.4 <sup>a,z</sup>	4.8 <sup>a,z</sup>
		E105	0.0 <sup>a,z</sup>	1.4 <sup>a,z</sup>	2.3 <sup>a,z</sup>	2.1 <sup>a,z</sup>	2.0 <sup>b,z</sup>
		E116	0.0 <sup>a,z</sup>	0.7 <sup>a,z</sup>	1.1 <sup>a,z</sup>	1.3 <sup>a,z</sup>	1.1 <sup>b,z</sup>
	32	MPC80	-	-	4.9 <sup>a,x</sup>	12.6 <sup>a,y</sup>	21.7 <sup>a,z</sup>
		E105	-	-	3.6 <sup>a,x</sup>	8.6 <sup>b,y</sup>	15.7 <sup>b,z</sup>
		E116	-	-	3.4 <sup>a,x</sup>	7.2 <sup>b,y</sup>	12.9 <sup>c,z</sup>

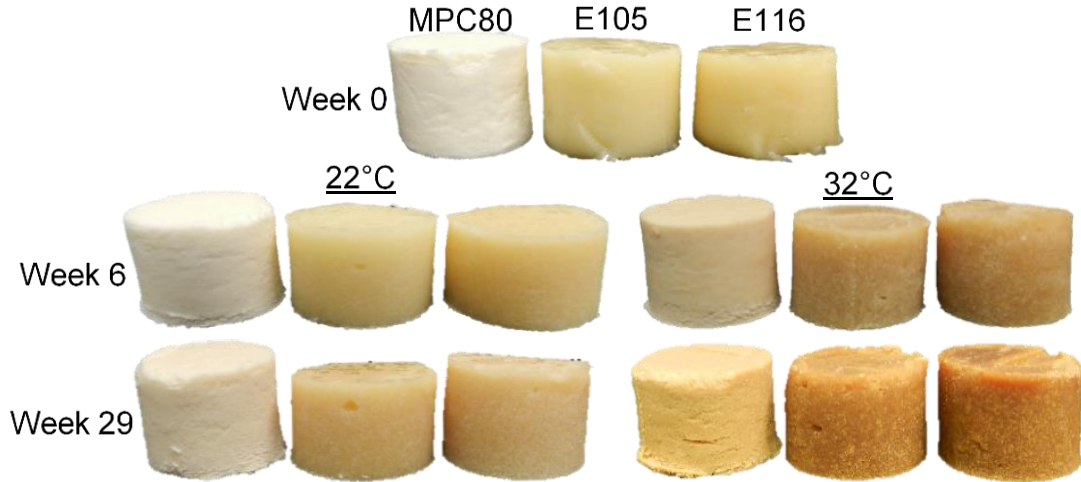
<sup>1</sup> MPC80, unmodified milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.

<sup>2</sup> Measurements taken after 3 day storage.

<sup>a-c</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column for each property at fixed temperature.

<sup>x,z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row for each property at fixed temperature.





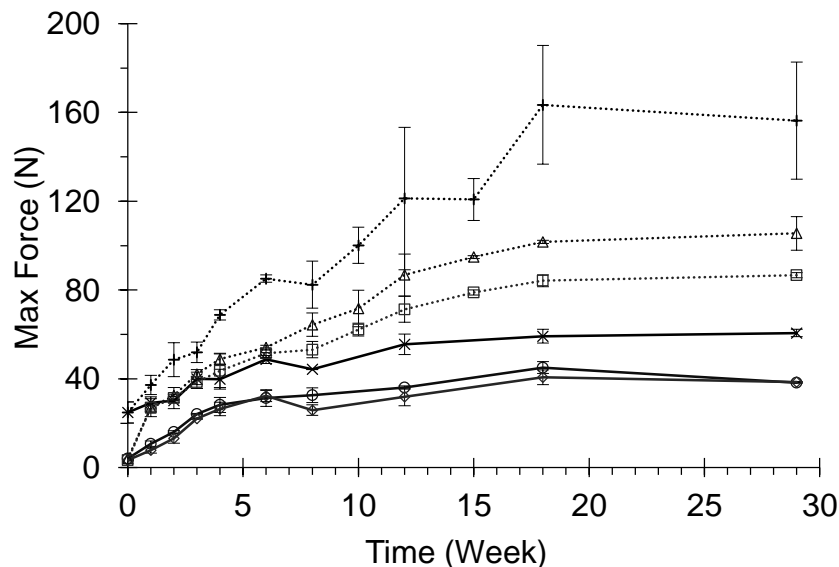
**Figure 4-2** Images of the model high-protein nutrition (HPN) bars on week 0, and on week 6 and week 29 after storage at 22°C or 32°C. MPC80, unmodified control milk protein concentrate with 80% protein was used to make the control HPN bar. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively, was used as the protein source in their respective HPN bars.

#### 4.5.3 High-protein Nutrition Bar Instrumental Texture

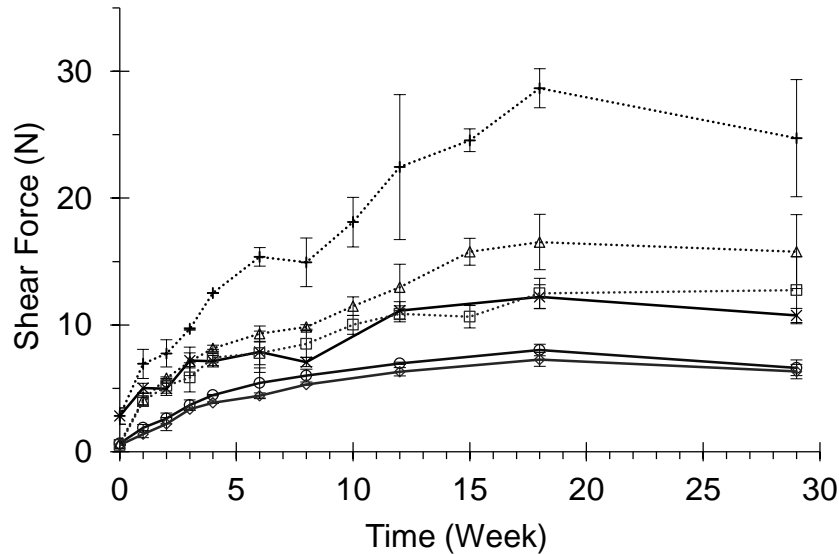
Select HPN bar instrumental attributes are reported based on convention in the field (i.e., max force, shear force), their relatability to the sensory panel measured attributes, and those TPA-generated attributes where differences between the samples were easily discerned. Protein ingredient, storage temperature, and storage time each had a significant effect ( $P < 0.05$ ) on max force (Figure 4-3), shear force (Figure 4-4), adhesiveness (Figure 4-5), cohesiveness (Figure 4-6), and crumbliness (Figure 4-7). Instrumental attribute correlation with the sensory responses are discussed in the following section.

Max force for MPC-formulated HPN bars was determined as the best instrumental output to represent sample firmness as perceived by a trained panel (Imtiaz and others 2012). HPN bar shearing was predicted to be more comparable to biting than puncture and TPA, and was used previously to describe hardness (McMahon and others 2009; Banach and others 2014). Max force and shear force showed that those samples formulated with extruded MPC80 remained softer than those formulated with unmodified

MPC80 (Figure 4-3 and Figure 4-4). At time 0, max force of the HPN bars formulated with extruded MPC80 was significantly lower than those formulated with control MPC80 ( $P < 0.05$ ). Max force increased with storage time and the increase was more pronounced at 32°C ( $P < 0.05$ ). Increasing shear force mirrored that of the max force, except that on day 0 there was no difference between the samples. The control always required more force to shear than the HPN bars prepared with extruded MPC80, of which the one made with E116 required less force to shear than the one formulated with E105. Significant differences in shear force between the control and extruded MPC80-formulated HPN bars were not observed until 12 and 4 weeks at 22°C and 32°C, respectively. Max and shear force measurement data showed that the HPN bars prepared with extruded MPC80 continued to remain softer than the control even as storage was extended to 7 months, which was much longer than, but in alignment with previous results (Banach and others 2014).



**Figure 4-3 Instrumental max force of the high-protein nutrition (HPN) bars during storage.** HPN bars formulated with MPC80 (×), E105 (○), and E116 (◇) were stored at 22°C (—). HPN bars formulated with MPC80 (+), E105 (Δ), and E116 (□) were stored at 32°C (···). MPC80, unmodified control milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.



**Figure 4-4 Instrumental shear force of the high-protein nutrition (HPN) bars during storage.** HPN bars formulated with MPC80 (×), E105 (○), and E116 (◇) were stored at 22°C (—). HPN bars formulated with MPC80 (+), E105 (△), and E116 (□) were stored at 32°C (···). MPC80, unmodified control milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.

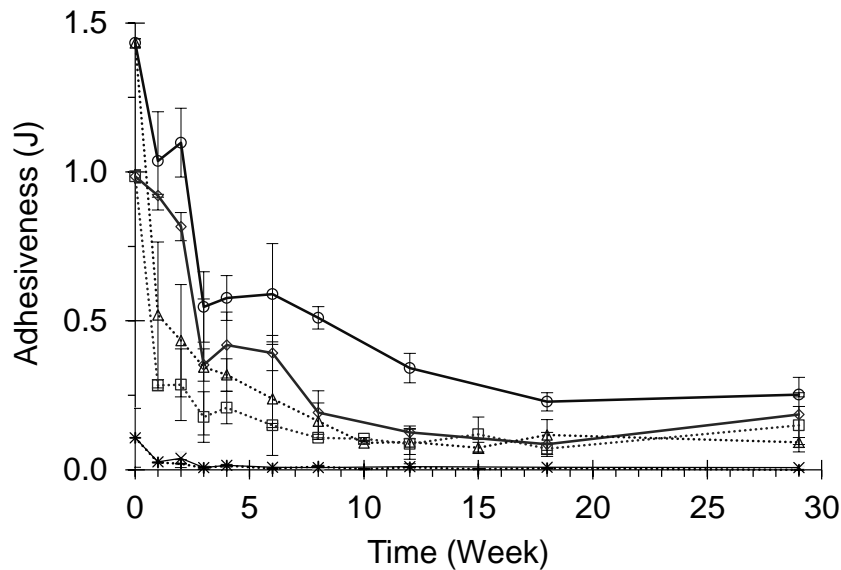
HPN bar and IMF literature has focused heavily on time dependent hardening.

Hardness is commonly measured using non-MPC formulated, hand-pressed samples in a sample cups and has been expressed as the peak force obtained while puncturing with a small diameter (3 to 5 mm) cylindrical probe to a predefined strain (35 to 50%) (Hogan and others 2012; Rao and others 2013b; Zhou and others 2008). Many other important texture attributes are overlooked using this methodology. Max force and force at maximum strain (i.e., 60%) convey important textural information. An elevated max force just prior to sample fracture followed by a weak force at 60% strain, a particularly common trait of the control HPN bar, indicated that a structural collapse occurred after initial fracture. A HPN bar of this nature would require a great deal of force to bite through, but would not contain much body or bar-like structure after the initial fracture. HPN bars prepared with extruded MPC80 rarely underwent this type of structural collapse during the early stages of the study. The degree to which a HPN bar holds

together without being too fluid has been referred to as “bar integrity” (Li and others 2008), but its quantification or that of related attributes such as cohesiveness or crumbliness, has been ignored by many HPN bar studies. Most studies have focused on whey protein utilization and since these proteins typically produce a more cohesive HPN bar than MPC, it is likely the main reason why “bar integrity” has been neglected and only hardening parameters have been reported.

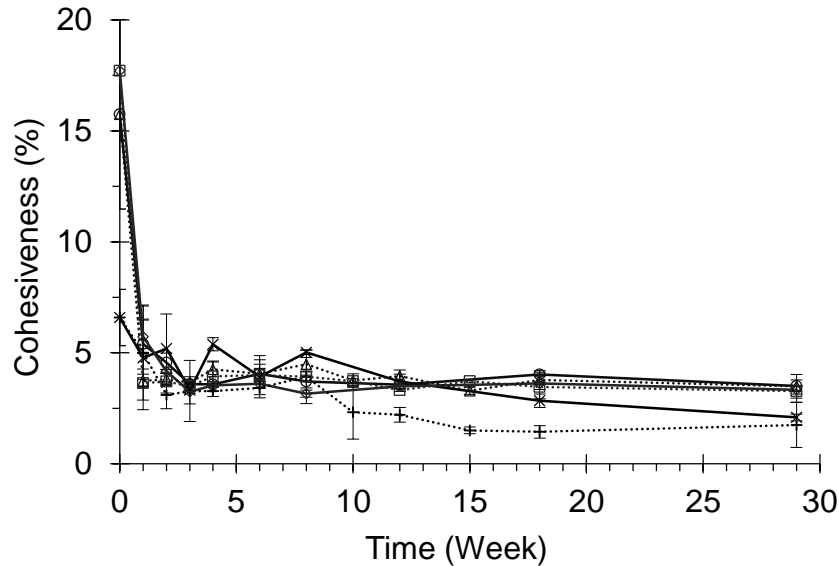
Instrumental probe withdrawal force and cohesiveness/crumbliness measured by a trained sensory panel were strongly correlated for MPC-formulated HPN bars (Imtiaz and others 2012). TPA withdrawal characteristics are related to adhesiveness (J) or the work necessary to overcome internal and external HPN bar attractive forces. A HPN bar that adheres to the probe also adheres to itself and forms a cohesive mass that holds its bar form. These three texture attributes, adhesiveness, cohesiveness, and crumbliness, are not always related and are reported separately in this study. Initial adhesiveness of the control was significantly lower than those HPN bars formulated with extruded MPC80, of which E105 produced a more adhesive system than E116 ( $P < 0.05$ ) (Figure 4-5). Adhesiveness of the HPN bars formulated with extruded MPC80 decreased quickly when stored at 32°C while at 22°C it slowly plateaued towards the same final value. At the end of storage, there were no differences between sample adhesiveness at 32°C ( $P > 0.05$ ), but at 22°C the HPN bar made with E105 was still the most adhesive ( $P < 0.05$ ). The HPN bars prepared with control MPC80 felt powdery to the touch and their adhesiveness values, which were near baseline, did not change significantly during storage ( $P > 0.05$ ). Excessive stickiness is not a favorable HPN bar attribute, but neither is powdery and dry. If increased adhesiveness translates to cohesiveness, extrusion would produce an

improved MPC80 ingredient since much criticism has focused on inducing unwanted crumbliness in HPN bars.



**Figure 4-5 Instrumental adhesiveness of the high-protein nutrition (HPN) bars during storage.** HPN bars formulated with MPC80 (×), E105 (○), and E116 (◇) were stored at 22°C (—). HPN bars formulated with MPC80 (+), E105 (Δ), and E116 (□) were stored at 32°C (···). MPC80, unmodified control milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.

TPA cohesiveness, or strength of internal interactions, measurements initially showed that extruded MPC80 produced HPN bars that were more cohesive than the control ( $P < 0.05$ ) (Figure 4-6). Unlike adhesiveness, TPA cohesiveness values decreased sharply after 1 week at both storage temperatures and were not differentiable for the remainder of storage. Around week 18 at 22°C and week 10 at 32°C, the control HPN bar became numerically less cohesive, based on TPA measurement, but the values were not significantly different from the other HPN bars. After one compression during the 2-bite test, the HPN bars were either permanently deformed or so crumbly that the area ratio was not well suited to differentiate cohesiveness.

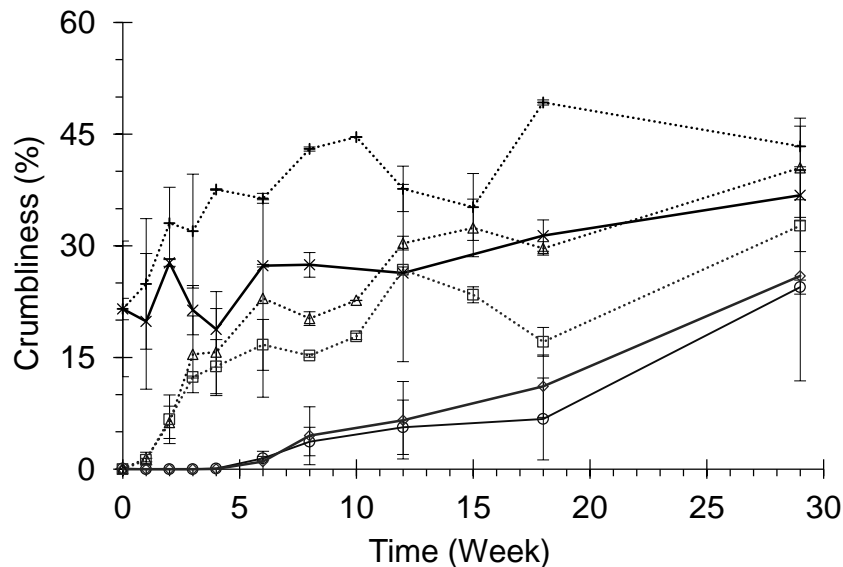


**Figure 4-6 Instrumental cohesiveness of the high-protein nutrition (HPN) bars during storage.** HPN bars formulated with MPC80 (×), E105 (○), and E116 (◇) were stored at 22°C (—). HPN bars formulated with MPC80 (+), E105 (△), and E116 (□) were stored at 32°C (···). MPC80, unmodified control milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.

Instead of relying on TPA cohesiveness or withdrawal force measurements for cohesiveness, an inverse relationship between crumbliness and cohesiveness was assumed. As HPN bar mass percentage passing the top sieve increased, crumbliness increased and in turn cohesiveness decreased. A large sieve aperture was selected since any crumb generation during a first or second bite would be undesirable and uncharacteristic of soft-textured HPN bars. Furthermore each HPN bar formulated with extruded MPC80 was completely retained on the top sieve until the sixth week at 22°C when underpass increased from essentially 0% to 1.2%. Sieved sample mass did not have normal distribution, therefore, geometric mean diameter was not calculated.

These crumbliness measurements (Figure 4-7) and its affiliated cohesiveness was better equipped to differentiate the HPN bars than TPA. Crumbliness of the HPN bars formulated with extruded MPC80 increased slowly while kept at 22°C whereas the increase was more pronounced at 32°C ( $P < 0.05$ ). At 22°C, the HPN bars formulated

with extruded MPC80 were always less crumbly than the control, but significance varied by time point when stored at 32°C. After 2 weeks at 32°C, which roughly simulated 17.3 weeks at ambient (Li and others 2008), crumbliness of the extruded MPC80 containing HPN bars increased to 6.5%. After 18 weeks at 22°C, average crumbliness of the HPN bars formulated with extruded MPC80 was 9.0%, and was quite similar to the value obtained at the simulated 17.3 weeks storage. Other texture attributes changed faster at elevated temperature storage and at many equivalent storage time points they were not differentiable from the control. After 29 weeks (~7 months) at room temperature, the HPN bars formulated with extruded MPC80 were less crumbly than the control and imparting cohesiveness makes extruded MPC80 more usable in these applications.



**Figure 4-7 Instrumental crumbliness of the high-protein nutrition (HPN) bars during storage.** HPN bars formulated with MPC80 (×), E105 (○), and E116 (◇) were stored at 22°C (—). HPN bars formulated with MPC80 (+), E105 (Δ), and E116 (□) were stored at 32°C (···). MPC80, unmodified control milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.

#### 4.5.4 High-protein Nutrition Bar Evaluation by the Trained Sensory Panel

The least squares means for sensory panel measured firmness, crumbliness, fracturability, hardness, and cohesiveness (Table 4-4) were significantly influenced by protein, temperature, and time ( $P < 0.05$ ). Panelists also measured mouth coating, or the powdery/chalky feeling left in one's mouth, but they were unable to distinguish any difference between the HPN bars ( $P > 0.05$ ). Commercial anchors for most texture attributes evaluated were readily available (Table 4-1). However, the in-mouth residual after swallowing or expectorating our HPN bars was not scalable using previously identified anchors (Meilgaard and others 2007). Our attempt to make anchors by varying the ratio of WPC80 to MPC80 in different HPN bars was not helpful for differentiating the samples. Similar properties (e.g., powderiness) were reported in other HPN bar sensory studies, as it cannot be measured by instrumental analysis (Childs and others 2007; Imtiaz and others 2012). Smoothness, stickiness, chewiness, dissolvability, tooth packing, denseness, adhesiveness, and visual appeal were not measured by the sensory panel, partly because they were not stressed during training and partly to avoid too many evaluation criteria.



**Table 4-4 Sensory attributes (cm) of the high-protein nutrition (HPN) bars during 6 weeks storage at 22°C or 32°C**

Attribute	°C	Protein <sup>1</sup>	Week 0	Week 1	Week 2	Week 3	Week 4	Week 6
Firmness	22	MPC80	6.7 <sup>a,y</sup>	9.4 <sup>a,z</sup>	10.2 <sup>a,z</sup>	9.0 <sup>b,z</sup>	9.5 <sup>a,z</sup>	9.3 <sup>a,z</sup>
		E105	2.0 <sup>b,x</sup>	3.6 <sup>b,xy</sup>	3.4 <sup>b,xy</sup>	4.8 <sup>b,yz</sup>	5.3 <sup>b,z</sup>	5.8 <sup>b,z</sup>
		E116	0.9 <sup>b,x</sup>	2.6 <sup>b,y</sup>	2.9 <sup>b,y</sup>	3.6 <sup>b,yz</sup>	3.6 <sup>c,yz</sup>	5.2 <sup>b,z</sup>
	32	MPC80	-	10.2 <sup>a,x</sup>	10.9 <sup>a,xy</sup>	12.0 <sup>a,yz</sup>	11.9 <sup>a,y</sup>	13.6 <sup>a,z</sup>
		E105	-	6.7 <sup>b,x</sup>	8.2 <sup>b,xy</sup>	9.9 <sup>b,z</sup>	9.3 <sup>b,yz</sup>	10.2 <sup>b,z</sup>
		E116	-	6.3 <sup>b,y</sup>	8.5 <sup>b,z</sup>	8.6 <sup>b,z</sup>	8.5 <sup>b,z</sup>	9.5 <sup>b,z</sup>
Crumbliness	22	MPC80	8.2 <sup>a,y</sup>	9.4 <sup>a,yz</sup>	10.7 <sup>a,z</sup>	10.0 <sup>a,yz</sup>	9.0 <sup>a,yz</sup>	9.7 <sup>a,yz</sup>
		E105	0.6 <sup>b,x</sup>	1.2 <sup>b,x</sup>	1.4 <sup>b,xy</sup>	2.1 <sup>b,xy</sup>	3.1 <sup>b,yz</sup>	4.2 <sup>b,z</sup>
		E116	0.3 <sup>b,y</sup>	0.9 <sup>b,xy</sup>	1.5 <sup>b,xy</sup>	2.1 <sup>b,xyz</sup>	2.4 <sup>b,yz</sup>	3.5 <sup>b,z</sup>
	32	MPC80	-	9.9 <sup>a,z</sup>	11.4 <sup>a,z</sup>	10.6 <sup>a,z</sup>	9.9 <sup>a,z</sup>	10.8 <sup>a,z</sup>
		E105	-	5.9 <sup>b,y</sup>	8.4 <sup>b,z</sup>	8.1 <sup>b,z</sup>	7.8 <sup>b,z</sup>	8.7 <sup>b,z</sup>
		E116	-	6.7 <sup>b,z</sup>	8.0 <sup>b,z</sup>	7.2 <sup>b,z</sup>	7.8 <sup>b,z</sup>	8.3 <sup>b,z</sup>
Fracturability	22	MPC80	5.2 <sup>a,z</sup>	6.9 <sup>a,z</sup>	6.7 <sup>a,z</sup>	5.9 <sup>a,z</sup>	6.1 <sup>a,z</sup>	6.9 <sup>a,z</sup>
		E105	1.8 <sup>b,y</sup>	1.9 <sup>b,y</sup>	2.2 <sup>b,yz</sup>	3.6 <sup>b,yz</sup>	3.8 <sup>b,yz</sup>	4.1 <sup>b,z</sup>
		E116	1.0 <sup>b,y</sup>	1.6 <sup>b,y</sup>	2.0 <sup>b,yz</sup>	2.8 <sup>b,yz</sup>	2.7 <sup>b,yz</sup>	3.8 <sup>b,z</sup>
	32	MPC80	-	6.2 <sup>a,y</sup>	8.0 <sup>a,yz</sup>	8.1 <sup>a,yz</sup>	7.7 <sup>a,yz</sup>	9.1 <sup>a,z</sup>
		E105	-	4.9 <sup>a,y</sup>	5.9 <sup>b,yz</sup>	6.6 <sup>a,yz</sup>	6.8 <sup>a,yz</sup>	7.7 <sup>ab,z</sup>
		E116	-	5.5 <sup>a,z</sup>	5.3 <sup>b,z</sup>	6.4 <sup>a,z</sup>	6.5 <sup>a,z</sup>	6.6 <sup>b,z</sup>
Hardness	22	MPC80	4.2 <sup>a,y</sup>	5.1 <sup>a,yz</sup>	5.1 <sup>a,yz</sup>	5.6 <sup>a,yz</sup>	5.2 <sup>a,yz</sup>	6.3 <sup>a,z</sup>
		E105	0.9 <sup>b,y</sup>	1.7 <sup>b,yz</sup>	1.7 <sup>b,yz</sup>	2.6 <sup>b,z</sup>	2.2 <sup>b,yz</sup>	2.6 <sup>b,z</sup>
		E116	0.5 <sup>b,y</sup>	1.5 <sup>b,yz</sup>	1.7 <sup>b,yz</sup>	2.0 <sup>b,yz</sup>	1.8 <sup>b,yz</sup>	2.5 <sup>b,z</sup>
	32	MPC80	-	4.9 <sup>a,x</sup>	6.1 <sup>a,xy</sup>	6.8 <sup>a,y</sup>	6.5 <sup>a,y</sup>	10.0 <sup>a,z</sup>
		E105	-	2.7 <sup>b,y</sup>	3.2 <sup>b,yz</sup>	3.8 <sup>b,yz</sup>	4.1 <sup>b,yz</sup>	4.4 <sup>b,z</sup>
		E116	-	3.1 <sup>b,z</sup>	3.0 <sup>b,z</sup>	3.8 <sup>b,z</sup>	3.8 <sup>b,z</sup>	4.5 <sup>b,z</sup>
Cohesiveness	22	MPC80	8.4 <sup>b,z</sup>	7.0 <sup>b,z</sup>	6.3 <sup>b,z</sup>	6.3 <sup>b,z</sup>	6.5 <sup>b,z</sup>	5.8 <sup>b,z</sup>
		E105	12.2 <sup>a,z</sup>	11.3 <sup>a,yz</sup>	11.2 <sup>a,yz</sup>	11.0 <sup>a,yz</sup>	10.9 <sup>a,yz</sup>	9.4 <sup>a,y</sup>
		E116	11.6 <sup>a,z</sup>	11.1 <sup>a,z</sup>	11.4 <sup>a,z</sup>	11.3 <sup>a,z</sup>	10.5 <sup>a,z</sup>	10.1 <sup>a,z</sup>
	32	MPC80	-	7.8 <sup>a,z</sup>	5.7 <sup>b,yz</sup>	5.4 <sup>a,yz</sup>	5.3 <sup>a,yz</sup>	5.1 <sup>a,y</sup>
		E105	-	8.1 <sup>a,z</sup>	8.4 <sup>a,z</sup>	7.1 <sup>a,z</sup>	7.0 <sup>a,z</sup>	6.3 <sup>a,z</sup>
		E116	-	9.0 <sup>a,z</sup>	7.8 <sup>ab,yz</sup>	7.1 <sup>a,yz</sup>	6.7 <sup>a,yz</sup>	5.6 <sup>a,y</sup>

<sup>1</sup> MPC80, unmodified milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively.

<sup>a-c</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column for each attribute at fixed temperature.

<sup>x-z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row for each attribute at fixed temperature.

HPN bar firmness and crumbliness were evaluated as in-hand parameters. Since HPN bars have a difficult-to-chew reputation, it would not be uncommon for a consumer to press on a HPN bar before purchase or consumption. An excessively firm sample or one that easily crumbles would not be appealing. At equivalent temperature and time stored, the HPN bars formulated with extruded MPC80 were softer and more cohesive than those prepared with control MPC80 ( $P < 0.05$ ). The HPN bars, especially those formulated with control MPC80, firmed quicker at 32°C ( $P < 0.05$ ). At this temperature, firmness did not change significantly after the second and third weeks for the HPN bars made with E116 and E105, respectively. The control HPN bar became firmer after 1 week storage at 22°C ( $P < 0.05$ ), after which its firmness did not change. Firmness of the extruded MPC80-containing HPN bars continued to increase after week 1 while kept at 22°C. Firmness was strongly correlated with instrumental max force ( $r = 0.87$ ) and shear force ( $r = 0.87$ ), and thus both instrumental techniques are representative of in-hand firmness (Table 4-5). The HPN bars formulated with extruded MPC80 maintained lower firmness than the control, even after 1 year of simulated storage.

**Table 4-5 Pearson correlation coefficients (r) for the instrumental and sensory panel measured high-protein nutrition (HPN) bar texture attributes**

Instrumental Attribute	Sensory Attribute				
	Firmness	Crumbliness	Fracturability	Hardness	Cohesiveness
Max Force	0.87***	0.76***	0.85***	0.84***	-0.84***
Cohesiveness	-0.48***	-0.40**	-0.48***	-0.39*	0.43**
Adhesiveness	-0.82***	-0.85***	-0.84***	-0.79***	0.83***
Crumbliness	0.86***	0.85***	0.84***	0.89***	-0.84***
Shear Force	0.87***	0.76***	0.83***	0.84***	-0.84***

\*\*\*  $P < 0.0001$ ; \*\*  $P < 0.001$ ; \*  $P < 0.05$

The panelists easily distinguished that the control was more crumbly than those HPN bars formulated with extruded MPC80 at fixed storage time and the same storage temperature ( $P < 0.05$ ). Panelists were not able to detect any significant change in the

control's crumbliness during storage ( $P > 0.05$ ). HPN bar crumbliness increased from 0.6 to 5.9 cm and from 0.3 to 6.7 cm after 1 week at 32°C when formulated with E105 and E116, respectively, after which no further changes in crumbliness were detected. At 22°C, in-hand crumbliness slowly increased for these two HPN bars and values at week 6 approached those obtained after 1 week at 32°C, which was similar to the previous estimate of 1 week at 32°C being equivalent to 8.7 weeks at room temperature (Li and others 2008). Sensory panel crumbliness data were strongly correlated ( $r = 0.85$ ) with the instrumental crumbliness data (Table 4-5). Similar to Imtiaz and others (2012), instrumental withdrawal energy, in the present study it is labeled adhesiveness, was inversely correlated ( $r = -0.85$ ) with crumbliness. Pieces or crumbs were unlikely generated during analysis of a more adhesive HPN bar. These data support that sieve analysis and mass percent finer than a specified sieve can be used in lieu of panelists to measure HPN bar crumbliness.

The panelists measured fracturability, hardness, and cohesiveness as in-mouth attributes. Compared with the in-hand measurements, less of each attribute-specific 15-cm line scale was used to differentiate the samples. This indicated that the HPN bars had greater textural similarity when evaluated in one's mouth. HPN bars formulated with extruded MPC80 fractured with less force between the panelists' incisors than the control each week at 22°C ( $P < 0.05$ ), but significance varied by time point at 32°C. Instrumental shearing with a 45° chisel blade was predicted to mimic one's incisors. However, fracturability had the strongest correlation with max force ( $r = 0.85$ ), which was slightly stronger than its correlation with shear force ( $r = 0.83$ ). Other correlations with fracturability were also strong, but they were inherent to the HPN bars used in this study.

For example, fracturability was correlated with instrumental crumbliness ( $r = 0.84$ ), but only because the HPN bars with higher fracture force, mainly those formulated with control MPC80, also tended to be more crumbly. By no means would a HPN bar with high crumbliness be implicated with a high fracture force. This happened in our study, but it is not a global property of the instrumental crumbliness test. Snapping, breaking, and fracturing are not typical texture attributes found in soft textured HPN bars, and extruded MPC80 helped reduce their presence.

Hardness, which was evaluated between each panelist's molars, of the control was greater than the HPN bars formulated with extruded MPC80 at each time point ( $P < 0.05$ ). Each HPN bar hardened significantly during storage ( $P < 0.05$ ) except for the sample formulated with E116 and stored at 32°C, where hardness did not change significantly between week 1 and week 6. At 22°C, the panelists did not detect significant hardening of the control HPN bar until week 6 and magnitude of change (2.1 cm) was just slightly greater than those formulated with E105 (1.7 cm) and E116 (2.0 cm). Sensory hardness measurements correlated strongly with max force ( $r = 0.84$ ) and shear force ( $r = 0.84$ ) (Table 4-5). Strong correlations with hardness were observed with other instrumental parameters. While those relationships in these particular HPN bars make sense, they do not transfer to all HPN bars. When evaluated in-mouth, the HPN bars formulated with extruded MPC80 were softer than the control.

Cohesiveness of mass was measured after 3 chews and it decreased during storage at both temperatures ( $P < 0.05$ ). Initially, the HPN bars formulated with extruded MPC80 were more cohesive than the control, but cohesiveness quickly decreased at 32°C. Extruded MPC80 produced HPN bars that maintained their structure more so than

the control after three chews while stored at 22°C. Sensory measured cohesiveness was inversely correlated with instrumental crumbliness ( $r = -0.84$ ), but it had the weakest correlation with TPA cohesiveness ( $r = 0.43$ ). TPA cohesiveness values were not representative of HPN bar cohesiveness and the newly proposed instrumental crumbliness assay better approximated in-mouth perceived cohesiveness. Although sieve analysis required timely weighing and reweighing sieves and was more involved than TPA alone, it is advantageous in the sense that it does not require panelists, which eliminates training, panelist commitment, and allows for non-food-grade modifications or ingredients to be thoroughly evaluated in HPN bars.

#### **4.6 Conclusions**

Extruded MPC80 performed more favorably in a model HPN bar when compared to the control. Instrumentally-measured max force and shear force and sensory-measured firmness and hardness showed that the HPN bars hardened during storage. HPN bar adhesiveness, cohesiveness, and crumbliness also changed during storage and their change may negatively impact HPN bar quality just as much as hardening. Sensory-measured hardness parameters, including firmness, fracturability, and hardness were correlated with instrumentally-measured max force and shear force. Sensory-measured crumbliness and cohesiveness were strongly correlated with the instrumental results from the newly implemented HPN bar crumbliness assay and it may be used to measure these two attributes in future HPN bar studies. Instrumental TPA was able to measure most of the reported texture attributes as perceived by humans. Extruded MPC80 produced HPN bars that were softer, more stable, and more cohesive than those prepared with the spray-dried control MPC80 even after extended storage.

#### 4.7 Acknowledgement

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## CHAPTER 5. TEXTURAL PERFORMANCE OF CROSSLINKED OR CALCIUM-REDUCED MILK PROTEIN INGREDIENTS IN MODEL HIGH-PROTEIN NUTRITION BARS

Modified from a manuscript submitted to the *Journal of Dairy Science*

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### 5.1 Abstract

Transglutaminase (Tgase) crosslinking, and calcium-reduction were investigated as ways to improve the texture and storage stability of high-protein nutrition (HPN) bars formulated with milk protein concentrate (MPC) and micellar casein concentrate (MCC). MPC and MCC crosslinked at ‘none,’ ‘low,’ and ‘high’ levels, and a reduced-calcium MPC (RCMPC) were each formulated into model HPN bars. HPN bar hardness, crumbliness, moisture content, pH, color, and water activity were measured during accelerated storage. HPN bars prepared with MPC were harder and more cohesive than those prepared with MCC. Higher levels of Tgase crosslinking decreased HPN bar hardening and led to improved cohesiveness during storage. RCMPC produced softer, yet crumblier HPN bars. Small textural differences were observed for the HPN bars formulated with the transglutaminase crosslinked proteins or RCMPC when compared with their respective controls. However, modification only slightly improved protein ingredient ability to slow hardening while balancing cohesion and likely require further improvement for increased applicability in soft-texture HPN bars.

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## 5.2 Practical Application

Transglutaminase-crosslinked MPC and MCC produced slightly softer, but still brittle model high-protein nutrition bars compared to their respective controls. High-protein nutrition bars prepared with micellar casein concentrate were more crumbly than those prepared with milk protein concentrate. Although still crumbly overall, a higher level of transglutaminase crosslinking decreased the fines produced during instrumental compression and may offer improved cohesiveness in commercial high-protein nutrition bars. High-protein nutrition bars formulated with calcium reduced milk protein concentrate were softer, and more crumbly and powdery when compared with their respective control, but were still powdery and crumbly overall.

## 5.3 Introduction

High-protein foods are popular amongst consumers seeking satiety, increased muscle mass, or decreased risk of sarcopenia (Sloan 2012). Consumers are turning to high-protein nutrition (HPN) bars to conveniently add more protein to their diet. HPN bars have utilized new, trendy protein sources (e.g., insect), but have traditionally relied on dairy and soy ingredients such as concentrates, isolates, and hydrolysates. Protein content typically ranges from 20-50% (w/w) whereas carbohydrates (e.g., high-fructose corn syrup), polyols (e.g., glycerol), sugar alcohols (e.g., sorbitol), and lipids (e.g., palm oil) comprise the rest of the formulation (Imtiaz and others 2012; McMahon and others 2009).

It is well known that HPN bars, especially those prepared with high-protein milk protein concentrates (MPCs;  $\geq 80\%$  protein w/w), are texturally unstable during storage (Imtiaz and others 2012; Loveday and others 2009). Specifically, HPN bars formulated

at 30% protein (w/w) using MPC that contained 80% protein (MPC80) rapidly hardened and lost cohesiveness during storage (Banach and others 2016a, 2014). Nutritionally, MPCs maintain the casein-to-whey protein ratio (80:20) of typical bovine skim milk and are a complete protein with higher digestible indispensable amino acid score (1.18) than whey protein isolate (WPI; 1.09), whey protein concentrate (WPC; 0.97), soy protein isolate (SPI; 0.90), and pea protein concentrate (PPC; 0.82) (Rutherford and others 2015). MPCs' nutritional aspects and their ability to be ultra-filtered directly from skim milk independent of other processes make HPN bars a primary target application.

Micellar casein concentrates (MCCs) are produced by micro-filtering skim milk such that the final spray dried powder has an elevated casein-to-whey protein ratio (92:8) (Dairy Management Inc., 2015). MCCs, which are undefined by the global trade atlas and the United States Food and Drug Administration (FDA), are less studied than MPCs (Lagrange and others 2015). Model HPN bars (45% protein w/w) prepared with MCC remained softer than those formulated whey protein hydrolysate,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, WPI, or sodium caseinate after 10 d at 37°C (Hogan and others 2012). Agglomerated MCC produced HPN bars (40-50% MCC powder w/w) that were less dough-like and less prone to hardening than those prepared with non-agglomerated MCC over 7 d storage at 37°C (Hogan and others 2012). Further validation of MCC in HPN bars is needed since based on protein composition similar textural performance as MPCs would be expected in these applications.

HPN bar texture changes during storage cannot be attributed to one mechanistic cause, and although multicomponent (e.g., protein, carbohydrate, fats, minerals, vitamins), most work has focused on the protein source and ingredient type while the

system hardens. Suggested HPN bar hardening mechanisms include moisture migration between constituents, limited free water for complete protein plasticization, entropy-driven macronutrient phase separations, internal disulfide bond formations, and Maillard-induced protein aggregations (Zhou and others 2013, 2008; McMahon and others 2009; Loveday and others 2009). Mineral (e.g.,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ) addition or removal, including those natively associated with the protein (e.g.,  $\text{Ca}^{2+}$ ), may alter the protein's structure, increase internal moisture migration, and subsequently accelerate HPN bar texture change (Book 2008). Protein hydrolysis has been the main modification technique to impart textural stability during HPN bar storage (Rao and others 2016; McMahon and others 2009). Proprietarily modified (Imtiaz and others 2012) and extruded MPCs (Banach and others 2014) also improved textural stability when incorporated into model HPN bars. MPC and MCC must be modified to not only slow hardening, but also to maintain cohesion during HPN bar storage in order to be a preferred protein source for these applications.

Most protein powders, especially MPCs, are modified to improve solubility (Mao and others 2012; Sikand and others 2013) as well as dependent functional properties (e.g., emulsification, foaming). However, there is no clear relation between these properties and performance in intermediate-moisture foods (IMFs) such as HPN bars.

Transglutaminase (Tgase), an enzyme produced by *Streptovercillium mobaraense*, has been used to improve the texture of solid foods such as restructured meats, fish pastes, yogurts, breads, and confectionaries (Gaspar and de Góes-Favoni 2015; Kieliszek and Misiewicz 2014). Tgase builds texture by crosslinking glutamine residues with intra- or inter-protein lysine residues, which occurs faster and with greater specificity than its acyl

transfer and deamidation processes (Gaspar and de Góes-Favoni 2015; DeJong and Koppelman 2002). Tgase treatment has historically been applied to processed foods seeking textural improvement, but is not commonly used to functionalize protein ingredients for multiple applications (DeJong and Koppelman 2002). Previously, MPC and MCC were crosslinked by Tgase and functionality was evaluated in processed cheese and yogurt (Salunke and others 2013a, 2013b; Salunke 2013), but they were not evaluated in HPN bars.

Tgase crosslinked proteins typically have increased water holding capacity (WHC) (Gaspar and de Góes-Favoni 2015). The effect of increased WHC on HPN bar texture is unknown as water may move towards the protein as driven by water activity ( $a_w$ ) gradient (Hazen 2010; Book 2008; Li and others 2008; Gautam and others 2006) or towards the low molecular weight, poly-hydroxyl compounds by osmotic pull (Loveday and others 2009). Reduced-calcium MPC (RCMPC) was manufactured by carbon dioxide acidification of milk protein retentate during ultra-filtration which solubilized micellar calcium and phosphate (Marella and others 2015). RCMPC had improved solubility which may allow for more rapid hydration during HPN bar production that along with its lower calcium, ash, and net negative charge may limit moisture migration and slow moisture-induced hardening during HPN bar storage.

This study was designed to compare relative textural performance of Tgase crosslinked MPC and MCC, and RCMPC, in a previously used model HPN bar formulation (Banach and others 2014). Crosslinked protein ingredients will have fewer amine groups available for participation in the Maillard browning reaction (Gerrard 2002), which may limit formation of protein aggregates that have been associated with

HPN bar texture change (Zhou and others 2013; Banach and others 2016b). Model HPN bars (30% protein w/w) were prepared with MPC and MCC previously Tgase crosslinked at 'none,' 'low,' and 'high' levels and RCMPC, and hardness, crumbliness, moisture content, pH, color, and  $a_w$  were measured during storage.

## 5.4 Materials and Methods

### 5.4.1 Materials

The MPC and MCC powders with 'none' (N), 'low' (L), and 'high' (H) Tgase crosslink levels, including MPC-N (74.4% protein, 3.7% moisture, 8.9% lactose), MPC-L (74.4% protein, 3.9% moisture, 8.7% lactose), MPC-H (74.3% protein, 2.7% moisture, 8.6% lactose), MCC-N (77.6% protein, 3.2% moisture, 4.4% lactose), MCC-L (77.6% protein, 3.6% moisture, 4.5% lactose), and MCC-H (76.9% protein, 3.2% moisture, 4.5% lactose), and the RCMPC (71.9% protein, 3.4% moisture, 14.4% lactose) were previously produced (Marella and others 2015; Salunke 2013). Urea, SDS,  $\beta$ -mercaptoethanol, bromophenol blue, and glycerol (99.8% glycerol, 0.1% water) were obtained from Fisher Scientific (Waltham, MA). Supplies for SDS-PAGE, including tris, Precision Plus Protein™ Standard, Any kD™ TGX™ precast gels, Bio-Safe™ Coomassie Stain, and 10x tris/glycine/SDS running buffer, were obtained from Bio-Rad, Inc. (Hercules, CA). Lactose (200-mesh, 99.8% lactose, Glanbia Nutritionals, Twin Falls, ID), maltitol syrup (Lycasin®80/55, 51.7% D-maltitol, 3.0% D-sorbitol, 24.5% water, Roquette America, Keokuk, IA), non-hydrogenated palm oil (SansTrans®39, IOI Lodens Croklaan, Channahon, IL), and high-fructose corn syrup (CornSweet®55, 55% fructose, 41% dextrose, 4% higher saccharides, 23% water, Archer Daniels Midland, Decatur, IL) were donated for use in this study.

#### *5.4.2 Brief Description of Milk Protein Ingredient Modification*

A full description of protein powder production and modification is available elsewhere (Marella and others 2015; Salunke 2013; Salunke and others 2012). Low (i.e., MPC-L, MCC-L) and high (i.e., MPC-H, MCC-H) crosslinking was accomplished by treating retentates with 0.3 and 3.0 Tgase units per g protein, respectively, for 25 min at 50°C, which was followed by enzyme inactivation at 72°C for 10 min. The controls (i.e., MPC-N, MCC-N) were not treated with Tgase. Separately, RCMPC was produced by injecting skim milk with carbon dioxide gas (2,200 ppm), which was then ultrafiltered and diafiltered (pH 5.7), and, like all the protein powders used in this study, was spray dried.

#### *5.4.3 Transglutaminase Crosslink Verification by SDS-PAGE*

Modified Proteins were dissolved at 6.7 mg protein per mL in tris buffer (50 mM; pH 8.0) with denaturants (8 M urea, 2% SDS, 5%  $\beta$ -mercaptoethanol). After being mixed for 4.5 h, protein was diluted to ~4 mg per mL. The solutions were centrifuged at 15,000 $\times$ g for 15 min and the supernatant was diluted two-fold with 2x reduced sample buffer (125 mM tris, 8 M urea, 20% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue). Samples (4  $\mu$ L) and a molecular weight standard (10  $\mu$ L) were loaded onto precast gels and were electrophoresed at 100 V for 70 min. The proteins were fixed, stained, and de-stained as described elsewhere (Banach and others 2016b).

#### *5.4.4 Model High-protein Nutrition Bar Preparation*

HPN bars (30% protein w/w) were prepared (n = 3) with each control, Tgase crosslinked, and RCMPC ingredient serving as the sole protein source in each 250 g batch. Each HPN bar formulation was first standardized to 6% lactose (w/w) by

combining the protein powder (251-271 g) with lactose (0-28 g). 50.6 g Glycerol, 26.9 g maltitol syrup, and 1.0-2.2 g distilled water were stirred into the dry ingredients. Forty-three g non-hydrogenated palm oil and 21.8 g high-fructose corn syrup were heated together until all the fat melted, which was then mixed into the other constituents. HPN bar dough was pressed into cylindrical molds (ID = 21 mm; H = 13 mm) and  $a_w$  sample cups, and were transferred to 32°C storage the following day. More details about HPN bar production are available elsewhere (Banach and others 2014).

#### *5.4.5 High-protein Nutrition Bar Texture (Hardness and Crumbliness) Measurement*

Measurements were made on day 0, 7, 16, 28, and 42 after equilibrating the HPN bars to room temperature (22°C). Each cylindrical HPN bar sample was compressed two times (i.e., texture profile analysis; TPA) to 60% strain at crosshead speed of 2 mm s<sup>-1</sup> with a flat plate while force versus time data were recorded (TA-XT2, Texture Technologies, Scarsdale, NY). Hardness was reported as the maximum force (N) during the first compression. After compression, the sample was transferred to a stack of 3-inch sieves and was mechanically shaken for 30 s (speed 3, Shaker #18480, CSC Scientific Sieve, Fairfax, VA). HPN bar crumbliness was reported as the mass percent finer than the top sieve (No. 3.5) with 5.6 mm aperture (Banach and others 2016a). HPN bar samples that were too hard for the analyzer to compress to 60% strain were not analyzed for crumbliness. When texture analyzer's load cell maxed out, hardness was specified as the force just prior to stopping. Additional sample measurements ( $n \geq 3$ ) were attempted as availability allowed.



#### 5.4.5 High-protein Nutrition Bar Color, Water Activity, pH, and Moisture Content Measurement

HPN bar color and  $a_w$  were measured on day 0, 2, 7, 16, and 42 as previously described (Banach and others 2014).  $a_w$  was also measured immediately after manufacture (day -1). HPN bar dispersions were prepared in Millipore water (20% w/w) and pH was measured after mixing for 16 h. 2 g of each HPN bar ( $n = 2$ ) was dried at 102°C for 24 h on day 0, 7, 16, and 42 and moisture content was calculated by difference.

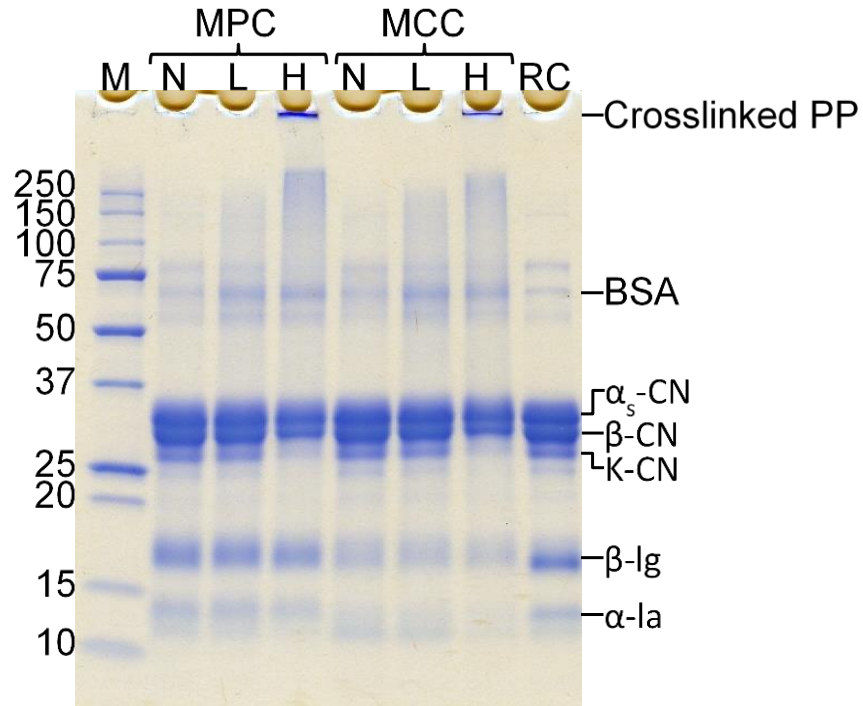
#### 5.4.6 Statistical Analyses

All statistical analyses were conducted using SAS® software (version 9.4, SAS Institute Inc., Cary, NC). Log-transformed hardness measurements were analyzed using the Lifereg procedure. Protein (i.e., MPC, MCC), crosslink level (i.e., none, low, high), storage day (i.e., 0, 7, 16, 28, 42), all two-way interactions, and preparation were the independent variables. In instances when the load cell maxed out (~240 N), the measurement was designated as the right-censoring value. Differences between least squares means (ls-means) were determined, unless otherwise stated, using Tukey's adjusted  $P$ -value ( $P < 0.05$ ). For HPN bar crumbliness analysis, protein, crosslink, and day were categorized into one variable since some protein  $\times$  crosslink  $\times$  storage day combinations were inestimable. That is every HPN bar sample tested on that day from each preparation failed to fracture. Ls-mean estimate statements were written to determine if differences between relevant ls-means were significant ( $P < 0.05$ ). Moisture content,  $a_w$ , pH, and  $L^*$  measurements of all the HPN bars were modeled using the mixed procedure. Protein ingredient (i.e., MPC-N, MPC-L, MPC-H, MCC-N, MCC-L, MCC-H, RCMPC) and time were the independent variables, and HPN bar preparation was set as the random effect.

## 5.5 Results and Discussion

### 5.5.1 Verification of Transglutaminase Mediated Crosslink Formation with SDS-PAGE

As expected, the SDS-PAGE profiles of the controls and RCMPC did not contain any polymerized or aggregated proteins (Figure 5-1). MPC and MCC were both crosslinked by Tgase and the portion of crosslinked protein increased with applied enzyme concentration. Highly crosslinked protein polymers, with molecular weight greater than 250 kDa, were unable to enter the gel and were only found in MPC-H and MCC-H. Vertical protein band smearing, an indicator of protein polymerization (Hsieh and Pan 2012), occurred between the 50 kDa marker through just above or just below the 250 kDa marker for the high-level or low-level Tgase crosslinked protein ingredients, respectively. A ten-fold increase in Tgase application increased protein polymer formation between 50-250 kDa, as visualized by increased stain intensity, and produced high molecular weight polymers incapable of permeating into the gel. However, when MPC-L and MCC-L are compared to their controls, that is MPC-N and MCC-N, respectively, they each contained a higher concentration of crosslinked protein with molecular weight between 50-250 kDa.



**Figure 5-1 Reduced SDS-PAGE of transglutaminase crosslinked milk protein concentrate (MPC), micellar casein concentrate (MCC), and reduced-calcium MPC (RCMPC).** N, L, and H, indicate none, low, and high transglutaminase crosslink levels, respectively. M, a molecular weight marker (kDa). Crosslinked PP, transglutaminase crosslinked protein polymers too large to enter the gel. CN, caseins from high to low molecular weight include:  $\alpha_{s2}$ ,  $\alpha_{s1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -Ig and  $\alpha$ -la, beta-lactoglobulin and alpha-lactalbumin, respectively.

$\beta$ -,  $\kappa$ -,  $\alpha_{s1}$ -, and  $\alpha_{s2}$ -casein in MPC and MCC served as the primary substrates for Tgase to crosslink since the globular whey proteins, including  $\beta$ -lactoglobulin ( $\beta$ -Ig),  $\alpha$ -lactalbumin ( $\alpha$ -la), and bovine serum albumin (BSA), are less crosslinkable due to structural constraints (Hsieh and Pan 2012). Since MCC is richer in casein compared to MPC, it should be more susceptible to Tgase crosslinking, but this was not readily apparent by SDS-PAGE. Corresponding with the newly formed protein polymer concentration, Tgase only slightly polymerized the caseins when applied at a low concentration and hence the SDS-PAGE protein profiles of MPC-L and MCC-L closely matched their controls. Tgase treatment polymerized essentially all the  $\kappa$ -casein in MPC-H and MCC-H, whereas the  $\beta$ -casein and the  $\alpha_s$ -caseins were only partially crosslinked.

Fresh raw skim milk casein susceptibility to Tgase crosslinking was previously determined as  $\beta > \kappa > \alpha_{s1} > \alpha_{s2}$  (Hsieh and Pan 2012). Another study revealed  $\kappa$ -casein was polymerized prior to all the  $\beta$ -casein in Tgase-treated reconstituted milk (Smiddy and others 2006). MPC and MCC  $\kappa$ -casein was polymerized more easily than the other caseins since it preferentially exists on the outside of the micelle and was more accessible to Tgase than the interiorly located caseins (Smiddy and others 2006). A truncated Tgase polymerization time of 30 min, which is more conducive for mass production, was insufficient to crosslink all the  $\beta$ -casein in either the MPC or MCC retentate, even though a portion of it is located on the micelle's exterior (Smiddy and others 2006).  $\beta$ -lg and  $\alpha$ -la were also polymerized by Tgase, as was previously observed (Hsieh and Pan 2012), but not nearly to the same extent as the caseins as their bands persisted on SDS-PAGE gel. Whey protein polymerization might contribute to the increased concentration of crosslinked protein polymers in MPC-H when compared to MCC-H.

SDS-PAGE analysis confirmed that MPC and MCC were both crosslinked at 'high' and 'low' levels. It is not possible to predict protein ingredient performance in HPN bars based solely upon their SDS-PAGE profiles. Protein hydrolysates soften initial HPN bar texture (Rao and others 2013), but with lower molecular weight and no protein aggregates, the system exists in the rubbery state which is prone to disulfide and Maillard browning induced protein aggregations that have been related to textural hardening during storage (Zhou and others 2013, 2008). Tgase modified MPC and MCC possess altered functionality (Salunke 2013) which will alter HPN bar texture. HPN bar stability might be conferred by limiting chemical reactivity by way of increased molecular weight and by preventing the internal production of Maillard- induced protein aggregates.

### 5.5.2 High-protein Nutrition Bar Moisture Content, pH, and L\* Color Values during Storage

HPN bar moisture content, averaged across days 0 and 42, was 16.7% and was not significantly influenced by protein ingredient or storage time (Table 5-1), which ruled out moisture loss as a contributor to texture change. HPN bar pH did not change during storage ( $P > 0.05$ ) (Table 5-1). On days 0 and 42, the HPN bar made with RCMPC, which was acidified during protein ingredient production, had lower pH than the other HPN bars ( $P < 0.05$ ). L\* lightness values decreased ( $P < 0.05$ ) as the samples browned by the Maillard reaction during storage (Table 5-1). On days 0 and 42, the HPN bar prepared with RCMPC had the lowest L\* value since slightly acidified dairy powders brown faster during storage (Dattatreya and Rankin 2006). Similar to L\*, the a\* and b\* color values (data not shown) of each HPN bar did not differ from their control after equivalent storage. Lower pH of RCMPC and fewer free amines present in the crosslinked protein ingredients did not slow the visual aspect of Maillard browning. Color compounds do not show through until the late stages of the reaction; regardless, it was unlikely that the development of Maillard-induced protein aggregates (Zhou and others 2013) was slowed by using these modified protein ingredients. After equivalent storage, each HPN bar likely contained a similar concentration of Maillard-induced protein aggregates and any apparent textural differences would be attributable to another aspect of the modified protein ingredient. The aesthetic aspect of color change is of minor importance as it and any potential off-flavors generated are masked by colorings and flavorings added to commercial products (Rao and others 2013).

**Table 5-1 High-protein nutrition (HPN) bar (30% protein w/w) moisture content (%), pH, and L\* color values on day 0 and after 42 d at 32°C**

Protein <sup>1</sup>	Moisture		pH		L*	
	Day 0	Day 42	Day 0	Day 42	Day 0	Day 42
MPC-N	16.1 <sup>a,z</sup>	16.7 <sup>a,z</sup>	6.6 <sup>a,z</sup>	6.5 <sup>a,z</sup>	87.5 <sup>a,z</sup>	79.5 <sup>ab,y</sup>
MPC-L	16.4 <sup>a,z</sup>	17.0 <sup>a,z</sup>	6.5 <sup>a,z</sup>	6.5 <sup>a,z</sup>	87.7 <sup>a,z</sup>	78.5 <sup>bc,y</sup>
MPC-H	17.5 <sup>a,z</sup>	16.3 <sup>a,z</sup>	6.5 <sup>a,z</sup>	6.4 <sup>a,z</sup>	88.5 <sup>a,z</sup>	79.0 <sup>abc,y</sup>
MCC-N	17.0 <sup>a,z</sup>	16.8 <sup>a,z</sup>	6.3 <sup>ab,z</sup>	6.5 <sup>a,z</sup>	88.3 <sup>a,z</sup>	79.9 <sup>ab,y</sup>
MCC-L	16.5 <sup>a,z</sup>	17.0 <sup>a,z</sup>	6.6 <sup>a,z</sup>	6.6 <sup>a,z</sup>	87.7 <sup>a,z</sup>	81.3 <sup>a,y</sup>
MCC-H	16.9 <sup>a,z</sup>	17.4 <sup>a,z</sup>	6.4 <sup>a,z</sup>	6.5 <sup>a,z</sup>	88.9 <sup>a,z</sup>	81.2 <sup>a,y</sup>
RCMPC	16.4 <sup>a,z</sup>	16.0 <sup>a,z</sup>	6.0 <sup>b,z</sup>	5.9 <sup>b,z</sup>	84.4 <sup>b,z</sup>	76.8 <sup>c,y</sup>

<sup>1</sup> The HPN bars (30% protein w/w) were formulated with milk protein concentrate (MPC), micellar casein concentrate (MCC), or reduced-calcium MPC (RCMPC). N, L, and H, indicate none, low, and high transglutaminase crosslink levels, respectively.

<sup>a-c</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>y-z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row for each attribute.

**Table 5-2 High-protein nutrition (HPN) bar (30% protein w/w) water activity ( $a_w$ ) during storage at 32°C**

Protein <sup>1</sup>	Day -1 <sup>2</sup>	Day 0	Day 2	Day 7	Day 16	Day 42
MPC-N	0.39 <sup>b,x</sup>	0.43 <sup>bc,y</sup>	0.44 <sup>b,yz</sup>	0.44 <sup>bcd,yz</sup>	0.46 <sup>ab,z</sup>	0.45 <sup>a,z</sup>
MPC-L	0.39 <sup>ab,x</sup>	0.42 <sup>cd,y</sup>	0.44 <sup>b,z</sup>	0.44 <sup>cd,z</sup>	0.45 <sup>b,z</sup>	0.45 <sup>a,z</sup>
MPC-H	0.41 <sup>a,x</sup>	0.44 <sup>b,y</sup>	0.45 <sup>ab,yz</sup>	0.45 <sup>abc,yz</sup>	0.47 <sup>ab,z</sup>	0.46 <sup>a,yz</sup>
MCC-N	0.41 <sup>a,y</sup>	0.46 <sup>a,z</sup>	0.47 <sup>a,z</sup>	0.46 <sup>a,z</sup>	0.47 <sup>a,z</sup>	0.47 <sup>a,z</sup>
MCC-L	0.40 <sup>ab,x</sup>	0.44 <sup>b,b</sup>	0.46 <sup>ab,z</sup>	0.45 <sup>ab,z</sup>	0.46 <sup>ab,z</sup>	0.46 <sup>a,z</sup>
MCC-H	0.40 <sup>ab,x</sup>	0.43 <sup>b,y</sup>	0.46 <sup>ab,z</sup>	0.46 <sup>ab,z</sup>	0.46 <sup>ab,z</sup>	0.46 <sup>a,z</sup>
RCMPC	0.36 <sup>c,w</sup>	0.40 <sup>d,x</sup>	0.42 <sup>c,yz</sup>	0.43 <sup>d,z</sup>	0.43 <sup>c,yz</sup>	0.41 <sup>b,xy</sup>

<sup>1</sup> The HPN bars were formulated with milk protein concentrate (MPC), micellar casein concentrate (MCC), or reduced-calcium MPC (RCMPC). N, L, and H, indicate none, low, and high transglutaminase crosslink levels, respectively.

<sup>2</sup> Day -1 indicates the day of HPN bar manufacture whereas day 0 was when samples were moved into 32°C storage.

<sup>a-d</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

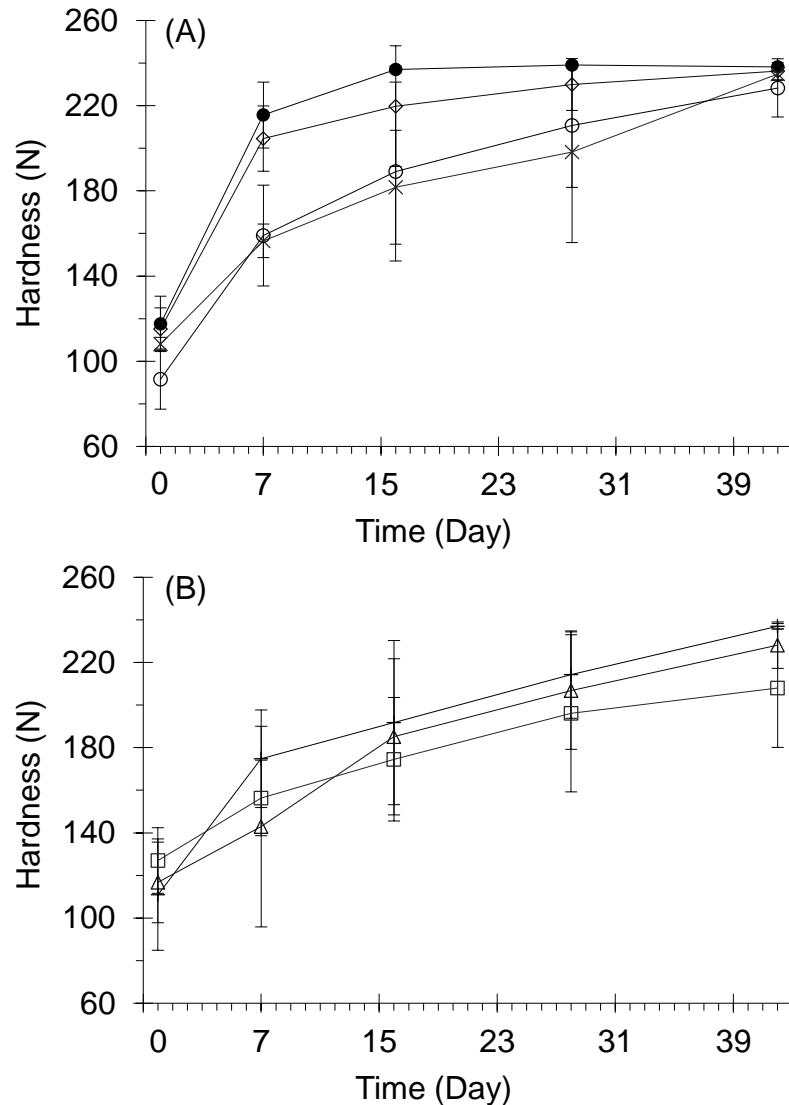
<sup>y-z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

Average HPN bar  $a_w$  on day manufacture (i.e., day -1) was 0.40, which increased to 0.43 in less than 24 h (i.e., day 0) and to 0.45 after 2 days (Table 5-2). These low magnitude increases in  $a_w$  were similar to those observed for other HPN bars, but such a small increase is difficult to relate to overall texture change (Banach and others 2014; McMahon and others 2009).  $a_w$  of each HPN bar was lower than expected, which may have factored into the low level of sample browning.

### *5.5.3 Texture (Hardness and Crumbliness) Changes in High-protein Nutrition Bar during Storage*

#### *5.5.3.1 Transglutaminase Crosslinked MPC and MCC*

The HPN bars hardened during storage (Figure 5-2) and in addition to time, hardness was significantly influenced by protein, crosslink level, and their two-way interactions ( $P < 0.05$ ). The HPN bars hardened quicker than expected based on a previous report (Banach and others 2014). Incompressibility occurred earlier in storage, around day 16, for the HPN bars formulated with MPC-N, for which additional sample measurements did not initiate sample fracture. HPN bars from different preparations became too hard for the texture analyzer on different testing days which was due to the effect of preparation ( $P < 0.05$ ). When additional samples were measured, some tended to fracture while others remained incompressible. Inconsistency made it statistically unjustified to include the three-way interaction term (i.e., protein  $\times$  crosslink  $\times$  day) in the Lifereg model and limited hardness contrasts to main effects and two-way interactions.



**Figure 5-2 High-protein nutrition (HPN) bar mean hardness during storage at 32°C.** HPN bars were formulated at 30% protein (w/w) using MPC-N (●), MPC-L (◇), MPC-H (×), RCMPC (○), MCC-N (+), MCC-L (△), or MCC-H (□). MPC, milk protein concentrate (A). MCC, micellar casein concentrate (B). N, L, and H, indicate none, low, and high transglutaminase crosslink levels, respectively. RCMPC, reduced-calcium milk protein concentrate. Error bars represent  $\pm 1$  SD ( $n = 3$ ).

HPN bar storage for 42 d at 32°C has been used to approximate 1 year at 22°C (Li and others 2008, McMahon and others 2009) and at that rate 1 week at 32°C is ~8.7 weeks or ~2 months at 22°C. Any substantial hardening within 2 months of manufacture would be unacceptable for a product whose target shelf life is 1 year. On day 0, MPC formulated HPN bars had mean hardness of 113 N and were not significantly ( $P > 0.05$ )

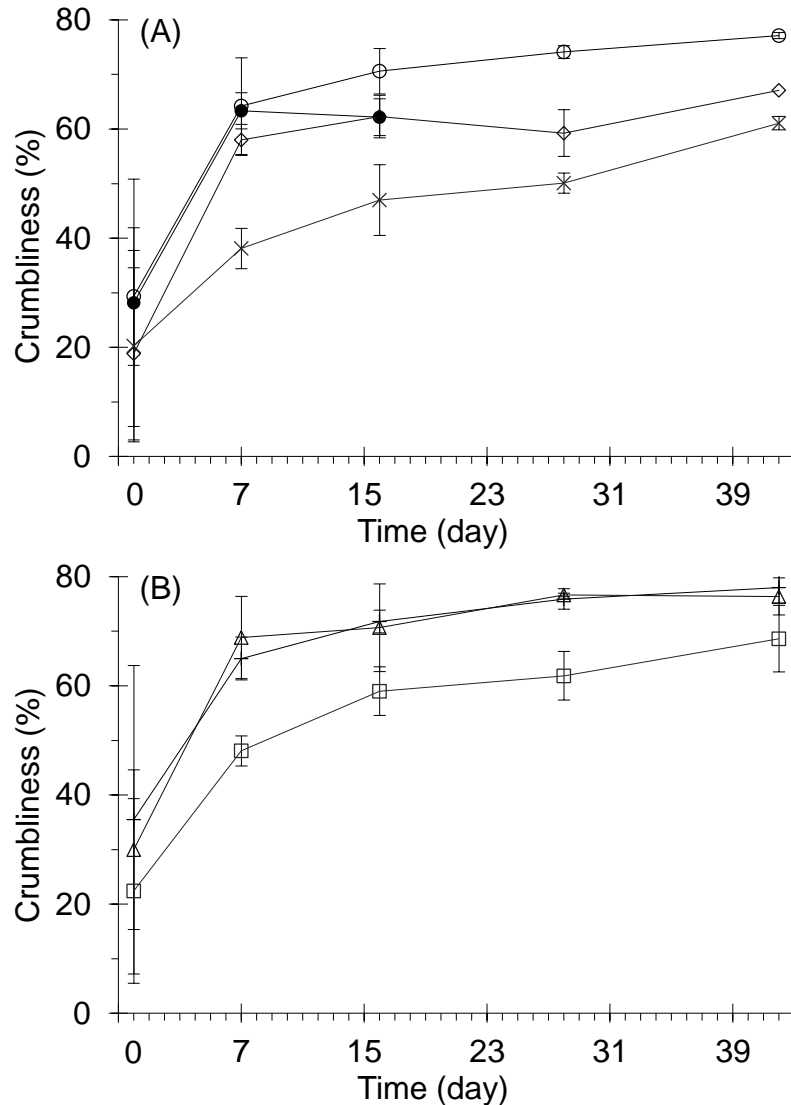


softer than the MCC formulated HPN bars that had mean hardness of 121 N. On all other days tested, the HPN bars prepared with MCC were softer than those prepared with MPC ( $P < 0.05$ ). MCC produced softer HPN bars than several other dairy proteins (Hogan and others 2102), but those particular MCC-formulated samples hardened substantially less over 10 d at 37°C than the present samples did over 6 d at 32°C. MPC-H hardened more gradually than MPC-N and MPC-L (Figure 5-2A) and more similar to the MCC formulated HPN bars (Figure 5-2B). On average, the HPN bars formulated with MPC-N were harder ( $P < 0.05$ ) than those formulated with MPC-H and MPC-L. Although significant, the small magnitude difference between MPC-N and MPC-L has no practical ability to reduce HPN bar hardness on each storage day (Figure 5-2A). Even the practicality of MPC-H to reduce HPN bar hardness on each day could be questioned, but it does produce a softer ( $P < 0.05$ ) HPN bar than MPC-N and MPC-L when averaged over the storage period. There was no difference in HPN bar hardness between MCC-L and MCC-H, but they were both softer ( $P < 0.05$ ) than the MCC-N. After equivalent storage, hardness of the MCC-formulated HPN bars all but matched one another (Figure 5-2B) and such small differences imparted by Tgase crosslinking did not impart practical softening.

Average HPN bar hardness was inversely related with level of crosslink, increasing from 175 N for MPC-H/MCC-H to 193 N for MPC-L/MCC-L to 218 N for MPC-N/MCC-N, and all the contrasts between levels were significant ( $P < 0.05$ ). The day  $\times$  crosslink interactions were compared using Bonferroni's adjustment. The different levels of Tgase crosslinking did not have an effect ( $P > 0.05$ ) on day 0 HPN bar hardness and if use of Tgase crosslinked proteins did not affect textural stability, this would be

seen on each testing day. However, after 7 d the 'high' Tgase crosslinked proteins produced softer HPN bars than the non-crosslinked proteins ( $P < 0.05$ ), but maintained similar hardness to those prepared with the 'low' crosslinked proteins ( $P > 0.05$ ). On day 42 the HPN bars formulated with 'low' and 'high' Tgase crosslinked proteins were both softer ( $P < 0.05$ ) than those made with non-crosslinked proteins, but there was no difference ( $P > 0.05$ ) between the Tgase levels. Tgase crosslinked proteins induced HPN bar brittleness and since max force during compression frequently occurred at the point of fracture, the modification imparted a softening effect. MPC-H/MCC-H each contained high molecular weight protein polymers (Figure 5-2) that imparted structural heterogeneity which created internal weak spots and allowed the system to fracture under lower compressive force (Purwanti and others 2010). HPN bars formulated with low molecular weight hydrolysates are soft and pliable, but they are susceptible to chemical changes, such as disulfide bond formations (Zhou and others 2008) and Maillard-induced protein aggregations (Zhou and others 2013), that occur with hardening. These changes, as well as free amine reduction, were not related to the texture change of MPC-formulated HPN bars, but they did occur during storage (Banach and others 2016b; Loveday and others 2009). Tgase crosslinking of the protein ingredients increases their average molecular weight, but decreases their molecular mobility and internal chemical reactivity. If these reactions do in fact play a role in HPN bar texture change, this would mean that disulfide bond formations and Maillard-induced protein aggregations would be slowed. Maillard browning-induced protein aggregations would also be slowed since the Tgase crosslinked proteins have lower initial free amine content when made into HPN bars.

Overall, the model HPN bars prepared with either MPC or MCC were crumbly and lacked cohesion. Crumbliness and cohesiveness are sparsely reported in the HPN bar based literature. Results from a sieve analysis of twice-compressed HPN bars were previously correlated with trained panel measured in-hand crumbliness and in-mouth cohesiveness (Banach and others 2016a). HPN bar crumbliness increased substantially after 1 week and then increased at a much slower rate (Figure 5-3). MCC produced HPN bars that were, on average, more crumbly than those made with MPC ( $P < 0.05$ ). A drawback of using MPCs in HPN bars is that they decrease cohesiveness (Banach and others 2016a; Imtiaz and others 2012) and the MCC under current study only worsened this texture attribute. Proprietarily functionalized WPC added to MPC decreased crumbliness and increased cohesiveness of a HPN bar (Imtiaz and others 2012). Whey proteins are removed during MCC production; since they possess an ability to impart cohesiveness, it was not surprising that MCC produced crumblier HPN bars.



**Figure 5-3 High-protein nutrition (HPN) bar mean crumbliness during storage at 32°C.** HPN bars were formulated at 30% protein (w/w) using MPC-N (●), MPC-L (◇), MPC-H (×), RCMPC (○), MCC-N (+), MCC-L (Δ), or MCC-H (□). MPC, milk protein concentrate (A). MCC, micellar casein concentrate (B). N, L, and H, indicate none, low, and high transglutaminase crosslink levels, respectively. RCMPC, reduced-calcium milk protein concentrate. Error bars represent  $\pm 1$  SD ( $n = 3$ ).

Tgase crosslinking of protein was expected to improve HPN bar cohesiveness/crumbliness by adding structure. Tgase crosslinked proteins produced HPN bars that were less crumbly than the control ( $P < 0.05$ ). The higher level of crosslinking imparted greater cohesion than the lower level of crosslinking ( $P < 0.05$ ). Data required careful analysis since HPN bars became incompressible at different storage times. Some crumbliness estimates were based on a single preparation while others were inestimable,

for example, the HPN bar formulated with MPC-N after day 16. Mechanical force generated during sieving/shaking was insufficient to break an incompressible sample and it was completely retained on the top sieve. While not crumbly in terms of the assay, these samples would be deemed unacceptable by hardness alone, and being texturally irrelevant, crumbliness was not reported for samples that did not break during compression. Crumbliness of the HPN bars prepared with MCC-N and MCC-L did not differ ( $P > 0.05$ ) on each day tested (Figure 5-3B). HPN bar crumbliness values of MPC-H were compared with MPC-L and those for MCC-H were compared with MCC-L. HPN bars formulated with MPC-H or MCC-H regularly fractured during TPA and while fines persisted, they were more cohesive than MPC-L or MCC-L, respectively, yet contrast significance varied with testing day. MPC-H or MCC-H HPN crumbliness was not different ( $P > 0.05$ ) than MPC-L or MCC-L on day 0, respectively, but on day 7 and day 16 those differences were significant ( $P < 0.05$ ). The HPN bar formulated with MCC-H was also less crumbly than MCC-L on day 28 ( $P < 0.05$ ). HPN bar crumbliness leveled off as day 42 approached and on that day, no difference ( $P > 0.05$ ) were found between MPC-H or MCC-H and MPC-L or MCC-L, respectively. Using Tgase crosslinked protein ingredients in HPN bars reduced the rate in which crumbliness developed and improved overall cohesiveness. Tgase was inactivated after MPC and MCC were crosslinked and so internal Tgase crosslinking does not occur within the HPN bar. Tgase improved the cohesiveness of an emulsified meat system when added in its active form (Herrero and others 2008). Since the HPN bars had low moisture (Table 5-1), low  $a_w$  (Table 5-2), and stable pH (Table 5-1), protein gelation cannot occur during storage. Caseinate gels produced by glucono-delta-lactone (GDL) acidification were

more cohesive when produced with Tgase-crosslinked caseinate (Song and Zhao 2013). Other than inhibition or slowing of the texture change mechanisms discussed for hardening, it was not possible to pinpoint why MPC-H and MCC-H produced a more cohesive HPN bar.

#### 5.5.3.2 *Reduced-Calcium MPC*

RCMPC produced a HPN bar that was more powdery, drier to the touch, and less adhesive (data not shown) on each testing day when compared with all the other model HPN bars. It was important to balance constituents for shelf stability (i.e.,  $a_w < 0.65$ ) while maintaining a formula suitable for all the protein ingredients being evaluated in the current study, yet similar to those previously used for MPC-formulated HPN bars (Banach and others 2014; Imtiaz and others 2012). MPC-N was not produced from the same lot of skim milk as RCMPC, but it sufficed as its control in this study. RCMPC slowed HPN bar hardening (Figure 5-2A), especially when compared with MPC-N, but values still approached the maximum measurable by the texture analyzer utilized as storage time neared 42 d. Standard deviation between preparations was high and thus it was unlikely that the hardness of the RCMPC formulated HPN bar differed with the MPC-N on day 0, 16, 28, and 42. Apparently its hardness was only lower than MPC-N on day 7 (Figure 5-2) or ~2 months at 22°C. While RCMPC produced a softer HPN bar for the short term, it was the crumbliest one evaluated in this study (Figure 5-3A). While softness was imparted initially, RCMPC did not improve HPN bar cohesiveness and thus reducing the calcium content of MPC will not improve its ability to serve as a predominant protein in these applications. However, RCMPC might be blended with

other protein ingredients to potentially impart softening or, in an instance desired, a crumbling effect.

## **5.6 Conclusions**

In this study, MPC and MCC, previously crosslinked at 'low' and 'high' levels, plus one RCMPC were texturally evaluated in a model HPN bar. MPC and MCC produced HPN bars that progressively hardened and lost cohesion during storage. Overall, those formulated with MPC were harder and more cohesive than those made with MCC. Tgase crosslinked proteins decreased HPN bar hardness and decreased the development of crumbliness during storage. More protein crosslinking lowered peak force during compression, after which the sample was characterized as being less crumbly. However, as storage time progressed, the HPN bars formulated with the modified protein ingredients behaved with greater textural similarity as their respective controls. The RCMPC produced a softer and crumblier HPN bar when compared with control MPC. We conclude that the small magnitude changes in HPN bar texture that resulted from utilizing Tgase crosslinked MPC or MCC, or RCMPC, did not improve stability during storage and that these modified protein ingredients have no practical advantage over their unmodified controls in HPN bars.

## **5.7 Acknowledgement**

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## CHAPTER 6. EXTRUSION-MODIFIED PHYSICO-CHEMICAL PROPERTIES OF MILK PROTEIN CONCENTRATE FOR IMPROVED HIGH-PROTEIN NUTRITION BAR TEXTURE

Modified from a manuscript to be submitted to the *Journal of Food Science*

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### 6.1 Abstract

Milk protein concentrate with 80% protein (MPC80) was extruded and jet-milled, and select functional properties with relevance to performance in high-protein nutrition (HPN) bars were evaluated. Extrusion at die-end melt temperature greater than 95°C decreased protein solubility, water holding capacity, free sulfhydryl content, and free amine content of MPC80. Initially, extrusion-modified MPC80 had higher water-protein contact angle and dynamic analysis showed that water spread more easily on its pressed surface compared to the control. While no significant difference was found for the rates at which the proteins absorbed water, the extruded MPC80s appeared to absorb water more readily. Chemical changes that occurred during previous storage of HPN bars formulated with extrusion-modified MPC80 were also measured. Protein free sulfhydryl content did not change significantly during storage whereas free amine content decreased ( $P < 0.05$ ). SDS-PAGE revealed protein aggregations over the course of 7 months HPN bar storage. These HPN bar relevant protein functional properties are discussed in terms of their impact on chemical changes during HPN bar storage as well as their influence on

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previously reported texture and stability of HPN bars formulated with extrusion-modified MPC80.

## 6.2 Practical Application

Extrusion modified the functional properties of MPC80. It also decreased its free sulfhydryl and free amine content. It was previously used to make high-protein nutrition (HPN) bars with greater textural stability than the control. Altered texture and improved stability were due to its ability to interact more readily with water and were not due to limited chemical reactivity within the HPN bars during storage.

## 6.3 Introduction

Extrusion imparts shear, heat, and pressure during processing, and denaturation and altered functionality are expected during extrusion of protein containing foods. Starchy matrices are easily extruded to produce puffed snacks with low nutritional quality. Adding protein to boost the nutritional value decreases processability and negatively impacts textural quality (Onwulata and others 2001). Literature has focused on protein-starch interactions by varying the protein, starch, and/or blend ratio prior to extrusion, and then the extrudate's properties (e.g., expansion index, hardness) are analyzed. Applications for extruded starch-protein blends other than puffed snacks are sparsely reported (Zhang and others 2016a). Proteins are extruded to produce crisps (Tremaine and Schoenfuss 2012) and meat analogs (Lin and others 2002). Recently, extrusion has been used to modify the functionality of protein ingredients such as milk protein concentrate (MPC) (Banach and others 2013), pea protein isolate (PPI) (Osen and others 2015), whey protein concentrate (WPC) (Nor Afizah and Rizvi 2014), and soy protein isolate (SPI) (Fang and others 2014). Protein denaturation during extrusion

decreases its solubility and this affects dependent functional properties (e.g., gelation, emulsification, water holding capacity).

Tuning the functionality of protein ingredients can improve their usability in specific applications. Onwulata (2010) used extruded whey protein isolate (WPI) to improve the quality attributes of puffed corn meal when compared to the same product formulated with spray dried WPI. Extruded MPC80 produced soft textured, non-baked HPN bars at 30% protein (w/w) that were less prone to hardening than those formulated with dry-heat toasted or control MPC80 during 42 d accelerated temperature storage (Banach and others 2014). Extruded MPC80 also improved HPN bar cohesion and textural stability over ~7 months storage at 22°C or 32°C (Banach and others 2016a). Extrusion altered the functional properties of MPC80 for improved textural performance in HPN bars. The specific functionalities that changed in the latter study still require investigation.

Extrusion-modified MPC80 may prevent or slow the protein aggregation mechanisms that are used to describe the time-dependent texture change of high-protein systems when they are used as the main protein source. Matrix hardening occurred as the proteins formed disulfide bonds (Zhou and others 2008) and Maillard-induced aggregates (Zhou and others 2013). Disulfide linked protein aggregates (DLPA) also formed in a MPC80 formulated HPN bars during accelerated storage, but their formation and texture change were not consistent in those HPN bars formulated with extruded MPC80 (Banach and others 2016b). Another study found minimal formation of DLPA in MPC80 formulated protein bars kept for 50 d at 20°C (Loveday and others 2009).

Protein powder properties, such as solubility, degree of hydrolysis, density, size, and morphology affect HPN bar texture (Cho 2010). Extrusion cooking did not hydrolyze PPI (Osen and others 2015), but resultant protein denaturation does decrease protein solubility (Banach and others 2013). The following study measured the solubility, density, and particle size of extrusion-modified MPC80 that previously produced HPN bars with greater textural stability (Banach and others 2016a). HPN bar relevant protein functional properties including, water holding capacity (WHC), surface hydrophobicity, and wettability were assessed to describe powder-water interactions that might influence initial hydration as well as time-dependent moisture migration between HPN bar constituents, another proposed mechanism for texture change (Loveday and others 2009; Li and others 2008). Extrusion-modified MPC80's free amine and free sulfhydryl contents, which have the potential to influence Maillard-induced and DLPA, respectively, when used in HPN bars were also measured. After HPN bar storage, protein free sulfhydryl and free amine content were measured and soluble protein aggregates that formed were discussed in terms of previously reported texture (Banach and others 2016a).

## **6.4 Materials and Methods**

### *6.4.1 Materials*

MPC80 (78.5% protein, 4.3% fat, 6.7% ash, 4.9% moisture, 5.6% lactose, Milk Specialties Global, Eden Prairie, MN) was previously extruded at die-end melt temperature of 95, 105, and 116°C to make the respective protein powders: E95 (74.0% protein, 7.6% moisture), E105 (74.3% protein, 7.5% moisture), and E116 (74.4% protein, 7.4% moisture) (Banach and others 2016a). E105, E116, and MPC80 were used as the

sole protein source in model HPN bars (30% protein w/w) that were kept at 22°C or 32°C for 0, 6, or 29 weeks prior to being frozen in liquid nitrogen and stored at -80°C (Banach and others 2016a). The Pierce™ BCA protein assay kit, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), urea, EDTA, SDS, boric acid, sodium chloride, sodium tetraborate decahydrate, isopropanol, and β-mercaptoethanol were purchased from Fisher Scientific (Waltham, MA). Dithiothreitol (DTT), O-phthalaldehyde (OPA), N<sub>α</sub>-acetyl-L-lysine, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). SDS-PAGE supplies, including 2x Laemmli sample buffer, Precision Plus Protein™ Standard, AnyKD™ Mini-Protean® TGX™ precast gels, Bio-Safe™ Coomassie Stain, and 10x tris/glycine/SDS running buffer, were obtained from Bio-Rad, Inc. (Hercules, CA).

#### *6.4.2 Brief Description of MPC80 Extrusion, Drying, Milling, and Particle Size Measurement*

MPC80 was fed at 25 kg/h into the co-rotating (350 rpm) twin-screw extruder (DN DL 44, Bühler AG, Uzwil, Switzerland) while water was added at 13, 11, or 10 kg/h to produce three extrudates with die-end melt temperatures of 95, 105, or 116°C, respectively (i.e., E95, E105, and E116). Extruder generated specific mechanical energy (SME) for each extrudate is reported. After drying 26 h at 40°C in a forced draft oven, the extrudates were jet-milled into powders. Each powder was dispersed (n = 2) into isopropanol and particle size was measured by laser diffraction (Banach and others 2016a).

#### *6.4.3 Protein Powder Density, Interstitial Air, and Occluded Air Measurement*

Thirty g protein powder was transferred into a glass 100-mL graduated cylinder and was mechanically tapped 1,250 times (Autotap™, Quantachrome Instruments, Boynton Beach, FL). Powder volume after 0, 100, and 1,250 taps was used to calculate

( $n = 3$ ) loose ( $\rho_{\text{loose}}$ ), tapped ( $\rho_{100x}$ ), and extremely tapped ( $\rho_{1250x}$ ) density, respectively. Particle density ( $\rho_{\text{particle}}$ ) was measured ( $n = 2$ ) by helium pycnometry (G-DenPyc 2900, Gold APP Instruments Corporation, Beijing, China). MPC80 solids density ( $\rho_{\text{solids}}$ ) was calculated at  $1.38 \text{ g/cm}^3$  using component (i.e., fat, protein, lactose, ash) densities as detailed elsewhere (Crowley and others 2014; Walstra and others 2005). Occluded ( $V_{\text{oa}} = 100/\rho_{\text{particle}} - 100/\rho_{\text{solids}}$ ) and interstitial ( $V_{\text{ia}} = 100/\rho_{100x} - 100/\rho_{\text{particle}}$ ) air volumes (mL/100 g) were also calculated (Crowley and others 2014).

#### 6.4.4 Protein Powder Solubility

Powder was dispersed (0.8% protein w/w) in Millipore water and pH was adjusted to 2.0, 3.5, 4.6, 5.5, 6.8, 8.0, 9.5, or 11.0 with hydrochloric acid or sodium hydroxide while stirring at 650 rpm ( $n = 3$ ). pH was checked after 15, 45, and 75 min, and if needed was adjusted back to the specified value. Ninety min after the initial pH adjustment, the dispersions were centrifuged at  $15,000 \times g$  for 15 min. Supernatants were filtered through Whatman No. 1 filter paper. Supernatants were diluted with Millipore water such that soluble protein concentration, as measured ( $n = 2$ ) by the BCA assay, fell within the linear range of the BSA standard curve (0.125-1.5 mg/mL). Protein solubility (%) was calculated by dividing the soluble protein concentration by the total dispersed protein concentration (8 mg/mL).

#### 6.4.5 Protein Powder Water Holding Capacity

Protein powder WHC (water (g)/dry powder (g)) was measured ( $n = 3$ ) using the procedure explained by Quinn and Paton (1979). Based on preliminary WHC estimates, MPC80 (3.6 g), E95 (5.1 g), E105 (5.2 g), and E116 (5.3 g) were each weighed into 4 separate 50-mL centrifuge tubes. Eight, 9, 10, and 11 g Millipore water were added to



each set of four tubes containing extruded MPC80 and 9.5, 10.5, 11.5, and 12.5 g Millipore water were added to the four tubes holding control MPC80. After mixing the protein powder and water with a spatula for 2 min, the tubes were centrifuged at  $3,900\times g$  for 10 min and any visible supernatant was decanted. Water occluded (g) by each sample was determined by difference and WHC for each tube was calculated:  $WHC = (\text{water occluded (g)} + \text{native protein powder water (g)})/\text{dry powder (g)}$ . The WHC of the tube with the lowest volume supernatant and the WHC capacity of the supernatant-less tube analyzed with 1 g less water added were averaged for WHC measurement.

#### *6.4.6 Protein Powder Dynamic Contact Angle*

One-tenth g protein powder was loaded into a 13-mm pellet die (model 3619, Carver, Inc., Wabash, IN) and was pressed (model 4350, Carver, Inc., Wabash, IN) to and maintained at  $8,000 \text{ kg}_f$  for 2 min (Crowley and others 2015). A  $4 \mu\text{L}$  Millipore water droplet was dispensed (Gilmont GS-1200 Micrometer Syringe, Cole-Parmer, Vernon Hills, IL) onto each pressed protein surface ( $n = 4$ ) and a goniometer (model 250, Ramé-hart Instrument Co., Succasunna, NJ) was used to acquire profile images (5 images/s) immediately after placement. After 20 s, the acquisition rate was adjusted to 1 image/s. Images captured up to 25 s were reprocessed by the DROPimage® software (version 2.8.02, University of Oslo, Norway) and water droplet volume ( $\mu\text{L}$ ) remaining and average contact angle ( $^\circ$ ) were reported over time.

#### *6.4.7 Protein Free Sulfhydryl Measurement*

Free sulfhydryl extraction buffer (pH 8.5) contained 8 M urea, 4.1 mM EDTA, and 2% (w/v) SDS dissolved in borate buffer (100 mM boric acid, 75 mM sodium chloride, and 25 mM sodium tetraborate decahydrate). Protein powder (0.78 to 0.82 g) plus 8 mL free

sulfhydryl extraction buffer with or without SDS were mixed at 900 rpm for 2 h prior to diluting to volume (10 mL) (n = 3). Each previously prepared (n = 2) and aged HPN bar (1.6 g) was mixed with 14.4 g free sulfhydryl extraction buffer containing SDS for 2 h at 750 rpm (n = 2). All dispersions were centrifuged at 15,000×g for 20 min. Supernatant free sulfhydryl content was measured using Ellman's assay as described elsewhere (Banach and others 2016b; Beveridge and others 1974). A cysteine standard curve ( $R^2 > 0.998$ ) encompassing net sample absorbance was used to calculate supernatant free sulfhydryl concentration ( $\mu\text{M}$ ). Two measurements were made per extraction and results were divided by BCA assayed soluble protein (g/L) to report free sulfhydryl concentration in  $\mu\text{mole per g protein}$ .

#### *6.4.8 Reduced and Non-reduced SDS-PAGE*

HPN bar extracts from the free sulfhydryl assay were diluted to 4 mg protein/mL and were then diluted 1 to 2 with either non-reducing or reducing 2x Laemmli sample buffer. Three  $\mu\text{L}$  of each sample and 10  $\mu\text{L}$  of the molecular weight standard were loaded onto precast gels and were electrophoresed for 45 min at 150 V. Details about SDS-PAGE are provided elsewhere (Banach and others 2016b).

#### *6.4.9 Protein Free Amine Content Measurement*

Twenty-three mL free amine buffer (50 mM boric acid, 37.5 mM sodium chloride, 12.5 mM sodium tetraborate decahydrate, 1% SDS (w/v), 0.1% DTT (w/v), pH 9.0) was added to 0.16-0.17 g protein powder. After stirring for 2 h at 900 rpm, the dispersions were diluted to 25 mL. Approximately 0.31 g (100 mg protein) of each HPN bar was mixed with 10 mL free amine buffer in 25-mL flasks for 2 h at 650 rpm. All dispersions were centrifuged at 15,000×g for 20 min and supernatants were filtered

through Whatman No. 4 filter paper. Supernatant protein was measured using the BCA assay and was diluted to 1 mg/mL. One-hundred  $\mu\text{L}$  sample was mixed with 900  $\mu\text{L}$  OPA reagent (0.8 mg OPA/mL free amine buffer) and absorbance was measured at 335 nm (Banach and others 2014; Loveday and others 2009). Linear ( $R^2 > 0.9999$ ) 3-point (500-1500  $\mu\text{M}$ ) and 4-point (100-1000  $\mu\text{M}$ )  $\text{N}_\alpha$ -acetyl-L-lysine standard curves were used to measure the free amine content ( $\mu\text{M}$ ) of the protein powders and HPN bars, respectively, after subtracting the OPA reagent absorbance from each sample. Free amine concentration was reported as  $\mu\text{mole per g protein}$  after dividing the result by the soluble protein concentration (1 g/L).

#### 6.4.10 Statistical Analyses

Protein powder functionality data were analyzed using the generalized linear mixed model (GLMM) in SAS® (version 9.4, SAS Institute Inc., Cary, NC). Protein ingredient was the only independent variable in WHC and free amine analysis whereas pH, SDS, and categorical time were added to the models for solubility, free sulfhydryl content, and dynamic contact angle measurement, respectively. Random error terms were assigned to account for assay replication as well as the replicate attribute measurement for each specific powder. Contact angle and droplet volume were also modeled with time set as a continuous variable and average rate of change for each was determined. Rate of change (slope values) were corrected for multiplicity using the simulate adjustment ( $\alpha = 0.05$ ). HPN bar free amine and free sulfhydryl content were modeled using the GLMM. Protein ingredient, storage time, storage temperature, and all interaction terms were set as the independent variables. Assay replicate as well as the

replicate nested preparation of each HPN bar were set as the random error terms. All statistical contrasts were significant if the adjusted  $P$ -value was less than 0.05.

## 6.5 Results and Discussion

### 6.5.1 Protein Powder Particle Size, Density, and Occluded and Interstitial Air

Control MPC80, which was spray dried, had larger particle size diameters than the jet-milled extrusion-modified MPC80s ( $P < 0.05$ ), whose diameters generally decreased in the order of E116, E105, and E95 (Table 6-1). Although  $D_{4,3}$  values were only separated by 18 micron, the particle size span (i.e.,  $(D_{90}-D_{10})/D_{50}$ ) for E95 (4.0), E105 (5.7), and E116 (3.3) showed that these powders had broader particle size distribution than control MPC80 (2.1). Particle size dispersity of E105, E116, and MPC80 was previously discussed as a factor affecting HPN bar texture (Banach and others 2016a), but its affect on their functional properties has not been discussed. Previously, smaller milk protein isolate (MPI) particles were less able to absorb water and were less wettable than larger and agglomerated MPI particles (Li and others 2016; Ji and others 2015). Therefore, the functionality of the extruded MPC80s, especially E95 which was significantly finer than the other powders, may be altered by particle size reduction alone.

Mean  $\rho_{\text{loose}}$ ,  $\rho_{100X}$ ,  $\rho_{1250X}$ , and  $\rho_{\text{particle}}$  of the extruded MPC80s were 0.52, 0.60, 0.64, and 1.32 g/cm<sup>3</sup>, respectively, and each was individually greater ( $P < 0.05$ ) than the same specified densities of control MPC80 (Table 6-1). Extruded MPC80 contained, on average, 91 and 3.3 mL/100 g  $V_{\text{ia}}$  and  $V_{\text{oa}}$ , respectively, and each was individually lower than the control ( $P < 0.05$ ). MPC80 had higher  $V_{\text{oa}}$  (17.8 mL/100 g), higher  $V_{\text{ia}}$  (189 mL/100 g), and lower densities due to being spray dried.

**Table 6-1 Protein powder loose ( $\rho_{\text{loose}}$ ), tapped ( $\rho_{100\text{X}}$ ), extremely tapped ( $\rho_{1250\text{X}}$ ), and particle ( $\rho_{\text{particle}}$ ) densities ( $\text{g/cm}^3$ ), occluded ( $V_{\text{oa}}$ ) and interstitial ( $V_{\text{ia}}$ ) air volumes ( $\text{mL}/100 \text{ g}$ ), and particle size diameters ( $\mu\text{m}$ )**

Protein <sup>1</sup>	Density				Volume		Particle Size Diameter <sup>2</sup>			
	$\rho_{\text{loose}}$	$\rho_{100\text{X}}$	$\rho_{1250\text{X}}$	$\rho_{\text{particle}}$	$V_{\text{oa}}$	$V_{\text{ia}}$	D <sub>10</sub>	D <sub>50</sub>	D <sub>90</sub>	D <sub>4,3</sub>
MPC80	0.31 <sup>b</sup>	0.36 <sup>c</sup>	0.39 <sup>c</sup>	1.11 <sup>b</sup>	17.8 <sup>a</sup>	189 <sup>a</sup>	16 <sup>a</sup>	49 <sup>a</sup>	121 <sup>c</sup>	61 <sup>a</sup>
E95	0.52 <sup>a</sup>	0.60 <sup>ab</sup>	0.65 <sup>a</sup>	1.32 <sup>a</sup>	3.5 <sup>b</sup>	89 <sup>c</sup>	2 <sup>c</sup>	25 <sup>c</sup>	103 <sup>d</sup>	43 <sup>d</sup>
E105	0.51 <sup>a</sup>	0.59 <sup>b</sup>	0.63 <sup>b</sup>	1.33 <sup>a</sup>	2.9 <sup>b</sup>	95 <sup>b</sup>	2 <sup>c</sup>	25 <sup>c</sup>	147 <sup>a</sup>	52 <sup>c</sup>
E116	0.53 <sup>a</sup>	0.61 <sup>a</sup>	0.65 <sup>a</sup>	1.32 <sup>a</sup>	3.4 <sup>b</sup>	88 <sup>c</sup>	3 <sup>b</sup>	38 <sup>b</sup>	132 <sup>b</sup>	57 <sup>b</sup>

<sup>1</sup> MPC80, control spray dried milk protein concentrate with 80% protein. E95, E105, and E116, MPC80 extruded at die-end melt temperature of 95°C, 105°C, and 116°C, respectively.

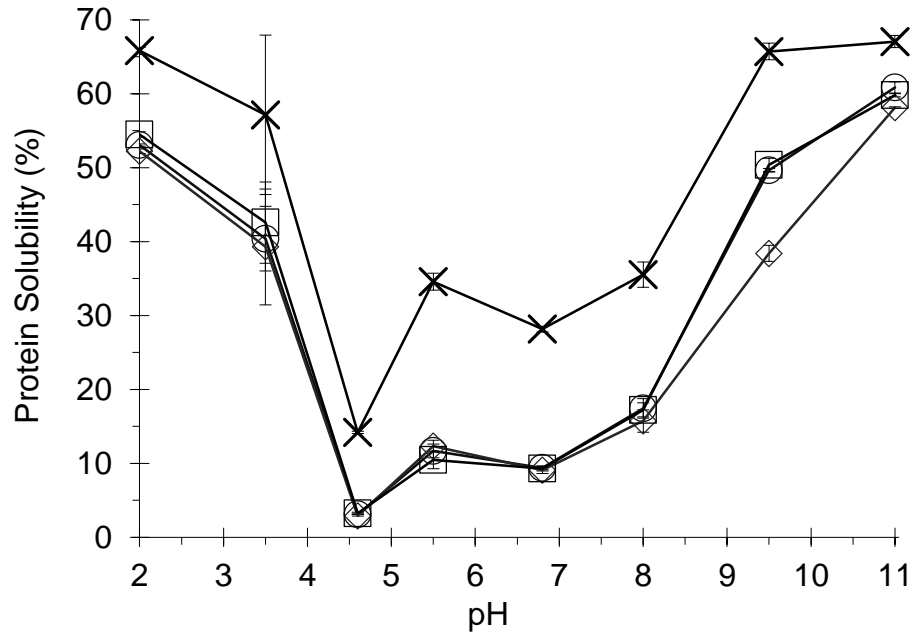
<sup>2</sup> Particle size diameters for MPC80, E105, and E116 were previously reported by Banach and others (2016a).

<sup>a-d</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

Protein powder particle size, densities,  $V_{\text{ia}}$ , and  $V_{\text{oa}}$  are not functionally relevant when fully dissolved, and although they affect dissolution rate, they are not reported in MPC solubility studies. However, MPC80 particle size and shape were not fully lost during model HPN bar production and their presence was noted in the final product (Loveday and others 2009). HPN bars produced using E105 or E116 were more dense and cohesive than those prepared with control MPC80 (Banach and others 2016a). Lower HPN bar density was due to low particle density, high  $V_{\text{oa}}$ , and the powder structure of control MPC80 being maintained in the HPN bar.  $V_{\text{ia}}$  is essentially the volume of air that exists between powder particles in the dry state. Lower  $V_{\text{ia}}$  in the extrusion-modified MPC80 means that smaller particles fill voids occupied by air in the control. If this powder attribute transfers to HPN bars, it becomes clear that the control would incorporate more air and would have limited particle-particle interactions, a likely reason for decreased product cohesion.

### 6.5.2 Protein Powder Solubility

Protein solubility of high-protein powders is related to total powder solubility and its insolubility after processing is an indicator of protein denaturation. Extrusion reduced MPC80's solubility at each pH tested ( $P < 0.05$ ) (Figure 6-1). Extrusion SME (W/kg) for E95, E105, and E116 were 216, 238, and 253, respectively. Higher SME and melt temperature did not affect extrudate solubility at any pH ( $P > 0.05$ ), except at pH 9.5 where E116 (38%) was less soluble than E95 (50%), E105 (50%), and control MPC80 (66%) ( $P < 0.05$ ). Protein denaturation is less dependent on temperature as processing concentration increases (Wolz and Kulozik 2015), and so the 21°C melt temperature increase switching from E95 to E105 to E116 did not have an effect. MPC80 was 14% soluble at pH 4.6, casein's isoelectric point, where complete whey protein dissolution or 20% protein solubility was expected. At the same pH, protein solubility of the extrusion-modified MPC80 decreased to 3%, which suggested whey protein denaturation and was consistent with the solubility values reported for whey proteins extruded at temperatures greater than 90°C (Nor Afizah and Rizvi 2014; Qi and Onwulata 2011). Extrusion-modified MPC80 solubility profiles mirrored those of MPC80 extruded on a smaller unit (Banach and others 2013), and the lower solubility values in the present study were attributed to the starting material, processing conditions, and modifications made to the solubility assay.



**Figure 6-1 Protein solubility (%) versus pH for extruded and control MPC80.** MPC80 (×), control spray dried milk protein concentrate with 80% protein. E95 (□), E105 (○), and E116 (◇), MPC80 extruded at die-end melt temperature of 95°C, 105°C, and 116°C, respectively. Error bars indicate  $\pm 1$  SD from the sample mean (n = 3).

Protein ingredient solubility has an underlying effect on HPN bar texture. SPIs that were too soluble, that is soluble solids index (SSI)  $> 55\%$ , or too insoluble, that is SSI  $< 30\%$ , produced HPN bars (30% protein w/w) that were too hard or too crumbly, respectively, whereas SPI with 40% SSI appropriately balanced these attributes (Cho 2010). HPN bar pH ranged from 6.0 to 6.8 (Banach and others 2016a) and in the encompassing pH range of 5.5 to 6.8, MPC80's protein solubility was between 35% and 28%. The control HPN bar from Banach and others (2016a) may have lacked cohesiveness by not possessing enough solubilized protein to hold the system together. However, the extruded MPC80s were 24% and 19% less soluble than MPC80 at pH 5.5 and 6.8, respectively, and their respective HPN bars were cohesive (Banach and others 2016a). Another suggestion is that proteins with higher solubility possess greater ability to pull water away from other HPN bar constituents during storage which subsequently

causes texture change by way of internal moisture migration (Cho 2010). Extrusion decreased MPC80's solubility and this difference in functionality was partially responsible for its improved textural performance in HPN bars (Banach and others 2016a, 2014).

### 6.5.3 Protein Powder Interaction with Water: Holding Capacity, Contact Angle, and Absorption

The extruded MPC80s interact with water differently than the spray dried control. Extrusion decreased MPC80's WHC by 42% ( $P < 0.05$ ), but no significant difference existed between extrudates ( $P > 0.05$ ) (Table 6-2). A comparable WHC decrease was previously observed for extruded MPC80 (Banach and others 2013). Protein powder occluded air (Table 6-1) served as reservoir for water to be held during WHC analysis. The extruded MPC80s lost this air and sponge-like functionality from processing, which resulted in lower WHC than the spray dried, non-extruded control.

**Table 6-2 Protein powder water holding capacity (WHC; water (g)/dry powder (g)), contact angle ( $\theta$ ; °), and water droplet volume (V;  $\mu\text{L}$ )**

Protein <sup>1</sup>	WHC	Contact Angle			Water Droplet Volume		
		$\theta_{0s}$	$\theta_{25s}$	Slope (°/s)	$V_{0s}$	$V_{25s}$	Slope (nL/s)
MPC80	3.3 <sup>a</sup>	66 <sup>b,z</sup>	61 <sup>a,y</sup>	-0.19 <sup>b</sup>	3.11 <sup>a,z</sup>	3.08 <sup>a,z</sup>	-1.31 <sup>a</sup>
E95	1.9 <sup>b</sup>	86 <sup>a,z</sup>	64 <sup>a,y</sup>	-0.88 <sup>a</sup>	3.49 <sup>a,z</sup>	3.34 <sup>a,y</sup>	-6.24 <sup>a</sup>
E105	1.9 <sup>b</sup>	85 <sup>a,z</sup>	62 <sup>a,y</sup>	-0.91 <sup>a</sup>	3.45 <sup>a,z</sup>	3.28 <sup>a,y</sup>	-6.81 <sup>a</sup>
E116	1.8 <sup>b</sup>	90 <sup>a,z</sup>	62 <sup>a,y</sup>	-1.12 <sup>a</sup>	3.64 <sup>a,z</sup>	3.56 <sup>a,y</sup>	-3.26 <sup>a</sup>

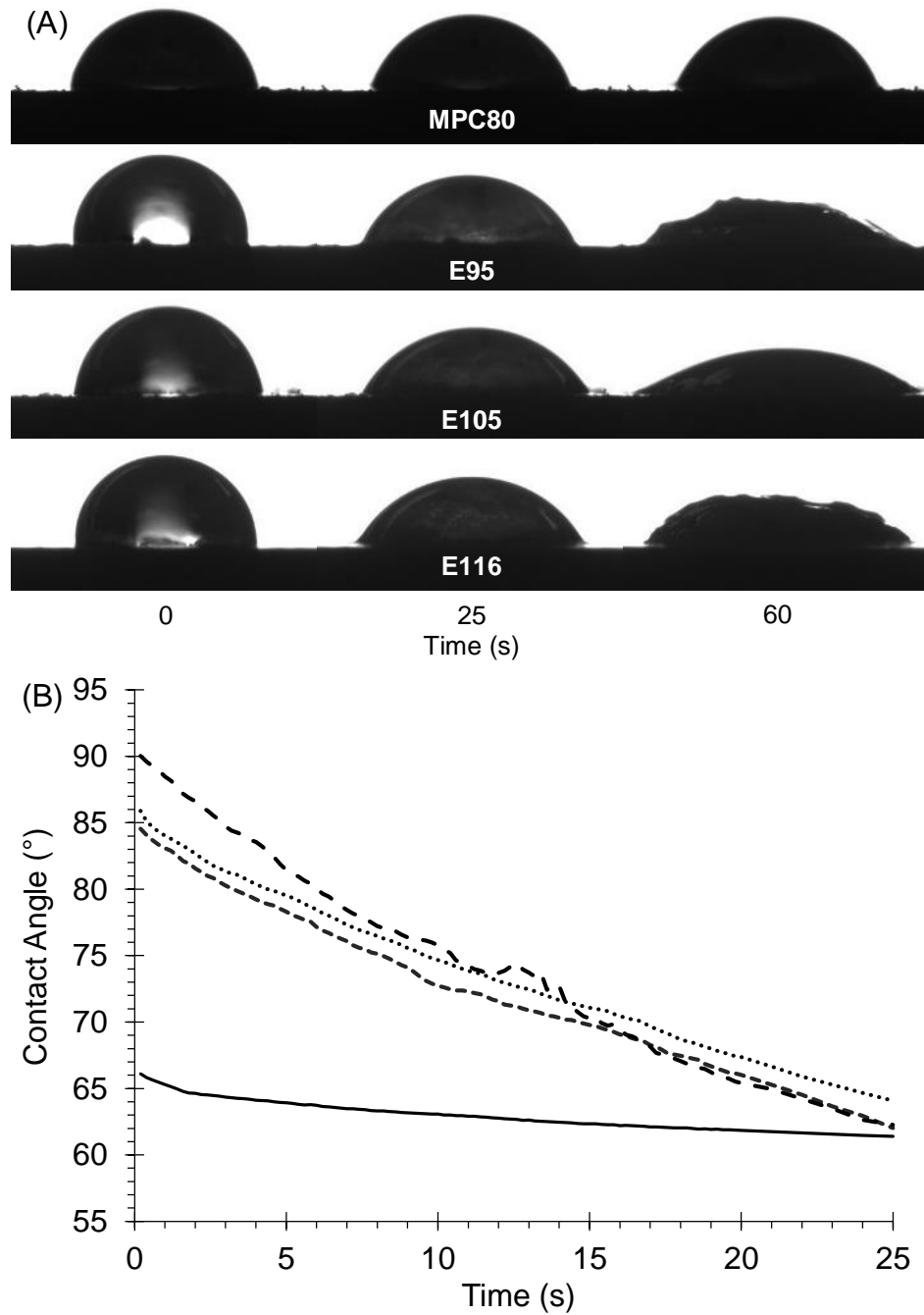
<sup>1</sup> MPC80, control spray dried milk protein concentrate with 80% protein. E95, E105, and E116, MPC80 extruded at die-end melt temperature of 95°C, 105°C, and 116°C, respectively.

<sup>a-b</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>y,z</sup> Contact angle or water droplet volume least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.



Apparent dynamic contact angle measurement was used to compare surface hydrophobicity and wettability of the protein powders. Initially ( $\theta_{0s}$ ), the extruded MPC80s had higher contact angle than the control (Table 6-2), but that quickly changed as the droplet spread across and was imbibed by the pressed protein surface (Figure 6-2). After 25 s ( $\theta_{25s}$ ), water droplet contact angles on each protein surface were statistically equivalent ( $P > 0.05$ ) (Table 6-2). Contact angle change rate (slope) on the control was slower than the rate for the extruded MPC80s ( $P < 0.05$ ) (Table 6-2). E95, E105, and E116 absorbed the water droplet and measurable droplet volume decrease was observed after 25 s ( $P < 0.05$ ), but no significant water absorption was detected for the control ( $P > 0.05$ ) (Table 6-2). The extrusion-modified MPC80s appeared to absorb water more quickly, but there was no significant difference ( $P > 0.05$ ) between absorption rates (Table 6-2). Droplet collapse, the point when the liquid-vapor interface lost its convex shape, occurred at ~60 s on the extruded MPC80s and did not occur on the control until well after 60 s (Figure 6-2A). Liquid-vapor surface tension decreased as particles entered and protein dissolved into the water droplet (Lazghab and others 2005).



**Figure 6-2** Representative side view (A) and apparent contact angle (B) of a water droplet on each protein powder pressed into a flat surface. MPC80 (—), control spray dried milk protein concentrate with 80% protein. E95 (···), E105 (---), and E116 (- - -), MPC80 extruded at die-end melt temperature of 95°C, 105°C, and 116°C, respectively.

Higher initial contact angle indicates a less wettable surface (Crowley and others 2015) whereas rapidly decreasing contact angle, by droplet spread and absorption, suggests lower surface hydrophobicity (Lazghab and others 2005). Control MPC80 had lower ( $\sim 9^\circ$ ) initial contact angle ( $\theta_{0s}$ ) than previously measured (Crowley and others 2015). Differences were attributed to MPC source and unaccounted sessile drop confounders such as powder compaction, surface roughness, and porosity (Alghunaim and others 2016). High-protein MPCs were less wettable and water droplet spread was less pronounced compared to their low-protein, high-lactose counterparts (Crowley and others 2015). Water droplet spread and absorption on E95, E105, and E116 was similar to that observed on the low-protein MPCs and suggested that surface hydrophobicity was decreased. Extrusion-modified MPC80 may also pull water into its surface such that water spread and contact angle decrease are due to capillary force. Spray dried MPC80 powders form water-impermeable crusts (Fyfe and others 2011) and this barrier limited both spread across and absorption into the control MPC80 during dynamic contact angle measurement. Processing MPC80 disrupted this hydrophobic barrier, decreased its surface hydrophobicity, and improved the ability of the powder to interact with water.

Protein powder WHC and these other related properties have not been fully recognized for their potential impact on HPN bar performance. Protein ingredients with low WHC are less able to absorb water from other components within the HPN bar and this keeps all components hydrated while maintaining texture (Cho 2010). With lower WHC, extruded MPC80 would help mitigate the pull of water molecules towards the protein component. However, slightly increasing  $a_w$  during HPN bar storage (Banach and others 2016a, 2014; McMahon and others 2009) indicates that water becomes less

associated with protein and potentially allows for protein-protein aggregations and macronutrient phase separation to occur. During HPN bar manufacture, extruded MPC80 powder particles hydrate more easily than the control as was suggested by rapidly decreasing contact angle (Figure 6-2B). As powder hydration increases, its glass-rubber transition temperature ( $T_{gr}$ ) decreases, above which particle structure is lost and the proteins plasticize or make the HPN bar more rubber-like (Hogan and others 2016). Increased E105 and E116 plasticization translated into HPN bars that were softer, more cohesive, and less crumbly than the control MPC80 whose particle properties were maintained within the model system (Banach and others 2016a). HPN bar crumbliness develops as water moves away from the protein (i.e.,  $a_w$  increases), which in turn increases  $T_{gr}$ , and shifts the individual particles and the system to a state that is less rubber-like and more prone to crumble. Protein hydrolysates readily hydrate during HPN bar production and cohesiveness, which is rarely reported, should be maintained as  $a_w$  increases during storage were minimal (McMahon and others 2009). With higher initial hydration, lower overall protein solubility, and lower WHC, extruded MPC80s are less able to pull water from other constituents than spray dried MPC80, which only partially hydrates during HPN bar production and slowly absorbs water during HPN bar storage. Extrusion-modified MPC80 interacted more favorably with water and this improved its ability to produce soft, cohesive, and texturally stable HPN bars (Banach and others 2016a).

#### 6.5.4 Protein Powder Free Sulfhydryl Content and its Change during High-protein Nutrition Bar Storage

MPC80 contains lower free sulfhydryl concentration (4  $\mu$ mole per g soluble protein) (Banach and others 2016b) than WPC80 (25  $\mu$ mole per g soluble protein) (Nor Afizah and Rizvi 2014) since casein, the predominate protein in MPC, does not contain any cysteine residues that are not part of a native disulfide bond. To increase DTNB's accessibility to MPC80's buried free sulfhydryls and thus elicit a higher response during Ellman's assay, SDS was included in the assay buffer even though it was excluded previously (Banach and others 2016b). Inclusion of SDS in the free sulfhydryl extraction buffer increased the protein solubility of E116 ( $P < 0.05$ ), but had no effect on the solubility of the other powders ( $P > 0.05$ ). Protein powder soluble protein, with or without SDS, ranged from 29.9 to 32.8 mg/mL and with similar solubility, any observed free sulfhydryl differences were attributed to structural or chemical (e.g., oxidation, disulfide bond formation) induced changes. Extrusion-modified MPC80 had lower free sulfhydryl content than the control ( $P < 0.05$ ) (Table 6-3). Extrudate melt temperature did not have a significant effect ( $P > 0.05$ ). The free sulfhydryl concentration of E116 was numerically lower than E95 and E105, which suggested slightly more protein denaturation through disulfide bond formation (Zhang and others 2016b) and/or free sulfhydryl oxidations (Banach and others 2016b, 2014) at higher melt temperature. The inclusion of SDS in the free sulfhydryl assay buffer did not have a significant effect ( $P > 0.05$ ) on the response variable. Toasted MPC80 with increased and extruded MPC80 with decreased free sulfhydryl exposure previously produced HPN bars that were texturally unstable and stable, respectively (Banach and others 2016b, 2014).

**Table 6-3 Free sulfhydryl (R-SH) content ( $\mu\text{mole/g}$  protein) of the protein powders measured with (+) and without (-) sodium dodecyl sulfate (SDS) and their corresponding high-protein nutrition (HPN) bars after storage at 22°C or 32°C for 0, 6, and 29 weeks**

Protein <sup>1</sup>	Powder R-SH		HPN bar R-SH after storage <sup>2</sup>				
			22°C			32°C	
	-SDS	+SDS	Week 0	Week 6	Week 29	Week 6	Week 29
MPC80	5.2 <sup>a,z</sup>	6.0 <sup>a,z</sup>	5.8 <sup>a,y</sup>	5.8 <sup>a,y</sup>	5.5 <sup>a,y</sup>	5.3 <sup>a,y</sup>	13.7 <sup>a,z</sup>
E95	2.9 <sup>b,z</sup>	2.9 <sup>b,z</sup>	-	-	-	-	-
E105	2.8 <sup>b,z</sup>	2.1 <sup>b,z</sup>	3.0 <sup>b,z</sup>	1.0 <sup>b,yz</sup>	0.4 <sup>b,y</sup>	0.6 <sup>b,y</sup>	3.1 <sup>b,z</sup>
E116	1.4 <sup>b,z</sup>	1.5 <sup>b,z</sup>	1.2 <sup>b,y</sup>	1.1 <sup>b,y</sup>	0.4 <sup>b,y</sup>	0.4 <sup>b,y</sup>	4.0 <sup>b,z</sup>

<sup>1</sup> MPC80, control spray dried milk protein concentrate with 80% protein. E95, E105, and E116, MPC80 extruded at die-end melt temperature of 95°C, 105°C, and 116°C, respectively.

<sup>2</sup> HPN bars (30% protein w/w) were previously prepared using MPC80, E105, or E116 and were previously stored by Banach and others (2016a).

<sup>a-b</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>y,z</sup> Protein powder or HPN bar least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

After storing the HPN bars 29 weeks at 32°C, protein solubility decreased to 6.9, 7.5, and 14.6 mg/mL and insolubility ( $\pm$ SD), with respect to week 0 solubility, was 70% ( $\pm$  2), 67% ( $\pm$  7), and 44% ( $\pm$  3) for the models formulated with E105, E116, and MPC80, respectively. These protein solubility values were significantly lower than those obtained from the same HPN bar kept at all other storage conditions ( $P < 0.05$ ), which ranged between 22.9-23.4, 22.8-23.2, and 26.1-27.2 mg/mL when formulated with E105, E116, and MPC80, respectively. More protein was extractable from the control HPN bar than the extrusion-modified MPC80 formulated samples at equivalent storage conditions ( $P < 0.05$ ). Protein solubility decreases as internal aggregations occur during HPN bar storage.

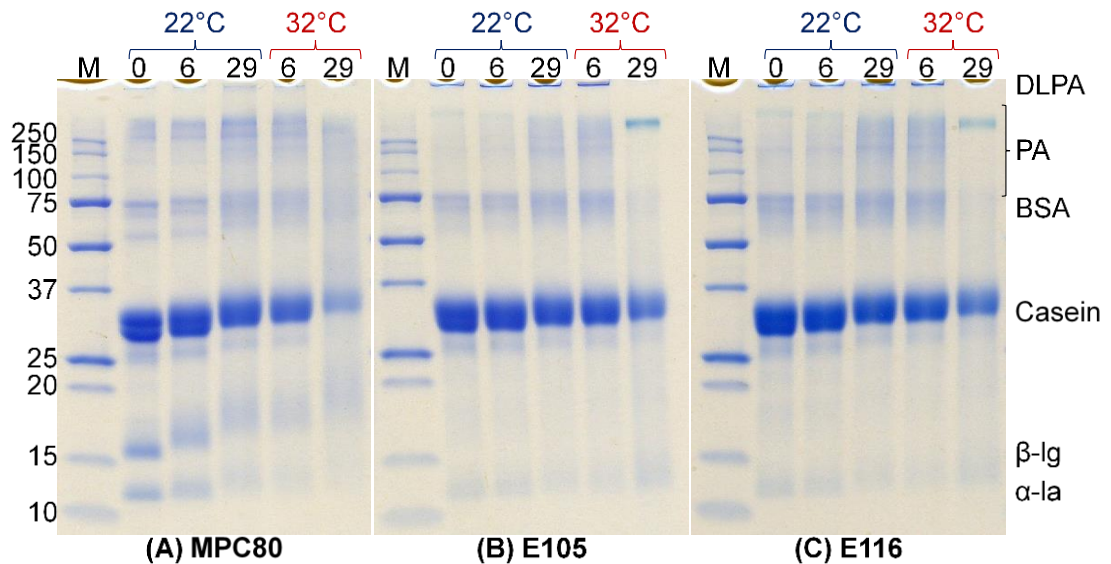
HPN bar free sulfhydryl content on week 0 was comparable to that of the protein powder ingredient with which it was formulated and this showed that the extra constituents do not interfere with Ellman's assay (Table 6-3). Excluding the samples kept for 29 weeks at 32°C, the free sulfhydryl content of the HPN bars formulated with

MPC80 and E116 did not change during storage ( $P > 0.05$ ). The free sulfhydryl content of the E105 HPN bar decreased after storage for 29 weeks at 22°C or 6 weeks at 32°C ( $P < 0.05$ ). The free sulfhydryl content of the control HPN bar was always greater than those prepared with extrusion-modified MPC80 ( $P < 0.05$ ). The measureable free sulfhydryl content in each HPN bar increased after 29 weeks at 32°C ( $P < 0.05$ ). Under these storage conditions, total mole free sulfhydryl, determined using net sample absorbance (i.e.,  $A_{\text{sample+DTNB}} - A_{\text{sample}}$ ), increased while soluble protein decreased. Decreasing free sulfhydryl content during HPN bar storage was expected if disulfide bonds form with time. Free sulfhydryl measurement was also influenced by selective protein solubility as discussed in the following SDS-PAGE section. This longer study corroborated shorter storage results (Banach and others 2016b) and showed that HPN bar free sulfhydryl content did not change during ~7 months at 22°C ( $P > 0.05$ ) even though texture changed during that time (Banach and others 2016a).

#### *6.5.5 Reduced and Non-reduced SDS-PAGE of the High-protein Nutrition Bars during Storage*

Non-reduced SDS-PAGE was used to look at the soluble proteins present in the free sulfhydryl assay buffer. Increasing protein free sulfhydryl content during shorter HPN bar storage was due to increasing  $\beta$ -lg (C121) concentration as casein solubility decreased (Banach and others 2016b). In the HPN bars formulated with E105 (Figure 6-3B) and E116 (Figure 6-3C),  $\beta$ -lg was initially soluble at a low concentration. Certainly the soluble  $\beta$ -lg concentration in the control extract was higher than that in E105 and E116 HPN bar extracts. The protein band for  $\beta$ -lg grew more disperse with time stored, which was likely due to increasing molecular weight by way of glycation of one or more of 19 potential sites (Chen and others 2012), and this made it difficult to tell

if its extractable concentration actually changed during storage. However, the soluble  $\beta$ -lg results aligned with the higher and lower free sulfhydryl contents obtained for the control and extrusion-modified MPC80 HPN bars, respectively, through 6 weeks storage at 32°C (Table 6-3).

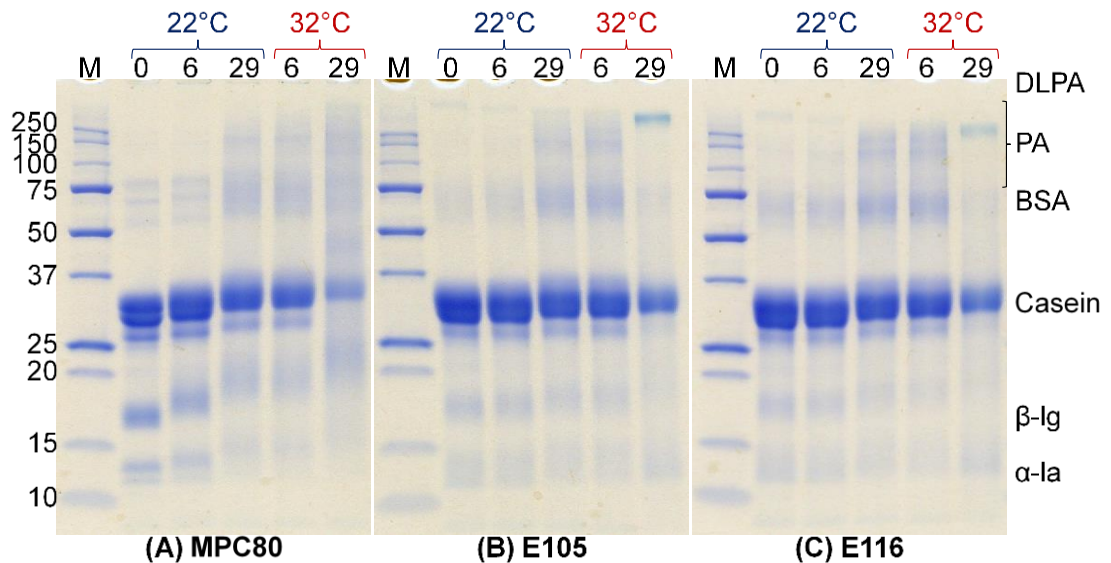


**Figure 6-3 Non-reduced extraction/non-reduced SDS-PAGE of the proteins in the model high-protein nutrition (HPN) bars (30% protein w/w) formulated with MPC80 (A), E105 (B), or E116 (C) after storage for 0, 6, and 29 weeks at 22°C or 32°C.** MPC80, control spray dried milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include:  $\alpha_{S2}$ ,  $\alpha_{S1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -lg, beta-lactoglobulin.  $\alpha$ -Ia, alpha-lactalbumin.

As HPN bar storage time progressed, the caseins became more glycosylated, less soluble, and less separable. Extensive glycation was prevalent in the control HPN bar after 29 weeks storage at 32°C as non-reducible protein aggregates, that is they persisted after reducing agent addition (Figure 6-4), were vertically distributed within its respective lane (Figure 6-3A). A more noticeable effect of storage on the caseins was that they became less soluble and less separable with time on the non-reduced SDS-PAGE gel, which was clearly seen for the control HPN bar (Figure 6-3A). Development of casein insolubility was more prevalent in the control HPN bar. This sample was more powder-



like than those formulated with extruded MPC80 and it is well known that MPC powders, particularly their micellar casein components, continually lose solubility during storage whereas the whey proteins retain solubility (Haque and others 2015; Gazi and Huppertz 2015). Casein insolubility after HPN bar storage for 29 weeks at 32°C meant that the soluble whey proteins, including  $\beta$ -lg (C121), should be relatively more concentrated in solution and should be more accessible to DTNB during Ellman's assay. The  $\beta$ -lg band was not apparent on the SDS-PAGE gel for the E105 or E116 HPN bar after such long storage. Its molecular weight may have changed due to glycation and involvement in non-reducible protein aggregates. Such aggregates were present between 75 kDa through just greater than 250 kDa on both the non-reduced and reduced SDS-PAGE gels and formed a concentrated protein band in the E105 and E116 HPN bar after 29 weeks at 32°C.



**Figure 6-4** Non-reduced extraction/reduced SDS-PAGE of the proteins in the model high-protein nutrition (HPN) bars (30% protein w/w) formulated with MPC80 (A), E105 (B), or E116 (C) after storage for 0, 6, and 29 weeks at 22°C or 32°C. MPC80, control spray dried milk protein concentrate with 80% protein. E105 and E116, MPC80 extruded at die-end melt temperature of 105°C and 116°C, respectively. M, a molecular weight marker (kDa). DLPA and PA, disulfide linked protein aggregates and protein aggregates, respectively. BSA, bovine serum albumin. Caseins, from high to low molecular weight, include:  $\alpha_{S2}$ ,  $\alpha_{S1}$ ,  $\beta$ , and  $\kappa$ .  $\beta$ -lg, beta-lactoglobulin.  $\alpha$ -la, alpha-lactalbumin.

Reducible and non-reducible soluble protein aggregates formed during HPN bar storage. Extruding MPC80 aggregated  $\beta$ -lg into high molecular weight ( $> 250$  kDa), DLPA that did not enter the non-reduced gel (Figure 6-3). After reduction these protein aggregates were disassociated, and  $\beta$ -lg and  $\kappa$ -casein, which formed soluble protein aggregates during MPC production (Donato and Guyomarc'h 2009) or during its extrusion, entered the gel (Figure 6-4). A portion of the protein aggregates (PA) (Figure 6-3 and Figure 6-4) were also DLPA since vertical band smearing in this region was less intense after reducing agent addition. The PA that remained after reduction were due to covalently crosslinked, Maillard-induced aggregations that were previously related to HPN bar texture change (Zhou and others 2013). Casein resolution in E105 and E116 improved with time stored on the reduced SDS-PAGE gel and was likely due to protein glycation. However, casein solubility remained low, which confirmed that insolubility in the free sulfhydryl assay buffer was not due to the formation of DLPA. Disulfide bonds and Maillard-induced aggregates formed in the control HPN bar over  $\sim 7$  months storage whereas the latter protein aggregation was more prevalent in the extrusion-modified MPC80 HPN bars. The HPN bars formulated with extruded MPC80 changed texturally during that time (Banach and others 2016a) even though internal disulfide bond formation was limited since the proteins were pre-aggregated as DLPA. SDS-PAGE revealed that some disulfide bonds form during HPN bar storage. This result did not agree with Ellman's assay, especially for the control the control HPN bar, which had no free sulfhydryl content change, but was the most prone to DLPA formation during storage. HPN bar texture changed during storage (Banach and others 2016a) whether or not DLPA were forming internally. However, texture changed more slowly when the

extrusion-modified MPC80s, which were pre-aggregated as DLPA by extrusion, were used in the HPN bars. Internal protein-protein disulfide bond formation partially contributes to HPN bar texture change, but PA that form due to Maillard browning likely play a greater role as they continually developed as HPN bar texture changed.

#### *6.5.6 Protein Powder Free Amine Content and its Change during High-protein Nutrition Bar Storage*

Although OPA registers both  $\epsilon$ - and  $\alpha$ -amino groups, this method is favored for measuring reactive or nutritionally active lysine over the total lysine technique which includes nutritionally unavailable lysine (Brestenský and others 2014; Moughan and Rutherford 2008; Carpenter and others 1989). Extrusion processing is known to decrease reactive lysine, with the extent of decrease dependent on the processing conditions, of which melt temperature plays a key role (Llopart and others 2014; Saalia and Phillips 2011; Konstance and others 2002). Protein solubility ranged from 3.1 to 4.2 mg/mL and, with similar solubility, differences in free amine content are attributable to the different processing conditions. Extrusion-modified MPC80 had significantly lower free amine content than the control and increasing melt temperature led to a more significant decrease ( $P < 0.05$ ) (Table 6-4).

**Table 6-4 Free amine (R-NH<sub>2</sub>) content (μmole/g protein) of the protein powder and their corresponding high-protein nutrition (HPN) bars after storage at 22°C or 32°C for 0, 6, or 29 weeks**

Protein <sup>1</sup>	Powder R-NH <sub>2</sub>	HPN bar R-NH <sub>2</sub> after storage <sup>2</sup>					
		Week 0	22°C			32°C	
			Week 6	Week 29	Week 6	Week 29	
MPC80	877 <sup>a</sup>	828 <sup>a,z</sup>	615 <sup>a,y</sup>	367 <sup>a,x</sup>	380 <sup>a,x</sup>	264 <sup>a,w</sup>	
E95	775 <sup>b</sup>	-	-	-	-	-	
E105	748 <sup>c</sup>	713 <sup>b,z</sup>	585 <sup>b,y</sup>	358 <sup>a,x</sup>	355 <sup>ab,x</sup>	229 <sup>b,w</sup>	
E116	695 <sup>d</sup>	667 <sup>c,z</sup>	560 <sup>b,y</sup>	348 <sup>a,x</sup>	346 <sup>b,x</sup>	242 <sup>ab,w</sup>	

<sup>1</sup> MPC80, control spray dried milk protein concentrate with 80% protein. E95, E105, and E116, MPC80 extruded at die-end melt temperature of 95°C, 105°C, and 116°C, respectively.

<sup>2</sup> HPN bars (30% protein w/w) were previously prepared using MPC80, E105, or E116 and were previously stored by Banach and others (2016a).

<sup>a-d</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>w-z</sup> HPN bar least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

HPN bar soluble protein ranged from 7.6-7.8, 7.7-7.8, and 7.5-8.0 mg/mL when prepared with E105, E116, and MPC80, respectively, through 6 weeks storage at 32°C (≈52 weeks at 22°C) and no significant ( $P > 0.05$ ) differences in solubility were detected after equivalent storage. However, protein solubility decreased ( $P < 0.05$ ) to 2.1, 1.9, and 3.6 mg/mL for the same respective HPN bars after 29 weeks at 32°C. At that time, protein insolubility, with respect to week 0 solubility, was 73% ( $\pm 2$ ), 75% ( $\pm 2$ ), and 52% ( $\pm 3$ ) for the HPN bars formulated E105, E116, and MPC80, respectively. Protein aggregation occurred during HPN bar storage (Zhou and others 2013) and this decreased protein solubility. These aggregations were not strictly due to disulfide bond formation since the reducing agent DTT did not restore protein solubility during the free amine assay. Non-disulfide based protein aggregates previously formed during MPC80 powder storage as advanced Maillard browning products (e.g., glutaraldehyde) and di-carbonyls (e.g., glyoxal) crosslinked proteins and decreased their solubility (Le and others 2013, 2012, 2011). A similar occurrence happened during HPN bar storage.

Despite having drastically reduced protein solubility after 29 weeks at 32°C, HPN bar free amine content consistently decreased during storage ( $P < 0.05$ ) (Table 6-4). Unlike the free sulfhydryl assay, where free sulfhydryl content increased with drastically reduced protein solubility, the free amine content of each HPN bar was the lowest measured ( $P < 0.05$ ). HPN bar week 0 free amine content was similar to their formulating protein ingredient. Decreasing free amine content, without substantial changes in solubility, were likely due to glycation of the lysine residues with glucose, fructose, and lactose. Decreasing OPA absorbance was also seen in other model HPN bars (Banach and others 2014; Loveday and others 2010, 2009), and was attributed to crosslink formation or Maillard-induced glycation, although these may or may not be a significant contributor to HPN bar texture change (McMahon and others 2009). Sugar alcohols, which do not participate in Maillard browning reactions, may potentially slow HPN bar texture change by not participating in glycation, thus, limiting the formation of advanced browning products and crosslinked protein aggregates (Liu and others 2009). While there remains discrepancy about Maillard browning's effect on HPN bar texture change, glycation of lysine decreases its nutritional value as the glycated products are not recognized by digestive enzymes (Brestenský and others 2014). The sulfur containing amino acids are limiting in MPC and thus lysine glycation during extrusion and HPN bar storage should not be a major nutritional concern (Rutherford and others 2015). A freshly prepared HPN bar might be more nutritious from an essential amino acid standpoint than one that has been stored for an extended period.

## 6.6 Conclusions

Extrusion-modified MPC80 had higher density, including particle and tapped, and lower occluded air than the spray dried control. Extrusion decreased WHC, protein solubility, and surface hydrophobicity, but improved MPC80's overall ability to interact with water. This allows for rapid powder hydration during HPN bar production and allows the protein component to stay consistently hydrated during storage making it a better option for improved textural stability. Maillard-induced protein aggregations caused free amine decreases, which were more prevalent during HPN bar storage than the formation of disulfide linked protein aggregates and changes in free sulfhydryl content. Protein induced chemical changes occurred during HPN bar storage, but texture and stability were more heavily influenced by the differences in protein powder functionality brought out by extrusion, especially its ability to interact well with water.

## 6.7 Acknowledgement

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## CHAPTER 7. PARTICLE SIZE OF MILK PROTEIN CONCENTRATE POWDER AFFECTS THE TEXTURE OF HIGH-PROTEIN NUTRITION BARS DURING STORAGE

Modified from a manuscript to be submitted to the *Journal of Food Science*

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### 7.1 Abstract

Milk protein concentrate with 85% protein (MPC85) was jet-milled at two levels of particle size reduction, and separately, its morphology was altered by freeze-drying. These physical modifications reduced the water holding capacity and increased the dispersibility index of MPC85. Water had larger contact angles (CA) on the modified MPC85 and the water droplet profiles changed, by both spread and absorption, at a slower rate compared to the control. High-protein nutrition (HPN) bars were prepared with the control, jet-milled, and freeze-dried MPC85, and textural and physical attributes including hardness, fracturability, crumbliness, adhesiveness, color, water activity, and moisture content were measured during storage. All the HPN bars hardened and lost cohesion during storage. Those prepared with finely jet-milled MPC85 were firmer, more cohesive, and less susceptible to texture change during storage than the control. Jet-milling and freeze-drying altered the functional properties of MPC85 and these alterations produced HPN bars with favorable texture and improved stability. Protein powder particle size should be considered when preparing HPN bars with high-protein MPCs.

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## 7.2 Practical Application

Milk protein concentrate (MPC) powder particle size significantly impacts the texture of high-protein nutrition (HPN) bars. Finely jet-milled MPC85 produced HPN bars with increased firmness and decreased crumbliness. Particle size reduction improved HPN bar textural stability and this physical modification has the potential to extend the textural shelf life of MPC-formulated HPN bars.

## 7.3 Introduction

The main function of protein in nutritional bars, specifically high-protein nutrition (HPN) bars (20-50% protein w/w), is to nurture the consumer, but at such a high level of inclusion, this macromolecule also affects the product's textural attributes. It is well known that high-protein (i.e.,  $\geq 80\%$ ) milk protein concentrate (MPC) powders produce HPN bars that lack cohesion and quickly harden during storage (Banach and others 2016a; Imtiaz and others 2012; Loveday and others 2009). On the other hand, whey protein concentrate (WPC) or isolate (WPI), specifically their hydrolysates, produce HPN bars with greater textural stability (McMahon and others 2009). Hydrolysates have lower glass transition temperature ( $T_g$ ) than their intact counterparts, which allows for protein powder plasticization during HPN bar production, the rubbery textural state to be maintained during storage, and potential texture-changing reactions to be slowed by increased system viscosity (Rao and others 2013). MPC powders contain  $\sim 20\%$  whey and  $\sim 80\%$  casein on a protein-basis. Each casein (i.e.,  $\alpha_{s1}$ ,  $\beta$ ,  $\kappa$ ,  $\alpha_{s2}$ ) has higher molecular weight (i.e., 24, 24, 19, 25 kDa) than  $\beta$ -lactoglobulin ( $\beta$ -lg; 18 kDa) and  $\alpha$ -lactalbumin ( $\alpha$ -la; 14 kDa), the two major proteins that makeup WPC and WPI. With higher net molecular weight, MPCs have elevated glass-rubber transition temperature ( $T_{gr}$ ), a value

analogous to the thermodynamically determined  $T_g$  (Hogan and others 2016). MPC's  $T_{gr}$  increases with powder protein content (Kelly and others 2015). With elevated  $T_{gr}$ , high-protein MPC powder particles are less likely to collapse, lose their structure, and produce a plasticized HPN bar compared to protein powders with lower  $T_{gr}$  (Hogan and others 2016). This explains why particle structure persisted in a model HPN bar formulated at 20% MPC80 (w/w) (Loveday and others 2009).

MPC powder particle size and distribution, shape, and surface composition influence their behavior when used in solid-type, intermediate moisture food (IMF) systems such as HPN bars (Li and others 2016; Huppertz and Hogan 2015). High-fructose corn syrup (HFCS) and other polyols (e.g., glycerol, sorbitol, maltitol) are used in HPN bar formulations to bind the system together and impart textural stability while maintaining microbe-inhibiting water activity ( $a_w \leq 0.65$ ) (Liu and others 2009). Smaller sized, higher polydispersity WPI powder particles increased the apparent viscosity less than larger, more uniform WPI powder particles when added to HFCS at the same volume fraction and required a higher volume fraction for the fluid HFCS to transition to a solid-like HFCS/WPI system (i.e., solidification) (Hogan and others 2016). Larger, agglomerated micellar casein concentrate (MCC), with approximate casein-to-whey protein ratio of 92:8 (Dairy Management Inc. 2015), particles produced HPN bars that were powdery yet texturally more stable than the dough-like control formulated with non-agglomerated, spray dried MCC (Hogan and others 2012). Spray dried high-protein MPC powder particle surfaces are the preferential location for fat and protein whereas hydrophilic lactose and minerals are interiorly located (Kelly and others 2015). MPC

powder particle size and surface composition have not garnered any attention as to how altering these properties influences their textural performance in HPN bars.

Spray dried MPC powders are relatively finer and in order to preserve the dry state, further size reduction requires either a ball-mill or jet-mill (Sanguansri and Augustin 2006). Superfine WPC powder was produced by ball-milling, but it required 4-8 h of processing, an obvious limitation of particle size reduction using this technique (Sun and others 2015b). Jet-milling is a continuous operation where size reduction occurs by particle-particle and particle-wall collisions within the grinding chamber by high velocity airflow (Saleem and Smyth 2010). Jet-milling is not a new unit operation in food processing, and while several powders have exhibited new functionality after particle size reduction, jet-mills are rarely utilized to modify the functionality of protein concentrates or isolates (Muttakin and others 2015; Hayakawa and others 1993). Jet-milled wheat flour had increased water holding capacity (WHC) and lighter color, and when baked into bread, it reduced specific volume, luminosity, moisture content, glycemic index, and increased crumb hardness (Protonotariou and others 2015, 2014). Superfine soy flour had higher WHC, solubility, swelling, fat binding, and improved sensory properties compared to the un-milled control (Muttakin and others 2015). Finely milled WPCs had increased solubility, hydrophobicity, oil binding, and foaming properties, but in this case, particle size reduction decreased WHC (Sun and others 2015a, 2015b). Hayakawa and others (1993) found that casein and egg white powder surface hydrophobicity increased as particle size decreased. Additionally, jet-milling can alter protein powder functionality by application of high compressive and shear forces (Hayakawa and others 1993). It is currently unknown how jet-milling will affect the

HPN bar relevant functional properties of high-protein MPCs, but alteration of those described will influence its performance in HPN bars.

Literature protein powder functionality discussions focus on protein solubility and its dependent properties (e.g., emulsification, foaming, gelation, heat stability). Such properties and structure-function information are only relevant in high-moisture liquid (e.g., beverages) and semi-liquid (e.g., soft gels, yogurt) foods. Properties other than solubility need to be considered in low-moisture (e.g., protein powders) and IMFs (e.g., HPN bars). For example, a protein powder does not need a soluble solids index (SSI) of 100% to function in a HPN bar formulation (Cho 2010) whereas insolubility would be problematic in beverages. Protein powder WHC and surface hydrophobicity, which are both relevant in HPN bars, were influenced by particle size reduction as previously discussed (Sun and others 2015a, 2015b; Hayakawa and others 1993). High WHC is thought to be a driving force behind moisture migration to the protein component during HPN bar storage, one of several proposed mechanisms for texture change (Hazen 2010; Loveday and others 2010, 2009). Protein powder surface hydrophobicity may affect the rate of particle hydration during HPN bar manufacture as well as moisture migration during storage if particle structure is maintained. In the following study, particle size distribution, densities, occluded and interstitial air volumes, WHC, dispersibility index (DI), surface hydrophobicity, and wettability were measured for particle size reduced (i.e., jet-milled) and morphologically altered (i.e., freeze-dried) MPC85. These functional properties were used to explain the HPN bar textural differences observed during storage in model HPN bars formulated with these physically modified MPC85s.

## 7.4 Materials and Methods

### 7.4.1 Materials

MPC85 (NutraPro®85, 85.2% protein, 4.3% moisture, 1.9% fat, 7.0% ash, 1.6% lactose, Grassland Dairy Products, Inc., Greenwood, WI), maltodextrin (Maltrin®180, 16.5-19.9 dextrose equivalent, 6% moisture, Grain Processing Corporation, Muscatine, IA), HFCS (CornSweet®55, 55% fructose, 41% dextrose, 4% higher saccharides, 23% water, Archer Daniels Midland, Decatur, IL), maltitol syrup (Lycasin®80/55, 51.7% D-maltitol, 3.0% D-sorbitol, 24.5% water, Roquette America, Keokuk, IA), non-hydrogenated trans-free palm oil (SansTrans®39, IOI Loders Croklaan, Channahon, IL), and low-viscosity liquid lecithin (Beakin®LV1, 0.8% moisture, Archer Daniels Midland, Decatur, IL) were donated. Glycerol (99.8% glycerol, 0.1% water) was purchased from Fisher Scientific (Waltham, MA).

### 7.4.2 Jet-milling and Freeze-drying MPC85

MPC85 powder was jet-milled by a service lab using an Aveka 100/20 jet mill/air classifier system (Aveka CCE Technologies, Cottage Grove, MN). Coarse (JM-Coarse) and fine (JM-Fine) powders were obtained by changing the classifier rotor speed from 1,000 to 2,500 rpm, respectively. Separately, MPC85 powder was rehydrated (5% protein w/w) in room temperature Millipore water for 2 h with continual overhead mixing. Rehydration continued for 5 h at 4°C, after which the solution was frozen overnight (-20°C) and freeze-dried the following day (VirTis Genesis 25 LE, SP Scientific, Warminster, PA). The freeze-dried material was mechanically milled (L'Equip NutriMill, St. George, UT) into powder (FD).



#### 7.4.3 Protein Powder Characterization and Functional Property Evaluation

Protein content was measured ( $n = 2$ ) by Dumas nitrogen combustion (AOAC 1998). Moisture content was determined ( $n = 3$ ) by mass difference after drying for 16 h at 102°C. Particle size was measured ( $n = 2$ ) by laser diffraction (Mastersizer 2000, Malvern Inc., Worcestershire, United Kingdom) after dispersing a sample of each powder in isopropanol (Banach and others 2016a). Protein powder (30 g) was transferred into a glass 100-mL graduated cylinder and loose ( $\rho_{\text{loose}}$ ), tapped ( $\rho_{100\text{X}}$ ), and extremely tapped ( $\rho_{1250\text{X}}$ ) density were calculated ( $n = 3$ ) based on volume ( $\text{cm}^3$ ) after 0, 100, and 1,250 taps, respectively (Autotap™, Quantachrome Instruments, Boynton Beach, FL). Particle density ( $\rho_{\text{particle}}$ ) was measured ( $n = 2$ ) by helium pycnometry (G-DenPyc 2900, Gold APP Instruments Corporation, Beijing, China). MPC85 solids density ( $\rho_{\text{solids}}$ ) was calculated to be 1.39  $\text{g}/\text{cm}^3$  based on its component (i.e., fat, protein, lactose, ash) densities (Crowley and others 2014; Walstra and others 2005). Occluded ( $V_{\text{oa}} = 100/\rho_{\text{particle}} - 100/\rho_{\text{solids}}$ ) and interstitial ( $V_{\text{ia}} = 100/\rho_{100\text{X}} - 100/\rho_{\text{particle}}$ ) air volumes ( $\text{mL}/100 \text{ g}$ ) were also calculated (Crowley and others 2014). Protein powder WHC was obtained ( $n = 3$ ) using the water saturation technique described by Quinn and Paton (1979). Dispersibility index (DI) was reported ( $n = 3$ ) as the percent solids that passed a 212-micron mesh (No. 70) after dispersing the protein powder (10 g) in Millipore water (100 mL) with a spatula for 25 s (Bouvier and others 2013; Schuck and others 2012).

Surface hydrophobicity and wettability were probed ( $n = 4$ ) by measuring the dynamic contact angle and absorption rate of water on a pressed surface made from each protein powder. Powder (0.10 g) was loaded into a 13-mm pellet die (model 3619, Carver, Inc., Wabash, IN) and was held at 8,000  $\text{kg}_f$  for 2 min (model 4350, Carver, Inc.,

Wabash, IN). A 4  $\mu\text{L}$  Millipore water droplet was placed (Gilmont GS-1200 Micrometer Syringe, Cole-Parmer, Vernon Hills, IL) on the pellet and images were captured every 0.1, 1, and 10 s between 0-1, 1-10, and 10-420 s, respectively, using a goniometer (model 250, Ramé-hart Instrument Co., Succasunna, NJ). Images were reprocessed (DROPimage® software, version 2.8.02, University of Oslo, Norway) and mean contact angle ( $^{\circ}$ ), and surface water droplet volume ( $\mu\text{L}$ ) and average volume-percent remaining were reported over 420 s.

#### *7.4.4 Model High-protein Nutrition Bar Preparation*

HPN bars (700 g) were prepared ( $n = 3$ ) at 30% protein (w/w) using either control, JM-Fine, or FD MPC85. HFCS (39.6 g), glycerol (146.1 g), maltitol syrup (72.5 g), and distilled water (50.2 g) were heated  $60^{\circ}\text{C}$  and were combined with melted palm oil (105.1 g)/lecithin (3.5 g) (Banach and others 2016a). Protein powder (248 g) blended with maltodextrin (35.1 g) were slowly added to the lipid/polyol blend over 4.5 min of low-speed mixing with the paddle attachment (K5SS, Kitchen Aid, St. Joseph, MI). HPN bar dough was pressed at constant height into a pan ( $18.4 \times 22.2 \times 1.27$  cm) and into  $a_w$  sample cups. Cylindrical (dia. = 1.91 cm) samples were cut from the sheeted dough and were sealed in metallized bags (S-16891, Uline, Pleasant Prairie, WI).

#### *7.4.5 Model High-protein Nutrition Bar Testing*

HPN bars were kept at room ( $22^{\circ}\text{C}$ ) or elevated ( $32^{\circ}\text{C}$ ) temperature for up to 42 d storage. Six HPN bar samples for each protein, storage temperature, and storage time (0, 6, 13, 20, 29, and 42 d) combination were compressed using 2-bite texture profile analysis (TPA) (Banach and others 2016a). Hardness (N) was the force at maximum strain (60%), fracturability (N) was where the sample yielded or cracked during the first

compression, and maximum compressive force (N) was the larger of the two attributes for each measurement. Instrumental adhesiveness (J) was recorded as the absolute area under the curve generated during crosshead withdrawal after the first compression. In addition to the mean ( $n = 3$ ), percent change of each TPA attribute was calculated with respect to the specific HPN bar average on day 0. After TPA, HPN bar samples were transferred three at a time to a sieve stack that was mechanically shaken for 30 s on speed 3 (Shaker #18480, CSC Scientific Sieve, Fairfax, VA). Average HPN bar crumbliness ( $n = 3$ ) was reported as the mass-percent passing the top mesh (No. 3.5; 5.6 mm aperture) (Banach and others 2016a).

HPN bar color values ( $L^*$ ,  $a^*$ ,  $b^*$ ) were acquired ( $n = 3$ ) using a colorimeter (LabScan XE, Hunter Laboratory Associates, Inc., Reston, VA) and total color change ( $\Delta E$ ), with respect to day 0 for each HPN bar, was calculated.  $a_w$  was measured ( $n = 3$ ) with a dew point based analyzer (Aqua Lab 4TE Duo, Decagon Devices Inc., Pullman, WA). HPN bar moisture content was measured ( $n = 3$ ) on day 0 and day 42 by oven drying 1 g at 102°C for 26 h. After 42 d, the height of each HPN bar was measured, its volume was calculated, and density was estimated by dividing by sample mass. The mean ( $n = 3$ ) for each attribute was calculated across the 3 HPN bar preparations and was reported.

#### 7.4.6 Statistical Analyses

Powder particle sizes, densities, volumes, and functional properties were modeled as a function of the protein powder using the generalized linear mixed model (GLMM) in SAS® (version 9.4, SAS Institute Inc., Cary, NC). Sample replicate was set as the random error term. For contact angle and water droplet volume, protein powder, time,

and their interaction were the independent variables and sample replicate was set as the random error term. Least squares means were significantly different if Tukey's adjusted  $P$ -value was less than 0.05. Contact angle and water droplet volume were also modeled with time set as a continuous variable and average rate of change for each was determined. Simulate was used to adjust for multiplicity and contrasts between least squares means were evaluated at  $\alpha = 0.05$ . HPN bar texture attributes, color values, water activities, and moisture contents were compared across HPN bar preparations using the GLMM. The preparation of each HPN bar was set as the random error term and least squares means were compared after Tukey's adjustment ( $\alpha = 0.05$ ).

## 7.5 Results and Discussion

### 7.5.1 Powder Protein and Moisture Content

Jet-milling or freeze-drying MPC85 did not change its average as-is protein content (84.5%). Substantial differences in moisture content were not expected. However, the high airflow rate and short-lived exposure to elevated temperature during jet-milling or the more thorough dehydration by freeze-drying might produce drier powders. Moisture content of FD (1.6%) was lower ( $P < 0.05$ ) than the statistically equivalent ( $P > 0.05$ ) control (2.6%), JM-Coarse (3.5%), and JM-Fine (3.1%).

### 7.5.2 Protein Powder Particle Size

High-protein MPCs have smooth, spherical particles with fewer wrinkles than their low-protein counterparts (Kelly and others 2015). Jet-milling demolished this geometry whereas freeze-drying likely produced more plate-like particles (Gong and others 2016). Within each diameter category (Table 7-1), the control had the largest size followed respectively by FD, JM-Coarse, and JM-Fine ( $P < 0.05$ ).  $D_{4,3}$  illustrated the

extent of particle size reduction brought about by adjusting the classifier speed from 1,000 (JM-Coarse) to 2,500 (JM-Fine) rpm during jet-milling. The particle size diameters of FD were always smaller than the control and differences in functionality and HPN bar textural performance can only be partially attributed to this drying technology.

**Table 7-1 Protein powder particle size diameters ( $\mu\text{m}$ ), loose ( $\rho_{\text{loose}}$ ), tapped ( $\rho_{100\text{X}}$ ), extremely tapped ( $\rho_{1250\text{X}}$ ), and particle ( $\rho_{\text{particle}}$ ) densities ( $\text{g}/\text{cm}^3$ ), and occluded ( $V_{\text{oa}}$ ) and interstitial ( $V_{\text{ia}}$ ) air volumes ( $\text{mL}/100 \text{ g}$ )**

MPC85 <sup>1</sup>	Particle Size Diameters				Density				Volume	
	D <sub>10</sub>	D <sub>50</sub>	D <sub>90</sub>	D <sub>4,3</sub>	$\rho_{\text{loose}}$	$\rho_{100\text{X}}$	$\rho_{1250\text{X}}$	$\rho_{\text{particle}}$	V <sub>oa</sub>	V <sub>ia</sub>
Control	18 <sup>a</sup>	67 <sup>a</sup>	179 <sup>a</sup>	86 <sup>a</sup>	0.32 <sup>b</sup>	0.37 <sup>b</sup>	0.41 <sup>c</sup>	1.08 <sup>c</sup>	20.5 <sup>a</sup>	182 <sup>a</sup>
JM-Fine	1 <sup>c</sup>	7 <sup>d</sup>	16 <sup>d</sup>	8 <sup>d</sup>	0.38 <sup>a</sup>	0.39 <sup>b</sup>	0.48 <sup>b</sup>	1.33 <sup>a</sup>	3.3 <sup>c</sup>	181 <sup>a</sup>
JM-Coarse	2 <sup>c</sup>	19 <sup>c</sup>	44 <sup>c</sup>	22 <sup>c</sup>	0.33 <sup>b</sup>	0.37 <sup>b</sup>	0.47 <sup>b</sup>	1.31 <sup>b</sup>	4.7 <sup>b</sup>	192 <sup>a</sup>
FD	11 <sup>b</sup>	39 <sup>b</sup>	97 <sup>b</sup>	49 <sup>b</sup>	0.41 <sup>a</sup>	0.50 <sup>a</sup>	0.56 <sup>a</sup>	1.33 <sup>a</sup>	3.3 <sup>c</sup>	127 <sup>b</sup>

<sup>1</sup> Control, milk protein concentrate with 85% protein (MPC85). JM-Fine, finely jet-milled MPC85. JM-Coarse, coarsely jet-milled MPC85. FD, freeze-dried MPC85.

<sup>a-d</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

The control MPC85 powder particles in the current study were larger ( $D_{4,3} = 86 \mu\text{m}$ ) than MPC80 ( $D_{4,3} = 61 \mu\text{m}$ ) used in a previous HPN bar study (Banach and others 2016a) and MPC85 ( $D_{4,3} = 31 \mu\text{m}$ ) analyzed by another group (Kelly and others 2015). Particle size differences in spray dried powders are attributable to retentate properties (e.g., percent solids, viscosity) and dryer conditions (e.g., inlet and outlet temperatures, atomization) (Chew and others 2014). MPC85s with the same composition, but larger or smaller particle size distributions will have different functionalities, especially since rehydration and dissolution rates are influenced by particle size. Solubility of casein-based protein powders (e.g., MPC, MCC) is limited by dissolution rather than wetting and smaller particles are recommended to improve this property (Schuck and others 2007).

### 7.5.3 Protein Powder Densities and Air Volumes

Reduction in particle size increased the  $\rho_{\text{loose}}$  of JM-Fine and FD ( $P < 0.05$ ) compared to the statistically equivalent JM-Coarse and control (Table 7-1).  $\rho_{100x}$  of control, JM-Fine, and JM-Coarse were statistically equivalent ( $P > 0.05$ ), but  $\rho_{1250x}$  of the jet-milled MPC85s was greater than the control ( $P < 0.05$ ). FD had particle size most similar to the control, but its  $\rho_{\text{loose}}$ ,  $\rho_{100x}$ , and  $\rho_{1250x}$  were all significantly greater, and in most instances the same was true when compared to the jet-milled MPC85s.  $\rho_{\text{particle}}$  increased with the level of jet-milled particle size reduction and it also increased after freeze-drying ( $P < 0.05$ ). Jet-milling and freeze-drying both decreased  $V_{\text{oa}}$  in comparison to the control ( $P < 0.05$ ) (Table 7-1). Only FD had significantly lower  $V_{\text{ia}}$  ( $P < 0.05$ ), a measure of air entrained between powder particles.

### 7.5.4 Protein Powder Water Holding Capacity and Dispersibility Index

Jet-milling and freeze-drying decreased the WHC of MPC85 (Table 7-2). Surface area for water absorption during WHC analysis increased with particle size reduction. However,  $\rho_{100x}$  and  $\rho_{1250x}$  of the jet-milled MPC85s indicated that particle size reduction increased compactability. Centrifugal force applied during the WHC assay compacted the jet-milled MPC85s more than the control and this limited water held between adjacent particles. Lower  $V_{\text{oa}}$  in the modified MPC85s meant less inner-particle space for water to be entrapped during WHC analysis. Reduction in WHC for both jet-milling and freeze-drying was not as large as when MPC80 was first denatured by extrusion. However, the WHC of control MPC85 and that of unmodified MPC80 only differed by 0.1 water (g)/dry powder (g) (unpublished data).

**Table 7-2 Protein powder water holding capacity (WHC; water (g)/dry powder (g)) and dispersibility index (DI; %)**

MPC85 <sup>1</sup>	WHC	DI
Control	3.4 <sup>a</sup>	44.8 <sup>b</sup>
JM-Fine	3.2 <sup>ab</sup>	65.6 <sup>a</sup>
JM-Coarse	3.0 <sup>b</sup>	72.4 <sup>a</sup>
FD	3.1 <sup>b</sup>	68.8 <sup>a</sup>

<sup>1</sup> Control, milk protein concentrate with 85% protein (MPC85). JM-Fine, finely jet-milled MPC85. JM-Coarse, coarsely jet-milled MPC85. FD, freeze-dried MPC85.

<sup>a,b</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

Physical modification increased DI ( $P < 0.05$ ), but no significant difference ( $P > 0.05$ ) was found between jet-milling and freeze-drying (Table 7-2). DI decreased in the order JM-Coarse  $>$  FD  $>$  JM-Fine ( $P > 0.05$ ). Protein powder size reduction alone may have increased particle passage through the mesh during DI analysis. Another spray dried MPC85 had DI of 38% and after modification by extrusion-porosification, which led to slight particle size reduction, its DI was increased to 96% (Bouvier and others 2013). Porosification by slowly freezing rehydrated MPC85 and freeze-drying may have similarly increased the DI of FD. This freeze-drying process previously improved the solubility of MPC80 between pH 5.5-7.0, an inclusive range of model HPN bar pH, and increased its gel strength and decreased its surface hydrophobicity (Banach and others 2013). Casein-based powders are known to be poorly dispersible and this limits their dissolution (Bouvier and others 2013; Schuck and others 2012). Both jet-milling and freeze-drying MPC85 improved its DI and while this should improve its solubility, it may also be indicative of improved dispersibility and rehydratability in the lipid/polyol blend during HPN bar production.

### 7.5.5 Protein Powder Dynamic Contact Angle: Surface Hydrophobicity and Wettability

Initial water droplet contact angle was used to infer about surface hydrophobicity, and its change over time by spreading over and absorption into the pressed protein surface indicated wettability (Alghunaim and others 2016). The initial contact angle on JM-Fine (76°C) was greater than JM-Coarse (67°) and FD (67°) ( $P < 0.05$ ), but did not differ significantly ( $P > 0.05$ ) from the control (69°) (Table 7-3). Thus, JM-Fine had higher surface hydrophobicity and this agreed with Hayakawa and others (1993), who found that jet-milling casein increased its ANS-measured hydrophobicity by exposure of previously buried hydrophobic residues. Even after an extended 420 s observation period, the water droplets persisted on each surface (Figure 7-1A). At that time, contact angle ( $\theta_{420s}$ ) on the control was lower ( $P < 0.05$ ) than that on all the other statistically equivalent proteins. This indicated that upon approaching wetted equilibrium, the physically modified MPC85 powders maintained greater hydrophobicity than the control.

**Table 7-3 Protein powder apparent contact angle ( $\theta$ ; °) and water droplet volume (V;  $\mu$ L) at the beginning (0 s) and end of analysis (420 s)**

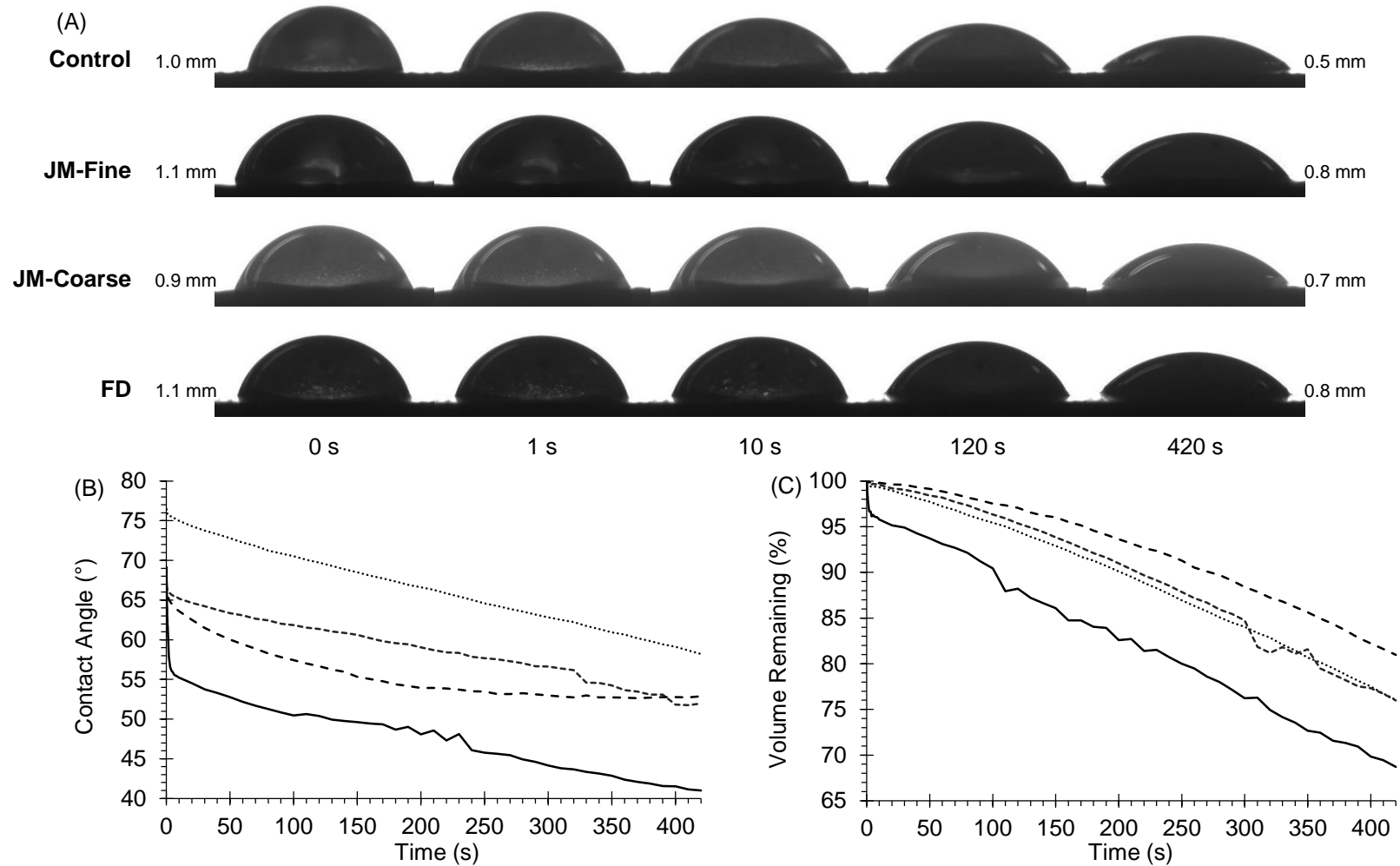
MPC85 <sup>1</sup>	Contact Angle			Water Droplet Volume		
	$\theta_{0s}$	$\theta_{420s}$	Slope (°/min)	V <sub>0s</sub>	V <sub>420s</sub>	Slope ( $\mu$ L/min)
Control	69 <sup>ab,z</sup>	41 <sup>b,y</sup>	-2.23 <sup>a</sup>	2.9 <sup>b,z</sup>	2.0 <sup>b,y</sup>	-0.12 <sup>ab</sup>
JM-Fine	76 <sup>a,z</sup>	58 <sup>a,y</sup>	-2.39 <sup>a</sup>	4.2 <sup>a,z</sup>	3.2 <sup>a,y</sup>	-0.14 <sup>a</sup>
JM-Coarse	67 <sup>b,z</sup>	52 <sup>a,y</sup>	-1.89 <sup>a</sup>	3.6 <sup>ab,z</sup>	2.7 <sup>ab,y</sup>	-0.13 <sup>ab</sup>
FD	67 <sup>b,z</sup>	53 <sup>a,y</sup>	-1.49 <sup>a</sup>	4.1 <sup>a,z</sup>	3.3 <sup>a,y</sup>	-0.11 <sup>b</sup>

<sup>1</sup> Control, milk protein concentrate with 85% protein (MPC85). JM-Fine, finely jet-milled MPC85. JM-Coarse, coarsely jet-milled MPC85. FD, freeze-dried MPC85.

<sup>a,b</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>y,z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row for each property.





**Figure 7-1** Representative side view (A), apparent contact angle (B), and volume remaining (C) of a water droplet on a pressed surface made from control, jet-milled, and freeze-dried milk protein concentrate with 85% protein (MPC85). Control (—), unmodified spray dried MPC85. JM-Fine (···), finely jet-milled MPC85. JM-Coarse (---), coarsely jet-milled MPC85. FD (- - -), freeze-dried MPC85.

Water droplet contact angle on the control decreased rapidly within 1 s, but then stabilized and decreased at a rate similar to the jet-milled and freeze-dried MPC85s (Figure 7-1B). The average slope for contact angle change over time (Table 7-3), which included the non-linear data points, was apparently greater for JM-fine and the control, but no significant difference was observed between the protein powders ( $P > 0.05$ ). Larger magnitude slope indicates a more wettable material. While some accounts indicate that MPCs are wettable (Dairy Management Inc. 2015), most would claim that these high-protein powders are non-wettable (Crowley and others 2014; Schuck and others 2012). Contact angle measurement by sessile drop technique is susceptible to a number of different errors (Alghunaim and others 2016) and qualitative water droplet profile comparisons are also useful (Figure 7-1A). Water droplet profile rapidly changed on the control and the initial contact angle was likely larger than measured. Each protein surface absorbed the water droplet and after 420 s, the goniometer-measured droplet volume significantly decreased with respect to its initially measured volume ( $P < 0.05$ ). Average slope for droplet volume ( $\mu\text{L}$ ) versus time (min) for JM-Fine was greater than FD ( $P < 0.05$ ), but significant difference was not found between the proteins (Table 7-3). Average volume percent remaining (Figure 7-1C) on the control decreased at the beginning of analysis and indicated that water was quickly absorbed. FD, which like the control underwent a rapid contact angle change during the first seconds of analysis, did not appear to absorb as much water as the other protein powders (Figure 7-1C).

#### *7.5.6 High-protein Nutrition Bar Production Characteristics*

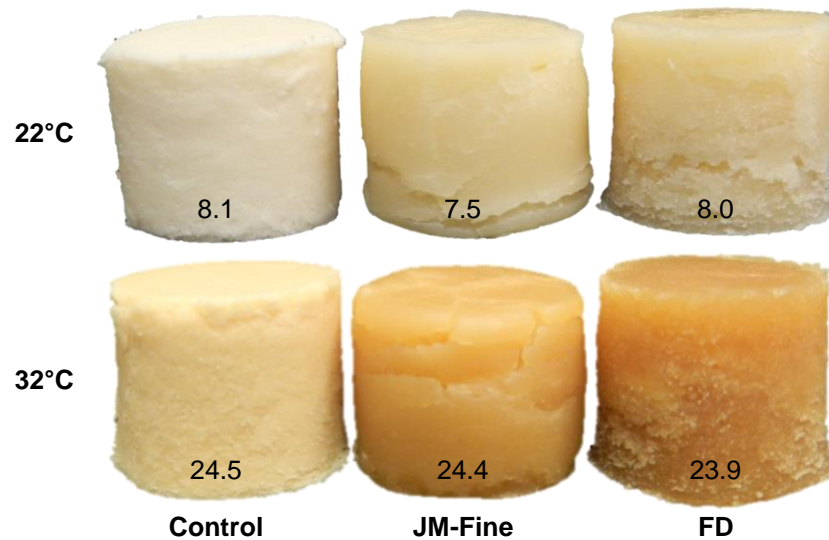
Dough made with FD and JM-Fine maintained greater fluidity than the control during HPN bar production. The smaller particles in these two powders were more easily

suspended in the lipid/polyol blend (Hogan and others 2016). Sheeted JM-Fine HPN bar dough quickly became solid-like such that it was difficult to penetrate with the cylindrical cutter and it resisted flow such that the cut samples were difficult to expel from the cutter while maintaining uniform geometry. JM-Fine HPN bars tended to split when pushed from the cutter and were gently hand formed back to the desired cylindrical shape. The HPN bars prepared with control or FD were easily sheeted and cut without shape distortion.

Particle size reduction and/or morphology changes resulted in denser HPN bars compared to the control. HPN bar mean ( $\pm$  SD) densities ( $\text{g}/\text{cm}^3$ ) were 0.81 (0.01), 0.96 (0.02), and 0.96 (0.01) when formulated with the control, FD, and JM-Fine MPC85, respectively. This suggested that some MPC85 particle structure was retained during HPN bar production as was seen in MPC80 formulated HPN bars (Loveday and others 2009). Without structural collapse of the powder particles during HPN bar manufacture, the larger particles present in the control were unable to pack as tightly together in the dough as the smaller sized particles found in JM-Fine and FD. For example, very small particles (i.e.,  $D_{10} = 1 \mu\text{m}$ ) in the latter two HPN bars filled void volume between larger sized particles and positioned themselves in closer vicinity to each other. Higher  $V_{\text{oa}}$  in the control powder also contributed to its HPN bar being low density. Using protein powders with lower  $V_{\text{oa}}$  reduced the amount of air incorporated into the HPN bars and this is why the products made with JM-Fine and FD were denser than the control. During model HPN bar production, it was impossible to increase the density of the control by pressing more mass into the fixed volume pan as was previously discussed (Banach and others 2016a).

### 7.5.7 High-protein Nutrition Bar Color and Water Activity

Protein ingredient particle size did not significantly affect  $\Delta E$  of the HPN bars (Figure 7-2), but it was significantly influenced by temperature and storage time ( $P < 0.05$ ). Average  $\Delta E$  after 42 d at 22°C and 32°C were 7.8 and 24, respectively. On day 0, whiteness ( $L^*$ ) decreased significantly in the order of control, JM-Fine, and FD, whereas yellowness ( $b^*$ ) of JM-Fine and FD were greater than the control ( $P < 0.05$ ). The same order was maintained throughout HPN bar storage while  $L^*$  decreased and  $b^*$  increased as all samples darkened and yellowed, respectively. There was no difference between  $a^*$  values on day 0, but after 42 d at 32°C redness ( $a^* > 0$ ) of JM-Fine and FD HPN bars was greater than the control ( $P < 0.05$ ). Jet-milling and freeze-drying, both of which increased total surface area available for the Maillard reaction, did not affect  $\Delta E$ , but increased sample browning.



**Figure 7-2** Images of the high-protein nutrition (HPN) bars after 42 day storage at 22°C or 32°C. HPN bars were formulated at 30% protein (w/w) using milk protein concentrate with 85% protein (MPC85). Control, unmodified spray dried MPC85. JM-Fine, finely jet-milled MPC85. FD, freeze-dried MPC85. Average total color change ( $\Delta E$ ) values, with respect to day 0, are listed on each HPN bar.

On the day of model HPN bar production, average  $a_w$  was 0.60 and after 42 d at 22°C or 32°C, it was 0.61. Storage time had an effect on  $a_w$  ( $P < 0.05$ ), but all other independent variables and their interaction terms were insignificant ( $P > 0.05$ ). Small yet significant increases in  $a_w$  previously occurred early on during in HPN bar storage (Banach and others 2016a, 2014) and suggested water molecule migration from intermediary association with the protein to the bulk phase. Larger  $a_w$  increases occurred in HPN bars more susceptible to texture change (McMahon and others 2009). On day 0, HPN bar moisture content of the control (26%) was higher ( $P < 0.05$ ) than JM-Fine (24%) and FD (24%). There was no significant ( $P > 0.05$ ) HPN bar moisture content change during storage at either temperature, which confirmed that the observed texture changes (below) were not due to moisture loss. Higher moisture and  $a_w$  in the present system, with respect to HPN bars previously formulated with MPC80 (Banach and others 2016a, 2014), might have masked the movement of water molecules between constituents by reducing internal  $a_w$  gradients. Dew point based  $a_w$  measurement lacks sensitivity and no detectable change during storage does not fully rule out movement of water molecules.

#### *7.5.8 High-protein Nutrition Bar Texture*

HPN bar storage for 42 d at 32°C has routinely been used to simulate 52 weeks at room temperature (Li and others 2008) and at that rate 6 days at 32°C would approximate 7.4 weeks or 1.4 weeks longer than the samples were actually kept. Statistical comparisons for each texture attribute (Table 7-4 to Table 7-7) were made between HPN bars on each storage day at fixed temperature (i.e., column) and within a HPN bar over time with storage at 32°C being used to simulate times longer than 6 weeks (i.e., row).

**Table 7-4 High-protein nutrition (HPN) bar hardness<sup>1</sup> (N) evaluated during storage at 22°C or 32°C**

MPC85 <sup>2</sup>	Day 0	22°C					32°C				
		Day 6	Day 13	Day 20	Day 29	Day 42	Day 6	Day 13	Day 20	Day 29	Day 42
Control	15 <sup>c,z</sup>	18 <sup>c,z</sup>	19 <sup>c,z</sup>	17 <sup>c,z</sup>	17 <sup>c,z</sup>	15 <sup>c,z</sup>	18 <sup>c,z</sup>	18 <sup>c,z</sup>	21 <sup>b,z</sup>	20 <sup>c,z</sup>	18 <sup>c,z</sup>
JM-Fine	56 <sup>a,w</sup>	56 <sup>a,w</sup>	58 <sup>a,wx</sup>	61 <sup>a,wxy</sup>	67 <sup>a,y</sup>	65 <sup>a,xy</sup>	56 <sup>a,w</sup>	56 <sup>a,w</sup>	62 <sup>a,wxy</sup>	62 <sup>a,wxy</sup>	77 <sup>a,z</sup>
FD	33 <sup>b,z</sup>	34 <sup>b,z</sup>	33 <sup>b,z</sup>	33 <sup>b,z</sup>	33 <sup>b,z</sup>	34 <sup>b,z</sup>	28 <sup>b,z</sup>	28 <sup>b,z</sup>	29 <sup>b,z</sup>	32 <sup>b,z</sup>	31 <sup>b,z</sup>

<sup>1</sup> Hardness (N) was the compressive force at 60% strain during the first compression.

<sup>2</sup> HPN bars were formulated at 30% protein (w/w) using milk protein concentrate with 85% protein (MPC85). Control, unmodified spray dried MPC85. JM-Fine, finely jet-milled MPC85. FD, freeze-dried MPC85.

<sup>a-c</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>w-z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

**Table 7-5 High-protein nutrition (HPN) bar fracturability<sup>1</sup> (N) evaluated during storage at 22°C or 32°C**

MPC85 <sup>2</sup>	Day 0	22°C					32°C				
		Day 6	Day 13	Day 20	Day 29	Day 42	Day 6	Day 13	Day 20	Day 29	Day 42
Control	15 <sup>a,u</sup>	19 <sup>a,uv</sup>	23 <sup>a,uvw</sup>	22 <sup>a,uv</sup>	24 <sup>a,uvw</sup>	27 <sup>a,vw</sup>	26 <sup>a,vw</sup>	31 <sup>a,wx</sup>	39 <sup>a,xy</sup>	46 <sup>a,y</sup>	56 <sup>a,z</sup>
JM-Fine	23 <sup>a,w</sup>	25 <sup>a,wx</sup>	27 <sup>a,wx</sup>	28 <sup>a,wx</sup>	30 <sup>a,wxy</sup>	32 <sup>a,xy</sup>	28 <sup>a,wx</sup>	32 <sup>a,wxy</sup>	33 <sup>a,xy</sup>	38 <sup>a,y</sup>	49 <sup>a,z</sup>
FD	19 <sup>a,v</sup>	23 <sup>a,vw</sup>	24 <sup>a,vwx</sup>	24 <sup>a,vwx</sup>	26 <sup>a,vwx</sup>	26 <sup>a,vwx</sup>	25 <sup>a,vwx</sup>	28 <sup>a,wxy</sup>	33 <sup>a,xy</sup>	36 <sup>a,yz</sup>	43 <sup>a,z</sup>

<sup>1</sup> Fracturability (N) was the compressive force where the sample yielded or cracked during the first compression.

<sup>2</sup> HPN bars were formulated at 30% protein (w/w) using milk protein concentrate with 85% protein (MPC85). Control, unmodified spray dried MPC85. JM-Fine, finely jet-milled MPC85. FD, freeze-dried MPC85.

<sup>a-c</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>u-z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

**Table 7-6 High-protein nutrition (HPN) bar maximum compressive force<sup>1</sup> (N) evaluated during storage at 22°C or 32°C**

MPC85 <sup>2</sup>	Day 0	22°C					32°C				
		Day 6	Day 13	Day 20	Day 29	Day 42	Day 6	Day 13	Day 20	Day 29	Day 42
Control	16 <sup>c,v</sup>	20 <sup>b,vw</sup>	23 <sup>b,vw</sup>	22 <sup>b,vw</sup>	24 <sup>b,vw</sup>	27 <sup>b,vw</sup>	26 <sup>b,vw</sup>	31 <sup>b,wx</sup>	39 <sup>b,xy</sup>	46 <sup>b,yz</sup>	56 <sup>b,z</sup>
JM-Fine	56 <sup>a,y</sup>	56 <sup>a,y</sup>	58 <sup>a,y</sup>	61 <sup>a,y</sup>	67 <sup>a,yz</sup>	65 <sup>a,y</sup>	56 <sup>a,y</sup>	56 <sup>a,y</sup>	62 <sup>a,y</sup>	62 <sup>a,y</sup>	77 <sup>a,z</sup>
FD	33 <sup>b,yz</sup>	34 <sup>b,yz</sup>	34 <sup>b,yz</sup>	33 <sup>b,yz</sup>	34 <sup>b,yz</sup>	34 <sup>b,yz</sup>	30 <sup>b,y</sup>	32 <sup>b,y</sup>	35 <sup>b,yz</sup>	41 <sup>b,yz</sup>	44 <sup>b,z</sup>

<sup>1</sup> Maximum compressive force (N) was either HPN bar hardness or fracturability, whichever value was greater for that measurement.

<sup>2</sup> HPN bars were formulated at 30% protein (w/w) using milk protein concentrate with 85% protein (MPC85). Control, unmodified spray dried MPC85. JM-Fine, finely jet-milled MPC85. FD, freeze-dried MPC85.

<sup>a-c</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>v-z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

**Table 7-7 High-protein nutrition (HPN) bar adhesiveness<sup>1</sup> (J) evaluated during storage at 22°C or 32°C**

MPC85 <sup>2</sup>	Day 0	22°C					32°C				
		Day 6	Day 13	Day 20	Day 29	Day 42	Day 6	Day 13	Day 20	Day 29	Day 42
Control	0.04 <sup>c,z</sup>	0.03 <sup>c,z</sup>	0.02 <sup>c,z</sup>	0.01 <sup>c,z</sup>	0.02 <sup>c,z</sup>	0.01 <sup>c,z</sup>	0.02 <sup>c,z</sup>	0.02 <sup>c,z</sup>	0.01 <sup>b,z</sup>	0.01 <sup>b,z</sup>	0.00 <sup>b,z</sup>
JM-Fine	1.19 <sup>a,z</sup>	1.01 <sup>a,yz</sup>	1.07 <sup>a,z</sup>	0.68 <sup>a,vwx</sup>	0.85 <sup>a,xy</sup>	0.80 <sup>a,xy</sup>	0.74 <sup>a,vwx</sup>	0.54 <sup>a,v</sup>	0.54 <sup>a,v</sup>	0.70 <sup>a,vwx</sup>	0.63 <sup>a,vw</sup>
FD	0.44 <sup>b,z</sup>	0.41 <sup>b,yz</sup>	0.40 <sup>b,yz</sup>	0.40 <sup>b,yz</sup>	0.40 <sup>b,yz</sup>	0.38 <sup>b,xyz</sup>	0.27 <sup>b,xyz</sup>	0.26 <sup>b,xyz</sup>	0.24 <sup>b,xyz</sup>	0.22 <sup>b,xy</sup>	0.18 <sup>b,x</sup>

<sup>1</sup> Adhesiveness (J) was the absolute area under the curve during crosshead withdrawal after the first compression.

<sup>2</sup> HPN bars were formulated at 30% protein (w/w) using milk protein concentrate with 85% protein (MPC85). Control, unmodified spray dried MPC85. JM-Fine, finely jet-milled MPC85. FD, freeze-dried MPC85.

<sup>a-c</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same column.

<sup>v-z</sup> Least squares means are significantly different ( $P < 0.05$ ) if they do not share a common superscript within the same row.

HPN bar hardness (Table 7-4), fracturability (Table 7-5), and maximum compressive force (Table 7-6) were reported separately. This was necessary because on day 29 and later, including samples analyzed after storage at 32°C, the control HPN bar always fractured and crumbled prior to 60% strain. The JM-Fine HPN bar only yielded during the first compression and conversely maximum compressive force was always obtained at maximum deformation. The FD HPN bar had fracture behavior in between the other two HPN bars. On day 0, FD HPN bar fractured/yielded during compression, but all samples obtained maximum compressive force at 60% strain. After 42 d at 22°C and 32°C, 4 and 13 of the 18 FD HPN bar samples required more force to induce initial fracture than compress at 60% strain, respectively. These textural differences would be missed if “hardness” were only described by maximum compressive force.

HPN bar hardness and maximum compressive force were significantly influenced by the protein powder used in their formulation ( $P < 0.05$ ), but it did not significantly affect fracturability. Fracturability, hardness, and maximum compressive force changed over time ( $P < 0.05$ ) and were affected by storage temperature ( $P < 0.05$ ) and their interaction term ( $P < 0.05$ ). Hardness (Table 7-4) was not significantly affected by storage temperature. At each evaluation time point, HPN bar hardness increased ( $P < 0.05$ ) in the order of control, FD, and JM-Fine, which was the order of decreasing protein powder particle size (Table 7-1). Cho (2010) also found that smaller protein powder particles produced firmer HPN bars. JM-Fine HPN bars never suffered catastrophic failure during instrumental compression and was the only HPN bar in which hardness increased significantly during storage ( $P < 0.05$ ).

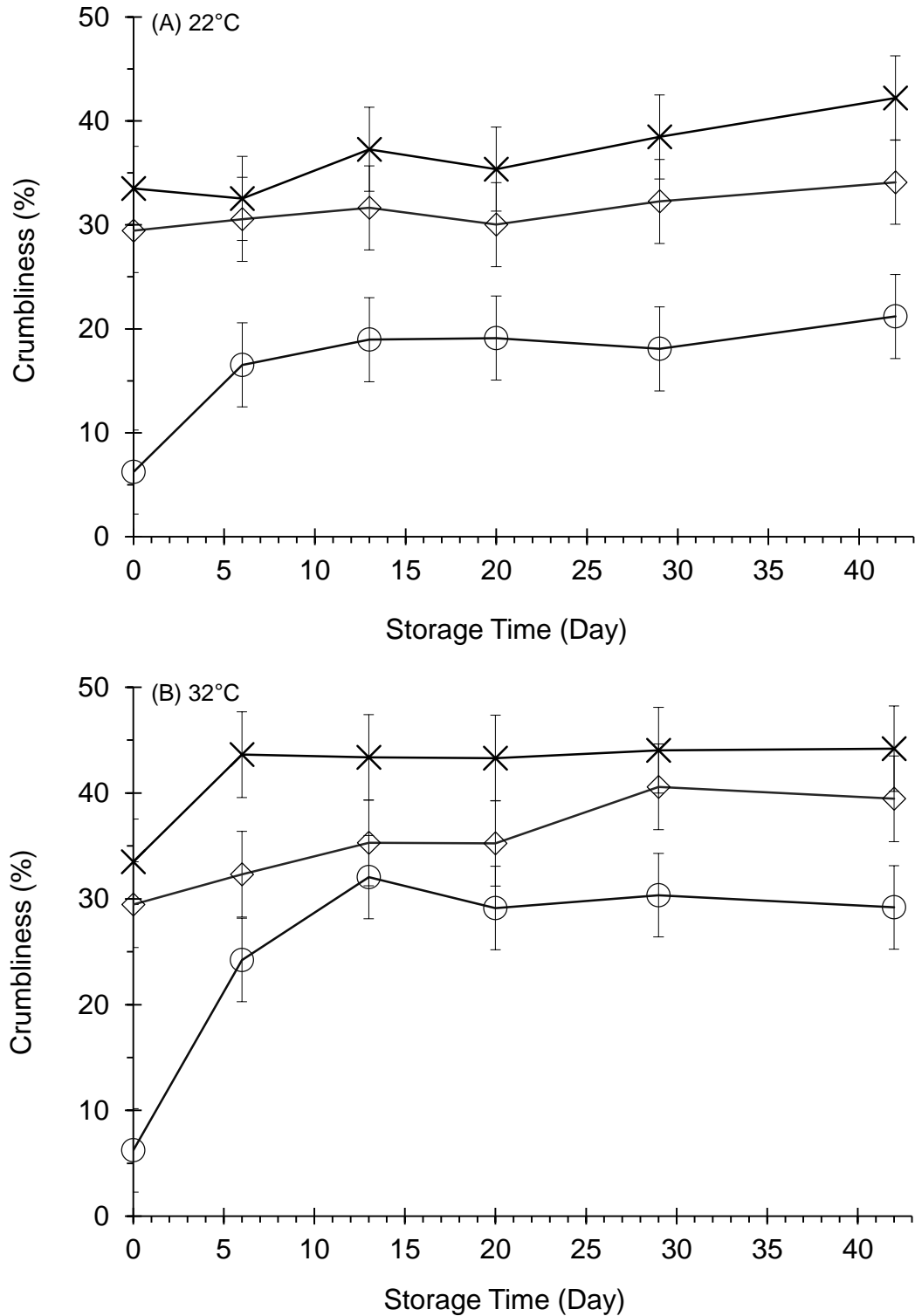


The control and JM-Fine HPN bars had significantly increased fracturability after 42 d at 22°C ( $P < 0.05$ ) and fracturability of FD HPN bar did not increase significantly day 13 at 32°C (Table 7-5). There were no significant differences in HPN bar fracturability when compared at equivalent storage temperature and time conditions ( $P > 0.05$ ). Despite having different hardness, all the HPN bars either fractured and crumbled or yielded under similar compressive force. Maximum compressive force (Table 7-6) for the control and FD HPN bars behaved similarly after storage at the same conditions. The JM-Fine HPN bar always had higher maximum compressive force than the other two HPN bars ( $P < 0.05$ ). With respect to day 0, maximum compressive force did not increase during storage at room temperature, rather a significant increase was measured after 13 d, 42 d, and 42 d at 32°C for the HPN bars formulated with control, JM-Fine, and FD, respectively.

Adhesiveness (Table 7-7), that is work necessary to overcome sample attractive forces external surfaces, was previously correlated with sensory panel cohesiveness and crumbliness; a more adhesive sample was also less crumbly (Banach and others 2016a). HPN bar adhesiveness (J) was influenced by protein ingredient, storage temperature, storage time, and all the interaction terms ( $P < 0.05$ ) except for time  $\times$  temperature. The control HPN bar lacked adhesiveness throughout storage and this aligned with the adhesiveness of a MPC80-formulated HPN bar (Banach and others 2016a). The JM-Fine HPN bar was more adhesive than both control and FD throughout storage ( $P < 0.05$ ). FD was more adhesive than the control through day 13 at 32°C ( $P < 0.05$ ). HPN bar adhesiveness decreased during storage yet it was better maintained by JM-Fine and FD

than for previously analyzed HPN bars formulated with extruded MPC80 (Banach and others 2016a).

Instrumental crumbliness (Figure 7-3) was a better measure of HPN bar crumbliness/cohesiveness than adhesiveness (Banach and others 2016a). Crumbliness was significantly affected by the protein powder used, storage time and temperature, and protein powder  $\times$  storage temperature interaction ( $P < 0.05$ ). JM-Fine produced a less crumbly HPN bar, that is, less mass passed through the uppermost mesh compared to those formulated with control and FD. JM-Fine HPN bar crumbliness increased from 6% to 17% ( $P < 0.05$ ) after 1 week at 22°C. No significant changes in this sample were noted again until 13 d at 32°C ( $\approx$ 16 week at 22°C) when crumbliness increased to 32% ( $P < 0.05$ ) and then finally plateaued for the remainder of storage ( $P > 0.05$ ). In comparison, HPN bars formulated with extrusion-modified MPC80 had crumbliness values of 1% and 20% after 6 weeks storage at 22°C and 32°C, respectively (Banach and others 2016a), and were more cohesive due to protein denaturation that occurred from extrusion. The control HPN bar under current study had higher moisture content and  $a_w$  than the MPC80-formulated control previously studied (Banach and others 2016a) and yet had higher crumbliness since it was formulated larger sized protein powder particles. JM-Fine powder produced a more cohesive HPN bar than the control and since this important attribute has rarely been reported, more in-depth comparisons with other protein powders was not currently possible.



**Figure 7-3 High-protein nutrition (HPN) bar crumbliness evaluated during storage at 22°C (A) or 32°C (B).** HPN bars were formulated at 30% protein (w/w) using milk protein concentrate with 85% protein (MPC85). Control (x), unmodified spray dried MPC85. JM-Fine (o), finely jet-milled MPC85. FD (◇), freeze-dried MPC85. Error bars represent  $\pm$  SE.

Particle size reduction by jet-milling and morphology change by way of freeze-drying influenced both initial HPN bar texture and its change during storage. In terms of the “hardening” attributes (i.e., hardness, fracturability, maximum compressive force), JM-Fine produced the firmest HPN bar and if softer texture is the main goal, then particle size reduction would not be a viable modification to improve the performance of high-protein MPCs for use in HPN bars. However, based on percent change with respect to day 0, the HPN bar texture attributes were less prone to change when formulated with JM-Fine or FD when compared to control MPC85 (Figure 7-4). Fracturability (Figure 7-4B) and maximum compressive force (Figure 7-4C) for the control HPN bar kept at 32°C for 42 d increased by 266% and 242%, respectively. The respective increases in the JM-Fine HPN bar were 115% and 38% and for the FD HPN bar were 128% and 33%. Physically modifying MPC85 by jet-milling and freeze-drying produced HPN bars with enhanced textural stability. Increasing HPN bar storage stability is a useful property of FD and JM-Fine MPC85, whereas the latter might be preferred for its added ability to maintain cohesion.

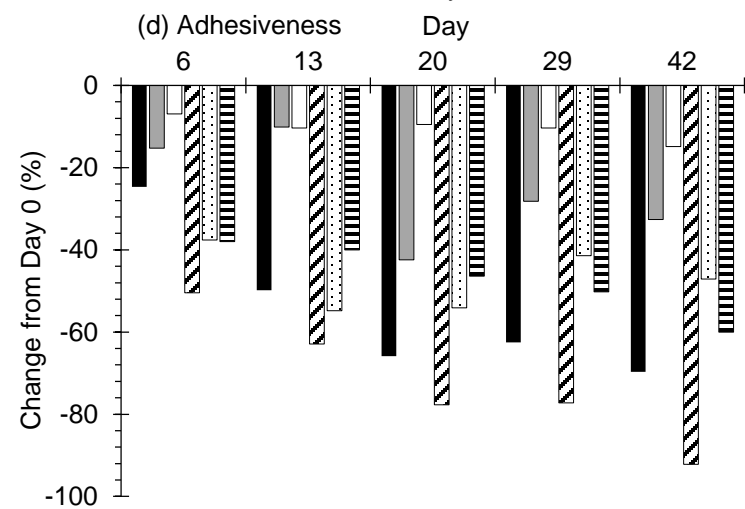
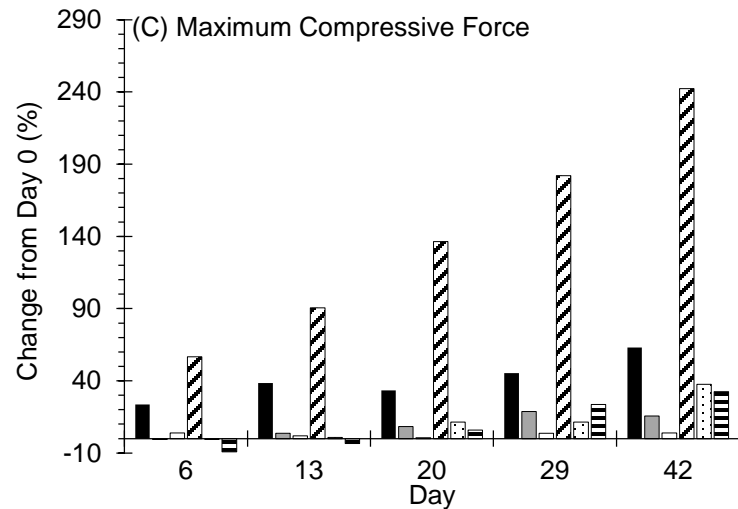
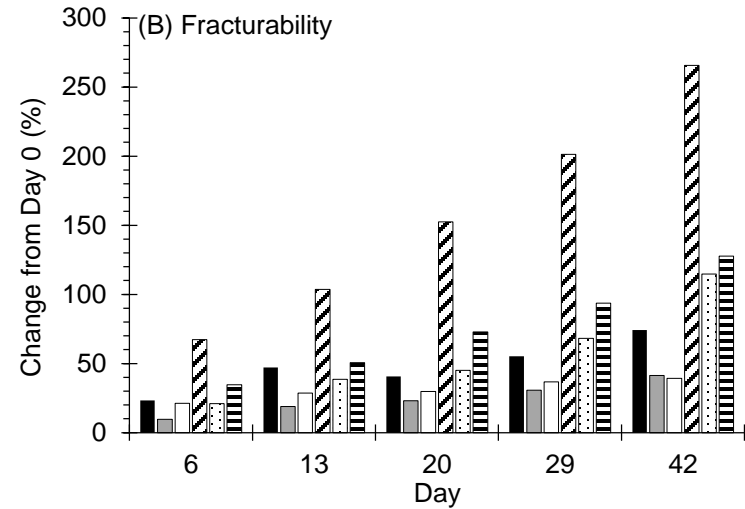
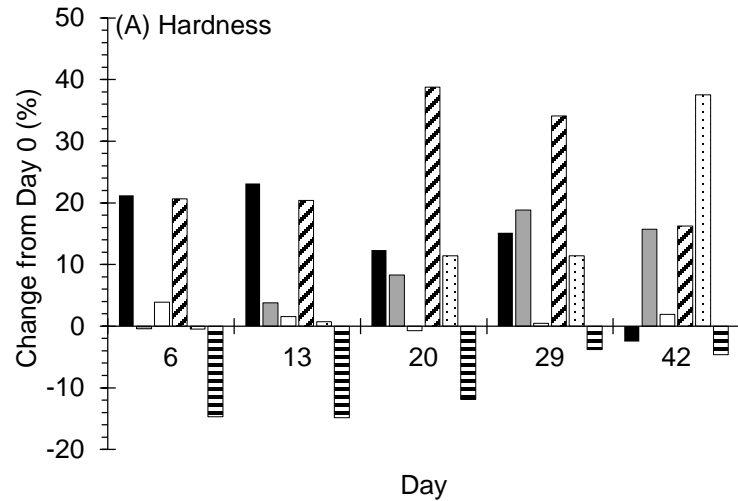


Figure 7-4 Average percent change in high-protein nutrition (HPN) bar hardness (A), fracturability (B), maximum compressive force (C), and adhesiveness (D) after storage at 22°C or 32°C for the days indicated with respect to day 0. HPN bars were formulated at 30% protein (w/w) using milk protein concentrate with 85% protein (MPC85). Control, unmodified spray dried MPC85. JM-Fine, finely jet-milled MPC85. FD, freeze-dried MPC85. 22°C storage: ■ Control, ■ JM-Fine, □ FD. 32°C storage: ▨ Control, ▨ JM-Fine, ▨ FD.

### 7.5.9 Explanation for Texture Changes in High-protein Nutrition Bars Formulated with High-protein MPCs

Based on present observations and literature (Banach and others 2014; Loveday and others 2009), when spray dried, high-protein MPCs are used in HPN bars their particle structure is maintained. Protein powder particle collapse and fusion into a continuously plasticized mass via particle-particle bridge formation occurs when its temperature exceeds its  $T_{gr}$  (Hogan and others 2016; Zhou and others 2014). Compared to lower protein MPCs, MPC85 had higher  $T_{gr}$ , which decreased from  $\sim 76^{\circ}\text{C}$  to  $\sim 53^{\circ}\text{C}$  as powder  $a_w$  increased from 0.11 to 0.44 (Kelly and others 2015). During model HPN bar production, the MPC85 powder particles were temporarily exposed to elevated temperature when mixed into the preheated ( $\sim 60^{\circ}\text{C}$ ) lipid/polyol blend and this allowed for surface rehydration as well as partial particle collapse. Elevated temperature exposure was short-lived and with limited free moisture, it was not possible for all MPC85 powder particles to proceed through glass-rubber transition. Thus, MPC85 in the HPN bar persisted as both structurally intact and partially plasticized particles.

High-protein MPC-formulated HPN bar texture and its time-dependent change are influenced by the fraction of un-plasticized versus plasticized protein powder particles, time the HPN bar spends in the rubbery state, and the rate that the proteins return to the glassy state. Upon cooling to and “setting up” at  $22^{\circ}\text{C}$ , the un-plasticized particles retain their structure and their presence contributes to HPN bar crumbliness. The proteins are chemically unreactive in this glassy state and this is why MPC-formulated HPN bar hardening during storage was not heavily influenced by chemical changes (Banach and others 2016b; Loveday and others 2009). HPN bar chemical changes are not completely

inhibited as low molecular mobility persists within this state (Roudaut and others 2004) and since a fraction of the MPC particles are plasticized during model production.

Texture changes occur as the partially plasticized, rubber-like, and chemically more reactive proteins return to the glassy state during HPN bar storage at a temperature less than its  $T_{gr}$ . As the HPN bar loses plasticization, it becomes firmer (Figure 7-4) and more crumbly (Figure 7-3). Conversely, whey protein hydrolysates have suppressed  $T_{gr}$  and produce texturally stable HPN bars by maintaining the rubbery state throughout storage despite being chemically more reactive (Rao and others 2016, 2013). Protein  $T_{gr}$  increases during HPN bar storage as water migrates away from the protein (i.e.,  $a_w$  increases) and as high molecular weight protein aggregates (i.e., disulfide bond, Maillard-induced) form (Loveday and others 2010; Zhou and others 2008a, 2008b). This accelerates the shift back to the glassy state and further contributes to texture change.

In the present study, day 0 texture was evaluated the day after HPN bar preparation and thus rapid changes may have been missed. However, the control HPN bar was the least texturally stable (Figure 7-4) and with poor powder rehydration characteristics, control MPC85 likely had the highest  $T_{gr}$  of the proteins studied. Even though the control had the highest WHC (Table 7-2), rapid contact angle change (Figure 7-1B), and better water absorption (Figure 7-1C), it did not help plasticize the system and the resultant HPN bar was always more crumbly than the other two (Figure 7-3). Particle size reduction by jet-milling or freeze-drying decreased  $V_{oa}$  and increased the specific surface area for water sorption which subsequently decreased the  $T_{gr}$  and increased particle collapse during HPN bar production. This contributed to the higher fluidity of the JM-Fine HPN bar dough during model HPN bar manufacture and produced a more

cohesive HPN bar. Moreover, smaller particles are by nature more adhesive (Schwarzwalder and others 2014) and that may have factored into improved cohesiveness when the HPN bars were formulated with FD or JM-Fine.

## 7.6 Conclusions

High-protein MPC powder particle size and morphology affects the initial texture and stability of HPN bars when used as the sole protein source in the formulation. Finely jet-milled MPC85 powder produced HPN bars that were firmer and more cohesive than the control. More importantly, the same HPN bar and the one formulated with freeze-dried MPC85 were less prone to texture change over the storage period. Particle size reduction removed occluded air from the spray dried MPC85 and allowed for denser particle packing in the HPN bars. Reducing the particle size of MPC85 improved its ability to rehydrate during HPN bar production, which translated to improved plasticization and HPN bar cohesion. A texturally stable, less-crumbly HPN bar can be produced with MPC85 if particle size is reduced. High-protein MPC particle size needs to be considered when formulating HPN bars.

## 7.7 Acknowledgement

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## CHAPTER 8. GENERAL CONCLUSIONS

### 8.1 Summary

High-protein milk protein concentrates (MPCs), such as MPC80 (80% protein w/w) and MPC85 (85% protein w/w), produced high-protein nutrition (HPN) bars (30% protein w/w) that hardened and lost cohesion during storage. Previously, the most detrimental aspect of HPN bar texture change was thought to be hardening, but in HPN bars formulated with high-protein MPCs, loss of cohesion is even more detrimental. Milled extruded MPC80 fared well in HPN bars by both slowing hardening and imparting cohesion. Transglutaminase crosslinked and calcium-reduced MPC did not impart any practical textural improvement for use in HPN bars. Reducing the particle size of MPC85 by jet-milling led to HPN bars that were denser, firmer, and more cohesive and texturally stable than those prepared with native MPC85 powder.

Milled extrusion-modified MPC80 had different physicochemical properties when compared to the native spray dried control. Extrusion decreased the free sulfhydryl content, free amine content, water holding capacity (WHC), protein solubility, surface hydrophobicity, and the occluded air of MPC80. Extruded powders had higher densities and had improved ability to interact with water. When extruded MPC80s were used in HPN bars, they increased denseness and textural stability of the final product. Chemical changes such as disulfide bond formation and Maillard-induced protein aggregations occurred in these HPN bars during storage, and the latter was more relatable with texture change. Extruded MPC80 produced HPN bars that were microstructurally more stable than the control and prevention of macronutrient phase separation translated into textural stability.

MPC powder particle size was not previously considered to have an effect on HPN bar texture. Reducing the particle size of MPC85 improved its ability to hydrate during HPN bar manufacture. Improved hydration coupled with the attraction that smaller particles naturally have for each other produced more cohesive HPN bars. Jet-milled MPC85 produced HPN bars that were denser, firmer, and more cohesive than the control initially and after 1 year accelerated storage. These HPN bars also exhibited much greater textural stability. Particle size reduced MPC85 did not produce the same level of cohesion as the extrusion-modified MPC80. This showed that protein denaturation by extrusion processing not only slowed hardening, but also decreased HPN bar crumbliness.

## **8.2 Recommendations**

MPC processing, including extrusion and particle size reduction, can be used to alter the texture of HPN bars formulated with these proteins. When high-protein MPCs are used in a HPN bar formulation, careful attention must be paid to determine if the particle structure collapses or if it is maintained. If the majority of the powder particles collapse, the HPN bar will be more cohesive and internal chemical reactions will likely proceed at an accelerated rate. These chemical changes (e.g., disulfide bond formation, Maillard-induced aggregations) were not related to texture change in HPN bars formulated with high-protein MPCs. If MPC powder particle structure is maintained, the HPN bar will be crumbly, and textural changes will be influenced by physical interactions between particles in the system. MPC particle size should be considered in future HPN bar studies, as variation exists between sources. Smaller particles likely hydrate better leading to collapse, and if not, the smaller particles will be more fluid in

the system and their HPN bars will have higher cohesiveness. On the other hand, the larger particles in coarser MPCs may serve as weak points when formulated into HPN bars and while they won't impart cohesion, these HPN bars will fracture under lower stress.