

# POTENTIAL APPLICATION FOR GENIPIN-MODIFIED GELATIN IN LEATHER PROCESSING<sup>\*,§</sup>

by

M. M. TAYLOR<sup>\*\*</sup>, L. P. BUMANLAG, W. N. MARMER AND E. M. BROWN

*United States Department of Agriculture, Agricultural Research Service*

*Eastern Regional Research Center*

600 EAST MERMAID LANE

WYNDMOOR, PA 19038

## ABSTRACT

Genipin is an iridoid compound extracted from gardenia fruits. Because of its low cytotoxicity, genipin can be used to replace both glutaraldehyde and formaldehyde as a crosslinking reagent. In recent years, research into the utilization of genipin for the modification of gelatin, particularly in the area of biomedical products, has increased. In prior research we had shown the potential of chemical (glutaraldehyde) and enzyme (transglutaminase) modified gelatin as fillers for leather. In this present study, we investigated whether genipin-modified gelatin products would be applicable. We initially determined optimal reaction conditions of genipin with gelatin for the purpose of creating products with suitable molecular weight distributions, viscosities and melting temperatures that would be appropriate for them to be used as fillers in leather processing. We applied the products to blue stock and, using epifluorescent microscopy, verified that these products were uniformly distributed through the blue stock and were not removed during washing. We scaled up these treatments and applied them to different areas of the hides; subsequently the pieces were retanned, colored and fatliquored (RCF), mechanical properties were determined and subjective evaluation was carried out. It was found that the mechanical properties were not significantly different from those of the control pieces and, with respect to subjective evaluation (handle, fullness, break and color) the treated products fared better than the controls. We also investigated the hydrothermal stability of the blue stock and RCF samples and found that there was an improvement in the shrink temperature in the genipin/gelatin-treated samples. SEM showed that the fibers appeared to be coated with the product, a phenomenon that we had observed in

studies using transglutaminase-modified proteins. Thus, genipin-modified gelatin has the potential to provide another environmentally safe alternative to the more conventional post tanning processes.

## RESUMEN

Genipín es un compuesto iridoide extraído de los frutos de la gardenia. Debido a su disminuida cito-toxicidad, el genipín puede ser utilizable para reemplazar ambos glutaraldehído y formaldehído como reactivo reticulante. En los últimos años, investigaciones acerca de la utilización del genipín para la modificación de gelatina, particularmente en el ramo de productos biomédicos, ha aumentado. En investigaciones anteriores hemos demostrado la posibilidad del uso de gelatina químicamente (glutaraldehído) y enzimáticamente (transglutaminasa) modificada, como rellenos para el cuero. En el presente estudio, investigamos si productos basados en gelatina modificada por genipín, serían utilizables. Inicialmente determinamos las condiciones óptimas de reacción con el propósito de crear productos con apropiadas distribuciones de peso molecular, viscosidades y temperaturas de fusión que fueran adecuadas para su utilización como rellenos en el procesamiento del cuero. Aplicamos los productos a wet-blue, y por medio de microscopia de epi fluorescencia, determinamos que los productos estuviesen uniformemente distribuidos a través del wet-blue y que no fuesen removibles durante el lavado. Estos tratamientos fueron efectuados a escalas superiores y aplicados a diferentes zonas de las pieles [sic]; subsecuentemente los pedazos fueron recurtidos, teñidos y engrasados (RET), propiedades mecánicas determinadas y evaluación subjetiva efectuada. Se encontró que las propiedades mecánicas no fueron

\*Presented in part at the 104<sup>th</sup> Annual Meeting, American Leather Chemists Association, June 19-22, 2008, Greensboro, NC.

\*\*Corresponding author – E-mail: maryann.taylor@ars.usda.gov; Tel (215) 233-6435

§Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Manuscript received August 12, 2008, accepted for publication August 17, 2008.

significativamente diferentes a las de los trozos para controles y que con respecto a la evaluación subjetiva (tacto, llenura, quiebre de flor y color) los productos así tratados fueron superiores a los controles. También investigamos la estabilidad hidrotérmica del material wet-blue y las muestras RET y se encontró que hubo mejoría en la temperatura de contracción en las muestras tratadas por medio de genipín/gelatina. Microscopia electrónica por barrido [SEM] ilustró que las fibras aparentemente estaban recubiertas por el producto, un fenómeno que habíamos observado en los estudios donde se utilizaron proteínas modificadas por medio de transglutaminasas. Gelatina modificada por genipín podría proveer otra alternativa ecológicamente sin peligro a los procesos convencionales de postcurtición.

## INTRODUCTION

Genipin is a naturally occurring iridoid compound extracted from gardenia fruits (*Gardenia jasminoides* Ellis). Because of its low cytotoxicity, it is replacing both glutaraldehyde and formaldehyde as a crosslinking reagent.<sup>1</sup> Genipin has been shown to have wide use in herbal medicine<sup>2</sup> and among its properties is a dark blue color that results upon reaction with amino acids and proteins and hence one of its common uses is in food dyes.<sup>3</sup> Additionally, it has been shown that genipin-fixed tissue has good resistance to enzymatic degradation.<sup>4</sup> Researchers, in recent years, have successfully demonstrated the effective use of genipin in the modification of gelatin, particularly for its use in biomedical products.<sup>5-9</sup> Nickerson et al. have reported on the physical properties of genipin-crosslinked gelatin-maltodextrin hydrogels<sup>10-11</sup> as well as on the possible mechanisms and optimal reaction conditions for fixation between genipin and gelatin.<sup>12</sup> Most recently a study reported on the rather simple but ingenious preparation of unique genipin-gelatin scaffolds that have the ability to be used in tissue engineering.<sup>13</sup>

Prior research from our laboratory has demonstrated that glutaraldehyde-modified gelatin could be used as a filler in leather processing.<sup>14</sup> We have also shown that transglutaminase-modified gelatin, alone or in combination with dairy proteins (casein, whey or whey protein isolate) could also be used as fillers.<sup>15</sup> The enzyme treatments were economical and environmentally safe whereas glutaraldehyde treatments, although effective, require the handling of a toxic reagent. We decided to examine genipin-modified gelatin products and determine if these would also be applicable as fillers.

In recent publications, we have reported the appropriate conditions, such as temperature (35°C), pH (7.0-7.5) and concentration (5%), necessary for reaction of genipin with hide powder and intact hides.<sup>16-18</sup> In this present study, we applied those reported conditions, except for genipin

concentration which had been significantly lower when applied to gelatin, as reported by Nickerson.<sup>12</sup> We investigated the crosslinking capability of genipin with gelatin and the parameters necessary to obtain products that could be used as fillers, products with physical properties which would be compatible at temperatures used during further leather processing (28-50°C). We next applied the appropriate products to blue stock, retanned, colored and fatliquored (RCF) the hides, and evaluated the leather. We also report in this paper the physical properties of the genipin/gelatin products as well as mechanical properties and subjective evaluation of the treated, finished leather.

## EXPERIMENTAL

### Materials

Genipin (MW = 226.23, 98% by HPLC) was purchased from Challenge Bioproducts Co. Ltd., Taiwan, ROC, and used without further preparation. Commercial Type B gelatin from bovine skin, characterized in this laboratory as 175 grams Bloom, was obtained from Sigma, St Louis, MO. Trutan PA-65 and PRP-77 were obtained from the former Pilar River Plate Corp. (Newark, NJ); Havana Dye (Derma Havana R Powder) and Sandozol Green 5BT powder were obtained from Clariant Corporation (Charlotte, NC); Altasol-CAM and Eureka 400R were obtained from Atlas Refinery, Inc. (Newark, NJ). Chrome-tanned blue stock (upholstery weight) was purchased from a local tannery. All other chemicals were analytical grade and used as received.

### Polymer Preparation

Gelatin samples (175 Bloom; 6 g) (Figure 1), were suspended in water (44 ml), and allowed to swell for about 2 h at room temperature; they were then stored overnight at 4°C. Next, they were placed in a bath at 65°C until dissolved. Control samples, without addition of genipin, were also run. The pH was adjusted to 7.5 with 1 N NaOH. In the time study, 0.5% genipin was prepared in 10 ml of water and these solutions were added with stirring to the protein solutions to give a final protein concentration of 10% w/w for gelatin. Aliquots (10 ml) of the reaction mixture were added to test tubes for melting point determination and the remainder of each solution was poured into appropriate containers for determining gel strength. These samples were warmed to 35°C in a shaker bath and the reaction was carried out for 0 to 24 h. In the concentration study, from 0 to 0.7% w/w genipin was prepared in 10 ml of water and the samples were treated as described above but were run for 4 h. When the reactions were complete, all samples were then cooled to room temperature and chilled for 17 h at 10°C in a constant temperature bath. Physical analyses (gel strength, melting point and viscosity) were run on these samples. Aliquots of the samples were lyophilized and molecular weight distribution was determined. Sodium azide (70  $\mu$ l of 1% solution) was added as a preservative and the samples were stored, tightly covered, at 4°C until use.

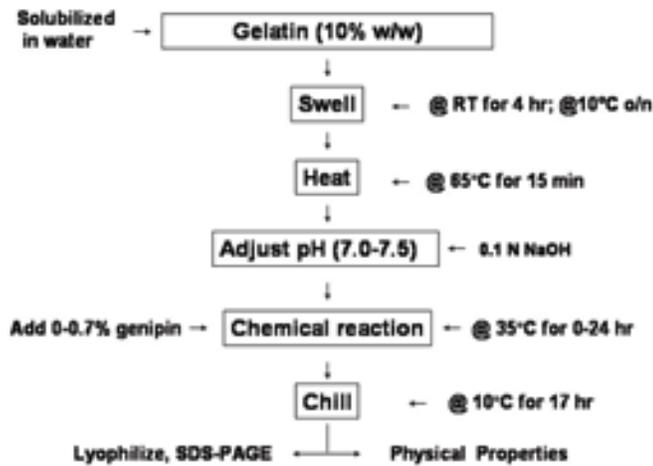


Figure 1: Schematic for genipin modification of gelatin.

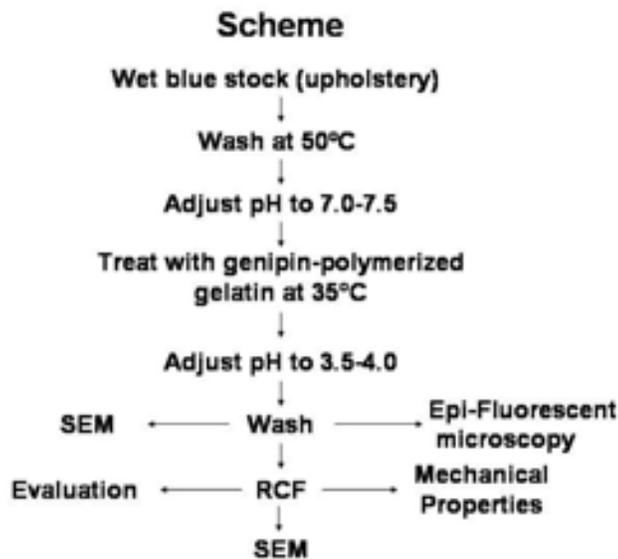


Figure 2: Schematic for treatment of wet blue with genipin/gelatin product.

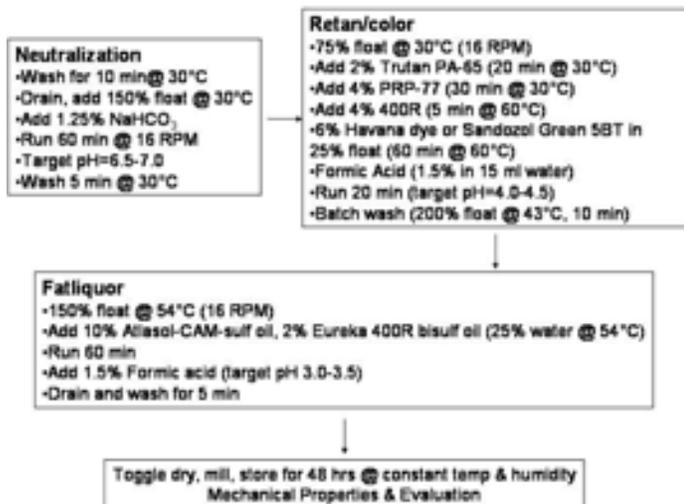


Figure 3: Schematic for retan, color, and fatliquor (RCF) of treated and untreated blue stock.

**Application of Filler to Wet Blue Leather (for Epi-fluorescent Evaluation)**

Wet blue samples (4 pieces/drum, ~25g each, 400% float) (Figure 2), were washed by drumming in a Dose drum (Model PFI 300-34, Dose Maschinenbau GmbH, Lichtenau, Germany) for 30 min at 50°C, drained and refloat in a 400% float; 4% sodium bicarbonate was added to adjust the pH to 7.0-7.5. The samples were drummed at ambient temperature (25-28°C) until the pH stabilized. The float was drained and the genipin-polymerized gelatin (diluted to give a 400% float based on wet blue weight) was added to the drums. The samples were then drummed for 1 h at ambient temperature and then for 4 h at 35°C. The pH was then adjusted to 3.5-4.0 with 4.0 M acetic acid. The floats were drained and the samples were washed twice for 10 min at 50°C (400% float), drained, patted dry and stored at 4°C in a dark place until examination under the microscope.

**Application of Filler to Wet Blue Leather (for RCF, Evaluation, and Mechanical Properties)**

Samples prepared for evaluation were treated as outlined in Figure 2 and as described above for the epi-fluorescent study, with the following changes. Six pieces of blue stock (~100g each), two pieces each from the butt, belly and neck area, were divided into tests and controls, and placed in two Dose drums. After pH treatment, the floats were drained and the prepared genipin-modified gelatin solutions (5% product loading, based on the wet blue weight and diluted to give a 400% float based on wet blue weight) were added to the test drums; water (400% float) was added to the control samples. After treatment, pH adjustment and washing, the samples were patted dry and stored in preparation for RCF. Three trials in which blue stock was treated were run (Experiments A, B, and C).

**Retan/Color/Fatliquor (RCF) and Drying**

The filled samples and the controls were placed in two Dose drums and the samples were retanned, colored and fatliquored as shown in Figure 3. When completed, all pieces were toggled and left to dry at ambient temperature and humidity. They were wet back, put into plastic bags for one day, then staked twice, and milled for 16-18 h. No finishing operations were done to the hides and they were kept on a shelf in the conditioned room at 20°C and 65% relative humidity (RH) for at least 3 days.

**Physical Properties and Molecular Weight Distribution**

Gel strength, melting point (MP), viscosity, and molecular weight distribution (by SDS-PAGE) of the enzyme-treated proteins were determined as described in previous publications.<sup>19-20</sup> Percent extractables in RCF samples was determined as described in ASTM 3495-83.

### Optical Microscope Equipped with Epi-fluorescent Attachment

The treated blue stock samples were sectioned, using a razor (grain to flesh) and mounted onto a glass slide. They were examined using an Eclipse 6600 Polarizing Microscope (Nikon Instruments Company, Melville, NY), at 4X magnification, operating in optical mode. The instrument was equipped with a X-Cite™ 120 Fluorescence Illuminator System that was fitted with a metal halide lamp (EXFO Photonic Solutions, Inc., Mississauga, ON, Canada), with two filter cubes or optical blocks containing epi-fluorescence interference and absorption filter combinations and including an excitation filter, dichromatic beamsplitter (often referred to as a mirror), and a barrier (or emission) filter,<sup>21</sup> and with a digital camera (DXM 1200).

### Subjective Evaluation

Each treated and untreated sample was evaluated with respect to handle, break (grain), fullness and color. A value from 1 to 5 was assigned for each parameter, with 1 being the worst and 5 being the best. From these ratings, an overall evaluation was determined and this value (from 1 to 5) was also assigned.

### Yellowing Test

Two three-inch (76 mm) square pieces were cut from each of the treated and untreated samples. One square of each sample was placed in an oven, at 120°C, for 72 h. After this time period, the heated samples were then compared to the unheated samples and evaluated with respect to color change. They were rated on a scale of 1 to 5, with 1 being the worst (greatest color change) and 5 being the best (least affect on color).

### Mechanical Properties

The samples were stored in a conditioned room at 20°C and 65% RH according to ASTM D1610-01. Mechanical property measurements were performed parallel to the backbone with a strain rate of 10 in/min and a gage length of 4 inches. The mechanical property measurements included: tear strength, tensile strength, elongation, and Young's Modulus. The tear strength, defined as the load at which the initial tear occurs in the sample, was determined according to ASTM D4704 and was normalized by dividing the tear load by the thickness of the sample and is presented with the units of N/mm. Dog-bone shaped samples were cut out and the tensile strength, defined as the stress required to rupture the leather, was determined according to ASTM D2209. Elongation is defined as the maximum strain at rupture. Young's modulus is a physical quantity representing the stiffness of the material. It is determined by measuring the slope of a line tangent to the initial stress-strain curve.<sup>22</sup> An upgraded Instron mechanical property tester, model 1122, and Testworks 4 data acquisition software (MTS Systems Corp., Minneapolis, MN) were used throughout this work. Each test was conducted on five samples of untreated and filled RCF blue stock; the average was calculated, and from the mean and standard deviation (STD), error bars were determined.

### Scanning Electron Microscopy (SEM)

The treated blue stock samples and their respective RCF samples were cut into small strips (6.5 cm × 1 cm) and freeze-dried. Two pieces (1.5 mm) were cut from each of the dry samples and were mounted onto the surfaces of carbon adhesive tabs with the help of Duco cement. After drying for at least 1 h, silver paint was applied to the exposed surface area around the samples. The samples were sputter-coated with a thin layer of gold using a Scancoat Six Sputter coater (Samples A and B, blue stock and RCF, 180 sec, Sample C, blue stock 180 sec, RCF, 240 sec). Samples were viewed using a Quanta 200 FEG Environmental Scanning Electron microscope, FEI Company (Hillsboro, OR) in high vacuum-secondary electron imaging mode at an accelerating voltage of 10 kV (spot size 3.0, pressure 0.3 torr). Digital images were collected at 50, 250 and 1000× magnification.

### Hydrothermal Stability (Shrinkage Temperature)

The shrinkage temperature ( $T_s$ ) of the treated and untreated wet blue samples, as well as the corresponding RCF samples, was determined as described by Fein et al.<sup>23</sup> A 7/32 × 2-1/4 inch (5.6 × 57.2 mm) piece of each sample was cut using a specialty die, the sample was inserted into an appropriate holder and then hung on a the edge of a beaker (800 ml) that had been placed on a stirring hot plate. About 600 ml of water was added and, with stirring, the temperature was raised at approximately 2°C per minute; at the first definite indication of shrinkage, the temperature of the water was recorded.

## RESULTS AND DISCUSSION

### Genipin/Gelatin Product Characterization

Recent research<sup>5-12,16-18</sup> has defined some of the parameters for genipin reaction with gelatin and collagen, such as optimal pH and temperature. Our ultimate goal, the preparation of a filler for leather, necessitated we prepare a product the physical properties, in particular its viscosity, of which would reflect not too high a degree of polymerization and thus would allow the product to be easily handled at temperatures in which leather was processed (e.g., 28 to 50°C). Therefore, starting with the reported conditions, our first objective was to react 175 Bloom gelatin with 0.5% genipin, and determine the effect of time on physical properties, e.g. gel strength, viscosity and melting point. Next, while keeping the pH, temperature, and time constant, we examined the effect of concentration on these physical properties.

The effect of time, at constant pH and temperature on the gel strength (a), melting point (b) and viscosity (c) of genipin-treated 175 Bloom gelatin, was compared to an untreated control (Figures 4a-c). As time increases, the gel strength of both the treated and untreated samples decreases (Figure 4a), which demonstrates the reported effect of temperature over a period of time on disruptions of the intermolecular

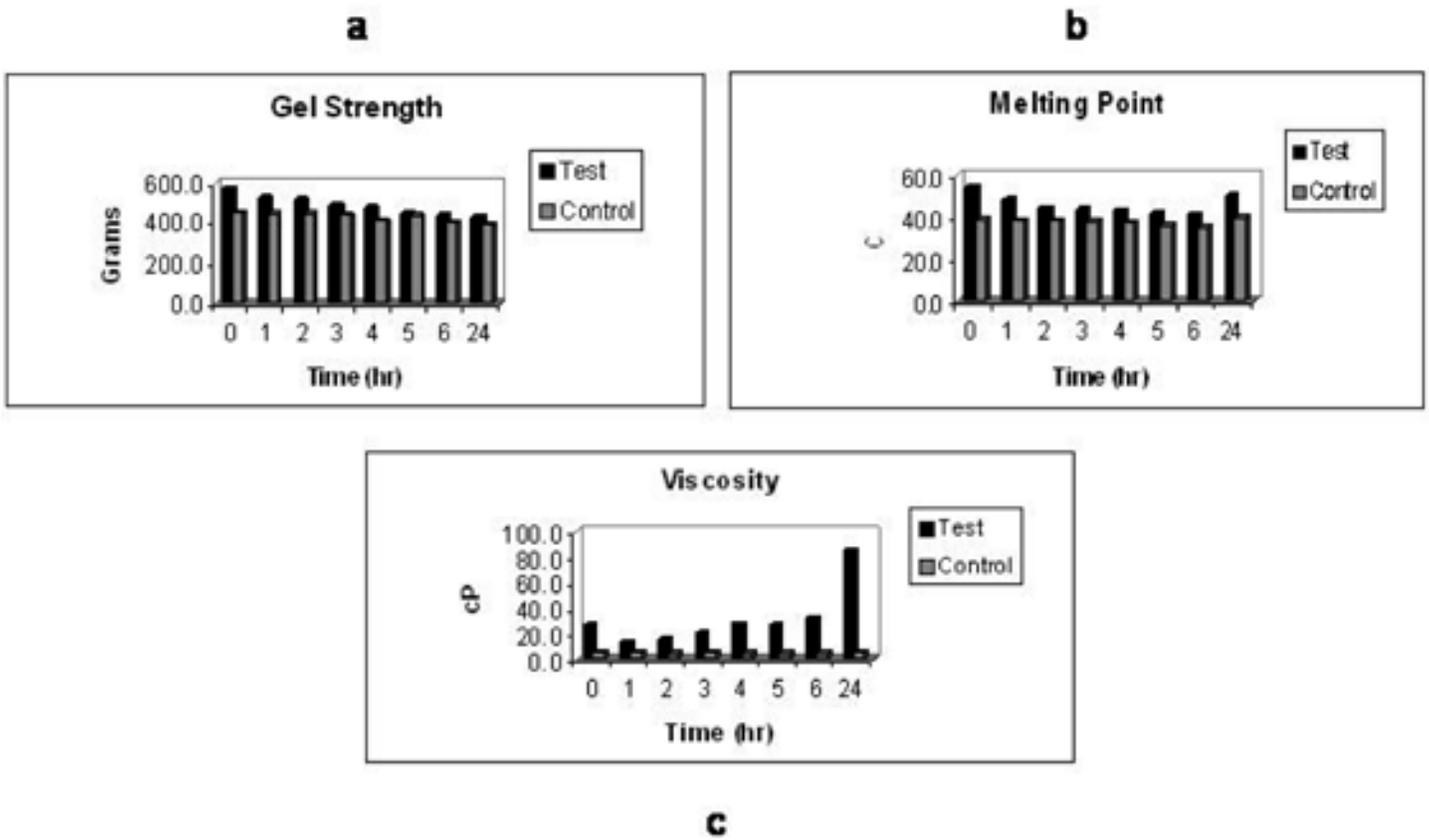


Figure 4: Gel strength (a), melting point (b), and viscosity @ 60°C (c) of 175 Bloom gelatin, 10% w/w concentration, controls (unmodified) and tests (modified with 0.5% genipin), incubated 0-24 h, pH 7.0-7.5 @ 35°C

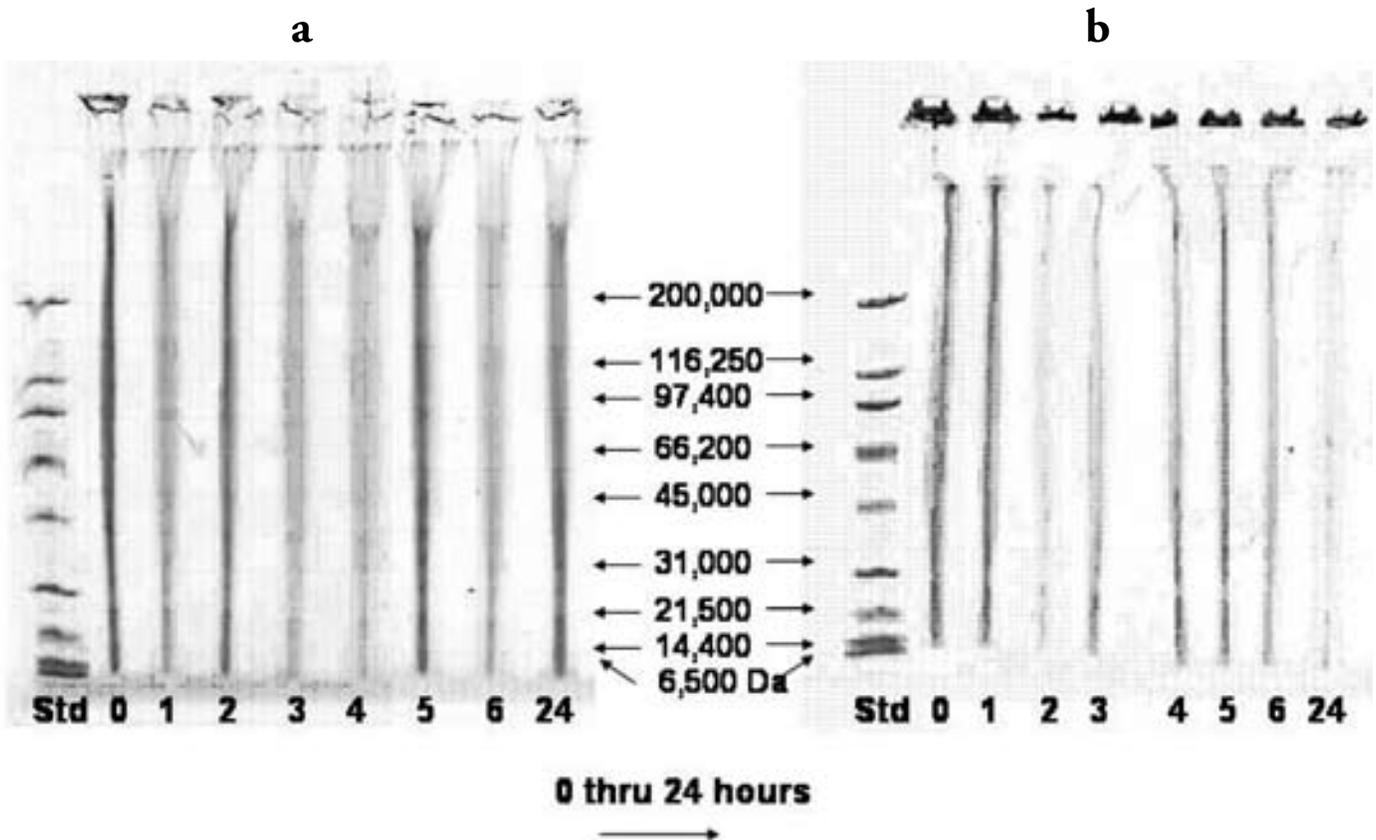


Figure 5: SDS-PAGE of 175 Bloom gelatin, 10% w/w concentration, incubated 0-24 h, pH 7.0-7.5 @ 35°C; (a) untreated control and (b) modified with 0.5% genipin; molecular weights are shown in Da.

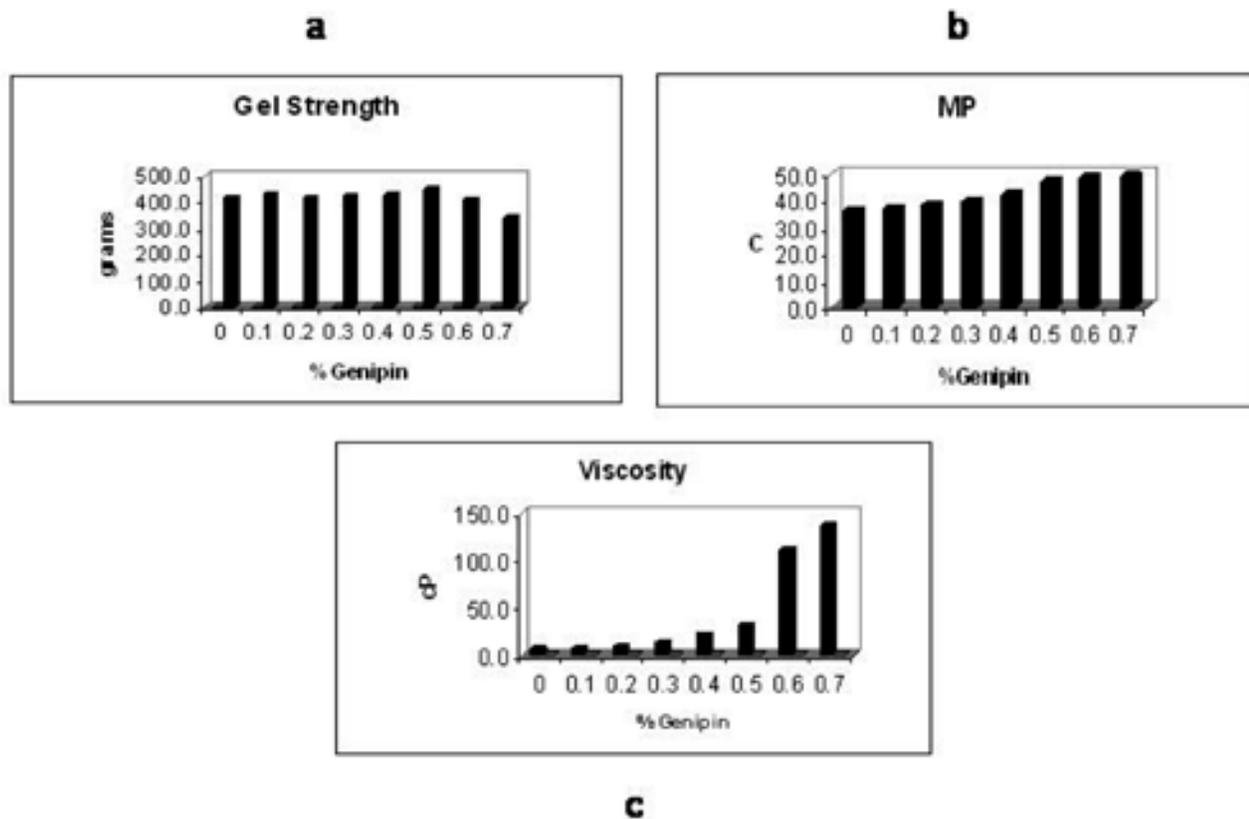


Figure 6: Gel strength (a), melting point (b), and viscosity @ 60°C (c) of 175 Bloom gelatin, 10% w/w concentration, modified with 0-0.7% genipin, reacted for 4 h, pH 7.0-7.5 @ 35°C

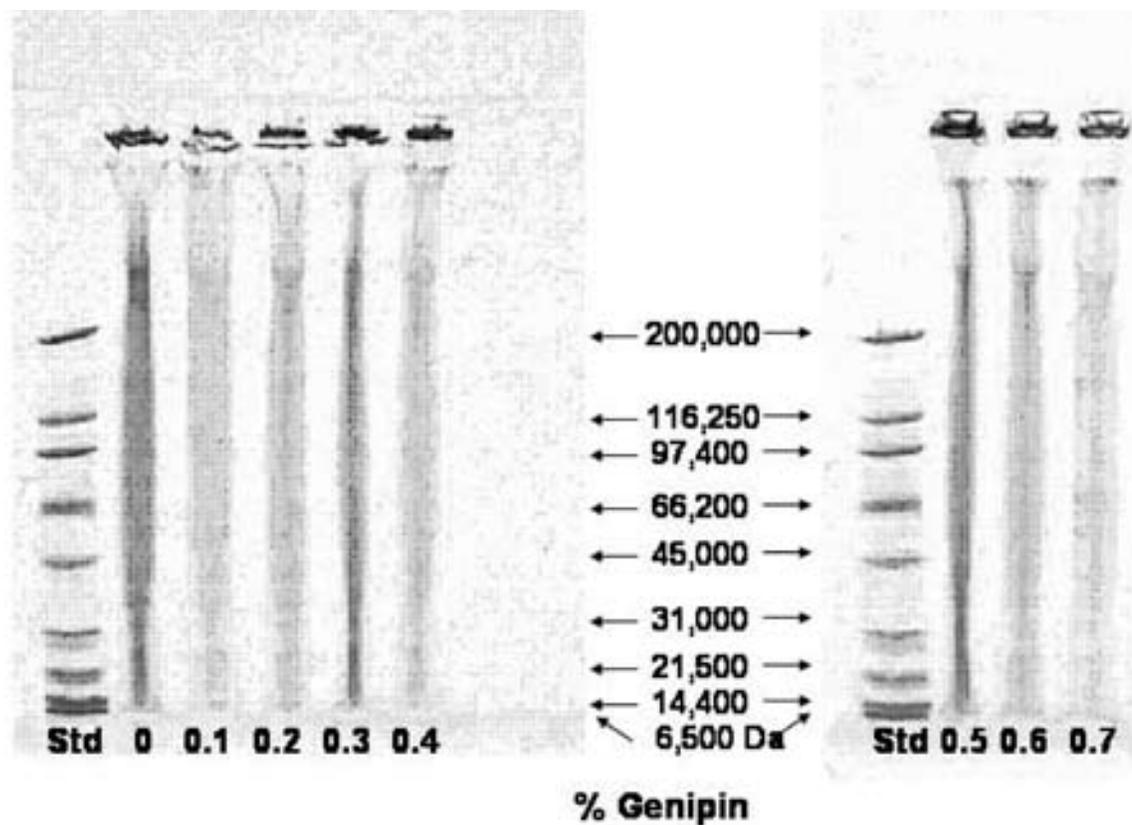


Figure 7: SDS-PAGE of 175 Bloom gelatin, 10% w/w concentration, modified with 0-0.7% genipin, reacted for 4 h, pH 7.0-7.5 @ 35°C; molecular weights are shown in Da.

crosslinks.<sup>24</sup> However, one can see that initially and throughout the reaction time, the genipin-treated samples have a higher gel strength, which can be attributed to the additional crosslinking of the protein. With respect to the melting point (Figure 4b) we are seeing the same trend, except for the 24 hour sample, in which there is a slight increase in this property. The effect of time on the viscosity is shown in Figure 4c. The untreated control's viscosity remained constant during the 24-hour period, but the treated rose dramatically at the 24-hour mark.

We also examined the effect of time on the molecular weight distribution of the genipin-treated gelatin as compared to untreated gelatin (Figures 5a and b). One can observe in the untreated gelatin (Figure 5a) that over a period of time, the band that does not enter the gel becomes less intense, suggesting that the gelatin is deteriorating (see above) when being held at this temperature. If one compares this to the gel of the genipin-treated gelatin (Figure 5b), this band does not diminish, suggesting that crosslinking has taken place.<sup>25</sup> This band does appear to lessen in the 24-hour sample, possibly due to the formation of a very high molecular weight moiety. Sharma *et al.*<sup>26</sup> suggest that the protein has become so highly polymerized that it cannot be resolved in the SDS gel; they further demonstrated that even though the highly polymerized products did not appear on the gels, their presence was confirmed by HPLC. These observations correlate with the physical properties (see Figures 4a, b, and c), particularly with respect to the viscosity of the 24-hour sample, which has risen to 86.3 cP.

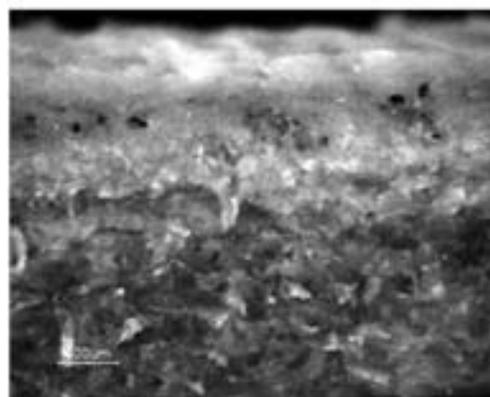
Based on the results, particularly those from determination of viscosity, we concluded that a four-hour reaction (viscosity @ 28.3 cP) would be a logical starting point to examine the effect of genipin concentration on physical properties. Keeping the temperature (35°C), time (4 h), and pH (7.5) constant, we next examined the effect of genipin concentration on the physical properties (Figures 6a-c). The gel strength of gelatin treated with increasing amounts of genipin is shown in Figure 6a. As the concentration of genipin increases, the gel strength also increases from 417 grams to 452 grams, until the 0.6% genipin level is reached, where it starts to decrease, possibly due to increasing number of crosslinks which can have an adverse effect on the strength.<sup>25</sup> With respect to the melting point, it increases from 36°C to about 49°C (Figure 6b) and at the same time the viscosity increases from 6 cP to 138 cP (Figure 6c).

The effects of increasing genipin concentration on the molecular weight distribution were evaluated (Figures 7a and b). As the genipin concentration increases, the bands indicative of the gelatin (from 14.4 KDa to 116.2 KDa) grow lighter, whereas the band that does not enter the gel increases in intensity,<sup>25</sup> until the 0.7% concentration is reached; again we may be observing that a high molecular weight moiety is formed, but cannot be resolved in the SDS gel.<sup>26</sup>

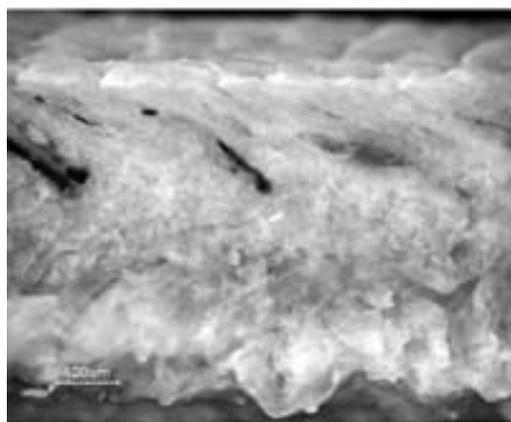
The physical properties and SDS-PAGE data suggest that a genipin/gelatin product made by reacting 10% gelatin with 0.4% genipin for a period of 4 h will give a product the properties of which would be amenable to the temperatures at which blue stock is further processed (28-50°C).

### Epi-fluorescent Microscopic Evaluation of Filler in Blue Stock

In previous studies, we labeled the different proteins that make up the biopolymer fillers with AlexaFluor® 488 and AlexaFluor® 568, and we subsequently monitored their distribution in treated blue stock. Initial experiments with labeled gelatin treated with genipin indicated that these labels could not be seen. In the literature it was found that when genipin reacts with protein, it can form a fluorogenic product, a phenomenon described in a paper in which genipin was used to detect fingerprints.<sup>27</sup> The paper reports that the product of reaction of genipin with the protein exhibits an emission around 610 nm, and this being true we would not see a label that had an emission near this wavelength (AlexaFluor® 568, 603nm). We tried labeling with AlexaFluor® 488, the emission wavelength of which is lower (~519 nm), but still did not see the fluorescence and we are assuming that the genipin/gelatin product is quenching this signal also.



a



b

Figure 8: Epi fluorescent microscopic images of blue stock (a) control (treated with pH-adjusting agents alone) and (b) test (treated with genipin-modified gelatin), emission at 610 nm ( $\bar{=}$  400  $\mu$ m).

Using the fluorescent properties of the genipin/gelatin product, we designed an experiment in which blue stock was treated, as shown in Figure 2, with a product that had been prepared by reacting 0.4% genipin in a 10% w/w solution of gelatin for a period of 4 h at 35°C and a pH of 7.5. The treated blue stock was examined using epi-fluorescent microscopy on samples removed before and after washing, from the edge of the hide, and from the center of the hide sample (to monitor diffusion). We found that, compared to an untreated control, the samples showed that the filler product was distributed evenly throughout the hide and was not removed by washing; representative images of an untreated control (a) and blue stock treated with the genipin/gelatin product (b) are shown in Figure 8.

#### Application of Fillers to Wet Blue and RCF

In preparation for scale-up, genipin/gelatin polymer solutions (gelatin solution, 10% w/w, 0.4% genipin, 35°C, pH 7.5, and 4 h) were prepared and characterized. The physical properties (Table I), when compared to an untreated control, show that the gel strength, melting point, and viscosity had increased from 411.6 g, 37.3°C, and 5.72 cP, respectively. Molecular weight distribution was determined on these samples and the results were similar to those found in the 0.4% study (Figure 7a; bands indicative of gelatin, from 14.4 KDa to 116.2 KDa, diminished and the band that does not enter the gel increased in intensity).

Blue stock pieces were treated with the products (Figure 2); control pieces, which were only subjected to pH adjustment, were also run. All samples were then RCF (Figure 3). The control samples and the filled samples were again run in separate drums, so as to prevent competing uptake of retan, dye, and fatliquor. After drying at ambient temperature and conditioning, the samples were milled for 16-18 h; the time was increased to accentuate any differences between the test pieces and the controls.

**TABLE I**

#### Physical Properties of Genipin-Modified Gelatin<sup>a</sup>

	Average <sup>b</sup>	Std. Dev
Gel Strength (g)	502.2	54.2
MP (°C)	42.3	0.5
Viscosity (cP)	17.2	2.2

<sup>a</sup>10%gelatin, 0.4%genipin, pH=7.5, temp=35°C, time=4h. <sup>b</sup>n=3.

#### Subjective Evaluation

All samples were evaluated with respect to handle, break (grain), fullness and color. The samples were rated on a scale of 1 to 5, with 1 being the worst and 5 being the best. From these ratings an overall rating was given. The numbers shown in Table II were calculated by subtracting the ratings of the control samples from the ratings of the test samples. In this table, those comparisons of the test to the control in which the test was superior are shown by positive numbers, those equal to the control, by zero, and those inferior to control samples by a negative number. To summarize, out of 45 comparisons, 38 test samples (84%) were equal to or better than the controls; to further break down these comparisons, 16 test samples (36%) were better than and 22 test samples (49%) were equal to control samples. In general, these evaluations showed that the test samples fared better than the controls, particularly in the neck area.

**TABLE II**

#### Evaluation of Treated, RCF Blue Stock

	Butt	Belly	Neck
Handle			
A <sup>a</sup>	0 <sup>b</sup>	0	0
B	-1	0	1
C	0	0	0
Fullness			
A	0	1	1
B	0	0	0
C	4	2	3
Break			
A	1	-1	2
B	0	1	0
C	-1	0	1
Color			
A	0	0	-1
B	0	2	2
C	-1	0	0
Overall			
A	0	-1	2
B	0	1	2
C	-1	0	2

<sup>a</sup>Trial. <sup>b</sup>rating of test minus rating of control.

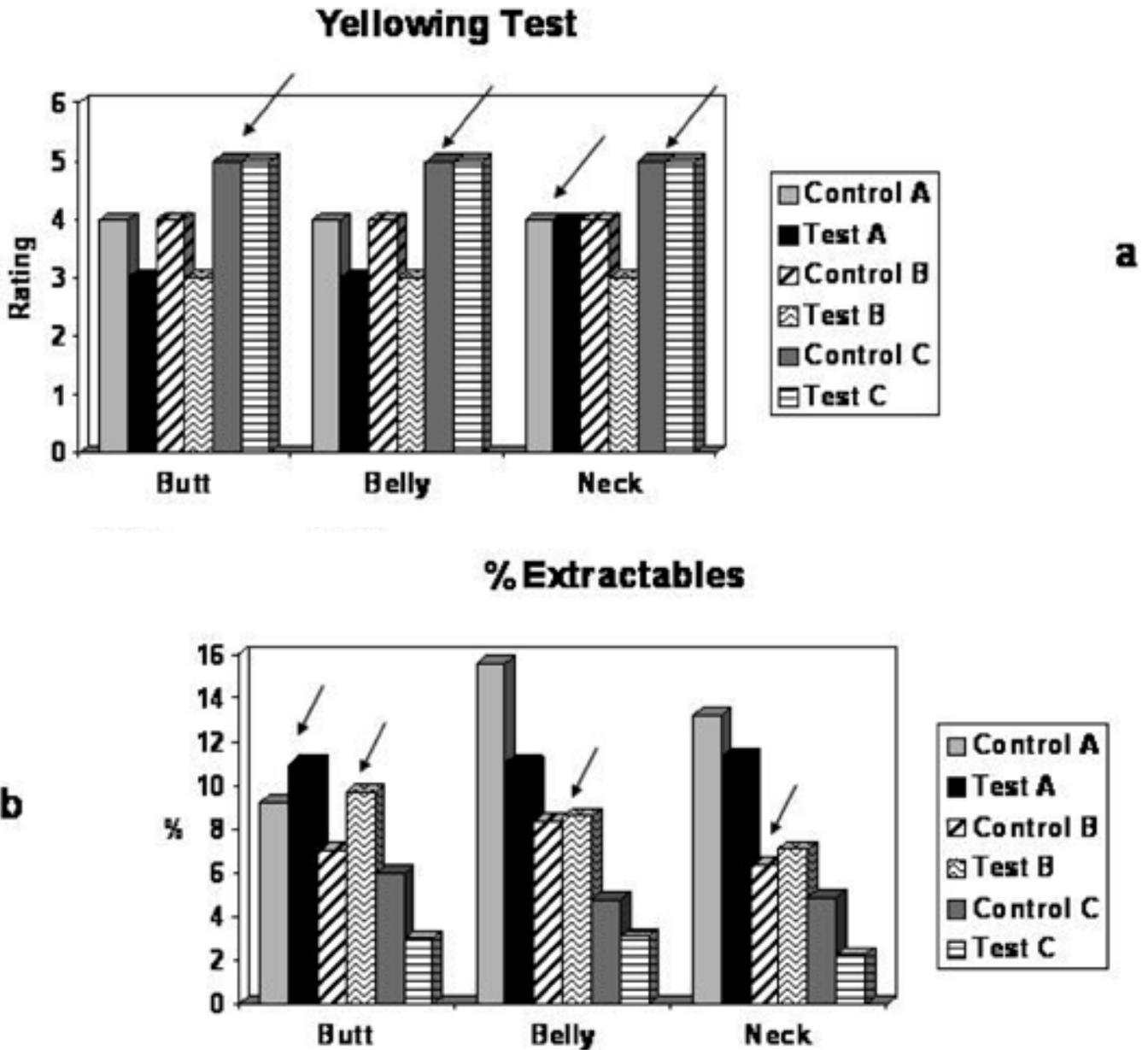


Figure 9: Yellowing test (a) and percent extractables (b), of blue stock, controls (treated with pH-adjusting agents alone) and tests (treated with genipin-modified gelatin), and then retanned, colored and fatliquored (RCF); data are from three trials (Experiments A, B, and C).

**Yellowing Test and Extractables**

The yellowing test was performed on all samples (Figure 9a). The samples from experiments A and B were treated with a light brown dye, and, as has been observed before,<sup>15</sup> the test samples did more poorly than the controls because the addition of proteins after tanning may have an adverse effect on this test, particularly if a light dye is used. In experiment C, the samples were treated with a dark green dye and one could not see a difference between the tests and the controls in these samples.

Percent extractables were carried out on all the RCF samples (Figure 9b). In 4 of the 9 comparisons, the test samples picked up more fatliquor than the controls, two in the butt area and one each in the belly and neck. In Experiment C, all of the controls did better than the tests, but even the control samples only picked up a small amount of the fatliquor and this may be due to the age of the blue stock. For improved fatliquor pick-up, the formula possibly should be altered; this could ultimately lead to products with enhanced properties, which would correlate with the subjective evaluation.

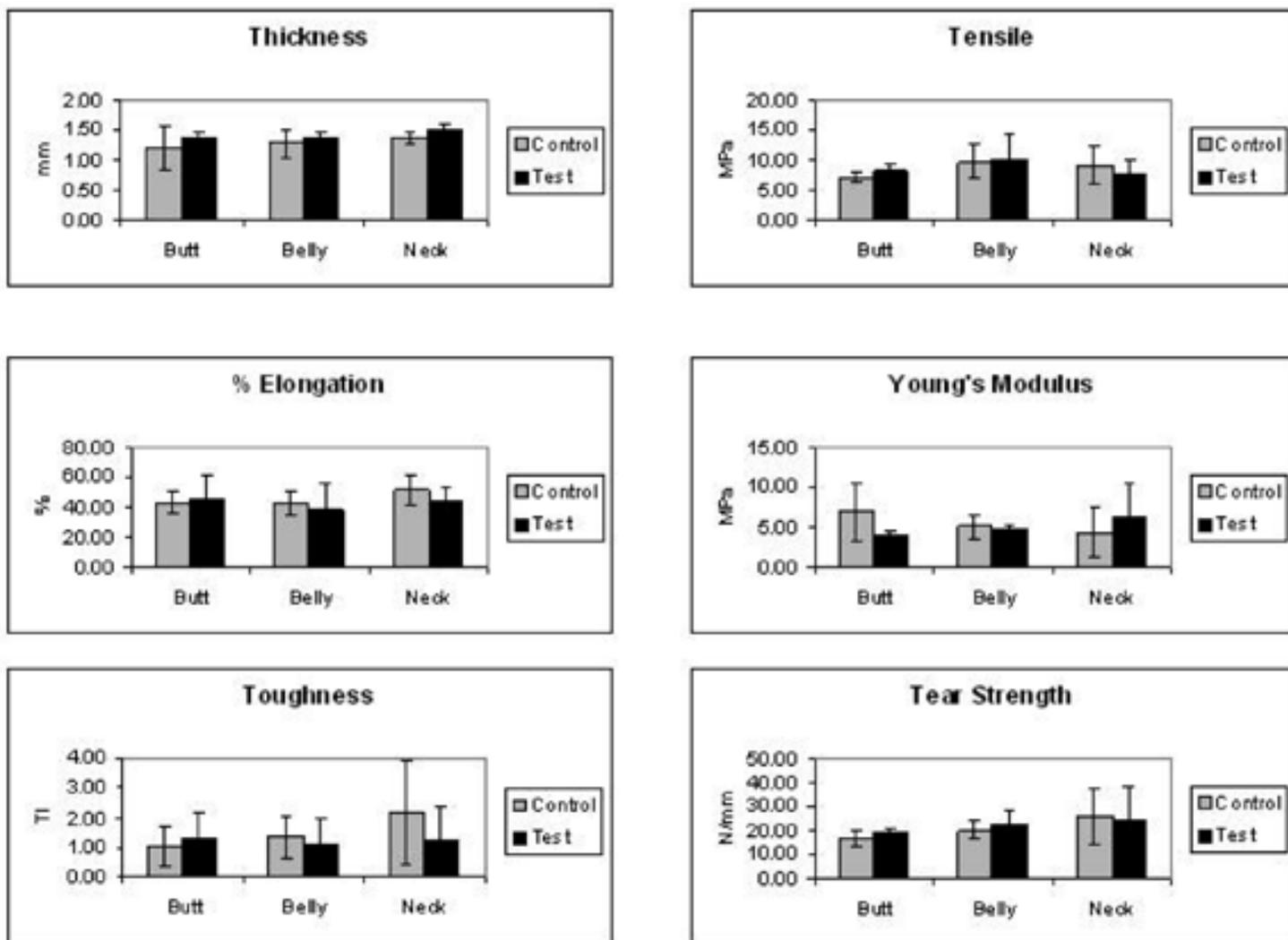


Figure 10: Mechanical properties (with Std Dev) of blue stock, treated with pH-adjusting agents alone (controls) and with genipin-modified gelatin (tests); average of data from three trials (Experiments A, B, and C).

TABLE III

### Summary of Mechanical Property Data

Mechanical property	Control	Test
Thickness		+++ <sup>a</sup>
Tensile	+	
Elongation	+	+
Young's modulus	+++	+
Toughness	+	+
Tear strength	+	+

<sup>a</sup>Samples (from 9 pairs analyzed) that are significantly different.

### Mechanical Properties

Control samples, which were only subjected to the same pH adjustment as the test samples and to which no genipin/gelatin polymer was added, were studied along with the test samples. The mechanical properties of RCF control and test samples from the butt, belly and neck areas were determined and are presented as average values from the three trials (Figure 10). These average values (and their standard deviations) were calculated from the mean of 5 tests for each sample. The error bars indicate if there are significant differences between tests and controls. Data from the individual trials that were analyzed are also shown (Table III), and those pairs that were statistically significant are indicated by a plus (+) sign. The data shows that from the nine pairs of samples analyzed from each trial, the test samples are thicker in three comparisons, and the Young's Modulus of the tests is higher, also in three pairs, and thus stiffer. The remainder of the data for each parameter is showing one pair in both the control and test that is significantly different and in two parameters (control thickness and test tensile) there are no differences. When the

data from each area for each mechanical property (thickness, tensile strength, percent elongation, Young's Modulus, toughness index (TI) and the calculation of the tear strength) are averaged, the data from three experiments show no significant difference between the test samples and the control samples (Figure 10). Taken as a whole, the mechanical properties of the genipin/gelatin-filled leather samples are not significantly different from those of the control samples, indicating that the addition of the filler does not adversely affect these properties.

### SEM Examination

All samples of the genipin/gelatin filled blue stock, their respective RCF samples as well as their controls were sampled for SEM investigation. Representative images of the micrographs of blue stock that were examined (Figure 11a-f) are showing blue stock from the butt, belly and neck areas, both control and treated samples. If one compares the control samples to the tests (1000× magnification), one can see that the structure of the filled samples is more open; the fibers appear to be separated. RCF polymer-treated blue stock and controls were also examined using SEM (Figure 12), but these samples demonstrated that it was difficult, in most cases, to distinguish differences between controls and tests after the samples were RCF, an observation that we have previously seen and described.<sup>15</sup>

### Hydrothermal Stability

The hydrothermal stability or shrinkage temperature of genipin/gelatin-treated blue stock, untreated control samples and their respective retanned, colored and fatliquored samples was determined (Table IV). It was found that the shrinkage temperature of the treated samples increased almost four degrees to 100°C, and sustained a three minute boil. However, after retanning, coloring and fatliquoring, the shrinkage temperature of the samples dropped, a phenomenon described earlier by Kronick et al.<sup>28</sup> This drop in hydrothermal stability was observed in both the control and test samples, but the test samples' shrinkage temperature still remained higher than the controls. The fact that the shrinkage temperature was higher in the treated samples could cause one to speculate that there may be enough unreacted genipin remaining in the prepared polymer which could possibly be reacting with the collagen to increase the hydrothermal stability, an attribute of genipin and combination genipin tannages, described by Ding et al. in recent publications.<sup>16-17</sup>

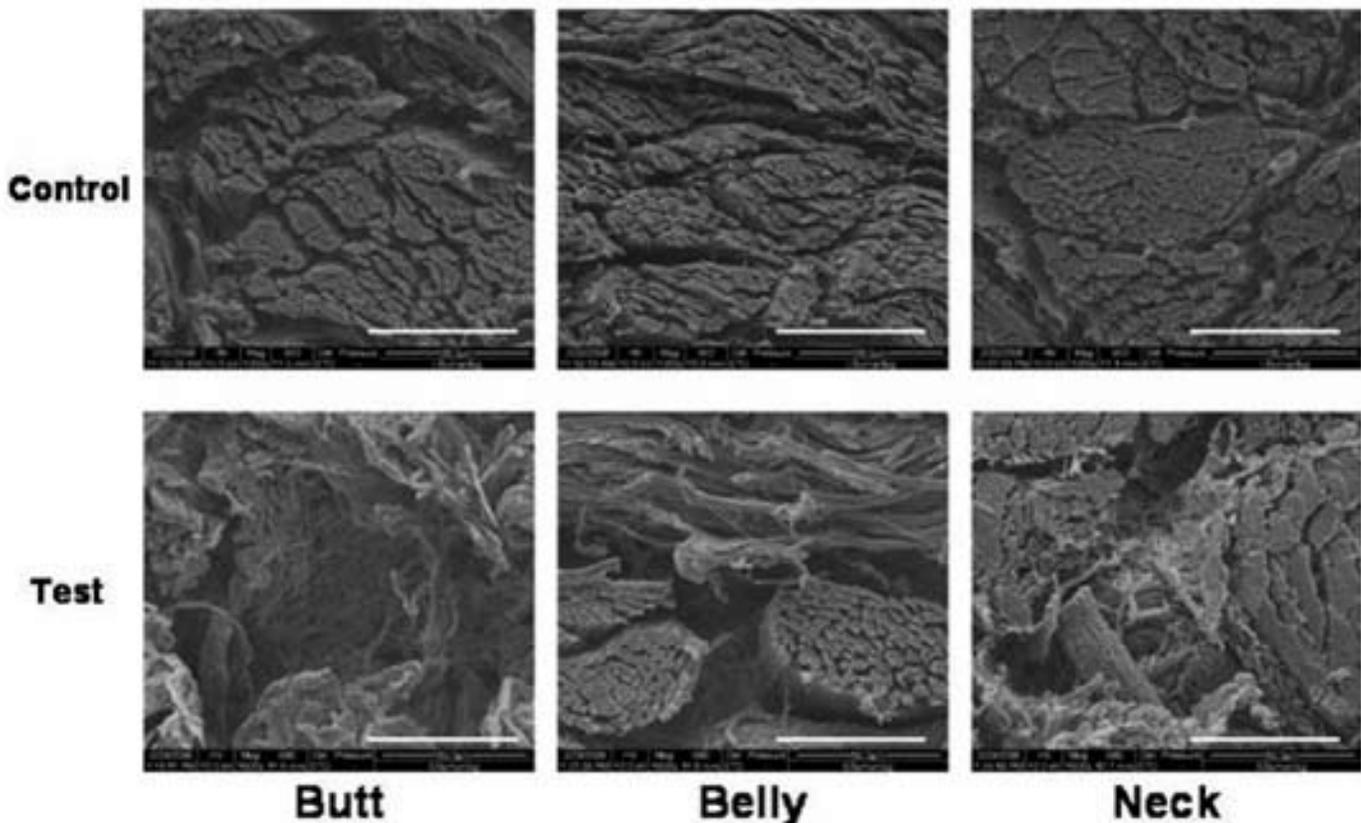


Figure 11: Scanning Electron Microscope (SEM) images of blue stock from butt, belly and neck areas: controls (treated with pH-adjusting agents alone) and filled blue stock (treated with genipin/gelatin), 1000X ( $\approx 50 \mu\text{m}$ ).

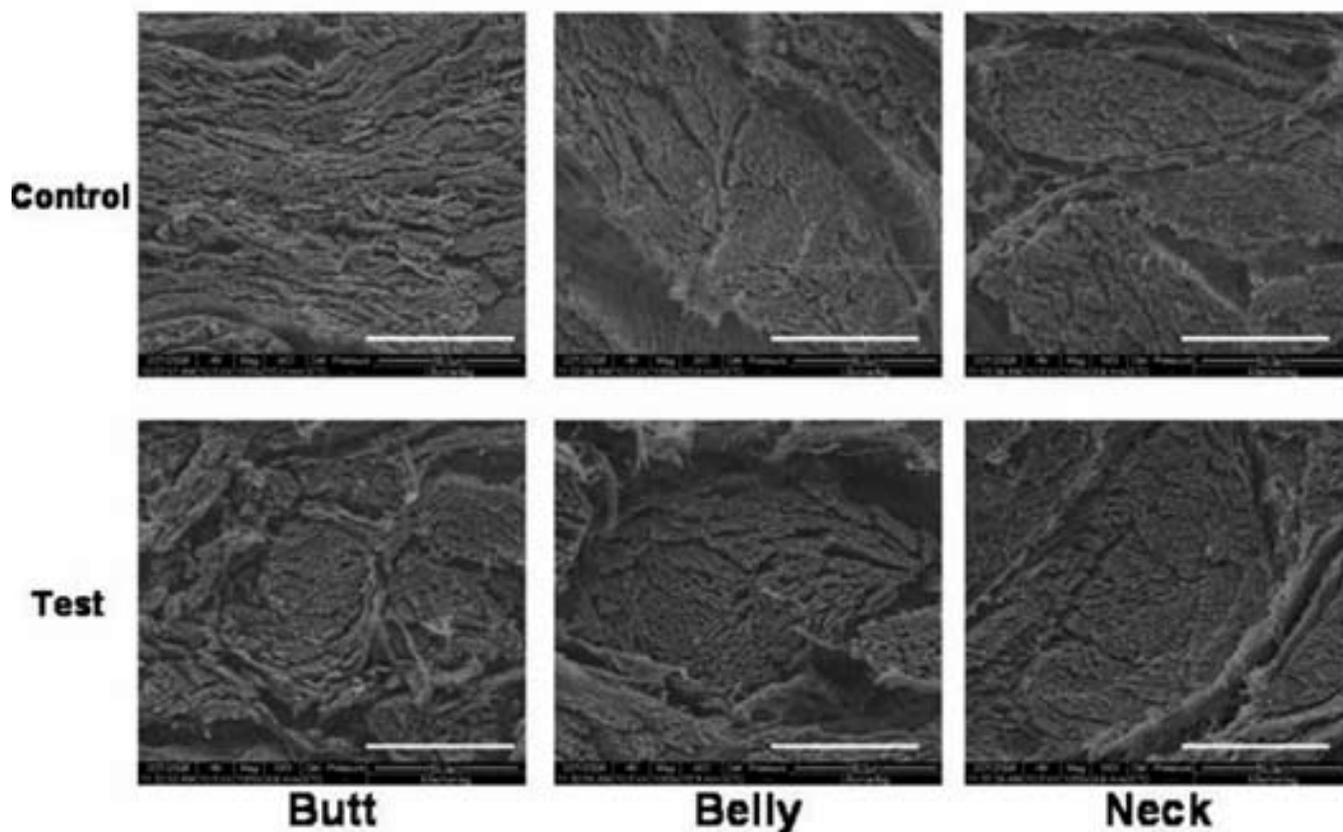


Figure 12: Scanning Electron Microscope (SEM) images of retained, colored, fatliquored blue stock from butt, belly and neck areas: controls (treated with pH-adjusting agents alone) and filled blue stock (treated with genipin/gelatin), 1000X (– = 50  $\mu\text{m}$ ).

**TABLE IV**

**Shrink Temperature ( $T_s$ )  
Genipin/Gelatin-Treated Blue Stock**

Sample <sup>a</sup>	Blue stock <sup>b</sup>	$T_s$	RCF <sup>c</sup>
Control (pH adj)	95.5°C		81.5°C
Test	100°C <sup>d</sup>		83°C

<sup>a</sup>Washed 2x's, <sup>b</sup>N=4; <sup>c</sup>N=2; <sup>d</sup>Sustained a 3 minute boil.

**CONCLUSIONS**

This study has shown that genipin-modified gelatin has the potential to be used as a filling agent for leather. Initially we determined the optimum parameters for producing a product that would be amenable (melting point and viscosity) to working into the post tanning processing of leather. We then demonstrated that the filling effect of this product could be monitored using the epi-fluorescent microscope, for the combination of genipin and gelatin gave a product with fluorogenic properties. We then evaluated the filler by applying it to butt, belly and neck areas of the hide, RCF the stock and then determined mechanical properties as well as

doing a subjective evaluation (handle, fullness, break and color). It was found that the mechanical properties were not significantly different from those of the control pieces and, with respect to subjective evaluation, the treated products fared better than the controls. The hydrothermal stability of the blue stock and RCF samples was also determined; there was a significant improvement in the shrink temperature in the genipin/gelatin-treated blue stock samples. In the RCF samples, there was a drop in hydrothermal stability in both the control and test samples, but the test samples' shrinkage temperature still remained higher than the controls. SEM showed that the fibers of the filled blue stock samples appear to be separated and the structure is more open, a phenomenon that we observed in studies using transglutaminase-modified proteins. Thus, genipin-modified gelatin has the potential to provide another environmentally safe alternative to the more conventional post tanning processes.

**ACKNOWLEDGEMENTS**

The authors would like to acknowledge the assistance of the following: Paul Pierlott, Dr. Peter Cooke, Guoping Bao, Eduard Hernandez, Dr. Cheng-Kung Liu, Nick Latona and Renée (Wildermuth) Latona. Finally, the authors would like to thank our Industrial Specialists for Leather, Gary DiMaio and Joe Lee, for their helpful advice, their guidance, and for their subjective evaluations of the leather.

## REFERENCES

1. Sung, H.-W., Huang, D.-M., Chang, W.-H., Huang, R.-N., and Hsu, J.-C.; Evaluation of gelatin hydrogel crosslinked with various crosslinking agents as bioadhesives: In vitro study. *Journal of Biomedical Materials Research* **46**, 520-530, 1999.
2. Akao, T., Kobashi, K., and Aburada, M.; Enzymic studies on the animal and intestinal bacterial metabolism of geniposide. *Biological and Pharmaceutical Bulletin* **17**, 1573-1576, 1994.
3. Touyama, R., Inoue, K., Takeda, Y., Yatsuzuka, M., Ikumoto, T., Moritome, N., Shingu, T., Yokoi, T., and Inouye, H.; Studies on the blue pigments produced from genipin and methylamine. II. On the formation mechanisms of brownish-red intermediates leading to the blue pigment formation. *Chemical and Pharmaceutical Bulletin* **42**, 1571-1578, 1994.
4. Sung, H.-W., Huang, R.-N., Huang, L.L.H., Tsai, C.-C., and Chiu, C.-T.; Feasibility study of a natural crosslinking reagent for biological tissue fixation. *Journal of Biomedical Materials Research* **42**, 560-597, 1998.
5. Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N., and Rubini, K.; Stabilization of gelatin films by crosslinking with genipin. *Biomaterials* **23**, 4827-4732, 2002.
6. Liang, H.C., Chang, W.H., Liang, H.F., Lee, M.H., and Sung, H.W.; Crosslinking structures of gelatin hydrogels crosslinked with genipin or a water-soluble carbodiimide. *Journal of Applied Polymer Science* **91**, 4017-4026, 2004.
7. Yao, C.-H., Liu, B.-S., Chang, C.-J., Hsu, S.-H., and Chen, Y.-S.; Preparation of networks of gelatin and genipin as degradable biomaterials. *Materials Chemistry and Physics* **83**, 204-208, 2004.
8. Chiou, B.-S., Avena-Bustillos, R.J., Shey, J., Yee, E., Bechtel, P.J., Imam, S.H., Glenn, G.M., and Orts, W.J.; Rheological and mechanical properties of cross-linked fish gelatins. *Polymer* **47**, 6379-6386, 2006.
9. Liu, T.-Y., Hu, S.-H., Liu, K.-H., Liu, D.-M., and Chen, S.-Y.; Preparation and characterization of smart magnetic hydrogels and its use for drug release. *Journal of Magnetism and Magnetic Materials* **304**, e397-e399, 2006.
10. Nickerson, M. T., Farnworth, R., Wagar, E., Hodge, S.M., Rousseau, D., and Paulson, A.T.; Some physical and microstructural properties of genipin-crosslinked gelatin-maltodextrin hydrogels. *International Journal of Biological Macromolecules* **38**, 40-44, 2006.
11. Nickerson, M.T., Paulson, A.T., Wagar, E., Farnworth, R., Hodge, S.M., and Rousseau, D.; Some physical properties of crosslinked gelatin-maltodextrin hydrogels. *Food Hydrocolloids* **20**, 1072-1079, 2006.
12. Nickerson, M.T., Patel, J., Heyd, D.V., Rousseau, D., and Paulson, A.T.; Kinetic and mechanistic considerations in the gelation of genipin-crosslinked gelatin. *International Journal of Biological Macromolecules* **39**, 298-302, 2006.
13. Lien, S.-M., Li, W.-T., and Huang, T.-J.; Genipin-crosslinked gelatin scaffolds for articular cartilage tissue engineering with a novel crosslinking method. *Materials Science and Engineering: C* **28**, 36-43, 2008.
14. Chen, W., Cooke, P.H., DiMaio, G., Wildermuth, R., Taylor, M.M. and Brown, E.M.; Modified collagen hydrolysate, potential for use as a filler for leather. *JALCA* **96**, 262-267, 2001.
15. Taylor, M.M., Marmer, W.N., and Brown, E.M.; Effect of fillers from enzymatically modified proteins on mechanical properties of leather. *JALCA* **103**, 128-137, 2008.
16. Ding, K., Taylor, M.M., and Brown, E.M.; Effect of genipin on the thermal stability of hide powder. *JALCA* **101**, 362-367, 2006.
17. Ding, K., Taylor, M.M., and Brown, E.M.; Genipin -aluminum or -vegetable tannin combinations on hide powder. *JALCA*, **102**, 164-170, 2007.
18. Ding, K., Taylor, M.M., and Brown, E.M.; Tanning effects of genipin -aluminum or vegetable tannin combinations" *JALCA* **103**, 377-382, 2008.
19. Taylor, M.M., Cabeza, L.F., Marmer, W.N., and Brown, E.M.; Enzymatic modification of hydrolysis products from collagen using a microbial transglutaminase. I. Physical Properties. *JALCA* **96**, 319-332, 2001.
20. Taylor, M.M., Marmer, W.N., and Brown, E.M.; Molecular weight distribution and functional properties of enzymatically modified commercial and experimental gelatins. *JALCA* **99**, 129-140, 2004.
21. <http://www.microscopyu.com/articles/fluorescence/filtercubes/filterindex.html>.
22. Liu, C.-K. and McClintick, M.D.; Measurements of the initial strain energy of leather. *JALCA* **92**, 157-171, 1997.
23. Fein, M. L., Harris, Jr., E. H., and Calhoun, Jr., R. R.; Determination of Ts on suspended leather specimens. *JALCA* **60**, 15-30, 1965.
24. Stainsby, G. The gelatin gel and the sol-gel transformation. in *The Science and Technology of Gelatin*, Ward, A.G., and Courts, A. eds. Academic Press, New York, pp. 179-207, 1977.
25. Nonaka, M., Toiguchi, S., Sakamoto, H., Kawajiri, H., Soeda, T., and Motoki, M.; Changes caused by microbial transglutaminase on physical properties of thermally induced soy protein gels. *Food Hydrocolloids* **8**, 1-8, 1994.
26. Sharma, R., Zakora, M., and Qvist, K.B.; Characteristics of oil-water emulsions stabilized by an industrial  $\alpha$ -lactalbumin concentrate, cross-linked before and after emulsification, by a microbial transglutaminase. *Food Chem.* **79**, 493-500, 2002.
27. Almog, J., Cohen, Y., Azoury, M., and Hahn, T.-R.; Genipin-A novel fingerprint reagent with colorimetric and fluorogenic activity. *J. Forensic Sci.* **49**, 255-257, 2004.
28. Kronick, P.L.; Destabilization of collagen in hide and leather by anionic surfactants. I. Differential scanning calorimetry of complexes with sulfates. *JALCA* **91**, 246-251, 1996.