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Antibacterial Activity of Sophorolipids Produced by Candida bombicola on Gram-positive and Gram-negative Bacteria Isolated from Salted Hides

by

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Abstract

Salted hides and soaked hides treated with certain antibacterial agents, may still contain different proteolytic and lipolytic Gram-positive and Gram-negative bacteria that affect the quality of leather adversely. The prevalence of bacteria resistant to antimicrobial agents in the leather industry has drawn attention of scientists to search new and effective antimicrobial agents. Examination of antimicrobial glycolipids such as sophorolipids for their effectiveness against proteolytic and lipolytic hide-degrading microorganisms, may offer important information. Hence, we describe a research that evaluates the susceptibility of various hide-degrading bacteria to sophorolipids. These extracellular glycolipids were produced by fermentation using Candida bombicola ATCC 22214. Palmitic acid, stearic acid, and oleic acid were used respectively to produce SL-p, SL-s, and SL-o. The minimal inhibitory concentrations (MICs) of SL-p, SL-s, and SL-o against Gram-positive endospore-forming bacteria (Bacillus licheniformis, B. pumilus and B. mycoides), Gram-positive bacteria (Enterococcus faecalis, Aerococcus viridans, Staphylococcus xylosus, S. cohnii and S. equorum), Gram-negative bacteria (Pseudomonas luteola, Enterobacter cloacae, E. sakazakii and Vibrio fluvialis), and mixed culture of these isolates were examined using an agar dilution method. The MICs of both SL-p and SL-o against the test bacteria were determined as 19.5 µg/mL, with an exception that E. cloacae was inhibited by SL-o at a MIC of 9.76 µg/mL. Although MICs of SL-p did not change against the test bacteria, the MICs of SL-s (ranging from 4.88 µg/mL to 19.5 µg/mL) changed according to species of the test bacteria. The MICs of SL-s were found to be 4.88 µg/mL against B. licheniformis, B. pumilus, P. luteola, S. xylosus and B. mycoides. The MICs of SL-p, SL-s, and SL-o against the mixed bacterial culture were detected as the same (19.5 µg/mL). In conclusion, SL-p, SL-s, and SL-o inhibited the growth of 12 different hide bacteria and their mixed culture, and have broad-spectrum activity. The results obtained in the present study may be valuable for the development of SL-p, SL-s, and SL-o as antimicrobial surfactants in the preservation and soaking processes of hides and skins.

Introduction

The leather industry is a significant commercial sector contributing to the world economy. Almost 23 billion square feet of leather is produced annually, with an estimated total value of more than 100 billion USD.1-3 Ineffective preservation techniques therefore lead to important substantial financial losses in the industry and World economy. Raw hides and skins are preserved with salt, boric acid and antibacterial agents such as didecyldimethylammonium chloride, isothiazolinone, glutaraldehyde, 2-bromo-2-nitropropane-1,3-diol, naphthalene and 1,2-dichlorobenzene, trichloro-s-triazinetrione and sodium sulphate, methylene bis(thiocyanate) and 2-(thiocyanomethylthio)benzothiazole to prevent bacterial growth and subsequent damage on hides or skins during storage and transportation.4-6 Despite preservation of hides or skins with salt, salted hides/skins may still contain a wide variety of Gram-positive and Gram-negative bacteria originating from faeces, soil, air, water, manure, feeds, and extraneous filth.7,8 We previously reported that a diverse range of Gram-positive bacteria (12 genera, 47 different species 396 isolates) and Gram-negative bacteria (21 genera, 46 different species and 256 isolates) were isolated from 10 salted hides belonging to different

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.
Due to the economic importance of hides and skins, in the present study we wanted to examine the antibacterial effect of sophorolipids against different species of bacteria isolated from the salted hides.

Sophorolipid (SL) is a class of microbial glycolipids that are synthesized by certain yeast species. Among the reported SL-synthesizing organisms, C. bombicola is by far the most studied yeast in term of SL production chiefly because of the ability to achieve high production yields. C. bombicola requires the simultaneous presence of carbohydrate and fatty acid substrates in order to produce large quantities of SLs. The most commonly used fermentation co-substrates for SL production by C. bombicola are glucose and oleic acid, which leads to predominantly the synthesis of SLs having the structures of 17-L-[2’-O-β-glucopyranosyl-β-D-glucopyranosyl]-oxy]-9-octadecenoic acid 1’-4’-lactone 6’,6’-diacetate (SL-1; Figure 1) and its free-acid form (SL-1A; Figure 1).

Ashby et al. (2008) subsequently showed that by substituting palmitic acid or stearic acid for the oleic acid in the fermentation, the fatty acid moiety of the SLs can be altered. Thus, the use of palmitic acid substrate resulted in SLs containing 15- and 16-hydroxyhexadecanoic acids as the hydrophobic moiety, while stearic acid yielded SLs having 17-hydroxydecanoic acid as the hydrophobic moiety. Since SLs are amphiphilic compounds, they possess good surface active properties that make them potentially useful as biobased surfactants in many industrial applications. More importantly, SL was found to have strong antimicrobial activity either in solution or when embedded in films. Based on the surface-active and antimicrobial properties of SLs, Ramos et al. (2011, 2012) had studied the application of these molecules in the treatment and preservation of animal hides. SL is advantageous over the traditional surfactants and antimicrobial agents. Because SLs are biosynthesized through microbial fermentation process using renewable feedstocks, it is a preferred “green” biobased material that can be manufactured in a sustainable manner. Furthermore, unlike commonly used antimicrobial agents in medical field against which many bacteria have developed resistance, SL is an emerging antimicrobial agents that are potentially effective against many antibiotic-resistant bacteria.

Hence, the objective of the present study was to determine MICs of SL-Glu/Oleic, SL-Glu/Stearic and SL-Glu/Palmatic against B. licheniformis, B. pumilus, B. mycoides, E. faecium, S. xylosus, A. viridans, S. cohnii, S. equorum, P. luteola, E. cloacae, V. fluvialis, E. sakazakii, and mixed culture of these isolates. These microorganisms were isolated from salted hides in previous studies.

**Experimental**

**Test Microorganisms**

C. bombicola ATCC 22214 was acquired from American Type Culture Collection (Manassas, VA). Endospore-forming Gram-positive bacteria (B. licheniformis, B. pumilus and B. mycoides), Gram-positive bacteria (E. faecium, S. xylosus, A. viridans, S. cohnii and S. equorum), Gram-negative bacteria (P. luteola, E. cloacae, V. fluvialis and E. sakazakii), and mixed culture of these isolates were used as test bacteria in this study. These bacteria were obtained from the culture collections of Division of Plant Diseases and Microbiology, Department of Biology, Faculty of Arts and Sciences, Marmara University. Test microorganisms were isolated from salted hides and identified using API test kits (BioMérieux, Inc, France) in the previous studies.

![Figure 1](image-url) Structures of sophorolipids predominantly produced by C. bombicola grown on glucose+oleic acid. The 17-L-[2’-O-β-glucopyranosyl-β-D-glucopyranosyl]-oxy]-9-octadecenoic acid 1’-4’-lactone 6’,6’-diacetate (SL-1) and its free-acid form (SL-1A).
Production of Sophorolipids

Sophorolipids (SLs) were produced by fermentation using C. bombicola ATCC 22214 as the producing organism, and glucose + fatty acid of choice as co-substrates according to a previously described protocol. For the present study, we used palmitic acid, stearic acid, and oleic acid to produce SL-p, SL-s, and SL-o, respectively. Briefly, fermentations were performed in a 12-L capacity vessel containing 10 L of culture medium in a bench-top fermenter (Bioflo 3000 Batch/Continuous Bioreactor, New Brunswick, NJ). The basal Candida Growth Media (CGM) consists of glucose (10%, w/v), yeast extract (1%, w/v), urea (0.1%, w/v). An amount of the appropriate fatty acid co-substrate was then added to the basal CGM to attain a concentration of 2% (w/v). The 10-L CGM+fatty acid medium was inoculated with 50-mL C. bombicola inoculum culture that had been previously prepared in bulk and stored in -80°C freezer in the form of 15% (v/v) glycerol-containing stocks. All fermentations were performed using the following settings: temperature, 26°C; impeller speed, 700 rpm; aeration rate, 2 L/min; and no pH control. Additional substrates were added on day 2 [glucose, to 7.5% (w/v); fatty acid, to 2% (w/v)] and day 5 [fatty acid only, to 1% (w/v)]. On day 7, the culture as a whole (i.e., cells and medium) was distributed in several 2-L capacity lyophilization jars and subsequently subjected to lyophilization. The lyophilized dry-culture was transferred to Erlenmeyer flasks containing ethyl acetate in excess to extract the SLs. Extraction was carried out at room temperature for 2 days with constant shaking in a shaker-incubator. The extraction mixture was filtered through Whatman No. 2 filter paper, and the solids were returned to the Erlenmeyer flasks for two additional extractions. The combined ethyl acetate extracts containing the SLs were concentrated by evaporation and precipitated in 1-L aliquots of hexane to obtain pure SLs. The SLs were recovered on a Whatman No. 2 filter paper and vacuum-dried in a desiccator. The structures of the SLs were confirmed by LC-MS as described previously.

Agar Dilution Method

Firstly, the test microorganisms were streaked and grown on Mueller Hinton Agar (MHA) (Merck, Darmstadt, Germany) at 37°C for 24 h. Overnight cultures of these isolates were prepared by inoculating 10 mL Mueller Hinton Broth (MHB) with 2-3 colonies of each test microorganism taken from MHA. The inoculated broths were incubated 12 h at 37°C. A 0.5 McFarland standard was used to adjust each bacterial suspension to a density equivalent to 10⁶ CFU/mL. Then, each bacterial suspension of test microorganisms was diluted in 0.85% saline to obtain 10⁷ CFU/mL. In addition, the mixed culture of the test isolates was prepared from these bacterial suspensions. Then, each of the test agents (SL-o, SL-s and SL-p) was separately dissolved in 70% ethanol to a final concentration of 10.000 µg/mL. Series of two-fold dilutions of these test agents, ranging from 10.000 µg/mL to 0.076 µg/mL, were prepared in MHA containing 3% NaCl. One mL volumes of 17 different concentrations of SL-p, SL-s, and SL-o solutions were separately added to 19 mL volumes of molten MHA. Next, these agar media were mixed thoroughly and poured into sterile petri dishes. One µL (an inoculum of 10⁴ CFU/spot) of diluted bacterial suspension of each test isolate and their mixed culture was separately transferred to agar plates containing 17 different concentrations (10.000 µg/mL, 5000 µg/mL, 2500 µg/mL, 1250 µg/mL, 625 µg/mL, 312.5 µg/mL, 156 µg/mL, 78 µg/mL, 39 µg/mL, 19.5 µg/mL, 9.75 µg/mL, 4.88 µg/mL, 2.44 µg/mL, 1.22 µg/mL, 0.61 µg/mL, 0.30 µg/mL, 0.15 µg/mL, 0.076 µg/mL) of the test agents. SL-p, SL-s, and SL-o free control media were also used in all experiments. Later, the plates were incubated at 37°C for 18 h, and MICs of antibacterial agents against the test bacteria were determined. Inhibitory effects of 70% ethanol on the test bacteria and mixed culture were also examined on MHA containing the same volume of ethanol concentrations as other agar media containing antimicrobial agents. All test bacteria and the mixed culture grew in the series of ethanol found in MHA. Dilutions of three antimicrobial agents were prepared according to the method explained in the EUCAST Definitive Document E. Def 3.1 (2000).

Results and Discussion

Proteolytic and lipolytic microorganisms on the salted hides and skins may provoke hair slip, discoloration, serious grain peeling, fiber disintegration, odor, looseness, weakness and holes in leather. Research has shown that among the microorganisms found in animal hides and skins, B. licheniformis, B. pumilus, E. faecium, P. luteola, A. viridans, B. mycoides and S. cohnii were shown to exhibit both protease and lipase activities. Although E. cloacae, V. fluvialis and S. equorum were protease positive and lipase negative, S. xylosus and E. sakazakii were protease negative but lipase positive.

Our test isolates in the present study were therefore the commonly found microorganisms on the salted hides that cover different Gram-positive and Gram-negative, proteolytic, and lipolytic isolates. While B. licheniformis and B. pumilus were isolated from 9 salted hide samples, B. mycoides were isolated from 4 hide samples. Both E. faecium and A. viridans were isolated from 10 salted hides, but S. cohnii and S. xylosus were isolated respectively from 8 and 7 salted hide samples. Although E. cloacae and P. luteola were isolated from 10 salted cured hide samples, V. fluvialis and E. sakazakii were found on 8 and 6 salted hide samples, respectively.

Despite the salt curing process of hides, a wide variety of genera of Aerococcus, Aneurinibacillus, Bacillus, Brevibacillus, Enterococcus, Geobacillus, Kocuria, Lactococcus, Paenibacillus, Streptococcus, Staphylococcus and Virgibacillus belonging to
Gram-positive bacteria and genera of Acinetobacter, Aeromonas, Alcaligenes, Burkholderia, Citrobacter, Comamonas, Edwardsiella, Enterobacter, Escherichia, Hafnia, Klebsiella, Mannheimia, Pasteurella, Proteus, Pseudomonas, Salmonella, Serratia, Sphingomonas, Stenotrophomonas, Vibrio and Yersinia belonging to Gram-negative bacteria were isolated from ten salted hides. In addition, a high percentage of these isolates showed proteolytic and lipolytic activities.

Our test microorganisms such as *E. sakazakii*, *E. cloacae*, *P. luteola*, *E. faecium*, *A. viridans*, *S. xylosus*, and *S. cohnii* were also isolated from main soak liquor treated with antibacterial agent containing 0.8 g/L of didecyldimethylammonium chloride. These studies proved that proteolytic and lipolytic bacterial populations on salted and soaked hides cannot be effectively prevented by commonly used antibacterial agents. Microbial spoilage of hide usually results from the failure to eliminate these microorganisms during salt and brine curing or soaking processes. Careful selection of antimicrobial agents facilitates high quality leather production. Hence, in this study we concentrated on finding an antibacterial agent that may prove potent against proteolytic and lipolytic hide bacteria.

Antibacterial activities of SL-p, SL-s and SL-o against the test bacteria are presented in Table I. The MICs were determined as the lowest concentration of antibacterial inhibiting the visible growth of each microorganism on the agar media containing 3% NaCl. The MICs of SL-p and SL-o against all test bacteria and their mixed culture were found as 19.5 µg/mL, except *E. cloacae*. Although the MIC of SL-p against *E. cloacae* was 19.5 µg/mL, the MIC of SL-o against this test microorganism was 9.76 µg/mL. The MICs of SL-s against each test bacteria were lower than those of SL-p and SL-o, with the exception that *E. cloacae* and *S. equorum* were inhibited by SL-o at MICs of 9.76 µg/mL and 19.5 µg/mL, respectively. The MICs of SL-s against *B. licheniformis*, *B. subtilis*, *P. luteola*, *S. xylosus* and *B. mycoides* were found to be 4.88 µg/mL, while the MICs of SL-s against *E. faecium*, *E. cloacae*, *V. fluvialis*, *A. viridans*, *S. cohnii* and *E. sakazakii* were 9.76 µg/mL. However, the MICs of SL-s against both *S. equorum* and the mixed culture (19.5 µg/mL) were found higher than those of the other test bacteria. Among the test bacteria, the highest MIC values belonging to three sophorolipids were detected against *S. equorum* (19.5 µg/mL). The MICs of SL-p, SL-s and SL-o against the mixed culture were found to be same (19.5 µg/mL) (Table I).

These three sophorolipids were fairly active against Gram-positive endospore-forming bacteria, Gram-positive bacteria, Gram-negative bacteria and their mixed culture. In comparison, Shah et. al. (2007) reported that SLs were more effective against Gram-positive bacteria than Gram-negative bacteria. In our study, the variance in antibacterial activity of sophorolipids between Gram-positive and Gram-negative bacteria was negligible except for *S. equorum* (Table I). Although further studies are needed to find the exact reason(s), the precise culture environment such as the presence of NaCl in our study might be a factor. In the leather processing such as brine curing ([25% (w/v)]) and soaking [3% (w/v)], NaCl is always added into these processes. Our results showed that SL-p, SL-s and SL-o were highly effective to inhibit proteolytic and lipolytic hide bacteria even in the test media containing organic substances and 3% NaCl (w/v).

Our research results were similar to the findings of Hommel et al. (1994) Lactonic sophorolipids obtained from *Candida apicola* IMET 43747 were found effective against both Gram-positive bacteria (*Azotobacter chroococcum*, *B. subtilis*, *Micrococcus luteus*, *Mycobacterium rubrum* and *S. aureus*) and Gram-negative bacteria (*Escherichia coli*, *Proteus vulgaris* and *P. aeruginosa*, *Serratia marcescens*). MICs of lactonic sophorolipids against *A. chroococcum*, *B. subtilis*, *M. luteus*, *M. rubrum* and *S. aureus* were respectively 1.95 µg/mL, 0.12 µg/mL, 0.48 µg/mL, 0.12 µg/mL and >800 µg/mL, while MICs of lactonic sophorolipids against *E. coli*, *P. vulgaris*, *P. aeruginosa* and *S. marcescens* were 7.8 µg/mL, >313 µg/mL, 7.8 µg/mL and 1.95 µg/mL, respectively.

It has been known that bacterial cells have diverse abilities to survive and grow in the presence of antibacterial agents. Therefore, it is usually difficult to inactivate the mixed cultures of different species of bacteria using antibacterial agents. Our previous studies showed that total bacterial counts in 34 hide-soak liquors treated with different antibacterial agents were between 10^3-10^8 CFU/mL. Total proteolytic and lipolytic bacterial counts in these liquors were between 10^8-10^9 CFU/mL. In another study, although the antibacterial agent containing didecyldimethylammonium chloride (0.4 g/L) was used in soaking process of hides for 8 h, we found total counts of all bacteria, proteolytic, and lipolytic bacteria to be 1x10^8 CFU/mL, 1x10^7 CFU/mL and 1x10^6 CFU/mL, respectively. A total of 26 different bacterial species belonging to genera of *Enterobacter*, *Pseudomonas*, *Enterococcus*, *Lactococcus*, *Aerococcus*, *Vibrio*, *Kocuria*, *Staphylococcus* and *Micrococcus* were isolated from main soak liquors of hides treated with antibacterial agent containing didecyldimethylammonium chloride (0.8 g/L).

Shah et al., (2007) compared the antibacterial activities of sophorolipids obtained from *C. bombicola* grown in medium containing glucose, fructose, xylose, ribose, lactose, mannose, arabinose, or galactose against *Rhodococcus erythropolis*, *B. subtilis*, *S. epidermidis*, *Streptococcusagalactiae*, *Moraxella* sp., *P. putida*, *E. aerogenes* and *E. coli*. These researchers observed that all sophorolipids obtained from different sugar-containing media were effective against the test bacteria, but they differ in the levels of their activities against the tested organisms. SLs obtained from arabinose-containing medium were found to be...
more effective against *R. erythropolis, B. subtilis, S. agalactiae,* and *Moraxella* sp. than SLs obtained from glucose-containing medium. On the other hand, while sophorolipid obtained from arabinose medium did not prevent the growth of *E. coli,* sophorolipid obtained from lactose-containing medium were found to be the most effective against *B. subtilis.*

Kitamoto et al. (1993) compared antimicrobial properties of two kinds of microbial glycolipids, i.e., mannosyIerythritol lipids (MEL-A and B), produced by *Candida antarctica T-34,* against *B. subtilis, M. luteus, M. rhodochrous, S. aureus, P. aeruginosa, P. rivoflavina and E. coli.* Unlike our study showing the antimicrobial activity of SLs against both the Gram-positive and Gram-negative test bacteria, their results showed that both MEL-A and B were fairly effective against Gram-positive bacteria but only mildly active against Gram-negative bacteria.

Sleiman et al. (2009) reported that the MICs of the ethyl ester diacetate derivative of sophorolipid against *E. coli, S. aureus, Klebsiella pneumoniae, P. aeruginosa, S. pneumoniae,* and *Proteus mirabilis* were >128µg/mL, which is higher than the values we observed in our study. Interestingly, Joshi-Navare and Prabhune (2013) determined that MICs of sophorolipid and tetracycline against *S. aureus* as 400 µg/mL and 150 µg/mL, respectively. They further found that a mixture of sophorolipid (300 µg/mL) and tetracycline (15 µg/mL) was effective to inhibit this microorganism in 6 h. It was also observed that this mixture formed pores and therefore caused damage to the bacterial cell membrane. Their results showed that a synergistic effect could result in the effective use of SLs and another antibiotic at low concentrations to achieve a similar or better antibacterial end-point.

In this study, we have successfully demonstrated that the "green" biosurfactant sophorolipids produced by *Candida bombicola* have antibacterial activity against a broad spectrum of bacteria found in hides. This opens up many research areas for the use of sophorolipids in the hides and leather industries. For example, aside from the SL-p, SL-s, and SL-o already tested here, we could compare their antibacterial activities with the sophorolipid produced by *Rhodotorula bogoriensis,* which has long hydroxy fatty acid chain of 22 carbons. Another area of important research is to determine the antimicrobial activities of sophorolipids against the extremely halophilic archaea often found on salt cured hides and skins ("red heat" condition). Furthermore, since sophorolipids are biosurfactants and therefore have washing/cleaning property, they can be used to develop washing and/or soaking bath that not only clean hides, skins, or leather but also help kill the bacteria. In summary, this study opens up exciting possibilities for us to further pursue development of applications of these "green" and antimicrobial biosurfactants sophorolipids in the hides and leather industries.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Minimum inhibitory concentrations of SL-p, SL-s and SL-o against 12 different hide bacteria and their mixed culture (µg/mL).</th>
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<tr>
<td></td>
<td><em>Bacillus licheniformis</em></td>
</tr>
<tr>
<td>SL-p</td>
<td>19.5</td>
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<tr>
<td>SL-o</td>
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SL, Sophorolipids, p, Palmitic; o, Oleic; s, Stearic
Conclusion

This is the first study that determines the MICs of SL-p, SL-s, and SL-o against the hide isolates (B. licheniformis, B. pumilus, B. mycoides, E. faecium, S. xylosus, A. viridans, S. cohnii, S. equorum, P. luteola, E. cloacae, V. fluvialis, E. sakazakii and the mixed culture of all isolates) on MHA containing 3% (w/v) NaCl. The present study affirms that SL-p, SL-s, and SL-o inhibit the growth of Gram-positive endospore-forming bacteria, Gram-positive bacteria, Gram-negative bacteria and their mixed culture on MHA. MIC values of SL-p, SL-s, and SL-o against the mixed culture were found as 19.5 µg/mL. It was also determined that SL-p, SL-s, and SL-o have broad-spectrum activity. Sophorolipids are environmentally friendly, readily biodegradable, and ecologically benign to aquatic life. Therefore, these antimicrobial glycolipids may be used by the leather industry in preservation and soaking processes of hides and skins to prevent proteolytic and lipolytic activities.

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References

Modified Smart Collagen Biomaterials for Pharmacy and Adhesive Applications

by

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Abstract

Collagen has widespread use for preparation of cell cultures, dermal cosmetics, food and medicines, while achieving biocompatibility with the biological environment. Unmodified native collagen is relatively difficult to process for intended applications. This work presents potential application possibilities of modified collagen biopolymer for pharmacy and adhesive applications.

Firstly, the application possibilities of collagen in the system of controlled drug release were verified. Dissolution profile of the matrix Ambroxol hydrochloride and Venlafaxine confirmed potential of crosslinked and plasticized collagen as a pharmaceutical agent for the solid medicinal product. This product was prepared by direct compression with controlled drug release within the 8-11 hours with almost zero-order kinetics.

Secondly, low temperature plasma was applied to achieve the biocompatibility and to modify the film surface of the collagen type I. Collagen film was treated in two plasma environments; low temperature plasma N2/H2 was used for grafting the amino groups and CO2 plasma ensured grafting the carboxyl groups. The surface functional groups of collagen were applied in further reactions (e.g., antimicrobial pretreatment with strong biocide properties). Functionalized collagen film provided stronger adhesion and compatibility. Prepared non-formaldehyde collagen thermoplastic adhesive was tested for the technical applications. The results have shown that the collagen adhesive bond gained high strength, flexibility and required strong gluing, e.g. books or wood veneered materials.

Introduction

In recent years, the environmental processing of secondary raw materials from various industrial productions to obtain products with high-added value has gained very high attention. Fibril proteins of skin, mainly collagen and keratin are perspective biopolymers for biomedicine, pharmacy, cosmetics and other technical applications (e.g. bonding). Food and leather industry are among the biggest polluters of the environment. Biopolymer waste from manufacturing of meat and skins is not recovered complexly, and remains as the environmental load. Other wastes such as chromium shavings, limed fleshings, animal fat, wool, hair, feathers, keratin hydrolysate from skins processing contain a mixture of various substances of the chemical and biological origin. These wastes can be on one hand inert and biodegradable, and on the other hand possibly hazardous. They contribute to global environmental pollution, and therefore, the main attention focuses on their further processing and use. The last couple of years are characterized by the rapid development of advanced technologies aimed specifically on new applications, quality parameters, and pricing of new biologically active natural substances.1-5

Almost all secondary raw materials described above contain collagen protein because this biopolymer is widespread throughout the animal kingdom. Collagen protein is one of the technically most important proteins. Collagen is the main component of connective tissues where affects their proper function-ability and determines mechanical properties. This is due to its specific structure and a high degree of the internal molecule organization.6

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Collagen represents 25-30% of all proteins in the body and represents the main organic component of skin, bone and cartilage. Moreover, it is an important part of blood vessel walls, basement membrane and corneas. It also has the support and protection functions and belongs to the key life protein processes in a healthy organism mainly as a component of the extracellular matrix proteins.\textsuperscript{7,8}

Collagen with other proteins has the amphoteric polyelectrolyte nature that causes ionic reactions to occur depending on the pH of the environment. This means that some side chain groups are ionized in the alkaline and some in the acid pH. The collagen molecule charge changes with pH. It has a positive charge in strong acids and a negative charge in strong alkalai. The isoelectric point of the native collagen occurs at pH 7 and under slight chemical affect could be changed within the range of 4.5-8.0. Most of the physical and chemical properties show the extreme values in this range.\textsuperscript{9}

Collagen is one of the transitional colloidal system – gel from the physical and chemical point of view. Its most important feature is the swelling ability. After immersion in water, the collagen fiber partially swells (exothermic process). This process is accompanied by a change of the fiber volume, length and flexibility. Some water in the swollen collagen can be mechanically removed, the other water type is moisturizing that is colloidally bounded and removable only by drying.\textsuperscript{10}

Numerous articles have been published about applications of collagen as a drug carrier, but only a few collagen-based systems are currently available or get into clinical testing. Therefore, there is a continuing effort to improve collagen products and find new processing ways and use. Advantages of the collagen application as a drug carrier are following:

- good biocompatibility and well characterized, low antigenic properties,
- degradation to physiologically well tolerated compounds,
- individual preparation steps take place in the aqueous environment,
- improved cell penetration and wound healing.

However, the collagen application has also some disadvantages:

- the high cost of pure collagen type 1 preparation,
- the composition variability of the insulated collagen (crosslinking density, fiber size, the trace contamination, etc.),
- hydrophilicity, which results in the higher swelling and more rapid drug release compared to synthetic polymers,
- variability in the rate of the enzymatic degradation compared to hydrolytic decomposition,
- complicated manipulation.

Benefits of collagen applications will lead to the intensive future research and new product development in tissue engineering. The interest in collagen medical applications in the formulation of dosage drug forms has been widely investigated.\textsuperscript{11-14}

In order to apply collagen in pharmacy or as an industrial adhesive, it needs to be modified by, for example, crosslinking or plasticizing. The main disadvantage of the chemical agents used in the collagen crosslinking for pharmaceutical or cosmetics applications is a potential toxic effect of residues. Therefore, the research focuses on the alternative physical methods such as dry heat, UV and gamma-radiation. The increase of denaturation temperature and resistance against degradation by collagenases is achieved by collagen exposure to dry heat and UV-radiation with the wavelength of 254 nm. However, partial denaturation is expected during this process. When using the heat, reduction of the actual water content to the minimum before preparations is very important because even a small amount of moisture can cause collapse of the original structure and lead to proteolysis.\textsuperscript{15}

Little dehydration induces the formation of amides and esterification. Dry heat sterilization causes partial denaturation and formation of crosslinked bonds. Although, tensile strength can be achieved by dry heat, degradation in vivo can be substantially changed. A combination of degradation and crosslinking enables non-specific enzymes to attack and solubilize the crosslinked fragments. The sensitivity of collagen to trypsin was increased at heat treatment, while degradation by pepsin and lysosomal enzymes was reduced.\textsuperscript{16}

Crosslinking under UV – radiation is initiated by free radicals, which are forming on aromatic residues of amino acids. They can create only a limited crosslink density due to the low content of tyrosine and phenylalanine in collagen.\textsuperscript{17} Therefore, the exposure doses are short and the maximum value of crosslinking is rapidly reached. The mechanical strength is also increased under UV – radiation.

Collagen is also possible to use as a modifier of polyvinyl alcohol intended for thermoplastic processing to blow extruded films. Proteins such as wheat gluten, corn zein, soy protein, myofibrillar proteins, and whey proteins have been successfully formed into films using thermoplastic processes such as compression molding and extrusion. Thermoplastic processing can result in a highly efficient manufacturing method with commercial potential for large-scale production of edible films due to the low
moisture levels, high temperatures, and short times used. Another way of modification of the properties and process-ability of collagen is plasticizing and compounding with suitable types of polymers.  

Another way of modification of the properties and process-ability of collagen is plasticizing and compounding with suitable types of polymers. The possibility of usage of the biodegradable polymer film of ethylene-vinyl acetate (EVAc) and modified protein as a bio-based and biodegradable hot-melt adhesive has also been studied.  

Thermo-oxidative stability of different materials and biopolymers was tested by differential scanning calorimetry (DSC). The method is based on determination of the end of induction period, or the beginning of the main oxidation process. Films of EVAc and collagen copolymers have been modified with plasma, and the influence of low-temperature atmospheric discharge plasma on the change of surface properties has been studied. The chemical changes of collagen films modified by plasma were analyzed using Fourier Transform Infra Red – Attenuated Total Reflection (FTIR-ATR) spectroscopy.  

The aim of the experiments is focused on verification of the possibility of collagen application not only as the potential binder (adhesive) of tablets, but also as an original pharmaceutic excipient based on physically crosslinked collagen for the purposes of the formulation, manufacturing and other usage of the solid dosage forms with the controlled drug release. Matrix tablets containing drug, Ambroxol hydrochloride and Venlafaxine are to be prepared by direct pressing. The technical applications are focused on the preparation of non-formaldehyde collagen thermoplastic adhesive with required quality for gluing of books or wood veneered materials.

**Experimental**

Experimental investigation was carried out using the published data about collagen as the perspective biomaterial for drugs (in various dosage forms) and transport systems related to solid medicament forms. Used measurement methods were performed according to European Pharmacopoeia.

**Material and Methods**

*Used Drug and Excipients:*

**Ambroxol hydrochloride** – Antitussive; white or yellowish crystalline powder, soluble in methanol and water, practically insoluble in methylene chloride, pH = 4.5-6.0, heavy metals max. 20 ppm.

**Venlafaxine hydrochloride** – antidepressant, racemic mixture, easily soluble in water and methanol, soluble in absolute ethanol, low solubility, practically insoluble in acetone, molar mass 277.402 g/mol, melting point 215-217°C.

**Collagen BS** – prepared from porcine skins, crosslinked, plasticized, the particle distribution of 45 Mesh - max. 0.355 mm prior the crosslinking and plasticization, pH 5.1, Bloom 220 at the concentration of 6.67% and temperature of 10°C – subsequently crosslinked and plasticized.

**Collagen HS** – prepared from bovine hides, heat-treated, plasticized, particle size of 45 Mesh - max. 0.355 mm prior the crosslinking and plasticization, pH 5.5, Bloom 176 at the concentration of 6.67% and temperature of 10°C – subsequently crosslinked and plasticized.

**Collagen K 12** – characterized as a collagen heat-treated powder. The sample is a mixture of particles less than 200 Mesh - 0.074 mm and the particles distribution of 60 Mesh - max. 0.25 mm. Collagen K 12 is light brown powder, plasticized, compressible, with the ability to produce matrix tablets.

**Ethylene Vinyl Acetate** – Evatane® 1080 VN 5 Ethylene Vinyl Acetate

**Methocel K 100 McR** – cellulose ethers, water-soluble polymers derived from cellulose, viscosity 75 000-140 000 cP, hydroxypropoxyl (HP) substitution 9.5-11.5%, methoxyl (MeO) substitution 22.0-24.0%, particle size through 100 mesh – 0.125 mm min 90%.

**Measurement Methods:**

*Tablet Pressing*

Tablets were pressed on universal press (VEB Elmo-Thurm), under the pressure of 1000 – 1500 kPa, with tablet diameter of 12 mm.

*Mechanical Testing of Tablets*

The tablet resistance test to fracture determines the force required to break the tablet under defined conditions. The used device (Schleuniger 2E Hardness Tester) was calibrated according to European Pharmacopoeia.
The test of friability of uncoated tablets was carried out on the tablet friability tester (Erweka TA) under defined conditions. The permitted weight loss of the tablet after exposure on friability test is 1%.

The test of mass uniformity was carried out on the laboratory weight (Mettler AE 163).

**Disintegration of Tablets**
This test investigates if tablets are disintegrated within the determined time, in liquid medium and in determined experimental conditions. Tablets are placed in a basket below the level of water at the temperature of 37 ± 2°C and the basket is rotated at 30 rpm. The end of the test is when no residue of the tablet remains in a basket. If the tablet does not disintegrate within 15 minutes, it is marked as non-compliant.

**Dissolution of Tablets**
The test quantifies the release rate of active substances from tablets and it was done under the following conditions:

- **dissolution medium:** purified water,
- **media volume:** 900 ml,
- **media temperature:** 37°C ± 0.5°C,
- **basket rotation:** 100 per min.

Drug concentration was determined spectrophotometrically (Philips PU 8620 UV/VIS/NIR) at a wavelength of 246 nm, and the flow cell of 10 mm.

**Surface and Adhesion Properties of EV Ac Films with Collagen**
The contact angles were determined by measurements of five test liquids with different polarity. Eight replicated measurements were used to test the contact angle for each testing liquid. The test liquids drops (V = 5 μl) were placed on the surface of the film samples with a micropipette (Biohit, Finland). The contact angle measurements were performed using a professional device equipped with a web camera (Advex, Czech Republic) and the appropriate software.

The shear strength of adhesion joints was tested on dynamometer (Instron 4301, USA) using overlapped sheets of aluminum with 20×10 mm dimensions of overlapping.

**Plasma Modification**
The surface of the film samples was treated under air, N_2/H_2 and CO_2 as the processing gases. The atmospheric Diffusive Coplanar Surface Barrier Discharge (DCSBD) plasma was applied in a laboratory-scale plasma system operated at a reduced pressure of 80 Pa. The system consists of two circular brass electrodes with dimensions of 240 mm in diameter and 10 mm in thickness. They were placed in a parallel orientation, between which DCSBD plasma was induced.

**ATR-FTIR Spectroscopy Measurements**
ATR-FTIR measurements were performed with an FTIR™NICOLET spectrometer (Thermo Scientific, USA) using a single bounce ATR accessory equipped with a Ge crystal. For each measurement, the spectral resolution was 2 cm⁻¹ and 64 scans were performed.

**Plywood Preparation**
Rotary-cut veneer sheets of birch wood (Betula verrucosa Ehrh.) free from defects were used for the experiments with the following dimensions: 300×300 mm with 1.5 mm thickness and the moisture content of approximately 6%. Three-layer experimental plywood panels were laboratory-prepared using prepared thermoplastic films. The film was placed between veneers, then laid up and hot-pressed in a laboratory press using the pressing temperature of 130°C, pressure of 1.8 MPa, and time of 5 min. The shear strength of plywood samples was tested according to EN 314-1 and 314-2. After pressing, plywood was cooled under the steel plate for 1 hour, ready panels were conditioned for 7 days and strength of samples (Figure 1) was tested after immersion in water for 24 hours.

**Marking of Prepared Films:**
- **No. 77** – (80% EVA copolymer + 20% modified collagen) – thickness 0.10 mm
- **No. 84** – (70% EVA copolymer + 30% modified collagen) – thickness 0.13 mm
- **No. 98** – (50% EVA copolymer + 50% modified collagen) – thickness 0.09 mm

**Results and Discussion**

1. **Applications of Modified Collagen in Pharmacy**
The aim was to prepare and verify the functionality of pharmaceutical excipients not only as the filler of solid medicament forms – uncoated tablets, but also as the binder and mainly as an additive of the matrix of tablets with the controlled drug release.
Non-crosslinked Collagen Hydrolysate as the Tablet Binder

Non-crosslinked “Collagen BS” hydrolysate was tested as an adhesive binder. The tablets with collagen concentration of 5%, 10%, 11%, 15%, and 22% were pressed from prepared granules. The effect of collagen concentration on basic parameters was evaluated, i.e. disintegration of tablets, friability of uncoated tablets – mechanical resistance to abrasion, and resistance of tablets to fracture – mechanical compression strength. Disintegration of tablets meet the requirements of European Pharmacopoeia – tablets were disintegrated within 15 minutes, the disintegration time (from 56.6 s to 680.3 s) was extended with an increase of concentration of collagen in the tablet, see Figure 2.

The resistance of tablets to breakage (radial strength of tablets in pressure) was satisfactory over collagen concentration of 11% in the granulate (from 72.5 N at 11% concentration to 138.6 N at 22% concentration), see Figure 3.

Mechanical resistance of uncoated tablets to abrasion (friability) – weight loss from 7.76% to 0.34% decreased with increasing concentrations of collagen, see Figure 4.

Applications of the non-crosslinked collagen hydrolysate confirmed the following:

- The tablet disintegration process meets the requirements of European Pharmacopoeia. Tablets were disintegrated within 15 min, which corresponds to requirements for uncoated tablets. The disintegration time was extended with the increasing dry matter content of collagen in the tablet.

- Resistance of tablets against fracture (radial tensile strength of tablets) was satisfactory for the samples prepared from granules with the collagen content higher than 11%. The same is valid also for friability of uncoated tablets.

- A strong binding effect of collagen was confirmed on the physical and technological parameters of prepared granules. The optimal fraction with regard to the tablet preparation process occurred at about 0.5 mm.

Non-crosslinked collagen with concentrations of 11%, 15% and 22% was confirmed as an effective adhesive. Granules and tablets complied with the parameters required by Pharmacopoeia.

Based on the obtained results, Zentiva Hlohovec pharmaceutical company (Slovakia) prepared tablets “Gastrogel” in the amount of 5 kg where the non-cross-linked collagen hydrolysate with concentration of 20% was applied as a binder of granules. After final treatment, the granulate obtained required properties for the tabletation process, Table I.

The molded sample tablets performed the comparable quality parameters as reference samples from the regular production.
Stability of tablets in an aqueous medium was investigated as the concentration of dissolved collagen in a standard solution of 0.1 mol/dm³ hydrochloric acid and evaluated with a biuret agent. The sensitivity of the proposed dissolution methodology of collagen is from concentration of 0.5 g/dm³. To determine the effect of molecular weight on the dissolution time of non-crosslinked collagen, the following samples were tested:

- No. 1 Collagen HS (bovine, the particle size of 0.355 mm - 45 Mesh, 176 Bloom),
- No. 2 Collagen BS (porcine, the particle size of 0.355 mm - 45 Mesh, 220 Bloom).

Measurements have shown (Figure 5) that the solubility of collagen samples is independent on the origin of the raw material and the molecular weight. The samples were dissolved within 2 hours, and the dissolubility process was almost linear. To enhance the stability, collagen was modified by dry heat in laboratory conditions. Prior to the experiment, there was important to reduce the actual water content to a minimum. Based on the obtained results, temperature of 150°C was chosen for crosslinking of samples no. 1 and no. 2 for 120 min exposure time.

Measurements have shown that the crosslinked samples no. 1 and 2:

- reduced dissolution of collagen compared with the non-crosslinked samples,
- the influence of collagen origin was not observed,
- the influence of molecular weight (measured as Bloom) was not observed.

<table>
<thead>
<tr>
<th>Physical parameters of tablets</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>diameter</td>
<td>12 mm</td>
</tr>
<tr>
<td>weight</td>
<td>0.6004 g</td>
</tr>
<tr>
<td>height</td>
<td>3.43 mm</td>
</tr>
<tr>
<td>resistance against fracture</td>
<td>227 N</td>
</tr>
</tbody>
</table>
| abrasion resistance            | after 4 min 0.06%  
                             | after 10 min 0.08% |
| disintegration                 | 2 min 30 s |

Tests of Controlled Release of Ambroxol from the Tablet Matrix

The analytical method was developed to determine the controlled dissolution of Ambroxol HCl and the wavelength was determined to measure the concentrations of Ambroxol in UV spectra at 246 nm.

Ambroxol HCl drug controlled release was tested from tablets made with non-crosslinked collagen. The tablets did not exhibit any controlled drug dissolution and they disintegrated within 2 hours in each used solution, as shown in Figure 6.

In the next step, tablet samples were prepared using crosslinked collagen to achieve the controlled drug release from the tablet.
matrix. Time dissolution of Ambroxol from tablets with additionally treated collagen by heat can be seen in Figure 7.

The dissolution time of Ambroxol HCl from tablets based on non-crosslinked and crosslinked collagen was measured, and the following was found:

- tablet samples based on non-crosslinked collagen ensured the drug release within the time range of max. 120 min. The release was not linear, but steep with limit approaching to 100% of the released drug,
- the time of release was extended for tablet samples after collagen crosslinking, within 8 hours 84% of the drug was released in the environment of 0.1 mol/dm³ HCl, and only 68% in a solution of pH 6.8. This means a possibility of controlled release of this modification up to 12 hours.

Tests of crosslinking of collagen tablets have shown that the physical methods of crosslinking can be effective. Physically crosslinked collagens for the excipients applications as for the controlled drug release must be modified by plasticization because their binding effect decreased significantly. For the tablet preparation, new collagen samples were prepared and the ability to form tablet matrix by dry compression was assessed.

Measurements of the time dissolution of drug Ambroxol from the tablets prepared of different types of collagen are in Figures 8, 9 and 10.

Based on the achieved results and evaluation of physical parameters, it can be concluded that the tablet skeleton with samples of modified collagen has satisfactory physical parameters.

Mechanical and physical parameters of tablets Collagen HS:
- Test “fracture toughness” – 61.8 N,
- Test “friability of uncoated tablet” – 2.4476%.

Mechanical and physical parameters of tablets Collagen BS:
- Test “fracture toughness” – 70.6 N,
- Test “friability of uncoated tablets” – 1.908%.

Mechanical and physical parameters of tablets Collagen K12:
- Test “fracture toughness” – 71.2N.
- Test “friability of uncoated tablets” – 1.373%.

Figure 7. Dissolution time of Ambroxol from tablets with additionally heat-treated collagen.

Figure 8. Dissolution profile of Ambroxol drug from the tablets prepared by direct compression of heat-treated and plasticized collagen “COL” BS.

Figure 9. Dissolution profile of Ambroxol drug from the tablets prepared by direct compression of heat-treated and plasticized collagen “COL” HS.

Figure 10. Dissolution profile of Ambroxol drug from the tablets prepared by direct compression at 1000 and 1100 kPa of heat-treated and plasticized collagen “COL” K 12.
The results of dissolution of heat-treated and plasticized collagens have confirmed:

- crosslinked collagen samples (COL HS, COL BS and COL K12) are suitable for application of the controlled drug release,
- crosslinked collagen samples (COL HS, COL BS and COL K12) have a comparable dissolution time of drug Ambroxol HCl from tablets,
- the assumption of the drug dissolution time extension with a pressure increase was not confirmed.

To verify the drug dissolution time extension, collagen mixtures “COL” (BS, HS and K12) with Ambroxol drug were modified with supplementary additives with 10% of weight.

- COL HS + additive 1 based on Ca²⁺,
- COL HS + additive 2 based on Mg²⁺,
- COL BS + additive 1 based on Ca²⁺,
- COL BS + additive 2 based on Mg²⁺,
- COL K 12 + additive 1 based on Ca²⁺,
- COL K 12 + additive 2 based on Mg²⁺,
- COL HS + additive 3 with a buffering effect,
- COL BS + additive 3 with a buffering effect,
- COL K 12 + additive 3 with a buffering effect.

The dissolution results have revealed:

- dissolution profile of the drug release from tablets can be changed by additives,
- to extend the dissolution up to 18 h, addition of 10% of alkali additive based on Ca²⁺ into the matrix of modified collagens “COL” 12, HS and BS is needed,
- similarly, the dissolution profile is improved by Mg²⁺ based additives,
- the dissolution time of the controlled drug release is shorter with additive 3 with a buffering effect.

Skeleton of tablets from heat-treated and plasticized collagen “COL” K12 were prepared to test the dissolution profile of Venlafaxin HCl. These skeletons were compared with a standard, which is based on modified cellulose Methocel K 100 McR. Dissolution profiles can be seen in Figure 11.

Tests confirmed that the modified collagen “COL” K12 provides a controlled release of Venlafaxin up to 6 hours.

---

**Table II**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Type of film</th>
<th>unmodified</th>
<th>DCSBD plasma N₂/H₂</th>
<th>Plasma CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>collagen film + keratin</td>
<td>97.4 ± 4.6</td>
<td>80.2 ± 2.6</td>
<td>64.2 ± 1.6</td>
</tr>
<tr>
<td>2</td>
<td>collagen film</td>
<td>88.2 ± 3.5</td>
<td>76.2 ± 2.1</td>
<td>58.1 ± 2.2</td>
</tr>
<tr>
<td>84</td>
<td>EVAc film + 30% collagen</td>
<td>94.0 ± 2.3</td>
<td>64.4 ± 1.9</td>
<td>56.1 ± 2.0</td>
</tr>
<tr>
<td>98</td>
<td>EVAc film + 50% collagen</td>
<td>87.8 ± 2.1</td>
<td>61.1 ± 1.7</td>
<td>52.2 ± 2.3</td>
</tr>
</tbody>
</table>

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*Figure 11. Dissolution profiles of the drug Venlafaxin.*
2. Surface and Adhesion Properties of the Film Samples with Collagen

Surface and Adhesion Properties of Collagen Based Thermoplastic Hot-melt Films

In the further research of collagen application, thermoplastic hot-melt films based on collagen biopolymer were prepared. The experiments were concentrated on the surface and adhesion properties of the samples EVAc films with collagen, and on the adhesive bond strength under shear load. Films no. 84 and 98 were modified by plasma, and the impact of discharge atmospheric low-temperature plasma on the surface properties modification was studied.

The selection and amount of additives changed the parameters of thermoplastic films, e.g. elongation of the film, hydrophilicity, hydrophobicity, viscosity, melting and solidification points. The static contact angles of re-distilled water were measured at different points on the film surface with collagen and they are presented in Table II.

Sample 2 – collagen film is more hydrophilic than the sample 1 according to the water contact angle. A similar difference was shown for samples 84 and 98. The sample 98 that contained 50% of collagen was more hydrophilic, and its hydrophilicity value from the wetting perspective was similar as hydrophilicity of collagen.

Comparison of the values of hydrophilicity and polarity of plasma-treated samples in various gases has shown that the most effective treatment was obtained using a CO₂ process gas. Using this treatment the highest wetting of the sample film surface was obtained.

The shear strength of the adhesive bonds was tested for samples 84 and 98 with aluminum adhesive joints. Adhesively bonded aluminum-collagen samples were prepared at the temperature of 120°C, and aluminum sheets were fixed by overlapping in a hydraulic press at a pressure of 50 N.cm⁻² and results are presented in Table III.

![Figure 12. FTIR spectra of EVAc film no. 84.](image)

![Figure 13. FTIR spectra of EVAc film no. 98.](image)

<table>
<thead>
<tr>
<th>Table III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results of the shear strength of adhesive bonds with aluminum.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Type of film</th>
<th>Unmodified</th>
<th>DCSBD plasma N₂/H₂</th>
<th>Plasma CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
<td>EVAc film + 30% collagen</td>
<td>0.8 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>98</td>
<td>EVAc film + 50% collagen</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Sample 84 – the adhesive bond strength increased after plasma modification
Sample 98 – the adhesive joint failed to measure
FTIR-ATR spectra of thermoplastic hot-melt film sample no. 84 based on collagen modified by plasma in various atmospheres are shown in Figure 12 where:

- curve A is unmodified sample no. 84,
- curve B is sample modified with DCSBD plasma in a N\textsubscript{2}/H\textsubscript{2} process gas,
- curve C is sample modified with DCSBD plasma in a CO\textsubscript{2} process gas.

FTIR spectra of the sample no. 84 are typical spectra of polyethylene. The content of acetate units in copolymer is most likely under the detection limit and therefore, it is not registered in the spectra. After plasma affection (in air vs. O\textsubscript{2}), the formation of new absorption bands can be seen in the area between 1800 and 1600 cm\textsuperscript{-1}. They belong to the oxidation products (carbonyl, carboxyl, hydroxyl, peroxy groups, and potential degradation products containing the double bond – the band with max at approx. 1640 cm\textsuperscript{-1}, which can also be assigned to the OH groups).

FTIR-ATR spectra of thermoplastic hot-melt film sample no. 98 based on collagen modified by plasma in various atmospheres are shown in Figure 13 where:

- curve 1 – unmodified sample no. 98,
- curve 2 – sample modified with DCSBD plasma in a N\textsubscript{2}/H\textsubscript{2} process gas,
- curve 3 – sample modified with DCSBD plasma in a CO\textsubscript{2} process gas.

The bands typical for the acetate group (1736 cm\textsuperscript{-1}) are present in the spectra of the sample 98, as well as the band amino-group that belongs to collagen (1640 cm\textsuperscript{-1}). Plasma treatment of samples in air vs in oxygen leads to changes in the area of the carbonyl and amino groups (the change of shape of absorption bands comparing to the changes of the intensity ratio of the functional groups (acetate and amino). Under the action of plasma in the oxygen, the volume of the amino-groups has decreased, resulting in a decrease of the absorption band intensity. Similarly, in the area under 1400 cm\textsuperscript{-1} the formation of new absorption bands was observed, most likely caused by oxidation of the material.

### Table IV
Shear strength of plywood and statistic evaluation.

<table>
<thead>
<tr>
<th>Plywood samples after the dry test</th>
<th>Avg x (MPa)</th>
<th>Std dev s (MPa)</th>
<th>Coeff of var (v_k) (%)</th>
<th>Value min (MPa)</th>
<th>Value max (MPa)</th>
<th>Number of samples (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>2.88</td>
<td>0.20</td>
<td>7.1</td>
<td>2.66</td>
<td>3.08</td>
<td>15</td>
</tr>
<tr>
<td>84</td>
<td>2.27</td>
<td>0.59</td>
<td>25.9</td>
<td>1.63</td>
<td>2.73</td>
<td>15</td>
</tr>
<tr>
<td>98</td>
<td>1.77</td>
<td>0.11</td>
<td>5.98</td>
<td>1.64</td>
<td>1.86</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plywood samples after immersion in water 24 h</th>
<th>Avg x (MPa)</th>
<th>Std dev s (MPa)</th>
<th>Coeff of var (v_k) (%)</th>
<th>Value min (MPa)</th>
<th>Value max (MPa)</th>
<th>Number of samples (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>1.64</td>
<td>0.22</td>
<td>13.1</td>
<td>1.26</td>
<td>1.95</td>
<td>15</td>
</tr>
<tr>
<td>84</td>
<td>1.47</td>
<td>0.13</td>
<td>9.1</td>
<td>1.19</td>
<td>1.68</td>
<td>15</td>
</tr>
<tr>
<td>98</td>
<td>0.35</td>
<td>0.04</td>
<td>10.8</td>
<td>0.25</td>
<td>0.39</td>
<td>15</td>
</tr>
</tbody>
</table>

Note. European standard EN 314-2 requires the value of shear strength to be 1.0 MPa.
Determination of the Bond Quality of Plywood Boards with Collagen Films

The shear strength test is commonly used as a fundamental indicator of the adhesive performance in plywood. Investigated non-formaldehyde collagen thermoplastic adhesive film samples based on collagen have different effects on the shear strength of plywood, as stated in Table IV. Performance of plywood samples from veneer bonded with samples no. 77 and 84 were corresponding to the requirements, but the strength of the sample no. 98 was low and do not meet the requirements of EN standard.

Conclusions

There is a large interest in the application of biopolymers especially from the perspective of the novel dosage forms with the controlled drug release. These medicament formulations represent significant added values from the perspective of comfort and benefits to patients. Replacement of the currently used chemically modified excipients (cellulose- and starch-based) by proteins such as collagen and keratin helps to reduce burden on the human body. Moreover, these two proteins are sources of amino acids essential for life. This feature significantly increases potential for the application of biopolymers in the pharmacy.

Non-crosslinked collagen hydrolysates with concentrations of 5%, 10%, 11%, 15% and 22%, were tested as an adhesive binder. Tablets were pressed from the prepared granules. The impact of collagen concentration on the basic parameters (disintegrating of the tablet, friability of uncoated tablets, mechanical resistance to abrasion of tablets, tablets resistance to fracture, mechanical strength in compression) was evaluated. Non-crosslinked collagens with concentrations of 11%, 15% and 22% were shown to work well as binders, and granules and tablets performed parameters in accordance with the requirements of European Pharmacopoeia. Tablets prepared from non-crosslinked collagen hydrolysate did not show extended release of Ambroxol. The release of drug was not linear and tablets disintegrated within 2 hours.

Prepared samples of thermally crosslinked collagen tablets extended the time of controlled drug release for more than 8 hours. To use the physically crosslinked collagens as excipients for the drug controlled release, they must be modified by plasticization because the binding effect decreased significantly. The controlled dissolution time was achieved for Ambroxol during 6-10 hours. The dissolution profile of drug release from tablets can be changed by additives. It can be extended up to 18 hours by addition of 10% of alkaline additive based on Ca2+ and Mg2+ into a matrix of modified collagens "COL" K12, HS and BS or it can be shortened with buffering effect additives. Tests confirmed that modified collagen “COL” K12 provides controlled release of the drug Venlafaxin up to 6 hours.

These tablets meet the requirements of the European Pharmacopoeia. This confirmed the potential of crosslinked collagen to be used as a pharmaceutical excipient for solid medicaments with the controlled drug release.

The surface properties of films with collagen were measured by the static contact angles. Comparison of the values of hydrophilicity and polarity of plasma-treated samples at various process gases showed that the most effective treatment was achieved by using CO2 as a process gas. The surface of samples was strongly polarized and hydrophilized, and the best wettability of the surface by water was reached.

Investigations of the adhesion properties and quality of plywood bonding with collagen thermoplastic film confirmed that standard requirements for the adhesive bond shear strength are fulfilled for the samples no. 77 (film with 20% of modified collagen) and no. 84 (film with 30% of modified collagen). In the case of the sample no. 98 (film sheet with 50% of modified collagen), the required shear strength of the adhesive bond was not met.

Acknowledgements

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References


An Experimental Comparative Study on Silicone Oil and Polyethylene Glycol as Dry Leather Treatments

by

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Abstract

The application of leather dressing and lubricants is one of the important challenges in conservation of historic dry leathers, due to their effects on structural and visual properties and stability of leathers. This study aimed to investigate influence and stability of silicone oil and polyethylene glycol (PEG) on treated dry leathers, and application assessment of ascorbic acid (AA) as an antioxidant additive for PEG. The polymers, untreated and treated leather samples were submitted to heat accelerated ageing process. FTIR spectroscopy, colorimetry, pH measurements, investigation of mechanical properties and shrinkage temperature (Ts) were used to explain effect of treatments. Also, the oxidation of polymers during ageing process was monitored by FTIR spectroscopy. Results showed that silicone oil has better stability against thermal oxidation with compared to PEG. Ascorbic acid inhibited the PEG oxidation. Moreover, results revealed that the silicone oil has a better performance in treatment of dry leather than PEG or PEG+AA, due to its high stability and minimum changes in visual, structural and mechanical properties in treated leathers.

Introduction

The leather products have been useful materials since the dawn of human history.1, 2 Collagenous materials, such as leather, have been used for thousands of years due to the availability of such a natural resource and its resilience and flexibility.3 As leather is widely used for historic clothing, upholstery, bookbinding and etc., historical and archaeological leathers constitute an important part of museum and archival objects.

These objects and artifacts are valuable treasures of historical information and cultural interest due to the history they represent.2 However, leather is still considered as one of the most sensitive materials towards environmental hazards, hard use, etc.,4 and their preservation is one of the biggest challenges in conservation. Wide variety application of leather, imposes a particular set of conditions, which can bring about deterioration in the leather.5 In general, deterioration of leather is a chemical process in which there are a great number of contributing factors. These factors, in combination with the leather and tanning chemistry, leads to very diverse and complex degradation mechanisms.6

One of the major problems facing the historical leathers is excessive desiccating and drying. A common way to dealing with this phenomenon and its complications, such as brittleness, is application of leather dressing and lubricant treatments. For a long time, dressings used to be the standard treatment used in conservation of leathers. The dressings are usually applied in an attempt to slow deterioration, improve the appearance of leather, and perhaps restore some of its former strength and flexibility.5-8

There are, to date, several different methods of conserving leathers. In the course of different conservation studies, a variety of lubricants and dressings have been introduced and investigated. Some of these have included neat’s-foot oil, lanolin, glycerin, British museum leather dressing (BML), Bavon, Marney’s leather dressing, pliantine, and SC6000,6,8-10 and or leather cream recommended by Larsen in 2007, the Dutch emulsion recommended by the Koninklijke Bibliotheek, The Hague, a modified leather dressing suggested by Fuchs in 2005 and Cire in 2013 recommended by the National Library of France.11

The effect of dressings on leather permanence has been studied, and almost invariably the researchers conclude that the dressings, as mentioned above, have no preservative effect7, 11, 12. Their main compounds are usually oils, fats and waxes.11 Overuse of these materials, since the base components are fatty substances containing varying concentrations of unsaturated fatty acids, can cause oxidation and stiffening, discoloration and staining, a tacky surface that attracts dust and dirt, encouraging of microorganism-growth, depositing of spew on the surface, and hampering of future conservation efforts.6-11

Also, these materials do not contribute to the preservation of the constituent moisture and, on the other hand, destabilize collagen filaments, which dispose to overdrying.13 The growing

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production of the leather lubricants, accompanied by the arrival of new synthetic materials that both moisturize and resistant. Hence, the application of synthesis polymer material has developed by leather conservators.

Polyethylene glycol (PEG) and silicone oil are common synthetic polymers in conservation, as treatments of both dry and waterlogged leathers. Several studies have been dedicated to the introduction and investigation of PEG or silicone oil in conservation of leather during the last years. Ershad-Langroudi and Mirmontahai investigated the influence of PEG–nano-378 Treatments for Historic Dry Leathers about lubrication of new leather with these material

suitable for the treatment of leather. Also, there are many papers BML. Their obtained results indicated that silicone oil is more leather, in comparison to two other treatments, Klucel G and PEG with average molecular weight of 400 (Merck Co.), PEG with polymers in the leather behavior. Also, application of ascorbic acid as an antioxidant additive (0.5% w/v in dimethyl silicone as silicone oil (KCC Co.) were studied as leather treatment, samples artificially were aged again. Accelerated Aging of Lubricants

It is very important that used treatments have a long-term stability and do not have the capacity to break down or formation of harmful products over a long period of time. However, PEG can be oxidized easily at environment conditions, and its decomposition can cause changes in leather properties. Hence, accurate assessment of material, before of treatment, is essential for appropriate conservation purposes.

Therefore, the aim of this study is to investigate the application of silicone oil and polyethylene glycol in treatment and lubrication of historic dried leather with the interaction of these polymers in the leather behavior. Also, application of ascorbic acid as an antioxidant of PEG was evaluated.

Materials and Methods

Accelerated Aging of Lubricants

PEG with average molecular weight of 400 (Merck Co.), PEG with ascorbic acid as an antioxidant additive (0.5% w/v; Acros Co.) and dimethyl silicone as silicone oil (KCC Co.) were studied as leather lubricants. Heat accelerated aging was used for thermal degradation evaluation. Samples were heated in an oven at 100°C for 72 hours, 130°C for 48h and 150°C for 48h. Thermal degradation was analyzed by ATR-FTIR spectroscopy during the aging process.

Leather Treatment and Accelerated Aging

Samples were prepared from goat leather which was vegetable tanned traditionally and was lubricated with animal tallow three years ago in a village of southern Khorasan province of Iran. They were aged to simulate the historic leathers. The aging process was accomplished according to ISO 17228:2005.

Samples were placed in oven at a 100°C for 72 hours, before and after treatment. Aged samples (3 repeat) were treated with silicone oil, polyethylene glycol (30% v/v in ethanol) and polyethylene glycol with ascorbic acid as an antioxidant additive (0.5% w/w in 30% v/v PEG in ethanol) in a vacuum tank at 170 mm Hg pressure for 21 hours. Then, excessive treatment materials were removed by dryer paper and samples were dried in a desiccator. After treatment, samples artificially were aged again.

Colorimetry

The colorimetric properties of aged and treated 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)]. The total color difference (ΔE), the chroma coordinate (C*) and the hue angle (h*) were calculated according equations 1, 2 and 3. Color values were measured ten times for each leather sample, and their average was considered as CIE Lab color coordinates.

\[ ΔE = \sqrt{[(ΔL)^2 + (Δa)^2 + (Δb)^2]} \]

\[ C = \sqrt{a^2 + b^2} \]

\[ h = \arctan(b/a) \]

pH Measurement

The pH was measured according to ASTM D2810-01. 2gr of leather samples were cut to small pieces and soaked in 40 mL of distilled water (20 times more than weight of the samples) for 12 hours. pH of the leather-water mixtures was assessed by using a Metrohm 744 pH meter calibrated between buffers pH 4 and 7.

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 zinc selenide (ZnSe) crystal plate. All Spectra were collected in the range of 4000-650 cm⁻¹ at 4 cm⁻¹ resolution with 32 numbers of scan.

Shrinkage Temperature

Shrinkage temperatures of the leather samples were determined according to ASTM D6076-03. The sample specimens, in the form of 12.5×76 mm 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.

Mechanical Properties

Mechanical Properties of dumbbell shape samples were assessed according to ASTM D2209-00 and D2211-00. A ZWICK tensile tester machine, model 1446-60 was used for evaluation.
Results and Discussion

ATR-FTIR technique has been applied to investigate structural changes of PEG, PEG with ascorbic acid (PEG+AA) and Silicone oil (Figure 1). Spectra of PEG and PEG+AA showed peaks at 3700-3200 cm⁻¹ (O-H), 2866 cm⁻¹ (CH₂ stretching), 1456 cm⁻¹ (CH₂), 1350 cm⁻¹ (CH₃), 1249 cm⁻¹ (C-O-C), 1200-1000 cm⁻¹ (C-O-C stretching), and silicone oil showed absorptions at 1258 cm⁻¹ (Si-CH₃), 1082 cm⁻¹ (Si-C), 1011 cm⁻¹ (Si-O), 900-730 cm⁻¹ (Si-C), 701 cm⁻¹ (Si-O), 686 cm⁻¹ (H₃C-Si-CH₃) and 662 cm⁻¹ (Si-C-H). Accelerated aging resulted to characteristic changes in 1780-1680 cm⁻¹ for PEG. This new band is assigned to the carbonyl stretching vibration and indicates that new carbonyl groups were produced due to thermal degradation.

Thermo-oxidation mechanism of polyethylene glycol is shown in Scheme 1, regarding to Lai and Liau (2003). In the beginning, PEG is oxidized to α-hydroperoxide form. Due to thermally labile of this peroxide, it can be decomposed by a radically mechanism. The degradation products are ultimately transformed to formic esters, a product with carbonyl groups, which can result to formation of formic acid.

Formation of carbonyl groups as a scale of PEG thermal degradation has been assessed, according to both height and area of peak intensities (Figure 2). It indicates an increase in PEG degradation during accelerated aging. But the ascorbic acid prevented oxidation of PEG, because the spectra of PEG+AA do not show any visible change in the absorptions compared with the PEG. Moreover, the spectra of silicone oil do not show any change during the accelerated aging. It indicates the high thermal stability of silicone oil.

Figure 3 shows diagrams of ΔL, Δa, Δb, ΔC and the total color difference (ΔE) of leather samples. It signifies a low change in colorimetric parameters of treated leather with silicone oil. Results indicate a great color stability and better efficiency of silicone oil for preserving of visual and aesthetic values of leather relics after accelerated aging process with compared to PEG (with or without ascorbic acid). In addition, ascorbic acid was not effective as an antioxidant for color stability of PEG treated leathers. Moreover, it has caused to decrease in color stability.

The average of pH contents and shrinkage temperatures (Ts) are shown in Figure 4. It signifies a little decrease of pH for untreated leathers from 4.82 to 4.76. Silicone oil resulted a very low pH change in leathers from 4.78 to 4.81. It is decreased to 4.79 after accelerated aging. It indicates the stability of the treated leathers with silicone oil. However, the PEG treatment particularly with ascorbic acid has reduced the pH of leathers to 4.65 and 4.53, which has more pH reduction compared to untreated and treated leathers with silicone oil. The decrease causes to protein hydrolysis, and diminution of hydrothermal stability of leather.

The untreated samples exhibited a wide range of Ts between 64-65°C, before and after aging. The use of PEG, pure or with ascorbic acid, has decreased the Ts to 60-62°C. As following the aging, it was observed that the average shrinkage temperature of
the PEG treated samples decreased from 62 to 50°C. Similarly, the average Ts of the PEG+AA treated samples reduced from 60 to 52°C. Accordingly, the use of PEG does not improve performance on hydrothermal stability of leather and accelerate the destruction rate, while silicone oil improved the hydrothermal properties of leathers and Ts has increased to about 69°C. Also, due to the high stability of the silicone oil, only a little change in Ts is observed after aging process.

Tensile strength is one of the most important mechanical properties of leather. Lubrication or fat liquoring greatly affects the physical properties. The mechanical properties of samples are shown in Figure 5. The results indicate a relative increase in mechanical strength of untreated samples after aging. Also, the physical properties of leather have improved by PEG treatment. But these properties have dropped sharply after aging due to the oxidation of PEG and its effects on the leather. However, the use of ascorbic acid, as antioxidants, prevents the reduction in PEG (+AA) treated samples. Whereas, silicone oil treatment does not lead to significant changes in mechanical strength of leather, before and after the aging.

The FTIR spectra of all samples were baseline corrected on 900-1800 cm⁻¹ wavenumber range. Figure 6 shows the FTIR spectrum of untreated and unaged sample (Or).

The peaks at 1650 cm⁻¹ for the amide I and 1550 cm⁻¹ for the amide II absorptions are interesting. The Δν (νₘ₁ - νₘ₂) value is corresponding to collagen denaturation. The difference in wavenumbers between the A₁ and AⅡ band positions in FTIR spectra of leather samples (corium layer) are shown in Figure 7. The PEG treatment, with or without ascorbic acid, has led to an increase in the Δν value. Whereas, FTIR spectrum of silicone oil treated leather shows small shift in peak positions of amide I and II. In other words, the denaturation of collagen has been increased after the use of PEG treatment, whereas silicone oil treated leather have a better structural stability.

**Conclusion**

In this study, silicone oil and polyethylene glycol, with and without ascorbic acid, were compared for their effectiveness in the treatment of dry leathers as well as their chemical stability. The FTIR results showed that aging process causes to severe oxidation in PEG, whereas ascorbic acid inhibits the change, while, silicone oil had great heat stability. The results confirmed that the treatment of leather samples with polyethylene glycol decreased

![Scheme 1. Mechanism of thermo-oxidation of polyethylene glycol, regarding to Lai and Liau 2003.](image)

![Figure 2. Increase the height and area of peak intensities between 1800 and 1650 cm⁻¹ indicates the production of carbonyl groups, as a scale for oxidation of PEG, during the accelerated aging.](image)

![Figure 3. ΔL, Δa, Δb, ΔC and total color difference (ΔE) of leather samples. *Or: Untreated sample; PT: Treated sample with PEG; PAnT: Treated sample with PEG+AA; SiT: Treated sample with silicone oil; A:- Aged sample; AA:- Twice aged sample.](image)

![Figure 4. The average data of Ts and pH of leather samples.](image)
the color stability, pH and shrinkage temperature of leather. The physical properties have improved by PEG, but dropped sharply after aging. However, the use of ascorbic acid prevents reduction strength in PEG treated leather. Whereas, treated leather with silicone oil exhibit great improvements in hydrothermal stability without any significant changes in pH, color and physical properties. Also, the FTIR results indicate that the PEG treatment increased denaturation of collagen; whereas silicone oil treatment has a good structural stability. Therefore, silicone oil could be selected as a better treatment for historic or new dried leathers.

Figure 5. Average of mechanical properties of leather samples.

Figure 6. ATR-FTIR spectrum of untreated and unaged sample (Or) with functional groups according to Koochakzaei and Achachluei (2015).41

Figure 7. The difference between $\nu_{\text{Am I}}$ and $\nu_{\text{Am II}}$ ($\Delta \nu$) as denaturation index in FTIR of corium layer; * Tr.: treatment.

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Dyeing of Chamois Leather using Water Soluble Sulphur Dyes
by
A. Jaya Prakash, R. Aravindhan, N. Nishad Fathima and J. Raghava Rao

Abstract

Chamois leathers find application in fuel filtration and cleaning. Recently, use of chamois leathers is explored in the development of sports garments, gloves and other personal wear. However, one of the consumer demand viz. different colors on the end product is not met by chamois leathers. Hence, dyeing is essential to make it more attractive for end user applications. In the present work an attempt has been made to develop colored chamois leather by employing water soluble sulphur dyes. The offer of sulphur dyes have been optimized based on dyeing characteristics, color fastness and color values. The results indicate that offer of 1 and 2% of the dyes show better colour value and fastness to rubbing. Hence, it could be inferred that coloring of chamois leathers not only improved the aesthetic appeal but also helped in adding value without altering inherent properties of chamois leather.

Introduction

Chamois leathers are made by in-situ oxidation of unsaturated oils, after which the skins turn to golden yellow color. The leathers are then washed using alkali and dried. Chamois leathers have gained importance as cleaning material since commercial production of glass have begun and they are widely used for cleaning high-end lenses. Not only as a cleaning material, chamois leather find numerous applications in making garments and sportswear. Color of chamois is yellow, which further will not be colored by tanners. However, application of chamois leather as garment and other wearable articles demand pleasing aesthetics, so there is a need for colouring of chamois, which would add value to the leathers.

Sulphur dyes are traditionally used for coloring cotton and cellulose fibers. Solubilized sulphur dyes are thiosulphate derivatives, which are applied to cellulosic fibers in the presence of alkali and reducing agent. These dyes produce heavy depth shades of moderate to good light fastness, good wet rub fastness.

Several reports are available on the use of sulphur dyes as a coloring agent for cellulosic and textile fabrics. However, very few reports are available on the use sulphur dyes for coloring of leather. The alkaline reducing environment required for the use of these dyes restricts the use in leather processing. Though exact reactive mechanism is unknown, dyeing of leather with insoluble sulphur dyes has been attempted. Reducing agents were used for strengthening of dye in combination with other water soluble colorants. Recently, a report is also available on the use of solubilized sulfur dyes for coloring chrome tanned leathers and tone-in-tone dyeing was shown.

Still, dyeing of chamois leather using soluble sulfur dyes has not been attempted earlier using soluble sulphur dyes from Bunte salts. The main advantage of using this dye in the chamois process is their operational pH (8-11). Another advantage is that the dye can be used directly without addition of alkali or reducing agents and can be fixed using mild acid in order to avoid serious effluent problems.

Objective of the present study is to employ commercially available water soluble sulphur dyes for dyeing of chamois leather. Use of sulphur dyes improves the aesthetic value of chamois leather and makes it attractive to end user. The offer of sulphur dyes has been optimized and the leathers are evaluated in terms of color values, fastness properties and organoleptic properties.

Experimental Section

Materials

Four water soluble sulphur dyes, Tancol Black LSG (C.I. Solubilized Sulphur Black 1), Tancol Prune LBR (C.I. Solubilized Sulphur Red 6), Tancol Brown LSG and Tancol Chocolate Brown LFP (C.I. Solubilized Sulphur Brown 14) samples were provided by SF Dyes Pvt. Ltd., India. Both brown dyes used in this study have same CI number. Structures of base material used for making sulphur dyes are provided in Figure 1 along with Color Index (CI) numbers. Chamois leathers were procured from Sai chamois Pvt. Ltd., India. All other chemicals used for leather processing are of commercial grade.

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Methods
Dyeing of Chamois Leather
Dye application procedure is given in Table I. Different trials have been carried out by changing the concentration of dye. All chemicals were offered on dry weight of the leather. Entire leather processing was done in a rotating drum at 4-6 revolutions per minute. Initially, chamois leathers were wetted and sulphur dyes were applied in two different concentrations and fixed using acetic acid. Acetic acid was used because of the operational pH of sulphur dyes varies from 8 to 11. The amount of acid required is usually less and need to be mild. After the fixing of the dye, liquor was drained and leathers were rinsed in fresh water.

Color Measurement of Chamois Leathers
Color of the dyed leathers was measured using Spectra Scan 5100A (Premier Colorsan Instruments India Pvt. Ltd) dual beam spectrophotometer equipped with pulse xenon light source having scan wavelength interval of 360 to 740 nm having wavelength accuracy of 0.1 nm and instrument was operated at 25°C with relative humidity <90% (Non-condensing). Prior to the color measurement instrument was calibrated using standard white slab provided by the equipment manufacturer. The perceptive change in color to human eye was analysed by ΔE values (Equation 1), which also denotes Euclidean distance between two color stimuli in CIELUV space:\(^1\)

\[
\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}
\]

Where, difference ΔL is the measure of lightness. Similarly, Δa values indicate difference to red and green; Δb values indicate difference to yellow and blue.

Color Fastness Analysis
Color fastness to rubbing both dry and wet were tested using STD 422 Crock meter instrument from SATRA UK in accordance to IUF 450.\(^2\) This method of testing chamois leathers was intended to determine the degree of surface color transferred from the material during mild dry or wet rubbing. A specimen of dyed leather material was rubbed by a cotton felt, which was linearly moved forward and backwards under the constant force. After a set number of rubs the cotton felt was assessed with the Greyscale for color transfer from the test material.

Wash fastness to washing machine was measured in accordance with IUP 435\(^3\) and final leathers after testing were rated using Greyscale.

Table I
Process for dyeing chamois leathers.

<table>
<thead>
<tr>
<th>Process</th>
<th>Chemical</th>
<th>Percentage (%)</th>
<th>Duration (min)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washing</td>
<td>Water</td>
<td>600</td>
<td>15</td>
<td>Drain/Wash/Drain</td>
</tr>
<tr>
<td>Dyeing</td>
<td>Water</td>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulphur Dye</td>
<td>1,2</td>
<td>60</td>
<td>Check penetration</td>
</tr>
<tr>
<td>Fixing</td>
<td>Acetic acid</td>
<td>2</td>
<td>In 3 feeds for 10 each + 30</td>
<td>In 1:10 dilution, set to pH 6-6.5*</td>
</tr>
</tbody>
</table>

\(^*\)Check exhaustion, Drain and hook to dry
Soluble Sulphur Dyes for Chamois

Water Absorption
Control un-dyed chamois leather and dyed chamois leathers were analysed for static absorption of water in accordance with IUP 7. This method calculates water absorbed by 100g of leather for a duration of 60 minutes (Equation 2). Here Q represents percentage absorption, v is the volume of water absorbed and m is the mass of leather.

\[ Q = \frac{100v}{m} \]  

Organoleptic Properties
Dyed leathers were evaluated for organoleptic properties; experienced leather technologists rated the leather for softness, visual appearance and dye penetration. Rating was given on scale of 1 to 10, higher value meaning better properties.

Results and Discussion

Dyeing of Leather Matrix
Dyeing of chamois leathers has been found to be uniform and penetration throughout the cross section has been achieved within 30-40 min. This has been confirmed by visual inspection of the cross-section of the leathers. Fixing of dye is achieved by reducing the pH to 6-6.5, where dyes tend to fix to the leather.

Reaction Mechanism and Effect of pH
Sulphur dyes are known to be soluble at alkaline pH and fix with collagen or cellulose fibers permanently after aerial oxidation. Sulphur dyes are believed to have high fastness and resistance to bleaching due to covalent interaction with substrate. Sodium carbonate (Soda ash) is used for washing of chamois after oxidation of oil, hence the alkaline conditions required for dyeing using sulphur dyes is maintained. These conditions also facilitate dye to penetrate throughout the leather matrix. Sulphur dye is applied to leather in the form of soluble leuco compound (containing bunte salt) that subsequently oxidize and gets converted to parent sulphur dye. Plausible interaction of sulphur dye with collagen is represented in Figure 2.

Stability of Dye Solution Towards Acid, Alkali and Hard Water
Stability of dyes solution towards acid, alkali and hard water is an important parameter to understand the behavior of dye in different environments. Though sulphur dyes operate in alkaline region, studying the effect of acids on these dyes gives information about dyes stability to color change and precipitation. From Table II, all the dyes used in the study exhibited good resistance towards acid, alkali and hard water.

Color Measurement of Chamois Leather
Reflectance measurements have been carried out for chamois leathers and *L, *a, *b values are provided in Table III. It is clear from the table that the color intensity increases with an increase in concentration of dye. The decrease in lightness value L was noticed when dye concentration was increased from 1 to 2%, meaning that the color was intensifying. It was observed that ΔE values were higher than 2.9, which give the measure of just noticeable difference (JND) of color, indicating that the color change is perceptible to human eye with increase in dye concentration. This also gives information that darker shades can be produced by increasing the concentration of dyes.

Color Fastness
Color fastness to rubbing was analyzed using Grey scale and rating from 1-5 were awarded based on colour of the felt. Rating close to 5 meaning leathers exhibited superior fastness property. From Table IV, it is observed that, leathers dyed with sulphur dyes showed excellent fastness to dry rubbing and moderate fastness to wet rubbing. Color fastness to washing machine values shows the leather staining towards washing with multi-fabric containing layers of acetate, cotton, nylon, polyester, acrylic and wool fibers (Table V). Staining with cotton is lower for Tanacol Prune LBR and Tanacol Chocolate Brown LFP, apart from that leather showed good fastness with respect to other fabric materials. Overall it can be concluded that the water soluble sulphur dyes were promising for dyeing chamois leathers.
Water Absorption
Chamois leathers were tested for static water absorption. Results showed that dyed chamois leather has similar water absorption to that of the control un-dyed chamois leather. Water absorption values for dyed leathers are in the range of 331 to 345%, while control leather sample exhibited 351% with an error of ±5%. This observation indicates that the water absorption capabilities of leathers are not affected due to dyeing.

Evaluation of Organoleptic Properties
Organoleptic properties of dyed chamois leathers were rated by experienced leather technologists and are compared in Figure 3. It is clear that dye penetration is excellent for all the dyes. Softness of leather is unchanged with respect to the un-dyed control chamois leather. There is no change in odor for dyed chamois leathers. Photograph shows some of the dyed chamois leathers in Figure 4.

Table II
Dye resistance to acid, alkali and hard water.

<table>
<thead>
<tr>
<th>Resistance towards</th>
<th>Acid</th>
<th>Alkali</th>
<th>Hard water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tancol Black LSG</td>
<td>4-5</td>
<td>4-5</td>
<td>4-5</td>
</tr>
<tr>
<td>Tancol Brown LSG</td>
<td>4-5</td>
<td>3-4</td>
<td>4-5</td>
</tr>
<tr>
<td>Tancol Prune LBRS</td>
<td>5</td>
<td>4-5</td>
<td>4-5</td>
</tr>
<tr>
<td>Tancol Chocolate Brown LFP</td>
<td>4-5</td>
<td>4</td>
<td>4-5</td>
</tr>
</tbody>
</table>

Table III
Color values of chamois leathers dyed with sulphur dyes.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Concentration (%)</th>
<th>*L</th>
<th>*a</th>
<th>*b</th>
<th>ΔE</th>
<th>Shade card</th>
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<td>Tancol Black LSG</td>
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*L* represents lightness, *a* represents redness-greenness and *b* represents blueness-yellowness of the color
Constitution

Chamois leathers were colored using sulphur dyes. The colored chamois leather was assessed for various parameters. It was observed that the application of 1 and 2% sulphur dye showed good colour value and better fastness to rubbing. Also, dyeing of leathers did not have any effect on water absorption capabilities. Hence, it could be concluded that the sulphur dyes can be effectively used as coloring agent for chamois leather without affecting the quality of the leather. At the same time, it is believed that this approach may add economic value for chamois leather making.

Table IV

<table>
<thead>
<tr>
<th>Dye</th>
<th>Concentration (%)</th>
<th>Dry Rub 10 Rubs</th>
<th>Wet Rub 10 Rubs</th>
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<td>4</td>
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Table V

<table>
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<th>Dye</th>
<th>Concentration (%)</th>
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<th>A</th>
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<th>N</th>
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<tr>
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</tbody>
</table>

<sup>*</sup>Leather staining towards different fibers, A = Acetate, C = Cotton, N = Nylon, P = Polyester, Ac = Acrylic, W = Wool
<sup>*</sup>Color change on, L= Leather
Acknowledgements

Financial support from CSIR under Supra-institutional project STRAIT (CSC 0201) is acknowledged. One of the authors A. Jaya Prakash acknowledges CSIR for awarding Senior Research Fellowship. Special thanks to Ms. Malathy Jawahar, Senior Scientist, Leather Processing Division and Mr R Mohan, Principal Scientist, Shoe Design and Development Centre (SDDC) – CLRI, K N Ramesh, Technical development manager, SF Dyes Ind. Pvt. Ltd., and M. Tamizhazhagan, Department of Leather Technology, Anna University and CLRI.

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Peter Duchovic, see JALCA 106, 33, 2011

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