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A Novel Ester-bonded Gemini Quaternary Ammonium Salt with Good Antimicrobial Activity and Anti-mold Performance for Wet Blue Leather

by

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Abstract

Herein, we have developed a kind of novel ester-bonded gemini quaternary ammonium salts microbicides named QAS(n+m) for leather application, in which n and m denotes the number of $-CH_2$ between the ester groups on the spacer, and the length of alkyl chain connected to ester groups on the hydrophobic tail chain, respectively. Results indicate that the hydrophobic tail lengths and the spacer lengths in the QAS(n+m) structures affect their antimicrobial activities very differently. QAS(4+10) was used to investigate the antimicrobial mechanism, cytotoxicity, and anti-mold performance for wet blue leather as it exhibits the strongest antimicrobial effect. It shows QAS(4+10) is capable of inactivating microorganisms mainly by disrupting the integrity of their cell membranes. Compared to commercial leather microbicide product 2-thiocyanomethylthiobenzothiazole (TCMTB), QAS(4+10) exhibits comparable mold resistance and lower toxicity. The present work gives positive insight into the development of novel candidate microbicides for the preservation of wet blue leather.

Introduction

Natural leather has been popularly used for fashion, footwear, upholstery and many crafts due to its breathability and durability.[1] Leather is made from hides and skins, which are basically constituted of 60% ~ 70% water, approximately 30% of proteins and a certain amount of fat.[2] These nutrient substances can serve as metabolic substrates for bacteria and fungi. Microorganisms are capable of contaminating hides at any leather processing stage ranging from slaughter to finished leather,[3] resulting in undesirable pigmentation, non-uniformity of dyeing and finishing, fatty acids production, poor physical-mechanical properties of leather.[4] Therefore, the proper use of microbicides, i.e. preservatives and fungicides, is considered as a facile and effective strategy to control microorganisms.[5] Generally, preservatives protect hides and skins against bacterial invasion (such as Halophilic bacteria, *Micrococcus, Bacillus, Pseudomonas, Proteus* and *Escherichia*),[3] while fungicides protect leather from fungal contamination (such as *Aspergillus, Penicillium*, *Paecilomyces, Scopulariopsis, Trichoderma and Rhizopus*).[3] To prevent the growth of these microorganisms, many antimicrobial agents have been used in leather industry, including phenols (e.g. 4-chloro-3-methylphenl, trichlorophenol), esters (e.g. dimethyl fumarate), aldehydes (e.g. glutaraldehyde) heterocyclic compound (e.g. N-octilisotiazolinona and 2-thiocyanomethylthiobenzothiazole), quaternary ammonium salts (QAS) and so on. Among them, the most widely used preservatives pentachlorophenol (PCP) and trichlorophenol (TCP) and fungicides dimethyl fumarate (DMF) have been restricted because of their high toxicity and ecological hazards.[6,7] Nowadays, 2-thiocyanomethylthiobenzothiazole (TCMTB) has become one of the main microbicides used in leather industry due to its excellent antimicrobial property. However, its toxicity and hazard assessments are also attracting great concern. It has been reported that TCMTB has potential sensitization[8] and reproductive toxicity[9] and can cause potential environmental problems.[10,11] More importantly, the TCMTB content in leather products has been limited to below 250.0 mg/kg in the latest testing standards *LEATHER STANDARD by OEKO - TEX®*.[12] Therefore, it is highly desirable to exploit novel nontoxic or low-toxic leather microbicides.

In the past decades, quaternary ammonium salts with single tail and single polar head (QAS) have been intensively investigated owing to their good antimicrobial activity. As developed, gemini QAS which is composed of two conventional QAS connected through the spacer[13] are emerging as the new generation of QAS compounds because they have lower critical micelle concentration (CMC),[14] unusual aggregation behavior,[15] lower cytotoxicity,[16] better water solubility, antimicrobial activity and broad-spectrum compared with the conventional ones.[17,18] Most of studies about gemini QAS focus on synthesis, structures and applications such as in agriculture,[19] textile industry[20] and leather industry.[21] As reported, introducing various functional groups and altering the hydrophobic tail lengths or spacer lengths can magnificently affect the properties of gemini QAS.[22] For instance, the melting points of the gemini QAS will be reduced by introducing hydroxyl groups,[23] and the introduction of heteroatoms into the gemini QAS can decrease its aquatic toxicity.[24,25]

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Currently, ester-bonded gemini QAS has drawn increasing interest owing to their better biodegradability and compatibility with the environment.[26,27] Nevertheless, reports on the studies of gemini QAS containing multiple ester groups are considerably insufficient, the details of relations between the structures especially alkyl chain lengths and their antimicrobial property, and their ability to preserve leather against microbes contamination remain unclear, which is important for their applications in leather industry.

In this study, we synthesized nine novel gemini QAS compounds with four ester groups. In our approach, alcohol compounds were functionalized with acyl group via a nucleophilic substitution reaction, and the ester groups were hereby incorporated into the hydrophobic tail and spacer of gemini QAS. We choose the most common microorganisms (*S. aureus*, *E. coli* and *A. niger)* present in hides or leather as the testing strains to study the antimicrobial effect of gemini QAS. Their antimicrobial mechanism, cytotoxicity and anti-mold performance for wet blue leather were further investigated. Our aim is to provide a novel gemini QAS microbicide with lower toxicity for leather industry.

Materials and Methods

1,2-ethanediol (≥99%), 1,3-propanediol (≥99%), 1,4-butanediol (98%), n-octanol (≥99%), n-decanol (≥99%), n-dodecanol (98%), chloroacetyl chloride (98%), bromoacetyl bromide (97%), diethylamine (≥99%), agar (BR), peptone (BR) as well as beef extract (BR) were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd., China. Dodecyl trimethyl ammonium bromide (DTAB, AR), dodecyl trimethyl ammonium chloride (DTAC, AR), dichloromethane (AR), tetrahydrofuran (AR) and acetone (AR) were provided by Chengdu Kelong Chemical Reagent Co., Ltd., China. Commercial product 2-thiocyanomethylthiobenzothiazole (TCMTB) with an active content of 30 wt% was bought from Fo Shan Lan Feng Auxiliary Limited Co., Ltd. Malt extract broth (MEB, BR) was supplied by Japan Institute of Biological Sciences Co., Ltd. *SYTO 9* (BR) and pyridinium iodide (*PI*, BR) were purchased from Thermo Fisher Biochemical Products Beijing Co., Ltd., China. Dulbecco's modified eagle medium (DMEM, BR) fetal bovine serum (BR) and penicillin/streptomycin antibiotics were obtained from HyClone Co., America.

S. aureus (CICC 21600) and *E. coli* (CICC 10389) were purchased from China Center of Industrial Culture Collection. *A. niger* (ATCC 10864) used in the study was acquired from Biofeng Co., Ltd., China.

Syntheses of gemini quaternary ammonium salts QAS(n+m)

Nine novel gemini QAS compounds with four ester groups were synthesized through a four-step procedure as shown in Figure 1. Firstly, 1,2-ethanediol, 1,3-propanediol and 1,4-butanediol, was reacted with chloracetyl chloride to generate ester-based dichlorosubstitutes ($ClCH_2COO(CH_2)_nOOCCH_2Cl$, n = 2, 3, 4), respectively.[14] Secondly, ester-based dichlorosubstitutes further reacted with diethylamine to obtain intermediate ester-based di-tertiary amine ($((C_2H_5)_2NCH_2COO(CH_2)_nOOCCH_2N(C_2H_5)_2$,

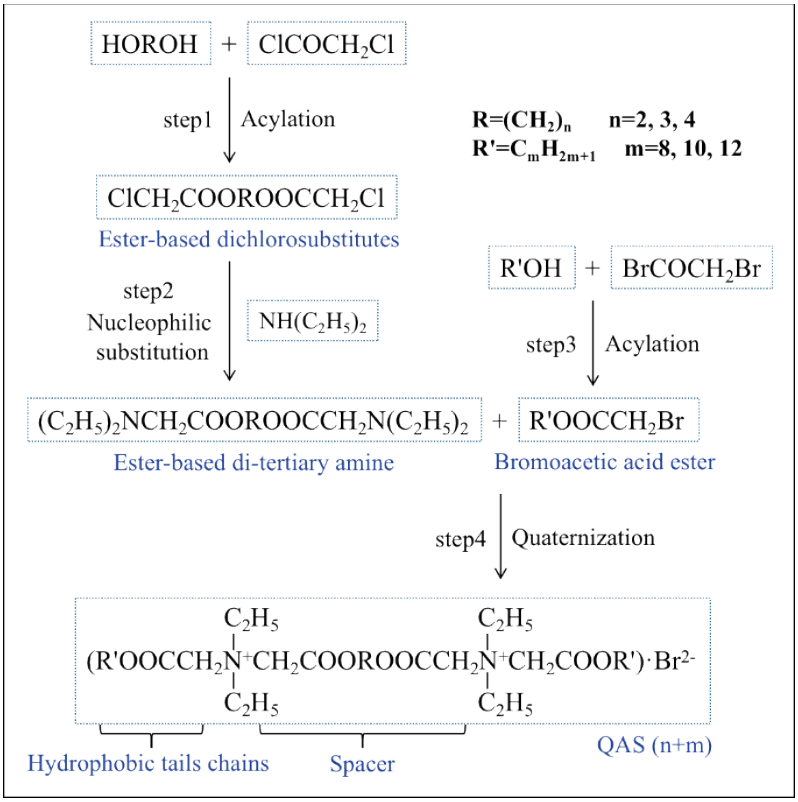

Figure 1. Synthesis process of QAS(n+m)

n = 2, 3, 4). Thirdly, n-octanol, n-decanol and n-dodecanol was esterified with bromoacetyl bromide to form bromoacetic acid ester ($H_{2m+1}C_mOOCCH_2Br$, m =8, 10, 12), respectively.[28] Finally, the gemini QAS(n+m) ($H_{2m+1}C_mOOCCH_2(C_2H_5)_2N^+CH_2COO(CH_2)_nOOCCH_2N^+(C_2H_5)_2CH_2COOC_mH_{2m+1}$)·2Br, n = 2, 3, 4 and m =8, 10, 12) were obtained by quaternization of ester di-tertiary amine with bromoacetic acid ester,[26] where n denotes the number of CH_2 between the ester groups on the spacer, and m denotes the length of alkyl chain connected to ester groups on the hydrophobic tail chains. The resulting QAS(n+m) was purified by recrystallization and drying. The structures of QAS(n+m) have been identified by Fourier transform infrared spectrum (FT-IR), proton nuclear magnetic resonance (^{1}H-NMR) and mass spectrometry (MS).

Antimicrobial activity evaluation

The minimum inhibitory concentration (MIC) of QAS(n+m) determined by the broth dilution method was used to evaluate the antimicrobial activities of QAS(n+m) against Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. col*i, *Escherichia*) and fungi (*A. niger*).[29] The test medium for *S. aureus* and *E. coli* (i.e. beef-peptone liquid medium) was prepared according to the most conventional formula (i.e. 3.0 g beef extract, 10.0 g peptone and 1000 mL water). *S. aureus* and *E. coli* was incubated in beef-peptone liquid medium until the culture reached exponential phase (1×10^{10} CFU/mL), respectively. Then 200 μL bacterial suspension was dispersed in 3.8 mL beef-peptone liquid medium containing different concentrations of QAS(n+m) and incubated for 24 h at 37°C. The final concentrations of QAS(n+8), QAS(n+10) and QAS(n+12) in the experiment was in the range of 0.74~375.00 μM, 0.59~300.00 μM and 0.07~37.50 μM, respectively. *A. niger* was grown on Malt Extract Agar medium (MEA) and incubated for 5 d at 30°C. After the incubation period, the spores of *A. niger* were re-suspended in Malt extract broth (MEB). 200 μL *A. niger* spores suspensions (1×10^6 CFU/mL) was dispersed in 3.8 mL MEB containing different concentrations of QAS(n+m) ($C_{QAS(n+m)}$ = 0.59~300.00 μM) and incubated for 48 h at 30°C. MIC value is defined as the lowest concentration of the QAS(n+m) at which there was no visible growth of microorganisms in the suspension. There was no QAS(n+m) in the negative and positive control and no strains in the negative control. TCMTB was used as test control group for the antifungal activity evaluation.

In addition, we further determined the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of QAS(n+m) by following the procedure previously reported.[30] The test medium for *S. aureus* and *E. coli* (i.e. beef-peptone agar medium) was prepared by following the most conventional formula (i.e. 3.0 g beef extract, 10.0 g peptone, 20.0 g agar and 1000 mL water). After bacterial suspension (1×10^{10} CFU/mL) was treated with different concentrations of QAS(n+m) as described above for 24 h at 37°C, the mixtures were streaked and grown on beef-peptone agar medium for 24 h at 37°C. MBC is defined as the lowest concentration of the QAS(n+m) at which there was no visible growth of bacteria on the agar medium.[30] The MFC values were determined by the same procedure using MEA as the medium and *A. niger* as the testing microorganism.

Antimicrobial mechanism study

Scanning electron microscopy (SEM) observation

To examine the antimicrobial mechanism of gemini QAS, bacteria of exponential phase and A. niger spores suspensions were treated with QAS(4+10), respectively. The bacterial samples were incubated for 4 h at 37°C, and the samples of *A. niger* spores were incubated for 8 h at 30°C. Then the suspensions were centrifuged for 10 min at 8000 rpm to obtain the precipitates and resuspended in 2.5% glutaraldehyde solution for 4 h at 4°C to keep the cell morphology intact. Subsequently, various alcohol concentrations (30%, 60%, 90% and 100%) were used to dehydrate the cells in sequence and each dehydration process was kept for 10 min. Finally, the alcohol solutions containing the cells were dropped on conductive glass for SEM scanning (Inspect F50, FEI Co., Ltd., America). All the samples were sputter-coated with gold before scanning.[31]

Determination of K^+ and soluble proteins content

In addition, to detect the destruction of *A. niger* spores by QAS(4+10), the K^+ content and the soluble proteins content in the supernatant of *A. niger* spores suspensions treated with QAS(4+10) for 8 h were determined by ion chromatography (IC) and Coomassie blue staining method, respectively.

Live/dead bacteria staining assay

200 μL bacterial suspension of exponential phase was mixed with 200 μL QAS(4+10) in 1.5 mL sterile Eppendorf tubes and the final QAS(4+10) concentration of the mixture was kept at MIC values. After culturing at 37°C for 4 h, 5 μL of *SYTO 9* (3.34 μM) and *PI* (20 μM) (v/v = 1:1) were added to the medium and the dying process was kept for 15 min in the dark.[32] Finally, the sample was dropped on the slide grid and observed by fluorescence microscope (CKX53, Olympus Co., Japan).

Cytotoxicity assay (CCK-8 test)

The CCK-8 test was used to evaluate the cytotoxicity of QAS(4+10), antimicrobial agent TCMTB, DTAB and DTAC. L929 cells were stored in the complete medium consisting of 10% fetal bovine serum, 1% penicillin/streptomycin antibiotics and 89% DMEM. 100 μL cell suspensions (1×10^4 cells/well) was put into the 96-well plates and cultured for 24 h at 37°C in CO_2 incubator. Then 100 μL fresh medium with different concentrations of QAS(4+10), TCMTB, DTAB and DTAC was used to replace the original medium, respectively. After incubating for another further 48 h at the same condition, the original medium was removed and replaced with 90 μL fresh complete medium and 10 μL CCK-8 solutions (5 mg/mL). The incubation was further conducted for 1.5 h at the same condition. There was only L929 cells and medium in the positive control and no L929 cells in the negative control.[33] Enzyme-labeled Instrument

(Bajiu Co., Ltd., China) was used to measure the absorbance of each well solution and the wavelength was set at 450 nm. The following equation was used to calculate the relative growth rates (RGRs):

$$\text{Relative growth rates (\%)} = \frac{(OD_t - OD_n)}{(OD_p - OD_n)} \times 100\%$$

Where OD_t, OD_n and OD_p represents the absorbance of the test group, negative control group and positive control group, respectively.

Anti-mold performance of QAS(4+10) for wet blue leather

The anti-mold test of QAS(4+10) for wet blue leather were carried out by following the method described in China light industry standards QB/T 4199-2011.[34] One piece of wet blue leather without any microbicides during the production process was taken as control group. Another two pieces of wet blue leather soaked in 0.5 wt.% TCMTB solution (active content) and 0.5 wt.% QAS(4+10) solution for 24 h respectively, were taken as the test groups. The three leather samples were placed equidistantly on MEA medium, and 30 µL *A. niger* spores suspensions (5×10^5 CFU/mL) was dropped at the center of each piece of leather, respectively. The incubations were conducted at 30°C in a chamber with relative humidity (RH) of 90% for 10 d and the samples were photographed at different incubation days.

Furthermore, the anti-mold performance was also tested by the inhibition zone method. The above wet blue leathers were placed equidistantly on MEA medium which had been completely inoculated with *A. niger* spores suspensions by spread plate method and the plate was incubated at 30°C in a chamber (RH =90%). The samples were observed and photographed accordingly.

Results and Discussion

Antimicrobial activity evaluation

The antimicrobial activity of QAS(n+m) was investigated by MIC, MBC and MFC values as listed in Table I. We chose *S. aureus, E. coli and A. niger* as the test microorganisms because they are common microorganisms on hides or leather. It shows that all the as-prepared gemini QAS exhibit good antimicrobial effect against *S. aureus*, *E. coli* and *A. niger*. The resistance of the three strains to QAS(n+m) varies in the following order: *A. niger* > *E. coli* > *S. aureus,* which is consistent with the previous study.[35] *A. niger* is usually more resistant to chemical disinfectants than bacteria because filamentous Aspergillus have better ability to form biofilm, thus protecting them from damage by biocides.[36,37] Additionally, QAS(n+m) are more active toward *S. aureus* than *E. coli*. It is possibly attributed to the different ultra-structures in their cell walls. Compared with thick but porous cell wall of Gram-positive bacteria (*S. aureus*), the cell wall of Gram-negative bacteria (*E. coli*) composed a thinner peptidoglycan layer and an outer lipopolysaccharide membrane has a stronger tendency to restrict the penetration of small molecules like QAS.[38,39]

From Table I it can be seen that both hydrophobic tail lengths and the spacer lengths affect the antimicrobial activity of QAS(n+m) markedly, and QAS(n+10) demonstrates the stronger antibacterial efficacy. It is possibly because that the hydrophobic tail chain lengths of QAS(n+10) is the most similar to the phospholipid bilayer of bacterial cell membrane, which is favorable for the interactions between QAS(n+10) and bacteria.[40] Likewise, QAS(n+10) also

Table I

The MIC, MBC and MFC values of QAS(n+m) against different microorganisms

QAS(n+m)	*S. aureus*		*E. coli*		*A. niger*	
	MIC	MBC	MIC	MBC	MIC	MFC
QAS(2+8)	46.88	187.50	187.50	>375.00	>300.00	>300.00
QAS(2+10)	18.75	37.50	37.50	75.00	300.00	>300.00
QAS(2+12)	18.75	37.50	>37.50	>37.50	150.00	>300.00
QAS(3+8)	11.72	46.88	46.88	375.00	>300.00	>300.00
QAS(3+10)	2.34	9.38	9.38	9.38	150.00	150.00
QAS(3+12)	18.75	37.50	>37.50	>37.50	150.00	300.00
QAS(4+8)	11.72	23.44	23.44	93.75	300.00	>300.00
QAS(4+10)	2.34	4.69	4.69	9.38	75.00	150.00
QAS(4+12)	9.375	37.5	>37.50	>37.50	75.00	150.00
QAS(5+10)	1.17	2.34	4.69	9.38	37.50	150.00
TCMTB	---	---	---	---	4.69	37.50

Notes: The unit is µM. "---" means no data.

exhibits the stronger antifungal effect, which is closely related to the proper hydrocarbon tail lengths. Either the shorter hydrocarbon tail or the longer tail tend to affect the penetration of QAS molecules into the spores membranes, resulting in lower antimicrobial activity.[35]

As for all the QAS(n+10) (n = 2, 3, 4) compounds, QAS(4+10) exhibits the best antimicrobial activity. On one hand, the electrostatic repulsions between the head groups (N^+) of gemini QAS diminishes when the spacer lengths gets longer, thus contributing to the adsorption of QAS on microbial cell surface.[23] On the other hand, when the spacer-$(CH_2)_n$ group is short (<4), the presence of conjugation effect of two ester groups in the spacers possibly leads to the averaging of electron clouds, thus weakening their induced effect on the head groups (N^+) and unfavorably affecting the adhesion of QAS on the cell surfaces. To verify the above analysis, QAS(5+10) was further synthesized. Its antimicrobial activity against *E. coli* is comparable to that of QAS(4+10) and slightly better than that of QAS(4+10) against *S. aureus* and *A. niger*. It further indicates that the spacer lengths of QAS(n+10) may change the polarity of the head groups of QAS, thus affecting the antimicrobial effect.

Antimicrobial mechanism study

The antimicrobial mechanism of gemini QAS was studied by SEM observation, the live/dead bacteria staining assay and determination of spillage content of *A. niger* spores. As illustrated in Figure 2A, the untreated microbial cells exhibit their own regular and typical morphology, i.e. plump and smooth surface with uniform size and distribution for bacteria,[31,41] and normal shape for *A. niger* spores.[42] In contrast, incomplete or deformed shapes, irregularly wrinkled and coarse outer surfaces are observed for the bacteria strains treated with QAS(4+10) (Figure 2B). The *A. niger* spores treated with QAS(4+10) also shrink (Figure 2B).

Figure 3 shows that fluorescence microscopy observation of the tested bacteria untreated and treated with QAS(4+10). DNA or RNA of microbes can generate red fluorescence when specifically combined with *PI* and green fluorescence when bonded with *SYTO9*.[43] Meanwhile, *PI* staining can identify dead cells as it is impermeant to living cells.[43] As for the *E. coli* and *S. aureus* untreated with QAS(4+10) (Figure 3A), no red fluorescence but much green fluorescence is observed, signifying that almost all the bacteria are alive. As for the bacteria treated with QAS(4+10) at MIC value for 4 h (Figure 3B), the presence of strong red fluorescence indicates the excellent antimicrobial activity of QAS(4+10).

Additionally, after treatment with QAS(4+10), the contents of K^+ and soluble protein in *A. niger* spores suspensions increased up to 0.75 ppm and 0.054 mg/mL, respectively (Table II). It indicates that the membrane structures of *A. niger* spores were damaged by QAS(4+10), leading to the release of the intracellular substances. The above results confirm that as-prepared gemini QAS compounds are membrane active substances and their effective destruction on the integrity of the bacteria cell membranes or plasma membrane of fungi spores cause the death of microbes.

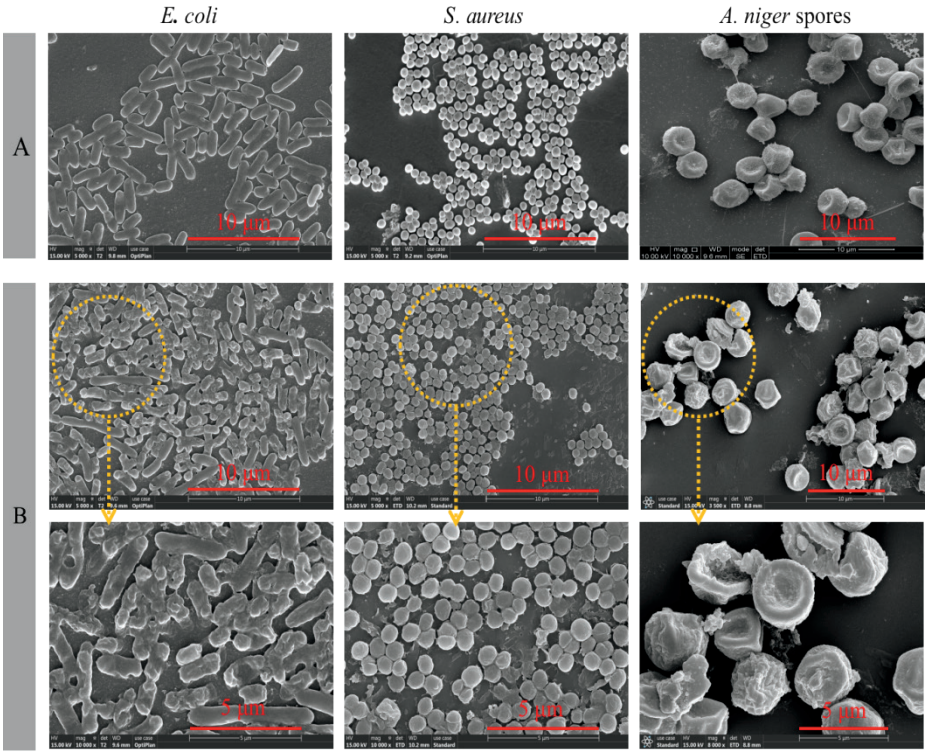

Figure 2. SEM images representing the morphology of the tested microorganisms untreated **(A)** and treated **(B)** with QAS(4+10) at MIC value

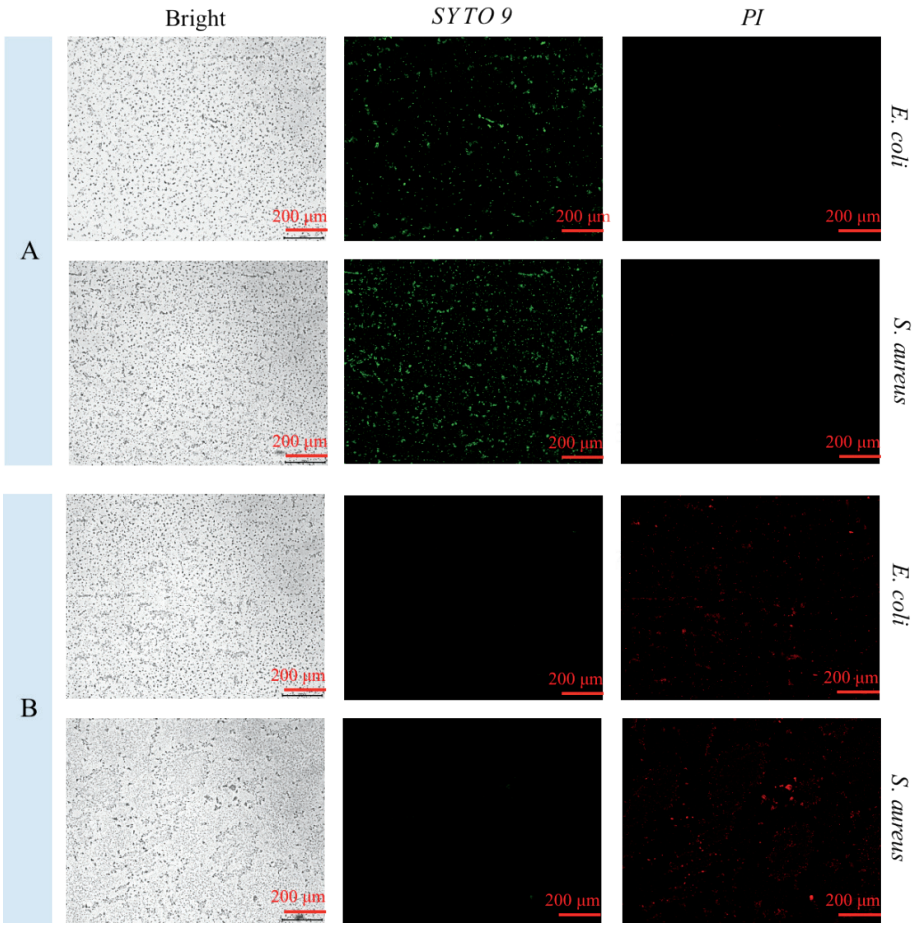

Figure 3. Fluorescence microscopy observation images of the tested bacteria untreated **(A)** and treated **(B)** with QAS(4+10) at MIC value

Cytotoxicity evaluation

Figure 4 and Figure 5 show the cell morphology of L929 and their cell relative growth rates (RGRs) in culture medium containing QAS(4+10), antimicrobial agent TCMTB, traditional quaternary ammonium salts DTAB and DATC, respectively. As for QAS(4+10) treated samples ($C_{QAS(4+10)}$≤75 μM), the cell morphology shows the same typical shuttle-shape as the control group (Figure 4A-D). By contrast, the cell shapes of DTAB, DATC and TCMTB treated samples change obviously, indicating that QAS(4+10) has lower cytotoxicity than these commercial leather microbicides. It can be further quantitatively proven by the results of RGRs.

The colorimetric assay (CCK-8 test) is widely used to examine the cytotoxicity of compounds to the living cells.[33] It is well accepted that the RGRs over 80% indicates non-cytotoxicity of the testing samples.[44] As shown in Figure 5, the cytotoxicity of the microbicides is highly related to their concentrations. The RGRs of L929 cells samples treated with QAS(4+10) ($C_{QAS(4+10)}$≤10 μM) is higher than 80%, revealing that QAS(4+10) at lower concentration exhibits no cytotoxicity. However, DTAB, DTAC (C = 10 μM) and TCMTB (C = 5 μM) have significant cytotoxicity as proved by the lower RGRs. As described in the above Table I, both the MIC and MBC of QAS(4+10) towards *S. aureus* and *E. coli* were lower than 10 μM, meaning that

Table II

The K^+ content and the soluble proteins content in the supernatant of *A. niger* spores with different treatment

The testing components	K^+	Soluble protein
A. niger spores without any treatment	0	0
A. niger spores treated with QAS(4+10)	0.75 ppm	0.054 mg/mL

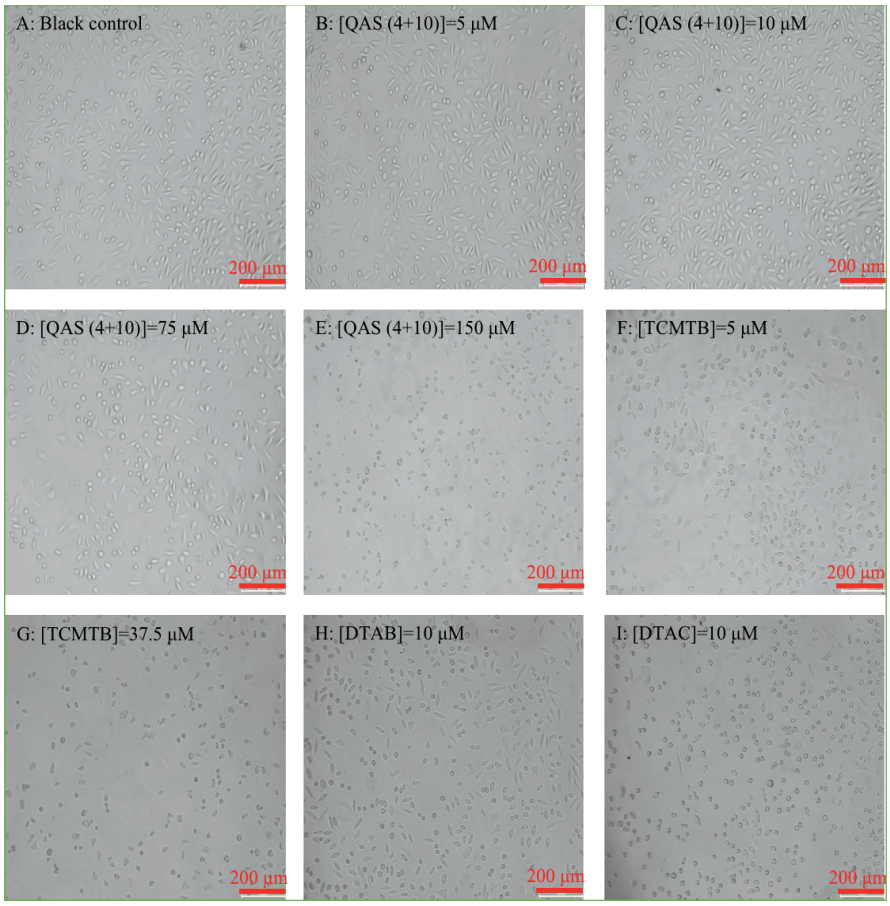

Figure 4. Cell morphology of L929 after 48 h of incubation with the QAS(4+10), TCMTB, DTAB and DATC with different concentrations (**A:** Black control; **B:** [QAS(4+10)]=5 μM; **C:** [QAS(4+10)]=10 μM; **D:** [QAS(4+10)]=75 μM; **E:** [QAS(4+10)]=150 μM; **F:** [TCMTB]=5 μM; **G:** [TCMTB]=37.5 μM; **H:** [DTAB]=10 μM; **I:** [DTAC]=10 μM)

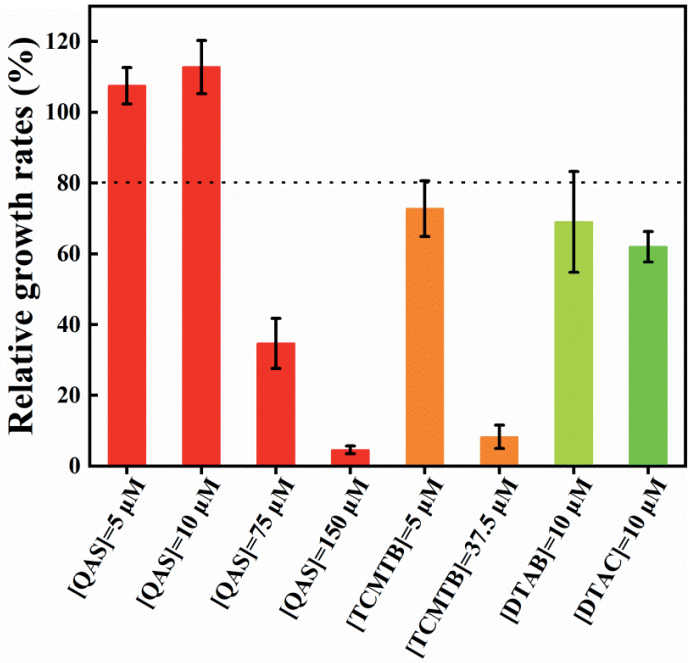

Figure 5. Relative growth rates of L929 after 48 h of incubation with the QAS(4+10), TCMTB, DTAB and DATC with different concentrations

the excellent bactericidal activity and non-toxicity can be achieved by choosing QAS(4+10) as microbicides.

Anti-mold performance of QAS(4+10) for wet blue leather

Figure 6 shows the anti-mold property of QAS(4+10) for wet blue leather within 10 days. *A. niger* are clearly observed on the control group after 5 days incubation, indicating the validity of the anti-mold experiments. In contrast, no *A. niger* appears on the TCMTB or QAS(4+10) treated samples, indicating that the wet blue leather treated with 0.5% microbicides exhibits excellent anti-mold performance. With the time extending (10 d), the control group is totally covered by black colonies of *A. niger*, whereas there is still no mold growth on the microbicides treated samples, indicating that the QAS(4+10) has comparable mold resistance compared with the widely used leather microbicide TCMTB. This result is very encouraging for its potential application in leather industry.

Similar result is also found by the inhibition zone method. As shown in Figure 7, no *A. niger* are observed on the surfaces of both TCMTB and QAS(4+10) treated leather, further affirming the good antifungal activity. Additionally, it is worth noting that a significant inhibitory

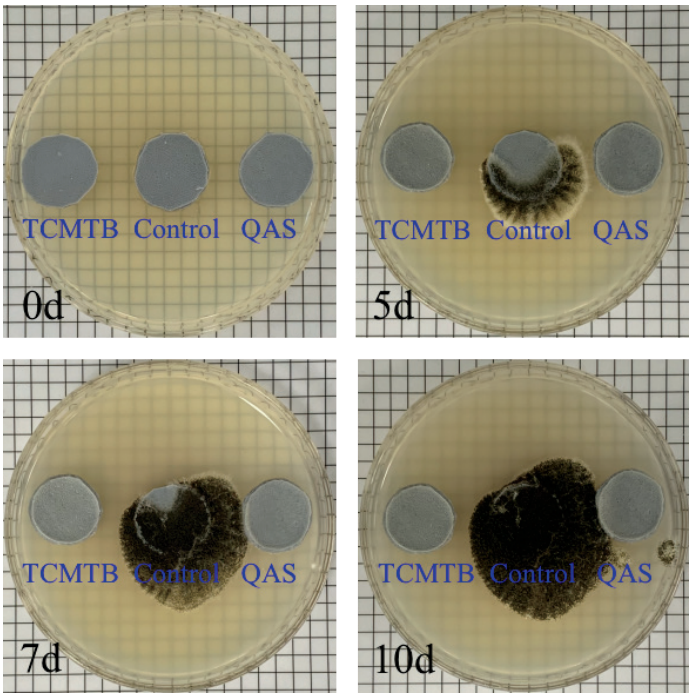

Figure 6. The growth of *A. niger* on the surface of wet blue leather treated with different microbicides: left sample treated with 0.5 wt.% TCMTB, right sample treated with 0.5 wt.% QAS(4+10) and middle sample without any treatment as control group

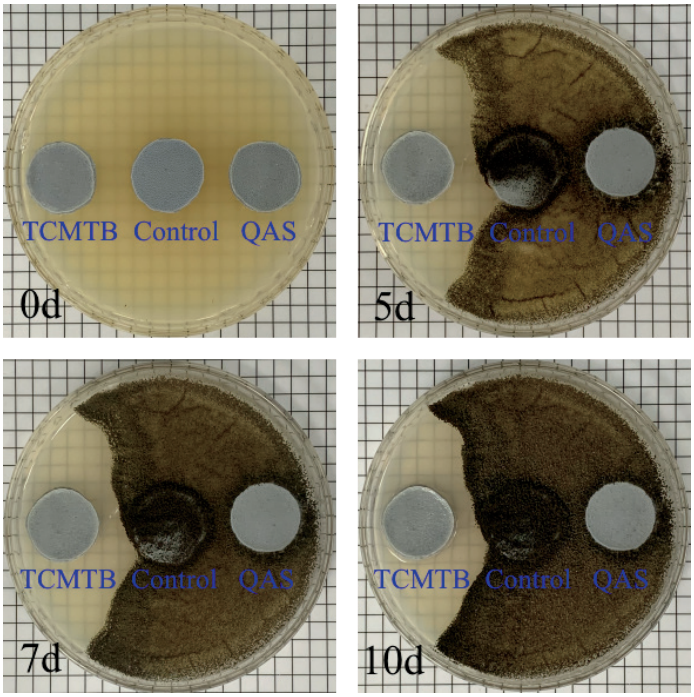

Figure 7. The result of the anti-mold property of QAS(4+10) by inhibition zone method: left sample treated with 0.5 wt.% TCMTB, right sample treated with 0.5 wt.% QAS(4+10) and middle sample without any treatment as control group

circle is observed for TCMTB treated sample, indicating that TCMTB absorbed by wet blue leather is more likely to leach in comparison with QAS(4+10). It is understandable because TCMTB is always used in the form of emulsions due to its poor water solubility, thus demulsificaiton and exudation of TCMTB upon contact with leather is unavoidable. By comparison, hydrophilic QAS(4+10) has stronger binding affinity with leather and is more stable in the practical application, which is favorable to endow leather with long-lasting anti-mold performance.

Conclusion

In conclusion, nine kinds of ester-bonded gemini QAS with different structures were synthesized and their antimicrobial actions, cytotoxicity and anti-mold performance for wet blue leather were studied. Results show that the hydrophobic tail lengths and spacer lengths in the gemini QAS structures can markedly affect their antimicrobial activity. All the as-synthesized QAS exhibit strong antimicrobial effect against *S. aureus*, *E. coli* and *A. niger*, and QAS(4+10) shows the optimal performance. SEM and fluorescence microscopy observation show that the integrity of the bacteria and *A. niger* spores cell membranes are effectively disrupted by QAS(4+10). Compared with the most widely used leather microbicide TCMTB, QAS(4+10) has comparable anti-mold performance for leather and better cyto-compatibility. It is reasonably believed that QAS(4+10) proposed in this study has the potential to be a highly effective and low-toxic leather microbicide.

Acknowledgements

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Synthesis of Multi-Site Polyether Amine Triazine Derivative for Sustainable Leather Manufacturing

by

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Abstract

In this work, a multi-site polyether amine triazine derivative (ET) was prepared via a one-step process and its structure was confirmed using FT-IR and ^{1}H NMR analyses. Then, the ET was applied to the tanning process and the ET tanning approach optimized and evaluated. The result indicated that after tanning with 8% ET at pH 6 for 6 h, the shrinkage temperature of leather was 83.9°C and the thickness change rate was 97.5%. The XRD, DSC, TG, SEM and Zeta potential analyses demonstrated that ET could couple with the amino groups of the collagen side chains and breaking partial hydrogen bonds between fibers, brought significant improvement to the softness of leather without obvious damage to the high-level structure of collagen. Residual Cl- concentration in the wastewater decreased 42.1% in the tanning process compared to the conventional chrome tanning method. The emissions of COD and TDS in the tanning process reduced by 40.5% and 27.2%, respectively. It is conceivable that the ET tanning approach provides an improved approach to promote leather industry to develop sustainably.

Introduction

As the most critical step in leather manufacturing, tanning is a process in which chemicals are used to introduce stable cross-linking between collagen and convert raw hide into leather. Chrome tanning is the most popular tanning method in the leather industry due to the prominent tanning performance of chrome. However, traditional chrome tanning technology and its subsequent wet finishing process could bring about consumption of the nonrenewable chrome salt and chrome-containing solid wastes and wastewaters[1] which could lead to consumption of resources and environmental pressure. In recent years, with the establishment of strategies of sustainable development, extensive studies have been conducted to develop substitute tanning agents of chrome. Aldehyde derivatives (such as modified glutaraldehyde,[2] oxazolidine,[3] organic phosphorus[4] have received special attention among researchers due to the significant potential in protein cross-linking. The aldehyde derivative tanning could endow wet-white with excellent hydrothermal stability and physical properties[5] which has been regarded as an appropriate alternative of conventional chrome tanning. Although an eco-friendly tanning technology can be achieved through aldehyde derivatives; there are some specific problems that can still be encountered. The principle of aldehyde derivative tanning is basically the aldehyde group or hydroxymethyl group coupling with the amino group of collagen side chain.[6] But the cracking of hydroxymethyl groups and presence of aldehyde groups could lead to formaldehyde emission in the wet-white during storage.[7, 8] Because of the biological toxicity of formaldehyde, it has been classified as a potential human carcinogen by the International Agency for Research on Cancer (IARC) and the products that contain aldehyde are facing stringent environmental regulations.[9] To overcome the aforementioned limitations, a novel tanning agent is needed to be employed without chrome or aldehyde that has good tanning properties to meet the needs of the market.

Cyanuric chloride (TCT) is an important chemical intermediate which has many applications in the synthesis of reactive dyes.[10, 11] The chlorine group in TCT has a strong reactivity with collagen under the action of a triazine ring. Specifically, the three chlorides can be substituted with the amino groups of the collagen side chain at 0-5°C, 35-40°C, 75-80°C, respectively.[12, 13] When the TCT reacts with proteins, the optimal pH for the reaction of the second Cl- on the TCT with collagen is 9.4 and the main reaction site is the amino group on the lysine residue,[14] where significant improvement has been found in the thermal stability and physical and mechanical properties of the protein treated with TCT.[15, 16] Besides, the rigid structural of the triazine ring in TCT could also bring stability of crosslinking.[17] However, the poor water solubility of TCT makes it unachievable to directly apply in the aqueous phase of tanning, The commonly used methods is to couple TCT with sulfonated aromatic compounds to enhance the water solubility,[15] but the introduction of aromatic compounds could increase the molecular size of tanning agent and prevent the penetration of tanning agent in skin. Besides, the commercial triazine tanning agent has insufficient tanning property due to the low active chlorine content.[18]

In this work, a novel chrome-free tanning agent based on polyether amine triazine derivatives (ET) was synthesized. The reaction between collagen and ET is shown in Figure 1. The structure of ET was characterized and the effect of ET alone when used as the

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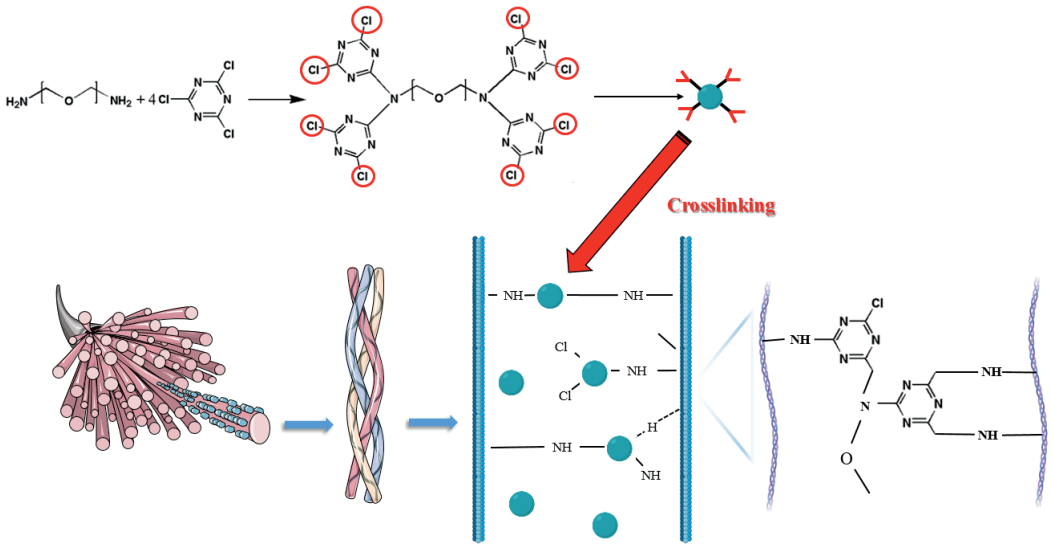

Figure 1. The crosslinking process and reaction of ET and collagen

main tanning agent was investigated and evaluated by comparing it with a commercial triazine derivative tanning agent (CTDT) and a chrome tanning agent. Based on the experimental results, a novel chrome-free and aldehyde-free approach for eco-friendly wet-white manufacturing was developed.

Experimental

Materials and Equipment

Raw cattle hides were used for leather processing. Polyether amine (ED) was purchased from Changke New Materials Technology Co., Ltd, Suzhou China. Cyanuric chloride was purchased from Aladdin Reagent Co., Ltd, Shanghai China. The chemicals employed in the subsequent operations were those normally used in leather industry.

Stainless Experimental Drum (GSD400-4, Wuxi Xinda Light Industry Machinery CO., Ltd, Wuxi, China); Fourier transform infrared spectrometer (Nicolet IS10, Nicolet CO., Ltd, Beijing, China); Nuclear magnetic resonance spectrometer (ARX400, Brucker CO., Ltd, Beijing China); Digital leather shrinkage temperature tester (MSW-YD4, Shaanxi University of Science & Technology, Shaanxi, China); Differential scanning calorimetry (DSC 200 PC, NETZSCH CO., Ltd, Beijing China); Thermal gravimetric analyzer (TG 209 F1, NETZSCH CO., Ltd, Beijing China); Potentiometric analyzer (SZP-10, BTG CO., Ltd, Beijing China); Scanning Electron Microscope (JSM-7500F/X-MAX50, JEOL CO., Ltd, Beijing China); Portable Colorimeter (CR-13, Konica Minolta CO., Ltd, Beijing China); Chloride ion selective electrode (9617BNWP, Thermofisher CO., Ltd, Beijing China); AES-ICP (QP 9100, Jena CO., Ltd, Beijing China); X-ray diffractometer (EMPYREAN, PANalytical B.V. CO., Ltd, Beijing China)

Preparation of polyether amine triazine derivatives (ET)

In a 300 ml 3-necked flask with stirring bar, 18.4g of cyanuric chloride and 100 mL of THF (dehydrated) and 0.25 mol of sodium carbonate (dehydrated) was charged. To this flask, 22.4g of ED (dehydrated) was added dropwise for a total of 0.5 h under the ice bath. After the addition of ED, the reaction took placed at 25°C for 4 h under a stirrer of 300rpm. The viscous liquid ET was obtained by removing THF through rotary evaporation.

Fourier Transform Infrared Spectroscopy (FT-IR)

The functional groups present in the ET were characterized using FT-IR. The FT-IR was used to determine the pure sample at room temperature by using Nicolet iS10 infrared spectrometer. The sample was ground and pressed with spectrally pure KBr and scanned 32 times in the wavenumber range of 400 to 4000 cm^{-1}. The resolution was 2 cm^{-1}.

Nuclear Magnetic Resonance spectroscopy (^{1}H NMR)

After purification and drying, the product was analyzed by a 400MHz Brucker ARX400 nuclear magnetic resonance spectrometer. The solvent was deuterated chloroform ($CDCl_3$) and the internal standard was tetramethylsilane (TMS).

Leather tanning processing

The tanning process were carried out after conventional liming, unhairing (Sulfide), deliming (Ammonia sulfate) and softened (Trypsin), the ratio of leather chemicals for the tanning process was calculated according to the limed split pelt (2.5mm) weight and the cumulative input of tanning process is presented in Table I. A range of 4%-16% of ET was used for tanning and the tanning pH was adjusted to 3 to 9 and tanning temperature was controlled at 25°C, 35°C and 45°C.

Table I

ET tanning process

Processes	Chemical	Dosage/%	Temperature/°C	Time/min	Remarks
Wash	water	400	25	20	Repeat twice
Tanning	water	50			
	ET	X		30	Initial pH 6.0
Basification	Na_2CO_3	1.5		180	Designated pH Y
	water	150	Z	150	overnight
Horse up					

X: The dosage of ET depending on the experiment: 4%, 8%, 16%; Y: The pH depending on the experiment: 3-9;
Z: The Temperature depending on the experiment: 25°C, 35°C, 45°C.

Table II

CTDT tanning process

Processes	Chemical	Dosage/%	Temperature/°C	Time/min	Remarks
Wash	water	400	25	20	Repeat twice
Tanning	water	50			
	CTDT	9		240	
	water	30	50	240	pH5
	Fungicide	0.1		60	overnight
Horse up					

The control process was carried out using a commercial triazine derivative tanning agent (CTDT) and the tanning process is presented in Table II.

The subsequent neutralization and fatliquoring processes were carried out according to the conventional process of cattle sofa leather.

Determination of the shrinkage temperature

The leather samples was measured by leather shrinkage temperature tester (MSW-YD4 Sunshine Electronic Research Institute of Shaanxi University of Science and Technology). Each experimental plot was obtained from an average of three samples.

Determination of the exhaustion rate of tanning agent

The waste liquor before and after tanning was collected and filtered, then digested with acid at 150°C for 2 hrs. The digestion solutions were appropriately diluted and the chemical oxygen demand (COD) determined by using the DR1010 COD detector. The exhaustion rate of tanning agent was calculated according to the following formula:

$$\text{Exhaustion rate (\%)} = 100\% \times \frac{a2 - a1}{a1}$$

a1: COD in waste liquid before tanning (ppm)
a2: COD in waste liquid after tanning (ppm)

Determination of the percent thickness increase

Select four corners and five different parts in the middle of the leather were measured for thickness with thickness meter and the average determined by taking the arithmetic mean. The δ(percent thickness increase) was calculated according to the following formula:

$$\delta\ (\%) = 100\% \times \frac{d2 - d1}{d1}$$

d1: thickness before tanning (mm)
d2: thickness after tanning (mm)

Thermal analysis

Thermogravimetric analysis (TG) measurements were carried out on the tanned leather. The untanned hide was used as a control sample. The dried samples were put into ceramic crucibles and heated at 10°C/min in a N_2 atmosphere. The range of temperature was from 40°C to 800°C. Thermal gravimetric analyzer was used for the determination.

Differential scanning calorimetry (DSC) measurements were carried out on the tanned leathers. The untanned hide was used as the control sample. The dried samples were put into aluminum crucibles and heated at 10°C/min in a N_2 atmosphere. The range of temperature was from 0°C to 200°C. Differential scanning calorimeter was used for the determination after vacuum drying and ground.

Zeta potential analyses

The leather was cut into small pieces, freeze-dried and crushed to powder, then dissolved in deionized water. The isoelectric point was determined by potentiometric analyzer.

Morphological analyses

The morphologies of crosslinked collagen fibers and grain patterns were observed using Desktop Scanning Electron Microscope. Samples of each tanning group were freeze-dried and then observed with an accelerating voltage of 5 kV.

Whiteness analysis

The Lab value of tanned leather was determined by CR-13 Portable Colorimeter (Konica Minolta CO., Ltd, Beijing China) and compared with the standard reference. The total color difference ΔE was calculated according to the following formula:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

ΔL: Lab value of black/white
Δa: Lab value of red/green
Δb: Lab value of yellow/blue

X-ray diffraction (XRD) measurement

The sample was analyzed by EMPYREAN X-ray diffractometer (PANalytical B.V. CO., Ltd, Beijing China). The untanned hide was used as control simple. Cuk as the ray source, the voltage of the tube was 40kV, the current of the tube was 30mA and the scanning speed was 1°/ min, angle range 5°-55°.

Determination of physical and mechanical properties

The leather from different groups were kept under standard atmospheric conditions for 48 h prior to testing. The physical and mechanical properties i.e. the tensile strength, elongation at break, tear strength as well as the bursting strength of the resultant leather were determined. These tests were performed according to the standards of IUP 2.[28]

Environmental impact assessment

The chemical oxygen demand (COD) was analyzed by using the standard procedure (AWWA, 1998) for the spent tanning liquors. Total dissolved chlorides (TDC) was determined using a chloride ion selective electrode. The total dissolved solids (TDS) was calculated after one hour desiccation at 104°C. The content of chromium in spent liquor was determined using Inductively Coupled Plasma Emission Spectrometer (QP 9100, Jena CO., Ltd, Beijing China) after digestion.

Results and Discussions

Characterization of ET

FT-IR analyses

Figure 2 shows the FT-IR spectrum of TCT and ET. It can be seen from the figure that the TCT and ET showed obvious stretching vibration absorption of C-Cl bond at 532 cm^{-1}, while the characteristic absorption peak of triazine ring appears at 1303 cm^{-1}, 1353 cm^{-1} and 1546 cm^{-1}. The C-N characteristic peak of ET at 1103 cm^{-1} could not be observed in the structure of TCT which indicates that the polyether amine was introduced into the structure of the product.

^{1}H NMR analyses

Figure 3 shows the ^{1}H-NMR spectra of ET while using TMS as internal standard and deuterated chloroform as solvent. As can be seen from the figure, the chemical shift at 7.26 ppm belongs to the deuterated chloroform, the chemical shift at 1.81 ppm was caused by the residual solvent of tetrahydrofuran, the chemical shift at 3.70 ppm was caused by the ethyl hydrogen in the structure of polyether amine. It can be seen that the number of hydrogen atoms of the

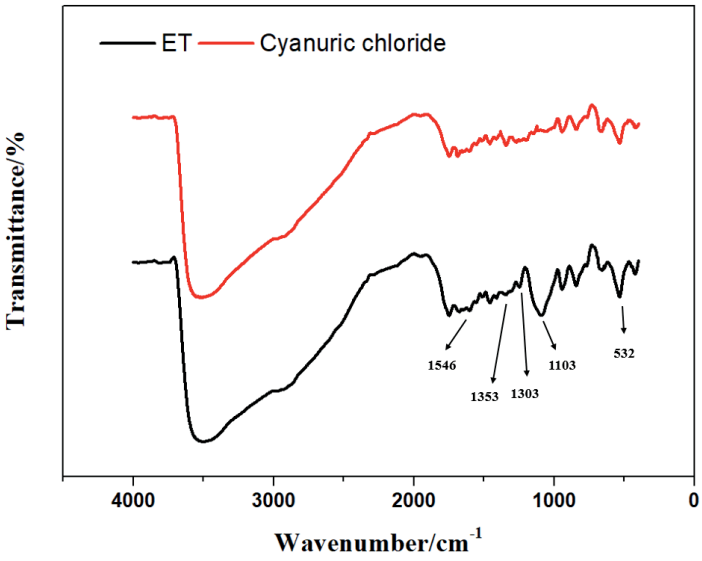

Figure 2. FT-IR spectrum of ET

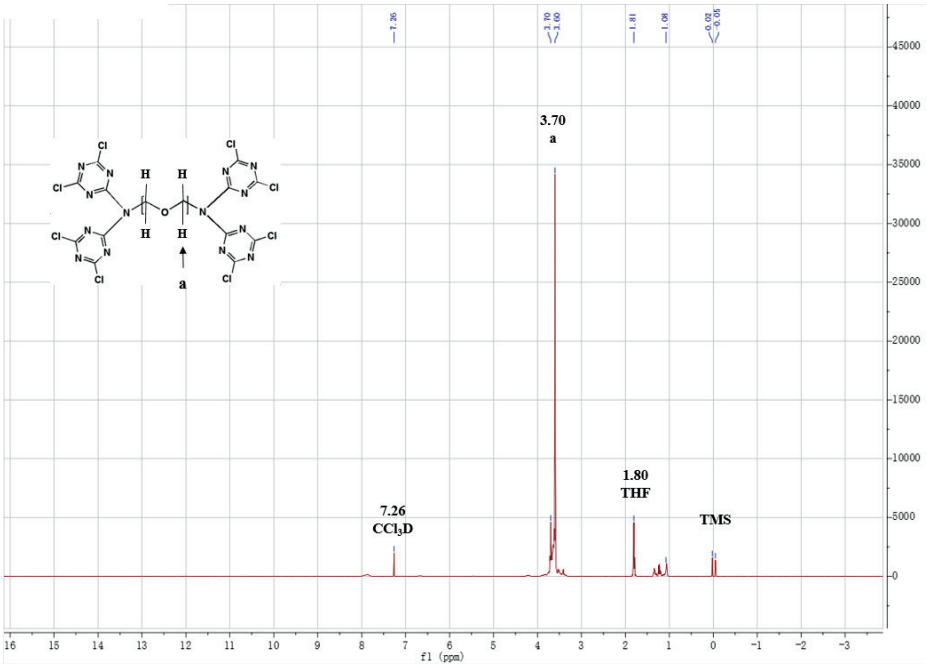

Figure 3. ^{1}H NMR spectrum of ET (CCl_3D, 400MHz)

product was consistent with the target product, which proves the existence of ET.

Optimization of the ET tanning conditions

Optimization of the ET tanning pH

The active chlorine in cyanuric chloride structure could be hydrolyzed at pH over 6, to prevent the excessive hydrolysis of tanning agent at the initial stage of tanning, the pH of tanning system was controlled at 6% and 8% of ET for tanning. With the reaction of skin collagen and ET, the formed HCl will lead to the decrease of bath pH. Therefore, sodium carbonate solution was used to adjust the pH and the percent thickness increase and shrinkage temperature of wet-white (after main tanning) was investigated. Figure 4 shows the percent thickness increase and shrinkage temperature of wet-white after tanning at pH of 3 to 9. It can be seen from the figure that during the tanning process, the shrinkage temperature and percent thickness increase of wet-white slightly increased as the pH increased from 3.0 to 6.0 and rapidly decreased at a higher pH. The shrinkage temperature reached the maximum of 79.8°C and the percent thickness increase reached the maximum of 86% at the pH 6. This is due to the unique hydrolysis-binding equilibrium theory of TCT.[12] When the pH is lower than 6, the binding ability of active chlorine to collagen is insufficient, resulting in weak tanning property, low shrinkage temperature and percent thickness increase; When the pH is higher than 6, high pH will lead to the rapid hydrolysis of TCT,[19] resulting in the loss of tanning property and the decrease of cross-linking rate. Therefore, it is an appropriate choice to tan at pH 6.

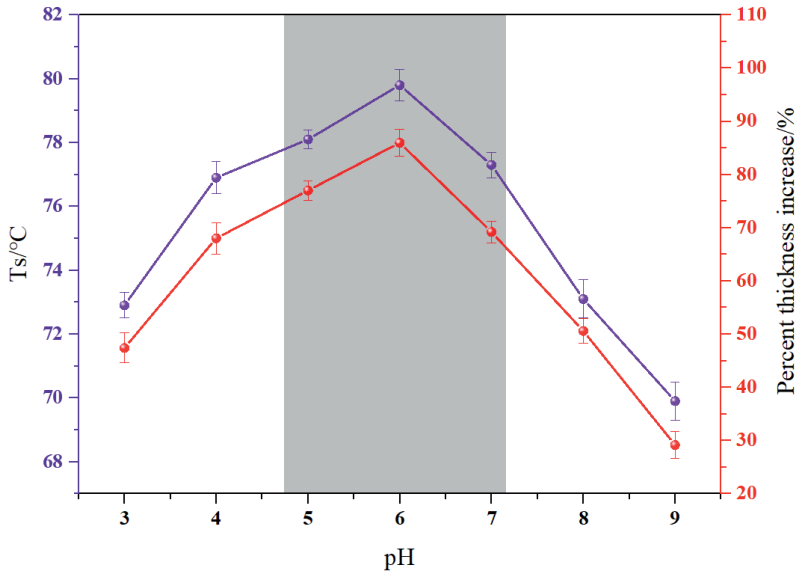

Figure 4. Shrinkage temperature and percent thickness increase of wet-white tanned with ET at different pH

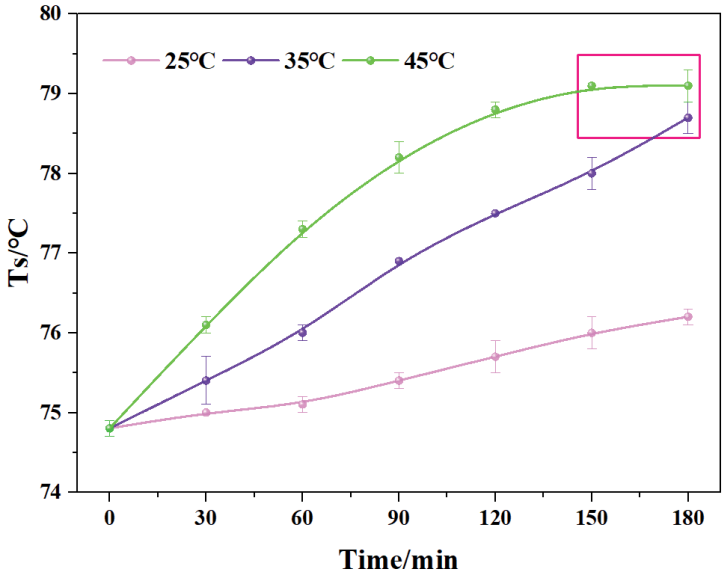

Figure 5. Shrinkage temperature of wet-white tanned with ET at different temperature

Optimization of the ET tanning temperature

Temperature is another important factor affecting the reaction between ET and collagen. For leather manufacturing, a lower temperature is usually used to promote the penetration of tanning agents in the initial tanning stage, and a higher temperature is used to promote the combination of tanning agents and collagen in the final tanning stage, which makes the tanning effect uniform. In order to investigate the effect of temperature at the final tanning stage using ET tanning, 8% ET was used at room temperature for 3h at the initial stage of tanning, and varying temperatures of 25°C, 35°C and 45°C were used at the final stage of tanning. The shrinkage temperature of wet-white was investigated at different times. The result in Figure 5 shows that the temperature increase at the final stage of tanning had a significant promoting effect on the shrinkage temperature of wet-white. Higher temperatures not only could accelerate the shrinkage temperature increase of wet-white, but also increases the final shrinkage temperature of wet-white to a certain extent. The shrinkage temperature of wet-white could reach 79°C after being treated at 45°C for 150 min. Considering that further heating may cause the destruction of the collagen structure and excessive hydrolysis of ET, it is suitable to treat at the temperature of 45°C for 150 min at the final stage of tanning.

Optimization of the ET dosage

The tanning agent of 4%, 8% and 12% was used for tanning according to the process mentioned in Table I. The subsequent neutralization and fatliquoring process was carried out according to the conventional process. As the Table III shows, with the increase of ET dosage, the shrinkage temperature and thickness of wet-white increased obviously. When the dosage of ET reached 8%, the shrinkage temperature of crust leather was 83.9°C, the percent thickness increase was 87.3% and no significant increase was achieved with the further increase of ET dosage. As for the exhaustion of tanning agent, when the ET dosage was higher than 8%, the exhaustion rate of ET decreases obviously, which indicates that the combination of ET and leather was reaching saturation. Further increasing the ET dosage contributes little to the leather properties. Besides, a large amount of ET remaining in the effluent liquor could increase the difficulty of post treatment. In order to ensure the effective utilization of materials and control cost, it is considered that 8% of ET dosage could be a more appropriate choice.

Thermal analysis

The thermodynamic analysis of leather is a reliable method to evaluate the thermal effect of chrome-free tanned leather. It can also

Table III

Tanning effect of different ET dosage

ET dosage/%	4	8	12
Ts of the wet-white /°C	73.9±0.1	78.9±0.1	79.3±0.2
Ts of the crust leather /°C	76.1±0.2	83.9±0.0	84.1±0.2
percent thickness increase /%	57.1±0.8	87.3±0.7	90.1±0.4
exhaustion rate %	81.6±0.7	79.6±0.5	59.3±0.9
Softness	7.6±0.0	8.8±0.0	8.9±0.1

reflect the effect of crosslinking on the advanced structure of leather. In this section, the thermal performance of wet-white tanned with ET was analyzed via TG and DSC, the untreated collagen was used as the control.

The thermal decomposition of leather is related to the cross-linking degree of collagen fibers. Covalent crosslinking in collagen could improve the thermal stability of skin, which is reflected in higher thermal decomposition temperature.[20] Figure 6 showed the TG curves of the ET and the corresponding characteristic parameters of them are given in Table IV. The first stage (I) is the vaporization of free water and bound water, the second stage (II) is the decomposition of collagen fibers, polymers and organic chemical materials used in tanning, and the third stage (III) is the decomposition of chemical bonds of polymers with collagen.[21] According to the results, the crosslinking of ET can obviously slow down the thermal decomposition during the heating process, this is because the triazine ring structure in ET can form stable sites between the collagen chains and play a role in fixing the collagen chains.

The hide before and after tanning (after freeze drying) was crushed and analyzed by DSC. The thermal stability of different leather powders before and after tanning can be judged by the position, width, strength and area of the peak in DSC spectrum. The differential scanning calorimeter (DSC) has been used to elucidate the degradation profile of wet-white tanned with ET in response to heat and the thermal stability of collagen before and after tanning can be judged by the position, width, strength and area of the peak in DSC spectrum. As showed in Figure 7, the peak of denaturation temperature of wet-white rises to 97.5°C while the peak of denaturation temperature of untanned collagen at 69.9°C and the position of the peak obviously drifts to the high temperature zone and gets wider. That is because the existence of triazine structure on the polypeptide macromolecular network could affect the three dimensional linkages and leads to the enhancement of heat resistance of collagen.

Zeta potential analysis

Collagen is a typical amphoteric polyelectrolyte. The blocking state of -COOH and -NH_2 in its structure can be described through zeta potential. Specifically, with the change of the pH value, different collagen structures become ions with many positive or negative charges. When the pH is lower than the isoelectric point of collagen, carboxyl groups are blocked, the amino groups are protonated and

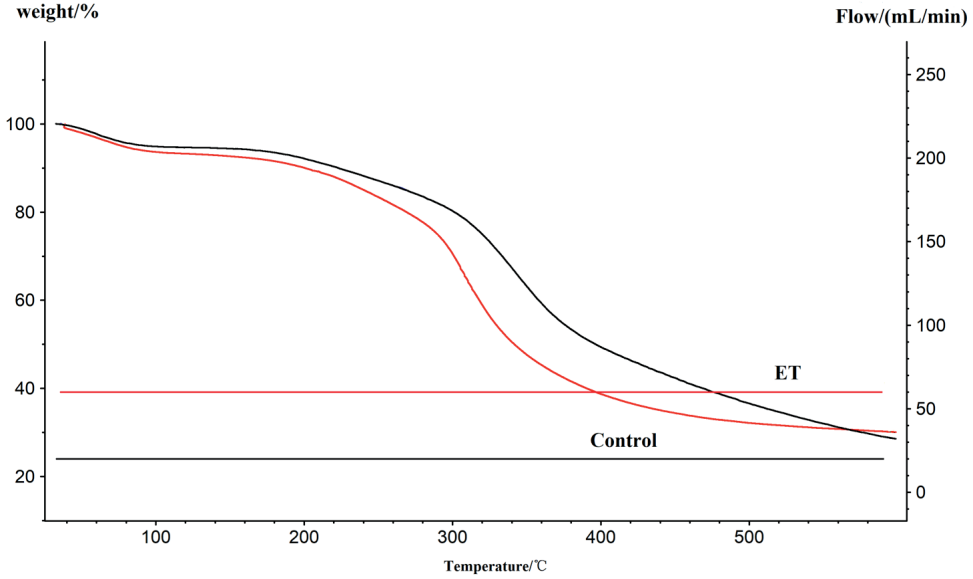

Figure 6. TG curve of wet-white tanned with ET

Table IV

Weight loss in different thermogravimetric stage

Simple	Thermal decomposition stages		
	I/wt%	II/wt%	III/wt%
ET tanning	8.03 (Room temperature-210.8°C)	50.11 (210.8°C-493.8°C)	9.88 (493.8°C-600.0°C)
Control	7.19 (Room temperature-209.2°C)	51.0 (209.2°C-397.3°C)	8.63 (397.3°C-600.0°C)

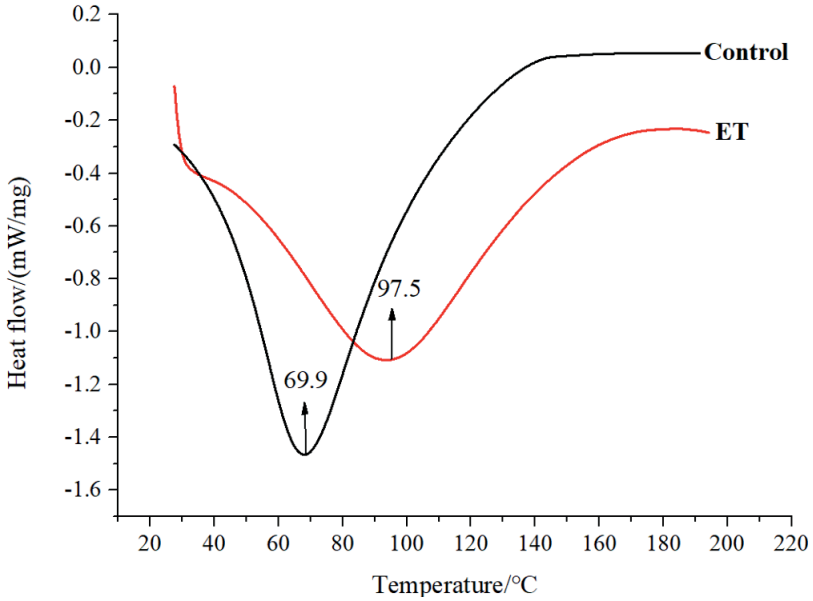

Figure 7. DSC curve of wet-white tanned with ET

the molecules carry a positive charge; when the pH is higher than the isoelectric point of collagen, the carboxyl groups are ionized and the molecules carry a negative charge. When the concentration of negatively charged carboxyl groups are equal to that of positively charged amino groups in solution, collagen will be in a state of equal charge.[22]

In this study, the Zeta potential of wet-white tanned with ET was investigated and the untreated collagen was used as the control. Figure 8 shows the Zeta potential of wet-white tanned with ET at different pH values. After tanning with ET, the isoelectric point of collagen decreased from 4.71 to 2.85, that means ET could react with the amino groups of collagen, a large number of amino groups in the side chain of collagen were blocked, which is consistent with the results shown in Figure 4.

XRD analysis

In order to reveal the dispersion of ET in collagen, the white-wet tanned with ET was characterized by XRD, and the untreated collagen was used as the control group. In Figure 9, there were two obvious diffraction peaks at 8° (A) and 22° (B). The (A) peak reflects the distance between the molecular chains of the collagen fibers, and belongs to the α-helix region with more ordered structure.[23] The fact that peak shape of (A) got sharper indicated that the tanning process of ET broke part of the hydrogen bonds and van der Waals forces between collagen fibers and lead to the loosening of the fibers. The (B) peak was caused by numerous structural layers inside the collagen fibers, such as the b-fold. After tanning with ET, the (B) peak moved to the direction of low diffraction angle, which indicated that the introduction of ET breaks the original weak interaction between collagen, reduces the randomness of fibers, and macroscopically

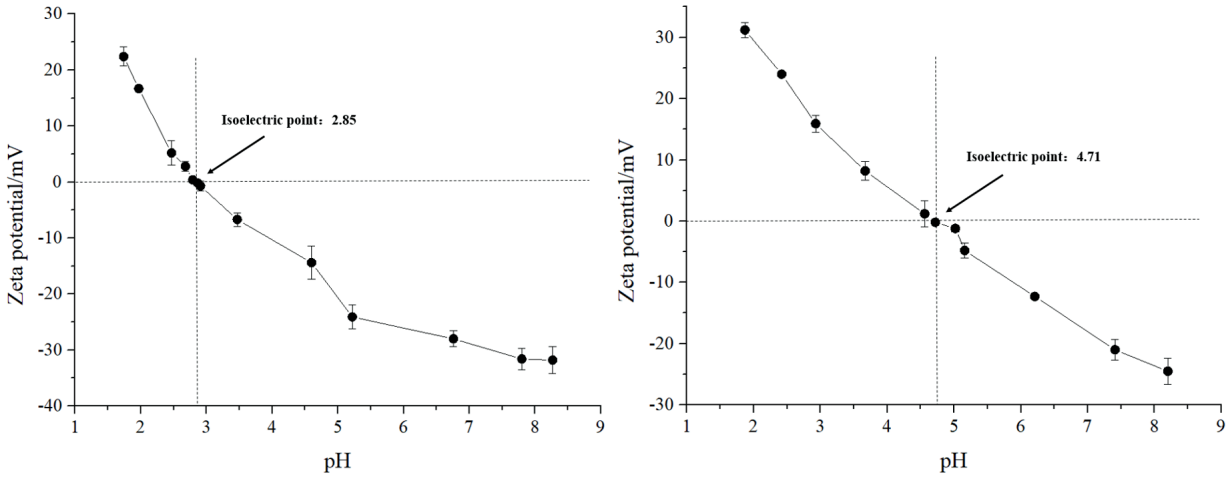

Figure 8. Zeta potential of wet-white tanned with ET

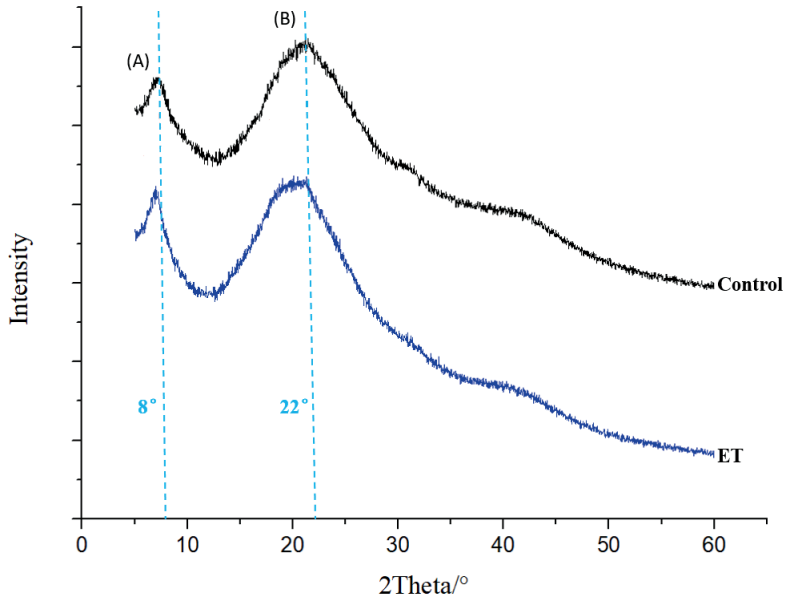

Figure 9. XRD curves for wet-white tanned with ET.

reflects the increase of softness. There was no obvious change of the diffraction peak at 43°, which indicated that ET tanning did not cause a change in the collagen crystalline region.

Physical performance of leather

In order to evaluate the physical performances of wet-white tanned with ET, wet-white was prepared with CTDT (9%) according to the process mentioned in Table II and wet-blue was prepared with conventional chrome tanning method (6.5%).[25] The subsequent neutralization and fatliquoring process was carried out according to the conventional process and the results were shown in Table V. As the results show, the physical performance of ET tanned leather were better than that of CTDT tanned leather, which was close to the physical performances of chrome tanning leather. This is because there were more active chlorine in ET structure than CTDT, which could be regarded as crosslinking sites when ET react with collagen. In addition, ET tanning can significantly improve the softness of leather, which was due to the supporting effect of triazine structure between leather fibers, and the lubricating effect of polyether chain segments in the gap between collagen fibers,[26] which gives leather excellent softness.

Whiteness analysis

The Lab values of leather (without dying and retanning) were determined and compared with the standard reference to evaluate the whiteness of several leathers tanned with different methods. It can be seen from Table VI that the color difference between ET tanned wet-white and standard white is little, which is similar to that of CTDT tanned wet-white and obviously better than that of chrome tanned wet-blue. Besides, triazine derivatives can be coupled with subsequent dyes lead to a fixation effect, which present a wide application prospect.

Table V

Physical performances of leather tanned with different method

Tanning agent	ET	CTDT	Chrome
Shrinkage temperature (°C)	83.9±0.2	74.3±0.3	109.2±0.2
Tensile strength (MPa)	16.19±0.00	14.1±0.11	18.31±0.07
Elongation (%)	65.1±0.3	66.9±0.1	65.2±0.1
Tear strength (N/mm)	47.93±0.24	38.9±0.17	60.21±0.30
Softness (mm)	8.8±0.0	9.1±0.0	7.8±0.2
Fineness of grain	+ + + +	+ + +	+ + + +
Fullness	+ + + + +	+ + + +	+ + + + +

Table VI

Whiteness of wet-white tanned by different methods

Tanning method	L	a	b	ΔE*
Standard white	91.60	-0.47	5.10	——
Chrome tanning	42.71	-0.18	0.88	49.07
CTDT tanning	79.30	-0.26	3.80	12.37
ET tanning	79.82	-0.49	3.77	11.85

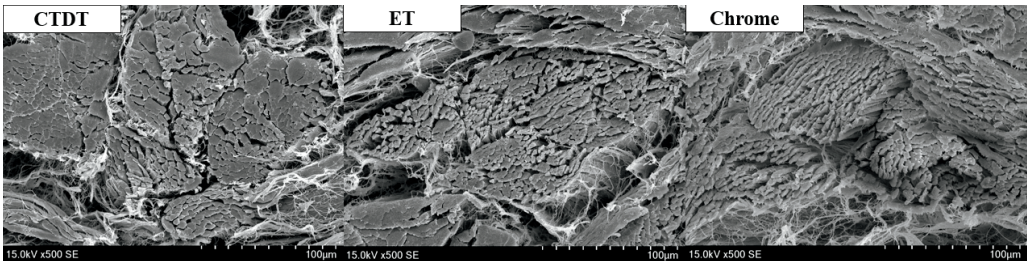

Figure 10. SEM observation of fiber patterns of the leather with different tanning method.

Morphology analysis

Figure 10 shows the SEM observation of leather tanned using three different methods. It can be seen from the result that the fibers of wet-white tanned with ET were more dispersed compared with the CTDT tanned leather, there was no obvious adhesion between fiber bundles, and the boundary of basic fibers was clear. Compared with the wet-blue tanned with chrome, the looseness of the fiber was similar, which indicates that the introduction of ET could break the adhesion between fibers, increase the sliding property of fibers and improve the softness of leather.

Environmental impact assessments

To evaluate the impact of the ET tanning process on the environment, biochemical analysis of leather processing waste liquor was carried out to assess its ecological risks to the environment.[27] The ET tanning effluent was evaluated to measure the environmental impact parameters of the ET tanning method and compare it with the CTDT and conventional chrome tanning methods. It is shown in Figure 11 that the residual Cl^- concentration in wastewater decreased 42.1% in tanning process compared to conventional chrome tanning method. That was because no pickling was needed in tanning process of ET and CTDT, the residual chloride ion in the waste liquid comes

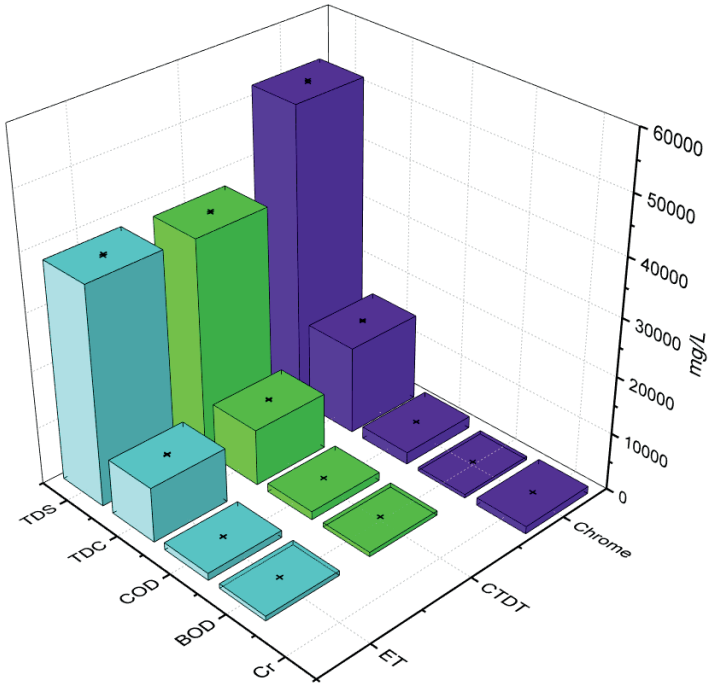

Figure 11. Environmental testing parameters of three tanning methods

from HCl formed by the reaction of tanning agent and collagen. The emissions of COD and TDS in waste liquor of ET were only 1242mg/L and 38118mg/L compared with 2088mg/L and 52300mg/L of chrome tanning waste liquor. The COD, TDS and TDC of ET tanning effluent were slightly lower than that of CTDT tanning effluent. ET tanning technology have a better biodegradability and no neutral salt was introduced in the process, which indicated that the lower environmental administration budget. ET as a novel tanning agent could provide guidance for develop sustainably and cleaner leather manufacturing.

Conclusion

Nowadays, a growing number of countries have implemented strict regulations to promote sustainable development. New criterion for clean leather manufacturing have been put forward for chrome-free tanning. In this work, polyether amine triazine derivative (ET) was prepared via a one-step process and a novel chrome-free tanning approach based on ET has been established and evaluated comprehensively. ET has been confirmed to combine with the amino groups of collagen mainly in the form of covalent bonds and produced crosslinking networks in the collagen structure, which greatly improved the thermal and physical properties of collagen. Due to the existence of polyether chain in ET structure, part of hydrogen bonds and van der Waals forces in collagen were broken, fibers loosened, and increased the softness of leather. After tanning with 8% of ET at pH 6 for 6 h, the wet-white present shrinkage temperature of 83.9°C (due to the introduction of covalent crosslinking) and percent thickness increase of 87.3% (due to the support effect of hydrophobic group of ET on fiber). The environmental impact assessments showed ET tanning shows better environmental protection than conventional chrome tanning as well as CTDT tanning.

Acknowledgment

Thanks also go to Chunxiao Zhang, a researcher in Sichuan University, for technical support.

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Antibacterial Properties of Several Lichen Extracts against Two Moderately Halophilic Bacteria from Salted Sheepskins

by

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Abstract

It is well known that possible undesirable defects in finished leathers can result from microbial activities on the salted raw hides/skins during storage. The traditionally used salt curing method can control bacterial activities on the raw stock, but it does not seem possible to completely eliminate microbial attacks. Moderately halophilic bacteria can cause serious damage to hides/skins. From this point of view, potential procedures such as applying new antibacterial agents in the leather industry should be considered. Since some lichen species have been indicated for their promising biological efficacies in the literature; most researchers have focused on their potencies in various fields including leather industry from ancient times. In this study, the bacterial growth of proteolytic and lipolytic Gram-negative moderately halophilic bacteria, *Chromohalobacter canadensis* (YN6) and *Halomonas eurihalina* (BL5), from salted sheepskin samples were tested with the extracts of *Usnea* sp., *Plastismatia glauca, Ramalina farinacea, Evernia divaricata, Bryoria capillaris, Hypogymnia tubulosa, Pseudevernia furfuracea* and *Lobaria pulmonaria*. Some of these lichen species were found to be successful to inhibit the bacterial growth of *C. canadensis* (YN6) and *H. eurihalina* (BL5). In conclusion, lichen extracts may be utilized in stacked raw hides/skins in tanneries or warehouses to control moderately halophilic bacteria which causes several defects on leather.

Introduction

Since leather is the most traded product, it has great importance in the world economy.[1] It has been reported that the international trade value of the leather sector is about $80 US billion dollars annually. China, Italy, Korea, India, Russia, and Brazil have a major role in leather production, import, and export worldwide. In particular, footwear takes place at top of the market share.[2] According to The Food and Agriculture Organization (FAO) reports, approximately, seven million tons of hides and one million tons of skins are utilized for the conversion into finished leather every year.[3]

During leather-making processes, every precaution has to be taken to produce the best quality leather. Previous studies have demonstrated that the bacterial population on hides may cause serious defects in the finished product.[4-8] These bacteria can be either halophilic or non-halophilic bacteria depending on different steps in the leather-making process.[9-11] Unfortunately, applied preservation methods cannot prevent these organisms and several problems may be encountered on the finished leather.[6, 7, 12-22] The prevention of these bacterial activities is of high importance, as it means that new problems arise later in the other stages of leather making due to the unhindered bacterial population. To avoid these defective bacterial populations in the storage step of raw hides/skins, the salt curing method is generally preferred in developing and undeveloped countries.[10] However, it seems that this application does not work as expected. The need for alternative applications in the protection of raw hides/skins has arisen due to economic losses experienced in the sector.

As known, commonly encountered defects such as red discolorations, bad odor, holes, and deterioration on raw hides/skins are attributed to the activities of halophilic bacteria.[23] Amongst halophilic bacteria, moderately halophilic ones are known to grow in hypersaline environments due to their ability to produce compatible solutes which help to maintain cytoplasmic homeostasis. Since the optimal conditions for these bacterial population are defined as 3-15% NaCl and pH 5-10, they may easily grow on salted hides/skins.[24] There are several studies focused on these moderately microorganisms in respect to their numbers or their potential defects. The number of moderately and extremely halophilic archaea was reported to be 10^5-10^8 colony-forming unit/gram (CFU/g) from 131 hide samples of 34 different tanneries.[6] In another study, 10^5-10^8 CFU/g of moderately halophilic bacteria and 10^5-10^7 CFU/g of extremely halophilic archaea were determined in four salted sheepskins with discolorations, slimy surface, bad smell, and hair slips.[17] In the same study, a total of 78 moderately halophilic bacterial isolates representing 7 species (*Alkalibacillus halophilus*, *Pseudomonas halophila*, *Acinetobacter johnsonii*, *A. salilacus*, *Salimicrobium salexigens*, *Marinococcus luteus* and *Staphylococcus equorum* subsp. *equorum*), and a total of 101 extremely halophilic archaeal isolates representing 12 species (*Halorubrum tebenquichense*, *H. saccharovorum*, *Halococcus dombrowskii*, *H. qingdaonensis*, *Natrinema pellirubrum*, *H.*

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morrhuae, *H. kocurii*, *H. terrestre*, *H. lipolyticum*, *Halostagnicola larsenii*, *Haloterrigena saccharevitans* and *N. versiforme*) were identified.[17] More detailed studies revealed that new moderately halophilic species, *Thalassobacillus pellis* sp. nov. and *Salimicrobium salexigens* sp. nov., may also be found on salted hide samples.[25,26] Thirteen species of moderately halophilic bacteria (*Salimicrobium luteum, Marinococcus halophilus, Halomonas koreensis, S. album, S. halophilum, H. elongata, H. halmophila, H. eurihalina, H. alimentaria, Oceanobacillus picturae, Thalassobacillus devorans, Chromohalobacter salexigens and Alkalibacillus salilacus*) were reported by researchers from the salt-pack cured hides.[16] Thirty-one moderately halophilic species belonging to the genus of *Staphylococcus, Salimicrobium, Bacillus*, *Salinicoccus, Planococcus, Alkalibacillus*, *Gracilibacillus*, *Oceanobacillus*, *Marinococcus, Halomonas*, *Salinivibrio*, *Chromohalobacter,* and *Idiomarina* and fourteen moderately halophilic bacteria belonging to the genus of Staphylococcus, *Bacillus, Gracilibacillus, Salinicoccus, Halomonas,* and *Chromohalobacter* were isolated and identified from salted sheepskins and salted goatskins, respectively.[18, 27] Due to their proteolytic and lipolytic activities of moderately halophilic bacteria on the salted goat skins,[27] the quality of leather may be affected negatively.

As mentioned earlier, it is of great importance to correctly combat the bacterial population prior to further steps to obtain a valuable product without economic loss. In this view, the bacterial population has to be under control from the beginning of the leather-making processes including the storage of raw hides/skins in warehouses. For this purpose, to control moderately halophilic bacteria with possible degradative and defective effects, different approaches were reported by the researchers such as direct and alternating electric current application,[28-30] bacteriocins,[31] antimicrobial agents such as alkyltrimethylammonium bromide, and a combination of alkyltrimethylammonium bromide, and chlorhexidine digluconate.[19, 22] However, bacteria may develop resistance to commonly used antimicrobial agents. The intrinsic or acquired resistance of bacteria to traditionally utilized antibacterial agents has potential. It has previously been demonstrated that some bactericides cannot be effective against the bacteria with proteolytic and lipolytic activities in soak liquors.[11, 32, 33]

In recent years, natural compounds from plants, microorganisms and lichens have been evaluated as promising alternatives for resistant bacteria.[34] Especially, lichens are reported with their various biological properties. They have been used for medicines, spices, foods, perfumes, and dyes. Studies have revealed that the healing properties of lichens used in the prevention of diseases are due to the acidic secondary metabolites in their structures.[35-37] Approximately 1050 unique secondary metabolites produced by lichens as metabolism products and these metabolites have various features (antimicrobial, antioxidant etc.).[38] Some antibiotic effective compounds are produced by the utilization of secondary metabolites obtained from lichens. Aslan et al. (2006), stated the antibacterial efficacy of methanol extracts of *Evernia divaricata* against *S. aureus, E. faecalis, E. coli* and *P. aeruginosa*.[39] However, it was observed that the antibacterial effects showed differences due to the bacterial species. Çobanoğlu et al. (2010) investigated that the extracts of five lichen species (*Alectoria sarmentosa, Bryoria fuscescens, E. divaricata, Platismatia glauca* and *Ramalina farinacea*), against *P. aeruginosa, E. coli* and *Acinetobacter* and the researchers found effective results depending on the bacteria.[40] In the study conducted by Esimone and Adikwu (1999), *R. farinacea* had antibacterial and antifungal effects.[41]

Lichens that are used for tanning belong to the genus *Cetraria islandica* and *Lobaria pulmonaria*.[42, 43] The efficiency of extracts belonging to *Pseudevernia furfuracea (L.) Zopf.* has been tested in the leather industry.[42] More recently, the potential antimicrobial effects of several lichen extracts against some bacterial species isolated from soak liquor samples have been reported.[44-46]

Considering the problems caused by the halophilic bacterial population in the stages of leather processing, especially in the storage period of raw hides, which affects further steps of the manufacturing, the need for new natural antibacterial agents come into prominence. The presence of the high number of halophilic bacteria in raw hides/skins preservation occurs due to the failure to provide the desired conditions during storage in warehouses and the extra bacterial load that may come from the salts used in salt curing with potential lipolytic and proteolytic activities. Furthermore, there is an adverse environmental impact of salt curing method and the subsequent soaking procedure which results in the discharge of excessive salt. Natural resources can be a solution to minimize the use of salt in raw hide curing, at least these resources can be applied together with salt. Thus, potentially harmful bacteria arising from salt can be kept at a minimum level. It is important to test the antibacterial activity of extracts obtained from natural sources on halophilic bacteria. In the literature, there are few studies on the antimicrobial potency of lichen extracts against moderately halophilic bacteria with proteolytic and lipolytic activities. Researchers isolated some proteolytic and lipolytic Gram-negative moderately halophilic bacteria from salted sheepskin samples.[18] It was stated that the main cause for the presence of bad odor, cream and yellow discolorations, sticky appearance maybe due to these bacterial species.[18] From this point, we tested the antimicrobial properties of the extracts of *Usnea sp.*, *P. glauca, R. farinacea, E. divaricata, Bryoria capillaris, Hypogymina tubulosa, P. furfuracea* and *Lobaria pulmonaria* as natural resources against *Chromohalobacter canadensis* (YN6) and *Halomonas eurihalina* (BL5) which were isolated from salted sheepskin samples in the previous study.[18]

Materials and Methods

Moderately Halophilic Test Bacteria

Moderately halophilic test bacteria (*C. canadensis,* YN6 and *H. eurihalina,* BL5) obtained from the culture collection of the Division of Plant Diseases and Microbiology, Biology Department, Faculty of Arts and Sciences, Marmara University (Turkey) were used in this study. These bacteria were isolated from salted sheepskin samples imported from Greece and Bulgaria and identified with molecular methods in the previous study of Caglayan et al. (2017).[18] While optimal growth conditions for *H. eurihalina* were detected as 10% NaCl, 37°C, and pH 7, *C. canadensis* showed optimum growth at 7.5%-10% NaCl, 30°C-37°C, and pH 7.[18] Complex Agar Medium I (CMI) prepared with 0.5% yeast extract and 2% agar was utilized as the growth medium of test bacteria. The final salt concentration of CMI was 10% with the following composition (SW10, saline water): 0.7% $MgCl_2$, 0.96% $MgSO_4$, 8.1% NaCl, 0.2% KCl, 0.036% $CaCl_2$, 0.0026% NaBr and 0.006% $NaHCO_3$.[48] The colonies of *C. canadensis* (YN6) and *H. eurihalina* (BL5) grown on Complex Agar Medium I were checked for purity and pure colonies were used for the experiments.

Lichen Samples

The samples belonging to *P. glauca, R. farinacea, E. divaricata, B. capillaris, H.tubulosa, Usnea sp., P. furfuracea* and *L. pulmonaria* were collected from Bursa Aladağ region. The classical taxonomic method via microscopic examination was utilized in the identification of lichen samples.

Photos of lichen samples are given below.

Extraction of Lichen Samples

The extraction processes were started by washing and drying the collected lichen samples. These samples were placed in sterile bottles and acetone (ACS, ISO, Reag. Ph Eur) solvent was added to them and kept in a dark environment for 24 hours. After 24 hours, the samples were filtered through filter paper. Acetone was removed by evaporation with the help of a rotary evaporator, and acetone extracts of lichens were obtained and stored for use at +4°C.

Antibacterial Tests

C. canadensis (YN6) and *H. eurihalina* (BL5) were grown in Tryptic Soy Agar supplemented with salt and yeast extract at 37°C for 24 h. Tryptic Soy Broth (including salt and yeast extract) was utilized for the antibacterial tests of acetone extracts of tested lichen samples against test bacteria. Antibacterial efficacy was determined using 96-well microplates (Greiner Bio-One, CellStar, F-bottom, with lid). Firstly, 50 µL of medium was placed into each well in 96-well microplates. Then, 50 µL of the tested lichen extract were added. Two-fold dilution concentrations of the tested lichen extract were made in every subsequent well. After serial dilutions were made, 50 µL of overnight bacterial culture of YN6 and BL5 with an optical density (OD) 600 nm of 0.01 were added to the wells. Therefore, final volume was 100 µL in each well. The acetone extracts of lichen samples were initially added in five dilutions to Tryptic Soy Broth in each well. The acetone extracts were applied at the concentrations of 240,120, 60, 30 and 15 µg/mL (5 dilutions) or 240, 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.9375, and 0.43875 µg/mL (10 dilutions). Depending on the species of lichen or bacteria, some lichen extracts showed efficacy up to 5 dilutions, while others showed efficacy up to 9 dilutions. It was not necessary to decrease the concentration in the groups that did not have an antibacterial effect below 15 µg/mL. Control

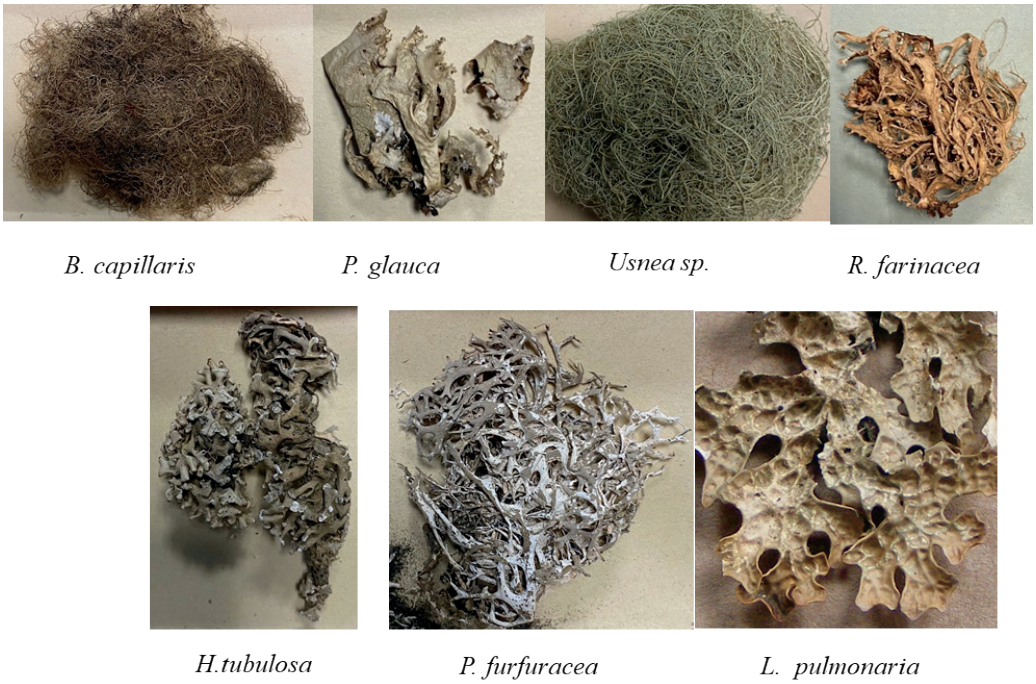

Figure 1. Photos of lichen samples.

(untreated group (medium and bacteria)) and blank (only medium) wells were also included in the experiments. Experiments were done in triplicate. The bacterial growth was evaluated every 20 minutes for 24 h using Cytation 3 multimode microplate reader (Biotek), by measuring the absorbance (OD, 600 nm) The measurement of absorbance provides information on changes in the optical density of the bacterial population. The antibacterial effects of acetone extracts of lichen samples against the test samples were compared with the control ones.[44-47] To test bactericidal or bacteriostatic effects of the tested lichen extracts against the test bacteria, first 10 µL of the test medium were taken from 24 h incubated-96 well microplates which include extracts and bacteria (test groups) and then spread onto the Tryptic Soy Agar medium containing salt and yeast extract. After incubation of the agar medium at 37°C for 24 h, bactericidal or bacteriostatic effects of the tested lichen extracts against the test bacteria were evaluated according to the presence or absence of bacterial colonies on the agar medium.

Statistical Analyses

Statistical analyses were evaluated by SPSS version 16.0 software program with One-way ANOVA (Tukey) to find significant differences between varying concentration groups of extract and untreated groups. A p-value below 0.05 was accepted as significant. The same letters in the figures indicate that there is no significant difference between the concentrations, while different letters indicate a significant difference.

Results and Discussion

As known, salt curing is a traditionally used preservation method to control halophilic and non-halophilic bacteria in different countries. However, these bacteria may cause destructive problems to the finished product. The potential efficacy of lichen extracts against moderately halophilic bacteria isolated from salted skins has not yet been studied in the literature. From this point, the potential antibacterial efficacy of some lichen species against two moderately halophilic bacteria such as *C. canadensis* (YN6) and *H. eurihalina* (BL5), which were isolated and identified from salted sheepskin samples in the previous study[18] has been examined. Therefore, the isolates mentioned above were chosen and the efficiency of the lichen extracts was tested on these microorganisms.

According to the results of the previous study,[18] the isolates, YN6 and BL5 were Gram negative bacteria, and positive for the biochemical activities of catalase, protease, lipase, methyl red degradation, and production NH_3 from peptone. Whereas YN6 was oxidase negative, BL5 was oxidase positive. Both strains were found to be positive for β-galactosidase activity and reduced nitrate to nitrite. Amylase and caseinase were not produced by the strains. BL5 was urease positive and produced H_2S whereas YN6 was indole positive. They were both citrate negative. Both YN6 and BL5 utilized L-arginine, L-glycine, L-alanine, L-tyrosine, and L-proline. In molecular analyses, these strains were assigned to *C. canadensis* (YN6) and *H. eurihalina* (BL5) with similarity percentages of 99.8 and 99.2%.[18] Overall these findings may suggest that *C. canadensis* (YN6) and *H. eurihalina* (BL5) may play an active role in skin biodegradation due to their proteolytic ability and utilization of the amino acids, which are main components of skin structure. As mentioned before, harmful activities of bacteria and skin degradation are unwanted situations for the finished leather as it causes loss of quality.

The need for potential antibacterial agents has been indicated in the literature. In this view, the antibacterial effect of lichen samples against various bacteria was analyzed in most studies. The antibacterial activity of the extracts from *P. furfuracea* lichen against several bacteria and fungi (*Staphylococcus aureus, Bacillus subtilis, B. cereus, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa, Aspergillus niger, A. fumigatus, A. candidus, A. flavus, Penicillium jensenii, Geotrichum candidum* and *Candida albicans)* growing on the raw skin and chrome-tanned leather was carried out by Türkan, et al. (2013).[42] Furthermore, in recent studies, Berber (2020) and Berber et al. (2020) reported antibacterial activities of some lichen species including *H. physodes, E. divaricata, P. furfuracea, Usnea sp., P. sulcata* and *H. tubulosa* against several bacterial isolates which were obtained from soak liquor samples.[44-47] Berber (2020) demonstrated the potency of the acetone extracts of *H. physodes, E. divaricata, P. furfuracea* and *Usnea sp.* against *B. toyonensis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. velezensis, B. cereus*, and *B. licheniformis*.[44] More recently, Berber et al. (2020) screened six lichen species for the potential efficiency against *Enterococcus durans*, which was isolated from soak liquor samples.[45] The researchers reported that only the acetone extracts of *P. sulcata*, amongst these lichen extracts, had no antibacterial effect. While other lichen extracts had a considerable antibacterial effect against *E. durans*, *Usnea* sp. in particular had prominent efficacy. In another study, *H. tubulosa* extracts were reported to be more efficient against the aforementioned isolates when compared to the acetone extracts of *P. sulcata*.[46]

In the present study, the acetone extracts of *P. glauca*, *H. tubulosa*, *R. farinacea*, *Usnea sp.*, *E. divaricata*, *L. pulmonaria*, *B. capillaris*, *P. furfuracea* were examined against both test bacteria. Considering that *P. sulcata* extracts had no potential activity on isolates tested from soaked liquor samples in previous studies, *P. sulcata* was not included in this study. On the other hand, to the best of our knowledge, *P. glauca*, *R. farinacea*, *L. pulmonaria* and *B. capillaris* were firstly tested on isolates obtained from salted sheepskins in the leather industry. The acetone extracts were applied at the concentrations of 240-15 µg/mL (5 dilutions) or 240-0.46875 (10 dilutions) µg/mL. Depending on the lichen or bacteria, some lichen extracts showed efficacy up to 5 dilutions, while others showed efficacy up to 9 dilutions. Acetone as a solvent was chosen for the extraction of lichen samples due to its potency of comprehensive extraction of the compounds with various bioactivities. All of the extracts of lichen species were firstly screened

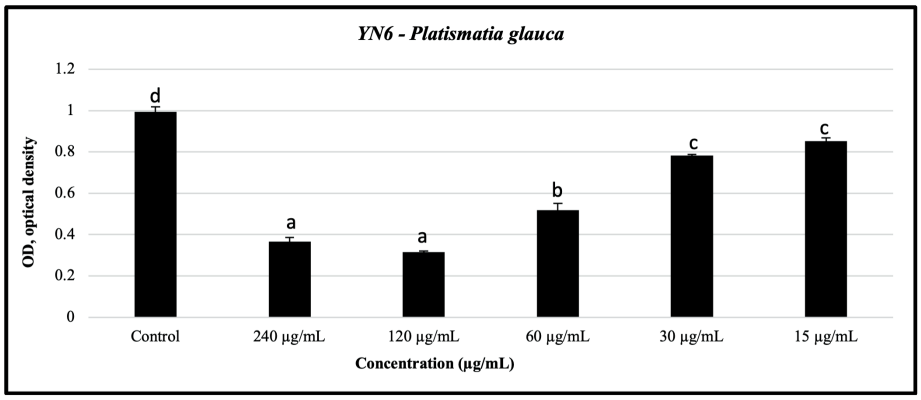

Figure 2. The antibacterial effects of the extracts of *P. glauca* against *C. canadensis* (YN6).

for the possible antibacterial efficacy on both bacteria. According to these preliminary screening studies, the extracts of *L. pulmonaria, B. capillaris* and *P. furfuracea* did not have any antibacterial effect on tested isolates. Otherwise, the extracts of *P. glauca, R. farinacea, Usnea sp., E. divaricata* and *H. tubulosa* were recorded to have various potential antibacterial efficacies against test bacteria.

The acetone extracts of *P. glauca* at concentrations of 240 and 120 µg/mL were shown to have average antibacterial activity against *C. canadensis* (YN6) when compared to the control group with inhibition rates of 63.14 and 68.22%, respectively. On the other hand, the inhibition percentages of *P. glauca* extracts for bacterial growth of YN6 were 47.83, 21.19 and 14.13% at tested concentrations of 60, 30 and 15 µg/mL. In statistical analyses for *P. glauca* extracts against *C. canadensis* (YN6), 240-15 µg/mL groups were found to be statistically significant compared to control groups ($p<0.05$). Significant differences were also detected in 240 µg/mL group when compared to all other treatment groups ($p<0.05$) except 120 µg/mL. In the statistical comparison of 120 µg/mL group, the differences were found to be significant between all groups ($p<0.05$). On the other hand, there were significant differences between 60 µg/mL and 30 µg/mL groups ($p<0.05$). But there was no difference in 30 and 15 µg/mL (Figure 2).

A great inhibition was detected in the bacterial growth of *C. canadensis* (YN6) by the acetone extracts of *H. tubulosa* at the concentration of 240 µg/mL. The inhibition percentages for *H. tubulosa* extracts were 81.12, 57.44, 67.26, 72.13 and 66.73 for the tested concentrations of 240-15 µg/mL, respectively (Figure 2). Statistically significant differences were detected in all treatment groups of *H. tubulosa* against *C. canadensis* (YN6) when compared to control groups ($p<0.05$). Similarly, each treatment group of 240 and 120 µg/mL differed significantly among themselves from the other low concentration groups ($p<0.05$). In 60 µg/mL treatment group, a significant difference has been detected with 30 µg/mL group ($p<0.05$) whereas no difference was observed with 15 µg/mL group. Also, 30 µg/mL group has demonstrated significant difference with 15 µg/mL group ($p<0.05$). This statistical information was included in Figure 3.

The acetone extracts of *R. farinacea* showed average antibacterial effects against YN6 as in *P. glauca* extracts. The concentrations of 240, 120 and 60 µg/mL were more successful according to the other tested ones with the inhibition ratios of 65.37, 71.37 and 54.57%, respectively. No noteworthy antibacterial effect (27.26 and 12.39%) was recorded at the concentrations of 30 and 15 µg/mL (Figure 3). In treatment groups with *R. farinacea* extracts, there were statistically significant differences when compared to control groups ($p<0.05$) except the 15 µg/mL group. The 240 µg/mL treatment group showed no significant difference with 120 and 60 µg/mL but significantly different from 30 and 15 µg/mL groups. In 60 µg/mL treatment group, significant difference has been detected with 30 and 15 µg/mL groups ($p<0.05$). Also, 30 µg/mL group has demonstrated significant difference with 15 µg/mL group ($p<0.05$). This statistical information was included in Figure 4.

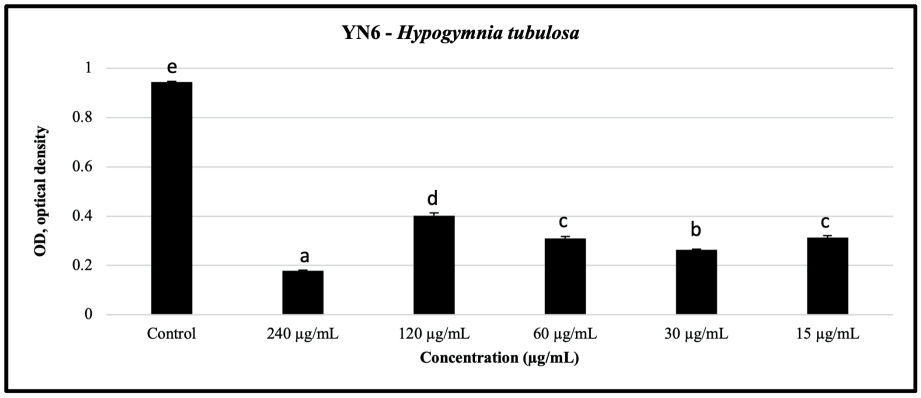

Figure 3. The antibacterial effects of the extracts of *H. tubulosa* against *C. canadensis* (YN6).

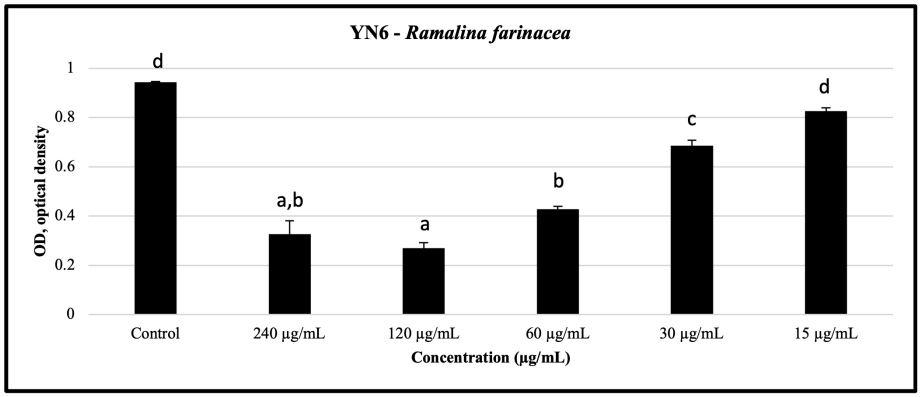

Figure 4. The antibacterial effects of the extracts of *R. farinacea* against *C. canadensis* (YN6).

YN6 isolate was also inhibited by more than 50% by the extracts of *Usnea* sp. at concentrations of 240, 120, and 60 µg/mL. The inhibition ratios were recorded as 89.5, 64.89, 63.58, 44.65, and 37.2% for all concentrations, respectively. Statistically significant differences were observed when all treatment groups were compared with the control group ($p<0.05$). Similarly, a comparison of 240 µg/mL group with the other treatment groups revealed a significant difference ($p<0.05$). At lower concentrations, there was no significant difference between the 120 and 60 µg/mL groups as well as 30 and 15 µg/mL groups (Figure 5).

The antibacterial efficacy was also observed for the extracts of *E. divaricata* against YN6 isolate. At the concentration of 240 µg/mL, 90.6% inhibition was detected on the bacterial growth. Also, antibacterial effects were observed at the concentrations of 120 and 60 µg/mL with inhibition ratios of 62.52% and 59.62%, respectively. The concentrations of 30 µg/mL and 15 µg/mL did not show notable efficiency against YN6 isolate with the inhibition ratio of 38.1 and 14.2, respectively (Figure 6). While no significant difference was found between 120 and 60 µg/mL treatment groups, a significant difference was observed between all other groups ($p<0.05$).

According to our results, the acetone extracts of *H. tubulosa* had considerable efficacy against *H. eurihalina* (BL5) at the concentrations of 240, 120, 60, 30, 15 and 7.5 µg/mL. The growth of BL5 was inhibited by the inhibition ratio of 84.52, 85.5, 90.41, 92.89, 97.12, 99.12%,

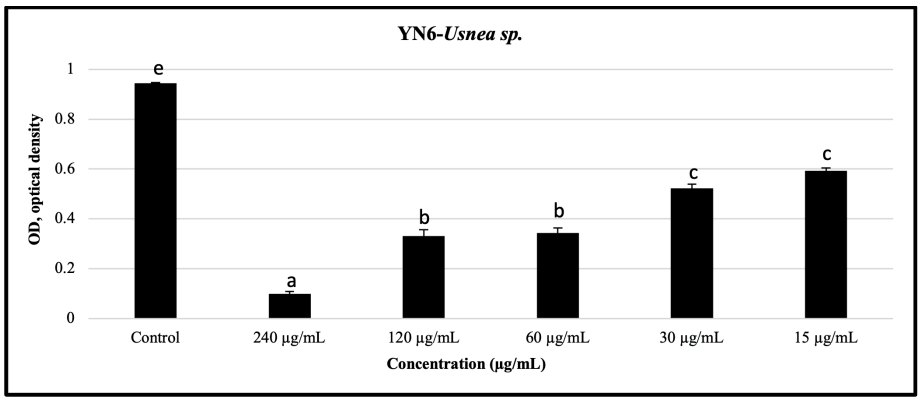

Figure 5. The antibacterial effects of the extracts of *Usnea* sp. against *C. canadensis* (YN6).

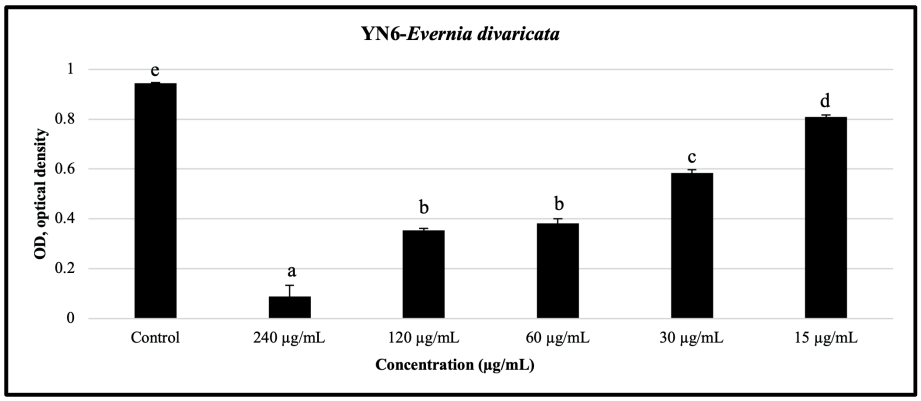

Figure 6. The antibacterial effects of the extracts of *E. divaricata* against *C. canadensis* (YN6).

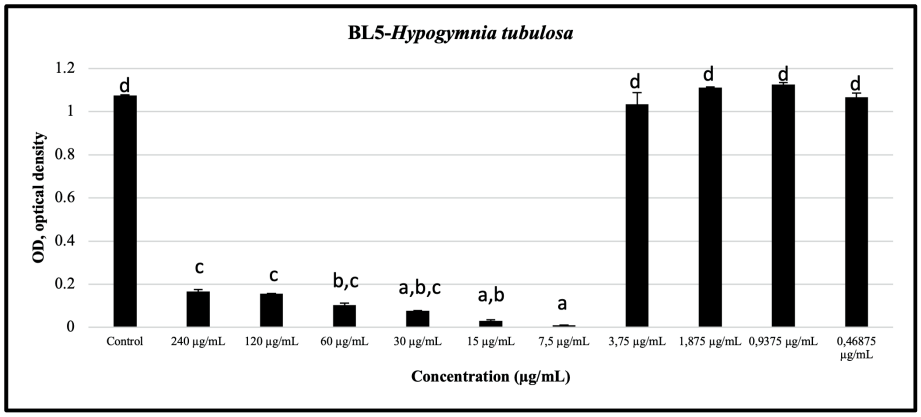

Figure 7. The antibacterial effects of the extracts of *H. tubulosa* against *H. eurihalina* (BL5).

respectively. As seen in Figure 7, the lower concentrations from 7.5 µg/mL (3.75, 1.875, 0.9375, and 0.46875 µg/mL) had no antibacterial efficacy against BL5. There was no significant difference between the 3.75 µg/mL treatment group and the lower concentration groups. In addition, there was no statistically significant difference between the 240 µg/mL treatment group and the 120, 60, 30 µg/mL groups, the 60 µg/mL treatment group and 30 and 15 µg/mL groups, and the 30 µg/mL treatment group and 15 and 7.5 µg/mL groups ($p<0.05$). Apart from these, it was observed that there was a significant difference between all other groups.

The most outstanding results were obtained with *Usnea* sp. extracts against *H. eurihalina* (BL5). As seen in Figure 8, the extracts were successful for the inhibition of BL5 bacterial growth up to 9 dilutions. However, at the last dilution increasing effect was recorded. Compared to control groups, detected inhibition rates were 82.65, 63.26, 87.73, 90.81, 91.72, 91.11, 91.68, 91.21 and 91.13% for 9 dilutions. At the last tested concentration, bacterial growth of BL5 was observed to increase at the percentage of 22.83. In statistical analyses for *Usnea* sp. extracts, all treatment groups, except 0.46875 µg/mL group, showed significant differences when compared to the control group ($p<0.05$). In the 120 µg/mL group, statistically significant differences were observed with the 30, 15, 7,5, 3,75, 1,875, 0,9375 µg/mL groups. There was no significant difference between the 240, 120, and 30 µg/mL groups. The 60 µg/mL group had no significant difference from the 30 and 15 µg/mL groups. Similarly, no significant difference was obtained among the 30 µg/mL group, 15 and 7.5 µg/mL groups.

Our results showed that the extracts of *R. farinacea*, especially at the concentrations of 240-30 µg/mL, had a great suppressive effect on bacterial growth of BL5 with the inhibition percentages of 98.01, 92.20, 98.12, 96.47. On the other hand, lower concentrations from 30 µg/mL, the inhibitory effect was not observed (between 30.84 and 4.31%) (Figure 9). All treatment groups, except the 7.5 µg/mL group, showed significant differences when compared to the control group ($p<0.05$). There was no statistically significant difference between 240, 120, 60, and 30 µg/mL treatment groups. Statistically, significant difference was observed between 60 µg/mL treatment group and the lower concentrations from 15 µg/mL group ($p<0.05$). The 15 µg/mL group showed statistically significant difference with the 7.5, 3.75, 1.875, 0.9375 and 0.46875 µg/mL groups ($p<0.05$). There was also a significant difference between the 7.5 µg/mL group and 3.75-1.875 µg/mL groups ($p<0.05$). On the other hand, there was no significant difference between 3.75 and 1.875, 0.9375 and 0.46875 µg/mL groups. Additionally, no difference was observed for 7.5 µg/mL and 0.9375 and 0.46875 µg/mL groups (Figure 8).

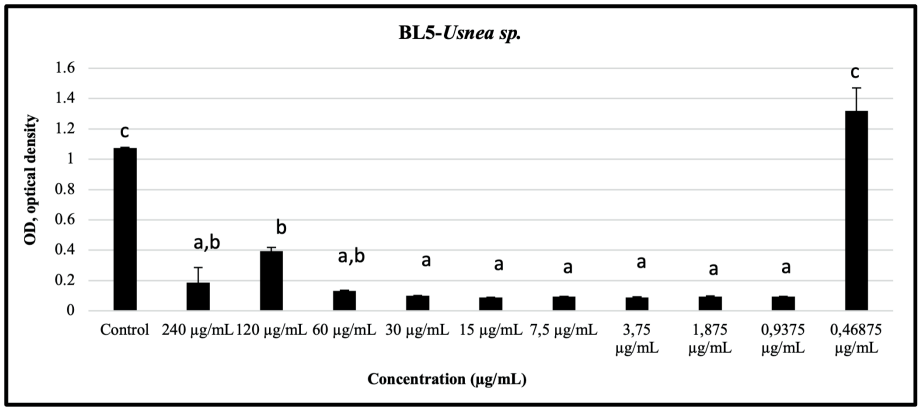

Figure 8. The antibacterial effects of the extracts of *Usnea* sp. against *H. eurihalina* (BL5).

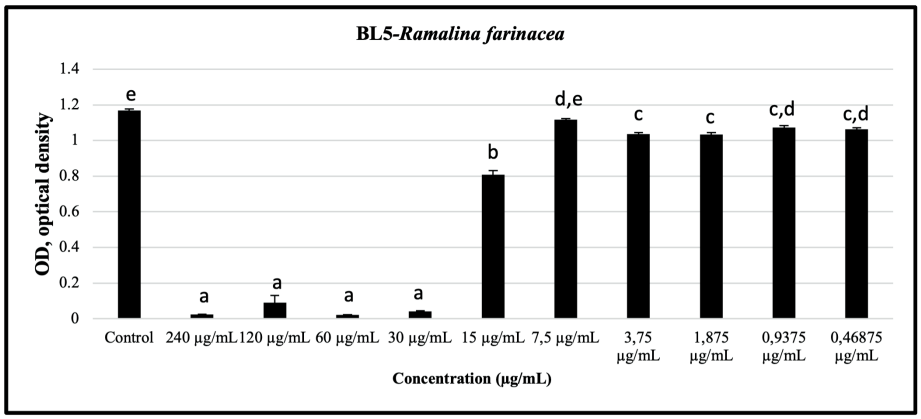

Figure 9. The antibacterial effects of the extracts of *R. farinacea* against *H. eurihalina* (BL5).

At the concentrations of 240, 120, 60, 30 and 15 µg/mL of *E. divaricata* extracts, there was also a notable antibacterial effect against *H. eurihalina* with the inhibition ratios of 91.25, 87.02, 91.04, 98.45, and 98.06%, respectively. 7.5 µg/mL and lower concentrations of *E. divaricata* extracts did not show any efficacy (between 25.70 and 5.89%) against *H. eurihalina*. Data were given in Figure 10. In the comparison of 3.75, 1.875, and 0.9375 treatment groups and the control group, there was no significant difference. On the other hand, other treatment groups showed statistically significant differences when compared to the control group ($p<0.05$). The treatment groups of 3.75, 1.875, 0.9375 and 0.46875 µg/mL have no significant difference among them (Figure 10).

We observed that the lichen extracts had bactericidal activities at the highest concentrations that the potential antibacterial activities were observed, whereas the lower but effective concentrations had bacteriostatic effects against test bacteria.

Overall, these results indicate that lichen extracts have varying effects against tested bacteria. In this respect, lichen extracts being both ecological and effective antibacterial agents against these bacteria may have a potential solution for antibacterial ineffectiveness in the leather industry. Some bacterial strains may cause a quality loss in the final product due to antibacterial resistance that occurred during leather-making processes. Therefore, natural products, especially from plants, have been investigated for their antibacterial potential on the growth of bacteria that may be encountered in the leather industry. In previous studies, antibacterial efficacy for essential oils of *Lavandula officinalis* and *Origanum minutiflorum* was reported. Moreover, myrtle oil (1%) was found to be effective in the soaking process.[48-50] On the other hand, there is no study focused on lichen extracts and/or their compounds against *C. canadensis* (YN6) and *H. eurihalina* (BL5). However, similarly to previous studies, *Usnea* sp. extracts were found to be effective for the suppression of bacterial growth even in very small concentrations.[44-45,47] The extracts of several lichens were known to have more prominent effects against Gram-positive bacteria in the literature. Although test bacteria are Gram-negative in the present study, sufficient antibacterial effects were demonstrated. In this regard, not only Gram-positive but also Gram-negative bacteria may be controlled by the lichen extracts or their compounds with antibacterial properties may be utilized in this sector. As well known, the secondary metabolites of most lichens are responsible for this mentioned antibacterial potential against several bacteria. These active chemical groups of

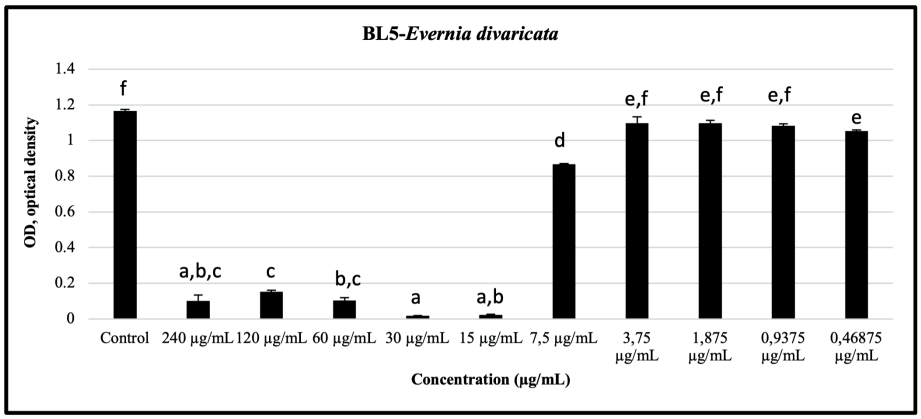

Figure 10. The antibacterial effects of the extracts of *E. divaricata* against *H.eurihalina* (BL5).

lichen - are reported as phenols, terpenes, steroids, anthraquinones, depsides, and depsidones. Some metabolites with antibacterial activities which are mentioned in the literature are usnic acid, lecanoric acid, atranorin, salazinic acid, olivetoric acid, stictic acid fumarprotocetraric acid, protocetraric acid, usnic acid, vulpinic acid, evernic acid, lobaric acid, physodic acid, barbatic acid, divaricatic acid, diffractaic acid, emodin, vulpic acid, psoromic acid, sekikaic acid, caperatic acid, homosekikaic acid etc.[51] For example, usnic acid, diffractaic acid, lecanoric acid, barbatic acid from *Usnea* sp., olivetoric acid, atranorin, chloroatranorin, physodic, oxyphysodic acid, virensic acid, and olivetoric acid acid from *P. furfuracea* and stictic acid, isidiophorin, rhizonaldehyde, rhizonyl alcohol, pulmonarianin, vesuvianic acid, ergosterol peroxide, usnic acid, and diffractaic acid from *Lobaria pulmonaria* (L.) Hoffm. were reported in the literature.[52-55] These results, which vary depending on the bacteria and lichen species, may be due to the differences in secondary metabolites of lichen species. Overall, further detailed studies may clarify which compounds are responsible for these potential antibacterial activities.

Conclusion

In this study, some lichen extracts were found to be potent for the inhibition of bacterial growth of *C. canadensis* (YN6) and *H. eurihalina* (BL5). In this respect, these natural biosources may also be used during storage periods of raw hides in warehouses. Moreover, in recent years, there has been an orientation towards nature in every sector. Lichen extracts are both ecological and non-synthetic. There is a need for further studies to investigate the feasibility of the application for compounds from lichens in raw hides/skins or subsequent processes in leather processes. In this respect, we made a preliminary experiment with acetone extracts of effective lichen species on salted sheepskin samples (data not shown). According to our first data, salted sheepskin samples treated with lichen extracts had considerably inhibitory zones on agar plates. These data suggest that detailed studies have to be performed to show the applicability of these natural extracts/their chemical compounds in the leather industry such as skin preservation.

Acknowledgement

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An Ensemble of Fine-Tuned Deep Learning Networks for Wet-Blue Leather Segmentation

by

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Abstract

As part of industrial quality control in the leather industry, it is important to segment features/defects in wet-blue leather samples. Manual inspection of leather samples is the current norm in industrial settings. To comply with the current industrial standards that advocate large-scale automation, visual inspection based leather processing is imperative. Visual inspection of wet-blue leather features is a challenging problem as the characteristics of these features can take on a variety of shapes and colour variations to constitute various normal and abnormal surface regions. The aim of this work is to automatically segment leather images to detect various features/defects along with the background through visual analysis of the surfaces. To accomplish this, a deep learning-based technique is developed that learns to segment wet-blue leather surface features. On our own curated leather images dataset, the proposed ensemble network performed well, with an F1-Score of 74 percent.

1 Introduction

The leather industry is one of the most important historic industries. The leather is mostly supplied to downstream leather products companies, which utilise it as a raw material to make leather shoes, handbags, luggage, gloves, belts, and sofas, among other things.[1] Inspectors examine the leather by hand, physically inspecting it and marking any defects with chalk. Because manual inspection might lead to fatigue and misidentification, the final judgement must be double-checked and approved by numerous inspectors. As a result, a quick, thorough, and noninvasive leather assessment technique has become necessary.[1]

Despite the fact that current manufacturing procedures fulfill high technological standards, quality inspection of leather products has room for improvement in terms of effectiveness and efficiency. The leather materials have a lot of natural defects/features because they are made from animal skin (cowhide, sheepskin, pigskin, and so on). On treated leather surfaces, scars, stains, wrinkles, cuts, and colour variations are common defects, whereas common normal features include creasing and folds that can be of interest due to their frequent occurance. As a result, developing a systematic procedure for evaluating leather surface faults/features is crucial for maintaining high-quality product consistency.

Artificial intelligence (AI) is the study of theories, methods, and systems that will allow machines to mimic human intelligence. Neural networks are a first step in this direction, seeking to mimic the capabilities of the human brain. Image processing is an area of AI that focuses on replicating human vision behaviour. While several AI techniques have helped in the replication of simple human vision, tasks like handwritten digit identification, number plate reading, and visual recognition, neural networks have made great progress.[2] Neural networks have demonstrated their efficacy on a wide range of image processing applications including image enhancement, compression, image segmentation, object recognition and image understanding.[3] Image enhancement, compression, image segmentation, object recognition, and image understanding are just a few of the applications for neural networks.[2]

For visual inspections of leather, non-neural network based AI approaches have already been investigated.[4,5] Despite their excellent performance, current technologies are still a long way from providing a generic solution for large-scale visual inspection. Furthermore, the full potential of CNN-based (a form of neural network) approaches for visual leather evaluation has yet to be realised. The unavailability of data sets, which is a major hindrance to expansion in this field, is one probable reason. The data from previous studies has not been made publically available for comparison.

The purpose of this research is to develop an automated defect detection system that can segment irregular areas of defects and interesting normal features. To analyse the test dataset and design a robust architecture, different instance segmentation deep learning models are used, such as UNet,[6] Segnet,[7] and Fully Convolutional Network (FCN).[8] The major objective, as set out in this paper, is to to propose a systematic ensembling of state-of-the-art CNNs and their adaptation for leather image segmentation.

In this study, we provide a systemically constructed ensemble convolutional neural network for robust leather sample segmentation based on empirical assessments. We also put together a new high-resolution wet-blue leather dataset with two classes containing

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images with abnormal feature types, whereas the third class contains normal leather images with interesting surface features, which occur frequently and therefore can not be neglected. So the classification problem becomes a generic one that is to learn three feature types based on the visual characteristics they present. The images were taken in a controlled environment with a digital camera. Appropriate lighting, distance from the leather surface, range of vision adjustment, and high-definition photos are all required for image capturing with a digital camera equipment.

The major contributions of the proposed work are:

- a new ensemble method for robust leather defect/feature segmentation,
- a thorough comparative evaluation of the proposed method with benchmark deep learning based segmentation methods,
- introduction of a new high-resolution leather images dataset for stimulating research in the field.

The rest of the paper is organized as follows. Section 2 contains a literature review on macihne learning based leather defect inspection. Section 3 describes the proposed method. Section 4 explains the experimental design. In section 5 all the results are presented. Class Activation Maps are explained in section 6. Finally, we conclude our work in section 7.

2 Literature Review

In this section, we review several machine learning based methods proposed in the literature for leather defect segmentation. The task of instance segmentation, which demarcates the image's regions of interest, has received minimal attention from the research community. Lovergine et al.[9] conducted one of the earliest studies on defect localization and segmentation. A black and white CCD camera was used to detect and determine the defective areas. The texture orientation features of the leather are reconstructed using a morphological segmentation[10,11] approach applied to the obtained images. The study has a few qualitative conclusions, but no quantitative methodologies or numerical data with which to evaluate the proposed methodology. Lanzetta and Tantussi[12] provide a laboratory prototype for trimming a leather's external part. Binarization, opening, and laplacian mask approaches are used to locate the trimming path from the leather sample images in order to find the background and troublesome regions. The proposed defect detection system correctly detects the majority of flaws on different types of leather. The surface polish and colour, on the other hand, are still the most essential factors that can influence the inspection's outcome.

A segmentation approach by Liong et. al.[13] was utilised to automatically capture the leather image and locate defects. The total amount of image data obtained from a single piece of leather was 584, with the tick bite defect being mostly included in the little leather patches. To find the damaged regions, the spotting approach Mask R-CNN (Regional Convolutional Neural Network)[14] was employed. When looking at training data, the average segmentation accuracy was 91.5 percent.

3 The Proposed Method

In this paper, we investigate deep learning architectures for defect segmentation in wet-blue leather. A Nikon Coolpix P300 camera was used to capture the photos. To increase the generalisation of segmentation models on unknown data, training images are augmented to add new image variants. Augmented data is used to train the models. For the inference stage, the best weights of the trained models are saved. The test data and the trained model are used in the inference phase to generate model predictions, which are then used to evaluate the model's performance in the test stage. The generic workflow of the proposed method is shown in Figure 1.

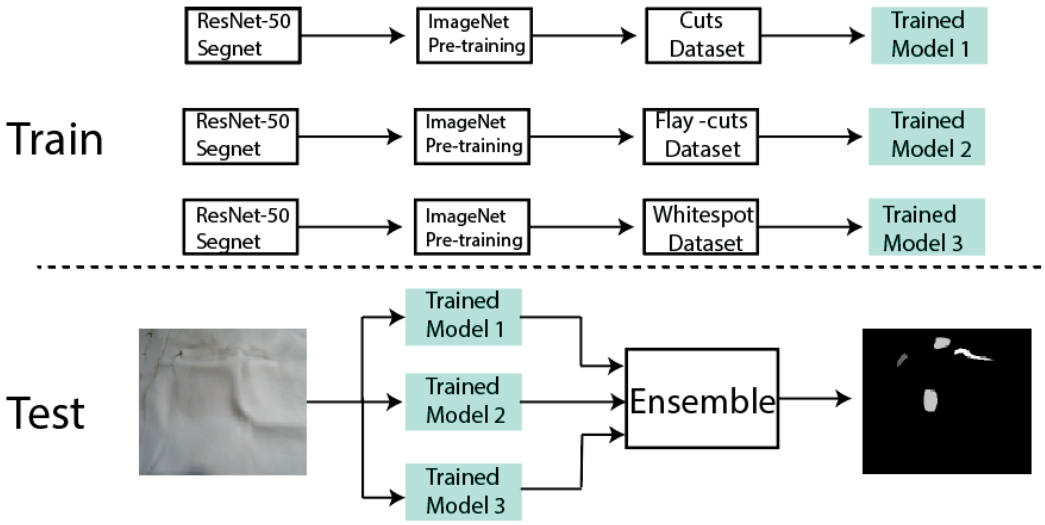

Figure 1. The generic workflow of the proposed method. The training phase is depicted on the top and the inference phase is shown on the bottom.

3.1 Data Augmentation

In order to train the network with different variations of the input images by artificially generating new images for the training, the data augmentation module was added to our workflow. In order to minimise network overfitting and enhance model generalisation, the data augmentation effect has been practically demonstrated.

The images are first resized to 512 × 384 before data augmentation. The chosen augmentation techniques employed in our experiments are horizontal flipping, vertical flipping and rotation using a random angle in the range [90, 360]. After the data augmentation process, a total of 960 training images were collected. The training data was split randomly into training, validation sets with a 80:20 ratio.

3.2 Architectures

Pixel-wise masks for each region of interest must be produced to denote the item discovered during the segmentation process. (FCN)[8] is the first work to train FCN end-to-end for pixel-wise prediction. The key idea of FCN[8] is to replace the fully connected layer of typical classification neural networks with convolutional layers so that a network output can be a two dimensional heat map, rather than class probability prediction. FCN[8] implements "skip architecture", meaning that shallow layer's outputs are merged to deeper layers so that the network can maintain both local and coarse information. SegNet[7] consists of an encoder network, a corresponding decoder network followed by a pixel-wise classification layer. Each encoder layer has corresponding decoder layer. The decoder used pooling indices computed in a max pooling step of the corresponding encoder to perform non-linear up-sampling. Since positional or boundary information are lost during the max pooling operations in the encoder network, maintaining positional information for each up-sampling operation in the decoder network is critical for accurate pixel-wise segmentation.

UNet[6] is used to indicate the precise position of the discovered defect. To summarise, UNet employs a network architecture that supports both downsampling and upsampling. Another name for it is the encoder-decoder structure. The pooling layer is used by the encoder to gradually reduce the spatial dimension of the input data, while the deconvolution layer is used by the decoder to restore the target's details and the proper spatial dimension. Most of the time, the encoder and decoder will share information directly. This is to help the decoder get the information it needs as quickly as possible. The instance segmentation system employs a four-level network structure. In summary, the encoder is made up of several building blocks, each with two convolutional operation layers and one pooling operation layer. Below the encoder, two convolution layers and a dropout layer are added to serve as a bridge to the decoder. In each block of the decoder design, a maxunpooling layers and two convolution layers follow the decoding method. Surprisingly, after each maxunpooling layer, the processed data is required to perform a depth concatenation with the output produced by the second convolution layer from the associated encoder block. Finally, a convolution layer, a softmax layer, and a pixel classification layer are employed for output prediction. To ensure that the input and output volumes are always the same, padding is utilised on all convolutional layers.

3.2 Ensembling Convolutional Neural Networks

Ensemble approaches combine several segmentation models, and it has been found that it is possible to obtain greater precision results than a single model. To boost the segmentation performance we trained three models on each class and then combined these modes to form one segmentation model. Instead of training models on complete dataset we trained each model on individual class to make it a binary problem instead of multi-class problem. We trained three UNet[6] and SegNet[7] models with ResNet-50 backbone. We fused their results in the inference phase for a given test image.

There have been few papers on the application of deep learning for handling the problem brought about by a small dataset. For small datasets, all these articles use the ensemble technique. When only a limited dataset is employed, over-fitting might occur, resulting in poor segmentation. As a result, the model's ability to generalize will be limited. To overcome this problem ensemble technique is used for small datasets.[15-21] In this work, we experimented with various combinations of standard state-of-the-art networks, including UNet,[6] Segnet[7] and Fully Connected Network(FCN),[8] in order to find the ideal collection for segmenting leather defects. The training and testing workflow diagram of the proposed ensemble network is shown in Figure 1. In both related and unrelated image domains, ensemble approaches have previously proven to be the most successful tool. In ensemble techniques, different segmentation models are used, and it has been found that they can achieve higher precision results than a single model. To combine cutting-edge designs, UNet,[6] SegNet[7] and the Fully Connected Network (FCN)[8] were used. Because there are no documented representative ensemble approaches for leather defect segmentation in the literature, ensemble combinations of two network architectures were investigated, and combinations that stand out in terms of learnt representations were chosen for this study. Figure 1 shows three SegNet models, each of which is trained separately on each of the three classes. All of the models are individually trained. Following training, all of the models are integrated to form an ensemble that can be utilised to solve a multi-class segmentation problem.

4 Experimental Design

4.1 Dataset

Wet blue leather data was collected at the Fitzherbert Science Centre, Dairy Farm Road, Manawatu-Wanganui, by the Leather and Shoe Research Association of New Zealand (LASRA). Wet blue leather refers to unfinished hides that have been dehaired and chrome tanned to preserve the leather. Because of the chromium tanning chemicals, these skins are commonly referred to as "wet blue". The images were taken with a Nikon Coolpix P300 camera with a 12MP AF sensor and under ideal lighting conditions. The regions containing the features constitute approximately 5-10 % of the entire

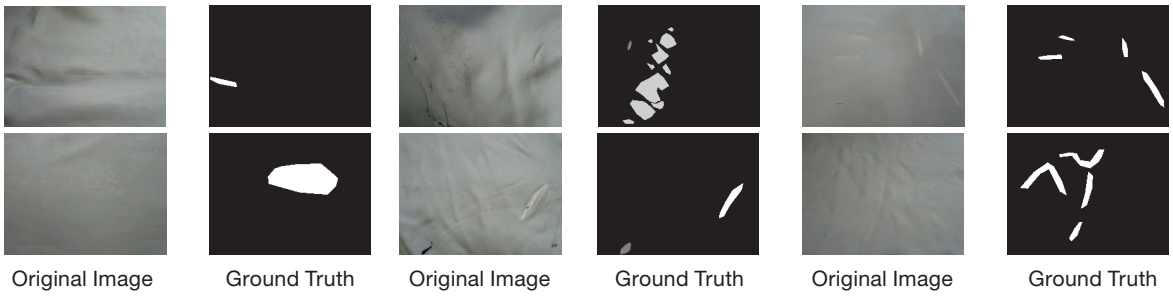

Figure 2. Dataset images with their corresponding ground truth.

image. In the dataset for each original image, the corresponding mask is provided. The mask image is considered to be the ground truth. A few example images from dataset are shown in Figure 2.

4.2 Feature/Defect Types

In this analysis, images having three types of surface features divided into three classes, repectively. The features of the first class have a set of characteristics close to cuts, termed as class A in this work. The class B contain images that include surface features that are in close resemblance to flay-cuts. Finally, the class C comprises of images containing regions that have interesting but normal features. Class C has a combination of surface features having the characteristics of creasing and folds. Although, these are not defects but have visually appealing characteristics, different appearance as compared to plain leather regions and a high frequency of occurance. The features in class A are usually elliptic in shape and do not carry much textural information, with the exception of at the edges. Class B visual features, on the other hand, have a rich texture and distinct colour tones in general that set them apart. The images from class C are characterized by bright regions which appear either due to leather bends or folds from the pressing of the wet blue on a slamming felt. These regions of high saturation usually have an elongated shape and occur at a high degree of varying scale. The majority of images incude only one feature class, however a small percentage of images have multiple class variations.

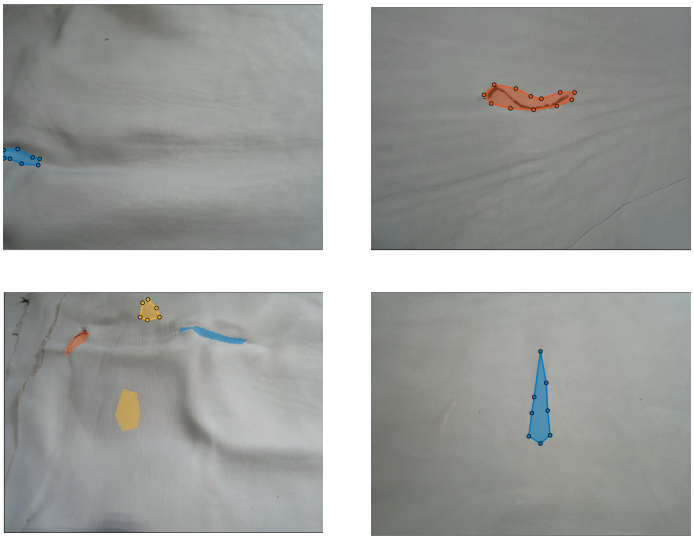

Figure 3. Representative examples of ground truth annotations. Blue color represents class C, orange colour represents class A and yellow color represents class B images.

4.3 Ground Truth Labelling

Image labeller, a popular MATLAB annotation tool, is used to create the dataset's ground truth annotations. The ability to annotate defects at various scales was a significant problem in the defect labelling process, necessitating the distinction between coarse and fine annotations. This was solved by establishing a balance of coarse and fine annotations. The grourd-truth labelled images are shown in Figure 3.

4.4 Experiment configuration

The experiments are conducted using MATLAB 2021a on an Intel(R) Xeon(R) E51620 v2 3.70 GHz processor, RAM 32GB. All the images used in the segmentation task are resized to 512 × 384. Concretely, all three FCN,[8] UNet[6] and SegNet[7] architectures are trained with Adam as the optimization algorithm. The initial learning rate is set to 0.001 and the epoch value is 20. Pixel-wise classification layer is used as the last layer in all the algorithms for the segmentation mask output.

4.5 Quantitative measures

When performing classification predictions, four types of outcomes could occur

- **True Positive** (*TP*): When a defected leather sample is predicted as defected by the model,
- **True Negative** (*TN*): When a leather sample without any defects/features is predicted as non-defected by the model,
- **False Negative** (*FN*): When defects/features were present in leather but the model could not recognize them; it is also called as a Type 2 error,
- **False Positive** (*FP*): When the leather sample was non-defective but the model predicted it as defective (containing features); it is also known as a Type 1 error.

However, TN is not very meaningful in this research because the percentage of defects/features in this dataset is approximately 5-10%. Thus, TN is always high, even when the model does not predict defects/features well. The F1 and IOU scores are more important metrics in standard segmentation evaluation.

The precision of the segmentation model indicates what proportion of positive predictions were deemed correct and is given as

$$\text{Precision} = \frac{TP}{TP + FP} \tag{9}$$

A related measure is recall which measures the proportion of actual positives which were identified correctly

$$\text{Recall} = \frac{TP}{TP + FN} \tag{10}$$

The F1-score is the average of the precision and recall

$$\text{F1} - \text{score} = 2\,\frac{\text{Precision} \times \text{Recall}}{\text{Precision} + \text{Recall}} \tag{11}$$

Consider the segmentation B_{gt} of a target object with a ground-truth bounding box and a B_{pr} prediction bounding box. Regardless of confidence level, a perfect match is defined as the projected and ground-truth boxes having the same area and location. The IOU is determined by multiplying the amount of overlap (intersection) in the object detection scope between the predicted bounding box B_{pr} and the ground-truth bounding box B_{gt} by the area of their union, which is given in equation

$$J(B_{pr}, B_{gt}) = \text{IOU} = \frac{\text{area}\,(B_{pr} \cap B_{gt})}{\text{area}\,(B_{pr} \cup B_{gt})}$$

5 Results

In this section, we begin by presenting a quantitative comparison of state-of-the-art segmentation models. Next, we present qualitative comparison of the employed segmentation models on representative images from the proposed dataset.

5.1 Quantitative Comparison

Table I shows the comparison of precision, recall, F1-score and IoU of state-of-the-art segmentation models on multi-class segmentation. UNet[6] achieves 0.47 precision, 0.38 recall and 0.42 F1-score and IoU is 34. SegNet[7] achieves 0.75 precision, 0.48 recall and 0.58 F1-score and IoU is 42. This shows that shallow networks are able to achieve better AP as compared to the deeper models.

The models' performance on multi-class segmentation had room for improvement, therefore the next step was to train the models on each class separately and evaluate how they performed. As a result, we divided the primary dataset into three datasets, one for each class, and trained the models on these datasets. The comparison of these classes is shown in Table II. Because FCN[8] was unable to provide sufficient results in the binary class, its results are not included in Table II. The main reason for this is because the FCN[8] model was over-fitting as the data was further separated into three sections, and it failed to produce any useful results because of small dataset and intra class variation which lead to over-fitting of FCN model.

In Table II, we can see the comparison of models on each class and we can see that SegNet performance was better than UNet[6] in every class. For classA, SegNet was able to achieve F1-Score of 0.86 and IoU of 0.79 while UNet performance was also similar to SegNet. For class B, both models got identical F1-Score and IoU. On class C images, again SegNet outperformed UNet and achieved an F1-Score of 0.7 and an IoU of 0.61. As SegNet performance was better than UNet in each class. For ensemble model we combined all three models of SegNet. The result of our ensemble model which is also the proposed

Table I

Comparison of the segmentation performance of our proposed method with other state-of-the-art segmentation models for the wet-blue leather dataset

Serial #	Model	Precision	Recall	F1-Score	IoU
1	UNet[6]	0.47	0.38	0.42	0.34
2	SegNet[7]	0.75	0.48	0.58	0.42
3	FCN[8]	0.85	0.46	0.60	0.44
4	Proposed	0.85	0.71	0.74	0.69

Table II

Class-wise comparison of the segmentation performance of state-of-the-art segmentation methods

Defect	Model	Backbone	Precision	Recall	F1-Score	IoU
Class A	SegNet[7]	ResNet-50	0.83	0.90	0.86	0.79
	UNet[6]	ResNet-50	0.85	0.83	0.84	0.76
Class B	SegNet	ResNet-50	0.88	0.63	0.71	0.63
	UNet	ResNet-50	0.77	0.67	0.71	0.63
Class C	SegNet	ResNet-50	0.83	0.65	0.70	0.61
	UNet	ResNet-50	0.84	0.6	0.66	0.58

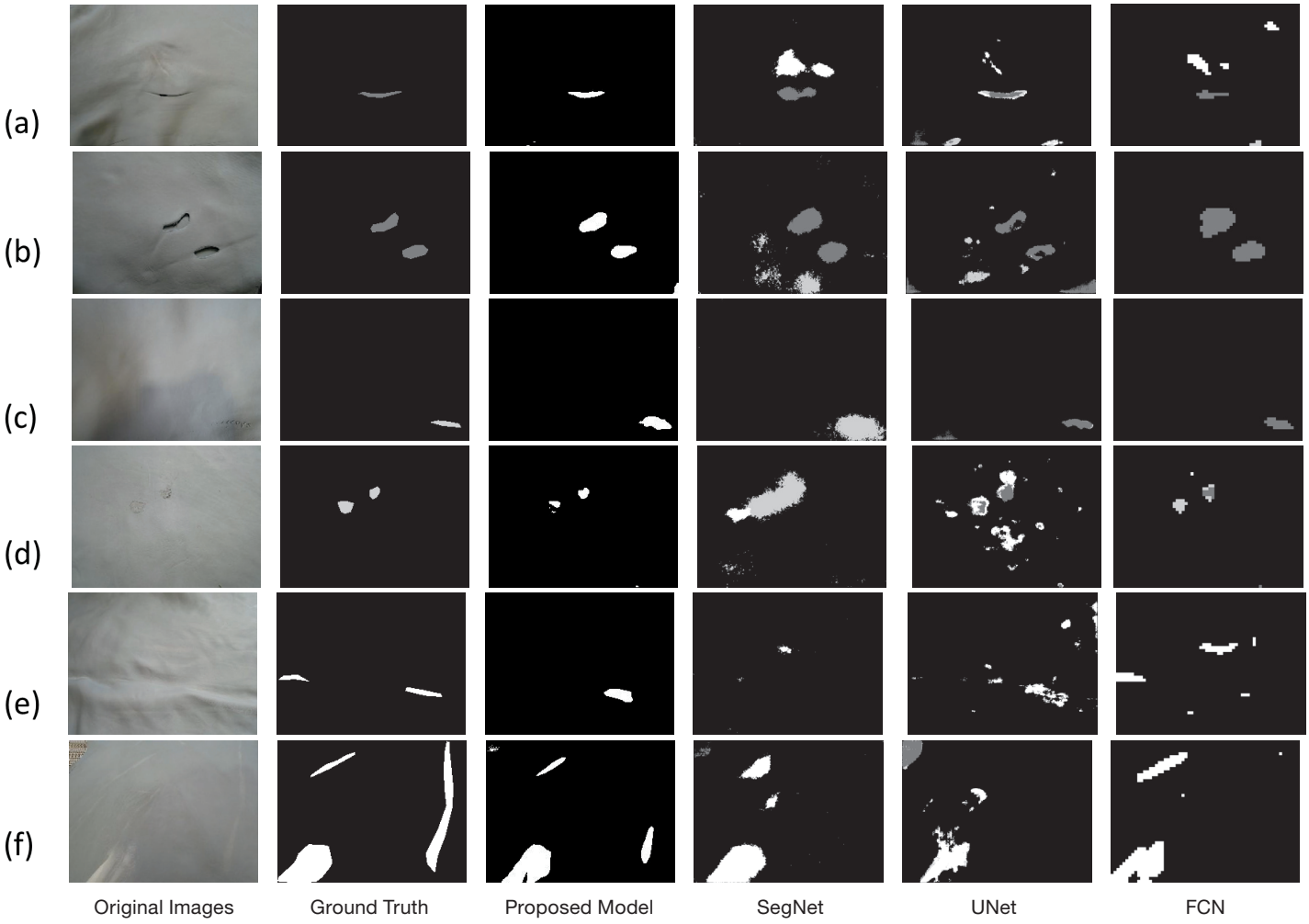

Figure 4. Visual comparison of the state-of-the-art methods for leather defect detection. **(a)** and **(b)** rows represent segmentation results of class A, **(c)** and **(d)** rows represent segmentation results of class B, **(e)** and **(f)** represents segmentation results on class C images.

model is given in Table I. We can see in Table I that proposed model outperformed all other state-of-the-art models.

5.2 Qualitative Comparison

Figure 4 shows the experimental results of different algorithms for defect/feature segmentation. In the first column (a) at number one is the original image that contains class A features, second is the ground truth, third is the result of the proposed method which obtained a close segmentation. The fourth and fifth images contain results of SegNet[7] and Unet, respectively. Both were able to segment the cut but there were a few false segmentations as well. Similarly, the sixth image contains segmentation results of FCN,[8] which is similar to both UNet and SegNet.[7] If we look at the results of column (b) (proposed model) and FCN, both segmented the class A image and do not give false positive results, whereas Unet and SegNet again gave a few false positives as well. In column (c), except for Unet, all the models predicted class B where as Unet, while able to segment the class B region, gave few false positives as well. In column (d), again only the proposed method was able to give the correct segmentation, and again the other models were not able to give the correct segmentation. Column (e) and (f) contain regions with class C features, which is a very difficult class because of intra class variation. Again, the proposed model performed better than other models but even the proposed model failed to give exact segmentation.

6 Conclusion

Automated visual inspection of leather in an industrial setting has recently received a lot of interest. There have been numerous machine learning algorithms proposed in the past, but convolutional neural network-based approaches remain rare. In this paper, we provide an ensemble convolutional neural network for visual inspection of wet-blue leather. In terms of F1-Score and IoU, our proposed method outperformed three state-of-the-art deep learning-based segmentation algorithms. A F1-Score of 74 percent and IoU of 66.7 percent were achieved by our model. Regardless of its competitive performance, the proposed method would need to be converted for real-time use, which would include thorough video data tweaking. A

significant future direction is the development of a system that can segment leather data in a real-world industrial scenario. Finally, the development of such systems that can characterise different defect types in terms of their properties could lead to artificial intelligence-based automated quality grading of leather samples.

Acknowledgment

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LEATHER

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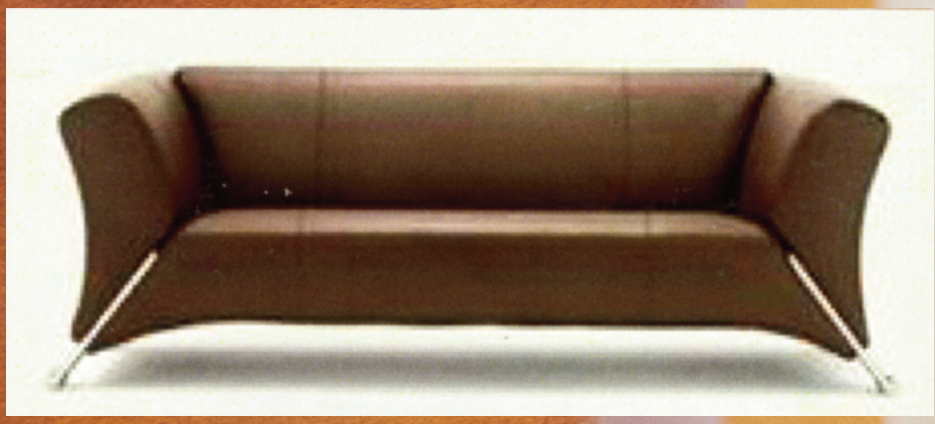

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Geoffrey Holmes is a leather technologist who obtained his Degree in Applied Science from Kingston University in 1986 and HND in Leather Technology from the University of Northampton's Institute for Creative Leather Technologies in 1989. He has over 30 years' experience in the leather industry in various production and technical roles, and for the last 14 years has been involved in research at the New Zealand Leather and Shoe Research Association (LASRA). He is the current LASRA Director.

Rafea Naffa is a scientist at the Leather and Shoe Research Association of New Zealand based in Palmerston North. He finished his PhD in Biochemistry from School of Fundamental Sciences at Massey University in 2017. His research focus is the analysis of collagen in skins and hides where he developed several analytical methods for the separation and quantitation of collagen crosslinks and advanced glycation end products using LC-MS. He published more than 18 peer-reviewed papers since 2017 including 6 method papers. Currently, he is studying the extraction, purification and characterization of collagen from different animal species for several applications.

Obituaries

Robert A. 'Bob' Denbow, 84, passed away February 14, 2022, surrounded by his loving family. Born in Greenville, Maine, December 27, 1937, he was the second of eight children of Eugene Irving and Agatha Blanchard Denbow.

In 1958 he married Mary Catherine 'Kay' Scott and they went on to have five children. They made their homes in Pittsfield, Palmyra, Newport, St. Albans, Welton, AZ, Brenda, AZ, and finally back home to Waldoboro.

Bob graduated from Maine Central Institute in Pittsfield in 1956 and joined General Electric in Lynn, MA before beginning his tanning career with Irving Tanning Company in January 1957. He took correspondence courses in analytical chemistry and the ALCA Leather Technology Course. H was a laboratory technician for five years and then laboratory supervisor for the next 15 years. He moved from Assistant director, to Technical Director, to his last position of Assistant Vice President Technical Services before retiring on October 16, 1998. During his almost 42 years at Irving Tanning Company in Hartland, he was involved with leather making from the hair to the finished produce with emphasis on retanning and coloring. He worked extensively with color drum computer automation. He served on the Steering Committee of the New England Tanners Club for six years and was a member of the ALCA since 1982.

Bob and Kay, with friends Keith and Marleen Bubar, founded and ran a woodworking business, St. Albans Slab & Sawdust, where they produce everything from Canadian Rockers to tanning drums for the tannery.

Bob loved music, both writing and playing. He played as a lounge entertainer at Squaw Mountain Resort and Lovley's Motel. He was also the musical director and organist for the Maine State Junior Miss Pageant. Bob and Kay loved to travel and play Bingo every chance they could. They also liked to garden and produced a lot of vegetables and grapes even though they lived in the desert.

After retiring in 1998, Bob and Kay moved to Welton, AZ, where they lived a couple years before settling in Brenda, AZ. In 2021, they returned to Maine.

In Brenda, Bob really had time to pursue his passion for music. There were several places in town where he could play at a jam almost any day. While living in Arizona, he was a member of five local bands. The last one was the Notables that included his best friend, Chuck Morris.

Other than his parents, Bob was predeceased by two sisters. He is survived by his wife of more than 63 years, Kay; his children, Kathryn Grant and her husband Peter of Waldoboro, Robert B. Denbow and his wife Michelle of St. Albans, Jeffrey Denbow and his wife Debra of Readfield, Peter Denbow and his wife Kim of St. Albans, Carol Millett and her husband Scott of Corinna; 14 grandchildren; 23 great-grandchildren; one sister and four brothers, as well as many nieces and nephews.

Bob requested there be no services at this time. As a lifelong member of the Catholic Church, a Funeral Mass and committal service will be held at a later date. Those who wish may make memorial donations to the American Cancer Society – (ME), P.O. Box 350, Westbrook, ME 04092-3438.

Thomas Emmerling, 66, of Barrington, passed away peacefully on Tuesday, September 14, 2021.

Tom was a member of the ALCA from 1988 until 2004. He returned to the ALCA in 2015 and was an active member at the time of his death. Tom graduated from German Tanning School, Reutlingen in 1983 with degree in leather technology. Over the years, he worked for Prime Tanning, Bayer AG, Coach Leatherware and Leather Blvd. and Schill and Seilacher for GmbH.

Young Leather Scientist Grants Announced

The Executive Committee of the IULTCS is pleased to announce the winners of the 2022 IUR research grants to be awarded to two young scientists, under the age of 35. The monetary awards help support the work of young talent in the leather sector.

This is the eighth year of the grants which have been generously supported by industry and IULTCS alike. The Selection Committee of the IULTCS Research Commission (IUR), chaired by Dr Michael Meyer, is pleased to announce the following recipients:

Young Leather Scientist Grant 2022 Basic Research

Fitsum Etefa Ahmed from the Ethiopian Institute of Textile and Fashion Technology. IULTCS has provided the monetary sponsorship for a single sum of €1,500 grant to Basic Research. The title of the project is 'Study of anti-ectoparasitic activity of medicinal plant extracts in terms of reducing cockle damage on sheep and goat skin'.

Dr Mike Redwood Young Leather Scientist Grant 2022 Sustainability / Environmental Award

Louret Atsenga Andalo, from Dedan Kimathi University of Technology, Kenya will be the beneficiary of the generosity of Leather Naturally who have sponsored the €1,000 grant for the project entitled 'Extraction of natural dye (batalain) from beetroot peel (Beta Vulgaris) and application in leather dyeing using bio-mordant'.

The grants have been very successful and well received by industry. Referring to the awardees of the 2021 grants Dr Meyer said "We are very happy that the research results of last year's two awardees were presented at the perfectly organised IULTCS Congress in Addis Ababa, Ethiopia last November. Although the number of physical participants was limited, due to the pandemic, we had the chance to attend the remote presentations delivered by Dr Caroline Agustini (Brazil) and Dr Nilay Ork (Turkey). This year we again received some strong new proposals and two of them have been selected. We look forward to seeing the research outcomes from Fitsum and Louret of the projects we are supporting and wish them every success as they contribute to expanding our industry knowledge."

The two proposals can be downloaded from the IULTCS website, https://iultcs.org/commissions/iur-research-commission/

It is also the opportunity to announce the next call for applications for YLSG 2023. The submission deadline of the next round is 30 November 2022. Details of the eligibility requirements and application forms will be available on the IULTCS website (www.iultcs.org) in May. The IULTCS requests that readers of this announcement forward the information to those institutions and individuals who could benefit from the award.

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Tentative Schedule

Tuesday, June 21

Golf Tournament, Opening Reception and Dinner

Wednesday, June 22

John Arthur Wilson Memorial Lecture
All Day Technical Sessions, Fun Run
Reception and Dinner, Activities - Bowling, Pool, Darts and an Open Bar

Thursday, June 23

All Day Technical Sessions, Annual Business Meeting
Activities Awards Luncheon
Social Hour, ALCA Awards Banquet

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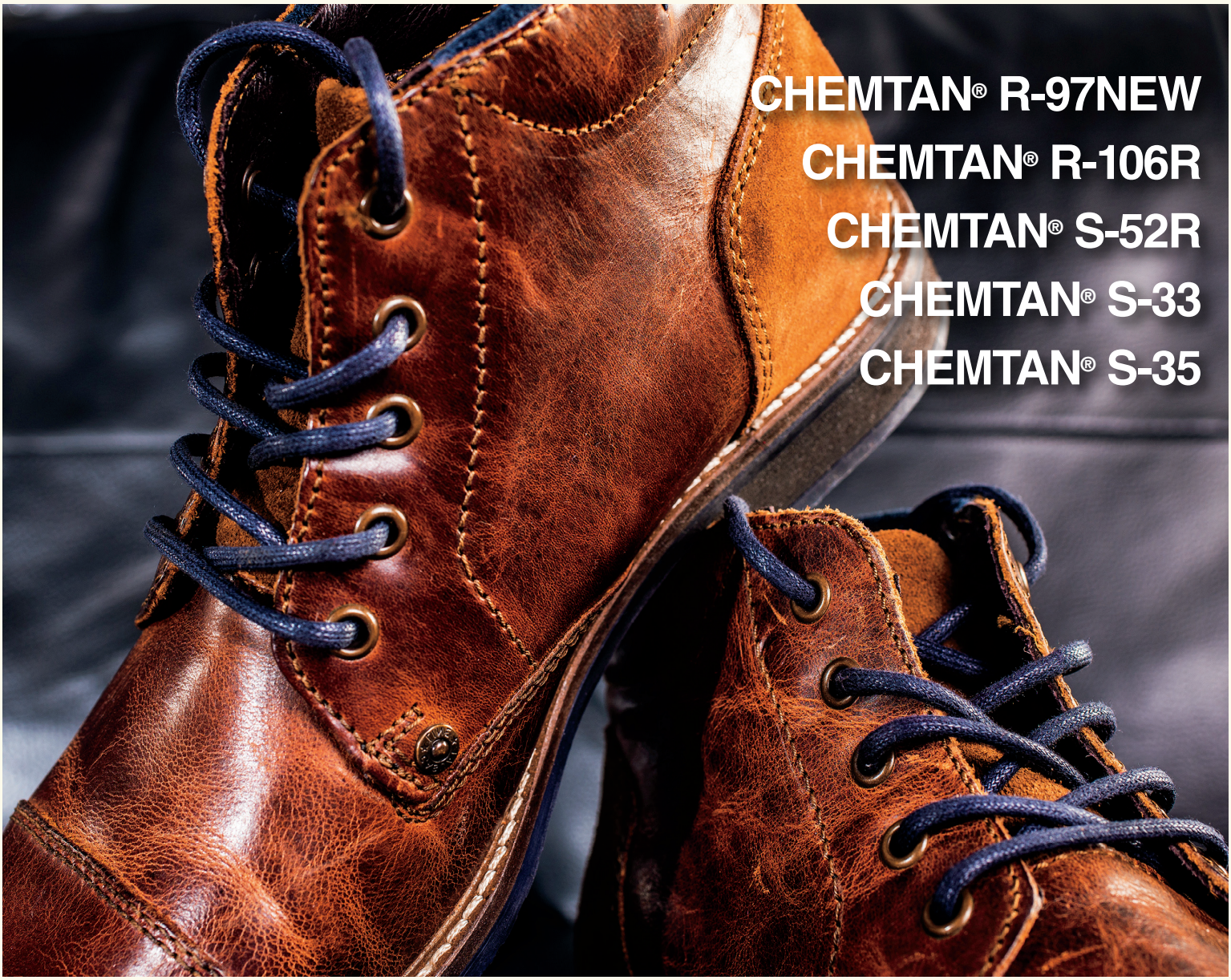

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