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Characterization and Control of Antibiotic-Resistant Camalti Saltern's Isolates with Bacteriocins

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Abstract

Camalti Saltern is the largest solar saltern in Izmir, Turkey. The salt obtained from Camalti Saltern is used in food and leather industries to prevent bacterial growth. In this saltern, seawater is pumped into shallow ponds. Then, the sun and wind cause evaporation and crystallization, finally sea salt is produced. Due to the fact that Camalti Saltern contains various halophilic bacteria, the goals of the present study were to isolate and identify haloversatile bacteria from Camalti Saltern's brine samples, to examine their antibiotic resistance profiles, to determine antimicrobial activities under optimum environmental conditions, to determine bacteriocin concentration by Bradford Method, to detect Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of bacteriocins against multidrug-resistant isolates, and to observe the cell structure of bacteriocin-treated bacteria under SEM. Sixteen bacterial isolates were recovered from Camalti Saltern's brine samples and were identified as 14 different species (*Bacillus haynesii*, *Bacillus simplex*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus*, *Staphylococcus petrasii* subsp. *jettensis*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Kocuria sediminis*, *Rhodococcus enclensis*, *Marinobacter hydrocarbonoclasticus*, *Vibrio olivae*, *Marinomonas communis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis*, *Vibrio neocaledonicus*). Percentages of antibiotic resistance of isolates were 63% to aztreonam, 50% to amoxicillin/clavulanic acid, 44% to ampicillin, 44% to cefadroxil, 31% to imipenem, 19% to ampicillin/sulbactam, 6% to chloramphenicol, 6% to tetracycline, 6% to mupirocin, 6% to meropenem. The bacteriocin concentrations of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were measured as 1.02 mg/mL and 1.25 mg/mL, respectively. Bacteriocins of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*, which were not resistant to any antibiotics tested, exhibited the inhibitory effect against *Kocuria sediminis* resistant to ten antibiotics and *Bacillus pumilus* resistant to four antibiotics. Bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* also demonstrated the inhibitory effect against *Pseudomonas psychrotolerans* resistant to five antibiotics. Scanning electron micrographs showed that cell morphologies of bacteriocin-treated isolates (*Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*) were damaged. In conclusion, bacteriocins produced from the haloversatile Camalti saltern isolates may be used in the leather industry to prevent the growth of antibiotic-resistant haloversatile bacteria.

Introduction

Camalti Saltern is the biggest seawater-based saltern in Izmir (Turkey). In this saltern, 500,000 ton of NaCl per year is produced since 1863. There is a multipond system with 182 ponds covering 58 km^2 area.[1] Solar evaporation is the oldest salt production method. In this system, seawater from the Aegean Sea is pumped at certain times into these shallow ponds in the Camalti Saltern.[2,3] The sun and dry wind cause evaporation of seawater. While the seawater salinity is about 0.35%, the salinity is reached to 26.5% after holding in the pools. Then, the salt begins to crystallize once supersaturation is reached. The salt obtained from Camalti Saltern is used in pickling, brine curing, olive salting, curing fish, as well as hide and skin preservation. The food, hide and skin materials are ideal environments for bacteria found in salt. When the salt containing harmful bacteria is used for food and hide preservation, undesirable changes in smell, taste, appearance, hair, color, grain surface may result from bacterial growth.[4,5] These problems will negatively affect economy, people and environment.

Microbial adaptation has a major role in multipond solar salterns that have a wide range of salt concentrations. Halophiles can thrive in saline environments such as salt lakes, sea, seawater-based salterns, salterns, saline soils, salted hides, and salt mines.[6-10] Among the halophilic microorganisms, haloversatile bacteria are capable of growth in the absence of salt and in saturated salt concentrations (>3M NaCl). These bacteria are able to grow optimally in media with 0.2-0.5 M NaCl.[8,11]

Although the presence of halophilic microorganisms in the Camalti Saltern was reported in previous studies,[1,12-14] the presence of bacteria showing haloversatile properties in Camalti Saltern's brine samples and their antibiotic susceptibilities were not reported yet. Only in two research papers, the haloversatile bacteria were reported in salt samples collected from Camalti Saltern.[15,16]

Antibiotics, natural microbial products, are commonly used in animals and humans for disease treatment, growth promotion

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and meat production.[17-20] According to the joint results of World Organisation for Animal Health,[21] World Health Organization,[22] the Food and Agriculture Organization of the United Nations,[23] antibiotic resistance was reported as a global health problem due to the misuse and overuse of antimicrobial agents.[21-23] It was recommended that antibiotics should not be routinely used in the food industry, animal husbandry, veterinary and human medicine.[21-23] Although less information is available about antibiotic resistance of bacteria in natural environments, it is known that the antibiotic-resistant bacteria present in manure and wastewater may pass through the soil, and from there to water sources.[24] These bacteria may transfer to humans and animals during washing and cleaning, air-conditioning, transportation, storage, packaging of food products, and leather processing.[25,26]

Due to the overuse of antibiotics in human and animal health, bacteriocins which are ribosomally synthesized by different microorganisms are used to kill or inhibit microorganisms as an alternative to antibiotics in recent years.[27,29,30] Bacteriocins may be potential candidates instead of antibiotics and chemicals in food preservation, hide and skin preservation, cancer therapy, treating multidrug-resistant bacteria in the food industry, leather industry and medicine.[27-30]

Since antibiotic-resistant bacteria may be found in the Camalti Saltern, the goals of the present study were to investigate: 1) haloversatile bacteria at species level using phenotypic and genotypic characteristics found in Camalti Saltern's brine samples; 2) their antibiotic resistance profiles against different antibiotics; 3) antimicrobial activities of test isolates against each other; 4) optimum environmental conditions for maximum bacteriocin production by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*; 5) effect of temperature, pH, NaCl concentration, effect of enzymes (Proteinase K and Lipase) on antimicrobial activities of bacteriocins obtained from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*; 6) Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of bacteriocins against multidrug-resistant isolates; 7) the cell structure observation of bacteriocin-treated bacteria under SEM.

Experimental

Sample collection

Five brine samples were collected from the surfaces of shallow seawater pools at the Camalti Saltern, Izmir/Turkey into sterile bottles in June 2018. Then, the brine samples were placed in a lightproof insulated box containing ice-packs and immediately transported to the laboratory.

Isolation of bacteria

All brine samples were diluted in a physiological saline solution containing 1.17% (w/v) NaCl (0.2 M NaCl). An aliquot of 0.1 mL of each direct and serial dilutions (10^{-1}-10^{-6}) was spread onto surface of Oligotrophic Agar Medium (OAM) plates (18.2 g R2A, 5 g agar, 11.7 g NaCl, 1000 mL distilled water).[15] The plates were incubated at 32°C for 24 hours. Then, different bacterial colonies were selected and restreaked several times to obtain pure cultures.[31,32]

Characterization of the isolates

Exponentially grown isolates were examined for cell morphology and pigmentation. Catalase, oxidase activities, Gram staining were performed using earlier described procedures.[33] Salt requirement and salt tolerance of the test isolates were investigated on both OAM plates without salt and OAM plates containing different NaCl concentrations such as 0.17 M, 0.2 M, 0.34 M, 0.51 M, 0.85 M, 1.36 M, 1.7 M, 2 M, 2.5 M, 3 M, 3.4 M, 4.2 M and 5.1 M. The pH tolerance of isolates were tested on OAM plates (0.2 M NaCl) and adjusted to pH values 4, 5, 6, 7, 8, 9, 10, 11, 12, 13. The pH values of the media were measured using a pH meter (Sartorius Professional Meter PT-10P, Goettingen, Germany). To determine optimum growth temperature of isolates, the plates inoculated with each isolate were incubated at different temperatures (4, 10, 20, 25, 28, 32, 37, 40, 45, 50, 55, 60 °C).

Amplification and sequencing of 16S rRNA genes

Chromosomal DNA was isolated and purified by QIAamp DNA Mini Kit (Qiagen) and QIAquick PCR Purification Kit (Qiagen). The 16S rRNA genes of the isolates were amplified by PCR using universal primers: 1492R (5'TACGGYTACCTTGTTACGACTT3') and 27F (5'AGAGTTTGATCMTGGCTCAG3').[34] The 16S rRNA gene sequences (1000-1380 bp) were determined by IONTEK Laboratory (Turkey). It was further determined 16S rRNA gene similarities (98.7%-100%) between isolates and closely related species using ChromasPro and EzTaxon-e tool.[35]

Nucleotide accession number

16S rRNA sequence data of the brine isolates SW1a, SW1b, SW2a, SW2b, SW3a, SW3b, SW3c, SW3d, SW3e, SW4a, SW4b, SW4c, SW4d, SW5a, SW5b, SW5c, reported in this article have been deposited in NCBI and GenBank nucleotide sequence database under the respective accession numbers: MH748797, MH748778, MH748707, MH748694, MH753648, MH748717, MH753655, MH748716, MH748723, MH748691, MH748690, MH748687, MH748684, MH748683, MH748680, MH748674.

Antibiotic susceptibility tests

Resistance to antibiotics was determined by the Kirby-Bauer disc diffusion method.[36] The isolates were grown in Mueller Hinton Broth (MHB) containing 1.17% NaCl. After incubation at 32°C, the optical density of overnight culture was adjusted to McFarland Standard No 0.5 (1×10^8 CFU/mL) with sterile saline solution (1.17% NaCl). Then, suspensions of isolates were evenly spread on Mueller Hinton Agar (MHA). Chloramphenicol, cefadroxil, ampicillin, tetracycline, mupirocin, imipenem, meropenem, aztreonam, ampicillin/sulbactam and amoxycillin/clavulanic acid discs (Oxoid,

UK) were placed on the surface of inoculated MHA plates. The plates were incubated at 32°C for 24h. At the end of the incubation period, the diameters of the inhibition zones were measured.

Preparation of cell-free supernatant

The isolates were grown in 20 mL Nutrient Broth (NB) (pH 7.0, 1.17% NaCl) at 32°C for 24 hours. Bacterial cells were removed by centrifugation (8000 g for 15 min at 4°C) to obtain a cell-free supernatant (CFS). The pH of CFS was adjusted to 7.0 using 1N NaOH to prevent the inhibitory effect of organic acids on bacterial growth. Each supernatant was filtered through a 0.22 µm membrane filter (Millipore) to produce sterile crude bacteriocin.[37]

Screening of antimicrobial activity among the test isolates

Antimicrobial activity of bacteriocin against each test isolate was examined. Each bacterial concentration was separately adjusted to 10^8 CFU/mL. Aliquots of 100 µL of the each of the adjusted bacterial suspensions were spread on the petri plates containing Nutrient Agar (NA) medium. Aliquots of 3 µL of each bacteriocin were placed on these inoculated petri plates. After 24 hours incubation at 32°C, the inhibition zone diameters (mm) of the bacteriocin substance against each test isolate were measured.[38] According to the obtained experimental results, bacteriocin-producing isolates, bacteriocin-sensitive and bacteriocin-resistant isolates were determined. Later, bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were investigated against multidrug-resistant isolates *Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*.

Determination of protein concentration using Bradford Method

The Bradford method was used to determine the amounts of protein in the supernatants of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* at 32°C, pH 7, 1.17% NaCl concentration.[39] Bradford reagent and Quick Start Bovine Serum Albumin (BSA), which are commercial product of BIO-RAD (BIO-RAD Laboratories, Hercules CA, USA), were used in this experiment. The optical density of bacteriocins were measured at 595 nm with spectrophotometer (UV Mini 1240, Shimadzu, Kyoto, Japan).

Optimum conditions for bacteriocin production from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

To investigate the impact of environmental factors on bacteriocin production by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*, bacterial concentration of each isolate was adjusted to 10^8 CFU/mL. Then, 3 mL of each bacterial solution was inoculated into flasks containing 50 mL of NB and the flasks were incubated at different temperatures (20, 28, 30, 32, 37, 40°C), different pH values (5.0, 6.0, 7.0, 8.0, 9.0, 10.0), different NaCl concentrations (1.17, 3, 5, 10, 15, 20, 25%) for 24 hours. Bacterial concentration of the multidrug-resistant isolates was separately adjusted to 10^8 CFU/mL. Subsequently, 100 µL aliquots from each multidrug-resistant isolate were spread on the petri plates containing NA. Aliquots of 3 µL of each bacteriocin were placed on the inoculated petri plates. The inhibition zone diameters of bacteriocin against each test isolate were measured after 24 hours incubation at 32°C.[38] The experimental results were evaluated to detect the optimum temperature, pH and salt concentration required for the highest bacteriocin production by isolates.

Effect of heat on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

The bacteriocin of each isolate was incubated at different temperatures (4, 10, 20, 25, 28, 32, 37, 40, 50, 60, 70, 80, 90, 100°C) for 15 min. Later, the heat-treated bacteriocin samples were tested for antimicrobial activity against multidrug-resistant isolates by agar spot diffusion assay. The plates were incubated at 32°C for 24 hours. After the incubation period, the inhibition zone diameters of the bacteriocin against the multidrug-resistant test isolates were measured.

Effect of pH on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

The pH of bacteriocin belonging to each isolate was adjusted to 4-12. Bacteriocin samples were kept at 25°C for 4 hours. Then, the bacteriocin samples were tested for antimicrobial activity against multidrug-resistant isolates by agar spot diffusion assay. The inoculated petri plates were incubated at 32°C for 24 hours. After incubation period, the inhibition zone diameters of the bacteriocin against the test isolates were measured.

Effect of NaCl concentration on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

NaCl concentration of the bacteriocin of each isolate was adjusted to 1.17, 3, 5, 10, 15, 20, 25% NaCl. The bacteriocin samples were tested for antibacterial activity against multidrug-resistant isolates by agar spot diffusion assay. The plates were incubated at 32°C for 24 hours. After incubation, the inhibition zone diameters were measured. The results were evaluated to determine the effect of heat, pH and NaCl concentration on the bacteriocins.

Effect of enzymes (Proteinase K and Lipase) on bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis*

The effects of protease and lipase enzymes on bacteriocins produced by *R.enclensis* and *S.costicola* subsp. *vallismortis*, proteinase K of *Tritirachium album* and lipase of *Candida rugosa* were purchased from Sigma-Aldrich (St Louis, MO).[37] Proteinase K and lipase were respectively prepared in 10 mM phosphate buffer (pH 7) and 0.05 M Tris HCl, 0.01 M $CaCl_2$, pH 8, and filtered through 0.22 µm pore size filter paper (HiMedia, Mumbai, India). One mL of sterile enzyme solution was added to the supernatant and this mixture was incubated for two hours at 37°C. The activities of bacteriocins treated with enzyme were examined by the agar spot diffusion method using

the multidrug-resistant isolates. The plates were incubated at 32°C for 24 hours. After incubation period, the inhibition zone diameters of the bacteriocin against the test isolates were measured. Phosphate buffer (10 mM) and Tris HCl buffer (0.05 M) were used as negative controls of Proteinase K and lipase, respectively.[40]

Minimum inhibitory concentrations and minimum bactericidal concentrations of bacteriocins against multidrug-resistant isolates

In order to detect the Minimum Inhibitory Concentrations of bacteriocin produced from *Rhodococcus enclensis* against *Kocuria sediminis* and *Bacillus pumilus*, and bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* against *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans*, broth microdilution in 96-well plate method was performed. Two-fold serial dilutions of each bacteriocin (Column 1: 1/2, Column 2: 1/4, Column 3: 1/8, Column 4: 1/16, Column 5: 1/32, Column 6: 1/64, Column 7: 1/128, Column 8: 1/256, Column 9: 1/512, Column 10: 1/1024) were prepared in 96-well microdilution plate containing Mueller Hinton Broth. A multichannel pipette was used to transfer and mix bacteriocin from Columns 1-10. While Column 11 contained 200 µL Mueller Hinton Broth only as a sterility control, Column 12 contained 100 µL Mueller Hinton Broth and 100 µL of each test isolate (*Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*) for the normal bacterial growth. The multidrug-resistant test isolates (*Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*) were streaked onto Mueller Hinton Agar plate and incubated at 32°C for 24 hours. Then, each suspension of test isolate was prepared in sterile Mueller Hinton Broth and adjusted to equal turbidity of 10^8 CFU/mL. An aliquot of 100 µL bacterial suspension of multidrug-resistant test isolates was added into each well on the Columns 1-12 (except Column 11 which is accepted as sterilization control) (totally 200 µL per well). After 24 hours incubation at 32°C, 30 µL of resazurin solution (0.015%) was added into each well on the Columns 1-12. Subsequently, 96-well plates were left for 4 hours at 25°C and the wells were checked for visible growth (turbity and pink colour).[41] Resazurin, which has a blue color, is irreversibly reduced to the pink-color by the respiration of metabolically active bacterial cells, and it is used as an indicator of cell viability.[41] Also, in which well the color of resazurin did not turn from blue to pink, it means that in that well the bacterial cells are killed or controlled by the bacteriocin, and that bacteriocin concentration is accepted as Minimum Inhibitory Concentration (MIC). The lowest concentration that inhibited the growth of the test isolates (blue colour) in the wells were accepted as MIC of bacteriocins. In order to determine Minimum Bactericidal Concentration (MBC), the visible growth of *Kocuria sediminis* and *Bacillus pumilus* effected by bacteriocin produced from *Rhodococcus enclensis*, and the visible growth of *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* effected by bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* were investigated from the wells belonging to MIC endpoint. Hence, 100 µL of each suspension from the each test well belonging to MIC (containing blue color resazurin) was spread on the Mueller Hinton Agar plates with glass spreader. The plates were incubated at 32°C for 24 hours. After incubation period, when the bacterial colonies did not observed on the plates, it was accepted as the bacteriocins have bactericidal effect on the test isolates. All experiments were performed in triplicate.

Preparation of bacteriocin-treated and bacteriocin-untreated cells for Scanning Electron Microscopy

Bacteriocin-treated (from wells belonging to MIC endpoint) and untreated (control) cultures of the multidrug-resistant bacterial isolates were separately dropped on filter membranes (0.45 µm) and air dried. The filter membranes were fixed in 2.5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for 1 hour. The filters were rinsed three times with 0.1 M PBS buffer for 15 min, and then treated with 1% OsO_4 in 0.1 M PBS buffer at room temperature for 1 hour. The filters were rinsed with distilled water for 15 min and then, were dehydrated with an ethanol series (30%, 50%, 70%, 80%, 100%), 10 min for each rinse. Then, the samples were examined using a Scanning Electron Microscope (Fei Quanta 450 FEG ESEM SEM) sample stub with double-sided sticky tape.[42]

Results and Discussion

In the present study, bacterial isolates were found in all the brine samples of shallow seawater ponds obtained from the Camalti Saltern, which is the largest seawater-based saltern in Izmir (Turkey). The temperature, pH and salt concentration of brine samples were respectively recorded between 24°C-26°C, 8.43-8.67, and 4.2%-5.0%. 14 different bacterial species such as *Bacillus haynesii* (isolates SW1a, SW3a, SW3c), *Staphylococcus petrasii* subsp. *jettensis* (isolate SW1b), *Kocuria sediminis* (isolate SW3b), *Bacillus simplex* (isolate SW3d), *Bacillus subtilis* subsp. *stercoris* (isolate SW3e), *Marinobacter hydrocarbonoclasticus* (isolate SW2a), *Vibrio olivae* (isolate SW2b), *Bacillus pumilus* (isolate SW4c), *Rhodococcus enclensis* (isolate SW4e), *Marinomonas communis* (isolate SW4f), *Staphylococcus saprophyticus* subsp. *saprophyticus* (isolate SW4g), *Pseudomonas psychrotolerans* (isolate SW5a), *Salinivibrio costicola* subsp. *vallismortis* (isolate SW5b), *Vibrio neocaledonicus* (isolate SW5c) were isolated and identified from five brine samples. The isolated species in this study were first isolated and identified from brine samples obtained from the Camalti Saltern. While the most abundant species in the water samples was *Bacillus haynesii* (n=3), the other species were not abundant (n=1). All isolates were able to grow at 0-3 M NaCl concentrations, but optimally grew at 0.2 M NaCl (Table I). Therefore, these isolates were accepted as haloversatile.[43-45] All isolates were able to grow at pH 5-12 and between 10-55°C (Table I). Gram reactions, cell morphologies, effects of NaCl, temperature and pH on bacterial growth, oxidase and catalase activities were shown in Table I.

Some of the isolated strains in the present study were previously reported by other researchers. For instance, *Bacillus haynesii*,

Table I

Characteristics of haloversatile bacteria isolated from brine samples of the Camalti Saltern

Characteristics	*Bacillus haynesii*	*Staphylococcus petrasii subsp. jettensis*	*Marinobacter hydrocarbonoclasticus*	*Vibrio olivae*	*Kocuria sediminis*	*Bacillus simplex*	*Bacillus subtilis subsp. stercoris*
Isolate number	3	1	1	1	1	1	1
Gram staining	+*	+	–**	–	+	+	+
Cell morphology	rod-shaped	coccus	rod-shaped	curved-rod shape	coccus	rod-shaped	rod-shaped
NaCl range (M)	0-3	0-3	0-3	0-3	0-3	0-3	0-3
Optimum NaCl (M)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Temperature range (°C)	20-55	20-55	10-55	20-45	10-50	10-55	10-55
Optimum temperature (°C)	32	32	28-32	32	32	32	32
pH range	5-12	5-12	5-12	5-12	6-12	5-12	5-12
Optimum pH	6-8	7-8	7-8	7-8	7	7-8	6-8
Oxidase	–	+	+	+	+	–	–
Catalase	+	+	+	+	+	+	+

Characteristics	*Bacillus pumilus*	*Rhodococcus enclensis*	*Marinomonas communis*	*Staphylococcus saprophyticus subsp. saprophyticus*	*Pseudomonas psychrotolerans*	*Salinivibrio costicola subsp. vallismortis*	*Vibrio neocaledonicus*
Isolate number	1	1	1	1	1	1	1
Gram staining	+	+	–	+	–	–	–
Cell morphology	rod-shaped	rod-coccoid	rod-shaped	coccus	rod-shaped	curved-rod shape	curved-rod shape
NaCl range (M)	0-3	0-3	0-3	0-3	0-3	0-3	0-3.0
Optimum NaCl (M)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Temperature range (°C)	20-45	10-45	20-45	20-45	10-50	10-45	20-55
Optimum temperature (°C)	32	32	28-32	32	32	32	32
pH range	5-11	5-12	6-12	5-11	6-12	5-11	5-12
Optimum pH	6-8	6-8	7-8	6-8	7-9	7-8	6-8
Oxidase	–	–	+	+	–	+	+
Catalase	+	+	+	+	+	+	+

+*: Positive, –**: Negative

Staphylococcus petrasii subsp. *jettensis*, *Kocuria sediminis*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus* and *Staphylococcus saprophyticus* subsp. *saprophyticus* were isolated from salt samples of Camalti Saltern and they were reported as haloversatile.[15] In another study, a raw salt sample collected from Camalti Saltern was examined, and six haloversatile bacteria (*Arthrobacter ginsengisoli*, *Arthrobacter psychrochitiniphilus*, *Pseudarthrobacter polychromogenes*, *Glutamicibacter arilaitensis*, *Arthrobacter agilis*) were isolated from that raw salt sample.[16] According to the experimental results, it is clear that the haloversatile bacteria found in the brine of Camalti Saltern, may contaminate the salt crystals during salt production process in evaporation ponds and crystallization ponds.

Among the test isolates, *Bacillus haynesii*, *Bacillus simplex*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus* have endospores which are resistant to UV radiation, antimicrobial agents, cold, heat, desiccation.[46-49] In another study, *Staphylococcus petrasii* subsp. *jettensis* was found to be resistant to penicillin, cefoxitin, gentamicin, erythromycin and clindamycin.[32] *Salinivibrio costicola* subsp. *vallismortis*, *Pseudomonas psychrotolerans*, *Marinomonas communis*, *Vibrio olivae*, *Vibrio neocaledonicus*, *Rhodococcus enclensis*, *Staphylococcus saprophyticus* subsp. *saprophyticus* and *Kocuria sediminis* were able to use different carbon and energy sources with different enzymes.[48,50-57] *Marinobacter hydrocarbonoclasticus* uses several hydrocarbons as the sole source of carbon and energy.[58] Other studies also suggest that these isolates are metabolically active. While the salt containing these isolates is used in the preservation of hides and skins in the leather industry, they may damage the structure of hides and skin. Bad odor, red heat, hair slip, grain damage are seen due to the catabolic activity of bacteria on the skin and hide structures.[59-61] Due to the huge economic importance of the leather industry in the world, destructive and antibiotic resistant hide and skin bacteria found in the preservation salt should be killed to prevent their damage to the structures of hides and skin, and to prevent financial losses to the leather industry.

Susceptibility of bacteria to antibiotics is also determined by standardized antimicrobial disc diffusion results, in which the inhibition zones around the antibiotic discs are measured and evaluated according to the standards reported by the Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing.[62,63] However, there is no standard regarding the antibiotic susceptibilities of the test isolates. Hence, the antibiotic test results were expressed as inhibition zone diameter measurements (mm) in the present study. The experimental results of the resistance of test bacteria to ten different antibiotics are presented in Table II. Multidrug-resistant bacteria, which are able to survive and grow in the presence of more than two antibiotics were detected in the present study. Among the test isolates, *Kocuria sediminis* was resistant to all antibiotics tested (Table II). However, *Vibrio olivae*, *Bacillus subtilis* subsp. *stercoris*, *Marinomonas communis* were resistant to only one antibiotic (Table II). *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were susceptible to all antibiotics tested (Table II).

A low percentage (35.7%) of the isolates produced bacteriocin against to each other. Although *Bacillus haynesii*, *Staphylococcus petrasii* subsp. *jettensis*, *Marinobacter hydrocarbonoclasticus*, *Vibrio*

Table II

Inhibition zone diameter measurements (mm) of haloversatile bacteria isolated from brine samples of the Camalti Saltern

	Bacillus haynesii	*Staphylococcus petrasii subsp. jettensis*	*Marinobacter hydrocarbonoclasticus*	*Vibrio olivae*	*Kocuria sediminis*	*Bacillus simplex*	*Bacillus subtilis subsp. stercoris*	*Bacillus pumilus*	*Rhodococcus enclensis*	*Marinomonas communis*	*Staphylococcus saprophyticus subsp. saprophyticus*	*Pseudomonas psychrotolerans*	*Salinivibrio costicola subsp. vallismortis*	*Vibrio neocaledonicus*	**Total number of isolates**
Number of isolates	3	1	1	1	1	1	1	1	1	1	1	1	1	1	**16**
Antibiotics															R%
Chloramphenicol (30 µg)	17	28	23	32	0	20	31	17	22	32	28	21	32	24	**6**
Cefadroxil (30 µg)	29	11	0	0	0	0	36	28	11	0	11	0	12	0	**44**
Ampicillin (10 µg)	14	0	0	20	0	0	24	11	10	28	0	0	20	0	**44**
Tetracycline (30 µg)	15	26	20	30	0	22	26	16	23	31	26	24	30	24	**6**
Mupirocin (20 µg)	39	21	18	20	0	20	30	28	20	31	21	16	20	14	**6**
Imipenem (10 µg)	0	25	24	34	0	22	42	0	23	37	25	18	34	18	**31**
Meropenem (10 µg)	34	25	24	29	0	23	36	34	23	35	25	19	29	11	**6**
Aztreonam (30 µg)	0	0	20	34	0	0	0	0	17	28	0	0	34	15	**63**
Ampicillin/sulbactam (10/10 µg)	15	19	16	29	0	13	27	0	13	27	19	0	29	12	**19**
Amoxycillin/clavulanic acid (20/10 µg)	0	0	12	23	0	0	22	0	10	21	0	0	23	0	**50**
Number of antibiotics that the species are resistant	**3**	**3**	**2**	**1**	**10**	**4**	**1**	**4**	**0**	**1**	**3**	**5**	**0**	**3**	

R (0 mm): Resistance

olivae, *Bacillus simplex*, *Bacillus subtilis* subsp. *stercoris*, *Bacillus pumilus*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Vibrio neocaledonicus* did not produce bacteriocin, *Kocuria sediminis*, *Rhodococcus enclensis*, *Marinomonas communis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis* produced bacteriocin (Table III). In this study, *Kocuria sediminis*, *Rhodococcus enclensis*, *Marinomonas communis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis* respectively produced bacteriocin against *Bacillus subtilis* subsp. *stercoris*; *Kocuria sediminis*, *Bacillus pumilus*, *Marinomonas communis*; *Bacillus pumilus*; *Kocuria sediminis*, *Bacillus pumilus*, *Salinivibrio costicola* subsp. *vallismortis*; *Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans*, *Vibrio neocaledonicus*. While bacteriocins of *Kocuria sediminis*, *Marinococcus tarijensis* and *Marinomonas communis* showed narrow antibacterial spectrum (inhibiting 1 test isolate), whereas bacteriocins of *Rhodococcus enclensis*, *Pseudomonas psychrotolerans*, *Salinivibrio costicola* subsp. *vallismortis* presented a wide antibacterial spectrum (inhibiting 3-4 test isolates) (Table III). These results are consistent with what has been reported in previous studies.[27-29] The researchers reported that 99% of bacteria produced at least one bacteriocin.[27]

To determine the effects of environmental conditions (°C, pH, NaCl concentration) on bacteriocin production, the bacteriocins produced by *Rhodococcus enclensis*, and *Salinivibrio costicola* subsp. *vallismortis* against antibiotic-resistant *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* were further studied (Table IV). The inhibition zone diameters of the isolates were measured as 5-10 mm, 8-12 mm, 14-17 mm, 9-13 mm, 5-7 mm respectively for 28°C, 30°C, 32°C, 37°C, 40°C; 6-8 mm, 14-17 mm, 8-11 mm respectively for pH 6, pH 7, pH 8; 14-17 mm, 10-14 mm, 8-11 mm, 5-9 mm respectively for 1.17%, 3%, 5%, 10% NaCl concentration. However, bacteriocin was not produced at 20°C, pH 5.0, 9.0, 10.0 and 15%, 20%, 25% NaCl concentration (Table IV). The

Table III

Inhibitory effect of bacteriocin produced by bacterial isolates against each other

	Bacteriocin producers	***Bacillus haynesii***	***Staphylococcus petrasii* subsp. *jettensis***	***Marinobacter hydrocarbonoclasticus***	***Vibrio olivae***	***Kocuria sediminis***	***Bacillus simplex***	***Bacillus subtilis* subsp. *stercoris***	***Bacillus pumilus***	***Rhodococcus enclensis***	***Marinomonas communis***	***Staphylococcus saprophyticus* subsp. *saprophyticus***	***Pseudomonas psychrotolerans***	***Salinivibrio costicola* subsp. *vallismortis***	***Vibrio neocaledonicus***	**Number of bacteriocin affected by isolate**
Species affected from bacteriocins	Bacillus haynesii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Staphylococcus petrasii subsp. *jettensis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Marinobacter hydrocarbonoclasticus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Vibrio olivae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Kocuria sediminis	–	–	–	–	–	–	–	–	14	–	–	10	15	–	3
	Bacillus simplex	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Bacillus subtilis subsp. *stercoris*	–	–	–	–	16	–	–	–	–	–	–	–	–	–	1
	Bacillus pumilus	–	–	–	–	–	–	–	–	15	13	–	10	17	–	4
	Rhodococcus enclensis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Marinomonas communis	–	–	–	–	–	–	–	–	14	–	–	–	–	–	1
	Staphylococcus saprophyticus subsp. *saprophyticus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
	Pseudomonas psychrotolerans	–	–	–	–	–	–	–	–	–	–	–	–	12	–	1
	Salinivibrio costicola subsp. *vallismortis*	–	–	–	–	–	–	–	–	–	–	–	12	–	–	1
	Vibrio neocaledonicus	–	–	–	–	–	–	–	–	–	–	–	–	10	–	1

largest inhibition zone (17 mm) was measured for *Bacillus pumilus* against bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* at 32°C, pH 7.0 and 1.17% NaCl. According to these results, optimum temperature, pH and NaCl concentration values of highest bacteriocin production were detected as 32°C, pH 7.0, 1.17% NaCl concentration (Table IV). The concentrations of crude bacteriocins produced by *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were respectively found as 1.02 mg/mL and 1.25 mg/mL. The amount of bacteriocins were found to be directly proportional to the inhibition zones of *Rhodococcus enclensis* (15 mm) and *Salinivibrio costicola* subsp. *vallismortis* (17 mm).

The antibacterial activities of bacteriocins produced from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* did not change after the treatments of heat temperatures (10-50°C for 15 min), pH ranges (6-8), NaCl concentrations (1.17-15%) (Table V). Although the antimicrobial activities of bacteriocins against all test microorganisms were not detected after treatment with Proteinase K at 37°C for 2 hours, these bacteriocins were resistant to treatment with lipase enzyme at 37°C for 2 hours. These results suggest that the antimicrobial compounds were proteinaceous substances (Table V).

Minimum Inhibitory Concentrations (MIC) of bacteriocins against multidrug-resistant isolates (*Kocuria sediminis*, *Bacillus pumilus*,

Table IV

Inhibition zone diameters (mm) of multidrug-resistant haloversatile bacteria against bacteriocins obtained from *Rhodococcus enclensis*, *Salinivibrio costicola* subsp. *vallismortis* at different incubation temperatures, pH and NaCl concentrations

Antibiotic resistant bacteria		*Kocuria sediminis*		*Bacillus pumilus*		*Pseudomonas psychrotolerans*
Bacteriocin producing isolates		*Rhodococcus enclensis*	*Salinivibrio costicola subsp. vallismortis*	*Rhodococcus enclensis*	*Salinivibrio costicola subsp. vallismortis*	*Salinivibrio costicola subsp. vallismortis*
Incubation temperature (°C)	20	–*	–	–	–	–
	28	8	6	5	10	5
	30	12	10	8	12	8
	32	**14**	**15**	**15**	**17**	**12**
	37	11	9	12	13	9
	40	6	5	7	6	6
pH	5	–	–	–	–	–
	6	8	8	6	7	5
	7	**14**	**15**	**15**	**17**	**12**
	8	9	11	8	9	6
	9	–	–	–	–	–
	10	–	–	–	–	–
NaCl concentration (%)	1.17	**14**	**15**	**15**	**17**	**12**
	3	10	12	13	14	10
	5	8	9	8	11	8
	10	5	5	6	9	5
	15	–	–	–	–	–
	20	–	–	–	–	–
	25	–	–	–	–	–

mm: milimeter, –*: absence of antibacterial effect

Table V

The effects of different temperatures, pH, NaCl concentrations, proteinase K and lipase enzymes on antibacterial effect of bacteriocins

		Kocuria sediminis		*Bacillus pumilus*		*Pseudomonas psychrotolerans*
		Rhodococcus enclensis	*Salinivibrio costicola* subsp. *vallismortis*	*Rhodococcus enclensis*	*Salinivibrio costicola* subsp. *vallismortis*	*Salinivibrio costicola* subsp. *vallismortis*
Temperature (°C) (15 min)						
Incubation temperature (°C)	4	+*	+	–**	+	–
	10	+	+	+	+	+
	20	+	+	+	+	+
	25	+	+	+	+	+
	28	+	+	+	+	+
	30	+	+	+	+	+
	32	+	+	+	+	+
	37	+	+	+	+	+
	40	+	+	+	+	+
	50	+	+	+	+	+
	60	–	+	–	+	–
	70	–	+	–	–	–
	80	–	–	–	–	–
	90	–	–	–	–	–
	100	–	–	–	–	–
pH (25°C, 4 h)						
pH values	4	–	–	–	–	–
	5	–	–	–	–	–
	6	+	+	+	+	+
	7	+	+	+	+	+
	8	+	+	+	+	+
	9	+	+	–	–	+
	10	–	–	–	–	–
	11	–	–	–	–	–
	12	–	–	–	–	–
	13	–	–	–	–	–
	14	–	–	–	–	–
% NaCl concentration (pH 7.0)						
Salt concentration (%)	1.17	+	+	+	+	+
	3	+	+	+	+	+
	5	+	+	+	+	+
	10	+	+	+	+	+
	15	+	+	+	+	+
	20	–	+	–	–	+
	25	–	–	–	–	–
Enzyme (2 h, 37°C)						
Proteinase K	–	–	–	–	–	–
Lipase	+	+	+	+	+	+

+*: presence of antibacterial effect; –**: absence of antibacterial effect

Table VI

The Minimum Inhibitory Concentrations of bacteriocin produced from *Rhodococcus enclensis* against *Kocuria sediminis* and *Bacillus pumilus*

Column number of 96-well plate	1	2	3	4	5	6	7	8	9	10	11	12
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	medium sterilization control	bacterial growth control
Kocuria sediminis	b[a]	p[b]	p	p	p	p	p	p	p	p	b	p
Bacillus pumilus	b	p	p	p	p	p	p	p	p	p	b	p

b[a]: Blue; p[b]: Pink

Pseudomonas psychrotolerans) were also detected in the present study. The MIC of bacteriocin produced from *Rhodococcus enclensis* against *Kocuria sediminis* and *Bacillus pumilus*, and the MIC of bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* against *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* were found as 1/2 (Tables VI-VII). The blue (**b**) colors in the Column 1 (Tables VI-VII) showed that the color of resazurin did not change to pink color due to the concentration of bacteriocins (1/2) were effective on the test bacteria and the cells of test bacteria were not alive. This bacteriocin concentration (1/2), which is the lowest concentration that inhibited the growth of the test isolates in the wells were accepted as MIC of bacteriocins (Tables VI-VII).

The growth of *Kocuria sediminis* and *Bacillus pumilus* which were exposed to the bacteriocin of *Rhodococcus enclensis*, and the growth of *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans* which were exposed to the bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* were examined from the wells of Column 1 belonging to MIC endpoint to detect Minimum Bactericidal Concentration (MBC) (Tables VI-VII). Although, the tested bacteriocin concentrations of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* from 1/4 to 1/1024 did not effected the multidrug-resistant bacteria, the 1/2 bacteriocin concentration of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* was found to be effective on the test isolates. The bacterial growth was not observed on the plates after plating from the MIC (1/2) endpoint wells. Therefore, it was accepted that the bacteriocins of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* have bactericidal effect on the test isolates (Tables VI-VII).

To examine morphological changes in bacteriocin-treated isolates, the multidrug-resistant test bacteria were examined for morphological changes using a scanning electron microscope after the treatment of bacteriocin (Figures 1-3). The length of untreated *Kocuria sediminis*, *Bacillus pumilus*, *Pseudomonas psychrotolerans* cells were respectively recorded as 1.38 µm, 6.77 µm, 2.61 µm. After bacteriocin treatment, the round cell structure of *Kocuria sediminis* was lost (Fig.1a-c).

Table VII

The Minimum Inhibitory Concentrations of bacteriocin produced from *Salinivibrio costicola* subsp. *vallismortis* against *Kocuria sediminis*, *Bacillus pumilus* and *Pseudomonas psychrotolerans*

Column number of 96-well plate	1	2	3	4	5	6	7	8	9	10	11	12
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	medium sterilization control	bacterial growth control
Kocuria sediminis	b	p	p	p	p	p	p	p	p	p	b	p
Bacillus pumilus	b	p	p	p	p	p	p	p	p	p	b	p
Pseudomonas psychrotolerans	b	p	p	p	p	p	p	p	p	p	b	p

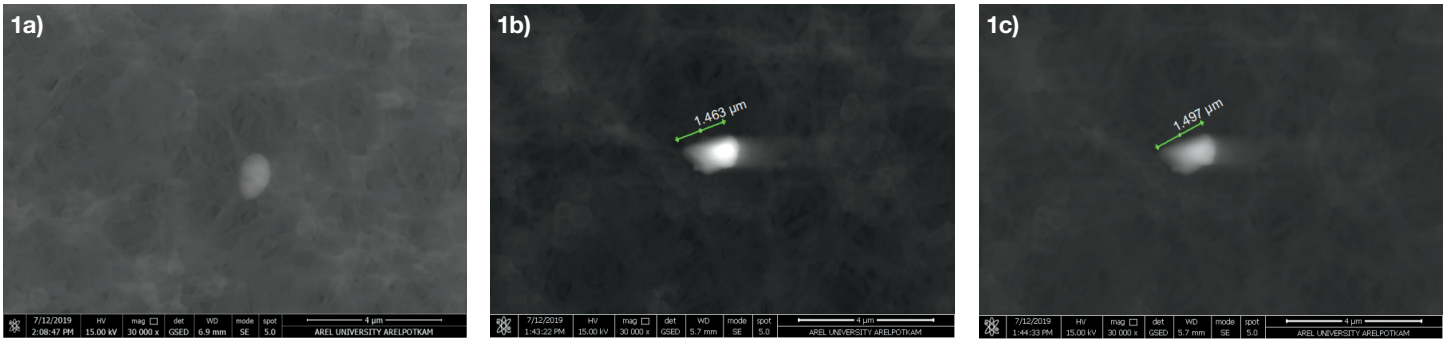

Figure 1. SEM micrographs of *Kocuria sediminis* on membrane filters without bacteriocin treatment **(a)**, after treatment with bacteriocin of *Rhodococcus enclensis* **(b)**, after treatment with bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* **(c)**. The bar = 4 µm (a, b, c).

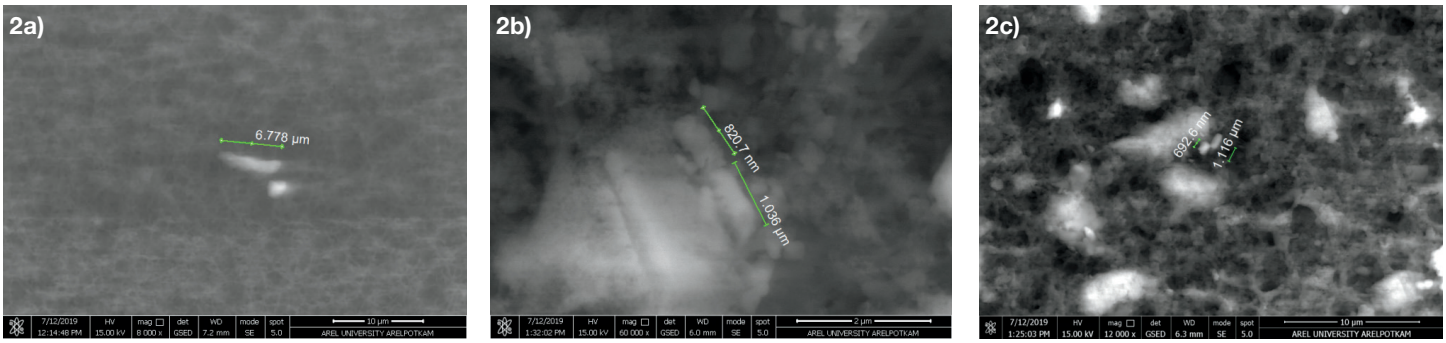

Figure 2. SEM micrographs of *Bacillus pumilus* on membrane filters without bacteriocin treatment **(a)**, after treatment with bacteriocin of *Rhodococcus enclensis* **(b)**, after treatment with *Salinivibrio costicola* subsp. *vallismortis* **(c)**. The bar = 10 µm (a), 2 µm (b), 10 µm (c).

The length of *Bacillus pumilus* cells after treatment with bacteriocin of *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* were respectively measured as 1.03 µm-820 nm and 1.11 µm-692 nm (Fig.2a-c).

The length of *Pseudomonas psychrotolerans* cells after treatment with bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* were recorded as 1.24 µm-692 nm (Fig.3a-b).

In leather industry, although salt is used for curing process, the experimental data of the researchers showed that salt contaminates hides and skins with various microorganisms. Mesophilic bacteria[64,65], moderately halophilic bacteria[32,33,66,67], enteric bacteria[68], halotolerant bacteria[49], extremely halophilic archaea[66] were previously reported on the salted hides, salted skins and preservation salt samples. In addition, antibiotic-resistant faecal indicator bacteria[69], antibiotic-resistant *Enterobacteriaceae*[70], antibiotic-resistant moderately halophilic bacteria[71] isolated from salted and soaked hides and skins were reported.

It has been known that antimicrobial agents are widely used in different industrial processes to prevent the bacterial growth and damage. Owing to randomly and misuse of these antimicrobial agents, some of the microorganisms may be resistant to these agents. Bacteria resistant to commonly used antimicrobial agents in different industries have been isolated in previous

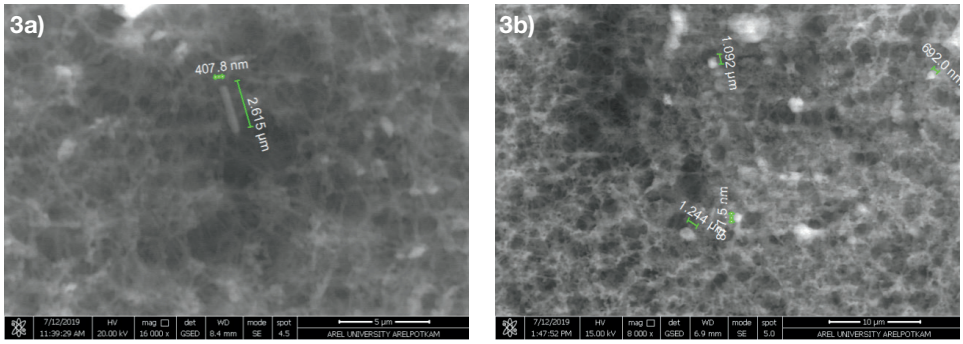

Figure 3. SEM micrographs of *Pseudomonas psychrotolerans* on membrane filters without bacteriocin treatment **(a)**, after treatment with bacteriocin of *Salinivibrio costicola* subsp. *vallismortis* **(b)**. The bar = 5 µm (a), 10 µm (b).

studies. These bacteria may have become resistant to antibiotics that entering their natural environment in different ways. To prevent spread of antibiotic resistance among bacteria in natural ecosystems, effective wastewater treatment, water disinfection and environmental regulations are essential. The researchers emphasized that bacteriocins may be an alternative to other antibacterial compounds due to their potency, low toxicity, broad or narrow spectrum of antibacterial activity, inhibition of pathogens and amenability to molecular manipulations.[28] The application of some bacteriocins were reported in previous studies. For instance, subtilosin A produced by *Bacillus subtilis* was used as anti-viral agent.[72] In another studies, it was reported that nisin inhibited the growths of *Listeria*, *Bacillus*, *Micrococcus*, *Alicyclobacillus*, *Pediococcus*, *Clostridium*, *Sporolactobacilllus*, *Desulfotomaculum*, *Enterococcus*, *Lactobacillus* and *Leuconostoc*[73] and it was used as biopreservative agent[74]. Sakacin A produced by *Lactobacillus curvatus* was used for controlling of *Listeria monocytogenes* and pediocin PA produced by *Pediococcus acidilactici* was used as a meat starter.[75,76] The applications of bacteriocins as cleaner-preservation methods to reduce salt pollution in leather industry were also reported by the researchers.[77-79] Bacteriocin produced from *Lactobacillus plantarum* was found to be effective against goat skin contaminated with *Pseudomonas aeruginosa* and *Bacillus putrefaciens*.[77] Bacteriocins produced by moderately halophilic non-enzyme producing *Salimicrobium salexigens*, *Halomonas halodenitrificans* and *Halomonas venusta* were inhibited the growth of moderately halophilic enzyme-producing *Gracilibacillus dipsosauri*, *Staphylococcus arlettae*, *Planococcus rifietoensis*, *Marinococcus tarijensis*, *Salinivibrio costicola subsp. alkaliphilus*, *Halomonas halmophila*, *Idiomarina loihiensis*, *Halomonas eurihalina*.[79] Rapid developement in genetics will allow to develope bacteriocins into the next generation antibiotics.[76]

Conclusion

This is the first study that contributes in screening and detection of bacteriocin producing haloversatile bacteria and multidrug-resistant haloversatile bacteria in the brine samples of Camalti Saltern. The experimental results showed that multidrug-resistant haloversatile bacterial species were found in brine samples collected from the Camalti Saltern. These species may survive during evaporation and salt production process at the Camalti Saltern due to their ability to live in harsh conditions, high salt concentrations, and wide ranges of pH and temperature values. When this salt is used in the leather industry, it may contaminate hide products with antibiotic-resistant bacteria. In order to combat antibiotic resistance, inappropriate and frequent antibiotic use must be reduced. Before discharging the wastewater to the sea, antibiotics and antibiotic resistant bacteria should be effectively removed from the wastewater. Due to the antimicrobial activities of bacteriocins and the stability of antimicrobial properties under various conditions, the bacteriocins produced by haloversatile bacteria may have potential application in different industries. Bacteriocins produced from *Rhodococcus enclensis* and *Salinivibrio costicola* subsp. *vallismortis* showed broad spectrum inhibition and they may play an important role to decrease detrimental effects of multidrug resistant or enzyme-producing bacteria in leather industry during salting, brine curing or soaking processes of hides and skins. These results highlight their potential use in different industries such as leather and food.

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Performance Evaluation of Hybrid Formulations Consisting of Antioxidant and Crosslinking Agents for the Treatment of Acid Degradation in Historic Vegetable-Tanned Leathers

by

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Abstract

This study investigated the performance of several new formulations for the treatment of acid degradation in historic leathers made with condensed tannins. This investigation was performed for four formulations consisting of collagen-stabilizing agents (oxazolidine E, resorcinol, nano alumina, and nano silica) and an antioxidant agent (Songnox 1010) in the acidic environment. The compounds were applied to 19th-century leathers with a brush. The properties of the leathers before and after treatment and accelerated aging were determined by measuring pH, shrinkage temperature, and color and conducting ATR-FTIR and FORS. For accelerated aging, the samples were exposed to an atmosphere with 95ppm SO_2, 40°C, and 51% relative humidity for 14 days. The results showed that all of the tested formulations slowed down the acid degradation process. The combined use of collagen stabilizing agents provided good treatment properties and adding Songnox 1010 antioxidant agent to this combination further enhanced the effect of treatment. Among the tested formulations, the one containing oxazolidine+resorcinol+nano alumina+S1010 provided the best properties for controlling acid degradation.

Introduction

Leathers and leather products tend to get damaged and degrade as they age. The rate of degradation progress depends on the characteristics of the leather and its surrounding environment. Leather degradation, which has destroyed a significant portion of historic artifacts over time, can have chemical, biological, and physical causes.[1] However, leather degradation is commonly considered a chemical process whose initiation and development are influenced by a variety of factors. In general, the leather-making process and the environment to which it is exposed both play a key role in the formation of complex degradation mechanisms.

Traditional leather-making processes and tanning methods are very diverse. Before the 1850s, common tanning agents were alums, plant-based tanning agents, or a combination of them.[2] The most common method was vegetable tanning with plant materials that contain hydrolysable tannins.[2,3] But starting from the turn of the 19th century and especially after the 1850s, the industrial revolution and rising demand fundamentally changed the traditional vegetable tanning processes. These changes included the widespread use of condensed tannins, sulfur compounds, synthetic dyes, and strong mineral acids (e.g., sulfuric acid) in the leather-making process, which result in the production of less durable leathers.[4-6] Also, the increasing emission of industrial air pollutants, especially SO_2, turned into one of the important causes of leather degradation.[7] The combination of these factors can cause severe chemical degradation in leather products in a short time. The most important cause of this degradation is acidic hydrolysis or in other words, acid degradation, which in its advanced form turns into red rot. It should be noted that similar damage has also been seen in some leathers produced before the 19th century.[8-10]

This aggressive, rapid, and irreversible degradation, which is a combination of oxidation and hydrolysis, is among the greatest concerns in the conservation of leather artifacts.[1,11-13] Acid degradation leads to the decomposition of collagen-tannin complexes, loss of tannins, degradation of collagen structure, and pH and hydrothermal stability reduction, which result in brittleness, poor durability, tearing, discoloration, and appearance of red spots and white deposits on the leather surface.[11,13-15] Considering the severe impacts of acidic degradation, it is important to study this process in order to understand it and determine how it can be prevented. The research on this subject has been ongoing since the early works of Faraday and his colleagues in the mid-nineteenth century and still continues today.[1,12,16] This highlights the importance and necessity of understanding acid degradation and protecting leather artifacts against it. A significant number of studies in this field have been focused on developing and evaluating treatment methods, which could be preventive measures or intervention measures. Over the years, a wide variety of materials have been used for treating leather, including buffer salts, gases, waxes, lubricants, and consolidants, many of which are now considered obsolete because of poor effectiveness.[17] In general, these treatment materials can be classified into three categories based on how they affect leather: stabilizing agents, consolidation agents, and surface coatings.[13] The previously

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common treatment materials that are now obsolete include potassium lactate, imidazole, and pliantex, parylene (polyparaxylylene), lankrothane 1304 (polyurethane). Some treatment materials such as ammonia vapor, aluminum alkoxide, klucel g, red-rot cocktail, polyethylene glycol (PEG), renaissance wax, SC6000 and certain leather lubricants and dressings are still occasionally used.[13,17,18] Each of these treatments has its own advantages and disadvantages and there is still no definitive solution to control this degradation. The major drawbacks of existing treatment methods and materials include staining and discoloration, dust absorption, inhibition of future conservation procedures, oxidation and hardening of leather, catalysis of degradation processes, softening of the finishing and decorative layer, creation of a sticky surface, effect on adjacent materials, surface flaking, white spue, biodegradation, problematic solvents, lack of long-term stability, difficult and complex application, flammable, toxic solvents, alkaline decay, and irreversibility.[1,13,17,19-23]

In general, it can be stated that since acid degradation is a step-by-step process, it can be somewhat controlled by countering each of these steps. One major factor of this process is SO_2 in the atmosphere, which upon oxidization to SO_3, provides a source for the formation of H_2SO_4.[2] Therefore, controlling this oxidation process can be effective in slowing down the degradation process. The formation of H_2SO_4 or its presence in the leather-making process is believed to be one of the most important causes of leather degradation.[14] When exposed to moisture, acids in leather dissolve and form positive hydronium ions, which may cause the pH to drop below the stability level of peptide chains and break the bonds between amino acids.[11] The pH in acid degraded leathers usually drops below 3,[24, 25] which accelerates the degradation process and the breakdown of the collagen-tannin complex.[14] Therefore, another key step in controlling acid degradation is to control acidity. Hydronium ions also weaken the leather structure by breaking the bonds between amino acids in collagen chains,[17] which in some cases causes shrinkage temperature (T_s) to drop to about 30°C [24]. Therefore, another important objective of treatment is to improve hydrothermal stability, which can be considered the best measure of the overall stability of leather.[26]

Treatment of acid degradation has always been an extremely important issue for the conservation of historic leather artifacts. Considering the drawbacks and poor effectiveness of many existing treatment methods, further research is still needed to find better alternatives. Therefore, the present study, which is a continuation of our previous study,[27] evaluated the ability of a number of treatment formulations composed of antioxidant and crosslinking agents to control the acid degradation process, improve the properties of weakened leather structures, and prolong the lifespan of leather.

Materials and Methods

Samples Preparation and Treatment

After applying a total of 17 formulations containing collagen stabilizing agents, antioxidants, and acid scavengers to new mimosa-tanned leathers, four of the formulations that contained collagen stabilizing agents and antioxidants were selected for testing on historical samples.[28] The antioxidant and collagen stabilizing agents used in the formulations were oxazolidine E (Sigma-Aldrich), resorcinol (Merck), nano alumina (US Research Nanomaterials), nano silica (US Research Nanomaterials), and Songnox 1010 (tetrakis[methylene-3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate] methane; Songwon), which were prepared with concentrations of 10% in isopropanol, 3% in isopropanol, 1% in isopropanol, 2% in isopropanol and 0.5% in white spirit, respectively. The treatments were applied through step-by-step impregnation with a brush (Table I). The historical samples, which were labeled S1, S2, and S3, were 2×10cm pieces of leather collected from the removed and discarded leather bookbinding of antique books belonging to the late Qajar era, from 1789 to 1925.

Accelerated Aging

The accelerated aging was conducted by exposing the samples to a concentration of SO_2 (95±5 ppm) at 40±1°C and 50±1% relative humidity for 2 weeks.

ATR-FTIR Spectroscopy

ATR-FTIR analysis was carried out using a Nicolet 470 FTIR spectrometer and OMNIC 6.1a software (Nicolet instrument corporation, USA) equipped with PIKE MIRacle attenuated total reflectance (ATR) accessory with diamond crystal plate. All Spectra were collected in the range of 1800-525 cm^{-1} at 4 cm^{-1} resolutions with 32 numbers of scan.

Table I

Formulation of hybrid treatments applied on leathers

Treatment	Treatment Formulation (steps and materials)			
	1	2	3	4
T1	Oxazolidine E	Nano alumina	-	-
T2	Oxazolidine E	Nano silica	-	-
T3	Oxazolidine E	Nano alumina	S1010	-
T4	Oxazolidine E	Resorcinol	Nano alumina	S1010

Colorimetry

The colorimetric properties of leather samples were analyzed with Salutron® Colortector Alpha apparatus as a portable colorimeter in terms of CIE Lab color coordinates [L* (brightness), a* (red - green) and b* (yellow - blue)]. Color values were measured five times for each leather sample, and their average was considered as CIE Lab color coordinates.

Fiber-optics Reflectance Spectroscopy (FORS)

The UV-VIS-NIR reflectance spectra were obtained using an AvaSpec-2048 fiber optic spectrometer, an AvaLight-DHc compact deuterium-halogen light source, and a glass fiber reflection probe (Avantes Inc., Netherlands), operating in the 200–1050 nm. Three spectra (with an average of 5 spectra each) were recorded from each sample, taken from different spots on the grain side. The average of reflectance percentage for each wavelength was calculated in Excel and the final spectrum was obtained in the range of 215–915 nm.

Shrinkage Temperature

The shrinkage temperatures of the leather samples were determined according to ASTM D6076-03. The sample specimens, in the form of 12.5 × 76mm strips, were soaked in water tank equipped with a vacuum pump. The wet specimens were inserted into the bath of water at room temperature. The water was heated at 3-4°C/min rate and the temperature at the first definite sign of shrinking was recorded.

pH Measurement

For pH measurement, 0.1g of leather samples were cut into small pieces and soaked in 2ml of distilled water (with 7±0.15 pH) for 12±1 hours. pH of the leather-water mixtures was assessed by using a Metrohm 744 pH meter calibrated between buffers pH 4 and 7.

Results and Discussion

The mean pH of the samples is presented in Figure 1. In the control samples, there was no certain pattern in pH changes after accelerated aging. Accelerated aging increased the pH of the untreated (control) S2 sample, but it decreased the pH of the untreated S3 and made no noticeable change in the pH of the untreated S2. This clearly indicates that pH is not a reliable indicator of degradation level. It has already been shown that sometimes the intense degradation of leather leads to increased pH, which is what we observed in S2. However, applying the treatments also raised the pH. This increase was more pronounced in S1 samples, especially S1-T1, where it was about 0.8, and ranged from 0.4 to 0.75 in other samples. An interesting result was the stable pH of the treated samples in the post-aging stage, where we observed only a slight decrease in the pH of S1-T3 and S3-T4 and even a smaller decrease in the pH of S3-T2. Overall, the post-aging pH of all treated samples was higher than that of their untreated (control) counterparts.

In addition to pH, the T_s measurements are also presented in Figure 1. Typically, vegetable tanning raises the shrinkage temperature of collagen to about 75-85°C. However, the shrinkage temperature of all three untreated leathers was around 48°C. This means a more than 30°C reduction in their shrinkage temperature, which is indicative of severe degradation. Accelerated aging further reduced the shrinkage temperature of these samples by 1-2°C. More specifically, T_s decreased from 47°C to 45°C in the S1, from 46°C to 45°C, in the S2, and from 48°C to 46°C in the S3.

Among the treated samples, only S1-T1 and S1-T3 showed a decrease in T_s after treatment (~2°C). In the case of S1-T1, T_s did not change after aging, but in S1-T3, aging decreased T_s from 45°C to 44°C. While these two samples had the lowest T_s values, they also had the highest pH, which again suggests that pH may not be a good indicator of degradation. These two treatments (T1 and T3) had a different impact on S3, where they increased T_s to 52°C and 50°C, respectively. After aging, the T_s of both of these samples increased to 53°C, which could be due to increased cross-linking of treatment agents and collagen during accelerated aging. It has been shown that oxazolidine performs better in reacting with collagen and increasing T_s in higher pH values (alkaline conditions).[29] Therefore, considering the acidic pH of leathers, the reaction of oxazolidine with collagen has been probably slow.

The treatment T2, which was composed of oxazolidine and nano silica, increased the shrinkage temperature of S2 and S3 by 2.5°C (from 46 to 48.5°C) and 3.5°C (from 48 to 51.5°C), respectively. After accelerated aging, the T_s of both of these samples decreased by 0.5°C.

The treatment T2 also increased the shrinkage temperature of these samples by 3°C. After accelerated aging, the shrinkage temperature of S2 and S3 further increased by 5.5°C and 3°C, respectively, most likely because of the treatment-induced cross-linking. The post-aging shrinkage temperatures of S2-T2 and S3-T2 were 54.5°C and 54°C, which were the highest among the tested treatments. In addition to oxazolidine E, this treatment also includes resorcinol, which is a cross-linking agent for collagen. In terms of chemical structure, resorcinol has two hydroxyl groups in the meta position, which is the main cause of its high reactivity.[30] Using resorcinol in combination with oxazolidine E enhances its effectiveness and leads to improved tanning.[31] While using oxazolidine E as the sole tanning agent increases the T_s of collagen to about 85°C, combining it with resorcinol can increase this temperature to over 100°C.[32] This indicates that the combined use of the two agents can offer better outcomes in terms of controlling the acid degradation of leather.

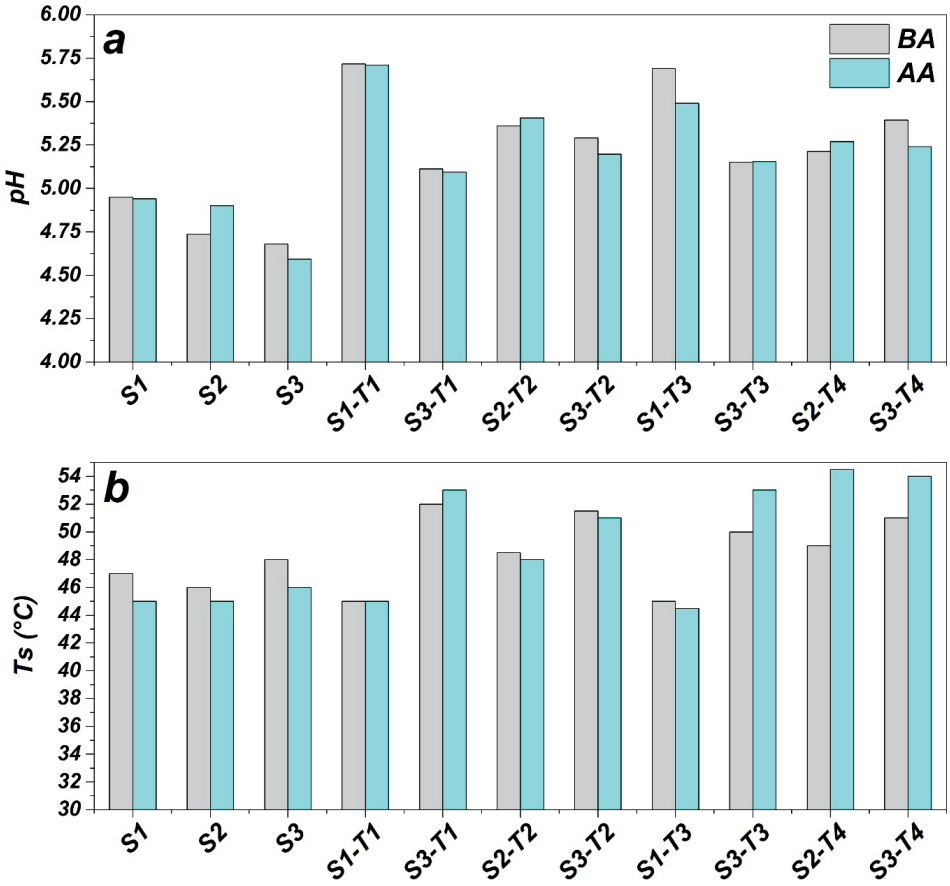

Figure 1. pH **(a)** and Ts **(b)** values of control and treated leather samples, before (BA) and after accelerated aging (AA)

The changes at the surface of the samples were examined by Fiber Optics Reflectance Spectroscopy (FORS). The mean spectra of the samples are shown in Figure 2. Since FORS is a surface analysis method, the obtained spectrum and its changes after accelerated aging are directly related to the composition and characteristics of the leather's surface layer. In the reference samples, in addition to the peak at 180nm, there is a peak at about 360nm, which can be related to copper or iron acetate and the dye applied to the leather.[33-35] In proteins, absorbance in this range is typically related to $\pi^{®}\pi^{*}$ and $n^{®}\pi^{*}$ transitions in C=O, NH, and CONH groups.[36] It has been reported that absorbance below 250nm can be related to non-aromatic amino acids, but absorbance in the range of 250-350nm is related to aromatic amino acids.[37] It has also been shown that collagen has absorbance in this range because of its triple-helical structure.[38] In

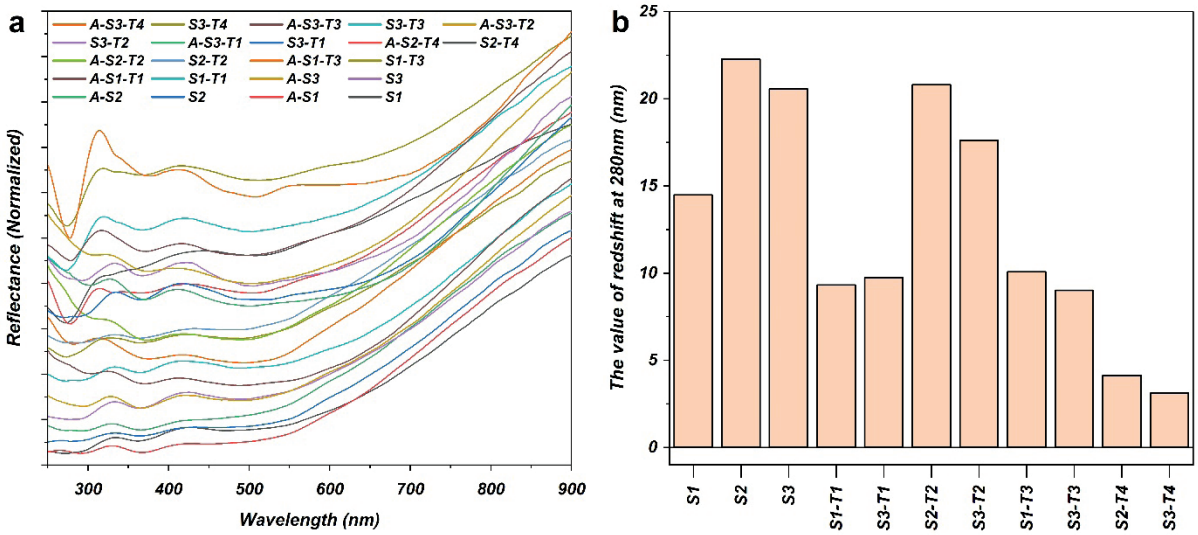

Figure 2. Mean FORS spectra of leathers surface, before and after aging **(a)** and the rate of shift at the peak 280nm after accelerated aging **(b)**

general, changes in structural properties cause peaks to move, and those changes that cause reduced structural consistency tend to shift the peaks to higher wavelengths. Research has shown that red shift occurs when collagen and its triple-helical structure are destroyed and denatured.[39,40] In the reference samples, accelerated aging caused a bathochromic shift in the peak of 280nm, or in other words, moved this peak to higher wavelengths. Therefore, the shift of this particular peak was considered as the measure of surface changes due to accelerated aging.

The shift of the 280nm peak of samples during the aging process is shown in Figure 2. In the untreated S1, S2, and S3 samples, accelerated aging moved the peak by about 14, 22, and 20nm, respectively. However, the applied treatments reduced the magnitude of this shift. Among the tested treatments, T2 had the greatest shift, which was still smaller than the shift in the control sample. In the T1 and T3 samples, the shift was about 9-10nm, but in T4 it was about 3-4nm. These results suggest that all four treatments managed to reduce the surface changes of the samples due to accelerated aging. However, T4 was most successful and T2 was least successful in this area.

The results of the color examination of the samples after accelerated aging are presented in Figure 3. The untreated S1 and S2 samples, which originally had a brownish color, turned slightly darker and redder after aging, but the untreated S3, which was originally blackish, became slightly brighter after aging. According to the results, all treatments showed an acceptable level of effectiveness in controlling the changes in parameters *L* and *a* during aging. Furthermore, examining the aging-induced total color difference showed substantially limited color changes in the treated samples compared to the control (untreated) samples, which is indicative of the good long-term color stability of the treated leathers.

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) was used to examine structural changes in the leather grain and corium layers. Typically, the interesting absorption bands in the FTIR spectrum of leather are those around 1650cm^{-1} for amide I, 1550cm^{-1} for amide II, 1220 cm^{-1} for amide III, and 1450cm^{-1}.[10] The degree of hydrolysis of polypeptide chains can be assessed semi-quantitatively using the absorption intensity ratio of amide I to II (AI/AII).[36,41-43] This ratio is about 1.25-1.30 for new leathers but increases with degradation. The integrity of the triple-helical structure of collagen can also be studied using the ratio of the absorption intensity of amide III to 1450cm^{-1}.[41] This ratio is equal to or greater than 1 for the intact structure and about 0.5 for denatured collagen. Furthermore, the difference between the absorption bands position of amides I and II can be an indicator of collagen gelatinization.[27, 41] This difference is about 90-100cm^{-1} for new leathers. Occasionally, this difference has also been used to express the extent of collagen denaturation.[36,42,43,44,45] The increased absorption intensity in the range of 2800-3000cm^{-1}, which is related to CH_2 stretching vibrations, can also be used as the measure of collagen denaturation.[11]

In this study, the differences between the positions of peaks of amides I and II and their intensity ratios were used as the measure of denaturation/gelatinization and hydrolysis of collagen at the surface and in the corium, respectively. The data obtained before and after accelerated aging of the samples are presented in Figure 4. The increased Δν in the spectra of the control samples surface indicates

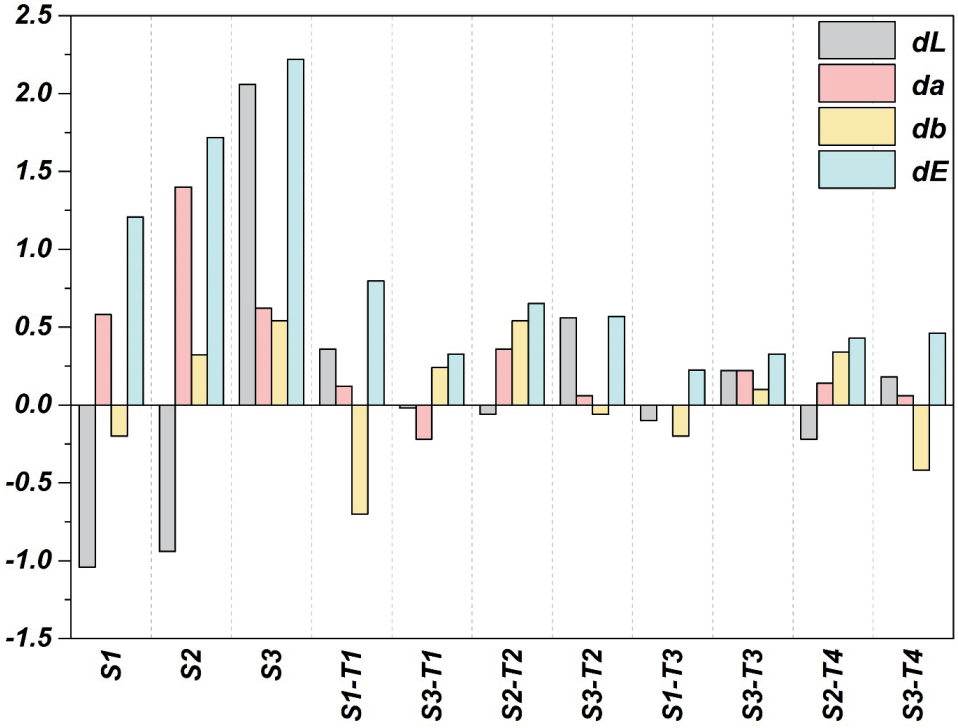

Figure 3. Color difference of leather samples, before and after accelerated aging based on ΔL*, Δa*, Δb* and ΔE

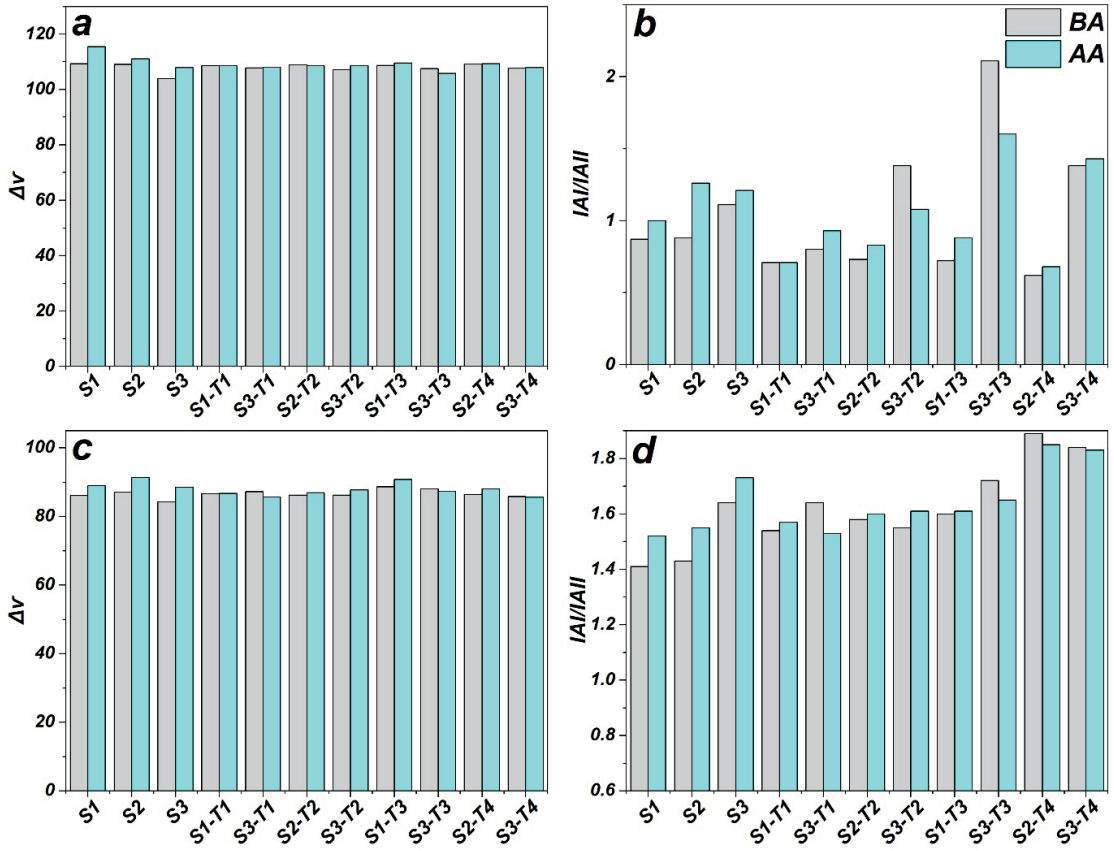

Figure 4. Data obtained from ATR-FTIR spectra of leathers, before (BA) and after accelerated aging (AA); **a:** difference in position of amide I and II (Δv) in the spectra of grain; **b:** Amide I to II intensity ratio (IAI/IAII) in spectra of grain; **c:** difference in position of amide I and II (Δv) in the spectra of the corium layer; **d:** Amide I to II intensity ratio (IAI/IAII) in the spectra of the corium layer

increased collagen denaturation/gelatinization due to accelerated aging. In comparison, the treated samples showed much smaller Δv changes. Among these samples, T4 and T1 had the lowest Δv changes, which reflect their ability to control collagen denaturation/gelatinization on the leather surface. Similar to the grain layer, the corium of the untreated leathers showed an increase in Δv after accelerated aging, which is indicative of intensified collagen denaturation/gelatinization. As before, applying the treatments significantly reduced this denaturation/gelatinization, leading to substantially smaller Δv changes in the treated samples compared to their untreated counterparts.

In all control samples, the hydrolysis index of the leather surface increased after accelerated aging. In S3-T2 and S3-T3, accelerated aging reduced this ratio. All other treated samples also showed signs of reduced hydrolysis at the surface. Among the treated samples, S1-T3 showed the highest increase in the AI/AII intensity ratio, which was almost the same as in the untreated S1. As with Δv, the samples treated with T1 and T4 showed the least change in the AI/AII intensity ratio of the surface layer after aging. As for the corium, accelerated aging also intensified hydrolysis in this layer of the untreated samples, leading to an increased AI/AII ratio. In S3-T1, S3-T2, and S2-T4, however, this ratio decreased after accelerated aging. The change in the AI/AII ratio of S3-T2 was particularly remarkable compared to the untreated samples. In S1-T1, S2-T2, S1-T3, and S3-T4, the aging-induced changes in this ratio were very small, which indicates more inhibited collagen hydrolysis. Overall, the results of ATR-FTIR showed that the treatments significantly reduced collagen denaturation, gelatinization and hydrolysis both on the surface and in the corium. Furthermore, using resorcinol in combination with oxazolidine E appears to increase leather stability.

Conclusion

This study investigated the combined performance of cross-linking and antioxidant agents in controlling acid degradation in historic leather artifacts. The studied compounds included oxazolidine, resorcinol, nano alumina, nano silica, and S1010 in four formulations, which were applied to the leathers with a brush. The leathers used in the experiments were selected from discarded bookbindings related to the 19th century.

Our pH assessments showed that, in general, pH cannot be considered a reliable measure of degradation severity. However, the treated samples showed a slight increase in pH compared to the control samples. T_s values measurements showed that resorcinol enhanced the effect of oxazolidine in improving the shrinkage temperature of

leather. When resorcinol and oxazolidine were used together, the increase in T_s continued even after aging; an effect that can probably be attributed to increased crosslinking during accelerated aging. The results of Fiber Optics Reflectance Spectroscopy (FORS) showed that it is a suitable tool for studying the surface changes of leather in the UV range. These results also showed that while aging led to structural degradation and loss of structural consistency as indicated by the bathochromic shift of the peak positioned around 280nm, all treatments except T2 were effective in inhibiting this effect. Among the tested treatments, the combination of oxazolidine, resorcinol, nano alumina, and S1010 provided the highest level of stability in leather, as was also the case with T_s. The comparison of color changes of treated and untreated samples before and after aging showed the effectiveness of all tested treatments in controlling color changes. Using the position shift and intensity ratio of amide I and II absorption bands in the FTIR spectra of the surface and corium, it was concluded that all treatments reduced collagen denaturation/gelatinization and hydrolysis in the acidic condition.

In summing, the results suggest that the combined use of antioxidant and collagen stabilizing agents will be effective in controlling acid degradation in vegetable-tanned leathers. Among the formulations tested in this study, those containing a combination of oxazolidine, resorcinol, nano alumina, and S1010 were the most effective in controlling this degradation.

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The Impact of Potassium Persulfate on Linseed Oil Tanning

by
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Abstract

The tanned collagen is the stabilized form of skin. Tanning collagen with oil is a particular class of tanning known as chamois tanning. Chemically the oil tanning involves autoxidation of unsaturation present in the oil. This study focuses on potassium persulfate as a catalyst to accelerate the oxidation rate of unsaturated bonds in the linseed oil with an increase in water absorption capacity (586%) of oil-tanned leather. Results indicate eccentrically reduction in the duration of the chamois process from 15 to 2 days. Shrinkage temperature, tensile strength and other organoleptic properties of experimental leathers are better than the control leathers.

Introduction

Tanning stabilizes collagen fibers through crosslinking with tanning agents. Various tanning agents are used for tanning purposes, such as oil, chromium and its complexes, vegetable barks and powders, and metal, along with their compounds. Each tanning method has its significance and limitations.[1]

Oil tanning is a particular class of tanning where highly unsaturated oils are utilized to stabilize skin fibers. Chamois leathers used to make various soft leather products such as filters, cleaners, gloves, and garments due to their high-water absorption properties.[2,3,4]

Chemically oil tanning is an oxidation reaction where fatty acids undergo a chain reaction involving initiation, propagation, and termination steps.[5] Various oils are used for the oil tanning, such as fish oil,[6] linseed oil,[7] rubber seed oil,[8] animal tallow,[9] fleshing oil,[10] egg oil,[11] jatropha [12] epoxidized oil,[13,14] castor oil [9], and sunflower oil.[9] However, highly unsaturated oils such as fish oil and linseed oil are preferred because of their high affinity towards oxidation.

Linseed oil is known for its high unsaturation and odor-free characteristics, making it suitable for the chamois process.[7] The most common pathways associated with the oxidation of linseed oil are autoxidation and photo-oxidation of fatty acids.[5] The mentioned oxidation pathways require 10 to 15 days to complete the process.

Various oxidizing agents have been used to accelerate the tanning process, such as hydrogen peroxide,[15,16,17] sodium percarbonate,[18] ozone,[19,20] benzoyl peroxide,[7] and benzenecarboperoxoic acid.[21] Each oxidizing agents has their specific operating mechanisms, restrictions, and importance depending upon the reaction conditions and parameters.

The present study predominantly focuses on the effect of different percentages of potassium persulfate on the duration and properties of chamois leather.

Materials and Methods

Materials

Linseed oil was procured from a local supplier, Chennai. Potassium persulfate was procured from Sigma-Aldrich, Chennai. All the other chemicals were analytical grade. For pre-tanning, 20 defect-free wet salted sheepskins were taken.

Method of oil tanning

For the experimental process, linseed oil (25%), soda ash (0.5%), and potassium persulfate (0.25, 0.5, 0.75, and 1% separately) were pre-mixed in a beaker with the help of a stirrer for complete mixing of the chemicals. Further, the mixture was applied to the leather in a rotating drum to distribute the oil throughout the surface. The process was carried out for 2 h continuously. The skins were hung up for oxidation in open drying stands. The completion of oil tanning was visually judged by the color of the skins turning to golden yellow. Then, the leathers were washed with water (100%), soda ash (1%), and wetting agent (1%) for the complete removal of unfixed oil. Final leathers were dried and subjected to staking, buffing, and milling. Control chamois leather was made as explained above without potassium persulfate. Detail description of leather processing is seen in Table I.

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Table I
Oil Tanning Process

Process	Chemical	Percentage (%)	Time	Remarks
Soaking	Water	300	One day	
	Preservatives	0.25		
	Wetting agents	0.50		
Unhairing and Liming	Water	20	Two days	
	Lime	10		
	Sodium sulfide (60%)	3		
Reliming	Water	300		
	Lime	10		
Fleshing				
Deliming	Water	100		
	Ammonium chloride	2	40 min	Check de-liming using phenolphthalein
	Alkaline bate	0.5	30 min	Drain
Washing	Water	200	10 min	Wash and drain
Partial pickling	Water	80		
	Salt	8	30 min	
	Formic Acid	0.5	30 min	In 1:10 dilution with water
	Sulfuric Acid	0.2		In three feeds with 1:10 dilution with water, adjust pH to 4
Depickling	Sodium bicarbonate	1		
Glutaraldehyde tanning	Glutaraldehyde	1	90 min	Drain, pile for overnight
	Soda ash dissolved	2		
Next day				
	Linseed oil	25		
	potassium persulfate (experiment)	0.25, 0.5, 0.75 and 1		
	Sodium carbonate	0.5		Mix using stirrer, make paste. add to drum along with skin

Scanning electron microscope analysis
SEM analysis was carried out to understand better the morphology of leather fibers. The Phenom Pro desktop scanning electron microscope (SEM) was used to analyze the fiber structure of the leather.

Water absorption
The most important property of chamois leather is its water absorption capacity. The higher the water absorption of the chamois leather, the better its quality. The standard procedure determined the water absorption of experimental leather.[22]

Shrinkage temperature measurement
Shrinkage temperature measurement provides information about the leather resistance towards heat. The leathers were subjected to shrinkage temperature as per standard test procedures.[23]

Strength and organoleptic properties of the chamois leather
The experimental leathers were examined for physical characteristics such as tensile strength organoleptic properties.[24]

Results and Discussion

The potassium persulfate acts as a catalyst for oil oxidation, which shortens the reaction duration from 15 days to 2 days. The experimental leathers have been characterized for various physical, chemical, and organoleptic properties, and the results are discussed in the following sections.

Plausible chemistry
The autoxidation of monounsaturated fatty acid (oleic acid) is achieved at high temperatures, while polyunsaturated fatty acids

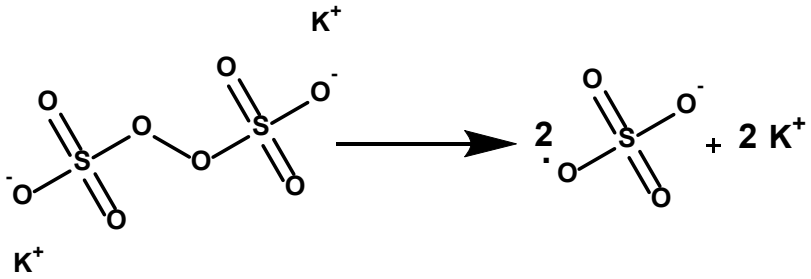


Figure 1. Generation of sulfate-free radical

undergo rapid oxidation even at room temperature.[25] Linseed oil contains polyunsaturated fatty acids, consisting of more active methylene groups, which start the chain reaction through free radical generation.[26,27] As shown in figure 1, potassium persulfate readily dissociates into the sulfate-free radicals, which act as free radical initiators for the oil tanning process. These free radicals stimulate the active methylene groups present in the oil moiety.[28]

These polyunsaturated fatty acid (PUFA) radicals quickly get attached with oxygen to form resonating stabilized peroxyl radicals. The peroxyl radical adds hydrogen atom from another polyunsaturated fatty acid chain of linseed oil to create the primary oxidation product, a lipid hydroperoxide (LOOH), leaving behind another reactive PUFA radical that can again start the process, and the propagation will continue.[29] LOOH on further decomposition generate alkoxyl radical and several secondary oxidized products such as saturated aldehydes, unsaturated aldehydes, short-chain ketones, alcohols, acids, esters, ethers, and hydrocarbons.[30] The aldehydes, a highly diffusible compound, interact with skin amino acids and form stabilized cross-linked protein.[31]

Water absorption

Water absorption is an essential property of chamois leather. The results for water absorption of experimental and control leathers are provided in Table II. The table indicates that leather tanned with 0.25% offer of potassium persulfate show better water absorption value (586%) than control (441%)[7] and other experimental leathers. Moreover, from the table, it could be seen that an offer of 0.50, 0.75, and 1% of potassium persulfate has shown the decreasing values of water absorption, respectively. The conclusion may be drawn from the observation that the offer of 0.25% of potassium persulfate is sufficient for complete oxidation of linseed oil. Much addition of potassium persulfate may not affect the results of water absorption.

Insight on role of potassium persulfate

The 0.25% of potassium persulfate with linseed oil shows a water absorption value (586%) which is better than the 0.25% of benzoyl peroxide with linseed oil (463%)[7]. The standard oxidizing potential value of potassium persulfate is 2.01V[32] which is higher than that of benzoyl peroxide (+1.5 V) therefore, the oxidizing power of potassium persulfate is found to be higher than that of benzoyl peroxide.

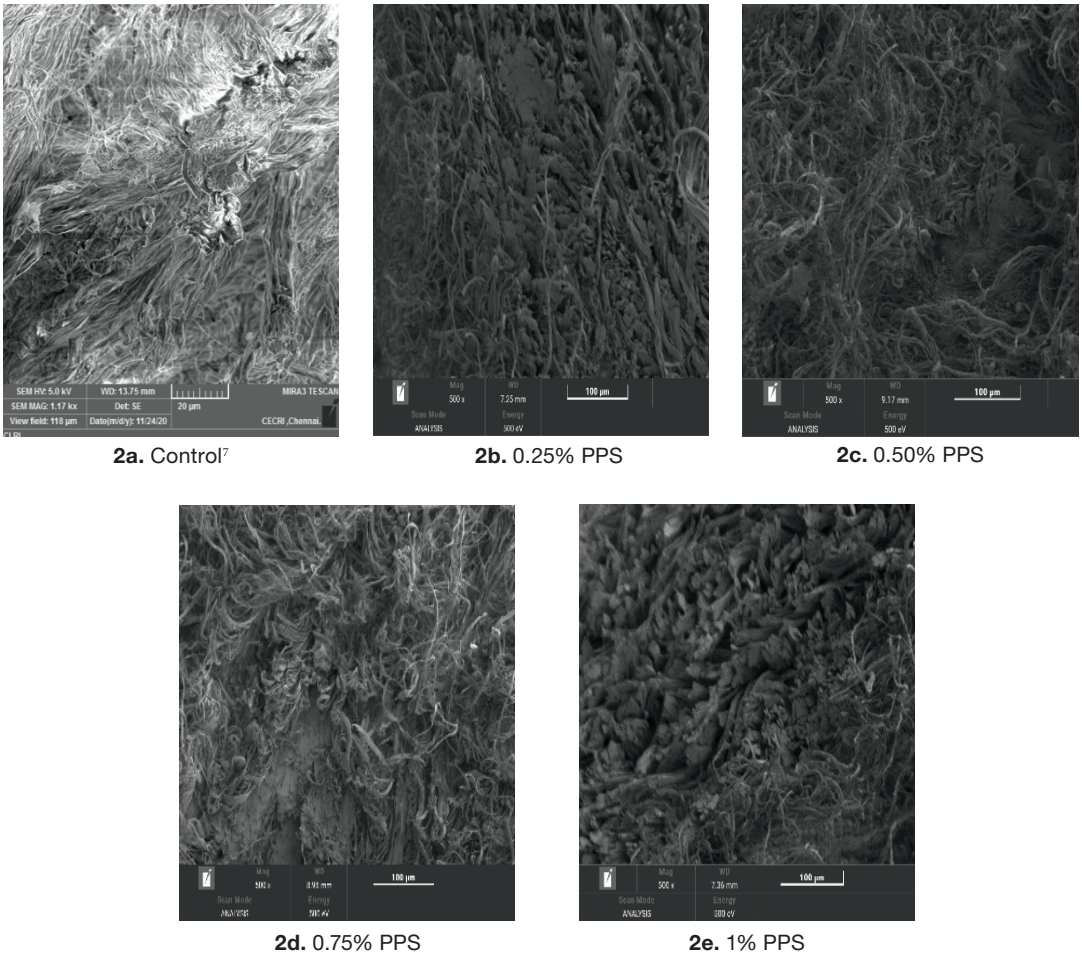
The free radical initiation chain reaction depends upon the ease of generation of free radicals and their half-life.[33] The half-life of potassium persulfate relies on the pH of the solution and operating temperature. At pH 1, the half-life is found to be 20 hours at temperature 50°C, whereas at pH 10, it showed 210 hours.[34] In the case of benzoyl peroxide, it is one hour at 92°C and one minute at 131°C.[35] Therefore, the less percentage (0.25%) of potassium persulfate can bring the desired properties of chamois leather within two days than that of benzoyl peroxide

Scanning electron microscopy analysis of chamois leathers

Scanning electron microscopy images of control and experimental leathers are shown in Figures 2 (a-e). The fiber compactness in

Table II

S No		Water Absorption (%)
1	Control[7]	441±20
2	Potassium persulfate (1.00%)	500±20
3	Potassium persulfate (0.75%)	550±20
4	Potassium persulfate (0.50%)	570±20
5	Potassium persulfate (0.25%)	586±20



2a. Control[7] **2b.** 0.25% PPS **2c.** 0.50% PPS

2d. 0.75% PPS **2e.** 1% PPS

Figure 2. a-e. SEM analysis of experimental chamois leathers against control[7]

control[7] and experimental leathers are very much aligned. Therefore, it may be inferred that the addition of potassium persulfate will not affect the morphology of leather fibers.

Physical testing data of chamois leathers

Experimental leathers were tested for strength and organoleptic properties. Table III indicates that the tensile strength of the chamois leather is almost 60 to 70% more than that of control where skin is treated only with linseed oil.

Shrinkage temperature measurement

Shrinkage temperature measurement of chamois leather gives information about the resistance of the leather due to hydrothermal shrinkage. Table IV indicated the increase in the shrinkage temperature of experimental chamois leathers obtained from 0.50% to 1%. Moreover, experimental leathers with 0.25 and 0.50% show the same shrinkage temperature value (78 °C), more significant than the control values (74°C).

Table III

Physical testing data of chamois leathers

S. No.		Tensile strength (N/mm²)
1	Control	14±2
2	Potassium persulfate (1.00%)	21±2
3	Potassium persulfate (0.75%)	22±2
4	Potassium persulfate (0.50%)	21±2
5	Potassium persulfate (0.25%)	24±2

Table IV

Shrinkage Temperature measurement of chamois leather

S. No.		Shrinkage Temperature (°C)
1	Control	74±1
2	Potassium persulfate (1.00%)	76±2
3	Potassium persulfate (0.75%)	78±2
4	Potassium persulfate (0.50%)	78±1
5	Potassium persulfate (0.25%)	78±1

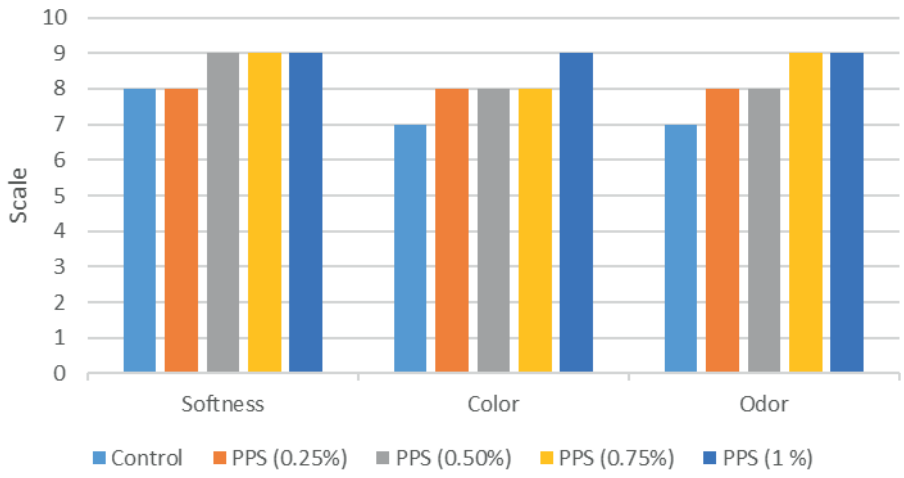


Figure 3. Organoleptic properties of chamois leathers

Organoleptic Properties

Chamois leather organoleptic properties were evaluated for softness, color, and odor. From Figure 3, observation can be drawn that the softness of chamois leathers improved with the increase in the percentage of oxidizing agents. Similarly, the color of the experimental chamois leathers with 1% of potassium per persulfate showed lighter yellow compared to the golden yellow of the control leathers. Although chamois leathers are made, using linseed oil as the leading tanning agent, the odor is one of the essential qualities to assess.

Conclusions

The present study focuses on the accelerated linseed oil tanning process with an optimized offer of 0.25% of potassium per persulfate as an accelerant. The study also emphasis on completion of oil tanning process within two days. The oil-tanned leather shows better water absorption and physical strength than control leathers. The conclusion may be drawn from the study that the use of 0.25% potassium per persulfate in chamois making, reduces the duration of oil tanning to within two days with better water absorption properties.

Acknowledgments

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Improved Method for Accurate and Efficient Analysis of Chlorophenols in Leather Compared with Conventional Steam Distillation Operation Specified by ISO 17070:2015

by
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Abstract

An improved analytical method was built based on the conventional standard method ISO 17070-2015 for the determination of 19 chlorophenols in leather. This developed method involved ultrasonic assisted extraction using methanol containing formic acid, derivatization using acetic anhydride, solid-phase extraction (SPE) cleanup with silica-gel cartridge. Final analysis of the chlorophenols compounds was performed by Gas Chromatography - Mass Spectrometry (GC-MS). Optimum conditions for sample extraction, such as time, temperature and formic acid content in methanol were studied. Satisfactory recoveries in the range of 92-105% (RSD, 2.1-7.3%) for the targets were obtained, and detection limits were in the range of 0.09-0.15 mg/kg. The developed procedure was evaluated and compared with ISO 17070-2015 which uses steam distillation for sample extraction. The method was successfully applied to determine 19 chlorophenols in series of leather samples from different origin. This study demonstrated that the ultrasonic assisted extraction, followed by acetyl derivatization and silica-gel cleanup coupled with GC-MS, can be used as an alternative to ISO 17070-2015, for detecting chlorophenols in leather.

Introduction

Chlorophenols (CPs) are well known ubiquitous pollutants in the environment for many decades.[1] Generally, CPs consist of three monochlorophenols (MCPs), six dichlorophenols (DCPs), six tri- chlorophenols (TrCPs), three tetrachlorophenols (TeCPs), and pentachlorophenol (PCP). Due to their antimicrobiological properties, CPs are used as herbicides, insecticides and fungicides in agriculture, and also as wood preservatives and intermediates in paper and leather industries.[2] CPs are persistent toxic substances which cause histopathological changes and mutations in aquatic life, and some are probable human carcinogens, e.g., PCP.[3] CPs have been classified by the International Agency for Research on Cancer (IARC) as possible carcinogenic agents.[4] Meanwhile, the negative effect of CPs on human health has led to their categorization and inclusion by the US Environmental Protection Agency (EPA) and the Commission of the European Communities in the lists of priority pollutants.[5-6]

Although the use of CPs has been officially restricted, they can still be found in many different matrices like food, water, sludge, soil, wood, textile and leather.[7-8] The presence of CPs in leather presents a health risk if there is an exposure to skin especially at a high temperature, for example, in car seats or sofa because of their lipophilic character.[1] There is no doubt that their analysis is of great importance in controlling the quality of leather. Therefore, providing an appropriate method is essential to screen residues of CPs in leather samples. Though, their analysis in leather has not been studied enough and few articles are available in the literatures.

The current standard method for analyzing 19 CPs in leather samples is ISO 17070-2015, which was totally transformed from the earlier edition ISO 17070-2006 specified for PCP determination. This method uses a steam distillation extraction, followed by acetyl derivatization and GC analysis. The methodology was initially used for analyzing CPs in soils and wood samples.[9] Steam distillation extraction gives relatively clean extracts, although the process takes a few hours or longer. To our knowledge, the validation of this procedure for leather has not yet been carried out. This method is time consuming, making it unsuitable when testing large numbers of leather samples. In addition, thermal decomposition of some halogenic dyes to release chlorophenol (as 4-MCP) due to the high temperature during the steam distillation, which might give false results.[10-11]

To solve the time consuming problem associated with steam distillation in ISO 17070-2015, a direct method has been reported based on Accelerated Solvent Extraction (ASE) technique followed by solid-phase extraction (SPE) cleanup and HPLC analysis.[8] Although this procedure presented a quick and efficient operation, the equipment of ASE was seldom employed in normal laboratory compared with Soxhlet or ultrasonic apparatus. Besides, the high pressure and temperature (100°C) might easily cause the release of CPs from certain halogenic dyes.[11] Comparatively, ultrasonic assisted solvent extraction is commonly preferred due to its high efficiency, simplicity and low extraction temperature. Ultrasonic assisted extraction has been officially recommended and applied for solid sample extraction as rubber, textile and leather in the normal laboratory (EPA 3550C, ASTM G136).

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Although HPLC might be desirable for directly detecting CPs without further derivatization, peak resolution of CPs has often been poor because of the co-elution of isomers as well as the interference of co-extractives.[12] LC-MS has been investigated for screening CPs and presented satisfactory results,[11-13] but the procedure is rarely employed in the normal laboratory due to the high cost of the equipment. Comparatively, method including acetyl derivatization based on GC is still predominant for routine analysis of CPs due to GC popular use, as well as its high resolution and sensitivity,[14] even if the acetylation operation requires extra time.

The aim of this work is to develop an improved procedure based on ISO 17070-2015, for determining CPs in leather with higher efficiency. The procedure is characterized by accuracy, adaptability, and reduced execution time. To improve extraction efficiency, extraction by ultrasonic assisted methanol containing formic acid is applied. The extracted CPs are further derivatized with acetic anhydride, purified and enriched by SPE, and detected by GC-MS. A validation procedure has been proposed and the optimized method can be applied to determine the 19 CPs (as listed in Table I) in real leather samples. The characteristics of the approach were compared with those obtained by ISO 17070-2015.

Experimental

Reagents

Acetone, n-hexane and methanol used to dissolve standards, and to prepare the extracts were of pesticide-grade obtained from Fisher Scientific (Shanghai, China). Acetic anhydride, anhydrous potassium carbonate (K_2CO_3), formic acid (88%), triethylamine and anhydrous sodium sulfate (Na_2SO_4) were of analytical grade from Anpel Co. Ltd (Shanghai, China). Na_2SO_4 was heated at 150°C for 24 h prior to use.

Reference standards of 19 CPs (in Table I), and internal standard Tetrachloro-o-Methoxyphenol (TCG), were obtained from Sigma-Aldrich (Shanghai, China), and their stock solutions (1000 mg/L) were prepared by dissolving appropriate amounts of the commercial products in acetone and stored in glass-stoppered bottles at 4°C. Appropriate volumes of the stock solutions were diluted to prepare solutions containing chlorophenols at 0.2-10 mg/L by n-hexane/acetone (2:1, v/v). Table I lists the CPs with numbers and abbreviations identifying the compounds.

Apparatus

The following apparatus were used in the experiment: Shaking machine designed especially for separating funnel (Haode in Hunan, China), votex for mixing (NuoMi in Changzhou, China), ultrasonic water-bath with ultrasonic frequency 45/60/100 kHz and power 200 W (Kunshan, China), steam generator with power 4 kW (Sespur in Foshan, China), PTFE membrane filter of 0.45 μm ×10 mm (Shanghai, China), and Solid Phase Extraction (SPE) system, with Silica-gel cartridge 0.5 g/6 mL (Agela in Tianjin, China).

Sample preparation

Eighty-five leather samples were analyzed, comprising shoe leather (25), sofa leather (32), garment leather (23) and car seat leather (5). These samples were obtained from the market located in Haining China-Leather City. Leather samples with moisture of 9-13% w/w, were suitably prepared by cutting into pieces (~3 mm × 3 mm) and sealed in bags lined with aluminum foil, which were ready for use.

The negative leather samples were prepared by the following operation. Sample pieces were immersed in ~10-fold methanol containing formic acid (0.5%) and treated by ultrasonic for 60 min at 45-55°C. Then the leather pieces were filtered and retreated twice under the same conditions. Further, the leather pieces were immersed in distilled water for 5 min. After filtering, the pieces were left standing at standard atmosphere for at least 7 days until the moisture content was at the range of 9-13% w/w.

The spiked samples were prepared by adding aliquots of CPs stock solutions to the negative leather pieces of 2 g. The stock solution was added to the sample slowly while verifying that the solution was absorbed by the pieces completely. Then the samples were conditioned in a sealed glass bottle for at least 7 days at room temperature, to allow the full adsorption of the CPs by leather fiber.

Sample extraction

Leather samples (2 g) were placed in a 50 mL screw-capped glass bottle. Methanol (20 mL) containing formic acid (0.5%, w/w) was added, the bottle was then sealed and immersed into the ultrasonic water bath (45 kHz) and treated continuously for 30 min at 45-55°C. After cooling to room temperature, the methanol was retrieved and the leather pieces were re-extracted with another 20 mL methanol containing formic acid (0.5%, w/w) with ultrasonic for 10 min. The extracts were then pooled into a pear-shaped flask and basified with 2 mL K_2CO_3 solution (1 mol/L) to prevent the loss of CPs. The solution was then concentrated at ~50°Cwith a rotary evaporator under reduced pressure, to yield residues about ~2 mL.

Derivatization

The derivatization of CPs was according to the routine operation described in ISO 17070-2015 and other references,[16-17] with minor modifications. Briefly, the residue in the flask was dissolved in 20 mL K_2CO_3 solution (0.1 mol/L) and transferred into a separating funnel. Then 4 mL aliquot of n-hexane, 0.1 mL triethylamine and 0.3 mL acetic anhydride was introduced into the funnel in that

order. The funnel was then fixed onto a shaking machine. It was initially shaken manually until the evolution of CO_2 subsided. Afterwards, it was shaken vigorously for 30 min, to yield acetylated CPs. The n-hexane extracts were then retrieved and the remaining water solution was re-extracted with another 4 mL n-hexane. The n-hexane extracts were pooled and dehydrated by Na_2SO_4, in readiness for cleanup.

Cleanup

The commonly used silica-gel cartridge (0.5 g/6 mL) was chosen for cleanup.[15,18] The cartridges were first cleaned and conditioned with 2 × 3 mL of n-hexane, then the dehydrated n-hexane extracts (~30 mL) was transferred into the cartridge. The solution was allowed to run through the cartridge freely at a flow rate of approximately one drop per second. The cartridge was then instantly washed using 5 mL of n-hexane, followed a gentle purge by a rubber pipette bulb, or short pulse of vacuum, to remove excess of washing solution. Elution was performed with 1 × 1 mL mixture of n-hexane/acetone (2:1, v/v), and eluants were pooled into a 2.0 mL volumetric flask and filled with n-hexane to the mark. The solution was then filtered on a 0.45 μm PTFE filter, which was now ready for GC-MS analysis.

A flowchart for the GC-MS analysis of CPs in leather is shown in Figure 1.

Sample test according to ISO 17070-2015

For method comparison, samples were tested according to the description in ISO 17070-2015. The samples (1 g) were first extracted with steam distillation and the eluted CPs were captured by K_2CO_3 (5 g), which were finally made up to 500 mL using distilled water. Then 100 mL of the target solution was transferred into a separating funnel for acetyl derivatization. For analytical tool, GC-MS was used because of the insensitivity of MCP/DiCP by Electron Capture Detector (ECD). Besides, cleanup after acetyl derivatization, were also included to improve the detection limits. The final solution of 2.0 mL was collected and filtered for GC-MS analysis.

GC-MS analysis

Agilent 8890 gas chromatograph equipped with 5977A mass detector operated in the positive electron impact mode (EI+) was used. The separation was performed on a fused silica capillary column (DB-5 ms; film thickness, 0.25 μm; 30 m × 0.25 m i.d. (J&W Science)). Splitless injection (1 μL) with purge time 0.8 min was made by an autosampler. The temperature of the injector was 260°C. The initial oven temperature was 60°C, and it was programmed to 100°C at 15°C/min and then to 220°C at 8°C/min, followed by a 50°C/min to 270°C with 5 min hold. The MS parameters were as follows: carrier gas, helium; flow rate, 1.0 mL/min; transfer line temperature, 260°C; solvent delay time, 2 min; ion source temperature, 150°C; quadrupole temperature, 230°C.

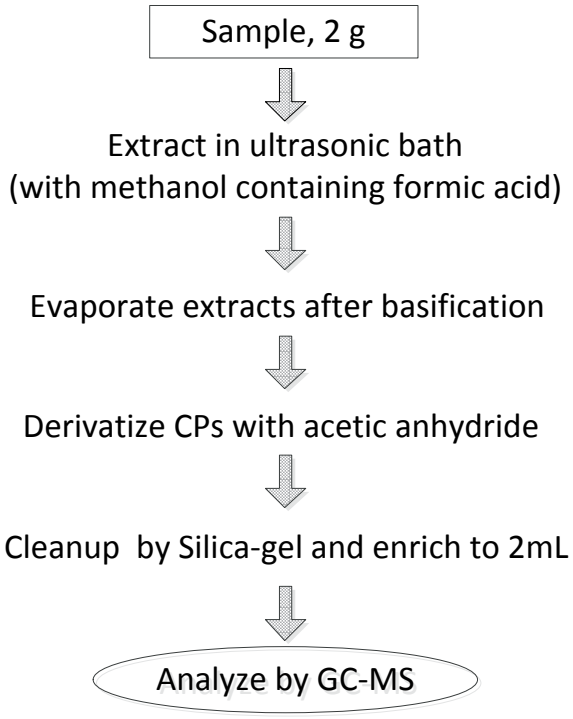

Figure 1. General flowchart of analytical procedure developed in present work

Table I

Target CPs with their CAS No., pK_a, retention time and detection ions

No	Compound	CAS. No	pK_a [a)]	Retention time (min)	Selected ions (m/z)	
					Quantitation ion	Confirmation ion
1	2-MCP	95-57-8	8.56	5.22	128	130
2	3-MCP	108-43-0	-	5.56	128	130
3	4-MCP	106-48-9	9.18	5.67	128	130
4	2,6-DiCP	87-65-0	6.79	6.97	162	164
5	2,4-DiCP	120-83-2	7.89	7.27	162	164
6	2,5-DiCP	583-78-8	-	7.27	162	164
7	3,5-DiCP	591-35-5	-	7.45	162	164
8	2,3-DiCP	576-24-9	-	7.73	162	164
9	3,4-DiCP	95-77-2	-	8.10	162	164
10	2,4,6-TrCP	88-06-2	6.23	8.65	196	198, 200
11	2,3,6-TrCP	933-75-5	5.80	9.30	196	198, 200
12	2,3,5-TrCP	933-78-8	-	9.43	196	198, 200
13	2,4,5-TrCP	95-95-4	7.40	9.51	196	198, 200
14	2,3,4-TrCP	15950-66-0	-	10.14	196	198, 200
15	3,4,5-TrCP	609-19-8	7.84	10.34	196	198, 200
16	2,3,5,6-TeCP	935-95-5	5.22	11.25	232	230, 234
17	2,3,4,6-TeCP	58-90-2	6.35	11.34	232	230, 234
18	2,3,4,5-TeCP	4901-51-3	5.14	12.13	232	230, 234
19	PCP	87-86-5	4.70	13.75	266	268, 264
20	TCG	2539-17-5	6.16	14.13	262	260, 247

[a)] pK_a Acid-base dissociation constant.[15,19]

Results and Discussion

Analysis of CPs with GC-MS

The standards of 19 CPs and TCG (internal standard) were analyzed with GC-MS at the concentration of 1.0 mg/L. Identity of each analyte peak was carried out by comparing GC retention time and the confirmation-to-quantification ion ratio to reference standards. The selected ions and the retention time for the analytes were summarized in Table I. For quantitation, chromatograms were registered using selective ion monitoring (SIM) of the main characteristic fragment ions for each of the analytes. It was noted that 2,4-DiCP and 2,5-DiCP were co-eluted as one peak (7.27 min) due to their closely characteristics, and the two were presumed one analyte in the investigation.

Optimization of extraction process

For determining CPs in leather, the first step is to isolate them from the solid matrix. In this work, ultrasonic assisted solvent extraction was selected due to its ease of operation and simplicity. For the extraction solvent, methanol was chosen because of its relatively low toxicity, availability, as well as the compatibility with the wide range of CPs polarity (3.2-4.8, $LogK_{ow}$).[19] Furthermore, methanol was desirable for eluting targets in leather (see ISO 16189-2013, and ISO 19070-2016) due to its less solubility for the matric substances as oils and polymers which co-exist in the leather fiber, thus, to reduce interfering species in the final solution for GC analysis. On the other hand, the wide range of CPs acidity (4.1-9.2, pK_a as listed in Table I) requires the extraction solvent to be acidified, to ensure all CPs were in their neutral form, thus, to increase their solubility in the methanol and enhance the extraction efficiency. Based on this consideration, a portion of formic acid (0.1-1.0% w/w) was introduced into the methanol used in the experiments. The evaluation of all the results was based on the 19 targets recoveries which should be at the range of 90-110%. With the systematically investigation of spiked samples with all CPs concentrations of 1.0 mg/kg, it was found the optimized extraction temperature for the total CPs was 45-55°C with extraction times of 2 × 30 min under ultrasonic frequency 45 kHz, and suitable proportion of formic

Table II

Optimized parameters of the extraction conditions

Variable	Parameters tested	Optimal
Ultrasonic frequency (kHz)	45, 60, 100	45
Extraction temperature (°C)	25, 35, 40, 45, 50, 55	45-55
Extraction time (min)	2 × 20, 2 × 30, 2 × 40	2 × 30
Formic acid concentration (w/w)	0.1%, 0.3%, 0.5%, 0.7%, 1.0%	0.5%

acid in methanol is 0.5%. The optimized parameters were listed in Table II.

The release of mono chlorophenol (as 4-MCP) from halogenated AZO dyestuff in high temperature has been reported,[10-11] and it was thought that the reason lies in the break of C-N bond of the AZO dye during the distillation process. It was apparent that the optimized extraction temperature (listed in Table II) was significantly lower than the steam temperature used in ISO 17070-2015, indicating these extraction conditions are quite gentle, and as such would not lead to breakdown of dyestuffs in the matrix, especially the release of MCP from certain halogenated AZO dyestuffs. In fact, it was noted that leathers are commonly dyed at the temperature of 50-55°C or higher in the tannery,[20] and these dyestuffs would easily be abandoned, if they can be broken at 45-55°C.

Effects of methanol on derivatization

In this work, concentration of sample extracts was performed prior to acetyl derivatization. Notably, methanol might remain in the residues if the evaporation is not carried out thoroughly, which might affect the derivatization process. Therefore, the influence of methanol on the acetyl derivatization was investigated. The experiments were performed by addition of methanol (0.2-1.8 mL) into 20 mL K_2CO_3 solution (0.1 mol/L) containing the 19 CPs standards. CPs in this solutions were acetylated [16] after mixed with hexane (4 mL), triethylamine and (0.1 mL) and acetic anhydride (0.3 mL). The recovery of each CP was calculated and compared based on their peak area, to evaluate the derivatization efficiency. As presented in Figure 2, when the volume of methanol is less than 0.6 mL in the system, the recoveries of the CPs remained relatively constant (90-105%), indicating limited or no unfavorable effects of methanol on the derivatization process. However, higher volume as 0.6 mL of methanol began to cause an obvious decline in the recoveries, especially for MCP and DiCP with lower chlorine contents, demonstrating that methanol more than 0.6 mL brought adverse effect on their derivatization step. The reason might be that extra methanol (> 0.6 mL) changes the interface behavior between n-haxane and water,[16-17] slowing down or preventing the acetylation reaction. Hence, the methanol volume in the evaporation residues should be controlled smaller than 0.6 mL. In fact, this problem could be easily resolved by extending evaporation time. Practical operation indicates that 11 min evaporation (-0.07 MPa, 50°C) is

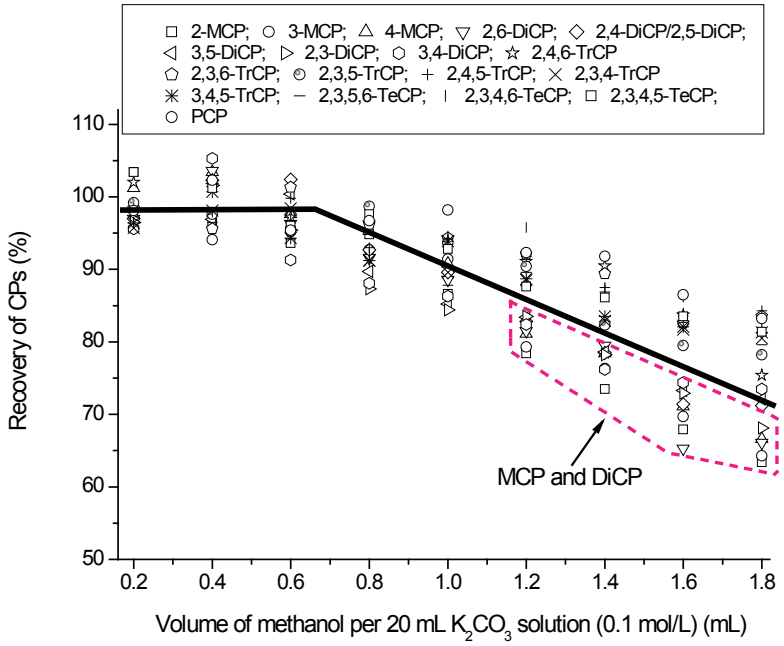

Figure 2. Effects of methanol on the acetyl derivatization efficiency

enough to remove the methanol in the mixture, leaving ~2 mL aqueous residues which was mainly from the 2 mL K_2CO_3 solution introduced prior to evaporation.

Cleanup with silica-gel cartridge

Purification of extracts to reduce matrix disturbance plays a key role for ensuring sensitivity of a method. It is especially important for samples containing complex organics (oils, polymers, dyestuff) such as leather. In this investigation, Silica-gel cartridges (0.5 g/6 mL) were selected according to the references,[15] and applied to clean up the n-hexane extracts isolated from the derivatization system. Tests showed that acetylated CPs (0-30 μg) in n-hexane extracts (~8 mL) are totally absorbed by the silica-gel frits. These acetylated CPs cannot be eluted during the washing process using ~5 mL of n-hexane for removing the disturbance, but they can be totally eluted by using a small volume (1 × 1 mL) of n-hexane/acetone (2:1, v/v) in the next elution operation. Thus, it could be considered that this cleanup technique can effectively reduce the matrix interference indicated by the circle box in Figure 3A, as well as pre-concentrate the acetylated CPs. The representative chromatogram of the extracts after silica-gel cleanup was shown in Figure 3B, which was in definite contrast with the original extracts presented in Figure 3A.

Method validation

The developed method was validated by determining the linearity, sensitivity and reproducibility. These characteristics were also compared with these obtained by ISO 17070-2015, as listed in Table III.

Linearity

The linearity of the method was determined by performing internal calibration curves with negative leather sample spiked with increasing concentrations of the 19 CPs standard mixtures, as well as a constant concentration of TCG internal standard. The spiking levels were at 0.2-10 mg/kg range for the 19 CPs. With the developed method, the linear range of CPs was at 0.4-10 mg/kg range with 1 mg/kg for TCG. While with ISO 17070-2015, the linear range of CPs was changed to 2-10 mg/kg range with 3 mg/kg for TCG, revealing a wider test concentration range of this improved procedure. In all cases, the relative responses of CPs were linear at the range of concentrations studied and the correlation coefficients (r^2) were all more than 0.993. However, based on the known amounts of CPs spiked, the range of the mean recoveries for the CPs were at 92-105% for this developed method, and 70-107% for ISO 17070-2015, as summarized in Table III. The data indicated a relatively low recovery for ISO 17070-2015, especially for TeCP and PCP with high boiling points.

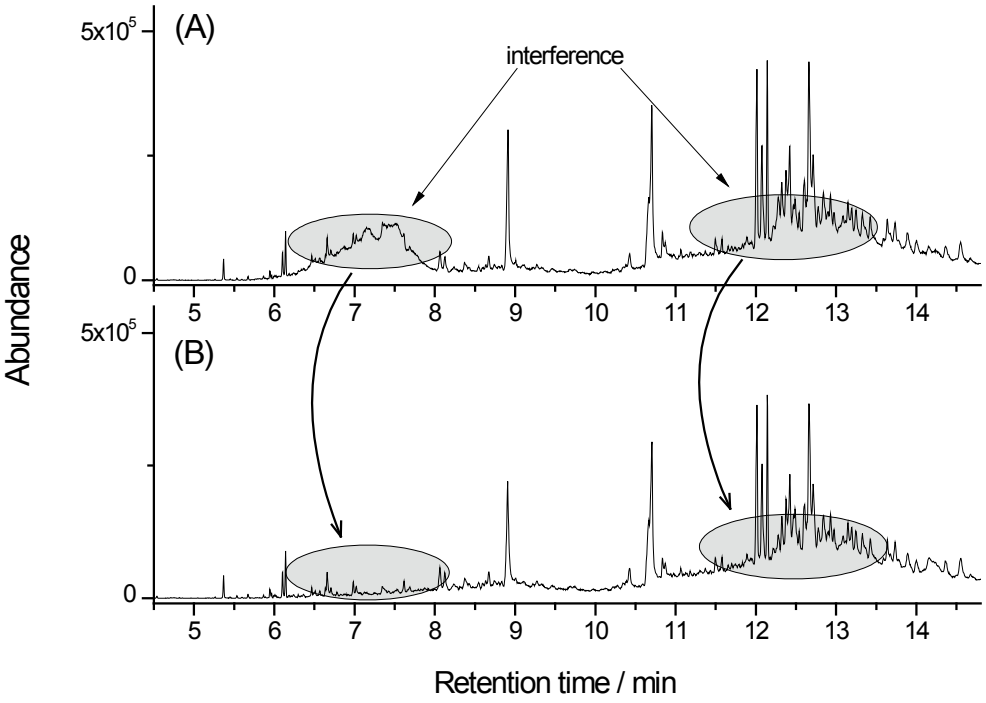

Figure 3. Total ion chromatograms of Sample extract **(A)** before and **(B)** after clean-up with Silica-gel cartridge

Reproducibility

Precision of the methods was assayed by repeatability studies for the peak area measurements of the negative samples with spiking level 1 mg/kg for each CPs (n = 6). In each case, reproducibility, expressed as relative standard deviation (RSD) based on the peak area of acetylated CPs, was obtained at 2.1-7.3% with this developed method, and 6.6-22.5% with ISO 17070-2015. The results indicated the robustness of the developed method due to the ultrasonic assisted extraction operation which is more reliable than steam distillation specified in ISO 17070-2015.

Limits of detection (LOD)

Negative samples spiked with mixed standards of CPs were used for the measurements of LOD, which were determined according to the instrumental detection limits, as well as weight of the sample and volume of the final extracts. LOD for each CPs determined by considering signal-to-noise of 3:1, ranged from 0.09 mg/kg (MCP) to 0.15 mg/kg (PCP) with the developed method, and from 0.5 mg/kg (MCP) to 1.5 mg/kg (PCP) with ISO 17070-2015 with GC-MS. This indicated that LODs obtained in the developed method were enhanced by a factor ranging between 5-10 with respect to ISO 17070-2015. These results demonstrated a greater sensitivity for this investigated method with ultrasonic extraction and SPE cleanup.

Comparison with ISO 17070-2015

Besides the contrasts in Table III, this developed procedure was further compared with ISO 17070-2015 with respect to extraction time, solvent use, handling, cost and batch test, as listed in Table IV. As discussed above, the major disadvantages of ISO 17070-2015 are long time extraction and failure for batch test. Although this proposed approach is relatively costly due to the silica-gel cartridge and solvent use, it provides a more sensitive and highly efficient way of testing CPs in leather, to reach the increasingly stringent requirements by European Union (EU) REACH regulation, National Compulsory Standards of China, or by Organizations such

Table III

Analytical parameters for this investigated procedure and ISO 17050-2015 with GC-MS

	Method of this work (GC-MS)				ISO 17070-2015 (GC-MS)			
Analyte	Pretreatment efficiency (%)[a)]	Linear range (mg/kg)	LOD (mg/kg)	RSD (%)[b)]	Pretreatment efficiency (%)[a)]	Linear range (mg/kg)	LOD (mg/kg)	RSD (%)[b)]
2-MCP	104.1	0.4-10	0.10	3.1	103.3	2-10	0.50	9.8
3-MCP	93.5	0.4-10	0.10	4.2	102.3	2-10	0.50	12.3
4-MCP	95.7	0.4-10	0.10	3.9	103.6	2-10	0.50	10.1
2,6-DiCP	97.4	0.4-10	0.10	4.3	93.2	2-10	0.50	6.6
2,4-DiCP/2,5-DiCP	95.7	0.4-10	0.10	2.9	106.6	2-10	0.50	7.5
3,5-DiCP	96.6	0.4-10	0.10	2.1	97.1	2-10	0.50	8.3
2,3-DiCP	99.1	0.4-10	0.10	6.7	102.6	2-10	0.60	9.2
3,4-DiCP	102.3	0.4-10	0.09	5.5	96.3	2-10	0.60	10.1
2,4,6-TrCP	98.2	0.4-10	0.10	3.9	93.9	2-10	0.60	13.5
2,3,6-TrCP	104.7	0.4-10	0.10	3.8	87.6	2-10	0.60	14.8
2,3,5-TrCP	95.3	0.4-10	0.10	5.5	83.5	2-10	0.60	16.7
2,4,5-TrCP	93.2	0.4-10	0.10	7.3	88.9	2-10	0.60	8.8
2,3,4-TrCP	102.5	0.4-10	0.09	6.1	86.4	2-10	0.60	12.1
3,4,5-TrCP	101.7	0.4-10	0.10	5.2	85.5	3-10	0.80	13.6
2,3,5,6-TeCP	93.2	0.5-10	0.15	4.6	73.3	4-10	1.2	15.9
2,3,4,6-TeCP	98.7	0.4-10	0.10	5.8	76.5	3-10	1.0	21.3
2,3,4,5-TeCP	92.6	0.4-10	0.10	6.7	77.9	3-10	1.0	22.5
PCP	93.5	0.5-10	0.15	6.4	70.1	4-10	1.5	19.7

[a)] Expressed as mean recoveries of n = 6.
[b)] Relative standard deviations.

Table IV

Comparison of the investigated method with ISO 17070-2015

Subjects	Method of this work	ISO 17070-2015
Extraction time	60-70 min	Up to 120 min
Batch test	Yes	No
Handling	Easy	Relatively easy
Solvent use	Methanol (40 mL), hexane (15 mL)	Hexane (40 mL)
Cost	Relatively costly	Relatively low

as OEKO-TEX® Association, AAFA (American Apparel & Footwear Association), among others.

This developed procedure was not further compared with other related methods as reported with ASE extraction and HPLC analysis,[8] because mono-chlorophenols were not included in the investigation, and the qualification assurance of method with HPLC-DAD is generally inferior to GC-MS. Although the quantification assurance with HPLC-DAD is superior to GC-MS, the internal calibration in this method can sufficiently make the test results reliable.

Analysis of real samples

The developed procedure was applied to determine the studied CPs in series of leather samples of different origin, including sheep-skin, goat-skin and cattle-hide (designed for garments, sofa, shoes and car seat) to confirm its applicability and feasibility. Figure 4 shows the typical GC-MS chromatograms obtained from cattle-hide sofa leather. The peak of 2,4,6-TrCP was easily identified by retention time and mass spectrum. The SIM detection (Figure 4B) allowed to further disregard the matrix observed in the total ion current (TIC) chromatograms (Figure 4A) and helped the quantitation using area response. This demonstrated the high selectivity and reliability of the method.

A total of 85 different samples comprising shoe leather (25), sofa leather (32), garment leather (23), car seat leather (5) were analyzed in this study. Six samples were found containing DiCP or TrCP, indicating the ratio of positive samples were about 7%. The other CPs were not detected. DiCP was detected in sheep-skin garment leather with concentration of 3.8 mg/kg, and TrCP was found in 5

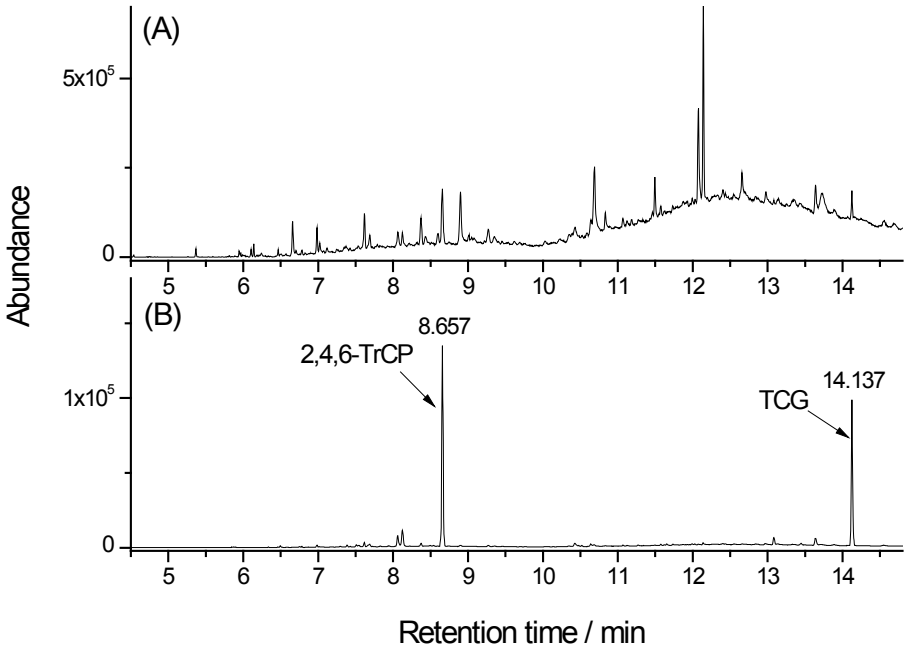

Figure 4. Total ion current **(A)** and selective ion monitoring **(B)** chromatograms of TrCP in the extracts of cattle-hide shoe leather (concentration of 0.88 mg/kg)

Table V

Levels of the CPs found in leather samples

Compounds	Number of positive Leather sample detected.				Conc. Range (mg/kg)
	Shoe	Sofa	Garment	Car seat	
2,4-DiCP	0	0	1	0	3.8
2,4,6-TrCP	1	2	2	0	0.88-14.6

samples with concentration range at 0.88-14.6 mg/kg, as shown in Table V. These results revealed the current levels of CPs in leather, and proved the occurrence of 2,4,6-TrCP in leather.

Conclusions

An improved method for detecting 19 chlorophenols in leather was investigated to solve the problems of time consuming and failure of batch test associated with ISO 17070-2015. The developed procedure consists of ultrasonic assisted extraction, acetyl derivatization and SPE cleanup followed by GC-MS detection. The ultrasonic assisted extraction with methanol containing formic acid (0.5%) presents high extraction efficiency, and this extraction technique is especially suitable for batch operation. The cleanup with silica-gel cartridge is effective to remove matrix disturbance, as well as enrich the analytes in the final solution for GC-MS analysis. The optimized method was validated and presented desirable recovery with lower RSD and higher sensitivity. The obtained results were compared with ISO 17070-2015, and indicated the method could be a good alternative to the steam distillation method in the analysis of 19 chlorophenols in leather. Finally, the procedure was applied to series of practical leather, and the preliminary results from 85 samples demonstrated current occurrence and concentrations of these targets, as well as the potential presence of 2,4,6-TrCP.

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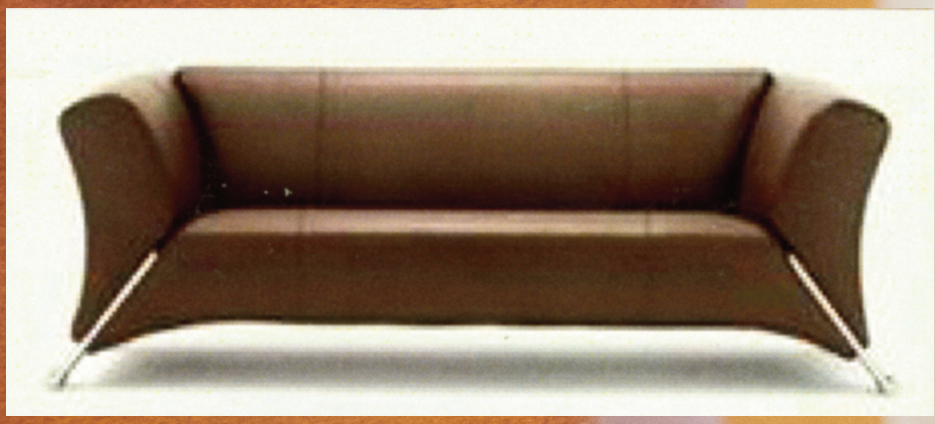

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Bindia Sahu, see JALCA **114**, 359, 2019

Gladstone C. Jayakumar, see *JALCA* 106, **68**, 2011

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Industry News

Tyson Foods to Sponsor International Union of Leather Technologists and Chemists Societies (IULTCS) Young Leather Scientist Grant in 2023

The Executive Committee of the IULTCS is delighted to announce that Tyson Foods will be joining the prestigious list of sponsors who support our young leather research community. This will be the 9th year that grants have been awarded and each year the research projects aim to address industry needs.

"Tyson Foods is proud to sponsor the 2023 IULTCS grant for young scientists," states Mike Larson, VP Hides & Tannery, Tyson Foods. "We're involved in the first step in the leather-making process and committed to producing high quality hides in a sustainable and responsible way. We're pleased to support innovative leather research to help the industry continue to learn, understand and advance."

IULTCS President Jean-Pierre Gualino expressed his thanks saying "We are very happy to have the support of Tyson Foods as they represent the starting point of leather production. Without good quality raw material we cannot produce good quality leather. We hope that we can attract young scientists who will direct their studies at understanding more fully, how best to utilise this valuable commodity in a sustainable way. We really appreciate the grant being provided."

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