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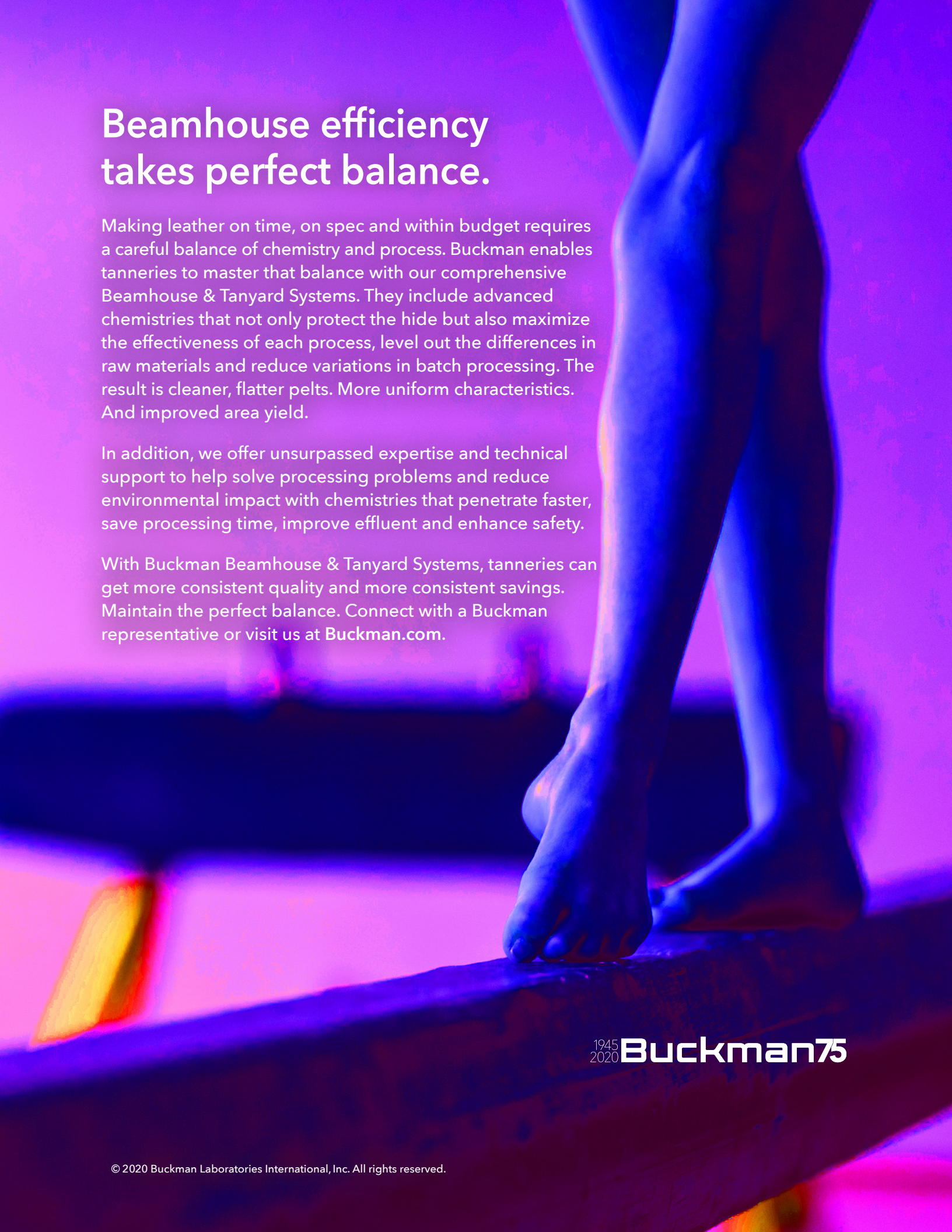

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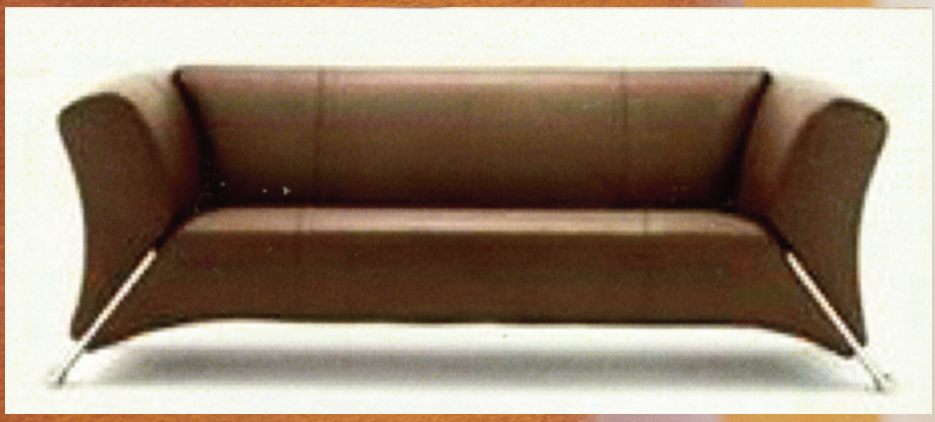

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Comparative Analysis of the Proteomic Profile of Cattle Hides that Produce Loose and Tight Leather using In-Gel Tryptic Digestion followed by LC-MS/MS

by

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Abstract

Looseness is a defect found in leather that reduces its quality by causing a wrinkly appearance in the finished product, resulting in a reduction in its value. Earlier studies on loose leather using microscopy and Raman spectroscopy reported a change in the collagen structure of loose leather. In this study, proteomics was used to investigate the possible molecular causes of looseness in the raw material, the first time such a study has been carried out. Proteins extracted from two regions of raw hide using two different methods were analysed; those taken from the distal axilla, an area prone to looseness, and those taken from the backbone which is less prone to looseness. Analyses using 1DE-LC-MS/MS showed that although the overall collagen concentration was similar in both areas of the hide, the distribution of the different types of collagen differed. Specifically, concentrations of type I collagen, and the collagen-associated proteoglycan decorin were lower in samples taken from the distal axilla, symptomatic of a collagen network with excess space seen for these samples using confocal microscopy. This study suggests a possible link between the molecular components of raw cattle hide and looseness and more importantly between the molecular components of skin and skin defects. There is therefore potential to develop biomarkers for looseness which will enable early preventative action.

Introduction

Leather is a durable and flexible material that is made by tanning degradable animal skins or hides (by-products of the agricultural industry), to produce a material that is stable and no longer subject to bacterial degradation.[1] The product is classed as high value and is used to make clothing, footwear and furniture.[1, 2] Cattle hide, most commonly used to make leather, is one of the biggest exports in New Zealand, reaching a total of NZD $353 million annually according to the 2018 Meat Industry Annual Report.[3]

Looseness is a defect found in cattle hide that causes a wrinkly appearance in the finished leather resulting in reduced leather quality.[4-6] Previous studies have investigated looseness in cattle hides using a combination of microscopy,[4, 6] small angle X-ray scattering,[4] ultrasonic imaging[5] and Raman spectroscopy[7] on wet blue or finished leather samples. In both these studies the hides have undergone a process designed to remove the hair and most of the non-collagenous proteins from the hide involving extremes of pH. Studies by Wood and Wells *et al.*[4, 8] showed there is a larger separation between the fiber bundles in loose leather while Wells *et al.* and Liu *et al.*[4-6] reported that loose leathers have a gap between the grain and corium layers that is absent in tight leathers. At the molecular level, Mehta *et al.*[7] detected differences in protein and lipid Raman fingerprints of loose and tight wet blue samples. It is accepted that defects in the hide can result from scarring and insect infestation, careless preparation of hides and skins for tanning such as flay-cuts and gouges, putrefaction, heat damage or poor tanning practices during tanning processing.[2, 9] It is also possible that defects are due to a change in the molecular components of hide caused by poor nutrition, stress, disease or genetic factors.[10]

Cattle hide is made up from many different macromolecules. The most abundant of these is protein, with collagen accounting for more than 70% of hide total dry weight.[11, 12] Proteins provide the structural scaffold that makes up hide and is directly responsible for all of its biological functions.[13-15] Collagen, elastin, proteoglycans and glycoproteins are the predominant proteins in hide, and have a significant and known impact on leather quality.[1, 2] However, due to the rapid advances in mass spectrometry (MS) and proteomic techniques a wide range of other proteins have been identified in low concentrations in animal skins, including that of humans. Such studies have shown that a wide range of proteins are affected by disease, ageing and stress.[16-19]

Proteomics is more frequently being used as a tool to identify biomarkers in animals for a range of conditions including infectious diseases such as mastitis, metabolic disorders and the presence of banned compounds in meat and milk.[20, 21] However, to the best of our knowledge only two studies have been published using proteomics to link different molecular components to leather quality.[22, 23] Both of these studies focused on sheepskin rather than cattle hide and no previous publication has used proteomics to investigate potential biomarkers for looseness in cattle hide.

This study used one dimensional gel electrophoresis with liquid chromatography and tandem mass spectrometry (1DE LC-MS/MS) to investigate the proteomic profile of two different regions of raw

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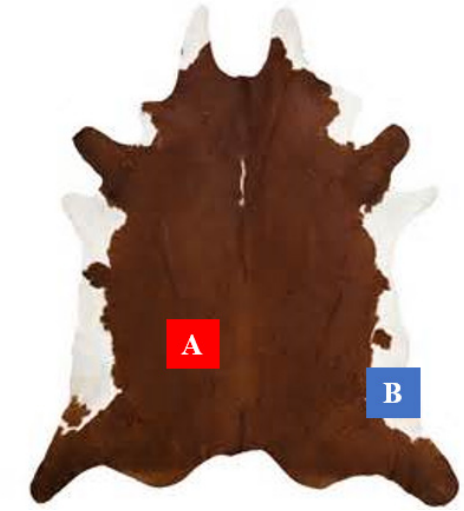

Figure S1. Diagram illustrating the location of the two sampling regions **(A)** official sampling position (OSP) and **(B)** distal axilla (DA).

cattle hide; the official sampling position (OSP) which is located near the lower backbone and the distal axilla (DA) which is located in the rear armpit as shown in Figure S1.

These regions have shown variations in mechanical properties,[24] such as laxity and extensibility [24, 25] as well as their appearance, pH, temperature, moisture content and microbiome.[26] Furthermore, prior studies have shown that the DA region is more prone to looseness than other areas of the hide.[4]

Proteomic studies on the total protein composition of tissues has been traditionally carried out using either gel-based (2D- and 1D-gel LC-MS.MS) or gel-free (1D- and 2D-LC-MS/MS (MudPIT) methods.[27] 1D-gel LC-MS/MS was used in this study because it has been shown to enable large-scale analyses of biological systems[28, 29] and was shown to result in a higher number of detected peptides in skin samples in preliminary experiments (results not shown). As hide is known to be very difficult to solubilise,[30] two different protein extraction methods were used prior to proteome analysis. One used a traditional lysis buffer whilst the other used a high salt extraction followed by urea extraction.

The discovery of a correlation between the proteomic profile of tight and loose cattle hide will enable a test for defective hides to be developed as well as adding to the skin proteome bank of knowledge about skin proteins and their changes during development, appearance and disease.

Experimental

Chemicals

All chemicals used for trypsin digestion and analysis were mass spectrometry grade (Optima® LC/MS) chemicals purchased from Fisher Scientific. Exceptions to this include; MS grade Trypsin Gold purchased from Promega; Wisconsin, USA, cOmplete® protease inhibitor tablets from Roche Diagnostics; Mannheim, Germany. DL-Dithiothreitol (DTT) from Gold Biotechnology; USA and iodoacetamide, urea and thiourea from GE Healthcare; Buckinghamshire, UK. Coomassie blue G-250 and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) from Biorad; California USA. The following chemicals were purchased from Sigma Aldrich; St. Louis, USA; glass beads (acid washed) and norleucine and stock amino acid standard solution containing 2.5mM of each amino acid except proline and hydroxyproline at 12.5mM and cystine at 1.2mM. 6-Aminoquinolylcarbamyl (AQC) from Synchem, Germany. All other chemicals were analytical grade.

Sample Preparation

Four raw hides obtained from Tasman Tannery; Whanganui, NZ were cut in half and 3 samples cut from the OSP and DA region of one half of each hide, then stored at -20°C for later analysis. The other half of the hide was processed to finished leather using conventional methods.[4] Looseness was measured using the SATRA STD 174 break/pipiness scale (SATRA Technology; Northampshire, UK) which consists of a graded selection of leather replicas numbered one to eight with one having the least severe wrinkles and eight having the most severe.

Protein extraction

Raw hide samples were shaved to remove the hair, then cut into approximately 1 cm^2 blocks. These were sliced into 10µm thick sections using the Leica CM 1850 UV cryostat (Leica Biosystems; Wetzlar, Germany) and approximately 50mg of grain, grain to corium junction and corium layers were collected as shown in Figure S2.

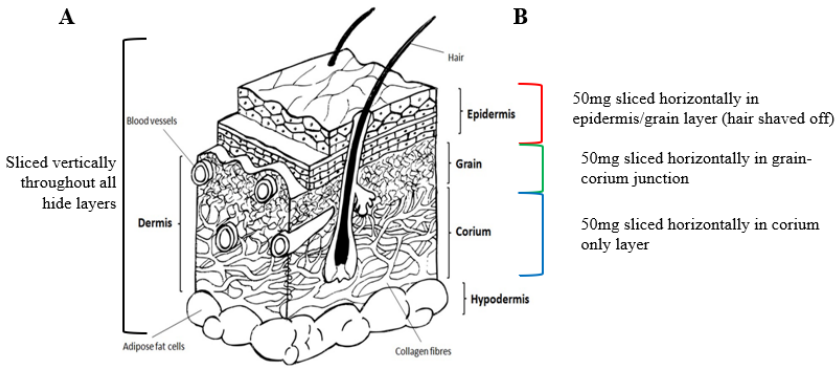

Figure S2. Diagram of how the hide was sliced **(A)** for confocal microscopy images (60µM thick slices) and **(B)** for protein extraction (10µM thick slices).

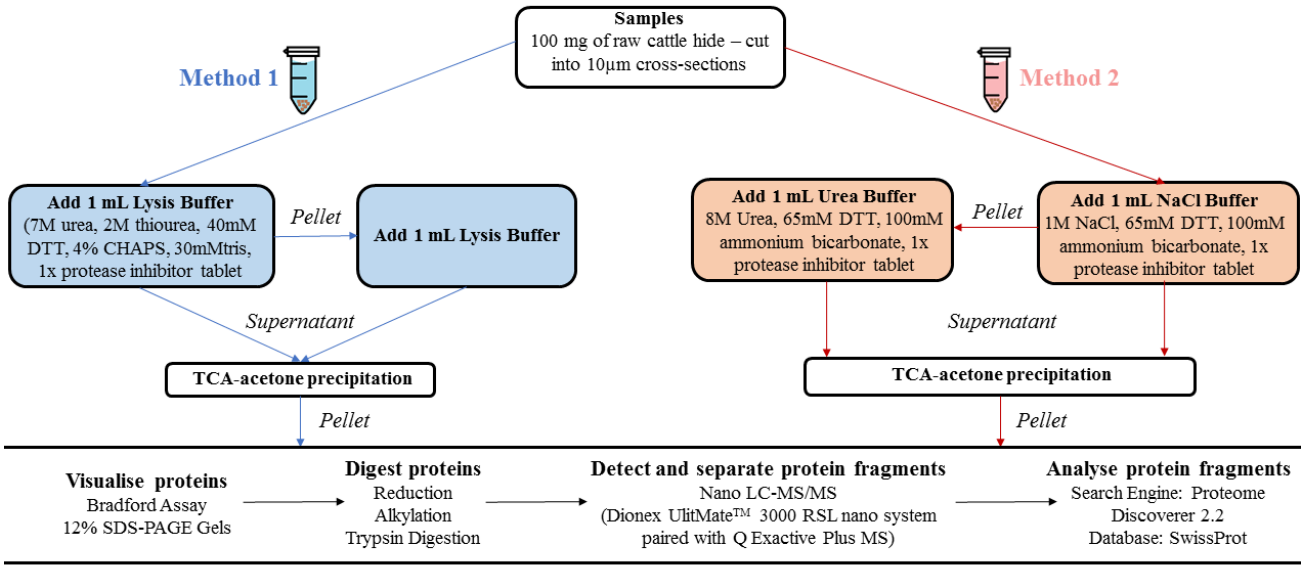

Figure 1. Flow diagram of protein extraction from cattle hide samples followed by in-gel tryptic digestion and LC-MS/MS analysis.

The samples were placed in 1.5 mL Eppendorf tubes then immersed in extraction buffer; either Lysis (7M Urea, 2M thiourea, 40mM DTT, 4% CHAPS, 30mM tris and 1x cOmplete® protease inhibitor tablet used according to the manufacturer's instructions, pH 7-9) or NaCl (1M NaCl, 65mM DTT, 100mM ammonium bicarbonate and 1x cOmplete® protease inhibitor tablet, pH 8) for 24 hours at 4°C. The extraction was aided by mechanical action provided through adding glass beads to each tube which was then placed on a rotating wheel (LABNET, USA) overnight. After this time, residual hide was removed from the protein solution by centrifugation at 16,500 x g for 30 minutes. The pellet was then treated with a second lot of extraction buffer either a repeat of lysis buffer or Urea buffer (8M Urea, 65mM DTT, 100mM ammonium bicarbonate and 1x cOmplete® protease inhibitor, pH 8) as shown in Figure 1. The supernatants from both lysis buffer extractions were pooled as were the supernatants from the sequential extraction and the proteins precipitated by the addition of 25% TCA in acetone in a (v/v) ratio of 1:9. After incubation at -20°C for at least 2 hours precipitated proteins were pelleted by centrifugation at 5,000 x g for 20 minutes, and the resulting pellets washed 3 times in cold acetone before being resuspended in the minimum volume of sample solution (7M Urea, 2M thiourea, 40mM DTT, 4% CHAPS and 1x cOmplete® protease inhibitor tablet, pH 7-9).

Protein digestion

The concentrations of the samples were measured using the standard Bradford assay protocol.[31] An equal volume of sample was mixed with the same volume of sample loading buffer (10% (v/v) SDS, 50% (v/v) glycerol, 100mM DTT, 0.25M Tris-HCL, 0.05% (w/v) bromophenol blue) and run on 12% Tris-glycine SDS-PAGE gels at 150V for approximately 90 minutes alongside precision plus protein™ dual xtra standards ranging in molecular weight from 250kDa to 20kDa from BioRad. Following electrophoresis, the gels were fixed in ethanol: acetic acid (40:10 (v/v)) for 15 minutes before being stained overnight with Colloidal Coomassie brilliant blue G250.[32]

Each lane was manually cut out of the gel using a sterilised scalpel blade then sliced into 6 even pieces (Figure S3). After cutting each band into small pieces, they were destained using 50% methanol, 5% acetic acid and dehydrated in 200µL acetonitrile. The gel pieces were air dried before being reduced by the addition of 50µL of 10mM DTT in 100mM ammonium bicarbonate. After 1 hour at room temperature, the solution was removed and replaced with 50µL of 200mM iodoacetamide in 100mM ammonium bicarbonate and the tubes incubated for 1 hour at room temperature in the dark. After this time the alkylating solution was removed, and the gel pieces washed in acetonitrile and dehydrated as before. They were then rehydrated and subjected to in-gel digestion with 6 µL 100µg/mL MS grade Trypsin Gold in 50 µL ammonium bicarbonate, 1mM $CaCl_2$, 10% (v/v) acetonitrile at 37°C overnight. The supernatant was carefully removed from the gel pieces and placed in a Lo-Bind Eppendorf tube. Any trapped peptides were extracted from the gel pieces by sonication in 40µL 50% (v/v) acetonitrile, 5% (v/v) formic acid. The resulting supernatant was added to the first before being concentrated to a final volume of 20µL using vacuum centrifugation.[33]

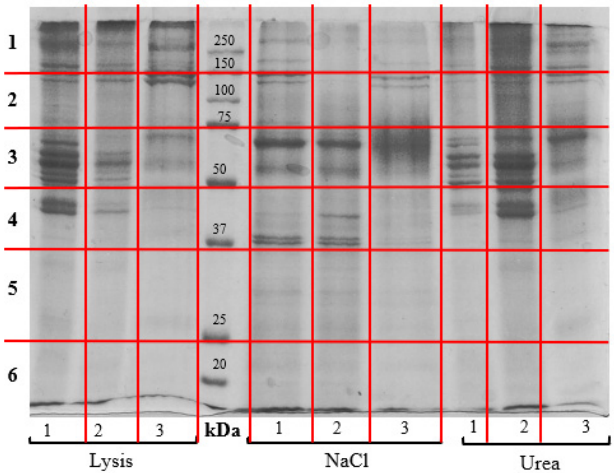

Figure S3. Diagram of how the SDS-PAGE gels were manually cut.

LC-MS/MS analysis

2µl of each sample (4 biological replicates with 3 technical replicates each) were injected on a 1.0mm × 5 mm PepMap 100 C_{18} trap column, 5µm particle size, at a flow rate of 25µl/min then onto a 75 µm × 50 cm PepMap C_{18} column, 3µm particle size, at a flow rate of 300nl/min using a Dionex Ultimate™ 3000 RSL nano system (Thermo Fisher Scientific, Massachusetts, USA). The mobile phase was 3% acetonitrile, 0.1% formic acid in MS grade H_2O. Peptides were eluted using a linear gradient from 3-30% acetonitrile, 0.1% formic acid over 55 minutes. The peptides eluted from the column were analysed using a Q Exactive Plus mass spectrometer with a Nano Flex ionization source operating with Xcalibur acquisition software (Thermo Fisher Scientific, Massachusetts, USA). The mass spectrometer was externally calibrated and operated in data-dependent mode. Full MS1 scans were acquired over a mass range of 375-1,500 m/z with a resolution setting of 70,000, while fragment ion spectra were acquired at a resolution of 17,500. For data dependent acquisition of HCD spectra, the top ten most intense ions were selected for fragmentation in each scan cycle and full MS and fragment ion spectra were detected by the Orbitrap mass analyser. Exclusion conditions were optimised according to the observed peak width (typically 10s).

Protein Identification

Processing of the raw data generated from LC-MS/MS analysis was carried out using Proteome Discoverer version 2.2 (Thermo Fisher Scientific; Massachusetts, USA). For the analysis, the grain, grain-corium junction and corium data from each extraction were combined. The following search parameters were used for protein identification: peptide mass tolerance 10 ppm, MS/MS mass tolerance 0.02 Da, up to two missed cleavages allowed, minimum peptide length, six amino acids, carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine, lysine and proline, acetylation of the N-terminal residue, and galactosyl, glucosylgalactosyl modifications of lysine were set as variable modifications. For each protein, the minimal number of unique peptides identified was set to two and the false discovery rate was set at 1%. The number of proteins initially identified was reduced from over 10,000 to approximately 1,000 using these criteria. Data were searched against the UniProtKB-SwissProt database (taxonomy: Bovine, release 10/2016).

Statistical analysis

Analysis of each sample was performed in three separate experiments. Statistical differences between the groups OSP and DA were determined using one-way student *t*-tests and volcano plots. In order to be classified as significantly different the p-value had to be less than 0.05 and the fold change greater than 2. Data analysis *via* principal component analysis (PCA) plots and visualization *via* heatmaps was carried out using the publicly available MetaboAnalyst 4.0 software (https://www.metaboanalyst.ca/).

Total collagen concentration

Total collagen concentration in the OSP and DA samples was analysed based on a method previously reported by Naffa *et al.*[11, 12] with slight modifications. 100 mg of lyophilized 40 µm slices of each skin sample were hydrolysed in 5 ml of 6 M HCl containing 3% (w/v) phenol for 24 hours at 110°C. The hydrolysate was filtered, concentrated by lyophilisation then dissolved in. 1.0 ml of 0.1 M HCl, it was then diluted 1:100 with MilliQ H_2O before being derivatized with AQC (10 µl sample, 100 pmol/µl norleucine, 15 pmol/µl AQC in 0.2 M borate buffer, pH 8.85) for 10 minutes at 55°C. After a further 1:10 dilution, 1 µl of sample or 1-5 µl of amino acid standard were injected on to a 150 × 4.6 mm Gemini C_{18}, 5µ HPLC column (Phenomenex; California, USA). Solvent A was 5 mM ammonium acetate, 1% acetic acid, pH 5.05 A and solvent B was 60% (v/v) acetonitrile in water. Solute was eluted using gradient elution (0-100% B), over 90 minutes at a flow rate of 1.00 ml/min and a column temperature of 37°C. Eluted peaks were separated and monitored using a Dionex UltiMate™ HPG-3400RS rapid separation binary pump with fluorescence detector (Dionex RF 2000). The excitation and emission wavelengths were set at 245 nm and 395 nm respectively. Amino acid concentrations were determined using calibration curves calculated using Dionex CHROMELION version 6.80 SR13 Build 3967.

3D Confocal Microscopy

Picrosirius red was used to stain the collagen network, using the slightly modified method of Naffa *et al.*[11] Briefly, 2 × 2 cm hide samples were fixed in buffered formalin (40% formaldehyde, 30mM di-sodium hydrogen orthophosphate and 30mM sodium dihydrogen orthophosphate, pH 7.4) for 24 hours before being sliced into 40 µm thick cross sections using the Leica CM 1850 UV cryostat. The slices were carefully placed on microscope slides then rinsed with H_2O, before being placed in a 1% (w/v) potassium permanganate solution for 5 minutes. They were then rewashed with H_2O, before being placed in a 1% (w/v) oxalic acid solution until they became colourless. After washing in H_2O, they were placed in 0.2% (w/v) phosphomolybdic acid for 10 minutes, then stained with 1.2% (w/v) picric acid containing 0.1g sirius red F3B for 60 minutes. After this time, the slides were placed in 0.01 N HCl for 15 minutes, washed with ethanol then placed in xylene (100%). Coverslips were attached using DPX containing dibutyl phthalate (10-20%) and xylene (100%) as the mounting solution.

A Leica SP5 DM6000B scanning Confocal Microscope with LAS AF software (version 2.7.1.9723) was used to visualize the collagen network using the parameters previously published by Vogel *et al.*[34] Images were acquired using a 20× lens with a 3× optical zoom and standard filters set at an excitation and emission wavelengths of 561 nm and 571 - 653 nm respectively.

Results and discussion

Proteomic profiles: A comparison of the extraction methods

A wide range of methods have been reported for protein extraction from different tissues, including hide.[30, 35-37] Efficient extraction of protein from hide is difficult because by its very nature, hide has limited solubility, thus it is common to use a combination of mechanical and

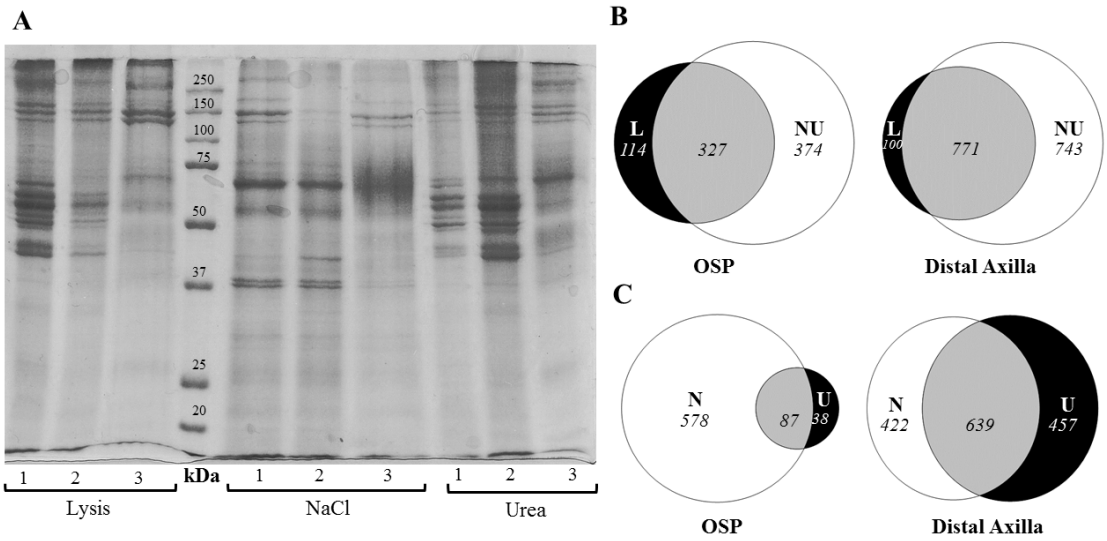

Figure 2. Proteomic profiles of cattle hide using different extraction methods. **A)** SDS-PAGE gel of proteins extracted from cattle hide using lysis, NaCl and Urea extraction buffers (1-grain, 2-grain-corium junction and 3-corium). **B)** Venn diagrams illustrating the number of proteins identified via the lysis (L) and NaCl/Urea (NU) extraction methods in both the OSP and DA regions. **C)** Venn diagrams illustrating the number of proteins identified individually by the NaCl (N) and urea (U) sequential extraction method in both the OSP and DA regions.

chemical methods.[30] This study used glass beads with rotation to provide mechanical action followed by sequential extraction with two different extraction buffers to investigate which was more effective.

Method 1 (Fig 1) used a traditional lysis buffer containing urea/thiourea to denature the proteins, a reducing agent (DTT) to reduce disulfide bonds, the detergent CHAPs for solubilisation of poorly soluble proteins and a protease inhibitor (cOmplete) to control undesirable proteolysis in a Tris buffer system, pH 7-9. Method 2 (Fig 1) used a high salt (NaCl) buffer followed by a buffer containing a high concentration of urea. NaCl is known to increase the concentration of extracted proteins as well as the number of higher molecular protein bands such as collagen[38] and contained a reducing agent and protease inhibitor in ammonium bicarbonate buffer (pH 8). The urea buffer contained a relatively high concentration of urea, known to efficiently denature and solubilise proteins, a reducing agent (DTT) and protease inhibitor (cOmplete) in an ammonium bicarbonate buffer. What was not done in this study was to use more than one protease to produce a greater coverage of the proteome as has been done in other studies,[39] however despite stringent filters over 400 proteins were identified with high confidence.

The protein profile of samples extracted using the lysis extraction buffer and the sequential NaCl/urea methods were different as shown by SDS-PAGE (Fig 2a) with the sequential method producing a greater number of bands. Not surprisingly, there were a greater number of proteins identified from this extraction by LC-MS/MS (Fig 2b). When comparing the proteins extracted from the individual steps of the sequential extraction, very different protein bands were seen on the gel. This was especially apparent in the OSP where only 87 proteins were common to the NaCl and urea extraction (Fig 2c). On the other hand, proteins extracted using the lysis buffer and the urea buffer had similar banding patterns on the gel. Although the sequential NaCl/Urea buffer extracted a greater number of proteins compared to the lysis buffer there were still a significant number of proteins unique to the lysis extraction suggesting that combining different fractionation and extraction methods results in a more complete proteome coverage. In all three extraction methods differences in banding pattern were observed between the three different layers with the grain having the most diverse banding pattern and the corium the least. This is most likely due to the corium being more collagen rich whilst the grain had more non-collagenous proteins. These layers were combined when analysing the mass spectrometry data.

Differences between the OSP and DA regions of raw cattle hide

The looseness grade of the OSP and DA regions from the half cattle hides processed to leather were analysed using the SATRA break scale. On average, the DA was significantly looser than the

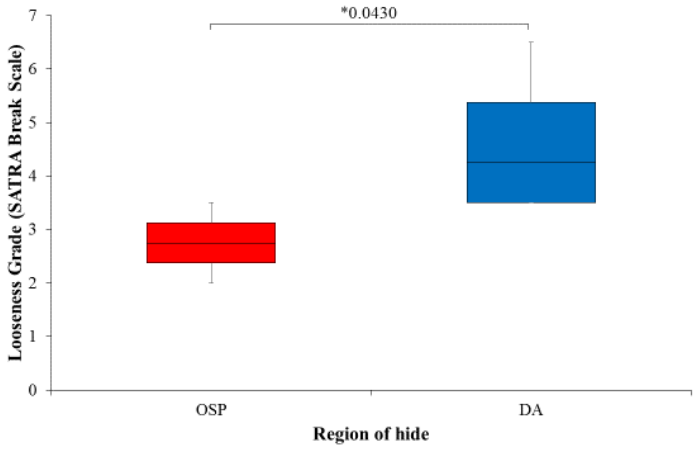

Figure 3. Looseness grade of OSP and DA region of leather samples, * p-value.

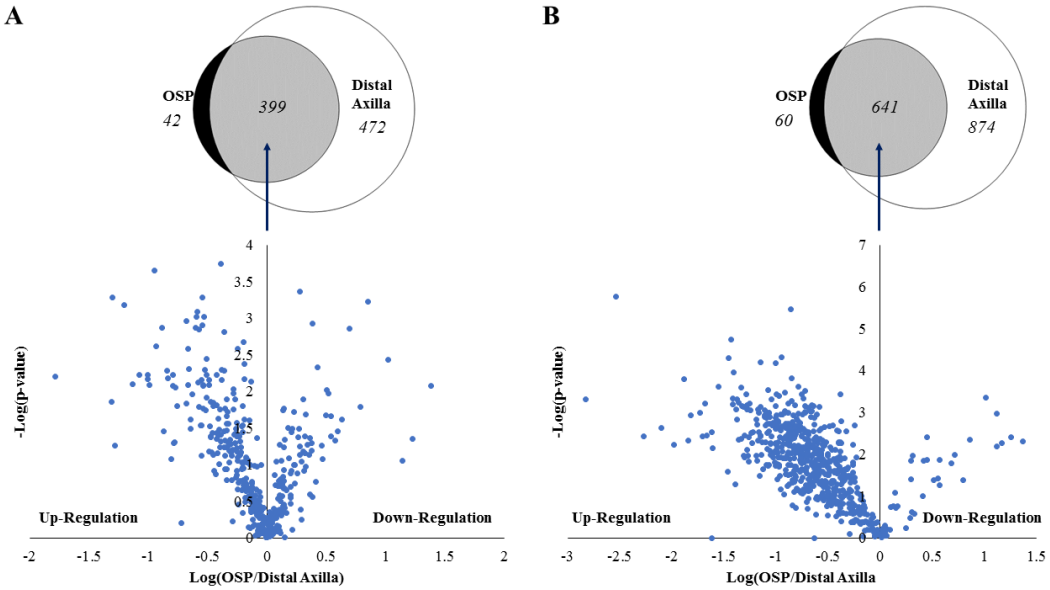

Figure 4. Venn diagrams comparing the number of proteins identified in OSP and DA samples and volcano plots comparing the statistical significance *vs* fold change in the abundance of proteins found in the OSP and DA for **A)** lysis extraction and **B)** sequential NaCl/Urea extraction.

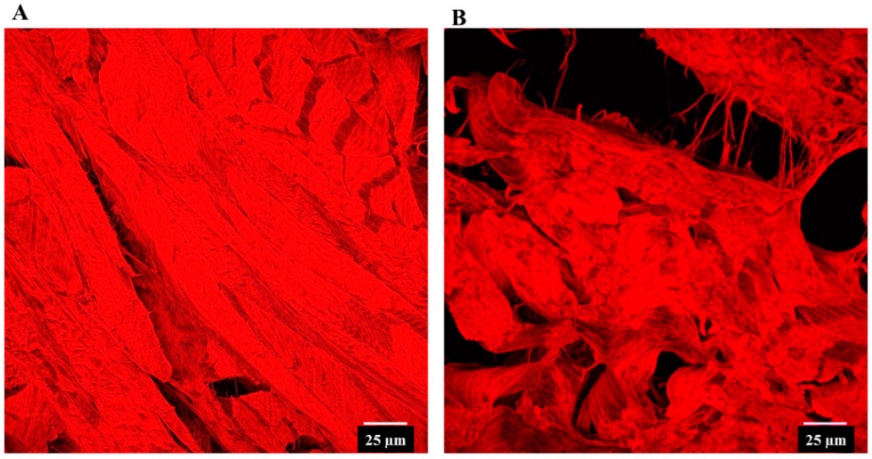

Figure 5. 3D confocal microscopy images of **A)** OSP region and **B)** distal axilla region of cattle hide.

OSP region (p-value 0.0430) for the four hides tested (Fig 3). This is consistent with previous reports by Wells *et al.*[4] and Mehta *et al.*[7] which state that the DA region is more prone to looseness in cattle hide. The proteomic profiles of the OSP and DA regions from the raw hide half were then analysed using in-gel LC-MS/MS.

Proteomic analyses identified 439 proteins with high confidence from the lysis extraction and 701 proteins with high confidence from the NaCl/urea extraction for samples taken from the OSP region. Samples from the DA region yielded 868 identifications using lysis buffer extraction and 1515 proteins extracted using NaCl/Urea (Fig 4). Interestingly, the proteins that were common to both the OSP and DA regions were typically up-regulated in the OSP region suggesting that regardless of the more complete extraction of proteins from the DA region there are higher concentrations of these proteins in the OSP region compared to the DA as shown in Fig 4.

The greater number of proteins identified in the DA region is potentially due to the increased space between the collagen fibers that is seen using confocal microscopy (Fig 5) and has been reported by others.[4,8] A looser arrangement of fiber bundles would enable easier access of the solubilisation reagents to the protein fiber network, resulting in an increased number of proteins extracted. The fact that similar observations were made both in this study and other studies[4, 8] suggests that the large gaps seen between the fiber bundles in loose leather are present in the raw material and are not caused by poor tanning practices.

PCA plots analysing all data (Fig 6) and heat maps containing the 50 most abundant proteins (Fig 7) were used to display the overall results of LC-MS/MS data. The PCA plots show distinct clustering of groups of proteins from the OSP and DA samples (Fig 6), strongly

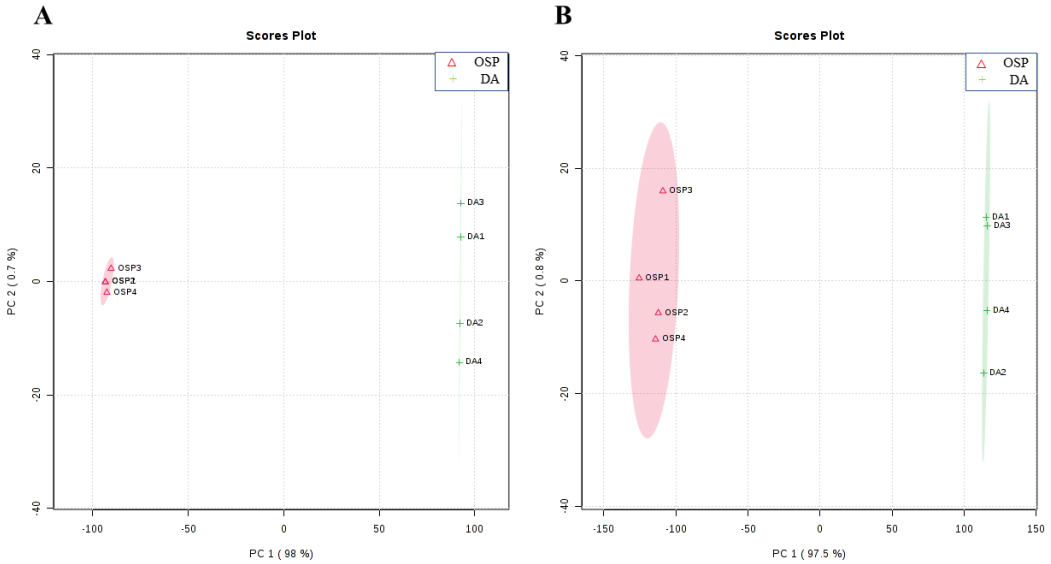

Figure 6. 2D score plots of four OSP and DA region samples for **A)** Lysis extraction and **B)** NaCl/urea extraction based on two principal components.

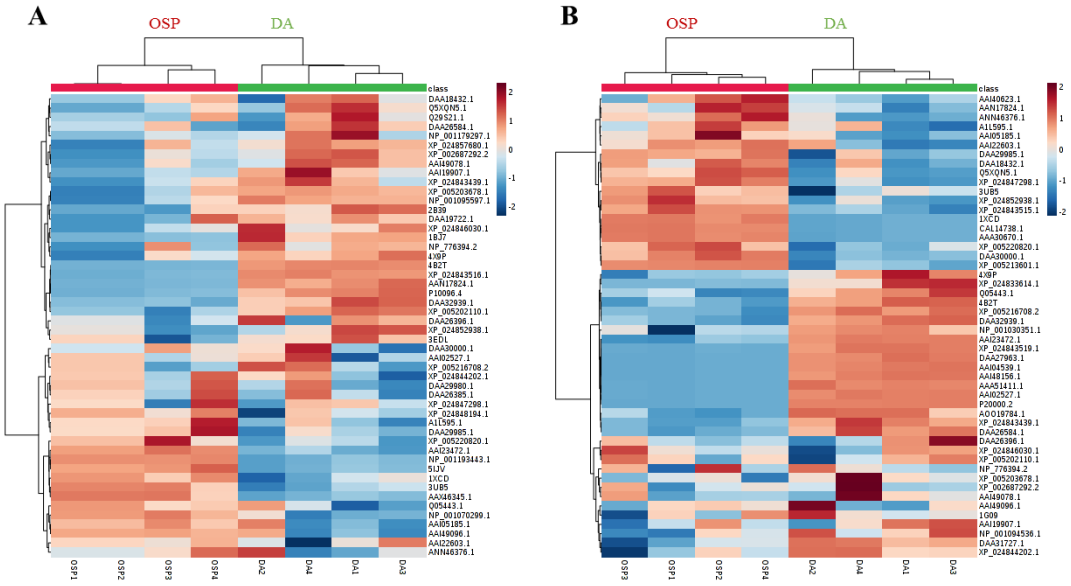

Figure 7. Heat maps of 50 most abundant proteins as identified by accession number from **A)** Lysis extraction and **B)** NaCl/urea extraction.

suggestive of a real difference in the protein composition between the DA and the OSP regions of the hide. The heat map supports this finding, showing significant differences between the relative concentrations of some protein groups in the DA and the OSP samples (Fig 7).

All proteins that were common to both the OSP and DA region (399 and 641 for the Lysis and NaCl/Urea extractions respectively) were analysed to determine whether there were any significant differences between the relative concentrations in the two regions. In order to be categorised as significantly different the proteins had to have p-values below 0.05 and a fold change equal to or greater than 2. Only 38 proteins met these constraints and all were down-regulated in the DA samples. Proteins included fibrous collagen, proteoglycans and other ECM proteins, keratins, cellular proteins, enzymes and serum proteins and are listed in Table I.

Fibrous collagens type I and III are down-regulated in DA samples. Fibrous collagen is the most abundant collagen in hide and provides mechanical and structural support to the hide with type I being more prevalent in the corium and type III in the grain.[40, 41] A decrease in the fibrous collagen may result in a less organised collagen network, as seen in figure 5, which could contribute to the development of looseness. However, the overall collagen content of OSP and DA samples was not significantly different when calculated using the hydroxyproline concentration measured by amino acid analysis[42] (Fig. 8). Because LC-MS/MS measures only the soluble protein in contrast to amino acid analysis which measures both soluble and

insoluble collagen, this is not entirely unexpected. Further validation steps are needed to confirm whether fibrous collagen is indeed down-regulated in the DA region, using immunological detection.

The proteoglycan decorin and proteoglycan associated protein glial hyaluronate binding protein are also down-regulated in DA samples. The core protein of decorin interacts with specific surface amino acid residues on type I collagen fibrils, the interaction being stabilised by electrostatic interactions between collagen and the sulfates of the GAG.[43] This interaction is necessary for assembly of collagen microfibrils and prevents the cleavage of collagen fibrils by matrix metalloprotease I.[43] As such, lower concentrations could affect collagen fiber bundle architecture. The glial hyaluronate binding protein is believed to be a proteolytic product of versican.[44] Versican is a hyalectan that binds to both hyaluronic acid and lectins and has roles in regulation of cell adhesion, migration and proliferation, ECM assembly and fibrillogenesis of elastic fibers.[45]

As seen in Table I many keratins were down-regulated in the OSP compared to the DA. As keratins are removed during the dehairing stage of leather processing it is unlikely that they contribute to looseness. It is therefore possible that the difference in the

Table I

Proteins that are significantly down regulated in the DA

	Protein	Accession	p-value	OSP/DA	Extraction Method
Fibrous Collagen	Collagen type I: alpha 1	AAI05185.1	0.0396	1.99	NU
	Collagen type 1: alpha1 CN8	0910139A	0.0474	3.77	L
	Precursor of collagen type III: alpha-1	NP_001070299.1	0.0213	3.15	L
Proteoglycans and ECM proteins	A Chain A, Decorin	1XCD	0.0204	2.11	L
	Glial hyaluronate-binding protein	AAB20399.1	0.0381	3.61	NU
Keratin	Keratin 31	DAA18488.1	0.0239	4.27	L
	Keratin 82	DAA29986.1	0.0216	3.46	L
	Keratin 84	DAA29999.1	0.0409	3.46	L
	Keratin 86	DAA30000.1	0.0144	1.98	NU
	Keratin 83	AAI23472.1	0.0037	10.54	L
	Keratin I: cytoskeletal 27	DAA18462.1	0.0006	7.10	L
	Keratin I: cytoskeletal 39	XP_010814574.2	0.0083	24.44	L
Cellular proteins	A Chain A, Actin, Cytoplasmic 1	3UB5	0.0047	2.67	L
	Actin, gamma-enteric smooth muscle	NP_001013610.1	0.0147	2.64	NU
	Annexin I	AAB25084.1	0.0441	16.87	L
			0.0412	3.31	NU
	Histone H2B type 1-K	DAA16155.1	0.0014	5.00	L
	Myosin-11	NP_001095597.1	0.0065	13.23	NU
	Isoform X1 of Periostin	XP_005213601.1	0.0045	7.29	NU
	Isoform X13 of Tropomyosin alpha-1 chain	XP_024853024.1	0.0164	4.89	NU
	Isoform X3 of Tropomyosin beta chain	XP_005210126.1	0.0138	3.77	NU
	Tubulin alpha 1C chain-like	XP_024838025.1	0.0049	23.66	NU
	Tubulin alpha 4a	AAI18200.1	0.0138	2.82	NU
	Tubulin beta 4B chain	NP_001029835.1	0.0011	13.34	NU
	Isoform X1 of V-set and immunoglobulin domain-containing protein 8	XP_010801062.1	0.0096	3.20	L
Enzymes	ADP/ATP translocase 3	DAA33747.1	0.0410	2.11	L
	Alpha-1-antiproteinase	P34955.1	0.0039	2.81	NU
	Bovine Mitochondrial F1-Atpase	2W6F	0.0054	14.72	NU
	Cathepsin C	AAI02116.1	0.0127	2.01	L
	Fatty acyl-CoA reductase 2	DAA29455.1	0.0353	3.99	L
	Precursor of Protein-lysine 6-oxidase	DAA27688.1	0.0164	6.13	L
	Pyruvate Kinase 2	AAI02827.1	0.0107	2.08	NU
	Isoform X2 of Serpin B6	XP_015315506.2	0.0004	10.37	NU
Serum Proteins	A Chain A, Bovine Fab E03 Light Chain	5IJV	0.0102	5.22	NU
	Albumin	754920A	0.0415	6.26	NU
	Alpha-2-macroglobulin	Q7SIH1.2	0.0039	18.34	NU
	Isoform X1 of Complement component C8 gamma chain	XP_005213573.2	0.0012	2.44	L
	Precursor of Complement component C9	NP_001030441.1	0.0431	2.30	L
	Immunoglobulin J chain	AAB03643.1	0.0105	3.31	L

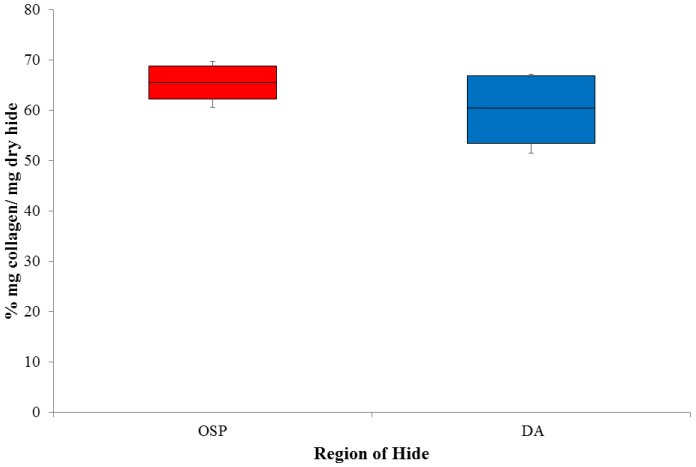

Figure 8. Collagen concentration of OSP and DA regions calculated from hydroxyproline concentration.

concentration of keratins could also be due to sampling issues. However, it must be noted that in cells of the dermis, keratin filaments and other intermediate filaments function as part of the cytoskeleton to mechanically stabilise the cell against physical stress. So, a decrease in these filaments could cause a less mechanically stable cell structure. Other cellular proteins that have a role in supporting the structure of dermal cells include annexin, tubulin and myosin.

Of the enzymes that were down-regulated in the DA region, protein-lysine-6-oxidase was of the most interest. It is an enzyme essential for the formation of crosslinks between tropocollagen molecules as well as various extracellular matrix proteins including elastin.[46] In humans, the lack of vitamin C, an essential cofactor of this enzyme, leads in the worst cases to scurvy, a disease first recorded in 1550 BCE whose symptoms are impaired wound healing and broken skin among others.[47] Down regulation of this enzyme would result in defective fibrillogenesis leading to the increased gaps and less organised structure of the collagen fibers seen in the DA samples (Fig 5). There was also a decrease in the lysosomal enzyme cathepsin C which activates serine proteases as well a decrease in the serine protease inhibitors alpha 1 anti-proteinase and isoform X2 of Serpin B6.

Conclusions

In this study, the DA region of the hide was used as a model for loose hide, with the OSP region being used as a control. Analysis of samples prepared using two different methods to extract the proteins showed advantages of this approach as it resulted in a more complete protein profile of hide than would have been achieved using a single method. Over 400 proteins were identified with high confidence and there were clear differences between the two regions tested some of which provided a molecular explanation for the differences in the collagen structure observed using confocal microscopy. It was particularly interesting that four of the proteins that were significantly down regulated in the DA are involved in or influence the arrangement of collagen microfiber bundles that are responsible for the physical properties of the hide. The decrease in these proteins are likely responsible for the increased gaps and less organised structure of the collagen fibers seen with confocal microscopy. Although these results need to be validated, the preliminary studies indicate that there are molecular differences in the raw hides that produce loose and tight leather. Understanding the molecular causes of loose leather may enable biomarkers to be developed for its early detection.

Acknowledgements

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Tanning Chemicals' Influence in Leather Tensile and Tear Strength Review

by
Ricardo Tournier*

Abstract

For over 100 years of tanning research, it is still arguable whether tanning chemicals weaken, strengthen or have an effect on the skin's original collagen fibers.

The current paper is a review of the literature regarding the impact of the tanning process on the mechanical properties of leather specifically, tensile and tear strength, that raises several questions about this topic. A call is made for the scientific and technical community to address these questions.

Findings about Hides and Leathers

Since the beginning of last century, it was broadly accepted in the trade as a fact that vegetable and chrome tanned leathers diminished their original hides strength. In 1920, Bowker and Churchill[1], found that the lower the tannage degree, the higher the tensile strength value of vegetable harness leather. Later, Downing[2] reported the fact found in his tannery, that the tensile strength value of vegetable belting leather produced during 1925 and 1926, decreased with the increase of the degree of tannage. In 1947, Highberger[3] considered that the decrease in the tensile strength value of collagen occurring during tanning, was inconsistent with the formation of strong crosslinks.

In 1949, regarding chrome tanned leather, Noerr and Classen,[4] observed that the strength of chrome tanned leather decreased as the chrome (Cr_2O_3) content was increased.

In 1951, Stather and Schmidt[5] found that for each tanning process there was a reduction in absolute tensile strength of about 10 to 25% compared to delimed pelts.

In 1952, Kanagy et al.[6] showed that chrome-retanned leathers were weaker than those that had been straight chrome tanned.

From 1953 onwards, discrepancies among different researchers and technicians were evident. Benskin[7] reported that the absolute tensile strength of limed and splitted hides, vegetable tanned, increased by 18-19% compared to the pelt from which it was made, while tear strength decreased 16%. Similar results were found for chrome tanned.

In 1959, Toth and Ribli[8] drew opposite conclusions than those of Stather and Schmidt[5] under the same trial conditions, according to the first. They found an increase in both tensile strength and stitch tear resistance. Lower values were measured only for the resistance to continuous tear. In both works from Benkins and Toth et al., the samples were first cut from limed hides, the thickness was measured, then tanned and resistance tests were carried out on the samples in the wet state.

Zissel[9] in 1974 studied the influence of three types of tanning on the mechanical resistance of leather. His findings demonstrated that in leathers that were not deliberately fatliquored, and air dried at 25°C, compared to chrome tanning, glutaraldehyde tanning causes lower absolute and relative tensile strength values. Vegetable tanning, on the other hand, lowers the relative tensile strength value only due to its strong filling effect.

As for absolute and relative tear resistance, Zissel found that when compared to chrome tanning, vegetable tanning and glutaraldehyde have the same effect on reducing resistance.

In 1977, Leberfinger[10] et al. did not find any reduction in absolute tensile and tear strength neither in chrome nor in vegetable tanning. On the contrary, the relative resistances showed a decrease in their values due to the associated increase in the thickness of the leather. They worked with pickled pelts, after tanning the leathers did not receive further post-treatment or fatliquoring. Fatliquored leathers are subject to a certain amount of fiber adhesion and this residual adhesion can be of different magnitude depending on the type and intensity of the tanning process. In order to eliminate this factor, instead of normal drying, dehydration with acetone and anhydrous calcium chloride was carried out.

In 1978, Bitcover and Everett,[11] found significant inverse correlations between chrome content and tensile strength (in both directions) and slit tear strength (in one direction). The authors minimize the possible influence of different raw hides used in the experiments and emphasize the influence of chemical composition of crust leather, to arrive to these conclusions. Nevertheless, they recognized that their results would have been more reliable if the experiments had been designed specifically to study the effects of varying chrome content on leather strength at a constant fat content and the effects

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of varying fat content at a constant chrome content. It would have been valuable also to know the original strength of raw or pickled hides either in wet state or dried with acetone, to verify the levels of starting strength values.

Findings about Collagen Fibers

Regarding the behavior of individual tanned collagen fibers and their physical resistances, the findings are equally contradictory. In 1950, Mao and Roddy[12] and later Roddy[13] in 1952, arrived at the conclusion, working with individual collagen fibers, that there was no loss of resistance after both vegetable and mineral tannages. In 1953, Michailov[14] demonstrated that the tensile strength of individual fibers increased considerably with tannage. The same conclusion was reached by Okamura and Shirai[15] tanning with cationic chrome complexes.

In 1960, Morgan and Mitton,[16] working for the BLMRA* with single raw fibers, found that chrome tanned fibers were about 25% weaker than raw collagen fibers and that vegetable tannage did not alter the strength of the fibers significantly.

In 1983, Bienkiewicz[17] came up with the concept that with the introduction of tanning agents into the hide, some "dilution" effect of native collagen properties could be expected.

Possible causes of confusion

It seems to be several reasons or causes that promote such confusions, divergences and contradictions.

One of them may be semantic, when naming the term "tanning". Some refer to tanning processes and others, to the tanning process in particular. When, for example, vegetable harness leather is mentioned, it includes several chemical and physical processes that raw hides are put through, that have the harness leather as a final result, and in which only one of them is the vegetal tanning process itself.

Another cause of confusion can be the term "leather resistance or leather strength". It is important to differentiate between absolute tensile or tear strength from the same relative, N/cm² and N/cm respectively. In this regard, a tannage that does not affect the absolute strength of a hide before and after being processed but increases its substance, will show a decrease in its relative strength.

Benskin[7] points out that differences of their findings with other workers "may be due to the fact that they may have worked on one or two hides so that variations in raw material may have offset differences caused by experimental changes."

In addition, Leberfinger[10] states: "The influence on tensile strength and progressive tear is based on very complex relationships. Both measurements depend not only on the type and quantity of the tanning agent, but also on other factors (condition of the skin, topographical sampling, work in the tanneries, fatliquoring, retanning, conditioning, finishing, etc.). Therefore, if you want to know about the influence of tanning alone, you have to eliminate the secondary factors."

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In 2015, Tournier[18] studied changes in tear resistance of bovine hides along the processes of a certain tannery, up to wet blue state. The author followed a protocol for the assessment of the tear strength absolute and relative of four fresh hides, processed in normal tannery lots, by means of a statistical sequential sampling. The full sampling and testing methodology is outlined in Tournier.[18] The tongue (or trouser) tear method (ALCA method E10) was used to measure tearing strength. The samples were tested in the wet state in fresh, limed, lime split, chrome tanned, chrome tanned full substance and wet blue split. The study showed, among other findings, that in this particular tannery, the processes from raw hides up to chrome tanned, decreased substantially the original fresh hides tear resistance in both processes, lime split and wet blue split.

The author noted that this tannery had opportunities for improvement on the tanning processes, namely, in deliming, bating, pickling and chrome tanning itself. Tournier suggested the tannery technicians using the new developed methodology to determine the impact of each one of the processes mentioned above in the decrease of the tearing resistance of their leather and to act later on, in reverting it.

In 2016, Sizeland[19] et al. studied the effect of tanning agents on collagen structure and response to strain in leather. Pickled pelts were tanned as standard, with 4,5% chromium sulfate, retanned with 4% of mimosa extract and fatliquored with 5% of two different types of oils. Zirconium leathers were tanned with 5% of zirconium sulfate, retanned with 3% of a commercial syntan and same fatliquor of chrome tanned. THPS* leathers were tanned with 2% of THPS, retanned with 2% of the commercial syntan and fatliquored with the same fatliquor of chrome tanned. Oxazolidine + Mimosa leathers were tanned with 2% of the commercial syntan, 6% of mimosa extract and 2% of oxazolidine. After the addition of a further 8% of mimosa extract, the standard fatliquor followed.

Regarding the results of tear and tensile strength of these leathers the authors found that "chromium or zirconium tanned leathers were higher than those of oxazolidine or THPS".

*British Leather Manufacturers' Research Association

*Tetrakis hydroxymethyl phosphonium sulfate

These results give the idea that metal tannages yield stronger leathers than metal-free ones. With so many different products used, in different proportions and applied in different manners, without mentioning the crusting procedures, this statement seems, at least, risky, and misleading.

In 2019, Kaijun Li et al.[20] reported the action of a new tanning agent, Chromium loaded PPA*copolymer Nanoparticles (Cr-PPA NPs). This product was used for tanning one sample of pickled sheep skin and comparing it with two other samples of the same sheep skin tanned with commercial chrome tanning as control (one sample with 4% and the other one with 8%). The control samples were cut from the left side of the skin, and the trial sample from the right side of the skin. Wet blue leather samples were further treated with 16% of a fatliquoring agent. It was not specified if all samples were treated together or separately and there was no information about crusting procedures. The authors claim higher hydrothermal stability of the novel product vs. chrome tanned (4 and 8%), and highest tearing strength, whereas the tensile strength and breaking elongation were nearly the same.

It is curious that the chrome tanned leathers reached a shrink temperature of only 89.5° and 98.1°C respectively. Also, regarding the physical properties, it would have been of interest to consider the following:

- a statistical design of experiments
- standard sample preparation: samples would have been cut in such a way that bilateral symmetry differences were minimized,
- to provide more information about crusting processes,
- to report chrome and oil content of each leather samples,
- to report tearing and tensile absolute values.

In 2020, Xiu He et al.[21] assessed the correlation between fiber dispersion and physical properties of chrome tanned leather with different quantities of chrome sulphate powder. As a parallel finding, they reported that the tensile strength, tear strength and elongation at break of crust leathers increased with the increase of chrome powder.

The sample preparation consisted in four pieces of pickled pelts that were tanned with 2, 4, 6 and 8% of chrome powder, the wet blue obtained was wrung and sampled. Afterwards, the remaining wet blue were rewetted, neutralized and fatliquored (each piece separately[22]) while following post-tanning processes of horsing-up, drying and staking, and finally, sampling of the crust leather

Table IV
Mechanical properties of crust leathers.

Crust leather	Tensile strength (N/mm2)	Tear strength (N/mm)	Elongation at break (%)
Cr-2	22.84±0.68	96.94±0.53	47.21±5.55
Cr-4	24.86±1.63	97.27±1.17	53.21±4.87
Cr-6	25.24±0.58	117.82±3.12	55.50±1.15
Cr-8	23.29±2.04	110.28±4.11	64.32±2.87

The data about mechanical properties is shown in Table IV of their paper and reproduced here with authors[21] permission. It seems that the conclusion reported, regarding the physical properties increasing with increasing chrome powder, is not accurate due to some weakness in the methodology. The author of this manuscript humbly suggests that this method would have provided better information about the real influence of chrome itself, if it had included in the table:

- the absolute and relative strength data of the original pickled pelts,
- the absolute and relative strength data of the tanned pelts in wet blue state, before going on with the fatliquor and the rest of the crusting processes,
- if instead of informing the chrome powder offered in each trial, the amount of chromium actually uptaken by the pelts was informed.

Conclusions

There is no doubt that there are many unanswered questions and issues to be clarified in the critical field of leather strength and its relationship with tanning products.

There are lots of publications regarding the structure of leather fibers and collagen, and research trying to elucidate which are the characteristics that define the strength of fibers, but not that many to explain the effect of tanning materials on them. A lot of work is needed in the scientific area, in laboratories and tanneries to shed light on these questions.

For tanneries in particular, the method suggested by the author[18] can be very useful to know how robust their formulations are and take advantage of an easy internal research method to surpass competition in this respect. Special care must be taken by scientists and technicians in the design of their tests and experiments as well as in the expression of results and conclusions.

It is both important and urgent to clarify the action of tanning agents on collagen and hides and their influence on tensile and tear resistance as new products are constantly being developed and released to the market in the need of less environmental impact.

*Poly (PEG-co-AA)

Perhaps, IULTCS could take the lead on this subject by defining rules or a standard test method to systematize experimental design, statistical sampling and assessment of results. This may help in doing research and the expression of results specifying whether certain products are tanning or not and whether they increase the physical properties of collagen fibers and hides or not.

In the meantime, technicians working in the shop floor must take care of the critical points of the processes where it is known for sure, that native collagen fibers can be damaged. Bear in mind the concept of Bienkiewicz[17], mentioned above, and act in consequence, constantly checking the results.

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Bioaccumulation of Chromium(III) from Aqueous Solutions of a Leather Wastewater Treatment Plant by *Saccharomyces cerevisiae* Yeast

by

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Abstract

With many industries discharging heavy metals into natural water resources, heavy metals have been found to accumulate in various living organisms which can ultimately threaten human life and pose a big threat to the environment. Thus, in the pursuit of a solution to the above mentioned problem, bioaccumulation has emerged as an interesting option for the removal of heavy metals from wastewater. In this paper, the effectiveness of the yeast *Saccharomyces cerevisiae* in the bioaccumulation of Cr^{3+} has been tested. Also, different factors influencing Cr^{3+} uptake have been discussed. This work has demonstrated that Saccharomyces cerevisiae is an effective Cr^{3+} biosorbent for tannery wastewater. The conditions of use of this yeast to achieve optimal chromium (III) absorption are: i) when a growth of the biosorbent equivalent to a similar concentration of Cr^{3+} is obtained, which contains the residual water that needs to be treated; ii) the smaller the biosorbent is the better the biosorption; iii) the uptake of Cr^{3+} is more efficient when no extra growth medium is added to the wastewater; iv) the longer the exposure period of the yeast to Cr^{3+}, the bigger the Cr^{3+} reduction. Since *Saccharomyces cerevisiae* is an inexpensive, readily available source of biomass, this discovery could be of great use for a low-budget and efficient wastewater treatment system.

Introduction

Industrialization is to a great degree responsible for the contamination of the environment especially water, where lakes and rivers are brimmed with a large number of toxic substances. Compared with other toxic substances, heavy metals are reaching hazardous levels. Their continuous release leads to overconsumption and accumulation. Many industries (leather, fertilizers, pesticide, metallurgy, photography, aerospace, electroplating, mining, iron and steel, surface finishing, energy and fuel production, appliance manufacturing, metal surface treating, electrolysis and electro-osmosis) discharge waste containing heavy metals either indirectly or directly into the water resources.[1] Toxic heavy metals of concern are lead (Pb), mercury (Hg), chromium (Cr), nickel (Ni), arsenic (As), zinc (Zn), copper (Cu), cobalt (Co), cadmium (Cd), and so on. Due to the fact that these metals are not biodegradable, they tend to accumulate in the living organisms and lead to various disorders and diseases which ultimately threaten human life. Bioaccumulation has emerged as an interesting option over conventional methods for the removal of heavy metal ions from effluents discharged from various industries.[2]

Bioaccumulation is an active, metabolism-mediated process where the metal ions accumulate intracellularly in the living cells.[3] As mentioned by Diep et al., bioaccumulation is a natural biological phenomenon where microorganisms use proteins to uptake and sequester metal ions in the intracellular space to utilize in cellular processes (e.g., enzyme catalysis, signaling, stabilizing charges on biomolecules). Recombinant expression of these import-storage systems in genetically engineered microorganisms allows for enhanced uptake and sequestration of heavy metal ions.The process occurs in two steps: firstly, the adsorption of metal ions onto cells, which is quick and identical to biosorption, and the second step is slower and it includes the transport of metal ions inside the cells by active transport.[4] The process of bioaccumulation occurs by cultivating the biomass of a microorganism in a solution that contains the metal that will be accumulated. Since the solution contains the growth medium, the organism begins its metabolic processes and activates the intracellular transport systems for the accumulation of the sorbate.[5] However, the major limitation of the process is that the nutritive medium for growth of the microorganism contains organic carbon sources.[6] Part of the biosorbate accumulates inside the cell which enables the biomass to increase and bind greater amounts of metal ions. The organisms which are capable of resisting high loads of metal ions are best suited for accumulating metal species. They do not possess any mechanisms for blocking the accumulation of metal ions in large quantities. They may possess special mechanisms for synthesizing special intracellular binding regions rich in thiol groups as a response to metal ions in their surviving environment.[7]

Bacteria, microalgae, yeasts and fungi all showed the ability to remove Cr^{3+} in bioacuumulation processes[8] but in this work we were focused on the removal ability of the yeast. Yeasts have been

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little studied for use in bioaccumulation processes. However, they are versatile microorganisms since they develop in both aerobic and anaerobic environments, principally the *Saccharomyces* species. In addition to their versatility they show low-cost nutritional requirements, they are safe microorganisms and can be applied both dead and alive.[9]

Saccharomyces cerevisiae is an inexpensive, readily available source of biomass for bioremediation of waste-water.[3] It has been shown to accumulate heavy metals, such as Cr^{3+}, Co^{2+} and Cd^{2+} via two distinct processes. There is an initial rapid accumulation step that is metabolism and temperature independent and is thought to involve cation binding at the surface. This step is followed by a second, much slower, process that is metabolism-dependent and can accumulate larger quantities of cation than the first process. This second process is believed to involve cation internalization into the cell. The uptake system that allows for accumulation of Co^{2+} and Cd^{2+} cations appears to be a general one with only limited specificity, since competition for uptake of cations occurs. The following processes contributing to the mechanism of bioaccumulation includes intracellular accumulation and oxidation or reduction reactions. The process is very complex and depends of several factors (which are almost identical as the factors influencing the cultivation of an organism): initial metal concentration, biosorbent dose, contact time (time the yeast has spent in the presence of the heavy metal), the composition of the growth medium, wastewater content, pH, temperature, the presence of other pollutants (which are growth inhibitors, as well) or other inhibitors. Further investigations demonstrated that yeasts are capable of accumulating other cations such as Cu^{2+}, Mn^{2+} and Ni^{2+} and are superior metal accumulators compared to certain bacteria.[13]

In this paper we have tested if the yeast *Saccharomyces cerevisiae* can be used as a biosorbent to eliminate Cr^{3+} from waste-waters and analyzed how various factors such as composition of medium, biosorbent dose, contact time and adaptation of biosorbent to Cr^{3+} affected the Cr^{3+} absorbtion.

Experimental

Microorganisms and growth conditions

Commercially available yeast *Saccharomyces cerevisiae* was routinely maintained in Erlenmeyer flasks containing YPD broth composed of (g L^{-1}): glucose 20, yeast extract 10 and bacto peptone 20. Erlenmeyer flasks and pipette tips were autoclaved for 20 min at 120°C. Growth medium was inoculated by transferring organisms to 250 mL Erlenmeyer flasks containing 100 mL growth medium and aeration was maintained by shaking at 200 rpm at 25°C. Growth of this medium in the absence of heavy metals was defined as the control run.

Preparation of the Chromium standard solution

A 1000 mg/L commercial stock solution (chromium standard solution 1000 mg/L Cr, chromium (III) nitrate in nitric acid 0.5 mol/L, CR02220100, Scharlau) was used to dose the adequate volume to the 100 mL flask containing the growth media to obtain 0, 5, 10 and 30 mg Cr/L (0.1, 0.5, 1 and 3 mL of commercial stock solution to 100 mL of growth media).

Cr^{3+} uptake experiments with nonadapted and adapted Saccharomyces cerevisiae

To study the bioaccumulation properties of *Saccharomyces cerevisiae*, chromium standard solution 1000 mg/L was added to a 100 mL of growth media. Stock solutions of chromium were prepared and appropriate volumes of stock solution were supplemented to the media to give final Cr^{3+} concentrations of 0, 1, 5, 10 and 30 ppm. The accumulation medium was also inoculated by transferring nonadapted microorganisms. Adaptation to chromium ion was achieved by subculturing the cells at increasing concentration of metal ions corresponding to 1, 5, 10 and 30 ppm of Cr(III). A 2.5 mL of sample taken from the previous culture containing 1 ppm Cr^{3+} ions was used for the inoculation of 100 mL culture medium having 5 ppm metal ions. When the adapted culture reached its exponential growth, the same amount of culture medium (2.5 mL) was used again to inoculate the next 100 mL of culture, which has 10 ppm metal ions. Finally, this procedure was repeated for cultures supplemented with the concentration of 30 ppm of Cr(III). Thus yeast cells exposed to Cr^{3+} in increasing concentrations developed Cr^{3+} resistance. For adapted and nonadapted viable microorganisms, cultures were grown at 25°C in a shaking incubator at a 200 rpm constant stirring rate for a 96 h exposure period. After 96 h the OD660 was analysed for each flask and the amount of yeast cells was calculated. An exact amount of cells were taken from each flask (1,785 × 10^6 cells) and they were used to inoculate 100 mL of wastewater. Cultures were left in the wastewater at 25°C in a shaking incubator at a 200 rpm constant stirring rate for a 96 h exposure period. After 96 h the samples where filtered (90 mm and 0.45 μm pores) and digested. Digestion of yeast samples was performed using a multi-block heater (Lab Line Instruments). Washed yeast cells were digested directly inside the filter plates (100 μL well–1nitric acid, ~88 °C for 40–45 min) using the heating block. After the digestion, the samples were filtered again and analyzed for Cr^{3+} using a Unicam 929 atomic absorption spectrophotometer. The bioaccumulated metal ion amounts were determined as the difference between the initial Cr(III) concentration and concentration in the filtrate.

Cr^{3+} uptake experiments with different biosorbent doses

To study the bioaccumulation properties of *Saccharomyces cerevisiae* with different biosorbent doses, 4 Erlenmeyer flaks containing 100 ml of wastewater each were inoculated with different amounts of

yeast cells (1 mL, 2.5 mL, 5 mL and 10 mL) adapted to 30 ppm of Cr^{3+} to obtain different dilutions of the biosorbent (1:100, 1:40, 1:20 and 1: 10). Cultures were grown at 25°C in a shaking incubator at a 200 rpm constant stirring rate for a 96 h exposure period. After 96 h the samples where filtered (90 mm and 0.45 μm pores) and digested. After the digestion, the samples were filtered again and analyzed for Cr^{3+} using a Unicam 929 atomic absorption spectrophotomete. The bioaccumulated metal ion amounts were determined as the difference between the initial Cr^{3+} concentration and concentration in the filtrate.

Cr^{3+} uptake experiments with different compositions of growth medium

To study the bioaccumulation properties of *Saccharomyces cerevisiae* in different growth medium conditions the growth medium was added to 3 Erlenmeyer flasks containing different amounts of wastewater (100 mL, 75 mL, 50 mL) by adding ultrapure water until achieving a final volume of 100 mL. The wastewaters where inoculated with 2.5 mL of yeast cells adapted to 30 ppm Cr^{3+} and left at 25°C in a shaking incubator at a 200 rpm constant stirring rate for a 96 h exposure period. After 96 h the samples where filtered (90 mm and 0.45 μm pores) and digested. After the digestion, the samples were filtered again and analyzed for Cr^{3+} using a Unicam 929 atomic absorption spectrophotometer. The bioaccumulated metal ion amounts were determined as the difference between the initial Cr^{3+} concentration and concentration in the filtrate.

Cr^{3+} uptake experiments with different contact times

To study the bioaccumulation properties of *Saccharomyces cerevisiae* with different contact time with the Cr^{3+} ions, 4 Erlenmeyer flaks containing 100 mL of wastewater each were inoculated with 5 mL of yeast cells adapted to 10 ppm of Cr (III). Cultures were left in the wastewater at 25°C in a shaking incubator at a 200 rpm constant stirring rate for different exposure periods (1 h, 3 h, 5 h and 24 h). After the specific exposure period finished for each sample, the samples where filtered (90 mm and 0.45 μm pores) and digested. After the digestion, the samples were filtered again and analyzed for Cr^{3+} using a Unicam 929 atomic absorption spectrophotometer. The bioaccumulated metal ion amounts were determined as the difference between the initial Cr(III) concentration and concentration in the filtrate.

Results and discussion

Cr^{3+} uptake experiments with non-adapted and adapted Saccharomyces cerevisiae

It is well known that yeast is readily adapted to new environmental factors.[14] Also, in the literature it is stated that yeasts which grow in a medium containing heavy metals is adapted to them and absorb them more effectively, compared to yeasts grown without the presence of heavy metals.[15] For that reason we decided to test this hypothesis by growing our yeast in the presence of different concentrations of Cr^{3+} (from 0 ppm to 30 ppm) After that we transferred these adapted and non-adapted yeasts to the wastewater and after 96 hours we could see that there are some differences in the Cr^{3+} absorption. In Figure 1 it is noticed that non-adapted yeast diminished the amount of Cr^{3+} by 24% where the yeasts adapted to 10 ppm and 30 ppm diminished the amount of Cr^{3+} by 48% and 47%, respectively. The yeast adapted to 5 ppm achieved the best result in Cr^{3+} uptake and diminished the amount of Cr3+ by 60%. We can conclude that adapted yeast absorb Cr^{3+} more effectively than non-adapted yeast. It is important to notice that the concentration of the Cr^{3+} in the wastewater was between 0.01-5 ppm and we can clearly see that yeast adapted to similar conditions achieved the best results in Cr^{3+} uptake.

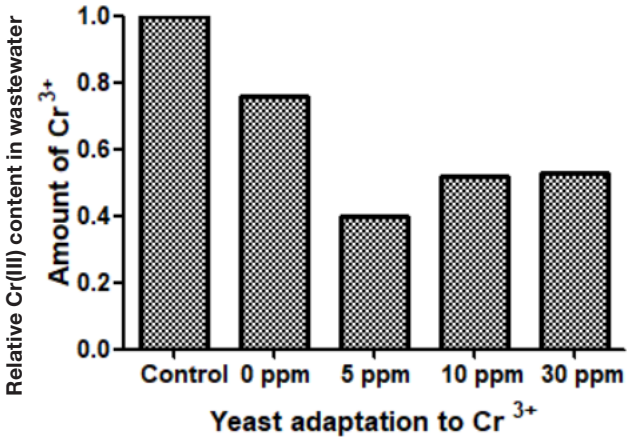

Figure 1. Chromium (III) uptake from wastewater is more effective with adapted than non-adapted yeast.

Cr^{3+} uptake experiments with different biosorbent dose

Once we confirmed that the Cr^{3+} uptake from wastewater is more effective with adapted yeast, we wanted to test how changing the dose of this adapted biosorbent will affect the chromium uptake. In this case, flasks containing 100 mL of wastewater each were inoculated with different amounts of yeast cells (1 mL, 2,5 mL, 5 mL and 10 mL) adapted to 5 ppm of Cr^{3+} to obtain different dilutions of the biosorbent (1:100, 1:40, 1:20 and 1: 10). The results (**Figure 2**) indicate that the amount of yeast in the wastewater is inversely proportional to Cr^{3+} uptake. When the yeast was diluted 10 times, the Cr^{3+} amount was diminished by 21%, on the other hand, when it was diluted 100 times, the Cr^{3+} amount was diminished by 62%. For the dilutions of 20 and 40 times, similar results were obtained as for the 1:10 dilution which are 25% and 26%, respectively. To sum it up, it can be said that the smaller the biosorbent dose is, the better the biosorption. The reason for that can be the fact that yeasts are known to introduce and metabolize Cr^{3+} only in the Log phase of growth which is the phase when cells are growing as fast as they can and that can only happen when there is a small amount of cells in

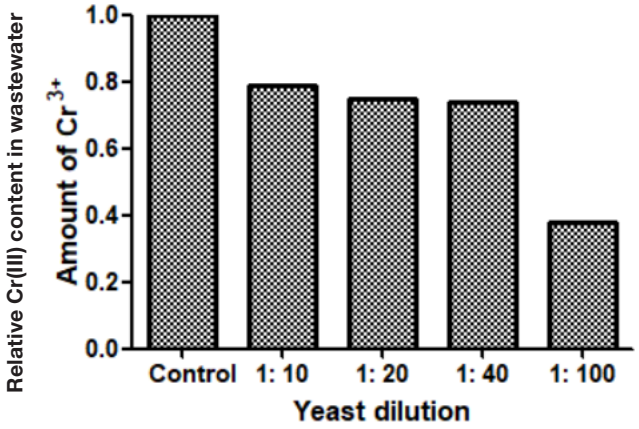

Figure 2. Chromium (III) uptake from wastewater is more effective with a smaller concentration of the biosorbent.

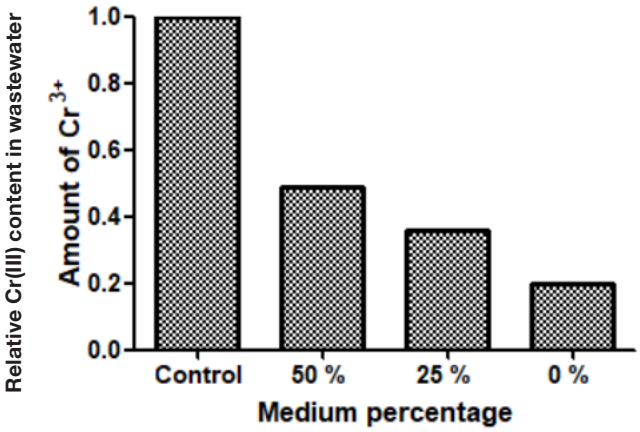

Figure 3. Chromium (III) uptake from wastewater is more effective when there is no extra growth medium added to the solution.

the solution and enough nutrients. As soon as they grow into a large quantity of cells, they start to run down on nutrients and slow down growth therefore also slowing down Cr^{3+} uptake. For that reason it is possible that if the wastewater is inoculated with a big amount of yeast cells they will not grow further and therefore they will not eliminate Cr^{3+} that efficiently.

Cr^{3+} *uptake experiments with different composition of growth medium*

To check how the biosorbent dose affects Cr^{3+} absorption, we decided to test the effect of the composition of the growth medium on the uptake of chromium (III). For this experiment we inoculated different amounts of wastewater (50, 75, 100 mL) with yeast adapted to 30 ppm of Cr^{3+} and added different amounts of medium (50, 25, 0 mL) to obtain a final volume of 100 mL. In Figure 3, it can be seen that the Cr^{3+} uptake is inversely proportional to the amount of growth medium in the wastewater, which means that the more growth medium the smaller is the Cr^{3+} uptake. It can be seen that when the solution is comprised of 50% growth medium and 50% wastewater, the Cr^{3+} amount was diminished by 51%, while there was less medium in the solution (25% and 0%), better results where obtained, which are 64% and 80% less chromium (III), respectively. The reason why this might be happening is that in the medium there is carbon which can also absorb heavy metals like Cr (III), but can't be filtered, so once the samples are digested and analyzed, all the Cr^{3+} absorbed by the carbon from the medium will stay in the solution. To conclude it can be said that the uptake of Cr^{3+} is more efficient when no extra growth medium is added to the wastewater.

Cr^{3+} *uptake experiments with different contact time*

Finally, we wanted to investigate how the exposure duration of the adapted yeast affects the Cr^{3+} uptake from the wastewater. To conduct this experiment, flask containing 100 mL of wastewater each were inoculated with 5 mL of yeast cells adapted to 5 ppm of Cr(III). Cultures were left in the wastewaters for different exposure periods (1 h, 3 h, 5 h and 24 h). As it can be seen in Figure 4, the best results where obtained after 24 h contact time, where the amount of Cr^{3+} was diminished by 39%. Shorter exposure periods such as 1 h and 3 h, showed a Cr^{3+} reduction of 18% and 20 % respectively, while the contact time of 5 h showed similar results as the contact time of 24 h with a Cr^{3+} reduction of 36%. From the results it is obvious that the longer the exposure period of the yeast to Cr (III), the bigger the Cr^{3+} reduction. However, the time required to attain maximum biosorption depends on the type of biosorbent, metal ion, and their combination. The rate of biosorption is rapid initially (within an hour) because all the active sites are vacant and available for metal ion biosorption. But with increase in time the rate of biosorption decreases due to increase in percentage saturation by metal ions remaining in the solution.

The results obtained were quantified in Image J program and normalized to the initial Cr^{3+} concentration.

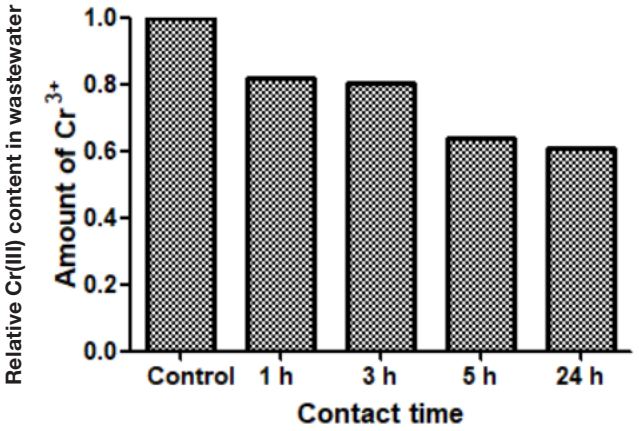

Figure 4. Chromium (III) uptake from wastewater is more effective when the exposure period is longer.

Conclusions

In this study, an alternative way for chemical treatment has been found to reduce the concentration of chromium in the wastewater from the treatment plant of the tanneries of Leather Cluster Barcelona. The application of microorganisms such as *Saccharomyces cerevisae* appears to be a low-cost biotechnological tool. In this paper it has been demonstrated that the yeast *Saccharomyces cerevisiae* can be used as a biosorbent to eliminate Cr^{3+} from wastewater and it has been shown how various factors such as composition of medium, biosorbent dose, contact time and adaptation of biosorbent to Cr^{3+} affect the Cr^{3+} absorbtion. It has been proven that the Cr^{3+} absorption is best in the conditions when the used biosorbent is pre-adapted to a similar concentration of Cr^{3+} like in the wastewater that needs to be treated, when there is a smaller concentration load of the biosorbent in the wastewater, when there is no extra medium added to the solution and long exposure period.

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Improvement of Leather Flame Retardancy through Nano Clay Addition

by
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Abstract

Leather is widely used in various industries including apparel, safety clothing, aircraft and automotive, due to its unique properties such as softness, air permeability, chemical resistance, high flexibility and reasonable mechanical resistance. Since leather products usually contain flammable organic compounds such as tanning, fatliquoring, dyeing and finishing materials, improvement of its flame retardancy is very important. A lot of flame retardants have been synthesized and applied to improve flame retardancy of leather. One of the best materials is nanoclay because it is easily available, environment-friendly and has a low cost. In this research, we propose a process that reduces the burning length of the leather and increases its thermal stability. For this purpose, clay nanoparticles by 1, 3, and 5 mass percent (relative to wet leather mass) were added to wet-blue bovine and goat leather under certain (temperature and time) conditions during the re-tanning process. The results of the TGA test on bovine leather samples showed that by increasing the amount of nanoclay, the thermal stability of samples was increased. SEM images prepared from the grain and cross-sections of bovine and goat leather samples showed that with a high percentage of nanoclay, particle agglomeration is partially visible. The results of the vertical flammability test also showed that the presence of clay nanoparticles reduced the burning length of leather samples. Tensile strength of bovine leather samples containing clay nanoparticles increased compared to the control sample. However, the tear strength of them did not differ significantly.

Introduction

Anti-fire or flame retarding characteristic of textiles has become very important due to the development in urbanization and technology advancements; specially regarding the use of electrical appliances which has increased the possibility of ignition. Leather is a material that due to its special properties such as softness, air permeability, chemical resistance and high flexibility, is widely used in various industries, including apparel, aircraft and automotive. Although leathers have much better flame resistance properties than fabric and plastic materials, its use requires improvement in flame resistance properties,[1] because the leather products after tanning, fat liquoring, dyeing and finishing processes could contain some inflammable and harmful organic compounds.[2]

In recent years, the use of nanotechnology in leather production processes to improve leather performance and lower production costs has increased.[3]

The use of nanomaterials, when properly distributed in a polymer structure, can improve thermal, mechanical and fire resistance properties. One of the best nanomaterials, nanoclay, has received much attention as a reinforcing material for polymers due to its potentially high aspect ratio and unique intercalation characteristics.[4] Also it is easily available, environmentally friendly and has a low cost.[5] Adding a small amount of nanoclay into a polymer matrix exhibits an unexpected shift in properties including reduced gas permeability, improved solvent resistance, being superior in mechanical properties and thermal stability and enhanced flame retardancy.[4]

The optimal dispersion of nanoclay in the samples causes it to act as a barrier and prevent the penetration of oxygen and heat into the sample and prevent the release of flammable products. Montmorillonite (Mt), a special kind of clay, has become the subject of considerable interest over the past few years for its lamellar structure and larger surface area.[6]

Several studies have been made about nanoclay as a reinforcing material. Khalid Saeed et al.[7] studied the thermal properties of nanoclay/PEO. In another study, Binu et al.[8] concentrated on the analysis of the effect of nano filler (Cloisite15A) on the glass fiber mat reinforced polyester. They studied the effect of use various weight percentages of nano filler on mechanical, thermo mechanical properties and thermal degradation. In another study,[9] organoclay based acrylic polymer nanocomposites were used as filling agents for leather. That study was done by Essambo et al.

Articles are available about finishing natural leather to achieve improved functionality e.g. antibacterial or anti fogging leather. Pollini et al.[10] deposited silver coatings on the natural leather used in public transport system seats. Carvalho et al.[11] studied about antimicrobial activity of leathers covered with Ag–TiO2NPs.

There are some articles about the improvement of leather flame retardancy. Jiang et al.[2] reported the use of Montmorillonite and IFR nanocomposite on pig skin. Their research results

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showed that the nanocomposite has good charring effect, can effectively improve non-flammability of leather and has good flame resistance properties. Olivares et al.[3] examined the effect of sodium montmorillonite on properties such as flame resistance; mechanical and thermal properties of bovine leather. They reported that the burning length of leather was reduced and the presence of clay mineral improved the thermal stability of leather. Wegene and Thanikaivelan reported a method to produce flame resistance leather using organic nanoclay and ammonium dihydrogen phosphate as flame retardant additives through surface coating. They showed that the flame resistance properties of leathers improved after treatment with flame retardant additives.[1] Lyu et al.,[6] used a nanocomposite containing montmorillonite as a fatliquoring agent. They showed that the flame retardancy and Limited Oxygen Index (LOI) value of a leather sample treated with nanocomposite could be increased. Yang et al.[12] reported the use of melamine resin on wet-blue leather. Their research results showed that the oxygen index and thermal stability of leather were improved significantly. Zhang et al.[13] examined the effect of phosphorus-nitrogen flame retardant retanning agent on properties of wet-blue goat leather. They reported that the oxygen index increased, but mechanical properties decreased. Baorong et al.[14] investigated the effect of phosphorus flame retardant (PFR), nitrogen and phosphorus intumescent flame retardant (NPIFR) and nitrogen and phosphorus flame retardant (NPFR) on the flame retardancy of leather. The results showed that the three types of flame retardants can enhance the fire retardancy of leather. Antifogging properties and flame retardancy of leather treated with modified layered double hydroxide (MLDH) and modified zanthoxylum bungeanum maxim seed oil (MZBMSO) was studied by Bin et al. They reported that the leather samples treated with those materials exhibited remarkable improvements in fogging value and reduction in the length of charring and smoke density.[15]

In the present work, wet-blue bovine and goat leather were examined and the effect of sodium montmorillonite, added during the re-tanning process, on the flame retardancy was investigated.

The main objective of this research was to figure out a method to improve the leather flame retardancy that would have the least dependency on the machinery and materials currently used in a plant. Therefore the materials used in this research were of commercial grade and were directly supplied from leather production lines.

Experiments

Materials and equipment

Wet chrome tanned bovine (0.1 g/cm^2) and goat (0.05 g/cm^2) leather were supplied from tanning plants located in Charmshahr industrial zone, Varamin, Iran. The supplied leather samples were produced through the tanning plants conventional leather processing and no change was made in their process with the aim of achieving a method that would be easily used in those leather plants.

Sodium formate, acrylic resin, vegetal tanning (Mimosa), neutralizer, acidic dyes as dyeing product, natural and synthetic fatliquors were purchased from Takshimi Co., Iran. Formic acid 98-100% and acetic acid 100% were purchased from Merck.

Sodium montmorillonite K10 with a cation exchange capacity of 48 meq/100gr and density 0.5-0.7 gr/cm^3, was purchased from NanoSany, Iran.

To investigate the effect of nanoclay presence on the thermal behavior of samples, thermal analysis (TGA) was carried out with heating rate of 10°C / min under nitrogen atmosphere on three samples of bovine leather using TA-instrument analyzer, manufactured in USA (ca. 1-2mg).

In case of grain surface images, XL30 SEM device, manufactured in Netherlands and 1x1 cm specimens were used. In case of cross sectional images, South Korea's AIS2100 SEM device and 1x2 mm specimens were used. Images with different magnifications were taken. Elemental analysis of samples was also performed.

Five specimens of each sample were prepared and their mechanical properties were evaluated using an Instron tensile testing machine at 100 mm/min crosshead speed according to Standard Numbers 3376 and 3377-1 of the International Organization for Standardization (ISO). The samples were placed in room conditions for 24 hours before the test and their thickness was measured at three different points.

Flame retardancy properties were evaluated through sixty seconds vertical flammability test according to Title 14-Aeronautics and Space, Part 25-Airworthiness standard, Appendix F to Part 25, Part I-Test Criteria and Procedures, published by the United States Department of Transportation of the Federal Aviation Administration suitable for testing the flammability of materials in vertical configuration (covered by paragraph (a) (1) (i) of this appendix).[2]

Specimens, 5×16 cm, were prepared and kept in room condition for 24 hours before the test. Samples' thickness was measured at three different points. The flame used in this test was a flame from a mixture of propane and butane gases (3.81 cm length). The flame was applied once to the center line of the lower edge of the specimen (1.90 cm above the top edge of the burner)[3] and was removed from the bovine samples after 60 seconds and from the goat samples after 12 seconds; due to the lower thickness. After the test, the burning length of the specimens was measured from the beginning

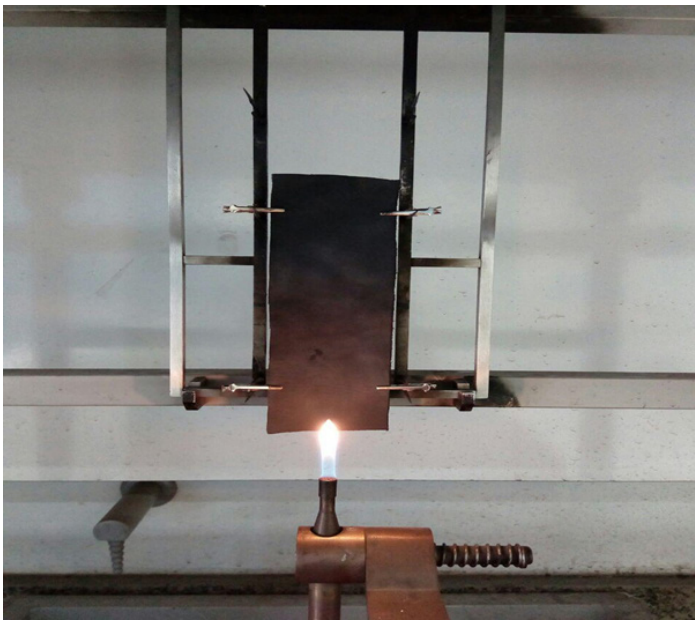

Figure 1. Sample placement for vertical flammability test.

of the burn to the burning peak. 3 specimens of each sample were prepared for this test. Fig. 1 shows sample placement for vertical flammability test.

Procedure

A dispersion of 5 mass percent of nanoclay in water was prepared using an ultrasonic mixer (frequency: 24 kHz, power: 200W, amplitude: 60%) for one hour. The prepared solution was placed on a magnetic stirrer at 250 rpm for 180 min before being used. This dispersion was added to the leather processing bath during the re-tanning step.

The order of the process steps and the percentage of materials added in each step are presented in Table I. This process is a commonly used version in Iran's leather plants and is based on BASF solutions for leather technologists.

At first, pieces of previously prepared wet blue leather (bovine and goat) were rewetted and weighed. This weight was used in calculating the amount of materials added at each step. In order to improve the material penetration into the leather structure, the baths were stirred every 10 minutes using a glass stirrer, and the leather pieces were pressed every 10 minutes by-hand.

The process steps, in accordance with table I, were as follows:

a) Washing

Wet blue pieces were washed in a bath containing acetic acid and water at 30°C for 5 min. After 5 minutes, the bath float was drained.

b) Neutralization

At this step wet blue pieces were placed in a bath containing water, sodium formate, neutralizer and acetic acid at 40°C for 40 minutes. At the end of this step, using scissors, the corner of leather pieces was cut and using a torrent paper, the pH of the leather cross section was measured. The pH at this stage should be 4.2. At the end of this step, the bath float was not drained and the next step materials were added to the same bath.

c) Retanning

Acrylic resin was added at this step to enhance the leather handle. The wet blue pieces remained in the bath at 40°C for 40 minutes. At the end of this time, Mimosa, neutralizer and acrylic resin were added to the bath and the wet blue leathers remained at the same temperature for 60 minutes.

Table I

Outline formulation of process.

Process	+	%	Product	°C	Dilution/ °C	Time (min)	pH, etc
Wash		200	Water	30			
		0.2	Acetic acid			5	6 Be
Drain float							
Neutralization	+	100	Water	40			
		1	Sodium formate				
		1	Neutralizer			40	pH 4.2
Retannage	+	4	Acrylic resin			40	
	+	1	Neutralizer				
		4	Acrylic resin				
		4	Mimosa			60	
	+	0.3	Formic acid 85%		1:10	10	
Drain float							
Short rinse							
Add nanoclay	+	100	Water	40		60	
			Nano clay				
Dyeing	+	100	Water				
		1	Acidic dye	50		20	
Fatliquoring	+	8	Natural and synthetic			40	
	+	0.5	Formic acid 85%		1:10	20	
	+	1	Acidic dye			20	
	+	0.5	Formic acid 85%		1:10	10	
Drain float							
Rinse beriefly							

Table II
Samples description

Sample Identification	Na^+Mt content [mass%]
Leather	0
Leather-Na^+Mt_1%	1
Leather-Na^+Mt_3%	3
Leather-Na^+Mt_5%	5
Leather-Na^+Mt_7%	7

d) Fixation
At the end of the previous step, formic acid (85%) was diluted 1:10 in water and added to the bath. After 10 minutes, the bath was drained and the leather pieces were rinsed for a short time with cold water.

e) Nano clay addition
At this step, only for flame retardant samples, baths containing nanoclay were prepared and retanned leather pieces were placed in these baths at 40°C for 1 hour. At the end of this step, the bath was drained and the samples were entered into the dyeing and fatliquoring baths. The amount of clay added for each sample is shown in Table II.

f) Dyeing and fatliquoring
The dyeing of the samples was done in two steps and after each step, the diluted formic acid was added to the bath. In the first step of dyeing, the samples were placed in a bath containing dye at 50°C for 20 minutes. At the end of this time, the fatliquor was added to the bath to soften the samples, and the time required to absorb the fatliquor was 40 minutes. After that, diluted formic acid was used for 20 minutes.

In the second step of dyeing, the dye was added to the previous bath and the samples were dyed again for 20 minutes at 50°C and finally the diluted formic acid was used for 10 minutes.

g) Washing and drying
After dyeing and fatliquoring step, the samples were rinsed with cold water and were dried using a hair dryer for 10 minutes, and then placed in room temperature to complete the drying step.

Results and Discussion

Thermal stability
The thermal stability of leather can be affected by the presence of nanoclay. For this purpose and to investigate the effect of nanoclay presence on the thermal behavior of samples, thermal analysis (TGA) was carried out in nitrogen.

It has been reported that the thermal degradation of sodium montmorillonite proceeds in two steps: first, the sample is dehydrated when the temperature increases from room temperature to 270°C and the water molecules adsorbed in pores and clay galleries are removed. The major decomposition region of the sodium montmorillonite is between 300-450°C, in which clay decomposes into aluminum oxide and silicon dioxide.Then, the sample undergoes dehydroxylation of structural –OH groups, which occurres between 500 and 700°C.[16]

Fig. 2 shows the TGA and DTG curves of three bovine leather samples. As can be seen in Fig 2, the decomposition mechanism of leather samples in nitrogen is a one-step process and the initial temperature of their decomposition, from the control sample, to the leather-Na^+Mt_5% and leather-Na^+Mt_7% samples

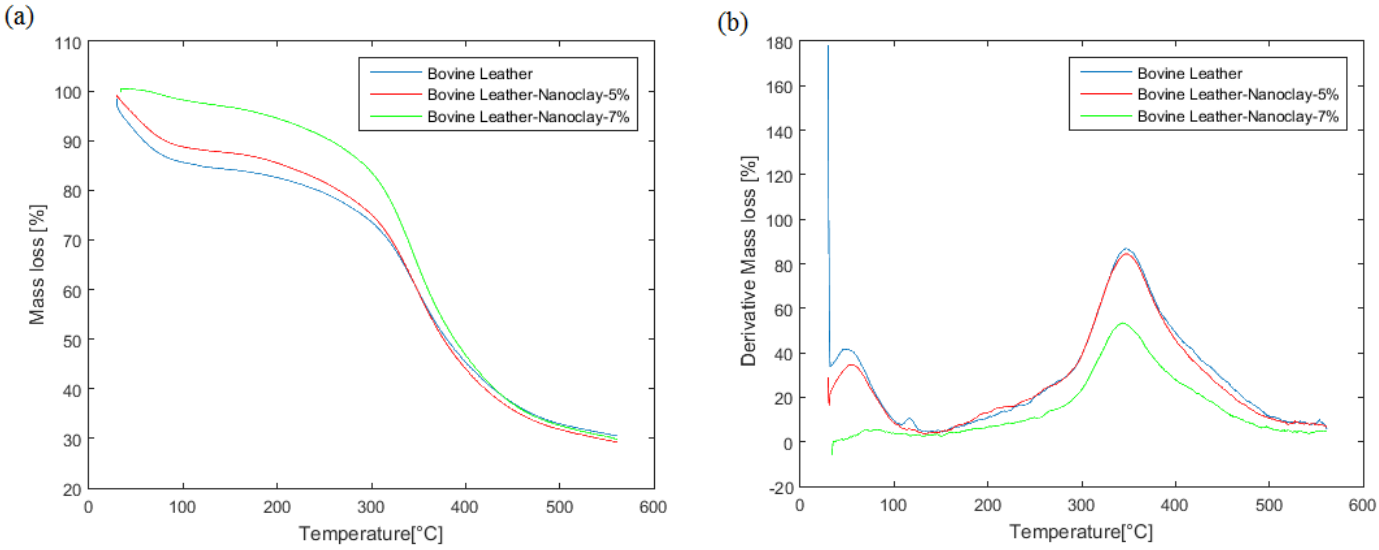

Figure 2. (a) TGA and **(b)** DTG curves of leather samples having different clay content in nitrogen.

increased. This increase in temperature indicates that the presence of nanoclay, protects leather samples against thermal decomposition. The greatest mass reduction of the samples is in 300-400°C, which can be attributed to collagen degradation in the samples. For better comparison, it can be said that 5 mass percent reduction for the control sample, the leather-Na^+Mt_5%, and leather-Na^+Mt_7%, happens at 42°C, 54°C and 192°C respectively which shows that by increasing nanoclay percentage, the thermal stability of the samples has increased.

Lowering the initial mass of the samples, which occurred between room temperature and 100°C, can be considered as loss of water and moisture.

According to the DTG curves, the temperature related to the highest amount of mass reduction, for the control sample is 346°C, for leather-Na^+Mt_5% is 349°C and for leather-Na^+Mt_7% is 344°C. Although T values are almost equal, the lost mass for samples at the relevant temperatures is 86.7%, 84.4% and 53.5%, respectively which shows that the sample with the highest amount of nanoclay, has more thermal stability and has the lowest mass reduction.

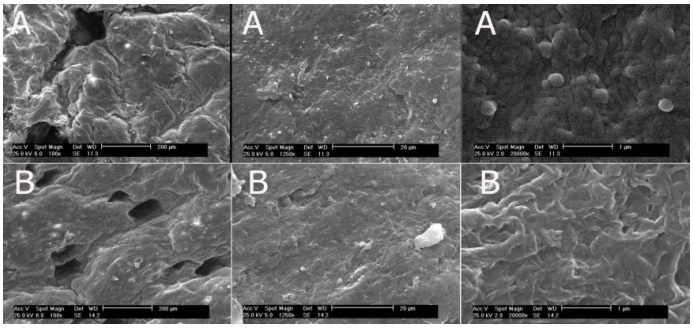

Figure 3. Grain surface SEM images of goat leather at 100, 1250 and 20000× magnification, respectively; **(A)** leather and **(B)** leather-Na^+Mt_3%.

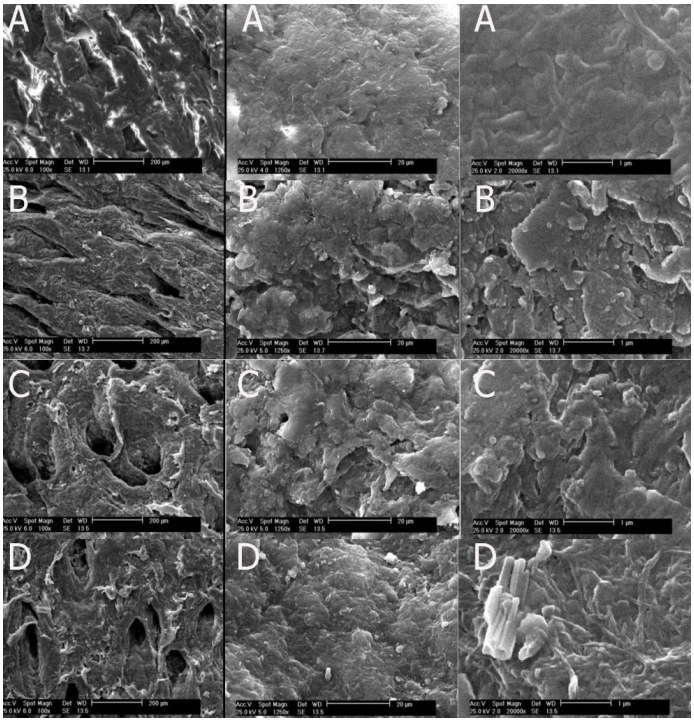

Figure 4. Grain surface SEM images of bovine leather at 100, 1250 and 20000× magnification, respectively; **(A)** leather, **(B)** leather-Na^+Mt_1%, **(C)** leather-Na^+Mt_3% and **(D)** leather-Na^+Mt_5%.

Morphology

SEM images were prepared from the grain and cross section of bovine leather, leather-Na^+Mt_1%, leather-Na^+Mt_3%, leather-Na^+Mt_5% and goat leather and leather-Na^+Mt_3% samples to observe their surface changes, as well as the presence of nanoparticles.

Fig 3(a) and (b), represent the grain surface of control goat leather and leather-Na^+Mt_3% samples, respectively. In Fig 3(a) the agglomeration of nanoparticles is not seen. However the agglomeration of nanoparticles in the sample containing nanoclay is seen compared to the control sample.

In Fig 4(b), which shows the grain surface of bovine leather-Na^+Mt_1% sample, there is no significant difference from Fig 4(a) which indicates the control sample. However, nanoparticles agglomeration are seen in the samples with 3% and 5% nanoclay.

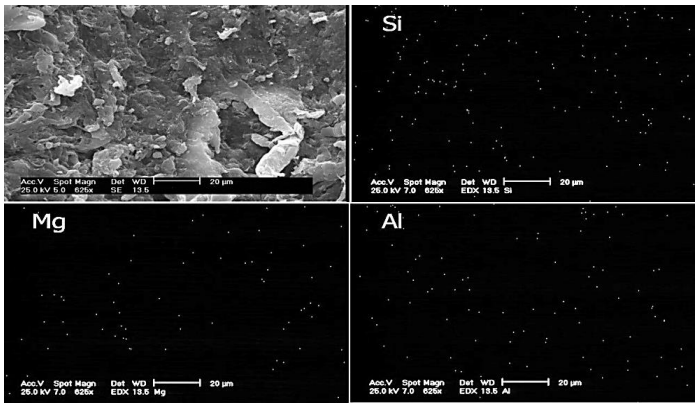

Figure 5. Elemental analysis of sample: leather-Na^+Mt_5%.

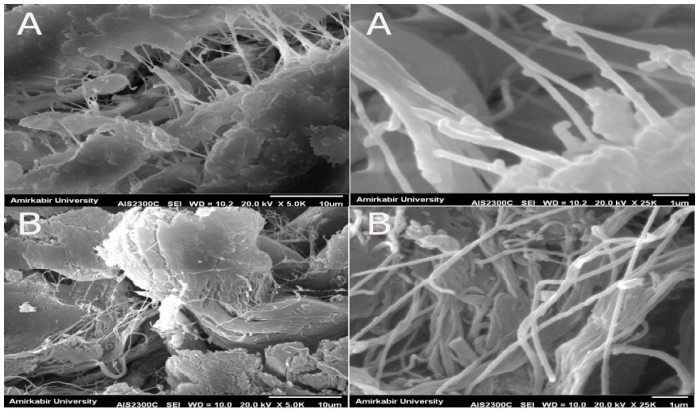

Figure 6. Cross sectional SEM images of goat leather at 500 and 2000× magnification, respectively; **(A)** leather **(B)** leather-Na^+Mt_3%.

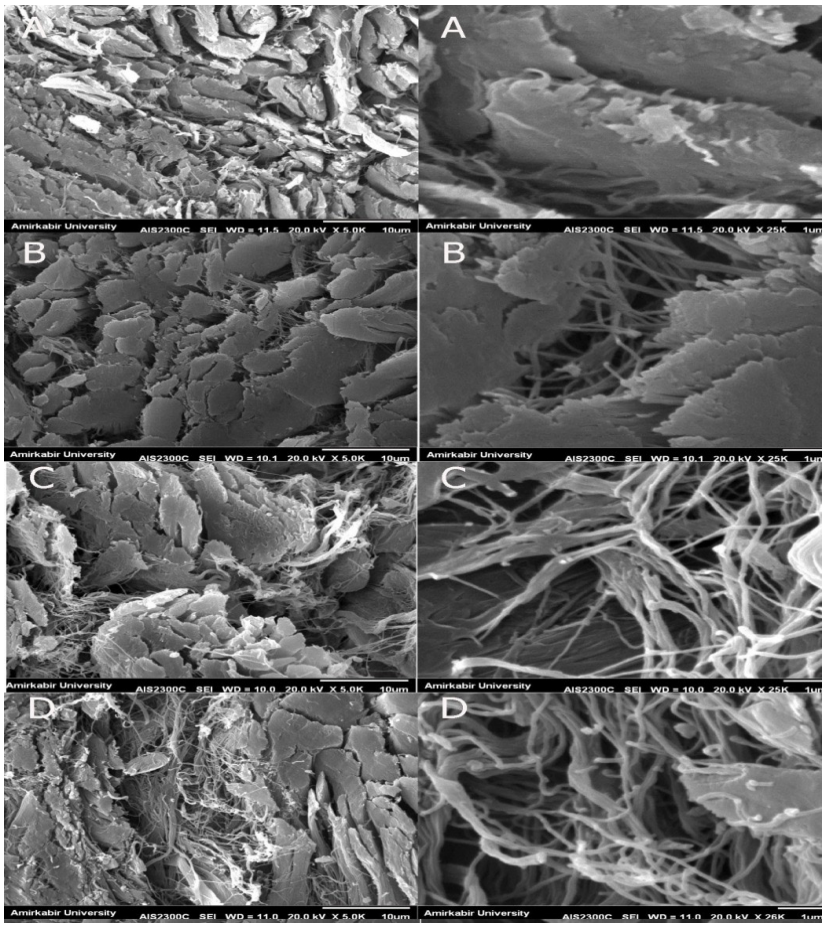

Figure 7. Cross sectional SEM images of bovine leather at 500 and 2000X magnification, respectively; **(A)** leather, **(B)** leather-Na^+Mt_1%, **(C)** leather-Na^+Mt_3% and **(D)** leather-Na^+Mt_5%.

According to the images of the electron microscope, it is observed that the size of samples' pores is microscopic. Because in the 100x magnification, these pores are well observed. Therefore, the distribution of clay nanoparticles in these pores is expected.

Fig. 5 represents the mapping of some elements found in the leather-Na^+Mt_5%. Si, Al and Mg, the main components of Na^+Mt, are homogeneously dispersed within the network of leather.[3]

Fig. 6 and 7 show cross-sectional SEM images of goat and bovine leather samples. Comparing the images of control and nanoclay containing samples, no significant agglomeration in the cross section of the samples can be observed.

According to the SEM images, it can be concluded that by increasing nanoclay percentage, the agglomeration of nanoparticles increases. However, this agglomeration is mostly seen on the grain of samples and images of samples cross-section do not show this agglomeration. Hence probably the clay nanoparticles have dispersed homogeneously within leather structure.

Tensile and tear strength

The tensile strength of leather depends on some factors such as the strength of the collagen fibers, the angle of the fibers with each other, the amount and type of material that is placed between the fibers, and so on.

By increasing the entanglement of collagen fibers, the modulus increases.

In order to investigate the effect of nanoclay on the strength of leather samples, tensile and tear strength of samples were evaluated using an Instron tensile testing machine.

Table III shows the mechanical properties of the leather samples.

As can be seen, by the addition of nanoclay, the modulus of the samples has increased, which indicates that the nanoclay performs as filler and increase entanglement of fibers and does not reduce the strength of the leather samples. In addition, the electrically-charged surface of clay that is negative due to the presence of

Table III

Mechanical properties of leather samples.

Sample	Average thickness (mm)	Tensile strength (MPa)	CV%	Average thickness (mm)	Tear strength (N)	CV%
Leather	2.04	13.17	26.37	2.22	63.02	11
Bovine Leather Na+Mt_1%	2.7	37.94	31.60	2.03	49.93	13
Bovine Leather Na+Mt_3%	2.43	20.18	43.19	2.02	61.55	15
Bovine Leather Na+Mt_5%	1.9	23.00	29.88	2.18	52.87	22

oxygen groups[17] creates a series of hydrogen bondings between collagen and nanoclay, which increases the tensile strength. However, the highest strength is related to the treated sample with 1% nanoclay. Increasing nanoclay amount to 3% and 5% may result in nanoclay agglomeration, which acts as a stress center and reduces the samples' strength. In addition, this increase in the amount of nanoclay probably reduces the entanglement of collagen fibers and reduces the strength of the leather compared to the treated sample with 1% nanoclay.

Tear strength actually shows the strength of the collagen fibers and the amount of adhesion bonding between them and is directly related to the quality of skin maintenance. If the skin is not kept properly, the tear strength will be low.

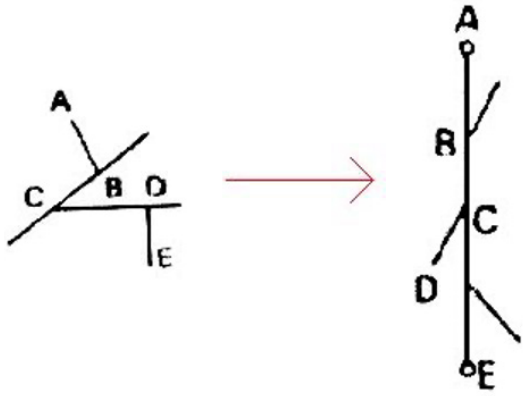

Figure 8. Formation of long fibers from entanglement short collagen fibers by applying force

Table IV

Burning Length of Bovine Leather

Sample	Average thickness (mm)	Average burn length (cm)	CV%	Average char from burning (%)	CV%
Leather	3	8.7	37.84	28.76	21.68
Bovine Leather-Na^+Mt_1%	2.4	5.9	14.77	14.32	6.78
Bovine Leather-Na^+Mt_3%	2.5	7.4	7.95	22.51	31.62
Bovine Leather-Na^+Mt_5%	2.3	7.3	24.25	26.40	30.43

Table V

Burning Length of Goat Leather

Sample	Average thickness (mm)	Average burn length (cm)	CV%	Average Char from burning (%)	CV%
Leather	1.2	6.1	38.41	42.28	36.39
Goat Leather-Na^+Mt_1%	1.4	4.9	8.89	9.57	33.78
Goat Leather-Na^+Mt_3%	1.4	4.7	8.66	6.77	9.51
Goat Leather-Na^+Mt_5%	1.4	5.3	28.14	12.25	48.13

By increasing the thickness of the leather, first increases its tear strength. However, if the empty spaces between the fibers are completely filled with solids, the thickness of leather increases too much and the tear strength will start to decrease. Because when we apply a force to stretch the fibers, short fibers cannot form the long fibers in the direction of force. Fig.8 shows the formation of long fibers from entanglement short collagen fibers by applying force.

The amounts of tear strength of samples can be seen in Table III. It can be said, the slight difference between the tear strength is due to the difference between the thicknesses of the samples.

However for more explanation about this slight difference, we can say that the presence of nanoclay between collagen fibers has reduced the tear strength of leather samples due to the reduction of the formation of longer fibers.

Flame retardancy properties

The vertical flammability test is one of the most important tests in terms of flammability. During a vertical flammability test, a material for its burning length (after the igniting flame is removed) and the amount of the char left is observed.

In order to investigate the effect of nanoclay presence on bovine and goat leather samples, the burning length of samples and their mass before and after burning were measured and the average of these lengths as well as the percentage of the average amount of char of the samples, are reported in Tables IV and V.

According to the data in Table IV and V, it can be concluded that the addition of nanoclay in the samples reduces their burning length and the average percentage of leather charcoal that can indicate the damage severity to a sample. In addition to the performance of nanoclay during fire, non-combustible gases, such as steam and carbon dioxide released by decomposition of collagen during burning, can also help to dilute the concentration of oxygen gas. This is confirmed by Jiang and his colleagues.[2]

As shown in Table IV, the average burning length and percentage of charcoal are the highest for control bovine leather, and the lowest for bovine leather with 1% nanoclay.

By increasing in the amount of nanoclay from 1% to 3% and 5% for bovine leather, the average burning length increased. It can be said that the leather treated with 1% nanoclay has a good distribution

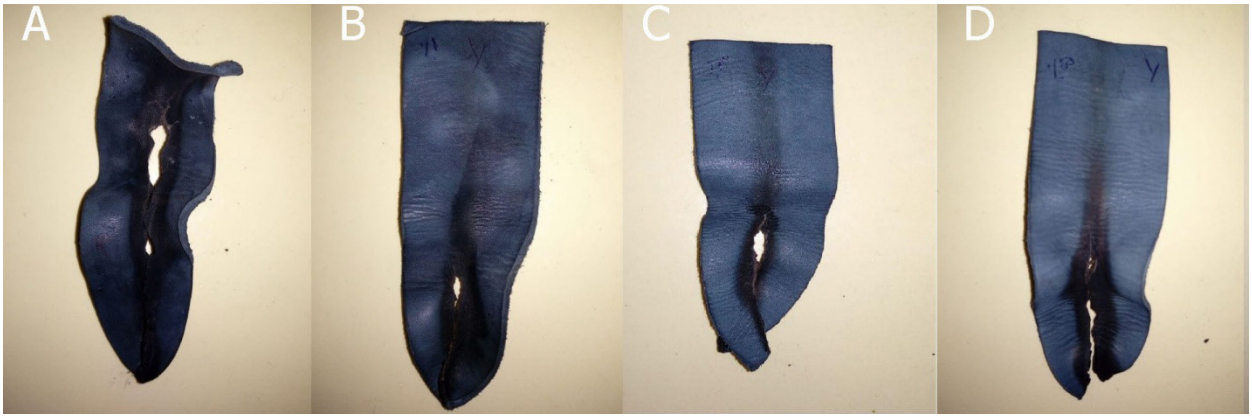

Figure 9. Bovine samples evaluated by flammability vertical test according to 14 C.F.R. Appendix F to Part 25 Part I (a) (1) (i). **(A)** leather, **(B)** leather- Na+Mt_1%, **(C)** leather- Na+Mt_3% and **(D)** leather- Na+Mt_5%.

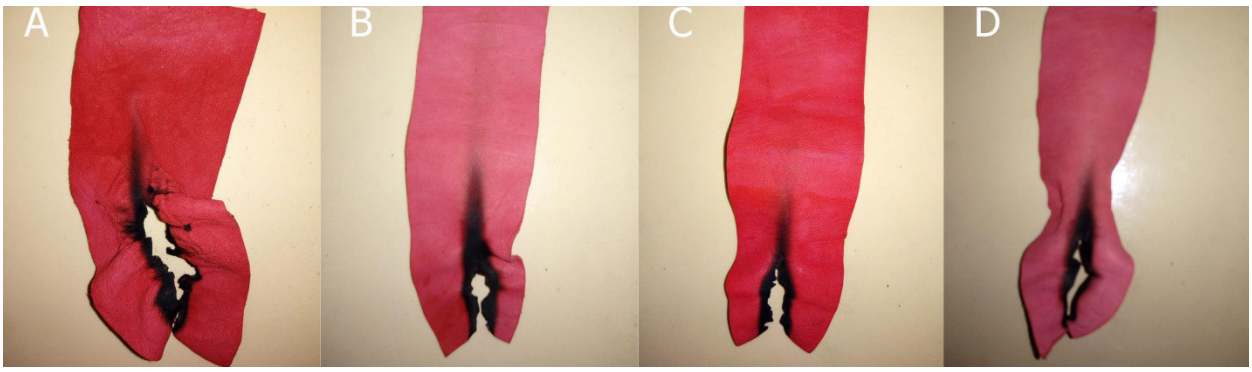

Figure 10. Goat samples evaluated by flammability vertical test according to 14 C.F.R. Appendix F to Part 25 Part I (a) (1) (i). **(A)** leather, **(B)** leather- Na+Mt_1%, **(C)** leather- Na+Mt_3% and **(D)** leather- Na+Mt_5%.

of this material between collagen fibers, however by increasing nanoclay percentage to 3% and 5%, clay nanoparticles probably agglomerate between collagen fibers and this agglomeration may not be in the burn path. Therefore, they do not reduce the burning length.

As can be seen in Table V, the highest burning length and charcoal are for control goat leather and the lowest are for goat leather with 3% nanoclay.

Since goat leather has more pores than bovine, the distribution of nanoclay in it, is better than bovine leather and more nanoclay is needed to achieve the optimal average burning length. Therefore, it has been expected that the optimal reduction of average burning length is obtained for goat leather with more nanoclay.

Also from the images of the burning length test, it can be concluded that the deformation and damage of the control samples of goat and bovine leather are higher than the other samples.

In Fig. 9 and 10, the comparison of the burning length of bovine and goat leather samples is possible.

Conclusion

In this study, the effect of clay nanoparticles on selected characteristics of bovine and goat leather was investigated. Sodium montmorillonite, a special type of clay, was selected for this study because it has no environmental hazards and its low price. It also improves flame retardancy by acting as a barrier and prevents the penetration of oxygen and heat into the samples and prevents the release of flammable products. In addition, it does not reduce the tensile strength of the leather. The final product also has an economical advantage. Some materials used in other studies are more expensive than nanoclay or have environmental hazards. In some cases they also reduce the tensile strength of leather. However, the main concern in this work was to figure out a process that can be applied in the leather production plants with only slight changes in the current machinery and chemicals.

In this study, clay nanoparticles by 1, 3, and 5 mass percent were added to wet-blue bovine and goat leather during the re-tanning process under the specified temperature and time conditions.

The result of TGA tests on bovine leather samples showed that samples with 5% and 7% nanoclay had higher thermal resistance than the control sample.

SEM images prepared from the grain and cross-sectional of bovine and goat leather samples showed that with a high percentage of nanoclay, particles agglomeration was partially visible. However, this agglomeration was not observed in samples with 1% nanoclay.

Tensile strength of bovine leather samples containing clay nanoparticles increased compared to control sample and the optimum amount of this increase was obtained with 1% nanoclay, so that the average modulus of control bovine leather samples and samples with 1% nanoclay were 13.17 and 37.94 MPa, respectively. However, the tear strength of the bovine leather containing nanoparticles did not differ significantly compared to the control samples.

The results of vertical burning test also showed that the presence of clay nanoparticles reduced the average burning length of leather samples. However, the optimum amount of this reduction was obtained for bovine and goat leather with 1% and 3% nanoclay respectively. The average burning length of bovine samples was 8.7 cm for control samples and 5.9 cm for samples with 1% nanoclay, and these values for goat control and leather- $Na^{+}Mt$_3%, were 4.9 and 1.6 cm, respectively.

Proper selection of materials used such as fatliquor, filler, dye, etc. can affect the flame retardancy of leather, so the process and materials of this research were designed and selected without changing the normal routine of leather factories. Therefore, only the effect of adding nanoclay can be examined. Since the results of this study indicate an improvement in the leather flame retardancy, without adversely affecting its strength, it is necessary to optimize the choice of materials and production conditions.

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Lifelines

Catherine Maidment is a research associate at New Zealand Leather and Shoe Research Association with research activities focusing on the study of molecular components in skin and hide, particularly proteins and glycosaminoglycans, and how they affect leather quality. In 2019 she graduated from Massey University, New Zealand with a master's degree in biochemistry.

Meekyung Ahn graduated from Massey University with a PhD in synthetic organic chemistry in 2007, followed by a post-doctoral position working on the proteomic characterization of processed hides. From 2009 to 2017, Meekyung was employed as a research scientist by LASRA (Leather and Shoe Research Association, NZ) and was part of a team trying to relate the molecular building blocks of animal hides and skins to their physical properties. She was also interested in and researched the use of microbes to convert waste materials from the leather industry into high value materials.

Rafea Naffa, see *JALCA* 114(1) 38, 2019

Trevor Loo is a laboratory manager that oversees the operation of the mass spectrometry facility at Massey University and a core member of the protein structural biology group, plays an active role in research and involves in regulatory compliance, maintenance and equipment training around School of Fundamental Sciences. Trevor majored in biochemistry and has extensive experience in cloning, expression, purification and characterization of proteins from bacterial, fungal and mammalian sources by mass spectrometry, biological assays and structural approaches.

Gillian Norris, see *JALCA* 110, 379, 2015

Ricardo Tournier earned a degree in Chemical Engineering from Universidad de la República, Montevideo, Uruguay in 1968 and a MSc in Chemical Engineering from University of South Carolina, USA in 1971. In 1974 he attended a Dyestuff Course at the Chemistry Department of Turin University and a Practical Course on hide tanning at Instituto "Baldracco," Turin, Italy. Started in the leather industry in 1971 at Lanza Tannery, Uruguay, for 9 years. Later he worked for 20 years as Technical Manager at Paycueros Tannery, a member of SADESA Group. From 2000 to 2012 he was Technical Assistant to the General Manager at Zenda Leather, Uruguay. His works on leather problems and defects have been published in regional journals. He is currently working as a freelance consultant.

Patricia Janković graduated in Biotechnology at the Department of Biotechnology, University of Rijeka (Croatia) in 2017. She received the Master in Pharmaceutics and Drug Design from the Department of Biotechnology, University of Rijeka (Croatia) in 2019. During her studies, she has worked as an intern at the Medical University of Lodz in 2018 and at the A3 Leather Innovation Center in 2019.

Reno Spinosi is Doctor in chemical science at the School of Advanced Studies of the University of Camerino. I have an undergraduate degree in Chemistry fromthe University of Camerino. Since 2017 he performed a post-doctoral stay at the A3 Leather Innovation Center. In the last year, the two projects in which he was involved were focused on the evaluation and reuse of residue coming from the leather sector.

Sílvia Sorolla is Doctor in Chemical Engineering by Universitat Politècnica de Catalunya. Since 2012 she has being working as a Researcher and Project manager at A3 Leather Innovation Center. She is mainly involved in the development of R&D projects at National and European level focused on the evaluation and reuse of residues from the leather sector.

Anna Bacardit graduated in Industrial Engineering Organization at the Technical University of Catalonia (Spain) in 2003. She got his Ph.D. degree in Chemistry at the University of Barcelona in 2005. She received the Master in Tanning Technical Management from the Technical University of Catalonia (Spain) in 2001. From 1995 to 1999 she has been working in the Technical Laboratory at BASF-Curtex in L'Hospitalet (Spain). Since 1999 she has being working as a lecturer at the Igualada Industrial Engineering Technical School – Igualada Tanning School at the Technical University of Catalonia. Now is full professor at Escola Politècnica Superior (UdL). She is mainly involved in the development of cleaner and innovative leather processing methods.

S. Sepehri received her B.Sc (2013) from Isfahan University of Technology and her M.Sc. (2018) from Amirkabir University of Technology (Tehran PolyTechnic) in Textile Engineering. Her thesis was *Improvement of Leather Flame Retardancy Properties* under the supervision of Prof. Amani Tehran. She has worked as a researcher in textile companies. She is currently doing research in person. Her research interests are Leather Production Improvement, Nano fibers and Nano composites.

M. Amani Tehran is a professor in Textile Department of Amirkabir University of Technology, Tehran, Iran. He received his B.Sc. (1987) from the Isfahan University of Technology and his M.Sc. (1990) from Sharif University of technology in Chemical Engineering. His Ph.D. (2001) is in Color Technology from Amirkabir University of Technology. In 1991 he employed as lecturer in Amirkabir University of Technology and now he continues working as a professor there.

F. Zeighami received her Ph.D (2016) in Textile Engineering from Amirkabir University of Technology (Tehran Polytechnic). She is currently the R&D manager in a textile company and teaches in Elmi-karbordi and Honar universities as a visiting Professor. Her research interests include Nanofibers, Electrospinning, Color Physics and Leather Production Improvement.

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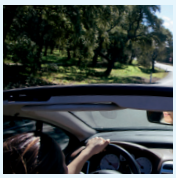

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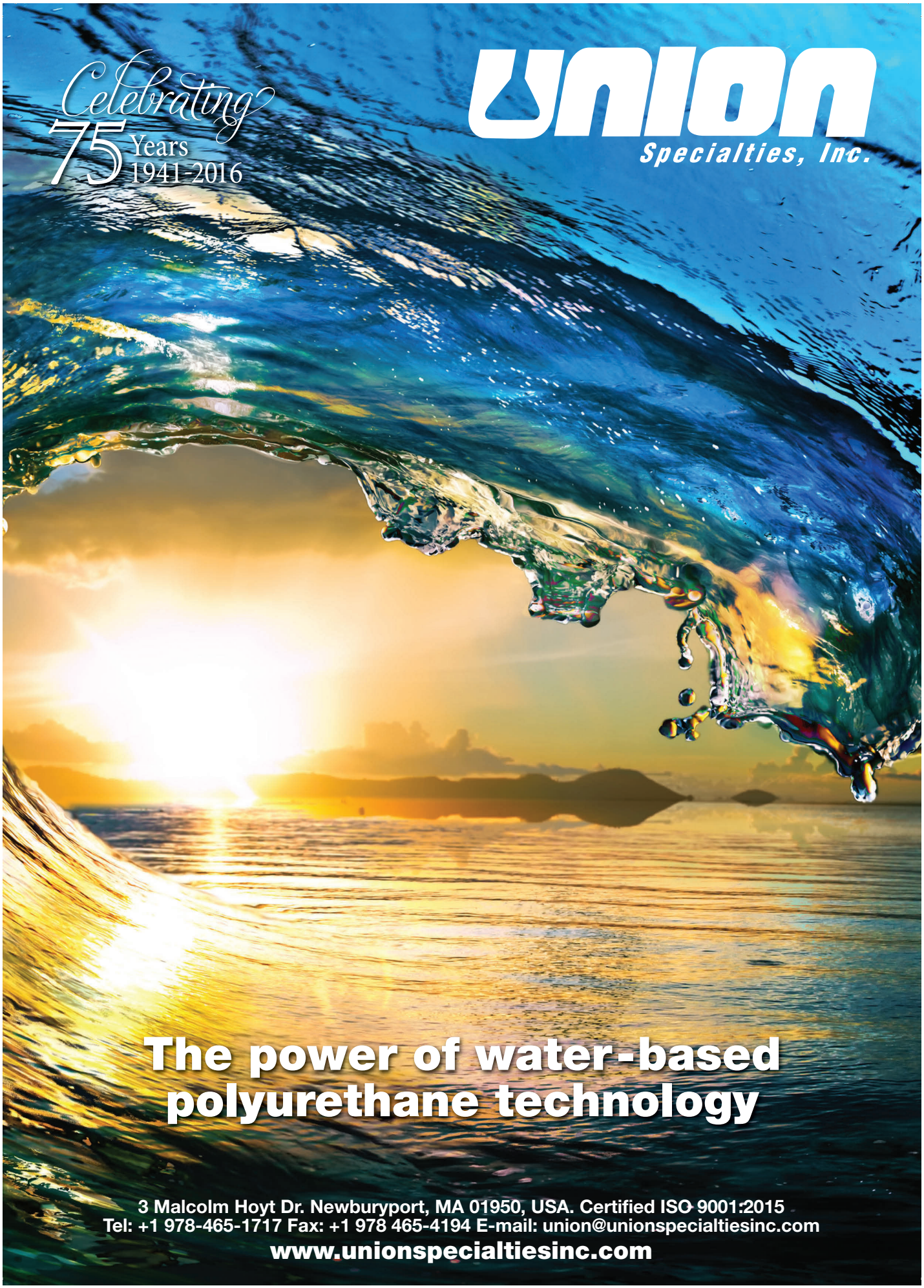
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Obituary

David Robert Small died peacefully on July 24, 2020, at the Royal Megansett nursing home in North Falmouth, MA, following a brief illness. He was two weeks short of his 97th birthday. David's wife, Doris, predeceased him in 2018. During nearly 70 years of marriage they lived in many places, including Wilmington, DE, Concord, NH, Topsfield, MA, Baton Rouge, LA, and Middleboro, MA. Born in Boston, MA, David attended the Brookline Public Schools, then earned a Bachelor's of Chemical Engineering at Rensselaer Polytechnic Institute in Troy, NY. During WWII, he studied meteorology at New York University and served as a Second Lieutenant in the U.S. Army Air Force. For more than four decades, David worked in the leather industry. In 1947, he joined the Allied Kid Company in Wilmington, DE. Many years later, he became an innovator in alligator tanning in Baton Rouge, LA. As a young volunteer, David was appointed to the Advisory Commission to the New Hampshire Department of Resources and Economic Development. After his retirement, he volunteered as a SCORE counselor, advising entrepreneurs on Boston's South Shore. David's diverse interests included skiing, golfing, photography, bird watching, gardening, weather forecasting, progressive politics, and music. An avid jazz fan, he was a guest host on Lew Carter's jazz radio show in Baton Rouge. He also played the violin in many local ensembles, including the Cape Ann Symphony in Gloucester and the Massasoit Senior Orchestra in Brockton. Three weeks before his death, he entertained family by playing part of the Mendelssohn Violin Concerto — twice — from memory. He was a gentle man, known for his kindness, friendliness and great love for his wife and family. David is survived by four children, Lyn Walfish and her husband, Joel, of Lynnfield, MA, Debbie Baylin and her husband, Steven, of Calgary, Alberta, Steven Small and his wife, Marianne, of Woods Hole, MA and Bradenton, FL, and Michael Small and his wife, Cindy Ruskin, of New York City. He had five grandchildren, Jonathan Small and his wife, Elizabeth, Melissa Baylin, Jessica Small and her husband, Pierre, Napert-Frenette, Jared Walfish and Ethan Walfish. He had two great-grandchildren, Anna Pax Small and Simone Cady Small. He will also be missed by numerous nieces and nephews. The family wants to acknowledge the thoughtful care from the Royal Megansett staff by asking that donations be sent in David's memory to Royal Megansett, c/o Sue Parkinson, Executive Director, 209 County Rd., North Falmouth, MA 02556. Services private.

In 1955 David joined the ALCA and became a life member in 1995. He served the Association as Chair of the Alsop Award Committee in 1970, council member from 1971 to 1972, member of the Editorial Board from 1971 to 1987, member of the subcommittee on Studying Journal Advertising in 1972, member of the Education Committee from 1982 to 1987, member of the 1978 Wilson Lecture Selection Committee, and member of the 1983 O'Flaherty Service Award Committee.

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