ABSTRACT

SCHNEIDER, MARGARET ELIZABETH. Formation of Whey Protein-Polyphenol Aggregates and Incorporation into Various Food Structures (Under the direction of Drs. E. Allen Foegeding and Mary Ann Lila)

There is a high demand for healthier food options, in particular, foods containing greater amounts of protein as well as fruits and vegetables. Polyphenols, such as those found in fruits and vegetables, have a natural affinity for proteins. Therefore, our first objective was to exploit known interactions between proteins and polyphenols to form protein-polyphenol particles with industry relevant raw materials: whey protein isolate and berry fruit juice concentrates. We hypothesized that the natural association between proteins and polyphenols during aggregate/particle formation would impact protein functionality. In turn, through modification of interaction conditions, the functionality could be tailored for certain applications, such as foam stabilization and reduction of protein bar hardening.

Particles containing both whey protein and cranberry, blackcurrant, or muscadine grape juice were prepared by combining juices containing either 50, 250, or 500 μg total phenolics per g of juice, with dry protein powder to equate to 20% w/w protein dispersion. After mixing for 4 hr, the pH was adjusted to 4.5 and particles were collected by centrifugation (8,000 x g for 20 min). The process of particle formation proved to be complex. Increasing juice polyphenol concentration did not increase the amount of polyphenols precipitated in the pellet, but increased the amount of protein in the pellet. This suggests a critical juice dilution for maximum yield of polyphenols in the pellet. Particles made with 250 μg/g total phenolic juice were selected for functionality testing. These particles were less soluble near the isoelectric point of the whey protein (pH 4-5). When incorporated into foams, particles increased stability (drainage half-life) and yield stress. The

increase in foam stability appeared to be a combined result of polyphenols and non-polyphenol components within the juice. The particles also reduced bar hardening that occurs during shelf life of protein bars. The reduction in hardening was associated with the large size and insoluble nature of the particles. Overall, we showed that through native interaction of proteins with berry juice components, diverse functional particles could be formed and used as structural components in foods.

Formation of Whey Protein-Polyphenol Aggregates and Incorporation into Various Food Structures

by
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BIOGRAPHY

Maggie Schneider started her education at the University of North Carolina at Wilmington where she received a B.S. in Chemistry and B.A. in Physics. She enjoyed the basic sciences, but had little interested in pursuing a career in pharmacy, as most chemistry students did, and felt physics was a challenging field to be in as it sometimes lacked a connection to other humans. It was through an errant internet search that she found the major "food science." As soon as she read the description she was hooked, as she grew up loving to cook and who does not relate to food⁉ So, she set forth to explore food science through whey protein at North Carolina State University under the direction of Dr. Allen Foegeding. While, Maggie's initial interest in protein stemmed from 1) her passion for consuming protein and 2) the positive nutritional benefits associated with it, her interested quickly spiraled deeper as she learned about the functionality and purpose of protein in food structures. She completed her Master's and was ready to explore a career in the industry, but a perfect Ph.D. project came along combining her new love for protein functionality and health associated with plant polyphenols. Through this project she has learned to appreciate natural protein and polyphenols interactions. Following the completion of her degree, Maggie will continue to build on her knowledge of ingredient functionality by working at Nestlé.

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1.1. Introduction

Health has shifted to the forefront of food research. Two driving forces are the increasing demand for foods for a growing population and need to ensure that foods maintain valuable nutrient quality. Another is the ongoing battle against obesity and chronic disease, as foods are being sought after as preventative and treatment agents. Eating the proper food can also help satiate individuals combating hunger pangs during weight loss. For those with chronic disease, such as type II diabetes, heart disease, and cancer, simply improving the diet can help reduce excess inflammation and stress to the body. Often times, eating better involves lowering the amount of lipid and carbohydrate and increasing the amount of protein, fiber, and supplementary nutrients to balance the diet. As such, foods with greater protein and whole fruit components are in high demand.

Although polyphenols, a major phytochemical class which accumulates in many fruits, are not classified as essential nutrients, they have anti-inflammatory, immune protective, and cancer protective properties. Hence, fruit polyphenols could also serve to mitigate and prevent negative health effects associated with obesity, chronic disease, and special health conditions (Yousef et al., 2014). Although natural, potent, anti-inflammatory phytochemical components are available through consumption of certain berry fruits and vegetables (Bakker et al., 2010; Lyall et al., 2009; Nieman, Stear, Castell, & Burke, 2010), and despite dietary guidelines which clearly encourage daily intake of fruits and vegetables, less than 1% of the US population actually consumes the recommended levels (NPD, 2013). Multiple factors

interfere with compliance, including consumer dissatisfaction with the bulk and perishability of garden produce, the perception that fresh produce is more costly than packaged foods, pungent flavors and/or bitter tastes, or simply the perceived inconvenience associated with culinary preparation of raw phytochemically-rich vegetables or fruits (Yousef et al., 2014). A recent meta-analysis in Britain showed a robust inverse association between produce consumption and risk of cancer and cardiovascular disease mortality by consuming up to seven servings/day. However, the benefits did not extend to canned products or juices which contain extraneous sugars (Oyebode, Gordon-Dseagu, Walker, & Mindell, 2014).

The challenges of providing adequate protein and fruits in the diet are exacerbated due to inconvenient food sources, taste, and difficulty in transporting. In addition, concentrated, convenient, and portable food sources are particularly needed for individuals with highly mobile lifestyles, such as high performance athletes, backpackers, campers, or even combat soldiers. As such, a concentrated dietary source of protein and fruit polyphenols appears to be a feasible solution to the stated health and consumer demands. Therefore, this review will discuss potential raw ingredients to use in forming a convenient high protein-polyphenol food source and the physical-chemical properties necessary to achieve a stable food structure.

1.2. Overview of Protein and Protein Food Structures

1.2.1. Whey Proteins

The protein composition of bovine milk is 80% caseins and 20% serum or whey proteins (Eigel et al., 1984). Whey proteins are known for their nutritional and functional properties. Compared to other dietary protein sources, whey proteins are fast absorbing and increase circulating amino acid content, cholecystokinin, and glucagon-like peptides (Hall,

Millward, Long, & Morgan, 2003). Whey proteins are also high in branch chain amino acids, such as leucine. When consumed, these branch chain amino acids quickly initiate the anabolism of muscle, important for building muscle following heavy exercise (Shimomura et al., 2006). Consuming whey protein is beneficial for athletes as well as elderly, since consumption of fast-absorbing protein leads to muscle development and protects against sarcopenia (muscle wasting) by strengthening skeletal muscle (Dangin et al., 2003).

Whey proteins, and proteins in general, are biopolymers with unique chemical and physical properties based on a diversity of structure, attributed to having 20 amino acids as their building blocks. Amino acids can have hydrophobic, polar not charged, polar negatively charged, or polar positively charged side groups, lending these properties to the overall protein characteristics. In turn, proteins have amphiphilic properties meaning that they have polar, water soluble groups and are therefore capable of interacting with lipids, polysaccharides, and other proteins. Therefore, proteins can partition at an interface between polar and non-polar phases. These properties are what allow them to be used in replacement of fat, sugar and other ingredients. Proteins can contribute to the structure of a food through network formation or serve as a potential ingredient to aid in modifying mouth feel with removal of fat and/or sugar, as well as qualify as a "clean" (non-"additive") ingredient. For example, micro-particulated proteins such as *Simplesse*® have been successful at imitating fat and improving texture and flavor in cheese (Schenkel, Samudrala, & Hinrichs, 2013), or *Dairy-Lo*® replacing fat in yogurt (Sandoval-Castilla, Lobato-Calleros, Aguirre-Mandujano, & Vernon-Carter, 2004).

Whey protein ingredients can be split into three main categories, whey protein isolate (WPI, ≥ 90% protein), whey protein concentrate (WPC, < 90% protein), and enzyme modified whey proteins (hydrolyzed, crosslinked, or glycosylated). Further discussion will be in reference to WPI as it is the ingredient of interest for use in developing a whey protein-polyphenol ingredient. Whey protein isolate consists predominantly of β-lactoglobulin, α-lactalbumin, and bovine serum albumin, which contain 162, 123, and 582 amino acids respectively. β-lactoglobulin has a molecular weight of 18.367 kDa (A form) or 18.281 kDa (B form) , α-lactalbulin has a molecular weight of 14.175 kDa (A form), 14.175 kDa (B form), and bovine serum albumin has a molecular weight of 66.267 kDa (Eigel et al., 1984). If not removing during processing, glycomacropeptide from κ-casein is a major protein found in WPI. Lactoferrin, lactoperoxidase, and immunoglobulin are present in whey protein isolates in small amounts, in addition to small amounts of fat, lactose, and minerals.

Whey protein ingredients are produced by processes that remove water, salts, lactose and lipid. Whey can be either "sweet" (from rennet-based cheeses at mid pH ranges) or "acid" (lower pH from cottage cheese and Greek yogurt), with sweet whey being the predominant one used to make whey protein ingredients (Morr & Ha, 1993). Whey protein isolate can be produced by processes that rely mainly on membrane filtration or ion exchange (L. Huffman, 1996). Whey protein isolates made by membrane filtration contains greater amounts of α-lactalbumin than whey protein isolate made by ion exchange (L. M. Huffman & Harper, 1999). In addition, whey protein isolate from membrane filtration contains glycomacropeptide, while this fraction is often removed in the ion exchange process. Glycomacropeptide is the part of κ-casein that is cleaved by chymosin hydrolysis in cheese

making and can constitute ~ 20% of the protein in whey isolate (L. M. Huffman & Harper, 1999). Depending on the degree of glycosylation, glycomacropeptide can range for 7-8.6 kDa and a pI near 3 (T. Wang & Lucey, 2003). A deeper discussion of whey processing is beyond the scope of this review, but can be found in text written by Mulvihill and Ennis (2003) (Mulvihill & Ennis, 2003).

Protein functionality is a term used to describe how a protein affects the physical structure and stability of a food. Proteins cans impact food structures by being in a native, denatured, soluble aggregate, or insoluble aggregate form. Aggregates of proteins participate in food structures on a meso-scale level which can lead to macroscopic structures that, in turn, impart flavor release and textural components of food. The desired size and inter-particle interactions of aggregates depends on the application. Protein aggregates or particles can be designed for specific applications based on particle size/shape, density, and surface properties. In protein-fortified beverages, the goal is to form nano-scale aggregates that produce a stable sol that is not destabilized by thermal processing (minimal inter-aggregate interactions). For foams and emulsions, nano and micro-scale aggregates can increase stability by a variety of mechanisms. With gels, the goal may be to weaken or strengthen the network, requiring no or strong interactions with the gel network, respectively. In order to appropriately approach making a food structure, one must first understand the in physico-chemical properties relevant to maintain structure.

1.2.2. Foams

Multiple foods contain a foam framework, including ice cream, breads, cakes, champagne, meringues, whipped yogurts, and whipping cream. By definition, a foam is formed when a gas, typically air or CO_2 in food systems, is dispersed in a liquid or solid by whipping or sparging (injection of gas bubbles into a solution). For this review, the continuous phase is assumed to be liquid, unless otherwise noted. The amount of gas dispersed into the liquid, or air phase volume, determines the geometry of the foam. There are two distinct geometries: spherical, which form under dilute gas conditions, and polyhedral, which form in concentrated gas conditions as the spherical shape is deformed during packing (Hailing & Walstra, 1981). Polyhedral foams are considered *true foams* (Bikerman, 1965) and are characterized as having three main elements: films (intersection of continuous and discontinuous phase), channels or lamella (intersection of two bubbles), and nodes (intersection of three bubbles) (Koehler, Hilgenfeldt, & Stone, 2000). Ideally, three bubbles of a polyhedral foam each meet at 120°. However, this angle will deviate from 120° in foams with a distribution of bubble sizes or what is termed an intermediate foam- a foam less packed than a true polyhedral foam but more packed than a true spherical foams (Bikerman, 1965). The exchange of liquid and gas among the elements drives separation into gas and liquid bulk phases and therefore influences the stability of a foam. The predominant destabilizing forces of a foam are: 1- disproportionation, the diffusion of gas from a small bubble to larger bubble; 2-coalescence, which is capillary flow rupturing the lamella fluid between two bubble; and 3- drainage, the redistribution of the liquid from the channels and

nodes into a bulk liquid phase. Physical properties of the continuous phase and presence of interface active compounds influence the reaction of a foam to destabilizing forces (A. Bos & van Vliet, 2001). Physical characteristics important to foam stability include but are not limited to viscosity of the continuous phase, surface active components, interfacial film strength, interfacial film electrostatic or steric repulsion forces, and bubble size distribution (Myers, c1999.).

1.2.2.1. Disproportionation

Disproportionation is driven by the difference between the internal pressure among bubbles and the external pressure (Murray & Ettelaie, 2004). The difference in pressure among gas bubbles is caused by variation in bubble size. The Laplace equation (Eqn 1.) describes the excess internal pressure (ΔP) in a bubble with a fixed surface tension (γ) and radius (r) (Dickinson, 1992).

$$\Delta P = 2\gamma/r \qquad \text{Eqn 1.}$$

According to the Laplace equation (Eqn. 1), for two bubbles with equal interfacial elasticity, but different sizes, the excess internal pressure (Δp) is inversely proportional to the radius of the bubble, and would be higher in smaller bubbles. Therefore, this pressure differential drives gas from a small bubble into the surrounding fluid. Depending on the solubility of the gas in the fluid, it will move into the fluid and then into a nearby larger bubble with lower pressure. This coarsening of the foam perpetuates disproportionation and leads to increased drainage and coalescence.

Disproportionation is the most challenging to prevent in foams (Murray & Ettelaie, 2004). One method to prevent disproportionation is the incorporation of an insoluble

compound into the dispersed phase of the foam. Additionally, the elasticity or viscosity of the continuous phase can be increased to physically inhibit the transfer of gas between bubbles, but this simply slows the process (Kloek, van Vliet, & Meinders, 2001; Murray & Ettelaie, 2004). Prevention of disproportionation can be achieved by altering the interfacial elasticity, reducing film shrinkage, or decreasing the permeability of the interface to the gas in the bubble (Kloek et al., 2001).

1.2.2.2. Coalescence

Coalescence is not to be confused with disproportionation, as coalescence is the simple rupture of the lamella between two bubbles resulting in one larger bubble, which takes place on the time scale of <1 sec., whereas disproportion occurs on the time scale of hours (Monin, Espert, & Colin, 2000). Coalescence tends to increase with decreasing lamellae or film thickness. Coalescences is reserved for discussion last as it is a direct result of drainage which decreases lamellae thickness. Lamellae thin with increasing phase volume of a foam, therefore over whipping can lead to coalescence during foam formation (Murray & Ettelaie, 2004). Therefore the predominant contributing force to coalescence is the energy of the interface (Bikerman, 1965). Similarly to disproportionation, increasing the film elasticity decreases the likelihood of two bubbles coalescing. In turn, adding surface-active compounds increases the interfacial elasticity and decreases the potential energy for rupture. Particles can also be added to foam as they take up space in the lamella, widening the lamella, preventing coalescence of bubbles and packing the space between two bubbles (Hailing & Walstra, 1981). When bubbles coalesce, there is an excess of drainage liquid which can lead to the final destabilizing force in foams (Hailing & Walstra, 1981).

1.2.2.3. Foam Drainage

Foam drainage results when pressure from gravity and capillary action work together to destabilize foams by directing fluid flow down and away from two neighboring foam bubbles (Koehler et al., 2000). The drainage rate can be define with Eqn. 2, where g is the gravity constant, ρ density difference between continuous and discontinuous phase, w distance between two bubbles or channel width, η the continuous phase viscosity, and δ the thickness of the film or Plateau-border (Myers, c1999.):

$$\frac{dV}{dt} = \frac{g\rho w\delta^3}{12\eta} \qquad \text{Eqn 2.}$$

If each of these variables can be controlled, drainage can be stopped or at least slowed. A simple means to prevent drainage is by increasing the viscosity of the continuous phase. While altering each variable in the drainage rate equation to slow draining is tempting, care must be taken not to increase propensity for coalescence or disproportionation. For example, decreasing the channel width, or lamellae, would increase coalescence.

1.2.2.4. Proteins

Foams need surface active agents (surfactants) to form interfacial films and increase stability. There is a wealth of information on small molecular weight surfactants that will not be discussed, as this review will focus on the potential of proteins to stabilize foams. In foam formation, proteins reduce the free energy of the interface and thereby aid in forming small bubbles (Damodaran, 2005). Proteins are often added at an amount that exceed the surface coverage of proteins so a portion remains dispersed in the continuous phase. Proteins contribute to continuous phase viscosity, which aids to prevent drainage and

disproportionation. The amphiphilic nature of proteins allows the proteins to adsorb to the interface of a foam. The rheological properties (viscosity and elasticity) and permeability of the interfacial film formed by proteins will alter the rate of disproportionation and coalescence. If a portion of the protein at the surface extends into the continuous phase, then steric stabilization between bubbles could preventing rupture (coalescence).

Protein aggregates and microgel particles can improve foam performance (Rawel & Muschiolik, 1994; Rullier, Novales, & Axelos, 2008; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007). Aggregates act as a physical barrier against disproportionation and prevent coalescence by steric hindrance and increased viscosity (Dickinson, 2010; Hunter, Pugh, Franks, & Jameson, 2008). Rawal and Muschiolik (1994) showed that the secondary aggregates of mechanically processed soy proteins, ranging from1-40 μm in diameter were key to improving foam volume and drainage time (Rawel & Muschiolik, 1994). Schmitt et al. formed soluble whey protein gel particles ranging in diameter from 290-330 nm with NaCl and found that particles formed above pH 6.6 were active at bubble interfaces and improving foam stability (Schmitt et al., 2007).

1.2.3. Bars

The recommended daily amount of protein for the average individual between ages 19 and 70 years of age is ~46 g/day or 56 g/day for 97-97% of healthy women and men respectively (Institute of Medicine (US). Panel on Micronutrients, 2005). In the United States, the average individual meets and exceeds the amount needed, but that is not the case for athletes who need extra protein for exercise recovery, or individuals combating muscle

wasting due to either aging or illnesses causing bed-ridden conditions. The typical dietary protein content for these individuals can make it difficult to achieve their protein needs. In addition, the muscles of the aging population, inactive individuals, or active individuals who expose themselves to strenuous exercise experience muscle wasting (Bortz, 1982; Evans, 2010) which requires a diet with additional protein to recover and facilitate muscle catabolism (Paddon-Jones et al., 2004). Protein bars are an efficient way to deliver a high amount of protein and a balanced source of required daily nutrients for such individuals.

Protein bars can be defined as a slab that consists of 20-45% protein, 40-55% carbohydrate, and 10-15% lipid (Loveday, Hindmarsh, Creamer, & Singh, 2009; McMahon, Adams, & McManus, 2009; Zhu & Labuza, 2010). These are usually made by combining dry ingredients by mixing, sometimes with an additional baking operation, ultimately resulting in a bar with a water activity of 0.50-0.70 and minimal organized structure. The high solids content and minimal mixing means that molecules in a bars are not equilibrated and will migrate. The alterations in bar structure over time causes a phenomenon called *bar hardening*. Bar hardening occurs over time; a bar that starts out soft and palatable becomes hard, brittle, and chewy. Protein bar hardening is a complex process, thought to depend on a combination of moisture migration, protein aggregation and network formation, and sugar crystallization (Childs, Yates, & Drake, 2007; Loveday et al., 2009; Loveday, Hindmarsh, Creamer, & Singh, 2010; McMahon et al., 2009; Zhu & Labuza, 2010).

1.2.3.1. Jammed State

The physical state of a protein bar is generally represented as either a viscous liquid, semisolid, or solid. Most protein bars are a viscoelastic material, meaning they contain both

liquid (viscous) and solid (elastic) structural components. Over time the degree elasticity becomes greater. A soft matter physics approach can help elucidate qualities of bars, as ideally we would like to maintain the food as a soft solid. This approach provides insight into the mesoscale of a food. On the mesoscale food can be formed by four main methods: destabilizing food components, creating new phases, destabilizing phase with mechanical sheer or flow, and/or stabilizing the newly developed phase through the jammed state (van der Sman & van der Goot, 2009). In the case of bars, the two predominate actions of forming a structure shearing the raw ingredients (protein powder, liquid oil, and liquid sugar) which forms a jammed state due to the reduction in water activity of the water ingredients as they become agglomerated with the dry powder. Jammed materials have a yield stress, below which the material will support forces, above which slow flow will occur. By definition, the jammed state is an amorphous structure. There is a critical volume fraction (φ), at which point a material becomes jammed and behaves predominantly as a solid, but may still be flowing at an infinitely small rate. The critical volume fraction is often compared to the glass transition (T_g) temperature of a material, but may still be flowing at an infinitely small rate (van der Sman, 2012). The state of a jammed food is predominately dependent on particle/ingredient interactions, volume fraction, and ratio of mechanical forces to the yield stress of the food (van der Sman & van der Goot, 2009). To understand these variables, the composition, phase, and aggregated state of the food must be considered, which provides for thought on three different length scales. On the micro scale, the composition effects the biochemistry or interaction between individual molecules, which consist of water, protein, sugar, and lipids for a bar. The aggregation of ingredients and phases can occur on the meso-

scale, and ultimately impacts the macrostructure (van der Sman & van der Goot, 2009). Therefore, the microscale reactions of bar ingredients will be discussed and related to resulting meso- and macro-scale changes in bar structure.

1.2.3.2. Protein Source

Typically, soy, egg, casein, and/or whey proteins are used individually or in combination as the protein source of a high protein bar. The type of protein selected for formulations can depend on the desired health benefits but, more importantly for consumer acceptance, the bar flavor and texture. Many researchers strive for solutions that allow for incorporation of health beneficial proteins without sacrificing texture, although one is often sacrificed for the other; for example, soy and whey proteins are high quality proteins known for their satiating properties, while casein is used due to its stability. However, the digestibility and amino acid composition of casein is less than that of whey proteins (Shrestha, 2012). Protein hydrolysates can reduce hardness and can be used in food for individuals with food protein allergies. While this solves the physical property of bar, but the problem of flavor arises, as hydrolysates are often bitter and brothy (Leksrisompong, Miracle, & Drake, 2010).

It has been well established that hydrolysates produce a less firm bar than their un-hydrolyzed form (S. Hogan, O'Loughlin, & Kelly, 2016; McMahon et al., 2009; Rao, Rocca-Smith, & Labuza, 2013), but discrepancies can be found when comparing between dairy proteins and across proteins types. Li et al. surveyed numerous dairy and soy protein powders in bar formulations made with sugar syrup, polyol syrup, or reduced sugar syrup. Regardless of sugar ingredient, milk protein isolate produced the least firm bar, so much so that integrity was compromised (Li, Szlachetka, Chen, Lin, & Ruan, 2006). A common

finding is that a mixture of protein types/powders can result in synergistic behavior reducing bar hardness below that of bars made with either protein alone (Imtiaz, Kuhn-Sherlock, & Campbell, 2012; Li et al., 2006; Rao et al., 2013). For example, synergistic effects were observed between some soy-soy, soy-calcium caseinate, and soy-whey mixtures in which the mixture produced bars less firm than bars form with a single starting ingredient (Li et al., 2006).

Loveday et al. (2010) found calcium caseinate bars to be firmer than bars formed with whey protein isolate (Loveday et al., 2010). The inverse was found in evaluation by Li et al. (2006) who observed calcium caseinate to form less hard bars than whey protein isolate. One reason why these two studies showed opposite trends is that there were differences in physical properties of the powder, such as the bulk density and dry particle size as suggested by Hogan et al. (2016) (S. Hogan et al., 2016). In addition, bars were formed with sugar syrup, polyol syrup, or reduced sugar syrup by Li et al. (2006), while bars formed by Loveday et al. (2010) contained glucose and glycerol, which could also contribute variations. It should be noted that comparing bars formed by different investigators can be very difficult as the formulations contain protein ingredients from varying suppliers, various carbohydrate and lipid ingredients, and drastically different formulations ratios (Table 1).

Table 1. Bar formulations, methods and storage conditions. SP-soy protein, MPI-milk protein isolate, WPI-whey protein isolate, CaCN-calcium caseinate, MPC –milk protein concentrate, HWPI-hydrolyzed whey protein isolate, HFCS-high fructose corn syrup, NMR-nuclear magnetic resonance, SDS-PAGE- sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a_w- water activity, FT-IR-Fourier transform infrared spectrometry, CLSM-confocal laser scanning microscopy, SEM –scanning electron microscopy.

Paper	Protein Source	%	Carbohydrate Source	%	Lipid Source	%	Water (%)	Methods	Storage Conditions
Li et al. (2008)	SP, MPI, WPI, and CaCN	30	Sugar syrup (50.4% 63 DE corn syrup, 41.25% HFCS, 8.35% gyrcerine) , polyol syrup (35% glycerine, 35 % maltitol, 20% polydextrose, 10% soribitol), and reduced sugar syrup(50:50 sugar syrup polyol syrup)	-	-	-	-	NMR, punch test, sensory, aw	32 °C 42 days
Loveday et al. (2009)	MPC	20	Glucose, Glycerol	40/15	Cocoa Butter	10	15	NMR, Aunch test, CLSM, SDS-PAGE, reactive lysine assay	20 °C 50 days
McMahon et al. (2009)	WPI, HWPI	38	Mono and diglycerides and HFCS, sorbitol	43	Crisco, fully hydrogenated cottonseed oil, partially hydrogenated cottonseed and soybean oils	15		NMR, CLSM, color,	32 °C 42 days
Loveday et al. (2010)	CaCN, WPI	20	Glucose, Glycerol	40/15	Cocoa Butter	10	15	NMR, punch test, CLSM, aw, SDS-PAGE, chemically available amine assay	20 °C 50 days
Zhu et al. (2010)	WPI Cysteine, N-ethylmaleimide in pH 6.8 buffer at a 2:3 ratio (powder:buffer)	-	-	-	-	-	-	aw, punch test, SDS-PAGE, solubility, SEM	45 °C 35 days
Imtiaz et al. (2012)	WPI, 2 MPC	30	Glucose.Glycerine/Maltodextrine	34.9/ 17.4/3-3.5	hydrogenated palm kernal oil/lecithin	5.7-6.7/0.5	0.3-1.6	Punch test, sensory, aw	20 °C 12 days
Hogan et al. (2012)	NaCN, WPI, β-lactoglobulin, α-lactalbumin, MC	45	HFCS/glycerol	45/10	-	-	-	Punch test, aw, CLSM, FT-IR, powder characterization	37 °C 14 days
Rao et al. (2012)	Hen egg white, hen egg white hydrolysate	75	-	-	-	-	25	Punch test, color, free amino groups	23, 35, and 45 °C 70 days
Zhou et al. (2013)	WPI	45	Frcutose or sorbitol, gycerol	25/17.5	-	-	12.5	Punch test, color, LC-MS, protein solubility, SDS-PAGE	
Hogan et al. (2016)	WPI, heat treated hydrolyzed whey, heat treated, filtered hydrolyzed whey, heat treated, permeate hydrolyzed whey	45	HFCS/glycerol	45/10	-	-		Punch test, aw, glass-rubber transition temperature, oscillatory testing, viscosity, SEM, powder characterization	40 °C 21 days

As previously mentioned in regards to the jammed state, the glass transition and φ of a bar determines the hardness of a bar (van der Sman, 2012). The molecular weight of an ingredient, say protein, has a critical impact on the glass transition, with larger proteins having a higher glass transition (Ubbink, Burbidge, & Mezzenga, 2008). Therefore, if the protein is broken down into smaller peptides, the T_g can be reduced. This is applied in industry when using hydrolyzed protein powders. In addition, Potes et al. (2014) found that olive oil and sunflower oil to increase the T_g of WPI-glucose-fructose mixtures (Potes, Kerry, & Roos, 2014). In a non-jammed food, the carbohydrate T_g would be most relevant to understanding the food structure. But in a bar, the protein and lipid component augment the overall T_g, therefore, the carbohydrate T_g cannot be the sole consideration, instead all ingredients behavior contribute to a T_g for the whole.

While whey proteins are ideal for imparting health benefits, they are susceptible to bar hardening more than soy or casein. The reactivity of the proteins and tendancy towards rearrangement and aggregation are thought to cause this increased propensity for hardening. Zhu and Labuza (2010) suspected that disulfhyde and thiol rearrangement form insoluble aggregates that could be a potential mediator in bar hardening. This supported by aforementioned increases in T_g with increasing molecule weight, in this case the increase in molecular weight of a protein is caused by aggregation. The increase in hardness associated with disulfhyde and thiol rearrangments led Zhu and Labuza (2010) to investigate the ability of the disulfide blockers cysteine and N-ethylmaleimide to modify bar hardness. Cysteine is a sulfhydral-containing amino acid and would compete with protein sulfhydrals in disulfide-sulfhydral exchange, while N-ethylmaleimide covalently binds to cysteine residues and

prevents disulfide formation (Zhu & Labuza, 2010). Zhu and Labuza found that adding cysteine to β-lactoglobulin and α-lactalbumin prevents interaction with the thiol-disulfide interchanges in α-lactalbumin, reducing bar hardening but adding excess cysteine increased bar hardening, possibly because excess cysteine increases rearrangement of thiol and sulfhydryl groups. When N-ethylmaleimide, a thiol group blocker, was added, hardness was reduced and maintained over time. Lui et al. also studied specific protein interactions, but in pure α-lactalbumin bars (Liu, Zhou, Liu, & Labuza, 2011). From these investigation, calcium improved α-lactalbumin stability preventing aggregation while increased in pH accelerated aggregation leading to firmer bars (Liu et al., 2011). The combined work showed that protein aggregation causes or contributed to bar hardening; resulting from either increases in molar mass and altering the T_g, or an overall network formation. If aggregation is the sole mechanism causing bar hardening, then it can be controlled with ingredients that alter pH, salt content, or available amino acids.

1.2.3.3. Maillard Reaction

The Maillard reaction is a non-enzymatic browning reaction that takes place between reducing sugars (such as glucose, fructose, or lactose) and unprotonated amino groups of a proteins. Amino acids such as lysine, tryptophan, and arginine tend to be more reactive in this reaction. The reaction progresses in three phases, but is dependent on available reactants, temperature and solvent conditions. The first phase involves the condensation of the reducing sugar with the amino groups, and the second involved the formation of advanced Maillard reaction products (Van Boekel, 2001). The third phase involves further polymerization of the protein and the formation of melanoidins. In the case of bars, the slow reaction rate of the

Maillard reaction (due to low temperature) can result in polymerization and network formation of the proteins in the presence of reducing sugars. Therefore, the type of sugar can significantly influence the potential for protein network formation over time.

In addition to the type of sugar in a bar, conditions such as pH, water activity, temperature, and salt influence the Maillard reaction. The Maillard reaction occurs more efficiently as pH and temperature increases. At lower pHs the amino group is protonated making it less reactive with the sugar group. The Maillard browning reaction is greatest in systems with a water activity between 0.5-0.8, which is the typical water activity of high protein bars (Owusu-Apenten, 2004). Therefore, bars have an ideal water activity to facilitate Maillard browning. Although salt is not a common ingredient in protein bar formulations, sodium chloride has been shown to reduce the rate of the Maillard reaction (Yamaguchi et al., 2009), and should be considered in bars made with sodium or calcium caseinate.

In bars, Maillard reaction can be optically detected after two weeks of storage in bars made with whey protein isolate and fructose, while glycosylated β-lactoglobulin and α-lactalbumin were measured with mass spectrometry after 7 days (Loveday et al., 2009; Zhou, Guo, Liu, Liu, & Labuza, 2013). The amount of glycosylated proteins increased with increasing storage temperature and incubation time. The solubility of protein was measured in parallel, but only decreased in bars stored at 45 °C and therefore not directly linked with glycosylation unless at higher temperatures (Zhou et al., 2013). The hardness and browning of bars made with fructose was mitigated with the use of sorbitol, a sugar alcohol, in place of fructose. In addition, bars made with sorbitol showed little to no large-insoluble aggregates suggesting the large aggregates formed in bar fructose bars are a result of Maillard reactions

(Zhou et al., 2013). The difference between the hardness of bars containing sorbitol versus fructose was accentuated by an increase in storage temperature. This was expected as the increase in temperature increases the reaction rate of the Maillard reaction. Li et al. (2006) discussed and types of sugar used as a carbohydrate source as acting as a plasticizer in bar systems. Bars containing sugar syrup (corn syrup, HFCS, glycerin) were ~2-3 times harder than bars produced with polyol (glycerin, maltitol, polydextrose, soribitol) or reduced sugar syrup (50:50 sugar syrup polyol syrup) for all protein powders except one of the three soy protein powders. In agreement, McMahon et al. (2009) and Zhou et al. (2013) showed glycerol and sorbitol to reduce bar hardening compared to bar formulated with fructose as a sugar source (McMahon et al., 2009; Zhou et al., 2013).

1.2.3.4. Sugar Source

Loveday et al. (2009) provided evidence that glucose crystallization and a loss of molecular mobility was associated with bar hardening (Loveday et al., 2009). Sugar crystallization is a processes in bars that is dependent on four main variables: water activity/moisture content, sugar, temperature, and time. Within the sugar component, the type of sugar and sugar concentration are important. Sugars vary in solubility, for example at room temperature fructose is most soluble in water, followed by sucrose, invert sugar, glucose and lactose (Pancoast & Junk, 1980). When the temperature or solvent changes the order of solubility and degree of solubility also changes, in part due to the mutarotations or molecular forms of reducing sugars being altered by these changes (Lowry & Faulkner, 1925). The higher the temperature the greater the rate of mutarotation, the greater the propensity for/against crystal formation, depending on the type of sugar. In addition, the

actual concentration and temperature, of sugar solution influences the affinity for crystallization, whether it is in the soluble, metastable, or labile state (Hartel & Shastry, 1991). In bars sugars are in the metastable region, an area in which the sugar concentration is just above saturation, or supersaturated, but just below the point of nucleation, or the point at which a solid crystalline phase develops. Nucleation is, again, dependent on solvent characteristics such as pH, temperature, agitation, and additives (Hartel & Shastry, 1991). In the reference frame of bars, the sugar crystallization is complicated by the addition of other additives/solvents (protein/lipid). For example the presences of calcium chloride allows for sucrose to be more soluble, but larger molecules such as proteins, which compose 25+ % of a bar, can decrease solubility.

1.2.3.5. Lipid Ingredient

The lipid component of a protein bar is typically neglected in the literature, this is likely for two reasons: one being that lipid does not compete for water as the protein and sugar ingredients do and second, lipid storage and oxidation are well studied in the literature on its own. There is little motivation to study lipid effect for these reasons, and those who do study lipid in bar systems observe little to no new effects. Loveday et al., observed no significant differences in lipid mobility detected with proton NMR spectra (Loveday et al., 2009). Although the lipid component is less discussed in literature, it is still a variable to consider to understand protein bar quality over time. It is well established that over time, lipids will oxidize with exposure to water, light, and oxygen. But more importantly for bars, when lipids become oxidized, the resulting oxygen and free radical can be reactive with

proteins, which can accelerate protein oxidation, cross-linking, and polymerization with fat. The role that the lipid ingredients play in bar hardening has not been thoroughly explored.

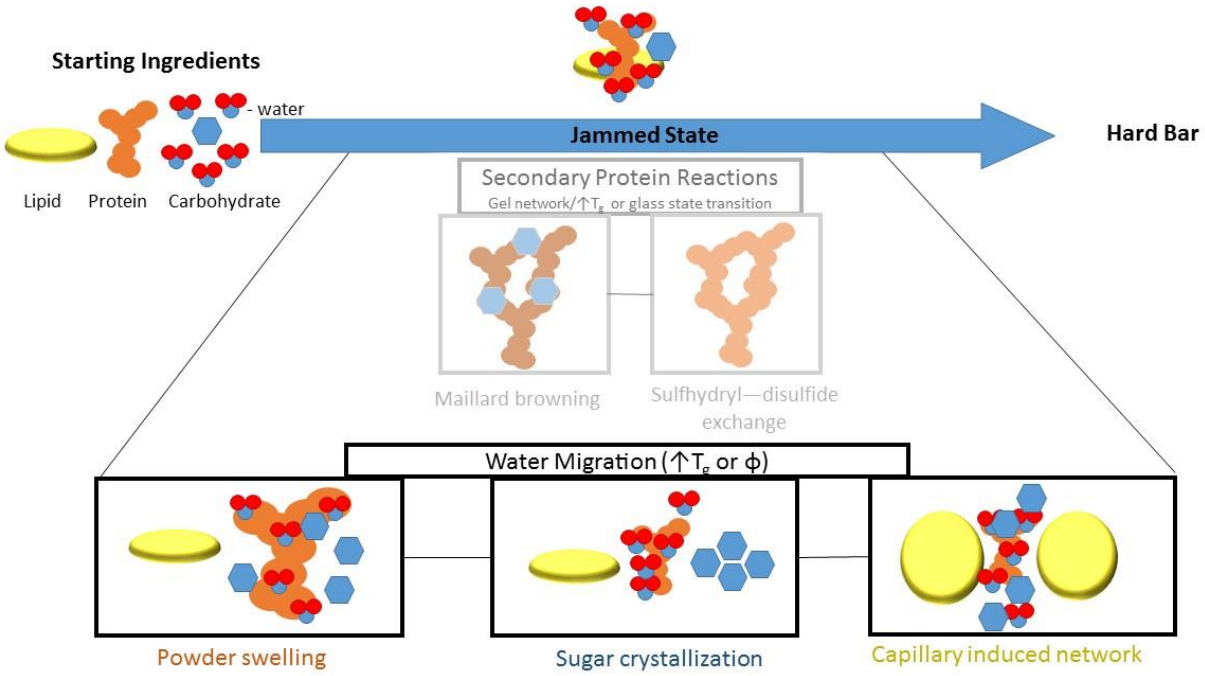

Figure 1. Overview of bar hardening mechanisms

1.2.3.6. Water

Immediately after mixing a bar will be soft, but after a time as short as one week, changes occur and a bar can harden. Loveday et al. found both slow and fast relaxation rate constants for water and polyhydroxy compounds during the first 5 days of storage in bars containing milk protein concentrate, which suggests that during this time protein powder particles become hydrated and solubilized (Loveday et al., 2009). In addition, a stark decrease in reactive lysine content was seen in the first 10 days of bar storage, but did not coincide with bar hardening over time. In support, after the first week of storage,

glycosylation of whey proteins via Maillard browning could be detected, although not until week two browning was visibly detected (Zhou et al., 2013). After the first 5 days in bars containing calcium caseinate and milk protein, there was a decrease in fast relaxation rate constants for water and polyhydroxy compounds, an indication of slow water migrations, but reduction of dissolution activity as the bars become set (Loveday et al., 2009). Hardening and reactions that contribute to hardening involved the migration of ingredients into different phases, reduced mobility as individual components adsorb water, and reactivity of ingredients with each other to form networks. Over longer times, sugar begins to crystalize and water is completely adsorbed by ingredient. Multiple researchers have observed that water activity decreases with bar storage (Potes et al., 2014) and therefore the following section will explore in more detail the contribution of water to bar hardening.

Water is arguably the most important and influential food ingredient that contributes to the state of a protein bar. Throughout time, investigators have attempted to understand water in food. Prior to 1950, the cause for considering water content was to determine potential cell growth (especially food poisoning bacteria). Eventually it was necessary to understand the structure of food as it relates to water *availability* for cell growth, so the understanding of water progressed to considering water *activity* (a_w) as an indicator of potential cell growth. Water activity expresses available water as a function of the vapor pressure where P is the vapor pressure in the food material, and P_0 is the vapor pressure of pure water all at a given temperature.

$$a_w = \frac{P}{P_0} \quad (1)$$

Assumptions in using Eqn. 1 make it less accurate, therefore, more recently, the relative vapor pressure (RVP) instead of a_w, which is related to the equilibrium relative humidity (ERH) of the sample surroundings (Damodaran, Parkin, & Fennema, 2007):

$$RVP = \frac{P}{P_0} = \frac{\%ERH}{100} \quad (2)$$

In high a_w systems, water participates in a food as a solvent and other molecules can be viewed as solutes. However, in systems with low a_w, the state of water is more complex and can be represented as being highly attracted to hydrophilic components. The state of water in food is a highly debated area of research. One approach is to consider water in two different states: either hydration water, that which participates in hydration shells, or free water, the water remaining when hydration water is excluded (Lewicki, 2000). More recently, water is understood to behave on a spectrum from "free water" to "bound" water in food systems, and likely not purely one or the other, but as a mobile component of food interacting on different scales (T. Labuza et al., 2007). Understanding the state of water in a protein bar can reduce potential of water conditions to cause bar hardening.

Water activity is also a regulator of chemical reactions and reaction rates between ingredients (T. P. Labuza, 1980). Foods with higher water contents have higher reaction rates between ingredients, due to mobility resulting in an increase in molecular collisions (Nelson & Labuza, 1994). Disregarding the a_w requirements for food safety, if the water content of a bar is increased, the increase in mobility could prevent sugar crystallization, but promotes reactivity between proteins that could cause aggregation and network formation. Therefore, in addition to considering the amount of free water within a bar, processors must also

consider chemical reactions taking place in the bar and the necessary amount of water to prevent or promote each reaction accordingly.

1.2.3.7. Particle size

Hogan et al. (2012) explored the impact of factors such as protein powder density, particle size, and water activity on bar hardness (S. A. Hogan, Chaurin, O'Kennedy, & Kelly, 2012). Sodium casein was either agglomerated (160 µm) or not (57 µm) and the agglomerated particles produced softer bars. In addition the density of powder particles (porosity) was inversely associated with bar hardening. The density of solids within the bulk powder was thought to cause this effect because of the physical barrier for reactions to occur. Moisture migration has been shown to be an effect of pore size, as larger pores were associated with greater rates of moisture migration than smaller pore, simply due to the available path for water to traverse (T. Labuza & Hyman, 1998). The raw ingredients used to formulate bars contribute chemical potential for reaction during storage, but the physical attribute of the ingredients should also be consider to understand the probability of interactions.

1.2.3.8. Mechanistic Overview

It is difficult to promote or discount any one of the several mechanisms proposed for bar hardening. Indeed, they are often interrelated and hardening could occur by several mechanisms. Water availability is important from a static view of how it reflects overall structure and from a dynamic view of how it contributes to reaction rates. What is consistent across the literature is that bar hardening is caused by reactive proteins that either form inter-molecular bonds (larger molar mass or network formation) or participate in phase separation

(altering the jammed state) (Figure 1). Once bars are produced, gradient-driven processes can change the structural arrangement of ingredients and directly push the system onto a crystalline, glassy, or jammed state that causes hardening. In addition, there are chemical reactions (e.g., Maillard reaction) that can either cause hardening directly – such as network formation – or lower barriers to forming crystalline, glassy, or jammed states. For example, the sugar component affects whether Maillard browning will occur. Bars must be understood on a multiscale level to effectively reduce hardening. On a microscopic scale the interactions between proteins, carbohydrates, and lipids to react with each other, but then consider the meso-scale as these interaction impact the phase and phases within a food that eventually lead to the macroscope scale of foods structure.

1.3. Overview of Protein –Polyphenol Interactions

1.3.1. Polyphenols

Polyphenols, naturally present in berries and other fruits and vegetables, are rich in anti-inflammatory activity which protect against chronic diseases including CVD, cancer, diabetes, and age-related declines (Basu, Rhone, & Lyons, 2010; Lau, Shukitt-Hale, & Joseph, 2005; Molan, Lila, & Mawson, 2008). In addition, the gut microbiota have been shown to break down polyphenol into metabolites that contribute to anti-carcinogenic effects along with maintaining a health gut microflora (Selma, Espín, & Tomás-Barberán, 2009). Bioactive polyphenolics can also combat obesity by up-regulating energy expenditure, which has been demonstrated in cell, animal, and clinical trials (Meydani & Hasan, 2010). More specifically, berry fruit anthocyanins have been shown to provide anti-inflammatory properties that may involve NF-κB inhibition and target proteins of the oncogenic AKT

pathway (key in cancer cell metabolism) (Karlsen et al., 2007; K. W. Lee, Bode, & Dong, 2011; J. Wang & Mazza, 2002). Last, but not least, polyphenols can decrease LDL-cholesterol and inhibit alpha-glucosidase, which contributes to improved glycemic levels in diabetic individuals (Basu et al., 2010; Boath, Stewart, & McDougall, 2012).

Cranberry

Fresh cranberries (*Vaccinium marcocarpon*) on average contain 120-315 mg phenolics per one hundred grams of fresh weight (Grace, Massey, Mbeunkui, Yousef, & Lila, 2012a). The phenolics consist of a mixture of flavonols, anthocyanins , proanthocyanidins, and phenolic acids (Grace, Massey, Mbeunkui, Yousef, & Lila, 2012b). The top flavanols found in cranberries are epicatechins while the top flavonols are myricetin and quercetin (Harnly et al., 2006). The anthocyanin glucosides present in cranberries are peonidin and cyanidin. Cranberries are unique to other berries in that they are high in A-type proanthocyanidins. A-type proanthocyanidins are flavan-4-ols bound by at least one C2 and C7 linkage (Feliciano, Krueger, Shanmuganayagam, Vestling, & Reed, 2012). These compounds have been shown to provide protection against urinary tract infections. The protection against urinary tract infections is mechanistically thought to be due to the A-type bond which makes the compounds better at adhering to the bacteria which cause urinary tract infections (Howell, 2007). Cranberry juice as a whole can reduce insulin levels of individuals with type II diabetes, reduce oxidative stress and therefore is thought to be a preventative of cardiovascular disease.

Blackcurrant

Blackcurrant (*Ribes nigrum*) juice contains a high concentration of anthocyanins and epicatechin, and small amounts of ellagic acid, gallic acid, proanthocyanidins, and linolenic acid (Gopalan et al., 2012; Kapasakalidis, Rastall, & Gordon, 2006; Määttä, Kamal-Eldin, & Törrönen, 2001).The top anthocyanins in blackcurrant are delphinidin and cyanidin flavonols myricetin and quercetin and kaempferol. The top hydroxycinnamic acid is caffeic acid. In addition to phytochemicals, blackcurrants also contain a significant amount of polyunsaturated acids (located in the seed of the berry) and ascorbic acid (vitamin C) (Gopalan et al., 2012). The polyunsaturated acids from blackcurrants have been found to give minor relief to patients with rheumatoid arthritis (Cameron, Gagnier, & Chrubasik, 2011; Watson, Byars, McGill, & Kelman, 1993). Similar to cranberry polyphenols, blackcurrant polyphneols are also thought to impart positive effects on oxidative stress and endothelial function as it relates to cardiovascular diseases (Khan et al., 2014). The health benefits could also be associated with the vitamin C present in blackcurrants. Anthocyanins and other blackcurrant flavonoids temper both acute and chronic inflammatory responses provoked by tumor necrosis factor alpha (Kumazawa, Kawaguchi, & Takimoto, 2006) and lipopolysacchride-stimulated inflammatory responses *ex vivo*. They also attenuated exercise-induced inflammation for untrained human subjects enduring moderate exercise intensity (Lyall et al., 2009).

Muscadine

Muscadine (*Vitis rotundifolia*) grapes are a common species grown in the southeastern United States (J.-H. Lee, Johnson, & Talcott, 2005). Unlike most grapes,

muscadines are high in hydrolyzable tannins such as ellagic acid and hydroxybenzoic acids such as gallic acid and flavanols, anthocyanins, and condensed tannins. Ellagic acids, which are either present or formed when tannins are hydrolyzed, form crystals and precipitate in juices and wines (Boyle & Hsu, 1990). These crystals are thought to be a defect of these products, but are considered beneficial from a health perspective as *in vitro* studies confirm high antioxidant capabilities and improved vascular function (Larrosa, García-Conesa, Espín, & Tomás-Barberán, 2010). The predominant form of flavanols in mucadines is epicatechins. The predominate condensed tannins in muscadines are B-type proanthocyanidins. B-type proanthocyanidins are flavon-3-ols bound between one molecules C4 and the others C8 or C6, with a ratio of (3:1) of C8:C6 linkages (De Bruyne, Pieters, Deelstra, & Vlietinck, 1999). The primary anthocyanins in muscadines are in the diglucoside forms of delphinidin, cyanidin, petunidin, peonidin, and malvidin. The anythocyanins in muscadine grape have potential for inhibiting cancer as shown in *in vitro* studies by Yi and Akoh (2005) (Yi, Fischer, & Akoh, 2005), as well as decreasing blood glucose, insulin, and glycated hemoglobin in diabetic individuals as shown in an *in vivo* study performed by Banini et al. (2006) (Banini, Boyd, Allen, Allen, & Sauls, 2006).

Health-protective polyphenolics are delivered whenever fruits are juiced, extracted, or made into teas. However, they are generally present in relatively low amounts, so ways to increase their concentration, as well as separating them from sugars or other non-healthy natural fruit components, would allow for a relevant application in foods. One way to increase the delivery concentration of polyphenols is foods is to first use their affinity for

protein to separate them from sugars in juice and concentrate them on a stable protein matrix, as has been recently been achieved (Roopchand et al., 2012; Yousef et al., 2014).

1.3.2. Interaction Mechanisms

Defining specific polyphenols was difficult prior to development of advanced analytical techniques, due to the lack of full understanding of stability, extraction, and post harvest reactions of polyphenols (Russell, 1935). Although identification and study of polyphenols was underdeveloped in the 1930's, one fact about a specific group of polyphenols, tannins, could be identified: tannins precipitate proteins in aqueous (acidic) solutions (Jordan-Lloyd, 1935; Russell, 1935). This was initially discovered as tannins had been used to precipitate proteins and allow for drying of animal hides (Jordan-Lloyd, 1935). In addition, protein-tannin interactions were being studied as early at the 1920's for their physiological astringency and the role in the digestion (Sollmann, 1921). During the 1950's, attention was brought to tannin-protein precipitation as it related to "vegetable" beverage haze, such as beer and wine (Koch & Sajak, 1959; "Process for clarifying and stabilizing vegetable beverages," 1954). Tannins are a plant's defense mechanism in various hazardous conditions. One such purposes is believed to be a bitter taste to prevent animals from consuming the plant (Wrangham & Waterman, 1981). In addition to bitterness, compounds in fruit juices containing polyphenolic tannins, such as cranberry, pomegranate, and persimmon, are characterized by a rough, dry, puckering sensation upon consumption, defined as astringency (Bate-Smith, 1954; Breslin, Gilmore, Beauchamp, & Green, 1993). It is thought that in response to the plant's bitter compounds, animals who consume such plants in turn have their own defense mechanism-proline rich oral proteins. The mechanism of

tannin astringency has been defined as tannin interaction between salivary proteins resulting in precipitation of salivary proteins and mucus polysaccharides (Luck et al., 1994). Oral proteins are postulated to aggregate with tannins making them unavailable to digestion and protect the animal from any harmful side effects of these compounds (Mole, Butler, & Iason, 1990).

The early studies of tannin and protein aggregations have led to studies to understand the binding mechanisms between the two. Investigation of hide tanning suggested that the carboxylic acid groups of a protein/amino acid interact with hydroxyl groups of the tannins (Jordan-Lloyd, 1935). Larger proteins with greater hydrophobic amino acids are more likely to interact with tannins than small proteins containing disulfide bonds (Mehansho, Butler, & Carlson, 1987). Early work by Brown and Wright suggests that green tea polyphenols interact with whey proteins forming soluble and insoluble complexes (Brown & Wright, 1963). Charlton et al. evaluated the binding of four tannins (epigallocatechin gallate, epicatechin gallate, pentagalloylglucose, and trigalloylglucoase) to mouse salivary proteins and various proline rich peptides. Results indicated tannins bind at proline, arginine, and phenylalanine side chains and the larger the peptide the greater the strength of binding. The increase in binding with peptide size was suggested to be a result of an increase in potential binding sites to allow for a single polyphenol to wrap around the peptides and interact at multiple binding sites (Charlton et al., 2002).

Oh et al. suggested that tannins have potential to interact with protein via hydrophobic interactions, which are solvent dependent (H. Oh, Hoff, Armstrong, & Haff, 1980). Furthermore, Oh and Hoff studied grape tannin interactions with bovine serum

albumin, ovalbumin, hemoglobulin, β-lactoglobulin, gelatin and various digestive enzymes through turbidity measurements. Turbidity was evaluated across a range of pHs and in high salt solutions. Turbidity increased around the isoelectric point of the proteins, suggesting the formation of more complexes at the isoelectric point (H.-I. Oh & Hoff, 1987). In addition to pH, Luck et al. also found interactions between and various proteins to be salt dependent. No precipitation was observed for mixtures of β-lactoglobulin or bovine serum albumin and pentagalloylglucose unless 1 M of sodium chloride was added. Dairy proteins and tea or coffee polyphenols have been exhaustively studied regarding binding parameters (e.g., stoichiometry and binding constants) due to the nature in which they are typically consumed together (see Table 1 in Bandyopadhyay et al., 2012) (Bandyopadhyay, Ghosh, & Ghosh, 2012).

As discussed previously, more recent studies suggest that tannins and other plant compounds are beneficial to human health. However, controversy remains high regarding the benefits of having protein and polyphenol bound to each other. Many *in vitro* studies show reduced antioxidant capacity of polyphenols when consumed with proteins (Ozdal, Capanoglu, & Altay, 2013). Yet, as more elaborate *in vivo* studies involving polyphenol absorption by the body are considered, results have been equivocal. For example, in at least four studies, the addition of milk to tea, cocoa, or coffee did not affect the amount of tea polyphenols in blood plasma (Kyle, Morrice, McNeill, & Duthie, 2007; Neilson et al., 2009; Renouf et al., 2010; van der Burg-Koorevaar, Miret, & Duchateau, 2011, p. -). However, it should be noted that the aforementioned studies primarily focus on dairy proteins and tea,

cocoa, or coffee polyphenols. Therefore, in the area of fruit polyphenol research, there remains much to be discovered.

1.3.3. Complexes for Use in Food

Recently, interest in utilizing protein as a delivery vehicle for polyphenols, which includes small molecules to larger bioactives such as plant polyphenols, has come to the forefront of protein-polyphenol research (Chen, Remondetto, & Subirade, 2006; Livney, 2010). The most common approach has been to form polyphenol-protein particles by various encapsulation techniques (Betz & Kulozik, 2011; El-Salam & El-Shibny, 2012; Fang & Bhandari, 2010; Von Staszewski, Jagus, & Pilosof, 2011). Particles created in this fashion range from <1 μm to 3 mm in size (Fang & Bhandari, 2010). Viljanen et al. formed 10% w/w oil-in-water emulsions via sonication in an ice bath with blackcurrant polyphenols and whey proteins. The emulsions with blackcurrant polyphenols contained more stable protein due to reduced oxidation in the presence of the antioxidant anthocyanins (Viljanen, Kylli, Hubbermann, Schwarz, & Heinonen, 2005). More recently, work has shown that gel particles of β-lactoglobulin, one of the main whey proteins, and epigallocatechin-3-gallate (EGCG), a polyphenol found in green tea, led to reduced oxidation of polyphenols, protecting EGCG against degradation (Shpigelman, Israeli, & Livney, 2010). In addition, green tea polyphenols were mixed with β-lactoglobulin and found to maintain polyphenol anti-proliferation activity in tumor cell lines (von Staszewski et al., 2012).

Multiple studies have investigated encapsulation of polyphenols as a way to preserve stability and bioactivity (Fang & Bhandari, 2010; Ozdal et al., 2013). For example, Von Staszewski et al. produced whey protein concentrate-tea polyphenol gel particles at pH 4.5

and 6, resulting in two distinct particle types. Particles formed at pH 4.5 were larger, and had a ξ-potential of -0.37 mV, while the pH 6 particles were smaller and had a ξ-potential of -34.9 mV. The large ζ-potential at pH 6.0 would favor stability in a protein-containing beverage. In addition, gel firmness and adhesiveness increased with addition of polyphenol (Von Staszewski et al., 2011).

Few studies have investigated the impact of polyphenols on protein foaming. One such study by Rodriguez et al. prepared nano particles with green tea polyphenols and β-lactoglobulin. The nano particles reduced surface elasticity of foams and increased foam drainage rate (Rodríguez, von Staszewski, & Pilosof, 2015). Sarker et al. also studied β-lactoglobulin foams, but evaluated foams destabilized with Tween 20. Foams with catechin added at an amount up to 0.1 molar ratio of catechin to protein produced more stable foams destabilized with Tween 20 without catechin; above this ratio foams exhibited a decrease in foam stability (Sarker, Wilde, & Clark, 1995). Additionally, Sausse et al. determined the impact of tea polyphenols on β-casein absorption at interfaces as it relates to foaming (Sausse, Aguié-Béghin, & Douillard, 2003). The adsorption rate was slowed as polyphenol concentration was increased; surprisingly there was an increase in β-casein surface concentration when 15-45 mg/L of polyphenols were added, but reduced at lower and higher concentrations (Sausse et al., 2003). These results suggest protein-polyphenol foams need to be further studied with a focus on protein polyphenol ratio and concentration. Our goals are to investigate protein polyphenol particle formation, these modifications will impact how these particles impact food structures such as protein foams and bars.

If particles can be formed with different ζ -potentials, and sizes, then particles can be designed for various functional applications. Additionally, if the particles impart different textural qualities, then current and new products can be enhanced with addition of proposed ingredient. Most studies on the impact of polyphenols on protein structure focus on tea polyphenols; but, few have looked at berry polyphenols and whey protein particles in the food system. In addition, research is often done with pure proteins and polyphenols, which is unrealistic in a food system, therefore studies with multiple proteins and polyphenols must be executed.

1.4. Conclusion

Individually, both whey proteins and polyphenols are known to impart a range of positive health benefits. We hypothesized that the ability of polyphenols to complex with and aggregate proteins can be used as a process to produce protein-polyphenol particles with functional properties desired for foams and bars. The functional goals are to increase stabilization of foams and slowing or preventing of bar hardening. The nutritional/health goals are to deliver the best mix of bioactive polyphenolic phytochemicals and proteins. This requires the desired quantity of compounds formed into structures that protect bioactivity and bioavailablity. To date, a considerable amount of work has been done on interaction between polyphenols and proteins with the goal of stabilizing and delivering polyphenols, but the methods are complex and involve isolated compounds (proteins/polyphenols) to form emulsions, capsules or micro/nano-gel particles. Protein-polyphenol affinity is primary due to hydrogen bonds, hydrophobic interactions, and the extent and type of interaction depends on pH, specific proteins and polyphenols involved, concentration ratios and reaction time.

Previous research on polyphenol and protein interactions has suggested that formation of protein-polyphenol complexes may result in altered protein solubility, overall enhanced protein functionality, and arguably, increased bioactivity of polyphenols. Previous studies have shown that proteins and polyphenols behave in a synergistic manner when complexed together, increasing bioactivity and bioefficacy of blueberry, cinnamon, and cranberry polyphenols (Ahmed et al., 2014; Grace et al., 2013; Roopchand et al., 2012, 2013). In this work, we will not only delve into the interaction of berry juices with whey proteins, but more importantly into the impact that these interactions have on structural components of foods.

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2. Chapter 2: Formation of whey protein-polyphenol meso-structures as a natural means of creating functional particles

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Formation of whey protein–polyphenol meso-structures as a natural means of creating functional particles

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Whey proteins provide structure and nutritional properties in food, while berry juices are thought to have biological activity that can impart anti-inflammatory health effects. In combination, the two could be an excellent source of necessary and supplemental nutrients as well as expand the functionality of whey proteins in food structures. The objectives of this investigation were to (1) develop an approach for particle formation between whey protein and cranberry, blackcurrant, or muscadine grape juices, (2) determine resulting particle composition and physical characteristics, and (3) evaluate properties related to food structure stability and maintenance of phytochemical bioactivity. Particles were formed by combining 20% w/w whey protein with juice containing 50, 250, or 500 μg g^{-1} total phenolics, adjusting pH to 4.5, and centrifuging to collect aggregated particles. Particles had an approximate molar ratio of 9–50 proteins per polyphenol, and the ratio increased with increasing phenolic content of the juice used to create the particles. Particle size ranged from 1–100 μm at pH 4.5, compared to 10 μm particles that formed when whey protein isolate alone was precipitated at pH 4.5. Polyphenols and other juice components, such as acids and sugars appeared to be involved in particle formation. Particles improved foam stability, and the anti-inflammatory properties of entrapped polyphenols were maintained in the particles. Highly functional protein–polyphenol particles can be designed to stabilize food structures and simultaneously deliver polyphenols associated with health benefits.

1. Introduction

Consumer trends are shifting towards healthier dietary options, low in fat and sugar, but high in protein.[1] Reduction of fat and sugar while increasing protein may achieve nutritional goals, but can alter food structure such that product acceptability is decreased.[2] Ideally, proteins would replace the structural contribution of fat or sugar. This is often a challenge because proteins form and stabilize food structures (*e.g.*, foams, emulsions, and gels); consequently, they may create their own structure rather than replace the one initially provided by the fat and sugar. Therefore, replacement of fat and sugar with protein can impart negative characteristics, such as gelling of beverages or hardening of bars. If the goal is to increase protein content of a food without changing food structural elements associated with sensory acceptance, then proteins need to be in a form that is less active as an ingredient so that it does not disrupt essential elements of food structure or reduce stability.

The premise of maintaining desirable food quality while altering molecular composition is only possible if quality is related to food structure in general and not an absolute requirement of specific molecules. This fits the approach of viewing foods as a form of soft matter. A basic concept of soft matter physics models is that the macroscopic properties, such as food texture, are not predicted directly from molecular properties, but associated with meso-structures observed at the nano- or micro-scales.[3,4] Therefore, an approach to accommodate various consumer desires (*e.g.*, gluten free, fat free, or sugar free) is to use different molecules to generate similar meso-scale structures that provide the desired property in foods. Proteins can be formed into micro- or nano-length scale particles with specific size, shape, and surface properties that provide a range of functional roles in food structure.[5,6] Protein particles can be produced by singular or combined processes including phase separation, emulsification, pH adjustment, addition of salt, and thermal treatments.[5,6] This investigation offers an approach that uses a combination of pH adjustment and addition of berry juices to create protein–polyphenol particles with functionality based on their meso-structures and

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the additional benefit of containing bioactive plant polyphenols.

Multiple studies have investigated encapsulation of polyphenols in a protein matrix, producing particles ranging from 1 nm to 100 μm in diameter.[7–15] Proteins are thought to interact with polyphenols *via* hydrophobic interactions between the aromatic nuclei of the phenolics and the aromatic and aliphatic side groups of the amino acids.[16] These interactions can result in reduced or enhanced protein aggregation, resulting in a range of outcomes including precipitation of protein, enhanced protein functionality, or increased bioactivity of polyphenols.[17–20] For example, early work by Brown and Wright suggests that green tea polyphenols interact with whey proteins forming soluble and insoluble particles.[21] von Staszawski and others showed that nano-particle morphology in whey protein–tea polyphenol mixtures depended on pH, and maintained the foaming capabilities of the protein.[8,14,15] Particles formed at pH 4.5 were larger and had a ζ-potential of −0.37 mV, while pH 6 particles were smaller and had a ζ-potential of −34.9 mV. Bovine serum albumin has been shown to form particles with a mixed range of polyphenols including proanthocyanidins, pentagalloyl glucose and epicatechin$_{16}$ (4 → 8) catechin.[20] These investigations show the potential for protein–polyphenol mixtures to be adjusted to produce a range of particles (meso-structures) for food applications.

Most studies on protein–polyphenol particles have focused on forming nano- or micro-scale particles as a means to protect the bioactivity of polyphenols, rather than producing particles with specific functional applications in foods.[9] In this investigation, we consider juice as a processing co-factor to produce functional particles with the added benefit of adding bioactive polyphenols. The protein source was whey protein isolate (**WPI**) and the particles formed were designated whey protein–polyphenol (**WP-P**) particles. Three juices, blackcurrant, muscadine grape, and cranberry, were selected for comparison based on their unique and diverse polyphenolic profiles. Blackcurrant juice has high concentrations of anthocyanins and epicatechin, and minor amounts of ellagic acid, gallic acid, and proanthocyanidins;[22,23] cranberry juice is characterized by high concentrations of anthocyanins and A-type proanthocyanidins;[24] and muscadine grape juice is a rich source of anthocyanins, ellagic and gallic acids, epicatechin and B-type proanthocyanidins.[25] The objective of this investigation was to determine: the conditions required for particle formation, the composition and physical characteristics of particles, foam functionality, and the potential biological activity of the total polyphenols (**TP**) in the particles.

2. Experimental

2.1. Materials

Whey protein isolate (brand name BiPro) was supplied by Davisco Foods International, Inc. (Le Sueur, MN, USA). It contained 93.19% protein, based on Dumas method for nitrogen analysis with a conversion factor of 6.38% protein to % nitrogen.[26] Elemental analysis by inductively coupled plasma spectroscopy showed the powder contained 1.23% P, 0.35% K, 2.02% Ca, 0.11% Mg, 0.7% S, and 0.10% Na. Cranberry juice concentrate (50 °Bx, 5.2 mg mL^{-1} TP) was obtained from Ocean Spray ® (Lakeville-Middleboro, MA, USA), muscadine juice concentrate (70 °Bx, 3.6 mg mL^{-1} TP) from The Muscadine Products Corporation (Wray, GA, USA), and blackcurrant juice concentrate (60 °Bx, 28.2 mg mL^{-1} TP) from The New Zealand Blackcurrant Co-operative LTD (Nelson, NZ). High performance liquid chromatography (**HPLC**) grade 0.2 μm filtered methanol, ACS grade acetic acid, and sodium hydroxide pellets were purchased from Fisher Scientific (Fairlawn, NJ, USA). Hydrochloric acid (34–37%) was purchased from VWR (West Chester, PA, USA) and L-tartaric acid (99%) from Acros Organics (Morris Plains, NJ, USA). Anhydrous citric acid citrate, tartrate, and lab grade anhydrous dextrose were sourced from Fisher Scientific (Nazarath, PA, USA). Fructose was purchased from Avantor Performance Materials Inc. (Philipsburg, NJ, USA) and D-phenylalanine (99%) from Alfa Aesar (Heysham, England). Nile Blue dye was purchased from Invitrogen Molecular Probes (Eugene, OR, USA) and was dissolved in glycerol from Sigma (St. Louis, MO, USA). Solvents for HPLC analysis – methanol, acetic acid, acetonitrile, and formic acid – were HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). RAW 264.7 murine macrophage cells (ATCC TIB-71) were purchased from American Type Culture Collection (Livingstone, MT, USA) and growth material, Dulbecco's modified eagle's medium (**DMEM**), phosphate buffered saline (**PBS**), and fetal bovine serum were purchased from Life Technologies (Carlsbad, CA, USA) along with TRIzol reagent, cDNA transcription kit, and SYBR green qPCR master mix. Penicillin and streptomycin for growth media were purchased from Fisher Scientific (Fairlawn, NJ, USA) and the sample vehicle dimethyl sulfoxide (**DMSO**) and lipopolysaccharide from *E. coli* strain 0111:B4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The molecular probe 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (**H_2DCFDA**) was purchased from Thermo Scientific (Waltham, MA, USA).

2.2. Effect of total phenolic concentration on particle formation and yield

Whey protein–polyphenol particles were formed with cranberry, blackcurrant, and muscadine concentrates diluted with deionized water (>17 MΩ) to achieve TP concentrations of 50, 250, or 500 μg g^{-1}. Dry WPI was then added to diluted juices to attain a concentration of 20% w/w protein and mixed for 4 h to fully disperse the powder. The pH of all mixtures was adjusted to 4.5 with either 3 M HCl or 3 M NaOH, and mixed for 30 min, to allow for maximum particle formation prior to centrifugation at 7000*g* for 20 min at 15 °C to collect the particles. A portion of the pellet was dried in a convection oven for 2 h at 110 °C to determine moisture content by weight differential. Protein and polyphenol content of the wet pellet was measured using the Dumas method for protein analysis

and Folin–Ciocalteu assay for total polyphenolic content (sections 2.5 and 2.6). Another portion of the pellet was dispersed at 10% w/w protein in deionized water for 1 h and used for particle size analysis (section 2.7). Three replicates of each treatment were prepared. Mixtures of juices and protein that undergo pH adjustment are referenced as whey protein-cranberry juice (**WP-C**), whey protein-blackcurrant juice (**WP-B**), or whey protein-muscadine juice (**WP-M**) suspensions. Following centrifugation, the particles formed in the pellet are referred to as whey protein-cranberry polyphenol (**WP-CP**), whey protein-blackcurrant polyphenol (**WP-BP**), or whey protein-muscadine polyphenol (**WP-MP**) particles.

2.3. Standard particle formation procedure

Large batches of particles made at TP concentrations of 250 μg g^{-1} of cranberry, blackcurrant, and muscadine grape juice were formed as described above (section 2.2). Particles were re-suspended in deionized water at ~10% w/w protein and spray dried with a BÜCHI B-290 mini spray dryer (BÜCHI Labortechnik AG, Flawil, Switzerland) with an inlet temperature of 190 °C ± 5 °C and an outlet temperature of 90 °C ± 5 °C (100% aspiration and 40° spray angle). Particles formed from 250 μg g^{-1} TP juice are referred to as "standard particles." Spray dried particles were analyzed for dispersibility, particle size, composition, *in vitro* anti-inflammatory properties (section 2.7–2.15), and sent to Medallion Laboratory (Minneapolis, MN, USA) for sugar analysis according to AOAC 977.20.[27] Particles were formed on three different days and spray dried separately. Each preparation represented a sample "lot" or replication.

2.4. Polyphenol free imitation juice formulation and particle formation

A polyphenol free imitation juice formulation was used to determine the effects of juice acids and sugars on particle formation. Formulations were made to imitate the buffering capacity, pH and sugar content of the three juices at 250 μg mL^{-1} TP dilutions. Formulations for imitation juices are presented in Table 1. Acid and sugar content were estimated based on measured Brix and buffering capacity and literature values were used for the ratio of specific sugars and acids.[28,29] Imitation juice solutions were mixed with 20% w/w protein, centrifuged, and the pellet spray dried as in section 2.3 for the standard particles formed with juice.

Table 1 Formulations for imitation juice particle formation

Composition of imitation juice	Buffer		Sugar	pH
	Acid	Base		
Cranberry	0.88 M citric acid	0.88 M citrate	1.6% fructose: 8.3% glucose	3.06
Blackcurrant	0.67 M citric acid	0.67 M citrate	2.6% fructose: 3.9% glucose	2.95
Muscadine grape	0.28 M tartaric acid	0.28 M tartrate	7.5% fructose: 7.5% glucose	3.20

2.5. Protein concentration

Protein concentration was determined based on nitrogen content measured by the Dumas method, with an Elementar vario MACRO cube CHN (Mt Laural, NJ, USA). The instrument was calibrated against three phenylalanine standards during each use. Liquid samples weighing 50–80 mg were enclosed in tin capsules and dry samples weighing 50–80 mg were enclosed in tin sheets for testing. Protein concentration was calculated by multiplying the percent nitrogen by 6.38; the nitrogen-to-protein conversion factor for whey proteins.[26] There was no detectable nitrogen in a 50–80 mg juice sample; therefore all nitrogen was assumed to be from whey protein isolate. Each protein sample replicate was run in duplicate.

2.6. Polyphenolic extraction and measurement

The polyphenols were extracted using 8 mL of 75% methanol: 2% acetic acid v/v per gram of powder. Extracting solvent and sample were sonicated for 5 min and centrifuged for 10 min at 4000*g*. The supernatant was collected in a 25 mL volumetric flask and the extraction (addition of methanol–acetic acid and centrifugation) was repeated twice more. The volume of the collected supernatant was brought up to 25 mL with methanol–acetic acid solvent. The total polyphenolic content of each extraction was measured using the Folin–Ciocalteu method.[30] The extracts were also used for individual polyphenol analysis and cell culture in sections 2.8, 2.14, and 2.15. A sample of unmodified WPI powder was extracted in the same fashion for use as a WPI control in cell culture assays (see section 2.14 and 2.15). Three lots were extracted in triplicate and TP content of each extraction was measured in triplicate.

2.7. Particle size measurement

Particle size was determined using laser diffraction with a Mastersizer 3000 (Malvern, Worcestershire, UK). Dry particles were dispersed in deionized water at 10% w/w protein. When dispersed, all particles resulted in a solution pH of 4.5 ± 0.1. The sample flask was stirred at 1500 rpm and particle dispersions were added to the sample flask until an obscuration of 10–13% was achieved, at which point samples were measured for particle size and reported in equivalent volume weighted diameters ($D[4, 3]$). Three lots of WP-P powders were analyzed and each sample was read five times.

2.8. HPLC analysis of anthocyanins (ANC) and proanthocyanidins (PAC)

The amount of PAC and ANC were determined using two Agilent Technologies 1200 series HPLCs (Santa Clara, CA, USA) each affixed with a photodiode array detector. Chemstation software (Agilent Technologies, Santa Clara, CA, USA) was used to control the HPLCs and complete data analysis. Total phenolic extracts were filtered through a 0.2 μm PTFE syringe filter (Fisher Scientific, Fair Lawn, NJ, USA) and injections of 10 μL of juice dilutions and filtered WP-P TP extracts were loaded on the columns. One HPLC was affixed with a normal phase Phenomenex Develosil Diol column (250 nm × 4.6 mm × 5 μm,

Torrance, CA, USA) for PAC analysis. The mobile phase used for separation of PACs was 2% acetic acid in deionized water (solvent A) and 50% acetonitrile in 0.5% acetic acid in deionized water (solvent B). Sample results were analyzed in type-A1 proanthocyanidin equivalence using a standard curve produced across the range of 0.03–1.00 mg mL^{-1}. A gradient of 10% at 0 min, 15% at 13 min, 25% at 20 min, 55% at 50 min, 100% at 54 min and 10% at 60 min of solvent B was achieved with a flow rate of 1 mL min^{-1}. The second HPLC was used for ANC separation, which was achieved using a reversed-phased Phenomenex Synergi 4 μm Hydro-RO 80A column (250 nm × 4.6 mm × 5 μm, Torrance, CA, USA). The gradient was 5% formic acid in water (solvent A) and 100% methanol (solvent B) gradient 10% at 0 min, 15% at 5 min, 20% at 15 min, 25% at 20 min, 30% at 25 min, 60% at 45 min, and 10% at 60 min solvent B. The results are represented in cyanidin-3-*O*-glycoside equivalence established from a standard curve between 0.1–0.5 mg mL^{-1}. Three different lots were extracted and each extraction was run once through the HPLC.

2.9. Protein dispersibility

Dispersions were prepared by adding 10% w/w protein of WP-P particles to deionized water and mixing at 300 rpm using a magnetic stirrer for 4 h at room temperature (24 ± 2 °C). Samples were adjusted to pH 2, 3, 4, 5, 6, 7, or 8 using 2 M HCl or 3 M NaOH and stirred for 30 min to allow for equilibration before being centrifuged for 15 min at 17 200*g* at 15 °C. One sample was collected pre-centrifugation to account for any discrepancy in protein concentration due to addition of acid or base. Dispersibility was calculated as the percent protein (protein was measured according to section 2.5) remaining in the supernatant following centrifugation. Three replicates (established by three separate lots of WP-P powders) of dispersibility experiments were completed in duplicate.

2.10. Confocal microscopy of rehydrated standard particles

Confirmation of particle size was achieved with confocal microscopy. Nile Blue was prepared at a concentration of 0.2% w/w in glycerol and filtered through at 0.45 μm filter to remove undissolved dye and residual particulates. A mixture of dye solution and WP-P particle solution (at 10% w/w protein) was made at a 1 : 2 ratio of dye to WP-P particle solution. Specially made slides containing a 15 mm hole were used as a sample carrier with a cover slide affixed over the hole with vacuum grease. A drop of the mixture was placed on a cover slip and viewed using and a 40× water immersion objective (LDC-Apochromat 40× N.A.-1.1 W Korr M27). Images were captured with a LSM 710 confocal laser light scanning microscope (Zeiss, Jena, Germany) with an inverted stage. Images were taken in triplicate and images were selected based on most representative sample section.

2.11. ζ-Potential of rehydrated standard particles

The samples of WP-P (from section 2.9) were suspended in deionized water at a concentration of 0.5 mg mL^{-1} protein. The pH of samples was adjusted using 0.5 M NaOH or 0.5 M HCl. A Wyatt Möbiuζ(Wyatt Technology, Santa Barbara, CA, USA) was used to calculate the ζ-potential of samples. The samples were read five times per load and three different lots of each WP-P powder were run to achieve three replicates.

2.12 Foam formation and drainage half-life

Foams were formed according to Pernell *et al.*[31,32] as outlined below. The wet WP-P particles were used to form foams. Solutions of 10% w/w protein were dispersed for 2 h before pH was adjusted to 7 with 1 M NaOH and mixed for an additional 2 h before use for preparing foams. Portions (200 g) of each protein solution were whipped for 20 min on speed 8 in a Kitchen Aid Power Mixer (Kitchen Aid, St. Joseph, Michigan, USA). The mixer bowl had a 6 mm drainage hole near the base that was covered with tape during whipping. Following mixing, the bowl was immediately placed over a weigh boat sitting on a scale and the tape removed to allow drainage. The drainage half-life was recorded as the time for 100 g of liquid to drain from the mixing bowl.

2.13 Cell culture growth, viability, and dosage determination

Mouse macrophage (RAW 264.7) cells were grown in DMEM growth media enhanced with 100 IU mL^{-1} penicillin/100 μg mL^{-1} streptomycin and 10% fetal bovine serum. The cells were allowed to grow at a confluence not exceeding 90% and kept in a humidified incubator at 37 °C with 5% CO_2. Samples of WP-CP, WP-BP, WP-MP, and WPI were extracted with methanol: acetic acid (section 2.6) and were prepared by evaporating off the methanol, acetic acid, and water with a rotary evaporator (BÜCHI Corporation, New Castle, DE, USA). The dried extracts were then solubilized in DMSO at 50 mg mL^{-1} and stored at −20 °C until use.

Cell viability of RAW 264.7 cells was determined by seeding cells in a 96-well plate (5×10^4 cells per well) and treating cells with 50, 100, or 200 μg mL^{-1} of each extract (WPI, WP-CP, WP-BP, or WP-MP) followed by exposure to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**) for 24 h according to Mosmann (1983).[33] Viability was quantified as the absorbance value at 550 nm after 24 h (Synergy H1 microplate reader, BioTek).

2.14 Anti-inflammatory properties of standard particle extracts in cell culture

The cells were seeded in 24-well plates (5×10^5 cells per well) and allowed to grow to 90–95% confluence for 24 h before pretreatment for 1 h with WP-CP, WP-BP, WP-MP, and WPI extracts at 100 and 200 μg mL^{-1}, as well as a positive control (dexamethasone (**DEX**) at 10 μM), and no treatment as a control for 1 h before cells were elicited with 1 μg mL^{-1} lipopolysaccharide (**LPS**) for an additional 4 h. After 4 h the total RNA was extracted from macrophages with TRIzol reagent according to manufacturer's instructions with storage at −80 °C overnight. RNA quantity and quality was evaluated using the Synergy H1/Take 3 spectrophotometer (BioTek, Winooski, VT, USA) where absorbance at 280 nm was used for quantification of RNA and the ratio of the absorbencies at

Table 2 Gene and respective forward and reverse primers used for cDNA amplification

Gene	Forward primer	Reverse primer
Reporter gene, β-actin	5′-AAC CGT GAA AAG ATG ACC CAG AT-3′	5′-CAC AGC CTG GAT GGC TAC GT-3′
COX-2	5′-TGG TGC CTG GTC TGA TGA TG-3′	5′-GTG GTA ACC GCT CAG GTG TTG-3′
iNOS	5′-CCC TCC TGA TCT TGT GTT GGA-3′	5′-TCA ACC CGA GCT CCT GGA A-3′
IL-6	5′-TAG TCC TTC CTA CCC CAA TTT CC-3′	5′ TTG GTC CTT AGC CAC TCC TTC-3′
IL-1β	5′-CAA CCA ACA AGT GAT ATT CTC CAT G-3′	5′-GAT CCA CAC TCT CCA GCT GCA-3′

260/280 nm was used for RNA quality. Only RNA with a quality >1.8 was used for further synthesis of cDNA. For each sample, 2 μg of RNA were used to synthesize cDNA using a high-capacity cDNA Reverse Transcription kit, according to manufacturer's instructions (Life Technologies) on an ABI GeneAMP 9700 (Life Technologies). The cDNA was amplified by real-time qPCR with SYBR green PCR Master Mix for quantification. To prevent genomic DNA interference, intron-overlapping primers were chosen using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA) as recorded in Table 2. Amplifications were performed on an ABI 7500 fast real-time PCR machine (Life Technologies) programed to run the first cycle at 50 °C for 2 min, the second cycle at 95 °C for 10 min, 40 intermediate cycles at 95 °C for 15 s each, and a final extension cycle at 60 °C for 1 min. A dissociation protocol was added following the last cycle with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. Gene expression was analyzed with 7500 Fast System SDS software v1.3.0 (Life Technologies) using the comparative Ct method where samples were normalized with respect to the β-actin housekeeping genes.[34] A value of 1 was assigned to samples treated with LPS as this treatment shows maximum gene expression. Results with values <1 are indicative of inhibition of gene expression, and values >1 indicative of overexpression of a gene, in excess of LPS stimulation. Melting curve profiles confirmed sufficient amplification of specific transcripts. Two replicates of the experiment were completed and samples run in triplicate.

2.15 Statistical analysis

Statistical analysis was completed using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA) using ANOVA and Tukey's multiple comparison of means.

3. Results and discussion

3.1. Particle formation and characterization

3.1.1. Effect of total phenolic concentration on particle formation yield. Protein–polyphenol particles/encapsulates are typically formed with individual proteins and polyphenols, and are intended to stabilize the bioactivity of the polyphenols. In this work, mixtures of whey proteins and juices, containing a range of polyphenols, were formulated as a means to produce food grade functional particles to stabilize and enhance food structures. Whey protein-juice polyphenol particles were formed by mixing WPI with juices and adjusting pH to 4.5 (for consistency between juices and maximum yield). Particles were collected by centrifugation. The juice plus protein mixtures yielded particles with varying amounts of protein and total phenolics depending on the ratio and amount of initial juice TP and added protein. The composition of WP-P particles ranged in protein from 672–898 mg g^{-1} and 0.47–2.00 mg g^{-1} TP on a dry weight basis (values above bars in Fig. 1). Protein yield in the aggregate particles ranged from 28–56% w/w of the initial protein added to the juice, and yield increased as the polyphenol content of the initial juice increased (Fig. 1). Since protein yield in the pelleted particles increases with increasing juice TP content, we can conclude that the combination of juice components and protein enhanced protein aggregation and precipitation. The TP yield in the particles ranged from 28–84% w/w of initial TP weight. For all juices, the greatest TP yield in the particles was achieved with an initial juice TP concentration of 50 μg g^{-1}, and the lowest yield resulted when the TP concentration in the initial juice was 500 μg g^{-1} (Fig. 1). Increasing initial juice polyphenol content resulted in a smaller portion of polyphenols being adsorbed to proteins. This behavior was also observed by Siebert *et al.*,[35] where haze formed in a concentration and ratio dependent manner and plateaued, at which point increased protein or polyphenol did not increase haze formation due to interactions between catechin and gliadin or gelatin. Likewise, Frazier *et al.*[36] observed tannin binding to reach a saturation point when binding to gelatin or bovine serum albumin. Therefore, for the current application, a balance must be met between TP and protein ratios and concentration to achieve a maximum yield of both starting ingredients. The yield of polyphenols bound to particles formulated with the 500 μg g^{-1} TP juice was insufficient to justify further use. Juices with 250 μg g^{-1} TP yield a higher protein yield in the aggregate particles, as well as a greatest overall TP content. To maximize the protein and TP yield, forming particles in juices at 250 μg g^{-1} TP concentration was selected as the standard particle forming procedure for further investigation.

Particle size contributes to a range of functional properties, therefore the size of particles in the pellet (WP-P) was measured after dispersing in deionized water at 10% w/w protein. In addition, to gain insight into particle formation, particle size was measured at an intermediate step of the process, after the whey protein and juice were mixed and adjusted to pH 4.5 but before centrifugation; this step is referenced as the suspension. The size of the particles formed in whey protein juice suspensions containing 50 μg g^{-1} TP were at around 10 μm for all juices (Fig. 2A). As the starting polyphenol concentration increased, the size of suspension particles shifted to groups mainly at 4 and 40 μm. Following centrifugation and re-suspension, particles dispersed from the pellet had groupings around 1, 4, and 40 μm, and a new prominent

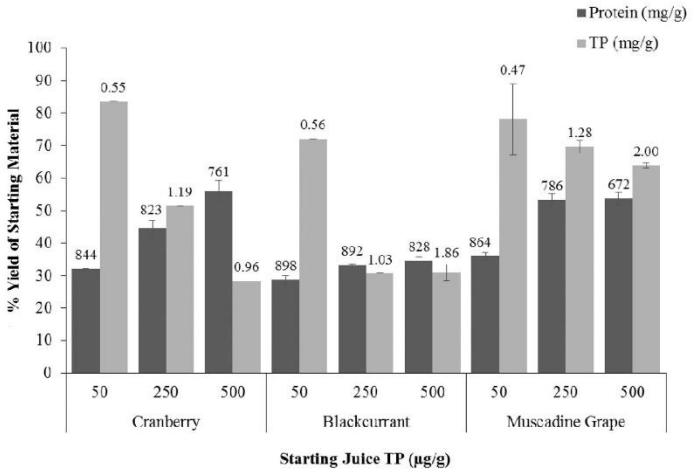

Fig. 1 Yield (bars) of protein and total polyphenolics (TP) in particles formed in suspensions of juices containing 50, 250, and 500 μg g^{-1} TP and 20% protein w/w. Amount of total dry weight of protein or polyphenol is given above the bars in mg g^{-1}.

group of particles greater than 100 μm (Fig. 2B). The size of particles increased with increasing polyphenol concentration and decreasing pH of the suspension after the protein was initially dispersed (Fig. 2). This trend in particle size aligns with the increasing protein yield with increasing starting TP. The larger particles may be secondary aggregates that form when more TP are present.

The particles formed in this investigation are similar in size to those formed in other investigations that used additional processing or ingredients other than polyphenols.[11,13,37,38] The particle forming processes in other studies required incorporation of polymers, such as alginate, pectin, or gelled whey proteins, as well as additional procedural steps such as emulsion formation.[11,13,37,38] In contrast, this work successfully produced micrometer scale protein–polyphenol particles by simply complexing whey protein and polyphenols under defined conditions without the need for additional processes or additives. While particle size achieved in this work with juices and whey protein was comparable to sizes achieved using other particle-forming processes, the particles formed were larger than those formed from mixtures of just proteins and isolated polyphenols.[9,12]

The particle size for the pellet and the suspension was measured at pH 4.5, the pH of all suspensions when particles were collected by centrifugation. However, a trend was observed when considering the pH of the suspensions before pH adjustment to 4.5. Particles appear to shift toward the large class sizes when the suspension pH was below pH 6, nearing the isoelectric point of the proteins (Fig. 2A and B). The apparent isoelectric-point dependent behavior of WP-P particle formation suggests that the pH of the suspension during the initial mixing of whey proteins with juice determined the particle size and subsequent adjustment to pH 4.5 after 4 h of mixing WPI and juice had minimal effects on particle size.

3.1.2. Composition of standard particles. Standard particles were formed from juice concentrates diluted to 250 μg g^{-1} TP with 20% w/w added WPI, centrifugation, and spray drying. This treatment was selected to achieve maximum yield of TP and protein. The standard particle powders contained TP averaging around 1 mg g^{-1} on a dry basis (Fig. 3; no significant differences were found in TP content among WP-P). We can deduce that 0.2 mg g^{-1} or 16% of the polyphenols present in the wet pellet (Fig. 1) are degraded or covalently bound to the protein and not available as free polyphenols during extraction. The protein content of WP-BP particles was 870 mg g^{-1}, which was significantly greater than that of the WP-CP and WP-MP powders (Fig. 3). As expected, on a dry weight basis, the protein content of the particles before and after drying did not change.

Taking a closer look at non-protein composition, anthocyanins (ANC), A-type proanthocyanidins (PAC-A), B-type proanthocyanidin, and individual sugars were measured. These polyphenols were selected for measurement because cranberry, muscadine, and blackcurrant juices contain large quantities of anthocyanins, cranberry juice contains A-type PACs, and muscadine juice contains B-type proanthocyanidins.[22–25] B-type proanthocyanidins were not detected at significant level in any of WP-P particles and only WP-CP had significant amounts of A-type PACs. All treatments contained substantial amounts of ANC, averaging around 600 μg g^{-1} (Fig. 3). In congruence with berry sugar literature,[28,29] the only sugars detected in the powders were glucose and fructose, and WP-MP contained the most with a total of 6.5% sugar (Fig. 3).

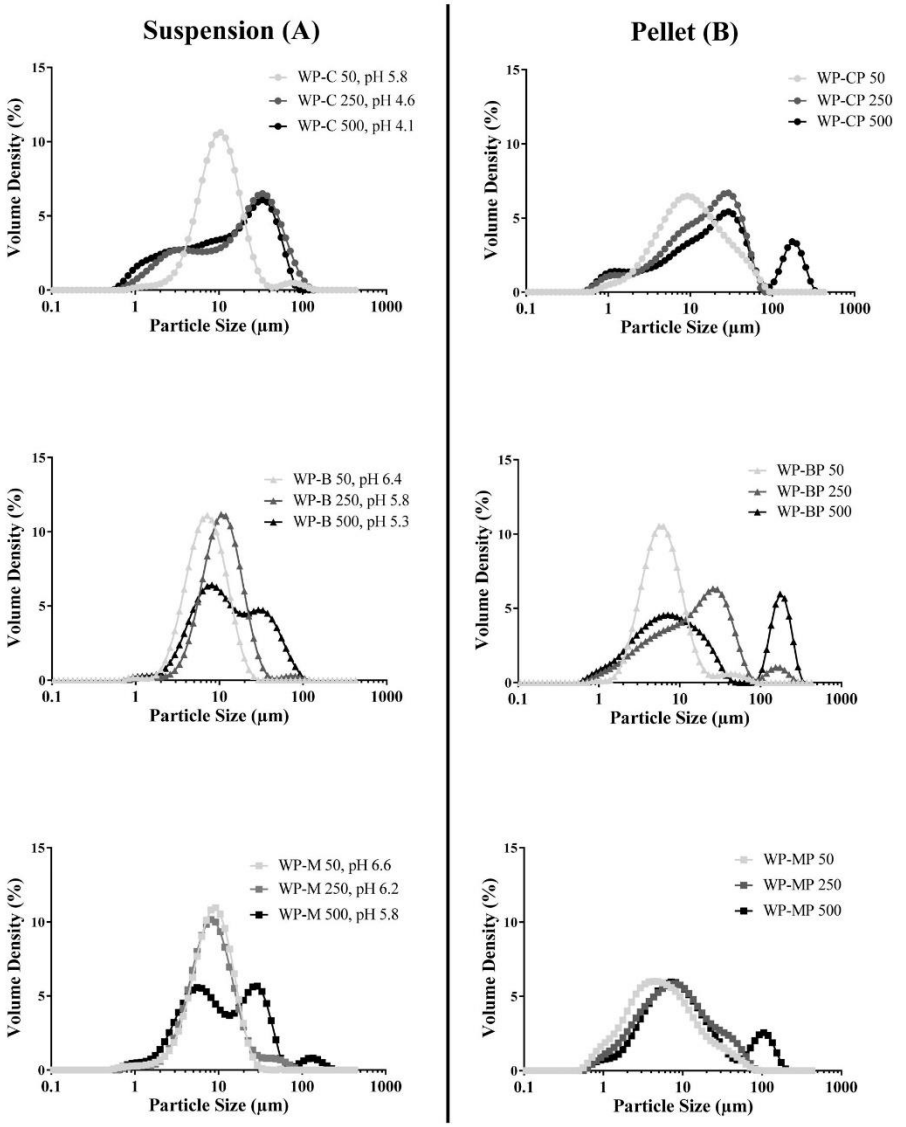

Fig. 2 Particle size of the suspension mixture (A) and resulting pellet after centrifugation (B). Particles were dispersed in deionized water at pH 4.5. The pHs next to treatment labels represent suspension pH before pH adjustment and centrifugation. Samples were whey protein-cranberry juice suspensions (WP-C), whey protein-blackcurrant juice suspensions (WP-B), whey protein-muscadine juice suspensions (WP-M), whey protein-cranberry polyphenol pellet (WP-CP), whey protein-blackcurrant polyphenol pellet (WP-BP), or whey protein-muscadine polyphenol pellet (WP-MP) particles with starting juice total phenolics of 50, 250, or 500 μg mL^{-1}.

3.1.3. Rehydrated standard particle size. Particle size was measured at pH 4.5 to coincide with the pH used to normalize solutions prior to centrifugation. All measurable particles were within the range of 2–200 μm, with the majority of particles peaking around 10 μm (Fig. 4). The WP-CP and WP-BP particles contained a second larger particle peak at 100 μm, indi-

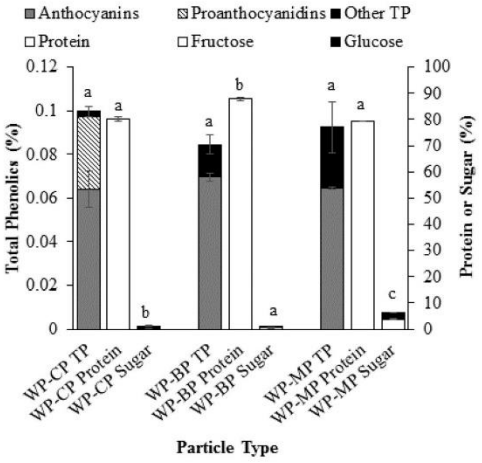

Fig. 3 Dry particle protein and total polyphenol (TP) composition on a dry weight basis. The polyphenol component is partitioned into anthocyanin content, A-type proanthocyanidin content, or other type of polyphenol. Samples were whey protein-cranberry polyphenol (WP-CP), whey protein-blackcurrant polyphenol (WP-BP), or whey protein-muscadine polyphenol (WP-MP) particles. Difference in letter indicates significant difference between total TP, protein, or sugar across samples ($p < 0.01$).

cating two particle groups. Confocal imaging showed blackcurrant and cranberry to have larger particulates around 40–50 μm, compared to muscadine solutions, supporting particles size measured with laser diffraction method (Fig. 5). However, the amount of particles in each size class observed in confocal imaging appear different from particle distributions by particle size analysis, as cranberry appears to have a greater percentage of large particles compared to blackcurrant. This is because of inherent differences between these methods in how particles are detected that would alter apparent distributions.

Particle formation may be driven by polyphenols interacting with proteins, changes in pH, other juice components, or a combination of all factors. To determine the effect of non-polyphenol juice variables, polyphenol-free imitation juice formulations were prepared according to Table 1 to obtain the same buffering capacity, pH, and sugar content of the juices. These imitation juices were used to create particles by the same process used for the juice treatments referred to as imitation juice particles (**WP-I**). The imitation juices produced particles averaging around 10 μm and did not form the larger class around 100 μm (Fig. 4). Therefore, the closest match was with muscadine juice, which lacked the 100 μm particles (Fig. 4).

3.1.4. Dispersibility of standard particle. Protein dispersibility was determined after particle dispersions at 10% w/w protein were adjusted to pH 2, 3, 4, 5, 6, 7, or 8 and mixed for 12 h at room temperature. Protein dispersibility was highest for the unmodified WPI (Fig. 6A). This reflects the amount of protein that remained dispersed after adjusting hydrated WPI powder (pH ~ 6.8) to the various pH values and centrifuging to remove aggregates. Unmodified WPI is a control showing maximum dispersibility of what should be mostly individual protein molecules or aggregates produced in processing or at a given pH. In comparison, the particles were formed, dried, and then re-dispersed. All WP-P particles were similar in dispersibility to unmodified WPI at pH 2, 3, 7, and 8 (Fig. 6). These regions correspond to high levels of positive (pH 2 and 3) or negative (pH 7 and 8) charge and increased dispersibility is likely related to dissolving the large aggregates generated during particle formation. Between pH 3 and 7, the dispersibility of WP-P particles was lower than the unmodified WPI (Fig. 4). Samples of WP-P between pH 4 and 6 developed larger particles >1 μm, composing ≥50% of the dispersed mass (data not shown). Comparatively, WPI in this pH range developed aggregates ~0.5 μm but only near the isoelectric point, and these accounted for <10% of the total mass (data not shown). The larger particle size of WP-P particles between pH 4–6 supports the reduced dispersibility in this pH range. This suggests the polyphenols or juice components are interacting with proteins, producing primary complexes that are more prone to aggregation and resulting in larger particles.

Juices contain other compounds besides polyphenols that may be altering aggregation and particle formation. Therefore, a comparison of particles from juice with those from imitation juice should suggest differences due to polyphenols. There were differences in dispersibility between juice and imitation juice treatments (Fig. 6B–D); however, they did not directly align with particle size results (Fig. 4). Dispersibility also depends on the density of particles, which is not accounted for in laser diffraction particle size analysis. Considering just the juice components, the WP-CP particles were the only particles more dispersible than the imitation particles (Fig. 6B). The dispersibility of WP-BP and WP-MP particles are very similar to those made with imitation juice, except for lower dispersibility at pH 6.0 (Fig. 6C & D). The non-polyphenol juice components appear to account for the reduction in dispersibility of WP-BP and WP-MP particles, while the WP-CP particles showed effects of polyphenols and juice components.

3.1.5. ζ-Potential of standard particle. The ζ-potential of re-suspended dry particles across a range of pH values was not significantly different for WP-P at pH 5, 6, 7 to that WPI (Fig. 7). The lower ζ-potential in WP-P particles between pH 3–4 could be due to a shielding of the charge by the polyphenols, but a molecular explanation is not clear. von Staszewski *et al.*[8] found similar behavior, where particles that contained polyphenols and whey protein concentrate (~35% protein) had very little charge at pH 4.5. However, dissimilar behavior was found at pH 6 in which polyphenol particles had greater negative charge (−50 mV) compared to whey protein concentrate free of polyphenols (−34.9 mV). This is expected because the specific polyphenols in the juice that interact with the protein cannot be controlled like those in other studies. Thus, if charged polyphenols are interacting with the proteins

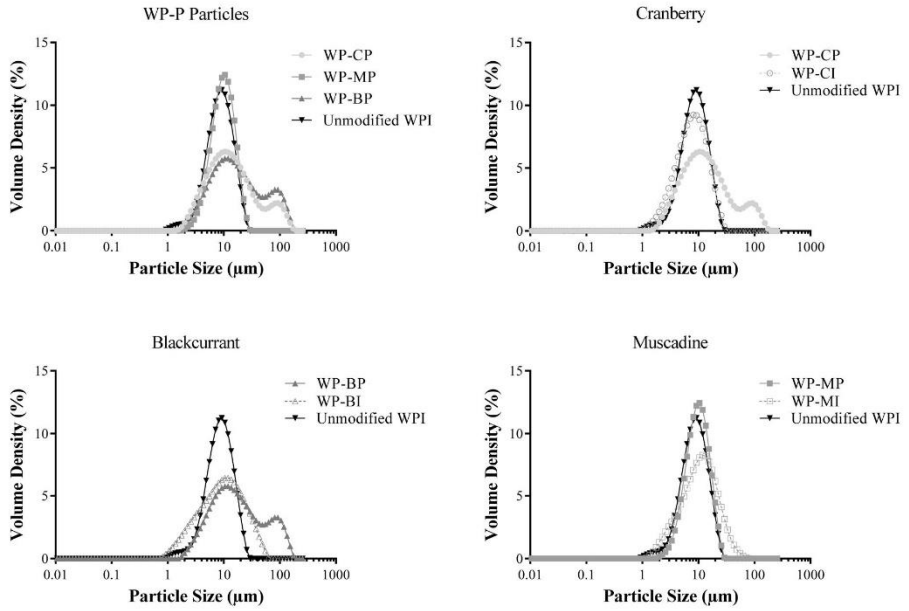

Fig. 4 Particle size of spray dried whey protein polyphenol particles. Particles were dispersed in deionized water at pH 4.5. Treatments included whey protein-cranberry polyphenol (WP-CP), whey protein-blackcurrant polyphenol (WP-BP), whey protein-muscadine polyphenol (WP-MP), whey protein-imitation cranberry control (WP-CI), whey protein-imitation blackcurrant control (WP-BI), whey protein- imitation muscadine control (WP-MI) particles, or the whey protein isolate used to make particles (unmodified WPI).

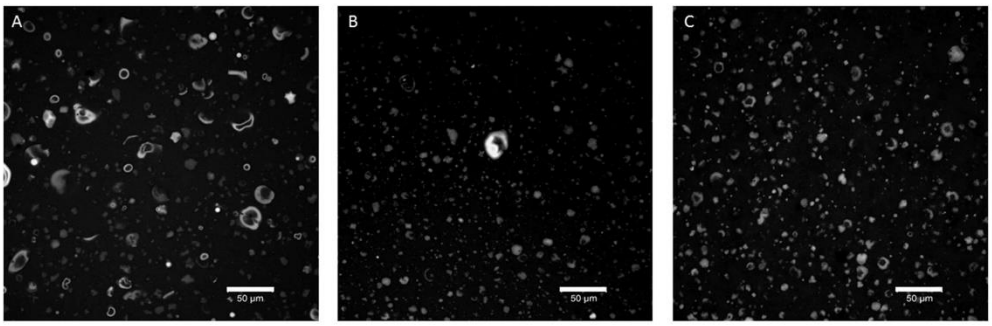

Fig. 5 Confocal images of dispersed whey protein-cranberry particles (A), whey protein-blackcurrant particles (B), and whey protein-muscadine particles(C). Open circles are an indication of surface bleaching from other layers of absorption or particles that have not been fully penetrated by the dye due to insolubility of particulates in dye solution.

the charges likely balanced out to net zero charge where as other researchers such as Staszewski were using green tea polyphenols predominately composed of negatively charged epigallocatechin gallate for their experiments. The discrepancies could be due to the use of whey protein isolate (predominately protein) verses whey protein concentrate (predominately lactose and contains more salts).

3.2. Particle functionality

3.2.1. Foam stabilization with standard particles. Foams were formed with each set of wet 250 mg g^{-1} WP-P particles using 10% protein solutions. There was no difference in overrun among foams (data not shown). All foams made with WP-P were more stable than foams made from WPI (Fig. 8).

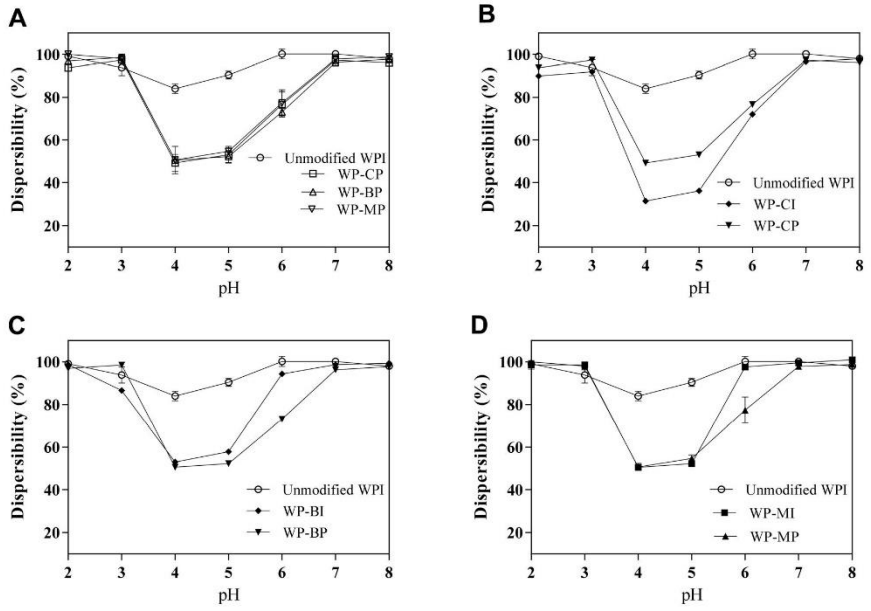

Fig. 6 Dispersibility of whey protein polyphenol particles over a range of pH values. Lines drawn between points for visual clarity. Particles containing polyphenols are whey protein-cranberry polyphenol (WP-CP), whey protein-blackcurrant polyphenol (WP-BP), or whey protein-muscadine polyphenol (WP-MP). Control particles formed without polyphenols are whey protein-cranberry imitation juice (WP-CI), whey protein-blackcurrant imitation juice (WP-BI), or whey protein-muscadine imitation juice (WP-MI).

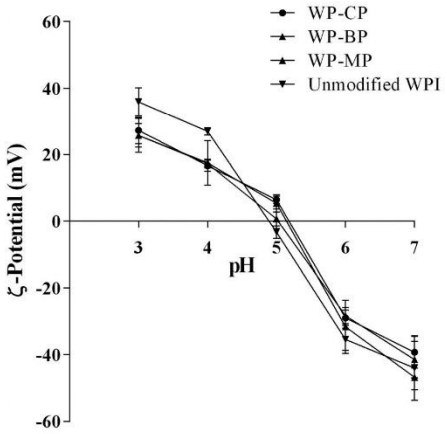

Fig. 7 ζ-potential of dried particles, formed with 250 μg mL^{-1} TP juice, across a range of pHs. Samples were suspended in pH solutions at 0.5 mg mL^{-1} protein. Samples were whey protein-cranberry polyphenol (WP-CP), whey protein-blackcurrant polyphenol (WP-BP), or whey protein-muscadine polyphenol (WP-MP) particles. Treatments are compared to the whey protein isolate used to make particle and referenced as unmodified WPI Only WP-CP treatments at pH 3 and 4 are significantly form other between treatments at the same pH ($p < 0.01$).

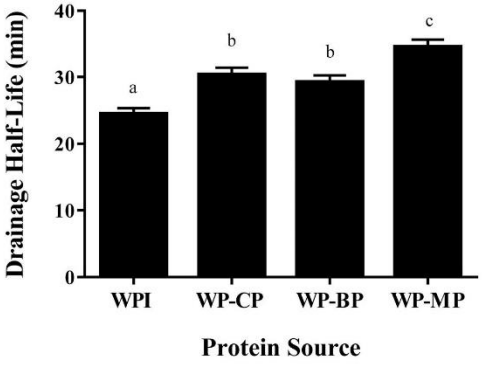

Fig. 8 Drainage half-life of foams produced with whey protein polyphenol particles. Particles containing polyphenols are whey protein-cranberry polyphenol (WP-CP), whey protein-blackcurrant polyphenol (WP-BP), whey protein-muscadine polyphenol (WP-MP), or whey protein isolate (WPI) were incorporated into foams contain 10% w/w protein. Different letters indicate significant differences between treatments ($p < 0.01$).

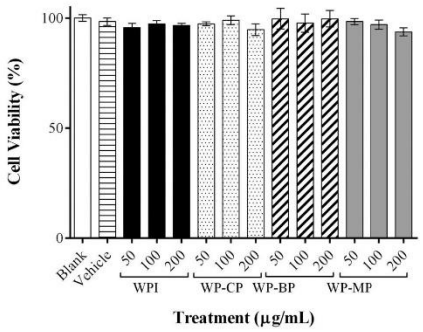

Fig. 9 Effects of treatment extracts on cell viability. Treatments were unmodified whey protein isolate (WPI), whey protein-cranberry polyphenol (WP-CP), whey protein-blackcurrant polyphenol (WP-BP), or whey protein-muscadine polyphenol (WP-MP) particle extracts. The blank consisted of untreated cells, and the vehicle consisted of dimethyl sulfoxide, which was the carrier of sample extracts.

This is indicated by a longer drainage half-life, or time for half of the starting volume to drain from the foam. The WP-MP particles produced foams with the greatest half-life. Increased stability may have been influenced by the 6% sugar content of the starting material. Overall, foam and dispersibility results support the notion that WP-P can be formed and have altered functionality compared to unmodified WPI. Similar increases in foam stability were seen by Schmitt *et al.*[37] when whey protein micro gels, of similar size to WP-P particles, were incorporated into foams. The microgels prevented gas permeation through the bubble interfaces to protect against foam coarsening. The improvement of foam stability with WP-P particles supports the increase in functionality, yet the mechanism needs to be fully evaluated with exploration of other factors contributing to stability such as continuous phase viscosity and interfacial rheology.

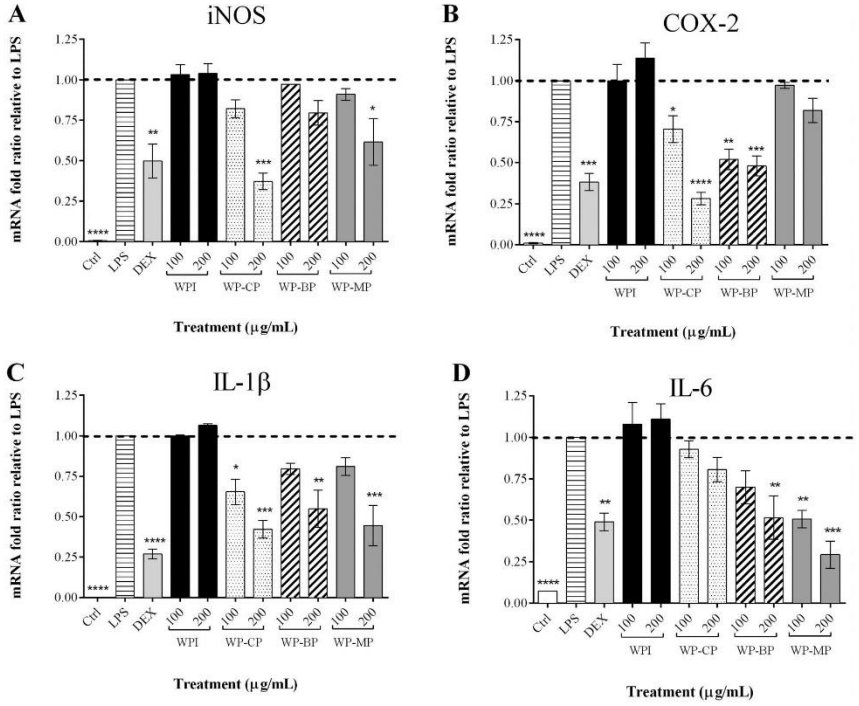

Fig. 10 Anti-inflammatory bioactivity of samples. Effects of 100 and 200 μg mL^{-1} extracts of unmodified whey protein isolate (WPI), whey protein-cranberry polyphenol (WP-CP), whey protein-blackcurrant polyphenol (WP-BP), or whey protein-muscadine polyphenol (WP-MP) particle extracts on iNOS (A), COX-2 (B), IL1β (C), and IL-6 (D). Dexametasone (DEX) was used as positive control at a concentration of 10 μM, lipopolysaccharide (LPS) as a negative control, and cell media as an unstimulated control (Ctrl). Samples marked with an asterisk are significantly different compared to LPS treated group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3.2.2. *In vitro* anti-inflammatory capacity of particle polyphenol extracts. Berry phytochemicals are known for their anti-inflammatory properties.[39–41] Previous research shows that protein can impact the bioavailability of polyphenol depending on the type of protein and type of polyphenols.[42] Therefore, it was imperative to evaluate if polyphenols were still in their biologically active state after being formed into protein–polyphenol particles. The WP-P formulations were evaluated with *in vitro* anti-inflammatory cell cultures by gauging cell response of exposure to LPS, an inflammatory compound found in the outer membrane of Gram-negative bacteria. The response of macrophage cells to LPS is to initiate a cascade of inflammatory responses.[43] Similar responses are seen in states of acute and chronic inflammation such as cancer, obesity, and heart disease.[44] Therefore, if compounds can mitigate the cascade of inflammatory responses the same compounds could be used to prevent and manage inflammatory conditions.[41] Inflammatory compounds such as LPS can cause macrophage cells to activate enzymes cyclooxynase-2 (**COX-2**) and inducible nitric oxide synthase (**iNOS**) that then aid in the metabolism of inflammatory arachidonic acid and nitric oxide, respectively. Macrophages will also release signals to communicate to other immune cells that there is a problem; these compounds are called cytokines. The increase of mRNA to code for expression of the enzymes iNOS and COX-2, in addition to the cytokines interleukin-1β (**IL-1β**) and interleukin-6 (**IL-6**) were measured in response to TP extracts from standard particles. This was achieved using LPS-stimulated murine RAW 264.7 macrophage cells. The MTT assay results showed that samples in the 50–200 μg mL^{-1} dose range did not reduce cell viability (Fig. 9). Therefore, treatments will not contribute cytotoxicity at these concentration and any effects that are observed can be attributed to polyphenolic biological activity.

The extracts from WP-CP and WP-MP suppressed iNOS expression at 200 μg mL^{-1} WP-CP (Fig. 10A), and WP-BP at 100 and 200 μg mL^{-1} suppressed COX-2 expression (Fig. 10B). This suggests that the enzymatic anti-inflammatory activity of the extracts is effective in the metabolism of either arachidonic acid, nitric oxide, or both in the case of WP-CP extract. The WP-CP (100 and 200 μg mL^{-1}), WP-BP (200 μg mL^{-1}), and WP-MP (200 μg mL^{-1}) extracts suppressed IL-1β genes (Fig. 10C) and WP-BP (200 μg mL^{-1}) and WP-MP (100 and 200 μg mL^{-1}) suppressed IL-6 expression (Fig. 10D). This result may suggest that the macrophages will not only act to reduce inflammatory compounds, but also reduce signaling, IL-1β and IL-6, to other cells regarding the inflammation. These results are consistent with other work involving cranberry polyphenols (especially cranberry PACs), blackcurrant polyphenols, and muscadine polyphenols in which macrophage cells exposed to LPS show reduced expression of iNOS, COX-2, IL-1β, and IL-6 with increasing amounts of polyphenols.[45–48] Therefore, between HPLC polyphenol characterization and cell culture results these particles have the potential to impart health benefits similar to that of the starting juice.

4. Conclusion

Whey protein–polyphenol aggregate particles were formed under food grade conditions and their functionality was evaluated by assessing physicochemical attributes. The yield of protein and polyphenols in particles was dependent on the concentration of TP in the initial juice. When the TP in the juice increased, the protein content in the aggregate particles also increased. The large size and reduced surface charge near pH 4.5 explained a reduction in dispersibility near pH 4.5. The dispersibility of particles was dependent on both polyphenols and other juice components such as sugars and acids. However, the size of the particles formed was clearly driven by the presence of polyphenols, and not other juice components. The WP-P particles increased foam stability, and phenolic extracts from the protein–polyphenol particles reduced the expression of genes associated with chronic inflammation. These results suggest that protein–polyphenol micro-scale particles can be made that benefit food quality, as in increased foam stability, and at the same time supply additional health benefits.

Acknowledgements

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3. Use of whey protein-polyphenol particles for foam formation and stabilization

3.1. Introduction

Foams are a colloidal system in which a gas is dispersed in a liquid or solid. Foam structures provide unique textures and are the defining structural component of foods such as bread, marshmallows, mousse, meringues, and whipped cream (Campbell & Mougeot, 1999). Foams are designed to function in various applications based on their structure (bubbly or polyhedral), gas phase volume, and stability. Foams are destabilized by anti-foaming forces via four main processes: drainage in polyhedral foams or creaming in bubbly foams, coalescence, and disproportionation (Hailing & Walstra, 1981; Koehler, Hilgenfeldt, & Stone, 2000). Stabilizing compounds are targeted to form interfacial films, increase viscosity of the continuous phase, or resist bubble movement. Incorporation of protein particles or microgel particles into a foam structure is one strategy to improve stabilization (Dickinson, 2010; Hunter, Pugh, Franks, & Jameson, 2008; Murray & Ettelaie, 2004; Rawel & Muschiolik, 1994; Rullier, Novales, & Axelos, 2008; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007; Studart, Gonzenbach, Akartuna, Tervoort, & Gauckler, 2007).

Particles and protein aggregates can function in foams by either absorbing at the interface or increasing the continuous phase viscosity. In addition, particles and aggregates can act as physical barriers in the continuous phase and slow disproportionation and coalescence by steric hindrance (Dickinson, 2010; Hunter et al., 2008). Rawal & Muschiolik (1994) showed that aggregates of mechanically processed soy proteins, ranging from 1-40 μm in diameter, were key to increasing foam volume and drainage time (Rawel &

Muschiolik, 1994). Soluble whey protein gel particles ranging in diameter from 290-330 nm were active at bubble interfaces and improved foam stability (Schmitt et al., 2007).

Polyphenols may be added to foams directly or as an ingredient component (e.g. fruit juice). Most studies that investigated the impact of polyphenols on protein foams indicated that stability increased with addition of small amounts of polyphenols (Prigent et al., 2009; Sarker, Wilde, & Clark, 1995; Sausse, Aguié-Béghin, & Douillard, 2003; Wu, Clifford, & Howell, 2007). Low concentrations of catechin stabilized β-lactoglobulin foams that had been destabilized by Tween 20 (Sarker et al., 1995). When procyanidin mixtures were added to an α-lactalbumin foaming solution, increasing degrees of procyanidin polymerization resulted in increased foaming properties (Prigent et al., 2009). Inversely, increased epigallocatechin gallate (EGCG) content up to 60 mg/L reduced the rate of β-casein absorption to the interface, suggesting that protein foams would be destabilized by protein-polyphenol aggregates in these conditions (Sausse et al., 2003). It would appear that there is an optimum concentration of polyphenols when it comes to stabilization.

Recently, we reported on the formation of whey protein-polyphenol particles in the size range of 10-200 μm, created by adding whey protein isolate to blackcurrant, cranberry, or muscadine juice under controlled conditions (protein concentration, juice concentration, and pH) (Schneider, Esposito, Lila, & Foegeding, 2016). The particles were used in preparing foams and caused an increase in stability (decreased drainage rate) and did not alter foam overrun. The mechanisms causing increased stability were not investigated. In the current work, the ability of whey protein-cranberry juice particles to alter macroscopic properties of foam drainage rate and yield stress were determined. The advantages to using a

protein ingredient and juices to stabilize foam formation are twofold. First, the use of a mixture of proteins and polyphenols allows for a molecular complexity such that effects of minor components are possible. Previous investigations with individual proteins and purified, isolated polyphenols are good for mechanistic work, but may be irrelevant when other compounds are present. Second, the mixture of juice and whey protein can be applied to food systems where the two ingredients are typically used, such as whipped products containing fruits and dairy proteins. Overall, the goal of this research is to evaluate effects of cranberry juice and its components on whey protein foams.

3.2. Materials and Methods

3.2.1. Materials

The whey protein isolate (WPI) powder was gifted by Glanbia Nutritionals (Provon ®, 89% protein w/w based on amount of nitrogen determine by the Dumas method; Twin Falls, ID, USA). Cranberry juice concentrate (50.2 °Brix and 5200 µg/ml total phenolics (TP)) and pomace was gifted by Ocean Spray® (Lakeville-Middleboro, MA, USA). Anhydrous citric acid, citrate, tartrate, NaOH, HCl, and anhydrous dextrose were sourced from Fisher Scientific (Fairlawn, NJ, USA). Fructose was purchased from Avantor Performance Materials Inc. (Philipsburg, NJ, USA)

3.2.2. *Methods*

3.2.2.1. Pomace Extract

Freeze dried cranberry pomace was blended 1:10 with 50:50 deionized water and ethanol in a Vitamix mixer (Cleveland, OH, USA) until fully homogenous. The mixture was

heated in a water bath at 80 °C for 2 hr. The resulting slurry was filtered through cheesecloth to remove any large particulates. The ethanol was removed through rotary evaporation. The total polyphenolic (TP) content of the extract was measured using the Folin-Ciocalteu method and used to make respective dilutions for each treatment (Singleton, Orthofer, & Lamuela-Raventós, 1999). The extract was diluted with deionized water to contain 200 mg TP per 138 g of solution, which is referred to as a treatment concentration of "100%".

3.2.2.2. Imitation Juice Preparation

Imitation juice solutions were made to mimic the buffering capacity, pH and sugar content of cranberry juice. The pH and titratable acidity of cranberry juice diluted to contain 200 mg TP per 138 g of solution was measured to determine the appropriate buffering properties of the solution, and literature values were used to determine the ratio of glucose to fructose in cranberry juice (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2012). Based on those values, stocks of 0.16 M citric acid 0.16 M citrate solutions each containing 1.6% glucose and 0.3% fructose, were prepared. The stock citrate and citric acid solutions were combined (1:10 ratio) to achieve a solution pH of 3.09. The solution pH 3.09 was chosen, as this was the pH of cranberry juice diluted to contain 200 mg TP per 138 g. The stock solution was used at the "100%" imitation juice concentration.

3.2.2.3. Solution Preparation

Foaming solutions were prepared to contain 10% w/w protein either cranberry juice concentrate, cranberry pomace extract, or imitation juice stock diluted to equate to 0, 25, 50, 75, or 100%. The 100% juice treatment was prepared by diluting the cranberry juice concentrate with deionized water to contain 200 mg TP per 138 g of solution. These

concentrations result in ratios of protein to polyphenol (by weight) of 0, 25, 50, 75, and 1 in foaming solutions containing juice and cranberry pomace extract. Whey protein powder was mixed with the solution for 16 hr at room temperature (25 ± 2 °C) to hydrate. Solutions were prepared with 20% less of the total volume to allow for additional volume from base or acid for pH adjustment (this was also accounted for in the initial dilutions, hence the 138 g in the denominator of "100%" treatments). Solutions were adjusted to either pH 4 or pH 7 using 3 M HCl or 1 M NaOH, respectively. Solutions were weighed before and after pH adjustment and deionized water was added to achieve final weight. Mixtures were equilibrated for 1 hr before foam formation.

3.2.2.4. Apparent Viscosity

Apparent viscosity was determined using a modular compact rheometer (MCR 302, Anton-Paar, Gratz, Austria) with a cup (C-CC27/T200) of 28.940 cm diameter and a cylindrical bob (SN 42278) with a length of 40.008 cm, diameter of 26.659 cm, and cone angle of 120 °. Samples were exposed to a pre shear of 50 s^{-1} for 60 s and allowed to rest for 15 s before testing. The apparent viscosity was measured across shear rates of 1-500 s^{-1}. Measurements were taken every 2 s, resulting in 100 data points between 0-500 s^{-1}. Temperature was controlled to 23 °C using a circulating water bath.

3.2.2.5. Particle size

Laser diffraction was used to determine particle size with a Mastersizer 3000 (Malvern, Worcestershire, UK). Both Mie and Fraunhofer diffraction theories were used to calculated particles size. Mie theory was used on measurements using a blue light source (10 mW LED, 470nm) to detect small particles < 50 µm, while the Fraunhofer theory was used

on measurement using a red light source (4mW He-Ne, 632.8nm) to detect large particles > 50 µm (de Boer, de Weerd, Thoenes, & Goossens, 1987). The foam solutions (pre-foaming) were added to the sample flask until an obscuration of 10-13% was achieved, at which point measurements commenced.

3.2.2.6. Surface Tension

Foaming solutions were centrifuged at 23,000 *x g* for 15 min. to remove large particulates that cause variability. An automated contact angle goniometer (Rame-Hart Inc., Mount Lake, NJ) was used to perform measurements and data was programmatically collected using DROPimage software. A pendant drop method was used to evaluate interfacial tension (Myrvold & Hansen, 1998). Drops of pre-foaming solution equating to 40 µL were hung from a stainless steel capillary within a humid chamber to prevent evaporation. The software calculated the surface tension every 2 s over a period of 600 s. Experiments were performed at room temperature (22 ± 2 °C) and the instrument was calibrated daily with the spherical portion of a Precision Combo Calibration Device (P/N 100-27-31, Rame-Hart Inc., Mount Lake, NJ).

3.2.2.7. Foam Formation and Rheology

Foams were prepared according to Pernell et al. (2000) (Pernell, Foegeding, & Daubert, 2000). A 10 mm diameter hole was drilled into the bottom of a Kitchen Aide mixing bowl and covered with tape during foam preparation. A 200 g aliquot of foam solution was placed in the bowl and foamed with a Kitchen Aide Ultra Power Mixer (Kitchen Aid, St. Joseph, MI, USA) set at a speed of 8 for 20 min (planetary rpm of 225 and beater rpm of 737). Immediately after foam formation, yield stress was measured using vane

rheometry (Pernell et al., 2000). A vane measuring 10 mm in diameter and 40 mm in length attached to a Brookfield 23xLVTDV-ICP viscometer (Middleboro, MA, USA) was used. The vane was rotated at 0.3 rpm and the maximum torque was recorded. The yield stress (τ) was calculated from the maximum torque (M_0) as follows where h was the height of the vane, d was the diameter of the vane:

$$\tau = \frac{M_0}{\left[\frac{h}{d}+\frac{1}{6}\right]\left(\frac{\pi d^3}{2}\right)} \qquad \text{Eqn. 1}$$

Two additional yield stress measurements were taken in undisturbed areas of the foam. The overrun of each foam was determined according to Phillips et al. (1987) (Phillips, Haque, & Kinsella, 1987). Portions of foam were placed into a weigh boat with maximum capacity of 100 mL. A spatula was used to scrape the surface of foam level to the top of the weigh boat, being sure not to exert any force that would collapse the foam. This process was repeated 4 times for each foam. The overrun was used to calculate the air phase fraction as follows:

$$\%\ Overrun = 100 \times \frac{(Weight\ of\ 100\ mL\ dispersion) - (Weight\ of\ 100\ mL\ of\ foam)}{(Weight\ of\ 100\ mL\ of\ foam)}$$

$$Air\ Phase\ Fraction = \frac{\%\ Overrun}{Overrun + 100}$$

After the mixer was turned off, a timer was started for measurement of drainage half-life. Following yield stress measurements, the mixer bowl was placed above a scale and the tape removed from atop the hole to allow for fluid to flow through to the scale. The time for half of the foaming solution to drain was recorded.

3.3. Results & Discussion

As shown previously in making whey protein-cranberry juice polyphenol particles, the polyphenol and non-polyphenol components of the juice can influence protein aggregation. Therefore, insight into foams made with whey protein and cranberry juice was gained through analysis of foams prepared with cranberry juice, with polyphenols extracted from cranberry pomace, or with an imitation cranberry juice (non-polyphenol components). The cranberry juice was prepared by diluting cranberry juice concentrate to appropriate concentrations of total polyphenols. Cranberry pomace was extracted to collect an alternative source of polyphenols, similar to those found in cranberry, but devoid of the sugars that would be present in juice, containing greater amounts of polymerized tannins, and containing less water soluble polyphenols. The pomace extract was therefore useful to isolate the effects of polyphenols on foam formation, as opposed to effects contributed by sugars or other components in a juice. The imitation juice foams were prepared to examine non-polyphenol (acid, sugar, and pH) contributions to protein foams. Each pomace polyphenol or imitation juice treatment was normalized to equate to the respective juice composition (acid, sugar, pH, or polyphenols) when juice is diluted to contain 25, 50, 75, or 100%. The solution containing 0% was simply a 10% w/w whey protein isolate solution.

3.4. Yield Stress & Drainage Half-life

Foams formed from pH 7 solutions had increased drainage half-life with increasing treatment concentrations (Figure 1). Juice treatments of 25% were not significant difference from the control WPI foam, but foams prepared in 50, 75, and 100% juice had significantly longer drainage half lives than control WPI foams. Although subtle, foams prepared with

imitation juice had shorter drainage half-lives than foams prepared with pomace polyphenols at 25, 50, and 75% levels. Overall, cranberry juice, or both main juice components, increased foam stability.

Foams are usually whipped to some consistency, i.e., a soft or stiff peak, so yield stress was used to measure foam textural properties. Yield stress of foams increased then decreased with juice concentration at pH 7, and a similar trend of lower magnitude was seen with imitation juice (Figure 1). The yield stress of foams prepared with cranberry pomace polyphenols remained relatively constant. The similarity in trends observed in foams containing juice and imitation juice, suggest the pH, buffering capacity, or sugar contribute to this behavior. Oh et al. (1987) observed various proteins, including β-lactoglobulin, interactions with grape tannins. Protein-tannin interactions were greatest at low pHs and peaked near the protein's isoelectric point (Oh & Hoff, 1987). Cranberry juice and WPI mixtures ranged from pH 3.5-4.5, and pH 5-6 for cranberry pomace polyphenol and WPI mixtures, before adjusting to pH 7 for foaming. Therefore, greater protein-polyphenol interactions are expected in the juice mixtures (vs polyphenol mixtures) as the mixture pHs are very close to the isoelectric points of the predominate whey proteins, 4.2-5.2 (Morr & Ha, 1993), but foaming solutions containing pomace polyphenols never are exposed to this pH range.

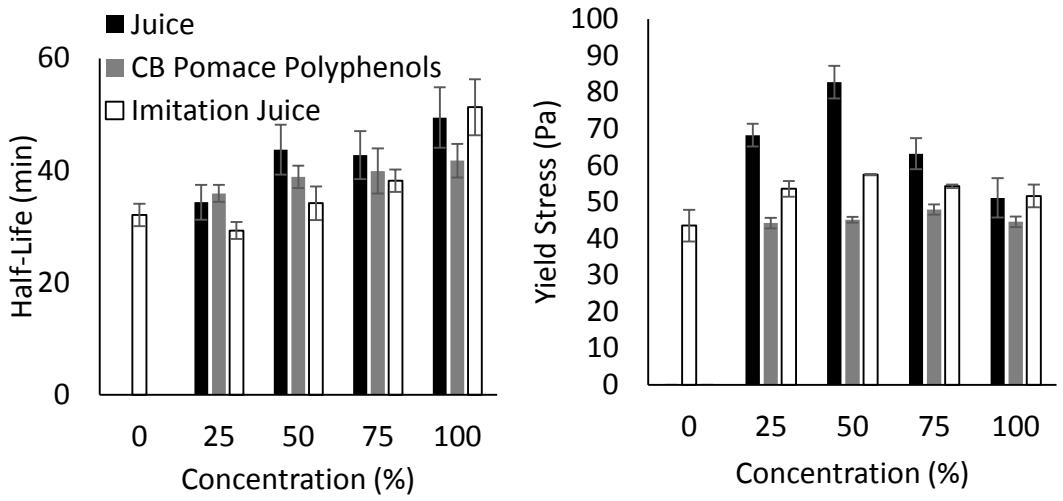

Figure 2. The yield stress and half-life of foams produced with varying amounts of added cranberry juice, cranberry (CB) pomace polyphenols, or imitation cranberry juice. All foams were made with 10% protein w/w at pH 7.

Foams prepared with 25% and 50% cranberry juice at pH 7 had increased yield stress but yield stress declined at higher concentrations, and foams prepared with a 100% cranberry juice did not appear different from the WPI control. Yield stress in polyhedral foams can theoretically be explained based on the mean bubble diameter (D[3,2], volume to surface area), interfacial tension, air phase volume fraction, and a fitting factor (Davis, Foegeding, & Hansen, 2004; Princen & Kiss, 1989). No differences were observed in airphase fraction results for foams contain juice (Figure 2). Therefore, there are many factors that could have been altered by the juice and juice components such as interfacial tension, or bubble diameter. In our foams, the surface tension of the foams decreased with the initial addition of juice to the foams, but increased with increasing juice concentration (Figure 3). This does not support the yield stress results. The differences between the drainage, yield stress, and surface tension trends with increasing juice concentration indicates different factors are at

play in stabilizing whey protein foams with added juice. This is likely due to the non-ideal conditions that are assumed when making theoretical mechanisms of foam strength.

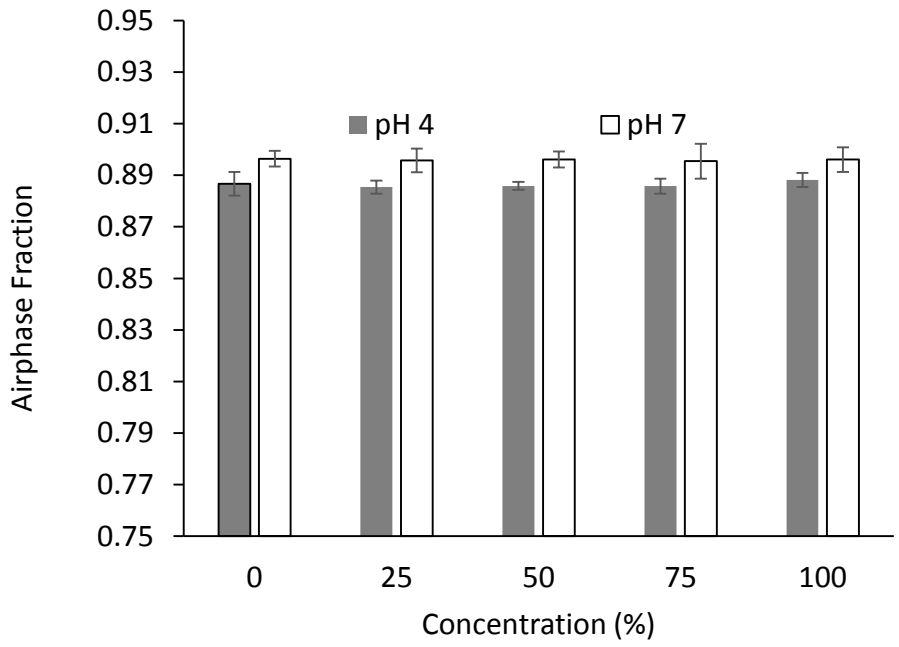

Figure 2. The airphase fraction of foams contain juice diluted to either 0, 25, 50, 75, or 100% polyphenols at pH 4 and 7.

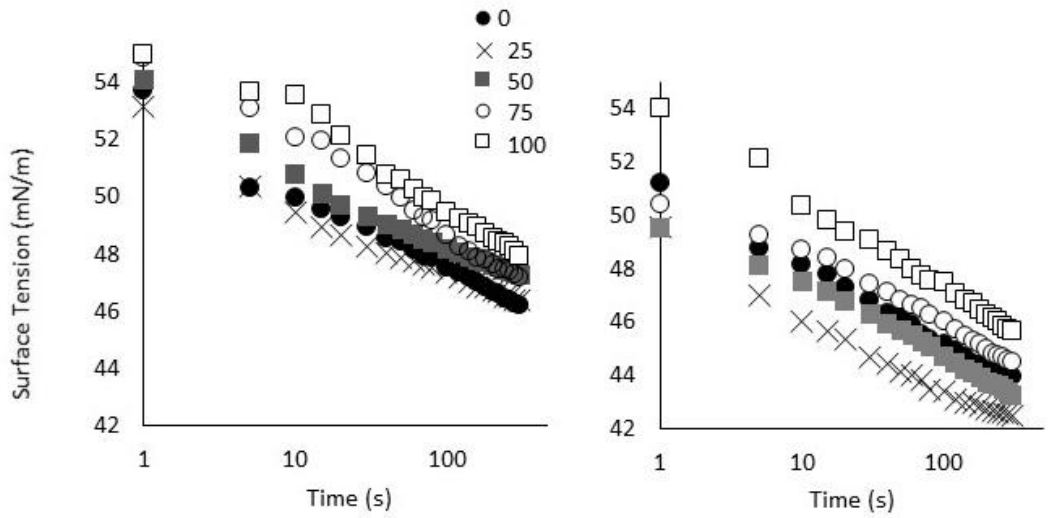

Figure 3. Changes in surface tension over time for foaming solutions containing 0, 25, 50, 75, or100% juice at pH 7(left) or pH 4 (right).

At pH 4, the drainage half-life was drastically impacted by the presence of cranberry juice (Figure 4). This behavior could not be explained by either the imitation juice or polyphenol content alone, as the changes in half-life in the component treatments did not compare to foams made with cranberry juice. Instead, this can be explained by colloidal interactions between proteins and polyphenols in acidic conditions, which will be discussed further in section 3.2. The yield stress of foams at pH 4 was less than that of the control (0%) at 25% and 50% total polyphenols, but increased above the control at 75% and 100% juice content. At pH 4, a large increase in yield stress was observed at 75 and 100% treatments.

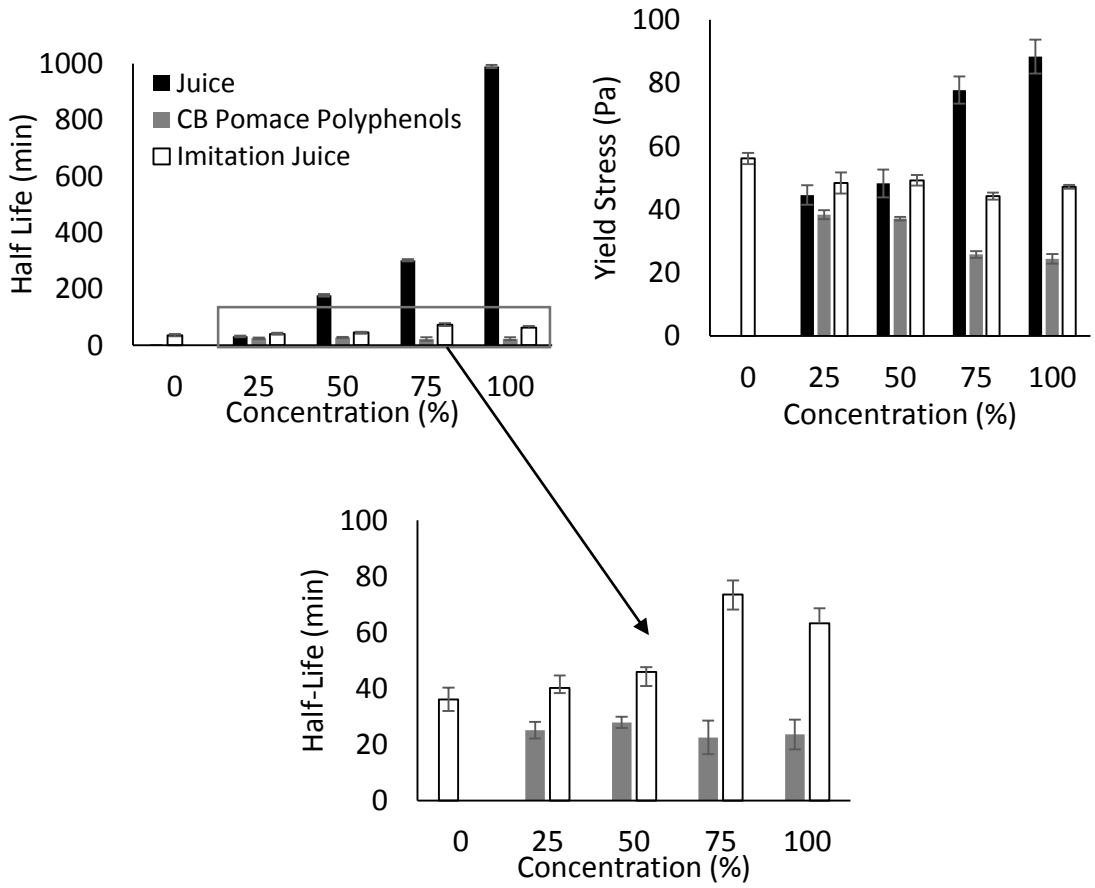

Figure 3. The yield stress and half-life of foams produced with varying amounts of added cranberry juice, cranberry (CB) pomace polyphenols, or imitation cranberry juice. All foams were made with 10% protein w/w at pH 4.

Overall, foams containing just pomace cranberry polyphenols (and none of the other components that might be present in cranberry juice) at pH 4 had a drastically depressed yield stress and drainage half-life, however these same foams containing pomace-derived polyphenols were not significantly different than controls at pH 7. This difference in foams prepared with juice and foams prepared with pomace-derived polyphenol extracts suggests there could be a difference in polyphenol composition in cranberry juice verses the pomace polyphenol rich extract. The pomace used to make cranberry polyphenols is the residual skin

and flesh of cranberries post juice processing, therefore while the extract will have a similar polyphenol profile it is slightly higher in proanthocyanidins (Grace, Massey, Mbeunkui, Yousef, & Lila, 2012). But, the difference in polyphenol composition does not explain foam difference, as an increase in proanthocyanidins (polymerized anthocyanidins) would increase foaming as observed by Prigent et al. (2009) (Prigent et al., 2009). Therefore, the poor foaming observed in foams prepared with the pomace-derived cranberry polyphenol extracts is likely not just an effect of the pH, as proposed with pH 7 foams, but the lack of other juice components such as the buffering capacity and sugars non-acidic extract, which reduces protein-polyphenol interaction. Nevertheless, these results can be applied to processing environments in which stable foams are not ideal conditions, such as beverage processing. In these environments, polyphenol extracts could be used in place of juice to control or reduce foaming. On the contrary, if the objective is to increase foam stability, then whole fruit juice should be used to enhance the foaming properties of whey proteins, particularly at pH 4.

3.5. Viscosity & Particle Size

One of the simplest ways to decrease drainage rate, thereby increasing foam stability, is to increase the viscosity of the continuous phase surrounding the bubbles. Increases in continuous phase viscosity delay the flow of drainage out of the foams; therefore, the viscosity of foam solutions prior to whipping was measured. Indeed, the viscosity of the treatments aligned with drainage behavior: an increase in viscosity was detected with increasing treatment concentration for cranberry juice and imitation juice and respective drainage times (Figure 5). This is expected as the amount of solids in a solution impact the

viscosity. Only minimal differences were detected in pH 7 foams made with cranberry polyphenols, which support minimal difference in drainage half-life of these foams (Figure 1). Large differences in viscosity were observed for increasing concentrations of juice at pH 4, compared to any other treatment. This suggests changes in colloidal structure, such as aggregate formation between proteins and polyphenols.

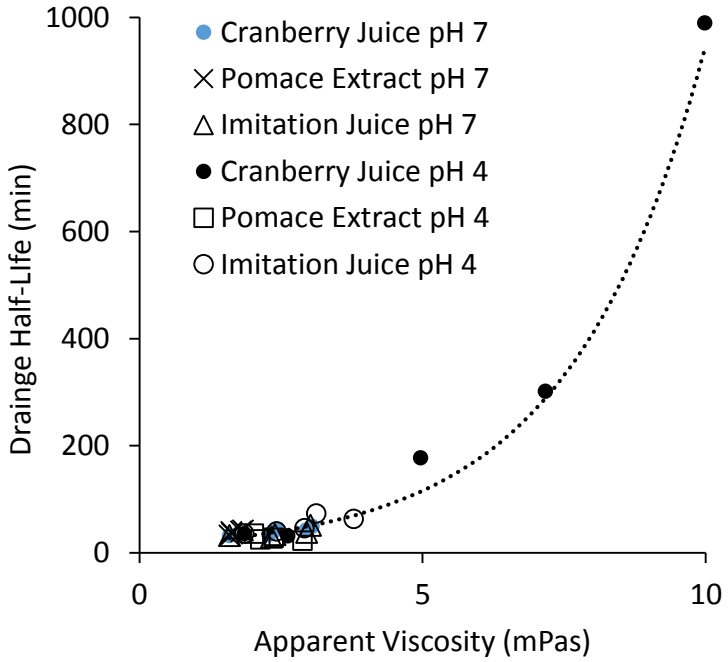

Figure 4. Relationship between drainage half-life and apparent viscosity.

The particle size was measured as an indicator of colloidal changes at pH 4 (Figure 6 & 7). The increases in viscosity were supported by particle size analysis as the distributions showed an overall increase in particles size as polyphenol concentration increased. The largest particles were observed for pH 4 cranberry juice-protein solutions as the main aggregate peak shifted from 5 µm in pomace extract and imitation juice aggregates to 25 µm for juice-whey aggregates. The degree of increase in viscosity cannot be represented fully by

the particle size information. This is because the method used does not detect soluble aggregates that form relatively clear solutions (but may be viscous). However, it suggests the possibility of large aggregates being present in the continuous phase and contributing to the decreased drainage rate.

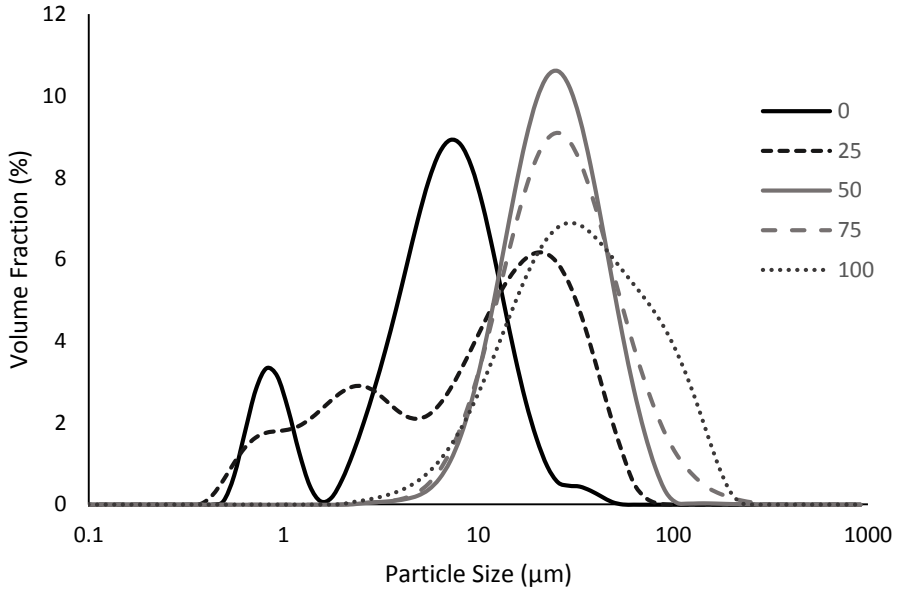

Figure 5. Particle size of whey protein-cranberry juice foaming solutions at various concentrations (%) prior to foaming at pH 4.

Many other studies showed an increase in foaming properties in the presence of protein aggregates (Murray & Ettelaie, 2004; Rullier et al., 2008; Schmitt et al., 2007). In addition, some studies observe foam stability with particles based on Pickering stabilization where particles form a very stable interfacial layer (Berton-Carabin & Schroën, 2015; Binks & Clint, 2002; Du et al., 2003; Lam, Velikov, & Velev, 2014). For a Pickering stabilized foam to be formed, particles tend to range in size from 10-30 nm in diameter, and stability is further enhanced by aggregates that form on the interface ranging from 100-300 nm in

diameter, which is much smaller than aggregates formed in this study (Binks & Clint, 2002; Dickinson, 2010; Du et al., 2003). In addition, Pickering foam particles must be wettable by both hydrophobic and hydrophilic materials to, in effect, have a portion of the particle in the interface and a portion in the discontinuous phase, and there is no evidence to support this behavior. Instead, this study suggests foams were stabilized by the presence of larger protein aggregates which could form a "disorder layer/network of particles" between bubble particles, preventing bubble collapse as mentioned by Dickinson 2010 (Dickinson, 2010).

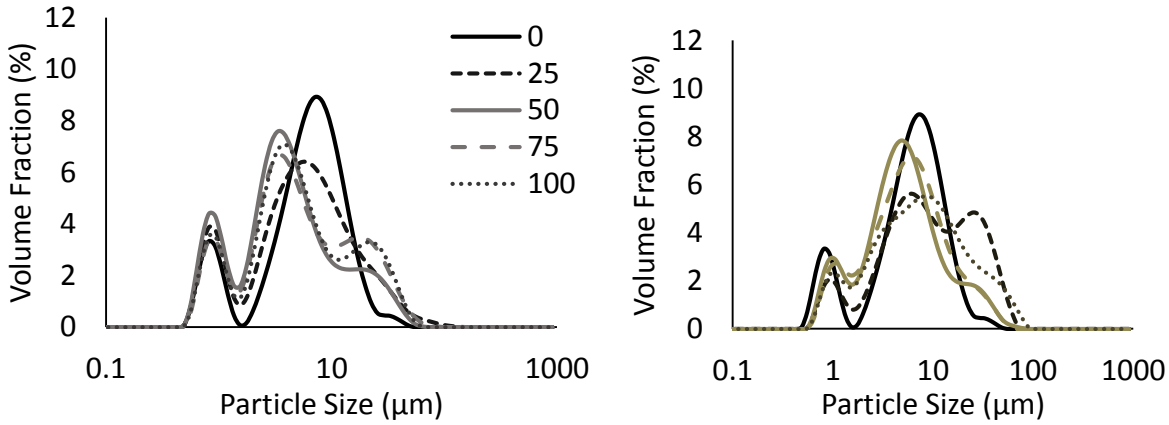

Figure 6. Particle size of whey protein-cranberry pomace polyphenol (left) protein-imitation juice (right) foaming solutions at various concentrations (%) prior to foaming at pH 4.

3.6. Conclusion

The aim of this study was to evaluate the impact of adding cranberry juice and isolated individual juice components to whey protein foams. On their own polyphenols contributed minimal, if any improvements in foam drainage and yield stress, and non-phenolic components of juice made small increases in foam drainage and yield stress. However, when these components were combined in whole juice, significant increases in yield stress and

drainage half-life were observed especially at pH 4. This behavior is likely due to the acidic conditions allowing for maximum protein-polyphenol interactions, which lead to aggregates that stabilize foams through 1-increasing viscosity and 2-network formation between bubbles.

3.7. Acknowledgements

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4 Chapter 4: Mitigation of protein bar hardening with insoluble whey protein-polyphenol particles

4.1 Introduction

Consuming the necessary amount of protein to reach dietary needs can be challenging, particularly for individuals who are recovering from strenuous exercise or dieting. Therefore, it is crucial to have diverse, convenient protein-dense and calorie-rich food sources available to supplement nutrition. Protein bars can be formulated with fruits, nuts, grains, and vegetables to address nutritional and health requirements, but may fail to meet shelf life needs, due to a phenomenon known as bar hardening. Over time, soft, chewy bars transform into hard, brittle solids with undesirable texture. This hardening reaction is associated with various transformations of ingredients in bars. Protein-dense bars have been formulated using a range of ingredients and processes, and several different hypotheses have been put forth as to the cause of bar hardening. Proposed mechanisms include protein aggregation & network formation, sugar crystallization, phase hydration, glass transition, and water migration (Hogan, Chaurin, O'Kennedy, & Kelly, 2012; Loveday, Hindmarsh, Creamer, & Singh, 2009; Zhu & Labuza, 2010).

Ingredient substitution can be used to at least partially alleviate bar hardness. For example, McMahon et al. 2009 discovered that substituting whey protein hydrolysate for whey protein isolate decreased bar hardness (McMahon, Adams, & McManus, 2009). It was hypothesized that the hydrolysate participated in the sugar phase, while whey protein isolate formed its own phase that competed for water and facilitated protein aggregation. Although Hogan et al. 2016 found similarly softer bars made with hydrolyzed whey protein, they

suggested that this effect was related to the lower volume fraction of the hydrolyzed powder compared to whey protein isolate powder (S. Hogan, O'Loughlin, & Kelly, 2016). In addition to replacing whey protein isolate with whey protein hydrolysate, McMahon et al. 2009 replaced high fructose corn syrup with a non-reducing sugar ingredient, sorbitol, which also reduced bar hardness (McMahon et al., 2009). This finding is supported by other studies and suggest that Maillard reactions are occurring in bars made with reducing sugars. Maillard reactions cause proteins to aggregate and crosslink over time (Loveday et al., 2009; Zhou, Guo, Liu, Liu, & Labuza, 2013). Li et al. 2006 adjusted ratios of various proteins to reduce hardness and observed synergistic interactions between soy protein isolate and either calcium caseinate or whey protein isolate (Li, Szlachetka, Chen, Lin, & Ruan, 2006). Other studies have sought to define hardening mechanisms, and therefore have used formulations that would not be appropriate for a food-grade consumer bar. For example, Zhu et al. (2010) included cysteine and N-ethylmaleimide to study disulfide interaction (Zhu & Labuza, 2010).

We hypothesize the use of a functionally "inert" protein particle to use in protein bars, which will resist protein-protein or protein-water interactions that cause bar hardening. In contrast to previous work, we describe a method to create a food-grade modified whey protein ingredient used to mitigate bar hardening. Particles were formed using a "green" chemistry approach where aggregation is facilitated by mixing whey protein isolate with fruit juices. This method for forming particles has been introduced in work by Schneider et al. (2016), in which protein particles formed with whey proteins and either cranberry, blackcurrant, or muscadine juices had decreased solubility near pH 4.5 (Schneider, Esposito, Lila, & Foegeding, 2016). In the current work, we evaluated the potential for combining

proteins and polyphenols into meso-scale particles designed primarily for reducing bar hardness. This has the added benefit of adding health-relevant phytochemicals.

4.2 Materials and Methods

4.2.1 Materials

Whey protein isolate (WPI) was gifted by Glanbia Nutritionals (Twin Falls, ID, USA; Provon 190®) and contained 89 % protein w/w based on nitrogen content determined by Dumas method (Wiles, Gray, & Kissling, 1997). Cranberry juice concentrate (50.2 °Brix and 5200 µg/mL total phenolics (TP)), cranberry juice, and cranberry pomace were gifted by Ocean Spray® (Lakeville-Middleboro, MA, USA). Soy protein isolate was acquired from DuPont (Supro® 340, New Century, KS, USA) and NaOH and HCl were from Fisher Scientific (Fairlawn, NJ, USA). Honey (Food Lion, Salisbury, NC, USA) and Crisco pure vegetable oil (Smucker's, Orrville, OH, USA) were sourced from a local grocery store. Folin-Ciocalteu reagent was from Sigma-Adrich (St. Louis, MO, USA). Pepsin (1:10,000), trypsin (1:250), and α-chymotrypsin were from MP Biomedicals, LLC (Solon, OH, USA).

4.2.2 Methods

4.2.2.1 Powder Formation

Juice concentrate was diluted to 250 µg TP per mL in deionized water to create 1 mg TP per g of dry powder (WP-1CP), or diluted to 500 µg TP per mL for 2 mg TP per g of dry powder (WP-2CP). The diluted juices were stirred for 4 hr with 10% w/w protein. The protein-juice mixture pH was adjusted to pH 4.5 with 1 M NaOH and centrifuged at 7000 *x g* for 20 min. The supernatant was collected for future use and the pellet containing whey

protein-polyphenol particles was re-suspended in deionized water at 10% w/w protein. The re-suspended pellet was spray dried to create WP-CP particle powders using a lab scale spray dryer (B-290, Büchi Corporation, DE, USA). Solutions were spray dried with an outlet temperature of 90 °C and an inlet temperature of 190 °C. For the mechanistic experiments, powders were made from various solutions (Table 1) and all were spray dried under the same conditions. The powders were suspended at 105 w/w protein in deionized water at respect bar pHs. The solutions were centrifuged at 17,200 *xg* for 15 min at 15 °C. The protein content of the supernatant was determined and used to calculate the percent soluble protein.

Table 2. Liquid formulations for spray drying powders.

Treatment ID	Solution Formulation
Unmodified WPI	10% w/w protein Unmodified WPI (pH 6.5)
WP-1CP	Pellet of 250 µg TP per mL +WPI mixture (centrifuged), suspended in deionized water at 10 % w/w protein
WP-2CP	Pellet of 500 µg TP per mL +WPI mixture (centrifuged), suspended in deionized water at 10 % w/w protein
Mixture	Juice diluted to 250 µg TP per mL +WPI (not centrifuged)
Supernatant	Supernatant of juice diluted to 250 µg TP per mL +WPI (centrifuged)
Polyphenol Control	WPI at 10 % w/w protein reconstituted in water containing cranberry pomace extract (pure source of cranberry pomace polyphenols) at 250 µg TP per mL
pH Control	WPI reconstituted at 10% w/w protein in deionized water and adjusted to pH 4.7

The pH control was formulated so that a bar made with the protein powder had a pH of 4.8. This required spray drying a solution adjusted to pH 4.7 (with 1 M HCl). Cranberry pomace extract was prepared by blending freeze-dried pomace1:10 with 50:50 ethanol: deionized water in a blender (Vitamix, Cleveland, OH, USA). The mixture was heated in a water bath at 80 °C for 2 hr and allowed to cool to room temperature. Once cooled, the mixture was filtered through cheesecloth. The ethanol was removed from the filtered mixture with a rotary evaporator, leaving a solution of polyphenols in water. The total phenolics content of the extract was measured using the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999).

4.2.2.2 Particle Size

Powders were suspended in deionized water at 10% w/w protein concentration by mixing at room temperature (25 ± 2 °C) for 4 hrs. Particle size was determined by laser diffraction using a Malvern Mastersizer 3000 (Worchestershire, UK). Scattered light intensity was used to determine particle size according to both Mie (blue laser, small particles < 50 µm) and Fraunhofer (red laser, large particles > 50 µm) diffraction theories (de Boer, de Weerd, Thoenes, & Goossens, 1987). Suspensions were added to deionized water in the sample flask until obscuration of 10-13% was achieved to assure accurate readings.

4.2.2.3 Total Phenolics

Polyphenols were extracted from WP-CP powders with 75 % methanol: 5 % acetic acid in water. WP-CP powders were washed three times with methanol: acetic acid and insoluble protein removed via centrifugation (7,000 *x g* for 20 min). The resulting

supernatant was collected and TP determined according to the Folin-Ciocalteu method. (Singleton et al., 1999).

4.2.2.4 Protein Powder Digestibility

Protein digestions were carried out according to Hsu et al. (1977) (Hsu, Vavak, Satterlee, & Miller, 1977). Enzyme stock was prepared at 0.13 mg/mL pepsin, 0.16 mg/mL trypsin, and 0.31 mg/mL α-chymotrypsin, adjusted to pH 8, and put on ice. A protein mixture of 6.25 mg/mL protein of the samples was prepared, adjusted to pH 8, and incubated at 37 °C. The amount of protein powder added to the digestion was normalized to 6.25 mg/mL across all powders. The protein powder nitrogen content was determined using the Dumas method (vario Macro cube CHN, Elementar Mt. Laural, NJ, USA), and a protein to nitrogen conversion ratio of 6.38 was used to calculate protein content. The digestion was started with the addition of the stock enzyme solution added to the protein solution at a ratio of 1:10. The change in pH was recorded at each minute for 10 minutes following the addition of the enzyme to the protein solution. To maintain temperature, the sample was kept in a 37 °C water bath. The pH meter was also calibrated to standards warmed to 37 °C prior to the start of the digestion.

4.2.2.5 Bar Formulation and Storage

Bars were formed by hand mixing 25:50:25 (w/w) lipid: carbohydrate: protein powder ratio for two min. Ingredient ratios were determined so that a bar could deliver the appropriate macronutrients to replace a balanced meal for an astronaut (Cooper, Douglas, & Perchonok, 2011). Vegetable oil was used as the lipid source and honey as the carbohydrate source. Bars were wrapped in parchment paper and rolled out to a 2.25 mm height, vacuum

sealed, and stored at accelerated storage conditions (32 °C) according to that of Li et al. (2008), where 3.5 days equated to 1 week at 25 °C (Li et al., 2006). On day one, the bar water activity was measured with an AquaLab water activity meter (Pullman, WA, USA). All bars measured 0.73 ± 0.01. Bar pH was measured with an accuTupH Rugged Bulb pH Combination Electrode (Fischer Scientific, Fairlawn, NJ, USA). The probe was calibrated with pH 4, 7, and 10 standard buffers. The probe was compressed into bar samples until pH was stable (~2-4 min), at which point the pH was recorded.

4.2.2.6 Rheology

Bars were cut into 2 mm thick disks of 22 mm diameter. A Stress Tech controlled-stress rheometer (ATS Rheosystems, Bordentown, NJ), with serrated parallel plate geometry, was used to to run a creep recovery test. Preliminary stress sweeps were conducted to confirm creep recovery test parameters were within the linear viscoelastic range of each sample. Creep recovery was determined by applying a 100 Pa stress and recording strain for 20 s, then removing the stress and allowing the material to recover for 7 min. Creep Recovery (%) was calculated according to equation 1 where J_{max} is compliance after 20 s, J_{min} is minimum compliance post stress release, and J_r is the compliance after recovery or the difference between J_{max} and J_{min}:

$$\text{Creep Recovery (\%)} = \left(\frac{J_{max} - J_r}{J_{max}}\right) * 100 \qquad \text{Eqn. 1}$$

4.2.2.7 Descriptive Sensory Analysis

A descriptive analysis panel of 7 individuals between the ages of 35-50 was trained for a minimum of 1000 hrs. The panel evaluated firmness (first bite), sour, fruit flavor,

rancidity/oxidation, astringent mouthfeel and tooth packing according to Childs et al. (2007) (Childs, Yates, & Drake, 2007). The panel evaluated each attribute on a 15 point Spectrum® scale (Meilgaard, Civille, & Carr, 1999). Panelists trained on commercial bar samples prior to testing (PowerBar® Protein Plus Bar, MET-RX® protein plus, Quest®). Bar samples were cut into rectangular cubes of ¾" x ¾" x ¼". The panel performed tests at room temperature (22 ± 2 °C) in a positive pressure, odor free room. Panelists were given 6 pieces of Ta sample to evaluate terms described in table 2. Between each sample panelist cleansed pallet with deionized water.

Table 3. Definition used by panel to define descriptive analysis terms.

	TERM DECRIPTION
HARDNESS	Force required to completely bite through sample with molars
TOOTH PACKING	Likeliness of sample to stick in molars during mastication
CHEWINESS (# CHEWS)	Number of chews until swallow
SWEET	Total sweetness of sample
SOUR	Total sourness of samples
OXIDIZED / RANCID / PAINTY	Total oxidized, fishy, rancid, painty flavor of sample
ASTRINGENT MOUTHFEEL	Total dry puckering sensation during mastication and immediately following swallow
OVERALL FRUIT FLAVOR	Any attributes associated with fruity aromatics

4.2.2.8 Statistical Analysis

Statistical analysis was completed using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA) using ANOVA and Tukey's multiple comparison of means.

4.3 Results

4.3.1 WP-CP Powders and Bars

Protein polyphenol powders were made to contain 1 and 2 mg of TP per gram of powder. The resulting powders contained a distribution of particles (Figure 1). Both powders

contained a peak around 1 µm as well as larger aggregates around 10 µm and 30 µm. The WP-2CP particles also contained a larger class of particles at around 174 µm.

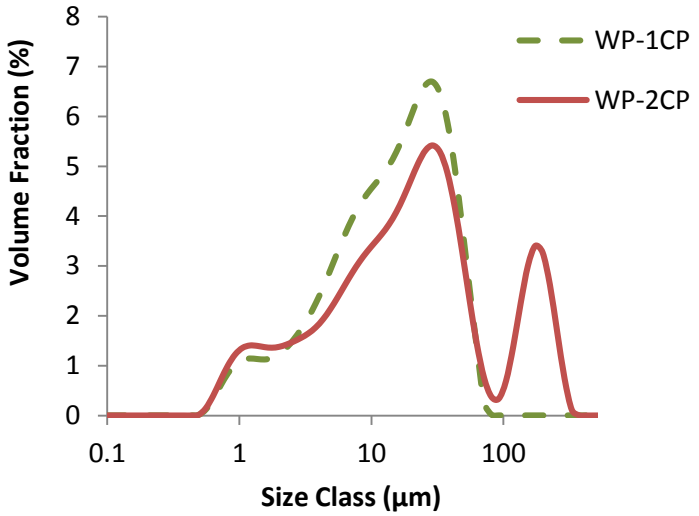

Figure 7. Wet particle size of whey protein-cranberry polyphenol (WP-CP) aggregates

The sensory panel detected a significant reduction in hardness and toothpacking due to incorporation of the WP-CP particles in the bars on day one (Table 3). Increases and sweet, sour and hints of fruit were detected but overall no negative flavor/texture attributes were described for a protein bar contain WP-CP particles. This reduction in firmness was supported by creep recovery results. Both the WP-1CP and WP-2CP particles reduced bar hardness on day one compared to bars formed with the unmodified WPI powder, as indicated by a reduction in creep recovery and an increase in maximum compliance (J_{max}) (Figure 2& 3). The higher the creep recovery, the more elastic (solid like) characteristic in a material, whereas a lower creep recovery indicates a more viscous or fluid like material. The J_{max} is inversely related to firmness, as a greater J_{max} indicates a more viscous material than a material with a lower J_{max}. Over time, bars with WP-CP aggregates maintained a decreased

creep recovery compared to unmodified WPI bars. Although the bars made with WP-1CP and WP-2CP differentiated with time; the bars containing WP-2CP had significantly lower creep recovery and higher J_{max} than those with WP-1CP.

Table 4. Descriptive Analysis results for WP-CP and WPI bars on day one. Letters indicate significantly different values across a row ($p < 0.05$)

	WPI	WP-1CP	WP-2CP
HARDNESS	5.1 [a]	4.2 [b]	4.0 [b]
SWEET	5.6 [a]	6.0 [a]	6.7 [b]
OVERALL FRUIT FLAVOR	0.3 [b]	0.0 [a]	0.5 [b]
TOOTH PACKING	10.2 [a]	9.6 [b]	9.5 [b]
OXIDIZED / RANCID / PAINTY	0.0 [a]	0.0 [a]	0.0 [a]
SOUR	0.1 [a]	0.2 [a]	0.6 [a]
ASTRINGENT MOUTHFEEL	2.8 [a]	2.5 [a]	2.8 [a]
CHEWINESS (# CHEWS)	28.2 [a]	27.2 [a]	24.3 [a]

A WP-2CP:WPI bar was formulated by mixing 50:50 WPI and WP-2CP powder as the protein source to equate to equal bar polyphenol content of bars formulated with WP-1CP. This WP-2CP:WPI was used to evaluate the potential of using the WP-CP powder as a supplemental protein source to WPI in a bar formulation. Indeed, the bars containing 50:50 WP-2CP:WPI maintained a lower creep recovery which was not significantly different than the bars made with WP-1CP.

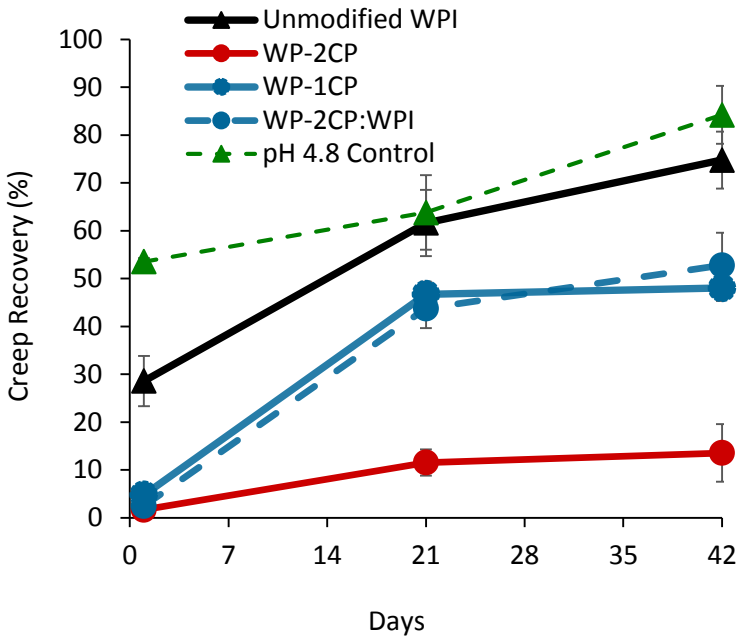

Figure 8. Creep recovery of protein bars containing unmodified whey protein isolate (WPI), whey protein-cranberry polyphenol powder containing 1 (WP-1CP) or 2 (WP-2CP) mg of total phenolics per g of powder, WP-2CP:WPI, and pH 4.8 control powder during storage.

One important variable was thought to be the change in pH of bars made with WP-CP powders, as when this powder was used, the bar pH was lowered to 4.8. Therefore, pH control bars were formed using a powder made from a WPI solution adjusted to pH 4.7 and dried, that produced a bar at pH 4.8 without any juice components. The pH control bar did not have comparable rheological properties to that of the WP-1CP or WP-2CP. On day one, the pH 4.8 control bars were actually firmer than bars formulated with WPI (no pH adjustment). Although, over time, this differentiation dissipated and pH 4.8 control bars were equivalent to bars made with unmodified WPI.

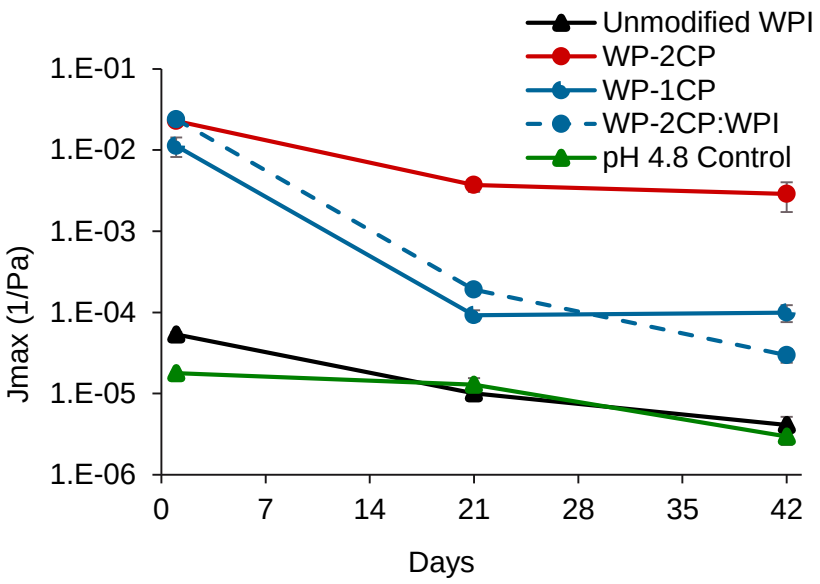

Figure 9. Maximum compliance (J_{max}) of protein bars containing unmodified whey protein isolate (WPI), whey protein-cranberry polyphenol powder containing 1 (WP-1CP) or 2 (WP-2CP) mg of total phenolics per g of powder, WP-2CP:WPI, and pH 4.8 control powder during storage.

Mechanistic Exploration

A major hindrance to the use of WP-CP powders to produce softer bars is the low yield of the method, meaning there is a high raw ingredient input (protein and juice) for a relatively low amount of produced product with yields of 45-50% for protein and 30-50% for polyphenols (Schneider et al., 2016). Therefore, it is crucial to understand the mechanism by which the WP-CP particles are reducing hardness in order to develop alternative, practical approaches for creating the particles. If the mechanism can be determined, a technique may be developed to form particles in a more sustainable manner while maintaining success in reducing bar hardness. Therefore, various powders were formulated to isolate the contributions of cranberry polyphenol content, pH, and protein solubility. To simplify the amount of data, treatments were made in comparison to unmodified WPI as the negative

control and WP-1CP as a positive control. In the standard practice for making WP-CP particles, the insoluble particles are separated by centrifugation and the supernatant, which contains protein and polyphenols, is discarded. The mixture (not separated) and supernatant were made into powders and incorporated into bars. The creep recovery of the bars made with supernatant powder actually increased in comparison to the unmodified WPI, while the creep recovery of the mixture was numerically lower but not significantly different from the unmodified WPI bars (Table 4).

Table 5. Creep recovery on day one for bars made with various treatment powders and protein solubility of powders at bar pH

Treatment ID	Creep Recovery	Protein Solubility
Unmodified WPI	28.58[c]	82[c]
WP-1CP	4.77[b]	45[a]
WP-2CP	2.49[a]	43[a]
Mixture	20.78[c]	58[b]
Supernatant	58.56[e]	96[d]
Polyphenol Control	58.54[e]	86[c]
pH Control	53.5[d]	82[c]

Different letters indicate significantly different values ($P < 0.01$). See Table 1 for full definition of treatments.

The WP-CP particles were formed using cranberry juice, meaning the particle functionality could be related to the basic juice components (sugar, pH, and buffering capacity), the phenolic compounds, or a combination of both. Therefore, to isolate the impact of polyphenols on bar texture, powders were made with polyphenol extracts co-dried with WPI (Polyphenol Control). Cranberry pomace was used as the source of polyphenols because, as a byproduct, it is an economical source of polyphenols. The polyphenol control treatment also increased the creep recovery, indicating more bar hardening. The polyphenol control treatment was similar to the cranberry juice mixture treatment and they produced similar changes in rheological properties. The pomace is composed of cranberry skin, seeds, and flesh, but free of sugar allowing for isolation of polyphenols sans sugar. The skin, seeds, and flesh of cranberries contains higher amounts of proanthocyanidins than the juice, therefore the pomace extract, while relatively similar in polyphenol profile to that found in juice, has higher amounts of proanthocyanidins which are more polymerized (Grace, Massey, Mbeunkui, Yousef, & Lila, 2012). Although, this cannot be completely dismissed as the cause of increased creep recovery, the particle size/insolubility should be increased due to more proanthocyanidins (polymerized polyphenols), since studies have shown degree of polymerization to increase protein-polyphenol interactions (Prigent et al., 2009). Instead, the differences between WP-CP and polyphenol control in bars is likely due to the low content of insoluble particles, as indicated by the overall higher protein solubility. The non-polyphenol juice components are likely providing an environment that facilitates protein-polyphenol interactions and formation of insoluble particles (H. Oh, Hoff, Armstrong, & Haff, 1980; H.-I. Oh & Hoff, 1987).

The force and time used in the centrifugation process will determine which particles are separated as a pellet. According to Stokes' law, the velocity of sedimentation as dependent on dispersed particle size, continuous phase viscosity, density difference between particle and continuous phase, and forces being exerted on the particle (Stokes, 1901). From a practical perspective in examining proteins in food applications, the apparent solubility of a protein is directly related to Stokes' law considerations determining the sedimentation rate of the dispersed protein. Therefore, we know that WP-CP particles are more dense and/or larger than the proteins remaining in the supernatant, and can be considered insoluble particles if they are precipitated by the mild centrifugal forces exerted on a particle at 7,000 *x g*. The reduced solubility of proteins used to make bars appears to be a major reason for the reduction in bar hardness (Table 4). The WP-CP powders have the least amount of soluble protein. For treatments with a high amount of soluble proteins, such as the polyphenol control the creep recovery was also greater. In addition, once solubility is decreased, the particle size appears to also play roll.

The particle size is directly related to surface area, which in turn is related to how a particle is hydrated. The particle size distribution for WP-1CP and WP-2CP particles were different and may be a contributing factor in bar hardening. Therefore, we considered the particle size of dispersed powders when evaluating firmness on day one results. Particles less than or equal to 1 μm are present in the supernatant, mixture, pH 4.8 control, and polyphenol control (Figure 4). Bars made with these powders were equally or more firm than unmodified WPI bars according to creep recovery results (Table 4). Bars that did not contain particles less than 1μm, WP-CP, were the only bars to reduce bar hardening on day one. Therefore, it

is critical that the particle size and solubility be considered to understand bar hardening mechanisms.

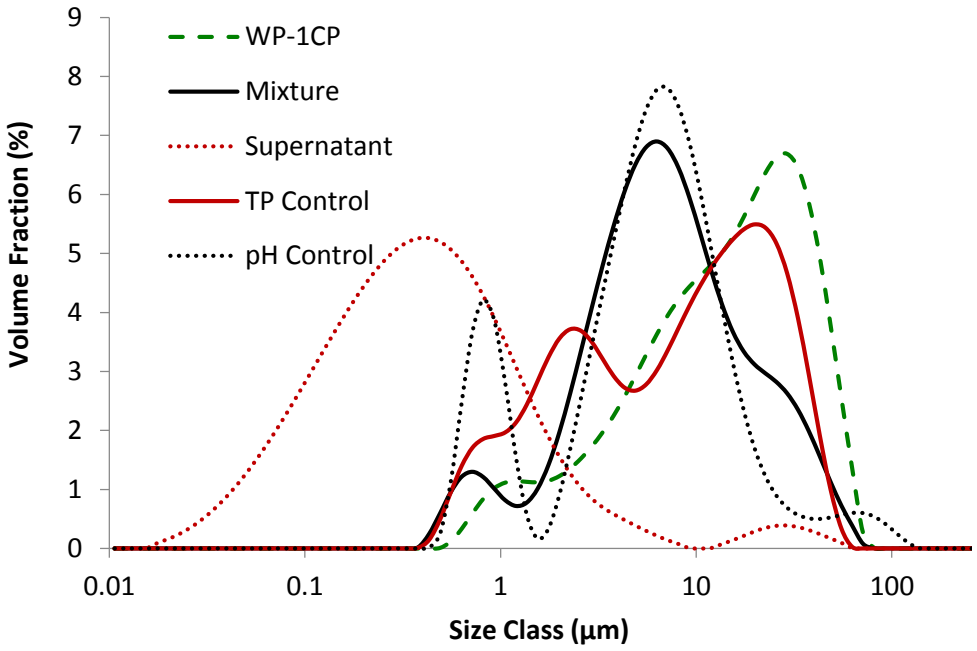

Figure 10. Wet particle size of various protein sources.

4.3.2 WP-CP Digestion

Previous studies demonstrate reduced digestibility of protein after addition of polyphenols (Ozdal, Capanoglu, & Altay, 2013). Therefore, a crude measure of protein digestibility was determined for WP-CP, unmodified WPI, and soy protein isolate (Figure 5). Soy was used as a benchmark to compare the degree of change in digestibility with the addition of polyphenols, as it is a common bar protein ingredient (Childs et al., 2007; Li et al., 2006). The protein in the WP-CB powder digested slower than WPI, but faster than soy protein isolate.

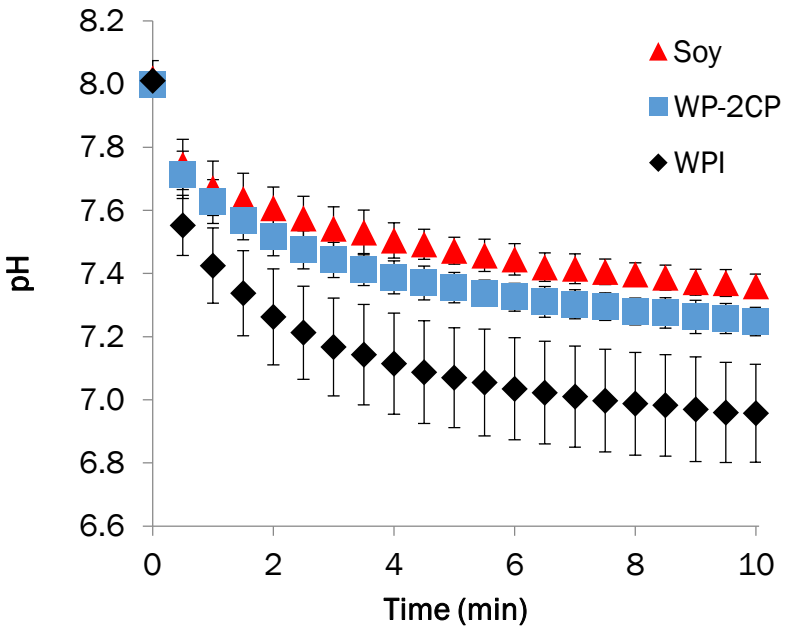

Figure 11. Change in pH during protein digestion of whey protein-cranberry juice aggregates containing 2 mg per g powder (WP-2CP) powder, and whey protein isolate (WPI).

4.4 Discussion

4.4.1 WP-CP Powders and Bars

Adding whey protein-polyphenol particles that contained WP-1CP or WP-2CP particles to bars made softer bars that showed less hardening over time (Figures 2 and 3). The protein particle could have been altering the physical properties of the bars due to shifting the pH, an effect of the polyphenols, or by having the protein in an insoluble form. The pH 4.8 control powder failed to decrease bar hardness, therefore the effect is likely a result of an increase in juice concentration producing larger, inactive particles. Our previous work showed that particle size and solubility is determined by the total phenols and other components of the juice (Schneider et al., 2016). Conditions that produce large, insoluble

particles appear to reduce bar hardness. Other investigations have suggested that protein aggregation increases bar hardness (Zhou et al., 2013); however, that was aggregation during bar storage, not pre-aggregation of particles.

4.4.2 Mechanistic Discussion

A critical step in forming particles is centrifugation, which, through the pelleting process, allows for the isolation of insoluble aggregates. The nature of being insoluble could suggest that the proteins are "pre-reacted" and therefore not available for aggregation or network formation once incorporated in a bar. Bar hardening mechanisms related to phase equilibration, phase separation, and network formation requires a protein-water phase(s) and some degree of molecular mobility. The supernatant resulting from WP-1CP particle formation contains a similar ratio of proteins to polyphenols to that of the WP-1CP (data not shown). Therefore, the hypothesis that solubility is important is supported by the increase in creep recovery in bars made with the supernatant compared to bars made with unmodified WPI. In a sense, the supernatant could be considered a soluble protein-juice components control. In addition, soy and other products such as milk protein isolate tend to produce less hardening than whey proteins in bars, but also contain less water soluble proteins compared to whey protein (Webb, Naeem, & Schmidt, 2002; Wohlt, Sniffen, & Hoover, 1973). Therefore, the critical step may not be increasing particle size, but, rather, *removal* of small reactive/native whey proteins. Zhou et al. (2008) demonstrated that protein aggregation during storage resulted in an altered microstructure of the bar (Zhou, Liu, & Labuza, 2008). We propose that pre-aggregating protein reduces the change in microstructure because the starting ingredient has less potential to aggregate over time. This is in agreement with

findings of Zhu et al. that addition of cysteine to whey protein bars reduced hardening due to a decreased potential for protein sulfhydryl-disulfide exchanges, which produces protein aggregates and possibly networks. In effect, Zhu et al. reduced the reactivity of the protein at the start of bar formulations to prevent aggregation over time. Therefore, we also suspect that the insoluble nature of the WP-CP particle could reduce water migration or other factors that cause bar hardening.

4.4.3 WP-CP Digestion

The digestion rate of the WP-CP powder was slower than the whey protein isolate, a finding supported in the literature (Bravo, 1998; Ozdal et al., 2013). The use of WP-CP powders should not be excluded for use in bars simply because of a decrease in protein digestibility from unmodified WPI. The initial methods paper by Hsu et al. (1977) showed soy protein isolate to digest slower than WPI, but faster than casein (Hsu et al., 1977). Therefore, we can deduce that WP-CP would still digest faster than soy and casein, which are the two most predominant protein sources, besides whey protein, used in bar recipes. If bar formulators intended to use whey protein isolate due to the rapid digestibility, WP-CP could still be an option. If one wanted prolong the effective release of proteins within a bar, WP-CP could be exploited in this way, as it could be used as an intermediate digesting protein or used in addition to another protein in a bar formulation. Results for the WP-2CP:WPI control showed a reduction in bar hardness with 50:50 WP-2CP and WPI. Ultimately, the amino acid composition, which is what gives whey proteins their high protein quality, would be maintained, and the ingredient would have the added health benefit of the polyphenols. Polyphenols have been shown to impart anti-inflammatory, anti-cancer, and cardiovascular

protective properties and may be a justifiable addition in exchange for in protein digestibility (Ahmed et al., 2014; Bravo, 1998; Duthie, 2007; Mozaffarian, Appel, & Van Horn, 2011).

4.5 Conclusions

The use of whey protein-cranberry polyphenol particles allowed for a reduction in bar hardness with increasing juice concentration increasing the reduction in bar hardness. This was explained by the protein-polyphenol particles keeping the protein in an insoluble, dispersed phase that was less likely to interact and form essentially larger polymer or a protein network during storage. The digestion rate of the whey protein was decreased in the presence of polyphenols, but remains greater than soy protein and therefore allows for manipulation of protein digestibility. This permits the option to substitute a fraction of WPI with WP-CP in a bar formulation to provide both a quick and slow digesting protein, *as well as* a softer bar. These results suggest that bar hardening can be mitigated by forming protein-polyphenol particles that act as an insoluble dispersed phase and do not participate in reactions associated with bar hardening.

4.6 Acknowledgements

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APPENDICES

Appendix A: Preliminary Foam Experiments

Materials

Whey protein isolate (WPI) (Bipro, 93.19% protein, supplied by Davisco Foods International, Inc., Le Sueur, MN, USA), obtained from The Cranberry Network, LLC (Wisconsin Rapids, WI, USA,), muscadine juice concentrate obtained from The Muscadine Products Corporation (Wray, GA, USA), or 1:19 (v:v), blackcurrant juice concentrate obtained from The New Zealand Blackcurrant Co-operative LTD (Nelson, New Zealand). The NaOH, citric acid, sodium acetate, acetic acid, fructose, and glucose were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Particle Formation

Whey protein powder was mixed overnight with diluted 1:4 (v:v) cranberry juice concentrate, 1:9 (v:v) muscadine juice concentrate, or 1:19 (v:v) juice concentrate in water for a final protein concentration of 20%. The pH of the mixture was adjusted to pH 4 using 3 M sodium hydroxide. Protein-polyphenol particles were collected by centrifugation at 7,000 x *g* for 20 min at 15 °C. The protein content was measured using a vario MACRO cube CHN (Elementar, Mt. Laural, NJ, USA) which uses the Dumas method to determine nitrogen content (Wiles, Gray, & Kissling, 1997). The protein concentration was calculated with a whey protein to nitrogen ratio of 6.38 (Jones, 1941). Polyphenol content was determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965). Anthocyanin content was determined using the pH differential method (Lee, Durst, & Wrolstad, 2005).

Whey protein control particles (WCP) which were formed without juice. WPC were formed by treating the proteins the same as for polyphenol-protein particulates, except a

mock juice (same buffering capacity, pH, and sugar as cranberry juice) without polyphenols was used (Table 1A).

Table 6A. Whey protein control particle solution formula

Composition of Imitation Juice	Buffer: Acid	Buffer: Base	Sugar	pH
Cranberry	0.88 M Citric Acid	0.88 M Citrate	1.6 % Fructose: 8.3% Glucose	3.06
Blackcurrant	0.67 M Citric Acid	0.67 M Citrate	2.6% Fructose: 3.9% Glucose	2.95
Muscadine Grape	0.28 M Tartaric Acid	0.28 M Tartrate	7.5% Fructose: 7.5% Glucose	3.20

Particle size

Particle size was determined using Fraunhofer diffraction theory (Mastersizer 3000; Malvern, Worcestershire, UK) at pH 4 and 5. Particles were dispersed in pH 4 or 5 acetate buffers at 10% w/w protein. Buffers were composed of 0.4M sodium acetate and acetic acid at ratios of 1:6 and 5:2, respectively. The dispersed particles were added to the sample flask until obscuration reached 10-15% before taking measurements.

Foam Formation

Foam formation and analysis of physical properties were determined according to the methods of Pernell et al. (2000 & 2002)(Pernell, Foegeding, & Daubert, 2000; Pernell,

Foegeding, Luck, & Davis, 2002). A fixed weight of 200 g of 10% w/w protein solutions is whipped in a mixer for a 20 min. Whey protein isolate was the standard protein and particles were substituted for 25, 50, 75 and 100% of the protein (i.e., total protein concentration maintained at 10% w/w with varied composition of un-reacted whey proteins and particles). The particles were stirred into foaming solutions from wet pellet form. After whipping, a hole in the bottom of the bowl was removed and drainage time is started. Simultaneously, the yield stress of the foam was determined using a vane fixture that was inserted in the foam.

Results

The molar ratio of protein to total phenolics for each juice was estimated to be 1-1.5 assuming a molecular weight of 500 Da for the average polyphenol and 18 kDa for whey proteins. The range produced was dependent on the juice source (Table 2A), and will likely vary with particle formation conditions.

Table 7A. Dry particle composition (mg/g) and initial juice composition (mg/mL) using cranberry, blackcurrant, and muscadine grape juice. Protein content of particles is in units of mg/g. TP= Total Phenolics (gallic acid equivalent), ANC = Anthocyanins (cyanidin-3-O-glucoside equivalent).

	Juice TP	Particle TP	Juice ANC	Particle ANC	Particle Protein
Cranberry	1.14	0.40	0.23	0.14	853
Blackcurrant	1.66	0.60	0.58	0.19	903
Muscadine Grape	1.20	0.56	0.68	0.09	843

Each juice produced a distribution of particles between 1 μm and 100 μm (Figure 1A). Therefore, all were in the category of "suspendable particles" (> 200 nm). The particle size tended to be smaller when formed at pH 4 as compared to pH 5. We suspect there are also particles below 1 μm that are not being detected by this method but will be seen in dynamic light scattering measurements.

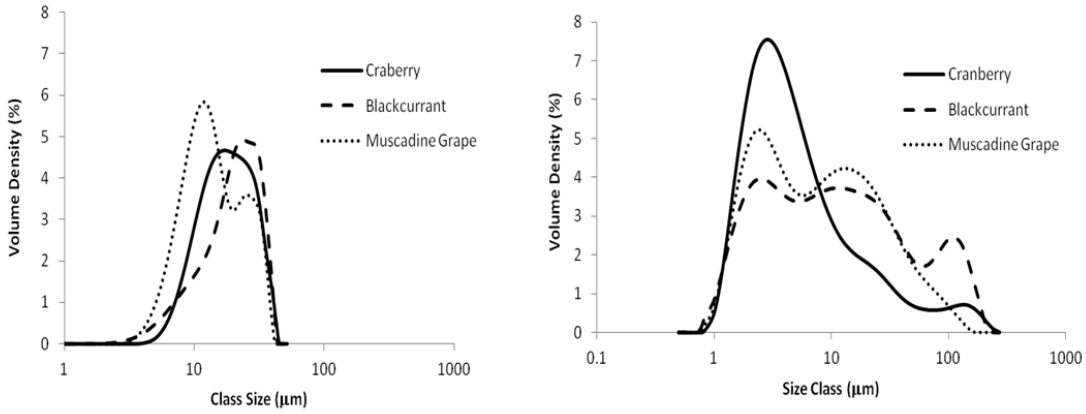

Figure 12A. Comparison of average particle size distribution for polyphenol-whey protein particles at pH 4 (left) and pH 5 (right).

Foam drainage half-life and yield stress increased with addition of juice polyphenols to plain whey protein. Upon further analysis to varying polyphenol content between juice polyphenols a concentration effect is seen (Table 1A) as blackcurrant appears to produce larger particles and contain the most polyphenols. Foams produced with these particles increased foam drainage half-life and yield stress (stiffness of peaks) when incorporated into whey protein foams to contribute 50% of the total foam protein (Figure 2A).

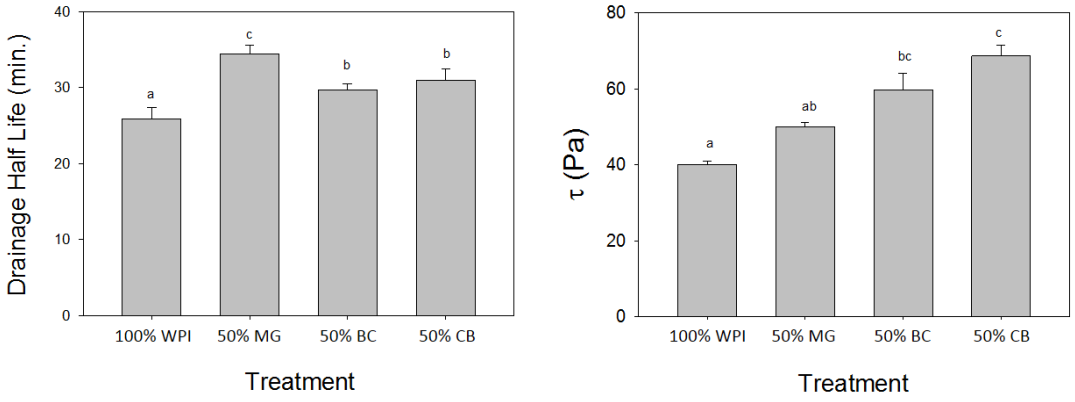

Figure 13A. Drainage half-life (indicating stability) and yield stress (τ) of foams. Foaming solutions were a control (no particles, 100% WPI) or treatments, containing 50% of the protein as particles containing polyphenols. All solutions contained 10% w/w protein polyphenol sources were: MG- muscadine, BC-blackcurrant, and CB-cranberry. Bars with different lowercase letters are significantly different ($p < 0.05$).

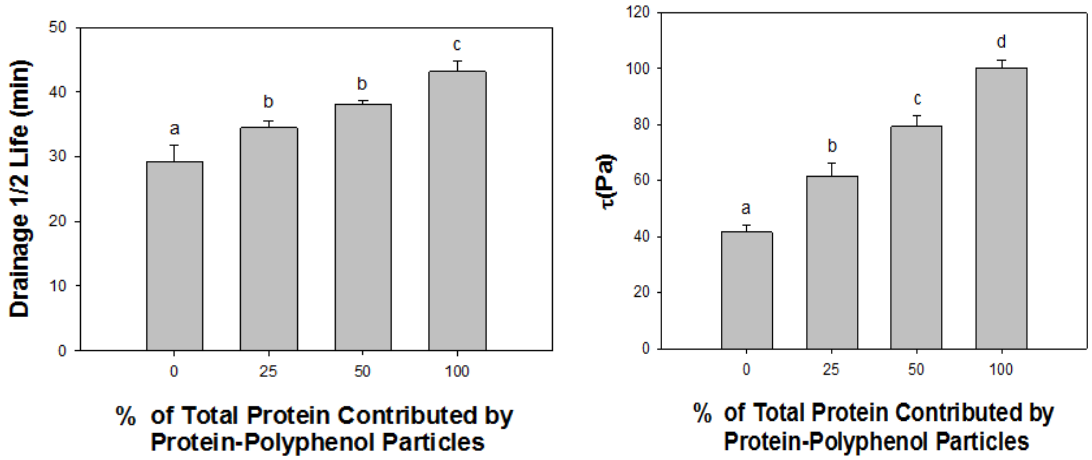

Figure 14A. Drainage half-life (indicating stability) and yield stress (τ) of foams. Foaming solutions were a control (no particles, 100% WPI) or treatments, containing 25, 50, or 100% of the protein as particles containing polyphenols. All solutions contained 10% w/w protein polyphenol sources were from cranberry juice. Bars with different lowercase letters are significantly different ($p < 0.05$).

To determine if there is a concentration of effect on foams when addition these particles the cranberry particles were added in increasing amounts to the foams (Figure 3A).

A stepwise result was observed, as yield stress and drainage half life increased with increasing particle concentration.

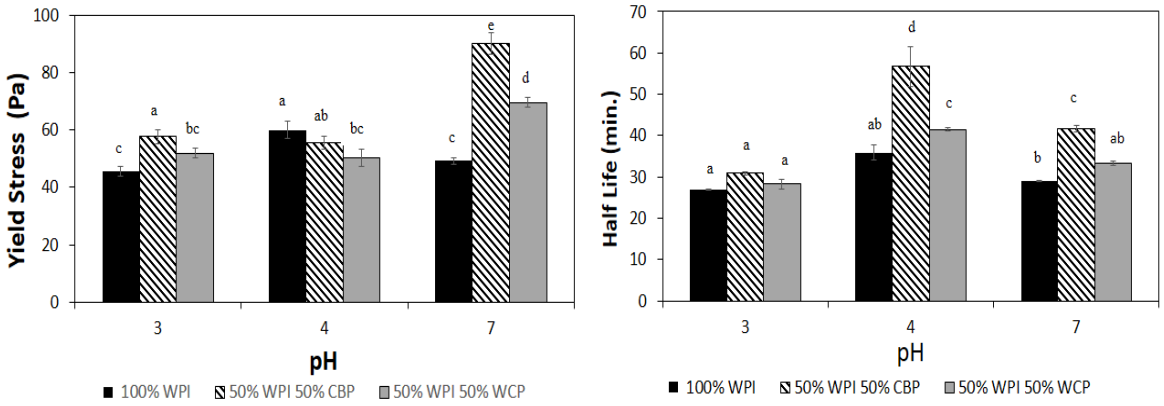

Figure 15A. Changes in half life (left) and yield stress (right) of foams made from solutions containing 10% w/w protein. The protein sources were either 100% WPI; 50% of the protein source from cranberry polyphenol protein particles (CBP) and 50% WPI, or 50% whey protein control particles (WCP) and 50% WPI. Bars with different lowercase letters are significantly different ($p < 0.05$).

The impact of foaming solutions containing whey protein control particles (WCP) were compared to foams containing cranberry polyphenol protein particles (CBP). Formulations contained 50% of either treatment and 50% of unmodified WPI. Whey protein control particles were used to isolate the effects of cranberry juice pH, buffering capacity and sugar content on foams. Yield stress more than doubled, going from 40 to 90 Pa for foams containing 50% CBP at pH 7; and a slight increase occurred at pH 3 (Figure 4A). In both cases, the protein-polyphenol particles increased yield stress compared to foams containing WCP. Likewise, adding CBP produced the most stable foams at pH 4 and 7. This demonstrates that the addition of CBP has the ability to increase stability and rigidity of foams, and it is not simply a result of the juice buffering capacity, pH, and sugar content.

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Appendix B-Exploratory Bar Formulations

All methods for particle/powder formulation, protein solubility, bar formulation, and rheology were performed according to section 4.2.2, unless otherwise noted. These are preliminary experimental explorations, therefore only one replicate was completed for the following results. The protein used to form powders was whey protein isolate (WPI) sourced from Glanbia Nutritionals (89 % protein, Twin Falls, ID, USA)

Cranberry juice concentrate and freeze dried blueberry juice co-dry powders

Experiments were performed to determine the difference between bars formulated with equal protein content compared to equal protein power content. This was evaluated using new powders, that were formed by co-drying cranberry juice or freeze dried blueberry juice with whey protein isolate. Blueberry powder was gifted from the Wild Blueberry Association (2.9% total phenolics, 1.9% anthocyanins, 0.8% moisture, Momnece, IL, USA). Cranberry juice concentrate (50.2 °Brix and 5200 µg/ml total phenolics (TP)) was gifted by Ocean Spray® (Lakeville-Middleboro, MA, USA). The co-drying solutions were prepared according to Table 1B.

Table 1B. Solutions used to form co-dried juice and whey protein powders

Treatment	Solution Formulation for Spray Drying
Ctrl (x g)	No modification to WPI (89% protein)
CB 50	1:7:2 w/w of cranberry juice: deionized water: WPI (Dry powder 86.4% protein)
CB 100	1:3:1 w/w of cranberry juice: deionized water: WPI (Dry powder 72.8% protein)
BB	3:37:10 w/w dry blueberry juice: deionized water: WPI (Dry powder- 45.5% protein)

Bars formulated with the powders did not show potential for reducing bar hardness (Figure 1B). The particle size for these powders were also evaluated (Figure 2B), but not found to correlate with J_{max} values, likely due to the importance of particle number, which would require solubility calculations, which were not obtained.

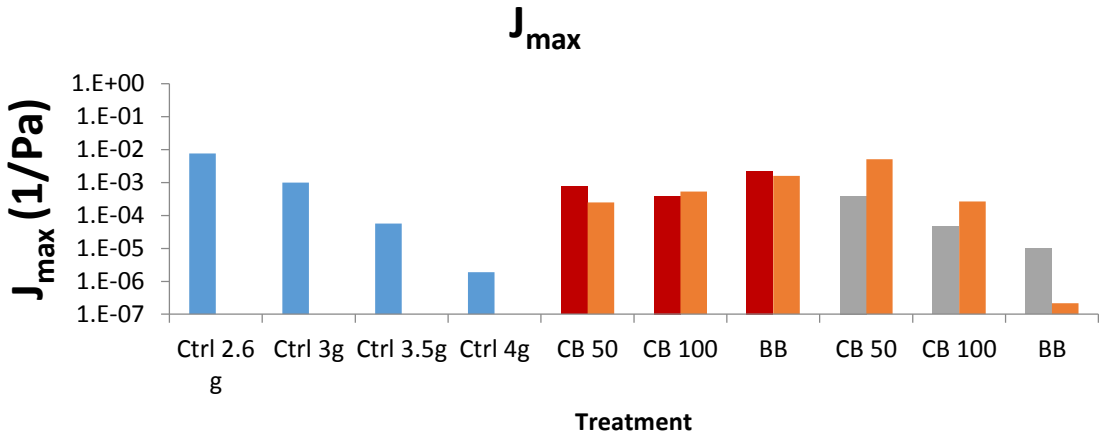

Figure 1B. Maximum compliance (J_{max}) of protein bars formulated with experimental powders. Blue indicates control (Ctrl) treatments made with 2.6 g whey protein isolate(standard weight of powder used in preliminary bar experiment recipes) and 3, 3.5, or 4 g to formulate a standard curve for various protein/powder weights. The red bars are protein bars formulated with equal powder weight and grey bars are proteins bars formulated with equal protein weight. Using the control results, theoretical J_{max} values for respective powder weights (next to grey bars) and protein contents (next to red bars) were determined using a standard curve made with the Ctrl results.

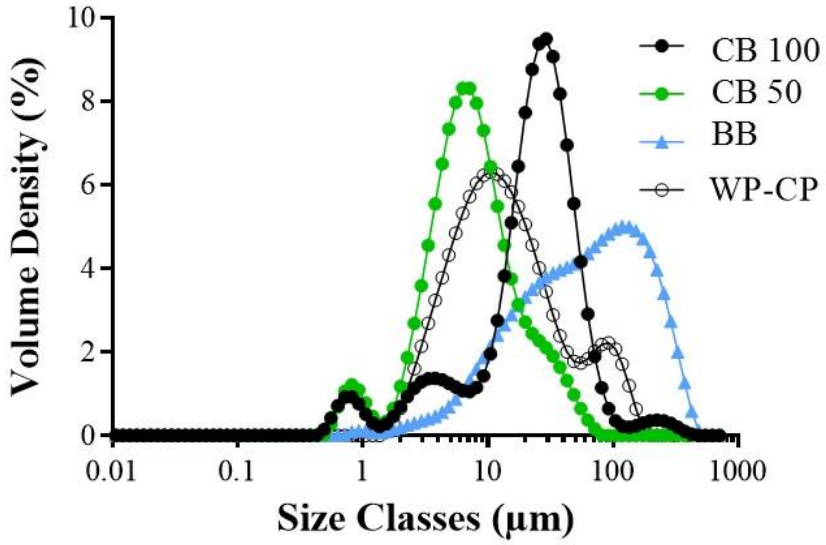

Figure 2B. Wet particle size of particles formed as a result of formulations in Table 1B.

Blackcurrant polyphenol extract powders

Preliminary experiments investigated the use of blackcurrant polyphenol extract powder as a source of polyphenols for forming protein-polyphenol powders. Blackcurrant polyphenol extract powder was obtained from Just the Berries Ltd. (Nelson, New Zealand). Dry powders were formulated to contain two different blackcurrant polyphenol contents (1 or 5% of dry powder) at two different pHs (no pH adjustment or pH adjusted to 5). Bars were formulated with either 100% of each powder or 50% treatment powder and 50% unmodified WPI (% according to protein content of powders and bar). The treatment with 50:50 treatment: unmodified WPI was denoted as the "half" treatment.

Table 2B. Solutions used to form co-dried juice and whey protein powders

Treatment	Solution Formulation for Spray Drying
Control	No modification to WPI (89% protein)
1 % TP no pH adj	10 % WPI in a 1% w/w blackcurrant extract powder in deionized water (pH 6.15)
1 % TP pH 5	10 % WPI in a 1% w/w blackcurrant extract powder in deionized water adjusted to pH 5
5% TP no pH adj	10 % WPI in a 5% w/w blackcurrant extract powder in deionized water (pH 5.3)
5% TP pH 5	10 % WPI in a 5% w/w blackcurrant extract powder in deionized water adjusted to pH 5

Bars formulated with protein-blackcurrant extract polyphenol powders did not show potential for reducing bar hardness (Figure 3B). The solubility and particles size of powders was also evaluated (Figure 4B & 5B). Although larger particle between 4-50 µm, the very high solubility across a range of pHs suggests the number of larger particles low.

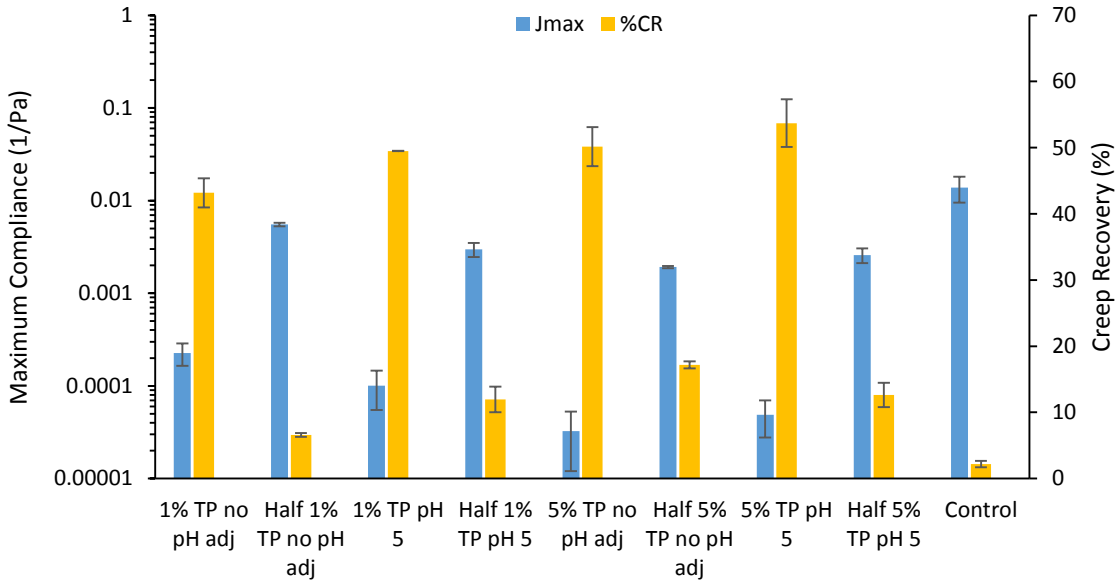

Figure 3B. Maximum compliance (J_{max}) and % creep recovery (% CR) of protein bars formulated with experimental powders. The control represents bars formulated with unmodified whey protein isolate.

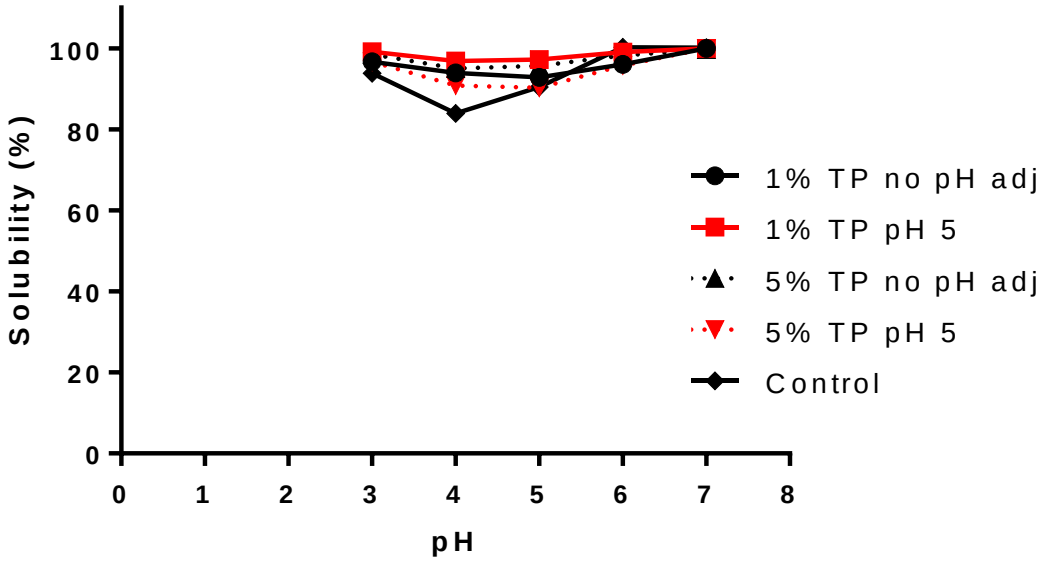

Figure 4B. Protein solubility of powders dispersed at 10% w/w protein in deionized water adjusted to pH 3, 4, 5, 6, or 7.

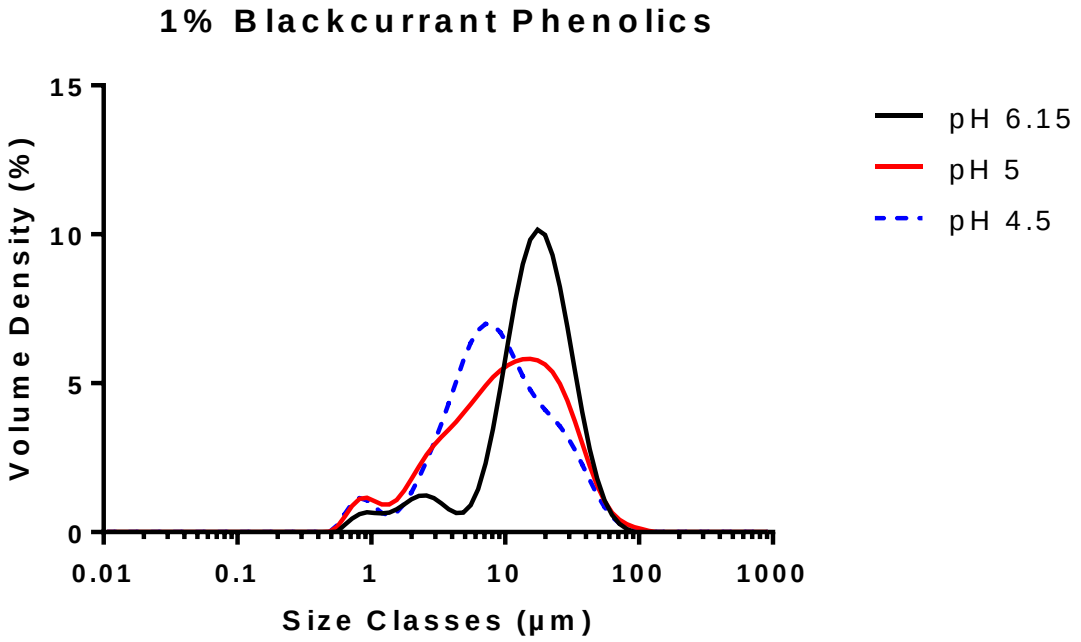

Figure 5B. Particle size of 1% blackcurrant polyphenol extract powders and 10% w/w protein mixtures, pre-spray drying at various pHs.

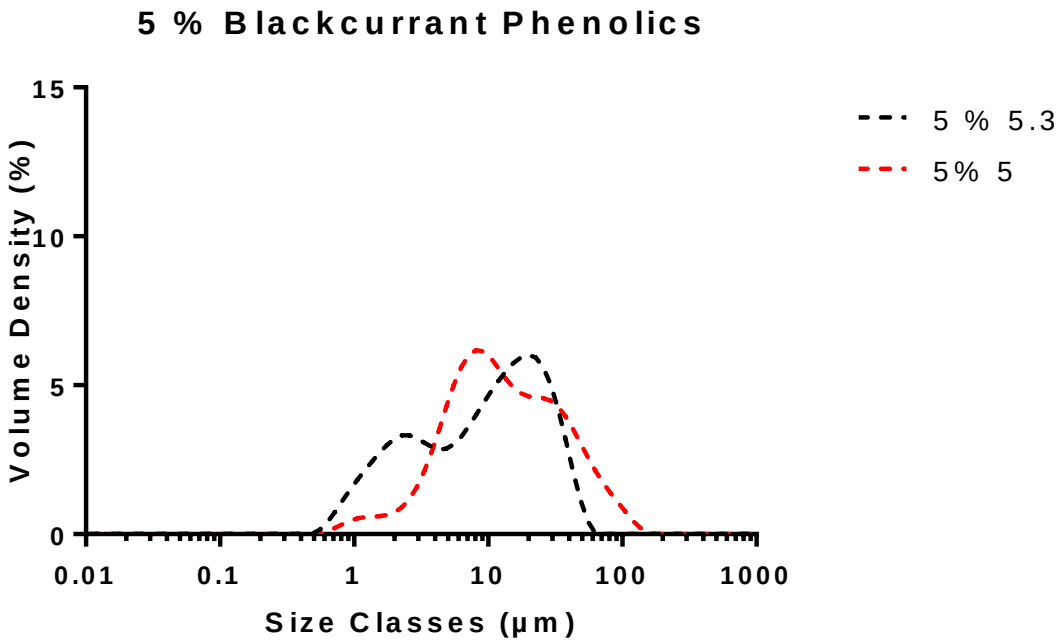

Figure 6B. Particle size of 5% blackcurrant polyphenol extract powders and 10% w/w protein mixtures, pre-spray drying at various pHs.