THE EFFECT OF GELATIN BLOOM STRENGTH ON DRY EXTRUDED PET FOOD AND INJECTION MOLDED TREATS

by

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Abstract

Pet food is a $23 billion industry that continues to grow. Owners continue to humanize their pets and their dietary needs, thus the pet food industry tends to mirror human dietary trends. Currently, pet food is trending towards higher levels of protein, thus lower levels of starch. Decreasing starch, one of the main structure forming ingredients in extruded foods, creates issues in terms of lower rates of expansion and decreased kibble durability. Consumers tend to dislike ingredients that do not serve a dual nutritional purpose; therefore gelatin may be a plausible binding ingredient for high protein pet foods.

Gelatin is a pure protein derived from collagen and is sold as a dry, odorless, tasteless powder. High-bloom gelatins find numerous uses in the human food as a stabilizer, foaming agent, and capsule base among other uses. Low-bloom gelatin may find a value-adding opportunity as a nutritional binder in the pet food market.

Four extrusion experiments were performed to test this hypothesis. Experiment 1 compared gelatin at 0%, 5%, 10%, and 15% inclusion and 15% gelatin at 3 different extruder screw speeds. Results showed a decrease in expansion but an increase in hardness and pellet durability index (PDI); however there may have been inadequate preconditioning. It was unclear whether the decrease in expansion or presence of gelatin improved product durability. Experiment 2 analyzed two levels of gelatin, 0% and 10%, under two extruder screw speeds, 300 rpm and 500 rpm, and two hydration ratios, 17% and 28%. In this experiment, there were no differences in density, expansion, hardness, or PDI. This indicated that preconditioning was more ideal and may indicate gelatin does not decrease product expansion. Experiment 3 analyzed two levels of gelatin, 0% and 10%, at two target densities, low and high. Results indicated that gelatin created a more expanded product when processed under similar conditions as a control

formula. Experiment 4 analyzed different strengths of gelatin to determine if the low-bloom gelatin experiments were repeatable with more conventional strength gelatins. Treatments were a control with no gelatin, and a 100 bloom, 175 bloom, and 250 bloom gelatin. Results showed increased gelatin strength increased product expansion, likely through a foaming effect. However, durability declined with mid- and high-bloom gelatins; thus, low-bloom gelatin may be the most promising to improve product characteristics and preserve durability.

Two additional experiments were performed in order to explore gelatin bloom strength in injection molded treat processing. A lab-scale experiment was performed to optimize an initial formula. Tensile strength, strain at break, Young's Modulus, puncture force, and peaks were measured. It was determined that equal parts gelatin, gluten, and glycerin were most ideal for further testing purposes. Determination of gelatin bloom strength effects with three bloom strength gelatins were used to produce beadlets on a pilot-scale twin-screw extruded and production model injection molding system. Differences were noted between treatments; wherein high bloom gelatin created a softer, more stretchy treat and low bloom gelatin created a tougher, more rubbery treat.

Low-bloom gelatin may find use as a nutritional binder in high protein pet foods and may be an alternative to high-bloom gelatin in injection molded dental treats.

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Acknowledgements

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Chapter 1 - Gelatin in Food and Feed: A Review

Pet food is a nearly $23 billion per year industry and growing (APPA 2015). As of 2015, over 54% of homes own a dog and nearly 43% of homes own a cat (APPA). In total, 77.8 million dogs and 85.8 million cats are owned in the United States. Owners of these dogs and cats spend $269 and $246 per year on food, respectively (APPA 2015).

Pet ownership and spending on pet food continues to increase regardless of the economy, suggesting that the pet food industry is one of the more recession-resistant segments of our economy. Some of this increase in pet food spending has been attributed to the continual humanization of pets; including attention given to their dietary needs. Some of the most popular human diet trends include the Atkins or Paleo diets, both of which encourage increased protein intake while eschewing carbohydrates – particularly cereal grains. This trend is mirrored in the pet food market. Wherein, several pet food brands promote products as "grain-free." This has moved from a niche selling point to a near mainstream standard. These major brands have created both grain-free formulations and continue to tout increasing levels of protein or higher levels of animal protein sources. This trend is certainly popular from a marketing standpoint, but can create issues during processing. High levels of protein cause a decrease in extruded product density and durability. Increasing protein creates a product matrix that is less elastic, therefore less expandable, and more apt to crumble and create fines during further processing.

To understand how this trend becomes a processing issue, the various roles of ingredients must be discussed. According to the Guy Classification System, there are 7 different classes of ingredients used for extrusion (Guy, 2001): structure forming, dispersed phase fillers, plasticizer and lubricants, soluble solids, nucleating agents, flavoring agents, and coloring agents. One additional class of ingredients omitted from this system includes nutritional additives such as vitamins, minerals, or any nutraceuticals. Of these ingredient classes, structure forming ingredients and dispersed phase fillers play some of the stronger roles in the final structure of a pet food extrudate. Structure forming ingredients are those that undergo a phase change within the extruder, starting as a glassy solid, melting into a rubber state, and then finally experiencing rapid expansion upon steam flash-off at the die exit. The unique characteristic of structure forming ingredients is the ability to quickly become rigid during steam flash-off. This characteristic allows the product to maintain a porous, expanded texture. Starches and some plant-based proteins are typical structure forming ingredients.

Dispersed phase fillers perform the inverse action of structure forming ingredients and serve to disrupt the formation of air cells in the final product. This disruption occurs by either puncturing cell walls as they form or decreasing the elasticity of the overall extrudate. Animal-based proteins, which are commonly used in the pet food industry, are typical examples of dispersed phase fillers.

The balance of structure forming ingredients and dispersed phase fillers has a strong impact on the characteristics of pet food. While literature on the inclusion of higher levels of protein, specifically in pet food, is difficult to find, literature does exist regarding more complex human food extrudates. The inclusion of protein can increase the nutrient density of extruded foods but decreases expansion, increases bulk density, and shifts the texture from crispy to crunchy (Day and Swanson, 2013). Density and textural changes are related to product expansion. Textural changes in particular are dependent on both expansion in general and the changes in the internal structure. The inclusion of added protein, either plant or animal-based, can cause the collapse of internal voids (Onwulata et al., 2013) and can cause a disruption in the creation of cell walls and lead to a decrease in both shearing and breaking strength of the final product (Day and Swanson, 2013).

Much of the extrusion literature in human foods focuses on the use of plant-based proteins sources, such as soy or gluten (Brennan et al., 2013). However, the trend in pet food is to use animal-based proteins in even more complex formulations than typical human food extrudates (Beaton, 2015). It is generally understood that animal-based protein sources, such as chicken by-product meal, will always act as a dispersed phase filler ingredient, thus always reducing the expansion and durability of the final product. Rather than attempting to alter the conditions used to produce pet food, perhaps research efforts would be better spent finding an alternative ingredients that can act as some sort of structure enhancer.

A potential structure enhancing ingredient is gelatin. Gelatin is a pure-protein that is used in many different ways within the food industry. To understand how gelatin may serve as a binder in extruded foods, one must start with the history of gelatin.

The History and Modernization of Gelatin

Gelatin has a rather extensive history, partly due to the fact that "It is relatively cheap to produce in quantity, and there is a ready supply of suitable raw material" (Johnston-Banks, 1990). This statement holds true 25 years later because as long as there is a market for meat consumption there will always be a steady supply of bones, skin, and hide to serve as gelatin raw material.

However, the history of gelatin begins with the production of animal glue (Johnston-Banks, 1990). The manufacture of animal-derived glues dates back to at least the 1800s. The manufacturers of animal glue relied on the hydrolysis of collagen sourced from bone, skin, or hide. The transition to gelatin production likely stemmed from a desire to increase the value of their end-products, according to Schreiber and Gareis (2007).

The first patent for the gelatin manufacturing process was established in 1845 by a Mr. Peter Cooper (US4084; Cooper, 1845), but it would take another 45 years before the patent would be put into action. The transition to gelatin production began between 1890 and 1891. C. Knox and E. Rousselot both began their namesake companies by changing over some of their current glue manufacturing plants to gelatin manufacturing plants (Schreiber and Gareis, 2007). While this change-over process was not thoroughly documented, it is likely the transition took minimal effort as both glue and gelatin manufacturing begins with the extraction of collagen from slaughterhouse by-products, followed by a refining process.

C. Knox's "Knox Gelatin" and E. Rousselot's "Rousselot" companies still exist today. Knox Gelatin has since been sold to Kraft foods, while Rousselot, now owned by Darling Ingredients International, has grown to be one of the largest gelatin manufacturers (Schreiber and Gareis, 2007).

Today, gelatin has numerous uses particularly within the food and pharmaceutical industries – the variety of which will be addressed in a later section. However, it should be noted that within the U.S. greater than 50% of the gelatin tonnage produced is used in gelatin-based desserts, and approximately 10% of the gelatin tonnage produced in any developed country is used in pharmaceutical applications of either capsule or emulsion medications (Djagny et al., 2001).

Clarity, transparency, and degree of purity are all significant to gelatins sold specifically for food and pharma-grade use (Baziwane & He, 2003). While degree of purity cannot be altered, the clarity and transparency can be aided by certain additives. Decolorizing agents and adsorbents such as aluminum sulfate and aluminum hydroxide can improve the clarity of gelatin. Monocalcium phosphate can be used to improve the transparency of gelatin (Figure 1). The pH is also a factor that determines if gelatin is used in desserts; wherein, the ideal pH of dessert gelatin is between 3.0 and 3.5 in order to provide a palatable tartness (Djagny et al., 2001).

While many characteristics are important to the quality of gelatin and determine its use, the primary characteristic that determines how it is sold and marketed is bloom strength. Bloom strength is a measure of the strength or toughness of the final gel. The typical bloom classifications are high bloom (250-300 bloom), medium bloom (150-200 bloom), and low bloom (50-100 bloom; Johnston-Banks, 1990). The official principle for measuring bloom strength as defined by the Gelatin Manufacturers Institute of America is as follows (GMIA, 2013): "The gel strength of gelatin is a measure of rigidity of a gel formed from a 6.67% solution and prepared according to certain arbitrary prescribed conditions. Bloom is a measure of the force (weight) required to depress a prescribed area of the surface of a sample a distance of 4 mm."

The method used to measure bloom strength requires a relatively low concentration solution and covers the 3 necessary steps to form a quality gel:

1. The gelatin must be allowed to "bloom". This blooming step is the full hydration of the gelatin before the application of heat.
2. Heat is applied to activate the gelatin. A gelatin will not form a gel if heat is not applied.
3. The gelatin is cooled and allowed to fully set. Gelatin is one of the few cold-set hydrocolloids.

Gelatin bloom strength is determined based on the specifics of the raw material source and the exact manufacturing process used.

Gelatin Manufacturing

To discuss the manufacture of gelatin, one must start with the raw material – collagen. Collagen is a protein that is defined by the following characteristics (Baziwane & He, 2003):

1. Three helical polypeptide chains with the repeating sequence "Gly-X-Y" wound in a stable triple helix (Figure 2);
2. One-third of the 20 amino acids present are glycine;
3. Wide-angle X-ray diffraction pattern exhibits a 2.86Å meridional arc and a 12Å equatorial reflection;
4. The molecule possesses a high negative optical rotation;
5. The molecule contains hydroxyproline and hydroxylysine – imino and amino acids, respectively, that are almost unique to collagen.

Collagen is the most prevalent protein in the animal kingdom, accounting for about 30% of the protein in humans and a similar content in most species. The exact amino acid content differs by species, but all collagen has a glycine on every third site on the chain. This particular composition of amino acids and structure allows collagen to add strength and support to the tissues and organs of all animals (Johnston-Banks, 1990).

The differences in specific amino acid composition create at least 10 known types of collagen, however only two of these types of collagen are used for deriving commercial gelatin: Type I and III (Hudson, 1994). Type I collagen is the sole type of collagen found in bones and is the prominent form of collagen in skins and hides. This form of collagen has a more rigid, three-dimensional network that creates a strong structure in the animal body. Type III collagen is also found in skins and hides but only accounts for 10-20% of collagen in this organ. This type of collagen forms a two-dimensional network that still allows for the flexibility of the structure of skin or hides (Johnston-Banks, 1990).

Collagen is structurally arranged in a fashion similar to muscles. Collagen is first arranged in fibrils, which are bundles consisting of four to five collagen molecules. These fibrils then associate in increasingly larger bundles (Figure 3). These bundles are stabilized by cross-linking of lysine or hydroxylysine residues at the terminal ends of the collagen chains (Figure 4). The bundling effect and cross-linking of chains creates the structural stability of collagen. Furthermore, the cross-linking creates water insolubility and nearly complete protection from enzymes. The only enzyme that can break down collagen is collagenase (Johnston-Banks, 1990). The cross-linking also becomes stronger as the source of collagen becomes more mature. The strength of these cross-links dictates the type and severity of processing needed to extract the collagen from the raw materials (Johnston-Banks, 1990).

There are many raw material sources of collagen. The most popular and available sources of collagen are pig skin, cattle hides, and bones of either species (Baziwane and He, 2003). However, gelatin can also be extracted from other animal sources. Gelatin derived from marine fish skins is increasing in popularity due to religious dietary restrictions; Judaism and Islam both prohibit the consumption of pork and pork products, and beef can only be consumed if processed according to strict religious requirements (Choi and Regenstein, 2000). Some research has been done to compare the characteristics of fish gelatin to more well-known pork gelatins. Choi and Regenstein (2000) used both instrumental and sensory techniques to compare fish and pork gelatins. Their results showed that fish gelatin had a lower melting point, but resulted in fewer off-flavors than pork gelatin. This reduction in off-flavors allows the final gelatin to have a better release of aroma and a stronger final flavor. However, these differences were rather minimal and their overall conclusion was that fish and pork gelatin were similar. Rawdkuen et al. (2013) compared two sources of fish gelatin, catfish and tilapia, to beef gelatin. The authors compared these fish gelatins and beef gelatin through proximate analysis and the analysis of functional properties. These authors also concluded that fish gelatin was chemically and functionally similar to commercial beef gelatin. Though fish gelatin is gaining popularity, information about porcine and bovine-sourced gelatin is more widely available. Therefore, this review will continue to focus on pork and beef-based gelatins.

Though bones, cattle hides, and pig skin are the most common sources of gelatin, the preparation of these raw materials for collagen extraction is vastly different (Schreiber & Gareis, 2007). The highly cross-linked, Type I collagen of bones requires the most aggressive pre-extraction treatment. Bones are first chipped into approximately 0.5cm cubes. The reduction in particle size increases and equalizes the surface area of the bone fragments to allow for faster and more uniform extraction. These bone cubes, or bone chips, are degreased in hot water for 30 minutes using strong mechanical agitation. After degreasing, the bone chips are dried and sorted by particle size. By-products of this stage in processing are fat and meat, as well as bone meal used for fertilizer.

Bone chips that are of a suitable size to continue through gelatin manufacturing processes are then treated with a 4-6% HCl solution that solubilizes the calcium phosphate and calcium carbonate portions of the bone. This demineralization process results in a residue known as ossein which is the true raw material needed for further processing. Ossein is the proteinaceous structure that provides the framework of bones (Schreiber and Gareis, 2007). This demineralization process is time consuming and requires the multiple steps of treat, soak, rinse, and repeat. The processing time can be decreased with the inclusion of heat and pressure via an autoclave-like machine, but these forces result in lower quality gelatins (Schreiber and Gareis, 2007).

For hide derived gelatin, preparation begins with separating the layers of the hide. There are essentially three layers to cattle hides that all serve different purposes. The outer layer is removed for leather production and the inner fatty layer is separated for applications as beef tallow. The center layer of the skin, called the split, is the most valuable for gelatin production as it is almost purely collagen. The thickness of each layer is highly dependent on the climate where the cattle are raised – cattle raised in warmer climates tend to have thinner hides and vice versa. The separation and breakdown of hide splits is aided by the use of an alkali solution, either a lime or caustic soda solution (Baziwane and He, 2003). The alkali solution is more effective when hides have been reduced to a more uniform size (Schreiber and Gareis, 2007).

Pigskin is perhaps the most important, and most used, raw material in modern gelatin manufacturing (Grand View Research, 2014). Pigskin can be processed similarly to cattle hides by being separated into splits but it is more commonly left whole. Modern swine are processed at a young age (less than 12 months) which means the collagen in their skin is weakly cross-linked. This allows skin to be separated from the fatty tissue during meat processing and directly processed using a weak acid solution. During this acid processing any remaining fat attached to the pigskin easily floats to the surface and can be skimmed off (Schreiber and Gareis, 2007).

As noted previously, different raw materials require either acid-based or alkali-based pretreatment. These two different pre-treatment methods are the difference between creating Type A or Type B gelatin. Type A gelatin is derived from acid pre-treated raw material and has an isotonic point of 7-9 (Baziwane and He, 2003). It is important to note that sulfuric or hydrochloric acids are more commonly used because they do not impart off-odors and flavors in the final gelatin, unlike cheaper phosphoric or organic acids (Schreiber and Gareis, 2007). Acid pre-treatment is a less aggressive processing method and can be accomplished in an 18-24 hour period (Johnston-Banks, 1990). Type B gelatin is derived from alkali pre-treated raw materials and has an isotonic point of 4-5 (Baziwane and He, 2003). A lime solution is the most common method of alkaline pre-treatment. Up to a 3% lime solution can be combined with calcium chloride or caustic soda. With calcium chloride, the pre-treatment process can take up to 8 weeks or more, but when caustic soda is used the process can be shortened to 10-14 days (Johnston-Banks, 1990).

Both acid and alkaline pre-treatments can be done using batch, continuous, or semi-continuous extraction methods. All extraction methods use drinking quality water heated to 50-100°C. Batch extraction requires collagen to be washed for 4-7 hours multiple times until extraction is complete. Washing is done over an extended period of time to prevent the emulsion of any remaining fat. This residual fat can decrease the clarity and overall quality of the final gelatin product if it cannot be removed. The first wash will result in the highest bloom, most colorless gelatin. With each subsequent wash, the bloom strength of the final gelatin decreases and the final gel color increases due to some Maillard browning (Schreiber and Gareis, 2007).

Continuous extraction uses a counter-current feeding of raw material in to the continuous extraction system. This method of feeding the raw material helps to increase the final bloom strength of the product. Bovine hides are the typical raw material for continuous extraction systems. This type of system must be occasionally purged to remove any insoluble material that gathers in the system. This purged material can also be re-extracted to produce a low bloom gelatin (Schreiber and Gareis, 2007).

Semi-continuous extraction also uses a continuous method of feeding raw material in to the system but involves multiple extraction collection stages. With each extraction stage the final gelatin bloom strength decreases (Schreiber and Gareis, 2007). This method combines the continuity of the continuous extraction method with the flexibility in final product bloom strength found in the batch extraction method.

After the collagen is extracted, the remainder of the gelatin process is the same for each type of raw material (Figure 5; Schreiber and Gareis, 2007). Gelatin is filtered to remove any undissolved solids and residual fat and deionized to remove additional mineral salts and reduce the overall ash content. The solution is then concentrated by reducing the water content to approximately 50% through evaporation, sterilized using direct steam heat or indirect plate heat exchangers, and finally dried to a final moisture content of 10-15%

While prepared gelatin it a good host for microbe growth, dry gelatin powder is very shelf-stable. Dry gelatin powders are typically labeled with a 5 year shelf-life. However, as long as no moisture is introduced the shelf-life can extend past a 5-year date (Schreiber and Gareis, 2007). This extended shelf-life makes gelatin convenient and popular to use in many applications.

The Health Benefits of Gelatin

Gelatin is poised to increase in use and popularity over the next several years, particularly as the overall world population continues to increase. The population is increasing faster in the underprivileged sector which makes the resource of food and the availability of good nutrition a concern. Gelatin is a relatively inexpensive pure protein allowing it to be a good source of protein and certain amino acids to the underprivileged population (Schreiber and Gareis, 2007). While gelatin may be deficient in tryptophan and low in methionine, it is very high in lysine and overall highly digestible (Baziwane and He, 2003). Additionally, gelatin sourced from fish would be a viable option for those sectors of the population with religious dietary restrictions. Further, not only is the overall population increasing, but the percentage of the population considered elderly continues to increase (UN, 2015). There is considerable growth in both the supplement and cosmetic industries that focus on anti-aging (Schreiber and Gareis, 2007). Gelatin can be a natural source of substances that promote healthy joints and a younger appearance.

In addition to growing and aging, society is continually becoming more conscious about health. As more research is published in nutritional fields about dietary needs and supplement benefits, consumers become more willing to alter their diets and lifestyles in the name of living longer, more fulfilling lives. Current diet trends heavily focus on decreasing the consumption of fats, carbohydrates, and overall Calories. Gelatin can be easily substituted in many formulations where fat or carbohydrates must be removed (Schreiber and Gareis, 2007). The gelling properties of gelatin can maintain a smooth mouth feel in the absence of fats and can provide a binding substitute in the absence of carbohydrates (Gad and Mohamad, 2014).

Some of the health benefits of gelatin come from the specific amino acid content. On average, the amino acid profile of gelatin is as follows (Hudson, 1993): 27% glycine, 25% proline or hydroxyproline, 10% glutamic acid, 9% alanine, 8% arginine, 6% aspartic acid, 15% other amino acids.

The concentration of proline or hydroxyproline makes gelatin an interesting substance to study from a digestibility standpoint. Gelatin is understood to be a highly digestible substance, which is why it is so appealing to add to foods meant for nutritionally-deficient individuals. In an older study by Hueckel and Rogers (1970) they found that a basic diet including or excluding gelatin tested on humans, rats, hamsters, dogs, and monkeys made it easier to study the digestibility of certain amino acids. The hydroxyproline of gelatin can both be absorbed in the intestine or excreted based on whether or not it is bound by digestive-resistant peptides. Their findings were that humans made the best model to understand the absorption of amino acids as humans are the most efficient at using proline and hydroxyproline.

Finally, some research demonstrates the antioxidant properties of some fractions of collagen. Ao and Li (2012) noted that certain peptides can scavenge free radicals, chelate transition metal ions, and inhibit lipid peroxidation. The authors specifically studied the major constituent amino acids of glycine, proline, glutamine, hydroxyproline, and alanine, as well as the minor amino acids of cysteine, tyrosine, methionine, and histidine. Overall, the authors found that basic, rather than acidic, amino acids exhibit stronger chelating properties and are more strongly related to the antioxidant activity of a protein.

Collectively, these health benefits make gelatin very attractive to use in human foods and a promising ingredient to include in pet foods.

Use of Gelatin in Food and Feed

Gelatin has numerous uses within the food industry. The wide variety of gelatin strengths and viscosities lend well to a wide range of applications, which are described by Baziwane and He (2003). Perhaps the oldest application of collagen is as an adhesive. The animal adhesive process was refined in order to turn collagen in to the food source now known as gelatin. Gelatin is commonly used as an additive or main ingredient that aids in crystal formation in frozen foods, foam formation in confectionary applications, or the clarification of fruit juices or wines. Gelatin also aids in the texture of foods. Gelatin can be used to stabilize foods such as yogurt and prevent the syneresis of water, improve the mouth feel of syrups and soups, and aid in emulsifying water and oil. Outside of the food industry, gelatin finds use in the pharmaceutical industry. Gelatin can be used to make a thin film that is ideal for microencapsulation of liquids or creating hard or soft capsule medications and supplements.

Minimal research exists in the area of gelatin applications to pet food and animal feed. A study by Wulansari et al. (1999) looked at starch conversion during extrusion and the impact of adding gelatin to their product matrix. Gelatin and starch mixtures are common in some confectionary applications. These confectionary applications can also utilize extrusion processing. To test the impact of the gelatin on the conversion of starch during extrusion, Wulansari et al. (1999) used a simple product matrix of gelatin – a 225 bloom gelatin originating from alkaline-processed hides – and waxy maize starch. Waxy maize is a low amylose starch which was used in this simple ingredient matrix to prevent the creation of amylose-lipid complexes. The formation of amylose-lipid complexes can confound analysis of starch gelatinization. Gelatin and starch were mixed in the following ratios of gelatin to starch: 8:2, 6:4, 4:6, and 2:8. These treatments were extruded on a co-rotating, intermeshing, twin-screw extruder (Clextral) fitted with a slit-shaped die. The resulting extruded ribbons did not expand. These unexpanded ribbons were ground, the gelatin was extracted using an enzymatic process, and were analyzed using differential scanning calorimetry (DSC), X-ray scattering, and viscoelastic rheological measurements. From this the authors concluded that gelatin inhibited full starch gelatinization as they found higher levels of native or ungelatinized starch in products that contained higher levels of gelatin. These findings were considered important to the confectionary industry as the ratio of gelatin to starch could be used to control the gelatinization of starch in confectionary products. However, this can also give us some insight as to the use of gelatin in animal feed and pet food. While this matrix was simple compared to the potential ingredient matrix of pet food, it may still give some insight as to the additional considerations to processing parameters that could be necessary for the use of gelatin in pet food. Additional thermal or mechanical energy may be needed to hydrate gelatin and ensure complete starch gelatinization.

Conclusions

Pet food is a large and growing industry. Current trends in pet food focus on increasing the protein content, but this increase in protein can decrease the expansion and durability of the final product and thus have an impact on overall consumer appeal. Gelatin is used in numerous applications in the food industry, and some confectionary extrusion experiments show that gelatin may be more functional than a typical animal-based protein. This potential functionality, in combination with the potential health benefits and flexibility of use, should make gelatin an attractive ingredient to consider for pet food formulations.

Figures

Figure 1.1 Increasing yellowing of the color of gelatin due to the extraction stage (Schreiber & Gareis, 2007).

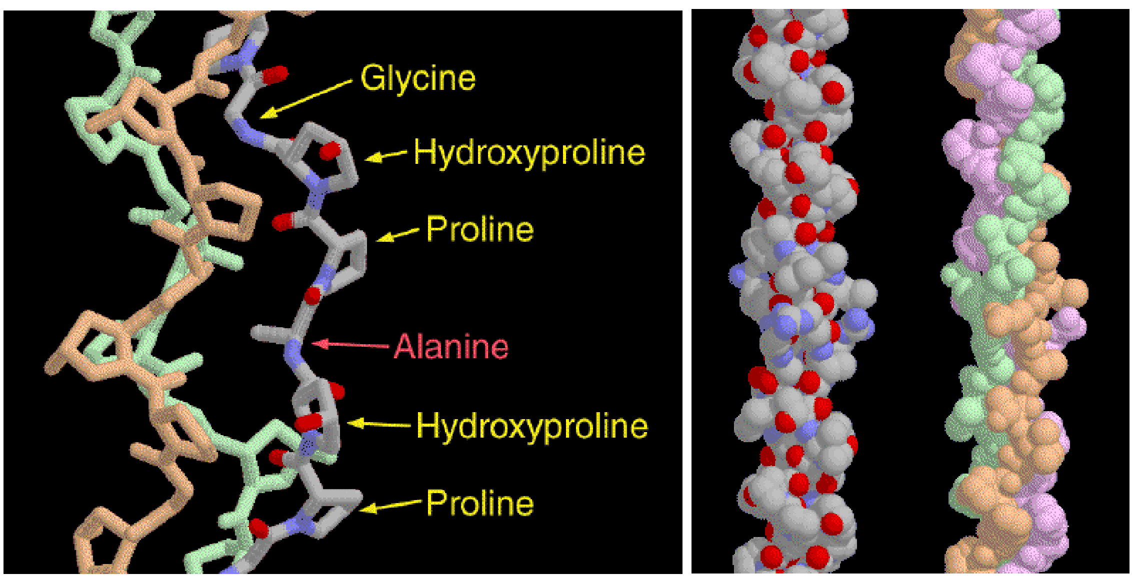

Figure 1.2 Computer-generated images representing the Glycine amino acid at every third location on a collagen chain, and the resulting triple helix structure of a collagen molecule (Goodsell, 2000)

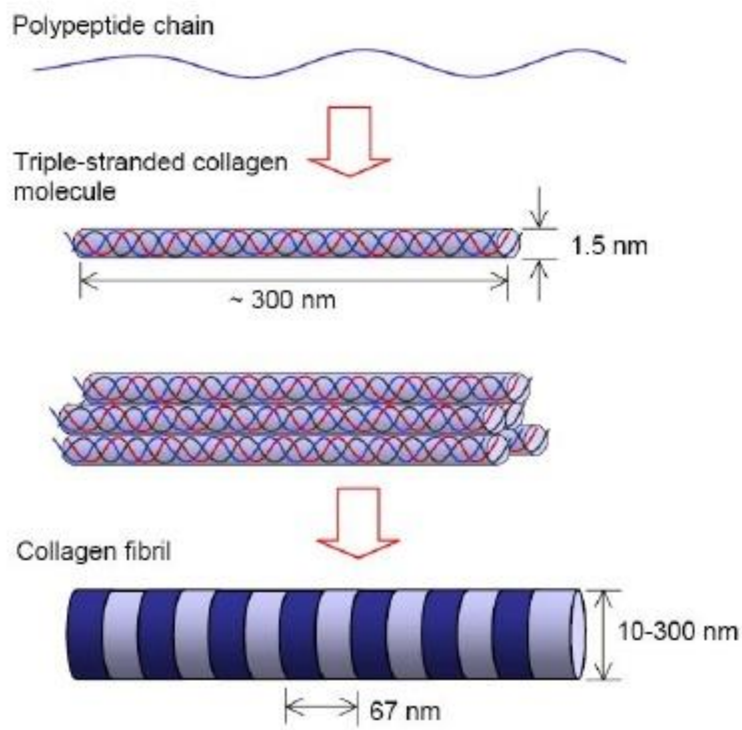

Figure 1.3 The arrangement of collagen starting from a polypeptide chain up through the association to a fibril (Scheff, 2010).

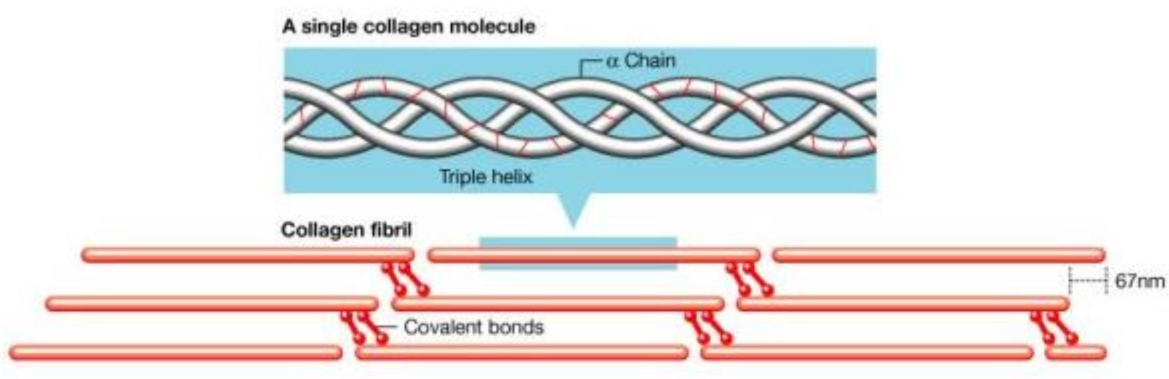

Figure 1.4 The crosslinking of collagen fibrils by covalent bonds (Schultz et al, 1992).

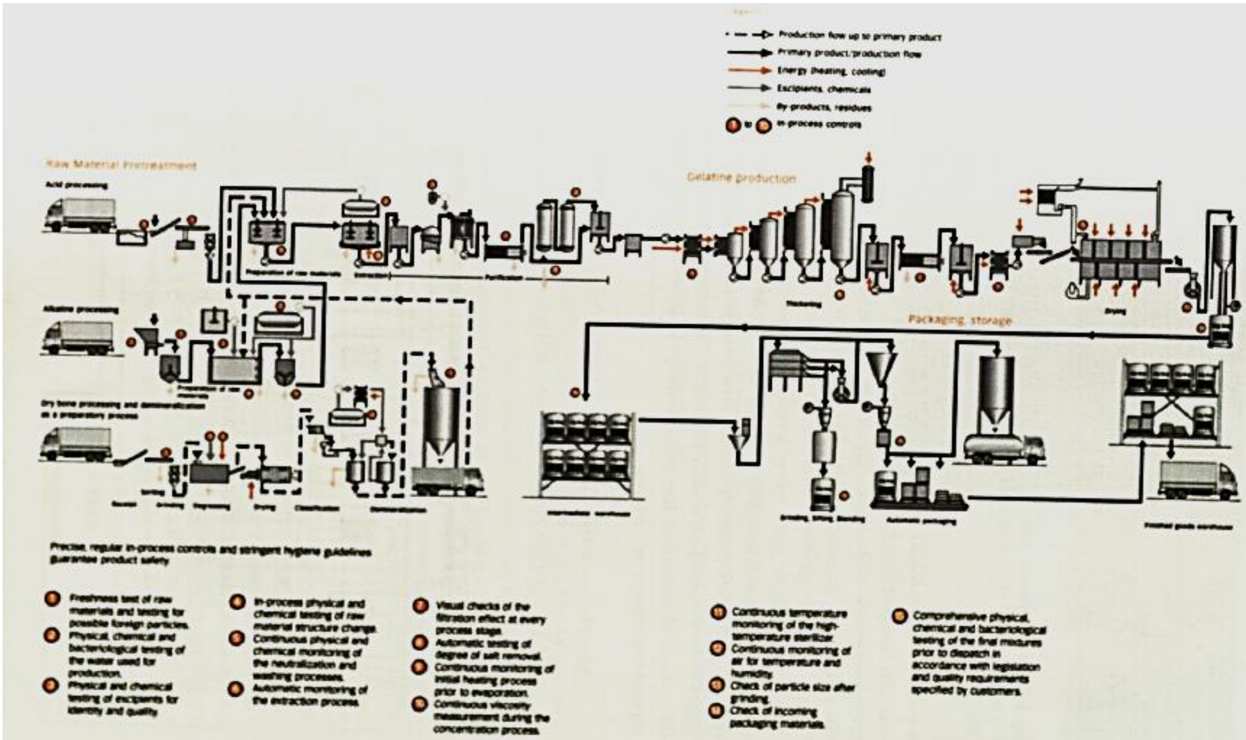

Figure 1.5 A schematic of the gelatin manufacturing process (Schreiber & Gareis, 2007).

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Chapter 2 - The Effect of Gelatin Inclusion in High Protein Extruded Pet Food on Kibble Physical Properties

1.0 Introduction

Pet food is a rapidly growing industry. The industry grew from $22.26 billion in 2014 to just over $23 billion estimated in 2015. In the current economy, few industries continue to see the steady growth like the pet food industry. Pet owners are spending an average of $269 per year on dog food and $246 per year on cats (APPA, 2015).

Humanization of pets spurs trends that drive the growth of the industry. Human food trends are currently driven popular diets that promote increased protein consumption and tend to eschew traditional cereal grain sources of carbohydrates (Mileen et al., 2015). Naturally, these trends have been paralleled in the pet food industry due to the close connections between owners and their pets.

The issue with increasing the protein content of pet food is apparent during processing. Increasing the protein content inherently decreases the starch content. When starch sources or plant-based proteins are replaced by animal proteins, products become less expanded, denser, and more fragile. This effect is described by Zhu et al. (2010). Zhu et al. (2010) studied combinations of high amylose corn starch and soy protein concentrate, and found that high levels of soy protein caused low levels of expansion due to starch-protein interactions where the proteins interfered with the continuous matrix of the product. In pet food, this is an issue in multiple ways from processing through to consumer use. Typically, extruded products do not use binders or ingredients strictly used to improve the cohesion of the internal matrix. In part because starches are usually in high enough proportions to provide significant structural enhancement and, with the push towards more natural pet foods, consumers are leery about ingredients that have no nutritional value. If they are missing because of market drivers, then we need an alternative like low-bloom gelatin to fill the void.

While low bloom gelatin has yet to be used in extruded foods, there may be an opportunity for its incorporation as a binding ingredient in higher protein pet food. As a nearly pure protein, gelatin provides nutritional value and it may have a functional use within a food matrix to improve structural durability. Therefore, our objectives were to determine the effect of low bloom gelatin in a moderate protein extruded pet food diet on processing parameters and physical properties of the kibble.

2.0 Materials and Methods

Two initial experiments were performed to determine the changes in kibble physical properties when gelatin was used at different inclusion rates in a dry extruded pet food. Experiment 1 (Table 1) tested 4 levels of gelatin – 0% (OG), 5% (5G), 10% (10G), and 15% (15G) – and three different extruder screw speeds on the 15G diet – a high screw speed of 500 rpm (15GH), a moderate screw speed of 400 rpm (15GM), and a low screw speed of 300 rpm (15GL). Experiment 2 (Table 2) tested 2 levels of gelatin – 0% and 10% - 2 screw speeds – low speed at 300 rpm and high speed at 500 rpm – and two different hydration ratios – 17% and 28%. Hydration ratio was expressed as the proportion of total water injected as water into the extruder.

Two additional experiments were performed to determine the relationship between gelatin level and product density and to determine the effect of increasing gelatin bloom strength on kibble properties. Experiment 3 (Table 3) compared 0% gelatin (0G) with 10% gelatin (10G) at a low target density (LD) and a high target density (HD). Experiment 4 (Table 4) compared a control diet with 0% gelatin (OG) with 3 strengths of gelatin – low bloom (100G), mid bloom

(175G) and high bloom (250G). These diets were processed once through a circle die (C) and once through a triangle die (T).

2.0.1 Diet Preparation

Diets for all experiments were formulated according to the American Association of Feed Control Officials Publications dog food nutrient profiles for all life stages (AAFCO, 2015). Four diets were formulated with four different levels of a low-bloom gelatin (Table 5): 0G was the control formula and contained no gelatin, 5G contained 5% gelatin, 10G contained 10% gelatin, and 15G contained 15% gelatin. Gelatin was added at the expense of chicken by-product meal to maintain consistent levels of starch in all diets. Select micro-ingredients were added to 5G, 10G, and 15G to keep the entire diet isonutritional. Further, in Experiment 1, only a small amount of chicken fat was also added to 5G, 10G, and 15G (Table 6).

The low-bloom gelatin used was a 100-bloom pork bone gelatin (Pro-Bind Plus 100, Sonac, Darling Ingredients International, Irving, TX). Mid-bloom and high bloom gelatins were 175-bloom pig skin and 250-bloom pig skin (Pig Skin 175 and Pig Skin 250, Rousselot, Darling Ingredients International, Irving, TX). All other ingredients were purchased from a local mill (Lortscher Animal Nutrition, Bern, KS). Brewers’ rice, corn, wheat, and beet pulp were ground to pass through a 0.50 mm screen (Fitzmill hammermill, Continental Agra Equipment Inc., Newton, KS). These ingredients, along with chicken by-product meal and gelatin, were weighed on a large digital scale with 0.01 kg sensitivity and then mixed in a double ribbon mixer for 5 minutes. Potassium chloride, salt, choline chloride, DL methionine, dry natural antioxidant, trace mineral premix, vitamin premix, calcium carbonate, taurine, monosodium phosphate, and L-tryptophan were weighed according to the formula using a laboratory scale with 0.001 g sensitivity and combined in a small container and added to the double ribbon mixer and mixed for an additional 3 minutes. In experiment 1, chicken fat was added to 5G, 10G, and 15G slowly into the ration while the mixer was running. Once the fat was added, the diet was mixed for an additional 2 minutes. In all other experiments, chicken fat was excluded from the formulation as it would typically be added topically to the kibbles following processing. Diets were then stored in labeled 25kg bags.

2.0.2 Extrusion Conditions Applicable to all Experiments

Diets for all experiments were processed through a differential diameter cylinder preconditioner, single screw pilot-scale extruder, and double pass dryer (DDC Preconditioner, X-20 Extruder, and 4800 Series Dryer, Wenger Manufacturing, Sabetha, KS). The screw configuration used in both experiments consisted of the following: extruder inlet, single flight uncut, small steam lock, single flight uncut, small steam lock, single flight uncut, medium steam lock, double flight uncut, large steam lock, and double flight uncut cone. The dryer was set at 104°C with 5 minute for each of the three passes.

2.1.0 Experiment 1 Processing Conditions

The following were the processing conditions used in experiment 1: feeder screw speed – 18.0 rpm, preconditioner cylinder speed – 400 rpm, preconditioner water – 18.0kg/hr, preconditioner steam – 15.0 kg/hr, extruder water – 6.0kg/hr, temperature zone 1 - 50°C, temperature zone 2 - 70°C, temperature zone 3 - 90°C, and knife speed – 1360 rpm. Extruder screw speed varied according to the treatment, with 4 treatments being processed at a moderate screw speed of 400 rpm, one treatment being processed at a low screw speed of 300 rpm, and one treatment being processed at a high screw speed of 500 rpm. The extruder was fit with a 3.19mm diameter circle die and a hard knife.

2.2.0 Experiment 2 Processing Conditions

The following were the processing conditions used in experiment 2: feeder screw speed – 18.0 rpm, preconditioner cylinder speed – 400 rpm, temperature zone 1 - 50°C, temperature zone 2 - 70°C, temperature zone 3 - 90°C, and knife speed – 1428 rpm. Extruder screw speed, preconditioner steam, preconditioner water, and extruder water varied according to treatment. Extruder screw speed was 300 rpm for two treatments and 500 rpm for four treatments. Preconditioner steam, preconditioner water, and extruder water was varied according to treatment to achieve the 17% and 28% hydration ratios. The 17% hydration ratio used for 4 treatments was achieved by injecting 11 kg/hr of preconditioner steam, 14 kg/hr of preconditioner water, and 5 kg/hr of extruder water. The 28% hydration ratio used for 2 treatments was achieved by injecting 9 kg/hr of preconditioner steam, 11 kg/hr of preconditioner water, and 8 kg/hr of extruder water. The extruder was fit with a 3.19 mm diameter circle die and a hard knife.

2.3.0 Experiment 3 Processing Conditions

The following were the constant processing conditions used: dry feed rate – 174 kg/hr, feeder screw speed – 18.0 rpm, preconditioner steam – 16.0 kg/hr, preconditioner water – 14.0 kg/hr, extruder water – 11.0 kg/hr, temperature zone 1 – 50°C, temperature zone 2 – 70°C, temperature zone 3 – 90°C, and knife speed – 1793 rpm. The extruder was fit with a 4.6 mm diameter circle die and a hard knife. The dryer was set at 104°C with 5 minute passes.

Two different product densities were achieved by varying the extruder screw speed and the opening of a throttle valve fitted between the end of the extruder and the die assembly. The OGHD was achieved with an extruder screw speed of 500 rpm and the throttle valve turned 5½ times resulting in an average die pressure of 550 psig. The OGLD was achieved with an extruder screw speed of 550 rpm and the throttle valve turned 7 times resulting in an average die pressure of 500 psig. 10GHD was achieved using an extruder screw speed of 500 rpm and the throttle valve turned 4 times resulting in an average die pressure of 380 psig. 10GLD was achieved using an extruder screw speed of 550 rpm and the throttle valve turned 5½ times resulting in an average die pressure of 390 psig.

2.4.0 Experiment 4 Processing Conditions

Diets were processed through a differential diameter cylinder (DDC) preconditioner, X-20 single screw pilot-scale extruder, and 4800 Series double pass dryer (Wenger Manufacturing, Sabetha, KS). The screw configuration used in both experiments is as follows: extruder inlet, single flight uncut, small steam lock, single flight uncut, small steam lock, single flight uncut, medium steam lock, double flight uncut, large steam lock, double flight uncut cone. The extruder was also fit with a throttle valve between the end of the extruder and the die assembly.

The following are the processing conditions used across all treatments: feeder screw speed – 16.2 rpm, preconditioner cylinder speed – 400 rpm, preconditioner steam – 16.0 kg/hr, preconditioner water – 15.0 kg/hr, extruder screw speed – 545 rpm, extruder water – 16.0 kg/hr, temperature zone 1 – 50°C, temperature zone 2 – 70°C, and temperature zone 3 – 90°C. The throttle valve was turned down 5 turns for all treatments, and the resulting die pressure ranged from 440-500 psi. All 4 diets were first processed through a 4.5 mm diameter circle die in a randomized order. The extruder was then shut down and the die was replaced with a 5.3 mm base by 4.9 mm height triangle and all 4 diets were processed a second time in a randomized order. Knife speed for all circle-shaped treatments was 2,046 rpm and knife speed for all triangle-shaped treatments was 2,038 rpm.

2.0.3 Product Analysis

All samples were stored in a freezer (0°C) after drying and prior to testing to prevent moisture loss. Samples were warmed to room temperature for at least 2 hours prior to analysis.

Products in Experiment 1 were analyzed for post-extrusion and post-drying moisture, post-extrusion and post-drying expansion ratio, post-extrusion and post-drying specific length, post-drying piece density, post-drying hardness, and post-drying pellet durability index (PDI).

Products in Experiment 2 were analyzed for post-extrusion and post-drying moisture, post-extrusion and post-drying expansion ratio, and post-drying specific length.

Products in Experiments 3 and 4 were analyzed for bulk and piece density, radial expansion, specific length, hardness, and pellet durability index (PDI).

Moisture was analyzed using the Association of Official Analytical Chemists (AOAC) method 934.01 (AOAC, 2010). Extrudates were first ground, and then 2 g of sample was dried at 95-100°C and the loss in weight was reported as moisture. Moisture was expressed in percent.

Expansion ratio represents the ratio of the size of the product relative to the size of the die to capture the degree of expansion that occurs in the radial or axial direction. Calipers were used to measure the diameter of the sample in triplicate. Expansion ratio (ER) was then calculated using the following formula:

$$ER = D_e^2 / D_d^2$$

Where D_e^2 is the average diameter of the extrudate squared and D_d^2 is the average diameter of the die squared. A higher value for expansion ratio corresponds to a more expanded, less dense product. Expansion ratio was expressed in mm^2/mm^2.

Specific length represents expansion in the longitudinal direction and is a ratio of the length of the sample to the weight of the sample. Calipers were used to measure the length of the samples, and a laboratory scale with 0.001 g sensitivity was used to weigh the samples. Specific length was expressed in cm/g.

Piece density represents the ratio of the weight of the product to the volume of the product on a single piece basis. Calipers were used to measure the diameter and height or length of the product. A lab scale with 0.001g sensitivity was used to weigh the samples. Piece density (PD) was then calculated using the following formula:

$$PD = Weight / (\pi r^2 h)$$

Where $\pi r^2 h$ is the volume of a cylinder and r is the radius and h is the height, or in the case of extruded product the length. Piece density is expressed in g/cm^3.

Hardness was determined using a texture analyzer (TA-XT2 with Exponent 32 software, Stable Micro Systems, Godalming, UK) fitted with a 38.1 mm diameter plastic compression probe (Texture Technologies, Hamilton, MA). A compression test with a pre-test and test speed of 1.0 mm/s, post-test speed of 2.0 mm/s, and 60% strain was used on 20 pieces of sample.

Pellet durability index (PDI) was analyzed using a pellet durability tester (Holmen NHP 100, Tekpro, Norfolk, UK) with 100 g of sample for 90 seconds. This instrument holds the sample in a perforated metal basket and forces air through the sample, causing the sample to impact the sides of the basket. The pneumatic force also carries any fines away from the remaining sample. At the end of the test, the remaining intact sample is weighed, and PDI is expressed as a percent based on the amount of intact kibble relative to the starting sample weight.

2.0.4 Statistical Analysis

Experiment 1 treatments were arranged to explore initial levels gelatin and extruder settings for future experiments by adding incremental levels of gelatin (0, 5, 10, and 15%) and extruder screw speeds (low, medium, and high rpm on the 15% gelatin treatment). Results were analyzed using the GLIMMIX procedure of SAS (SAS Institute, Cary, NC). Main effect means for gelatin level and extruder screw speed were separated by least squared means and considered significant at an alpha of 5%.

Experiment 2 was arranged in a fractional factorial arrangement of treatments evaluating two levels of gelatin (0 and 10%), extruder screw speeds (300 and 500 rpm), and hydration ratio (17 and 28%) after Collins et al., (2009). Results were analyzed using the GLIMMIX procedure of SAS (SAS Institute, Cary, NC). Main effect means were separated by least squared means and considered significant at an alpha of 5%.

Experiment 3 was arranged in a 2x2 factorial design with two levels of gelatin (0 and 10%) and two target densities (High and Low). Results were analyzed using the GLM procedure of SAS (SAS Institute, Cary, NC). Main effect means and the interaction of the two were separated by least squared means and considered significant at an alpha of 5%.

Experiment 4 was arranged in a 4x2 factorial arrangement of treatments with four levels of gelatin strength (0 gelatin, and 100, 175, and 150 bloom) and two die shapes to create replication. Results were analyzed using the GLM procedure of SAS (SAS Institute, Cary, NC). Main effect means for bloom strength are presented and differences were determined by least squared means and considered significant at an alpha of 5%.

3.0 Results

3.1 Experiment 1

No differences were noted ($P>0.05$) in post-extrusion moisture, post-drying moisture, and piece density in the presence of different levels of gelatin or under different extruder screw speeds (Table 7 and Table 8, respectively).

Post-extrusion and post-drying expansion ratios were influenced by gelatin level (Figure 1). Wherein, post-extrusion expansion ratio was similar for 0G and 5G (3.55 mm^2/mm^2 for 0G, and 3.64 mm^2/mm^2 for 5G), but lower ($P<0.05$) than 10G (6.49 mm^2/mm^2), and greater ($P<0.05$) than 15G (2.02 mm^2/mm^2). In a similar fashion, post-drying expansion ratio was lower ($P<0.05$; 4.27 mm^2/mm^2 for 0G, 3.31 mm^2/mm^2 for 5G) than 10G (6.65 mm^2/mm^2), but greater ($P<0.05$) than 15G (2.02 mm^2/mm^2).

Post-extrusion and post-drying expansion ratios were also influenced by extruder screw speed Figure 2). Post-extrusion expansion ratio was the lowest at 400 rpm and the highest at 500 rpm and intermediate at 300 rpm ($P<0.05$; 2.02 mm^2/mm^2 for 400 rpm, 6.27 mm^2/mm^2 for 500 rpm, and 5.56 mm^2/mm^2 for 300 rpm , and). Post-drying expansion ratio followed a similar pattern where it was intermediate at 300 rpm, lowest at 400 rpm and highest at 500 rpm ($P<0.05$; 5.83 mm^2/mm^2 , 2.40 mm^2/mm^2 and 7.02 mm^2/mm^2, respectively).

Post-extrusion length was not influenced by gelatin (average 3.58), but post-drying specific length was (Figure 3). Wherein, post-drying specific length for 0G, 5G, and 10G were similar to each other, but shorter than 15G (4.21, 4.05, and 4.16 cm/g vs. 4.83 cm/g for, respectively).

Post-extrusion and post-drying specific length was also affected by extruder screw speed (Figure 4). Wherein, post-extrusion specific length was greater ($P<0.05$) for 300 rpm than 400 rpm which was greater ($P<0.05$) than 500 rpm (4.57 cm/g, 3.83 cm/g, and 3.51 cm/g, respectively). Post-drying specific length was greater ($P<0.05$) for 300 rpm than for 500 rpm and 400 rpm was intermediate between them (6.26 cm/g vs. 4.38 cm/g and 4.83 cm/g, respectively).

Hardness increased with addition of gelatin ($P<0.05$), but additional gelatin did not cause a change ($P>0.05$; Figure 5). Wherein, hardness was 5.15 kg for 0G and averaged 9.30 for 5G, 10G, and 15G. Likewise the PDI increased ($P<0.05$) in with added gelatin but there was no effect due to increasing gelatin level. The hardness was also affected by screw speed wherein it was lowest at 9.27 kg for 300 rpm, and increased ($P<0.05$) when increased to 400 and 500 rpm (9.70 and 10.85 kg, respectively). The corresponding PDI was did not differ due to extruder rpm.

3.2 Experiment 2

In the second experiment the post-extrusion and post-drying moisture were not affected ($P>0.05$) by gelatin level, screw speed, or hydration ratio (Table 9). Likewise, post-extrusion and post-drying expansion ratio was not affected ($P>0.05$) by gelatin level, screw speed, or hydration ratio (Table 10). Specific length was not affected ($P>0.05$) by gelatin level or screw speed, but was affected ($P<0.05$) by hydration ratio (Table 11). In this case, specific length decreased ($P<0.05$) as the proportion of water in the extruder increased (4.12 cm/g for 17% vs. 3.76 cm/g for 28%).

3.3 Experiment 3

The bulk density and piece density were lower ($P<0.05$) for 10% gelatin (Table 12). Bulk and piece density decreased (351.83 g/L vs. 280.67 g/L and 0.52 g/cm^3 vs. 0.39 g/cm^3, respectively). Radial expansion increased ($P<0.05$) from 2.86 mm^2/mm^2 for 0G to 3.56 mm^2/mm^2 for 10G and the specific length also increased from 4.12 cm/g for 0G to 4.47 cm/g for 10G. Though hardness was unaffected, PDI decreased ($P<0.05$) in the gelatin containing diet (77.57% vs. 52.25% for 0G and 10G, respectively).

High target density corresponded to higher bulk density ($P<0.05$; 345.83 vs. 266.67 g/L for high density and low density, respectively). Piece density had a similar relationship to target (Piece density was 0.460 g/cm^3 vs. 0.441 g/cm^3 for high and low density, respectively). There was no effect ($P>0.05$) on radial expansion, specific length, or hardness (Table 13). However, the

PDI was lower for the low target density (72.17% vs. 57.65% for high and low target density, respectively).

3.4 Experiment 4

Bulk and piece density decreased ($P<0.05$) with each incremental increase in bloom strength (347.6, 310.65, 242.4, and 212.3 g/L and 0.56, 0.48, 0.39, and 0.33 for 0, 100, 175, and 250 gelatin bloom strength treatments, respectively; Table 14). The cross-sectional expansion and specific length increased with the corresponding increase in bloom strength. Hardness was lower for 0G than 100G, but greater than 175G and 250G (5.93 vs. 7.38, 4.57 and 3.59 kg, respectively). The PDI was similar ($P>0.05$) between 0G and 100G, and greater ($P<0.05$) than each of 175G and 250G (88.49% and 87.57% vs. 64.55% and 30.01%, for 0G and 100G vs. 175 G and 250G, respectively).

4.0 Discussion

The issue with increasing the protein content of pet food can be apparent during processing; wherein increasing the protein content directly decreases the starch content. This has an impact on the kibble physical stability or integrity. This relationship has been characterized previously. The classification system established by Guy (Guy, 2001) for ingredients in extruded products describes the importance of both starches and proteins. These ingredients fall under either the “structure forming” or “dispersed phase filler” categories, respectively (Guy, 2001). Structure forming ingredients typically have high starch content and are characterized by the way they melt inside the extruder and upon exiting the die quickly become rigid. This quick return to rigidity after the steam flash-off retains a highly expanded, porous texture typically expected of extruded products. Structure forming ingredients encompass typical cereal grain products such as corn meal and wheat flour, other sources of starch such as potato and tapioca, as well as some vegetable proteins such as soy protein or wheat gluten (Guy, 2001). Dispersed phase fillers tend to function opposite of structure forming ingredients. While dispersed phase fillers tend to hold more nutritional value than most starchy structure forming ingredients, they disrupt the expansion of the final product. Dispersed phase fillers are less elastic, thus do not become as fluid inside the extruder and do not stretch upon steam flash-off or quickly become rigid to retain a porous structure. Most animal-based proteins fall in to this category of ingredients (Guy, 2001).

When starch sources or plant-based proteins are replaced by animal proteins, products become less expanded and denser and more fragile (Storebakken et al., 2015). In pet food, this is an issue in multiple ways. A decrease in expansion creates issues in maintaining an appropriate bag fill which can cause consumer dissatisfaction. A change in expansion is also reflected in the texture of the product and may impact palatability depending on species (Houpt and Smith, 1981; Zaghini and Biagi, 2005). While this decrease in expansion may create a product with a harder, crunchier texture, this hardness in texture is not necessarily reflected in product durability. The decrease in starch creates an internal matrix that is not as tightly bound as a similar product with higher starch content, as starch content and starch type influence product characteristics (Chinnaswamy and Hanna, 1988). This weaker product matrix may create more fines as the product moves through the handling systems, dryer, cooler, bagging system, and transportation process. Increased fines become a processing concern as they take up bin space and are typically only reused in low inclusion levels. Fines are also undesirable to the consumer as it impacts the perceived value of the product, and are undesirable from a palatability standpoint.

Typically, extruded products do not use binders or ingredients strictly used to improve the cohesion of the internal matrix, as food extrusion usually relies on starches to create product structure (Guy, 2001). With the push towards more natural pet foods consumers are becoming leery about ingredients that are perceived to have minimal nutritional value like starches (Swanson et al., 2013). If they are removed because of market drivers, then something must takes its place. An alternative ingredient like low-bloom gelatin might fill the void.

Gelatin is a nearly pure-protein extracted from the collagen found in the bones and skin of cattle and swine. The collagen protein is extracted using either an acid or alkaline pretreatment soak. Collagen is then refined into gelatin through a multi-step process of filtration and clarification, deionization, concentration, sterilization, and drying (Schreiber and Gareis, 2007). This process creates a product that is a dry, odorless, tasteless powder (Baziwane and He, 2003). This gelatin powder is sold on the basis of a gelatin-specific quality measurement called bloom strength. Bloom strength is defined as a measure of rigidity of a gel formed from a 6.67% solution and prepared according to certain arbitrary prescribed conditions and is expressed in grams (GMIA, 2013). Simply put, bloom strength is a measure of how hard or tough a gel will form from the gelatin powder being sold.

Bloom strength varies according to the extraction stage at which the collagen is removed from bones or skin. Collagen is extracted in multiple stages in order to collect as much proteinaceous material as possible. Collagen extracted in the beginning stages has higher bloom strength and as the process continues the bloom strength decreases with each extraction stage. High bloom strength is greater than 250, medium or moderate bloom strength is typically between 150 and 200 but really encompasses any strength gelatin between 100 and 250, and low bloom strength gelatin is less than 100 (Johnston-Banks, 1990). Moderate and high bloom strength gelatins find multiple uses within the food industry as a gelling, thickening, stabilizing, or clarifying agent (Baziwane and He, 2003). However, low bloom gelatin is less often used in the food industry. It may find use as a clarifying agent for juices or wines (Baziwane and He, 2003), but overall it creates too weak a gel to function singly. However, low bloom gelatin has found some use in Europe as a binder for pelleted animal and aquatic feeds; wherein, low bloom gelatin was reported in a trade bulletin to be as effective as other more "typical" pellet binders while still providing some nutritional value (van der Velden and van den Bosch, 2011).

4.1 Experiment 1

In Experiment 1, 0%, 5%, 10%, and 15% low-bloom gelatins were incorporated into an isonutritional formula. All 4 formulas were processed under the same conditions, and the 15% gelatin formula was processed under 2 additional extruder screw speeds. Only the 15% gelatin diet was further processed due to resource constraints. As samples were pulled from the preconditioner it was observed visually that the increase in gelatin level led to increasing particle agglomeration. Gelatin is a very hydrophilic substance as it has been reported to absorb 5-10 times its volume of water (Baziwane and He, 2003). This was apparent during the preconditioning phase as the gelatin portions of the mixtures appeared to absorb the majority of water injected. The water absorption was not quantified during this experiment, but this may have been reflected in other final product characteristics such as product expansion; wherein, piece density did not change (Table 7 and 8). If the preconditioning moisture was primarily absorbed by the gelatin then the starches in the ration were likely not hydrated sufficiently for proper expansion (Dogan et al., 2010). Improper hydration during preconditioning impacts overall starch gelatinization and leads to reduced product expansion and textural changes (Chuang and Yeh, 2004). Further, there were no differences in moisture content for both post-extrusion and post-drying, across all treatments. So, gelatin inclusion, particularly as high as 15%, did not seem to impact moisture retention.

Expansion ratio and specific length were each affected by gelatin level and extruder screw speed (Figure 1 – 4). The true reason behind the inconsistent expansion ratio with increases in gelatin and (or) screw speed is not fully understood. It may relate to the inadequate hydration of the raw material observed from the preconditioner mash samples which is known to directly affect radial expansion of starches (Chuang and Yeh, 2004).

Furthermore, the relationship between expansion ratio and screw speed was not typical. Usually, increasing extruder screw speed creates more mechanical energy and more pressure behind the die which results in a higher rate of expansion due to the added steam flash-off (Dogan et al., 2010). Conversely, lower screw speeds may increase residence time within the extruder, but don’t create as much added pressure to increase expansion. In this experiment, expansion increased at both a lower screw speed of 300 rpm and a higher screw speed of 500 rpm relative to the moderate screw speed of 400 rpm. The higher expansion at the 500 rpm screw speed was expected. It was hypothesized that that slowing the extruder screw speed to 300 rpm may have increased the residence time and in turn allowed for more contact between the preconditioned material and the added water injected in the extruder. It was also noted during the experiment that preconditioning could have been incomplete due to the presence of gelatin and its more rapid absorption of the moisture relative to the starches. Therefore the increase in residence time in the extruder may have acted as an additional conditioning step and aided in the gelatinization of the starches; thus, increasing the final product expansion. Based on these results, gelatin may improve the expansion of pet food, but more exploration is needed to clarify the ideal preconditioner and extruder settings to optimize processing with formulations including gelatin.

The relationship between gelatin level and specific length suggests that gelatin may have a plasticizing effect which is more prominent with a higher level of gelatin in the formula (Wulansari et al, 1999). This added lubrication of the extruder barrel might have decreased the amount of mechanical energy incorporated into the product. While the length of the product could be adjusted by altering the knife speed, the increased rate of velocity of product through the die might also contribute to the low radial expansion of the 15% gelatin formula.

Extruder screw speed also affected specific length. Typically, an increase in extruder screw speed would be expected to increase specific length as the added mechanical energy increases pressure behind the die and thus increases product velocity. However, the presence of gelatin may create a plasticizing effect that is exaggerated at a lower screw speed. Furthermore, the lower screw speed may have allowed for added hydration of the gelatin. The longer residence time and added period of hydration may have allowed the gelatin to form more of a gel that coated the barrel for the "plasticizing-like" effect.

The increase in hardness and PDI with the addition of gelatin indicated that gelatin could improve the durability of high protein kibble (Figure 5 and 6). However, a decrease in product expansion inherently makes a product harder and more durable because the internal air cells decrease in size and the cell walls thicken naturally strengthening the product matrix. Further experiments are needed to clarify if the increase in durability was caused by the inclusion of gelatin or if it was simply a factor of the decrease in expansion.

4.2 Experiment 2

Experiment 2 was the beginning of additional exploration of this relationship; wherein, the treatments were organized to evaluate two levels of gelatin (0 and 10%), processed under two screw speeds (300 and 500 rpm) and two hydration ratios (17 and 28% of total moisture added at the preconditioner; Tables 9-11). The post-extrusion and post-drying moisture levels were unaffected by treatment. It should be noted that a change in moisture might be expected with the change in hydration ratio, but total moisture added was not altered. Rather, the proportion of water added in the preconditioner rather than extruder was the sole change. Even yet, it was surprising that there were no changes in either expansion ratio or specific length despite formulation and processing changes. However, all products had a rather high expansion ratio. In the previous experiment, the inclusion of gelatin led to a decrease in expansion; whereas, in this experiment, the inclusion of gelatin had no effect. This may indicate that the overall hydration of the raw material was adequate in this experiment, but preconditioning was still inadequate in the presence of gelatin.

Screw speed also had no effect on post-extrusion or post-drying expansion ratio. In the previous experiment, the slower screw speed increased product expansion likely because of the increase in conditioning perhaps due to the longer residence time. In this experiment, the product expansion similarity despite screw speed changes also indicates a more adequate preconditioning process.

Specific length previously increased with the addition of gelatin indicating it may have imparted a plasticizing effect. In this experiment, specific length was not affected by gelatin level or screw speed (Table 11). There was a slight difference in specific length due to changes in hydration ratio. Typically, increasing the amount of water in the extruder, as was done with the 28% hydration ratio, would have increased specific length because water can act as a plasticizer. However, the reverse was observed in this experiment and again may be reflective of the different preconditioning requirements for gelatin.

These two experiments provided a baseline of information about the use of low-bloom gelatin in a pet food, but demonstrated that more clarity was needed to further determine the relationship between gelatin inclusion and extruder processing conditions. Clarification was specifically needed regarding the relationship between gelatin inclusion and final product density. Based on experiments 1 and 2, the effect of gelatin on density may be dependent on the target density range. Experiment 1 demonstrated that gelatin may improve product durability in a low density range, but experiment 2 demonstrated that gelatin may improve expansion in a high density range.

4.3 Experiment 3

Experiment 3 was performed to compare 0% and 10% gelatin inclusion under similar processing conditions to achieve two distinctly different densities and provide more insight into the effect of gelatin on final product characteristics.

First, it was noted during extrusion that a higher inclusion of water, particularly in the preconditioning step, resulted in what appeared to be a higher degree of cook as the kibble had a darker, more intertwined center. This greater addition of water may have influenced some of the following results. Additional water likely allowed the gelatin to fully hydrate in the preconditioner while still hydrating starches and begin the gelatinization process as well.

The inclusion of gelatin affected bulk and piece density, expansion both radially and longitudinally, and PDI, but surprisingly not hardness (Table 12). Bulk density and piece density decreased with the inclusion of gelatin. As was expected with this trend in density, radial expansion and specific length both increased in the presence of gelatin. The more complete hydration of the both the gelatin and the starches likely allowed for a more complete gelatinization thus greater rate of expansion with the 10G formula. These decreases in density and subsequent increases in expansion support a hypothesis of a protein foaming effect when gelatin was added (Schonauer et al., 2004; Muller-Fischer and Windhab, 2004; Miquelim et al., 2009). Gelatin, and some grain-based proteins, in the presence of a plasticizer have been shown to create a stable foam, particularly when carbon dioxide or nitrogen is introduced via a gas foaming process (Salerno et al., 2007). While water and steam were the only plasticizers (Guy, 2001) used in this experiment and there was no introduction of gases, it is possible the steam flash-off at the die may have had a similar effect to gas foaming.

Typically, more expanded products are less durable. In this experiment, a decrease in PDI was seen, but interestingly there was no change in hardness. The higher rate of expansion may explain the decrease in PDI, however there may still be some effect of matrix enhancement due to gelatin inclusion that preserved hardness values. This hypothesis is somewhat reaffirmed when results were analyzed for the effects of high versus low density targets; wherein, low density led to a reduction in PDI but no change to hardness (Table 13). The decrease in PDI may be a result of the added expansion achieved with the addition of gelatin. This added expansion may be great enough that PDI cannot be preserved, while hardness is still somewhat aided by the binding ability of gelatin.

The hypothesis established after experiment 2 regarding the possible dual effect of gelatin at different density ranges may be further supported by the results of experiment 3 in which the effect of gelatin appeared to be dependent on both formulation and processing conditions.

4.4 Experiment 4

The fourth and final experiment was conducted to determine if the results observed in the first three experiments using low-bloom gelatin would be the same using mid- and high-bloom gelatin. Stronger gelatins currently find more use in the food industry.

Two die shapes were used to add replication to the study. Some differences occurred; however it is likely that this was a function of slight differences in die open area and not a true treatment effect. Therefore, data for the die shape were pooled and main effect means for gelatin strength are reported (Table 14). As gelatin strength increased, density (bulk density and piece density) decreased and expansion increased (cross-sectionally and longitudinally). These changes in density, and subsequently in expansion, are again representative of the potential of a protein foaming effect. High-bloom gelatin, like the 250-bloom used in this experiment, is typical for confectionary applications such as marshmallows. The gelatin, when combined with heat and air, creates a stiff foam that remains rigid as it cools (Baziwane and He, 2003). This effect would explain why the high-bloom gelatin created the most expansion and the expansion decreased as the strength of the gelatin lessened.

Typically, this increase in internal air cells and thinning of cell walls creates weaker product. This relationship was seen when PDI was measured. However, hardness had a more interesting relationship, as it was the strongest for the 100 bloom formula rather than the 0 bloom control. This indicated a stronger potential for binding capability when low-bloom gelatin was used.

Overall, it appeared that the use of gelatin in pet food increased product expansion and this improved with the gelatin strength. Low-bloom gelatin showed the most promise for preserving or even enhancing product durability simultaneous to an increase in expansion. However, it should be noted that the rate of expansion for mid- and high-bloom gelatins was much higher than the average expansion ratio for pet food, particularly pet food with higher protein content. Therefore it may be possible to achieve the same durability preservation with mid- and high-bloom gelatins if used in lesser concentrations.

5.0 Conclusions

In experiment 1, the inclusion of low-bloom gelatin increased kibble durability. However, gelatin inclusion may have led to inadequate preconditioning thus decreased expansion. In experiment 2, the effect of preconditioning was further explored, and in the presence of adequate preconditioning water, gelatin inclusion had no effect on product expansion. In experiment 3, the relationship between gelatin inclusion and product density was further explored. Gelatin inclusion increased expansion under gentler processing conditions relative to the gelatin-free formulation. In experiment 4, increasing gelatin bloom strength caused increases in expansion, but only low-bloom gelatin preserved kibble durability relative to no gelatin.

Overall, gelatin increased the expansion of high protein pet foods and improved durability in most experiments, but may have different effects in different density ranges. Based on these experiments, low-bloom gelatin increased expansion while preserving the durability of the kibble. While gelatin appears to be a potential solution for durability and expansion issues experienced when producing higher protein pet foods. However, further experimentation is needed to evaluate gelatin at lower levels more practical for industry use and to evaluate gelatin in diets that have a protein content greater than 30% that also follow the grain-free trend.

6.0 Tables and Figures

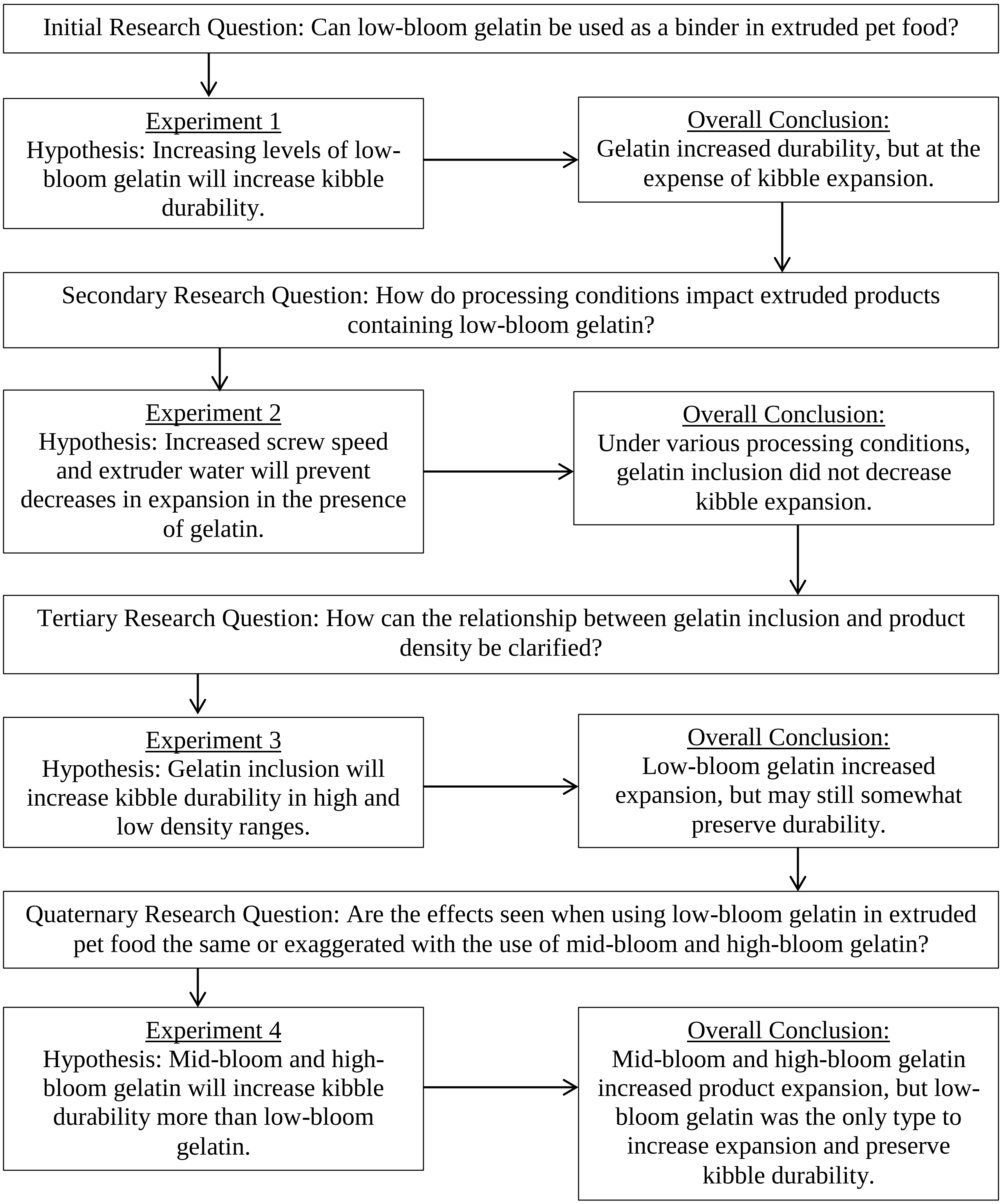

Figure 2.1 Flow diagram of experiment layout.

Table 2.1 Treatments for Experiment 1 pilot-scale extrusion processing comparing four levels of gelatin at three different extruder screw speeds.

Treatment Name	Gelatin Level (%)	Extruder RPM Target
0G	0.0	400
5G	5.0	400
10G	10.0	400
15GM	15.0	400
15GH	15.0	500
15GL	15.0	300

Table 2.2 Treatments for Experiment 2 to evaluate pilot-scale extrusion processing to compare two levels of gelatin processed at two different extruder screw speeds and 2 different hydration ratios*.

Treatment Name	Gelatin Level%	Extruder RPM Target	Hydration Ratio (%)*
0GL17	0	300	17
0GH17	0	500	17
0GH28	0	500	28
10GL17	10	300	17
10GH17	10	500	17
10GH28	10	500	28

*Hydration ratio is expressed in the percentage of total water added as liquid and steam into the extruder. The 17% hydration ratio was achieved with 5 kg/hr water in the extruder, 11 kg/hr steam in the preconditioner, and 14 kg/hr water in the preconditioner. The 28% hydration ratio was achieved with 8 kg/hr water in the extruder, 9 kg/hr steam in the preconditioner, and 11 kg/hr water in the preconditioner.

Table 2.3 Treatments for experiment 3 comparing two levels of gelatin at two different target densities.

Treatment Name	Gelatin Level	Target Piece Density
0GHD	0%	High
0GLD	0%	Low
10GHD	10%	High
10GLD	10%	Low

Table 2.4 Treatments for experiment 4 comparing different strengths of gelatin in a dry extruded pet food.

Treatment Name	Gelatin Bloom Strength	Die Shape
0GC	No Gelatin	Circle
100GC	100	Circle
175GC	175	Circle
250GC	250	Circle
0GT	No Gelatin	Triangle
100GT	100	Triangle
175GT	175	Triangle
250GT	250	Triangle

Table 2.5 Ingredient composition of control and experimental diets containing incremental levels of low-bloom strength gelatin.

Ingredient (%)	0G	5G	10G	15G
Pro-Bind Plus Gelatin	0.00	5.00	10.00	15.00
Chicken By-Product Meal	41.36	32.17	25.37	18.68
Brewers Rice	17.77	17.07	17.14	17.00
Corn	17.77	17.07	17.14	17.00
Wheat	17.77	17.07	17.14	17.00
Beet Pulp	4.23	4.00	4.00	4.00
Calcium Carbonate	0.00	0.00	0.00	0.36
Potassium Chloride	0.26	0.25	0.28	0.39
Salt	0.26	0.25	0.25	0.25
Choline Chloride	0.21	0.20	0.20	0.20
DL Methionine	0.06	0.15	0.25	0.34
Taurine	0.00	0.00	0.01	0.03
Monosodium Phosphate	0.00	0.00	0.49	1.03
L-Tryptophan	0.00	0.00	0.02	0.06
Natural Antioxidant, Dry	0.04	0.03	0.03	0.03
Trace Mineral Premix	0.11	0.10	0.10	0.10
Vitamin Premix	0.16	0.15	0.15	0.15
Chicken Fat	0.00	-	-	-

Table 2.6 Calculated nutritional values for four experimental diets containing incremental levels of low-bloom strength gelatin.

Nutrient	0G	5G	10G	15G
Moisture (%)	8.64	10.00	10.00	10.00
Crude Protein (%)	32.39	30.00	30.00	30.00
Crude Fat (%)	7.15	12.00	12.00	12.00
Ash (%)	7.68	6.20	5.79	5.85
Crude Fiber (%)	2.67	2.35	2.21	2.07
NFE (%)	42.17	39.99	40.43	40.40
ME (kcal/kg)	3217.44	3469.64	3484.91	3484.03

Table 2.7 Main effect means for post-extrusion and post-drying moisture and post-drying piece density in the presence of different levels of gelatin in Experiment 1*.

	0G	5G	10G	15G	SEM	P
Post-Extrusion Moisture (%)	24.35	23.63	22.85	25.00	1.53	0.64
Post-Drying Moisture (%)	9.59	10.73	10.83	10.72	0.93	0.78
Piece Density (g/cm^3)	0.44	0.58	0.69	0.69	0.01	0.20

*Evaluated at an extruder screw speed of 400 rpm.

Table 2.8 Main effect means for post-extrusion and post-drying moisture and post-drying piece density processed under different screw speeds in Experiment 1*.

	300 rpm	400 rpm	500 rpm	SEM	P
Post-Extrusion Moisture (%)	27.28	23.96	23.93	0.77	0.10
Post-Drying Moisture (%)	11.89	10.47	9.83	0.45	0.17
Piece Density (g/cm^3)	0.72	0.60	0.60	0.01	0.71

*Main effects of variables processed by different extruder screw speeds were evaluated at a gelatin level of 15%.

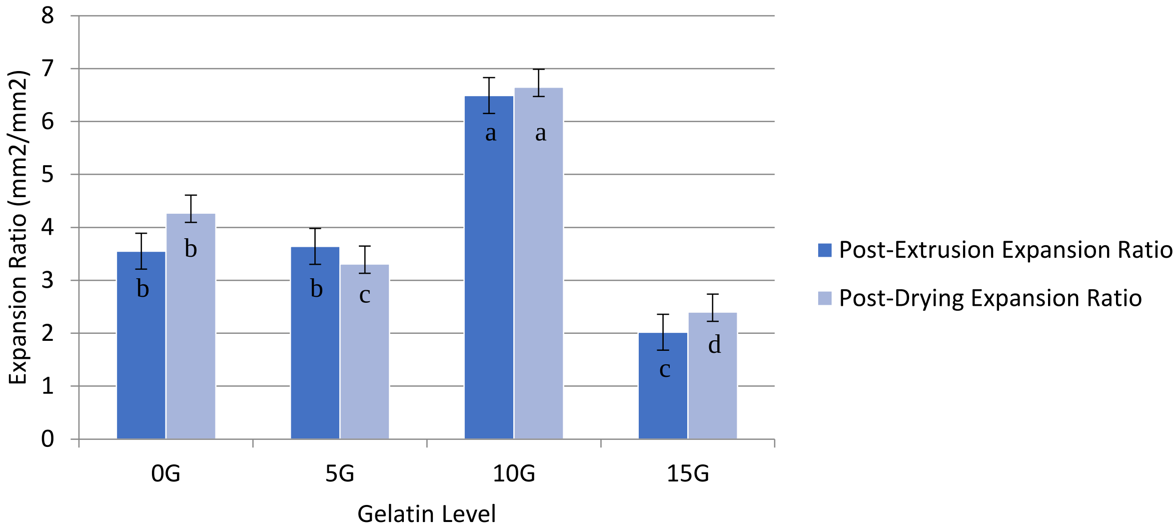

Figure 2.2 Main effect means of post-extrusion and post-drying expansion ratio for each gelatin level tested in Experiment 1.

[abcd]Columns within a variable with unlike superscripts differ by P<0.05.

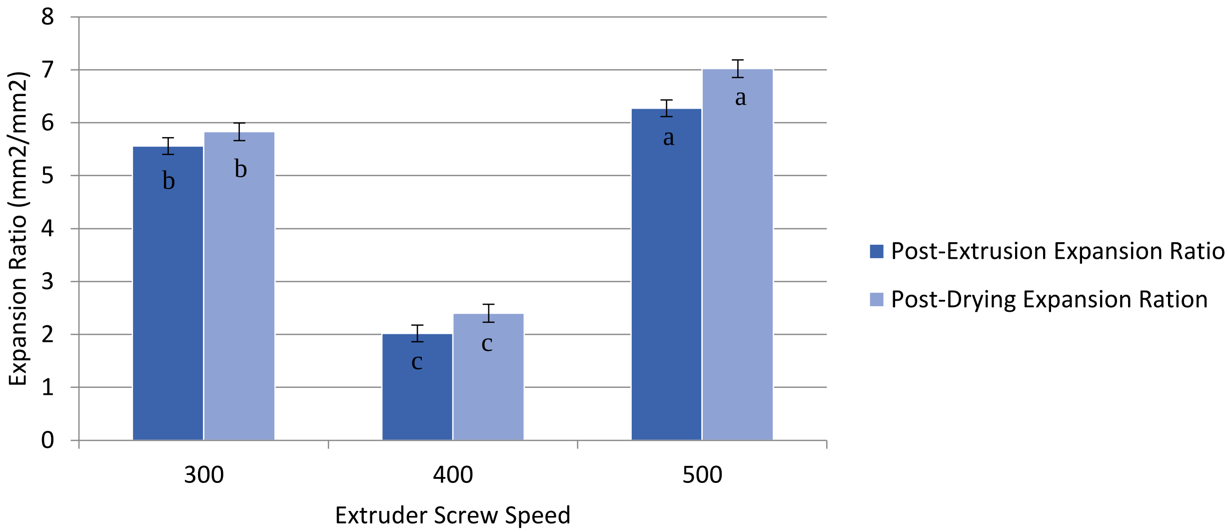

Figure 2.3 Main effect means of post-extrusion and post-drying expansion ratio dependent on extruder screw speed in Experiment 1.

[abcd]Columns within a variable with unlike superscripts differ by P<0.05.

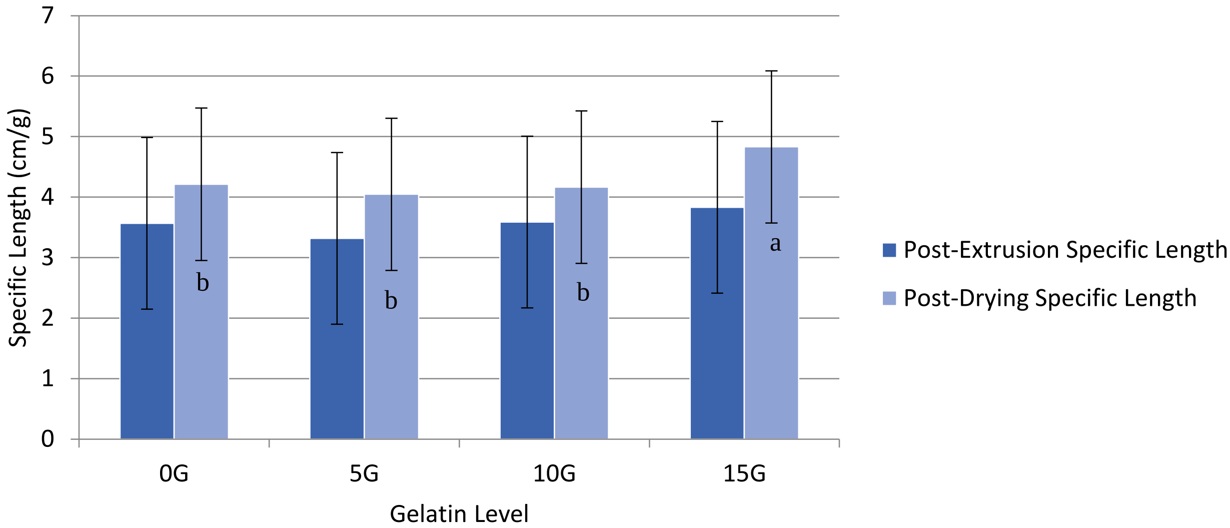

Figure 2.4 Main effect means of post-extrusion and post-drying specific length dependent on gelatin level in Experiment 1.

[ab]Columns within a variable with unlike superscripts differ by P<0.05.

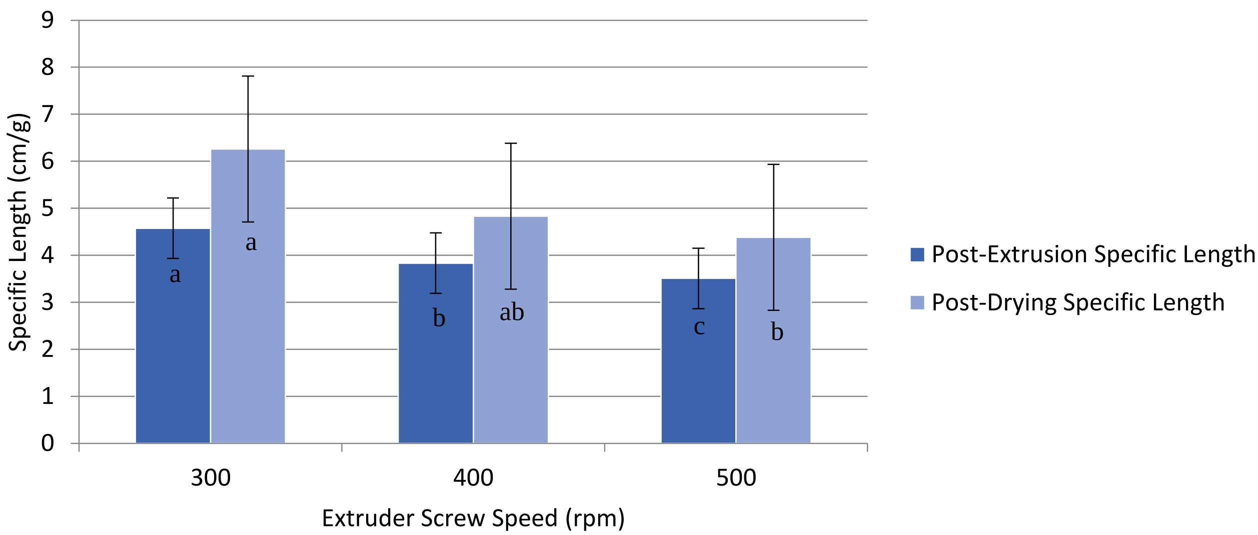

Figure 2.5 Main effect means of post-extrusion and post-drying specific length dependent on extruder screw speed in Experiment 1.

[abc]Columns within a variable with unlike superscripts differ by P<0.05.

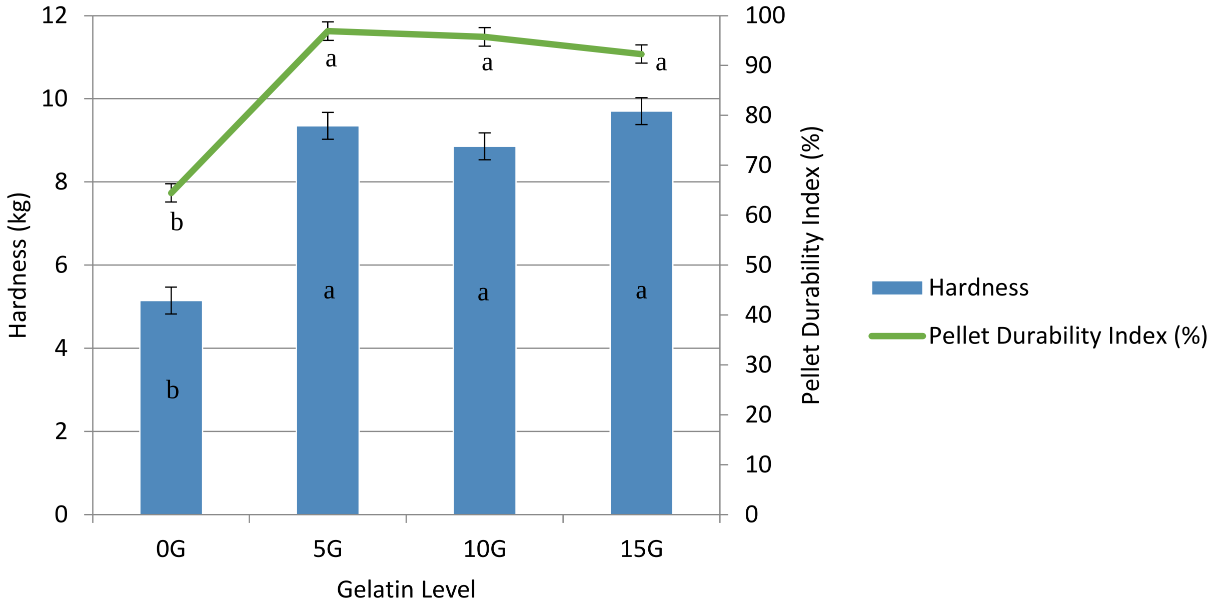

Figure 2.6 Main effect means of hardness and pellet durability index dependent on gelatin level in Experiment 1.

[ab]Columns within a variable with unlike superscripts differ by P<0.05.

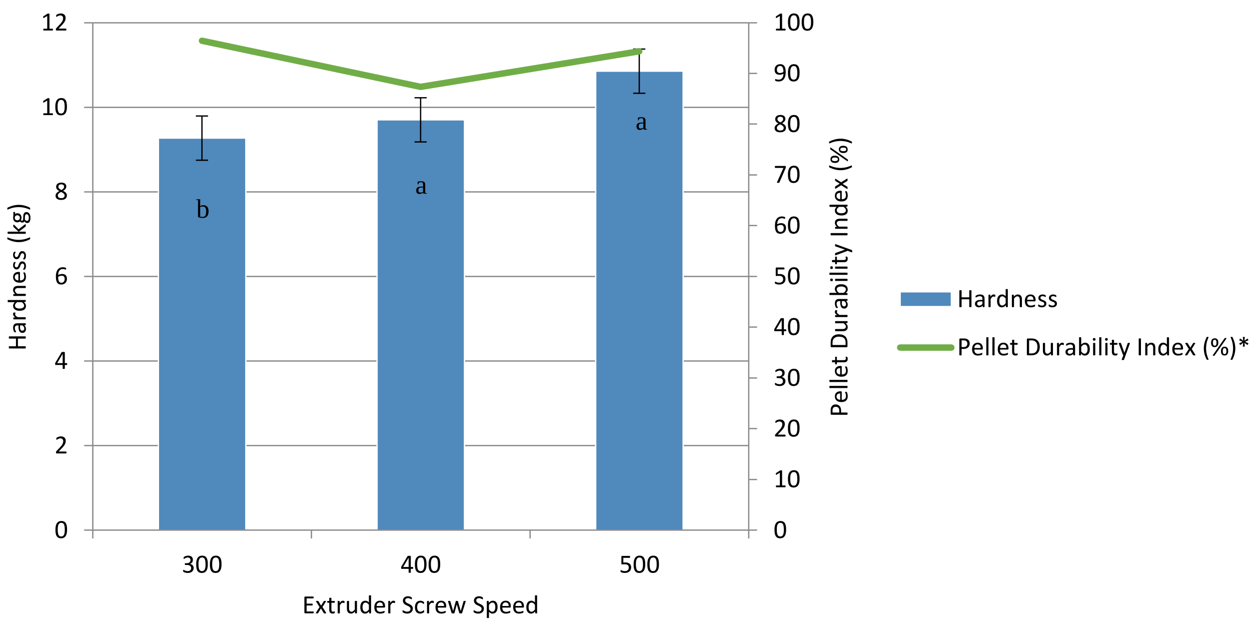

Figure 2.7 Main effect means of hardness and pellet durability index due to extruder screw speed in Experiment 1.

[ab]Columns within a variable with unlike superscripts differ by P<0.05.
*PDI had an SEM of 12.81.

Table 2.9 Main effect means for post-extrusion and post-drying moisture for different levels of gelatin, extruder screw speeds, and hydration ratios in Experiment 2.

		Post-Extrusion Moisture (%)	SEM	P	Post-Drying Moisture (%)	SEM	P
Gelatin Level	0%	17.91	1.144	0.91	8.79	0.721	0.11
	10%	18.14			6.01		
Extruder Screw Speed	300 rpm	18.56	1.013	0.53	7.91	1.484	0.676
	500 rpm	17.47			6.89		
Hydration Ratio	17%	16.92	0.789	0.67	6.34	0.751	0.65
	28%	17.47			6.89		

Table 2.10 Main effect means for post-extrusion and post-drying expansion ratio for different levels of gelatin, extruder screw speeds, and hydration ratios in Experiment 2.

		Post-Extrusion Expansion Ratio (mm^2/mm^2)	SEM	P	Post-Drying Expansion Ratio (mm^2/mm^2)	SEM	P
Gelatin Level	0%	4.58	0.227	0.548	4.43	0.266	0.806
	10%	4.35			4.32		
Extruder Screw Speed	300 rpm	4.30	0.189	0.331	4.24	0.234	0.493
	500 rpm	4.64			4.51		
Hydration Ratio	17%	4.80	0.397	0.803	4.64	0.421	0.853
	28%	4.64			4.51		

Table 2.11 Main effect means for specific length at different levels of gelatin, extruder screw speed, and hydration ratios in Experiment 2.

		Specific Length (cm/g)	SEM	P
Gelatin Level	0%	3.95	1.60	0.74
	10%	3.86		
Extruder Screw Speed	300 rpm	4.05	0.79	0.12
	500 rpm	3.76		
Hydration Ratio	17%	4.12[b]	0.79	0.008
	28%	3.76[a]		

[ab]Values with unlike superscripts differ by $P<0.05$

Table 2.12 Main effect means of gelatin level on kibble physical properties for diets in Experiment 3.

Variable	n	0%	10%	MSE	P
Bulk Density (g/L)	13	351.83[a]	280.67[b]	115.70	<0.0001
Piece Density (g/cm^3)	120	0.52[a]	0.39[b]	0.002	<0.0001
Radial Expansion (mm^2/mm^2)	120	2.86[b]	3.56[a]	0.15	<0.0001
Specific Length (cm/g)	60	4.12[b]	4.47[a]	0.03	<0.0001
Hardness (kg)	60	4.40	4.09	0.87	0.2036
PDI (%)	20	77.57[a]	52.25[b]	16.82	<0.0001

[ab]Values with unlike superscripts differ by P<0.05

Table 2.13 Main effect means for target densities (High vs. Low) on kibble physical properties produced in Experiment 3.

Variable	n	High	Low	MSE	P
Bulk Density (g/L)	13	345.83[a]	266.67[b]	115.70	<0.0001
Piece Density (g/cm^3)	120	0.460[a]	0.441[b]	0.002	0.0384
Radial Expansion (mm^2/mm^2)	120	3.18	3.25	0.15	0.2950
Specific Length (cm/g)	60	4.31	4.29	0.03	0.64
Hardness (kg)	60	4.39	4.11	0.87	0.2529
PDI (%)	20	72.17[a]	57.65[b]	16.82	<0.0001

[ab]Values with unlike superscripts differ by P<0.05

Table 2.14 Main effect means for gelatin bloom strength on kibble physical properties in Experiment 4.

Variable	n	0	100	175	250	MSE	P
Bulk Density (g/L)	35	347.6[a]	310.65[b]	242.4[c]	212.3[d]	143.76	<0.0001
Piece Density (g/cm^3)	180	0.56[a]	0.48[b]	0.39[c]	0.33[d]	0.01	<0.0001
Cross-Sectional Expansion (mm^2/mm^2)	180	3.05[d]	3.54[c]	4.29[b]	4.92[a]	1.01	<0.0001
Specific Length (cm/g)	120	4.27[c]	4.26[c]	4.49[b]	4.74[a]	0.14	<0.0001
Hardness (kg)	120	5.93[b]	7.38[a]	4.57[c]	3.59[d]	1.10	<0.0001
PDI (%)	39	88.49[a]	87.57[a]	64.55[b]	30.01[c]	3.56	<0.0001
Throughput (kg/hr)	16	1.56	1.59	1.53	1.61	0.0048	0.4048

[abcd]Values with unlike superscripts differ by P<0.05

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Chapter 3 - The Effect of Gelatin Bloom Strength on Physical Properties of Injection Molded Dental Chews for Dogs

1.0 Introduction

The pet food industry has seen continual growth year after year, particularly in more affluent countries (PFI, 2013). People spend more on their companion animals because they see themselves as parents, rather than owners, and their pets more like a child rather than an animal (Squires-Lee, 1995). With this humanization of their pets, owners feel the need to supplement their pet's diet with treats (German, 2006). In 2013, owners spent $2.6 billion on dog treats and $0.56 billion on cat treats (PFI, 2013).

However, treats are no longer just training motivators or simple snacks. More treats are being fortified to provide additional health benefits. Perhaps one of the more popular treat benefits currently on the market is dental health. A July 2015 search on petco.com provided 32 options for dental treats and an additional 8 products categorized as dental chews.

Several of these treats intended for dental health are injection molded to provide a rubbery, plastic-like texture. The idea is that the chewing action with a tough treat helps to remove plaque from the surface of teeth. Wang and Chen of the Natural Polymer International Corporation were issued a patent in 2002 for a "Protein-based Chewable Pet Toy" with the general characteristics of tensile strength between 20 and 40 MPa and a Young's Modulus between 800 to 4000 MPa (Wang & Chen, 2002).

Minimal research can be found regarding the characteristics and manufacturing of this type of treats for pets. However, pseudo-plastic products have been studied in the field of polymer science. Gelatin has been studied alone and in combination with synthetic polymers as a means to increase the biodegradability of packaging (Koepff, 1992). In a study by Koepff

(1992), twin-screw extrusion was used to process gelatin, without the aid of water or glycerin, into thermoplastic granules which could then be injection molded to create biodegradable packaging material. The water solubility of gelatin makes it a less-than-idea substance to use alone to create packaging; however, when combined with starch or polymers, such as polyethylene, it can create durable, yet recyclable material (Koepff, 1992).

However, there is interest in recreating these food formats in order to evaluate ingredient options for future products within the category. Therefore, the objective of this research was to determine preliminary standards for the formulation, extrusion, and injection molding of plant-based dental chew treat. Additionally, the objectives of this research were to compare low, mid, and high bloom gelatins and evaluate the differences in toughness and malleability of different formulations in the injection molded chew type treat product.

2.0 Materials and Methods

2.1 Lab-Scale Experiment

The lab-scale experiment was performed to determine the optimal formula for creating an injection molded dog treat with low-bloom strength gelatin. The levels of gelatin, vital wheat gluten, and glycerin were varied at 5%, 10%, and 15% in order to add up to 30% of the total formulation (Table 1). A complete formula was created to replicate a "Greenies®"-style treat like the product sold by Mars Petcare (Table 2 and 3). However, this formula was simplified for the purpose of performing a small scale experiment (Table 4).

According to the respective formula all ingredients for each treatment mix were hydrated to two different initial moisture levels – 15% and 20%. The following calculation was used to determine the quantity of water to add to each formula:

(% dry matter, actual) / (% dry matter, target) * starting weight of material = material plus water;

Then, Material plus water – starting weight of material = weight of water to be added

An example calculation for hydrating a 1.00 kg diet starting at 10% moisture and increasing to 15% moisture follows:

$$0.90 / 0.85 * 1.00 = 1.06$$

$$1.06 – 1.00 = 0.06 \text{ kg}$$

0.06 kg or 60 mL of water needs to be added

All treatment mixes for both hydration levels were extruded twice – once at a low temperature profile and once at a high temperature profile. Treatments were labeled according to formula number – which represented the levels of gelatin, vital wheat gluten, and glycerin – initial moisture content, and processing temperature (Table 5).

2.1.1 Diet Preparation

To determine the optimal levels of gelatin, vital wheat gluten, and glycerin in an injection molded treat 7 experimental formulas were produced (Table 5). The gelatin used was a low-bloom gelatin (Pro-Bind Plus 100; provided by Sonac, a subsidiary of Darling Ingredients International, Irving, TX). Vital wheat gluten and oat bran (Bob's Red Mill; Milwaukie, OR) were purchased from an online grocer. Pea protein isolate was sourced from the Pet Food Research Center at Kansas State University and had been used in previous experiments within the lab. Wheat flour, potassium chloride, dog vitamin premix, and natural antioxidant were purchased from a local mill (Lortscher Animal Nutrition, Bern, KS). Glycerin was purchased from a laboratory supplier (ChemWorld, Kennesaw, GA). Ingredients were mixed with a bench-top mixer (N50, Hobart, Troy, OH). Gelatin, gluten, oat bran, pea protein, and wheat flour were weighed with a large laboratory scale with 0.01 g sensitivity. These ingredients were added to the mixer and blended for 3 minutes. Potassium chloride, vitamin premix, and a natural antioxidant (Naturox; Kemin Industries, Des Moines, IA) were weighed with a small laboratory scale with 0.001 g sensitivity. These ingredients were added to the mixer and combined for an additional 2 minutes. Glycerin was weighed using a large laboratory scale with 0.01 g sensitivity and was slowly poured into the mixer while it was running. Once fully added, the mix was combined for an additional minute. Water was measured volumetrically and slowly added to the mixer while it was running. Once the last ingredients were added, the contents were mixed for an additional minute.

Diets were stored in sealed plastic zip-top bags labeled according to formula and hydration level. Bags were sealed and stored in a refrigerator to prevent moisture loss prior to extrusion.

2.1.2 Extrusion Conditions

Formulas were extruded on a lab-scale twin screw extruder (Micro-18, Leistritz, Somerville, NJ) fitted with a 3 mm diameter circle die and a conveying screw profile (Table 6). The following processing conditions were used for all treatments: feeder screw speed – 487 rpm, and extruder screw speed – 141 rpm. The temperature profile used for the high processing temperature treatment was as follows: temperature zone 1 – 30°C, temperature zone 2 – 50°C, temperature zone 3 – 70°C, temperature zone 4 – 90°C, temperature zone 5 – 110°C, and temperature zone 6 – 120°C. The temperature profile used for the low processing temperature treatment was as follows: temperature zone 1 – 30°C, temperature zone 2 – 50°C, temperature zone 3 – 70°C, temperature zone 4 – 80°C, temperature zone 5 – 90°C, and temperature zone 6 – 100°C.

The Micro-18 extruder did not have a knife attachment, so samples were collected in ropes and cut from the extruder by hand. Samples were not dried and were transferred to a refrigerator for cooling and storage.

2.1.3 Sample Preparation and Compression Molding

Samples were ground using a bench-top grinder fit with a 1 mm screen (Retsch Ultra Centrifugal Mill ZM200, Verder Group, Haan, Germany). Samples were stored in a refrigerator pre- and post-grinding to prevent moisture loss.

Samples were molded on a lab-scale compression molder (Auto Series Standard Auto CH 15-Ton Capacity Press Molder, Carver Inc., Wabash, IN). The top and bottom plates were set to 160°C and the compression period was set for 5 minutes with 907 kg of force.

The mold consisted of 6 pieces: a flat plate base, a solid rectangular bar, a small bar shaped insert, 4 small washers, a thicker piece with a bar shaped hollowed in the center, and another thicker metal piece with a bar shape protruding from the center. The flat base plate and solid rectangular bar were wrapped in aluminum foil to prevent material adhesion. The small bar shaped insert was not assembled with the rest of the mold, but used to cut a piece of aluminum foil that was assembled with the rest of the mold.

The solid rectangular bar was inserted into the thick metal piece with the hollowed out bar shape, and this was placed on the flat base plate. Ground sample was weighed out (8g) and poured into the hollowed out center of the mold (Figure 1). The sample was leveled-out to fill every corner of the mold. The sample was topped with the aluminum foil bar-shaped cutout. The 4 washers were placed in the corners of the mold, and the entire assembly was placed on top with the bar-shaped protrusion being placed over the sample.

The mold assembly was placed on the bottom plate of the press molder and allowed to warm to temperature for 5 minutes. The press molder was then closed and the test was completed according to the program settings.

After the press was completed, the mold was allowed to cool and the sample was gently ejected from the mold. Compressed bars were stored in a humidity chamber (Electro-Tech Systems, Glenside, PA) at 24°C and 50% relative humidity. Samples remained in the humidity chamber until ready to be analyzed.

2.1.4 Product Analysis

Sample bars were first analyzed for tensile strength, Young's Modulus, and strain at break on a tensile tester (Universal Type Tensile Tester 4465 with Series IX Software, Instron, Norwood, MA). The center portion of the compressed bars was measured for width and thickness in triplicate using a standard pair of calipers. The average width and thickness of the samples was recorded and recorded into the apparatus software (Series IX, Version 8.06.00, Instron, Norwood, MA). The samples were then placed vertically in the tensile tester, where clamps held the wider top and bottom portions.

After the sample was placed in the tensile tester, the test was started using the software command routine. After the sample had been fully pulled apart by the tensile tester, the test was manually ended using the software commands. The software recorded the tensile stress and Young's Modulus in MPa and the strain at the breaking point in percent. This was repeated for each of the 5 samples per treatment.

Samples were also analyzed for puncture force using a texture analyzer (TA-XT2 Texture Analyzer with Exponent 32 Software, Stable Micro Systems, Godalming, U.K.). An aluminum cone-shaped probe with a 20 mm base, 17 mm height, and 60° angle at the tip was used with a single compression test with a pre-test and test speed of 1 mm/s and a post-test speed of 2 mm/s. Instead of a full puncture, the force was measured using a 90% strain test. The wider portion at the end of the bars was used to complete the puncture test. This test was repeated with 10 samples. The data from the puncture tests was used to create force deformation graphs of all samples. These graphs were used to determine the number of peaks created during the puncture test as multiple peaks indicates an undesirable crunchy texture.

2.1.5 Statistical Analysis

The lab-scale experiment was arranged in a 7x2x2 factorial design without replication. Results were analyzed using the GLM procedure of SAS (SAS Institute, Cary, NC). Main effects of gelatin level, gluten level, glycerin level, moisture content, and processing temperature profile were analyzed, along with all subsequent 2-way, 3-way, 4-way and 5-way interactions. For clarity, results for the main effects of gelatin, gluten, and glycerin levels and the 3-way interaction of gelatin, gluten, and glycerin levels are reported. Differences were determined by least square means and considered significant at an alpha of 5%.

2.2 Pilot-Scale Experiment

The pilot-scale experiment was conducted to validate the lab-scale formula optimization on larger equipment that is more consistent with that used in product production. A single formula was chosen from the lab-scale experiment to evaluate 3 different gelatin strengths: low-bloom, mid-bloom, and high-bloom. The objective was to determine the effects of different gelatin bloom strengths on the production potential for injection-molded treats and the resulting physical characteristics resulting from the production process.

2.2.1 Treatment Formula Preparation

Gelatins were sourced from direct manufacturers: Low-bloom gelatin (Pro-Bind Plus 100, Sonac; a subsidiary of Darling Ingredients International, Irving, TX) and mid-bloom and high-bloom gelatins (Pig Skin 175 and Pig Skin 250, Rousselot; a subsidiary of Darling Ingredients International, Irving, TX). Wheat flour was purchased from a local mill (Fairview Mills, Auburn, NE). Likewise, all other ingredients were purchased from another local ingredient supplier (Lortscher Animal Nutrition, Bern, KS). All ingredients were purchased pre-ground to pass through a 4.76mm or US No.4 screen. Major ingredients (wheat flour, gelatin, vital wheat gluten, rice flour, oat bran, pea protein isolate, and potato protein) were weighed using a large scale with 0.1 kg sensitivity and added to a 90 kg double ribbon mixer and mixed for 5 minutes. Minor ingredients (flaxseed, dicalcium phosphate, lecithin powder, dry digest, apple pomace, tomato pomace, calcium carbonate, potassium chloride, choline chloride, vitamin premix, and trace mineral premix) were weighed on a small scale with 0.1 g sensitivity and added to the mixer and mixed for an additional 3 minutes. Treatments were stored in 25 kg paper bags labeled by gelatin type.

2.2.2 Extrusion Conditions

Treatments were extruded on a pilot-scale twin screw extruder (TX-52, Wenger Manufacturing, Sabetha, KS) fitted with a 3.5 mm diameter circle die. The processing parameters were as follows: feeder screw speed - 9.30 rpm; preconditioner screw speed - 404 rpm; preconditioner water flow - 1.36 kg/hr; preconditioner steam flow - 0.05 kg/hr; preconditioner downspout temperature - 23.2°C; extruder screw speed - 259 rpm; zone 1 temperature - 31.9°C; zone 2 temperature - 50.5°C; zone 3 temperature - 56.8°C; zone 4 temperature - 66.6°C; extruder water flow - 4.04 kg/hr; extruder motor load - 27.29%; die temperature - 114.0°C; knife speed – 2,324.30 rpm.

Glycerin was pumped into the discharge end of the preconditioner at a rate of 6 kg/hr to account for 10% of the extruded formula.

The screw profile consisted of the following: 9 unit full pitch, 9 unit full pitch, 9 unit ¾ pitch, 3 unit forward lobe, 9 unit ¾ pitch, 9 unit ¾ pitch, 3 unit forward lobe, 9 unit ¾ pitch, 6 unit half pitch, 6 unit half pitch, 3 unit reverse lobe, 9 unit ¾ pitch, 3 unit reverse lobe, 9 unit half pitch, 9 unit half pitch, and 9 unit ¾ pitch cone.

Upon exiting the extruder, the beadlets were conveyed by pneumatic transfer to a double-pass convection dryer (4800 Series, Wenger Manufacturing, Sabetha, KS) which was set to ambient temperature with 5 minute passes through each of the three zones.

2.2.3 Injection Molding Conditions

Treatments were molded on a pilot-scale injection molder (Boy 22S, Boy Machines, Exton, PA). Both heating barrels were set at 100°C and all pressure dials were left at neutral settings. Treatments were molded using a semi-automatic setting with a 45 second injection time and 10 second holding time. The dimension of the bars was 110 mm total length by 17 mm total width with a center portion that is 35 mm long by 7 mm wide (Figure 1).

2.2.4 Product Analysis

Five bars of each treatment were molded and trimmed of excess product around the border. All bars were stored in a humidity chamber (Electro-Tech Systems, Glenside, PA) at 24°C and 50% relative humidity for 24 hours prior to analysis.

Samples were first analyzed for tensile strength, Young's Modulus, and strain at break on a tensile tester (Universal Type Tensile Tester 4465 with Series IX Software, Instron, Norwood, MA). The center portion of the compressed bars was measured for width and thickness in triplicate using a standard pair of calipers. The average width and thickness of the samples was entered in to the software (Series IX, Version 8.06.00, Instron, Norwood, MA). The samples were then placed vertically in the tensile tester, where clams held the wider top and bottom portions.

After the sample was placed in the tensile tester, the test was started using the software commands. After the sample had been fully broken by the tensile tester, the test was manually ended using the software commands.

Samples were also analyzed for puncture force using a texture analyzer (TA-XT2 Texture Analyzer with Exponent 32 Software, Stable Micro Systems, Godalming, U.K.). An aluminum cone-shaped probe with a 20 mm base, 17 mm height, and 60° angle at the tip was used with a single compression test with a pre-test and test speed of 1 mm/s and a post-test speed of 2 mm/s. Instead of a full puncture, the force was measured using a 90% strain test. The wider portion at the end of the bars was used to complete the puncture test. This test was repeated with 10 samples.

2.2.5 Statistical Analysis

The pilot-scale experiment was arranged in a completely randomized design with replication, where the 3 treatments were extruded twice in a randomized order, and injection molded in a random order. Results were analyzed using the GLM procedure of SAS (SAS Institute, Cary, NC). The main effect of gelatin strength was evaluated. Differences were determined using least squared means and considered significant at an alpha of 5%.

3.0 Results

3.1 Lab-Scale Experiment

The tensile strength, Young's Modulus, puncture force, and number of peaks increased with each added increment of gelatin level ($P<0.05$). The exception was strain at break which tended ($P=0.07$) to decline with each increment of gelatin. (Table 7).

When evaluating the main effect of gluten level the picture was not as consistent; wherein, tensile strength was not affected ($P<0.05$) by gluten level (Table 8). However, Strain at break declined (<0.05) with each increment of gluten. The measure of Young's Modulus increased from 5% gluten to 10%, but did not increased beyond this to the 15% gluten containing treatment (217.7 vs. 333.01 and 367.23, respectively). The puncture force was not affected by gluten level, but peaks were lowest for the 5% gluten and increased for both the 10 and 15% levels (1.12 vs. 1.35 and 1.43).

The main effect of increasing glycerin levels was nearly opposite that of gelatin (Table 9). Wherein, the tensile strength decreased ($P<0.05$) with each increment of glycerin, strain at break increased ($P<0.05$), and Young's modulus decreased ($P<0.05$). Puncture force and number of peaks both decreased ($P<0.05$) with each increment of glycerin.

The three way interaction of gelatin, gluten, and glycerin levels affected ($P<0.05$) each of the physical parameters tested: tensile strength, strain at break, Young's Modulus, puncture force, and peaks. Tensile strength was the highest ($P<0.05$) for 15% gelatin with 10% gluten and 5% glycerin, was the lowest for 5% gelatin with 10% gluten and 15% glycerin, with the 10% gelatin+10% gluten+10% glycerin and 15% gelatin+5% gluten+10% glycerin treatments intermediate. Strain at break was the highest ($P<0.05$) for the 10% gelatin+5% gluten+15% glycerin treatment, the lowest for the 10% gelatin+15% gluten+5% glycerin treatment, and intermediate for the 10% gelatin+10% gluten+10% glycerin and 15% gelatin+5% gluten+10% glycerin treatments . Young's Modulus was the highest ($P<0.05$) for the 15% gelatin+10 gluten+5% glycerin treatment, the lowest for the 5% gelatin+10% gluten+15% glycerin treatment, and intermediate for 10% gelatin+10% gluten+10% glycerin and 15% gelatin+5% gluten+10% glycerin treatments. Puncture force was the highest ($P<0.05$) for the 15% gelatin+10% gluten+5% glycerin treatment, the lowest for the 5% gelatin+10% gluten+15% glycerin treatment, and intermediate for the 5% gelatin+15% gluten+10% glycerin and 15% gelatin+5% gluten+10% glycerin treatments. The number of peaks was the highest ($P<0.05$) for the 10% gelatin+15% gluten+5% glycerin treatment, the lowest for both the 5% gelatin+10% gluten+15% glycerin and 10% gelatin+5% gluten+15% glycerin treatments, and intermediate for the 10% gelatin+10% gluten+10% glycerin and 15% gelatin+5% gluten+10% glycerin treatments.

3.2 Pilot-Scale Experiment

Gelatin bloom strength had no effect ($P>0.05$) on post-extruder bulk density (Table 11). There was a slight decrease ($P<0.05$) in post-dryer bulk density, post- extruder and post-dryer piece density as gelatin bloom strength increased. Conversely, the post-extruder and post-dryer expansion ratio increased with increasing bloom strength. The post-extruder and post-dryer specific length were greatest for the PB100 and declined as gelatin bloom strength increased (Table 11). There was a trend ($P=0.06$) for puncture force to decrease as gelatin bloom strength increased. Tensile strength was not affected by treatment. Gelatin type affected ($P<0.05$) strain at break wherein PB100 and PS175 were similar but lower than PS250. The Young's Modulus for PB100 and PS175 were similar, but greater than the PS250 (128.11 and 97.81 vs. 44.21 MPa, respectively)

4.0 Discussion

4.1 Lab-Scale Experiment

The objective for the laboratory-scale experiment was to determine the optimal combination of gelatin, gluten, and glycerin in which to use in larger, pilot-scale series of experiments. In the initial steps to determine the optimum formula, the goal was to obtain a product with the best combination of a high tensile strength, high strain at break, high puncture force, low Young's Modulus, and low number of peaks - not the extremes for any one combination. Tensile strength refers to the force in kg required to pull the tabs of the test bar fully apart, or the maximum stress on a strain-stress diagram (Dupen, 2014). Strain at break is a percentage representing the length to which the bar was stretched before breaking relative to the original length of the bar. This particular measure of strain refers to normal strain, or a change in length of a material relative to the initial length (Dupen, 2014) Puncture force in kg is the force required for a cone-shaped probe to punch a hole 90% of the way through the tabs of the bar and is intended to replicate a biting force. The cone-shaped probe was used to resemble a dog-like tooth, and a value of 90% deformation was used for two reasons: 1) strain values greater than 70% can be reflective of the chewing process (Dogan and Kokini, 2007); and 2) a value closer to 100% was more representative of a complete bite, rather than a simple chewing action. Young's Modulus, or elastic modulus, measures the stiffness of a material and is a relationship between the tensile strength and strain and represents the slope of the linear elastic portion of the curve (Dupen, 2014). "Peaks" refers to the number of peaks on the force deformation graph created during the testing of puncture force (Dogan and Kokani, 2007). An ideal force deformation graph would have only one peak representing the force level reached at 90% of maximal strain. Additional peaks would be indicative of a change in the crispiness of the product (Devi et al., 2013). Crispiness is indicative of product remaining in a glassy-state, rather than having transitioned to a rubbery-state (Dogan and Kokini, 2007). Crispy textures are undesirable in this particular style of dental treat, which is supposed to have a more rubber-like texture.

While main effects and interactions were analyzed for gelatin level, gluten level, glycerin level, initial moisture content, and processing temperature, results for the main effect means of gelatin level, gluten level, and glycerin level, and the interaction of these three variables provide the most deliberate approach to selecting an optimal level of each.

As gelatin level increased, so did tensile strength and puncture force. Tensile strength increased ($P<0.05$) for each additional increment of gelatin. This is consistent with the findings of Fakhoury et al. (2012). Fakhoury et al. (2012) studied the impact of gelatin and manioc starch combinations used to create edible and biodegradable films and found that increased gelatin concentration increased tensile strength and attributed this to the increase in protein chains that enhanced intermolecular interactions. Puncture force also increased ($P<0.05$). Increasing gelatin levels had a negative impact on strain at break and Young's Modulus. Strain at break decreased ($P<0.05$). Young's Modulus increased ($P<0.05$). Increasing gelatin also had a negative impact on the number of peaks wherein peaks increased ($P<0.05$). Overall, increasing the level of gelatin increased the strength and hardness of the product but this was at the detriment to product elasticity. While minimal literature exists regarding the impact of gelatin level on mechanical properties of food products, gelatin has been studied in a simple formulation with starch for the purpose of forming film-based capsules. In a study by Zhang et al. (2013), decreasing levels of gelatin, thus increasing starch content, created rigidity and brittleness. Fakhouri et al. (2013) had similar results in a study comparing various processing methods of starch-gelatin films, where the increase in gelatin created a more rigid film. Perhaps the still relatively high starch content of the experimental formulation limited the ability of gelatin to aid in product elasticity. Based on this, preliminary assessment 10% gelatin inclusion appeared to be an ideal level to begin evaluations.

Increasing gluten level had no impact on tensile strength or puncture force but caused a decrease ($P<0.05$) in strain at break and an increase ($P<0.05$) in both Young's Modulus and the number of peaks. Strain at break decreased ($P<0.05$). Young's Modulus increased ($P<0.05$) and peaks increased ($P<0.05$). This suggests that increasing gluten tends to toughen the molded product, but not increase product strength. Preliminary assumptions were that lower levels of gluten in the 5-10% range would be optimal.

Glycerin levels had a strong impact on product flexibility. Increasing the level of glycerin decreased tensile strength, Young's Modulus, and number peaks and increased strain at break. Tensile strength decreased ($P<0.05$), Young's Modulus decreased ($P<0.05$), strain at break increased ($P<0.05$), and number of peaks decreased ($P<0.05$). Puncture force had a more curvilinear relationship as it increased ($P<0.05$) 21.36 kg at 5% glycerin to 23.73 kg at 10% glycerin and decreased ($P<0.05$) to 18.57 kg at 15% kg. Notably, a decrease in tensile strength follows the findings of Hanani et al. (2013) and Mo et al. (1999). Hanani et al. (2013) studied the effect of plasticizer content on gelatin-based films, and found that higher levels of plasticizers, like glycerin, decrease tensile strength due to a reduction in intermolecular forces between protein molecules. This action decreases tensile strength, but improves flexibility and extensibility (Hanani et al., 2013), which follows the findings of this experiments where Young's Modulus decreased and strain at break increased. Mo et al. (1999) noted that tensile bars prepared from soy protein had increased flexibility and decreased tensile strength in the presence of added water and glycerol. Overall, additional glycerin increased the flexibility and stretch of the molded bars but at the detriment to product strength. However, flexibility is important to the proper texture of this type of treat. Additionally, as a plasticizer, glycerin may be important to the flow of material through the molder into the die. Preliminary assumptions were that glycerin level would be optimal at 10-15%.

These main effects were confirmed by the 3-way interaction to determine the optimal formula to be used in the pilot-scale experiment. While all combinations of gelatin, gluten, and glycerin differed, main effect means suggested that the ideal level of gelatin was 10%, the ideal level of gluten was 5-10%, and the ideal level of glycerin was 10-15%. This narrowed the formula combinations to 10% gelatin x 5% gluten x 15% glycerin (10x5x15) and 10% gelatin x 10% gluten x 10% glycerin (10x10x10).

Considering the interactions when comparing these two formulas, 10x5x15 had a higher strain at break, lower Young's Modulus, and the number of peaks was at the ideal 1.00. This combination should be evaluated in the future. For the purposes of the pilot scale the 10x10x10 had a higher ($P<0.05$) tensile strength and puncture force and was therefore selected for the pilot-scale experiment because it offered these advantages. It was hypothesized that the temperature used to mold the samples might have been too high. While samples did not burn, this high temperature may have limited the flexibility of the final products. The 10x10x10 formula still had intermediate values for strain at break and a peaks value of 1.38.

4.2 Pilot-Scale Experiment

The objective of the pilot-scale experiment was to determine the effect of different gelatin bloom strengths on molded treats. This is not a single step process. It first requires the mixed ingredients be extruded to create resinous beadlets. During this process measurements of extrusion parameters and the resulting beadlets were taken to provide a framework for interpretation if differences were encountered. Previous extrusion experiments where gelatin was extruded in a typical pet food formulation resulted in increased expansion both radially and longitudinally. This effect also increased as the strength of the gelatin increased (Manbeck et al, 2015). The extrusion parameters during beadlet production were set to minimize expansion. However, some expansion was noted.

Both bulk and piece density were higher with the low-bloom gelatin beadlets versus the other two treatments; but, no difference between the mid-bloom and high-bloom gelatin containing beadlets was observed. Post-extruder and post-dryer piece density showed the same relationship. This was reflected in the changes to expansion ratio. Expansion ratio, which represents expansion in the radial direction, increased from low-bloom to mid-bloom and subsequently to high-bloom. The additional expansion seen in post-dryer products relative to post-extruder products is likely due to water migration during drying under low-temperature settings. The ambient air, rather than heated air, was slower to dry products and may have created some residual expansion due to the travel of water from the center of the product to the outside. Specific length saw a decrease as gelatin strength increased. The added radial expansion seen with the inclusion of higher bloom strength gelatin may have decreased the length as product exited the die because the energy created during the steam flash-off. This may have been applied to outward expansion rather than forward momentum. Regardless all treatments produced resin beadlets suitable for injection molding.

The increasing gelatin strength appears to create a foaming or expansion effect even under gentle processing conditions. While beadlet expansion was not a priority for this experiment and may be detrimental to a product intended for injection molding, it was interesting to note the differences in products due to the type of gelatin used.

While these differences were interesting to observe, the more relevant outcome relates to the injection molded bars resulting from the different beadlet formulations. Minimal literature can be found regarding the use of plant-based injection molded products and none exist regarding use of gelatin to our knowledge. Most injection molding is done with plastic or plastic-like resins which melt at much higher temperatures than plant-based materials. Furthermore, melt temperatures are relatively well established for plastic and plastic-like materials (e.g. polylactic acid, polyethylene) because there is more published research regarding these materials and these materials are inherently more consistent in composition. Plant-based materials have not been studied as extensively to establish known melting temperatures. Additionally, plant composition varies based on growing conditions, weather changes, harvest variation, and processing inconsistencies. This creates natural variation between batches of certain materials. Some of this variation can be overcome through a certain degree of blending, but plant-based materials will never be completely homogenous.

This combination of minimal published research and natural ingredient variation led to the trial and error approach for testing temperature set points. Barrel temperatures were evaluated at 160°C, the set point used for temperature when compression molding samples in the lab scale experiment, and with failed iterations were decreased eventually to 95°C. When the barrel temperature was between 120°C-160°C there was some degree of burning or overcooking. This was evidenced through observations of a much darker brown color, a dry surface, less pliable texture, and a stronger burning smell during the injection phase. The darkening of color was particularly noticeable between 140-160°C

Products molded between 95°C-115°C were fairly similar. Products were a more neutral brown color with a smoother surface as seen on the sample product in Figure 1. This temperature range resulted in a more malleable product that was much more visually similar to the reference, thus all products used for further analysis were processed at 100°C on both barrel sections.

Additional visual observations were noted during the injection molding process. Two issues were apparent during this process. First, there was an issue with product leaking from the end of the screw during injection to the die. This may have resulted for multiple reasons: the end of the screw might not have been in full contact with the die hole; the rotation speed of the screw might have been too high forcing too much product for the size of the die hole; or there may have been an issue with particle size of some of the base ingredients or an inadequate inclusion of a plasticizer. The first two potential reasons are technical considerations that may be solved by mechanical adjustments to the molder or through more experiment repetitions. Alterations in screw speed or pressure settings may also help to resolve this issue. Ensuring the injection apparatus is in full contact with the die may prevent leakage, as would slowing the screw speed. A slower screw speed may allow for a better metering of product through the die opening. A finer grind on the ingredients prior to extrusion might also prevent any particles from clogging the die opening and obstructing the flow of material in to the die.

A finer grind of ingredients might also help solve another issue: material remaining in the die opening after the completion of the injection phase. Warm-up runs with polyethylene beadlets did result in clogging at the die opening. Thus clogging of the die opening may be a result of larger particles clogging the opening or also an issue of screw speed.

Another issue was inadequate die fill. This is likely due to a short injection time. However, the flow of material may have also been hindered the product wasted from leaking prior to the die or from a slower flow of the material due to inherent viscosity and uneven particle size of the initial mix. Wherein, the product viscosity may have been impeding the penetration of material through to the full capacity of the die. This may suggested that additional plasticizing from a higher level of glycerin may be necessary. When gelatin is used in film-forming applications, glycerin is important for mobilizing the protein molecules of the gelatin. The mobilization of protein molecules through plasticization aids in forming continuous films (Krishna et al., 2012). This concept may be applicable in this experiment, as increasing glycerin levels may improve the cohesiveness of the final products.

There were also issues with the flowability of the beadlets extruded with the high-bloom strength gelatin. The increased expansion with the PS250 beadlets caused issues with bridging in the hopper and flowability through the opening to the injection apparatus. This issue could easily be fixed by increasing the knife speed of the extruder or by using a smaller die during the extrusion of the beadlets.

Despite these operational issues, 5 bars of each formula were molded and analyzed for puncture force, tensile strength, strain at break, and Young's Modulus. These products were not analyzed for peaks, as was done in the lab-scale experiment, because all samples only had one peak on a force deformation graph indicating no internal crunchy texture.

Puncture force showed a decreasing trend as gelatin strength increased. This indicates that low bloom strength gelatin results in a tougher treat than high bloom strength gelatin. Tensile strength was not truly different but also showed a numerical decrease. Strain at break yielded an inconsistent relationship wherein percent strain showed a numerical decrease. Young's Modulus resulted in an incremental decrease ($P<0.05$) as gelatin strength increased ($P<0.05$). These values indicate the most flexible product was molded using the PS250. Likely there is a continual increase in product flexibility as gelatin strength increases, but more samples are needed to evaluate if this is truly a quadratic relationship or more of a linear relationship.

5.0 Conclusions

The lab-scale experiment resulted in a workable formulation for injection molded treats and consisted of equivalent proportions of gelatin, gluten, and glycerin. This was determined to be the best combination of a high tensile strength, puncture force, and strain at break with a low Young's Modulus and number of peaks on the force deformation graph for the ratios tested. However, further pilot-scale experiments should be conducted to evaluate formula alterations that contain 10% gelatin, 5-10% gluten, and 10-15% glycerin. Additionally, further lab-scale experiments should be done to determine the ideal formula for different types of gelatin, as this experiment only used low-bloom gelatin as the frame of reference.

On a pilot-scale, low-bloom gelatin in an injection molded treat results in a tougher, less malleable product, while high-bloom gelatin results in a softer, more flexible treat. These differences were interesting to note, but it would be valuable to find a method in which to compare these experimental treats to an injection molded dental treat that is currently available on the market (e.g. Greenies).

Finally, more refinement is needed regarding the injection molder operational settings. Additional adjustments should be made to address issues with the flow of material into the die cavity.

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6.0 Tables and Figures

Table 3.1 Formula treatment identifications used in the lab-scale evaluation of an injection molded chew treat based on the differing levels of gelatin, vital wheat gluten, and glycerin.

Formula Number	Gelatin Level (%)	Vital Wheat Gluten Level (%)	Glycerin Level (%)
1	5	15	10
2	5	10	15
3	10	10	10
4	10	5	15
5	10	15	5
6	15	5	10
7	15	10	5

Table 3.2 The complete ingredient composition for a predicted an injection molded chew treat formula.

Ingredient Name	Percent
Wheat Flour	30.00
Glycerin*	8.96
Vital Wheat Gluten*	8.87
Gelatin*	8.79
Rice Flour	8.70
Oat Bran	8.61
Pea Protein Isolate	8.53
Potato Protein	8.44
Flaxseed	2.00
Dicalcium Phosphate	1.00
Lecithin Powder	1.00
Dry Dog Digest	0.99
Apple Pomace	0.98
Tomato Pomace	0.97
Calcium Carbonate	0.96
Potassium Chloride	0.50
Choline Chloride	0.25
Dog Vitamin Premix	0.15
Green Tea Extract	0.10
Chlorophyll Powder	0.10
Trace Mineral Premix	0.10

*Levels of gelatin, vital wheat gluten, and glycerin were estimated using formulation software. These levels were adjusted according to the specific formula variation used in the experiment. Actual levels of glycerin, vital wheat gluten, and glycerin varied at 5%, 10%, and 15% and these three ingredients accounted for 30% of the total formulation.

Table 3.3 Calculated nutritional composition of the complete formula used to recreate an injection molded chew treat.

Nutrient	Amount
Moisture	7.8%
Crude Protein	34.1%
Crude Fat	3.5%
Ash	4.0%
Crude Fiber	2.8%
NFE	47.7%
ME	3159.9 kcal/kg

Table 3.4 Simplified formula used in the lab-scale recreation of an injection molded chew treat.

Ingredient	Amount (%)
Gelatin	*
Vital Wheat Gluten	*
Glycerin	*
Wheat Flour	33
Oat Bran	15
Pea Protein Isolate	15
Potassium Chloride	3
Dog Vitamin Premix	3
Naturox	1

*The levels of gelatin, vital wheat gluten, and glycerin accounted for 30% of the total simplified formula. The specific levels of these three ingredients varied at 5%, 10%, and 15% depending on the specific version of the formula.

Table 3.5 Treatment labels used in the lab-scale experiment and the levels of gelatin, gluten, and glycerin for each in the production of an injection molded chew treat.

Treatment Label	Formula Number	Gelatin Level (%)	Vital Wheat Gluten Level (%)	Glycerin Level (%)	Initial Moisture Content (%)	Processing Temperature Profile
F115H	1	5	15	10	15	High
F115L	1	5	15	10	15	Low
F120H	1	5	15	10	20	High
F120L	1	5	15	10	20	Low
F215H	2	5	10	15	15	High
F215L	2	5	10	15	15	Low
F220H	2	5	10	15	20	High
F220L	2	5	10	15	20	Low
F315H	3	10	10	10	15	High
F315L	3	10	10	10	15	Low
F320H	3	10	10	10	20	High
F320L	3	10	10	10	20	Low
F415H	4	10	5	15	15	High
F415L	4	10	5	15	15	Low
F420H	4	10	5	15	20	High
F420L	4	10	5	15	20	Low
F515H	5	10	15	5	15	High
F515L	5	10	15	5	15	Low
F520H	5	10	15	5	20	High
F520L	5	10	15	5	20	Low
F615H	6	15	5	10	15	High
F615L	6	15	5	10	15	Low
F620H	6	15	5	10	20	High
F620L	6	15	5	10	20	Low
F715H	7	15	10	5	15	High
F715L	7	15	10	5	15	Low
F720H	7	15	10	5	20	High
F720L	7	15	10	5	20	Low

Table 3.6 Screw configuration for the Micro-18 lab scale extruder for production of resin ropes to evaluate levels of gelatin, gluten, and glycerin.

Screw Element	Length (mm)
Spacer	30
Full pitch, Forward	120
¾ pitch, Forward	60
Forward Kneading Block	20
¾ pitch, Forward	60
Forward Kneading Block	20
½ pitch, Forward	90
Neutral Kneading Block	20
¾ pitch, Forward	60
½ pitch, Forward	60

Table 3.7 Main effect means of gelatin level on the physical characteristics of a compression molded treat.

Variable	n	5	10	15	MSE	P
Tensile Strength (MPa)	110	1.99^{c}	3.34^{b}	5.05^{a}	2.79	<0.0001
Strain at Break (%)	110	2.36^{a}	2.46^{a}	1.49^{b}	2.54	0.07
Young's Modulus (MPa)	110	194.79^{c}	331.09^{b}	492.60^{a}	38766.31	<0.0001
Puncture Force (g)	220	20.64^{b}	21.17^{b}	23.61^{a}	34.71	0.03
Peaks (n)	220	1.06^{c}	1.36^{b}	1.68^{c}	0.46	<0.0001

^{abc}Means in a row with unlike superscripts differ by P<0.05

Table 3.8 Main effect means of gluten level on the physical characteristics of a compression molded treat.

Variable	n	5	10	15	MSE	P
Tensile Strength (MPa)	110	2.67	3.41	3.24	3.89	0.25
Strain at Break (%)	110	3.10^{a}	2.27^{b}	1.36^{c}	2.25	<0.0001
Young's Modulus (MPa)	110	217.70^{a}	333.01^{b}	367.23^{b}	46614.35	0.02
Puncture Force (g)	220	20.56	21.70	21.82	35.56	0.42
Peaks (n)	220	1.12^{b}	1.35^{a}	1.43^{a}	0.50	0.04

^{abc}Means in a row with unlike superscripts differ by P<0.05

Table 3.9 Main effect means of glycerin level on the physical characteristics of a compression molded treat.

Variable	n	5	10	15	MSE	P
Tensile Strength (MPa)	110	5.74^{a}	3.14^{b}	1.89^{c}	2.14	<0.0001
Strain at Break (%)	110	1.31^{b}	1.56^{b}	3.57^{a}	1.63	<0.0001
Young's Modulus (MPa)	110	617.96^{a}	320.52^{b}	145.33^{c}	22252.27	<0.0001
Puncture Force (g)	220	21.36^{b}	23.73^{a}	18.57^{c}	30.38	<0.0001
Peaks (n)	220	2.03^{a}	1.27^{b}	1.00^{c}	0.38	<0.0001

^{abc}Means in a row with unlike superscripts differ by P<0.05

Table 3.10 The interaction means of gelatin, gluten, and glycerin levels on the physical characteristics of a compression molded treat.

Variable	n	5x10x15	5x15x10	10x5x15	10x10x10	10x15x5	15x5x10	15x10x5	MSE	P
Tensile Strength (MPa)	110	1.55^{e}	2.42^{d}	2.23^{de}	3.68^{c}	4.89^{b}	3.51^{c}	6.59^{a}	1.87	<0.0001
Strain at Break (%)	110	3.32^{a}	1.40^{b}	3.83^{a}	1.68^{b}	1.27^{b}	1.63^{b}	1.36^{b}	1.66	<0.0001
Young's Modulus (MPa)	110	117.92^{d}	271.66^{c}	172.73^{d}	375.82^{b}	558.36^{a}	307.63^{bc}	677.57^{a}	21061.35	<0.0001
Puncture Force (g)	220	17.64^{b}	23.64^{a}	19.49^{b}	24.35^{a}	18.18^{b}	22.68^{a}	24.53^{a}	28.56	<0.0001
Peaks (n)	220	1.00^{c}	1.13^{bc}	1.00^{c}	1.38^{b}	2.05^{a}	1.35^{b}	2.00^{a}	0.38	<0.0001

*5x10x15 and 10x5x15 are also significantly different

^{abcde}Means in a row with unlike superscripts differ by P<0.05

Table 3.11 Main effect means of gelatin type of physical characteristics of extruded beadlets prior to injection molding.

Variable	PB100	PS175	PS250	SEM	P
Post-Extruder Bulk Density (g/L)	671.58	667.08	664.17	7.43	0.78
Post-Dryer Bulk Density (g/L)	724.00^{a}	669.83^{b}	670.33^{b}	3.26	<0.0001
Post-Extruder Piece Density (g/cm^3)	1.03^{a}	0.97^{b}	0.94^{b}	0.02	0.004
Post-Dryer Piece Density (g/cm^3)	0.95^{a}	0.88^{b}	0.88^{b}	0.02	0.01
Post-Extruder Expansion Ratio (mm^2/mm^2)	1.88^{c}	2.30^{b}	2.48^{a}	0.04	<0.0001
Post-Dryer Expansion Ratio (mm^2/mm^2)	1.86^{c}	2.47^{b}	2.63^{a}	0.04	<0.0001
Post-Extruder Specific Length (cm/g)	5.48^{a}	4.70^{b}	4.52^{c}	0.05	<0.0001
Post-Dryer Specific Length (cm/g)	6.03^{a}	4.82^{b}	4.54^{c}	0.06	<0.0001

[abc]Means in a row with unlike superscripts differ by P<0.05

Table 3.12 Main effect means of gelatin type on physical characteristics of injection molded treats.

Variable	PB100	PS175	PS250	SEM	P
Puncture Force (kg)	15.46	14.11	12.59	0.80	0.06
Tensile Strength (MPa)	3.03	2.53	2.33	0.27	0.20
Strain at Break (%)	7.43^{b}	5.94^{b}	14.08^{a}	0.78	<0.0001
Young's Modulus (MPa)	128.11^{a}	97.81^{a}	44.21^{b}	14.15	0.004

[abc]Means in a row with unlike superscripts differ by P<0.05

Figure 3.1 Mold used to create compression molded treats using a simplified formula of varying levels of low-bloom gelatin, gluten, and glycerin.

Figure 3.2 Injection molded bar from pilot-scale production of dental treats.

Ruler for scale.

7.0 References

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Appendix A - Raw Data of Extrusion Experiments

Units for measured values are as follows:

Moisture: %
Expansion Ratio: mm^2/mm^2
Specific Length: cm/g
Piece Density: g/cm^3
PDI: %
Hardness: kg

Table A.1 Raw data from Experiment 1 comparing various levels of gelatin at different screw speeds.

Treatment +	OE Expansion Ratio*	OD Expansion Ratio*	OE Specific Length*	OD Specific Length*	Piece Density	Moisture	PDI	Hardness
OG400	3.55	4.27	35.67	42.73	0.000437	9.59	64.47	5.46
OG400	3.55	4.27	36.97	42.11	0.000437	9.59	64.47	6.61
OG400	-	-	-	-	-	-	-	6.03
OG400	-	-	-	-	-	-	-	4.75
OG400	-	-	-	-	-	-	-	4.93
OG400	-	-	-	-	-	-	-	6.37
OG400	-	-	-	-	-	-	-	5.08
OG400	-	-	-	-	-	-	-	4.93
OG400	-	-	-	-	-	-	-	4.27
OG400	-	-	-	-	-	-	-	4.90
OG400	-	-	-	-	-	-	-	4.22
OG400	-	-	-	-	-	-	-	6.04
OG400	-	-	-	-	-	-	-	4.33
OG400	-	-	-	-	-	-	-	5.60
OG400	-	-	-	-	-	-	-	5.46
OG400	-	-	-	-	-	-	-	4.50
OG400	-	-	-	-	-	-	-	5.54
OG400	-	-	-	-	-	-	-	4.42
OG400	-	-	-	-	-	-	-	5.37
OG400	-	-	-	-	-	-	-	4.09
5G400	3.64	3.31	33.17	40.46	0.000584	10.73	96.90	10.17
5G400	3.64	3.31	33.17	40.46	0.000584	10.73	96.90	8.09
5G400	-	-	-	-	-	-	-	11.00
5G400	-	-	-	-	-	-	-	11.05
5G400	-	-	-	-	-	-	-	7.34
5G400	-	-	-	-	-	-	-	10.22
5G400	-	-	-	-	-	-	-	11.26
5G400	-	-	-	-	-	-	-	8.38

5G400	-	-	-	-	-	-	-	8.97
5G400	-	-	-	-	-	-	-	8.91
5G400	-	-	-	-	-	-	-	11.82
5G400	-	-	-	-	-	-	-	8.24
5G400	-	-	-	-	-	-	-	8.26
5G400	-	-	-	-	-	-	-	9.41
5G400	-	-	-	-	-	-	-	8.88
5G400	-	-	-	-	-	-	-	9.08
5G400	-	-	-	-	-	-	-	6.53
5G400	-	-	-	-	-	-	-	11.24
5G400	-	-	-	-	-	-	-	8.60
5G400	-	-	-	-	-	-	-	9.45
10G400	2.64	2.77	35.89	41.64	0.000694	10.83	95.73	7.33
10G400	5.67	6.70	36.72	42.77	0.000694	10.83	95.73	9.61
10G400	6.62	6.79	36.29	40.10	-	-	-	10.87
10G400	6.30	6.39	35.02	45.18	-	-	-	7.52
10G400	6.51	6.88	35.53	39.72	-	-	-	8.96
10G400	6.42	6.29	-	40.44	-	-	-	8.41
10G400	6.41	6.83	-	-	-	-	-	10.63
10G400	7.29	6.71	-	-	-	-	-	8.31
10G400	6.67	6.63	-	-	-	-	-	10.38
10G400	6.27	-	-	-	-	-	-	8.18
10G400	6.71	-	-	-	-	-	-	8.12
10G400	-	-	-	-	-	-	-	11.30
10G400	-	-	-	-	-	-	-	6.55
10G400	-	-	-	-	-	-	-	8.74
10G400	-	-	-	-	-	-	-	9.79
10G400	-	-	-	-	-	-	-	7.35
10G400	-	-	-	-	-	-	-	8.66
10G400	-	-	-	-	-	-	-	9.41
10G400	-	-	-	-	-	-	-	9.63
10G400	-	-	-	-	-	-	-	7.44
15G400	2.02	2.40	38.30	48.30	0.000687	10.72	92.30	4.40
15G400	2.02	2.40	41.32	47.63	0.000687	10.72	92.30	9.47
15G400	-	-	36.16	47.71	-	-	-	9.80
15G400	-	-	37.45	49.50	-	-	-	10.93
15G400	-	-	38.29	48.91	-	-	-	9.10
15G400	-	-	-	47.75	-	-	-	8.19
15G400	-	-	-	-	-	-	-	8.64
15G400	-	-	-	-	-	-	-	8.97
15G400	-	-	-	-	-	-	-	8.74
15G400	-	-	-	-	-	-	-	9.79
15G400	-	-	-	-	-	-	-	13.58

15G400	-	-	-	-	-	-	-	11.32
15G400	-	-	-	-	-	-	-	10.33
15G400	-	-	-	-	-	-	-	10.66
15G400	-	-	-	-	-	-	-	10.94
15G400	-	-	-	-	-	-	-	8.07
15G400	-	-	-	-	-	-	-	7.05
15G400	-	-	-	-	-	-	-	12.39
15G400	-	-	-	-	-	-	-	11.77
15G400	-	-	-	-	-	-	-	9.80
15G300	1.93	2.12	45.72	52.58	0.000715	11.89	96.43	8.98
15G300	5.46	5.83	45.11	53.30	0.000715	9.85	96.43	7.75
15G300	5.82	5.73	47.03	56.96	-	13.92	-	7.35
15G300	5.65	5.52	44.96	45.06	-	-	-	13.88
15G300	5.74	5.87	45.02	51.97	-	-	-	8.43
15G300	5.68	5.91	46.59	55.69	-	-	-	9.17
15G300	6.27	6.02	-	-	-	-	-	8.21
15G300	5.38	5.74	-	-	-	-	-	9.28
15G300	5.42	6.09	-	-	-	-	-	7.05
15G300	4.90	5.67	-	-	-	-	-	7.00
15G300	5.29	5.90	-	-	-	-	-	6.66
15G300	-	-	-	-	-	-	-	8.02
15G300	-	-	-	-	-	-	-	14.47
15G300	-	-	-	-	-	-	-	16.13
15G300	-	-	-	-	-	-	-	8.28
15G300	-	-	-	-	-	-	-	10.91
15G300	-	-	-	-	-	-	-	7.47
15G300	-	-	-	-	-	-	-	12.05
15G300	-	-	-	-	-	-	-	8.28
15G500	2.46	3.07	35.05	43.69	0.000597	9.83	94.33	10.56
15G500	6.31	6.68	36.14	48.18	0.000597	9.96	94.33	14.38
15G500	6.51	6.41	34.07	41.96	-	9.69	-	14.96
15G500	6.22	7.12	34.75	46.07	-	-	-	5.66
15G500	6.38	7.41	34.70	38.57	-	-	-	9.41
15G500	6.29	6.63	35.70	44.23	-	-	-	9.69
15G500	6.47	7.43	-	-	-	-	-	11.97
15G500	6.24	7.39	-	-	-	-	-	10.75
15G500	5.92	6.68	-	-	-	-	-	12.38
15G500	6.31	7.48	-	-	-	-	-	8.97
15G500	6.04	6.93	-	-	-	-	-	9.50
15G500	-	-	-	-	-	-	-	13.36
15G500	-	-	-	-	-	-	-	12.11
15G500	-	-	-	-	-	-	-	9.87
15G500	-	-	-	-	-	-	-	9.76

15G500	-	-	-	-	-	-	-	9.91
15G500	-	-	-	-	-	-	-	9.92
15G500	-	-	-	-	-	-	-	9.26
15G500	-	-	-	-	-	-	-	12.28
15G500	-	-	-	-	-	-	-	12.33

+Treatments are as follows: 0G400 = 0% gelatin at 400 rpm; 5G400 = 5% gelatin at 400 rpm; 10G400 = 10% gelatin at 400 rpm; 15G400 = 15% gelatin at 400 rpm; 15G300 = 15% gelatin at 300 rpm; 15G500 = 15% gelatin at 500 rpm
*OE refers to “out of the extruder” and OD refers to “out of the dryer”

Table A.2 Average data values from Experiment 2 comparing gelatin level, screw speed, and hydration ratio.

Treatment +	OE Moisture*	OD Moisture*	OE Expansion Ratio*	OD Expansion Ratio*	OD Specific Length*
0G 300 17%	19.46	9.81	4.26	4.06	41.62
0G 500 17%	16.36	7.77	4.90	4.79	37.39
0G 500 28%	17.03	6.93	5.29	5.16	41.37
10G 500 28%	16.80	5.74	4.30	4.11	40.95
10G 500 17%	18.58	6.01	4.37	4.23	37.85
10G 300 17%	17.65	6.00	4.33	4.41	39.43

+Treatments are abbreviated as “percent gelatin” [0% or 10%] “screw speed” “hydration ratio”
*OE refers to “out of the extruder” and OD refers to “out of the dryer”

Table A.3 Raw data from Experiment 3 comparing gelatin inclusion and target density.

Treatment*	Bulk Density	Radial Expansion	Specific Length	Piece Density	Hardness	PDI
0GLD	320.00	3.05	3.85	0.512	5.483	61.86
0GLD	300.00	3.48	3.99	0.434	4.124	61.76
0GLD	321.00	3.23	4.29	0.435	3.206	62.85
0GLD	319.00	2.77	3.98	0.546	4.011	64.21
0GLD	.	2.90	4.29	0.483	4.445	65.03
0GLD	.	2.78	4.10	0.528	4.356	.
0GLD	.	3.09	3.97	0.491	3.595	.
0GLD	.	3.06	4.13	0.476	4.908	.
0GLD	.	2.66	4.17	0.543	6.010	.
0GLD	.	3.48	4.13	0.419	2.676	.
0GLD	.	3.28	4.32	0.425	3.542	.
0GLD	.	3.13	4.11	0.468	3.641	.
0GLD	.	3.12	4.15	0.464	4.849	.
0GLD	.	2.77	4.48	0.485	4.355	.
0GLD	.	3.20	4.04	0.465	2.864	.

0GLD	.	2.98	.	0.525	.	.
0GLD	.	2.95	.	0.512	.	.
0GLD	.	2.62	.	0.537	.	.
0GLD	.	3.09	.	0.490	.	.
0GLD	.	3.20	.	0.438	.	.
0GLD	.	3.15	.	0.466	.	.
0GLD	.	2.99	.	0.506	.	.
0GLD	.	3.02	.	0.482	.	.
0GLD	.	3.51	.	0.411	.	.
0GLD	.	2.69	.	0.541	.	.
0GLD	.	2.93	.	0.476	.	.
0GLD	.	2.85	.	0.513	.	.
0GLD	.	2.75	.	0.527	.	.
0GLD	.	3.34	.	0.402	.	.
0GLD	.	2.90	.	0.513	.	.
0GHD	400.00	2.57	4.16	0.563	3.185	91.04
0GHD	397.00	2.93	3.85	0.533	5.445	91.01
0GHD	369.00	2.69	4.18	0.535	3.733	94.93
0GHD	.	3.13	4.25	0.452	3.822	91.39
0GHD	.	2.99	4.16	0.484	5.650	91.62
0GHD	.	2.59	4.17	0.558	5.927	.
0GHD	.	2.69	4.17	0.536	5.420	.
0GHD	.	3.02	4.13	0.482	3.642	.
0GHD	.	2.68	3.90	0.576	3.983	.
0GHD	.	2.71	4.27	0.520	4.937	.
0GHD	.	2.72	4.03	0.549	3.970	.
0GHD	.	2.71	4.05	0.549	3.760	.
0GHD	.	2.79	4.20	0.514	6.543	.
0GHD	.	3.05	4.13	0.478	4.876	.
0GHD	.	2.47	4.08	0.597	5.172	.
0GHD	.	2.78	.	0.521	.	.
0GHD	.	2.85	.	0.548	.	.
0GHD	.	2.52	.	0.572	.	.
0GHD	.	2.32	.	0.611	.	.
0GHD	.	2.88	.	0.504	.	.
0GHD	.	2.34	.	0.617	.	.
0GHD	.	2.44	.	0.591	.	.
0GHD	.	2.58	.	0.565	.	.
0GHD	.	2.64	.	0.584	.	.
0GHD	.	2.44	.	0.577	.	.
0GHD	.	2.82	.	0.530	.	.
0GHD	.	2.31	.	0.644	.	.
0GHD	.	2.62	.	0.548	.	.

0GHD	.	2.76	.	0.528	.	.
0GHD	.	2.83	.	0.521	.	.
10GLD	260.00	2.88	4.75	0.440	6.602	48.95
10GLD	265.00	3.75	4.48	0.358	3.670	52.19
10GLD	250.00	4.20	4.23	0.339	5.202	52.00
10GLD	.	3.15	4.28	0.446	3.502	52.64
10GLD	.	3.66	4.37	0.376	3.718	55.03
10GLD	.	3.76	4.09	0.392	3.977	.
10GLD	.	2.88	4.50	0.465	3.405	.
10GLD	.	3.94	4.12	0.371	5.006	.
10GLD	.	4.36	4.58	0.301	3.225	.
10GLD	.	3.10	4.53	0.429	2.697	.
10GLD	.	4.18	4.49	0.321	3.349	.
10GLD	.	3.79	4.69	0.338	3.313	.
10GLD	.	3.35	4.42	0.406	3.447	.
10GLD	.	3.89	4.74	0.327	4.603	.
10GLD	.	3.88	4.29	0.362	5.537	.
10GLD	.	3.95	.	0.321	.	.
10GLD	.	2.90	.	0.462	.	.
10GLD	.	3.38	.	0.421	.	.
10GLD	.	4.19	.	0.336	.	.
10GLD	.	2.85	.	0.484	.	.
10GLD	.	3.09	.	0.477	.	.
10GLD	.	3.06	.	0.437	.	.
10GLD	.	3.63	.	0.403	.	.
10GLD	.	2.70	.	0.486	.	.
10GLD	.	3.63	.	0.366	.	.
10GLD	.	3.30	.	0.407	.	.
10GLD	.	3.09	.	0.415	.	.
10GLD	.	2.93	.	0.464	.	.
10GLD	.	3.09	.	0.412	.	.
10GLD	.	3.40	.	0.413	.	.
10GHD	300.00	3.09	4.72	0.412	3.302	47.80
10GHD	308.00	3.13	4.77	0.403	3.778	48.80
10GHD	301.00	3.22	4.52	0.413	4.339	49.12
10GHD	.	4.66	4.78	0.270	2.723	50.12
10GHD	.	3.92	4.34	0.354	4.614	65.85
10GHD	.	3.77	4.50	0.355	5.113	.
10GHD	.	4.71	4.53	0.282	3.907	.
10GHD	.	3.51	4.39	0.391	4.764	.
10GHD	.	3.26	4.20	0.440	4.562	.
10GHD	.	3.30	4.33	0.422	4.536	.
10GHD	.	3.56	4.49	0.376	3.840	.

10GHD	.	3.25	4.40	0.421	3.158	.
10GHD	.	2.93	4.73	0.435	4.257	.
10GHD	.	3.34	4.42	0.408	4.622	.
10GHD	.	2.99	4.33	0.464	4.076	.
10GHD	.	3.85	.	0.331	.	.
10GHD	.	4.15	.	0.304	.	.
10GHD	.	4.04	.	0.330	.	.
10GHD	.	3.19	.	0.395	.	.
10GHD	.	3.42	.	0.406	.	.
10GHD	.	3.25	.	0.412	.	.
10GHD	.	3.29	.	0.404	.	.
10GHD	.	3.32	.	0.413	.	.
10GHD	.	3.82	.	0.375	.	.
10GHD	.	4.00	.	0.347	.	.
10GHD	.	4.52	.	0.296	.	.
10GHD	.	4.00	.	0.342	.	.
10GHD	.	4.25	.	0.300	.	.
10GHD	.	4.20	.	0.324	.	.
10GHD	.	3.70	.	0.376	.	.

*0GLD = 0% gelatin, low-density; 0GHD = 0% gelatin, high density; 10GLD = 10% low-bloom gelatin, low density; 10GHD = 10% low-bloom gelatin, high density

Table A.4 Raw data from Experiment 4 comparing different strengths of gelatin in extruded pet food using die shape to create replication.

Treatment*	Bulk Density	Radial Expansion	Specific Length	Piece Density	Hardness	PDI
OGC	365.00	3.54	4.23	0.420	6.841	89.26
OGC	338.00	3.87	3.97	0.409	7.491	92.88
OGC	325.00	4.10	4.51	0.340	6.778	89.71
OGC	333.00	4.25	4.34	0.341	6.688	89.57
OGC	325.00	3.64	4.55	0.379	7.015	90.17
OGC	.	3.74	4.66	0.361	5.805	.
OGC	.	3.85	5.00	0.327	6.114	.
OGC	.	4.11	4.41	0.348	5.366	.
OGC	.	2.55	4.35	0.568	7.840	.
OGC	.	4.10	4.20	0.365	7.631	.
OGC	.	2.68	4.65	0.504	6.452	.
OGC	.	4.07	3.86	0.400	5.811	.
OGC	.	2.84	3.92	0.563	5.545	.
OGC	.	2.95	4.45	0.479	5.853	.
OGC	.	3.77	4.62	0.361	5.122	.
OGC	.	3.02	.	0.492	.	.
OGC	.	3.09	.	0.512	.	.
OGC	.	3.21	.	0.435	.	.

OGC	.	3.04	.	0.478	.	.
OGC	.	2.50	.	0.552	.	.
OGC	.	2.23	.	0.606	.	.
OGC	.	2.51	.	0.501	.	.
OGC	.	2.55	.	0.561	.	.
OGC	.	3.49	.	0.414	.	.
OGC	.	2.85	.	0.525	.	.
OGC	.	4.32	.	0.313	.	.
OGC	.	3.39	.	0.481	.	.
OGC	.	3.75	.	0.428	.	.
OGC	.	3.99	.	0.354	.	.
OGC	.	2.71	.	0.502	.	.
100GC	320.00	4.89	4.75	0.271	8.087	87.18
100GC	310.00	3.83	4.24	0.387	6.459	87.55
100GC	302.00	4.79	3.98	0.330	11.470	87.55
100GC	302.00	4.89	4.58	0.281	8.794	87.95
100GC	290.00	5.33	4.60	0.257	8.545	.
100GC	.	3.87	4.15	0.392	7.743	.
100GC	.	5.47	4.40	0.262	9.449	.
100GC	.	4.87	4.63	0.279	7.609	.
100GC	.	4.84	3.89	0.334	7.914	.
100GC	.	3.30	3.71	0.514	8.782	.
100GC	.	3.78	4.50	0.370	6.722	.
100GC	.	4.31	4.48	0.326	6.011	.
100GC	.	4.85	4.05	0.320	8.269	.
100GC	.	4.08	4.37	0.353	6.734	.
100GC	.	3.18	4.17	0.475	5.543	.
100GC	.	2.57	.	0.516	.	.
100GC	.	2.68	.	0.555	.	.
100GC	.	3.37	.	0.469	.	.
100GC	.	2.58	.	0.532	.	.
100GC	.	3.19	.	0.429	.	.
100GC	.	3.11	.	0.489	.	.
100GC	.	3.04	.	0.471	.	.
100GC	.	3.10	.	0.439	.	.
100GC	.	2.76	.	0.587	.	.
100GC	.	4.50	.	0.376	.	.
100GC	.	3.31	.	0.422	.	.
100GC	.	3.00	.	0.468	.	.
100GC	.	3.22	.	0.482	.	.
100GC	.	2.83	.	0.508	.	.
100GC	.	5.00	.	0.302	.	.
175GC	257.00	3.81	4.22	0.392	4.210	68.33

175GC	235.00	6.35	4.23	0.234	7.111	68.58
175GC	230.00	5.57	5.28	0.214	5.097	70.44
175GC	236.00	6.52	4.37	0.221	6.227	71.10
175GC	240.00	5.85	4.25	0.253	4.127	72.51
175GC	.	4.15	4.09	0.371	6.260	.
175GC	.	6.42	4.45	0.220	3.321	.
175GC	.	6.13	4.18	0.245	4.413	.
175GC	.	6.41	4.46	0.220	6.688	.
175GC	.	6.44	4.03	0.243	6.411	.
175GC	.	3.08	5.32	0.384	7.377	.
175GC	.	6.44	4.59	0.213	5.179	.
175GC	.	4.81	5.53	0.236	3.316	.
175GC	.	6.27	4.40	0.228	5.521	.
175GC	.	5.70	4.38	0.252	5.182	.
175GC	.	6.52	.	0.229	.	.
175GC	.	3.31	.	0.448	.	.
175GC	.	2.48	.	0.480	.	.
175GC	.	3.55	.	0.405	.	.
175GC	.	3.43	.	0.432	.	.
175GC	.	6.38	.	0.241	.	.
175GC	.	3.40	.	0.415	.	.
175GC	.	3.54	.	0.425	.	.
175GC	.	4.78	.	0.295	.	.
175GC	.	3.98	.	0.392	.	.
175GC	.	5.10	.	0.232	.	.
175GC	.	3.60	.	0.381	.	.
175GC	.	3.78	.	0.301	.	.
175GC	.	3.72	.	0.384	.	.
175GC	.	3.33	.	0.432	.	.
250GC	208.00	6.77	5.00	0.186	3.835	23.11
250GC	206.00	7.75	4.44	0.183	3.358	22.68
250GC	212.00	6.61	4.69	0.203	2.918	27.50
250GC	206.00	4.13	5.60	0.272	5.357	29.15
250GC	.	4.86	4.63	0.280	3.809	27.84
250GC	.	3.64	4.50	0.384	3.034	.
250GC	.	4.61	4.56	0.299	2.763	.
250GC	.	7.05	5.00	0.178	2.642	.
250GC	.	5.41	4.69	0.248	5.181	.
250GC	.	4.00	4.46	0.353	2.775	.
250GC	.	5.93	5.79	0.183	4.079	.
250GC	.	4.63	4.67	0.291	2.936	.
250GC	.	3.59	4.41	0.397	2.903	.
250GC	.	4.66	4.87	0.277	5.459	.

250GC	.	4.10	4.74	0.324	3.172	.
250GC	.	4.06	.	0.310	.	.
250GC	.	4.13	.	0.344	.	.
250GC	.	4.79	.	0.280	.	.
250GC	.	5.51	.	0.204	.	.
250GC	.	6.58	.	0.207	.	.
250GC	.	8.33	.	0.168	.	.
250GC	.	7.83	.	0.176	.	.
250GC	.	3.58	.	0.352	.	.
250GC	.	6.42	.	0.209	.	.
250GC	.	5.94	.	0.238	.	.
250GC	.	4.74	.	0.229	.	.
250GC	.	7.61	.	0.177	.	.
250GC	.	6.38	.	0.223	.	.
250GC	.	8.80	.	0.147	.	.
250GC	.	9.07	.	0.146	.	.
0GT	366.00	2.73	4.15	0.679	4.147	85.06
0GT	328.00	2.44	4.23	0.746	3.902	85.86
0GT	378.00	2.53	4.05	0.752	5.065	88.04
0GT	360.00	2.63	3.83	0.766	4.200	86.23
0GT	.	3.49	4.01	0.552	4.943	88.11
0GT	.	3.36	4.23	0.541	6.536	.
0GT	.	2.85	4.10	0.658	6.523	.
0GT	.	2.74	4.07	0.690	7.171	.
0GT	.	2.52	4.33	0.707	6.064	.
0GT	.	3.31	4.08	0.571	6.217	.
0GT	.	2.33	4.32	0.765	4.863	.
0GT	.	2.36	4.15	0.786	3.883	.
0GT	.	2.66	4.09	0.709	6.358	.
0GT	.	2.52	4.44	0.688	5.862	.
0GT	.	2.71	4.19	0.677	5.818	.
100GT	319.00	3.19	3.93	0.613	6.234	86.40
100GT	314.00	3.07	4.29	0.586	6.468	86.74
100GT	314.00	3.15	4.00	0.612	7.210	87.18
100GT	319.00	3.45	4.02	0.555	7.377	91.13
100GT	.	3.27	4.18	0.564	5.183	86.43
100GT	.	3.45	4.26	0.525	7.597	.
100GT	.	2.99	4.36	0.590	6.087	.
100GT	.	3.29	4.40	0.532	6.748	.
100GT	.	3.28	4.68	0.502	7.549	.
100GT	.	3.53	4.05	0.538	8.390	.
100GT	.	3.30	4.11	0.568	6.327	.
100GT	.	2.99	4.50	0.573	6.318	.

100GT	.	3.48	4.00	0.554	6.220	.
100GT	.	3.20	4.26	0.565	8.600	.
100GT	.	3.31	4.26	0.546	7.029	.
175GT	261.00	3.06	4.74	0.530	3.790	57.30
175GT	238.00	3.58	4.36	0.493	2.805	57.48
175GT	244.00	3.73	4.81	0.429	2.889	58.79
175GT	238.00	4.09	4.43	0.424	5.158	58.95
175GT	.	4.07	4.75	0.398	2.997	61.98
175GT	.	3.76	4.70	0.435	3.827	.
175GT	.	2.96	4.40	0.592	3.477	.
175GT	.	4.21	5.03	0.364	4.477	.
175GT	.	3.93	4.40	0.445	4.259	.
175GT	.	3.89	4.26	0.465	3.567	.
175GT	.	4.05	4.09	0.465	4.007	.
175GT	.	3.70	4.30	0.484	4.598	.
175GT	.	3.63	4.60	0.460	2.684	.
175GT	.	3.85	4.41	0.453	3.887	.
175GT	.	3.82	3.74	0.539	4.091	.
250GT	213.00	4.96	4.64	0.335	3.898	35.08
250GT	215.00	3.89	3.86	0.512	2.748	31.15
250GT	225.00	4.41	4.46	0.391	3.400	32.94
250GT	213.00	4.23	4.49	0.406	2.678	34.12
250GT	.	3.99	4.91	0.393	3.030	36.49
250GT	.	4.42	4.87	0.358	4.020	.
250GT	.	4.61	4.16	0.402	2.827	.
250GT	.	3.43	6.77	0.331	2.619	.
250GT	.	3.17	4.60	0.528	5.011	.
250GT	.	4.75	4.79	0.339	3.869	.
250GT	.	3.93	4.42	0.442	4.615	.
250GT	.	4.26	4.64	0.389	3.607	.
250GT	.	4.33	4.29	0.415	3.615	.
250GT	.	3.93	4.55	0.430	2.885	.
250GT	.	3.57	4.82	0.448	4.765	.

*0GC = 0% gelatin, circle die; 100GC = 10% 100-bloom gelatin, circle die; 175GC = 10% 175-bloom gelatin, circle die; 250GC = 10% 250-bloom gelatin, circle die; 0GT = 0% gelatin, triangle die; 100GT = 10% 100-bloom gelatin, triangle die; 175GT = 10% 175-bloom gelatin, triangle die; 250GT = 10% 250-bloom gelatin, triangle die

Appendix B - All Main Effect and Interaction Analysis of Lab-Scale Injection Molding Project

All Main Effects

Table B.1 Main effect means of gelatin level in the lab-scale injection molding experiment.

Variable	n	5	10	15	MSE	P
SME (KJ/kg)	62	516.10	558.53	530.08	11955.42	0.60
Die Temperature (°C)	62	123.00	123.41	121.60	11.97	0.89
Die Pressure (PSI)	62	247.44[a]	182.33[b]	142.00[b]	49031.22	0.002
Tensile Strength (MPa)	110	1.99[c]	3.34[b]	5.05[a]	64.05	<0.0001
Strain at Break (%)	110	2.36[a]	2.46[a]	1.49[b]	7.09	0.07
Young's Modulus (MPa)	110	194.79[c]	331.09[b]	492.60[a]	609974.73	<0.0001
Puncture Force (g)	220	20.64[b]	21.17[b]	23.61[a]	123.23	0.03
Peaks (n)	220	1.06[c]	1.36[b]	1.68[c]	5.24	<0.0001

[abc]Means in a row with unlike superscripts differ by P<0.05

Table B.2 Main effect means of gluten level in the lab-scale injection molding experiment.

Variable	n	5	10	15	MSE	P
SME (KJ/kg)	62	454.42[b]	578.67[a]	553.14[ab]	89235.76	0.017
Die Temperature (°C)	62	124.22	122.73	121.79	24.76	0.79
Die Pressure (PSI)	62	139.06[c]	202.87[b]	281.43[c]	79831.38	<0.0001
Tensile Strength (MPa)	110	2.67	3.41	3.24	5.48	0.25
Strain at Break (%)	110	3.10[a]	2.27[b]	1.36[c]	22.72	<0.0001
Young's Modulus (MPa)	110	217.70[a]	333.01[b]	367.23[b]	190104.60	0.02
Puncture Force (g)	220	20.56	21.70	21.82	31.12	0.42
Peaks (n)	220	1.12[b]	1.35[a]	1.43[a]	1.66	0.04

[abc]Means in a row with unlike superscripts differ by P<0.05

Table B.3 Main effect means of glycerin level in the lab-scale injection molding experiment.

Variable	n	5	10	15	MSE	P
SME (KJ/kg)	62	594.87[a]	581.54[a]	478.25[b]	88183.45	0.02
Die Temperature (°C)	62	122.33	121.24	124.68	80.09	0.47
Die Pressure (PSI)	62	170.00[b]	245.60[a]	173.54[b]	39712.82	0.006
Tensile Strength (MPa)	110	5.74[a]	3.14[b]	1.89[c]	96.64	<0.0001
Strain at Break (%)	110	1.31[b]	1.56[b]	3.57[a]	55.88	<0.0001
Young's Modulus (MPa)	110	617.96[a]	320.52[b]	145.33[c]	1493475.93	<0.0001
Puncture Force (g)	220	21.36[b]	23.73[a]	18.57[c]	593.18	<0.0001
Peaks (n)	220	2.03[a]	1.27[b]	1.00[c]	14.15	<0.0001

[abc]Means in a row with unlike superscripts differ by $P<0.05$

Table B.4 Main effect means of moisture content in the lab-scale injection molding experiment.

Variable	n	15	20	MSE	P
SME (KJ/kg)	62	674.85[a]	471.10[b]	562444.25	<0.0001
Die Temperature (°C)	62	123.60	122.62	12.41	0.73
Die Pressure (PSI)	62	281.50[a]	164.26[b]	186219.48	<0.0001
Tensile Strength (MPa)	110	2.91	3.31	4.06	0.31
Young's Modulus (MPa)	110	286.98	324.56	35953.20	0.40
Puncture Force (g)	220	22.81[a]	20.63[b]	243.18	0.009
Peaks (n)	220	1.21	1.36	1.17	0.13

[ab]Means in a row with unlike superscripts differ by $P<0.05$

Table B.5 Main effect means of processing temperature in the lab-scale injection molding experiment.

Variable	n	High	Low	MSE	P
SME (KJ/kg)	62	508.54	565.11	49590.11	0.14
Die Temperature (°C)	62	132.61[a]	113.29[b]	5787.11	<0.0001
Die Pressure (PSI)	62	200.03	204.13	260.14	0.86
Tensile Strength (MPa)	110	3.33	2.99	3.19	0.37
Young's Modulus (MPa)	110	332.29	289.50	50353.77	0.31
Puncture Force (g)	220	22.01	20.83	76.06	0.14
Peaks (n)	220	1.26	1.35	0.45	0.34

[ab]Means in a row with unlike superscripts differ by $P<0.05$

All 2-Way Interactions

Table B.6 Interaction of Gelatin Level and Gluten Level (%Gelatin x %Gluten) in the lab-scale injection molding experiment.

Variable	n	5x10	5x15	10x5	10x10	10x15	15x5	15x10	MSE	P
SME (KJ/kg)	62	500.57^{bc}	539.38^{bc}	452.50^{c}	684.76^{a}	587.52^{abc}	459.42^{bc}	600.75^{ab}	65291.04	0.004
Die Temperature (°C)	62	124.73	120.40	124.62	121.10	125.25	123.20	120.00	41.33	0.89
Die Pressure (PSI)*	62	204.40^{b}	312.00^{a}	137.92^{c}	231.00^{b}	205.00^{bc}	142.00^{c}	142.00^{c}	36484.68	<0.0001
Tensile Strength (MPa)	110	1.55^{e}	2.42^{d}	2.23^{de}	3.68^{c}	4.89^{b}	3.51^{c}	6.59^{a}	38.98	<0.0001
Young's Modulus (MPa)	110	117.92^{d}	271.66^{c}	172.73^{d}	375.82^{b}	558.36^{a}	307.63^{cb}	677.57^{a}	533104.322	<0.0001
Puncture Force (g)	220	17.64^{b}	23.64^{a}	19.49^{b}	24.35^{a}	18.18^{b}	22.68^{a}	24.53^{a}	282.63	<0.0001
Peaks (n)	220	1.00^{c}	1.13^{cb}	1.00^{c}	1.38^{b}	2.05^{a}	1.35^{b}	2.00^{a}	4.96	<0.0001

*5x10 and 10x5 are also significantly different
[abcd]Means in a row with unlike superscripts differ by P<0.05

Table B.7 Interaction of Gelatin Level and Glycerin Level (%Gelatin x %Glycerin) in the lab-scale injection molding experiment.

Variable	n	5x10	5x15	10x5	10x10	10x15	15x5	15x10	MSE	P
SME (KJ/kg)	62	539.38^{bc}	500.57^{bc}	587.52^{abc}	684.76^{a}	452.50^{c}	600.75^{ab}	459.52^{bc}	65291.04	0.004
Die Temperature (°C)	62	120.40	124.73	125.25	121.10	124.62	120.00	123.20	41.33	0.89
Die Pressure (PSI)*	62	312.00^{a}	204.40^{bc}	205.00^{bc}	231.00^{b}	137.92^{c}	142.00^{c}	142.00^{c}	36484.68	<0.0001
Tensile Strength (MPa)	110	2.42^{d}	1.55^{e}	4.89^{b}	3.68^{c}	2.23^{de}	6.59^{a}	3.51^{c}	38.87	<0.0001
Young's Modulus (MPa)	110	271.66^{c}	117.92^{d}	558.36^{a}	375.82^{b}	172.73^{d}	677.57^{a}	307.63^{bc}	53304.32	<0.0001
Puncture Force (g)	220	23.64^{a}	17.64^{b}	18.18^{b}	24.35^{a}	19.49^{b}	24.53^{a}	22.68^{a}	282.63	<0.0001
Peaks (n)	220	1.13^{bc}	1.00^{c}	2.05^{a}	1.38^{b}	1.00^{c}	2.00^{a}	1.35^{b}	4.96	<0.0001

*5x15 and 10x15 are also significantly different
[abcde]Means in a row with unlike superscripts differ by P<0.05

Table B.8 Interaction of Gelatin Level and Initial Moisture Content (%Gelatin x %Moisture) in the lab-scale injection molding experiment.

Variable	n	5x15	5x20	10x15	10x20	15x20	MSE	P
SME (KJ/kg)	62	611.51[b]	452.49[c]	738.20[a]	452.84[c]	530.08[bc]	172088.60	<0.0001
Die Temperature (°C)	62	124.40	122.07	122.80	123.77	121.60	15.62	0.97
Die Pressure (PSI)	62	352.00[a]	177.73[bc]	211.00[b]	165.47[bc]	142.00[c]	73331.86	<0.0001
Tensile Strength (MPa)	110	237[cd]	1.60[d]	3.44[b]	3.23[bc]	5.05[a]	35.58	<0.0001
Young's Modulus (MPa)	110	246.52[bc]	143.07[c]	327.44[b]	333.53[b]	492.60[a]	331853.63	<0.0001
Puncture Force (g)	220	23.43[a]	17.84[c]	22.19[ab]	20.49[b]	23.61[a]	235.01	<0.0001
Peaks (n)	220	1.08[c]	1.05[c]	1.35[bc]	1.37[b]	1.68[a]	2.62	0.0003

[abc]Means in a row with unlike superscripts differ by P<0.05

Table B.9 Interaction of Gelatin Level and Processing Temperature (%Gelatin x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5xHigh	5xLow	10xHigh	10xLow	15xHigh	15xLow	MSE	P
SME (KJ/kg)	62	515.58	516.58	517.56	602.65	466.46	593.70	22640.23	0.43
Die Temperature (°C)	62	133.00[a]	113.77[b]	132.79[a]	113.31[b]	131.20[a]	112.00[b]	1162.12	<0.0001
Die Pressure (PSI)*	62	241.33[ab]	253.08[a]	190.36[abc]	173.69[bc]	128.00[c]	156.00[bc]	20551.00	0.02
Tensile Strength (MPa)	110	2.06[d]	1.91[d]	3.54[bc]	3.14[c]	5.33[a]	4.77[ab]	26.39	<0.0001
Young's Modulus (MPa)	110	210.90[de]	178.69[e]	351.52[bc]	310.67[cd]	527.01[a]	458.19[ab]	254971.72	<0.0001
Puncture Force (g)	220	21.02[ab]	20.26[b]	21.94[ab]	20.41[b]	24.17[a]	23.04[ab]	65.86	0.10
Peaks (n)	220	1.13[bc]	1.00[c]	1.32[b]	1.40[b]	1.40[b]	1.95[a]	2.80	<0.0001

*5xHigh and 10xLow are also significantly different
[abcde]Means in a row with unlike superscripts differ by P<0.05

Table B.10 Interaction of Gluten Level and Glycerin Level (%Gluten x %Glycerin) in the lab-scale injection molding experiment.

Variable	n	5x10	5x15	10x5	10x10	10x15	15x5	15x10	MSE	P
SME (KJ/kg)	62	459.42^{bc}	452.50^{c}	600.75^{ab}	684.76^{a}	500.57^{bc}	587.52^{abc}	539.38^{bc}	65291.04	0.004
Die Temperature (°C)	62	123.20	124.62	120.00	121.10	124.73	125.25	120.40	41.33	0.89
Die Pressure (PSI)*	62	142.00^{c}	137.92^{c}	142.00^{c}	231.00^{b}	204.40^{bc}	205.00^{bc}	312.00^{a}	36484.68	<0.0001
Tensile Strength (MPa)	110	3.51^{c}	2.23^{de}	6.59^{a}	3.68^{c}	1.55^{e}	4.89^{b}	2.42^{d}	38.98	<0.0001
Young's Modulus (MPa)	110	307.63^{bc}	172.73^{d}	677.57^{a}	375.82^{b}	117.92^{d}	558.36^{a}	271.66^{c}	533104.32	<0.0001
Puncture Force (g)	220	22.68^{a}	19.49^{b}	24.53^{a}	24.35^{a}	17.64^{b}	18.18^{b}	23.64^{a}	282.63	<0.0001
Peaks (n)	220	1.35^{b}	1.00^{c}	2.00^{a}	1.38^{b}	1.00^{c}	2.05^{a}	1.13^{bc}	4.96	<0.0001

*5x15 and 10x15 are also significantly different

^{abcde}Means in a row with unlike superscripts differ by P<0.05

Table B.11 Interaction of Gluten Level and Initial Moisture Content (%Gluten x %Moisture) in the lab-scale injection molding experiment.

Variable	n	5x15	5x20	10x15	10x20	15x15	15x20	MSE	P
SME (KJ/kg)	62	638.13^{a}	362.57^{c}	723.95^{a}	506.03^{b}	307.20^{ab}	531.51^{b}	163037.09	<0.0001
Die Temperature (°C)	62	122.33	125.17	125.40	121.40	121.00	121.10	38.35	0.88
Die Pressure (PSI)	62	176.67^{d}	120.25^{e}	285.00^{b}	161.80^{d}	430.00^{a}	222.00^{c}	79438.76	<0.0001
Tensile Strength (MPa)	110	2.90^{ab}	2.53^{b}	2.83^{ab}	3.80^{a}	3.07^{ab}	3.33^{ab}	4.74	0.30
Young's Modulus (MPa)	110	248.19^{ab}	202.46^{b}	277.51^{ab}	370.10^{a}	344.69^{ab}	378.49^{a}	100886.63	0.06
Puncture Force (g)*	220	21.87^{b}	19.90^{b}	21.46^{b}	21.86^{b}	26.47^{a}	19.49^{b}	153.22	0.0005
Peaks (n)	220	1.00^{b}	1.18^{b}	1.35^{ab}	1.35^{ab}	1.15^{b}	1.58^{a}	1.23	0.03

*10x20 and 15x20 are also significantly different

^{abcde}Means in a row with unlike superscripts differ by P<0.05

Table B.12 Interaction of Gluten Level and Processing Temperature (%Gluten x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5xHigh	5xLow	10xHigh	10xLow	15xHigh	15xLow	MSE	P
SME (KJ/kg)	62	401.20^{b}	520.95^{ab}	579.33^{a}	578.09^{a}	520.35^{ab}	585.92^{a}	51455.06	0.04
Die Temperature (°C)	62	132.70^{a}	113.63^{b}	133.36^{a}	113.44^{b}	131.00^{a}	122.57^{b}	1163.60	<0.0001
Die Pressure (PSI)*	62	139.50^{b}	138.50^{b}	200.43^{b}	205.00^{b}	285.71^{a}	277.14^{a}	32016.08	0.0005
Tensile Strength (MPa)	110	2.66	2.65	3.74	3.08	3.32	3.17	3.30	0.53
Young's Modulus (MPa)	110	221.81^{bc}	213.59^{c}	379.54^{a}	286.48^{abc}	364.01^{abc}	370.44^{ab}	97857.39	0.07
Puncture Force (g)	220	21.04^{ab}	20.07^{b}	23.21^{a}	20.19^{b}	20.97^{b}	22.67^{ab}	69.53	0.08
Peaks (n)	220	1.10^{b}	1.13^{ab}	1.28^{ab}	1.42^{ab}	1.40^{ab}	1.47^{a}	0.78	0.17

*5xHigh and 10xLow are also significantly different

^{abc}Means in a row with unlike superscripts differ by P<0.05

Table B.13 Interaction of Glycerin Level and Initial Moisture Content (%Glycerin x %Moisture) in the lab-scale injection molding experiment.

Variable	n	5x20	10x15	10x20	15x15	15x20	MSE	P
SME (KJ/kg)	62	594.87^{b}	747.75^{a}	503.33^{c}	626.25^{b}	367.25^{d}	240341.32	<0.0001
Die Temperature (°C)	62	122.33	122.25	120.77	124.50	124.81	43.21	0.81
Die Pressure (PSI)	62	170.00^{cd}	346.25^{a}	198.24^{bc}	238.33^{b}	124.94^{d}	71695.11	<0.0001
Tensile Strength (MPa)	110	5.74^{a}	3.52^{b}	2.89^{bc}	2.29^{cd}	1.49^{d}	52.10	<0.0001
Young's Modulus (MPa)	110	617.96^{a}	375.69^{b}	283.74^{c}	198.26^{d}	92.39^{e}	800127.16	<0.0001
Puncture Force (g)	220	21.36^{b}	24.49^{a}	23.22^{ab}	21.13^{b}	16.00^{c}	437.94	<0.0001
Peaks (n)	220	2.03^{a}	1.43^{b}	1.17^{c}	1.00^{c}	1.00^{c}	7.47	<0.0001

^{abcde}Means in a row with unlike superscripts differ by P<0.05

Table B.14 Interaction of Glycerin Level and Processing Temperature (%Glycerin x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5xHigh	5xLow	10xHigh	10xLow	15xHigh	15xLow	MSE	P
SME (KJ/kg)	62	561.92^{abc}	621.23^{ab}	541.97^{abc}	624.42^{a}	462.27^{c}	494.24^{bc}	46751.45	0.06
Die Temperature (°C)	62	133.50^{a}	113.40^{cd}	130.23^{b}	111.50^{d}	134.57^{a}	114.79^{c}	1197.51	<0.0001
Die Pressure (PSI)*	62	190.00^{ab}	154.00^{b}	233.08^{ab}	259.17^{a}	172.21^{b}	174.86^{b}	17320.39	0.05
Tensile Strength (MPa)	110	6.23^{a}	5.25^{a}	3.22^{b}	3.07^{b}	2.02^{c}	1.76^{c}	40.61	<0.0001
Young's Modulus (MPa)	110	636.46^{a}	599.47^{a}	349.28^{b}	291.75^{b}	158.96^{c}	131.69^{c}	608519.85	<0.0001
Puncture Force (g)	220	21.56^{ab}	21.15^{ab}	23.98^{a}	23.48^{a}	19.76^{bc}	17.37^{c}	261.81	<0.0001
Peaks (n)	220	1.60^{b}	2.45^{a}	1.34^{bc}	1.20^{cd}	1.00^{d}	1.00^{d}	7.20	<0.0001

^{abcd}Means in a row with unlike superscripts differ by P<0.05

Table B.15 Interaction of Initial Moisture Content and Processing Temperature (%Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	15xHigh	15xLow	20xHigh	20xLow	MSE	P
SME (KJ/kg)	62	658.60^{a}	691.11^{a}	437.10^{b}	505.11^{b}	205434.73	<0.0001
Die Temperature (°C)	62	132.30^{a}	114.90^{b}	132.76^{a}	112.52^{c}	1942.27	<0.0001
Die Pressure (PSI)	62	276.00^{a}	290.00^{a}	165.29^{b}	163.24^{b}	62569.50	<0.0001
Tensile Strength (MPa)	110	2.97	2.84	3.54	3.08	2.64	0.57
Young's Modulus (MPa)	110	317.60	256.35	340.68	308.44	30553.94	0.61
Puncture Force (g)	220	22.88^{a}	22.75^{a}	21.51^{ab}	19.74^{b}	117.90	0.02
Peaks (n)	220	1.30^{ab}	1.13^{b}	1.24^{b}	1.49^{a}	1.28	0.05

^{abc}Means in a row with unlike superscripts differ by P<0.05

All 3-Way Interactions

Table B.16 Interaction of Gelatin, Gluten, and Glycerin Levels (%Gelatin x %Gluten x %Glycerin) in the lab-scale injection molding experiment.

Variable	n	5x10x15	5x15x10	10x5x15	10x10x10	10x15x5	15x5x10	15x10x5	MSE	P
SME (KJ/kg)	62	500.57[bc]	539.38[bc]	452.50[c]	684.76[a]	587.52[abc]	459.42[bc]	600.75[ab]	65291.04	0.004
Die Temperature (°C)	62	124.73	120.40	124.62	121.10	125.25	123.20	120.00	41.33	0.89
Die Pressure (PSI)*	62	204.40[bc]	312.00[a]	137.92[c]	231.00[b]	205.00[bc]	142.00[c]	142.00[c]	36484.68	<0.0001
Tensile Strength (MPa)	110	1.55[e]	2.42[d]	2.23[de]	3.68[c]	4.89[b]	3.51[c]	6.59[a]	38.98	<0.0001
Strain at Break (%)	110	3.32[a]	1.40[b]	3.83[a]	1.68[b]	1.27[b]	1.63[b]	1.36[b]	19.21	<0.0001
Young's Modulus (MPa)	110	117.92[d]	271.66[c]	172.73[d]	375.82[b]	558.36[a]	307.63[bc]	677.57[a]	533104.32	<0.0001
Puncture Force (g)	220	17.64[b]	23.64[a]	19.49[b]	24.35[a]	18.18[b]	22.68[a]	24.53[a]	282.63	<0.0001
Peaks (n)	220	1.00[c]	1.13[bc]	1.00[c]	1.38[b]	2.05[a]	1.35[b]	2.00[a]	4.96	<0.0001

*5x10x15 and 10x5x15 are also significantly different

[abcde]Means in a row with unlike superscripts differ by P<0.05

Table B.17 Interaction of Gelatin and Gluten Levels and Initial Moisture Content (%Gelatin x %Gluten x %Moisture) in the lab-scale injection molding experiment.

Variable	n	5x10 x15	5x10 x20	5x15 x15	5x15 x20	10x5 x15	10x5 x20	10x10 x15	10x10 x20	10x15 x20	15x5 x20	15x10 x20	MSE	P
SME (KJ/kg)	62	614.38[bc]	424.70[f]	607.20[bc]	494.17[de]	638.13[b]	293.39[g]	888.31[a]	549.07[cd]	587.52[bc]	459.42[ef]	600.75[bc]	121208.05	<0.0001
Die Temperature (°C)	62	126.67	123.44	121.00	120.00	122.33	126.57	123.50	119.50	125.25	123.20	120.00	38.42	0.97
Die Pressure (PSI)*	62	300.00[b]	140.67[gf]	430.00[a]	233.33[cd]	176.67[ef]	104.71[g]	262.50[bc]	210.00[de]	205.00[de]	142.00[gf]	142.00[gf]	42646.95	<0.0001
Tensile Strength (MPa)	110	1.68[e]	1.43[e]	3.07[c]	1.78[de]	2.90[cd]	1.56[e]	3.98[bc]	3.39[c]	4.89[b]	3.51[c]	6.59[a]	25.33	<0.0001
Young's Modulus (MPa)	110	148.34[ef]	87.51[f]	344.69[bc]	198.63[def]	248.19[cde]	97.28[f]	406.69[b]	344.95[bc]	558.36[a]	307.63[bcd]	677.57[a]	345673.56	<0.0001
Puncture Force (g)	220	20.40[cd]	14.88[f]	24.47[a]	20.81[cd]	21.87[bc]	17.12[ef]	22.52[bc]	24.53[ab]	18.18[de]	22.68[bc]	24.53[ab]	268.00	<0.0001
Peaks (n)	220	1.00[c]	1.00[c]	1.15[c]	1.10[c]	1.00[c]	1.00[c]	1.70[ab]	1.05[c]	2.05[a]	1.35[bc]	2.00[a]	3.40	<0.0001

[abcdefg]Means in a row with unlike superscripts differ by P<0.05

Table B.18 Interaction of Gelatin and Gluten Levels and Processing Temperature (%Gelatin x %Gluten x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x10xHigh	5x10xLow	5x15xHigh	5x15xLow	10x5xHigh	10x5xLow	10x10xHigh	10x10xLow	10x15xHigh	10x15xLow	15x5xHigh	15x5xLow	15x10xHigh	15x10xLow	MSE	P
SME (KJ/kg)*	62	524.86^{bcd}	479.32^{cd}	502.58^{bcd}	576.18^{abcd}	399.68^{d}	514.12^{bcd}	663.69^{ab}	705.83^{a}	564.77^{abcd}	610.27^{abcd}	404.73^{cd}	541.45^{abcd}	559.07^{abcd}	628.54^{abc}	37698.10	0.04
Die Temperature (°C)	62	135.57^{a}	115.25^{e}	129.40^{d}	111.40^{f}	133.57^{abc}	114.17^{e}	130.80^{cd}	111.40^{f}	135.00^{ab}	115.50^{e}	130.67^{cd}	112.00^{ef}	132.00^{bcd}	112.00^{ef}	464.27	<0.0001
Die Pressure (PSI)**	62	196.57^{b}	211.25^{b}	304.00^{a}	320.00^{a}	147.86^{b}	126.33^{b}	230.00^{ab}	232.00^{ab}	240.00^{ab}	170.00^{b}	120.00^{b}	175.00^{b}	140.00^{b}	143.33^{b}	17723.27	0.002
Tensile Strength (MPa)	110	1.65^{g}	1.45^{g}	2.47^{efg}	2.37^{efg}	2.39^{efg}	2.07^{fg}	3.97^{cd}	3.40^{de}	5.00^{bc}	4.77^{bcd}	3.21^{def}	3.82^{cde}	7.45^{a}	5.72^{b}	18.84	<0.0001
Young's Modulus (MPa)	110	137.00^{gh}	98.85^{h}	284.80^{ef}	258.52^{gef}	180.93^{fgh}	164.54^{fgh}	436.63^{cd}	604.68^{ab}	522.45^{bc}	594.26^{ab}	303.56^{def}	311.70^{def}	750.46^{a}	604.68^{ab}	257757.55	<0.0001
Puncture Force (g)***	220	19.28^{bcde}	16.00^{e}	22.75^{ab}	24.52^{a}	20.25^{bcd}	18.74^{cde}	25.89^{a}	22.80^{ab}	17.41^{de}	18.95^{bcde}	22.63^{abc}	22.74^{abc}	25.72^{a}	23.34^{ab}	153.31	<0.0001
Peaks (n)	220	1.00^{c}	1.00^{c}	1.25^{bc}	1.00^{c}	1.00^{c}	1.00^{c}	1.45^{b}	1.30^{bc}	1.70^{b}	2.40^{a}	1.30^{bc}	1.40^{bc}	1.50^{b}	2.50^{a}	2.93	<0.0001

^{abcdefgh}Means in a row with unlike superscripts differ by P<0.05

*5x15xLow and 10x5xHigh are also significantly different

**10x5xLow and 10x10xLow; 10x10xLow and 15x5xHigh; 10x5xLow and 10x10xHigh; 5x10xLow and 10x5xLow are also significantly different pairs

***5x10xHigh and 15x10xLow; 5x10xHigh and 10x10xLow; 5x10xLow and 5x15xHigh are also significantly different pairs

Table B.19 Interaction of Gelatin and Glycerin Levels and Initial Moisture Content (%Gelatin x %Glycerin x %Moisture) in the lab-scale injection molding experiment.

Variable	n	5x10 x15	5x10 x20	5x15 x15	5x15 x20	10x5 x20	10x10x 15	10x10x 20	10x15x 15	10x15x 20	15x5 x20	15x10x 20	MSE	P
SME (KJ/kg)	62	607.20 [bc]	459.42 [ef]	614.38[bc]	424.70[f]	587.52[bc]	888.31[a]	549.07[cd]	638.13[b]	293.39[g]	600.75[bc]	459.42[ef]	121208.05	<0.0001
Die Temperature (°C)	62	121.00	120.00	126.67	123.44	125.25	123.50	119.50	122.33	126.57	120.00	123.20	38.42	0.97
Die Pressure (PSI)*	62	430.00[a]	233.33[cd]	300.00[b]	140.67[gf]	205.00[de]	262.50[bc]	210.00[de]	176.67[ef]	104.71[g]	142.00[fg]	142.00[fg]	42646.95	<0.0001
Tensile Strength (MPa)	110	3.07[c]	1.78[de]	1.68[e]	1.43[e]	4.89[b]	3.98[bc]	3.39[c]	2.90[cd]	1.56[e]	6.59[a]	3.51[c]	25.33	<0.0001
Young's Modulus (MPa)	110	344.69[bc]	198.63 [def]	148.34[ef]	87.51[f]	558.36[a]	406.69[b]	344.95[bc]	248.19 [cde]	97.28[f]	677.57[a]	307.63 [bcd]	345673.56	<0.0001
Puncture Force (g)	220	26.47[a]	20.81[cd]	20.40[cd]	14.88[f]	18.18[de]	22.52[bc]	26.18[a]	21.87[bc]	17.12[ef]	14.88[f]	22.68[bc]	268.00	<0.0001
Peaks (n)	220	1.15[c]	1.10[c]	1.00[c]	1.00[c]	2.02[a]	1.70[ab]	1.05[c]	1.00[c]	1.00[c]	2.00[a]	1.35[bc]	3.40	<0.0001

[abcdefg]Means in a row with unlike superscripts differ by P<0.05

Table B.20 Interaction of Gelatin and Glycerin Levels and Processing Temperature (%Gelatin x %Glycerin x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x10 x High	5x10 x Low	5x15 x High	5x15 x Low	10x5 x High	10x5 x Low	10x10x High	10x10x Low	10x15x High	10x15x Low	15x5 x High	15x5 x Low	15x10x High	15x10x Low	MSE	P
SME (KJ/kg)*	62	502.58^{bcd}	576.18^{abcd}	524.86^{bcd}	479.32^{cd}	564.77^{abcd}	610.27^{abcd}	663.69^{ab}	705.83^{a}	399.68^{d}	514.12^{bcd}	559.07^{abcd}	628.54^{abc}	404.73^{cd}	541.45^{abcd}	37698.10	0.04
Die Temperature (°C)**	62	129.40^{d}	111.40^{f}	135.7^{a}	115.25^{e}	135.00^{ab}	115.50^{e}	130.80^{cd}	111.40^{f}	133.57^{abc}	114.17^{e}	132.00^{bcd}	112.00^{ef}	130.67^{cd}	112.00^{ef}	464.27	<0.0001
Die Pressure (PSI)+	62	304.00^{a}	320.00^{a}	196.57^{b}	211.25^{b}	240.00^{ab}	170.00^{b}	230.00^{ab}	232.00^{ab}	147.86^{b}	126.33^{b}	140.00^{b}	143.33^{b}	120.00^{b}	175.00^{b}	17723.27	0.002
Tensile Strength (MPa)	110	2.47^{efg}	2.37^{efg}	1.65^{g}	1.45^{g}	5.00^{bc}	4.77^{bcd}	3.97^{cd}	3.40^{de}	2.39^{efg}	2.07^{fg}	7.45^{a}	5.72^{b}	3.21^{def}	3.82^{cde}	18.84	<0.0001
Young's Modulus (MPa)	110	284.80^{ef}	258.52^{efg}	137.00^{gh}	98.85^{h}	522.45^{bc}	594.26^{ab}	436.63^{cd}	315.01^{de}	180.93^{fgh}	164.54^{fgh}	750.46^{a}	604.68^{ab}	303.56^{def}	311.70^{def}	257757.55	<0.0001
Puncture Force (g)++	220	22.75^{ab}	24.52^{a}	19.28^{bcde}	16.00^{e}	17.41^{de}	18.95^{bcde}	25.89^{a}	22.80^{ab}	20.25^{bcd}	18.74^{cde}	25.72^{a}	23.34^{ab}	22.63^{abc}	22.74^{abc}	153.31	<0.0001
Peaks (n)	220	1.25^{bc}	1.00^{c}	1.00^{c}	1.00^{c}	1.70^{b}	2.40^{a}	1.45^{b}	1.30^{bc}	1.00^{c}	1.00^{c}	1.50^{b}	2.50^{a}	1.30^{bc}	1.40^{bc}	2.93	<0.0001

^{abcdefgh}Means in a row with unlike superscripts differ by P<0.05

*5x10xLow and 10x15xHigh are also significantly different

**10x10xHigh and 10x15xHigh; 5x15xLow and 15x5xLow are also significantly different pairs

+10x10xLow and 10x15xLow; 10x10xLow and 15x10xHigh; 10x10xHigh and 10x15xLow; 5x15xLow and 10x15xLow are also significantly different pairs

++5x15xHigh and 15x5xLow; 5x15xHigh and10x10xLow; 5x10xHigh and 5x15xHigh are also significantly different pairs

Table B.21 Interaction of Gelatin Level, Initial Moisture Content, and Processing Temperature (%Gelatin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x15x High	5x15x Low	5x20x High	5x20x Low	10x15x High	10x15x Low	10x20x High	10x20x Low	15x20x High	15x20x Low	MSE	P
SME (KJ/kg)	62	610.86^{bc}	612.15^{bc}	447.51^{e}	456.85^{e}	706.33^{ab}	770.07^{a}	412.68^{e}	498.01^{cde}	466.46^{de}	593.70^{bcd}	85572.67	<0.0001
Die Temperature (°C)	62	133.60^{a}	115.20^{b}	132.57^{a}	112.88^{b}	131.00^{a}	114.60^{b}	133.78^{a}	112.50^{b}	131.20^{a}	112.00^{b}	652.08	<0.0001
Die Pressure (PSI)*	62	320.00^{a}	384.00^{a}	185.14^{bc}	171.25^{bc}	226.00^{b}	196.00^{bc}	170.56^{bc}	159.75^{bc}	128.00^{c}	156.00^{bc}	34332.50	<0.0001
Tensile Strength (MPa)	110	2.70^{cde}	2.05^{de}	1.43^{e}	1.78^{de}	3.25^{cd}	3.63^{bc}	3.74^{bc}	2.81^{cd}	5.33^{a}	4.77^{ab}	16.20	<0.0001
Young's Modulus (MPa)	110	300.87^{bc}	192.16^{dc}	120.92^{d}	165.21^{cd}	334.34^{bc}	320.54^{bc}	362.67^{b}	304.09^{bc}	527.01^{a}	458.19^{ab}	160771.70	0.0002
Puncture Force (g)	220	23.88^{a}	22.99^{a}	18.15^{c}	17.54^{c}	21.87^{ab}	22.51^{a}	21.98^{a}	19.00^{bc}	24.17^{a}	23.04^{a}	122.39	0.0002
Peaks (n)	220	1.15^{bcd}	1.00^{d}	1.10^{cd}	1.00^{d}	1.45^{bc}	1.25^{bcd}	1.23^{bcd}	1.50^{b}	1.40^{bcd}	1.95^{a}	1.70	0.0002

^{abcde}Means in a row with unlike superscripts differ by P<0.05

Table B.22 Interaction of Gluten and Glycerin Levels and Initial Moisture Content (%Gluten x %Glycerin x %Moisture) in the lab-scale injection molding experiment.

Variable	n	5x10 x20	5x15 x15	5x15 x20	10x5 x20	10x10x 15	10x10x 20	10x15x 15	10x15x 20	15x5 x20	15x10x 15	15x10x 20	MSE	P
SME (KJ/kg)	62	459.42^{ef}	638.13^{b}	293.39^{g}	600.75^{bc}	888.31^{a}	549.07^{cd}	614.38^{bc}	424.70^{f}	587.52^{bc}	607.20^{bc}	494.17^{de}	121208.05	<0.0001
Die Temperature (°C)	62	123.20	122.33	126.57	120.00	123.50	119.50	126.67	123.44	125.25	121.00	120.00	38.42	0.97
Die Pressure (PSI)*	62	142.00^{fg}	176.67^{ef}	104.71^{g}	142.00^{fg}	262.50^{bc}	210.00^{de}	300.00^{b}	140.67^{fg}	205.00^{de}	430.00^{a}	233.33^{cd}	42646.95	<0.0001
Tensile Strength (MPa)	110	3.51^{c}	2.90^{cd}	1.56^{e}	6.59^{a}	3.98^{bc}	3.39^{c}	1.68^{e}	1.43^{e}	4.89^{b}	3.07^{c}	1.78^{de}	25.33	<0.0001
Young's Modulus (MPa)	110	307.63 bcd	248.19 cde	97.28^{f}	677.57^{a}	406.69^{b}	198.63 def	148.34^{ef}	87.51^{f}	558.36^{a}	344.69^{bc}	198.63 def	345673.56	<0.0001
Puncture Force (g)	220	22.68^{bc}	21.87^{bc}	17.12^{ef}	24.53^{ab}	22.52^{bc}	26.18^{a}	20.40cd	14.88^{f}	18.18^{de}	26.47^{a}	20.81^{cd}	268.00	<0.0001
Peaks (n)	220	1.35^{bc}	1.00^{c}	1.00^{c}	2.00^{a}	1.70^{ab}	1.05^{c}	1.00^{c}	1.00^{c}	2.05^{a}	1.15^{c}	1.10^{c}	3.40	<0.0001

^{abcdefg}Means in a row with unlike superscripts differ by P<0.05

Table B.23 Interaction of Gluten and Glycerin Levels and Processing Temperature (%Gluten x %Glycerin x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x10x High	5x10x Low	5x15x High	5x15x Low	10x5x High	10x5x Low	10x10 x High	10x10 x Low	10x15 x High	10x15 x Low	15x5x High	15x5x Low	15x10 x High	15x10 x Low	MSE	P
SME (KJ/kg)*	62	404.73 cd	541.45 abcd	399.68 d	514.12 bcd	559.07 abcd	628.54 abc	633.69 ab	705.83 a	524.86 bcd	479.32 cd	564.77 abcd	610.27 abcd	502.58 bcd	574.18 abcd	37698.10	0.04
Die Temperature (°C)**	62	130.67 cd	112.00 ef	133.57 abc	114.17 e	132.00 bcd	112.00 ef	130.80 cd	111.40 f	135.57 a	115.25 e	135.00 ab	115.50 e	129.40 d	111.40 f	464.27	<0.0001
Die Pressure (PSI)+	62	120.00 b	175.00 b	147.86 b	126.33 b	140.00 b	143.33 b	230.00 ab	232.00 ab	196.57 b	211.25 b	240.00 ab	170.00 b	304.00 a	320.00 a	17723.27	0.002
Tensile Strength (MPa)	110	3.21 def	3.82 cde	2.39 efg	2.07 fg	7.45 a	5.72 b	3.97 cd	3.40 de	1.65 g	1.45 g	5.00 bc	4.77 bcd	2.47 efg	2.37 efg	18.84	<0.0001
Young's Modulus (MPa)	110	303.56 def	311.70 def	180.93 fgh	164.54 fgh	750.64 a	604.68 ab	436.63 cd	315.01 de	137.00 gh	98.85 h	522.45 bc	594.26 ab	284.80 ef	258.52 efg	257757.55	<0.0001
Puncture Force (g)++	220	22.63 abc	22.74 abc	20.25 bcd	18.74 cde	25.72 a	22.34 ab	25.89 a	22.80 ab	19.28 bcde	16.00 e	17.41 de	18.95 bcde	22.75 ab	24.52 a	153.31	<0.0001
Peaks (n)	220	1.30 bc	1.40 bc	1.00 c	1.00 c	1.50 b	2.50 a	1.45 b	1.30 bc	1.00 c	1.00 c	1.70 b	2.40 a	1.25 bc	1.00 c	2.93	<0.0001

abcdefgh Means in a row with unlike superscripts differ by P<0.05

*5x15xHigh and 15x10xLow are also significantly different

**5x15xHigh and 10x10xHigh; 10x5xLow and 10x15xLow are also significantly different pairs

+5x15xLow and 10x10xLow; 5x10xHigh and 10x10xLow; 5x15xLow and 10x10xHigh; 5x15xLow and 10x15xLow are also significantly different pairs

++10x5xLow and 10x15xHigh; 10x10xLow and 10x15xHigh; 10x15xHigh and 15x10xHigh are also significantly different pairs

Table B.24 Interaction of Gluten Level, Initial Moisture Content, and Processing Temperature (%Gluten x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x15x High	5x15x Low	5x20x High	5x20x Low	10x15x High	10x15x Low	10x20x High	10x20x Low	15x15x High	15x15x Low	15x20x High	15x20x Low	MSE	P
SME (KJ/kg)*	62	585.02^{bcd}	691.24^{abc}	322.41^{f}	418.78^{ef}	739.47^{a}	708.42^{ab}	490.36^{de}	518.84^{de}	566.78^{bcde}	647.62^{abcd}	501.77^{de}	561.24^{cde}	80090.65	<0.0001
Die Temperature (°C)**	62	130.33^{b}	114.33^{cd}	133.71^{ab}	133.20^{cd}	134.60^{a}	116.20^{c}	132.67^{ab}	112.18^{d}	129.50^{b}	112.50^{cd}	131.60^{ab}	112.60^{cd}	538.02	<0.0001
Die Pressure (PSI)	62	193.33^{d}	160.00^{de}	116.43^{e}	125.60^{e}	286.00^{c}	284.00^{c}	152.89^{de}	169.09^{d}	360.00^{b}	500.00^{a}	256.00^{c}	188.00^{d}	39234.11	<0.0001
Tensile Strength (MPa)+	110	2.94^{ab}	2.86^{ab}	2.52^{b}	2.55^{b}	2.57^{b}	3.09^{ab}	4.52^{a}	3.08^{ab}	3.81^{ab}	2.32^{b}	3.07^{ab}	3.60^{ab}	4.33	0.36
Young's Modulus (MPa)	110	267.25^{ab}	229.13^{ab}	199.09^{b}	205.83^{b}	279.93^{ab}	275.10^{ab}	445.96^{a}	294.07^{ab}	443.31^{a}	246.08^{ab}	324.37^{ab}	432.62^{a}	76115.87	0.10
Puncture Force (g)++	220	22.97^{abc}	20.76^{bcd}	20.07^{cd}	19.73^{cd}	20.97^{bcd}	21.94^{bc}	24.71^{ab}	19.02^{cd}	26.59^{a}	26.34^{a}	18.16^{d}	20.83^{bcd}	123.38	<0.0001
Peaks (n)#	220	1.00^{c}	1.00^{c}	1.15^{bc}	1.20^{bc}	1.45^{abc}	1.25^{bc}	1.17^{bc}	1.53^{ab}	1.30^{abc}	1.00^{c}	1.45^{abc}	1.70^{a}	0.88	0.06

^{abcdef}Means in a row with unlike superscripts differ by P<0.05
*5x20xLow and 15x20xLow are also significantly different
**10x15xLow and 15x20xLow are also significantly different
+10x20xHigh and 10x20xLow are also significantly different
++10x15xHigh and 10x20xHigh; 10x20xHigh and 15x20xLow are also significantly different pairs
#10x20xHigh and 10x20xLow are also significantly different

Table B.25 Interaction of Glycerin Level, Initial Moisture Content, and Processing Temperature (%Glycerin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x20x High	5x20x Low	10x15x High	10x15x Low	10x20x High	10x20x Low	15x15xHigh	15x15xLow	15x20x High	15x20x Low	MSE	P
SME (KJ/kg)	62	561.92^{c}	621.23^{bc}	727.54^{ab}	767.96^{a}	459.49^{d}	552.64^{c}	612.63^{c}	639.87^{bc}	349.50^{e}	385.01^{de}	112941.64	<0.0001
Die Temperature (°C)	62	133.50^{ab}	113.40^{d}	130.75^{bc}	113.75^{d}	130.00^{c}	110.38^{e}	133.33^{ab}	115.67^{d}	135.50^{a}	114.13^{d}	671.53	<0.0001
Die Pressure (PSI)	62	190.00^{cde}	154.00^{de}	317.50^{ab}	375.00^{a}	195.56^{cd}	201.25^{cd}	243.33^{bc}	233.33^{c}	118.88^{e}	131.00^{e}	33033.15	<0.0001
Tensile Strength (MPa)	110	6.23^{a}	5.25^{a}	3.68^{b}	3.36^{bc}	2.91^{bcd}	2.88^{bcd}	2.26^{cde}	2.32^{cde}	1.78^{de}	1.20^{e}	23.94	<0.0001
Young's Modulus (MPa)	110	636.46^{a}	599.47^{a}	422.36^{b}	329.02^{bc}	300.56^{cd}	266.91^{cd}	212.84^{cde}	183.69^{def}	105.08^{ef}	79.70^{f}	362986.74	<0.0001
Puncture Force (g)*	220	21.56^{bcd}	21.15^{cd}	23.68^{abc}	25.30^{a}	24.19^{ab}	22.26^{abcd}	22.07^{abcd}	20.19^{de}	21.56^{bcd}	14.54^{f}	217.22	<0.0001
Peaks (n)	220	1.60^{b}	2.45^{a}	1.60^{b}	1.25^{bc}	1.17^{c}	1.17^{c}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	4.26	<0.0001

^{abcdef}Means in a row with unlike superscripts differ by P<0.05

*10x15xLow and 10x20xLow are also significantly different

All 4-Way Interactions

Table B.26 Interaction of Gelatin, Gluten, and Glycerin Levels and Initial Moisture Content (%Gelatin x %Gluten x %Glycerin x %Moisture) in the lab-scale injection molding experiment.

Variable	n	5x10x 15x15	5x10x 15x20	5x15x 10x15	5x15x 10x20	10x5x 15x15	10x5x 15x20	10x10x1 0x15	10x10x1 0x20	10x15x5 x20	15x5x 10x20	15x10x5 x20	MSE	P
SME (KJ/kg)	62	614.38^{bc}	424.70^{f}	607.20^{bc}	494.17^{de}	638.13^{b}	293.39^{g}	888.31^{a}	549.07^{cd}	587.52^{bc}	459.42^{ef}	600.75^{bc}	121208.05	<0.0001
Die Temperature (°C)	62	126.67	123.44	121.00	120.00	122.33	126.57	123.50	119.50	125.25	123.20	120.00	38.42	0.97
Die Pressure (PSI)	62	300.00^{b}	140.67^{fg}	430.00^{a}	233.33^{cd}	176.67^{ef}	104.71^{g}	262.50^{bc}	210.00^{de}	205.00^{de}	142.00^{fg}	142.00^{fg}	42646.95	<0.0001
Tensile Strength (MPa)	110	1.68^{e}	1.43^{e}	3.07^{c}	1.78^{de}	2.90^{cd}	1.56^{e}	3.98^{bc}	3.39^{c}	4.89^{b}	3.51^{c}	6.59^{a}	25.33	<0.0001
Young's Modulus (MPa)	110	148.34^{ef}	87.51^{f}	344.69^{bc}	198.63^{def}	248.19^{cde}	97.28^{f}	406.69^{b}	344.95^{bc}	558.36^{a}	307.63^{bcd}	677.57^{a}	345673.56	<0.0001
Puncture Force (g)	220	20.40^{cd}	14.88^{f}	26.47^{a}	20.81^{cd}	21.87^{bc}	17.12^{ef}	22.52^{bc}	26.18^{a}	18.18^{de}	22.68^{bc}	24.53^{ab}	268.00	<0.0001
Peaks (n)	220	1.00^{c}	1.00^{c}	1.15^{c}	1.10^{c}	1.00^{c}	1.00^{c}	1.70^{ab}	1.05^{c}	2.05^{a}	1.35^{bc}	2.00^{a}	3.40	<0.0001

^{abcdefg}Means in a row with unlike superscripts differ by P<0.05

Table B.27 Interaction of Gelatin, Gluten, and Glycerin Levels and Processing Temperature (%Gelatin x %Gluten x %Glycerin x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x10x 15x High	5x10x 15x Low	5x15x 10x High	5x15x 10x Low	10x5x 15x High	10x5x 15x Low	10x10x 10x High	10x10x 10x Low	10x15x 5xHigh	10x15x 5xLow	15x5x 10x High	15x5x 10x Low	15x10x 5xHigh	15x10x 5xLow	MSE	P
SME (KJ/kg)*	62	524.86^{bcd}	479.32^{cd}	502.58^{cde}	576.18^{abcd}	399.68^{d}	514.12^{bcd}	663.69^{ab}	705.83^{a}	564.77^{abcd}	610.27^{abcd}	404.73^{cd}	541.45^{abcd}	559.07^{abcd}	628.54^{abc}	37698.10	0.04
Die Temperature (°C)**	62	135.57^{a}	115.25^{e}	129.40^{d}	111.40^{f}	133.57^{abc}	114.17^{e}	130.80^{cd}	111.40^{f}	135.00^{ab}	115.50^{e}	130.67^{cd}	112.00^{ef}	132.00^{bcd}	112.00^{ef}	464.27	<0.0001
Die Pressure (PSI)+	62	196.57^{b}	211.25^{b}	304.00^{a}	320.00^{a}	147.86^{b}	126.33^{b}	230.00^{ab}	232.00^{ab}	240.00^{ab}	170.00^{b}	120.00^{b}	175.00^{b}	140.00^{b}	143.33^{b}	17723.27	0.002
Tensile Strength (MPa)	110	1.65^{g}	1.45^{g}	2.47^{efg}	2.37^{efg}	2.39^{efg}	2.07^{fg}	3.97^{cd}	3.40^{de}	5.00^{bc}	4.77^{bcd}	3.21^{def}	3.82^{cde}	7.45^{a}	5.72^{b}	18.84	<0.0001
Young's Modulus (MPa)	110	137.00^{gh}	98.85^{h}	284.80^{ef}	258.52^{efg}	180.93^{fgh}	164.54^{fgh}	436.63^{cd}	315.01^{de}	522.45^{bc}	594.26^{ab}	303.56^{def}	311.70^{def}	750.46^{a}	604.68^{ab}	257757.55	<0.0001
Puncture Force (g)++	220	19.28^{bcde}	16.00^{e}	22.75^{ab}	24.52^{a}	20.25^{bcd}	18.74^{cde}	25.89^{a}	22.80^{ab}	17.41^{de}	18.95^{bcde}	22.63^{abc}	22.74^{abc}	25.72^{a}	23.34^{ab}	153.31	<0.0001
Peaks (n)	220	1.00^{c}	1.00^{c}	1.25^{bc}	1.00^{c}	1.00^{c}	1.00^{c}	1.45^{b}	1.30^{bc}	1.70^{b}	2.40^{a}	1.30^{bc}	1.40^{bc}	1.50^{b}	2.50^{a}	2.93	<0.0001

^{abcdefgh}Means in a row with unlike superscripts differ by P<0.05

*5x15x10xLow and 10x5x15xHigh are also significantly different

**10x5x15xHigh and 10x10x10xHigh; 5x10x15xLow and 15x10x5xLow are also significantly different pairs

+10x5x15xLow and 10x10x10xLow; 10x10x10xLow and 15x5x10xHigh; 10x5x15xLow and 10x10x10xHigh; 5x10x15xLow and 10x5x15xLow are also significantly different pairs

++5x10x15xHigh and 15x10x5xLow; 5x10x15xHigh and 10x10x10xLow; 5x10x15xHigh and 5x15x10xHigh are also significantly different pairs

Table B.28 Interaction of Gelatin and Glycerin Levels and Initial Moisture Content and Processing Temperature (%Gelatin x %Glycerin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x 10x 15x High	5x 10x 15x Low	5x 10x 20x High	5x 10x 20x Low	5x 15x 15x High	5x 15x 15x Low	5x 15x 20x High	5x 15x 20x Low	10x 5x 20x High	10x 5x 20x Low	10x 10x 15x High	10x 10x 15x Low	MSE	P
SME (KJ/kg)	62	566.79^{cdef}	647.62^{bc}	459.78^{gh}	528.56^{efg}	640.25^{bc}	588.50^{cdef}	438.32^{h}	413.82^{h}	564.77^{cdef}	610.27^{cdef}	888.31^{a}	888.31^{a}	61698.36	<0.0001
Die Temp* (°C)	62	129.50^{c}	112.50^{efg}	129.33^{c}	110.67^{gh}	136.33^{a}	117.00^{d}	135.00^{a}	114.20^{ef}	135.00^{ab}	115.50^{de}	132.00^{bc}	115.00^{def}	293.36	<0.0001
Die Pressure (PSI)	62	360.00^{b}	500.00^{a}	266.67^{cdef}	200.00^{ghij}	293.33^{cd}	306.67^{c}	124.00^{mno}	154.00^{klm}	240.00^{efgh}	170.00^{ijklm}	275.00^{cde}	250.00^{defg}	22247.61	<0.0001
Tensile Strength (MPa)	110	3.81^{cdef}	2.32^{fghij}	1.14^{j}	2.42^{fghij}	1.58^{hij}	1.77^{ghij}	1.73^{ghij}	1.13^{j}	5.00^{bc}	4.77^{bcd}	3.56^{cdef}	4.40^{bcde}	13.57	<0.0001
Young's Modulus (MPa)	110	443.31^{bcde}	246.08^{ghij}	126.26^{ijk}	270.97^{fghi}	158.43^{hijk}	138.24^{ijk}	156.56^{ijk}	59.46^{k}	522.45^{bc}	594.26^{ab}	401.42^{cdefg}	411.96^{cdef}	183161.64	<0.0001
Puncture Force (g)	220	26.59^{b}	26.34^{b}	19.92^{efg}	22.70^{bcde}	21.17^{cdef}	19.63^{defg}	17.39^{gf}	12.38^{h}	17.41^{fg}	18.95^{efg}	20.78^{cdefg}	24.26^{bc}	165.95	<0.0001
Peaks (n)	220	1.30^{de}	1.00^{e}	1.20^{de}	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.70^{cd}	2.40^{ab}	1.90^{bc}	1.50^{cde}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by $P<0.05$

*10x10x20xLow and 15x5x20xLow are also significantly different

Table B.29 Interaction of Gelatin and Glycerin Levels and Initial Moisture Content and Processing Temperature (%Gelatin x %Glycerin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	10x 10x 20x High	10x 10x 20x Low	10x 15x 15x High	10x 15x 15x Low	10x 15x 20x High	10x 15x 20x Low	15x 5x 20x High	15x 5x 20x Low	15x 10x 20x High	15x 10x 20x Low	MSE	P
SME (KJ/kg)	62	513.95^{fg}	584.19^{cdef}	585.02^{cdef}	691.24^{a}	260.68^{j}	337.00^{i}	559.07^{cdef}	628.54^{bcd}	404.73^{hi}	541.45^{defg}	61698.36	<0.0001
Die Temp* (°C)	62	130.00^{c}	109.00^{h}	130.33^{c}	114.33^{def}	136.00^{a}	114.00^{ef}	132.00^{bc}	112.00^{fgh}	130.67^{c}	112.00^{fgh}	293.36	<0.0001
Die Pressure (PSI)	62	200.00^{ghij}	220.00^{fghi}	193.33^{hijk}	160.00^{jklm}	113.75^{no}	92.67^{o}	140.00^{lmno}	143.33^{lmn}	120.00^{mno}	175.00^{ijkl}	22247.61	<0.0001
Tensile Strength (MPa)	110	4.38^{bcde}	2.39^{fghij}	2.94^{efgh}	2.86^{efghi}	1.84^{ghij}	1.27^{ij}	7.45^{a}	5.72^{b}	3.21^{defg}	3.82^{cdef}	13.57	<0.0001
Young's Modulus (MPa)	110	471.84^{bcd}	218.06^{hijk}	267.25^{fghi}	229.13^{hij}	94.61^{jk}	99.95^{jk}	750.46^{a}	604.68^{ab}	303.56^{efgh}	311.70^{defgh}	183161.64	<0.0001
Puncture Force (g)	220	31.01^{a}	21.35^{cdef}	22.97^{bcde}	20.76^{cdefg}	17.52^{fg}	16.71^{g}	25.72^{b}	23.34^{bcd}	22.63^{bcde}	22.74^{bcde}	165.95	<0.0001
Peaks (n)	220	1.00^{e}	1.10^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.50^{cde}	2.50^{a}	1.30^{de}	1.40^{cde}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by P<0.05

*10x10x20xLow and 15x5x20xLow are also significantly different

Table B.30 Interaction of Gelatin and Gluten Levels and Initial Moisture Content and Processing Temperature (%Gelatin x %Gluten x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x 10x 15x High	5x 10x 15x Low	5x 10x 20x High	5x 10x 20x Low	5x 15x 15x High	5x 15x 15x Low	5x 15x 20x High	5x 15x 20x Low	10x 5x 15x High	10x 5x 15x Low	10x 5x 20x High	10x 5x 20x Low	MSE	P
SME (KJ/kg)	62	640.25^{bc}	588.50^{cdef}	438.32^{h}	413.82^{h}	566.79^{cdef}	647.62^{bc}	459.78^{gh}	528.56^{efg}	585.02^{cdef}	691.24^{b}	260.68^{j}	337.00^{i}	61698.36	<0.0001
Die Temp* (°C)	62	136.33^{a}	117.00^{d}	135.00^{ab}	114.20^{ef}	129.50^{c}	112.50^{efg}	129.33^{c}	110.67^{gh}	130.33^{c}	114.33^{def}	136.00^{a}	114.00^{ef}	293.36	<0.0001
Die Pressure (PSI)	62	293.33^{cd}	306.67^{c}	124.00^{mno}	154.00^{klm}	360.00^{b}	500.00^{a}	266.67^{cdef}	200.00^{ghij}	193.33^{hijk}	160.00^{jklm}	113.75^{no}	92.67^{o}	22247.61	<0.0001
Tensile Strength (MPa)	110	1.58^{hij}	1.77^{ghij}	1.73^{ghij}	1.13^{j}	3.81^{cdef}	2.32^{fghij}	1.14^{j}	2.42^{fghij}	2.94^{efgh}	2.86^{efghi}	1.84^{ghij}	1.27^{ij}	13.57	<0.0001
Young's Modulus (MPa)	110	158.43^{hijk}	138.24^{ijk}	115.56^{ijk}	59.46^{k}	443.31^{bcde}	246.08^{ghij}	126.28^{ijk}	270.97^{fghi}	267.25^{fghi}	229.13^{hij}	94.61^{jk}	99.95^{jk}	183161.64	<0.0001
Puncture Force (g)	220	21.17^{cdef}	19.63^{defg}	17.39^{fg}	12.38^{h}	26.59^{b}	26.34^{b}	18.92^{efg}	22.70^{bcde}	22.97^{bcde}	20.76^{cdefg}	17.52^{fg}	16.71^{g}	165.95	<0.0001
Peaks (n)	220	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.30^{de}	1.00^{e}	1.20^{de}	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by P<0.05

*5x10x20xHigh and 15x10x20xHigh; 5x10x20xHigh and 10x10x15xHigh are also significantly different pairs

Table B.31 Interaction of Gelatin and Gluten Levels and Initial Moisture Content and Processing Temperature (%Gelatin x %Gluten x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	10x 10x 15x High	10x 10x 15x Low	10x 10x 20x High	10x 10x 20x Low	10x 15x 20x High	10x 15x 20x Low	15x 5x 20x High	15x 5x 20x Low	15x 10x 20x High	15x 10x 20x Low	MSE	P
SME (KJ/kg)	62	888.31^{a}	88831^{a}	513.95^{fg}	584.19^{cdef}	564.77^{cdef}	610.27^{bcde}	404.73^{hi}	541.45^{defg}	559.07^{cdef}	628.54^{bcd}	61698.36	<0.0001
Die Temp* (°C)	62	132.00^{bc}	115.00^{def}	130.00^{c}	109.00^{h}	135.00^{ab}	115.50^{de}	130.67^{c}	112.00^{fgh}	132.00^{bc}	112.00^{gh}	293.36	<0.0001
Die Pressure (PSI)	62	275.00^{cde}	250.00^{defg}	200.00^{ghij}	220.00^{fghi}	240.00^{efgh}	170.00^{ijklm}	120.00^{mno}	175.00^{ijkl}	140.00^{lmno}	143.33^{lmn}	22247.61	<0.0001
Tensile Strength (MPa)	110	3.56^{cdef}	4.40^{bcde}	4.38^{bcde}	2.39^{fghij}	5.00^{bc}	4.77^{bcd}	3.21^{defg}	3.82^{cdef}	7.45^{a}	5.72^{b}	13.57	<0.0001
Young's Modulus (MPa)	110	401.42^{cdefg}	411.96^{cdef}	471.84^{bcd}	218.06^{hijk}	522.45^{bc}	594.26^{ab}	303.56^{efgh}	311.70^{defgh}	750.46^{a}	604.68^{ab}	183161.64	<0.0001
Puncture Force (g)	220	20.78^{cdefg}	24.26^{bc}	31.01^{a}	21.35^{cdef}	17.41^{fg}	18.95^{efg}	22.63^{bcde}	22.74^{bcde}	25.72^{b}	23.34^{bcd}	165.95	<0.0001
Peaks (n)	220	1.90^{bc}	1.50^{cde}	1.00^{e}	1.10^{e}	1.70^{cd}	2.40^{ab}	1.30^{de}	1.40^{cde}	1.50^{cde}	2.50^{a}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by P<0.05 *5x10x20xHigh and 15x10x20xHigh; 5x10x20xHigh and 10x10x15xHigh are also significantly different pairs

Table B.32 Interaction of Gluten and Glycerin Levels and Initial Moisture Content and Processing Temperature (%Gluten x %Glycerin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	5x 10x 20x High	5x 10x 20x Low	5x 15x 15x High	5x 15x 15x Low	5x 15x 20x High	5x 15x 20x Low	10x 5x 20x High	10x 5x 20x Low	10x 10x 15x High	10x 10x 15x Low	10x 10x 20x High	10x 10x 20x Low	MSE	P
SME (KJ/kg)	62	404.73^{hi}	541.45^{defg}	585.02^{cdef}	691.24^{a}	260.68^{j}	337.00^{i}	559.07^{cdef}	628.54^{bcd}	888.31^{a}	888.31^{a}	513.95^{fg}	584.19^{cdef}	61698.36	<0.0001
Die Temp (°C)	62	130.67^{c}	112.00^{fgh}	130.33^{c}	114.33^{def}	136.00^{a}	114.00^{ef}	132.00^{bc}	112.00^{fg}	132.00^{bc}	115.00^{def}	130.00^{c}	109.00^{h}	293.36	<0.0001
Die Pressure (PSI)	62	120.00^{mno}	175.00^{ijkl}	193.33^{hijk}	160.00^{jklm}	113.75^{no}	92.67^{o}	140.00^{lmno}	143.33^{lmn}	275.00^{cde}	250.00^{defg}	200.00^{ghij}	220.00^{fghi}	22247.61	<0.0001
Tensile Strength (MPa)	110	3.21^{defg}	3.82^{cdef}	2.94^{efgh}	2.86^{efghi}	1.84^{ghij}	1.27^{ij}	7.45^{a}	5.72^{b}	3.56^{cdef}	4.40^{bcde}	4.38^{bcde}	2.39^{fghij}	13.57	<0.0001
Young's Modulus (MPa)	110	303.56^{efgh}	311.70^{defgh}	267.25^{fghi}	229.13^{hij}	94.61^{jk}	99.95^{jk}	750.46^{a}	604.68^{ab}	401.42^{cdefg}	411.96^{cdef}	471.84^{bcd}	218.06^{hijk}	183161.64	<0.0001
Puncture Force (g)	220	22.63^{bcde}	22.74^{bcde}	22.97^{bcde}	20.76^{cdefg}	17.52^{fg}	16.71^{g}	24.26^{bc}	23.34^{bcd}	20.78^{cdefg}	24.26^{bc}	31.01^{a}	21.35^{cdef}	165.95	<0.0001
Peaks (n)	220	1.30^{de}	1.40^{cde}	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.50^{cde}	2.50^{a}	1.90^{bc}	1.50^{cde}	1.00^{e}	1.10^{e}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by P<0.05

Table B.33 Interaction of Gluten and Glycerin Levels and Initial Moisture Content and Processing Temperature (%Gluten x %Glycerin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment.

Variable	n	10x 15x 15x High	10x 15x 15x Low	10x 15x 20x High	10x 15x 20x Low	15x 5x 20x High	15x 5x 20x Low	15x 10x 15x High	15x 10x 15x Low	15x 10x 20x High	15x 10x 20x Low	MSE	P
SME (KJ/kg)	62	640.25^{bc}	588.50^{cdef}	438.32^{h}	413.82^{h}	564.77^{cdef}	610.27^{bcde}	566.79^{cdef}	647.62^{bc}	459.78^{gh}	528.56^{efg}	61698.36	<0.0001
Die Temp (°C)	62	136.33^{a}	117.00^{d}	135.00^{a}	114.20^{ef}	135.00^{ab}	115.50^{de}	129.50^{c}	112.50^{efg}	129.33^{c}	110.67^{h}	293.36	<0.0001
Die Pressure (PSI)	62	293.33^{cd}	306.67^{c}	124.00^{mno}	154.00^{klm}	240.00^{efgh}	170.00^{ijklm}	360.00^{b}	500.00^{a}	266.67^{cdef}	200.00^{ghij}	22247.61	<0.0001
Tensile Strength (MPa)	110	1.58^{hij}	1.77^{ghij}	1.73^{ghij}	1.13^{j}	5.00^{bc}	4.77^{bcd}	3.81^{cdef}	2.32^{fghij}	1.14^{j}	2.42^{fghij}	13.57	<0.0001
Young's Modulus (MPa)	110	158.43^{hijk}	138.24^{ijk}	115.56^{ijk}	59.46^{k}	522.45^{bc}	594.26^{ab}	443.31^{bcde}	246.08^{ghij}	126.28^{ijk}	270.97^{fghi}	183161.64	<0.0001
Puncture Force (g)	220	21.17^{cdef}	19.63^{defg}	17.39^{fg}	12.38^{h}	17.41^{fg}	18.95^{efg}	26.59^{b}	26.34^{b}	118.92^{efg}	22.70^{bcde}	165.95	<0.0001
Peaks (n)	220	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.70^{cd}	2.40^{ab}	1.30^{de}	1.00^{e}	1.20^{de}	1.00^{e}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by P<0.05

5-Way Interaction

Table B.34 Interaction of Gelatin, Gluten, and Glycerin Levels and Initial Moisture Content and Processing Temperature (%Gelatin %Gluten x %Glycerin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment – Part 1.

Variable	n	5x 10x 15x 15x High	5x 10x 15x 15x Low	5x 10x 15x 20x High	5x 10x 15x 20x Low	5x 15x 10x 15x High	5x 15x 10x 15x Low	5x 15x 10x 20x High	5x 15x 10x 20x Low	10x 5x 15x 15x High	10x 5x 15x 15x Low	10x 5x 15x 20x High	10x 5x 15x 20x Low	MSE	P
SME (KJ/kg)	62	640.25^{bc}	588.50^{cdef}	438.62^{h}	413.82^{h}	566.79^{cdef}	647.62^{bc}	459.78^{gh}	528.56^{efg}	585.02^{cdef}	691.24^{b}	260.68	337.00^{i}	61698.36	<0.0001
Die Temp (°C)*	62	136.33^{a}	117.00^{d}	135.00^{ab}	114.20^{ef}	129.50^{c}	112.50^{efg}	129.33^{c}	110.67^{gh}	130.33^{c}	114.33^{def}	136.00^{a}	114.00^{ef}	293.36	<0.0001
Die Pressure (PSI)	62	239.33^{cd}	306.67^{c}	124.00^{mno}	154.00^{klm}	360.00^{b}	500.00^{a}	266.67^{cdef}	200.00^{ghij}	193.33^{hijk}	160.00^{jklm}	113.75^{no}	92.67^{o}	22247.61	<0.0001
Tensile Strength (MPa)	110	1.58^{hij}	1.77^{ghij}	1.73^{ghij}	1.13^{j}	3.81^{cdef}	2.32^{fghij}	1.14^{j}	2.42^{fghij}	2.94^{efgh}	2.86^{efghi}	1.84^{ghij}	1.27^{ij}	13.57	<0.0001
Young's Modulus (MPa)	110	158.43^{hijk}	138.24^{ijk}	115.56^{ijk}	59.46^{k}	443.31^{bcde}	246.08^{ghij}	126.28^{ijk}	270.97^{fghi}	267.25^{fghi}	229.13^{hij}	94.61^{jk}	99.95^{jk}	183161.64	<0.0001
Puncture Force (g)	220	21.17^{cdef}	19.63^{defg}	17.39^{fg}	12.38^{h}	26.59^{b}	26.34^{b}	18.92^{efg}	22.70^{bcde}	22.97^{bcde}	20.76^{cdefg}	17.52^{fg}	16.71^{g}	165.95	<0.0001
Peaks (n)	220	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.30^{de}	1.00^{e}	1.20	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	1.00^{e}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by P<0.05 *5x10x15x20xHigh and 15x10x5x20xHigh; 5x10x15x20xHigh and 10x10x10x15xHigh are also significantly different pairs

Table B.35 Interaction of Gelatin, Gluten, and Glycerin Levels and Initial Moisture Content and Processing Temperature (%Gelatin %Gluten x %Glycerin x %Moisture x Processing Temperature) in the lab-scale injection molding experiment – Part 2.

Variable	n	10x 10x 10x 15x High	10x 10x 10x 15x Low	10x 10x 10x 20x High	10x 10x 10x 20x Low	10x 15x 5x 20x High	10x 15x 5x 20x Low	15x 5x 10x 20x High	15x 5x 10x 20x Low	15x 10x 5x 20x High	15x 10x 5x 20x Low	MSE	P
SME (KJ/kg)	62	888.31^{a}	888.31^{a}	513.95^{fg}	584.19 ^{cdef}	564.77 ^{cdef}	610.27 ^{bcde}	404.73^{hi}	541.45 ^{defg}	559.07 ^{cdef}	626.54 ^{bcd}	61698.36	<0.0001
Die Temp (°C)*	62	132.00^{bc}	115.00^{def}	130.00^{c}	109.00^{h}	135.0^{ab}	115.50^{de}	130.67^{c}	112.00^{fgh}	132.00^{bc}	112.00^{fg}	293.36	<0.0001
Die Pressure (PSI)	62	275.00^{cde}	250.00^{defg}	200.00^{ghij}	220.00^{fghi}	240.00^{efgh}	170.00^{ijklm}	120.00^{mno}	175.00^{ijkl}	140.00^{lmno}	143.33^{lmn}	22247.61	<0.0001
Tensile Strength (MPa)	110	3.56^{cdef}	4.40^{bcde}	4.38^{bcde}	2.39^{fghij}	5.00^{bc}	4.77^{bcd}	3.21^{defg}	3.82^{cdef}	7.45^{a}	5.72^{b}	13.57	<0.0001
Young's Modulus (MPa)	110	401.42^{cdefg}	411.96^{cdef}	471.84^{bcd}	218.06^{hijk}	522.45^{bc}	594.26^{ab}	303.56^{efgh}	311.70^{defgh}	750.46^{a}	604.68^{ab}	183161.64	<0.0001
Puncture Force (g)	220	20.76^{cdefg}	24.26^{bc}	31.01^{a}	21.35^{cdef}	17.41^{fg}	18.95^{efg}	22.63^{bcde}	22.74^{bcde}	25.72^{b}	23.34^{bcd}	165.95	<0.0001
Peaks (n)	220	1.90^{bc}	1.50^{cde}	1.00^{e}	1.10^{e}	1.70^{dc}	2.40^{ab}	1.30^{de}	1.40^{cde}	1.50^{cde}	2.50^{a}	2.05	<0.0001

^{abcdefghijklmno}Means in a row with unlike superscripts differ by P<0.05

*5x10x15x20xHigh and 15x10x5x20xHigh; 5x10x15x20xHigh and 10x10x10x15xHigh are also significantly different pairs

Appendix C - Differential Scanning Calorimetry of Gelatin, Gluten, and Glycerol

Objective: To evaluate the glass transition (Tg) temperature of Pro-Bind Plus 100 against a conventional gelatin in a basic injection molding formula.

Treatment Structure:

Treatment Number	Type of Gelatin	Gluten? Y/N	Amount of Glycerol
1	Pro-Bind Plus 100	No	0%
2	Pig Skin 225	No	0%
3	Pro-Bind Plus 100	No	10%
4	Pro-Bind Plus 100	No	15%
5	Pig Skin 225	No	10%
6	Pig Skin 225	No	15%
7	None	Yes	10%
8	None	Yes	15%
9	Pro-Bind Plus 100	Yes	10%
10	Pro-Bind Plus 100	Yes	15%
11	Pig Skin 225	Yes	10%
12	Pig Skin 225	Yes	15%

Instrument Start-Up

1) Open the gas cylinders attached to the DSC instrument.
2) Turn on the power to the DSC instrument – there are 2 switches: one on the front of the instrument and one on the back.
3) Turn on the computer and login with username: purple, password: password.
4) Open the TA Instrument Explorer software.
5) Double click on the icon, exit out of the popup, and minimize the window to continue in to the software.
6) Click "Next" then "Finish" to continue to the operations page.
7) Go to the "Control" drop down tab, select "Event", click "On".
8) Go to the "Control" drop down tab, click "Standby Temperature" to initiate instrument warm-up.
9) Now that the instrument is started, continue to sample preparation.

Sample Preparation

- Test moisture levels of both gelatins and the wheat gluten to ensure equal moisture content between all samples
- For treatments containing glycerol, glycerol will be added to a small sample and mixed in a small bowl with a wire whisk the day before analysis.

*When handling pan elements, DO NOT touch with your bare hands as this will contaminate your samples. ALWAYS handle unsealed pans and pan elements with tweezers.

1) Prepare an empty pan:
    a. Remove a lid, pan, and rubber ring from their respective jars using the tweezers.
        i. The lid will have thinner, straight walls. The pan will have a slightly rounded top edge.
    b. Place the rubber ring in the lid using the tweezers, and ensure the rubber ring is completely along the flat bottom of the lid.
    c. Place the pan on the insert of the sample sealer, place the lid on the pan, place the insert in the sample sealer, and press the handle of the sample sealer to seal the pan.
    d. Place the empty pan in the first slot of the sample holder.
2) Prepare samples:
    a. Repeat steps 1a. and 1b. above, then set the lid aside.
    b. Place the pan on the micro scale and tare.
    c. Weight out 5-10 grams of your sample in to the pan. Record the weight in an Excel spreadsheet.
    d. Remove the pan and gently tap the pan on a paper towel to wipe off the bottom and level out the sample.
    e. Repeat steps 1c. and 1d. above to seal the pan.
    f. Prepare 2 pans of each sample (i.e.: 10 treatments x 2 pans = 20 total).

Sample Testing

1) Fill in the following boxes:
    a. Mode → Standard
    b. Test → Custom
    c. Sample → Should correspond to the sample name in your Excel file
    d. Sample Size → the dry weight of the sample in the pan
    e. Data File Name → browse to create a new file to save your results
2) Under the “Procedure” tab, name your test “Glass Transition for Gelatin”, then fill in the following steps:
    a. 1) Eq @ 10°C
    b. 2) Ramp 10°C/min to 150°C
    c. 3) Mark end of cycle 1
    d. 4) Ramp 25°C/min to 10°C
    e. 5) Mark end of cycle 2
    f. 6) Ramp 10°C/min to 150°C
    g. 7) Mark end of cycle 7
3) Save the procedure file under your new folder for your results.
4) Under “Notes” fill in the following:
    a. Op → this means the operator, so fill in your name
    b. Pan type → high volume
5) Under the “Control” drop down menu select “Lid” and then “Open”.

6) Place your empty pan and sample pan on the appropriate pegs. The empty pan should be centered on the peg farther away from you, and the sample pan should be center on the peg closer to you. DO NOT TOUCH THE WHITE SENSOR IN THE MIDDLE.
7) Under the “Control” drop down menu select “Lid” and “Close”.
8) Click the green start button or under the “Control” drop down menu select “Start” to begin your test.
    a. NOTE: the flange temperature must be ≤-80°C to safety start the instrument
9) After the test is completed, wait until the temperature of the instrument has cooled to approximately 30°C to open the lid and remove samples (see step 5 and 7 for opening and closing procedure).

Data Analysis

1) Open the TA Universal Analysis software and open the file that corresponds to your results.
2) Under the “Graph” drop down menu select “Data Limits” and “Cycle”.
3) Change the cycle option to “3 to 3” and click OK. This should show a curve going from 10 to 150 and back to 10.
4) Enlarge the graph by dragging a box over the area.
5) Click on the Tg icon on the toolbar. This should cause a small and large cross to pop up.
6) Move the large cross to the baseline of the Tg peak, and move the small cross to the upper baseline after the Tg peak.
7) Right click on any blank space and select “Accept Limits”.
    a. This should give you 3 points and 2 temperatures → an onset and ending temperature as well as a middle temperature with an enthalpy.
8) Select “Save”, “File”, “Export PDF file” and save this to your results folder.
9) Select “Save”, “File”, “Export Data File”, “File and Plot Signals” and “Finish” and save this to your results folder WITHOUT RENAMING.

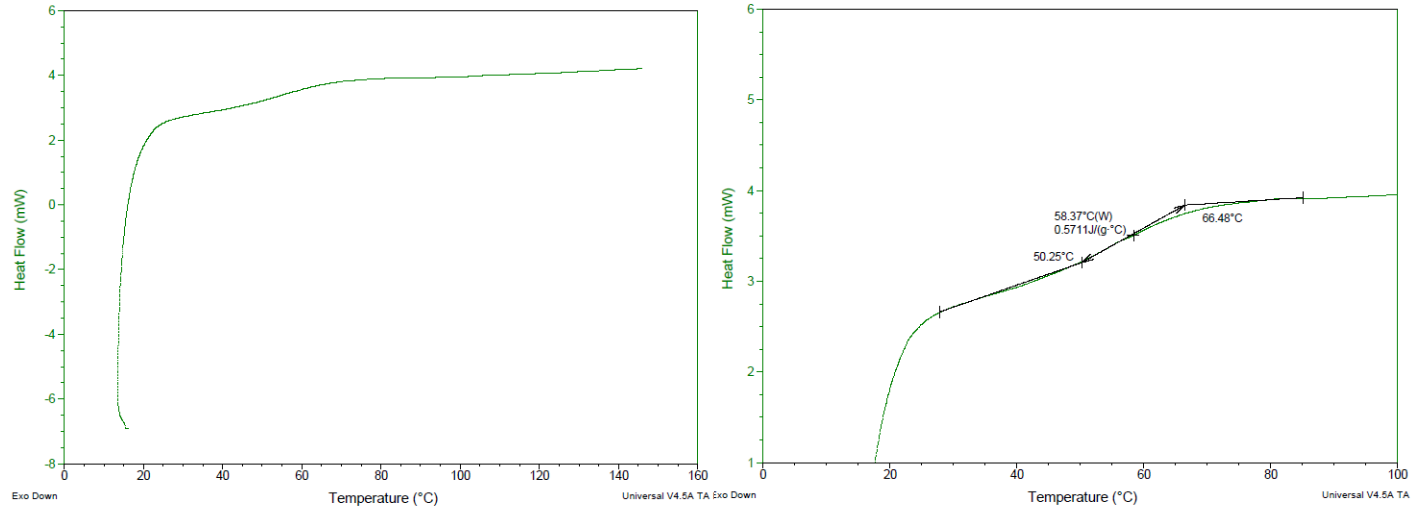
DSC Shut-Down Procedure

1) Under the “Control” drop down menu select “Event” and “Off”. You should hear the instrument fan stop.
2) Under the “Control” drop down menu select “Standby Temp”.
    a. NOTE: Do NOT proceed until the flange temp has come up to approximately 20°C.
3) Turn off the gas cylinders.
    a. NOTE: Do NOT do this if the flange temp has yet to come up to approximately 20°C.
4) Turn off BOTH the front and back power switches.

Close the software and shut down the computer

Figure C.1 DSC of Pro-Bind 100 – No Glycerol.

Sample 1



Sample 2

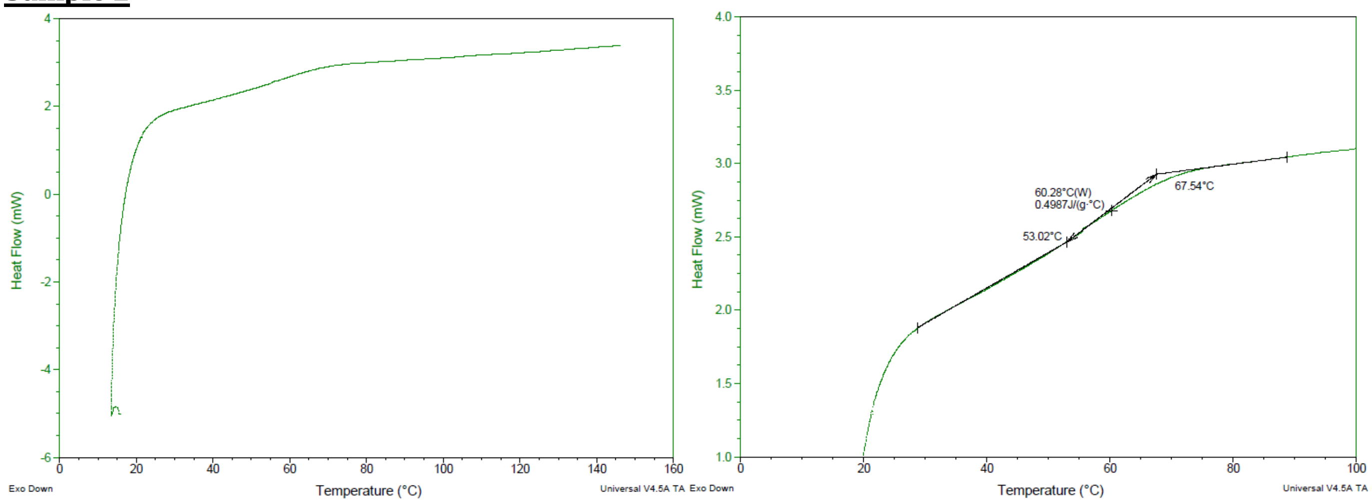
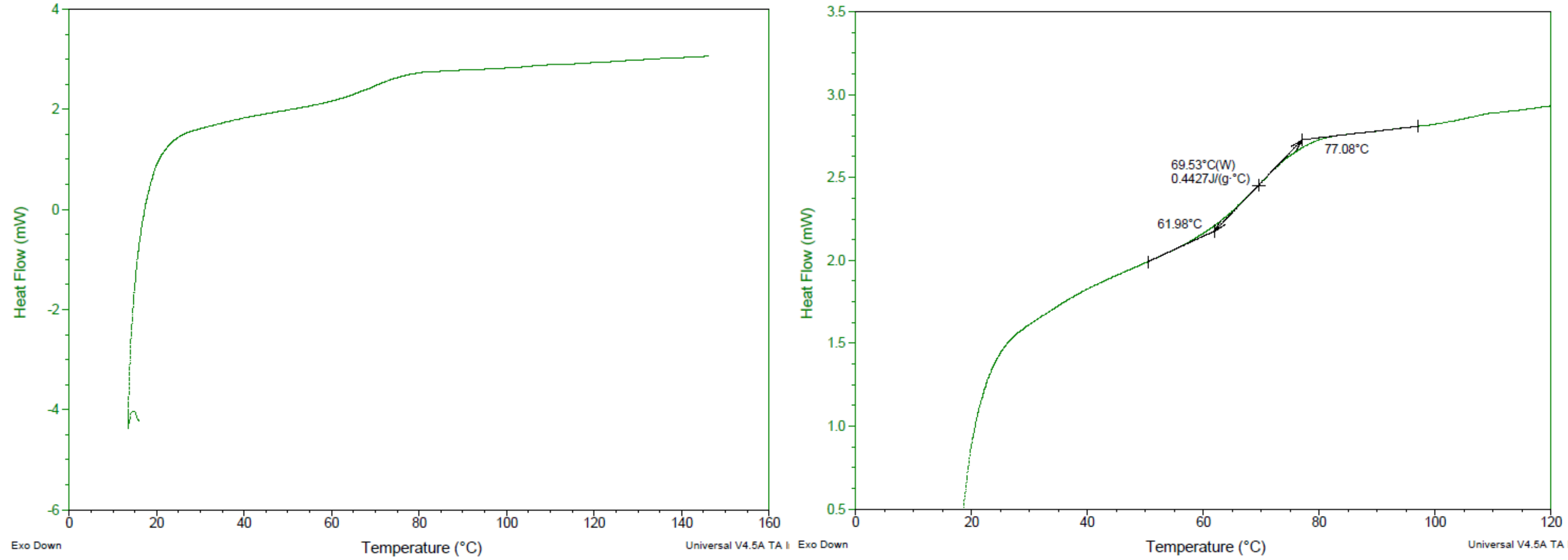


Figure C.2 DSC of Pig Skin 225 – No Glycerol.

Sample 1



Sample 2

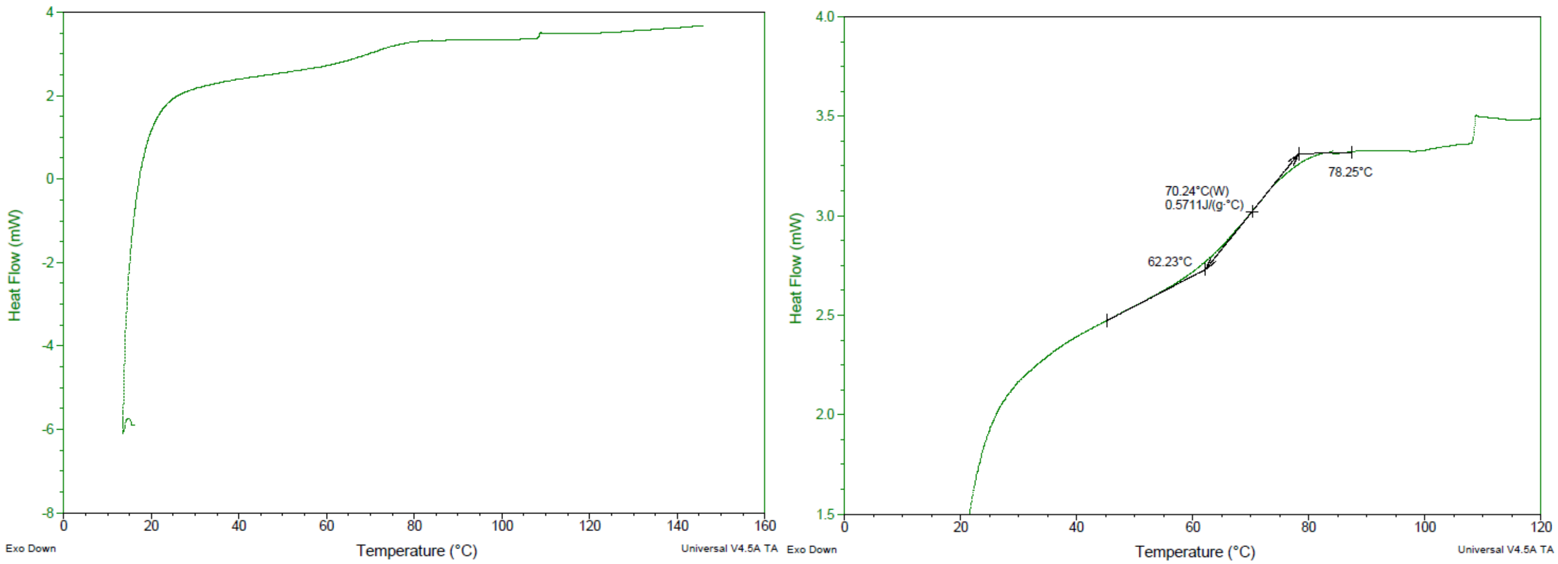
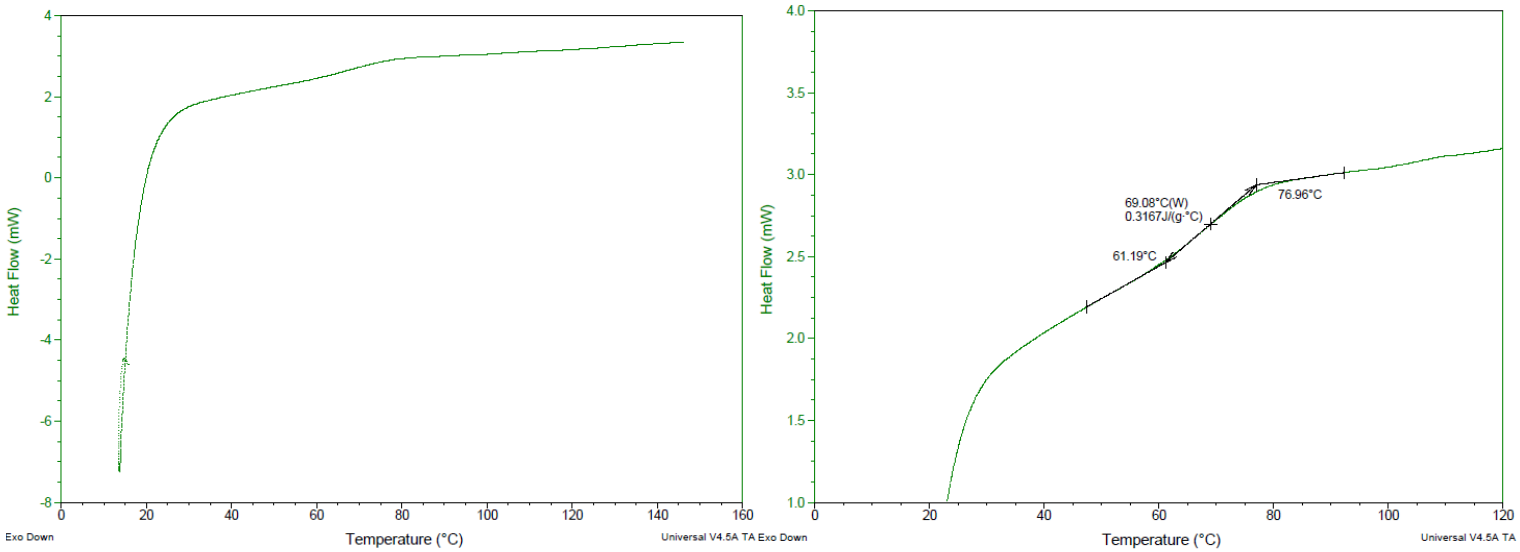


Figure C.3 DSC of Pro-Bind 100 + 10% Glycerol (PB100+Gly10).

__Sample 1__



__Sample 2__

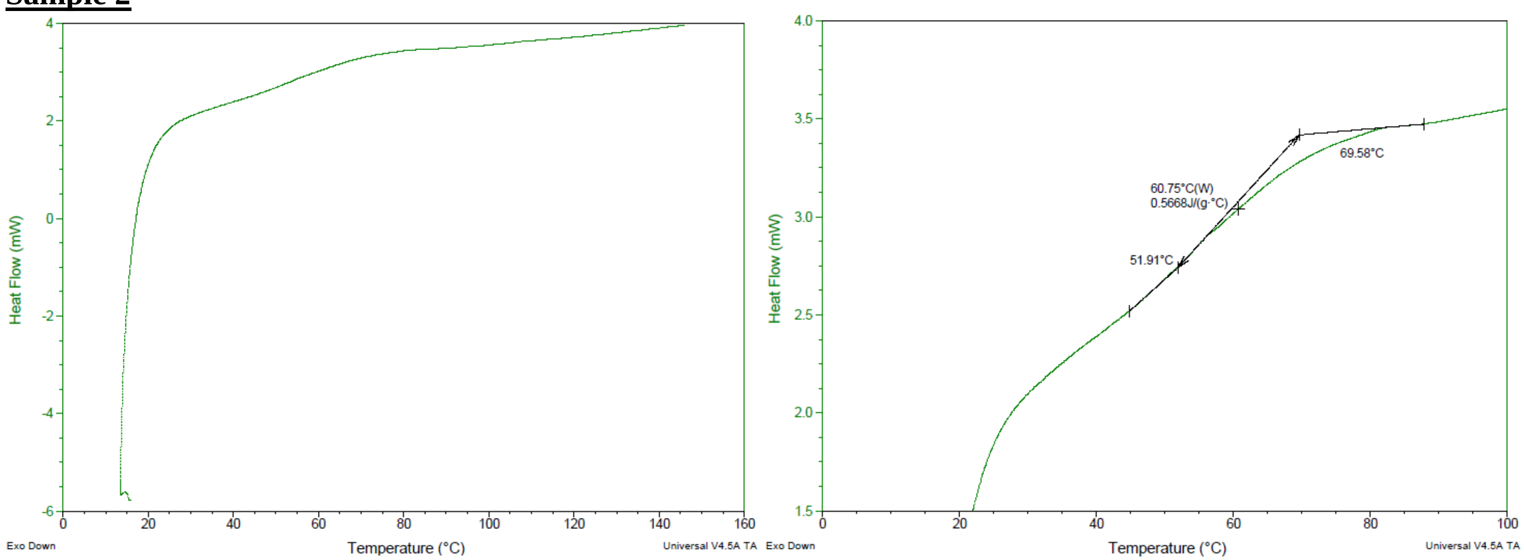
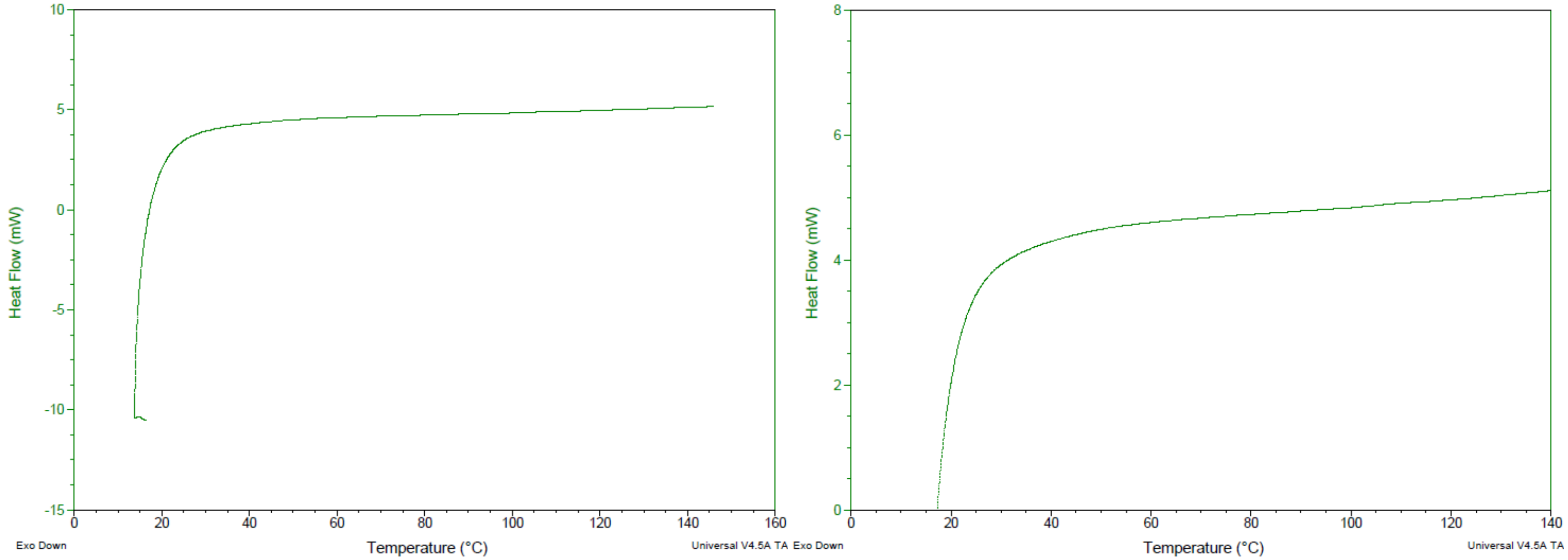


Figure C.4 DSC of Pro-Bind 100 + 15% Glycerol (PB100+Gly15).

Sample 1



Sample 2

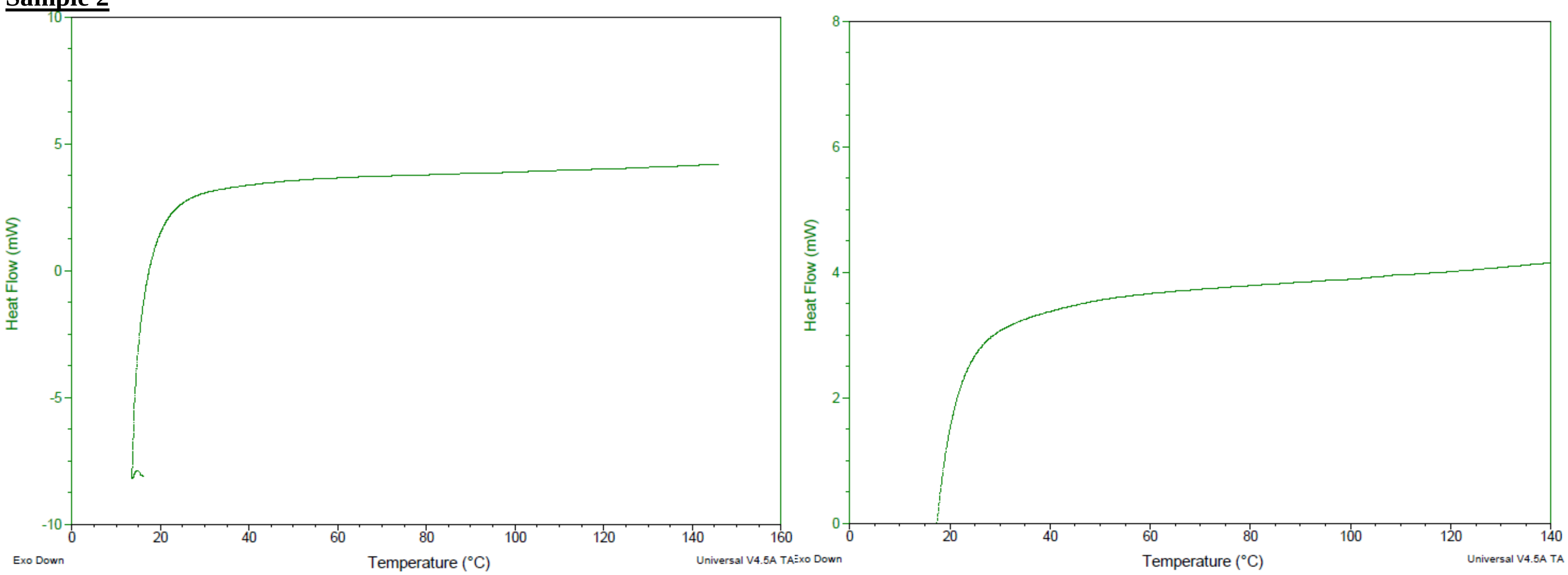
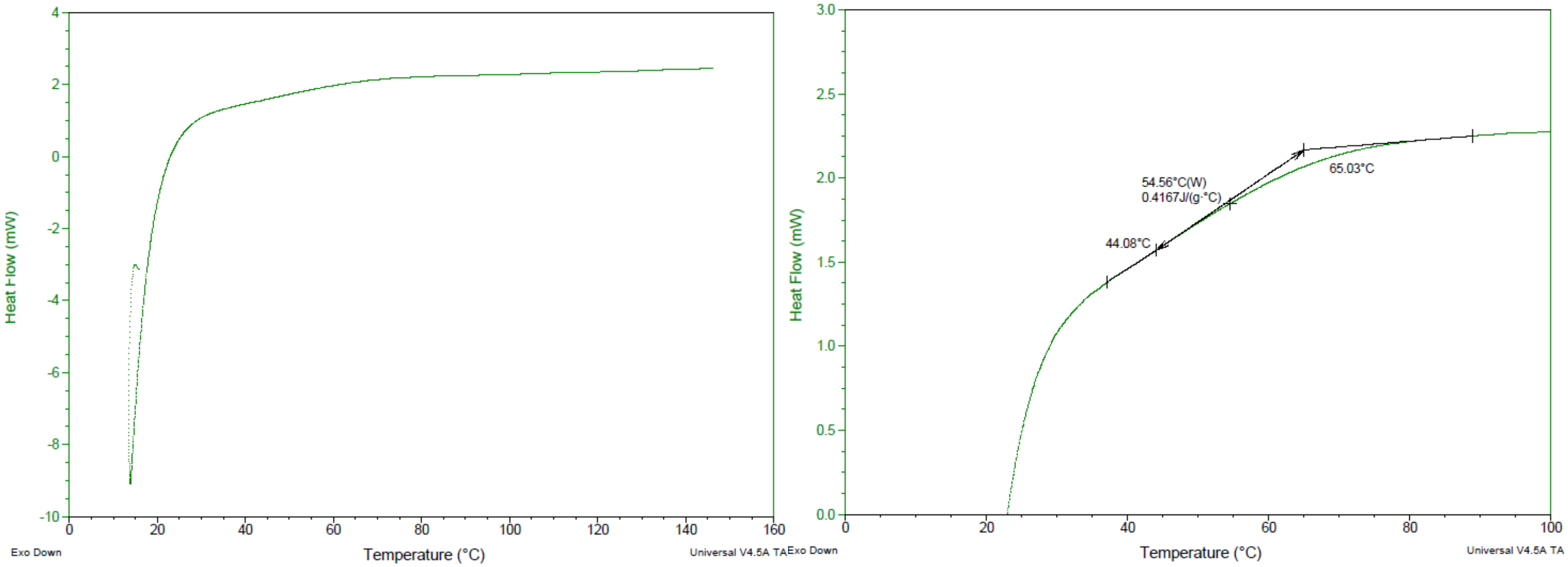


Figure C.5 DSC of Pig Skin 225 + 10% Glycerol (PS225+Gly10).

Sample 1



Sample 2

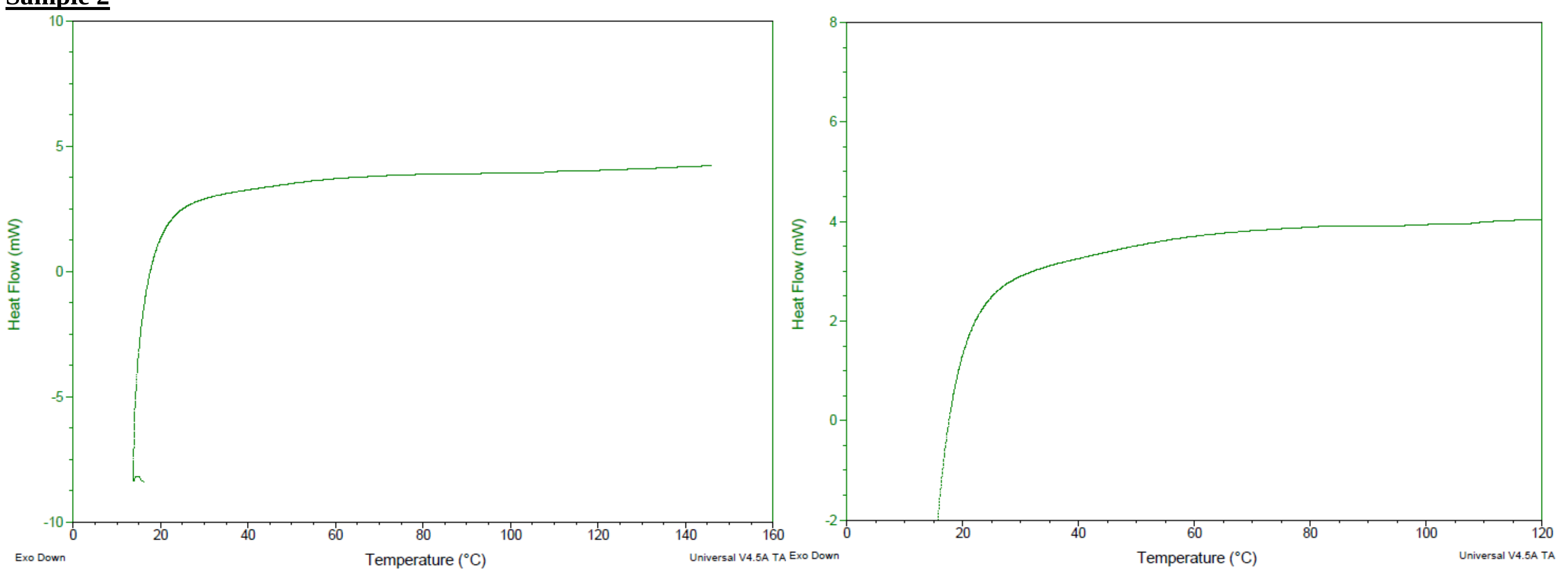
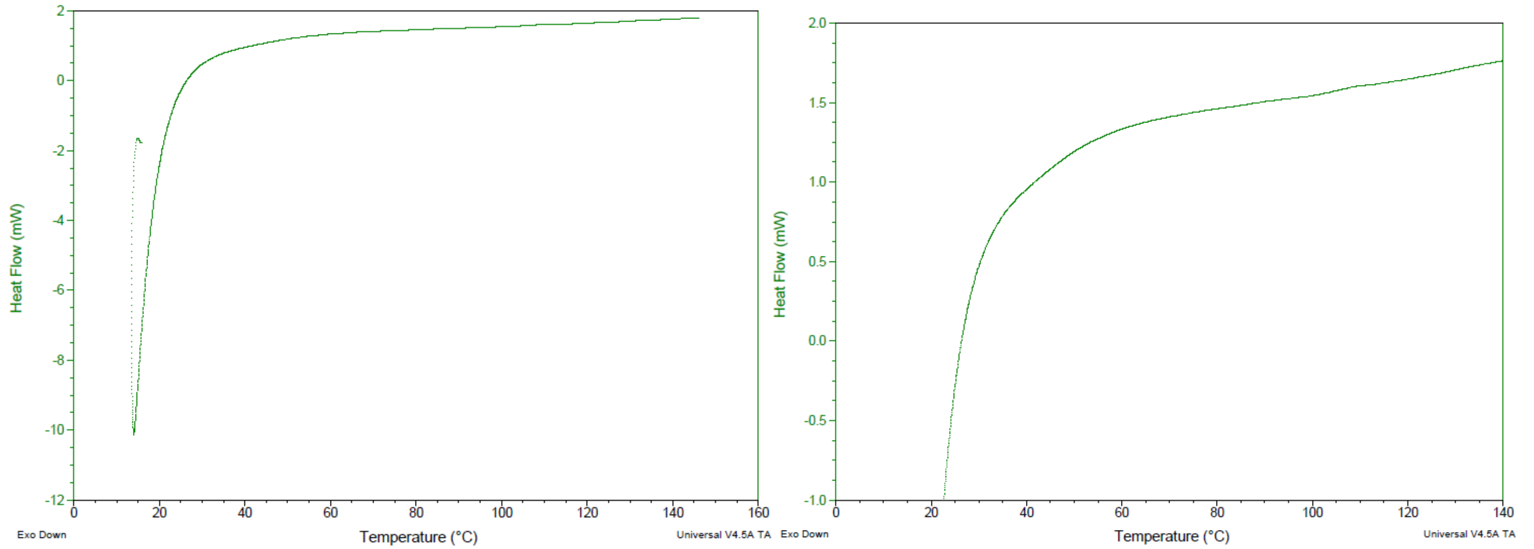


Figure C.6 DSC of Pig Skin 225 + 15% Glycerol (PS225+Gly15).

Sample 1



Sample 2

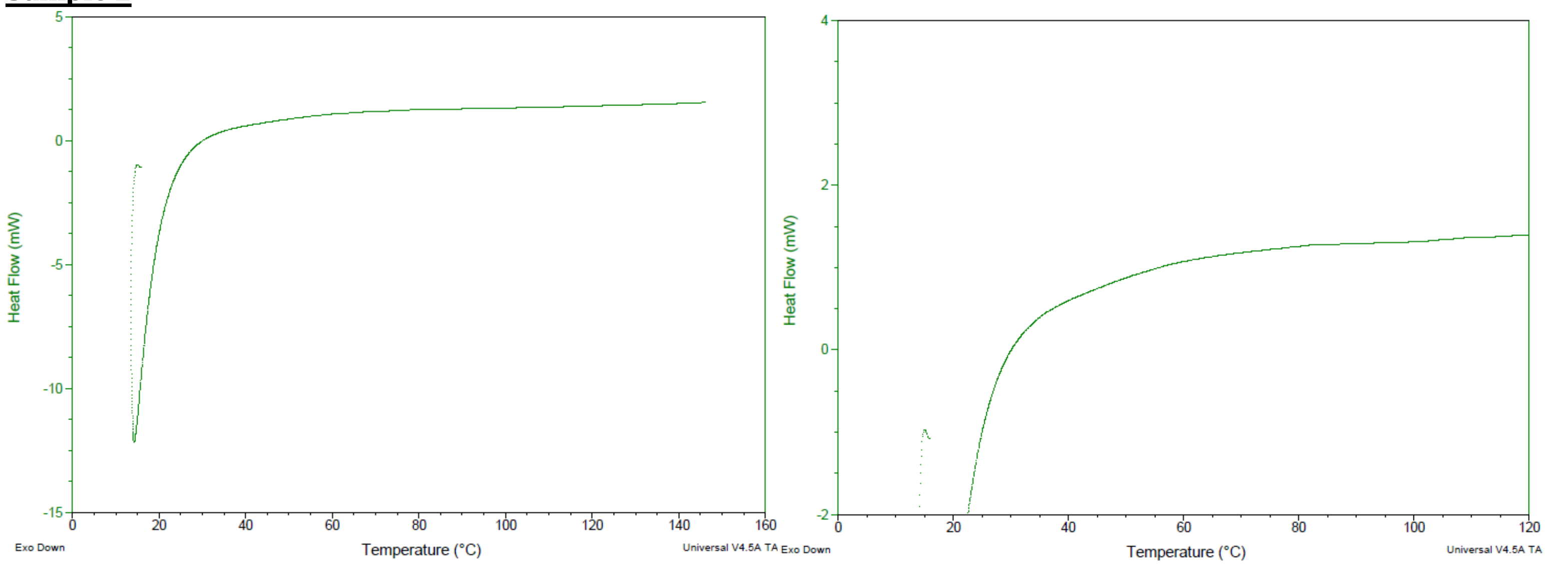
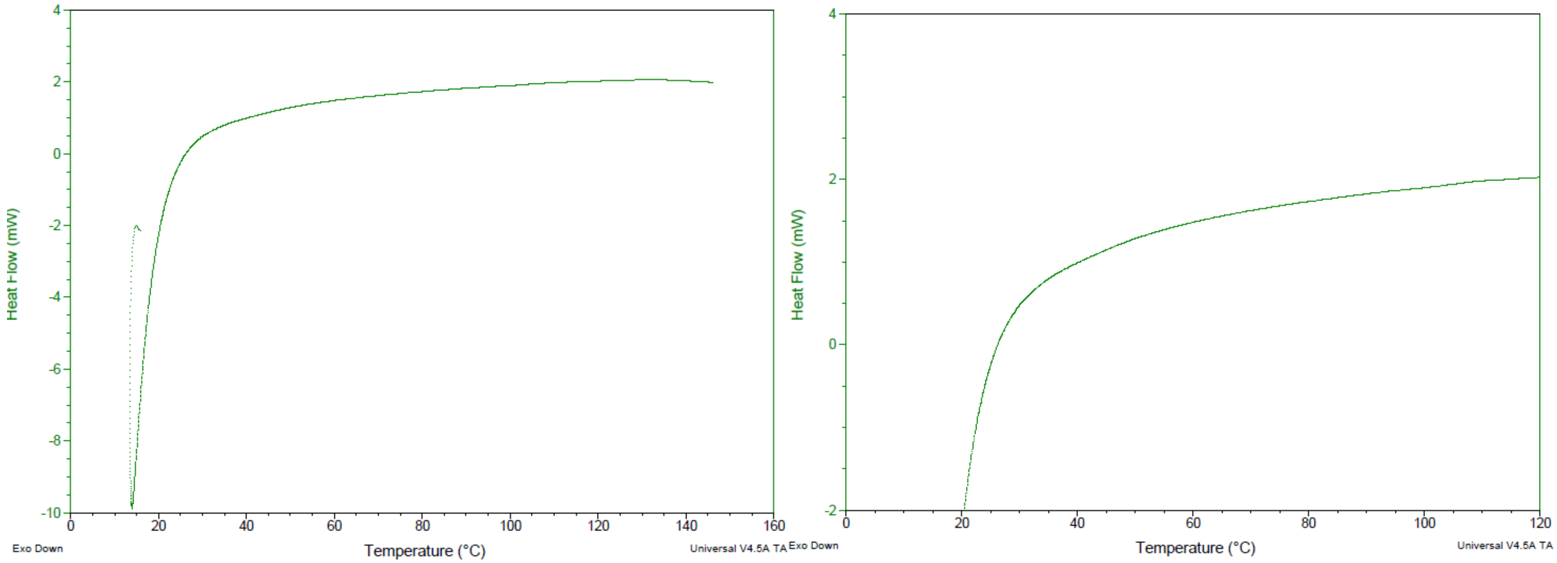


Figure C.7 DSC of Wheat Gluten – No Glycerol, No Gelatin.

<u>Sample 1</u>



<u>Sample 2</u>