

## A Molecular Dissection of Quality

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### Abstract

The early stage processing of skins and hides is believed to have the greatest effect on structure and composition, as strong reducing chemicals are applied in high concentrations to assist in the removal of hair, non-collagenous proteins and other non-structural components. In this study a ‘proteomics’ approach has been taken to analyse the changes in the protein complement of ovine skin during the beamhouse process. Methods for the extraction of protein from ovine and other mammalian skins have been developed and validated and qualitative proteomics methods have been developed which have been shown to be effective in identifying proteins present in the different skin systems. To determine the loss of proteins at each stage of processing samples have been taken at predetermined stages throughout a conventional beamhouse/tanning process. Protocols have been developed to efficiently extract the protein from skin, and proteomic methods used to monitor the change in skin protein composition through early processing. In addition, similar methods were used to investigate differences in the protein composition of pickled skins from different animals, with a view to relating these to the physical properties of the resultant crusted-out skin. Results show that while collagen and keratin dominate the tissue samples, as expected, there was a surprisingly complex mix of proteins in the processed skin tissue sampled just prior to tanning. Comparison of the protein complement of pickled skins from different animal species showed that whilst the same proteins were dominant in all samples there were unexpected differences in their molecular weights, which may be indicative of differential protease activity in skin depending on species. This result is supported by the unexpected finding that endogenous protease inhibitors are present in skin samples just prior to bating. We are in the process of determining the significance of these proteins to the properties of leather, with the aim of developing specific processes that will enhance specific traits such as strength and appearance in the finished product.

### Introduction

Leather manufacture has long been considered both an art as well as a technological process. Part of the reason for this is the variety in the raw material provided to the leather technologist necessitating modifications to the protocols used for processing the skin. To move the leather

industry from an artisan based understanding where improvements in leather properties and performance happen slowly and incrementally to a situation in which large step changes can occur requires a fundamental understanding of the molecular changes that take place during the production of leather together with information on how these changes affect finished leather properties and performance.

New Zealand produces large quantities of sheep pelts that are processed into leather that because of its strength is only suitable for the garment industry. Raw sheep skins are however, strong, a property that appears to be lost during conventional processing. Much research has been carried out into the physical structure and arrangement of collagen fibres/fibrils of leather and the effects of this on strength (Chan et al. 2009; Sizeland et al. 2013). Less is understood about the chemical characteristics and molecular composition of leather. Although fibrous collagen is the major component of leather, other proteins are present albeit in lower concentrations. The identity of these minor components is not known, nor is their role in leather structure.

During the early stages of conversion of skin into leather by the conventional process (known as “liming”), the fibre network is opened up and unwanted skin components such as glycosaminoglycans (GAGs) and proteoglycans (PGs) are removed in order to produce the desired physical properties (especially softness and flexibility) of leather (Aldema-Ramos et al. 2012; Dettmer et al. 2012). It is possible, however, that some of these components may play a role in protecting the skin structure from physical damage during processing and that their early removal detrimentally affects leather properties such as strength. Once it is understood which aspect(s) of processing are responsible for the loss of strength and why, it should be possible to modify processing to maximise strength. Similarly a comparison of the protein complement of pickled hides from different animals may also identify proteins important for strength.

We have also compared the proteomes of raw and pickled (post-liming) sheep skins with those of pickled skins from other animals (cattle, deer and goats) frequently used for leather. The strength hierarchy of leathers from these species is sheep < cattle < goat < deer (Sizeland et al. 2013). We predict that differences in the pickled skin proteomes will reflect the differences in physical properties.

## **The skin**

The skin is one of the largest organs in a mammal consisting of a sheet that covers the whole body. It acts as physical barrier between the organism and the environment and is adapted to withstand desiccation in a dry environment as well as many mechanical, chemical, and microbial challenges. On top of this it contains a complex neuro-receptor network to communicate environmental information to the organism and in mammal's acts as a thermal regulatory system (Goldsmith 1983). There are two major tissue layers in the skin. The topmost layer is termed the epidermis, and the lower layer the dermis. The third layer, the hypodermis, is technically not part of the skin although for leather making purposes is considered part of the skin system.

The epidermis, the outermost layer of the skin consists of ‘cornified’ non-living cells, in which the dominant protein is keratin. During leather manufacture the epidermis is removed. In fact any epidermis present in the final finished leather is considered a defect in processing (Covington 2009).

The dermis is a fibro-elastic connective tissue consisting of collagen fibres, elastic fibres, and an interfibrillar network of glycosaminoglycans (Orgel et al. 2011). Interwoven among the bundles of collagen fibres is a network of elastic fibres which help restore the shape of skin following deformation by external pressures (Cain et al. 2008; de Vega et al. 2009).

The thin layer between the epidermis and the dermis called the basement membrane contains a complex assembly of type IV, VI, VII, IX, XV and XVIII collagens, laminin, and heparan sulfate proteoglycans.

The hypodermis is technically not a part of the skin, but for the purposes of leather manufacture it is considered to be part of the skin system. Its main purpose is to attach the dermis to the body. Like the dermis the hypodermis consists of bundles of connective tissue and elastic fibre networks (Jor et al. 2011). It also contains nerves and blood vessels. Fat is also stored in the hypodermis within lipocytes. In finished leather, the hypodermis is called the ‘corium’ layer. This loose interweaving structure provides the bulk of the leather and contributes most to the final strength of leather (Boote et al. 2002; Oleary and Attenburrow 1996).

The components of the skin that are thought to have the biggest effect on its properties are collagen, elastin, and glycosaminoglycans. Collagen is the main structural protein in skin being responsible for approximately 70% of its dry weight and for its strength (Prockop and Kivirikko 1995). The collagen superfamily of proteins currently contains at least 28 distinct members, although types I and III are the primary collagens present in skin (Fratzl 2008). Elastic fibres are responsible for the resilience of skin, permitting long-range deformability and passive recoil (Kielty et al. 2002; Muiznieks et al. 2010). The core of the elastic fibre is elastin, a polymer of monomeric tropoelastin. Non-elastin contributors to elastic fibres include fibrillins, fibulins, and microfibril associated glycoproteins which make up 10% of the elastic fibre (Cain et al. 2006).

Glycosaminoglycans are long unbranched polysaccharides of repeating disaccharide units. They are often (although not always) attached to a protein core forming a proteoglycan. Proteoglycans associate *via* the protein core with collagen fibrils (Lujan et al. 2007) and along with glycosaminoglycans, play a key role in regulating collagen fibril size during fibrillogenesis (Reed and Iozzo 2002). They also play a role in cross-linking collagen fibrils (Lujan et al. 2007) and are responsible for the viscoelastic properties of collagen (Lujan et al. 2009; Scott 2003).

## **Skin proteomics**

The proteomics of skin poses several challenges (Huang et al. 2005). Skin as a tissue is largely dominated by collagens and keratins both of which have repetitive sequences and are insoluble in aqueous solution. These two factors make proteomic analyses difficult. Additionally skin contains a significant population of glycoproteins which on their own can be challenging to analyse (Wilson and Bateman 2008). Considering how important the skin as an organ is there are surprisingly few papers using proteomics to examine the changes that occur within skin, or even to broadly survey the proteins present within skin.

While studies examining the proteome of skins are limited and have been mainly restricted to human and mouse skins, the use of proteomics in leather has received little attention except for investigations into the proteome of pickled skins (Choudhury et al. 2006; Edmonds et al.

2008). By looking at the proteomic methods used to analyse other extracellular matrix dominated tissues, the full complement of proteins in ovine skin may be identified allowing both analyses and comparisons to be made between skins of different animal species used to make leather as well as to follow the changes in skin components as skin is subjected to the processes necessary to change it into leather.

Proteomic extraction protocols were developed based on a strategy developed for cartilage (Wilson and Bateman 2008) and extracted proteins separated using two different techniques. The first of these was used to investigate the differences between pickled skins from different animal species and used a 2D approach that combined sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) with nano reversed phase HPLC (RPHPLC) and MSMS. The second method used nano-RPHPLC only and MSMS.

## Materials and Methods

Proteomics grade 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate (CHAPS), Tris(hydroxymethyl)aminomethane, thiourea, urea, iodoacetamide, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), dithiothreitol (DTT), sodium bicarbonate were purchased from Sigma Aldrich. Trichloroacetic acid (TCA) and protein molecular weight marker (Broad Range, prestained) were obtained from BDH Chemicals Ltd and BioRad, respectively. Mass spectrometry grade trypsin was purchased from Promega. Complete tablet was obtained from Roche Diagnostics (USA). Acetonitrile (MeCN) and dichloromethane (DCM) were from SDS chemicals (France). All other chemicals and solvents were analytical grade. Fresh sheep skin from New Zealand Romney cross lamb was used as the raw material for this experiment. All chemicals used for leather processing were commercial grade.

## Standard tanning procedure

Fresh ovine skins were obtained and pickled sheep pelts produced using the methods reported by Sizeland et al (2013). Other animal skins were processed from a commercial fellmongery. Samples for analysis were cut from the official sampling position as defined in ISO 2418:20002. All sheep materials were from New Zealand Romney cross.

## Gel analysis methods

Proteins were extracted using two different methods, to compare extraction efficiencies. For both methods, 100 mg of fresh or pickled animal skins were used.

**Method 1.** Finely chopped skin pieces were incubated in 5 volumes of 1M NaCl with mixing at 4°C overnight then centrifuged for 30 minutes at 16,000 x g at 4°C and the soluble fraction carefully removed (NS1). The insoluble fraction was re-suspended in the same volume of 6M urea and incubated with mixing at 4°C overnight. The clear supernatant (US2) was separated from the insoluble material by the centrifugation at 16,000 x g at 4 °C for 30 minutes.

**Method 2.** Finely sliced skin pieces were suspended in 5 volumes of lysis solution (7M urea, 2M thiourea, 4% CHAPS, 2% IPG buffer (pH 4-7), 40mM DTT, and Complete<sup>TM</sup> (wide spectrum protease inhibitor used according to the manufacturer's instructions) in 30mM Tris/HCl pH 7.5). After incubation with mixing at 4°C overnight supernatant (LS1) was separated from insoluble material by centrifugation (16,000 x g at 4 °C for 30 minutes). The

insoluble skin material was re-suspended in lysis solution for a second extraction and incubated with mixing at 4°C overnight. The clear supernatant (LS2) was again separated from the insoluble material by centrifugation (16, 000 x g at 4 °C for 30 minutes) (Berkelman and Stenstedt 1998).

**Protein precipitation.** 9 volumes of cold (-20°C) TCA/Acetone (25% TCA in acetone) were added to the supernatant from each extraction (LS1, LS2, NS1, and US2). The mixture was incubated at -20°C overnight then centrifuged for 30 minutes at 16,000 x g at 4°C. The supernatant was discarded and the pellet washed twice with cold acetone, then air-dried until acetone was completely evaporated. Protein was then dissolved in sample buffer containing 7M urea, 2M thiourea, 4% CHAPS, 40mM DTT, and protease inhibitor (Complete™) in water. Protein concentrations were determined by the modified Bradford method using BSA as the standard.

## 1-D SDS-PAGE

5-10µg of animal skin proteins were loaded on 1D 4-20% gradient gels (Bio-Rad) to analyse proteins in different animal skin samples. Protein molecular mass markers between 5 and 250 kDa were used for calibration. Separation was achieved on a Mini Protean II (Bio-Rad) at 150V for 5 minutes then at 200V until the dye front reached the bottom of the gel. The proteins were visualized by staining with colloidal Coomassie blue (Berkelman and Stenstedt 1998).

## In-gel digestion

The protein bands were manually excised from 1D SDS gels using a clean scalpel blade and transferred into 0.5mL LoBind Eppendorf tubes, destained with 50% methanol and 5% acetic acid at 37°C, dehydrated in acetonitrile, and air-dried. Dried gel pieces were then reduced in 10mM DTT in 100mM ammonium bicarbonate for 1 hour and alkylated in 100mM iodoacetamide in 100mM ammonium bicarbonate in dark place for 1 hour. After dehydrating in acetonitrile and air-drying, gel pieces were rehydrated with digesting solution (1mM calcium chloride in 50mM ammonium bicarbonate) for 10 minutes, before again being dehydrated by acetonitrile and air-dried. The gel pieces were finally rehydrated in digesting solution, containing 600 ng of trypsin (mass spectrometry grade, Promega), on ice for 10 minutes, then incubated overnight at 37°C. Digestion was stopped and peptides extracted by the addition of 0.5% formic acid in 50% acetonitrile and the mixture was concentrated under vacuum (Savant Speed-vac, SC-100) to give a final volume of ~50µL. The concentrate was then stored at -80°C until further use.

## LC-MS/MS analysis

For LC-MS/MS, samples of 8 µL were injected by auto-sampler (Agilent 1260 Infinity High Performance Micro Autosampler) held at 4° C into an Agilent 6520 nanoflow HPLC-Chip Q-TOF mass spectrometer using an Agilent ProtID-Chip 43 (II) comprised of a 40 nL enrichment column and a 43 mm 300 Å C<sub>18</sub> nano column. The mobile phase was comprised of Solvent A (water with 0.1% formic acid) and solvent B (90% acetonitrile, 10% water with 0.1% formic acid). Each run comprised the following steps:

**Table 1.** Nanoflow pump solvent and flow gradient for gel extracted peptides.

Time (minutes)	%B	Flow nL/min
0	0	400
35.0	65	400
37.0	65	400
37.5	95	400
44.5	95	400
45.0	15	400
55.0	0	400

### Protein identification and data analysis

Mass data was converted to m/z data using Agilent Mass Hunter B. 04.00 software, and proteins were identified with Mascot Daemon (sys-mascot.wehi.edu.au) using a SwissProt protein database search for tryptic peptides in all *Mammalia*. A mass tolerance of  $\pm 20$  ppm for MS and a tolerance of  $\pm 50$  mmu for MS/MS were used and one missed cleavage was allowed. Carbamidomethylated cysteines were set as a fixed modification and oxidized methionine, lysine, and proline as variable modifications. All matched results showed a false discovery rate below 5% with at least 2 independent peptide identifications for protein IDs. Only proteins that were observed in 2 different samples were considered for further analysis.

### Processing trial

Twenty skins were obtained fresh. Experiments were conducted at LASRA's pilot tannery using the dose drums. Samples of skin and liquor were taken throughout the process for each individual skin and stored frozen for further proteomic analysis. After pickling all skins were combined together and processed to crust using the standard LASRA process. The final leather was assessed for subjective properties and sampled for physical testing (tear strength).

### Protein extraction

The protein extraction protocol was based on a protocol developed for proteomics on cartilage (Wilson and Bateman 2008) and produced three different extracts: a high salt soluble fraction (S1), a high UREA soluble fraction (S2), and an extract resulting from Cyanogen Bromide solubilisation (C1). Sampling points were at the following stages: 1, green skin; 2, dewooled slat; 3, initial liming (60 minutes); 4, final liming (overnight); 5, washout of lime; 6, delimed/bated; 7, wash out of bate; 8; pickled pelt.

5 mg of freeze dried ground skin (or 50 mg of wet skin) in 1500  $\mu$ L of extraction buffer A (1M NaCl, 100 mM ammonium bicarbonate) were homogenised by the action of  $\sim 0.7$  mL of acid-washed glass beads in a 2 mL screw-capped Eppendorf tube on a bead mill (Fast prep 120 cell disruptor, Thermo Savant, Qbiogene, USA) at 4° C (speed 4, 20 sec x 3). Proteins were extracted by constant mixing in the extraction buffer at 4°C overnight. Tubes were then centrifuged at 16,000 x g at 4°C for 10 minutes and the supernatant (S1) carefully removed. The protein concentration was measured using the Bradford assay, following the manufacturer's instructions and retained. A further 1000  $\mu$ L of 100mM ammonium bicarbonate was added to the tube and mixed by vortexing. Insoluble material was pelleted

by centrifugation at 4°C (16,000 x g for 20 minutes) and the supernatant carefully discarded. The pellet was washed once more following the same procedure before being subjected to a second extraction.

1000 µL of extraction buffer B (8M urea, 100 mM ammonium bicarbonate) was added to the pellet which was re-homogenised using the same conditions as above. After overnight mixing at 4°C insoluble material was pelleted by centrifugation at 4°C (16,000 x g for 20 minutes) and the supernatant carefully removed, labelled (S2) and retained. Protein concentration was measured using the Bradford assay. The pellet was washed in water as in the first extraction before being subjected to cyanogen bromide (CNBr) digestion.

The Eppendorf tube was wrapped in foil to exclude light, then 500 µL of formic acid was added and the tube purged with nitrogen before the addition of 5 µL of 1g/mL CNBr in formic acid. After O/N incubation in the dark at ambient temperature with constant mixing, the reaction was halted by the addition of 500 µL of ice cold 30% ammonia solution. The mixture was centrifuged at 16,000 x g for 20 minutes at 4°C and the supernatant carefully removed and labelled (C1). The protein concentration was measured using the Bradford assay as above. 900 µL of ice cold ethanol was then added to 100 µL of recovered supernatants containing 20-50 µg of protein and incubated at -20°C for 1 hour to precipitate the protein from solution.

The protein was pelleted by centrifugation at 16,000 x g for 20 minutes at 4°C, the supernatant removed and discarded, and any remaining ethanol removed by Speed-vac (Savant, SC-100). 20 µL of 50 mM ammonium bicarbonate, 1 mM CaCl<sub>2</sub> was added to the dried pellet and mixed gently then heated in a boiling water bath for 5 minutes to reduce and solubilise the protein mixture. 5 µL of 200 mM iodoacetamide in 50mM ammonium bicarbonate was then added and the solution gently mixed, then incubated for 1 hour at ambient temperature. 20 µL of 50 mM dithiothreitol (DTT) in 50mM ammonium bicarbonate was added to consume any un-reacted alkylating agent, mixed gently and incubated for 1 hour.

After this time 10 µL of trypsin solution (100 ng/µL in 50 mM ammonium bicarbonate, 1 mM CaCl<sub>2</sub>) was added and the mixture incubated with gentle shaking at 37°C overnight. Digestion was halted by adding 5 µL of 10% formic acid. The sample was centrifuged at 16,000 x g for 20 minutes at 4°C to remove any insoluble material and the supernatant transferred to HPLC sample vials.

### Mass Spectrometry

Using an Agilent qTOF / ChipCube a 45 minute LC/MSMS method (Table 2) was used to analyse each extracted fraction and the resulting spectra processed as to identify proteins present.

**Table 2.** Nanoflow pump solvent and flow gradient for solution extracted peptides.

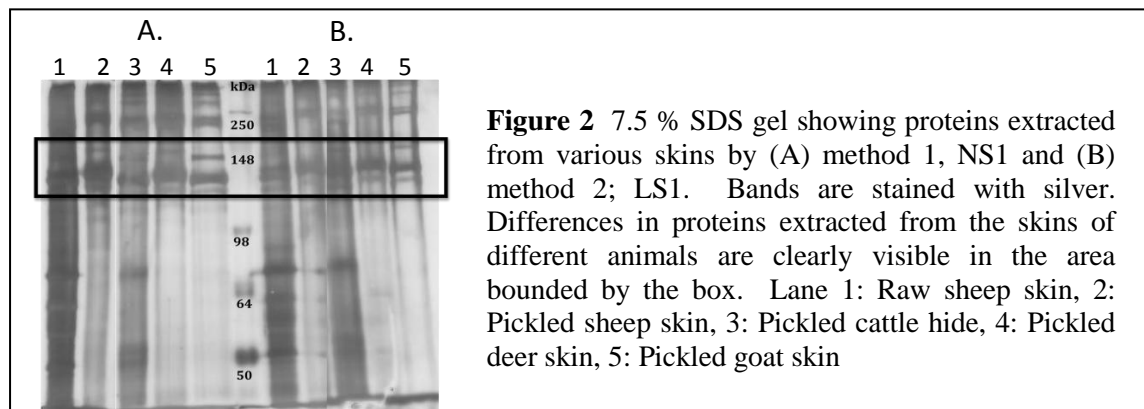
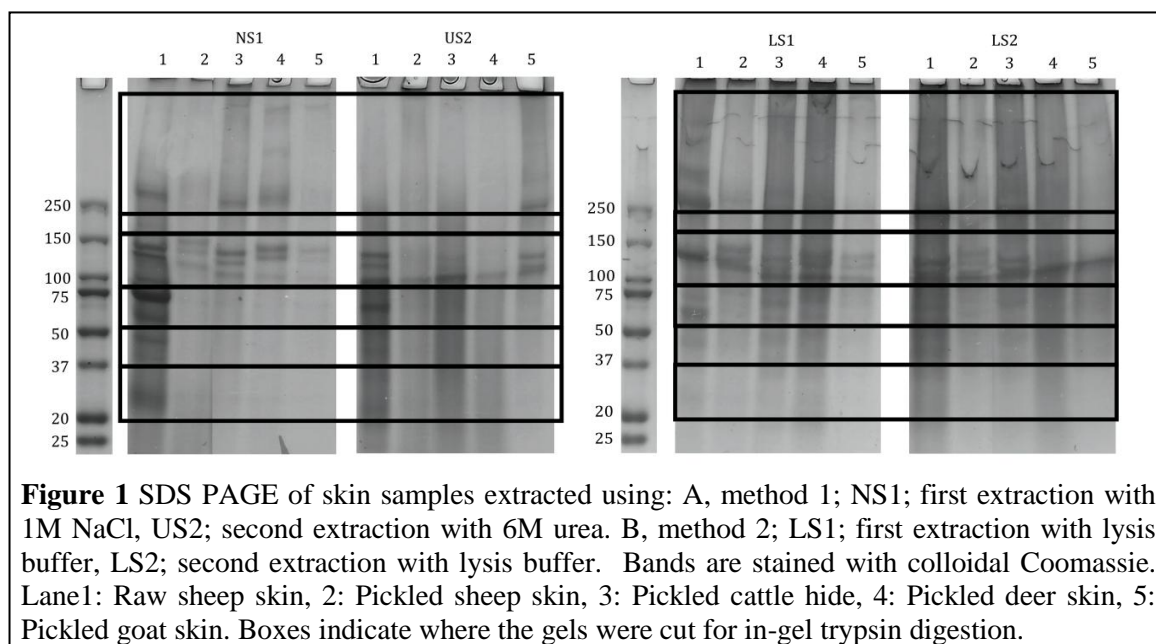
Time (minutes)	%B	Flow nL/min
0	8	400
30.0	45	400
30.5	90	400
34.0	90	400
34.5	8	400
45.0	8	400

## Results and Discussion

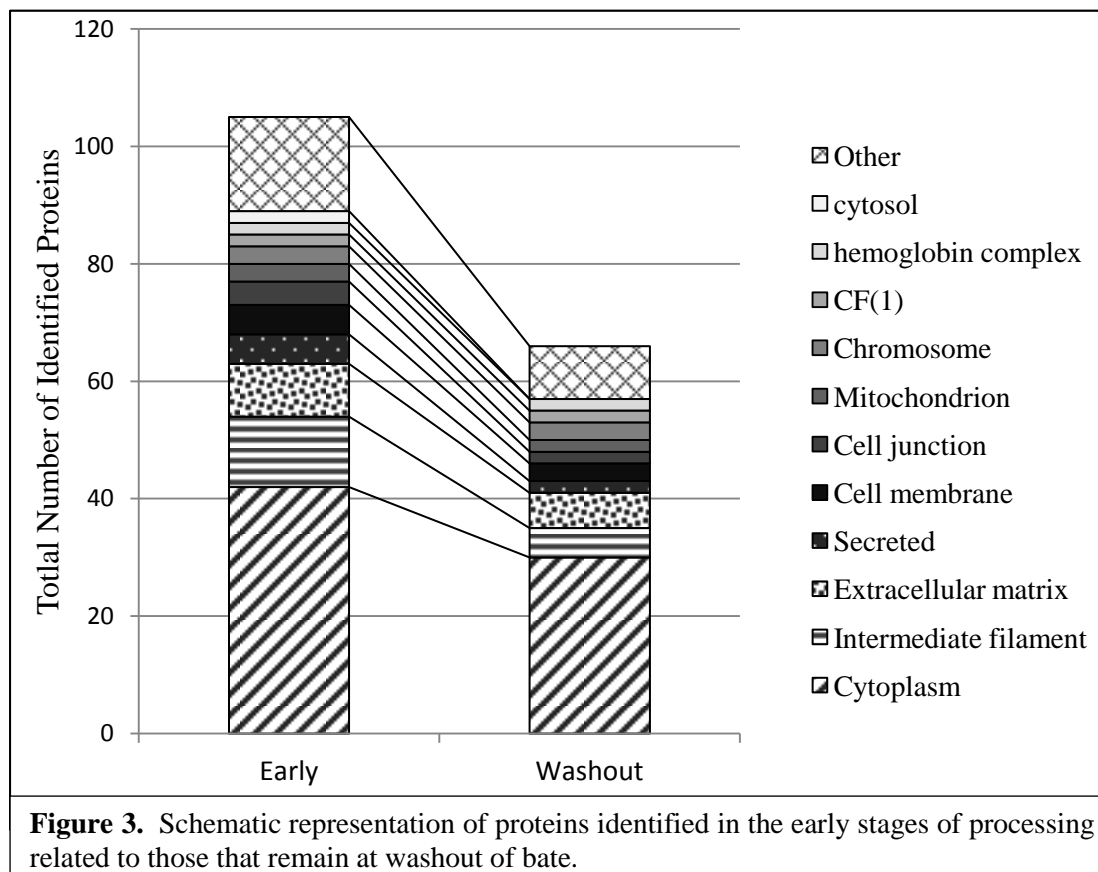
The SDS PAGE analysis of skin proteins from the pickled skins of sheep, cow, goat and deer showed differences in their banding patterns for all four extractions. (Figure 1) Perhaps the most noticeable were the differences between samples extracted with NaCl (NS1) and urea (US2).

When gels were stained with the more sensitive silver stain, these differences were more easily visualised as shown in Figure 2 which compares samples from the different skins from the first extraction of method 1 (NS1) and the first extraction of method 2 (LS1).

These differences were corroborated by the mass spectrometry results which showed that although bands in the gels appeared to have different molecular weights, they belonged to the same proteins (Table 3). This table clearly shows that pickled sheep skin, the weakest of the four skins analysed appears to be deficient in Collagen  $\alpha 1$  type (II) peptides implying that the protein has been removed from the pickled skin during processing. In contrast there seem to be a greater number of keratin type I and type II peptides in pickled sheep skin compared to pickled cattle and more particularly pickled goat and deer skins, skins that produce stronger leather than ovine skin.



There could be numerous reasons for this; there may be differences in the grain to corium ratio for each skin, or there could be genuine differences in the way the different “hair/wool” fibres respond to the lime/bate process. Further work will be necessary to resolve the molecular bases of these observations. Apart from keratin type II cytoskeletal 7, 73 and 79, few keratin peptides were identified in samples from goat and deer skins, implying that they may be detrimental to leather structure.



The differences in the distribution of molecular weights of these keratin peptides is also interesting bearing in mind that they had not been exposed to any exogenous protease when analysed by SDS PAGE. It is well known that proteases are present in skin where they play roles in remodelling, inflammation and disease. While there was no evidence of proteases in the raw skin samples, there was evidence of the presence of endogenous protease inhibitors alpha-1-antiproteinase, and various serpins which are serine protease inhibitors. It is therefore possible that the difference in molecular weights observed for some of these proteins in the gels may be due to differential processing of proteins in the skin system that is species specific. The impact of differently processed proteins on the fibrillar structure of the skin is not known, but will be an area of investigation in the future.


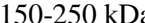

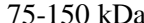

Analysis of the proteins removed from skin during processing yielded some surprises. It has long been assumed that the quality of leather relies on an almost complete removal of all proteins other than collagens before tanning. Our results clearly show that there are a significant number of proteins left in the skin at the later stages of processing. 105 proteins were identified in raw skin and at the early stages of processing in the processing trial. These could be broken down into the categories shown in figure 3. At the late stages of processing

or after wash-out of bating, there were still 66 proteins left in the skin samples. Some of the more unexpected and interesting of these were  $\alpha$ -1-antiproteinase, cystatin-B and serpin H1, all protease inhibitors. Two of these were also identified in pickled skin using SDS PAGE and it is interesting to speculate on why they are present, and their effect on bating.

An interesting observation in comparing early versus late processing is the disappearance of proteoglycans, which are known to modulate collagen properties and have been linked to changes in quality, during processing (Aldema-Ramos et al. 2012). It is not yet clear if the presence of specific GAGs is desirable in processed leather, but if it is found to be so, fine tuning of bating solutions may be required to ensure it is not removed during processing.

Protein	Sheep	Cattle	Goat	Deer
Actin (smooth muscle)				
Actin cytoplasmic				
Collagen $\alpha$ 1(I)				
Collagen $\alpha$ 2(I)				
Collagen $\alpha$ 1(II)				
Collagen $\alpha$ 1(III)				
Decorin				
Lumican				
Mimecan				
Keratin type (I) cytoskeletal 10				
Keratin type (I) cytoskeletal 14				
Keratin type (I) cytoskeletal 15				
Keratin type (I) cytoskeletal 17				
Keratin type (I) cytoskeletal 19				
Keratin type (I) microfibrillar 47.6 kDa				
Keratin type (I)microfibrillar component 8C-1				
Keratin type (II)cytoskeletal 5				
Keratin type (II)cytoskeletal 7				
Keratin type (II)cytoskeletal 71				

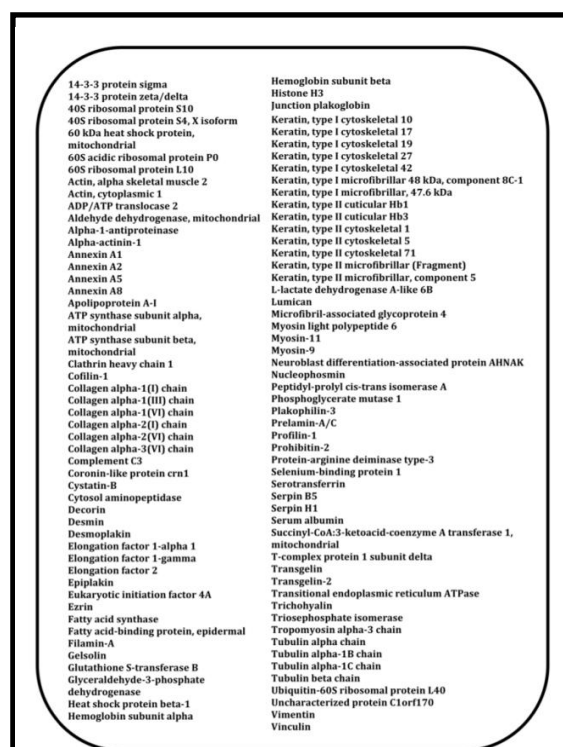
Keratin type (II)cytoskeletal 73				
Keratin type (II)cytoskeletal 75				
Keratin type (II)cytoskeletal 79				
Keratin type (II) microfibrillar fragment				
Keratin type (II) microfibrillar component 7C				

**Table 3.** Distribution of peptides from proteins present in pickled skins of sheep, cattle, goat and deer. The molecular weight distribution of the peptides is as follows.  $M_{wt} > 250$  kDa; , 150-250 kDa;  75-150 kDa;  50- 75 kDa;  37-59 kDa;  <37 kDa

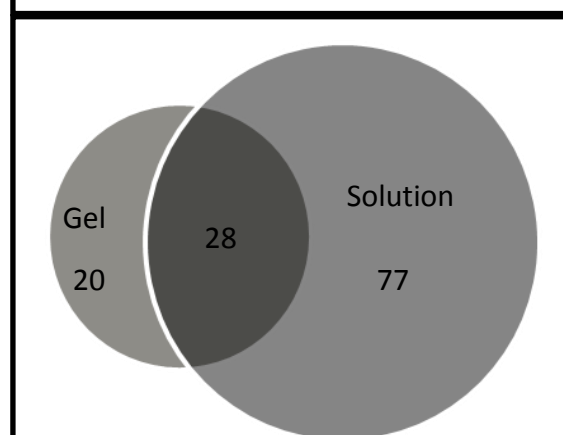
A list of all the proteins found in raw (early stage) ovine skin using both the SDS PAGE gel and solution approaches is shown in Figure 4. Elastin, which is known to be present in skin, is conspicuous by its absence. This is because of the high stability of elastin which makes it impervious to this type of proteomic analysis. Venn analysis of proteins identified in ovine skin using the two methods described in this work shows the value of such an approach (Figure 5). Here it is shown that only 28 proteins were identified by both methods out of the total of 125; the remaining 97 were exclusively identified by only one technique.

## Conclusions

Using gel based proteomics we compared the proteomes of pickled skins from different species. While SDS PAGE showed different banding patterns for each of the skins, analysis of the peptides produced from the bands showed remarkably little variation in protein type. One interpretation of these results is that individual proteins are being differentially processed “in skin”, to produce proteolytically modified proteins of different sizes that are species specific and which may impact on overall skin structure. There was a larger number of keratin peptides in the so called ‘weaker’ skins, which may be a consequence of the leather-making process on the wool/hair assembly of the different animal species. It is possible that keratins and fragments of keratins interfere with



**Figure 4.** Total proteins identified in raw (early stage) ovine skin from both RPHPLC and SDS-PAGE / RPHPLC methods



**Figure 5.** A Venn analysis of the distribution of proteins from ovine skin identified from solution and gel based proteomics

the structure and arrangement of collagen fibres, affecting their alignment and therefore the strength of the skin. Interestingly only one proteoglycan was detected in the pickled skins of sheep, cow and deer, but not goat, implying that the presence of proteoglycan's do not lead to increased strength. Also noted was the apparent complete absence of collagen  $\alpha$  1 type (II) from pickled sheepskin and the different molecular weight distributions of this protein in the other skins. The significance of this finding is not yet clear.

Using a proteomics protocol developed specifically for skin, 105 proteins were identified in tissue samples sampled throughout the leather making process. The number of proteins identified was reduced from 102 to 66 (just prior to pickling) then to 22 in pickled skin. Although collagens and keratins dominate these reduced subsets of proteins there is still a significant number of muscle and blood related proteins present at late stage processing, suggesting that leather is more complex than a simple collagen matrix. Proteoglycans, which are known to modulate collagen properties, were not detected after the bating process using the solution approach, although they were detected in pickled skin using the gel approach. Another interesting observation was that protease inhibitors were shown to persist in the skin right up to the bating process. The role(s) that these may play in modulating bating enzymes and endogenous skin enzymes remains unclear as does their influence on skin structure. This work was begun with the aim of identifying the contribution(s) of different proteins in leather to leather properties. It has however posed more questions than it has answered, providing a focus for future research as we work towards understanding the molecular determinants of skin and leather properties.

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