EFFECT OF RADIO FREQUENCY DIELECTRIC HEATING ON FUNCTIONALITY OF NONFAT DRY MILK

by

LU WANG

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Approved by:

Major Professor
Karen Schmidt

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Abstract

Radio frequency dielectric heating (RFDH) can provide rapid and uniform heating throughout the products' mass, and can be used to bake, dry, and defrost foods. Studies have shown that when RFDH induced a 5-log reduction of *Salmonella* spp. in nonfat dry milk (NDM), whey protein nitrogen index (WPNI) decreased, suggesting that functional properties of the NDM might be impacted. This research was conducted to determine if RFDH affected the functional properties of NDM [high-heat (HH) and low-heat (LH)]. Nonfat dry milk were treated to 75, 80 and 85°C in the RFDH unit, then were held for 125, 63 and 43 min for LH-NDM or 115, 52 and 43 min for HH-NDM, and cooled to ~23 ± 1°C. Powders were evaluated for WPNI , nitrogen solubility index, and color. Maillard browning and functional properties of NDM samples were evaluated after NDM was rehydrated to 3.5% protein with deionized water, and adjusted to pH 7.00. Glucono-delta-lactone was added in rehydrated NDM (3.5% protein; natural pH) as an acidifying agent to form milk gels, and the physical properties of the gels were determined. Two replications were conducted and data were analyzed with two-way ANOVA (RFDH and NDM) and Tukey mean differentiations ($p \leq 0.05$). Results showed that LH-NDM (collapsed for RFDH treatments) had 5.7% less viscosity, 20.9% less overrun, 27.4% less foam stability, as well as 15% less water holding capacity compared with HH-NDM (collapsed for RFDH treatments). This can be explained by the natural whey protein denaturation differences in the HH-NDM and LH-NDM. Viscosity and surface tension were impacted by the RFDH treatment. NDM (HH and LH) treated to 85°C had 10% greater viscosity than the control, and the NDM treated to 75°C had less surface tension compared with samples treated to 80°C, 85°C and the control. Overall, RFDH decreased WPNI in LH-NDM, but not HH-NDM. The SDS-PAGE gel images provided supportive evidence to the WPNI results. RFDH is a processing

technology that could change a few functional properties of NDM in this study, which makes it a promising method that may be further exploited for various food applications, such as emulsifiers, foaming agents, etc. However, potential negative impacts, such as color change caused by Maillard reaction, loss of WPNI in LH-NDM, cannot be neglected.

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Chapter 1 - Literature Review

1.1 Introduction of radio frequency dielectric heating

Commercial pasteurization and sterilization are applied to reduce spoilage and eliminate pathogens in the food industry (Wang et al., 2003). Conventional heating methods (using steam, water bath, or hot room), which require heat energy to be generated eternally and then transferred to food products by convection, conduction, or radiation, are widely used (Zhao et al., 2000; Wang et al., 2003; Boreddy et al., 2016). Ma et al. (2009) reported that conventional heating (using water bath) at 90°C for less than 30 min was not sufficient to cause a 5-log reduction of *Salmonella* in highly contaminated peanut butter due to its relatively low thermal diffusivity. Boreddy et al. (2016) reported that a novel method – radio frequency thermal processing (ranging from 4 h at 90°C to 72 h at 60°C), not only met pasteurization requirements for egg white powder (EWP) and caused no changes in quality (color and solubility) as the conventional heating method (hot room at 58°C for at least 14 d), but also was less expensive and less time consuming compared with the conventional heating method. Radio frequency dielectric heating (RFDH) provides a more energy-efficient heating due to increased heat transfer efficiency, especially for low-moisture foods, resulting in more rapid and uniform heating throughout a product's mass compared with the conventional heating methods (Zhao et al., 2000).

During RFDH, a high frequency alternating electric field is applied between two metal capacitor plates. Dielectric molecules, which try to align themselves with the polarity of the electric field, rotate in response to the rapid-changing electric field. The resulting kinetic energy and friction caused by colliding neighboring molecules generates heat within the product (Zhao et al., 2000; Piyasena et al., 2003).

Wang et al. (2003) used a model solid food, consisting of 20% whey protein concentrate (WPC), 2% glucose, 0.59% sodium chloride, with the remainder distilled water, to compare the sterilization performance between conventional retort and RFDH methods. A 2.7-kg capacity polymeric tray (292 × 229 × 49 mm, with a 1.6-mm-thick wall) was filled with the model food and sealed with a 0.1-mm thick aluminum foil lid. To assess severity of heat treatment, a chemical marker assay, which yielded the thermally-produced compound, 2,3-dihydro-3,5-dihydroxy-6-methyl-(4H)-pyran-4-one, as a time-temperature integrator to investigate food degradation, was added. A pilot-scale radio frequency system was set at 6 kW, 27.12 MHz with plate applicators. The researchers reported that the RFDH for 30 min delivered similar lethality to a 90 min conventional retort process, as the core of both model foods reached 121°C. After RFDH, they found the chemical marker yields were around 0.87 peak area/g on average at different locations in the 3 layers of the model food. However, marker yields were less at the core (1.18 peak area/g) and greater at the edges (1.64 peak area/g) if conventionally retorted. The average concentration of chemical marker among all 33 samples was 1.39 ± 0.16 peak area/g, which was greater than 0.87 ± 0.07 peak area/g for the RFDH-treated samples. They concluded that RFDH had less adverse impact on product quality and provided more uniform heating than the conventional retort. Further, RFDH offered the possibility of faster heating in foods (Ramaswamy and Tang, 2008; Wang et al., 2012).

Over the past 70 years, many studies have been done on RFDH (0.003-300MHz) as well as microwave heating (300-300,000MHz) to show that both techniques can potentially improve the quality of heated foods (Ramaswamy and Tang, 2008). In the food industry, popular applications of microwave heating include tempering and pre-cooking meat products, while RFDH is commonly used to dry freshly baked products (Ramaswamy and Tang, 2008).

Microwave heating has limitations however (Wang et al., 2003). Guan et al. (2004) compared power penetration depth in mashed potatoes when treated with microwave and RFDH. They found when moisture content of the potatoes was 81.7% and process temperature was 60°C, power penetration depth was 45.9 and 15.33 mm for RFDH (40MHz) and microwave heating (915 MHz), respectively. As RFDH had greater penetration than microwave heating, this limitation of microwave heating might be overcome by using radio frequency energy (Wang et al., 2003).

RFDH has been used to defrost, dry and bake foods (Piyasena et al., 2003). The first attempts were to use RFDH to cook processed meat, to bake bread and to dehydrate vegetables (Piyasena et al., 2003). Demeczky (1974) successfully showed that bottled juices moving on a conveyer belt through a radio frequency unit had better flavor scores (4.1 vs 3.5 on a 5-point scale) and less operation costs (~60%) than juices treated by conventional thermal methods.

Also, RFDH has been reported to significantly inactivate *Bacillus cereus* and *Clostridium perfringens* vegetative cells and spores in pork meat, *Clostridium botulinum* in scrambled eggs, and *Escherichia coli* in apple cider and orange juice (Luechapattanaporn et al., 2005; Geveke et al., 2007; Geveke and Brunkhorst, 2008; Byrne et al., 2010). Byrne et al. (2010) reported that after cooking pork luncheon meat at 80°C for 33 min using RFDH, followed by cooling (to 4°C), the microbial enumeration results showed a reduction in *B.cereus* vegetative cells and spores of 5.4 and 1.8 $\log_{10}$ cfu g^{-1}, respectively. The radio frequency electric field (RFEF) nonthermal-process has been developed for inactivating bacteria in apple juice. Unlike RFDH which yields heat to inactivate pathogens, the RFEF nonthermal-process applies a voltage across the cell membrane in an electric field. This causes the membrane to thin because the opposite charges on either side of the membrane attract. At high field strengths, pores form in the membrane and the

cell ruptures (Geveke and Brunkhorst, 2008). Geveke and Brunkhorst (2008) applied RFEF to pasteurize inoculated apple cider (6-7 log cfu/ml *E.coli* K12). An 80 kW RFEF system was used to provide different frequencies of 21, 30, and 41 kHz. Treatment times varied from 140 to 420 μs. The results showed that RFEF inactivation of *E. coli* K12 improved (1.3 vs. 5.0 log) as the temperature increased from 50 to 60°C. However, when treating the apple cider using ohmic heating at 50-60°C for the same heating times as the RFEF method, there were no changes in the populations of *E. coli* K12, except for 60°C, which suggested that the vast majority of the RFEF inactivation was due to the non-thermal effects. In addition, the microbial inactivation was found to be independent of frequency (Geveke and Brunkhorst, 2008).

Michael et al. (2014) studied the destruction of *Cronobacter sakazakii* and *Salmonella* spp. in nonfat dry milk (NDM) via conventional heating and RFDH. They reported that similar lethality of organisms can be reached regardless of the heating system (conventional vs. RFDH), but RFDH shortened the overall lethality treatment compared with conventional heating (e.g. 7.94 vs. 4.26 min to 75°C; 9.51 vs. 4.54 min to 80°C; 8.86 vs. 4.95 min to 85°C; 10.72 vs. 5.50 min to 90°C). Further 5-log reactions in the pathogens *Cronobacter sakazakii* and *Salmonella* spp. were achieved, making RFDH an alternate post-process lethality treatment. Chen et al. (2013) studied changes in whey protein nitrogen index (WPNI) and nitrogen solubility index (NSI) of low-heat (LH) and high-heat (HH) NDM that had been treated by RFDH to 75, 80, 85 and 90°C and subsequently held in a convection oven for up to 125.67, 57.75, 25.0, and 11.50 min, respectively. They reported that the WPNI for HH-NDM decreased only if processed at 80°C for 57.75 min, or 85°C for 10 or 25 min compared with the control HH-NDM (HH-C). However, the WPNI of LH-NDM decreased significantly when treated at ≥ 80°C compared with the control LH-NDM (LH-C). The NSI for HH-NDM decreased significantly when treated at ≥

80°C compared with HH-C. However, all RFDH-treated LH-NDM samples showed significant decrease in NSI compared with LH-C, except for samples treated to 75° C using RFDH without holding in the oven. This study suggested that the functional properties of NDM might be impacted via RFDH by influencing protein solubility.

1.2 Nonfat dry milk - production, category, use, and change during storage

Milk is a nutritious food, rich in proteins, minerals, fat, lactose, vitamins and enzymes; but it is highly perishable. Converting milk into milk powder increases its shelf life to ~1 year without substantial loss of quality, and eliminates the need for refrigeration (Sharma et al., 2012). Nonfat dry milk (NDM) is made by removing almost all fat and water from milk, followed by drying. The resulting powder contains ≤ 5% moisture and ≤ 1.5% fat (21CFR 131.125; FDA, 2016). Based on 7CFR 58.248 (GPO, 2016), NDM shall meet the requirements of U.S. Extra Grade or U.S. Standard Grade. According to American Dairy Products Institute (2009), Extra Grade NDM (spray drying) and Standard Grade NDM (spray drying) shall meet the specifications displayed in Table 1-1.

Table 1-1. Specifications for Extra Grade and Standard Grade nonfat dry milk (spray drying)

	Extra Grade nonfat dry milk	Standard Grade nonfat dry milk
Milkfat	≤ 1.25%	≤ 1.5%
Moisture	≤ 4.0%	≤ 5.0%
Titratable acidity	≤ 0.15%	≤ 0.17%
Solubility index	≤ 1.2 ml	≤ 2.0 ml
Bacterial estimate	≤ 10,000 per g	≤ 50,000 per g
Scorched particles	≤ disc B (15.0 mg)	≤ disc B (22.5 mg)

In the production of NDM, the raw milk is clarified in conjunction with fat separation (Bylund, 1995). Then skim milk is then preheated according to the classification standard, fed to an evaporator to increase the concentration of total solids, and dried. During drying, the milk is dispersed as a mist into a rapidly moving hot air stream in the drying chamber, causing the mist droplets to instantly evaporate, and milk powder falls to the bottom of the chamber to be collected (United Nations Environment Programme, 2000).

Milk powder is classified into categories related to the typical processing conditions the skim milk has undergone prior to evaporation and drying (Bylund, 1995). The most common analytical parameter used for classification of NDM is whey protein nitrogen index (WPNI), defined as undenatured whey proteins (mg) / powder (g) (Kelly et al., 2003). A summary of the categories and matching applications of spray-dried NDM is shown in Table 1-2.

Table 1-2. Heat treatment classification of nonfat dry milk and appropriate application

Classification	Typical processing treatment of skim milk[1]	WPNI[2] (mg/g)	Recommended applications[2]
Low-heat	≤160°F for 2 minutes	≥ 6.0	Buttermilk, cottage cheese
Medium-heat	160-175°F for 20 minutes	1.51-5.99	Ice cream, confectionery
High-heat	190°F for 30 minutes	≤ 1.5	Recombined evaporated milk, baked goods

[1]Adapted from ADPI (2009).

[2]Whey protein nitrogen index.

[3]Adapted from Kelly et al. (2003).

NDM provides a wide range of functions in foods, including emulsification, thickening, gelling and foaming (Canadian Dairy Commission, 2011), so it is an important ingredient in many food formulations, such as yogurt, bread, ice cream, etc. (Reger et al., 1951; Bylund, 1995; Isleten and Karagul-Yuceer, 2006).

During storage of NDM, deterioration might occur which can jeopardize the quality. Lactose, which consists of ~ 50% in NDM, plays an important role in storage stability and quality of NDM (ADPI, 2009). Lactose exists often as an amorphous glass which is stable below its glass transition temperature (T_g). Lactose is hygroscopic and may adsorb water from the environment, resulting in plasticization and decreasing of the T_g. A storage temperature above T_g increases molecular mobility and decreases viscosity, causing caking and lactose crystallization, which deteriorates the quality of NDM (Jouppila and Roos, 1994).

The Maillard browning reaction is also a common deteriorative reaction in foods, eventually causing discoloration and various losses in nutritive value (Le et al., 2011b). This chemical reaction is initiated by condensation of lactose (reducing sugar) with lysine, which is an abundant amino acid in milk proteins, forming lactulosyllysine, also known as an Amadori rearrangement product (Figure 1-1). This product is further degraded via different pathways to produce various intermediate compounds followed by brown pigments (melanoidins) when the reaction reaches its final stage (Le et al., 2011a) (Figure 1-1). According to Le et al. (2011a), to investigate the progress of the Maillard reaction, furosine (an initial-stage Maillard reaction product, derived from lactulosyllysine), free hydroxymethylfurfural (a mid-stage Maillard reaction product), and color (late stage) can be measured. On the molecular level, the formation of brown pigments is due to production of high molecular weight melanoidins, which form in the late stage of the Maillard reaction (Thomsen et al., 2005). The rate of Maillard reaction in milk

products greatly depends on the conditions of heat treatment or storage temperature and humidity and pH, as well as milk composition (Le et al., 2011a). Liu and Metzger (2007) studied color changes after storage of LH-NDM at 4, 22, 35 and 50°C for 8 weeks. They reported that no color differences were observed from the LH-NDM samples stored at 4, 22, and 35°C. However, a decrease in whiteness and increases in redness and yellowness were observed in LH-NDM samples stored at 50°C, suggesting that Maillard reaction might have occurred when NDM was stored at 50°C.

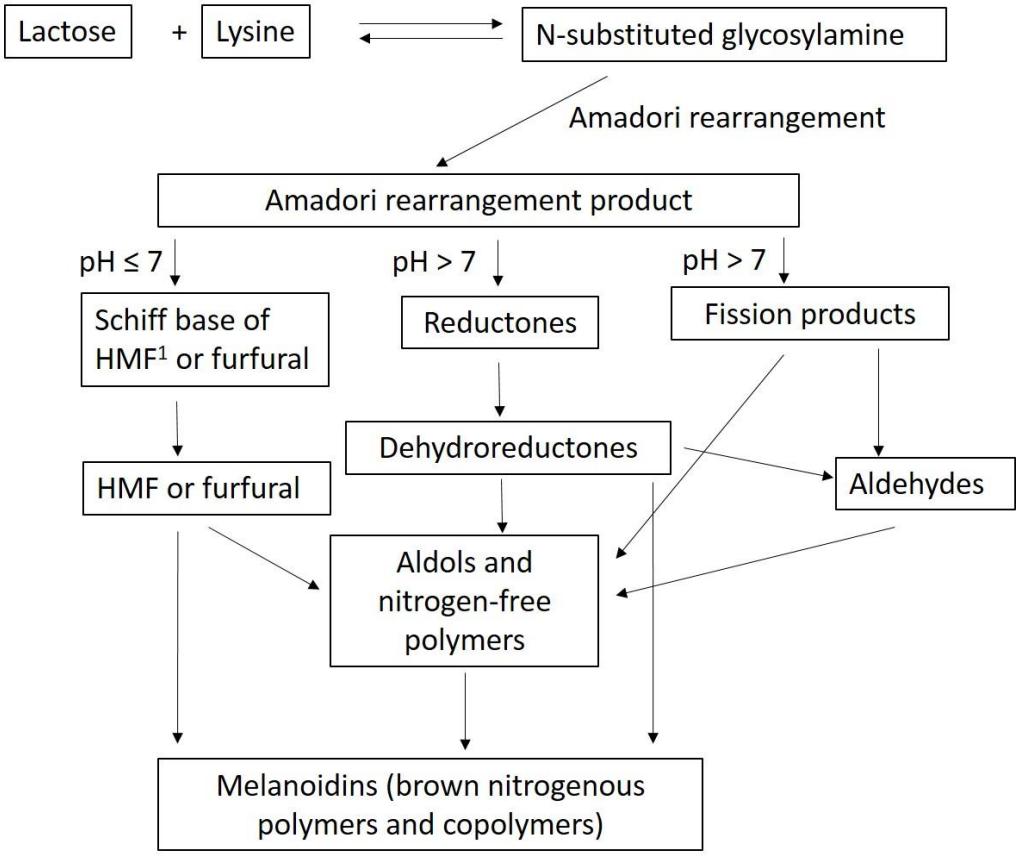


Figure 1-1. Maillard reaction scheme.

[1]Hydroxymethylfurfural.

Adapted from Zhang et al. (2015).

Additionally, NDM is very sensitive to light oxidation because of the presence of riboflavin (vitamin B_2), which is a strong photosensitizer and can induce oxidation reactions, leading to significant losses of nutrients, such as vitamins and amino acids; to discoloration; as well as to formation of strong off-flavors (Mestdagh et al., 2005). Typically, Maillard reaction and oxidation are measured by chemical methods, including loss of lysine availability, development of Amadori product and advanced glycosylation end products, and decrease in riboflavin content. However, Liu and Metzger (2007) reported that front-face fluorescence spectroscopy (FFFS) has potential as an analytical technique to monitor changes in NDM samples during storage. After stored at 4, 22, 35, and 50°C for 8 weeks, they observed an increase in the peak intensity around 337 nm in the Maillard excitation spectra when NDM samples were stored at 50°C, suggesting the formation of Maillard reaction products during storage at 50°C in NDM (Liu and Metzger, 2007).

Dry heating can initiate glycation between α-lactalbumin (α-La) and maltopentaose through the Maillard reaction (Enomoto et al., 2009). Enomoto et al. (2009) reported that sugar content in α-La increased 12.3%, indicating that α-La was conjugated with maltopentaose when exposed to pH 8.0 and 50°C (65% RH) for 3 d. They interpreted these results as the initiation of the Maillard reaction.

Ukeda et al. (1998) proposed to monitor the Maillard reaction in a model system based on the reduction of tetrazolium salt 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro) benzensulfonic acid hydrate (XTT) by reactions with Maillard browning products (some nitrogen containing heterocyclic compounds such as pyrazinium and pyridinium) (Figure 1-1). The tetrazolium salt XTT can be reduced to water-soluble formazan, which is suitable for spectrophotometric measurement at 492 and 600 nm (Shimamura et al., 2000). Ukeda et al.

(1998) found both XTT and NBT (conventional indicator of Maillard reaction) reducibility gradually increased with temperature from 100 to 140°C, which indicated XTT assay could estimate the degree of thermal stress delivered to the milk as well as the NBT value (Shimamura et al., 2000).

1.3 Milk proteins – composition, structure and physical state in milk

The major milk proteins are casein and whey, which represent ~79.5 and 19.3%, respectively, of the total protein (Byland, 1995).

1.3.1 Casein proteins

Casein is actually comprised of four casein fractions -- α_{s1}-, α_{s2}-, β- and κ-caseins, which represent approximately 38, 10, 35 and 15%, respectively, of total casein (Fox, 2003). The major casein components have several genetic variants and contain variable numbers of phosphoseryl residues, especially α_{s2}-casein, which exhibits a large variability (10-13 seryl phosphate groups) in phosphorylation (Fox, 2003). α_{s1}-Caseins contain 8-10 seryl phosphate groups, while β-casein contains about 5 phosphoserine residues, and is more hydrophobic than the α_s-caseins and κ-caseins (κ-CN) (Phadungath, 2005). The high content of phosphate groups causes the α_{s1}-, α_{s2}- and β-caseins to have a strong tendency to bind Ca^{2+} in milk and become insoluble in certain circumstances (calcium concentration greater than ~ 6 mM at > 20°C). On the other hand, κ-CN, which only contains one organic phosphate group, binds calcium weakly and is soluble at all calcium concentrations found in dairy products (Fox, 2003).

The caseins easily form polymers containing several identical or different types of molecules. The polymers consist of hundreds and thousands of individual molecules and form a colloid ranging in size from 50-500 nm (average ~150 nm) in diameter and a molecular mass from 10^6 to 3×10^9 Da (average ~10^8 Da) (Fox, 2003). These molecular complexes are known as

casein micelles (Bylund, 1995). A typical casein micelle contains a complex of sub-micelles, most of which form thermodynamically stable complexes with nanoclusters of amorphous calcium phosphate (Holt et al., 2013). κ-Casein is located on the outside of casein micelles for stabilization (Dalgleish, 1998).

Casein micelles are very stable at high temperatures, coagulating only at 140°C for 15-20 min at the native pH (6.7) of milk. When the pH is 4.6 and 20°C, the caseins aggregate and precipitate. Precipitation at this pH is temperature-dependent, i.e. does not occur at temperatures < 5-8°C (Fox, 2003).

1.3.2 Whey proteins

Whey proteins have distinctive nutritional and functional properties, which can provide unique properties in the foods (Dissanayake et al., 2013). In bovine milk, the principal whey proteins by weight are β-lactoglobulin (β-Lg) and α-La, which comprise approximately 9.8% and 3.7%, respectively, of the total proteins, while minor whey proteins include blood serum albumin (1.2%), immunoglobulins (2.1%), proteose-peptones (2.4%) (Bylund, 1995).

1.3.2.1 Categories - α-Lactalbumin and β-Lactoglobulin

α-La is present in milk from all mammals and plays a significant part in the synthesis of lactose in the udder (Bylund, 1995). α-La, from most mammals, contains 123 amino acid residues, and is the predominant protein in human milk (concentration in mature milk about 2.5 g L^{-1}) and the second most abundant whey protein in bovine milk, with a concentration of 1-1.5 g L^{-1} (Liskova et al., 2010). In its native form, α-La contains one mole of bound calcium per mole of protein which stabilizes the tertiary structure of the protein (Bramaud et al., 1997). Overall, α-La has an approximate molecular mass of 14.2 kDa (Liskova et al., 2010).

β-Lg is the most abundant whey protein in bovine milk, which contains all 20 amino acids in relative amounts that enhances the nutritional value. Bovine β-Lg has two genetic variants, A and B. At 24 ± 1°C and between pH 5 and 7, β-Lg occurs as a dimer consisting of two identical subunits, each with a molecular weight of 18,400 Da (Kelly and Larsen, 2010). The β-Lg contains two disulphide bridges and one free thiol group per monomer. The thiol group is capable of interacting to form new disulphide bonds (De Wit and Klarenbeek, 1984). Such reactions can lead to gel formation and may be involved in the coagulation of sterilized milk (Kelly and Larsen, 2010). Bovine β-Lg seems to denature through an initial dissociation of dimer to monomer followed by a change in the polypeptide chain conformation, and subsequent aggregation. The factors that induce denaturation can be heat, pH, pressure, and salt. (Sawyer, 2003).

1.3.2.2 Whey protein denaturation

Heat treatment, as one of the major processing operations of milk, can cause the denaturation of whey proteins, resulting in changes in their functional properties (Singh and Havea, 2003). The denaturation of whey proteins is assumed to be a two-step process: modification of the native state of the protein to an activated state or unfolding, which may be reversible or irreversible, then aggregation, which follows irreversible unfolding (De Wit, 1998; Singh and Havea, 2003).

The transformation from the initial native molecule to the unfolded state is cooperative and reversible. Unfolding of globular proteins is accompanied by an endothermal heat treatment, which can be observed by differential scanning calorimetry (DSC) (De Wit, 1998). Using DSC, the temperature of unfolding can be measured either as the onset temperature of an endotherm, or as the endotherm peak temperature. De Wit and Klarenbeek (1984) reported that different whey

proteins unfold at different temperatures in aqueous state, with the initial denaturation temperature of 62°C for α-La and 78°C for β-Lg. However, according to Wijayanti et al. (2014), when temperature is > 70°C, irreversible aggregation reactions of β-Lg are initiated, which leads to formation of 2 types of aggregates: small (via –SH group oxidation and/or –SH/S-S interchange) and large aggregates (via nonspecific interactions without involvement of –SH groups).

When heating in a dry state, the molecular motion of compounds is retarded because of the decreased water content, resulting in an increase in the denaturation temperature of proteins compared with the same proteins in aqueous states. When whey proteins are thermally denatured, several intermediate conformations may exist between the native and the final, unfolded state, which have a compact structure with a native-like secondary structure but tertiary structure similar to that of the unfolded state (Singh and Havea, 2003). A "molten" structure might be a unique intermediate state with a partially folded conformation, which can impact functional properties of whey proteins, such as foaming and emulsifying properties, due to exposure of hydrophobic groups and enhanced protein-protein interactions (Kato et al., 1989; Ibrahim et al., 1993; Bals and Kulozik, 2003). When whey proteins unfold at least partially, the hydrophobic amino acid residues buried deep within the native structure are exposed, increasing the reactivity of such groups. Besides, an increased reactivity of sulphydryl groups is involved, resulting in the formation of disulphide bonds or sulphydryl-disulphide interchange reactions. Unfolded protein molecules may associate with each other to form aggregates through sulphydryl-disulphide interchange and hydrophobic interactions.

Denaturation of whey proteins may or may not affect solubility. According to Gulzar et al. (2011), after dry heating whey protein isolate (WPI) (pH 6.5) at 100°C for 8 h, the amount of

native whey proteins (at pH 4.6) dropped from 90 to 49%, and soluble aggregates (pH 7) increased from 10 to 51%, compared with the control WPI (pH 6.5). No insoluble aggregates formed in either sample type, as solubility values were equivalent, at 100%. Dissanayake and Vasiljevic (2009) studied the changes in solubility of whey protein retentate (10%, wt/wt) under heat and microfluidization in an aqueous state. They reported that under the same microfluidization treatment (1 pass), samples treated at 90°C for 20 min in an aqueous state had a 73% decrease in solubility compared with the non-heated samples. They interpreted that as a result of heat treatment, the globular conformation of whey proteins was irreversibly changed to a more random structure by exposing the hydrophobic groups. Free thiol oxidation and sulfhydryl/disulfide interchange reactions between exposed hydrophobic groups lead to protein aggregation and precipitation, thus decreasing the solubility (Dissanayake and Vasiljevic, 2009).

Denaturation of whey proteins can be impacted by the pH in both aqueous and dry state (Singh and Havea, 2003). Dissanayake et al. (2013) reported that a loss in solubility (%) was induced by heating whey protein dispersions at 140°C for 20 sec; systems at pH 4 were less soluble (~29%) than those at pH 5 and 6 (~33% and 35%, respectively). They reported that this could have resulted from the increased overall positive charge, which enhanced repulsion and hindered aggregation. To study effects of heat treatment and pH on protein aggregation in a dry state, Gulzar et al. (2011) reconstituted whey protein isolate (WPI) (pre-heated at 100°C for 0, 8, 16 or 24 h at pH 2.5, 4.5 or 6.5) at 10 g/l in distilled water containing 0.12 M sodium chloride, followed by protein fractionation to separate insoluble aggregates, soluble aggregates (at pH 7), and soluble protein (at pH 4.6). Gulzar et al. (2011) reported that when heating time was below 8 h, no insoluble aggregates (at pH 7) were found in samples at all pH values. As heating time increased to 16 or 24 h, lower quantities of soluble proteins (at pH 4.6) and higher quantities of

insoluble aggregates were observed if whey proteins were dry-heated at pH 4.5 and 6.5 compared with those dry-heated at pH 2.5. Increasing the pH for dry heating increased the rate of denaturation/aggregation of whey proteins because proteins exhibited a greater propensity for polymerization through sulphydryl/disulphide interchange reactions (Gulzar et al., 2011).

At neutral pH, β-Lg exists primarily as a dimer. When temperature exceeds 40°C, the dimers start to dissociate, which is the most important species of β-Lg during heat denaturation (Kelly, 2010). As temperature increases, monomer unfolding occurs so as to permit more rapid sulphydryl reactivity that can lead to disulphide interchange and aggregation (Sawyer, 2003). The effect of heating β-Lg in the presence of other milk components has shown that lactose inhibits β-Lg denaturation, in contrast to κ-CN which destabilizes β-Lg and facilitates denaturation (Park and Lund, 1984; Garrett et al., 1988). When pH is at 8-9, alkali denaturation of β-Lg becomes significant, and dissociation occurs (Sawyer, 2003). The rate of denaturation rises rapidly with increasing pH and leads eventually to the formation of aggregates (Sawyer, 2003).

The ability of thermally denatured β-Lg to interact with casein has been studied. A number of factors such as temperature, β-Lg to casein ratio, and pH impact the rates of aggregation (O'Connell and Fox, 2003). When heated at ~70°C, complexes between β-Lg and κ-CN start to form due to hydrophobic bonding. As temperature increases, the interaction increases rapidly; when heated at 90 and 120°C for 20 min, about 75 and 95% of total β-Lg complexes with casein micelles, respectively (Smits and van Brouwershaven, 1980). Whether sulphydryl-disulphide interchange reactions are involved at such high temperatures is still in disagreement. Haque et al. (1987) proposed that when heated at 70°C, β-Lg forms a trimer via hydrophobic bonding which then interacts with κ-CN, initially through hydrophobic bonding but eventually

through sulphydryl-disulphide interchange reaction. However, Wong et al. (1996) suggested that during the aggregation step, there is a non-specific association between β-Lg and κ-CN and no disulfide bonding is involved.

1.3.2.3 Whey protein nitrogen index

Whey protein nitrogen index (WPNI) measures the amount of undenatured whey protein present in the nonfat dry milk, and it is a relative indication of heat treatment the skim milk had received prior to drying (Kelly et al., 2003; Sikand et al., 2008). Lower levels of undenatured whey protein in the powder indicate exposure to higher temperatures (Smith, 2008). Chen et al., (2013) treated LH-NDM and HH-NDM using RFDH treatment to cause a 5-log reduction of *Salmonella* spp. They reported that the RFDH had less effect on the WPNI of HH-NDM than that of LH-NDM. For HH-NDM, the WPNI decreased by 11% after samples were treated at 85°C for 25 min compared with the control HH-NDM (Chen et al., 2013). However, under the same temperature/time treatment, the WPNI of LH-NDM decreased by 19% compared with the control LH-NDM. RFDH significantly reduced WPNI in all LH-NDM samples when the treatment temperature was ≥ 80°C compared with the control while HH-NDM did not (Chen et al., 2013).

1.3.2.4 Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) is an ordinary gel-based electrophoresis technique, which is important and universally used for protein separation (Zhang et al., 2011). Electrophoretic mobility of protein is relevant only to its mass because the use of SDS denatures the original proteins and eliminates the protein's original surface charge and form, producing a mass-based SDS-protein complex. Thus, the mobility of the SDS-protein complex in a polyacrylamide gel is determined by its mass and the gel

permeability (Zhang et al., 2011). If however, a reducing agent, for example, β-mercaptoethanol (BME) is used to cleave disulfide bonds, the tertiary and quaternary structures of some proteins are disrupted, which is useful in protein analysis as it can help identify soluble aggregates when comparing samples with and without BME (Nguyen et al., 2012).

Interactions of protein can be inferred by SDS-PAGE technology. Jovanovic et al. (2007) investigated the effects of different heat treatments (75, 85, 90°C for 20 min) on soluble proteins in reconstituted milk, and reported that heat-induced high molecular weight complexes were formed, as evidenced by the observation of 50-141 kDa bands via densitometric analysis of bands. This was interpreted as disulfide interactions had occurred and co-aggregates had formed during heat treatment. The intensity of bands can indicate the concentration of proteins. Gulzar et al. (2011) reported that when dry-heated WPI (100°C for 0, 8, 16, and 24 h) was rehydrated and then analyzed by SDS-PAGE, the band intensities corresponding to monomers of α-La and β-Lg decreased as the heating time increased, which was interpreted as increased whey protein denaturation caused by dry heating.

1.3.3 Protein solubility

Protein solubility is commonly expressed as water-soluble nitrogen, nitrogen solubility index, water-soluble protein, or protein dispersibility index (Morr et al., 1985). Solubility is a primary property of proteins and can significantly influence functional properties, especially in foams, emulsions and gels (Zayas, 1997; Wong and Kitts, 2003; Pelegrine and Gomes, 2012). Solubility of milk proteins is influenced by various environmental factors, such as pH, solvent polarity, temperature and processing conditions (Pace et al., 2004; Anema et al., 2006; Dissanayake and Vasiljevic, 2009).

1.3.3.1 Evaluation of protein solubility - nitrogen solubility index (NSI)

According to standard method (IDF, 2002a), dried milk protein products are rehydrated, adjusted to pH 7.00, and then centrifuged at 3,000 ×g at 22°C. Filtration is performed after centrifugation to remove the sediment fraction. The nitrogen content of the supernatant (soluble components) and that of the original aqueous dispersion are determined by the Kjeldahl method, and the quotient was calculated and recorded as NSI (IDF, 2002a).

The Dumas combustion method (Beljkaš et al., 2010; IDF, 2002b) is an alternative method to determine nitrogen in samples. Compared with the Kjeldahl method, the Dumas combustion method is quicker and safer (Beljkaš et al., 2010). The principle of the Dumas combustion method is the conversion of all nitrogen into gaseous nitrogen oxides by complete combustion at 950°C, followed by the reduction of NO_x gasses to N_2 and quantification of N_2 by thermal conductivity (Beljkaš et al., 2010).

Webb et al. (2002) studied protein solubility in aqueous systems for deamidated wheat protein (DWP), sodium caseinate (SC), soy protein isolate (SPI), and WPI. The NSI values are displayed in Table 1-2. Solubility varies in different protein powders, suggesting that they may have different applications.

Table 1-3. Means of solubility[1] of 3% protein dispersions of sodium caseinate (SC), soy protein isolate (SPI), deamidated wheat protein isolate (DWP), and whey protein isolate (WPI)[2]

Functional properties	SC	SPI	DWP	WPI
Solubility (%)	$86.67^{b} \pm 2.89$	$60.67^{c} \pm 1.53$	$79.67^{b} \pm 4.51$	$96.33^{a} \pm 1.15$

[1]Adapted from Webb et al. (2002)

[2]Means with different superscripts, differ. ($P < 0.05$)

1.3.3.2 Heating affects protein solubility

Protein solubility changes with heat treatment in an aqueous state (Zayas, 1997). Dissanayake et al. (2013) studied the solubility of 17.5% whey protein dispersions by heating at 140°C for 20 s (solubility was tested every 5 s) at pH 4, 5 and 6. The results showed a significant drop in solubility during the first 5 s of heating for all whey dispersions (at pH 4, 5, and 6), but the solubility of pH 4 samples remained greater (~ 56%) compared with the samples at pH 5 and 6 (~ 52 and 50%) (Dissanayake et al., 2013), which indicated that pH impacted the solubility. Moreover, effects of heating on proteins in dry state have been studied. Gulzar et al. (2011) reported that after WPI (pH 6.5) was dry-heated at 100°C for 24 h, the percentage of soluble protein (at pH 4.6) decreased from 90 to 23%. Using a modified Lowry method, Ibrahim et al. (1993) reported that the solubility of whey proteins was maintained after exposure to 80°C for 5 days in dry state (7.5% moisture content), although at day 7, the solubility decreased (by 5-8%). Chen et al. (2013) reported that when LH-NDM and HH-NDM were RFDH-treated to 75, 80, 85 and 90°C and held in a convection oven for 125.67, 57.75, 25.0 and 11.50 min, respectively, NSI of all HH-NDM samples was found to significantly decrease by 1.2-3.0% compared with the non-treated HH-NDM except the sample treated to 75°C without any hold time in the oven. When the RFDH temperature was ≥ 80°C, NSI of all LH-NDM samples decreased significantly by 3.4-5.4% compared with the non-treated LH-NDM (Chen et al., 2013).

1.4 Functional properties of proteins

1.4.1 Emulsifying properties

Emulsifying properties are important functional properties of proteins (Euston and Hirst, 2000). The main characteristics used to describe emulsifying properties of proteins can be separated into two categories. The first category is emulsifying activity (EA), which measures

the ability of the protein to disperse the oil phase by estimating the dispersed particle size. The second category is emulsion stability (ES), which is the ability of emulsion droplets to remain dispersed without coalescing, flocculating, or creaming (Hung and Zayas, 1991). Proteins are effective surface-active agents because they can lower the interfacial tension between hydrophobic and hydrophilic components in foods. The stability of an oil-in-water emulsion is controlled by three important parameters: surface coverage, adsorbed layer dimensions, and surface viscosity. In general, emulsions with a higher surface coverage, a higher surface viscosity and more adsorbed layer dimensions would be expected to have a greater stabilizing effect on the emulsion droplets (Euston and Hirst, 2000).

1.4.1.1 Emulsifying properties evaluation

Several methods have been recognized to evaluate EA of proteins, such as a turbidimetric technique first proposed by Pearce and Kinsella (1978), which promoted the emulsifying activity index (EAI) to represent the ability of proteins to form an emulsion. Particle-size analysis can be used to determine ES by monitoring changes in the number and size of the fat particles using a Coulter Counter, which will detect particles as small as 0.5 μm-diameter (Groves and Freshwater, 1968). Emulsion stability can also be measured using multiple light scattering, with a near-infrared light source (Panaras et al., 2011). Kato et al. (1985) developed a simple and reliable method to determine the emulsifying properties, including EA and ES, of proteins from the changes in the conductivity of an emulsion, which was more time-saving compared with the turbidimetric method created by Pearce and Kinsella (1978). Simple linear regression analysis showed a good correlation between the EA determined by both methods with a correlation coefficient of 0.89 ($p < 0.01$). A good correlation was also observed between the ES determined by both methods with a correlation coefficient of 0.91 ($p < 0.01$) (Kato et al., 1985).

1.4.1.2 Heating affects emulsifying properties

Millqvist-Fureby et al. (2001) revealed that when whey protein dispersions are thermally processed between 60 to 90°C for a maximum of 1000 s, the fat droplet sizes increased with increased heat treatment, which might lead to inefficient coverage of fat droplets, causing emulsion instability. Dissanayake and Vasiljevic (2009) compared emulsifying properties of the control and heat-denatured (90°C for 20 min) whey protein retentate samples, after microfluidization at 140 MPa, 1 pass using the Pearce and Kinsella (1978) method. The results showed the EAI of emulsions increased significantly ($P < 0.05$) by 83.6%. Dissanayake and Vasiljevic (2009) explained that thermal-induced irreversible protein denaturation might reduce emulsifying properties, whereas the thermal-induced partial protein unfolding improved its interfacial properties by exposing hydrophobic portions and therefore emulsifying ability.

Ibrahim et al. (1993) reported that both EA and ES of α-La increased by ~50% after dry-heating at 80°C for 5 days using the conductivity method (Kato et al., 1985). Kato et al. (1989) hypothesized that during dry heating, a "molten globule" structure that is partially unfolded and more flexible than the native form may be formed, which in turn increased the surface hydrophobicity due to the exposure of previously hidden hydrophobic domains. This would improve emulsifying properties (Dissanayake and Vasiljevic, 2009). The heat denaturation of proteins was greatly suppressed in the absence of free water (Ibrahim et al., 1993). Whey proteins were completely insolubilized in aqueous solution during heating at 80°C, while the heat treatment at 80°C for 5 days in a dry state resulted in unchanged solubility and greater emulsion stability (~50%) and foaming stability (~140%) of α-La (Ibrahim et al., 1993). This provides evidence that dry-heating can cause different protein behavior compared with heating in an aqueous system.

1.5.4 Rheological properties

Knowledge of viscosity and other rheological properties of protein dispersions such as storage modulus, are of practical significance in food processing and in many other applications, for example, texturization of yogurt, salad dressing, etc. Viscous behavior is particularly important in the processing of fluid foods, such as skim milk, which includes flow rates, pumping capacity, heat exchange, energy input for mixing, etc. (Kinsella and Morr, 1984).

1.5.4.1 Rheological properties evaluation

Apparent viscosities can be measured by rheometers. For liquids, the use of rotational viscometry is popular, which may operate in steady shear or oscillation (Daubert and Foegeding, 2010). Steady shear is a condition in which the sheared fluid velocity remains constant at any single position. Typically a concentric cylinder and the cone and plate are used as test fixtures in steady shear rotational viscometry. Oscillation testing is conducted with amplitude of strain or stress at a constant frequency (Daubert and Foegeding, 2010).

1.5.4.2 Heating affects rheological properties

Jeurnink and De Kruif (1993) measured the viscosity of skim milk heated at 60 and 90°C for various holding times. They reported that the viscosity did not change if heated at 60°C for 600 s, while a 10% increase in viscosity was observed if heated at 90°C for 450 s, after which a plateau was reached. Because no appreciable denaturation of whey proteins occurs below 70°C, as expected, the viscosity did not change after heating at 60°C for 600 s (Jeurnink and De Kruif, 1993). When samples were heated at 90°C, 450 s, effective volume fraction increased, caused by the association of denatured whey proteins with casein micelles, resulting in increased viscosity (Jeurnink and De Kruif, 1993; Bienvenue et al., 2003).

1.5.5 Foaming properties

The property of proteins to form stable foams is important in the production of a variety of foods, such as ice cream, bread, etc. The size distribution of air bubbles in a foam influences the foam product's appearance and textural properties, which results in different smoothness and lightness of food products, from cakes and breads to champagne, beer, meringues and ice cream (Berry et al., 2009). Foam can be defined as a two-phase system consisting of air cells separated by a thin continuous liquid layer called the lamellar phase (Zayas, 1997).

During the whipping process, air comes into the solution to form bubbles and the hydrophobic regions of proteins facilitate the adsorption at the interface, a process that is followed by partial unfolding (surface denaturation). This change in the molecular configuration results in the loss of solubility or precipitation of some proteins. The attendant reduction in surface tension facilitates the forming of new interfaces and more bubbles. These partially unfolded protein molecules then associate to form a stabilizing film around the bubbles, which is essential for the stability of the foam (Lomakina and Mikova, 2006). A good foaming agent should stabilize foams rapidly and effectively at low concentrations, and perform effectively in the medium with foam inhibitors such as fat, alcohol or flavor substances (Zayas, 1997).

Foam can be obtained by a high blending or whipping treatment, as well as injecting air or gas through the protein solution (Ross, 1946). The foaming properties of proteins are influenced by multiple factors: protein structure, temperature, pH, isoelectric point (pI), fat content, protein concentration, mixing time, and method of foaming (Kamath et al., 2008; Lomakina and Mikova, 2006; Marinova et al., 2009; Patel and Kilara, 1990). Marinova et al. (2009) studied foamability of WPC and sodium caseinate (SC) solutions as the pH changed from 3 to the natural pH (6.4-6.8). The SC dispersion had minimum foamability at the isoelectric pH

(~ 4.5), whereas WPC exhibited maximum foamability at pH = 4, which is around the effective pI (~ 4.2).

1.5.5.1 Evaluation of foaming properties

There are two major properties measured to assess foaming properties: foam overrun and stability (Huang et al., 1997). Foam overrun reflects the capacity of protein to generate foam and is measured by the foam volume produced. There are two main methods to determine overrun: bubbling and whipping (Phillips et al., 1987). Bubbling is reproducible, gives uniform bubble size, and allows ease of monitoring of foam formation (Kinsella and Morr, 1984). Whipping is the most common method of making food foams and is much more useful for evaluating practical applications (Kinsella and Morr, 1984). Foam stability reflects the ability to retain the gas for a certain period of time. The measurement of foam stability often focuses on volume collapse of foam during a certain period of time (Ross, 1946) or conductivity decrease with time (Raymundo et al., 1998).

1.5.5.2 Heating affects foaming properties of proteins

According to Dissanayake and Vasiljevic's study (2009), the foaming properties of whey proteins can be detrimentally affected by previous heating in solution. After heating at 90°C for 20 min, foaming capacity of whey protein dispersions decreased from 1,187% to 0%, meanwhile foam stability dropped from 23.7 to 0 s, which suggested that the extensive aggregation of whey proteins caused by heat denaturation may have reduced the ability of proteins to produce a stable film. Ibrahim et al. (1993) revealed that after heating at 80°C for 7 days in a dry state, the foam stability of dry-heated β-Lg increased by ~220%, compared with the control β-Lg. Moreover, fluorometric analysis showed increased fluorescence emission intensity and a red-shift in the wavelength of maximum emission with the increasing heating time, indicating that dry-heated

whey proteins had undergone conformational changes. The results suggested that conformational changes (partially protein unfolding) might be induced by the dry heating and protein-protein interactions might be enhanced due to the formation of soluble aggregates; thus, forming a strengthened foam film (Ibrahim et al., 1993).

1.5.6 Surface properties

Surface properties are important characteristics of emulsifiers and foaming agents. Surface tension (ST) is defined as the amount of energy required to increase the surface area between two phases, while interfacial tension (IT) can be defined as the amount of energy required to increase the interfacial area a specified amount between two immiscible fluids (McClements, 1998). The main difference between ST and IT is the type of phases.

1.5.6.1 Evaluation of surface properties

ST and IT can be measured with a tensiometer. Two types of measurements can be achieved, dynamic and static. Dynamic measurements are carried out on systems that are not at equilibrium, while static measurements are collected on samples that are at equilibrium (McClements, 1998).

The different measuring techniques for ST and IT may influence the results. For example, the dynamic drop number method gives invariably higher results than the static Du Nouy ring method because of the time dependency of diffusion of surface-active components to the interface (Kristensen et al., 1997). By using the Wilhelmy plate method, Kristensen et al. (1997) found the ST of skim milk decreased from 49.72 to 42.22 mN/m when temperature increased from 10°C to 40°C.

1.5.6.2 Heating affects surface properties

Lam and Nickerson (2015) studied the IT between canola oil and a 0.1% w/w α-La dispersion (pH 5.00 or 7.00). Samples were heated at 25, 65 and 95°C for 30 min in a water bath, cooled to 22 ± 1°C, and evaluated for IT using a Du Nouy ring method. They reported that α-La lowered IT by ~ 10% at pH 7.00 and ~ 12% at pH 5.00, when the α-La dispersion was heated to 95°C compared with 25°C. This indicated that heating, together with pH, might induce conformational changes of proteins in dispersions, which influenced the interface properties by increasing the hydrophobicity of proteins.

1.6 Formation and physical properties of milk gels

Gelation of milk proteins is a crucial process in both cheese and yogurt manufacturing. In milk, gelation can be induced by enzyme (chymosin) action, and/or heat. The gel's physical properties, such as water holding capacity (WHC), syneresis, and firmness are very important in evaluating quality of cheese and yogurt (Lucey, 2002).

1.6.1 Syneresis

In set-style yogurt, syneresis is an aspect that affects overall quality of yogurt. Syneresis, or whey separation, is extraction or expulsion of whey from a milk gel, which appears as a liquid on the gel surface. Rearrangement of the network just after gel formation may be responsible for syneresis (Lucey, 2001). Lucey et al. (1998a) reported that high heat treatment (> 75°C) in solution before acidification made gels more brittle and susceptible to rearrangements and fracture, thus increased syneresis.

Syneresis of milk gels can be assessed by several methods including drainage, centrifugation and siphon (Amatayakul et al., 2006). The amount of syneresis could be influenced by method of determination (Lucey et al., 1998a). The siphon method would be more appropriate in the determination of the level of spontaneous whey separation on the surface of set

type yogurt because no external forces are applied on the gel (Lucey et al., 1998a; Amatayakul et al., 2006).

1.6.2 Water holding capacity

The water held in a gel can be divided into two main types: 1) absorbed water, which is bound by the proteins and no longer available as a solvent and 2) retained water, which is trapped in the protein matrix (Kneifel et al., 1991). Although a substantial portion of the water is bound by the proteins, most of the solution is physically entrapped within the 3-dimensional gel network (Blecker et al., 2000). The WHC is a measure of both absorbed and retained water (Kneifel et al., 1991).

Enzymes, solids content, and heating can greatly affect WHC of yogurt (Imm et al., 2000). Imm et al. (2000) reported that WHC of acid gels prepared from transglutaminase-treated skim milk powder (TG-SMP) was significantly higher than that of control skim milk powder (C-SMP) at all solid levels (12, 14, and 16%), with a 47.26, 46.86, and 42.02% increase, respectively. This research indicated that the introduction of new cross-links by TG, which catalyzed an acyl-transfer reaction between glutamine and lysine residues, was responsible for the increased WHC. As solids content increased from 12 to 16%, WHC of C-SMP increased from 34.17 to 47.76%, while that of TG-SMP increased from 50.32 to 67.83% , since the protein network became denser and bound more water. When heating reconstituted SMP at 90°C for 30 min prior to acidification (at 12% solids), WHC of gels prepared from pre-heated C-SMP increased by 9.7% compared with C-SMP; for TG-SMP, WHC of pre-heated samples increased by 9.9% compared with the control samples. According to Lucey et al. (1998c), heat-induced changes in orientation and continuity of protein strands may facilitate immobilization of water, which increased WHC of pre-heated acid gels.

WHC of yogurt gels can be determined by the centrifugation procedure (Parnell-Clunies et al., 1986). Yogurt samples are centrifuged, drained of supernatant, and pellet weights determined. The WHC is expressed as percent pellet weight relative to the original weight of yogurt (Imm et al., 2000; Parnell-Clunies et al., 1986).

1.6.3 Firmness

Firmness is one of the most important textural characteristics of yogurt (Hassan et al., 1996). Adequate firmness without syneresis is essential for a top-quality product (Kroger, 1975). The firmness, also referred as gel strength, of yogurt is related to the cumulative effects of the chemical interactions (Krasaekoopt et al., 2004). The specific association of β-Lg and α-La on the casein micelle surface seems to be responsible for the increase of gel strength (Mottar et al., 1989). The gel strength of yogurt can be measured by an Instron testing machine where maximum force to a penetration limit is measured, or by using a texture analyzer by compression test where peak force is recorded (Krasaekoopt et al., 2004)

1.6.4 Use of glucono-δ-lactone in yogurt manufacturing

Glucono-δ-lactone (GDL) has been used to model the acidification of milk where the hydrolysis of GDL to gluconic acid results in a reduction in pH (Lucey et al., 1998b). The use of GDL in model studies avoids some of the difficulties associated with starter bacteria including variable activity and variation with type of culture used. Model studies on the formation of acid milk gels with GDL have normally used a low gelation temperature (30°C) compared with the usual temperature used for yogurt fermentation, which usually ranges from 40 to 45°C (Lucey et al., 1998b).

1.6.5 Heating affects gel structures

Dry heating strongly affects the gelling properties of whey proteins. Gulzar et al. (2012) dry-heated WPI at 80, 100 and 120°C for 144, 24 and 3h, respectively, to induce a similar level of whey protein denaturation. Then they rehydrated WPI and fractionated to residual native, soluble aggregates, and insoluble aggregates, followed by protein determination using the Lowry method (Lowry et al., 1951). Gulzar et al. (2012) revealed that the percentage of soluble aggregates in WPI solution had a positive correlation (0.83) with WHC in the range 0-80% soluble aggregates. The gel strength of dry-heated proteins increased to a maximum point at 35% soluble aggregates under dry heating conditions (80, 100 and 120°C for 144, 24 and 3h, respectively), then decreased due to excessive denaturation/ aggregation of whey proteins resulting in insoluble aggregates.

Lucey et al. (1998a) studied effects of heat treatment on whey separation in acid skim milk gels using GDL as the acidifying agent. They found that when gelation temperature remained at 35°C, increasing heating temperature of milk to 93°C would increase whey separation in gels by 20% compared with 82.5°C. They concluded that high heat treatment of milk made acid gels unstable and sensitive to spontaneous whey separation (syneresis) since those conditions favor more rearrangement of the network just after formation of gels.

Lucey et al. (1997) used reconstituted skim milk (made from ultra-low-heat skim milk powder) to make acid milk gels. Reconstituted skim milk was heated at 75, 80, 85, and 90°C for 15 min prior to gel formation, and a decreasing trend in apparent yield strain was observed with increasing temperature. The smaller yield strain in gels formed from high-heated milk made these gels more brittle and susceptible to rearrangements and fracture, compared with gels formed from unheated or milk heated at lower temperature. Storage modulus (G') of acid milk

gels made from reconstituted skim milk increased with increasing temperature (75, 80, 85, and 90°C for 15 min) before gelling. This is because the denatured whey proteins can associate with casein micelles and form aggregates, and the number and strength of bonds between protein particles would increase. However, the slight decrease in G’ was observed when skim milk was heated at 90°C for 30 min, compared with the sample heated at 85°C for 30 min. It may have been due to formation of large whey protein–casein aggregates which may alter the process of gel formation during acidification of heated milk.

Chapter 2 - Research Objectives

To determine if nonfat dry milk that has been treated via radio frequency dielectric heating and convection oven heating to deliver a 5-log reduction of *Salmonella* spp. will exhibit changes in functional properties.

Chapter 3 - Materials and Methods

3.1 Experimental design

Extra-grade low heat-nonfat dry milk (LH-NDM) manufactured by Dairy America (1H-41316-01, Fresno, CA), and extra-grade high heat-nonfat dry milk (HH-NDM) manufactured by ConAgra Foods (B150/1000913, Menomonie, WI) were obtained and analyzed for composition (Specification sheets are in Appendix A). Approximately 350 g of HH-NDM and LH-NDM were heated to 75°C, 80°C and 85°C in a radio frequency dielectric heating (RFDH) unit (Food Engineering Lab, University of Nebraska, Lincoln) (Figure 3-1), then held for 115, 52, and 43 min for HH-NDM and 125, 63, and 43 min for LH-NDM, respectively (Michael et al., 2014) (Table 3-1); the control HH-NDM and LH-NDM (HH-C and LH-C) samples were not treated. All samples were taken to Manhattan, KS after RFDH treatments. Samples were analyzed for color, WPNI, and NSI at Kansas State University. Rehydrated samples (3.5% protein, w/v) adjusted to pH 7.00, were evaluated for functional properties. In addition, rehydrated samples were evaluated by SDS-PAGE and Maillard browning analysis by XTT assay. Two measurements were made of each treatment, and the average was reported. NDM samples were split, processed using RFDH on two different days and were regarded as two replications (the third replication was found to have a significantly longer heating time in RFDH unit compared with the first and second replication, so it was not considered as a valid replication). All tests conducted on NDM for each replication was completed within 7 days after RFDH treatment.

3.1.1 RFDH treatment

The RFDH unit (Strayfield Limited, Theale, Berkshire, England) was set at 27.12 MHz frequency and 120 mm electrode distance from the surface of the sample (Figure 3-1), which was different from Michael et al. (2014), where the distance from electrode to the bottom of the

samples was set at 120 mm. The NDM samples were placed in a circular high-density polyethylene tray (0.3 cm thick, 19.0 cm diameter, and 2.7 cm height for LH-NDM and 0.3 cm thick, 22.4 cm diameter, and 2.1 cm height for HH-NDM) (Dynalab Corp., Rochester, NY) (Appendix B), leveled with a straight-edge and placed in the RFDH unit (Figure 3-2). Two circular trays were used in order to treat both HH-NDM and LH-NDM on the same day (using the same tray would introduce another source of variation as it would take 2 days to complete a replication, resulting in an incomplete block design). Two fiber-optic probes (T1, Neoptix Inc., Québec, Canada) were carefully placed in the center and 3 cm to the edge of the plastic tray, and connected to a data logger (Reflex, Neoptix Inc.) to monitor the temperature throughout the RFDH process (Figures 3-3 and 3-4 show temperature vs. time graphs for HH-NDM and LH-NDM from 30 to 75°C, the remaining temperature vs. time graphs are shown in Appendix C). Once the center point achieved the target temperatures, trays holding NDM were immediately transferred to a convection oven (Thelco, GCA/Precision Scientific, Schaar Scientific Company, Chicago, IL) (Figure 3-5) pre-set at the designated temperatures for the designated holding times (Table 3-1). Treatment conditions were based on a 5-log reduction of *Salmonella* spp. (calculations are shown in Appendix D), according to Michael et al. (2014).

RFDH treatment (the overall heating) includes two steps: 1. heating NDM to the target temperatures in the RFDH unit; 2. transferring the NDM to a convection oven and holding it for the designated times. The hold times in the oven for NDM are displayed in Table 3-1. The time to reach the target temperatures in the RFDH unit was a monitored variable.

Table 3-1. Hold times* in a convection oven for high heat (HH) and low heat (LH) nonfat dry milk (NDM) based on a 5-log reduction of *Salmonella* spp.

	Holding time (min)			
	Control	75°C	80°C	85°C
HH-NDM	0	115	52	43
LH-NDM	0	125	63	43

*Calculated based on Michael et al. (2014).

Figure 3-1. Radio frequency dielectric heating unit (Strayfield Limited, Theale, Berkshire, England) at University of Nebraska-Lincoln.

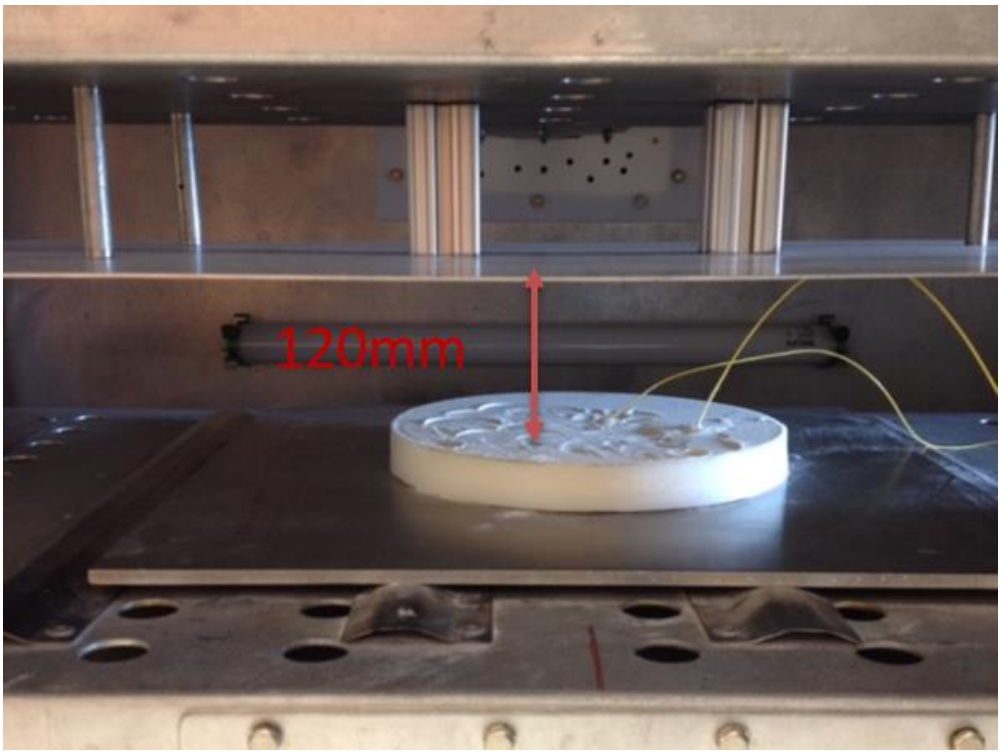
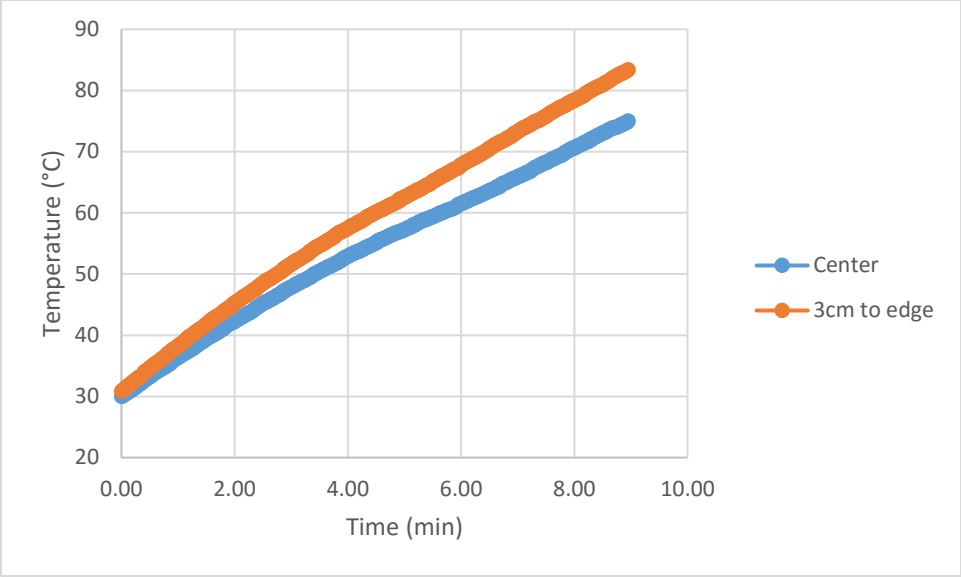
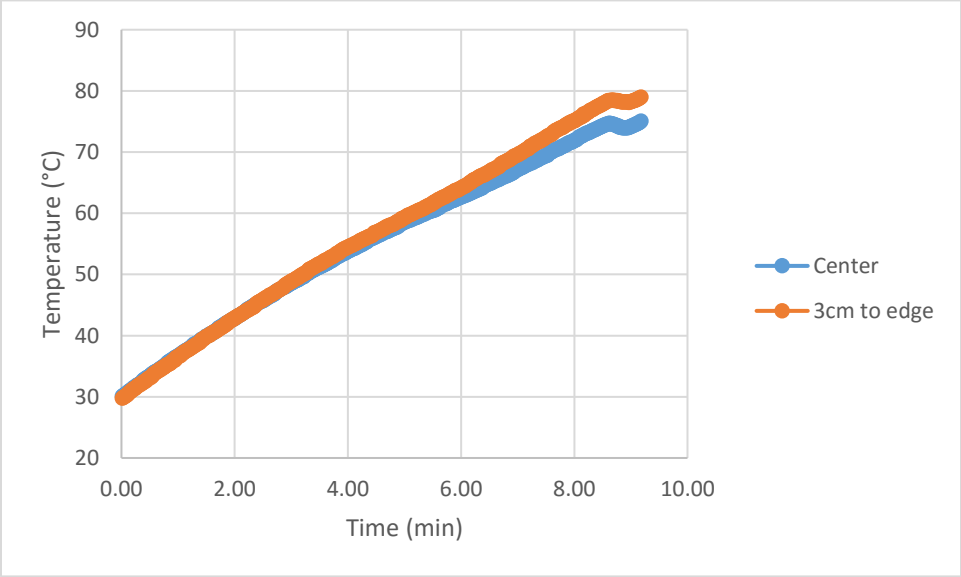


Figure 3-2. Radio frequency dielectric heating unit (Strayfield Limited, Theale, Berkshire, England) with approximately 350 g of nonfat dry milk samples placed in a circular plastic tray (Dynalab Corp., Rochester, NY). Two fiber-optic probes (T1, Neoptix Inc. Québec, Canada) were placed in the center and 3 cm to the edge of the tray. Red arrow shows the distance from the electrode probe to the surface of nonfat dry milk samples.

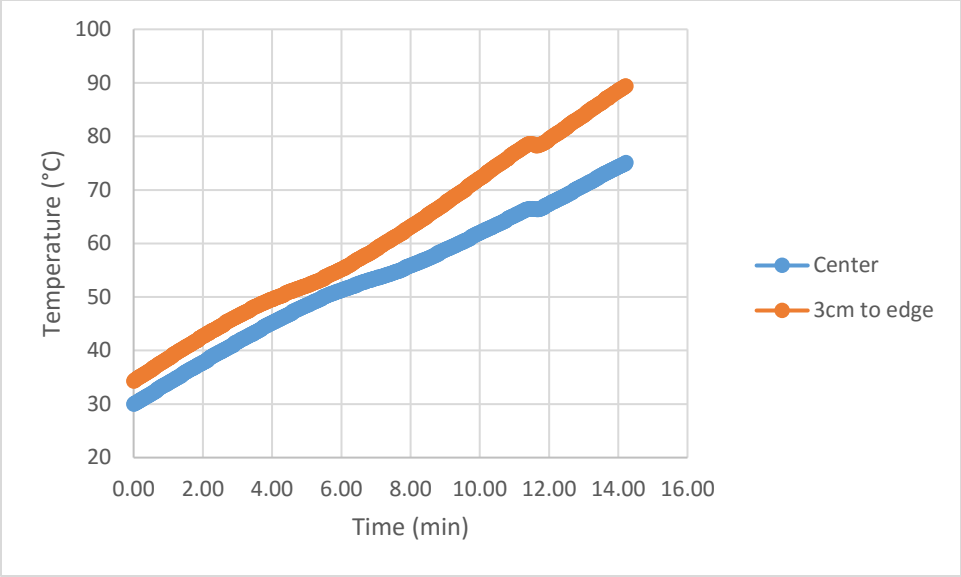


(a)

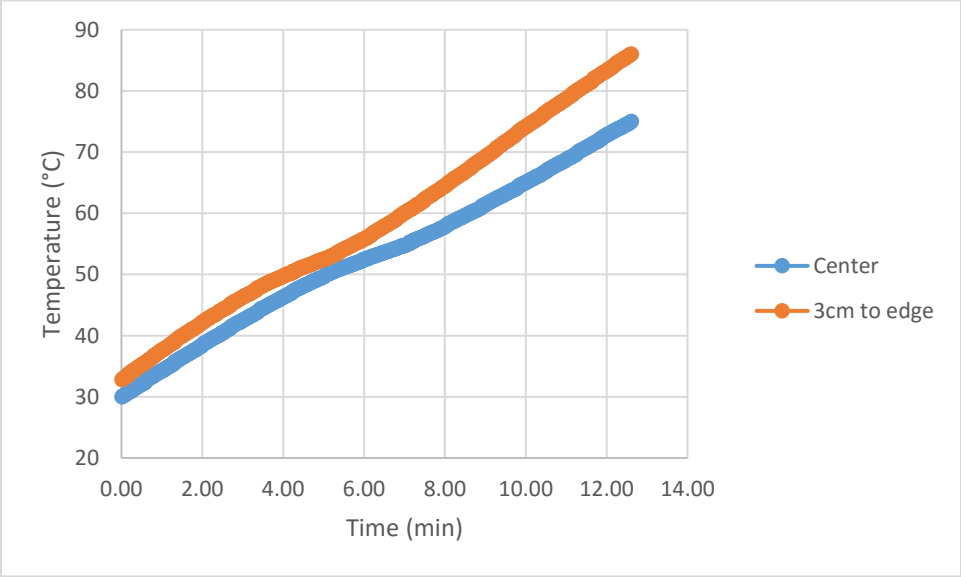


(b)

Figure 3-3. Radio frequency dielectric heating of high heat nonfat dry milk from 30 to 75°C (target temperature for the center probe) in a circular polypropylene tray. The legend shows the location of two fiber-optic probes for monitoring temperatures. (a) Rep 1; (b) Rep 2.



(a)



(b)

Figure 3-4. Radio frequency dielectric heating of low heat nonfat dry milk from 30 to 75°C (target temperature for the center probe) in a circular polypropylene tray. The legend shows the location of two fiber-optic probes for monitoring temperatures. (a) Rep 1; (b) Rep 2.

Figure 3-5. The convection oven (Thelco, GCA/Precision Scientific, Schaar Scientific Company, Chicago, IL) used to hold nonfat dry milk for designated times at University of Nebraska - Lincoln.

3.1.2 Collection and Storage of Samples

After the hold period ended, ~200 g of NDM were removed from the center of the tray, cooled to 23 ± 1°C and double-bagged in re-sealable polyethylene bags (0.93L Ziploc® freezer bags, S.C. Johnson & Son, Inc., Racine, WI), labeled, and stored in environmental incubator (Equatherm®, Lab-Line Instruments, Inc., Melrose Park, IL) in Manhattan at -2 ± 1°C until assessment.

3.2 NDM: compositional analysis

3.2.1 Nitrogen

Total nitrogen of the control LH-NDM (LH-C) and HH-NDM (HH-C) was determined according to IDF Standard 185 (IDF, 2002b) by the combustion method using a LECO® FP-2000 Nitrogen/Protein Determinator (Laboratory Equipment Co., St. Joseph, MI). The crude protein was calculated by a multiplication factor of 6.38 (IDF, 2006) (Shown in Appendix E).

3.2.2 Moisture

Moisture contents of LH-C and HH-C were determined using the forced air drying method (AOAC International, 2002; method 990.20) using an Isotemp atmospheric oven (Model 750F, Fisher Scientific, Pittsburgh, PA).

3.2.3 Ash

Ash contents of LH-C and HH-C were determined by ignition at 550°C in a Thermolyne™ tabletop muffle furnace (Cat. F30428C, Thermo Fisher Scientific) following AOAC method 954.46 (AOAC International, 2002).

3.2.4 Fat

Ten grams of NDM were rehydrated with 90 g of DI water. Fat contents were measured by microwave drying followed by nuclear magnetic resonance using the CEM, SMART Trac™

Fat and Moisture Analyzer (CEM Corporation, Matthews, NC), following the method specifically created for skim milk (Samarakoon, 2016).

3.2.5 Carbohydrates

The carbohydrates content was calculated by difference (AOAC International, 2002).

$$Carbohydrate\% = 100\% - protein\% - moisture\% - fat\% - ash\% \quad (1)$$

3.3 Protein characterization

3.3.1 Whey Protein Nitrogen Index (WPNI)

Standard reference LH-NDM and HH-NDM samples (~125 g each) were obtained from American Dairy Products Institute (ADPI) (Elmhurst, IL) with whey protein nitrogen values of 7.74 mg/g and 0.63 mg/g, respectively (Shown in Appendix A). A standard curve [transmittance (T%) vs. WPN (mg/g)] was prepared using the standard reference NDM samples and ADPI method for WPNI (ADPI, 2009). The standard curve equation was calculated as $y = -5.6505x + 99.818$ ($R^2 = 0.99$) by Excel 2013 (Microsoft, Redmond, WA) (shown in Appendix F). This standard curve was used for all samples in this experiment.

3.3.2 Nitrogen Solubility Index (NSI)

Nitrogen solubility index was estimated according to IDF Standard 173 (IDF, 2002a). NDM was rehydrated with deionized (DI) water to 3.5% (w/v) protein. LH-NDM (9.67g) and HH-NDM (9.91g) were transferred separately to 100 ml glass beakers and 75 ml DI water was added into each beaker. Dispersions were centrifuged using Marathon 21000R (Fisher Scientific) at 3,000g and 22°C for 10 min. The supernatant was filtered using Whatman™ No.1 filter papers (Cat No. 1001-150, GE Healthcare UK Limited, Buckinghamshire, UK), and the nitrogen contents of supernatant and original dispersion were determined using a Leco FP-2000 protein

analyzer (Laboratory Equipment Co.) according to IDF Standard 185 (IDF, 2002b). NSI was calculated as:

$$NSI\% = \frac{Nitrogen\ content\ of\ supernatant}{Nitrogen\ content\ of\ original\ dispersion} \times 100 \qquad (2)$$

3.3.3 SDS-PAGE

Proteins of the NDM dispersions were analyzed by SDS-PAGE according to Liu et al. (2012) with some modifications. Two types of diluted NDM dispersions (with or without 2-mercaptoethanol) were prepared to compare on the same polyacrylamide gel. 2-Mercaptoethanol (BME) (0.05%) (60242, ACROS Organics™, Geel, Belgium), which is used as a reducing agent, can be used to determine if additional protein aggregation occurred during the RFDH treatment (LH-NDM and HH-NDM were on separate gels). The NDM dispersions were diluted to 2 mg/ml protein with the 2× Laemmli sample buffer (pH 6.8 at 20°C) (#161-0737, Bio-Rad Laboratories, Inc., Hercules, CA) with or without 0.05% BME. Diluted samples were heated for 5 min at 95°C using an AccuBlock™ digital dry bath (Model D1100, Labnet International, Inc., Woodbridge, NJ) and immediately cooled in a freezer at -18°C (Westinghouse 18.0 Cu. Ft. Top Freezer Refrigerator, Westinghouse Electric Corporation, Pittsburgh, PA) for 1-2 min. After vortex mixing (Labnet VX100, Labnet International, Inc., Edison, NJ), 15µl of samples were loaded in the wells of 4-15% Mini-PROTEAN® TGX™ precast polyacrylamide gel (Cat. 456-1083, Bio-Rad Laboratories, Inc). Seven µl of Precision Plus Protein™ Dual Color Standards (Cat. 161-0374, Bio-Rad Laboratories, Inc.) were loaded as the marker. The gel sat in diluted 10× Tris/Glycine/SDS running buffer (Cat. 161-0732, Bio-Rad Laboratories, Inc.), and electrophoresis was performed at 120 V for 1.5 hr using a PowerEase™ 500 Power Supply (Novex, San Diego, CA) as the power source. After electrophoresis was completed, the gel was washed for 3-5 min with 100-200 ml of lab grade double distilled (DD) water (109 Call Hall,

Kansas State University). Fifty milliliters of Bio-Safe™ Coomassie G-250 Stain (Cat. 161-0786, Bio-Rad Laboratories, Inc) was added and the gel was stained for 1 hr with gentle shaking. The stain was washed out with DD water and the gel was placed in DD water at 4 ± 1°C (Westinghouse 18.0 Cu. Ft. Top Freezer Refrigerator, Westinghouse Electric Corporation) overnight. The SDS gel was digitally scanned and quatified using a Bio-Rad software Gel Doc XR + Imager (Bio-Rad Laboratories, Inc) as described by Liu et al. (2012), and the molecular weight for each band was determined by a software GelAnalyzer 2010 (GelAnalyzer.com) (Appendix G).

3.4 Color and browning reaction analysis

3.4.1 Color

The NDM samples were evaluated for color using a Hunter Lab MiniScan EZ 4500L spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA) on the CIE L*, a*, and b* scale. The spectrophotometer was calibrated with the instrument's standard tile (X: 80.49 Y: 85.30 Z: 88.35) (Serial No. MSEZ0396, Hunter Associates Laboratory, Inc.). A 100×15mm sterile polystyrene petri dish (Fisher Scientific) was filled with NDM powders without pressing, and the surface was leveled using a straight edge, then three readings were taken at every 120° angle and averaged. Color difference (ΔE^*) was calculated as described by Sun-Waterhouse et al. (2010).

$$\Delta E^* = \left[\left(L_1^* - L_2^*\right)^2 + \left(b_1^* - b_2^*\right)^2 + \left(a_1^* - a_2^*\right)^2\right]^{1/2} \quad (3)$$

Where L_1^*, L_2^* represent lightness from black (0) to white (100) for the control sample and the heated sample, respectively; a_1^*, a_2^* represent red (+) or green (-) for the control sample

and the heated sample, respectively; b_1^*, b_2^* represent yellow (+) or blue (-) for the control sample and the heated sample, respectively.

3.4.2 Reduction of tetrazolium salt XTT

Reduction of tetrazolium salt 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro) benzensulfonic acid hydrate (XTT) was measured according to Shimamura et al. (2000) with slight modifications. Six hundred μl of the 0.5mM XTT sodium salt (X4626, Sigma-Aldrich, Co., St. Louis, MO) (rehydrated with deionized water) and 400 μl of NDM dispersions (3.5% protein; pH 7.00) were added into Fisherbrand™ polystyrene, disposable, plastic cuvette (1.5 ml) (Cat. 14955127, Fisherbrand™, Fisher Scientific) and mixed well. The wavelength of UV-Visible wavelength spectrophotometer (Genesys5, Thermo Electron Corporation, Madison, WI) was adjusted to 492 nm and zeroed with 0.5 mM XTT. The absorbance of dispersion was recorded at 492 nm. Then the wavelength was changed to 600 nm, again zeroed with 0.5 mM XTT and the reading of absorbance was recorded. After 20 min, the absorbance was read again at 492 nm and 600 nm, respectively, as described above.

$$Reduction\ of\ XTT = \left(A_{492}^{0} - A_{600}^{0}\right) - \left(A_{492}^{20} - A_{600}^{20}\right) \qquad (4)$$

Where A_j^i means absorbance at i min and j nm of the dispersions.

3.5 Functionality of rehydrated nonfat dry milk

3.5.1 Rehydration of NDM

Nonfat dry milk was rehydrated with DI water to 3.5% (w/v) crude protein content. LH-NDM (48.35g) and HH-NDM (49.55g) were carefully transferred separately to 1000 ml glass beakers and 400 ml DI water was added into each beaker. Beakers were placed on hot plates (Fisher Scientific) and constantly mixed at 600 rpm with a magnetic stir bar (Fisher Scientific). Dispersions were stirred for 1hr at 24 ± 1°C with beakers covered by aluminum foil (Reynolds

Kitchens, Richmond, VA). Protein dispersions were stored at 4 ± 1°C (Westinghouse 18.0 Cu. Ft. Top Freezer Refrigerator) overnight before the test day.

3.5.2 pH

On the test day, pH of samples were determined using a pH/mV/Ion meter (Accumet® portable AP63, Fisher Scientific) that had been calibrated with standardized buffer solution, pH 4.00 and 7.00 (S25849A/B, Fisher Science Education), and then adjusted to 7.00 ± 0.01 with 0.1N sodium hydroxide (SS276-1, Fisher Scientific), made to the mark with DI water in 500 ml volumetric flasks. Dispersions were used immediately for the following tests.

3.5.3 Emulsion activity (EA) and emulsion stability (ES)

EA and ES were determined using the method described by Kato et al. (1985) with the following modifications. The dispersions were diluted to 0.35% (w/v) with DI water at 24 ± 1°C, homogenized with corn oil (Mazola, ACH Food Company, Inc., Memphis, TN) at a 3:1 ratio using a handheld homogenizer (CAT X120, PolyScience, Niles, IL) at speed 1 for 1 min. Fifteen seconds after homogenization, the conductivity was measured on a conductivity/TDS/°C/°F meter (Accumet® AP75, Fisher Scientific), which had been calibrated by Traceable® conductivity standard certified reference material (Cat. 09-328-2/3, Fisher Scientific). Readings were recorded at 1, 2, 3, 4, and 5 min. EA and ES were calculated (Kato et al., 1985);

$$EA = C_0 - C_1 \quad (5)$$

$$ES = 4(C_0 - C_1)/(C_5 - C_1) \quad (6)$$

Where C_0, C_1, C_5 represents conductivity at 0, 1, and 5 min, respectively.

3.5.4 Apparent viscosity

A rheometer (Model ATS-CO22, ATS RheoSystems®, Bordentown, NJ) equipped with cup and bob was used to measure viscosity. Eleven ml of reconstituted NDM was used in each

measurement. Pre-measurement temperature was set at 24°C, equilibrium time was 20.0 s. Measurement settings were as follows: measurement interval 2.000E+1 s; shear rate 1.000E+0 – 1.000E+2 1/s; delay time 1.000E+1 s; integration time 1.000E+1 s; approach to steady state +- 0.010; regulator strength 100.0%. After each measurement was completed, a plot of shear stress (mPa) vs. shear rate (s^{-1}) (intercept was set at 0) was generated using Excel 2013 (Microsoft), the slope was recorded as apparent viscosity (Kristensen et al., 1997). Graphs are shown in Appendix H.

3.5.5 Foaming properties

Overrun was measured according to the method of Berry et al. (2009) using a KitchenAid® Mixer (Model K45SSWH, Kitchen Aid, St. Joseph, MI) with a wire whip attachment (K45WW, KitchenAid®). One hundred milliliters of dispersion were poured into a 4.3 L stainless steel bowl (KitchenAid®) and whipped for 15 min at a speed setting of 10 at 24 ± 1°C. Overrun was determined by the following formula using a 59 ml soufflé cup:

$$\%Overrun = \frac{Wt\ of\ \ protein\ dispersion - Wt\ of\ \ foam}{Wt\ of\ \ foam} \times 100\,\% \qquad (7)$$

Foam stability was evaluated according to Webb et al. (2002) with slight modifications. After 15 min of whipping, foam was transferred into a 59 ml soufflé cup (Solo® Brand Products Co., Lake Forest, IL) and foam stability was reported as the time (min) for 50% of the initial weight of foam to drain at 24 ± 1°C.

3.5.6 Surface properties

Surface and interfacial tension between corn oil (Mazola, ACH Food Companies, Inc.) and protein dispersion were measured using the DuNouy drop ring tensiometer (Model 70545, CSC Precision Tensiometer, CSC Scientific Co., Inc., VA) at 24 ± 1°C (Webb et al., 2002). Twenty ml of dispersion was placed in a 50 ml glass beaker, transferred to the tensiometer

platform at 24 ± 1°C, and evaluated for surface tension after 20 min. Twenty mL of corn oil (Mazola) was added carefully into the 50 ml glass beaker and was set at 24 ± 1°C for 30 min before the measurement of interfacial tension. The correction factor was calculated by the following formula:

$$F = 0.725 + \sqrt{0.01452 \times P / \left(C^2 \left(D - d\right)\right)} + 0.04534 - 1.679 r / R \qquad (8)$$

Where F = correction factor, P = apparent surface or interfacial tension (dynes/cm), C = 6.104 (cm), D = density of the lower phase (g/cm^3), d = density of the upper phase (g/cm3), r/R = 1/49.6 (cm) (CSC Scientific Co., Inc).

Densities of the samples were determined using a Fisherbrand^{TM} pycnometer (5 × 2.5 cm) (Cat. 03247Q, Fisher Scientific) at 24 ± 1°C (Webb et al., 2002). The pycnometer was weighed, tared, and filled with distilled water or sample, which had been equilibrated at 24 ± 1°C for 2 h before weighing. The calculation is as follows:

$$Density = \frac{Wt\ \ pycnometer\ and\ the\ sample - Wt\ \ dry\ \ pycnometer}{Wt\ \ pycnometer\ and\ \ water - Wt\ \ dry\ \ pycnometer} \qquad (9)$$

3.6 Physical properties of acid milk gels

3.6.1 Formation of an acid milk gel

Rehydrated NDM was prepared at 3.5% of protein with DI water and sodium azide (S227I-1, Fisher Scientific). The final concentration of sodium azide was 0.1% (w/v) to prevent bacterial growth (Lucey et al., 1998a). The beaker was covered with aluminum foil (Reynolds Kitchens) and placed in a water bath (Isotemp 220, Fisher Scientific) at 30 ± 1°C. When the samples reached 30°C (~10 min), 1.3% (w/v) delta-gluconolactone (GDL) (271050010, ACROS Organics^{TM}, Springfield Township, NJ) was added to the dispersion, which was constantly stirred with a glass rod for 2 min. Twenty ml of acidified milk sample was transferred into 50 ml

polypropylene centrifuge tubes (Cat. 05-539-8, Fisherbrand™, Fisher Scientific) for evaluation of water-holding capacity (WHC). Eighty ml of acidified milk sample was transferred into each of two 120 ml specimen containers (Cat. 16-320-731, Fisherbrand™, Fisher Scientific) for determination of syneresis and firmness. All samples were incubated for 12 hr at 30°C (final pH 4.60 ± 0.05) in an Isotemp incubator (Model 650D, Fisher Scientific) (Lucey et al., 1998a) and then placed at 4 ± 1°C (Westinghouse 18.0 Cu. Ft. Top Freezer Refrigerator) for 2 hr. According to Lucey et al. (1998a), incubating 3.9% protein dispersion (reconstituted NDM) with 1.3% GDL at 30°C took 16 hr to reach pH 4.5. Less protein content (3.5%) and higher target pH (4.6) in the current study reduced incubation time by 4 hr compared with Lucey et al. (1998a).

3.6.2 Water-holding capacity

Polypropylene centrifuge tubes (50 ml) (Fisher Scientific) with 20 ml of GDL-added dispersion were removed from the refrigerator (Westinghouse Electric Corporation). WHC was determined according to Parnell-Clunies et al. (1986). Samples were centrifuged (Centrifuge Model J2-21, Beckman) at 13,500 ×g for 30 min at 4°C, drained of supernatant for 10 min, and pellet weight determined. The WHC was calculated as percent pellet weight relative to the original weight of the acid milk gel.

$$WHC\% = (W_2 - W_0)/(W_1 - W_0) \times 100 \qquad (10)$$

Where W_0 is the weight of the tube

W_1 is the weight of tube and initial sample weight

W_2 is the weight of tube and pellet

3.6.3 Syneresis

Syneresis was determined according to Amatayakul et al. (2006) with slight modifications. The specimen containers (Fisher Scientific) filled with 80 ml of GDL-added

dispersion were removed from a refrigerator (Westinghouse Electric Corporation) after storage for 2 hr. Samples were weighed, and kept at an angle of 8° instead of 45° to prevent surface cracking of gels, which destroyed the gel matrix. After storage at 4 ± 1°C for 2 hr, the expelled whey was siphoned with a syringe (Cat. 14-823-435, Fisher Scientific) within 20 s and the remaining gel and container was weighed. Syneresis was calculated as follows:

$$Syneresis = (W_1 - W_2)/(W_1 - W_0) \times 100 \qquad (11)$$

Where W_0 is the weight of container

W_1 is the weight of container and initial sample weight

W_2 is the weight of container and sample after draining

3.6.4 Firmness

The specimen containers (Cat. 16-320-731, Fisherbrand™, Fisher Scientific) that were filled with 80 ml of GDL-added dispersion were removed from a refrigerator (Westinghouse Electric Corporation) after storage for 2 hr. A texture analyzer (Stable Micro System, Model TZ-XT2, Texture Expert, UK) was used to determine the firmness of the acid milk gels. A 25 mm acrylic probe was attached to the texture analyzer and force calibration was performed using a 300 g weight. “TA settings” were chosen as follows: test velocity at 2.0 mm/s, distance at 5.0 mm. The sample was placed on the platform centered with the probe then a test was run. After analysis was done, “extrusion” macro was selected to obtain the value of firmness using the manufacturer software provided (Stable Micro System).

3.7 Statistical analysis

All data were analyzed using SAS v. 9.2 (SAS Institute Inc., Cary, NC). Compositional differences between low heat (LH) and high heat (HH) nonfat dry milk (NDM) were analyzed

using Tukey's significant difference test (Kuehl, 1999), with three repeated measurements (n = 3).

A two-way ANOVA for RFDH (control, 75, 80, and 85°C) and NDM (HH and LH) was conducted. Significant interactions (NDM and RFDH) were compared by Tukey's significant difference test (Kuehl, 1999). Significant main effects for NDM and RFDH were determined using Tukey's significant difference test, and were blocked by replication (n = 2). Significance for all tests was completed at a $p \leq 0.05$.

Lastly, one-way ANOVA for RFDH in HH-NDM and LH-NDM were individually conducted. Significant means were differentiated with Tukey's significant difference test, blocked by replication (n = 2).

Chapter 4 - Results and Discussion

4.1 NDM: compositional analysis and classification

Composition analyses (total protein, moisture, fat, ash, carbohydrates) based on weight of powder, NSI, and WPNI of control HH-NDM (HH-C) and LH-NDM (LH-C) are displayed in Table 4-1. Tukey's significant difference tests were used to analyze the compositional difference between HH-C and LH-C. LH-C had slightly more protein (2.5%), moisture (54.3%), but less carbohydrates (4.14%) compared with HH-C. The WPNI and NSI varied in LH-C and HH-C as well. According to ADPI (2009), Standard Grade NDM must not exceed 5% moisture nor 1.5% of fat. Both LH-C and HH-C met these standards. However, as the fat contents for both NDM exceeded 1.25%, these powders would not meet Extra Grade Standards (Table 4-1).

Table 4-1. Composition analyses, nitrogen solubility index, and whey protein nitrogen index of the control high heat (HH-C) and low heat (LH-C) nonfat dry milk[1]

Item	HH-C	LH-C
Total protein (%)	$35.30^{b} \pm 0.01$	$36.19^{a} \pm 0.06$
Moisture (%)	$2.45^{b} \pm 0.04$	$3.78^{a} \pm 0.06$
Fat (%)	1.33 ± 0.06	1.27 ± 0.12
Ash (%)	7.74 ± 0.03	7.78 ± 0.01
Carbohydrates (%)	$53.18^{a} \pm 0.04$	$50.98^{b} \pm 0.13$
NSI[2] (%)	96.3 ± 0.3^{b}	98.5 ± 0.1^{a}
WPNI[3] (mg/g)	1.78 ± 0.02^{b}	7.30 ± 0.04^{a}

[a-b]Means within a row with different superscripts, differ ($p \leq 0.05$).

[1]Mean ± SD, n = 3.

[2]Nitrogen solubility index.

[3]Whey protein nitrogen index.

Nonfat dry milk is classfied into high heat, medium heat, and low heat based on heat treatment before spray drying. The WPNI values for LH-C and HH-C are shown in Table 4-1. LH-C met the standard classification for LH-C (> 6.0 mg/g), but HH-C exceeded 1.5 by 0.28 mg/g, which was slightly higher than the stated WPNI value for HH-C. Since WPNI value is calculated based on the weight of NDM powder, variations in the percentage of total protein could affect the WPNI values. The WPNI was about 3 × greater in LH-C than HH-C, while the NSI in LH-C was only 0.02 × greater than the HH-C (Table 4-1). The different process conditions during manufacture may cause this level of variation in WPNI and NSI of LH-C and HH-C. According to Bylund (1995), LH-NDM receives a cumulative heat treatment ≤ 160°F for 2 minutes, while HH-NDM is preheated to 190°F for 30 minutes during drying. Higher temperature and extended holding times used during HH-C manufacture is known to induce greater whey protein denaturation, resulting in lower WPNI values (Table 4-1). According to Bienvenue et al. (2003), greater denaturation of whey proteins facilitates associations between whey proteins, especially β-lactoglobulin (β-LG), with casein micelles to form larger aggregates (soluble/insoluble), which in turn lowers protein solubility compared with LH-C.

4.2 Heating times in the RFDH unit and oven

Times required for HH-NDM and LH-NDM to reach the target temperatures in the RFDH unit are shown in Table 4-2. The replications were similar ($p = 0.1123$) (Table L-1 in Appendix L). LH-NDM had 53.4% longer heating time in the RFDH unit compared to HH-NDM ($p < 0.0001$), perhaps due to the differences in composition (Table 4-1), leading to differences in dielectric properties between HH-NDM and LH-NDM. However, Michael et al. (2014) reported that the time required for NDM to increase from ~ 27°C to the target temperatures (75, 80, or 85°C) ranged from 4.26 to 4.95 min, which was much shorter than the current study. According

to Michael et al. (2014), the distance from the electrode to the bottom of the NDM sample was set at 120 mm, however, in this study, the system was set so that 120 mm was the distance from the electrode to the surface of NDM samples. This greater electrode distance might have caused longer heating times in the RFDH unit, and may need to be investigated in a future study.

Table 4-2. Times* required for high heat (HH) and low heat (LH) nonfat dry milk to reach the target temperatures (monitored by center probe) in the radio frequency dielectric heating (RFDH) unit

		Time (min)	
Temperature (°C)	Rep	HH	LH
75	1	8.95	14.22
	2	9.18	12.62
80	1	9.92	16.13
	2	9.77	15.48
85	1	11.07	17.12
	2	10.97	16.27

*Time required to reach the target temperature, starting from 30 ± 0.1°C.

Figure 4-1 displays the total heat exposure times including times starting from 30 ± 0.1°C to designated temperatures in RFDH unit (red bars) and oven hold times (blue bars). However, Chen et al. (2013) set the starting temperature at 22°C while Michael et al. (2014) set that at 27°C. The interaction between RFDH temperature and NDM was significant ($p < 0.0001$). The overall heating times (RFDH unit + oven) were different for each sample. At each temperature, the overall heating time for LH-NDM was greater than that for HH-NDM. And for each powder,

overall heating times were different depending on treatment temperatures. These differences are expected as the RFDH time/temperature treatments were identified as to provide a 5-log reduction of *Salmonella* spp. (Michael et al., 2014).

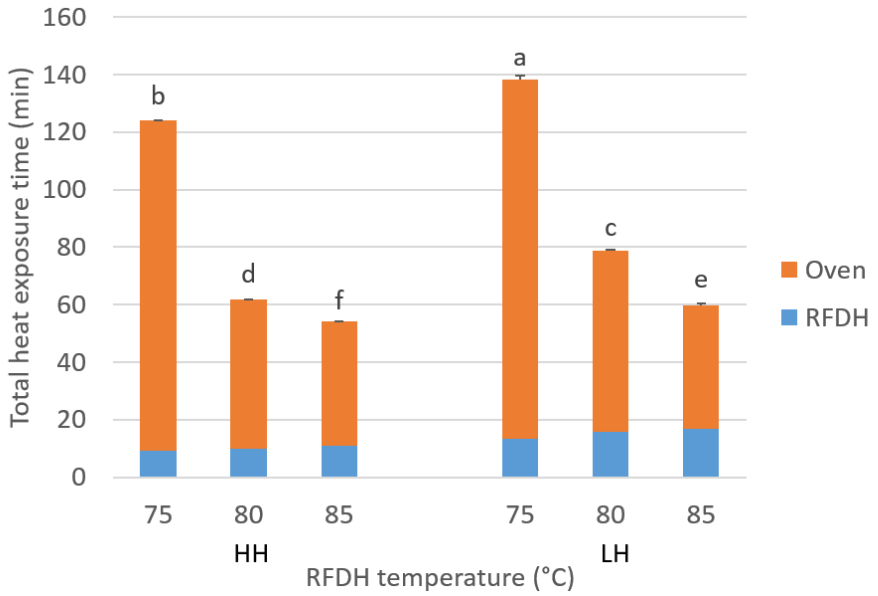


Figure 4-1. Total heat exposure times (min) including radio frequency dielectric heating (RFDH) and convection oven hold times for high heat (HH) and low heat (LH) nonfat dry milk from 30 ± 0.1°C to 75, 80, or 85°C.

Bars (n = 2) with different superscripts, differ ($p < 0.05$) (Appendix K).

Error bars represent standard deviations.

4.3 P values

The p values from the two-way ANOVA for physico-chemical and functional properties are summarized in Table 4-3. P values for NDM were < 0.05 for all measurements except for a*, b*, emulsion activity and emulsion stability, suggesting HH-NDM and LH-NDM differed in most physico-chemical and functional properties. On the other hand, the RFDH effect was significant for WPNI, viscosity, surface tension, gel water holding capacity (WHC), gel firmness, and color b* ($p < 0.05$). Only 3 properties had a significant interaction between NDM and RFDH, these are WPNI, WHC (gel), and b* (color), which suggested LH-NDM and HH-NDM might behave differently under RFDH treatments.

Based on a recommendation from the Statistical Consulting Lab (Room 11, Dickens Hall, Kansas State University, Manhattan, KS), variables with significant interactions were analyzed using Tukey's significant difference test to compare all LH-NDM and HH-NDM samples. Variables without significant interactions were analyzed for significant main effects, which were also differentiated by Tukey's significant difference test (Kuehl, 1999).

Table 4-3. Two-way ANOVA - p values for NDM[1], RFDH[2], NDM*RFDH for various physico-chemical and functional properties

		P value		
System	Property	NDM	RFDH	RFDH*NDM
Powder				
	L*	0.0005**	0.1395	0.1872
	a*	0.5417	0.8601	0.2461
	b*	0.0658	0.0012*	0.0138*
	WPNI[3]	< 0.0001***	< 0.0001***	< 0.0001***
	NSI[4]	< 0.0001***	0.7518	0.8863
Dispersion (pH adjusted)				
	Foam stability	0.0025*	0.1817	0.9867
	Overrun	< 0.0001***	0.0629	0.7076
	IT[5]	0.0211*	0.4140	0.4506
	ST[6]	0.0123*	0.0081*	0.5175
	EA[7]	0.7616	0.8576	0.8403
	ES[8]	0.1195	0.0680	0.7234
	Viscosity	0.0236*	0.0491*	0.4820
Dispersion				
	pH	0.0034*	0.3690	0.3690
	XTT[9]	0.0001**	0.5859	0.0941
Gel				
	Firmness	< 0.0001***	0.0002**	0.6905
	Syneresis	0.0191*	0.1435	0.2054
	WHC[10]	< 0.0001***	0.0022*	0.0017*

[1]Nonfat dry milk.
[2]Radio frequency dielectric heating.
[3]Whey protein solubility index.
[4]Nitrogen solubility index.
[5]Interfacial tension.
[6]Surface tension.
[7]Emulsion activity.
[8]Emulsion stability.
[9]Reduction of tetrazolium salt.
[10]Water holding capacity.
*P < 0.05.
**P < 0.001.
***P < 0.0001.

4.4 WPNI

According to Table 4-3, RFDH and NDM had a significant interaction for WPNI ($p < 0.05$), so the pairwise comparisons are shown in Table 4-4. The RFDH-treated LH-NDM samples had less WPNI compared with the LH-C. WPNI for LH-NDM samples treated at 75°C, 125 min (LH-75) and 80°C, 63 min (LH-80) decreased by 11.4 and 9.8% compared with the LH-C, between which no significant difference was found, while WPNI for LH-NDM treated at 85°C, 43 min (LH-85) decreased by 5.7% compared with the LH-C (Table 4-4). Because WPNI is an index which reflects undenatured (native) whey proteins (ADPI, 2009), the results indicated that some native whey protein may have denatured during this dry heating process. Since higher values of WPNI would indicate greater amounts of undenatured whey proteins, LH-85 had less whey protein denaturation than LH-75 and LH-80 (Table 4-4), which suggests that the time may be a more important factor than the temperature to induce whey protein denaturation, when the temperature exceeds the minimum whey protein denaturation temperature.

The WPNI values for all HH-NDM samples were less than those of LH-NDM samples, and RFDH did not influence the WPNI for HH-NDM (Table 4-4). The possible reason is that HH-C had much less undenatured whey proteins (1.78 ± 0.02) compared with LH-C (7.30 ± 0.04), the changes of which was too minor to be detected (Table 4-1).

Table 4-4. Whey protein nitrogen index (WPNI) for high heat (HH) and low heat (LH) nonfat dry milk (NDM) as a function of radio frequency dielectric heating treatment, resulting in a 5-log reduction in *Salmonella* spp.

	Temperature (°C)	Oven hold time (min)	WPNI (mg/g)[1]
HH	Control	0	1.79 ± 0.01[d]
	75	115	1.89 ± 0.01[d]
	80	52	1.90 ± 0.08[d]
	85	43	1.97 ± 0.13[d]
LH	Control	0	7.31 ± 0.06[a]
	75	125	6.48 ± 0.13[c]
	80	63	6.59 ± 0.03[c]
	85	43	6.89 ± 0.08[b]

[a-d]Means with different superscripts, differ ($p \leq 0.05$).

[1]Mean ± SD, n = 2.

These results contradict those reported by Chen et al. (2013). Using the same RFDH unit and the same settings as Michael et al. (2014), Chen et al. (2013) treated LH-NDM and HH-NDM using different temperature/time combinations (to achieve 1, 2, and 5 log reductions in *Salmonella* spp. and *Cronobacter sakazakii* in nonfat dry milk), but had no significant changes in WPNI if LH-NDM was treated at 75°C for 125.67 min (7.32 ± 0.05), compared with LH-C (7.24 ± 0.06), which is not in agreement with results from this study, where LH-NDM treated at 75°C for 125 min showed 11% decrease in WPNI compared with LH-C (6.48 ± 0.13 vs. 7.31 ± 0.06). According to Chen et al. (2013), when LH-NDM was treated at 80°C for 57.75 min, the WPNI decreased by 12% (6.35 ± 0.04), compared with LH-C (7.24 ± 0.06), but the WPNI for LH-NDM

treated at 80°C for 63 min decreased by 9.8% (from 7.31 ± 0.06 to 6.59 ± 0.03) from the current study (Table 4-4). The WPNI for HH-NDM treated at 80°C for 57.75 min and 85°C for 25.00 min were 1.34 ± 0.01 and 1.32 ± 0.03, respectively, which decreased by 9.5 and 11% compared to HH-C (1.48 ± 0.02). These are different from results in this study – WPNI values were equivalent for all RFDH-treated HH-NDM and HH-C. NSI was not affected by the RFDH treatment in this study (Table 4-3). This suggests that the insoluble aggregates might not have been formed in either LH-NDM or HH-NDM during RFDH treatment since NSI is a measurement of native proteins and soluble aggregates. However, Chen et al. (2013) reported that loss of nitrogen solubility occurred when the RFDH temperature reached 75°C for HH-NDM and 80°C for LH-NDM.

Those differences might be caused by difference in pairwise comparison methods used. Chen et al. (2013) used Dunnett's mean comparison to compare 16 treatments (n = 3) with the control, however, in this study, Tukey's test was applied, and 4 treatments (n =2) were compared with each other, showing fewer degrees of freedom compared with Chen et al. (2013). In addition to the fewer treatments and fewer degrees of freedom in my study, Chen et al. (2013) split HH-NDM and LH-NDM and compared separately, however, in this study, HH-NDM and LH-NDM were compared together, making it harder to pick up small differences compared with the method from Chen et al. (2013). Additionally, Tukey's adjustment is more conservative than Dunnett's (Kuehl, 2000), which led to more "conservative" results than those from Chen et al. (2013).

4.5 SDS-PAGE

Rehydrated NDM samples were analyzed by SDS-PAGE as shown in Figures 4-2 and 4-3. All samples were reconstituted to an equivalent protein concentration (2 mg/ml), so that band

intensities could be compared. As shown in Figure 4-2, lane 1 is the protein marker; lanes 2-5 show reconstituted HH-NDM samples without reducing agent - 2-mercaptoethanol (BME) (lanes 2-5: HH-C, HH-75, HH-80, HH-85); lanes 6-9 show reconstituted samples with reducing agent (lanes 6-9: HH-C, HH-75, HH-80, HH-85).

The SDS-PAGE results showed that six major bands were detected in reconstituted HH-NDM without reducing agent (lanes 2-5), including a 115 kilo Dalton (kDa) band, a 69 kDa band, α-casein (α-CN) and β-CN (β-CN) (26-33 kDa), κ-casein (κ-CN) (23 kDa), β-Lg (16 kDa), and α-La (13 kDa) from top to bottom in order (Table 4-5; Figure 4-2). When a reducing agent was added, six bands were detected in all reconstituted HH-NDM (lane 6-9), including a 152 kDa band, a 82 kDa band, a 68 kDa band, α-CN and β-CN (26-33 kDa), κ-CN (23 kDa), β-Lg (16 kDa), and α-La (13 kDa) from top to bottom in order (Table 4-5; Figures 4-2). The molecular weight for each band was determined by a software GelAnalyzer (gelanalyzer.com) (calculations are shown in Appendix G). As shown in lane 2-5 (Figures 4-2), clear protein bands corresponding to β-Lg and α-La were consistently observed in RFDH-treated HH-NDM and HH-C, which indicates no substantial changes in these two proteins, occurred from the RFDH. This agrees with WPNI results, in which HH-NDM was not affected by RFDH. However, greater band intensities were observed corresponding to β-Lg and α-La in the reducing agent added samples compared with non-reducing agent added samples (Figure 4-2), suggesting that the content of native β-Lg and α-La increased when BME was added in samples. Since aggregated proteins (thermal-induced by associations between casein micelles and whey proteins by disulfide bonds) were cleaved by the BME, β-Lg and α-La dissociated from protein aggregates could contribute to the increased concentration of the soluble β-Lg and α-La (Dissanayake and

Vasiljevic, 2009). For casein bands, it was difficult to distinguish between non-reducing agent added and reducing agent added samples since the casein bands merged together.

Table 4-5. Molecular weight of proteins corresponding to bands on the SDS-PAGE gels for high heat (HH) and low heat (LH) nonfat dry milk (non-treated and RFDH[1]-treated) calculated based on the standard protein marker (Appendix G)

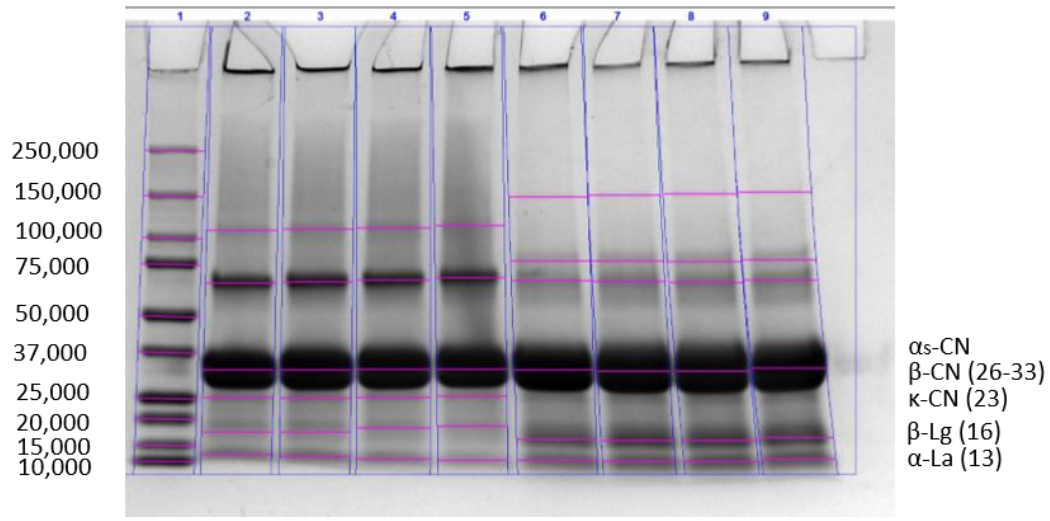
	HH[2]		LH[3]	
Molecular weight (kDa)	Reducing agent[4]	Non-reducing agent	Reducing agent	Non-reducing agent
	152	115	152	115
	82	69	82	69
	68	26-33	68	26-33
	26-33	23	60	23
	23	16	26-33	16
	16	13	23	13
	13		16	
			13	

[1]Radio frequency dielectric heating.

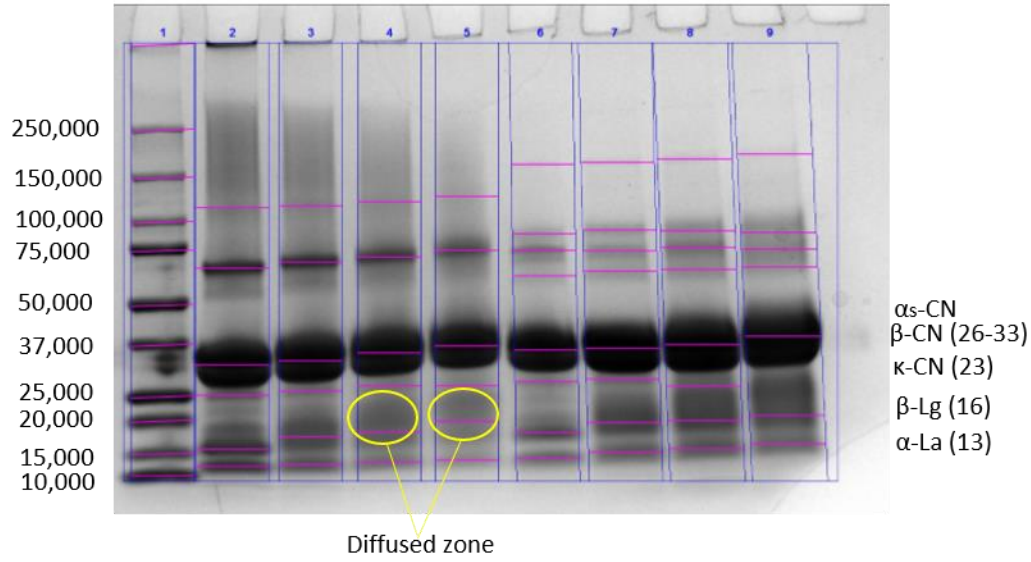
[2]HH: Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively.

[3]LH: Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.

[4]β-mercaptoethanol.



(a)



(b)

Figure 4-2. SDS-PAGE gel images for HH-NDM samples.

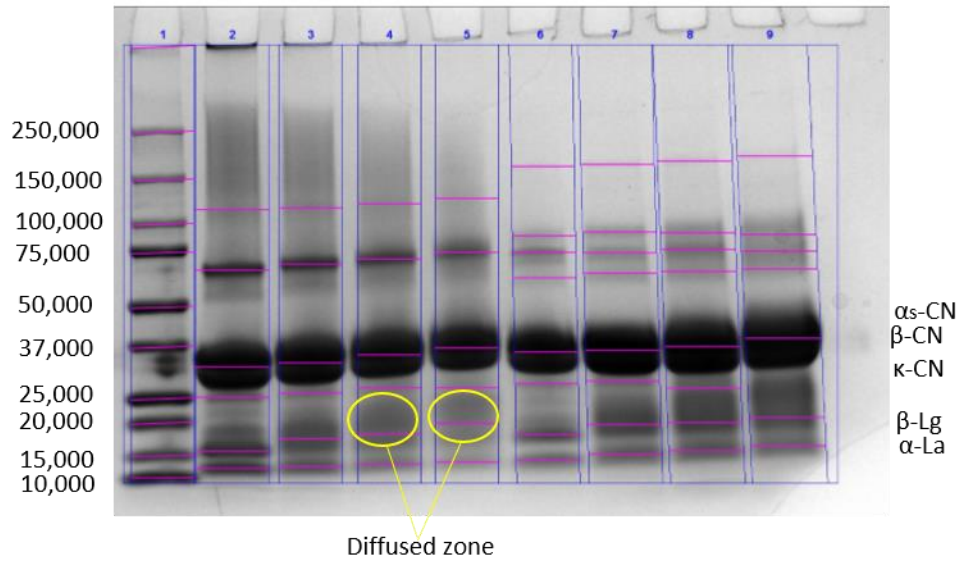
Image (a) represents Rep 1, and image (b) represents Rep 2.

Left to right on the SDS-PAGE gel image: Standard protein marker (Lane 1); reconstituted rehydrated high heat nonfat dry milk (HH-NDM) (Lane 2) and heated HH-NDM (Lane 3-5) without reducing agent - 2-mercaptoethanol (BME); reconstituted HH-NDM (Lane 6) and heated HH-NDM (Lane 7-9) with reducing agent. HH-NDM was RFDH-treated at 75°C, 115 min (Lane 3, 7), 80°C, 52 min (Lane 4, 8), and 85°C, 43 min (Lane 5, 9), reconstituted to 3.5% protein, and diluted to 2 mg/ml protein.

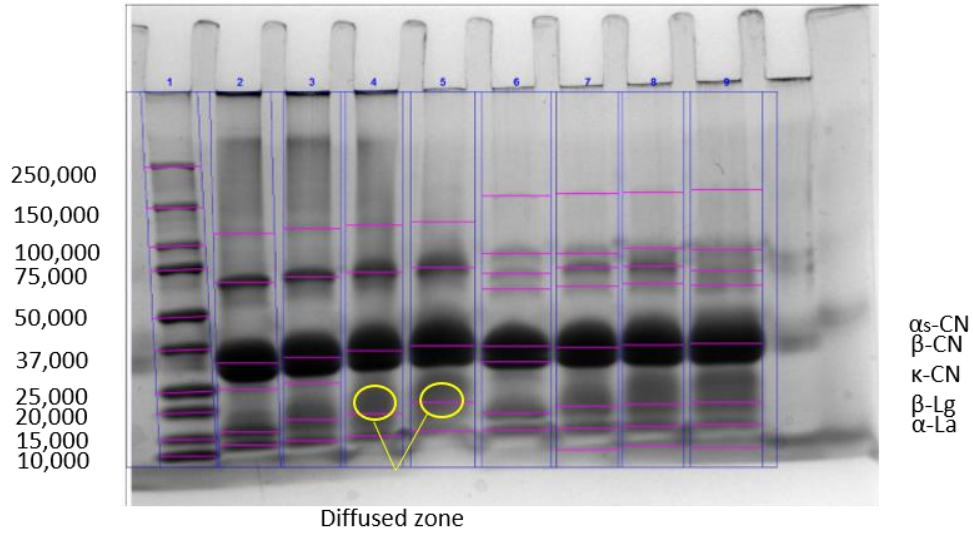
The SDS-PAGE gel images of reconstituted LH-NDM showed that six major protein bands were detected without reducing agent for all samples (LH-C, LH-75, LH-80, LH-85), including a 115 kDa band, a 69 kDa band, α-CN and β-CN (26-33 kDa), κ-CN (23 kDa), β-Lg (16 kDa) and α-La (13 kDa) from top to bottom in order (Table 4-5; Figure 4-3). After adding reducing agent to the samples, eight bands were detected in all reconstituted LH-NDM, including a 152 kDa band, a 82 kDa band, a 68 kDa band, a 60 kDa band, α-CN and β-CN (26-33 kDa), κ-CN (23 kDa), β-Lg (16 kDa), and α-La (13 kDa) from top to bottom in order (Table 4-5; Figure 4-3). The LH-C sample without reducing agent shows clear bands corresponding to β-Lg and α-La, while the RFDH-treated samples only show a clear band corresponding to α-La, which has less visual intensity compared with the LH-C. Additionally, diffused zones are visible on the gels in the location corresponding to β-Lg in LH-75, LH-80, and LH-85.

Jovanovic et al. (2007) reported that a diffused zone on a SDS-PAGE gel is a sign of protein denaturation. The decreasing intensity of bands corresponding to β-Lg and α-La and occurrence of diffused zones suggested that whey protein denaturation might have occurred during RFDH treatments of the LH-NDM. When adding reducing agent to LH-NDM samples, no significant changes in band intensity were found in β-Lg and α-La regardless of RFDH conditions, which would support that the large soluble aggregates of casein and whey proteins were cleaved by BME, releasing β-Lg and α-La in the solution (Dissanayake and Vasiljevic, 2009). Comparing bands from reducing agent added samples with non-reducing agent added samples, greater intensity bands were found in β-Lg and α-La in the reducing agent added samples for LH-75, LH-80, and LH-85. However, due to the deformation of the gel caused by overheating during electrophoresis (Saxena, 2010), bands corresponding to α-La were distorted

at the bottom (lane 2-5), making it hard to observe. This problem might be solved by decreasing the voltage level in further studies.



(a)



(b)

Figure 4-3. SDS-PAGE gel images for LH-NDM samples.

Image (a) represents Rep 1, and image (b) represents Rep 2.

Left to right on the SDS-PAGE gel image: Standard protein marker (Lane 1); reconstituted low heat nonfat dry milk (LH-NDM) (Lane 2) and heated LH-NDM (Lane 3-5) without reducing agent - 2-mercaptoethanol (BME), and reconstituted LH-NDM (Lane 6) and heated LH-NDM (Lane 7-9) with reducing agent. LH-NDM was RFDH-treated at 75°C, 125 min (Lane 3, 7), 80°C, 63 min (Lane 4, 8), and 85°C, 43 min (Lane 5, 9), reconstituted to 3.5% protein, and diluted to 2 mg/ml protein.

4.6 Color and XTT reduction

The color results are shown in Table 4-6. Results from the two-way ANOVA showed all p-values for a^* were > 0.05. However, NDM effect was significant for L^* (lightness). HH-NDM exhibited 2.01% greater L^* compared with LH-NDM (92.73 vs. 90.11), indicating HH-NDM was lighter than LH-NDM. A significant interaction between RFDH and NDM was found in b^* (yellowness) ($p < 0.05$). According to results from all pairwise comparisons, HH-NDM samples showed no change in yellowness regardless of RFDH. However, LH-80 and LH-85 showed more yellow than LH-C, with an increase of 15.5 and 25.9%, respectively. All HH-NDM samples had equivalent b^* values to all LH-NDM samples except LH-85. These results indicated reactions might have been initiated during the RFDH of the LH-NDM samples, such as Maillard browning (Chen et al., 2013). During heating, carbonyl groups in lactose and free amine groups in the amino acids (mainly lysine) can react with each other and lead to the formation of brown color (Gonzales et al., 2010).

Table 4-6. Color properties of high heat (HH) and low heat (LH) nonfat dry milk (NDM) as a function of radio frequency dielectric heating (RFDH) treatment

NDM	Temperature (°C)	Color[1,2] L*	a*	b*	ΔE*
HH[3]					
	Control	92.78 ± 1.37	-2.13 ± 0.07	13.50 ± 0.54[bc]	
	75	92.75 ± 1.17	-2.07 ± 0.16	14.06 ± 0.25[bc]	0.70 ± 0.60
	80	92.75 ± 1.63	-2.16 ± 0.15	14.35 ± 0.55[bc]	0.89 ± 0.00
	85	92.64 ± 1.59	-2.19 ± 0.00	14.25 ± 0.69[bc]	0.79 ± 0.10
LH[4]					
	Control[5]	90.95 ± 0.32	-2.16 ± 0.14	12.93 ± 0.12[c]	
	75	90.50 ± 0.23	-2.31 ± 0.00	13.86 ± 0.23[bc]	1.14 ± 0.09
	80	90.79 ± 0.10	-2.10 ± 0.12	14.94 ± 0.15[ab]	2.06 ± 0.23
	85	88.19 ± 1.97	-2.12 ± 0.10	16.28 ± 0.70[a]	4.50 ± 0.58

[A-B]Means within a column (main effect) with different superscripts, differ ($p \leq 0.05$).

[a-c]Means within the column for b* (RFDH*NDM) with different superscripts, differ ($p \leq 0.05$).

[1]Mean ± SD, n = 2.

[2]L* represents lightness; a* represents red or green; b* represents yellow or blue; ΔE* represents color difference: $\Delta E^* = \left[\left(L_1^* - L_2^*\right)^2 + \left(b_1^* - b_2^*\right)^2 + \left(a_1^* - a_2^*\right)^2\right]^{1/2}$.

[3]HH: Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively.

[4]LH: Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.

[5]Non-treated sample.

Many studies have set the acceptance limit for color matching at 3.7 units (ΔE*), beyond which the differences are clinically visible (Eliades et al., 2001; Sun-Waterhouse et al., 2010). The ΔE* for all HH-NDM samples was less than 3.7 units, which meant no color change could be observed visually between RFDH-treated and the control HH-NDM. LH-75 and LH-80

showed no observable color change ($\Delta E^* < 3.7$), however, the ΔE^* for LH-85 was 4.50, indicating that visible color changes occurred. As mentioned before, the color change was attributed to b^*, which meant LH-85 had more yellow color than the LH-C. However, Chen et al. (2013) reported that using RFDH to increase HH-NDM temperature to 90°C, without transferring to oven afterwards caused an increased b^* value compared to the control, while samples treated at 85°C, 25 min showed no change in b^*. Further, the three RFDH-treated LH-NDM samples had a significant color change: samples treated at 75°C, 25 min, and 85°C, 10 min were greener (greater a^*), whereas the sample treated at 90°C, 0 min (no hold time) showed more yellow than the control. No change in a^* was observed in the current study, so was in Chen et al. (2013).

The XTT reduction of LH-NDM was 0.10 ± 0.04, which was 55% less than that of HH-NDM (0.22 ± 0.03) (Appendix J), probably due to different process conditions during manufacture, which would induce the Maillard browning reactions (Ukeda et al., 1998). The XTT reduction was not found to be impacted by RFDH in the current study (Table 4-3). Shimamura et al. (2000) studied the Maillard reaction of lactose with butylamine in a model system using the XTT reduction method. They heated a mixture of sample solution at 80, 90, and 100°C for up to 30 min, and found that XTT reduction for samples treated at 100°C were always greater than samples treated at 80 and 90°C. This suggests that temperature may have been the main factor that impacted the Maillard reaction as opposed to the heating time. Realistically, the XTT reduction method only provides limited information about the Maillard reaction. The degree of Maillard reaction cannot be evaluated by XTT reduction itself, since the reaction rate needs to be taken into consideration as well.

4.7 Functional properties of reconstituted nonfat dry milk

Based on p-value (Table 4-3), Tukey's mean comparisons for functional properties of NDM dispersions, collapsed for RFDH, are shown in Table 4-7. When comparing LH-NDM with HH-NDM, the LH-NDM had less viscosity (5.7%), overrun (20.9%), and foam stability (27.4%), but had greater surface tension (1%) and interfacial tension (12%) than the HH-NDM (Table 4-7). No differences in emulsifying properties were found between HH-NDM and LH-NDM (Appendix J).

Greater whey protein denaturation tends to cause more associations of whey proteins with casein micelles, forming larger micelles; thus greater volume fraction, which leads to greater viscosity (Bienvenue et al., 2003). Since HH-NDM had greater whey protein denaturation than LH-NDM (as indicated by the WPNI results), the viscosity of HH-NDM should be greater than that of LH-NDM (Jeurnink and de Kruif, 1993), which was validated in the results.

As expected, the HH-NDM had greater foaming properties (overrun and foam stability) than LH-NDM, which can be explained also by different degrees of whey protein denaturation in LH-NDM and HH-NDM (Ibrahim et al., 1993). Heating during production of powders can initiate the unfolding of whey proteins, which would increase flexibility of proteins and enhance protein-protein interactions, forming a strengthened film at the air-water interface (Ibrahim et al., 1993; Kato et al., 1989). For surface tension and interfacial tension, greater values were found in LH-NDM than HH-NDM. According to Dissanayake and Vasiljevic (2009), thermal denaturation can lead to an increase in surface hydrophobicity of proteins, resulting in a greater tendency to be adsorbed at air-water interface, which in turn lowered surface tension. Because HH-C had lower WPNI than LH-C (Table 4-1), less surface and/or interfacial tension were expected.

Table 4-7. Mean differentiations[1] for viscosity, surface tension (ST), interfacial tension (IT), overrun, foam stability of reconstituted high heat (HH) and low heat (LH) nonfat dry milk (NDM) (3.5% protein), collapsed for radio frequency dielectric heating treatment

Functional properties	HH[2]	LH[3]
Apparent viscosity (mPaS)	2.09 ± 0.09^{a}	1.97 ± 0.12^{b}
ST (dynes/cm)	46.6 ± 0.5^{b}	47.1 ± 0.8^{a}
IT (dynes/cm)	4.1 ± 0.2^{b}	4.6 ± 0.4^{a}
Overrun (%)	817 ± 32^{a}	646 ± 34^{b}
Foam stability (min)	76.86 ± 10.01^{a}	55.78 ± 8.02^{b}

[a-b]Means within a row with different superscripts, differ ($p \leq 0.05$).

[1]Mean ± SD, n = 8.

[2]HH: Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively.

[3]LH: Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.

The functional properties of RFDH-treated NDM (collapsed for NDM) are shown in Table 4-8 ($p < 0.05$). The control NDM had viscosity of 1.94 mPaS, which was higher than skim milk (~ 1.5 mPaS) (Bakshi and Smith, 1984), probably due to greater total solids in rehydrated NDM in this study (total solids for the control HH-NDM and LH-NDM were 9.67 and 9.42%, based on a preliminary total solids test on other batches from the same NDM suppliers; minimum for the milk solid not fat in skim milk is 8.25%) (21CFR 131.110; FDA, 2016). NDM treated at 85°C had 10% greater viscosity than did the control, which can be explained by greater volume fraction caused by growing micelle size followed by whey protein denaturation (Jeurnink and De Kruif, 1993). The NDM treated at 75°C had less surface tension, compared with the control sample. Thermal denaturation in NDM might account for the changes in surface tension. As previously mentioned, LH-75 had lower WPNI value compared with LH-C (Table 4-4), whereas WPNI for HH-NDM was not affected during the RFDH. Kato et al. (1989) proposed

that a "molten" protein structure might be formed during dry heating, in which proteins are partially unfolded, resulting in higher surface hydrophobicity. The NDM samples treated at 75°C for 115 or 125 min might have had greater surface hydrophobicity compared with the control sample, resulting in greater tendency to be adsorbed at air-water interface, which lowered surface tension (Dissanayake and Vasiljevic, 2009). Surface tension for samples RFDH-treated at 80 and 85°C did not change probably because treatment times were shorter than samples treated at 75°C.

Table 4-8. Mean differentiation[1] for viscosity and surface tension (ST) of dispersions made from nonfat dry milk (NDM) treated as varied radio frequency dielectric heating (RFDH)[2] temperatures, collapsed for NDM

RFDH temperature (°C)	Control	75	80	85
Viscosity (mPaS)	1.94 ± 0.14^{b}	2.03 ± 0.10^{ab}	2.01 ± 0.07^{ab}	2.14 ± 0.08^{a}
ST (dynes/cm)	47.1 ± 0.7^{a}	46.2 ± 0.4^{b}	47.0 ± 0.8^{a}	47.1 ± 0.6^{a}

[a-b]Means within a row with different superscripts, differ ($p \leq 0.05$).

[1]Means ± SD, n = 4.

[2] HH: Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively; LH: Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.

No significant differences were observed in emulsifying properties in NDM under all RFDH treatments (Table 4-3). Casein micelles, which represents ~80% of the total protein, play the major role to stabilize fat droplets in the emulsion. Whey proteins also have been reported to have good emulsifying properties (Hung and Zayas, 1991). However, in the current study, whey

protein denaturation occurred in LH-NDM due to the RFDH treatment based on WPNI and SDS-PAGE results, but no changes in emulsifying properties was found. Perhaps the conductivity method is not sensitive to reveal minor differences among proteins.

4.8 Physical properties of acid milk gels

According to Table 4-3, a significant interaction existed for water holding capacity (WHC) of the acid gels, and the pairwise comparison results are shown in Table 4-9. LH-75 had 8.3% greater WHC than LH-C, which indicated that greater amount of soluble aggregates might have formed under dry heating at 75°C, 125 min (Gulzar et al., 2012). Interestingly, HH-80 and HH-85 had 11%, and 7.7% greater WHC compared with HH-C, respectively. However, no indication of protein conformational changes in HH-80 and HH-85 were observed in SDS-PAGE gel image analysis (Figure 4-2). Parnell-Clunies et al. (1986) reported that yogurt made from vat (low temperature pasteurization) milk, which was extensively denatured (88 to 100%), had lower WHC compared with yogurt mixes that were subjected to HTST and UHT treatments, indicating denaturation is not necessarily a precursor to improved WHC. According to Parnell-Clunies et al. (1986), slight increases in WHC due to protein denaturation have been attributed to the exposure of charged groups and increased surface area, resulting in enhanced protein-water interactions. However, if the association between β-LG and κ-casein occurs upon heating of milk, then the steric effect could mask effects on WHC attributed to unfolding of whey proteins (Parnell-Clunies et al., 1986).

Table 4-9. Water holding capacity (WHC)[1] for high heat (HH) and low heat (LH) nonfat dry milk (NDM) as functions of radio frequency dielectric heating (RFDH) temperature[2]

WHC (%)		RFDH temperature (°C)			
	NDM	Control	75	80	85
	HH[2]	15.5 ± 0.5^{b}	15.6 ± 0.7^{b}	17.2 ± 0.5^{a}	16.7 ± 0.4^{a}
	LH[3]	13.3 ± 0.3^{d}	14.4 ± 0.4^{c}	13.8 ± 0.3^{cd}	13.7 ± 0.3^{cd}

[a-d]Means with different superscripts, differ ($p \leq 0.05$).

[1]Mean ± SD, n = 2.

[2]HH: Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively.

[3]LH: Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.

Tukey's mean comparisons for physical properties of gels made from NDM dispersions, collapsed for RFDH, are shown in Table 4-10. The acid gels formed by HH-NDM samples had 18% greater WHC than those formed by LH-NDM samples, suggesting more water was absorbed or retained in the protein matrix formed by HH-NDM dispersion. Gulzar et al. (2012) dry-heated WPI at 80, 100, and 120°C for up to 6 days, 24 h and 3 h. As dry heating time was extended, the percentage of residual native proteins continuously decreased, whereas the percentage of insoluble aggregates increased. They also reported that the quantity of soluble aggregates is positively correlated (r = 0.83) with WHC in a heat-set gel, because the structure and properties of soluble aggregates formed during dry heating provided excellent ability to reduce water release from heat-set gels. In the current study, the soluble and insoluble protein fractions in LH-NDM and HH-NDM are unknown, but it is hypothesized that because of greater whey protein denaturation in HH-NDM, more soluble aggregates might have been formed in HH-NDM compared with LH-NDM during RFDH treatment, resulting in greater amount of water retained in gel matrix, thus increasing WHC (Gulzar et al., 2012). Further tests need to be

done to quantify native proteins, soluble aggregates, and insoluble aggregates to prove this hypothesis.

Table 4-10. Physical properties of acid milk gels made from high heat (HH) and low heat (LH) nonfat dry milk collapsed for radio frequency dielectric heating treatment

Physical properties[3]	HH[1]	LH[2]
Syneresis (%)	2.46 ± 0.22[a]	1.44 ± 1.20[b]
WHC (%)	16.3 ± 0.8[a]	13.8 ± 0.5[b]
Firmness (g)	35.76 ± 3.84[a]	29.68 ± 3.94[b]

[a-b]Means within a row with different superscripts, differ ($p \leq 0.05$).

[1]HH: Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively.

[2]LH: Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.

[3]Mean ± SD, n = 8.

According to Lucey et al. (1998a), heat treatments (> 75°C) of skim milk before acidification might make gels unstable and more prone to syneresis because those conditions favor the gel network to rearrange more, just after formation. HH-NDM showed 70.8% greater syneresis than LH-NDM, which indicated that gels formed from HH-NDM dispersions might be more brittle and susceptible to rearrangements and fracture, compared with those formed from LH-NDM. However, in the current study, syneresis was not affected by the RFDH treatment (Table 4-3). The possible reason is that during dry heating, the heat denaturation of proteins was more suppressed in the absence of free water compared with reactions occurring in aqueous

solution (Ibrahim et al., 1993). So the short exposure times (43 to 125 min) of the RFDH treatment did not have significant effects on syneresis.

The firmness of gels was affected by RFDH treatment (Table 4-11). The gels made from NDM treated at 80 and 85°C were 9.90 and 25.1% less firm than the control NDM, respectively; whereas gels made by samples treated at 75°C had equivalent firmness to the control NDM. Parnell-Clunies et al. (1986) reported a positive relationship between firmness and percent denaturation in heated milk with a correlation coefficient of 0.831. However, in the current study, gels made from the control NDM, and with NDM treated at 75°C, had the greatest firmness. Lucey et al. (1997) reported that post-acidified gels formed from high-heated (85°C for 30 min) milk were less firm and more susceptible to rearrangements and fracture, compared with gels formed from unheated or mildly heated (75°C for 15 min) skim milk. Generally, the number and strength of bonds between protein particles increases as treatment temperature increases before gelling, owing to the associations between the denatured whey proteins with casein micelles and the formation of aggregates (Lucey et al., 1997). But Lucey et al. (1997) also reported that the storage modulus (G') of acid milk gel decreased when skim milk was heated at 90°C for 30 min compared with 85°C for 30 min, which suggested the formation of large whey protein–casein aggregates may alter the process of gel formation during acidification of heated milk.

Table 4-11. Firmness of acid milk gels made from rehydrated nonfat dry milk (NDM) as a function of radio frequency dielectric heating (RFDH) temperature[2], collapsed for NDM

RFDH temperature (°C)	Control	75	80	85
Firmness (g)	36.46 ± 4.19[a]	34.25 ± 2.86[ab]	32.85 ± 3.96[b]	27.32 ± 4.23[c]

[a-b]Means with different superscripts, differ ($p \leq 0.05$).

[1]Mean ± SD, n = 4.

[2]HH: Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively; LH: Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.

Other than NDM, egg white protein (EWP) has also been treated by RFDH. Boreddy et al. (2016) reported that RFDH improved gelling properties of EWP. They heated standard EWP to 60, 70, 80, and 90°C in a RFDH unit, followed by holding in a hot air oven from 4 h at 90°C to 72 h at 60°C, then compared the quality and functional properties of EWP with those treated traditionally (heat treatment in a hot room at 58°C for at least 14 d). They found that the WHC and gel-firmness of RFDH-assisted thermally processed standard EWP at 90°C for 16 and 24 h were significantly greater compared with the traditionally hot-room processed standard EWP. Moreover, the gel firmness reached the level of high-gelling EWP, indicating that RFDH might be a new approach to improve gelling properties of EWP.

4.9 RFDH effect on HH-NDM and LH-NDM

When reviewing the electrophoresis and WPNI results, the HH-NDM samples seemed less impacted by RFDH treatment than did the LH-NDM samples. These results suggested that the initial whey protein denaturation differences between HH-NDM and LH-NDM might have made it more difficult to ascertain the RFDH effect. Thus HH-NDM and LH-NDM data were

separated and re-analyzed to get a better understanding of effect of the RFDH treatment. The means from these analyses are shown in Table 4-12 (HH-NDM) and Table 4-13 (LH-NDM).

When analyzed alone, the HH-NDM samples exhibited changes in gelling properties as a function of RFDH treatment, in particular gel firmness and water holding capacity (Table 4-12). HH-NDM samples treated at 85°C, 43 min had a decreased firmness compared with the control HH-NDM, while WHC increased when RFDH temperature was ≥ 80°C compared with the control (Table 4-12). Since all HH-NDM samples had equivalent WPNI and NSI, the degree of whey protein denaturation and the amount of soluble proteins in the HH-NDM samples should be similar as well as the amount of soluble aggregates (Gulzar et al., 2011). So these results may not be explained by the "soluble aggregates theory" (Gulzar et al., 2012). But, Kato et al. (1989) explained that a "molten" structure of whey proteins might form during dry heating, which caused an increase in the hydrophobicity on the protein's surface. I hypothesize that changes in surface hydrophobicity might lead to a difference in interactions between caseins and whey proteins when forming a gel matrix. Further tests would need to be done to understand the role of surface hydrophobicity on the gelling characters.

Table 4-12. Mean differentiations[1] for various physico-chemical and functional properties of high heat nonfat dry milk as a function of radio frequency dielectric heating (RFDH) temperature[1]

		RFDH temperature (°C)			
System	Property	Control	75	80	85
Powder					
	L^*	92.78 ± 1.37	92.75 ± 1.17	92.75 ± 1.63	92.64 ± 1.59
	a^*	-2.13 ± 0.07	-2.07 ± 0.16	-2.16 ± 0.15	-2.19 ± 0.00
	b^*	13.50 ± 0.54	14.06 ± 0.25	14.35 ± 0.55	14.25 ± 0.69
	WPNI[3] (mg/g)	1.79 ± 0.01	1.89 ± 0.01	1.90 ± 0.08	1.97 ± 0.13
	NSI[4] (%)	96.4 ± 0.4	96.3 ± 0.0	96.2 ± 0.4	96.0 ± 0.1
Dispersion (pH adjusted)					
	Foam stability (min)	71.96 ± 6.55	72.97 ± 1.11	77.22 ± 3.39	85.32 ± 20.58
	Overrun (%)	799 ± 13	802 ± 12	838 ± 13	829 ± 67
	IT[5] (dynes/cm)	4.2 ± 0.1	4.1 ± 0.4	4.2 ± 0.1	4.1 ± 0.0
	ST[6] (dynes/cm)	46.9 ± 0.8	46.2 ± 0.3	46.7 ± 0.9	46.8 ± 0.1
	EA[7] (μs/cm)	316 ± 34	321 ± 16	324 ± 25	328 ± 8
	ES[8]	24.3 ± 1.3	18.1 ± 2.6	23.4 ± 3.8	19.8 ± 1.5
	Viscosity (mPas)	2.06 ± 0.01	2.08 ± 0.14	2.03 ± 0.08	2.19 ± 0.05
Dispersion					
	pH	6.62 ± 0.06	6.60 ± 0.04	6.61 ± 0.06	6.61 ± 0.06
	XTT[9]	0.23 ± 0.05	0.23 ± 0.04	0.22 ± 0.02	0.20 ± 0.02
Gel					
	Firmness (g)	39.46 ± 4.07[a]	36.60 ± 1.59[a]	36.15 ± 1.65[ab]	30.84 ± 1.97[b]
	Syneresis (%)	2.34 ± 0.03	2.42 ± 0.44	2.53 ± 0.07	2.56 ± 0.30
	WHC[10] (%)	15.5 ± 0.5[b]	15.6 ± 0.7[b]	17.2 ± 0.5[a]	16.7 ± 0.4[a]

[a-b]Means within a row with different superscripts, differ ($p < 0.05$).
[1]Mean ± SD, n = 2.
[2]Control, 75, 80, 85°C and oven hold times of 0, 115, 52, 43 min, respectively.
[3]Whey protein solubility index.
[4]Nitrogen solubility index.
[5]Interfacial tension.
[6]Surface tension.
[7]Emulsion activity.
[8]Emulsion stability.
[9]Reduction of tetrazolium salt.
[10]Water holding capacity.

When analyzing the LH-NDM alone, significant differences were found for b*, WPNI, surface tension, and gelling properties, which includes gel firmness and WHC, as a function of the RFDH treatment. The LH-NDM treated at 75°C, 125 min and 80°C, 63 min had the lowest WPNI among all LH-NDM samples (Table 4-13), suggesting the greatest degree of whey protein denaturation occurred in these samples. However, NSI did not change as a function of RFDH treatment, indicating that the amount of soluble proteins (native proteins and soluble aggregates) was equivalent in all LH-NDM samples.

The LH-NDM samples treated at 75°C, 125 min had less surface tension compared with the control, which suggested that the whey protein denaturation caused increased surface hydrophobicity and greater tendency to adsorb to water-air interface, thus lowering surface tension. Moreover, the LH-NDM treated at 75°C, 125 min exhibited greater WHC compared with the control, suggesting that greater amounts of soluble aggregates might have formed due to whey protein denaturation, which in turn reduced water release from the gel. The amount of soluble aggregates is related to gelling properties (Gulzar et al., 2012). The optimal point for water holding capacity requires higher amounts of soluble aggregates than does optimal gel firmness (Gulzar et al., 2012). The decrease in gel firmness in the LH-NDM samples treated by RFDH might agree with this theory, but surface hydrophobicity may have also had a role during gel formation. No changes were found in XTT reduction, even though samples treated at 85°C had the greatest value ($p > 0.05$).

Table 4-13. Mean differentiations[1] for various physico-chemical and functional properties of low heat nonfat dry milk as a function of radio frequency dielectric heating (RFDH) temperature[2]

		RFDH temperature (°C)			
System	Property	Control	75	80	85
Powder					
	L^*	90.95 ± 0.32	90.50 ± 0.23	90.79 ± 0.10	88.19 ± 1.97
	a^*	-2.16 ± 0.14	-2.31 ± 0.00	-2.10 ± 0.12	-2.12 ± 0.10
	b^*	12.93 ± 0.12^{c}	13.86 ± 0.23^{bc}	14.94 ± 0.15^{ab}	16.28 ± 0.70^{a}
	WPNI[3] (mg/g)	7.31 ± 0.06^{a}	6.48 ± 0.13^{c}	6.59 ± 0.03^{c}	6.89 ± 0.08^{b}
	NSI[4] (%)	98.5 ± 0.1	98.7 ± 0.7	98.9 ± 0.9	98.4 ± 0.7
Dispersion (pH adjusted)					
	Foam stability (min)	48.14 ± 9.50	51.99 ± 5.11	57.72 ± 0.84	65.26 ± 0.46
	Overrun (%)	616 ± 4	619 ± 4	665 ± 16	685 ± 31
	IT[5] (dynes/cm)	4.4 ± 0.5	4.3 ± 0.1	5.0 ± 0.2	4.7 ± 0.5
	ST[6] (dynes/cm)	47.4 ± 0.7^{a}	46.3 ± 0.6^{b}	47.3 ± 1.0^{a}	47.5 ± 0.6^{a}
	EA[7] (μs/cm)	315 ± 7	329 ± 7	319 ± 3	316 ± 13
	ES[8]	27.4 ± 5.3	20.0 ± 0.6	23.8 ± 3.5	24.8 ± 4.0
	Viscosity (mPas)	1.80 ± 0.08	1.98 ± 0.04	1.98 ± 0.07	2.10 ± 0.10
Dispersion					
	pH	6.68 ± 0.01	6.68 ± 0.02	6.68 ± 0.03	6.63 ± 0.01
	XTT[9]	0.08 ± 0.01	0.07 ± 0.00	0.08 ± 0.03	0.16 ± 0.02
Gel					
	Firmness (g)	33.46 ± 0.28^{a}	31.91 ± 0.16^{b}	29.55 ± 0.86^{b}	23.81 ± 0.57^{c}
	Syneresis (%)	1.01 ± 0.16	0.92 ± 0.03	0.89 ± 0.04	2.95 ± 2.00
	WHC[10] (%)	13.3 ± 0.3^{c}	14.4 ± 0.4^{a}	13.8 ± 0.3^{b}	13.7 ± 0.3^{b}

[a-c]Means within a row with different superscripts, differ ($p < 0.05$).
[1]Mean ± SD, n = 2.
[2]Control, 75, 80, 85°C and oven hold times of 0, 125, 63, 43 min, respectively.
[3]Whey protein solubility index.
[4]Nitrogen solubility index.
[5]Interfacial tension.
[6]Surface tension.
[7]Emulsion activity.
[8]Emulsion stability.
[9]Reduction of tetrazolium salt.
[10]Water holding capacity.

In summary, HH-NDM and LH-NDM showed different changes when treated by RFDH. The degree of whey protein denaturation may be one important factor but more experiments are needed to identify and understand other driving factors to those changes, such as surface hydrophobicity.

Chapter 5 - Summary

High heat (HH) and low heat (LH) nonfat dry milk (NDM) are widely used in foods and beverages due to their appealing functional properties (Jervis et al., 2012). Radio frequency dielectric heating (RFDH), which was demonstrated by Michael et al. (2014) to be capable of achieving a 5-log reduction in *Salmonella* spp., caused changes in functional properties of NDM, but only apparent viscosity, surface tension, and water holding capacity (acid gels). Whey protein denaturation is considered to be the driving factor to those changes, as the LH-75, LH-80, and LH-85 exhibited decreased WPNI (by 11, 10, and 6%, respectively) when compared to the control LH-NDM. Despite decreases in WPNI, LH-NDM still maintained its classification as WPNI not smaller than 6.0 mg/g. It is important to note that a color change was observed in LH-85 samples suggesting that the Maillard reaction might have been initiated during the RFDH treatment. However, no changes in XTT reduction were found in LH-85 samples, probably due to the unknown browning reaction stage. HH-NDM showed no changes in WPNI as a function of RFDH, which is interpreted as no additional protein denaturation occurred during RFDH treatment. The SDS-PAGE results support these interpretations.

LH-NDM and HH-NDM (control) differed in most functional properties of dispersions and physical properties of acid gels due to the difference in protein denaturation. Most functional properties of HH-NDM and LH-NDM dispersions were not impacted by the RFDH treatment, such as foaming and emulsifying properties. Gels made from RFDH-treated HH-NDM exhibited significant changes only in gel firmness and water holding capacity, while b*, surface tension, gel firmness and water holding capacity for LH-NDM were affected in the RFDH-treated samples.

Using the XTT reduction as an indicator of Maillard browning, results suggested that this reaction might not have been initiated by the RFDH treatment. However, it would be important to verify that in a shelf life study.

Chapter 6 - Conclusions

As nonfat dry milk (NDM) is commonly used as functional ingredient in foods, knowing how external factors influence the functional properties of NDM is of great importance. In this study, heat is the concern. NDM is often added in baked goods, such as granola bars, breads, etc. (Canadian Dairy Commission, 2011), and the baking process is often the critical control point to eliminate pathogens and drive off moisture. But meanwhile, quality changes can also occur, including the Maillard reaction, protein denaturation and coagulation. To have better control of product quality, understanding how ingredients react under heat is crucial. If NDM acts as a foaming agent in ice cream, the overrun of ice cream could be impacted as well as the texture and mouthfeel, if a heat treatment to induce a 5-log reduction in *Salmonella* spp. is applied to NDM during the manufacture.

Further studies are needed to fully understand this phenomena. For example, extending the overall heating times may improve functional properties of low heat (LH) NDM; changing frequency level may impact heating rate in RFDH unit, causing variations during the process. Using RFDH instead of a conventional heating process of skim milk during manufacture as a new approach may reduce energy needs.

Besides milk powder, RFDH can also be applied to other low-moisture food products, such as wheat flour. The food industry is using continuous treatment systems to treat in-package powder using RFDH to eliminate pathogens and thus, increase food safety. However, cost-performance ratios need to be taken into consideration when a company makes decisions on whether to utilize RFDH technology to replace traditional methods. There would be concerns including cost of new equipment and training, energy consumption, environmental influence, sterilization efficiency, degradation of product quality. Besides various applications in food

industry, RFDH can also be used for drying lumber and gypsum wallboard and for preheating in molding plastics and glasslike materials.

Overall, RFDH, is a promising post-process lethality method to treat low-moisture foods and can be used to tailor functional properties of protein powders. However, there are still questions to be answered. Based on results from the current studies, it cannot be concluded that significant whey protein denaturation occurred in HH-NDM during this RFDH treatment. These data suggest that the surface hydrophobicity of HH-NDM might be altered which led to changes in gel firmness. Further study is needed to determine whether there is any conformational change on the surface of whey proteins in HH-NDM.

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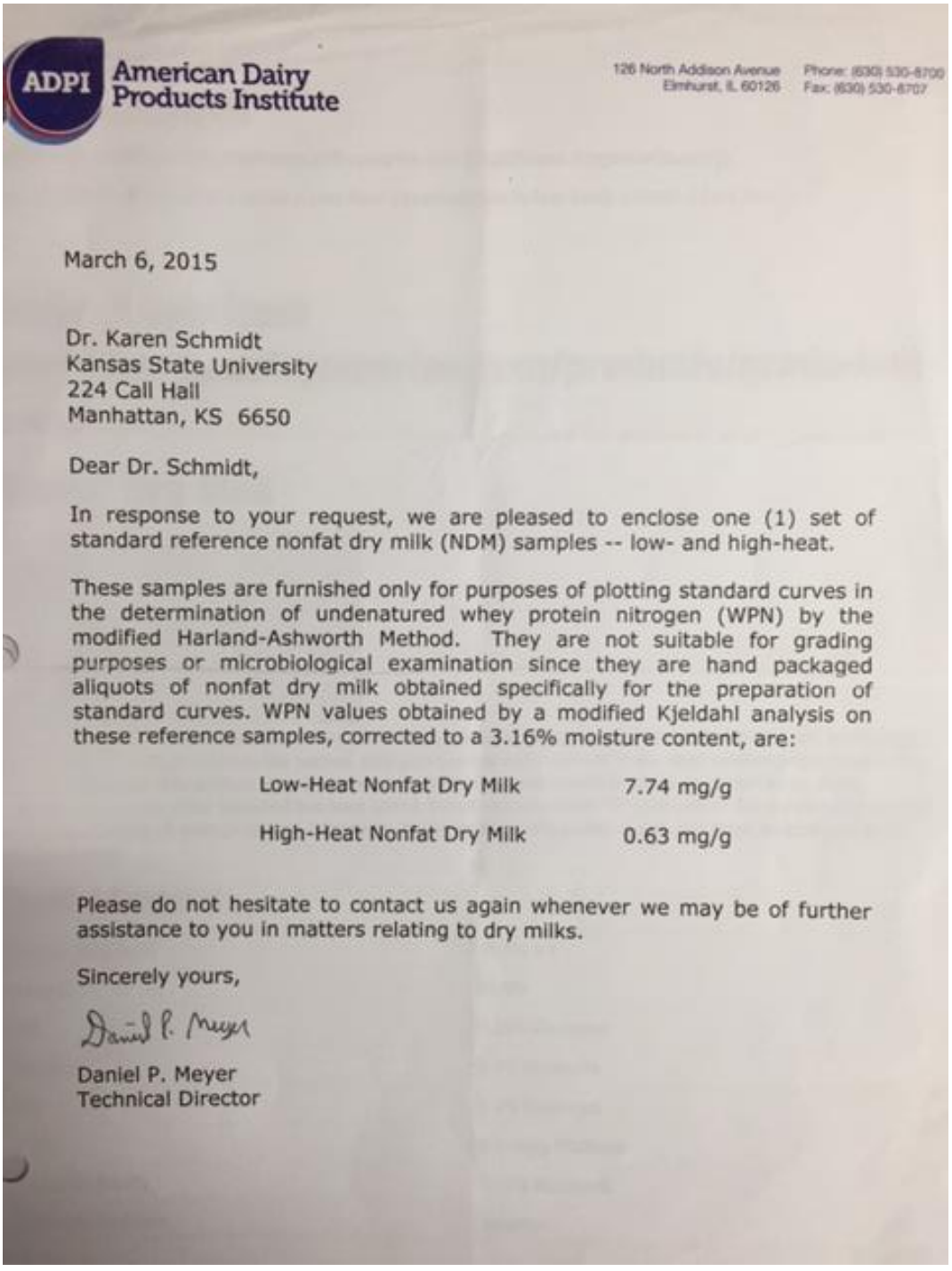
Appendix A - **Specification sheets for LH- and HH-NDM and standard reference nonfat dry milk sheet**



ADPI American Dairy Products Institute

126 North Addison Avenue
Elmhurst, IL 60126
Phone: (630) 530-8700
Fax: (630) 530-8707

March 6, 2015

Dr. Karen Schmidt
Kansas State University
224 Call Hall
Manhattan, KS 6650

Dear Dr. Schmidt,

In response to your request, we are pleased to enclose one (1) set of standard reference nonfat dry milk (NDM) samples -- low- and high-heat.

These samples are furnished only for purposes of plotting standard curves in the determination of undenatured whey protein nitrogen (WPN) by the modified Harland-Ashworth Method. They are not suitable for grading purposes or microbiological examination since they are hand packaged aliquots of nonfat dry milk obtained specifically for the preparation of standard curves. WPN values obtained by a modified Kjeldahl analysis on these reference samples, corrected to a 3.16% moisture content, are:

Low-Heat Nonfat Dry Milk	7.74 mg/g
High-Heat Nonfat Dry Milk	0.63 mg/g

Please do not hesitate to contact us again whenever we may be of further assistance to you in matters relating to dry milks.

Sincerely yours,

Daniel P. Meyer

Daniel P. Meyer
Technical Director

Figure A-1. Specification sheet for standard reference nonfat dry milk.

DairyAmerica

Published on *DairyAmerica* (http://www.dairyamerica.com (http://www.dairyamerica.com))

Home (/) > Grade A Low Heat > Grade A Low Heat (/products/grade-low-heat) > Grade A Low Heat

Grade A Low Heat (http://www.dairyamerica.com/products/grade-low-heat)

Nonfat Dry Milk

Spray Dried Grade "A" Low Heat Nonfat Dry Milk is the powder resulting from the removal of the fat and water from fresh Grade "A" Milk. It contains the lactose, milk proteins and milk minerals in the same relative proportions as they occur in fresh milk. The product is made from fresh, pasteurized nonfat milk to which no preservative, alkali, neutralizing agent or other chemical has been added. DairyAmerica's Grade "A" Low Heat NFDM is commonly used in the manufacturing of: cottage cheese, buttermilk, frozen desserts, margarine, soups, puddings, cultured products, and beverages.

Typical Analysis

Protein (Nx6.38)%	33.0% ± 2
Lactose	51.0%
Fat	1.25% Maximum
Moisture	4.0% Maximum
Ash	8.2% Maximum
WPN	6.0 mg/g Minimum
Titratable Acidity	0.15% Maximum
Antibiotic Residues	Negative

Microbiological Standards

Standard Plate Count	10,000/g Maximum
Coliform	≤10/g
Salmonella	Negative

Physical Properties

Scorched Particles	Disc B (15.0mg), Maximum
Solubility Index	1.2 ml, Maximum
Color	Light cream to white
Flavor	Clean, cooked flavor
Odor	Fresh,no off odor

Storage and Shipping

Product should be stored in a dry, cool, clean warehouse free of odors with a temperature below 80 degrees F and relative humidity below 65%. Stored under these conditions, it is recommended that the product be consumed in less than eighteen months to assure fresh tasting product.

Packaging

ultiwall kraft bags with polyethylene inner liner or other approved closed container, i.e., "tote bags", etc.

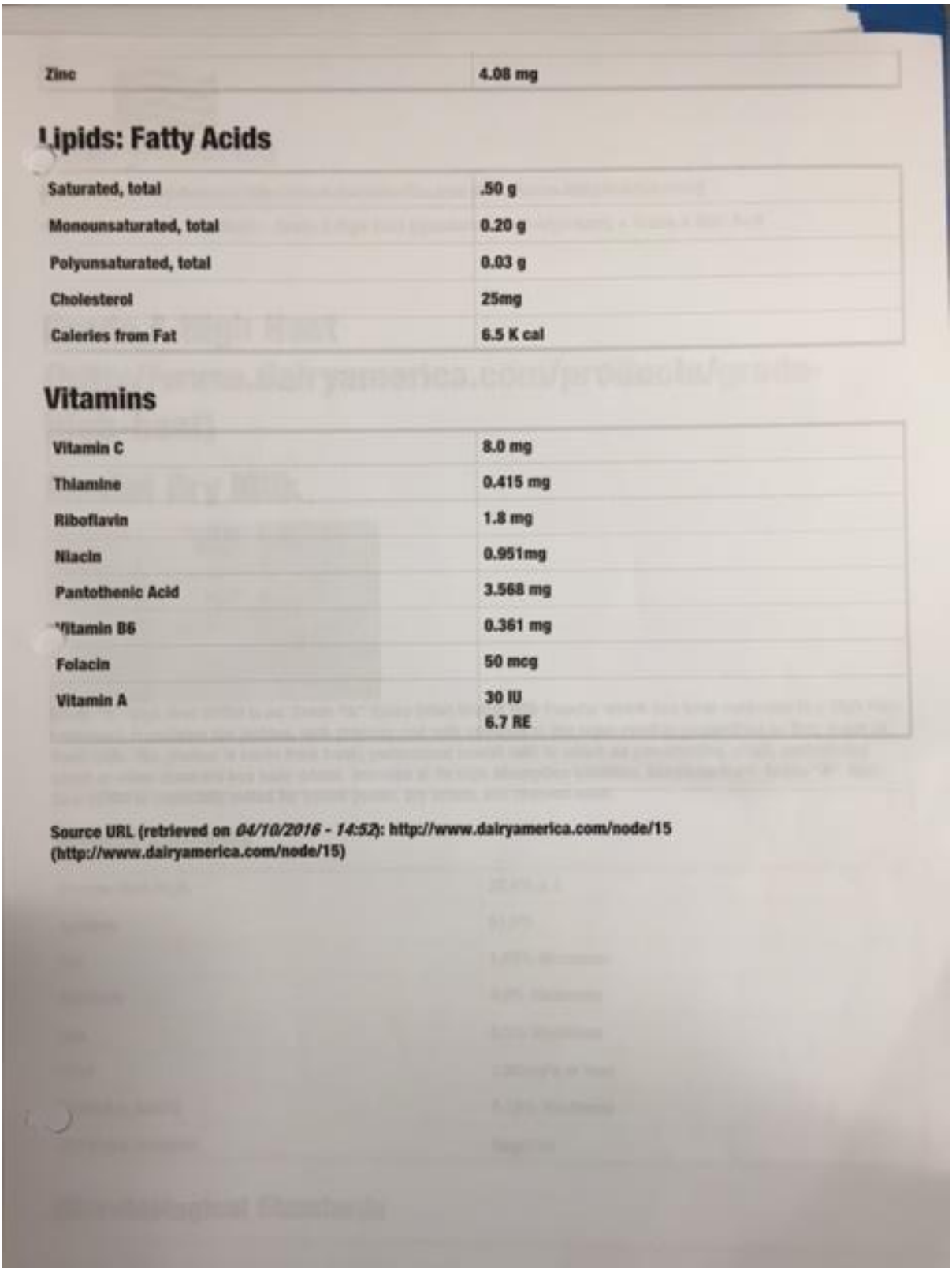
Nutrient Information

The nutrients found in 100 grams of products are as follows:

Protein (Nx6.38)	34 g
Lactose	51 g
Fat	0.8 g
Water	4.0g Maximum
Ash	8.0 g
Calories	359 K cal

Minerals

Calcium	1248 mg
Iron	0.4 mg
agnesium	110 mg
Phosphorus	993mg
Potassium	1674 mg
Sodium	494 mg



Zinc	4.08 mg

Lipids: Fatty Acids

Saturated, total	.50 g
Monounsaturated, total	0.20 g
Polyunsaturated, total	0.03 g
Cholesterol	25mg
Calories from Fat	6.5 K cal

Vitamins

Vitamin C	8.0 mg
Thiamine	0.415 mg
Riboflavin	1.8 mg
Niacin	0.951mg
Pantothenic Acid	3.568 mg
Vitamin B6	0.361 mg
Folacin	50 mcg
Vitamin A	30 IU 6.7 RE

Source URL (retrieved on *04/10/2016 - 14:52*): http://www.dairyamerica.com/node/15
(http://www.dairyamerica.com/node/15)

Figure A-2. Specification sheet for Grade A low heat nonfat dry milk. (N/A for high heat nonfat dry milk) Downloaded at http://www.dairyamerica.com/products/grade-low-heat, April 29th.

Appendix B - **Impact of tray on RFDH heating time**

Table B-1. Times in the radio frequency dielectric heating unit for high heat (HH) and low heat (LH) nonfat dry milk samples to reach target temperatures, as functions of tray

		Time (min)[1]	
Temperature (°C)	Tray	HH	LH
75	Big[2]	16.93	17.75
	Small[3]	12.38	18.06
80	Big	19.81	20.33
	Small	14.11	20.20
85	Big	23.08	23.08
	Small	15.96	22.30

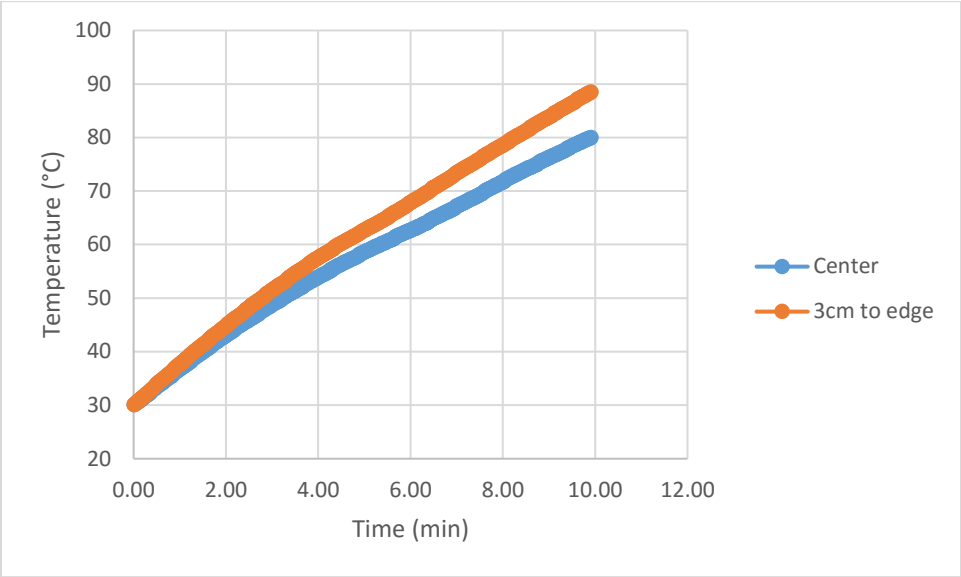
[1]Time required to reach the target temperature, starting from 30 ± 0.1°C.

[2]represents the circular polypropylene tray with inner diameter of 22.4 cm.

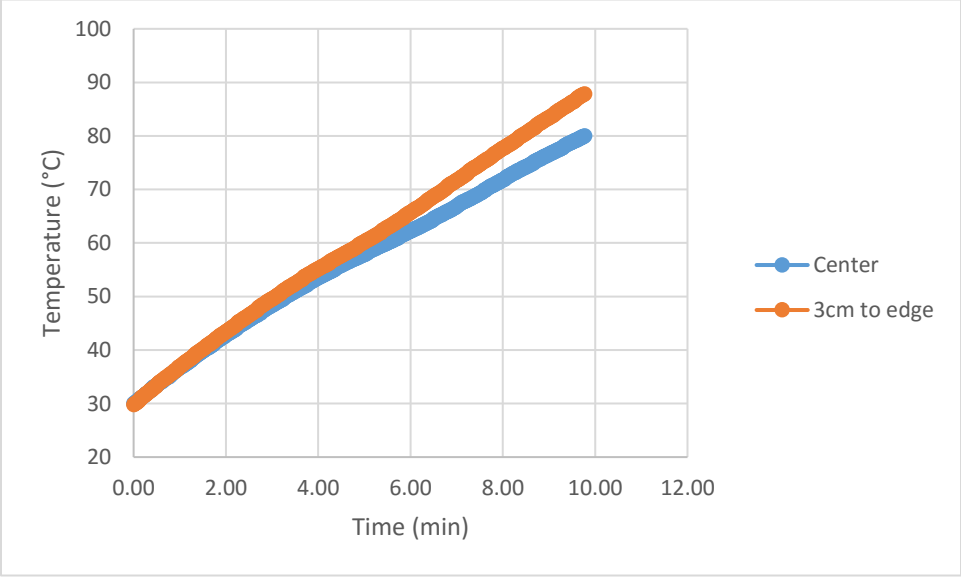
[3]represents the circular polypropylene tray with inner diameter of 19.0 cm.

When using big tray for HH-NDM and small tray for LH-NDM, differences between HH-NDM and LH-NDM samples in RFDH heating times were 1.13 min at 75°C, 0.39 min at 80°C, and 0.78 min at 85°C, whereas using small tray for HH-NDM and big tray for LH-NDM led to greater differences in RFDH heating times: 5.37 min at 75°C, 6.22 min at 80°C, and 7.12 at 85°C. Since heating time in RFDH unit is an important variable that might impact functionality of NDM, using big tray for HH-NDM and small tray for LH-NDM would be the best option.

Appendix C - **RFDH heating curve**

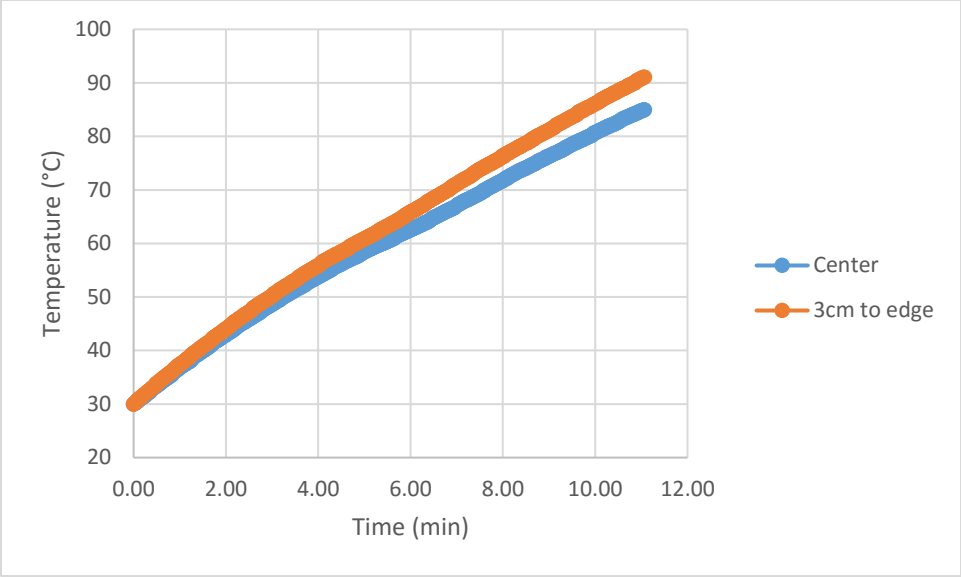


(a)

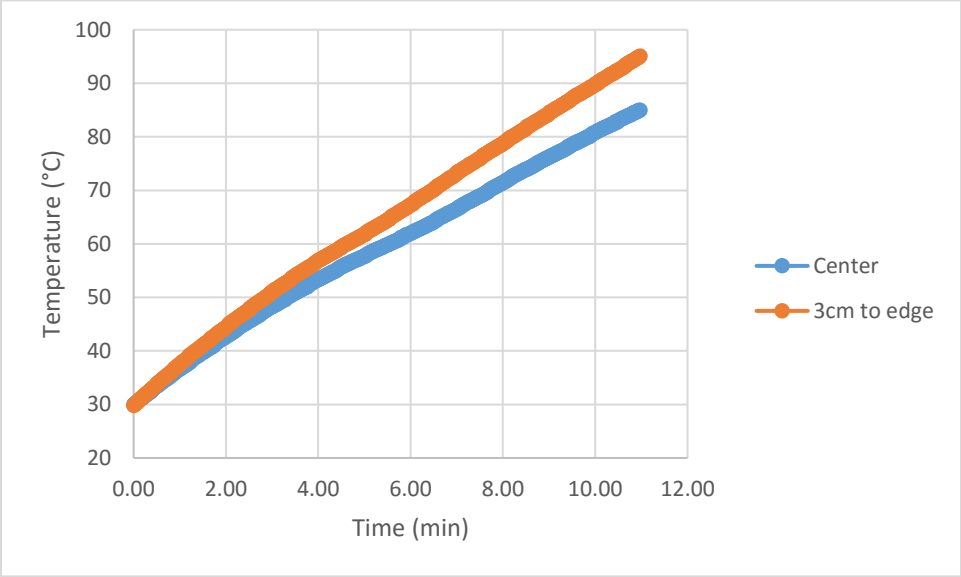


(b)

Figure C-1. Radio frequency dielectric heating of high heat nonfat dry milk from 30 to 80°C (target temperature for the center probe) in a circular polypropylene tray. The legend shows the location of two fiber-optic probes for monitoring temperatures. (a) Rep 1; (b) Rep 2.

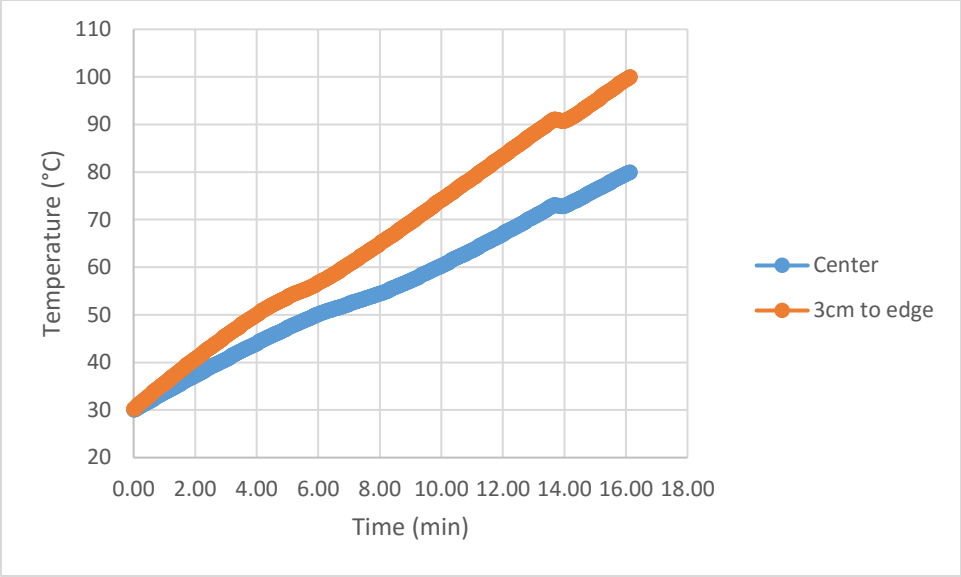


(a)

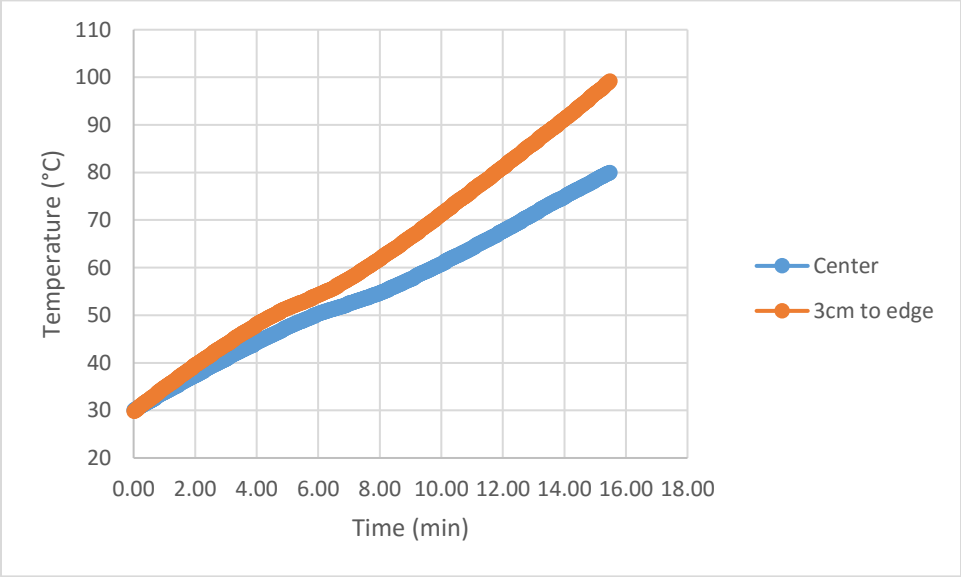


(b)

Figure C-2. Radio frequency dielectric heating of high heat nonfat dry milk from 30 to 85°C (target temperature for the center probe) in a circular polypropylene tray. The legend shows the location of two fiber-optic probes for monitoring temperatures. (a) Rep 1; (b) Rep 2.

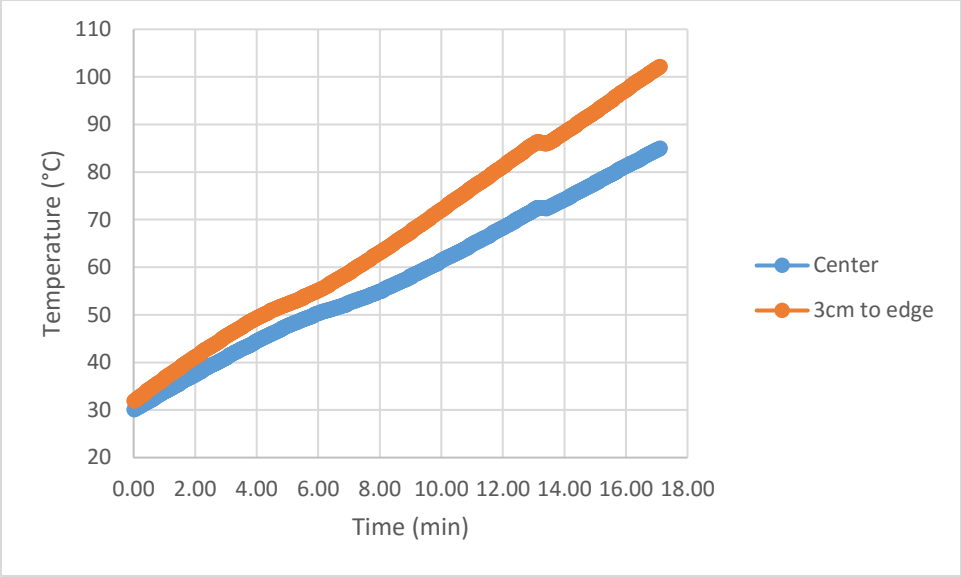


(a)

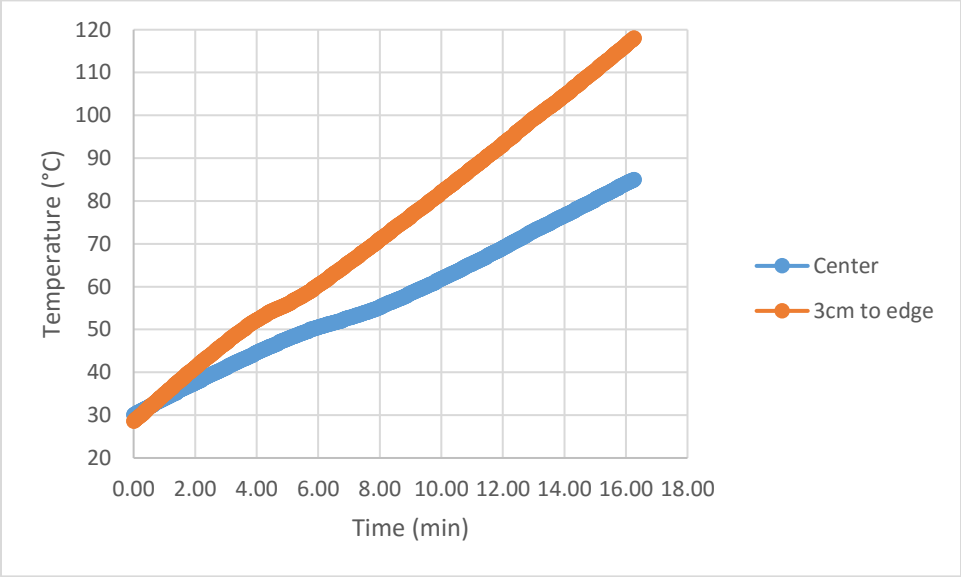


(b)

Figure C-3. Radio frequency dielectric heating of low heat nonfat dry milk from 30 to 80°C (target temperature for the center probe) in a circular polypropylene tray. The legend shows the location of two fiber-optic probes for monitoring temperatures. (a) Rep 1; (b) Rep 2.



(a)



(b)

Figure C-4. Radio frequency dielectric heating of low heat nonfat dry milk from 30 to 85°C (target temperature for the center probe) in a circular polypropylene tray. The legend shows the location of two fiber-optic probes for monitoring temperatures. (a) Rep 1; (b) Rep 2.

Appendix D - **D-values**

Table D-1. The D-values for *Salmonella* spp. in high heat (HH) and low heat (LH) nonfat dry milk (NDM) heated in thermal-death-time disks taken from Michael et al. (2014), and calculated heat process times to reach 5-log reductions of *Salmonella* spp.

	Temperature (°C)	D-values (min)[1]	Heat process times (min)[2]
HH			
	75	23.02	115.1
	80	10.45	52.25
	85	8.63	43.2
LH			
	75	24.94	124.7
	80	12.54	62.70
	85	8.68	43.4

[1]Decimal reduction time.

[2]Calculated by D-value × 5.

Appendix E - **Converting nitrogen to protein content**

Table E-1. Nitrogen content for low heat (LH) and high heat (HH) nonfat dry milk (NDM) and conversion to protein content

NDM	Rep	Nitrogen (%)[1]	Factor	Protein (%)[2]
HH	1	5.53	6.38	35.29
HH	2	5.53	6.38	35.31
HH	3	5.53	6.38	35.31
LH	1	5.68	6.38	36.25
LH	2	5.67	6.38	36.19
LH	3	5.66	6.38	36.14

[1]Obtained by Leco FP-2000 protein analyzer (Laboratory Equipment Co.).

[2]Calculated by nitrogen (%) × factor.

Appendix F - **WPNI standard curve and equation**

Table F-1. WPNI standard curve

Tube	T%				Average T%	WPN (mg/g)
	M1*	M2	M3	M4		
1	58.8	58.8	55.1	54.8	56.875	7.74
2	61.7	62.5	64.8	64.8	63.45	6.318
3	69	71.8	70.4	71.9	70.775	4.896
4	81.8	83.7	81.2	81.6	82.075	3.474
5	87.9	87.6	87	86.7	87.3	2.052
6	96.6	96.5	96.6	96.5	96.55	0.63

*M1-4 represent measurements 1-4.

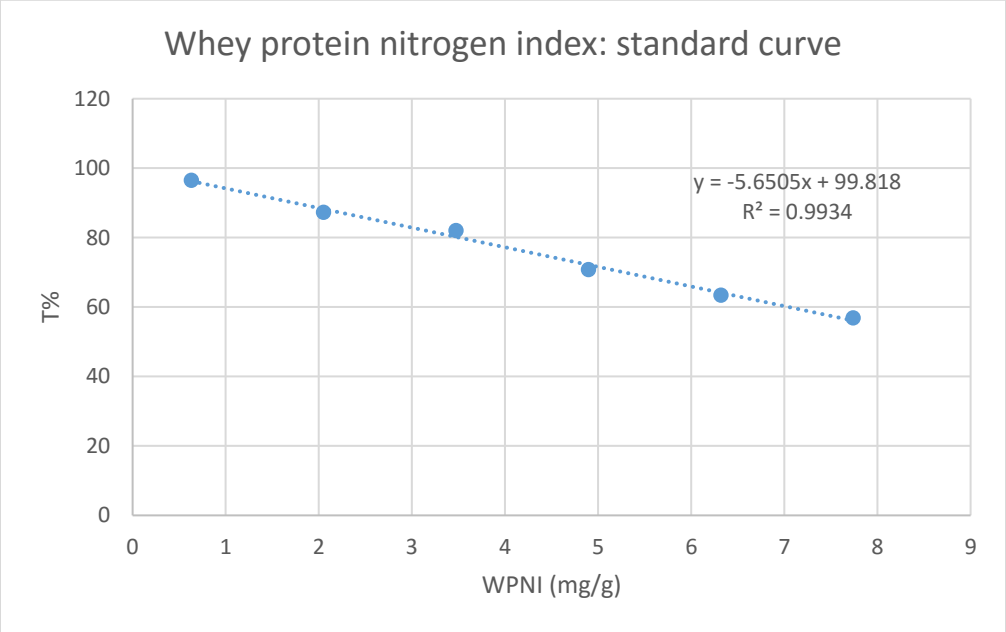


Figure F-1. Whey protein nitrogen index (WPNI) standard curve plotted by T% vs. WPNI

Table F-2. Transmittance% (T%) and whey protein nitrogen index (WPNI) values for all high heat nonfat dry milk samples as a function of radio frequency dielectric heating temperature[1]

HH		T%				Average T%	WPNI (mg/g)
	Temperature (°C)	M1[2]	M2	M3	M4		
Rep1							
	C[3]	89.7	89.7	89.7	89.7	89.7	1.79
	75	89.1	89.1	89.1	89.1	89.18	1.88
	80	89.4	89.4	89.4	89.4	89.45	1.83
	85	89.2	89.2	89.2	89.2	89.2	1.88
Rep2							
	C	90	89.7	89.5	89.9	89.8	1.78
	75	89.3	89.2	89.1	89.0	89.2	1.89
	80	89.3	89.1	88.4	88.4	88.8	1.95
	85	88.2	88.2	88.2	88.0	88.2	2.06

[1]Control, 75, 80, 85°C for 0, 115, 52, 43 min, respectively.

[2] M1-4 represent measurements 1-4 (two measurements were done on each filtrate).

[3]Represents the control.

Table F-3. Transmittance% (T%) and whey protein nitrogen index (WPNI) values for all low heat (LH) nonfat dry milk samples as a function of radio frequency dielectric heating temperature[1]

LH		T%				Average T%	WPNI (mg/g)
	Temperature (°C)	M1[2]	M2	M3	M4		
Rep1							
	C[3]	58.5	58.4	58.9	59.1	58.7	7.27
	75	63.6	63.9	63.5	63.8	63.7	6.39
	80	62.4	62.6	62.7	63.0	62.7	6.57
	85	61.9	62.1	60.3	60.4	61.2	6.84
Rep2							
	C	58.3	58.4	58.1	58.3	58.3	7.35
	75	62.0	62.1	63.3	63.4	62.7	6.57
	80	62.7	62.6	62.2	62.4	62.5	6.61
	85	60.4	60.4	60.8	60.7	60.6	6.95

[1]Control, 75, 80, 85°C for 0, 125, 63, 43 min, respectively.

[2] M1-4 represent measurements 1-4 (two measurements were done on each filtrate).

[3] Represents the control.

Appendix G - **Molecular weight determination of bands on SDS-PAGE gels**

Table G-1. The relative distance[1] vs. molecular weight standard curve for high heat nonfat dry milk using standard protein marker

Weight (kDa)	250	150	100	75	50	37	25	20	15	10
Distance	0.195	0.308	0.413	0.477	0.61	0.696	0.812	0.862	0.929	0.967

[1]Defined in Figure F-2.

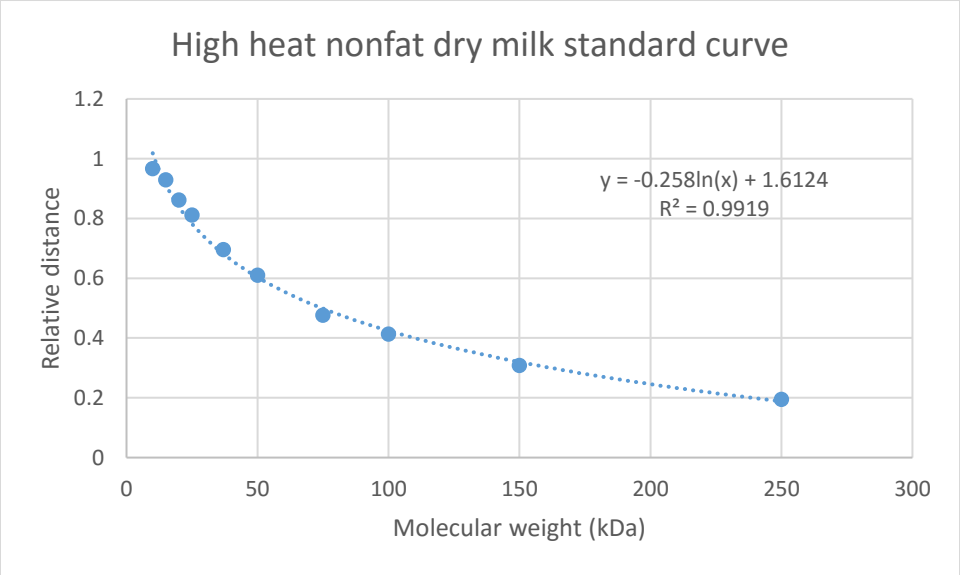


Figure G-1. The relative distance vs. molecular weight standard curve for high heat nonfat dry milk (plotted using Microsoft Office Excel 2013)

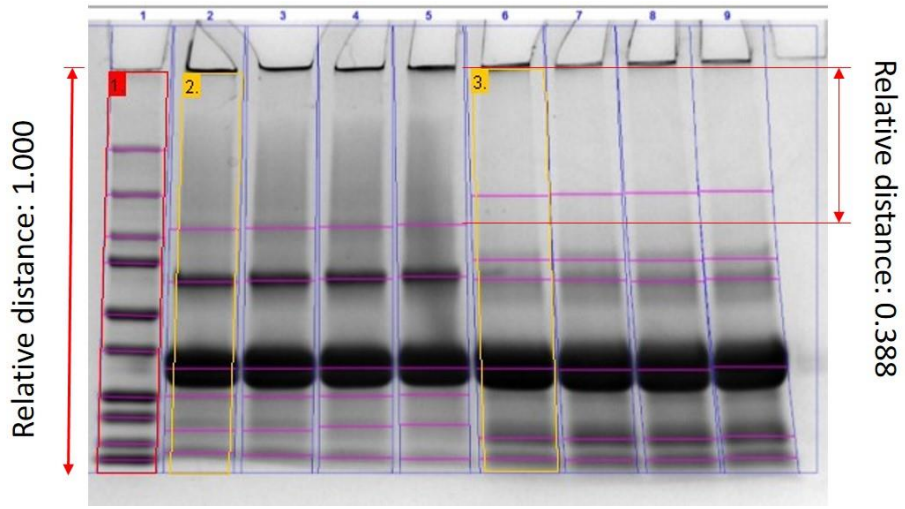


Figure G-2. Definition of relative distance on SDS-PAGE gel for high heat nonfat dry milk.

*Purple lines are used to mark bands.

Table G-2. Calculation[1] of molecular weights of bands based on standard curve

Distance	0.316	0.388	0.474	0.519	0.523	0.709	0.769	0.803	0.891	0.947
Weight (kDa)	152	115	82	69	68	33	26	23	16	13

[1]$y = -0.258 \ln(x) + 1.6124$; x = molecular weight (kDa), y = relative distance.

Table G-3. The relative distance[1] vs. molecular weight standard curve for high heat nonfat dry milk using standard protein marker

Weight (kDa)	250	150	100	75	50	37	25	20	15	10
Distance	0.199	0.303	0.409	0.471	0.591	0.682	0.799	0.854	0.926	0.977

[1]Defined in Figure F-4.

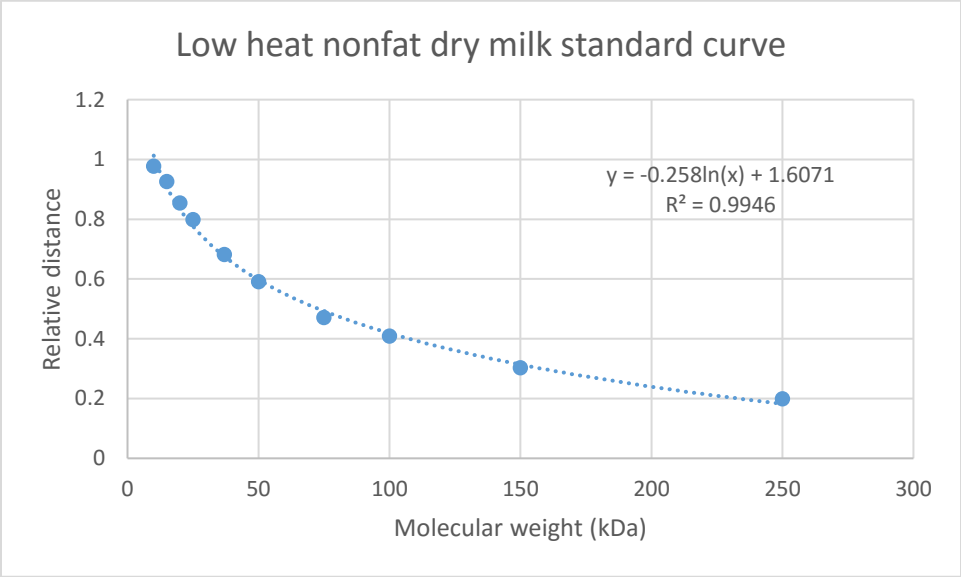


Figure G-3. The relative distance vs. molecular weight standard curve for low heat nonfat dry milk (plotted using Microsoft Office Excel 2013)

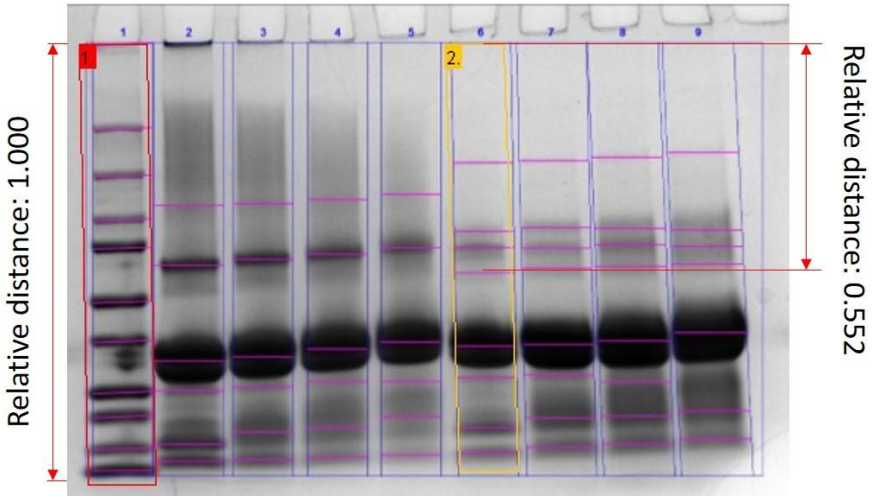


Figure G-4. Definition of relative distance on SDS-PAGE gel for low heat nonfat dry milk.

*Purple lines are used to mark bands.

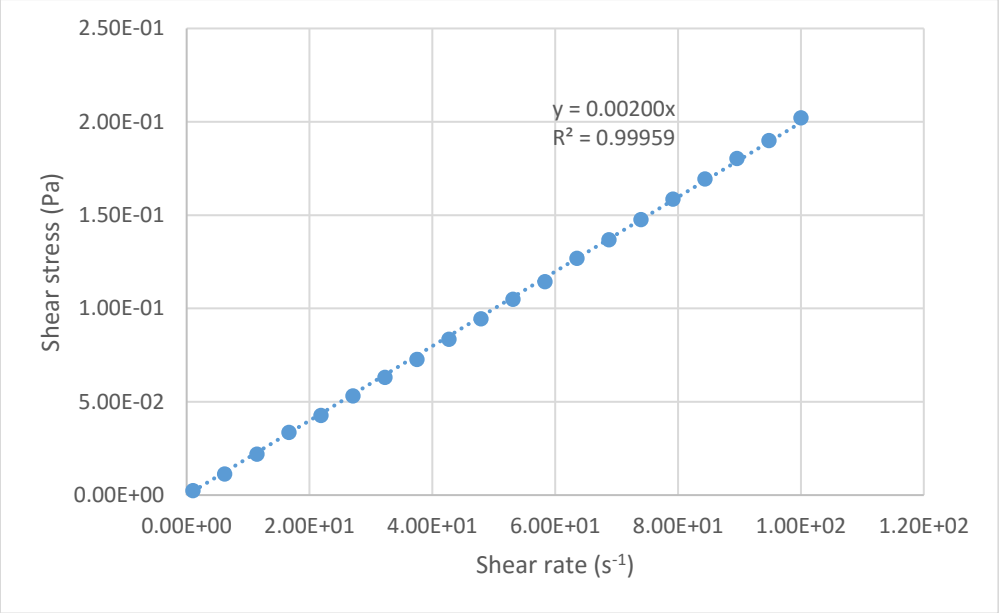
Table G-4. Calculation[1] of molecular weights of bands based on standard curve[*]

Distance	0.552
Weight (kDa)	60

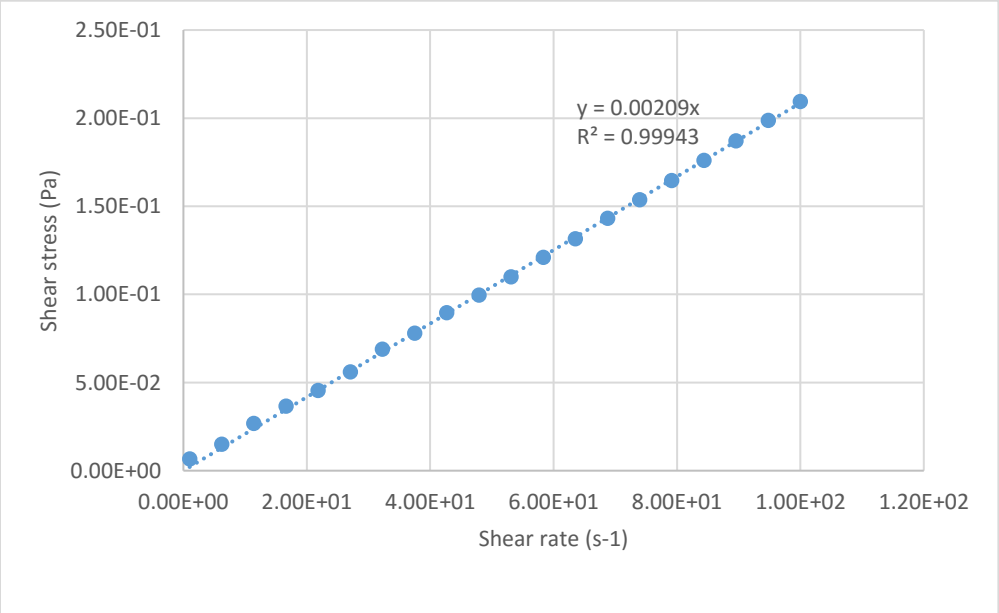
[1]y = -0.258 ln(x) + 1.6124; x = molecular weight (kDa), y = relative distance.

[*]Only shows the band that doesn't exist in high heat nonfat dry milk samples

Appendix H - **Apparent viscosity**



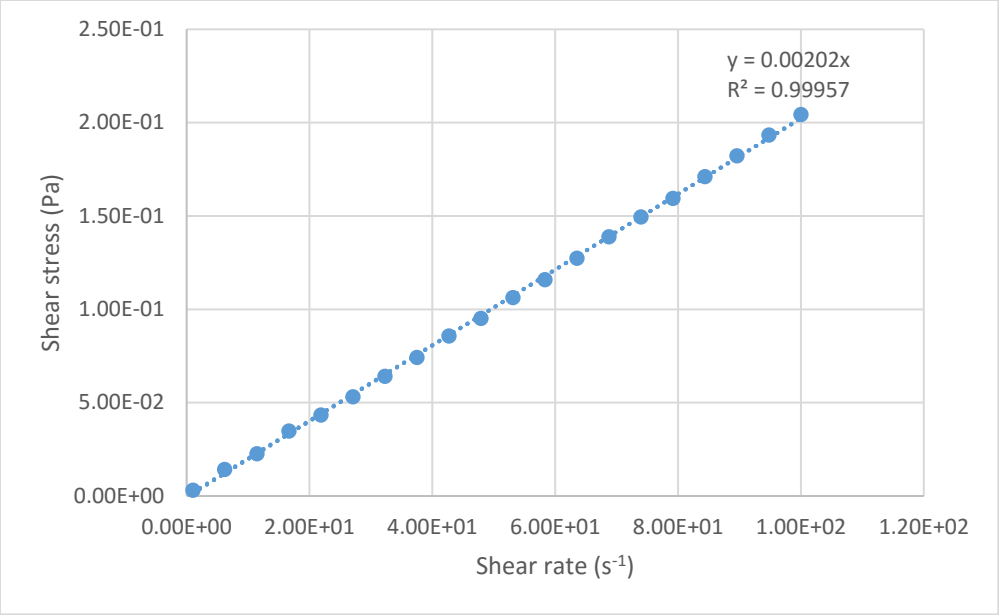
(a)



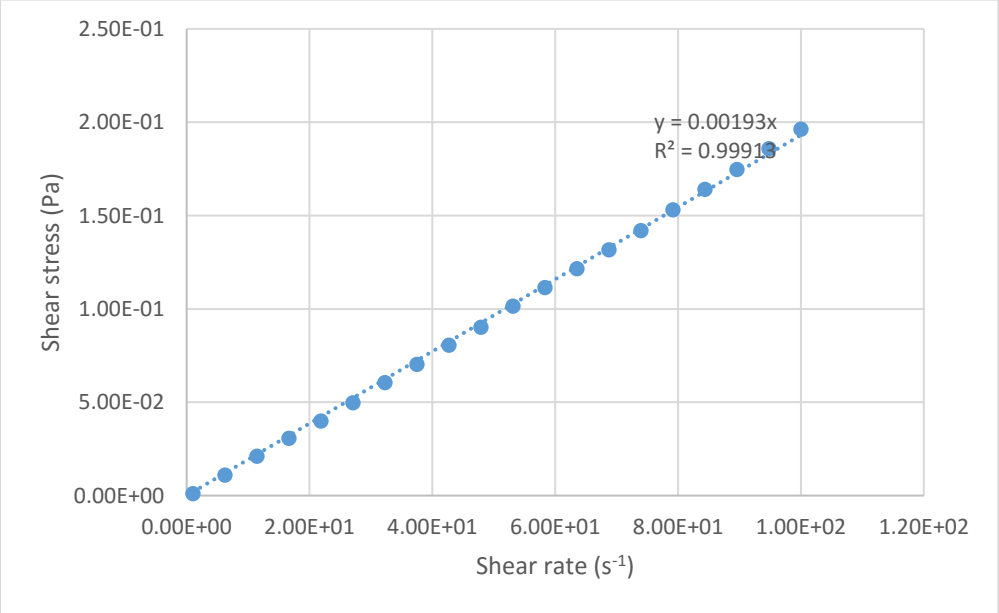
(b)

Figure H-1. Shear stress vs. shear rate for the dispersion made from the control (non-treated) high heat nonfat dry milk samples (Rep 1).

(a) Measurement 1; (b) Measurement 2.



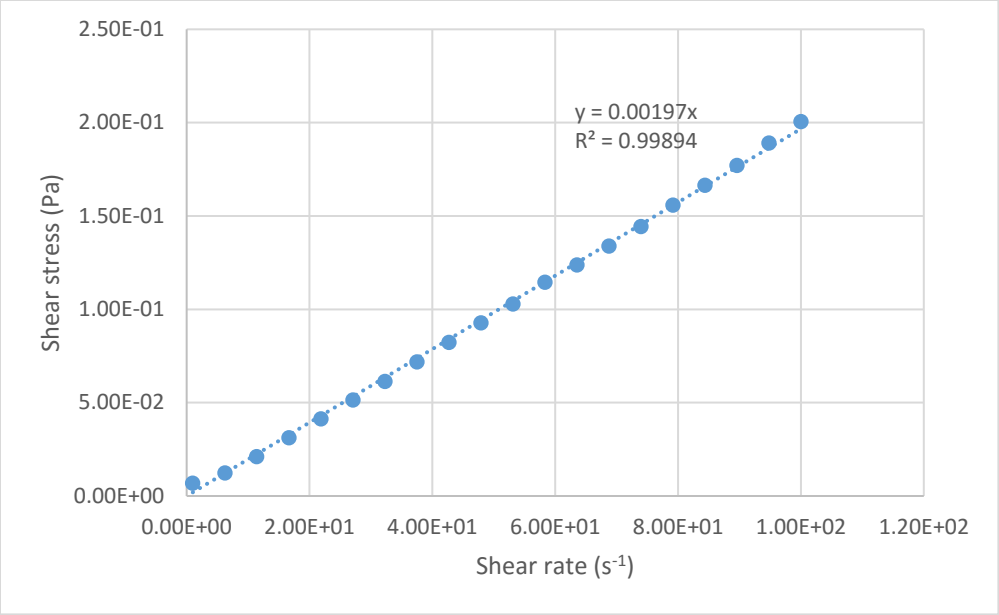
(a)



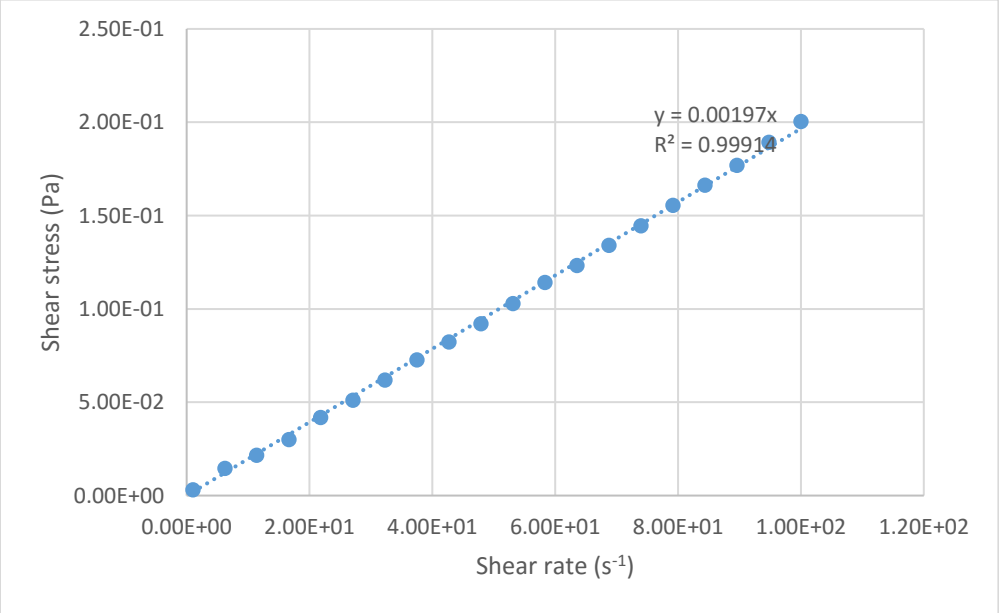
(b)

Figure H-2. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 75°C in a RFDH unit and held in a convection oven for 115 min (Rep 1).

(a) Measurement 1; (b) Measurement 2.



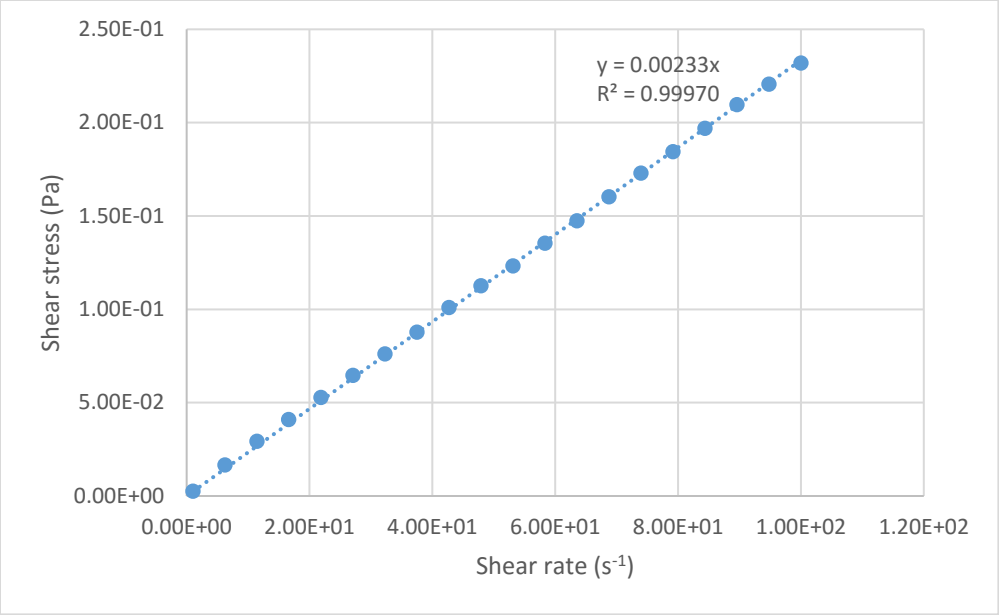
(a)



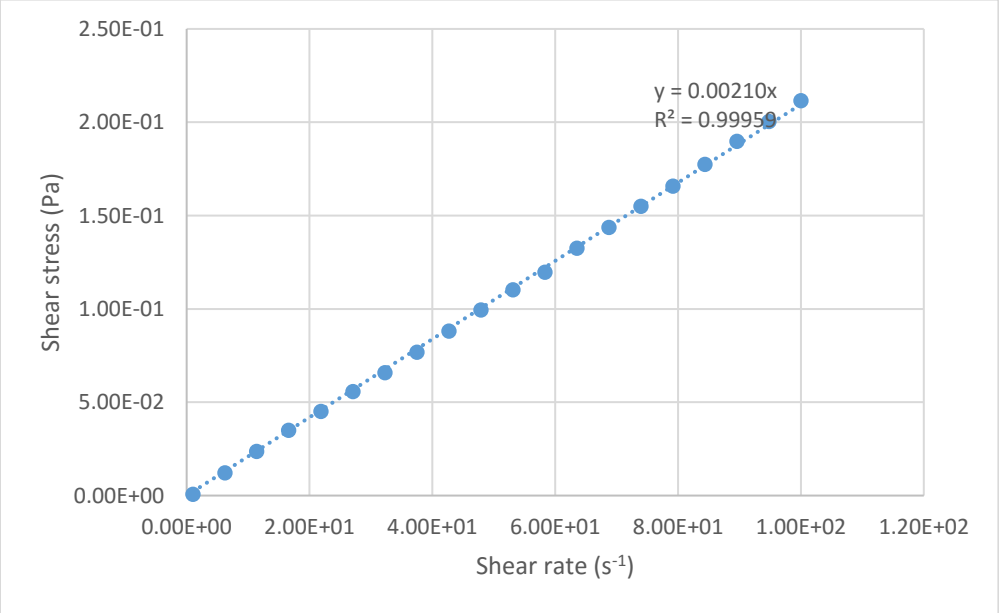
(b)

Figure H-3. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 80°C in a RFDH unit and held in a convection oven for 52 min (Rep 1).

(a) Measurement 1; (b) Measurement 2.



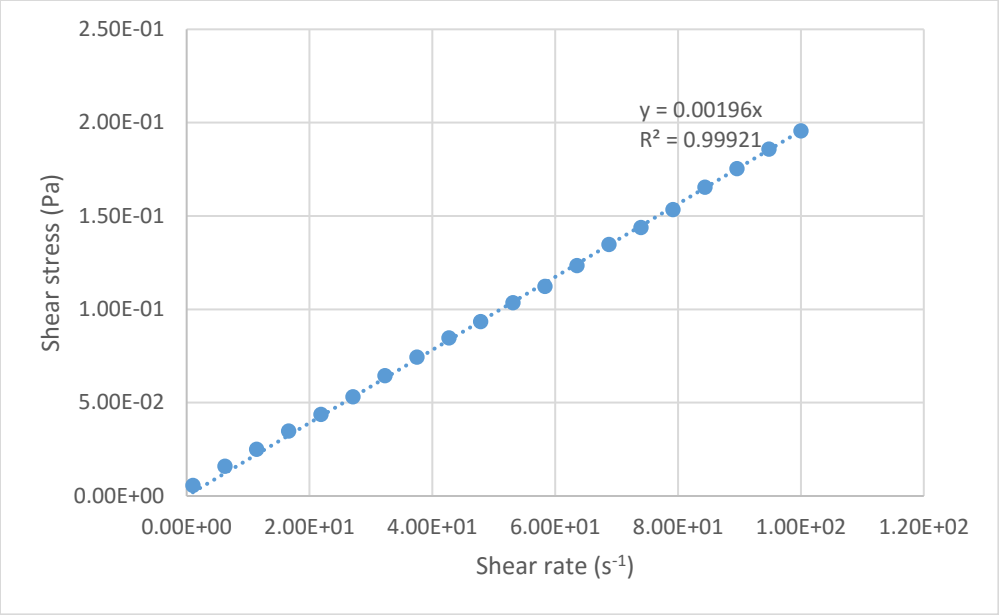
(a)



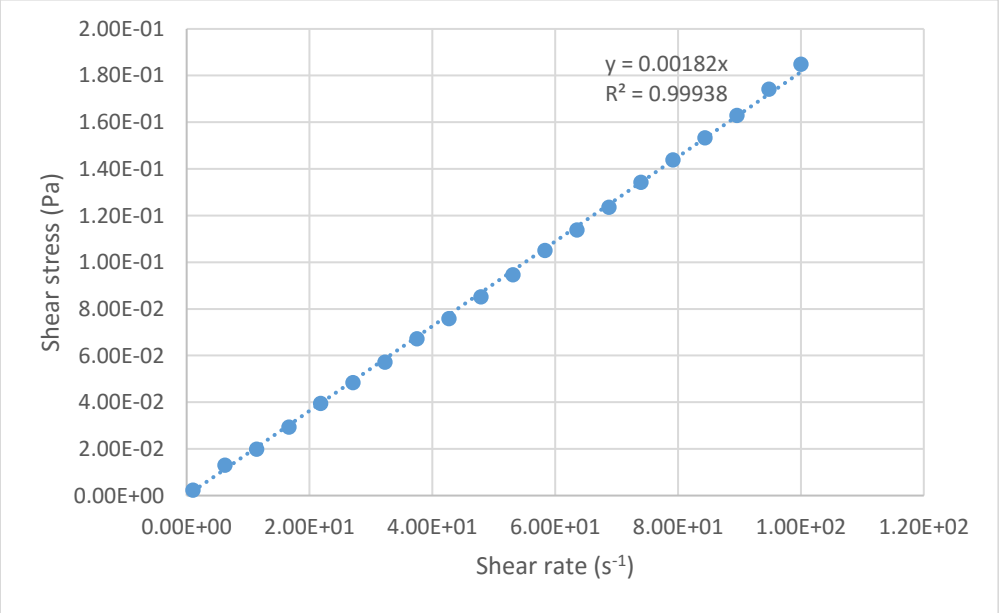
(b)

Figure H-4. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 85°C in a RFDH unit and held in a convection oven for 43 min (Rep 1).

(a) Measurement 1; (b) Measurement 2.



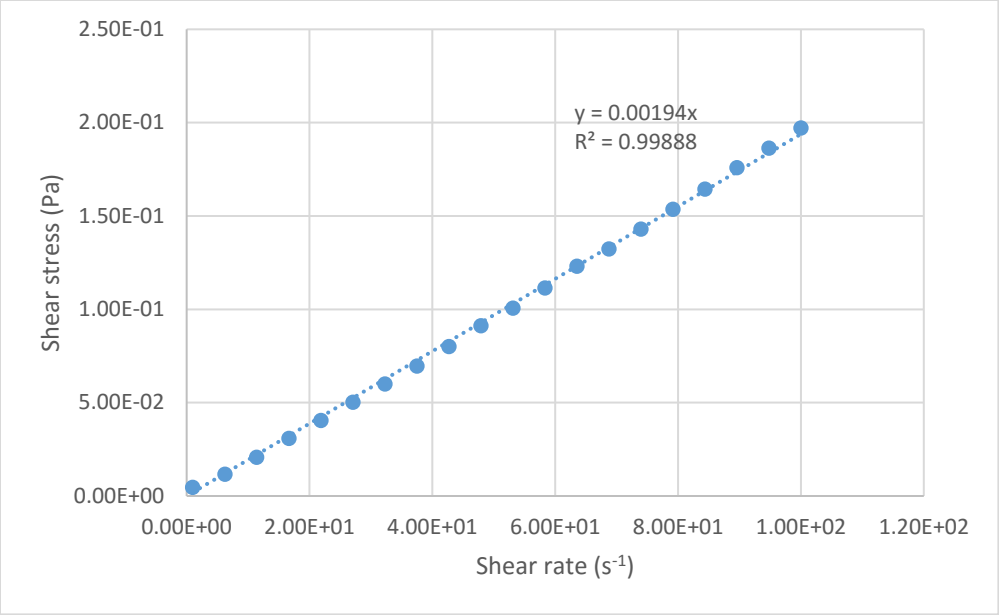
(a)



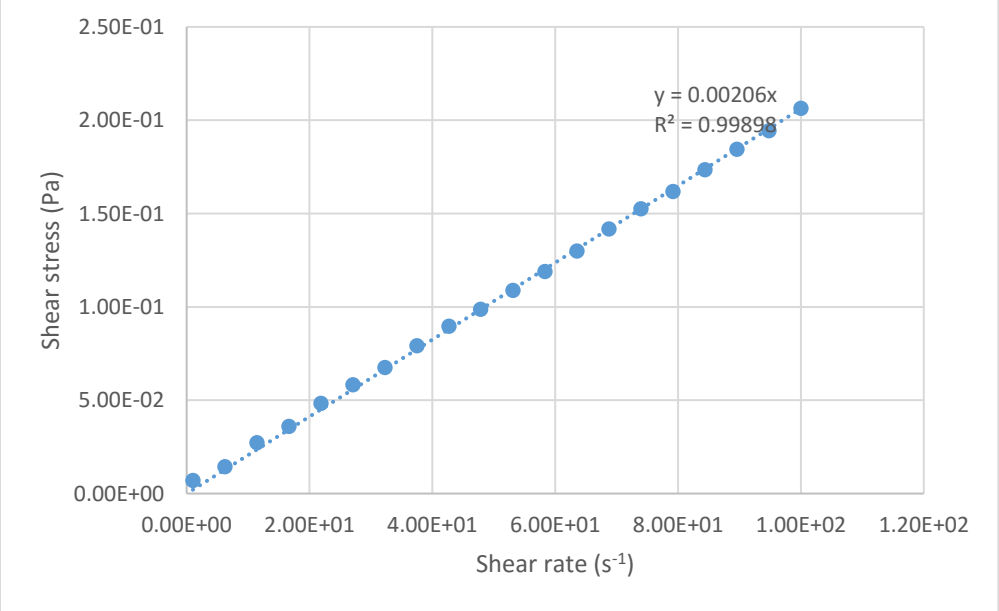
(b)

Figure H-5. Shear stress vs. shear rate for the dispersion made from the control (non-treated) low heat nonfat dry milk samples (Rep 1).

(a) Measurement 1; (b) Measurement 2.



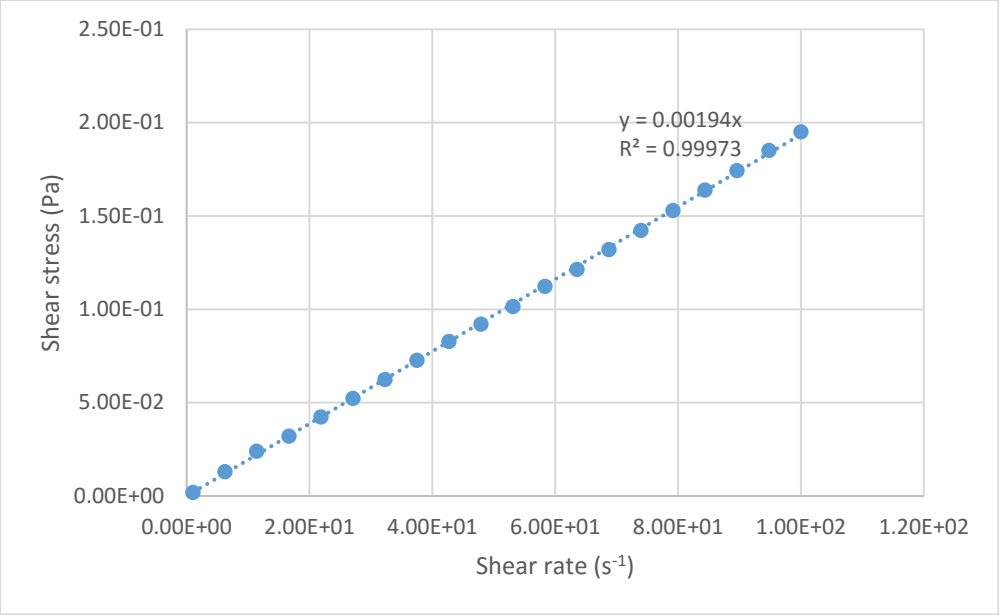
(a)



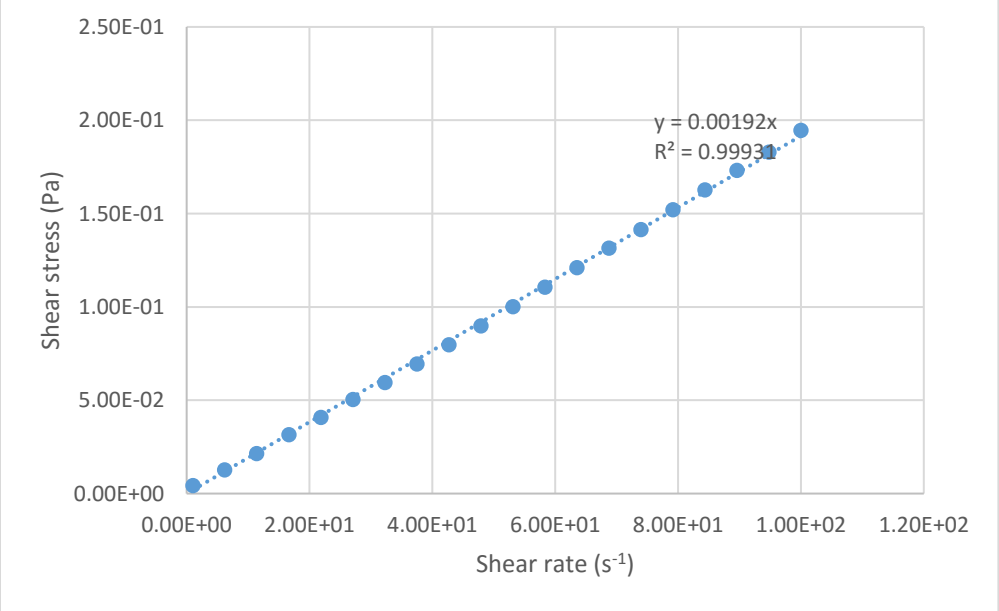
(b)

Figure H-6. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 75°C in a RFDH unit and held in a convection oven for 125 min (Rep 1).

(a) Measurement 1; (b) Measurement 2.



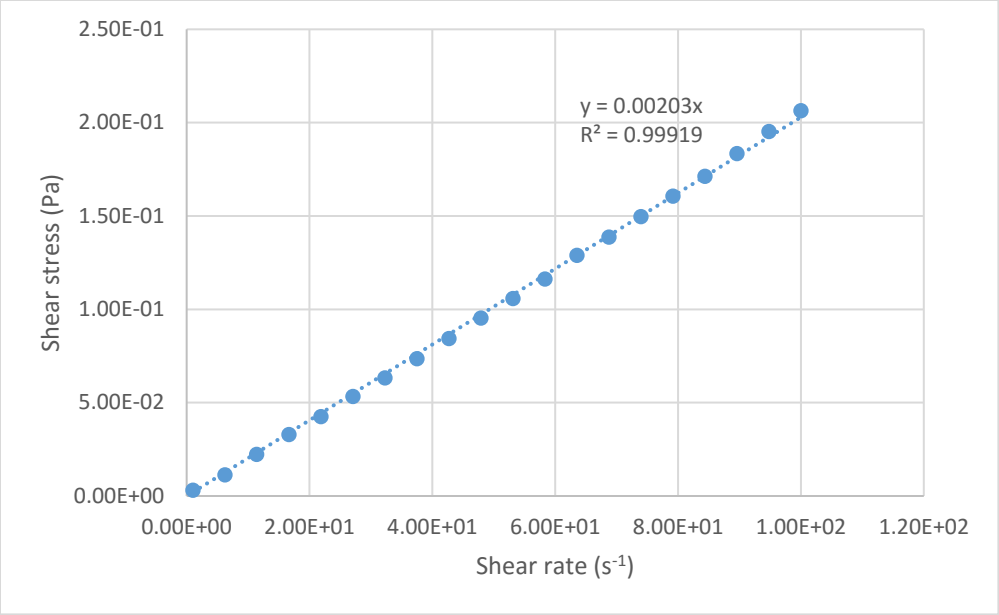
(a)



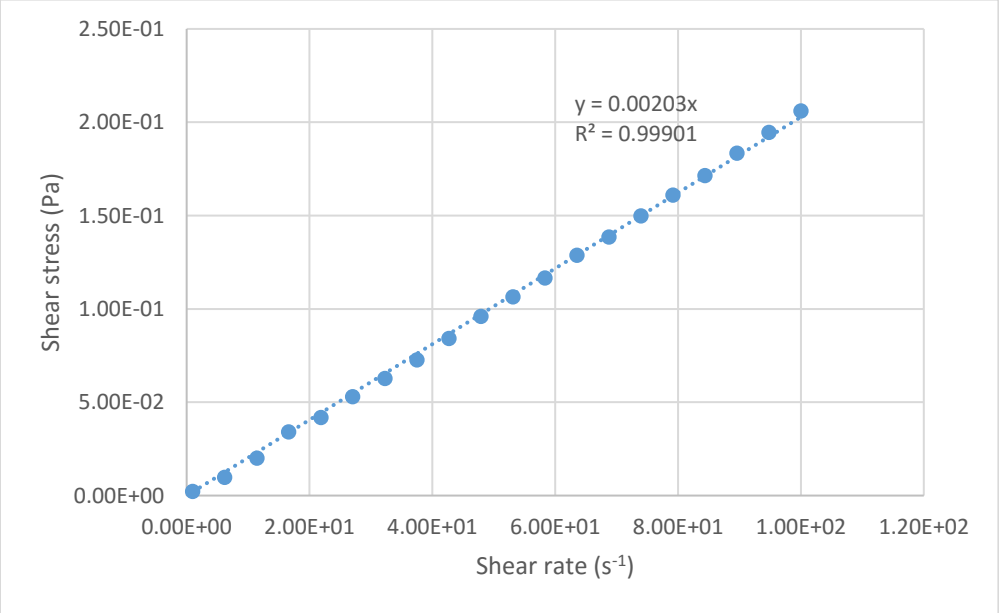
(b)

Figure H-7. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 80°C in a RFDH unit and held in a convection oven for 63 min (Rep 1).

(a) Measurement 1; (b) Measurement 2.



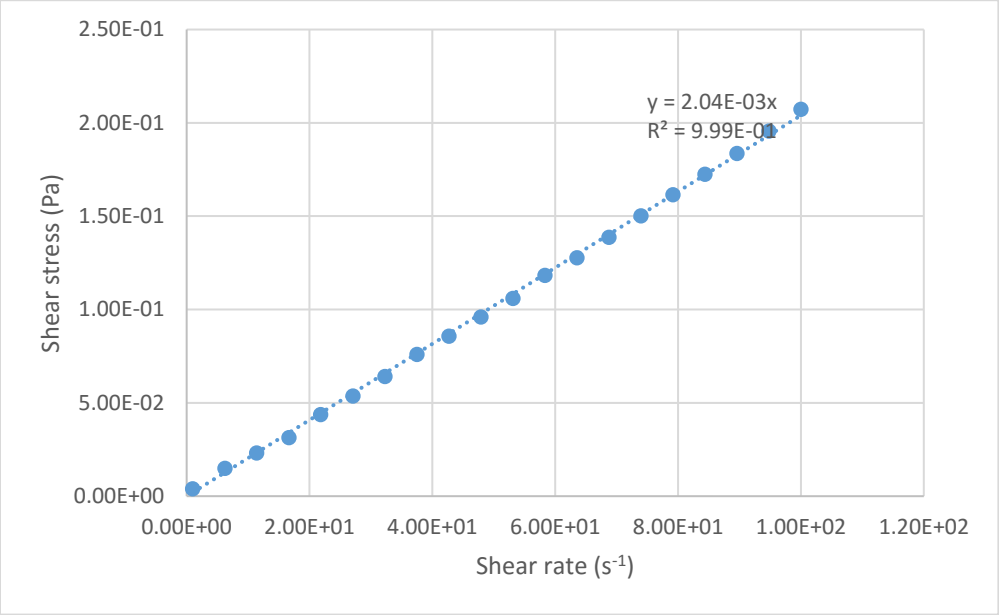
(a)



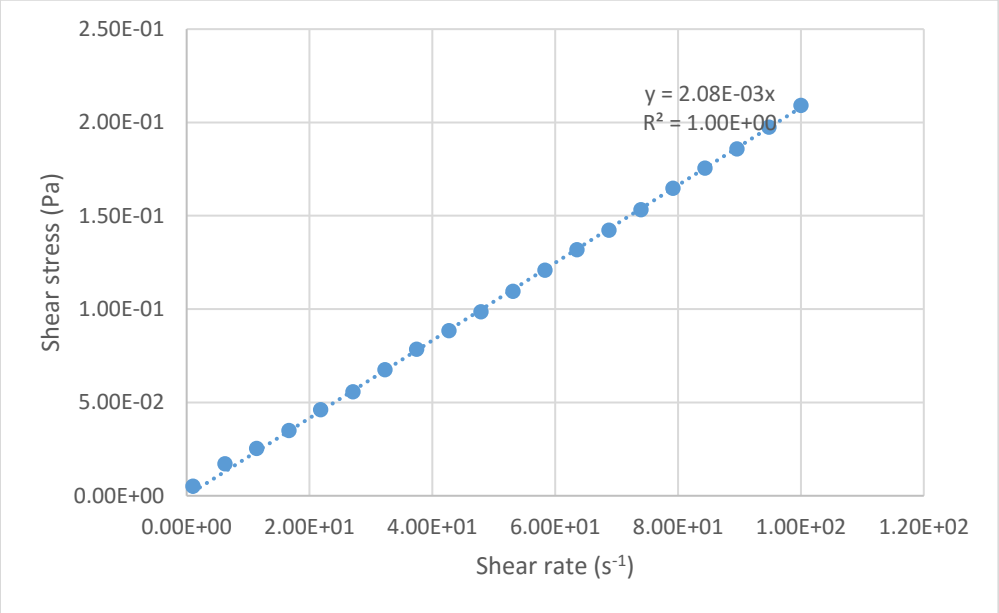
(b)

Figure H-8. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 85°C in a RFDH unit and held in a convection oven for 43 min (Rep 1).

(a) Measurement 1; (b) Measurement 2.



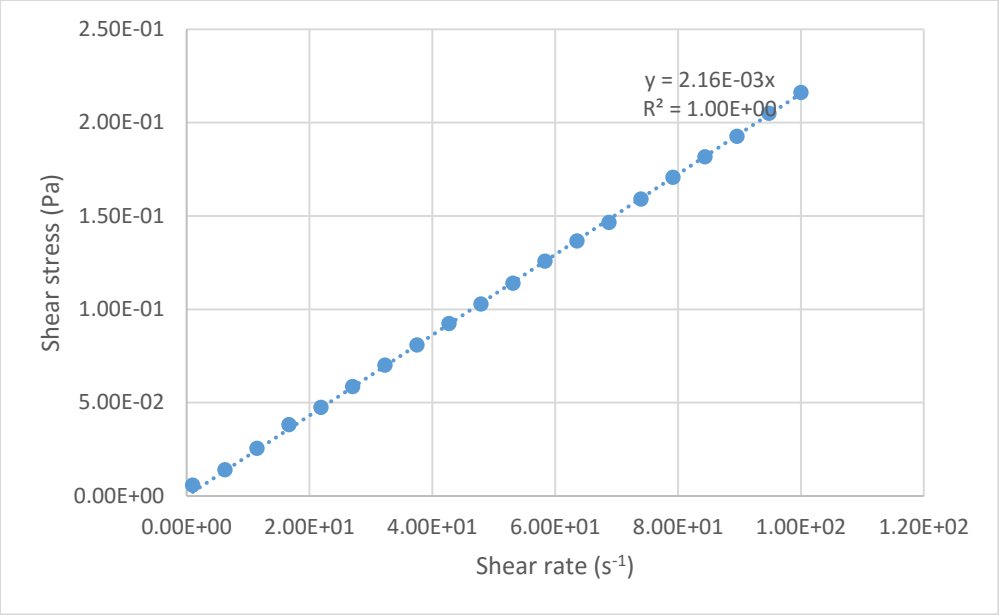
(a)



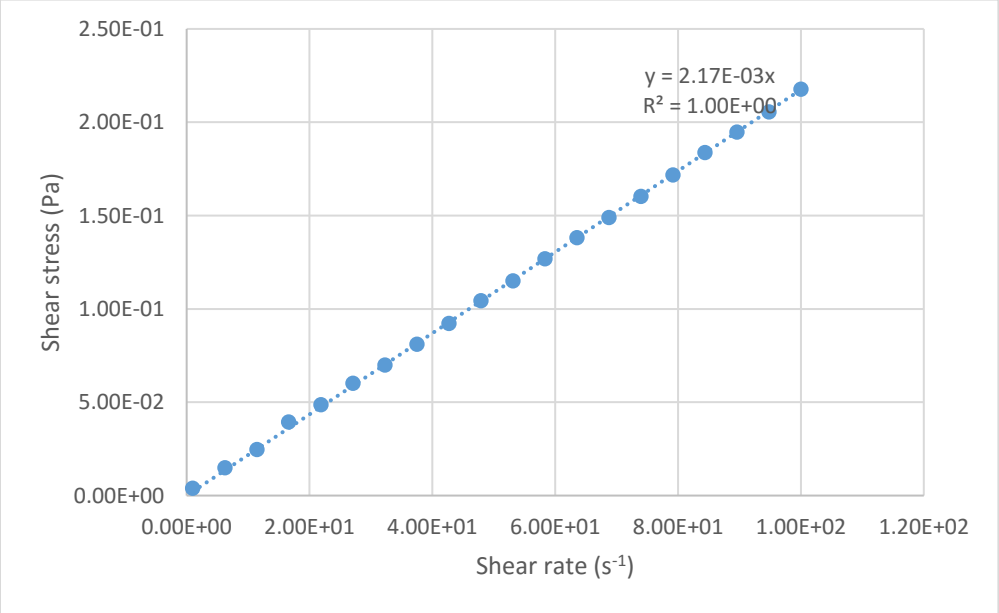
(b)

Figure H-9. Shear stress vs. shear rate for the dispersion made from the control (non-treated) high heat nonfat dry milk samples (Rep 2).

(a) Measurement 1; (b) Measurement 2.



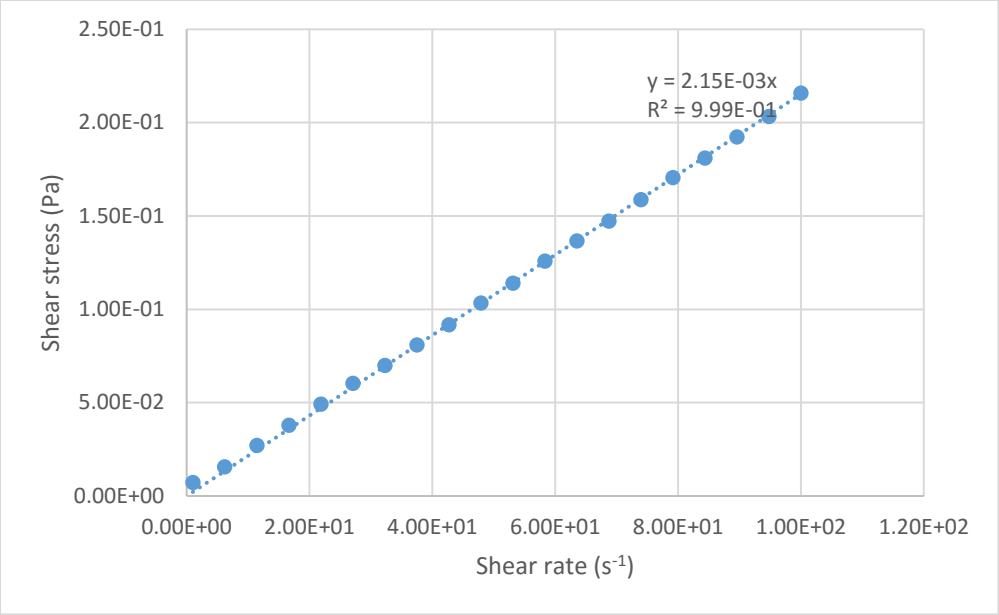
(a)



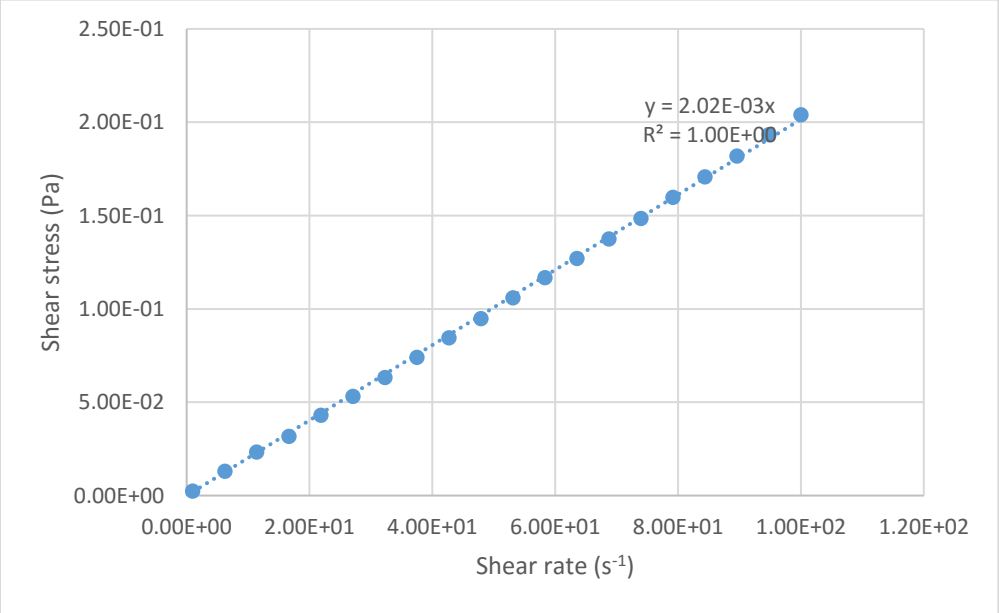
(b)

Figure H-10. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 75°C in a RFDH unit and held in a convection oven for 115 min (Rep 2).

(a) Measurement 1; (b) Measurement 2.



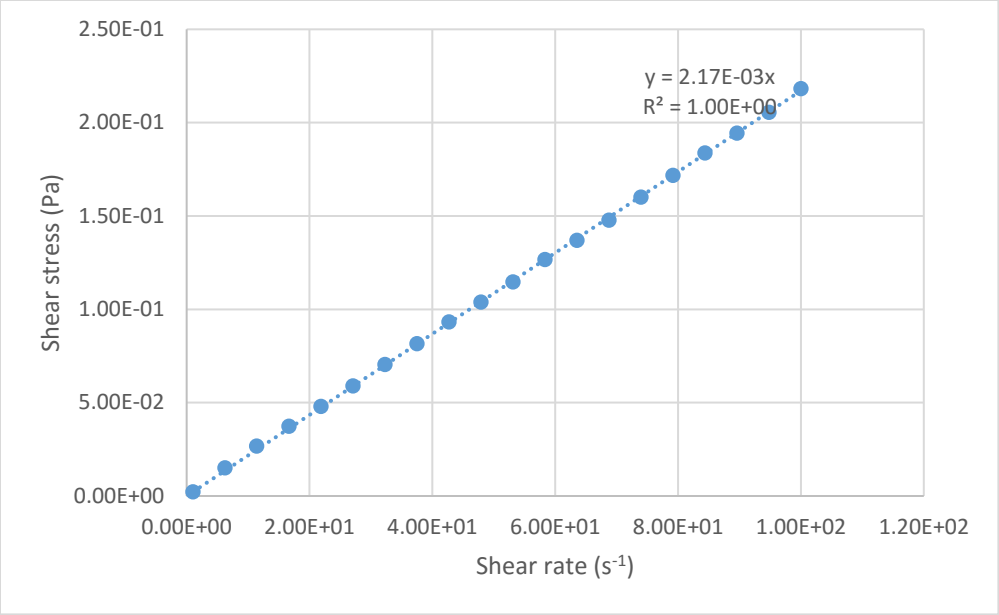
(a)



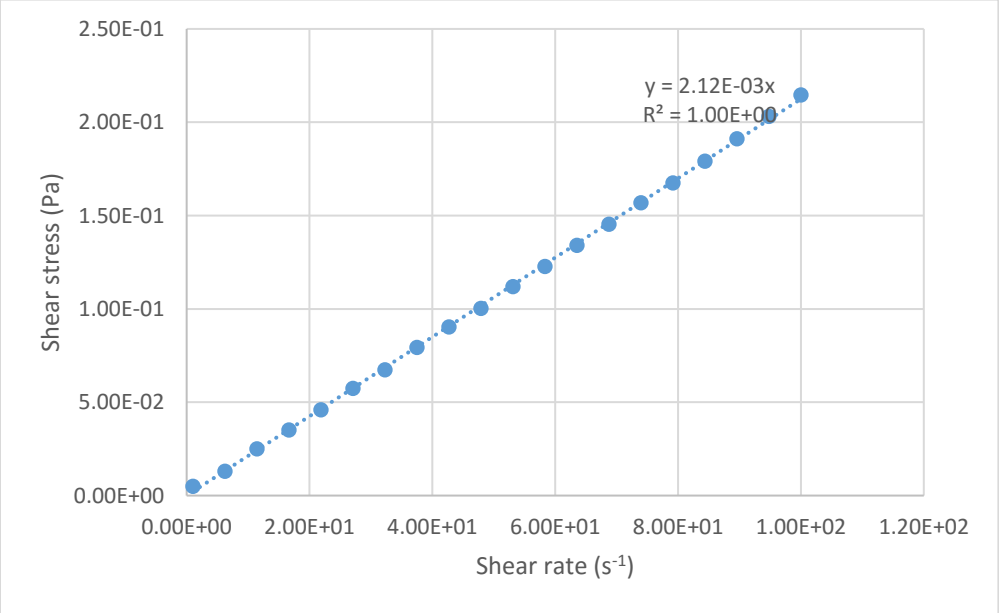
(b)

Figure H-11. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 80°C in a RFDH unit and held in a convection oven for 52 min (Rep 2).

(a) Measurement 1; (b) Measurement 2.



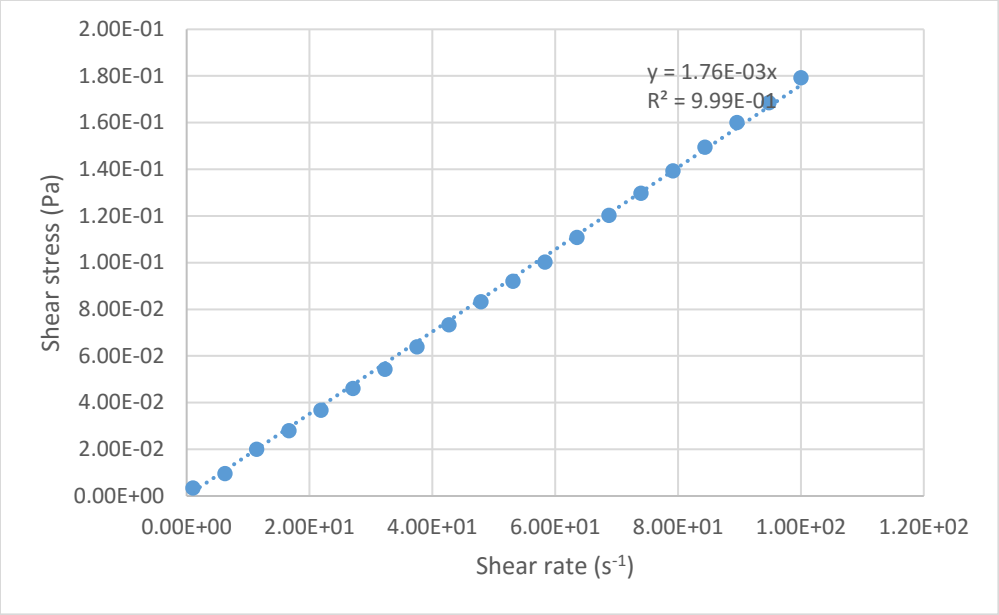
(a)



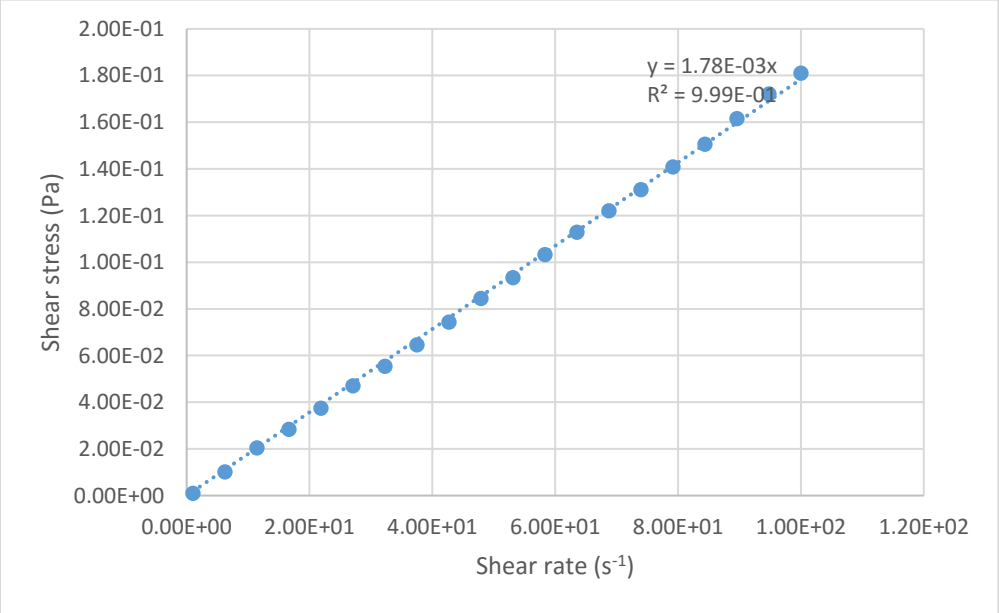
(b)

Figure H-12. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 85°C in a RFDH unit and held in a convection oven for 43 min (Rep 2).

(a) Measurement 1; (b) Measurement 2.



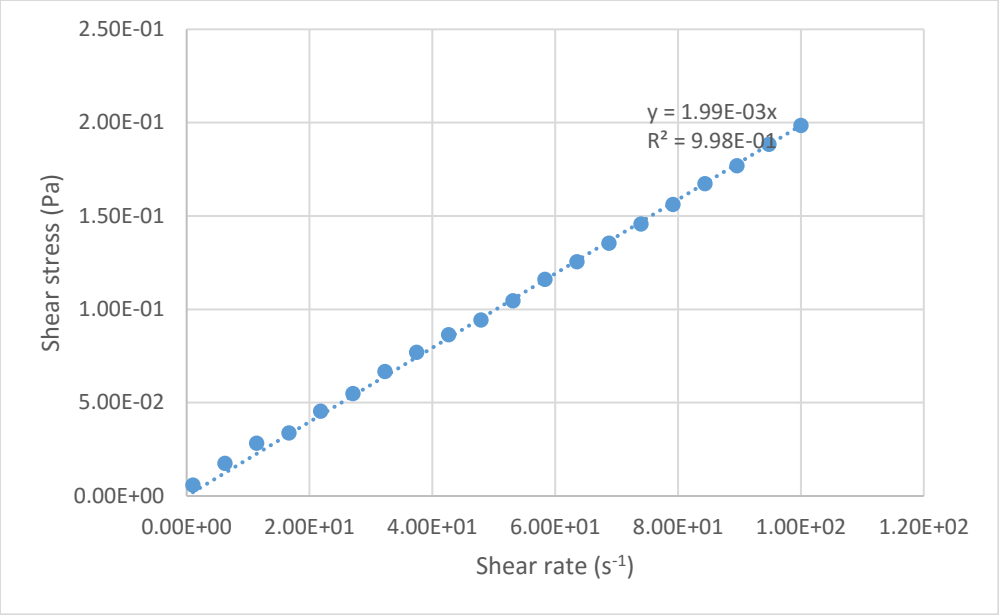
(a)



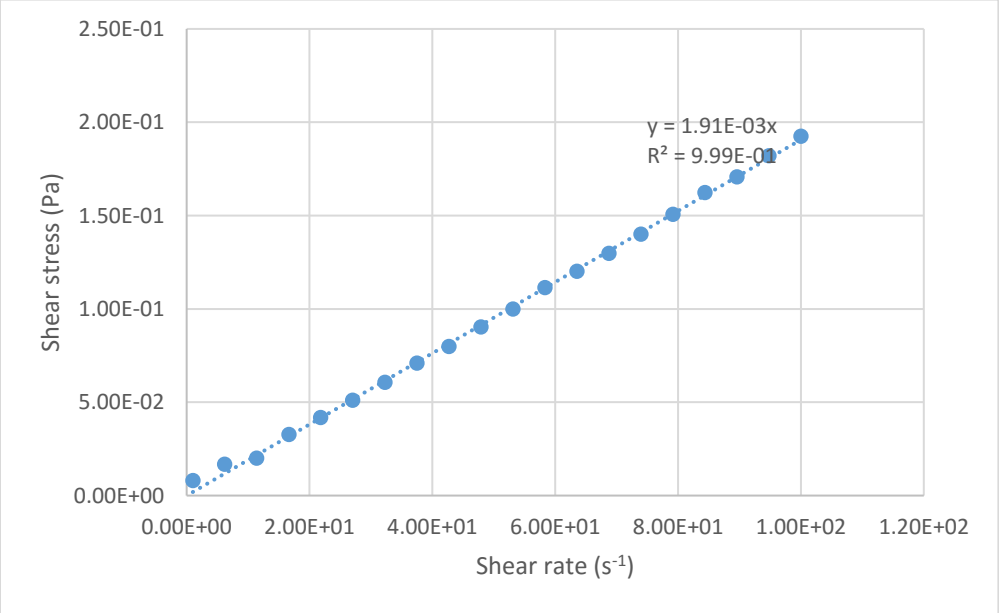
(b)

Figure H-13. Shear stress vs. shear rate for the dispersion made from the control (non-treated) low heat nonfat dry milk samples (Rep 2).

(a) Measurement 1; (b) Measurement 2.



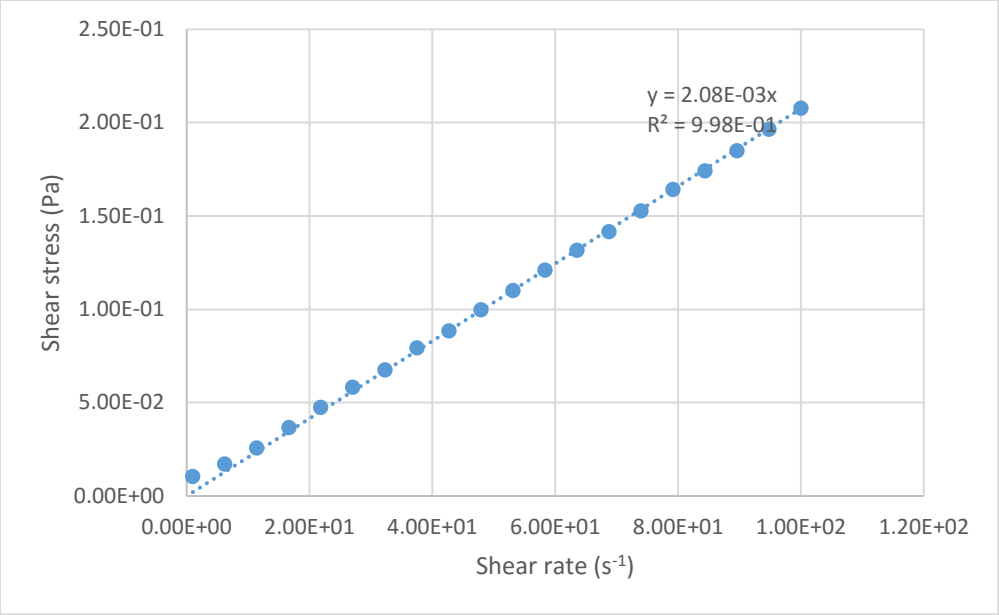
(a)



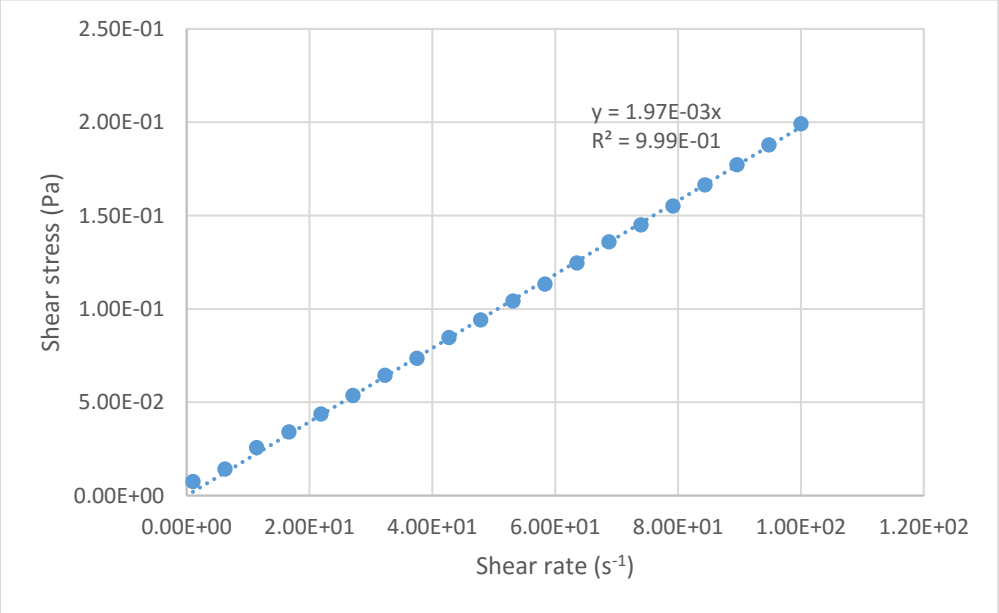
(b)

Figure H-14. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 75°C in a RFDH unit and held in a convection oven for 125 min (Rep 2).

(a) Measurement 1; (b) Measurement 2.



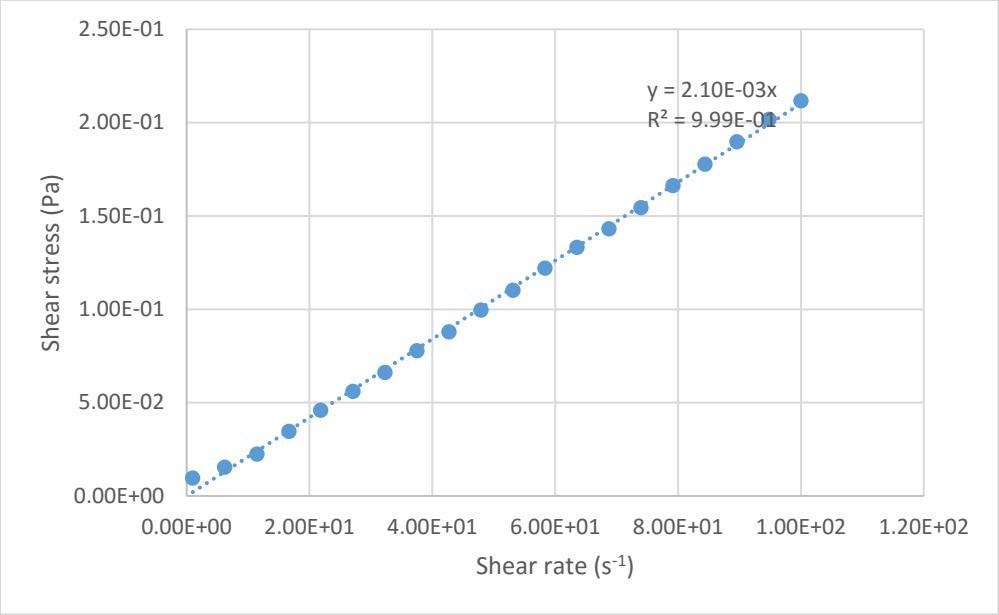
(a)



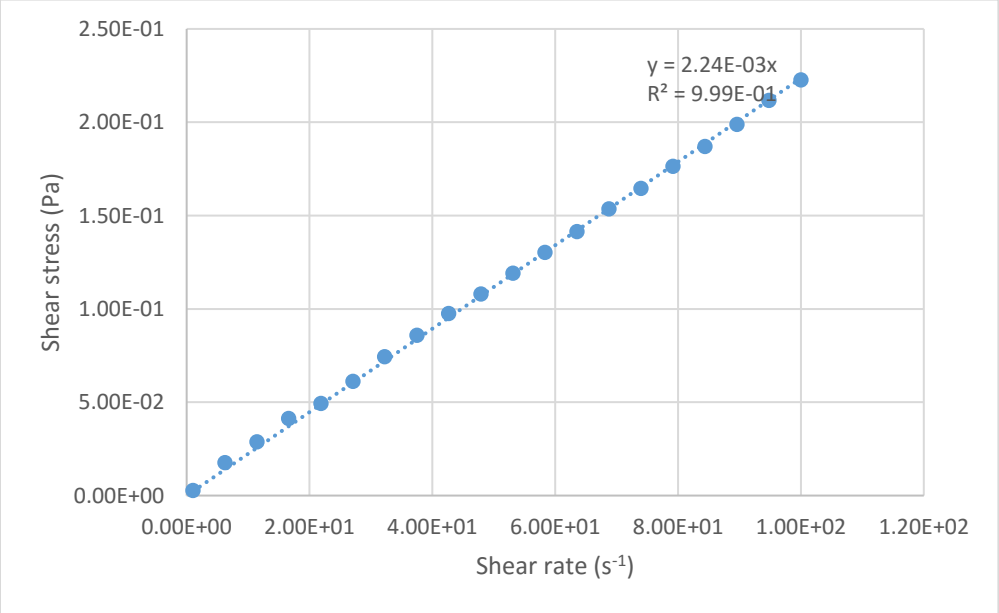
(b)

Figure H-15. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 80°C in a RFDH unit and held in a convection oven for 63 min (Rep 2).

(a) Measurement 1; (b) Measurement 2.



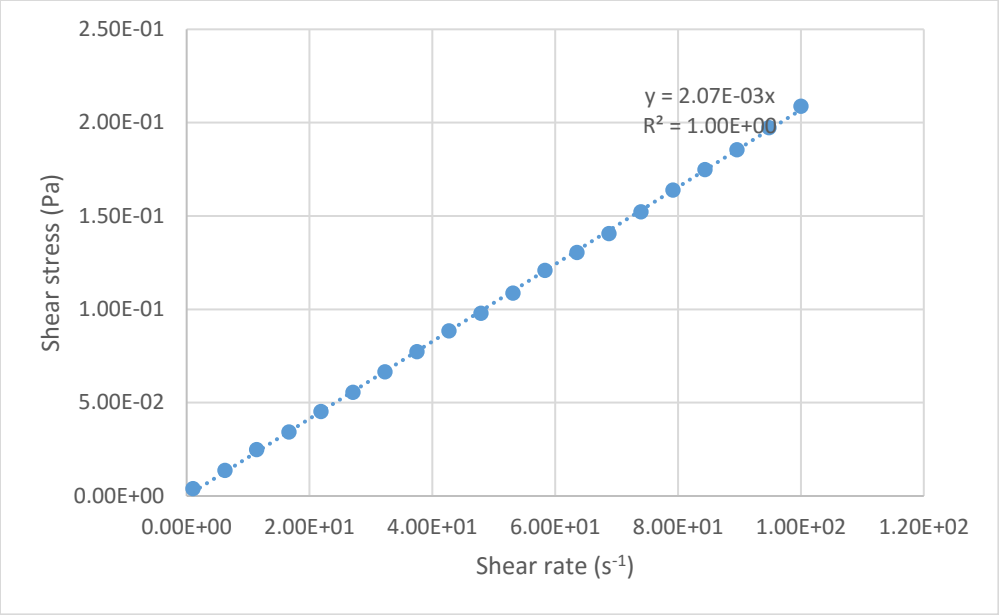
(a)



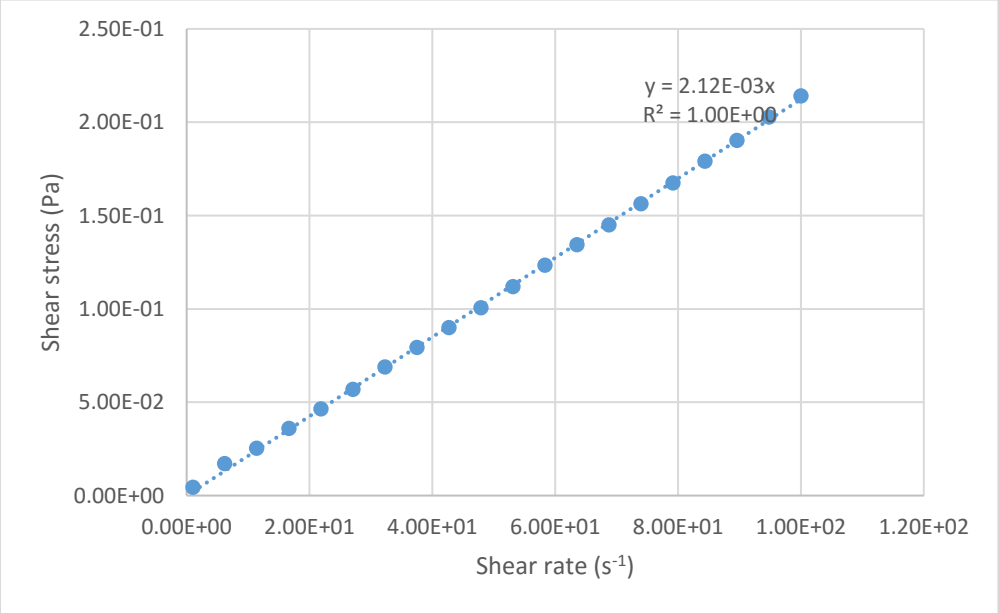
(b)

Figure H-16. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 85°C in a RFDH unit and held in a convection oven for 43 min (Rep 2).

(a) Measurement 1; (b) Measurement 2.



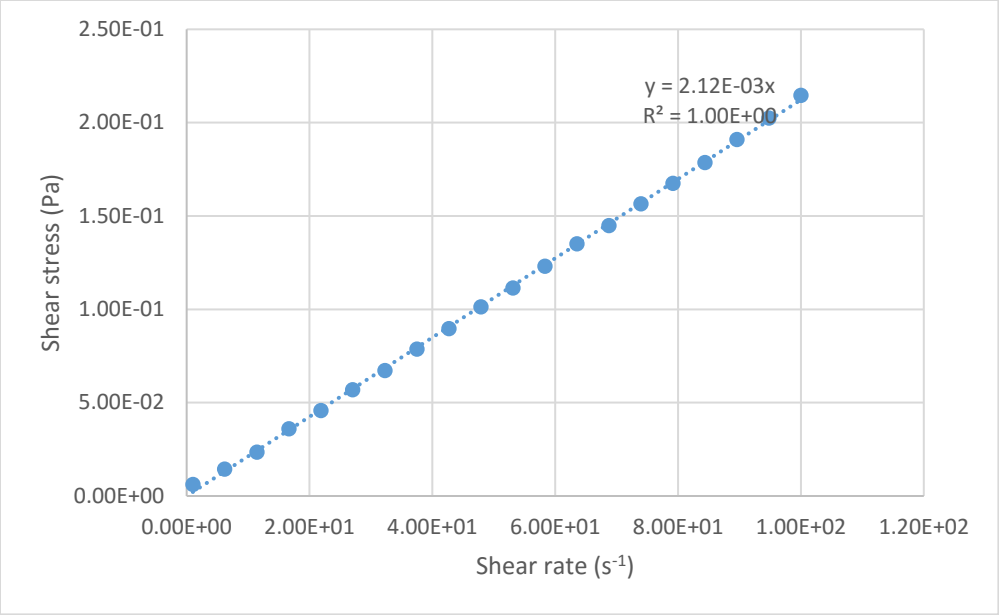
(a)



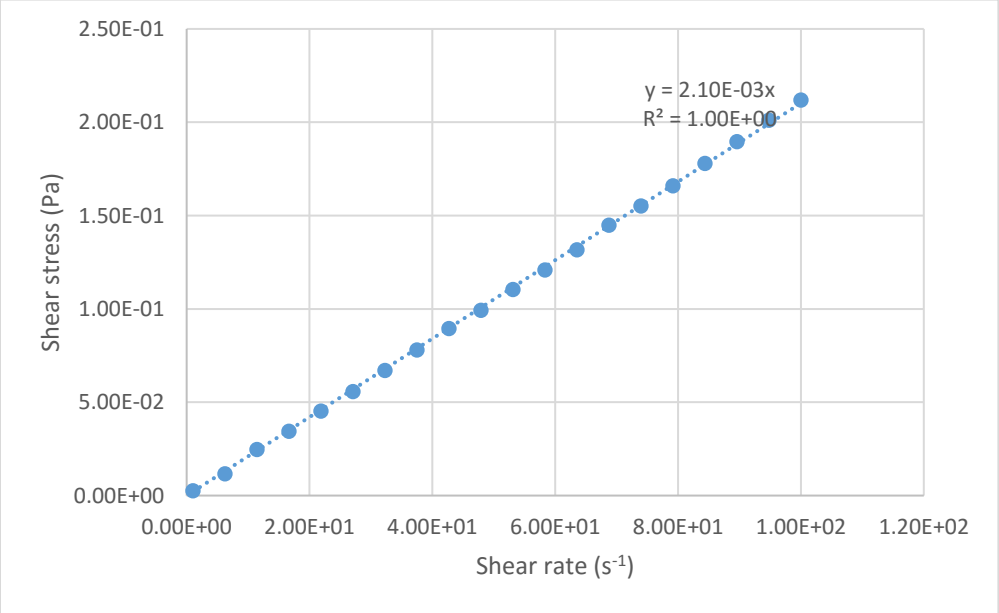
(b)

Figure H-17. Shear stress vs. shear rate for the dispersion made from the control (non-treated) high heat nonfat dry milk samples (Rep 3).

(a) Measurement 1; (b) Measurement 2.



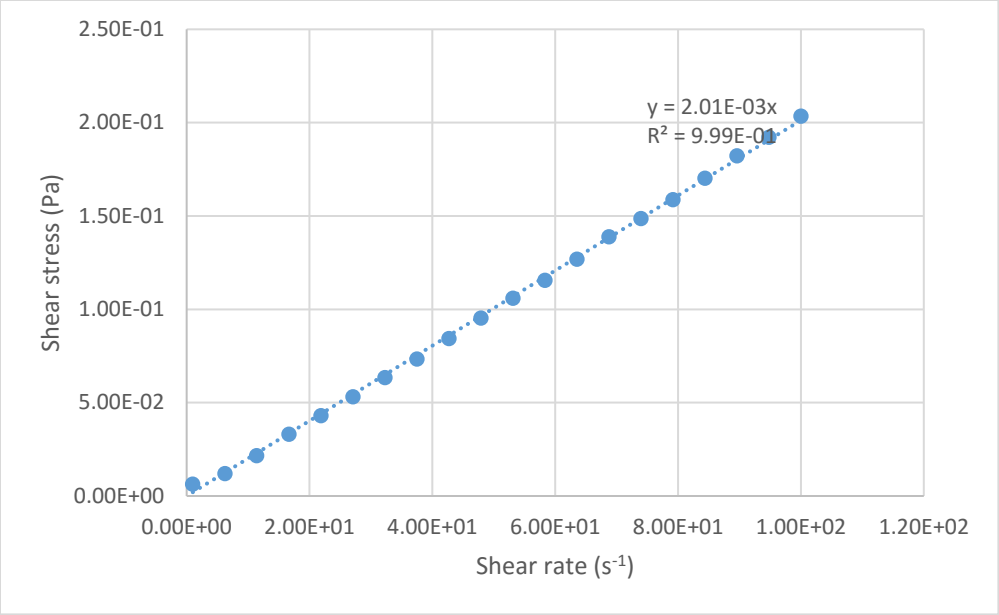
(a)



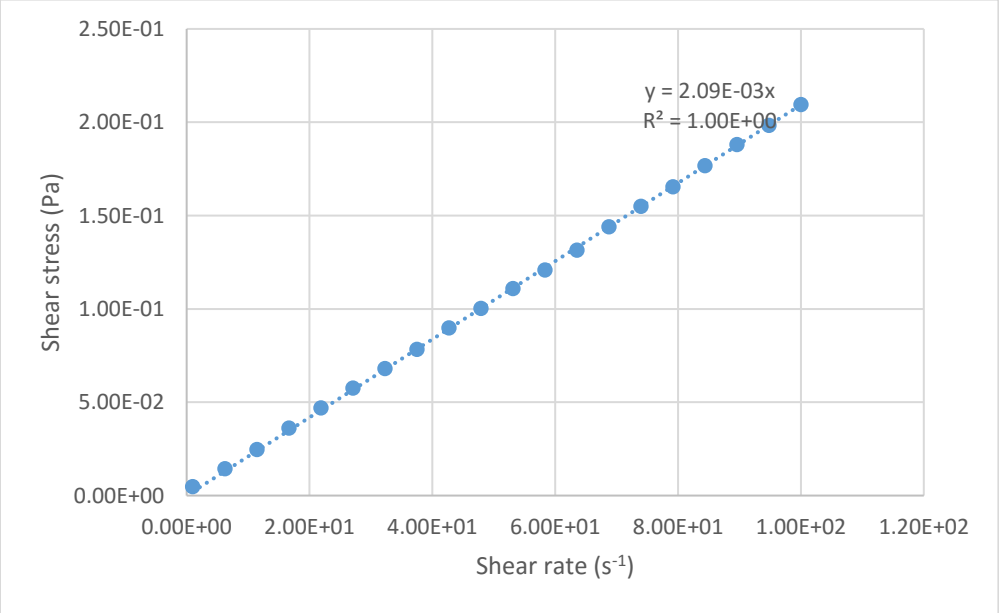
(b)

Figure H-18. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 75°C in a RFDH unit and held in a convection oven for 115 min (Rep 3).

(a) Measurement 1; (b) Measurement 2.



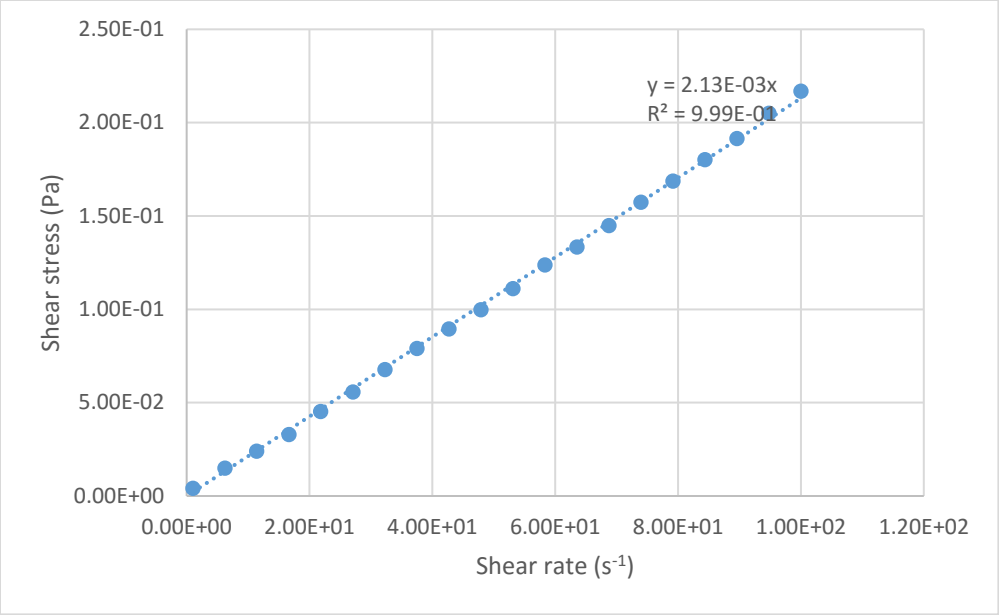
(a)



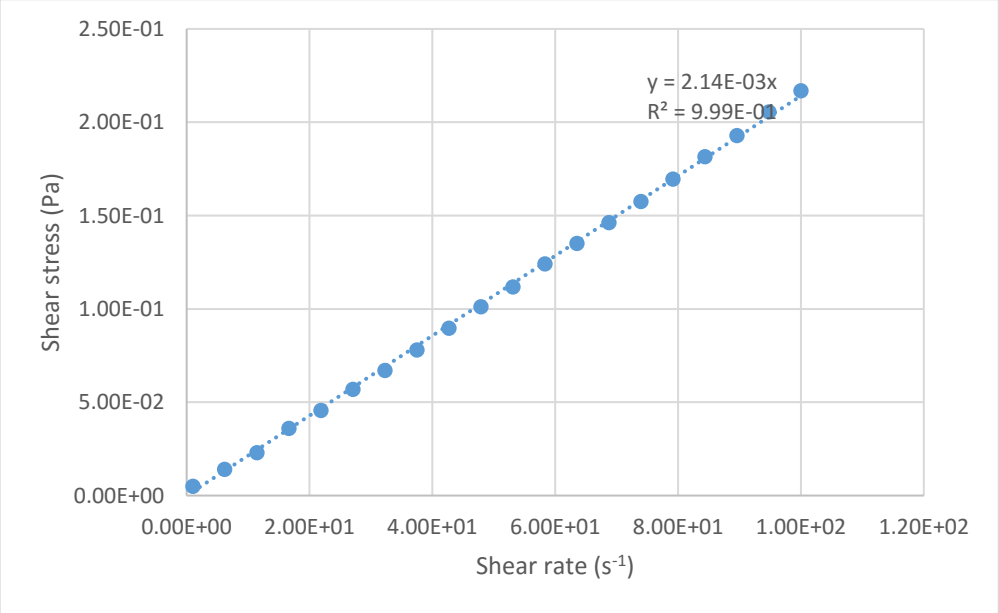
(b)

Figure H-19. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 80°C in a RFDH unit and held in a convection oven for 52 min (Rep 3).

(a) Measurement 1; (b) Measurement 2.



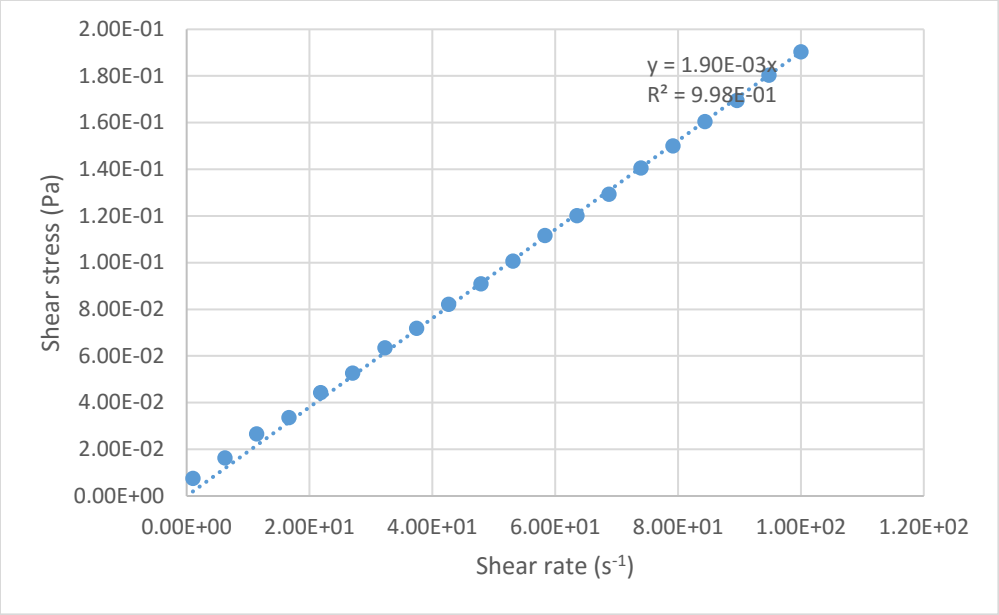
(a)



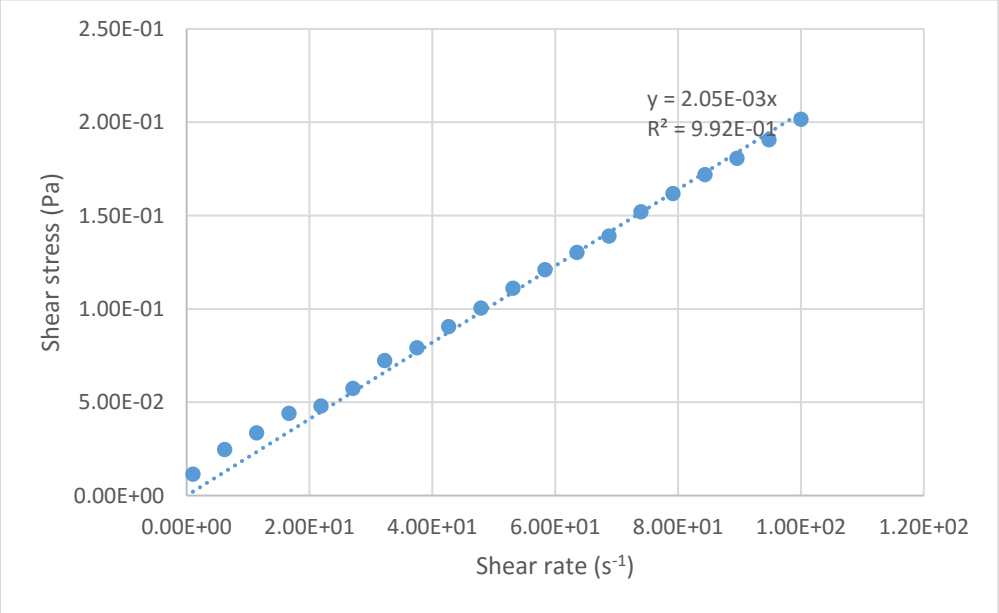
(b)

Figure H-20. Shear stress vs. shear rate for the dispersion made from high heat nonfat dry milk treated to 85°C in a RFDH unit and held in a convection oven for 43 min (Rep 3).

(a) Measurement 1; (b) Measurement 2.



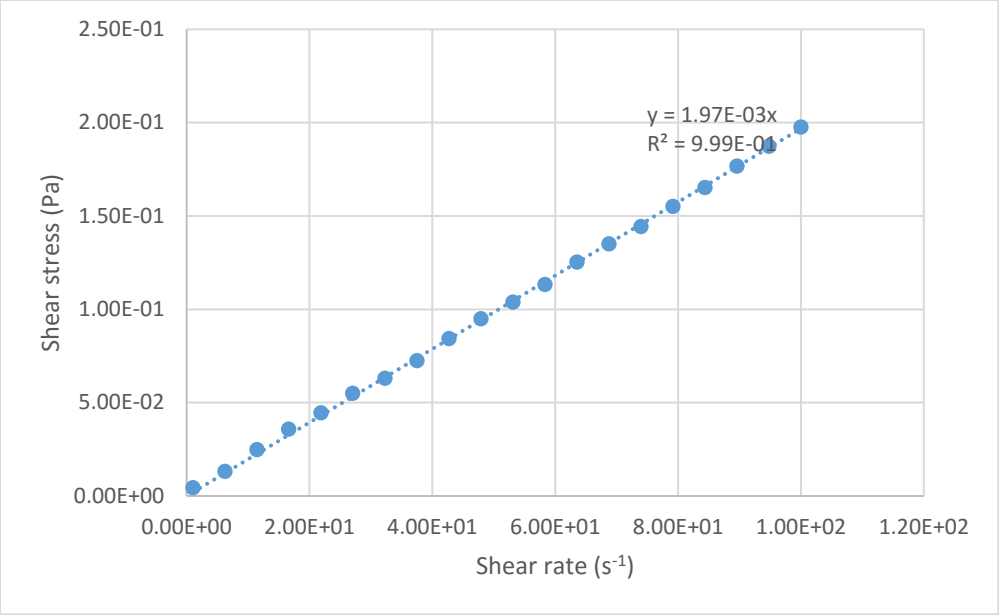
(a)



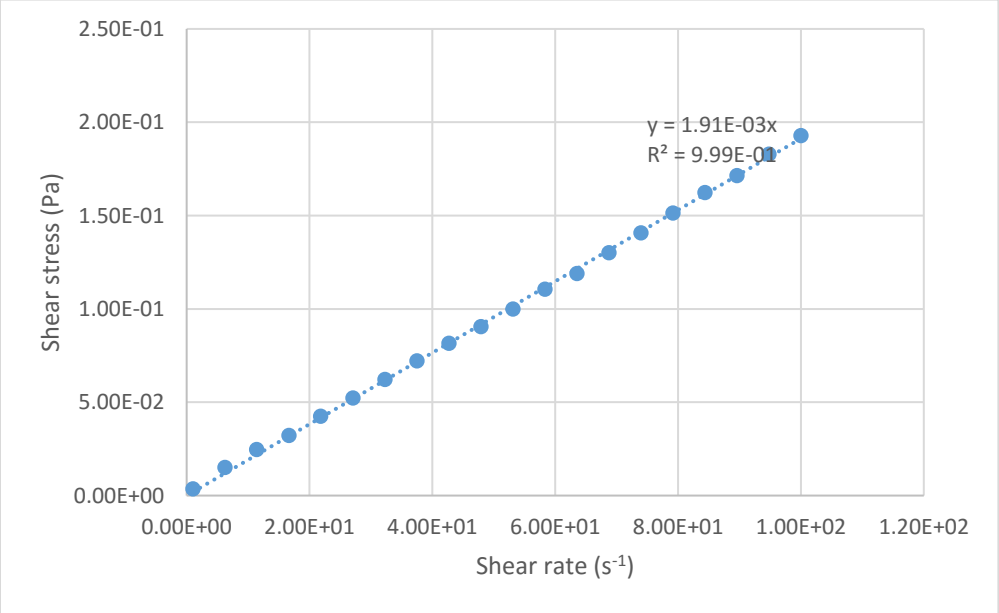
(b)

Figure H-21. Shear stress vs. shear rate for the dispersion made from the control (non-treated) low heat nonfat dry milk samples (Rep 3).

(a) Measurement 1; (b) Measurement 2.



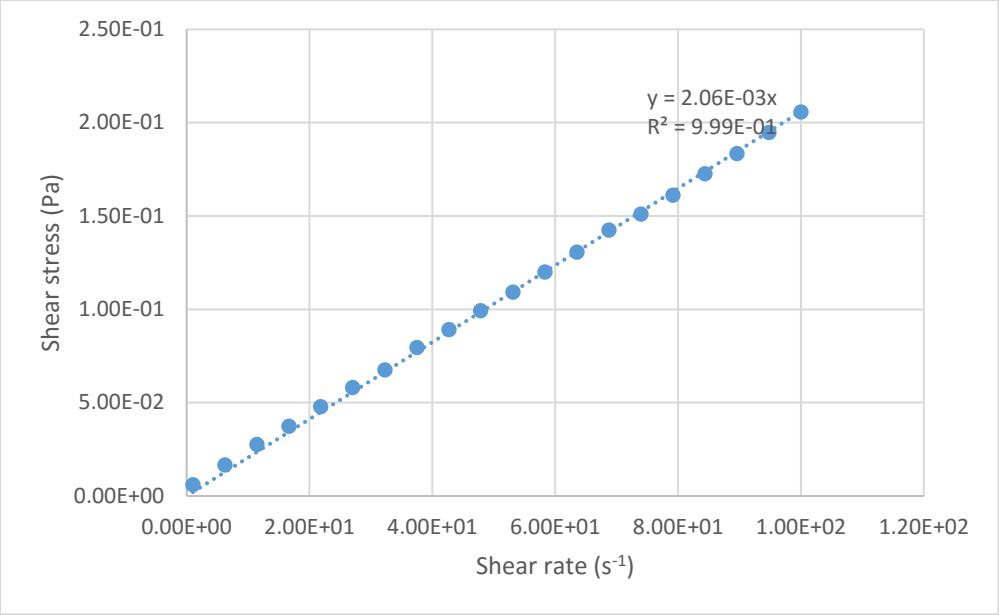
(a)



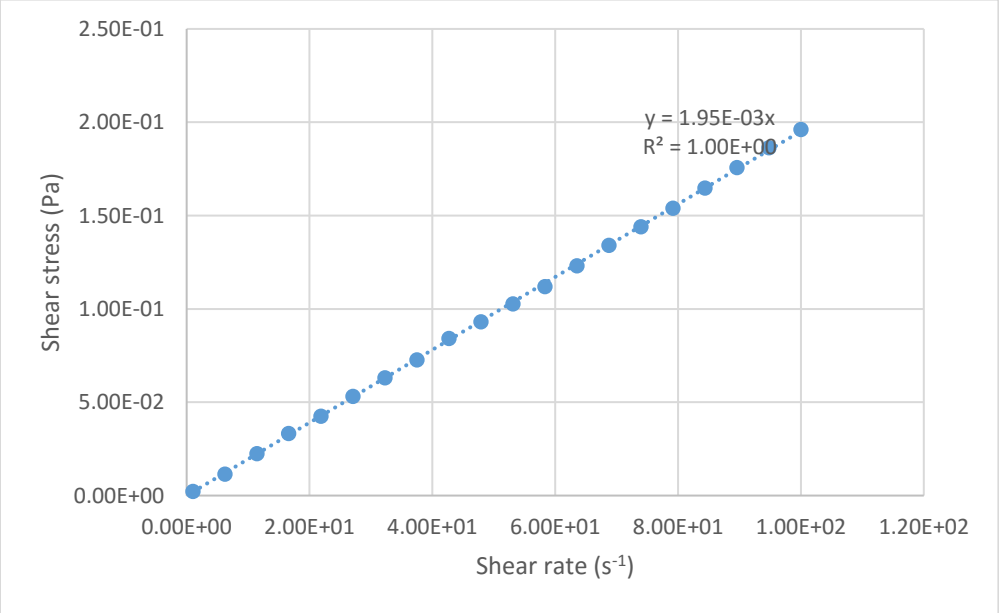
(b)

Figure H-22. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 75°C in a RFDH unit and held in a convection oven for 125 min (Rep 3).

(a) Measurement 1; (b) Measurement 2.



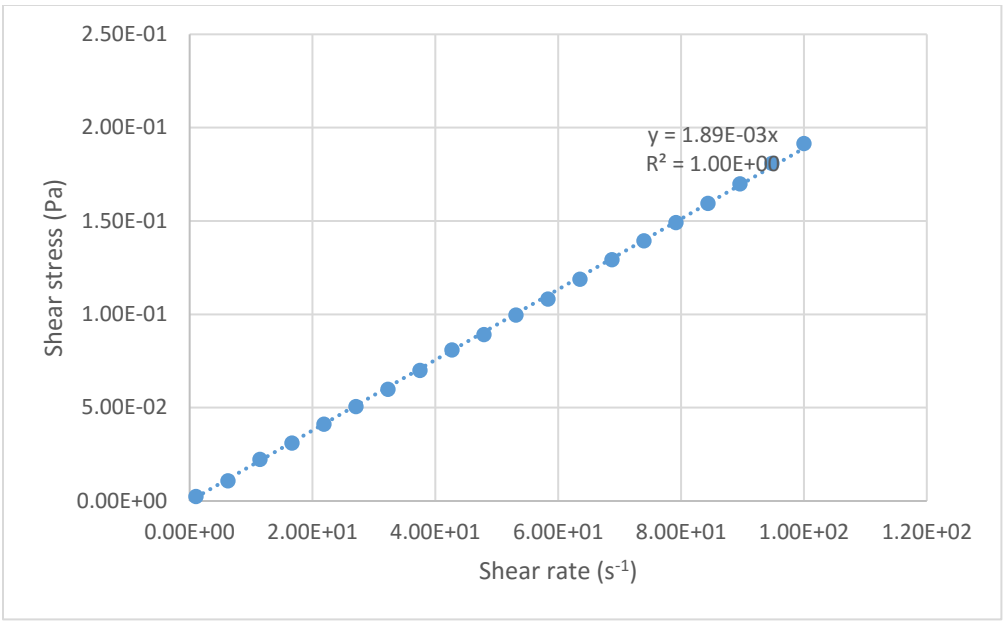
(a)



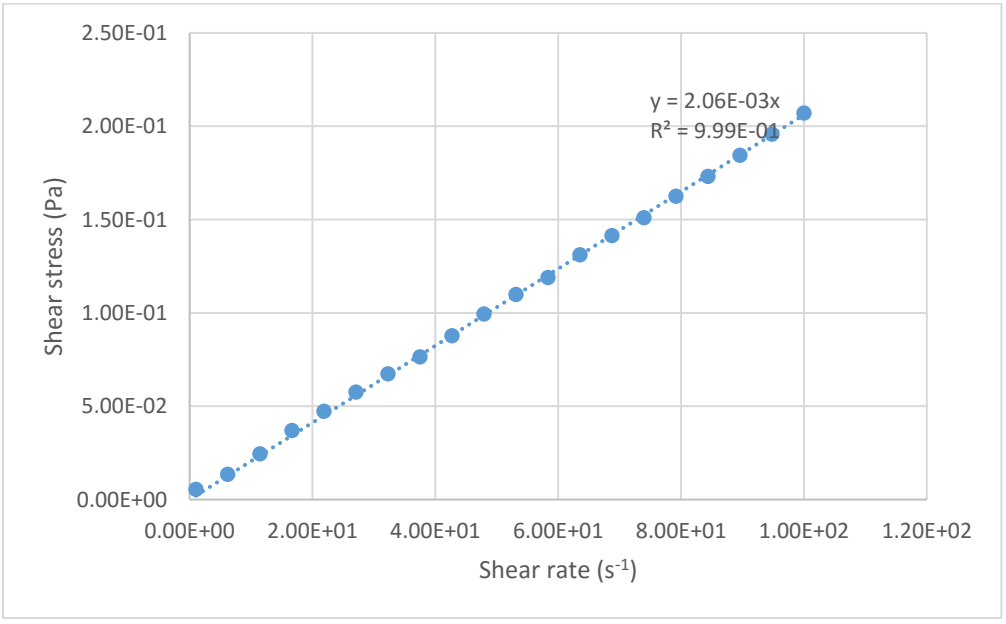
(b)

Figure H-23. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 80°C in a RFDH unit and held in a convection oven for 63 min (Rep 3).

(a) Measurement 1; (b) Measurement 2.



(a)



(b)

Figure H-24. Shear stress vs. shear rate for the dispersion made from low heat nonfat dry milk treated to 85°C in a RFDH unit and held in a convection oven for 43 min (Rep 3).

(a) Measurement 1; (b) Measurement 2.

Appendix I - **Data lists**

Table I-1. Composition of high heat (HH) and low heat (LH) nonfat dry milk (NDM)

Rep	NDM	Fat (%)	Moisture (%)	Ash (%)	Protein (%)	Lactose (%)
1	HH	1.4	2.41	7.71	35.29	53.19
2	HH	1.3	2.50	7.78	35.31	53.12
3	HH	1.3	2.45	7.74	35.31	53.19
1	LH	1.2	3.82	7.78	36.25	50.94
2	LH	1.2	3.71	7.77	36.19	51.13
3	LH	1.4	3.79	7.78	36.14	50.88

Table I-2. Radio frequency dielectric heating (RFDH) treatment for high heat (HH) and low heat (LH) nonfat dry milk (NDM) to cause a 5-log reduction in *Salmonella* spp.

Rep	NDM	Temperature (°C)	RFDH unit (min:sec)	Oven holding (min)	Overall heating (min:sec)
1	HH	75	8:57	115	123:57
		80	9:55	52	61:55
		85	11:04	43	54:04
2	HH	75	9:11	115	124:11
		80	9:46	52	61:46
		85	10:58	43	53:58
1	LH	75	14:13	125	139:13
		80	16:08	63	79:08
		85	17:07	43	60:07
2	LH	75	12:37	125	137:37
		80	15:29	63	78:29
		85	16:16	43	59:16

Table I-3. WPNI[1], NSI[2], color[3], and XTT[4] reduction of high heat (HH) and low heat (LH) nonfat dry milk (NDM) as a function of radio frequency dielectric heating temperature

Rep	NDM	Temperature (°C)	WPNI (mg/g)	NSI (%)	L*	a*	b*	XTT
1	HH[5]	Control	1.79	96.6%	91.81	-2.08	13.12	0.19
		75	1.88	96.3%	91.92	-2.18	14.24	0.2
		80	1.84	95.9%	91.59	-2.26	13.96	0.23
		85	1.88	96.0%	91.51	-2.19	13.76	0.21
2	HH	Control	1.78	96.1%	93.75	-2.18	13.88	0.26
		75	1.89	96.2%	93.58	-1.96	13.88	0.26
		80	1.95	96.5%	93.90	-2.05	14.74	0.2
		85	2.06	96.0%	93.77	-2.19	14.74	0.18
1	LH[6]	Control	7.27	98.4%	90.72	-2.06	12.84	0.09
		75	6.39	98.2%	90.66	-2.31	14.02	0.07
		80	6.57	98.2%	90.86	-2.18	15.05	0.1
		85	6.83	97.9%	86.79	-2.19	15.78	0.14
2	LH	Control	7.35	98.6%	91.17	-2.26	13.01	0.07
		75	6.57	99.2%	90.34	-2.31	13.69	0.07
		80	6.61	99.5%	90.72	-2.01	14.83	0.06
		85	6.95	98.9%	89.58	-2.05	16.77	0.17

[1]Whey protein solubility index.

[2]Nitrogen solubility index.

[3]L^* represents lightness; a^* represents red or green; b^* represents yellow or blue.

[4]Tetrazolium salt.

[5]HH: Control, 75, 80, 85°C for 0, 115, 52, 43 min, respectively.

[6]LH: Control, 75, 80, 85°C for 0, 125, 63, 43 min, respectively.

Table I-4. Functional properties of high heat (HH) and low heat (LH) nonfat dry milk (NDM) as a function of radio frequency dielectric heating temperature

Rep	NDM	Temperature (°C)	Viscosity (mPas)	ST[1] (dynes/cm)	IT[2] (dynes/cm)	Overrun (%)	Foam stability (min)	EA[3] (μs/cm)	ES[4]	pH
1	HH[5]	Control	2.05	47.4	4.2	789	67.33	340	23.4	6.66
		75	1.98	46.4	3.8	810	73.75	332	19.9	6.62
		80	1.97	47.3	4.2	847	79.61	342	26.1	6.65
		85	2.22	46.8	4.1	781	70.77	322	20.8	6.65
2	HH	Control	2.06	46.3	4.1	808	76.59	292	25.2	6.58
		75	2.17	45.9	4.4	793	72.18	310	16.2	6.57
		80	2.09	46.1	4.1	828	74.82	306	20.7	6.57
		85	2.15	46.7	4.0	876	99.87	334	18.7	6.57
1	LH[6]	Control	1.89	47.9	4.7	619	54.85	320	31.1	6.67
		75	2.00	46.7	4.2	616	55.60	334	20.4	6.69
		80	1.93	48	4.8	654	58.32	321	21.3	6.70
		85	2.03	47.9	5.0	663	64.94	307	27.6	6.63
2	LH	Control	1.77	46.9	4.1	613	41.42	310	23.6	6.69
		75	1.95	45.8	4.3	622	48.37	324	19.6	6.66
		80	2.03	46.6	5.1	677	57.13	317	26.2	6.66
		85	2.17	47.0	4.3	707	65.58	325	22	6.62

[1]Surface tension.

[2]Interfacial tension.

[3]Emulsion activity.

[4]Emulsion stability.

[5]HH: Control, 75, 80, 85°C for 0, 115, 52, 43 min, respectively.

[6]LH: Control, 75, 80, 85°C for 0, 125, 63, 43 min, respectively.

Table I-5. Physical properties of acid milk gels made from high heat (HH) and low heat (LH) nonfat dry milk (NDM) as a function of radio frequency dielectric heating temperature

Rep	NDM	Temperature (°C)	Syneresis (%)	WHC[1] (%)	Firmness (g)
1	HH[2]	Control	2.36	15.3	42.33
		75	2.11	15.7	37.72
		80	2.48	17.1	37.31
		85	2.34	16.9	32.23
2	HH	Control	2.32	15.7	36.58
		75	2.73	15.5	35.47
		80	2.58	17.3	34.98
		85	2.77	16.5	29.44
1	LH[3]	Control	0.89	13.5	33.66
		75	0.90	14.7	32.02
		80	0.86	14.0	30.16
		85	1.53	13.9	23.41
2	LH	Control	1.12	13.1	33.26
		75	0.94	14.1	31.79
		80	0.91	13.6	28.94
		85	4.36	13.5	24.21

[1]Water holding capacity.

[2]HH: Control, 75, 80, 85°C for 0, 115, 52, 43 min, respectively.

[3]LH: Control, 75, 80, 85°C for 0, 125, 63, 43 min, respectively.

Appendix J - **Mean differentiation for various properties**

Table J-1. Means[1] for emulsion activity (EA), emulsion stability (ES), pH, and XTT reduction of reconstituted high heat (HH) and low heat (LH) nonfat dry milk (NDM) (3.5% protein), collapsed for radio frequency dielectric heating treatment

Functional properties	HH[2]	LH[3]
EA	322 ± 18	320 ± 9
ES	21.4 ± 3.3	24.0 ± 4.0
pH	6.61 ± 0.04[b]	6.67 ± 0.03[a]
XTT reduction	0.22 ± 0.03[a]	0.10 ± 0.04[b]

[a-b]Means within a row with different superscripts, differ ($p \leq 0.05$)。

[1]Mean ± SD, n = 8

[2]HH: Control, 75, 80, 85°C for 0, 115, 52, 43 min, respectively.

[3]LH: Control, 75, 80, 85°C for 0, 125, 63, 43 min, respectively.

Appendix K - **P value**

Table K-1. Two-way ANOVA - p values for heating time in radio frequency dielectric heating (RFDH) unit and overall heating time (RFDH unit + oven): 75, 80, 85°C

P value	NDM	RFDH	NDM*RFDH
RFDH unit	< 0.0001	0.0014	0.1161
Overall time	< 0.0001	< 0.0001	< 0.0001

Appendix L - **ANOVA tables**

Table L-1. ANOVA tables (Rep, NDM, RFDH, NDM*RFDH) for various physico-chemical and functional properties of nonfat dry milk

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep[1]	1	7.49390625	7.49390625	10.40	0.0145
NDM[2]	1	27.53625625	27.53625625	38.23	0.0005
RFDH[3]	3	5.49441875	1.83147292	2.54	0.1395
NDM*RFDH[4]	3	4.56251875	1.52083958	2.11	0.1872

(a) L*

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.01210000	0.01210000	1.02	0.3471
NDM	1	0.00490000	0.00490000	0.41	0.5417
RFDH	3	0.00887500	0.00295833	0.25	0.8601
NDM*RFDH	3	0.06210000	0.02070000	1.74	0.2461

(b) a*

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.47955625	0.47955625	2.70	0.1441
NDM	1	0.84180625	0.84180625	4.75	0.0658
RFDH	3	9.36656875	3.12218958	17.60	0.0012
NDM*RFDH	3	3.97956875	1.32652292	7.48	0.0138

(c) b*

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.6071007	0.6071007	3.30	0.1123
NDM	1	63.9333507	63.9333507	347.23	<.0001
RFDH	3	493.1849133	164.3949711	892.85	<.0001
NDM*RFDH	3	22.7958854	7.5986285	41.27	<.0001

(d) Heating time in radio frequency dielectric unit

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.03150625	0.03150625	12.09	0.0103
NDM	1	97.36755625	97.36755625	37359.3	<.0001
RFDH	3	0.34006875	0.11335625	43.49	<.0001
NDM*RFDH	3	0.52151875	0.17383958	66.70	<.0001

(e) Whey protein nitrogen index

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.00007656	0.00007656	3.80	0.0922
NDM	1	0.00232806	0.00232806	115.63	<.0001
RFDH	3	0.00002469	0.00000823	0.41	0.7518
NDM*RFDH	3	0.00001269	0.00000423	0.21	0.8863

(f) Nitrogen solubility index

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	7.290000	7.290000	0.09	0.7770
NDM	1	1779.152400	1779.152400	21.15	0.0025
RFDH	3	543.545537	181.181846	2.15	0.1817
NDM*RFDH	3	11.100087	3.700029	0.04	0.9867

(g) Foam stability

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.13140625	0.13140625	1.85	0.2160
NDM	1	11.57700625	11.57700625	162.98	<.0001
RFDH	3	0.83086875	0.27695625	3.90	0.0629
NDM*RFDH	3	0.10186875	0.03395625	0.48	0.7076

(h) Overrun

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.02250000	0.02250000	0.24	0.6370
NDM	1	0.81000000	0.81000000	8.76	0.0211
RFDH	3	0.30250000	0.10083333	1.09	0.4140
NDM*RFDH	3	0.27500000	0.09166667	0.99	0.4506

(i) Interfacial tension

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	3.15062500	3.15062500	37.11	0.0005
NDM	1	0.95062500	0.95062500	11.20	0.0123
RFDH	3	2.33187500	0.77729167	9.15	0.0081
NDM*RFDH	3	0.21187500	0.07062500	0.83	0.5175

(j) Surface tension

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	625.0000000	625.0000000	2.49	0.1588
NDM	1	25.0000000	25.0000000	0.10	0.7616
RFDH	3	190.0000000	63.3333333	0.25	0.8576
NDM*RFDH	3	209.0000000	69.6666667	0.28	0.8403

(k) Emulsion activity

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	21.16000000	21.16000000	2.46	0.1608
NDM	1	27.04000000	27.04000000	3.14	0.1195
RFDH	3	96.86000000	32.28666667	3.75	0.0680
NDM*RFDH	3	11.69000000	3.89666667	0.45	0.7234

(l) Emulsion stability

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	6.4E-9	6.4E-9	1.00	0.3496
NDM	1	5.29E-8	5.29E-8	8.30	0.0236
RFDH	3	8.3825E-8	2.7941667E-8	4.39	0.0491
NDM*RFDH	3	1.745E-8	5.8166667E-9	0.91	0.4820

(m) Apparent viscosity

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	12.5493063	12.5493063	6.15	0.0423
NDM	1	147.6832562	147.6832562	72.34	<.0001
RFDH	3	181.8362687	60.6120896	29.69	0.0002
NDM*RFDH	3	3.0975188	1.0325063	0.51	0.6905

(n) Firmness

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.00011342	0.00011342	2.49	0.1586
NDM	1	0.00041820	0.00041820	9.18	0.0191
RFDH	3	0.00034169	0.00011390	2.50	0.1435
NDM*RFDH	3	0.00027083	0.00009028	1.98	0.2054

(o) Syneresis

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.00002025	0.00002025	3.40	0.1079
NDM	1	0.00240100	0.00240100	402.56	<.0001
RFDH	3	0.00025900	0.00008633	14.48	0.0022
NDM*RFDH	3	0.00028300	0.00009433	15.82	0.0017

(p) Water holding capacity

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.00765625	0.00765625	11.42	0.0118
NDM	1	0.01265625	0.01265625	18.87	0.0034
RFDH	3	0.00246875	0.00082292	1.23	0.3690
NDM*RFDH	3	0.00246875	0.00082292	1.23	0.3690

(q) pH

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Rep	1	0.00010000	0.00010000	0.11	0.7524
NDM	1	0.05760000	0.05760000	62.03	0.0001
RFDH	3	0.00192500	0.00064167	0.69	0.5859
NDM*RFDH	3	0.00885000	0.00295000	3.18	0.0941

(r) Reduction of Tetrazolium salt XTT

[1]Replication.

[2]Nonfat dry milk.

[3]Radio frequency dielectric heating.

[4]Interaction between nonfat dry milk and radio frequency dielectric heating.

Appendix M - **SAS codes**

1. Composition as functions of powder

```
data powder;
input Rep$ Item$ fat moisture ash protein lactose WPNI NSI;
datalines;
1     LH    1.2   3.82  7.78  36.25 50.94 7.27  0.9842
2     LH    1.2   3.71  7.77  36.19 51.13 7.35  0.9858
3     LH    1.4   3.79  7.78  36.14 50.88 7.29  0.9855
1     HH    1.4   2.41  7.71  35.29 53.19 1.79  0.9663
2     HH    1.3   2.50  7.78  35.31 53.12 1.78  0.9606
3     HH    1.3   2.45  7.74  35.31 53.19 1.76  0.9634
;
run;

proc print data=powder;
run;

proc glimmix data=powder;
class Rep Item;
model fat = Item;
lsmeans Item/plot=meanplot(cl);
lsmeans Item/ pdiff adjust=Tukey;
run;

proc glimmix data=powder;
class Rep Item;
model moisture = Item;
lsmeans Item/plot=meanplot(cl);
lsmeans Item/ pdiff adjust=Tukey;
run;
proc glimmix data=powder;
class Rep Item;
model ash = Item;
lsmeans Item/plot=meanplot(cl);
lsmeans Item/ pdiff adjust=Tukey;
run;
proc glimmix data=powder;
class Rep Item;
model protein = Item;
lsmeans Item/plot=meanplot(cl);
lsmeans Item/ pdiff adjust=Tukey;
run;
proc glimmix data=powder;
class Rep Item;
model lactose = Item;
lsmeans Item/plot=meanplot(cl);
lsmeans Item/ pdiff adjust=Tukey;
run;
proc glimmix data=powder;
class Rep Item;
model WPNI = Item;
lsmeans Item/plot=meanplot(cl);
lsmeans Item/ pdiff adjust=Tukey;
```

```
run;
proc glimmix data=powder;
class Rep Item;
model NSI = Item;
lsmeans Item/plot=meanplot(cl);
lsmeans Item/ pdiff adjust=Tukey;
run;
```

2. Two- way ANOVA: Rep, NDM, RFDH, NDM*RFDH

```
data property;
input Rep$ NDM$ RFDH$ WPNI NSI Viscosity ST IT Overrun
Foam_stability EA ES Syneresis WHC Firmness pH L a b XTT Time;
datalines;
1     LH    Control     7.27  0.984 1.89E-03    47.9  4.7   6.19  54.85
320   31.1  0.0089      0.135 33.66 6.67  90.72 -2.06 12.84 0.09  0
1     LH    75    6.39  0.982 2.00E-03    46.7  4.2   6.16  55.6  334
20.4  0.0090      0.147 32.02 6.69  90.66 -2.31 14.02 0.07
14.21666667
1     LH    80    6.57  0.982 1.93E-03    48    4.8   6.54  58.315
321   21.3  0.0086      0.14  30.16 6.7   90.86 -2.18 15.05 0.1
16.13333333
1     LH    85    6.83  0.979 2.03E-03    47.9  5.0   6.63  64.935
307   27.6  0.0153      0.139 23.41 6.63  86.79 -2.19 15.78 0.14
17.11666667
2     LH    Control     7.35  0.986 1.77E-03    46.9  4.1   6.13  41.42
310   23.6  0.0112      0.131 33.26 6.69  91.17 -2.26 13.01 0.07  0
2     LH    75    6.57  0.992 1.95E-03    45.8  4.3   6.22  48.37 324
19.6  0.0094      0.141 31.79 6.66  90.34 -2.31 13.69 0.07
12.61666667
2     LH    80    6.61  0.995 2.03E-03    46.6  5.1   6.77  57.13 317
26.2  0.0091      0.136 28.94 6.66  90.72 -2.01 14.83 0.06
15.48333333
2     LH    85    6.95  0.989 2.17E-03    47.0  4.3   7.07  65.58 325
22    0.0436      0.135 24.21 6.62  89.58 -2.05 16.77 0.17
16.26666667
1     HH    Control     1.79  0.966 2.05E-03    47.4  4.2   7.89  67.33
340   23.4  0.0236      0.153 42.33 6.66  91.81 -2.08 13.12 0.19  0
1     HH    75    1.88  0.963 1.98E-03    46.4  3.8   8.1   73.75 332
19.9  0.0211      0.157 37.72 6.62  91.92 -2.18 14.24 0.2   8.95
1     HH    80    1.84  0.959 1.97E-03    47.3  4.2   8.47  79.61 342
26.1  0.0248      0.171 37.31 6.65  91.59 -2.26 13.96 0.23
9.916666667
1     HH    85    1.88  0.960 2.22E-03    46.8  4.1   7.81  70.77 322
20.8  0.0234      0.169 32.23 6.65  91.51 -2.19 13.76 0.21
11.06666667
2     HH    Control     1.78  0.961 2.06E-03    46.3  4.1   8.08  76.59
292   25.2  0.0232      0.157 36.58 6.58  93.75 -2.18 13.88 0.26  0
```

```
2     HH    75    1.89  0.962 2.17E-03    45.9  4.4   7.93  72.18 310
16.2  0.0273      0.155 35.47 6.57  93.58 -1.96 13.88 0.26
9.183333333
2     HH    80    1.95  0.965 2.09E-03    46.1  4.1   8.28  74.82 306
20.7  0.0258      0.173 34.98 6.57  93.90 -2.05 14.74 0.2
9.766666667
2     HH    85    2.06  0.960 2.15E-03    46.7  4.0   8.76  99.87 334
18.7  0.0277      0.165 29.44 6.57  93.77 -2.19 14.74 0.18
10.96666667
;
run;

proc glm data=property;
class Rep NDM RFDH;
model WPNI NSI Viscosity ST IT Overrun Foam_stability EA ES
Syneresis WHC Firmness pH L a b XTT Time= Rep NDM RFDH NDM*RFDH;
run;
```

3. Pairwise comparison (significant main effect, interactions) for all samples using Tukey's significant difference test

```
data RFDH;
input Rep$ NDM$ RF$ WPNI NSI Viscosity ST IT Overrun
Foam_stability
EA ES Syneresis WHC Firmness pH L a b XTT;
datalines;
1     LH    Control     7.27  0.984 1.89E-03    47.9  4.7   6.19  54.85
320   31.1  0.0089      0.135 33.66 6.67  90.72 -2.06 12.84 0.09
1     LH    75    6.39  0.982 2.00E-03    46.7  4.2   6.16  55.6  334
20.4  0.009 0.147 32.02 6.69  90.66 -2.31 14.02 0.07
1     LH    80    6.57  0.982 1.93E-03    48    4.8   6.54  58.315
321   21.3  0.0086      0.14  30.16 6.7   90.86 -2.18 15.05 0.1
1     LH    85    6.83  0.979 2.03E-03    47.9  5.0   6.63  64.935
307   27.6  0.0153      0.139 23.41 6.63  86.79 -2.19 15.78 0.14
1     HH    Control     1.79  0.966 2.05E-03    47.4  4.2   7.89  67.33
340   23.4  0.0236      0.153 42.33 6.66  91.81 -2.08 13.12 0.19
1     HH    75    1.88  0.963 1.98E-03    46.4  3.8   8.1   73.75 332
19.9  0.0211      0.157 37.72 6.62  91.92 -2.18 14.24 0.2
1     HH    80    1.84  0.959 1.97E-03    47.3  4.2   8.47  79.61 342
26.1  0.0248      0.171 37.31 6.65  91.59 -2.26 13.96 0.23
1     HH    85    1.88  0.960 2.22E-03    46.8  4.1   7.81  70.765
322   20.8  0.0234      0.169 32.23 6.65  91.51 -2.19 13.76 0.21
2     LH    Control     7.35  0.986 1.77E-03    46.9  4.1   6.13  41.42
310   23.6  0.0112      0.131 33.26 6.69  91.17 -2.26 13.01 0.07
2     LH    75    6.57  0.992 1.95E-03    45.8  4.3   6.22  48.37 324
19.6  0.0094      0.141 31.79 6.66  90.34 -2.31 13.69 0.07
2     LH    80    6.61  0.995 2.03E-03    46.6  5.1   6.77  57.13 317
26.2  0.0091      0.136 28.94 6.66  90.72 -2.01 14.83 0.06
```

```
2     LH    85    6.95  0.989 2.17E-03   47.0  4.3   7.07  65.58 325
22    0.0436      0.135 24.21 6.62  89.58 -2.05 16.77 0.17
2     HH    Control     1.78  0.961 2.06E-03   46.3  4.1   8.08  76.59
292   25.2  0.0232      0.157 36.58 6.58  93.75 -2.18 13.88 0.26
2     HH    75    1.89  0.962 2.17E-03   45.9  4.4   7.93  72.18 310
16.2  0.0273      0.155 35.47 6.57  93.58 -1.96 13.88 0.26
2     HH    80    1.95  0.965 2.09E-03   46.1  4.1   8.28  74.82 306
20.7  0.0258      0.173 34.98 6.57  93.90 -2.05 14.74 0.2
2     HH    85    2.06  0.960 2.15E-03   46.7  4.0   8.76  99.87 334
18.7  0.0277      0.165 29.44 6.57  93.77 -2.19 14.74 0.18
;
run;

proc print data= RFDH;
run;

proc glimmix data= RFDH;
class Rep NDM RF;
model WPNI = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model NSI = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model Viscosity = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model ST = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
```

```
proc glimmix data= RFDH;
class Rep NDM RF;
model IT = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model Overrun = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model Foam_stability = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model EA = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model ES = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model Syneresis = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
```

```
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model WHC = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model Firmness = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model pH = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model L = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model a = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model b = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
```

```
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
proc glimmix data= RFDH;
class Rep NDM RF;
model XTT = NDM RF NDM*RF ;
random Rep;
lsmeans NDM*RF/ pdiff adjust=Tukey
plot=meanplot(sliceby=RF join);
lsmeans NDM/ pdiff adjust=Tukey;
lsmeans RF/ pdiff adjust=Tukey;
run;
```

4. Low heat: one-way ANOVA

```
data LHNDM;
input Rep$ RFDH$ WPNI NSI Viscosity ST IT Overrun Foam_stability
EA ES Syneresis WHC Firmness pH L a b XTT;
datalines;
1     Control     7.27  0.9842      0.00189     47.9  4.7   6.19  54.85
320   31.1  0.0089      0.135 33.66 6.67  90.72333333 -2.06 12.84 0.09
1     75    6.39  0.9817      0.002 46.7  4.2   6.16  55.6  334   20.4
0.009 0.147 32.02 6.69  90.66 -2.31 14.01666667 0.07
1     80    6.57  0.982 0.00193     48    4.8   6.54  58.315      321
21.3  0.0086      0.14  30.16 6.7   90.85666667 -2.183333333      15.05
0.1
1     85    6.83  0.9792      0.00203     47.9  5     6.63  64.935
307   27.6  0.0153      0.139 23.41 6.63  86.79 -2.19 15.77666667 0.14
2     Control     7.35  0.9858      0.00177     46.86218933 4.059747372
6.13  41.42 310   23.6  0.0112      0.131 33.26 6.69  91.17333333 -2.26
13.01 0.07
2     75    6.57  0.992 0.00195     45.83742921 4.346990125 6.22  48.37
324   19.6  0.0094      0.141 31.79 6.66  90.34 -2.31 13.69 0.07
2     80    6.61  0.9954      0.00203     46.58526262 5.082063374 6.77
57.13 317   26.2  0.0091      0.136 28.94 6.66  90.72 -2.01 14.83333333
0.06
2     85    6.95  0.989 0.00217     47.01882484 4.34385203  7.07  65.58
325   22    0.0436      0.135 24.21 6.62  89.57666667 -2.046666667
16.77333333 0.17
;
run;

proc print data=LHNDM;
run;

proc glimmix data=LHNDM;
class Rep RFDH;
model WPNI= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
```

```
run;

proc glimmix data=LHNDM;
class Rep RFDH;
model NSI= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;

proc glimmix data=LHNDM;
class Rep RFDH;
model Viscosity= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model ST= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model IT= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model Overrun= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model Foam_stability= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model EA= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model ES= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
```

```
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model Syneresis= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model WHC= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model Firmness= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model pH= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model L= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model a= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model b= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=LHNDM;
class Rep RFDH;
model XTT= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
```

```
run;
```

5. High heat: one-way ANOVA

```
data HHNDM;
input Rep$ RFDH$ WPNI NSI Viscosity ST IT Overrun Foam_stability
EA ES Syneresis WHC Firmness pH L a b XTT;
datalines;
1     Control     1.79  0.966 2.05E-03    47.4  4.2   7.89  67.33 340
23.4  0.0236      0.153 42.33 6.66  91.81 -2.08 13.12 0.19
1     75    1.88  0.963 1.98E-03    46.4  3.8   8.1   73.75 332   19.9
0.0211      0.157 37.72 6.62  91.92 -2.18 14.24 0.2
1     80    1.84  0.959 1.97E-03    47.3  4.2   8.47  79.61 342   26.1
0.0248      0.171 37.31 6.65  91.59 -2.26 13.96 0.23
1     85    1.88  0.960 2.22E-03    46.8  4.1   7.81  70.77 322   20.8
0.0234      0.169 32.23 6.65  91.51 -2.19 13.76 0.21
2     Control     1.78  0.961 2.06E-03    46.3  4.1   8.08  76.59 292
25.2  0.0232      0.157 36.58 6.58  93.75 -2.18 13.88 0.26
2     75    1.89  0.962 2.17E-03    45.9  4.4   7.93  72.18 310   16.2
0.0273      0.155 35.47 6.57  93.58 -1.96 13.88 0.26
2     80    1.95  0.965 2.09E-03    46.1  4.1   8.28  74.82 306   20.7
0.0258      0.173 34.98 6.57  93.90 -2.05 14.74 0.2
2     85    2.06  0.960 2.15E-03    46.7  4.0   8.76  99.87 334   18.7
0.0277      0.165 29.44 6.57  93.77 -2.19 14.74 0.18
;
run;


proc print data=LHNDM;
run;

proc glimmix data=HHNDM;
class Rep RFDH;
model WPNI= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;

proc glimmix data=HHNDM;
class Rep RFDH;
model NSI= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;

proc glimmix data=HHNDM;
class Rep RFDH;
model Viscosity= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
```

```
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model ST= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=hHNDM;
class Rep RFDH;
model IT= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model Overrun= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model Foam_stability= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model EA= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model ES= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model Syneresis= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model WHC= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
```

```
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model Firmness= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model pH= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model L= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model a= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model b= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
proc glimmix data=HHNDM;
class Rep RFDH;
model XTT= RFDH;
random Rep;
lsmeans RFDH/plot=meanplot(cl);
lsmeans RFDH/ pdiff adjust=Tukey;
run;
```