

AN INVESTIGATION OF THE MECHANICAL, MICROSTRUCTURAL AND THERMO-MECHANICAL PROPERTIES OF COLLAGEN FILMS CROSS-LINKED WITH SMOKE CONDENSATE AND GLUTARALDEHYDE

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ABSTRACT

Collagen films were produced from five commercial collagen dispersions used for in-line sausage co-extrusion production. Films were prepared by partially dehydrating in a salt solution (30%) and cross linked with smoke condensate (15%) or glutaraldehyde (GA; 0-1%). Both treatments increased the tensile strength (0.32 to 0.91 MPa) and reduced % elongation while differences among the dispersions were observed. Overall, % elongation generally decreased with a higher degree of cross linking. Transmission electron micrographs revealed that collagen fibers were swollen to varying degrees, likely influencing the mechanical behaviours. Protein concentration affected the transparency of the films with a difference of 100% in light transmission between the clearest and most opaque film studied. Cross-linking with GA appeared to thermally stabilize films up to 80°C. Aldehydes in the smoke condensate were identified by gas chromatography showing highest concentration of benzaldehyde, 4-hydroxy-3,5-dimethoxy.

Keywords: casings, coating, collagen, cross-linking, glutaraldehyde, smoke condensate

1. INTRODUCTION

Sausage products rely on casings to contain, form and bind the raw ground/emulsified meat placed inside them. After cooking (heat induced gelation) of the meat proteins, the casings can be removed from the product by the food processor (e.g., frankfurters produced in cellulose casings), by the consumer (salami produced in plastic casings) or consumed with the product (pepperettes produced in natural casings). Prior to the early 20th century almost all casings were derived from animal intestines. Since then, advancements in casing technology and manufacturing have led to several types of synthetic casings (e.g., cellulose, plastic) that can be tailored to a given process and product. With increasing sophistication, the production of manufactured casings was moved to designated plants, where collagen derived from hides is extracted, formed into tubes and then crosslinked with a strong crosslinking agent such as glutaraldehyde. When co-extrusion was more recently commercialized, on a large industrial scale (SAVIC and SAVIC, 2016), direct casing application was moved to the meat processing facilities. However, the issue of finding an acceptable mild crosslinking agent, that will also enable operating at a high speed production line, is still a challenge today (i.e., glutaraldehyde is not permitted to be used in food production areas, and in the off-premises casing manufacturing plants it is thoroughly washed away prior to shipping) and hence the need for the current investigation.

Co-extrusion is the process of extruding a cylindrical core of sausage meat, while simultaneously extruding an outer layer of casing material directly on to the meat's surface. Subsequent processes include: brining, smoking, drying and cooking. They are needed to stabilize and harden the casing material by osmotically removing water, and later chemically cross-linking the building blocks of the casing material (e.g., collagen, alginate). Stabilizing the casing is essential, as the casings require strength and elasticity to undergo linking, packaging, storage and reheating.

Since casings are formed directly on the meat's surface, co-extrusion systems must use a suitable brining (e.g., concentrated salt solution) and cross-linking agents that will set the casings fairly quickly (MORGAN *et al.*, 1998). Smoke condensates are commonly used as chemical cross-linking agents, as their aldehydes form covalent linkages between collagen molecules and fibers. Exposure to smoke condensates enhances the mechanical properties of the casing, and also provides the sausage with colour, flavour and some antimicrobial properties. In co-extrusion, smoke condensate is favoured over traditional smoking (burnt wood shavings) because of the ability to work with higher concentrations, product's uniformity, and the intense colour they can provide during a short exposure time (MORGAN *et al.*, 1998; BONTJER *et al.*, 2011). Smoke condensates are produced by combustion of hard woods (e.g., cherry, oak), while the smoke is moved through a shower of water droplets that collect the aldehydes, ketones, furans, phenols, and acids (TOLEDO, 2007).

When dry regenerated collagen casings are produced (in special plants), glutaraldehyde (GA) is used as a cross-linking agent, and is later thoroughly washed off the casings before the drying process (AVERY and BAILEY, 2008; MORGAN *et al.*, 1998; SAVIC and SAVIC, 2016). Glutaraldehyde is also used as a leather tanning agent, production of medical constructs made from collagen, and tissue fixative (COVINGTON, 1997; CHEUNG and NIMNI, 1982). It is able to form stable bonds among proteins and increase the mechanical properties of collagen fibers. The mechanism of glutaraldehyde's reactions has been studied quite a lot in order to help improve the modification of collagenous materials (CHEUNG and NIMNI, 1982; BARBUT, 2015). Overall, it has been shown that GA reacts with amine groups, specifically lysine, to form heterocyclic compounds. Subsequent oxidation reactions produce pyridine rings (ENGLERT *et al.*, 2007). Although GA is a

highly functional cross-linking agent, cytotoxic effects have limited its direct food applications, including in semi-liquid casing application as used in co-extrusion. However, as mentioned above in designated casing producing plants it is used as part of the manufacturing of regenerated collagen casings that subsequently go through an extensive washing process to remove all the GA residues.

Overall, there has been a substantial effort to understand the properties of collagenous materials and the effects of different cross-linking agents (O'SULLIVAN *et al.*, 2006; WOLF *et al.*, 2006; TOMIHATA *et al.*, 1994; OLDE DAMIK *et al.*, 1995). However, little has been published about the effectiveness of cross-linking agents on the performance of commercial co-extrusion collagen products. This study was designed to address this area in two steps. First, the effects of smoke condensate (currently used in meat processing plants for the wet co-extrusion process), and glutaraldehyde (used in special plants to make regenerated dry manufactured collagen casing) concentration and contact time were examined by evaluating the mechanical properties of collagen films. This was done to provide an insight into potential manipulation of the mechanical properties of co-extrusion collagen casings. The second study examined the mechanical, microstructural and thermal properties of cross-linked films to compare the similarities and differences in functionality of different commercial collagen dispersions.

2. MATERIAL AND METHODS

2.1. Study I. Manipulation of cross-linking conditions

2.1.1 Preparation of films

Five commercial collagen dispersions used for sausage extrusion processes were obtained from major manufacturers. The dispersions were labeled as: Collagen 1 through 5 (C1, C2, C3, C4, C5). Protein content was determined by the Dumas method (Leco FP528, St Joseph, MI, USA) using a nitrogen factor of 6.25. Moisture content was determined by drying triplicate samples (5 g) at 105°C for 24 h.

The method of film formation was adapted from the work of Harper *et al.* (2013) with alginate solutions and was performed at 4°C to reduce adhesion during film formation. Briefly, the samples were degassed using a vacuum packager (Multivac Canada Inc., Woodbridge, ON, CAN) 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) to remove gas bubbles that were incorporated during processing, as they can create weak spots in the films. While still in the vacuum pack bags, the dispersions were mixed by rolling the dispersions 10 times in adjacent directions. Later, approximately 3 g portions were rolled on a stainless steel board between two layers of plastic film, 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 collagen film on it was then placed in a 30 wt.% sodium chloride (prepared in deionized water) for 5 min, in order to dehydrate the film. The NaCl treatment mirrors the industrial practice of dehydrating the film (AVERY and BAILEY, 2008). 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 moved to the cross-linking solution.

The goal of the first study was to determine the best exposure time and cross-linking agent concentration to obtain a good quality film/casing. For this part we used Collagen 2, which is one of the most common dispersions currently used by the meat industry. The partially dehydrated films (i.e., by the salt solution) were cross-linked with either liquid

smoke condensate (Charsol Select 24P Liquid Smoke, Red Arrow Products, Manitowoc, WI, USA) or glutaraldehyde (EM Grade, Canemco, Canton de Gore, QC, CAN). The application of liquid smoke is a standard treatment used by the meat industry, while glutaraldehyde was used here as a control treatment with a known aldehyde (cross-linker) concentration. The films were immersed in a 15 vol.% smoke condensate in deionized water, i.e., based on industry recommendation/procedure. Films were held in the diluted smoke condensate bath for 10, 20, 40 or 80 s. Following cross-linking, films were covered with a plastic sheet to avoid drying before testing. As indicated above, glutaraldehyde (GA) was used because its mechanism of cross-linking is better understood, thus its effects could be used as a reference/standard. Films were cross-linked in solutions of 0.1, 0.5 and 1.0 vol.% GA in 1M HEPES buffer at pH 7.4 (Fisher Scientific, Ottawa, ON, CAN). The partially dehydrated films were immersed in the GA solution for 5, 10 or 20 min intervals (note: longer times were used because of the low GA concentrations), followed by a 5 min rinse in water to remove GA residues. Similar to cross-linking with smoke condensate, the films were later covered with plastic to avoid drying before testing.

2.1.2 Mechanical properties

The standard tensile test (ASTM, 2010) was used, by employing a texture analyzer (TA-XT2i, Texture Technologies Corporation, Scarsdale, NY, USA) with a gripper distance set at 50 mm, trigger force at 5 g, test speed at 2 mm/s and test distance of 25 mm. Six measurements were made to calculate an average thickness, using a digital micrometer (Testing Machines Inc., Islandia, NY, USA), of each film in each of the three trials. Films were cut into 75 mm × 25 mm strips. The average thickness and width of the films were used for the tensile stress calculations. Tensile strength (maximum stress the film endured prior to breaking) and the percent elongation (maximum elongation the film reached prior to breaking) were determined from the generated stress–strain curve.

Puncture force was also examined using the texture analyzer. In this test, a 5 mm stainless steel 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 force–distance curve. A total of eighteen measurements were used for each of the treatments (six measurements per trial).

2.1.3 Experimental design and statistical analysis

The experiment was designed as a randomized complete block with 3 independent trials. Each trial consisted of evaluating six samples per treatment. 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.

2.2. Study II. Evaluation of cross-linked collagen dispersions

2.2.1 Preparation of films

Collagen films were prepared from the five dispersions, as described before. In this part they were cross-linked with either 15 vol.% smoke condensate in deionized water, for 40 s, or with 0.5 vol.% GA in 1M HEPES buffer at pH 7.4 for 5 min, followed by a 5 min rinse.

2.2.2 Characterization of smoke condensate

The smoke condensate was diluted in methanol and injected directly to a gas chromatograph (GC-MS 1200, Varian, Palo Alto, CA, USA) to determine both volatile and semi-volatile compounds. Compounds were tentatively identified based on the NIST library provided with the instrument.

2.2.3 Transmission electron microscopy (TEM)

TEM was performed on collagen films, raw collagen and cellulose fibers as controls. The films were fixed in 2.0% glutaraldehyde, buffered with HEPES (pH 7.4) for 90 min. This was followed by fixing in osmium tetroxide and dehydration through a series of graded ethanol solutions (50%, 70%, 80%, 90%, and 100%), each for 10 min. Once the films were dehydrated, they 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 h at 60°C. Samples were then cut into 70 to 90 nm sections on a microtome (Reichert Ultracut S, Leica Microsystems Inc., Concord, ON, CAN), fixed on grids, stained with saturated uranium acetate and lead citrate (Hayat, 2000).

Negative staining was used to prepare raw collagen and cellulose fibers as controls. The collagen control was prepared by hydrating dried bovine hide sample (provided by the Meat Laboratory at the University of Guelph) for 24 h at 23°C. The pH was then lowered to 2 and blended to aid in dispersion and fiber swelling. 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 and the excess solution was removed with a filter paper. A drop of 2% uranium acetate was then applied to stain the sample. After 30 s, the excess uranium acetate was removed with a filter paper and the grids were dried, on the bench top.

Samples were examined by a TEM (Philips CM 10, Philips Scientifics, Eindhoven, NB, NL) and photographed (Olympus Morada camera, Olympus Soft Imaging System, Berlin, GER) using iTEM imaging software (Item Software, Whiteley, HPH, UK).

2.2.4 Optical property

The light transmission (380–780 nm) of the films was evaluated 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.

2.2.5 Mechanical properties of thermally treated films

Cross-linked films from Collagen 2 (C2) 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 test was performed as previously described. Once again, a total of eighteen measurements were used for each of the thermal treatments (six measurements per trial).

2.2.6 Experimental design and statistical analysis

The experiment was designed as a completely randomized block with 3 independent trials. Each trial consisted of evaluating six sub-samples per treatment. The statistical analysis was performed as indicated above.

3. RESULTS AND DISCUSSION

3.1. Study I. Manipulation of cross-linking conditions

3.1.1 Mechanical properties

The goal of the first study was to determine the best exposure time to obtain a good self-supporting collagen film, and for this we focused on Collagen 2, which is currently the most commonly used preparation. The results of the tensile test (Fig. 1) demonstrate that there were no significant differences ($p > 0.05$) in the tensile strength and % elongation when films were exposed to smoke condensate for 10 to 80 s. The results were obtained by using Collagen 2, which is one of the most commonly used collagen dispersions in the industry. The pH of the dispersion was 2.21 and is in line with the values of the other dispersions (C1=2.06; C3=2.01; C4=2.67; C5=2.04). According to manufacturers, the swelling agent for C3 was HCl, for C4 it was HCl/acetic acid; this information was not provided for C1, 2 and 5. Overall, the results reveal that after 10 s exposure the film gains a certain degree of cross-linking, and further exposure does not have a significant effect. It should be pointed out that after 10 s the film is removed from the solution but not rinsed so the amount of liquid smoke picked up by the film keeps on working. Overall, cross-linking, by intramolecular bonds, are formed between collagen molecules and fibers. The formation of covalent cross-links prevents collagen fibers from sliding past each other, thus reducing the film's ability to undergo deformation (RAULT *et al.*, 1996; PAUL and BAILEY, 2003). From a practical point of view, the results indicate that the meat processor would not be able to significantly modify the tensile properties of their casings by increasing the contact time, within this range (10 to 80 s). As noted in the Method section, the concentration of smoke condensate used here was based on industry practices/recommendations.

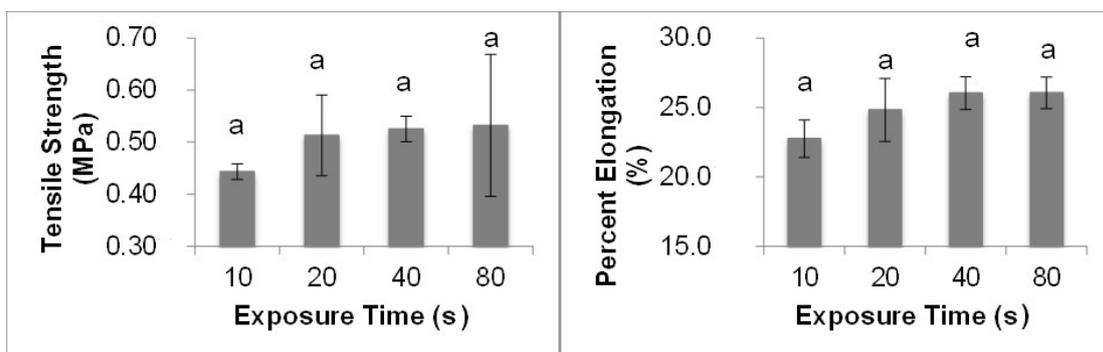


Figure 1. Mechanical properties of collagen films produced with increasing exposure times to smoke condensate (15 vol.% in deionized water). Samples made from Collagen 2. Means with the same letter are not significantly different ($p > 0.05$).

This study employed a commonly used liquid smoke condensate, currently used by various meat processors for co-extrusion applications. Overall, it is known that the composition of smoke condensates can vary, because of variations in the proportion of compounds such as aldehydes and phenols in the hard wood used to produce the condensates (GUILLÉN and MANZANOS, 1999; MONTAZERI *et al.*, 2013). Since aldehyde compounds are linked to the smoke's cross-linking ability, variations in their concentration could potentially produce different results related to tensile strength. In order to address this point, laboratory grade glutaraldehyde (GA) with a known aldehyde concentration was also used in our study.

The mechanical properties of the glutaraldehyde-treated films (Table 1) show that the tensile strength and % elongation were basically in the same range as obtained by the smoke condensate. When using GA there were no significant interactions between the GA concentration and exposure time. Also, the results indicate that there was no significant difference ($p > 0.05$) in the percent elongation between 5 and 20 min exposure (Fig. 2).

Thus, exposure time appears to have a greater effect on tensile strength when cross-linking with GA. This difference might be attributed to the concentration of the cross-linking agent in the GA solution. The results also suggest that manipulating the concentration of GA will have significant differences ($p < 0.05$) on the tensile and puncture properties of the collagen films. The most notable effect of increasing GA concentration (0.1 to 1.0 vol.%) was the significant decrease in percent elongation and distance to break (Fig. 2). These observations are consistent with those discussed by AVERY and BAILEY (2008). Overall, it appears that over cross-linking with GA would make the films less stretchable.

Table 1. Mechanical properties of collagen films produced with increasing exposure time and concentration of glutaraldehyde; samples made from Collagen 2.

Concentration vol.%	Time min	Tensile Strength MPa	Percent Elongation %	Distance to Break mm	Work to Break N*mm
0.1	5	0.564±0.14	22.475±2.11	3.012±0.26	1.537±0.56
	10	0.765±0.09	23.213±1.72	2.911±0.31	1.590±0.28
	20	0.833±0.12	20.741±0.96	2.787±0.35	1.566±0.31
0.5	5	0.502±0.06	20.137±1.21	2.517±0.24	1.068±0.05
	10	0.664±0.02	20.381±1.43	2.338±0.44	1.058±0.37
	20	0.577±0.05	19.661±3.28	1.952±0.48	0.672±0.19
1.0	5	0.708±0.08	20.921±2.96	2.071±0.63	1.630±0.90
	10	0.680±0.15	15.877±2.80	1.784±0.72	1.276±0.61
	20	0.764±0.16	17.591±3.68	1.634±0.59	1.135±0.69

3.2. Study II. Evaluation of cross-linked collagen dispersions

3.2.1 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, 2016). For example, during traditional production, casings must withstand tensile stresses during stuffing, and hanging in the smokehouse. In addition, casings must provide compressive strength during meat protein gelation (heating) and exhibit elasticity during cooling the product (SAVIC and SAVIC, 2016).

Examining properties such as percent elongation, and tensile strength, can help to predict the success of a casing.

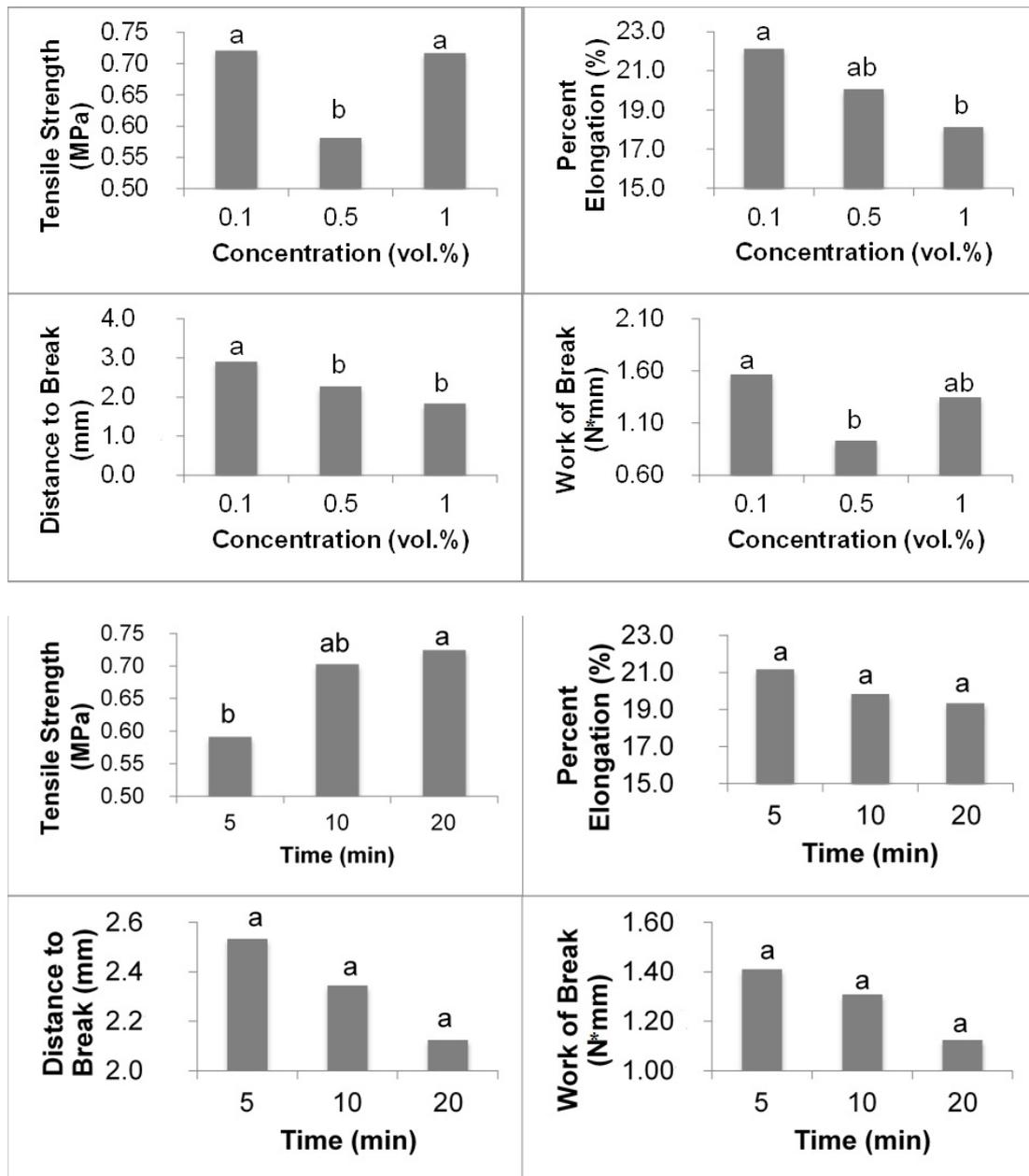


Figure 2. Mechanical properties of collagen films produced with increasing contact times to glutaraldehyde and concentration of glutaraldehyde. Samples made from Collagen 2. The contact time means were averaged across all concentration (0.1, 0.5, 1.0 vol.%) and the concentration means were averaged across all contact times (5, 10 and 20 min). Means in the same graph with same letter are not significantly different ($p > 0.05$).

In the second study, the five different collagen dispersions were compared. Overall, they all had a protein content or $4.3 \pm 0.8\%$ (C1=5.1; C2=3.6; C3=3.7; C4=4.4; C5=4.8%) and moisture content of $94.5 \pm 1.0\%$ (C1=93.5; C2=95.5; C3=93.5; C4=94.0; C5=93.7%). After partially drying the casings, by exposing them to a salt solution (30% salt as is also done by the industry), all the films showed a pretty similar moisture content ($76.0 \pm 1.5\%$, where

C1=74.5; C2=77.5; C3=74.5; C4=75.0; C5=77.0%). Tensile strength and percent elongation results (Table 2) showed that there were some significant differences among the various collagen films. The film produced with Collagen 4 (C4) had the lowest tensile strength (0.32 MPa) when cross-linked with smoke condensate, and C3 (0.41 MPa) when cross-linked with GA. In any case, both C3 and C4 showed low tensile strength under both cross-linking treatments. SAVIC and SAVIC (2016) indicated that a greater degree of native and intact fibrillar structures produce higher strength and elasticity in collagen casings. The C3 and C4 collagen dispersions may have undergone further degradation during processing, resulting in their lower tensile properties. For instance, excessive mechanical or alkaline modification, during corium separation, can result in greater degradation of the native collagen fibers (SAVIC and SAVIC, 2016). The influence of protein concentration on the mechanical properties was also investigated (Table 3). When comparing adjusted mechanical properties (i.e., per % protein), again C3 and C4 were found to result in low tensile strength values when cross-linked with smoke condensate. When cross-linked with GA, C3 showed the lowest value (0.077 MPa/% protein). When looking at % elongation, the samples that showed the lowest values when tested as received (i.e., their original protein content) also showed the lowest values when compared on a % protein basis (sample C5 treated with smoke condensate, and sample C3 treated with GA; Tables 2 and 3). These results indicate that the mechanical properties of films may be more dependent on the quality of protein, rather than on protein content. Another factor that may have led to differences in mechanical properties was the concentration of cellulose fibers within the partially dried films tested here. MATHEW *et al.* (2012) observed that interactions between cellulose nanofibers and collagen significantly increased the strength and break strain of fully dry collagen films. Note: see below additional discussion and micrographs concerning cellulose fibers. It is important to note that cross-linking has a dramatic effect on the mechanical properties of partially dehydrated films (i.e., with salt solution). Cross-linking with smoke condensate or GA appeared to increase the tensile strength of collagen films by approximately 100% and 200% (respectively) over the values obtained after salt solution partial drying (BARBUT, unpublished data), which were in the range of 0.2 to 0.3 MPa. In addition, the distance to break was reduced by 30% and 60%, when cross-linked with smoke condensate and GA, respectively. As suggested by others (AVERY and BAILEY, 2008; COVINGTON, 1997; PAUL and BAILEY, 2003) cross-links increase the mechanical strength by forming bonds among the collagen molecules/fibers and preventing them from sliding past each other, creating a stiff but brittle network within the films. Overall, there were some differences in the mechanical properties between films cross-linked with smoke condensate and GA. In general, films cross-linked with GA showed greater tensile strength than those cross-linked with liquid smoke (Table 2). Smoke condensates typically contain over 300 compounds (TOLEDO, 2007) therefore, the differences observed in tensile strength can likely be attributed to the purity of the cross-linking agent. The smoke condensate was analyzed to determine the type and concentration of its aldehyde compounds (Table 4). It was observed that the aldehyde compounds were all mono-functional aldehydes. The effect of various mono- and di-aldehydes (formaldehyde, gluteraldehyde, crotonaldehyde and glyoxal) on the thermal and conformational stability of type I collagen has been investigated by FATHIMA *et al.* (2004). Their work suggested that the aldehydes differ in their ability to improve the thermal stability of collagen. It was observed that the increase in thermal stability followed the order: formaldehyde > gluteraldehyde > glyoxal > crotonaldehyde, thus demonstrating that di-aldehydes are not necessarily more functional than mono-aldehydes.

Table 2. Mechanical properties of cross-linked films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5).

Collagen	Cross-linker ¹	Tensile Strength ² MPa	Percent Elongation ² %	Distance at Break ³ mm	Work to Break ³ Nmm	Thickness mm
C1	SC	0.67±0.04 ^a	24.80±1.23 ^{ab}	3.85±0.22 ^a	2.75±0.48 ^a	0.35±0.01 ^{ab}
C2	SC	0.53±0.02 ^{ab}	26.32±1.18 ^a	3.39±0.18 ^a	1.52±0.14 ^a	0.30±0.01 ^b
C3	SC	0.38±0.05 ^b	22.41±0.78 ^{abc}	3.21±0.30 ^a	1.24±0.34 ^a	0.34±0.01 ^{ab}
C4	SC	0.32±0.07 ^b	21.37±0.99 ^{bc}	3.27±0.35 ^a	1.23±0.31 ^a	0.36±0.02 ^a
C5	SC	0.39±0.16 ^b	18.81±2.08 ^c	2.59±0.04 ^a	0.85±0.10 ^a	0.38±0.03 ^a
C1	GA	0.91±0.17 ^d	26.26±4.65 ^d	2.77±0.52 ^d	1.79±0.57 ^d	0.36±0.03 ^d
C2	GA	0.66±0.02 ^e	20.38±1.43 ^d	2.34±0.44 ^d	1.06±0.37 ^d	0.45±0.01 ^d
C3	GA	0.41±0.13 ^f	18.95±2.56 ^d	2.66±0.39 ^d	1.35±0.27 ^d	0.38±0.05 ^d
C4	GA	0.61±0.20 ^{ef}	24.18±3.72 ^d	2.74±0.29 ^d	1.52±0.11 ^d	0.38±0.04 ^d
C5	GA	0.60±0.18 ^{ef}	22.04±2.60 ^d	2.87±0.60 ^d	1.91±0.76 ^d	0.39±0.05 ^d

¹Smoke Condensate (SC), Glutaraldehyde (GA). ²Tensile test. ³Puncture test.

⁴Means in columns with same letter are not significantly different p > 0.05: letters ^{a-c}refer to Smoke Condensate treated films; ^{d-e}Glutaraldehyde treated films.

Table 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).

Collagen	Cross-linker ¹	Tensile Strength ² MPa / %protein	Percent Elongation ² % / %protein	Distance at Break ³ Mm / %protein	Work to Break ³ N*mm / %protein
C1	SC	0.092±0.01 ^{ab}	3.36±0.17 ^{bc}	0.38±0.07 ^a	0.24±0.08 ^a
C2	SC	0.101±0.00 ^a	4.98±0.23 ^a	0.45±0.08 ^a	0.20±0.07 ^a
C3	SC	0.062±0.01 ^b	3.67±0.13 ^b	0.44±0.06 ^a	0.22±0.04 ^a
C4	SC	0.056±0.01 ^b	3.68±0.17 ^b	0.47±0.05 ^a	0.26±0.02 ^a
C5	SC	0.058±0.02 ^b	2.77±0.31 ^c	0.42±0.09 ^a	0.28±0.11 ^a
C1	GA	0.140±0.03 ^d	4.05±0.72 ^d	0.43±0.08 ^d	0.28±0.09 ^d
C2	GA	0.131±0.00 ^d	4.02±0.28 ^d	0.46±0.09 ^d	0.21±0.07 ^d
C3	GA	0.077±0.03 ^e	3.59±0.48 ^d	0.50±0.07 ^d	0.26±0.05 ^d
C4	GA	0.120±0.04 ^{de}	4.77±0.73 ^d	0.54±0.06 ^d	0.30±0.02 ^d
C5	GA	0.118±0.04 ^{de}	4.34±0.51 ^d	0.57±0.12 ^d	0.38±0.15 ^d

¹Smoke Condensate (SC), Glutaraldehyde (GA). ²Tensile test. ³Puncture test.

⁴Means in columns with same letter are not significantly different p > 0.05: letters ^{a-c}refer to Smoke Condensate treated films; ^{d-e}Glutaraldehyde treated films.

It was suggested that the number of cross-links formed influence the stability of the cross-links, therefore the reactivity of the aldehyde is of greater importance. The reactivity of mono and di-aldehydes is thought to stem from stereochemical factors and pH (increase uptake at higher pH) as was indicated by BOWES and CATER (1968). Although according to our calculation the smoke condensate had a higher percentage of different aldehydes (0.95%; Table 4), compared to the 0.5% glutaraldehyde used in the experiment, it appears that glutaraldehyde is a more reactive cross-linker that overall provided higher tensile strength values to the films (Tables 2 and 3).

It should be mentioned that the pH of the cross-linking solution may have also affected the mechanical properties of the films. MORGAN *et al.* (1998) suggested that neutralizing the acidic collagen dispersion would result in water loss. Similar to dehydrating films with a salt brine, the removal of water will result in shorter distances between the collagen molecules and hence improve the stability of the collagen structure. The smoke condensate was used as is (*i.e.*, not buffered; at pH 3.5) while the GA was buffered (pH 7.4). Thus the buffered solution could have increased the neutralization of the collagen gels, and in turn, improving stabilization.

Table 4. Tentative identification of aldehyde compounds found in the commercial smoke condensate, the relative concentration (%) and approximate concentration in the cross-linking bath.

Tentative ID	Relative %	Cross-linking Bath ¹ %
2,4-Diethoxybenzaldehyde	0.47	0.07
4-Methyl 2,5-dimethoxybenzaldehyde	1.24	0.19
Benzaldehyde, 4-hydroxy-3,5-dimethoxy	3.22	0.48
2-Oxa-6-azatricyclo[3.3.1.1(3,7)]decane-6-carboxaldehyde	0.47	0.07
3,5-Dimethoxy-4-hydroxycinnamaldehyde	0.92	0.14
(Aldehyde in Smoke Condensate)	(6.32)	(0.95)

¹The original Smoke Condensate received was diluted to 15 vol.% using deionized water.

3.2.2 Electron microscope imaging

The collagen fibers appear to have varying degrees of swelling or hydration (Fig. 3). Overall in natural tendon tissue, collagen fibrils are long, slender and show cylindrical structures, but can differ in length, diameter, uniformity, and telopeptide size, as a result of collagen type and interactions (WESS, 2008; CAMERON *et al.*, 2002). The alignment of collagen molecules in a staggered array conformation develops areas of overlap and gaps. 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 sample (obtained by us from beef hide) was imaged to compare the likeness of the banded fibers (Fig. 4A). It appeared that, at this magnification, the banded structures were indicative of collagen fibers. In addition, the bulk material, presented in this micrograph, shares similar characteristics to the collagen tissue studies by MEYER *et al.* (2005).

Although some collagen fibers display a banding pattern or cross-striations (Fig. 3), the majority of the fibers presented 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 cross-links varies depending on animal age, growing conditions, overall muscle activity, etc. Overall, there is an increase in natural cross-links,

with increasing age and therefore the solubility also decreases (MEYER *et al.*, 2005). In addition, the connective tissue extraction process would be expected to result in varying degree of fiber degradation.

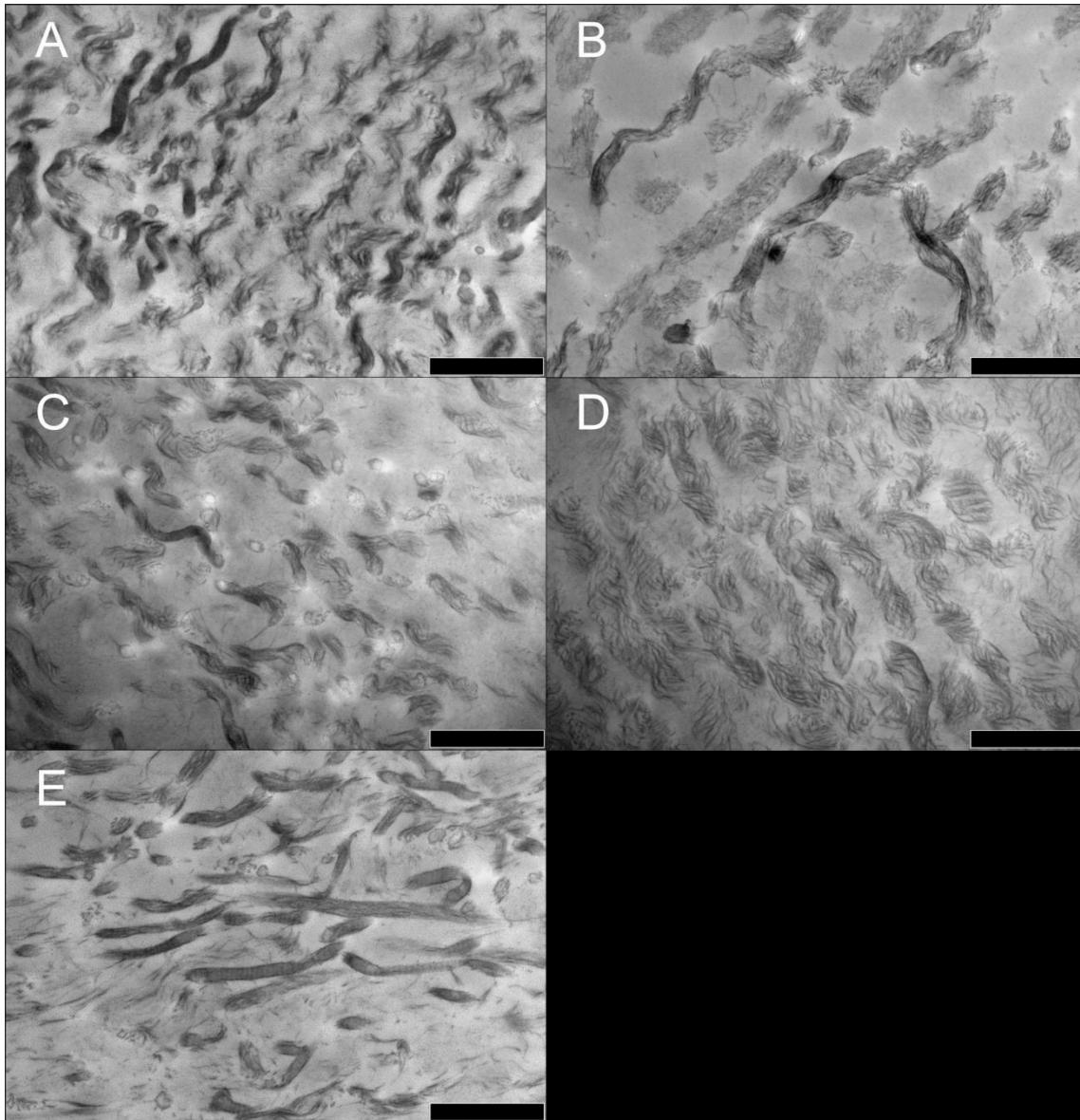


Figure 3. Transmission electron micrographs of collagen film: Collagen C1 (A), Collagen C2 (B), Collagen C3 (C), Collagen C4 (D), Collagen C5 (E). Black bar represents 1 μm .

The final collagen structure supports the mechanical properties of the film because a more intact fibrillar structure produces higher strength and elasticity in collagen casings (SAVIC and SAVIC, 2016). The C4 appeared to have the most swollen or hydrated fibers, with little visible banded fibrils remaining (Fig. 3D). Since C4 generally revealed lower tensile strength and percent elongation (Table 2), one may presume that greater swelling and hydration during collagen extraction, with HCl, and acetic acid 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.

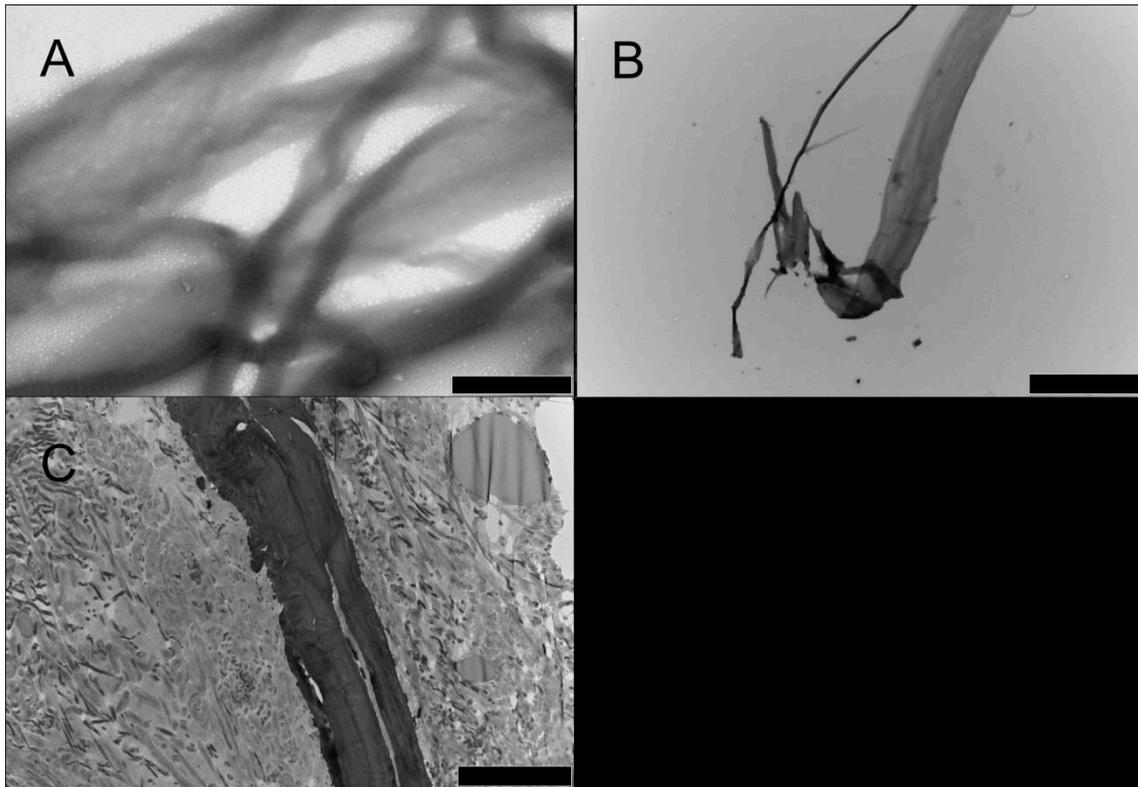


Figure 4. Transmission electron micrographs of a collagen control obtained from beef hide (A), a cellulose control (B) and a cellulose fiber embedded in a collagen film network (C). Black bar represents 1 μm in micrograph (A) and 5 μm in micrographs (B) and (C).

Another interesting observation was that there appears to be differences in the linearity of fibers. The fibers in C1 appear to be arranged in a less linear way compared to the other treatments. C1 also appears to have a higher concentration of more condensed fibers, which may have hindered alignment of the fibers during film preparation by rolling. It should be pointed out that during the formation of commercial co-extruded casings, a counter rotating extrusion head applies shear forces, which orientates and elongates the collagen fibers (HOOGENKAMP *et al.*, 2015). Orienting the fibers improves the mechanical properties of casing by reducing splitting (BONTJER *et al.*, 2011). Overall, it would be interesting to further investigate this topic to further assist the industry.

Microstructure imaging the films did not suggest significant differences in the concentration of cellulose in the five dispersions. As previously mentioned, cellulose fibers are commonly added to modify the mechanical properties and porosity of collagen casings (SAVIC and SAVIC, 2016; BARBUT, 2010). To verify that the larger fiber structures were derived from cellulose, a cellulose control (Fig. 4B) was imaged, demonstrating the difference in structure and magnitude. An example of a cellulose fiber embedded in the collagen network of one of the dispersions can be seen in Fig. 4C. Overall, cellulose fibers can be distinguished based on their larger size, lack of striation and electron density.

3.2.3 Optical properties of films

The degree of light transmission through casings affects consumer's ability to evaluate the meat product inside (SAVIC and SAVIC, 2016). Light transmission measurements were

taken to evaluate the transparency of cross-linked collagen films (Fig. 5). Among the collagen films, C5 was the least transparent and C2 was the most. Simple visual inspection also confirmed that C5 was more opaque than the other samples. The micrograph of the C5 films also showed high concentration of individual fibrils and sub-fibrils, which could result in higher light scattering. Light transmission is also affected by the composition of each casing (SAVIC and SAVIC, 2016). C2 was on the lower end of protein content (3.6%), which may have contributed to it being the most transparent.

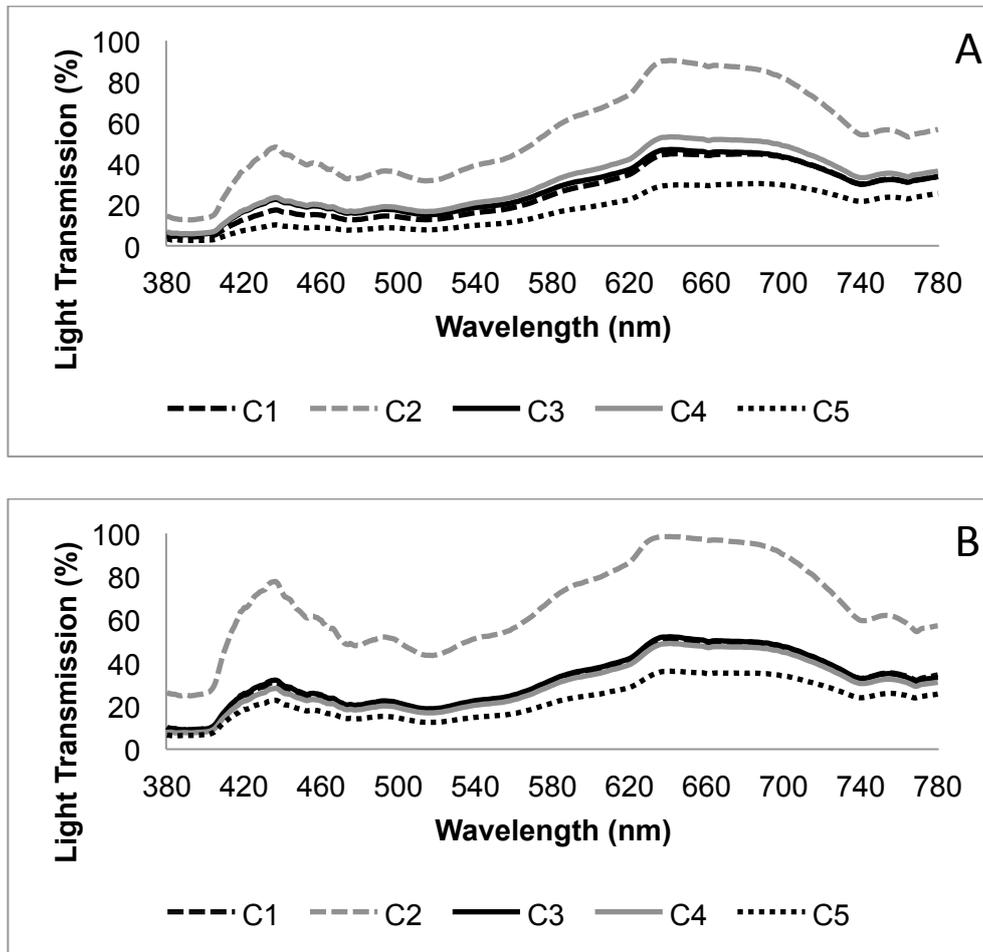


Figure 5. The light transmission (380 to 780 nm) through cross-linked collagen films; C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5). A - films cross-linked with liquid smoke; B - films cross-linked with glutaraldehyde.

Films treated with liquid smoke tended to have lower transmission than films treated with glutaraldehyde (Fig. 5). This observation is likely a result of some dark smoke components that became deposited on the films. There was no difference in the ranking of transparency between the liquid smoke and glutaraldehyde treated films. This observation is somewhat inconsistent with TANAKA *et al.* (2011), who observed that increased cross-linking results in lower transmission of wet collagen films cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide.

3.2.4 Mechanical properties of thermally treated films

Table 5 shows the puncture distance and work to puncture as an effect of heating temperature. In general, as heating increases, puncture distance significantly decreased and work to puncture showed a trend of decreasing. This is in line with the expectation that collagen tissue, in muscle foods, softens as temperature goes above the collagen denaturation temperature of 60°C (MILES *et al.*, 2005). Overall, there were no measurements recorded from testing the films cross-linked with smoke condensate because the films became too fragile to be evaluated 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 *et al.* (2005) suggested that the increased thermal stability is a result of the water content of the fibers. They observed that cross-linking reduced the axial separation between molecules, thus reducing the amount of entrapped water. AVERY and BAILEY (2008) observed that cross-linking agents, having different cross-link length, had no effect on denaturation temperature when hydration was done in the same way.

Table 5. Mechanical properties (puncture distance and work to puncture) of collagen films cross-linked with glutaraldehyde and thermally treated. Films produced with Collagen 2.

Thermal Treatment	Puncture Distance mm	Work of Puncture mJ
No Treatment	3.781±0.75 ^a	2.576±0.82 ^a
40°C	3.180±0.17 ^{ab}	2.053±0.16 ^a
50°C	3.151±0.57 ^{ab}	2.584±0.45 ^a
60°C	2.838±0.25 ^{ab}	1.844±0.63 ^a
70°C	2.423±0.13 ^b	1.265±0.30 ^a
80°C	2.578±0.23 ^b	1.518±0.40 ^a

^ameans in columns with a similar letter are not significantly different ($p > 0.05$).

In the present study, collagen may have not undergone full thermal denaturation by 80°C when films were cross-linked with GA (AVERY and BAILEY, 2008). Cross-linking with GA has been observed to increase the denaturation temperature of collagen to approximately 100°C (MILES *et al.*, 2005). Our results may suggest that the thermal stability of cross-linked casings depend more on moisture content than cross-links.

4. CONCLUSIONS

It was observed that manipulating the smoke condensate contact time (10, 20, 40, 80 s) did not result in significant differences ($p > 0.05$) in the mechanical properties of collagen films. Similarly, the GA contact time (5, 10, 20 min) did not result in significant differences in mechanical properties, when averaged across the GA concentrations employed. Although contact time had little effect, increasing the concentration of cross-linking with GA (0.1, 0.5 1.0%) significantly decreased the % elongation (22 to 17%) and distance to break (3.0 to 1.6 mm). This suggests that increasing the GA concentration increases cross-linking and brittleness of the film.

There were some significant differences between the mechanical properties of the different cross-linked films. It was suggested that fiber structure, cellulose content and pH of the cross-linking agent could influence the mechanical properties. Therefore manufacturers of regenerated collagen casings and producers of co-extruded sausages should consider these differences when selecting a source of collagen for their casings.

In addition, meat processors should monitor the concentration and pH of their cross-linking solution as solutions from a continuous liquid smoke drip/spray application are known to become diluted over time. As discussed in the paper, these changes may significantly affect the mechanical properties of the collagen films. Overall, proper understanding is required when selecting the raw collagen dispersion and the conditions for the co-extrusion process. Furthermore, if products are going to be cooked, the casings may require further heating and dehydration, which will also change their mechanical properties.

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