ABSTRACT

JACKSON, MAKENZIE ELIZABETH BRYSON. Developing Methods for Measuring the Physical Stability of Protein Ingredients Alone or in Beverages (Under the direction of Dr. E. Allen Foegeding).

Protein beverages are a rapidly growing segment in the area of functional foods. It has been shown that consumers desire ≥ 20 grams of protein per serving, with a preference for whey proteins. However, an issue presents itself as protein beverages are colloidal systems and the thermal process denatures proteins and can cause unwanted phase separation, gelation, or large aggregates. The physical destabilization process over time is not well understood, thus the purpose of this study is to investigate the *meso-* scale for physical stability of high-protein beverages by analysis of protein particles immediately post processing for *thermal stability* and over the course of eight weeks for understanding of *shelf stability*.

This study was split into two phases. In phase one, high-protein beverages (6.3% and 10.5% (wt/wt) protein) either classified as high-casein or high-whey protein were ultra-high temperature processed (141°C for 2.5 seconds) and analyzed for eight weeks under storage at 4°C using particle size analysis by laser diffraction, soluble protein fractionation, and size exclusion chromatography methods. Particle size analysis and mass balance of soluble protein showed that casein-enriched beverages were more thermally stable. Size exclusion chromatography showed that over the eight weeks, the casein-enriched beverages also had less aggregation over time, indicating a higher level of stability.

In the second phase, high-protein solutions (6% and 11% (wt/wt) protein) containing either milk protein concentrate or a blend of equal parts milk protein concentrate and whey protein isolate were retort processed (121°C for 20 minutes) and stored at 25°C. This study did not include soluble protein fractionation but repeated the measurements of particle size analysis by laser diffraction, size exclusion chromatography, and included using a z-dimension

turbidimetric scanner that allowed for analysis of settling behavior. Particle size analysis showed some differences in thermal stability, with milk protein concentrate alone being more thermally stable, but no temporal results were seen. Size-exclusion chromatography demonstrated that secondary aggregation was occurring in all samples, and that the blend of whey protein and milk protein aggregated more rapidly. Settling behavior showed two patterns of destabilization, an increase in particle size overall and settling of particles over time. The most stable protein solution was milk protein concentrate at 6% (wt/wt) protein, at 11% (wt/wt) protein, the solution was too viscous to measure. The blend of milk protein concentrate and whey protein isolate was also more stable at 6% (wt/wt) protein, at 11% (wt/wt) protein, a greater settling pattern was recorded, and a greater extent of secondary aggregation was also recorded.

Both experiments highlight that the physical stability of dairy proteins in beverages or alone in solution can be measured on the particle size length scale and that differentiation could be made between combinations of proteins. These experiments show the importance of using multiple measurements on physical stability through settling patterns, thermal stability, and secondary aggregation. The goal of this thesis was met to develop and evaluate a method that could be used to gain more insight into physical destabilizations of protein beverages.

Developing Methods for Measuring the Physical Stability of Protein Ingredients Alone or in Beverages

by
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BIOGRAPHY

Makenzie Jackson is a Raleigh native and NC State University alum. After graduation from high school and spending a half-year abroad teaching in Burundi, Africa, she discovered the discipline of food science while pursuing a degree in nutrition science. After taking Dr. Foegeding's Food Chemistry course, it sealed the deal: food science was what she wanted to study, especially analytical chemistry. After three years of interning with the Analytical team at the Golden Leaf Biomanufacturing Training and Education Center with Dr. Nathaniel Hentz, she interned for a summer at PepsiCo in Valhalla, New York in the forensics lab. When the opportunity came for her to work under the guidance of Dr. Foegeding as a graduate student, it was a perfect fit.

During her time as a graduate student, she has met and married her husband, who has been the most supportive spouse in enabling her to pursue her passions.

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CHAPTER 1. LITERATURE REVIEW

1. INTRODUCTION

Protein beverages are a growing segment within functional foods (Beverage Industry, 2017). The format of protein beverages is a popular option for those who are seeking to increase their protein intake. Beverages such as protein-fortified smoothies, meal replacement drinks, and pre-and-post workout beverages all fall under the umbrella of functional food. One major challenge with protein beverages is that they are colloidal systems rather than true solutions, and therefore are inherently unstable and constantly undergoing phase separation. Protein beverages are sols, a solid particle phase dispersed in a continuous fluid, and the solid phase is undergoing settling and possibly aggregation.

There are two general forms of physical stability described in reference to protein beverages – *thermal stability* reflecting the ability of a formulation to undergo thermal processing and remain a homogeneous sol and *shelf stability* that can be defined as the time post processing that the sol remains fluid and visually homogeneous. The goal of this thesis was to develop and evaluate a method that could be used to gain more insight into physical destabilizations of protein beverages.

1.1. An industrial problem: shelf stability of thermally processed protein beverages

Dairy protein is a growing commodity, with 51% of U.S. consumers trying to incorporate more protein into their diets (Gerdes, 2012). Additionally, the Food and Agriculture Organization (FAO) projects a 33% increase in the world population, rising to 9.8 billion by 2050 (UnitedNations, 2017). With this increase, food production will need to be able to meet the demands for sources of high-quality proteins. An excellent source of protein can be found in bovine milk, rated as 10-30% higher quality on the Digestible Amino Acid Score over plant proteins such as soy, pea, and rice (Lagrange et al., 2015). Because dairy products are considered a complete protein source and can improve muscle protein synthesis, they are an ideal choice for everyone from athlete to infant (Litwin et al., 2015).

Recent market research projects a compound annual growth rate of 5 percent in the ready to drink protein beverages market over the years 2018-2022, with whey protein-based beverages taking a 41 percent share of the market (Entertainment Close-Up, 2018). While there is no current legal standard for protein content for a drink to qualify as a "protein beverage," Oltman et al. (2015) found that consumers desire ≥ 20 grams of protein per serving and have a preference for whey as the primary protein source.

These beverages must be processed to inactivate spoilage and pathogenic microorganisms, which presents a challenge because thermal processing may influence colloidal stability of beverages by denaturing proteins and causing various extents of aggregation depending on conditions such as pH, ionic strength, and protein concentration (Singh et al., 2015). While types of thermal treatments vary, most heating processes are above the denaturation temperature of whey proteins (~ 60°C) and also may cause changes in caseins (Qi et al., 2004; de Wit, 2009). Furthermore, while denaturation (unfolding) of proteins can increase

viscosity, it is aggregation that produces the undesirable states of precipitation, phase separation, or gelation.

2. MILK PROTEINS AND MILK INGREDIENTS

2.1. Bovine milk proteins

Milk protein ingredients may be in the form of individual proteins, protein classes, or combinations of proteins. Dairy proteins have been studied since the start of protein chemistry. In 1838, the scientist who coined the term "protein," J.G. Mulder, created a method for the preparation of proteins from milk using acid (McSweeney and O'Mahony, 2016). The acid-induced coagulum was referred to as casein (McSweeney and O'Mahony, 2016). It was believed that the proteins extracted were the only proteins found in milk; however, Olav Hammarsten showed in 1880 that the proteins in milk can be fractionated into two well-defined groups by dropping milk to a pH of 4.6 (Hammarsten, 1883). The proteins that precipitate at pH 4.6 were referred to as isoelectric casein while those which remained soluble in these conditions were referred to as whey or serum proteins.

In bovine milk, the ratio of casein to whey proteins is approximately 80:20. It was initially assumed that casein consisted of only one type of protein, but further investigation revealed this casein was actually a heterogenous mixture of proteins (Osborne and Wakeman, 1918). Following research revealed that isoelectric casein is comprised of α- and ß-caseins. Within the α-casein, it was determined that there were calcium sensitive (α_s) fractions that were two separate proteins, α_{s1}- and α_{s2}-. A calcium insensitive fraction (κ-) was also found that is proposed to exist on the exterior of the micelle as a electrostatic and steric stabilizer (Huppertz et al., 2017). The ß-casein is often on the interior of the micelle and hydrophobic. The individual proteins that make up casein will be covered further in section 2.3.

Up until the 1950s, the whey fraction of milk proteins was typically discarded as it was considered the byproduct (waste stream) of cheesemaking; however, advancements in separation, concentration, and purification techniques have allowed whey to become the source of several valuable ingredients (Smithers, 2008). Cheese whey (or milk serum) contains a variety of proteins, including: β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin, lactoperoxidase and others, with β-lactoglobulin, α-lactalbumin, serum albumin being the most abundant (Hahn et al., 1998). The functionality of whey protein ingredients will be covered in section 2.4.

2.2. Dairy protein ingredients

Dairy protein ingredients are typically prepared from a whey stream or skim milk. Whole milk may be used, but issues may arise with using whole milk because fat may interfere with some separation procedures (O'Mahony and Fox, 2014). Dairy ingredients are based on either simply drying the initial fluid (milk or a whey stream) or are the result of removing some components and thereby concentrating others. Several methodologies of casein and whey separation have been developed, creating different categories and terminology for ingredients enriched in either casein or whey proteins. The methods for creating each dairy ingredient are discussed under the subheadings below for the three primary dairy ingredients used in this investigation: micellar casein, milk protein concentrate, and whey/serum proteins.

Ingredients can be in the form of concentrates, which are above 25 percent protein, or isolates that have 90 percent protein or more on a dry mass basis. The nomenclature generally used for these ingredients is based on the amount of protein in the product using an abbreviation followed by a number. For example, MPC70 is milk protein concentrate with 70 percent protein on a dry mass basis.

2.3. Casein-based ingredients, including milk protein concentrates

As mentioned in section 2.1, individual casein proteins are classified α_{s1}-, α_{s2}-, ß-, and κ-caseins. They are individually on the size order of about 20 kDa but appear in milk as micelles. Casein micelles are colloidal particles containing both protein and minerals with an average diameter of 120 nm, but the size ranges from 50-500 nm (Fox and Brodkorb, 2008). The native structure and function of casein micelles has been extensively discussed (de Kruif, C. G. and Holt, C., 2003; Fox and Brodkorb, 2008; Dalgleish and Corredig, 2012; De Kruif and Huppertz, 2012; Holt et al., 2013; Huppertz et al., 2017).

While the specific structural arrangement of the micelle is still not agreed upon, it is accepted that that the κ-casein appears on the outer part of the micelle and stabilizes the micelles electrostatically and sterically. Destabilization of the κ-casein from the micelle by charge neutralization combined with the subsequent release of calcium ions present in the micelle is the basis for creating both acid and rennet casein (Huppertz et al., 2017). Additional factors that may destabilize the micelle include addition of alcohol, anionic detergents, and high pressure, but the micelle has been shown to be heat stable (Fox and Brodkorb, 2008).

The recovery of caseins has traditionally been based on the above described disruption of κ-casein via precipitation-based isolation in rennet-based casein, acid-based casein, and creation of sodium caseinate and calcium caseinate. The oldest method of separation, rennet casein, is created by the addition of a mixture of proteolytic enzymes known as rennet, traditionally used in cheese-making. These enzymes cause the release of glycomacropeptides (GMP) by cleavage of a peptide bond in the κ-casein. Glycomacropeptides are highly negatively charged and their removal decreases the steric and electrostatic stabilization on the casein micelle. Electrostatic stabilization is further decreased by adding calcium ions, causing aggregation of micelles (Carr and Golding, 2016).

Isoelectric precipitation, also known as acid coagulation, is typically done with direct addition of hydrochloric acid, sulfuric acid or the addition of a *Lactococcus* culture which converts lactose to lactic acid. In all cases, the pH is dropped to the isoelectric point of the casein (pH 4.6) at temperatures around 30°C.

Historically, for both acid and rennet casein, a cooking step takes place following precipitation. This is followed by de-wheying and washing. Finally, the curds are dewatered, dried, tempered, and ground to the desired particle size. Dried caseins are packaged according to particle size, typically 30, 60, 80, or 90 mesh, with 90 being the finest grain. Carr and Golding (2016) provide a complete review of these processes.

Calcium-caseinates and sodium caseinates are manufactured typically from acid casein. Before drying, calcium hydroxide or sodium hydroxide are added to raise the pH, followed by drying. These have uses in products such as dried creamers and meat emulsions and have excellent water-binding capacity and heat stability (Patel, 2016).

The above casein-extraction methods disrupt the native casein micelle structure and require additional processing to render them soluble. As bovine casein micelles in their native state are electrostatically and sterically stabilized, they are better suited for beverage applications. Therefore, milk protein ingredients intended for beverage applications should be processed in a way that keeps the native micelle structure as intact as possible. The methods below give an overview of processing that does not disrupt the micelle.

For lab-scale work, caseins can be sedimented out in their native form at 100,000 x*g* for 1 h, but this is not realistic for industrial applications (Fox et al., 2015). However, when the use of membrane technology became widely applied on an industrial scale in the 1980s it allowed for the physical separation of caseins in their native micellar state (Tamime, 2013). The principle of membrane filtration is that the milk is pumped through a permeable membrane of a given pore

size. The smaller particles such as salts, lactose, and water flow through (permeate), while those too large to pass through the pore are retained (retentate). For casein-only retentate, a membrane size of about 0.1 μm is used. But a variety of membrane pore sizes are available, therefore manufacturers may choose to retain or remove whey proteins, allowing for the production of milk protein concentrate, containing both whey and casein, or micellar casein products (Carr and Golding, 2016).

Milk protein concentrates (MPC) are generally obtained via ultrafiltration by the methods outlined above using a membrane with a pore size in the range of 0.05 μm to 0.1 μm which retains both the whey and casein proteins while allowing soluble salts and lactose to be removed. The retentate of this process contains proteins in approximately the same ratio found in bovine milk. The retentate is then spray dried (Meena et al., 2017). For specific detail on membrane processing as it relates to the dairy industry, Tamime (2013) gives a thorough overview.

Milk protein concentrate functionality has been well-reviewed and studied alongside casein-based ingredients (Martin et al., 2010; Huppertz and Gazi, 2015; Carr and Golding, 2016; Meena et al., 2017; Carter et al., 2018). In Table 1.1, the ingredients are listed as MPC followed by a number that indicates the percentage of protein in the ingredient on a dry mass basis. It can be seen that the lactose content decreases with increasing protein content and that moisture, fat, and ash stay relatively the same.

2.4. Whey/serum proteins

As mentioned in section 2.1, whey from cheese or casein manufacturing was long considered a waste stream and disposal was difficult. The historical journey of whey from byproduct to coproduct is well-documented by Smithers (2008). Section 2.3 outlined the process of precipitation of caseins, which was the first basis for the recovery of whey proteins, as they

remained in the liquid portion of the acid or rennet milk, along with soluble salts and lactose. There are two types of whey obtained from these processes, acid whey and sweet (rennet) whey, both have a standard of identity in the United States Code of Federal Regulations (21CFR184.1979).

Sweet whey is more commonly produced between the two types of whey (Kilara, 2009); however, the amount of acid whey produced has increased due to the popularity of Greek-style yogurt. In this process, 75% of the milk volume used in making Greek yogurt is removed as whey (Boynton and Novakovic, 2013). The composition of sweet and acid whey depends on the processes occurring before whey separation. For example, in yogurt processing heating will cause whey proteins to aggregate with casein micelles and the resulting whey stream will be lower in protein (Nishanthi et al., 2017). If whey proteins are not heat processed prior to precipitation of casein, both acid and sweet whey are similar with the exceptions that sweet whey contains the negatively charged glycomacropeptides (GMP) from κ-casein (Huppertz et al., 2017) and is at a pH of 5.9 - 6.0 while acid whey is pH 4.6 - 4.7 (Królczyk et al., 2016).

All whey-based ingredients follow a combination of general processing steps: 1) clarification, 2) separation of fat, 3) concentration of solids, and 4) fractionation of solids for the purposes of protein recovery, recovering or removal of lactose, and demineralization (Bansal and Bhandari 2016). The following is an overview of the commonly found whey-based ingredients.

Whey powders require the least amount of processing and can be classified as sweet or acid whey as described above. Both are processed by spray drying and there are options in processing to reduce mineral content as well as lactose for these powders. Table 1.2 shows the general composition of these powders. However, the high lactose content of sweet and acid whey favors Maillard browning, thus these are not ideal for a product undergoing a high heat thermal process such as a thermally processed beverage (Van Boekel, 1998). The delactosed whey has

less lactose by design, but it is still a high amount for a high heat application such as thermally processed beverages. Demineralized whey has lower mineral content than dried whey, but still a high lactose concentration. As these ingredients were not used in this research, the reader is referred to Bansal and Bhandari (2016) for a detailed overview for the production of these whey powders as well as considerations for each.

2.4.1. Whey protein concentrates

The starting liquid whey is approximately 0.6% protein and 93% water. Converting whey to whey protein concentrates (WPC) and isolates (WPI), which have 25% to 90% protein on a dry mass basis, requires large volumes of water and non-protein solids to be removed (Foegeding et al., 2011). The manufacture of WPCs is based on membrane separation techniques. Fat and residual casein may be first removed by a low pressure microfiltration (Ramchandran and Vasiljevic, 2013). The following step is ultrafiltration: whey is passed through a filter with a 1-200 kDa cut off size. Lactose, salts and water permeate while protein, fat, and any larger size particles are retained. The resulting whey protein concentrate may be in fluid, concentrate, or dried form. When dried, these have a final protein content of ≥ 25% as defined by the United States Code of Federal Regulations (21CFR184.1979). The nomenclature system for WPCs is the abbreviation followed by a number that denotes the protein content on a dry weight basis. These ingredients are typically commercially available as 35% or 80% protein (de Wit, 2001). Table 1.2 gives the typical composition of whey protein concentrates. Some applications for WPC are baking, yogurt, deli meats and infant formula. Ingredients can be chosen based on the functionality of the lactose, protein, and ash content while 80% protein WPCs are usually selected for their nutritional and functional properties (Foegeding et al., 2011).

2.4.2. Whey protein isolates

Whey protein isolates (WPI) are at a higher protein content and contain lower levels of lactose and minerals over WPCs. They are produced by membrane processing and ion exchange methods (Bansal and Bhandari, 2016). The basic principle of ion exchange chromatography is that pH-adjusted whey is passed through a column such that the proteins adsorb to the counter-ionic charge on the column while the lactose and minerals and pass through. Whey proteins are then eluted from the column by either changing pH or addition of counter ions. Stanic et al., (2012) provides an overview of this technology as well as the advantages and disadvantages of the ion-exchange methods.

A more recent approach is to remove whey proteins directly from milk, leaving a concentrated casein fraction (Nelson and Barbano, 2005; Marcelo and Rizvi, 2008). Whey protein ingredients isolated by this approach have been called native whey proteins, virgin whey proteins, and serum proteins, but recently the term *milk whey proteins* have been suggested. In this thesis, these will be referred to as serum protein isolates (SPI). The purpose of this burgeoning method is to prevent whey protein exposure to the potentially destructive cheesemaking process and start with the unmodified proteins (Foegeding et al., 2011). The processing is done via ultrafiltration and extensive diafiltration. More detail on membrane processing can be found in Hurt and Barbano, (2010).

As mentioned previously, whey/serum proteins include β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin, lactoperoxidase, and GMP is found present in sweet whey (Hahn et al., 1998). Each has been shown to have unique functional and nutritional properties, and methodologies for the fractionation of each exists (Foegeding et al., 2011). The individual functionalities and methods of isolation of each will not be covered in this review but

they have been well-reviewed (Madureira et al., 2010; Stanic et al., 2012; Arrutia et al., 2016; Mohanty et al., 2016).

3. PROTEIN BEVERAGE THERMAL PROCESSING

To create a desirable drink for consumers, manufacturers use a variety of processes that inactivate pathogenic organisms and reduce microbial load to increase shelf life. Beverage manufacturers use thermal processes with a combination of temperature and time to create a safe product, and hopefully, one that has maximum physical stability (Barraquio, 2014). Different thermal processing techniques, such as pasteurization, ultra-high temperature (UHT), retort, and ultrapasteurization, will result in different beverage properties. An overview of commonly used processes is presented below.

3.1. Pasteurization and ultra-pasteurization

Pasteurization is a mild heating process that is designed to inactivate pathogenic bacteria and reduce the microbial load of spoilage microorganisms to extend shelf life, here defined as the length of time a product can be stored and maintain acceptable quality (Cornell University, 2007). The USDA defines pasteurization as, “any process, treatment, or combination thereof, that is applied to a food to reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage” (Sugarman, 2004). Thus, the purpose of this heating method is to eliminate pathogenic organisms to protect consumers.

The first batch pasteurization procedures were instituted by Louis Pasteur and Franz von Soxhlet in the late 1800s. This method is now referred to as *low temperature long time* pasteurization, and the process for milk is defined as 63°C for 30 minutes (Partridge, 2016). In

the 1920s, continuous flow pasteurizers, also known as *high-temperature short-time* (HTST) pasteurizers, were developed (Lewis, 2003). They typically operate with the use of a positive displacement or centrifugal pump that moves product through holding tubes for 15 s at 72°C (Fellows, 2009). Different time-temperature combinations may be used because a logarithmic relationship exists between increased temperature and destruction of bacteria (Fellows, 2009). If pasteurized dairy beverages are properly cooled and stored under refrigeration, they may have a shelf life of 10-28 days (Partridge, 2016).

Pasteurization processes ≤100°C have minimal effect on flavor, color, and protein denaturation; however, the short shelf life of 10-28 days is the primary drawback (Livney et al., 2003). As dairy proteins relate to this thermal process, the casein micelle is largely unaffected by heating at the pasteurization temperatures of 70-100°C. The whey fraction, however, is sensitive to heating, particularly proteins β-lactoglobulin and α-lactalbumin, which are denatured and cause small levels of aggregation with other whey proteins or bind to casein micelles (Vasbinder and De Kruif, 2003).

Heating a beverage a minimum of 138°C for 2 seconds is classified as ultra-pasteurization or extended shelf life processing (Partridge, 2016). The term "ultra-pasteurized" is defined by the FDA in the Pasteurized Milk Ordinance (Food and Drug Administration, 2015). The use of aseptic packaging, sanitary fillers, and refrigeration in tandem with this heating method can create a shelf life of ~90 days under refrigeration. This allows for greater range of distribution, especially for small dairy farms, but products still require refrigeration (Partridge, 2016).

3.2. Commercial sterilization and retort processing

Commercial sterilization is required if a long shelf life at ambient (~25°C) temperatures is desired. It is defined by the United States Food and Drug Administration as, “the condition achieved by application of heat, chemical sterilant(s), or other appropriate treatment that renders the equipment and containers free of viable microorganisms having public health significance, as well as microorganisms of non-health significance, capable of reproducing in the food under normal nonrefrigerated conditions of storage and distribution." (Food and Drug Administration, 2018). Heat sterilization may be accomplished by in-container heating to inactivate microbes and enzymes or by thermal sterilization of the product followed by filling under aseptic conditions.

3.2.1. Retort processing

In-container sterilization, or retort processing, is one of the first preservation techniques that dates to 1795 when Nicolas Appert created a method for Napoleon Bonaparte’s food preservation competition. An entertaining history of the development of canning can be read in Allen (2016). Traditional retort processing involves heating at 114°C-120°C for 20-30 min in a container that is hermetically sealed, such to not allow the growth of organisms and prevent re-contamination post-sterilization (Awuah et al., 2007). The time and temperature selected for the process are based on several factors: heat-resistance of microorganisms, pH of the foods, size and shape of the heating container, and the physical state of the food (Holdsworth, 2004). The modern microbial basis for retort processing is the inactivation of *Clostridium botulinum*, as it grows in anaerobic conditions and produces toxins deadly to humans (Fellows, 2009). An overview of types of retorts and their limitations can be read in Bown (2003). Techniques to minimize heat impact on nutrients and quality are outlined in Awuah et al. (2007).

As retorting relates to dairy proteins, at temperatures > 120°C, protein destabilization has been observed because whey proteins bind to κ-casein and larger casein micelles may precipitate because they are no longer stabilized by the κ-caseins (O'Connell and Fox, 2001). Retort processing of dairy ingredients containing lactose also results in a distinctive cooked flavor and a darker coloring as a result of browning reactions (Deeth and Lewis, 2017).

3.2.2. Ultra-high temperature processing

Ultra-high temperature (UHT) processing is the concept of commercially sterilizing the food product using direct or indirect heating, holding to achieve desired lethality, cooling rapidly, and then filling into an aseptic package in a sterile environment (Awuah et al., 2007). This involves continuous heating at temperatures above 135°C for at least 1s followed by filling into an aseptic container. The advantage of this process is that the product is not exposed to high temperatures for as long as it is with retort processing. The temperature can be adjusted so long as the product is able to be shown to be commercially sterile and the process is filed with the FDA by a Process Authority (Lund and Singh 1993). Regulations for UHT processing may be found in Title 21 CFR sections 108 and 113.

Aseptically processed products are stored at ambient temperatures and have an expected shelf life of six months or greater; however, chemical and physical changes do occur over shelf life. Viscosity change in milk has been attributed to modification of κ-casein, causing potential destabilization in casein micelles. Age-gelation is commonly seen in UHT-dairy beverages and milks, with increased viscosity, gelation, and aggregation of micelles (Fellows, 2009). The extent of denaturation of whey proteins is 60-70% when using direct heating UHT and 75-80% in indirect heating UHT, with β-lactoglobulin being more sensitive to the heat process than α-lactalbumin (Ramesh, 1999). However, it has been seen that direct-heat processed UHT milks

tend to gel more rapidly than indirect-heat processed milks (Anema, 2017). In the following section, thermal and colloidal stability will be discussed in greater detail.

4. PHYSICAL STABILITY OF PROTEIN BEVERAGES

There are two forms of physical stability relating to protein beverages. The most commonly investigated is *thermal or heat stability*, which is defined as the ability of a protein solution, or beverage, to withstand thermal processing and remain a homogeneous fluid (colloidal state of a sol). The second form of physical stability is *shelf life,* which can be defined as the time period after thermal processing where the physical properties and appearance remain acceptable.

The colloidal state of matter is defined as consisting of, "a single dispersed phase of particles in a second continuous phase called the dispersion medium (Dickinson, 1992)." This is the simplest form, foods often have more complexity in the dispersed and continuous phases. For example, bovine milk contains two dispersed phases (casein micelles and milk fat globules) and a continuous phase solution with protein (whey proteins), mineral, and sugar solutes. One common feature of colloidal systems is that they are not inherently stable, and the dispersed phase is separating at a rate that is regulated by several factors. Food colloidal systems are investigated to determine 1) the conditions required to produce the desired colloidal structure and 2) how the colloidal structure may be designed to slow down destabilization. A *physically shelf stable beverage* is defined as one that has colloidal stability over the desired shelf life (Ryan and Foegeding, 2015). Thermal processing causes denaturation and aggregation and the physical characteristics of the resulting aggregates determine the shelf stability. The term "soluble aggregates" has been used to describe aggregates that are larger in size than the original individual protein molecules (or in the case of milk, protein micelles) but do not form a

precipitate or gel immediately after thermal processing (Foegeding, 2015). Therefore, physical stability depends on the formation of soluble aggregates that remain stable and suspended in solution over the shelf life of the product.

4.1. Thermal stability

Depending on the specific discipline, "protein stability" can mean many different things. In the context of a thermally processed beverage, the emphasis is on colloidal stability instead of maintaining protein structure as it would be for biopharmaceuticals. For the purposes of this study, *thermally stable* proteins (ingredients) are defined as those that remain dispersed in the beverage through thermal processing.

4.2. Colloidal shelf stability

The second form of physical stability is *shelf life*, which can be defined as the time period after thermal processing where the physical properties and appearance remain acceptable. The ability of a particle to remain suspended in solution is due to properties of the solvent and dispersed particles, as modeled by Stokes' law (Equation 1.4). There are three proposed mechanisms for physical destabilization in beverages that do not undergo polymer-polymer segregated phase separation: 1) phase separation based on Stokes' law, 2) phase separation based on secondary aggregation and Stokes' law, and 3) gel formation.

The first two destabilization mechanisms relate to when non-homogeneity is observed in a beverage. This is due to settling of the dispersed protein phase and can be analyzed based on the factors included in Stokes' law:

$$v_s = \frac{2(\rho_{particle} - \rho_{solvent})\, g\, r^2}{9\eta} \quad (1.4)$$

Where:

v_s is the velocity of sedimentation, m s^{-1}

ρ particle = density of soluble complex, kg m^{-3}

ρ solvent = density of continuous phase, kg m^{-3}

g = gravity, m s^{-2}

r = hydrodynamic radius, m

η = continuous phase Newtonian viscosity, Pa s

Stokes' law can be used to evaluate the effects of particle size, density differences between phases, and continuous phase viscosity in relationship to shelf life being terminated by formation of a visible precipitated phase in the bottom of a beverage. However, Stokes' law only precisely predicts settling velocity of hard spheres at phase volumes (ϕ) less than approximately 0.05, or ~ 0.5 percent (wt/wt) particles (Dickinson, 1992). Beverages generally have much larger phase volumes, a particle size distribution rather than being monodispersed, and often contain polysaccharides in the continuous phase that cause non-Newtonian flow. However, Stokes' law does define the variables that contribute to destabilization over time assuming no secondary aggregation, which would cause an increase in particle size with time. With the knowledge of Stokes' Law, the approach of many product developers is to increase the viscosity of the continuous phase, thus slowing the sedimentation velocity e.g. the addition of xanthan gum. Another approach product developers use is to form a weak "gel" that is solid until mixed, whereby it rapidly ruptures into a liquid e.g. the addition of carrageenan to chocolate milk beverages. Furthermore, the occurrence of secondary aggregation over time makes it difficult to predict destabilization based on initial physical properties.

Protein aggregation can also produce a continuous gel network. Often the beverage appearance remains homogenous but becomes a solid gel. This is related to secondary aggregation at a protein concentration above the critical gel concentration and the rate of secondary aggregation occurring rapidly enough that a gel is formed before settling as a precipitate.

4.3. Aggregation

Two or more colloidal particles sticking together is defined as aggregation (Dickinson 1992). Aggregation can occur at several steps of protein beverage processing. It is a universal term that encompasses all proteins that have formed inter-molecular covalent bonds or noncovalent interactions and create a protein particle (Mahler et al., 2009). It is a complex process, as there is no one pathway for aggregation that applies to all proteins (Roberts, 2007).

Aggregates may be soluble and remain dispersed over an appropriate time scale, or insoluble, leading to precipitation (Mahler et al., 2009). The methods of non-covalent aggregation are based on weak forces like electrostatic interactions, van der Waals interactions, hydrogen bonding, and hydrophobic interactions (Karshikoff, 2006). Covalent bonding in the form of disulfide bonds may also occur with the exposure of thiol groups or nondisulfide bonds may form as well via cross linking pathways (Cromwell et al., 2006). The aggregation process depends on the mixture of proteins, protein concentration, temperature of processing and storage, pH and salts (Laclair and Etzel, 2010; Dickinson, 2015; Ryan and Foegeding, 2015; Ho et al., 2018; Leeb et al., 2018).

4.4. Precipitation or sedimentation

Sedimentation is explained by Stokes' equation as particles fall out of the dispersed state and settle to the bottom of the container at a given velocity. Sedimentation has been commonly reported in dairy proteins and increases with time and temperature of heating (Ramsey and Swartzel, 1984). Regular disturbance of beverages will decrease sedimentation, as it resuspends the particles (Deeth and Lewis, 2016). The mechanisms for sedimentation in milk are not well-understood or characterized (Nieuwenhuijse and van Boekel, 2003).

4.5. Gelation and age-gelation

As mentioned previously, gelation occurs when protein concentration is above the critical gelation concentration and aggregation produces a continuous three-dimensional network (Panouille et al., 2005).

Another form of gelation seen over storage is age-gelation which is characterized by an irreversible gel that causes the beverage to lose fluidity and become a solid. The gel is present throughout the container and not as a layer on the bottom of the container (Anema, 2017). The mechanism of age-gelation is not completely understood (Nieuwenhuijse and van Boekel, 2003; Chavan et al., 2011). Age-gelation may be a product of several different mechanisms; one study found it was caused by protein proteolysis by enzymes not inactivated by UHT processing (plasmin and bacterial proteinases) but when the product was stored at temperatures above 30°C, gelation did not happen as quickly (Deeth and Lewis, 2016). Another mechanism proposed by Anema, (2017) is non-enzymatic but the age-gelation is caused by dissociation of κ-casein from casein micelles, the κ-casein-depleted micelles in turn cross-link, leading to age-gelation. The study showed that this was more likely to happen at high protein concentrations.

4.6. Factors contributing to stability

4.6.1. Protein Type and Concentration

It has been demonstrated that casein-based ingredients are more heat stable than whey protein-based ingredients. Milk is heat instable, the instability is due to the whey proteins denaturing and interacting with κ-casein, thus destabilizing the electrostatic stability of micelles (Singh et al., 2004). Specifically, β-lactoglobulin will bind to the κ-casein on the exterior of casein micelles (Gaspard et al., 2017).

One functional advantage of micellar casein concentrate (MCC) is the reduced amount of whey proteins. Sauer and Moraru (2012) studied heat stability of MCC at a pH range 6.5-7.3 and heating temperatures of 110°C-150°C. High temperatures caused change in mineral equilibrium and release of both κ-casein and α_s-casein, which are proposed to contribute to aggregation. Treatments at pH less than 6.7 consistently produced aggregation while treatments greater than pH 6.9 did not show aggregation across the temperature range. It was suggested that when high temperatures for processing are required, the pH of solution should greater than 6.9.

The aggregation of whey proteins, representing whey protein ingredients, has been well-studied, and many mechanisms of aggregation and gelation are well-understood (Roefs and Peppelman, 2000; Fitzsimons et al., 2007; Dissanayake and Vasiljevic, 2009; LaClair and Etzel, 2009; Wijayanti et al., 2014). Whey proteins aggregate due to non-covalent and disulfide bonding. β-Lactoglobulin is more prone to form aggregates than α-lactalbumin and disulfide-linked β-lactoglobulin oligomers are formed at neutral pH (Singh and Creamer, 2001). When α-lactalbumin is heated alone, little aggregation is seen, but in the presence of bovine serum albumin and β-lactoglobulin it forms large aggregates (Kessler and Beyer, 1991).

4.6.2. Effect of pH

Beverage pH is important to protein stability because it changes the protein's net charge. Low and high pH values are typically preferential because they provide electrostatic stabilization but the pH should not go to extremes that can cause denaturation (Pelegrine and Gasparetto, 2005). The isoelectric point (pI) is considered the point of least solubility for proteins because at this pH the electrostatic forces stabilizing the proteins are at a net neutral charge, creating a more favorable environment for aggregation. Each type of protein has its own unique pI based on the amino acid side chains (Kilara, 2009; Huppertz et al., 2017). When caseins and whey proteins are together in solution, the pH has a significant impact on how the proteins behave. At pH < 6.8, whey proteins are more associated with casein micelles, at pH > 6.8, whey proteins aggregate but remain dispersed (Singh et al., 2004).

5. CHARACTERIZING THERMAL AND SHELF STABILITY FOR PROTEINS IN BEVERAGES

As mentioned previously, *thermal stability* is the ability to survive thermal processing and remain a colloidal sol (solid dispersed in a liquid) and colloidal *shelf stability* is how long a beverage remains homogeneous in appearance and does not form an undesirable level of viscosity or be converted to a solid (gel). Babick (2016) outlines methods for observing the behavior of the dispersed particles and their theory in detail.

Investigations on thermal stability and colloidal shelf life stability of beverages and dairy protein ingredients use a variety of techniques to characterize particle size, aggregation, and gelation, then relate those results to stability. Whey proteins and caseins have been studied for heat stability, but few studies, other than those for age gelation of milk, have investigated shelf stability. The following sections will focus on colloidal shelf stability studies done and

methodologies applied. The general topic of thermal processing of whey and caseins has been addressed in several reviews (Schokker et al., 2000; Fitzsimons et al., 2007; Roberts, 2007; Mahler et al., 2009; Dalgleish and Corredig, 2012; Dissanayake et al., 2013).

Shelf stability has not been a large focus in the published literature. While studies on age gelation in milk are more prevalent (reviews found in (Datta and Deeth, 2001; Lakemond and van Vliet, 2008)), only a few studies exist on shelf stability of formulated milk protein beverages, which can be seen in Table 1.3 (Temelli et al., 2004; LaClair and Etzel, 2009; Villumsen et al., 2015; Le et al., 2016; Anema, 2017; Gaur et al., 2017). Since the goal of this investigation was to evaluate ways to measure thermal and shelf stability, the literature is organized based on methods used to follow stability.

5.1. Changes in solubility

It is important to note that because protein ingredients form aqueous dispersions of individual proteins and aggregates, protein solubility is operationally defined as the amount of protein that remains after a given centrifugation (Sikand et al., 2011). It is not the same definition of solubility as for a true solution, i.e. sucrose solubility. There are a range of methods proposed for measuring protein solubility that vary in centrifugal force, time, and temperature of operation. Comparing the starting concentration of protein to the concentration remaining in the supernatant will give percentage of soluble protein. The higher the percentage after a thermal process or storage time, the more stable the dispersion. This is a simple measurement, but difficult to compare across studies because of variations in times, temperatures, and forces among investigations. Therefore, solubility should be considered a relative value that will show differences within an investigation but not an absolute value for comparison among

investigations. Other factors being equal, solubility is proportional to particle size with greater force and time removing smaller particles.

Gaur et al., (2017) studied gravimetric sedimentation behavior after 8 weeks in UHT (144°C for 4s) milk at varying pH and ionic calcium levels and found that both decreased pH and increased calcium ions caused sediment to form more quickly. The sediment was measured by decanting the liquid milk and weighing the sediment, which consisted predominately of casein micelles without κ-casein. This highlighted the importance of steric stabilization of casein micelles.

Anema et al., (2017) studied age-gelation in UHT processed reconstituted milk powders and measured solubility of particles by centrifugation at 63,000 x *g* at 20°C, with no time given. It was seen that α- and β-caseins were less soluble than the whey proteins and κ-casein (Anema 2017).

5.2. Turbidity

Turbidity-based methods are based on larger particles causing an increase in turbidity (increased light scattering and decreased transmitted light). However, other factors contribute to turbidity, including particle size, shape, concentration, and refractive index of the particles and continuous medium (Mahler et al., 2009). An excellent overview of turbidity may be found in Kitchener et al., (2017). Figure 1.1 from Wagoner et al. (2015) illustrates the effect of pH on turbidity in WPI solution at the same concentration, as pH increased, the 1% (wt/wt) solution became more turbid.

Temelli et al., (2004) claimed to have created a stable pasteurized and refrigerated beverage containing whey proteins and β-glucans from barley; however, a marked decrease in turbidity and increase in sedimentation was observed in all beverages at two weeks. While it was

shown that the sediment could be re-dispersed with shaking, this beverage would *not* be considered stable by definitions in this literature review. Viscosity and pH were also monitored across the six weeks, but no significant differences were seen (Temelli et al., 2004). This study showed phase separation occurs with increasing protein concentration, but particles could be redispersed with agitation, indicative of Stokes' settling behavior of these particles.

Laclair and Etzel (2009) studied the aggregation of whey proteins in acidic beverages at 1.25% (wt/wt) protein over the course of six weeks. Turbidity and particle size were measured. They found all acidic whey protein beverages increased in turbidity over time, but those with aggregates from heating filtered out prior to bottling had a lower initial turbidity (Laclair and Etzel, 2009). They did not measure the protein content of the filtered beverages compared to the non-filtered, which would have added insight into turbidity meaurements.

Villumsen et al., (2015) investigated processing and storage conditions (up to six months at 4°C and 20°C) on formation of aggregates in acidic whey protein beverages (7.0% (wt/wt) protein). Aggregate formation was measured based on changes in turbidity and rheological properties, while the protein forming the aggregates were determined by 2D-gel electrophoresis and mass spectrometry. Only one sample showed an increase in viscosity and turbidity over storage. Aggregates from that sample contained an unidentified protein, proposed to be glycomacropeptide or another protein that had been enzymatically hydrolyzed. Le et al., (2016) expanded on the Vllusen et al., (2015) study and confirmed the presence of glycomacropeptides as the "unidentified protein" in soluble aggregates.

5.3. Particle size

Rather than measure particle size indirectly by solubility or turbidity, some investigations have directly measured particle size distribution. A challenge with measuring particle size in

protein beverages is that particles can span a range from a few nanometers to visible aggregates (Amin et al., 2014). There is not one analytical technique that covers such a range and each technique has limitations.

Laclair and Etzel, (2009) used UV-spectrophotometry and size-exclusion high performance liquid chromatography to measure changes in acidic whey protein beverages at 1.25% (wt/wt) protein over six weeks. They found a slow aggregation process controlled the rate of sediment formation and aggregation could be explained and predicted based on aggregates seen immediately post-processing. Prior to heating, half of the beverages were centrifuged (3000 x *g* for 30 mins at 4°C), all samples were hot-filled (88°C for 2 mins), and half were filtered with a 0.1 μm filter before bottling to remove aggregates from thermal processing. Initially after heating, all treatments showed the same percentages of soluble protein but over time, SEC-HPLC showed soluble protein peaks decreased and aggregates in the void volume increased over time and that increased storage temperature increased amount of aggregates. The suggestion from this finding was that primary aggregates larger than 0.1 μm act as nucleation sites for further aggregation and that a first- and second-order kinetic equation could potentially be applied to predict the effects of concentration, storage life, and temperature of storage on turbidity and aggregation. However, these experiments were run on solutions at 1.25% (wt/wt) protein, much lower than what is considered a, "good source of protein" by Code of Federal Regulations (21CFR2008) and there was no measurement of the amount of protein lost by the filtration step (Laclair and Etzel, 2009).

Anema et al., (2017) studied causes of age gelation in milk over 10 months using dynamic light scattering and zeta potential to characterize changes in particles. Surprisingly, increase in particle size or change in zeta potential did not predict gelation. A sudden

destabilization occured that caused gelation (Anema et al., 2017). Gelled material was composed of α- and β-caseins (Anema et al., 2017).

5.4. State diagrams

Wagoner et al. (2015) demonstrated the creation and utilization of state diagrams to represent the *thermal stability* of whey protein solutions across pH and protein concentration using turbidity, solubility, and macroscopic phase (sol, precipitate, gel) (Figure 1.2). These diagrams are useful for identifying pH and protein concentrations corresponding to regions of colloidal stability. This approach is applicable to individual protein ingredients as well as complex protein beverages (Wagoner et al. 2015). While Wagoner et al. (2015) established regions of heat stability, the colloidal shelf stability was not investigated.

6. MOTIVATIONS AND APPROACH

The literature suggests that a comprehensive approach to particle characterization could provide greater insight into destabilization based on Stokes' considerations. All shelf stability studies found in the literature on beverages have been on milk (~3.3% protein) or acidic whey beverages (pH<4.6). However, the behavior of high protein beverages (defined as > 42 g protein L^{-1} by United States Food and Drug Administration) at neutral pH involving both caseins and whey at different concentrations has received minimal investigations.

6.1. Beverage formulation

Two studies were conducted to evaluate heat stability and the initial phase of storage stability. In the first study, ingredients of serum (whey) protein isolate (SPI), micellar casein concentrate (MCC), and milk protein concentrate (MPC) were produced and used in liquid form (not dried and rehydrated). The ingredients were formulated into beverages (containing water,

carrageenan, cellulose gel, sucralose, dipotassium phosphate, vanilla flavoring, α-monohydrate lactose, and cream) using the following treatments: 100% MCC, 100% MPC, 18% SPI and 82% MCC, 50% MCC 50% SPI, and 50% MPC 50% SPI at 10.5% and 6.3% (wt/wt) protein. Heat stability was determined immediately after UHT processing and colloidal shelf stability was followed over 8 weeks of storage at 4°C.

The second study was on aqueous dispersions of hydrated milk protein concentrate (MPC85) and whey protein isolate (WPI) ingredients. Treatments were: 100% MPC85 and 50% MPC85 50% WPI at concentrations of 11% (wt/wt) and 6% (wt/wt) protein. Heat stability was determined immediately after retort processing and colloidal shelf stability was followed over 8 weeks of storage at 25°C.

The formulations were at a pH of 6.8 and at protein concentrations allowing for a claim of "high protein" as defined by the United States Food and Drug Administration as > 4.2% (wt/wt) protein.

6.2. Heating methods

As outlined in section 3, heating methods vary in their effect on protein denaturation and particle size change. For this study, two different heating methods were used. Study one used ultra-high temperature processing (141°C for 2.5 s) and retort processing (121°C for 20 minutes) was used in Study 2. This allowed for a comparison of two commercially relevant heating conditions.

6.3. Particle size by laser diffraction

Particle sizing by laser diffraction was used to give an approximation of the distribution of particle sizes. An advantage of this method is a simple operation (sample and dilute), but it

has complications in 1) assuring a proper refractive index is used, 2) it is limited in the range of particle size (0.1 μm to 100 μm), and 3) dilution/mixing may disrupt particles. Measurements were taken at time zero and across sampling timepoints to track changes.

6.4. Particle fractionation/mass balance

Particle size measurement doesn't allow for protein quantification (i.e., how much of the total protein is found in each particle size class), which is the justification for developing a fractionation method. Methods for particle fractionation are described in Babick (2016); however, they have not been used in reference to protein solubility. Figure 1.3 shows the principle of fraction based on a combination of centrifugation and filtration. This method showed the amount of protein within different levels of solubility defined by fractionation at each stage removing "insoluble" fractions. This results in three soluble fractions that can be tracked over time. It has the advantage that all protein is accounted for in soluble or insoluble fractions.

6.5. Size-exclusion high performance liquid chromatography

The last soluble fraction in the method described in section 6.4 can contain individual protein molecules as well as small aggregates. Therefore, size exclusion chromatography was used to characterize the "soluble" fraction, by using a high molecular weight ladder to compare size class of particles in the most soluble fraction, detected by UV absorbance at 280 nm.

6.6. Turbiscan z-plane turbidity measurement

In experiment two, sedimentation and gelation were measured along the height of a quiescent tube containing protein beverages. The principle of this is to be more quantitative and detect changes not seen with visual inspection. The changes in time can be computed to

determine differences in settling. There does not appear to be any published data on the use of Turbiscan for the purposes of protein beverages, it is commonly used for studying emulsion stability. This instrument scans the total length of a glass cylindrical cell with a detection head that pulses light at a wavelength of 850 nm. Two detectors are at 0° and 135° from the incident beam of light, the 0° measures transmission of light and the 135° detector measures backscattering of light. For complete review of the operating principles of the Turbiscan, the reader is referred to Mengual et al. (1999). Turbiscan has the advantage of following aggregation under quiescent conditions without the requirement for dilution.

6.7. Experimental objective

The purpose of this thesis is to determine if several measurements related to particle size distribution can be used to provide insight into thermal and shelf stability. Neutral pH, high protein beverages were used as they can contain a combination of casein and whey proteins and high protein concentrations are desirable. The goals were to 1) utilize several methods to more fully characterize the aggregated particles in neutral-pH beverages post-processing at two concentrations 2) observe these differences over the course of eight weeks, and 3) observe differences in formulations with varied ratios of whey and casein beverages. The data produced should lay the groundwork for future studies on shelf life of high-protein, neutral pH beverages.

7. FIGURES

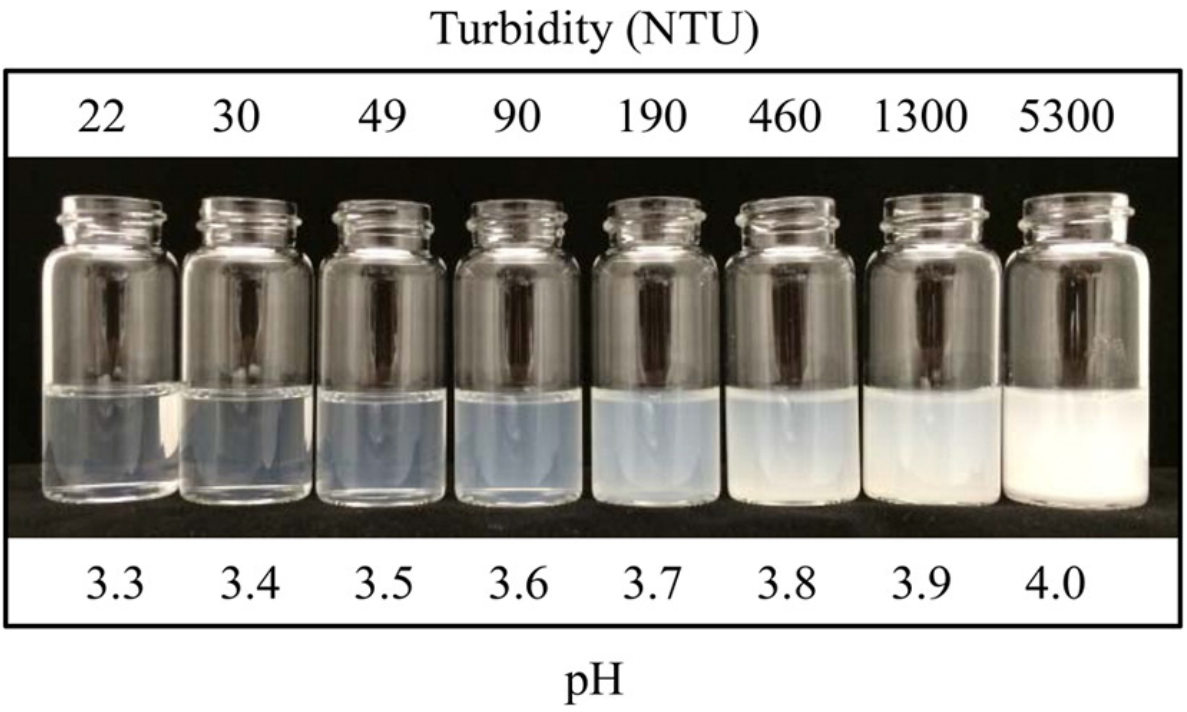

Figure 1.1. Turbidity of 1% (wt/wt) WPI solutions from pH 3.3 to 4.0 post-heating. The NTU is stated above the sample.

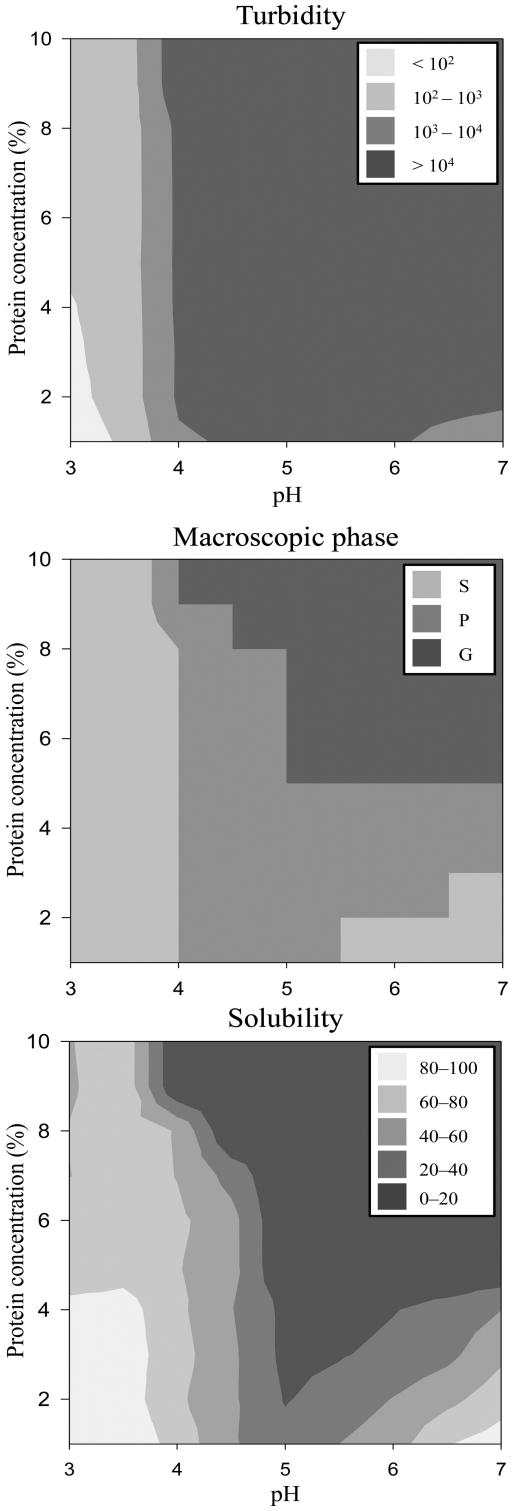

Figure 1.2. State Diagrams from Wagoner et al. (2015). Turbidity is given in logarithmic NTU, Macroscopic definitions are S, sol; P, precipitate; G, gel. Solubility shading is based on 20% differences in solubility.

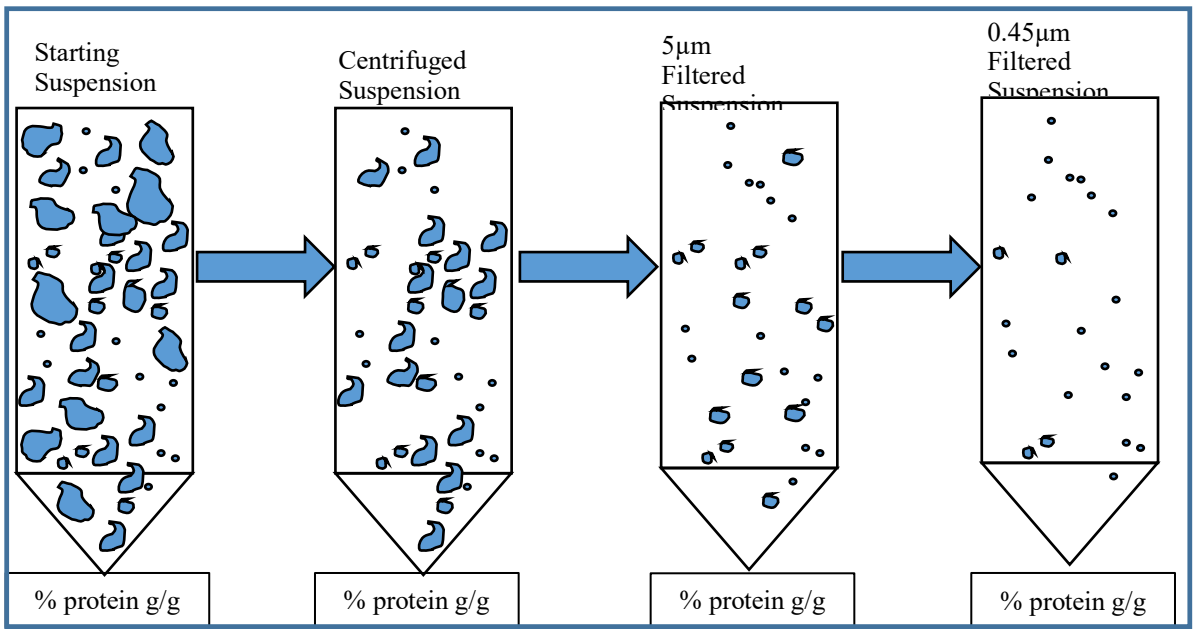

Figure 1.3. Illustration of principle for mass balance of proteins

8. TABLES

Table 1.1. Typical compositions of milk protein concentrate (g/100g)[1]

Ingredient	Moisture %	Fat %	Protein %	Lactose %	Ash %
MPC 42	3.5	1.0	42.0	46.0	7.5
MPC 70	4.2	1.4	70.0	16.2	8.2
MPC 75	5.0	1.5	75.0	10.9	7.6
MPC 80	3.9	1.8	80.0	4.1	7.4
MPC 85	4.9	1.6	85.0	1.0	7.1
MCC	5	1	83	1	7.8

[1]Sourced from Dairy Management Inc., 2003 and U.S. Dairy Export Council, 2015
MPC, Milk Protein Concentrate; MCC, Micellar Casein

Table 1.2. Composition of different whey powders (g/100g)[1]

Product	Moisture	Fat	Protein	Lactose	Minerals
Sweet Whey Powder	3-6	0.8-1.5	12-13	70-74	7.2-8.5
Acid Whey Powder	≤ 3.5	0.8	9-12	65-69	11-12
Reduced Minerals Whey Powder	≤ 3	≤ 1.5	≥11	75-84	≤4
Delactosed Whey Powder	2-3	1-4	18-25	40-60	11-27
WPC35	4.6	2.1	29.7	46.5	7.8
WPC50	4.3	3.7	40.9	30.9	6.4
WPC65	4.2	5.6	59.4	21.1	3.9
WPC80	4.0	7.2	75.0	3.5	3.1
WPI	4.5	1	92	0.5	2

[1]Sourced from de Wit, 2001; Foegeding et al., 2011; and Bansal and Bhandari, 2016
WPC, whey protein concentrate; WPI, whey protein isolate

Table 1.3. Summary of Shelf Life Studies

Study	*Methods Used*	*Duration of Study*	*Goal*
Temelli et al., 2004	Viscosity, pH, color, turbidity,	8 weeks	*Develop a formulation, examine shelf stability*
LaClair and Etzel, 2009	Turbidity, SEC-HPLC, Soluble protein UV-Spectrophotometry	6 weeks	*Understand relation between monomoer loss, agregation formation, and turbidity increase*
Villumsen et al., 2015	Oscillory Rheology, Turbidity by absorbance at 500 nm, 2-D gel electrophoresis, Mass Spectroscopy	6 months	*Assess heat treatment, storage conditions, mineral content impact on high protein acidicWPI beverages*
Wagoner et al., 2015	Turbidity, Electrophoresis, RP-HPLC, Observation of macroscopic state	Immediately post-processing	*To develop a state diagram approach to determine whey protein beverage functionality in beverages after thermal processing across a range of pH values.*
Le et al., 2016	1-D gel electrophoresis, 2-d gel electrophoresis, In gel-digestion, Mass spectroscopy	12 months	*Proteomics to identify and characterize chemical changes in WPI acidic drinks*
Anema et al., 2017	Visual inspection for gelling, gel electrophoresis, solubility, particle size, zeta potential, pH and serum mineral levels	10 months	*Understand mechanism of age-gelation in UHT milk*
Gaur et al., 2017	Visual, gravimetric, microfluidic chip analysis, pH and ionic calcium measurements	8 weeks	*Assesment for mechanism of settling behavior in UHT milk*

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CHAPTER 2. ANALYSIS OF PROTEIN PARTICLES REVEALS AGGREGATION PATTERNS ASSOCIATED WITH PHYSICAL STABILITY IN PROTEIN BEVERAGES

1. ABSTRACT

Thermal processing of beverages containing protein is a challenge because the resulting protein aggregation can cause undesirable effects. Extensive aggregation can cause beverage shelf life to be terminated by the development of an undesirable level of phase separation, sedimentation, or gelation. The mechanisms causing physical destabilization over time are not well characterized. In this study, an analytical method based on a combination of physical properties was used to provide a "protein aggregation fingerprint" for protein-containing beverages. The method was evaluated by analyzing five beverages immediately after thermal processing and over eight weeks of storage at 4 °C. The treatments consisted of: 100% micellar casein, 100% milk protein concentrate, a blend of 18% serum protein isolate and 82% micellar casein, a blend of 50% serum protein isolate and 50% micellar casein, and a blend of 50% serum protein isolate and 50% milk protein concentrate. Beverages were formulated at protein concentrations 6.3% (wt/wt) and 10.5% (wt/wt). All beverages were ultra-high temperature processed (141°C for 2.5 s) at pH 6.8. Beverages were characterized based on protein particle size; protein fractions determined by centrifugation and filtration; and gel permeation chromatography of the most soluble fraction. Changes were monitored over eight weeks and the experiment was duplicated.

The method was able to differentiate treatments based on patterns in size exclusion chromatography and laser diffraction particle size analysis. Beverages containing 50% of serum protein had larger initial aggregates that increased with time and less soluble protein. Treatments with 18% serum protein or less were stable based on appearance and particle size determined

immediately after processing; however, size exclusion chromatography of the most soluble protein fraction showed secondary aggregation occurring over time. For the beverage containing 100% micellar casein at 10.5% protein, a cold set-gel formed while the overall appearance remained homogenous.

In conclusion, all methods used showed differences in *thermal stability* of protein beverages. In contrast, detection of secondary aggregation, important to *shelf stability*, depended on the ingredient. Beverages high in casein did not show consistent trends in secondary aggregation with laser diffraction particle size analysis, but trends were observed in size exclusion chromatography. In high whey protein beverages, the opposite was true. The most soluble aggregates were already in the exclusion volume at time zero (e.g., thermal stability) so size exclusion chromatography did not show any changes in particle size. The combination of methods would appear to be useful in investigating thermal and shelf stability of protein ingredients or beverages.

2. INTRODUCTION

Protein beverage applications include protein-fortified smoothies, meal replacement drinks, and pre-and-post workout beverages, all of which fall under the umbrella of a functional food, defined as a product that provide health benefits beyond nutritional value. It is projected that the market for functional foods will grow, with the category of sports and protein drinks reaching about $8.5 billion by 2020 (Beverage Industry, 2017). While there is no current legal standard for protein content in these beverages, Oltman et al. (2015) showed that consumers desire ≥20 grams of protein per serving and have a preference for whey as the primary protein source.

Protein-containing beverages are a colloidal sol, with a protein particle phase dispersed in an aqueous solution containing a range of solutes. Beverages must be processed to inactivate pathogenic microorganisms, which presents a challenge because the normal approach - thermal processing - may influence the physical stability of the protein dispersion. Heating causes denaturation of proteins leading to various extents of aggregation depending on conditions such as pH, ionic strength, and protein concentration (Singh et al., 2015). Types of thermal treatments vary, but all heating processes are above the denaturation temperature of whey proteins (~ 60 °C) and may also cause changes in caseins (Qi et al., 2004; de Wit, 2009). This results in protein aggregation that can produce the undesirable states of precipitation, phase separation, or gelation.

There are two forms of physical stability relating to how proteins function in beverages. The most commonly investigated is *thermal or heat stability*, which is defined as the ability of a protein solution or beverage to withstand thermal processing and remain a homogeneous fluid (colloidal state of a sol). Wagoner et al. (2015) developed a state diagram approach for predicting thermal stability of protein ingredients or beverages. The effects of pH and protein concentration on thermal stability are displayed by state diagrams relating pH and protein concentration to protein solubility, solution turbidity, and macroscopic phases (solid, liquid, gel) after a given thermal process. This approach showed the combination pH and protein concentrations that produce a sol after thermal processing.

The second form of physical stability is *shelf life*, which can be defined as the time period after thermal processing where the physical properties and appearance remain acceptable. There are three proposed mechanisms for physical destabilization in beverages that do not undergo polymer-polymer segregative phase separation: phase separation based on Stokes' law, phase separation based on secondary aggregation and Stokes' law, and gel formation.

The first two destabilization mechanisms relate to when non-homogeneity is observed in a beverage. This is due to settling of the dispersed protein phase and can be analyzed based on the factors included in Stokes' law:

$$v_s = \frac{2\left(\rho_{particle} - \rho_{solvent}\right) g\, r^2}{9\eta}$$

Where:

v_s is the velocity of sedimentation, m s^{-1}

ρ particle = density of soluble complex, kg m^{-3}

ρ solvent = density of continuous phase, kg m^{-3}

g = gravity, m s^{-2}

r = hydrodynamic radius, m

η = continuous phase Newtonian viscosity, Pa s

However, Stokes' law only precisely applies to hard spheres at phase volumes (ϕ) less than approximately 0.05, or ~ 0.5 % (wt/wt) particles (Dickinson, 1992). Protein beverages targeted for functional foods have much higher dispersed phase (protein particles) volumes, have particle size distributions rather than being monodispersed, and often contain polysaccharides in the continuous phase that cause non-Newtonian flow. Therefore, methods used for analyzing factors related to physical stability should focus on the variables defined in Stokes' Law (density difference between phases, viscosity of the continuous phase, and size of dispersed particles), rather than direct use of the equation.

If no secondary aggregation occurs after thermal processing, then the variables found in Stokes' law should be sufficient to predict shelf life. If secondary aggregation does occur, then more rapid settling due to increase in particle radius or gelation are possible. The kinetics of aggregation and gelation depend on the processing conditions as well as the protein

concentration. It has been shown that the higher the protein concentration, the faster the reaction and the higher the molecular weight of the aggregates (Fitzsimons et al., 2007).

Bovine milk contains on average 3.25% (wt/wt) protein, with casein and serum proteins (more commonly called whey proteins) accounting for the largest fraction (Walstra, 1999). Serum proteins are called whey proteins because they are isolated from the whey produced during cheese and casein manufacturing; however, new processes are being used to isolate serum/whey proteins directly from milk (Hurt and Barbano, 2010). This new class of ingredients had several initial names, including "native" whey proteins, but the current recommendation is *milk whey protein*, to indicate that the proteins are not associated with whey from cheese or casein manufacturing (Evans et al., 2010). Whey proteins that have not been exposed to the cheese-making process have a milder flavor profile, which makes them applicable to beverage applications (Evans et al., 2010). In addition, removal of whey proteins from milk leaves a casein enriched fraction called micellar casein (MCC).

The casein micelle structure is generally stable to heating, though the disassociation of κ-casein from the micelle has been observed depending on pH at heating (Dalgleish and Corredig, 2012). When whey proteins and casein micelles are heated together, whey proteins can self-aggregate, bind to κ-casein at the surface of casein micelles, or bind with disassociated κ-casein (Singh et al., 2004).

As mentioned previously, consumers desire high levels of protein and favor whey proteins (Oltman et al., 2015). However, both conditions favor increased protein aggregation during thermal processing and decreased physical stability. We hypothesize that destabilization in beverages is based on the primary aggregation occurring during thermal processing (so called thermal stability) and secondary aggregation that takes place over storage. A method was developed to characterize thermal stability and follow physical destabilization over time. The

method was tested on beverages made with ingredients containing different levels of casein and whey proteins and at two protein concentrations to make it relevant to consumer interests.

3. MATERIALS AND METHODS

3.1. Ingredients

Micellar casein, milk protein concentrate, and all beverages were manufactured by the Drake lab for an investigation lead by Professor MaryAnne Drake. Some assistance was provided during beverage bottling, but the majority of the work was done by members of the Drake lab. Details are provided for each process.

3.1.1. Micellar casein

Micellar casein (MCC) was manufactured in the NC State Feldmeier Dairy Processing Lab according to methods from Cheng et al., (2018). Briefly, raw bovine skim milk from NC State Lake Wheeler Dairy Farm – Teaching and Research Unit was High Temperature Short Time (HTST) pasteurized at 72°C with a hold time of 16 s, then cooled to 4 °C and prefiltered using a Nexus T Filter (NXT 10-30U-M7S, Pall Corporation, Port Washington, NY). The pasteurized and pre-filtered skim milk was heated to 50°C and a 3-stage, 3× microfiltration process described by Zulewska and Barbano, (2014) was used to produce a 95% serum-protein-removed MCC with total protein concentration between 8.4 to 8.6%. Liquid MCC was stored at 4 °C in 1.89-liter light shielding milk jugs (Upstate Niagara Cooperative, Buffalo, NY). The MCC contained 11.95% protein, 0.57% fat, and 0.41% lactose based on Fourier-transform infrared spectroscopy (van de Voort, 1992).

3.1.2. Milk protein concentrate

Milk protein concentrate (MPC) was made in the NC State Feldmeier Dairy Processing Lab following the methods of Carter et al. (2018). Briefly, raw bovine skim milk from NC State Lake Wheeler Dairy Farm – Teaching and Research Unit was weighed and pasteurized as described previously for MCC. The pasteurized skim milk was heated with a plate heat exchanger to 50°C and concentrated via ultra-filtration. The ultra-filtration resulted in a concentration factor of 5× and 74% total protein solids (wt/vol). Diafiltration water was added to return the concentrated protein to return to the starting weight. This was followed by a concentration step and the resulting MPC was 15.15% protein, 0.25% fat, and 0.34% lactose based on Fourier-transform infrared spectroscopy (van de Voort, 1992).

3.1.3. Serum protein isolate

Frozen serum protein isolate (SPI) was purchased from South Dakota State University. It was stored at -20 °C and thawed at 4 °C in a walk-in cooler for 3 days until use in beverage preparation. The SPI consisted of 22.29% protein, 0.15% fat, and 0.52% lactose based on Fourier-transform infrared spectroscopy (van de Voort, 1992).

3.1.4. Additional ingredients and beverage formulations

Beverages were prepared with milk protein ingredients combined with water, 0.015% (wt/wt) carrageenan (Ticaloid 780, TIC Gums, Belcamp, MD, USA), 0.4% (wt/wt) cellulose gel (Ticaloid Pro HC 988, TIC Gums, Belcamp, MD, USA), 0.03% (wt/wt) sucralose (100% Sucralose Powder, Sweet Solutions LLC, Plainfield, IL, USA), 0.15% (wt/wt) dipotassium phosphate (Consolidated Chemical, Quakertown, PA, USA), and 0.5% (wt/wt) vanilla flavoring (Glanbia Nutritionals, Carlsbad, CA, USA). Alpha – monohydrate lactose (Hilmar 5120 Refined

Edible Lactose 200 mesh, 25 kg/bag, Hilmar Ingredients, Hilmar, CA, USA) and cream (NC State University Dairy Enterprise System, Raleigh, NC, USA) were used to standardize the beverages to 0.5% fat and 0.7% lactose. The protein content of each formulation can be seen in Table 1.

3.2. Beverage preparation

All ingredients were blended together, preheated to 65°C, and homogenized at 3000 PSI, followed by homogenization at 500 PSI, and cooled to 8°C. A MicroThermics unit (MicroThermics Inc., Raleigh, NC) was configured for heating with direct stem injection. Beverages were preheated to 92°C, heated to 141°C, and held for 2.5 s, followed by vacuum cooling to 92°C and two-stage homogenization: 3000 PSI for the first stage and 500 PSI for the second stage. Beverages were cooled to 8°C and filled into 250 mL sanitized plastic bottles (Upstate Niagara Cooperative, Buffalo, NY) using a laminar flow hood. Samples were stored at 4°C in a light protected environment. The entire experiment was replicated twice.

3.3. Sampling

Beverages were sampled at 0, 2, 4, 6, and 8 weeks. Bottles were randomly selected from 4°C storage, shaken to disperse any settling, and 45 mL poured into sterile 50 mL centrifuge tubes for analysis.

3.4. Particle size analysis

Size of the dispersed particles was measured by laser diffraction at a wavelength of 632.8 nm using a Malvern Mastersizer 3000 laser diffraction particle size analyzer (Malvern Instruments Limited, Worcestershire, UK) equipped with a Hydro MU sample dispersion unit

stirring at 2400 rpm with deionized water as the dispersion medium. Samples were mixed by ten inversions of centrifuge tubes and added to the dispersion medium dropwise to an obscuration rate of 7% ± 0.5%. Refractive indices for solvent, particle, and adsorption coefficient were set at 1.33, 1.45, and 0.01 respectively following the method of Ji et al., (2015). Quintet measurements were taken per sample. Results were reported as 90^{th} percentile particle size, 50^{th} percentile particle size, and 10^{th} percentile particle size based on the number distribution of particles by diameter.

3.5. Protein aggregate size fractionation

At each sampling time point, a 10 mL aliquot of sample was measured into in a tared 15-mL centrifuge tube and weighed. Solutions were centrifuged at 12,000 x *g* for 20 min at 25 °C. The supernatant (≤ 10 mL) was collected into a tared 15-mL centrifuge tube and the weight of supernatant and precipitate were recorded. The supernatant was labeled as soluble fraction-1 (SF-1). The SF-1 was filtered into through a 5-µm syringe filter (Whatman, GD/X syringe filters PDVF membrane, 25-mm diameter) and filtrate was collected in a tared 15-mL centrifuge tube, weighed, and designated as soluble fraction-2 (SF-2). Soluble fraction-3 (SF-3) was prepared by passing the 5 µm filtrate through a 0.45-µm syringe filter (Whatman, GD/X syringe filters PDVF membrane, 0.45 -µm pore size, 25-mm diameter) into a tared 15-mL centrifuge tube. Aliquots of 600 µL were taken of each soluble fraction for measurement of protein content. Each treatment was analyzed in triplicate at each timepoint.

3.6. Mass balance and total nitrogen of proteins

A mass balance of protein was used to follow the amount of protein partitioned into the fractions outlined in section 3.5. The soluble fraction weight and total nitrogen concentration was

used to calculate the mass of protein in each fraction. The data are presented as the percentage of protein in each fraction compared to the percentage of total protein. Total nitrogen of each protein class was determined using the Dumas combustion procedure on an Elementar rapid N-Exceed (Elementar Americas, Inc. Mt. Laurel, NJ). A protein conversion factor of 6.38 from Maubois and Lorient, (2016) was used.

3.7. Size-exclusion high performance liquid chromatography (SE-HPLC)

The filtrate from the 0.45-µm fraction (SF-3) was further analyzed by size-exclusion chromatography. Samples were separated on a Waters system (Waters Corporation, Milford, MA) using Yarra™ 3-µm SEC-2000 and Yarra™ 3-µm SEC-4000 LC (Phenomenex, Torrance, CA) columns run in series at 22°C. These columns provided a theoretical separation range of 1 kDa to 1,500 kDa. The columns were eluted with a mobile phase of 100 mM sodium phosphate buffer, pH 6.8, containing 0.025% sodium azide. Each treatment was run as a 20-µL injection with a flow rate of 0.5 mL/min for 75 min. Proteins were followed by measuring the absorbance at 220 nm and 280 nm for detection of peptide bonds and aromatic amino acids, respectively, using a Waters photodiode array detector (PDA) (Waters Corporation, Milford, MA). Retention times were compared to a high molecular weight ladder kit (GE Healthcare, Chicago, IL) with sizes from 44 kDa to 669 kDa. Void volume of the columns was determined by blue dextran 2000 (GE Healthcare, Chicago, IL).

4. RESULTS AND DISCUSSION

4.1. Particle Size

Average particle size of beverages unheated, immediately after thermal processing (0 time), and over 8-weeks of storage at 4 °C are seen in Tables 2.2, 2.3 and 2.4. Particle diameters

are reported as 10th (Dx(10)), 50th (Dx(50)), and 90th (Dx(90)) percentiles to determine the sensitivity of particle classes to changes with storage. A visual representation of these percentiles can be seen in Figure 2.1. The Dx(50), also known as the median, reports the diameter where half of the population lies below the value. The 90th percentile accounts for the particles below 90 percent of the total distribution; similarly, the 10th percentile data shows the maximum size particles in the lowest 10 percent of the total distribution. Unheated data compared to 0-time data shows shifts in particle size as a result of thermal processing. The 0-time values represent beverage thermal stability while changes over time indicate secondary aggregation. In all cases, a larger particle diameter is expected to reflect lower stability.

The treatment containing 100% MCC 10.5% (wt/wt) gelled at 4, 6, and 8 weeks and therefore measurements were not possible.

The Dx(90) values generally show a large increase on the order of one magnitude in particle diameter from unheated beverage to heated at time 0, with the exception of 100% MPC at both concentrations, and 50% MCC 50% WPI at both concentrations. This indicates that the thermal process is causing aggregation. The Dx(90) values did not show consistent temporal trends of particle size increasing or decreasing (Table 2.2); however, differences were seen among the ingredients and protein concentrations. Beverages containing mainly casein (MCC, MPC, and 18% SPI with MCC) did not show a difference between protein concentrations. Those made with 50% SPI containing 10.5% (wt/wt) protein had average particle sizes about two to three times greater than beverages containing 6.3% (wt/wt) protein. Overall, the beverages at 10.5% (wt/wt) containing 50% SPI were the lowest in heat stability. The general trend of major differences between *high-casein* beverages (MCC, MPC, and 18% SPI with MCC) and *high-whey protein* beverages (50% MCC + 50% SPI or 50% MPC + 50% SPI) were seen in other properties and those terms will be used in referring to the groups of beverages.

The Dx(50) unheated values were not significantly larger than the 0-time values for *high-casein* beverages, but the *high-whey* beverages did have an increase on the order of one magnitude at 6.3% (wt/wt) and two orders of magnitude at 10.5% (wt/wt). This increase indicates that the whey and casein may be forming a complex as has been seen in literature (Vasbinder and De Kruif, 2003). Like the Dx(90) values, there were not consistent temporal trends observed for Dx(50) values for *high-casein* beverages seen in Table 2.3. As was observed for Dx(90), beverages containing mainly casein did not show a difference between protein concentrations, but *high-whey protein* beverages had larger particles in the 10.5% (wt/wt) protein, two to five times in size compared to particles in beverages containing 6.3% (wt/wt) protein. What was different about the Dx(50) values is that both high and low concentrations of 50% MCC + 50% SPI and 50% MPC + 50% SPI showed an increase in the 50^{th} percentile over the 8 weeks, indicating secondary aggregation may be detected by this measurement. The Dx(10) values and trends were all similar to Dx(50) values, seen in Table 2.4. A marked difference is seen in *high-whey protein* beverages samples, indicating that even in the smallest percentile particle size, large aggregates are seen.

Overall, particle size was better at differentiating beverages according to their thermal stability than showing changes associated with shelf life. The inherent problem with particle size distribution measurement based on laser diffraction is that it does not account for the mass fraction of particles, only distributions, therefore a protein mass balance was evaluated.

4.2. Protein Mass Balance

The fraction of protein in each soluble fraction over storage is reported in Appendices 2.2 and 2.3. This information accounts for all protein in the system and is the only method that

showed the solubility of the protein at each fractionation timepoint. No temporal trends were observed, therefore the values were averaged over time and reported in Figures 2.3 and 2.4.

The general trends observed in particle size analysis were also seen in soluble protein fractions, but the amount of soluble protein provided some more information. Beverages containing 6.3% (wt/wt) protein (Figure 2.3) appeared to follow a similar pattern to the particle size data, the *high-casein* beverages all had similar trends. For all of the high casein treatments, the SF-1 recovery was above 80 percent, SF-2 was ~80 percent recovery, and SF-3 was ~50 percent recovery.

High-whey protein beverages at 6.3% (wt/wt) protein had SF-1 levels of ~60 percent recovery, SF-2 indicated a ~50 percent recovery. The SF-3 fraction was ~40 percent of the total protein in the treatment, 10 percent lower than recovery seen for *high-casein* beverages.

The soluble fraction protein recovery for beverages containing 10.5% (wt/wt) protein (Figure 2.4) had relative trends similar to those for the 6.3% (wt/wt) protein concentration (Figure 2.3); however, all values were lower. While unambiguous effects were not established based on particle size, the soluble fractions clearly showed larger amounts of soluble protein in *high-casein* beverages over soluble protein in *high-whey protein* beverages, but a decrease in soluble protein was seen in both classifications when protein concentration was increased. This is consistent with research done showing the thermal stability of casein micelles and the thermal instability of whey proteins (de Wit, 2009; Dalgleish and Corredig, 2012; Wijayanti et al., 2014).

4.3. Size-exclusion high performance liquid chromatography

Proteins in SF-3 were further fractionated using size-exclusion chromatography. It should be noted that this represented 26.8 to 56.7% of the total protein in the beverage (Appendix 1). A

typical chromatogram can be seen in Figure 2.2 of 100% micellar casein, 50% milk protein concentrate + 50% serum protein isolate, and molecular weight markers.

No data was collected for 100% MCC at 10.5% (wt/wt) because it was too viscous to be filtered and loaded onto the HPLC.

The molecular weight range of 669-44 kDa should reflect changes in aggregation at the smallest scale measured in this investigation (Figure 2.5). *High-casein* beverages had similar trends with a slight decrease or no changes seen in the 669-44 kDa fraction over the 8 weeks. Since this was an intermediate fraction, the change over time reflected particle growth from those < 44 kDa moving into the fraction and those > 669 kDa moving out of the fraction. The overall shift to larger particles was confirmed in Figure 2.6 by a general increase in particles > 669 kDa fraction increasing for *high-casein* beverages. Considering that the data represents sampling different beverage containers for each time point in duplicate, the trends observed in Figure 2.6 are a reasonable indication of secondary aggregation occurring during storage.

The *high-whey protein* beverages were predominately in the >669 kDa size fraction at time 0 immediately after thermal processing, seen in Figure 2.6. Little change is observed in either the 10.5 or the 6.3% (wt/wt) concentrations of 50% SPI treatments, indicative that the particles were too large to be characterized by this method.

5. CONCLUSIONS

Based on particle size analysis and soluble protein fraction, beverages made with 100% MCC, 100% MPC, and 82% MCC + 18% SPI were the most thermally stable and an increase in protein concentration decreased thermal stability. However, these methods showed no indication of secondary aggregation. Changes reflecting secondary aggregation were seen by changes measured by size-exclusion chromatography.

In contrast, the four beverages containing serum protein at 50% were more prone to aggregation based on particle size and soluble protein than samples containing 18% or less. Size exclusion chromatography of these whey-enriched beverages showed no changes in secondary aggregation because the soluble particles were in the exclusion volume at time zero. Particle size analysis by laser diffraction showed general trends of increase in particle size over time.

The approach of multiple methods was needed to evaluate thermal stability and secondary aggregation. The casein-dominant beverages had less extensive secondary aggregation not detected by laser diffraction particle size analysis, but secondary aggregation was observed in the 669-44 kDa size range the SEC-HPLC. In contrast, beverages with higher levels of whey proteins showed secondary aggregation via laser diffraction particle size analysis but not by SEC-HPLC, as all protein was already in the exclusion volume after thermal processing. This showed the importance of the utilization of methods with differing levels of sensitivity to characterize particle changes in beverages.

6. FIGURES

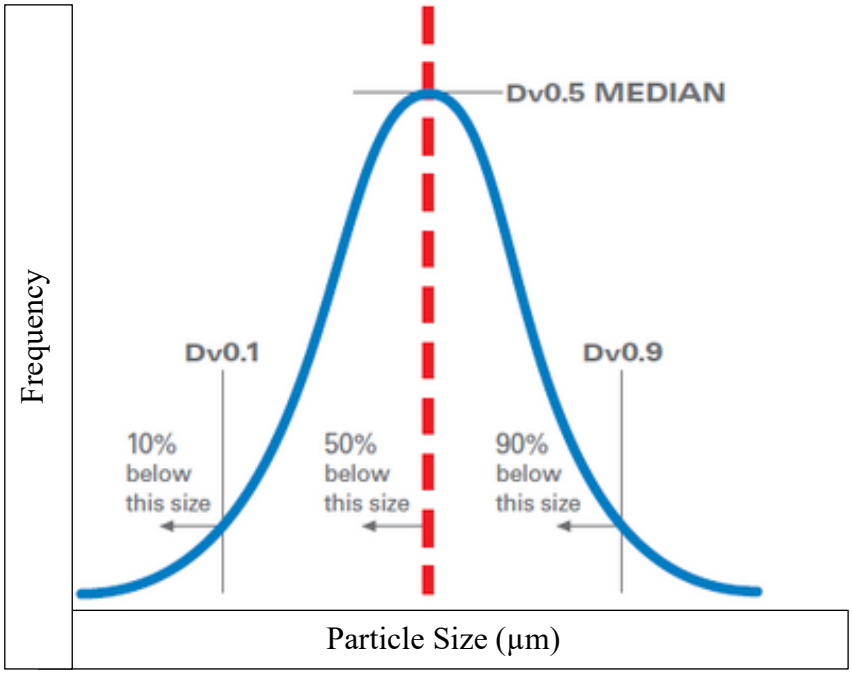

Figure 2.1. Distribution curve illustration the values of Dx(10), Dx(50) and Dx(90) represented on the distribution curve. Figure adapted from Understanding Particle Size Distribution Calculations - HORIBA

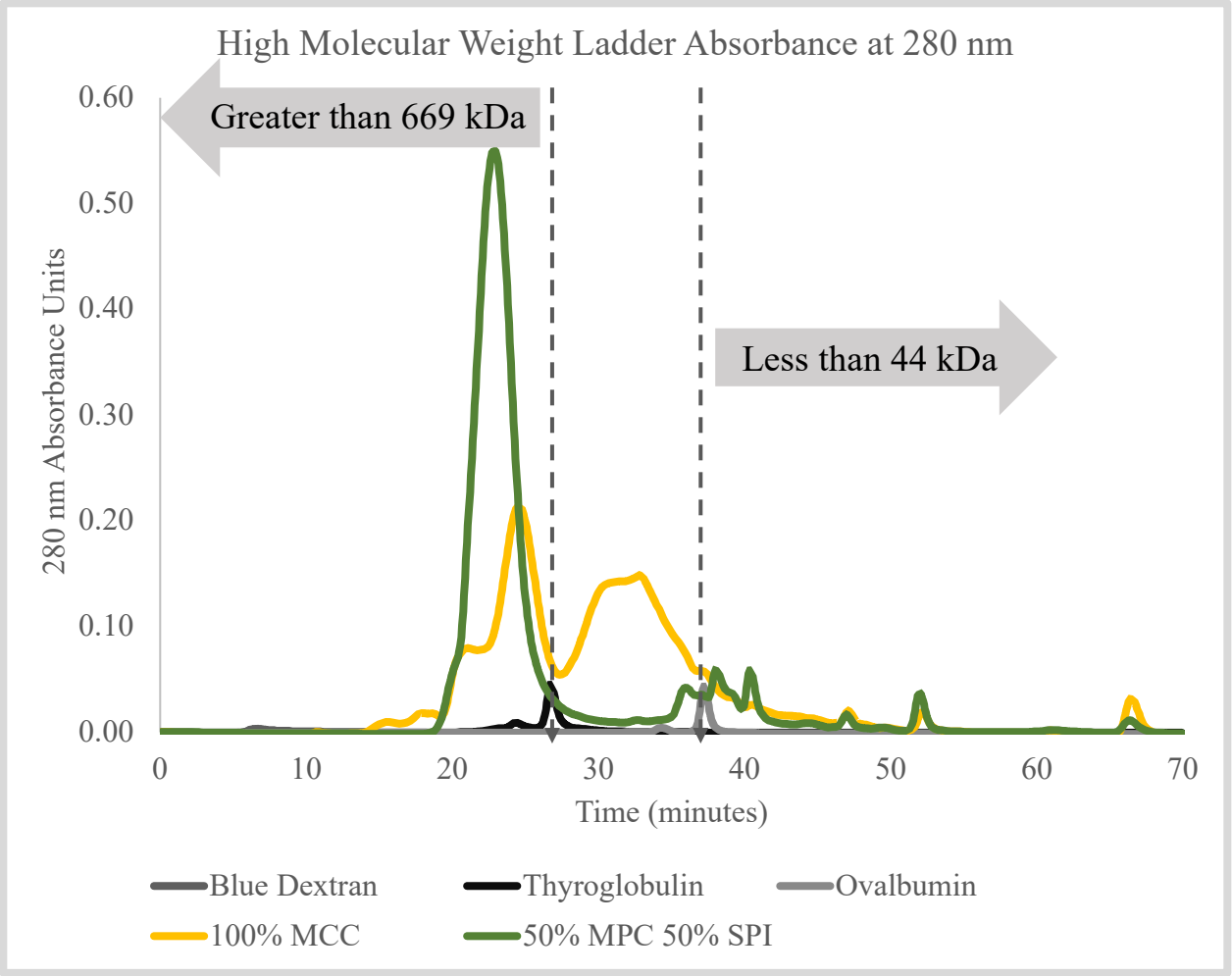

Figure 2.2. Example chromatogram showing the separation of protein aggregates.

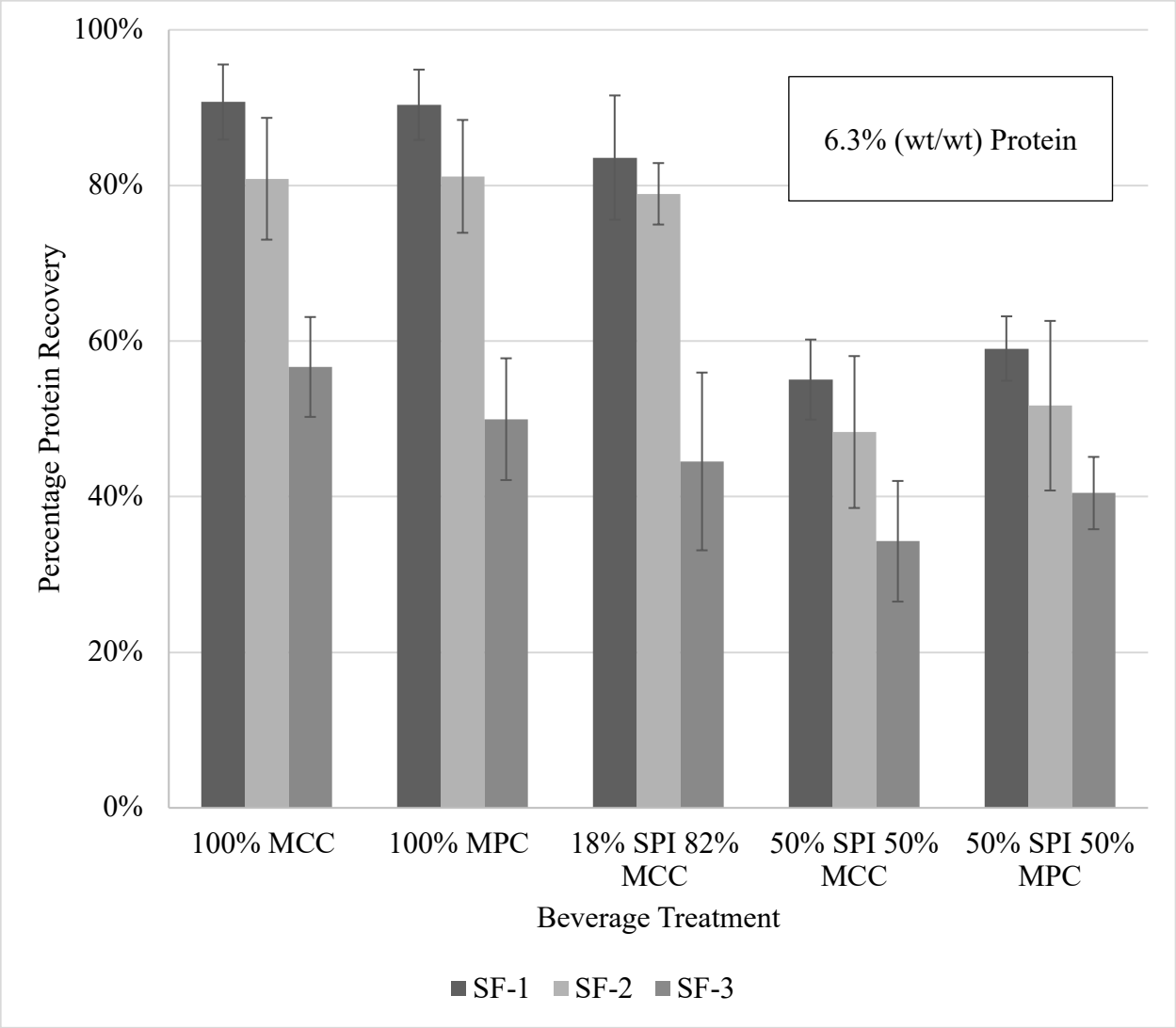

Figure 2.3. Protein recovered from beverages containing 6.3% (wt/wt) protein.

Each bar graph is a minimum of n=5 measurements per timepoint, with a total of five timepoints. The entire experiment was performed in duplicate. SF-1 SF-2 and SF-3 represent the three soluble fractions. The full data set can be seen in Appendix 2.

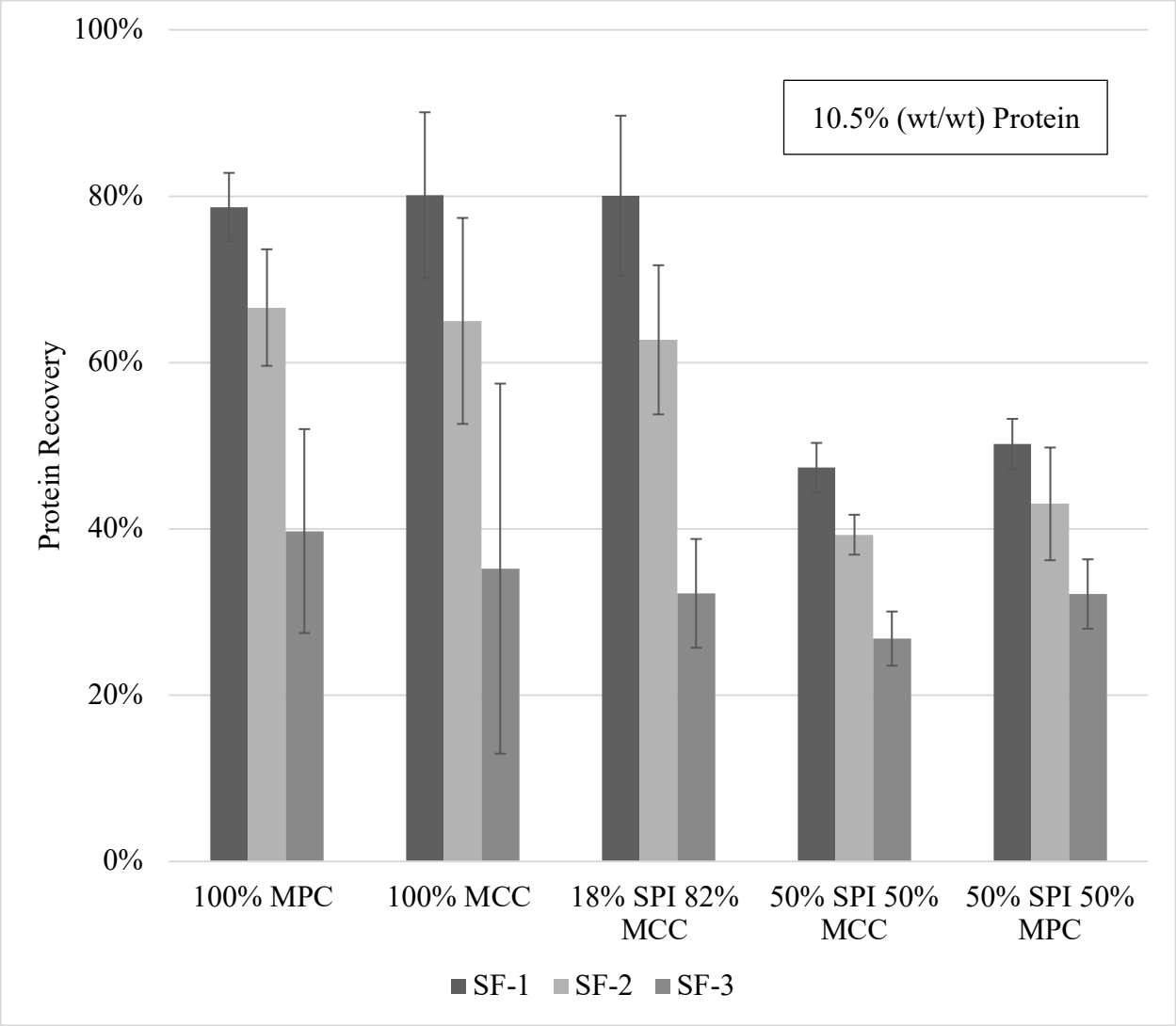

Figure 2.4. Protein recovered from beverages containing 10.5% (wt/wt) protein.

Each bar graph is a minimum of n=5 measurements per timepoint, with a total of five timepoints. The entire experiment was performed in duplicate. SF-1 SF-2 and SF-3 represent the three soluble fractions. The full data set can be seen in Appendix.3.

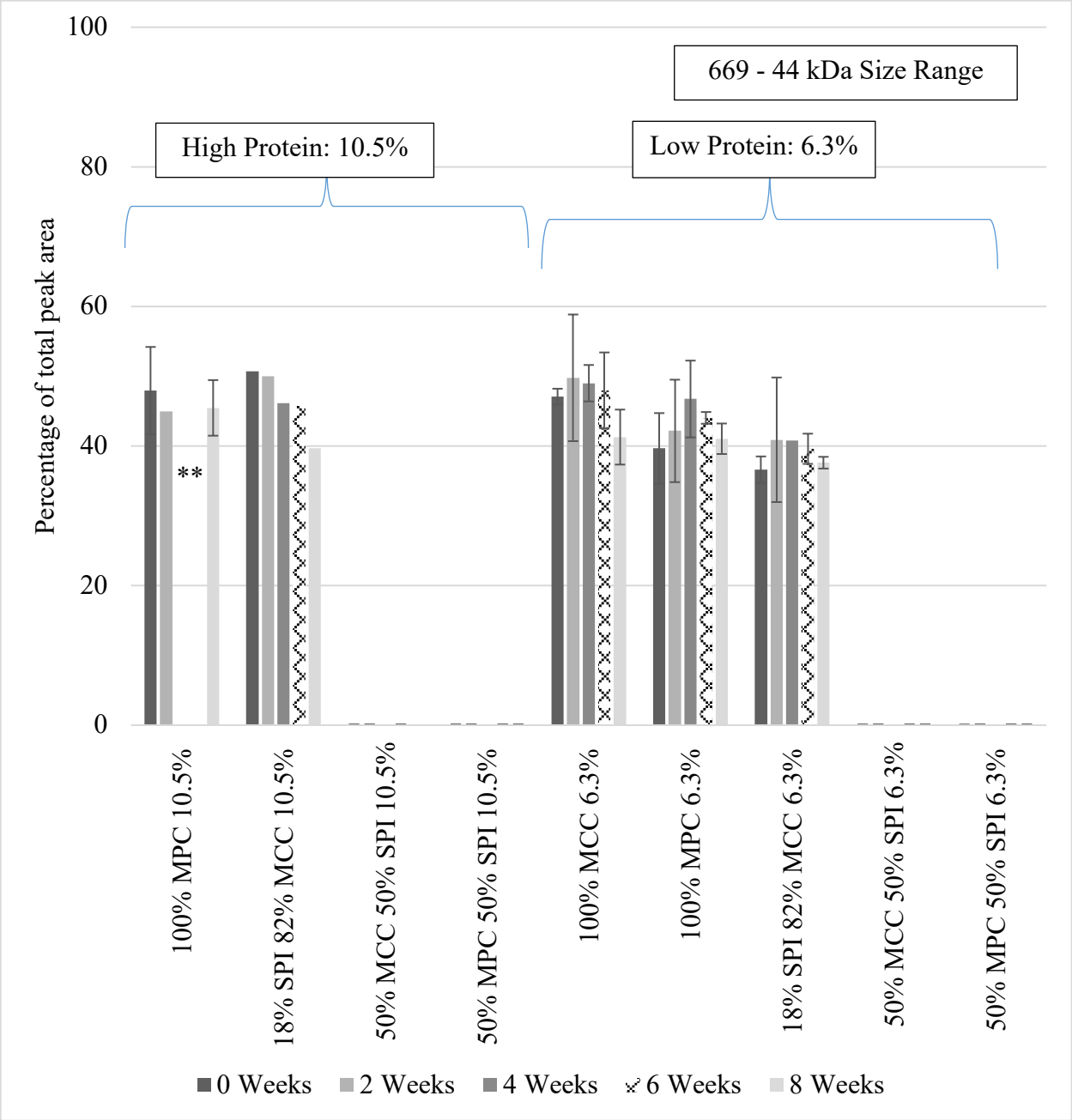

Figure 2.5. Changes in the 669-44kDa size class from size exclusion chromatography. Treatments are defined according to Table 2.1. Data from two replicates. *Denotes weeks with no data collected, namely MPC 100% 10.5% protein weeks 4 and 6.

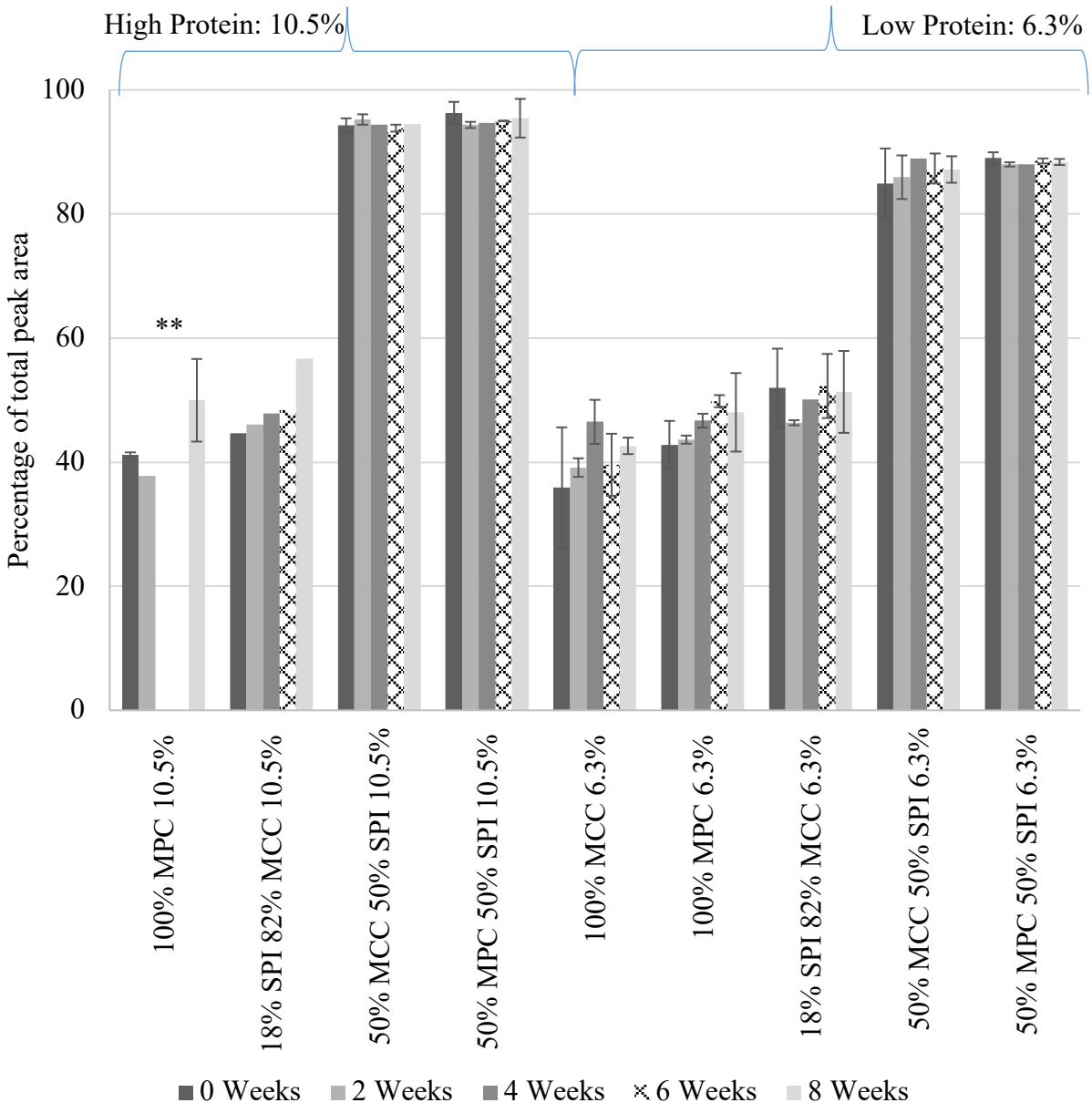

Figure 2.6. Changes in percentage of total peak area for >669kDa size class from size exclusion chromatography. Treatments according to Table 2.1 Data from two replicates. *denotes weeks with no data collected, namely MPC 100% 10.5% protein weeks 4 and 6.

7. TABLES

Table 2.1. Protein ingredient composition and protein concentration of beverages.

Treatment	Protein Source (Percentage)		
	SPI	MPC	MCC
100% MCC 10.5% (wt/wt)	0	0	100
100% MCC 6.3% (wt/wt)	0	0	100
100% SPI 10.5% (wt/wt)	100	0	0
100% SPI 6.3% (wt/wt)	100	0	0
100% MPC 10.5% (wt/wt)	0	100	0
100% MPC 6.3% (wt/wt)	0	100	0
82%/18% MCC/SPI 10.5% (wt/wt)	18	0	82
82%/18% MCC/SPI 6.3% (wt/wt)	18	0	82
50%/50% MCC/MPC 10.5% (wt/wt)	0	50	50
50%/50% MCC/SPI 6.3% (wt/wt)	0	50	50

Table 2.2. Particle diameter (μm) of beverages reported as Dx (90)

		Time (weeks)				
Beverage Treatment	Unheated	0	2	4	6	8
100% MCC 6.3%	2.28 ± 0.21	46.7 ± 34.8	7.9 ± 2.9	6.7 ± 1.9	13.4 ± 4.9	8.9 ± 1.9
100% MCC 10.5%	*	21.0 ± 26.4	0.6 ± 0.1	*	*	*
100% MPC 6.3%	7.70 ± 0.09	9.5 ± 3.5	10.2 ± 3.1	9.6 ± 1.1	12.4 ± 4.7	8.6 ± 3.6
100% MPC 10.5%	2.91 ± 0.12	0.6 ± 0.1	2.4 ± 1.6	2.5 ± 0.3	2.5 ± 2.0	23.5 ± 18.5
18% SPI 82% MCC 6.3%	6.01 ± 0.2	31.6 ± 19.5	10.1 ± 2.6	7.5 ± 0.8	13.7 ± 5.3	11.9 ± 4.1
18% SPI 82% MCC 10.5%	5.18 ± 2.7	42.9 ± 32.0	23.4 ± 34.6	4.2 ± 0.2	3.5 ± 3.2	8.5 ± 1.4
50% MCC 50% SPI 6.3%	43.66 ± 3.28	45.0 ± 28.5	23.45 ± 6.2	29.5 ± 12.5	51.0 ± 22.4	30.0 ± 10.0
50% MCC 50% SPI 10.5%	74.56 ± 7.85	50.4 ± 9.4	57.31 ± 15.2	71.9 ± 2.6	169.3 ± 52.0	87.7 ± 1.2
50% MPC 50% SPI 6.3%	5.19 ± 0.33	14.4 ± 6.2	13.2 ± 1.2	38.0 ± 34.2	28.9 ± 6.7	25.0 ± 2.4
50% MPC 50% SPI 10.5%	1.85 ±0.26	59.7 ± 42.2	85.8 ± 41.6	68.2 ± 32.1	88.0 ± 51.9	85.5 ± 43.7

The Dx(90) is the 90th percentile data indicating that 90% of the particles are below this value (see Figure 1).

* Denotes no data because sample cohesiveness was too high for proper dispersion in the mastersizer unit.

Table 2.3. Particle diameter (μm) of beverages reported as the Dx (50)

		Time (weeks)				
Beverage Treatment	Unheated	0	2	4	6	8
100% MCC 6.3%	0.32 ± 0	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.0	2.2 ±1.7	0.3 ± 0.2
100% MCC 10.5%	*	0.4 ± 0.2	0.4 ± 0.1	*	*	*
100% MPC 6.3%	0.50 ± 0.0	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.0	2.0 ± 2.0	0.4 ± 0.2
100% MPC 10.5%	0.36 ± 0.01	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.2 ± 0.2
18% SPI 82% MCC 6.3%	0.36 ± 0.0	0.4 ± 0.2	0.5 ± 0.1	0.3 ± 0.0	2.2 ± 1.9	0.4 ± 0.2
18% SPI 82% MCC 10.5%	0.29 ± 0.01	0.5 ± 0.3	0.7 ± 0.8	0.4 ± 0.0	0.5 ± 0.2	0.5 ± 0.1
50% MCC 50% SPI 6.3%	0.67 ± 0.0	6.3 ± 4.0	11.0 ± 3.9	12.0 ± 2.3	14.1 ± 3.5	14.5 ± 4.3
50% MCC 50% SPI 10.5%	0.49 ± 0.01	18.9 ± 7.7	24.3 ± 3.4	32.6 ± 1.0	73.4 ± 20.6	40.2 ± 0.4
50% MPC 50% SPI 6.3%	0.27 ± 0.0	5.8 ± 3.8	7.3 ± 0.7	9.2 ± 1.7	10.7 ± 1.6	10.1 ± 1.0
50% MPC 50% SPI 10.5%	0.38 ± 0.01	28.1 ± 18.4	42.7 ± 22.1	32.4 ± 13.3	46.8 ± 28.6	35.5 ± 16.3

The Dx(50) is the 50th percentile data indicating that 50% of the particles are below this value (see Figure 2.1). *Denotes no data because sample cohesiveness was too high for proper dispersion in the mastersizer unit.

Table 2.4. Particle diameter (μm) of beverages reported as the Dx (10)

		Time (weeks)				
Beverage Treatment	Unheated	0	2	4	6	8
100% MCC 6.3%	0.13 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
100% MCC 10.5%	*	0.2 ± 0.1	0.2 ± 0.1	*	*	*
100% MPC 6.3%	0.22 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.2	0.2 ± 0.1
100% MPC 10.5%	0.15 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.1
18% SPI 82% MCC 6.3%	0.14 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.2	0.2 ± 0.1
18% SPI 82% MCC 10.5%	0.11 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
50% MCC 50% SPI 6.3%	0.23 ± 0.0	1.7 ± 1.8	5.5 ± 2.3	6.1 ± 0.9	6.0 ± 0.9	7.0 ± 1.7
50% MCC 50% SPI 10.5%	0.14 ± 0.0	7.6 ± 2.2	9.7 ± 0.4	11.5 ±1.1	18.3 ± 3.5	12.5 ± 0.1
50% MPC 50% SPI 6.3%	0.1 ± 0.0	2.8 ± 1.9	3.3 ± 1.3	4.4 ± 0.3	4.4 ± 0.5	4.4 ± 0.4
50% MPC 50% SPI 10.5%	0.18 ± 0.0	9.7 ± 5.0	15.8 ± 6.3	12.8 ± 3.5	18.7 ± 10.6	13.0 ± 4.9

The Dx(10) is the 10^{th} percentile data indicating that 10% of the particles are below this value (see Figure 2.1). *No data because sample viscosity was too high for dispersion in the mastersizer unit.

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CHAPTER 3. INDICATORS OF PHYSICAL STABILITY IN RETORT PROCESSED BEVERAGES

1. ABSTRACT

Thermal processing of beverages containing protein is a challenge because the resulting protein aggregation can cause undesirable effects. Extensive aggregation during processing can prevent formation of a dispersed sol (low thermal stability) or settling or aggregation over time can cause phase separation, sedimentation, or gelation resulting in a termination of shelf life. This investigation examined thermal and shelf stability of retort processed dairy protein ingredient solutions at concentrations relevant to beverage applications. Treatments of 100% milk protein concentrate and a blend of 50% whey protein isolate and 50% milk protein concentrate were formulated at protein concentrations 6% and 11% (wt/wt), adjusted to pH 6.8, and retort processed (121°C for 20 min). Physical stability was characterized based on laser diffraction-based particle size analysis, size-exclusion chromatography (SEC), and degree of light backscattering measured along the height of a quiescent sample (Z-dimension light backscattering; Turbiscan instrument). Measurements were made immediately after processing and over eight weeks. Changes in amount of Z-dimension light backscattering produced two patterns. One pattern reflected a general top to bottom settling while the second showed a shift to overall coarsening over time. The latter was associated with a high level of viscosity. Changes in chromatograms were indicative of secondary aggregation. Particle size measurements only provided information regarding thermal stability. The combination of techniques was able to show differences in physical properties related to thermal and shelf stability.

2. INTRODUCTION

Beverages designed to be a good dietary source of protein include protein-fortified smoothies, meal replacement drinks, and pre-and-post workout beverages, all of which fall under the umbrella of a functional food (products that provide health benefits beyond nutritional value). It is projected that the market for functional foods will grow, with the category of sports and protein drinks reaching about $8.5 billion by 2020 (Beverage Industry, 2017). While there is no current legal standard for protein content in these beverages, Oltman et al., (2015) showed that consumers desire ≥ 20 grams of protein per serving and have a preference for whey as the primary protein source. A 20-gram serving requires a protein concentration of ≥ 5.6% in a 355 ml (12 fl. oz) serving or ≥ 8.4% in a 237 ml (8 fl. oz) serving.

As mentioned in Chapters 1 and 2, protein beverages are a colloidal sol and physical stability can be defined based on a formulation remaining a sol after thermal processing (thermal stability) and during storage (shelf stability). Destabilization takes the form of the dispersed phase either settling to an apparent precipitate phase, polymer-polymer phase separation, or gelation (Wagoner et al., 2015). There is a desire to have methods that can be used to characterize thermal stability and predict shelf stability.

In Chapter 2, a set of ultra-high temperature processed protein beverages were analyzed for thermal stability and secondary aggregation (aggregation occurring post-thermal processing). In theory, knowledge of thermal stability and the potential for secondary aggregation could be used to predict shelf stability. It was seen that high-casein beverages were the most thermally stable, as shown by particle size and amount of soluble protein. Changes reflecting secondary aggregation were shown by size exclusion chromatography. Increasing the amount of whey proteins decreased thermally stability and temporal increases in particle size were indicative of lower shelf stability. Size-exclusion chromatography was able to detect secondary aggregation in

high-casein beverages but at time zero the soluble protein in the high-whey beverages was all in the exclusion volume (> 669 kDa). Chapter 2 demonstrated the need for multiple methods for measuring a broad range of aggregate size and temporal changes in size to understand thermal and shelf stability.

For this study, protein solutions were used instead of a beverage formulation containing additional ingredients including polysaccharides. This allowed for analyzing the protein ingredients without the confounding effects of other compounds. In chapter 2, beverages were required to be stored at 4°C because processing was done via ultra-high temperature under semi-aseptic conditions. Retort processing, a more extreme heating method, was chosen for this investigation to create harsher conditions for protein aggregation and produce commercial sterilization. While casein-based ingredients are known to be more heat stable, consumers desire whey proteins (Oltman et al., 2015), therefore, milk protein concentrate (MPC) and a combination of MPC and whey protein isolate (WPI) were investigated.

Something not measured in chapter 2 was a measurement of settling behavior. A method was added to this experiment to measure settling over time as indicated by z-directional turbidity (backscattering). Protein solutions were processed in the tubes used for the instrument to allow for quiescent samples to be moved from storage to analysis without disturbing the structure.

3. MATERIAL AND METHODS

3.1. Protein ingredients

Protein ingredients used were whey protein isolate (WPI) (BiPRO, Agropur, Longueuil, Canada) and milk protein concentrate (MPC 85) (Idaho Milk Products, Jerome, ID) at respective protein concentrations of 93.8% and 82.5%. Protein content of ingredients was determined in triplicate using the Dumas method for total nitrogen and an Elementar N-Exceed instrument

(Elementar Americas, Inc. Mt. Laurel, NJ). A conversion factor of 6.38 was used to convert nitrogen content to protein. Mineral composition was determined by inductively coupled plasma atomic emission spectroscopy by the Environmental and Agricultural Testing Service laboratory, Department of Crop and Soil Sciences, at NC State University. Mineral composition of ingredients is seen in Appendix 4.

3.2. Protein solutions

Solution of 11% (wt/wt) and 6% (wt/wt) protein were chosen based on the study in Chapter 2 that showed the 6% (wt/wt) to be relatively stable and 11% (wt/wt) on the edge of stability and instability. These values were chosen because consumers desire ≥ 20 grams of protein per serving, requiring a protein concentration of ≥ 5.6% in a 355 ml (12 fl oz) serving or ≥ 8.4% in a 237 ml (8 fl. oz) serving. Stock solutions of 11% (wt/wt) protein were prepared. Whey protein isolate solution was made by mixing the powder and 80% of the total deionized water at a speed of 400 rpm for 3 h at 22°C and holding at 4°C overnight for full hydration. The following day, the solution was stirred at 400 rpm and pH was adjusted to 6.8 with 1 N NaOH or 1 N HCl and the final 20% of water was added by weight for an 11% (wt/wt) solution. Half of the solution was further diluted with deionized water to 6% (wt/wt) protein solution.

Milk protein concentrate was hydrated by adding MPC 85 in 100-g increments into deionized water in equilibrated to 55°C with a water bath while mixing with an overhead stirrer (Heidolph RZR 2021, Schwabach, Germany) at 400 rpm for 1 h. Once the powder appeared fully dispersed, it was further mixed for 2.5 h and stored at 4°C overnight for full hydration. The following day, the solution was mixed on a stir plate at 400 rpm and pH was adjusted to 6.8 with 1 N NaOH or 1 N HCl. Deionized water was added to create an 11% (wt/wt) solution. Half of

the solution was diluted to 6% (wt/wt) protein. A 50:50 mixture of WPI:MPC85 at 6% and 11% (wt/wt) protein was made by mixing equal parts of the solutions.

3.3. Retort processing

Fifteen-mL aliquots of protein solutions were placed in loosely capped autoclavable glass vials (Formulaction, Toulouse, France) that were tightened after heating and cooling. The vials were equilibrated to 22°C from 4°C storage. Thermal treatment was done using an autoclave (AMSCO Gravity Sterilizer, 3021-S, Steris Healthcare, Mentor, Ohio, USA) following the thermal processing schedule listed in Table 3.4.

The sterilization process was validated using BT Sure™ Biological indicators (Thermo Fischer Scientific, Waltham, MA). Samples were divided into two batches to accommodate the required number of samples. Between thermal processes a fan was used to cool the chamber to the starting temperature of 45°C, which required approximately 20 mins. Sample placement within the autoclave was determined using a random number generator in Excel (Microsoft Corporation, Albuquerque, NM). A map of positions in the autoclave can be seen in Appendix 5. Following heating, samples were cooled to room temperature, caps were tightened, and stored in an incubator at 25°C.

To determine moisture lost during heating, weight of heated and unheated sample was recorded and an average of ~2 g of water was lost during heating. Each heating batch was considered to be one replicate and was repeated for a total of two replicates, minimizing the variable of protein lots and mixing.

3.4. Z-Scanned back scattering measurement

The Turbiscan instrument (Formulaction, Toulouse, France) operates by shining a light at 880 nm on the sample and measuring the transmitted and backscattered light. The measurement is made from the bottom to top of the tube, thereby providing a Z-resolved pattern of transmitted and backscattered light. Twice a week, undisturbed vials were scanned on a Turbiscan LAB (Formulaction, Toulouse, France). Samples were gently moved from storage into the instrument such that the fluid remained undisturbed. A total of 10 scans were taken along the height of the tube over a period of 5 min. Measurements were taken at 25°C and reported as change in backscattering along the height of the tube. An additional 21 days of scanning was conducted on the Turbiscan.

3.5. Particle size analysis

Particle size measurement of dispersed particles was measured by laser diffraction at 632.8 nm using a Malvern Mastersizer 3000 (Malvern Instruments Limited, Worcestershire, UK). Samples were added to the Hydro MU sample dispersion unit which consisted of an 800 mL beaker containing deionized water at 25°C ± 3°C and stirring at 2400 rpm. Sample was added until an obscuration level of 7% was achieved. Quintet measurements were taken per sample. Refractive index and absorption coefficient for solvent (water) were 1.33 and 0.01 respectively. The refractive indexes for MPC and 50:50 mixture of MPC and WPI were measured according to the method of Saveyn et al., (2002). Protein solutions were prepared at 1%, 0.5%, 0.25% and 0.01% (wt/wt) and their refractive indexes were measured using an Abbe Refractometer (Abbemat 350, Anton Parr, Graz, Austria). The refractive indices were plotted and analyzed by a linear regression and found to have R^2 values of 0.999. Extrapolation of the linear equation to 100% concentration produced refractive indexes of 1.543 for MPC and 1.523 for

50% MPC 50% WPI. Particle sizes in diameter (μm) were reported as 90^{th}, 50^{th}, and 10^{th} percentile particle size based on the number distribution of particles by diameter.

3.6. Soluble protein quantification and aggregation based on size-exclusion chromatography

Samples were passed through a 0.45μm filter (SF-3 from chapter 2) and the total nitrogen content of the filtrate was determined and converted to total protein as described above. Size exclusion chromatography was done using a Waters system (Waters Corporation, Milford, MA) and Yarra™ 3 μm SEC-2000 and Yarra™ 3 μm SEC-4000 LC (Phenomenex, Torrance, CA) columns run in series at 22°C. These provide a theoretical separation range of 1 kDa to 1,500 kDa. The columns were eluted with a mobile phase of 100-mM sodium phosphate pH 6.8, 0.025% sodium azide. Each treatment was run as a 20-μL injection with a flow rate of 0.5 mL/min for 75 min. Data was collected by UV absorbance at 220 nm and 280 nm for detection of peptide bonds and aromatic amino acids, respectively, using a Waters photodiode array detector (PDA) (Waters Corporation, Milford, MA).

4. RESULTS AND DISCUSSION

4.1 Z-Scanned backscattering

All samples appeared homogenous in appearance throughout the 56 days of the experiment. The Z-scanned backscattering results from the Turbiscan LAB show changes occurring that were not detectable by the human eye. The results are first presented as the percent of change in backscattering (ΔBS%) along the height of the tube from the base (0 mm) to the top of the cylinder (~ 28 mm). Each timepoint scan is represented by a colored line that ranges from blue (representing time zero) to red (representing the most recent measurement). These scans are seen in Figures 3.1 a, b, c, and d. By this measurement, the most stable beverage

would be one that has minimal changes in backscattering over time. All samples showed changes over time that allowed for differentiation among the treatments. While the experiment was slotted for 8 weeks (56 days) of study, an added 21 days of scans were conducted on the Turbiscan, evaluating 77 days of settling behavior.

The most stable treatment based on scattering graphs was MPC at 6% (wt/wt) protein (Figure 3.1a). It was not without temporal changes but had the least amount of change among treatments. An increase in backscattering across time is seen from 0 mm to 5 mm at the bottom of the tube, and a slight decrease over time in the 22 to 30 mm range. This indicates settling from the top to the bottom. Temporal changes in the middle of the vial (6 to 22 mm) were slight with decreased backscattering observed, but not greater than 5% from time zero.

In Figure 3.1b, a similar increase at the bottom of the vial was observed for 50% MPC + 50% WPI 6% (wt/wt) protein treatment (Figure 3.1b). However, it differs from 6% (wt/wt) protein MPC by the temporal changes seen in the middle and top of the vial (between 6 and 32 mm), a marked decrease in backscattering is present, around -10% change in backscattering.

Increasing the protein concentration of the 50% MPC + 50% WPI treatment to11% (wt/wt) caused the same increase seen in the bottom of the vial, from 0 mm to 5 mm, but an even more dramatic decrease in backscattering from 5 mm to 32 mm. The increase in protein concentration increased the settling behavior of the protein particles (Figure 3.1c).

The scattering pattern for MPC at 11% (wt/wt) was unique compared to the other treatments (Figure 3.1d), indicating a different mechanism of destabilization. Backscattering is shifted positively across the entire tube, and over each timepoint, an increase of 10% in backscattering is seen from 2 mm to 30 mm. At the most recent scan, 77 days, the trend in backscattering remains. A dip in backscattering is present from 0 mm to 2 mm and from 30 to 32 mm, which may be a false change due to being at the edge of the tube or it may be indicative of

something else occurring, such as water movement. The overall increase would indicate that particle density had an overall increase, especially in the middle of the tube. These samples were extremely viscous.

The instrumental software calculated a Turbiscan Stability Index (TSI) according to equation 3.1. This is presented as an overall evaluation of stability with increases in TSI indicating a decrease in stability.

$$TSI = \frac{\sum_h |scan_i(h) - scan_{i-1}(h)|}{H} \quad \text{(Eq 3.1)}$$

All samples increased in TSI value over time, but the most unstable by this measurement was the 50% WPI 50% MPC at 6% (wt/wt) protein (Figure 3.2a). The most stable treatment according to global TSI was MPC at 6% (wt/wt) protein, which concurs with our assessment from the backscattering pattern. Because MPC at 11% (wt/wt) is a different pattern of instability, the TSI value may not be comparable with the other samples.

Assuming that accumulation of particles in the bottom of the tubes would be a universal measure of destabilization, the TSI values for the bottom region (0 to 6 mm) was calculated (Figure 3.2b). Again, all samples increased in TSI value over time, but this did not show treatment-specific trends.

The middle region of the tube (10 mm to 21 mm) was also analyzed by TSI (Figure 3.2c). The middle region does show transitioning particle behavior. The MPC 6% (wt/wt) treatments had the lowest TSI, indicating greatest stability; the 50% MPC + 50% WPI 6% (wt/wt) was second most stable followed by the 50% MPC + 50% WPI 11% (wt/wt). These values align with the scattering plots observed in Figure 3.1a,b,c, and d. Up until about day 40, the TSI values are distinctive from one another, but beyond day 40, the values begin to converge and become difficult to differentiate.

Backscattering patterns for all samples were plotted together to show differences in treatments (Figure 3.3). The top of the vial (part A, ~25-32 mm) shows the MPC 11% (wt/wt) increased in backscattering, MPC 6% (wt/wt) stayed close to zero change, and both WPI-containing treatments decreased in backscattering. From this portion of the graph, we would assume that MPC 6% (wt/wt) is the most stable, which correlates with the scattering plot for MPC 6% (wt/wt) (Figure 3.1a).

The middle of the vial, Part B of the figure (10-21 mm), has clear treatment differences for changes in backscattering. The MPC 6% (wt/wt) again shows the least amount of change, concurring with the scattering pattern seen in Figure 3.1a. The MPC 11% again shows an increase in backscattering. Both of the WPI-containing treatments had a similar level of decrease in backscattering in this mid-region.

Part C of Figure 3.3 shows the bottom of the vial (0-6.5 mm), which was expected to be a good indicator of stability by settling behavior. Surprisingly, there was no clear pattern that differentiated treatments.

4.2 Particle size analysis

Average particle size immediately after thermal processing (0 time) and over storage at 25 °C are seen in Tables 3.2, 3.3 and 3.4. Particle diameters are reported as 10^{th} (Dx(10)), 50^{th} (Dx(50)), and 90^{th} (Dx(90)) percentiles to determine the sensitivity of particle classes to changes with storage. The Dx(50), also known as the median, reports the diameter where half of the population lies below the value. The 90^{th} percentile accounts for the particles below 90 percent of the total distribution; similarly, the 10^{th} percentile data shows the maximum size particles in the lowest 10 percent of the total distribution. The 0-time values represent beverage thermal stability while changes over time indicate secondary aggregation. In all cases, a larger particle

diameter is expected to reflect lower stability. The Dx(90) values (Table 3.2) are what would be expected for thermal stability of the treatments – smaller particles were observed for MPC and 50% WPI 50% MPC at 6% (wt/wt). However, no consistent temporal changes were observed. Furthermore, Dx(50) and Dx(10) values did not show differences at time zero nor trends over time (Table 3.3 and 3.4).

4.3 Soluble protein quantification and aggregation by SEC

Over the course of analysis, there were no temporal changes observed for the amount of protein that passed through a 0.45-µm filter (seen in Appendix 6), therefore data were averaged over time. The percent soluble protein (corresponding to SF-3 in Chapter 2) was found to be > 80% for all treatments, much higher than what was observed in Chapter 2 (Figure 3.4 and 2.4). There are several possible reasons for this observation. It is important to note that heating caused ~ 2 g of sample loss and with the assumption of equal density, this further concentrated solutions to be at increased protein levels: 6% (wt/wt) protein increased to 6.9% (wt/wt) protein and 11% (wt/wt) protein increased to 12.6% (wt/wt) protein. Even with increased protein content, the recovery values in this study are higher than those seen in Chapter 2. The additional ingredients added to the beverages in Chapter 2 may have caused larger aggregates to form that were sedimented out by centrifugation.

Secondary aggregation was indicated by an increase in the amount of aggregates appearing in the exclusion limit (Figure 3.5) and a coinciding decrease in the 44-669 kDa size fraction (Figure 3.6). Very little protein was seen in the < 44-kDa fraction, with all values less than ten percent of the total peak area and not showing any trends over time. Therefore, it can be concluded that the decrease in the 44-669 kDa size fraction is an indication of secondary aggregation for all samples.

The MPC at 11% (wt/wt) protein was very viscous at time zero and difficult to filter, by week 3 it was too thick to pass through the filter, another sign of extensive aggregation. The 50% MPC 50% WPI treatment had a larger percentage of protein in the > 669 kDa fraction at 11% than at 6% (wt/wt) protein, as would be predicted. SEC appears to be the best indicator of secondary aggregation.

5. CONCLUSIONS

The analysis of undisturbed treatments by Z-scattering showed differences in settling behaviors even though the treatments all appeared visually homogenous. This offers the possibility of following early stages of physical destabilization *in situ* and developing predictive models. Furthermore, two backscattering patterns were observed that suggest sensitivity of this method to different mechanisms of destabilization.

Particle size analysis showed only differences in thermal stability and just in the Dx(90) fraction. No temporal changes were observed. In contrast, the SEC-HPLC data showed temporal changes in particle size that could be used to model secondary aggregation.

Z-scattering showed overall destabilization while analysis of SF-3 by SEC-HPLC was able to detect secondary aggregation. It is hypothesized that physical destabilization patterns both in *thermal stability* and in *shelf life stability* can be predicted based on combinations of these methods.

6. FIGURES

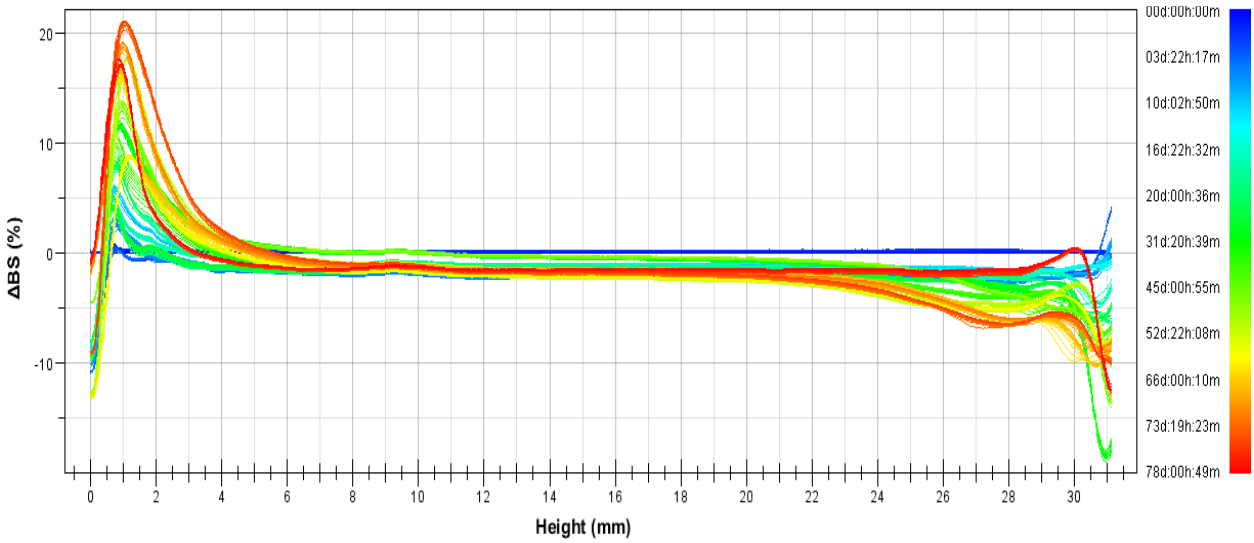

Figure 3.1a. Change in backscattering (ΔBS%) for 100% MPC at 6% (wt/wt) protein. The X-axis denotes the height of the vial from bottom to top in mm. Each line is an individual timepoint scan ranging from time zero (blue) to 77 days (red).

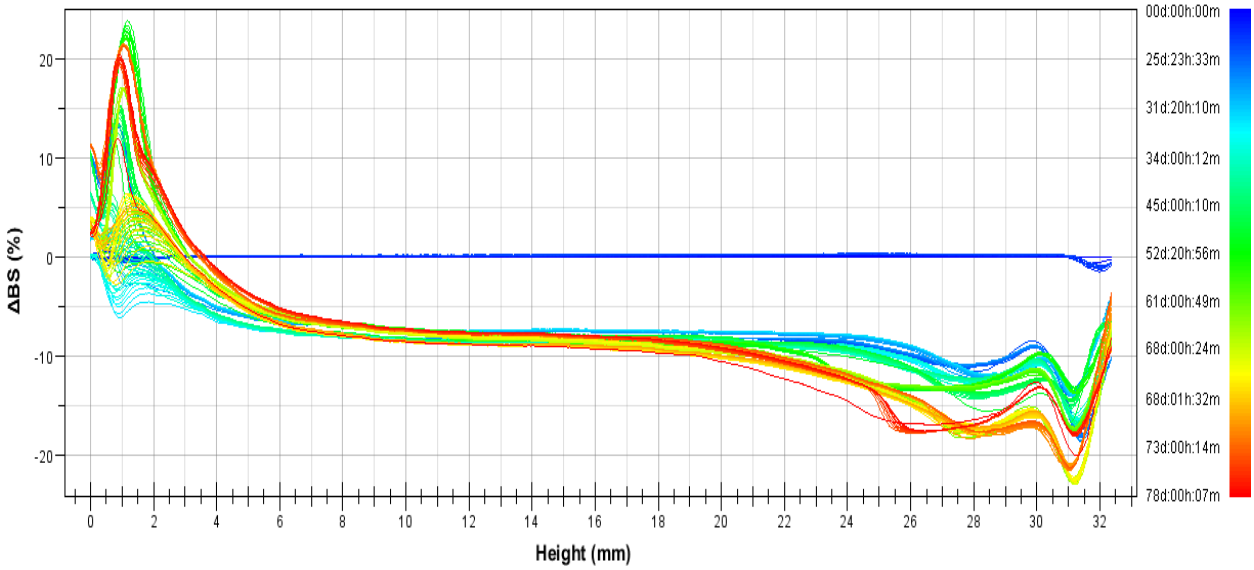

Figure 3.1b. Change in backscattering (ΔBS%) for 50% WPI 50% MPC at 6% (wt/wt) protein. The X-axis denotes the height of the vial from bottom to top in mm. Each line is an individual timepoint scan ranging from time zero (blue) to 78 days (red).

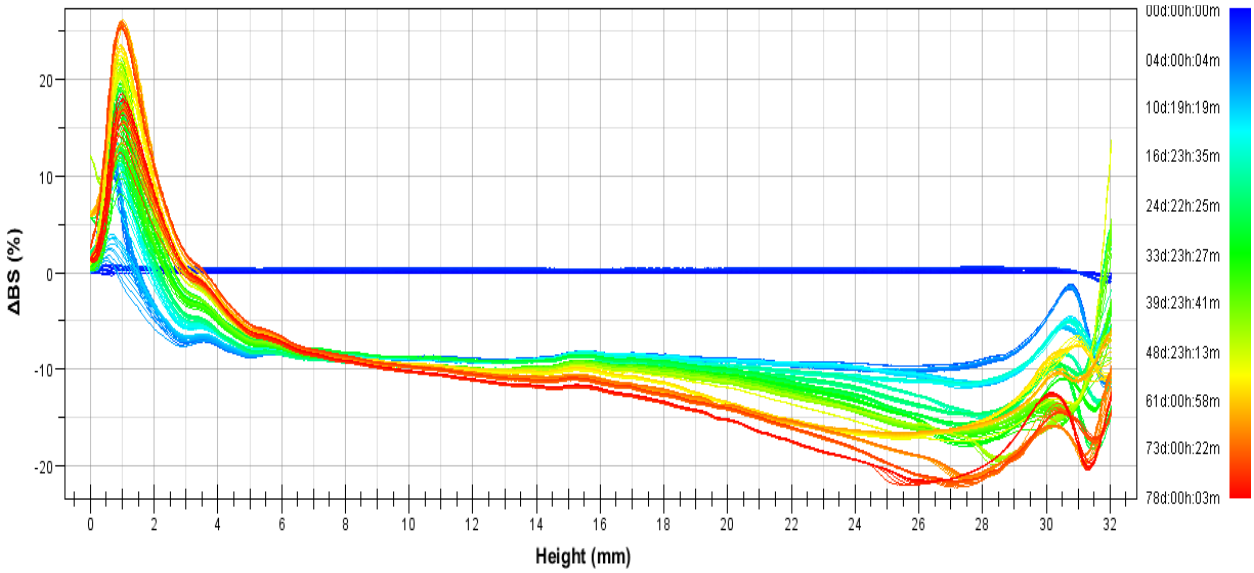

Figure 3.1c. Change in backscattering (ΔBS%) for 50% WPI 50% MPC at 11% (wt/wt) protein. The X-axis denotes the height of the vial from bottom to top in mm. Each line is an individual timepoint scan ranging from time zero (blue) to 78 days (red).

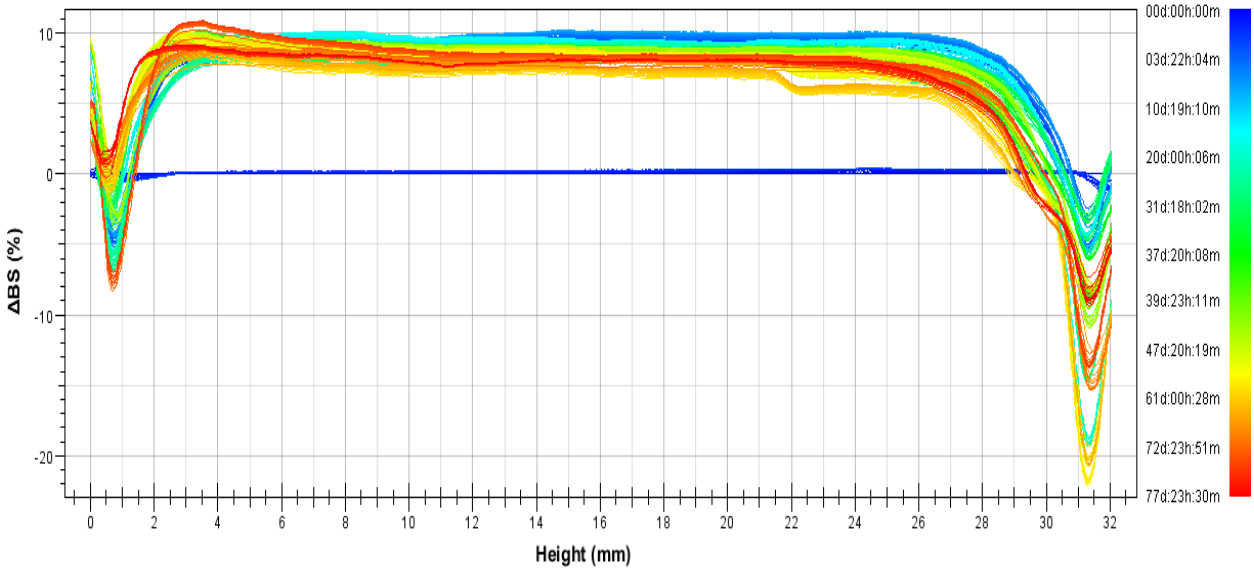

Figure 3.1d. Change in backscattering (ΔBS%) for 100% MPC at 11% (wt/wt) protein. The X-axis denotes the height of the vial from bottom to top in mm. Each line is an individual timepoint scan ranging from time zero (blue) to 78 days (red).

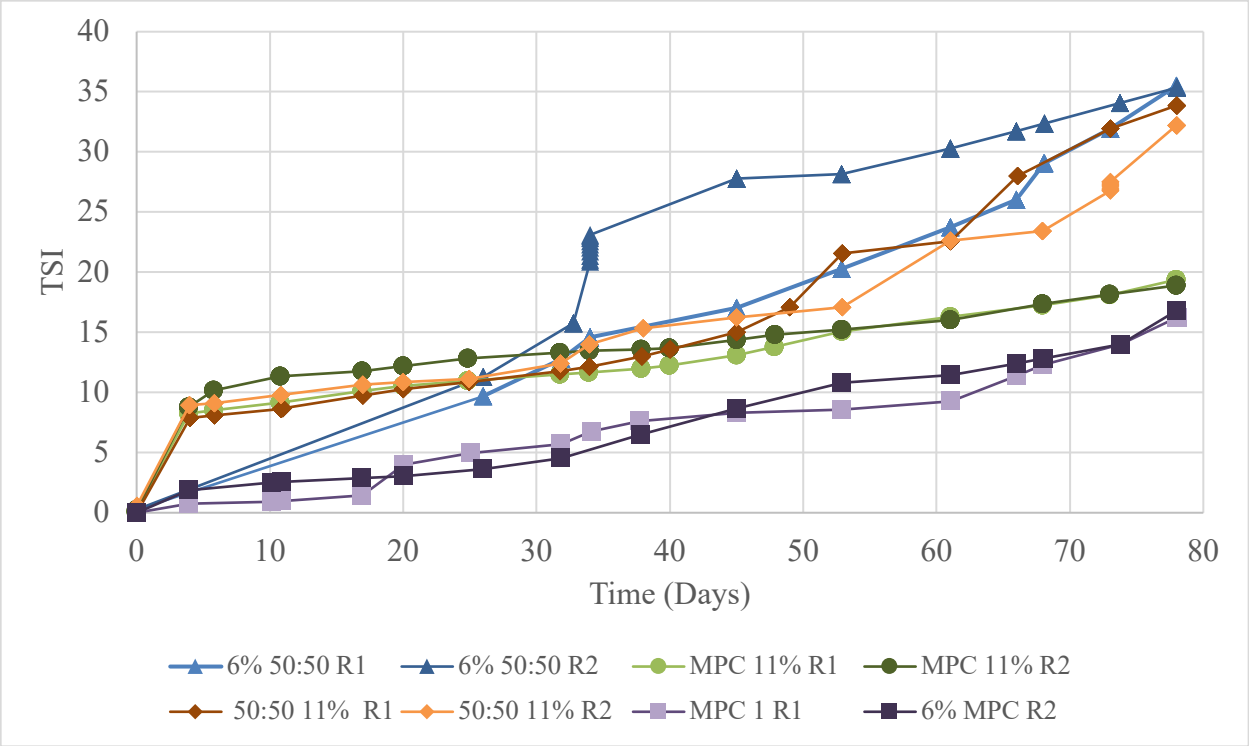

Figure 3.2a. Global Turbiscan Stability Index (TSI) for changes across the length of a tube observed for 79 days. Units are in TSI, Turbiscan stability index.

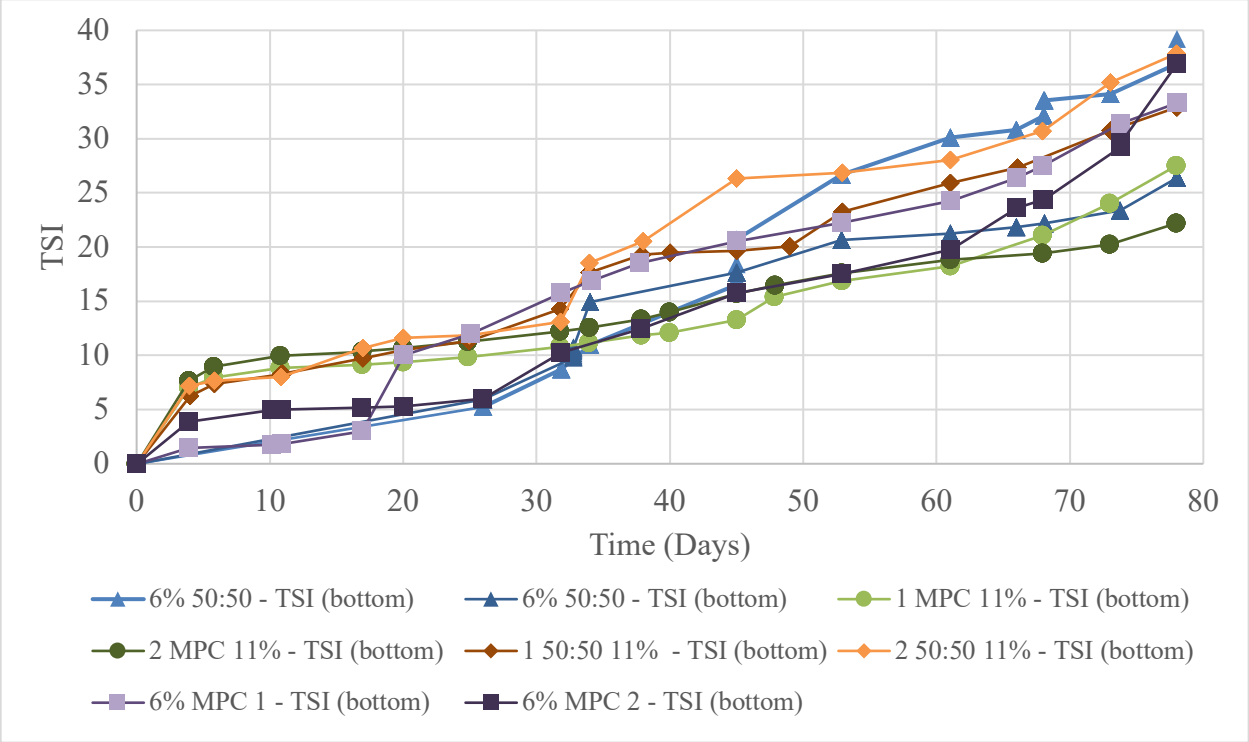

Figure 3.2b. Turbiscan Stability Index (TSI) for changes across the bottom portion of each tube observed over 79 days. Units are in TSI, Turbiscan stability index.

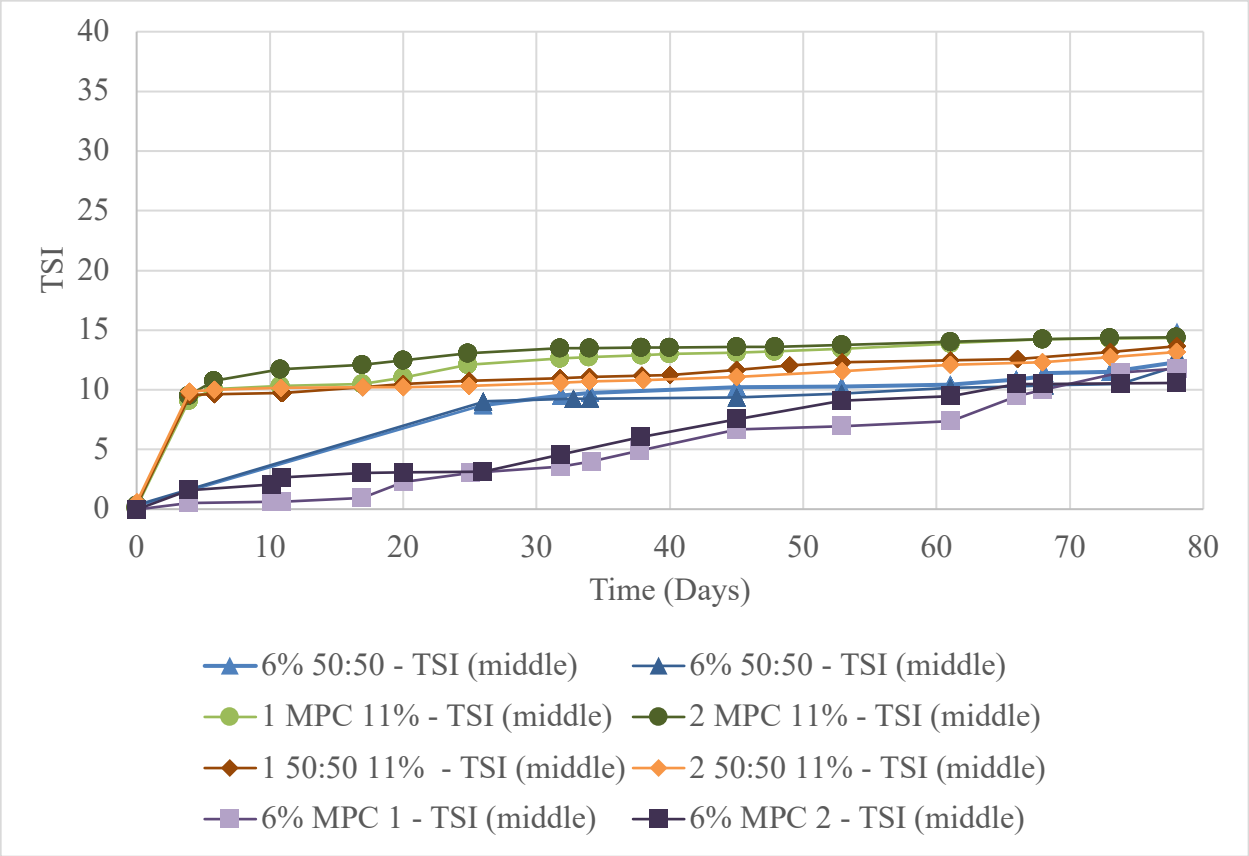

Figure 3.2c. Turbiscan Stability Index (TSI) for changes across the middle portion of each tube observed over 77 days. Units are in TSI, Turbiscan stability index.

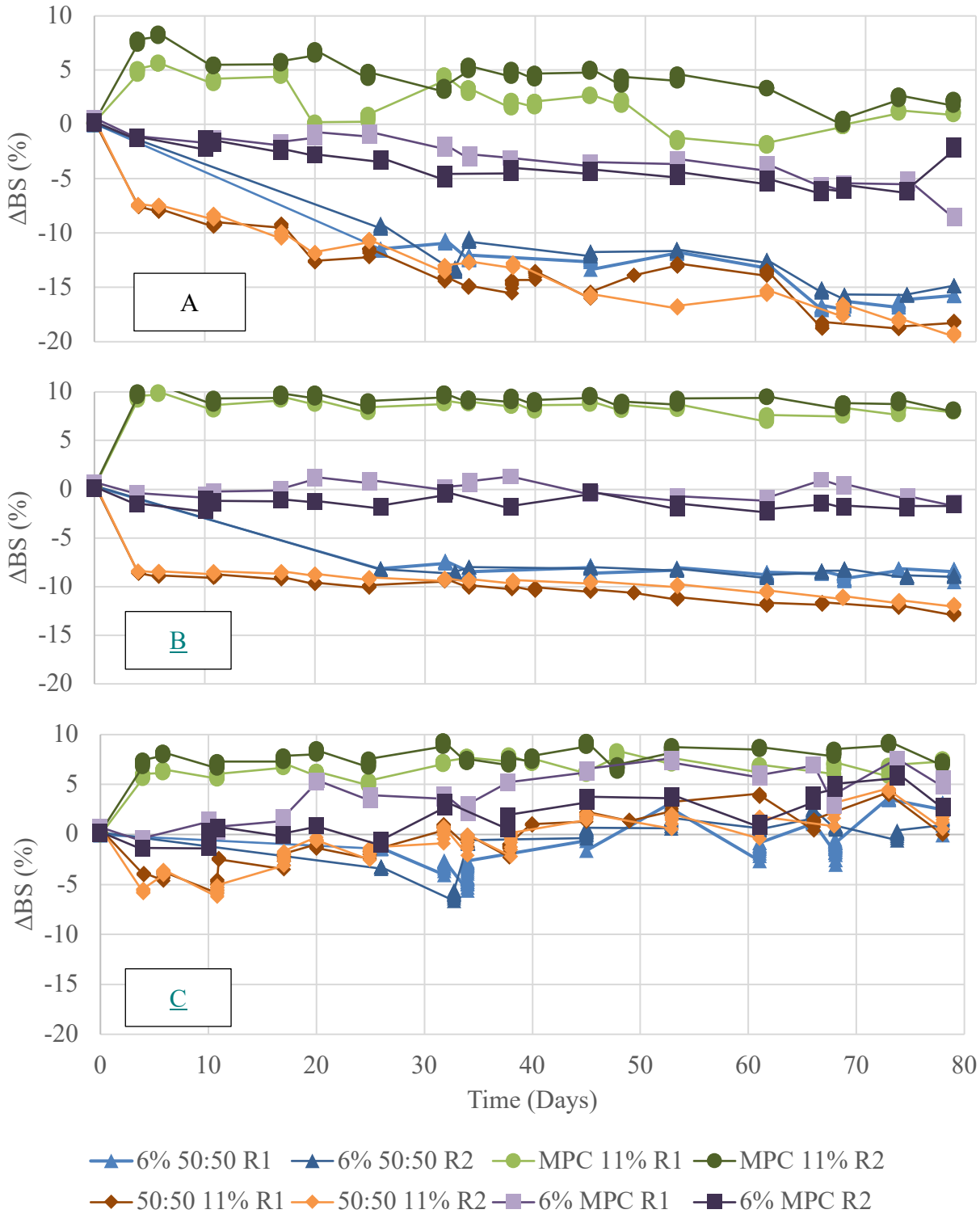

Figure 3.3. Recorded change in backscattering (ΔBS%) for all samples. Graph A shows changes in the top of the vial, graph B shows the middle section of the vial, and graph C is the bottom of the vial. The change in backscattering is relative to time zero measurement.

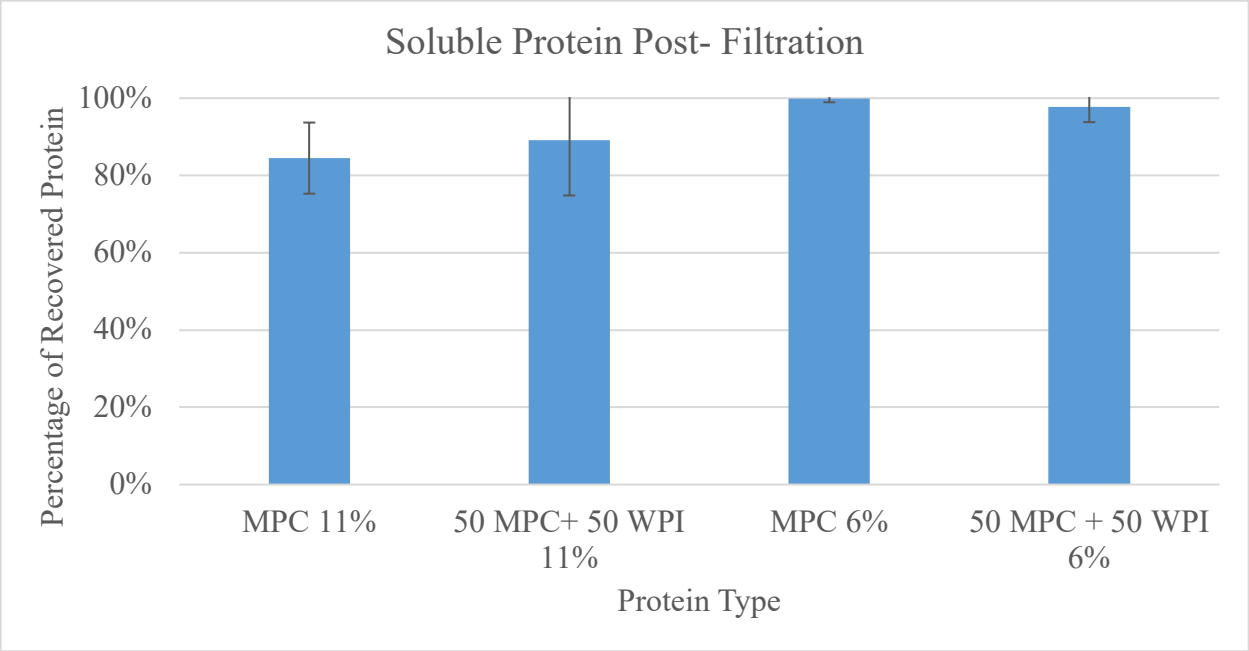

Figure 3.4. Recovered protein post 0.45 µm filtration prior to loading on SEC-HPLC averaged across 8 weeks.

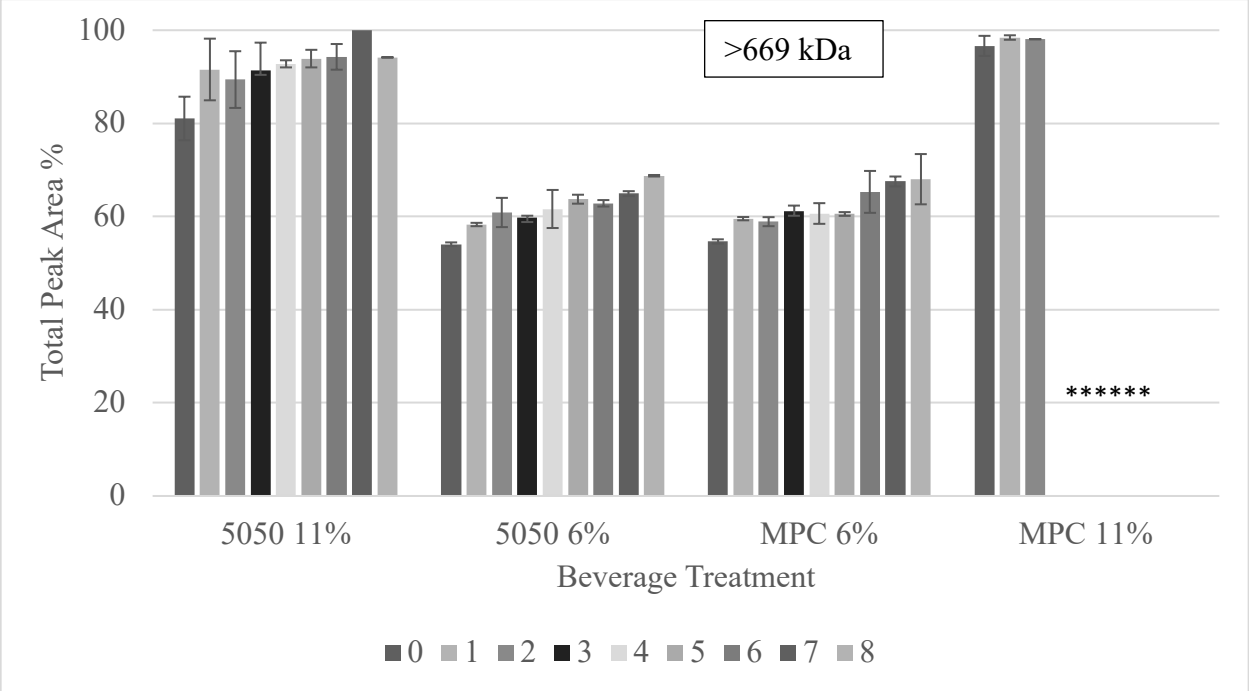

Figure 3.5. Size exclusion chromatography results for total percentage of peak area defined for > 669 kDa size class measured over 8 weeks.
Data from two replicates. *denotes weeks with no data collected due to sample gelation, MPC 11% weeks 3-8.

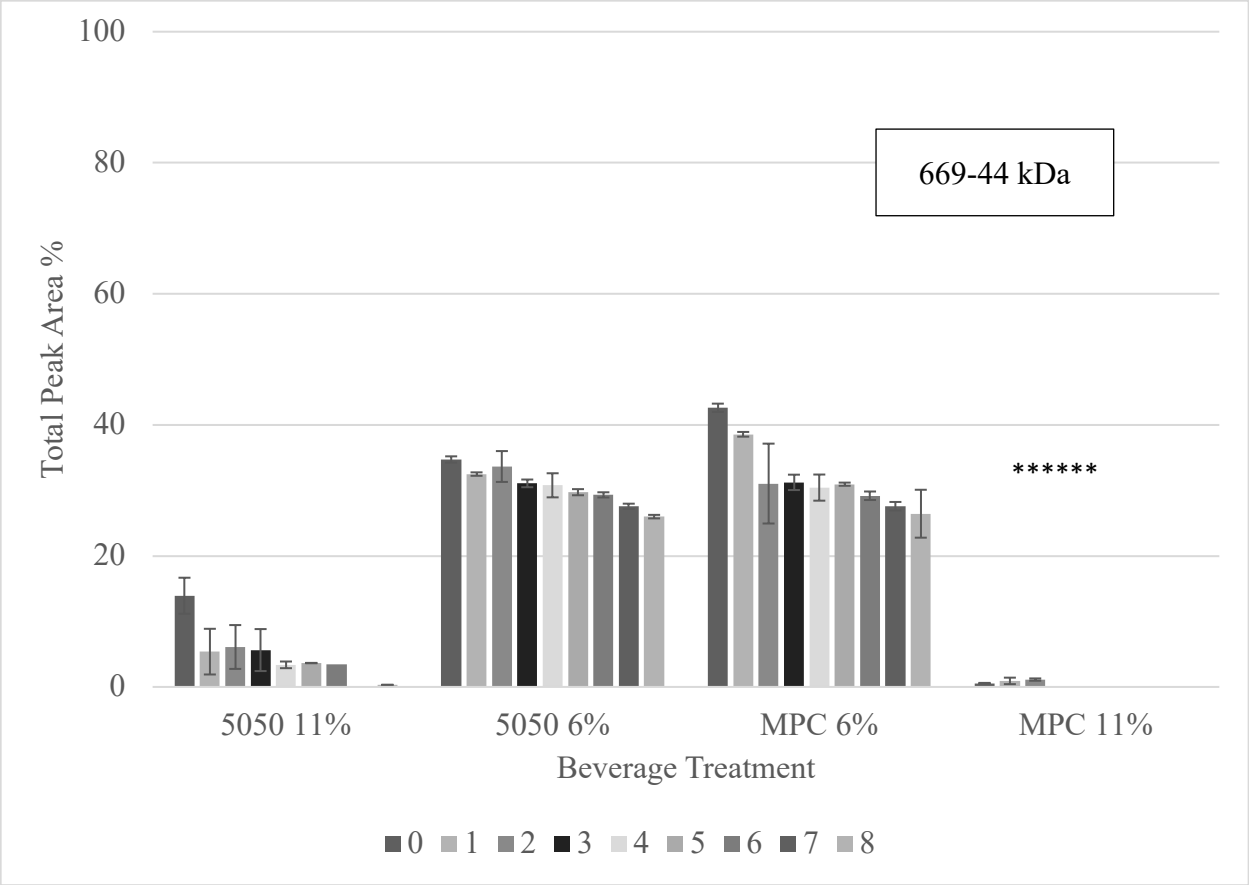

Figure 3.6. Size exclusion chromatography results for total percentage of peak area defined between 44kDa and 669kDa

Data from duplicates. *denotes weeks with no data collected due to gelation, MPC 11% weeks 3-8.

7. TABLES

Table 3.1. Retort thermal processing schedule

	Time (min : s)	Start Temp (°C)	End Temp (°C)
Warm Up	3:30 ± 0:30	105 ± 5.0	121.0
Sterilize	20:00	121.0	120.0 ± 2.0
Cool Down	14:30 ± 0:30	121.0 ± 2.0	105 ± 5.0

Adapted from SDFRC Methods

Table 3.2. Particle diameter at Dx(90) for retort processed beverages

Beverage Treatments / Time (weeks)	100% MPC 11% (wt/wt)	100% MPC 6% (wt/wt)	50% MPC + 50% WPI 11% (wt/wt)	50% MPC + 50% WPI 6% (wt/wt)
0	24.7 ± 25	0.5 ± 0	7.8 ± 13.5	0.5 ± 0
1	7.6 ± 3.3	5.6 ± 2.2	1.3 ± 0.8	0.5 ± 0
2	8.8 ± 11	3.3 ± 2.7	1.1 ± 0.8	0.4 ± 0
3	9.7 ± 21.8	0.7 ± 0.3	0.9 ± 0.1	0.4 ± 0.1
4	4.3 ± 1.2	4.1 ± 2.9	5.4 ± 5.6	0.9 ± 0.7
5	28.8 ± 21.4	8.7 ± 8.7	45.6 ± 16.9	0.9 ± 0.6
6	11.1 ± 16.4	0.6 ± 0.3	0.6 ± 0.1	0.5 ± 0
7	1.7 ± 0.8	2.4 ± 3.5	0.5 ± 0	0.4 ± 0
8	35.2 ± 33.3	2.3 ± 4.2	3.1 ± 5.0	0 ± 0

Data collected over 8 weeks. Each measurement taken in quintuplet for two separate replications. Numbers given are in μm particle size.

Table 3.3. Particle diameter at Dx (50) for retort processed beverages

Beverage Treatments / Time (weeks)	100% MPC 11% (wt/wt)	100% MPC 6% (wt/wt)	50% MPC + 50% WPI 11% (wt/wt)	50% MPC + 50% WPI 6% (wt/wt)
0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
1	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
2	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
4	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
5	0.4 ± 0.0	12.5 ± 39.2	0.2 ± 0.0	0.1 ± 0.0
6	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
7	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
8	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

Data collected over 8 weeks. Each measurement taken in quintuplet for two separate replications. Numbers given are in μm particle size.

Table 3.4. Particle diameter at Dx(10) for retort processed beverages

Beverage Treatments / Time (weeks)	100% MPC 11% (wt/wt)	100% MPC 6% (wt/wt)	50% MPC + 50% WPI 11% (wt/wt)	50% MPC + 50% WPI 6% (wt/wt)
0	0.1 ± 0.0	0.03 ± 0.0	0.04 ± 0.0	0.04 ± 0.0
1	0.1 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0
2	0.2 ± 0.0	0.08 ± 0.0	0.06 ± 0.0	0.06 ± 0.0
3	0.03 ± 0.0	0.04 ± 0.0	0.03 ± 0.0	0.03 ± 0.0
4	0.1 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0
5	0.1 ± 0.01	0.04 ± 0.01	0.04 ± 0.0	0.04 ± 0.0
6	0.1 ± 0.01	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0
7	0.1 ± 0.0	0.03 ± 0.0	0.04 ± 0.0	0.04 ± 0.0
8	0.1 ± 0.01	0.03 ± 0.0	0.04 ± 0.0	0.03 ± 0.0

Data collected over 8 weeks. Each measurement taken in quintuplet for two separate replications. Numbers given are in μm particle size.

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CHAPTER 4. CONCLUSIONS AND FUTURE WORK

1. CONCLUSIONS

The goal of this thesis was to evaluate methods for measuring meso-scale structures that are related to physical stability of proteins in beverages. The methods chosen were based on settling behavior as defined by variables outlined in the Stokes' equation:

$$v_s = \frac{2}{9} * \frac{(\Delta\rho) * g * r^2}{\eta} \qquad \text{Eq. 4.1}$$

v_s is the velocity of sedimentation, $m \bullet s^{-1}$

ρ particle = density of soluble complex, $kg \bullet m^{-3}$

ρ solvent = density of continuous phase, $kg \bullet m^{-3}$

g = gravity, $m \bullet s^{-2}$

r = hydrodynamic radius, m

η = continuous phase Newtonian viscosity, Pa•s

While the Stokes' equation is not directly applicable to protein beverages, due to high phase volume and size and shape heterogeneity of particles, the variables contained in the equation are applicable to understanding beverage stability. The experiments in this thesis set out to study sizes of the particles and through the use of multiple measurements it was possible to observe particle size changes. Furthermore, *thermal stability* was defined as the ability of a beverage formulation, or protein ingredient, to be thermally processed and remain a homogeneous sol. In contrast, *shelf stability* indicated the time the "thermal stable" beverage can remain at storage temperature without settling or aggregation producing an undesirable increase in viscosity, gelation, a visible precipitate, or a two-phase fluid.

It was demonstrated that multiple physical measurements are needed to understand the physical destabilization mechanisms occurring during thermal processing and over time. The expectation for the *thermal stability* of these treatments based on literature review were 1) beverages with lower protein content would have greater thermal stability (Sauer and Moraru, 2012; Dissanayake et al., 2013); 2) beverages based primarily on casein would be relatively thermally stable, and 3) increasing the ratio of whey proteins to casein would decrease thermal stability (Sauer and Moraru, 2012; Kelleher et al., 2018). The methods for measuring thermal stability did confirm these expectations in both chapters 2 and 3, the high-casein ratio beverages were more stable initially after processing and beverages with > 50% whey protein had larger aggregates after processing.

If protein aggregates do not undergo secondary aggregation during storage, then the variables found in the Stokes' equation should be sufficient to predict shelf stability. Secondary aggregation will increase particle size and may produce a gel network; therefore, it is a critical component in determining shelf life. Surprisingly, there are not many investigations on shelf stability of high-protein beverages as relates to measurements in the *meso*-scale. While age-gelation studies in bovine milk are plentiful, they have generally been done at protein concentrations relevant to fluid milk (~ 3-4% protein) (Datta and Deeth, 2001). Work has been done on acidic (pH > 4.6) beverages containing whey proteins, but not at protein levels used in this thesis. No studies have yet to be published on neutral pH (pH 6.8) beverages containing micellar casein. Due to the thermal stability of casein, it was assumed that beverages higher in casein would be more stable over time, this was confirmed with our results.

The overall challenge was to have a series of measurements that showed treatment differences in thermal stability and detected secondary aggregation. The efficacy of measuring so

called soluble protein was evaluated in Chapter 2. Beverages were separated into three soluble fractions based on centrifugation and two filtration steps. The amount of soluble protein after centrifugation was a good measurement of thermal stability, but no changes were seen over eight weeks of storage. However, it is possible that other centrifugal forces, times, and temperatures may separate aggregate fractions associated with secondary aggregation. The two additional soluble fractions created by filtration at 5 μm and 0.45 μm respectively did not reflect secondary aggregation, but size exclusion chromatography of the most soluble fraction (0.45 μm filtration) did show secondary aggregation in aseptically processed beverages (Chapter 2) and retort processed protein ingredient dispersions (Chapter 3).

An inherent problem with investigating storage stability of high protein beverages is that methods for characterizing particle size generally require mixing and diluting the sample. Therefore, weakly linked aggregates could be responsible for precipitation but dissociate in sample preparation for particle size analysis.

In Chapter 3, an experiment was designed so that protein solutions could be thermally processed and evaluated in the same container (glass tubes). This was designed to allow for using a Turbiscan LAB instrument, which measure backscattering and transmission across the height of the tube. This approach allowed for measuring changes in the undisturbed system *in situ* over time. Backscattering patterns showed settling before it could be visually observed and two patterns were seen that may indicate different mechanisms: Stokes' settling and either a high viscosity behavior or gelation. This interesting observation requires additional investigation. Changes in Z-direction backscattering was an effective way of showing settling behavior prior to visual observation and should be of value in predicting shelf life. The instrument also claims to measure particle size, although this feature was not evaluated in this investigation.

2. FUTURE WORK

There is a large amount of research still needed to move this approach into a method that can be used to predict shelf stability of protein beverages. Table 4.1 gives an overview of the methods used in this thesis and the pros and cons of each. For example, with milk ingredients the next step would be to test different ratios of whey to casein proteins over a range of protein concentrations. In Chapter 2, it was seen that somewhere between 18% and 50% whey (serum) proteins produced a beverage that was similar to the 100% micellar casein treatment. Furthermore, another dimension to investigate is the different ingredients that are used in beverages and how they impact stability and protein concentration. It is possible that the choice of polysaccharide(s) to increase stability will depend on the ratio of whey to casein proteins, as differences between these proteins were seen in our data set.

The Turbiscan vials were autoclavable making it possible to simulate retort processing. They could also be sterilized and filled under aseptic conditions, allowing for evaluating a range of thermal processing conditions.

One major challenge in moving to a full-scale shelf life investigation would be the number of samples evaluated at each time point. If storage was at temperatures allowed for the Turbiscan instrument, then the same sample could be followed over time for Z-scattering. All other measurements require “destroying” the sample and the number of samples analyzed at each time period would need to be established such that that an appropriate statistical power would be reached to evaluate hypotheses on mechanisms of destabilization.

This study has laid the groundwork for establishing a general method for evaluating thermal stability and shelf stability in protein beverages. Future work would include studying different ingredient impacts, increasing statistical power with more replicates, and evaluating over a range of thermal processing parameters and storage conditions.

3. TABLES

Table 4.1. Methods Used for Tracking Protein Stability in Chapters 1 and 2

Method (chapters used)	Property Measured	Pros	Cons
Turbiscan LAB, (Chapter 3)	Settling in Z-direction	Simple, quick	Sample had to remain undisturbed, filling needs to be sterile and is limited to retort presently.
Laser diffraction particle size analysis (Chapter 2, Chapter 3)	Particle size, Secondary aggregation	Simple, fast, easy	Aggregates had to have strong interactions to be measured, was not effective for protein solutions
Soluble fractions of Proteins (Chapter 2)	Particle Concentration	Insight into the size classes of proteins and solubility	Time-intensive; may require optimization of conditions for particle sizes associated with specific treatments
Size exclusion chromatography (Chapter 2, Chapter 3)	Particle size, secondary aggregation	Universal, detection possible at multiple wavelengths	Requires filtration that may filter out aggregates responsible for destabilization

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APPENDICES

Appendix 1. Soluble Protein Fractions (percent of total protein) For Beverages Averaged Over Eight Weeks of Storage.

Protein	SF-1	SF-2	SF-3		Protein	SF-1	SF-2	SF-3
100% MPC 6.3%	90.7	80.9	56.7	averages	100% MPC 10.5%	78.7	66.6	39.7
100% MCC 6.3%	90.4	81.2	50		100% MCC 10.5%	80.1	65	35.2
18% SPI 82% MCC 6.3%	83.6	78.9	44.5		18% SPI 82% MCC 10.5%	80.1	62.7	32.2
50% SPI 50% MCC 6.3%	55.1	48.3	34.3		50% SPI 50% MCC 10.5%	47.3	39.3	26.8
50% SPI 50% MPC 6.3%	59	51.7	40.5		50% SPI 50% MPC 10.5%	50.2	43	32.2
100% MPC 6.3%	4.8	7.8	6.4	SD	100% MPC 10.5%	4.1	7	12.3
100% MCC 6.3%	4.5	7.3	7.8		100% MCC 10.5%	10	12.4	22.2
18% SPI 82% MCC 6.3%	8	4	11.4		18% SPI 82% MCC 10.5%	9.6	9	6.5
50% SPI 50% MCC 6.3%	5.1	9.8	7.8		50% SPI 50% MCC 10.5%	3	2.4	3.3
50% SPI 50% MPC 6.3%	4.1	10.9	4.7		50% SPI 50% MPC 10.5%	3	6.8	4.2
100% MPC 6.3%	5.3	9.7	11.3	RSD	100% MPC 10.5%	5.2	10.5	30.9
100% MCC 6.3%	5	8.9	15.7		100% MCC 10.5%	12.4	19.1	63.2
18% SPI 82% MCC 6.3%	9.6	5	25.7		18% SPI 82% MCC 10.5%	12	14.3	20.3
50% SPI 50% MCC 6.3%	9.3	20.2	22.6		50% SPI 50% MCC 10.5%	6.3	6.1	12.1
50% SPI 50% MPC 6.3%	7.0	21.1	11.5		50% SPI 50% MPC 10.5%	6.1	15.8	13

All data is averaged across 8 weeks for two replicates. Each timepoint was measured in triplicate to account for variability.

Appendix 2. Soluble Protein Fractions for Beverages Containing 6.3% (wt/wt) Protein

Protein	Time (weeks)	SF-1 Percentage of Protein Recovered			SF-2 Percentage of Protein Recovered			SF-3 Percentage of Protein Recovered		
100% MPC 6.30%	0	85.8	±	3.7	78.1	±	2.6	60.1	±	6.1
	2	94.2	±	2.1	87.6	±	2.5	61.2	±	7.3
	4	91.8	±	1.6	82.4	±	4.6	56.4	±	6.7
	6	92.2	±	5.4	83.3	±	5.1	52.9	±	2.8
	8	89.5	±	5.9	73	±	12.1	52.8	±	5.2
100% MCC 6.30%	0	86.5	±	6	81.3	±	4.9	43.8	±	6.3
	2	93.4	±	1.6	78.7	±	12.6	46.4	±	10.6
	4	90	±	1.8	83.4	±	2.2	51.9	±	4.7
	6	88.7	±	5.6	81.6	±	6.9	49	±	2.8
	8	93.2	±	1.6	80.9	±	7.5	58.7	±	4
18% SPI 82% MCC 6.30%	0	77.8	±	4.5	77.2	±	1	41	±	4.6
	2	87.7	±	3.6	84.4	±	2.8	45.5	±	10.6
	4	87	±	3.6	79.7	±	1.2	48.8	±	1.4
	6	78.7	±	12.6	75	±	2.8	46.5	±	8.2
	8	86.8	±	5	78.4	±	1.6	40.9	±	22.1
50% SPI 50% MCC 6.30%	0	54.2	±	8.2	52	±	5.3	34.4	±	6.7
	2	54.9	±	1.6	50.6	±	1.4	34.1	±	5.5
	4	55.3	±	4.1	41.7	±	20.6	31	±	15.2
	6	56.1	±	7.3	50.2	±	3.5	36.5	±	2.1
	8	54.7	±	2.4	47.1	±	2.6	35.4	±	4.3
50% SPI 50% MPC 6.30%	0	56	±	3.6	46	±	22.8	43.9	±	2.9
	2	59.8	±	2.9	55	±	3	37.8	±	6
	4	57.5	±	4.3	53.3	±	3.9	39.1	±	3.6
	6	61	±	5.1	53.8	±	4.4	38.9	±	4.9
	8	60.9	±	2.6	50.3	±	5.5	42.7	±	2.4

All measurements were taken in triplicate for two replications of data.

Appendix 3. Soluble Protein Fractions for Beverages Containing 10.5% (wt/wt) Protein

	Time (weeks)	SF-1 Percentage of Protein Recovered			SF-2 Percentage of Protein Recovered			SF-3 Percentage of Protein Recovered		
100% MPC 10.5%	0	80.2	±	1.9	71.6	±	2	53.8	±	5.93
	2	*	±	*	*	±	*	*	±	*
	4	*	±	*	*	±	*	*	±	*
	6	80.1	±	4.2	70.2	±	2.4	38.6	±	3.6
	8	75.9	±	5.3	58	±	4.3	26.8	±	1.3
100% MCC 10.5%	0	85.3	±	2.1	76.1	±	2.3	54.2	±	10.8
	2	77.9	±	11.5	61.7	±	13.6	32.4	±	19.3
	4	68	±	0.8	55.8	±	2.3	38.8	±	1.2
	6	90.5	±	1.5	82	±	1.8	*	±	*
	8	79	±	13.5	49.5	±	1.7	15.4	±	0.9
18% SPI 82% MCC 10.5%	0	83.6	±	4.6	71.6	±	6.3	35.5	±	9
	2	88.7	±	8.5	71	±	3.4	34.9	±	1.5
	4	88.3	±	1.7	64.6	±	4.7	34.8	±	2.3
	6	68.5	±	4.6	53.2	±	0.8	33.8	±	2.4
	8	71.1	±	4	53.3	±	1.5	22.3	±	0.3
50% SPI 50% MCC 10.5%	0	46.4	±	3	39.7	±	1.6	29.1	±	2.6
	2	45.1	±	1.5	37.2	±	2.3	26.3	±	1.4
	4	48.1	±	4.6	40	±	3	24.9	±	4.8
	6	48	±	1.9	40.4	±	2.3	28.8	±	2
	8	49.1	±	1.3	39.2	±	0.8	24.9	±	2
50% SPI 50% MPC 10.5%	0	48.1	±	3.7	44	±	4	34.8	±	4.3
	2	51.8	±	3	44.5	±	6.9	32.4	±	6.1
	4	52.7	±	3	47.1	±	2.9	34.1	±	2
	6	48.5	±	1.5	43.9	±	1.6	31.2	±	1.3
	8	49.8	±	1	35.5	±	10	28.3	±	3.5

All measurements were taken in triplicate for two replications of data.

*no data due to gelation

Appendix 4. Protein and mineral content of protein ingredients

Sample Lot Numbers	Name	% Protein	P %	K %	Ca %	Mg %	Na mg/kg	S %
MPC121704	MPC 85	82.5	1.39	0.36	2.08	0.10	1457	0.78
WPI121703	BiPRO WPI	92.8	0.11	0.10	0.16	0.02	5297	1.53

Mineral content data was collected in part at the Environmental and Agricultural Testing Service laboratory (EATS), Department of Crop and Soil Sciences, at North Carolina State University.

Appendix 5: Map of Autoclaved Samples

Rack 1

	A	B	C
1	50-6	50-6	M-11
2	0	0	0
3	50-6	M-11	M-6
4	M-6	M-11	0
5	M-11	M-6	M-6
6	M-11	50-11	50-6
7	50-11	50-6	0
8	50-11	M-6	50-6

Rack 2

	A	B	C
1	M-11	50-11	50-11
2	50-6	50-11	0
3	0	0	50-6
4	50-6	M-6	M-6
5	0	0	0
6	0	M-6	M-6
7	0	0	0
8	M-11	50-11	50-11

Rack 3

	A	B	C
1	50-6	M-6	M-11
2	0	50-11	M-6
3	50-6	50-11	0
4	0	0	0
5	0	M-6	M-11
6	M-11	M-11	50-11
7	0	M-11	50-6
8	0	0	50-11

Appendix 6. Recovery of protein from 0.45 μm filtration fraction for size exclusion chromatography

Time	MPC 11% (wt/wt) protein	MPC 6% (wt/wt) protein	50% MPC + 50% WPI 11% (wt/wt)	50% MPC + 50% WPI 6% (wt/wt)
0	95.1%	100.0%	96.0%	100.0%
1	79.2%	99.8%	71.3%	100.0%
2	79.1%	99.4%	**	100.0%
3	*	100.0%	**	88.9%
4	*	98.7%	91.3%	98.6%
5	*	99.7%	89.6%	97.0%
6	*	99.5%	89.8%	99.5%
7	*	**	96.9%	**
8	*	**	**	**

*denotes no sample filtered due to viscosity

**denotes either sample contamination (bacteria/mold), or nitrogen analysis error