ABSTRACT

PAGE, DYLAN LEWIS. Colloidal Stability of Whey Protein Isolate in Acidic Beverages. (Under the direction of Dr. E. Allen Foegeding).

Functional foods are food or beverage that provides a health benefit. One of the most popular functional food ingredients is whey protein because it is highly nutritious, a good source of essential amino acids, and has functional properties that allow them to stabilize emulsions and foams, form gel networks, and create beverages that maintain colloidal stability. Colloidal stability is a measure of the protein stability after thermal processing in terms of aggregation and precipitation and is measured by properties such as protein solubility and beverage turbidity. Beverages that are high in solubility and of an acceptable level of turbidity are considered to have colloidal stability.

Two popular types of protein fortified beverages are flavored water and fruit juices. They are typically formulated at pH values ≤ 4.6 and have a translucent appearance. In this work, the colloidal stability of a protein fortified lemon flavored water and fruit juice were measured across a range of protein concentrations and acidic pH levels. The protein fortified fruit juice was investigated as beverages made with individual juices of pomegranate, cherry, and apple, in addition to a juice mixture. The lemon flavored water was a simple system of whey protein and water with low concentrations of flavorants, acidulates, and preservatives and whey proteins would be expected to act very similar to being dispersed in water. The fruit juices offer a much different environment that includes polyphenols, which are known to interact with whey proteins and form soluble or insoluble complexes.

The objective of this work was to determine at what pH levels and protein concentrations a beverage maintains colloidal stability. In doing so, the effects of pH and protein concentration on colloidal stability were evaluated. Each beverage was thermally processed and made with varying protein concentrations, ranging from 2 to 8% (w/w) protein, and pH levels, ranging from 3.2 to 4.3. Protein solubility of the beverages was measured based on the percentage of nitrogen that remained dispersed after centrifugation to remove insoluble aggregates. Turbidity, due to lighter scattering caused by the number and size of aggregates, was measured using a turbidimeter. Based on general observation among all beverages, a beverage with colloidal stability was ≥90% soluble with turbidity ≤300 NTU. To provide further insight, particle analysis was measured using dynamic light scattering while zeta potential was calculated using electrophoretic mobility.

The lemon flavored water had colloidal stability at nearly all protein concentrations ≤ pH 3.8. The mixed fruit and pomegranate juices were only stable at pH 3.2 at higher protein concentrations and were the only beverages that showed the trend of increasing solubility and decreasing turbidity at pH 3.2 and 3.5. The cherry and apple juices were stable at pH 3.2 and within a limited range of protein concentrations at pH 3.5, depending on the fruit juice source. While pH and protein concentration affected the stability of all beverages, the specific fruit juices also altered colloidal stability. It was hypothesized that the most plausible reason was variations in the amount and types of polyphenols among fruit juices; however this was not proven. Variations observed in colloidal stability of beverages seen among fruit juices and between different sources of fruit juices indicate a need to understand

how each juice component can alter protein aggregation and thereby beverage colloidal stability.

The objectives of this work were completed. The colloidal stability of the lemon flavored water and fruit juices was established across a range of protein concentrations and pH levels. In addition, it was shown that juice components, such as the number and types of polyphenols, are critical to the colloidal stability of proteins in fruit juice beverages.

Colloidal Stability of Whey Protein Isolate in Acidic Beverages

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BIOGRAPHY

Dylan Page was born and raised in Mooresville, NC before attending NC State University from 2008-2012 earning his Bachelor's degree in food science. After completing his undergraduate degree, he worked for Carolina Beverage Group in Mooresville, NC as a quality analysis technician for just over year. Dylan returned to NC State in 2014 to continue his studies in food science in order to earn his Master's degree.

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1 Introduction

Whey is the liquid byproduct of cheese making or casein/caseinate processes that is created after the casein from milk has been coagulated into curds (Surroca et al., 2002; Smithers, 2008). The whey liquid that forms is either sweet whey or acid whey depending on the type of dairy product made from the milk. Sweet whey (pH 6.0-6.3) is the byproduct that comes from the production of cheese made using rennet, while acid whey (pH 4.3-4.6) is created during the production of cottage cheese and yogurt type products (Morr & Ha, 1993). Whey proteins, also called serum proteins, represent 20% of the proteins found in milk and their overall amount in whey depends on the extent of retention in the casein curd (Shah, 2000; Smithers 2008).

Whey proteins are concentrated into ingredients as either whey protein concentrates (25-80% protein) or whey protein isolates (≥90% protein) (Foegeding et al., 2002). Whey proteins are separated from the other whey liquid components through filtration techniques and/or other purifying operations, such as ion exchange chromatography, and then made into powder by evaporating and spray drying the proteins (de Wit, 1990; Palazolo et al., 2000). There is a mixture of proteins in whey. The most prevalent protein is β-lactoglobulin, which makes up 50% of whey protein, followed by α-lactalbumin and bovine serum albumin, which make up 20% and 5% of whey protein, respectively (Havea et al., 2001; Cornacchia et al., 2014).

Whey liquid was originally viewed as a waste product created during the cheese making process and was typically thrown out into bodies of water or the sewage system (Smithers, 2008). However, it was discovered that the whey liquid was causing a large pollution problem and resulted in governments passing regulations dealing with the disposal of the whey liquid which forced the cheese industry to begin to research new ways to use this waste product (Smithers, 2008). This led to the discovery that the whey proteins found in the liquid were very nutritious and had a high biological value and were a good source of essential amino acids (Smithers, 2008). With this discovery and the creation of methods to isolate the proteins, companies were presented with an opportunity to turn this waste product into a valuable commodity. Besides the nutrition contributions, whey proteins were also discovered to have functional properties, like emulsification and foaming, which could be used to improve food products (de Wit, 1990). Whey proteins also fit into the new consumer demand for "functional foods" that were very nutritious and healthy while being convenient and taste good (Smithers, 2008). With the discovery of these uses for whey protein, production of whey protein products has grown. In 2012, 441.1 million pounds of whey protein concentrate and 64.8 million pounds of whey protein isolate were produced, which was an increase of 2.4% and 7.1%, respectively, from 2011 (ADPI, 2012).

2 Whey Protein Processing

As mentioned previously, the two main techniques used in industry for whey protein production are filtration and ion exchange chromatography. Filtration is done using ultrafiltration and diafiltration, usually in conjunction, which use semipermeable membranes

to isolate the proteins in whey liquid (de la Fuente et al., 2002, Morr & Ha, 1993). The first step is ultrafiltration which uses polymeric or ceramic membranes, with pore sizes ranging from 20-200 nm, to concentrate the proteins in the whey liquid (Zydney, 1998; de la Fuente et al., 2002; Tunick, 2012). Proteins only make up 0.82% of whey liquid initially, but after ultrafiltration, proteins can be concentrated up to about 60% (Morr & Ha, 1993; Zydney 1998). After ultrafiltration, diafiltration can be used to remove more non-protein components of whey liquid like lactose and minerals to further concentrate the liquid (de la Fuente et al., 2002). Diafiltration is completed by repeatedly rinsing the whey proteins with deionized water to wash the protein and push non-protein components through the membrane until the desired protein concentration is reached (Zydney, 1998). Another type of filtration that can be used is microfiltration which can be done before ultrafiltration and diafiltration and uses a membrane with 0.2 to 5 μm pores to remove lipids from the whey liquid (Morr and Ha, 1993).

Ion exchange chromatography is the other widely used whey protein process in industry. There are two types of ion exchangers, stirred bed column and fixed bed column, with the stirred bed column being the most commonly used industry because it creates less waste and denatures proteins less (Morr & Ha, 1993). Ion exchange isolates proteins by running the whey liquid through columns that contain charged resins that reversibly react with the proteins (Greiter et al., 2002; Morr & Ha, 1993). The pH of the whey liquid is adjusted to give the proteins the desired charge needed in order for the proteins to be adsorbed by resin while the non-protein components continue to pass through the column (Greiter et al., 2002;

Morr & Ha, 1993). After the whey liquid has been run through the column, a new solution is run through the column that changes the pH and/or ionic strength of the solution and desorbs them from the resin (Greiter et al., 2002; Morr & Ha, 1993). This allows the proteins to pass through the column where they are then collected and spray dried into powder form (Morr & Ha, 1993).

While both of these techniques are widely used and effective at isolating whey proteins, there are some challenges associated with whey protein production. The first problem is there is variability between different batches of whey protein products. This variability is not only caused by differences in processing, but also from the sources of the whey protein which have different compositions of the individual whey proteins (de la Fuente et al., 2002). Secondly, both filtration and ion exchange can cause irreversible changes in the whey protein, like denaturation and aggregation, which affect the whey protein's functionality (Palazolo et al., 2000). In order to limit these irreversible changes, whey protein processing must be carefully controlled to try and reduce the variability between batches of whey protein products (Morr & Ha, 1993).

3 Whey Protein Components

3.1 ß-Lactoglobulin

ß-Lactoglobulin (ß-lg) is a globular protein that makes up 50% of the total protein found in whey protein and is often considered the main protein responsible for the functional properties of whey protein (Surroca et al., 2002). It is made up of 162 amino acid residues with a molecular mass around 18.3 kDa, depending on the genetic variation of the protein (Surroca et al., 2002; Farrell et al., 2004; de Wit, 2009). Bovine ß-lg has seven different genetic variations (A-G), with variants A (18.363 kDa) and B (18.277 kDa) being the most common (de Wit, 2009; Farrell et al., 2004). ß-Lactoglobulin has a structure made up of nine anti-parallel ß-sheets and one α-helix and has two disulfide bonds at C66-C160 and C106-C119 and a free thiol group at C121 that is buried in the hydrophobic region of the protein (Crogunnec et al., 2003). The native state of ß-lg is a non-covalently linked dimer at neutral pH and room temperature, but at temperatures above 65°C or pH levels below 3.5 or above 6.5, the dimer dissociates into two identical monomers (Palazolo et al., 2000; de Wit, 2009). The secondary and tertiary structures of ß-lg are stabilized by the two covalent disulfide bonds present in the protein structure as well as non-covalent interactions, like van der Waals, hydrogen bonds, and hydrophobic interactions, but these interactions are dependent on the pH and ionic strength (de Wit, 2009; Surroca et al., 2002).

3.2 α-Lactalbumin

α-Lactalbumin (α-la) is a globular protein that makes up 20% of the proteins found in whey protein (Hong & Creamer, 2002; Kuwajima, 1989). It is made up of 123 amino acid residues

with a molecular mass of around 14.2 kDa and contains four disulfide bonds (Brew et al., 1970; Permyakov & Berliner, 2000; Farrell et al., 2004). α-Lactalbumin has a structure that contains an α-helix domain as well as a ß-sheet domain that are connected through a calcium binding loop (Permyakov & Berliner, 2000). It is a metalloprotein that has one strong binding site for metal ions with Calcium (Ca^{2+}) having the strongest affinity for the site, but other metals like Manganese (Mn^{2+}) and Zinc (Zn^{2+}) can bind to the site as well (Apenten, 1995; Kronman et al., 1981; Farrell et al., 2004). α-Lactalbumin loses tertiary structure stability when a calcium ion is removed from the binding site, but will stabilize when a calcium ion attaches to the binding site again (Permyakov & Berliner, 2000, Kronman et al., 1981).

α-Lactalbumin has two main forms with the first being apo-α-la which does not have a calcium ion bound and the holo-α-la which does (Hong & Creamer, 2002; Apenten, 1995). The holo-α-la is considered more thermal stable than the apo form, because the protein structure becomes more stable to heat denaturation of α-la (Apenten, 1995; Permyakov & Berliner, 2000). At low pH levels, protons are able to bind with α-la at the calcium binding site and displace the calcium ions (Permyakov & Berliner, 2000). α-Lactalbumin in the holo form is stable when exposed to mild heat treatments and has minimal intermolecular disulfide bond formation due to a lack of free thiol groups like those found in ß-lg (Hong & Creamer, 2002).

3.3 Other Whey Proteins

While α-la and ß-lg are the two largest components of whey protein, there are many other different types of protein present too. Minor whey proteins include bovine serum albumin (BSA), lactoferrin, lactoperoxidase, lysozyme, and immunoglobulins. Bovine serum albumin account for 5% of the total whey protein, making it the third largest component (Havea et al., 2001; Cornacchia et al., 2014). Bovine serum albumin contains 583 amino acid residues with 17 disulfide bonds and has a molecular mass of 66.4 kDa (Morr & Ha, 1993; Ferrel et al., 2004). Bovine serum albumin has the ability to bind with long chain fatty acids and increase their solubility in aqueous solutions (Spector et al. 1969). Lactoferrin is made up of 708 amino acid residues with 17 disulfide bonds and a molecular weight of 76.1 kDa (Farrell et al., 2004) Its structure is composed of a single peptide chain that is folded into 2 globular lobes (Farrell et al., 2004). Lactoferrin has antimicrobial and antiviral properties and has iron binding capabilities (Smithers et al., 1996; Shah, 2000). Lysozyme is an antimicrobial enzyme that has a molecular mass of 15 kDa (Shah, 2000). Lactoperoxidase is a glycoprotein consisting of 595 amino acids with a molecular weight around 80 kDa, and is an enzyme that is a catalyst for the oxidation of thiocyanate (Shah, 2000; Jafary et al., 2013). Immunoglobulins are composed of four polypeptide chains linked together by disulfide bonds (Ferrel et al., 2004). Two of the chains in the protein are heavy chains that have a molecular weight ranging between 55 and 76 kDa, while the other two chains are light chains which weigh between 22.5 and 27.3 kDa (Ferrel et al., 2004). Immunoglobulins help newborns fight against infections (Shah, 2000). Glycomacropeptides is a peptide made of 64 amino acid residues that only comes from sweet whey when chymosin cleaves κ-casein (Wagoner et al., 2015; Mollé & Léonil, 2004). Glycomacropeptides do not aggregate as

much as the other proteins found in whey and can be removed from the whey protein through ion exchange chromatography, but not filtration (Wagoner et al., 2015).

4 Whey Protein Functionality

Whey proteins have functional properties, which refer to the physiochemical abilities the protein has in food systems; however, it is difficult to predict the properties, in part due to the ability of whey protein to interact with a variety of ingredients in a food (de Wit, 1990). Functional properties that whey proteins have include stabilizing emulsions and foams, creating gel networks, and creating stable sols in beverages (Foegeding et al., 2002). Environmental factors, like temperature and pH, greatly affect the functionality of whey proteins (Surroca et al., 2002). Functional properties are also affected by the presence of other ingredients in the system as well as the heat treatment used to process the food system (de Wit, 1990; Foegeding et al., 2002). Whey protein ingredients can contain different amounts of non-protein compounds as well as have differences in the protein composition that affects the functionality of whey protein (Dalgleish et al., 1997; Wong & Lucey, 2003).

Whey protein functionality can vary even with the same type of product, for example, WPC 80 from different manufacturers will be different in terms of functionality. This is because of factors like the source of the liquid whey, which will contain different types and amounts of non-protein components based on the product made from the milk (de la Fuente et al., 2002). The processing technique used also affects whey protein functionality because each

technique will remove different amounts of non-protein components and can cause changes in the protein through denaturation and aggregation (Palazolo et al., 2000).

4.1 Solubility

Many applications of whey proteins, such as whey protein fortified beverages, require a fully dispersed and hydrated powder that is generally measured by a variety of tests fitting under the general concept of "protein solubility." Protein solubility refers to the amount of protein in the aqueous phase of a solution/dispersion compared to the total protein (Pelegrine & Gasparetto, 2005). This is empirically measured in protein powders by first dispersing a given amount of powder (mixing apparatus, time, and temperature fixed) and then removing insoluble protein by centrifugation and measuring the protein content of the supernatant (Pelegrine & Gasparetto, 2005). At the individual protein level, protein solubility is determined by surface hydrophobic and hydrophilic interactions between the proteins themselves and the solvent, which makes solubility a hydrophilic property (Pelegrine & Gasparetto, 2005). Environmental factors, like pH, ionic strength, and temperature, greatly affect the solubility of proteins (Morr & Ha, 1993; Pelegrine & Gasparetto, 2005).

The isoelectric point (pI) of a protein refers to the pH at which a protein has a net charge equal to zero (Cannan, 1942). When whey protein solutions have a pH that is near or at the pI, the proteins are more likely to precipitate and reduce solubility, but when the pH is away from the pI, the solubility of whey protein solutions increases due to electrostatic

stabilization (Pelegrine & Gasparetto, 2005; Cornacchia et al., 2014). Temperature also has an effect on whey protein solubility where the solubility increases between 40 and 50ºC, but as the temperature continues to increase, the proteins begin to denature which exposes the hydrophobic region and free thiol and allows for interactions and aggregation among the proteins (Pelegrine & Gasparetto, 2005, Cornacchia et al., 2014). During evaporation and spray drying of whey proteins, the thermal processes used can cause the proteins to denature and interact in unwanted ways that could reduce solubility (de Wit, 1990; Morr & Ha, 1993).

4.2 Turbidity

Turbidity is the measurement of the cloudiness of a solution that is caused by particles of varying size scattering light in the solution (Mahler et al., 2009). These measurements are expressed in Nephelometric Turbidity Units (NTU) and, like solubility, are influenced by protein concentration, pH, and temperature, which influence the size and number of particles in a solution (Mahler et al., 2009). At higher temps and pH levels near the isoelectric point, proteins tend to aggregate which causes an increase in turbidity because larger particles scatter more light (Cornacchia et al., 2014, Mahler et al., 2009). Increases in turbidity tend to coincide with decreases in solubility.

5 Protein Aggregation

Aggregation is another functional property of whey proteins that must be considered when using protein in a food system, particularly in beverages. Aggregates are non-defined

multimeric particles that form due to covalent and noncovalent interactions between molecules or particles (Mahler et al., 2009). The protein concentration and availability of reactive amino acids greatly affect the ability of proteins to interact and aggregate with each other (Morr & Ha, 1993). The two main mechanisms for aggregation of whey proteins are hydrophobic interactions and disulfide bonds, but at acidic conditions hydrophobic interactions are the chief mechanism of aggregation (Hoffman & van Mil, 1999; Cornacchia et al., 2014). Cornacchia et al. (2014) used fluorescence spectrometry to measure surface hydrophobicity of whey proteins and found that the hydrophobicity increased as the pH decreased from 4.5 to 3.5 and they also found that the free thiol group became less accessible as the pH decreased from 4.5 to 3.5 by measuring the reactivity of the whey proteins with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). β-Lactoglobulin is the most abundant whey protein and has the most influence on whey protein aggregation, and according to Roefs & De Kruif (1994) and Hoffman & van Mil (1999), the covalent aggregation mechanism of β-lg at neutral pH follows a three-step process. The first step is initiation where the β-lg dimer disassociates into its monomers and the free thiol becomes exposed due to denaturation caused by temperature or pH. The next step is propagation which is the formation of a disulfide linkage between the free thiol group and the disulfide bond of another β-lg, which creates another free thiol group that can link with another β-lg. The proteins continue to cross-link and form aggregates till the termination step where two free thiol groups interact to form a polymer and leaves no free thiols remaining.

Environmental conditions, like pH and temperature don't only just affect a protein's ability to aggregate, but also the type of aggregates that form and if they are soluble or insoluble

(Prabakaran & Damodaran, 1997). Protein concentration also influences protein aggregation because the more protein present, the greater the possibility that proteins interact and aggregate (Mahler et al., 2009). If the protein concentration of the solution is high enough, the aggregates can form a gel (Prabakaran & Damodaran, 1997). The type of whey impacts aggregation because acid whey is more likely to aggregate than sweet whey because acid whey contains more ions (Morr & Ha, 1993).

5.1 pH Effects on Protein Aggregation

pH is one of the most important factors to consider in food products due to its effect on a multitude of functional and sensory properties as well as microbial growth. According to Covington (1985), pH is defined as the negative log of the hydrogen ion activity ($-\log(a_{H+})$). As the pH moves closer to the isoelectric point of a protein, the electrostatic repulsion between proteins is greatly decreased, leading to the formation of aggregates due to noncovalent interactions (Cornacchia et al., 2014; Palazolo et al., 2000). However, these interactions can be reversed by moving the pH away from the isoelectric point where the electrostatic repulsion between proteins in greater (Bernal & Jelen, 1985; Cornacchia et al., 2014). ß-Lactoglobulin has a pI around 5.1 while α-la has a pI near 4.5 (Farrell et al., 2004). Cornacchia et al. (2014) measured the zeta potential of whey protein solutions between pH 7 and 2 and determined the isoelectric point of whey protein was around pH 4.8 which was supported by turbidity measurements that showed turbidity of whey protein solutions increased as the pH moved closer to the isoelectric point. Majhi et al. (2006) also determined the effect of pH on protein aggregation using turbidity and stopped-flow spectrophotometry

and found that the greatest aggregation of the whey proteins occurred between pH 4.6 and 4.7, while little to no aggregation occurred above pH 5.2 and below pH 3.8.

5.2 Thermal Processing Effects on Protein Aggregation

Thermal processing is typically used in helping control the microbial growth in food products, but the temperature effects on whey protein must be considered when they are used in a food system. At temperatures above 65ºC, whey proteins begin to denature and aggregate which could be an undesirable or desirable effect, depending on the food (Morr & Ha, 1993). When whey protein solutions are heated, hydrophilic interactions become weaker, but between 60 and 80ºC, the whey proteins denature which exposes the hydrophobic regions of the proteins and increases the amount of hydrophobic interactions between proteins. (Relkin & Mulvihill, 1996; Morr & Ha, 1993). Also, when the whey proteins are heated and denatured, the free thiol group in the hydrophobic region becomes exposed and which leads to disulfide bonds forming between proteins and increasing aggregation (Mahler et al., 2009; Cornacchia et al., 2014). The aggregate size of proteins is heavily influenced by the amount of time the protein is exposed to heat. Typically, the longer proteins are heated, the larger the particles that will form (Ndoye et al., 2012). The rate of heating affects the aggregation abilities of whey proteins as well. During thermal processing, when whey protein is heated at higher temperatures for a shorter time, fewer aggregates form when compared to a process where the proteins are heated for longer at lower temperatures (de Wit, 1990).

When individual whey proteins are mixed together like they are in a WPC or WPI, the aggregation abilities of the proteins change greatly. When α-la is mixed with ß-lg in solution and heated, ß-lg forms larger aggregates compared to when it is heated alone (Havea et al., 2001). Also, when the two are mixed together and heated, α-la is able to form dimers with the help of the exposed free thiol group found in hydrophobic regions of ß-lg (Havea et al., 2001). This allows α-la to form irreversible disulfide bonds and create dimers, trimers, and other aggregates (Hong & Creamer, 2002). The two whey proteins can form soluble aggregates together through the formation of disulfide bonds and hydrophobic interactions, but the type of aggregate formed is dependent on the ratio of the individual proteins present during heating (Dalgleish et al., 1997). Bovine serum albumin can form aggregates, but tends to only interact with itself instead of α-la and ß-lg because they denature at higher temperatures than BSA (Havea et al., 2001).

5.2.1 *Thermal Process Effects on ß-Lactoglobulin Solutions*

At neutral pH, ß-lg dimers begin to dissociate at around 55ºC and forms a molten globule state between 60 and 70ºC (de Wit, 2009; Prabakaran & Damodaran, 1997). At temperatures above 65ºC, ß-lg begins to denature and form dimers, trimers, and small oligomers (Morr & Ha, 1993; de Wit, 2009). Using high performance gel permeation chromatography (HP-GPC), Croguennec et al. (2003) determined that at neutral pH, unheated β-lg exists primarily as native dimers and monomers, but when heated, the native β-lg concentration decreases while the oligomer concentration increases. According to Havea et al. (2001), when ß-lg is heated by itself at neutral pH, aggregation and gelation are mainly controlled by the

formation of disulfide bonds between the ß-lg monomers during heating. They determined this by heating 10% (w/w) solutions of β-lg at 75°C at different time intervals up to 10 minutes and analyzed the samples using SDS-PAGE and alkaline-PAGE electrophoresis. The results showed that the monomer and dimer bands decreased in intensity as the heating time increased, while the bands corresponding to disulfide linked aggregates grew in intensity after 4 minutes of heating. This work is further supported by Croguennec et al. (2003), who added N-ethylmaleimide (NEM) to β-lg solutions and measured the reactivity of the free thiol group in β-lg to NEM using mass spectrometry and found that all free thiols had reacted with NEM after 4 minutes of heating at 85°C.

Aggregates are formed at neutral pH because the high temperatures denature the proteins and expose the thiol group that is buried in the hydrophobic regions of the protein (Surroca et al., 2002; Prabakaran & Damodaran, 1997; Majhi et al., 2006). By heating β-lg solutions for varying amounts of time and using SDS-PAGE, Surroca et al. (2002) and Prabakaran & Damodaran (1997) determined that at neutral pH, ß-lg will only form polymers when the dimer and trimer concentration has reached their max concentration meaning dimers and trimers are the polymer building blocks and not monomers. When the temperature gets above 85ºC, the aggregates reduce in size due to the loss of hydrophobic interactions at higher temperatures (de Wit, 2009).

5.2.2 *Thermal Process Effects on α-Lactalbumin Solutions*

The denaturation temperature of α-Lactalbumin is dependent on its form with apo-α-la having a denaturation temperature around 35°C while the holo-α-la denatures at around 65°C (Morr & Ha, 1993; Relkin et al., 1993). α-Lactalbumin is able to form a molten globule state where the α-helix domain remains intact but develops a looser structure. The transition from native to molten globule state is reversible at temperatures below 90°C(Kuwajima, 1989; Hong & Creamer, 2002). McGuffey et al. (2005) used differential scanning calorimetry, native and SDS-PAGE, and turbidity measurements to describe the aggregation mechanism of α-la. When α-la is heated above its denaturation temperature, but at or below 90°C, the denaturation is primarily reversible when cooled. However, when heated above 90°C, α-la forms non-native monomers through changes in the disulfide bonds within the protein which creates a reactive, free thiol group that can interact with other free thiols of α-la to form aggregates.

The effect of α-la structure (holo or apo) on aggregation under similar heating conditions appears has been investigated. Hong and Creamer (2002) showed that apo-α-la forms more dimers and aggregates through alkaline and SDS-PAGE, but McGuffey et al. (2005) determined that holo-α-la forms more large aggregates through turbidity measurements. It appears that the differences in covalent aggregates, seen by Hong and Creamer (2002), did not produce larger aggregates when covalent and non-covalent interactions are involved (McGuffey et al., 2005).

6 Polyphenols

Polyphenols are secondary metabolites created when plants are exposed to environmental stress, but are not involved in the growth or energy metabolism of plants (Harnly et al., 2007). In the plant kingdom, polyphenols are the most abundant non-nutrient class (Harnly et al., 2007). Increasing polyphenol intake in diets has been connected to decreasing the risk of chronic diseases like cancer and heart disease (Vauzor et al., 2012). The basic polyphenol structure is an aromatic ring with at least one hydroxyl group and another compound attached to the ring and the number of hydroxyls group and type of compound differentiate the types of polyphenols (Xu & Chen, 2011). The main classes of polyphenol groups are phenolic acids and flavonoids (Vauzor et al., 2012; Ozdal et al., 2013). Phenolic acids include hydrolyzable tannins, hydroxybenzoic acid, and hydroxycinnamic acids, while flavonoids include flavanols and anthocyanins (Vauzor et al., 2012).

Tannins are a group of polyphenols found in plants and is divided into two main groups: condensed and hydrolyzable tannins (Seeram et al., 2005; Deaville et al., 2007). Condensed tannins, also known proanthocyanidins, are found in foods like tea, grapes, and cranberries (Seeram et al., 2005). Hydrolyzable tannins are further divided into either gallotannins or ellagitannins and found in foods like raspberries, strawberries, and pomegranates (Deaville et al., 2007; Seeram et al., 2005).

6.1 Polyphenol Types

6.1.1 *Flavonoids*

Flavonoids are one of the largest classes of polyphenols and are furthered divided into groups with the most prevalent groups being flavanols and anthocyanins. Flavonoids are made up of three aromatic rings and the difference in structures among flavonoids is based on the number and arrangement of the hydroxyl groups and if there is alkylation and/or glycosylation (Vauzor et al., 2012; Beecher 2003). Flavonoids have *in vitro* hydrogen donating properties due to their phenolic hydroxyl group, which make them antioxidants, but they may not be as effective *in vivo* (Vauzor et al., 2012).

6.1.1.1 Flavanols

Flavanols are either monomers (catechin) or polymers (proanthocyanidins) and are not glycosylated, unlike the other flavonoids (Vauzor et al., 2012; Kanakis et al., 2011). Proanthocyanidins are made up of the catechins and its derivatives, the monomer form of flavanols that are bound together through carbon-carbon bonds and ether linkages (Hagerman & Butler, 1981; Beecher 2003). The types of proanthocyanidins are distinguished by their monomer composition, the degree of polymerization, and the types of bonds that link the monomers (Bourvellec & Renard, 2012). Anthocyanins are water soluble polyphenols that are usually found as glycosides (Vauzor et al., 2012). They are made up of a central heterocyclic ring and two benzoyl rings with hydroxyl and sugar groups attached (Chung et al., 2015).

6.1.2 *Hydrolyzable Tannins*

Hydrolyzable tannins are known to cause astringency in the mouth because they interact with the proteins found in saliva and precipitate and coat the mouth (Arapitsas, 2012).They have simpler structures than condensed tannins and are made up of polyols, such as glucose, with phenolic acids esterified to it (Kawamoto et al., 1996; Bourvellec & Renard, 2012; Beecher 2003). Gallotannins have gallic acid groups esterified to its central polyol, while ellagitannins are created through oxidation reactions between gallic acid residues on the polyol (Mueller-Harvey, 2001; Bourvellec & Renard, 2012).

Ellagitannins are found more in nature than gallotannins and found in foods like strawberries, pomegranates, and walnuts (Arapitsas, 2012). Ellagitannins do not have as much conformational freedom as gallotannins because their aromatic rings are held in place by intramolecular biphenyl linkages (Deaville et al., 2007; Frazier et al., 2003).

6.2 Polyphenol-Protein Interactions

Polyphenols are able to bind to whey proteins, which can influence the stability of the protein (Prigent et al., 2003). For example, Prigent et al. (2003) used differential scanning calorimetry to analyze the effect of chlorogenic acid on the denaturation temperature of different whey proteins. They found that chlorogenic acid increases the denaturation temperature for BSA, decreases the denaturation temperature of lysozyme, and has not effect on the denaturation temperature of α-la. While protein-polyphenol interactions are influenced

by polyphenol and protein concentration, other factors that affect the interactions range from environmental parameters like pH and temperature, to protein factors like amino acid composition and isoelectric point, to polyphenol factors like their size, conformational freedom, and affinity for water (Bourvellec & Renard, 2012; Prigent et al., 2003; Ozdal et al., 2013).

Environmental factors impact the ability of polyphenols to bind with whey proteins. Temperature plays an important role in these interactions because an increase in temperature denatures the whey proteins, breaks hydrogen bonds, and encourages hydrophobic interactions (Ozdal et al., 2013). For example, anthocyanins are able to bind to the whey proteins after they have been denatured because they can interact with the exposed hydrophobic region (Chung et al., 2015). pH is another parameter that affects the binding and precipitation of protein-polyphenol complexes. At acidic pH, the two tend to bind through non-covalent interactions, but as the pH increases, covalent bonding becomes more prevalent (Ozdal et al., 2013).

Like with proteins, the characteristics of the polyphenols impacts the complexation of polyphenols and whey proteins. The hydrophobicity of the polyphenols is related to its ability to interact with whey proteins because more hydrophobic polyphenols will bind better to the proteins since it will not want to bind with water (Bourvellec & Renard, 2012). Polyphenol type and structure influence the polyphenols affinity towards protein as well as molecular

weight and the degree of methylation, hydroxylation, glycosylation, or hydrogenation (Ozdal et al., 2013).

Whey proteins and polyphenols can bind to form complexes which can be soluble or insoluble (Siebert et al., 1996). Polyphenols can reversibly or irreversibly interact with whey proteins with reversible interactions being caused by noncovalent interactions, like hydrogen bonding and hydrophobic interactions, while irreversible interactions are formed using covalent bonds, like those involving protein thiol groups (Bourvellec & Renard, 2012; Ozadal et al., 2013). The ability of polyphenols to form hydrophobic interactions with whey protein is dependent on the size of the polyphenols and the exposed surface area of the protein, while hydrogen bond formation is contingent on the number of binding sites on the protein (Hagerman et al., 1998). Polyphenols can also cross-link proteins which modifies the net charge and structure of the whey proteins (Ozdal et al., 2013). Polyphenols tend to bind better to proline-rich proteins, like gelatin, compared to the globular proteins found in whey protein because proline-rich proteins usually have more binding sites (Deaville et al., 2007; Frazier et al., 2010).

6.2.1 *Condensed Tannin-Protein Interactions*

When condensed tannins bind to whey proteins, it can influence the protein's functional properties, including solubility, which typically decreases with an increase in polyphenol-protein interactions (Ozdal et al., 2013). For example, von Staszewski et al. (2012) found that

adding condensed tannins not only decreased the denatured temperature of β-lg, but also could decrease the gelation temperature of β-lg at pH 4.5 and 6, while not having any effect at pH 3. Condensed tannins can bind to proteins through hydrogen bonding and hydrophobic interactions, but after these initial interactions, the tannins can act as polydentate ligands and cross-link proteins to form soluble or insoluble complexes (Hagerman & Butler, 1981; Carvalho et al., 2004). The number of hydroxyl groups on a polyphenol can affect its ability to bind with whey proteins because the more hydroxyl groups, the more likely it will bind (Carvalho et al., 2004). The pH of whey protein-polyphenol complexes affected their ability to precipitate. At pH levels near the isoelectric point of whey protein, the net charge on the proteins in near neutral which allows for more particle aggregation, while at pH levels away from the isoelectric point, the net charge on the proteins is high enough to cause electrostatic repulsion between proteins which limits aggregation and allows for the formation of soluble complexes (von Staszewski et al., 2012).

There is a stoichiometric effect on the complexation of condensed tannins and whey protein. According to Siebert et al. (1996) and Carvalho et al. (2004), if there are more polyphenols than protein binding sites, then only a small amount of polyphenols can bind to the protein which limits the amount of cross-linking and promotes the formation of soluble complexes. If the polyphenol content is similar to the number of protein binding sites, the proteins and polyphenols are able to interact more and cross-link to form large aggregates. If the number of polyphenols is less than the number of protein binding sites, more small aggregates are

created with some cross-linked aggregates because the protein molecules block the polyphenols and decrease the amount of cross-linking.

6.2.2 *Hydrolyzable Tannin-Protein Interactions*

Hydrolyzable tannin-protein interactions are very different from the interactions with condensed tannins. The precipitation of whey proteins by hydrolyzable tannins can be explained by a two-step mechanism where the galloyl groups on the tannin bind to the surface area of the protein and, if enough galloyl groups attach to the protein, precipitation occurs (Kawamoto et al., 1996; Kawamoto & Nakatsubo, 1997). Protein precipitation is dependent on the number of galloyl groups that binds to its surface and not the number of hydrolyzable tannins because multiple galloyl groups from a single hydrolyzable tannin can bind to a whey protein (Kawamoto et al., 1996). The structure and placement of the galloyl groups also plays a role in the hydrolyzable tannin's ability to precipitate whey proteins, but this is not as important as the number of galloyl groups (Kawamoto et al., 1996). In order for hydrolyzable tannins to cross-link proteins, at least three galloyl groups are needed on the tannin; however, cross-linking is not necessary to precipitate the whey proteins (Kawamoto et al., 1996).

Protein concentration and pH play a major role in the precipitation of whey proteins by hydrolyzable tannins. Near the isoelectric point of the whey protein, fewer tannins are needed to precipitate the whey proteins, and while the concentration does not affect the precipitation

ability of hydrolyzable tannins, it does affect the type of complexes formed when initially mixed with the tannins (Kawamoto & Nakatsubo, 1997). The type of hydrolyzable tannin impacts the ability of the tannin to bind to the surface of the whey protein. Gallotannins tend to interact more strongly with proteins than ellagitannins because they are more flexible which allows for more gallotannins to bind to the surface of the protein; however, both tannins bind to the protein the same way (Frazier et al., 2003). The reason why ellagitannins have less conformational freedom is because their aromatic rings are held in place by intramolecular biphenyl linkages which limits the tannins ability to bind to globular tannins (Deaville et al., 2007).

6.2.3 *Comparing Tannin-Protein Interactions*

Condensed tannins tend to interact with protein through hydrogen bonds while hydrolyzable tannins usually bind to proteins through hydrophobic interactions (Bourvellec & Renard, 2012; Hagerman et al., 1998). Increasing the temperature increases the rate of protein precipitation by hydrolyzable tannins, but does not noticeably affect condensed tannins (Hagerman et al., 1998). In order to precipitate the same amount of protein, a higher concentration of hydrolyzable tannins is needed compared to condensed tannins. While there are many differences in the interactions between the two tannins with whey protein, both tannins are controlled by a stoichiometric effect where a certain ratio of polyphenols and protein is required for precipitation to occur (Hagerman et al., 1998). The structure of both tannins impacts their ability to bind with whey proteins, but hydrolyzable tannins are

dependent on the number of galloly groups, while condensed tannins are controlled by the degree of polymerization (Bourvellec & Renard, 2012).

6.3 Polyphenol Composition of Apple, Cherry, and Pomegranate Juice

6.3.1 *Apple Polyphenols*

The two main groups of polyphenols in apple juice are hydroxycinnamic acid derivatives and flavonoids (Malec at al., 2014; Wong-Paz et al., 2015). The flavonoids found in apple juice are flavanols, anthocyanins, flavonols, and dihydrochalcones (Wong-Paz et al., 2015; Malec et al., 2014). Flavonoids make up the majority of the polyphenols found in apple juice with flavanols (catechin and proanthocyanidins) being the most abundant group of polyphenols (Malec et al., 2014; Beecher 2003).

6.3.2 *Cherry Juice Polyphenols*

Depending on the type of cherry used, either anthocyanins or hydroxycinnamic acids are the largest polyphenol group in cherry juices (Schüller et al., 2015; Han et al., 2007). The type of cherry juice used affects the amount of anthocyanins present in the juice with sour cherry juices having high anthocyanin contents than sweet cherry juices (Schüller et al., 2015). Flavanols and flavonols are the other flavonoids found in cherry juice (Schüller et al., 2015; Han et al., 2007).

6.3.3 *Pomegranate Juice Polyphenols*

The types of polyphenols found in pomegranate juice include ellagitannins, gallic and ellagic acid, anthocyanins, catechins and caffeic acid, but the composition of polyphenols in pomegranate differ greatly between the cultivars and the region the fruit was grown (Li et al., 2015; Fischer et al., 2011). The majority of polyphenols in pomegranate juice are hydrolyzable tannins, specifically ellagitannins, with punicalagin being not only the most abundant ellagitannin, but the most abundant polyphenol as well (Fischer et al., 2011; Li et al., 2015).

Punicalagin is a water soluble polyphenol that has a molecular weight of 1.1 kDa (Kulkarni et al., 2007). Punicalagin is mainly found in the pith and carpellary membrane of pomegranates and is extracted in commercial juices by hydrostatically pressing the entire fruit which releases the ellagitannins since they have hydrophilic properties (Seeram et al., 2005). Hydrolyzable tannins, specifically punicalagin, have higher antioxidant activity than the other polyphenols in pomegranate, which is most likely due to the high number of hydroxyl groups on punicalgin (Gil et al., 2000). Gallic acid has only 3 free hydroxyls and cyanidin-3-glucoside (anthocyanin) and ellagic acid have 4 free hydroxyls, while in comparison, punicalgin has 16 free hydroxyl groups which makes it very hydrophilic and reactive with proteins since it can form more hydrogen bonds with proteins than other polyphenols (Kulkarni et al., 2007; Gil et al., 2000).

7 Whey Protein Beverages

In whey protein beverages, protein stability refers to the colloidal stability of the thermally processed whey protein in the beverages and this colloidal stability can be determined using a multitude of tests, including solubility and turbidity (Wagoner et al., 2015). pH is one of the main factors used to control whey protein colloidal stability as well as other factors of the beverage (Wagoner et al., 2015; Beecher et al., 2008). At neutral pH levels, whey protein beverages are typically opaque while acidic beverages are more clear (Beecher et al., 2008). Acidic pH whey protein beverages require milder thermal processes than neutral whey protein beverages (Wagoner et al., 2015; Beecher et al., 2008). Astringency in whey protein beverages is a sensorial problem that creates and unpleasant aftertaste in the mouth (Smithers, 2008; Beecher et al., 2008). It is only a major problem for acidic beverages since astringency occurs at low pH levels and reaches its max at around pH 3.4, and at these low pH levels, sourness is another problem that has to be addressed as well (Beecher et al., 2008).

In order for a beverage to stand out in the crowded beverage industry today, a beverage must meet at least one of the needs of a consumer today: high sensory quality, quenches thirst, low price, or provide a health benefit (Chavan et al., 2015). Whey protein beverages are a functional food, which is a food product that overs some sort of health benefit when eaten (Smithers et al., 1996). Functional food sales have increased worldwide and become a 100 billion dollar industry (Smithers, 2008). Whey protein is a popular ingredient in functional foods due to its neutral taste and high nutritional value as well as its ability to be used in fruit and vegetable juices (Chavan et al., 2015). Whey protein enriched fruit juices are one of the

most widely manufactured whey beverages and is a very simple drink to make as it usually only consists of whey protein and fruit juice or fruit juice concentrate (Chavan et al., 2015). The difficulty with these beverages is that polyphenols in the fruit juices interact with the whey proteins to form complexes and if the complexes become large enough, they will scatter light, resulting in the formation of a haze which is a prevalent problem with these types of beverages (Siebert et al., 1996).

8 Thesis Goals

The primary goal of this thesis is to analyze the aggregation of whey protein and the colloidal stability of two different types of beverages found in the marketplace across a range of different pH and protein concentration levels. The two beverages tested were a clear, acidic protein fortified flavored water and a protein fortified fruit juice containing individual juices or combinations of pomegranate, cherry and apple juice. In order to study the colloidal stability of the beverages, the solubility and turbidity of the beverages at the different pH and protein concentration levels were measured. Both of these properties are influenced by the aggregation of whey proteins in the beverages because the larger the aggregates that form, the lower the solubility and higher the turbidity becomes. To further analyze the aggregation of the proteins, particle size analysis and zeta potential measurements were taken at critical pH and protein concentration levels that were determined based on the solubility and turbidity results. While the protein fortified lemon flavored water primarily focused on protein-protein interactions, the fruit juices provided an additional goal by analyzing the

effects of juice components, such as polyphenols, and the complexes they form with whey protein and how that effects aggregation of the proteins.

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CHAPTER 2: COLLOIDAL STABILITY OF PROTEIN FORTIFIED LEMON FLAVORED WATER

1 Introduction

Functional foods are food or beverage products that offer the consumer some sort of health benefit and whey protein has become a popular ingredient in functional foods not only because it's highly nutritious, but also due to its functional properties (Smithers, 2008; de Wit 1990). While whey protein provides numerous benefits to functional foods, it also provides challenges that must be addressed by manufacturers in order to create a beverage that maintains colloidal stability. Colloidal stability is based on the aggregation and precipitation of whey proteins in a beverage after thermal processing and is determined by testing different properties of the beverage like solubility and turbidity (Wagoner et al., 2015). In order to better understand colloidal stability in these types of beverages, an acidic protein enriched flavored water was selected as a model beverage due to its low concentration of non-protein ingredients and being the beverage that would most resemble whey protein in water.

Protein fortified flavored water is primarily made up of whey protein and water, but does contain some non-protein ingredients that serve different purposes. Flavorings and sweeteners are added to provide the overall flavor desired for a particular beverage, which in this case was lemon flavor and sucralose. Citric acid and its salts are used as preservatives in acidic beverages because they reduce the development of yeasts and molds, as well as limit the growth of numerous types of bacteria and also are an effective way to increase the mineral content of a beverage (Araújo et al., 2011). Potassium and sodium citrate were added

to the lemon flavored water used for testing. A vitamin blend was also incorporated in order to increase the nutrient content of the beverage. The last ingredient added was phosphoric acid and was used to adjust the pH of the beverage to the desired level for testing.

Thermal processing is a procedure used to ensure microbial safety in the beverages, but thermal processes differ among beverages based on factors like the pH and if the beverage is going to be shelf stable or refrigerated. According to 21 CFR 114, the lemon flavored water is considered an acidified beverage because its pH is at or below 4.6 and only requires a mild heat treatment (<100°C) in order to be sold as a shelf stable beverage. This is an important consideration because the thermal process greatly influences the colloidal stability of a beverage. Higher temperature thermal processes encourage protein denaturation and aggregation and can lead to precipitation (Relkin & Mulvihill, 1996; Morr & Ha, 1993). The amount of time whey proteins are subjected to a thermal process influences the size of the aggregates formed where typically, the longer the proteins are heated, the larger the aggregates that form (Ndoye et al., 2012).

Aggregation of proteins is the most influential reaction affecting the colloidal stability of a beverage (Wagoner et al., 2015). Since β-lg is the most abundant protein found in whey proteins, it has the largest influence over the aggregation of whey proteins. At neutral pH, β-lactoglobulin (β-lg) denatures and aggregates primarily through the three step process of initiation, propagation, and termination where disulfide linkages are made between the free thiol group of a protein with the disulfide bond of another protein (Roefs & De Kruif, 1994; Hoffman & van Mil, 1999). At acidic pH, the three step mechanism does occur in a limited

capacity, but the main method of aggregation is hydrophobic interactions (Cornacchia et al., 2014).

Aggregation can be controlled through a number of different factors including pH and protein concentration. As protein concentration increases, more whey proteins are present in the solution which increases the chances proteins interact and if the protein concentration is high enough, proteins can cross-link enough to form gels (Mahler et al., 2009; Prabakaran & Damodaran, 1997). Controlling aggregation through pH involves changing the electrostatic charge of the proteins. At pH levels near the isoelectric point of a protein (β-lg≈5.1, α-lactalbumin (α-la) ≈4.5), the electrostatic charge is near zero which reduces the electrostatic repulsion and promotes aggregation, but by moving the pH away from the isoelectric point, the electrostatic repulsion increases as the charge increases (Cornacchia et al., 2014; Palazolo et al., 2000; Bernal & Jelen, 1985).

The colloidal stability of the protein enriched lemon flavored water will be dependent on the protein concentration and pH of the beverage. Protein concentration will have a greater effect on the gelation of the lemon flavored water, while pH will have more of an influence on the aggregation and precipitation of the whey proteins. When the protein concentration reaches a critical point at a specific pH, the proteins will form into a gel. If the pH is far enough away from the isoelectric point of the whey proteins, the electrostatic repulsion will be great enough to limit aggregation of the whey proteins, even after thermal processing, which will result in high solubility and low turbidity for the lemon flavored water. However, as the pH

moves closer to the isoelectric point, the electrostatic repulsion will decrease and lead to lower solubility and higher turbidity values.

The effects of pH and protein concentration on thermally processed whey protein are known, but when whey proteins are placed in a beverage system, the pH and protein concentration effects have not been as thoroughly studied. The goal of this research was to measure the colloidal stability of whey proteins in a clear, acidic beverage across a range of protein concentration and pH levels. Colloidal stability was determined by using solubility and turbidity measurements, while particle size and zeta potential measurements were measured to help further explain the differences in solubility and turbidity. Using these testing methods, the pH and protein concentration levels that create a stable beverage were determined.

2 Materials and Methods

2.1 Materials

All materials used were food grade. Potassium and sodium citrate were obtained from Fischer Scientific (Fair Lawn, NJ, USA), lemon flavoring from Virginia Dare (Brooklyn, NY, USA), sucralose from Tate & Lyle (London, UK), vitamin blend from Fortitech Premixes (Schenectady, NY, USA), and phosphoric acid from ICL Performance Products (Lawrence, KS, USA). BiPRO, whey protein isolate (WPI), was gifted from Davisco Foods International (Le Sueur, MN, USA). The BiPRO lot used contained 92.83% protein, which was determined by measuring the percentage of nitrogen in the powder using inductively

coupled plasma atomic emission spectroscopy and multiplying the nitrogen percentage by a conversion factor of 6.38.

2.2 Whey Protein Isolate Stock Solution Preparation

Stock solutions of WPI at 12% (w/w) protein were made by adding powder to deionized water (DI water), 18 MΩ resistance, and stirring for 3 to 4 hours at room temperature (22±2ºC) until completely solubilized. After the solutions were solubilized, they were held at 4ºC overnight. Solutions were stirred while equilibrating to room temperature (22±2ºC) before use. This procedure was completed three times to create three replications.

2.3 Protein Fortified Lemon Flavored Water Beverage Preparation

2.3.1 *Non-WPI Ingredients Stock Solution*

A stock solution containing all the non-WPI ingredients listed in Table 2.1 was prepared. This stock solution contained four times the concentration required for the samples since the solution would be added as 25 g of a 100 g lemon flavored water treatment. The stock solution was made by combining the non-WPI ingredients with DI water and stirred until all ingredients were completely solubilized. It required approximately 30 minutes for the ingredients to solubilize at room temperature.

2.3.2 *Lemon Flavored Water Sample Preparation*

Lemon flavored water samples were made at pH values of 3.2, 3.5, 3.8, 4.1, and 4.3, and WPI concentrations of 2, 4, 6, and 8% (w/w) protein. The samples were created by combining the WPI stock solution, non-WPI ingredients stock solution, and DI water to approximately 99 g with the appropriate concentrations. After reaching 99 g, 75% phosphoric acid was used to adjust the pH to the desired level and after reaching the desired pH level, the samples were brought up to 100 g with DI water. The samples were then stirred for 30 minutes to ensure complete mixture.

2.4 Lemon Water Thermal Processing

2.4.1 *Development of Thermal Process*

The lemon flavored water samples require a thermal process of 85ºC for 15 seconds. This was accomplished by heating samples in a silicon oil bath set at 115ºC. Temperature change was measured with a thermocouple held at the geometric center of the sample in a glass test tube (16 x 100 mm) filled with 8 ml of the protein solution. To hold the samples in the oil bath, a metal rack was used that had 25 slots, but after some initial testing, only the five slots in the first row were used to reduce variability among temperature change in the samples. For each protein concentration, the five slots in the first row were tested twice to ensure a proper heat treatment was developed for the samples. The other four slots not being tested were filled with test tubes containing 8 ml of DI water. The most accurate procedure involved heating the samples to approximately 86ºC and then removing them from the oil bath and holding them for 15 seconds. To ensure that the beverage remained above 85°C for the 15

second hold time, the beverage needed to be heated to a temperature slightly higher than 85°C. The residual heat held the samples above 85ºC for the required 15 second hold time. A thermocouple and a stop watch were used to record the times and temperatures for each slot, and the thermal process was chosen to ensure all treatments reached the heating time and temperature requirements. The thermal process for all the protein concentrations was heating for 95 seconds at 115ºC and then pull the samples from the oil bath and holding for 15 seconds.

2.4.2 *Thermal Processing*

For each pH level of each protein concentration, ten test tubes filled with 8 ml of lemon flavored water samples were thermally processed by heating in a silicon oil bath at 115ºC for 95 seconds, removing and holding at room temperature (22±2ºC) for 15 seconds, then placed in an ice bath for 4 minutes. Ten test tubes for each pH and concentration treatment were pooled and held overnight at 4ºC before being tested. If samples formed a gel during thermal processing or cooling, no further analysis was conducted.

2.5 Analysis of physical and chemical properties

2.5.1 *Protein Thermal Stability (solubility after thermal processing)*

The amount of protein remaining soluble after thermal processing was used as a measure of protein thermal stability. Samples were brought from 4ºC to room temperature (22±2ºC) by slow stirring at room temperature for an hour. For each treatment, 1600 µl of sample was centrifuged for 20 minutes at 17,200 x *g* at 15ºC to pellet aggregated protein. The supernatant

was decanted with a transfer pipette and analyzed for nitrogen using an Elementar vario MACRO cube (Mt Laurel, NJ, USA) using the liquid waste method. If samples could not be analyzed within two days, they were stored in a freezer set at -20ºC for up to 60 days. Frozen samples were allowed to thaw completely and then mixed thoroughly before nitrogen determination. To account for any possible non-protein nitrogen, a standard was created for each protein concentration which contained the same percentage of protein and ingredients as the samples, but the pH was not adjusted and the standards were not centrifuged in order to ensure the maximum nitrogen percentage was measured. This was necessary because the amount of non-protein nitrogen in the samples was below the threshold of nitrogen analyzer. Two samples were taken from each of the three replications to give a total of six measurements for each sample point and standard. The soluble protein percentage was then calculated as the amount of nitrogen remaining dispersed dividing that by the amount in the standard. Initial analysis showed that nearly all protein concentrations at or below pH 3.8 had solubility values that were greater than 100% soluble. It was determined that a protein solution at pH 6.8 (pH of the dispersed powder) had a nitrogen value at low pH that was 105% of the value of the neutral standard, and this was used as a correction factor for all samples.

2.5.2 *Turbidity*

The thermally processed samples were brought from 4ºC to room temperature (22±2°C) by slow stirring at room temperature for an hour. All samples were ten-fold diluted (800 µl of the sample with 7200 µl of DI water) and mixed thoroughly prior to testing. Diluting the

samples changed the pH, but all samples will be referred to by their initial pH. The turbidity of the samples for each treatment was measured using a turbidity meter (Hach 2100AN Turbidimeter, Loveland, CO, USA). Measurements were made using the ratio setting and reported in units of Nephelometric Turbidity Units (NTUs). Since the samples were diluted by a factor of 10 prior to measurement, the turbidity measurements were multiplied by 10 to take into account the dilution factor. Two samples were analyzed from each of the three replications giving a total of six measurements for each sample point.

2.5.3 *Particle Size Analysis and Zeta Potential*

Thermally and non-thermally processed samples were brought to room temperature (22±2°C) from 4°C by slow stirring at room temperature (22±2°C) for an hour. Based on the solubility and turbidity results, it was determined that only samples at pH 3.8 would be used for this test since they had no protein precipitation and large differences in turbidity across the protein concentrations. Only 2, 4, and 6% protein were tested because particles in the 8% protein were too large for accurate measurement. Thermally and non-thermally processed samples (taken from the same lemon flavored water sample) were used to determine how thermal processing affected the zeta potential and particle size of aggregates formed in the beverage. For each sample, 1600 µl were pipetted into a centrifuge tube and spun at 17,200 x *g* for 20 minutes at 15°C to remove the same size/density of aggregates that were removed in thermal stability analysis (see above). After centrifugation, all samples were then diluted to 1mg/ml using HPLC grade water, 18Ω resistance, and mixed thoroughly before testing. Electrophoretic mobility, z diameter, and %mass and %intensity were measured using the

Wyatt Technologies Möbiuζ (Santa Barbara, CA, USA). Electrophoretic mobility was measured with massively-parallel phase analysis light scattering and using that measurement, the zeta potential (mV) was calculated using Smoluchowski's equation. Three peak ranges were established with peak 1 representing particle diameters that were 0.1-10 nm, peak 2 signifying 10-100 nm, and peak 3 characterizing particles that were 100-1000 nm. The z diameter, peak %intensity, and peak %mass distribution were calculated for comparison of relative abundance in each peak. The z diameter is the intensity weighted average particle diameter found in the beverage. The peak %intensity is the intensity of light that is scattered by the particles that fall within each peak range in the sample, while the %mass represents the approximate concentration of particles in the peak ranges. Where %intensity is a measured variable, the %mass is a calculated variable that requires an assumption of the shape model of the particles in order to be determined and the model used was Rayleigh spheres to represent globular proteins. Three replications were tested for each sample point and the Möbiuζ was set to measure the electrophoretic mobility and %mass and %intensity for each sample five times.

2.6 Statistical Analysis

Two-way analysis of variance was completed using PROC GLM in SAS version 9.2 (SAS Institute Inc., Cary, NC) to determine the overall significance of the model and the effects, protein concentration and pH, as well as their interaction. All tests were carried out at the α=0.05 significance level.

3 Results

3.1 Protein Thermal Stability

Table 2.2: Lemon Flavored Water Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	93.4	31.1	2.36	0.0908
pH	4	9570	2390	182	<.0001
Protein %*pH	8	140	17.5	1.33	0.2683
Rep	2	77.2	38.6	2.93	0.0688

The pH was the only factor that significantly affected the solubility, while the protein concentration and interaction between pH and protein concentration did not significantly influence solubility of the lemon flavored water (Table 2.2). At pH values less than or equal to 3.8, the protein solubility of the lemon water for each protein concentration was greater than 90%, but there was a significant decrease in solubility as the pH moved from pH 3.8 to 4.1 (Figure 2.1). Beverages at pH 4.1 for 8% and pH 4.3 for 4%, 6%, and 8% formed gels. The trend in the data showed that the solubility values were fairly similar for all protein concentrations when the pH was less than or equal to 3.8, but the solubility values were more varied at pH 4.1. Solubility drastically decreased between pH 3.8 and 4.1, as this was approaching the isoelectric point of whey protein (about pH 5.2).

3.2 Turbidity

Table 2.3: Lemon Flavored Water Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	209000	70000	0.00	0.9999
pH	4	5190000000	1300000000	29.89	<.0001
Protein %*pH	8	567000000	70900000	1.63	0.1570
Rep	2	134000000	66900000	1.54	0.2306

Like with solubility, pH was the only factor that significantly affected the turbidity of the lemon flavored water (Table 2.3). Forming large protein aggregates decreases the solubility and increases turbidity. Trends in turbidity increasing (Table 2.4) therefore coincide with decreases in solubility (Figure 2.1). There was a gradual increase in turbidity as the pH changes from 3.2 to 3.8 for all protein concentrations, in this situation; turbidity showed more changes than solubility. Between pH 3.2 and 3.8, turbidity increased with pH and protein concentrations; however, at pH 4.1 and 4.3 the turbidity was noticeably higher and more varied. Due to the high and varied turbidity values and gelled samples for pH 4.1 and 4.3, no consistent trends were observed.

3.3 Particle Size Analysis

Larger whey protein particles will result in a decrease in solubility and increase in turbidity. When comparing the z diameters of the unheated and heated lemon flavored water, there was no noticeable trend as the protein concentration increased, but the unheated proteins had larger z diameters than those that had been thermal processed (Table 2.5). The z diameters represent the weighted average diameter and, since the beverages had a distribution of

particle sizes, do not provide the detailed analysis of particles. In terms of %mass, the majority of the concentration of particles for both the heated and unheated lemon flavored water was in the peak 1 range, with the unheated lemon flavored water having a higher %mass in peak 1for all protein concentrations than the heated beverage. Peak 1 covers the size range of individual molecules and possibly the first stage of aggregation. The heated beverage contained a higher concentration of peak 2 particles than the unheated, while the peak 3 %mass values showed no real trends between the two beverages. The %intensity values increased across the peak ranges for the unheated beverage where the peak 1 %intensity was the lowest while the peak 3 %intensity was the highest. However, the heated beverage had the highest %intensity values within peak 2 while the peak 1 %intensities were the lowest and all under 1.5%.

3.4 Zeta Potential

Zeta potential is a measure of the electrostatic charge on particles, which affects the attraction or repulsion of the particles. As zeta potential moves farther away from zero, the more electrostatic repulsion that occurs between particles which results in a higher amount of electrostatic stabilization. For both the heated and unheated lemon flavored water beverage, the zeta potential increased as the protein concentration increased at pH 3.8 (Table 2.5). Like with particle size, thermal processing had a noticeable effect on the zeta potential of the beverage where the heated beverage had higher zeta potentials than unheated.

4 Discussion

4.1 Protein Solubility After Thermal Processing

As expected solution pH had a significant effect on protein solubility. The solubility of protein in the beverage at or below pH 3.8 was ≥ 90% soluble (Figure 2.1). This was most likely due to the higher electrostatic repulsion between the whey proteins which reduces the amount of aggregation. This is supported by the results found in Table 2.5, where at pH 3.8, the zeta potential values for samples at 2, 4, and 6% protein are highly positive. Based on the electrostatic properties of proteins, it can be inferred that the samples at the lower pH levels would have more positive zeta potential values due to the greater amount of positive charge because the pH was farther from the isoelectric point of whey protein. This idea is supported by Majhi et al. (2006) whose work showed that below pH 3.5, whey proteins do not aggregate at high rates because of high electrostatic repulsion.

As the pH of the samples moved closer to the isoelectric point, the solubility of the whey protein began to decrease rapidly or was more likely to form a gel (Figure 2.1). Due to the capabilities of the Möbiuζ, it was not possible to measure the zeta potential of samples at pH 4.1 and 4.3 due to the low solubility or gelled samples. However, based on the work of Thongkaew et al. (2014) and Souza et al. (2012), it can be postulated that samples at these pH values would have less positive zeta potential values, which would reduce the electrostatic repulsion and result in more aggregation of proteins which would precipitate and reduce protein solubility or form a gel.

In order for a whey protein solution to become a gel, a critical concentration must be reached where enough whey protein aggregates can cross-link in order to form a gel network (Prabakaran & Damodaran, 1997; McGuffey & Foegeding, 2001). At pH 3.8 and below, the critical protein concentration needed to form a gel was outside the experimental range tested, but at pH 4.1, the critical concentration was 8% while it was only 4% for pH 4.3, as seen in Figure 2.1 and Table 2.4. These results demonstrate that pH influences the critical concentration needed to form a gel. As the pH moved closer to whey protein's isoelectric point, the proteins were able to aggregate more easily which decreased the critical protein gelation concentration.

4.2 Turbidity

Turbidity is the measurement of the amount of light scattered by the particles in the solution and is influenced by the size and number of particles (Mahler et al., 2009). The turbidity results support the solubility results in that they follow a similar trend with all samples at or below pH 3.8 having turbidity values much lower than those at pH 4.1 and 4.3. However, the turbidity results show more of the difference between samples than the solubility results, since the solubility of the samples at or below pH 3.8 was ≥ 90% soluble at all protein concentrations. With every increase in the pH, the change in turbidity was amplified with the greatest change in turbidity occurring between 3.8 and 4.1, which means that the critical point for rapid turbidity increase lies somewhere between those two pH levels (Table 2.4). This same trend can be seen in the solubility results as well with the steep decrease in solubility occurring between pH 3.8 and 4.1. This is because as the pH moves closer to the

isoelectric point of whey protein, there is a decrease in electrostatic repulsion which allows protein to aggregate more and form larger particles which scatters more light and increases turbidity (Cornacchia et al., 2014; Mahler et al., 2009). Souza et al. (2012) studied the effect of pH on the zeta potential of heated and unheated whey protein that further validates this idea. They found that unheated solutions of whey protein concentrate decreased from 18.8 mV to 4.1 mV as the pH increased from 3.0 to 4.0, while the heated whey protein concentrate samples decreased from 26.1 mV to 10.0 mV within the same pH range.

For each pH level, there was an increase in the turbidity or the amount of gelled samples as the protein concentration increased. With the addition of more protein, there is an increase in the amount of aggregates that can form which increases the amount of light scattered within the sample. This is supported by the results in Table 2.5 which show that at pH 3.8 there was an increase in the peak 2 %mass and %intensity as the protein concentration increased indicating that there was an increase in the number of aggregates within peak 2 which resulted in the scattering of more light. However, changes in pH had a greater effect on turbidity than concentration.

It is important to remember that the production of the lemon flavored water in an industrial setting will be much different than what was used in the benchtop testing. While the benchtop method used in thermally processing the lemon flavored water is similar to an industrial practice where the beverage is placed in its container and then pasteurized, the size of the container and volume of beverage used in the benchtop testing are much smaller than would be seen in industry and would affect the thermal load experienced by the beverage in

processing. Differences in thermal load will affect the size of the protein aggregates that form in the beverage because higher temperatures and longer heating times promote more denaturation of the proteins which will affect the aggregation the proteins (Ndoye et al., 2012). Also, the shear that the beverage would experience in flowing through a heat exchanger in an industrial setting was not part of our benchtop heating. The benchtop testing is a good starting point and provides important insight into the effects of pH and protein concentration on the colloidal stability of the lemon flavored water; however, scaling up the beverage production to that seen in an industrial setting would require additional verification to assure proper pH and protein concentration levels that produce a stable beverage.

4.3 Particle Size Analysis

At pH 3.8, protein solubility remained high while the turbidity remained low. This is explained by the size and number of aggregated particles in the beverage. The majority of the particles in the beverage were within peaks 1 and 2 (Table 2.5). The particles within peak 1 were too small to influence changes in solubility or turbidity; however, the peak 2 particles could have contributed to the turbidity of the beverage while still remaining soluble (Wang & Ismail, 2012). According to the %mass values, even though there were a considerable number of peak 2 particles and some peak 3 particles, they were not large enough to decrease protein solubility, but were large enough to influence the turbidity of the beverage and increased the turbidity as the protein concentration increased. The %intensity values support this idea because the peak 2 particles were responsible for most of the scattering and the intensity increased as the protein concentration increased. Since the turbidity values at pH 3.2

and 3.5 were lower than those at pH 3.8, the beverages at the lower pH would be expected to have a combination of size and number of aggregates that was lower than at pH 3.8, and likewise greater at pH 4.1 and 4.3.

When making measurements using dynamic light scattering, it is important to take into account the possibility of multiple scattering. In dynamic light scattering, light is assumed to be reflected off particles and back to a detector; however, if the light reflects off more than one particle before reaching the detector, it will affect the particle size measurement and this is called multiple scattering. The proper dilution was determined by measuring the particle size of the different beverages across a range of dilutions to determine the lowest concentration that did not result in multiple scattering. However, due to the differences between the beverages, picking a concentration that worked best for all the beverages at each pH and protein concentration proved difficult. The dilution chosen (1 mg/ml) was the best option for sensitivity and decreasing the chance of multiple scattering.

Unheated samples were also tested and used to see the thermal processing effects on particle size. Across all three peaks, the unheated beverages had noticeably larger z diameters than the heated beverages, but the analysis of the %mass and %intensity provide the better picture of the particle size distribution. While the heated beverages contained mostly peak 1 particles according to %mass, the amount of light scattered in the beverage by the peak 1 particles was very low (as expected due to their small size). Most of the light scattering came from peak 2 particles that were present in substantial amounts according to the %mass data. The unheated lemon flavored water had even higher amounts of peak 1 particles, which had more of an

influence on the amount of light scattering than the heated beverage's peak 1 particles. The majority of the light scattering for the unheated beverages came from peak 3 particles, but was only a small portion of the concentration of particles. This data shows that by heating the lemon flavored water, the concentration of peak 1 particles decreased while the amount of peak 2 particles increased, which means more larger aggregates are forming that can scatter more light and increase turbidity. . This idea is supported by the work of Iametti et al. (1995), who determined that the formation of β-lg aggregates increased with increases in temperature and protein concentration using electrophoresis to follow aggregate size. At acidic pH levels, the main method of aggregation for whey protein is hydrophobic interactions (Cornacchia et al., 2014). When a whey protein solution is heated, the proteins denature which exposes the hydrophobic regions which promotes hydrophobic interactions between proteins to create aggregates (Relkin & Mulvihill, 1996; Morr & Ha, 1993).

4.4 Zeta Potential

Zeta potential is used to relate to electrostatic stabilization of particles, with a higher absolute value indicating greater electrostatic stability. There was a noticeable trend where the zeta potential of the lemon flavored water increased after thermal processing (Figure 2.5). The unheated beverages had lower zeta potential values than the heated beverages. Most of the light scattering in the unheated beverage was caused by the large peak 3 particles, while the heated beverage had higher zeta potential values and the majority of the light scattering was caused by peak 2 particles. This suggests the possibility of thermal processing forming more

positively charged aggregates (increased peak 2); however, separation and characterization of particles is required to validate this hypothesis.

4.5 Colloidal Stability

The colloidal stability of proteins in a beverage is evaluated based on if the dispersion remains a sol, or if it destabilizes into a gel or precipitate. Furthermore, the optical clarity of the sol is important as some beverages are designed to be clear/translucent while opacity is acceptable in others. Acidic beverages are often formulated to be clear. Since turbidity is a function of size and number of particles, a small fraction of large particles can increase turbidity. Stability is based on proteins not precipitating or forming a gel over time, so solubility of the beverage should be high. For the purpose of this experiment, we will assume that the beverage has to have protein solubility $\geq 90\%$ and the turbidity values of ≤ 300 NTU to be achieve colloidal stability. Based on these results, the lemon flavored water was a stable beverage at all protein concentrations for pH 3.2 and 3.5, but only stable at 2, 4, and 6% protein at pH 3.8. The 8% protein beverage at pH 3.8 was stable in terms of solubility (92% soluble), but not turbidity where it was over 500 NTU. All the protein concentrations at pH 4.1 and 4.3 were not stable. While the other beverages at pH 3.8 were stable, the amount of protein in the 8% beverage produced enough aggregates to increase turbidity beyond the target range.

5 Conclusion

When the pH of the lemon flavored water was at 3.8 or below, the beverage was at or near 100% protein solubility and had a low turbidity values, but between pH 3.8 and 4.1, the solubility decreased sharply and showed a large increase in turbidity. The decreases in solubility and increases turbidity was a result of an increase in particle size due to aggregation occurring during thermal processing. From a stability standpoint, the protein fortified lemon flavored water was a viable beverage at all protein concentrations for pH 3.2 and 3.5 and at 2, 4, and 6% protein. At pH 3.8 the solubility remained high but turbidity increased. At pH $\geq$ 4.1, the low solubility would predict an unstable beverage.

Table 2.1: Protein Fortified Lemon Flavored Water Formulation

Ingredient	Formulation % (w/w)
BiPRO (WPI)	2, 4,6,8%
Flavor	0.1%
Potassium Citrate	0.1%
Sodium Citrate	0.1%
Sucralose	0.01%
Vitamin Blend	0.007%
Phosphoric Acid	<1%
DI Water	Remainder

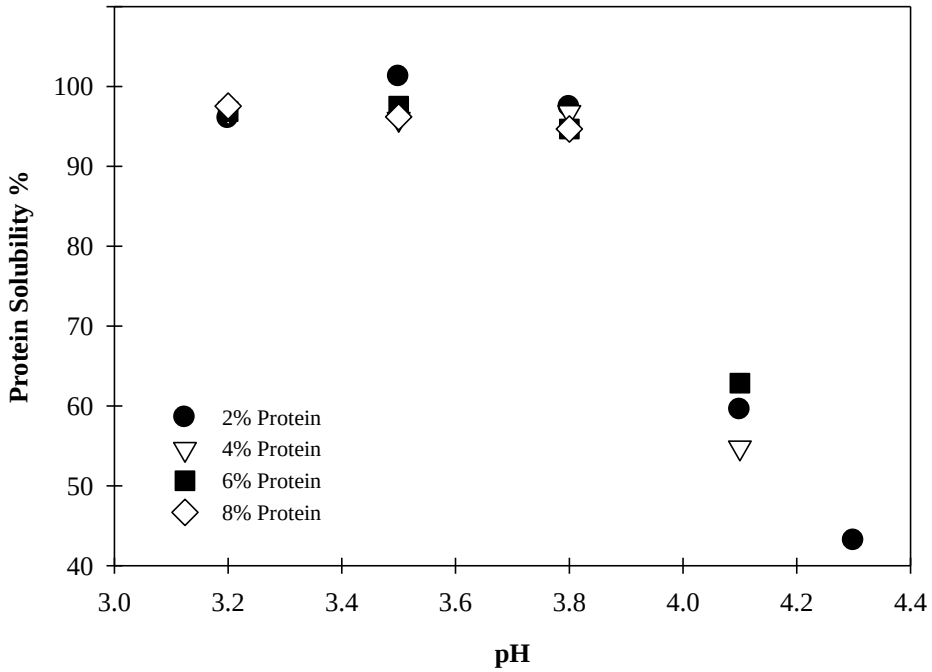

Figure 2.1: Relationship between pH and protein solubility after thermal processing at varying whey protein concentrations in a protein fortified lemon flavored water beverage. Gelled samples were not tested for solubility and do not show up on figure.

Table 2.4: Lemon Water Turbidity (in NTU) at Different pH and WPI Concentrations[1]

Protein Concentration	pH Level				
	3.2	**3.5**	**3.8**	**4.1**	**4.3**
2%	35.3±3.1	40.6±5.0	68.7±17.4	21600±1810	47000±1700
4%	49.3±1.4	55.0±3.6	113±36.8	42700±2910	gel
6%	65.3±5.2	79.5±10.2	258±105	38100±15000	gel
8%	85.5±5.2	118±15.0	533±309	gel	gel

[1]Mean values +/- standard error

Table 2.5: Protein Fortified Lemon Flavored Water Z Diameter, Peak %Mass, Peak %Intensity

Thermal Process	Protein %	pH	Zeta Potential (mV)	Z Diameter (nm)	Peak 1		Peak 2		Peak 3	
					%mass	%intensity	%mass	%intensity	%mass	%intensity
Unheated	2	3.8	18±1.9	98±1.5	95±0.1	8.7±0.2	1.2±0.4	19±9.7	2.8±0.7	54±11
	4		20±3.3	95±2.0	94±0.4	8.1±0.2	1.3±0.4	26±13	2.4±0.7	53±11
	6		24±1.2	100±9.3	95±1.0	6.7±0.2	0.75±0.1	14±5.0	3.0±0.2	60±5.7
Heated	2	3.8	26±3.0	62±4.2	73±2.6	1.4±0.3	14±1.7	59±6.8	7.0±1.7	32±3.3
	4		29±1.2	48±1.4	81±2.2	1.1±0.1	15±1.7	82±6.0	2.5±0.4	9.9±2.9
	6		31±1.1	58±2.1	68±8.7	0.5±0.2	28±9.8	88±5.8	1.6±1.6	2.4±2.4

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CHAPTER 3: COLLOIDAL STABILITY OF PROTEIN FORTIFIED FRUIT JUICES

1 Introduction

Whey protein fortified fruit juices are based on simple formulations to provide beverages that are enjoyable and have health benefits (Chavan et al., 2015). As with protein-fortified waters, protein aggregation is an important characteristic of the fruit juice beverage that must be controlled in order to assure colloidal stability (Wagoner et al., 2015).

Along with whey protein and water, the only other ingredient typically added to a protein fortified fruit juices are fruit juices or fruit juice concentrates added individually or in combination (Chavan et al., 2015). Fruit juices provide the flavor, color, and sweetness in beverages. The pH of protein fortified fruit juices is generally below pH 4.6, so only a mild thermal heat treatment (<100°C) is required if the goal is to sell as a refrigerated beverage (21 CFR 114). However, even a mild thermal process will affect colloidal stability of beverages by altering the extent of protein denaturation and aggregation (Relkin & Mulvihill, 1996; Morr & Ha, 1993).

Besides thermal processing, the beverage pH and protein concentration also determine the extent of aggregation and colloidal structure. Higher protein concentrations favor more extensive aggregation and increased aggregate size or possibly formation of a gel network, (Mahler et al., 2009; Prabakaran & Damodaran, 1997). One way to decrease aggregation is through electrostatic stabilization by shifting to a pH that maximizes charge (Bernal & Jelen, 1985; Cornacchia et al., 2014)). Controlling protein aggregation using pH is dependent on

the isoelectric point of proteins, the pH where the net charge is zero (Cannan, 1942). If the pH is near the isoelectric point, protein aggregation is at its greatest due to the lack of electrostatic repulsion, but as the pH moves away from the isoelectric point, the electrostatic repulsion increases which reduces the amount of protein aggregation (Cornacchia et al., 2014; Palazolo et al., 2000).

Fruit juices provide another challenge in maintaining colloidal stability because of the polyphenols found in the juices. Polyphenols are secondary metabolites found in plants that are known to have health benefits like reducing the risk of cancer and heart disease (Harnly et al., 2007; Vauzor et al., 2012). Polyphenols can affect the colloidal stability of the protein fortified fruit juice beverages because they can bind with whey proteins (Prigent et al., 2003). Tannins are one of the main groups of polyphenols found in fruit juices that interact with whey proteins (Seeram et al., 2005; Deaville et al., 2007). They can be sub-divided into *condensed* and *hydrolyzable* tannins.

Condensed tannins, also known as proanthocyanidins, are the polymer form of flavanols, which is a type of flavonoid, which are the largest class of polyphenols. Condensed tannins are made up of catechins, the monomer flavanol form, and its derivatives (Hagerman & Butler, 1981). Condensed tannins that bind to whey protein affect the aggregation of the proteins which influences functional properties like solubility (Ozdal et al., 2013). Condensed tannins are able to bind to whey proteins through hydrogen bonds and hydrophobic interactions, but can also cross-link proteins to form complexes (Hagerman & Butler, 1981; Carvalho et al., 2014). The complexation of condensed tannins and whey

proteins is based on a stoichiometric effect that is dependent on the number of polyphenols and protein binding sites (Siebert et al., 1996; Carvalho et al., 2004).

Hydrolyzable tannins are another class of polyphenols that have a simpler structure than condensed tannins, with the two main types being gallotannins and ellagitannins (Arapitsas, 2012; Bourvellec & Renard, 2012). Hydrolyzable tannins interact with proteins through hydrophobic interactions between the galloyl group of the tannin and the surface of the whey protein, and if enough galloyl groups attach to the protein, it can cause precipitation (Kawamoto et al., 1996; Kawamoto & Nakatsubo, 1997). Hydrolyzable tannins can cross-link proteins, but this is not a necessity to cause whey protein precipitation, which is primarily dependent on the total number of galloyl groups bound to the protein (Kawamoto et al., 1996). Using HPLC, Kawamoto et al. (1996) determined that bovine serum albumin will begin to precipitate after 30 galloyl groups have attached to the globular protein. Gallotannins are able to interact with proteins more than ellagitannins because they are more flexible which means more can bind to the surface of the protein (Frazier et al., 2003). Like with condensed tannins, there is a stoichiometric effect on the complexation of hydrolyzable tannins with whey protein and the ratio of the tannins to protein influences whether the complexes formed are soluble or insoluble (Hagerman et al., 1981; Kawamoto et al., 1996).

While there has been a lot of research on whey protein and polyphenol interactions, most focuses on the ability of polyphenols to precipitate whey proteins (Hagerman et al., 1998; Kawamoto et al., 1996), or the mechanism of interactions between polyphenols and whey proteins (Hagerman & Butler, 1981; Deaville et al., 2007; Siebert et al, 1996; Frazier et al,

2003; Frazier et al, 2010). There is little research on the effects of polyphenols on the colloidal stability of proteins in beverages where thermal processing and pH are adjusted to assure safety and optimize quality. The objective of this investigation was to determine the influence of three different fruit juices (pomegranate, cherry, and apple) on colloidal stability of whey protein isolate. To simulate a beverage, the amount of fruit juice was fixed and the pH and protein concentration varied. Colloidal stability was evaluated based on protein solubility and beverage turbidity after thermal processing.

2 Materials and Methods

2.1 Materials

All materials used were food grade. Sodium hydroxide was obtained from Fischer Scientific (Fairlawn, NJ, USA) and phosphoric acid from ICL Performance Products (Lawrence, KS, USA). Dennick Fruitsource (Tampa, FL, USA) gifted pomegranate, dark sweet cherry, and apple juice concentrate. Tree Top, Inc (Selah, WA, USA) gifted dark sweet cherry and apple juice concentrate and pomegranate juice concentrate was obtained from Brownwood Acres Foods, Inc (Eastport, MI, USA). Whey protein isolate (WPI) was provided by Davisco Foods International (Le Sueur, MN, USA). The WPI contained 92.8% protein, which was determined by measuring the percentage of nitrogen in the powder using inductively coupled plasma atomic emission spectroscopy and multiplying the nitrogen percentage by a conversion factor of 6.38.

2.2 Whey Protein Isolate Stock Solution Preparation

Stock protein solutions were made using the same method as in- *2.2 Whey Protein Isolate Stock Solution Preparation* found in Chapter 2.

2.3 Fruit Juice Brix Adjustments

Juice concentrates were adjusted to a Brix value of 45º for pomegranate and cherry and 40º for the apple juice concentrate. The juice concentrates were combined with deionized water (18Ω) mixed completely, and the Brix was measured to within 0.5º of their desired Brix value using the Brix method for the Anton Paar Abbemat 550 refractometer (Ashland, VA, USA).

2.4 Protein Fortified Fruit Juice Beverage Preparation

Formulations for the fruit juice and WPI solutions are listed in Table 3.1. Only fruit concentrates from Dennick Fruitsouce were used in the mixed fruit juice formulation. Solutions containing WPI and fruit juices were made at pH values of 3.2, 3.5, 3.8, 4.1, and 4.3 for protein concentrations of 2, 4, 6%, and 8% (w/w) protein. The appropriate amount of stock WPI solution and fruit juice were mixed and the pH adjusted to the desired levels using 75% phosphoric acid or 1 M sodium hydroxide. After adjusting the pH, the sample was brought to final weight with deionized water. The samples were allowed to stir for 30 minutes to ensure complete mixing before being heated. To test the effects of using a different thermal process, a second set of mixed fruit juice samples were made using the same formulation as in Table 3.1, but only for pH levels 3.2, 3.5, and 3.8.

A separate group of solutions was made with the entire fruit juice component (20% w/w) coming from individual fruit juices. For the individual fruit juices, samples using the fruit concentrates from Dennick Fruitsource are referred to as fruit juice A, while samples using the concentrates from TreeTop (cherry and apple) and Brownwood (pomegranate) were referred to as fruit juice B. Samples containing 2, 4, 6, or 8% (w/w) protein were made for all the individual fruit juice formulations. Fruit juice A samples were tested at pH levels of 3.2, 3.5, 3.8, and 4.1, while fruit juice B samples were tested only within the critical range of protein stability based on the results of the fruit juice A samples. For pomegranate juice B, only pH levels 3.2, 3.5, and 3.8 were tested while cherry and apple juice B were tested at pH 3.5 and 3.8. In discussing the results, the WPI-fruit juice treatments will be referred to as, for example, cherry juice A, so as to not add the wordiness of saying "cherry juice A with WPI."

2.5 Fruit Juice Thermal Processing

2.5.1 *Development of Thermal Process*

A process of 75ºC for 15 seconds was used as a typical thermal process employed in manufacturing these beverages. Processing conditions were established as was done for lemon flavored water (Chapter 2)- *2.4.1 Development of Thermal Process*, with the difference being 10 ml of sample was placed in the test tubes instead of 8 ml and the oil bath was at 100ºC. After testing all of the slots in the first row of the rack, the best procedure for pasteurization was to heat the samples for 110 seconds and then hold for 15 seconds.

2.5.2 *Thermal Processing*

For each pH value and protein concentration, five tests tubes filled with 10 ml each of sample were heated in a 100°C oil bath for 110 seconds, held for 15 seconds at room temperature (22±2°C), and then placed in an ice bath for four minutes to cool. The five test tubes for each pH level and protein concentration were pooled and held overnight at 4°C. If solutions formed a gel during thermal processing or cooling, no further analysis was conducted. This method was used for both the mixed and individual fruit juice formulations.

To test the effects of thermal processing treatments, a second set of mixed fruit juice samples were thermally processed using the same methods found in- 2.4.2 *Thermal Processing* in Chapter 2.

2.6 Analysis of physical and chemical properties

2.6.1 *Protein Thermal Stability (solubility after thermal processing)*

The solubility for the mixed and individual fruit juices were measured using the same method as in- *2.5.1 Protein Thermal Stability (solubility after thermal processing)* in Chapter 2. The same correction factor applied to the lemon flavored water was applied to all fruit juice samples.

2.6.2 *Turbidity*

The turbidity for the mixed and individual fruit juices were measured using the same method as in- *2.5.2 Turbidity* in Chapter 2.

2.6.3 *Particle Size Analysis and Zeta Potential*

The particle size analysis and zeta potential for the mixed and individual fruit juices were measured using the same method as in-2.5.3 *Particle Size Analysis and Zeta Potential* in Chapter 2. The mixed fruit juice and pomegranate juice samples were tested at pH 3.2 and 3.5 while the cherry and apple juice samples were tested at pH 3.5 and 3.8. Some samples within these pH ranges could not be tested due to having too large of particles for accurate measurement or due to the gelation, and are so noted in the results. The particle size analysis only showed a representation of the soluble aggregates, which relates to the solubility percentage, with the insoluble particles having been removed.

2.6.4 *Polyphenolic Analysis*

Polyphenol analysis of the fruit concentrates was carried out by Dr. Mary Grace from the Plants for Human Health Institute (Kannapolis, NC). Total phenolics were measured using the Folin-Ciocalteu assay based on the method developed by Singleton et al. (1999). The total phenolics were measured in units of milligrams per liter gallic acid equivalents based on a gallic acid standard curve. Normal phase HPLC-fluorescence analysis was used to determine the amount of proanthocyanidins in each of the fruit concentrates. An Agilent 1200 HPLC with a fluorescence detector and photodiode array detector (Aglient Technologies, Englewood, CO) with a Develosil Diol column (250 mm x 4.6 mm x 5μm) (Phenomenex Terrence, CA) was used to measure the proanthocyanidins based on the method developed by Brownmiller et al. (2009). A binary mobile phase was used with the first phase being a combination of acetonitrile/acetic acid (98:2 v/v) and the second phase

being methanol/water/acetic acid (95:3:2 v/v/v). The fruit juice concentrates were diluted with methanol and 20 µl of the diluted concentrates were injected into the column after being filtered through a 0.2 µm PTFE filter (Fischer Scientific, Pittsburgh, PA). An external standard curve was made with commercial standards, ranging from monomers (DP1) to tetramers (DP4), in order to calculate the concentration of proanthocyanidins in units of mg/ml. The fluorescence detector managed the elution of compounds with excitation at 230 nm and emission at 321 nm while the photodiode array detector monitored emission at 280 nm.

2.7 Statistical Analysis

Two-way analysis of variance was completed using PROC GLM in SAS version 9.2 (SAS Institute Inc., Cary, NC) to determine the overall significance of the model and the effects, protein concentration and pH, as well as their interaction. All tests were carried out at the α=0.05 significance level.

3 Results

3.1 Protein Thermal Stability

3.1.1 *Mixed Fruit Juice*

Table 3.2: Protein Fortified Mixed Fruit Juice (75°C for 15 secs) Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	66	22	3.11	0.0437
pH	4	1590	397	56.32	<.0001
Protein %*pH	6	228	38	5.39	0.0010
Rep	2	111	56	7.88	0.0021

Table 3.3: Protein Fortified Mixed Juice (85°C for 15 secs) Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	260	87	12.99	0.0001
pH	2	2699	1350	202.16	<.0001
Protein %*pH	3	159	53	7.95	0.0018
Rep	2	2.8	1.4	0.21	0.8134

The solubility of the beverages thermally processed to 75 and 85°C for 15 seconds both were significantly affected by pH and protein concentration, and the interaction between the two factors was also significant (Table 3.2 and 3.3). The replications only had a significant effect on the beverage thermally processed to 75°C for 15 seconds (Table 3.2). Protein concentration and pH played a significant role in protein solubility of fruit juice and whey protein beverages, but in a different way when compared to the protein fortified lemon water (Chapter 2). The protein solubility decreased at an almost linear rate as the pH increased from 3.2 to 4.3 (Figure 3.1), which is a stark difference to the lemon water whose protein

solubility remained greater than 90% up to pH 3.8 and decreased drastically at pH 4.1 and 4.3 (Figure 2.1).

The effect of protein concentration was pH specific and showed some unexpected trends. At pH 3.2 and 3.5, surprisingly, the protein solubility increased as the protein concentration increased (Figure 3.1). At pH 3.8 and above, protein solubility decreased or had minimal change as protein concentration increased.

Changing the thermal process also affected protein thermal stability. While maintaining a similar trend as the beverage processed to 75°C, the solubility of the mixed fruit juice heated to 85°C decreased at each pH level compared to those heated to 75°C, with the size of the decrease increasing as the pH level increased. The beverage processed at 85°C also had more samples form gels over the same pH and protein concentration range compared to those heated to 75°C (Figure 3.1).

3.1.2 *Individual Fruit Juices*

Table 3.4: Protein Fortified Pomegranate Juice A Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	231	77	13.72	<.0001
pH	3	232	77	13.76	<.0001
Protein %*pH	4	87	22	3.89	0.0170
rep	2	16	7.8	1.39	0.2732

Table 3.5: Protein Fortified Pomegranate Juice B Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	415	138	37.28	<.0001
pH	2	1195	598	161.17	<.0001
Protein %*pH	4	162	41	10.94	0.0001
Rep	2	1.7	0.87	0.23	0.7942

Table 3.6: Protein Fortified Cherry Juice A Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	61	20	2.48	0.0813
pH	3	2502	834	101.78	<.0001
Protein %*pH	8	108	13	1.64	0.1576
Rep	2	485	242	29.58	<.0001

Table 3.7: Protein Fortified Cherry Juice B Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	15	5.1	1.48	0.2702
pH	1	0.50	0.50	0.15	0.7083
Protein %*pH	2	2.4	1.2	0.35	0.7119
Rep	2	8.2	4.1	1.19	0.3363

Table 3.8: Protein Fortified Apple Juice A Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	96	32	3.90	0.0225
pH	3	462	154	18.72	<.0001
Protein %*pH	5	45	8.9	1.09	0.3949
Rep	2	19	9.5	1.15	0.3346

Table 3.9: Protein Fortified Apple Juice B Solubility Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	89	30	10.19	0.0008
pH	1	20	20	6.81	0.0206
Protein %*pH	3	18	6.0	2.05	0.1526
Rep	2	35	18	6.02	0.0130

Two different sources of juice were investigated to see if this may be a major variable. The protein solubility of pomegranate juice A and B were both significantly affected by pH, protein concentration, and the interaction between the two factors (Table 3.4 and 3.5). The pH and replication significantly affected the solubility of cherry juice A, but no factors had a significant effect on the solubility of cherry juice B (Table 3.6 and 3.7). While apple juice A and B solubility was significantly affected by the pH and protein concentration of the beverage, but the replication was only significant for apple juice B (Table 3.8 and 3.9).

Overall trends in protein solubility as a function of pH and protein concentration were compared. Pomegranate juice A had a similar trend to that of the mixed fruit juice with the solubility decreasing at a more linear rate than the lemon flavored water (Figure 2.1), but the solubility values were lower for pomegranate juice A at pH 3.2 and 3.5 (Figure 3.2A). Pomegranate juice A has the same protein concentration effect as the mixed fruit juice where the solubility increased as the protein concentration increased at pH 3.2 and 3.5. Pomegranate juice B (Figure 3.2B) showed trends similar to pomegranate juice A and the mixed fruit juice, but with some differences. Pomegranate juice B has a protein concentration effect at pH 3.2 and 3.5, where the solubility increases as the protein concentration increases above 2% protein. However, unlike the mixed fruit juice and pomegranate juice A, the solubility decreases as the protein concentration increases from 4% protein to 6 and 8% protein at pH 3.2, but 2% protein has the lowest solubility. At pH 3.5 for pomegranate juice B, the increase in solubility from 2% to the higher protein concentrations is the largest among the mixed fruit juices and both pomegranate juices and the solubility for 4, 6, and 8% protein are nearly identical to the solubility of pomegranate juice A at the same conditions. Both pomegranate

juices had more samples form a gel when compared to mixed fruit juice over the same pH and protein concentration ranges.

Cherry juice A had a trend in protein solubility that was more similar to the lemon flavored water (Figure 2.1) than the mixed fruit juice (Figure 3.1), where all samples between pH 3.2 and 3.8, except for 8% at pH 3.8, had a solubility value that was greater than 90% and a large decrease in solubility at pH 4.1 (Figure 3.3A). However, the decrease in solubility at pH 4.1 is not nearly as drastic as that of the lemon flavored water. Cherry juice A only had one sample form a gel at 8% protein for pH 4.1. Figure 3.3B shows that cherry juice B had protein solubility trends similar to cherry juice A at pH 3.5 and 3.8, with the main difference being that cherry juice B formed a gel at 8% protein for pH 3.8, unlike cherry juice A.

Like the cherry juices, the trend in protein solubility for apple juice A more closely resembles the lemon flavored water (Figure 2.1) than the mixed fruit juice (Figure 3.1). The protein solubility levels of apple juice A at pH levels 3.2, 3.5, and 3.8 were all greater than 90%, and then decreased at pH 4.1 (Figure 3.4A). The drop in solubility at pH 4.1 was not as large as the decrease for the lemon flavored water and cherry juice A. Apple juice A had samples form a gel at 4, 6, and 8% protein at pH 4.1 and at 8% protein for pH 3.8, which shows that pH and protein concentration had an effect on the gelation of samples at the higher pH levels. Apple juice B (Figure 3.4B) had similar solubility results as apple juice A at pH 3.5 and 3.8, but the solubility values were slightly lower for apple juice B and did not form a gel at 8% protein for pH 3.8.

3.2 Turbidity

3.2.1 *Mixed Fruit Juice*

Table 3.10: Protein Fortified Mixed Fruit Juice (75°C for 15 secs) Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	52800000	17600000	2.82	0.0587
pH	4	1950000000	488000000	78.18	<.0001
Protein %*pH	6	1740000000	290000000	46.42	<.0001
Rep	2	28600000	14300000	2.29	0.1211

Table 3.11: Protein Fortified Mixed Juice (85°C for 15 secs) Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	18000000	6010000	0.71	0.5621
pH	2	5380000000	2690000000	316.25	<.0001
Protein %*pH	3	472000000	157000000	18.50	<.0001
Rep	2	21700000	10800000	1.27	0.3065

The pH and interaction of pH and protein concentration had significant effects on the turbidity of both mixed fruit juices (Table 3.10 and 3.11). As with solubility, changes in turbidity are different than those observed for the lemon water (Table 2.4). Overall, turbidity values for the mixed fruit juices (Table 3.12) were higher than those of the lemon water and treatments with pH values greater than or equal to 3.8 followed the same trends as those of the lemon water where the turbidity increased as the protein concentration increased. However, at pH values of 3.2 and 3.5, turbidity in the mixed fruit beverage had the opposite trend of the lemon water. At pH 3.2 and 3.5, the turbidity decreased as the protein concentration increased. This suggests that the number of particles, size of particles, or combination of both decreased as the protein concentration increased.

3.2.2 *Individual Fruit Juice*

Table 3.13: Protein Fortified Pomegranate Juice A Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	124000000	41200000	91.63	<.0001
pH	3	773000000	258000000	572.64	<.0001
Protein %*pH	4	422000000	105000000	234.35	<.0001
Rep	2	1130000	563000	1.25	0.3075

Table 3.14: Protein Fortified Pomegranate Juice B Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	43300000	14400000	6.21	0.0044
pH	2	758000000	379000000	163.21	<.0001
Protein %*pH	4	186000000	46600000	20.07	<.0001
rep	2	8560000	4280000	1.84	0.1871

Table 3.15: Protein Fortified Cherry Juice A Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	1440000	481000	0.13	0.9442
pH	3	2720000000	906000000	236.32	<.0001
Protein %*pH	8	96300000	120000000	31.42	<.0001
Rep	2	7580000	3790000	0.99	0.3844

Table 3.16: Protein Fortified Cherry Juice B Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	5240	1750	0.02	0.9947
pH	1	608000	608000	8.30	0.0138
Protein %*pH	2	132000	66000	0.90	0.4316
Rep	2	315000	158000	2.15	0.1593

Table 3.17: Protein Fortified Apple Juice A Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	3580	1190	0.00	0.9999
pH	3	221000000	73700000	95.35	<.0001
Protein %*pH	5	361000	72200	0.09	0.9924
Rep	2	870000	435000	0.56	0.5777

Table 3.18: Protein Fortified Apple Juice B Turbidity Two-Way ANOVA

Source	DF	Type IV SS	Mean Square	F Value	Pr > F
Protein %	3	761000	254000	27.94	<.0001
pH	1	1290000	1290000	141.93	<.0001
Protein %*pH	3	687000	229000	25.22	<.0001
Rep	2	51600	25800	2.84	0.0921

For both pomegranate juices (Table 3.13 and 3.14), the protein concentration, pH, and interaction between the two factors had a significant effect on turbidity. The pH and interaction between protein concentration and pH significantly affected the turbidity of cherry juice A (Table 3.15), while only pH had a significant effect for cherry juice B (3.16). The turbidity of apple juice A (Table 3.17) was only significantly influenced by beverage pH, while apple juice B was significantly affected by pH, protein concentration, and their interaction (Table 3.18)

Overall trends in turbidity showed differences among individual juices. Both pomegranate juices had similar trends to that of the mixed fruit juice where the turbidity decreased as protein concentration increased for pH 3.2 and 3.5, with the opposite trend occurring at pH levels at and above pH 3.8 (Table 3.19). This trend is very different from the lemon flavored water, where the turbidity increased as protein concentration increased for all pH values (Table 2.4). The pomegranate juices had much higher turbidity values than the lemon flavored water; however, pomegranate juice A had much higher turbidity values than the mixed fruit juice, while pomegranate juice B had turbidity values that were more similar to the mixed fruit juice.

Cherry juice A had trends that were similar with both the lemon flavored water and mixed fruit juice (Table 3.20). Cherry juice A had low turbidity values that more resembled the lemon flavored water at pH 3.2 and 3.5, but was much higher in turbidity at pH 3.8. At pH 3.5, cherry juice A showed a decrease in turbidity as the protein concentration increased from 2-6%, which is like the trend seen in the mixed fruit juice and pomegranate juice, but the decrease in turbidity as well as the overall values were much lower for cherry juice A. Cherry juice B followed the same trend as the lemon flavored water where the turbidity increases as the protein concentration increases for each pH level (Table 3.20). Although the trend differs between the two cherry juices at pH 3.5, the turbidity values for the two are similar; however, cherry juice B was much lower in turbidity than cherry juice A at pH 3.8 for all protein concentrations except for 8% protein which formed a gel.

Results in Table 3.21 show that apple juice A displayed similar trends as cherry juice A, with low turbidity values at pH 3.2 and 3.5 and a large increase at pH 3.8, with pH 3.5 also having a decrease in turbidity as the protein concentration increased, but only when increasing from 2 to 4% protein, and the decrease was much smaller than that of cherry juice A. Apple juice B, in general, was lower in turbidity than apple juice A at pH 3.5 and 3.8. Apple juice B had a similar trend to apple juice A where the turbidity decreased as the protein concentration increased from 2 to 4%; however, unlike apple juice A, apple juice B decreased in turbidity as the protein concentration increased from 2 to 6% at pH 3.8, with the decrease in turbidity being much larger than that of pH 3.5. Neither apple juice exhibited trends that were like those found with lemon flavored water and only partially followed trends displayed by the

mixed fruit juice, with the decrease in turbidity as protein increased in some cases, but not on the same scale.

3.3 Particle Size Analysis

3.3.1 *Mixed Fruit Juice*

The Z diameter of particles in unheated, and both types of thermally processed mixed fruit juices decreased as the protein concentration increased at pH 3.2, but at pH 3.5, the Z diameter values remained fairly consistent for all the protein concentrations (Table 3.22). For the unheated mixed fruit juice, peak 1 had the highest %mass of particles while peak 3 had very low %mass values for both pH 3.2 and 3.5. The % intensity was greatest at peak 2 for both pH 3.2 and 3.5, except at 2% at pH 3.2 where peak 3 scattered the most light.

The highest % mass for the mixed fruit juice thermally processed to 75°C was found in peak 1 at pH 3.2, but at 2% protein, the %mass in peak 1 was much lower than the higher protein concentrations and had higher %mass values in peak 2 and 3. Like with pH 3.2, at pH 3.5, peak 1 made up most of the %mass, but there were substantial %mass values in peak 3 while peak 2 was only a small part of the %mass. In terms of %intensity, the majority of the light scattering was caused by peak 2 particles for pH 3.2 while peak 3 particles scattered the most for pH 3.5. The amount of light scattered by peak 1 particles was low for both pH levels.

Peak 1 particles made up the majority of the % mass at pH 3.2 and 3.5 for the mixed fruit juice thermally processed to 85°C, except for 2% protein at pH 3.2, where peak 2 had the

highest %mass of particles. While peak 1 had the largest %mass at pH 3.5, peak 2 and 3 also had substantial %mass values. Peak 2 had the largest %intensity for both pH levels while the amount of light scattered by peak 1 particles was equal to or less than 1.1% for all concentrations at both pH 3.2 and 3.5.

3.3.2 *Individual Fruit Juice*

For pomegranate juice A at pH 3.2 and pomegranate juice B at both pH 3.2 and 3.5, the z diameter of the beverages decreased as the protein concentration increased, but the z diameter increased as the protein concentration increased for pomegranate juice A at pH 3.5 (Table 3.23). The %mass of particles within peak 1 for pomegranate juice A increased at pH 3.2 and decreased at pH 3.5 as the protein concentration increased and had the highest %mass values for each concentration level, except for 4% protein at pH 3.2 whose largest %mass was found in peak 2. The peak 2 %mass decreased as the protein concentration increased at pH 3.2 and the %mass values were less than 1% at pH 3.5, while the opposite was found for the peak 3 %mass where pH 3.2 had the lowest values and pH 3.5 had the higher values. In terms of light scattering, both pH 3.2 and 3.5 had low %intensity values within peak 1 with the majority of the %intensity coming from peak 2 for pH 3.2 and peak 3 for pH 3.5. In general, the highest %mass numbers were found within peak 1 for both pH 3.2 and 3.5 for pomegranate juice B. And with the only exception being 8% at pH 3.2, the lowest %mass values were found in peak 3. However, the lowest %intensity for pomegranate juice B was in peak 1, and peak 2 had the highest %intensity with the exception of 8% protein at pH 3.2 where peak 3 particles were responsible for scattering the most light.

Cherry juice A had a decrease in z diameters as the protein concentration increased at pH 3.5, but showed consistent z diameter values at pH 3.8 for 2, 4, and 6% protein, but saw a large increase at 8% protein and a similar trend was seen for cherry juice B at pH 3.8 with the increase occurring at 6% (Table 3.24). The z diameters were fairly similar for all protein concentrations at pH 3.5 for cherry juice B. Peak 1 for cherry juice A contained the largest %mass numbers for both pH 3.5 and 3.8 while, in general, peak 3 contained the lowest %mass amounts. With %intensity, the least amount of light scattering was caused by the particles in peak 1 while the peak 2 particles were responsible for scattering the most light, with the exception of 8% protein at pH 3.8 where peak 3 particles scattered the most light. In cherry juice B, the peak 3 particles had the smallest %mass for pH 3.5 and 3.8 with peak 1 containing the highest %mass values for both pH levels. At pH 3.5, the lowest %intensity was caused by the peak 3 particles, but the peak 1 particles scattered the least amount of light at pH 3.8.

At pH 3.5, both apple juice A and B had z diameters that decreased as the protein concentration increased, while at pH 3.8, apple juice A did not show any real trends for z diameter, but the z diameters for apple juice B at pH 3.8 increased as the protein concentration increased (Table 3.25). For both apple juices, the largest %mass of particles were in peak 1 and the smallest %mass of particles were in peak 3 for both pH levels. Also, in general, the greatest light scattering was caused by peak 2 particles for both apple juices and pH levels, but peak 1 for pH 3.5 and peak 3 for pH 3.8 produced the lowest %intensity values.

3.4 Zeta Potential

Zeta potential values for all fruit juice beverages were plotted with their corresponding solubility value on one plot for each protein and pH combination over the pH range of 3.2 to 3.8, (Figure 3.5). This was done to determine if zeta potential had a noticeable effect on protein solubility. The zeta potential values ranged from around 11 mV to close to 34 mV. Nearly all the pH and protein concentration combinations maintained a solubility that was near or greater than 90% across the entire range of zeta potentials, with the exception of a few outliers. Based on this trend, it can be concluded that the zeta potential of soluble aggregates in the beverages at pH 3.2, 3.5, and 3.8 did not any trends in the percent soluble protein after thermal processing.

4 Discussion

4.1 Protein Solubility after Thermal Processing

4.1.1 *Mixed Fruit Juice*

Like with the lemon flavored water (Figure 2.1), the protein concentration and pH of the beverage affected the solubility of the protein fortified fruit juice beverage (Figure 3.1A). However, the protein concentration and pH effects were much different in the mixed fruit juice when compared to the lemon flavored water. For example, at pH 3.2 and 3.5, the protein solubility increased as the protein concentration increased, but reversed the trend at pH 3.8. In order to determine why these two beverages produced such different solubility curves, the differences between the two beverages had to be addressed.

When comparing the methods of how the two beverages were prepared, the first difference between the two was the thermal processing procedure used. To determine if the differences in thermal processing were responsible for the difference in protein solubility trends, the mixed fruit juice was thermally processed using the lemon flavored water method and the solubility was measured. If the thermal process was responsible for the difference in solubility trends between the beverages, the mixed fruit juice solubility curve would change to more closely resemble the solubility curve of the lemon flavored water. However, as Figure 3.1B shows, the trend in the solubility curve did not change to look like the lemon flavored water and actually decreased in solubility while maintaining the same trend as in Figure 3.1A.

With the thermal process not being responsible for the differences in solubility, the next difference to analyze was the ingredients in the two beverages, particularly the fruit juice concentrates. The differences between the solubility curves of the two beverages were most likely due to the presence of polyphenols or other components in the fruit juices. Polyphenols are secondary metabolites found in plants that have the ability to bind with whey proteins and form soluble or insoluble complexes (Harnly et al., 2007; Prigent et al., 2003). The concentration of proteins and polyphenols influence the complex formation between whey proteins and polyphenols (Bourvellec & Renard, 2012; Prigent et al., 2003; Ozdal et al., 2013). By measuring the solubility of proteins in the individual fruit juices across the same pH and protein concentration range as the mixed fruit juice, the fruit juice that has the greatest influence on the mixed fruit juice solubility curve can be determined.

4.1.2 *Individual Fruit Juices*

When comparing the solubility curves of the individual fruit juices, it became apparent that the pomegranate juices (Figure 3.2) had the solubility curves that most closely resembled the mixed fruit juice (Figure 3.1A). The pomegranate juice solubility had the same near linear decrease in solubility as the pH increased and showed the same effect at pH 3.2 and 3.5 where the solubility increased as the protein concentration increased. Whereas the cherry juice (Figure 3.3) and apple juice (Figure 3.4) beverages maintained solubility curves that were more similar to the lemon flavored water (Figure 2.1).

Polyphenols have the ability to bind to whey proteins and form soluble or insoluble complexes that would alter the colloidal stability of a protein enriched fruit juice beverage (Siebert et al., 1996; Ozdal et al., 2013). When comparing the total number of phenolic compounds in each of the fruit juices, both pomegranate juices contained the most total phenols, followed by the cherry juices, which were nearly three times less than the pomegranate juices, and lastly the apple juices which contained about half the amount of total phenols found in the cherry juices (Figure 3.6). The total number of phenols found in the fruit juices is an important characteristic to consider because there can be a stoichiometric effect on the complexation of whey proteins and polyphenols (Siebert et al., 1996; Carvalho et al., 2004; Kawamoto et al., 1996). In addition, while the total number of phenols is important, the types of polyphenols are also critical as they may increase or decrease the extent of protein aggregation and thereby influence solubility (Hagerman & Butler, 1981; Carvalho et al., 2004; Kawamoto et al., 1996).

The most abundant polyphenols in apple juice are hydrocinnamic acid derivatives and flavonoids, which includes proanthocyanidins (condensed tannins) and anthocyanins (Malec et al., 2014; Wong-Paz et al., 2015). Cherry juices contain anthocyanins, hydroxycinnamic acids, and flavanols, like proanthocyanidins, and the type of cherry juice can affect the amounts of these polyphenols (Schüller et al., 2015). Like with the other juices, pomegranate juice contains anthocyanins and proanthocyanidins, but pomegranate juice is the only one of the three to contain hydrolyzable tannins, specifically ellagitannins, which makes up the majority of the polyphenols in pomegranate juice (Li et al., 2015; Fischer et al., 2011). Figure 3.7 shows the amounts of proanthocyanidins in the fruit juices where cherry juices contained the most proanthocyanidins followed by the pomegranate juices and then lastly the apple juices, which were considerably lower than the other juices. Based on the literature and polyphenolic contents, condensed and hydrolyzable tannins will be discussed in relation to their influence on whey protein aggregation and solubility.

The condensed tannins (proanthocyanidins) found in all of the fruit juices are able to form complexes with whey protein but in a very specific way. The initial interactions between condensed tannins and whey proteins are typically hydrogen bonds and hydrophobic interactions; however, the tannins can act as polydentate ligands and cross-link proteins to form complexes, meaning there are at least two specific binding sites on the proanthocyanidins (Hagerman & Butler, 1981; Carvalho et al., 2004). Cherry juice had the highest amount of condensed tannins; however, the cherry juice solubility curve closely resembled the curve of the lemon water. Therefore it would appear that the amount of polyphenols is too low to greatly influence protein solubility. While there were minimal

changes in solubility, complexation may be forming soluble aggregates in cherry and apple juices, as will be seen more clearly in the turbidity results.

Hydrolyzable tannins bind to whey protein very differently than the condensed tannins. The complexation of hydrolyzable tannins and whey proteins is based on a two-step mechanism where the galloyl groups of the tannin bind to the surface of the protein through hydrophobic interactions and if enough galloyl groups attach to the protein, precipitation will occur which would reduce protein solubility (Kawamoto et al., 1996; Kawamoto & Nakatsubo, 1997). Hydrolyzable tannins can cross-link proteins, but requires a tannin with at least three galloyl groups; however, cross-linking is not necessary to precipitate whey proteins (Kawamoto et al., 1996). pH also has an influence on the complexation of whey proteins and hydrolyzable tannins. The closer the pH is to the isoelectric point, the lower the concentration of tannins required to precipitate proteins (Kawamoto & Nakatsubo, 1997). Based on literature and the polyphenolic composition, hydrolyzable tannins may explain the solubility curve of the pomegranate juice and mixed fruit juice. At pH 3.2 and 3.5, the pH appears to be far enough from the isoelectric point of whey protein to require a minimal amount of hydrolyzable tannins to bind and cause protein precipitation. This requirement is met at low protein concentrations, but as protein concentration increased, the tannin to protein ratio decreased, which would explain the increase in solubility. However, it appears that at pH 3.8, the pH is too close to the isoelectric point and fewer tannins are required to precipitate the proteins. This resulted in decreased solubility as protein concentration decreased at the higher pH levels. This is supported by the work of Kawamoto & Nakatsubo (1997), who measured the ability of hydrolyzable tanins to precipitate bovine serum albumin and found that the tannins

precipitated more protein as the pH moved closer to the isoelectric point of bovine serum albumin.

While polyphenols offer a plausible explanation for the differences in the colloidal stability of the beverages, there could be other components of the fruit juice that control the stability. For example, pectin is known to affect the complex formation between polyphenols and whey proteins (Thongkaew et al., 2014). Although the fruit juice concentrates were depectinized, if small amounts of pectin remain after processing, it could affect the complexation between the proteins and polyphenols. Also, Schneider et al. (2016) found that the different sugars and acids found in fruit juices can influence the complexes that polyphenols and whey proteins form; however, the main factor in the complex formation was the polyphenols. Further investigation would need to be conducted to validate if the polyphenols are the main parameter influencing the colloidal stability of the beverages or if another factor like pectin or acids can significantly affect the complexes that form between polyphenols and whey proteins.

4.2 Turbidity

4.2.1 *Mixed Fruit Juice*

The amount of aggregation that occurs affects whether an aggregate is soluble or insoluble. Solubility is only affected by the largest groups of aggregates which are pelleted by centrifugation, but turbidity is related to soluble and insoluble aggregates. A larger soluble aggregate may not decrease solubility, but would increase turbidity (Wang & Ismail. 2012).

The turbidity results for the mixed fruit juice (Table 3.12) supports the solubility results where there was a decrease in turbidity as the protein concentration increased at pH 3.2 and 3.5 and then the trend reversed at pH 3.8 and higher. Likewise, the mixed fruit juice that was thermally processed to 85°C for 15 seconds maintained the same trends as the mixed fruit juice thermally processed to 75°C for 15 seconds, but did have higher turbidity values over all (Table 3.12). This corresponds with the decreased overall solubility with the higher temperature process. Turbidity and solubility are both detecting groups of the aggregate population, so similar overall trends are expected.

4.2.2 *Individual Fruit Juice*

The turbidity of both pomegranate juices followed the same trends as the mixed fruit juice, but pomegranate juice A had turbidity values at pH 3.2 and 3.5 that were much larger than the turbidity values of pomegranate B (Table 3.19) and the mixed fruit juice (Table 3.12). The ratio of hydrolyzable tannins to proteins is an important factor in the ability of the tannins to precipitate whey protein (Kawamoto & Nakatsubo, 1997). When the ratio of hydrolyzable tannins to proteins is high, more tannins can bind to each protein through hydrophobic interactions and result in precipitation, but when the tannin to protein ratio is low, the amount of tannins that binds to each protein is reduced enough to prevent precipitation (Figure 3.8). By comparing the amounts of pomegranate juice and polyphenols that would be present in the beverages, it becomes plausible that hydrolyzable tannins are the most likely fruit juice component altering protein aggregation. When comparing the turbidity values of the mixed fruit juice (Table 3.12) with pomegranate juice A, the turbidity values

are much higher for the pomegranate juice A which contains 20% pomegranate juice in its formulation compared to the 13% pomegranate juice in the mixed fruit juice. This suggests that with less pomegranate juice, the number of hydrolyzable tannins decreases which results in lower turbidity values at pH 3.2 and 3.5 as the protein concentration increases because the ratio of tannins to proteins is more favorable towards colloidal stability. To further validate this idea, the turbidities of the two pomegranate juices can be compared with pomegranate juice A (Table 3.19) having a lot higher turbidity than pomegranate juice B, even though they both contain 20% pomegranate juice in the beverage. Figure 3.6 shows that pomegranate juice B had a noticeably smaller total phenolic composition compared to pomegranate juice A, which would mean that fewer hydrolyzable tannins would be present in pomegranate juice B.

Like with solubility, the turbidity values of the cherry (Table 3.20) and apple juices (Table 3.21) more closely resembled the turbidity values of the lemon water (Table 2.4), but the two fruit juices had higher turbidity values than the lemon water. While the number of phenols and condensed tannins may not have been high enough to greatly influence the solubility of the beverages, the effect of the polyphenol-whey protein complexation was seen more clearly in turbidity. The pH of the beverages affects the ability of the condensed tannins to bind with the proteins because near the isoelectric point, the electrostatic repulsion between the proteins is very low which promotes aggregation (von Staszewski et al., 2012). This idea supports the results of the cherry and apple juice turbidity values because as the pH moves closer to the isoelectric point, the turbidity increased greatly. The complexation of whey proteins and condensed tannins is also dependent on a stoichiometric effect. Using flow

nephelometric analysis to analyze the effects of grape seed tannins on bovine serum albumin, Carvalho et al. (2004) proposed that when there are more polyphenols than protein binding sites, then only a small amount of polyphenols can bind to the proteins which limits the amount of cross-linking that can occur and encourages soluble complex formation which would keep solubility high. If the number of polyphenols and protein binding sites are equivalent, then more cross-linked aggregates will form because the proteins and polyphenols are able to cross-link more which could result in a loss of solubility due to the precipitation of the aggregates. But if there is more protein binding sites than polyphenols, some cross-linked aggregates can form but more small aggregates will form because there are enough proteins to block the polyphenols from cross-linking (Figure 3.9).

4.3 Particle Size Analysis

4.3.1 *Mixed Fruit Juice*

Based on the particle size range for each of the peaks, peak 1 (0.1-10 nm) represents the soluble monomers, dimers, and oligomers in the beverage, while peak 2 (10-100 nm) and 3 (100-1000 nm) represent the soluble aggregates that will affect the turbidity of the beverage but not the solubility. Therefore, the discussion of particle size is just related to turbidity as insoluble aggregates were removed before completing particle analysis.

As expected, thermally processing the mixed fruit juices caused a change in the particle size distribution of the beverages. Based on the %mass values, the unheated mixed fruit juice generally had higher concentrations of peak 1 particles and lower concentrations of peak 2

and 3 particles than both thermally processed mixed fruit juices. This would be expected because thermally processing the beverages would result in more denaturation of the whey proteins and affect the formation of larger aggregates. Although the thermal processing influenced the differences in the distribution of particles, the majority of the %mass of soluble particles fell within peak 1, meaning that most of the whey proteins in the beverages are not forming higher order aggregates, but the thermal process did cause an increase in the concentration of peak 2 and 3 particles which would increase the turbidity of the beverages. This is also seen in the %intensity values of the mixed fruit juice beverage, where the majority of the light being scattered by the beverages was by the particles found in peak 2 and 3.

Based on the solubility and turbidity results, the amount of aggregates, soluble and insoluble, should have increased as the pH increased; however, the pH also influenced the solubility and turbidity trends across the protein concentrations and this should also be seen when analyzing the particle size distribution. At pH 3.2 and 3.5, the mixed fruit juices increase in solubility and decrease in turbidity as the protein concentration increased. The particle analysis data reinforced the trends seen at pH 3.2, where the z diameters and %mass of peak 2 and 3 particles decreased as the protein concentration increased (Table 3.22). However, although both pH 3.2 and 3.5 share similar trends in solubility and turbidity, the particle distribution of pH 3.5 does not share that same trends seen at pH 3.2. At pH 3.5, as the protein concentration was increased, the z diameters and %mass values were all similar and showed no noticeable trends.

4.3.2 *Individual Fruit Juice*

Both pomegranate juice A and B share the similar trend with the mixed fruit juice where the solubility increased and the turbidity decreased as the protein concentration increased at pH 3.2 and 3.5 and like the mixed fruit juice, the particle size analysis of the pomegranate juices supported the trends seen in solubility and turbidity. For both pomegranate juices at pH 3.2 the z diameters and the %mass of peak 2 and 3 particles decreased as the protein concentration increased, but the trends were not as clear at pH 3.5. At pH 3.5, similar trends could be seen, but not as clearly because for pomegranate juice A at pH 3.5, the particles could not be accurately measured at 2% and 4% protein because of the turbid nature of the samples even after removing the insoluble particles. The particle size distribution could be measured at 6% and 8% protein, indicating an increase in smaller aggregates that could be properly measured. Pomegranate juice had decreases in z diameter and peak 3 %mass as the protein concentration increased, which supports the results seen in turbidity even though %mass of peak 1 and 2 did not show any noticeable trends.

There was very little difference in solubility for all the apple and cherry juices at pH 3.5 and 3.8, but the turbidity for both juices increased as the pH increased. In general, the z diameters for the juices at pH 3.8 were larger than those at pH 3.5, except for apple juice A where the z diameters did not show any noticeable trends between the two pH levels. The turbidity of the beverages most likely was not influenced by the particles in peak 1 due to the small size of the particles. When analyzing the %mass and %intensity of peaks 2 and 3 individually, there were no noticeable trends to explain the trends seen in turbidity; however, when combining the %intensity of the two peaks together, the trend shows that the amount of scattered light

by peaks 2 and 3 together was higher for the beverages at pH 3.8. This trend corresponds with the increasing turbidity values as the pH increases.

4.4 Zeta Potential

Figure 3.7 displays the relationship between zeta potential and solubility for all of the mixed fruit juices at pH 3.2, 3.5, and 3.8. The zeta potential did not appear to have any noticeable relationship to protein solubility. The zeta potential ranged from around 11 mV to 34 mV and the solubility remained at or above 90% for nearly all of the beverage samples tested, with only a few exceptions. However, the zeta potential measurements reported were only for the soluble particles remaining in the beverage. While the zeta potential for the insoluble particles could not be measured, the data gathered does suggest that the lowest level of zeta potential needed to create a beverage with high solubility is ~ 11 mV but this needs to be further tested. Moreover, what is more interesting is that this shows a wide range for zeta potential in the beverages, which would directly influence secondary aggregation upon storage.

4.5 Colloidal Stability

Using the same requirements as for the lemon water, ≥90% soluble and ≤300 NTU turbidity, the colloidal stability of the fruit juice beverages were evaluated. The mixed fruit juice processed to 75°C for 15 seconds was only stable at 4, 6, and 8% protein at pH 3.2, while the mixed fruit juice processed to 85°C for 15 seconds was stable at 6 and 8% protein at pH 3.2. At pH 3.2, pomegranate juice A was stable only at 8% protein and pomegranate juice B was

stable at 4 and 6% protein. Neither mixed fruit juice nor pomegranate juice formed a stable beverage at any pH level above 3.2. Both cherry juices were stable at all protein concentrations at pH 3.2, but only cherry juice A produced a beverage that was stable at all protein concentrations at pH 3.5 while cherry juice B was only stable at 2% protein at pH 3.5. Like the cherry juices, all protein concentrations at pH 3.2 were stable for both apple juices and all protein concentrations were stable at pH 3.5 for apple juice A while only 6% protein was stable at pH 3.5 for apple juice B. None of the fruit juice beverages produced a beverage with colloidal stability at pH 3.8 and above.

5 Conclusion

All of the fruit juices were able to create beverages with colloidal stability at pH 3.2, but only the apple juices and cherry juices produced stable beverages at all protein concentrations at pH 3.2. Also, the apple and cherry juices were the only juices with stable beverages at pH 3.5. The cherry and apple juices had trends in solubility that were similar to those seen in the lemon flavored water, where the solubility remained high from pH 3.2 to 3.8 before decreasing at pH 4.1. However, the mixed fruit juices and pomegranate juices had a very different trend where the solubility increased as the protein concentration increased at pH 3.2 and 3.5 before reversing at pH 3.8. The turbidity trends corresponded with the solubility trends as the turbidity decreased as the protein concentration increased at pH 3.2 and 3.5, which suggests that the aggregates within the two types of juices became smaller as the protein concentration increased at those two pH levels. At pH 3.8 and 4.1, no beverages with colloidal stability were produced.

Table 3.1: Protein Fortified Mixed Fruit Juice Formulation

Ingredient	Formulation % (w/w)
Whey Protein Isolate	2, 4, 6, 8 (% protein)
Pomegranate Juice Concentrate (45° Brix)	13%
Dark Sweet Cherry Juice Concentrate (45° Brix)	5%
Apple Juice Concentrate (40° Brix)	2%
Phosphoric Acid	<1%
Sodium Hydroxide	<1%
DI Water	Remainder

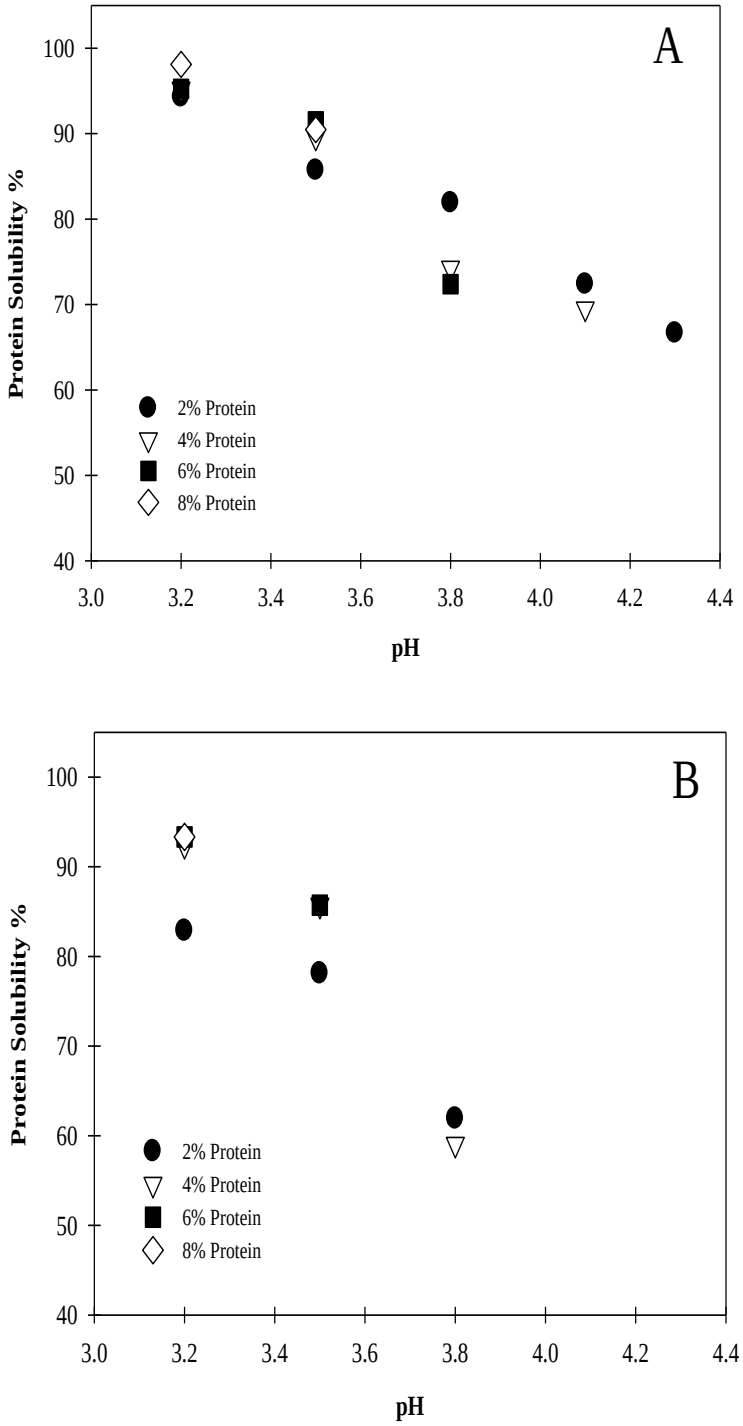

Figure 3.1: Relationship between pH and protein solubility at varying whey protein concentrations in a protein fortified mixed juice beverage thermally processed to (A) 75°C for 15 seconds and (B) 85°C for 15 seconds.

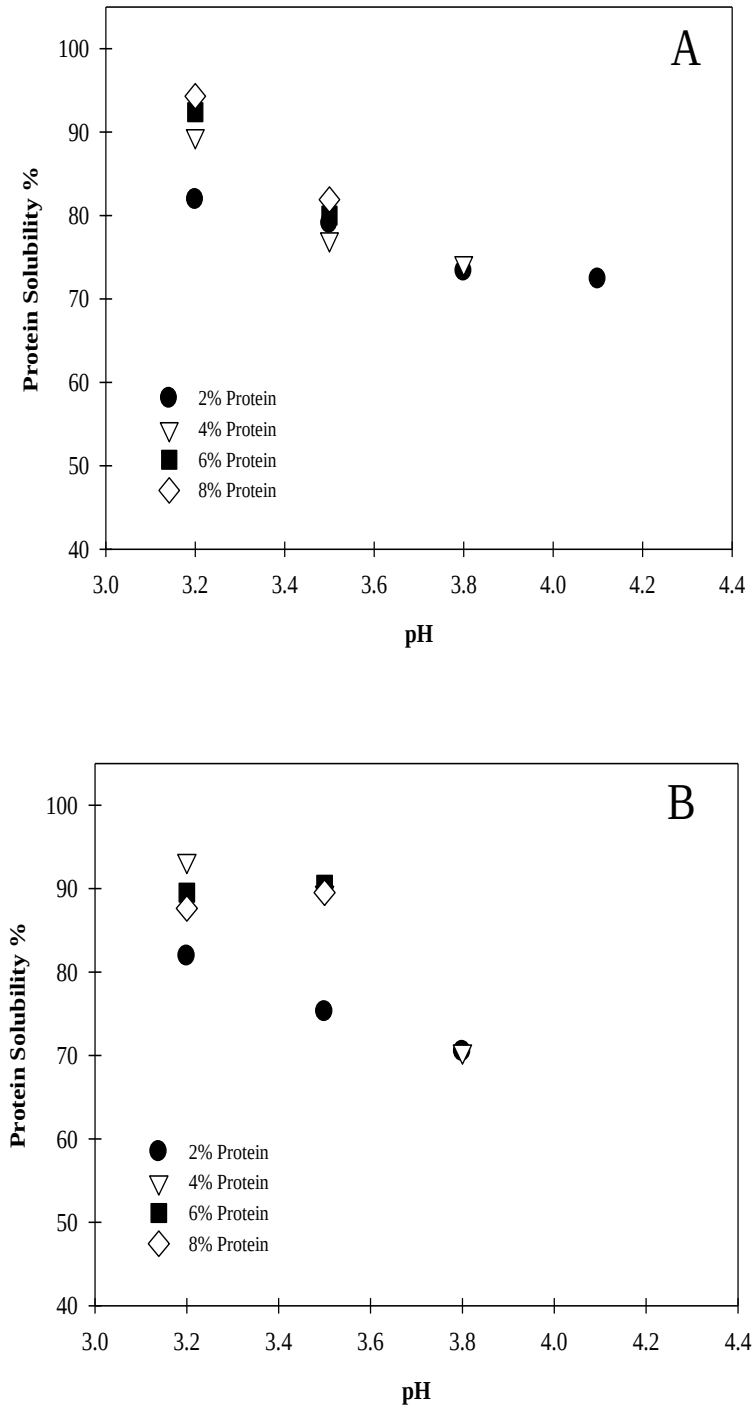

Figure 3.2: Relationship between pH and protein solubility at varying protein concentrations for thermally processed protein fortified pomegranate juice A and B.

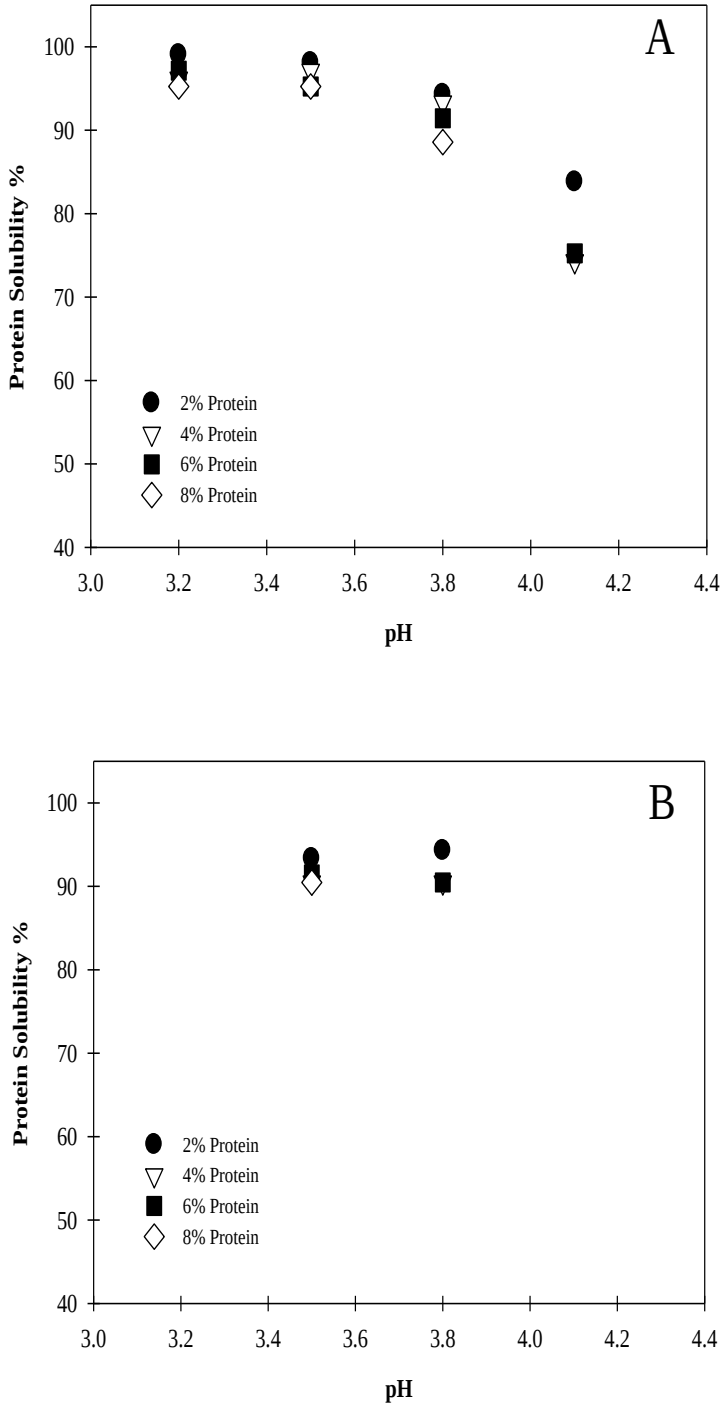

Figure 3.3: Relationship between pH and protein solubility at varying protein concentrations for thermally processed protein fortified cherry juice A and B.

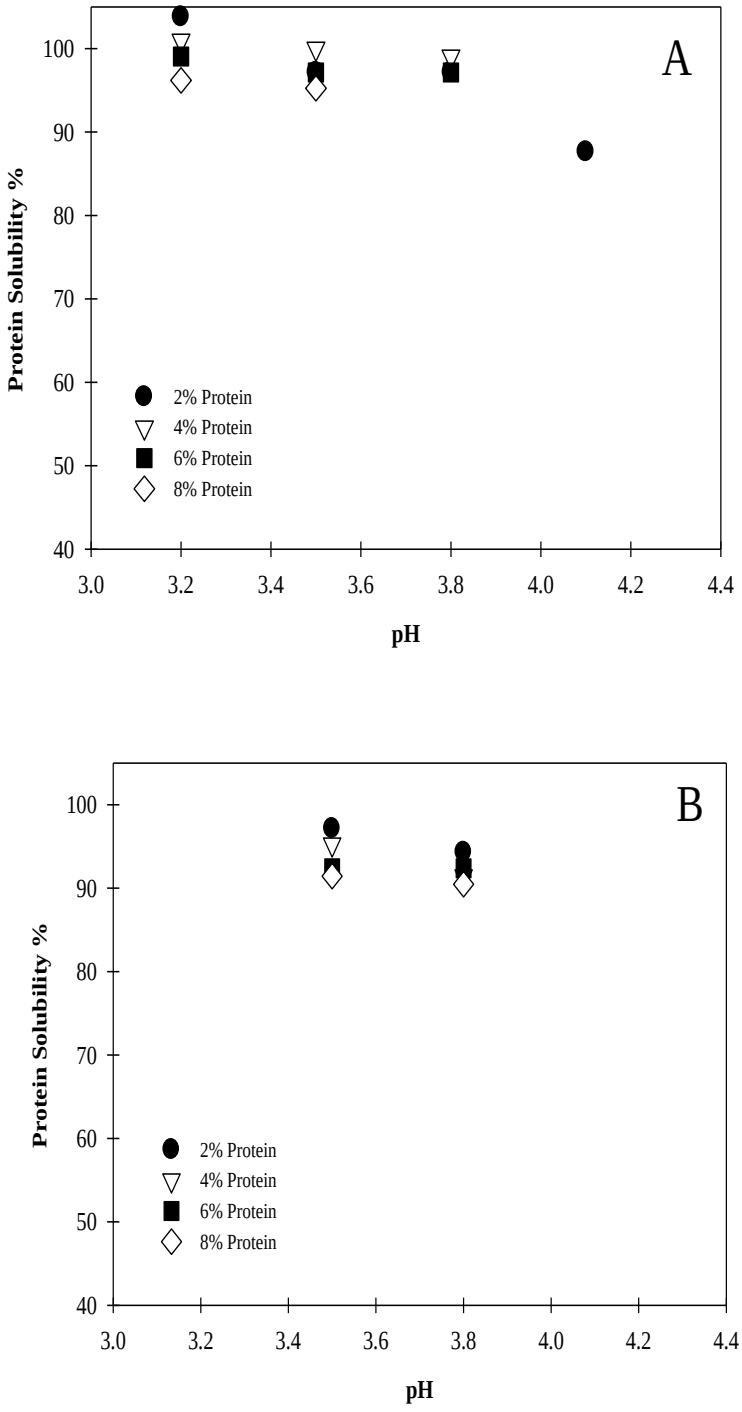

Figure 3.4: Relationship between pH and protein solubility at varying protein concentrations for thermally processed protein fortified apple juice A and B.

Table 3.12: Protein Fortified Mixed Fruit Juice Turbidity (in NTUs)[1]

Thermal Process	Protein %	pH				
		3.2	**3.5**	**3.8**	**4.1**	**4.3**
75°C for 15 sec	**2%**	2130±259	6690±440	10500±630	22800±1770	32900±2350
	4%	203±31.2	3430±267	29200±1220	51800±1490	Gel
	6%	159±3.2	1710±229	39700±4330	Gel	Gel
	8%	162±0.9	1110±145	Gel	Gel	Gel
85°C For 15 sec	**2%**	3240±300	6810±758	30700±3480		
	4%	1130±212	6110±1720	50200±3190		
	6%	257±6.4	4940±579	Gel		
	8%	218±19.9	Gel	Gel		

[1]Mean values +/- Standard Error

Table 3.19: Protein Fortified Pomegranate Juice Turbidity (in NTUs)[1]

Juice Source	Protein %	pH			
		3.2	**3.5**	**3.8**	**4.1**
A	**2%**	7340±30.3	11700±165	17400±375	28800±667
	4%	2660±101	11600±527	34200±480	Gel
	6%	494±59.9	8620±222	Gel	Gel
	8%	224±8.3	7300±710	Gel	Gel
B	**2%**	1950±180	5200±232	10500±468	
	4%	284±2.0	1920±42.3	21400±2840	
	6%	187±6.8	460±210	Gel	
	8%	169±5.9	373±49.4	Gel	

[1]Mean values +/- Standard Error

Table 3.20: Protein Fortified Cherry Juice Turbidity (in NTUs)[1]

Juice Source	Protein %	pH			
		3.2	**3.5**	**3.8**	**4.1**
A	**2%**	62.7±7.3	243±95.4	1150±383	6000±199
	4%	62.8±3.4	115±8.5	1410±478	20400±2490
	6%	69.9±2.6	103±4.2	1430±592	35300±3470
	8%	92.1±2.0	141±5.4	2690±429	Gel
B	**2%**		96.1±4.7	271±55.8	
	4%		114±4.1	450±115	
	6%		137±5.4	728±427	
	8%		150±1.8	Gel	

[1]Mean values +/- Standard Error

Table 3.21: Protein Fortified Apple Juice Turbidity (in NTUs)[1]

Juice Source	Protein %	pH			
		3.2	**3.5**	**3.8**	**4.1**
A	**2%**	48.3±22.3	118±81.8	618±280	10200±1640
	4%	50.7±7.2	103±48.9	690±316	Gel
	6%	74.7±13.1	105±26.8	1180±304	Gel
	8%	87.5±10.3	132±20.6	Gel	Gel
B	**2%**		109±6.2	1145±85.4	
	4%		78.4±2.2	368±28.6	
	6%		88.0±1.5	246±34.3	
	8%		127±6.2	498±143	

[1]Mean values +/- Standard Error

Table 3.22: Protein Fortified Mixed Fruit Juice Z Diameter, Peak %Mass, and Peak %Intensity

Thermal Process	Protein %	pH	Z Diameter (nm)	Peak 1 (0.1-10 nm)		Peak 2 (10-100 nm)		Peak 3 (100-1000 nm)	
				%Mass	%Intensity	%Mass	%Intensity	%Mass	%Intensity
Unheated	2%	3.2	160±16	83±3.4	3.1±0.9	5.9±5.8	33±32	5.6±3.0	61±31
	4%		110±3.7	87±0.8	0.95±0.1	8.4±1.2	74±16	0	0
	6%		76±5.0	85±7.5	0.76±0.1	12±6.5	84±12	0.08±0.08	0.20±0.20
	8%		73±2.9	86±6.9	2.0±0.1	12±6.6	84±9.9	0.12±0.08	0.92±0.9
	2%	3.5	N.A	N/A	N/A	N/A	N/A	N/A	N/A
	4%		98±1.2	95±0.3	7.2±1.0	2.4±0.9	60±22	1.5±1.1	31±22
	6%		87±5.2	92±0.8	3.0±0.9	6.1±1.1	90±5.4	0.06±0.06	0.13±0.13
	8%		99±12	87±6.2	1.1±0.3	8.4±4.3	84±6.9	0.27±0.27	0.44±0.44
75°C for 15sec	2%	3.2	160±4.4	49±2.8	0.64±0.1	22±15	27±17	26±13	66±24
	4%		80±1.6	87±1.9	0.48±0.1	12±2.3	99±0.4	0	0
	6%		64±3.3	87±2.3	1.1±0.1	13±2.2	94±5.1	0.46±0.2	036±0.2
	8%		54±2.8	81±6.7	0.85±0.1	15±5.4	69±6.6	0.12±0.12	0.29±0.29
	2%	3.5	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4%		160±5.3	75±3.1	0.69±0.2	0.27±0.1	2.2±0.5	24±3.3	97±0.4
	6%		160±6.4	77±0.8	0.79±0.2	0.13±0.03	2.7±0.5	23±0.6	96±0.7
	8%		160±25	53±12	0.72±0.1	1.4±0.7	8.4±4.4	45±12	91±4.4
85°C for 15sec	2%	3.2	120±2.0	29±0.6	1.0±0.2	59±3.2	91±5.4	2.3±2.3	1.2±1.2
	4%		77±12	68±11	0.70±0.1	14±5.6	64±21	14±11	25±17
	6%		51±0.5	88±1.4	1.1±0.1	7.9±1.1	79±5.3	1.3±0.9	4.7±2.4
	8%		48±0.6	92±0.6	0.95±0.2	6.8±0.8	83±10	0.25±0.2	1.2±1.2
	2%	3.5	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4%		94±8.1	62±12	0.42±0.1	17±4.9	88±9.7	10±8.0	6.2±5.1
	6%		90±13	63±8.5	0.68±0.1	20±6.4	80±19	12±9.8	19±18
	8%		Gel	Gel	Gel	Gel	Gel	Gel	Gel

N/A: Particles in beverage not able to be accurately measured

Table 3.23: Protein Fortified Pomegranate Juice Z Diameter, Peak %Mass, and Peak %Intensity

Juice Source	Protein %	pH	Z Diameter (nm)	Peak 1 (0.1-10 nm)		Peak 2 (10-100 nm)		Peak 3 (100-1000 nm)	
				%Mass	%Intensity	%Mass	%Intensity	%Mass	%Intensity
A	2%	3.2	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4%		130±8.1	31±13	0.74±0.2	45±10	67±17	3.4±3.4	13±13
	6%		96±3.8	57±5.9	0.95±0.4	37±6.4	92±6.0	0	0
	8%		68±2.7	89±3.2	0.53±0.1	11±2.8	93±6.0	0.17±0.17	0.93±0.93
	2%	3.5	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4%		N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6%		150±4.8	90±1.2	1.6±0.5	0.76±0.4	16±4.0	7.7±0.6	79±4.3
	8%		420±33	52±4.6	0.68±0.1	0.82±0.4	15±7.0	45±4.9	94±5.5
B	2%	3.2	97±4.5	46±5.3	1.0±0.2	46±5.6	98±0.6	2.2±2.2	0.45±0.45
	4%		52±1.1	86±2.2	2.2±0.6	7.6±0.8	88±3.8	0.78±0.2	2.4±1.5
	6%		52±3.7	81±5.7	1.9±0.3	13±4.9	86±2.0	0.66±0.3	1.5±0.5
	8%		43±1.1	93±1.2	2.9±0.7	5.5±0.5	79±3.4	0.16±0.1	0.70±0.5
	2%	3.5	190±6.2	74±5.4	0.92±0.1	0.55±0.3	5.3±3.5	22±5.2	93±3.1
	4%		100±4.0	61±1.4	0.74±0.1	33±3.0	92±7.0	0.10±0.1	0.24±0.24
	6%		75±12	84±5.1	0.79±0.2	13±6.2	97±0.7	0.20±0.1	0.94±0.6
	8%		88±5.5	70±13	0.37±0.01	22±15	71±15	0.55±0.55	0.78±0.78

N/A: Particles in beverage not able to be accurately measured

Table 3.24: Protein Fortified Cherry Juice Z Diameter, Peak %Mass, and Peak %Intensity

Juice Source	Protein %	pH	Z Diameter (nm)	Peak 1 (0.1-10 nm)		Peak 2 (10-100 nm)		Peak 3 (100-1000 nm)	
				%Mass	%Intensity	%Mass	%Intensity	%Mass	%Intensity
A	2%	3.5	81±3.1	93±0.8	8.7±1.2	3.1±0.7	51±16	1.5±1.0	23±16
	4%		67±2.4	80±7.1	5.3±1.9	16±6.8	37±5.6	0.50±0.5	10±10
	6%		58±0.9	68±5.8	3.0±0.4	30±6.8	55±14	0.62±0.05	9.1±3.9
	8%		52±1.8	75±11	2.2±0.2	24±11	66±7.4	0.49±0.3	6.8±3.4
	2%	3.8	82±12	74±12	1.4±0.6	21±12	63±10	0.45±0.45	2.2±2.2
	4%		73±0.1	84±1.7	0.89±0.2	11±1.6	93±5.6	0.96±0.96	1.5±1.5
	6%		82±2.6	85±0.6	0.76±0.03	12±1.2	98±0.5	0.49±0.49	0.49±0.49
	8%		200±68	56±19	0.81±0.2	5.9±4.8	41±30	36±23	58±29
B	2%	3.5	48±0.9	44±17	7.3±4.1	55±17	82±2.3	0	0
	4%		43±0.9	55±11	3.6±2.0	44±12	69±3.3	0	0
	6%		41±0.2	93±0.2	4.5±1.3	5.3±0.3	72±4.7	0	0
	8%		45±0.7	93±1.2	1.8±0.3	6.1±1.1	84±6.3	0.25±0.2	1.0±0.6
	2%	3.8	71±3.8	74±9.5	1.5±0.4	23±8.9	73±14	2.2±2.2	4.7±4.7
	4%		74±4.4	84±5.2	1.6±0.8	15±4.9	96±0.3	0	0
	6%		120±38	63±14	0.90±0.3	13±7.3	59±30	15±11	32±22
	8%		Gel	Gel	Gel	Gel	Gel	Gel	Gel

Table 3.25: Protein Fortified Apple Juice Z Diameter, Peak %Mass, and Peak %Intensity

Juice Source	Protein %	pH	Z Diameter (nm)	Peak 1 (0.1-10 nm)		Peak 2 (10-100 nm)		Peak 3 (100-1000 nm)	
				%Mass	%Intensity	%Mass	%Intensity	%Mass	%Intensity
A	2%	3.5	100±61	77±1.0	7.4±1.3	0.45±0.2	13±5.0	23±1.0	77±8.5
	4%		77±3.8	75±11	3.4±0.4	23±11	58±14	0.86±0.4	17±9.5
	6%		76±2.0	86±6.6	2.3±0.6	11±6.6	75±3.0	0	0
	8%		58±0.7	82±6.0	2.0±0.1	18±6.2	77±4.6	0	0
	2%	3.8	76±9.4	86±1.5	2.9±0.5	5.3±1.5	90±1.8	0.91±0.5	2.3±1.0
	4%		46±3.7	87±5.2	2.8±0.7	5.4±0.2	89±2.3	2.7±1.2	5.7±1.7
	6%		83±11	83±5.1	0.98±0.2	8.9±1.5	64±22	6.5±6.5	25±25
	8%		Gel	Gel	Gel	Gel	Gel	Gel	Gel
B	2%	3.5	52±1.0	86±6.2	2.9±0.5	11±5.5	93±0.6	0.06±0.06	0.11±0.11
	4%		40±1.0	62±6.2	2.8±0.5	37±6.6	63±11	0.15±0.15	0.83±0.83
	6%		37±0.3	63±17	3.0±0.8	36±17	64±8.0	0.11±0.06	0.50±0.3
	8%		38±0.7	95±0.1	3.3±0.9	4.1±0.1	63±2.4	0	0
	2%	3.8	62±5.4	89±1.7	2.6±0.4	8.7±1.7	97±0.4	0.31±0.31	0.11±0.11
	4%		68±5.3	72±6.3	3.2±1.3	26±6.8	94±2.8	1.6±1.6	2.7±2.7
	6%		69±8.4	85±5.6	1.5±0.6	14±4.6	96±1.1	0.34±0.18	0.95±0.5
	8%		94±6.8	83±1.8	0.78±0.2	15±2.8	93±5.8	0	0

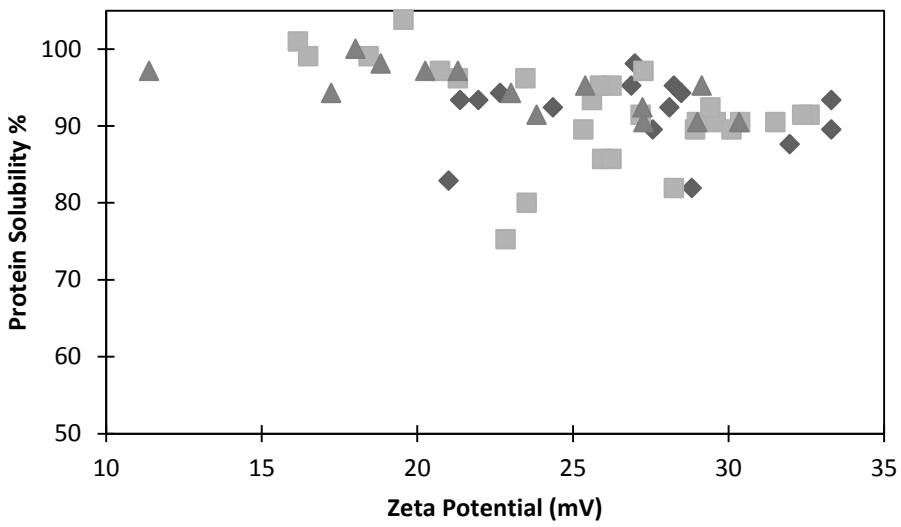

Figure 3.5: Relationship between zeta potential and solubility for all protein fortified juices. Results were divided by pH level 3.2 (♦), 3.5 (■), and 3.8 (▲) and used to determine overall trend of zeta potential on solubility in fruit juices.

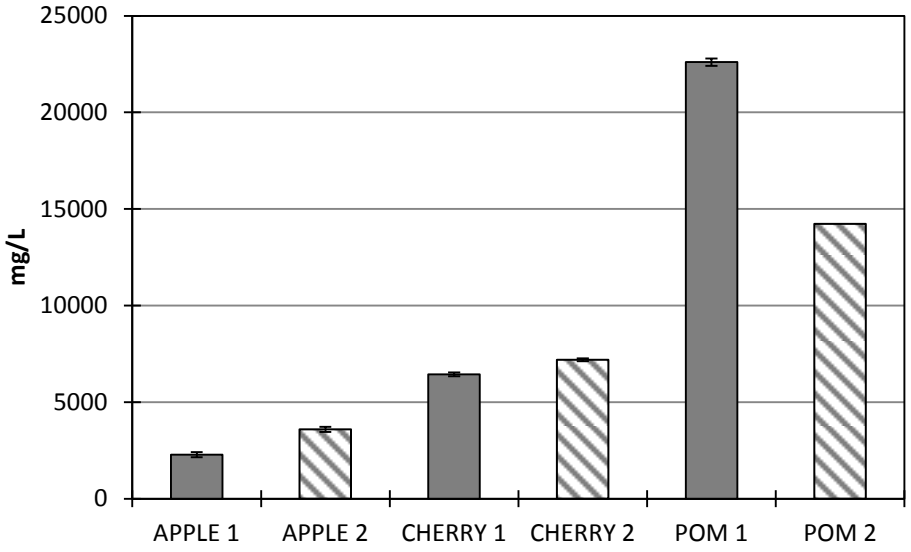

Figure 3.6: The total phenolic content of the fruit juice concentrates (POM indicated pomegranate juice).

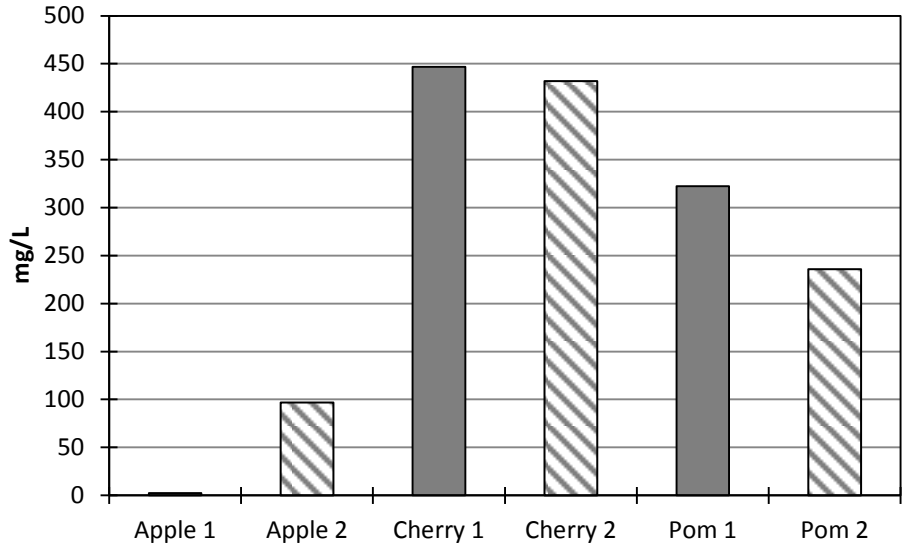

Figure 3.7: The proanthocyanidin content of the fruit juice concentrates (Pom indicates pomegranate).

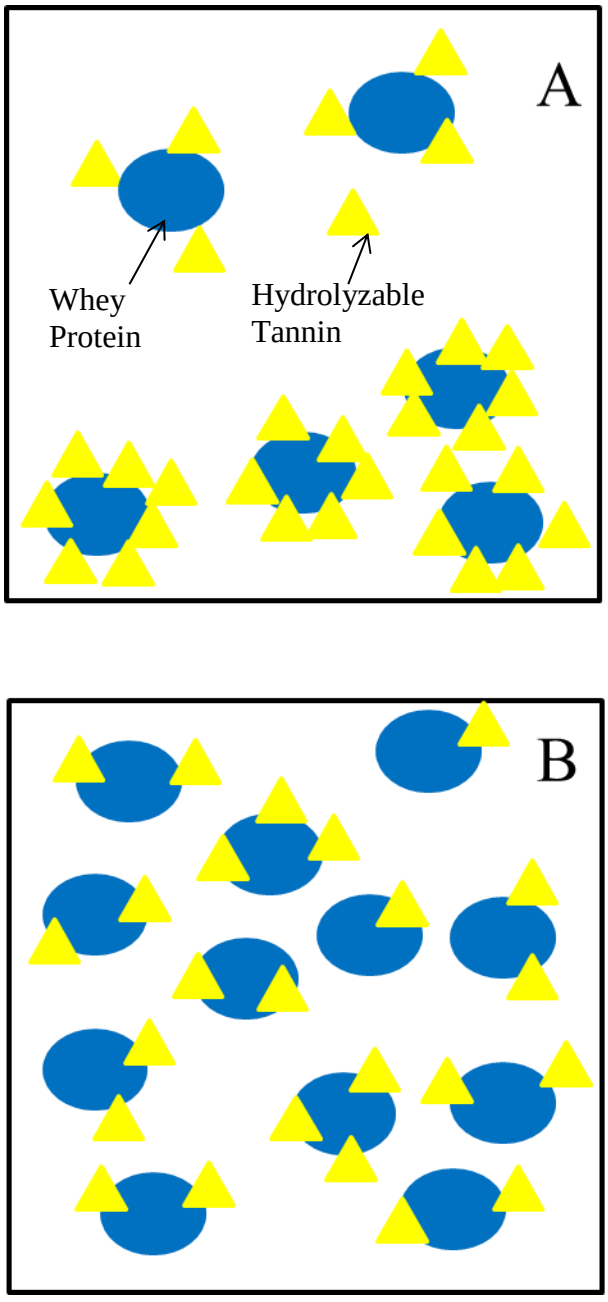

Figure 3.8: Model for whey protein and hydrolyzable tannin interactions at high polyphenol to protein ratio (A) and low polyphenol to protein ratio (B). When the polyphenol to protein ratio is high, more hydrolyzable tannins interact with whey protein and can lead to precipitation, but if ratio is low enough, precipitation can be limited. Hydrolyzable tannins bind through hydrophobic interactions to the exposed hydrophobic regions of denatured protein.

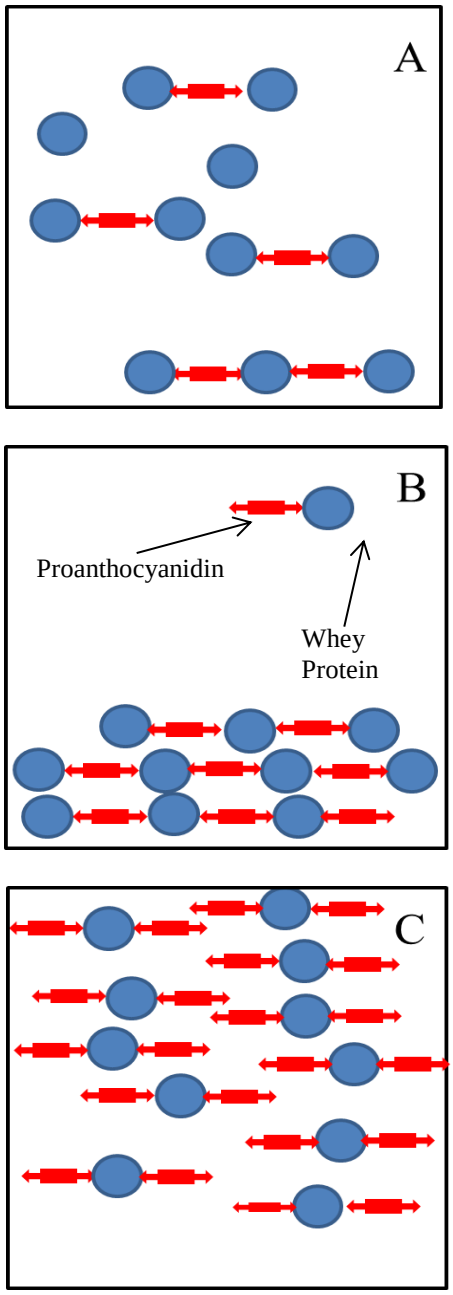

Figure 3.9: Models for interaction between proanthocyanidins and whey proteins at varying ratios. The complexes formed differ when proteins greatly outnumber polyphenols (A), proteins and polyphenols are equivalent (B), and when polyphenols greatly out number proteins (C). Model assumes that proanthocyanidins have two specific binding sites in order to cross-link whey proteins, but it is possible proanthocyanidins could have more than two binding sites.

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CHAPTER 4: OVERALL CONCLUSIONS AND FUTURE RESEARCH CONSIDERATIONS

When formulating a protein fortified beverage for production, a multitude of factors must be considered ranging from the ingredients that will be used to the pH of the beverage to what thermal process will be used. All of these factors will affect the extent of whey protein denaturation and aggregation within the beverage and will affect the colloidal stability. For a simple beverage, like the protein fortified lemon flavored water, the main ingredients are water and whey protein with small concentrations of flavorants, acidulants, and preservatives. With a beverage like that, the whey protein ingredient would be expected to act like the ingredient dispersed in water because other ingredients would not have an effect on the colloidal stability. The pH of the beverage is the main factor that influences the changes in solubility and turbidity because it influences the electrostatic repulsion between the proteins based on how close the beverage pH is to the isoelectric point of the protein. When the pH of the lemon flavored water was at or below pH 3.8, the solubility of the beverage remained very high (>90%), while the turbidity generally remained low (<300 NTU). This is what would be expected of WPI in water as well because when the pH of the solution is far enough away from the isoelectric point, the electrostatic repulsion is strong enough to limit the aggregation of the proteins. By understanding how WPI, or other whey protein ingredients, acts under different protein and pH conditions, the formulation of a simple beverage, like the lemon flavored water, with colloidal stability becomes easier to develop. Unfortunately not all beverages are as simple as the lemon flavored water and requires a better understand of how whey protein interact with different ingredients, like fruit juice.

Protein fortified fruit juices provide more challenges in creating a beverage with colloidal stability than the lemon flavored water because of the presence of polyphenols and possibly other juice components such as pectin or acids. When whey proteins are to the fruit juice, the polyphenols in the fruit juice can interact with the proteins to form soluble and insoluble complexes. With the introduction of polyphenols, the protein fortified fruit juices don't simply act like WPI in water, and further study of fruit components and how they may alter protein aggregation must be completed. For example, in the mixed fruit juices and pomegranate juices, the solubility increased and the turbidity decreased as the protein concentration increased at pH 3.2 and 3.5, but at pH 3.8 and above, the trend reversed. No trends like that were seen in the lemon flavored water at any protein concentration or pH level. However, the cherry and apple juices did have trends similar to the lemon flavored water where the solubility remained high at pH levels 3.2, 3.5, and 3.8 and saw a decrease in solubility as the pH increased to 4.1, but the decrease was not as large as it was for the lemon flavored water. In addition, while the lemon flavored water maintained colloidal stability at pH 3.8, neither cherry nor apple juice produced a beverage with colloidal stability at pH 3.8 because the turbidity was too high. Based on the research conducted, a hypothesis was developed explaining how not only does the amount of polyphenols in the fruit juice influence the colloidal stability of the beverage, but the type of polyphenols does as well. While this hypothesis is plausible, more research must be conducted to further prove the validity of it.

Future research that would need to be conducted would be to test the cherry and apple juices with concentrations of polyphenols that are equivalent to those found in the pomegranate juice to determine if the unique effects seen with pomegranate juice were due to overall concentration of specific polyphenols. If the effects are similar to the pomegranate juice, then it would show that the concentration of polyphenols is more important than the types of polyphenols. However, if the effects are different, then the next step would be to test other fruit juices that are high in hydrolyzable tannins to see if they share the same effects on colloidal stability as pomegranate juice. If the same trends are seen, then it can be assumed that any type of hydrolyzable tannin will create the same trends seen in the pomegranate juice. But if the same trends as the pomegranate juice are not seen in those fruits high in hydrolyzable tannins, then analyzing the differences in the types of hydrolyzable tannins in the pomegranate juice to other fruit would need to be conducted. Pomegranate juice contains hydrolyzable tannins that are not found in any other fruit, like punicalagin. By analyzing and testing the different types of tannins in the pomegranate juice, the specific tannin or tannins that cause the different complexation with whey protein from the other fruits could be determined. If no explanation for the differences between the colloidal stability of the fruit juices is found relating to the polyphenols, other factors such as the differences between the types of acids found within each fruit juice and any possible residual pectin that survived the clarification process would need to be investigated to determine if there is another causative factor besides polyphenols. The shelf stability of the fruit juice beverages should be tested to determine how long the complexes remain soluble because if the complexes do not remain soluble long enough to be put on shelves and sold, then further refinement of the beverage formulation would need to be conducted.