An Investigation of Commercial Collagen Dispersions and their use in Co-Extrusion Sausage Manufacturing

by

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The mechanical, microstructural and thermo-mechanical properties of five collagen dispersions were evaluated to identify differences between commercial products. Collagen films were produced from collagen dispersions, which are used in co-extruded sausage manufacturing. Collagen was rolled into films and then partially dehydrated (brine) or cross-linked (smoke condensate or glutaraldehyde (GA)). Manipulating the film forming conditions, concentration and contact time, demonstrated that there were some significant differences (p < 0.05) in the mechanical properties when partially dehydrated or cross-linked. The mechanical properties of the dispersions and films demonstrated that there were some differences (p < 0.05) between the commercial collagen preparations. Transmission electron microscopy (TEM) imaging revealed that collagen fibers were swollen to varying degrees. The dispersions composition and protein quality appeared to result in differences between commercial products.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
<th>Unit</th>
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<tr>
<td>(\Delta H)</td>
<td>Transition enthalpy</td>
<td>[J/g]</td>
</tr>
<tr>
<td>(\Delta r)</td>
<td>Difference in distance</td>
<td>[mm]</td>
</tr>
<tr>
<td>(A)</td>
<td>Area</td>
<td>[m²]</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
<td>[-]</td>
</tr>
<tr>
<td>C1</td>
<td>Collagen 1</td>
<td>[-]</td>
</tr>
<tr>
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<tr>
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<td>C5</td>
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</tr>
<tr>
<td>C5</td>
<td>Collagen 5</td>
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</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
<td>[-]</td>
</tr>
<tr>
<td>F</td>
<td>Force</td>
<td>[N]</td>
</tr>
<tr>
<td>GA</td>
<td>Glutaraldehyde</td>
<td>[-]</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
<td>[-]</td>
</tr>
<tr>
<td>(r_o)</td>
<td>Initial distance</td>
<td>[mm]</td>
</tr>
<tr>
<td>SC</td>
<td>Smoke Condensate</td>
<td>[-]</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
<td>[-]</td>
</tr>
<tr>
<td>(T_o)</td>
<td>Onset temperature</td>
<td>[°C]</td>
</tr>
<tr>
<td>(T_p)</td>
<td>Peak temperature</td>
<td>[°C]</td>
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<tr>
<td>(Y)</td>
<td>Young’s Modulus</td>
<td>[MPa/%]</td>
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Chapter 1 Introduction

Meat has been and continues to be a central element of diets in developed countries, with processed meat products (sausages, burgers and meat pies) accounting for almost half of the meat being consumed in these countries. Projections suggest that the global consumption of meat will continue to increase moderately over the next 40 years. This increase reflects the increasing demand for pork and poultry (Kearney 2010). With increasing market demand, meat processors are driven to invest in research and innovation to remain competitive (Chen and others 2013).

Meat processors must meet consumer preferences is one of the keys to developing a successful product (Chen and others 2013). There have been rapidly changing consumer demands in the food industry: improved health, safety, quality, convenience, value, experience as well as addressing ethical and environmental issues (Kearney 2010). The implementation of in-line production of collagen casings, through the co-extrusion process, has been one step in enhancing food safety, providing consumers with a convenient, cost effective product. This is because co-extrusion can be highly automated, thus reducing, human contact (reducing the risk of contamination), labour costs and the need for highly trained personnel (Savic and Savic 2012).

In addition to meeting consumer’s demand, it has also suggested that the success of products hinges on the consumers knowledge of new technology. One example of how a lack of knowledge has hindered the acceptance of food technology can be observed through the use of technologies like irradiation, genetically modified organisms and nanotechnologies (Chen and others 2013; Rollin 2011). Unfortunately, co-extrusion is also a fairly new technology and is associated to high capital costs and a complex process. As a result the acceptance of the co-extrusion technology relies on a better understanding of the materials and process. This provides an opportunity to improve our understanding of the production of collagen casing, as if they were formed through the co-extrusion process (Savic and Savic 2002). The objective of this research is to study conditions used in formation of experimental films, as well as the commercially prepared, collagen dispersions that are used in in-line production of co-extruded collagen casings. Research was divided into two main categories;

1. Investigating the properties and functionality of the commercially-available raw materials
2. Assessing the effects of partially-dehydrating and cross-linking on the mechanical, thermo-mechanical and optical properties

Information from this research could increase the acceptance of this technology with both industry members and consumers. In addition, research could guide manufacturers when selecting the materials and processes to convert collagen dispersions into stable casings or films.
1.1 References


2.1 Edible Films

2.1.1 Definition and Functionality

Edible films are continuous layers of material that are commonly used to compliment conventional packaging. Edible films have been used in many industries, as covers, wrappers, casings, capsules, pouches and bags (Krochta 2002). When applied appropriately, edible films can improve food’s quality, extend shelf life and replace some synthetic films. Improved food quality and preservation is typically accomplished by controlling mass transfers between the food and it’s environment. Mass transfers that can result in quality loss include: surface dehydration, moisture absorption, aroma and flavour gain, oxygen uptake and fat migration (Savic and Savis 2002).

2.1.2 Production

Overall, it can be said that the industry utilizes two general methods of producing edible films, which include solvent casting and extrusion. The fundamentals of casting, involve the evaporation of solvent, usually water or aqueous ethanol, from a solution of protein or carbohydrates. Heat treatment and pH adjustment is typically employed, during casting, to enhance film formation and functional properties. Extrusion involves pumping a viscous suspension of material through a die, into a neutralizing coagulation bath. The set film is then typically washed to remove residues and prepared for storage by drying (Krochta 2002).

The materials that are used in the production of edible films are commonly derived from biological sources. Biopolymers, such as, proteins, carbohydrates and lipids are commonly used, both on their own and in combination, to produce films (Fernandez-Pan and Caballero 2011). Since each application has specific requirements, it is important to select a material or system that has the desired properties (Krochta 2002). In addition to providing functional attributes, materials should not detract from the quality of the encased material. Quality loss may result from imparting colors, odors and flavours, microbial contamination and toxic effects (Krochta 2002).
2.1.3 Application in the Meat Industry

The meat processing industry is an example of where edible films the food industry uses. Sausages use edible and non-edible films, also known as casings, to provide a primary level of packaging. The separation of processed meat from the surrounding environment results in chemical, structural and volumetric changes (Savic and Savic 2002). By regulating these changes, casings can add value to less tender cuts of meat through the production of sausage meat products (Savic and Savic 2002). During processing, sausages are subjected to dramatic changes (e.g., cold filling, cooking, chilling, frying), thus casings should maintain their functionality, throughout processing. Some operations where conditions may change include, drying, smoking, cooking and storage. The value of casings can be better understood through the processes of sausage production.

2.2 Sausage Casings

2.2.1 Traditional Production

Processed meat products, such as sausages, burgers and meat pies account for approximately half of all the meat that is consumed in developed countries (Kearney 2010). Sausages can be simply described as formed meat products that have improved structure and flavour (Savic and Savic 2002). More specifically, they are encased protein products, which are efficient and effective vehicles of delivering portioned food products. Production of sausages typically starts with chopping/grinding meat into a fine emulsion or course-ground batter. Chopping incorporates meat proteins with fat, water, salt and other curing and extending additives. The second major phase of sausage production involves the conversion of batter into a number of independent sausage units. In this stage casing are filled or stuffed with the cured meat and finally linked. Encased meat proteins can then undergo heat induced gelation, developing an elastic structure of fairly uniform shape and size (Savic and Savic 2002).

2.2.2 Natural casings

Animal casings are the oldest form of casings used and are considered to have superior (high) moisture vapor transmission and have a wide range of mechanical properties, depending on the type and preparation of the casing (Savic and Savic 2002). The conversion of digestive tubes and bladders into casings is performed immediately after slaughter. The first stages of production involve cleaning and defatting. Defatting is performed immediately because the removal of solidified fat is more labour intensive and increases the risk of physical damage to
the casing. Sliming is the next stage of production and involves the removal of one or more intestinal layers, to increase permeability, elasticity and edibility. In addition, sliming also helps remove excess gut content that is not removed during cleaning. Casings are then soaked in ice water or a brine for blood removal. If the casings are not immediately used, they are then cured in salt and packaged (Savic and Savic 2002). In order to maintain mechanical properties and permeability, casings must undergo flushing, which is hydration stage, prior to stuffing.

Natural casings tend to lack some consistency because their functionality is dependent on their origin. For example, the age, breed, feed and upbringing conditions of the animal (sheep, pig) affect the attributes of casings (Savic and Savic 2002). In addition, contamination of pathogenic and spoilage bacteria are one major concern when using natural casings. The longer a casings goes without being thoroughly cleaned, after slaughter, the greater the loss of quality and safety. Additional processes, like irradiation, have been observed to successfully control microorganisms, to a degree (Kim and others 2012). However it should be recognized that processes, such as these, add additional processing and labour costs.

2.2.3 Manufactured Casings

In modern sausage production, manufactured casings are commonly produced in designated manufacturing facilities, away from the meat processing plant (Visser 2012). Collagen and cellulose are the most commonly used biopolymers, in the production of sausage casings. Regenerated collagen casings have gained popularity because they posses many advantages over traditional animal casings. Including: improved uniformity, strength flexibility and hygiene. In addition, they increase the ease of processes, such as filling, portioning and slicing (Osburn 2000; Savic and Savic 2002).

Regenerated collagen casings are composed of both fibrous and solubilized material that is extracted from cattle hide, bone and connective tissue (Ratanavaraporn and others 2008). Generally, collagen casing production involves corium separation, decalcification, homogenization/regeneration and extrusion. During collagen separation hides are washed and limed to remove impurities. Following separation, calcium is removed to promote uniform swelling of collagen fibrils and the material is ground. The dispersed collagen then undergoes regeneration, where swelling is promoted through the addition of acids. Depending on the method (dry or wet process) there may be subtle difference in the steps involved in the handling and extrusion of the regenerated casing (Savic and Savic 2002; Miller 1983). Hydrochloric acid is typically used in commercial products, other acids, such as acetic acid have been used
(Ratanavaraporn and others 2008). Finally, the swollen material is extruded through an annular die, crossed-linked and dried. Cross-linking is accomplished by brine immersion or extraction with gaseous ammonia. Drying is the final stage, during which it is important to keep the temperature low to avoid the denaturation of collagen (Savic and Savic 2002).

Like collagen, cellulose is a highly functional biopolymer that provides the functional properties required to make sausage casings. As cellulose is the principle component of all higher plants, it is the most abundant renewable, organic, raw material in the world. Generally, cellulose is commonly extracted from wood and cotton, through either an acid or alkaline procedure. Cellulose casings are produced from the wood or cotton short fibers. There are many varieties of cellulose casings that are tailored to their application. Cellulose and cellulose composites systems are commonly distinguished by their strength and stiffness. However, the application of cellulose casings is limited because there are non-eatable (Savic and Savic 2002).

2.2.4 Co-extrusion

Co-extrusion is a relatively new method of sausage production, which avoids intermediate stages of the preparation and storage of premade casings. Unlike traditional sausage production that involves stuffing prepared meat into natural or pre-fabricated casings, the casings are formed directly on the sausages surface (Figure 2.1). This is accomplished by stabilizing the casing material as it is simultaneously extruded with the meat. Natural polymers, like collagen and alginate have been successfully used with this method (Morgan and others 1998).
Collagen, derived from bovine hides, is a biopolymer that is frequently used as a casing material. Co-extrusion of collagen casings requires a dispersion of collagen with a high water content. Collagen dispersions are typically mixtures of soluble and insoluble collagen fibers at a pH of 2 – 3 so that they have maximum water uptake (Morgan and others 1998). During extrusion, a counter rotating extrusion head applies shear forces to orientate and elongate the collagen fibers in a transverse direction to the extrusion direction. Orienting the fibers improves the mechanical properties of casing by reducing the probability of the splitting of casings later on in the process. Direction and rotational speed of inner and outer surface dies results in entwining collagen fibers, which improves the casings strength. Optimal conditions will produce 40 to 100° angles between fibers (Bontjer and others 2011).

Producing co-extruded casings with polysaccharides, like alginate, requires a different extrusion system. Unlike collagen, there are no fibers therefore it is not necessary to provide orientation during extrusion. Other polysaccharides that have been used are gums, such as carrageenan, which provide some stability to the final casing (Bontjer and others 2011; Visser 2012).

Once the casings has been extruded, subsequent treatment is required to improve the functionality of the casing. Collagen casings require a system that sprays, drips or immerses the product into a salt brine. The brine dehydrates the casing and allows the collagen to conform to
the shape of the food material. This improves the sausage quality by also removing gaps that occasionally form between the food material and casing. Stabilization can be performed by air-drying, as well as cross-linking with smoke condensate and other agents. Most, if not all sausages are exposed to smoke because of the added flavour, colour and preservative qualities. Sausages that are produced with smoke generally have a thinner layer of collagen because crosslinking agents improve the strength of the casing. If the thickness of the casing is not reduced, the smoking process can make casings unacceptably tough. Bradshaw and Taylor (1971) provided some of the original research on the ideal co-extrusion processing parameters (Table 2.1).

**Table 2.1 Ideal collagen dispersion, brine and extrusion parameters based off industry protocol – adapted from Bradshaw and Taylor (1971)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Preferable</th>
<th>Most Advantageous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity</td>
<td>45 - 90 Poise</td>
<td>60 - 70 Poise</td>
</tr>
<tr>
<td>Collagen Solids</td>
<td>4.3 - 7.4 %</td>
<td>5.2 - 5.8 %</td>
</tr>
<tr>
<td>Extractability¹</td>
<td>7 - 16 %</td>
<td>9%</td>
</tr>
<tr>
<td>pH of Dough</td>
<td>2.8 - 3.5</td>
<td>2.9 - 3.1</td>
</tr>
<tr>
<td>Brine</td>
<td>4.3-7.4 %</td>
<td>&gt; 75 %</td>
</tr>
<tr>
<td>Brine Setting Temperature</td>
<td>Do not exceed 40°C</td>
<td>15 - 25 °C</td>
</tr>
<tr>
<td>Film Thickness</td>
<td>&gt; 0.050 mm</td>
<td>0.100 mm</td>
</tr>
</tbody>
</table>

¹ Extractability refers to the proportion of a given collagen sample that is acid soluble

After dehydrating and crossing linking processes, the casings are of sufficient strength to undergo crimping or linking. Linking is accomplished by pushing food material aside within the casing to define the individual sausages. The conventional twist linking, used for natural / regenerated casings, is not required to ensure adequate casing coverage/closure. The crimper consists of an upper and lower v-formed guides. The crimping process involves the v-cutouts slowly closing on the sausage. The guides displace the meat within the coating until there is only a thin neck of casing, which can then be cut (Bradshaw and Taylor 1971).

The co-extrusion process provides many benefits over traditional sausage processing but also results in new challenges in production and casing performance. Co-extrusion is fundamentally a continuous process, which can reduce labour and waste, energy consumption and improve quality from greater automation (Bontjer and others 2011). Since natural polymers are being formed directly on the food’s surface, co-extruded casings become a part of the meat, to a
greater degree. For this reason, the casing cannot be easily stripped from the meat product, and this is a desirable characteristic in their production as they are eatable casings (Visser 2012).

Processors must be aware of the quality of the brining solution. The removal of water from the casing can rapidly dilute the brine, therefore processors commonly have a supersaturated brine (75% NaCl) to account for dilution. In addition, the brine will potentially become contaminated, over time, with additives such as casing material and meat product. Reduction of this waste can be limited with improved brine collection and reconditioning systems. The brine must also be monitored for changes in the pH as acids may be extracted from the casings during dehydration. If the pH drops the collagen will changing the sensory characteristics of the product (Kobussen and others 2012).

Selecting an adequate cross-linker presents other challenges. Since the casing is cross-linked directly on the meat, the casing cannot be washed in the same way as prefabricated collagen and synthetic casings. Washing is performed to remove residues that may impart flavour, colour or toxic effects. For these reasons, smoke condensates, which have aldehyde cross-linking components, are used in the co-extrusion process (Visser 2012).

2.3 Functional Properties of Casings

Casings are continually being designed to accommodate sausage manufacture’s quality and processing needs. The ideal collagen sausage casings should provide sufficient strength to undergo processing, while still remaining tender, for consumption (Miller 1983). In addition, their barrier properties must accommodate smoking, drying and cooking operations. In order to catalog the different attributes of casings, a number of methods have been employed to objectively test the properties of casings (Harper and others 2012; Harper and others 2013; Miller 1983; Barbut 2010). Mechanical properties, barrier properties and other basic physical properties, such as uniformity of caliber/ thickness, shrink ability, light transparency, and thermal stability, are of interest to manufacturers (Savic and Savic 2002).

The mechanical properties are often of greatest interest to sausage manufactures. They affect structural integrity size, shape, volumetric changes, textural properties, and behavior during processing (Savic and Savic 2002). For example, during traditional production, casings must withstand the tensile stress during stuffing and cooking. In addition, casings must provide compressive strength and elasticity during meat gelation and shrinkage, respectively (Savic and
Examining properties like casing’s elongation, tensile strength, toughness, and the elastic modulus can help predict the success of a casing.

Casings are subjected to a considerable amount of tension, during operations like stuffing and cooking. When materials, such as casings, are subjected to a tensile force, the resulting deformation is typically elongation in the direction of the applied force. This elongation is also accompanied by a contraction of the material in the direction perpendicular to the applied stress. These deformations are characterized by stress and strain, which is the force applied, per unit area and the fractional change of length, respectively (Equation 1 and 2). Stress and strain are commonly expressed in N/m² or Pa and as a percentage, respectively (Savic and Savic 2002; Fratzl 2008).

Generally, a stress-strain relationship appears to be a linear increase stress with strain (Fratzl 2008). The measurement of these deformations can determine a film or casings tensile strength, percent elongation and elastic modulus. These properties are defined as the maximum stress required to break the film, the strain at failure, and the film stiffness as determined by the stress-strain ratio (Equation 3), respectively (ASTM D638).

\[
\text{Stress} = \frac{F}{A}, \text{ where } F \text{ is the force and } A \text{ is the area} \tag{1}
\]

\[
\text{Strain} = \frac{\Delta r}{r_0}, \text{ where } \Delta r \text{ is the distance to failure and } r_0 \text{ is the initial distance} \tag{2}
\]

\[
Y = \frac{\text{stress}}{\text{strain}} = \text{Elastic modulus} \tag{3}
\]

To some degree most materials have proportional stress and strain. When we increase the stress beyond the elastic limit, the material suffers an irreversible deformation and the recovery length is now greater than its original length. A material is considered mechanically elastic when the stress-strain curve, from the increasing load, follows the curve during the release of stress. Materials typically show elastic behavior under small deformations until a critical point. After that critical point, known as the elastic limit, the increase in stress will result in an irreversible deformation where the material will not return to its original shape. Some materials, like collagen, exhibit viscoelastic properties, which means that they slowly return to its original shape (Fratzl 2008). Once the elastic limit has been surpassed and enough stress is applied, the material will fail, which is considered to be the fracture point. The amount of stress that can be applied between the elastic limit and fracture point, determines whether a casing is considered ductile or brittle (Krochta 2002).
The resistance to deformation, when a material is subjected to a given stress, is the stiffness. Stiffness is commonly associated with the strength of a material but the mechanical strength is the maximum stress a material can sustain before breaking; casing materials typically need to be stiff and strong. Defects in the structure of a homogeneous material will have a different effect on stiffness and strength. A defect or 'weak link' that has half the stiffness and strength of a functional link, will reduce the overall material's stiffness and strength by 1% and 50%, respectively. This means that the strength of a material is heavily dependent on the local properties of a material. This dependence on material homogeneity is also translated in to the toughness of a material, which is the energy needed to propagate a crack through the material, to break it. The area under a stress strain curve gives an estimate of a material's toughness, when the material is loaded to failure (Fratzl 2008).

The permeability is another important property as it pertains to parameters such as water loss, compositional changes, fat hydrolysis, oxidation, pH, water activity, ripening of raw sausages and sensory properties (Savic and Savic 2002). Permeability describes the extent to which a permeating substance dissolves and then the rate at which the permeate diffuses through the film. This migration of permeant is ultimately driven by concentration gradients. Polarity of a given material is one factor that affects permeability. In general, protein films have high permeability to polar substances such as water vapour, and low permeability to nonpolar substances such as oxygen, aroma compounds and oils (Krochta 2002). Collagen is known to be a good oxygen barrier but not a good moisture barrier. Occasionally, composite technology can be employed in order to take advantage of polymers with different properties (Fernandez-Pan and Caballero 2011).

The degree of light transmission through a collagen casing governs consumer's ability to evaluate the product. Light transmission measurements can be evaluated in the visible light spectrum in order to evaluate the transparency of collagen casings. In sausages stuffed into several pre-manufactured casings (stand alone, large diameter casings), light transmission is commonly modified to reduce photochemical colour changes in the sausage meat (Savic and Savic 2002). Reducing light transmission with colouration can significantly decrease or prevent negative colour changes, due to oxidation of nitrosomyoglobin (Savic and Savic 2002). Casings can also contribute to differences in the colour parameters in products. In coarse ground products, it was found that there is no significant difference between natural and synthetic casings in L* value (lightness) but there was one in a* value (redness index). Natural casings had a significantly higher 'a' value (Conte and others 2012).
The thermal stability of casings is crucial in controlling volumetric changes of sausages. Cooking and freezing operations can result in an increase in volume of sausages with high water content. This expansion is sometimes accompanied with the casing shrinkage. Materials, like collagen fibers undergo contraction and shortening at elevated temperatures (Savic and Savic 2002). This contraction takes place when thermal energy denatures collagen structure to a more disordered state (Wess 2008).

The mechanical properties of casings produced with biological polymers are more variable and typically lower in stiffness and strength than those produced with synthetic polymers (Krochta 2002). It has been observed that natural and synthetic collagen casings do not exhibit significant differences in elongation at break, although there are some differences in water vapor permeability (Conte and others 2012). In addition, natural casings are often slightly thicker, thus reducing the degree of water loss from evaporation.

2.4 Collagen

The application of collagen, as a biomaterial, has been successful in many industries. Collagen has been used in personal care, health care and food applications (Sundar and others 2011). The practicality of collagen is a result of its abundance in animal byproducts and its functionality. Collagen’s application in sausage production, as natural and regenerated collagen casings, makes it the most commercially successful edible protein (Fernandez-Pan and Caballero 2011). Regenerated collagen casings were developed in Germany in the 1920s but only popularized in the United States during the 1960s (Jayathilakan and others 2012). Collagen casing production is continually being manipulated to improve yield, texture, extend shelf-life and alter colour and flavour.

Collagen is a complex protein, found in the living animal in fibrillar and amorphous scaffolds. Collagen is commonly found in connective tissue, in an insoluble fibrillar form, as long, slender, cylindrical, tapered fibrous structures (Wess 2008). In sausage production, collagen’s hierarchical organization is of significance because the cleavage of the collagen casing’s network, during mastication, has been suggested to be of the greatest importance for the overall tenderness sensation of sausages. The variations in strength, within and between casing varieties, can give consumers different impressions of tenderness. In addition to the basic protein structure an understanding of the hierarchical structure of collagen will provide insight into its mechanical properties (Fratzl and Weinkramer 2007).
2.4.1 Collagen Structure

The primary structure of collagen is rich in glycine, which makes up approximately one third of the amino acid residues. A high degree of glycine gives individual protein chains rotational mobility (Savic and Savic 2002). Collagen also has a high degree of hydroxyproline (unique to collagen and often used to measure collagen content) and proline. Steric repulsion that is produced by hydroxyproline and proline’s cyclic ring prevents the formation of alpha helix chains. As a result, the helical nature of the secondary structure is not as tightly coiled as an alpha helix (Arvanitoyannis 2002).

The assembly of collagen structures is dependent on small molecular variations (Savic and Savic 2002). Collagen’s basic organization can be understood through the tropocollagen unit (Figure 2.2). Tropocollagen is a super-helix, consisting of three collagen chains that have a repeating unit of approximately 100Å or 100 nm. Assembly of tropocollagen is spontaneously driven by lysine in the polypeptide chain, which promotes hydrophobic interactions (Savic and Savic 2002). In addition, amino acid sequencing of fibril forming collagen provides optimal electrostatic pairings between adjacent triple helices and maximizes the contact between hydrophobic regions.

![Figure 2.2 A generalized drawing of the hierarchical structure of polypeptides, triple helical tropocollagen molecules, collagen fibrils as staggered array of tropocollagen molecules, and collagen fibers — adapted from Buehler (2008)](image)

Although tropocollagen provides a simplified model, at the molecular level, there are many variations in the collagen structure. For example, there are over 25 collagen types of fibril-forming collagens. However, collagen I is the most commonly used in sausage casings as it is a
major structural component of bovine hide. It has been proposed that the differences in collagen type are to give additional functionality or govern molecular packing (Wess 2008). Since different collagen rich tissues have specific structural prerequisites, it is not uncommon for a single fibril to have a variety of collagen types (Wess 2008).

Tropocollagen molecules align in specific arrays to build microfibrils, subfibrils and fibrils. Since the collagen fibril (Figure 2.2) is the key to scaffolding structures in the body, it can be used to provide a basic understanding of collagen’s macrostructures. In general, fibrils are long, slender and cylindrical structures but can differ in length, diameter, uniformity, and telopeptide size, as a result of collagen type and interactions (Wess 2008; Cameron and others, 2002). As tropocollagen aligns in a staggered array conformation, they develop areas of overlap and gap. The overlap and gap regions of the parallel arrays help give collagen fibrils their distinctive striated pattern (Figure 2.3). The striations are regions of high and low electron density with a periodicity that measures about 640-700 Å (Wess 2008).

![Collagen fibrils](image)

**Figure 2.3** Fibril surface properties: electron micrographs of longitudinal sections of collagen sclera. Bar = 50 nm. The proteoglycans decorate the surface of the fibrils – adapted from Young (2000)

The stabilization of tropocollagen’s macromolecular structure is a result of telopeptide interactions (Wess 2008). Telopeptides are short, non-helical sections at the N- and C- terminals of each super helix. They contribute to axial stabilization of collagen chains through the development of molecular cross-links between telopeptides. In addition, telopeptides provide lateral associations between collagen chains through cross-linking. Lateral interactions appear much more variable and are not as well understood (Wess 2008). Electrostatic pairings between
adjacent triple helices and contact between hydrophobic regions also help link molecules both end to end and adjacently (Wess 2008).

The organization of fibrils into bundles contributes to collagen’s mechanical properties (Birk and Trelstad 1996). Variations in assembly will provide differences in mechanical properties, for instance fibril alignment can provide great tensile strength in the axial direction (Arvanitoyannis 2002). In addition to orientation, a high degree of proteoglycan (PG) and glycosaminoglycans (GAG) can increase the strength of the structure. PG and GAGs can be found on the interfaces of fibril bundles (Figures 2.3 and 2.4) and take part in fibril interactions by controlling interfacial shear and restricting fibril growth. This is of importance since factors such as diameter, and length have significance in mechanical properties (Wess 2008).

Figure 2.4 A representation of collagen fibril packing and collagen molecular arrangement; A - Representation of collagen fibrils packing laterally. B - An axial view of collagen fibril cross-sections, with associated proteoglycans and glycosaminoglycans (GAGs). The small circles on the fibrils surface are proteoglycans and the outward facing lines represent the GAG chains. C - Each bar is representative of a collagen molecule in the staggered array conformation, found within a fibril. D - The D-period of the collagen molecules represented by a gap and overlap area within the staggered array – adapted from Wess (2008)
2.4.2 Collagen’s Mechanical and Thermal Properties

The mechanical properties of collagenous tissues are a result of the optimization of their structure on many hierarchical levels. The interactions between collagen structures are critical to the overall mechanical and thermal properties (Fratzl, 2003). The deformations that occur at the molecular and fibril level provide some insight on the mechanical behaviors of collagen (Gupta 2008). As it has been previously discussed, collagen fibers show banded structures due to alignment of collagen molecules and fibrils. When a fiber undergoes strain, the initial deformation involves the removal of molecular kinks that are found in the gap regions. On a stress-strain curve, these deformations would result in a gradual upward slope. The length of these deformations is approximately 6% elongation but there are variations, due to differences in the degree of pre-straightening. Larger strains result in molecular elongation of the triple helices and eventually fibril gliding along the proteoglycan-rich regions. It is believed that interface interactions play a significant role in the strain of collagen rich materials. Consequently, gap and overlap regions of fibrils become increasingly disordered. This can be observed as a loss of striation pattern (Gupta 2008). During fibril elongation and sliding deformations, the stress-strain relationship is typically linear (Figure 2.5) as indicated by Fratzl and others (1998). The events that occur during fiber failure are not well understood (Gupta 2008).
Figure 2.5 Changes that occur to collagen at the fibrillar level when a tensile stress is applied. Molecular kinks are gradually straightened out prior to the stretching of the triple helices and gliding of the molecules. The stress–strain relationship becomes linear once all the kinks have been removed. The alignment of gap and overlap zones becomes less well-defined with increasing strain – adapted from Fratzl and others 1998

Some studies have been performed to observe the effects of natural crosslinks. Puxkandl and others (2002) compared naturally cross-linked animal tendons where natural cross-linking was inhibited by feeding test animals a diet of β-aminoproprionitrile. The results demonstrated that cross-link deficient collagen had a 10-20% reduction of tensile strength. Thus, cross-linking plays a crucial role in providing strength to collagen structures (Puxkandl and others 2002). In addition, it has been suggested that cross-link deficient fibrils have less elasticity in contrast to normally cross-linked collagen (Puxkandl and others 2002).

The thermal properties of collagen dispersions are sensitive to a number of factors. The temperature of denaturation can vary, as a result of thermal history and moisture content (Zhang and others 2006). The degree of cross-linking also increases denaturation temperature. Cross-linking with aldehydic reagents such as glutaraldehyde (commonly employed in the meat industry, as discussed above) has been observed to increase the denaturation temperature from 65°C to about 100°C. The increased thermal stability is thought to be a result of the water content within fiber (Miles and others 2005). Since cross-linking reduces the axial separation between molecules, there is a decrease in entrapped water and higher denaturation
temperature. To observe these effects, cross-linking agents, with different lengths, were used with no change in denaturation temperature, when hydration was the same. It has also been observed that the enthalpy of denaturation is not affected because cross-linking does not affect bound water (Avery and Bailey 2008).

2.4.3 Collagen Cross-linking

In the living cell, the mechanical properties of collagenous materials are dependent on the formation of cross-links. In natural tissues, cross-linking occurs in two stages, enzymic and non-enzymic. The initial stage involves the enzymic formation of divalent and trivalent cross-links at the head and tail of fibers. The second stage involves a non-enzymic reaction with glucose, which cross-links lysine and arginine resides in the triple helix. Cross-links, of this nature, increase the stiffness and brittleness of the collagen fiber’s network.

Chemical cross-links have been developed to modify and control the mechanical properties of collagenous materials in industrial, medical and cosmetic products. These cross-links utilize reactive groups, like lysine, glutamic acid and hydroxyl groups, which project from collagen molecules. Cross-linking these groups increase the mechanical strength of fibers by preventing the molecules and fibers from sliding past each other. Under stress, cross-linked, collagenous tissues exhibit an increase in breaking strength and stiffness (Covington 1997; Paul and Bailey 2003). In addition, collagen’s susceptibility to enzyme degradation and thermal denaturation is reduced, as a result of lower water content and accessibility for enzymes (Avery and Bailey 2008).

Aldehydes, like glutaraldehyde, are commonly employed cross-linking agents because they form stable bonds and significantly increase the mechanical properties of collagen fibers (Covington 1997). Glutaraldehyde complexes are believed to react with lysine to form heterocyclic compounds. Subsequent oxidation reactions produce pyridine rings (Figure 2.6). The use of glutaraldehyde induces cytotoxic effects, therefore aldehydes, present in liquid smoke, are used to cross-link collagen casings (Avery and Baily 2008, Savic and Savic 2002).
Smoke and smoke condensates are used globally in the meat industry to improve flavour, colour, preservation and the textural properties of processed meat products. Smoke and smoke condensates are produced through a process called pyrolysis. Pyrolysis is a controlled, chemical decomposition of wood or other fuel, which is performed under limited oxygen. The byproducts of pyrolysis are an array of compounds, including aldehydes, ketones, furans, phenols, acids, etc (Toledo 2007). The presence of aldehydes give smoke condensates their cross-linking functionality. The composition of smoke and smoke condensates can differ, because of the presence and proportion of major (cellulose, hemicellulose and lignin) and minor compounds (terpenes, fatty acids, other carbohydrates, polyhydric alcohols, nitrogen and phenols) in the fuel source. Compositional variations can also be derived from availability of oxygen and moisture of the fuel during smoke generation, in addition to the age of a smoke condensate (Guillen and Manzanos 1999; Montazeri and others 2013).

Smoke condensate has been reported to have 20 times the level of compounds than that of gaseous smoke. This suggests that some polymerization of smoke components may form in the liquid phase. Also, aging smoke condensates continue to change. Wasserman and Fiddler (1969) reported improved flavour when smoke condensates that were aged 1 to 2 month rather than 5 hours. In addition, they showed compositional changes (concentration of furans, phenolic and cyclic compounds) as a result of available oxygen, during smoke generation.

Traditionally, direct exposure was the primary method of applying smoke to foods. However, this method exposes food to the solid (soot, tar and ash) and liquid components (can polymerize to solid particles), in smoke, which do not contribute to the desirable smoke flavour and are high in polycyclic aromatic hydrocarbons, which are carcinogenic (Toledo 2007, Ruzum 2007). Sophisticated smoke generators and smoke condensates were developed in order to reduce the application of these components. Some of these developments employ refining process is to remove the solid and liquid phase. The simplest of method involves routing the smoke under a curtain of flowing water (Ruzum 2007).
Smoke condensates are commonly favoured over traditional smoking because they improve health (safer and fewer carcinogens) and cleanliness (reducing labour costs) of processing, as well as imparting more uniform colour and flavour (regulated specifications). Smoke condensates can be applied to foods through atomization and showering. In atomizing, the smoke condensate is applied using high air pressure, which makes really small droplets. The atomized droplets can then be circulated in a smokehouse, similar to traditional smoking methods. Showering pertains to a 5-50% solution in water, cascading over the food product. This is commonly used in large meat applications because it allows for greatly reduced cook times, while ensuring uniform colour and flavour (Ruzum 2007).

2.4.4 Collagen Film Forming

Proteins are sensitive to the environment (pH, temperature, etc) so their conditions should be closely monitored when producing biopolymer films. Environmental changes can affect both the film forming properties and the film’s performance. The pH of the solvent or liquid phase is one example of a condition that should be monitored when using proteins as biopolymers. Since proteins are zwitterions (possessing a negatively charged carboxyl and positively charged amine group) researchers typically adjust the pH away from the protein’s isoelectric point when producing films and coatings. The isoelectric point is avoided so that the proteins remain soluble in their solvent through electrostatic repulsion. The isoelectric points of collagen, gelatin and collagen hydrolysate was observed to be 8.26, 4.88 and 4.56, respectively (Zhang and others 2006; Friess and Schlapp 2001; Latinovic and others 2010). The differences in isoelectric points were thought to be a result of keeping side amide residues intact. Gelatin and collagen hydrolysate had higher density of carboxyl groups caused by the hydrolysis of side amide groups. Commercially, when producing collagen casings, the protein dispersions typically have their pH lowered to a value around 2-3 (Morgan and others 1998).

There may also be difference when the pH is more alkaline or acidic than the isoelectric point. The industry commonly lowers the pH because it has been suggested that collagen dispersions become more swollen and bind more water (Morgan and others 1998). The type of acid used to control the pH has also been observed to provide different properties. For example, the use of strong acids, (hydrochloric acid) rather than weak acids (acetic acid) have been observed to give collagen scaffolds different properties. It is suggested that differences in ionic strength could be attributed to an increase in swelling of collagen fibers that were extracted with Acetic acid (Ratanavaraporn and others 2008). In addition, Ratanavaraporn and others (2008) also
reported that collagen dispersions extracted with HCl were much more viscous than those extracted with acetic acid. The viscosity of protein dispersions also influences the film forming and film behavior. If the dispersion is too viscous then there may be problems producing a homogenous film and there may be a less drying efficiency.

Mechanical properties and structural integrity of films can be temperature dependent. This is why the thermal behavior of dispersions and gels was studied. Protein dispersion and solubility are required to produce a high quality film. Protein solubility and unfolding can be increased with different solvents (ethanol, pH adjusted). The degree of unfolding can be manipulated by the solvents pH (adjusting away from the isoelectric point of the protein) or through the reduction of disulfide bonds via reducing agents. In addition, the use of polar solvents can decrease the amount of hydrogen bonding (Gallstedt and others 2011).

2.4.5 Composite Film Technology and Modifiers

Composite film technology utilizes the functionality of two or more materials. For instance, polysaccharides, like cellulose, can improve the strength of collagen films, through physical entanglement of fibers (Krochta 2002; Mathew and others 2012). The use of polysaccharides has also been observed to have little affect of water vapor permeability but can increase oxygen permeability and tensile strength. In addition to composite technology, a number of modifiers have been used optimize the performance of protein films (Krochta 2002). Plasticizers are one group of modifiers that can be added to decrease the film stiffness and increase film elongation. Plasticizers also have varying effects on the barrier properties, like water vapor and oxygen permeability (Krochta 2002).

Protein films can also be modified through a number of simple treatments. As previously described, cross-linking with glutaraldehyde can alter the mechanical and thermal properties of collagen films. Adjusting the pH is another modification of the film forming solution that will alter the protein’s solubility. Thermal treatment can cause denaturation, which can alter the barrier and mechanical properties of the film (Krochta 2002).
2.5 References


Miles CA, Avery NC, Rodin V, Bailey AJ. 2005. The increase in denaturation temperature following cross-linking is caused by dehydration of the fibres. Journal of Molecular Biology 346:551–556.


Chapter 3 An Investigation of the Mechanical, Thermal and Microstructural Properties of Commercial Collagen Dispersions and Partially Dehydrated Films

3.1 Abstract

The mechanical, microstructural and thermo-mechanical properties of five commercially prepared collagen dispersions were investigated. The protein quality and pH may have contributed to these differences in the denaturation temperature of the dispersions. Partially-dehydrated films were produced to simulate sausage casings manufactured in the co-extrusion process. Materials were also studied in partially dehydrated film form. Manipulating the dehydration conditions (brine concentration; 15, 20, 25 or 30 wt.% sodium chloride in deionized water and contact time; 1.0, 2.5, 5.0 or 10.0 min) demonstrated that there were significant differences ($p < 0.05$) in the film tensile strength and percent elongation. The mechanical properties of the dehydrated collagen films also demonstrated differences ($p < 0.05$) between the commercial collagen dispersions. The quality and of collagen, swelling agent and added cellulose were thought to result in differences in film properties.

3.2 Introduction

A sausage casing is a necessary component in the conversion of comminuted meat into a finished product. Throughout history there have been a number of different technologies that have improved the processing, handling and functional properties of casings; uniformity, hygiene, strength, flexibility and stability during storage (Osburn 2000, Savic and Savic 2002). For instance, prior to the early 20th century almost all sausages were produced with natural casings that were derived from animal intestines. Although natural casings are still considered the ‘gold standard’, advances in casing technology have led to numerous types of precisely engineered casing. These modern casings can now be produced with a number of different materials, like regenerated biopolymers (collagen and cellulose) and even with thermoplastic materials (polyvinyl alcohol polymers). Manipulation of these materials has even made it possible to tailor casings to have specific functional attributes (Savic and Savic 2002; Wang 1986).

Until recently, the increased sophistication of modern casings required specialized production at designated manufacturing facilities (Karmas 1974). With the development of co-extrusion, the
need to prefabricate, store and prepare sausage casings, prior to stuffing, was eliminated. Co-extrusion is a continuous method of sausage production where a thin layer of casing material is simultaneously extruded on the comminuted meat surface. Immediately after formation, the casing material undergoes stabilization, through dehydration or cross-linking to enhance the mechanical properties of the casing. A brine application (spray, drip or immersion) dehydrates the casing and allows the material to conform to the shape of the meat. Further stabilization is commonly performed through cross-linking and air-drying as they provide the mechanical properties necessary for linking, cooking and packaging (Bradshaw and Taylor 1971, Morgan and others 1998).

Co-extruded casings can be produced with a dispersion or gel of fibrous and soluble collagenous material (Morgan and others 1998). The collagen is typically derived from cattle hide and connective tissue (Ratanavaraporn and others 2008). Generally, collagen casing production involves corium separation, decalcification and regeneration. During collagen separation hides are washed and limed (pH 11-13) to remove impurities. Following separation, calcium is removed to promote uniform swelling of collagen fibrils and then the material is chopped and ground. The collagen then undergoes regeneration, where swelling is promoted through the addition of acids (Savic and Savic 2002). Hydrochloric acid (HCl) is the most commonly used swelling agent in commercial products but other acids, such as acetic acid have also been used (Ratanavaraporn and others 2008). Finally, the swollen material is commonly mixed, prior to extrusion to reorient the fibers for added strength (Savic and Savic 2002).

There has been some of effort made to understand the film forming properties of collagen and the affects of different additives (Harper and others 2013, O’Sullivan and others 2006, Telis and others 2006, Tomihata and others 1994, Olde Damik and others 1995). However, the properties of commercially prepared collagen dispersions and co-extrusion processing conditions for casing production have yet to be assessed. The first goal of this research is to evaluate different processing conditions that can be used to partially dehydrate collagen films. This research will also investigate the differences between commercially prepared materials that are used in the production of co-extruded meat products. The differences were evaluated through the collagen dispersion and films mechanical, microstructure and thermo-mechanical properties. This study should help manufacturers in the conversion of extracted collagen into wet casings, through material selection and manipulation of brining operations. As co-extrusion manufacturing is continuous and automated, increasing the acceptance and the implementation of this technology could also improve food safety and help reduce waste (Osburn 2000).
3.3 Materials and Methods

3.3.1 Ingredients for film formation

Five commercial collagen dispersions were evaluated. The dispersions were labeled Collagen 1 through 5 (C1, C2, C3, C4, C5), as they are proprietary blends. Information that was provided to the researchers and compositional analysis can be found in Table 3.1. Protein content was determined by the Dumas method (Leco FP528, St Joseph, MI, USA) using a nitrogen factor of 6.25.

Table 3.1 Commercial collagen material specifications and work of extrusion through a 7mm die: C1 (collagen 1), C2 (collagen 2), C3 (collagen 3), C4 (collagen 4) and C5 (collagen 5).

<table>
<thead>
<tr>
<th>Collagen</th>
<th>pH</th>
<th>Protein¹</th>
<th>Swelling Agent</th>
<th>Work of Extrusion J</th>
<th>Work of Extrusion J/±protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>2.06</td>
<td>5.14</td>
<td>-</td>
<td>5.16 ± 0.19</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>C2</td>
<td>2.21</td>
<td>3.57</td>
<td>-</td>
<td>3.19 ± 0.21</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>C3</td>
<td>2.01</td>
<td>3.68</td>
<td>HCl</td>
<td>4.04 ± 0.05</td>
<td>1.10 ± 0.01</td>
</tr>
<tr>
<td>C4</td>
<td>2.67</td>
<td>4.37</td>
<td>HCl/Acetic Acid</td>
<td>3.63 ± 0.01</td>
<td>0.83 ± 0.01</td>
</tr>
<tr>
<td>C5</td>
<td>2.04</td>
<td>4.82</td>
<td>-</td>
<td>4.04 ± 0.13</td>
<td>0.84 ± 0.03</td>
</tr>
</tbody>
</table>

¹Protein content was determined the Dumas method (Leco FP528, St Joseph, MI, USA) using a nitrogen factor of 6.25

3.3.2 Preparation of film dispersions

The commercial collagen dispersions were degassed using a Multivac vacuum packager (Multivac Canada Inc., Woodbridge, ON, Canada) at 7.3 kPa for 25 sec then again at 7.3 kPa for 50 sec and 75 sec (settings 4, 6 and 8, respectively). This was performed to remove gas bubbles that were incorporated during processing, as they can create weak spots in the films. Following the degassing stage, dispersions were mixed to improve the homogeneity of the samples. While still in the vacuum pack bags, the dispersions were mixed by rolling the solutions 10 times in adjacent directions. It is important to note that commercial collagen dispersions were commercially prepared, therefore the processing conditions (mechanical chopping, swelling agent and pH adjustment) were not controlled by the researchers. Except degassing and mixing, the commercial products were examined as they were received. These decisions were made in order to accurately report any variability between the materials used in modern sausage casing production.
3.3.3 Mechanical properties of the collagen dispersions

The collagen dispersions were evaluated by using a texture analyzer (TA-XT2i Texture Technologies Corporation, Scarsdale, NY, USA) with the TA-93 (100 mL cell) forward extrusion fixture. Approximately 50 g of sample was loaded into the cell fitted with a 7 mm die, and brought to 4°C. The plunger compressed the dispersion at a rate of 1 mm/s. From a generated force-distance curve, the work of extrusion was calculated once the readings had stabilized (between 10-35 mm).

3.3.4 Rheology of film forming dispersion

Rheological analysis was performed on the collagen dispersions using a Bohlin CS50 (Malvern Instruments Ltd, Worscestershire, UK) with a 25 mm DIN coaxial cylinder bob and cup fixture. The bob was lowered into 13 g of collagen that was preloaded into the bottom of the cup. Excess collagen was removed and mineral oil was then applied on the top to keep the exposed surface from drying. The temperature of the collagen was increased from 20 to 55°C at 1.25°C/min, held for 2 min and returned to 20°C, at the same rate. The temperature was regulated with a circulated water bath (Neslab RTE). Continuous oscillating shear (1 Hz and 0.0012 strain) was applied during testing. Test parameters were set and a thermograph of the elastic modulus (G’) was recorded with Bohlin Zetasizer Series software, version 6.32 (Malvern Instruments Ltd, Worscestershire, UK). Elastic modulus readings were taken to determine the stiffness of the dispersions (Helary and others 2009).

3.3.5 Differential scanning calorimetry (DSC)

The melting profiles of the collagen dispersions and dehydrated films were evaluated using a differential scanning calorimeter (DSCQ2000, TA Instruments, New Castle, DE). Samples (~0.001 g) were placed in alodined-aluminum hermetically sealed pans. The temperature of the collagen was ramped from 20 to 80°C at a rate of 5°C/min. Samples were then held at 80°C for 2 mins and then cooled back to 20°C at 5°C/min. The same thermal profile was used to rescan samples for reversible peaks. The melting behavior was studied between the temperatures of 30 and 50 °C by integrating the endothermic peak. This was performed with the TA Universal Analysis 2000 Software (TA Instruments, New Castle DE) to determine the onset temperature and peak melting temperature and enthalpy. A total of three dispersions or films were tested for each of the treatments.
3.3.6 Light microscopy

The collagen dispersions were prefixed in 10% neutral buffered formalin for 10 h at room temperature and then dehydrated in 70% isopropanol for 2 h, 95% for 1 h, and 100% for 4 h. The dehydrated of samples was completed in xylene, prior to embedding in paraffin. Samples were cut into 4-6 μm cross sections. Masson stain was used to differentiate collagen from any meat proteins, whereas, Periodic-acid Schiff (PAS) stain was used in order to differentiate carbohydrates, specifically cellulose fibers. Masson Trichrome stain is a combination of acid dye solutions (different molecular weight and size) that permeate into tissues of different protein density. Acid dyes bind to proteins via van der Waals forces because collagenous tissues are acidophilic (Cook 2008). PAS staining is based on an oxidation reaction of the 1,2 glycol group (commonly found in carbohydrates like cellulose) resulting in dialdehydes (two aldehyde groups where vestigial diols were in the monosaccharide ring). Subsequent reactions between the Schiff reagents and aldehyde groups result in the stain colouration (Carson 1997).

A light microscope (Olympus BX 60, Olympus Corporation, Centre Valley, PA, USA) was used to examine the samples. Representative images (a total of six images per treatment) were taken using Image Pro Plus (Version 6.0, Media Cybernetics, Inc., Bethesda, MD, USA) software.

3.3.7 Dehydrated film formation

The method of film formation was adapted from Harper and others (2013) who worked with alginate solutions. Collagen dispersions were first cooled to 4°C to reduce the adhesion during the film formation. Approximately 3 g portions of the collagen were rolled on a stainless steel board between two layers of plastic sheets with a stainless steel roller. The roller had a recess of 0.50 mm in order to achieve uniform film thickness. The top plastic sheet was removed and the remaining plastic sheet with film was then placed in a salt bath. A preliminary study was performed on one commercial collagen dispersion (C2) to evaluate the effects of brine concentration and contact time on the textural properties of the films. Brine solutions were 15, 20, 25 and 30 wt.% sodium chloride in deionized water and films were immersed in the brine for 1.0, 2.5, 5.0 and 10.0 min intervals. The plastic sheet was folded onto the formed film to prevent further dehydration of the film before it was tested.

The films that were prepared to evaluate the different commercial collage dispersions were immersed in 30 wt.% sodium chloride in deionized water for 5 min, in order to dehydrate the
film. After 5 min, the film was strong enough to hold together when removed from the plastic sheet. Similar to the preliminary study, a plastic sheet was folded onto the formed film.

### 3.3.8 Mechanical properties and film thickness

The standard test method for testing tensile properties (ASTM-D882) was performed on the dehydrated films. Films were evaluated by using a texture analyzer (TA-XT2i, Texture Technologies Corporation, Scarsdale, NY, USA) with a gripper distance that was set at 50 mm, trigger force at 5 g, test speed at 2 mm/s and the test distance at 25 mm. The film’s thickness was determined by measuring each film three times (top, center and bottom) using a digital micrometer (Testing Machines Inc., Islandia, NY, USA). The three measurements were then averaged to give a thickness for each film. The films were cut into 75 mm × 25 mm strips (JDC Precision Sample Cutter, Thwang-Albert Instrument Comp, Philadelphia, PA, USA). The average thickness and width of the films were used for the tensile stress calculations. From the generated stress–strain curve, the tensile strength (maximum stress the film endured prior to breaking) and the percent elongation (the maximum elongation the film reached prior to breaking) were determined. A total of eighteen films were tested for each of the treatments (six films per trial).

The second method was a puncture test, which also used the texture analyzer. In this test, a 5 mm diameter ball probe was used to puncture films mounted in a film extensibility fixture with circular opening of 10 mm diameter (TA-108S5, Texture Technologies, Corporation, Scarsdale, NY, USA). The test speed was 1 mm/s and the trigger force was 5 g. The distance to puncture and work of puncture were determined from the generated force–distance graph. Again, a total of eighteen films were tested for each of the treatments (six films per trial).

### 3.3.9 Optical property

The light transmission of the films (380–780 nm) was evaluated by using a single beam spectrophotometer (USB 2000, Ocean Optics Inc., Dunedin, FL, USA). The following settings were used: integration time: 100 ms, scans to average: 2 and boxcar width: 4. The light transmission was measured on twelve films per sample.

### 3.3.10 Experimental design and statistical analysis

The experiment was designed as a completely randomized block with three independent trials. Each trial consisted of six measurements per dispersion for the mechanical properties of the
films (tensile and puncture). Statistical analysis could not be reported on the collagen dispersion tests (protein, forward extrusion, rheology and DSC) because the tests were performed on one batch of collagen dispersions.

The statistical analysis was performed using SAS Version 9.2 (SAS Inst., Cary, NC, USA). A General Linear Model was used for the analysis of variance (ANOVA). The film type means and interactions were compared by using Tukey’s multiple comparison analysis with a P-value ≤ 0.05, which was used to detect statistical significance.

3.4 Results and Discussion

3.4.1 Mechanical Properties

Casing manufacturers must consider the extrusion properties of the raw materials so that they can be adjusted to an optimal processing value. The forward extrusion test was performed to provide insight into whether there may be differences in the flow behaviours of dispersions. It appeared that samples with lower pH had a higher work of extrusion (Table 3.1). If these trends were validated, it may suggest that a greater degree of conformational changes in the collagen network may increase the stiffness of the dispersion. The conformational change discussed are a result of lowering the pH from 5 to 2 (away from pl = 8.26 and 4.56 collagen and collagen hydrolysate), which would increase fiber hydration and swelling (Wolf et al 2006).

Once the casing are formed and stabilized, casings are faced with a variety of different tensile stresses. It is crucial to impart sufficient strength shortly after extrusion so that the sausages can undergo subsequent treatment and processing (Kobussen and others 2012). In an industrial setting, the newly formed casing is stabilized by rapidly extracting water through brining. Dehydration is driven by osmosis, which is thought to increase the density of the collagen polymer chains, thus improving the mechanical stability (Kobussen and others 2000; Visser 2012).

The conditions used to dehydrate the collagen films were based on guidelines described in some industry protocols (Kobussen and others, 2000). In order to evaluate the mechanical properties of the wet films, as applied to the meat in the raw state, tensile and puncture tests were performed to determine the tensile strength, percent elongation, distance to break and work to break (Figure 3.1). Mechanical testing demonstrated that there were no significant interactions (p > 0.05) between the brine concentration and contact time. With the exception of concentration on tensile strain, there were some significant differences (p < 0.05) in the films
mechanical properties when modifying either the concentration or contact time (Tables 3.2 and 3.3). It was observed that the tensile strength, percent elongation and work to break increased with concentration and contact time. This suggests that further dehydration results in greater stabilization of the collagen network in films. These results also indicate that a processor would be able to significantly modify the mechanical properties of their casings by altering the concentration or time exposed to brine (independently or not) and help processors optimize their process.
Figure 3.1 Mechanical properties of collagen films produced with increasing concentration of sodium chloride and contact time.
Table 3.2 Mechanical properties of Collagen 2 films produced with increasing concentration of sodium chloride. Means were averaged across contact times; 1.0, 2.5, 5.0, 10.0 min.

<table>
<thead>
<tr>
<th>Concentration wt.%</th>
<th>Tensile Strength$^1$ (MPa)</th>
<th>Percent Elongation$^1$ (%)</th>
<th>Distance to Break$^2$ (mm)</th>
<th>Work of Break$^2$ (Nmm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.23 c</td>
<td>21.0 a</td>
<td>4.0 a</td>
<td>2.2 b</td>
</tr>
<tr>
<td>20</td>
<td>0.26 bc</td>
<td>21.1 a</td>
<td>4.0 ab</td>
<td>2.4 b</td>
</tr>
<tr>
<td>25</td>
<td>0.32 b</td>
<td>21.3 a</td>
<td>3.8 b</td>
<td>2.4 b</td>
</tr>
<tr>
<td>30</td>
<td>0.40 a</td>
<td>22.7 a</td>
<td>3.7 b</td>
<td>2.8 a</td>
</tr>
</tbody>
</table>

$^1$Tensile Test
$^2$Puncture Test
$^3$Means, within a column, with same letter are not significantly different p > 0.05

Table 3.3 - Mechanical properties of collagen films produced with increasing contact times to sodium chloride. Means were averaged across concentrations; 15, 20, 25, 30wt.% sodium chloride in deionized water

<table>
<thead>
<tr>
<th>Time min</th>
<th>Tensile Strength$^1$ (Mpa)</th>
<th>Percent Elongation$^1$ (%)</th>
<th>Distance to Break$^2$ (mm)</th>
<th>Work of Break$^2$ (Nmm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.19 c</td>
<td>20.0 b</td>
<td>3.9 a</td>
<td>1.9 b</td>
</tr>
<tr>
<td>2.5</td>
<td>0.28 b</td>
<td>21.5 ab</td>
<td>4.0 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td>5.0</td>
<td>0.36 a</td>
<td>23.1 a</td>
<td>3.9 ab</td>
<td>2.8 a</td>
</tr>
<tr>
<td>10.0</td>
<td>0.39 a</td>
<td>21.4 ab</td>
<td>3.7 b</td>
<td>2.6 a</td>
</tr>
</tbody>
</table>

$^1$Tensile Test
$^2$Puncture Test
$^3$Means, within a column, with same letter are not significantly different p > 0.05

Studying the effects of the dehydration conditions also helped the researchers select the conditions for the analysis of the different commercial collagen dispersions. It was observed that films produced with 30wt.% brine had the highest tensile strength and work of puncture (p < 0.05), across all contact times (Table 3.2). The higher concentration was selected for further research because the additional strength would help researchers avoid damaging films prior to testing. Although the highest concentration was used in this research, processors must consider maintaining highly concentrated brines. Highly concentrated salt solutions reduce the processing time by increasing the osmotic drying of the collagen. It has been proposed that decreasing the exposure time can reduce off flavours that are caused by salt migration into the
meat. Unfortunately, over time, brines become less concentrated and contaminated, thus processors must consider the overhead costs (Kobussen and others 2012). The results from the dehydration analysis also indicated that there was not a significant difference ($p > 0.05$) in mechanical properties between 5 and 10 min (Table 3.3). The speed of co-extrusion is one of the major benefits of the technology. Thus it is in a producer’s best interest to reduce the brining time, thus a 5 min exposure time was chosen to evaluate the commercial collagen films. (Bontjer and others 2011).

When evaluating the different commercial collagen dispersions, it was observed that there were some significant differences ($p < 0.05$) in the tensile strength and percent elongation between samples (Table 4). Films produced with C4 had the lowest tensile strength and percent elongation of the unadjusted, protein films (0.15 MPa and 16.33%, respectively). These observations may be correlated to the collagen structure and dispersions composition.
Table 3.4 – Mechanical properties of dehydrated films: C1 (collagen 1), C2 (collagen 2), C3 (collagen 3), C4 (collagen 4) and C5 (collagen 5)

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Tensile Strength\textsuperscript{1} MPa</th>
<th>Percent Elongation\textsuperscript{1} %</th>
<th>Distance at Break\textsuperscript{2} mm</th>
<th>Work to Break\textsuperscript{2} J</th>
<th>Thickness \textsuperscript{3} mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0.33 ± 0.03 \textsuperscript{a}</td>
<td>24.38 ± 3.82 \textsuperscript{a}</td>
<td>6.12 ± 0.51 \textsuperscript{a}</td>
<td>3.72 ± 0.88 \textsuperscript{a}</td>
<td>0.43 ± 0.01 \textsuperscript{a}</td>
</tr>
<tr>
<td>C2</td>
<td>0.29 ± 0.05 \textsuperscript{ab}</td>
<td>18.57 ± 2.42 \textsuperscript{ab}</td>
<td>5.57 ± 0.41 \textsuperscript{a}</td>
<td>2.75 ± 0.48 \textsuperscript{ab}</td>
<td>0.37 ± 0.00 \textsuperscript{b}</td>
</tr>
<tr>
<td>C3</td>
<td>0.19 ± 0.02 \textsuperscript{cd}</td>
<td>19.22 ± 2.35 \textsuperscript{ab}</td>
<td>5.26 ± 0.47 \textsuperscript{a}</td>
<td>1.99 ± 0.41 \textsuperscript{b}</td>
<td>0.41 ± 0.01 \textsuperscript{a}</td>
</tr>
<tr>
<td>C4</td>
<td>0.15 ± 0.03 \textsuperscript{d}</td>
<td>16.33 ± 1.37 \textsuperscript{b}</td>
<td>5.33 ± 0.42 \textsuperscript{a}</td>
<td>1.89 ± 0.34 \textsuperscript{b}</td>
<td>0.41 ± 0.02 \textsuperscript{a}</td>
</tr>
<tr>
<td>C5</td>
<td>0.24 ± 0.02 \textsuperscript{bc}</td>
<td>23.42 ± 1.81 \textsuperscript{a}</td>
<td>5.91 ± 0.24 \textsuperscript{a}</td>
<td>1.81 ± 0.26 \textsuperscript{b}</td>
<td>0.42 ± 0.01 \textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Tensile Test
\textsuperscript{2}Puncture Test
\textsuperscript{3}Means, within a column, with same letter are not significantly different p > 0.05
The extraction of collagen into a pure fibrillar form can be accomplished with a number of different methods. Once fibrillar collagen has been extracted, it is suspended with water and acidified, with either organic or inorganic acids, to increase swelling and water binding. Intact fibrillar structures produce higher strength and elasticity in collagen casings. The lower tensile strength and elasticity could be from excessive alkaline modification during corium separation. If alkaline modification, or liming, is not controlled then the extracted collagen may be of low molecular weight, which does not contribute to regeneration of collagen structures (Savic and Savic 2002).

Swollen collagen can also be modified with a number of other functional ingredients, including colourants, crosslinking agents (smoke condensate), fillers (cellulose fibers) and plasticizers (glycerol). The combination of dry matter collagen (commonly 3 to 8%) and other modifiers (can be 4 to 10%) typically results in dispersions with 3 to 25 wt.% dry matter (Kobussen and others 2000). With such a wide range in production methods and composition, it was expected that there would be differences between commercial samples. The concentration of cellulose in the films may have affected mechanical properties of the films. Mathew and others (2012) observed that physical entanglements of cellulose nanofibers can increase the tensile strength in dried collagen films. Therefore, film’s with higher tensile strength may have a slightly higher concentration of cellulose fibers.

3.4.2 Light Microscope Imaging

Light microscopy was used to identify and characterize the homogeneity and condition of fibers within the collagen dispersion. As previously discussed collagen dispersions are commonly mixtures of soluble and insoluble collagen as a result of the material’s origin and methods of extraction. Collagen dispersions were stained with Masson trichrome stain because it is frequently used to differentiate collagen from other proteins. It was our hope that differences in the relative condition of the collagen fibers would provide some indication of their mechanical and thermo-mechanical properties. Since cellulose is commonly added to casing, the samples were also stained with PAS to identify and characterize carbohydrates found in the dispersion (Carson 1997).

It was observed in the light microscope images that there were few observable differences between the collagen dispersions (Figure 3.2). Collagen C2 and C5 appeared to have a greater degree of small, circular pockets, which may be small gas bubbles that were not removed. This
could result from differences in consistency during degassing. Also, there were differences in the homogeneity of the collagen network as there are areas of varying stain intensity, throughout the network. C3 appeared to have the greatest homogeneity and required the highest work of extrusion (1.10 J/% protein). Therefore the work of extrusion may be attributed to the homogeneity. Helary and others (2009) reported that areas of non-homogeneity affect the mechanical properties of collagen hydrogels.
Figure 3.2 Light microscope images of commercial collagen dispersion: collagen 1 (A), collagen 1 under polarized light (B), collagen 2 (C), collagen 2 under polarization (D), collagen 3 (E), collagen 3 under polarized light (F), collagen 4 (G), collagen 4 under polarized light (H), collagen 5 (I), collagen 5 under polarized light (J). Black bar represents 100 μm.
All of the dispersions also appeared to have insoluble fibers, of similar size and morphology, suspended in the collagen network. The insoluble fibers were identified as to be cellulose because they had a similar ribbon-like morphology with twists down its length (Reddy and Yang 2005; Cranston and Gray 2008). Also, the fibers picked up the PAS stain, providing further evidence that these fibers are cellulose (Figure 3.3). The commercial collagen dispersions were reported to only have 0.5% cellulose fiber. If the insoluble fibers are cellulose, then it would appear, at first glance, that there may be a higher concentration than was reported. Since there does not appear to be major differences in the cellulose concentration, the differences in mechanical properties may not be attributed to the addition of cellulose. The stained material on the interface between the collagen matrix and cellulose appeared to be darker. This suggested that collagen might have developed interfacial interactions with the fibers (thought to be cellulose). Santana and others (2011) have suggested that soluble and insoluble collagen fibers provide stability to the interface of oil in water emulsions. The collagen matrix around the interface also appeared to pull off the insoluble fibers, which may be an artefact of sample preparation. This may have occurred from the dehydration steps, during sample preparation.

Figure 3.3 Light microscope image of a commercial collagen dispersion stained with Periodic Acid Schiff. Black bar represents 100 μm.

3.4.3 Thermo-mechanical Properties

The rheological tests demonstrated that between 30˚C and 40˚C all of the dispersions begin to display a rapid decrease in firmness (Figure 3.4). Helary and others (2009) noted that the protein concentration contributes to the rigidity of the collagen dispersion. In order to account for this, the mean thermograms were adjusted for protein concentration. It was observed that the collagen samples with higher pH (Table 3.1; C4 and C2) began to lose firmness at a higher temperature. This may be attributed to the fact that collagen undergoes significant conformational change as one lowers the pH from 5 to 2. As previously stated conformational
changes result in increased hydration and swelling, therefore it may have reduced the thermal stability of the dispersion (Wolf et al 2006).

![Rheological thermographs](image)

**Figure 3.4** Rheological thermographs (20 to 55°C at 1.25°C/min) of five, commercial collagen dispersion: C1 (collagen 1), C2 (collagen 2), C3 (collagen 3), C4 (collagen 4) and C5 (collagen 5).

DSC scans were also performed on the collagen dispersions and dehydrated films. The collagen dispersions exhibited an endothermic peak that started between 33.5 to 35.4°C, with a maximum temperature of 36.7 to 38.9°C and had a denaturation enthalpy of approximately 3.1 to 5.3 J/g (Table 3.5). Once the initial run was completed the samples were given an opportunity to cool to 4°C before a secondary run was completed to look for reversible and non-reversible changes. The second run of all dispersions resulted in no endothermic peaks. This suggests that irreversible denaturation occurred, which is similar to Friess and Lee’s (1996) observations of insoluble collagen fibers.
Table 3.5 Analysis of endothermic peaks from differential scanning calorimetry (DSC) thermograms. Five commercial collagen samples were tested in extracted collagen dispersion and partially dehydrated film form: C1 (collagen 1), C2 (collagen 2), C3 (collagen 3), C4 (collagen 4) and C5 (collagen 5).

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Treatment</th>
<th>Onset Temperature °C</th>
<th>Temperature of Denaturation °C</th>
<th>Enthalpy ΔH J/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Dispersion</td>
<td>33.54 ± 0.21</td>
<td>36.71 ± 0.51</td>
<td>5.33 ± 0.61</td>
</tr>
<tr>
<td>C2</td>
<td>Dispersion</td>
<td>34.59 ± 0.15</td>
<td>38.44 ± 0.06</td>
<td>3.05 ± 0.31</td>
</tr>
<tr>
<td>C3</td>
<td>Dispersion</td>
<td>34.26 ± 0.01</td>
<td>38.09 ± 0.08</td>
<td>4.12 ± 0.10</td>
</tr>
<tr>
<td>C4</td>
<td>Dispersion</td>
<td>35.41 ± 0.11</td>
<td>38.94 ± 0.02</td>
<td>3.93 ± 0.26</td>
</tr>
<tr>
<td>C5</td>
<td>Dispersion</td>
<td>33.45 ± 0.10</td>
<td>37.30 ± 0.21</td>
<td>4.45 ± 0.03</td>
</tr>
<tr>
<td>C1</td>
<td>Dehydrated Film</td>
<td>59.90 ± 0.23</td>
<td>64.87 ± 0.12</td>
<td>3.07 ± 0.55</td>
</tr>
<tr>
<td>C2</td>
<td>Dehydrated Film</td>
<td>58.40 ± 0.21</td>
<td>63.88 ± 0.57</td>
<td>1.76 ± 0.38</td>
</tr>
<tr>
<td>C3</td>
<td>Dehydrated Film</td>
<td>60.32 ± 1.61</td>
<td>65.00 ± 0.68</td>
<td>3.05 ± 0.21</td>
</tr>
<tr>
<td>C4</td>
<td>Dehydrated Film</td>
<td>58.22 ± 0.24</td>
<td>63.94 ± 0.61</td>
<td>3.06 ± 0.82</td>
</tr>
<tr>
<td>C5</td>
<td>Dehydrated Film</td>
<td>58.30 ± 0.40</td>
<td>65.34 ± 0.37</td>
<td>4.19 ± 0.37</td>
</tr>
</tbody>
</table>
Similar to the rheological observations, the collagen with lower pH displayed lower thermal stability in the DSC thermograms. The dispersions with a pH closer to 2 (Collagen C1, C3 and C5) appeared to have slightly lower temperatures of denaturation. Once again, conformational changes at a lower pH may result in a greater hydration (Telis and others 2006). Gioffre and others (2011) observed similar thermal denaturation behavior when the pH of wet gelatin films was decreased.

DSC was performed on dehydrated films to see if there was any effect on the thermal stability of the collagen. It was observed that dehydrating the films increased the stability. The thermograms of the films showed an endothermic peak that started between 58.2 to 60.3°C, with a maximum temperature of 63.9 to 65.3°C and a denaturation enthalpy of approximately 1.8 and 4.2 J/g (Table 3.5). These denaturation temperatures are fairly similar to Bernal and Stanley (1986) intact bovine tendon, which was reported to have a temperature of denaturation 61.55°C. Gioffre and others (2011) also observed an increase in the denaturation temperature when gelatin films were dried. The assembly of fibers may help explain the increased in thermal stability of dehydrated films. McPherson and others (1986) suggested that stronger association of collagen fiber structures is correlated to increased denaturation temperatures. It has been demonstrated that high ionic strength conditions resulted in a greater degree of packed collagen fibers and assembly (Williams and others 1978). Since the ionic strength is increased during film dehydration (migration of brine salts into film), there may be collagen fiber assembly, resulting in increased in thermal stability of the films.

3.4.4 Optical Transmission

The degree of light transmission through a collagen film governs consumer’s ability to evaluate the product (Savic and Savic 2002). Light transmission measurements were evaluated in the visible light spectrum in order to evaluate the transparency of the dehydrated films (Figure 3.5). Between the collagen films, it was apparent that C5 was the least transparent and C2 was the most. Light transmission is affected by the composition and thickness of each casing (Savic and Savic 2002). The lower transmission could have been a result of a significantly thinner film thickness than some of other dehydrated films (Table 3.4). C2 was also lower in protein content (Table 3.1), which may have also affected the transparency. This is because a lower concentration of collagen fibers would result in less light scattering.
3.5 Conclusion

Manipulating the dehydration conditions (brine concentration; 15, 20, 25, 30 wt.% sodium chloride in deionized water and contact time; 1.0, 2.5, 5.0, 10.0 min) has demonstrated that there are significant differences ($p < 0.05$) in mechanical properties when the brine concentrations were averaged across contact times and contact times were averaged across all concentrations. This allows manufactures to adjust the performance of their casings through the modification of either brine concentration or contact time.

The results also show differences between selected commercial collagen dispersions on the market and provide some actual values, as well as some potential explanation for the differences, that have not been reported before. The mechanical evaluation of dispersions and films demonstrated that there were may be differences in flow behavior as well as significant differences between the dehydrated film’s tensile strength and percent elongation. It was suggested that intact fibers (cross-striated) might give a film higher tensile strength and elasticity. Collagen dispersions with pH values closer to 2 may exhibit lower thermal stability, as
conformational changes in the fiber structure occur at lower pH. Furthermore, partially dehydrating collagen fibers may increase the temperature of denaturation.

It would appear that this research will provide manufacturers of co-extruded sausages with a better understanding of the conversion of extracted collagen into casings, through material selection and manipulation of brining operations.
3.6 References


Chapter 4 Investigation of the Mechanical, Microstructural and Thermo-mechanical Properties of Collagen Films Cross-linked with Smoke Condensate and Glutaraldehyde

4.1 Abstract

Collagen films were produced from five commercially manufactured collagen dispersions used for in line sausage casing production. Films were rolled and stabilized by partially dehydrating and cross-linking the collagen with smoke condensate (as used by the industry) or glutaraldehyde. There were significant decreases (p < 0.05) in the percent elongation and distance of break when films were cross-linked with different concentrations of glutaraldehyde (0.1, 0.5, or 1.0 vol.% glutaraldehyde in 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 7.4). There were some significant differences (p < 0.05) in tensile strength and elongation between films produced with different commercial materials. Transmission electron microscopy (TEM) imaging revealed that collagen fibers were swollen to varying degrees, among the commercial dispersions, likely influencing the mechanical behaviours of the films. Thickness and protein concentration appeared to have the greatest effect on the transparency of films. Cross-linking with GA appeared to thermally stabilize films up to 80°C.

4.1 Introduction

Sausage products rely on casings to contain, form and bind comminuted meat for further processing, shipping and merchandising. Prior to the early 20th century almost all casings were derived from animal intestines. Since then, advancements in casing technology and manufacture have led to several types of synthetic casings that they can be tailored to a given process, regenerated biopolymers (collagen and cellulose). With increasing sophistication, the production of synthetic casings was moved to designated manufacturing facilities, until fairly recently, when co-extrusion was commercialized (Karmas 1974; Savic and Savic 2002)

Co-extrusion is the process of extruding a cylindrical core of sausage meat, while simultaneously extruding an outer layer of casing material on to the meat’s surface. Subsequent processes, brining, smoking, drying and cooking, stabilize and harden the casing material by osmotically removing of water and chemically cross-linking. Stabilizing the casing material is a necessary process, as casings require the strength and elasticity to undergo linking, packaging and storage.
Since casings are formed directly on the meat’s surface, co-extrusion systems must use a suitable brining and cross-linking agent that is food grade and will not negatively affect the product (Morgan and others 1998). Smoke condensates are commonly used as chemical cross-linking agents in the co-extrusion of collagen casings, as they form covalent linkages between collagen molecules and fibers. Exposure to smoke condensates improve the mechanical properties of the casing, while also providing the sausage with colour, flavour and act as a preservative. In co-extrusion, smoke condensates are favoured over traditional smoking because of their increased uniformity of colour and flavour (regulated specifications), health (safer and fewer carcinogens) and cleanliness (reducing labour costs) (Morgan and others 1998; Bontjer and others 2011). Smoke condensates gain their functionality as a result of byproducts of combustion: aldehydes, ketones, furans, phenols, acids (Toledo 2007).

Glutaraldehyde is a common aldehyde cross-linking agent that has gained commercial acceptance as a leather tanning agent and tissue fixative (Covington 1997, Cheung and Nimni 1982). Glutaraldehyde is also used as a cross-linking agent in the production of dry collagen sausage casings produced in dedicated casing processing plants and not for in-line co-extrusion at a meat processing plant (Morgan and others 1998). Glutaraldehyde is able to form stable bonds and can significantly increase the mechanical properties of collagen fibers. The mechanism of glutaraldehyde’s reactions has been an area of interest to help improve the modification of collagenous material (Cheung and Nimni 1982). In short, it has been suggested that glutaraldehyde and its derivatives react with amine groups, specifically lysine, to form heterocyclic compounds. Subsequent oxidation reactions produce pyridine rings (Figure 4.1). Although glutaraldehyde is a highly functional crosslinking agent, cytotoxic effects have limited its application in the food industry and especially semi-liquid casing application as used in co-extrusion. Glutaraldehyde is more commonly employed in manufactured dry casing production because residues can be thoroughly washed out of the casing in the dedicated manufacturing plant. (Avery and Baily 2008; Savic and Savic 2002).

![Glutaraldehyde reaction](image)

**Figure 4.1** Proposed reaction of Glutaraldehyde and collagen – adapted from Englert and others (2007)
There has been a substantial effort to understand the properties of collagenous materials and the effects of different cross-linking agents (O’Sullivan and others 2006; Telis and others 2006; Tomihata and others 1994; Olde Damik and others 1995). However, little has been published about the effectiveness of cross-linking agents on commercial collagen products. This study was designed to address this area of research in two steps. First, the effect of the smoke condensate and glutaraldehyde concentration and contact time was examined through the mechanical properties of collagen films. This may provide manufacturers with an insight into how to manipulate the mechanical properties of co-extrusion collagen casings. The second step examined the mechanical, microstructural and thermal properties of cross-linked films to compare the differences in functionality of commercial collagen dispersions.

4.3 Materials and Methods

4.3.1 Study I – Manipulation of Cross-linking Conditions

4.3.1.1 Preparation of films

The commercial collagen dispersions were degassed using a Multivac vacuum packager (Multivac Canada Inc., Woodbridge, ON, Canada) at 7.3 kPa for 25 s then again at 7.3 kPa for 50 s and 75 s (settings 4, 6 and 8, respectively). This was performed to remove gas bubbles that were incorporated during processing. Following the degassing stage, dispersions were mixed to improve the homogeneity of the samples. While still in the vacuum pack bags, the dispersions were mixed by rolling the dispersions 10 times in adjacent directions. It is important to note that commercial collagen dispersions were prepared under different conditions. In this study, the researchers did not control the raw materials and methods that were used to prepare the collagen dispersions. Other than degassing and mixing, the commercial products did not undergo further preparation, prior to film formation. These decisions were made in order to accurately report the actual differences between the materials used in modern sausage casing production.

The method of film formation was adapted from Harper and others (2013) work with alginate solutions. Collagen dispersions were first cooled to 4°C to reduce the adhesion during the film formation. Approximately 3 g portions of the collagen were rolled on a stainless steel board between two layers of plastic with a stainless steel roller. The roller had a recess of 0.50 mm in order to achieve uniform film thickness. The plastic sheet on the roller side of the film was removed and the remaining plastic sheet with the film on it was then placed in a 30 wt.% sodium
chloride in deionized water for 5 min, in order to dehydrate the film. After 5 min the film was strong enough to hold together when removed from the plastic sheet. The plastic sheet was folded onto the formed film to prevent further dehydration of the film before it was tested.

Dehydrated films were cross-linked with smoke condensate (Charsol Select 24P liquid smoke Red Arrow Products, Manitowoc, WI, USA) or glutaraldehyde (EM Grade, Canemco, Canton de Gore, Quebec, Canada). The films were immersed in a 15 vol.% smoke condensate in deionized water, based on industry recommendation. Films were held in the diluted smoke condensate bath for 10, 20, 40 or 80 sec intervals. Following cross-linking, films were covered with plastic films to avoid drying before testing.

Glutaraldehyde was used because its mechanism of cross-linking is better understood, thus its effects could be used as a standard. Films were cross-linked in solutions of 0.1, 0.5 and 1.0 vol.% glutaraldehyde in 1M 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid HEPES buffer at pH 7.4 (Fisher Scientific, Ottawa, ON, CA). Dehydrated films were immersed in the glutaraldehyde cross-linking solution for 5, 10 or 20 min intervals, followed by a 5 min rinse in water to remove residues. Similar to cross-linking with smoke condensate, the films were covered with plastic to avoid drying before testing.

4.3.1.2 Mechanical properties

The test used to evaluate the films was a standard tensile test (ASTM-D882) using a texture analyzer (TA-XT2i, Texture Technologies Corporation, Scarsdale, NY, USA) with a gripper distance that was set at 50 mm, trigger force at 5 g, test speed at 2 mm/s and the test distance at 25 mm. Three thickness measurements (top, center and bottom) were taken using a digital micrometer (Testing Machines Inc., Islandia, NY, USA). The three measurements were used to calculate an average thickness of each film. The films were cut into 75 mm × 25 mm strips (JDC Precision Sample Cutter, Thwang-Albert Instrument Comp, Philadelphia, PA, USA). The average thickness and width of the films were used for the tensile stress calculations. From the generated stress–strain curve, the tensile strength (maximum stress the film endured prior to breaking) and the percent elongation (the maximum elongation the film reached prior to breaking) were determined. A total of eighteen films were tested for each of the treatments (six films per trial).

The puncture strength of the films was also examined using the texture analyzer. In this test, a 5 mm ball probe was used to puncture films mounted in an extensibility fixture with 10 mm
diameter, round openings (TA-108S5, Texture Technologies, Corporation, Scarsdale, NY, USA). The test speed was 1 mm/s and the trigger force was 5 g. The distance to puncture and work of puncture were determined from the generated force–distance graph. A total of eighteen measurements were used for each of the treatments (six measurements per trial).

4.3.1.3 Experimental design and statistical analysis

The experiment was designed as a completely randomized block with 3 independent trials. Each trial, consisted of six sub-sample measurements, for each of the tests (tensile and puncture), which were averaged. The statistical analysis was performed using SAS Version 9.2 (SAS Inst., Cary, NC, USA). A General Linear Model was used for the analysis of variance (ANOVA). The film type means and interactions were compared by using Tukey’s multiple comparison analysis with a P-value ≤ 0.05, which was used to detect statistical significance.

4.3.2 Study II – Evaluation of Cross-linked Commercial Materials

4.3.2.1 Ingredients for film formation

Five commercial collagen dispersions were evaluated. The dispersions were labeled Collagen 1 through 5 (C1, C2, C3, C4, C5). Information that was provided to the researchers and compositional analysis can be found in Table 3.1. Protein content was determined by the Dumas method (Leco FP528, St Joseph, MI, USA) using a nitrogen factor of 6.25.

4.3.2.2 Preparation of films

The commercial collagen dispersions were degassed, mixed, formed and dehydrated, using the methods described in 4.3.1. The cross-linking materials were from the same sources in 4.3.1. Dehydrated films cross-linked with smoke condensate were immersed in a 15 vol.% smoke condensate in deionized water, for 40 sec. Alternatively, dehydrated films were immersed in 0.5 vol.% glutaraldehyde in 1M Hepes buffer (pH 7.4) for 5 min, followed by a 5 min rinse. Cross-linked, films were covered with plastic to avoid drying before testing.

4.3.2.3 Mechanical properties and film thickness

Refer to the mechanical tests used in section 4.3.1.2 for the methods for the tensile and puncture tests.

Tensile and puncture tests were performed to determine the tensile strength, percent elongation, distance to puncture and work to puncture.
4.3.2.4 Transmission electron microscopy of collagen films

Transmission electron microscopy (TEM) was performed on collagen films, raw collagen and cellulose controls. The films were fixed in 2.0% glutaraldehyde, buffered with HEPES (pH 7.4) for 90 min. This was followed by osmium tetroxide stain and dehydration through a series of graded ethanol solutions (50%, 70%, 80%, 90%, and 100%), each for 10 min. Once the films were dehydrated, the films were run through a series of propylene oxide and Spurr’s resin solutions (1:0, 3:1, 1:1, 1:3 and 0:1) to ensure that the resin was thoroughly incorporated, prior to embedding. The resin was polymerized for 24 hr at 60°C. The samples were cut into thin slices of 70 to 90 nm on a microtome (Reichert Ultracut S, Leica Microsystems Inc, Concord, ON, CA), fixed on grids, stained with saturated uranium acetate and lead citrate (Hayat 2000).

Negative staining was used to prepare raw collagen and cellulose controls. The collagen control was prepared by hydrating raw hide (Summma, Jesus Maria, Mexico) for 24 h at 23°C to soften the hide. The pH was lowered to 2 and blended to aid in dispersion and fiber swelling. Finally, the blended material was diluted with deionized water to 0.1 wt.% of the original raw hide. The cellulose control was a 0.1 wt.% solution of powdered cellulose (Arbocel, JRS, Schoolcraft, MI, USA). One drop of the control solution was placed on a formvar-coated grid. After 30 seconds, the excess solution was removed with filter paper. A drop of 2% uranium acetate was then applied to stain the sample that was deposited on the formvar grid. Finally, after 30 seconds the excess uranium acetate was removed with filter paper and the grids were dried on the bench top.

The samples were examined on a TEM (Philips CM 10, Philips Scientifics, Eindhoven, Netherlands) and photographed (Olympus Morada camera, Olympus Soft Imaging System, Berlin, Germany) using iTEM imaging software (Item Software, Whiteley Hampshire, UK).

4.3.2.5 Optical property

The light transmission of the films (380–780 nm) was evaluated by using a single beam spectrophotometer (USB 2000, Ocean Optics Inc., Dunedin, FL, USA). The following settings were used: integration time: 100 ms; scans to average 2; and boxcar width 4. The light transmission was measured on twelve films per collagen sample.
4.3.2.6 Mechanical Properties of Thermally Treated Films

Cross-linked films were mounted in the puncture fixture and placed in a plastic bag. The fixture and bag were lowered into a water bath at 40, 50, 60, 70 or 80°C and held for 15 min. The puncture test was performed immediately after thermal treatment. The puncture equipment, parameters and methods of analysis (4.3.1.2) were used to measure the distance of puncture and work of puncture of thermally treated films. The films that were tested in this section were prepared using collagen sample C2, under the same conditions that were used to prepare cross-linked films, with smoke condensate and glutaraldehyde. Once again, a total of eighteen measurements were used for each of the thermal treatments (six measurements per trial).

4.3.2.7 Experimental design and statistical analysis

The experiment was designed as a completely randomized block with 3 independent trials. Each trial, consisted of six sub-sample measurements, for each of the tests (tensile and puncture), which were averaged. The statistical analysis was performed by using SAS Version 9.2 (SAS Inst., Cary, NC, USA). A General Linear Model was used for the analysis of variance (ANOVA). The film type means were compared by using Tukey’s multiple comparison analysis with a P-value ≤ 0.05, which was used to detect statistical significance.

4.4 Results and Discussion

4.4.1 Study I – Manipulation of Cross-linking Conditions

4.4.1.1 Mechanical Properties

The results of the tensile test demonstrated that there were no significant differences (p > 0.05) in the tensile stress and strain, when the films were exposed to smoke condensate for 10 to 80 s (Figure 4.2). This indicates that a manufacturer would not be able to significantly modify the tensile properties of their casings by increasing the contact time, within this range (10 to 80 sec). The concentrations used were based of industry recommendation but if higher concentrations resulted in the formation of more cross-links, increase the concentration of smoke condensate may have resulted in significant differences in the mechanical properties.

Although the films were not significantly different (p > 0.05) from one another, the mean tensile strength and percent elongation appeared to increase slightly with the time of exposure. These observations suggest that intramolecular bonds were being formed between collagen molecules and fibers, with increasing contact time. The formation of covalent cross-links would prevent
collagen fibers from sliding past each other, thus reducing the film’s ability to undergo deformation (Rault and others 1996, Paul and Bailey 2003).

![Figure 4.2](image)

**Figure 4.2 Mechanical properties of collagen films produced with increasing exposure times to smoke condensate (15 vol.% in deionized water). Means with the same letter are not significantly different p > 0.05.**

It should be acknowledged that this study was performed with one type of smoke condensate, commonly used in co-extrusion applications. The composition of smoke condensates can differ, because of the presence and proportion of major (cellulose, hemicellulose and lignin) and minor compounds (terpenes, fatty acids, other carbohydrates, polyhydric alcohols, nitrogen and phenols) in the fuel source, as well as variations that are derived from availability of oxygen and moisture during smoke generation (Guillen and Manzanos 1999; Montazeri and others 2013). Thus, the results of this study may not be indicative of the effects of all smoke types. Smoke condensates can differ in composition because of the method of production and the type of wood used. These differences in the composition are attributed to the presence and proportion of the compounds in wood, availability of oxygen during smoke generation and the age of a smoke condensate. (Guillen and Manzanos 1999). Since aldehyde compounds are linked to smoke’s cross-linking properties, variations in their concentration could produce different results of contact time on tensile properties.

The mechanical properties of the glutaraldehyde-treated films (Table 4.2) indicated that there were no significant interactions (p > 0.05) between the concentration of glutaraldehyde and exposure time. Furthermore, the results suggested that there was no significant difference (p > 0.05) in the percent elongation and puncture properties, between 5 and 20 min films (Figure 4.3). Thus, exposure time appears to have a greater effect on tensile stress when cross-linking
with glutaraldehyde. This difference could be attributed to the concentration of cross-linking agent or the interactions of other compounds found in smoke condensate.
Table 4.1 Mechanical properties of collagen films produced with increasing exposure time and concentration of glutaraldehyde.

<table>
<thead>
<tr>
<th>Concentration vol.%</th>
<th>Time min</th>
<th>Tensile Strength Mpa</th>
<th>Percent Elongation %</th>
<th>Distance at Break mm</th>
<th>Work of Break Nmm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5</td>
<td>0.564 ± 0.14</td>
<td>22.475 ± 2.11</td>
<td>3.012 ± 0.26</td>
<td>1.537 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.765 ± 0.09</td>
<td>23.213 ± 1.72</td>
<td>2.911 ± 0.31</td>
<td>1.590 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.833 ± 0.12</td>
<td>20.741 ± 0.96</td>
<td>2.787 ± 0.35</td>
<td>1.566 ± 0.31</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>0.502 ± 0.06</td>
<td>20.137 ± 1.21</td>
<td>2.517 ± 0.24</td>
<td>1.068 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.664 ± 0.02</td>
<td>20.381 ± 1.43</td>
<td>2.338 ± 0.44</td>
<td>1.058 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.577 ± 0.05</td>
<td>19.661 ± 3.28</td>
<td>1.952 ± 0.28</td>
<td>0.672 ± 0.19</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0.708 ± 0.08</td>
<td>20.921 ± 2.96</td>
<td>2.071 ± 0.63</td>
<td>1.630 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.680 ± 0.15</td>
<td>15.877 ± 2.80</td>
<td>1.784 ± 0.72</td>
<td>1.276 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.764 ± 0.16</td>
<td>17.591 ± 3.68</td>
<td>1.634 ± 0.59</td>
<td>1.135 ± 0.69</td>
</tr>
</tbody>
</table>
Figure 4.3 Mechanical properties of collagen films produced with increasing contact times to glutaraldehyde and concentration of glutaraldehyde. The contact time means were averaged across all concentration (0.1, 0.5, 1.0 vol.%) and the concentration means were averaged across all contact times (5, 10 and 20 min). Means in the same graph with same letter are not significantly different p > 0.05.
The results of the study also suggested that manipulating the concentration of glutaraldehyde will have some significant differences (p < 0.05) on the tensile and puncture properties of the collagen films. The most notable effect of increasing the concentration (0.1 to 1.0 vol.%) was the significant decrease (p < 0.05) in percent elongation and distance to break (Figure 4.3). These observations (decrease in percent elongation and distance to break, as a result of cross-linking) are consistent with those discussed by Avery and Bailey (2008). It would appear that over cross-linking would make the films brittle by inhibiting deformation.

4.4.2 Study II – Evaluation of Cross-linked Commercial Materials

4.4.2.1 Mechanical Properties

The mechanical properties of casings play a crucial role in sausage production. They affect sausages structural integrity, shape, volumetric changes, textural properties, and behavior during processing (Savic and Savic 2002). For example, during traditional production, casings must withstand tensile stresses during stuffing, hanging in the smokehouse and cooking. In addition, casings must provide compressive strength during meat gelation and exhibit elasticity during shrinkage (Savic and Savic 2002). Examining properties like percent elongation, tensile strength, can help to predict the success of a casing.

It was observed that there were some significant differences (p < 0.05) in the various collagen films tensile strength and percent elongation (Table 4.3). Films produced with C4 had the lowest tensile strength and percent elongation (0.15 MPa and 16.33%, respectively). A greater degree of native and intact fibrillar structures produce higher strength and elasticity in collagen casings (Savic and Savic 2002). The collagen in C4 may have undergone further degradation during processing, resulting in its lower tensile properties. For instance, excessive mechanical or alkaline modification during corium separation could have resulted in greater degradation of native fibers (Savic and Savic 2002). The influence of protein content on mechanical properties was also investigated (Table 4.4) to determine if protein content or quality contributed more to the mechanical properties of films. When comparing adjusted and unadjusted mechanical properties, there appeared to be few differences in the trends between the collagen samples. From these results, the mechanical properties of films may be more dependent on the quality of protein, rather than protein content.
<table>
<thead>
<tr>
<th>Collagen</th>
<th>Cross-linker(^1)</th>
<th>Tensile Strength(^2) MPa</th>
<th>Percent Elongation(^2) %</th>
<th>Distance at Break(^3) mm</th>
<th>Work to Break(^3) mJ</th>
<th>Thickness mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>SC</td>
<td>0.67 ± 0.04 (a)</td>
<td>24.80 ± 1.23 ab</td>
<td>3.85 ± 0.22 a</td>
<td>2.75 ± 0.48 a</td>
<td>0.35 ± 0.01 ab</td>
</tr>
<tr>
<td>C2</td>
<td>SC</td>
<td>0.53 ± 0.02 (ab)</td>
<td>26.32 ± 1.18 a</td>
<td>3.39 ± 0.18 a</td>
<td>1.52 ± 0.14 a</td>
<td>0.30 ± 0.01 b</td>
</tr>
<tr>
<td>C3</td>
<td>SC</td>
<td>0.38 ± 0.05 (b)</td>
<td>22.41 ± 0.78 abc</td>
<td>3.21 ± 0.30 a</td>
<td>1.24 ± 0.34 a</td>
<td>0.34 ± 0.01 ab</td>
</tr>
<tr>
<td>C4</td>
<td>SC</td>
<td>0.32 ± 0.07 (b)</td>
<td>21.37 ± 0.99 bc</td>
<td>3.27 ± 0.35 a</td>
<td>1.23 ± 0.31 a</td>
<td>0.36 ± 0.02 a</td>
</tr>
<tr>
<td>C5</td>
<td>SC</td>
<td>0.39 ± 0.16 (b)</td>
<td>18.81 ± 2.08 c</td>
<td>2.59 ± 0.04 a</td>
<td>0.85 ± 0.10 a</td>
<td>0.38 ± 0.03 a</td>
</tr>
<tr>
<td>C1</td>
<td>GA</td>
<td>0.91 ± 0.17 (d)</td>
<td>26.26 ± 4.65 d</td>
<td>2.77 ± 0.52 d</td>
<td>1.79 ± 0.57 d</td>
<td>0.36 ± 0.03 d</td>
</tr>
<tr>
<td>C2</td>
<td>GA</td>
<td>0.66 ± 0.02 (e)</td>
<td>20.38 ± 1.43 d</td>
<td>2.34 ± 0.44 d</td>
<td>1.06 ± 0.37 d</td>
<td>0.45 ± 0.01 d</td>
</tr>
<tr>
<td>C3</td>
<td>GA</td>
<td>0.41 ± 0.13 (f)</td>
<td>18.95 ± 2.56 d</td>
<td>2.66 ± 0.39 d</td>
<td>1.35 ± 0.27 d</td>
<td>0.38 ± 0.05 d</td>
</tr>
<tr>
<td>C4</td>
<td>GA</td>
<td>0.61 ± 0.20 (ef)</td>
<td>24.18 ± 3.72 d</td>
<td>2.74 ± 0.29 d</td>
<td>1.52 ± 0.11 d</td>
<td>0.38 ± 0.04 d</td>
</tr>
<tr>
<td>C5</td>
<td>GA</td>
<td>0.60 ± 0.18 (ef)</td>
<td>22.04 ± 2.60 d</td>
<td>2.87 ± 0.60 d</td>
<td>1.91 ± 0.76 d</td>
<td>0.39 ± 0.05 d</td>
</tr>
</tbody>
</table>

\(^1\)Smoke Condensate (SC), Glutaraldehyde (GA)  
\(^2\)Tensile test  
\(^3\)Puncture test  
\(^4\)Means in the columns with same letter are not significantly different \(p > 0.05\)  
\(^5\)a-c liquid smoke films, d-e glutaraldehyde films
Table 4.3 Protein adjusted mechanical properties of cross-linked films: C1 (collagen 1), C2 (collagen 2), C3 (collagen 3), C4 (collagen 4) and C5 (collagen 5)

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Cross-linker</th>
<th>Tensile Strength</th>
<th>Percent Elongation</th>
<th>Distance at Break</th>
<th>Work to Break</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MPa / %protein</td>
<td>% / %protein</td>
<td>mm / %protein</td>
<td>mJ / %protein</td>
</tr>
<tr>
<td>C1</td>
<td>SC</td>
<td>0.092 ± 0.01</td>
<td>3.36 ± 0.17</td>
<td>0.38 ± 0.07</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>C2</td>
<td>SC</td>
<td>0.101 ± 0.00</td>
<td>4.98 ± 0.23</td>
<td>0.45 ± 0.08</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>C3</td>
<td>SC</td>
<td>0.062 ± 0.01</td>
<td>3.67 ± 0.13</td>
<td>0.44 ± 0.06</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>C4</td>
<td>SC</td>
<td>0.056 ± 0.01</td>
<td>3.68 ± 0.17</td>
<td>0.47 ± 0.05</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>C5</td>
<td>SC</td>
<td>0.058 ± 0.02</td>
<td>2.77 ± 0.31</td>
<td>0.42 ± 0.09</td>
<td>0.28 ± 0.11</td>
</tr>
<tr>
<td>C1</td>
<td>GA</td>
<td>0.140 ± 0.03</td>
<td>4.05 ± 0.72</td>
<td>0.43 ± 0.08</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>C2</td>
<td>GA</td>
<td>0.131 ± 0.00</td>
<td>4.02 ± 0.28</td>
<td>0.46 ± 0.09</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>C3</td>
<td>GA</td>
<td>0.077 ± 0.03</td>
<td>3.59 ± 0.48</td>
<td>0.50 ± 0.07</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>C4</td>
<td>GA</td>
<td>0.120 ± 0.04</td>
<td>4.77 ± 0.73</td>
<td>0.54 ± 0.06</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>C5</td>
<td>GA</td>
<td>0.118 ± 0.04</td>
<td>4.34 ± 0.51</td>
<td>0.57 ± 0.12</td>
<td>0.38 ± 0.15</td>
</tr>
</tbody>
</table>

1Smoke Condensate (SC), Glutaraldehyde (GA)  
2Tensile test  
3Puncture test  
4Means in the columns with same letter are not significantly different p > 0.05  
5a-c liquid smoke films, d-e glutaraldehyde films
Another factor that may have led to differences in mechanical properties was the concentration of cellulose in the films. Mathew and others (2012) observed that interactions between cellulose nanofibers and collagen significantly increased the strength and strain break of dry collagen films. Although previous work (Chapter 3) did not confirm differences in the cellulose content, the concentration of cellulose and interactions may have contributed to differences in mechanical properties.

Cross-linking films appeared to have dramatic effects on the mechanical properties of dehydrated films. Cross-linking with smoke condensate or glutaraldehyde appeared to increase the tensile strength of collagen films by approximately 100% and 200% of their original value, respectively (Chapter 3, Table 3.4) In addition, the distance to break was reduced by 30% and 60%, when cross-linked with smoke condensate and glutaraldehyde, respectively. As suggested by others (Avery and Bailey 2008; Covington 1997; Paul and Bailey 2003) cross-links increase the mechanical strength of fibers by preventing the molecules and fibers from sliding past each other, creating a stiff but brittle network in the films.

There also appeared to be differences in the mechanical properties between films cross-linked with smoke condensate and glutaraldehyde. Films cross-linked with glutaraldehyde appeared to have greater tensile strength than those cross-linked with liquid smoke (Table 4.3). Smoke condensate is composed of hundreds of compounds, therefore this observation can likely be attributed to the purity of the cross-linking agent (Toledo 2007). The pH of cross-linking solvents may have also affected the mechanical properties of the films. Morgan and others (1998) suggested that neutralizing the acidic collagen dispersion would result in water loss. Similar to dehydrating films with brine, the removal of water would improve the stability of the collagen structure. The smoke condensate was not buffered (pH 3.5) to simulate the processes used in industry where as the glutaraldehyde was buffered (pH 7.4). Thus the buffered solution increased the neutralization of the collagen gels, in turn, improving the stabilization.

One final observation was that visually inspecting punctured films provided some evidence that the rolling method orientated the collagen fibers. As the films failed, the probe consistently split the films parallel to the roller direction. Directional variations in shear strength have been observed and documented in manufactured collagen casings (Harper and others 2012).
4.4.2.2 Electron Microscope Imaging

The collagen fibers also appear to have varying degrees of swelling or hydration (Figure 4.4). Generally in tendon tissue, collagen fibrils are long, slender and cylindrical structures but can differ in length, diameter, uniformity, and telopeptide size, as a result of collagen type and interactions (Wess 2008; Cameron and others, 2002). The alignment of collagen molecules in a staggered array conformation develops areas of overlap and gap. The overlap and gap regions of the parallel arrays give collagen fibrils their distinctive striated pattern with a periodicity of 640-700 Å (Wess 2008). The control collagen dispersion was imaged to compare the likeness of the banded fibers (Figure 4.5A). It appeared that, at this magnification, the banded structures were indicative of collagen fibers. In addition, the bulk material shares similar characteristics to the unbanded collagen, presented by Meyer and others (2005).
Figure 4.4 TEM images of collagen film C1 (A), C2 (B), C3 (C), C4 (D), C5 (E); C1 (collagen 1), C2 (collagen 2), C3 (collagen 3), C4 (collagen 4) and C5 (collagen 5). Black bar represents 1 μm.
Although some collagen fibers display a banding pattern or cross-striations, which is typical of native collagen, the majority of the fibers in the current work appear to be swollen. Since commercial collagen dispersions are extracted from hides that have varying degree of natural cross-linking, the degree of natural cross-links in collagenous tissues varies. Variations are a result of the age of animal, growing conditions and activity. There is an increase in natural cross-links, with increasing age and therefore the solubility decreases (Meyer and others 2005). Thus the extraction process would result in varying degree of fiber degradation.

The collagen structures support the mechanical properties of the films because intact fibrillar structures produce higher strength and elasticity in collagen casings (Savic and Savic 2002). C4 appeared to have the most swollen or hydrated fibers, with little visible banded fibrils remaining. Since C4 generally had lower tensile strength and percent elongation one may presume that greater swelling and hydration during collagen extraction with acetic acid (i.e. C3 is from the same manufacturer and similar raw material, but treated with HCl) resulted in lower mechanical
properties. However, definite conclusions cannot be drawn as the orientation and plane at which the fibrils are viewed is not known with certainty.

Another observation from the micrographs was that there appears to be differences in the linearity of fibers. The fibers in C1 appear to be less linear than the other treatments. C1 also appears to have a higher concentration of fibers, which may have hindered alignment of the fibers during rolling. Although there may be differences in the raw materials, the linearity may also be attributed to the plane at which the samples are sectioned, during preparation. During the formation of commercial co-extruded casings, a counter rotating extrusion head applies shear forces, which orientates and elongates the collagen fibers. Orienting the fibers improves the mechanical properties of casing by reducing splitting (Bontjer and others 2011). Since the direction of the section cannot be confirmed, the orientation of the collagen fibers is inconclusive from Figure 4.4.

TEM imaging the films did not suggest significant differences in the concentration of cellulose in the raw materials. As previously discussed cellulose fibers are also commonly added to improve the mechanical properties of collagen casings (Savic and Savic 2002). To verify that the larger fiber structures were derived from cellulose, a cellulose control (Figure 4.5B) was imaged, demonstrating the difference in magnitude. An example of the cellulose fiber in the collagen network can be observed in Figure 4.5C. It would appear that the cellulose fibers are easily distinguished because of their magnitude and electron density.

4.4.2.3 Optical Transmission

The degree of light transmission through a collagen film affects consumer’s ability to evaluate the product (Savic and Savic 2002). Light transmission measurements were also taken in the visible light spectrum in order to evaluate the transparency of cross-linked collagen films (Figure 4.6). Among the collagen films, it was apparent that C5 was the least transparent and C2 was the most. Upon visual inspection, C5 appeared to be more opaque than other samples. The TEM images of the C5 films appeared to have a higher concentration of individual fibrils and sub-fibrils. C5 may have a lower transmission if these fibrils resulted higher light scattering. Light transmission is also effected by the composition and thickness of each casing (Savic and Savic 2002). C2 was on lower end of protein content, which may have also affected the transparency (Table 4.1).
Figure 4.6 The light transmission spectra (light range 380 to 780 nm) of cross-linked films; C1 (collagen 1), C2 (collagen 2), C3 (collagen 3), C4 (collagen 4) and C5 (collagen 5). A - films cross-linked with liquid smoke, B - films cross-linked with glutaraldehyde.
Films treated with liquid smoke tended to have lower transmission than the dehydrated (Chapter 3, Figure 3.5) and glutaraldehyde films. These observations were likely a result of smoke components that became deposited within the films structure. There were no trends between the transparency of dehydrated and glutaraldehyde treated films. This observation is inconsistent with Tanaka and others (2011), who observed that increased cross-linking results in lower transmission, when cross-linking wet collagen films with 1-ethyl-3-(3-dimethylaminopropyl) carbodimide and N-hydroxysuccimide. Covington (1997) also discussed how tanning with glutaraldehyde imparts a yellow-orange colour to the leather. Therefore, if this pigmentation was developed when cross-linking collagen films, it would be expected to lower the transmission of light.

4.4.2.4 Mechanical Properties of Thermally Treated Films

There were no measurements recorded from testing the films cross-linked with smoke condensate because the films became too fragile to be detected by the texture analyzer. As previously suggested the collagen in films cross-linked with smoke condensate may have remained more swollen and had higher water content. The higher water content may have resulted in lower thermal stability of these films. Miles and others (2005) support this explanation as their work suggested that the increased thermal stability is a result of the water content of fibers. They observed that cross-linking reduces the axial separation between molecules, thus reducing the amount of entrapped water. These effects were observed when cross-linking agents (with different cross-link lengths) had no effect on denaturation temperature when hydration was the same (Avery and Bailey 2008).

It was observed that thermally treating the glutaraldehyde cross-linked films did not have a significant affect on the puncture distance and work of puncture (Table 4.5). The collagen may not have undergone thermal denaturation by 80°C when the films were cross-linked with glutaraldehyde (Avery and Bailey 2008). Cross-linking with glutaraldehyde, has been observed to increase the denaturation temperature of collagen, as significantly as 65°C to approximately 100°C (Miles and others 2005). These results suggest that the thermal stability of cross-linked casings may rely more on moisture content than cross-links.
Table 4.4- Mechanical properties of cross-linked with glutaraldehyde and thermally treated collagen films; films were produced with Collagen 2.

<table>
<thead>
<tr>
<th>Thermal Treatment</th>
<th>Puncture Distance mm</th>
<th>Work of Puncture mJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment</td>
<td>3.781 ± 0.75 a</td>
<td>2.576 ± 0.82 a</td>
</tr>
<tr>
<td>40˚C</td>
<td>3.180 ± 0.17 ab</td>
<td>2.053 ± 0.16 a</td>
</tr>
<tr>
<td>50˚C</td>
<td>3.151 ± 0.57 ab</td>
<td>2.584 ± 0.45 a</td>
</tr>
<tr>
<td>60˚C</td>
<td>2.838 ± 0.25 ab</td>
<td>1.844 ± 0.63 a</td>
</tr>
<tr>
<td>70˚C</td>
<td>2.423 ± 0.13 b</td>
<td>1.265 ± 0.30 a</td>
</tr>
<tr>
<td>80˚C</td>
<td>2.578 ± 0.23 b</td>
<td>1.518 ± 0.40 a</td>
</tr>
</tbody>
</table>

*Means in the columns with same letter are not significantly different p > 0.05*

4.5 Conclusion

It was observed that manipulating the smoke condensate contact time (10, 20, 40, 80 sec) did not result in significant differences (p>0.05) in mechanical properties of collagen films. Similarly, the glutaraldehyde contact time (5, 10, 20 min) did not result in significant differences in mechanical properties, when averaged across the concentrations. Although contact time had little effect, increasing the concentration of cross-linking agent (0.1, 0.5 1.0 vol.% glutaraldehyde in 1M Hepes buffer at pH 7.4) did significantly decrease (p < 0.05) the percent elongation and distance to break. This suggested that increasing the concentration of cross-linking agent increased the brittleness of the film.

It was also observed that there were some significant differences (p < 0.05) between the mechanical properties of cross-linked, commercial materials. Therefore manufacturers of regenerated collagen casings and co-extruded sausages must consider the differences when selecting a source of collagen for their product. The mechanical evaluation of films demonstrated that there were significant differences between the collagen film’s tensile strength and percent elongation. It was suggested that fiber structure, cellulose content and pH of cross-linking solvent might have influenced the mechanical properties. Furthermore, reducing the moisture content collagen’s films may have caused the increase in thermal stability.

The information from this research can be used by manufactures of co-extruded casings. Manufacturers should monitor the concentration and pH of their cross-linking solutions as recovered solutions from drip or spray application may become diluted and contaminated. As observed these changes may significantly change the mechanical properties of collagen films. In addition, the application of different casing materials may significantly affect the mechanical properties of co-extruded casings; therefore, selection deserves consideration of the process.
and product. Finally, if products are going to be cooked, the casings may require further dehydration.
4.6 References


Chapter 5 Conclusions & Recommendations

These studies were performed to determine if there are differences between the raw materials used in the production of co-extrusion sausage; commercial collagen dispersions. This research is the first to report some actual values, as well as, provide potential explanations for the differences between commercial products.

It was observed that the mechanical and thermo-mechanical properties may be different, as a result of the dispersions pH. Dispersions with a lower pH (approximately pH 2) appeared to have a higher work of extrusion and a lower temperature of denaturation. In addition there were significant differences between the dehydrated film’s tensile strength and percent elongation. It was suggested that a greater degree intact/native fibers (cross-striated) might give a film higher tensile strength and elasticity. Similar to the dispersions, films that originally had a pH value closer to 2 may exhibit lower thermal stability, as conformational changes in the fiber structure occur at lower pH. Furthermore, lower film stability (temperature of denaturation) likely resulted from less fiber and film dehydration.

It was also observed that there were some significant differences between the mechanical properties of cross-linked, dispersions. The mechanical evaluation of cross-linked films demonstrated that there were significant differences between the films tensile strength and percent elongation. These observations also suggested that fiber structure, cellulose content and pH of cross-linking solvent influence a film’s mechanical properties. Once again, the thermal stability of cross-linked films supported that influence of moisture content.

When forming films, the manipulation of the dehydration conditions (brine concentration; 15, 20, 25, 30 wt.% and contact time; 1.0, 2.5, 5.0, 10.0 min) resulted in several significant differences (p < 0.05) in mechanical properties. Generally there was an increase in tensile strength, percent elongation and work to break with increasing concentration and time. This suggests that the degree of dehydration plays a role in the mechanical properties of collagen films. In addition, it allows manufactures to adjust the performance of their casings through the modification of either brine concentration or contact time.

Manipulating the smoke condensate or glutaraldehyde contact time (10, 20, 40, 80 sec and 5, 10, 20 min, cross-linking with smoke condensate or glutaraldehyde, respectively) did not result in significant differences in mechanical properties of collagen films. Although contact time had
little effect, increasing the concentration of cross-linking agent (0.1, 0.5 1.0 vol.% glutaraldehyde in 1M HEPES buffer at pH 7.4) did significantly decrease the percent elongation and distance to break. It would appear that increasing the concentration of cross-linking agent also increased the brittleness of the film.

This research can be used by casing manufactures and meat processors because it was observed that that raw materials and processing conditions have a significant effect on the properties of collagen casings. The application of different casing materials can significantly affect the mechanical properties of co-extruded casings; therefore, selection deserves consideration of the process and product. In addition, co-extruded sausage manufactures should monitor the conditions of their brine and cross-linking solutions (concentration and pH) as they may become diluted and contaminated by the moisture and or acid being removed from the casings. As observed these changes may significantly change the mechanical properties of collagen films. Finally, if products are going to be cooked, the casings may require further dehydration.

It is suggested that supplementary studies involving more controlled manipulation of collagen dispersions (composition, mechanical processing, swelling agent and pH adjustment) are performed to support the explanations for the differences. In addition, the completion of in-line trials should be performed to confirm these observations, as well as study the effects of fiber orientation from the co-extrusion nozzle.
Figure 0.1 Sample preparation of commercial collagen dispersions; A – vacuum packed collagen dispersion, B – collagen dispersion after mixing with a roller.

Figure 0.2 Formation of films using commercial collagen dispersions; A – Collagen dispersion on plastic film, B – Collagen dispersion rolled between plastic sheets.
Figure 0.3 Moisture content of collagen dispersions and partially dehydrated films. Moisture was measured by calculating the relative weight loss when 5 g of sample was dehydrated for 24 h at 105°C.

Figure 0.4 Tensile test (ASTM-D882) using a texture analyzer (TA-XT2i, Texture Technologies Corporation, Scarsdale, NY, USA) with a gripper distance that was set at 50 mm; A – Dehydrated collagen film, loaded in grippers, B – Dehydrated collagen film after failure
Figure 0.5 Puncture test using a texture analyzer (TA-XT2i, Texture Technologies Corporation, Scarsdale, NY, USA) with an extensibility fixture with 10 mm diameter, round openings (TA-108S5, Texture Technologies, Corporation, Scarsdale, NY, USA); A – Dehydrated collagen film, loaded in fixture, B – Puncture test setup on texture analyzer.

Figure 0.6 Overlaid thermograms to show the difference in the endothermic peaks when collagen dispersions are partially dehydrated into a film. The collagen dispersion had an onset temperature, denaturation temperature and transition enthalpy of 33.2°C, 35.8°C and 1.3 J/g, respectively. The partially dehydrated film had an onset temperature, denaturation temperature and transition enthalpy of 51.4°C, 62.4°C and 3.2 J/g, respectively. Scans were performed on the same collagen material.
APPENDIX B

Materials and Methods

Sample Collection

Commercial sausages were produced with a co-extrusion casings system and shipped to the laboratory for analysis. The product was extruded immersed in brine (30 wt.% NaCl in water) for 30, 60 or 90 s at a temperature of 4 °C. The collagen network is stabilized by the brine, which partially dehydrates the casing via osmosis. After brining, products were exposed to smoke condensate for approximately 5 s. Exposing the product to smoke condensate adds flavour and colour but most importantly contributes to the stabilization of the collagen casing. Aldehyde compounds in the smoke are believed to covalently cross-link the collagen fibers and molecules (Morgan and others 1998). After cross-linking, the product was air dried. Air drying further stabilizes the casing and partially cooks the meat. Finally, a cooking stage brought the core temperature of the meat to 80°C and the samples were cooled and sealed plastic bags to prevent further drying. Samples were collected at random after brining, smoke treatment, drying or cooking stage. All samples were analyzed within 24 h of production.

Tensile and Puncture Analysis of Casings

Refer to the mechanical tests used in Section 3.3.8 for the methods for the tensile and puncture tests.

Tensile and puncture tests were performed to determine the tensile strength, percent elongation, distance to puncture and work to puncture. Tensile strength and percent elongation were calculated from the tensile test and can be defined as the maximum stress sustained during elongation (MPa) and elongation relative to the initial length (%), respectively (ASTM D638). Distance at break (mm) and the work of break (J) were calculated from the generated force-distance graph. The means of six film measurements were presented for each test.

Light microscopy

Light microscopy was performed on co-extruded casings after the smoke or drying treatment. Casings from the smoke treatment stage were removed from the meat and stabilized in the cassettes on foam pads. The casings after the drying stage were left on the meats surface to avoid damage during removal (i.e. already partially cooked and difficult to remove). Casings were prefixed in 10% neutral buffered formalin for 10 h at room temperature and then
dehydrated in 70% isopropanol for 2 h, 95% for 1 h, and 100% for 4 h. The dehydration of samples was completed with xylene, prior to embedding in paraffin. Samples cut into 4-6 μm cross-sections of the casing wall and stained with Masson to differentiate collagen from any other proteins. Masson Trichrome stain is a combination of acid dye solutions (different molecular weight and size) that permeate into tissues of different protein density. Acid dyes bind to proteins via van der Waals forces because collagenous tissues are acidophilic (Cook 2008).

A light microscope (Olympus BX 60, Olympus Corporation, Centre Valley, PA, USA) was used to examine the samples. Representative images (a total of six images per treatment) were taken using Image Pro Plus (Version 6.0, Media Cybernetics, Inc., Bethesda, MD, USA) software.

*Transmission electron microscopy of collagen films*

Transmission electron microscopy (TEM) was performed on a lab produced film and co-extruded films at the smoke treated and air-drying stages. Refer to Section 3.3.7 for methods of film formation and Section 4.3.2.4 for TEM sample preparation.
Results and Discussion

Tensile and Puncture Analysis of Casings

Figure 0.1 The mechanical properties of commercially produced, co-extrusion sausage casings. Casings were partially-dehydrated by passing the product through brine for 30, 60 or 90 sec. Casings were measured using a tensile and puncture test (See Section 3.3.3) after a brine or brine and smoke treatment. Films were analyzed the day of production.

The results of the tensile and puncture tests (Figure 1) suggests that increasing the brine contact time from 30 to 90 s would only slightly increase the mechanical properties of co-extruded collagen casings, after brining. It has been observed that increasing the brine contact time (1 to 10 min) can significantly increase the tensile strength of collagen films (Section 3.4.1),
therefore it would appear that these time intervals were not great enough to alter the mechanical properties of the films.

Another observation from the mechanical tests was that the smoke treatment appeared to increase the tensile properties of the casings. These results were consistent with Section 4.4.2.1 as it was observed that cross-linking with smoke condensate appeared to increase the tensile strength of collagen films by approximately 100% of their original partially-dehydrated value. Once again, this would suggest that covalent-crosslinks increase the mechanical strength of casings by preventing the molecules and fibers from sliding past each other (Avery and Bailey 2008; Covington 1997; Paul and Bailey 2003).
Light microscopy

Figure 0.2 Light micrographs of Masson stained cross-sections of commercially produced, co-extruded collagen casings: A and B - The casing was partially dried (30 sec in brine) and smoked; C and D - the casing was partially-dehydrated (30 sec in brine), treated with smoke condensate and air-dried. Micrographs B and D were images using a polarizer. Black bar represents 100 µm.

The light micrographs of the co-extruded casings demonstrated that thermal treatment during air drying may alter the collagen casings structure. It would appear that the collagen network in the air-dried casing appears to have a smoother texture (Figure 2A verses Figure 2C). Although it cannot be concluded, the smoothness may be a result of thermal denaturation.

Figure 2C also demonstrate that there a good contact between the meat and the casings. Any gaps that form between the meat and the casing can result in wrinkles or lipid migration (the formation of fat/jelly pockets) during cooking and storage; both of which commonly considered as production defects (Savic and Savic 2002).
Transmission Electron Microscopy (TEM)

Figure 0.3 TEM micrographs of A – lab produced partially dehydrated collagen film. The collagen film was dehydrated in a 30 wt.% NaCl bath for 5 min (Section 3.3.7). B - commercially produced co-extrusion sausage casing. The casing was partially-dehydrated (30 sec in brine) and treated with smoke condensate and C - commercially produced co-extrusion sausage casing. The casing was partially-dehydrated (30 sec in brine), treated with smoke condensate and air dried.

The TEM micrographs demonstrate that the lab produced films are somewhat similar to the co-extrusion produced films (Figure 7.3A and 7.3B). The micrographs show that the collagen material used in these collagen casings appeared to be highly swollen with very little banded structure, which is a characteristic morphology of native collagen (Wess 2008). One difference between the micrographs, is that Figure 7.3B appears to have a greater degree of fiber orientation than Figure 7.3A. This would suggest that rolling films does not impart as much fiber orientation than the counter rotating nozzle on the co-extruder. Finally, it was observed that almost all collagen fiber structure was lost when the casing was thermally treated by exposure to hot air (Figure 7.3C).
References


