THE Journal OF THE AMERICAN LEATHER CHEMISTS ASSOCIATION

October 2020 Vol. CXV, No.10 *JALCA* 115(10), 353–396, 2020

Contents

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An imprint of the University of Cincinnati Press

ISSN: 0002-9726

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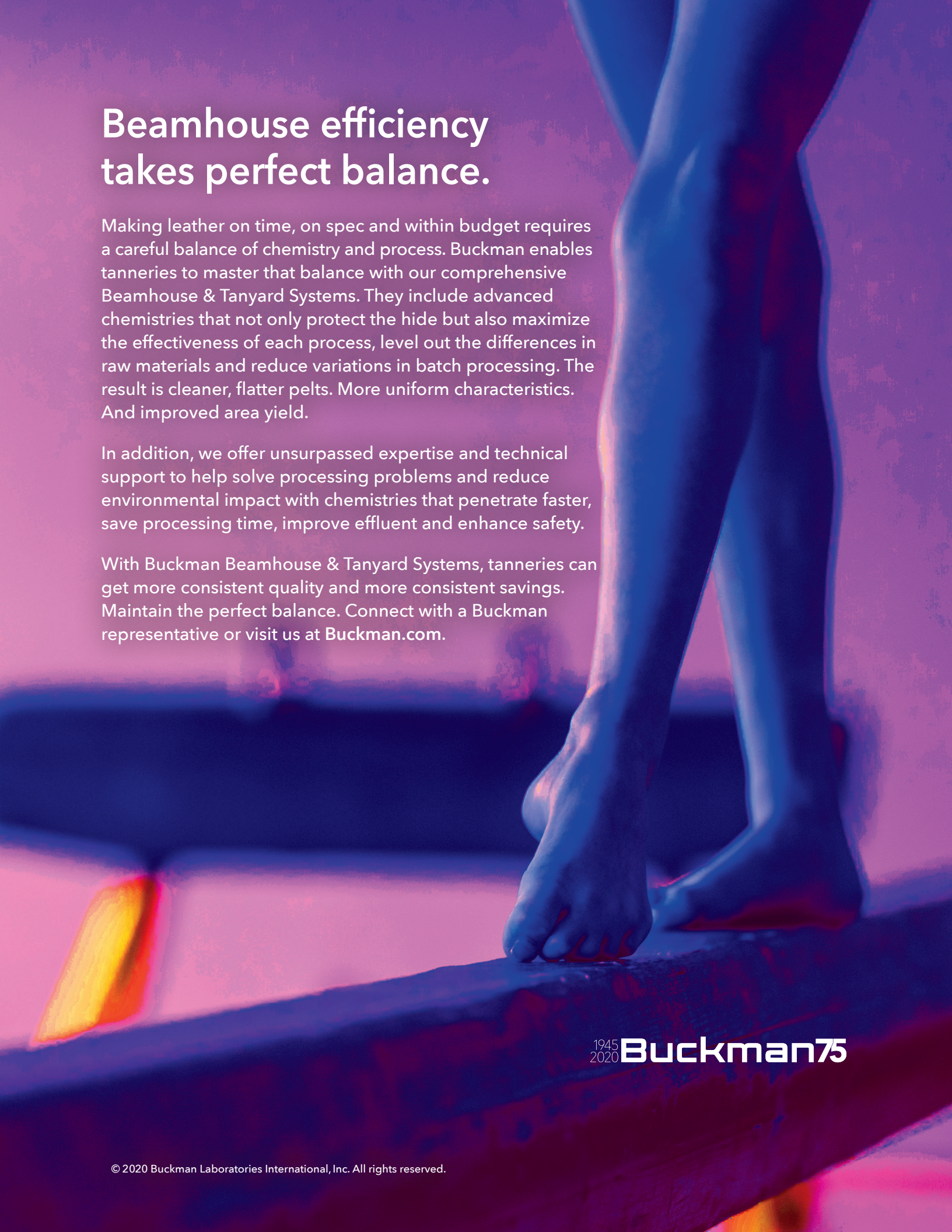

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JOURNAL OF THE
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THE JOURNAL OF THE AMERICAN LEATHER CHEMISTS ASSOCIATION (USPS #019-334) is published monthly by The American Leather Chemists Association, 1314 50th Street, Suite 103, Lubbock, Texas 79412. Telephone (806)744-1798 Fax (806)744-1785. Single copy price: $8.50 members, $17.00 non-member. Subscriptions: $185 for hard copy plus postage and handling of $60 for domestic subscribers and $70 for foreign subscribers; $185 for ezine only; and $205 for hard copy and ezine plus postage and handling of $60 for domestic subscribers and $70 for foreign subscribers.

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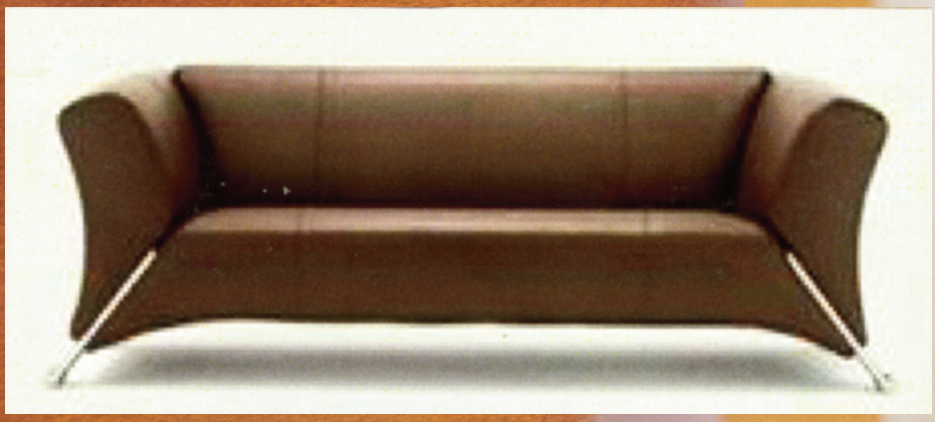

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Regeneration of Raw Hide Water Balance by Electrochemically Activated Water

by

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Abstract

Improving the collagen structure, which was impacted by the curing process, increases the efficiency of all subsequent structural transformations during leather production and results in the formation of a high-quality leather material. Herein, we studied the process of soaking green-salted horsehides in electrochemically activated aqueous solutions and the properties of resulting chrome-tanned leather. It was found that the process of soaking horsehides can be effectively carried out using an electrochemically activated solution (ECAS) – a mixture of catholyte and anolyte at a volume ratio of 5 : 1. Using this soaking solution, sodium sulfide (an environmentally harmful reagent), sodium carbonate, and detergent can be completely excluded from the technological solution. The use of ECAS at the soaking stage effectively regenerates the water balance of horsehides while maintaining a stable pH during the soaking process and increases the efficacy of liming and all subsequent processes. Considering the prominent structural differences (density and thickness) between the front and shell of horsehide, the developed method, which utilize ECAS for soaking, allows the entire process to be carried out on uncut horse hides instead of processing two parts separately, which is normally required. The chrome-tanned leather produced by the developed method in semi-industrial conditions has elastic-plastic properties which are superior compared to the leather produced by current technology. The developed method also results in increasing the area yield by 2.5%. The resulting elastic leather can be used for manufacturing a variety of articles, including clothing, accessories, and footwear.

Introduction

The development of innovative technologies to produce leather materials involves implementing effective ways to carry out a variety of chemocolloidal reactions during the processing of natural raw materials. Improving the structure of raw hides, particularly after the curing process during preparatory stages, increases the efficacy of all subsequent structural transformations and the formation of a high-quality final leather material. The use of aqueous solutions with novel chemical compositions can minimize the process length which is necessary to protect natural raw materials from biological damage by microorganisms, especially in the beginning of the hide processing.[1] These solutions should be also scalable and inexpensive considering the significant volumes which are used during multistage processing of raw hides.

Activated aqueous solutions can be used as new alternatives to technological solutions during the processing of raw hides. To produce the activated aqueous solutions, different methods, such as ultrasonic or electric fields, can be used.[2,3] Treatment of animal hides and skins with activated solutions has a number of advantages over other varieties of water treatments which utilize traditional technological solutions.[3–5] Particularly, activated solutions maintain their properties, nonequilibrium thermodynamic state and high oxidation-reduction potentials (ORP), for a prolong time. These properties contribute to the intensification of biotechnological processes.

Among activated solutions, electrochemically activated (ECA) solutions have a potential role as disinfectants in medicine and industry due to their biocidal properties, *in situ* generation from inexpensive materials (water and salts such as *NaCl*), and environmental compatibility (refer to the review [6]). ECA solutions, also denoted as electrolyzed oxidizing (EO) water, mixed oxidant (MIOX) solutions, and electrochemically activated water (ECAW), are produced by electrolysis of dilute salt solutions in an electrolytic cell where the cathode and anode are separated by a membrane. Electrochemical activation of water yields two fractions, catholyte and anolyte, with highly specific physicochemical properties regarding pH, ORP, electroconductivity, and the presence of chemically active radicals and ions. The main reactions that occur during electroactivation of aqueous sodium chloride solutions can be summarized as:

anode:	*cathode:*
$2H_2O - 4e^- \rightarrow O_2\uparrow + 4H^+$	$2H_2O + 2e^- \rightarrow H_2\uparrow + 2HO^-$
$2Cl^- - 2e^- \rightarrow Cl_2\uparrow$	

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Manuscript received April 10, 2020, accepted for publication May 12, 2020.

Depending on the operating parameters of electrolytic cell, the resulting anolyte solution will have a high ORP (oxidizing), a low pH (which can be neutralized by reconfiguring the electrolytic cell), and a variable concentration and type of chlorine-containing moieties. The catholyte will be characterized by low ORP (reducing) and a high pH. Catholyte and anolyte can be also combined in a specific proportion to achieve desirable properties of electroactivated solution. The relative volumes of catholyte and anolyte in a given solution will determine its final pH.

As an alternative to chlorine forms and other oxidants, ECA solutions also contain a mixture of reactive oxygen species and free radicals. Higher organisms possess antioxidant defense systems, whereas microorganisms generally do not. The presence of the free radicals, with their high oxidizing effects, in the solutions are considered of great importance to effectively destroy a wide variety of microorganisms. The active antimicrobial components of ECA solutions have been reported to include *HOCl*, hydroxyl radical, and other short-lived oxidative moieties like ClO_2.

Based on many reports, it has been shown that anolyte solutions have a broad spectrum of antimicrobial activity[6–17] while catholyte solutions are characterized by their cleaning properties, including biofilm removal.[10,12,16–19] In addition to antibacterial and sporicidal activity,[6,20,21] ECA solutions have antiviral and fungicidal activities[6,22] and inactivate toxins[6] while maintaining high biocompatibility[23] and antioxidative properties.[24,25]

ECA solutions have been used in many different areas,[6] specifically for (*i*) the treatment and prevention of wound infection and periodontal diseases; (*ii*) medical device disinfection, including dental unit water lines,[10] which if inadequately disinfected, may harbor polymicrobial biofilms containing potentially pathogenic organisms; and (*iii*) for disinfection of surfaces[12] and food products[26–28] which may help to control infection outbreaks.

Authors of the work[14] assessed the performance of sodium hypochlorite (*NaOCl*) and ECA water in terms of disinfection efficacy, disinfection by-products formation and the stability of the disinfectant with respect to storage.[14] In the absence of or at low concentrations of dissolved organic carbon, ECA solution showed better disinfection efficacy in terms of *Escherichia coli* inactivation at both pH 5 and 7 while forming fewer by-products. Also, ECA solution was more stable during storage compared with *NaOCl* solution. Acidic ECA solution retains useful bactericidal activity for more than 12 months, which can significantly expand its potential applications.[22] During the recovery of ECA solution, the chemical components of the solution will shift spontaneously from their thermodynamically unequilibrated conditions to a stable non-active form. Thus, ECA solution will slowly revert to a dilute salt solution without presenting any environmental hazards. Moreover, ECA solutions can be effectively inactivated by organic matter and are considered as "green biocides" which can help to reduce the consumption of free chlorine and replace other toxic chemicals.[22]

Many industrial processes can benefit from utilizing ECA solutions. For example, the high potential of catholyte as an environmentally friendly alternative to laundry detergents for sustainable consumption of cotton textiles have been described.[18] In addition to its cleaning properties, catholyte also exhibits a plasticization effect on the collagen structure of the dermis of leather materials which results in reduced stress at the final stage of product formation during footwear manufacturing.[29] As a result of the catholyte treatment of the leather part of fur and leather, the plasticity of the materials has been restored.[30, 31]

Experimentally, the use of both catholyte and anolyte in the processing of raw fur materials was reported previously.[32] The anolyte was effectively used in the process of restoring the collagen structure of the raw material and releasing inter-fiber proteins, polysaccharides and partially lipids.[32] Owing to anolyte bactericidal properties it was possible to carry out the process without adding antiseptic substances. At the second stage of soaking and degreasing the raw biomaterial, catholyte was used to remove the remaining lipids. The use of ECA solutions resulted in decreasing the time required to process fur materials without the application of toxic reagents.[33]

Thus, the results of the aforementioned studies specify the particular physicochemical, cleaning and antiseptic properties of the ECA solutions, especially, for processing plant and animal raw materials. This can indicate their promising application for the improvement and development of innovative methods for producing leather and fur. Utilizing solutions with a broad spectrum of antimicrobial activity at the preparatory stages becomes especially highly important after a new kind of bacterial defect on hides/skins and leather has been identified as a microbial biofilm which differs from the previously well-known bacteria-borne defects such as hair slip, red discoloration, and grain pilling.[1]

Thus, the goal of this work was to study the process of soaking green-salted horsehide using electrochemically activated solutions. To achieve this goal, the following tasks were defined:

– to determine the effect of various fractions of electrochemically activated water on the kinetics of water absorption by different areas (cuts) of horsehide;

– to establish the relationship between water sorption by horsehide and pH of the solutions used during the soaking;

– to determine the effect of the soaking process on the chemical composition and properties of the chrome-tanned leather which was produced by the developed method including soaking in electrochemically activated aqueous solutions in the semi-industrial conditions.

Materials and Methods

Leather, as a natural material, can differ significantly in properties not only between different types of hides and skins but also between its different regions.[34] The difference in properties between various regions is the most prominent for horsehides, yet the information on the tear strength variability and collagen fibril orientation together with other properties across horsehide are very scarce.

In this study, the green-salted horse raw hide with the thickness and initial moisture content in the areas of the horsefront and shell (Fig. 1) equal to 2.2–2.7 mm and 47.8% and 4.8–5.9 mm and 44.1%, respectively, were used.

Laboratory Testing

After raw hide fleshing and mechanical removal of its pelage and before soaking, three samples (approx. 100 g each) were cut out from the horsehides and washed with water (50% excess of the total sample mass) for an hour at 26–28°C. Washing and soaking were carried out at a rotational speed of 15–17 rpm (in a vertical plane) using custom-made laboratory equipment shown in Fig. 2.

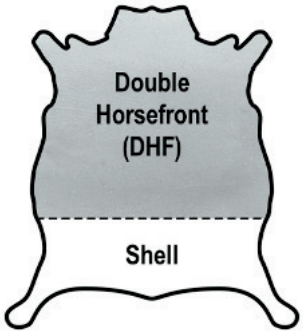

Figure 1. Standard horsehide leather cuts: front and shell.

Figure 2. Photo of custom-made laboratory equipment used for washing and soaking of horsehide samples.

Fractions of ECA water – anolyte and catholyte with a pH 3.3 and 10.3, respectively, were produced by electrolysis of 5 mM *NaCl* solution in an electrolytic cell (Ekovod, technical specifications: TU У29.1-1285006876.001-2000) with a silicon anode, a steel cathode, and an ion-exchange cotton membrane[35,36]. For the regeneration of water balance which was affected during the curing process and transportation, horsehide soaking in solutions based on ECA water was studied. Particularly, catholyte, catholyte supplemented with Na_2S (2 g/L) and 5:1 catholyte-anolyte mixture was used to study the kinetics of water absorption. For controls, distilled water and industrial soaking solution[37] containing non-ionic surface-active substance SPK-50 (0.25% of the sample mass, technical specifications: TU 2484-014-22284995-99), sodium carbonate (0.45% of the sample mass) and sodium sulfide (0.30% of the sample mass) were used.

The pH of the soaking solutions was measured with a pH meter (Gomel Plant of Measuring Devices, pH-340), while water content of the samples was determined gravimetrically.

For water absorption studies three samples per group were cut from the right and left half-skins of cured raw hides after their fleshing in the form of symmetrical strips close to the line separating front and shell. After removing the hair with a razor blade knife, the samples were weighed on scales (AXIS, AD200). After all the samples were washed simultaneously, they were carefully but thoroughly dried with filter paper to remove the excess surface water and weighed again. This procedure was repeated after 2, 4, 7, 17 h (Fig. 3) and 1, 3, 6, 16 h (Fig. 4). The water content in the samples was calculated according to the following formula:

$$WA = \frac{(m_1 - m_2)}{m_2} 100\%$$

where WA – water absorbed by the sample [%]; m_1 and m_2 – the sample weight before and after drying [g]. To determine the dry weight after measurements the samples (2–2.5 g) were dried at 102±2°C for 4 h, equilibrated for 30 min in a desiccator at room temperature and weighed.

Semi-Industrial Testing

After laboratory experiments, the horse hides were processed in semi-industrial conditions using the developed method and the current technology as a control. In the developed method a slightly alkaline ECA solution obtained by mixing catholyte and anolyte fractions at volume ratio 5 : 1 was used for soaking. In the developed method the soaking process was carried out using three halves (~1.2 m^2 each) of hides along the back bone line, while according to the current technology the horsefront and shell were processed separately yielding, respectively, elastic and boardy leather materials (Fig. 6).

For removal of the epidermis and hair from the horse pelts, the raw hide was further subjected to liming using a mixture of calcium hydroxide (4% of the sample mass) and sodium sulfide (3% of the

sample mass). The processing of horse raw hides was carried out in a revolving drum (Dose, 0.39 m^3) at a private joint-stock company "Chinbar" (Kyiv, Ukraine). Further processing of the limed hides, including deliming, pickling, tanning, shaving, neutralizing, tanning with chromium salts and plant extracts, fat-liquoring, samming, setting out and final drying were performed according to the previously described technology.[37] Briefly, the horsehide after tanning with chromium (III) compounds (technical specifications: TU 2141-033-54138686-2003) was skived to a uniform thickness of 1.4–1.5 mm; post-tanned with chromium (III) compounds in the amount of 2% of the hide mass; neutralized with a mixture of sodium formate and sodium bicarbonate with a weight ratio of 4 : 1; filled with organic compounds: acrylic polymer Retanal RCN-40 (Cromogenia Units SA, 4% of the hide mass) and quebracho (5% of the hide mass); and fat-liquored with Trupol RA (Trumpler, 8% of the hide weight mass). The filling-fat-liquoring process was completed after fixation of reagents in the structure of re-tanned hides with 10% formic acid at pH 4.2. After the drying-moisturizing processes,[38] the samples of the resulting semi-finished leather were set out.

Physicochemical Characterization

The chemical composition and physicomechanical properties of the leather samples were determined by the methods described in our previous work[39] after conditioning at 20±2°C and 65±5% relative humidity of the air (ASTM D1610–18). Briefly, the total ash was determined thermogravimetrically after oxidizing the sample (7.5 g) in air at 600±25°C until constant mass was reached (ASTM D2617–17a and GOST 938.2-67). The weighed residual matter, termed "ash", was calculated as a percentage from the original sample. Liquid pycnometry was utilized to measure the apparent density of the samples (10 g) at 22°C using kerosene as a solvent. Considering that collagen is the primary structural element in the extracellular matrix in animal hides/skins, the heat resistance of leather against shrinkage was characterized by the hydrothermal denaturation or shrinkage temperature (T_S) of collagen molecules[40] by gradually heating the sample in a mixture of glycerol and water with a 4 : 1 weight ratio at the heating rate of 2–3°C/min (ASTM D6076–18 and DSTU 2726-94). Before the measurements, the samples were thoroughly soaked in water for 4 h at 20±1°C. Chromium content was determined by iodometric titration and expressed as the mass fraction of chromium (III) oxide. Mechanical properties (tensile stress and elongation) were measured using a tensile testing machine (PM-250M) at extension rate 90 mm/min. Initial length of 10 mm wide leather samples between clamps was 50 mm. Content of the bound organic tannins (BOT) [%] in the leather samples was calculated using the following formula: $\omega_{BOT} = \omega_L - \omega_N$, where $\omega_L = 100 - (\omega_{TA} + \omega_{OS} + \omega_{OWS})$ – the mass fraction of leather substances calculated on the dry basis [%]; ω_{TA} – the mass fraction of total ash [%]; ω_{OS} – the mass fraction of organic substances (OS), unbound fatty acids, extracted with organic solvents (GOST 938.5-68) [%]; ω_{OWS} – the mass fraction of organic water-soluble substances (OWS) (GOST 938.6-68) [%]; ω_N – the mass fraction of nitrogen-containing water extractable materials (determined according to the previously described methodology,[38] ASTM D6016 – 17) [%]. The content of substances extracted with organic solvents was determined using a Zaivhenko extraction apparatus. A crushed sample (5 g) within a paper shell was put into the extractor and solvent extraction with tetrachloromethane was carried out for 1.5 h. After solvent evaporation under vacuum, the fatty substances were dried in an oven at 128–130°C for 1 h. After cooling the flask in the desiccator, the flask with unbound fatty acids was weighed and the mass fraction of OS was calculated. To calculate the yield, the surface area of leather was determined using specialized electromechanical machinery (Svit, model 07484/P1).

Result and Discussion

Structure and Composition of Raw Hides

Collagen is the main and the most abundant structural protein in the extracellular matrix of various animal tissues, including skin, bone and tendon. The fibrous collagen provides tensile strength, while glycosaminoglycans (GAGs), covalently linked to proteins to form proteoglycans, act as space-fillers and provide resistance to compression.[41] Skin components such as keratin, albumins, and globulins together with GAGs (e.g., hyaluronic acid), proteoglycans (e.g., dermatan sulfate), triglycerides and lipids are usually removed during the earlier stages of hide and skin processing.[42] Removal of these non-collagenous proteins is a necessary step to produce soft leather material as it allows the collagen fiber structure to split apart. This "opening up" effect results in a successful implementation of all subsequent structural transformations.

Many varieties of collagen have been identified; for example, mammals have about 20 different genes which code various forms of collagen.[41] As a function of structure and supramolecular organization, collagen can be classified as fibril-forming (types I, II, III, V, and XI), fibril-associated (types IX, XII, and XIV), or network-forming (types IV, VII, VIII, and X).[43] Tropocollagen, a collagen molecule, being a right-handed triple helix stabilized mostly by hydrogen bonding is the major structural element of collagen. Heterotrimers of two α1(I) and one α2(I) protein chains which only slightly differ in amino acid sequence are the dominant isoform of type I collagen. The polypeptide chains contain glycine at every third position and have a high proline and hydroxyproline content.[41,42] All collagen molecules in a precursor form, called procollagen, are synthesized intracellularly by connective-tissue cells and then secreted by exocytosis. Additional peptide extensions at each end of procollagen hinder premature assembly of collagen molecules. Extracellular enzymes, procollagen proteinases, cut off these terminal extensions to allow collagen assembly only after the molecules have been extracted. At each end of the helical region of the procollagen molecule, there is a non-helical region known as telopeptide. Collagen, being a multi-hierarchical structure, is organized in four levels of macromolecular structure: first the collagen molecules pack together into *collagen fibrils*, then the fibrils are further organized into larger *fibril bundles*, then they are arranged into *fascicles* and finally – into *fiber bundles*.

Water Absorption by Horsehide

The chemical treatments and mechanical processes used to produce leather from hides and skins result in structural changes in collagen fibrils and their supramolecular organization.[44] Even though the general chemistry used in hide and skin processing is well-known, the structural changes at the level of the collagen fibrils is just starting to be revealed utilizing x-ray scattering techniques.[45–47] Herein we focused on the macroscopic effect of the composition of the soaking solutions on water sorption by horsehide and the correlations with the properties of corresponding final leather material.

Generally the water sorption of collagen and other fibrous proteins in acidic and alkaline solutions is predominantly governed by the osmotic pressure difference arising between the protein phase and the external solution and by protein cohesion.[48] According to the literature data, the isoelectric point of collagen, which can be affected by the nature and ionic strength of the salts presented in the solution, is 4.7, although a second critical point in the collagen behavior has frequently been noted at pH 7.7.[49] The maximum equilibrium swelling is usually observed at an external pH between 2–3 and 11–12, while the minimum – at the isoelectric point.[50]

The kinetics of water absorption by green-salted horsehide was determined for technological solutions with different compositions (Fig. 3 and Fig. 4). It should be noted that the soaking process of the raw hide was carried out after washing the salt off the samples. To estimate the sorption of ECA solution by the dermis, the soaking process was also carried out using distilled water as an additional control (Fig. 4).

As it can be seen from the data, after rapid solution uptake within the first 3 h, a further increase in water content, which was not complete after 16–17 h, was observed. The results also indicate that solution

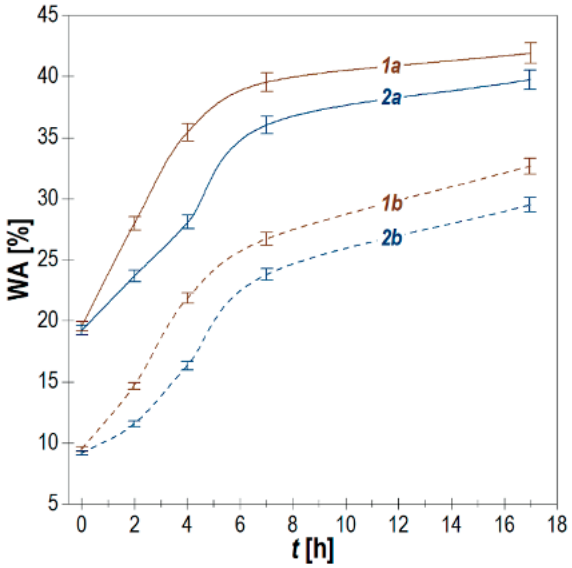

Figure 3. Kinetics of water absorption (WA) by the dermis of horsehide in a 5 : 1 mixture of catholyte and anolyte (1) and in a control soaking solution according to the existing technology (2) for horsefront (a, solid lines) and shell (b, dash lines).

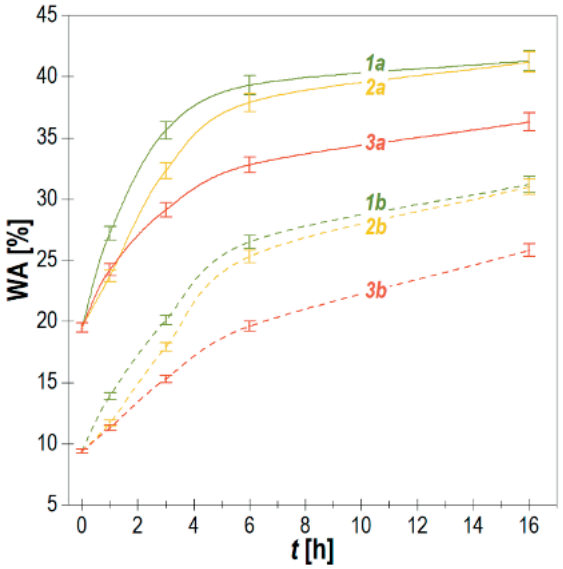

Figure 4. Kinetics of water absorption (WA) by the dermis of horsehide in catholyte (1), catholyte supplemented with Na_2S (2), and distilled water (3) for horsefront (a, solid lines) and shell (b, dash lines).

composition significantly affects the kinetics of water absorption and the final water content in the samples. The most prominent effect was observed for the sample soaked in the mixture of catholyte and anolyte with a 5 : 1 volume ratio (Fig. 3) and catholyte (Fig. 4). This is manifested by faster water absorption, especially in the beginning of the soaking, and a higher water content after 17 h of soaking compared to the control group (existing technology). After the first hour of soaking, the horsefront absorbs twice as much water as the shell. Additionally, the pH of the solution is reduced to a greater extent for the control group (ΔpH = 3.4) and for catholyte group (ΔpH = 3.1) compared with the mixture of catholyte and anolyte for which the pH difference was 1.1 (Fig. 5).

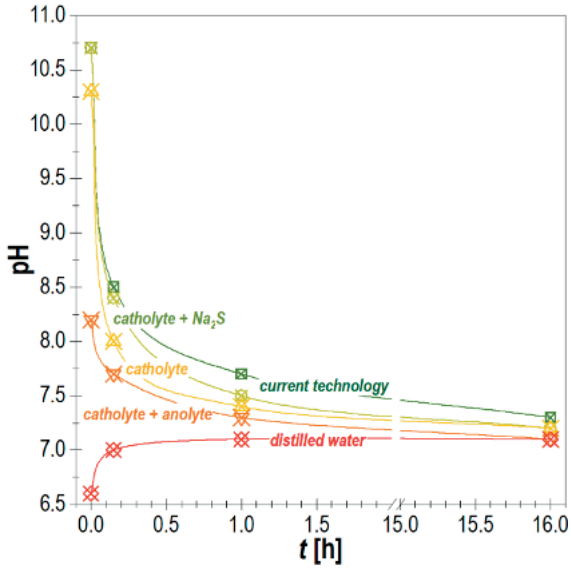

Figure 5. Change in the pH of technological solutions during soaking of horse raw hide.

The water sorption by horse raw hides is sensitive to the solution pH and its composition and increases with increasing pH until it reaches a maximum at 8.2 (Fig. 4 and Fig. 5). At pH lower than 8.2 the water content is decreased, while at higher pH it remains almost the same for horsefront or decreases for shell. The effect of solution composition can be seen comparing the kinetics of swelling horsehide in catholyte supplemented with Na_2S and in control soaking solution. Even though the initial pH of the solution is the same, the water sorption rate and the water content is lower for the samples swollen in the latter solution.

The water sorption by horsehides, in addition to hydration of hydrophilic groups of macromolecules, can be explained by the local change in the protonation degree of carboxyl groups of collagen, GAGs/proteoglycans, and other macromolecules in the beginning of the soaking.[51] The observed decrease in pH within the first few hours (Fig. 5) can be explained by the neutralization of hydroxyl anions presented in the solutions by protons which were released during dissociation of carboxyl groups. The electrostatic repulsion between negatively charged carboxylate groups and an increased ion concentration inside the dermis due to ionization will result in an increase in osmotic pressure which can cause the observed increase in the water content. In addition to the increased osmotic pressure, the increased mobility of the collagen macromolecules due to the disruption of some of its physical and chemical crosslinks and removing (washing out) of non-collagenous components of the dermis can also contribute to the increased water content. Furthermore, negatively charged polysaccharide chains of GAGs even at low concentrations can form hydrophilic gels: their multiple negative charges at carboxyl groups attract cations, such as Na^+, that are osmotically active, causing large amounts of water to be absorbed by the sample. This gives rise to a swelling pressure, which is balanced by tension in the collagen fibers interwoven with the proteoglycans.[41]

During the soaking process using an industrial soaking solution, uneven hydration (blistering), caused by the lower density of the outer layers and faster water absorption of alkaline solution (pH 10.7) which results almost in complete ionization of carboxyl groups, was observed. A similar effect of water absorption by the skin dermis was also found for the samples soaked in alkaline solutions of catholyte with and without sodium sulfide with pH 10.3 and 10.7, respectively. A uniform hydration, probably due to less rapid pH change and more effective and homogeneous water absorption by the whole dermis using catholyte and anolyte mixture can indicate deeper changes in the structure of the dermis on the microfibrillar level.

Generally, faster kinetics of water absorption was observed for the horsehides soaked in electroactivated solutions (Fig. 3 and Fig. 4). This effect can be explained by the declasterization of the water structure after its activation and additional changes in the water structure and water/macromolecule interactions in the presence of ions and free radicals.[32]

Thus, among tested soaking solutions, the catholyte and anolyte mixture provides uniform swelling without blistering and more effective regeneration of water balance of raw leather material while maintaining a stable pH during the soaking process. Higher water content in the horsefront and shell samples soaked in a mixture of catholyte and anolyte and a smaller difference between them compared with the control group (Fig. 3) indicates the possibility of simultaneous processing of the whole hide rather than processing the two parts separately (i.e., horsefront and shell).

Thus, in slightly alkaline solutions with moderate salt concentration (to avoid electrostatic screening and corresponding decrease in hydration) the water sorption will depend on solution solubilizing efficacy and its ability to decrease the collagen crosslinking degree.

The higher content of organic substances extracted from the raw material after soaking in ECA solution (Table I) compared with the control group (industrial soaking solution) also indicates more effective interaction of the activated water with the components of the dermis. This effect is more pronounced for the samples from shell which contains more soluble proteins and GAGs than the horsefront. Despite increased density of shell samples compared to the horsefront samples, 5.6% more organic substances were extracted from the former samples. It should be noted that the process of

Table I

Content of inorganic and organic substances in solutions after soaking of horsehide, apparent density, and hydrothermal stability.

Horsehide leather cut	Solution	Mass fraction of substances [%]		Density [g/cm³]	T_S [°C]
		Inorganic	Organic		
Horsefront	catholyte + anolyte	14.4 ± 0.3	85.6 ± 1.7	1.18 ± 0.04	64.0 ± 0.6
	control	15.4 ± 0.3	84.6 ± 1.7	1.18 ± 0.04	63.0 ± 0.6
Shell	catholyte + anolyte	13.4 ± 0.3	86.6 ± 1.7	1.24 ± 0.04	62.0 ± 0.6
	control	18.2 ± 0.4	81.1 ± 1.6	1.21 ± 0.04	63.0 ± 0.6

soaking the shell samples in activated solutions is accompanied by significant foam formation indirectly indicating the extraction of GAGs along with other water-soluble proteins.

Based on the results, soaking the horsehide in solutions of ECA water results in a higher efficacy of carrying out this process using a mixture of catholyte and anolyte at a volume ratio of 5 : 1 with pH 8.2. This eliminates the need to use the environmentally harmful sodium sulfide for soaking and facilitates the process with a smaller change in pH at the initial soaking stage. Higher water content and less variation in water content between the horsefront and the shell after soaking using a mixture of catholyte and anolyte compared with the control indicate the possibility of carrying out the entire technological cycle of manufacturing leather from green-salted raw hide using the whole horsehide.

Properties of the Horse Leather Produced in Semi-Industrial Conditions

To test the developed method in the semi-industrial conditions, the three half-skins from green-salted horse hides were used. The differences between developed method and current technology are summarized in Fig. 6.

The results of the liming process are shown in Table II and Table III. As it can be seen from the data, the pickled horsehide produced by the developed method contains 8.2% and 12.4% more inorganic substances for horsefront and shell areas compared with the existing technology. This indicates the profound structural changes in the soaked hides using ECA water, especially within the denser parts of raw hides – shell.

The properties of leather material are closely related to organization and supramolecular structure of collagen and results from the interplay of electrostatic, hydrophobic and van der Waals interactions in addition to hydrogen and covalent bonds.[43] During liming in a strongly alkaline environment further disruption of intra- and intermolecular bonds within fibrous collagen structure and the formation of weakly dissociated salts of calcium hydroxide with carboxyl groups were observed.[52] The hydrothermal stability of a leather material will reflect the stability of collagen structures at the molecular level and at several levels of supramolecular structure.[43] The shrinkage temperature of leather is affected by many different factors, most of which appear to alter the number and nature of crosslinks between adjacent polypeptide chains of the collagen protein macromolecules[53–56]. The value of the shrinkage temperature of leather is commonly used as an indicator of the type and degree of tannage or both. For both the developed and current method, this is testified by a decrease in the density after liming with a corresponding decrease in hydrothermal stability (Table III and Table IV). Based on shrinkage temperature, the pickled horse half-skins processed according to the developed method are structurally more "opened up" and prepared for the effective tanning-fat-liquoring.

Table II

Content of inorganic substances in the pickled horsehides processed in semi-industrial conditions.

Technology /Method	Horsehide leather cut	Total ash [%]
Developed	horsefront	0.92 ± 0.02
	shell	1.18 ± 0.02
Existing	horsefront	0.85 ± 0.02
	shell	1.05 ± 0.02

Table III

Properties of the pickled horsehides limed in semi-industrial conditions according to the existing technology.

Technology / Method	Density [g/cm³]		T_S [°C]	
	horsefront	shell	horsefront	shell
Developed	1.03 ± 0.03	1.12 ± 0.03	51.0 ± 0.5	57.5 ± 0.6
Existing	1.03 ± 0.03	1.12 ± 0.03	52.0 ± 0.5	59.0 ± 0.6

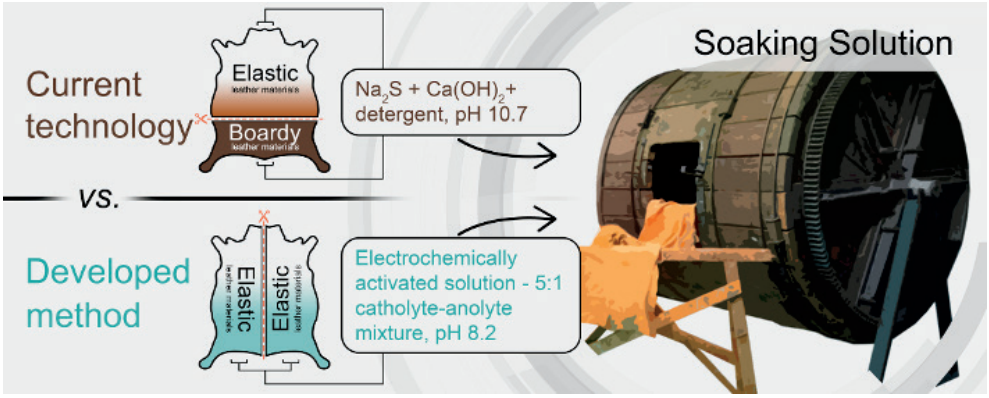

Figure 6. The differences in soaking stage between developed method and current technology during semi-industrial processing of horse raw hides.

The half-skins processed by the developed method and current technology in semi-industrial conditions after drying-moisturizing treatments were analyzed based on their chemical composition and physicomechanical properties (Table IV). Comparative analysis of leather samples obtained according to the developed method indicates a higher content of tanning compounds of chromium and organic matter than those obtained by existing technology. At the same time, the amount of unbound fatty substances is 6% and 12% higher for areas of horsefront and shell processed using the developed method. These data correlate with the heat resistance of leather samples. The filled leather obtained according to the developed method is 2–3 °C more hydrothermally stable.

The increased content of fatty substances and uniform distribution within the structure of the leather obtained by the developed method will promote the mobility of the collagen fibrils under deformation. This statement is confirmed by the observed decrease in the density and increase in elastic-plastic properties of the leather material. This is especially true for the tensile strength and deformation of leather from the horsefront and shell areas. Both characteristics increase by 9–11% (tensile strength) and 8–9% (elongation before fracture).

Overall, the developed method utilizing ECA solution for soaking provides an efficient regeneration of water balance of horse raw hide and its further processing. This is also evidenced by an increase in the area to 0.4 m^2 per 100 kg of raw hides. Based on the studied properties, the resulting leather can be used for manufacturing of clothing, accessories, and footwear.

Conclusions

It was established that the process of soaking of horse raw hides can be effectively carried out using the ECA solution – a mixture of catholyte and anolyte at a volume ratio of 5 : 1. The environmentally harmful reagent which is commonly used for leather soaking, such as sodium sulfide, together with sodium carbonate and surface-active substance(s) have been completely excluded from the composition of the soaking solution. Taking into account the peculiarities of the structure of horsehide, particularly, drastic difference in the thickness and the density of the horsefront and shell, an effective regeneration of water balance of the dermis takes place when a mixture of catholyte and anolyte is used to provide a stable pH environment during entire soaking process. The higher water content for the horsefront and shell samples during soaking and smaller difference between them compared to the samples processed using existing technology provide (1) the effective process of liming and (2) the basis for the processing of the whole horse raw hide at once into elastic leather. Soaking of green-salted horse raw hides in a mixture of catholyte and anolyte results in the formation of leather with the increased elastic-plastic properties and increase area yield by 2.5% compared with the existing technology which involves separate processing of the horsefront and shell. The leather made from the horse raw hides processed using the developed method meets the requirements of DSTU 3115-95 "Leather for garments - General specifications" and the international standard ISO 9001:2008 for "Quality management systems - requirements" and can be used for manufacturing of clothing, accessories and footwear.

Table IV

Characteristics of the filled leather.

Characteristic	Developed method	Current technology
– Mass fraction* [%]		
• chromium (III) oxide	4.1 ± 0.08 / 3.6 ± 0.08	3.8 ± 0.08 / 3.3 ± 0.08
• substances extracted with organic solvents	8.7 ± 0.2 / 6.6 ± 0.1	8.2 ± 0.2 / 5.9 ± 0.1
• bound organic tannings	12.9 ± 0.3 / 8.7 ± 0.2	12.2 ± 0.2 / 7.9 ± 0.2
– Shrinkage temperature, T_s [°C]	115 ± 1 / 111 ± 1	113 ± 1 / 108 ± 1
– Density [g/cm^3]	0.63 ± 0.02 / 0.66 ± 0.02	0.65 ± 0.02 / 0.70 ± 0.02
– Tensile strength [MPa]	19.7 ± 1.2 / 22.6 ± 1.4	17.7 ± 1.1 / 20.8 ± 1.2
– Relative elongation at 10 MPa load [%]	36 ± 2 / 25 ± 3	34 ± 2 / 24 ± 1
– Relative elongation before fracture [%]	67 ± 4 / 49 ± 3	62 ± 4 / 45 ± 3
– Yield [m^2/(100 kg of raw hides)]	15.1 ± 0.3	14.7 ± 0.3

Note: The numerator and denominator correspond to the values for horsefront and shell, respectively, while * denotes that the mass fraction was recalculated on the dry basis.

Acknowledgments

Authors thank Victor Marik and Olivia Lanier for participating in discussions and providing language help, writing assistance, and proofreading the paper and Creative Vector Studio (https://www.etsy.com/shop/CreativeVectorStudio), particularly Tatiana Chernenko, for the help with graphical illustrations (illustration to Figure 1 and Figure 6).

Funding

This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Cadirci, B. H.; Ozgunay, H.; Vural, C.; Yilmaz, O. A New Defect on Leather: Microbial Bio-Film. *JALCA* 105, 129–134, 2010.
2. Plutakhin, G. A.; Aider, M.; Koshchaev, A. G.; Gnatko, E. N. Theoretical Bases of Electrochemical Treatment of Aqueous Solutions (in Russian). *Sci. J. Kuban State Agrar. Univ.*, 92 (08), 1–25, 2013.
3. Fidaleo, M.; Moresi, M. Electrodialysis Applications in the Food Industry. *Adv. Food Nutr. Res.* 51, 265–360, 2006. https://doi.org/10.1016/S1043-4526(06)51005-8.
4. Hsu, S.-Y.; Kao, H.-Y. Effects of Storage Conditions on Chemical and Physical Properties of Electrolyzed Oxidizing Water. *J. Food Eng.* 65 (3), 465–471, 2004. https://doi.org/10.1016/j.jfoodeng.2004.02.009.
5. Shirahata, S.; Hamasaki, T.; Teruya, K. Advanced Research on the Health Benefit of Reduced Water. *Trends Food Sci. Technol.* 23 (2), 124–131, 2012. https://doi.org/10.1016/j.tifs.2011.10.009.
6. Thorn, R. M. S.; Lee, S. W. H.; Robinson, G. M.; Greenman, J.; Reynolds, D. M. Electrochemically Activated Solutions: Evidence for Antimicrobial Efficacy and Applications in Healthcare Environments. *European Journal of Clinical Microbiology and Infectious Diseases*. Springer-Verlag, 641–653, May 2012. https://doi.org/10.1007/s10096-011-1369-9.
7. Cloete, T. E.; Thantsha, M. S.; Maluleke, M. R.; Kirkpatrick, R. The Antimicrobial Mechanism of Electrochemically Activated Water against *Pseudomonas Aeruginosa* and *Escherichia Coli* as Determined by SDS-PAGE Analysis. *J. Appl. Microbiol.* 107(2), 379–384, 2009. https://doi.org/10.1111/j.1365-2672.2009.04233.x.
8. Wilsmann, D. E.; Carvalho, D.; Chitolina, G. Z.; Borges, K. A.; Furian, T. Q.; Martins, A. C.; Webber, B.; do Nascimento, V. P. Electrochemically-Activated Water Presents Bactericidal Effect against *Salmonella* Heidelberg Isolated from Poultry Origin. *Foodborne Pathog. Dis.*, 1–6, 2019. https://doi.org/10.1089/fpd.2019.2682.
9. Goncharuk, V. V.; Bahryi, V. A.; Arkhypchuk, V. V.; Chebotarov, R. D. Biotesting of Electrochemically Activated Water (in Russian). *J. Water Chem. Technol.*, 4, 399–411, 2005.
10. Marais, J. T.; Brözel, V. S. Electro-Chemically Activated Water in Dental Unit Water Line. *Br. Dent. J.,* 187(3), 154–158, 1999. https://doi.org/10.1038/sj.bdj.4800228.
11. Thorn, R. M. S.; Robinson, G. M.; Reynolds, D. M. Comparative Antimicrobial Activities of Aerosolized Sodium Hypochlorite, Chlorine Dioxide, and Electrochemically Activated Solutions Evaluated Using a Novel Standardized Assay. *Antimicrob. Agents Chemother.*, 57(5), 2216–2225, 2013. https://doi.org/10.1128/AAC.02589-12.
12. Swan, J. S.; Deasy, E. C.; Boyle, M. A.; Russell, R. J.; O'Donnell, M. J.; Coleman, D. C. Elimination of Biofilm and Microbial Contamination Reservoirs in Hospital Washbasin U-Bends by Automated Cleaning and Disinfection with Electrochemically Activated Solutions. *J. Hosp. Infect.*, 94(2), 169–174, 2016. https://doi.org/10.1016/j.jhin.2016.07.007.
13. Akbulut, M. B.; Ünverdi Eldeniz, A. *In Vitro* Antimicrobial Activity of Different Electrochemicallyactivated Solutions on *Enterococcus Faecalis*. *Eur. Oral Res.* 53(1), 44–50, 2019. https://doi.org/10.26650/eor.20194564125648.
14. Ghebremichael, K.; Muchelemba, E.; Petrusevski, B.; Amy, G. Electrochemically Activated Water as an Alternative to Chlorine for Decentralized Disinfection. *J. Water Supply Res. Technol., AQUA*, 60(4), 210–218, 2011. https://doi.org/10.2166/aqua.2011.034.
15. Gulabivala, K.; Stock, C. J. R.; Lewsey, J. D.; Ghori, S.; Ng, Y.-L.; Spratt, D. A. Effectiveness of Electrochemically Activated Water as an Irrigant in an Infected Tooth Model. *Int. Endod. J.*, 37(9), 624–631, 2004. https://doi.org/10.1111/j.1365-2591.2004.00867.x.
16. Pogorelov, A. G.; Suvorov, O. A.; Kuznetsov, A. L.; Panait, A. I.; Pogorelova, M. A.; Ipatova, L. G. Disintegration of Bacterial Film by Electrochemically Activated Water Solution. *Bull. Exp. Biol. Med.*, 165(4), 493–496, 2018. https://doi.org/10.1007/s10517-018-4202-y.
17. Solovyeva, A. M.; Dummer, P. M. H. Cleaning Effectiveness of Root Canal Irrigation with Electrochemically Activated Anolyte and Catholyte Solutions: A Pilot Study. *Int. Endod. J.*, 33(6), 494–504, 2000. https://doi.org/10.1046/j.1365-2591.2000.00342.x.
18. Cronjé, N.; Steyn, H. J. H.; Schall, R. Catholyte as an Environmentally Friendly Detergent: Effect on the Colourfastness of Black Dyed Textiles. *J. Text. Inst.* 2019. https://doi.org/10.1080/00405000.2019.1703489.
19. Pogorelova, M. A.; Kuznetsov, A. L.; Suvorov, O. A. Does Electrochemically Reduced Water Remove Bacterial Film? *Int. J. Pharm. Res. Allied Sci.*, 7(2), 139–142, 2018.
20. Rogers, J. V.; Ducatte, G. R.; Choi, Y. W.; Early, P. C. A Preliminary Assessment of *Bacillus Anthracis* Spore Inactivation Using an Electrochemically Activated Solution (ECASOL™). *Lett. Appl. Microbiol.*, 43(5), 482–488, 2006. https://doi.org/10.1111/j.1472-765X.2006.02002.x.
21. Robinson, G. M.; Lee, S. W.-H.; Greenman, J.; Salisbury, V. C.; Reynolds, D. M. Evaluation of the Efficacy of Electrochemically Activated Solutions against Nosocomial Pathogens and Bacterial Endospores. *Lett. Appl. Microbiol.*, 50(3), 289–294, 2010. https://doi.org/10.1111/j.1472-765X.2009.02790.x.
22. Robinson, G.; Thorn, R.; Reynolds, D. The Effect of Long-Term Storage on the Physiochemical and Bactericidal Properties of Electrochemically Activated Solutions. *Int. J. Mol. Sci.*, 14(1), 457–469, 2013. https://doi.org/10.3390/ijms14010457.
23. Michel, N. S. D.; Paletta, J. R. J.; Kerwart, M.; Skwara, A. Role of Electrochemically Activated Solution in Asepsis in Osteoblasts and Chondrocytes *in Vitro*. *J. Investig. Surg.*, 29(3), 157–166, 2016. https://doi.org/10.3109/08941939.2015.1098757.

24. Lee, M. Y.; Kim, Y. K.; Ryoo, K. K.; Lee, Y. B.; Park, E. J. Electrolyzed-Reduced Water Protects against Oxidative Damage to DNA, RNA, and Protein. *Appl. Biochem. Biotechnol.*, 135(2), 133–144, 2006. https://doi.org/10.1385/ABAB:135:2:133.
25. Kinjo, T.; Ye, J.; Yan, H.; Hamasaki, T.; Nakanishi, H.; Toh, K.; Nakamichi, N.; Kabayama, S.; Teruya, K.; Shirahata, S. Suppressive Effects of Electrochemically Reduced Water on Matrix Metalloproteinase-2 Activities and in Vitro Invasion of Human Fibrosarcoma HT1080 Cells. In *Cytotechnology*; Springer Netherlands, 64, 357–371, 2012. https://doi.org/10.1007/s10616-012-9469-7.
26. Yang, Z.; Li, Y.; Slavik, M. F. Antibacterial Efficacy of Electrochemically Activated Solution for Poultry Spraying and Chilling. *J. Food Sci.*, 64(3), 469–472, 1999. https://doi.org/10.1111/j.1365-2621.1999.tb15065.x.
27. Thorn, R. M. S.; Pendred, J.; Reynolds, D. M. Assessing the Antimicrobial Potential of Aerosolised Electrochemically Activated Solutions (ECAS) for Reducing the Microbial Bio-Burden on Fresh Food Produce Held under Cooled or Cold Storage Conditions. *Food Microbiol.*, 68, 41–50, 2017. https://doi.org/10.1016/j.fm.2017.06.018.
28. Surdu, I.; Vătuiu, D.; Jurcoane, Ş.; Olteanu, M.; Vătuiu, I. The Antimicrobial Activity of Neutral Electrolyzed Water against Germs and Fungi from Feedstuffs, Eggshells and Laying Hen House. *Rom. Biotechnol. Lett.*, 23(3), 2018.
29. Lutsyk, R. V.; Matvienko, O. A.; Bovsunovskyi, O. V. Possibilities of Using Electroactivated Water in Technological Processes for Shoe Production (in Ukrainian). *Bull. Kyiv Natl. Univ. Technol. Des.*, 2, 53–58, 2005.
30. Zorina, E. F.; Zeleva, G. M.; Nagornaia, Z. E. The Effect of the Nature of Tanning Agents and Water on the Plastic Properties of the Skin Tissues of Fur and Leather (in Russian). *J. Omsk Sci. Bull.*, 19, 140–141, 2002.
31. Zorina, E. F.; Zeleva, G. M. Dyeing of Fur with Various Dyes (in Russian). *J. Omsk Sci. Bull.*, 19, 138–139, 2002.
32. Danylkovych, A. G.; Lishchuk, V. I.; Romaniuk, O. O. Use of Electrochemically Activated Aqueous Solutions in the Manufacture of Fur Materials. *Springerplus*, 5(1), 1–11, 2016. https://doi.org/10.1186/s40064-016-1784-6.
33. Danylkovych, A. H.; Mokrynska, O. R.; Okhmat, O. A. *Technology and Materials for Leather Production (in Ukrainian)*; Danylkovych, A. H., Ed.; Phoenix: Kyiv, 2009.
34. Basil-Jones, M. M.; Edmonds, R. L.; Cooper, S. M.; Kirby, N.; Hawley, A.; Haverkamp, R. G. Collagen Fibril Orientation and Tear Strength across Ovine Skins. *J. Agric. Food Chem.*, 61(50), 12327–12332, 2013. https://doi.org/10.1021/jf4038375.
35. Apukhovskyi, A. Y.; Krutov, V. D.; Kosinov, B. V. Process for Producing Drinking and Medicinal Water. 49551, 2002.
36. Potapenko, S. I.; Kostiuk, O. M.; Kostiuk, I. O.; Marinchenko, V. O. Mechanism for Preparing Silicon Potable Water. 19633, 2006.
37. Danylkovych, A. H.; Chursyn, B. I. *Technological Methodology for Production of Variety of Leather Materials for Upper Shoe Parts and Shoe Lining, Leather Haberdashery from Cattle and Horse Hides (in Ukrainian)*; JSC Chinbar: Kyiv, 2003.
38. Danylkovych, A. H.; Korotych, O. I. Optimization of Leather Filling Composition Containing SiO_2 Nanoparticles. *JALCA* 114, 333–343, 2019.
39. Danylkovych, A. H.; Chursyn, V. I. *Analytical Control in Leather and Fur Production: A Laboratory Practicum (in Russian)*; Infra-M: Moscow, 2016.
40. Zhang, Y.; Mansel, B. W.; Naffa, R.; Cheong, S.; Yao, Y.; Holmes, G.; Chen, H.-L.; Prabakar, S. Revealing Molecular Level Indicators of Collagen Stability: Minimizing Chrome Usage in Leather Processing. *ACS Sustain. Chem. Eng.*, 6(5), 7096–7104, 2018. https://doi.org/10.1021/acssuschemeng.8b00954.
41. Alberts, B.; Bray, D.; Hopkin, K.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Essential Cell Biology*, Fourth.; Garland Science (Taylor & Francis Group): New York, 2013.
42. Covington, A. D. Modern Tanning Chemistry. *Chem. Soc. Rev.*, 26(2), 111–126, 1997. https://doi.org/10.1039/cs9972600111.
43. Brown, E. M. Collagen - a Natural Scaffold for Biology and Engineering. *JALCA* 104, 275–285, 2009.
44. Sizeland, K. H.; Edmonds, R. L.; Basil-Jones, M. M.; Kirby, N.; Hawley, A.; Mudie, S.; Haverkamp, R. G. Changes to Collagen Structure during Leather Processing. *J. Agric. Food Chem.*, 63(9), 2499–2505, 2015. https://doi.org/10.1021/jf506357j.
45. Buchanan, J. K.; Zhang, Y.; Holmes, G.; Covington, A. D.; Prabakar, S. Role of X-ray Scattering Techniques in Understanding the Collagen Structure of Leather. *ChemistrySelect*, 4(48), 14091–14102, 2019. https://doi.org/10.1002/slct.201902908.
46. Naffa, R.; Maidment, C.; Ahn, M.; Ingham, B.; Hinkley, S.; Norris, G. Molecular and Structural Insights into Skin Collagen Reveals Several Factors That Influence Its Architecture. *Int. J. Biol. Macromol.*, 128, 509–520, 2019. https://doi.org/10.1016/j.ijbiomac.2019.01.151.
47. Zhang, Y.; Ingham, B.; Cheong, S.; Ariotti, N.; Tilley, R. D.; Naffa, R.; Holmes, G.; Clarke, D. J.; Prabakar, S. Real-Time Synchrotron Small-Angle X-Ray Scattering Studies of Collagen Structure during Leather Processing. *Ind. Eng. Chem. Res.*, 57(1), 63–69, 2018. https://doi.org/10.1021/acs.iecr.7b03860.
48. Bowes, J. H.; Kenten, R. H. The Swelling of Collagen in Alkaline Solutions. 1. Swelling in Solutions of Sodium Hydroxide. *Biochem. J.*, 46(1), 1–8, 1950. https://doi.org/10.1042/bj0460001.
49. Highberger, J. H. The Isoelectric Point of Collagen. *JALCA* 61(9), 2302–2303, 1939. https://doi.org/10.1021/ja01878a010.
50. Knaggs, J. Studies on Collagen. The Changes Which Collagen Undergoes When Treated with Solutions of Hydrochloric Acid and Sodium Hydroxide1. *Biochem. J.*, 23(6), 1308–1327,1929. https://doi.org/10.1042/bj0231308.
51. Samchenko, Y.; Ulberg, Z.; Korotych, O. Multipurpose Smart Hydrogel Systems. *Adv. Colloid Interface Sci.*, 168(1–2), 247–262, 2011. https://doi.org/10.1016/j.cis.2011.06.005.
52. Basford, J. The Soaking, Unhairing and Liming Process: Part 1 of 3. The Practicalities of the Soaking Process. *World Leather*, 19(7), 23–26, 2006.
53. Fratzl, P. Collagen: Structure and Mechanics, an Introduction. In *Collagen: Structure and Mechanics*; Springer US,1–13, 2008. https://doi.org/10.1007/978-0-387-73906-9_1.
54. Miles, C. A.; Avery, N. C.; Rodin, V. V.; Bailey, A. J. The Increase in Denaturation Temperature Following Cross-Linking of Collagen Is Caused by Dehydration of the Fibres. *J. Mol. Biol.*, 346(2), 551–556, 2005. https://doi.org/10.1016/j.jmb.2004.12.001.
55. Witnauer, L. P.; Fee, J. G. Effect of Diluents on Fusion Temperature of the Crystalline Regions in Plain and Tanned Cowhide. *J. Polym. Sci.*, 26(113), 141–150,1957. https://doi.org/10.1002/pol.1957.1202611302.
56. Flory, P. J.; Garrett, R. R. Phase Transitions in Collagen and Gelatin Systems. *J. Am. Chem. Soc.*, 80 (18), 4836–4845, 1958. https://doi.org/10.1021/ja01551a020.

Antibacterial and Anti–Biofilm Activities of Acetone Extracts of *Usnea* sp. against Mixed Cultures of Bacteria from Soak Liquor Samples and Tank Surfaces

by

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Abstract

Long-term or improper use of antibacterial agents utilized in the soaking process has led to the resistance of some bacteria in the leather industry. New agents may be the solution to combat these antibacterial resistant bacteria in the soaking process. As a natural resource, lichens are known to have many biological activities. In previous studies, we demonstrated that the acetone extracts of several lichen species including *Usnea* sp. may have potential antibacterial and anti–biofilm properties against some *Bacillus* species, which were isolated from different soak liquor samples. In the present study, it was questioned whether the same bioactivities of acetone extracts of *Usnea* sp. can be seen in the mixed cultures of tank surface samples and pre-and main soak liquor samples, which were obtained from different tanneries. Although the extracts did not show noteworthy antibacterial effect against one of the tank surface samples (inhibition ratios; 6.5–16.22%), inhibition percentages were detected as 69.32 and 46.33 at the concentrations of 240 and 120 µg/mL for the other tank surface sample. The anti-biofilm potential of the extracts was tested on the sample where the antibacterial activity of the extracts was not observed. One of the mixed culture of samples from the tank surface could not be inhibited by the extracts in terms of bacterial growth. However, the extracts were tested on this biofilm-forming sample and detected more than 50% inhibition. Furthermore, the extracts inhibited the growth of the mixed culture of bacteria from pre-soak liquor by the percentages of 78.96, 61.5, 51.3, 45.1, and 33.4 at the concentrations of 240, 120, 60, 30 and 15 µg/mL, respectively. On the other hand, the same antibacterial efficacy could not be observed in the other mixed culture from pre-soak liquor sample obtained from a different tannery whereas this sample formed a biofilm structure. The mixed culture of samples from the main soaking process was inhibited by the extracts at the inhibition percentages of 62.13–78.17 at the concentrations of 240- 30 µg/mL. Similar results were also obtained for the other sample (64.6–76.5%) from main soak liquor sample obtained from a different tannery. In conclusion, lichen extracts may have potential antibacterial and anti-biofilm properties against the mixed culture of bacteria from tank surface, pre-and main soak liquor samples and maybe alternatively utilized in the leather industry.

Introduction

The leather industry has great economic value as one of the oldest industries in the world.[1] Bacterial microflora on hides/skins composes of many resident or transient bacteria which come from the animal itself or environmental sources. Whereas these bacteria are not harmful and do not cause any defects on hides of live animals under normal circumstances, the flaying process and a possible delay between the flaying and curing process trigger bacterial population growth. These bacterial population may have proteolytic and also other catabolic activities which cause to break down collagen network and diffuse into the hide and also bacterial attack to the grain surface. It was reported that protease and lipase producing bacteria cause hair-slip, putrefaction, grain peeling, loose grain, holes on the hides/skins, uneven dyeing, etc. Especially extremely halophilic archaea may cause red heat resulting in sueded grain.[2-5] It is well known that many bacteria can multiply in 1–3 h under optimal conditions and bacterial deterioration of hides/skins may occur within 5–6 h after flaying.[1, 6-8] It is obvious that bacterial density on raw hides/skins and in soak liquor may rapidly reach high levels in a short time period.[6] Hide preservation techniques ensures the destruction of harmful bacteria, prevention of bacterial activities and contamination; and resistance of hides/skins against putrefaction during transport and storage.[1] However, the adverse effects of bacterial activities on hides/skins in the production of high–quality leather have been emphasized in many studies.[4-6, 9-13] In this context, the accuracy of the preservation methods of raw hides/skins and also the proper soaking process gains importance. The researchers investigated the bacterial numbers from salt samples which were utilized for salt–curing, fresh, salted, and soaked hides and also from soak liquor samples.[2, 6, 9, 14, 15] The number of bacteria from fresh hides were reported to be 10^8 CFU/g.[14] Furthermore, the numbers of non–halophilic bacteria and extremely halophilic archaea were reported to be 10^4–10^8 CFU/g and 10^3–10^8 CFU/g on 36 salted hides. In the same study, the number of non-halophilic bacteria on soaked hides (10^5–10^8 CFU/g) and in soak liquors (10^5–10^7 CFU/mL) was also high.[2] On the other hand, it was reported that the number of bacterial populations in soak liquors should be up to 10^5 CFU/mL.[16] Therefore, the number of bacteria do not seem to be efficiently

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Manuscript received April 8, 2020, accepted for publication May 13, 2020.

controlled by curing methods and the use of common antibacterial agents. Unfortunately, the presence of a large number of bacteria on the hides/skins and soaking liquor also reveals some consequences. Most bacteria, which were isolated from the leather industry, have been reported to secrete degrading enzymes such as protease and lipase. These enzymatic activities may cause degradation of the hide substance.[2, 4, 5, 11, 17]

The researchers emphasized that the mixed culture of bacteria on salted hides/skins may act synergistically and may cause unwanted defects on leather quality.[6] It is well known that many bacteria coexist by interacting with other bacteria in their surrounding milieu and utilize various functions.[18] To survive or dominate, bacteria must compete with each other because of their nutritional needs.[19] Recently, researchers have reported that bacterial social behaviors such as intraspecies, interspecies or interkingdom interactions are regulated by quorum sensing system (QS) when bacterial population density reaches to higher levels in the environment. This bacterial communication is achieved by signaling molecules called autoinducers (AIs). Three types of AIs were identified: AI–1, also called homoserine lactones (HSLs) for Gram–negative bacteria, autoinducing peptides (AIP) for Gram–positive bacteria, and autoinducer–2 (AI–2) for Gram–positive and Gram–negative bacteria for interspecies interaction.[20-23] In some circumstances, aggregated multicellular communities can form a three-dimensional biofilm structure, which is associated with the QS system, via extracellular polymeric substances (EPS).[24-27] The biofilm structure ensures resistance to disinfectants, antibiotics, U.V., etc. in favor of bacteria so that these bacteria can easily escape from these conditions and continue to survive.[28] Furthermore, this biofilm form has more resistance up to 10–1000 times against antibiotics when compared to its planktonic (free–swimming) forms.[29] In general, research on biofilm structures are performed by using monocultures. However, these mono-cultures of biofilm communities are reported to be rarely encountered in nature.[30] Moreover, it has been reported that mixed–culture biofilms are more resistant to disinfectants than monoculture biofilms.[30] This antimicrobial resistance property of bacteria in such biofilm structures also leads to the multidrug resistance problems.[31] In this respect, high bacterial density on the hides/skins and/or in soak liquor may activate the QS system followed by secretion of some virulence factors (protease, etc.) and/or biofilm formation. Then, unwanted defects may be seen due to all these features.

Due to resistance (intrinsic or acquired) problem of most bacteria to commonly utilized antibacterial agents, it has been suggested that some bactericides may not have efficient inhibitory effects against both total bacteria, proteolytic and lipolytic bacteria in soak liquors because of high organic content in soak liquors.[2, 32] In a previous study supporting this suggestion, the presence of many non-halophilic bacteria was demonstrated despite the antimicrobial agent at a twofold increased concentration (0.8 g/L).[32] The mixed cultures are sub–samples of complex natural communities consisting of two or more bacterial strains and can be utilized as model communities.[33] Since bacterial cells can survive despite the use of antibacterial agents, the difficulty of inactivation in mixed bacterial cultures with these agents has been reported.[34] Moreover, the physiological behaviors of bacteria in their natural community, i.e. in a mixed culture can change.[33] Furthermore, the efficacy of antimicrobials may change in multi-species biofilms in comparison to single-species cultures and planktonic cells.[35]

Recently, new bioactive compounds from natural sources are thought to be an alternative to overcome the potential antimicrobial resistance of many bacteria. Lichens, symbiotic organisms between fungus and one or more algae or cyanobacteria, can synthesize many novel secondary metabolites with biological activities such as antibacterial, anti–biofilm, anti–oxidant, etc. against some Gram–positive and Gram–negative bacteria.[36-38] In the past decade, it was reported that preservation and storage and/or early stages of beam house processes are suitable for biofilm formation on hides/skins.[39] More recently, the potential antibacterial and anti–biofilm activities of several lichen species against some *Bacillus* species from soak liquor samples were demonstrated in our previous studies.[40, 41]

In this way, we aimed to examine the potential antibacterial and anti–biofilm activities of *Usnea* sp. on mixed culture, which is similar to the natural environment but is an uncomplicated and also sufficient community. For this purpose, the acetone extracts of *Usnea* sp. were tested for their antibacterial and anti–biofilm properties on mixed cultures of samples from tank surfaces, as well as mixed cultures of pre– and main soak liquor samples collected from different tanneries.

Experimental

Lichen Samples

The lichen samples belonging to the *Usnea* sp. were collected from fir trees of Kastamonu province in the north–west of Turkey by all of the authors. The identification of the samples was confirmed by classical taxonomic methods based on microscopic examination by Çobanoğlu G.[42-43]

Usnea sp.*:* Turkey, Kastamonu province, Kapaklı Village, 41.24492, 34.18330.

Extraction of Lichen Samples

The lichen samples were washed, dried in air and weighed. After the samples were taken into sterile bottles, acetone (ACS, ISO, Reag. Ph Eur) was added and kept in a dark place for 24 h followed by filtration through filter paper. The acetone was evaporated in a rotary evaporator and crude lichen acetone extracts were obtained.[40, 41, 44]

Samples

The mixed culture samples from pre– (two samples) and main (two samples) soak liquors, as well as tank surfaces (two samples) were tested in the present study. Each pre- and main-soak liquor sample and tank surface sample was collected from different tanneries in Leather Organized Tannery Region, Tuzla–İstanbul, Turkey to obtain comparative results amongst the tanneries. The mixed culture samples of pre- and main soak liquor were taken into sterile glass bottles. The mixed culture samples were taken from the tank surfaces with transport swabs. Then, these samples were immediately placed into sterile sample bags and carried on ice during transportation. The samples were brought to the laboratory as soon as possible and the experiments were started. The pre- and main soak liquor samples were transferred into 50 mL sterile falcon tubes in the sterile cabinet. After these samples were centrifuged twice at 10000 rpm for 10 minutes, the supernatants in the tubes were removed. Then, tryptic soy broth (TSB) medium added onto cell pellets. The optical density (OD) values for each sample was checked at 600 nm for the antibacterial assays. On the other hand, the mixed culture of tank surface samples was also inoculated into TSB medium and the OD values at 600 nm were measured. In this way, bacterial suspensions with 0.02 OD values were prepared to examine antibacterial and anti–biofilm assays.

Antibacterial Assays

The antibacterial assays were performed in 96–well microplates (Greiner Bio–One, Cell Star, F–bottom, with lid). TSB medium was added to each well and five-fold serial dilutions of the acetone extracts of *Usnea* sp. were made. The final concentrations of lichen extracts were 240, 120, 60, 30 and 15 µg/mL. The prepared bacterial suspensions were added to obtain a total volume of 100 µL. The untreated (TSB medium and test bacteria) and blind wells (only the TSB medium) were included in the study. The tests were performed in three replicates. The bacterial growth was evaluated at 24th h using Cytation 3 multimode microplate reader (Biotek), by measuring the absorbance. The antibacterial effects of acetone extracts of *Usnea* sp. against the test samples were compared with the untreated ones.[40]

Anti–biofilm Assays

The anti–biofilm potential of the acetone extracts of *Usnea* sp. were also tested against the mixed culture samples from pre– and main soak liquors and tank surfaces. The assays were performed in 96–well microplates (Greiner Bio–One, Cell Star, F–bottom, with lid). TSB medium was added to each well and fivefold serial dilutions of the acetone extracts of *Usnea* sp. were made. The final concentrations of lichen extracts were 240, 120, 60, 30, and 15 µg/mL. The prepared bacterial suspensions were added to obtain a total volume of 100 µL. These samples were incubated overnight at 37°C in TSB medium in 96–well microplates without shaking. After incubation, we confirmed that the extracts did not kill bacterial strains in the samples tested. For this purpose, bacterial growth ratios at OD 600 nm were checked in the microplates before biofilm staining procedure in a microplate reader (Cytation 3-BioTek). Then, the microplates, in which no antibacterial effects were observed, were washed by physiological saline solution and dried at 60^{0}C. Then, 200 µl of 0.1% crystal violet was added to each well for biofilm staining. The microplates were kept at room temperature for 10 min and washed three times. Following the second drying step, 200 µl ethanol was added and absorbance values were measured at OD 590 nm in a microplate reader (Cytation 3-BioTek). The experiments included untreated (the TSB medium and test bacteria) and blind wells (only the TSB medium). The anti–biofilm effect of acetone extracts of *Usnea* sp. against the test samples was compared with the untreated isolates. The tests were performed in three replicates.[38, 41, 45]

Results and Discussion

To the best of our knowledge, antibacterial and anti–biofilm effects of *Usnea* sp. against mixed–culture of bacteria from soak liquor and tank surface samples of leather processing plant have not been studied yet. In this study, we examined the acetone extracts of *Usnea* sp. for their antibacterial and anti–biofilm properties against mixed culture of bacteria from pre-and main–soak liquor samples and tank surface samples obtained from different tanneries.

The potential biological activities of various extracts obtained by different solvents from some lichen species have been indicated in the literature. In a recent study, the antibacterial properties of acetone extracts of *Hypogymnia physodes, Evernia divaricata, Pseudevernia furfuracea* and *Usnea* sp. at different concentrations were evaluated against *Bacillus toyonensis, Bacillus mojavensis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus cereus,* and *Bacillus licheniformis*, which were isolated from different soak liquor samples. In the same study, *Usnea* sp. acetone extracts were determined to have considerably high inhibitory effects on *Bacillus* species (86.6–97.9%) even at low concentrations.[40] In a continuation of this work, biofilm–forming *Bacillus* species were detected as *B. subtilis, B. amyloliquefaciens,* and *B. velezensis* and the acetone extracts of *Usnea* sp. have been detected to have potential anti–biofilm properties depending on the tested bacteria or concentrations applied.[41]

In this study, we firstly examined the potential antibacterial and anti–biofilm efficacies of acetone extracts of *Usnea* sp. against a mixed culture of bacteria, which were obtained from tank surfaces. As mentioned earlier, it is very important to control the number of bacteria on raw hides/skins or in soak liquor samples to produce high–quality leather because the number of bacteria from raw and soaked hides as well as soak liquor samples has been reported considerably high in the literature.[2, 6, 9] A high number of bacteria means that the bacterial activity will be high and, as a result, hide or skin will be damaged. It is quite difficult to overcome any bacterial degradative activity, which occurs during the storage period or

soaking process, in the subsequent stages of tanning operations until the production of finished leather. We suggested that many bacteria may also colonize and form a biofilm structure on the tank surfaces and these bacteria may cause spontaneous contamination in every soaking process. From this point, two mixed–culture samples (A1 and A2) of tank surfaces from different tanneries were tested. According to our results, the acetone extracts of *Usnea* sp. had almost no antibacterial effect against the sample A1. The inhibition ratios were detected between 6.5 and 16.22%. On the other hand, the potential antibacterial efficacy was observed in the sample A2. The maximum inhibition percentages were calculated as 69.32 and 46.33 at the concentrations of 240 and 120 µg/mL. At the lower concentrations (60, 30 and 15 µg/mL), inhibition ratios were between 38.34 and 43.86%. These results demonstrate that the acetone extracts of *Usnea* sp. may have a potential antibacterial effect against the bacterial population, which colonized on the tank surfaces, depending on the bacterial communities. Considering our previous studies, the mixed culture of the tank surface samples may have *Bacillus* species dominancy.[40, 41] Therefore, we could detect the antibacterial activity of the extracts in the sample A2, but not in the sample A1 (Figure 1).

We also tested these mixed cultures of samples obtained from tank surfaces for the capability of forming a biofilm. The experiments showed that a mixed culture sample of A1 formed a biofilm but the sample A2 did not. The difference between the two samples may be related to the bacterial composition of mixed culture. This result pointed out that disinfection techniques have to be performed regularly on tank surfaces. The anti–biofilm potential of the acetone extracts of *Usnea* sp. was only evaluated on the sample A1 because the growth of sample A2 was already inhibited by the extracts at varying percentages. It is well known that anti-biofilm assays are performed at sub-inhibitory concentrations of antibacterial agents below the minimum inhibitory concentration (MIC) value. To evaluate the anti-biofilm potential of the extracts, the same test concentrations were utilized because we detected that these concentrations had no antibacterial effects in the sample A1. The extracts suppressed more than 50% biofilm formation of the sample A1 at concentrations of 240, 30 and 15 µg/mL (Figure 2).

According to the results of our anti-biofilm experiments, the fluctuating effect by the varying concentrations of the extracts was observed. A similar effect was also determined in the biofilm formation of some *Bacillus* species in our previous study.[41] Therefore, the bacterial population that forms a biofilm structure could probably have caused this effect. Since biofilm formation is a multi-step process, it may also be suggested that the tested extracts might affect different targets. It is well known that the soaking process consists of two stages as pre–soaking and main soaking.[16] It has been reported that the duration of the main soaking process generally changes from 1.5 to 24 h, depending on curing methods and countries.[2, 16] In our previous questionnaire study, we determined that the

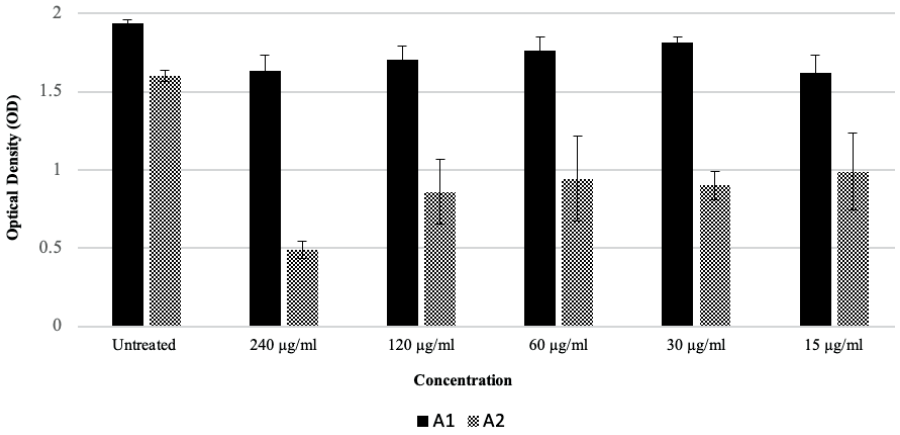

Figure 1. The antibacterial effects of the extracts against the samples A1 and A2.

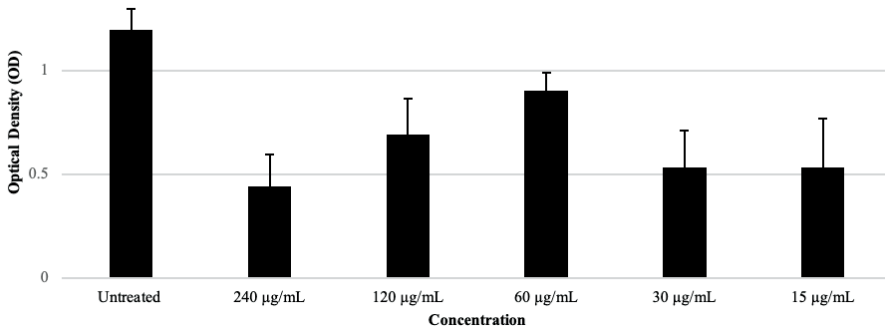

Figure 2. Anti–biofilm effect of the extracts against the biofilm–forming sample A1.

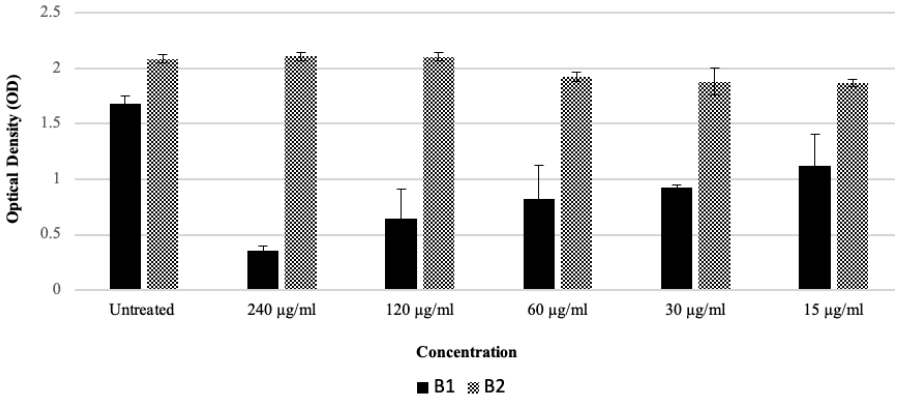

Figure 3. The antibacterial effects of the extracts against the samples B1 and B2.

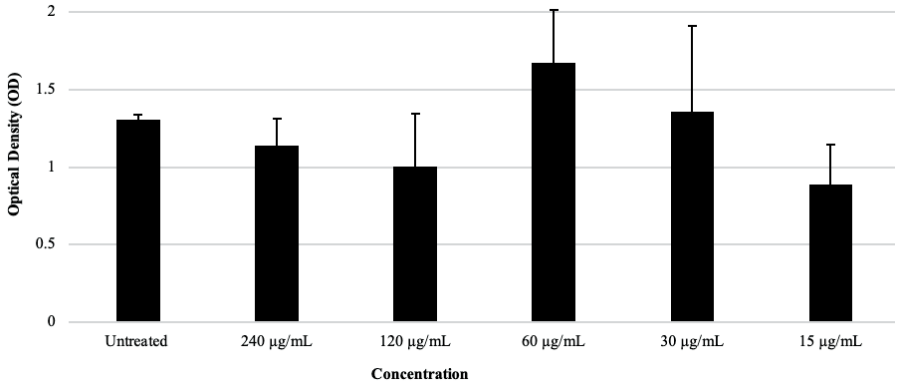

Figure 4. Anti–biofilm effect of the extracts against the biofilm–forming sample B2.

soaking process was generally carried out for 12–18 h in Turkey.[46] Such a quite long soaking process will inevitably trigger the growth of bacteria in the presence of high organic matter. Previously, the antimicrobial agent including didecyldimethylammonium chloride was found to be ineffective to decrease the number of bacteria in soak liquor samples at the manufacturers' recommended dose.[32] In the present study, we analyzed the antibacterial and anti–biofilm potential of the acetone extracts of *Usnea* sp. in two different samples of mixed culture bacteria from pre–soaking process. According to the results of the experiments evaluating antibacterial efficacy of the extracts, the sample B1, which was isolated from pre–soaking process, was inhibited by the percentages of 78.96, 61.5, 51.3, 45.1, and 33.4 at the tested concentrations of 240, 120, 60, 30 and 15 µg/mL, respectively. On the other hand, the same efficacy could not be observed in the sample B2. At the initial three concentrations, there was no antibacterial effect by the extracts. At the last three concentrations (60, 30 and 15 µg/mL), there was a slight inhibition on the bacterial growth of the sample B2, which was recorded as below 10.6% (Figure 3).

Also, the samples B1 and B2 were tested for the capability of biofilm formation. Our findings showed that the mixed culture sample of B1 did not form a biofilm structure whereas the sample B2 formed a biofilm. As in the mixed culture samples of tank surfaces, the difference between the two samples was probably due to the bacterial composition of mixed cultures. Moreover, the acetone extracts of *Usnea* sp. could not efficiently inhibit the biofilm formation of the sample B2. Slight inhibition ratios were determined and these ratios were recorded under 50% (Figure 4). These data suggest that a mixed culture of bacteria can survive in a biofilm structure despite the utilized antibacterial agents. Although our natural extracts do not have a remarkable effect on the inhibition of biofilm formation of mixed culture of bacteria from pre–soaking process, further studies for antibacterial agents in combination with lichen extracts, which are known to have several biological activities, may be performed for the evaluation of their antibacterial and anti–biofilm efficacies against bacteria in soak liquor.

We also evaluated the antibacterial and anti–biofilm potential of the acetone extracts of *Usnea* sp. on two different samples of mixed culture bacteria from main–soaking process, which were obtained from different tanneries. The mixed culture of sample C1 from the main–soaking process was inhibited by the extracts at the inhibition percentages of 62.13–78.17 at the initial four concentrations (240, 120, 60, 30 µg/mL). The inhibition ratio for the concentration of 15 µg/mL was recorded as 48.24. Similarly, the inhibitions by the

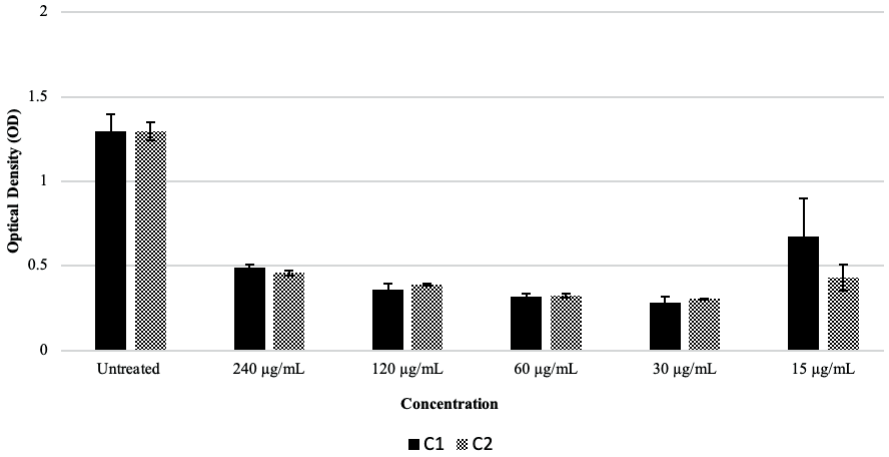

Figure 5. The antibacterial effects of the extracts against the samples C1 and C2.

acetone extracts of *Usnea* sp. were detected in the mixed culture of sample C2 from main soaking process. The inhibition percentages were between 64.6 and 76.5 (Figure 5). However, these samples did not form any biofilm structure (data not shown). These results might be related to the timing of sample collection because the main soak liquor samples were taken approximately at the 4 h of the main soaking process from the leather processing plant. Taking samples from the soak liquor periodically at certain intervals may be more useful to assess the potential of these extracts. Also, the differences in bacterial composition or the presence of resistant bacteria in the soak liquor samples may affect the biofilm formation.

There are also several studies in the leather industry that investigate the potential antibacterial effects of other natural products against bacterial growth. In a study evaluating the antibacterial effects of essential oils of *Lavandula officinalis* and *Eucalyptus globulus* on leather specimens, it was reported that samples treated with essential oil of *L. officinalis* had a more protective effect when compared to the samples cured with 2–(thiocyanomethylthio) benzothiazole (TCMTB) after 24 weeks. Also, the essential oil of *L. officinalis* was found to have a better antibacterial effect than *E. globulus*.[47] Furthermore, there are studies examining potential antibacterial agents from natural resources, especially in the soaking process. For example, myrtle oil (1%), which was added to soak liquor, was examined on the bacterial growth after 7 and 24 h soaking, and its effect was compared against the tested bactericide including 7–25% phenol, 4–chloro–3–methyl. The researchers detected similar bacterial counts in both treatment groups.[48] In another study, the potential antibacterial effect of *Origanum minutiflorum* was reported in comparison to the commercial bactericide, including 7–25% phenol, 4–chloro–3–methyl.[49] In this study, the potential antibacterial and anti-biofilm efficacies were also demonstrated for the acetone extracts of *Usnea* sp. In a previous study, it has been reported that biofilm formation causes dyeing defects in the leather industry.[39] Therefore, biofilm formation may have an important role in the production of the best value of leather. More recently, novel approaches such as anti-virulence strategy have come into prominence because most bacteria may secrete virulence factors such as protease and elastase. The production of virulence factors and also biofilm formation is associated with the QS system. The anti-QS potential of lichens was reported in the literature.[44] In this manner, the potential effects may be investigated in detail for anti-QS and anti-virulence properties of lichen extracts. These extracts and/or their compound(s) with anti-biofilm, anti-bacterial, and anti-QS properties may be applicable in the leather industry.

Conclusion

In the present study, it has been demonstrated that the acetone extracts of *Usnea* sp. may have antibacterial and anti–biofilm properties against a mixed culture of bacteria from tank surface and pre–or main soak liquor samples. The efficacy of the extracts varied depending on the samples. These differences may result from different bacterial compositions of mixed cultures obtained from soak liquor or tank surface samples. In this study, it has been also shown that mixed cultures of bacteria from the tank surface and pre-soaking process can form a biofilm structure. Biofilm formation may play an important role during leather–making processes. Further detailed studies may increase our knowledge about biofilm formation on hides/skins. Lichen extracts have advantages such as having potential antibacterial and anti–biofilm properties as well as being non–toxic, and ecological material. If the chemical(s) of these lichen extracts are comprehensively investigated, the major active ingredient may be discovered and may be utilized in the leather industry.

Acknowledgement

We express very profound gratitude to Meral Birbir, Pınar Çağlayan for their valuable supports. The authors are also grateful to Gülşah Çobanoğlu Özyiğitoğlu for confirmation of taxonomic identification of lichen species tested. The authors would like to thank Barış Gökalsın, Arhun Ali Balkan, and Ayla Yıldız for sharing their experiences during the experiments.

References

1. Gudro, I., Valeika, V., and Sirvaitytė, J.; Short Term Preservation of Hide Using Vacuum: Influence on Properties of Hide and of Processed Leather. *Plos One* **9** (11), 2014.
2. Berber, D., and Birbir, M.; Examination of Bacterial Populations in Salt, Salted Hides, Soaked Hides and Soak Liquors. *JALCA* **105** (10), 320–326, 2010.
3. Vreeland, R.H., Angelini, S. and Bailey, D.G.; Anatomy of Halophile Induced Damage to Brine Cured Cattle Hides. *JALCA* **93**, 121-131, 1998.
4. Birbir, M., and Ilgaz, A.; Isolation and Identification of Bacteria Adversely Affecting Hide and Leather Quality. *J. Soc. Leather Technol. Chem.* **80**, 147–153, 1996.
5. Bailey, D.G., and Birbir, M.; The Impact of Halophilic Organisms on the Grain Quality of Brine Cured Hides. *JALCA* **91**, 47–51, 1996.
6. Aslan, E., and Birbir, M.; Examination of Gram–Negative Bacteria on Salt–Pack Cured Hides. *JALCA* **107** (4), 106–115, 2012.
7. Bauman, R.W.; Microbiology with Diseases by Taxonomy, 3th Edition, Pearson Education, Benjamin Cummings, 2011.
8. Kanagaraj, J., Sundar, V.J., Muralidharan, C., and Sadulla S.; Alternatives to Sodium Chloride in Prevention of Skin Protein Degradation-A Case Study. *J. Clean. Prod.* **13** (8), 825–831, 2005.
9. Aslan, E., and Birbir, M.; Examination of Efficiency and Sufficiency of Salt–Pack Curing Method. *J. Soc. Leather Technol. Chem.* **95** (3), 98–103, 2011.
10. Bailey, D.G.; The Preservation of Hides and Skins. *JALCA* **98** (8), 308–319, 2003.
11. Orlita, A.; Microbial Biodeterioration of Leather and Its Control: A Review. *Int. Biodet. Biodeg.* **53**, 157–163, 2004.
12. Woods, D.R., Welton, R.L., Thomson, J.A., and Cooper, D.R.; Collagenolytic Activity of Cured Hide Bacteria. *J. App. Bacteriol.* **35** (1), 123–128, 1972.
13. Haines, M.B.; Quality Rawstock. *JALCA* **66** (4), 164–173, 1984.
14. Oppong, D., Bryant, S., Rangarajan, R., Steele, S., Radwell, D., and Hyllengren, L.; Application of Molecular Techniques to Identify Bacteria Isolated From the Leather Industry. *JALCA* **101,** 140–145, 2006.
15. Bailey, D.G., and Birbir, M.; A Study of the Extremely Halophilic Microorganisms Found on Commercially Brine–Cured Cattle Hides. *JALCA* **88**, 285–293, 1993.
16. Rangarajan, R., Didato, T.D., and Bryant, S.; Measurement of Bacterial Populations in Typical Tannery Soak Solutions by Traditional and New Approaches. *JALCA* **98**, 477–485, 2003.
17. Wu, J., Zhao, L., Liu, X., Chen, W., and Gu, H.; Recent Progress in Cleaner Preservation of Hides and Skins. *J. Clean. Prod.* **148**, 158–173, 2017.
18. Kato, S., Haruta, S., Cui, Z.J., Ishii, M., and Igarashi, Y.; Network Relationships of Bacteria in a Stable Mixed Culture. *Microb. Ecol.* **56** (3), 403–411, 2008.
19. Hibbing, M.E., Fuqua, C., Parsek, M.R., and Peterson, S.B.; Bacterial Competition: Surviving and Thriving in the Microbial Jungle. *Nat. Rev. Microbiol.* **8** (1), 15–25, 2010.
20. Fuqua, C., Parsek, M.R., and Greenberg, E.P.; Regulation of Gene Expression by Cell-To-Cell Communication: Acyl-Homoserine Lactone Quorum Sensing. *Annu. Rev. Genet.* **35** (1), 439–468, 2001.
21. Waters C.M., and Bassler, B.L.; Quorum Sensing: Cell-To-Cell Communication in Bacteria. *Annu. Rev. Cell Dev. Biol.* **21**, 319–346, 2005.
22. Ng, W.L., and Bassler, B.L.; Bacterial Quorum–Sensing Network Architectures. *Annu. Rev. Genet.* **43**, 197–222, 2009.
23. Rutherford, S.T., and Bassler, B.L.; Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harb. Perspect. Med.* **2** (11), 2012.
24. Parsek, M. R., and Greenberg, E.P.; Sociomicrobiology: The Connections between Quorum Sensing and Biofilms. *Trends Microbiol.* **13** (1), 27–33, 2005.
25. Grandclément, C., Tannières, M., Moréra, S., Dessaux, Y., and Faure, D.; Quorum Quenching: Role in Nature and Applied Developments. *FEMS Microbiol. Rev.* **40** (1), 86–116, 2015.
26. Jayathilake, P.G., Jana, S., Rushton, S., Swailes, D., Bridgens, B., Curtis, T., and Chen, J.; Extracellular Polymeric Substance Production and Aggregated Bacteria Colonization Influence the Competition of Microbes in Biofilms. *Front. Microbiol.* **8**, 1865, 2017.
27. Flemming, H. C., and Wingender, J.; The Crucial Role of Extracellular Polymeric Substances in Biofilms. Biofilms in Wastewater Treatment. IWA Publishing, London, United Kingdom, 178–210, 2003.
28. Hall–Stoodley, L., Costerton, J.W., and Stoodley, P.; Bacterial Biofilms: From the Natural Environment to Infectious Diseases. *Nat. Rev. Microbiol.* **2** (2), 95–108., 2004.
29. Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G., and Read, R.R.; Biofilm Bacteria: Formation and Comparative Susceptibility to Antibiotics. *Can. J. Vet. Res.* **66** (2), 86, 2002.
30. Jahid, I.K., Han, N.R., Srey, S., and Ha, S.D.; Competitive Interactions inside Mixed–Culture Biofilms of *Salmonella typhimurium* and Cultivable Indigenous Microorganisms on Lettuce Enhance Microbial Resistance of Their Sessile Cells to Ultraviolet C (UV-C) Irradiation. *Food Res. Int.* **55**, 445–454, 2014.
31. Lewis, K.; Persister Cells, Dormancy and Infectious Disease. *Nat. Rev. Microbiol.* **5** (1), 48, 2007.
32. Berber, D., Birbir, M., and Hacioglu, H.; Efficacy Assessment of Bactericide Containing Didecyldimethylammonium Chloride on Bacteria Found in Soak Liquor at Different Exposure Times. *JALCA* **105** (11), 354–359, 2010.
33. Garcia, S.L.; Mixed Cultures as Model Communities: Hunting for Ubiquitous Microorganisms, Their Partners, and Interactions. *Aquat. Microb. Ecol.* **77** (2), 79–85, 2016.
34. Solaiman, D.K.Y., Ashby, R.D., Birbir, M., and Caglayan, P.; Antibacterial Activity of Sophorolipids Produced by *Candida bombicola* on Gram–positive and Gram–negative Bacteria Isolated from Salted Hides. *JALCA* **111**, 358–364, 2016.
35. Lee, K.W.K., Periasamy, S., Mukherjee, M., Xie, C., Kjelleberg, S., and Rice, S.A.; Biofilm Development and Enhanced Stress Resistance of a Model, Mixed–Species Community Biofilm. *ISME J.* **8** (4), 894–907, 2014.
36. Mitrovic, T., Stamenkovic, S., Cvetkovic, V., Radulovic, N., Mladenovic, M., Stankovic, M., Topuzovic, M., Radojevic, I., Stefanovic, and O., Vasic, S.; *Platismatia glaucia* and *Pseudevernia furfuracea* Lichens as Sources of Antioxidant, Antimicrobial and Anti-biofilm Agents. *EXCLI Journal* **13**, 938, 2014.

37. Nithyanand, P., Shafreen, R.M.B., Muthamil, S., and Pandian, S.K.; Usnic acid, a Lichen Secondary Metabolite Inhibits Group A *Streptococcus* Biofilms. *Ant. Van Leeuw.* **107** (1), 263–272, 2015.
38. Gökalsın, B., and Sesal, N.C.; Lichen Secondary Metabolite Evernic Acid as Potential Quorum Sensing Inhibitor against *Pseudomonas aeruginosa*. *World J. Microbiol. Biotechnol.* **32** (9), 150, 2016.
39. Cadirci, B.H., Ozgunay, H., Vural, C., and Yılmaz, O.; A New Defect on Leather: Microbial Biofilm. *JALCA* **105** (4), 129–134, 2010.
40. Berber, D.; Antibacterial Activities of Lichen Derived Extracts against Different *Bacillus* Species from Soak Liquor Samples. *JALCA* **115** (03), 96–104, 2020.
41. Berber, D., Türkmenoğlu, İ., Birbir, M., and Sesal, N.C.; Efficacy of *Usnea* sp. Extracts in Preventing Biofilm Formation by *Bacillus* Species Isolated from Soaking Liquor Samples. 2020, *in press*.
42. Wirth, V., Die Flechten Baden-Württembergs: Teil 1, Stuttgart, Germany, Eugen Ulmer GmbH and Co. 1995.
43. Smith, C.W., Aptroot, A., Coppins, B.J., Fletcher, A., Gilbert, O.L., James, P.W. and Wolseley, P.A. The Lichens of Great Britain and Ireland. The British Lichen Society, London, UK. 2009.
44. Gökalsın, B., Berber, D., Özyiğitoğlu, G. Ç., Yeşilada, E., and Sesal, N.C.; Quorum Sensing Attenuation Properties of Ethnobotanically Valuable Lichens against *Pseudomonas aeruginosa*. *Plant Biosyst.* 1–13, 2019.
45. Başaran, T.I., Berber, D., Gökalsın, B., Tramice, A., Tommonaro, G., Abbamondi, G.R., Hasköylü, M.E. Öner, E.T., Iodice, C. and Sesal, N.C.; Extremophilic *Natrinema versiforme* against *Pseudomonas aeruginosa* Quorum Sensing and Biofilm. *Front. Microbiol.* **11**, 79, 2020.
46. Berber, D., and Birbir, M.; Determination of Major Problems of Raw Hide and Soaking Process in Leather Industry. *International Journal of Advances in Engineering and Pure Sciences* **31** (2), 118–125, 2019.
47. Sirvaityte, J., Siugzdaite, J., and Valeika, V.; Application of Commercial Essential Oils of *Eucalyptus* and *Lavender* as Natural Preservative for Leather Tanning Industry. *Rev. Chim.* **62** (9), 884–893, 2011.
48. Bayramoğlu, E.E.E.; Antibacterial Activity of *Myrtus communis* Essential Oil Used in Soaking. *J. Soc. Leather Technol. Chem.* **90** (5), 217–219, 2006.
49. Bayramoğlu, E.E.E. Natural and Environment–friendly New Bactericide for Leather Industry: Essential Oil of *Origanum minutiflorum*. *J. Biol. Sci*, **5** (4), 455–45, 2005.

Studies on Collagen Structure using X-Ray Scattering on a Closed-Loop Leather Process

by

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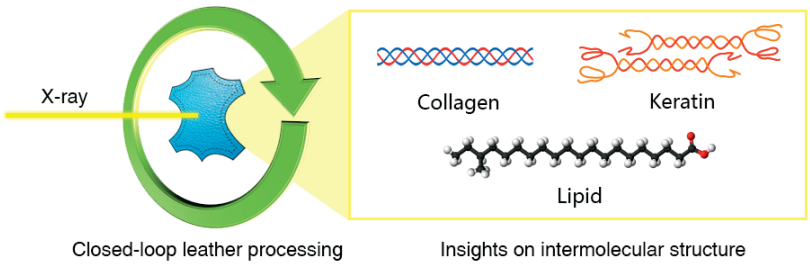
Abstract

Waste management in leather processing is crucial in limiting the excess use of hazardous materials that lead to environmental pollution and health concerns. A closed-loop approach was developed to recycle the spent solutions from leather processing to reduce waste in the effluent. The structural changes of collagen that accompany such processing are yet to be studied and is crucial in understanding the closed-loop process and its subsequent leather properties. In this study, we analysed the collagen structure at different processing stages across the closed-loop approach using synchrotron small-angle X-ray scattering. An increased filling effect in the collagen matrix was observed and attributed to the residual organic component and chromium species in recycled spent solutions. A high uptake of chromium was also observed from the increased scattering intensity from leathers treated with recycled chrome solution, indicating its efficient use. Additionally, the changes in scattering intensity from keratin and lipids indicated an effective unhairing process. Such findings on collagen structure changes will support the development of more environmentally and economically sustainable processing methods to benefit the leather industry.

Introduction

The processing of animal skins making wet blue leather involves a series of chemically intense steps such as liming, pickling and chrome tanning.[1] To improve the efficiency of these processes, an excess of reagents is often used, leaving the unused chemicals to be treated before discharging into the environment.[2] During the liming step, sodium sulphide (Na_2S) and sodium hydrosulphide (NaHS) are used as depilatories in breaking down the disulphide (S-S) bonds in the cystine (Cys) residues of the keratin.[1] The excess sulphide (S^{2-}) and hydrosulphide ions (HS^-) in the spent solution are oxidised to avoid the generation of toxic hydrogen sulphide (H_2S) gas, resulting in a high sulphate (SO_4^{2-}) concentration in the effluent.[3,4] The pickling step requires high concentrations of sodium chloride (NaCl) to prevent skins from swelling at low pH, leading to an excess of chloride ions (Cl^-) in the wastewater.[4] Further, the inefficient uptake of chromium sulphate in the chrome tanning step, results in the presence of unbound Cr^{3+} in the spent tanning solution requiring the effluent to be treated before being released into the enviroment.[4,5] According to previous investigations the wastewater produced during unhairing and liming contains 26,000 mg/L of SO_4^{2-} while the wastewater from the pickling and chrome tanning steps contains 59,000 mg/L of Cl^- and around 2000 mg/L of Cr^{3+}.[4] These values are much higher than the permissible limits for the direct discharge of wastewater according to effluent disposal standards (GB 30486-2013).[6] This has lead researchers to study methods to recover, recycle and reuse chemicals involved in leather processing.

Recycling could mean the recovery of chemicals from the spent solutions followed by reusing or the direct recycling of the spent solutions, although the latter is preferred due to an extra benefit from the reduced water usage.[2] Recently, a closed-loop approach was developed and utilized in leather tanneries.[7,8] In this method, the effluent from unhairing, liming, pickling and chrome tanning could be completely reused in the ensuing processing cycles, with the leather properties shown to be comparable to a conventional process.[7,8] As the main component of animal skins, collagen has its



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Manuscript received March 4, 2020, accepted for publication May 17, 2020.

intrinsic long-range ordered molecular and fibrillar structure that gives leather its characteristic organoleptic properties.[9] Because of the periodic arrangement of collagen molecules, X-ray scattering technologies are widely applied to analyse changes in its structure.[10,11] The intrinsic properties of collagenous tissues were studied and related to the collagen structure at different length scales from sub-nanometer to hundreds of nanometer using X-ray scattering techniques,[12-14] highlighting X-ray as an important tool for providing insights into the collagen structure. Previous X-ray scattering studies on collagen in leather specifically have shown that it is possible to follow molecular-level changes in the collagen structure during each stage of a conventional process from raw skin to leather.[13] However, leather processes based on closed-loop systems are yet to be studied, and can provide valuable information on the molecular interactions of the recycled solutions with the collagen in leather. The ability to directly resolve molecular-level changes quickly, accurately and with minimal sample preparation makes synchrotron SAXS an important technique to evaluate novel tanning processes.

In the current work we study the effect of recycling solutions on the collagen structure of skins and the resultant leathers. Results indicated an increase in chromium binding efficiency based on changes observed in the collagen intermolecular packing, D-period and peak intensities. Also, keratin and lipid components that were affected during hair removal supported the effectiveness of recycled liming solution. The results provide complementary information that adds to the chemistry knowledge of leather tanning and a more comprehensive understanding to the closed-loop tanning approach is established. The improvement of fundamental understanding on this recycling technology will also shed light on the improvement of the sustainability of industrial leather manufacturing processes.

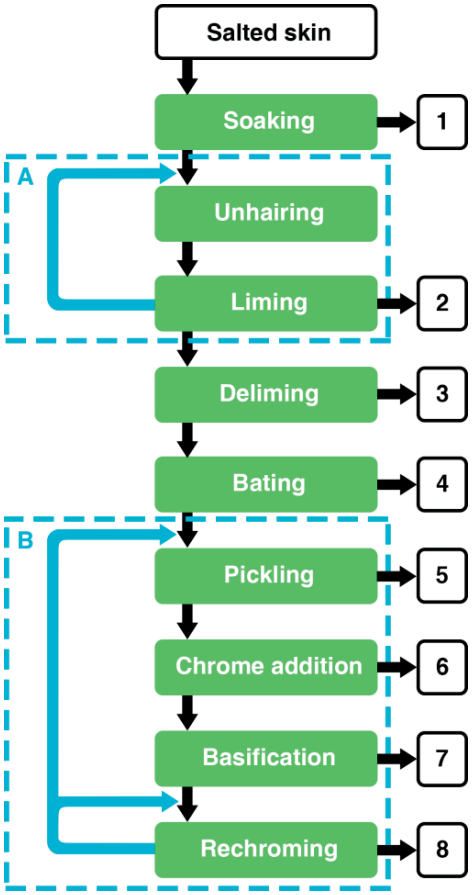
Experimental Methods

Sample preparation

Calf skins were prepared following the proprietary closed-loop processing method (Scheme 1)[15] in a local tannery in Hebei Province, China, and samples were taken during a number of different process steps (stage **1** to **8**). Firstly, the salted raw skins were soaked in water to allow them to rehydrate, following which they were mechanically processed to remove the flesh. The fleshed raw skins (**1**) were then loaded in the drum with 100 wt% (based on the weight of the fleshed raw skin, same until splitting) the recycled solution obtained from previous unhairing-liming cycles (part A, Scheme 1). The recycled liming solutions at pH around 12.5 contain in average 1500 ppm of S^{2-} and were relatively consistent over more than 300 cycles in the tannery. Then, 0.8 wt% of $Ca(OH)_2$, 0.5 wt% of NaHS and 0.15 wt% of degreasing agent were added to the drum at 25°C and ran for 1 h before filtration. During this unhairing step, hair became dislodged from the grain surface of skins and was removed by filtration while running for 1 h. The solution was then pumped back to the drums for liming. Chemicals for liming were subsequently added at 25°C, including 1.0 wt% of $Ca(OH)_2$, 0.8 wt% Na_2S to raise the pH to around 13 to open up the collagen fibre structure to facilitate chemical interaction during the following processing steps. During liming, skins can easily swell by up to 80%,[1] so fresh water is added to compensate for the reduced amount of solution at this stage to make up a 80 wt% solution, followed by an addition of 0.4 wt% $Ca(OH)_2$ and the skins run overnight at 25°C. Limed skins (**2**) were unloaded while the solution was filtered for reuse in the next cycle.

The skins were fleshed, split to a thickness of 2 mm, and delimed (**3**) to pH = 8.0 using 0.3 wt% of sodium metabisulfite ($Na_2S_2O_5$), 2.0 wt% of ammonium chloride (NH_4Cl) at 30°C for 2 h, and then treated with 0.3 wt% of bating enzyme ("bated") (**4**) at 30°C for 1 h. The purpose of deliming and bating is to remove dissolved proteins from skins as well as the potentially residual hair buried under the grain surface.

The pickling step is the starting point of the second closed-loop cycle (part B, Scheme 1), in which spent chromium solution is used for pickling and also during tanning. The recycled chromium solution at pH around 3.5 contained in average 2800 ppm of Cr(III) and was reused for more than 300 cycles in the tannery. The skins were first acidified to pH = 2.5 using a subsequent addition of 0.7 wt% of formic acid (HCOOH) and 1.1 wt% of H_2SO_4 in 50 wt% of recycled



Scheme 1. Flow chart of a closed-loop approach for leather processing with blue arrows highlighting the recycling of the spent solutions. (A) Unhairing-liming cycle; (B) Pickling-chrome tanning cycle. Samples are collected from different stages of the process for SAXS analysis: (1) raw; (2) limed; (3) delimed; (4) bated; (5) pickled; (6) Cr treated; (7) basified; (8) wet blue.

chromium solution at 25°C, under NaCl concentration of 2.0 wt% (to the limed split skin weight, same below) (**5**), then tanned with 5.0 wt% basic chromium sulphate ($Cr(OH)SO_4$, 25% Cr_2O_3, 33% basicity) for 2 h (**6**), followed by basification to pH = 4.0 in another 2 h (**7**). Next, two portions of 50 wt% of recycled chromium solution was added at 55-65°C to raise the final drum temperature to 40°C and the drums run for another 4 h to produce the "wet blue" leathers (**8**). Such a high temperature will facilitate the chemical exhaustion and interactions of the recycled chromium species with collagen. The spent solution was then filtered for reuse in the next cycle.

Samples collected at each stage were named as: (**1**) raw; (**2**) limed; (**3**) delimed; (**4**) bated; (**5**) pickled; (**6**) Cr treated; (**7**) basified; (**8**) wet blue. All samples were kept at 4°C prior to the structural analysis.

Small-angle X-ray scattering (SAXS)

Skin and leather samples were cut into squares sized 10 mm × 10 mm × 3 mm (L × W × H), kept between polyimide films, and sealed into sample cells to keep their moisture levels constant. SAXS experiments were conducted on Beamline 23A1 at the National Synchrotron Radiation Research Centre (NSRRC) in Hsinchu, Taiwan. The measurements were carried out at a 2.602 m sample-to-detector distance with an X-ray energy of 15 keV. The scattering intensity $I(q)$ is presented as a function of scattering vector, q, where $q = 4\pi\sin(\theta/2)/\lambda$, and where θ is the angle between incident and scattered radiation. Peak fittings were conducted following previous studies using SAXSFit and Fityk.[16-18] Relative diffraction peak intensity is calculated as $R_{n/m} = A_n/A_m$, where A_n and A_m stands for the area of peak order n and m. Peak positions were recorded and converted to real-space distance $d = 2\pi/q$.

Results and Discussion

The SAXS pattern of raw calf skin (Figure 1A) showed a group of well-resolved diffraction rings within the q region of 0.1–3.0 nm^{-1} originating from the long-range ordered packing of collagen molecules within the collagen fibrils.[13] During the processing of skins in the closed-loop system, the integrated SAXS data showed significant changes in the intensities and positions of the diffraction peaks (Figure 1B). The overall trend is in good accordance with conventional leather processing methods, indicating a comparable structure to standard leathers.[18,19] The third to eleventh order peaks dropped from the raw (**1**) to the limed stage (**2**), along with a shift towards higher q. This can be explained by the swelling of collagen fibres under alkaline conditions.[1] Additionally, a slight increase in background scattering is observed at the limed stage, which may be caused by the organic components in the recycled solution that have been absorbed by the skins during swelling. This is also supported by the relative decrease in the form factor scattering from collagen fibrils due to mass dilution. After deliming (**3**), the peak intensities increased, and remained the same at the bated stage (**4**). Deliming and bating brings the skins back to a neutral pH causing deswelling,[1] observed as a shift of peak position backward to lower q. However, the overall peak intensities of delimed and bated skins are similar to the limed skins, indicating that permanent changes in collagen structure were conferred to the skin during liming.[1] Overall peak intensities increased marginally when skins reached the pickled stage (**5**). During closed-loop processing, pickling was conducted using a recycled chromium solution. Normally the introduction of chromium species into the collagen matrix will cause a significant increase in diffraction peak intensities from the fourth order onwards.[18-20] However, in our case, as the chromium species are not activated, no binding occurs, hence there is no concomitant enhancement of the electron density contrast of the collagen structure. This further supports the assertion that only a low concentration of chromium species is left over from the previous cycles, which is suitable for recycling and avoids causing coarse grain on leather products.[21] After the addition of basic chromium sulphate (**6**), an increase in intensity of the fifth to eleventh order peaks was observed, along with the disappearance of the third order peak. This can be explained by the fact that the tanning effect of the chromium species changes the structure of collagen in the skins and thereby increases the electron density contrast in the matrix.[20] The intensity of the fifth to eleventh order peaks increased at basification (**7**) and wet blue (**8**) stages, at which point the third order peak then reappeared. The increase in overall peak intensity along with the reappearance of the third order peak suggested the exhaustion of active collagen amino acid residues and the increasing uptake of chromium into the collagen matrix.[18] Another broad feature was observed at high q region around 4.0 nm^{-1} (Figure 1C and 1D) which was attributable to the intermolecular lateral packing (ILP) of the collagen molecules according to previous studies.[22,23] This peak shifted significantly towards low q from raw (**1**) to limed (**2**), and then moved back gradually towards high q from the deliming (**3**) to wet blue (**8**) stages, The changes in ILP can also be attributed to the chemical treatments during the process, which will be discussed later with the peak fitting results.

Apart from the signals from collagen, we also observed a diffuse feature at q = 1.3 nm^{-1} in the raw skin SAXS pattern (Figure 1A). Further investigations on the hair of raw skins (**1'**) provided more resolved features (Figure 1C). According to the previous reports, the equatorial peaks at q = 0.7, 1.3 and 2.3 nm^{-1} (scale in real-space = 9.2, 4.7 and 2.8 nm) and the meridional peak at q = 0.9 nm^{-1} (scale in real-space = 6.8 nm) can originate from the lateral and axial packing of keratin intermediate filaments (KIF) in hair, respectively.[24-28] The intensity of the equatorial peaks from KIF usually follow a decreasing trend with increasing q,[25,28] however, we observed an exceptionally higher intensity of the peak at q = 1.3 nm^{-1} than the peak at q = 0.7 nm^{-1} (Figure 1D). This implies a superimposition from the scattering signal of other components in hair or skin. The crystalline lipid with layered stacking in skin hair follicles was reported to have an isotropic ring showing a real-space scale of 4.5 nm, which is in good accordance with our observation in this study.[25-27,29]

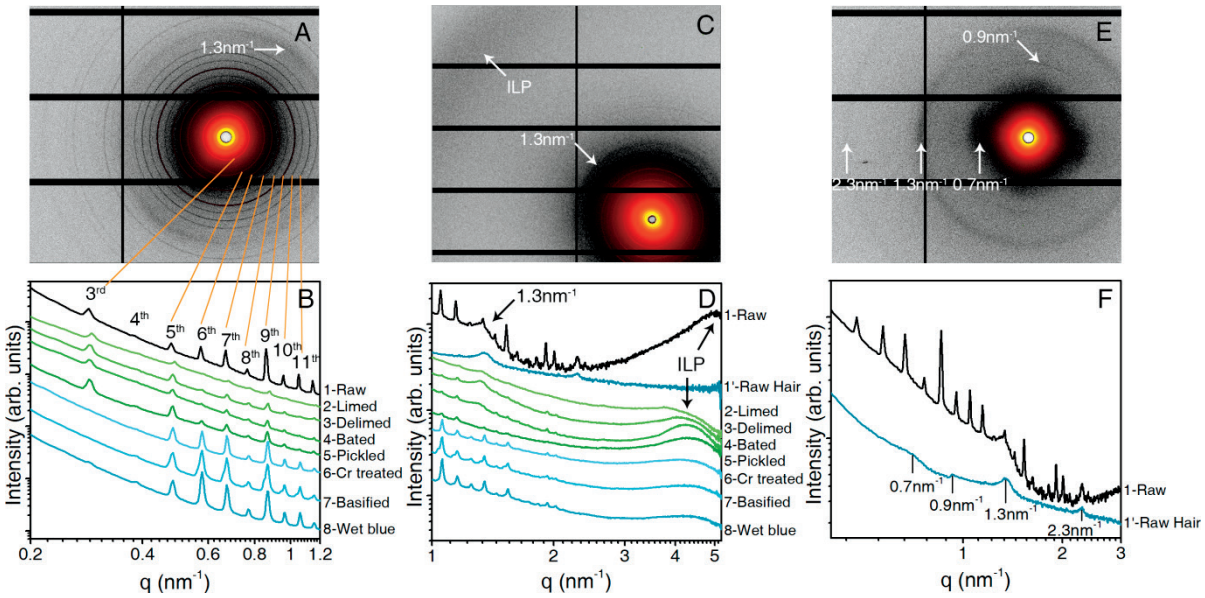


Figure 1. (A) SAXS pattern of a raw calf skin showing characteristic diffraction rings originating from the long-range ordered collagen structure. (B) Integrated data in the q region of 0.2–1.2 nm^{-1} from skins at different stages of processing using recycled solution. Selected peaks are labelled corresponding to $q = 2\pi n/D$ where n is the peak order and D is the collagen axial packing periodicity (D-period). (C) SAXS pattern of a bated skin sample showing the diffuse arc originating from the ordered intermolecular lateral packing (ILP) of the collagen molecules. (D) Integrated data in the q region of 1.0–5.2 nm^{-1} from skins at different stages of processing using the recycled solution. (E) SAXS pattern of raw calf hair showing diffuse features with orientation. (F) Integrated data in the q region of 0.4–3.0 nm^{-1} from raw calf skin and hair to highlight the position of the diffuse peaks.

We then monitored the changes of the aforementioned diffuse features during the processing of skins in the closed-loop system (Figure 1F). When the skins were limed, the peak at q = 1.3 nm^{-1} diminished and the peak at q = 0.7 and 2.3 nm^{-1} become almost undetectable. The surfaces of the skins were visually clean at this stage, so we could not collect hair samples to measure them separately from the skins. During the liming step, the majority of the hair around the surface of skin is broken down and removed from the solution by filtration,[1,7] causing a significant decrease in the scattering signal from KIF from hair on limed skin (**2**).[25] However, the presence of peak at q = 1.3 nm^{-1} suggests either the existence of minimal residual KIF or the retained crystallinity of lipid in the follicles under the surface of the skins. Nevertheless, this is direct evidence of the effectiveness of recycled liming solution to remove hair from skins in this closed-loop process. After deliming (**3**), the collagen matrix became more compact, therefore revealing a slightly stronger scattering of the peak at q = 1.3 nm^{-1} from the residual lipid or KIF. However, this peak displaced slightly to the low q in bated skin (**4**), indicating a disrupted intermolecular structure of the lipid in combination with the enzymatic effect from the broad-spectrum protease that facilitates the removal of potential residual keratin filaments at this stage of processing.[30] After pickling (**5**), most of the dissolved components are removed, leaving a clean grain with no residual hair.

Detailed studies on the structural changes of collagen during the closed-loop process were obtained from the fitting of peaks in the SAXS plots (Figure 2). The packing of collagen molecules in a fibril

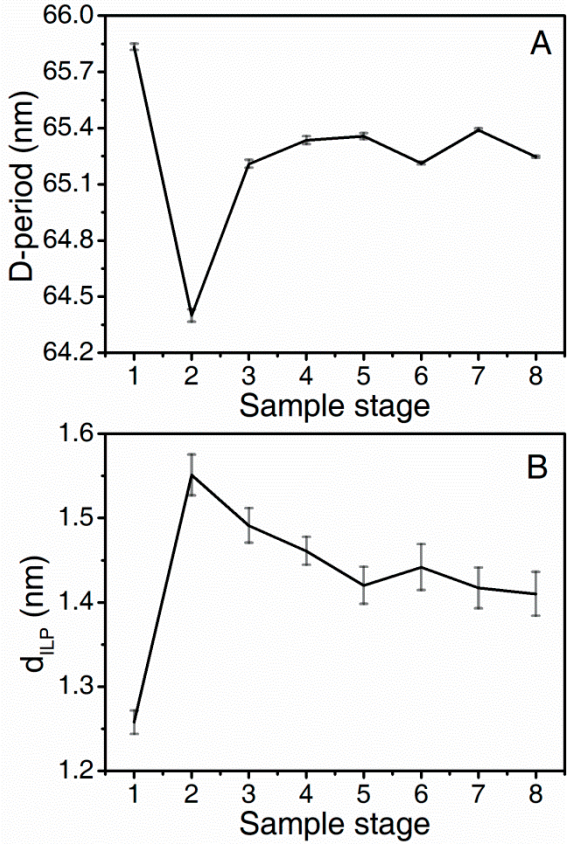


Figure 2. Calculated collagen (A) D-period and (B) intermolecular lateral packing distance (d_{ILP}) in calf skins from SAXS analysis at different stages of leather processing using the closed-loop approach: (1) raw; (2) limed; (3) delimed; (4) bated; (5) pickled; (6) Cr treated; (7) basified; (8) wet blue.

follows a long-range ordered quarter-stagger arrangement with a characteristic axial packing periodicity (*D*-period) and a lateral packing distance (d_{ILP}).[9,31] A sharp decrease in *D*-period from 65.8 to 64.4 nm between the raw (**1**) and limed (**2**) stages was observed along with a steep increase in d_{ILP} from 1.26 to 1.55 nm. This observation is in agreement with the visual shrinkage of the skin as well as the increase in its thickness at the limed stage. Under the highly alkaline conditions found during liming, the hydrolysis of amide groups on collagen side chains happens rapidly, generating more carboxyl groups.[1] This can result in a shift of the isoelectric point of collagen from 7.4 to 5.0-6.0, disrupting the existing salt links and hydrogen bonds due to alteration of charges on the side chains.[1] The weakened intermolecular linkages and the like-charge repulsion between negatively charged side chains on collagen could cause anisotropic swelling of the matrix, which is observed as a decrease in the *D*-period and an increase in the d_{ILP} during the liming stage.[23] Deliming (**3**) on the other hand, causes deswelling on skins at the macroscale, observed as an increase in the *D*-period to 65.2 nm and a decrease in d_{ILP} to 1.49 nm. The structure after deliming is different to the original structure in the raw stage suggesting a permanent opening-up effect in the fibrous structure of collagen during the liming process, which is supported by previous studies.[1] During bating (**4**) and pickling (**5**), the collagen intermolecular structure gradually became denser, especially in the lateral direction, supported by a continuous decrease in d_{ILP} from 1.49 to 1.42 nm, while the *D*-period increased slightly to 65.4 nm at the pickling stage. When basic chromium sulphate (**6**) was added to the skins, we observed a slight decrease in *D*-period from 65.4 to 65.2 nm. During basification (**7**), the *D*-period increased by 0.2 nm, which decreased again following the introduction of recycled chromium solution to bring the skins to wet blue (**8**). These observations agree with our earlier report on the conventional processing of bovine hides that the *D*-period decreased when tanned using basic chromium sulphate.[19,32] However, the decrease in *D*-period from pickled skin to wet blue leather is much less in this closed-loop approach (0.2 nm) than in the previously reported results (1.2 nm).[32] By reusing spent chromium solution that contains dissolved organic components such as protein as well as inactive chromium species, an enhanced filling effect can be expected. This molecular-level observation showed good accordance with the improved fullness of the wet blue leather produced from the closed-loop approach in comparison to conventional leathers in this trial.

To further investigate the closed-loop process, structural indicators of collagen were demonstrated based on the ratio of SAXS peak intensities (Figure 3), namely, fifth order to third order ($R_{5/3}$) for the tanning effect, and sixth order to fifth order ($R_{6/5}$) for the hydration level of collagen.[13] From raw (**1**) to limed (**2**), delimed (**3**), bated (**4**) and pickled (**5**), only marginal changes were observed in $R_{5/3}$, followed by a sharp increase after the addition of basic chromium sulphate (**6**) (Figure 3A). This can be attributed to the binding of metallic species to collagen and the covalent crosslinking between chromium species and collagen. The value remained constant

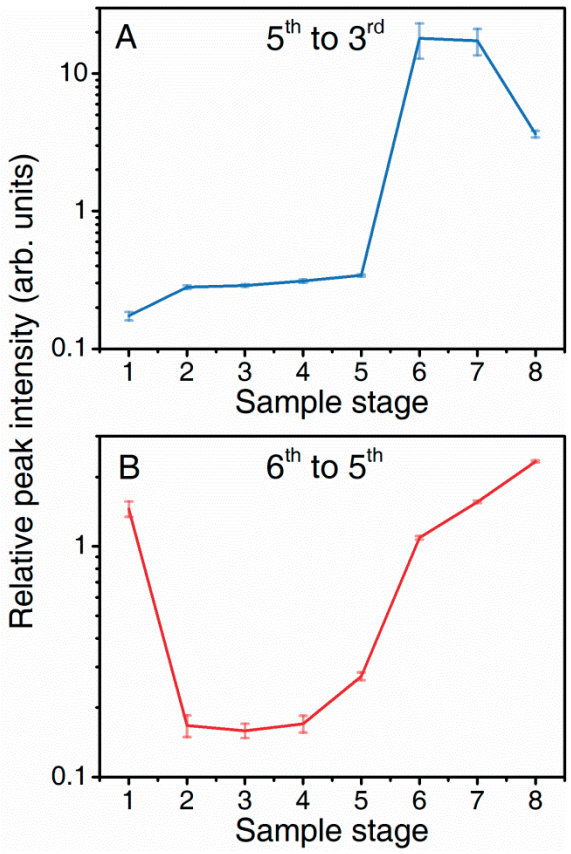


Figure 3. Relative intensities of the fifth to third ($R_{5/3}$) and sixth to fifth ($R_{6/5}$) order peaks at different stages of leather processing using the closed-loop approach: (1) raw; (2) limed; (3) delimed; (4) bated; (5) pickled; (6) Cr treated; (7) basified; (8) wet blue.

during basification (**7**). However, the value decreased at the wet blue (**8**) stage. It was proposed that the intensity of the fifth and third orders is affected by two events: (i) crosslinking of the collagen and (ii) the increase in electron density contrast due to the introduction of Cr^{3+} species.[18] While the former causes a decrease in the third order and increase in the fifth order, the latter leads to an increase in both peaks. Therefore, when adding recycled chromium solution to the skins after basification, the decrease in $R_{5/3}$ is attributable to the further increased uptake of Cr^{3+} species within the collagen matrix. When it comes to changes in $R_{6/5}$ (Figure 3B), a higher $R_{6/5}$ generally indicates a more dehydrated collagen matrix in the skins. As expected, a sharp decrease in $R_{6/5}$ from raw (**1**) to limed (**2**) indicated the opening up of the collagen structure associated with the influx of water into the collagen matrix during alkaline swelling. However, after deliming (**3**) and bating (**4**) the $R_{6/5}$ remained at low levels. This suggests that the removal of non-structural proteins as well as the increased carboxyl side chains during liming can potentially increase the hydrogen bonding propensity of water to the collagen molecules in the matrix. The $R_{6/5}$ then increases during pickling (**5**) using sulfuric acid diluted by recycled chromium solution. This can be explained by the lyotropic effect of high concentrations of Cl^- which weakens the hydrogen bonding interactions between collagen and water.[33] In addition,

due to the introduction of inactive chromium species, water can be substituted from the collagen matrix which would also lead to an increase in $R_{6/5}$.[18,34] A sharp increase was observed following the addition of basic chromium sulphate (**6**) which was followed by more moderate increases through to the wet blue (**8**) stage. This dehydration effect during chrome tanning is attributable to the covalent binding of chromium with collagen, which displaces the hydrogen-bonded water from the molecules.[18] The further improved uptake of chromium during basification and the addition of more recycled chromium solution at higher temperature can also result in dehydration due to the displacement of unbound water by the deposition of chromium species in the collagen matrix.[18,34]

In summary, the structure of collagen in skins predominantly showed the characteristic changes previously documented during conventional tanning,[13] while differences were highlighted. An increased filling effect from the dissolved organic component and the inactive chromium species are suggested by the intermolecular spacing changes, and may result in an improvement in fullness of the resulting leather. Changes in peak intensities also suggested a higher exhaustion during chrome tanning using recycled chrome solutions. Structural changes in keratin and lipid were also detected during the unhairing and liming stage, confirming the effectiveness of the recycled liming solutions following the closed-loop approach.

Conclusions

Recovery and reuse of spent solutions can substantially reduce the environmental burden and considerably improve the economic sustainability of the leather industry. Wet blue leathers processed using a closed-loop approach have been previously shown to produce similar macroscale properties and the advantages of this technology are indicated through the molecular-level structural analyses of samples at different process stages using synchrotron SAXS. Results indicated an increase in chromium uptake and fixation, as well as an increased filling effect. The effectiveness of unhairing and liming using recycled solution is also proven. The fundamental information revealed in this study extends our understanding of the effects of green processing technologies on collagen structure in skins and thereby helps guide the future development of more sustainable manufacturing methods for the leather industry.

Funding

The research was financially supported by the Ministry of Business, Innovation and Employment (MBIE) through LASRA Strategic Science Investment Funding (SSIF) Grant Number LSRX1801. Post-doctoral funding for B.M. was provided by the Ministry of Science and Technology, Taiwan under grant No. MOST 105-2221-E-007-137-MY3. The synchrotron SAXS beam time is funded by the Ministry of Science and Technology Taiwan (MOST).

Acknowledgements

S.P., G.H., Y.Z., and J.B., thank the Ministry of Business, Innovation and Employment (MBIE) for providing funding through grant LSRX-1801. B.M., would like to acknowledge the Ministry of Science and Technology Taiwan (MOST) for postdoctoral research funding. S.P., Y.Z. and B.M. thank NSRRC for merit access to the BL23A SAXS beamline.

References

1. Covington AD. *Tanning chemistry: the science of leather.* Cambridge: Royal Society of Chemistry; 2009.
2. Sreeram K, Ramasami T. Sustaining tanning process through conservation, recovery and better utilization of chromium. *Resources, conservation and recycling.* **38**(3),185-212, 2003.
3. Beauchamp R, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA, Leber P. A critical review of the literature on hydrogen sulfide toxicity. *CRC Critical Reviews in Toxicology.***13**(1), 25-97,1984.
4. Chowdhury M, Mostafa M, Biswas TK, Mandal A, Saha AK. Characterization of the effluents from leather processing industries. *Environmental Processes.* **2**(1),173-187, 2015.
5. Rao JR, Chandrasekaran B, Nair B, Ramasami T. Eco-benign management options for cleaner chrome tanning. *Journal of Scientific and Industrial Research.* **61**,912-926, 2002.
6. GB 30486-2013.Discharge standard of water pollutants for leather and fur making industry. Beijing: China Environmental Science Press; 2013.
7. Daniels R, Su J, Zhang F, Zhang Z. The elimination of effluent from liming, acid/salt pickling and the chromium tanning processes. *Journal of the Society of Leather Technologists and Chemists.***101**,105-111, 2017.
8. Daniels R, Su J, Zhang F, Zhang Z. Closed-loop Liming and Chrome Tanning Systems in Full-scale Wet Blue Manufacture. Operational Management, Technical and Environmental Advantages. *JALCA,* **113**(12), 418-423 2018.
9. Fratzl P. *Collagen: Structure and Mechanics.* New York: Springer Science & Business Media; 2008.
10. Claffey W. Interpretation of the small-angle X-ray diffraction of collagen in view of the primary structure of the alpha1 chain. *Biophysical journal.* **19**(1), 63-70, 1977.
11. Hulmes DJ, Miller A, White SW, Doyle BB. Interpretation of the meridional X-ray diffraction pattern from collagen fibres in terms of the known amino acid sequence. *Journal of molecular biology.***110**(4), 643-666,1977.
12. Sasaki N, Odajima SJJob. Stress-strain curve and Young's modulus of a collagen molecule as determined by the X-ray diffraction technique. **29**(5), 655-658, 1996.
13. Buchanan JK, Zhang Y, Holmes G, Covington AD, Prabakar S. Role of X-ray Scattering Techniques in Understanding the Collagen Structure of Leather. *ChemistrySelect.* **4**(48),14091-14102, 2019.
14. Goh KL, Hiller J, Haston JL, et al. Analysis of collagen fibril diameter distribution in connective tissues using small-angle X-ray scattering. *Biochim Biophys Acta.***1722**(2), 183-188, 2005.

15. Zhang Z, Inventor; (Shangqiu), assignee. Tannery process with effluent recycling. US patent US9776887B22017.
16. Ingham B, Li H, Allen EL, Toney MF. SAXSFit: A program for fitting small-angle X-ray and neutron scattering data. *arXiv.org,09014782,*1-5, 2009.
17. Wojdyr M. Fityk: a general-purpose peak fitting program. *Journal of Applied Crystallography.* **43**(5-1), 1126-1128, 2010.
18. Zhang Y, Mansel BW, Naffa R, et al. Revealing Molecular Level Indicators of Collagen Stability: Minimizing Chrome Usage in Leather Processing. *ACS Sustainable Chemistry & Engineering.* **6**(5),7096-7104, 2018.
19. Zhang Y, Snow T, Smith AJ, Holmes G, Prabakar S. A guide to high-efficiency chromium (III)-collagen cross-linking: Synchrotron SAXS and DSC study. *International journal of biological macromolecules.* **126**,123-129, 2019.
20. Zhang Y, Ingham B, Cheong S, et al. Real-Time Synchrotron Small-Angle X-ray Scattering Studies of Collagen Structure during Leather Processing. *Industrial & Engineering Chemistry Research*. **57**(1), 63-69, 2018.
21. Rao JR, Chandrababu N, Muralidharan C, Nair BU, Rao P, Ramasami T. Recouping the wastewater: a way forward for cleaner leather processing. *Journal of Cleaner Production*. **11**(5), 591-599, 2003.
22. Maxwell CA, Smiechowski K, Zarlok J, Sionkowska A, Wess TJ. X-ray studies of a collagen material for leather production treated with chromium salt. *JALCA*. **101**(1), 9-17, 2006.
23. Maxwell CA, Wess TJ, Kennedy CJ. X-ray diffraction study into the effects of liming on the structure of collagen. *Biomacromolecules.* **7**(8), 2321-2326, 2006.
24. Er Rafik M, Doucet J, Briki F. The Intermediate Filament Architecture as Determined by X-Ray Diffraction Modeling of Hard α-Keratin. *Biophysical Journal*. **86**(6), 3893-3904, 2004.
25. Rafik ME, Briki F, Burghammer M, Doucet J. In vivo formation steps of the hard α-keratin intermediate filament along a hair follicle: Evidence for structural polymorphism. *Journal of structural biology.***154**(1),79-88, 2006.
26. Soomro A, Alsop RJ, Negishi A, et al. Giant axonal neuropathy alters the structure of keratin intermediate filaments in human hair. *Journal of The Royal Society Interface*. **14**(129), 20170123, 2017.
27. Yang F-C, Zhang Y, Rheinstädter MC. The structure of people's hair. *PeerJ*. **2**, e619, 2014.
28. Stanić V, Bettini J, Montoro FE, Stein A, Evans-Lutterodt KJSr. Local structure of human hair spatially resolved by sub-micron X-ray beam. *Sci Rep*, **5**, 17347, 2015.
29. Fraser RDB, MacRae TP, Rogers GE, Filshie BK. Lipids in keratinized tissues. *Journal of Molecular Biology.* **7**(1), 90-IN10, 1963.
30. Wojciechowska E, Rom M, Włochowicz A, Wysocki M, Wesełucha-Birczyńska A. The use of Fourier transform-infrared (FTIR) and Raman spectroscopy (FTR) for the investigation of structural changes in wool fibre keratin after enzymatic treatment. *Journal of Molecular Structure*. **704**(1-3), 315-321, 2004.
31. Petruska JA, Hodge AJ. A subunit model for the tropocollagen macromolecule. *Proceedings of the National Academy of Sciences*. **51**(5):871-876, 1964.
32. Zhang Y, Buchanan JK, Holmes G, Mansel BW, Prabakar S. Collagen structure changes during chrome tanning in propylene carbonate. *Journal of Leather Science and Engineering.* **1**(1), 8, 2019.
33. Zhang Y, Cremer PS. Interactions between macromolecules and ions: the Hofmeister series. *Current Opinion in Chemical Biology.* **10**(6), 658-663, 2006.
34. Wu B, Mu C, Zhang G, Lin W. Effects of Cr3+ on the structure of collagen fiber. *Langmuir.* **25**(19), 11905-11910, 2009.

Mechanism of Collagen Processed with Urea Determined by Thermal Degradation Analysis*

by

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*Presented in part at the 30th International Congress of the IULTCS, Beijing, 2009.

Abstract

During the beamhouse process for nappa leather, pelts are usually limed with amino compounds such as urea, ethylenediamine, and triethanolamine. However, the interaction between amino compounds and collagen is not well known. In this work, collagen fibers were soaked in various concentrations of urea and the thermal degradation of collagen fibers were studied by the methods Horowitz-Metzger and Coats-Redfern. The mechanism of the reaction between urea and collagen fibers is discussed, wherein the thermal degradation activation energy first decreases and then increases. The lowest thermal degradation activation energy of urea processed collagen appears at 2-3 mol/L urea, suggesting that the stability of collagen is the poorest when the pelt is processed in the urea solution. At the urea concentration above 6 mol/L, the thermal degradation activation energy of the sample is similar to samples without urea processing and the higher concentrations does not have the same effect as lower concentrations of urea. The collagen fibers with a urea processing history were washed to remove the urea in them, and the samples were studied again for their thermal degradation behavior. The results indicated that the thermal degradation activation energy of the collagen fibers might recover to the unprocessed level. Therefore, it was suggested that the reaction between urea molecules and collagen fibers is reversible. Urea molecules might help to destroy some of the hydrogen bonds between collagen peptides in the urea solution. After the urea is washed out, the structure of the collagen will return to its original state, because the hydrogen bonds might be reconstructed.

Introduction

As an abundant natural polymer in animal skins and bones, collagen has found wide applications in many fields, such as leather, gelatin, glue, food, health products, medicine and the cosmetic industry because of its favorable triple helix structure and biochemical characters.[1] Leather is made from hides or skins through a complicated process, including soaking, degreasing, liming, reliming, deliming, bating, pickling, tanning, retanning, fatliquoring and finishing, in which liming is one of the most important ones in determining the feature and properties of resultant leathers.[2, 3] In liming, pelts are usually limed with amino compounds such as urea, ethylenediamine, and triethanolamine. However, the interaction between amino compounds and collagen is not well known.[3] Urea is a low-cost chemical and an important protein denaturant, which is frequently used in liming. So, the interaction mechanism of urea on collagen deserves a thorough study.

The influence of urea on shrinkage temperature, isometric tension, swelling behavior, tensile strength, and percentage extension of native rat tail tendon (RTT) were examined by *R Usha*.[5-6] They found that the lyotropic swelling increased the width of the fiber and was associated with the action of urea on the collagen fiber. The melting behavior and the swollen fascicles were clearly seen in scanning electron micrographs of 3 and 6M urea-treated RTT. The reduction in the dimensional stability of native RTT collagen fiber on treatment with urea demonstrated the role of secondary structure in the dimensional stabilization of collagen. In the DSC study of urea process on rat tail tendon (RTT) collagen fiber, the peak temperature and enthalpy changes decreased with increasing concentrations of urea, increasing chain length of alcohol and decreasing pH.[7] As to the effect of guanidinium salts on protein, lyotropic activity increased in the series formamide <urea <guanidinium ion, and in the guanidinium salts in the anion order of fluoride< sulphate< chloride< bromide< nitrate< iodide. Low activities of guanidinium fluoride and sulphate were attributable to counter-effects of the anions, which acted as structural stabilizers.[8] Urea might cause a decrease of venous tissue volume over the whole range of concentration with the exception that, beyond 1.0 osM, an increase appeared.[9] The renaturation rates of calfskin collagen were largely determined by the degree of "undercooling" irrespective of the particular perturbant present, although perturbants with hydrocarbon structure deviated progressively from the linear trend found for formamide and urea. A direct relationship existed between lyotropic activity and perturbant hydrogen bonding to collagen peptide bonds.[10]

From the thermal behaviors of a material, we may indirectly know some structural information of the material. In our reported

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Manuscript received May 24, 2020, accepted for publication June 30, 2020.

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works, the thermal degradation kinetics of collagen fibers tanned by different tanning agents and soaked in sweat were studied, as well as the interaction between collagen fibers and the tanning agents or sweat soaking was discussed from viewpoints of thermal degradation of collagen fibers.[11-15] It was found that chrome tanning might improve the thermal degradation stability obviously, while no significant changes were found for aldehyde tanning.

In this work, after being soaked in various concentrations of urea, the collagen fibers were washed to completely remove the urea. The thermal degradation kinetics of the samples before and after washing were studied by the thermal degradation activation energy of the samples using the methods of Horowitz-Metzger and Coats-Redfern. The action of urea on the aggregation structure of the collagen fibers and the interaction mechanism between urea and collagen fibers were considered.

Experimental

Materials and apparatus

Cattlehide collagen fibers (CCFs) were obtained from Sichuan University, China. Urea was analytically pure and purchased from Luoyang chemical reagent Co. Ltd, China. The thermal analysis system was from Netzsch Company, Germany. The constant temperature water bath oscillator, THZ-82, was made by Fuhua instrument Co. Ltd., Jiangsu, China.

Preparation of urea solutions

Various weights of urea were dissolved in 100 mL distilled water to yield urea solutions with the concentrations of 0.5 mol/L, 1.0 mol/L, 1.5 mol/L, 2.0 mol/L, 3.0 mol/L, 4.5 mol/L, 6.0 mol/L, 7.5 mol/L, and 9.0 mol/L, respectively.

Preparation of different CCFs

Urea-processed CCFs were soaked in various concentrations of urea for 7 days at room temperature. After being filtrated, the cakes were air-dried and put in a desiccator to constant weight, ready for subsequent thermal analysis.

Water-washed CCFs were soaked in 3.0 mol/L and 9.0 mol/L urea solutions for 7 days. The CCFs were then soaked in 20 times the weight of sample of distilled water for an hour, the samples were then filtrated and soaked in fresh distilled water again for a total of 7 cycles to dissolve out the urea. After the final wash, the samples were filtrated and the cakes were air-dried then put in a desiccator to reach constant weight, ready for subsequent thermal analysis.

TG/DTG analysis and data processing

For TG/DTG analysis, the samples were put in aluminium crucibles. Nitrogen was used as the protective gas, and the samples were heated at a heating rate of 10°C/min from room temperature to 650°C. The thermal analysis system gave the TG and DTG curves automatically. The thermal degradation activation energies of all the CCFs of urea processed and water washed were obtained with the help of the Horowitz-Metzger and Coats-Redfern methods.

Results and Discussion

Thermal degradation behaviors of urea processed collagen fibers

Thermal degradation curves

The TG and DTG curves of the CCFs processed by various concentrations of urea solutions are shown in Figure 1. It demonstrated that when the CCFs were heated, two obvious weight losses appeared. The first one appears before 100°C, which is the water molecules adsorbed by the samples. The other weight loss appeared at a

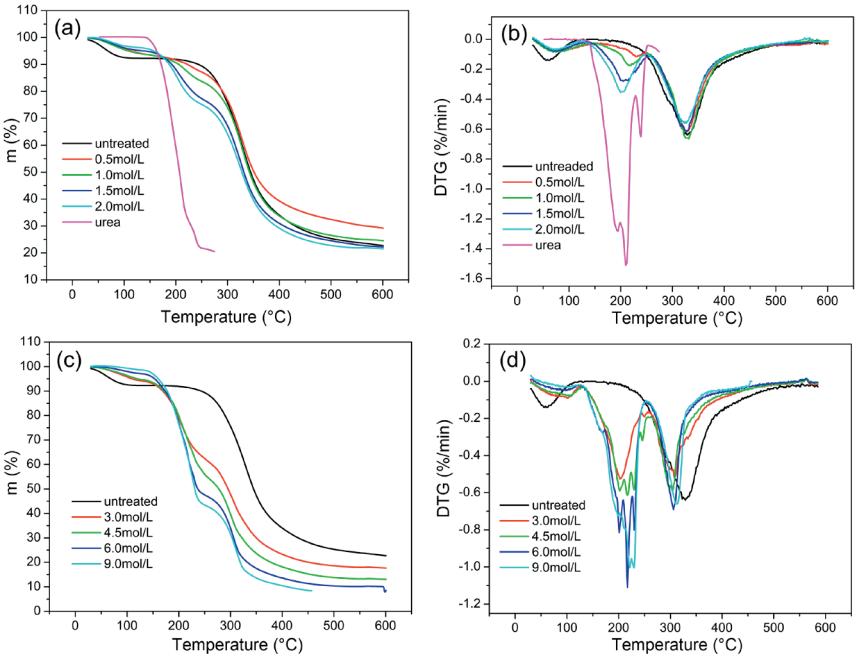

Figure 1. (a, c) TG and **(b, d)** DTG curves of CCFs processed in urea solutions with various concentrations (β=10 K/min)

temperature range from 250°C to 450°C, which is most likely due to the thermal decomposition of the CCFs. The decomposition of pure urea takes place in the temperature range from 130°C to 250°C. After CCFs are soaked in various concentrations of urea, the decomposition peak of urea should appear in the thermogravimetry curves of the samples. With an increase of urea concentration, the weight loss peak continued to grow, which suggested that the urea in the samples contributes to the whole thermal degradation curves. Regarding the weight loss peak of CCFs, it first decreased and then increased.

Thermal degradation activation energy and thermal degradation mechanism

Thermogravimetry (TG), differential scanning calorimetry (DSC) and differential thermal analysis (DTA) are usually used to determine the thermal kinetic parameters of polymers. Among them, thermogravimetry (TG) is the most widely used. Based on the TG data and curves, the thermal degradation activation energy of polymers might be calculated. Isothermal, non-isothermal and high-resolution analysis are the most popular methods to obtain the thermal degradation activation energy of polymers, based on the TG and DTG curves. There are some sub-methods in each one. In the present paper, the non-isothermal method, also known as the dynamic method, was chosen for the study of the thermal degradation behaviors of the samples with the weight loss by linearly heating. Based on the data in the TG curves, the methods of Coats-Redfern and Horowitz-Metzger were used to yield the thermal degradation activation energy (TDAE) of the various CCFs. The meaning of the symbols in this paper are as follows:

E or TDAE—Thermal degradation activation energy, kJ/mol;

A—Pre-exponential factor;

α—Conversion or reactive fracture at time t, %;

R—Universal gas constant, 8.314 J/mol K;

f(α)—Conversion in differential form;

G(α)—Conversion in integral form;

β—Heating rate, 10 K/min;

T_P—Temperature at which reaction rate is the greatest, K;

α_p—Conversion at which reaction rate is the greatest, %;

T_S—Corresponding temperature at $\alpha = \frac{1}{e}$;

T—Reaction temperature, thermodynamics temperature, K;

θ—Difference between T and T_S, $\theta = T - T_S$;

(1) Horowitz-Metzger method[7-8]

According to the theory of Horowitz-Metzger method, we have equation (1) as follows:

$$\ln G(\alpha) = \ln \frac{ART_S^2}{\beta E} - \frac{E}{RT_S} + \frac{E\theta}{RT_S^2} \quad (1)$$

The TDAE (or E here) of the sample might be calculated from the slopes of the plots of $\ln G(\alpha)$ versus θ [16-17].

There are thirty different forms of *G(α)* as shown in Table I.

Table I

Different integral forms of kinetics functions for *G(α)* [15-17]

No.	*G(α)*
1	α^2
2	$\alpha+(1-\alpha)\ln(1-\alpha)$
3	$(1-2/3\alpha)-(1-\alpha)^{2/3}$
4, 5	$[1-(1-\alpha)^{1/3}]^n$ (n = 2,1/2)
6	$[1-(1-\alpha)^{1/2}]^{1/2}$
7	$[(1+\alpha)^{1/3}-1]^2$
8	$[(1/(1+\alpha))^{1/3}-1]^2$
9	$-\ln(1-\alpha)$
10-16	$[-\ln(1-\alpha)]^n$ (n = 2/3, 1/2, 1/3, 4, 1/4, 2, 3)
17-22	$1-(1-\alpha)^n$ (n = 1/2, 3, 2, 4, 1/3, 1/4)
23-27	α^n (n = 1, 3/2, 1/2, 1/3, 1/4)
28	$(1-\alpha)^{-1}$
29	$(1-\alpha)^{-1}-1$
30	$(1-\alpha)^{-1/2}$

The original thermogravimetric data of the samples from the TG curves of the different samples in Figure 1 are shown in Table II.

Various functions in Table I were tried for the TG data in Table II. By fitting and comparing, the function best consistent with the data in Table II was chosen for subsequent study. $\ln G(\alpha)$ was plotted against θ, in which the slopes of the fitted lines were used to calculate the thermal degradation activation energy of various samples.

Table II

TG data for samples processed in different concentrations of urea solutions

Urea Concentration (mol/L)	$T_{\alpha=0.20}$ (K)	$T_{\alpha=0.25}$ (K)	$T_{\alpha=0.30}$ (K)	$T_{\alpha=0.35}$ (K)	$T_{\alpha=0.40}$ (K)	$T_{\alpha=0.45}$ (K)
0	562.6	574.4	584.4	593.2	601.2	609.2
0.5	560.0	577.4	587.5	596.3	604.6	613.4
1.0	551.0	569.9	582.3	590.6	599.5	607.2
1.5	505.2	542.2	565.0	578.2	587.8	596.4
2.0	471.2	481.0	492.0	509.2	537.2	556.2
3.0	492.0	524.2	554.2	570.3	582.2	591.8
4.5	472.7	481.4	490.3	499.7	509.9	528.0
6.0	468.7	475.6	483.0	489.2	494.3	501.9
9.0	468.2	475.2	482.1	488.2	493.8	498.9

In this study, $G(\alpha)=[-\ln(1-\alpha)]^4$ best fits the data, and the correction coefficients are reasonable for all the samples as shown in Table III.

Figure 2 was obtained by plotting $lnG(\alpha)$ against θ. The TDAE of the samples processed in various concentrations of urea was calculated based on the slopes of the lines in Figure 2, with the TDAE values in Figure 4.

(2) Coats-Redfern Method [16-17]

According to the theory of Coats-Redfern method, we have equation (2) as follows.

$$\ln [G(\alpha)/T^2] = \ln\left(\frac{AR}{\beta E}\right) - \frac{E}{RT} \quad (2)$$

If $\ln [G(\alpha)/T^2]$ is plotted against $1/T$ to yield straight lines, the thermal degradation activation energy of the different samples might be calculated from the slopes of the lines.[16-17]

Table III

Correlation coefficients of the linear fitting between $\ln G(\alpha)$ and θ by $G(\alpha)=[-\ln(1-\alpha)]^4$

Urea Concentration (mol/L)	0	0.5	1.0	1.5	2.0	3.0	4.5	6.0	9.0
Correlation Coefficient	0.9999	0.9973	0.9953	0.9782	0.9875	0.9639	0.9751	0.9970	0.9995

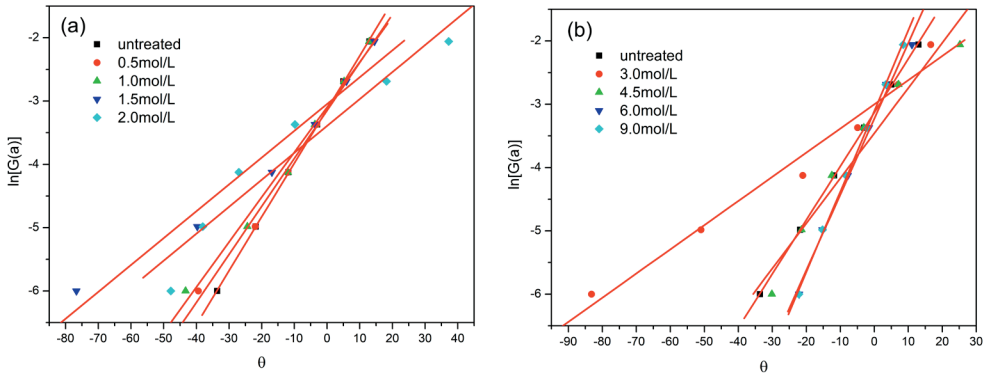

Figure 2. $lnG(\alpha)$ versus θ of samples processed in various concentrations of urea: **(a)** 0.5-2.0 mol/L; **(b)** 3.0-9.0 mol/L.

Table IV

TG data for samples processed in different concentrations of urea solutions

Urea concentration (mol/L)	$1/T_{\alpha=0.20}\times10^3$ (K^{-1})	$1/T_{\alpha=0.25}\times10^3$ (K^{-1})	$1/T_{\alpha=0.30}\times10^3$ (K^{-1})	$1/T_{\alpha=0.35}\times10^3$ (K^{-1})	$1/T_{\alpha=0.40}\times10^3$ (K^{-1})	$1/T_{\alpha=0.45}\times10^3$ (K^{-1})
0	1.777	1.741	1.711	1.686	1.663	1.641
0.5	1.786	1.732	1.702	1.677	1.654	1.630
1.0	1.815	1.755	1.717	1.693	1.668	1.647
1.5	1.979	1.844	1.770	1.729	1.701	1.677
2.0	2.122	2.079	2.032	1.964	1.861	1.798
3.0	2.032	1.907	1.804	1.753	1.717	1.689
4.5	2.115	2.077	2.039	2.001	1.961	1.894
6.0	2.133	2.102	2.070	2.044	2.023	1.992
9.0	2.136	2.104	2.074	2.048	2.025	2.004

The original TG data of different collagen fibers from the TG curves in Figure 1 are shown in Table IV.

Various functions in Table I were tried for the data in Table IV. By fitting and comparing, the function best consistent with the data in Table IV was chosen for the study. It was found that $G(\alpha)=[-\ln(1-\alpha)]^4$ is the most consistent with the data and with reasonable correction coefficients, as shown in Table V.

From Table V, we observed that the function of $G(\alpha)=[-\ln(1-\alpha)]^4$ presents a reasonable linear relation between $\ln[G(\alpha)/T^2]$ and $1/T$, so $G(\alpha)=[-\ln(1-\alpha)]^4$ was chosen for the study.

$\ln[G(\alpha)/T^2]$ was plotted with $1/T$ to yield Figure 3. The thermal degradation activation energy (TDAE) of the samples processed in different concentrations of urea was calculated with the slopes of the lines in Figure 4, with the results shown in Figure 4.

Table V

Correlation coefficients of the linear fitting between $\ln[G(\alpha)/T^2]$ and $1/T$ by $G(\alpha)=[-\ln(1-\alpha)]^4$

Urea Concentration (mol/L)	0	0.5	1.0	1.5	2.0	3.0	4.5	6.0	9.0
Correlation Coefficient	0.9998	0.9950	0.9914	0.9620	0.9677	0.9752	0.9798	0.9981	0.9998

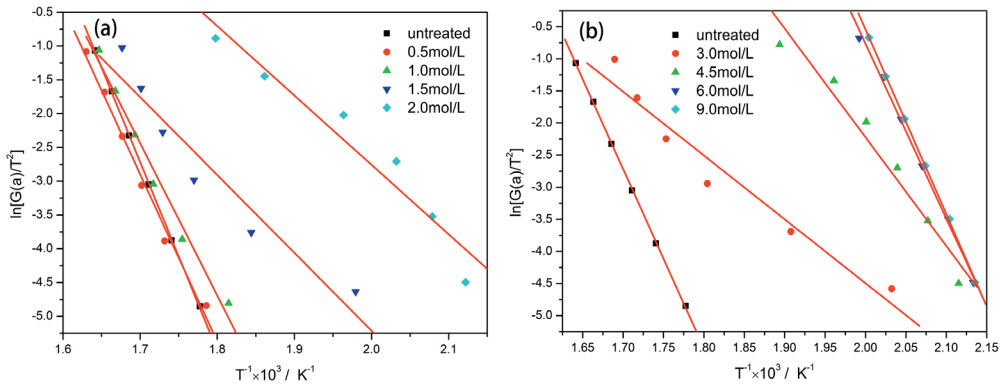

Figure 3. $\ln[G(\alpha)/T^2]$ versus $1/T$ of different samples.

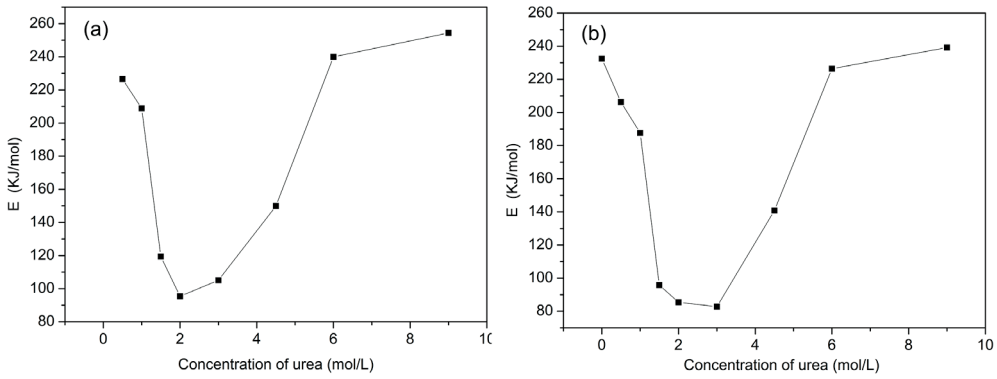

Figure 4. TDAE of different samples versus urea concentrations by the methods of **(a)** Horowitz-Metzger and **(b)** Coats-Redfern

Influence of urea concentration on the TDAE of collagen fibers

The relationship between the thermal degradation activation energies of CCFs processed in different concentrations of urea solutions are shown in Figure 4. In Figure 4, it is apparent that the changing trend in TDAE of CCFs is the same, no matter which method is used. The difference in the TDAE values by different methods is not obvious. With an increase in urea concentration, the TDAE of the CCFs firstly decreases and then increases. At the urea concentration ranged from 2.0 mol/L to 3.0 mol/L, the minimum value of the thermal degradation activation energy for the CCFs appeared. When the CCFs are soaked in a low concentration of urea solution, the hydrogen bonds inter- and intra- collagen peptides might be partly destroyed by the attacking of amino groups in the urea molecules, which will weaken the interaction between collagen peptides. So, the structure stability of the collagen will be decreased, resulting in poor heat resistance and lower thermal degradation activation energies for the urea processed collagen fibers. However, further increase of urea concentration from 3.0 mol/L to 9.0 mol/L, great damage might be caused for the CCFs by destroying the hydrogen bonds between adjacent collagen peptides. Some of the structure of CCFs might even be destroyed. Before the thermal degradation takes place, dry heat shrinkage will appear, causing the adjacent collagen peptides to get closer. In this case, the interaction between adjacent collagen peptide chains will be enhanced because of the mutual reaction between the reaction groups on the collagen chains such as carboxyl, hydroxyl, and amino, resulting in an increase in the thermal degradation activation energy of the CCFs (239.2 kJ/mol at 9.0 mol/L). Therefore, in liming, appropriate urea concentrations (lower than 1.0 mol/L) should be applied in order to get an excellent liming effect. In the reported works, the melting behavior and the swollen fascicles were clearly seen in scanning electron micrographs of 3 M and 6 M urea-treated RTT, and the reduction in the dimensional stability of native RTT collagen fiber on treatment with urea was demonstrated by the role of secondary structure in the dimensional stabilization of collagen.[6] Urea might cause a decrease of venous tissue volume over the whole range of concentration with the exception that, beyond 1.0 osM, an increase appeared.[9] The present work further proved the interaction of urea on the structure of collagen from viewpoint of thermal degradation activation energy.

Influence of water washing on the urea-processed CCFs

TG and DTG curves of urea-processed CCFs before and after water washing

Both TG and DTG curves of CCFs before and after water washing were shown in Figure 5 and Figure 6. Here, the urea concentrations were 3.0 mol/L and 9.0 mol/L, respectively. In Figure 5 and Figure 6, the TG and DTG curves of the CCFs processed with urea solutions are greatly different from those of the untreated ones. After being fully washed with water, the curves changed greatly again, which nearly

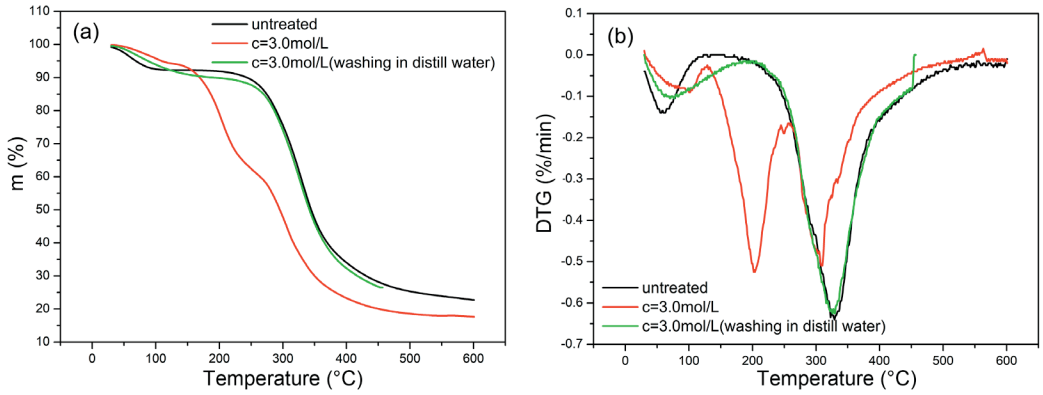

Figure 5. (a) TG and **(b)** DTG curves of urea-processed CCFs before and after water washing (c=3.0mol/L, β=10 K/min)

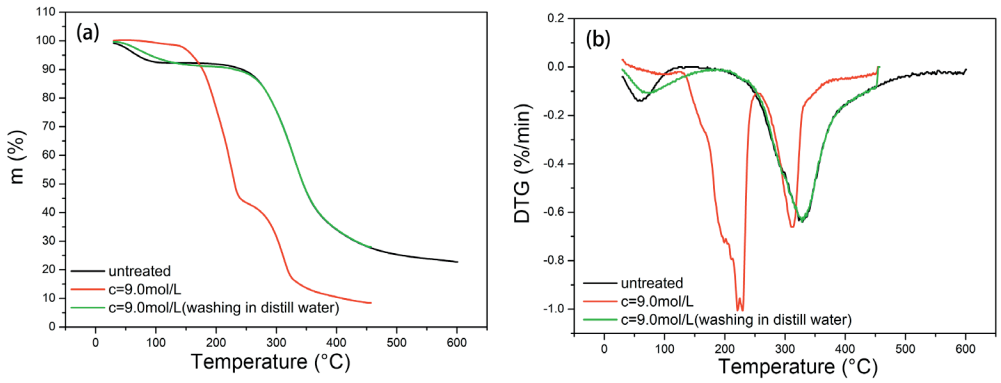

Figure 6. (a) TG and **(b)** DTG curves of urea-processed CCFs before and after water washing (c=9.0mol/L, β=10 K/min)

overlapped those of the untreated ones. Before urea processing, there are two weight losses in the TG and DTG curves of the CCFs. After the samples were processed in urea, a new weight loss profile was found at the temperature ranged from 130°C to 250°C, which should be due to the decomposition of urea in the samples. After being repeatedly washed with distilled water, the urea decomposition peak disappeared, which suggested that the urea in the samples was completely removed. It is interesting to note that the TG curves and DTG curves of the CCFs washed with distilled water nearly overlap those untreated with urea, which suggests the reversibility of the reaction between urea and collagen fibers. So, it could be concluded that no chemical reactions might take place here. In liming, the purpose to use urea is to open the collagen fiber bundles in order to help other chemicals such as protease and calcium hydroxide to be transferred into the hides and react with the collagen fibers. When the collagen fiber bundles are open, the urea is not needed anymore, and should be washed from the hides.

Thermal degradation activation energy and thermal degradation mechanism

(1) Horowitz-Metzger Method

The original thermogravimetric data in the TG curves for the urea-processed samples before and after water washing in Figure 5 and Figure 6 are shown in Table VI.

The functions in Table I were applied to the data in Table VI one by one. By fitting and comparing, the function that is best consistent with the data in Table VI was chosen for subsequent study. Here, $G(\alpha)=[-\ln(1-\alpha)]^4$ best fits the original data, with reasonable correction coefficients for all the CCFs as shown in Table VII. ln$G(\alpha)$ was plotted against θ to yield Figure 7.

Table VI

Thermogravimetric data for urea-processed and washed samples

Urea Concentration (mol/L)	$T_{\alpha=0.20}$ (K)	$T_{\alpha=0.25}$ (K)	$T_{\alpha=0.30}$ (K)	$T_{\alpha=0.35}$ (K)	$T_{\alpha=0.40}$ (K)	$T_{\alpha=0.45}$ (K)
0	562.6	574.4	584.4	593.2	601.2	609.2
3.0*	492.0	524.2	554.2	570.3	582.2	591.8
3.0**	559.3	571.1	581.1	590.0	598.1	606.2
9.0*	468.7	475.6	483.0	489.2	494.3	501.9
9.0**	562.9	574.2	584.1	593.1	601.1	609.2

*Before water washing, **After water washing

Table VII

Correlation coefficients of linear fitting between ln$G(\alpha)$ and θ by $G(\alpha)=[-\ln(1-\alpha)]^4$

Urea Concentration (mol/L)	0	3.0*	3.0**	9.0*	9.0**
Correlation Coefficient	0.9999	0.9639	0.9999	0.9795	0.9999

*Before water washing, **After water washing

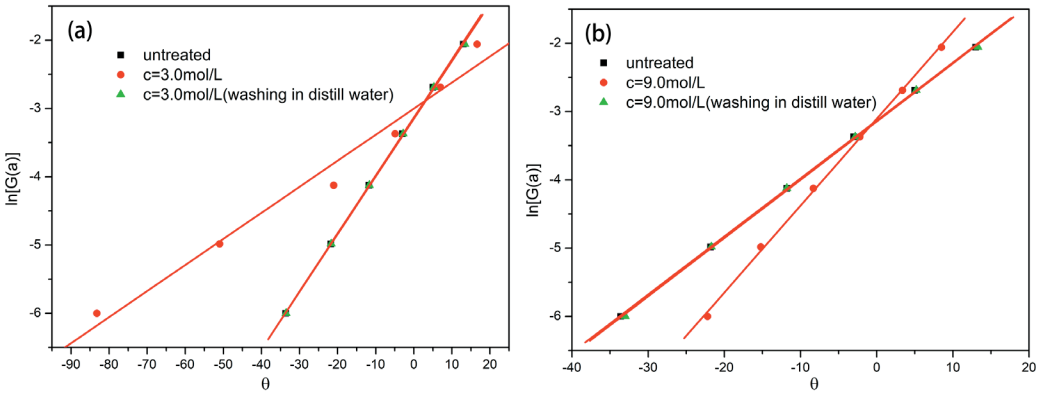

Figure 7. *lnG(a)* versus θ for different samples

Table VIII

TDAE of the various CCFs by Horowitz-Metzger method

Urea concentration (mol/L)	0	3.0*	3.0**	9.0*	9.0**
E(kJ/mol)	251.2	105.1	246.7	254.4	251.9

*Before water washing, **After water washing

With the slopes of the lines in Figure 7, the thermal degradation activation energy (TDAE) of the various CCFs was obtained as shown in Table VIII.

(2) Coats-Redfern method

The original thermogravimetric data in TG curves for the urea-processed samples before and after water washing in Figure 5 and Figure 6 is shown in Table IX.

The various functions in Table I were applied to the data in Table IX. By fitting and comparing, the one best consistent with the data in Table IX was chosen for subsequent study. In this study, the one of $G(\alpha) = [-\ln(1-\alpha)]^4$ was chosen with reasonable correction coefficients for all the CCFs, as shown in Table X. ln$G(\alpha)$ was plotted against θ to yield Figure 8.

Based on the slopes of the fitted straight lines in Figure 8, the thermal degradation activation energy (TDAE) was obtained with the results in Table XI.

As shown in Table VIII and Table XI, it was indicated that, if the CCFs are fully washed in distilled water after soaked in urea solutions, the thermal degradation activation energy of CCFs will return to the original level of CCFs without a urea processing history, no matter what urea concentration was used to process the samples. Soaking in 3.0 mol/L urea solution remarkably decreases the TDAE of CCFs, which might return to the original level with no

Table IX

Thermogravimetric data for urea-processed and washed samples

Urea Concentration (mol/L)	$1/T_{\alpha=0.20}\times10^3$ (K^{-1})	$1/T_{\alpha=0.25}\times10^3$ (K^{-1})	$1/T_{\alpha=0.30}\times10^3$ (K^{-1})	$1/T_{\alpha=0.35}\times10^3$ (K^{-1})	$1/T_{\alpha=0.40}\times10^3$ (K^{-1})	$1/T_{\alpha=0.45}\times10^3$ (K^{-1})
0	1.777	1.741	1.711	1.686	1.663	1.641
3.0*	2.032	1.907	1.804	1.753	1.717	1.689
3.0**	1.788	1.751	1.721	1.695	1.672	1.649
9.0*	2.136	2.104	2.074	2.048	2.025	2.004
9.0**	1.776	1.741	1.712	1.686	1.663	1.641

*Before water washing, **After water washing

Table X

Correlation coefficients of the linear fitting between $\ln[G(\alpha)/T^2]$ and 1/T by $G(\alpha) = [-\ln(1-\alpha)]^4$

Urea Concentration (mol/L)	0	3.0*	3.0**	9.0*	9.0**
Correlation Coefficient	0.9999	0.9639	0.9999	0.9995	0.9999

*Before water washing, **After water washing

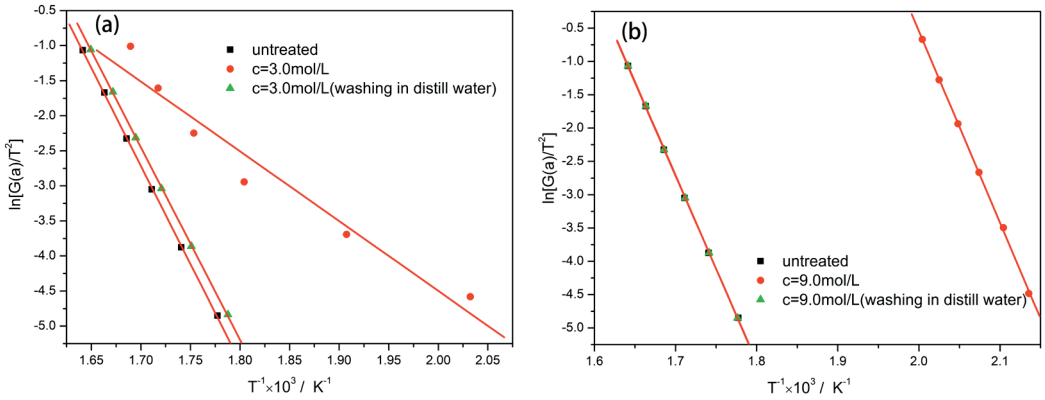

Figure 8. $\ln[G(a)/T^2]$ versus 1/T for various CCFs.

urea process if sufficient washing is conducted to completely remove the urea. From the viewpoint of thermal degradation activation energy, the reaction between urea and CCFs should be reversible, rather physical than chemical. Once the urea in the collagen fibers is completely removed by water washing, no indication of the urea process in the thermal degradation activation energy is detected. Therefore, the action of urea on CCFs should be a physical one, by forming new hydrogen bonds between urea molecules and collagen molecules instead of the original hydrogen bonds between adjacent collagen molecules. Therefore, the opening up of the collagen fiber bundles by urea process results in decreasing the thermal stability of the collagen fibers, with no chemical reaction.

Conclusions

Soaking in low concentrations of urea solution does not significantly change the thermal degradation activation energy of collagen fibers. Increasing the concentration of the urea solution, the thermal degradation activation energy will first decrease and then increase. When the urea concentration is in the range from 2 mol/L to 3 mol/L, the lowest thermal degradation activation energy for the collagen fibers appears. Further increasing the urea concentration to above 6.0 mol/L, however, urea soaking does not affect the thermal degradation activation energy, which is similar to the non-urea processed collagen fibers. The reaction between urea molecules and collagen fibers is temporary and reversible, from the viewpoint of the thermal degradation activation energy. When the urea in the collagen fibers is completely removed, the thermal degradation activation energy might return to the values without urea treatment history. The reaction of the urea process on CCFs should be a physical one, by forming new hydrogen bonds between urea molecules and collagen molecules instead of the original hydrogen bonds between adjacent collagen molecules.

Table XI

TDAE of various CCFs by Coats-Redfern method

Concentration of Urea (mol/L)	0	3.0*	3.0**	9.0*	9.0**
E(kJ/mol)	232.5	82.8	228.4	239.2	233.5

*Before water washing, **After water washing

Acknowledgments

The financial supports of this work from the National Natural Science Foundation Commission of China (No. 51673177, 51373097) and National Key R & D Program of China (No.2017YFB0308500) are greatly appreciated.

References

1. Dai, H., Zhang, X.S., Zhang, Z.C., Tian, Z. and Zhang, H.; The separation of hydrolysate of enzyme-soluble pigskin by chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Journal of the Society of Leather Technologists and Chemists* **87**(3), 110–112, 2003.
2. Tang, K. Y. Collagen Physics and Chemistry, Beijing, Science Press, 2012.
3. Liu, H., Yin, Z. H., Zhang, Q., Li, X. M., Tang, K. Y., Liu, J., Pei, Y., Zheng, X. J. and Ferah, C. E.; Mathematical modeling of bovine hides swelling behavior by response surface methodology for minimization of sulfide pollution in leather manufacture, *Journal of Cleaner Production* **237**, 1–9, 2019.
4. Huang, J. B., Liu, J., Tang, K. Y., Yang, P. Y., Fan, X. L., Wang, F., Du, J. and Liu, C-K.; Effect of cyclic stress while being dried on the mechanical properties and thermostability of leathers. *JALCA* **113**(10), 318–325, 2018.
5. Abernethy, J. L.; Lyotropy-With particular application to collagen. *Journal of Chemical Education* **44**(6), 364-370, 1967.
6. Usha, R. and Ramasami, T.; Effect of hydrogen-bond-breaking reagent (urea) on the dimensional stability of rat tail tendon (RTT) collagen fiber. *Journal of Applied Polymer Science* **84**, 975–982, 2002.
7. Usha, R. and Ramasami, T.; Influence of hydrogen bond, hydrophobic and electrovalent salt linkages on the transition temperature, enthalpy and activation energy in rat tail tendon (RTT) collagen fibre. *Thermochimica Acta* **338**, 17–25, 1999.
8. Russell, A. E. and Cooper, D. R.; Effect of compounds of the urea–guanidinium class on renaturation and thermal stability of acid-soluble collagen. *Biochemical Journal* **127**, 855–863, 1972.
9. Winkler, R.; On the existence of a lyotropic and osmotic type of collagen swelling produced by representative electrolytes and nonelectrolytes. *Pflugers Archiv-European Journal of Physiology* **345**, 37–42, 1973.
10. Russell, A. E. and Cooper, D. R.; Structural and functional factors in the lyotropic activity of amides and alkyl-substituted amides on acid-soluble collagen, *Biochemistry* **9**(14), 2802–2806, 1970.
11. Tang, K. Y., Wang, F., Liu, J. and Wang, Q. J.; Preliminary studies on the thermal degradation kinetics of cattlehide collagen fibers. *JALCA* **99**(10), 401–408, 2004.
12. Yang, P. Y., He, X. C., Zhang, W. J., Qiao, Y. X., Wang, F. and Tang, K. Y.; Study on thermal degradation of cattlehide collagen fibers by simultaneous TG-MS-FTIR. *Journal of Thermal Analysis and Calorimetry* **127**(3), 2005–2012, 2017.
13. Tang, K. Y., Wang, F., Jia, P., Liu, J. and Wang, Q.; Thermal degradation kinetics of sweat soaked cattlehide collagen fibers. *JALCA* **102**(2), 52–61, 2007.
14. Liu, J., Brown, E. M., Liu, C-K. and Tang, K. Y.; Characterization and thermal properties of polygenipin-crosslinked hide powders. *JALCA* **113**(4): 96–104, 2018.
15. Liu, J., Brown, E. M., Uknalis, J., Liu, C-K., Luo, L. and Tang K. Y.; Thermal stability and degradation kinetics of vegetable-tanned collagen fiber with in-situ precipitated calcium carbonate. *JALCA* **113**(11): 358–370, 2018.
16. Hu, R. and Shi, Q.;, Thermal analysis dynamics. Science Press, Beijing, China, 2001.
17. Chen, J. and Li, C.; Thermal analysis and its application. Science Press, Beijing, China, 1985.

Lifelines

Anatolii Danylkovych (orcid.org/0000-0002-5707-0419) is a professor at Kyiv National University of Technologies and Design (Department of Biotechnology, Leather, and Fur) where he has been working since 1980. He published more than 470 scientific works, among them 9 monographs, 4 textbooks, and 11 tutorials, and has 72 Ukrainian patents. His current scientific interests include physicochemical fundamentals and modeling of the technological processes of leather and fur production. The main areas of scientific research are focused on the optimization and development of new resource-saving ecologically-oriented technologies for leather and fur production and mathematical modeling of technological processes.

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Didem Berber graduated from Marmara University Atatürk Faculty of Education, Biology Department in 2001. She received MSc. degree from Pediatric Allergy-Immunology Department of Marmara University Medical Faculty in 2003 and PhD degree from Department of Biology, Faculty of Arts and Sciences of Marmara University in 2010. She has been studying as post doctorate researcher in Marmara University, Department of Biology, Faculty of Arts and Sciences from 2016 up to date. She contributed in international projects (COST and other bilateral collaboration projects) on bacterial quorum sensing and biofilm inhibition. Her research topics are hide microbiology, environmental microbiology, antimicrobial agents, fungi, quorum sensing, and biofilm formation.

İpek Türkmenoğlu graduated from Marmara University Atatürk Faculty of Education, Biology Department in 2012. She is continuing to the master program and she is studying as scholarship researcher with the support of TUBITAK on determination and utilization of species-specific allosteric inhibition zones in glycolytic enzymes in pharmaceutical design. Her research topics are hide microbiology, environmental microbiology, antimicrobial agents, quorum sensing, and biofilm formation.

Nüzhet Cenk Sesal graduated from Marmara University, Atatürk Faculty of Education, Biology Department. He has been working at Marmara University, Faculty of Arts and Sciences, Department of Biology since 2001. His research area is molecular microbiology. He has been working as a principal investigator, researcher and consultant in national and international projects, especially about molecular diversity, environmental microbiology, antimicrobial agents, quorum sensing, and biofilm formation.

Yi Zhang received his B.S. in chemistry from Fudan University in 2014 and his B.S.(Hons) from the University of Auckland in 2015. Since 2016, he has worked in Dr Sujay Prabakar's group at the Leather and Shoe Research Association of New Zealand, studying collagen structure using synchrotron SAXS.

Bradley W. Mansel is a Postdoctoral researcher at National Tsing Hua University in Taiwan. He received his PhD from Massey University in New Zealand in 2016. He is currently focused on researching the physical properties of biopolymers.

Jenna K. Buchanan has been working in Dr Sujay Prabakar's research group at the Leather and Shoe Research Association of New Zealand in Palmerston North since 2018. Her research is focused on developing novel crosslinking agents to replace or minimize chrome in leather processing. She received her BSc in chemistry and statistics in 2015 and her MSc in chemistry in 2017 from Massey University in Palmerston North.

Jiasheng Su see JALCA, 113 (12), 424, 2018

Zhuangdou Zhang see JALCA, 113 (12), 424, 2018

Geoff Holmes see JALCA, 110 (11), 379, 2015

Sujay Prabakar see JALCA, 111 (5), 200, 2016

Keyong Tang received his PhD degree in 1998 from Sichuan University, Chengdu, China. He is working for Zhengzhou University as a professor in the College of Materials Science and Engineering, Zhengzhou, China. His research interests include the leather structure and properties. He has published more than 100 papers, co-authored 4 books and edited 1 book in the field of leather chemistry and engineering.

Weilin Li received his Master's degree in 2005 from Henan University of Technology, China. He is now a PhD candidate in material physics and chemistry from Zhengzhou University, China. His current research interests include support design for enzyme immobilization, transesterification with immobilized lipase for bio-diesel, collagen-based hydrogels with drug release applications, development of natural fibers from both plants and animals.

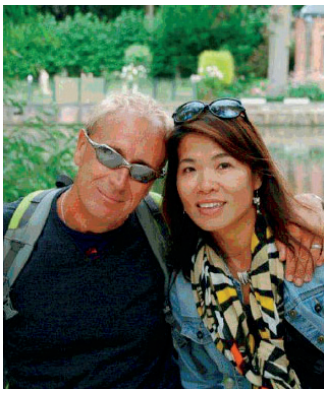
Jie Liu received his PhD degree in 2007 from Institute of Mechanics, Chinese Academy of Sciences, Beijing, China. He currently is an associate professor of School of Materials Science and Engineering at Zhengzhou University, Zhengzhou, China. From 2016 to 2017, he worked as a visiting scientist at ERRC, USDA in Cheng-Kung Liu's group. His current research interests focus on green composite materials based on natural polymers and their applications in packaging, leather making, biomedical and environmental fields.

Cheng-Kung Liu, Ph.D. see *JALCA* 94, 158, 1999

Hongbo Pan received his Master degree from Zhengzhou University, Zhengzhou, China, in Leather Chemistry and Engineering in 2010. His research interests mainly focus on effect of force on leather property and has contributed 5 publications in this field.

Obituaries

Rodney Hammond was born June 14, 1956. He passed away in the UK on August 13, 2020 at the age of 64. Services were held September 9 in Grantham, UK. His beloved wife Ivy Yuan preceded him in death on May 22, 2020. He is survived by his daughter Victoria Hammond and his son Benjamin Hammond, grandchildren Ché Holton, London Hammond, and Melodee Hammond, his sisters Margaret and Daphne and his brother Danny and his nephew James Cady.



Rodney's favorite photo with his wife.

Mr. Rodney Hammond was a 1977 Graduate of National College London, where he studied Chemistry & Leather technology. In 1977 he joined the Barrow Hepburn Leather Company in the United Kingdom and worked in a number of technical positions within that group of companies including Bjorlow Leather, Hodson Tanners & Walker Leather Factories. In 1980 Mr. Hammond emigrated to the USA and joined Granite State Leather in Nashua, New Hampshire as Technical Superintendent. In 1984 Mr. Hammond joined Bayer Corporation and subsequently held the positions of Technical Sales Representative, Product Manager leather chemicals, Regional Business Manager Leather & Textile coatings for North, Central & South America. In 1996 Mr. Hammond joined Seton Company and subsequently held the positions of Plant Manager Seton Newark, Managing Director Seton Europe, President Seton Argentina and in 1999 he was appointed VP Manufacturing-Americas. In September 2004 he moved to Singapore to be Managing Director for the Asia Pacific Region of Stahl until March 2007. In April 2007 he returned to the United States to work as VP and General Manager for Asia and the Americas for Seton Company until 2010. He then moved to Seoul, Korea to be the Chief Marketing Officer and Senior Managing Director for Chokwang Leather Co. until June of 2012. In February of 2013 he moved to Guangdong, China to be Chief Operating Officer for Impactiva S. de R.L. until July of 2015. For over ten years he was the owner of Harmony Base Enterprises Limited in the British Virgin Islands.

Rodney published several papers in various technical journals and made numerous presentations over the years. He was a past board member of the New England Tanners Club.

Rodney joined the ALCA 1986 and served as VP Elect from June 202 to June 2003, Vice President from June of 2003 to 2004, and then as President from June 2004 to October 2004 when he moved out of the country and felt he needed to resign as President. He also served the ALCA as the chair of the 2002 Fred O'Flaherty Service Award Committee and chair of the 2005 Lollar Prize Paper Award Committee.

Thomas William Krisko, 82, of Exeter, formerly of Hampton and Peabody, Mass. died, Sunday, Aug. 9, 2020 at Massachusetts General Hospital in Boston after a period of failing health.

He was born in Peabody, Mass. on Nov. 24, 1937. Tom graduated from Peabody High School with the Class of 1955 where he excelled in football as a quarterback. He was also a graduate of the former Bentley College of Accounting.

Tom spent most of his adult life working in the leather industry. He worked for Pearse Leather for 27 years. When Pearse relocated to Hampton in 1965, Tom became the general manager, a post he held until 1981. He then worked at various leather plants in Manchester, N.H., Saxton, Penn., Omaha, Neb., and finally, Suncook Leathers Inc. from which he retired in 2004. His next career was as a Professional Driver for eight years. Tom was proud of his expertise in leather and he enjoyed all of the relationships he formed while working in the industry. As a driver, he enjoyed meeting new people and also getting to drive all over the USA.

An avid golfer, Tom was a former member of Abenaqui Country Club in Rye where he served as vice president in the 1970s. He enjoyed watching all types of sports. Most of all, he was a very social person and loved spending time with his family, friends and his beloved wife, Lorraine R. (FitzGerald) Krisko of Exeter, with whom he shared 60 years of marriage.

In addition to his wife he leaves five children, Cheryl Robison and husband Jim of Austin, Texas; Diane Kamieneski and husband Gary of Hampton; Barbara Gagne and husband Michael of Hampton; Thomas Krisko of Nebraska City, Neb.; Linda Aurandt and husband Michael of Jacksonville, Fla.; eight grandchildren, three great grandchildren, his two brothers, Peter and Henry Krisko of Peabody, Mass. and many nieces and nephews.

Private services will be held at the convenience of the family. In lieu of traditional remembrances, donations may be made to AstraZeneca Hope Lodge Boston, 125 Huntington Avenue, Jamaica Plain, MA 02130.

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1. Publication Title	2. Publication Number	3. Filing Date
The Journal of The American Leather Chemists Association	0 1 9 _ 3 3 4	09-10-2020
4. Issue Frequency	**5. Number of Issues Published Annually**	**6. Annual Subscription Price**
monthly	12	$185

7. Complete Mailing Address of Known Office of Publication *(Not printer) (Street, city, county, state, and ZIP+4®)*
1314 50th Street, Suite 103
Lubbock, Lubbock County, Texas 79412-2940

Contact Person: Carol Adcock
Telephone *(Include area code)*: 806-744-1798

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same as Item 7

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