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Cross-linking of Gelatin with Epichlorohydrin in a Sodium-Acetate-Trihydrate/Urea Deep Eutectic Solvent System

by

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Abstract

To improve the cross-linking efficiency of gelatin molecules, sodium acetate trihydrate/urea deep eutectic solvent with 30% water (SAT/U-DES$_{30W}$, based on DES weight) was proposed as the solvent of gelatin in this paper. The system could overcome the gelation effect of gelatin at low temperature and expose more active groups of gelatin molecules, resulting in better cross-linking effect. Firstly, the optimal reaction concentration of epichlorohydrin (ECH) was determined by boiling water solubility of cross-linking products, and the cross-linking efficiency of gelatin under two systems was judged together with the Glass transition temperature method. Secondly, the water resistance of the cross-linked product was investigated by swelling capacity and water contact angle. The water absorption rate of GE$_W$ and GE$_{DES}$ decreased from 4614% to 1500% and 929% at 12 h, respectively, and the water contact angle also obviously decreased, indicating that the cross-linking efficiency of gelatin under DES system was higher. Finally, the cross-linking mechanism of gelatin and ECH were discussed and it was found the thermal stability of GE$_{DES}$ was significantly improved, which would provide a theoretical basis for gelatin-based materials.

Introduction

In the leather industry, a large amount of leather wastes is generated during tanning. Initially, leather wastes were disposed of by discarding in a landfill. This way not only caused a waste of resources, but also seriously polluted the environment. In this context, the researchers have transformed the waste into resources by hydrolyzing the leather shavings to obtain gelatin.[1] The molecular weight of gelatin acquired by the method was low but exhibited a wide distribution and its aqueous solution shows high viscosity. Gelatin is widely used in food, pharmaceuticals and cosmetics industry due to its good film-forming properties, emulsifying properties and biocompatibility. There are a large number of hydrophilic groups in gelatin molecules, the molecules will absorb water and swell rapidly when dissolved in water. And the elevated temperature will break the helix structure of molecular chains to promote its dissolution. However, the strong hydrophilicity and instability of gelatin limit its further application. Cross-linking reaction is generally regarded as an effective method to overcome these problems, because cross-linking reactions will form a dense structure among gelatin chains to enhance the water resistance and stability. But gelatin molecules exhibit an aggregated state in aqueous solution,[2] which forms a large number of hydrogen bonds and van der Waals forces between chains, resulting in the difficulty exposing active groups and hydrophobic bonds inside the molecules and low modification efficiency in water phase. In addition, there is always a certain amount of water in the bulk gelatin, named structural water, which contribute to the helix structure of gelatin. However, the presence of such water complicates the relationship between the macromolecular structure and its solution properties.[3] Cooling of a gelatin solution induces partial conformational change of gelatin chains into helix and then the sol-gel transition occurs, leading to the difficulty of the cross-linking reaction in gelatin molecules at room temperature.[4] Although gelatin can be dissolved in some organic solvents, the application of these solvent systems is still limited due to toxicity and instability. At present, scholars have begun to turn their attention to ionic liquids and deep eutectic solvents (DES) to solve these problems of gelatin molecules in aqueous solution. For instance, Horinaka et al have investigated the rheology behavior of gelatin/ 1-ethyl-3-methylimidazolium dimethyl phosphate.[5] Grønlien et al have explored the behavior of collagen in a natural DES solution.[6] However, most of these studies only focus on the interaction mechanism between gelatin and solvents, and few studies concentrate on the modification effect of gelatin in the systems.

In previous work of this laboratory, sodium acetate trihydrate (SAT) and urea were heated in a molar ratio of 1:2 to obtain SAT/U-DES. The changes of physicochemical properties and microstructure of "water-in-DES" system were investigated. Then SAT/U-DES was used to dissolve gelatin and it was found SAT/U-DES with 30% water (SAT/U-DES$_{30W}$), was the optimal dissolution system for gelatin. SAT/U-DES$_{30W}$ system could effectively overcome the gel effect of gelatin solution and did not destroy the molecular structure of gelatin.[7] Therefore, this work intends to carry out subsequent cross-linking reaction under the system.

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At present, modification methods for gelatin are mainly either physical or chemical with the later proving to be more effective. The common cross-linking agents include aldehydes, epoxides, carbodiimides, and natural molecules. Therein, the polarization of the epoxy charge and the epoxy ring tension of epichlorohydrin (ECH) make it highly reactive. Thus, ECH can be used to react with the reactive groups in gelatin molecule via its epoxy group to form new chemical bonds and a denser network, which can make the peptide structure of gelatin more stable and also improve the properties of gelatin-based products.

The objectives of this work were to cross-link gelatin with ECH in SAT/U-DES_{30W}, and to compare the cross-linking effect with that in water phase. It was expected that the SAT/U-DES_{30W} system can significantly improve the cross-linking efficiency of gelatin, the cross-linking products obtained showed higher water resistance and stability than those cross-linked under water phase, which could provide a certain theoretical basis for the diversified application of gelatin.

Material and Methods

Materials

Sodium acetate trihydrate (SAT), urea and epichlorohydrin (ECH) were purchased from Chengdu Jinshan Chemical Reagent Co. Ltd and they are all analytical purity. Gelatin (Industrial Grade, M_w = 20~60 kDa) was acquired from Key Laboratory of Leather Chemistry and Engineering, Sichuan University.

Cross-linking reactions under SAT/U-DES_{30W} and water system

On the basis of the dissolution system for gelatin previously explored in the laboratory, the SAT/U-DES with 30% water content was used to dissolve gelatin. Exactly 40.8 g of SAT, 36.0 g of urea and 30% w/w water content (23.04 g, based on SAT/U-DES weight) were mixed in a round-bottomed flask and heated at 60°C, with constant mechanical stirring for 1 h until forming a transparent homogeneous liquid. Then 20% w/w of gelatin was introduced into the system under continuous stirring until dissolved completely. Finally, ECH with different weight fractions (based on gelatin weight) was added to the system and cross-linked with gelatin molecules for 2 h under SAT/U-DES_{30W} system, and the sample was named GE_{DES}. What's more, the sample under water system was prepared under the same reaction conditions, and it was labeled as GE_W.

GE_{DES} was transferred to the 300 Da dialysis tubing for 24 h dialysis and dried in a polytetrafluoroethylene mold at 45°C to obtain solid GE_{DES} sample. The solid gelatin and GE_W samples were also prepared in the same way for subsequent performance tests.

Boiling water solubility

The solid GE_{DES} and GE_W samples were completely dried and placed in a boiling water bath at 100°C for 1 h, then taken out and dried to constant weight. The weight of the samples before and after boiling were determined, and the solubility was calculated to determine the cross-linking degree of gelatin.

Glass transition temperature

The solid gelatin, GE_W and GE_{DES} were dried completely and 5-10 mg of samples were taken under nitrogen atmosphere to test the glass transition temperature at a heating rate of 10°C/min and a scanning range of 30~150°C.

Swelling capacity and water contact angle

The swelling capacity of solid gelatin, GE_W and GE_{DES} were determined using the gravimetric method. The samples after drying were immersed in distilled water at room temperature for 12 h, they were weighed after blotting excess surface water with filter papers every two hours. The water absorption rate (%) of the samples was calculated as follows:

$$\text{Water absorption rate (\%)} = \frac{W_2 - W_1}{W_1} \times 100\% \quad (1)$$

where W_2 is the weight of the swollen sample and W_1 is the weight of the dried sample.

The water contact angle (WCA) of gelatin film, GE_W film and GE_{DES} film obtained were carried out by the sessile drop method using an optical tensiometer, equipped with the image analysis OneAttension software. A droplet of Mili-Q water (5 μL) was deposited on the film surface (2 cm^2, 25°C) with a precision syringe. The droplet image was recorded by a video camera (after 0s, 30s, 60s), and the droplet profile was numerically solved and fitted to Young-Laplace equation by the OneAttension software. The test was repeated three times for each sample and the results were averaged.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR was conducted using a Nicolet iS10 FTIR spectrometer (PerkinElmer, USA). The gelatin and solid GE_{DES} were oven dried and milled into powder. Then the dried samples were blended with potassium bromide powder and pressed into tablets before spectrum acquisition. The pellet was examined using a Spectrum One FTIR spectrometer. The scan was conducted between 4000 and 500 cm^{-1} with a resolution of 2 cm^{-1} and the background spectrum was collected before each scan.

X-ray diffraction (XRD)

The gelatin and GE_{DES} film were dried and made into 30 mm × 30 mm film, placed in an X-ray diffractometer to test the crystal structure. The voltage was 40 kV, the current was 40 mA, the diffraction Angle was 5°-50°, and the scanning rate was 2°/min.

Scanning electronical microgram (SEM)

The surfaces and cross-sections of gelatin film and GE_{DES} film were observed under field emission scanning electron microscope. The samples were treated with gold spraying in vacuum before observation and the test voltage was 5 kV.

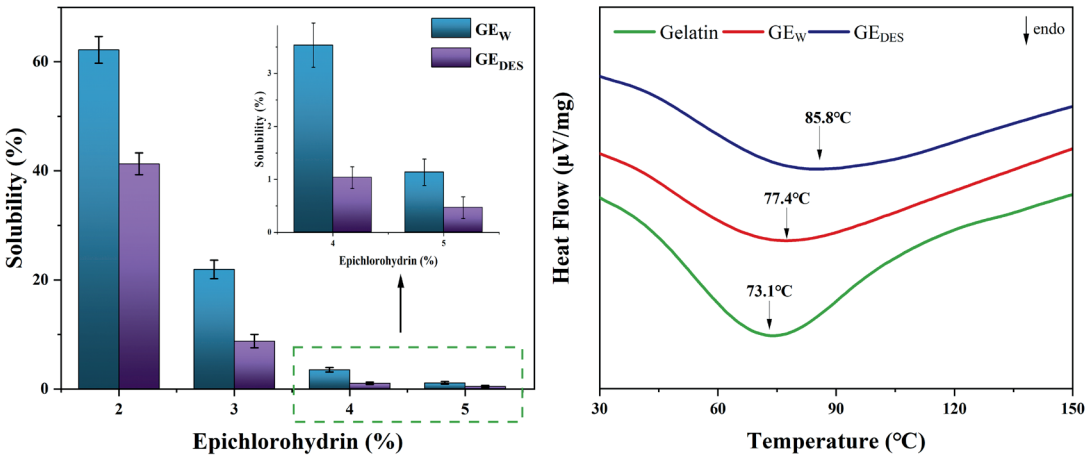

Figure 1. **(a)** The solubility of GE_W and GE_{DES} with different ECH concentrations in boiling water. **(b)** DSC curves of gelatin, GE_W and GE_{DES}

Thermo-gravimetric analysis (TGA)

Thermogravimetric analysis was carried out using a TG209F1 thermal analyzer (Netzsch, Germany). The dried samples were heated from 50°C to 600°C at a heating rate of 10°C/min under a continuous stream of nitrogen. During this pyrolysis, the apparatus automatically recorded TG curves and saved data for analysis.

Results and Discussions

Cross-linking degree analysis

Boiling water solubility

The cross-linking degree of ECH to gelatin can be estimated from the solubility of samples in boiling water. Figure 1(a) shows the solubility of solid GE_W and GE_{DES} samples in boiling water at 100°C. When the weight fraction of ECH was 2.0%, the solubility of GE_W reached 65.2%, indicating that the cross-linking degree of gelatin and ECH was too low and most of gelatin molecules still existed in the dissolved state. With the increase of ECH content, the degree of cross-linking increased and the solubility of cross-linking products gradually decreased. When the ECH content reached 4.0%, the solubility of GE_W reduced to 3.1%. It could be explained that ECH and gelatin molecules formed a dense network structure by cross-linking reaction and blocked the infiltration of water molecules. The solubility of GE_{DES} was only 0.8% under the same ECH content conditions (4.0%). With a further increase of ECH content, the solubility of GE_{DES} remained constant, suggesting that the cross-linking reaction between gelatin and ECH reached saturation as the content of ECH was 4.0% in SAT/U-DES_{30W} system. According to the overall solubility trend, gelatin and ECH can display higher cross-linking degree under SAT/U-DES_{30W} system than that in water phase, resulting in lower solubility of the products. For subsequent experiments and performance characterization, the products cross-linked with 4% ECH was adopted.

Glass transition temperature

The thermal stability of gelatin molecules will increase with the increase of cross-linking degree, so DSC can be used to characterize the Glass transition temperature of gelatin, GE_W and GE_{DES} to determine their cross-linking degree.[8] As was shown in Figure 1(b), the Glass transition temperature of gelatin was 73.1°C, representing the transition of gelatin molecules from glassy state to high elastic state.[9] The Glass transition temperature of GE_W increased to 77.4°C due to the chemical cross-linking between gelatin and ECH. When the external chemical cross-linking existed, the cross-linking agent will covalently bond among the molecules in gelatin, which makes it tend to be stable and difficult to be destroyed by high temperature. In addition, it was observed that the enthalpy changes in the gelatin denature process was significantly greater than that in the GE_W denature process. Therefore, compared with GE_W, the phase transition temperature of gelatin molecules in GE_{DES} was higher and reached 85.8°C, GE_{DES} showed better thermal stability than that of GE_W, indicating that gelatin and ECH have better cross-linking effect under SAT/U-DES_{30W} system.

Swelling capacity

Figure 2 showed the swelling capacity of solid gelatin, GE_W and GE_{DES} in distilled water. It could be observed that the swelling weight of the samples increased with the increasing length of

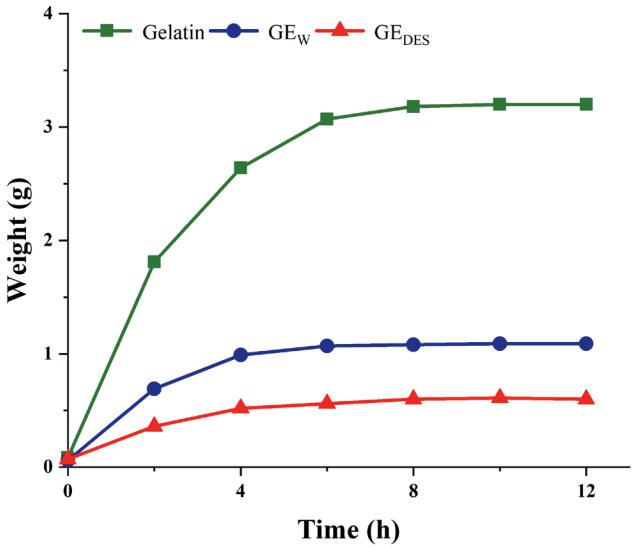

Figure 2. The swelling capacity of gelatin, GE_W and GE_{DES}.

time, and then leveled off until an equilibrium was reached. The initial weight of all three samples was 0.07 g and they reached different weights after 12 h water absorption expansion. The weight of gelatin reached 3.30 g after water absorption and the swelling rate reached 4614%. The surface of gelatin molecules contained a large number of hydrophilic groups, leading to its high swelling capacity. The water absorbed by gelatin could be divided into two states. First, water molecules were combined with the polar groups in gelatin by hydrogen bonds, called *hydration water*. Second, the free water existed among the gelatin chains, called *swelling water*.[10] After cross-linked with ECH, the weight of GE_W and GE_{DES} reached 1.12 g and 0.73 g, respectively, it could be explained that ECH can cross-link more groups inside gelatin molecules and form denser network than that in the water system. Compared with original gelatin, the cross-linked samples exhibited much lower swelling capacity, owing mainly to the network structure formed effectively via intermolecular chemical coupling. The structure prevented gelatin free-chains movement ability, leading to the water resistance increase. Moreover, the solid gelatin after cross-linked did not dissolve in water phase even at high temperature, revealing the improvement in water resistance.

Water contact angle

The surface hydrophobicity of gelatin film and GE_W film and GE_{DES} film under SAT/U-DES_{30W} and water system was determined by measuring the water contact angle (Table I). A high WCA ($\theta > 65°$) represents a hydrophobic surface, whereas a small WCA ($\theta < 65°$) indicates a hydrophilic one.[11] The WCA of the gelatin film was slightly below 65°, exhibiting low hydrophilicity. The functional groups of hydrophilic amino acids (serine, threonine, and glutamic acid) of gelatin remain positioned in the film-forming aqueous matrix while the hydrophobic ones (leucine, valine, and phenylalanine), driven by their hydrophobic nature, are preferably realigned towards the air-side interface, contributing to the formation of a hydrophobic surface.[12,13] ECH reacted with hydrophilic groups in gelatin molecules, such as amino and carboxy groups to form secondary amines and esters, greatly enhancing its hydrophobicity. And the decrease of the WCA of GE_W film and GE_{DES} film within 60 s was much smaller than that of gelatin film, because the cross-linking of ECH to gelatin matrix would delay the diffusion of water on the film and the absorption of water by the film. In addition, the WCA of GE_{DES} displayed smallest change trend within 60 s, which further indicated that SAT/U-DES_{30W} system can improve the reaction accessibility of gelatin.

FTIR

To explore the cross-linking mechanism between gelatin and ECH, the infrared spectra of gelatin and GE_{DES} were determined, as shown in Figure 3(a). The amide region (amide A, B, I, II, III) was the main characteristic peaks of protein, which can significantly exhibit the functional groups within the protein molecule. Firstly, amide A band was aroused from the characteristic of the stretching vibration of N-H group coupled with hydrogen bonds. Compared with gelatin, the absorption peak of N-H group in GE_{DES} shifted from 3382 cm^{-1} to 3425 cm^{-1} due to the reaction of ECH and amino group in gelatin molecule. Next, amide B band at 3081 cm^{-1} was attributed to N-H bending vibration.[14] The absorption band at 1652 cm^{-1} corresponded to the C=O stretch vibration in amide I, which represented a random coil structure of gelatin. The amide II at 1535 cm^{-1} and amide III at 1240 cm^{-1} were mainly attributed to the N-H in plane bend coupled with C-N stretching variation and N-H bending combined with the C-N stretching vibration.[15] The peak at 1403 cm^{-1} of GE_{DES} was slightly stronger than that of gelatin, this could arise from esters formed in the reaction of the epoxy groups of ECH with carboxyl groups in gelatin molecule, resulting in the formation of new cross-linked network structures, which in turn led to the observed enhanced water resistance.[16] The decrease in all of the absorption peak intensities of GE_{DES} also confirmed the reaction of ECH and gelatin. The absorption band of GE_{DES} at 1030 cm^{-1} was obviously broadened because new C-O-C absorption peak was generated by the addition of ECH. The cross-linking mechanism between gelatin and ECH is shown in Figure 3(c). Moreover, the absorption bands at 2933 cm^{-1} and 2873 cm^{-1}(-CH_2 asymmetric and symmetric stretching variation), 1449 cm^{-1} (-CH_2 bending variation), 1332 cm^{-1} (-CH_2 wag of proline & glycine) of gelatin and GE_{DES} were observed. It demonstrated that the main amide structure of gelatin modified by ECH under SAT/urea system has not changed or degraded. Instead, the presence of urea in DES would expose the polar groups inside the gelatin molecule and promote the degree of cross-linking with ECH.

Table I

The WCA of gelatin film, GE_W film, and GE_{DES} film

Time (s)	Water contact angle (θ, °)		
	Gelatin film	GE_W film	GE_{DES} film
0	92.8	100.2	99.3
30	66.1	89.4	97.3
60	59.2	88.9	96.5

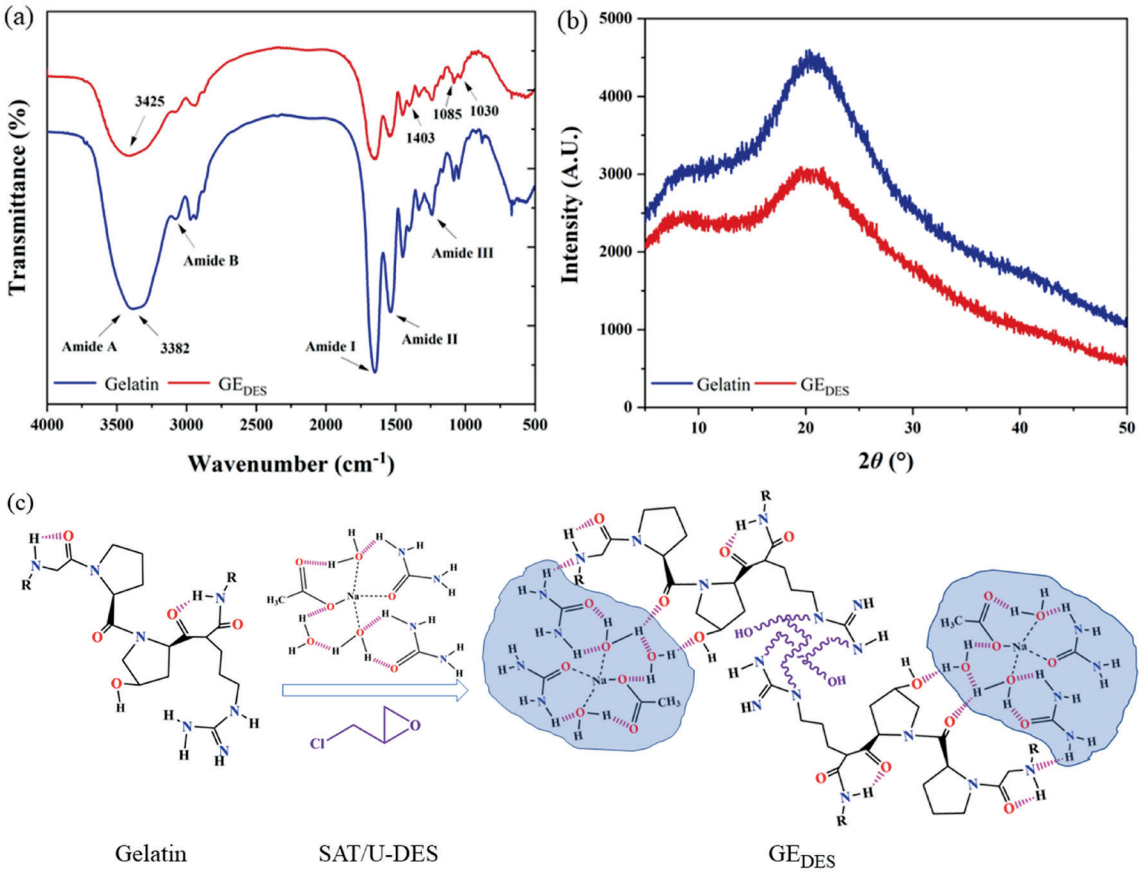

Figure 3. FTIR spectra **(a)** and X-ray diffraction patterns **(b)** of gelatin and GE_{DES}, **(c)** schematic diagram of cross-linking mechanism between gelatin and ECH.

XRD

The X-ray diffraction spectra of gelatin and GE_{DES} were shown in the Figure 3(b). Two major diffraction peaks were observed in both curves. The 2θ values of the first diffraction peak represented the distance among collagen molecules, the intensity of the diffraction peak represented relative triple helix content in gelatin.[17] The 2θ values of the first diffraction peaks of gelatin and GE_{DES} were 8.75 and 8.14. According to the Bragg equation (d(Å) = λ/2sin θ), the distances between collagen molecules in gelatin and GE can be calculated as 1.014 nm and 1.089 nm, respectively. The increase of distance among gelatin molecules might be due to the formation of covalent cross-linking between ECH and gelatin. Besides, the intensity of the first diffraction peak in GE_{DES} curve showed a smaller increase than that in gelatin curve after cross-linking by ECH. The literature has reported that covalent cross-links among collagen molecules might affect the measurement of triple helix content under neutral conditions.[8] The second diffraction peaks showed at 2θ = 20°, were one of the main characteristic peaks of gelatin. The gelatin molecule was long-range disorder and short-range order and the better the short-range order degree was, the narrower the diffraction peak will be.[18] It can be observed from the two curves that GE_{DES} showed a wider and weaker peak compared to gelatin. On the one hand, covalent cross-links formed new hydrogen bonds and hydrophobic bonds inside the gelatin molecules, which destroyed the amorphous structure of the gelatin molecule, leading to a decrease in the intensity of the diffraction peak. On the other hand, the cross-links made the short-range structure of gelatin molecules disordered, resulting in the broadening of diffraction peak.

SEM

Figure 4 shows the micromorphology of surfaces and cross-sections microstructure of gelatin film and GE_{DES} film. As can be seen from the Figure 4(a,b), the surfaces of gelatin film without cross-linking were relatively rough. After cross-linking, the GE_{DES} film displayed a smoother surface, which was attributed to the fact that cross-linking between molecules stabilized the longitudinal and transverse aggregation among collagen fibers, resulting in a more disorderly structure and a denser network structure. The cross-sections of gelatin film and GE_{DES} film were shown in Figure 4(c,d). It was observed that there existed many obvious cracks and pores on the cross-sections of gelatin film without cross-linking. The cracks and pores reduced significantly and the cross-sections became smoother after cross-linking. This was because ECH and gelatin molecules rearranged the structure of collagen fiber by covalently cross-linking. However, it was found that the GE_{DES} film exhibited uneven smoothness, which might be related to the difference of cross-linking effect caused by the aggregation of the cross-linking agent.[19]

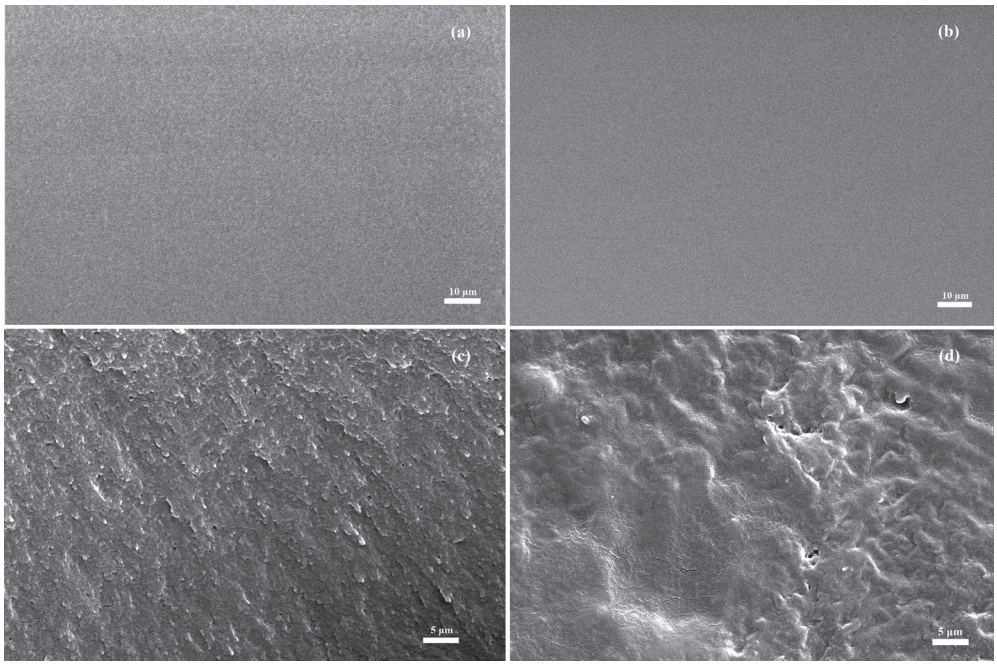

Figure 4. SEM images of surfaces **(a, b)** and cross-sections **(c, d)** of gelatin film and GE_{DES} film

TGA

Figure 5 revealed the thermal degradation behaviors of gelatin and GE_{DES}. The curves can be divided into two main stages ((I) 50~260°C, (II) 260~500°C). The first stage weight loss at the range of 50~260°C was observed on both curves, which associated with the evaporation of water inside gelatin molecules. The weight loss of GE_{DES} (12%) was lower than that of gelatin (20%) and the downward trend was relatively gentle, indicating the GE_{DES} displayed higher thermal stability at the range of 50~260°C. Its thermal stability in the temperature range can basically satisfy the demand for material application. In the second stage, major weight loss stage occurred from 260 to 500°C owing to the degradation of highly interacted peptides in gelatin molecules, the decomposition of unstable chemical bonds, and the breaking of intramolecular hydrogen bonds, electrostatic interactions and covalent bonds between amino acid residues.[20] Moreover, the temperature of gelatin with a maximum weight loss rate (311°C) was slightly lower than that of GE_{DES} (318°C). And its total weight loss (83.5%) was higher than that observed for original gelatin (67.5%) by 16%. The phenomenon could be explained that ECH cross-linked with the active groups in gelatin molecule to form dense network structure, making it difficult to decompose, and thus resulting in higher thermal stability.

Conclusion

In this paper, the cross-linking efficiency and mechanism of ECH and gelatin in SAT/U-DES_{30W} were investigated. First, the cross-linking efficiency was explored by denature-temperature and boiling water solubility of GE_W and GE_{DES}. Next, the water resistance of cross-linked products was analyzed by water contact angle and swelling capacity of GE_W and GE_{DES}. The results showed that the cross-linking efficiency of ECH to gelatin in DES system was better than that in

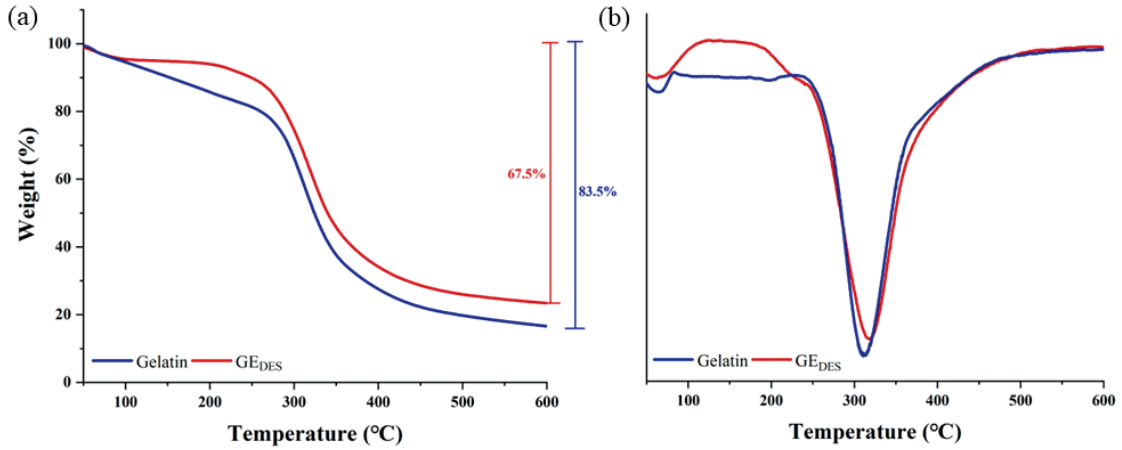

Figure 5. TG curves **(a)** and DTG curves **(b)** of gelatin and GE_{DES}.

water system. Finally, the cross-linking mechanism between gelatin and ECH was investigated by means of FT-IR, XRD and SEM. The epoxy group of ECH displayed high reactivity and could react with active groups such as amino groups in gelatin molecules, forming a dense network between gelatin molecular chains by covalently cross-linking. In addition, due to the intermolecular cross-linking, the thermal stability of cross-linked products has been significantly improved, which can meet the application in industrial production.

Credit authorship contribution statement

Yuming Cui: Investigation, Methodology, Data curation, Writing-original draft. Min He: Data curation, Formal analysis. Yanqing Wang: Methodology, Supervision. Hui Chen: Conceptualization, Methodology, Investigation, Writing-review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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An Improved Method for Accurately Determining Total Content of Four Fungicides (TCMTB, OIT, OPP And PCMC) in Leather

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Abstract

Determination of total contents of four fungicides (TCMTB, OIT, OPP and PCMC) in leather is required by Ecolabels due to health risks. Thus, an improved analytical procedure based on the current Standard ISO 13365-1-2020, was investigated to accurately test the total contents of the four fungicides in leather. It was verified that the ultrasonic assisted acetonitrile extraction described in this Standard is only effective for extracting TCMTB, but inefficient for PCMC, OPP and OIT. The extraction efficiency by acetonitrile was 93% for PCMC, 95% for OPP and 89% for OIT, respectively. Methanol was proven to be an optimal alternative of acetonitrile for sample extraction and presented satisfactory extraction efficiencies (~100%) for eluting the four fungicides at a wide content range (80-470 mg/kg). The detection limits were evaluated and well satisfy the requirement by the Ecolabels. Results of HPLC chromatograms from commercial leather samples demonstrated no obvious matrix interferences from methanol extraction. Series of leather samples from different origins were analyzed. TCMTB was found in 86% and OIT in 30% of the total tested samples, which were higher in content than the allowable limits by Ecolabels.

Introduction

Mold growth is a common problem for leather consumer products. It arises from the fact that leather is an organic material which has nutrient fats and proteins,[1] and is often exposed to mold under the daily storage and application conditions. Mold growth takes place particularly under humid conditions and at relatively high temperatures that enable mold to reproduce. Mold may hydrolytically degrade the collagen fibers and fat/oil components, thus impairing aesthetic, functional and other properties of leather. Researchers have stated that spue and some finishing defects in leather are closely related with mold growth.[2-3] Although precautions taken for leather can inhibit or slow down mold growth, the simple and precise solution is to use commercial preparations containing active ingredients called fungicides.[4]

Early fungicide products used in tanneries were chlorinated phenols (e.g. pentachlorophenol), or organo arsenic or mercury compounds (e.g. Phenyl mercuric acetate).[5] These products are effective for inhibiting the growth of mold, but they are also very toxic to human and environmental organisms. For example, pentachlorophenol has been proven to be a persistent toxic substance causing histopathological changes and mutations in aquatic life and is a probable human carcinogen.[6] Since the 1970s, the use of chlorophenols and organic compounds containing heavy metals as fungicides have been banned by legislation worldwide and have been gradually phased out.[7]

Organic pesticides are numerous and might be used as fungicides for leather. Their cost and anti-mold efficiency, as well as risk assessment by legislation, suggest that only a few pesticides are currently used in the leather industry.[8] In 2013, the European Biocidal Product Regulation (EU 528/2012) restricted the application of unregistered fungicides in leather manufacture. It led to four essential fungicides which are currently used. They are commonly known by their abbreviation PCMC, OPP, OIT and TCMTB, as listed in Table I. The four fungicides used in leather processing are also required to be controlled within a certain content range due to their toxicity and hazards, as indicated by the Ecolabels of OEKO-TEX®, BLUESIGN® and German BLUE ANGEL. The maximum limits of the four fungicides in leather recommended by BLUE ANGEL (DE-UZ 148, 2015) are 300, 500, 100, and 500 mg/kg for PCMC, OPP, OIT and TCMTB, respectively.

With this recognition of the allowable limits of the four fungicides in leather by brands, it is necessary to employ a robust procedure to detect their total content. The current standard method for analyzing the four fungicides in leather samples is ISO 13365-1-2020. It uses ultrasonic-assisted acetonitrile extraction followed by HPLC or LC-MS detection. This Standard has been in effect since 2011 due to its early edition ISO 13365-2011 and confirmed by the literature.[9] However, the full validation of this Standard has not yet been publicly reported, and the test results of the targets based on this procedure were found to be inconsistent with the desired contents in our laboratory. So, it is unsure whether the test results are real 'total contents' of these targets in leather, or not.

The aim of this study was to develop a reliable procedure for measuring the real 'total content' of the four targets based on ISO

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13365-1-2020. Firstly, leather samples containing known amounts of the four fungicides were carefully made according to leather-making processes.[10] These samples were then extracted according to the Standard method with acetonitrile, as well as with selected solvents as methanol, dimethylformide, ethanol, tetrahydrofuran and isopropanol. These results were finally analyzed and the extraction efficiencies were compared.

Experimental

Reagents and materials

Acetonitrile (ACN), methanol (MeOH), ethanol (EOH), tetrahydrofuran (THF), isopropanol (IPA) and N,N-dimethylformide (DMF) were used to dissolve standards, and to prepare the extracts. They were of HPLC-grade obtained from Aladdin (Shanghai, China). Reference standards of four fungicides (in Table I), were obtained from Sigma-Aldrich (Shanghai, China), and their stock solutions (1000 mg/L) were prepared by dissolving appropriate amounts of the commercial products in ACN and stored in glass-stoppered bottles at 4°. Appropriate volumes of the stock solutions were diluted to prepare solutions containing the four targets at 0.8-55 mg/L by the proper solvent. Table I lists the targets with numbers and abbreviations identifying the compounds.

The liquid commercial preparation containing the four fungicides was obtained from Kai-Mei Scientific Co. Ltd (China) with each concentration of ~8.5% (w/w). The preparation could well disperse and form an aqueous emulsion after being dropped into water. It was used for processing the leather crusts in drums to obtain samples containing the four targets.

Lime-split cattle-hides were obtained from a local tannery in Haining City (China). Commercial leather samples (50 pieces) were obtained from the market located in Haining China-Leather market. Leather samples with moisture content of 9-13% w/w, were cut into pieces (~3 mm × 3 mm) and sealed in bags lined with aluminum foil.

Apparatus

The following apparatuses were used: ultrasonic water-bath with frequency 40 kHz and power 200 W (Kunshan, China), PTFE membrane filter of 0.45 μm × 10 mm (Shanghai, China), and experimental drum with diameter 60 cm (Wuxi, China).

Sample preparation

Cattle-hide crusts were carefully prepared from limed hides at the leather laboratory (Jiaxing University), according to normal chrome-leather processing procedure.[10] After retanning and fatliquoring, the crusts were air dried to a moisture content of ~12% w/w and stored in sealed plastic bags at ~4°C. It was noted that during the processing, all the chemicals and auxiliaries were carefully selected and only those without the four fungicides were used, to ensure the crusts were not contaminated by those four targets. These crusts were used as negative controls.

Three positive samples (No. 1#, 2# and 3#) containing known amounts of the analytes were made by adding the preparation with 0.1%, 0.3% and 0.6% individually based on the negative crust weight in the experimental drums. The water amounts in the drum were 3-fold of the crust weight. The preparation used was first diluted with 10-fold water and then added gradually into the running drum within 3 min. After that the drum ran continuously for 120 min to allow the fungicides to penetrate into the crust thoroughly. The crusts were then taken out and lay on a porous mesh to dry under the room conditions for almost one week with a moisture content of ~12% w/w, and then stored in sealed plastic bags at 4°C. The distribution of the four targets in the three positive crusts was the same as in general leather processing.

The content of each fungicide in the positive samples was analyzed by also accounting for its residue in the float, as well as the amounts of the commercial fungicides used. For analyzing the float, this solution was first filtered with stainless meshes, and then directly analyzed with HPLC according to the procedure described in the following sample extraction. Then the real amounts of the four analytes in the three crusts were evaluated (as listed in Table III).

Prior to test, both the negative and positive crusts were cut into pieces (~ 3 mm × 3 mm) and conditioned for 24 h at standard atmospheric temperature of 20°C and relative humidity 65% (Temp. 20°C/R.H. 65%). The moisture contents of the samples were near to 12% w/w.

Sample extraction

Extraction of the four fungicides from leather samples was carried out by ultrasonic-assisted solvent extraction (single-cycle) according to ISO 13365-1-2020, but with the following modification. The operation was performed in a 50 mL screw-capped glass bottle charged with 2.0 g of accurately weighed sample pieces. After adding 40.0 mL aliquot of the selected solvent into the bottle, the contents were treated continuously in the ultrasonic water bath (40 kHz) at 25-35°C for 60 min. The bottles were then cooled to room temperature, and the extracts were filtered on a 0.45 μm PTFE filter, which were then ready for HPLC analysis. All tests were performed in triplicate. The calculations were all based on dry matter of leather sample.

For the evaluation of the real contents of the four targets in the positive samples, repeated extraction (3-cycles) with MeOH under the same ultrasonic conditions was also performed as follows. Firstly, 2.0 g of sample pieces were extracted with 40 mL MeOH for 60 min. Then the leather pieces were filtered and retreated with 40 mL MeOH for another 60 min. Finally, the leather pieces were further treated with 20 mL MeOH for 30 min. All the extracts retrieved were merged into a 100 mL conical flask. After filled to the mark with MeOH, the solutions were filtered and analyzed with HPLC.

Extraction efficiency

Extraction efficiency (E, %) with different solvents was evaluated according to:

$$E = 100 \times w_e/w_r$$

where w_e refers to the test result with the used solvent, and w_r is the real content of the target in the sample.

HPLC analysis

The HPLC system was Agilent 1260 equipped with Diode Array Detection (DAD) and a thermostat. The separation was performed on a Diamond C_{18} reversed-phase column (250 mm × 4.6 mm I.D.; 5 µm particles (Dikma, China)) with an isocratic elution program. The condition was ACN/water solution 70:30 (v/v) for 10 min. The flow rate was 1.0 mL/min and the column was maintained at 40°C. Injection volume was 10 µL.

External calibration plots were built in the 0.8-55 mg/L concentration range for the four analytes. The curves were fitted by linear-ship and the correlation coefficient r^2 was calculated from the linear regression, which was expected to be greater than 0.995.

Results and Discussion

Analysis of fours targets with HPLC

According to ISO 13365-1-2020, HPLC parameters were further optimized to ensure the four fungicides were satisfactorily separated from one another. The chromatogram of the injected standard mixture exhibited complete resolution under the chosen HPLC operating conditions. The use of DAD allowed the acquisition of all analyte's spectra and the selection of the optimum detection wavelength for each compound. Besides, the spectrum was a means to verify the identity of analytes by comparison with the Standards. Table I reported the elution order, retention time of each analyte. Figure 1 presented all the spectra recorded at the peak retention time. A relative absorption maximum was evident at 225 nm and 280 nm for TCMTB, 246 nm and 285 nm for OPP, 229 nm and 282nm for PCMC, and 280 nm for OIT, respectively. In general, a long UV wavelength (as 280 nm) is clearly better than the short one (as 225 nm or 246 nm) for recovery and precision due to the noise and interferences from matrix and solvent (as DMF). In view of this consideration, 280 nm of UV detection was chosen for detecting the four targets.

Table I

The four fungicides with their names, CAS No. and retention times

No.	Target	Structure name	CAS. No	Retention time / min
1	PCMC	4-chloro-3-methylphenol	59-50-7	3.89
2	OPP	2-phenylphenol	90-43-7	4.56
3	OIT	2-octylisothiazol-3(2H)-one	26530-20-1	5.53
4	TCMTB	2-(thiocyanomethylthio)- benzothiazole	21564-17-0	5.86

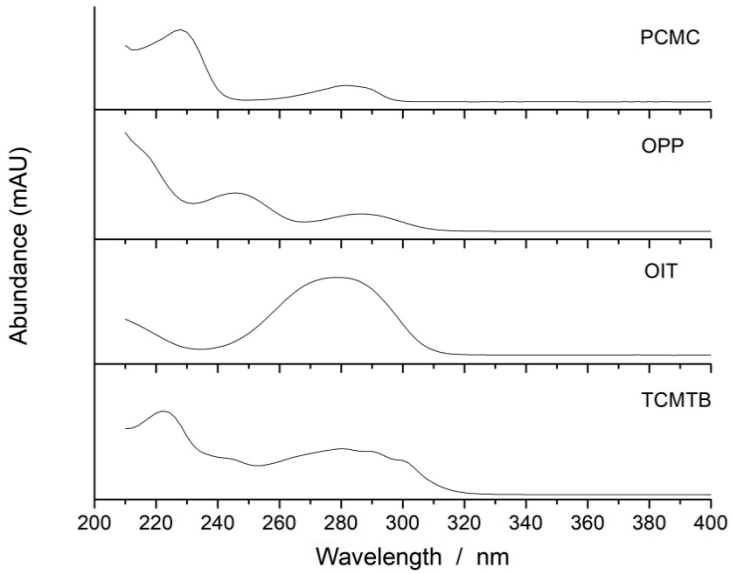

Figure 1. UV spectra of the four fungicides obtained with DAD

Calibration and detection limits

Linearity was tested based on the standard addition procedure by adding known amounts of the four fungicides to blank leather extracts. The negative leather sample was used as blank due to the absence of the four fungicides. External calibration curves (in terms of peak areas vs. concentration) were obtained in the range 0.8-55 mg/L at seven concentration levels. All calibrations were linear in the explored concentration range. Figure 2 presented the calibration plots relative to the four targets. It could be seen that the curves for the single target in ACN, MeOH, DMF, EOH, THF and IPA, almost completely coincided, indicating little interference of the six solvents for HPLC analysis. Thus, the calculation was based on one standard curve for one target even if different solvent was used in the following extraction experiment. The calibration parameters were reported in Table II.

Detection limits (LODs) were evaluated according to the instrumental detection limits, as well as weight of the sample and volume of the final extracts. LODs for targets determined by considering signal-to-noise of 3:1, were 20.0 mg/kg for PCMC, 10.0 mg/kg for OPP, 8.0 mg/kg for OIT and 4.5 mg/kg for TCMTB, respectively. These LODs can well satisfy the requirements by current Ecolabels.

Sample extraction

Repeated extraction (3-cycles) with MeOH was first carried out for measuring the total contents of the four targets. The results were compared with the real contents as described above. Three positive samples with different concentration levels were analyzed, and the data are listed in Table III. For any single target, the two results from one concentration level were close with each other, and their average deviation was less than 2%. Thus, it was believed that the repeated extraction (three times) with methanol could elute all of the four fungicides in leather. These results (in Table III) further verified the real contents of each target in the three positive samples and provided a basis for evaluating the extraction efficiencies of ACN, MeOH, DMF, EOH, THF and IPA in the followings investigation.

In contrast to repeated extraction, the single-cycle extraction operation as described in ISO 13365-1:2020 is obviously handy and efficient. However, the solvent used should be competitive for

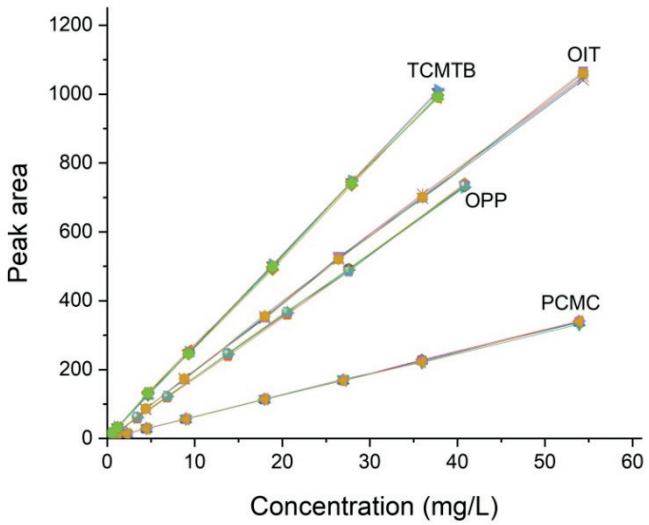

Figure 2. Calibration curves of the four fungicides in the six solvents: ACN, MeOH, DMF, EOH, THF and IPA, respectively (λ=280 nm)

Table II

Regression parameters and LODs for the four analytes

Analyte	Curve equation[a)]	r^2	LODs (mg/kg)
PCMC	$A_i = 6.33 \times C_i - 0.22$	0.9985	20.0
OPP	$A_i = 17.91 \times C_i - 1.01$	0.9991	10.0
OIT	$A_i = 19.49 \times C_i + 0.81$	0.9992	8.0
TCMTB	$A_i = 26.38 \times C_i + 0.44$	0.9989	4.5

[a)] A_i is the peak area, and C_i is the concentration, mg/L. The results were based on MeOH as solvent.

Table III

Contents of four fungicides in three positive samples

Sample No.	Contents based on float analysis (mg/kg)				Contents based on repeated extraction (mg/kg)			
	PCMC	OPP	OIT	TCMTB	PCMC	OPP	OIT	TCMTB
1#	78.0±4.5	80.7±5.2	80.2±4.3	81.1±4.4	80.1±3.1	81.4±3.8	81.8±3.0	82.3±2.2
2#	238.6±6.3	231.1±5.6	235.1±5.1	238.3±6.1	233.7±4.3	236.9±5.2	240.1±2.5	242.7±4.5
3#	462.1±5.1	457.8±5.7	464.0±4.7	463.1±5.6	470.4±5.0	450.2±6.3	457.3±4.1	468.1±5.1

solubilizing all the analytes to release them fully from the leather fiber. The solubility of target in solvent depends on their polarity, molecular structures, etc, as revealed by the Rule of the Like Dissolves Like in solubility. The four analytes and ACN are all polar compounds. Thus, other polar solvents as MeOH, EOH, THF, IPA and DMF should be comparative with ACN for dissolving the analytes. Especially for DMF, it is a Universal Solvent and possesses excellent solubility for the four analytes. So it is desired to fully elute the four targets from leather fibers.

The extraction efficiencies of MeOH, EOH, DMF, THF and IPA were tested and compared with ACN in one-cycle extraction. These results are reported in Figure 3. It can be seen that the three solvents (DMF, MeOH and THF) gave desired extraction efficiencies (~100%) for the four targets, indicating the total elution of the targets from the three positive samples. However, another three solvents (IPA, EOH and ACN) presented unsatisfactory results. For IPA, it only gave desired extraction efficiency (~100%) for OIT, but low results for PCMC (~90%), OPP (~95%) and TCMTB (~83%). For EOH, it presented relatively poor extraction efficiencies for PCMC (~90%), OPP (~88%), OIT (93%) and TCMTB (82%). For ACN, it gave a satisfied result (~100%) for TCMTB, but poor results for PCMC (93%), OPP (~95%) and OIT (~89%). These indicated that the three solvents (IPA, EOH and ACN) can't totally elute the four targets in one-cycle extraction. Thus, according to ACN extraction described in ISO 13365-1:2020, the test results of the three targets (PCMC, OPP and OIT) were not their 'total contents' in leather.

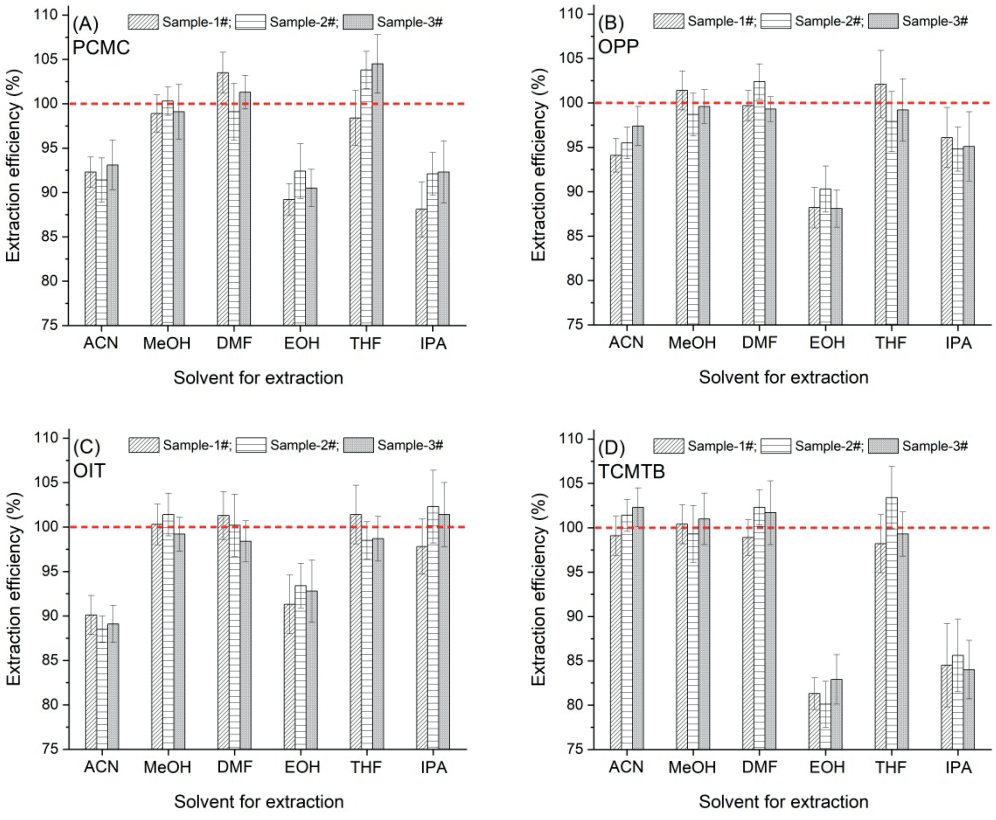

Figure 3. Extraction efficiencies of ACN, MeOH, DMF, EOH, THF and IPA for extracting **(A)** PCMC, **(B)** OPP, **(C)** OIT and **(D)** TCMTB from three positive samples in single-cycle extraction

The satisfied results determined by MeOH, DMF and THF rely on their polarity-based ability to dissolve the four targets. These data indicated the feasibility of using MeOH, DMF and THF to replace ACN for extraction. In comparison, MeOH should be given priority, because DMF and THF are very strong for solubilizing the leather fiber and leads to serious disturbance for HPLC analysis. Besides, MeOH has low toxicity, is readily available and a poor solvent of oils and polymers in the leather fibers. Thus, the new and improved procedure using ultrasonic assisted MeOH extraction was built and applied for testing real leather samples.

Analysis of real samples

Fifty commercial samples collected from garment, shoe and furniture leather, were tested by using the improved procedure, to verify its adaptability and to determine the contents of the four fungicides. All of the samples contained at least one of the four targets, and almost 40% of the samples contained two different compounds. Of all the tested samples, TCMTB was detected in 86%, OIT in 30%, OPP in 10% and PCMC in 2%. The concentrations ranged from 221 to 623 mg/kg for TCMTB, 79 to 211 mg/kg for OIT, 58 to 104 mg/kg for OPP, and 125 mg/kg for PCMC, particularly. These results demonstrate the popular application of TCMTB in current leather processing as well as its realistic levels. On the other hand, it was found that contents of TCMTB and OIT in 5 pieces were significantly higher than the allowable limits by Ecolabels from BLUE ANGEL (TCMTB < 500 mg/kg, OIT < 100 mg/kg). This may be a problem that should be addressed by leather tanneries.

Two samples each containing PCMC and OPP individually, and another sample containing both TCMTB and OIT, were further tested with ACN assisted ultrasonic extraction (single cycle, as described in ISO 13365-1-2020). These results were compared and listed in Table III. For PCMC, OPP and OIT, the contents based on ACN extraction were about 92.6%, 94.5% and 90.0% of that on MeOH extraction, respectively. This fact was quite similar with that obtained using positive samples above, and further verified the lower extraction efficiencies of ACN for the three targets.

Figure 4 showed the typical HPLC chromatogram with TCMTB and OIT. Their peaks were easily identified by their retention times

Table III

Comparison of ACN and MeOH extraction by testing commercial samples

Extraction solvent	Content of Targets[a] (mg/kg)			
	PCMC	OPP	OIT	TCMTB
MeOH	125.0±3.4	77.6±3.0	108.4±3.5	372.1±4.8
ACN	115.8±2.1	73.3±3.1	97.6±4.2	369.4±3.9

[a] PCMC and OPP were tested with each individual sample. OIT and TCMTB were tested together with another sample.

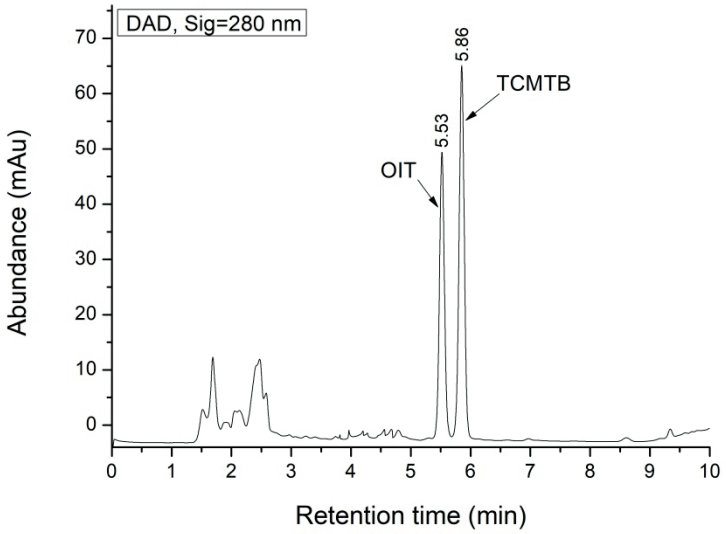

Figure 4. Chromatograms of OIT and TCMTB in MeOH extracts of real sample (concentrations of OIT and TCMTB were 181 and 176 mg/kg, respectively)

and UV spectra from DAD detection. The results demonstrate the absence of enhanced noise or interference from/in the MeOH extracts. These findings indicate the selectivity and reliability of this improved procedure.

Conclusion

The method of determining the total content of four fungicides (PCMC, OPP, OIT and TCMTB) in leather was investigated. The ultrasonic-assisted acetonitrile extraction method as described in ISO 13365-1:2020, was not effective for fully eluting the three fungicides (PCMC, OPP, and OIT) from leather. This problem is mainly due to the relative weak solubility of these fungicides in acetonitrile. By comparison, the alternative methanol extraction gave excellent results. This improved procedure was validated by testing samples with known contents of four fungicides and gave extraction efficiencies of almost 100% for the targets at wide concentration levels. The chromatogram of real samples indicated no obvious noise or interferences from methanol extracts. The detection limits were sufficient enough to satisfy the requirements of Ecolabels.

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Effect of Calcium in Delimed Hide on Leather Quality

by

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Abstract

The efficient removal of calcium from delimed hide is essential to leather quality, but few reports have elaborated on how residual calcium in delimed hide affects leather quality. In this study, four delimed hides containing different calcium content were first prepared by using ammonium sulfate and sodium gluconate. Energy dispersive X-ray spectrometry results showed that the atomic percentages of calcium on the grain surfaces of the four delimed hides were 8.17%, 4.82%, 2.34%, and 0.25%, respectively. The bating, chrome tanning, and post-tanning performances of the four delimed hides were then analyzed to evaluate the effect of calcium in delimed hide on leather quality. Results showed that the removal efficiency of non-collagenous protein from hide in enzymatic bating, the evenness of chromium distribution, the shrinkage temperature of wet blue, and the physical properties of crust leather all increased as the calcium content of delimed hide decreased. Furthermore, efficient calcium removal from the grain surface of delimed hide played a key role in the color brightness and uniformity of wet blue and crust leather. The results draw our attention to the importance of considering the effectual removal of calcium from delimed hide for obtaining high-quality leather.

Introduction

Leather quality is always attracting considerable attention from consumers and is vital to the price and sales of leather products. High leather quality is closely related to each process of leather manufacture, including soaking, liming, deliming, bating, pickling, tanning, post-tanning, dyeing, fatliquoring, and finishing.[1,2] The conventional liming process aims to remove hair and epidermis from the hide or skin and open up collagen fibers in the raw material using sodium sulfide or hydrosulfide and lime (calcium hydroxide) and is considered the most important process in the beamhouse, as it plays a key role in preparing for the tanning process.[3,4] The liming process increases the pH of the hide to more than 12 and introduces high amounts of calcium into the hide. The high pH and calcium content of limed hide are not suitable for the subsequent bating, pickling, and tanning.[5,6] Therefore, the deliming process is performed to decrease the pH of limed hide to 8–9 and remove calcium from the hide before the bating process.[7]

Existing deliming agents include ammonium salts,[8] boric acid,[9] organic acids,[10,11] and esters.[12] The extent of calcium removal from limed hide using these deliming agents is 50–80%. Sometimes, small amounts of ammonium salt or other weakly acidic substances are also used in the bating process to further remove the residual calcium in the delimed hide for maintaining the proteolysis activity of trypsin.[13] In addition, tanners have found that the insufficient removal of calcium from the hide probably increases the color difference of wet blue and crust leather. The efficient removal of calcium from limed hide is essential to leather quality. However, to the authors' knowledge, few reports have elaborated on how residual calcium in delimed hide affects leather quality.[13] Obviously, a clear understanding of the relationship between calcium in the hide and leather quality has a substantial implication for developing better deliming agents and technologies and ensuring leather quality.

This study focuses on how residual calcium in delimed hide affects the performances of the bating, tanning, and post-tanning processes and the organoleptic and physical properties of leather. Ammonium salts, such as ammonium sulfate and ammonium chloride, are usually used to perform the deliming owing to their high pH-buffering capacity, rapid penetration in the limed hide, and low cost.[14] However, the extent of calcium removal from hide is limited by ammonium salts. Sodium gluconate is applicable for the efficient removal of calcium from limed hide based on our previous work.[15] Therefore, different amounts of sodium gluconate were used with ammonium sulfate in the deliming to prepare delimed hides with different calcium content. The effects of residual calcium in delimed hide on the removal efficiency of non-collagenous protein from bated hide, chromium distribution, shrinkage temperature (T_s) and color of wet blue, and the color and physical properties of crust leather were analyzed in order to establish how the calcium content affects leather quality.

Experimental

Materials

Relimed cattle hide (pH 12.54) prepared by conventional soaking, liming (3% sodium sulfide and 8% lime, based on the weight of fleshed hide), splitting, and reliming (4% lime, based on the weight of limed grain split) processes was used in deliming, bating, pickling, chrome tanning, and post-tanning experiments. The sodium gluconate

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used for removing calcium from the limed hide was of analytical grade and purchased from Chron Chemicals Co., Ltd. (Chengdu, China). Trypsin from bovine pancreas was purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The chemicals used for analyses were of analytical grade. Lime, ammonium sulfate, chrome tanning agent, dye, and other chemicals used for leather processing were of commercial grade.

Deliming experiments and evaluation of deliming performance

Four pieces of relimed hides labeled SG-0%, SG-0.4%, SG-0.8%, and SG-1.2% were used for the four groups of deliming experiments. Each deliming experiment was performed in a drum with 2.0% ammonium sulfate, *X*% sodium gluconate (*X*=0, 0.4, 0.8, 1.2), and 100% water (based on the weight of relimed hide) at 32°C for 60 min. During the deliming process, the float pH values were measured using a precise pH meter (FE28-Standard, Mettler-Toledo, Switzerland), where the hides were delimed for 5, 10, 20, 40, and 60 min. After deliming for 20 min, the hides were cut with a scalpel, checked using a phenolphthalein indicator, and observed using a stereomicroscope (M205C, Leica, Germany) to determine the penetration of the deliming agents. After deliming for 60 min, the hydroxyproline (Hyp) concentrations of the deliming effluents were measured to assess collagen damage.[16] The measurement was repeated thrice to obtain the average values and standard deviation. The delimed hides were sampled to analyze the morphology and calcium content of grain surfaces by scanning electron microscopy (SEM; JSM-7500F, JEOL, Japan) and energy dispersive X-ray spectrometry (EDX; INCA X-MAX 50, Oxford, UK), respectively. EDX can be used to determine which chemical elements are present in a sample and estimate their relative abundance (semi-quantitative content).[17] In addition, the calcium contents of relimed and delimed hides were determined by inductively coupled plasma–atomic emission spectrometry (ICP–AES; Optima 8000DV, PerkinElmer, USA) after ashing and acid digestion of the hides as described in the literature,[9] and the measurement was repeated thrice to obtain the average values and standard deviation. The extents of calcium removal from the hides calculated using Formula (1):

$$\%\ \text{extent of calcium removal} = \frac{(C_1 - C_2)}{C_1} \times 100 \quad (1)$$

where C_1 is the calcium content of relimed hide (mg/g), and C_2 is the calcium content of delimed hide (mg/g).

Bating experiments and evaluation of bating performance

The four delimed hides were subsequently bated in a drum with 0.1% trypsin and 100% water (based on the weight of relimed hide) at 32°C for 40 min. The bating effluents were collected and centrifuged at 8000 rpm for 6 min. The total protein concentrations of the supernatants were measured according to the method described in the document.[8] In addition, the Hyp concentrations of the bating effluents were analyzed.[16] The measurements of the total protein and Hyp concentrations were repeated thrice to obtain the average values and standard deviation. The non-collagenous protein concentrations of the bating effluents were calculated using Formula (2):[18]

$$\text{Non-collagenous protein concentration (mg/L)} = P - H \times 7.4 \quad (2)$$

where P is the total protein concentration of the bating effluent (mg/L), H is the Hyp concentration of the bating effluent (mg/L), and 7.4 is the conversion factor from Hyp to collagen.

Tanning experiments and evaluation of tanning performance

The four bated hides were pickled and chrome tanned using conventional processes.[7] After chrome tanning, the T_s of wet blue was measured using a shrinkage temperature tester (MSW-YD4, Sunshine Electronic Research Institute, China). The measurement was repeated thrice to obtain the average values and standard deviation. Then, the wet blue after horsing up for 48 h was sampled for the following tests. The sample was divided into the grain, middle, and flesh layers, and each layer was dried to constant weight and digested as reported previously.[19] After cooling, the chromium concentration of the digestion solution was determined by ICP–AES. The measurement was repeated thrice to obtain the average values and standard deviation. The Cr_2O_3 content of each layer was calculated using Formula (3):

$$Cr_2O_3\ \text{content (\%)} = \frac{M(Cr_2O_3) \times c \times V}{2 \times M\,(Cr) \times w} \quad (3)$$

where $M(Cr_2O_3)$ is the molar mass of Cr_2O_3 (152 g/mol), c is the chromium concentration of the digestion solution (mg/L), V is the volume of the digestion solution (L), M(Cr) is the molar mass of Cr (52 g/mol), and w is the dry weight of wet blue (mg).

Moreover, the sample was freeze-dried to analyze the morphology and elemental (carbon, calcium, and chromium) distributions of cross-sections using SEM and EDX, respectively. The grain surface of the sample was directly observed using a digital camera, and their color parameters (L^*, a^*, and b^*) were recorded using a colorimeter (CR-13, Konica Minolta, Japan) by testing 10 different points in each sample.[20] The color difference (ΔE) was calculated using Formula (4):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (4)$$

where L is the brightness and describes the color in the range from black to white, a is the red/green value, and b is the yellow/blue value.

Post-tanning experiments and evaluation of crust leather performance

The wet blue was treated by conventional shaving, rewetting, neutralization, retanning, dyeing, fatliquoring, horsing up, samming, and milling, and four crust leathers with a thickness of 1.0 mm were prepared. The grain surface of the crust leathers were observed using a stereomicroscope. The color parameters (L^*, a^*, and b^*) of the grain surfaces were recorded using a colorimeter by testing 10 different

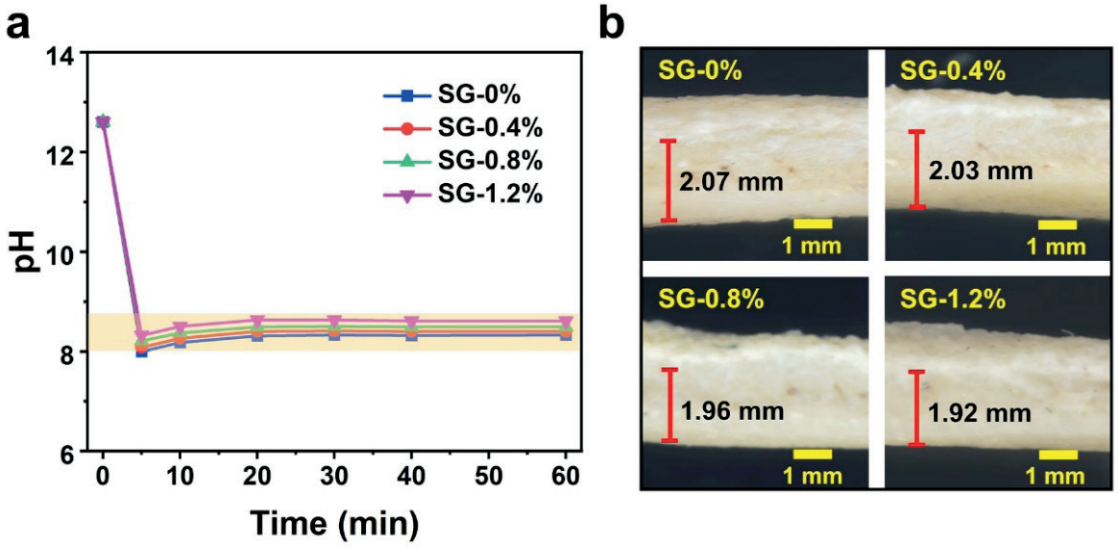

Figure 1. (a) Float pH during deliming process (The reliming float pH of 12.60 was recorded as the initial pH of deliming float) and **(b)** stereomicrographs of hide vertical sections after deliming for 20 min and checking with phenolphthalein.

points in each sample,[20] and ΔE was calculated using Formula (4) above. Besides, the four crust leathers were sampled and conditioned at 20°C and 65% relative humidity for 24 h (ISO 2418:2002), and their physical properties, including tensile strength (ISO 3376:2011), tear strength (ISO 3377-2:2002), bursting strength (ISO 3379:2015), and softness (ISO 17235:2015), were measured.

Results and Discussion

Preparation of delimed hides with different calcium contents

The main purposes of deliming are to reduce the pH of limed/relimed hide from 12–13 to 8–9 and remove calcium from the limed/relimed hide.[7] The effects of percentage of sodium gluconate (X% = 0, 0.4, 0.8,

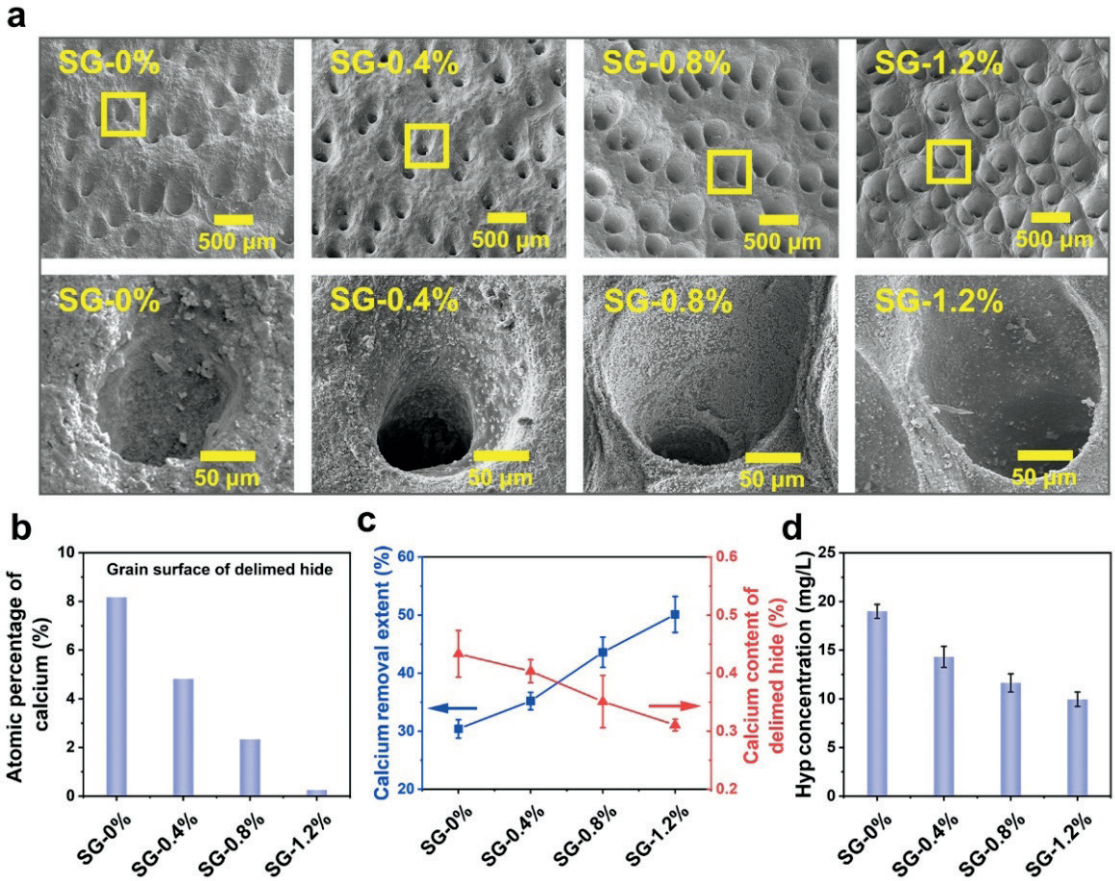

Figure 2. (a) SEM micrograph of the grain surface of delimed hide; **(b)** atomic percentage of calcium on the grain surface of delimed hide; **(c)** extent of calcium removal and calcium content of delimed hide (based on the dry weight of hide); **(d)** Hyp concentration of the deliming effluent.

1.2) on the pH of deliming float, the penetration of deliming agent, the calcium content of hide, and the damage to hide collagen are shown in Figures 1 and 2. The deliming float pH increased slightly with increasing sodium gluconate but was maintained in the range of 8.0–8.6 because of the high pH-buffering capacity of ammonium sulfate (Figure 1a). The four hides were all colorless after deliming for 20 min and checking with phenolphthalein (Figure 1b), which indicated that deliming agents SG-0%, SG-0.4%, SG-0.8%, and SG-1.2% penetrated the relimed hide rapidly.

The SEM observation of the grain surfaces of the delimed hides showed that the grain surface became smoother with more sodium gluconate (Figure 2a). The EDX data indicated that the atomic percentage of calcium on the surface decreased sharply with increasing sodium gluconate (Figure 2b). The SEM and EDX results revealed that the calcium precipitates on the surface could be converted into soluble calcium salts and removed using sodium gluconate.[15] The total extent of calcium removal from the hide also increased with the increase in sodium gluconate (Figure 2c). The results proved that delimed hides with different calcium content were obtained using different amounts of sodium gluconate. Specifically, the atomic percentages of calcium on the grain surfaces of delimed hides SG-0%, SG-0.4%, SG-0.8%, and SG-1.2% were 8.17%, 4.82%, 2.34%, and 0.25%, respectively (Figure 2b), and the calcium contents of the delimed hides SG-0%, SG-0.4%, SG-0.8%, and SG-1.2% were 0.43±0.04%, 0.40±0.02%, 0.35±0.05%, and 0.31±0.01% (based on the dry weight of hide), respectively (Figure 2c).

Moreover, the Hyp concentration of the deliming effluent decreased with increasing sodium gluconate (Figure 2d), indicating that deliming with sodium gluconate was useful in reducing collagen damage. The smooth surface (few calcium precipitates on the grain surface) caused by sodium gluconate could be the reason for the reduction in collagen damage.

Effect of calcium in delimed hide on bating performance

The effect of residual calcium in the delimed hide on the bating performance was investigated in this section. The aim of bating is mainly to soften the hide by removing non-collagenous protein from the hide and improve the smoothness of the grain.[6,21] Therefore, bating performance was evaluated by analyzing the concentration of non-collagenous protein in the bating effluent and the smoothness of the grain. As shown in Figure 3a, the non-collagenous protein concentration of the bating effluent increased as the calcium content of the delimed hide decreased. The results indicated that calcium removal from delimed hide was beneficial to remove non-collagenous protein and disperse collagen fibers. Additionally, we found that the grain surface with lower calcium content was smoother. But it is worth noting that the decrease in calcium content was also accompanied by an increase in the Hyp concentration of the bating effluent (Figure 3b), which implies that extreme calcium removal during deliming may damage the hide collagen. The results in Figure 3 imply that hide protein is easier to be hydrolyzed by trypsin than calcium-bound hide protein.

Effect of calcium in delimed hide on chrome tanning performance

Tanning is the key process of converting hide into leather, and chrome tanning is the most commonly used form of tannage because of its ability to produce soft and lightweight leathers with high hydrothermal stability.[22] In this section, the effect of residual calcium in the hide on the chrome tanning performance was investigated by analyzing the changes in the T_s and color of wet blue with different calcium content.

The data in Figure 4a show that the T_s of wet blue followed the order: SG-0% (92.3±1.3°C) < SG-0.4% (95.6±2.1°C) < SG-0.8% (101.2±1.4°C) < SG-1.2% (102.5±1.7°C). The main reason for this order is probably because the hide with a lower calcium content was bated more effectively and achieved a more uniform distribution of chrome tanning agent. The Cr_2O_3 content of each layer of wet blue

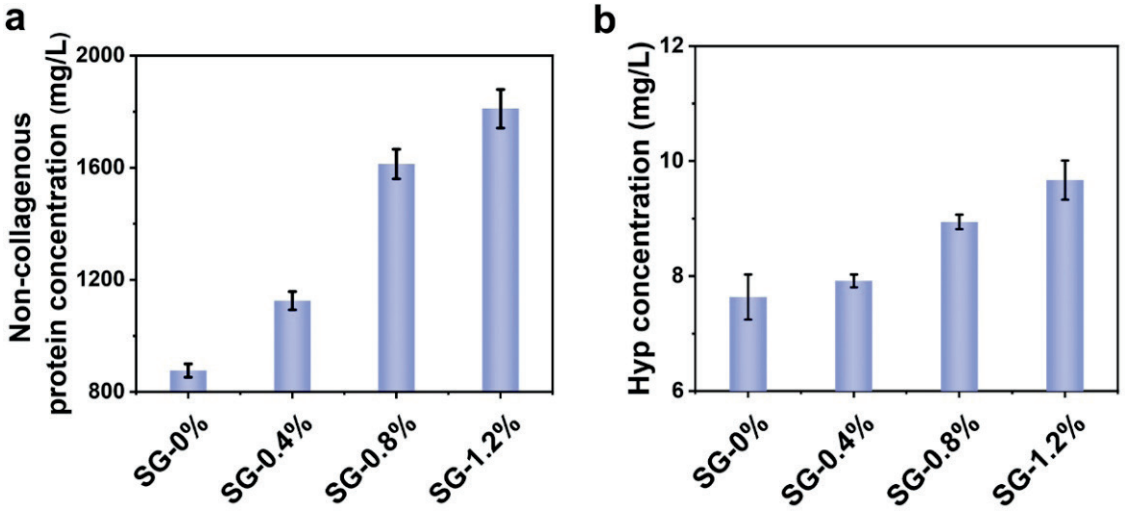

Figure 3. Concentrations of non-collagenous protein **(a)** and Hyp **(b)** in bating effluents.

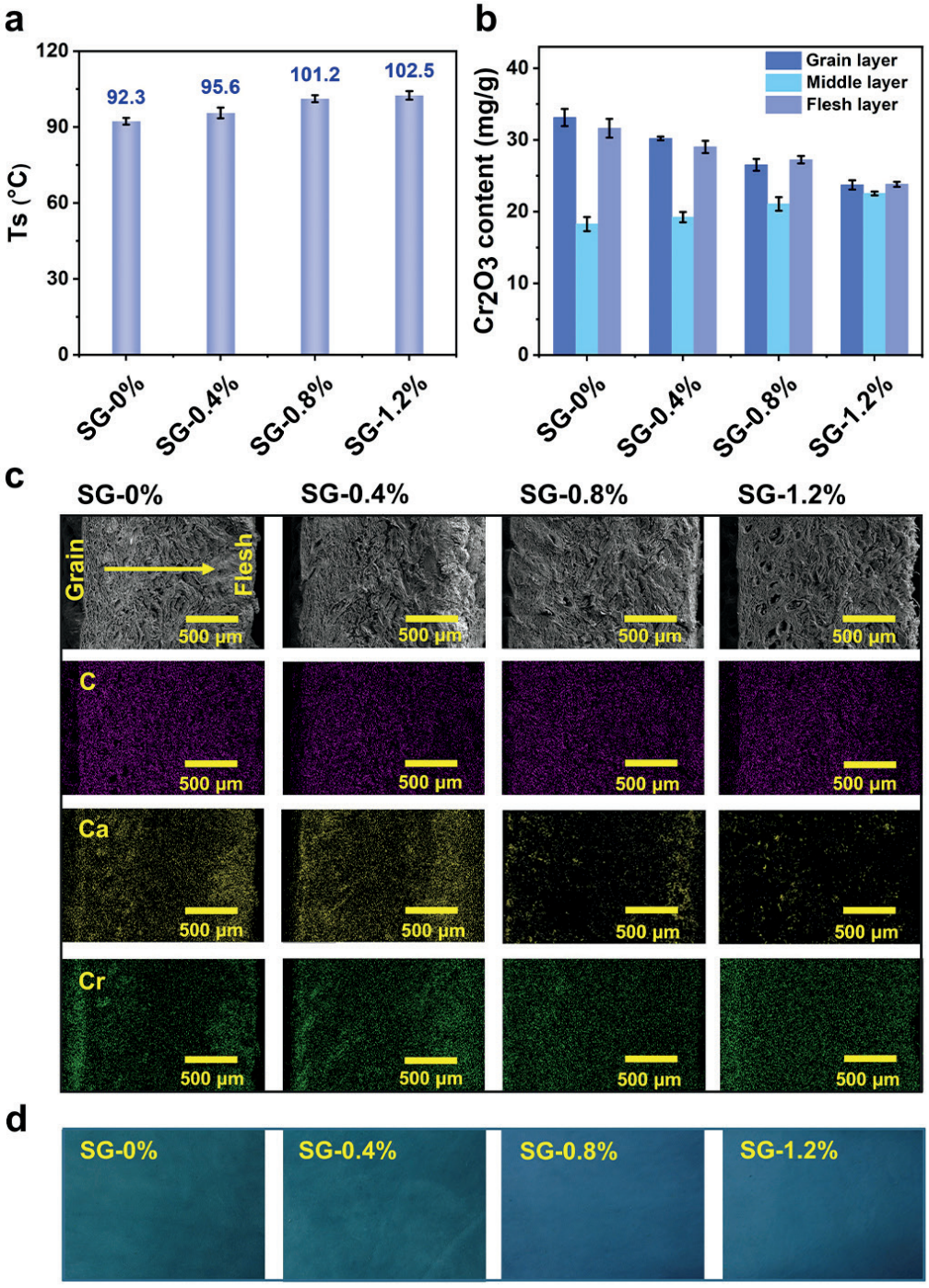

Figure 4. **(a)** T_s of wet blue; **(b)** Cr_2O_3 content of each layer of wet blue; **(c)** SEM micrograph and EDX elemental mapping images of C, Ca, and Cr distributions of the cross-section of wet blue; and **(d)** digital photo of wet blue.

in Figure 4b and the EDX results of wet blue in Figure 4c proved this point. The chromium content of the surface layers decreased and that of the middle layer increased with decreasing calcium content (Figure 4b), and the distribution of chromium in wet blue was more uniform when the hide had less calcium (Figure 4c). These phenomena supported the view that a low calcium content of the hide favors the uniform distribution of chromium in wet blue. Another reason may be that the calcium in the delimed hide would form calcium sulfate in the pickled hide and negatively affect the penetration of chrome tanning agent and its reaction with collagen fiber.[23]

In addition, the photos in Figure 4d and the a^* and b^* values in Table I show that the wet blue SG-0% and SG-0.4% were greener and darker compared with the wet blue SG-0.8% and SG-1.2% (lake blue).[24] The obvious differences in the ΔE_1 values of the four wet blue (Table

Table I

Color parameters of the wet blue

Sample	L*	a*	b*	ΔE_1 [a]	ΔE_2 [b]
SG-0%	46.84 ± 1.01	-8.68 ± 0.71	-3.06 ± 0.50	11.47 ± 0.94	2.41 ± 0.72
SG-0.4%	45.56 ± 1.00	-7.85 ± 0.80	-4.28 ± 0.40	10.15 ± 1.03	1.43 ± 0.65
SG-0.8%	46.62 ± 0.76	-5.23 ± 0.47	-5.36 ± 0.58	7.08 ± 0.67	1.24 ± 0.61
SG-1.2%	46.81 ± 0.54	-4.04 ± 0.38	-5.79 ± 0.42	0	1.22 ± 0.37

[a]ΔE_1 reflects the color difference among the four wet blue. The wet blue SG-1.2% was used as the calibration board.

[b]ΔE_2 reflects the color difference among various points in a single wet blue. One of the points on the wet blue was used as the calibration board.

I) show that the calcium content of hide greatly affects the color of wet blue. The ΔE_2 values of wet blue decreased with decreasing atomic percentage of calcium on the grain surface, indicating that an efficient calcium removal from the hide surface can improve the color uniformity of wet blue.

Effect of calcium in delimed hide properties of crust leather

The color and physical properties of leather directly affect the consumer experience and market sales.[25,26] High-quality leather requires a uniform color and excellent physical properties. Hence, the effect of residual calcium in delimed hide on the crust leather was investigated by analyzing the color and physical properties of crust leathers. The stereomicrographs in Figure 5 and the L^*, a^*, and b^* values in Table II indicate that the crust leather became brighter and yellower as the atomic percentage of calcium on the grain surface decreased. The change trend of the crust leather color was consistent with that of the wet blue color. The ΔE_1 values of the four crust leathers had great differences, showing that the calcium content of the hide is an important factor affecting the color of crust leather. The ΔE_2 values of crust leathers followed the order: SG-0% > SG-0.4% > SG-0.8% > SG-1.2%. This finding is likely because the calcium on the grain surface made the dye form a precipitate.[27,28] This finding indicates that an effectual calcium removal from the hide surface is important for the color uniformity of crust leather.

As shown in Table III, the tensile strength, tear strength, bursting strength, and softness of crust leathers followed the order, SG-0% < SG-0.4% < SG-0.8% < SG-1.2%, which is consistent with the removal extent of calcium from the delimed hide. The delimed hide with lower residual calcium content produced a crust leather with better

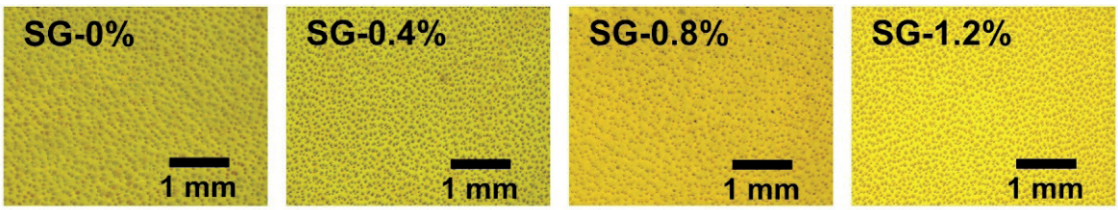

Figure 5. Stereomicrograph of the crust leather.

Table II

Color parameters of the crust leathers

Sample	L^*	a^*	b^*	ΔE_1 [a]	ΔE_2 [b]
SG-0%	57.43 ± 3.60	3.58 ±1.70	48.45 ± 4.24	23.24 ± 3.41	7.57 ± 3.07
SG-0.4%	53.29 ± 3.06	2.97 ± 1.63	59.42 ± 3.60	14.58 ± 4.01	5.80 ± 2.80
SG-0.8%	56.96 ± 1.41	2.38 ± 1.06	68.11 ± 2.04	5.77 ± 1.69	3.88 ± 1.54
SG-1.2%	56.06 ± 1.11	1.94 ± 0.88	73.38 ± 1.69	0	2.24 ± 0.78

[a]ΔE_1 reflects the color difference among the four crust leathers. The crust leather SG-1.2% was used as the calibration board.

[b]ΔE_2 reflects the color difference among various points in a single crust leather. One of the points on the crust leather was used as the calibration board.

Table III

Physical properties of the crust leathers

Sample	Tensile strength (N/mm^2)	Tear strength (N/mm)	Bursting strength (N/mm)	Softness (mm)
SG-0%	8.0 ± 1.0	54.6 ± 2.9	266.7 ± 11.2	4.6 ± 0.1
SG-0.4%	8.4 ± 0.8	58.1 ± 2.3	276.6 ± 10.9	5.0 ± 0.2
SG-0.8%	11.6 ± 1.4	69.4 ± 3.2	335.3 ± 11.6	6.2 ± 0.2
SG-1.2%	12.3 ± 1.0	71.9 ± 2.8	336.1 ± 15.1	6.6 ± 0.1

Values are means ± standard deviations of six determinations.

physical properties, probably because better bating and chrome tanning performances were obtained and promoted the penetration and fixation of post-tanning agents and fatliquors.

Conclusion

Calcium removal from delimed hide greatly affects the bating, chrome tanning, and post-tanning performances. Efficient calcium removal from delimed hide is beneficial to improve the enzymatic bating effectiveness, the uniform distribution of chrome tanning agent in wet blue, the color brightness and uniformity of wet blue and crust leather, and the physical properties of crust leather. Therefore, the ability of deliming agent to remove calcium and the extent of calcium removal from delimed hide should be considered to be important indicators to guarantee the high quality of leather.

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Objective Review: Advanced Testing and Toxicity of Restricted Substances for Sustainable Leather Industry

by
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Abstract

Leather industry uses a great number of processing chemicals to make leather from animal hides. The toxicity, health and environmental impacts of many of these chemicals have been established leading to significant regulatory certifications and programs helping in trade and policy making. The focus of this review are the hazardous chemicals and auxiliaries relevant to leather industry, their categories, possible origins, analysis and toxicity with respect to humans and the environment and the regulatory mechanisms suggested for them through manufacturing restricted substance list (MRSL) 2.0.** Informative guidelines are provided about the most probable leather processing stage for the origin of these chemicals. Some alternative technologies, chemicals and ideas gaining popularity are also suggested as probable remedies. Recommended test methods are stated for the adequate monitoring of the hazardous chemicals. The nature and severity of chemical toxicity and corresponding limits set for their allowed use in formulations are graphically expressed for clarity and ease of understanding. The maximum number of compounds / isomers belong to classes comprising chlorinated paraffins, perfluorooctanoic acids (PFOA), perfluorooctanesulfonates (PFOS) and banned aromatic amines. Similarly, most of the restricted chemicals are used during the finishing stage and may be considered for eco-friendly alternatives. Likewise, the analytical equipment covering most testing requirements is GC-MS among other hyphenated techniques. Additionally, most critical chemicals from toxicity point of view are arsenic, cadmium and chromium (VI), whereas navy blue colorants and chlorinated aromatic compounds may be considered as less toxic among the restricted chemicals under the current scope. Surprisingly, the latest UV absorbents and polymeric fatliquors are comparatively non-toxic. Similarly, the most relaxed formulation limits are given for UV absorbers and 2-methoxypropylacetate whereas strict limits have been set for PFOA, PFOS, mercury, triclosan permethrin, sensitizing dyes etc. in MRSL 2.0.

Introduction

Leather industry is one of the oldest industries known. The popularity of leather products is because of their durability, feel, strength, quality, long service life and natural looks that still make them attractive among high-end markets. It takes a large number of different processing chemicals, many important time taking steps and a team of leather experts to make good quality leather from raw hides.[1] With the commencement and frequency of various toxicological and case studies related to human health and environment, some chemicals were classified as unsuitable for human use and/or detrimental for the environment. Unfortunately, certain hazardous chemicals are still in use because suitable alternates are either not available or they are not as efficient as the conventional chemicals in use. The information related to safety and toxicity, upon its availability stressed on the establishment of permissible limits for the target chemicals in leather processing chemicals.[2] It also compelled leather manufacturers to frequently monitor leather products to ensure that the presence of such compounds is under pre-defined limits.

Regulatory Standards and Certifications for Leather Sector Sustainability

The leather industry has been a major stake holder on the manufacturing and export side in many developing countries. This emphasizes the importance of appropriate reforms necessary in the leather sector to achieve the sustainability development goals (SDGs) set by the member states by the year 2030.[3] It is interesting to note that the certification programs already developed and implemented so far, directly address 12 out of 17 SDGs.

The very first regulation was implemented by Germany for Pentachlorophenol (PCP) and Azo Dyes in 1990.[4] Later on a number of certification programs were introduced to address social, economic, environmental and equality, security, and public health related issues focusing on leather and allied industries and businesses. Bluesign is an endorsement focused on human health,

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air emissions, water management and client well-being.[5] Likewise, Content Claim Standard (CCS), as the title suggests, is related to the third party verification of product contents and certification. Also, Cradle to Cradle is a certification focusing on material health, material reuse, social responsibility and sustainable use of energy etc. The system offers grading of manufacturers according to their level of compliance.[6] Moreover, the Fairtrade certification program provides reforms in social, economic and environmental aspects of small and medium leather manufacturing units in developing countries.[7] Aiding the global efforts, the European Union (EU) initiated Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) in 2006 to address the management of hazardous chemicals in a more comprehensive way.[8]

Two certifications focusing on recycling are Global Recycle Standard (GRS) and Recycled Claim Standard (RCS). GRS is a certification program initially launched in 2008 for ecological and human health benefits. It emphasizes on the utilization of recycled materials along with checking and control of physical and chemical parameters before discharge of effluent into the environment. It also includes all the checks already suggested by Global Organic Textile Standard (GOTS)[9] and puts limitations on the use of synthetic substances. RCS is another standard focusing also on reuse and recycling of materials from input up to the final product. Similarly, the Higg Index was initiated by Sustainable Apparel Coalition (SAC) in 2012. Higg index works as a tool for the assessment of sustainability of the entire cycle of the end product, identifying room for necessary improvements.

Oeko-Tex is a globally acclaimed test standard initiated in Germany in the year 1995. Oeko-Tex certification is awarded to leather or leather products for one year. It ensures that the leather product and processing are both eco and human friendly.[10] Furthermore, Source Map ecolabel concentrates on the information on the starting points of a product's sections and also its social and natural footprints.[9] In the same way, Internationale Verband der Naturtextilwirtschaft (IVN) Natural Leather Standard was established in 1999. IVN provides two types of quality seals, Naturleder IVN certified and Naturtextil IVN certified BEST; mainly addressing social and environmental aspects.[9]

A prominent association of a group of leather manufacturing giants, retailers, brands and technical experts initiated the Leather Working Group (LWG) in 2005[11] and introduced a complete accreditation program to revolutionize the conventional leather making practices to green leather manufacturing. The standard addresses waste management, control of toxic chemicals, carbon footprint, energy and water usage optimization, and implementation of safety protocols.[12] The Leather Working Group has been a major driver of the industry towards sustainable leather manufacturing.[9]

With a similar reputation, the Worldwide Responsible Accredited Production (WRAP) program has been the world's biggest sector based program for leather consisting of compliance with 12 principles related to ethical workforce utilization, environmental sustainability, gender equality, health and security. In addition to these certification programs, two important socioeconomic certifications linking international trade are Sustainable Fair Trade Management System (SFTMS) and SA8000 Certification. The later has been an internationally recognized program for social reforms in the leather manufacturing sector, launched in 1997.[13]

Another pertinent development was the establishment of National Science Foundation (NSF) in 1994. The first assessment of NSF incorporates Zero Discharge of Hazardous Chemicals Manufactured Restricted Substances List (ZDHC MRSL). The second assessment called ToxFMD Screened Chemistry Program screens all chemicals used in the manufacturing process ensuring human well-being globally. Some other initiatives are Leather Sector – Sustainability Certification Program as an agreement between Institute of Quality Certification for Leather Sector (ICEC), Italy and Brazilian Leather Certification of Sustainability (CSCB) program in 2018 and Brazilian Carbon Disclosure Project (CDP).[11]

ZDHC MRSL 2.0

The first restricted substances list, ZDHC MRSL 1.0 was issued back in 2014. The next version, 1.1 was launched the next year.[8] The second revision of ZDHC MRSL (version 2.0) was published in January 2020.[2] It includes more than 450 compounds belonging to around 22 different classes of compounds (Table I).

The various chemical classes comprise of different number of compounds, isomers etc. Although, some chemicals contribute less in numbers but their screening is highly important due to their relative toxicity such as the metals like arsenic, chromium (VI) and chlorinated aromatic compounds. The actual compounds in each class have been detailed along with their chemical abstract service (CAS) numbers in the LWG chemical module.[2]

Table I

Details of analysis and formulation limits for restricted chemicals[14] relevant to leather

S. No.	Analyte / Class of Analyte	Test methods (if available)	Detection Techniques	Formulation Limits
1.	Nonylphenols (NP)	ISO 18218-2:2015 [IULTCS/IUC 28-2]	LC-MS or GC-MS	250 ppm
2.	Nonylphenolethoxylates (NPEO) and Octylphenolsethoxylates (OPEO)	EN ISO 18218-1:2015	LC-MS & GC-MS	500 ppm
3.	Octylphenols (OP)	ISO 18218-2 (2019)	LC-MS & GC-MS	250 ppm
4.	2-phenylphenol (OPP)	ISO 13365-1 & 2:2020	LC	5000 ppm
5.	Permethrin	Jinlan et. al., 2020[15]	LC-MS/MS & GC-MS/MS	250 ppm
6.	Triclosan	Jahangir et. al., 2020[16]	LC MS, DAD	250 ppm
7.	Dichlorobenzene	Similar to DIN 54232:2010	GC-MS	500 ppm
8.	Other isomers of chlorobenzene & chlorotoluene	Similar to DIN 54232:2010	GC-MS	Sum = 200 ppm
9.	Pentachlorophenol (PCP) and Tetrachlorophenol (TeCP)	EN ISO 17070	GC-MS	Sum = 20 ppm
10.	All other Chlorophenols	EN ISO 17070:2015	GC-MS	Sum = 50 ppm
11.	Dyes – Azo forming banned aromatic amines	ISO 17234-1 & 17234-2	LC, GC	150 ppm (for each)
12.	Navy blue colorants and Sensitizing Dyes	DIN 54231:2005	LC	250 ppm for both
13.	Restricted Dyes (Carcinogenic)	DIN 54231	HPLC	250 ppm
14.	Flame retardants	EN ISO 17881-1:2016	GC-MS	250 ppm
15.	Fatliquoring agent with Short-chain chlorinated paraffins, (SCCPs), (C_{10} – C_{13}) & Medium-chain chlorinated paraffins, (MCCPs), (C_{14} – C_{17})	prEN ISO 22699-2, ISO/DIS 18219-1(en)	GC-MS, GC/ECNI-MS	250 & 500 ppm respectively
16.	Glycols / Glycols Ethers	Wang, et. al., 2014[17]	GC MS [17], HPLC, LC-MS	50 ppm
17.	2-methoxypropylacetate	Based on US EPA 8270	HPLC, LC-MS, GC MS	1000 ppm
18.	Halogenated Solvents	Chorier 2014[18]	GC-MS	5 & 40 ppm
19.	Organotin Compounds	CEN ISO/ TS 16179:2012	GC-MS	1 – 20 ppm
20.	Polycyclic Aromatic Hydrocarbons (PAHs)	AFPS GS 2014	GC-MS	Sum = 200 ppm
21.	Benzo[a]pyrene (BaP) and Naphthalene	AFPS GS 2014	GC-MS	20 and 300 ppm respectively
22.	PFOS and PFOA	prISO FDIS 23702-1:2018	LC-MS	Sum = 2 ppm and 25 ppb respectively
24.	Phthalates	ISO/TS 16181	GC-MS	Sum = 250 ppm
25.	Arsenic (As), Cadmium (Cd), Mercury (Hg) and Lead (Pb)	DIN EN ISO 17072-1:2017	ICP-OES, AAS	50, 20, 4 and 100 ppm respectively
26.	Chromium (VI)	EN ISO 17075-1:2017	ICP-OES, AAS	10 ppm
27.	Antimony	DIN EN ISO 17072-1:2017	ICP	Dye 50/ Pigment 250 ppm
28.	UV Absorbers (Conventional)	DIN EN 62321-6:2016-05	LC-MS/MS, GC-MS	1000 ppm
29.	VOCs	DIN CEN ISO/TS 16189:2013	GC-MS	500 ppm
30.	Benzene	Similar to AFPS GS 2014	GC-MS	50 ppm

where

AAS = Atomic Absorption Spectroscopy
AFPS = German Product Safety Commission
DAD = Diode array detection
ECNI = Electron capture negative ion chemical ionization
ICP-OES = Inductively Coupled Plasma - Optical Emission Spectrometry
VOCs = Volatile organic compounds

Control at Process Inputs for Restricted Substances

An effective way of reducing hazardous chemicals in the product is to control them at the raw materials input level, i.e. during the initial stages of manufacturing. Even the water used for wet processing stages could introduce unwanted substances like PFOS and PFOA into the system, paralleled by retanning and fatliquoring stages. The probable origins of hazardous substances according to MRSL (ver. 2.0) are presented in Table II and Figure 1, highlighting the importance of input controls.

It can be observed that most chemical classes and individual chemicals fall in the finishing stage, followed by dyeing stage as well as product manufacturing. Other categories include curing, fatliquoring, pigment and chemical formulations, water used in processing etc. which can incorporate number of potentially toxic chemicals into the final product.

Alternate Processes and Chemicals

Alternates for many processing stages and chemicals have been successfully trialed but their economic feasibility and commercial

Table II

Probable sources of origin for various restricted substances in leather

Analyte / Class of Analyte	Most Probable Origin
Alkylphenols and alkylphenol ethoxylates	Detergents, degreasing agents, dye and pigment preparations, wetting agents, emulsifier/dispersing agent, impregnating agents etc.[2]
2-phenylphenol (OPP)	Some use is permitted as a preservative in formulations.[2]
Permethrin and Triclosan	*May be present in low quality anti-microbials and biocides used for preservation
All chlorophenols	*Used as anti-mould before being regulated.
Azo forming banned aromatic amines, Navy blue colorants and Sensitizing Dyes	*May originate from dyes and pigments
Restricted Dyes (Carcinogenic)	*May originate from dyes
Flame retardants (halogen containing)	*Banned, but may still be used in some chemical products used in leather finishing
SCCPs, (C_{10} – C_{13}) & MCCPs, (C_{14} – C_{17})	Fatliquors
Glycols / Glycols Ethers	*Possible origin through solvents for finishing or printing agents and fat/oil diluters or dissolving agents.[2]
Halogenated Solvents	May originate from finishing chemicals and solvents
Organotin Compounds	*Probable origin in leather products could be through plastics, glue and polyurethane
Polycyclic Aromatic Hydrocarbons (PAHs)	*PAHs are added in plastics, rubbers, coatings and lacquers. They could be present in carbon black dyestuffs as impurities. In footwear industry, PAHs are frequently found in the outsoles and in screen printing pastes.
PFOS and PFOA	*Possible origin could be oil, water and stain repellents.[2]
Phthalates	*May be detected in leather products, originating through any plastic parts or adhesives used.
Heavy metals: As, Cd, Hg and Pb	Heavy metals are not intentionally used in leather. Arsenic may originate from polluted water.[19] Cadmium, mercury and lead could originate through certain pigment formulations used during finishing stage.
Chromium (VI)	*Could be detected due to oxidation of chromium (III)
Antimony	*May originate through certain pigment
UV Absorbers (Conventional)	*Used in some chemical formulations for stability against UV and visible light.
VOCs	*They are associated with solvent based polyurethane coatings at finishing stage and glues and adhesives

*Not intentionally used in leather.

Figure 1. Likelihood of MRSL compounds emerging from different leather processing stages

success still needs to be figured out. Halophyte plants containing large amount of salts have been suggested as a replacement for salt as hide preservatives and can also act as biocides owing to their terpene contents.[20] Similarly, dehairing can also be performed through an effective way by oxidative method replacing sodium sulfide with sodium percarbonate.[21] Successful trials on enzymatic dehairing have also been carried out with proteases isolated from *Aspergillus tamarri* species,[22] combination of proteases and lipases[23] and activated proteases within lesser time.[24]

The demand for chrome free tanning has increased since the deleterious effects of hexavalent chromium have been established. A recent study utilizes an epoxy modified dialdehyde starch, producing a biomass based tanning agent; replacing chromium in the tanning process towards a good quality leather.[25] Furthermore, an attempt to replace toxic organic and inorganic tanning agents with a compact glyoxal based tanning system also aiding in upgradation of low grade skins has shown promising results.[26]

Fatliquors introduced during leather processing may contain medium and short chain chlorinated paraffins. A proposed way of avoiding them is to use fatliquors based on vegetable oils.[27] More recent work suggests using polymeric fatliquors that have considerably higher performance as compared to the conventional fatliquors and are also compatible with chrome-free leather manufacturing systems.[28]

Similarly, effective strategies for reducing the use of VOCs have also been reported.[29] The accidental presence of hexavalent chromium should always be kept in check as well as the mass fraction of fungicides should be within the permissible limits. Likewise, an example of possible utilization of ionic liquids in leather processing has been explored with fiber opening properties. In combination

Figure 2. Recommended Analysis Techniques for MRSL (2.0) chemicals and compound classes

with certain enzymes, they could be a promising alternate for replacing dangerous chemicals like sodium sulfide and calcium hydroxide.[30] Many similar processes, chemical and technology alternatives have been reviewed by Covington and Wise.[31]

The more recent advances have brought the concept of modern tannery 4.0 with the aim of achieving near zero discharges, lowering wastages and enhancing productivity through state-of-the-art automation employing information communication technology and internet of things.[32] This information could also be helpful in troubleshooting repetitive positive tests for specific restricted compounds leading to their most probable source of origin.

Analysis Recommendations

The modern advancements in technology have provided adequate technical support and literature on quality assurance and testing of various toxic chemicals.[33] The analysis methods may include either digestion or extraction depending on the type of analyte. For some compounds additional steps of derivatization or reduction may be required. Various analytical techniques have been recommended for the analyses of restricted chemicals. Owing to the capabilities of the technique to analyze a wide variety of organic compounds it is not surprising that GC-MS is the technique of choice for a large number of chemicals mentioned in MRSL (2.0) as shown in Table I and Figure 2. For metal analysis, the best option is ICP-OES followed by AAS. For compounds with large and small organic moieties, GC, LC, HPLC, GC-MS, LC-MS and LC-MS/MS have been recommended (Table I and Figure 2). For chemicals like organotins and SCCPs, low resolution mass spectrometry (LRMS) and ECNI-MS have been suggested respectively. Similarly, for the typical analysis of perfluoroalkyl compounds LC-MS is preferred. Some analytes could be quantified with multiple techniques like

glycols and glycol ethers, SCCPs and MCCPs, banned aromatic amines, UV absorbers (conventional), AP and APEO etc. A rough estimate of test costs could also be projected considering the general costs incurring on the recommended sophisticated hyphenated techniques. For some compounds the standard test methods for leather are still under development and will soon be available. However, supporting literature is available as analyses have been reported in other matrices (Table I).

Toxicity

Chemicals are widely used in leather industries such as basic chromium sulphate, synthetic tanning agents, fatliquors, resins, formaldehyde, biocides, surfactants, dyes, pigments and fire retardants, etc.[34,35] The use of hazardous leather chemicals may lead to acute and chronic toxicities in humans. The release of hazardous chemicals into the aquatic environment, i.e. seas, lakes and oceans, due to lack of proper management and effluent treatment prior to its discharge may lead to aquatic toxicity.[36] A comparative toxicity profile of the restricted compounds according to MRSL 2.0 is presented in Figure 3 in the form of a heat map chart with the effects of most toxic compounds highlighted by a reddish color tone and the least toxic by a dark greenish tone. The colors are assigned on the basis of the vitality of the body organs affected and the severity of the detrimental effect. The chemical classes and compounds are discussed in order of decreasing priority with the most toxic ones addressed first. The most harmful substances for the aquatic environment among the restricted substances are alkylphenol (AP), alkylphenol ethoxylates (APEO), chloro compounds and (PAHs).

Second to them are navy blue colorants, SCCPs and halogenated solvents, whereas organotin compounds are reported to have comparatively lower toxicities (Figure 3). According to toxicity studies exposure to chlorophenols[37], navy blue dyes[38], short chain chlorinated paraffins (SCCPs)[39] and some chlorobenzenes[39] may lead to chronic adverse effects on the aquatic life. SCCPs are categorized as persistent organic pollutants (POP). Certain organotins may also be persistent pollutants and could be harmful to the life in aquatic environment when exceeding specific concentration levels.[39] Certain PAHs[39] and dyes[40] can also cause toxicity of the aquatic environment. In the environment, alkylphenolethoxylates breakdown into alkylphenols, that are very noxious to aquatic environment if exceeding specific exposure levels.[39] Permethrin is toxic for bees being an environmental concern.[41]

The compounds posing threats to human health are monitored on priority basis and the most toxic compounds among the restricted substances are chromium (VI), arsenic and cadmium. The toxic effects of arsenic (especially in drinking water) are well known,[42] followed by mercury and lead as they affect brain and the central nervous system (CNS). Arsenic poisoning is a serious health issue disturbing millions of people worldwide. Short-term arsenic toxicity causes abdominal pain, vomiting, nausea and severe diarrhea. Additionally, peripheral neuropathy and encephalopathy are also on record. Long-term arsenic toxicity leads to multisystem illness.[43] Cadmium and hexavalent chromium are also classified as highly toxic compounds that could possibly cause cancer.

Cadmium exposure has been linked with cancers of the prostate, stomach, kidney, hematopoietic system and liver.[44,45] Some regulatory organizations such as the International Agency for Research on Cancer (IARC) and U.S. National Toxicology Program have identified cadmium compounds as human carcinogens.[46] Hexavalent chromium is a frequently reported job-related carcinogen linked with sinus, nasal and lung cancers.[47] Likewise, mercury can induce a variety of chronic and acute symptoms affecting several organs such as the heart, lungs, CNS and kidneys. It may also develop problems in liver, skin, muscles, gastrointestinal tract, and reproductive system and may cause harm to the newborn.[48] SCCPs are suspected of producing specific cancers. Continuous exposure to SCCPs could result in drying of skin and the formation of cracks.[39]

Among other severely toxic compounds are halogenated phenols[37] and halogenated aromatic solvents, banned aromatic amines[49], benzene, 2-thiocyanomethylthiobenzothiazole (TCMTB), restricted dyes, some flame retardants (halogen containing), PAHs, PFOA and OPP. Exposure to these compounds may lead to the development of cancers or tumors. Certain chlorobenzenes are noxious if inhaled or come in contact with the skin, when exceeding specific exposure levels.[39] Benzene shows carcinogenic potential for multiple organs in animals. Epidemiological research has revealed that it is leukemogenic in humans.[50] Dyes with carcinogenic or equivalent concern are reproductive toxicants or may pose mutagenic and/or skin contact threats.[40]

There are many biocides generally in use, however, the toxicity of only two is discussed, limiting to the scope of MRSL 2.0. Permethrin is a suspected thyroid disruptor and an irritant of skin and eyes. It has been designated as class 3, unclassifiable carcinogen by IARC.[41] Triclosan has been categorized as a possible carcinogen assigned to class 2B as suggested by IARC. It is highly corrosive in nature and exposure to the chemical may cause hypotension, pulmonary edema, myocardial failure, neurological changes, renal and liver toxicity, hemolysis and methemoglobinemia.[61]

Evidence has revealed that numerous halogen containing flame retardants are linked with detrimental health impacts on humans and animals including reproductive toxicity, thyroid and endocrine disruption, cancer, effects on the immune system, detrimental impacts on child and fetal, neurologic and development function.[55] In the case of PAHs, continuous exposure above specific concentrations could cause certain cancers to develop and some PAHs, may lead to infertility in humans or result in harm to fetus.[39] Likewise exposure

Figure 3. Toxicities of restricted substances expressed through heat map chart

to PFOS and PFOA can lead to developmental and reproductive problems in kidney, liver and immunological effects and tumors in animals. Most reliable conclusions are high levels of cholesterol in affected populations, with sparser findings relating to lower birth weights of babies, cancer (PFOA), thyroid hormone disturbance (PFOS) and effects on the immune system.[57] OPP has been linked to toxicity in humans, including reproductive carcinogenicity and neurotoxicity, developmental and acute toxicity.[60] Navy blue dyes are skin sensitizers.[38]

Certain other compounds have been connected to detrimental effects on neurologic functions and the human immune system such as some flame retardants (halogen containing), glycol ethers, organotin compounds, PFOA and PFOS, arsenic and OPP etc. Glycol ethers and lead may cause anemia and blood related disorders. In humans, acute exposure to high concentrations of glycol ethers causes pulmonary edema, narcosis and serious harm to kidney and liver. Continuous exposure to these compounds could cause blood and neurological effects, including anemia, tremor, fatigue, and

nausea. No evidence is available for any carcinogenic potential of glycol ethers in humans however, they have been connected with liver damage.[56]

Certain organotins, above specific exposure levels, may confer immunotoxic effects and some may lead to infertility in humans or result in harm to fetus.[39] Compounds affecting the pulmonary system and lungs include chlorotoluenes, glycol ethers, phthalates and VOCs. Continuous exposure to certain halogenated solvents (e.g. chlorotoluene), above specific concentrations, could lead to specific cancers. Certain halogenated solvents may be noxious to aquatic life when present above specific concentration levels.[39] Arsenic and lead can disrupt the stomach. Short-term exposure to lead causes kidney damage, brain damage and gastrointestinal illnesses, whereas long-term exposure could result in detrimental effects on vitamin D metabolism, blood, kidneys, blood pressure, and central nervous system.[46] In humans, there is strong proof suggesting that high concentrations of cresols (VOC) are absorbed quickly during oral or dermal exposure, producing extreme toxicity (being corrosive) that could be fatal. Inhalation could cause respiratory tract irritation. No evidence exists related to the long-term toxicity of these compounds and there is insufficient information about the carcinogenic capability of cresols.[59] Chemicals like AP & APEO, some flame retardants (halogen containing), organotin compounds, PAHs and OPP may confer reproductive disorders and even infertility. These compounds have also been linked with damage to human fetus with the addition of phthalates. NP could lead to infertility in humans and result in harm to fetus.[39] Conventional UV stabilizers and absorbers in general cause developmental toxicities.[62,63] However, many recently discovered UV absorbing compounds are actually eco-friendly. For e.g. some marine natural products such as micosporine-like amino acids have been shown to absorb harmful UV radiations in UVA and UVB regions.[64]

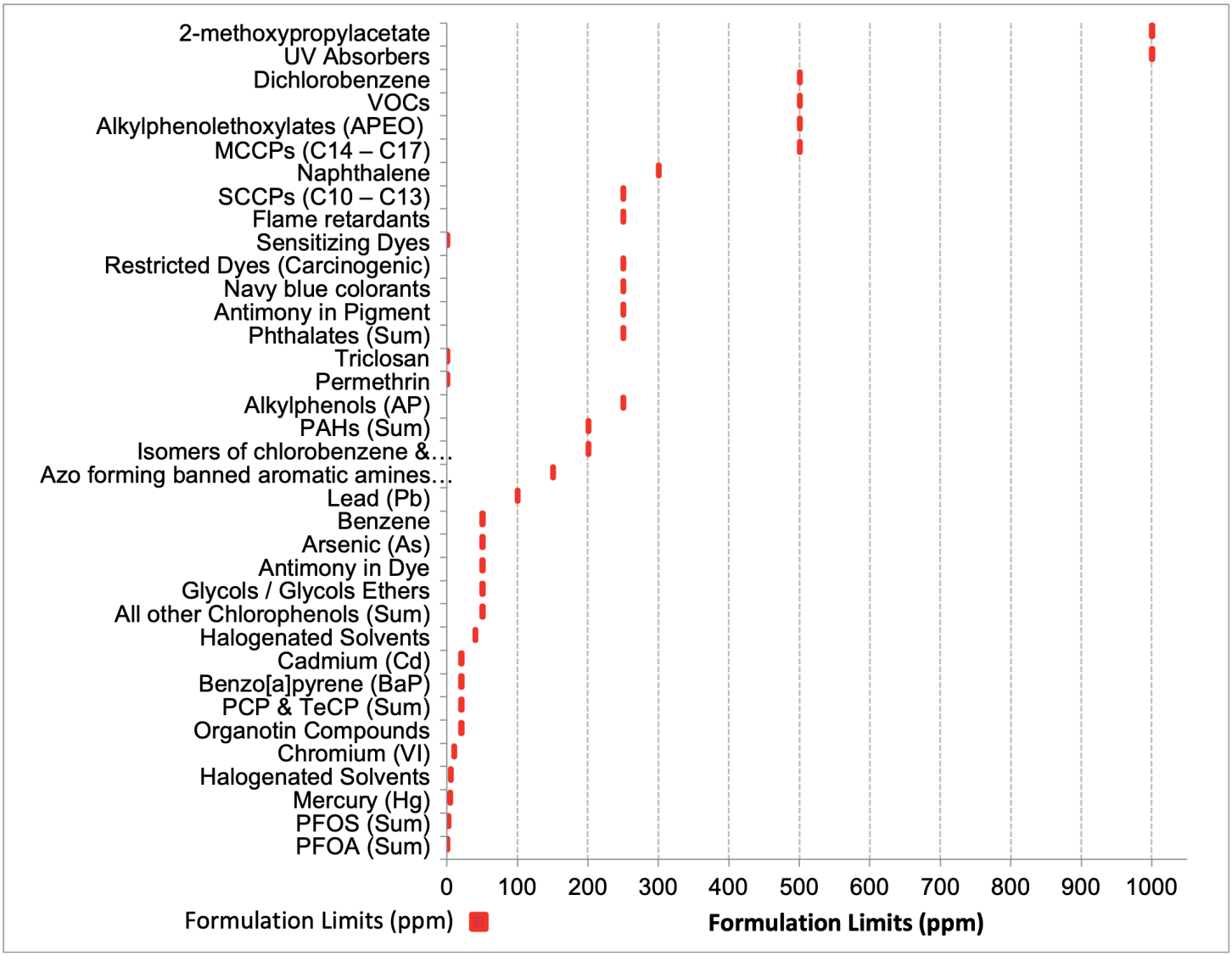

Figure 4. List of restricted substances (MRSL 2.0) with formulation limits (ppm)

There is evidence that phthalates affect male organs in newborn infants and may cause hormone imbalance, asthma and bronchial diseases.[58] Flame retardants (halogen containing), PFOS and phthalates have also been identified as hormone disruptors. Though, the newly developed flame retardants are comparatively non-toxic.[65] The comparatively less toxic compounds being those that do not penetrate the body deeply, affecting the skin only, such as colorants, SCCPs, OIT, *p*-chloro-*m*-cresol (PCMC) and some chlorinated benzene derivatives etc.

For human health, the level and pace of toxicity of the restricted chemicals and their property to invade human body serve as the decisive factors for setting their formulation limits (Figure 4). For the assessment of environmental concerns, the level of toxicity for the environment in general and the persistence of chemicals in the environment are considered.

Prospective Compounds

The listing of toxic compounds, their monitoring and search for alternates is a continuous process. In this regard a list has been formulated including aniline (free), diazene-1,2-dicarboxamide (ADCA), cyclic siloxanes, dimethylfumarate, carcinogenic dyes (or equivalent concern), flame retardants (halogen containing), formaldehyde, perfluorinated and polyfluorinated chemicals (PFCs), phenol, solvents and total heavy metals.[14] Another list of archived chemicals have been issued to strike out the use of chemicals banned earlier in order to avoid their reuse. Those include carcinogenic dyes (or equivalent concern), solvents and miscellaneous chemicals.[14]

Conclusion

The review is aimed to present a better and meaningful picture of the restrictions suggested in MRSL 2.0. Finishing has been identified as the leather processing stage having enormous potential for chemical replacements towards eco-friendly productions. The toxicity profiles of these chemicals also signify the importance of their screening and the justification for their formulation limits. GC-MS covers the analyses of most of the analytes highlighted in MRSL 2.0. The graphical presentation of data and grouping of information may lead to conclusive assessment, enabling leather manufacturers and chemical suppliers to have better controls and monitoring strategies for these chemicals. The guidelines for prospective chemicals in future revisions and testing most of the analytes under consideration are available where standard test methods have not been established.

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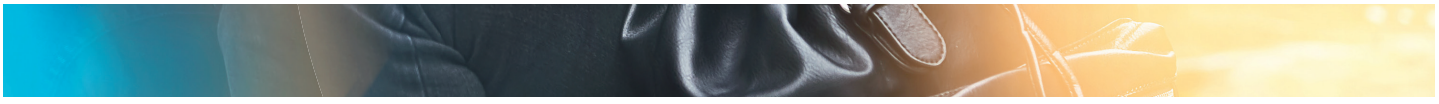

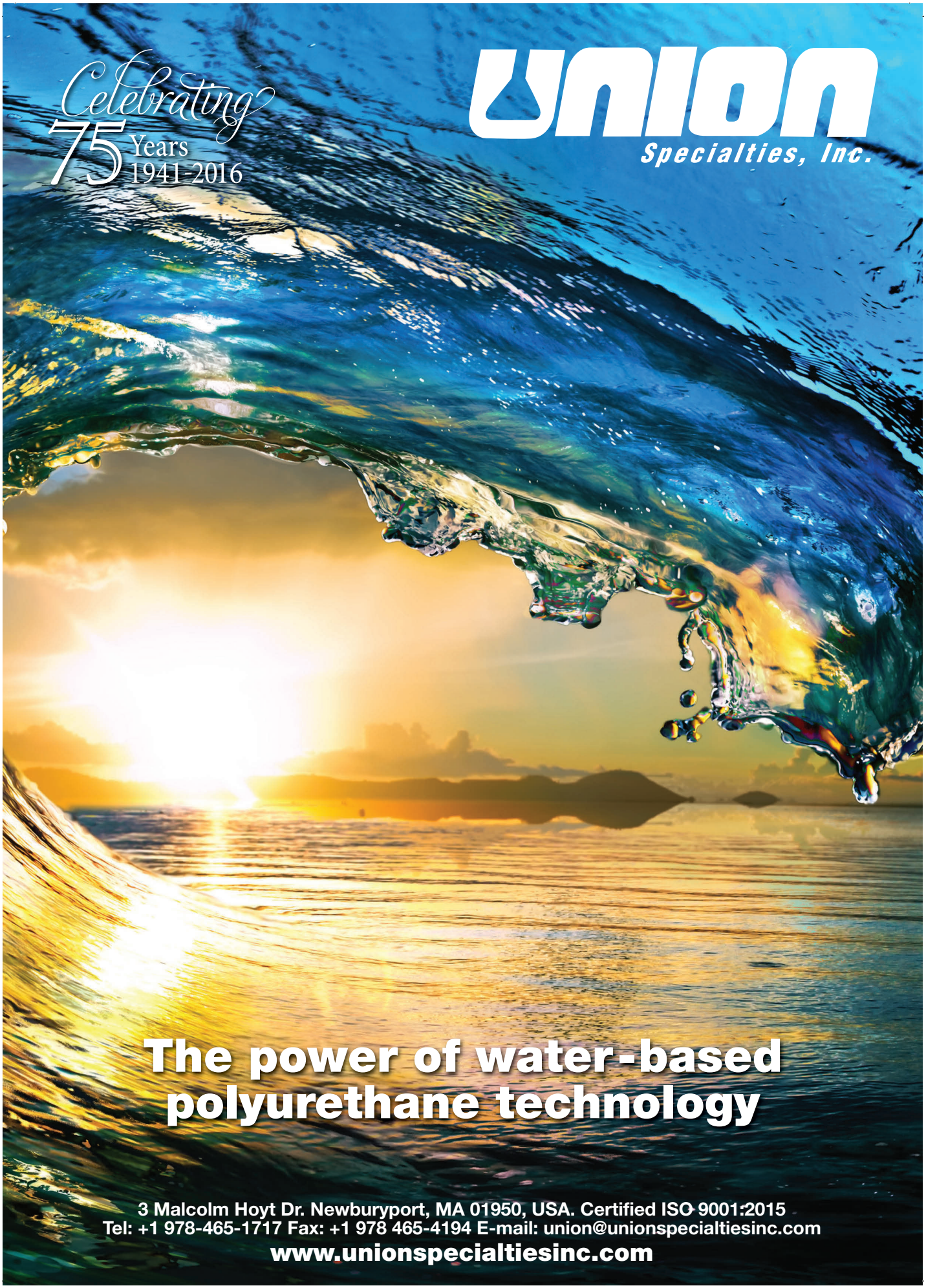
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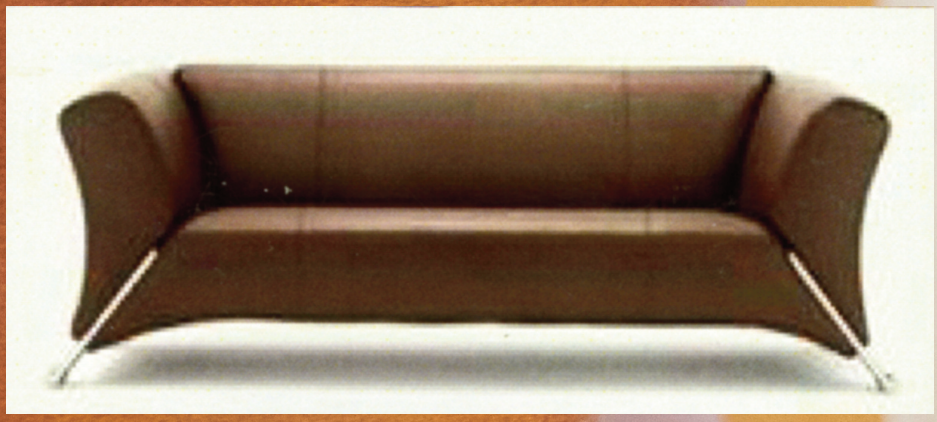

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Bi Shi, see *JALCA* **99**, 220, 2004

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(HEC Postdoctoral Study Program) to conduct research on 'Synthesis of eco-friendly dyes and metal complexes' with Prof. Dr Harold S. Freeman in North Carolina State University (NCSU), USA. Dr Kashif has been awarded with Research Productivity Allowance (RPA) for three times by Pakistan Council of Science & Technology (2004, 2005 &2006). Dr Kashif made several In-house R & D Projects and proposals (funded) mostly addressing Eco-friendly Synthesis of Leather and Textile auxiliaries for application in Leather & Textile industries. Dr Kashif has expertise in the testing of toxic and hazardous chemicals and substances in Leather and Textile. Dr Kashif has several national and international publications in reputed International Journals. His key areas of interest are eco-friendly and sustainable approaches in synthesis, processing and analysis of leather and textile materials.

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