The importance of elastin distribution and morphology in ovine leather

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Introduction

Elastin is the main extensible protein which allows reversible stretching of the skin (Mier and Cotton, 1976). The elastic recoil of elastin is provided by the hydrophobic domains within the protein. When elastin is stretched the hydrophobic regions come in contact with water decreasing entropy. Then, when the tension is released, the hydrophobic regions re-aggregate and expel the water, and the entropy increases which drives the movement (Mecham, 1999). Elastin can be histologically viewed using the stains orcein, or resorcin and fuchsin. It appears as a web like, branched fibrillar structure, in which very fine fibres branch from the main web of fibres in the dermis and extend into the epidermis as barely resolvable "fibrelets" (Montagna and Parakkal, 1974). More elastin appears in the grain region in comparison to deeper within the skin with some elastin appearing again at the flesh side of the corium region. Elastin comprises about 2.7% of the dry matter in the grain layer (Keller and Heidemann, 1989).

Most work on the importance of elastin has focussed on bovine material (Webster et al., 1987). However, more recent data presented on ovine material indicated specific differences in expected results between the two materials (Edmonds et al., 2005). Work done on ovine material has indicated that the retention of elastin during the processing of ovine skins is important in retaining good product performance characteristics including flatness and looseness (Cooper, 1998; Lowe et al., 2000; Edmonds et al., 2005).

It was unclear why the removal of elastin during processing should have negative results for ovine skins when this had never before been reported for the removal of elastin from bovine hide. Although it has been noted that the distribution and orientation of elastin in ovine skins is different to that of bovine material implying that the negative effects related to its removal in ovine material may be as a result of differences in the resulting orientation and distribution of elastin in the processed skins (Edmonds et al., 2005). Most importantly, measurements of total elastin content didn't fit with previous understandings of the impact of elastin removal, in particular on ovine leather

It is conjectured that the influence of elastin distribution, orientation, and subsequent processing on the physical properties of the ovine leather may explain the seemingly mutually exclusive observations to date. The aim of this work therefore is to use modern microscopy and image analysis techniques to investigate the validity of this conjecture and determine the nature of the impact of elastin distribution, orientation and processing on ovine skin properties.

Location of elastin and response to conventional processing

A method of viewing elastin based on immuno-histology has been previously used to clarify its location in skin during conventional processing (Edmonds, 2008). Positive staining indicated elastin throughout the grain which appeared as horizontal fibres a few microns in diameter. In the top 100µm of the pelt or so (also known as the grain enamel) the elastin appeared to take the form of vertical fibres with smaller diameter fibrils branching out and up to the epidermal dermal junction (figure 1).

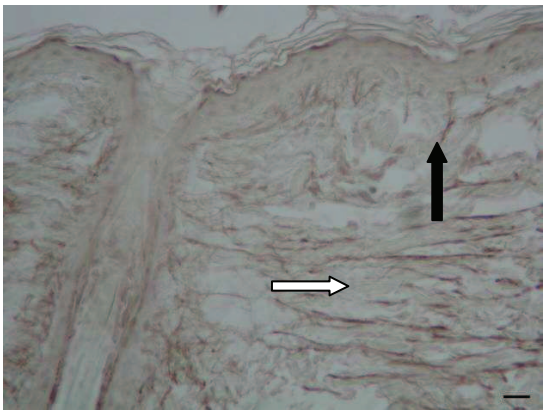
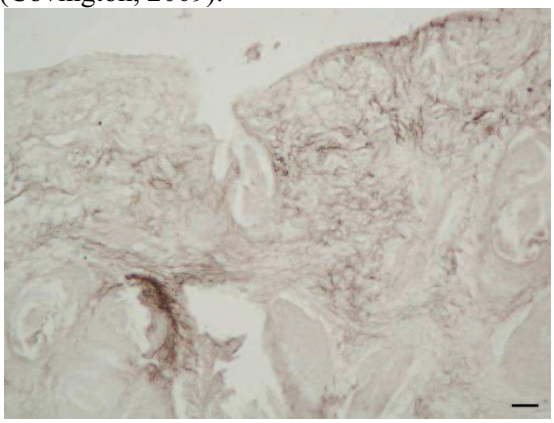


Figure 1: Immunohistology of elastin in raw skin, containing a wool root. Note the horizontal fibres in the grain (white arrow) and vertical fibres in the enamel (black arrow) (bar is 100 um)

After deliming the elastin was seen in the grain as horizontal fibres with some broken pieces (Figure 2). Again the elastin visible in the enamel existed as vertical fibres. It is worth noting that the depth of sample

Después del desencalado, la elastina aparecía en la flor en forma de fibras horizontales con algunos fragmentos rotos (Figura 2). De nuevo la elastina visible en el "esmalte" estaba en forma de fibras verticales. Es importante señalar que el ancho de muestra que contenía estas fibras verticales era de alrededor de 100 um, que coincide aproximadamente con el espesor del "esmalte" de la flor (Dempsey, 1984).

La elastina todavía fue visible en la piel piquelada en forma de algunas fibras horizontales que estaban rotas y esparcidas por la flor y algunas fibras verticales en el "esmalte". Sin embargo, algunas regiones no mostraron coloración indicando la ausencia de elastina en estas regiones, probablemente debido a la eliminación de elastina durante el proceso de rendido de carácter elastolítico (Covington, 2009).



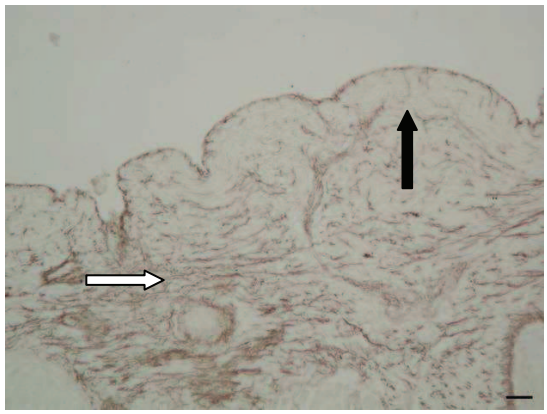


Figure 2: Immunohistology of elastin in delimed skin. Note the horizontal fibres in the grain (white arrow) and vertical fibres in the enamel (black arrow). (bar is 100 um)

containing these vertical fibres appears to be about 100 um, which is about the thickness of the grain enamel (Dempsey, 1984)

Figure 3: Immunohistology of elastin in pickled pelt. Note the lack of staining in some regions of the surface and the broken material scattered throughout. (bar is 100 um)

Elastin was still visible in the pickled pelt as some scattered broken horizontal fibres in the grain and some vertical fibres in the enamel. However, some regions did not show any staining indicating the absence of elastin in these regions, due perhaps to removal of elastin through an elastolytic bating process (Covington, 2009).

Unfortunately this staining process is not quantifiable due to the inability to quantitatively control the development of intensity of the stain. For this reason a staining technique was developed using carefully timed additions of binding-type stains which, while not absolutely quantifiable, at least provide a relative measure of the elastin found in the section.

Materials and methods

In order to examine a range of elastin arrangement variations, ovine skins were selected from early (6mth) and late (12mth) in the season. These skins were then processed using a standard process. In addition, a number of skins from the middle of the season were processed with either of two processes; with a high level of expected elastin removal, and one designed to retain elastin. Sections were taken from different positions on the skins; neck, belly, butt, and the official sampling position (OSP).

The conventional process to pickle was as follows: After removing the skin from the carcass, it was immediately cooled to 10 °C then transported to the laboratory, where any adhering fat and flesh was mechanically removed prior to processing. Fast acting (< 4 hour) lime sulphide depilatory paint comprising 200 g/L commercial flake sodium sulphide, 45g/L sodium hydroxide, 50 g/L hydrated lime and 23 g/L pre-gelled starch thickener, was applied to the flesh (inner) side of the skins at a rate of 400 g/m². The skins were then incubated at 20 °C for 16 hours (h) after which time the wool was manually removed. The skin was then processed in a drum with 0.8 volumes of water for 6 h after which it was washed with 4 changes of 2 volumes of water to remove the lime and sulphide. One volume of 2% (w/v) ammonium sulphate was then added, which lowered the pH to about 8, followed by the addition of 0.1% (w/v) Tanzyme®, (Tryptec Biochemicals Ltd.), a commercial pancreatic bate enzyme, in water. After a period of 75 min at 35°C, the treated skin was washed with 4 changes of 2 volumes of cold water (20 °C) and then pickled by the addition of one volume of pickle solution (20% w/v common salt and 2% w/v sulphuric acid diluted in water). The highly elastolytic process was carried out with this method with the following modifications: Carbon dioxide was used in place of ammonium sulphate to lower the pH to 8.0, followed by 0.2% Tanzyme used in the bating.

Pickled pelts prepared this way were then processed through to crust using standard high exhaust chrome tanning processes. Briefly; Pickled pelts were degreased by processing in a drum in 0.5 volumes of a degreasing solution being 10% common salt, 8% non-ionic surfactant, and 4% Oxazolidene E. After 30 min processing at 35°C the pickled pelts were neutralised; first by a 1%(pickled pelt weight (ppw)) addition of sodium formate followed by 20 min of processing at 35°C followed by three 1%(ppw) additions of sodium bicarbonate each of which received 20 min of processing in the drum after addition. At this point the pH was approximately 7.5. One more volume of water was added at 42°C and the pelts processed for another 60 min at which point the float liquor was drained. The neutralised pelts were then washed a further four times in 1.5 volumes of water at 42°C with 20 min processing time between each. Finally the neutralised, degreased pelts were prepared for tannage by processing for 10 min in 1 volume of water containing 1% disodium phthalate. 4.5%(ppw) chromium sulphate (33% basic) was then added and the pelts processed for 30 min and then fixed by raising the temperature to 40°C while processing overnight. The tanning solution was then drained and the wet blue pelts are washed with 1 volume of water at 25°C for 20 min.

The wet blue pelts were then processed as follows; wet blue pelts were first neutralised in 1 volume of water containing 1% sodium formate and 0.15% sodium bicarbonate for one hour. The neutralised wet blue was then washed in two volumes of water followed by retannage in 1 volume of water containing 2% synthetic retanning agent and 3% vegtan. Finally fatliquors were added (a total of 6% mixed fatliquors) and the leathers processed at 50°C for 45 min. The leathers were then fixed by the addition of 0.5% formic acid and processed for 30 min followed by draining and a wash of 3 volumes of cold water.

Image analysis

Since immunohistological stains cannot be quantified because the staining does not follow Beers law a quantification technique was developed using a histological staining technique developed at LASRA® which incorporated picric acid, aldehyde fuchsin, and fast green. Sections were stained using the

carefully monitored protocol carried out on sections cut to 30µm thickness in order to produce consistently coloured sections which could be analysed and compared statistically.

Staining

Samples of pickled pelt were cut and fixed in buffered formalin (0.0375M phosphate buffer at pH 6.65 in 0.8% w/w formaldehyde) for 24 h before being sectioned. The sections were cut from the prepared samples using a LeicaCM1850UV cryostat, dried onto superfrost slides for 24 hours then stained using the following protocol:

Slides were rinsed in two changes of water for 5 min each followed by 5 min in 1% potassium permanganate solution followed by another 5 min wash in water. The sections were then decolourised by immersion in 2% oxalic acid solution until no more colour came out. The sections were then stained with aldehyde fuschin: 2 min in water, 2 min in 70% ethanol, 7 min in aldehyde fuschin solution(1% fuschin, 1% Conc. HCl, 2% paraldehyde, 70% ethanol), 5 min in 70% alcohol, 2 min in water. The sections were then stained with fast green: 10 min in fast green solution (0.1% fast green in saturated picric acid), 10 min in 0.2% phosphomolybdic acid, 15 min in 0.01M HCl. The sections were then cleared with xylene and mounted in DPX.

A series of digital photomicrographs of the stained sections were made using an Olympus BX51 microscope, Olympus CAMEDIA C3040 zoom digital camera using 10x UplanFl objective to produce a series of images through the section each being 2048x1536 pixels and 1567 pixels per mm as in figure 4.

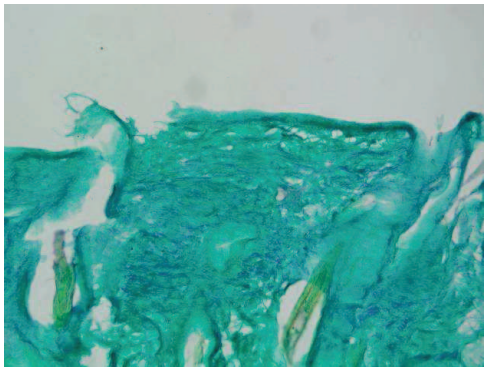


Figure 4: Elastin image of pickled pelt. Note keratin stained yellow, elastin stained purple and remaining proteins stained green

The obtained Images were then corrected and analysed using the free open source image analysis program ImageJ and associated plugins (Rasband, 2009). Firstly digital photomicrographs were corrected for bright field lighting (Landini, 2010) (Figure 5).

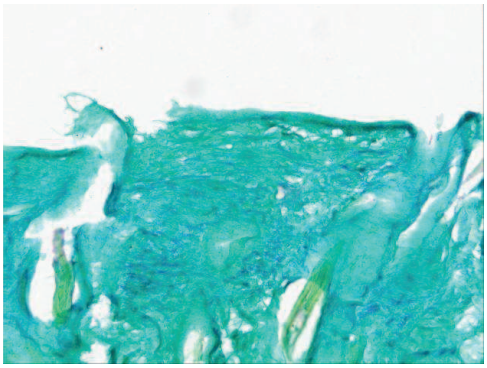
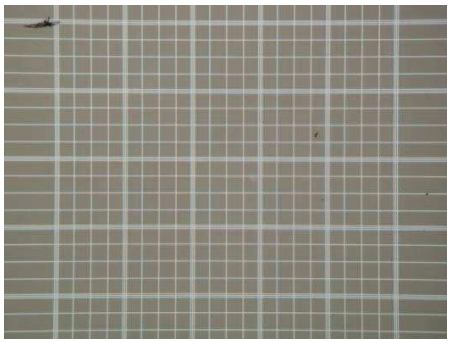


Figure 5: Photomicrograph of section corrected lighting and camera imperfections.

After bright field correction some lens distortion was still apparent (Figure 6a left). An algorithm was therefore incorporated into the microscope correction plugin to correct the lens distortion. A simple radial distortion equation was used to straighten the image (Bourke, 2002).



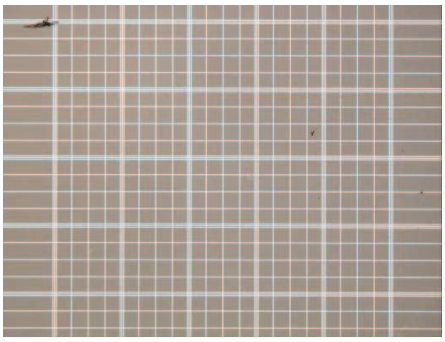


Figure 6: left: grid image illustrating lens distortion. right: image corrected for lens distortion

Once images were corrected for lighting and distortion they could be made into a mosaic (Figure 8)to produce a single image of the whole section at high resolution. This was carried out with MosaicJ (Thévenaz and Unser, 2007).
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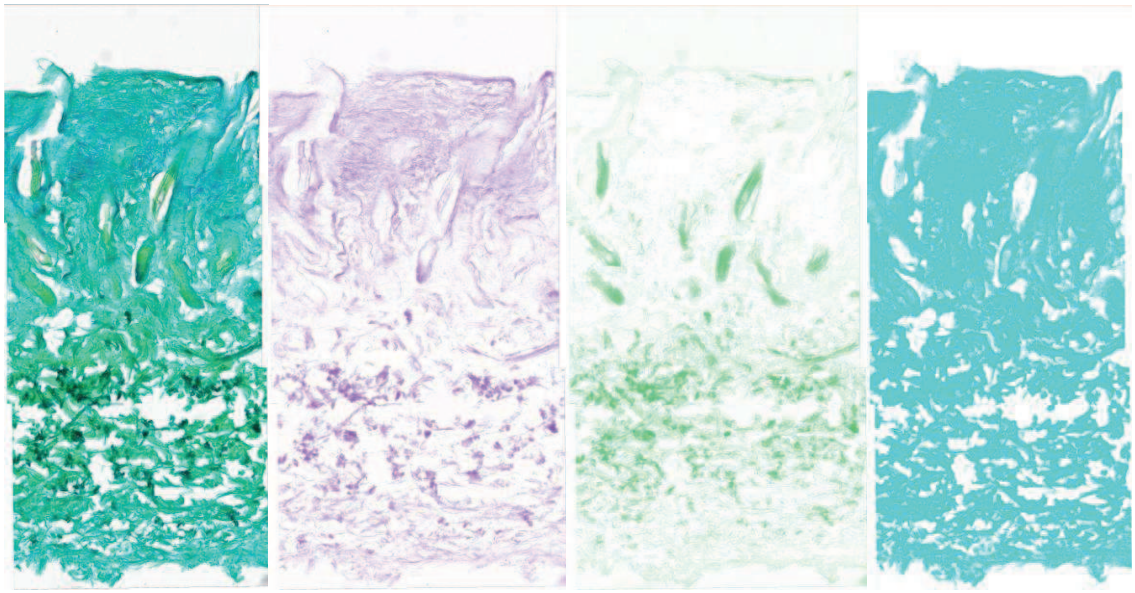

Original Color 1 Color 2 Color 3

Figure 7:Mosaic of corrected photomicrographs from section and that mosaic image separated into the 3 stain component colours: Colour 1, Colour 2, and Colour 3.

The corrected full section mosaic was then deconvolved into its constituent stain colours (Ruifrok and Johnston, 2001) (figure 7): where aldehyde fuchsin appears predominantly in Colour 1.

Finally non elastin structures in the aldehyde fuchsin prominent stain image which were not sufficiently separated using the colour deconvolution technique were mathematically subtracted from the image using the green colour as a template for non elastin material. Specifically a negative of a greyscale of Colour 2 is subtracted from colour 1 which removed artefacts from Colour 1 that were found in Colour 2. The resulting image is shown in figure 8

The elastin image could then be analysed for elastin distribution and orientation. Elastin distribution with depth was characterised by carrying out a colour density profile from the grain side to the flesh side as illustrated in figure 9.

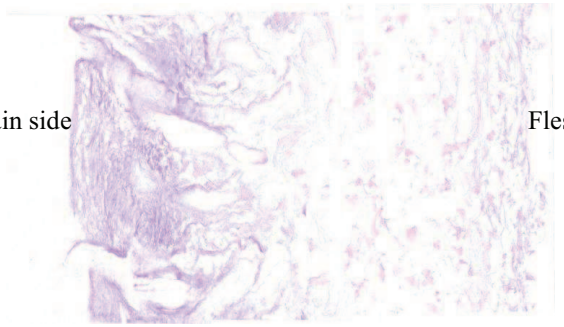

Figure 8: Elastin image of section after mathematical masking procedure to optimise elastin components and minimise non elastin magenta-coloured stained components as assessed by the features morphology. (Rotated 90° for illustrative purposes). Figure 9: Vertical profile of elastin density. Vertical axis represents the fraction of elastin density measured as a proportion of the total elastin colour density with 0% being no elastin colour detected and 100% being every pixel in the row being completely saturated with the elastin masked magenta stain.

Results and discussion

Measurement of elastin

Sections from the pickled pelts were analysed as described above and the results for the different factors are illustrated in figures 10-12:

As expected early season stock had lower amounts of elastin. The biggest difference being in the grain enamel. The increase in elastin measured between the two differently aged groups of pelts was significant for each depth in the skin ($p<0.05$).

Again levels of elastin were higher in the grain layer than in the enamel and corium layers

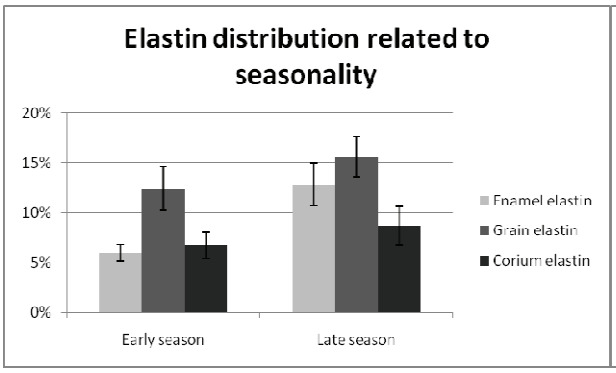

Figure 10: Impact of seasonality on elastin distribution in lambskins (n=40)

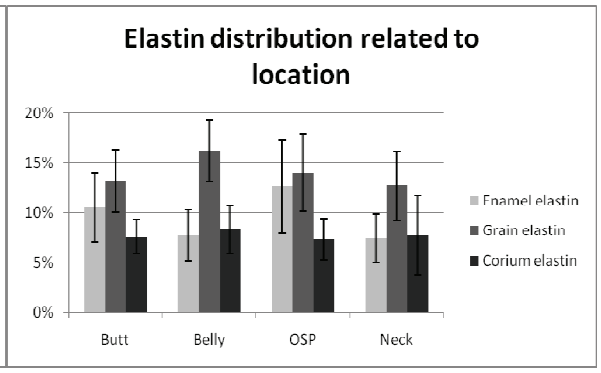

Figure 11: Distribution of elastin within and across lamb pelts (n=40)

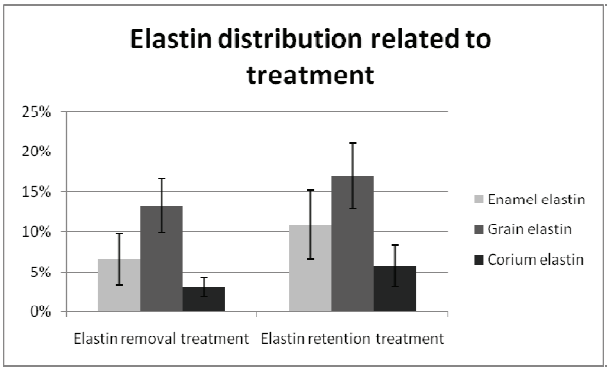

Figure 12: Effect of two different elastolytic treatments on the elastin distribution

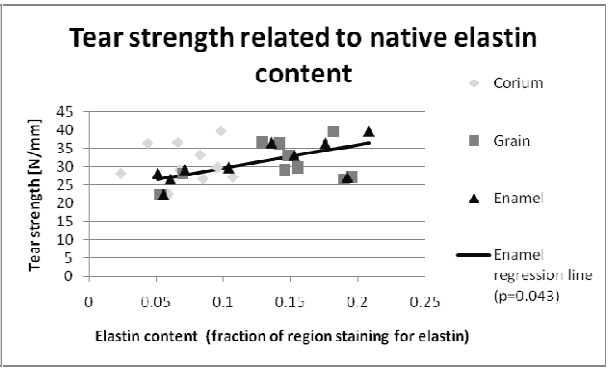

Figure 13: Impact of native elastin levels on tear strength.

The two elastolytic treatments applied to the pelts resulted in significantly different levels of elastin in all positions within the thickness of the skin (Figure 12). It is worth noting that analysis of the data suggests that the outer (enamel and corium) regions were affected to a greater degree, possibly due to processes such as bating acting over a relatively short period having more time to act on the elastin located closer to the surface.

Impact of native and residual elastin on physical properties of the crust leather

The skins from the two trials were examined for a selection of physical properties thought to be influenced by elastin and the physical results were correlated to either the native levels of collagen found from the early and late season stock or the levels of residual elastin found after applying processing regimes imparting different levels of elastolytic activity.

Strength

Tear strength was tested using the standard method (IUP_8, 2000) for the skins with a range of different native levels of elastin and the results are illustrated in figure 13. Different levels of native elastin do appear to influence the strength of the resulting leather. In particular, the level of elastin in the grain enamel correlated significantly with the resulting tear strength of the leather.
resultados se muestran en la figura 13. Parece realmente que un nivel diferente de elastina nativa afecta la resistencia del cuero resultante. En particular, el nivel de elastina en el “esmalte” de la flor correlacionaba significativamente con la resistencia al desgarro resultante del cuero.

Mottle

Mottle was assessed by comparing the regions of each skin (Neck, Belly, Butt) against a subjective scale for each region ranging from 1 (no mottle, completely flat) to 4 (an extreme mottle pattern). The results are illustrated in figure 14.

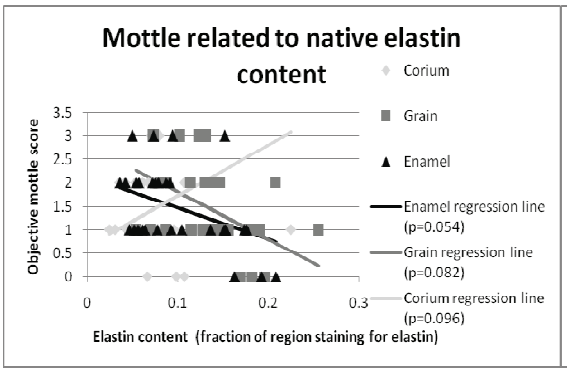

Figure 14: Impact of native elastin levels on mottle

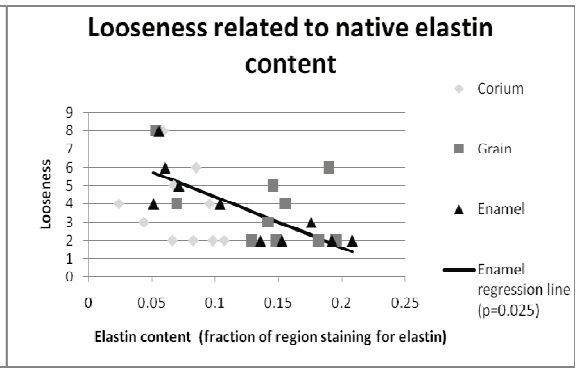

Figure 15: Impact of native levels of elastin on looseness

Multiple regression of this data was carried out and while the results are only significant to 90% it is worth noting the trends observed. Skins with higher levels of elastin near the grain surface correlated with a flatter leather whereas higher levels of elastin in the corium correlated with an increase in mottle. These findings support the concept that elastin in the grain surface serves to retain a flat shape in the skin during processing. The finding that elastin in corium served to increase the mottle is new and indicates that removal of elastin from the corium could be useful to improve flatness. For the first time work investigating the impact of elastin from different positions within the skin has lead to a possible conclusion that not just the level of elastin but its location is important for the physical properties of the resultant leather.

Looseness

Looseness was examined by manipulation of the leathers and direct comparison to the SATRA break scale where a score or 1 indicates a tight break or in the case of lamb leather low looseness up to a value of 8 indicating a loose break or in the case of lamb leather a loose leather. The results are illustrated in figure 15:

Analysis of the data as a whole indicated that overall a greater level of elastin in the skin resulted in a reduced amount of looseness (p=0.03) Further analysis using multiple regression of the data indicated that this effect was significant for the enamel (p=0.025).

Impact of variations in residual elastin after processing on leather physical properties

Strength

Tear strength was tested using the standard method (IUP_8, 2000) for the skins processed with two levels of elastolytic activity. There was no significant relationship found between tear strength and the level of residual elastin found after different levels of elastin removal.

Resistencia al desgarro

La resistencia al desgarro fue evaluada según la Norma IUP-8, 2000, para pieles procesadas con dos niveles de actividad elastolítica. No hubo una relación significativa entre la resistencia al desgarro y el nivel de elastina residual encontrada después de diferentes niveles de eliminación de elastina.

Mottle

Again mottle was assessed for the leathers generated with different levels of residual elastin by comparison of the leathers to an objective scale of mottle. The results showed no significant difference between skins which had more elastin removed in comparison to skins with less elastin removed. This finding is different from the impact of levels of native elastin on the generation of mottle and may be related to the point in processing at which mottle occurs. It is possible that mottle is actually formed during the highly swelling process of liming resulting in a permanent change in the structure.

The main elastolytic portion of the process occurs after liming via enzymatic removal of the elastin during delime and bating. It

therefore follows that if mottle occurs during the liming then changes in the elastolytic portion of the process would have less impact on the resulting mottle. It also suggests that attempts to modify the mottle result by targeting elastin in the grain at post-liming stages are unlikely to be successful since the damage has been done.

Looseness

Initial analysis of the data showed that greater levels of elastin resulted in greater levels of looseness (p=0.03) as shown in figure 16.

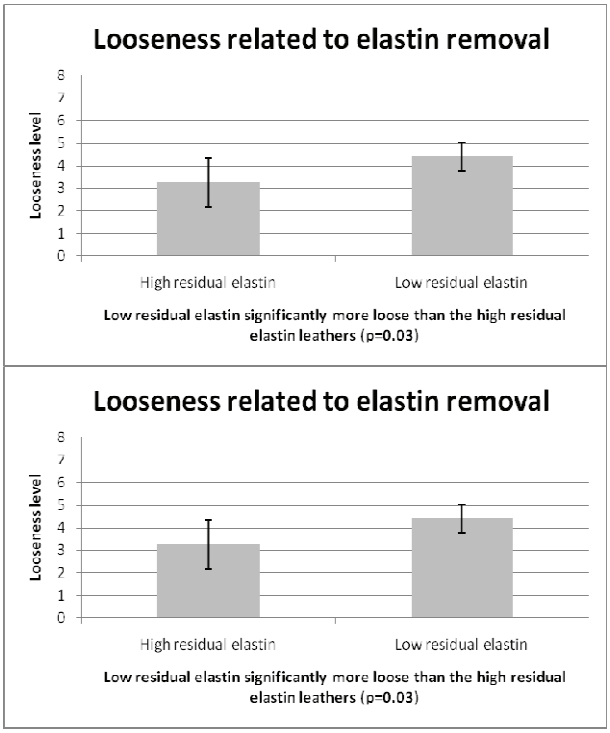

Figure 16: Impact of elastin removal on leather looseness

Further analysis using multiple regression indicated that high levels of residual elastin correlated with less looseness for each of the layers investigated. The trends for specific skin layers were not, however, statistically significant.

Suggested mechanism for the generation of mottle

Analysis of the elastin content in the different layers within lambskin has shown interesting results and supports the following possible mechanism for mottle and looseness formation as they relate to elastin. Analysis was done using both the different native levels of elastin and the differences induced by processing.

Mottle may be formed during the period of painting and pulling when the skins are at high pH and undergoing the stress of swelling. The presence of elastin may serve to restrict the swelling during this period resulting in less permanent change to the structure of the skin. Subsequently the elastin level has little effect on further mottle formation unless the skin undergoes additional swelling pressure. Further work investigating the specific forces involved in swelling and its retention by elastin fibres could shed more light on this interesting phenomenon.

Conclusions

The results indicate that mottle formation occurs in the liming process prior to the elastolytic process of bating. The highly swelling nature of the high pH achieved during painting and liming imparts stress to the collagen, particularly in the grain, which undergoes differential swelling with respect to the corium and in combination with elastin may result in the distinctive mottle pattern. The results indicated that higher levels of elastin in the grain serve to maintain a flat structure during this swollen period. Mottle appears to be caused by the swelling pressure to the grain surface during liming and the failure of elastin to retain flatness of the skin during that period. It is even possible that patterns of low levels of elastin found in the native skin may be what gives rise to the specific chicken wire pattern observed. After the swelling period is over the importance of elastin in mottle appears less, so removal during processing may be of lesser significance.

Looseness in crust leather however appears related to the amount of elastin retained in the crust leather throughout processing, regardless of whether the final residual elastin content is a result of initial native levels of elastin or a result of the levels of elastolytic processing.

This explanation of the effects of elastin in ovine skins explains many of the previous observations which initially appeared anomalous. It would be expected that skins with less native elastin would have greater levels of mottle since the skins would be both more prone to swelling and processing may

accentuate the native differences in elastin distribution. This has been observed.

It is already well known that proper removal of adhering flesh and fat is vital to ensure a flat pelt and it seems that the effect of mottle formation due to material on the flesh side continues with the elastin located at the flesh side also. It may therefore be possible to produce less mottle by designing a process which also removes the elastin found at the flesh side.

A further supposition is that differences in the native distribution of elastin between ovine and bovine skin would support the differences in elastin processing results observed between these two materials.

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