The use of milk protein concentrates with high water activity in the manufacture of high protein nutritional bars

by

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Abstract

Milk protein concentrate (MPC) is extensively used in food formulations due to its physical, chemical, sensory, and functional attributes. However, when utilized in nutrition bars, they are often not shelf stable longer than 6 months due to increased hardening. Matching the water activity of MPC to other ingredients in the nutritional bars can help mitigate moisture migration and aid in the reduction of bar hardening during storage. In this study, an adsorption and desorption method were used to produce high water activity MPCs. In the adsorption method, three lots of MPC 85 were split into control and treatment batches to produce 1-inch square high protein nutrition bars (HPNBs) with (1) no MPC modification with a water activity of approximately 0.2 or (2) MPC modification with a water activity of approximately 0.5. HPNBs were stored in two different temperatures: 25ºC and 36ºC and tested in triplicates on days 1, 2, 4, 6, 13, 22, and 31. In the desorption method, three lots of liquid MPC 85 were produced from skim milk, split into control and high-water activity batches and spray dried to produce 1-inch square HPNBs with (1) no MPC modification with a water activity of approximately 0.2 or (2) MPC with a high-water activity of approximately 0.5. HPNBs and were stored in two different temperatures: 25ºC and 36ºC. HPNBs were tested in triplicates on days 3, 6, 13, 22, and 28. The physical and chemical changes of HPNBs during storage were monitored using different analytical tests.

Compared to control HPNBs, the HPNBs produced with water activity adjusted MPC 85 or high-water activity MPC 85 showed no differences in chemical and physical characteristics during storage 25ºC. However, at 36ºC the water activity adjusted and high-water activity MPC did affect the rate of moisture migration and Maillard browning. Thereby, reducing the rate of HPNB hardening.

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Dedication

I dedicate this thesis to Linda Hamilton, who influenced countless people in her too-short time here on earth. Grandma, I miss you more than words can say. Thank you for believing in me and my dreams. You will inspire my life forever and I look forward to the day we meet again.

Chapter 1 - Introduction

Demand for high-protein nutrition bars as a meal supplement, energy fueled snack, or dieting staple has grown significantly in recent years. Containing approximately 20g to 50g of protein per 100g and other nutritional additives, protein bars are often desired over other snacks (Banach et al., 2014). However, inclusions and their different content levels can cause undesired effects such as changes in color and texture. Bar hardening is often an undetected feature until approximately 3 months of storage but has disastrous effects and is no longer shelf stable after 6 months (Hogan et al., 2012).

There are many different protein bars on the market containing a wide variety of protein sources. The incorporation of milk protein concentrate (MPC) has been found to provide more nutritional value and flavor compared to other sources. However, they perform poorly during storage due to rapid hardening, shorter than the desired shelf life. It has been suggested that bar hardening occurs because of protein aggregation and moisture migration during storage (Meng et al., 2019). The rate of hardening also increases with storage temperature. Maillard browning occurs at a faster rate when a reducing sugar is included in the formulation (Zhou et al., 2013). Glycation of protein molecules as well as the aggregation of proteins can occur. Overall, hardening is a correlation between the amount of available water in the system able to act as a plasticizer (McMahon 2009). There is no single cause responsible for hardening in protein bars. It can occur due to a wide range of chemical, physical, thermodynamic, and process related factors (Hogan et al., 2012). It is recognized though that the driving force behind hardness is the difference in osmotic potential amongst the ingredients. Swelling, molecular reorganization, and protein aggregation can change the structure overtime leading to increased hardness.

This study was carried out with the goal of finding new methods to mitigate bar hardening during storage. This was achieved by using different MPC85 production methods and adjusting the water activity to match other ingredients. Understanding the chemical and physical changes that govern structure formation will allow for greater control of high protein nutrition bars as well as other dairy based foods.

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Chapter 2 - Literature Review

Milk

Milk and its composition play critical roles in the production of high-quality dairy products. Milk contains key nutrients such as carbohydrates, proteins, fats, minerals, and vitamins (Foroutan et al., 2019). There has been an increased study of milk protein and its functionality in many products such as flavored milk, yogurt, ice cream, cheese, butter, and other dairy products. Important functional properties of milk protein include solubility, viscosity building, emulsification, heat stability, and aeration. The two main components of milk protein are casein and whey. Caseins, a phosphoprotein composes approximately 80% of the total protein in milk. Suspended in the aqueous phase, these colloidal particles contribute to the white color of milk and provide stability when heated (Fox & Brodkorb., 2008). Caseins are not denaturable by heat within normal ranges of pH, salt, and protein content. Whey protein composes the remaining 20% of the total protein in milk. The most added forms of whey protein utilized in products today are whey protein concentrate (WPC) and whey protein isolate (WPI). Whey proteins provide a high solubility, dispersibility, water binding, foaming, whipping, emulsification, gelation, buffering power, and are heat sensitive (Evans et al., 2010). Research of milk and milk components has led to growth and commercialization of milk products in the industry with a concentration of nutritional applications (Lucey et al., 2017).

Overview of milk protein concentrate powders

Milk protein concentrate is a dairy powder with approximately 35-90% total solids range. Compared to whole milk powder, nonfat dry milk, MPC powders have a higher protein content and contain less lactose. They are manufactured through ultrafiltration by the partial removal of

lactose and minerals from skim milk. A retentate obtained from ultrafiltration is further concentrated by evaporation and spray dried. The composition of milk protein concentrate includes whey proteins, caseins, lactose, fat, and minerals in the same concentrations as the milk used during manufacture (Havea, 2005). Caseins are heat-stabile milk proteins that encompass approximately 80% of total milk protein. MPCs can be used as an ingredient in many food applications such as yogurt, cheese, lactose, butterfat, and sour cream. Because of its desirable functional and nutritional benefits, MPC powder is an ideal ingredient. Table 2.1 shows the various compositions of MPC powders with different protein concentrations.

Table 2.1 MPC powder bulk composition

Component (%)	MPC35	MPC50	MPC60	MPC70	MPC80	MPC85	MPC90
Protein	35.4	49.9	60.8	68.2	79.1	84.0	85.8
Lactose	49.6	35.8	24.5	18.0	6.4	1.8	0.4
Fat	0.6	0.8	1.0	1.1	1.2	1.2	1.4
Ash	8.1	7.8	7.7	8.0	7.7	7.5	7.6
Moisture	3.4	3.8	4.0	3.6	4.6	4.8	4.2

Bulk composition adapted from Kelly et al. (2015)

Overview of dairy derived ingredients

Within the dairy industry there is a vast array of dairy derived ingredients that provide important nutritional benefits as well as functionality. Yogurt, cheese, lactose, butterfat, and sour cream are just several examples. Amongst these different products, the principal source of difference is protein. Most functional properties of proteins are described based on their usefulness (Craig, 1979). Modification of whey proteins or caseinates in combination with other ingredients can provide the necessary functionality or nutritional value. Four subcategories of caseins include $\propto_{s1}$, $\propto_{s2}$, β, and k caseins. Whey proteins are comprised of β-lactoglobulin and ∝-lactalbumin. In milk, whey proteins are in colloidal solution whereas caseins are in colloidal suspension. Substances such as salts destabilize colloidal systems by changing the water binding

and thereby reducing protein solubility, and factors such as heat, causing unfolding of the whey proteins and increased interaction between the proteins, or alcohol which may act by dehydrating the particles. If milk is heated to a high temperature, it turns brown and acquires a caramel taste. This process is called caramelization and is the result of a chemical reaction between lactose and proteins called the mallard reaction. The fat and protein contained in milk used to produce high protein nutrition bars may undergo chemical changes such as oxidation and lipolysis during storage. This can result in products having an off flavor, color, or increased hardening throughout storage.

Ultrafiltration

Ultrafiltration is the force of molecules through semipermeable membranes through a pressure gradient. Dependent on the pore size of the membrane some particles are unable to travel through. Ultrafiltration has been utilized in the dairy industry to produce whey protein concentrates and milk protein concentrates due to the selective concentration of protein in relation to the other components. Membranes used for whey processing are typically modified to allow salt, minerals, and lactose to pass through while proteins are retained (Craig, 1979). There is variation in the ratio of concentration between the whey components due to the retention of protein and selective permeation of lactose, minerals, water, and compounds with a low molar mass (Baldasso et al., 2011). The isolation of milk proteins and removal of lactose may make it possible to produce a high protein product at relatively low temperatures with no pH adjustment (Mistry & Hassan., 1991). In MPC powders, ultrafiltration is performed prior to concentration which can be performed by nanofiltration or evaporation (Park et al., 2016). It has been found that ultrafiltration temperature not only affects the size of casein micelles but also membrane performance with higher temperatures which results in smaller particle sizes (Luo et al., 2016).

For high protein powders, UF alone is not suitable to obtain the desired protein-to-solids ratio in the retentate. Therefore, diafiltration (DF) is also performed. Heat stability and solubility are just a few of the factors that must be taken into consideration when producing MPC to avoid producing a low-quality product.

Spray Drying

Spray drying is the action of atomizing milk into fine particles which are then mixed with hot air to remove water. The principle of spray drying milk concentrate is to remove the water as fast and at as low temperature as possible to minimize heat damage of the milk solids. The temperature of particles typically does not reach above 60ºC due to evaporative cooling (Park et al., 2016). During this process the water content, powder structure, and physiochemical characteristics are altered (Park et al., 2016). The surface composition of milk powders is determined to a large extent during spray drying. However, the subsequent fluidized bed drying procedure has little to no effect on the surface composition of milk powders. Surface free fat (SFF) is a fat not entirely coated by amphiphilic molecules or protected by a matrix of carbohydrates and protein throughout drying (Park et al., 2016). SFF can alter milk powder properties such as oxidative stability, wettability, dispersibility, solubility, flowability, and shelf life. In baked goods, milk and whey proteins assist keeping baked goods soft and results in more consistent browning of pastry crusts. It can also increase the overall shelf life of products and confections while simultaneously acting as a contributor to flavor. Spray drying can impact the physical and sensory properties of milk and whey protein concentrate. Lipid oxidation compounds are the primary source of off flavors. With increased inlet temperatures and feed solid concentrates a sweet aromatic flavor could be enhanced while decreasing the cardboard

flavor associated with volatile lipid oxidation products (Park et al., 2016). However, this heat treatment could decrease the nutritional quality of the powder resulting from mallard browning.

Functionality of MPC and WPC

Milk and milk powders are consumed mainly because of their high nutritional value. Milk can be consumed as a fluid or used in production of products such as butter, cheese, yogurt, and ice cream. Milk proteins are often subjected to heat during production. During thermal processing, it is possible that whey proteins undergo denaturation. Denaturation is the process of protein unfolding and the hydrophobic groups being exposed (Raikos, 2010). β-lactoglobulin and whey protein aggregates are also formed during increased heating and bind to the surface of casein micelles. pH effects protein denaturation and the interaction between whey proteins and casein micelles. At low pH values the whey proteins are most often found on the surface of casein micelles whereas at high pH values they are in the serum form (O'Kennedy, 2014). Heat treatment of milk can have different effects depending on the product being produced. For example, yogurt requires a high-water holding capacity to supply strength for the acid gel. However, too much water in a cheese rennet could result in gel formation and syneresis resulting in a high moisture curd.

Milk protein concentrate (MPC) is a dairy protein powder with a protein percentage in the 50-85% total solids range (Havea, 2006). Manufactured through the removal of lactose and minerals from skim milk thorough ultrafiltration. The powder is then further evaporated by spray drying. Often used as an ingredient in food, MPC is highly used in cheese and yogurt manufacture when reconstituted because of its high protein content and low carbohydrate concentration. MPC provides high solubility and dispersibility when used in dairy-based products. Overall, any food produced with milk protein will be affected by pH and temperature.

Nutritional Bars

High protein nutritional bars (HPNBs) have recently become popular as a nutritious snack due to the high protein and low carbohydrate content. With approximately 20-50% protein, on-the-go consumers and athletes are the most targeted customers. The majority of HPNBs belong to the category of medium-moisture foods (10-30%) with a shelf life of 6 to 12 months depending on the storage condition and ingredients. HPNBs consist of mainly protein with 10-30% carbohydrates, 5-10% fat, less than 1% water, and other components for flavor or stabilization (Jiang et al., 2021). Carbohydrates can include glucose, fructose, and other maltose syrups. High fructose corn syrup (HFCS) blended with maltitol syrup are also common sources of carbohydrate that provide enough water to form a dough (McMahon et al., 2009). The fat sources most used include vegetable shortening, cocoa butter, palm kernel oil, or vegetable oil.

Texture of food has great significance amongst consumers because it often influences their perception of quality. Storage of protein HPNBs is approximately 6 to 12 months because of its increased hardness and crumbly texture. These attributes are most likely due to moisture migration, limited free water, macronutrient phase separation, and sugar crystallization (Banach et al., 2014). With the absence of solvent or co-solvent protein interactions water migrates away from the protein and into other constituents with a lower water activity. This allows for aggregation and the creation of network formations which have been linked to HPNB hardening (Loveday et al., 2009). Maillard browning may also be involved in the hardening process when a reducing sugar is utilized in the product formulation (McMahon et al., 2009).

Changes During Storage

Physical Changes

Texture analysis is the mechanical testing of food to better understand its physical properties. This includes a wide range of characteristics such as how the food breaks, bends, flows, and sticks. Texture analysis is a financially affordable method to replicate mouth feel when chewing and can accurately be utilized to establish an index of quality. A texture analyzer moves in a downward position to compress a sample. The moving arm is connected to a load cell and records the force required to deform the sample. Texture analyzers provide researchers the opportunity to control almost all aspects of the chewing process. A wide range of probes and fixtures can aid in this process by connecting to the arm or base.

Texture analysis can be used to investigate the hardness of high protein nutrition bars during storage. Banach et al. (2014) compressed cylindrical high protein nutrition bar samples to 60% strain with a 0.05 N trigger force. It was found that there was no significant difference in hardness between samples on the day of manufacture. At a higher storage temperature, moisture loss and increased fluidity of the bar matrix occurs (Banach et al., 2014). It is important to note that bars formulated with MPC or MPI often have decreased cohesiveness and increased crumbliness. During the first day of storage more than 15% protein became insoluble and increased to 35% by day 3 (Zhou et al., 2013). As the storage time and temperature increases, whey proteins form aggregates. While the individual aggregates did not affect the texture significantly, the conglomeration of aggregates did cause a significant change in texture (Zhou et al., 2013).

Confocal laser scanning microscopy (CLSM) is an optical imaging technique used to increase optical resolution and contrast of a micrograph using a pinhole to block out of focus

light in image formation. An optical section contains information from only one focal plane. By moving the focal plane of the instrument, the depth of the specimen and a stack of optical sections can be recorded (Dürrenberger et al., 2001). These sections can be used to characterize the amorphous structure in highly charged colloidal systems which have undergone a gas-solid transition. The component of interest is stained with a fluorescent dye of Nile Blue or Fast Green FCF. Light reflected from the focal plane is partially reflected by the beam splitter towards the pinhole in front of the detector. The objective lens forms an image of what is positioned in front of the detector pinhole and the illuminating pinhole at the same position in the focal plane. Therefore, the images are said to be confocal with each other. Confocal microscopy can be used to investigate the structure of cells and the location of protein populations. MPC contains whey proteins and casein micelles in a ratio of 80:20, respectively (Loveday et al., 2009). The dissolved protein identified after mixing consists mainly of whey proteins because they are more soluble than caseins. A day after HPNB production, the protein becomes a cohesive solid and the clustering of protein particles and the disappearance of soluble protein can be observed (Loveday et al., 2009). As storage time increases, there is a loss of particle definition, increase in the homogenous matrix, and an increase in sample air voids. This is due to the displacement of occluded air from powder particles (Hogan et al., 2021). In a study performed by Loveday et al. (2009), MPC was used to produce high protein nutrition bars. It was observed the first day after manufacture that the protein bar material hardened from a batter-like pourable material into a soft but cohesive solid. During storage, small molecules in the gap between particles migrate into the proteins through the force of a potential energy gradient. Equilibrium is reached through the migration of water and is driven by the osmotic differential between high and low water activity ingredients. Depending on the sample and its preparation, the direction of water migration can

occur from proteins to the liquid phase containing glucose and glycerol or from liquid phase into proteins (Lu et al., 2016). The state and amount of sugar used in bar production is critical to which form of migration occurs. For example, if glycerol and sorbitol are premixed and dissolved then the water would migrate from liquid to protein phase. However, if the sugar did not fully hydrate after sample preparation, then there would be moisture migration from proteins to sugar (Lu et al., 2016). It was also found that high protein nutrition bars with non-agglomerated micellar casein were more densely arranged with smaller voids (Hogan et al., 2012).

Front face fluorescence can be used as a non-destructive tool to evaluate the quality of dairy products such as HPNBs (Shaikh & O'Donnell, 2017). Fluorescence spectroscopy can provide insight into the chemical, physical, and thermodynamic changes in HPNBs during storage. Babu & Amamcharla (2018) reported that the emission of tryptophan is sensitive to the environment and a longer storage period results in a decrease of peak intensities, indicating a change in dairy proteins over time. For Maillard emissions, an increase in the duration of storage results in increased peak intensities (Babu & Amamcharla, 2018).

Chemical Changes

Water activity is a measure of how much free or unbound water is available in the system and available for use. A water activity measurement of 0.65 or less in HPNBs indicates limited or no microbial growth (Banach et al., 2014). As water activity increases, the HPNBs harden at a faster rate (Li et al., 2008 and McMahon et al., 2009). The typical interaction between free water and the surface of proteins is usually weak (Malecki et al., 2022). A lack of free water molecules associating with proteins allows amino acids to form disulfide bonds, resulting in protein aggregation (Banach et al., 2014). The humidity exchanged between the surfaces of protein

molecules can also impact the rate of HPNB hardening. Malecki et al. (2022) reported a 4% increase in humidity during storage of HPNBs. Banach et al. (2014) reported that while there were increases in water activity of HPNBs with either toasted and extruded MPC, at higher temperature over a 40-day storage period, the increase was small and had no influence on HPNB hardening. It is hypothesized that a minimization of water activity between ingredients in HPNBs can reduce the rate of hardness during storage (Hogan et al., 2012). The differences in the chemical potential of water between ingredients is a significant driving force for the migration of water to reach equilibrium. When the water activity of MPC is close or matches the water activity of other ingredients, the osmotic equilibrium will take less time to be reached. Therefore, the differences in water vapor and the rate and extent at which moisture migration occurs is significantly reduced (Hogan et al., 2012).

Using a colorimeter, the L*, a*, and b* values of HPNBs are recorded to indicate the change between lightness and darkness as well as the saturation of colors during storage. Color change is often an indicator of quality (Banach et al., 2014). Banach et al. (2014) reported that there was no significant change in color when HPNBs were stored at 22°C. However, at 32°C and 42°C there was a significant increase in color change of HPNBs. It is hypothesized that change in color during storage is a result of amines participating in Maillard reactions (Banach et al., 2014). McMahon et al. (2009) reported that bars get darker during storage according to the amount of Maillard browning reactants. Generally, all HPNBs changed from a white or cream color to brown by the end of storage. This same trend was identified by Hassan (2020) when HPNBs were stored at 35°C for 43 days. It was also reported that a loss of free water able to act as a plasticizer could result in the loss of protein flexibility (Hassan, 2020). Thus, increasing the hardness and water activity, resulting in the brown-red color of HPNBs.

As hardness of HPNBs increases during storage, the amount of insoluble protein often increases as a result. The solubility of a 60mg HPNB sample was added to 10mL of phosphate buffer and the suspension was stirred prior to centrifugation. Using Leco, a decrease in the amount of soluble protein during storage would suggest the formation of insoluble protein (Zhou et al., 2008). It is known that temperature and time are major factors in the amount of protein aggregation. Zhou et al. (2008) reported that insoluble protein significantly increased during the first 3 days of storage before slowing continuing increasing through 100 days of storage at 45°C. At the end of storage, almost two thirds of the whey protein using in the manufacture of HPNBs was insoluble. Zhou et al. (2008) also reported that changes in whey protein after 3 months of storage could be determined using differential scanning calorimetry (DSC). After storage for 3 months at 23°C, the temperatures of the endothermic denaturation peaks increased but no change in denaturation enthalpy as observed (Zhou et al., 2008). However, at 45°C, the denaturation temperature of β-lactoglobulin increased and totally denaturation enthalpy decreased. Therefore, with an increased storage temperature proteins lose their tertiary structure, lowering the denaturation temperature and contributing for the formation of disulfide bonds that promote hardness of HPNBs during storage.

To illustrate the formation of Maillard induced protein aggregates in HPNBs, SDS-PAGE was performed and analyzed under reducing and non-reducing conditions. Maillard reactions can lead to high molecular mass polymers. With the addition of anticaking agents, protein aggregation in HPNBs was limited (Meng et al., 2019). However, the intensities of the larger protein bands remain unchanged during 35 days of storage. It was hypothesized by Meng et al. (2019) that the modification of proteins is slow, and weeks or months might be required to developed extensive protein aggregation. Loveday et al. (2009) performed reduced and non-

reduced SDS-PAGE on HPNBs. It was reported that high molecular weighted proteins remained at the top of the same well. However, this was not seen in reduced gels, indicating that disulfide bonded aggregates had formed (Loveday et al., 2009).

To monitor the changes in the protein structure during storage, the amide I region (1700 cm^{-1} - 1600 cm^{-1}) must be analyzed. Zhou et al. (2022) reported after 35 days of storage the β-sheet in HPNBs increased significantly while the β-turn decreased significantly ($P < 0.05$). These results were consistent with similar findings from Meng et al. (2019). Water molecules in HPNBs migrate during storage towards the proteins. The formation of β-sheet structures resulted in protein aggregation causing the HPNBs to become harder (Zhou et al., 2022). Mitigating the variations in water activity is hypothesized to reduce the rate of moisture migration during storage and the hardness of HPNBs.

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Chapter 3 - Research Objectives

This study was performed to have a better understanding of water activity adjusted MPC 85 that was used in the manufacture of high protein nutrition bars (HPNBs). The second objective of this study was to evaluate the chemical and physical changes of HPNBs during storage. HPNBs were stored at 25ºC and 36ºC and analyzed for changes in texture, color, water activity, insoluble protein, Fourier transform infrared (FTIR) spectroscopy, fluorescence, SDS-Page, and Differential Scanning Calorimetry (DSC).

Chapter 4 - High Protein Nutrition Bars: Water Activity Adjustment Through Adsorption

Abstract

High protein nutrition bars (HPNBs) containing milk protein concentrate powder (MPC) have increased physical, chemical, sensory, and functional properties. However, nutrition bars are often not shelf stable longer than 6 months due to increased hardening. Matching the water activity of MPC to other bar ingredients mitigates moisture migration and aids in the reduction of bar hardening during storage. In this study, an adsorption method was studied. Three lots of MPC 85 were split into control and treatment batches to produce 1-inch square HPN bars with (1) no MPC modification with a water activity of approximately 0.2 or (2) MPC modification with a water activity of approximately 0.5. HPN bars were stored at two different temperatures: 25ºC and 36ºC and tested in triplicates on days 1, 2, 4, 6, 13, 22, and 31. The physical and chemical changes of HPNBs during storage were monitored using different analytical tests.

Water activity adjusted HPNBs stored at 25ºC and 36ºC did not show different chemical and physical characteristics during storage. However, there was a significant difference in chemical and physical characteristics between storage temperatures. Literature has shown various methods for creating a HPNB with decreased hardness rates during storage, but often it requires expensive ingredients and equipment. Developing an effective method to reduce the rate of moisture migration between HPNB ingredients could impact the rate at which hardening of HPNBs occurs during storage.

Keywords: Milk protein concentrate powder, moisture migration, and hardness.

Introduction

Demand for HPNBs as a meal supplement, energy fueled snack, or dieting staple has grown significantly in recent years. Containing approximately 20g to 50g protein per 100g and other nutritional additives, protein bars are often desired over other snacks (Banach et al., 2014). The average protein content in most commercial HPNBs is approximately 30g per 100g. However, inclusions and different protein levels can cause undesired effects such as changes in color and texture during storage. Bar hardening is a detected feature after 2 to 3 months of storage and HPNBs are no longer shelf stable after 6 months (Hogan et al., 2012).

There are many different protein bars on the market containing a wide variety of protein sources such as whey protein concentrate, whey protein isolate, and other plant-based sources. The incorporation of milk protein concentrate (MPC) has been found to provide more nutritional value and flavor compared to other sources (Banach et al., 2014). However, MPCs perform poorly because of their crumbly texture and lack of cohesion during storage due to rapid hardening, shorter than the desired shelf life. There is no single cause responsible for hardening in protein bars. It can occur due to a wide range of chemical, physical, thermodynamic, and process related factors (Hogan et al., 2012). It has been suggested that bar hardening occurs because of protein aggregation, moisture migration, and glycation of protein molecules during storage (Meng et al., 2019). The rate of hardening also increases with storage temperature. Maillard browning occurs at a faster rate when water activity decreases and a reducing sugar is included in the formulation (Zhou et al., 2013). Overall, hardening is a correlation between the amount of free versus bound available in the system able to act as a plasticizer (McMahon 2009). The driving force behind hardness is the difference in osmotic potential amongst the ingredients cause moisture migration from high to low moisture ingredients (Hogan et al., 2012). Swelling

(Rawat et al., 2015, sugar crystallization (Jiang et al., 2021), and protein aggregation (McMahon et al., 2009) can change the structure overtime leading to an increased hardness. Other studied methods include the use of extruded and toasted MPC (Banach et al., 2016), hydrolyzed whey protein (McMahon 2009), and addition of anticaking agents (Meng et al., 2019).

This study was carried out with the goal of finding new methods to mitigate bar hardening during storage. This was achieved by using different MPC 85 production methods and adjusting the water activity to match other ingredients.

Materials and Methods

Experimental Design

Freshly manufactured MPC 85 was procured from a commercial manufacturer in the United States. The MPC had an initial water activity of approximately 0.23 ± 0.03. Each lot of MPC 85 was divided into two equal parts. The first part was used as an ingredient in the manufacture of control high protein nutrition bars (HPNBs). The water activity of the remaining part of MPC 85 was adjusted to approximately 0.5 ± 0.01 using the adsorption technique as described below. The water activity adjusted MPC 85 was used as an ingredient in the manufacture of water activity adjusted HPNBs. The control and water activity adjusted HPNBs were sealed and stored at 25 ºC and 36 ºC for up to 31 days and analyzed for physical and chemical properties during storage.

Water activity adjustment of MPC85 by adsorption

A laboratory scale adsorption system was developed and assembled in house. Fresh milk protein concentrates in powder form were procured from Idaho Milk Products and (Jerome, ID) and divided equally into two parts. The first part was used without modifications in the manufacturing of control HPNBs. The water activity of the second part of MPC 85 was adjusted

to approximately 0.50 and subsequently used in the manufacturing of HPNBs. For this purpose, approximately 200 g of MPC 85 was evenly spread in an aluminum tray (18 x 14 x 3") and placed in an incubator (Hettich HettCube 200R) set at 25ºC. In addition to temperature, the relative humidity of the incubator was increased using a humidifier (JISULIFE, model # JB08 Shenzhen, China). The MPC 85 absorbed the water vapor to increase the water activity from 0.23 to 0.5. Every 30 minutes, the tray containing the MPC 85 was removed from the incubator, mixed thoroughly, and the water activity of MPC 85 was measured using a water activity meter (model #HC2-AW-USB-SW-USB Water Activity Probe with HW4 Software, Rotronic, Hauppauge, New York). The process was repeated until the water activity reached 0.5. When the desired level of 0.5 was reached, the MPC 85 was packed in a vacuum bag (FoodSaver, model # FSFSBFLB216NP, Oklahoma City, Oklahoma) and sealed. The water activity was checked every 3 or 4 days for up to 3 weeks. If the water activity dropped below 0.5 during this resting period, the adsorption process was repeated by placing the MPC 85 back in the incubator. When the water activity remained above 0.5, it was repacked in a vacuum bag until the next testing period. After the water activity of the powder had been in equilibrium for approximately two to three weeks, the powders were used in the manufacture of nutritional bars.

Manufacture of High Protein Nutrition Bars (HPNBs)

HPNBs were formulated to contain 30 g protein per 100 g of bar using the method and formulation as suggested by Banach et al. (2013). The ingredients and their concentrations are provided in Table 4.1.

Table 4.1 Model high-protein nutrition bar (HPNB) formulation.

Ingredient	High-protein nutrition bar	
	Ingredient (g per 100 g)	Source
MPC85	37.39	Idaho Milk Products, Jerome, ID
Vegetable Glycerin	21.50	Raw Plus Rare, Garden Grove, CA
Palm Kernel Oil	18.46	Green Beauty, Maywood, NJ
Maltitol Syrup	12.00	Chef Rubber, Las Vegas, NV
High-fructose corn syrup	10.00	Good Food, Inc., Honey Brook, PA
Water	0.65	Kansas State University, Manhattan, KS

The unadjusted MPC 85 (control), glycerol, maltitol syrup, and water were added to a mixing bowl and mixed with a wire whip attachment on for 30 seconds on speed 2 followed by 2-minute mixing on speed 4 using a stand mixer (model # KSM75SL, Kitchen Aid, St. Joseph, MI). Palm kernel oil and HFCS were heated together until fat liquefaction and cooled to 55-60ºC before being mixed into the protein mixture on speed 4 for 2 minutes. Mixing was paused every 30 seconds to scrape the sides and bottom of the mixing bowl. This process was repeated with the water activity adjusted MPC 85 to manufacture water activity adjusted HPNBs.

HPNB dough was uniformly packed into a rectangular steel mold (1in x 2 in x 10 in). Bar dough in the mold was leveled with a wire cutter, removed from the mold, and sealed with plastic wrap overnight at room temperature. After resting overnight, the dough was removed from the plastic wrap and cut with a wire cutter into 1 x 1 x 1” cubes. Samples were randomly placed into 4 x 6 x 2” clear stand-up barrier pouches (S-19171, Uline, Pleasant Prairie, WI), labeled appropriately, and stored in 25 ºC or 36 ºC incubators.

Evaluation of model high-protein nutrition bars

High protein nutrition bar samples studied using the adsorption theory were analyzed after being stored at 25 ºC or 36 ºC for 1, 2, 4, 6, 13, 22, and 31 days.

Water Activity Measurement. The crushed sample was placed into a 14mm sample cup and water activity was measured with a water activity probe (model #HC2-AW-USB-SW-USB Water Activity Probe with HW4 Software, Rotronic, Hauppauge, New York). Water activity measurements were carried out in triplicate for each batch, storage temperature, and storage time combination. The water activity values are reported as the average of duplicate HPN bar batches.

Hardness Evaluation. Following the method provided by Banach et al. (2013) each texture test was carried out for each HPN bar batch at each storage time and temperature. Prior to testing, samples were allowed to equilibrate for 10 to 20 minutes at room temperature. Upon testing, samples were removed from their packaging and compressed at 22 mm s^{-1} with a flat plate (Stable Micro Systems TA-30A, Texture Technologies, Scarsdale, NY) to 60% strain with a 0.05 N trigger force (model #TA-XT2). Hardness was defined as the force (N) needed to compress 60%. This procedure was carried out on 7 independent samples and analyzed using Exponent Connect software.

Color Measurement. Color values for each HPN bar sample were acquired with a MiniScan EZ Colorimeter (Hunter Laboratory Associates, Inc., Reston, VA) using the method described by McMahon et al. (2009). The colorimeter was calibrated using black and white tile. The HPNBs were removed from their package and measurements were taken at different locations on the sample and a L*, a*, and b* value calculated by the colorimeter was recorded. Each trial day samples were measured in triplicate.

Determination of Insoluble Protein. Insoluble protein was determined using the method described by Liu et al. (2009) with some modifications. Insoluble aggregates were determined by the solubility of a protein bar sample in a 10 mM phosphate buffer with a pH of 7. Approximately 60 mg of nutrition bar sample was added to 10 mL of phosphate buffer. The

mixture was stirred at room temperature at 400 rpm for 60 minutes and then centrifuged (Thermo Fisher Scientific Marathon 21000R Multi-Purpose Benchtop Centrifuge, model # 64660557, Waltham, MA) at 10,000 g for 30 minutes. The concentration of soluble proteins in the supernatant was then determined using LECO analysis (Leco TruMac N Nitrogen Analyzer, St. Joseph, MI). A decrease in the percentage of soluble proteins would suggest the formation of insoluble proteins.

Confocal Laser Scanning Microscopy. Confocal laser scanning microscopy (CLSM) was used to study the microstructure of the proteins within the HPN bars during storage. Approximately 0.5 g of HPN bar sample was placed onto a glass slide. Protein and lipids were stained using Fast green FCF (Sigma-Aldrich, St. Louis, MO, USA) and Nile red (Molecular probes, Thermo Fisher Scientific, Waltham, MA, USA) stains, respectively. Stock solutions of Fast green (5 mg dye in 5 mL water) and Nile red (100 mg dye in 500 mL acetone) were mixed in a ratio of 1:3 and 10 μL of the mixed solution was applied to the sample for 5–10 min. The stained samples were analyzed with a LSM 5 PASCAL (Zeiss, Thornwood, NY, USA). The objectives used were a Plan Neofluar 10×/0.3 and Plan Neofluar 40×/0.75. Multitracking was used to minimize possible bleed through and/or cross talk of the two fluorescence stains. Track 1 used a 543 nm Helium-Neon laser line and a primary dichroic HFT /488/543/633 to excite Nile red and collect differential interference contrast (DIC) transmitted light images. The emission signal was collected with Channel 2 using a secondary dichroic NFT 635 and a BP 560–615 nm filter to detect Nile red (red). Track 2 used the 633 nm Helium-Neon laser line to excite Nile red. A primary dichroic-HFT/488/543/633 was used to excite Fast green FCF, while a secondary dichroic – NFT 635 was used to separate the emission signals of these 2 fluorescent stains. The

emission signal was collected with a Long Pass (LP) 650 nm filter prior to Channel 1 PMT for detecting Fast green FCF (green).

Front-Face Fluorescence Spectroscopy. Front-face fluorescence spectra of bar samples were collected using a Perkin-Elmer (Waltham, MA) LS50B Luminescence spectrometer with the front face accessory using the method described by Babu and Amamcharla (2018). The bar sample was loaded into a sample holder with a quartz window. To obtain the fluorescence spectra, the sample holder was mounted on a front-face accessory fitted to a Perkin-Elmer LS50B spectrometer, maintaining an incidence angle of excitation at 56°C. Three scans were performed on each bar sample to record the fluorescence emission spectra of tryptophan (305 to 450 nm) at an excitation wavelength of 290 nm, Maillard products emission spectra (380 to 480 nm) at an excitation wavelength of 360 nm, and the Maillard excitation spectra at a range of 260 to 350 nm at an emission of 410 nm. The slit widths were set at 9.0 and 4.0 nm for excitation and emission, respectively. Each bar sample was analyzed in triplicate and averaged. A total of 9 individual spectra were collected for each sample. The FL Data Manager Software (Perkin-Elmer) was used for the spectral data acquisition.

Fourier Transform Infrared Spectroscopy. FTIR spectra were collected from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} using a Nicolet Summit FTIR Spectrometer (Nicolet, Madison, WI, USA) with an Everest ATR Diamond accessory (Model# 869-168800). A background spectrum of the AR-coated diamond crystal was recorded before the sample measurement, and a total of 16 scans were recorded per spectrum. All FTIR experiments were done in triplicate, following the method of Hogan et al (2012). The amide I band (1600-1700 cm^{-1}) in each spectrum was analyzed using Ominic software (Version 1.9, Nicolet, Madison, WI, USA).

Differential Scanning Colorimetry. The measurements were done in duplicate using a DSC Q200 (Model # 2000-1055) that was calibrated using indium and sapphire for temperature and energy. 7-20 mg samples were heated from 37°C to 100°C at a heating rate of 5°C/min, following the method provided by Zhou et al (2008). The denaturation temperature of proteins was recorded as the peak temperature of the endothermic peak.

Polyacrylamide Gel Electrophoresis. The level and composition of the protein in the nutrition bar were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. Aqueous solutions of 5% (w/w) protein bar sample in deionized water was made by stirring the protein bar sample in deionized water with a stir bar in a 50mL beaker. During stirring, the solution was maintained at 400rpm for 30 minutes at 45°C. 20 micro-liters of sample and 40 micro-liters of Sigma sample buffer (Lammeli 2x concentrate) or Bio-Rad 2x Lammeli sample buffer were vortexed and placed in a 90°C water bath for 5 minutes then removed and placed into the freezer for 3 minutes. A 1x Reducing/Non-reducing SDS running buffer was prepared and poured into the gel slot. 20 micro-liters of each sample were added into a gel column and ran at a constant current of 70V for 15 minutes and then 100V for 1 hour or until solution reached the end of the gel. After electrophoresis, the gels were stained using Coomassie Brilliant Blue R-250 staining solution and shaken for 30 minutes. The staining solution was drained and replaced with a destaining solution of 100mL acetic acid, 100mL methanol, and 800mL deionized water. The gel was shaken overnight, scanned, and then stored in the refrigerator.

Results and Discussion

Three lots of MPC 85 were obtained from a commercial manufacturer in the United States. The average protein, moisture, fat, lactose, and ash content as per the certificate of

analysis provided by the supplier for the MPC were 86.77% (w/w), 4.97% (w/w), 1.12% (w/w), 4.77% (w/w), and 6.68% (w/w), respectively. Randomized complete block design with MPC 85 treatment (control and water activity adjusted) and temperature (25°C and 36°C) as independent factors were studied. The final water activity of control and water activity adjusted MPC 85 before manufacture of HPNBs was 0.52±0.01 and 0.23±0.03, respectively.

Changes in water activity during storage

Water activity of all HPNBs throughout the duration of this study were under 0.53. Since the water activity remained less than 0.6 in all samples, the HPNBs are within an acceptable range where little to no microbial growth can occur (Loveday et al., 2009). The water activity of control and water activity adjusted HPNBs stored at 25°C initially increased until day 6 as the ingredients moved towards osmotic equilibrium (Figure 4.1). Li et al. (2008) reported that an increase in water activity indicates movement of water molecules from the intermediate phase to bulk phase. At the bulk phase, the molecules act as a plasticizer, decreasing the viscosity to keep the HPNBs soft during storage. In the present study, water activity initially increased before becoming relatively unchanged through day 31. This initial increase in water activity was not observed in control and water activity adjusted HPNBs stored at 36°C and is seen in Figure 4.1. Control and water activity adjusted HPNBs stored at 25°C had a significant ($P < 0.05$) increase in water activity during 31-day storage. On other hand, HPNBs stored at 36°C had a significant decrease ($P < 0.05$) in water activity during storage at 36°C for 31 days. It was found by Zhou et al. (2008) that a lack of water molecules allowed amino acids to form disulfide bonds which can result in protein aggregation. Adjusting the water activity of milk protein concentrate to match the other ingredients was hypothesized to reduce the amount of moisture migration and protein

aggregation. Accounting for the water activity of each individual ingredient can improve bar stability and mitigate the rate at which hardening occurs. The water activity adjusted HPNBs stored at both temperatures were significantly lower in water activity throughout the duration of the experiment compared to the control HPNBs at their respective temperature. It is also known that sugar crystallization can increase the water activity (Diaz et al., 2021). Sugar crystallization can lead to HPNB hardening in two ways: through formation of aggregated sugar particles and at higher temperatures reduces the sugar available for use as a plasticizer (Lu and Zhou., 2019). Sugar crystallization occurred within both the control and water activity adjusted HPNBs stored at 25°C because the water activity increased during storage. However, this was not observed in HPNBs stored at 36°C because of the increased temperature causing sugar to be more soluble.

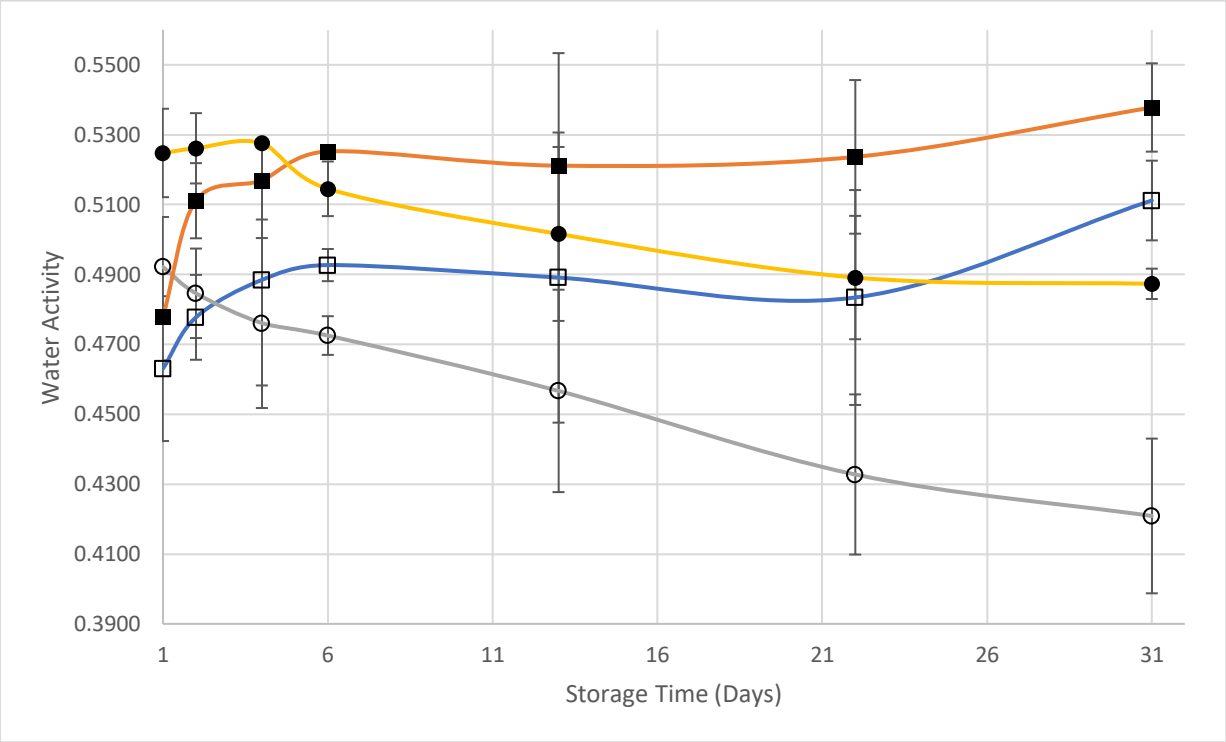

Figure 4.1 Changes in water activity of high protein nutrition bars with treated and untreated milk protein concentrate at 25°C and 36°C storage temperatures. Data are mean values of 3 measurements on three samples; with standard deviation indicated by vertical error bars. ■ 25°C water activity adjusted; ● 36°C water activity adjusted; □ 25°C control; ○ 36°C control.

Changes in hardness during storage

Figure 4.2 depicts a typical force deformation curve observed during uniaxial compression of HPNBs. The peak force, also known as hardness, represents the maximum force required to compress 60%. In HPNBs, the initial break in the force deformation curve represents the intial fracture during compression. Fracture does not always begin at the highest point on the curve, but the maximum force required to compress 60% is recorded as the peak. When the fracture is complete then the force falls to zero. Hogan et al. (2012) reported that during an accelerated storage, sharp initial increases in hardness followed by a decrease then slight increase was observed. In this study, the peak force of HPNBs at day 31 was greater than day 1, indicating hardness during storage.

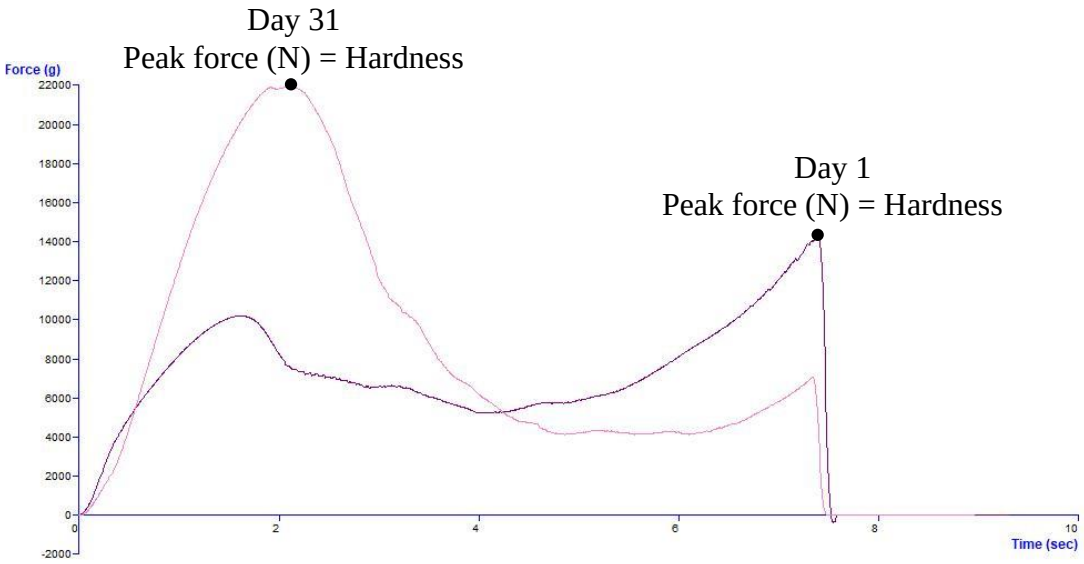

Figure 4.2 A typical force deformation curve obtained during compression of high protein nutritional bars on Days 1 and 31.

The hardness of control and water activity adjusted HPNBs stored at 25°C, did not significantly increase 31 days after manufacture. The hardness of the control and water activity adjusted HPNBs stored at 25°C were similar in hardness until day 13 (approximately 140 N). Change in hardness at 36°C may not follow the same mechanism as 25°C due to a faster rate of

moisture migration and increased fluidity of the HPNB matrix at higher temperatures (Banach et al., 2014). From day 1 to 31 of storage, control HPNBs stored at 36°C had approximately 200% increase in hardness. HPNBs prepared with water activity adjusted MPC maintained lower hardness at both the beginning and end of storage compared to the control samples. There was approximately 180% increase in hardness for water activity adjusted HPNBs stored at 36°C. This can be viewed in Figure 4.3. Both the control and water activity adjusted HPNBs are statistically significant ($P < 0.05$) between day 1 and day 31 of storage at 36°C. Signficant increase ($P < 0.05$) of hardness in control HPNBs stored at 36°C started from day 13. However, for water activity adjusted HPNBs this signficant increase ($P < 0.05$) in hardness did not occur until 22 days of storage. It can be concluded that as water activity increases the hardness decreases. Therefore, the water activitiy adjusted HPNBs will harden at a slower rate than control HPNBs at 36°C.

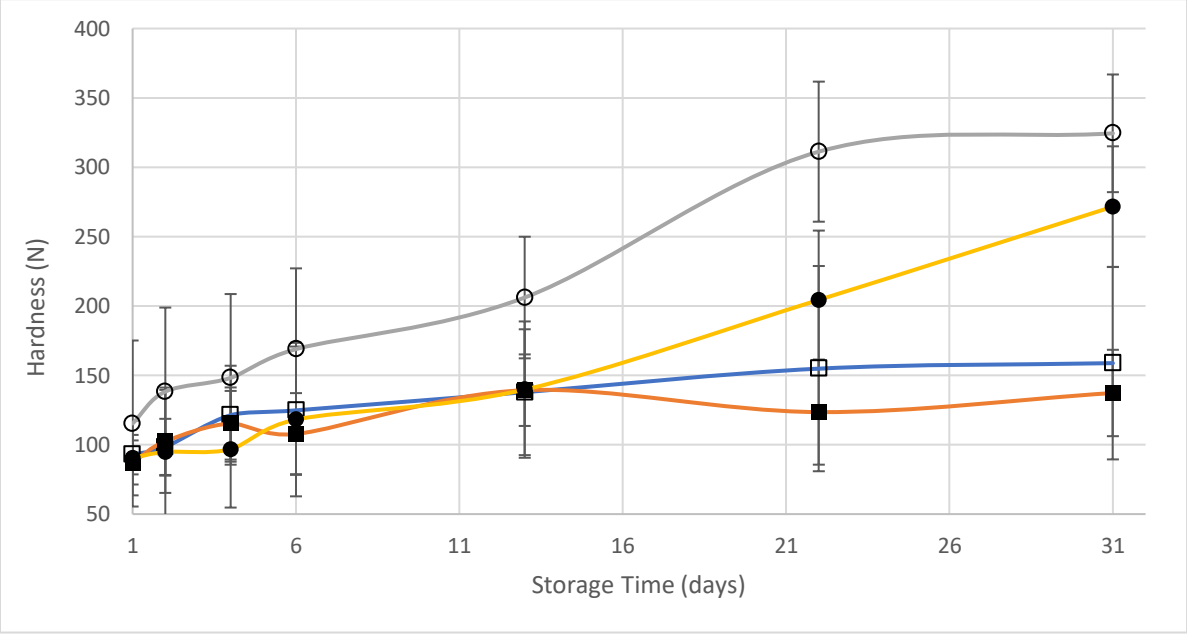

Figure 4.3 Changes in hardness of high protein nutrition bars with treated and untreated milk protein concentrate at 25°C and 36°C storage temperatures. Data are mean values of 7 measurements (N=3), with standard deviation indicated by vertical error bars. ■ 25°C water activity adjusted; ●36°C water activity adjusted;□ 25°C control; ○ 36°C control.

HPNBs formulated with water activity adjusted MPC 85 lacked initial cohesion. The dough was viscous, sticky, and had to sit covered at room temperature before being molded into a bar shape and placed into the airtight bag. Hogan et al. (2012) found that HPNBs with a more viscous texture contained non-agglomerated micellar casein (MC). Hogan et al. (2012) suggested that bars with non-agglomerated MC were more dense, resulting in less air pockets or voids between particles. The water activity adjusted MPC 85 was able to reduce moisture migration by creating a moisture barrier between ingredient particles through steric hindrance (Meng et al., 2019). The MPC 85 also competed for the available water, reducing the differences in water activity between the other ingredients. A smaller range of water activity between other ingredients means there is less of a drive for bar hardening. Discontinuities between the chemical and osmotic potential of water between ingredients plays a major role in hardening (Purwanti et al., 2010). Therefore, it is likely that the signficant decrease in hardness of HPNBs during storage was a result of using a lower storage temperature. While the water activity adjusted MPC 85 did aid in the decrease of texture, there was not a signficant difference between most control and water activity adjusted HPNBs, indicating it did not impact the hardness as much as expected.

Changes in color during storage

The mean L*, a*, b*, and delta E values of control and water activity adjusted HPNBs were used to evaluate the changes in color during storage at 25°C and 36°C (Tables 4.2 and 4.23). At 25°C, there was no significant change in L* values ($P > 0.05$) of control HPNBs from day 1 to day 31. The same trend was observed in water activity adjusted HPNBs until day 31 with no significant ($P > 0.05$) decrease. Similarly, Banach et al. (2014) reported no significant change in color of high protein nutrition bars stored at 22°C for 42 days. At 25°C, there was a

significant increase ($P < 0.05$) in a* of control and water activity adjusted HPNBs from day 1 to day 13. This increase is due to Maillard browning in the sample, causing HPNBs to turn a red-brown or caramel color (McMahon et al., 2009). This color change trend continues through day 31 for both samples and can be seen in Figure 4.4. At 25°C there was a significant increase ($P < 0.05$) in b* values of control and water activity adjusted HPNBs from day 1 to day 31. Similarly, McMahon et al. (2009) observed no change in HPNBs produced with hydrolyzed whey protein isolate through day 34. Overall, the variation in L*, a*, and b* values between control and water activity adjusted HPNBs stored at 25°C were not significantly ($P > 0.05$) mitigated through the water adjustment of MPC 85.

Table 4.2 Changes in color of high protein nutrition bars with and without untreated milk protein concentrate during storage at 25°C.

	25°C					
	Control			Water Activity Adjusted		
	L*	a*	b*	L*	a*	b*
Day 1	90.29 ± 0.66^{ab}	0.11 ± 0.11^{fg}	13.30 ± 1.01^{ef}	89.07 ± 2.08^{cde}	-0.16 ± 0.25^{g}	14.43 ± 0.71^{def}
Day 2	90.50 ± 1.03^{ab}	0.16 ± 0.11^{fg}	13.33 ± 0.81^{def}	90.31 ± 1.06^{abcde}	-0.06 ± 0.08^{g}	13.97 ± 0.62^{f}
Day 4	90.69 ± 0.73^{a}	0.07 ± 0.14^{fg}	13.08 ± 0.69^{f}	89.99 ± 1.45^{abcde}	0.27 ± 0.50^{def}	14.87 ± 1.70^{cdef}
Day 6	90.52 ± 0.39^{abc}	0.05 ± 0.18^{efg}	13.65 ± 0.58^{def}	88.82 ± 3.27^{de}	0.09 ± 0.20^{defg}	17.27 ± 4.39^{abc}
Day 13	89.47 ± 1.11^{abcde}	0.27 ± 0.24^{cde}	14.79 ± 1.50^{cdef}	89.13 ± 1.71^{e}	0.46 ± 0.14^{cd}	15.59 ± 1.20^{abcde}
Day 22	89.90 ± 0.40^{abcd}	0.66 ± 0.19^{bc}	15.58 ± 0.69^{bcde}	89.41 ± 0.26^{abcde}	0.78 ± 0.19^{b}	16.42 ± 0.55^{abcd}
Day 31	89.19 ± 0.91^{bcde}	1.31 ± 0.35^{a}	18.01 ± 1.09^{ab}	89.12 ± 0.97^{de}	1.31 ± 0.10^{a}	18.21 ± 0.55^{a}

Values were compared within column and with same superscript are not significantly different (P >0.05)
All values are expressed as mean ± SD. N=3

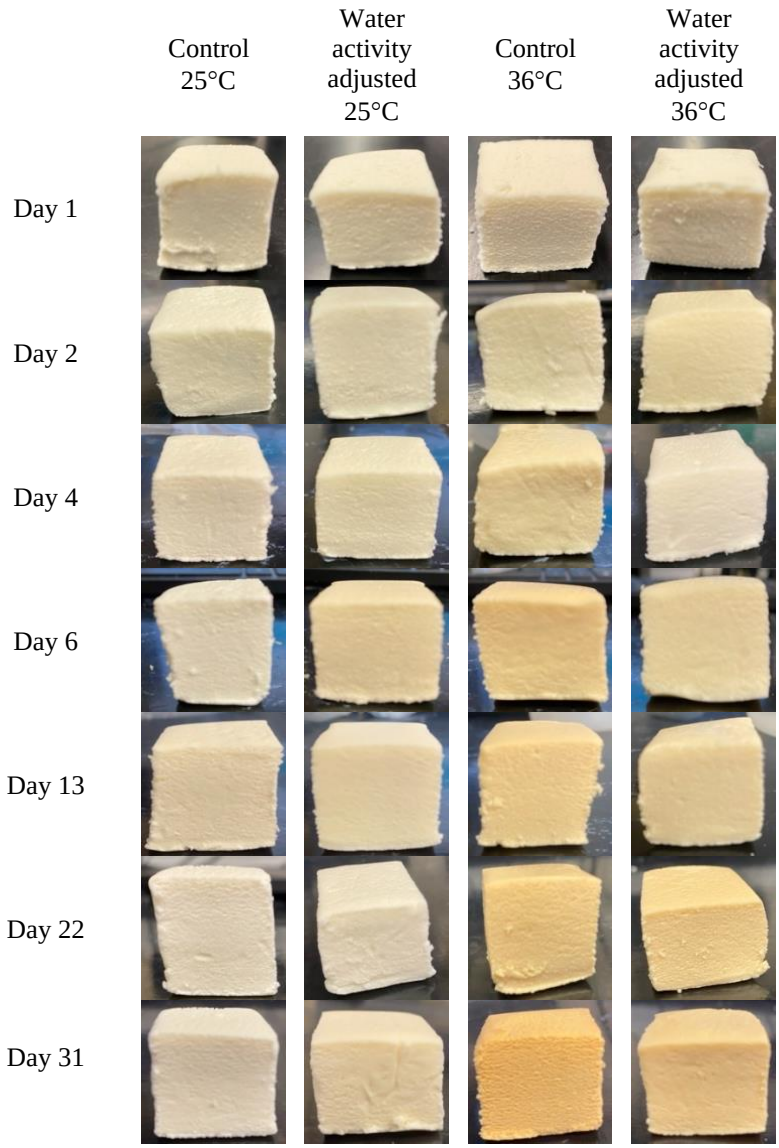

Figure 4.4 Model high protein nutrition bars (HPNB) color after Day 1, 2, 4, 6, 13, 22, and 31 of storage at 25°C and 36°C. Control identifies a model high protein bar made with unmodified milk protein concentrate (MPC) 85. Water activity adjusted identifies a model high protein bar made with modified MPC 85.

At 36°C, there was no significant change in L* values ($P > 0.05$) of control HPNBs from day 1 to day 31. Like the control HPNBs, the same trend was observed in water activity adjusted HPNBs until day 31 with no significant ($P > 0.05$) decrease. There was a significant change in a* ($P < 0.05$) for control and water activity adjusted HPNBs stored at 36°C from day 1 to day 13. This trend of significant color increase continues through day 31 and can be visualized in Figure

4.4. The water activity adjusted HPNBs stored at 36°C underwent a significant increase in a* vales ($P > 0.05$) between day 1 and 22. Similarly, Meng et al. (2019) found that the addition of anticaking agents could block the progress of Maillard browning. With the water activity adjustment of MPC 85, less moisture migration occurred, slowing the rate of the Maillard reaction. A significant increase ($P < 0.05$) in the b* value of control HPNBs occurred on day 13. The same trend was observed for water activity adjusted HPNBs stored at 36°C except the significant increase was seen on day 6. Overall, a decrease in L* and increase in a* and b* values demonstrate an increase in brown coloration and a decrease of lightness during storage. Banach et al. (2014) found that color change can be an indication of quality decline. Color developed at a different rate and extent for HPNBs made with control versus water activity adjusted MPC 85. As the bars reach a darker color, so does the decline in shelf stability. Shelf stability and consumer acceptability are positively correlated. Banach et al. (2014) also hypothesized that a progression in browning results from amines participating in a Maillard reaction. It can be concluded from this table that there is a significant change of color in water activity adjusted HPNBs during storage and a Maillard reaction is occurring amongst all samples.

Table 4.3 Changes in color of high protein nutrition bars with and without untreated milk protein concentrate during storage at 36°C.

	36°C					
	Control			Water Activity Adjusted		
	L*	a*	b*	L*	a*	b*
Day 1	90.35±1.69^{a}	-0.15±0.12^{d}	13.74±0.50^{f}	87.07±5.17^{ab}	0.12±0.13^{d}	14.28±0.13^{f}
Day 2	90.24±0.84^{a}	0.20±0.23^{d}	14.83±1.23^{f}	90.28±1.35^{a}	0.10±0.06^{d}	15.14±0.98^{f}
Day 4	88.14±2.17^{a}	1.30±1.37^{d}	17.98±4.28^{ef}	89.83±0.96^{a}	0.42±0.13^{d}	16.27±0.80^{f}
Day 6	87.95±2.09^{a}	1.88±1.75^{d}	18.52±4.60^{ef}	89.21±1.15^{a}	0.93±0.34^{d}	17.04±1.38^{de}
Day 13	80.88±8.76^{bc}	5.84±5.09^{bc}	26.01±10.15^{cd}	84.97±2.88^{ab}	3.95±1.49^{cd}	23.43±3.09^{cd}
Day 22	71.86±6.74^{de}	7.73±6.60^{bc}	36.07±1.77^{ab}	77.82±3.00^{dc}	7.79±1.37^{b}	31.42±1.75^{bc}
Day 31	67.14±4.34^{e}	12.62±1.65^{a}	39.29±1.39^{a}	74.75±1.65^{cd}	9.77±0.92^{ab}	35.65±1.13^{ab}

Values were compared within column and with same superscript are not significantly different (P >0.05)
All values are expressed as mean ± SD. N=3

The delta E results depicted that there was a significant increase ($P < 0.05$) between control HPNBs stored at 25°C on day 1 and day 22. The exact trend was also seen for the water activity adjusted HPNBs stored at 25°C. Signifying, there was little to no effect on the HPNBs when performing a water activity adjustment of MPC 85. Unlike the control HPNBs stored at 25°C, the HPNBs stored at 36°C had a significant increase in the delta E value by day 13. The same trend was identified for the water activity adjusted HPNBs. This signifies that the temperature does significantly influence the rate of color change in HPNBs but the water activity adjustment on MPC 85 does not.

Changes in insoluble protein during storage

Figure 4.5 shows the formation of insoluble protein in the HPNBs as the function of storage time and temperature. An increase of insoluble protein during storage of HPNBs resulted in increased hardness (Zhou et al., 2008). Zhou et al. (2008) reported that the texture of whey proteins signficantly increased when approximately 25% of proteins became insoluble on the third day of storage at 45°C. The amount of insoluble protein to cause hardening at 23°C or 34°C is 12% and 15%, respectively (Zhou et al., 2008). The insoluble protein content of the control HPNBs stored at 25°C had a decrease during storage (18.78%) compared to the water activity adjusted HPNBs with a 49.81% increase. The control HPNBs stored at 36°C had a increase of 7.47% compared to water activity adjusted HPNBs that had a 30.47% increase. Maillard reactions between the proteins and reducing sugars can cause glycosylation of protein molecules, resulting in proteins as insoluble aggregates (Zhou et al., 2022). Zhou et al. (2022) reported no signficant changes of insoluble protein for the first 21 days of storage, followed by a sharp increase of insoluble protein through day 35. The data shown in Figure 4.5 depicts a decrease of insoluble protein in HPNBs from day 1 to 13, followed by a increase through day 31 of storage.

An increase of insoluble proteins in HPNBs formulated with control and water activity adjusted MPC 85 was observed at 36°C, indicating that the formation of insoluble protein did affect bar hardening during storage. At 31 days of storage, the 30kg load cell of the texture analyzer was maximized for most HPNBs. The water activity of HPNBs stored at 36°C declined during storage. After 31 days of storage at 36°C, the lowest recorded water activity had been reached (Figure 4.1). However, water activity adjusted HPNBs stored at 36°C had a lower percentage of insoluble protein compared to control HPNBs. Indicating, water activity adjusted MPC 85 did reduce the rate of formation of insoluble protein at 36°C. Banach et al. (2016) predicted that as storage time increased past 35 days there would been an increased amount of insoluble protein and formation of protein aggregates. It is likely that as storage time increased, insoluble protein in HPNBs stored at 25°C would've significantly increased as well. Further storage studies of HPNBs are needed to confirm this theory.

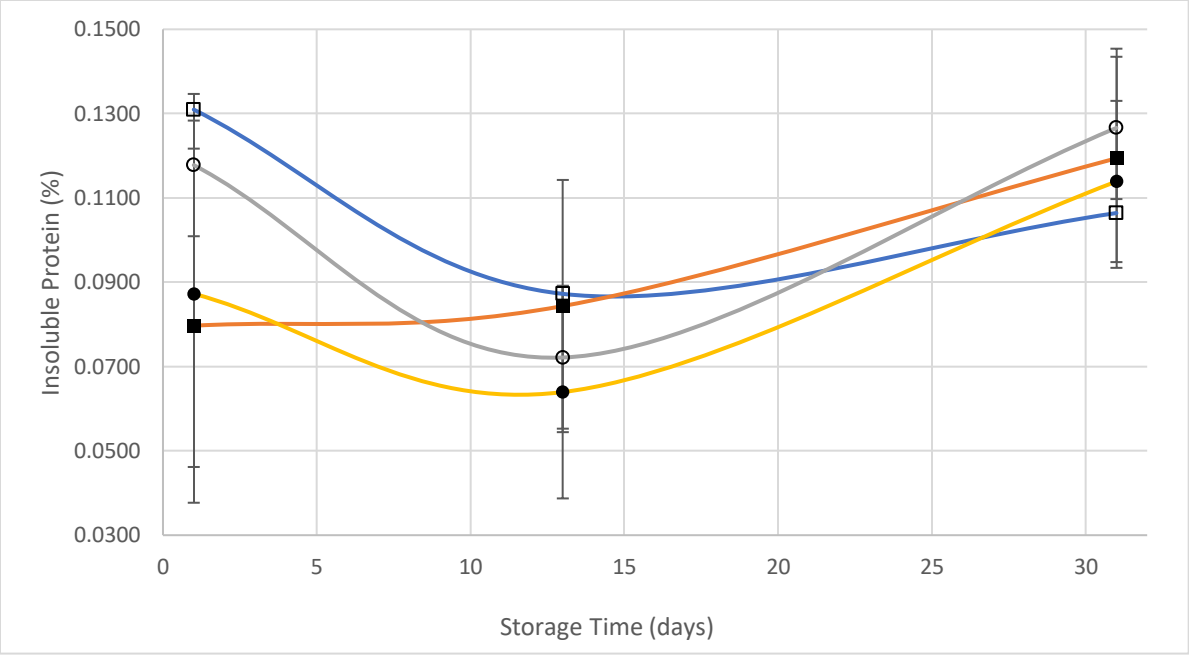

Figure 4.5 Formunation of insoluble protein in high protein nutrition bars with treated and untreated milk protein concentrate at 25°C and 36°C storage temperatures. Data are mean values of 3 independent lots (N=3), with standard deviation indicated by vertical error bars. ■ 25°C water activity adjusted; ● 36°C water activity adjusted; □ 25°C control;○ 36°C control.

Changes in Sodium Dodecyl Sulfate Polyacrylamide Get Electrophoresis (SDS-PAGE)

Protein profiles (SDS-PAGE) for high protein nutrition bars produced with control and water activity adjusted MPC 85 were analyzed under reducing conditions on the first (day 1), middle (day 13), and last day of storage (day 31). On day 1, the reduced protein patterns of control and water activity adjusted HPNBs appeared identical (Figure 4.6). By day 13, the major protein bands for control and water activity adjusted HPNBs stored at 25°C became slightly more prominent. There was no increase in molecular weight. On day 31, the protein bands of HPNBs stored at 25°C were faded compared with day 1 and 13, with no appearance of minor whey proteins.

Under non-reducing conditions, control and water activity adjusted HPNBs at 25°C on day 1 had similar protein bands (Figure 4.7). At day 13, little change had progressed. By day 31, the major protein bands had become smaller and moved up the lane. With low molecular weight and no protein aggregates the HPNBs exist in a rubbery state, prone to Maillard reactions resulting in textural hardness during storage (Banach et al., 2016 and Zhou et al., 2008). It is not possible to predict the performance of water activity adjusted MPC 85 based on only the SDS-PAGE results (Banach et al., 2016). However, the gels show no signficant differences between control or water activity adjusted HPNBs, implying that the water activity adjustment of MPC 85 did not impact the rate at which hardness occurs during storage.

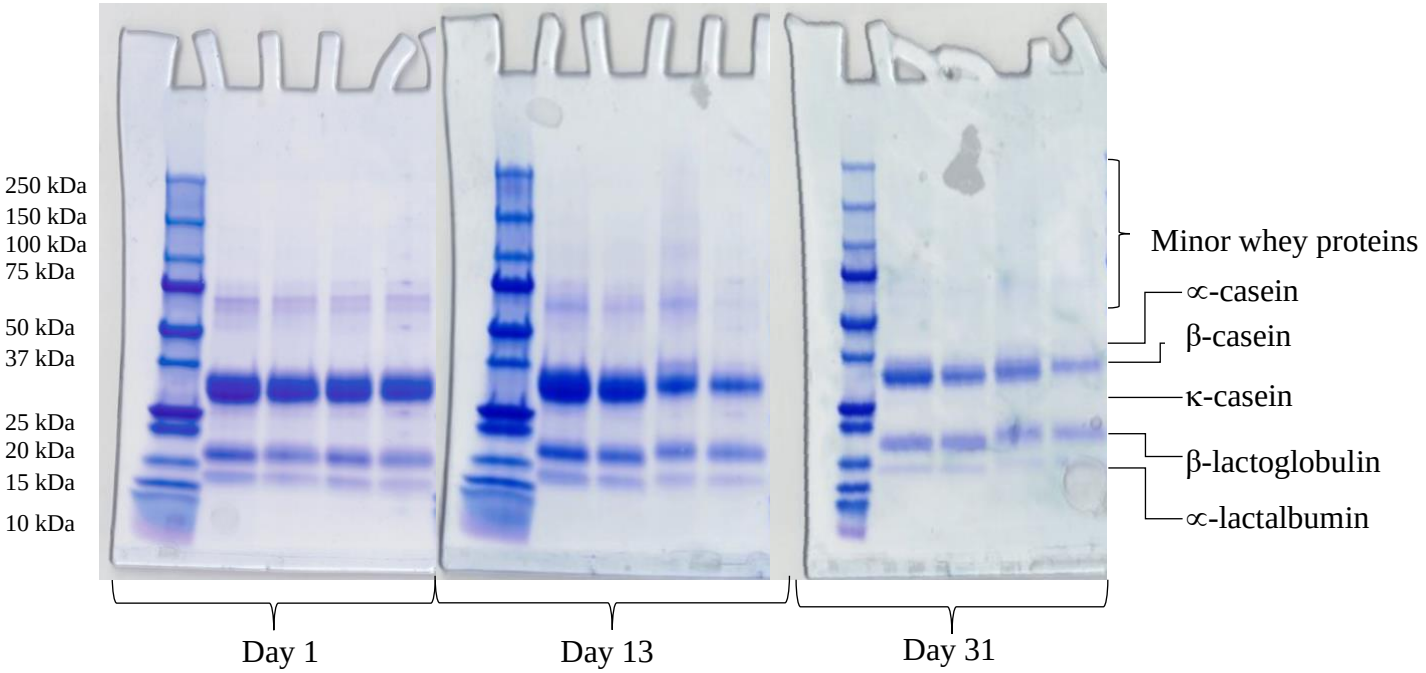

Figure 4.6 Reduced SDS-PAGE gel for high protein nutrition bars produced using control or water activity adjusted HPNBs stored at 25°C and 36°C. Lane 1 = molecular weight ladder; lane 2= 25°C control; lane 3= 25°C water activity adjusted; lane 4= 36°C control; lane 5= 36°C water activity adjusted.

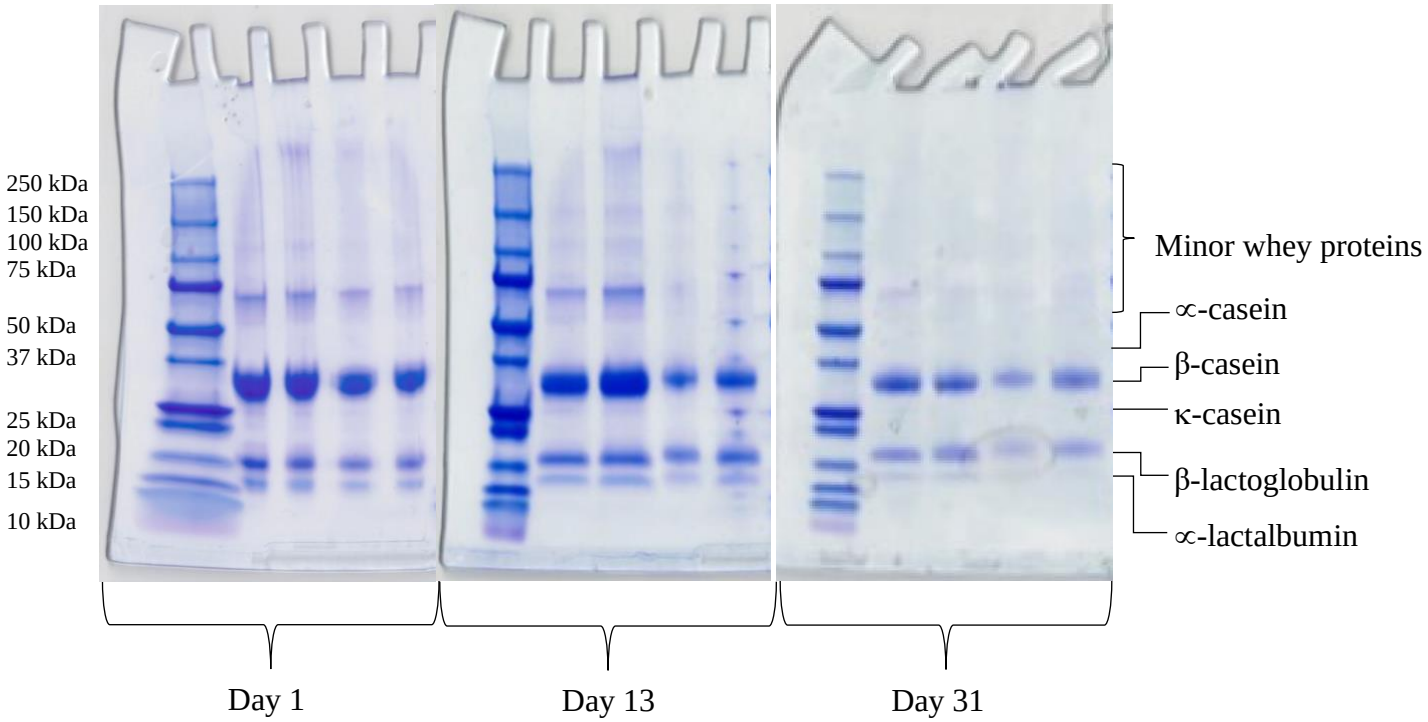

Figure 4.7 Non-Reduced SDS-PAGE gel for high protein nutrition bars produced using control or water activity adjusted HPNBs stored at 25°C and 36°C. Lane 1 = molecular weight ladder; lane 2= 25°C control; lane 3= 25°C water activity adjusted; lane 4= 36°C control; lane 5= 36°C water activity adjusted

On day 1, the reduced protein patterns of control and water activity adjusted HPNBs stored at 36°C appeared identical (Figure 4.13). On day 13, the control HPNB band became more diffuse and the molecular weight increased. A similar trend was observed for water activity adjusted HPNBs, however, the molecular weight had not increased as much. On day 31 of storage, the band for control and water activity adjusted HPNBs stored at 36°C reached the top of the gel. This observation confirmed that the molecular weight of the proteins was not changed by MPC treatment.

Anema et al. (2006) used reduced and non-reduced SDS-PAGE to determine the effects of storage temperature on the solubility of MPC 85 and noted Maillard reactions can lead to the appearance of high molecular mass polymers in proteins. With increasing temperature and storage time, the molecular mass of protein increased (Li et al., 2011). Meng et al. (2019) used reduced SDS-PAGE to illustrate the formation of Maillard-induced aggregates with and without anticaking agents. In their study it was noted that the bands of β-lactoglobulin and ∝-lactalbumin diffused and moved upwards during storage. With the addition of anticaking agents, the rate of diffusion and Maillard-induced protein aggregation decreased. However, the modification of whey proteins is relatively slow and could take months to develop extensive protein aggregation (Meng et al., 2019). Therefore, it can be concluded that very minimal to no protein aggregation occurred during storage under any conditions. HPNBs stored at 36°C did have higher mass polymers compared to HPNBs stored at 25°C, indicating a Maillard reaction. However, HPNBs manufactured with water activity adjusted MPC 85 did not provide any significant results.

Confocal Laser Scanning Microscopy

Confocal micrographs of HPNBs were taken after the bars were thawed for approximately 30 minutes (Figure 4.8 and 4.9). These images depict how proteins formed large bulbous strctures with smooth surfaces and in some cases contained spherical vacuoles. These vacuoles contain a mixture of air, water, glucose, or glycerol (Loveday et al., 2009). Overall, the protein particles were unchanged in shape but arranged more compactly after 31 days of storage.

MPC contains protein in the form of caseins and whey proteins with a ratio of approximately 80:20, respectively (Loveday et al., 2009). It is likely that the protein seen after 1 day of storage consisted of mostly whey proteins because they are more soluble than caseins. MPC powders are most soluble after initial manufacture. Loveday et al. (2009 and 2010) reported a decrease in protein solubility and increased particle clustering during storage as the HPNB batter turned into a firm dough matrix the day after manufacture. Solubility decreases as the storage time and temperature increase (Babu and Amamcharla 2018). The surface of the proteins are hydrated, but during storage a driving force pulles the moisture inwards to associate with the hydroxyl groups of molecules. This moisture migration from the surface inwards can result in a phase separation. A phase separation is a determinant of the HPNBs texture, stability, appearance, and taste due to the presence of multiple polymers in the matrix causing thermodynamic instability (McMahon et al., 2009 and Anema & De Kruif, 2016). In HPNBs, proteins are the only polymers present because there are no other macromolecular polysaccharides included in the matrix (McMahon et al., 2009). Therefore, fructose or maltitol is able to undergo a phase transition to form a glass state during storage (Tolstoguzov, 2003). A phase separation is mostly observed when conditions very humid or hot because of sugar crystallization. However, there were no crystals detected in the HPNBs, indicating that a change

in water activity or hardening during storage was not a result of crystallization. The hardening of HPNBs during storage was driven by the difference in osmotic pressure, resulting in the separation of proteins from water (Loveday et al., 2009).

Storge of control and water activity adjusted HPNBs at 25°C for 31 days resulted in a fusion of protein particles and development of a homogenous matrix. The average size of air voids also decreased during storage. This may have been due to the displacement of occluded air from the powder particles immediately after manufacture. A more complex protein matrix from day 1 to day 31 in HPNBs stored at 36°C can be seen in Figure 4.9. As the duration of storage increases, the control HPNBs rearranged into a more organized structure.

Overall, a reduced rate of matrix formation was seen with HPNBs containing water activity adjusted MPC 85. At 25°C, HPNBs produced with water activity adjusted MPC 85 had more air air pockets compared to HPNBs stored at 36°C. There is minimal change in the structure of the water activity adjusted HPNBs, indicating that it was not MPC treatment but temperature that effected the migration of small molecules, particularly water and moltose.

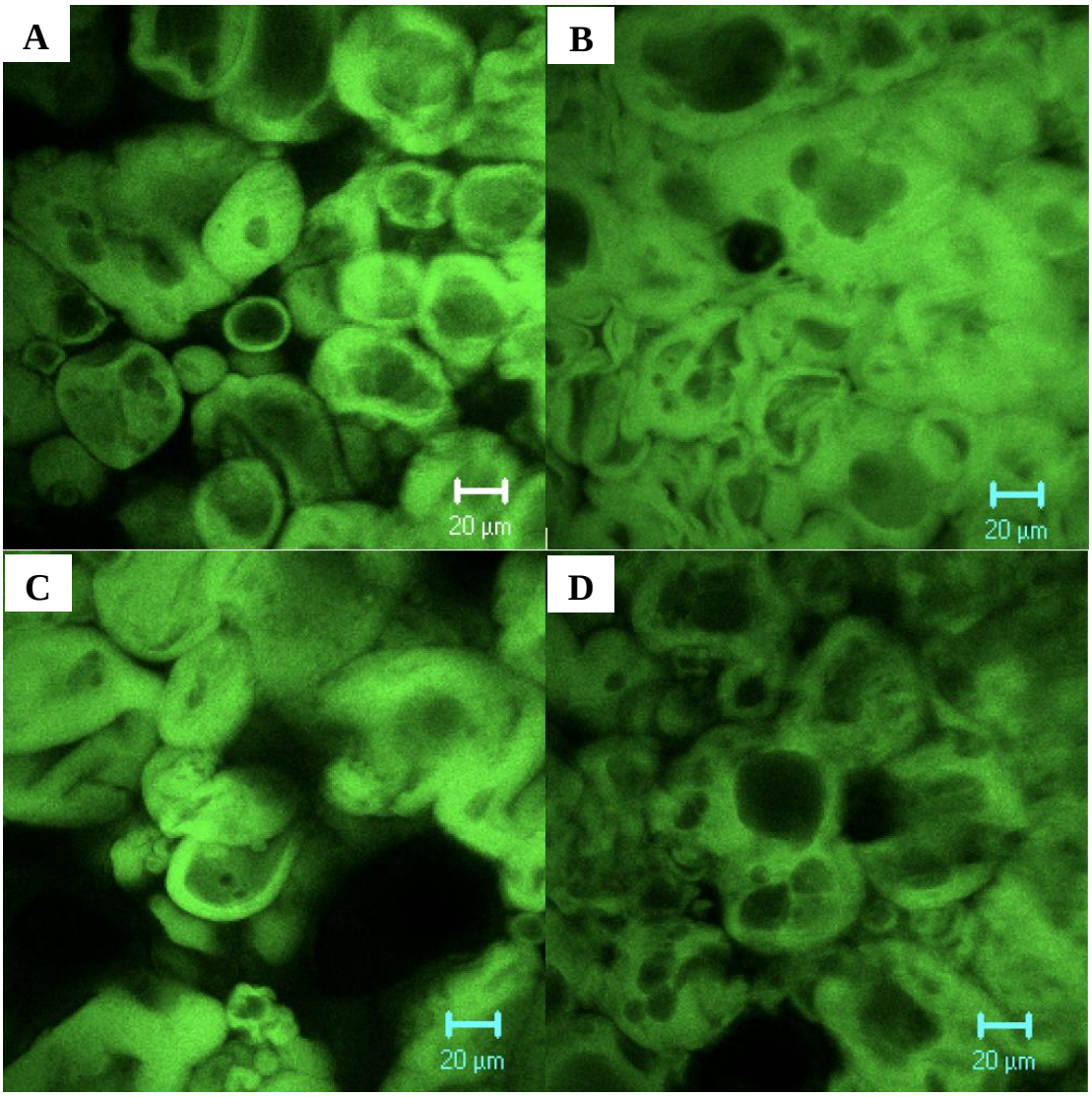

Figure 4.8 Confocal laser scanning microscopy images of day 1 control (A), day 1 water activity adjusted (B), day 31 control (C), and day 31 water activity adjusted (D) high protein nutrition bars produced using the adsorption method stored at 25°C. Green indicates Fast Green FCF staining (protein).

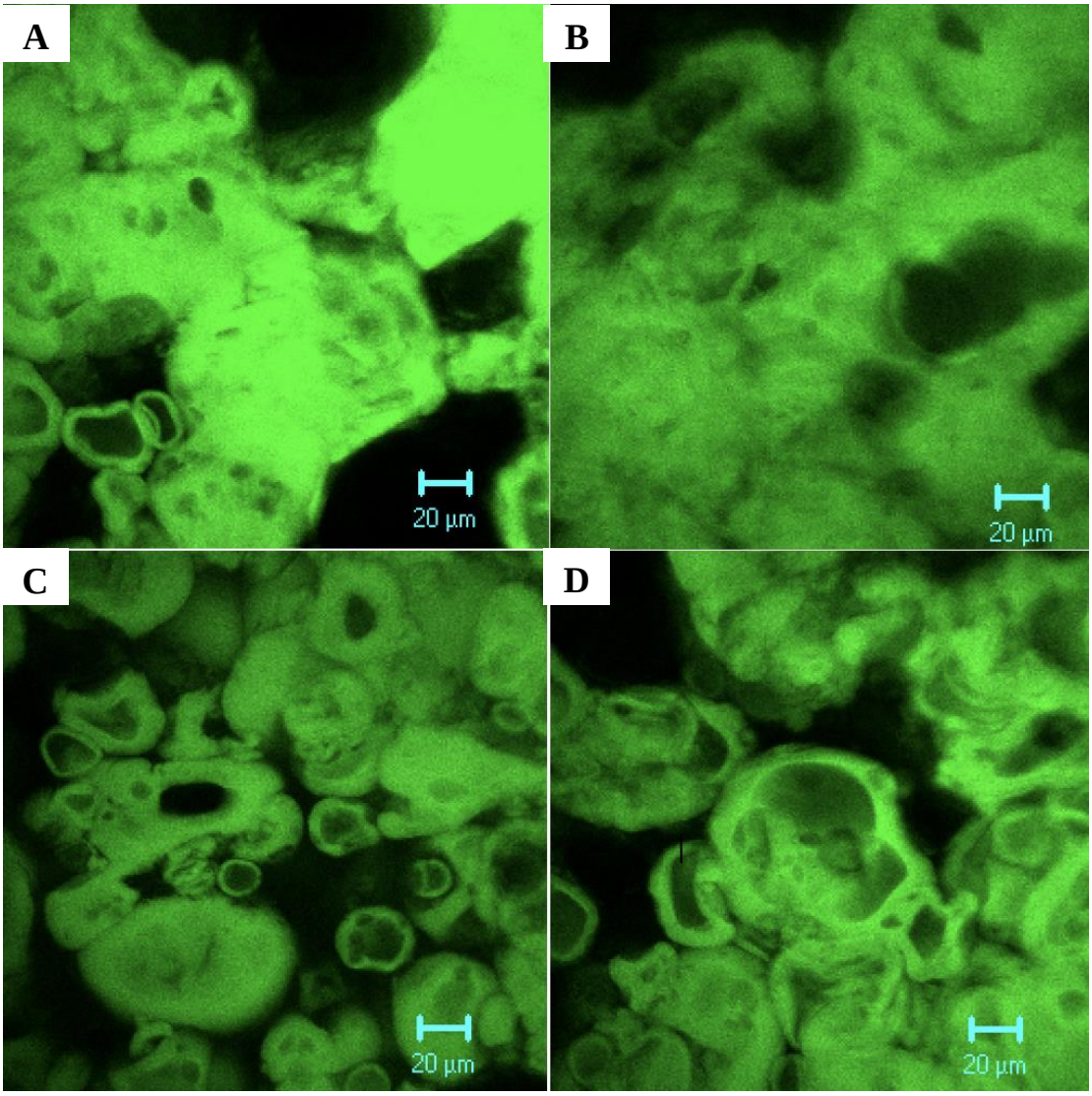

Figure 4.9 Confocal laser scanning microscopy images of day 1 control (A), day 1 water activity adjusted (B), day 31 control (C), and day 31 water activity adjusted (D) high protein nutrition bars produced using the adsorption method stored at 36°C. Green indicates Fast Green FCF staining (protein).

Front-Face Fluorescence Spectroscopy

Changes in the tryptophan emission spectra were observed during the storage of HPNBs (Table 4.4). The tryptophan maximum peak for both control and water activity adjusted HPNBs stored at 25°C was approximately 341 nm. There were minimal differences in tryptophan fluorescence between the control and water activity adjusted HPNBs stored at 25°C from day 1

to day 31 of storage. At 36°C, the average maximum tyrptophan peak for control and water activity adjuted HPNBs was 344 nm and 342 nm, respectively. There was no signficant difference between control or water activity adjusted peaks on day 1 and day 31. The absorbance units of control and water activity adjusted HPNBs were unchanged until day 13. At day 13, the absorbance of HPNBs stored at 36°C signficantly decreased through day 31 (Table 4.4). As the tryptophan emission intensity decreased, an increase in hardness and decrease in water activity is observed. The emission of tryptophan is highly sensitive to the environment (Lakowicz 2006). The spectral peak shifts indicate conformational changes of the proteins during storage while moisture equilibrated. Changes in spectra could be caused by protein-protein association or protein unfolding (Lakowicz, 2006). A signficant decrease in the peak intensities of control and water activity adjusted HPNBs from day 1 to day 31 stored at 36°C was observed, indicating a chemical and physical change of tryptophan in dairy proteins during storage. Compared with the control HPNBs stored at 36°C, the water activity adjusted HPNBs had lower absorbance units during storage. Until day 13, the absorbance of the water activity adjusted HPNBs stored at 36°C was similar to HPNBs stored at 25°C. This indicates that the water activity adjustment of MPC 85 did aid in the mitigation of hardness in HPNBs stored at 36°C during storage.

Table 4.4 Tryptophan emissions of high protein nutrtion bars produced with control and water activity adjusted milk protein concentrate using the adsorption method.

Storage Time	25°C				36°C			
	Control		Water Activity Adjusted		Control		Water Activity Adjusted	
	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)
1	73.12	341	64.45	340.5	81.47	340.5	77.74	341
2	76.93	341	76.94	341	73.78	341.5	77.74	340.5
4	85.71	339	75.61	339.5	60.28	343	75.36	341
6	87.55	340	90.39	341	56.39	343	72.88	341.5
13	73.54	339.5	70.39	342.5	19.52	349	43.15	343
22	84.4	344.5	67.3	341	11.85	345.5	26.11	343.5
31	72.16	340.5	68.07	340.5	10.83	344	17.66	345.5

WL = wavelength

The Maillard emission spectra of control and water activity adjusted HPNBs stored at 25°C and the water activity adjusted HPNBs stored at 36°C showed an increase in absorbance units from day 1 to day 31 of storage (Table 4.5). A decrease in absorbance units of control HPNBs during storage at 36°C was reported. There was little variation between the control and water activity adjusted HPNBs stored at 25°C. The variations in the Maillard emission spectra at 36°C could be explained using the b* values of color measurement (Table 4.3). An increased positive b* value indicates a color change from yellow to brown and is a measure of browning in the HPNBs. A significant increase ($P < 0.05$) in b* values of control and water activity adjusted HPNBs occurred during storage on day 13 and 6, respectively. This increase indicated that the color change from yellow to brown was caused by a Maillard reaction. Similarly, the maximum absorbance of all HPNBs stored at 36°C started within 6 days of storage which is when the significant changes of water activity, texture, and Maillard browning began to occur.

Table 4.5 Maillard emissions of high protein nutrtion bars produced with control and water activity adjusted milk protein concentrate using the adsorption method.

Storage Time	25°C				36°C			
	Control		Water activity adjusted		Control		Water activity adjusted	
	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)
1	9.69	436	11.45	436.5	13.33	436	14.27	436
2	10.91	435.5	13.24	435.5	14.67	433.5	15.88	436.5
4	13.72	435	13.85	435	16.83	434.5	17.96	435.5
6	14.76	435	17.61	433.5	18.72	434.5	19.34	434.5
13	15.53	435.5	16.56	436	13.15	437.5	17.79	436.5
22	19.27	436.5	18.82	435.5	11.54	435.5	17.42	434.5
31	21.42	435	21.2	435.5	11.89	437	15.99	435.5

WL = Wavelength

Lakowicz (2006) reported that the emission of tryptophan is often used as an indicator for protein conformational changes. The emission spectra shift results from the binding of ligands, protein-protein association, and protein unfolding (Lakowicz, 2006). Babu & Amamcharla (2018) reported a significant decrease in maximum absorbance of MPC when the storage temperature was increased from 20°C to 40°C. There was also a prominent decrease in tryptophan absorbance with an increase in storage time (Babu & Amamcharla, 2018). Similarly, results in this study depict a decrease in maximum absorbance in HPNBs stored at 36°C compared to 25°C. No significant difference in tryptophan spectra at 25°C was reported. However, at 36°C, the maximum absorbance of HPNBs decreased, indicating protein-protein association, or unfolding. Babu & Amamcharla (2018) reported an increase in absorbance as storage temperature and time increased for the Maillard emission spectra. In this study, an increase in absorbance during storage was seen in HPNBs at 25°C and the water activity adjusted HPNBs at 36°C. Compared to control HPNBs, water activity adjusted HPNBs stored at 36°C had a slower rate of change. It can be concluded that water activity adjustment of MPC 85 did reduce the rate at which spectral changes occurred in HPNBs stored at 36°C.

Fourier Transform Infrared (FTIR) Spectra

FTIR spectra of HPNBs on day 1 and day 31 of storage are shown in Figure 4.10 and 4.11. Similar spectra were found in HPNBs on day 31, regardless of the presence of water activity adjusted MPC 85. The main absorbance peaks were found at 3200-3300 cm^{-1} (amide A, the stretching of O-H groups and N-H groups), 2850-2990 cm^{-1} (amide B, the stretching of C-H groups), 1740 cm^{-1} (the stretching of C=O in ester bonds), 1600 cm^{-1} (amide I, the stretching of C-N and C=O in CONH groups), and 1530 cm^{-1} (amide II, the stretching of C-N groups and bending of N-H in CONH groups), and 1020 cm^{-1} (the stretching of C-O and C-N groups) (Meng et al. 2019). Maximum absorbance at each wavelength decreased during storage for HPNBs stored at 25°C and 36°C.

Amide I region (1600-1700 cm^{-1}) is associated with protein backbone comformation (Hogan et al., 2012). The C=O (70-85%) and C-N group (10-20%) stretching vibrations predominately occur in this region. The peaks at 1606 cm^{-1} (parallel β-sheet) and 1691 cm^{-1} (antiparallel β-sheet) in the HPNBs stored at 25°C on day 1 were shifted to 1635 and 1716 cm^{-1} in HPNBs on day 31, respectively. The peaks at 1608 cm^{-1} (parallel β-sheet) and 1743 cm^{-1} (antiparallel β-sheet) in the HPNBs stored at 36°C on day 1 were shifted to 1637 and 1749 cm^{-1} in HPNBs on day 31, respectively. A higher wavenumber suggests weaker crosslinking between via hydrogen bonds (Ramos et al., 2013). The formation of β-sheet structures is commonly found in aggregated proteins and relates to HPNB hardening (Nishanthi et al., 2017). Maillard reactions occurring in the HPNBs are responsible for the increase in β-sheet structures, implying that as the temperature increased more Maillard reactions occurred (Meng et al., 2019). However, the addition of water activity adjusted MPC 85 did not minimize the secondary structure

transformation indicating that the rate at which Maillard browning occurs appears to be unaffected.

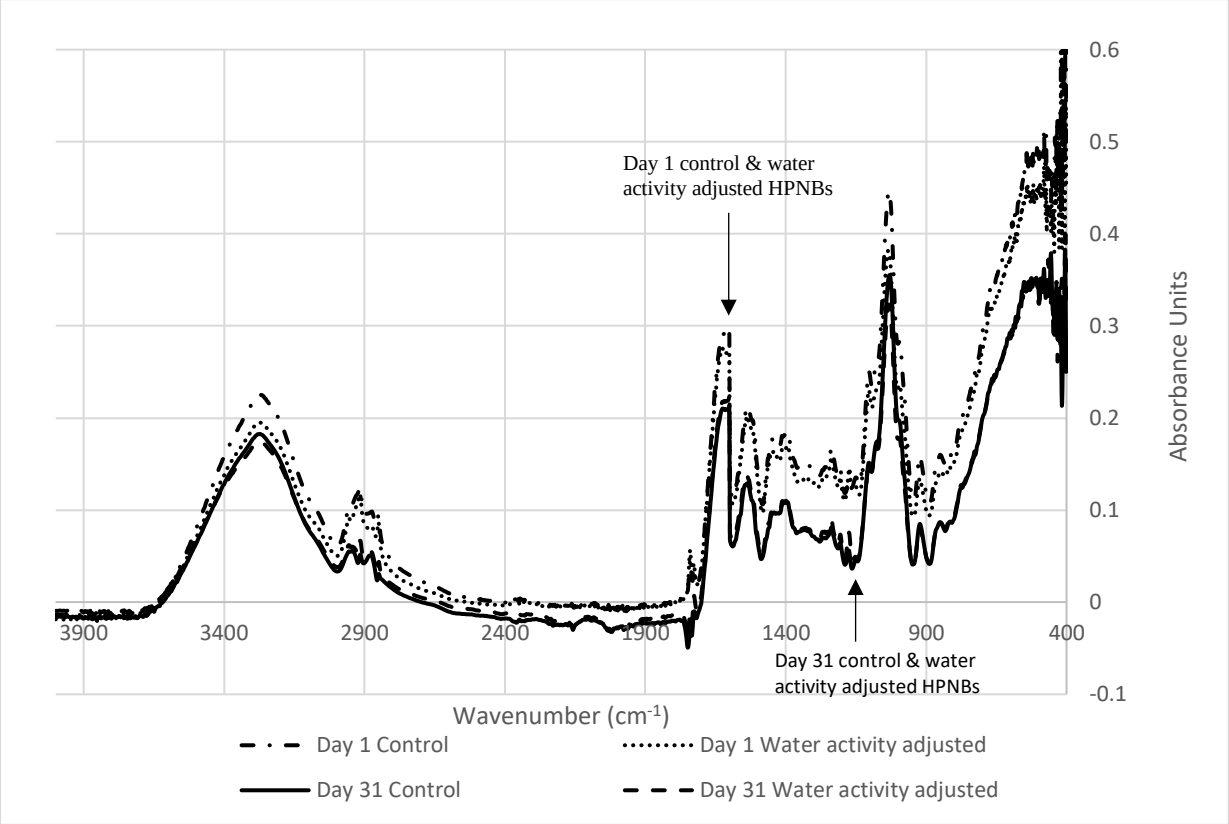

Figure 4.10 FTIR spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate in the 400-4000 cm^{-1} region stored at 25°C.

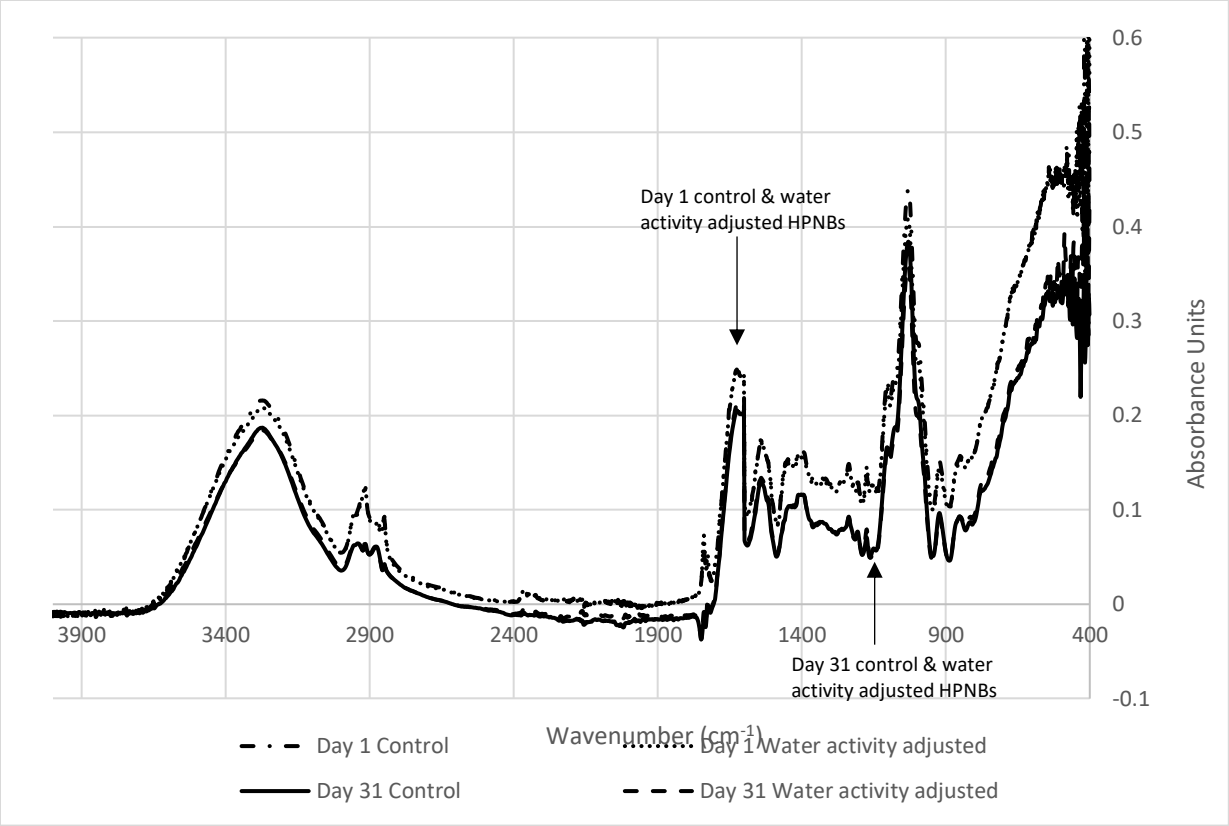

Figure 4.11 FTIR spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate in the 400-4000 cm^{-1} region stored at 36°C.

Differences observed in the amide I region (Figure 4.10 and 4.11) were dependent on the interaction between the protein and solvent (Hogan et al., 2012). Polarity, hydrophobicity, and viscoity are several factors likely to affect the rate at which diffusion of water in powder particles occurs. A rapid decrease in absorbance of all HPNBs occurs around 1630 cm^{-1}. This indicates an increase in the ∝-helix which is known to result in bar hardening (Hogan et al., 2012). At 25°C, a decrease in absorbance from day 1 to day 31 is shown. The water activity adjusted HPNBs have lower absorbance than control HPNBs, indicating that water activity adjustment of MPC 85 did not aid in the mitigation of hardness during storage. At 36°C, there is also a decrease in absorbance from day 1 to 31 of storage. At day 1, the control and water activity adjusted HPNBs appeared almost identical. However, at day 31, the water activity adjusted HPNBs had slightly higher absorbance values. Indicating that water activity adjustment of MPC 85 did positively

influence the rate at which Maillard browning reactions and hardening occurs in HPNBs stored at 36°C.

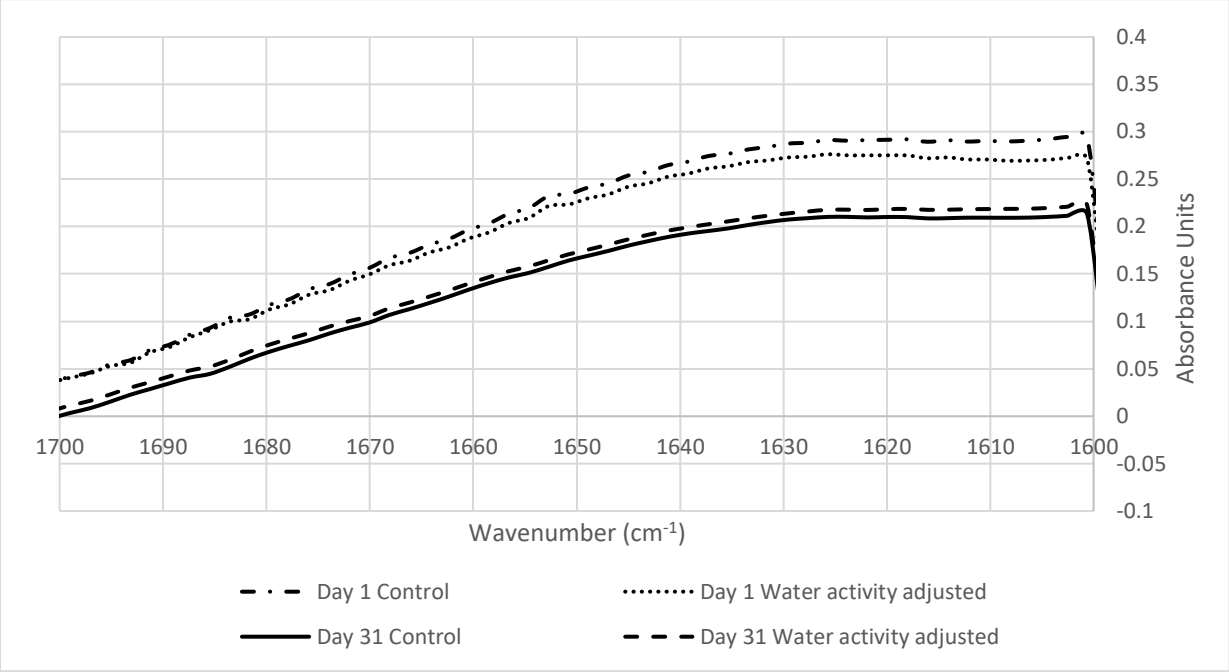

Figure 4.12 FTIR spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate in the 400-4000 cm^{-1} region stored at 36°C.

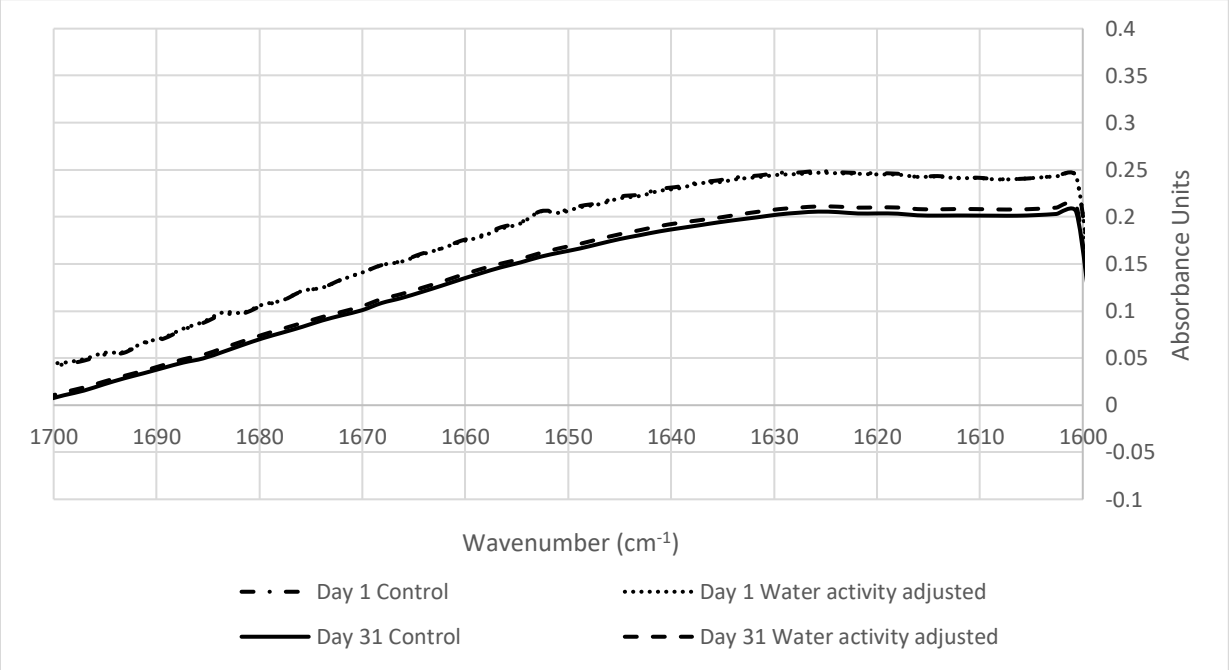

Figure 4.13 FTIR spectra showing amide I band of high protein nutrition bars produced with untreated and treated milk protein concentrate and stored at 36°C.

Differential Scanning Calorimeter (DSC)

The denaturation temperature of the protein contained in the HPNBs was recorded as the peak temperature of the endothermic peak (Figure 4.14 and 4.15). At 25°C on day 1 there were two endothermic peaks for the control and water activity adjusted HPNBs. The first denaturation peak corresponds to the denaturation of bovine serum albumin (BSA) and ∝-lactalbumin (Zhou et al., 2008). The second denaturation peak corresponds to the denaturation of β-lactoglobulin (Zhou et al., 2008). After storage at 25°C for 31 days, the heat flow and temperature required for denaturation were reduced. However, there was no significant difference between the control and water activity adjusted HPNBs.

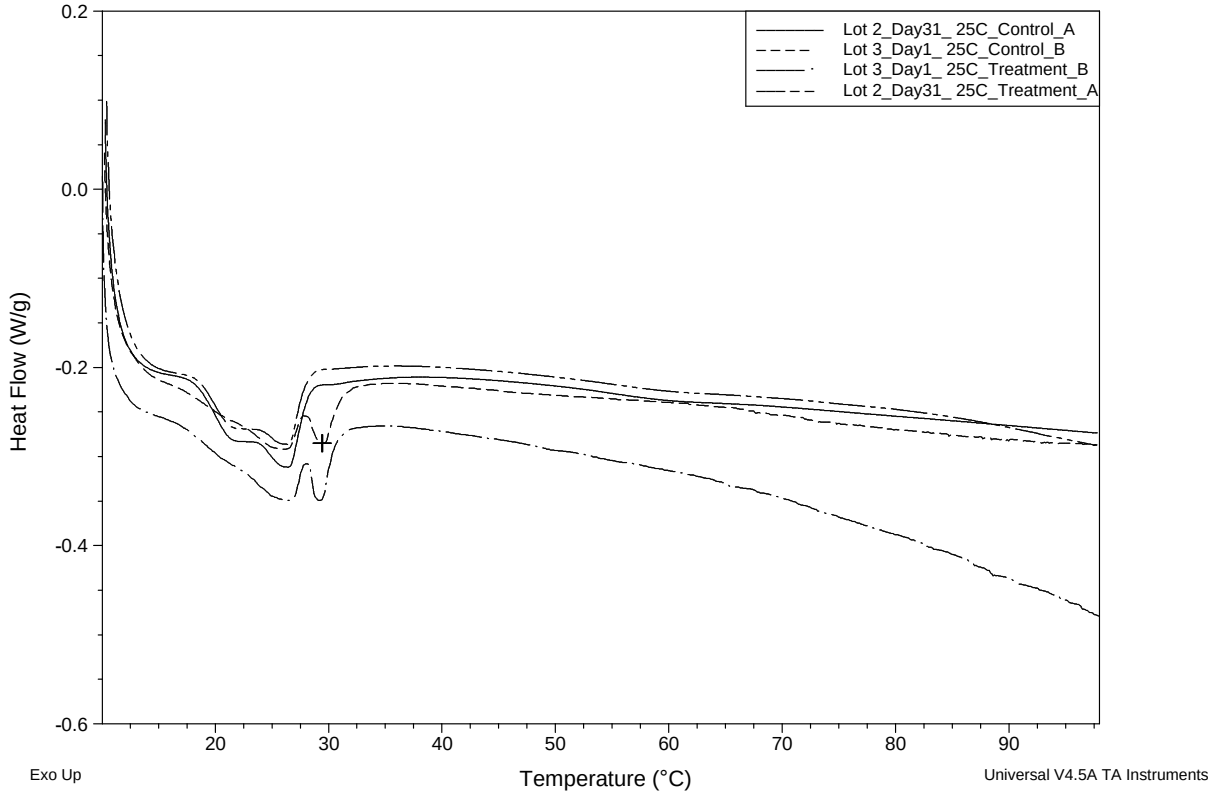

Figure 4.14 Differential Scanning Calorimeter scans of high protein nutrition bars stored for 31 days at 25°C.

On day 1 of analysis at 36°C, there were no significant changes in protein denaturation temperatures between the control and water activity adjusted HPNBs. By day 31, the control and water activity adjusted HPNB had peaks with an increased heat flow and a lower temperature. Research indicates that with an increase of storage temperature, the flexibility and mobility of protein peptides increase (Zhou et al., 2008). Therefore, HPNBs stored at higher temperatures (such as 36°C) would partly lose their tertiary structures and have lower denaturation temperatures than those stored at 25°C. On day 31, the control HPNBs at 36°C required slightly lower temperature to achieve denaturation compared to the water activity adjusted HPNBs. This indicates that the water activity adjustment of MPC 85 slightly impacted the rate at which protein denaturation occurs in HPNBs stored at 36°C.

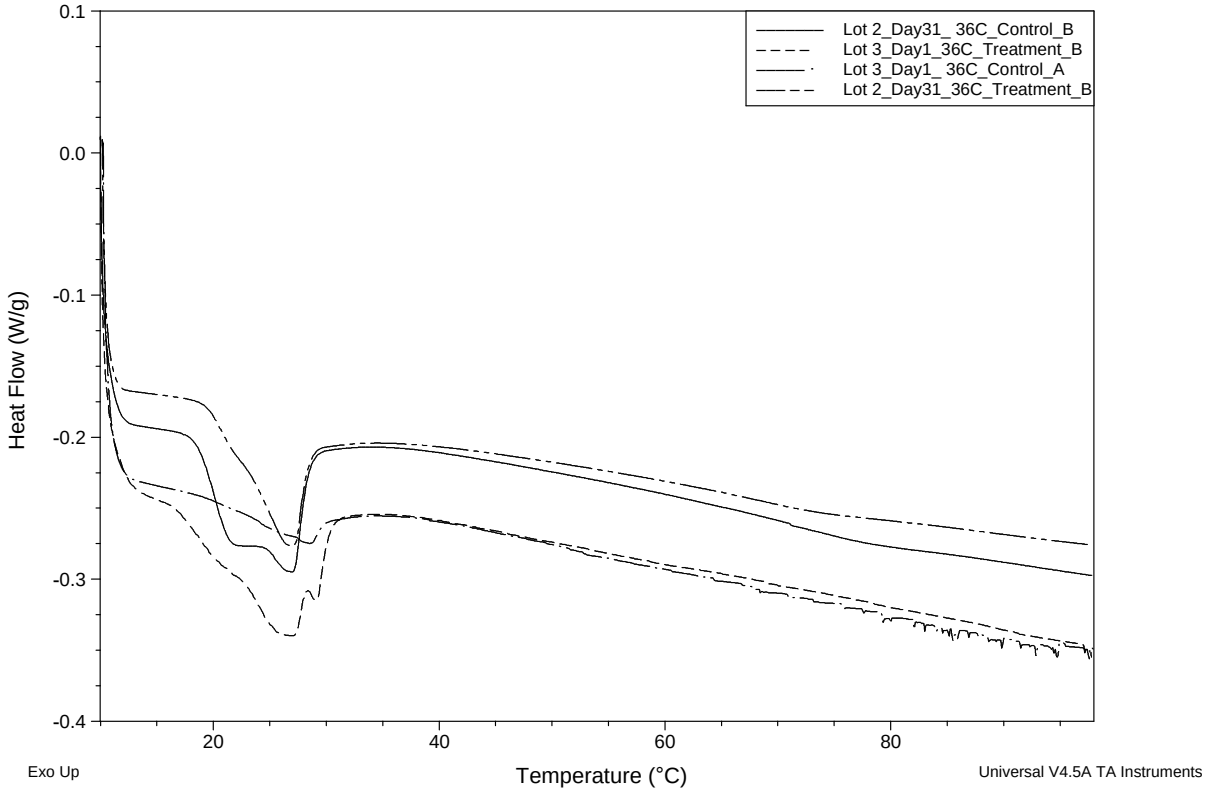

Figure 4.15 Differential Scanning Calorimeter scans of high protein nutrition bars stored for 31 days at 36°C.

Conclusion

HPNBs produced with control and water activity adjusted MPC 85 stored at 25°C had primarily increased textural, color, and water activity properties compared to 36°C. However, there was not a significant difference between control and water activity adjusted HPNBs at 25°C. At 36°C, the water activity adjusted HPNBs did perform slightly better than the control HPNBs. However, the increased cost and time spent adjusting the water activity of MPC 85 might not be worth the benefit. Overall, temperature had a greater impact on the physical and chemical properties causing HPNB hardening than water activity. It is possible that a different technique to adjust the water activity of MPC 85 might have been more effective to mitigate the rate of hardness at lower temperatures.

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Chapter 5 - High Protein Nutrition Bars: Water Activity Adjustment Through Desorption

Abstract

Milk protein concentrate (MPC) has increased flavor and is more nutritional compared to other protein sources used in the manufacture of HPNBs. However, with increased rates of hardening, the average shelf life of HPNBs is 6 months. Moisture migration between MPC and other ingredients can be mitigated by matching the water activity. A desorption method to mitigate the rate of hardness in HPNBs during storage was studied.

Three lots MPC 85 were produced through ultrafiltration of skim milk. Each lot of liquid MPC 85 was split into control and treatment batches. The control liquid MPC 85 was spray dried to reach a water activity of approximately 0.2. Using a decreased flow rate and reduced inlet temperature, the treatment liquid MPC 85 was spray dried and had a water activity of approximately 0.5. Using the spray dried MPC 85, HPNBs were stored in two different temperatures: 25ºC and 36ºC and tested in triplicates on days 3, 6, 13, 22, and 28. Physical and chemical changes of HPNBs during storage were monitored by observing the following: texture, color, water activity, insoluble protein, Fourier transform infrared (FTIR) spectroscopy, fluorescence, SDS-PAGE, and Differential Scanning Calorimetry (DSC).

High-water activity HPNBs stored at 25ºC did not show different chemical and physical characteristics during storage. However, there was a significant difference at 36ºC, indicating that high-water activity MPC 85 did aid in the mitigation of hardness in HPNBs at an increased temperature.

Keywords: Milk protein concentrate, moisture migration, and hardness.

Introduction

HPNBs provide many nutritional benefits and are often used by consumers as a meal supplement, energy fueled snack, or dieting staple. Compared to other snacks, HPNBs have an average protein content of 30g per 100g (Banach et a., 2014). The average content varies based on the inclusions incorporated during manufacture. Ingredients as well as inclusions can impact the color and texture of HPNBs during storage. Most changes during storage are undetectable for approximately 3 months and the product is no longer shelf stable after 6 months (Hogan et al., 2012).

Whey protein concentrate (WPC), whey protein isolate (WPI), milk protein concentrate (MPC), and other plant-based proteins are commonly found in HPNBs. Providing increased nutritional value and flavor, the addition of MPC into HPNBs is favored by producers and consumers alike. Unfortunately, MPCs undergo an increased rate of hardening compared to other dairy powder sources. Protein aggregation, moisture migration, glycation of protein molecules during storage, and increased temperature are the driving forces behind HPNB hardening (Meng et al., 2019). When a reducing sugar such as high fructose corn syrup is included in the formulation a Maillard reaction occurs at an increased rate (Zhou et al., 2013).

The amount of available water to act as a plasticizer in HPNBs during storage is correlated to the rate of hardening (McMahon, 2009). Hardening of HPNBs during storage is not a result of a singular cause, instead it occurs based on a wide range of chemical, physical, thermodynamic, and process related factors (Hogan et al., 2012). Differences in water activity between HPNB ingredients is what drives moisture migration, influencing the rate at which hardening occurs during storage. Other chemical and physical changes such as swelling,

molecular reorganization, sugar crystallization, and protein aggregation can influence the rate of hardening as well.

The goal of this study was to develop an efficient method to mitigate bar hardening during storage. This was achieved by spray drying MPC 85 to a water activity of approximately 0.5, matching other ingredients and storing HPNBs at different temperatures. Monitoring the chemical and physical changes of HPNBs during storage will allow manufacturers to create a longer lasting product.

Materials and Methods

Experimental Design

Three lots of fresh skim milk were procured and ultrafiltered at South Dakota State University to produce liquid MPC 85. Each lot of liquid MPC 85 was divided into two equal parts. The first part was spray dried under normal conditions to produce control MPC 85 and used as an ingredient in the manufacture of control high protein nutrition bars (HPNBs). The control MPC had a water activity and moisture content of approximately 0.18 ± 0.06 and 4%, respectively. The remaining liquid MPC 85 was spray dried to achieve a water activity of approximately 0.5 by adjusting the spray drying conditions. The average moisture content and water activity of the resultant MPC 85 was 12.5% and 0.53, respectively. The high-water activity MPC 85 was also used as an ingredient in the manufacture of high-water activity HPNBs. The control and high-water activity HPNBs were sealed and stored at 25ºC and 36 ºC for up to 28 days and analyzed for physical and chemical properties during storage.

Ultrafiltration and spray drying of MPC85

Three lots of pasteurized (72ºC /15 seconds) skim milk (1200 lb.) were obtained from the Davis dairy plant (South Dakota State University, Brookings, SD) and processed into MPC using

the UF process described by Salunke et al. (2021). Skim milk (800 lb.) was ultrafiltered to a final retentate volume of 138.78 lb., achieving a volume reduction ratio of approximately 3.8 (on a feed volume basis). The UF process was carried out using a 10-kDa polyether sulfone spiral-wound membrane (3838 element format with 43 mL spacers and 5.7m^2 area; Parker Hannifin Corp.) at a transmembrane pressure of 276 kPa. Once 400 lbs. of permeate is removed (2x concentration), diafiltration water (140% of the feed volume) was added at the same flow rate of permeation. Each lot of MPC retentate was divided into two equal parts.

The first half was spray dried using a pilot scale spray dryer (Niro Dryer, Cophenhagen, Denmark, serial # 7712) operating at an inlet and outlet temperatures of 170ºC and 90ºC. The feed flow rate was maintained at 150 mL/min and the feed temperature was maintained between 54ºC and 60ºC using an inline preheater. On the other hand, the high-water activity MPC 85 was manufactured at a feed flow rate of 234 mL/min at an inlet air temperature of 160ºC to obtain a MPC 85 at approximately 0.5 water activity.

After drying, the control and high-water activity MPC 85 was packed in a plastic bag (Associated Bag Co.) and shipped to Kansas State University for HPNB manufacturing and further analysis. At Kansas State University, the MPC 85 was resealed in vacuum bags (FoodSaver, model # FSFSBFLB216NP, Oklahoma City, Oklahoma) until manufacture of HPNBs.

Manufacture of High Protein Nutrition Bars

A similar process was used to produce HPNBs as described in Chapter 4 (page 22). The ingredient sources were given in Table 4.1. High protein nutrition bars were formulated to contain 30 g protein per 100 g (Banach et al., 2014).

Using a stand mixer (model # KSM75SL, Kitchen Aid, St. Joseph, MI) the control MPC 85, glycerol, maltitol syrup, and water were added to a bowl and mixed for 30 minutes on speed 2 with a wire whip attachment. The sides of the bowl were scraped followed by 2-minute mixing on speed 4. Stirring was halted every 30 seconds to scrape the bowl. Palm kernel oil and HFCS were heated together until fat liquefaction and cooled to 60ºC. After cooling, the palm kernel oil and HFCS were added to the bowl and stirred for 2 minutes on speed 4, pausing every 30 seconds to scrape the sides of the bowl. This process was repeated with high water activity MPC 85.

After mixing, HPNB dough was uniformly packed into a rectangular steel mold (1in x 2 in x 10 in). Using a wire cutter, the dough in the mold was leveled. The dough was then removed from the mold and sealed with plastic wrap overnight at room temperature. After resting overnight, the dough was removed from the plastic wrap and cut with a wire cutter into 1 x 1 x 1" cubes. Samples were placed into 4 x 6 x 2" clear stand-up barrier pouches (S-19171, Uline, Pleasant Prairie, WI), labeled appropriately, and stored in 25 ºC or 36 ºC incubators.

Evaluation of model high-protein nutrition bars

The changes during storage of control and high-water activity HPNBs were analyzed in terms of water activity, hardness, color, and insoluble protein using the methods described in Chapter 4. HPNBs were analyzed after being stored at 25 ºC or 36 ºC for 3, 6, 13, 22, and 28 days.

Results and Discussion

Three lots of control and high-water activity MPC 85 were produced at South Dakota State University. The average total solids of the control and high-water activity was 95% and 87.5%, respectively. A randomized complete block design with MPC 85 treatment (control and

high-water activity and temperature (25°C and 36°C) were evaluated as independent factors. Before manufacture of HPNBs, the average final water activity of control and high-water activity MPC 85 was 0.18±0.06 and 0.53±0.01, respectively. HPNBs were produced using the control and high-water activity MPC 85 and were stored at 25°C and 36°C for 28 days. Evaluation of chemical and physical properties of HPNBs during storage were on day 3, 6, 13, 22, and 28.

Changes in water activity of HPNBs during storage

Minimal to no microbial growth can occur in HPNBs when the water activity is under 0.6 (Loveday et al., 2009). All HPNBs had a water activity under 0.56 during this study, indicating very limited growth. From day 3 to day 28 of storage, both control and high-water activity HPNBs stored at 25°C had no significant change ($P > 0.05$) in water activity (Figure 5.1). On each testing day there was a significant difference ($P < 0.05$) between the control and high-water activity HPNBs. The high-water activity adjusted HPNBs stored at 25°C had increased water activity throughout the duration of storage compared to control HPNBs. Minimizing the differences in water activity between HPNB ingredients is a method to mitigate the rate hardening occurs during storage (Hogan et al., 2012). From day 3 to 28 of storage, the water activity of HPNBs stored at 36°C significantly decreased ($P < 0.05$). On each testing day there was a significant difference ($P < 0.05$) between the control and high-water activity HPNBs, respectively (Figure 5.1). In this study, increased water activity in HPNBs occurred because of water activity modification of MPC 85 during the spray drying process. At increased temperatures (36°C), the amount of available water able to act as a plasticizer decreases. Therefore, the sugar in the HPNB was more soluble, decreasing the water activity.

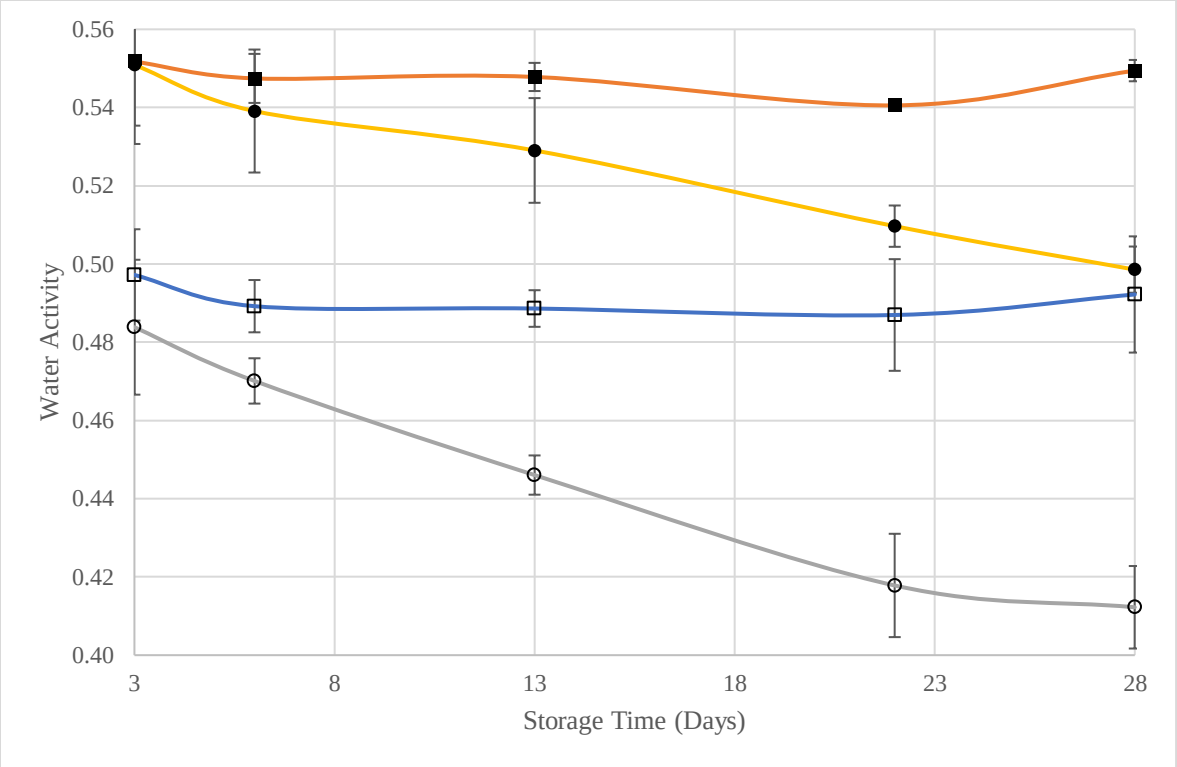

Figure 5.1 Changes in water activity of high protein nutrtion bars with treated and untreated milk protein concentrate at 25°C and 36°C storage temperatures. Data are mean values of 3 measurements on three samples; with standard deviation indicated by vertical error bars. ■ 25°C high water activity; ● 36°C high water activity; □25°C control; ○ 36°C control.

In Chapter 4, the control HPNBs at 25°C on day 1 and day 31 had a water activity of 0.46 and 0.51, respectively. However, in Chapter 5 the water activity of control HPNBs stored at 25°C maintained a water activity of 0.49 during storage for 28 days. This slight difference could've resulted during manufacture of MPC or HPNBs. The lots of MPC 85 used in the adsorption method came from a different manufacturer. It is also likely that when the HPNBs sat overnight there was a fluctuation in the room temperature. HPNBs produced using the adsorption method were produced in early spring whereas HPNBs produced using the desorption method were manufactured during the summer. The humidity and weather conditions inside the laboratory were not constant and impacted the rate at which water activity occurred.

Matching the water activity of MPC to match the other ingredients is hypothesized to reduce moisture migration and protein aggregation during storage, resulting in softer bars. HPNBs stored at 25°C had more free water available compared to HPNBs stored at 36°C. When less free water is available in the system, HPNBs can become unstable and a quicker rate of quality deterioration can occur (Banach et al., 2014). During storage the interaction between free water and the protein surface is weak (Malecki et al., 2022). As storage temperature increases, the vapor pressure will also increase as well as the rate of moisture migration. As moisture migrates between ingredients during storage, the HPNBs get harder. (Hogan et al., 2012). Using high-water activity MPC 85 in manufacture of HPNBs increased the overall water activity during storage at 36°C. Therefore, spray drying a high-water activity MPC 85 did positively influence the rate of moisture migration and hardness of HPNBs during storage at 36°C.

Changes in hardness during storage

A typical force deformation curve during uniaxial compression of HPNBs can be visualized in Figure 5.2. The hardness or peak force is identified as the maximum force required to compress an HPNB 60%. Fracture during compression of HPNBs is reprented by a break or decrease in the force deformation curve. When the HPNB is compressed 60% and fracture is complete the force falls to zero. Sharp increases in storage followed by a small decrease then further increase was observed by Hogal et al. (2012). In this study, the peak force of HPNBs at day 28 was greater than day 3, indicating hardness during storage.

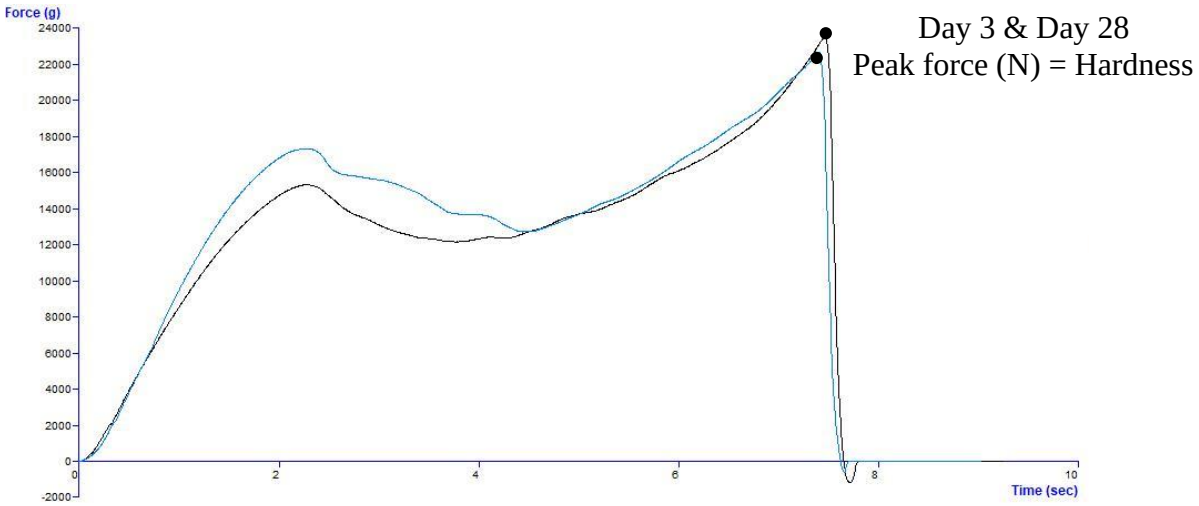

Figure 5.2 A typical force deformation curve obtained during compresion of high protein nutritional bars.

After 28 days of storage at 25°C, the hardness of control HPNBs did not significantly increase ($P > 0.05$). An increase of 7.8% was reported. High-water activity HPNBs did have a significant increase ($P < 0.05$) in hardness during 28 days of storage. An increase of 45.5% was reported. The rate of hardening at 36°C is not the same as 25°C because higher temperatures increse the fluidity of the HPNB matrix and speed the rate of moisture migration (Banach et al., 2014). At 36°C, the change in hardness of control HPNBs during storage for 28 days was 43.5%. HPNBs produced with the high water activity MPC 85 maintained a lower hardness overall compared to the control HPNBs. However, the change in hardness during storage was 86.1%. The changes during storage can be viewed in Figure 5.3. At 36°C, the hardness in control and high water activity HPNBs signficantly increased ($P < 0.05$) between day 3 and day 28 of storage. The signficant increase ($P < 0.05$) of hardness in control HPNBs did not occur until day 28 of storage. However, for high water activity HPNBs a signficant increance ($P < 0.05$) of hardness occurred on day 22. Overall, the high water activity HPNBs became harder at a faster rate compared to control HPNBs. Even with the increased rate of hardness, high water activity

HPNBs had a softer texture throughout the duration of storage. It can be concluded that incorporating a high water activity MPC 85 in the manufacture of HPNBs can reduce hardening during storage.

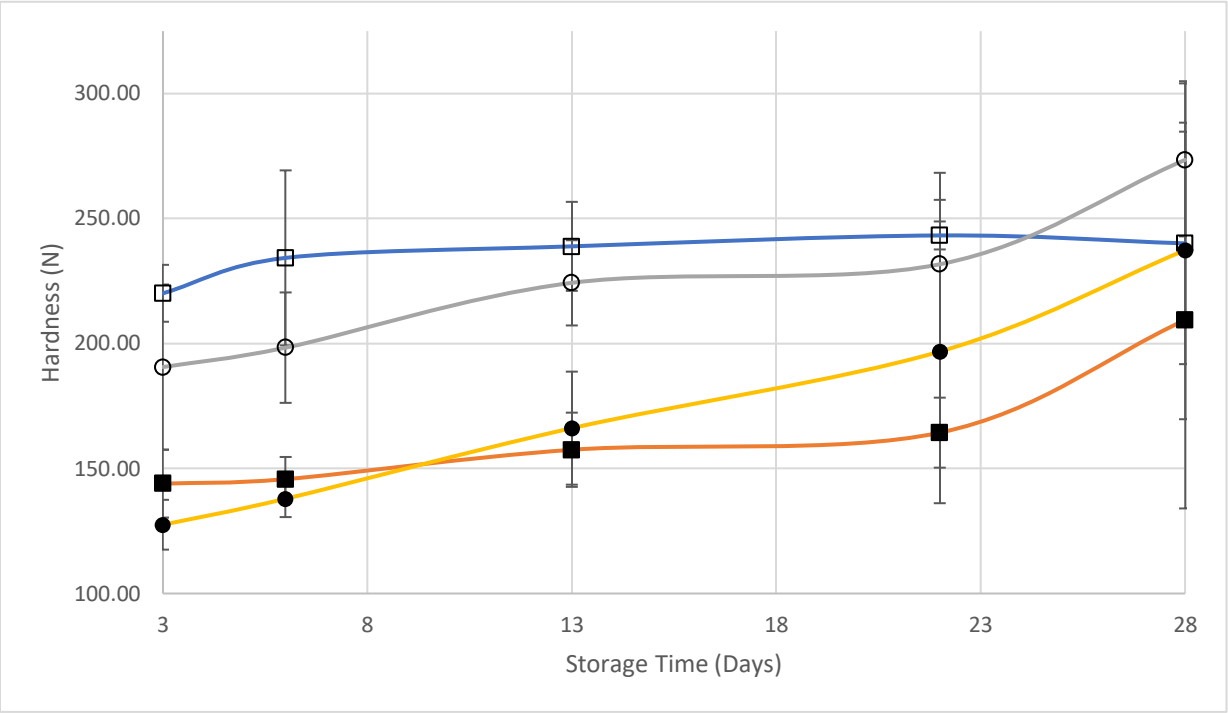

Figure 5.3 Changes in hardness of high protein nutrtion bars with treated and untreated milk protein concentrate at 25°C and 36°C storage temperatures. Data are mean values of 3 measurements on three samples; with standard deviation indicated by vertical error bars. ■ 25°C high water activity; ● 36°C high water activity; □ 25°C control; ○ 36°C control.

HPNBs are heat sealed in vacuum bags to prevent moisture loss and environmental interaction that could contribute to an increased hardness. Harder texture of HPNBs often results as moisture migrates from proteins to sugar (Jiang, 2021). Spray drying MPC 85 with a higher water activity created a moisture barrier between ingredient particles which resulted in decreased moisture migration (Meng et al., 2019). During storage, the MPC 85 in HPNBs competes for available water. The chemical and osmotic potential of water between ingredients signficantly impacts hardening (Purwanti et al., 2010). Since the water activity of MPC 85 was similar to the other ingredients, there was less competition for available water and softer HPNBs occurred as a

result. Lower storage temperature also played a signficant role in the decrease of hardness in HPNBs during storage. Overall, high-water activity MPC 85 did result in softer HPNBs during storage compared to control HPNBs, suggesting that matching the water activities of ingredients reduces moisture migration and the rate at which hardening occurs.

Color Evaluation

During storage at 25°C and 36°C, the mean L*, a*, b* and delta E values of control and high-water activity HPNBs were used to evaluate changes in color (Table 5.1 and 5.2). At 25°C, there was a significant decrease in L* values ($P < 0.05$) of control and high-water activity HPNBs from day 3 to day 28. There was also a significant difference ($P < 0.05$) between L* values of control and high-water activity HPNBs from day 3 to day 28 of storage at 25°C. At 25°C, there was no significant increase ($P > 0.05$) in a* of control HPNBs from day 3 to day 28. There was a significant increase ($P < 0.05$) in a* values for control HPNBs during storage for 28 days at 25°C. The increase in a* is an indicator of a Maillard reaction occurring, causing HPNBs to turn a red-brown or caramel color during storage (McMahon et al., 2009). This color trend can be seen in Figure 5.3. At 25°C, there was a significant increase in b* values of control and high-water activity HPNBs during 28 days of storage. Overall, at 25°C there was no significant variation between the L*, a*, and b* values of control and high-water activity HPNBs. Indicating that the high-water activity MPC 85 did not mitigate the rate of Maillard browning and other color changes during storage. It was reported by Zhou et al. (2013) that no significant increase in browning occurred during the first 2 weeks of storage at room temperature. Before HPNBs changed color, a short induction period without color formation occurred. The duration of the

induction period was dependent on water activity and temperature (Loveday et al., 2009). The higher the temperature, the shorter the induction period.

Table 5.1 Changes in color of high protein nutrition bars with control and high-water activity milk protein concentrate during storage at 25°C.

	25°C					
	Control			High water activity		
	L*	a*	b*	L*	a*	b*
Day 3	91.33±0.28^{a}	-0.19±0.07^{b}	9.59±0.66^{e}	89.67±0.54^{cd}	0.04±0.37^{b}	12.23±2.55^{cde}
Day 6	91.79±0.79^{a}	-0.16±0.06^{b}	10.41±0.92^{de}	89.67±1.13^{cd}	0.11±0.33^{b}	13.15±2.34^{bcd}
Day 13	90.86±0.81^{ab}	-0.19±0.08^{b}	11.80±0.62^{cde}	89.22±0.17^{cd}	0.16±0.46^{b}	14.52±1.94^{abc}
Day 22	90.97±0.80^{ab}	0.04±0.15^{b}	13.09±0.79^{bcd}	88.84±0.33^{de}	0.43±0.50^{ab}	15.35±1.42^{ab}
Day 28	89.99±0.41^{bc}	0.29±0.19^{ab}	13.78±0.61^{abc}	87.96±0.45^{e}	0.74±0.45^{a}	16.53±1.34^{a}

Values were compared within column and with same superscript are not significantly different (P >0.05)
All values are expressed as mean ± SD.

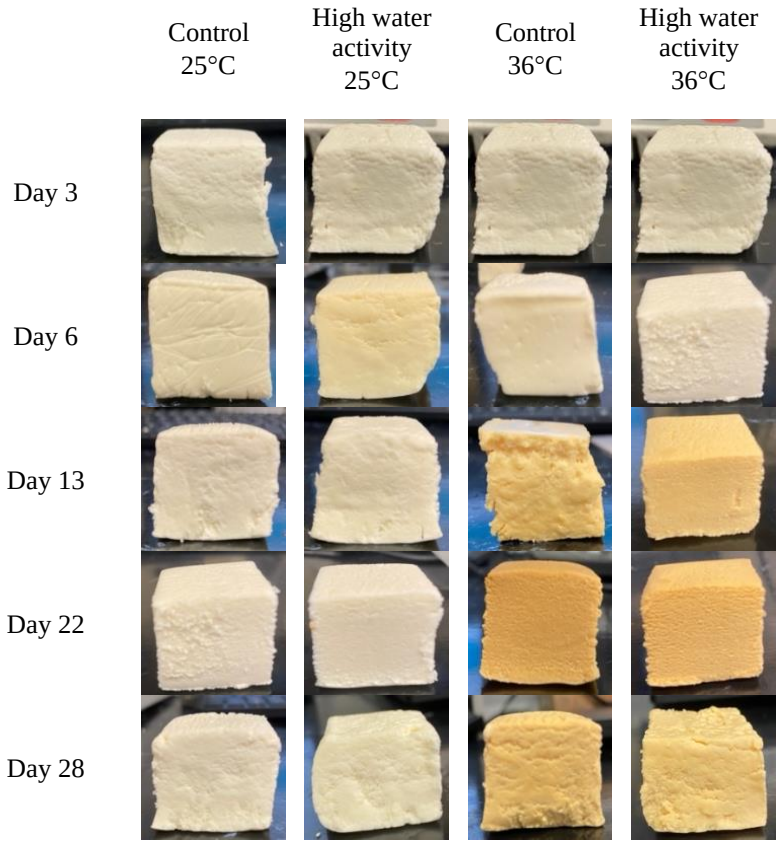

Figure 5.4 Model high protein nutrition bars (HPNB) color after Day 3, 6, 13, 22, and 28 of storage at 25°C and 36°C. Control identifies a model high protein bar made with unmodified milk protein concentrate (MPC) 85. High water activity identifies a model high protein bar made with modified MPC 85.

At 36°C, the L* values of control HPNBs significantly decreased ($P < 0.05$) from day 3 to day 13 and continued through day 28. This trend can be seen in Figure 5.4. For high water activity HPNBs the L* value significantly decreased ($P < 0.05$) from day 3 to day 22 and continued through day 28. Control and high-water activity HPNBs had a significant increase in a* values from day 3 to 13 of storage at 36°C. This trend continues through day 28. The b* values of control HPNBs significantly increased ($P < 0.05$) on day 6 and continued through day 28. A similar trend was observed for high water activity HPNBs stored at 36°C except a significant increase in b* values was not observed until day 13.

A decrease in L* values and increase of a* and b* values indicate a Maillard reaction resulting in a darker HPNBs with a red-brown color. It was reported that color change is an indication of quality decline (Banach et al., 2014). Development of color occurred at a similar rate for control and high-water activity HPNBs. As significant color change occurs in HPNBs, shelf-life decreases. It was hypothesized by Banach et al. (2014) that increased browning is a result of amines participating in a Maillard reaction. Table 5.2 reports a significant color change amongst both control and high-water activity HPNBs during storage, indicating that a Maillard reaction occurred.

Table 5.2 Changes in color of high protein nutrition bars with control and high-water activity milk protein concentrate during storage at 36°C.

	36°C					
	Control			High water activity		
	L*	a*	b*	L*	a*	b*
Day 3	90.03±0.73^{a}	0.05±0.27^{f}	13.02±0.67^{f}	88.99±0.55^{a}	0.13±0.53^{ef}	13.98±1.65^{ef}
Day 6	88.25±2.40^{a}	2.61±1.39^{def}	18.72±2.26^{cde}	87.95±0.93^{a}	0.82±0.32^{def}	16.48±1.31^{def}
Day 13	81.74±2.63^{bc}	3.85±3.65^{c}	21.46±8.09^{cd}	85.77±0.93^{ab}	3.09±0.42^{dc}	21.78±1.25^{c}
Day 22	73.93±3.95^{d}	9.39±0.98^{a}	32.58±0.78^{ab}	79.77±1.85^{c}	6.59±1.34^{b}	28.19±2.68^{b}
Day 28	71.78±3.05^{d}	10.15±1.06^{a}	33.89±1.15^{a}	75.57±5.23^{d}	7.78±1.72^{ab}	30.01±2.12^{ab}

Values were compared within column and with same superscript are not significantly different (P >0.05)
All values are expressed as mean ± SD. N=3.

Total color change represented by the delta E value, indicated a significant increase ($P < 0.05$) of control HPNBs on day 3 and day 13 of storage at 25°C. An identical trend was seen for the high-water activity HPNBs stored at 25°C. This suggests there is little to no effect of high-water activity MPC 85 on HPNBs at 25°C. The delta E value significantly increased ($P < 0.05$) by day 6 and day 13 for control and high-water activity HPNBs, respectively. At 36°C, the high-water activity MPC 85 does influence the rate of color change in HPNBs.

Determination of Insoluble Protein

The formation of insoluble proteins in HPNBs during storage can be seen in Figure 5.5. Zhou et al. (2008) reported that an increase of insoluble protein during storage is an indicator of increased hardness. The texture of whey proteins significantly increased on the third day of storage when 25% of proteins became insoluble at 45°C (Zhou et al., 2008). It was reported that the amount of insoluble protein needed to cause increased hardening at 23°C or 34°C is 12% and 15%, respectively (Zhou et al., 2008). Control HPNBs stored at 25°C had a 17.71% increase of insoluble protein during storage. High water activity HPNBs stored at 25°C had a 19.65% decrease. Control HPNBs stored at 36°C had a 18.85% increase of insoluble protein compared to high-water activity HPNBs with a 7.25% decrease. Zhou et al. (2022) reported no change of insoluble protein occurred for the first 21 days of storage, followed by a significant increase through 35 days of storage. Figure 5.5 depicts a similar trend where there little change of insoluble protein occurred for 28 days. A slight increase of insoluble proteins in control HPNBs and decrease in high water activity HPNBs can be seen in Figure 5.5. An increase of insoluble protein in control HPNBs stored at 25°C and 36°C indicated that the formation of insoluble protein did impact the rate at which bar hardening occurs during storage. High water activity HPNBs stored at 25°C and 36°C had lower concentrations of insoluble protein compared to

control HPNBs. This suggests that high water activity MPC 85 did reduce the rate of insoluble protein formation during storage. It was predicted by Banach et al. (2016) that past 35 days of storage, a significant amount of insoluble protein would form. Therefore, it is likely that as storage time increased in this study the number of insoluble proteins in high water activity HPNBs would form. Further studies are needed to confirm this theory.

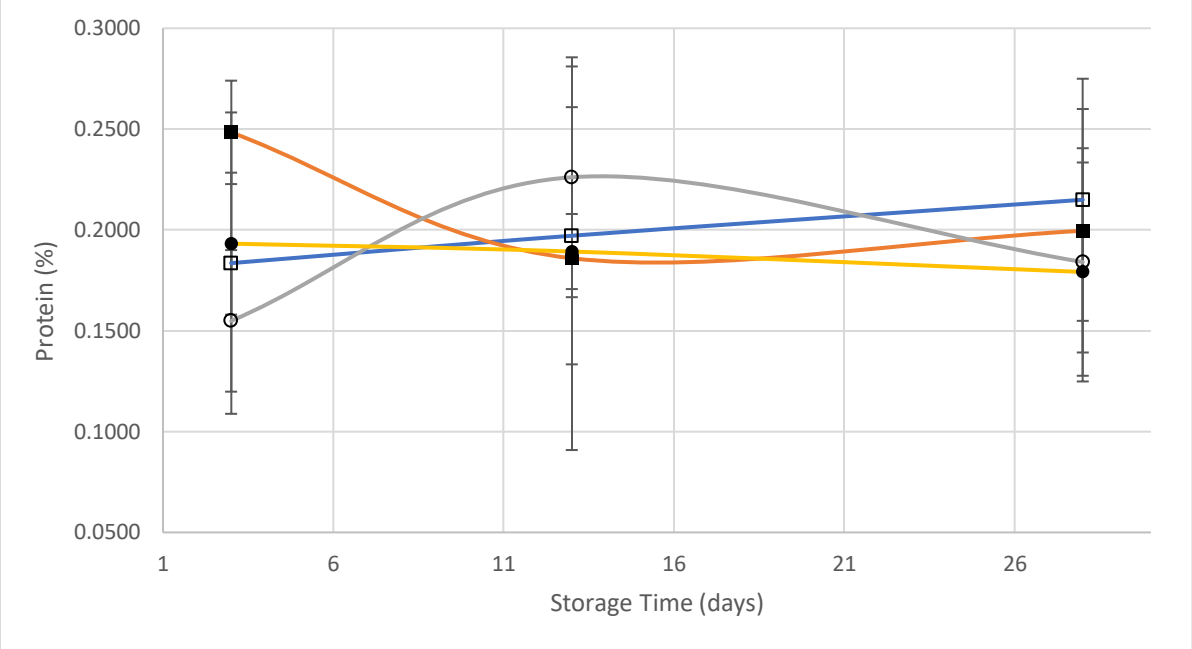

Figure 5.5 Formunation of insoluble protein in high protein nutrition bars with control and high water activity milk protein concentrate at 25°C and 36°C storage temperatures. Data are mean values of 7 measurements (N=3), with standard deviation indicated by vertical error bars. ■ 25°C high water activity; ● 36°C high water activity; □ 25°C control; ○36°C control.

Sodium Dodecyl Sulfate Polyacrylamide Get Electrophoresis (SDS-PAGE)

Protein profiles (SDS-PAGE) for high protein nutrition bars produced with control and high water activity MPC 85 were analyzed under reducing conditions on the first (day 3), middle (day 13), and last day of storage (day 28). The reduced protein patterns of control and high water activity HPNBs appeared identical on day 3 of storage (Figure 5.6). On day 13, the major protein bands for control and water activity adjusted HPNBs stored at 25°C became slightly less defined.

There was no increase in molecular weight. By day 28, the protein bands of HPNBs stored at 25°C were darker compared with day 3 and 13, with appearance of minor whey proteins up the column.

Under non-reducing conditions, control and high water activity HPNBs at 25°C on day 3 had similar protein bands (Figure 5.7). At day 13, little change had progressed. By day 31, the major protein bands had become smaller and moved up the lane. With low molecular weight and no protein aggregates the HPNBs exist in a rubbery state, prone to Maillard reactions resulting in textural hardness during storage (Banach et al., 2016 and Zhou et al., 2008). It is not possible to predict the performance of high water activity MPC 85 based on only the SDS-PAGE results (Banach et al., 2016). However, the gels show no signficant differences between control or high water activity HPNBs, implying that the water activity adjustment of MPC 85 during spray drying did not impact the rate at which hardness occurs during storage.

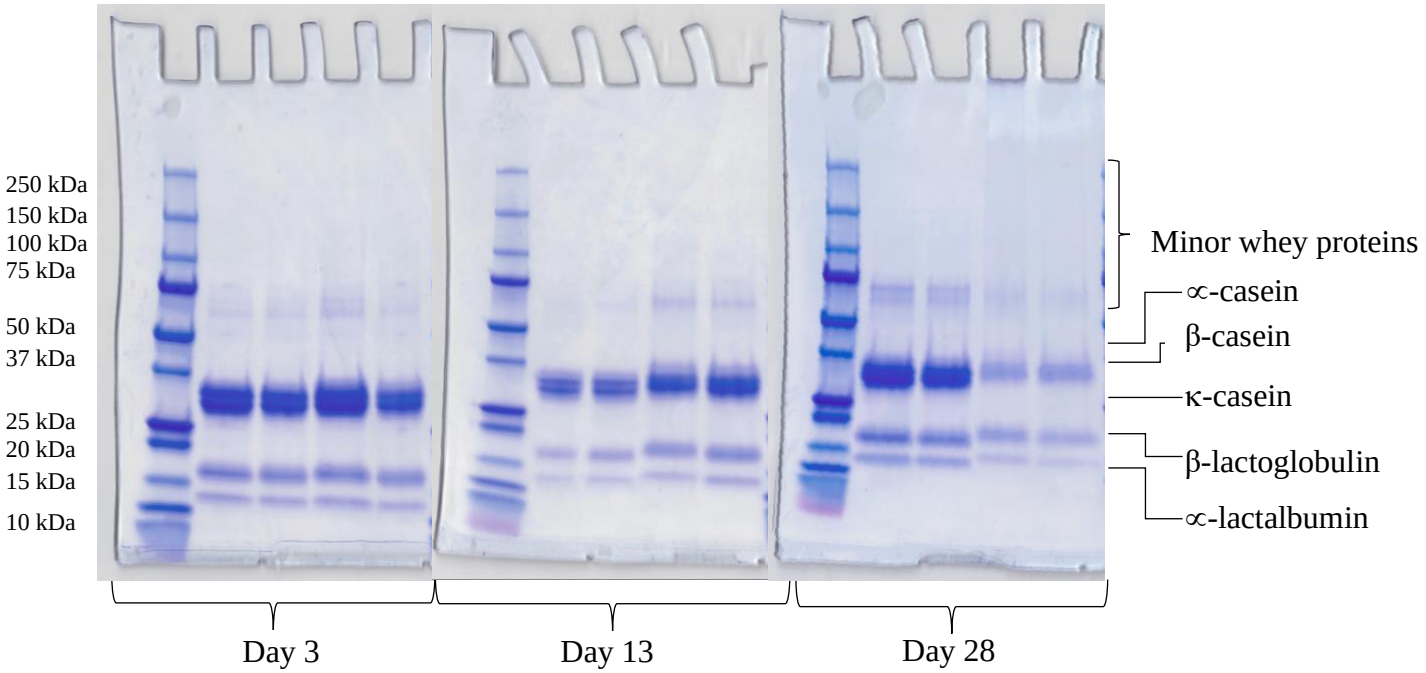

Figure 5.6 Reduced SDS-PAGE gel for high protein nutrition bars produced using control or high water activity HPNBs stored at 25°C and 36°C. Lane 1 = molecular weight ladder; lane 2= 25°C control; lane 3= 25°C high water activity; lane 4= 36°C control; lane 5= 36°C high water activity.

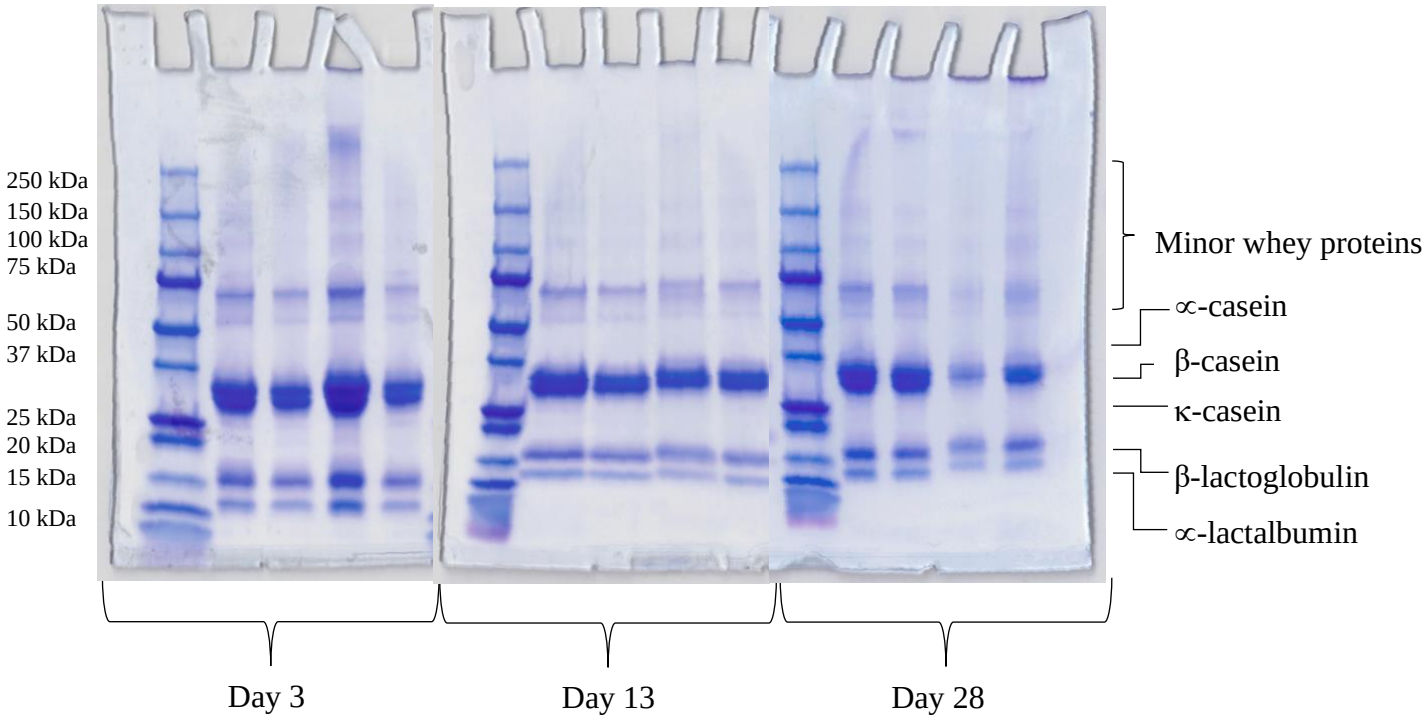

Figure 5.7 Non-Reduced SDS-PAGE gel for high protein nutrition bars produced using control or high water activity HPNBs stored at 25°C and 36°C. Lane 1 = molecular weight ladder; lane 2= 25°C control; lane 3= 25°C high water activity; lane 4= 36°C control; lane 5= 36°C high water activity.

On day 3, the reduced protein patterns of control HPNBs stored at 36°C appeared slightly darker with more minor whey proteins compared to the high water activity HPNBs (Figure 5.6). On day 13, the control and high water activity HPNB band appeared identical. The bands became more diffuse and the molecular weight increased. On day 28 of storage, the band for control HPNB stored at 36°C reached the top of the gel. This observation confirmed that the molecular weight of the proteins was changed by MPC treatment. Under non-reducing conditions, control HPNBs at 36°C on day 3 had a protein band reaching the top of the gel (Figure 5.7). The high water activity HPNBs had a band with increased molecular weight but not to the extent of the control band. At day 13, little change had progressed. By day 28, the major protein bands had become significantly smaller and moved up the lane. Both the control and high water activity HPNB bands reached the top of the gel. The gels show a signficant difference between control or high water activity HPNBs, implying that the water activity adjustment of MPC 85 during spray drying did impact the rate at which hardness occurs during storage at 36°C.

Anema et al. (2006) used reduced and non-reduced SDS-PAGE to determine the effects of storage temperature on the solubility of MPC 85 and noted Maillard reactions can lead to the appearance of high molecular mass polymers in proteins. With increasing temperature and storage time, the molecular mass of protein increased (Li et al., 2011). Meng et al. (2019) used reduced SDS-PAGE to illustrate the formation of Maillard-induced aggregates with and without anticaking agents. In their study it was noted that the bands of β-lactoglobulin and ∝-lactalbumin diffused and moved upwards during storage. With the addition of anticaking agents, the rate of diffusion and Maillard-induced protein aggregation decreased. However, the modification of whey proteins is relatively slow and could take months to develop extensive protein aggregation

(Meng et al., 2019). Therefore, it can be concluded that very minimal to no protein aggregation occurred during storage under any conditions. HPNBs stored at 36°C did have higher mass polymers compared to HPNBs stored at 25°C, indicating a Maillard reaction. Overall, HPNBs manufactured with high water activity MPC 85 did reduce the rate at which high molecular bands formed at 36°C.

Confocal Laser Scanning Microscopy

Confocal micrographs of the HPNBs were taken after the bars were thawed for approximately 30 minutes (Figure 5.8 and 5.9). These images depict how lipids formed large bulbous strctures with smooth surfaces and in some cases contained spherical vacuoles. These vacuoles contain a mixture of air, water, glucose, or glycerol (Loveday et al., 2009). Overall, the protein particles were unchanged in shape and arrangement from day 3 to 28 of storage at 25°C.

MPC contains protein in the form of caseins and whey proteins with a ratio of approximately 80:20, respectively (Loveday et al., 2009). It is likely that the protein seen after 1 day of storage consisted of mostly whey proteins because whey proteins are more soluble than caseins. MPC powders are most soluble after intial manufacture. Loveday et al. (2009 and 2010) reported a decrease in protein solubility and increased particle clustering during storage as the HPNB batter turned into a firm dough matrix the day after manufacture. Solubility decreases as the storage time and temperature increase (Babu and Amamcharla 2018). The surface of the proteins are hydrated, but during storage a driving force pulles the moisture inwards to associate with the hydroxyl groups of molecules. This moisture migration from the surface inwards can result in a phase separation. A phase separation is a determinant of the HPNBs texture, stability, appearance, and taste due to the presence of multiple polymers in the matrix causing thermodynamic instability (McMahon et al., 2009 and Anema & De Kruif, 2016). In HPNBs,

proteins are the only polymers present because there are no other macromolecular polysaccharides included in the matrix (McMahon et al., 2009). Therefore, fructose or maltitol is able to undergo a phase transition to form a glass state during storage (Tolstoguzov, 2003). A phase separation is mostly observed when conditions very humid or hot because of sugar crystallization. However, there were no crystals detected in the HPNBs, indicating that a change in water activity or hardening during storage was not a result of crystallization. The hardening of HPNBs during storage was driven by the difference in osmotic pressure, resulting in the separation of proteins from water (Loveday et al., 2009).

Storage of control and high water activity HPNBs at 25°C for 28 days resulted in a fusion of protein particles. The average size of air voids remained unchanged during storage. The addition of air voids from day 3 to day 28 in control HPNBs stored at 36°C can be seen in Figure 5.9. This indicates a phase separation occurred through the direction of water migration. Similarly to Meng et al. (2019), the direction was from the aqueous solution into the proteins. Water migration is also expected to hydrate proteins which causes swelling and crosslinking. There is less fusion of protein particles in water activity adjusted HPNBs stored at 36°C, indicating that adjusting water activity of MPC 85 during the spray drying did impact the migration of small molecules, particularly water and moltose.

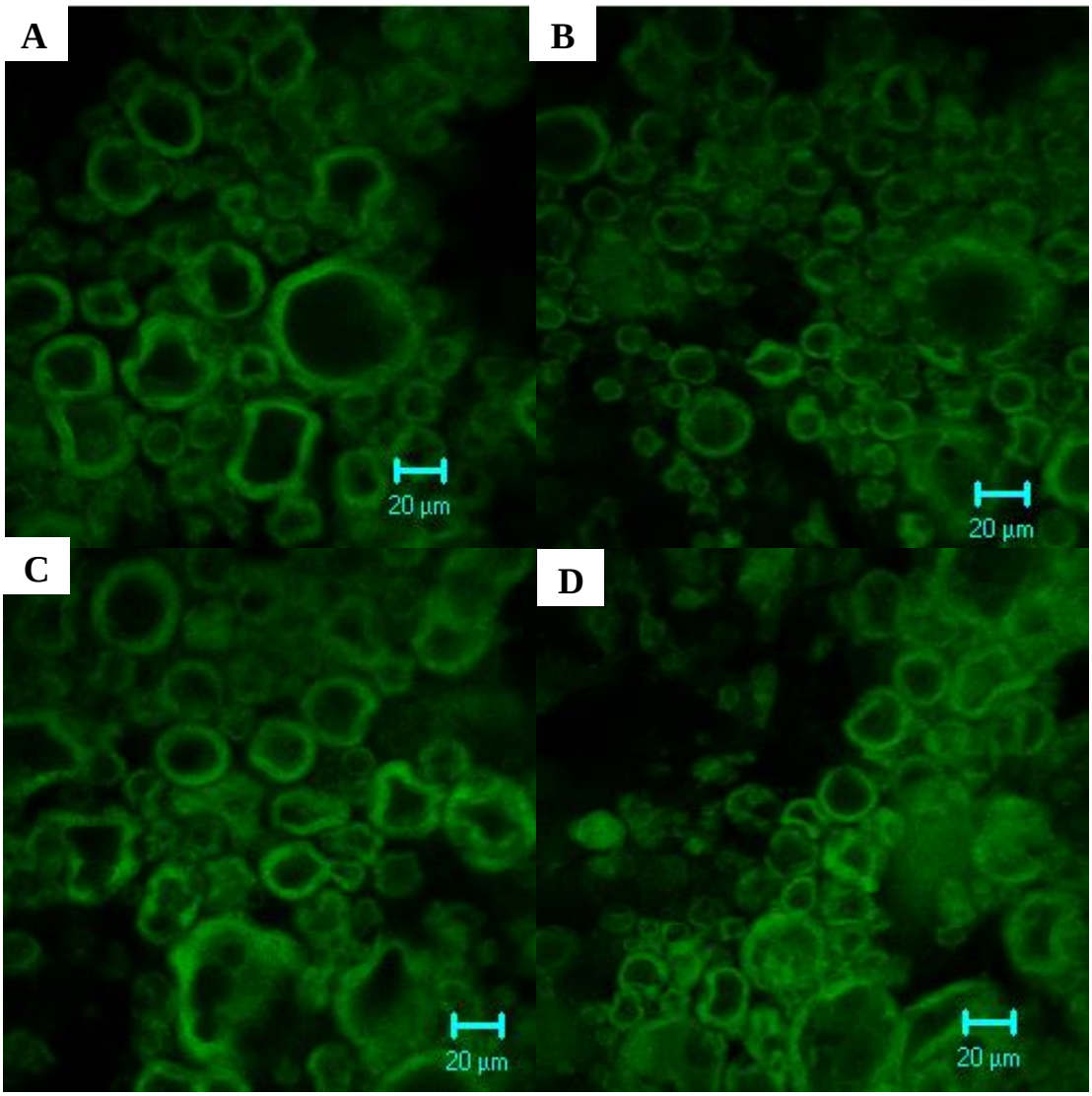

Figure 5.8 Confocal laser scanning microscopy images of control day 3, (A), high water activity (B), day 28 control (C), and day 28 high water activity (D) high protein nutrition bars produced using the desorption method stored at 25°C. Green indicates Fast Green FCF staining (protein).

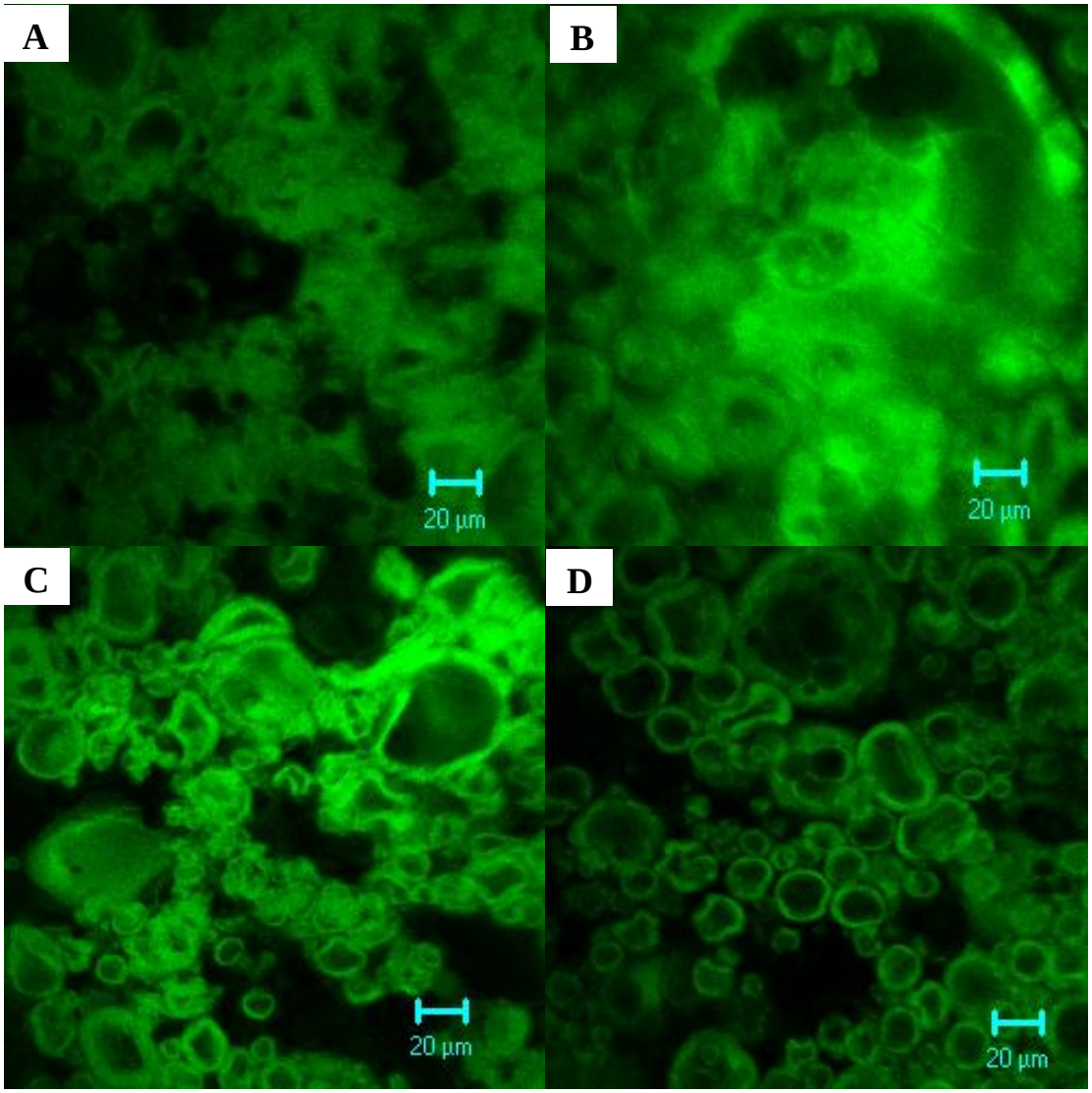

Figure 5.9 Confocal laser scanning microscopy images of control day 3 (A), high water activity (B), day 28 control (C), and day 28 high water activity (D) high protein nutrition bars produced using the desorption method stored at 36°C. Green indicates Fast Green FCF staining (protein).

Front-Face Fluorescence Spectroscopy

Changes of HPNBs during storage for the tryptophan emission spectra were observed in Table 5.3. The average tryptophan maximum peak for both control and high water activity HPNBs was 339 nm. Only slight differences were viewed in tryptophan fluorescence between the control and high water activity HPNBs stored at 25°C from day 3 to day 28 of storage. The average maximum tryptophan peak for control and high water activity HPNBs stored at 36°C

was 341 nm and 340 nm, respectively. On day 3 and 28 of storage there were no signficant differences between the peaks of control or high water activity HPNBs at 25°C. However, this was not the case for HPNBs stored at 36°C. On day 13, the absorbance of HPNBs stored at 36°C signficantly decreased through day 28 (Table 5.3). Similar to the findings in chapter 4, as the HPNBs getting harder during storage and the water activity decreases, a decrease tryptophan emission intensity is observed. Tryptophan emission is can vary substantially based on environmental factors such as temperature (Lakowicz 2006). As the peaks shift during storage, moisture migration is ongoing and creating changes within the proteins. These spectral shifts could've result from protein-protein association or protein unfolding (Lakowicz, 2006). From day 1 to day 31 of HPNB storage at 36°C, a signficant decrease in the maximum absorbance occurs. The decrease signifies a chemical and physical change is occuring amogst the dairy proteins. In chapter 4, the control HPNBs stored at 36°C had lower absorbance units during storage compared to the water activity adjusted HPNBs. Here the higher water activity HPNBs produced using the desorption method had a higher absorbance compared to the control HPNBs. A higher absorbance indicates that the higher water activity MPC 85 did aid in the mitigation of hardness in HPNBs stored at 36°C during storage.

Table 5.3 Tryptophan emissions of high protein nutrtion bars produced with control and water activity adjusted milk protein concentrate using the desorption method.

Storage Time	25°C				36°C			
	Control		High water activity		Control		High water activity	
	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)
3	69.82	337	57.53	339	57.61	339.5	54.24	338.5
6	83.51	339	68.38	338.5	49.00	339.5	52.70	339.5
13	59.90	338	48.07	339.5	20.17	341.5	31.81	340
22	70.87	339.5	49.76	338.5	10.35	343.5	20.80	342.5
28	63.53	339.5	54.53	339	7.19	341	15.00	341

WL = Wavelength

Control and high-water activity HPNBs stored at 25°C and high-water activity HPNBs stored at 36°C had slightly increased Maillard emission after 28 days of storage (Table 5.4). The maximum absorbance of control HPNBs had little to no change during storage at 36°C. Control and high-water activity HPNBs stored at 25°C had very similar maximum absorbance values. The absorbance values of high-water activity HPNBs stored at 36°C remained larger during storage compared to the control HPNBs. The same results were reported using the adsorption method. The slight increase in absorbance for high water activity HPNBs at 36°C indicates that a higher water activity of MPC 85 did reduce the rate at which browning occurs during storage.

Table 5.4 Maillard emissions of high protein nutrtion bars produced with control and water activity adjusted milk protein concentrate using the desorption method.

Storage Time	25°C				36°C			
	Control		High water activity		Control		High water activity	
	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)	Max Peak	WL (nm)
3	7.38	435.5	8.73	437	8.9	434.5	9.67	436
6	8.98	435.5	10.17	436	11.26	435	12.07	437
13	7.92	435	10.29	438.5	8.71	435	10.17	435.5
22	12.18	436	12.22	437	8.38	433	10.44	435
28	11.74	435.5	12.48	435	8.26	436.5	10.05	433.5

WL = Wavelength

Fluorescence spectroscopy is a sensitive technique used to measure trace substances in HPNBs containing fluorescent molecules with conjugated double bonds (Hassoun & Karoui, 2015). Tryptophan spectra vary largely according to storage time and temperature. As the storage time increases, the spectra decreased in all HPNBs. This result was also reported in chapter 4. For the Maillard emissions, Babu and Amamcharla (2018) reported an increase in absorbance when storage time and temperature increased. This was not seen in control HPNBs stored at 36°C. Therefore, the high-water activity MPC 85 used in the manufacture of HPNBs did slightly mitigate the rate of browning during storage at 36°C.

Fourier Transform Infrared (FTIR)

FTIR spectra of HPNBs on day 3 and day 28 of storage at 25°C and 36°C are shown in Figure 5.12 and 5.13, respectively. Control and high-water activity HPNBs had almost identical spectra at 25°C. Meng et al. (2019) reported the main absorbance peaks were found at 3200-3300 cm^{-1} (amide A, the stretching of O-H groups and N-H groups), 2850-2990 cm^{-1} (amide B, the stretching of C-H groups), 1740 cm^{-1} (the stretching of C=O in ester bonds), 1600 cm^{-1} (amide I, the stretching of C-N and C=O in CONH groups), and 1530 cm^{-1} (amide II, the stretching of C-N groups and bending of N-H in CONH groups), and 1020 cm^{-1} (the stretching of C-O and C-N groups). As storage time increased, the absorbance of HPNBs decreased.

The wavenumber range 1700-1600 cm^{-1} corresponds to the Amide I region or the backbone conformation of proteins (Hogan et al., 2012). The peaks at 1624 cm^{-1} (parallel β-sheet) and 1744 cm^{-1} (antiparallel β-sheet) in the HPNBs stored at 25°C on day 3 were shifted to 1634 and 1729 cm^{-1} in HPNBs on day 28, respectively. The peaks at 1624 cm^{-1} (parallel β-sheet) and 1742 cm^{-1} (antiparallel β-sheet) in the HPNBs stored at 36°C on day 1 were shifted to 1643 and 1736 cm^{-1} in HPNBs on day 31, respectively. β-sheet structures are commonly found in areas with aggregated proteins (Nishanthi et al., 2017). Therefore, increased β-sheet structures are correlated to the hardening of HPNBs during storage, especially at higher temperatures. The higher the temperature, the more Maillard reactions occur as well (Meng et al., 2019). At 25°C, the β-sheet structures of control and high water activity HPNBs was almost identical, indicating that the rate at which Maillard browning occurs was not impacted by a difference in water activity of MPC 85. Similar results were seen in chapter 4. At 36°C, the spectra of control HPNBs on day 28 was slightly higher than the high-water activity HPNBs. At higher wavenumbers, there is weaker crosslinking of hydrogen bonds, negatively influencing the rate of

hardness (Ramos et al., 2013). At 36°C the high-water activity HPNBs had a lower wavenumber, indicating that hydrogen bonds were stronger and could mitigate the rate at which moisture migration and Maillard browning occurs.

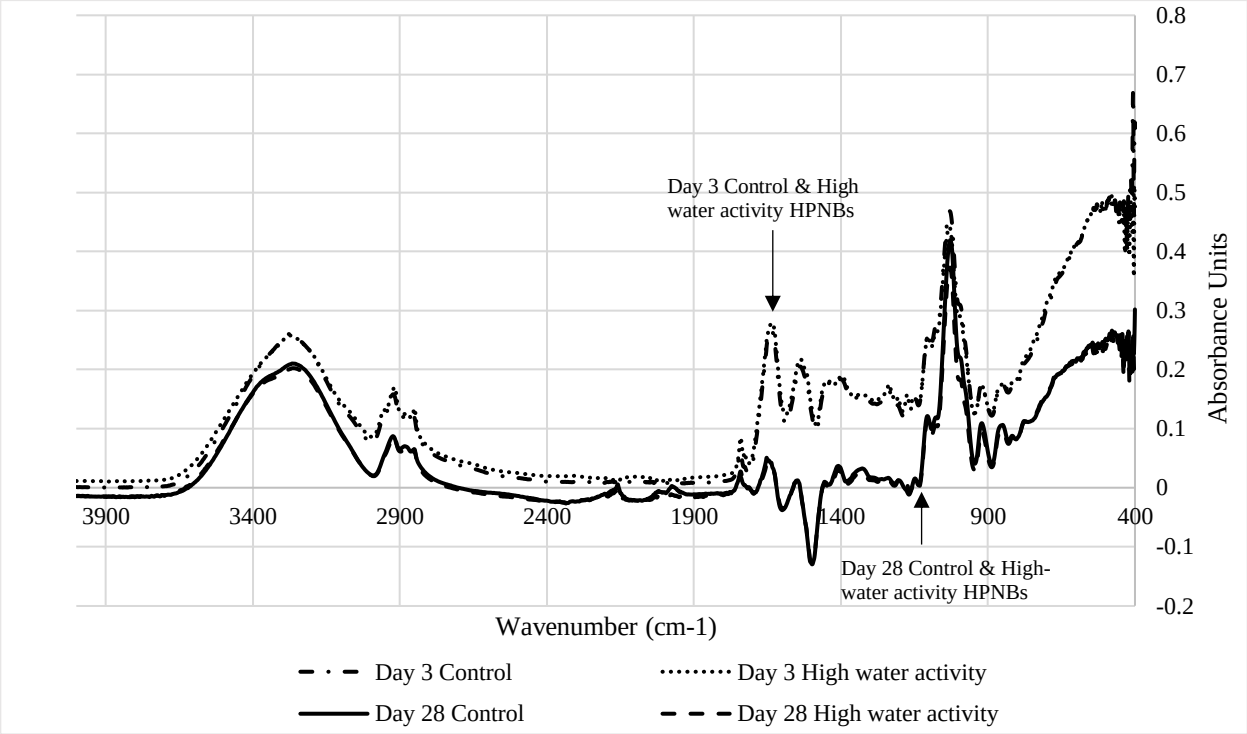

Figure 5.10 FTIR spectra of high protein nutrtion bars produced with untreated and treated milk protein concentrate in the 400-4000 cm^{-1} region stored at 25°C.

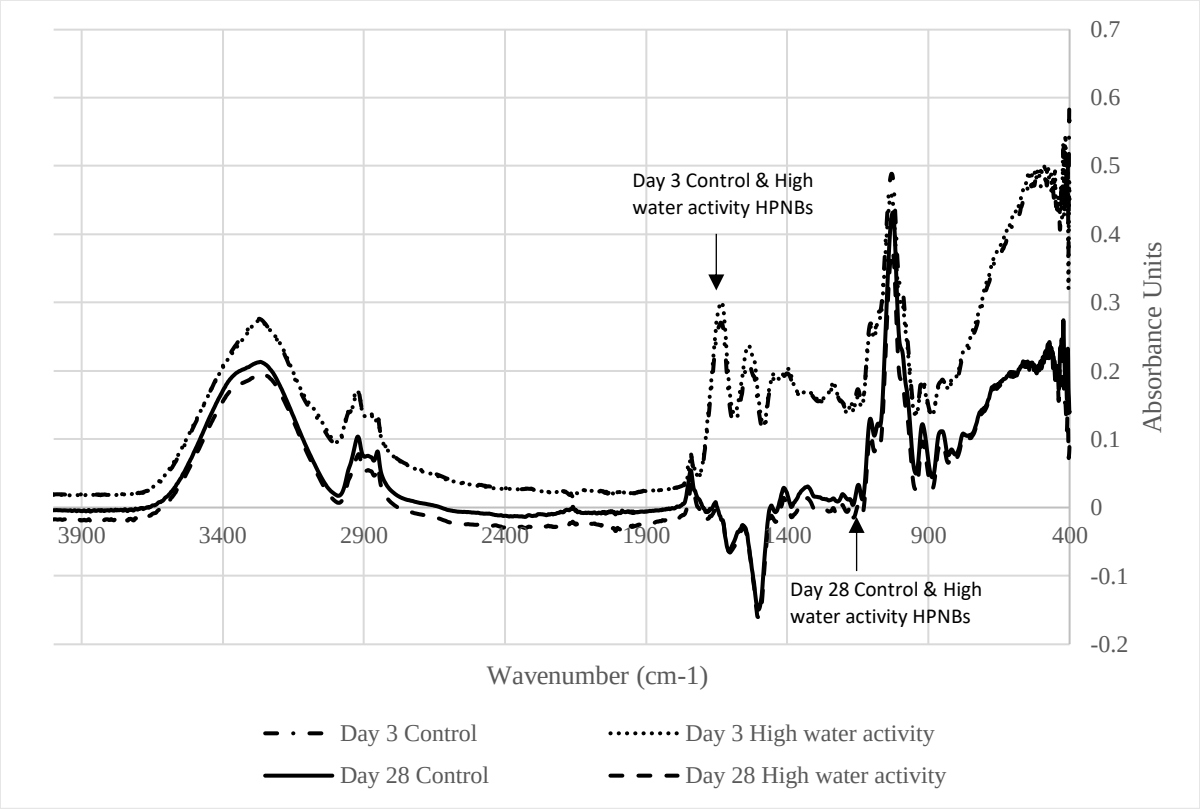

Figure 5.11 FTIR spectra of high protein nutrtion bars produced with untreated and treated milk protein concentrate in the 400-4000 cm^{-1} region stored at 36°C.

Differences in amide I region can be observed in Figure 5.10 and 5.11. During storage the absorbance of HPNBs decreases. At 25°C, the amide I bands for control and high-water activity HPNBs was almost identical. The high-water activity MPC 85 did not affect the rate of hardness or Maillard browning during storage. At 36°C, the control and high-water activity HPNBs appeared slightly different on both day 3 and day 28 of storage. On day 3, the high-water activity band had increased absorbance. However, on day 28 of storage the control HPNBs had slightly more absorbance from 1700-1650 cm^{-1}. From 1650-1600 cm^{-1} the amide I band appeared almost identical again. It was expected that the higher water activity band would have a higher absorbance on day 28 of storage because the HPNBs had a softer texture and lighter color. It can be concluded that the higher water activity MPC 85 did influence the rate at which Maillard

browning and increased hardening occurs. However, it did not affect the rate as much as expected.

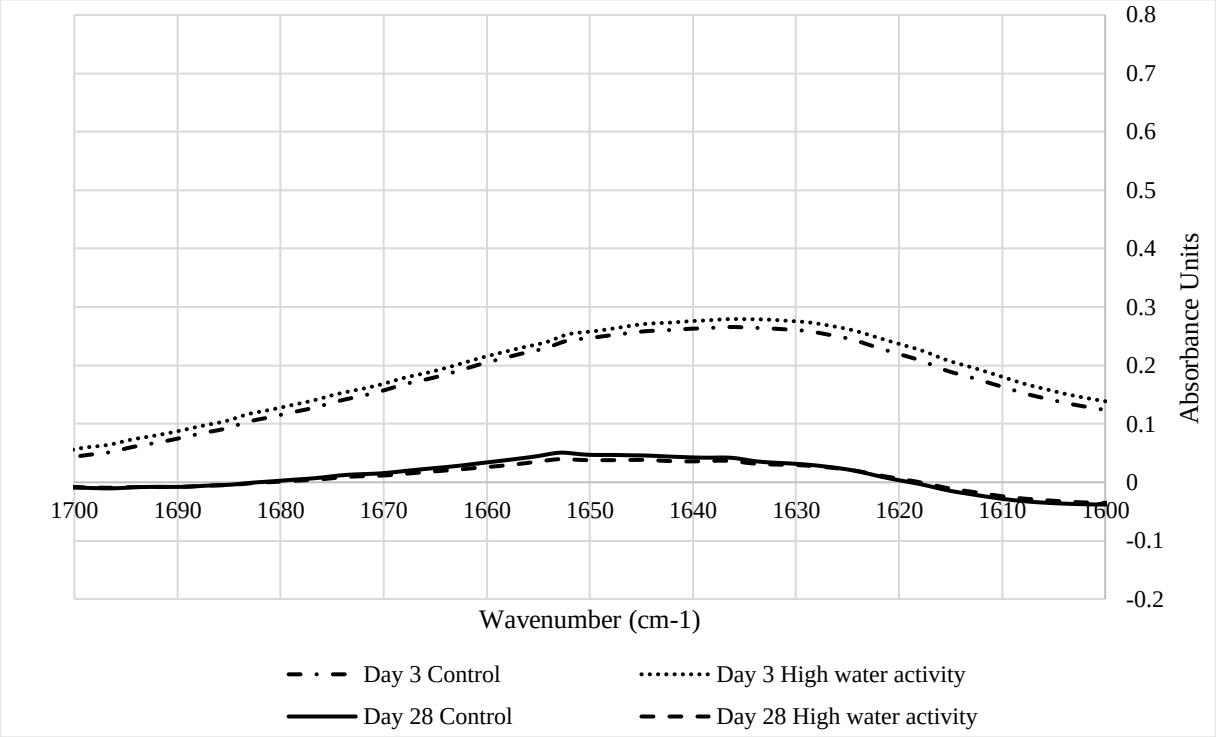

Figure 5.12 FTIR spectra showing amide I band of high protein nutrtion bars produced with untreated and treated milk protein concentrate and stored at 25°C.

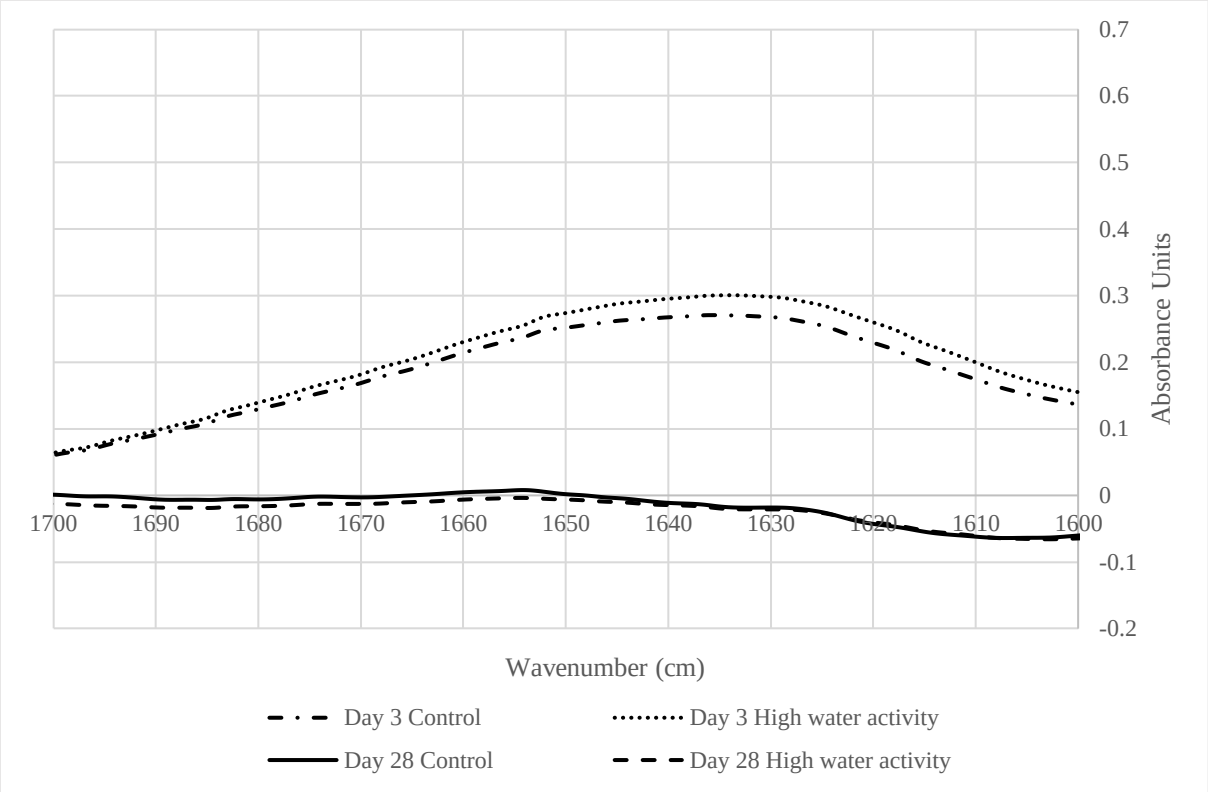

Figure 5.13 FTIR spectra showing amide I band of high protein nutrtion bars produced with untreated and treated milk protein concentrate and stored at 36°C.

Differential Scanning Calorimeter (DSC)

The peak temperature at the endothermic peak indicates the denaturation temperature of protein contained in HPNBs (Figure 5.14 and 5.15). Similar to the adsorption method, on the first and last day of analysis there wre two endothermic peaks for the control and high moisture HPNBs. Zhou et al. (2008) reported the first endothermic peaks represents the denaturation of bovine serum albumin (BSA) and ∝-lactalbumin. The second endothermic peak represents the denaturation of β-lactoglobulin (Zhou et al., 2008). After storage at 25°C for 28 days, the heat flow and temperature required for denaturation were reduced. However, there was no significant difference between the control and high-water activity HPNBs.

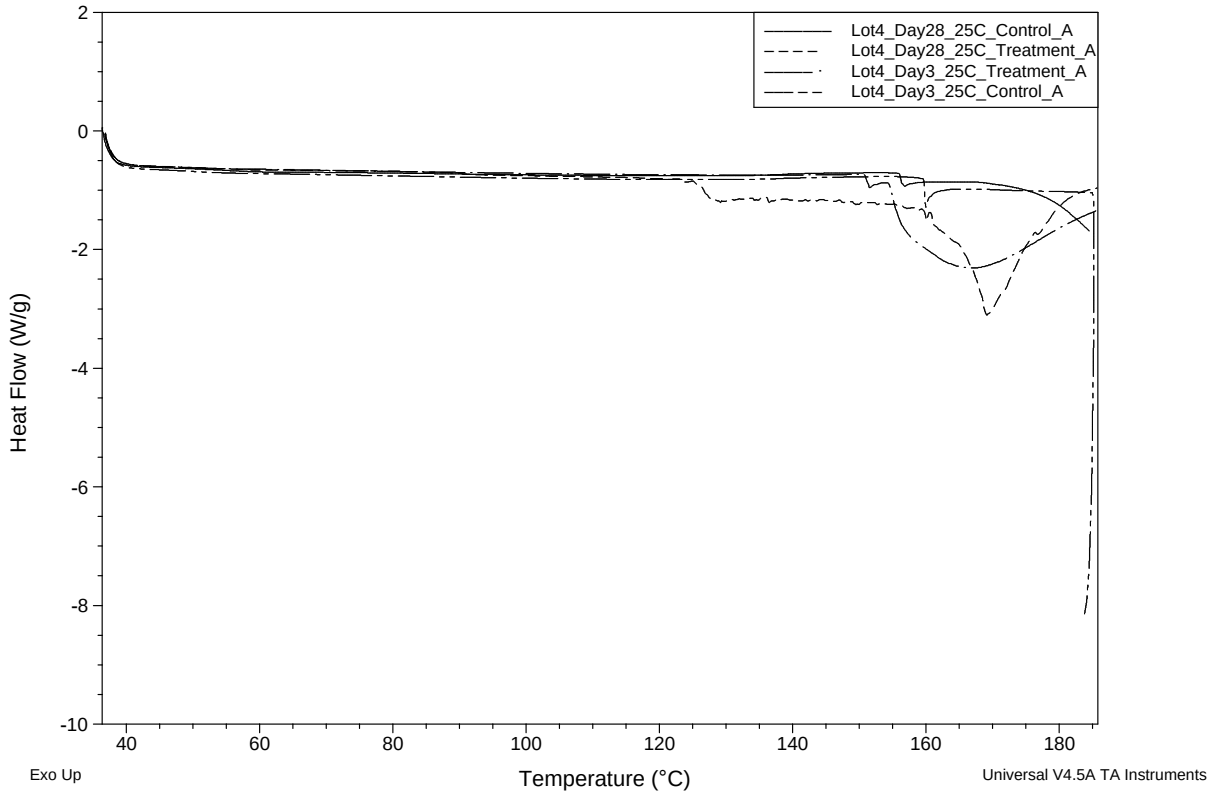

Figure 5.14 Differential Scanning Calorimeter scans of high protein nutrition bars stored for 28 days at 25°C.

On day 3 of storage at 36°C, the denaturation temperature of control HPNBs required slightly less heat for the protein to denature. On day 28 of storage, the control HPNBs had a significantly higher temperature required for protein denaturation. Zhou et al. (2008) reported that at a higher temperature, the mobility or flexibility of protein peptides increases resulting in a lower temperature required for protein denaturation. On day 28 of storage, the HPNBs stored at 36°C required slightly less heat to denature. The control HPNBs on day 28 required a higher temperature to denature than the high-water activity HPNBs. This indicates that the high-water activity of MPC 85 did impact protein denaturation at 36°C.

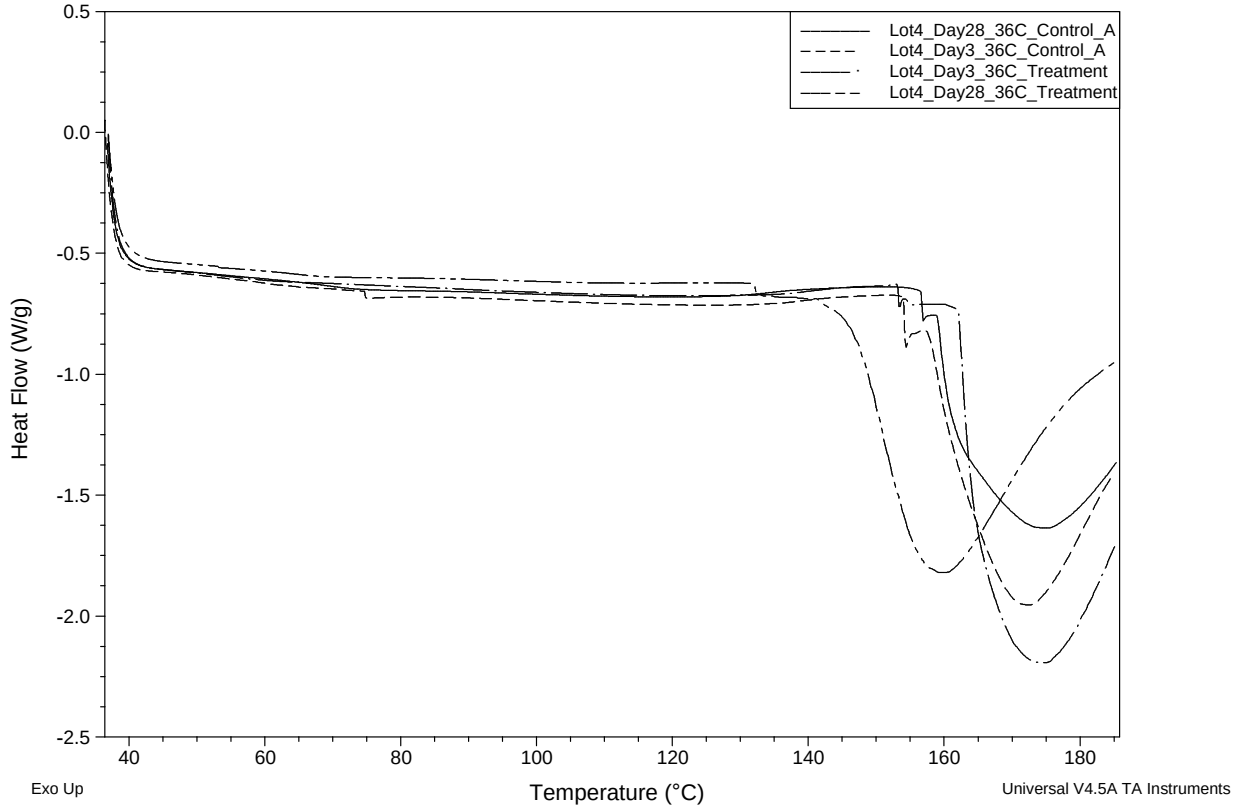

Figure 5.15 Differential Scanning Calorimeter scans of high protein nutrition bars stored for 28 days at 36°C.

Conclusion

Control and high-water activity HPNBs stored at 25°C had increased textural, color, and water activity characteristics compared to HPNBs stored at 36°C. However, at 25°C there was no significant difference between the control and high-water activity HPNBs. At 36°C, the high-water activity MPC 85 did impact the physical and chemical properties of HPNB hardening. While adjusting the water activity of MPC 85 during spray drying can improve the physical and chemical properties of HPNBs during storage, temperature played a more critical role. More research on different techniques to adjust the water activity of MPC must be conducted to better control HPNB hardening during storage.

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Appendix A - SAS Code for the analysis

The color, water activity, and hardness of HPNBs stored at 25°C manufactured using the using adsorption method. The repeated measures SAS code is below.

```
Data repeatedmeasures;
    Input Trt$ rep$ temp$ day$ l a b de Aw Hard;
Datalines;
Control 1 25 1 91.05 -0.02 13.76 0 0.4419 9210.72
Control 1 25 2 91.43 -0.05 14.07 0.8287 0.4738 2217.62
Control 1 25 4 91.51 -0.09 13.38 0.5743 0.5045 10015.22
Control 1 25 6 90.32 -0.01 14.23 1.0017 0.4943 9689.45
Control 1 25 13 90.55 0.31 14.84 1.2314 0.5115 9676.20
Control 1 25 22 90.31 0.58 16.04 2.7448 0.4981 11434.97
Control 1 25 31 89.26 1.1 17.89 5.0480 0.4987 12393.42
Treatment 1 25 1 86.75 -0.44 15.2 0 0.4663 13450.46
Treatment 1 25 2 91.09 -0.05 13.91 4.5483 0.5106 1057.09
Treatment 1 25 4 88.79 0.84 16.83 2.9100 0.5263 14826.02
Treatment 1 25 6 85.13 -0.13 22.33 7.3200 0.5256 14718.31
Treatment 1 25 13 89.18 0.29 14.51 2.6356 0.5354 14398.55
Treatment 1 25 22 89.32 0.82 15.88 2.9432 0.5296 17252.04
Treatment 1 25 31 89.48 1.25 17.7 4.0700 0.5244 15888.38
Control 2 25 1 91.05 -0.02 13.76 0 0.4639 17077.53
Control 2 25 2 91.43 -0.05 14.07 0.4877 0.4681 7998.18
Control 2 25 4 91.51 -0.09 13.38 0.5989 0.4536 10921.01
Control 2 25 6 90.32 -0.01 14.23 0.8697 0.4963 10381.76
Control 2 25 13 90.55 0.31 14.84 1.2325 0.4412 11356.12
Control 2 25 22 90.31 0.58 16.04 2.4732 0.4481 10558.85
Control 2 25 31 89.26 1.1 17.89 4.6350 0.5138 10916.20
Treatment 2 25 1 89.67 -0.09 13.81 0 0.4762 8649.37
Treatment 2 25 2 89.1 -0.14 13.39 0.3399 0.5006 8277.51
Treatment 2 25 4 89.58 -0.06 13.76 0.8580 0.5045 10998.67
Treatment 2 25 6 90.02 0.26 14.52 0.8601 0.5263 8703.15
Treatment 2 25 13 87.39 0.53 16.88 1.1944 0.4843 12822.75
Treatment 2 25 22 89.7 0.95 16.38 3.1577 0.4993 8342.55
Treatment 2 25 31 88.03 1.42 18.79 4.1520 0.5395 10732.42
Control 3 25 1 89.9 -0.09 14 0 0.4834 11041.83
Control 3 25 2 89.39 -0.15 13.45 0.7525 0.4914 12125.14
Control 3 25 4 90.09 0.08 13.56 0.5121 0.5072 16517.69
Control 3 25 6 90.97 0.11 13.65 1.1459 0.4875 18136.41
Control 3 25 13 88.33 0.49 16.26 2.8164 0.5146 19412.09
Control 3 25 22 89.5 0.88 15.91 2.1779 0.5040 24450.77
Control 3 25 31 88.25 1.72 19.16 5.7122 0.5210 24249.73
Treatment 3 25 1 89.67 -0.09 13.81 0 0.4910 7419.55
Treatment 3 25 2 89.1 -0.14 13.39 0.7901 0.5221 8248.51
```

```
Treatment 3 25 4 89.58 -0.06 13.76 0.1047 0.5200 9580.54
Treatment 3 25 6 90.02 0.26 14.52 0.8692 0.5237 9882.64
Treatment 3 25 13 87.39 0.53 16.88 3.8742 0.5437 12576.06
Treatment 3 25 22 89.7 0.95 16.38 2.7788 0.5420 13584.32
Treatment 3 25 31 88.03 1.42 18.79 5.4583 0.5495 14222.40
;
Run;

proc print;run;
proc mixed data= repeatedmeasures cl;
class Trt rep day;
model L =Trt day Trt*day /ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model a=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model b=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
```

```
class Trt rep day;
model de=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Aw=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Hard=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;
```

The color, water activity, and hardness of HPNBs stored at 36°C manufactured using the using adsorption method. The repeated measures SAS code is below.

```
Data repeatedmeasures;
    Input Trt$ rep$ temp$ day$ l a b de Aw Hard;
Datalines;
Control 1 36 1 89.09 -0.12 13.22 0.00 0.4793 4725.58
Control 1 36 2 90.62 -0.06 13.43 1.5500 0.4780 7363.42
Control 1 36 4 89.60 -0.10 13.18 0.5114 0.4481 8030.27
Control 1 36 6 90.17 -0.13 13.31 1.0858 0.4777 10655.82
Control 1 36 13 78.60 6.87 28.54 19.8416 0.4233 16218.27
```

```
Control 1 36 22 74.34 0.52 34.78 26.1274 0.4068 25860.59
Control 1 36 31 71.72 10.98 38.53 32.6418 0.4201 28715.62
Treatment 1 36 1 88.62 -0.28 14.43 0.00 0.5104 8752.65
Treatment 1 36 2 91.39 0.06 14.06 2.8217 0.5155 5757.44
Treatment 1 36 4 89.24 0.30 15.36 1.2542 0.4990 7166.23
Treatment 1 36 6 89.88 0.60 15.56 1.9063 0.5233 8212.05
Treatment 1 36 13 85.99 3.17 21.61 8.3830 0.4732 11092.97
Treatment 1 36 22 79.05 6.88 30.19 19.7774 0.4688 15927.95
Treatment 1 36 31 75.59 8.91 34.48 25.6109 0.4827 24303.81
Control 2 36 1 92.27 -0.04 13.77 0.00 0.4896 14960.20
Control 2 36 2 90.83 0.32 15.70 2.4368 0.4765 15474.07
Control 2 36 4 89.19 1.37 19.34 6.5212 0.4916 19185.62
Control 2 36 6 86.03 3.11 22.01 10.8025 0.4667 22196.40
Control 2 36 13 90.55 0.31 14.84 2.0585 0.4747 25061.55
Control 2 36 22 77.00 9.21 35.34 27.9977 0.4503 34037.99
Control 2 36 31 66.60 12.61 38.44 37.7801 0.3991 37366.95
Treatment 2 36 1 91.29 -0.03 14.17 0.00 0.5341 6752.64
Treatment 2 36 2 90.67 0.07 15.36 1.3491 0.5273 8313.86
Treatment 2 36 4 90.94 0.41 16.83 2.7171 0.5370 7631.55
Treatment 2 36 6 89.87 0.90 17.29 3.5474 0.5120 9416.31
Treatment 2 36 13 87.20 3.01 21.67 9.0697 0.5120 11644.25
Treatment 2 36 22 80.02 7.12 30.64 21.1998 0.5003 20539.71
Treatment 2 36 31 75.81 9.66 35.74 28.2659 0.4880 26101.43
Control 3 36 1 89.69 -0.28 14.23 0.00 0.5076 15610.87
Control 3 36 2 89.28 0.34 15.37 1.3655 0.4994 19498.61
Control 3 36 4 85.64 2.63 21.41 8.7413 0.4886 18152.44
Control 3 36 6 87.64 2.65 20.23 6.9877 0.4732 18860.44
Control 3 36 13 73.49 10.33 34.67 28.1594 0.4721 21813.37
Control 3 36 22 64.23 13.47 38.08 37.4994 0.4412 35379.12
Control 3 36 31 63.09 14.28 40.89 40.3830 0.4434 33242.21
Treatment 3 36 1 81.31 -0.06 14.25 0.00 0.5299 12188.28
Treatment 3 36 2 88.78 0.17 15.98 7.6726 0.5355 14938.65
Treatment 3 36 4 89.31 0.55 16.61 8.3636 0.5468 14816.94
Treatment 3 36 6 87.89 1.29 18.28 7.8303 0.5083 18536.35
Treatment 3 36 13 81.72 5.66 26.99 13.9771 0.5196 20039.62
Treatment 3 36 22 74.40 9.37 33.43 22.4610 0.4983 26106.84
Treatment 3 36 31 72.85 10.75 36.73 26.3390 0.4913 32732.03
;
Run;

proc print;run;
proc mixed data= repeatedmeasures cl;
class Trt rep day;
model L =Trt day Trt*day /ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
```

```
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model a=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model b=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model de=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Aw=Trt day Trt*day/ddfm=satterth;
```

```
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Hard=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;
```

The color, water activity, and hardness of HPNBs stored at 25°C manufactured using the using desorption method. The repeated measures SAS code is below.

```
Data repeatedmeasures;
    Input Trt$ rep$ temp$ day$ l a b de Aw Hard;
Datalines;
Control 1 25 3 91.39 -0.12 10.01 0.0000 0.4839 21467.35
Control 1 25 6 90.91 -0.12 11.38 1.4482 0.4880 19791.49
Control 1 25 13 90.53 -0.16 12.43 2.5630 0.4922 22347.66
Control 1 25 22 91.85 0.21 13.52 3.5518 0.4866 24268.03
Control 1 25 28 90.43 0.50 14.31 4.4401 0.4994 23002.97
Treatment 1 25 3 89.21 0.44 15.18 0.0000 0.5307 13798.45
Treatment 1 25 6 88.45 0.48 15.84 1.0103 0.5417 14403.02
Treatment 1 25 13 89.09 0.69 16.75 1.5947 0.5437 15275.87
Treatment 1 25 22 88.48 1.00 16.97 2.0134 0.5415 17312.95
Treatment 1 25 28 87.62 1.25 17.81 3.1781 0.5482 15245.02
Control 2 25 3 91.58 -0.25 9.94 0.0000 0.5056 22175.50
Control 2 25 6 92.42 -0.12 10.29 0.9130 0.4832 26217.63
Control 2 25 13 91.78 -0.13 11.79 1.8627 0.4833 25855.81
Control 2 25 22 90.28 -0.04 13.57 3.8686 0.4729 24786.50
Control 2 25 28 89.93 0.19 13.11 3.6076 0.4751 30000.00
Treatment 2 25 3 90.26 -0.02 10.84 0.0000 0.5728 13984.51
Treatment 2 25 6 90.68 0.05 11.91 1.1467 0.5541 14298.40
Treatment 2 25 13 89.41 -0.11 13.21 2.5120 0.5500 15122.38
Treatment 2 25 22 88.89 0.22 14.33 3.7514 0.5403 15150.01
```

```
Treatment 2 25 28 88.47 0.41 15.14 4.6755 0.5475 30000.00
Control 3 25 3 91.03 -0.21 8.83 0.0000 0.5022 23737.26
Control 3 25 6 92.04 -0.22 9.55 1.2416 0.4964 25708.30
Control 3 25 13 90.26 -0.28 11.19 2.4793 0.4903 24923.61
Control 3 25 22 90.79 -0.05 12.17 3.3524 0.5014 25408.96
Control 3 25 28 89.61 0.16 13.92 5.2977 0.5021 20486.52
Treatment 3 25 3 89.53 -0.29 10.67 0.0000 0.5517 16283.78
Treatment 3 25 6 89.89 -0.18 11.68 1.0763 0.5465 15916.42
Treatment 3 25 13 89.16 -0.09 13.60 2.9573 0.5498 17818.86
Treatment 3 25 22 89.14 0.08 14.76 4.1201 0.5399 17849.01
Treatment 3 25 28 87.79 0.57 16.64 6.2741 0.5526 18858.90
;
Run;

proc print;run;
proc mixed data= repeatedmeasures cl;
class Trt rep day;
model L =Trt day Trt*day /ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model a=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model b=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
```

```
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model de=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Aw=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Hard=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;
```

The color, water activity, and hardness of HPNBs stored at 36°C manufactured using the using desorption method. The repeated measures SAS code is below.

```
Data repeatedmeasures;
    Input Trt$ rep$ temp$ day$ l a b de Aw Hard;
```

```
Datalines;
Control 1  36  3  90.28  0.36  13.71  0.0000  0.4639  16331.28
Control 1  36  6  89.23  1.66  17.42  4.0735  0.4685  20120.05
Control 1  36  13  80.49  -0.16  12.43  9.8948  0.4403  21187.76
Control 1  36  22  69.38  10.52  33.49  30.5205  0.4107  26190.42
Control 1  36  28  71.84  10.33  34.51  29.5357  0.4098  24316.67
Treatment 1  36  3  89.34  0.71  15.56  0.0000  0.5339  11845.70
Treatment 1  36  6  88.58  1.17  17.62  2.2447  0.5216  13924.03
Treatment 1  36  13  86.05  2.89  21.95  7.5098  0.5161  15374.17
Treatment 1  36  22  80.91  5.56  25.89  14.1904  0.5060  17844.65
Treatment 1  36  28  79.77  6.39  28.29  16.9120  0.4959  16580.39
Control 2  36  3  90.60  -0.14  12.97  0.0000  0.4941  23026.31
Control 2  36  6  90.00  1.97  17.40  4.9440  0.4653  22552.33
Control 2  36  13  84.77  4.75  23.92  13.3341  0.4486  24663.72
Control 2  36  22  76.50  8.78  32.15  25.4244  0.4097  19356.97
Control 2  36  28  74.81  9.02  32.56  26.7773  0.4238  30000.00
Treatment 2  36  3  89.29  -0.02  12.27  0.0000  0.5644  13477.14
Treatment 2  36  6  88.38  0.55  15.05  2.9745  0.5519  14886.92
Treatment 2  36  13  86.53  2.80  20.45  9.0783  0.5428  15888.99
Treatment 2  36  22  80.78  6.09  27.54  18.5166  0.5073  15319.44
Treatment 2  36  28  77.22  7.23  29.38  22.1515  0.5053  30000.00
Control 3  36  3  89.21  -0.06  12.37  0.0000  0.4935  18987.73
Control 3  36  6  85.51  4.21  21.33  10.5817  0.4765  18049.04
Control 3  36  13  79.98  6.98  28.04  19.4896  0.4493  22807.33
Control 3  36  22  75.90  8.88  32.10  25.4158  0.4331  25384.92
Control 3  36  28  68.70  11.12  34.59  32.2314  0.4031  29402.18
Treatment 3  36  3  88.36  -0.32  14.10  0.0000  0.5544  13712.35
Treatment 3  36  6  86.89  0.75  16.78  3.2356  0.5438  13409.77
Treatment 3  36  13  84.74  3.56  22.93  10.2995  0.5282  19602.38
Treatment 3  36  22  77.64  8.11  31.13  21.8069  0.5157  27082.00
Treatment 3  36  28  69.72  9.70  32.38  27.9600  0.4945  26075.04
;
Run;

proc print;run;
proc mixed data= repeatedmeasures cl;
class Trt rep day;
model L =Trt day Trt*day /ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;
```

```
proc mixed data= repeatedmeasures cl;
class Trt rep day;
model a=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model b=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model de=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Aw=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
```

```
slice Trt*day/sliceby=Trt lines;
run;

proc mixed data= repeatedmeasures cl;
class Trt rep day;
model Hard=Trt day Trt*day/ddfm=satterth;
repeated day/type=cs subject=rep(Trt);
lsmeans Trt day Trt*day/pdiff;
store sasuser.MPC1;
run;
proc PLM restore=sasuser.MPC1;
lsmeans Trt*day/lines;
slice Trt*day/sliceby=Trt lines;
run;
```

Appendix B - Chapter 4

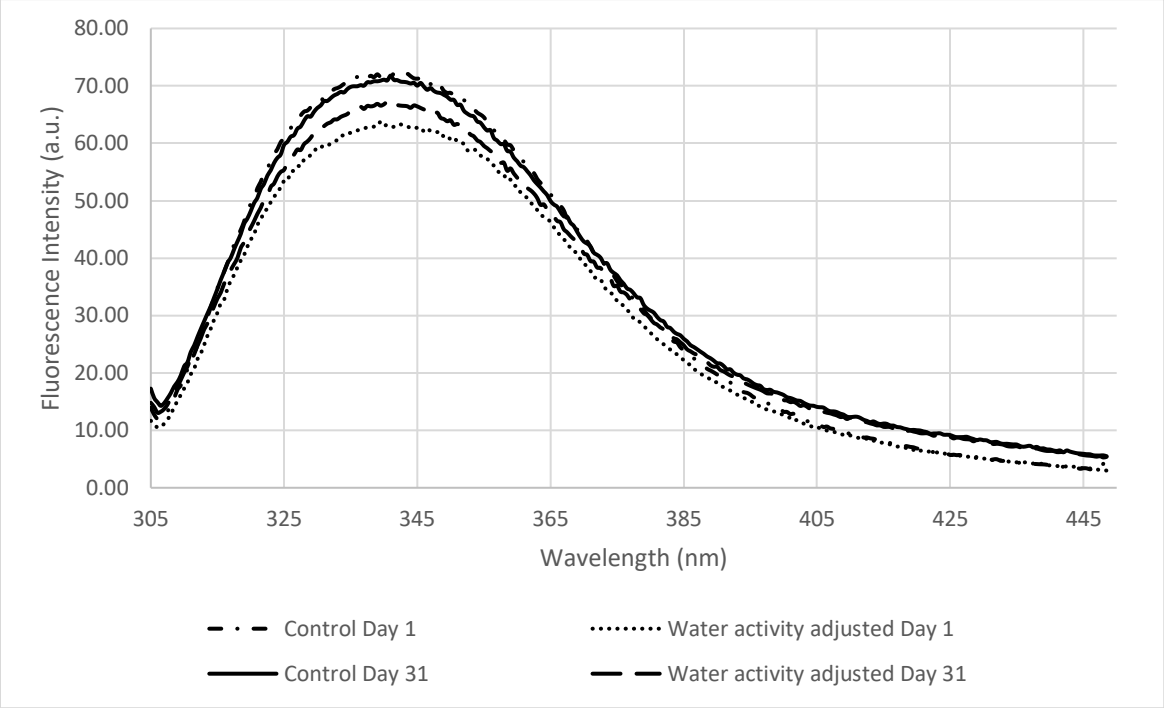

Figure B.1 Averaged tryptophan emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 25°C.

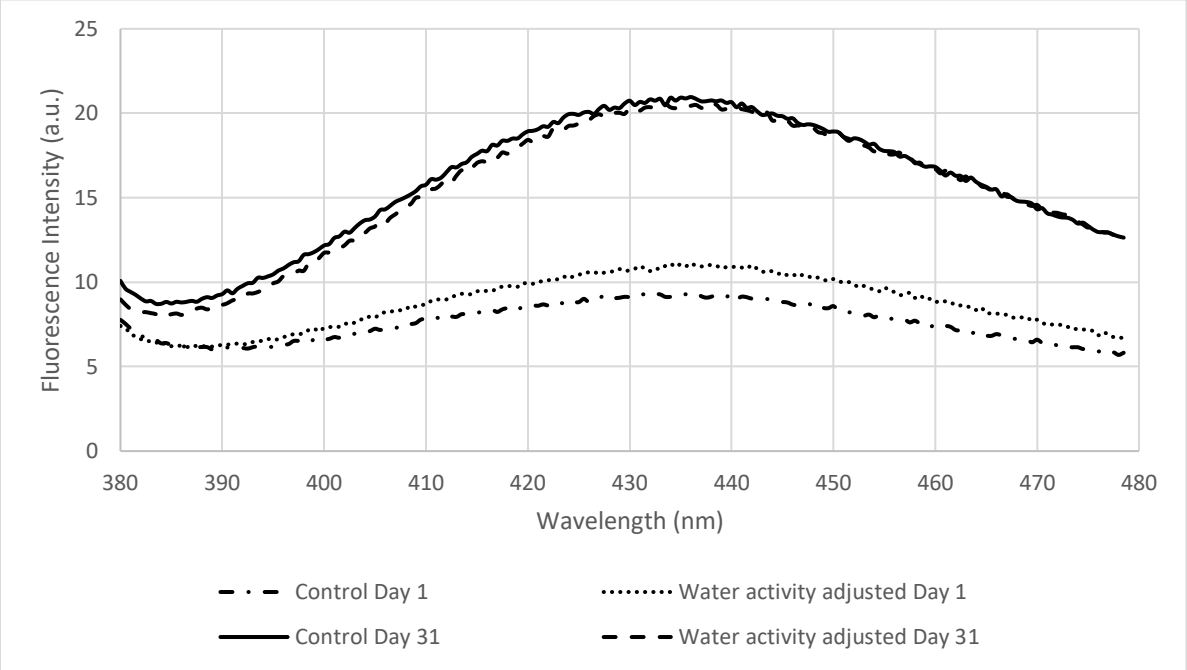

Figure B.2 Averaged Maillard emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 25°C.

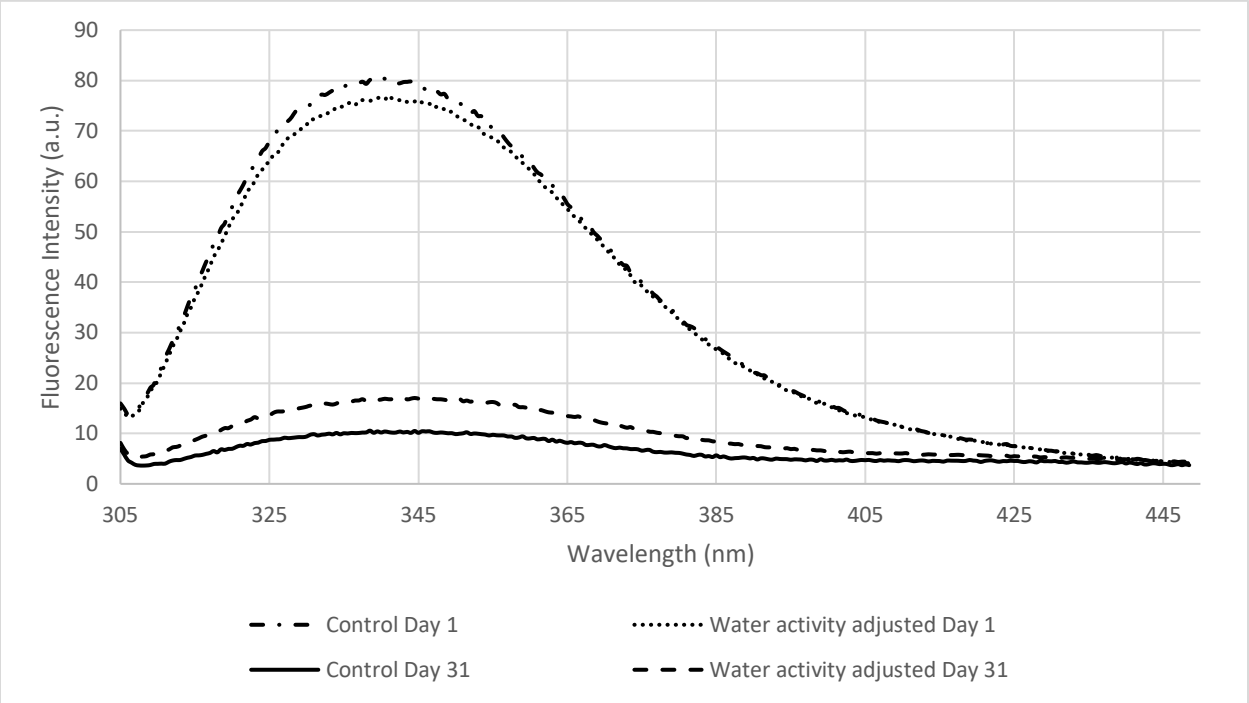

Figure B.3 Averaged tryptophan emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 36°C.

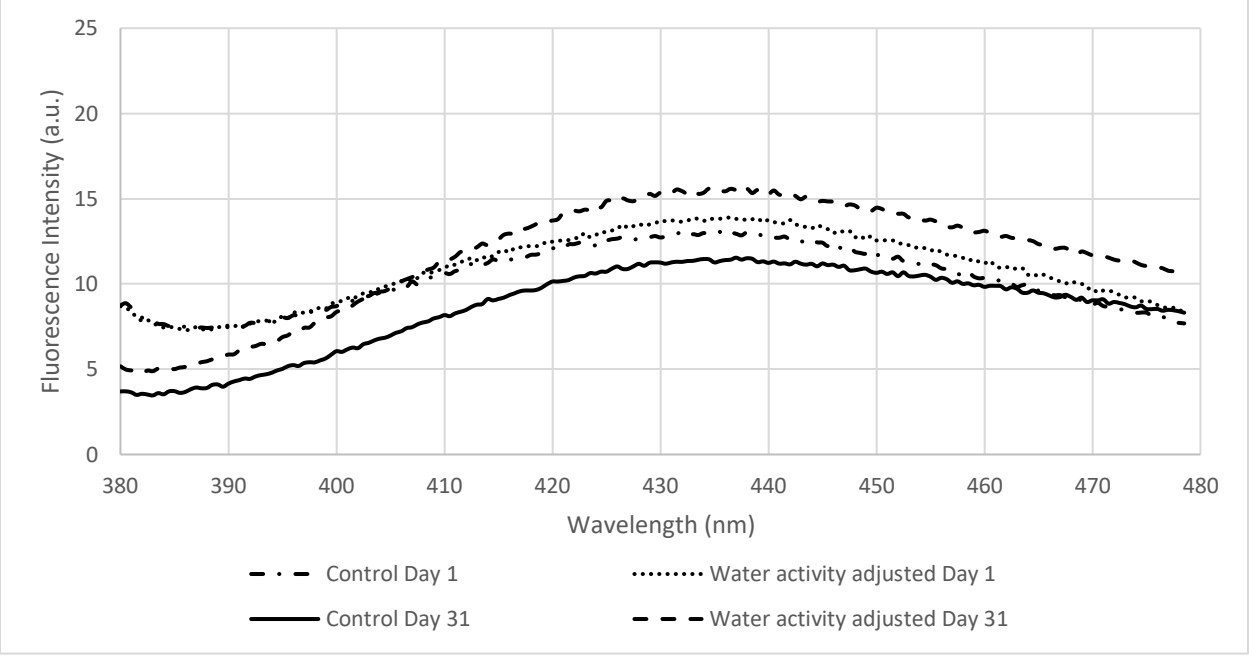

Figure B.4 Averaged Maillard emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 36°C.

Appendix C - Chapter 5

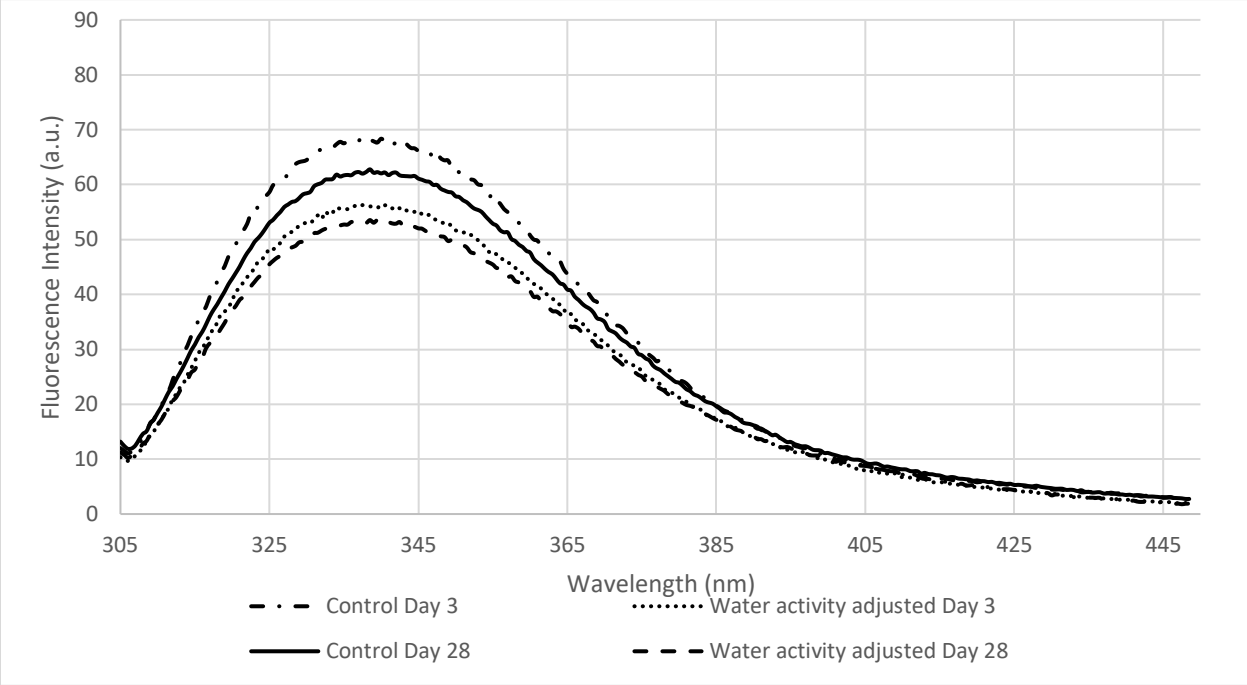

Figure C.1 Averaged tryptophan emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 25°C.

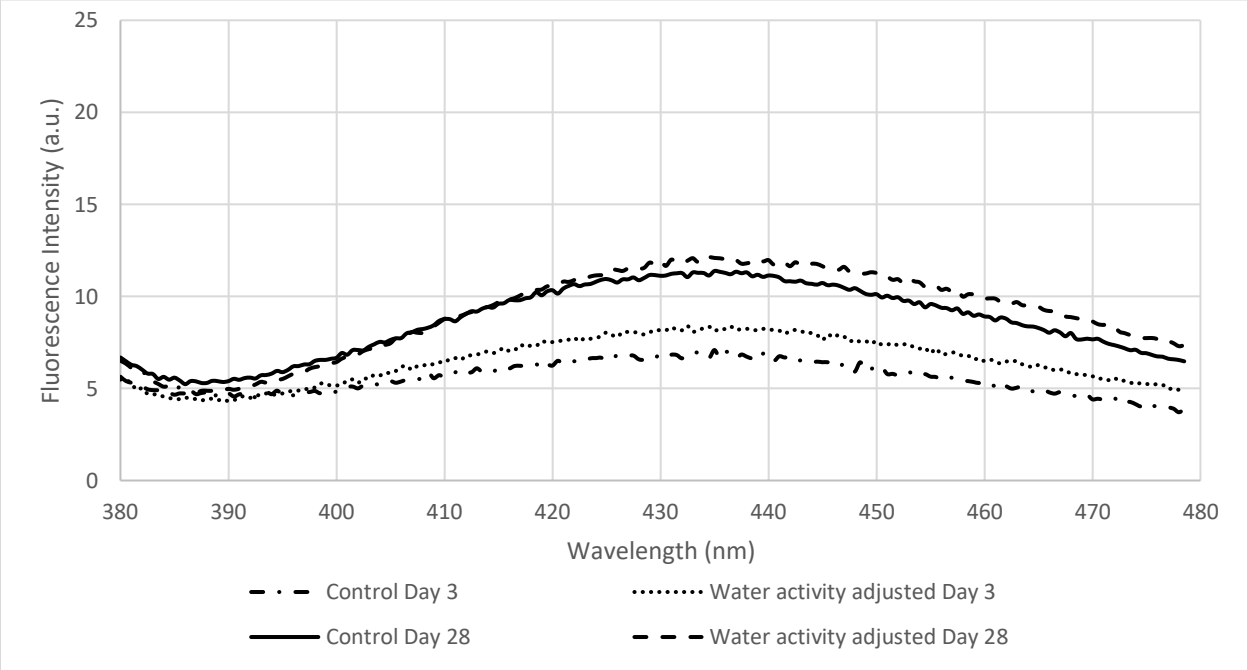

Figure C.2 Averaged Maillard emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 25°C.

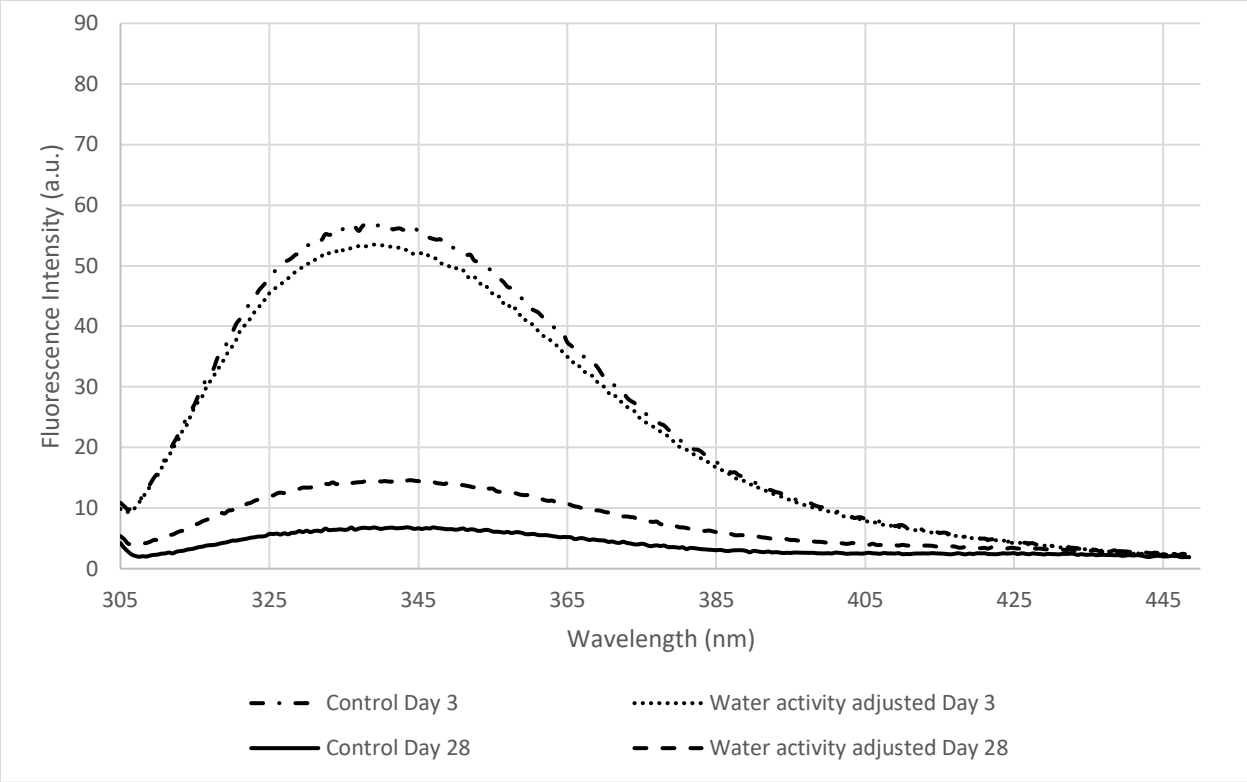

Figure C.3 Averaged tryptophan emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 36°C.

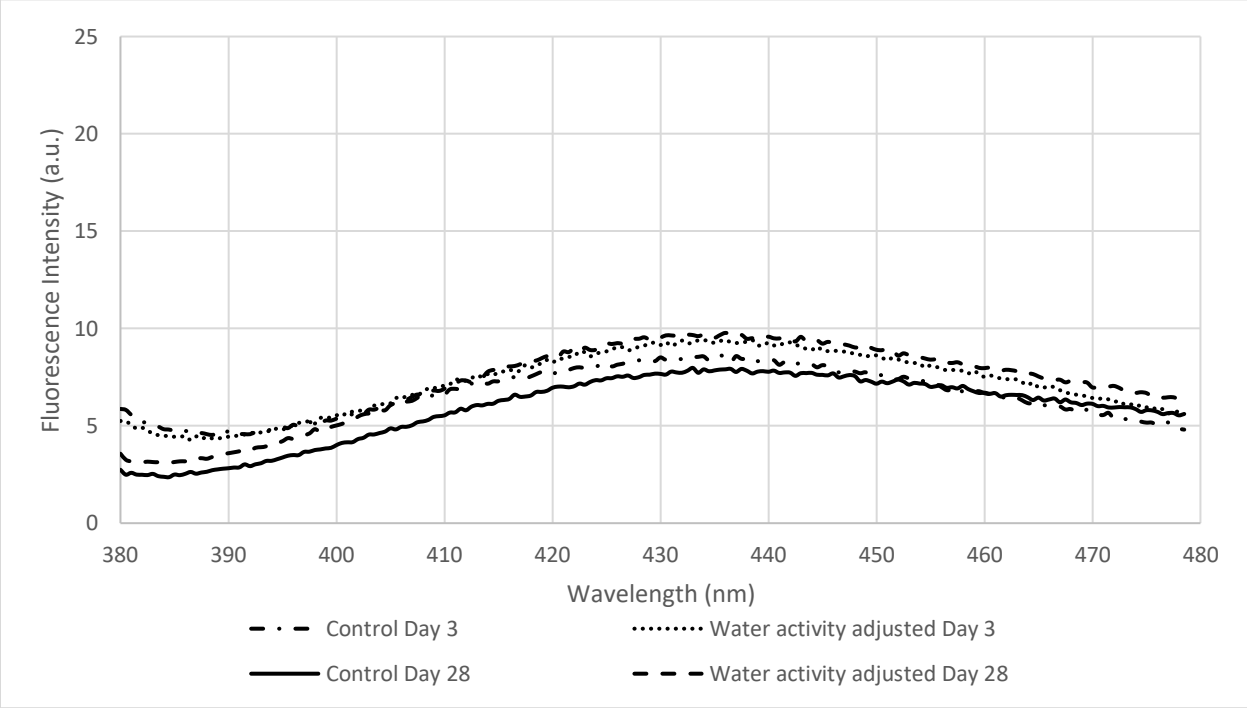

Figure C.4 Averaged Maillard emission spectra of high protein nutrition bars produced with untreated and treated milk protein concentrate (MPC) 85 stored at a temperature of 36°C.