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

by

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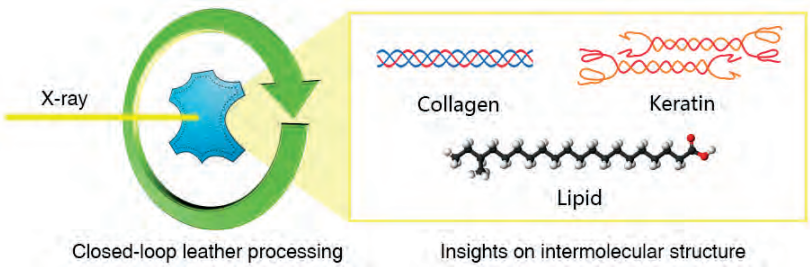
Abstract

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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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.

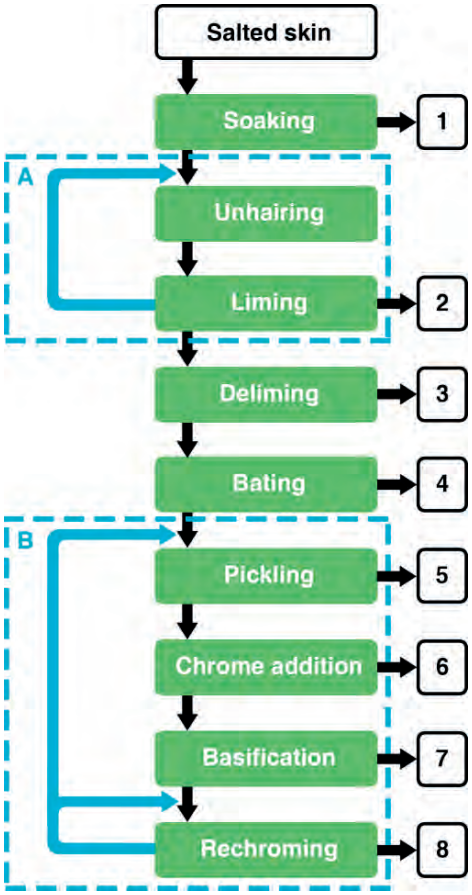
Experimental Methods

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] peak fitting method as described in the supporting information. 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]

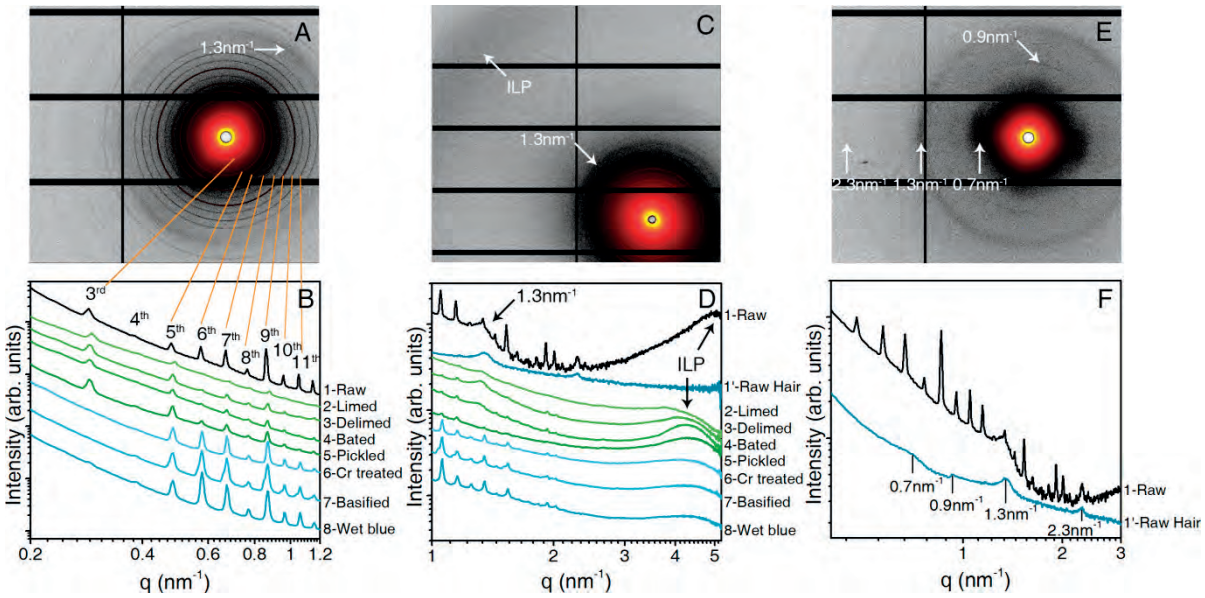


Figure 1. (A) SAXS pattern of a raw calf skin showing characteristic diffraction rings originating from the long-range ordered collagen structure. (B) Integrated data in the q region of 0.2–1.2 nm^{-1} from skins at different stages of processing using recycled solution. Selected peaks are labelled corresponding 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

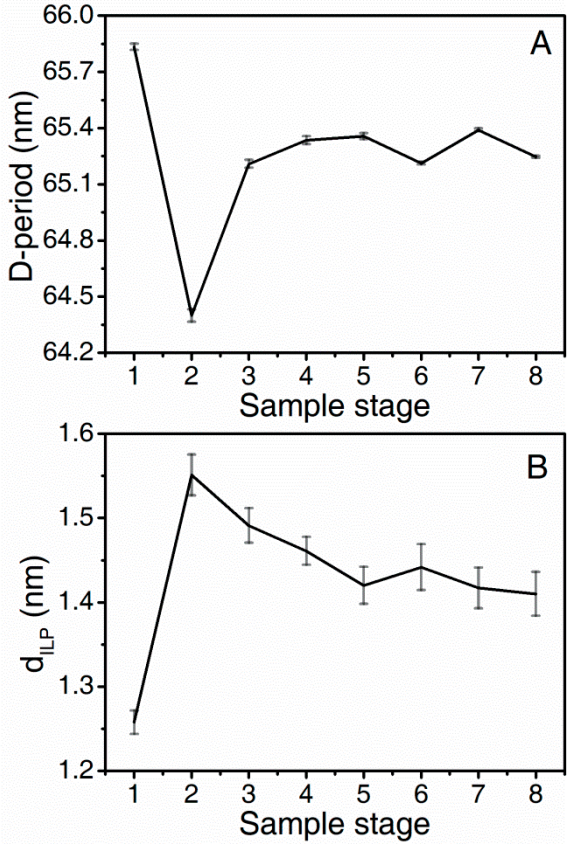


Figure 2. Calculated collagen (A) D-period and (B) intermolecular lateral packing distance (d_{ILP}) in calf skins from SAXS analysis at different stages of leather processing using the closed-loop approach: (1) raw; (2) limed; (3) delimed; (4) bated; (5) pickled; (6) Cr treated; (7) basified; (8) wet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

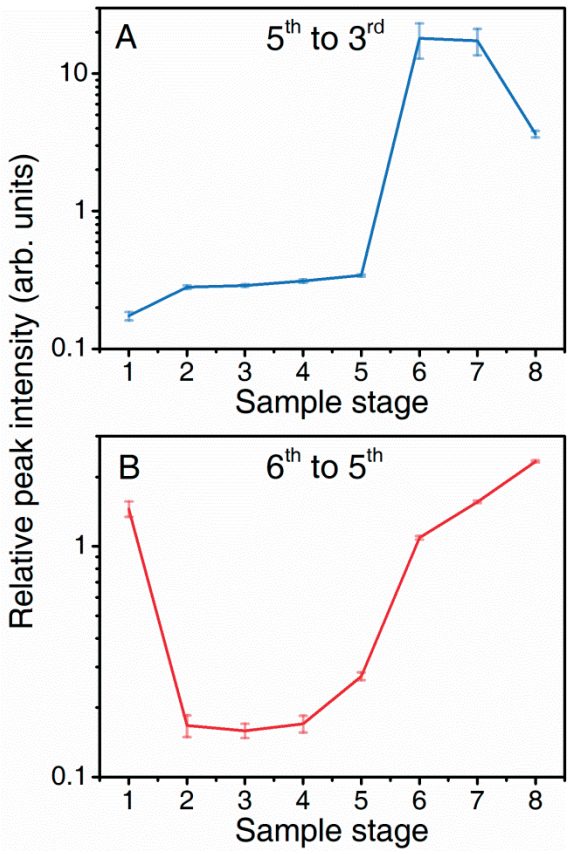


Figure 3. Relative intensities of the fifth to third ($R_{5/3}$) and sixth to fifth ($R_{6/5}$) order peaks at different stages of leather processing using the closed-loop approach: (1) raw; (2) limed; (3) delimed; (4) bated; (5) pickled; (6) Cr treated; (7) basified; (8) wet blue.

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.

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