The optimization and characterization of dairy-based agglomerated protein products using whey protein hydrolysate as a binding agent

by

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Abstract

Whey proteins are highly soluble and have many functions in food applications such as gelation, foaming, and emulsification. Many approaches were performed to modify the physiochemical characteristics of whey proteins such as enzymatic hydrolysis. The type and specificity of the enzyme influence the properties of the resultant hydrolysate. In a recent disclosure of invention, whey protein hydrolysate (WPH) was utilized as a binder in whey protein isolate (WPI) wet agglomeration process. The first objective of this study was to characterize the physical and chemical properties of three lots of commercial WPH. High-performance liquid chromatography (HPLC) and Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) confirmed a complete hydrolysis of whey proteins in the three lots, which indicated a consistent hydrolysis. Moreover, the degree of hydrolysis was not significantly different ($P>0.05$) among the lots. The second objective of the study was to optimize and evaluate the effectiveness of WPH as a binder in wet agglomeration of WPI. The second objective was carried out in two phases. In the first phase, a 3×3×2 factorial design was conducted with pre-wet mass (60, 100, and 140 g), WPH concentration (15, 20, and 25%), and flow rate (4.0 and 5.6 $mL.min^{-1}$) as independent variables, and the other processing parameters were kept constant. Agglomeration was carried out in a top-spray fluid bed granulator (Midi-Glatt, Germany). All the experiments were performed in triplicates and agglomerated WPI samples were stored at 25°C. In the second phase, agglomerated WPI samples were analyzed for functional and physical characteristics such as moisture content, water activity, emulsifying capacity, the agglomerates size and shape characteristics, and density. Moisture content of agglomerated samples was within the range of 3 – 15%. The treatment combinations of high pre-wet mass (140g) and flow rate (5.6 $mL.min^{-1}$) resulted in high moisture content and water

activity in the agglomerated WPI samples, consequently, clumps were formed during the agglomeration process. The size and shape characteristics of agglomerates were evaluated using Morphology G3-ID (Malvern Instruments Ltd, UK). The mean of circle equivalent diameter (CED), circularity, elongation, and convexity were measured. The CED of the WPI agglomerates was in between 13.63-17.96 μm. No significant differences ($P>0.05$) were observed for the CED and convexity for the main effects and their interaction. The utilization of WPH as a binder is a promising approach to produce “lecithin free” agglomerated high protein powders, which provides a product with a clean label. In addition, the shelf life of agglomerated powders can be increased as WPH is not susceptible to oxidation as lecithin. Overall, the results suggest that WPH may be used as an alternative of soy lecithin in agglomerating WPI.

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Dedication

To who gave me life, my wonderful mom (Kulthoum) and dad (Jameel): I may have never expressed in words how much I love you as I found no words that can befit such a feeling, but today while I am wrapping up two years of dedicated hard work, I am genuinely thankful for the wonderful gifts; your blessings, your enlightenment in making my life meaningful, and for the countless times you have glowed hope kindles in my heart since my birth, which made me competent to resist while facing the obstacles and hardship throughout my life in a brave, flexible, and also compassionate manner.

To my sisters (Maisa', Malak, Najah, Ghaida', Sereen, and our little angel Ghena) brothers (Suliman, Omar and Sa'ad) and all my friends….. Thank you all for being in my life.

With love,
Baheeja

Chapter 1 - Introduction

Whey is a co-product of cheese making that contains valuable and nutritious whey proteins. Currently, the dairy industry is utilizing whey to produce a variety of whey protein-based powders that are used as ingredients in other products such as infant formula, high protein beverages, and bars. Whey protein concentrates (35-80% protein) and whey protein isolates (>90% protein) are produced by partially removing lactose, minerals and other non-protein components. In addition to producing protein-rich powders, the dairy industry applies some processes/technologies to modify physical and functional characteristics of whey-based powders. For example, enzymatic hydrolysis is used to improve the functional and nutritional characteristics of whey protein powders. The functional and nutritional characteristics of hydrolyzed powders vary according to the type and specificity of the enzyme used in the hydrolysis. The improved functionality of hydrolyzed whey protein is an area of interest. Palmer et al. (2016) showed that the hydrolysate worked as an excellent binder in whey protein isolate agglomeration process and it is considered as a substitute for soy lecithin. Therefore, a clean label “lecithin-free” of high-protein dairy ingredients can be provided, which decreases consumers concerns about allergens contents.

High-protein dairy ingredients should have a high solubility and good reconstitution properties. Agglomeration is the most commonly used technique to improve the rehydration properties and other functional and physical characteristics. Palmer et al. (2016) showed the effectiveness of using whey protein hydrolysate as a binder in wet agglomeration process of whey protein isolate to replace the conventionally used lecithin. However, more studies are needed to have a better understanding of physical, chemical, and functional properties of whey

protein hydrolysate. In addition, more investigation is required to optimize the agglomeration process conditions and study their effects on WPI agglomerated powder characteristics.

Chapter 2 gives an overview of whey proteins, application and functionality of whey proteins, enzymatic hydrolysis processes and the use of the hydrolysate. Chapter 3 outlines the research objectives. Chapter 4 is focused on the physical and chemical characterization of whey protein hydrolysate and its use as a binding agent in whey protein isolate agglomeration process. Whereas, chapter 5 is focused on investigating the effect of whey protein hydrolysate as a binder on the morphology of agglomerated whey protein isolate.

References

Palmer, N.J., B.L. Petersen, and L.S. Ward. 2018. Agglomerated protein products and method for making. US Pat. No. US20180070624A1.

Chapter 2 - Literature Review

Milk is produced in the mammary glands of all mammals. Milk composition is highly dependent on its source (cow, sheep, etc.), milking season, genetic makeup, feed of producing animal, and stage of lactation. Whey is a soluble fraction in milk. It is a co-product of cheese making process. Whey compositions are also affected by the same factors that changes the milk compositions. In addition, the type of cheese that is produced and the enzyme that is used are also contributing factors affecting whey composition. Whey retains about 55% of milk nutrients and contain approximately 93% water, 5% lactose, 0.8-1% whey protein, and 0.7 % minerals such as calcium, phosphates and chlorides (Tsakali et al., 2010). Two different types of whey can be attained depending on the type of the product produced which are sweet and acid whey. For example, sweet whey can be obtained by manufacturing rennet type cheese such as cheddar. While acid whey can be obtained from cottage cheese or Greek yogurt. Sweet whey contains about 6.0-10 g/L protein, while acid whey has approximately 6.0-8.0 g/L as shown in Table 2.1 (Božanić et al., 2014).

Table 2.1. Sweet and acid whey compositions (g/100g)

Whey	Water	Lactose	Proteins	Fat	Minerals
Sweet	3-6	70-74	12-13	0.8-1.5	7.5-8.5
Acid	≤3.5	65-69	9-12	0.8	11-12

Composition adapted from Bansal and Bhandari, (2016).

Whey protein compositions

The major components of whey proteins (WPs) are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), blood serum albumin (BSA), and immunoglobulins (Ig) as shown in Table 2.2. β-Lg has a secondary structure that is made up of about 15% α-helix, 50% β-sheet, and 15–20% reverse turn as was first noted by Creamer et al. (1981). The molecular weight of β-LG is

18.3 kDa, and it contains 162 AA (Hambling et al., 1992). β-LG is the most abundant protein in whey at 40 to 55% of total whey proteins and it is a rich source of all amino acids such as Leucine, lysine, and glutamic acid (Etzel, 2004). β-Lg exhibit a high affinity binding site of retinols (Fugate and Song, 1980). α-LA is a smaller protein (14 kDa) compared to β-LG and consist of 123 AA. It is a calcium metal protein (Hiraoka et al., 1980) which has an important function in the mammary secretory cells. It is one of the two components of lactase synthase that play a role in lactose synthesis in milk produced in the mammary gland. Acharya et al. (1991) presented the structure of α-LA; it consists of two domains: a large α-helical and a small β-sheet domain. The two domains are held together and stabilized by four disulfide bridges at four different locations (6–120, 61–77, 73–91, and 28–111), (Permyakov and Berliner, 2000). Ig is the largest component in WPs (15-1000 kDa). Ig in whey has an antimicrobial and antifungal activities (Madureira et al., 2007). BSA is not synthesized in the mammary gland, it follows the passive leakage from the blood stream, and it has a vital role in maintaining the osmotic pressure and pH in blood (Huang et al., 2004). BSA has a three dimensional structure that plays a significant role in making it a transporter for many endogenous and exogenous compounds such as fatty acids, metals, and amino acids (Carter et al., 1989). In conclusion, all whey components have unique structures and physiochemical properties that make it a highly nutritious and a functional ingredient in food products.

Table 2.2. The compositions and physiochemical properties of proteins in whey

Protein	Molecular weight (Da)	Isoelectric point	Concentration (g/L)	Proportion of total whey (%)
β-lactoglobulin	18,300	5.35 to 5.46	3.0	40 to 55
α-lactalbumin	14,000	4.2 to 4.5	1.2	11 to 20
Immunoglobulins	15,000-1,000,000	5.5 to 8.3	0.6	8 to 11
Blood serum albumin	69,000	5.13	0.3	4 to 12
Lactoferrin	77,000	7.8 to 8.0	0.1	1
Lactoperoxidase	77,500	9.2 to 9.9	0.002	1

Adapted from Goodall et al. (2008).

The functional and physiological benefits of whey proteins

WPs have various biological functions that are well studied by scientists. Whey protein isolate (WPI) contains about 14g of Leucine per 100 g of protein. It was reported that the branched-chain amino acids, especially leucine play numerous metabolic roles that function in proportion with cellular concentration such as maintenance of glucose homeostasis (Layman, 2003). It was also observed that WPI supplementation had increased the eccentric strength after training in young but not in older adults. However, a molecular evidence was provided that WPI supplementation enhanced the activation of translation initiation with combining WPI intake and chronic resistance training in older participants (Farnfield et al., 2005). In another study, it was reported that WPs played a significant role as an antioxidant in the cells. For example, sheep WP exhibited scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), diammonium (ABTS+) and hydroxyl (OH) radicals. Whereas, Bumrungpert et al. (2018) discovered that WP supplementation improved the nutritional status in cancer patients by increasing the albumin level. Moreover, their data indicated that WPs supplementation increased glutathione levels, and immune function in cancer patients during chemotherapy.

Besides all of the biological benefits, WPs are considered a highly functional ingredient in food products due to their unique physiochemical properties such as solubility, foaming capacity, emulsifying capacity, water holding capacity, and gelation (Foegeding et al., 2002). WPs are used as ingredients to enhance functional characteristics in the final products (Table 2.3). for example, WPs improve baking quality and texture, enrich high protein beverages, increase cheese yield, can be used as an emulsifier and texturizer in the dips, and as a stabilizer in yogurt (Yadav et al., 2015). As proposed previously, WPs have remarkable biological

functions and physiochemical properties, which directed the dairy industry's attention towards using concentration and fractionation techniques to increase the value of whey.

Table 2.3. Functions and applications of dairy proteins in food products

Function	Benefit	Application
Browning/color	Accentuates color development during cooking and baking. Enhances the color of viscous products such as sauces, soups. Improves opacity in low fat foods.	Baked goods, confections, recombined milk, nutritional beverages, sauces, soups, salad dressings.
Flavor enhancement	Provides baked flavor during baking and heating. Provides creamy dairy notes.	Meat and same as above.
Emulsification	Prevents fat globules from forming lumps. Improves product appearance.	Same as above.
Gelling and heat setting	Improves mouthfeel, helps provide the creamy, smooth texture of fat important for low-fat products.	Confections, recombined milk, meat, prepared foods.
Solubility	Some milk powders disperse well in food systems. Prevents sedimentation in beverages, soups, and sauces.	Bakery, beverages, confections, frozen desserts, infant formula, soup and sauces, yogurt.
Water binding and viscosity building	Provides fat-like attributes in products. Allows a reduction in fat content. Improves product texture.	Baked goods, confections, recombined milk, nutritional beverages, prepared foods, sauces, soups.
Whipping, foaming, and aeration	Maintains foam properties which enhance visual appeal as well as taste and texture.	Baked products, confections, recombined milk, nutritional beverages.

Adapted from Sharma et al. (2012).

Nature of WPs (cheese vs. native whey)

WPs can be separated from liquid whey that is a co-product in cheese manufacturing. Whey can also be separated from skim milk using microfiltration (MF) and the resultant whey is called "serum proteins" (Nelson and Barbano, 2005a). Because of a lack of familiarity with the term serum proteins, these proteins were also referred as "native whey" (Maubois, 2002). There is some processing, compositional and functional differences between cheese and native whey.

For example, native whey does not contain any added rennet, thermoduric bacteria or dead cells with their enzymatic activates or bacteriophages (Maubois, 2002). In addition, native whey doesn't contain any glycomacropeptides (GMPs) that are peptide fragments produced by enzymatic cleavage of k-casein by chymosin, which makes it a good ingredient in mother's milk substitute (Heino et al., 2007). From a Processing perspective, native whey can be separated form milk by a two stage MF process then ultrafiltration (UF), while cheese whey goes through UF only. In addition, native whey is more resistant to enzymatic hydrolysis due to the globular structure of β-LG that makes the cleavage sites unavailable to the enzymes (Kim et al., 2007). They observed that hydrolysis was greater in heated whey protein concentrate (WPC) than it is in native WPC at all incubation times. In terms of the functional properties, Heino et al. (2007) reported that the gel strength, emulsifying capacity and foaming properties of native WPC were significantly better compared to traditional WPC. The state of the protein native or denatured also affects protein solubility, besides other environmental factor such as processing temperature and pH (Pelegrine and Gasparetto, 2005).

WPC and WPI production

The dairy industry processes whey into different WPCs (25-80% proteins) and WPI (>90% protein). The basic steps in manufacturing these products are whey pretreatment (fat separation, pasteurization), UF/ diafiltration, concentration by vacuum evaporation and spray drying (Abd El-Salam et al., 2009). Because WPI contains at least 90% protein, it requires more separation steps by either ion exchange chromatography or microfiltration. The processing steps of WPI are shown in Figure 2.1 below.

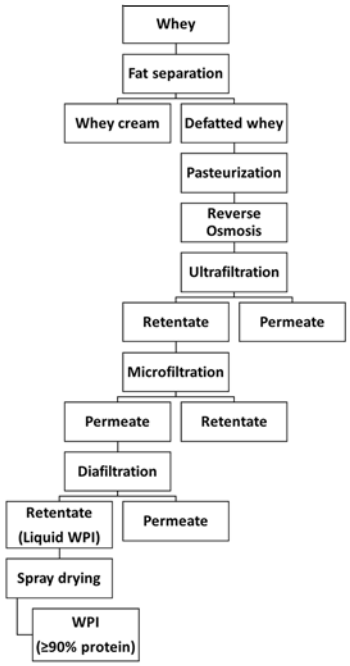

Figure 2.1. The typical processing steps of WPI form cheese whey in dairy industry

Processing challenges of WPs

WPs are globular in structure, which makes it relatively heat liable. The exposure of WPs to high temperatures can cause protein denaturation, aggregation, and gelation. The thermal transition of WPs were estimated by (Rüegg et al., 1977) using the differential scanning calorimetry (DSC). They found that the denaturation temperatures near neutral pH were about 73, 65, 62°C for β-LG, α-LA, and BSA, respectively. In addition, there was no detectable renaturation of β-LG and BSA, but about 80-90% of α-LA was renatured. β-Lg is the primary protein in whey, it contributes greatly in whey thermal sensitivity. When β-Lg is heated at a temperature in between 50 to 80°C, denaturation starts occurring which alters the tertiary structure of β-Lg and increases the reactivity of the thiol group that results in protein aggregation (Cayot and Lorient, 2017). The thiol group in the modified monomer can induce thiol/ disulfide exchange reactions which leads to the formation of disulfide-linked aggregates (Hoffmann and van Mil, 1997). The thiol/disulfide exchange reactions that leads to the formation of

intermolecular disulfide bonds is responsible of the heat induced aggregation and gelation of β-Lg (Sawyer, 1968). The behavior and nature of those aggregates are dependent on heating temperature, pH, protein concentration, and ionic strength. Gelation properties of WPI were significantly improved at neutral pH by limited hydrolysis with *Bacillus licheniformis* (BLP) protease (Ju et al., 1995). Prior to heating, a soft gel was formed by BLP hydrolyzed WPI, which confirms that limited hydrolysis is one way to increase thermal stability by altering heat-induced gelation (Otte et al., 1996, Foegeding et al., 2002).

Hydrolysis

The dairy industry is applying different approaches such as physical, chemical, and enzymatic hydrolysis to modify whey protein functionality. Proteases used in industrial and laboratory-scale production of WPH originated mainly from animal, plant, and microbial sources. Examples include fungal protease and papain (Sinha et al., 2007); trypsin and pepsin (Kim et al., 2007); chymotrypsin and thermolysin (Damodaran and Li, 2017); *Bacillus licheniformis* (BLP) protease (Creusot and Gruppen, 2008); alcalase (Wu et al., 2018); pancreatin (Silvestre et al., 2012). The functional and nutritional characteristics of hydrolyzed powders vary according to the type and specificity of the enzyme used in the hydrolysis. Additionally, hydrolysis conditions such as temperature, pH and enzyme to substrate ratio also influence the resultant peptides and degree of hydrolysis (DH). A low DH of less than 10% is required in order to improve the physiochemical properties of whey proteins. Whereas, a DH (>10%) is better in the purpose of improving the biological functions such as antimicrobial, antihypertensive and immunomodulatory functions of the resultant peptides (Agyei et al., 2016; Dullius et al., 2018). The use of only one enzyme can give the desirable outcomes. However, a mixture of different enzymes can also be useful in producing a hydrolysate with desired

functional or physiological properties. For example, Torkova et al.(2017) demonstrated the use of multi-enzyme cocktail in obtaining hydrolysates with acceptable organoleptic properties and predetermined antioxidant (400–500 μM TE/g protein) and antihypertensive (IC50 537–2500 mg protein/L) activities. Many studies were reported on finding the optimal conditions of hydrolysis, but we can conclude that more studies are needed to embrace as many enzymes and potential functional improvements as possible without any increase in production cost or decrease in product quality and stability.

Methods of hydrolysis

A wide range of enzymes can be utilized for production of WPH. The soluble enzyme is usually used for batch processes, while the immobilized form is utilized in continuous operations (Haider and Husain, 2009). Batch reaction is the most commonly used method in WPH production. It is based on incubating the substrate and proteolytic enzyme at high temperature between 40-60ºC. The DH is monitored using pH stat, when the desired DH reached, the reaction is stopped by using specific enzyme inhibitors (Kuipers et al., 2007) or using a suitable combination of pH, temperature and time. There are several challenges involved in using batch reactors including high operating costs (Mannheim and Cheryan, 1990).

Continuous reactors have the ability to separate products from the reaction media in order to increase the yield. The soluble enzyme is confined in the retentate side of the membrane, where it is in contact with the substrate (which is retained as well), while the product is small enough to permeate through the membrane. Although, batch reactor is more commonly used, higher processing efficiency can be obtained in continuous membrane reactors. At least 50% of enzyme can be saved using the membrane reactor in comparison with batch hydrolysis and the resultant WPH does not contain residual enzyme and unhydrolyzed proteins (Mišún et al., 2008).

Prieto et al. (2008) observed that the temperature of hydrolysis affects the amount of enzyme used in the process. They found that the optimal operation temperature of 60ºC resulted in lower enzyme consumption. In order to attain the desired functional and nutritional properties in the hydrolysate, we have to choose the optimal hydrolysis conditions, such as temperature, pH, enzyme concentration, type and specificity of proteolytic enzyme, DH and enzyme to substrate ratio. Furthermore, choosing the best hydrolysis method is critical and plays a significant role in reducing the cost, maximizing the hydrolysis efficiency and delivering a product with expected functionality and bioactivity.

Applications of the hydrolysate

WPH can be used as an ingredient in food products for their unique functional properties in sustaining food quality and stability. For example, WPH is widely used as an emulsifier and stabilizer in the food industry. It was noted by Christiansen et al. (2004) that the controlled enzymatic hydrolysis can produce peptides that enhance the role of WPs as an emulsifier and a stabilizer in making high-pressured processed dressings. In addition, WP peptides are considered a potential emulsifier in food nano-emulsions (Adjonu et al., 2014). Therefore, hydrolysis generates peptides with improved emulsifying capacity and stability compared with WPs. Lieske and Konrad (1996) showed that the limited hydrolysis (DH=3.0%) was more effective in improving the physio-functional properties (foaming and emulsifying) than the higher ones.

Additionally, hydrolysis improves the solubility of WPs. The solubility of the hydrolysate was investigated by Severin and Xia (2006). They noted that the solubility increases with the higher DH due to the increase in the ionizable groups (NH_4^+, COO^-) number with concomitant increase in hydrophilicity and net charge of the resulting hydrolysates, promoting

hydrolysate water interactions and enhancing their solubility. The enhanced solubility of the hydrolysate was also caused by their smaller molecular size.

WPH can be used as an ingredient in high protein bars while maintaining high quality and stability during storage. McMahon et al. (2009) reported that high protein bars made by WPH were softer than the WPI ones. Hydrolysis degrades WPs into smaller peptides that are easier and faster to be digested and absorbed in the intestines than free amino acids (Boza et al., 2000). Those peptides can function as an antimicrobial, antioxidant, antihypertensive or immunomodulatory (Dullius et al., 2018). It was reported by Mann et al. (2014) that WPH can be used as natural biofunctional ingredients in enhancing antioxidant activity in milk drinks. In recent research, it was observed that the controlled enzymatically-hydrolyzed WP can more directly influence the immune system (Parker et al., 2020). In addition, Silvestre et al. (2012) was able to identify some bioactive peptides that has an effective ACE inhibitory activity. Moreover, Farup et al. (2016) noted that plasma amino acid appearance of WPH samples was higher than casein. Although, the hydrolyzed samples were highly digestible, the biological value in these samples decreased with the increase of the degree of hydrolysis. Therefore, it's important to have a controlled hydrolysis in order to sustain the biological value of WPs.

β-LG can induce an allergic reaction in human infants because of the absence β-LG in human milk (Zeiger et al., 1986). Therefore, enzymatic hydrolysis of whey proteins reduces its antigenicity (Kim et al., 2007), which makes WPH a suitable ingredient in infant formula (Exl, 2001). WPH can also be used as an encapsulant for hydrophobic and hydrophilic components as well as probiotics (Augustin and Oliver, 2014). Another application of WPH was introduced by Palmer et al. (2016), as they used the hydrolysate as a binder in WPI agglomeration process. In conclusion, enzymatic hydrolysis is a very beneficial strategy in producing bioactive peptides.

However, the functional and nutritional characteristics of hydrolyzed powders vary according to the type of protein and extent of denaturation, degree of hydrolysis, conditions of hydrolysis (pH, temperature, enzyme concentration), and type and specificity of enzyme used in the hydrolysis.

The challenges of WP hydrolysis

Bitter taste is one of the biggest issues in WP hydrolysate. It occurs in the hydrolysate due to the resultant small peptides. The intensity of the bitterness in WPH increases with the increase of the DH and the higher concentration of low molecular weight peptides (Leksrisompong et al., 2010). There are many factors that affect developing the bitterness in the hydrolysate such as degree of hydrolysis, type of enzyme used and enzymes mixture, processing time and source of protein. It is important to note that there are some techniques that can be followed to debitter the resultant hydrolysate such as the absorption of bitter peptides on activated carbon, chromatographic removal using different matrices and selective extraction with alcohols (FitzGerald and O'Cuinn, 2006). However, this technique causes loss of some amino acid's residues from hydrolysate. Another technique can be applied in order to reduce bitterness is using exopeptidases (Raksakulthai and Haard, 2003). The specificity of those enzymes and reaction conditions can highly affect their efficiency in reducing the hydrolysate bitterness. One more approach for debittering is adding other flavors that masks the bitterness such as sweeteners or polysaccharides. The bitterness is not the only concern in manufacturing the hydrolysate. The loss of protein bioavailability is the other issue faced while making the hydrolysate. Farup et al. (2016) noted that the biological value in the hydrolyzed samples decreased with the increase of the degree of hydrolysis. It is very crucial to determine the purpose of hydrolysis first, in order to produce a hydrolysate with proper characteristics.

Rehydration of high dairy powders and agglomeration

Drying is an important process in food products as it increases products shelf life, lowers transportation and storage costs. Rehydration is the most essential characteristics of powders as it is the first step towards using it in other food applications. Rehydration consists of steps which are; wetting and swelling, sinking, dispersion and dissolution as shown in Figure 2.2.

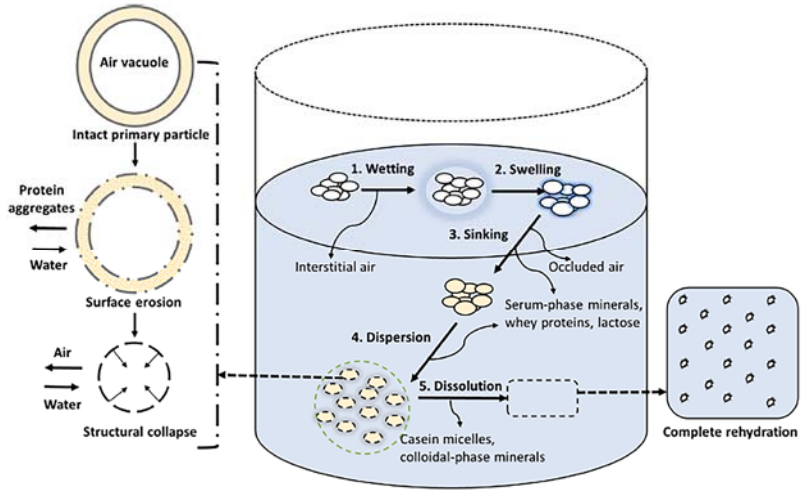

Figure 2.2. A schematic illustration of rehydration mechanism for an agglomerated high protein dairy powder (adapted from Crowley et al., 2016)

Fang et al. (2007) had defined each rehydration phases as the following; wettability is the ability of powder particles to overcome the surface tension between themselves and water. After the powder has been wetted, the gaseous phase surrounding each particle is gradually replaced by the aqueous phase as the particles began to sink (sinkability). Once the agglomerated particles are wetted and have sunk, they would immediately start to disperse uniformly as individual particles, while the agglomerates cease to exist (dispersion). Solubility is the final step of powder dissolution and is considered as the key determinant of the overall reconstitution quality (dissolution). It is important to note that these different phases often overlap as shown in Figure 1.2.

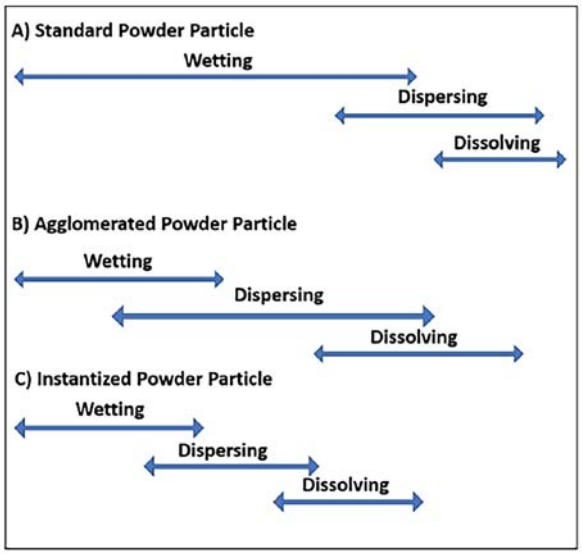

Figure 2.3. A schematic diagram of dissolution timeline for different types of food powder showing the overlaps between different phases with time (adapted from Fang et al., 2007)

Solubility of proteins based powders can be influenced by many factors such as pH and temperature of the solution, state of protein, the surface composition of the powders, degree of agglomeration and the structure of agglomerates (Chen and Özkan, 2007). Agglomeration technologies are commonly used by the food industry to enlarge the particles size that leads to optimize several properties such as density and flowability and also to shorten the reconstitution time of commercial powders (Forny et al., 2011). Agglomeration is also called "Granulation" and it consists of three sets of phases: wetting and nucleation, consolidation and growth, and breakage and attrition.

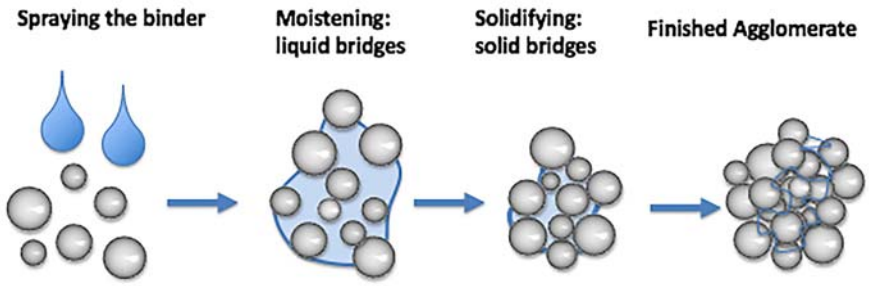

Figure 2.4. A schematic illustration of wet agglomerations process

From industrial perspectives, powders should flow freely and rehydrates easily. Some of the desired properties of granulated products include reduced dustiness which minimizes losses, inhalation and explosion risks, improved flow and handling which facilitates controlled metering; controlled dissolution rates; and the co-mixing of particles which would otherwise segregate during handling. On the other hand, improper granulation causes problems in down-stream processes such as caking, segregation and poor tableting performance (Iveson et al., 2001). In industrial practice, agglomeration of spray-dried powders is performed either outside the drying chamber in a fluid bed where the particle surface is wetted with sprayed water (or another binder solution), or by recycling dry fines (particles of a diameter smaller than 100 μm) into the drying chamber (Gianfrancesco et al., 2008). Soy lecithin is the most commonly used binder in agglomerating WPs (Rogers, 2011). However, it was reported that the consumption of soy lecithin on daily basis had some health side effects. In addition, consumers demand of having dairy products with a "clean label" had increased the need of lecithin-free agglomerated powder. Therefore, finding a soy lecithin substitute is an area of study.

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Chapter 3 - Research Objectives

This study was performed to have a better understanding of physical, chemical and functional properties of commercial whey protein hydrolysate that was successfully used as binder in WPI agglomeration process. The second objective of this study was to optimize the agglomeration process parameters and characterize the physical and functional properties of the resultant agglomerated WPI. WPI agglomeration was carried out in a top-spray fluid bed granulator (Midi-Glatt, Germany). Agglomerated WPI samples were stored at 25°C and analyzed for moisture, water activity, relative dissolution index (RDI), particle size and shape, color, microstructure, bulk and tapped densities. and emulsifying capacity.

Chapter 4 - Characterization of a commercial whey protein hydrolysate and its use as a binding agent in whey protein isolate agglomeration process

Abstract

Enzymatic hydrolysis is used to improve the functional characteristics of whey proteins. The type and specificity of the enzyme influence the properties of the resultant hydrolysate. In a recent disclosure of invention, the whey protein hydrolysate (WPH) was utilized as a binder to facilitate the agglomeration of whey protein isolate (WPI). The first objective was to characterize the chemical properties of three lots of WPH obtained from a commercial manufacturer. The degree of hydrolysis (DH) of WPH was between 13.82 and 15.35% and not significantly ($P>0.05$) different between the lots. From Matrix Assisted Laser Desorption/Ionization- Time of Flight (MALDI-TOF), 10 to 13 and different peptides were observed in the range of 2.5 – 5 kDa and 5 – 8 kDa, respectively. Additionally, it was observed from the High-performance liquid chromatography (HPLC) that the major whey proteins were completely hydrolyzed indicating a consistent hydrolysis between the lots. The second objective of the study was to evaluate the effectiveness of WPH as a binder in WPI wet agglomeration. For this purpose, a 3×3×2 factorial design was conducted with pre-wet mass (60, 100, and 140 g), WPH concentration (15, 20, and 25%), and flow rate (4.0 and 5.6 $mL.min^{-1}$) as independent variables. WPI agglomeration was carried out in a top-spray fluid bed granulator (Midi-Glatt, Germany). All the experiments were performed in triplicates using three lots of WPH. All data was analyzed based on the Randomized Complete Block Design (RCBD) using PROC GLIMMIX in SAS Studio (version

9.4; SAS Inst., Cary, NC). Agglomerated WPI samples were stored at 25°C and analyzed for moisture, water activity, relative dissolution index (RDI), and emulsifying capacity.

Moisture content (MC) of agglomerated samples was in the range of 3 – 15%, whereas water activity was within the range of 0.08 – 0.80. There was a significant ($P<0.05$) difference in both of moisture content and water activity among the treatments. Per-wet mass, flow rate and the WPH concentration had significant ($P<0.05$) effect on the MC. Moreover, all interactions among the main effects had also a significant ($P<0.05$) effect on MC. High MC and water activity were observed for the treatments with higher pre-wet volume and higher flow rate and also resulted in clumping of the powders. The treatment that has 60 g of pre-wet, 20% WPH concentration and 5.6 $mL.min^{-1}$ flow rate combination had the highest RDI among all the samples. In conclusion, WPH can be used as a potential alternate to soy lecithin in WPI wet agglomeration.

Keywords: Agglomeration, Whey, Hydrolysate

Introduction

Whey is a co-product obtained during manufacturing of cheese. It was considered as a waste for decades until research studies showed its functionality and nutritional value. Sweet whey contains about 6.0 to 10 g/L proteins (Božanić et al., 2014). The major proteins in whey include β-lactoglobulin (β-LG), α-lactalbumin (α-LA), blood serum albumin (BSA), and immunoglobulins (Ig) (Goodall et al., 2008). Whey proteins (WPs) are highly soluble and have unique physio-chemical characteristics that influence its functionality in food applications such as gelation, emulsification, and foaming (Foegeding et al., 2002). This makes it a feasible ingredient in various food products. However, some approaches are performed to modify WPs functional and physiochemical properties such as chemical, physical and enzymatic treatments.

The most research on protein modification include Millard conjugation (Jiménez-Castaño et al. 2007); physical modification such as high pressure treatment (Bouaouina et al., 2006), heat-induced polymerization (thermal treatment) (Ryan et al., 2013); and enzymatic modifications (Kim et al., 2007). Modification can also be carried out by applying approaches such as enzymatic and physical treatments (Lozano-Ojalvo et al., 2017). Enzymatic hydrolysis of WPs is measured by degree of hydrolysis and it is defined as the percentage of peptides bonds cleaved (Adler-Nissen, 1979). A low DH (<10%) is sufficient for improving the physiochemical properties of WPs. Whereas, a DH (>10%) is more suitable for improving the biological functions of the resultant peptides such as antimicrobial, antioxidant, antihypertensive, and immunomodulatory functions (Agyei et al., 2016; Dullius et al., 2018).

Enzymatic hydrolysis of WPs was extensively studied (Ismail and Gu, 2010). It was proven that hydrolysis improves WPs digestibility and nutritional value and reduces

allgergenicity (Agyei et al., 2016), which makes it a suitable ingredient in infant formula (Exl, 2001). Hydrolysis also improves the solubility (Severin and Xia, 2006) and the emulsifying capacity at alkaline pH of the whey hydrolysate (Chobert et al., 1988), which makes it a desirable ingredient in high-protein beverages and bars. Moreover, the hydrolysate can be used as an encapsulant for protection and delivery of bioactive components such as Omega-3 oil and probiotic organisms (Augustin and Oliver, 2014). In a recent disclosure of invention, Palmer et al. (2018) found that the hydrolysate worked as an excellent binder in WPI agglomeration process as an alternative to soy lecithin.

Agglomeration improves the reconstitution properties of the powders due to incorporation of air between powder particles, which makes the water penetration into these particles easier during subsequent rehydration. Therefore, the agglomerates readily disperse and dissolve quickly (Dhanalakshmi et al., 2011; Carić, 1994) compared to non-agglomerated powders. More studies are needed to have a better understanding of the physical and chemical properties of this whey protein hydrolysate. In addition, the agglomeration process conditions need to be optimized and their effects on the resultant powder properties studied. Therefore, the first objective of this study is to characterize the physical and chemical properties of three lots of a commercial WPH. Subsequently, optimizing and evaluating the effectiveness of WPH as a binder in WPI wet agglomeration were investigated as a second objective.

Materials and methods

Experimental design

Three lots of WPH and one lot of WPI were obtained from a commercial manufacturer (Glanbia Nutritionals, Twin falls, ID). The lots were randomly selected and were manufactured on March, April and May of 2018. Initially, chemical and physical properties of WPH and WPI

were analyzed in terms of peptides characterization, degree of hydrolysis, zeta potential, color, bulk and tapped densities in order to evaluate the consistency of the enzymatic hydrolysis. After determining the similarities and differences among WPH lots, the effectiveness of using WPH as a binder in WPI wet agglomeration was evaluated. For this purpose, a 3×3×2 factorial design was conducted with pre-wet mass (60, 100, and 140 g), WPH concentration (15, 20, and 25%), and flow rate (4.0 and 5.6 $mL.min^{-1}$) as independent variables. The other processing parameters such as the nozzle pressure, fluid bed pressure and fluid bed temperature were set at 0.65 bar (9.43 psi), 0.45 bar (6.53 psi) and 60°C, respectively.

The pre-wet mass represents weight of water (g) used in wetting the WPI powder as the first step of agglomeration. WPH concentration is the concentration (w/w) of the binder solution. Lastly, the flow rate is amount of water and binder solution pumped during agglomeration and expressed as $mL.min^{-1}$. WPI agglomeration was performed in a top-spray fluid bed granulator (Midi-Glatt, Binzen, Germany) as shown in Figure 4.1.

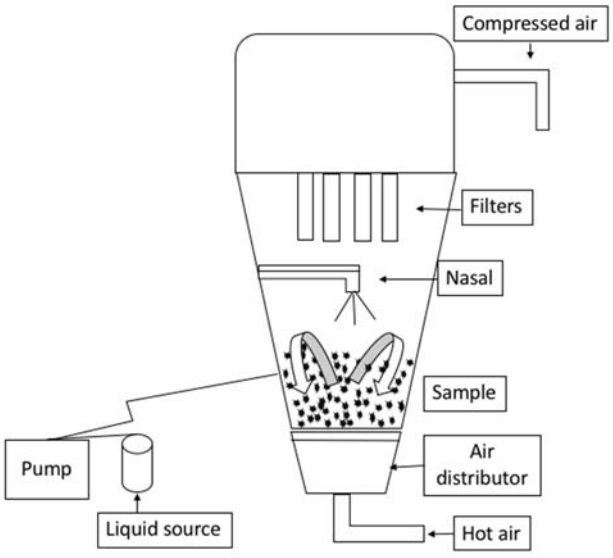

Figure 4.1. The top-spray fluid bed granulator (Midi-Glatt) that was used in agglomerating WPI powder

All the experiments were performed in triplicates using the three lots of WPH. Agglomeration was stopped when the temperature of the end powder reached 45°C. Agglomerated WPI samples were stored at 25°C and analyzed for moisture, water activity, relative dissolution index (RDI), and emulsifying capacity.

WPH and WPI chemical characterization

HPLC and MALDI-TOF mass spectrometry

About 1 g of WPH powders were dissolved in distilled deionized water and dialyzed using 2 kDa cut off cassettes (Slide-A-Lyzer® Dialysis Cassettes, Thermo Scientific, Waltham, MA). The dialyzed WPH solution was passed through 0.45 µm filter units (MF-Millipore™ membrane, Carrigtwohill Co., Ireland). Then, protein concentrations in dialyzed solution were determined by measuring the absorbance at 280 nm using a Cary 50 bio UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA). Then their concentrations were standardized by taking the number of tyrosine and tryptophan residues from α-LA, β-LG and BSA into account with their extension coefficient (Ninfa et al., 2010). Samples were stored at -18 ºC until use.

HPLC analysis was done based on the method described by Bonfatti et al.(2008) with some modifications. Separation of whey proteins was performed by RP-HPLC at 25°C with a Beckman Coulter- System Gold® (Beckman Coulter, Fullerton, CA), attached with 126 solvent module pump, 168 System Gold® detector and Beckman 32 Karat software data acquisition system. ZORBAX 300SB-C8 column, 2.1x150mm with 5 µm pore size was used in the analysis (Agilent Technologies Co. Englewood, CO). A 50 µL aliquot of 20 µM sample was injected into the column. Solvent A of the mobile phase was 0.1% trifluoroacetic acid (TFA, American Bioanalytical Co., Natick, MA) in 99.9% H_2O, and solvent B was 0.1% TFA, 90% acetonitrile (Fisher Scientific-HPLC grade) and 9.9% H_2O. The gradient of solvent B started at 10% for 5

min, then increased to 30% over 5 min. Subsequently, the gradient continuously increased to 80% over 30 min, then to 100% over 5 min. Lastly, the gradient was held at 100% of solvent B for 10 more min, and then brought back down to 10% to re-equilibrate for the next run. The flow rate was 0.5 mL/min. When the peak appeared on HPLC, samples were collected in Eppendorf tubes and the peptides were analyzed on MALDI-TOF. α-LA, β-LG and BSA standards were purchased from Sigma-Aldrich (St. Louis, MO). Detection was performed at wavelength of 168-220 nm.

MALDI-TOF (Ultraflex II MALDI-TOF/TOF, Burker, Germany) was used in analyzing the peptides from HPLC fractions (Sheoran et al., 2018). A 20 mg/mL of 2,5- Dihydroxybenzoic acid (DHB) matrix (Sigma Aldrich, St. Louis, MO) in a solution of 50%. 1% TFA in acetonitrile was used in spotting the samples for MS analysis.

Degree of hydrolysis (DH)

DH was measured using 2,4,6 trinitrobenzene sulfonic acid (TNBS) method as described by (Adler-Nissen, 1979). A 50 ml of 0.02% of protein solution was prepared by dissolving 1 of WPH powder in distilled water. Protein solutions were stirred for 30 min to ensure that the powder is fully dissolved. Then, a 1 g of protein solution was added to 9 g of 1% (w/w) sodium dodecyl sulfate (SDS) solution (Sigma Aldrich, St. Louis, MO). In test tubes, a 0.1 mL aliquot of protein solution, 2 mL of phosphate buffer (pH 8.2), and 2 mL of 0.1% (w/w) TNBS solution (Sigma Aldrich, St. Louis, MO) were added. A leucine standard linear curve was obtained by diluting 530 mg/L leucine standard (SigmaAldrich, St. Louis, MO) in 1% SDS (w/w) to obtain 42.4, 84.8, 127.2, 169.6, and 212 mg/L amino nitrogen leucine standards. All tubes were vortexed and placed in the water bath at 50 ºC for 60 min. The reaction was stopped by adding 4 mL of 0.1 N HCL (Chemicals, Gibbstown, NJ) to each tube. Standards and samples were

measured at 340 nm using UV/VIS spectrophotometer (Metash Instruments Inc., Shanghai, China) against 1% SDS blank solution. Leucine standard curve was plotted, then calculations were done accordingly.

WPH and WPI physical characterization

Water activity

Water activity of powders was measured at 25°C using an Aqua Lab Series 3 model TE instrument (Pullman, WA, USA). The measurements were performed in triplicates. Same procedures were followed in analyzing the water activity of agglomerated powders.

Mean particle size and zeta potential (ζ)

The particle size distribution and Zeta potential were measured using dynamic light scattering (DLS) (DelsaMax PRO, Beckman, Germany) for the reconstituted WPH or WPI solutions. Initially, 5 g of WPH or WPI was dissolved in 95 g of distilled water and stored in the fridge overnight for a complete dehydration. The 5% solutions were diluted 1:100 with distilled water and then slowly injected in the DLS flow cell. The measurements were performed in triplicates.

Bulk and tapped densities (g/ cm^3)

Bulk and tapped densities were measured by Hosokawa Micron PT-R powder tester (Hosokawa Micron Corp., Osaka, Japan). A 100 cm^3 cup, and 180 vertical taps were applied to measure tapped density. Standard steps were used in conducting both of the analysis. The measurements were performed in triplicates.

Color

The CIE LAB values L*, a*, and b* were measured using a Hunter-Lab Mini Scan colorimeter (Hunter Associates Laboratory, Reston, VA). About 2 g of powder was placed in a

transparent plastic container to take the measurement. The analysis was performed in triplicates. L* value indicates the whiteness/darkness of the sample, a* indicates the redness/greenness, and b* shows the yellowness/blueness of the powder.

The use of WPH as a binder in agglomerating WPI

A top-spray fluid bed granulator (Midi-Glatt; Glatt Process Technology, Binzen, Germany) was used, attached with a peristaltic pump (Model 1B.1003-R/65, Petro Gas, Germany) as shown in Figure 4.1. A 480 g of WPI was weighed in the agglomerator chamber. WPH solution was prepared at 25°C, and two drops of the SUPPRESSOR 3569 defomer (Hydrite Chemical Co. Brookfield, WI.) were added to prevent foam formation. Processing parameters such as pre-wet mass (60, 100, and 140 g), WPH concertation (15, 20, and 25%), and flow rate (4.0, 5.6 $mL.min^{-1}$) were set according to the factors-levels combination for each trial. The other processing parameters were fixed. The nozzle pressure, fluid bed pressure and fluid bed temperature were set at 9.43 psi (0.65 bar), 6.53 psi (0.45 bar), and 60°C, respectively. Distilled water was added as pre-wetting step, followed by inserting the WPH solution to the system. When 20 mL of the WPH solution remained, the fluid bed temperature was increased from 60 to 80°C. The process was stopped when the temperature of agglomerated powder reached 45°C. Subsequently, samples were collected in plastic containers (48 oz, PET square grip container, Container and Packaging Co., KY) and stored at 25°C. Samples were analyzed for moisture, water activity, solubility index, relative dissolution index, and emulsifying capacity. All the experiments were performed in triplicates using the three WPH lots.

Agglomerated WPI characterization

Moisture content (MC)

MC of agglomerated samples was determined using the direct forced oven following the AOAC official method 990.20 (AOAC, 2000). Aluminum pans were dried overnight at 102 ºC then placed in a desiccator to cool down. Two grams of powder was weighed in a pan and placed in the direct forced oven (Fisher Scientific Isotemp oven 737G, NJ) at 100ºC for 4 hrs. Analysis was performed in duplicates.

Relative dissolution index (RDI)

Dissolution characteristics of agglomerated WPI samples were evaluated using Focused Beam Reflectance Measurement (FBRM), following the method proposed by Hauser and Amamcharla (2016)with some modifications. In a 500- mL glass beaker, 5% (wt/wt) protein solutions of agglomerated WPI were prepared by dissolving powders in distilled water, while temperature was maintained at 25ºC. FBRM instrument is equipped with an overhead stirrer 4-blade impeller (Caframo, Georgian Bluffs, Ontario, Canada) that was rotating at 700 rpm during the powder addition (~40-45 sec), then it was set at 400 rpm for data collection. The dissolution test was held for 30 min, using the iC FBRM software (version 4.3.391, Mettler-Toledo AutoChem Inc., Columbia, MD) to gather the data. The software program enabled to collect the number of particles in the category of <10 μm chord length that is characterized as fine particles. Particles count was plotted against dissolution time. Subsequently, the area under the fine particles count curve was calculated to determine the dissolution using the trapezoidal rule. RDI of the agglomerated powders was determined using equation (1) below (Babu and Amamcharla, 2018):

$$\text{RDI (\%)} = \frac{\text{Area under the curve for the sample}}{\text{The highest area under the curve among agglomerated samples}} \times 100$$

Solubility index (SI)

Twenty g of the 5% (w/w) protein solutions that were prepared in the FBRM analysis were weighed into 50-ml centrifuge tubes. Samples were centrifuged at 700 × g for 10 min at 25°C in a Marathon 21000R centrifuge (Fisher Scientific, Pittsburgh, PA). Aluminum pans were dried overnight at 102ºC and left in a desiccator to cool down. SI was determined based on the total solid of the supernatant. Three grams of the supernatant were weighed in the pan then placed in the oven at 100ºC for 4 hrs. Final weights were recorded. Subsequently, the amount of soluble material (σ) was calculated following equation (2) below:

$$\sigma = \frac{Weight \text{ of dry material}}{\text{Weight of solution}} \times 100$$

Emulsifying capacity (EC)

EC of samples were determined following Webb et al. (1970) method. At room temperature, 120 mL of 0.05% protein solution was prepared in a 600 mL beaker. The conductivity meter electrode (Accumet™ AP75; Fisher Scientific, PA), and CAT Scientific X120 Handheld Homogenizer Drive (PolyScience, Niles, IL, USA) were placed in the beaker. With continuous blending, vegetable oil -that was purchased locally- addition started by opening the 125 mL separatory funnel valve all the way up. Blending process was started at slow rate (speed 1), then it was increased to speed 3 when the emulsion became more viscous. Oil addition was stopped when the emulsion breakpoint was detected. This point is defined as the sudden increase in electrical resistance of the dispersion that occurs upon emulsion collapse. Emulsification capacity was expressed as the total g of oil emulsified per (60 mg) of soluble protein Acton and Saffle (1972).

Statistical analysis

All data was analyzed based on the Randomized Complete Block Design (RCBD) using PROC GLIMMIX in SAS Studio (version 9.4; SAS Inst., Cary, NC) and Tukey's test to determine any significant differences between treatment levels, which were declared significant when $P \leq 0.05$. WPH lots were the blocking factor in our experimental design. In addition, PROC RSREG was used to get the response surface models for moisture content and relative dissolution index. The general model equation is $\hat{Y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + {}_+\beta_{11}(x_1)^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{22} (x_2)^2 + \beta_{23} x_2 x_3$; where $\beta_0, \beta_1, \beta_2, \beta_3, \beta_{11}, \beta_{22}, \beta_{12}, \beta_{23}, \beta_{13}$ are the constant coefficients, and x_1 is the pre-wet mass (g), x_2 is the WPH concentration (w/w), and x_3 is the flow rate ($mL.min^{-1}$).

Results and discussion

The chemical compositions of WPI and WPH lots were provided from the manufacturer (Glanbia Nutritionals, Twin Falls, ID). Moisture content of the WPI sample was 3.61%, whereas WPH moisture content was within the range of 2.67-2.99% (Table 4.1). WPI protein content was 94.02% and WPH protein content was within the range of 91.57-92.28%.

Table 4.1. Moisture content (%), protein content (%), and degree of hydrolysis of WPH and WPI samples

Samples	Moisture content (%)	Protein content (%)	Degree of hydrolysis (%)
WPH lot 1	2.99	91.96	14.80±0.35
WPH lot 2	2.67	91.57	15.35±0.64
WPH lot 3	2.88	92.28	13.82±0.39
WPI	3.61	94.02	-

[a-c] Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ± SD.

According to the American Dairy Products Institute (ADPI, 2002) standards for dry whey products, moisture content should not exceed 6% by wt and should contain at least 89.5% protein

on dry matter basis. Therefore, both of WPI and WPH samples met the ADPI requirements of dry whey products compositions.

WPH chemical characterization

HPLC and MALDI-TOF mass spectrometry

The standards of BSA, β-LG, and α-LA were identified on the HPLC and their peaks occurred at 26.3, 27.2, and 29.9 min, respectively. The major components of WPs (β-LG, α-LA, and BSA) were observed on the WPI-HPLC chromatogram. In addition, two unidentified peaks were also observed. However, none of those peaks were detected on the WPH samples chromatogram (Figure 4.2), which suggests the complete hydrolysis of these major whey proteins in the WPH lots.

This finding confirmed with MALDI-TOF results. The MW of BSA, β-LG and α-LA are about 69, 18.3, and 14 kDa, as it was noted by Goodall et al. (2008). The peptides on MALDI-TOF spectrum were classified as small (2.5-5 kDa), medium (6-10 kDa) and large (>10 kDa). In WPI sample, about 25% of the peptides that found are small peptides and same for the medium size ones. The remaining 50% of the peptides were classified as large peptides. These proteins had a MW of 18.3 and 14 kDa, which are the β-LG, and α-LA WPs. In WPH samples, about 75% of the peptides that were observed classified as small peptides and 25% as medium peptides. In addition, no peptides were observed above10 kDa, which confirms the HPLC findings that the major whey proteins were completely hydrolyzed.

Moreover, the largest MW of peptide observed in all WPH samples was 8 kDa. These results were similar to the findings that reported by Adjonu et al. (2014), all peptides were <8 kDa and 52% of the obtained peptides were in the range of 2.1-8.0 kDa when chymotrypsin was used as an enzyme after 24 h of hydrolysis.

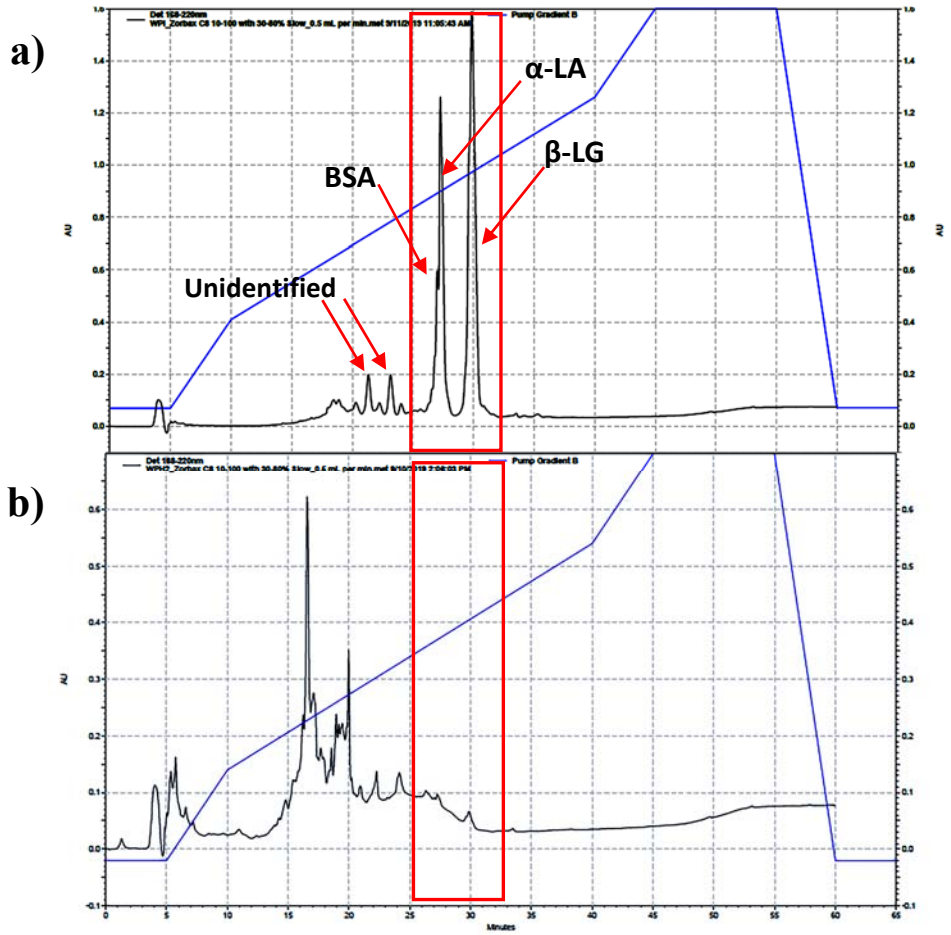

Figure 4.2. a) The RP-HPLC chromatogram of WPI, shows the major WPs in the unhydrolyzed sample. **b)** The RP-HPLC chromatogram of WPH. No peaks were observed in the times of 26.3, 27.2, 29.9 min indicating a complete hydrolysis of the major WPs in WPH samples.

The HPLC chromatograms for all WPH lots were similar. No peaks were observed in the times of 26.3, 27.2, and 29.9 min indicating a complete hydrolysis of the major WPs in WPH samples. This finding was confirmed with MALDI-TOF results as the major WPs peptides were detected in WPI sample but not in WPH samples. Both HPLC and MALDI-TOF results showed that the major WPs were completely hydrolyzed indicating a consistent hydrolysis.

Degree of hydrolysis (DH)

It was noted by Spellman et al. (2003) that TNBS method is the most suitable for quantifying the DH in WPH compared with the *o*-phthaldialdehyde (OPA) and pH-stat methods. TNBS reacts with amino groups forming a chromophore with a maximum absorbance at 340 nm

(Adler-Nissen, 1979). The DH of WPH samples was between 13.82 and 15.35% and it was not significantly (P>0.05) different between the lots (Table 4.1). Limited hydrolysis improves the physio-functional properties of the hydrolysate, as it was concluded from Lieske and Konrad (1996) study that the optimal DH is 3% to improve the foaming and emulsifying capacity. Partial hydrolysis improved the thermal stability of WPH due to the loss of the secondary structure (Foegeding et al., 2002). Moreover, a DH (>10%) is more suitable for improving the biological functions of the resultant peptides such as antimicrobial, antioxidant, antihypertensive and immunomodulatory functions (Agyei et al., 2016; Dullius et al., 2018).Adjonu et al. (2014) had used the OPA method in measuring the DH and reported that WPs hydrolysis had significantly increased the biological functions of WPs such as oxygen radical absorbance capacity and ACE-inhibition activity. However, extended hydrolysis (up to 24 hrs) had no significant effect on the DH and the molecular weight profiles (P > 0.05) but in some instances caused a reduction in the antioxidant activity of WPI hydrolysates.

WPH and WPI physical characterization

Water activity

Water activity of WPH samples was within the range of 0.16-0.21 (Table 4.2). The water activity of WPH samples was higher than WPI sample due to the differences in the chemical compositions such as proteins and moisture content. For example, WPI contained 94.02% proteins compared with about 91.57% in lot 2 of WPH. The higher protein content resulted in a lower water activity. There was a significant difference (P<0.05) between lot 2 and 3 samples.

Mean particle size and zeta potential (ζ)

Mean particle size and zeta potential were measured for the rehydrated samples. The mean particle size was within the range of 150.67-198.93 μm, and it was significantly different

(P<0.05) among the three WPH lots (Table 4.2). The mean particle size of WPH samples was bigger than the WPI due to the aggregation behavior of the hydrolysate. The hydrolysate may cause aggregation, but not necessarily induce gelation as Spellman et al. (2005) noted.

Zeta potential is an indicator of the surface charges of the particles and its stability to aggregation. The mean of zeta potential was ranged from -22.88 to -24.04 mV, and there was no significant difference (P>0.05) among the WPH lots (Table 4.2). Zeta potential of the hydrolysate samples was higher than the intact WPI. This is due to the increase of net charge on protein hydrolysate (Mahmoud et al., 1992). The increase of negative charges might be due to the increase in the number of hydrophobic amino acid side chains after enzymatic hydrolysis (Duan et al., 2014). In addition, as the absolute value of zeta potential increases, the stability of dispersion increases (Wu et al., 2014). Both egg yolk and whey proteins are globular proteins, which explains why both had similar results of zeta potential when hydrolyzed.

Table 4.2. Water activity, mean particle size, and zeta potential of WPH and WPI samples

Samples	Water activity	Mean particle size (nm)	Zeta potential (mV)
WPH lot 1	0.20 ± 0.02^{ab}	181.23 ± 0.60^{b}	-23.86±2.03
WPH lot 2	0.21 ± 0.01^{a}	150.67 ± 3.65^{c}	-24.04±1.40
WPH lot 3	0.16 ± 0.00^{b}	198.93 ± 9.77^{a}	-22.88±1.16
WPI	0.12±0.01	112.17±14.40	-19.59±1.58

[a-c] Means with different superscripts are significantly different (P<0.05).
All values are expressed as mean ± SD. N=3

Bulk and tapped densities

The average of bulk density values of WPH samples were within the range of 0.31- 0.33 g/cm^3 (Table 4.3). Additionally, the mean of tapped density was in the range of 0.42-0.45 g/cm^3. WPH powders were statistically different (P<0.05) in both bulk and tapped densities. However, these values are pretty similar practically. WPH samples had slightly higher bulk and tapped densities than the intact WPI densities, this is due to the lower volume of occluded air in WPH samples (Kelly et al., 2016a).

Color

The mean of L*, a*, and b* values were used to evaluate the differences in color intensity among the WPI and WPH samples. The average of L* was 92.15, and there was no significant difference ($P>0.05$) among the three WPH lots in the L* value (Table 4.3). Two samples had similar a* and b* values with a mean of 0.15 and 7.39, respectively. However, the third lot was significantly different ($P<0.05$) from the other two lots with a* and b* values of 1.54 and 10.52, respectively. This variation might have been caused by some minor manufacturing variation such as degree of hydrolysis and/or moisture content. Lot three had the lowest moisture content and degree of hydrolysis among WPH lots. These differences are very minor and don't have any effect on the use of these lots as a binding agent in the agglomeration process.

Table 4.3. Bulk and tapped density, color of WP1 and WPH samples

Samples	Bulk Density (g/cm^3)	Tapped Density (g/cm^3)	Color		
			L*	a*	b*
WPH lot 1	0.33 ± 0.00^{a}	0.45 ± 0.00^{a}	91.95 ± 0.83	0.15 ± 0.04^{b}	7.49 ± 0.15^{b}
WPH lot 2	0.31 ± 0.00^{b}	0.42 ± 0.00^{c}	92.39 ± 0.21	0.16 ± 0.01^{b}	7.29 ± 0.15^{b}
WPH lot 3	0.32 ± 0.01^{ab}	0.44 ± 0.00^{b}	92.11 ± 0.25	1.54 ± 0.03^{a}	10.52 ± 0.11^{a}
WPI	0.30 ± 0.00	0.42 ± 0.00	92.73 ± 0.49	1.22 ± 0.06	10.39 ± 0.04

$^{a-c}$ Means with different superscripts are significantly different ($P<0.05$).
All values are expressed as mean ± SD. N=3

Agglomerated WPI characterization

Wet agglomeration involves spraying a liquid binder on the powder in a fluidized bed chamber causing adhesion of wet particles due to viscous bridges between the particles. With the continuous supply of hot air from fluid bed, the viscous bridges consolidate. The resultant agglomerates have a porous structure that improves dissolution rate, flowability and decrease apparent bulk density (Forny et al., 2011). In this study, agglomerated powders were tested for moisture content, water activity, solubility index, relative dissolution index (RDI), and emulsifying capacity.

Moisture content (MC)

According to the ADPI (2002) standards, MC should not exceed 6% by wt in dry whey products. MC for all treatments (Table 4.5) was in the normal range (<6%), except in treatments 14(140 g, 15% and 5.6 $mL.min^{-1}$), 16 (140 g, 20% and 5.6 $mL.min^{-1}$) and 18 (140 g, 25% and 5.6 $mL.min^{-1}$) that contained a MC of 14.79, 7.56, and 7.41%, respectively. In these treatments, clumps were formed, therefore monitoring the temperature of the end product was difficult, which resulted in a large variation of MC in these treatments. On the other hand, MC in the treatments that have the combination of high moisture content (140 g) and low flow rate (4.0 $mL.min^{-1}$) was in between 3.30-3.56% (Table 4.5), which matches the standards requirements of dry whey products. In treatments 2 (60 g, 15% and 5.6 $mL.min^{-1}$), 4 (60 g, 20%, and 5.6 $mL.min^{-1}$), and 6 (60 g , 25% and 5.6 $mL.min^{-1}$), MC of those samples were 4.15, 4.25, 5.37%, which is slightly higher than the ones that had the same pre-wet mass (60 g), and low flow rate (4.0 $mL.min^{-1}$), combination. However, in both cases the MC was still in the normal range (<6%). Statistically, the main effects; pre-wet mass, flow rate and WPH concentration, and their interactions had significant ($P<0.05$) effects on the MC. MC was also significantly different ($P<0.05$) among the replications.

Figure 4.3. The resultant agglomerated powder in treatments that had the combination of 140 g of pre-wet mass and 5.6 $mL.min^{-1}$ flow rate. The resultant powder did not meet the industrial specification of WPI

The regression coefficients for the statistically significant models are given in Table 4.4. The MC model equation is $\hat{Y}= 9.0147-0.2080x_1-0.1828x_2+0.5160x_3+0.0007(x1)^2\ 0.0038x_1x_2+ 0.0319(x_2)^2 +0.0131\ x_1x_3-0.0657x_2x_3$. The influence of pre-wet mass, WPH concentration and flow rate on MC is shown in Figure 4.4.

Table 4.4 The coefficients of moisture content and relative dissolution rate models for the response variables

Parameter	MC (%)	RDI (%)
Intercept (β_0)	9.0147	-10.3740
Linear	P(<.0001)	P(0.0011)
Pre-wet mass (β_1)	-0.2080	0.8782
WPH Concentration (β_2)	-0.1828	2.5652
Flow rate (β_3)	0.5160	NS
Quadric	P(0.0028)	P(0.3129)
Prewet*Prewet (β_{11})	0.0007	NS
WPH Concentration * WPH Concentration (β_{22})	0.0319	NS
Cross product	P(<.0001)	P(0.0039)
Pre-wet mass * WPH Concentration (β_{12})	-0.0038	-0.0202
Pre-wet mass*Flowrate (β_{13})	0.0131	NS
WPH Concentration*Flowrate (β_{23})	-0.0658	NS
P (Model)	<.0001	0.0002

NS=not significant (P>0.05)

The increase of pre-wet mass had highly influenced the MC Figure 4.4 (a and b). Flow rate had also increased the MC in agglomerated WPI, whereas, WPH concentration had a slight effect on the MC in agglomerated WPI. High pre-wet mass and high flow rate in these treatments caused a collapse in fluid bed, that can be explained with the plasticisation of the entire particle surface due to an increasing humidity of the air inside the fluid bed. Large clumps were formed and settled in the bottom of the fluid bed chamber (Figure 4.3), because the shear forces acting on the particles in the fluid bed are no longer sufficient to destroy the numerous sinter bridges generated which leads to a rapidly progressing cake formation (Palzer, 2009). High MC in the powder may influence the shelf-life due to Millard reaction, creating the lumps and leading to microbial growth (Pisecky, 1997).

Table 4.5. Moisture content (%), water activity, solubility index (%), emulsifying capacity (g of oil/mg of protein), and relative dissolution Index of all agglomerated WPI treatments as per experimental design

Treat-ment	Pre-wet mass (g)	WPH concentration (%)	Flow rate ($mL.min^{-1}$)	Moisture content (%)	Water activity	Solubility index (%)	Emulsifying capacity (g of oil/mg of protein)	Relative dissolution Index (%)
1	60	15	4.0	3.36 ± 0.15^{c}	0.13 ± 0.01^{d}	95.36 ± 0.02	4.66 ± 0.29	69.324 ± 3.79^{def}
2	60	15	5.6	5.37 ± 1.60^{bc}	0.21 ± 0.08^{cd}	95.43 ± 0.08	4.58 ± 0.34	73.37 ± 6.48^{bcdef}
3	60	20	4.0	3.35 ± 0.52^{c}	0.12 ± 0.01^{d}	95.34 ± 0.03	4.59 ± 0.29	63.20 ± 0.94^{f}
4	60	20	5.6	4.15 ± 0.16^{c}	0.11 ± 0.01^{d}	95.48 ± 0.19	4.40 ± 0.42	95.57 ± 4.32^{a}
5	60	25	4.0	3.48 ± 0.52^{c}	0.10 ± 0.02^{d}	95.33 ± 0.02	4.40 ± 0.52	84.12 ± 5.02^{abc}
6	60	25	5.6	4.25 ± 0.07^{c}	0.13 ± 0.01^{d}	95.38 ± 0.01	4.33 ± 0.42	86.56 ± 3.03^{ab}
7	100	15	4.0	4.48 ± 0.73^{c}	0.18 ± 0.00^{d}	95.41 ± 0.05	4.93 ± 0.20	81.83 ± 3.24^{abcd}
8	100	15	5.6	5.39 ± 0.37^{bc}	0.21 ± 0.00^{cd}	95.45 ± 0.03	4.75 ± 0.11	78.05 ± 0.67^{bcdef}
9	100	20	4.0	4.26 ± 0.41^{c}	0.16 ± 0.00^{d}	95.41 ± 0.03	4.80 ± 0.13	82.83 ± 5.19^{abcd}
10	100	20	5.6	3.77 ± 0.25^{c}	0.14 ± 0.00^{d}	95.39 ± 0.00	4.82 ± 0.20	75.25 ± 5.55^{bcdef}
11	100	25	4.0	3.73 ± 0.45^{c}	0.14 ± 0.02^{d}	95.39 ± 0.02	4.73 ± 0.27	79.13 ± 1.56^{bcde}
12	100	25	5.6	3.66 ± 0.16^{c}	0.14 ± 0.03^{d}	95.39 ± 0.05	4.47 ± 0.55	76.50 ± 6.62^{bcdef}
13	140	15	4.0	3.30 ± 0.13^{c}	0.12 ± 0.01^{d}	95.36 ± 0.01	4.63 ± 0.11	74.19 ± 4.91^{bcdef}
14	140	15	5.6	14.79 ± 4.50^{a}	0.67 ± 0.10^{a}	95.94 ± 0.31	4.52 ± 0.30	76.64 ± 6.17^{bcdef}
15	140	20	4.0	3.51 ± 0.06^{c}	0.12 ± 0.01^{d}	95.36 ± 0.05	4.75 ± 0.29	67.98 ± 4.58^{ef}
16	140	20	5.6	7.56 ± 1.32^{b}	0.33 ± 0.07^{bc}	95.51 ± 0.07	4.82 ± 0.20	75.30 ± 4.48^{bcdef}
17	140	25	4.0	3.56 ± 0.10^{c}	0.12 ± 0.01^{d}	95.34 ± 0.04	4.76 ± 0.25	70.64 ± 4.18^{cdef}
18	140	25	5.6	7.41 ± 3.46^{b}	0.34 ± 0.10^{b}	95.51 ± 0.15	4.71 ± 0.39	75.37 ± 10.42^{bcdef}

[a-j] Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ± SD. N=3

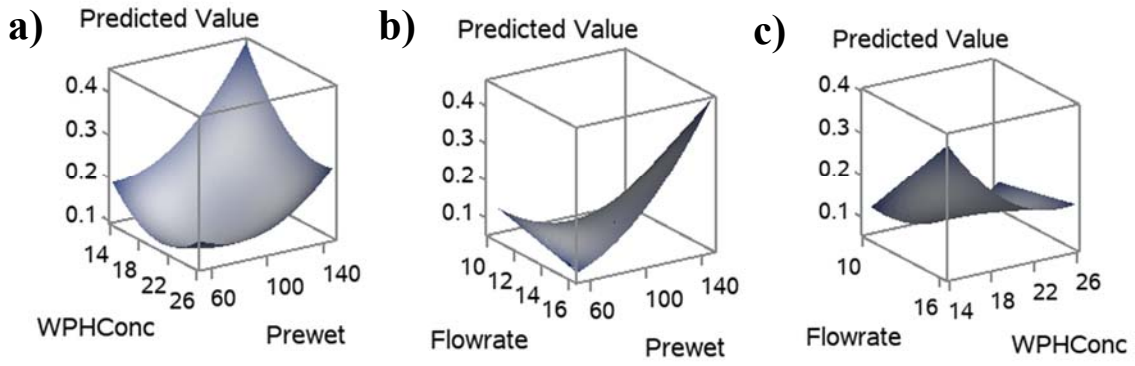

Figure 4.4. Response surface for MC, for a) pre-wet mass vs WPH concentration, b) flow rate and pre-wet mass and c) WPH concentration and flow rate

It was reported by (Gaiani et al., 2007) that agglomerated powder contained a slightly lower MC than the non-agglomerated WPI. In this study, non-agglomerated WPI sample contained about 3.61% MC, some of the agglomerated WPI samples contained a slightly lower MC than 3.61%, while the others did not, and the highest MC detected among the agglomerated samples was 14.79% as in treatment 14 (Table 4.5). This might be due to the differences in processing temperatures or the higher flow rate that were followed in this study.

Water activity

Water activity was positively correlated with the powders MC. In agglomerated powders, the higher MC resulted in a higher water activity. Water activity was significantly different ($P<0.05$) among the treatments, it was in the range of 0.67-3.30. As it was observed in MC, water activity had the highest values in treatments 14, 16 and 18. These treatments had the combination of high per-wet mass (140g) and high flow rate (5.6 $mL.min^{-1}$), and their water activity values were 0.67, 033, and 0.34, respectively. In these treatments, large and hard clumps were formed and settled in the bottom of the fluid bed chamber (Figure 4.3). Clumps formation made it hard to monitor the temperature of the end product. This resulted in a large variation of water activity in these treatments. The rest of the agglomerated samples had a water activity of ≤

0.20. All main effects and their interactions had significantly ($P<0.05$) influenced the water activity.

Relative dissolution index (RDI)

The number of fine particles with a chord length of <10 μm was plotted vs rehydration time. Figure 4.5 shows a typical FBRM plot obtained during the rehydration of agglomerated WPI with WPH as binder. As can be seen from Figure 4.5, the initial increase in the fine particle count during the first 142.5-171.7 s of rehydration can be attributed to breakage/ dissociation of the agglomerated WPI particles. Subsequently, a gradual decrease in the fine particle count was observed at 319.2-348.3 s into the dissolution of agglomerated WPI. As the dissolution continued further, the fine particle count continued to decrease below 0.5 μm, which is below the FBRM detection limit that specified by the manufacturer. In between those two stages, it was interesting to observe a slight decrease, followed by a slight increase in fine particle count during dissolution in all agglomerated WPI. More studies are needed to understand the phenomena. It was interesting to observe a striking contrast between the dissolution behavior of agglomerated WPI and milk protein concentrates (MPC) as observed from the fine particle count obtained from the FBRM. Hauser and Amamcharla (2016) observed a continuous increase in the fine particles count during the dissolution of MPC 90. The fine particle count continued to increase until the first 900 s of MPC 90 dissolution and did not observe any further increase of the fine particle count. On the other hand, the dissolution of agglomerated WPI followed a different trend as it is shown in Figure 4.5 that can be due to the compositional differences in between WPI (mostly whey proteins) and MPC (caseins and whey proteins) powder.

The mean RDI of the agglomerated WPI samples manufactured as per the experimental design was in between 63.20-95.57% (Table 4.5). Treatment 4 (60 g, 20 %, 5.6 $mL.min^{-1}$) had

the highest RDI of 95.57%, followed by treatment 6 (60 g, 25%, 5.6 mL.min^{-1}) and treatment 5 (60 g, 25%, 4.0 mL.min^{-1}) that had an 86.56 and 84.12%, respectively. There was no significant difference (P >0.05) among these treatments. Treatment 3 (60 g, 20%, 4 mL.min^{-1}) had the lowest RDI of 63.20 among the resultant agglomerated samples, followed by treatment 15 (140 g, 20%, 4 mL.min^{-1}), and 1 (60 g,15%, 4 mL.min^{-1}) 67.98 and 69.32 %, respectively. There was also no significant difference (P >0.05) among those treatments.

Pre-wet mass and flow rate had a significant effect (P<0.05) on RDI. WPH concentration did not have a significant (P>0.05) difference as a main factor. However, its interactions with other factors were significantly different (P<0.05). The interactions; pre-wet ×WPH concentration, pre-wet × Flow rate, WPH concentration × Flow rate, and pre-wet ×WPH concentration ×Flow rate were all significantly (P<0.05) different (Appendix B).

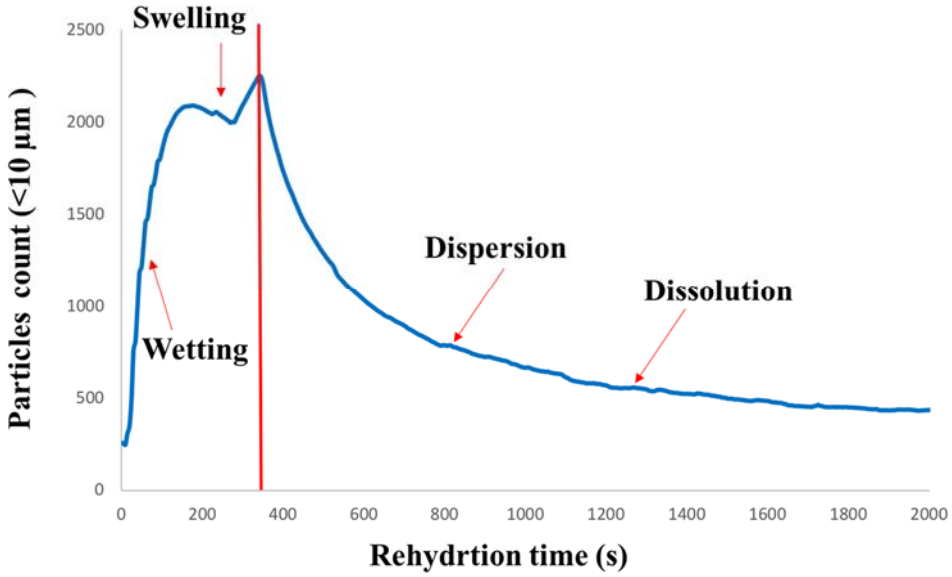

Figure 4.5. A typical fine particle count of chord length <10 µm plot vs rehydration time with showing the four rehydration phases (wetting, swelling, dispersion, and dissolution)

In the case of 60 g and 140 g of pre-wet, it was observed that higher flow rate (5.6 mL.min^{-1}) resulted in higher RDI in agglomerated powders. On the other hand, high flow rate resulted in a decrease of the RDI in treatments with 100 g of pre-wet (Table 4.5). Vengateson

and Mohan (2016) reported that increasing binder flow rate caused the formation of bigger granules with more flowability and decreased bulk density and friability, which consequently improved the rehydration. The influence of pre-wet mass, WPH concentration and flow rate on RDI is shown in Figure 4.5. Response surfaces Figure 4.6 (a and b) shows that the increase of pre-wet mass had drastically decreased the RDI. Whereas, the flow rate and WPH concentration has a positive influence on the RDI as they increased the RDI had increased Figure 4.6 (a, b and c). Prewet mass and flow rate had significantly affected the RDI might be due to their influence on the particles structure and shape. The regression coefficients for the statistically significant models are given in Table 4.4. The RDI model equation is $\hat{Y} = -10.3740 + 0.8782x_1 - 2.5652x_2 - 0.0201x_1x_2$ (Table 4.4). As noted by (Gaiani et al., 2011), the size associated with the shape descriptors could be important factors influencing milk powders rehydration properties.

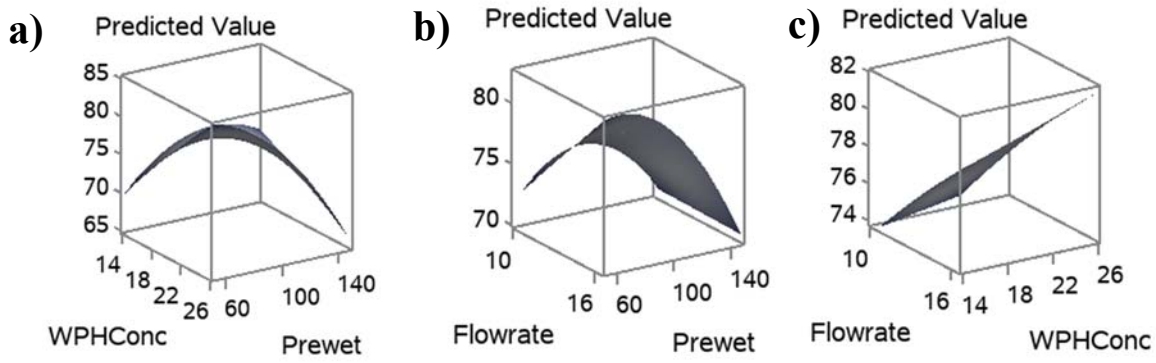

Figure 4.6 Response surfaces for RDI, for a) pre-wet mass vs WPH concentration , b) flow rate and pre-wet mass, and c) WPH concentration and flow rate

Solubility index (SI)

SI measures the status of the powder after 30 min of rehydration, followed by centrifugation to remove the undissolved particles. In all agglomerated samples, SI was 95%. As expected, agglomeration processing conditions did not have any significant effect on the solubility index of the powders ($P>0.05$). None of the processing main effects nor their

interactions had significantly affected (P>0.05). Chever et al. (2017) reported also that agglomeration did not affect the solubility index of milk powders.

Emulsifying capacity (EC)

EC of agglomerated samples is shown in Table 4.5, it was within the range of 4.33-4.93 g of oil/mg of protein, and there was no significant difference (P>0.05) among the treatments. The highest EC was observed in the treatment 7 (100g, 15%, and 4.0 $mL.min^{-1}$). In contrast, the lowest EC was observed in the treatment 6 (60g, 25%, and 5.6 $mL.min^{-1}$). Pre-wet mass is the only main factor that had a significant effect (P<0.05) on the EC. EC was decreased with the increase of flow rate and WPH concentration. However, it was increased with the increase of pre-wet mass. In the comparison of pre-wet levels, 60 and 100 g of pre-wet mass were the ones that were significantly different (P<0.05) from each other's. Hence, there was no significant difference (P>0.05) among the main effect's interactions. Turgeon et al. (1991) had used the same method in determining the EC of WPC, the EC was about 4.6 g of oil/mg of protein. Which is matching our results of WPI agglomerated samples.

Conclusion

WPH samples had similar chemical and physical properties indicating a consistent manufacturing conditions and process. Agglomeration conditions especially the pre-wet mass and the flow rate have affected primarily the moisture content, water activity and the RDI of agglomerated samples. Some of the agglomerated samples have failed to meet the industrial and ADPI standards of dry whey products. We believe that the WPH concentration has more impact on the physical properties of the agglomerates than it does on the functional properties. Finally, the hydrolysate can be used as a binder and an alternative to soy lecithin. Therefore, the possible

health side effects can be minimized, a clean label can be provided, and the shelf life of agglomerated powders can be increased.

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Chapter 5 - The effect of whey protein hydrolysate as a binder on the morphology of agglomerated whey protein isolate

Abstract

Wet agglomeration involves spraying a liquid binder on the powder in a fluidized bed chamber causing adhesion of wet particles due to the viscous bridges formed between the particles. The viscous bridges are then consolidated by the continuous supply of hot air to form agglomerated particles. The agglomerates have a porous structure that play a role in improving the dissolution rate, flowability and decreasing apparent bulk density. The objective of this study was to evaluate the effect of using whey protein hydrolysate (WPH) as a liquid binder on the physical properties of the agglomerated whey protein isolate (WPI). Three lots of WPH were obtained from a commercial manufacturer. A 3×3×2 of Randomized Complete Block Design (RCBD) experiment was performed. A top-spray fluid bed granulator (Midi-Glatt, Germany) was used. Size and shape characteristics of agglomerates were evaluated using Morphology G3-ID (Malvern Instruments Ltd, UK). The mean circle equivalent diameter (CED), circularity, elongation, and convexity were 15.18 μm, 0.74, 0.273 and 0.95, respectively. No significant differences ($P>0.05$) were observed for the CED and convexity for the main effects. The WPH concentration, pre-wet mass, and flow rate had significantly ($P<0.05$) influenced the elongation of the WPI agglomerates. The mean bulk density of agglomerated WPI samples manufactured as per the experimental design was in between 0.22 and 0.31 g/cm^3, and it was significantly ($P<0.05$) influenced by the pre-wet mass. Overall, pre-wet mass had the major effect on the agglomerates physical properties followed by the flow rate and the WPH concentration.

Key words: Agglomeration, Whey, Physical Characteristics

Introduction

Agglomeration or granulation is a size enlargement process that converts small powder particles into larger agglomerates. Agglomeration is a commonly used approach in the food industry to modify the physical characteristics such as density and flowability and also to improve the reconstitution properties of commercial powders by forming larger particles with a porous structure (Pisecky, 1997; Iveson et al., 2001; Turchiuli et al., 2005; Forny et al., 2011). The porous structure in agglomerated powder makes the water penetration easier during subsequent rehydration. Therefore, the agglomerates readily disperse and dissolve quickly (Carić, 1994) compared to non-agglomerated powders.

In the food industry, a variety of agglomeration technologies are available to increase the powder particles' size. These technologies can be classified into two categories: pressure agglomeration and wet-controlled growth agglomeration. In pressure assisted agglomeration, a static pressure is applied on powders to cause the adhesion between the particles. The static pressure can be applied using roller compaction, tableting, and extrusion. On the other hand, wet-controlled growth agglomeration involves utilizing a liquid binder over agitated powder bed to cause the collision of particles leading to agglomeration. The wet-controlled agglomeration can be achieved by using mechanical mixing (disk and drum-type mixers) and pneumatic mixing (fluid bed, spray drying, and steam jet). Heat is applied to remove excess water in the pneumatic mixing wet agglomeration technologies (Cuq et al., 2013).

In wet agglomeration process, particles are exposed to three different sets of rate processes including wetting and nucleation, consolidation and growth, and breakage and attrition. During wetting and nucleation phase, the liquid binder gets in contact with the dry powder particles in the fluid bed chamber to initiate nuclei granules formation.

During consolidation and growth phase, collisions and adhesion between granules occurs leading to granule compaction and growth of agglomerates. In attrition and breakage phase, wet or dried agglomerates disintegrate due excessive mixing and agitation when the granules are too wet or too dry. The attrition and breakage may also continue to occur during the subsequent poor product handling (Iveson et al., 2001). Therefore, pneumatic wet agglomeration involves spraying the liquid binder on the powder under constant mixing and agitation in a fluidized bed chamber causing adhesion of wet particles due to the formation of viscous bridges. The viscous bridges are then consolidated by continuous supply of hot air to form agglomerated particles. Agglomerates grow rapidly as a result of simultaneous mixing, wetting, collision, adhesion, and drying steps until it reaches to the final size. There are many operating parameters that can potentially influence the agglomeration characteristics in wet agglomeration process including pre-wet mass, binder solution concentration, flow rate, nozzle pressure, fluid bed pressure, amount of non-agglomerated powder in the fluid bed chamber, and fluid bed temperature. In this study, only pre-wet mass, binder concentration, and flow rate were selected as independent factors. Pre-wet mass (g) is the amount of water used in wetting the WPI powder in the first stage of agglomeration. In addition, the concentration of binder solution (w/w) can also be controlled. Lastly, the flow rate is amount of water and binder solution enters to the system and it is expressed as $ml.min^{-1}$.

In the dairy industry, agglomeration is applied on milk protein and whey protein-based powders to improve the functionality of those powders. It was first noted by Rogers (2011) that egg lecithin worked as a good binder in agglomerating whey protein powders. However, in the dairy industry, soy lecithin is the most commonly used binder in agglomerating whey proteins, because it is more abundant and cheaper than egg lecithin. With the aim of removing soy

lecithin, another allergen, from whey protein-based powders Palmer et al. (2018) evaluated the use of whey protein hydrolysate as a binding solution and found that whey protein hydrolysate can be used as an alternative to soy lecithin in WPI agglomeration process. The powder rehydration behavior is highly affected by the agglomerates shape and size. Therefore, the objective of the present study was to understand the effect of WPH as a binder on morphology and flowability of agglomerated WPI.

Materials and methods

Experimental design

Three lots of WPH and one lot of WPI were obtained from a commercial manufacturer and used in agglomeration process. To evaluate the effect of WPH as a liquid binder on the physical properties of the agglomerated WPI, a 3×3×2 factorial design was conducted with pre-wet mass (60, 100, and 140 g), WPH concentration (15, 20, and 25%), and flow rate (4.0 and 5.6 $mL.min^{-1}$) as independent variables. Other processing parameters such as the nozzle pressure, fluid bed pressure, and fluid bed temperature were set at 0.65 bar (9.43 psi), 0.45 bar (6.53 psi) and 60°C, respectively. WPI agglomeration process was carried out in a top-spray fluid bed granulator (Midi-Glatt, Binzen, Germany) as explained in the previous chapter. All treatments were performed in triplicates using the three lots of WPH. Agglomeration was stopped when the temperature of the end powder reached 45°C. Agglomerated WPI samples were collected in plastic containers (48 oz, PET square grip container, Container and Packaging Co., Louisville, KY) and stored at 25°C. Agglomerated WPI samples were analyzed for particle size and shape, color, microstructure, bulk and tapped densities.

The agglomerate shape and size

The agglomerate shape and size characteristics were obtained using Malvern Morphology G3-ID (Malvern Instruments Ltd, Worcestershire, UK). Agglomerated powder (5 mm^3) was loaded in the dispersion unit of the instrument to evenly disperse on a glass slide as one layer using compressed air at a pressure 1 bar. The instrument was centered on the plate and programmed to measure 10,000 particles in a $15mm^2$. The mean value of 10,000 particles were used to quantify the shape and size parameters of the agglomerated powders. Particle size was indicated by the circle equivalent diameter (CED). Circularity, convexity, and elongation parameters were used to describe the shape properties of the agglomerated powder. The circularity indicates how close the particle shape to the equivalent circle. The convexity indicates smoothness of the particle surface with a value closer to 1 for less spiky surface agglomerates. Finally, the elongation is based on the aseptic ratio of particle, so its value is close to 1 when the particle shape is similar to a needle or fibril.

Microstructure

The agglomerated WPI microstructure was obtained using a scanning electron microscope (SEM) as described by Mimouni et al. (2010). The agglomerated WPI samples were placed onto a carbon double-sided adhesive tape that was mounted on microscopy stubs. Samples were platinum coated using a Denton Vacuum Desk II sputter coater (Denton Vacuum, NJ) for 20 min in order to have a 15-20 nm thin layer of the platinum. Images were captured using a S-3500N (Hitachi Science Systems Ltd., Tokyo, Japan) and detected by a secondary electron detector (SED) operating at 8 kV.

Flowability parameters

Angle of repose, bulk and tapped density were measured using Hosokawa Micron PT-R powder tester (Hokosawa Micron Corp., Osaka, Japan). Bulk and tapped densities were measured after180 vertical taps in a 100 cm^3 cup to measure tapped density following the manufacturer's instructions.

The compressibility (Carr's index) was calculated based on the bulk and tapped densities using the following equation (1):

$$Compressibility = 100 \times \frac{Tapped\ denity - bulk\ density}{Tapped\ density}.$$

Hausner ratio was calculated based on the bulk and tapped densities using the following equation (2):

$$Hausner\ ratio = 100 \times \frac{Tapped\ denity}{Bulk\ density}$$

Color

The color L*, a*, and b* values were measured using a Hunter-Lab Mini Scan colorimeter (Hunter Associates Laboratory, Reston, VA). L* value evaluates the whiteness/darkness of the sample, a* measures the redness/greenness, and b* represents the yellowness/blueness of the powder. Analyses performed in duplicates.

Statistical analysis

All data was analyzed based on the Randomized Complete Block Design (RCBD) using PROC GLIMMIX in SAS Studio (version 9.4; SAS Inst., Cary, NC). WPH lots were considered as the blocking factor. In addition, PROC RSREG was used to get the response surface models for agglomerates' circularity, bulk density, compressibility and Hausner ratio . The general model equation is $\hat{Y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + {}_+\beta_{11}(x_1)^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{22}(x_2)^2 + \beta_{23} x_2 x_3$; where

β_0 ,β_1, β_2, β_3, β_{11}, β_{22}, β_{12}, β_{23},β_{13} are the constant coefficients, and x_1 is the pre-wet mass (g), x_2 is the WPH concentration (w/w), and x_3 is the flow rate (mL.min^{-1}

Results and discussion

The agglomerate size and shape

Technological properties of powders (bulk density, flowability, and surface area) as well as the potential areas of their application depend on the particles size and shape (Chegini and Taheri, 2013). The mean of CED of the agglomerated WPI samples manufactured as per the experimental design was within the range of 13.63-17.96 µm (Table 5.2). Treatment 5 (60 g, 25%, and 4 mL.min^{-1}) had the highest CED of 17.96 µm, followed by treatment 14 (140 g, 15%, and 5.6 mL.min^{-1}) and treatment 16 (140 g, 20%, and 5.6 mL.min^{-1}) that had a CED of 16.44 and 15.80 µm, respectively. There was no significant difference (P>0.05) among these treatments. It is important to note that high pre-wet mass and high flow rate in treatments 14 (140 g, 15%, and 5.6 mL.min^{-1}) and 16 (140 g, 20%, and 5.6 mL.min^{-1}) caused a collapse in fluid bed due to the increase in humidity of the air inside the fluid bed that resulted in the formation of large clumps (Palzer, 2009). Therefore, these treatments did not meet the ADPI (2002) and industrial standards of dry whey products. Treatment 1(60 g, 15%, and 4 mL.min^{-1}) had the lowest CED of 13.63 µm followed by treatment 13 (140 g, 15%, and 5.6 mL.min^{-1}) and treatment 7 (100 g, 15%, and 4.0 mL.min^{-1}) that had CED of 14.39, 14.32 µm, respectively. There was also no significant difference (P >0.05) among those treatments. The main effects (pre-wet mass, WPH concentration, and Flow rate) and their interactions had no significant effect (P>0.05) on CED. Treatments with low flow rate (4 mL.min^{-1}) and higher pre-wet mass (140 g) required more time (~13 min or more) for the agglomeration process and consequently gave the agglomerates more time to consolidate their structures in the agglomerator.

Table 5.1. The particles size (CE diameter) and shape characteristics (HS circularity, elongation, convexity) of all agglomerated WPI treatment combinations

Treatment	Pre-wet mass (g)	WPH concentration (%)	Flow rate ($mL.min^{-1}$)	CE Diameter (μm)	HS Circularity	Elongation	Convexity
1	60	15	4.0	13.63±1.81	$0.72±0.01^{de}$	$0.30±0.01^{abc}$	0.95±0.01
2	60	15	5.6	15.52±1.44	$0.74±0.03^{abcd}$	$0.27±0.02^{cd}$	0.95±0.01
3	60	20	4.0	14.71±2.16	$0.74±0.04^{abcd}$	$0.27±0.04^{cd}$	0.95±0.01
4	60	20	5.6	15.68±1.32	$0.78±0.02^{ab}$	$0.22±0.02^{ef}$	0.96±0.01
5	60	25	4.0	17.96±4.93	$0.77±0.02^{abc}$	$0.22±0.01^{ef}$	0.95±0.01
6	60	25	5.6	15.68±2.96	$0.79±0.03^{a}$	$0.21±0.02^{f}$	0.96±0.01
7	100	15	4.0	14.32±1.29	$0.74±0.01^{bcde}$	$0.27±0.01^{cd}$	0.95±0.00
8	100	15	5.6	15.20±1.16	$0.74±0.02^{cde}$	$0.27±0.01^{cd}$	0.95±0.01
9	100	20	4.0	14.94±3.40	$0.72±0.01^{de}$	$0.29±0.01^{abcd}$	0.95±0.01
10	100	20	5.6	14.75±2.29	$0.71±0.02^{de}$	$0.29±0.02^{abc}$	0.95±0.01
11	100	25	4.0	15.48±2.24	$0.72±0.01^{de}$	$0.29±0.01^{abcd}$	0.95±0.00
12	100	25	5.6	14.52±0.92	$0.73±0.01^{de}$	$0.29±0.01^{abcd}$	0.95±0.00
13	140	15	4.0	14.39±1.78	$0.72±0.01^{de}$	$0.31±0.00^{ab}$	0.95±0.00
14	140	15	5.6	16.44±2.15	$0.75±0.02^{abcd}$	$0.26±0.02^{de}$	0.95±0.00
15	140	20	4.0	15.18±3.94	$0.72±0.02^{de}$	$0.30±0.01^{abc}$	0.95±0.01
16	140	20	5.6	15.80±1.09	$0.73±0.01^{de}$	$0.27±0.01^{bcd}$	0.95±0.00
17	140	25	4.0	14.58±1.42	$0.71±0.01^{e}$	$0.31±0.01^{a}$	0.95±0.01
18	140	25	5.6	14.58±1.92	$0.74±0.01^{cde}$	$0.27±0.02^{cd}$	0.95±0.00

[a-j] Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ± SD. N=3

Higher WPH concentration had more total solids which resulted in a slightly bigger agglomerates than the lower binder concentration. Turchiuli et al. (2005) reported that the agglomerates formed at a lower binder concentration were larger, more irregular, and less fragile than the agglomerated powders produced at higher binder concentration. Which contradicts with our finding, this might be due to the differences in both the studies in terms of binder composition, particle size of the binder, and the agglomerated material. Turchiuli et al. (2005) used carbohydrate-based binder, maltodextrin, and agglomeration resulted in median particles diameter of 475 and 210 µm. On the other hand, the current study used a protein-based binder.

The shape characteristics were determined by measuring the circularity, elongation, and convexity, all their values were within the range of 0 to 1 (Figure 5.1). The mean of circularity among agglomerated WPI samples manufactured as per the experimental design is within the range of 0.71-0.79 (Table 5.2). Treatment 6 (60 g, 25%, 5.6 $mL.min^{-1}$) had the highest circularity of 0.79, followed by treatment 4 (60 g, 20%, and5.6 $mL.min^{-1}$) and treatment 5 (60 g, 25%, and 4 $mL.min^{-1}$) of 0.78 and 0.77, respectively.

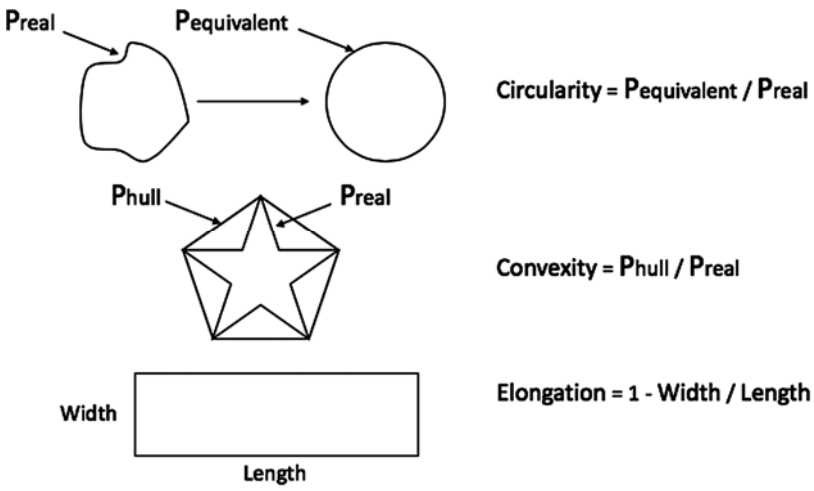

Figure 5.1. The definitions of agglomerates shape parameters: circularity, convexity and elongation measured by Malvern Morphology G3-ID

Pre-wet mass and flow rate had a significant (P<0.05) influence on the circularity. None of the interactions were significantly different (P>0.05) except for pre-wet mass × WPH concentration and pre-wet mass × flow rate. As the flow rate and WPH concentration increased, the circularity increased Figure 5.2 (a, b and c). However, the pre-wet mass had a negative effect on the circularity Figure 5.2 (a and b). The regression coefficients for the statistically significant models are given in Table 5.1. The circularity model equation is $\hat{Y}= 0.7443-0.0007x_1-0.00009x_2+0.00001(x_1)^2-0.00008x_1x_2$.

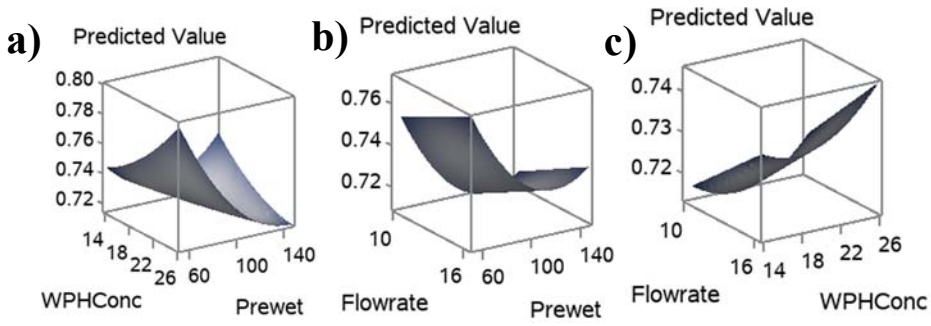

Figure 5.2. Response surfaces for circularity: a) pre-wet mass vs WPH concentration, b) flow rate and pre-wet mass and c) WPH concentration and flow rate

Table 5.2 The coefficients of circularity, bulk density, compressibility and Hausner ratio models for the response variables

Parameters	Circularity	Bulk density	Compressibility	Hausner ratio
Intercept (β_0)	0.7443	0.4543	33.9868	0.0002
Linear	P(<.0001)	P(0.0037)	P(0.0001)	P(0.0002)
Pre-wet mass (β_1)	-0.0008	0.0008	-0.3091	-0.0060
WPH Concentration (β_2)	-0.0001	-0.0138	NS	NS
Flow rate (β_3)	NS	-0.0105	NS	NS
Quadric	P(0.0025)	P(0.0262)	P(0.2275)	P(0.1787)
Prewet*Prewet (β_{11})	<0.0001	<-0.0001	NS	NS
WPH Concentration*WPH Concentration (β_{22})	NS	NS	NS	NS
Cross product	P(<.0001)	P(<.0001)	P(0.7846)	P(0.8692)
Pre-wet mass * WPH Concentration (β_{12})	-0.0001	0.0001	NS	NS
Pre-wet mass*Flowrate (β_{13})	NS	-0.0001	NS	NS
WPH Concentration*Flowrate (β_{23})	NS	0.0008	NS	NS
P (model)	<.0001	<.0001	0.0020	0.0027

NS=not significant (P>0.05)

The other shape property analyzed was elongation. The same samples that had the highest circularity, had the lowest elongation. The mean of elongation among agglomerated WPI samples manufactured as per the experimental design was within the range of 0.21-0.31 (Table 5.2). All main factors and their interactions had significantly affected the agglomerates elongation except WPH concentration × flow rate. The increase in the pre-wet mass had positively affected the elongation. On the other hand, when the WPH concentration and flow rate the elongation decreased and the agglomerates shape was more circular (regular).

The mean of convexity among agglomerated WPI samples manufactured as per the experimental design is 0.95. Pre-wet mass had significantly affected the convexity, and none of the other main factors or their interactions had significantly ($P>0.05$) influenced the convexity. The increase in the pre-wet mass had negatively affected the convexity. On the other hand, when the WPH concentration and flow rate the convexity increased.

Microstructure

The resultant powders were imaged using the SEM analysis as it is shown in Figure 5.3. In case of using 60 g pre-wet mass and low flow (Figure 5.3a) was applied, more agglomerates were observed with more hollow particles appeared in those samples. However, high flow rate combination (Figure 5.3b), it resulted in rounder and lesser number of big agglomerates and fine particles observed. In case of using 100 g pre-wet mass with high flow rate (5.6 mL.min-1) as in Figure 5.3c, more fine particles were observed. Hence, with low flow rate bigger agglomerates were formed.

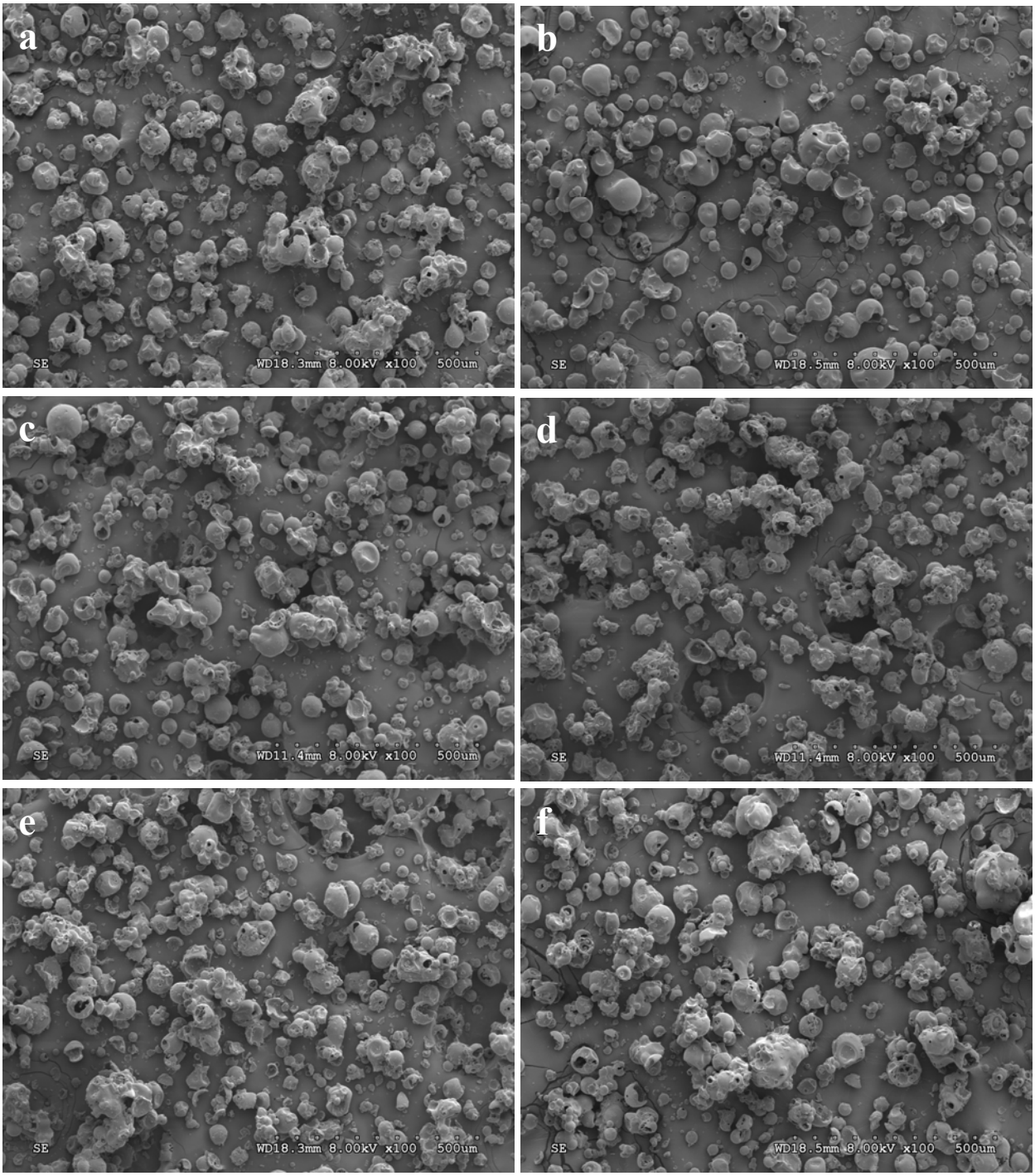

Figure 5.3. Scanning electron micrographs (×100) of agglomerated WPI. a) Treatment 3 (60g, 20 WPH % and 4 mL.min^{-1}), b) Treatment 4 (60g, 20 WPH % and 5.6 mL.min^{-1}), c) Treatment 8 (100g, 15 WPH % and 5.6 mL.min^{-1}), d) Treatment 9 (100g, 20 WPH % and 4 mL.min^{-1}), e) Treatment 15 (140g, 20 WPH % and 4 mL.min^{-1}), f) Treatment 16 (140g, 20 WPH % and 5.6 mL.min^{-1})

In addition, same attributes were observed in the case of using 140 g, however higher number of fine particles was observed when low flow rate was applied, which resulted in having a dustier powder. It was also noted by (Kelly et al., 2016b) that utilizing the hydrolysate resulted in having rougher surfaces than the samples that treated with the intact powder. Suggesting that low molecular mass amorphous proteins are liable to plasticization and deformation (Netto et al., 1998). Based on the flow rate, samples with low flow rate (4 $mL.min^{-1}$) had fuzzier (irregular) surfaces (Figures 5.3a, d, and e). On the other hand, when high flow rate (5.6 $mL.min^{-1}$) resulted in a bigger and smoother, and more hollow agglomerated particles (Figures 5.3b, c, and f).

Flowability parameters

Angle of repose, bulk and tapped density, compressibility and Hausner ratio of agglomerated WPI results are shown in Table 5.3. Agglomeration causes a drop in the bulk density and influences the powder properties such as flowability and instant properties (Pisecky, 1997). In addition to bulk density, moisture content, particle composition, and particles size and shape can also directly influence the powder flowability.

Angle of repose

Angle of repose is the steepest slope of the unconfined material, measured from the horizontal plane on which the material is heaped without collapsing (Al-Hashemi and Al-Amoudi, 2018). Angle of repose is important for the design of processing, storage, and conveying systems of particulate materials (Teferra, 2019). The mean of angle of repose among agglomerated WPI samples manufactured as per the experimental design is within the range of range of 42.88-52.05º (Table 5.3). The highest value of angle of repose was observed in treatment 14 (140 g, 15 %, 5.6 $mL.min^{-1}$), while the lowest value of angle of repose was observed in treatment 15 (140 g, 20 %, 4.0 $mL.min^{-1}$). These treatments 14 and 15 were

significantly different ($P<0.05$) from each other. However, the other treatments combinations were similar, and their values were within the range of 42.88-52.05º (Table 5.3). Chegini and Taheri (2013) that powders properties such as bulk density and flowability as well as the potential areas of their application depend on the particles size and shape. Treatments 14 and 15 showed differences in the agglomerate's circularity and elongation. In addition, the bulk density of treatments 14 and 15 was 0.23 and 0.30 g/cm3, respectively.

Based on the bulk density results, treatment 14 should've had the highest flowability, but we observed the opposite due to having large clumps in this treatment that made it less flowable. According to Carr classification of flowability of powder based on their angle of repose (Riley and Hausner, 1970), the agglomerated WPI powders flowability is classified from fair to passable flow (38-45º) to cohesive (45-55º). Treatments that classified as a fair to passable flow were treatments 1, 8, 10, 11, 12, 13, 15, 17 and 18, the remained treatments were classified as cohesive powders.

Density

Bulk density of milk powders is a very important property, from the point of view of economy, functionality and market requirements. When shipping milk powder in bulk over long distances the producer is interested in high bulk density to reduce shipping costs, since in most cases transportation costs relate to volume. Also, high bulk density saves packaging material for a given weight shipment (Pisecky, 1997). The bulk density of non-agglomerated WPI was 0.30 g/cm^3, while the mean of bulk density among agglomerated WPI samples manufactured as per the experimental design is within the range of 0.22 and 0.31 g/cm^3 (Table 5.3).

Table 5.3. Flowability parameters; angle of repose, bulk and tapped density, compressibility, and Hausner ratio for all agglomerated WPI treatment combinations

Treatment	Pre-wet mass (g)	WPH concentration (%)	Flow rate ($mL.min^{-1}$)	Angle of repose	Bulk density (g/cm^3)	Tapped density (g/cm^3)	Compressibility (%)	Hausner ratio
1	60	15	4.0	43.35±3.96	$0.31±0.01^{a}$	$0.40±0.00^{a}$	$22.83±2.44^{bc}$	$1.30±0.04^{bc}$
2	60	15	5.6	46.70±1.61	$0.27±0.02^{abcd}$	$0.40±0.03^{a}$	$32.30±1.86^{a}$	$1.48±0.04^{a}$
3	60	20	4.0	45.35±8.18	$0.26±0.04^{bcdef}$	$0.37±0.06^{a}$	$29.53±2.31^{ab}$	$1.42±0.05^{ab}$
4	60	20	5.6	49.17±3.09	$0.25±0.01^{def}$	$0.32±0.00^{bcd}$	$23.92±1.00^{bc}$	$1.31±0.02^{bc}$
5	60	25	4.0	47.95±3.93	$0.22±0.03^{f}$	$0.32±0.02^{cd}$	$29.88±6.06^{ab}$	$1.44±0.13^{ab}$
6	60	25	5.6	48.03±4.42	$0.26±0.02^{cdef}$	$0.36±0.00^{abc}$	$29.99±6.17^{ab}$	$1.42±0.13^{ab}$
7	100	15	4.0	45.17±5.72	$0.27±0.02^{abcd}$	$0.37±0.01^{a}$	$27.52±5.92^{abc}$	$1.39±0.11^{abc}$
8	100	15	5.6	44.45±2.32	$0.28±0.01^{abcd}$	$0.36±0.00^{abc}$	$21.01±1.34^{c}$	$1.27±0.02^{c}$
9	100	20	4.0	47.68±4.01	$0.26±0.02^{abcde}$	$0.36±0.01^{abc}$	$25.13±4.40^{abc}$	$1.34±0.08^{abc}$
10	100	20	5.6	44.6±5.82	$0.30±0.02^{ab}$	$0.40±0.02^{a}$	$24.02±2.04^{bc}$	$1.32±0.03^{bc}$
11	100	25	4.0	43.80±6.33	$0.28±0.01^{abcd}$	$0.37±0.01^{ab}$	$24.18±2.81^{bc}$	$1.32±0.05^{bc}$
12	100	25	5.6	43.77±4.95	$0.30±0.00^{ab}$	$0.39±0.01^{a}$	$23.38±1.99^{bc}$	$1.31±0.03^{bc}$
13	140	15	4.0	43.15±2.41	$0.29±0.01^{abc}$	$0.36±0.01^{abc}$	$20.08±3.39^{c}$	$1.25±0.05^{c}$
14	140	15	5.6	52.05±2.94	$0.23±0.03^{ef}$	$0.29±0.02^{d}$	$22.01±3.66^{bc}$	$1.28±0.06^{bc}$
15	140	20	4.0	42.88±4.56	$0.30±0.00^{ab}$	$0.40±0.01^{a}$	$23.09±2.97^{bc}$	$1.30±0.05^{bc}$
16	140	20	5.6	48.30±2.85	$0.29±0.02^{abcd}$	$0.38±0.02^{a}$	$25.43±5.10^{abc}$	$1.35±0.09^{abc}$
17	140	25	4.0	43.93±6.00	$0.30±0.01^{ab}$	$0.40±0.00^{a}$	$24.81±2.79^{abc}$	$1.33±0.05^{abc}$
18	140	25	5.6	44.02±3.89	$0.28±0.03^{abcd}$	$0.38±0.06^{a}$	$25.52±6.44^{abc}$	$1.35±0.12^{abc}$

[a-f] Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ±SD. N=3

Agglomeration affected the powder density as agglomerated samples had a lower bulk density than the non-agglomerated sample. In general, treatments treated with 60 g of pre-wet mass level recorded the lowest bulk densities among agglomerated samples such as treatment 5 (60 g, 25%, and 4 $mL.min^{-1}$) has the lowest bulk density of 0.22 g/cm^3. The second lowest bulk density was 0.23 g/cm^3 for treatment 14 (140 g, 15%, and 5.6 $mL.min^{-1}$) followed by treatment 4 (60 g, 25%, and 5.6 $mL.min^{-1}$). No significant difference ($P<0.05$) was found among those samples. However, all treatments with 140 g pre-wet mass recorded the highest bulk density among agglomerated samples with an exception of treatment 14. In this treatment, with the combination of high flow rate (5.6 $mL.min^{-1}$) and pre-wet mass (140 g), it resulted in high moisture content (>5%) and large clumps formation. Therefore, sample treatment did not meet the standard and industrial requirements of dry whey products. Treatment 1 (60 g, 15%, and 4 $mL.min^{-1}$) had the highest bulk density that was 0.31, followed by treatments 10 (100 g, 20%, 5.6 $mL.min^{-1}$), 12 (100 g, 25%, 5.6 $mL.min^{-1}$), 15 (140 g, 20%, and 4.0 $mL.min^{-1}$) and 17 (140 g, 25%, and 5.6 $mL.min^{-1}$), all of these treatments had the same bulk density which was 0.30 g/cm^3. No significant difference ($P<0.05$) was found among those agglomerated samples.

Treatments treated with 60 g of pre-wet mass had the lowest amount of solution added to the system that resulted in having a short drying time (about 7min on average). Consequently, the agglomerates were exposed to less time of agitation and mixing. This might result in maintaining the porous structure of the agglomerates as less wet granule breakage occurred. While in the case of using 140g pre-wet mass with the combination of low flow rate, the resulted agglomerated samples were very dusty. This might be due to the long drying time (about 36min on average) that caused breakage and attrition of the dry agglomerates due to the constant agitation. That resulted in generating more dusty fines (Iveson et al., 2001).

Pre-wet mass had mainly affected the bulk density at a confidence level of 0.05. In addition, pre-wet mass × WPH concentration, pre-wet mass × flow rate, WPH concentration × flow rate interactions were all significant (0.05). The influence of main effects on bulk density was highly dependent on the other treatment conditions (prewet mass, flow rate and WPH concentration) Figure 5.4. For example, when the pre-wet mass combined with WPH concentration resulted in an increase of the bulk density. However, when it combined with flow rate, it showed an increase then a decrease in bulk density as high moisture content and large clumps were formed in treatment 14, for example. WPH concentration had highly influenced agglomerated samples bulk density when low pre-wet mass (60 g) was used Figure 5.4 (a). b and c). However, the pre-wet mass had a negative effect on the circularity Figure 5.2 (a and b). The regression coefficients for the statistically significant models are given in Table 5.1. The bulk density model equation is $\hat{Y}= 0.4542+0.0008x_1-0.0138x_2-0.0105x_3-0.000008(x_1)^2+0.0001x_1x_2-0.0001x_1 x_3-0.0008x_2x_3$ (Table 5.1).

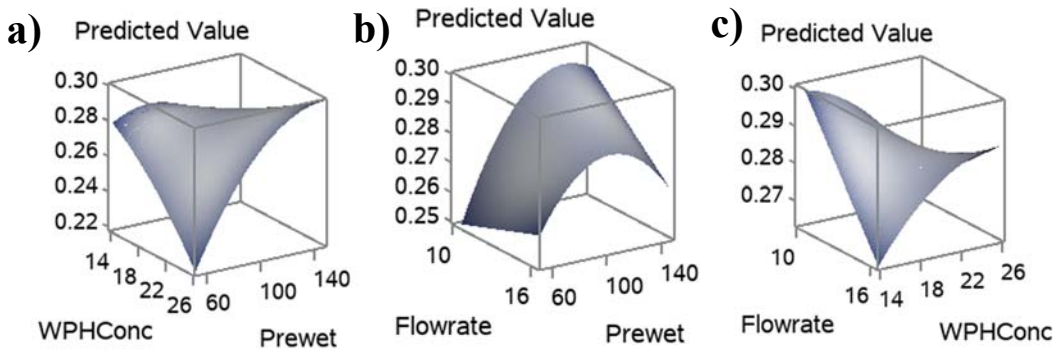

Figure 5.4. Response surfaces for bulk density: a) pre-wet mass vs WPH concentration, b) flow rate and pre-wet mass and c) WPH concentration and flow rate

Tapped density of non-agglomerated WPI was 0.42 g/cm^3 and the mean of tapped density among agglomerated WPI samples manufactured as per the experimental design is within the range of was in the range of 0.29- 0.40 g/cm^3 for agglomerated samples (Table 5.3). Same samples that had the lowest bulk density have also had the lowest tapped density (treatments 5,

14 and 4). No significant difference (P<0.05) was found among those samples. Treatments; 1 (60 g, 15%, and 4 mL.min^{-1}), 2 (60 g, 15%, and 5.6 mL.min^{-1}), 10 (100 g, 20%, 5.6 mL.min^{-1}), 15 (140 g, 20%, and 4.0 mL.min^{-1}) and 17 (140 g, 25%, and 5.6 mL.min^{-1}), all had the highest tapped density which was 0.40 g/cm^3. No significant difference (P>0.05) was found among those agglomerated samples.

None of the main factors and their interactions had a significant (P>0.05) effect on tapped density except for pre-wet mass × WPH concentration, pre-wet mass × flow rate and WPH concentration × flow rate interactions. In addition, there was a significant difference among the treatment's replicates in tapped density value. Agglomerates density was influenced by amount of binder solution entering the system, total solids of the binder solution, flow rate, agitation and length of the process.

Compressibility (Carr's index)

The mean of compressibility among agglomerated WPI samples manufactured as per the experimental design is within the range of 20.08-32.30% (Table 5.3). The highest compressibility was observed in treatment 2 (60 g, 15%, 5.6 mL.min^{-1}), followed by treatment 3 (60 g, 20%, 4.0 mL.min^{-1}) and treatment 5 (60 g, 25%, 4.0 mL.min^{-1}) of 32.30, 29.53 and 29.88, respectively. No significant difference (P<0.05) was found among those samples. The lowest compressibility was observed for treatment 13 (140 g, 15%, 4.0 mL.min^{-1}), followed by treatment 8 (100 g, 15%, 5.6 mL.min^{-1}) and treatment 14 (140 g, 15%, 5.6 mL.min^{-1}) of 20.08, 21.01 and 22.01%, respectively. No significant difference (n0.05) was detected among those samples. Based on the Carr's index, agglomerated samples flowability from fair to very poor. The highest compressibility was observed in treatment 2 (60 g, 15%, 5.6 mL.min^{-1}) of 32.30%, and it was categorized as fair to flow. On the other hand, the lowest compressibility was

observed for treatment 13 (140 g, 15%, 4.0 mL.min^{-1}) of 20.08, and it was classified under very poor flowability. All remained agglomerated samples were in between possible to poor flowability (Table 5.4).

Pre-wet is the main factor that significantly ($P<0.05$) affected the compressibility. None of the main effect interactions was significant except the pre-wet mass × flow rate interaction. This interaction had significantly affected all the powder physical characteristics such as bulk density, tapped density and circularity of the agglomerates. b and c). However, the pre-wet mass had a negative effect on the circularity Figure 5.2 (a and b). The regression coefficients for the statistically significant models are given in Table 5.1. The Compressibility model equation is $\hat{Y} = 33.9868\text{-}0.3090x_1$ (Table 5.1). As the flow rate increased, it helped with increasing the compressibility up to 100 g of pre-wet mass, then a decrease in the compressibility was observed in the treatment combinations where 140 g were used Figure 5.5b.

Table 5.4. Specifications for Carr's index and Hausner ratio

SI.no.	Flowability	Carr's index (%)	Hausner ratio
1	Excellent	0-10	1.00-1.11
2	Good	10-15	1.12-1.18
3	Fair	16-20	1.19-1.25
4	Possible	21-25	1.26-1.34
5	Poor	26-31	1.35-1.45
6	Very poor	32-37	1.46-1.59
7	Very, very poor	>38	>1.60

Adapted from Lebrun et al. (2012).

However, an increase in agglomerated WPI compressibility with the increase of WPH concentration was observed 5.5a. Flow rate had a negative effect on the compressibility, as it increased, it resulted in a decrease in the compressibility due to increase in the breakage rate especially in treatments of high flow rate (5.6 mL.min^{-1}) and pre-wet mass (140 g) combination. Recall, those treatments were very dusty and had a relatively high bulk density when compared with other agglomerated samples.

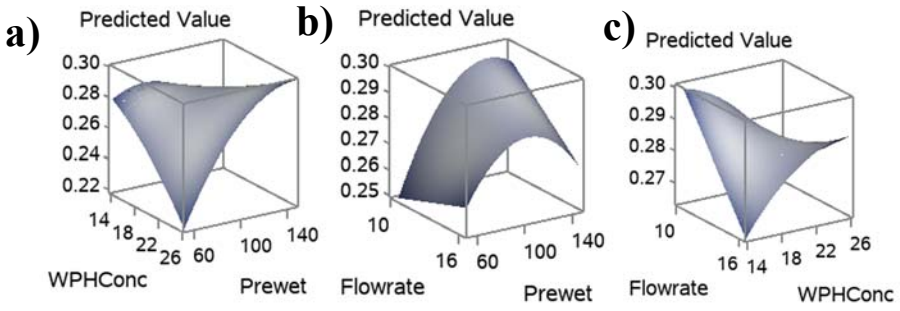

Figure 5.5. Response surfaces for compressibility: a) pre-wet mass vs WPH concentration, b) flow rate and pre-wet mass and c) WPH concentration and flow rate

Hausner ratio (HR)

The mean of HR among agglomerated WPI samples manufactured as per the experimental design is within the range of 1.25-1.48% (Table 5.3). The lower HR value indicates a better flow of the powder. The powder compressibility indicated that the WPI agglomerated samples flowability is ranging from fair to very poor, which agrees with Hausner ratio classification for WPI agglomerated samples. One sample was classified as fair to flow which was in treatment 13 (140 g, 15%, 4 $mL.min^{-1}$) which had the lowest Hausner ratio of 1.25, followed by 1.27 and 1.28 for treatments 8 (100 g, 15%, 5.6 $mL.min^{-1}$) and 14 (140 g, 15%, 5.6 $mL.min^{-1}$). Another one sample was classified as very poor to flow which was in treatment 2 (60 g, 15%, 5.6 $mL.min^{-1}$), and the rest of the WPI agglomerated samples were classified as possible to poor to flow.

Pre-wet is the only main factor that significantly ($P<0.05$) affected HR. None of the main effect interactions was significant except the pre-wet mass × flow rate interaction. This interaction had significantly affected all the powder physical characteristics such as bulk density, tapped density and circularity of the agglomerates. Higher pre-wet mass resulted in decreasing HR (Figure 5.6b) due to increase in the breakage rate especially in treatments of high flow rate (5.6 $mL.min^{-1}$) and pre-wet mass (140 g) combination which resulted in dustier and had relatively higher bulk density when compared with other agglomerated samples. As the flow rate

increased, it helped with increasing in HR value. An increase in HR values among agglomerated WPI was observed with the increase of WPH concentration was observed (Figure 5.6c). b and c). However, the pre-wet mass had a negative effect on the circularity Figure 5.2 (a and b). The regression coefficients for the statistically significant models are given in Table 5.1. The HR model equation is $\hat{Y}= 1.6022-0.006x_1$(Table 5.1).

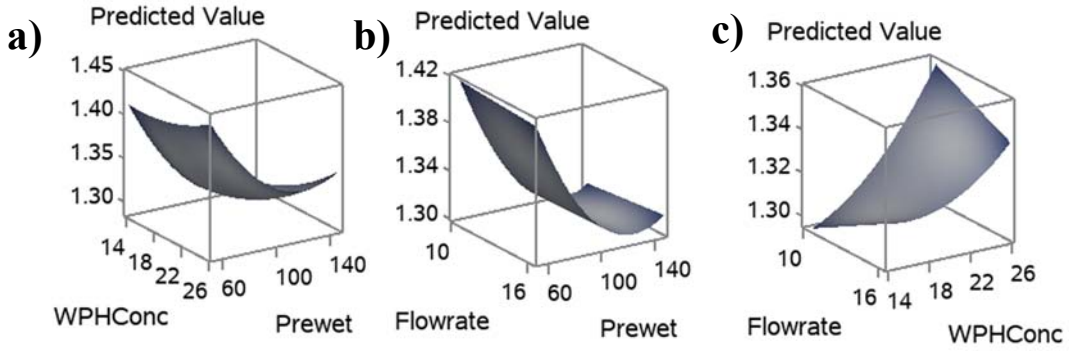

Figure 5.6. Response surfaces for HR: a) pre-wet mass vs WPH concentration, b) flow rate and pre-wet mass and c) WPH concentration and flow rate

Color

Powder color is evaluated using L*, a* and b* values. L* value indicates the whiteness/darkness of the sample, a* indicates the redness/greenness, and b* shows the yellowness/blueness of the powder (Table 5.5). The mean of L* value among agglomerated WPI samples manufactured as per the experimental design was within the range of 91.71-92.80. The lowest L* value was observed for treatment 16 (140 g, 20%, 5.6 $mL.min^{-1}$). While the highest L* value was recoded in treatment 6 (60 g, 25%, 5.6 $mL.min^{-1}$). A significant difference ($P<0.05$) was found between those two agglomerated samples, whereas the rest of the samples were all similar. Pre-wet mass was the only main factor that significantly ($P<0.05$) affected the L* value of agglomerated samples. however, the three ways interaction of all main factors was significant ($P<0.05$).

The mean of a* value among agglomerated WPI samples manufactured as per the experimental design was within 1.05-1.48 (Table 5.4). The lowest a* value was observed in treatment 5 (60 g, 25%, and 4 $mL.min^{-1}$), while the highest value was in treatment 14 (140 g, 15%, and 5.6 $mL.min^{-1}$). There was a significant difference among the agglomerated samples ($P<0.05$). The mean of b* value among agglomerated WPI samples manufactured as per the experimental design was within 10.63-11.91. The lowest b* value was observed in treatment 3 (60 g, 20%, and 4 $mL.min^{-1}$), while the highest value was in treatment 16 (140 g, 20%, and 5.6 $mL.min^{-1}$). There was a significant difference among the agglomerated samples ($P<0.05$). All main effects had significantly influenced the a* and b* values in addition to pre-wet mass × flow rate interaction ($P<0.05$). Moreover, a significant different ($P<0.05$) was detected among the replicates for a* value. The amount of liquid inserted to the system is the factor that influence the resultant agglomerated powder color the most, because it influences the length of processing and moisture content of agglomerated WPI.

Conclusion

The agglomerates circular equivalent diameter was similar in all treatment that were manufactured as per experimental design. Treatments with the lowest pre-wet mass, and higher binder concentration and high flow rate had bigger agglomerates with a more regular shape (circular) than the opposite treatment conditions. SEM imaging showed that all agglomerated samples had rough particles surfaces, and it was also observed that the low flow rate samples had fuzzier surfaces than the high flow rate ones. Agglomeration resulted in decreasing the bulk and tapped densities of WPI. The amount of pre-wet mass, binder solution, flow rate are all factors affected the agglomerated WPI densities.

Table 5.5. Color; L*, a* and b* values for all agglomerated WPI treatment combinations

Treatment	Pre-wet mass (g)	WPH concentration (%)	Flow rate ($mL.min^{-1}$)	Color		
				L*	a*	b*
1	60	15	4.0	92.51 ± 0.38^{ab}	1.19 ± 0.00^{defg}	11.02 ± 0.21^{de}
2	60	15	5.6	92.33 ± 0.28^{ab}	1.14 ± 0.00^{efgh}	10.94 ± 0.16^{de}
3	60	20	4.0	92.67 ± 0.42^{ab}	1.07 ± 0.07^{h}	10.63 ± 0.34^{e}
4	60	20	5.6	91.80 ± 0.40^{b}	1.08 ± 0.08^{gh}	11.19 ± 0.31^{bcde}
5	60	25	4.0	92.13 ± 0.51^{ab}	1.05 ± 0.03^{h}	10.94 ± 0.17^{de}
6	60	25	5.6	92.80 ± 0.89^{a}	1.13 ± 0.09^{fgh}	10.94 ± 0.26^{de}
7	100	15	4.0	92.24 ± 0.13^{ab}	1.23 ± 0.07^{cdef}	11.34 ± 0.32^{bcde}
8	100	15	5.6	92.11 ± 0.34^{ab}	1.28 ± 0.10^{bcd}	11.67 ± 0.53
9	100	20	4.0	92.11 ± 0.37^{ab}	1.21 ± 0.05^{def}	11.34 ± 0.21^{bcde}
10	100	20	5.6	92.55 ± 0.29^{ab}	1.24 ± 0.03^{cdef}	11.15 ± 0.19^{cde}
11	100	25	4.0	92.62 ± 0.35^{ab}	1.24 ± 0.02^{cdef}	11.35 ± 0.37^{bcde}
12	100	25	5.6	92.55 ± 0.62^{ab}	1.25 ± 0.03^{bcde}	11.33 ± 0.20^{bcde}
13	140	15	4.0	92.35 ± 0.23^{ab}	1.28 ± 0.06^{bcd}	11.74 ± 0.24^{bcd}
14	140	15	5.6	92.09 ± 0.57^{ab}	1.48 ± 0.14^{a}	12.68 ± 0.66^{a}
15	140	20	4.0	92.17 ± 0.65^{ab}	1.27 ± 0.08^{bcd}	11.66 ± 0.43^{bcd}
16	140	20	5.6	91.71 ± 0.54^{b}	1.37 ± 0.07^{ab}	11.91 ± 0.63^{abc}
17	140	25	4.0	92.43 ± 0.63^{ab}	1.25 ± 0.09^{bcde}	11.26 ± 0.34^{bcde}
18	140	25	5.6	91.99 ± 0.31^{ab}	1.34 ± 0.06^{bc}	11.97 ± 0.71^{ab}

[a-f] Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ±SD. N=

Based on the Carr's index and Hausner ratio, agglomerated samples flowability from fair to very poor. All processing parameters were evaluated had influenced the agglomerates shape, density and agglomerated powders flowability.

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Chapter 6 - Conclusions

Whey protein-based powders are widely used as ingredients in numerous food product formulations including infant formulas, high protein dairy bars, and beverages to improve nutritional and functional properties of the finished product. Many approaches are performed to modify the physio-chemical characteristics of whey proteins including enzymatic hydrolysis. The type and specificity of the enzyme influence the properties of the resultant hydrolysate. WPH has been shown to work as a binder in WPI wet agglomeration. However, more studies are needed to have a better understanding of the physical, chemical and functional properties of WPH, optimizing the agglomeration process conditions and studying their effects on the resultant agglomerated WPI characteristics. The physical and functional properties of WPH were investigated. The degree of hydrolysis (DH) of WPH was between 13.82 and 15.35% and not significantly ($P>0.05$) different between the lots. From the MALDI-TOF, 10 to 13 peptides were observed in the range of 2.5 – 5 kDa, and another two peptides were observed in the range of 5 – 8 kDa. It was also noticed from the HPLC data that the major whey proteins (α-LA, β-LG and BSA) were completely hydrolyzed indicating a consistent hydrolysis. Therefore, it was concluded that all three WPH lots were similar in their chemical characteristics. However, WPH samples were statistically different ($P<0.05$) in their physical properties such as particle size distribution, color, density, and water activity yet their values were practically similar.

WPH was used as a binder in WPI agglomeration process carried out in a top-spray fluid bed granulator (Midi-Glatt, Germany). Agglomerated WPI samples were stored at 25°C and analyzed for moisture, water activity, relative dissolution index (RDI), emulsifying capacity, agglomerates size and shape, Color, microstructure, and bulk and tapped densities. There was a significant ($P<0.05$) difference in moisture content (range: 3 – 15%) and water activity (range:

0.08 – 0.80) among the agglomerated powders. All agglomerated samples met the moisture content requirements with no clumps formation, except the treatments with combination of the highest pre-wet mass (140g) and high flow rate (5.6 $mL.min^{-1}$). The highest RDI was observed among the agglomerated WPI was 95.57%, followed by 86.56% and 84.12%. Pre-wet mass and Flow rate had the major significant ($P<0.05$) effect on RDI. The mean of agglomerates circle equivalent diameter (CED) was within the range of 13.63-17.96 μm with no significant difference observed for the CED ($P>0.05$) among the agglomerated samples for the main effects and their interactions. The agglomerates shape characteristics were determined by measuring the circularity, elongation, and convexity. The agglomerated samples circularity was within the range of 0.71-0.79 and there was a significant difference ($P<0.05$) among the agglomerated samples for the main effects and their interactions, except the WPH concentration × Flow rate interaction. Bulk density of the agglomerated samples was in between 0.22 and 0.31 g/cm^3. Agglomeration decreased the bulk density of WPI and had no effect on the tapped density. In conclusion, the use of WPH as a binder is a promising approach to produce "lecithin free" agglomerated high protein powders, which provides a product with clean label and minimized the possible health side effects of consuming lecithin-containing powders. In addition, the shelf life of agglomerated powders can be increased as WPH is not susceptible to oxidation as lecithin. Overall, the results suggest that WPH may be used as an alternative of soy lecithin in agglomerating WPI.

Appendix A - SAS code for the analysis

All data was analyzed using PROC GLIMMIX in SAS Studio (version 9.4; SAS Inst., Cary, NC). Here is an example of how the data was organized in SAS and the codes used in the analysis:

```
data MoistureContent;
   Input Num block Prewet WPHConc Flowrate MC aw @@;
   datalines;
1 1 100 15 11 3.7892 0.141   1 1 100 15 11 3.7103 0.161
2 2 100 15 11 5.3669 0.220   2 2 100 15 11 5.3509 0.243
3 3 100 15 11 4.3353 0.157   3 3 100 15 11 4.3081 0.182
4 1 100 15 16 5.7649 0.210   4 1 100 15 16 5.5933 0.234
5 2 100 15 16 5.6002 0.208   5 2 100 15 16 5.5422 0.231
6 3 100 15 16 4.9105 0.179   6 3 100 15 16 4.9346 0.201
7 1 100 20 11 4.6779 0.175   7 1 100 20 11 4.7925 0.193
8 2 100 20 11 4.1610 0.149   8 2 100 20 11 4.2793 0.173
9 3 100 20 11 3.8937 0.127   9 3 100 20 11 3.7552 0.149
10 1 100 20 16 4.0607 0.139   10 1 100 20 16 4.1026 0.152
11 2 100 20 16 3.6793 0.119   11 2 100 20 16 3.6793 0.135
12 3 100 20 16 3.5504 0.123   12 3 100 20 16 3.6127 0.153
13 1 100 25 11 4.2225 0.147   13 1 100 25 11 4.3910 0.165
14 2 100 25 11 3.4530 0.136   14 2 100 25 11 3.4854 0.161
15 3 100 25 11 3.3586 0.118   15 3 100 25 11 3.4858 0.138
16 1 100 25 16 3.7504 0.122   16 1 100 25 16 3.7039 0.146
17 2 100 25 16 3.8109 0.140   17 2 100 25 16 3.8051 0.192
18 3 100 25 16 3.4789 0.122   18 3 100 25 16 3.4389 0.143
19 1 140 15 11 3.2748 0.104   19 1 140 15 11 3.1533 0.126
20 2 140 15 11 3.2099 0.107   20 2 140 15 11 3.2677 0.139
21 3 140 15 11 3.4127 0.126   21 3 140 15 11 3.4996 0.135
22 1 140 15 16 20.2130 0.839   22 1 140 15 16 20.5033 0.784
23 2 140 15 16 13.6955 0.710   23 2 140 15 16 13.7667 0.698
24 3 140 15 16 10.1167 0.483   24 3 140 15 16 10.4739 0.483
25 1 140 20 11 3.6084 0.117   25 1 140 20 11 3.4827 0.125
26 2 140 20 11 3.4624 0.124   26 2 140 20 11 3.5680 0.130
27 3 140 20 11 3.4465 0.103   27 3 140 20 11 3.4816 0.122
28 1 140 20 16 7.6964 0.346   28 1 140 20 16 7.7059 0.313
29 2 140 20 16 6.0355 0.243   29 2 140 20 16 6.0149 0.259
30 3 140 20 16 8.7969 0.420   30 3 140 20 16 9.1011 0.392
31 1 140 25 11 3.4488 0.109   31 1 140 25 11 3.5023 0.128
32 2 140 25 11 3.4261 0.113   32 2 140 25 11 3.4835 0.129
33 3 140 25 11 3.7057 0.131   33 3 140 25 11 3.7734 0.138
34 1 140 25 16 10.7925 0.524   34 1 140 25 16 10.952 0.501
```

```
35 2 140 25 16 8.0900 0.384   35 2 140 25 16 8.1611 0.356
36 3 140 25 16 3.2879 0.120   36 3 140 25 16 3.1692 0.147
37 1 60 15 11 3.4901 0.119   37 1 60 15 11 3.3122 0.137
38 2 60 15 11 3.3540 0.117   38 2 60 15 11 3.5451 0.142
39 3 60 15 11 3.1376 0.112   39 3 60 15 11 3.3127 0.137
40 1 60 15 16 6.8451 0.288   40 1 60 15 16 6.9394 0.272
41 2 60 15 16 5.7723 0.246   41 2 60 15 16 5.8789 0.242
42 3 60 15 16 3.3260 0.112   42 3 60 15 16 3.4749 0.127
43 1 60 20 11 2.8252 0.092   43 1 60 20 11 2.8797 0.132
44 2 60 20 11 3.1977 0.107   44 2 60 20 11 3.2350 0.126
45 3 60 20 11 3.9175 0.118   45 3 60 20 11 4.0616 0.116
46 1 60 20 16 4.2029 0.118   46 1 60 20 16 4.1446 0.120
47 2 60 20 16 4.3047 0.116   47 2 60 20 16 4.2980 0.123
48 3 60 20 16 4.0473 0.104   48 3 60 20 16 3.8737 0.104
49 1 60 25 11 4.0004 0.120   49 1 60 25 11 3.9413 0.117
50 2 60 25 11 3.6167 0.105   50 2 60 25 11 3.6571 0.114
51 3 60 25 11 2.8523 0.083   51 3 60 25 11 2.8378 0.088
52 1 60 25 16 4.2736 0.132   52 1 60 25 16 4.2049 0.136
53 2 60 25 16 4.2129 0.118   53 2 60 25 16 4.1972 0.122
54 3 60 25 16 4.3791 0.129   54 3 60 25 16 4.2404 0.127
;

proc print data=MoistureContent;
run;

proc glimmix data = MoistureContent;
       class block prewet wphconc flowrate;
       model MC= block prewet|wphconc|flowrate;
       lsmeans prewet|wphconc|flowrate / adjust=tukey lines;
run;

proc rsreg data=MoistureContent out=cont plots=surface;
  model MC=Prewet WPHConc Flowrate/predict;
run;

proc rsreg data=MoistureContent out=cont plots=surface(3d);
  model MC=Prewet WPHConc Flowrate/predict;
run;

proc glimmix data = MoistureContent;
       class block prewet wphconc flowrate;
       model aw= block prewet|wphconc|flowrate;
       lsmeans prewet|wphconc|flowrate / adjust=tukey lines;
run;
```

```
proc rsreg data =MoistureContent out=cont plots=surface;
  model aw=Prewet WPHConc Flowrate/predict;
run;

proc rsreg data=MoistureContent out=cont plots=surface(3d);
  model aw=Prewet WPHConc Flowrate/predict;
run;
```

Appendix B - Chapter 4

Table B.1. The mean square and probabilities of main effects and their interactions for moisture content, water activity, relative dissolution rate, and emulsifying capacity

	MC (%)		Water activity		RDI (%)		EC (%)	
Factor	DF	MS	DF	MS	DF	MS	DF	MS
Block	2	14.19 (<0.01)	2	0.02 (<0.01)	2	48.81 (0.33)	2	0.05 (0.62)
Pre-wet mass	2	80.47 (<.0001)	2	0.22 (<.0001)	2	340.01 (<0.01)	2	0.66 (<0.01)
WPH concentration	2	35.73 (<.0001)	2	0.10 (<.0001)	2	83.25 (0.15)	2	0.17 (0.19)
Pre-wet mass* WPH concentration	4	10.31 (<0.01)	4	0.02 (<0.01)	4	266.62 (<0.01)	4	0.19 (0.13)
Flowrate	1	181.50 (<.0001)	1	0.39 (<.0001)	1	494.19 (<0.01)	1	0.24 (0.13)
Pre-wet mass* Flow rate	2	103.76 (<.0001)	2	0.28 (<.0001)	2	665.37 (<.0001)	2	0.03 (0.75)
WPH concentration* Flow rate	2	33.09 (<.0001)	2	0.07 (<.0001)	2	252.56 (<0.01)	2	0.03 (0.78)
Pre-wet mass* WPH concentration* Flow rate	4	13.42 (<.0001)	4	0.03 (<.0001)	4	278.47 (<0.01)	4	0.04 (0.83)

Appendix C - Chapter 5

Table C.1. The mean square and probabilities of main effects and their interactions for CE diameter, HS circularity, elongation, and convexity

	CE Diameter (μm)		HS Circularity		Elongation		Convexity	
Factor	DF	MS	DF	MS	DF	MS	DF	MS
Block	2	4.03 (0.49)	2	0.00 (0.46)	2	0.00 (0.33)	2	0.00 (0.84)
Pre-wet mass	2	3.98 (0.49)	2	0.01 (<.0001)	2	0.01 (<.0001)	2	0.00 (<0.01)
WPH concentration	2	2.75 (0.61)	2	0.00 (0.15)	2	0.00 (<.01)	2	0.00 (0.73)
Pre-wet mass* WPH concentration	4	8.32 (0.22)	4	0.00 (<.0001)	4	0.00 (<.0001)	4	0.00 (0.34)
Flowrate	1	2.97 (0.47)	1	0.01 (<.0001)	1	0.01 (<.0001)	1	0.00 (0.44)
Pre-wet mass* Flow rate	2	2.28 (0.67)	2	0.00 (<0.01)	2	0.00 (<.0001)	2	0.00 (0.54)
WPH concentration* Flow rate	2	16.31 (0.06)	2	0.00 (0.91)	2	0.00 (0.37)	2	0.00 (0.27)
Pre-wet mass* WPH concentration* Flow rate	4	1.96 (0.84)	4	0.00 (0.26)	4	0.00 (0.09)	4	0.00 (0.92)

Table C.2. The mean square and probabilities of main effects and their interactions for bulk density, tapped density, and color

	Bulk density		Tapped density		Color (L*)		Color (a*)		Color (b*)	
Factor	DF	MS	DF	MS	DF	MS	DF	MS	DF	MS
Block	2	0.00 (0.15)	2	0.00 (0.02)	2	0.11 (0.63)	2	0.09 (<.0001)	2	0.14 (0.39)
Pre-wet mass	2	0.01 (<.0001)	2	0.00 (0.10)	2	0.73 (0.04)	2	0.44 (<.0001)	2	7.75 (<.0001)
WPH concentration	2	0.00 (0.50)	2	0.00 (0.38)	2	0.57 (0.09)	2	0.04 (<.0001)	2	0.81 (0.01)
Pre-wet mass* WPH concentration	4	0.01 (<.0001)	4	0.01 (<.0001)	4	0.21 (0.45)	4	0.01 (0.12)	4	0.28 (0.13)
Flowrate	1	0.00 (0.07)	1	0.00 (0.09)	1	0.55 (0.12)	1	0.09 (<.0001)	1	2.12 (<0.01)
Pre-wet mass* Flow rate	2	0.01 (<.0001)	2	0.01 (<.0001)	2	0.51 (0.11)	2	0.03 (<.0001)	2	0.89 (<0.01)
WPH concentration* Flow rate	2	0.00 (<.01)	2	0.00 (<.01)	2	0.29 (0.28)	2	0.00 (0.75)	2	0.10 (0.53)
Pre-wet mass* WPH concentration* Flow rate	4	0.00 (0.01)	4	0.00 (<.0001)	4	0.90 (<0.01)	4	0.01 (0.01)	4	0.43 (0.03)

Table C.3. The mean square and probabilities of main effects and their interactions for angle of repose, compressibility, and Hausner ratio

	Angle of repose		Compressibility		Hausner ratio	
Factor	DF	MS	DF	MS	DF	MS
Block	2	17.43 (0.44)	2	9.11 (0.55)	2	0.00 (0.61)
Pre-wet mass	2	30.86 (0.24)	2	218.90 (<.0001)	2	0.08 (<.0001)
WPH concentration	2	10.51 (0.60)	2	36.30 (0.10)	2	0.01 (0.11)
Pre-wet mass* WPH concentration	4	37.25 (0.14)	4	27.69 (0.13)	4	0.01 (0.13)
Flowrate	1	105.81 (0.03)	1	0.09 (0.94)	1	0.00 (0.95)
Pre-wet mass* Flow rate	2	84.40 (0.02)	2	55.63 (0.03)	2	0.02 (0.03)
WPH concentration* Flow rate	2	32.52 (0.22)	2	21.49 (0.25)	2	0.01 (0.25)
Pre-wet mass* WPH concentration* Flow rate	4	23.37 (0.36)	4	92.65 (<0.01)	4	0.03 (<0.01)