REVISTA

DE PIELĂRIE ÎNCĂLŢĂMINTE

Leather and Footwear Journal

December/ Decembrie 2020
Volume / Volumul 20
No / Numărul 4

INCDTP - SUCURSALA INSTITUTUL DE CERCETĂRI PIELĂRIE ÎNCĂLŢĂMINTE
INCDTP - DIVISION: LEATHER AND FOOTWEAR RESEARCH INSTITUTE

PIELĂRIE/LEATHER ÎNCĂLŢĂMINTE/FOOTWEAR BUNURI DE CONSUM DIN CAUCIUC/RUBBER GOODS

AIMS AND SCOPE

Revista de Pielarie Incaltaminte / Leather and Footwear Journal (Print ISSN 1583-4433) is published 4 times a year, by Leather and Footwear Research Institute (ICPI) Bucharest, Romania, Division of The National Research and Development Institute for Textiles and Leather (INCDTP), CERTEX Press.

Revista de Pielarie Incaltaminte / Leather and Footwear Journal aims to present current science and technology developments as well as initiatives in Romania and South Eastern Europe region. The Journal publishes original research papers of experimental and theoretical nature, followed by scientific, technical, economic and statistic information, reviews of local and foreign conferences, congresses, symposia, with the purpose of stimulating the dissemination of research results.

Revista de Pielarie Incaltaminte / Leather and Footwear Journal focuses particular attention on the key areas of new systems and technologies applied in leather, footwear and rubber goods sectors; biomaterials, collagen-based medical devices, biochemistry of collagen; environment; innovation; leather and parchment cultural heritage; management and marketing, quality control; applications of IT field in these sectors, and other related fields.

OPEN ACCESS STATEMENT

Revista de Pielarie Incaltaminte / Leather and Footwear Journal is a peer reviewed, open access journal. All articles published open access will be immediately and permanently free for everyone to read, download, copy and distribute, under the provisions of a Creative Commons Attribution (CC BY) which lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

PEER-REVIEW PROCEDURE

Submission of a manuscript to *Revista de Pielarie Incaltaminte* implies that the work described has not been published before (except in the form of an abstract or as part of a published lecture, review, or thesis); that it is not under consideration for publication elsewhere; that its publication has been approved by all coauthors, if any.

Submitted manuscripts are single-blind peer-reviewed by two qualified reviewers selected by the Editorial Board. The manuscript will be accepted for publication provided reviews are favorable. In case of diversified opinions of the reviewers, the Editorial board will make the final decision. If the paper is not accepted for publication, the manuscripts and the figures will not normally be returned. The authors will be notified as soon as possible of the result of the paper evaluation (to be published, to be modified, rejected).

JOURNAL SPONSORSHIP

Edited with the sponsorship from the Ministry of Education and Research. We are pleased to acknowledge support from the following: The Confederation of National Associations of Tanners and Dressers of the European Community – COTANCE, Belgium; Romanian Leather and Fur Producers Association, APPBR, Romania; SFERA FACTOR, Romania.

COPYRIGHT

ABSTRACTING AND INDEXING

Revista de Pielarie Incaltaminte / Leather and Footwear Journal is acknowledged in Romania by the National University Research Council (CNCSIS) in B+ Category (code 565), and is indexed in Chemical Abstracts Service (CAS) Database, USA, CAB Database (CAB International, UK), Elsevier's Compendex and SCOPUS, CrossRef, EBSCO, CiteFactor, Research Bible, Index Copernius and listed in AcademicKeys, Environmental XPRT, MIAR, Electronic Journals Library, Cosmos Impact Factor, Science Library Index and SCIPIO.

INCDTP-ICPI is a member of The Publishers International Linking Association, Inc. (PILA), a nonprofit corporation doing business as Crossref. As a PILA member, we have the right to assign Digital Object Identifiers (DOIs) to journal content. DOIs are persistent links to an object/entity and can be used to cite and link to any article existing online, even if full citation information is not yet available. We strongly recommend that authors cite references using DOIs where possible. For details regarding citation format and examples, please visit the Instructions for Authors section on *Revista de Pielarie Incaltaminte / Leather and Footwear Journal*'s website, http://www.revistapielarieincaltaminte.ro

FEES AND SUBSCRIPTIONS

Revista de Pielarie Incaltaminte / Leather and Footwear Journal requires article processing charges of 200 EURO per article, for accepted manuscripts, payable by the author to cover the costs associated with publication. There are no submission charges.

Hard copies of journal issues are available for purchase at subscription rates of 160 EURO for companies and 100 EURO for individual subscribers, while the rate for a single issue is 40 EURO. Subscriptions (include mailing costs) can be made at the editorial office, to the following address: INCDTP – DIVISION: LEATHER AND FOOTWEAR RESEARCH INSTITUTE, 93 Ion Minulescu Street, postal code 031215, sector 3, Bucharest, Romania, Europe.

Both article processing charges and subscription fees are to be paid in the following account:
Account holder: INCDTP – Division: Leather and Footwear Research Institute; Address of the account holder: 93 Ion Minulescu Street, postal code 031215, sector 3, Bucharest, Romania, Europe
IBAN Code: RO25 RNCB 0074029208380005
Bank code: 300413024
Swift bank address: RNCBROBU; Bank: BCR sector 3 (ROMANIAN COMMERCIAL BANK – SECTOR 3); Bank address: 11 Decebal Blvd., Bl. S14, sector 3, Bucharest, Romania.

CORRESPONDENCE

Editor-in-Chief – Dana Gurău
INCDTP – Division: Leather and Footwear Research Institute (ICPI), 93, Ion Minulescu Street, Bucharest, sector 3, postal code 031215, Romania, Europe; tel./fax: +40 21 323 52 80, e-mail: jlfjournal@gmail.com

CERTEX Publishing House – Bucharest, 16 Lucreţiu Pătrăşcanu St., sector 3; Tel./ Fax: (0040) 21 340.55.15; certex@ns.certex.ro

Website: http://www.revistapielarieincaltaminte.ro
Facebook: http://www.facebook.com/LeatherFootwearJournal
Title DOI: https://doi.org/10.24264/lfj

REVISTA DE PIELĂRIE ÎNCĂLŢĂMINTE LEATHER AND FOOTWEAR JOURNAL

ISSN: 1583-4433
Volume 20, No. 4, December 2020
https://doi.org/10.24264/lfj.20.4

DETERMINATION OF THE TOLERANT CONCENTRATION OF EISENIA FETIDA TO CHROME SOLID RESIDUES FROM TANNERY

Robert BULEJE DEL CARPIO*, Liliana MARRUFO SALDAÑA*

Productive Innovation and Technological Transfer Center of Leather, Footwear and related industries (CITEccal Lima). Technological Institute of Production (ITP), Caquetá Ave. 1300, Rímac, 15094, Lima, Perú, rbuleje@itp.gob.pe, lmarrufo@itp.gob.pe

Received: 02.06.2020 Accepted: 14.10.2020 https://doi.org/10.24264/lfj.20.4.1

DETERMINATION OF THE TOLERANT CONCENTRATION OF *Eisenia fetida* TO CHROME SOLID RESIDUES FROM TANNERY

ABSTRACT. The tanning industry, although it is characterized by the use of the skin waste originated by the cattle raising, in contrast, produces huge volumes of waste, among which chrome shavings stand out due to their high percentage of chromium. The objective of this research was to determine the concentration where earthworms (*Eisenia fetida*) could tolerate chrome shavings, in order to evaluate the potential for degradation of these wastes through biological treatment. To determine the tolerant concentration, an experimental design was established that included as factors, the time of exposure in weeks (0-11) and the concentrations of exposure: 0.01, 0.02, 0.04, 0.08, 0.12 and 0.16 grams of shavings per grams of substrate. The response variable was the mortality rate. Each treatment was performed in triplicate and a negative control was included. Statistical treatment was performed using ANOVA and multiple comparison tests at 95% confidence with the statistical complement Real Statistics, Statgraphics and Yupana software. The tolerant concentration established in the study was 0.04 g/g (grams of shavings per grams of substrate) which is equivalent to 636 mg/kg (based on dry weight) expressed in weight of chrome per weight of compost.

KEY WORDS: chromium, chrome shavings, *Eisenia fetida*, bioassay

DETERMINAREA CONCENTRAȚIEI DE REZIDUURI SOLIDE DE CROM DIN TĂBĂCĂRIE TOLERATE DE *Eisenia fetida* ÎN VEDEREA DEGRADĂRII ACESTORA

REZUMAT. Industria de pielărie, deși se caracterizează prin utilizarea deșeurilor de piele provenite de la creșterea bovinelor, produce, în schimb, volume imense de deșeuri, dintre care se remarcă răzătura de piele cromată datorită procentului ridicat de crom. Obiectivul acestei cercetări a fost de a determina concentrația în care râmele (*Eisenia fetida*) ar putea tolera răzătura de piele cromată, pentru a evalua potențialul de degradare a acestor deșeuri prin tratament biologic. Pentru a determina concentrația tolerată, s-a conceput un experiment care a inclus ca factori timpul de expunere în săptămâni (0-11) și concentrațiile de expunere: 0,01, 0,02, 0,04, 0,08, 0,12 și 0,16 grame de răzătură per grame de substrat. Variabila de răspuns a fost rata mortalității. Fiecare tratament a fost efectuat de trei ori și a fost inclus un martor negativ. Analiza statistică a fost efectuată utilizând testul ANOVA și teste de comparație multiple la un nivel de încredere de 95% folosind programele complementare de analiză statistică Real Statistics, Statgraphics și Yupana. Concentrația tolerată stabilită în studiu a fost de 0,04 g/g (grame de răzătură de piele cromată per grame de substrat), care este echivalentă cu 636 mg/kg (pe baza greutății substanței uscate) exprimată în greutate crom per greutate compost.

CUVINTE CHEIE: crom, răzătură de piele cromată, *Eisenia fetida*, bioanaliză

DÉTERMINATION DE LA CONCENTRATION DE RÉSIDUS SOLIDES DE CHROME TOLÉRÉE PAR *Eisenia fetida* POUR LA DÉGRADATION DE CES DÉCHETS

RÉSUMÉ. L'industrie du cuir, bien que caractérisée par l'utilisation de déchets de cuir provenant de l'élevage du bétail, produit à la place d'énormes volumes de déchets, dont les copeaux de cuir chromé sont perceptibles en raison du pourcentage élevé de chrome. Le but de cette recherche était de déterminer la concentration dans laquelle les vers de terre (*Eisenia fetida*) pouvaient tolérer les copeaux de cuir chromé, d'évaluer le potentiel de dégradation de ces déchets par traitement biologique. Pour déterminer la concentration de tolérance, une expérience a été conçue qui incluait comme facteurs le temps d'exposition en semaines (0-11) et les concentrations d'exposition : 0,01, 0,02, 0,04, 0,08, 0,12 et 0,16 gramme de copeaux par gramme de substrat. La variable de réponse était le taux de mortalité. Chaque traitement a été effectué trois fois et un contrôle négatif a été inclus. L'analyse statistique a été réalisée en utilisant le teste ANOVA et des tests de comparaison multiples à un niveau de confiance de 95% en utilisant les programmes d'analyse statistique complémentaires comme Real Statistics, Statgraphics et Yupana. La concentration de tolérance établie dans l'étude était de 0,04 g/g (grammes de copeaux de cuir chromé par gramme de substrat), ce qui équivaut à 636 mg/kg (sur la base du poids de la matière sèche) exprimée en poids de chrome par poids de compost.

MOTS CLÉS : chrome, copeaux de cuir chromé, *Eisenia fetida*, essai biologique

** Correspondence to: Liliana MARRUFO SALDAÑA and Robert BULEJE DEL CARPIO, Productive Innovation and Technological Transfer Center of Leather, Footwear and related industries (CITEccal Lima). Technological Institute of Production (ITP), Caquetá Ave. 1300, Rímac, 15094, Lima, Perú, lmarrufo@itp.gob.pe, rbuleje@itp.gob.pe*

INTRODUCTION

Tanning industry is a manufacturing activity, which, together with the footwear and related industries, is important due to the employability it generates and the use it makes of the skin waste originating from livestock farming. However, due to the complexity of its processes and the chemical products used, it produces large volumes of solid waste and effluents with a high pollution load. Among the solid waste generated, the large volumes of wet blue chrome shavings, solid waste generated in the trimming process. Wet blue leather is characterized by its high chromium content, acquired after the leather passes through the tanning stage, which could compromise environmental and human health [1].

For each ton of leather produced, 200 to 250 kg of tanned solid waste would be generated [2]. These waste products occupy important spaces in the industries, giving rise to an idle installed capacity and whose final disposal is not necessarily adequate. The danger of these wastes lies mainly in their chromium content, which comes from the basic salts of this metal, which is the most widely, used and diffused tanning agent, as it makes the leather imputrescible, durable and resistant, characteristics that no other tannery agent has managed to match.

Chromium is naturally present in the earth's crust, however, high concentrations of it can be attributed to the wastewater discharges generated in the metallurgical industry and tanneries [3], is also a trace element, a metal that is important for the metabolism of living beings, however, at high concentrations [4], such as those found in chrome shavings, it becomes a hazard due to the toxicity generated by its +3 and +6 species. Although the +3 species is more stable and less toxic, it has been shown that under oxidative conditions it is susceptible to conversion to Cr+6. This species has been classified as carcinogenic [5]. Traditionally, this waste has been disposed of in an inappropriate manner in landfills or outdoors, however, with the emergence of environmental regulations worldwide, the rigour of disposal has increased. Some are disposed of as low-cost non-hazardous waste and others as high-cost hazardous waste. For this reason, companies have chosen to reduce the amount of solid waste generated as far as possible, and with the rest, they have opted for ways of recovering it (taking advantage of collagen) and treating it to reduce its dangerousness and, consequently, its cost of disposal [6, 7].

Among the treatment alternatives, the biological pathway always stands out, due to its low installation and operation costs, even reaching comparable results with the physical-chemical methods, the space they require being its only limitation. However, systematic information regarding the use of earthworms to treat these wastes and their interaction with chrome is limited [3, 8, 9]. The aim of this research work was to determine the tolerant concentration of *Eisenia fetida* to chrome shavings, which is the starting point for evaluating the potential for degradation of these wastes through biological treatment.

EXPERIMENTAL

This research was developed in the facilities of the Productive Innovation and Technological Transfer Center of Leather, Footwear and related industries (CITEccal Lima).

Materials and Methods

Earthworms

The earthworms (*E. fetida*) were acquired from the National Agrarian University La Molina (UNALM), Lima, Peru, and were selected earthworms with visible clitellum, the presence of which indicates that the earthworms are sexually mature. This was done in order to have a homogeneous population for testing [10, 11].

Before any test was carried out on the earthworms, the harmlessness of the new support medium (mature compost) was verified by means of a survival test. This test consisted of placing 20 earthworms on a portion of the new medium and waiting for them to be introduced into it, otherwise the medium was declared uninhabitable. Once the safety was

verified, a container with the new medium was conditioned to contain all the earthworms used in the investigation. The humidity of the medium was controlled moisture 80-85% at all times [12].

Chrome Shavings

Chrome shavings were collected from a tannery that processes bovine skin, located in the district of San Juan de Lurigancho, Lima, Peru.

Experimental Design

In order to observe the behaviour and mobility of earthworms in the face of moderate exposure to shavings, a preliminary experiment was designed with 6 grams of shavings, 150 grams of soil and 250 grams of compost resulting in a concentration of 0.015 grams of shavings per gram of substrate. The shavings, soil and compost were systematically distributed as shown in Table 1. Only in the first container were the three substrates mixed. All containers were inoculated with 10 earthworms.

The total exposure time was set at four days. A daily review was conducted to check the toxic effect of the shavings on the earthworms; however, no mortality was reported in any container. For the following tests, the distribution by mixture (Box 1, Table 1) was established as it offers the greatest interaction between the shavings and the earthworms and the distribution by layer (Box 3, Table 1) was established as it offers the least interaction possible between the earthworms and the shavings.

Table 1: Experimental design, exposure to different distributions

Box	Distribution
1	Total mix of the substrate
2	Stratum 1: a mixture of 75 g of soil and 125 g of compost
	Stratum 2: a mixture of 75 g of soil, 125 g of compost and 6 g of chrome shavings
3	Stratum 1°: mix 150 g of soil and 250 g of compost.
	Stratum 2: 6 g of chrome shavings
4	Stratum 1°: mix 50 g of soil, 50 g of compost and 1 g of chrome shavings
	Stratum 2: 50 g of soil, 100 g of compost and 2 g of chrome shavings
	Stratum 3: 50 g of soil, 100 g of compost and 3 g of chrome shavings

In order to determine a threshold concentration for exposure of earthworms to shavings, a preliminary experimental exposure design was established, where exposure concentrations were 0.075, 0.100, 0.150 and 0.200 grams of shavings per gram of substrate in mixture distribution (Table 2).

Test was conducted in individual containers with 10 earthworms per concentration. Identical substrates were used. The total exposure time was established to 4 days, in constant monitoring of the activity of the earthworms and the visible effects of the shavings on them.

Table 2: Experimental design, preliminary exposure to different concentrations

Box	Soil (g)	Compost (g)	Chrome shavings (g)	Concentration (g of chrome shavings/g of substrate)
1	100	300	30	0.075
2	100	200	30	0.100
3	100	100	30	0.150
4	100	50	30	0.200

From the observation of the behavior of the earthworms (permanence or not in the substrate) a maximum viable concentration of exposure equal to 0.16 grams of chrome shavings per grams of substrate was established, from which the concentrations of the experimental design of exposure in decreasing geometric progression were determined, such concentrations were: 0.01, 0.02, 0.04, 0.08, 0.12 and 0.16 grams of chrome shavings per grams of substrate (Table 3) [10, 11]. These concentrations were made at the humidity of each component, and then the mixture was taken to 80-85% moisture (dry basis).

As mentioned above, it was decided on two configurations in the distribution of the substrates. It started with the distribution by layers (mixed substrate covered with shavings), however, when it was verified that the earthworms did not have apparent contact with the shavings, a mixture of the substrates was made, to guarantee the interaction of the earthworms with the chrome shavings. This mixing was done after 7 (D-7), 14 (D-14) and 28 (D-28) days of the earthworms' stay in the stratified substrate.

A chronic exposure period (greater than 28 days) was established for this experiment, no additional food was added during testing. A weekly moisture control was performed to verify its value within the optimal range (80-85%) [13]. Each container was inoculated with 20 sexually mature earthworms. To control the quality of the test, a negative control (substrate without the presence of chrome shavings) was carried out in parallel for each group evaluated (D-7, D-14 and D-28), and each concentration and negative control was carried out in triplicate. The quality control to validate the data was set to obtain a survival above 90% in the negative controls [10, 11].

The number of earthworms in the containers was counted weekly. The counts were done in an enclosed area with limited natural light to minimize stress on the earthworms.

Statistical treatment to analyze significant differences between the data groups (response variable: mortality) was performed using the multifactorial ANOVA test and Tukey multiple comparisons with the statistical complement Real Statistics and the Statgraphics and Yupana software. Tests were performed at 95% confidence.

Table 3: Experimental design, systematic evaluation of earthworm exposure to shavings

Name of the concentration	Soil (g)	Compost (g)	Chrome shavings (g)	Concentration (g of chrome shavings/g of substrate)
C-01	75	300	3.8	0.01
C-02	75	300	7.5	0.02
C-04	75	300	15.0	0.04
C-08	75	300	30.0	0.08
C-12	75	300	45.0	0.12
C-16	75	300	60.0	0.16
C-00 (white)	75	300	0	0.00
Moisture (dry basis)	0%	33.4%	25.5%	

RESULTS AND DISCUSSIONS

Preliminary Study

The survival of negative controls was above 90%, therefore the quality control required for this type of trial was found. The results obtained, shown in Figure 1, indicate that in the samples coming from the previous exposure to 7 days per stratum (D-7), the concentrations 0.12 g/g and 0.16 g/g cause the total death of the earthworms from week 1, on the other hand, the concentration 0.08 g/g exerts the gradual increase of the mortality through the weeks. The samples with previous exposure of 14 days (D-14) (Figure 2) and 28 days (D-28) (Figure 3) showed a similar behavior to the previous group, however, the concentrations 0.12 g/g and 0.16 g/g began to cause total mortality from the fifth and second week respectively, which would indicate that previous exposure to the environment is a factor that influences the response of the earthworms, because the earthworms are sensitive to changes in the environment. For this reason, the guidelines recommend a minimum adaptation time to the test substrate or living environment [10].

Statistical analysis with multifactorial ANOVA in Statgraphics software, at 95% confidence, to evaluate the contribution of the previous exposure time factor, indicated that there were no significant differences between groups D-14 and D-28, unlike D-7 which did present significant differences with the mentioned groups.

In negative control and the concentrations 0.01 g/g, 0.02 g/g and 0.04 g/g the mortality

was maintained at the same level during all the weeks, in the same way, no significant differences existed between these treatments and between the groups of previous exposure (D-7, D-14 and D-28), which was established by means of comparisons Tukey at 95% confidence. This behavior led to estimate that the tolerant concentration, which causes no effect on earthworms (NOEC), is around 0.04 g/g.

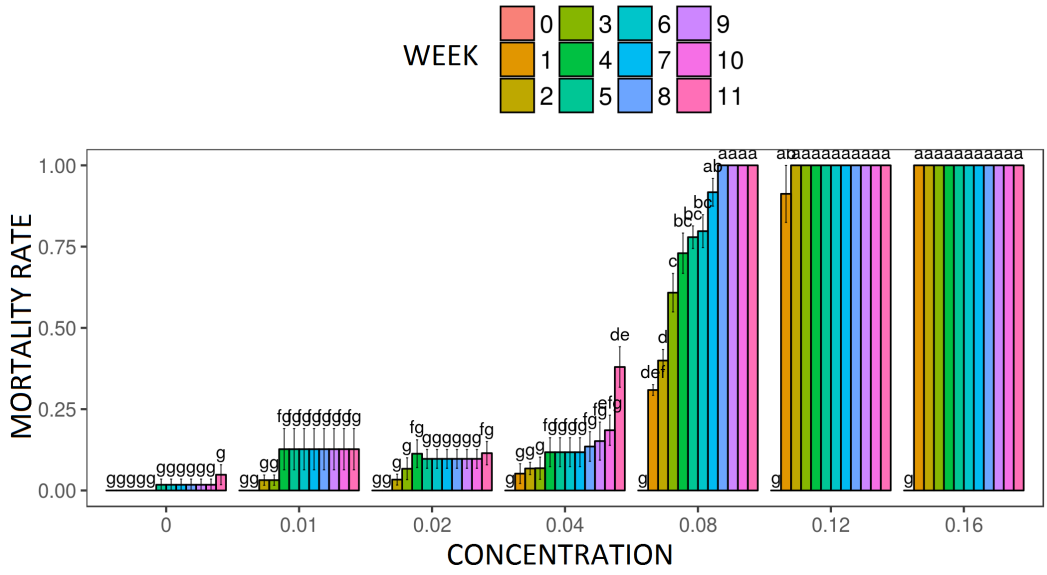


Figure 1. Answers from Group D-7

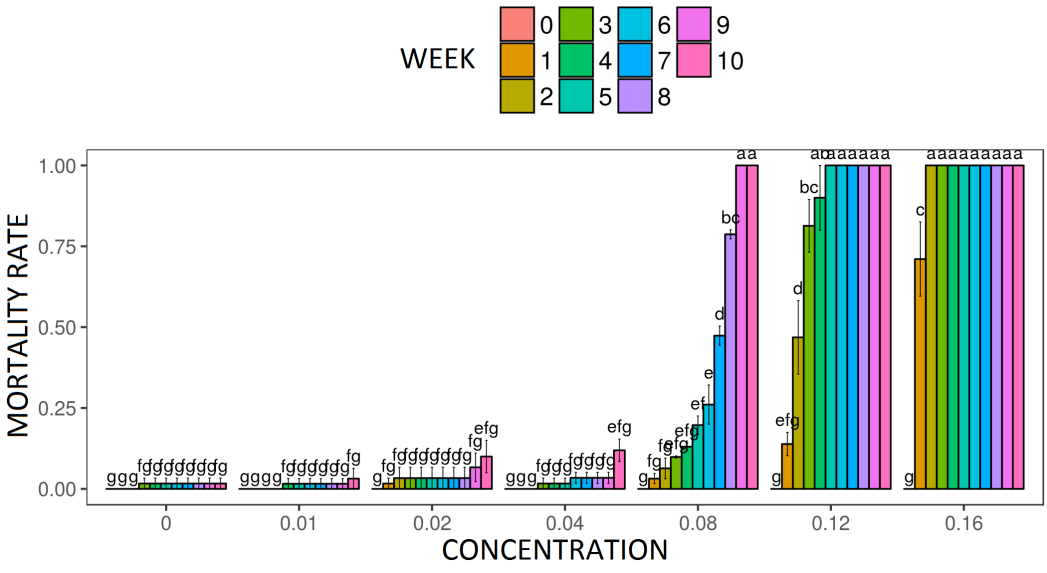


Figure 2. Answers from Group D-14

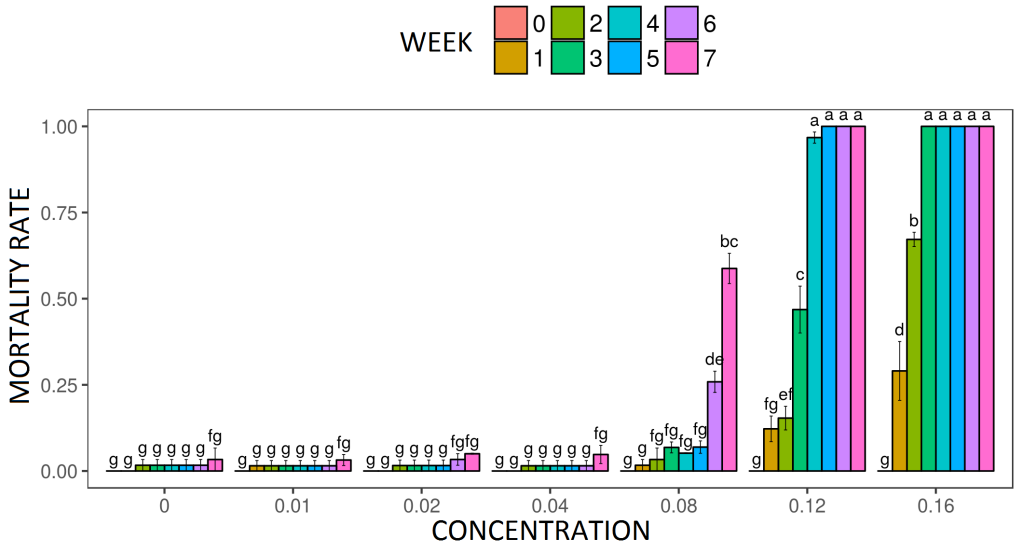


Figure 3. Answers from Group D-28

Chrome shavings have an average chromium concentration near to 16323.73 mg/kg on dry weight [14], therefore, the concentration of 0.04 g/g would be equivalent to 636 mg/kg expressed in weight of chromium per weight of substrate, a concentration lower than the range determined by Sivakumar and Subbhuraam [3] as average lethal concentration (LC50-14days) ranging from 1656 mg/kg to 1902 mg/kg in different types of soil and organic matter, which supports the tolerant concentration (NOEC) determined in this research. However, it disagrees with the value reported by Lock and Janssen [8], where no mortality of *Eisenia fetida* is recorded at a concentration of 1800 mg/kg of chromium (dry basis) per substrate (developed according to OECD guidelines), this suggests that organic matter present in the substrate may have increased the toxicity of chromium on earthworms. It should also be borne in mind that chromium in the shavings is encapsulated, and although it is considered to be the problem element in the investigation, it is not the only agent that could be exerting toxicity on earthworms.

The survival of the earthworms to long periods (chronic exposure), without any food, suggests that the earthworms have taken advantage of the collagen present in the shavings, being the route of entry of the chromium the oral route, in contrast, the concentrations C-12 and C-16, suggest the toxic effect through the skin of the earthworms, since it was found in the containers to the first and second week (acute exposure), dead individuals, sectioned and/or decomposed [15].

During the counts, the presence of cocoon up to tolerant concentration was reported, including the presence of some developed juveniles, due to the length of the test. However, values close to the tolerant concentration showed a decreasing trend in the presence of cocoons [8, 9].

CONCLUSIONS

This study established as tolerant concentration 0.04 g/g (grams of chrome shavings per grams of substrate), which was the highest concentration that caused no effect on the earthworm population and did not compromise their survival over time. The substrates and the shavings provided the necessary food for the survival of the earthworms.

This tolerant concentration corresponds to a chrome concentration of approximately 636 mg/kg (dry basis). The previous time of adaptation to the environment is an important factor to consider in studies of chrome removal from chrome shavings using earthworms. Ensuring the survival and development of earthworms within shaved media is vital for

future bioaccumulation studies and evaluation of potential uses of chrome shavings compost.

Acknowledgements

We acknowledge to the Productive Innovation and Technological Transfer Center of Leather, Footwear and related industries (CITEccal Lima) and all its staff for the support provided to carry out this project.

REFERENCES

1. Chowdhury, M., Mostafa, M.G., Biswas, T.K., Saha, A.K., Treatment of leather industrial effluents by filtration and coagulation processes, *Water Resour Ind*, **2013**, 3, 11–22, https://doi.org/10.1016/j.wri.2013.05.002.
2. Mella, B., Glanert, A.C., Gutterres, M., Removal of chromium from tanning wastewater and its reuse, *Process Saf Environ Prot*, **2015**, 95, 195–201, https://doi.org/10.1016/j.psep.2015.03.007.
3. Sivakumar, S., Subbhuraam, C.V., Toxicity of chromium(III) and chromium(VI) to the earthworm *Eisenia fetida*, *Ecotoxicol Environ Saf*, **2005**, 62, 1, 93–8, https://doi.org/10.1016/j.ecoenv.2004.08.006.
4. Singh, H.P., Mahajan, P., Kaur, S., Batish, D.R., Kohli, R.K., Chromium toxicity and tolerance in plants, *Environ Chem Lett*, **2013**, 11, 3, 229–54, https://doi.org/10.1007/s10311-013-0407-5.
5. Domènech, X., Fundamentos de química Ambiental, Madrid: Editorial Sintesis, **2014**.
6. Bautista, M.E., Pérez, L., García, M.T., Cuadros, S., Marsal, A., Valorization of tannery wastes: Lipoamino acid surfactant mixtures from the protein fraction of process wastewater, *Chem Eng J*, **2015**, 262, 399–408, https://doi.org/10.1016/j.cej.2014.10.004.
7. Oruko, R.O., Selvarajan, R., Ogola, H.J.O., Edokpayi, J.N., Odiyo, J.O., Contemporary and future direction of chromium tanning and management in sub Saharan Africa tanneries, *Process Saf Environ Prot*, **2020**, 133, 369–86, https://doi.org/10.1016/j.psep.2019.11.013.
8. Lock, K., Janssen, C.R., Ecotoxicity of Chromium (III) to *Eisenia fetida*, *Enchytraeus albidus*, and *Folsomia candida*, *Ecotoxicol Environ Saf*, **2002**, 51, 3, 203–5, https://doi.org/10.1006/eesa.2001.2122.
9. van Gestel, C.A.M., Dirven-van Breemen, E.M., Baerselman, R., Accumulation and elimination of cadmium, chromium and zinc and effects on growth and reproduction in *Eisenia andrei* (*Oligochaeta*, *Annelida*), *Sci Total Environ*, **1993**, 134, 585–97, https://doi.org/10.1016/S0048-9697(05)80061-0.
10. OECD, Test No. 207: Earthworm, Acute Toxicity Tests [Internet], **1984**, available from: https://www.oecd-ilibrary.org/content/publication/9789264070042-en
11. EPA, Ecological Effects Test Guidelines OCSPP 850.3100: Earthworm Subchronic Toxicity Test, **2012**.
12. Schuldt, M., Testa, H., Reconversion of hop plant residues by vermicomposting. Substrate acceptance on trays and reproductive biology (P5L Test). *REDVET - Rev electrón vet - Revista electrónica de Veterinaria* [Internet], **2011**, 12, 9, available from: https://recyt.fecyt.es/index.php/REDVET/article/view/14043
13. Domínguez, J., State-of-the-Art and New Perspectives on Vermicomposting Research, in: Earthworm ecology, CRC press; **2004**, 401–424, https://doi.org/10.1201/9781420039719.ch20.
14. Barra, J., Marrufo, L., Optimization of Alkaline Hydrolysis of Chrome Shavings to Recover Collagen Hydrolysate and Chromium Hydroxide, *Leather and Footwear Journal*, **2020**, 20, 1, 15–28, https://doi.org/10.24264/lfj.20.1.2.

15. Rodríguez, A.R., Marchese, M., Ojea, N., Efecto del Cromo en *Eisenia Fetida* (Savigny, 1826) (*Oligochaeta: Lumbricidae*): Toxicidad y Bioacumulación, *Natura*, **2005**, 1, 33, 55–63, https://doi.org/10.14409/natura.v1i33.3791.

LASER MODIFICATION OF LEATHER AND FUR SURFACE TO IMPROVE ITS QUALITY WHEN CONDUCTING FINISHING OPERATIONS

Akmal Yusupovich TOSHEV[1*], Mariya Ivanovna MARKEVICH[2], Tulkin Jumayevich KODIROV[1], Valentina Illarionovna ZHURAVLEVA[3]

[1]Tashkent Institute of Textile and Light Industry, 100100, Shohzhahon-5, Tashkent, Republic of Uzbekistan, email: akmal-toshev-yu@mail.ru

[2]Physical-Technical Institute of the NAS of Belarus, 220141, Academician Kuprevich street 10, Minsk, Belarus 712518272

[3]Military Academy of the Republic of Belarus, 220057, Minsk, Independence Ave 220, Minsk, Belarus

Received: 16.07.2020 Accepted: 09.10.2020 https://doi.org/10.24264/lfj.20.4.2

LASER MODIFICATION OF LEATHER AND FUR SURFACE TO IMPROVE ITS QUALITY WHEN CONDUCTING FINISHING OPERATIONS

ABSTRACT. The article discusses the problems of activation of the surface of the leather tissue by laser exposure, changes in the morphology of the surface of the leather tissue during laser processing, and subsequent covering dyeing of the leather. It is shown that laser treatment changes the structure of the surface of the leather tissue, the bundles of collagen fibers are split, the structure becomes loose, which increases the diffusion of the coating dye reagents, and their reactivity increases without chemical modification. In the process of grinding leather fabric, it is finely cleaned which improves adhesion of the coating paint to the surface of the leather fabric, which improves the quality of leather fabric products. It was shown that laser exposure under the conditions found (pulses with an interval of 3 μs, a duration of 10 ns) does not cause destruction and configuration changes in collagen.

KEY WORDS: leather tissue, pigment concentrate, casein, acrylic aldehyde, copolymer emulsion of butyl acrylate, methyl methacrylate and acrylic acid, coating dyes, laser radiation, diffusion, surface structure of leather tissue, collagen fibers, elemental analysis, magnetic resonance, adhesion

MODIFICAREA CU LASER A SUPRAFEȚEI PIEILOR ȘI BLĂNURILOR PENTRU ÎMBUNĂTĂȚIREA CALITĂȚII ACESTEIA LA EFECTUAREA OPERAȚIUNILOR DE FINISARE

REZUMAT. În articol se discută problemele legate de activare a suprafeței pielii prin expunerea la laser, modificările morfologiei suprafeței pielii în timpul prelucrării cu laser și vopsirea ulterioară a pielii. S-a demonstrat că tratamentul cu laser schimbă structura suprafeței pielii, mănunchiurile de fibre de colagen se scindează, structura devine slăbită, ceea ce crește difuzarea reactivilor din coloranți, iar reactivitatea acestora crește fără modificări chimice. În procesul de șlefuire a pielii, aceasta este curățată fin, ceea ce îmbunătățește aderența vopselei pe suprafața pielii, îmbunătățind în cele din urmă calitatea produselor din piele. S-a demonstrat că expunerea la laser în condițiile specificate (impulsuri cu un interval de 3 μs, durată de 10 ns) nu provoacă distrugeri și modificări în structura colagenului.

CUVINTE CHEIE: țesutul pielii, pigment concentrat, cazeină, aldehidă acrilică, emulsie de copolimer de acrilat de butil, metacrilat de metil și acid acrilic, coloranți pentru vopsire, radiații laser, difuzie, structura suprafeței pielii, fibre de colagen, analiză elementară, rezonanță magnetică, aderență

MODIFICATION AU LASER DE LA SURFACE DU CUIR ET DE LA FOURRURE POUR AMÉLIORER LA QUALITÉ DE LA SURFACE LORS DE LA RÉALISATION DES OPÉRATIONS DE FINITION

RÉSUMÉ. L'article traite des problèmes liés à l'activation de la surface du cuir par exposition au laser, aux changements de morphologie de la surface du cuir pendant le traitement au laser et à la teinture ultérieure du cuir. Il a été démontré que le traitement au laser modifie la structure de la surface du cuir, les faisceaux de fibres de collagène se séparent, la structure s'affaiblit, ce qui augmente la diffusion des réactifs dans les colorants, et leur réactivité augmente sans changement chimique. Lors du ponçage du cuir, celui-ci est finement nettoyée, ce qui améliore l'adhérence de la peinture sur la surface du cuir, améliorant finalement la qualité des produits en cuir. Il a été démontré que l'exposition au laser dans les conditions spécifiées (impulsions avec un intervalle de 3 μs, durée 10 ns) ne cause pas de dommages ou de changements dans la structure du collagène.

MOTS CLÉS : tissu de cuir, concentré de pigments, caséine, aldéhyde acrylique, émulsion de copolymère d'acrylate de butyle, méthacrylate de méthyle et acide acrylique, colorants de revêtement, rayonnement laser, diffusion, structure de la surface du cuir, fibres de collagène, analyse élémentaire, résonance magnétique, adhérence

INTRODUCTION

The variety of existing forms of plasma discharges (smoldering, RFE, RFI, arc, laser, etc.) provides this method of processing with a wide and multi-directional application. An analysis of the types of plasma discharges showed the promise of using some forms for processing a leather semi-finished product, with the aim of

* *Correspondence to: Akmal Yusupovich TOSHEV, Tashkent Institute of Textile and Light Industry, 100100, Shohzhahon-5, Tashkent, Republic of Uzbekistan, email: akmal-toshev-yu@mail.ru*

forming a set of new unique characteristics for it to ensure its competitiveness in the world market [1].

The authors of [2] activated the surface of dyed genuine leather using diffuse air plasma treatment. Plasma was generated by a diffuse coplanar surface barrier discharge (DCSBD). It was found that a plasma treatment time of 10 s is sufficient to reduce the contact angle of water from 85° to 45°. Improving wettability is important for bonding stained leather and better adhesion to leather. An X-ray photoemission spectroscopy study showed that the percentage of oxygen-containing bonds responsible for hydrophobicity increases significantly when treated with plasma. The effect of plasma treatment on the mechanical properties of dyed leather was evaluated using tensile strength measurements. No significant changes in surface morphology and mechanical properties were observed. The results showed that the DCSBD method can provide the high throughput, technical simplicity and economy required by the leather and fur industry.

Currently, the problems of improving the quality of domestic products are relevant. Quality requirements for leather products include not only strength properties, but also an attractive appearance. The quality of products can be improved by creating new materials or giving them new properties by modifying them [3-5]. One of the promising areas for the modification of genuine leather is its processing by laser radiation. The advantage of this method is that it allows you to change the structure of surface layers [6-7]. Such processing is one of the new technologies that allows you to create leather materials with desired characteristics. Cover dyeing of leather fabric is the main finishing process of the technology. The leather then acquires a good appearance and defects in leather fabric hide. Laser exposure allows you to purposefully change the structure and thereby change the properties of natural materials. This technology relates to environmentally friendly processing methods.

The purpose of the work is to establish the features of laser modification of the surface of the leather tissue in the dual pulse mode on the morphology of the surface of the leather tissue in the process of coating dyeing.

EXPERIMENTAL PART

Objects of Study

Leather

Leather is a strong, flexible and durable material obtained by dressing hides in a traditional economy or industrial enterprise. A separate category is fur production. Leather is used in various fields, from the production of shoes and clothing to the binding of books and the manufacture of furniture upholstery and leather wallpapers. Many varieties of leather with various properties are produced. Most often, under these concepts of cattle or small cattle, all types of classical smooth or embossed, but not exotic leather, are meant. Namely, in everyday life for cattle or small cattle - the leather of a cow, bull, buffalo, calf, goat, sheep, lamb, etc.

Cattle skin - the leathers of castrated gobies weighing more than 17 kg in pairs. Depending on the mass, the calf is divided into two groups: light calf - weighing from 17 to 25 kg inclusive, heavy - weighing more than 25 kg. The thickness of the calf is not the same: in the rump 3.5–5.5 mm, in the head 2.5–4.5 mm, in the floors 2-3 mm. With a mass of 17-19 kg, the area of the bovine reaches 300 dm^2, with a mass of more than 30 kg - 500 dm^2. The leathers have a length of 1.75 to 2.4 m, and a width of 1.5-2 m.

Fur Sheepskin

Leathers of adult and semi-adult sheep of fine wool, semi-fine-wool and semi-coarse-haired breeds, as well as crossbreeds, are referred to the fur sheepskin. In this regard, distinguish fine-fleece, half-fine, semi-rough sheepskin.

The production of fur sheepskin comes in approximately the following proportions,%: in terms of wool - wool and half-woolen 88.1, low-wool 11%; by canning methods - wet salted and acid-salted 65.1; dry-salted and fresh-dry 34.9;

according to the fineness of the hair - fine-fleece 19.8, half-fine-crowned 67.3, semi-rough 12.9; by grades - I - 10.1, II - 28, III - 44.4, IV - 17.5.

The quality of the sheepskin depends on the breed, age, conditions of keeping, feeding the sheep, as well as on the quality of the primary processing of sheepskin. Fur sheepskins are characterized by large sizes, their weight varies depending on the degree of contamination, quality of removal, degree of humidity, length and density of wool.

Chrome Tanned Leather

For the study, unpainted chrome leather wastes were used, with the following physicochemical parameters. In %: humidity - 52.4; total ash - 4.8; fatty substances - 3.2; Goal substance - 76.83; chromium oxide - 5.2; and hydrothermal destruction of 92.0 °C.

Methodology and Research

Laser Radiation

In this work, we used laser processing in the regime of double pulses of a sample of genuine leather. An LS-2134D yttrium-aluminum grenade laser (LOTIS, Belarus) with a wavelength of 1064 nm was used, which generated in a two-pulse mode (pulses were separated by a time interval of 3 μs, pulse duration 10 ns). The sample was treated with laser radiation in the energy range 5–40 J at exposure times of 5–40 s [8].

SEM Research and Elemental Analysis

The study of the surface morphology of the leather was carried out using a MIRA-3 scanning electron microscope (Czech Republic) with a system of micro analyzers from Oxford Instruments (Great Britain). The device allows you to simultaneously study the surface morphology of the material, determine the distribution of chemical elements of the sample, and also obtain an image of the object in a wide range of magnifications. The thickness of the leather sample is ~ 500 μm [9].

Magnetic Resonance

Magnetic resonance studies were carried out on a specialized small-sized EPR analyzer Minsk 22 at room temperature. The working wavelength is 3 cm. The maximum value of the magnetic field induction is 450 mT. The modulation frequency of the magnetic field is 30 kHz. To calibrate the signal intensity of the objects of study, we used a sample from a ruby single crystal (Al_2O_3: Cr_3^+). The optimal parameters for recording the working magnetic resonance spectra were chosen in the range of g-factors from 1.5–4.0. During measurements, an additional control of the stability of the spectrometer was carried out by measuring the calibration material of divalent manganese ($MgO{\cdot}Mn_2^+$) [8].

RESULTS AND DISCUSSION

In this work, we used laser processing of leather tissue in the dual pulse mode. An LS-2134D yttrium aluminum garnet laser (LOTIS, Belarus) with a wavelength of 1064 nm was used, which generated in a two-pulse mode (pulses were separated by a time interval of 3 μs, pulse duration 10 ns) [8, 9]. The sample was treated with laser radiation in the energy range 5–40 J at exposure times of 5–40 s. According to [4–5, 7], the evaporation of matter occurs under the influence of the first laser pulse, and a region with an increased temperature and a lower density of air particles forms in the surface layer, which leads to a more complete use of the energy of the second pulse for laser ablation [8].

The study of the surface morphology of leather tissues was studied using a MIRA-3 scanning electron microscope (Czech Republic) with a system of micro analyzers from Oxford Instruments (Great Britain). The device allows you to simultaneously study the surface morphology of the material, determine the distribution of the chemical elements of the sample, and also obtain an image of the object in a wide range of magnifications.

The leather tissue of the fur was also investigated. The thickness of the leather tissue sample is 1.2 mm.

Figure 1 shows the structure of the leather tissue before laser exposure.

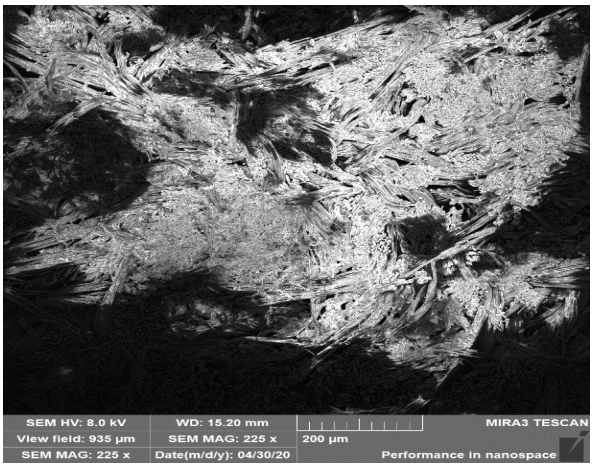


Figure 1. The structure of the leather tissue before laser exposure

From the figure it was established that the structure of the dermis is dense, collagen fibers are adjacent to each other. Moreover, the pore size varies from 20 to 70 microns.

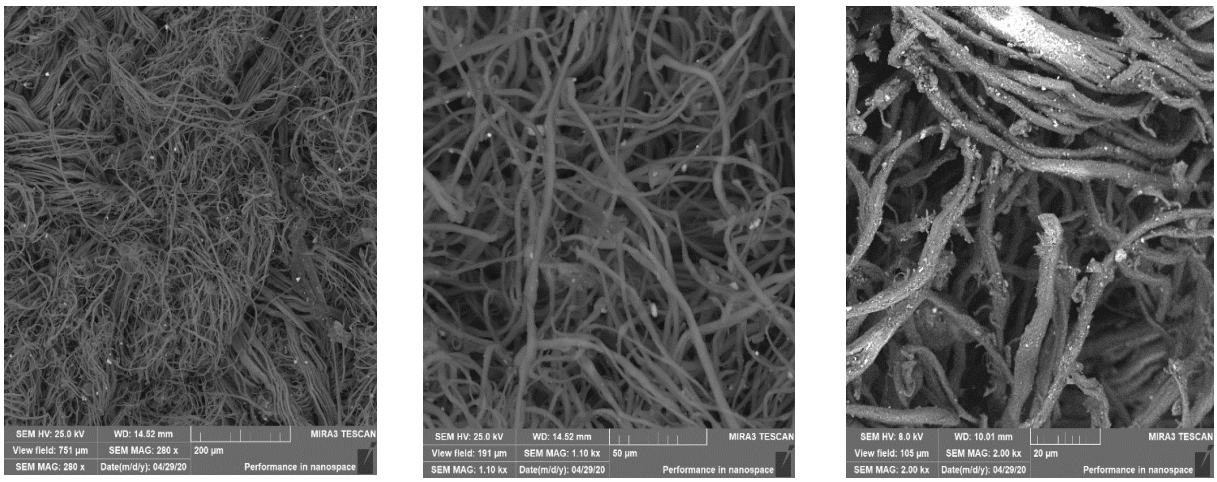
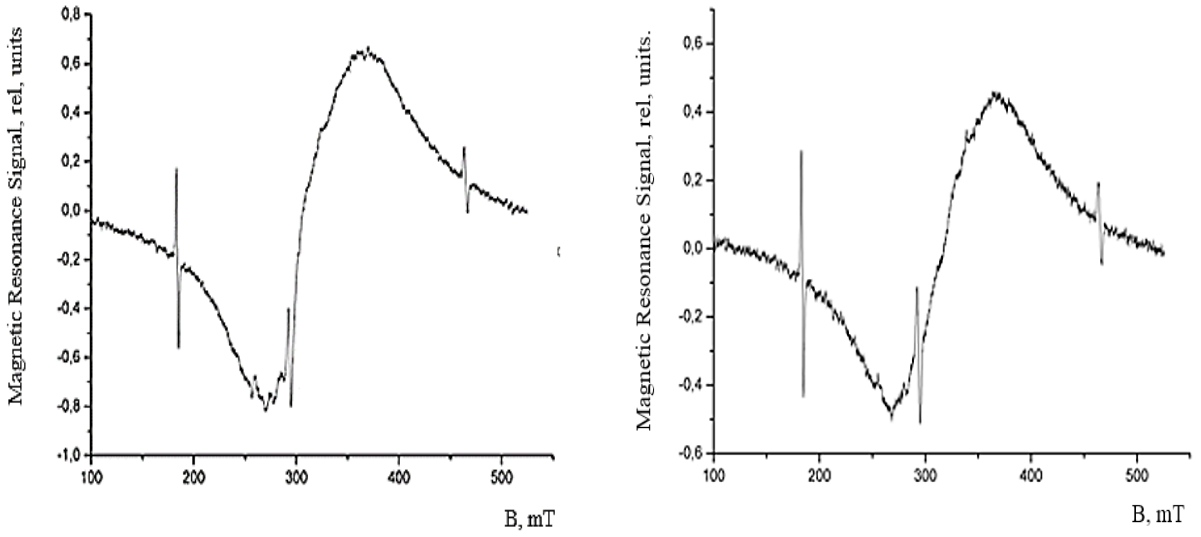


Figure 2. The surface structure of the leather after laser exposure (t=40 s; E=40 J)

From Figure 2 it follows laser irradiation, changes in the structural elements of collagen bundles occur, the structure loosens, the surface relief changes, smoothest. From Figure 2, it follows that the thickness of an individual collagen fiber varies from 2.5 to 5 µm. A comparative analysis of the structure of the leather tissue before and after laser treatment shows that it leads to the splitting of bundles of collagen fibers and changes in the microstructure, which are expressed in the breaking of weak inter fibrillar hydrogen bonds, which contributes to an increase in chemical activity during further covering staining of the leather tissue due to the formation of free radicals (see Figure 3).



a) b)

Figure 3. EPR signal for a sample of leather tissue before (a) and after (b) laser irradiation (energy input 40 J, exposure time 40 s).

An inhomogeneous broadened resonance line with effective g-factor values of 2.3 ± 0.1 and a line width of 96 mT after laser irradiation is recorded on the magnetic resonance spectrum. The wide line after laser exposure indicates the activation of the surface and the chemical activity of the leather tissue for further processing.

In addition, in the process of laser resurfacing, leather tissue is cleaned, which also contributes to improved adhesion during subsequent coating dyeing of the leather.

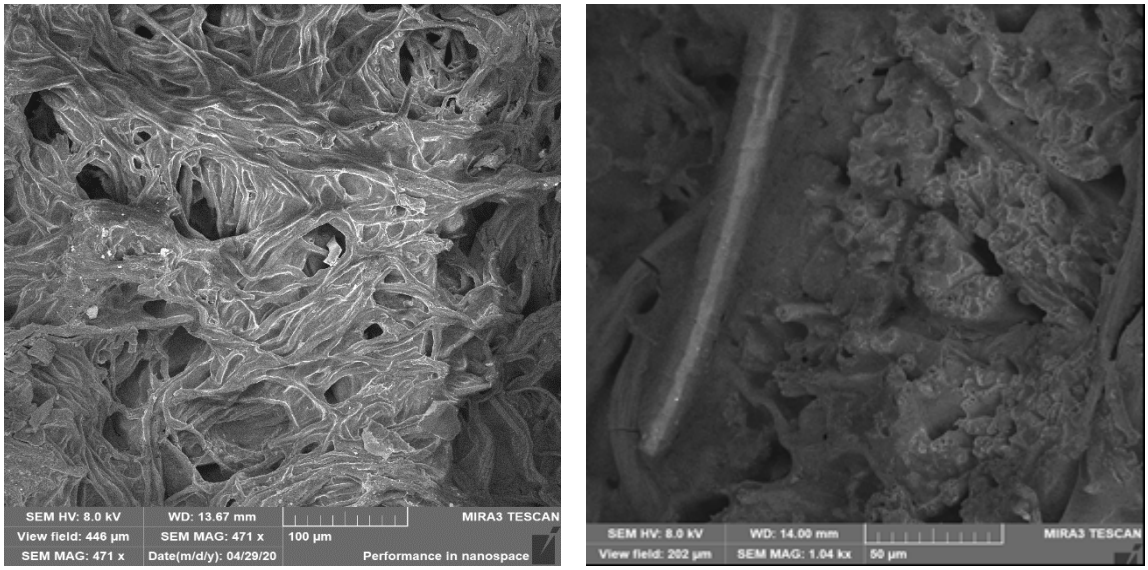
Coatings that are used to finish leather fabric are divided into 4 groups, determined by the type of film former [10-12]: nitrocellulose, acrylate and protein, polymerization (emulsion or latex). None of the film formers are used in their pure form. This is because each of them, along with positive properties, has a number of disadvantages that do not allow creating a coating with a full range of useful properties [13].

In accordance with the technology of coating dyeing, the leather tissue was coated using a copolymer emulsion of butyl acrylate, methyl methacrylate and acrylic acid neutralized with ammonia to pH = 7.0-8.0 with the addition of polyhydrosiloxane - GKZh-94 as a hydrophobizing agent.

The ratio of the components of the film-forming composition was calculated based on the dry residue of the film-forming agent and the pigment content in the coating concentrate. The concentration of pigments in coating compositions ranged from 5 to 10 parts by weight. From the mass fraction of the polymer, namely 5, 6, 7, 8, 9, 10 parts by weight.

Upon receipt and study of the properties of coating compositions, the problem was solved to improve the adhesive properties during long-term operation of the products. The coating compositions studied included a pigment concentrate, casein, acrylic aldehyde, a copolymer emulsion of butyl acrylate, methyl methacrylate and acrylic acid (in a ratio of 35.37: 46.68: 17.95, respectively).

Figure 4 shows the morphology of the surface of the leather tissue after laser exposure and coating dyeing (at various magnifications).



a) b)

Figure 4. The morphology of the surface of the leather tissue after laser exposure and coating dyeing

As can be seen from Figure 4, topcoat dyeing of leather tissue decorates and slightly changes its appearance. In the process of painting, the fibrils are glued together, the size of the bundles varies from 12 to 40 microns. Figure 5 shows the elemental analysis of skin tissue after laser treatment and coating dyeing. Black topcoat was applied to the surface of the sample 2 times, and after drying, further research was conducted.

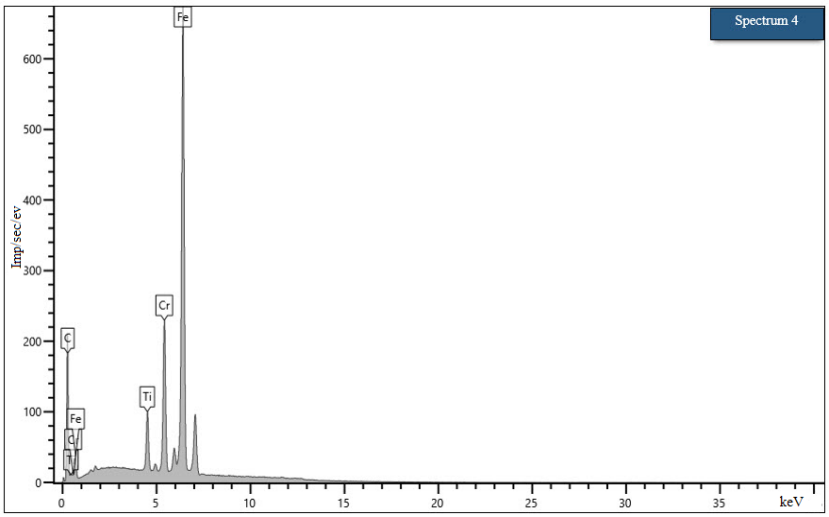


Figure 5. Elemental analysis of skin tissue after coating dyeing

Table 1: Spectrum results depending on the ratio of elemental analysis

№	Result Type, Spectrum Label	Spectrum 4, mass /%
1.	C	41.25
2.	Ti	3.08
3.	Cr	9.91
4.	Fe	45.76
	Total	100.00

As can be seen from the analysis (Figure 5 and Table 1), Ti, Cr, Fe are present on the surface of the sample.

The results of the study of the influence of the chemical nature of the coating dyes on the gasoline resistance of a multilayer coating of leather tissue are presented in Table 2.

Table 2: Coating adhesion coating dyeing obtained at room temperature

Index	Laser free impacts	After laser impact
The amount of swelling of the sample in gasoline for 2 hours, %	82	24
Adsorption of water, ml	0.33	0.46
Adhesion, N/m		
in gasoline	548	813
in dry condition	2148	2578

Analyzing the data obtained in the table, it can be noted that the proposed samples treated with a coating dye after laser exposure form films having almost four times less swelling than samples treated with a coating dye without laser exposure. Reducing the amount of swelling of the lower coating layer reduces the amount of swelling of the coating as a whole, which possibly slows down the further diffusion of gasoline, and thereby reduces its accumulation at the skin-fabric coating interface and reduces the wedging effect of the aggressive environment. This is facilitated by the better compatibility of the coating dyes with the front surface after laser exposure. The value of the adhesion of the coating and its gas resistance, along with the indicated factors, can be associated with the interaction of the laser exposure of the skin tissue with the coating dyes.

CONCLUSIONS

Laser modification of a sample of the surface of the skin tissue was performed using a laser generating in a double-pulse mode (pulses separated by a time interval of 3 μs, pulse duration 10 ns) with a wavelength of 1064 nm with an input energy of 5-40 J and an exposure time of 5-40 s followed by a coating dyeing. It was shown that laser treatment changes the structure of the surface of the skin tissue, the bundles of collagen fibers are split, the structure becomes loose, which increases the diffusion of the coating dye reagents, and their reactivity increases without chemical modification. In the process of grinding leather fabric, it is finely cleaned, which helps to improve the adhesion of topcoat paint to the surface of leather fabric, which will improve the quality of leather fabric products. It is shown that laser exposure under the conditions found does not cause destruction and configurational changes in collagen.

REFERENCES

1. Pankova, E.A., Scientific and technological fundamentals of fur finishing using plasma-chemical processing, nanomaterials and nanocoatings: abstract, Dis. Doct. Tech. Sciences, Kazan, **2011**, p. 38.
2. Stepanova, V., Kelar, J., Slavicek, P., Chlupova, S., Stupavska, M., Jurmanova, J., Cernak, M., Surface modification of natural leather using diffuse ambient air plasma, *Int J Adhes Adhes*, **2017**, 77, 198-203, https://doi.org/10.1016/j.ijadhadh.2017.05.004.
3. Voznesensky, E.F., Khasanshin, R.R., Krasina, I.V., Abdullin, I.Sh., Safin, R.R., Aminov, L.I., General principles for the modification of natural fibrous materials of various origin in high-pressure plasma - low pressure discharge, *Bulletin of Kazan Technological University*, **2009**, № 1/5, 304-307.
4. Strakhov, I.P., Shestakova, I.S., Kutsidi, D.A., Chemistry and technology of leather and fur, M- Legprombytizdat, **1985**, p. 496.
5. Tikhonova, N.V., Abdullin, I.Sh., Mahotkina, L.Yu., High-frequency plasma of reduced pressure in the manufacture of shoes, *Bulletin of Kazan Technological University*, **2009**, № 1/5, 131-136.
6. Kodirov, T.J., Markevich, M.I., Malyshko, A.N., Zhuravleva, V.I., Sodikov, N.A., Researches of Morphological Structure, Element Composition and Natural Leather Adsorption

by Exposure to Laser Radiation, *Int J Recent Technol Eng*, **2020**, 8, 6, 4880-4884, https://doi.org/10.35940/ijrte.F8853.038620.
7. Narica, P., Laizāne, R., Pacejs, A., Mežinska, S., Analysis of Laser Processing of Artificial Leather, Environment. Technology. Resources. Proceedings of the 12th International Scientific and Practical Conference, Rezekne, Latvia, **2019**, Vol. III, 177-183, https://doi.org/10.17770/etr2019vol3.4040.
8. Markevich, M.I., Zhigulin, D.V., Zhuravleva, V.I., Stelmakh, V.F., Chaplanov, A.M., Impact of pulsed laser radiation on basalt fabric, *Polymer Materials and Technologies*, **2018**, 4, 3, 51-56, https://doi.org/10.32864/polymmattech-2018-4-3-51-56.
9. Adashkevich, S.V., Markevich, M.I., Malyshko, A.N., Zhuravleva, V.I., Stelmakh, V.F., Chaplanov, A.M., Surface morphology and magnetic resonance absorption of microwave energy by foam treated with pulsed laser radiation, *Polymer Materials and Technologies*, **2018**, 4, 1, 27–31.
10. Bedenko, V.G., Bezyazychnaya, L.A., Lazebnaya, O.M., Materials for the leather and fur industry, *Leather and Footwear Industry*, **2006**, № 6, 22-23.
11. Kazas, V.M., Poelueva, A.P., Fur production, M: Folk art, **2006**, P 304.
12. Toshev, A., Kodirov, T., Influence of Inorganic Pigments on the Properties of Acrylic Coatings for Finishing Fur Sheepskin, *Int J Adv Sci Technol*, **2020**, 29, 5, 12547-12555.
13. Chichvarina, L.I., Tikhomirova, E.N., The use of modern hydrophobic preparations to improve the operational properties of fur velor, Development of the fur industry of Russia: theses and reports of the VI Interregional scientific and practical conference, M.: NIIMP, **2004**, 43-44.

EFFECTIVENESS OF PRESSURE-RELIEVING SHOES/INSOLES ON LOWERING THE PLANTAR PRESSURE OF DIABETIC FOOT: A META-ANALYSIS

Xinrong ZHI[1], Weiping WANG[2], Bo XU[1], Jin ZHOU[1*]

[1]National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University; Chengdu 610065, P. R. China; 1436160237@qq.com, 228447319@qq.com, zj_scu@scu.edu.cn

[2]452 Hospital of PLA; Chengdu 610065, P. R. China; wwp021006@sina.com

Received: 15.05.2020 Accepted: 28.08.2020 https://doi.org/10.24264/lfj.20.4.3

EFFECTIVENESS OF PRESSURE-RELIEVING SHOES/INSOLES ON LOWERING THE PLANTAR PRESSURE OF DIABETIC FOOT: A META-ANALYSIS

ABSTRACT. Since current reports demonstrated a higher prevalence of foot ulcers in diabetic patients who suffer from foot complication, the preventing occurrence of foot ulcers were the primary target in foot care. Clinical consensus introduced a variety of pressure-relieving products to diabetic patients and clinicians prescribed these products to their patients and recommended them used in daily life. However, available data were still controversial and whether these products could effectively reduce plantar pressure or not were uncertain. Thereby, this meta-analysis aimed first to summary all relevant findings in current database and secondly to explore whether pressure-relieving insoles/shoes can really relieve plantar pressure and what's differences between customized products (shoes/insoles) and standard ones in reducing plantar pressure. We first searched published articles cited from Web of Science, Medline via OVID, CINAHL, SCOPUS, INFORMIT, Cochrane Central and EMBASE via OVID. Then we filtered observational studies reporting experimental effect of pressure-relieving insoles/shoes. Meanwhile, we set up primary outcome as overall mean peak plantar pressure (MPP) and secondary outcomes as MPP at various plantar regions and MPP at insoles/shoes with various structure designs. Our results show that pressure-relieving products (shoes/insoles) did lower the amplitude of pressure concentration; effect of custom-made and pre-fabricated products on pressure-relieving were similar. These findings suggested that no matter pressure-relieving products were custom-made or prefabricated standard one, if they were designed targeting to increase overall plantar contact areas, such as designed based on plantar model, or to provide extra arch supports or plug-in structures to transfer pressure concentration, they were all useful in diabetic foot care to prevent occurrence of ulceration. Overall, it is recommended that diabetic patients shall wear pressure-relieving insoles/shoes while walking.

KEY WORDS: diabetes mellitus, footwear, foot ulcer, plantar pressure

EFICACITATEA ÎNCĂLȚĂMINTEI / BRANȚURILOR ÎN REDUCEREA PRESIUNII PLANTARE ÎN CAZUL DIABETICILOR: O META-ANALIZĂ

REZUMAT. Întrucât rapoartele actuale au demonstrat o prevalență mai mare a ulcerelor piciorului la pacienții diabetici care suferă de complicații ale piciorului, prevenirea apariției ulcerațiilor a fost ținta principală în îngrijirea piciorului. În urma consensului clinic s-a introdus o varietate de produse pentru ameliorarea presiunii la pacienții cu diabet zaharat, iar clinicienii au prescris aceste produse pacienților și le-au recomandat utilizarea acestora în viața de zi cu zi. Cu toate acestea, datele disponibile încă sunt controversate și este incert dacă aceste produse ar putea reduce efectiv presiunea plantară sau nu. Prin urmare, această meta-analiză a avut ca scop mai întâi să treacă în revistă toate constatările relevante din baza de date actuală și, în al doilea rând, să determine dacă branțurile / încălțămintea cu funcție de reducere a presiunii pot ameliora cu adevărat presiunea plantară și care sunt diferențele dintre produsele personalizate (încălțăminte / branțuri) și cele standard în ceea ce privește reducerea presiunii plantare. S-au căutat mai întâi articole indexate în Web of Science, Medline via OVID, CINAHL, SCOPUS, INFORMIT, Cochrane Central și EMBASE via OVID. Apoi s-au filtrat studiile observaționale care raportează efectul experimental al branțurilor / încălțămintei cu funcție de reducere a presiunii. Între timp, s-a stabilit ca rezultat primar media generală a maximelor de presiune plantară (MPP) și ca rezultate secundare, MPP în diferite regiuni plantare și MPP la branțuri / încălțăminte cu diferite modele structurale. Rezultatele arată că produsele de reducere a presiunii (încălțăminte / branțuri) au scăzut amplitudinea concentrației de presiune, iar efectul produselor personalizate și prefabricate în ceea ce privește ameliorarea presiunii a fost similar. Aceste constatări au sugerat că, indiferent dacă produsele de reducere a presiunii au fost fabricate la comandă sau prefabricate standard, dacă au fost concepute pentru a crește suprafața de contact în zona plantară, cum ar fi cele proiectate pe baza modelului plantar, sau pentru a oferi suport plantar suplimentar sau structuri „plug-in" pentru a transfera concentrația de presiune, toate produsele au fost utile în îngrijirea piciorului diabetic pentru a preveni apariția ulcerațiilor. În general, se recomandă ca pacienții cu diabet zaharat să poarte branțuri / încălțăminte pentru ameliorarea presiunii în timpul mersului.

CUVINTE CHEIE: diabet, încălțăminte, ulcerul piciorului, presiunea plantară

EFFICACITÉ DES CHAUSSURES / SEMELLES DE DÉCHARGE SUR LA RÉDUCTION DE LA PRESSION PLANTAIRE DU PIED DIABÉTIQUE : UNE MÉTA-ANALYSE

RÉSUMÉ. Étant donné que les rapports actuels ont démontré une prévalence plus élevée des ulcères du pied chez les patients diabétiques qui souffrent de complications du pied, la prévention des ulcères du pied était la principale cible des soins des pieds. Le consensus clinique a introduit une variété de produits anti-pression pour les patients diabétiques et les cliniciens ont prescrit ces produits à leurs patients et les ont recommandés dans la vie quotidienne. Cependant, les données disponibles étaient encore controversées et la question de savoir si ces produits pouvaient effectivement réduire la pression plantaire ou non était incertaine. Ainsi, cette méta-analyse visait d'abord à résumer tous les résultats pertinents de la base de données actuelle et, d'autre part, à explorer si les semelles / chaussures de décharge peuvent vraiment soulager la pression plantaire et quelles sont les différences entre les produits personnalisés (chaussures / semelles) et les produits standard pour réduire la pression plantaire. On a d'abord recherché des articles cités à partir de Web of Science, Medline via OVID, CINAHL, SCOPUS, INFORMIT, Cochrane Central et EMBASE via OVID. Ensuite, on a filtré les études observationnelles rapportant l'effet expérimental des semelles / chaussures de décharge. Pendant ce temps, on a défini le résultat principal en tant que pic de pression plantaire moyenne

** Correspondence to: Assoc. Prof. Dr. Jin Zhou. National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University; Chengdu 610065, P. R. China; Fax: 008613880467494. zj_scu@scu.edu.cn*

(MPP) globale et les résultats secondaires en tant que MPP dans diverses régions plantaires et MPP au niveau des semelles / chaussures avec différentes conceptions de structure. Les résultats montrent que les produits de soulagement de la pression (chaussures / semelles) ont réduit l›amplitude de la concentration de pression ; l'effet des produits sur mesure et préfabriqués sur le soulagement de la pression était similaire. Ces résultats suggèrent que peu importe que les produits de soulagement de la pression soient fabriqués sur mesure ou préfabriqués en standard, s'ils ont été conçus pour augmenter les zones de contact plantaires globales, telles que les produits conçus sur la base d'un modèle plantaire, ou pour fournir des supports plantaires supplémentaires ou des structures enfichables pour transférer la concentration de pression, ils étaient tous utiles dans les soins du pied diabétique pour éviter l'apparition d'ulcères. Dans l'ensemble, il est recommandé aux patients diabétiques de porter des semelles / chaussures anti-pression tout en marchant.
MOTS CLÉS : diabète, chaussure, ulcère du pied, pression plantaire

INTRODUCTION

More than 0.5 billion patients worldwide are diagnosed with diabetic mellitus (DM), which severely lowers the quality of life and even threatens the life of patients. DM cannot be cured and can only be controlled in a general level; however, poor control measures can result in complications, such as nephropathy and diabetic foot, which contribute to other serious consequences [1].

Diabetic foot, usually found in the lower limbs, is characterised by diabetic sensory neuropathy, limited joint activity, poor immune function, peripheral artery disease, foot ulcer and Charcot joint disease [2]. These complications provide an ideal environment for unrecognised tissue injury, which leads to ulceration [3]. Currently, the prevalence of foot ulcer ranges from 4% to 10% in the DM group, and the annual incidence by population ranges from 1.0% to 4.1% [4]. Furthermore, foot ulcers are the main cause of amputation, and the possibility of ulceration is 10–30 times higher in patients with DM than in healthy individuals [5, 6]. In fact, one person is amputated every 30 s in the world because of diabetes [7].

Clinical consensus approved that abnormal pressure and pressure concentration are highly correlated with ulceration [8, 9]. Those abnormal pressures might be attributed to foot deformities, wearing unsuitable shoes or trauma caused by accident. Sites with abnormal pressure then develop muscle/soft tissue constrains, which might further deteriorate as pressure ulcers and even amputations. According to existing literature, the foot pressure of patients with diabetes is higher than that of people without diabetes [10-13]. In particular, ulcer sites, either the new one or with previous ulcer history, are recorded with high pressure distribution [10, 13]. Hence, avoiding high pressure load and implementing pressure relief are the primary tasks in diabetic foot care.

Protective shoes and insoles are prescribed to patients with DM because they lower pressure amplitudes and thus avoid foot ulcers [14]. However, a descriptive study following ulcerated patients over 2 years found that re-ulceration occurs in 72% of patients who resumed wearing their own footwear compared with 26% of patients who continued wearing prescribed footwear [15]. Other studies disclosed the positive effectiveness of various protective shoes/insoles on pressure relief [16-18]. Thereby, the International Consensus for Diabetic Foot [19] suggested that wearing correct shoes/insoles is a direct and effective protocol in diabetic foot care. However, a few controversial outcomes were observed in the literature [20]. In specific, (1) whether or not pressure-relieving products (shoes/insoles) actually lower the pressure concentration under feet and (2) whether or not customised products (shoes/insoles) are superior to standard pressure-relieving ones remain uncertain.

Therefore, this study assessed the current literature by meta-analysis and then quantitatively evaluated the pressure-relieving effect of shoes and insoles and those with varied design characteristics, such as custom made or standard one.

METHODS

Search Strategy and Quality Assessment

The following databases published until 18th March 2019 were selected: Web of Science (1994–2018), Medline via OVID (1994–2018), CINAHL (1994–2018), SCOPUS (1994–2018), INFORMIT, Cochrane Central (2000–2018) and EMBASE via OVID (1994–2018). A primary author conducted the searching action; thereafter, this author repeatedly searched in August 2019 to identify any new articles of relevance. Keywords were elected according to a meeting among authors of this study: 'diabetes*', 'diabetic foot',

'shoe*', 'pressure*', 'insole', 'ulcer', 'relieving', 'offload*', 'random', 'custom-made' and 'prevention'.

Two blinded authors applied the tools in Review Manager (Version 5.3, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) for risk of bias assessment. A quality assessment tool was also adapted from validated measures with addition of questions specific to the pressure-relieving function of shoes/insoles [21]. Quality scores of ≥45, 30–45, 20–30 and ≤20 were defined as excellent, good, fair and poor, respectively [10]. A primary author checked both records of risk of bias and quality of studies for consistency.

Study Selection

Studies were included in the meta-analysis if they met all the below inclusion criteria. Potential studies identified for inclusion were reviewed independently by two authors using those inclusion criteria. Group discussions were held to resolve any disagreements in the inclusion of studies.

- An observational study;
- Subjects included had no current ulcers or ulcers in their feet already recovered;
- The study was reported in or available in the English language;
- Plantar pressure values were reported in two groups: one included pressure-relieving shoes/insoles, including custom-made and standard products, and the other included control products.
- Plantar pressure values were reported as the mean peak plantar pressure (MPP) in any acceptable pressure unit (KPa, N/kg^2 or similar);
- Overall plantar pressure or regional ones, such as fore foot, mid foot and rear foot were reported
- Experimental shoes or insoles were reported with details.
- Studies were excluded if they meet any of the following criteria:
- Other pressure-relieving protocols rather than insoles/shoes, such as callus debridement and podiatry.
- Foot pressure data did not provide extractable mean and standard deviation (SD);
- Foot pressure data were not provided by sufficiently large samples. Studies only reported one person's data;
- No randomised experiments were conducted in the study;
- Only pressure plate measures were reported, as pressure plate recorded the pressure distribution of outsoles.
- Full-text manuscripts could not be acquired.

Primary and Secondary Outcomes

Three primary outcomes were set up: overall MPP, MPP in pressure-relieving insoles and MPP in pressure-relieving shoes. Then, seven secondary outcomes were nominated: MPP at the fore foot, MPP at the mid foot, MPP at the rear foot, MPP in custom-made insoles, MPP in standard pressure-relieving insoles, MPP in custom-made shoes and MPP in standard pressure-relieving shoes. We defined that the toes, MTH1-5, hallux were included into fore foot regions and the heel was divided into rear foot regions.

Data Extraction and Synthesis

Data extraction was first completed by one primary author, and the extracted data were checked by another primary author for any omissions. At first, descriptive data such as age, sample size, types and structures of shoes/insoles were recorded for each study. Afterwards, numerical data (mean and SD) for each plantar pressure variable were carefully identified and extracted. When studies assessed insoles/shoes with a variety of structure designs or modifications and more than one comparison were reported, each comparison was extracted and included for analysis. Considering that plantar pressure data were measured and reported in terms of feet unit, we extracted each foot data. Anatomical locations were unspecified, and we defaulted that all the included studies have a unified criterion for anatomical definition.

Statistical Methods

Standardised mean differences (SMD) were calculated by Cohen's d [22] and then input in meta-analyses. Results were expressed as SMD with 95% confidence intervals (95%CI)

and p-values. Furthermore, weighted means (according to the sample size factor) were randomly calculated for reported variables. The Z test and I2 statistics were used to assess statistical heterogeneity between studies. I2 with values of 25%, 50% and 75% were considered as low, moderate and high heterogeneity, respectively [23]. All meta-analysis models were executed by the primary author using the software package of Review Manager.

RESULTS

Search Results

The flowchart of literature filtering is shown in Figure 1, and 18 studies were retained for further analysis [20, 24-40].

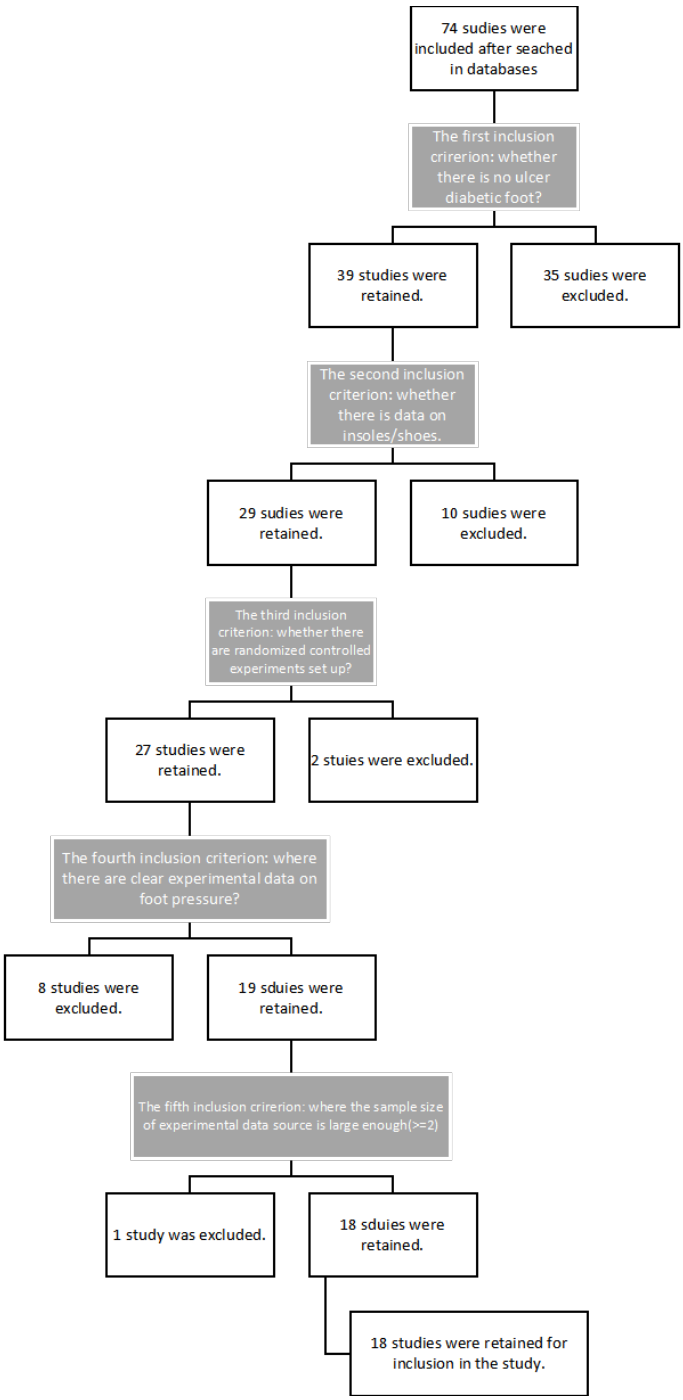
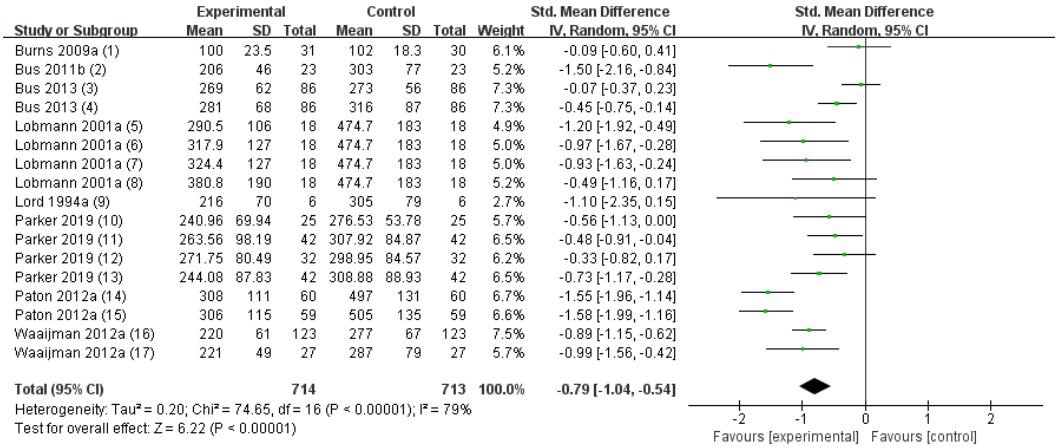


Figure 1. Flowchart of filtering of literature

Primary Outcomes

Overall MPP was reported by all 17 comparisons from 8 studies. Meta-analysis combining the data from 17 comparisons (pressure-relieving insoles n = 714; standard control insoles n = 713) suggested that pressure-relieving shoes/insoles significantly reduced the MPP for patients with diabetes in comparison with the control ones (SMD = −0.74, 95% CI = −1.00–(−0.49), Z = 5.70, P < 0.0001). The heterogeneity between studies was high (I2 = 80%) (Figure 2).



Footnotes
(1) Custom orthoses Vs Sham
(2) fully customized footwear Vs custom molded insoles in an extradepth shoe
(3) Improved footwear Vs Usual care
(4) Improved footwear Vs Usual care
(5) 2 weeks after insole protection Vs Baseline
(6) 8 weeks after insole protection Vs Baseline
(7) 6 months after insole protection Vs Baseline
(8) 1 year after insole protection Vs Baseline
(9) molded inserts Vs flat inserts
(10) Traditional Supply Chain: Orthotic Vs Control
(11) Traditional Supply Chain: Orthotic Vs Control
(12) Digital Supply Chain: Orthotic Vs Control
(13) Digital Supply Chain: Orthotic Vs Control
(14) Custom-made functional insole: Baseline Vs after 6-months
(15) Custom-made group Vs Prefabricated group
(16) custom-made insoles in custom-made shoes Vs custom-made insoles in offthe-shelf extra-depth shoes
(17) custom-made insoles in custom-made shoes Vs custom-made insoles in offthe-shelf extra-depth shoes

Figure 2. Forest plot of the overall Peak Plantar Pressure (MPP)

Pressure-relieving insoles significantly reduced the MPP for patients with diabetes in comparison with the control shoes (SMD = −1.19, 95%CI = −1.45–(−0.93), Z = 9.01, P < 0.00001, I2 = 87%). Similarly, pressure-relieving insoles significantly reduced the MPP for patients with diabetes in comparison with the control insoles (SMD = −0.42, 95% CI = −0.55–(−0.29), Z = 6.35, P < 0.00001, I2 = 85%).

Secondary Outcomes

MPP at Various Plantar Regions

MPP at the forefoot was significantly lowered (SMD = −0.70, 95% CI = −0.85–(−0.54), Z = 8.79, P < 0.00001). The heterogeneity between studies was high (I2 = 85%).

MPP at the rear foot was also significantly attenuated by pressure-relieving insoles (SMD = −1.00, 95% CI = −1.27–(−0.74), Z = 7.41, p < 0.00001, I2 = 73%).

Although a slight pressure-relieving effect on the mid foot area was observed in contrast with the control insoles (SMD = 0.00, 95% CI = −0.18–0.19, Z = 0.03, P = 0.97), heterogeneity between studies was low (I2 = 46%).

MPP at Various Shoes

MPP with custom-made shoes was divided by three studies, and four comparisons were reported. Meta-analysis combining the data from four comparisons (pressure-relieving shoes n = 211; standard control shoes n = 211) implied that with the favour of custom-made shoes, the

MPP with custom-made shoes was significantly lowered (SMD = −0.75, 95% CI = −1.33–(−0.17), Z = 2.52, P = 0.01). The heterogeneity between studies was high (I2 = 86%) (Figure 3).

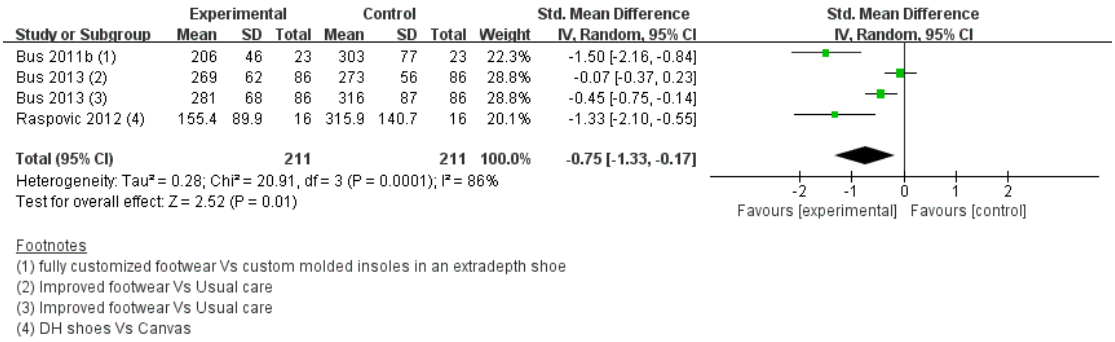
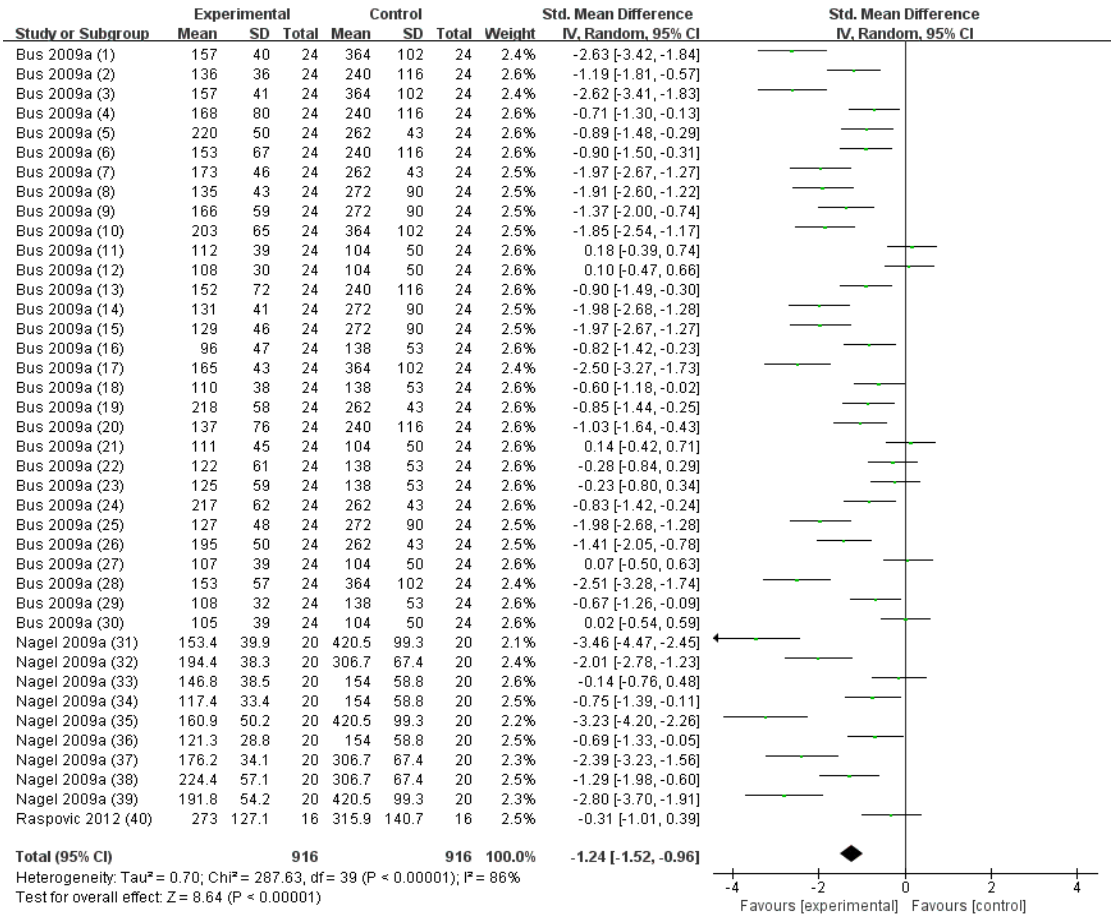


Figure 3. Forest plot of the Peak Plantar Pressure (MPP) in custom-made pressure-relieving shoes

The same tendency was also found for standard pressure-relieving shoes and meta-analysis combining data 49 comparisons from three studies (pressure-relieving insoles n = 1096; standard control insoles n = 1096) indicated that the MPP under the standard insoles was also slightly attenuated by pressure-relieving insoles (SMD = −1.34, 95% CI = −1.60–(−1.07), Z = 9.89, p < 0.00001, I2 = 87%) (Figure 4).



Study or Subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Bus 2009a (1)	157	40	24	364	102	24	2.4%	-2.63 [-3.42, -1.84]
Bus 2009a (2)	136	36	24	240	116	24	2.6%	-1.19 [-1.81, -0.57]
Bus 2009a (3)	157	41	24	364	102	24	2.4%	-2.62 [-3.41, -1.83]
Bus 2009a (4)	168	80	24	240	116	24	2.6%	-0.71 [-1.30, -0.13]
Bus 2009a (5)	220	50	24	262	43	24	2.6%	-0.89 [-1.48, -0.29]
Bus 2009a (6)	153	67	24	240	116	24	2.6%	-0.90 [-1.50, -0.31]
Bus 2009a (7)	173	46	24	262	43	24	2.5%	-1.97 [-2.67, -1.27]
Bus 2009a (8)	135	43	24	272	90	24	2.5%	-1.91 [-2.60, -1.22]
Bus 2009a (9)	166	59	24	272	90	24	2.5%	-1.37 [-2.00, -0.74]
Bus 2009a (10)	203	65	24	364	102	24	2.5%	-1.85 [-2.54, -1.17]
Bus 2009a (11)	112	39	24	104	50	24	2.6%	0.18 [-0.39, 0.74]
Bus 2009a (12)	108	30	24	104	50	24	2.6%	0.10 [-0.47, 0.66]
Bus 2009a (13)	152	72	24	240	116	24	2.6%	-0.90 [-1.49, -0.30]
Bus 2009a (14)	131	41	24	272	90	24	2.5%	-1.98 [-2.68, -1.28]
Bus 2009a (15)	129	46	24	272	90	24	2.5%	-1.97 [-2.67, -1.27]
Bus 2009a (16)	96	47	24	138	53	24	2.6%	-0.82 [-1.42, -0.23]
Bus 2009a (17)	165	43	24	364	102	24	2.4%	-2.50 [-3.27, -1.73]
Bus 2009a (18)	110	38	24	138	53	24	2.6%	-0.60 [-1.18, -0.02]
Bus 2009a (19)	218	58	24	262	43	24	2.6%	-0.85 [-1.44, -0.25]
Bus 2009a (20)	137	76	24	240	116	24	2.6%	-1.03 [-1.64, -0.43]
Bus 2009a (21)	111	45	24	104	50	24	2.6%	0.14 [-0.42, 0.71]
Bus 2009a (22)	122	61	24	138	53	24	2.6%	-0.28 [-0.84, 0.29]
Bus 2009a (23)	125	59	24	138	53	24	2.6%	-0.23 [-0.80, 0.34]
Bus 2009a (24)	217	62	24	262	43	24	2.6%	-0.83 [-1.42, -0.24]
Bus 2009a (25)	127	48	24	272	90	24	2.5%	-1.98 [-2.68, -1.28]
Bus 2009a (26)	195	50	24	262	43	24	2.5%	-1.41 [-2.05, -0.78]
Bus 2009a (27)	107	39	24	104	50	24	2.6%	0.07 [-0.50, 0.63]
Bus 2009a (28)	153	57	24	364	102	24	2.4%	-2.51 [-3.28, -1.74]
Bus 2009a (29)	108	32	24	138	53	24	2.6%	-0.67 [-1.26, -0.09]
Bus 2009a (30)	105	39	24	104	50	24	2.6%	0.02 [-0.54, 0.59]
Nagel 2009a (31)	153.4	39.9	20	420.5	99.3	20	2.1%	-3.46 [-4.47, -2.45]
Nagel 2009a (32)	194.4	38.3	20	306.7	67.4	20	2.4%	-2.01 [-2.78, -1.23]
Nagel 2009a (33)	146.8	38.5	20	154	58.8	20	2.6%	-0.14 [-0.76, 0.48]
Nagel 2009a (34)	117.4	33.4	20	154	58.8	20	2.5%	-0.75 [-1.39, -0.11]
Nagel 2009a (35)	160.9	50.2	20	420.5	99.3	20	2.2%	-3.23 [-4.20, -2.26]
Nagel 2009a (36)	121.3	28.8	20	154	58.8	20	2.5%	-0.69 [-1.33, -0.05]
Nagel 2009a (37)	176.2	34.1	20	306.7	67.4	20	2.3%	-2.39 [-3.23, -1.56]
Nagel 2009a (38)	224.4	57.1	20	306.7	67.4	20	2.5%	-1.29 [-1.98, -0.60]
Nagel 2009a (39)	191.8	54.2	20	420.5	99.3	20	2.3%	-2.80 [-3.70, -1.91]
Raspovic 2012 (40)	273	127.1	16	315.9	140.7	16	2.5%	-0.31 [-1.01, 0.39]
Total (95% CI)			**916**			**916**	**100.0%**	**-1.24 [-1.52, -0.96]**

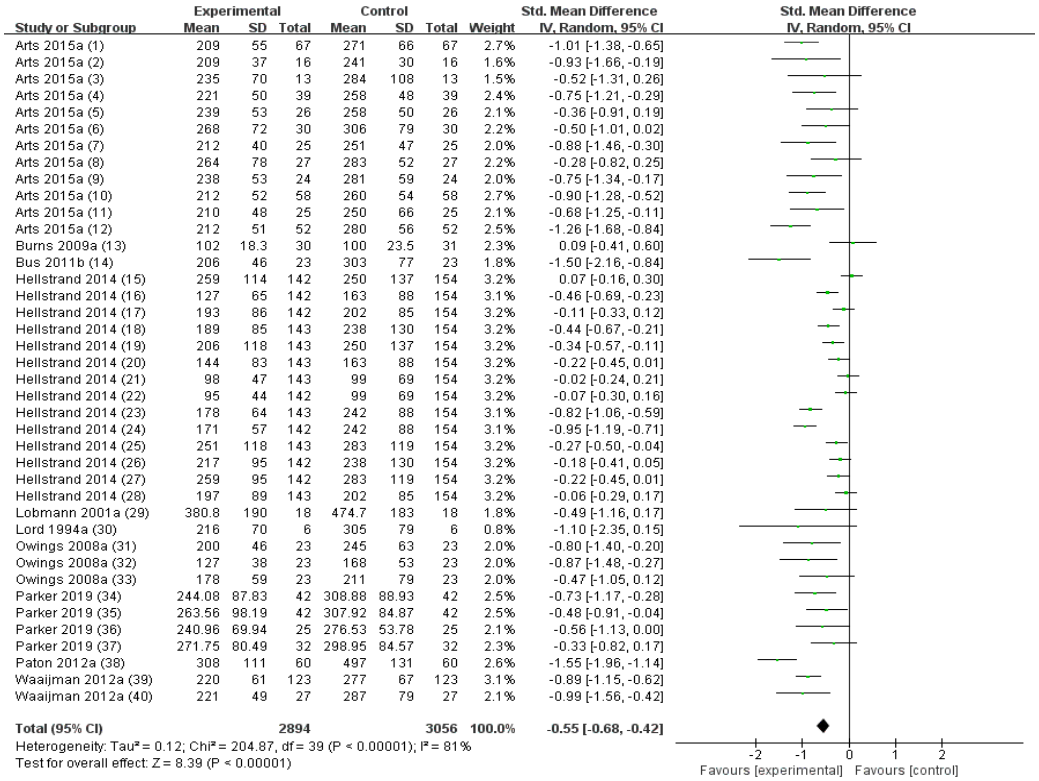
Heterogeneity: Tau² = 0.70; Chi² = 287.63, df = 39 (P < 0.00001); I² = 86%
Test for overall effect: Z = 8.64 (P < 0.00001)

Footnotes
(1) Rattenhuber Talus FOS Vs Control shoe
(2) Thanner Cabrio FOS Vs Control shoe
(3) Thanner Cabrio FOS Vs Control shoe
(4) Fior&Gentz Hannover FOS Vs Control shoe
(5) Thanner Cabrio FOS Vs Control shoe
(6) Mabal cast shoe Vs Control shoe
(7) Mabal cast shoe Vs Control shoe
(8) Fior&Gentz Hannover FOS Vs Control shoe
(9) Mabal cast shoe Vs Control shoe
(10) Mabal cast shoe Vs Control shoe
(11) Fior&Gentz Luneburg FOS Vs Control shoe
(12) Fior&Gentz Hannover FOS Vs Control shoe
(13) Fior&Gentz Luneburg FOS Vs Control shoe
(14) Thanner Cabrio FOS Vs Control shoe
(15) Rattenhuber Talus FOS Vs Control shoe
(16) Rattenhuber Talus FOS Vs Control shoe
(17) Fior&Gentz Hannover FOS Vs Control shoe
(18) Mabal cast shoe Vs Control shoe
(19) Fior&Gentz Luneburg FOS Vs Control shoe
(20) Rattenhuber Talus FOS Vs Control shoe
(21) Mabal cast shoe Vs Control shoe
(22) Fior&Gentz Luneburg FOS Vs Control shoe
(23) Fior&Gentz Hannover FOS Vs Control shoe
(24) Rattenhuber Talus FOS Vs Control shoe
(25) Fior&Gentz Luneburg FOS Vs Control shoe
(26) Fior&Gentz Hannover FOS Vs Control shoe
(27) Rattenhuber Talus FOS Vs Control shoe
(28) Fior&Gentz Luneburg FOS Vs Control shoe
(29) Thanner Cabrio FOS Vs Control shoe
(30) Thanner Cabrio FOS Vs Control shoe
(31) Post-operative shoe (POS) Vs Off-the-shelf footwear (OSF)
(32) VACOdiaped-Plus (low-cut) Vs Off-the-shelf footwear (OSF)
(33) VACOdiaped-Plus (low-cut) Vs Off-the-shelf footwear (OSF)
(34) Post-operative shoe (POS) Vs Off-the-shelf footwear (OSF)
(35) VACOdiaped (high-cut) Vs Off-the-shelf footwear (OSF)
(36) VACOdiaped (high-cut) Vs Off-the-shelf footwear (OSF)
(37) VACOdiaped (high-cut) Vs Off-the-shelf footwear (OSF)
(38) Post-operative shoe (POS) Vs Off-the-shelf footwear (OSF)
(39) VACOdiaped-Plus (low-cut) Vs Off-the-shelf footwear (OSF)
(40) Standard shoes Vs Canvas

Figure 4. Forest plot of the Peak Plantar Pressure (MPP) in standard pressure-relieving shoes

MPP at Various Insoles

The insoles can be classified into custom-made and standard ones. The MPP under the custom-made insoles was divided by 10 studies, and 33 comparisons were reported. Meta-analysis combining data from 33 comparisons (pressure-relieving insoles n = 2894; standard control insoles n = 3056) implied that the MPP under custom-made insoles was significantly lowered (SMD = −0.48, 95% CI = −0.61–(−0.34), Z = 6.73, P < 0.00001). The heterogeneity between studies was high (I2 = 84%) (Figure 5).



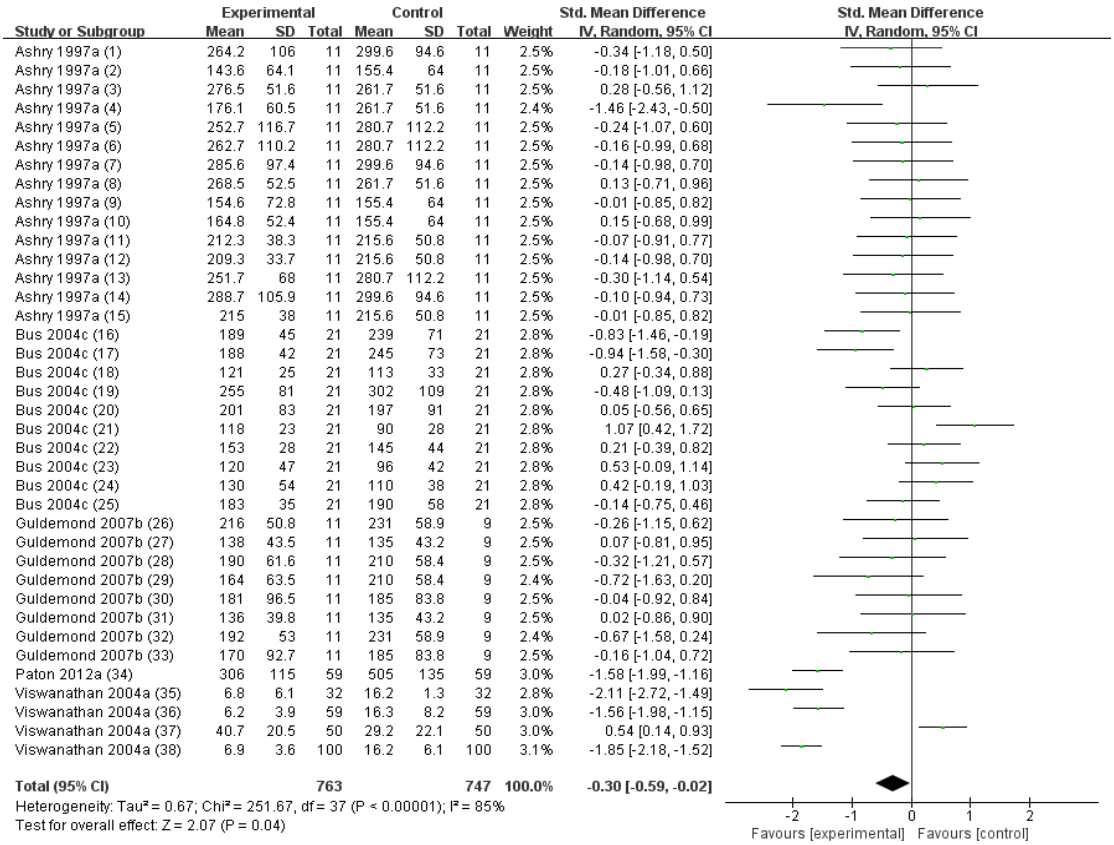
Study or Subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Arts 2015a (1)	209	55	67	271	66	67	2.7%	-1.01 [-1.38, -0.65]
Arts 2015a (2)	209	37	16	241	30	16	1.6%	-0.93 [-1.66, -0.19]
Arts 2015a (3)	235	70	13	284	108	13	1.5%	-0.52 [-1.31, 0.26]
Arts 2015a (4)	221	50	39	258	48	39	2.4%	-0.75 [-1.21, -0.29]
Arts 2015a (5)	239	53	26	258	50	26	2.1%	-0.36 [-0.91, 0.19]
Arts 2015a (6)	268	72	30	306	79	30	2.2%	-0.50 [-1.01, 0.02]
Arts 2015a (7)	212	40	25	251	47	25	2.0%	-0.88 [-1.46, -0.30]
Arts 2015a (8)	264	78	27	283	52	27	2.2%	-0.28 [-0.82, 0.25]
Arts 2015a (9)	238	53	24	281	59	24	2.0%	-0.75 [-1.34, -0.17]
Arts 2015a (10)	212	52	58	260	54	58	2.7%	-0.90 [-1.28, -0.52]
Arts 2015a (11)	210	48	25	250	66	25	2.1%	-0.68 [-1.25, -0.11]
Arts 2015a (12)	212	51	52	280	56	52	2.5%	-1.26 [-1.68, -0.84]
Burns 2009a (13)	102	18.3	30	100	23.5	31	2.3%	0.09 [-0.41, 0.60]
Bus 2011b (14)	206	46	23	303	77	23	1.8%	-1.50 [-2.16, -0.84]
Hellstrand 2014 (15)	259	114	142	250	137	154	3.2%	0.07 [-0.16, 0.30]
Hellstrand 2014 (16)	127	65	142	163	88	154	3.1%	-0.46 [-0.69, -0.23]
Hellstrand 2014 (17)	193	86	142	202	85	154	3.2%	-0.11 [-0.33, 0.12]
Hellstrand 2014 (18)	189	85	143	238	130	154	3.2%	-0.44 [-0.67, -0.21]
Hellstrand 2014 (19)	206	118	143	250	137	154	3.2%	-0.34 [-0.57, -0.11]
Hellstrand 2014 (20)	144	83	143	163	88	154	3.2%	-0.22 [-0.45, 0.01]
Hellstrand 2014 (21)	98	47	143	99	69	154	3.2%	-0.02 [-0.24, 0.21]
Hellstrand 2014 (22)	95	44	142	99	69	154	3.2%	-0.07 [-0.30, 0.16]
Hellstrand 2014 (23)	178	64	143	242	88	154	3.1%	-0.82 [-1.06, -0.59]
Hellstrand 2014 (24)	171	57	142	242	88	154	3.1%	-0.95 [-1.19, -0.71]
Hellstrand 2014 (25)	251	118	143	283	119	154	3.2%	-0.27 [-0.50, -0.04]
Hellstrand 2014 (26)	217	95	142	238	130	154	3.2%	-0.18 [-0.41, 0.05]
Hellstrand 2014 (27)	259	95	142	283	119	154	3.2%	-0.22 [-0.45, 0.01]
Hellstrand 2014 (28)	197	89	143	202	85	154	3.2%	-0.06 [-0.29, 0.17]
Lobmann 2001a (29)	380.8	190	18	474.7	183	18	1.8%	-0.49 [-1.16, 0.17]
Lord 1994a (30)	216	70	6	305	79	6	0.8%	-1.10 [-2.35, 0.15]
Owings 2008a (31)	200	46	23	245	63	23	2.0%	-0.80 [-1.40, -0.20]
Owings 2008a (32)	127	38	23	168	53	23	2.0%	-0.87 [-1.48, -0.27]
Owings 2008a (33)	178	59	23	211	79	23	2.0%	-0.47 [-1.05, 0.12]
Parker 2019 (34)	244.08	87.83	42	308.88	88.93	42	2.5%	-0.73 [-1.17, -0.28]
Parker 2019 (35)	263.56	98.19	42	307.92	84.87	42	2.5%	-0.48 [-0.91, -0.04]
Parker 2019 (36)	240.96	69.94	25	276.53	53.78	25	2.1%	-0.56 [-1.13, 0.00]
Parker 2019 (37)	271.75	80.49	32	298.95	84.57	32	2.3%	-0.33 [-0.82, 0.17]
Paton 2012a (38)	308	111	60	497	131	60	2.6%	-1.55 [-1.96, -1.14]
Waaijman 2012a (39)	220	61	123	277	67	123	3.1%	-0.89 [-1.15, -0.62]
Waaijman 2012a (40)	221	49	27	287	79	27	2.1%	-0.99 [-1.56, -0.42]
Total (95% CI)			**2894**			**3056**	**100.0%**	**-0.55 [-0.68, -0.42]**

Heterogeneity: Tau² = 0.12; Chi² = 204.87, df = 39 (P < 0.00001); I² = 81%
Test for overall effect: Z = 8.39 (P < 0.00001)

Footnotes
(1) fully custom-made footwear Vs semi-custom-made footwear
(2) fully custom-made footwear Vs semi-custom-made footwear
(3) fully custom-made footwear Vs semi-custom-made footwear
(4) fully custom-made footwear Vs semi-custom-made footwear
(5) fully custom-made footwear Vs semi-custom-made footwear
(6) fully custom-made footwear Vs semi-custom-made footwear
(7) fully custom-made footwear Vs semi-custom-made footwear
(8) fully custom-made footwear Vs semi-custom-made footwear
(9) fully custom-made footwear Vs semi-custom-made footwear
(10) fully custom-made footwear Vs semi-custom-made footwear
(11) fully custom-made footwear Vs semi-custom-made footwear
(12) fully custom-made footwear Vs semi-custom-made footwear
(13) Custom orthoses Vs Sham
(14) fully customized footwear Vs custom molded insoles in an extradepth shoe
(15) 55 shore EVA insoles Vs prefabricated insoles
(16) 55 shore EVA insoles Vs prefabricated insoles
(17) 55 shore EVA insoles Vs prefabricated insoles
(18) 35 shore EVA insoles Vs prefabricated insoles
(19) 35 shore EVA insoles Vs prefabricated insoles
(20) 35 shore EVA insoles Vs prefabricated insoles
(21) 35 shore EVA insoles Vs prefabricated insoles
(22) 55 shore EVA insoles Vs prefabricated insoles
(23) 35 shore EVA insoles Vs prefabricated insoles
(24) 55 shore EVA insoles Vs prefabricated insoles
(25) 35 shore EVA insoles Vs prefabricated insoles
(26) 55 shore EVA insoles Vs prefabricated insoles
(27) 55 shore EVA insoles Vs prefabricated insoles
(28) 35 shore EVA insoles Vs prefabricated insoles
(29) 1 year after insole protection Vs Baseline
(30) molded inserts Vs flat inserts
(31) insoles X in the rigid shoes Vs insoles X in the fiflexible shoes
(32) insoles Z in the rigid shoes Vs insoles X in the fiflexible shoes
(33) insoles Y in the rigid shoes Vs insoles X in the fiflexible shoes
(34) Digital Supply Chain: Orthotic Vs Control
(35) Traditional Supply Chain: Orthotic Vs Control
(36) Traditional Supply Chain: Orthotic Vs Control
(37) Digital Supply Chain: Orthotic Vs Control
(38) Custom-made functional insole: Baseline Vs after 6-months
(39) custom-made insoles in custom-made shoes Vs custom-made insoles in offthe-shelf extra-depth shoes
(40) custom-made insoles in custom-made shoes Vs custom-made insoles in offthe-shelf extra-depth shoes

Figure 5. Forest plot of the Peak Plantar Pressure (MPP) in custom-made pressure-relieving insoles

Similarly, the MPP under the standard insoles was also slightly attenuated (SMD = −0.43, 95% CI = −0.78–(−0.09), Z = 2.46, p = 0.01, I2 = 85%) on the basis of 28 comparisons (pressure-relieving insoles n = 553; standard control insoles n = 537) from four studies (Figure 6).



Footnotes
(1) Arch Pad Vs Plastizote
(2) Metatarsal-Arch Pad Vs Plastizote
(3) Metatarsal Pad Vs Plastizote
(4) Metatarsal-Arch Pad Vs Plastizote
(5) Metatarsal Pad Vs Plastizote
(6) Arch Pad Vs Plastizote
(7) Metatarsal Pad Vs Plastizote
(8) Arch Pad Vs Plastizote
(9) Metatarsal Pad Vs Plastizote
(10) Arch Pad Vs Plastizote
(11) Metatarsal-Arch Pad Vs Plastizote
(12) Arch Pad Vs Plastizote
(13) Metatarsal-Arch Pad Vs Plastizote
(14) Metatarsal-Arch Pad Vs Plastizote
(15) Metatarsal Pad Vs Plastizote
(16) CMI Vs Flat insole
(17) CMI Vs Flat insole
(18) CMI Vs Flat insole
(19) CMI Vs Flat insole
(20) CMI Vs Flat insole
(21) CMI Vs Flat insole
(22) CMI Vs Flat insole
(23) CMI Vs Flat insole
(24) CMI Vs Flat insole
(25) CMI Vs Flat insole
(26) Standard arch support Vs Basic
(27) Standard arch support Vs Basic
(28) Standard arch support Vs Basic
(29) Extra arch support Vs Basic
(30) Standard arch support Vs Basic
(31) Extra arch support Vs Basic
(32) Extra arch support Vs Basic
(33) Extra arch support Vs Basic
(34) Prefabricated insole: Baseline Vs after 6-months
(35) molded footwear: follow up Vs first visit
(36) polyurethane foam-insoled footwear: follow up Vs first visit
(37) prescribed sandals: follow up Vs first visit
(38) MCR insoles: follow up Vs first visit

Figure 6. Forest plot of the Peak Plantar Pressure (MPP) in standard pressure-relieving insoles

DISCUSSION

In this study, we used a meta-analysis to evaluate 18 studies and 346 comparisons between pressure-relieving products (shoes/insoles) and standard ones. Then, we analysed the overall MPP, MPP at various plantar regions and MPP at insoles/shoes with various structure designs. Our findings confirmed several positive agreements that pressure-relieving products (shoes/insoles) really lower the amplitude of pressure concentration. In addition, the pressure-relieving effects of custom-made and prefabricated products were similar.

Abnormal peak pressure is considered a major cause of diabetic foot ulcer; thus, pressure relief is a fundamental measure in diabetic care [19, 41, 42]. According to foot biomechanics, foot is the only body part contacting with the ground, where a large amount of ground reaction forces is concentrated on. Two principles are followed in lowering plantar pressure: one is increasing contact areas, and the other is transferring the peak pressure from risk areas to relatively safe ones, such as areas with occurrence of foot deformities, abnormal gait, wearing wrong shoes or shoes with foreign bodies (e.g. scree) [25].

In the first situation, current studies directly approved that plantar pressures are significantly reduced by using pressure-relieving products [43, 44], where experiment shoes and insoles performed closely. Moreover, two types of product designs were available: one was custom made [45, 46], and the other was prefabricated standard or modular assembled [31, 35]. Usually, the custom-made ones use static or dynamic foot impressions in a foam box or by digital 3D scan, from which a positive plaster cast of the plantar surface is created [28]. The custom-made shoes or insoles were developed based on the patient's plantar surface. This style of products has the largest contact area with the foot; thus, the overall MPP can be distributed.

In the second situation, extra structure designs were provided to both shoes and insoles. Standard pressure-relieving products provide extra structures, such as arch support, heel cup, wedges, metatarsal pad and hollow treatment [18, 47, 48], and they also aim to achieve pressure exchange. By contrast, standard pressure-relieving products (SMD = −1.34 for shoes and SMD = −0.43 for insole) performed superior than custom-made ones (SMD = −0.75 for shoes and SMD = −0.48 for insole). Thereby, we postulated that efficient pressure-relieving measures should first consider shoes before insoles. These findings once again support the description in international consensus for diabetic foot [19]: wearing the pressure-relieving shoes/insoles can effectively reduce plantar pressure and avoid ulceration.

By considering the heterogeneity of our results, majority of findings displayed a moderate-to-high heterogeneity (I2 > 70%). The I2 value at the mid foot was lower than 50% because this area beard extra loading by the arch support structure which transferred pressure from other parts of plantar regions. The high heterogeneity further supported validity of this meta-analysis [10].

Risk of bias for the included studies was assessed. The overall agreement between the two quality assessors was good, with the variation of scores ranging from zero to three points. In general, all studies used an appropriate study design and accounted for potential confounders. However, one study did not report data on a primary outcome measure for at least 85% of the participants, none of the studies calculated the power of the sample and only two studies identified the presence of PAD or excluded those with PAD. The highest score for the method- and participant-specific questions was given to the study which addressed issues such as number of steps used in measurements, number of walking trials and the measurement of factors which potentially affected plantar pressure, such as diabetes duration and type of diabetes [29].

This study has some limitations. In a single study, researchers reported two measures between pressure-relieving and control products. One was two or more types of pressure-relieving shoes versus control shoes (See [31, 32]); the other was continuing modification for custom-made insoles (See [34, 49]). Regarding the two conditions, we counted each measure as individual record and input each of them in the meta-analysis. This method might introduce bias by increasing the sample size.

CONCLUSION

This meta-analysis confirmed that pressure-relieving shoes/insoles perform well in

lowering plantar pressure distribution. Pressure-relieving products, regardless if they are custom made or pre-fabricated, can prevent ulceration and help in diabetic foot care if they are designed to increase overall plantar contact areas or to provide extra arch supports or plug-in structures to transfer pressure concentration.

Acknowledgement

The authors appreciate all the children and adults who participated in this study. This work was supported by Sichuan Science and Technology Program: 2020YFH0068. We also thank the National Natural Science Foundation of China (31700813).

Conflict of Interest Statement

There are no conflicts of interest with other authors and institutions. No subjects or animals were included in this study. Neither participants nor informed consent were included in the study.

REFERENCES

1. Bus, S.A., van Netten, J.J., Lavery, L.A., Monteiro-Soares, M., Rasmussen, A., Jubiz, Y., Price, P.E., IWGDF guidance on the prevention of foot ulcers in at-risk patients with diabetes, *Diabetes Metab Res Rev*, **2016**, 32, 16-24, WOS: 000369134100004, https://doi.org/10.1002/dmrr.2696.
2. Waaijman, R., de Haart, M., Arts, M.L.J., Wever, D., Verlouw, A.J.W.E., Nollet, F., Bus, S.A., Risk Factors for Plantar Foot Ulcer Recurrence in Neuropathic Diabetic Patients, *Diabetes Care*, **2014**, 37, 6, 1697-1705, WOS: 000337746100047, https://doi.org/10.2337/dc13-2470.
3. Lavery, L.A., Oz, O.K., Bhavan, K., Wukich, D.K., Diabetic Foot Syndrome in the Twenty-First Century, *Clin Podiatr Med Surg*, **2019**, 36, 3, 355-359, MEDLINE: 31079602, https://doi.org/10.1016/j.cpm.2019.02.002.
4. Singh, N., Armstrong, D.G., Lipsky, B.A., Preventing foot ulcers in patients with diabetes, *J Am Med Assoc*, **2005**, 293, 2, 217-228, WOS: 000226171000038, https://doi.org/10.1001/jama.293.2.217.
5. Siitonen, O.I., Niskanen, L.K., Laakso, M., Siitonen, J.T., Pyörälä, K., Lower-Extremity Amputations in Diabetic and Nondiabetic Patients: A population-based study in eastern Finland, *Diabetes Care*, **1993**, 16, 1, 16-20, https://doi.org/10.2337/diacare.16.1.16.
6. Trautner, C., Haastert, B., Giani, G., Berger, M., Incidence of lower limb amputations and diabetes, *Diabetes Care*, **1996**, 19, 9, 1006-1009, https://doi.org/10.2337/diacare.19.9.1006.
7. Boulton, A.J.M., Vileikyte, L., Ragnarson-Tennvall, G., Apelqvist, J., The global burden of diabetic foot disease, *Lancet*, **2005**, 366, 9498, 1719-1724, WOS: 000233206400028, https://doi.org/10.1016/S0140-6736(05)67698-2.
8. Veves, A., Murray, H.J., Young, M.J., Boulton, A.J.M., The risk of foot ulceration in diabetic patients with high foot pressure: a prospective study, *Diabetologia*, **1992**, 35, 7, 660-663, https://doi.org/10.1007/BF00400259.
9. Stess, R.M., Jensen, S.R., Mirmiran, R., The role of dynamic plantar pressures in diabetic foot ulcers, *Diabetes Care*, **1997**, 20, 5, 855, https://doi.org/10.2337/diacare.20.5.855.
10. Fernando, M.E., Crowther, R.G., Pappas, E., Lazzarini, P.A., Cunningham, M., Sangla, K.S., Buttner, P., Golledge, J., Plantar Pressure in Diabetic Peripheral Neuropathy Patients with Active Foot Ulceration, Previous Ulceration and No History of Ulceration: A Meta-Analysis of Observational Studies, *PLoS One*, **2014**, 9, 6, 10, WOS: 000340947700054, https://doi.org/10.1371/journal.pone.0099050.
11. Bus, S.A., Innovations in plantar pressure and foot temperature measurements in diabetes, *Diabetes Metab Res Rev*, **2016**, 32, 221-226, WOS: 000369134100023, https://doi.org/10.1002/dmrr.2760.
12. Pitei, D.L., Foster, A., Edmonds, M., The effect of regular callus removal on foot pressures, *J Foot Ankle Surg*, **1999**, 38, 4, 251-306, MEDLINE: 10464719, https://doi.org/10.1016/S1067-2516(99)80066-0.
13. Owings, T.M., Apelqvist, J., Stenstrom, A., Becker, M., Bus, S.A., Kalpen, A., Ulbrecht, J.S., Cavanagh, P.R., Plantar pressures in diabetic patients with foot ulcers which have remained healed, *Diabet Med*, **2009**, 26, 11, 1141-1146, WOS: 000271493900010, https://doi.org/10.1111/j.1464-5491.2009.02835.x.
14. McCabe, C., Stevenson, R.C., Dolan, A.M., Evaluation of a diabetic foot screening and

protection program, *Diabet Med*, **1998**, 15, 1, 80-84, https://doi.org/10.1002/(SICI)1096-9136(199801)15:1%3C80::AID-DIA517%3E3.0.CO;2-K.
15. Reiber, G.E., Who Is At Risk of Limb Loss And What To Do About It?, *J Rehabil Res Dev*, **1994**, 31, 4, 357-362.
16. Praet, S.F.E., Louwerens, J.W.K., The influence of shoe design on plantar pressures in neuropathic feet, *Diabetes Care*, **2003**, 26, 2, 441-445, WOS: 000185505000032, https://doi.org/10.2337/diacare.26.2.441.
17. Cheung, J.T.-M., Zhang, M., Parametric design of pressure-relieving foot orthosis using statistics-based finite element method, *Med Eng Phys*, **2008**, 30, 3, 269-277, WOS: 000254685000001, https://doi.org/10.1016/j.medengphy.2007.05.002.
18. Actis, R.L., Ventura, L.B., Lott, D.J., Smith, K.E., Commean, P.K., Hastings, M.K., Mueller, M.J., Multi-plug insole design to reduce peak plantar pressure on the diabetic foot during walking, *Med Biol Eng Comput*, **2008**, 46, 4, 363-371, WOS: 000254237800007, https://doi.org/10.1007/s11517-008-0311-5.
19. Connor, H., International Consensus on the Diabetic Foot - Book review, *Pract Diabetes*, **2000**, 17, 6, 642A, https://doi.org/10.1002/1528-252X(200009)17:6%3C194::AID-PDI94%3E3.0.CO;2-G.
20. Lobmann, R., Kayser, R., Kasten, G., Kasten, U., Kluge, K., Neumann, W., Lehnert, H., Effects of preventative footwear on foot pressure as determined by pedobarography in diabetic patients: a prospective study, *Diabet Med*, **2001**, 18, 4, 314-319, WOS: 000169753600010, https://doi.org/10.1046/j.1464-5491.2001.00482.x.
21. Sanderson, S., Tatt, I.D., Higgins, J.P.T., Tools for assessing quality and susceptibility to bias in observational studies in epidemiology: a systematic review and annotated bibliography, *Int J Epidemiol*, **2007**, 36, 666–676, https://doi.org/10.1093/ije/dym018.
22. Cohen, J., Statistical Power Analysis for the Behavioral Sciences, Lawrence Erlbaum Associates, **1988**.
23. Fernando, M.E., Crowther, R.G., Pappas, E., Lazzarini, P.A., Cunningham, M., Sangla, K.S., Buttner, P., Golledge, J., Plantar pressure in diabetic peripheral neuropathy patients with active foot ulceration, previous ulceration and no history of ulceration: a meta-analysis of observational studies, *PLoS One*, **2014**, 9, 6, e99050, https://doi.org/10.1371/journal.pone.0099050.
24. Raspovic, A., Landorf, K.B., Gazarek, J., Stark, M., Reduction of peak plantar pressure in people with diabetes-related peripheral neuropathy: an evaluation of the DH Pressure Relief Shoe (TM), *J Foot Ankle Res*, **2012**, 5, WOS: 000310812500002, https://doi.org/10.1186/1757-1146-5-25.
25. Bus, S.A., Ulbrecht, J.S., Cavanagh, P.R., Pressure relief and load redistribution by custom-made insoles in diabetic patients with neuropathy and foot deformity, *Clin Biomech*, **2004**, 19, 6, 629-638, https://doi.org/10.1016/j.clinbiomech.2004.02.010.
26. Paton, J.S., Stenhouse, E.A., Bruce, G., Zahra, D., Jones, R.B., A comparison of customised and prefabricated insoles to reduce risk factors for neuropathic diabetic foot ulceration: a participant-blinded randomised controlled trial, *J Foot Ankle Res*, **2012**, 5, 11, WOS: 000314610300001, https://doi.org/10.1186/1757-1146-5-31.
27. Burns, J., Wegener, C., Begg, L., Vicaretti, M., Fletcher, J., Randomized trial of custom orthoses and footwear on foot pain and plantar pressure in diabetic peripheral arterial disease, *Diabet Med*, 2009, 26, 9, 893-899, WOS: 000269366400008, https://doi.org/10.1111/j.1464-5491.2009.02799.x.
28. Waaijman, R., Arts, M.L.J., Haspels, R., Busch-Westbroek, T.E., Nollet, F., Bus, S.A., Pressure-reduction and preservation in custom-made footwear of patients with diabetes and a history of plantar ulceration, *Diabet Med*, **2012**, 29, 12, 1542-1549, WOS: 000311054600019, https://doi.org/10.1111/j.1464-5491.2012.03700.x.
29. Hellstrand Tang, U., Zugner, R., Lisovskaja, V., Karlsson, J., Hagberg, K., Tranberg, R., Comparison of plantar pressure in three types of insole given to patients with diabetes at risk of developing foot ulcers - A two-year, randomized trial, *J Clin Transl Endocrinol*, **2014**, 1, 4, 121-132, MEDLINE: 29159093, https://doi.org/10.1016/j.jcte.2014.06.002.

30. Bus, S.A., van Deursen, R.W.M., Kanade, R.V., Wissink, M., Manning, E.A., van Baal, J.G., Harding, K.G., Plantar pressure relief in the diabetic foot using forefoot offloading shoes, *Gait Posture*, **2009**, 29, 4, 618-622, https://doi.org/10.1016/j.gaitpost.2009.01.003.
31. Owings, T.M., Woerner, J.L., Frampton, J.D., Cavanagh, P.R., Botek, G., Custom therapeutic insoles based on both foot shape and plantar pressure measurement provide enhanced pressure relief, *Diabetes Care*, **2008**, 31, 5, 839-844, WOS: 000256016300001, https://doi.org/10.2337/dc07-2288.
32. Guldemond, N.A., Leffers, P., Schaper, N.C., Sanders, A.P., Nieman, F., Willems, P., Walenkamp, G.H.I.M., The effects of insole configurations on forefoot plantar pressure and walking convenience in diabetic patients with neuropathic feet, *Clin Biomech*, **2007**, 22, 1, 81-87, https://doi.org/10.1016/j.clinbiomech.2006.08.004.
33. Ashry, H.R., Lavery, L.A., Murdoch, D.P., Frolich, M., Lavery, D.C., Effectiveness of diabetic insoles to reduce foot pressures, *J Foot Ankle Surg*, **1997**, 36, 4, 268-71; discussion 328-9, MEDLINE: 9298441, https://doi.org/10.1016/S1067-2516(97)80071-3.
34. Parker, D.J., Nuttall, G.H., Bray, N., Hugill, T., Martinez-Santos, A., Edwards, R.T., Nester, C., A randomised controlled trial and cost-consequence analysis of traditional and digital foot orthoses supply chains in a National Health Service setting: application to feet at risk of diabetic plantar ulceration, *J Foot Ankle Res*, **2019**, 12, WOS: 000455264900001, https://doi.org/10.1186/s13047-018-0311-0.
35. Bus, S.A., Waaijman, R., Arts, M., de Haart, M., Busch-Westbroek, T., van Baal, J., Nollet, F., Effect of Custom-Made Footwear on Foot Ulcer Recurrence in Diabetes A multicenter randomized controlled trial, *Diabetes Care*, **2013**, 36, 12, 4109-4116, WOS: 000327211500065, https://doi.org/10.2337/dc13-0996.
36. Nagel, A., Rosenbaum, D., Vacuum cushioned removable cast walkers reduce foot loading in patients with diabetes mellitus, *Gait Posture*, **2009**, 30, 1, 11-15, WOS: 000266905000003, https://doi.org/10.1016/j.gaitpost.2009.02.007.
37. Bus, S.A., Haspels, R., Busch-Westbroek, T.E., Evaluation and Optimization of Therapeutic Footwear for Neuropathic Diabetic Foot Patients Using In-Shoe Plantar Pressure Analysis, *Diabetes Care*, **2011**, 34, 7, 1595-1600, WOS: 000293261200030, https://doi.org/10.2337/dc10-2206.
38. Viswanathan, V., Madhavan, S., Gnanasundaram, S., Gopalakrishna, G., Das, B.N., Rajasekar, S., Ramachandran, A., Effectiveness of different types of footwear insoles for the diabetic neuropathic foot - A follow-up study, *Diabetes Care*, **2004**, 27, 2, 474-477, WOS: 000188739900029, https://doi.org/10.2337/diacare.27.2.474.
39. Arts, M.L.J., de Haart, M., Waaijman, R., Dahmen, R., Berendsen, H., Nollet, F., Bus, S.A., Data-driven directions for effective footwear provision for the high-risk diabetic foot, *Diabet Med*, **2015**, 32, 6, 790-797, WOS: 000354637600013, https://doi.org/10.1111/dme.12741.
40. Lord, M., Hosein, R., Pressure Redistribution by Molded Inserts in Diabetic Footwear - A Pilot-Study, *J Rehabil Res Dev*, **1994**, 31, 3, 214-221, WOS: A1994PE45700005.
41. Randolph, A.L., Nelson, M., Akkapeddi, S., Levin, A., Alexandrescu, R., Reliability of measurements of pressures applied on the foot during walking by a computerized insole sensor system, *Arch Phys Med Rehabil*, **2000**, 81, 5, 573-578, WOS: 000086867800006, https://doi.org/10.1016/S0003-9993(00)90037-6.
42. Drerup, B., Beckmann, C., Wetz, H.H., Effect of body weight on plantar peak pressure in diabetic patients, *Orthopade*, **2003**, 32, 3, 199-206, WOS: 000182200300003, https://doi.org/10.1007/s00132-002-0444-4.
43. Janisse, D.J., Janisse, E., Shoe modification and the use of orthoses in the treatment of foot and ankle pathology, *J Am Acad Orthop Surg*, **2008**, 16, 3, 152-158, WOS: 000253744500006, https://doi.org/10.5435/00124635-200803000-00006.
44. Jarl, G., Tranberg, R., An innovative sealed shoe to off-load and heal diabetic forefoot ulcers - a feasibility study, *Diabet Foot Ankle*, **2017**, 8, 1, 1348178, MEDLINE: 28804593, https://doi.org/10.1080/2000625X.2017.1348178.

45. Veitenhansl, M., Hierl, F.X., Landgraf, R., Pressure reduction through various premanufactured shoe models with insoles in diabetic foot syndrome to prevent ulceration: a prospective randomised study, *Diabetologia*, **2003**, 46, A4-A5, WOS: 000185242100007.
46. Kastenbauer, T., Sokol, G., Auinger, M., Irsigler, K., Running shoes for relief of plantar pressure in diabetic patients, *Diabet Med*, **1998**, 15, 6, 518-522, WOS: 000073898400014, https://doi.org/10.1002/(SICI)1096-9136(199806)15:6%3C518::AID-DIA623%3E3.0.CO;2-J.
47. Zequera, M., Stephan, S., Paul, J., Effectiveness of moulded insoles in reducing plantar pressure in diabetic patients, 2007 Annual International Conference of the Ieee Engineering in Medicine and Biology Society, Vols 1-162007, pp. 4671, https://doi.org/10.1109/IEMBS.2007.4353382.
48. Lavery, L.A., LaFontaine, J., Higgins, K.R., Lanctot, D.R., Constantinides, G., Shear-Reducing Insoles to Prevent Foot Ulceration in High-Risk Diabetic Patients, *Adv Skin Wound Care*, **2012**, 25, 11, 519-524, WOS: 000310361800007, https://doi.org/10.1097/01.ASW.0000422625.17407.93.
49. Paton, J., Bruce, G., Jones, R., Stenhouse, E., Effectiveness of insoles used for the prevention of ulceration in the neuropathic diabetic foot: a systematic review, *J Diabetes Complications*, **2011**, 25, 1, 52-62, WOS: 000285899600009, https://doi.org/10.1016/j.jdiacomp.2009.09.002.

CHARACTERIZATION AND PRODUCTION OPTIMIZATION OF KERATINASE FROM THREE *Bacillus* STRAINS

Raden Lukas Martindro Satrio Ari WIBOWO[1], Ragil YULIATMO[2*]

[1]Laboratory of Microbiology and Enzyme, Politeknik ATK Yogyakarta, Special District of Yogyakarta, 55281, Indonesia

[2]Laboratory of Beam House Operation and Tanning, Politeknik ATK Yogyakarta, Special District of Yogyakarta, 55281, Indonesia, ragilyuliatmo@atk.ac.id

Received: 12.08.2020 Accepted: 26.11.2020 https://doi.org/10.24264/lfj.20.4.4

CHARACTERIZATION AND PRODUCTION OPTIMIZATION OF KERATINASE FROM THREE *Bacillus* STRAINS

ABSTRACT. Indonesia has large coastal areas. The fisheries are good for exploitation. In the previous studies, bacteria producing keratinase were isolated from fish market waste. Keratinase enzyme is able to degrade keratin on the skin. Enzyme activity is influenced by external conditions, such as pH, temperature, and incubation time. The study aimed to investigate the characteristics and the optimal conditions of the keratinase production. The materials used were keratinase from three *Bacillus* strains: *Bacillus thuringensis* BRAW_PT, *Bacillus aerius* BRAW_PB, and *Bacillus subtilis* BRAW_PI. The keratinase was investigated by sodium dodecyl sulfate (SDS PAGE) and nondenaturing polyacrylamide gel electrophoresis (Native PAGE). Conditions of the production were optimized by pH, temperature and incubation time on enzyme activity. The molecular weights of all keratinases from *Bacillus* species were 94.803 kDa and 70.115 kDa. The optimum activity of keratinase from *B. thuringensis* BRAW_PT and *B. firmus* BRAW_PI was obtained at pH 8, while keratinase from *B. aerius* BRAW_PB was optimal at pH 6-8. Keratinase from *B. thuringensis* BRAW_PT has maximum activity at 25°C, whereas keratinase from *B. aerius* BRAW_PB and *B. firmus* BRAW_PI at 29°C. All keratinases from *Bacillus* species are optimal at 90 minutes incubation. Based on the principal component analysis (PCA), *B. thuringensis* BRAW_PT was discriminated from the other enzymes.

KEY WORDS: production optimization, keratinase enzyme, unhairing, fish market waste

CARACTERIZAREA ȘI OPTIMIZAREA PRODUCȚIEI DE KERATINAZĂ DIN TREI TULPINI DE *Bacillus*

REZUMAT. Indonezia are mari zone de coastă, iar pescăriile pot fi exploatate. În studiile anterioare, s-au izolat bacterii producătoare de keratinază din deșeurile de pe piața de pește. Enzima keratinază este capabilă să degradeze keratina din piele. Activitatea enzimatică este influențată de condiții externe, cum ar fi pH-ul, temperatura și timpul de incubație. Studiul a urmărit să investigheze caracteristicile și condițiile optime de producție a keratinazei. Materialele utilizate au fost keratinaza din trei tulpini *Bacillus*: *Bacillus thuringensis* BRAW_PT, *Bacillus aerius* BRAW_PB și *Bacillus subtilis* BRAW_PI. Keratinaza a fost investigată utilizând electroforeza în gel de dodecil sulfat de sodiu poliacrilamidă (SDS PAGE) și cea fără denaturare (Native PAGE). Condițiile de producție au fost optimizate în ceea ce privește pH-ul, temperatura și timpul de incubație pentru activitatea enzimatică. Greutatea moleculară a keratinazelor din speciile *Bacillus* a fost de 94,803 kDa și 70,155 kDa. Activitatea optimă a keratinazei din *B. thuringensis* BRAW_PT și *B. firmus* BRAW_PI a fost obținută la pH 8, în timp ce keratinaza din *B. aerius* BRAW_PB a fost optimă la pH 6-8. Keratinaza din *B. thuringensis* BRAW_PT are activitate maximă la 25°C, iar keratinaza din *B. aerius* BRAW_PB și *B. firmus* BRAW_PI la 29°C. Toate keratinazele din speciile *Bacillus* sunt optime la 90 de minute de incubație. Pe baza analizei componentei principale (PCA), *B. thuringensis* BRAW_PT s-a evidențiat din rândul celorlalte enzime.

CUVINTE CHEIE: optimizare producție, enzimă keratinază, îndepărtarea părului, deșeuri de pe piața de pește

CARACTÉRISATION ET OPTIMISATION DE LA PRODUCTION DE KÉRATINASE À PARTIR DE TROIS SOUCHES de Bacillus

RÉSUMÉ. L'Indonésie a de vastes zones côtières et les pêcheries sont bonnes pour l'exploitation. Dans l'étude précédente, des bactéries produisant de la kératinase ont été isolées des déchets du marché aux poissons. L'enzyme kératinase est capable de dégrader la kératine sur la peau. L'activité enzymatique est influencée par des conditions externes, telles que le pH, la température et la durée d'incubation. L'étude visait à étudier les caractéristiques et les conditions optimales de la production de kératinase. Les matériaux utilisés étaient la kératinase de trois souches de *Bacillus* : *Bacillus thuringensis* BRAW_PT, *Bacillus aerius* BRAW_PB et *Bacillus subtilis* BRAW_PI. La caractérisation de la kératinase a été étudiée par électrophorèse sur gel de polyacrylamide en présence de dodécylsulfate de sodium (SDS PAGE) et par électrophorèse non dénaturante (Native PAGE). Les conditions de production ont été optimisées par le pH, la température et le temps d'incubation sur l'activité enzymatique. Les poids moléculaires de toutes les kératinases de l'espèce *Bacillus* étaient de 94.803 kDa et 70.115 kDa. L'activité optimale de la kératinase de *B. thuringensis* BRAW_PT et *B. firmus* BRAW_PI a été obtenue à pH 8, tandis que la kératinase de *B. aerius* BRAW_PB a été optimale à pH 6-8. La kératinase de *B. thuringensis* BRAW_PT a une activité maximale à 25°C, tandis que la kératinase de *B. aerius* BRAW_PB et *B. firmus* BRAW_PI à 29°C. Toute la kératinase des espèces de *Bacillus* est optimale à 90 minutes d'incubation. Sur la base de l'analyse des composants principaux (PCA), *B. thuringensis* BRAW_PT se démarque des autres enzymes.

MOTS CLÉS : optimisation de la production, enzyme kératinase, épilage, déchets du marché du poisson

** Correspondence to: Ragil YULIATMO, Laboratory of Beam House Operation and Tanning, Politeknik ATK Yogyakarta, Special District of Yogyakarta, 55281, Indonesia, ragilyuliatmo@atk.ac.id*

INTRODUCTION

Indonesia is the world's largest archipelagic state with 54,716 km of coastline, and 17,508 islands (of which 6,000 are inhabited), and the world's fourth most populous nation (247.5 million) [1]. After China, Indonesia is the second largest fish producer in the world, with capture fisheries and aquaculture production. Fish supply in Indonesia has been increasing over the past 50 years, from 0.8 million tons in 1,960 to 10.7 million tons in 2014 [2]. The proportion of the catch is consumed in dried, salted, smoked, boiled or fermented form, while 46 percent is consumed fresh from fish market [1].

In the previous study, Wibowo *et al*. [3] have isolated the bacteria producing keratinase enzymes from fish market waste. The enzyme is used in the unhairing process for environmentally friendly fish skin tanning. According to Tamersit and Bouhidel [4], unhairing generates heavily polluted solutions. Dettmer *et al*. [5] also stated that the conventional lime-sulfide in unhairing process leads to the destruction of the hair, causing emissions with high chemical oxygen demand (COD), biological oxygen demand (BOD), and total suspended solid (TSS) loads in the effluent of these industries. Moreover, during this step in leather industry, strong chemicals are used, such as sodium sulphide and lime, which represent approximately 80-90% of total pollution of leather manufacturing [6]. Keratinase enzyme is important for the environmentally-friendly tanning process by reducing the usage of sodium sulfide as chemical [7].

Utilization of enzyme in leather making is a promising application. Researchers discovered the keratinolytic enzymes, most of them produced from *Bacillus* strain, such as *Bacillus aerius* NSMk2 [8], *Bacillus* BPKer and BAKer [9], *Bacillus cereus* and *Bacillus polymyxa* [10], and *Bacillus suptillis* [11]. Enzyme production can be influenced by several factors, such as temperature, pH, and incubation time. Optimizing conditions is a crucial aspect of enzyme production [12]. In the current study, we report the characterization and optimization of keratinase from three different *Bacillus* strains as an innovative solution in supporting the cleaner production of the tannery.

EXPERIMENTAL

Production of Keratinase Enzyme

Preparation of Inoculum for Enzyme Production

The materials used in this study were: *B. thuringensis* BRAW_PT, *B. aerius* BRAW_PB, and *B. subtilis* BRAW_PI collected from isolation results of a previous study [3]. The fermentation media was: 0.5 g/l sodium chloride, 0.3 g/l potassium hydrogen phosphate, 0.4 g/l potassium dihydrogen phosphate. Stock solution (1 g/100 ml Yeast extract; 1 g/100 ml Biological peptone; 0,5 g/100 ml NaCl and 100 ml distilled water), ammonium sulfate, 20 mM Tris HCl pH 8, 12 kDa dialysis bag, 1 mM EDTA, $NaHCO_3$, distilled water. One dose of pure culture result isolate was inoculated from agar media into 5 ml preculture medium, then incubated in a shaker at 120 rpm overnight.

Enzyme Production

The enzyme production was based on the method of Hoq *et al*. [13]. One and a half milliliters isolate were inoculated into 50 ml liquid medium, then incubated in a shaker at 120 rpm overnight. Enzyme production is characterized by yellowing. Separation of the isolates from extracellular enzyme was performed by centrifuge at 4°C and 3500 rpm for 15 minutes. The resulting supernatant was raw enzyme source whose enzyme activity can be measured. The collected enzyme was measured for enzyme activity.

Enzyme Purification

One liter of fermentation product culture was centrifuged at 10.000g for 15 minutes at 4°C. The formed supernatant was separated from the pellet. The supernatant was an enzyme extract ready to be concentrated. Purification of enzyme by precipitated by 60% saturation of ammonium sulfate. Saturation was performed using ammonium sulfate [14]. Ammonium sulfate crystals were added slowly while stirring until dissolved. The solution was left for 24 hours at 4°C, then centrifuged at 10.000g for 15 minutes at 4°C. This step in order to purifying protein by removing other nutrient such as saccharides and minerals [15].

Characterization of Eznyme

Determination of Molecular Weight of Protein by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Materials for SDS-PAGE include 70% alcohol, sterilized water, acrylamide solution, Tris HCl, SDS, d H2O, TEMED, ammonium persulfate, glacial acetic acid, Coomassie blue, methanol. The buffers were sodium phosphate, glycine-NaOH, Tris acetate. The materials used to test keratinase activity were keratin azure, Tris HCl pH 7.5 and 10% TCA solution. Protein separation by SDS-PAGE method aims to separate protein in a sample based on molecular weight. The basic principle of SDS-PAGE is protein denaturation by sodium dodecyl sulfate, followed by separating molecules based on molecular weight by electrophoresis method using gel, in this case, polyacrylamide. Identification and analysis of SDS PAGE compared protein band which was previously separated with standard protein [16].

Protease Activity by Native or Nondenaturing PAGE Polyacrylamide Gel (Native-PAGE)

Native-PAGE was performed by Hiol *et al*. [17]. Materials for 10% concentrated Native-PAGE were 30% acrylamide solution; 0.8 % methylenebisacrylamide (Bis); 1.5 M tris HCl pH 8.8; 1.0 M. Tris HCl pH 6.8; N, N, N',N',tetramethyl-ethylenediamine (TEMED); 10% APS (amonium peroxidisulfate); 0.1% casein; 50% glycerol; A and B staining solutions; Tefco clear dry; Running buffer (1.5 g tris aminomethane; 7.2 g glycine, and 500 ml distilled water); 70 mm Advantec filter paper.

Determination of Vmax and Km

Kinetic parameters of the enzyme were determined by measuring the keratinase activity at different substrate concentrations. Lineweaver – Burk plot was drawn to determine the values of Km and Vmax of the enzyme [18].

Optimization of Enzyme Production

Keratinase Activity

Keratinase activity test was performed using keratin azure (Sigma-Aldrich, St. Louis, USA) as keratin substrate from wool and given azo stain. Keratinase activity test was performed based on Wang *et al*. [19]. Five hundred micro litres enzyme sample was incubated in 5 mg keratin azure solution in 500 µL 50 mM sodium phosphate buffer for 30 minutes at 30°C with constant agitation 180 rpm using shaker incubator. Enzyme reaction was stopped by adding 1 ml 10% TCA solution. The solution was put in ice for 30 minutes, then centrifuged at 13.000g for 5 minutes. The absorption of azo stain which was removed in supernatant was measured with 595 nm wavelength and compared with control tube. The control tube was given the same treatment except enzyme sample was replaced with sodium phosphate buffer.

The Effects of pH, Temperature, and Incubation Time on Keratinase Activity

Keratinase activity in purified enzyme was measured at pH 6; 7; 8; 9; and 10 using the following buffers: sodium acetate (pH 4,0-6,0), sodium phosphate (pH 7,0-8,0), and TrisNaOH buffer (pH 9.0-10.0). The optimum temperature was determined by incubation reaction combination at different temperatures, i.e. 25, 27, 29, 31 and 33°C. Beside temperature and pH, incubation time for keratinase characteristic was optimized for 30; 60; 90; 120 and 150 minutes [20].

Data Analysis

The results obtained from the production optimization were analyzed by analysis of variance (ANOVA) using IBM SPSS Statistics 25. Correlations among the variables were analyzed by Principal Component Analysis (PCA) using Minitab 18 Statistical Software.

RESULTS AND DISCUSSIONS

Characterization of Keratinase

SDS PAGE

B. thuringensis strain BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* strain BRAW_PI had similar molecular weights of enzyme between 72 kDa and 95 kDa. Electrophoresis method was used broadly in protein characterization, including determining molecular weight of

protein. The molecular weight of enzyme can be measured by calculating the molecular weight of protein which is electrophoresed by polyacrylamide sodium dodecyl sulfate (SDS-PAGE) gel and comparing it with molecular weight of standard protein. Bacteria from the genus *Bacillus*, generally secrete two types of extracellular peptidase, an alkaline peptidase and a neutral peptidase [19, 20]. This is in accordance with the results obtained by Mazotto *et al*. [21] Generally, all *Bacillus* spp. genus had keratinases with molecular weights between 13.8 and 140 kDa. The molecular weights of keratinase of the three isolates are presented in Figure 1. The keratinases were detected on various species of *Bacillus* spp. With molecular weight of 45 to 80 kDa on *B. subtilis* 1270, 15 to 100 kDa on *B. subtilis* 1273, and 63 to 140 kda on *B. licheniformis* 1274 [22]. Other extracellular keratinases such as on *B. subtilis* ks-1, *B. pumilus*, and *B. cereus* had molecules with masses of 25.4, 65, and 45 kDa [21, 23].

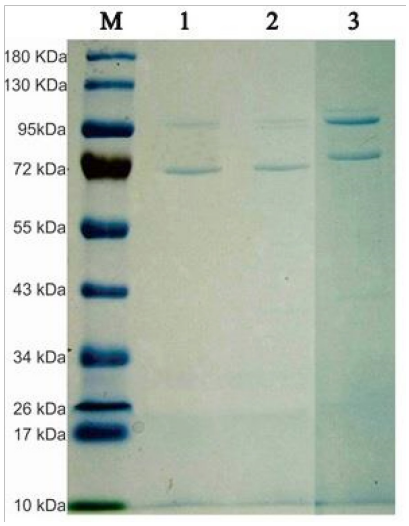


Figure 1. Molecular weight of keratinase from *B. thuringensis* BRAW_PT (1), *B. aerius* BRAW_PB (2), and *B. subtilis* BRAW_PI (3)

Native-PAGE

Native-PAGE analysis using casein substrate (Figure 2) demonstrated that the enzymatic extract obtained from three *Bacillus* strains was able to hydrolyze protein. Protein band of *B. thuringensis* BRAW_PT was very clear, followed by *B. aerius* BRAW_PB, while *B. firmus* BRAW_PI showed thin band. Native-PAGE method was used to determine protein bands, in this case protease activity of certain bacteria. Sattayasai [24] state that many proteins can be stained in gel by using their enzyme activity, native-PAGE are compatible with activity stains. Wilson and Walker [25] also stated that the sample in Native-PAGE process is not denaturized because it can make bonds in the secondary structure of protein to be destructed.

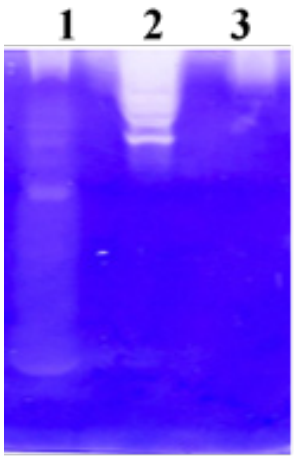


Figure 2. Native-PAGE results of enzyme keratinase from *B. thuringensis* BRAW_PT (1), *B. aerius* BRAW_PB (2), and *B. firmus* BRAW_PI (3)

Kinetics of Keratinase

The Lineweaver–Burk plot was represented against different concentrations of the substrate with the Michaelis–Menten plot (Fig. 3). The Michaelis constant (Km) of keratinase from *B. thuringensis* BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* BRAW_PI were found to be 1.09, 0.46, and 0.10 mg/ml, respectively and the maximum velocity of the reaction (Vmax) were 0.83, 7.29, and 37.4 mg/ml/min. Kinetic of enzyme was investigated to determine the enzyme reaction rate on different concentrations of the substrate through the Michaelis–Menten equation (Km). Keratinase produced by *Pseudomonas aeruginosa* KS-1 found higher Km which 1.66 mg/ml and Vmax which 3.1 mg/ml/min [26]. Purified keratinase from *B. thuringiensis* presented higher Km (5.97 mg/ml) [27]. The estimated Km and Vmax values for feather keratin were 6.6 mg/ml and 5.0 mg/ml/min, respectively [28].

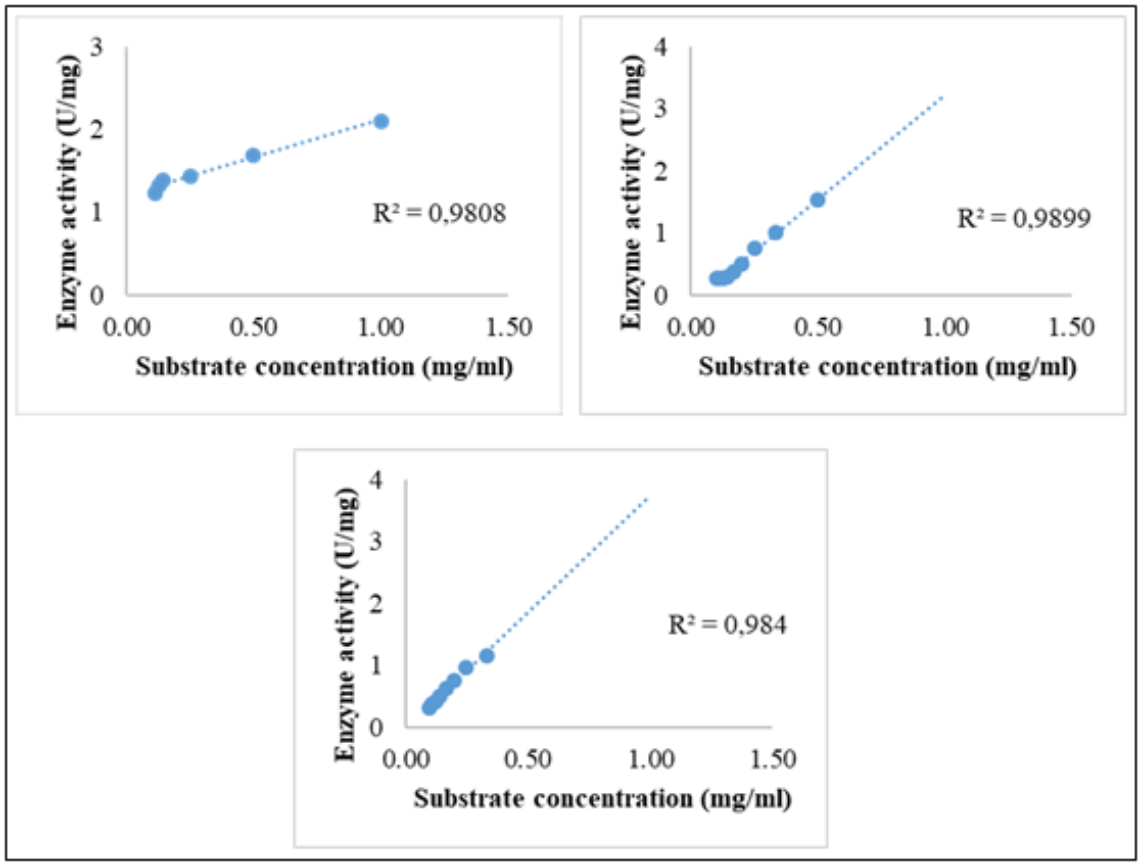


Figure 3. Graph of Line weaver - Burk plot of keratinase

Optimization of Keratinase Production

The effect of temperature on enzyme activity is depicted in Fig 4. The determination of optimum temperature of keratinase activity was performed by incubating at 25°C – 33°C. Enzyme from *B. aerius* BRAW_PB shows the highest enzyme activity at 29°C (9.39±0.05 U/mg). Enzyme from *B. firmus* strain BRAW_PI also had optimum activity at 29°C (8.25±0.10 U/mg). The optimum temperature of enzyme activity from these *Bacillus* sp stains were almost the same with the results of Balakumar *et al.* [29]. They state that *Bacillus subtilis* inoculated in medium and optimized the production at different temperature, and the increased production was identified at 30°C. However, enzyme from *B. thuringensis* BRAW_PT was optimum at 25°C, then its activity decreased when the temperature increased.

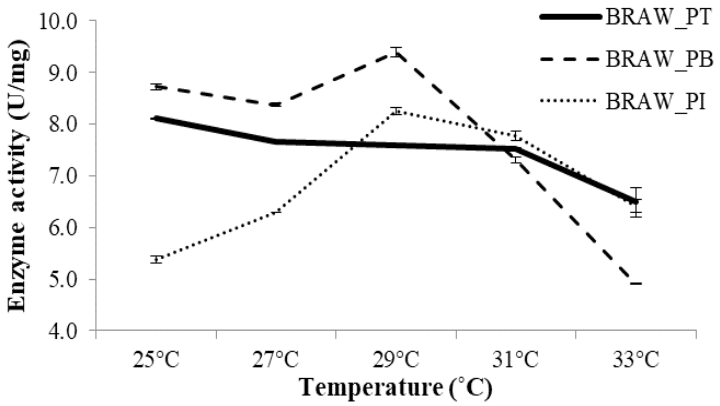


Figure 4. The effect of temperature on keratinase activity

Keratinase from *B. thuringensis* BRAW_PT and *B. firmus* BRAW_PI activities were optimal at pH 8 (Fig 5). However, keratinase activity of *B. aerius* BRAW_PB was optimal at pH in the range of 6-8. Selvam *et al*. [30] indicating that keratinase were produced by *Bacillus* sp at pH between 7 and 8. Keratinase from these bacteria strains can be categorized as alkaline protease because the optimum activity of the enzyme at alkaline pH. The result was almost similar to alkaline protease from APR-4 *Bacillus* sp which has optimum activity at pH 9 [23]. This result of keratinase activity of *B. aerius* BRAW_PB is in accordance with the optimum at pH 7.5 reported previously for azo keratin hydrolysis to hydrolyze fur with keratinase [31, 32]. Keratinases from *Meiothermus taiwanensis* WR-220 were also active in a broad range, which is between pH 4-11 [33].

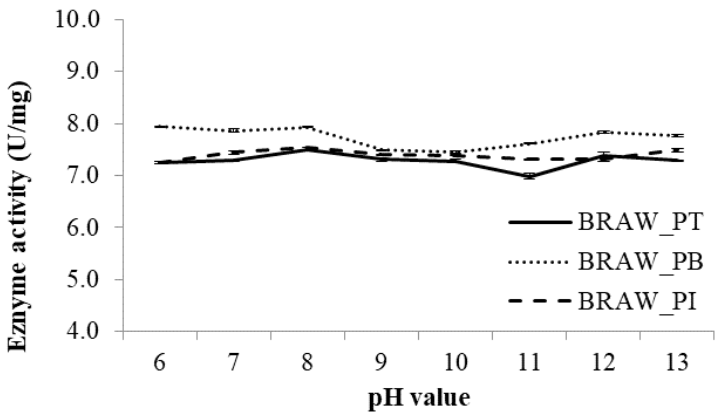


Figure 5. The effect of pH value on keratinase activity

Activity of all types of enzymes increased for 90 minutes, then it decreased by 9% after 90 minutes (Fig. 6). The highest activity is that of enzyme from *B. thuringensis* BRAW_PT (7.72±0.01 U/mg). Gupta *et al*. [28] have the same results with the optimum time incubation of keratinase from these bacteria strains, keratinase from *B. subtilis* stabilized up to 90 minutes, and decreased by 11% after 120 minutes. A different result was found in proteolytic enzyme of *B. subtilis* BLBc 11 [5] and commercial keratinase [34] that had stable activity for 120 minutes. Gessesse *et al*. [35] reported that enzyme from *B. pseudoformis* sp. was inactive after 20 minutes, moreover Ogino *et al*. [36] discovered proteolytic enzyme from *Bacillus* species that was inactive after only 10 minutes of incubation.

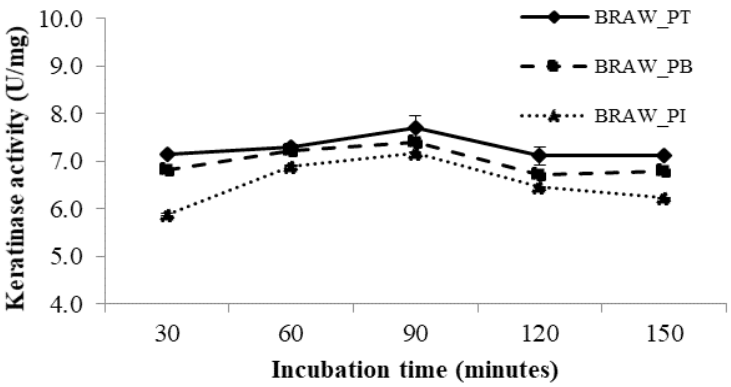


Figure 6. The effect of pH value on keratinase activity

Correlation among Variables Using Principle Component Analysis

Principal component analysis (PCA) is a statistical analysis method of combining several indexes into a few comprehensive ones. PCA is a calculation method allowing the reduction of variables, correlation among variables, and visualization data [37]. Reducing the amount of variables involves changing the initial set of variables into the new, reduced in number set of the so-called principal components [38]. In addition, the PCA makes the new variables be independent from each other, to achieve the purpose of simplification [39]. The variables that effected the principle component of enzyme activity are shown in Table 1. Temperature is the most effected to the first principle component (PC1), while the incubation time is the most effected second principle component (PC2). Table 1 provides the information about that there is a reducing variable, from 3 variables (temperature, pH value, and incubation time) to 2 variables (PC1 and PC2). The temperature is the most effected to the first principle component (PC1), while the incubation time is the most effected second principle component (PC2).

Table 1: Correlation between principle components

Variable	PC1	PC2
pH	0,555	-0,660
Temperature	0,643	-0,045
Time Incubation	0,527	0,750

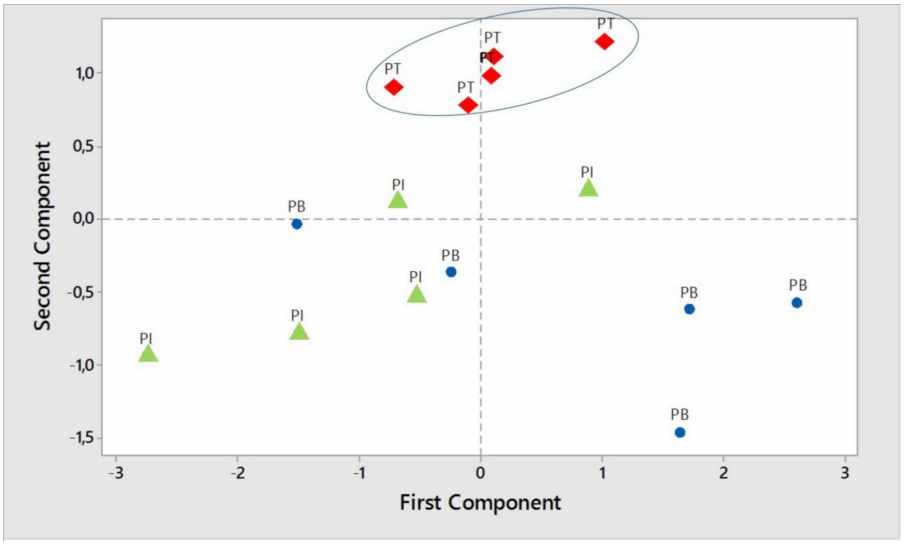


Figure 7. Score plot of type of bacterial producing keratinase strain. PT: Keratinase from *B. thuringensis* BRAW_PT; PB: Keratinase from *B. aerius* BRAW_PB; PI: Keratinase from *B. firmus* BRAW_PI

Moriya *et al*. [40] used PCA to discriminate the different enzymes. In this study, PCA showed a clear discrimination between keratinase from *B. thuringensis* BRAW_PT and other enzymes (Fig 6). Figure 7 showed the difference discriminant component of the variables of the bacterial strain. Keratinase from *B. thuringensis* BRAW_PT have a discriminant with other kinds of keratinase (from *B. aerius* BRAW_PB and *B. firmus* BRAW_PI).

CONCLUSIONS

B. thuringensis strain BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* strain BRAW_PI had similar molecular weights of enzyme between 72 kDa and 95 kDa. Protein band on native-PAGE of *B. thuringensis* BRAW_PT was very clear, followed by *B. aerius* BRAW_PB, while *B. firmus* BRAW_PI showed thin band. The Michaelis constant (Km) of keratinase from *B. thuringensis* BRAW_PT, *B. aerius* BRAW_PB, and *B. firmus* BRAW_PI were found to be 1.09, 0.46, and 0.10 mg/ml, respectively and the maximum velocity of the reaction (Vmax) were 0.83, 7.29, and 37.4 mg/ml/min. The optimum activity of keratinase from *B. thuringensis* BRAW_PT and *B. firmus* BRAW_PI was obtained at pH 8, while keratinase from *B. aerius* BRAW_PB at pH 6-8. Keratinase from *B. thuringensis* BRAW_PT has maximum activity at 25°C, whereas keratinase from *B. aerius* BRAW_PB and *B. firmus* BRAW_PI at 29°C. All keratinases are optimal at 90 minutes incubation. Keratinase has optimum activity at 29°C, pH 8 to 12, and 90 minutes of incubation time. The Principal Component Analysis (PCA) resulted some correlations among variables, and discriminated *B. thuringensis* BRAW_PT among other enzymes.

Acknowledgements

This work has received financial support from research grants from Politeknik ATK Yogyakarta, Ministry of Industry of Republic Indonesia.

REFERENCES

1. FAO, Fishery and Aquaculture Country Profiles of The Republic of Inonesia, **2019**, available at: http://www.fao.org/fishery/facp/IDN/en.
2. Tran, N., Rodriguez, U.P., Chan, C.Y., Phillips, M.J., Mohan, C.V., Henriksson, P.J.G., Koeshendrajana, S., Suri, S., Hall, S., Indonesian aquaculture futures: An analysis of fish supply and demand in Indonesia to 2030 and role of aquaculture using the AsiaFish model, *Mar Policy*, **2017**, 79, May, 25–32, https://doi.org/10.1016/j.marpol.2017.02.002.
3. Wibowo, R.L.M.S., Bachruddin, Z., Fitriyanto, N.A., Nakagawa, T., Hayakawa, T., Pertiwiningrum, A., Screening and Characterization of Keratinolytic Bacteria from Puffer Fish Skin Waste, *Pakistan J Nutr*, **2017**, 16, 488–496, https://doi.org/10.3923/pjn.2017.488.496.
4. Tamersit, S., Bouhidel, K.E., Treatment of tannery unhairing wastewater using carbon dioxide and zinc cations for greenhouse gas capture, pollution removal and water recycling, *J. Water Process Eng*, **2020**, 34, December 2019, 101120, https://doi.org/10.1016/j.jwpe.2019.101120.
5. Dettmer, A., Cavalli, É., Ayub, M.A.Z., Gutterres, M., Environmentally friendly hide unhairing: Enzymatic hide processing for the replacement of sodium sulfide and delimig, *J Clean Prod.*, **2013**, 47, 11–18, https://doi.org/10.1016/j.jclepro.2012.04.024.
6. Dettmer, A., Coelho Cavalheiro, J., Cavalli, E., Misturini Rossi, D., De Souza Gusatti, C., Záchia Ayub, M.A., Gutterres, M., Optimization of the Biotechnological Process for Hide Unhairing in Substitution of Toxic Sulfides, *Chem Eng Technol*, **2012**, 35, 5, 803–810, https://doi.org/10.1002/ceat.201100350.
7. Kandasamy, N., Velmurugan, P., Sundarvel, A., Jonnalagadda Raghava, R., Bangaru, C., Palanisamy, T., Eco-benign enzymatic dehairing of goatskins utilizing a protease from a *Pseudomonas fluorescens* species isolated from fish visceral waste, *J Clean Prod*, **2012**, 25, 27–33, https://doi.org/10.1016/j.jclepro.2011.12.007.
8. Bhari, R., Kaur, M., Singh, R.S., Thermostable and halotolerant keratinase from *Bacillus aerius* NSMk2 with remarkable dehairing and laundary applications, *J Basic Microbiol*, **2019**, 59, 6, 555–568, https://doi.org/10.1002/jobm.201900001.

9. Gegeckas, A., Šimkutė, A., Gudiukaitė, R., Čitavičius, D.J., Characterization and application of keratinolytic paptidases from *Bacillus* spp., *Int J Biol Macromol*, **2018**, 113, 2017, 1206–1213, https://doi.org/10.1016/j.ijbiomac.2018.03.046.
10. Laba, W., Rodziewicz, A., Biodegradation of hard keratins by two Bacillus strains, *Jundishapur J Microbiol*, **2014**, 7, 2, 1–7, https://doi.org/10.5812/jjm.8896.
11. Mousavi, S., Salouti, M., Shapoury, R., Heidari, Z., Optimization of keratinase production for feather degradation by *Bacillus subtilis*, *Jundishapur J Microbiol*, **2013**, 6, 8, 6–10, https://doi.org/10.5812/jjm.7160.
12. Mechri, S., Kriaa, M., Ben, M., Berrouina, E., Benmrad, M.O., Jaouadi, N.Z., Rekik, H., Bouacem, K., Bouanane-darenfed, A., Chebbi, A., Sayadi, S., Chamkha, M., Bejar, S., Jaouadi, B., Optimized production and characterization of a detergent-stable protease from *Lysinibacillus fusiformis* C250R, *Int J Biol Macromol*, **2017**, 101, 383–397, https://doi.org/10.1016/j.ijbiomac.2017.03.051.
13. Hoq, M.M., Siddiquee, K.A.Z., Kawasaki, H., Seki, T., Keratinolytic activity of some newly isolated *Bacillus species*, *Journal of Biological Sciences*, **2005**, 193–200, https://doi.org/10.3923/jbs.2005.193.200.
14. Tatineni, R., Doddapaneni, K.K., Potumarthi, R.C., Vellanki, R.N., Kandathil, M.T., Kolli, N., Mangamoori, L.N., Purification and characterization of an alkaline keratinase from *Streptomyces* sp., *Bioresour Technol*, **2008**, 99, 1596–1602, https://doi.org/10.1016/j.biortech.2007.04.019.
15. Yuliatmo, R., Fitriyanto, N.A., Bachruddin, Z., Erwanto, Y., Increasing of angiotensin converting enzyme inhibitory derived from Indonesian native chicken leg protein using *Bacillus cereus* protease enzyme, *Int Food Res J*, **2017**, 24, 4, 1799–1804.
16. Laemmli, U.K., Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature*, **1970**, 227, 5259, 680–685, https://doi.org/10.1038/227680a0.
17. Hiol, A., Jonzo, M.D., Druet, D., Comeau, L., Production, purification and characterization of an extracellular lipase from *Mucor hiemalis f. hiemalis*, *Enzyme Microb Technol*, **1999**, 25, 80–87, https://doi.org/10.1016/S0141-0229(99)00009-5.
18. Park, C.H., Lee, S.J., Lee, S.G., Lee, W.S., Byun, S.M., Hetero- and autoprocessing of the extracellular metalloprotease (Mpr) in *Bacillus subtilis*, *J Bacteriol*, **2004**, 186, 19, 6457–6464, https://doi.org/10.1128/JB.186.19.6457-6464.2004.
19. Wang, R.U.I., Min, C., Haiming, C., Li, Z., Enzyme Unhairing - An Eco-Friendly Biotechnological Process, *J Soc Leath Tech Ch*, **2009**, 93, 2, 51–55.
20. Tang, X.M., Lakay, F.M., Shen, W., Shao, W.L., Fang, H.Y., Prior, B.A., Wang, Z.X., Zhuge, J., Purification and characterisation of an alkaline protease used in tannery industry from *Bacillus licheniformis*, *Biotechnol Lett*, **2004**, 26, 18, 1421–1424, https://doi.org/10.1023/B:BILE.0000045642.19299.3f.
21. Mazotto, A.M., Coelho, R.R.R., Cedrola, S.M.L., De Lima, M.F., Couri, S., Paraguai De Souza, E., Vermelho, A.B., Keratinase production by three Bacillus spp. using feather meal and whole feather as substrate in a submerged fermentation, *Enzyme Res.*, **2011**, 2011, 1, https://doi.org/10.4061/2011/523780.
22. Mazotto, A.M., de Melo, A.C.N., Macrae, A., Rosado, A.S., Peixoto, R., Cedrola, S.M.L., Couri, S., Zingali, R.B., Villa, A.L.V., Rabinovitch, L., Chaves, J.Q., Vermelho, A.B., Biodegradation of feather waste by extracellular keratinases and gelatinases from *Bacillus* spp., *World J Microbiol Biotechnol*, **2011**, 27, 6, 1355–1365, https://doi.org/10.1007/s11274-010-0586-1.
23. Kumar, A.G., Swarnalatha, S., Gayathri, S., Nagesh, N., Sekaran, G., Characterization of an alkaline active - Thiol forming extracellular serine keratinase by the newly isolated *Bacillus pumilus*, *J Appl Microbiol*, **2008**, 104, 2, 411–419, https://doi.org/10.1111/j.1365-2672.2007.03564.x.
24. Sattayasai, N., Protein Purification, in Deniz Ekinci (ed.), Chemical Biology, IntechOpen, **2012**, available at: http://www.intechopen.com/books/chemical-biology/protein-purification, https://doi.org/10.5772/35425.
25. Wilson, K., Walker, J., Principles and Techniques of Practical Biochemistry,

Cambridge, UK: Cambridge University Press, **2010**.
26. Sharma, R., Gupta, R., Substrate specificity characterization of a thermostable keratinase from *Pseudomonas aeruginosa* KS-1, **2010**, 785–792, https://doi.org/10.1007/s10295-010-0723-8.
27. Sivakumar, T., Shankar, T., Ramasubramanian, V., Purification Properties of *Bacillus thuringiensis* TS2 Keratinase Enzyme, **2012**, 12, 12, 1553–1557, https://doi.org/10.5829/idosi.aejaes.2012.12.12.1905.
28. Gupta, S., Nigam, A., Singh, R., Purification and characterization of a *Bacillus subtilis* keratinase and its prospective application in feed industry, *Acta Biol Szeged*, **2015**, 59, 2, 197–204.
29. Balakumar, S., Mahesh, N., Arunkumar, M., Sivakumar, R., Hemambujavalli, V., Optimization of keratinase production by Keratinolytic organisms under submerged fermentation, *Int J PharmTech Res*, **2013**, 5, 3, 1294–1300.
30. Selvam, K., Vishnupriya, B., Yamuna, M., Isolation and description of keratinase producing marine actinobacteria from South Indian Coastal Region, *African J Biotechnol*, **2013**, 12, 1, 19–26, https://doi.org/10.5897/AJB12.2428.
31. Lin, X., Shih, J.C.H., Swaisgood, H.E., Hydrolysis of feather keratin by immobilized keratinase, *Appl Environ Microbiol*, **1996**, 62, 11, 4273–4275, https://doi.org/10.1128/AEM.62.11.4273-4275.1996.
32. Lin, X., Lee, C.G., Casale, E.S., Shih, J.C.H., Purification andcharacterization of a keratinase from a feather-degrading Bacillus licheniformis strain, *Appl Environ Microbiol*, **1992**, 58, 3271–5.
33. Wu, W.L., Chen, M.Y., Tu, I.F., Lin, Y.C., Eswarkumar, N., Chen, M.Y., Ho, M.C., Wu, S.H., The discovery of novel heat-stable keratinases from *Meiothermus taiwanensis* WR-220 and other extremophiles, *Sci Rep*, **2017**, 7, 1, 1–12, https://doi.org/10.1038/s41598-017-04723-4.
34. Dettmer, A., Ayub, M.A.Z., Gutterres, M., Hide unhairing and characterization of commercial enzymes used in leather manufacture, *Brazilian J Chem Eng*, **2011**, 28, 3, 373–380, https://doi.org/10.1590/S0104-66322011000300003.
35. Gessesse, A., Hatti-Kaul, R., Gashe, B.A., Mattiasson, B., Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather, *Enzyme Microb Technol*, **2003**, 32, 5, 519–524, https://doi.org/10.1016/S0141-0229(02)00324-1.
36. Ogino, H., Otsubo, T., Ishikawa, H., Screening, purification, and characterization of a leather-degrading protease, *Biochem Eng J*, **2008**, 38, 2, 234–240, https://doi.org/10.1016/j.bej.2007.07.008.
37. Brereton, R.G., Chemometrics for Pattern Recognition, **2009**, John Wiley & Sons, Ltd, https://doi.org/10.1002/9780470746462.
38. Łojewski, M., Muszyńska, B., Smalec, A., Reczyński, W., Opoka, W., Sułkowska-Ziaja, K., Development of Optimal Medium Content for Bioelements Accumulation in *Bacopa monnieri* (L.) *In Vitro* Culture, *Appl Biochem Biotechnol*, **2014**, 174, 4, 1535–1547, https://doi.org/10.1007/s12010-014-1095-8.
39. Zhang, W.Y., Yao, D.X., Zhang, Z.G., Yang, Q., Zhao, K., An, S.K., Characteristics of soil enzymes and the dominant species of repair trees in the reclamation of coal mine area, *J Coal Sci Eng*, **2013**, 19, 2, 256–261, https://doi.org/10.1007/s12404-013-0223-3.
40. Moriya, R.Y., Gonçalves, A.R., Faria, F.P., Enzymatic bleaching of organosolv sugarcane bagasse pulps with recombinant xylanase of the fungus *Humicola grisea* and with commercial cartazyme HS xylanase, *Appl Biochem Biotechnol*, **2005**, 121, 195–203, https://doi.org/10.1385/ABAB:121:1-3:0195.

DETERMINATION OF 24 PESTICIDES RESIDUES IN LEATHER PRODUCTS BY SOLID-PHASE MICROEXTRACTION COUPLED WITH GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Jinlan DAI[1,2,3], Honglei YIN[3], Hang WEI[4], Lei ZHOU[5], Minghua LIU[6*]

[1]College of Chemical Engineering, Fuzhou University, Fuzhou, 350116, Fujian Province, China; 29171239@qq.com

[2]Technical Center of Fuzhou Customs District of P.R.China, 350003, China

[3]Fujian Key Laboratory for Technology Research of Inspection and Quarantine, 350003, China; 21558049@qq.com

[4]Institute of Quality Standards & Testing Technology for Agro-products, Fujian Academy of Agricultural Sciences/Fujian Key Laboratory of Agro-products Quality and Safety, Fuzhou 350003, China; 349537891@qq.com

[5]Technical Center for Safety of Industrial Products of Tianjin Customs District of P .R.China, 300308, China; zhouleiciq@126.com

[6]College of Environment and Resource, Fuzhou University, Fuzhou 350116, Fujian Province, China; mhliu2000@fzu.edu.cn

Received: 09.09.2020 Accepted: 26.11.2020 https://doi.org/10.24264/lfj.20.4.5

DETERMINATION OF 24 PESTICIDES RESIDUES IN LEATHER PRODUCTS BY SOLID-PHASE MICROEXTRACTION COUPLED WITH GAS CHROMATOGRAPHY–MASS SPECTROMETRY

ABSTRACT. Considering the high content of oil and complex residual additives in leather samples, a new analytical method based on the solid-phase extraction technique and gas chromatography-selected ion monitoring mass spectrometry (GC-SIM-MS) was developed to determine 24 organic compounds involving the organochlorine pesticides (OCPs), organophosphorous pesticides (OPPs) and pyrethroids pesticides residues in leather. The extraction conditions (such as the extraction solution, purification procedure and solid-phase extraction column) were optimized using the positive leather samples based on the recovery rates of the pesticides. The best extraction solution, solid-phase extraction column and chromatography column were n-hexane and ethyl acetate (1+1, volume) mixed solution, Carb-PSA (1.0 g, 6mL) and DB-1701 (length: 30 m, inside diameter: 0.25 mm, film thickness: 0.25 μm). The optimized extraction time and temperature were 20 min and 25°C, respectively. The detection limits of 24 pesticide residues range from 0.05 to 0.10 mg/kg, and the recoveries range from 74% to 116%. The relative standard deviations (RSD, n=6) range from 5.42% to 12.00%. The developed method presented a simple, rapid, sensitive, and inexpensive method to detect 24 pesticides in skin and leather and was successfully applied to the detect them in leather products (cowhide, sheep leather and pig leather).

KEY WORDS: leather, organochlorine pesticides, organophosphorous pesticides, pyrethroids pesticides, GC-MS

DETERMINAREA A 24 DE REZIDUURI DE PESTICIDE ÎN PRODUSELE DIN PIELE PRIN MICROEXTRACȚIE ÎN FAZĂ SOLIDĂ CUPLATĂ CU CROMATOGRAFIE DE GAZE - SPECTROMETRIE DE MASĂ

REZUMAT. Având în vedere conținutul ridicat de ulei și aditivi reziduali complecși din probele de piele, s-a dezvoltat o nouă metodă analitică bazată pe tehnica de extracție în fază solidă și cromatografie de gaze cuplată cu spectrometria de masă, prin monitorizarea ionilor selecționați (GC-SIM-MS) pentru a determina reziduurile a 24 de compuși organici printre care pesticide organoclorurate (OCP), pesticide organofosforice (OPP) și piretroizi. Condițiile de extracție (cum ar fi soluția de extracție, procedura de purificare și coloana de extracție în fază solidă) au fost optimizate folosind probe de piele pozitive pe baza ratelor de recuperare a pesticidelor. Cea mai bună soluție de extracție, coloană de extracție în fază solidă și coloană de cromatografie au fost soluția mixtă de n-hexan și acetat de etil (1+1, volum), Carb-PSA (1,0 g, 6 ml) și DB-1701 (lungime: 30 m, diametru interior: 0,25 mm, grosime film: 0,25 μm). Timpul și temperatura de extracție optimizate au fost de 20 min și respectiv 25°C. Limitele de detecție a celor 24 de reziduuri de pesticide variază de la 0,05 la 0,10 mg/kg, iar recuperările variază de la 74% la 116%. Abaterile standard relative (RSD, n=6) variază de la 5,42% la 12,00%. Metoda dezvoltată pentru a detecta 24 de pesticide în piele este simplă, rapidă, sensibilă și ieftină, și a fost aplicată cu succes pentru a detecta aceste reziduuri în produse din piele (de vacă, de oaie și de porc).

CUVINTE CHEIE: piele, pesticide organoclorurate, pesticide organofosforice, pesticide piretroide, GC-MS

DÉTERMINATION DE 24 RÉSIDUS DE PESTICIDES DANS LES PRODUITS EN CUIR PAR MICROEXTRACTION EN PHASE SOLIDE COUPLÉE AVEC LA CHROMATOGRAPHIE GAZEUSE - SPECTROMÉTRIE DE MASSE

RÉSUMÉ. Compte tenu de la teneur élevée en huile et en additifs résiduels complexes dans les échantillons de cuir, une nouvelle méthode analytique basée sur la technique d'extraction en phase solide et la chromatographie en phase gazeuse couplée avec la spectrométrie de masse à contrôle d'ions sélectionnés (GC-SIM-MS) a été développée pour déterminer 24 composés organiques impliquant les pesticides organochlorés (OCP), les pesticides organophosphorés (OPP) et les pesticides pyréthrinoïdes dans le cuir. Les conditions d'extraction (telles que la solution d'extraction, la procédure de purification et la colonne d'extraction en phase solide) ont été optimisées en utilisant les échantillons de cuir positifs basés sur les taux de récupération des pesticides. La meilleure solution d'extraction, la colonne d'extraction en phase solide et la colonne de chromatographie étaient une solution mixte de n-hexane et d'acétate d'éthyle (1+1, volume), Carb-PSA (1,0 g, 6 ml) et DB-1701 (longueur : 30 m, diamètre à l'intérieur : 0,25 mm, épaisseur du film : 0,25 μm). Le temps et la température d'extraction optimisés étaient respectivement de 20 min et 25°C. Les limites de détection de 24 résidus de pesticides vont de 0,05 à 0,10 mg/kg et les taux de récupération vont de 74% à 116%. Les écarts types relatifs (RSD, n=6) vont de 5,42% à 12,00%. La méthode développée pour détecter 24 pesticides dans la peau est une méthode simple, rapide, sensible et peu coûteuse et a été appliquée avec succès pour les détecter les résidus dans les produits en cuir (vache, mouton et porc).

MOTS CLÉS : cuir, pesticides organochlorés, pesticides organophosphorés, pesticides pyréthrinoïdes, GC-MS

** Correspondence to: Minghua LIU, College of Environment and Resource, Fuzhou University, Fuzhou 350116, Fujian Province, China; mhliu2000@fzu.edu.cn*

INTRODUCTION

Skins and leather were widely used in clothing, shoes, automobile, packaging, and decoration industry [1-3]. To obtain good quality of leather, a variety of pesticides were often used in the different stage of leather storage and production [4, 5]. In 1950s, when the raw skin was preserved by air drying in Africa, DDT and lindane were used widely to protect raw skin from putrefaction. Organochlorine and dieldrin were applied to protect against ectoparasites. Lindane was widely used to protect hides and skins from insects in 1990s [6, 7].

Pesticide is a substance or mix-up substances which were used to prevent, destroy, repel, or lessen the damage of any injurious insects. According to the source of raw materials, there are many kinds of pesticide, such as chemical substance, biological agent (such as virus or bacteria), antimicrobial or disinfectant. Some of the used pesticides are persistent organic pollutants (POPs). Compared to conventional pollutants, POPs can cause more hazards on human health and the natural environment due to their persistence, bioaccumulation and high toxicity [8, 9]. The persistence of these compounds in the environment is due to their low degradation by biotic and abiotic process, leading to a long half-live time [8]. Because most of the pesticides in leather, especially OCPs are lipophilic, can be absorbed by living organisms through alimentation, breathing and the skin. After absorption, these compounds usually distributed in various tissue including blood [10-12]. The toxicity of these contaminants is very complex and is specific to each compound. Therefore, multiple toxic responses can occur according to the species, gender and organ affected [13-15]. Previous reports showed that high levels of DDT and pentachlorophenol were detected in the children and adolescents of Germany, because of a leather jacket impregnated with some pesticides, pyrethroid, organophosphorus insecticides, pentachlorophenol (PCP), lindane (γ-HCH), and dichloro(diphenyl)ethylene (DDE) [16, 17].

The trace pesticides in leather may come from livestock breeding process, delivery in the animal food chain, used as preservatives in storage, transportation or processing [18]. These additives may remain in the final products as volatile and semi-volatile organic compounds [8].

The critical substances potentially presented in footwear and footwear components are listed in ISO/TR 16178:2012 [19]. But there are very few sensitive analytical methodologies published and only few related standards and methods for testing pesticides residue in textiles [20-23]. Until now, there has been no responding fast detection method and standard for many kinds of pesticides involved in shoe materials, especially in leather. To protect environment and human health [24-27], it is urgent and necessary to develop methods and international detection standard to monitor and detect these substances.

This paper described a method for the quantitative analysis of 24 pesticide residues in leather. Using small sample volumes, solid phase extraction combined with GC–MS was used to identify 24 pesticide residues at trace concentrations.

MATERIALS AND METHODS

Chemicals and Reagents

Certified standards of the 24 kinds of pesticide (purity of 97 % or more) were obtained from Dr.E of Germany. Their retention time, quantitative and qualitative ions and ion abundance ratios were shown in Table 1. The standard stock solution of pesticides was 1 μg/ml in n-hexane. The standard stock solution was stored at 4°C, and the shelf life is one month. The standard calibration solutions of pesticides are in the range of 10 ng/ml to 1000 ng/ml, and were prepared before using. Chromatographic pure grade of N-hexane, ethyl acetate, acetonitrile, toluene were purchased from TEDIA USA. Solid phase extraction (SPE) column is graphitized carbon black - ethylenediamine - N - propyl methyl silane (Carb – PSA, 1.0 g, 6mL), Carb-NH2, Carb-PSA, PSA, Carb-NH2, Florisil and Alumina N. All the analytical solvents were supplied by Sigma–Aldrich.

Table 1: Retention time, quantitative and qualitative ions and ion abundance ratios of the 24 pesticides

Series Number	Pesticides	Retention time /min	Characteristic fragment ions /amu		
			Quantitative ion	Qualitative ion	Abundance ratio
1	Pentachloroanisole	10.52	280	265,237,263	100:100:82:63
2	α-BHC	11.26	181	183,217,254	100:97:69:4
3	Lindane	12.41	181	183,217,254	100:98:64:11
4	Aldrin	13.78	263	265,261,293	100:67:65:38
5	Chlorothalonil	14.64	266	264,268	100:78:48
6	β- BHC	14.87	181	183,217,254	100:98:74:9
7	δ- BHC	15.65	181	183,217,254	100:97:70:8
8	Malathion	15.84	173	158.256.285	100:48:8:5
9	Dichlofluanide	16.05	123	224,226,332	100:37:26:5
10	Ethylparathion	16.10	275	220,247,232	100:82:77:68
11	Heptachloroepoxide	16.36	353	355,351,317	100:81:52:68
12	o,p'-DDE	16.74	246	318,176,248	100:34:29:65
13	α-Endosulfan	17.16	241	265,277,339	100:63:57:40
14	Tolyfluanide	17.85	238	240,181	100:69:63
15	p,p'-DDE	17.97	318	316,246,248	100:78:130:84
16	Dieldrin	18.55	263	277,345,380	100:79:27:24
17	o,p'-DDD	19.21	235	237,165,199	100:65:44:17
18	o,p'-DDT	19.72	235	237,165,199	100:65:40:15
19	p,p'-DDD	21.10	235	237,165,199	100:64:42:12
20	β-Endosulfan	21.30	241	265,237,339	100:49:84:35
21	p,p'-DDT	21.68	235	237,165,199	100:65:39:12
22	Mirex	22.85	272	274,237,332	100:80:53:11
23	Methoxychlor	23.41	227	228,212,274	100:20:17:15
24	Permethrin	25.70	183	163,165,184	100:20:17:15
		26.13	183	163,165,184	100:27:22:15

Instrument

SPE was performed using a Bond Elut Carbon column (Agilent technologies, USA). GC-MS analysis was achieved using GC with mass-selective detector (MSD) and electron impact source (EIS). Separation was performed on Agilent DB-1701 capillary column (30 m × 0.25 mm, 0.25 µm film thickness). The optimized GC–MS conditions were as follows: injection mode: without split; injector port temperature: 280°C; injection volume: 1.0 µL; carrier gas: helium with flow rate 1.2 mL min^{-1}. Oven program: 50°C for 2 min, increased to 185°C at 30.0°C min^{-1}, held for 1 min and raised to 240°C at a rate of 4.0°C min^{-1} and raised to 270°C at a rate of 20.0°C min^{-1} then held for 5 min. Finally, selected 280°C and held for 2 min to remove impurities. All data were analyzed using Agilent Chem Station software.

Methods

Preparation of Positive Samples

The positive leather samples were prepared and just added the 24 pesticides to the samples in Jinjiang Chenxin Tannery according to usual process, meanwhile blank leather samples were prepared with the same process without pesticides.

Sample Extraction and Purification

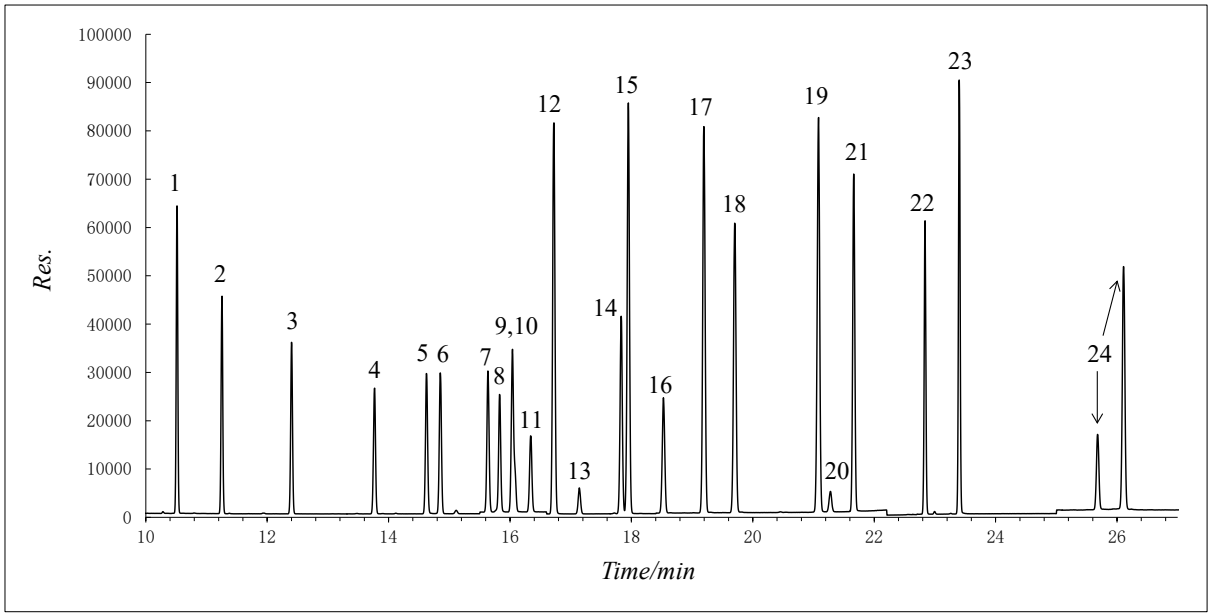
Blank and leather samples 2.0 g was extracted with 20 mL hexane-ethyl acetate (v/v 1:1), vortex oscillated for 10 min and then

extracted for 20 min by ultrasonic extraction under 25°C±2°C. The extraction was centrifuged for 5 min at 5000rpm, and the supernatant was transferred into a brown conical flask. The above procedure was repeated twice. Then combined supernatant was concentrated by a vacuum rotary evaporator at 35°C ± 5°C to approximately 2 ml to 5 ml for further purification.

Before injection into the GC-MS system, the sample solution needs to be concentrated and purified. Firstly, SPE Carb column was pre-rinsed with acetonitrile- toluene (3:1, v/v), and the conical flask was rinsed twice with 3 mL acetonitrile and toluene (3:1, v/v) mixed solution and transfer the washing solutions to the Carb column.

Secondly, the extraction solution flew through the pre-prepared SPE Carb column, 15 mL acetonitrile-toluene (3:1, v/v) as the eluent. Combined and concentrated the solution, then added 5 mL hexane to exchange the solvent and evaporated to be dry at 35°C± 5°C (Solvent displacement was used to change the polarity of the solvent to protect chromatographic column). Repeat this operation. Finally, 2.0 mL hexane was used to dissolve the analytes and the sample was filtered with 0.22 μm membrane for further GC-MS analysis. The 100% ion abundance of fragment ion in the mass spectrum of each underivatized drug was chosen as the quantitative ion. The selective representative ions of 24 kinds of pesticides for quantification were shown in Table 2.

Under the optimized instrument conditions, the chromatograms of 24 pesticides in the standard solutions were shown in Figure 1.



1-Pentachloroanisole,10.52min	2-α-BHC, 11.26min	3-Lindane, 12.40min	4-Aldrin, 13.77min
5-Chlorthalonil, 14.63min	6-β-BHC, 14.85min	7-δ-BHC, 15.64min	8-Malathion, 15.83min
9-Dichlofluanide, 16.04min	10-Ethylparathion, 6.09min	11-Heptachloroepoxide, 16.35min	12-o,p'-DDE, 16.73min
13-α-Endosulfan, 17.14min	14-Tolyfluanide, 17.84min	15-p,p`-DDE, 17.95min	16-Dieldrin, 18.53min
17-o,p`-DDD, 19.20min	18-o,p`-DDT, 19.70min	19-p,p`-DDD, 21.08min	20-β-Endosulfan, 21.28min
21-p,p`-DDT, 21.67min	22-Mirex, 22.84min	23-Methoxychlor, 23.40min	24-Permethrin, 25.68 and 26.11min

Figure 1. The chromatograms of 24 pesticides

RESULTS AND DISCUSSION

Method Development

Through preliminary test, it was found out that hexane-ethyl acetate system was the suitable extraction solvent. So then the effect of extraction solvent on the efficiency was studied by different ratio of hexane and ethyl acetate (5:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:5, v/v). The results showed that the recoveries of p, p'-DDD, p, p'-DDT, methoxychlor were lower than 60% when the ratio was 5:1,3:1, or 2:1. Besides, when the ratio was 1:2, 1:3, or 1:5, the recoveries of p, p'-DDD, p, p'-DDT, methoxychlor were 85-107%, which satisfied the requirement, but those of chlorothalonil, dichlofluanide, tolyfluanide were very low. Generally considering the total analytes, the ratio 1:1 was chosen as the optimum ratio of hexane and ethyl acetate solvent system. The experimental results were shown in supplemental materials (Table S2).

To optimize the extraction efficiency, three extraction methods (votex oscillation, sonication, mixed votex and sonication) were evaluated. The experiments results showed that the extraction efficiency of the analytes was the highest when hexane-ethyl acetate (1:1, v/v) was used as the solvent and sonicated for 20 min after votex oscillation for 10 min at 25°C.

The extraction efficiency at different extraction time was studied. The test samples were extracted for 10 min, 20 min, 30 min, 40 min, 50 min and 60 min at the temperature of 25°C ± 2°C. Each sample was tested twice, and the results are shown in Figure 2. From the results, it was found that with the extraction time increasing, the pesticide content increased in the beginning, and then decreased. For most of the 24 kinds of pesticide (except tolyfluanide and chlorothalonil), the concentration reached the highest when the extraction time was 20 min. So 20 min was chosen as the optimum extraction time.

To study the effect of the temperature on the extraction efficiency, the test samples were extracted at the temperatures of 25°C, 30°C, 35°C, and 40°C for 20 min, respectively. It was found that the highest extraction content was obtained at the temperature of 25°C. So the temperature of 25°C was chosen as the optimized extraction temperature. The result for each test piece was the average content of the two-test piece, and the test results are shown in Figure 3.

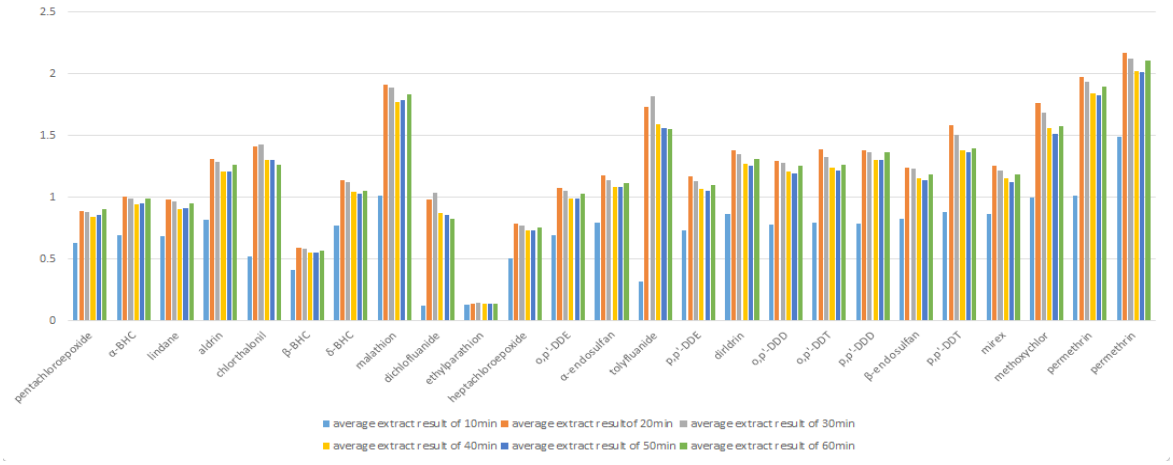


Figure 2. Extraction results at different extraction time

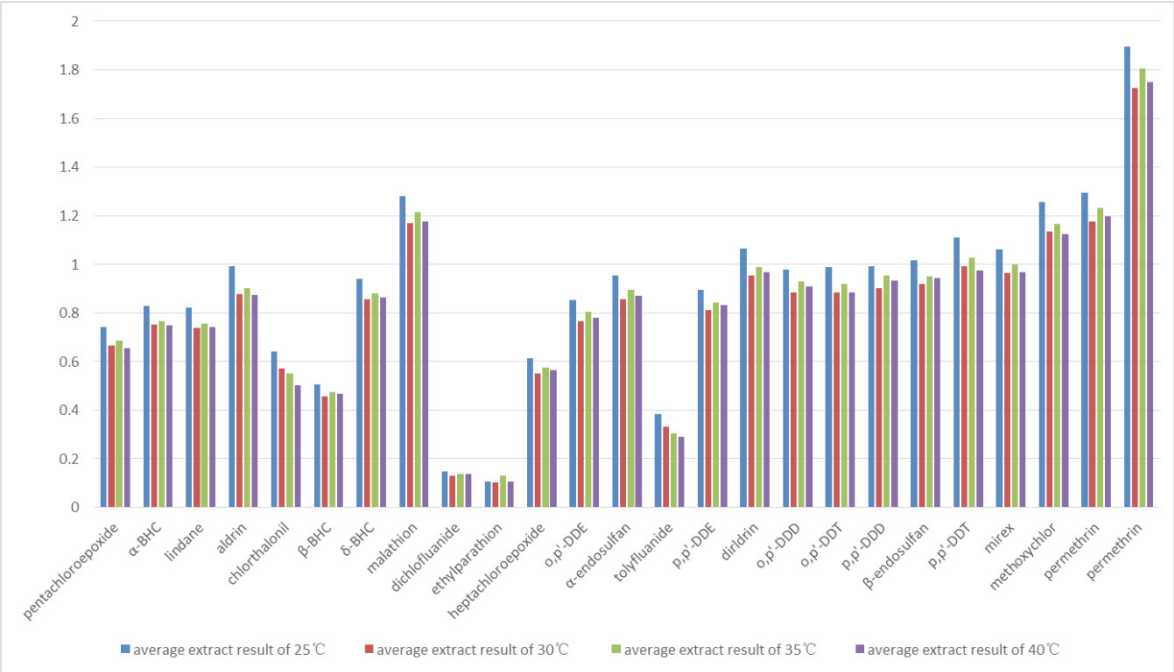


Figure 3. Extraction results at different extraction temperature

Linearity and limit of detection (LOD)

A series of mixed standard working solutions (10, 20, 50, 100, 200, 500, 1000 ng mL^{-1}) were prepared and all determined under the optimal pretreatment and instrument conditions. Then the standard curves were plotted between the peak areas and corresponding concentrations and it showed linear regression relationship in the certain range. Limit of detection (LOD) and limit of quantitation (LOQ) were performed using spiked standard samples method and LOQ of the method for the analytes were calculated at signal-to-noise (S/N) ratio of 3 and 10. All the above experimental results, retention time, quantitative and qualitative ions and ion abundance ratios of 24 pesticide residues were listed in Table 3.

From the results in Table 3, it was revealed that 24 analytes presented good linearity, with coefficient of determination (R^2) between 0.9983 and 0.9998. The LODs were in the range of 0.3–15.0 ng/mL (0.01-0.15 mg/kg), and the LOQ ranged from 2.0 to 50.0 ng/mL.

Matrix Effects

For GC–MS analysis, it is necessary to evaluate method matrix effects for each analyte, because they may result from various physical and chemical processes which are difficult to eliminate in analysis. To estimate matrix effects, the slopes of the matrix-matched calibration curves are compared with those obtained in solvent without matrices, and matrix effect (ME%) is evaluated as the following equation:

$$ME\ (\%) = B/A \times 100 \quad (1)$$

where A and B are the slopes of calibration curve without matrix and with different matrix [15-17]. ME% values suggest that the ionization signals of target compounds were enhanced and suppressed by the matrix, respectively. Generally, ME% ranging from 85% to 115% indicate that the signal enhancement or suppression is acceptable, while ME values < 85% or >115% refer to strong matrix effects.

From Table 3, it was known that ME% of most of pesticides were between 85% and 115% in solvent, blank, cow leather, sheep leather, pig leather, PU and PVC and the matrix effects were very small and could be ignored according to the requirements. The ME% of malathion, ethylparathion, o,p'-DDD, p,p'-DDD was more than 115%, and it meant that they had strong matrix enhancement effect. Meanwhile, the ME% of α-endosulfan, β-endosulfan, o,p'-DDT, p,p'-DDT, and methoxychlor was less than 85%, and it showed that these analytes had strong signal suppression effect by matrix. To obtain the results more accurately, it was necessary to use the blank negative sample to calibrate the working curve.

Table 3: Overview of the methodological characteristics including linear range, linear equation, coefficient of determination (R^2), matrix effect (ME), LOD, and LOQ for each analyte in different samples

The analyte	Quantitative ion m/z	matrix	Linear range (ng/mL)	Linear equation	Coefficient R^2	ME (%)	LOD ng/mL	LOQ ng/mL
Pentachloroanisole (1)	280	solvent	10~1000	Y=31.58X+2.004	0.9998	100.00	1	3
		blank	10~1000	Y=30.48X+229.1	0.9996	96.52	3	10
		cowhide	20~1000	Y=33.74X+127.8	0.9978	106.84	5	15
		Sheep leather	20~1000	Y=27.87X+345.9	0.9934	88.25	6	20
		Pig leather	50~1000	Y=27.94X+722.1	0.9956	88.47	10	30
		PU	20~1000	Y=29.03X-738.1	0.9965	91.93	5	15
		PVC	20~1000	Y=27.98X+913.3	0.9949	88.60	5	15
α-Benzenehexachloride (α-BHC, 2)	181	solvent	10~1000	Y=32.17X-34.99	0.9997	100.00	2	6
		blank	20~1000	Y=30.68X+38.54	0.9974	95.37	8	25
		cowhide	50~1000	Y=35.08X+39.66	0.9997	109.05	10	30
		Sheep leather	50~1000	Y=27.85X+57.12	0.9965	86.57	15	50
		Pig leather	50~1000	Y=27.49X+331.1	0.9971	85.45	15	50
		PU	50~1000	Y=35.64X-1004	0.9971	110.79	10	30
		PVC	50~1000	Y=30.52X+382.7	0.9983	94.87	10	30
Lindane (3)	181	solvent	10~1000	Y=27.36X-37.28	0.9998	100.00	2	6
		blank	20~1000	Y=25.36X+163.3	0.9972	92.69	8	25
		cowhide	50~1000	Y=28.67X-626	0.9989	104.79	10	30
		Sheep leather	50~1000	Y=27.89X-424	0.9962	101.94	15	50
		Pig leather	50~1000	Y=26.92X-81.29	0.9969	98.39	15	50
		PU	50~1000	Y=30.88X-672	0.9964	112.87	10	30
		PVC	50~1000	Y=24.27X+570.7	0.9981	88.71	10	30
Aldrin (4)	263	solvent	10~1000	Y=18.12X-23.22	0.9998	100.00	2	6
		blank	10~1000	Y=18.26X+76.58	0.9981	100.77	8	25
		cowhide	50~1000	Y=20.67X-60.11	0.9994	114.07	15	50
		Sheep leather	100~1000	Y=19.87X+234	0.9924	109.66	30	100
		Pig leather	100~1000	Y=18.31X+73.76	0.9978	101.05	30	100
		PU	50~1000	Y=20.46X-478.3	0.9972	112.91	10	30
		PVC	50~1000	Y=18.53X+371	0.9968	102.26	10	30
Chlorothalonil (5)	266	solvent	50~1000	Y=23.75X-1291	0.9978	100.00	15	50
		blank	100~1000	Y=26.09X-147	0.9989	109.85	23	75
		cowhide	100~1000	Y=27.39X+5290	0.9903	115.33	30	100
		Sheep leather	100~1000	Y=19.13X-1323	0.9931	80.55	30	100
		Pig leather	100~1000	Y=20.38X+3425	0.9921	85.81	30	100
		PU	100~1000	Y=17.16X-1467	0.9951	72.25	23	75
		PVC	50~1000	Y=26.38X-2422	0.9954	111.07	15	50
β-BHC (6)	181	solvent	10~1000	Y=25.83X-61.41	0.9999	100.00	2	6
		blank	50~1000	Y=24.88X+82.35	0.9981	96.32	10	30
		cowhide	50~1000	Y=28.35X-11.34	0.9996	109.76	15	50
		Sheep leather	50~1000	Y=22.64X-2441	0.9932	87.65	15	50
		Pig leather	50~1000	Y=23.03X+691	0.9954	89.16	15	50
		PU	50~1000	Y=29.54X-704.8	0.9967	114.36	10	30
		PVC	50~1000	Y=22.24X+684.9	0.9943	86.10	10	30
δ-BHC (7)	181	solvent	10~1000	Y=23.09X-106.5	0.9997	100.00	2	6
		blank	50~1000	Y=21.11X+258.9	0.9951	91.42	10	30
		cowhide	50~1000	Y=25.72X+443.3	0.9984	111.39	15	50
		Sheep leather	50~1000	Y=24.71X+148.4	0.9927	107.02	15	50
		Pig leather	50~1000	Y=21.23X+868.2	0.9931	91.94	15	50
		PU	50~1000	Y=26.46X+167.1	0.9964	114.60	10	30
		PVC	50~1000	Y=26.51X-220	0.9983	114.81	10	30

The analyte	Quantitative ion m/z	matrix	Linear range (ng/mL)	Linear equation	Coefficient R^2	ME (%)	LOD ng/mL	LOQ ng/mL
Malathion (8)	173	solvent	10~1000	Y=36.24X-515.1	0.9987	100.00	2	6
		blank	20~1000	Y=41.95X+309.1	0.9965	115.76	6	20
		cowhide	50~1000	Y=57.42X-912.5	0.9987	158.44	15	50
		Sheep leather	100~1000	Y=45.78X-893	0.9952	126.32	20	60
		Pig leather	100~1000	Y=46.51X-1840	0.9957	128.34	30	100
		PU	50~1000	Y=55.75X-747.1	0.9976	153.84	10	30
		PVC	20~1000	Y=50.51X+1106	0.9986	139.38	6	20
Dichlofluanide (9)	123	solvent	10~1000	Y=51.09X-126.4	0.9976	100.00	3	10
		blank	50~1000	Y=43.79X-1112	0.9961	85.71	30	100
		cowhide	100~1000	Y=60.91X-1854	0.9988	119.22	30	100
		Sheep leather	100~1000	Y=45.87X-1242	0.9967	89.78	30	100
		Pig leather	100~1000	Y=41.87X+2126	0.9975	81.95	30	100
		PU	100~1000	Y=62.87X-4773	0.9994	123.06	30	100
		PVC	100~1000	Y=59.14X-1769	0.9989	115.76	20	60
Ethylparathion (10)	275	solvent	20~1000	Y=3.879X-14.57	0.9976	100.00	6	20
		blank	20~1000	Y=4.639X+30.11	0.9943	119.59	20	60
		cowhide	50~1000	Y=8.189X-42.11	0.9989	211.11	23	75
		Sheep leather	100~1000	Y=7.231X-452	0.9936	186.41	30	100
		Pig leather	100~1000	Y=7.041X+218.6	0.9934	181.52	30	100
		PU	100~1000	Y=7.907X-267.9	0.9953	203.84	20	60
		PVC	50~1000	Y=7.642X-33.66	0.9971	197.01	10	30
Hepta-chloroepoxide (11)	353	solvent	20~1000	Y=3.445X+1.98	0.9997	100.00	6	20
		blank	20~1000	Y=3.734X+3.592	0.9989	108.39	15	50
		cowhide	50~1000	Y=3.926X-4.895	0.9992	113.96	15	50
		Sheep leather	50~1000	Y=3.532X+32.56	0.9974	102.53	15	50
		Pig leather	50~1000	Y=3.405X+42.79	0.9962	98.84	15	50
		PU	50~1000	Y=3.489X-90.61	0.9959	101.28	10	30
		PVC	50~1000	Y=3.567X+77.34	0.9981	103.54	10	30
o,p'dichloro (diphenyl) ethylene (o,p'-DDE, 12)	246	solvent	10~1000	Y=84.93X-81.78	0.9998	100.00	0.3	1
		blank	10~1000	Y=86.34X+304.3	0.9981	101.66	3	10
		cowhide	10~1000	Y=95.54X-156.8	0.9996	112.49	3	10
		Sheep leather	20~1000	Y=84.67X-245	0.9972	99.69	3	10
		Pig leather	20~1000	Y=88.01X+789.3	0.9966	103.63	3	10
		PU	10~1000	Y=79.06X+570.1	0.9923	93.09	3	10
		PVC	10~1000	Y=87.77X+3034	0.9985	103.34	3	10
α-Endosulfan (13)	241	solvent	50~1000	Y=3.992X+17.7	0.9998	100.00	8	25
		blank	50~1000	Y=4.263X+182.2	0.9986	106.79	30	100
		cowhide	100~1000	Y=3.156X-243.9	0.9966	79.06	30	100
		Sheep leather	100~1000	Y=3.032X+563	0.9951	75.95	30	100
		Pig leather	100~1000	Y=2.681X+244.7	0.9943	67.16	30	100
		PU	100~1000	Y=3.606X+207	0.9944	90.33	30	100
		PVC	100~1000	Y=4.235X+152.1	0.9962	106.09	30	100
Tolyfluanide (14)	238	solvent	10~1000	Y=21.51X-465.9	0.9978	100.00	3	10
		blank	50~1000	Y=20.53X-445	0.9988	95.44	15	50
		cowhide	100~1000	Y=22.39X-181.1	0.9987	104.09	30	100
		Sheep leather	100~1000	Y=21.63X-242.1	0.9963	100.56	30	100
		Pig leather	100~1000	Y=20.89X+170.8	0.9939	97.12	30	100
		PU	50~1000	Y=21.89X-6996	0.9972	101.77	15	50
		PVC	50~1000	Y=24.55X-159.4	0.9973	114.13	10	30

The analyte	Quantitative ion m/z	matrix	Linear range (ng/mL)	Linear equation	Coefficient R^2	ME (%)	LOD ng/mL	LOQ ng/mL
p,p'-DDE (15)	318	solvent	10~1000	Y=51.26X-53.69	0.9997	100.00	0.6	2
		blank	10~1000	Y=54.29X+105.1	0.9991	105.91	1	3
		cowhide	10~1000	Y=56.69X+2.21	0.9996	110.59	3	10
		Sheep leather	10~1000	Y=42.89X+345	0.9962	83.67	3	10
		Pig leather	10~1000	Y=43.47X+1612	0.9938	84.80	3	10
		PU	10~1000	Y=48.58X+272.8	0.9932	94.77	2	6
		PVC	10~1000	Y=45.47X+1791	0.9934	88.70	2	6
Dieldrin (16)	263	solvent	10~1000	Y=8.776X+51.73	0.9995	100.00	4	10
		blank	10~1000	Y=9.157X+50.29	0.9953	104.34	15	50
		cowhide	50~1000	Y=7.971X-495.6	0.9924	90.83	15	50
		Sheep leather	50~1000	Y=7.672X+241	0.9935	87.42	15	50
		Pig leather	50~1000	Y=8.471X+1.368	0.9976	96.52	15	50
		PU	50~1000	Y=8.522X-32.01	0.9931	97.11	15	50
		PVC	50~1000	Y=10.11X+1.474	0.9981	115.20	15	50
o,p'-dichloro bis(4-chlorophenyl) ethane (o,p'-DDD, 17)	235	solvent	10~1000	Y=97.91X-150	0.9997	100.00	0.6	2
		blank	10~1000	Y=113.4X+243.5	0.9989	115.82	2	6
		cowhide	20~1000	Y=122.5X-410.7	0.9997	125.11	6	20
		Sheep leather	50~1000	Y=117.3X-652	0.9954	119.80	10	30
		Pig leather	50~1000	Y=121.8X+513.2	0.9964	124.40	10	30
		PU	10~1000	Y=121.3X+496.6	0.9933	123.89	2	6
		PVC	10~1000	Y=120.7X+3018	0.9974	123.28	2	6
o,p'-dichloro (diphenyl) trichloroethane (o,p'-DDT, 18)	235	solvent	10~1000	Y=76.68X-1140	0.9989	100.00	1	3
		blank	10~1000	Y=71.71X+306.4	0.9981	93.52	3	10
		cowhide	20~1000	Y=77.69X+138.3	0.9998	101.32	5	15
		Sheep leather	50~1000	Y=54.82X+2834	0.9931	71.49	10	30
		Pig leather	50~1000	Y=51.02X+2938	0.9929	66.54	10	30
		PU	20~1000	Y=68.87X+606.4	0.9934	89.81	5	15
		PVC	10~1000	Y=54.46X+1291	0.9926	71.02	3	10
p,p'-DDD (19)	235	solvent	10~1000	Y=106.1X-335	0.9997	100.00	0.6	2
		blank	10~1000	Y=127.3X+225.9	0.9984	119.98	2	6
		cowhide	10~1000	Y=133.5X-87.84	0.9998	125.82	3	10
		Sheep leather	50~1000	Y=132.4X+4522	0.9935	124.79	10	30
		Pig leather	50~1000	Y=126.1X+2374	0.9925	118.85	10	30
		PU	10~1000	Y=116.7X+334	0.9937	109.99	2	6
		PVC	10~1000	Y=120.3X+5296	0.9944	113.38	2	6
β-Endosulfan (20)	241	solvent	50~1000	Y=2.642X+120.1	0.9991	100.00	15	50
		blank	50~1000	Y=2.151X+616.6	0.9955	81.42	15	50
		cowhide	100~1000	Y=2.127X-73.08	0.9905	80.51	30	100
		Sheep leather	100~1000	Y=2.034X+1312	0.9902	76.99	30	100
		Pig leather	100~1000	Y=1.903X+1049	0.9918	72.03	30	100
		PU	100~1000	Y=1.797X+1765	0.9913	68.02	20	60
		PVC	100~1000	Y=2.253X+390	0.9979	85.28	20	60
p,p'-DDT (21)	235	solvent	10~1000	Y=77.75X-1764	0.9976	100.00	1	3
		blank	10~1000	Y=67.17X+223.9	0.9964	86.39	3	10
		cowhide	10~1000	Y=67.99X+1329	0.9997	87.45	3	10
		Sheep leather	50~1000	Y=53.45X+5631	0.9937	68.75	10	30
		Pig leather	50~1000	Y=44.01X+6144	0.9957	56.60	10	30
		PU	10~1000	Y=66.03X+289.5	0.9925	84.93	3	10
		PVC	10~1000	Y=47.62X+1058	0.9929	61.25	3	10

The analyte	Quantitative ion m/z	matrix	Linear range (ng/mL)	Linear equation	Coefficient R^2	ME (%)	LOD ng/mL	LOQ ng/mL
Mirex (22)	272	solvent	10~1000	Y=48.08X+22.72	0.9996	100.00	3	10
		blank	10~1000	Y=47.28X+62.23	0.9985	98.34	3	10
		cowhide	10~1000	Y=41.22X-164.1	0.9998	85.73	3	10
		Sheep leather	20~1000	Y=43.67X+452	0.9978	90.83	6	20
		Pig leather	20~1000	Y=42.76X+649.2	0.9961	88.94	6	20
		PU	10~1000	Y=41.98X+268.6	0.9934	87.31	3	10
		PVC	10~1000	Y=44.73X+961.3	0.9972	93.03	3	10
Methoxychlor (23)	227	solvent	10~1000	Y=140.4X-2725	0.9983	100.00	0.6	2
		blank	10~1000	Y=126.4X+98.56	0.9985	90.03	2	6
		cowhide	10~1000	Y=129.1X+1090	0.9998	91.95	3	10
		Sheep leather	20~1000	Y=132.4X+1343	0.9976	94.30	6	20
		Pig leather	20~1000	Y=102.8X+6422	0.9935	73.22	6	20
		PU	10~1000	Y=117.1X+1178	0.9942	83.40	3	10
		PVC	10~1000	Y=110.4X+265.1	0.9983	78.63	2	6
Permethrin (24)	183	solvent	10~1000	Y=116.6X+26.87	0.9998	100.00	3	10
		blank	10~1000	Y=128.1X+622.8	0.9952	109.86	3	10
		cowhide	20~1000	Y=125.2X+1069	0.9999	107.38	5	15
		Sheep leather	20~1000	Y=127.7X+2234	0.9987	109.52	6	20
		Pig leather	20~1000	Y=119.5X+7337	0.9977	102.49	6	20
		PU	20~1000	Y=111.9X+1962	0.9937	95.97	6	20
		PVC	10~1000	Y=124.4X+3987	0.9992	106.69	3	10

Recovery and Precision Test

Blank negative leather samples (including cow leather, sheep leather, and pig leather) at 100, 300, 600 μg/kg levels of mixed standard solutions were selected to perform the recovery experiment. Precisions were evaluated by using the relative standard deviation (RSD) of six measurements. The average recoveries for 24 analytes were in the range of 75.49%-131%. The precision was 1.14%-14.37%. The experimental results were shown in supplemental materials (Tables S2A, S2B and S2C). These results can satisfy the requirement of testing the pesticide residues in leather product.

Determination of 24 Pesticide Residues in Leather Samples

The developed and validated method was applied to detect the presence and quantify 24 pesticide residues in three positive samples. Results were shown in Table 4. The experimental results showed that the concentrations of most pesticide residues in these samples were about 200, 500, 1000μg/kg. And the RSD was the range of 2%~13%. However, chlorothalonil, dichlofluanide, and tolyfluanide were not detected because of degradation.

Table 4: The average content of 24 pesticide residues in three positive leather samples

Analyte	Sample number	Content (μg/kg) 1	2	3	4	5	6	Average (μg/kg)	RSD (%)
1	4#	205.7	216.7	194.2	198.7	204.3	197.3	202.8	3.97
	5#	508.6	482.6	486.4	493.1	477.6	493.1	490.2	2.21
	6#	949.3	953.7	1015.3	986.1	948.7	986.1	973.2	2.78
2	4#	186.1	176	176.1	182.7	177.4	194.2	182.1	3.95
	5#	417.3	443.9	417.7	487.5	480.7	404.8	442.0	7.94
	6#	932.2	932.9	1079	986.5	938	977.2	974.3	5.79
3	4#	197.4	173.7	166.4	172.5	209.3	163.4	180.5	10.27
	5#	463.2	438	450.6	416.6	450.8	418.6	439.6	4.29
	6#	818.5	1006.9	1044	1025.4	1001.9	1025.4	987.0	8.50

Analyte	Sample number	Content (μg/kg)						Average (μg/kg)	RSD (%)
		1	2	3	4	5	6		
	4#	219.4	213.2	225.2	213	211.7	213.4	216.0	2.43
4	5#	557.8	543.4	542	542.8	538.4	542.8	544.5	1.24
	6#	1072.3	1069.3	1167.9	1142.2	1064.3	1142.2	1109.7	4.15
	4#	--	--	--	--	--	--	--	--
5	5#	--	--	--	--	--	--	--	--
	6#	--	--	--	--	--	--	--	--
	4#	213.7	191.3	201.2	188.2	211.6	162.4	194.7	9.71
6	5#	451.1	424.8	408.8	445.4	367	445.4	423.8	7.56
	6#	1142.5	925.2	903	1151.5	892.6	863.1	979.7	13.39
	4#	145.8	165.4	143.8	140.2	120.6	136.2	142.0	10.25
7	5#	298.6	229.8	220.3	254.1	224.8	254.1	247.0	11.83
	6#	563.6	606.7	665.2	683.7	601.7	683.7	634.1	7.93
	4#	82	86.3	72.8	75.5	58.7	81.4	76.1	12.89
8	5#	171.4	192.7	194	191.6	187.7	191.6	188.2	4.51
	6#	540.5	537.6	521.6	569.9	532.6	569.9	545.4	3.68
	4#	--	--	--	--	--	--	--	--
9	5#	--	--	--	--	--	--	--	--
	6#	--	--	--	--	--	--	--	--
	4#	54.65	56.46	50.5	44.85	58.61	65.6	55.1	12.85
10	5#	90.2	79.5	84.4	78.9	74.5	78.9	81.1	6.75
	6#	229.3	230.4	248.4	242.4	225.4	242.4	236.4	3.89
	4#	178	170.6	143.2	150.7	155.7	150.9	158.2	8.42
11	5#	390.5	402.2	437.4	407.9	397.2	407.9	407.2	3.99
	6#	872.3	854	928.5	930.7	849	930.7	894.2	4.47
	4#	178.6	174.9	175.5	166.2	169.5	168.2	172.2	2.83
12	5#	447.3	441.7	480.6	464.3	436.7	464.3	455.8	3.67
	6#	945.7	947	1025.3	997.1	942	997.1	975.7	3.62
	4#	336.2	271.2	351.8	307	325	291.1	313.7	9.51
13	5#	582.5	659	715.2	587.7	654	587.7	631.0	8.54
	6#	1211.2	1222.2	1347.2	1249.4	1217.2	1249.4	1249.4	4.05
	4#	--	--	--	--	--	--	--	--
14	5#	--	--	--	--	--	--	--	--
	6#	--	--	--	--	--	--	--	--
	4#	151.1	145.2	141	126.4	134.3	126.6	137.4	7.34
15	5#	364.5	359.4	398.2	389	354.4	389	375.8	4.91
	6#	943.4	945.1	1006.5	972	940.1	972	963.2	2.66
	4#	234.4	227.9	170.7	184.6	221.7	218.3	209.6	12.28
16	5#	524	526.2	566.4	574.5	521.2	570.7	547.2	4.71
	6#	1054.2	1036.3	1098.5	1095	1047.7	1103.5	1072.5	2.77
	4#	169.6	168.1	162.4	155.8	159.4	157.9	162.2	3.45
17	5#	443.1	436.7	463.4	443.7	423	454.3	444.0	3.15
	6#	864.5	863.4	909.1	881.2	858.4	881.2	876.3	2.13

Analyte	Sample number	Content (μg/kg)						Average (μg/kg)	RSD (%)
		1	2	3	4	5	6		
	4#	178.3	184.3	180.2	179	179.9	179.7	180.2	1.17
18	5#	453.4	444	504.3	500.3	439	500.3	473.6	6.58
	6#	1126.5	1117.1	1196.3	1155.5	1112.1	1155.5	1143.8	2.78
	4#	177.6	171.3	160	157.8	167.4	157.1	165.2	5.02
19	5#	439.8	427.9	455	443.6	422.9	443.6	438.8	2.66
	6#	708.1	690.8	768.6	687.1	685.8	687.1	704.6	4.61
	4#	548	440.2	399.1	519.8	497.4	491	482.6	11.24
20	5#	1213.1	1135	1085.6	1206.9	1091.4	1162.8	1149.1	4.80
	6#	2080.3	2022.6	2200.3	1979.2	2064.1	2025.3	2062.0	3.71
	4#	215.4	226.9	181.5	209.6	202.4	210	207.6	7.31
21	5#	571.2	580.9	649.8	638.6	575.9	638.6	609.2	6.02
	6#	1537	1511.5	1582.1	1515.5	1506.5	1515.5	1528.0	1.86
	4#	186.5	181	174.4	163.9	177.6	164	174.6	5.24
22	5#	431.1	425.1	481.9	475.5	420.1	475.5	451.5	6.40
	6#	1010.9	1005.5	1059.4	1019.8	1000.5	1019.8	1019.3	2.07
	4#	180.3	188.4	181	171	181.4	176	179.7	3.25
23	5#	449.1	446.4	481.8	478.3	441.4	478.3	462.6	4.05
	6#	979	963.7	1009.6	983.4	958.7	983.4	979.6	1.84
	4#	223.97	213.12	204.5	208.5	220.44	205.91	212.7	3.75
24	5#	514.36	502.69	568.55	562.16	497.69	562.16	534.6	6.18
	6#	1175	1178.6	1198.2	1153.3	1173.6	1153.3	1172.0	1.45

CONCLUSIONS

This study contributed to a validated method detection standard for quantitative detecting common pesticide residues in leather. Small sample volumes, solid phase extraction and GC–MS was used to identify these 24 pesticide residues at trace concentrations. The method established has been successfully applied in detecting the pesticide residues in cowhide, sheep leather and pig leather. In the future, the supervisor can use this method to monitor the content of the harmful chemicals. It will gain more practical applications in shoe materials and provide the reference and advises for the detection of these pesticides residues in other industry products.

Author Contributions

Method development, Jinlan Dai and Honglei Yin; Experiment, Jinlan Dai and Hang Wei; Application, Jinlan Dai, and Lei Zhou, Director, Minghua Liu.

Acknowledgement

This project was financially supported by the key project of Fuzhou Customs District of P.R. China (FK2013-42) and the project of General Administration of Customs of P.R. China (2016IK038), and Natural Science Foundation of Tianjin, China (General Program, No. 14JCYBJC16800).

REFERENCES

1. Kim, W., Lee, Y., Lee, J.H., Shin, G.W., Yun, M.H., A comparative study on designer and customer preference models of leather for vehicle, *Int J Ind Ergonom*, **2018**, 65, 110-121, https://doi.org/10.1016/j.ergon.2017.07.009.
2. Adigüzel Zengin, A.C., *Örk*, N., Yiğit, Ü., Bitlisli, B.O., Leather as a potential liner for the prosthetic leg users, *Leather and Footwear Journal*, **2018**, 18, 4, 321-326, https://doi.org/10.24264/lfj.18.4.7.

3. Indian Leather Group, Furniture and Automotive Leathers, **2015**, 49, 5-8.
4. Kvitko, K., Bandinelli, E., Henriques, J.A.P., Heuser, V.D., Rohr, P., da Silva, F.R., Schneider, N.B., Fernandes, S., Ancines, C., da Silva, J., Susceptibility to DNA damage in workers occupationally exposed to pesticides, to tannery chemicals and to coal dust during mining, *Genet Mol Biol*, **2012**, 35, 4 Suppl, 1060–1068, https://doi.org/10.1590%2Fs1415-47572012000600022.
5. Lin, F., Chen, X.C., Li, X.Y., Wu, Z.Y., Chen, H.L., Application status of biocides and fungicide in shoe material and its determination methods, *China Leather*, **2010**, 10, 110-115.
6. Corning, D.R., Cory, N.J., Sykes, R.L., Studies on pesticides used for the protection of skins during storage and transportation. Part 2: Analytical studies and environmental impact, *J Soc Leath Tech Ch*, **1993**, 77, 3-13.
7. Golob, P., Robinson, F.V., Gutu, S., Studies on pesticides used for the protection of skins during storage and transportation. Part 1: Protection of goat skins against beetle damage using insecticides in Zimbabwe, *J Soc Leath Tech Ch*, **1992**, 76, 83-88.
8. Jin, J., Yu, L.Y., Dong, W., Wu, M.R., Liu, S.R., Study on determination of carbendazim, chlorothalonil and thiophanate-methyl in Leather, *Leath Ch*, **2014**, 31, 16-20.
9. Li, Z.J., Yang, W.H., Qiu, M.J., Guangdong chemical industry, **2002**, 2, 17-19.
10. Nunes, R.R., Bontempi, R.M., Mendonça, G., Galetti, G., Rezende, M.O.O., Vermicomposting as an advanced biological treatment for industrial waste from the leather industry, *J Environ Sci Health B*, **2016**, 51, 5, 271-277.
11. De La Cruz, A.R.H., Ferreira, L.D.S.C., Andrade, V.P., Gioda, A., Biomonitoring of Toxic Elements in Plants Collected Near Leather Tanning Industry, *J Braz Chem Soc*, **2019**, 30, 2, 256-264, https://doi.org/10.21577/0103-5053.20180174.
12. Shi, J.J., Chen, H.L., Arocena, J.M., Whitcombe, T., Thrig, R.W., Memiaghe, J.N., Elemental sulfur amendment decreases bio-available Cr-VI in soils impacted by leather tanneries, *Environ Pollut*, **2016**, 212, 57-64, https://doi.org/10.1016/j.envpol.2016.01.045.
13. Balaji, A.P.B., Sastry, T.P., Manigandan, S., Mukherjee, A., Chandrasekara, N., Environmental benignity of a pesticide in soft colloidal hydrodispersive nanometric form with improved toxic precision towards the target organisms than non-target organisms, *Sci Total Environ*, **2017**, 579, 190-201, https://doi.org/10.1016/j.scitotenv.2016.10.240.
14. Sundaramoorthy, R., Velusamy, Y., Balaji, A.P.B., Mukherjee, A., Chandrasekaran, N., Comparative cytotoxic and genotoxic effects of permethrin and its nanometric form on human erythrocytes and lymphocytes in vitro, *Chem Biol Interact*, **2016**, 257, 119-124, https://doi.org/10.1016/j.cbi.2016.08.001.
15. Fritschi, L., Benke, G., Risch, H.A., Schulte, A., Webb, P.M., Whiteman, D.C., Fawcett, J., Neale, R.E., Occupational exposure to *N*-nitrosamines and pesticides and risk of pancreatic cancer, *Occup Environ Med*, **2015**, 72, 9, 678-683, https://doi.org/10.1136/oemed-2014-102522.
16. Mikoczy, Z., Hagmar, L., Cancer incidence in the Swedish leather tanning industry: updated findings 1958–99, *Occup Environ Med*, **2005**, 62, 7, 461-464, https://doi.org/10.1136/oem.2004.017038.
17. Heudorf, U., Angerer, J., Drexler, H., Current internal exposure to pesticides in children and adolescents in Germany: Blood plasma levels of pentachlorophenol (PCP), lindane (γ-HCH), and dichloro(diphenyl)ethylene (DDE), a biostable metabolite of dichloro(diphenyl) trichloroethane (DDT), *Int J Hyg Environ Health*, **2003**, 206, 6, 485-491.
18. Heudorf, U., Angerer, J., Drexler, H., Current internal exposure to pesticides in children and adolescents in Germany: urinary levels of metabolites of pyrethroid and organophosphorus insecticides, *Int Arch Occup Environ Health*, **2004**, 77, 67-72,

https://doi.org/10.1007/s00420-003-0470-5.
19. ISO/TR 16178:2012 Footwear Critical substances potentially present in footwear and footwear components.
20. GB/T 18412.1-2006 The detection of pesticide residues in textiles Part one: 77 kinds of pesticides.
21. GB/T 18412.2-2006 The detection of pesticide residues in textiles Part two: organochlorine pesticides.
22. GB/T 23204-2008 The detection of 519 kinds of pesticide residues and related chemicals in Chinese tea by GC-MS.
23. Gong, T.S., Dong, S.M., Lin, F., Lian, X.B., Determination of Salicylanilide in Leather Fungicides by HPLC, *Leather Science and Industry*, **2011**, 21, 43-47.
24. Frenich, A.G., Romero-González, R., *Gómez-Pérez, M.L.*, Martinez Vidal, J.L., Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry, *J Chromatogr A*, **2011**, 1218, 28, 4349–4356, https://doi.org/10.1016/j.chroma.2011.05.005.
25. Xian, Y., Dong, H., Wu, Y., Guo, X., Hou, X., Wang, B., QuEChERS-based purification method coupled to ultrahigh performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) to determine six quaternary ammonium compounds (QACs) in dairy products, *Food Chem*, **2016**, 212, 96–103, https://doi.org/10.1016/j.foodchem.2016.05.151.
26. Xian, Y., Guo, X., Hou, X., Wang, L., Wu, Y., Chen, L., Dong, H., Wang, B., A modified quick, easy, cheap, effective, rugged, and safe cleanup method followed by liquid chromatography-tandem mass spectrometry for the rapid analysis of perchlorate, bromate and hypophosphite in flour, *J Chromatogr A*, **2017**, 1526(Supplement C), 31–38, https://doi.org/10.1016/j.chroma.2017.10.047.
27. Xian Y., Wu, Y., Dong, H., Guo, X., Wang, B., Wang, L., Dispersive micro solid phase extraction (DMSPE) using polymer anion exchange (PAX) as the sorbent followed by UPLC–MS/MS for the rapid determination of four bisphenols in commercial edible oils, *J Chromatogr A*, **2017**, 1517(Supplement C), 35–43, https://doi.org/10.1016/j.chroma.2017.08.067.

SUPPLEMENTAL MATERIALS

Table S1: Detailed information of 24 pesticides

Series Number	Pesticide	CAS number	Chemical formula
18	o,p'-DDT	789–02–6	$C_{14}H_9Cl_5$
21	p,p'-DDT	50–29–3	$C_{14}H_9Cl_5$
17	o,p'-DDD	53–19–0	$C_{14}H_{10}Cl_4$
19	p,p'-DDD	72–54–8	$C_{14}H_8Cl_4$
12	o,p'-DDE	3424–82–6	$C_{14}H_8Cl_4$
15	p,p'-DDE	72–55–9	$C_{14}H_8Cl_4$
2	α-BHC	319–84–6	$C_6H_6Cl_6$
6	β-BHC	319–85–7	$C_6H_6Cl_6$
7	δ-BHC	319–86–8	$C_6H_6Cl_6$
3	Lindane	58–89–9	$C_6H_6Cl_6$
8	Malathion	121–75–5	$C_{10}H_{19}O_6PS_2$
23	Methoxychlor	72–43–5	$C_{16}H_{15}Cl_3O_2$
4	Aldrin	309–00–2	$C_{12}H_8Cl_6$
16	Dieldrin	60–57–1	$C_{12}H_8Cl_6O$
10	Ethylparathion	56–38–2	$C_{10}H_{14}NO_5PS$
13	α-Endosulfan	115–29–7	$C_9H_6Cl_6O_3S$
20	β-Endosulfan	33213–65–9	$C_9H_6Cl_6O_3S$
22	Mirex	2385–85–5	$C_{10}Cl_{12}$
9	Dichlofluanide	1085–98–9	$C_9H_{11}Cl_2FN_2O_2S_2$
11	Heptachloroepoxide	1024–57–3	$C_{10}H_5Cl_7O$
1	Pentachloroanisole	1825–21–4	$C_7H_3Cl_5O$
24	Permethrin	52645–53–1	$C_{21}H_{20}Cl_2O_3$
14	Tolyfluanide	731–27–1	$C_{10}H_{13}Cl_2FN_2O_2S_2$
5	Chlorothalonil	1897–45–6	$C_8Cl_4N_2$

Table S2: The extraction effect of different ratio of n-hexane-ethyl acetate on 24 pesticide residues in leather sample

Analyte	Concentration (added) ng mL^{-1}	Recovery (%) Ratio (n-hexane-ethyl acetate)						
		5:1	3:1	2:1	1:1	1:2	1:3	1:5
1	200	104.99	98.51	88.60	93.24	81.09	82.34	78.46
2	200	100.78	97.57	84.05	101.75	85.72	87.86	84.93
3	200	96.56	87.19	76.52	110.41	92.88	92.70	92.99
4	200	94.36	88.54	85.45	105.98	87.90	93.11	91.09
5	200	107.82	42.40	55.44	57.66	45.33	53.45	39.20
6	200	94.44	89.55	92.92	99.81	83.66	86.03	85.45
7	200	129.78	119.05	111.83	117.92	104.36	109.34	104.68
8	200	132.41	121.99	114.86	127.30	115.29	118.32	116.72
9	200	53.49	53.14	55.72	53.94	36.75	23.64	27.11
10	200	142.86	131.11	124.11	126.81	112.13	114.13	118.79
11	200	92.14	85.07	79.99	96.68	86.44	92.69	88.78
12	200	91.31	85.71	86.32	103.70	84.83	94.50	89.10
13	200	138.75	162.17	130.85	112.79	149.26	147.74	126.97

14	200	95.41	57.25	46.34	57.29	44.17	29.61	31.04
15	200	97.04	87.37	83.97	99.91	85.19	91.74	89.24
16	200	98.00	84.55	81.43	109.29	86.50	85.50	83.50
17	200	113.23	106.95	100.65	104.25	93.18	97.53	93.69
18	200	55.05	42.23	37.00	120.70	91.48	98.33	105.27
19	200	124.64	113.29	105.25	111.34	101.00	104.92	97.56
20	200	142.04	141.02	129.22	109.96	105.81	89.64	100.15
21	200	37.85	23.78	20.62	114.72	83.86	92.14	106.92
22	200	77.31	67.89	55.64	93.00	77.19	83.76	83.86
23	200	44.29	29.60	27.31	110.22	84.68	94.79	101.00
24	200	101.22	136.02	117.00	107.80	94.05	119.83	129.82

Table S2A: Recovery and precision test in Cowhide sample

Analyte	Added (μg/kg)	Found Average (n=6, μg/kg)	Average recovery (%)	SD (μg/kg)	RSD (%)
	100	107.1	107.1	2.41	2.25
1	300	322.6	107.5	8.61	2.66
	600	666.6	111.1	22.3	3.35
	100	109.9	109.9	5.98	5.44
2	300	331.8	110.6	8.62	2.61
	600	676.3	112.7	20.4	3.01
	100	80.0	80.0	4.20	5.25
3	300	303.1	101.0	16.9	5.58
	600	636.4	106.1	21.6	3.40
	100	113.3	113.3	7.21	6.37
4	300	337.7	112.6	9.61	2.84
	600	700.9	116.8	19.1	2.73
	100	78.4	78.4	5.68	7.24
5	300	312.3	104.1	34.8	11.16
	600	733.0	122.2	62.3	8.50
	100	83.6	83.6	3.87	4.63
6	300	306.7	102.2	9.00	2.93
	600	660.2	110.0	24.9	3.77
	100	91.8	91.8	6.05	6.60
7	300	336.1	112.0	10.2	3.02
	600	681.6	113.6	20.8	3.05
	100	114.9	114.9	10.8	9.40
8	300	394.8	131.6	35.2	8.90
	600	675.8	112.6	24.2	3.59
	100	114.3	114.3	6.09	5.33
9	300	330.7	110.2	28.4	8.60
	600	732.4	122.1	14.9	2.03
	100	114.4	114.4	9.36	8.18
10	300	335.0	111.7	6.2	1.84
	600	702.3	117.0	13.4	1.91
	100	117.1	117.1	4.26	3.64
11	300	343.1	114.4	7.41	2.16
	600	709.2	118.2	20.9	2.95
	100	109.0	109.0	4.76	4.37
12	300	337.4	112.5	11.0	3.27
	600	693.7	115.6	21.8	3.14
	100	115.2	115.2	3.85	3.34
13	300	394.0	131.3	22.3	5.67
	600	762.2	127.0	33.8	4.43

Analyte	Added (µg/kg)	Found Average (n=6, µg/kg)	Average recovery (%)	SD (µg/kg)	RSD (%)
	100	110.2	110.2	10.1	9.14
14	300	341.9	114.0	39.2	11.46
	600	574.5	95.8	19.6	3.41
	100	118.2	118.2	3.91	3.31
15	300	340.2	113.4	9.72	2.85
	600	695.5	115.9	23.0	3.31
	100	90.6	90.6	7.80	8.60
16	300	217.1	72.4	24.3	11.18
	600	482.1	80.3	37.9	7.85
	100	127.5	127.5	6.49	5.09
17	300	392.8	130.9	21.7	5.52
	600	737.0	122.8	45.3	6.15
	100	108.7	108.7	3.95	3.63
18	300	323.9	108.0	15.3	4.72
	600	653.1	108.8	24.9	3.81
	100	113.5	113.5	8.84	7.79
19	300	368.7	122.9	25.73	6.98
	600	680.2	113.4	49.4	7.26
	100	95.2	95.2	6.72	7.05
20	300	290.2	96.7	32.3	11.14
	600	552.6	92.1	51.3	9.29
	100	106.0	106.0	9.11	8.59
21	300	314.8	104.9	27.5	8.75
	600	616.3	102.7	52.3	8.48
	100	104.6	104.6	2.37	2.27
22	300	318.4	106.1	7.1	2.23
	600	655.9	109.3	17.2	2.63
	100	102.5	102.5	9.59	9.36
23	300	325.1	108.4	12.9	3.98
	600	645.0	107.5	26.1	4.04
	100	124.8	124.8	8.35	6.69
24	300	373.9	124.6	12.8	3.41
	600	763.7	127.3	25.7	3.37

Table S2B: Recovery and precision test in sheep leather sample (n=6)

Analyte	Added (µg/kg)	Found Average (n=6, µg/kg)	Average recovery (%)	SD (µg/kg)	RSD (%)
	100	107.97	107.97	1.42	1.32
1	300	325.42	108.47	4.77	1.46
	600	667.72	111.29	10.76	1.61
	100	100.90	100.90	3.06	3.03
2	300	326.18	108.73	4.58	1.40
	600	676.63	112.77	13.22	1.95
	100	105.94	105.94	3.58	3.38
3	300	323.86	107.95	5.70	1.76
	600	667.29	111.22	12.32	1.85
	100	114.16	114.16	2.63	2.30
4	300	342.56	114.19	5.11	1.49
	600	700.92	116.82	11.80	1.68
	100	78.36	78.36	1.97	2.52
5	300	231.27	77.09	11.65	5.04
	600	492.19	82.03	44.17	8.97

Analyte	Added (μg/kg)	Found Average (n=6, μg/kg)	Average recovery (%)	SD (μg/kg)	RSD (%)
	100	107.61	107.61	2.43	2.26
6	300	333.12	111.04	6.36	1.91
	600	687.42	114.57	16.79	2.44
	100	106.23	106.23	5.94	5.59
7	300	311.20	103.73	6.50	2.09
	600	640.76	106.79	16.96	2.65
	100	111.49	111.49	3.77	3.39
8	300	387.82	129.27	5.97	1.54
	600	746.45	124.41	16.27	2.18
	100	72.78	72.78	5.70	7.84
9	300	240.82	80.27	13.07	5.43
	600	480.29	80.05	14.65	3.05
	100	87.98	87.98	3.90	4.43
10	300	363.85	121.28	8.10	2.23
	600	649.37	108.23	14.74	2.27
	100	118.06	118.06	4.40	3.72
11	300	348.46	116.15	7.72	2.21
	600	653.41	108.90	14.48	2.22
	100	118.33	118.33	3.38	2.86
12	300	351.02	117.01	5.77	1.64
	600	653.99	109.00	13.68	2.09
	100	103.37	103.37	6.18	5.98
13	300	377.60	125.87	20.07	5.32
	600	679.32	113.22	12.90	1.90
	100	81.10	81.10	7.51	9.26
14	300	262.55	87.52	25.76	9.81
	600	528.26	88.04	16.33	3.09
	100	118.80	118.80	2.42	2.03
15	300	358.31	119.44	6.69	1.87
	600	664.75	110.79	15.21	2.29
	100	103.82	103.82	3.16	3.04
16	300	329.67	109.89	13.56	4.11
	600	659.88	109.98	16.17	2.45
	100	104.65	104.65	7.96	7.60
17	300	380.88	126.96	22.95	6.03
	600	737.28	122.88	45.95	6.23
	100	100.54	100.54	8.60	8.56
18	300	304.30	101.43	24.92	8.19
	600	560.08	93.35	41.59	7.43
	100	98.32	98.32	10.58	10.76
19	300	395.68	131.89	30.27	7.65
	600	724.84	120.81	55.60	7.67
	100	79.73	79.73	6.22	7.80
20	300	304.06	101.35	15.80	5.20
	600	617.20	102.87	15.47	2.51
	100	81.23	81.23	2.57	3.16
21	300	318.60	106.20	6.92	2.17
	600	561.11	93.52	26.22	4.67
	100	109.71	109.71	4.83	4.40
22	300	327.41	109.14	4.18	1.28
	600	604.07	100.68	7.49	1.24

Analyte	Added (µg/kg)	Found Average (n=6, µg/kg)	Average recovery (%)	SD (µg/kg)	RSD (%)
	100	93.40	93.40	9.56	10.23
23	300	280.94	93.65	27.78	9.89
	600	511.95	85.33	46.06	9.00
	100	107.68	107.68	4.05	3.76
24	300	364.06	121.35	8.80	2.42
	600	657.94	109.66	17.62	2.68

Table S2C: Recovery and precision test in pig leather sample (n=6)

Analyte	Added (µg/kg)	Average content (n=6, µg/kg)	Average recovery (%)	SD (µg/kg)	RSD (%)
	100	80.53	80.5	5.96	7.40
1	300	228.53	76.18	2.93	1.28
	600	509.46	84.91	9.88	1.94
	100	111.32	111.3	9.66	8.68
2	300	239.73	79.91	9.69	4.04
	600	535.16	89.19	9.28	1.73
	100	104.72	104.7	7.33	7.00
3	300	291.07	97.02	26.79	9.21
	600	585.58	97.60	12.26	2.09
	100	110.65	110.65	9.92	8.97
4	300	282.99	94.33	5.86	2.07
	600	602.71	100.45	9.53	1.58
	100	82.92	82.92	8.57	10.33
5	300	347.82	115.94	19.41	5.58
	600	578.61	96.44	15.99	2.76
	100	100.20	100.2	9.12	9.10
6	300	335.92	111.97	12.80	3.81
	600	542.62	90.44	49.73	9.17
	100	116.45	116.5	11.98	10.29
7	300	331.24	110.41	8.46	2.55
	600	573.56	95.59	15.60	2.72
	100	110.17	110.2	11.47	10.41
8	300	330.24	110.08	20.94	6.34
	600	682.62	113.77	56.30	8.25
	100	90.58	90.58	9.65	10.66
9	300	270.29	90.10	36.70	13.58
	600	545.94	90.99	14.30	2.62
	100	123.00	123.00	12.09	9.83
10	300	298.87	99.62	7.88	2.64
	600	607.05	101.17	11.69	1.93
	100	119.42	119.42	6.61	5.54
11	300	289.43	96.48	6.44	2.23
	600	604.65	100.77	9.24	1.53
	100	99.87	99.87	4.34	4.34
12	300	280.76	93.59	10.43	3.72
	600	608.38	101.40	8.64	1.42
	100	82.89	82.89	11.91	14.37
13	300	381.80	127.27	12.92	3.38
	600	618.21	103.04	24.07	3.89
	100	92.92	92.92	12.09	13.01
14	300	280.07	93.36	18.58	6.63
	600	565.14	94.19	16.42	2.91

Analyte	Added (μg/kg)	Average content (n=6, μg/kg)	Average recovery (%)	SD (μg/kg)	RSD (%)
	100	118.77	118.77	4.59	3.87
15	300	257.68	85.89	6.89	2.67
	600	533.98	89.00	7.56	1.42
	100	84.36	84.36	11.88	14.08
16	300	274.41	91.47	15.69	5.72
	600	603.08	100.51	29.49	4.89
	100	123.49	123.49	12.61	10.21
17	300	335.66	111.89	4.34	1.29
	600	727.10	121.18	7.91	1.09
	100	104.94	104.94	7.65	7.29
18	300	235.10	78.37	2.80	1.19
	600	452.92	75.49	5.37	1.18
	100	129.02	129.02	9.60	7.44
19	300	324.75	108.25	3.71	1.14
	600	713.24	118.87	8.22	1.15
	100	116.48	116.48	13.40	11.50
20	300	312.64	104.21	32.49	10.39
	600	645.48	107.58	39.55	6.13
	100	89.25	89.25	7.00	7.84
21	300	258.12	86.04	7.66	2.97
	600	443.00	73.83	7.23	1.63
	100	105.16	105.16	2.66	2.53
22	300	258.73	86.24	6.25	2.42
	600	541.44	90.24	8.75	1.62
	100	103.27	103.27	4.37	4.23
23	300	222.43	74.14	3.22	1.45
	600	462.89	77.15	6.64	1.43
	100	125.62	125.62	4.16	3.31
24	300	304.41	101.47	4.31	1.42
	600	662.45	110.41	8.21	1.24

ESTIMATION OF THE ALLOWABLE CONCENTRATION OF CHLORIDES IN SOAKING EFFLUENTS FROM THE TANNING PROCESS USING ECOTOXICOLOGICAL TOOLS

Ruth MOGOLLON ROPA, Liliana MARRUFO SALDAÑA*, Julio BARRA HINOJOSA*, Rosa CONTRERAS PANIZO, Robert BULEJE DEL CARPIO

Productive Innovation and Technological Transfer Center of Leather, Footwear and related industries (CITEccal Lima). Technological Institute of Production (ITP), Caquetá Ave. 1300, Rímac, 15094, Lima, Perú, rmogollonr@uni.pe, lmarrufo@itp.gob.pe, julio.barra.h@uni.pe, rcontreras@itp.gob.pe, rbuleje@itp.gob.pe

Received: 19.06.2020 Accepted: 15.10.2020 https://doi.org/10.24264/lfj.20.4.6

ESTIMATION OF THE ALLOWABLE CONCENTRATION OF CHLORIDES IN SOAKING EFFLUENTS FROM THE TANNING PROCESS USING ECOTOXICOLOGICAL TOOLS

ABSTRACT. The use of sodium chloride for the preservation of pelts and skins destined for tanning is a very widespread technique in Peru, therefore the quantities used of this salt represent high concentrations of chlorides in the liquid effluents that are generated, which can affect the environmental quality of ecosystems and human health. The present study aimed to estimate an allowable concentration of chlorides in tanning effluents using ecotoxicological tools so that it would serve as a guide for the tanning industry and the authorities in their efforts to establish better conservation practices and effluent control parameters, which are currently absent from national environmental legislation. The permissible concentration of chlorides was determined using bioassays with seeds of *Lactuca sativa* species (lettuce), which was exposed to soaking effluents obtained from local tanneries. The studied species was determined based on sensitivity tests to zinc sulfate ($ZnSO_4$) applied to three commercial lettuce species. The selected lettuce seed presented a germination rate higher than 90%, a CV lower than 30% and an IC_{50} of 38.97 mg/L of Zn^{+2} with a confidence interval between 19.30 mg/L and 58.65 mg/L of Zn^{+2}. The admissible guide concentration determined for chlorides in soaking effluents from IC_{50} was 582.30 mg/L with a minimum value of 258.45 mg/L and a maximum of 906.15 mg/L. Additionally, because of the presence of other pollutants in the soaking effluents, such as bactericides, organic matter, enzymes and salts added in soaking process, tests with synthetic solutions of sodium chloride at the same concentrations of the soaking effluents were conducted, to evaluate the effect produced by those substances.

KEY WORDS: waste valorization, chrome shavings, alkaline hydrolysis, tanning process

ESTIMAREA CONCENTRAȚIEI ADMISIBILE DE CLORURI ÎN EFLUENȚII DE LA ÎNMUIERE DIN CADRUL PROCESULUI DE TĂBĂCIRE FOLOSIND INSTRUMENTE ECOTOXICOLOGICE

REZUMAT. Utilizarea clorurii de sodiu pentru conservarea blănurilor și a pieilor pentru tăbăcire este o tehnică foarte răspândită în Peru, prin urmare cantitățile de sare utilizate înseamnă concentrații mari de cloruri în efluenții generați, ceea ce poate afecta calitatea ecologică a ecosistemelor și sănătatea umană. Prezentul studiu are ca scop estimarea concentrației admisibile de cloruri în efluenții de la tăbăcire utilizând instrumente ecotoxicologice, astfel încât să servească drept ghid pentru industria de pielărie și pentru autorități în eforturile acestora de a stabili practici mai bune de conservare și parametrii de control al efluenților, care lipsesc în prezent din legislația națională de mediu. Concentrația admisibilă de cloruri a fost determinată folosind teste biologice cu semințe din specia *Lactuca sativa* (salată), care au fost expuse la efluenții de înmuiere obținuți din tăbăcăriile locale. Specia studiată a fost determinată pe baza testelor de sensibilitate la sulfatul de zinc ($ZnSO_4$) aplicate la trei specii de salată comercială. Semințele de salată selectate au prezentat o rată de germinare mai mare de 90%, un coeficient de variație (CV) mai mic de 30% și o concentrație inhibitorie maximă 50% (IC_{50}) de 38,97 mg/l de Zn^{+2} cu un interval de încredere între 19,30 mg/l și 58,65 mg/l de Zn^{+2}. Concentrația orientativă admisă determinată pentru cloruri în efluenții de înmuiere la IC_{50} a fost de 582,30 mg/l cu o valoare minimă de 258,45 mg/l și maxim de 906,15 mg/l. În plus, din cauza prezenței altor poluanți în efluenții de înmuiere, cum ar fi bactericidele, materia organică, enzimele și sărurile adăugate în procesul de înmuiere, s-au efectuat teste cu soluții de clorură de sodiu sintetică la aceleași concentrații ale efluenților de înmuiere, pentru a evalua efectul produs de aceste substanțe.

CUVINTE CHEIE: valorificarea deșeurilor, răzătură de piele cromată, hidroliză alcalină, proces de tăbăcire

ESTIMATION DE LA CONCENTRATION ADMISSIBLE DE CHLORURES DANS LES EFFLUENTS DE TREMPAGE DU PROCESSUS DE TANNAGE À L'AIDE D'OUTILS ÉCOTOXICOLOGIQUES

RÉSUMÉ. L'utilisation de chlorure de sodium pour la conservation des fourrures et des peaux destinées au tannage est une technique très répandue au Pérou, donc les quantités utilisées de ce sel représentent des concentrations élevées de chlorures dans les effluents liquides générés, ce qui peut affecter la qualité environnementale des écosystèmes et la santé humaine. La présente étude visait à estimer une concentration admissible de chlorures dans les effluents de tannage à l'aide d'outils écotoxicologiques afin qu'elle serve de guide à l'industrie du tannage et aux autorités dans leurs efforts pour établir de meilleures pratiques de conservation et des paramètres de contrôle des effluents, qui sont actuellement absents de législation environnementale nationale. La concentration admissible de chlorures a été déterminée à l'aide d'essais biologiques avec des graines d'espèces de *Lactuca sativa* (laitue), qui ont été exposées à des effluents de trempage provenant de tanneries locales. L'espèce étudiée a été déterminée sur la base de tests de sensibilité au sulfate de zinc ($ZnSO_4$) appliqués à trois espèces de laitues commerciales. La graine de laitue sélectionnée présentait un taux de germination supérieur à 90%, un coefficient de variation (CV) inférieur à 30% et une concentration inhibitrice médiane (CI_{50}) de 38,97 mg/L de Zn^{+2} avec un intervalle de confiance entre 19,30 mg/L et 58,65 mg/L de Zn^{+2}. La concentration guide admissible déterminée pour les chlorures dans les effluents de trempage à partir de la CI_{50} était de 582,30 mg/L avec une valeur minimale de 258,45 mg/L et un maximum de 906,15 mg/L. De plus, en raison de la présence d'autres polluants dans les effluents de trempage, tels que les bactéricides, la matière organique, les enzymes et les sels ajoutés dans le processus de trempage, des essais avec des solutions synthétiques de chlorure de sodium aux mêmes concentrations des effluents de trempage ont été effectués, pour évaluer l'effet produit par ces substances.

MOTS CLÉS : valorisation des déchets, copeaux de cuir chromé, hydrolyse alcaline, procédé de tannages

* *Correspondence to: Liliana MARRUFO SALDAÑA and Julio BARRA HINOJOSA, Productive Innovation and Technological Transfer Center of Leather, Footwear and related industries (CITEccal Lima). Technological Institute of Production (ITP), Caquetá Ave. 1300, Rímac, 15094, Lima, Perú, lmarrufo@itp.gob.pe, julio.barra.h@uni.pe.*

INTRODUCTION

The tanning industry generates pollutants with significant dangerous effects on the environment because traditional leather processing procedures involve large amounts of chemicals and water throughout their different stages [1, 2]. In the beamhouse operations, the initial stage, more water is needed than in any of the other stages because the skins that have previously gone through a conservation process have to be re-hydrated; this conservation is carried out with layers of salt (sodium chloride) to avoid its natural decomposition [3, 4] and consequently the effluents generated in this stage presents large amounts of sodium chloride (NaCl) which, if spilled into the environment, can cause damage to the soil and even impact waterbodies, altering the normal development of the living organisms that inhabit them [2].

Different studies had been carried out to evaluate the toxicity of tannery effluents; Hussain *et al.* [5] analyzed the effect of tannery effluents on seeds germination and growth of two sunflower cultivars in Pakistan, their results showed that the raw effluent caused the reduction in biomass accumulation and reproductive growth of sunflower cultivars, and that one of them was more resistant to lower concentrations, but due to the presence of chemicals, the effluents are not suitable for inclusion in irrigation system. Calheiros *et al.* [6] tested tannery effluents with high salinity treated by an activated sludge system on the germination and seedling growth of red clover (*Trifolium pretense*), where growth was inhibited by concentrations over 25% and undiluted effluent caused a complete germination inhibition. Kohli and Malaviya [7] evaluated the impact of tannery effluents with high salinity (45.97 ppm) at different concentrations on the germination pattern of ten varieties of wheat (*Triticum aestivum*), where some varieties exhibited more tolerance at lower concentrations of tannery effluents, showing a potential to grow in tannery effluents contaminated soils.

Currently, Peru has environmental legislation for the regulation of physical and chemical parameters in the effluents generated by the tanning industry. However, despite the harmful consequences that high concentrations of chlorides on waterbodies can generate [8], this parameter is not being regulated, and when a tannery treats its effluents, they don't know how much this parameter must be reduced to establish its treatment level. One of the methods used for stablishing maximum allowable concentrations is the use of bioassays [9, 10], where the toxicity of an effluent sample is tested by measuring its effect on the growth of bioindicators organisms; in view of this, the present study aimed at proposing a maximum allowable concentration of chlorides, supported on the basis of toxicity bioassays with seeds of *Lactuca sativa* species exposed to local tannery effluents to determine the half maximal inhibitory concentration (IC50) [11, 12]. Likewise, the toxicity of synthetic samples of sodium chloride was evaluated to determine the effect of other substances from the soaking process. The analysis of the bioassay results was done by the statistical complement REAL STATISTICS, ANOVA tests and multiple comparisons.

EXPERIMENTAL

Materials and Methods

Sample

The tanning effluents used in this research corresponded to those generated in the soaking phase of the tanning processes of local tanneries in the city of Lima. The samples were kept at a temperature below 4ºC, immediately after being taken.

Determination of Chloride Concentration in Effluents

The determination of the concentration of chlorides in mg/L in the soaking effluent was carried out in a laboratory accredited by INACAL using the EPA 300.0 method. Rev. 2.1:1993. Determination of Inorganic Anions by Ion Chromatography.

Toxicity Test

The toxicity tests were carried out in the R&D Laboratory of the Center for Productive Innovation and Technology Transfer of the Leather, Footwear and Related Industries (CITEccal Lima) and included the application of sensitivity tests for the selection of the test

species from commercial *Lactuca sativa* seeds [11, 13, 14], the toxicity analysis of soaking effluents and synthetic samples of sodium chloride with the selected seed.

The chemical reagents used were sodium bicarbonate for analysis ACS,Reag. Ph Eur MERCK, magnesium sulfate heptahydrate for analysis EMSURE® ACS, Reag. Ph Eur MERCK, potassium chloride for analysis Reag. Ph Eur SCHARLAU for the preparation of the reconstituted water. Anhydrous zinc sulfate, brand name, for the sensitivity test. As for the equipment, an analytical balance AND, model GF-1000 was used; an oven JP SELECTA, model DRY - BIG and an incubator VELP, model FTC 90I; all calibrated. In all procedures, reconstituted water was used and the materials used were washed with neutral detergent, EXTRAN, rinsed with tap water and distilled water. The volumetric glass materials were dried in an oven at a temperature not exceeding 50ºC and the petri dishes were dried at a temperature of 100ºC to sterilize them.

The reconstituted water was prepared with distilled water and had a composition of 192 mg/L of sodium bicarbonate, 120 mg/L of anhydrous magnesium sulfate and 8 mg/L of potassium chloride. The stock solution of zinc sulfate had a concentration of 500 mg/L from which the following concentrations were obtained by dilution with reconstituted water: 8.15 mg/L, 16.30 mg/L, 32.40 mg/L, 48.89 mg/L and 65.19 mg/L. The synthetic samples of sodium chloride were prepared from a 28.57 g/L chloride stock solution using reconstituted water as the solvent.

The selection of the species for the toxicity tests was made from the study of red lettuce seeds from the Hydroponics Unit of the National Agrarian University La Molina (UNALM), romaine lettuce seeds from the bio garden of the UNALM and long light green romaine lettuce seeds, butter variety, from BATLLE S.A. 08750 Molins de Rei, Barcelona, Spain. Seeds were exposed to reconstituted water and to the diluted concentrations of the $ZnSO_4$ stock solution, being determined for each of them the germination percentage, the coefficient of variation between the replicates and the IC_{50}.

After the selection of the test organism, the IC_{50} of the tanning effluents was determined by exposing them to tanning effluent dilutions corresponding to 1%, 3%, 10%, 30% and 100% prepared with reconstituted water. The exposure of the seed to the synthetic sodium chloride solution was made from dilutions of the stock solution corresponding to 285.7 mg/L, 857.1 mg/L, 2857.1 mg/L, 8571.4 mg/L, 28 571, 4 mg/L.

The procedure of exposing the seeds to reconstituted water, stock solution dilutions and effluent dilutions was initiated by placing filter paper discs on the plates, then using a pipette to saturate each of the plates with 2 mL of the solutions to prevent air bubbles. Later, using a tweezer, 20 seeds were carefully placed in each plate, leaving enough space between the seeds to allow the elongation of the roots. The plates were then covered and placed in plastic bags to prevent them from losing humidity, and finally they were placed in the incubator for 120 hours (5 days) at a temperature of 22 ± 2ºC. Each concentration or dilution was worked in triplicate [10, 15, 16].

For all cases the final evaluation consisted of comparing the effects generated in the test organisms exposed to the sample (zinc sulphate, soaking effluent and synthetic sodium chloride sample) with the response of the exposed organisms to the negative control (reconstituted water) under the same test conditions. Once the exposure period was completed (120 hours), the effect on germination and elongation of the radicle was quantified. Samples with necrosis, fungi or other aspects that may have interfered with germination and growth were observed. Seed elongation was performed by measuring root growth.

The germination percentage (GP) was determined by comparing the sown seeds with the germinated ones according to the following equation:

$$GP\ (\%) = \frac{Number\ of\ total\ germinated\ seeds}{Total\ number\ of\ seeds\ tested}\ x\ 100 \quad (1)$$

The radicle growth inhibition percentage (RIGP) was estimated with the average elongation for each dilution with respect to the average elongation of the negative control.

$$RIGP\ (\%) = \left(\frac{\bar{x}\ elongation\ at\ dilution\ \tau - \bar{x}\ control\ elongation}{\bar{x}\ control\ elongation}\right)x100 \quad (2)$$

The half maximal inhibitory concentration (IC_{50}) is the concentration where the 50% of the test organisms are inhibited, in this study, where the elongation of the radicle is inhibited at 50%. The IC_{50} was determined on the basis of a fitting equation with a regression coefficient greater than 80% which is derived from the graph of the percentage inhibition vs. the diluted concentrations of the samples (dose-response curve). From the fitting equation:

$$y = ax + b, with\ R^2 > 0.8$$

$$x = CI50\ when\ y = 50\%, then\ CI50 = \frac{50 - b}{a} \quad (3)$$

Where:
x: Dilution
y: Radicle growth inhibition percentage (RIGP)

The coefficient of variation (CV) was determined by determining the elongation standard deviation of a series of measurements between the average elongations.

$$CV\ (\%) = \left(\frac{standard\ deviation\ of\ elongation\ data}{Average\ elongation}\right)x100 \quad (4)$$

For the statistical analysis of the results, the analysis of the normality of the data for each dilution with the Shapiro Wilk test was considered. Likewise, by means of an ANOVA analysis and multiple comparison tests through Tukey Test and Dunnett Test, it was evaluated if there were significant differences between the sample dilutions of each toxicity test with the negative control and between the dilutions. This analysis was performed with the supplement Real Statistics for MS. Excel.

RESULTS AND DISCUSSIONS

Evaluation of the Seeds

Table 1 shows the germination results for the three species evaluated. The highest average percentage of germination in reconstituted water corresponds to light green romaine lettuce with a value of 93.89%. On the other hand the lowest average value for the coefficient of variation, also corresponds to this species and is equal to 24.53% according to the methodology of Acute Toxicity Test with seeds of *Lactuca sativa L.* [10, 17] it is considered that there is a good percentage of germination if this is greater or equal to 90% and an adequate CV if this is less or equal to 30%.

Table 1: Comparison between the results of percentage of germination, coefficient of variation and CI50 for the species of lettuce studied

Day	Red lettuce			Romaine lettuce			Light green romaine lettuce		
	% of germination	Root CV	IC_{50} (mg/L)	% of germination	Root CV	IC_{50} (mg/L)	% of germination	Root CV	IC_{50} (mg/L)
01	70.00	33.38	95.87	55.00	48,35	82.79	91.67	29.07	121.77
02	85.00	44.33	80.13	66.67	63.88	88.94	98.33	22.40	71.83
02	65.00	34.24	45.63	88.33	38.95	42.57	91.67	22.12	56.03

In terms of species sensitivity analysis, long light green romaine lettuce has the highest sensitivity expressed as an average IC_{50} of 48.07 mg/L. It should be noted that the correlation coefficients of the dose response curves were greater than 0.80 for all the trials, and in their determination the statistical conditions were met that there is normality in the data and significant differences between the concentrations or dilutions with the negative control and among themselves.

Sensitivity Test - Positive Control

Table 1 shows the germination results for the three species evaluated. The highest average percentage of germination in reconstituted water corresponds to light green romaine lettuce with a value of 93.89%. On the other hand the lowest average value for the coefficient of variation, also corresponds to this species and is equal to 24.53% according to the methodology of Acute Toxicity Test with seeds of *Lactuca sativa L.* [10] it is considered that there is a good percentage of germination if this is greater or equal to 90% and an adequate CV if this is less or equal to 30%.

To establish the reproducibility of the applied methodology, sensitivity tests were performed with the selected species from the following Zn (II) concentrations: 8.15 mg/L, 16.30 mg/L, 32.40 mg/L, 48.89 mg/L and 65.19 mg/L. The IC_{50} of Zn (II) determined from root growth assessment was 38.97 mg/L with a 95% confidence interval between 19.30 mg/L and 58.65 mg/L. Figure 1 shows the established control chart for this test.

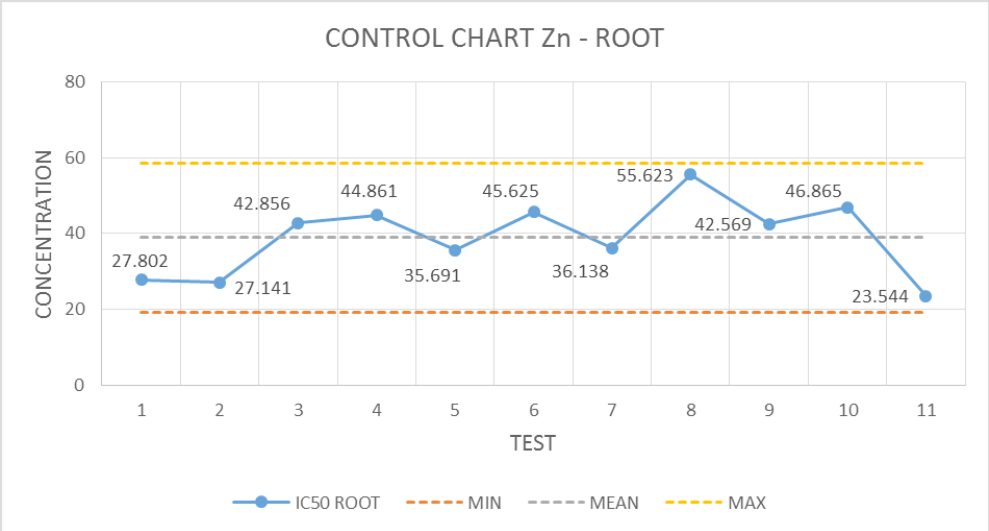


Figure 1. Control Chart Zn-Root

Bohórquez P. and Campos C. [16] determined an IC_{50} for *L. sativa* of 24.48 mg/L of Zn^{+2} with 95% confidence interval between 14.60 mg/L and 34.36 mg/L.

Toxicity Tests on Soaking Effluents and Proposal of a Permissible Value for Chlorides in Tanning Effluents

Table 2 presents the results of the chloride concentration in soaking effluents from tanneries in Metropolitan Lima as well as the respective IC_{50} obtained in the toxicity tests. On average, an IC_{50} of 582.30 mg/L and a range with a minimum value of 258.45 mg/L and a maximum value of 906.15 mg/L are determined. In order to determine the admissible concentration to be proposed, the safety factor or assessment factor approach was considered. This approach is applied to ensure the protection of the most sensitive organisms and is inversely proportional to the quantity and quality of the toxicity data available. However, when comparing the IC_{50} value obtained with the value of the Environmental Quality Standards for water in national legislation, which considers a value of 500 mg/L of chlorides for irrigation of vegetables and animal beverages (category 3) and with the value considered by international legislation for the case of Bogotá, which establishes a maximum value of chlorides in tanning effluents of 3,000 mg/L, the conclusion was reached that the value of 582.30 mg/L of chloride is sufficiently restrictive to ensure that tanning effluents that come into contact with a water body do not affect the normal development of the species, therefore the safety factor applied was 1.

Table 2: Chloride and IC_{50} concentration of soaking effluents from tanneries in Metropolitan Lima

Sample code	Chloride concentration (mg/L)	IC_{50} (mg/L)
PC-11	9 428.86	551.76
PC-12	16 578.55	477.08
PC-13	9 626.20	393.87
PC-15	4 082.95	695.29
PC-16	16 418.50	793.49

Table 3: IC_{50} in synthetic samples of sodium chloride

Sample code	IC_{50} (mg/L) for sodium chloride in synthetic samples	IC_{50} (mg/L) for chloride in synthetic samples
1	1 040.59	631.23
2	1 688.22	1 024.08
3	1 016.98	616.91

Table 3 shows the IC_{50} values determined for synthetic sodium chloride samples. It can be seen that these values are higher than those obtained with the soaking effluent samples, this is because the synthetic sample does not contain other substances that normally result from a soaking effluent. The chemicals used in the soaking process are: sodium hydroxide, sodium hypochlorite, surfactants, bactericides and enzymatic preparations, these by their nature add to the final effluent a higher toxicity, on the other hand, besides salt, blood and dirt adhered to the skin are dragged away. This difference in toxicity would evidence the effect of salt in its pure and mixed state, which was also observed by Lyu *et al.* [18] who performed toxicity tests on *Lactuca sativa L.* with pure compounds and industrial effluent from Soyo and Daejeon in Korea, determining that the Zn concentration required to inhibit root elongation in *L. sativa* by 50% (IC_{50}) was higher in Daejeon effluent than pure Zn, concluding that Zn mixed with the

effluent is less toxic than in pure form. Therefore, for the acceptable concentration to be proposed to be valid for the tanning industry, the joint effect of the chloride immersed in the soaking effluent should be considered. On the other hand, Campagna-Fernandes, Marin, & Penha [12], determined that the effects of sodium chloride on root growth are detected from a concentration of 1,000 mg/L of NaCl, that is, at 606,655 mg/L of Cl-, values that coincide with those established in the present investigation.

CONCLUSIONS

The species of *Lactuca sativa L.* that validates the toxicity test is the long light green romaine lettuce, because it has a high percentage of germination (more than 90 %) that is preserved in time, a low variability of root elongation and hypocotyl (CV < 30 %) and presents the lowest values of IC_{50}, that is to say, it is the most sensitive among those evaluated. The IC_{50} calculated by sensitivity tests with $ZnSO_4$ for *Lactuca sativa L.* is 38.97 mg/L of Zn^{+2} with confidence interval between 19.30 mg/L and 58.65 mg/L of Zn^{+2}. Analysis with synthetic samples of industrial sodium chloride led to the conclusion that chlorides have a more toxic effect if they are discharged to the environment as part of the steeping effluent than in their pure state, since the IC_{50} values of chlorides in synthetic samples were higher than the IC_{50} values of chlorides in steeping effluents.

The maximum allowable concentration of chlorides for soaking effluent determined in this work is 582.30 mg/L with a minimum value of 258.45 mg/L and a maximum of 906.15 mg/L. This value is the starting point for establishing a limit and minimizing the effects of chlorides when discharged into the environment. The reduction of the chloride levels in the soaking effluents, implies for the tanneries to implement source reduction measures, applying the Cleaner Production process focused on minimization and reuse, with the last alternative being to treat the effluent at the end of the process.

It is important to strengthen the present study with a greater number of samples, representative of the national tanneries, to validate the findings of the present investigation. For this purpose, a more in-depth study should be carried out to analyze samples of tanning effluents in the main areas where this industry is developed in Peru, which is not only Lima, but also Trujillo and Arequipa, and thus have a national scope. It is suggested that at the legislative level, the maximum permissible limit values for tanning effluents be updated to include a limit value for chlorides based on studies that consider the toxicity of the element, as is the case with the present research, and even promote research that analyzes toxicity with species of different trophic levels.

Acknowledgements

We aknowledge to the Productive Innovation and Technological Transfer Center of Leather, Footwear and related industries (CITEccal Lima) and all its staff for the support provided to carry out this project.

REFERENCES

1. Aragón Guzmán, M., Sistema de referenciación ambiental (SIRAC) para el sector curtiembre en Colombia, Proyecto gestión ambiental en la industria de curtiembres, **2004**.
2. Emmer, V., del Campo, M.J., Guía de Producción Más Limpia en el Sector Curtiembres, Vol. 53, Dinama, **2014**, 1689–1699 p.
3. China Leather and Footwear Industry, Developing countries training course on eco-leather manufacture technology, Beijing, China, **2007**.
4. Escoto-Palacios, M.J., Pérez-Limiñana, M.Á., Arán-Ais, F., From leather waste to functional leather, **2016**, 60 p.
5. Hussain, F., Malik, S.A., Athar, M., Bashir, N., Younis, U., Mahmood-ul-Hassan, Mahmood, S., Effect of tannery effluents on seed germination and growth of two

sunflower cultivars, *African J Biotechnol*, **2010**, 9, 32, 5113–20.
6. Calheiros, C.S.C., Silva, G., Quitério, P.V.B., Crispim, L.F.C., Brix, H., Moura, S.C., Castro, P.M.L., Toxicity of High Salinity Tannery Wastewater and Effects on Constructed Wetland Plants, *Int J Phytoremediation*, **2012**, 14, 7, 669–80, https://doi.org/10.1080/15226514.2011.619233.
7. Kohli, R., Malaviya, P., Impact of tannery effluent on germination of various varieties of wheat (*Triticum aestivum* L.), *J Appl Nat Sci*, **2013**, 5, 2, 302–5, https://doi.org/10.31018/jans.v5i2.321.
8. Mwinyihija, M., Ecotoxicological Diagnosis in the Tanning Industry [Internet], New York, NY: Springer New York; **2010**, 157 p., available from: http://link.springer.com/10.1007/978-1-4419-6266-9, https://doi.org/10.1007/978-1-4419-6266-9_4.
9. Planes, E., Fuchs, J., Cuáles son los aportes de la ecotoxicología a las regulaciones ambientales, *Cienc Invest*, **2015**, 65, 2, 18.
10. Sobrero, M.C., Ronco, A., Ensayo de toxicidad aguda con semillas de lechuga *Lactuca sativa* L. Ensayos toxicológicos y métodos de evaluación de calidad de aguas, **2004**.
11. OECD, Terrestrial Plants, Growth Test, in: Guidelines for the Testing of Chemicals, **1984**, p. 6.
12. Campagna-Fernandes, A.F., Marin, E.B., Penha, T.H.F.L., Application of root growth endpoint in toxicity tests with lettuce (*Lactuca sativa*), *Ecotoxicol Environ Contam*, **2016**, 11, 1, 27–32, https://doi.org/10.5132/eec.2016.01.05.
13. USEPA, Ecological Effects Test Guidelines OPPTS (850.4200), Seed Germination/ Root Elongation Toxicity Test, United States; **1996**, p. 1–8.
14. Sánchez Ortiz, K., Sánchez Melo, L., Determinación De La Concentración De Inhibición Media (Ce50-120) Del Bario, Hierro Y Manganeso Mediante Bioensayos De Toxicidad Acuática Sobre Semillas De Lechuga (*Lactuca Sativa* L.) [Internet], Universidad de La Salle; **2009**, available from: http://repository.lasalle.edu.co/bitstream/handle/10185/14913/T41.09 S55de.pdf?sequence=1
15. Sobrero, M.C., Estudio de la fitotoxicidad de metales pesados y del herbicida glifosato en ambientes acuáticos, Bioensayos con plantas vasculares como organismos diagnóstico, Universidad Nacional de La Plata; **2010**.
16. Bohórquez-Echeverry, P., Campos-Pinilla, C., Evaluación de *Lactuca sativa* Y *Selenastrum capricornutum* como indicadores de toxicidad en aguas, *Univ Sci*, **2007**, 12, 2, 83–98.
17. Ronco, A.E., Ensayos toxicológicos y métodos de evaluación de calidad de aguas, **2004**, 190 p.
18. Lyu, J., Park, J., Kumar Pandey, L., Choi, S., Lee, H., De Saeger, J., Depuydt, S., Han, T., Testing the toxicity of metals, phenol, effluents, and receiving waters by root elongation in *Lactuca sativa* L., *Ecotoxicol Environ Saf*, **2018**, 149 (November 2017), 225–32, https://doi.org/10.1016/j.ecoenv.2017.11.006.

DEVELOPING INNOVATIVE FOOTWEAR DESIGNS: EMPIRICAL EVIDENCE FROM INDONESIA

David Sukardi KODRAT, Tina MELINDA*, Denpharanto A. KRISPRIMANDOYO

Universitas Ciputra, Citraland – Surabaya, Indonesia, david.kodrat@ciputra.ac.id, tina.melinda@ciputra.ac.id, agungkris@ciputra.com

Received: 22.08.2020 Accepted: 19.11.2020 https://doi.org/10.24264/lfj.20.4.7

DEVELOPING INNOVATIVE FOOTWEAR DESIGNS: EMPIRICAL EVIDENCE FROM INDONESIA

ABSTRACT. This research aims to develop innovative footwear designs using Modified Design Thinking. This research is a qualitative research with Focus Group Discussion (FGD) as data source. Additionally, Modified Design Thinking was used to analyse the research data. The research findings reinforce the use of Modified Design Thinking in developing innovative footwear designs by identifying the needs of potential customers. Furthermore, local culture is believed to be the most influential factor in footwear design. As such, the innovation process in Indonesia is unique as it incorporates local culture into the creation of footwear design. This research enriches the existing footwear design innovation from the customers' perspective by using local culture as the main innovation element.

KEY WORDS: innovation, footwear design, Modified Design Thinking, local culture

DEZVOLTAREA UNOR MODELE DE ÎNCĂLȚĂMINTE INOVATOARE: DOVEZI EMPIRICE DIN INDONEZIA

REZUMAT. Această cercetare își propune să dezvolte modele de încălțăminte inovatoare folosind metoda Modified Design Thinking. Această cercetare este una calitativă, având ca sursă de date Focus Group Discussion (FGD). În plus, metoda Modified Design Thinking a fost utilizată pentru a analiza datele cercetării. Rezultatele cercetării consolidează utilizarea metodei Modified Design Thinking în dezvoltarea de modele de încălțăminte inovatoare prin identificarea nevoilor potențialilor clienți. În plus, cultura locală pare să fie cel mai influent factor în designul încălțămintei. Ca atare, procesul de inovare din Indonezia este unic deoarece încorporează elemente din cultura locală în crearea designului încălțămintei. Această cercetare îmbogățește inovația existentă în designul încălțămintei din perspectiva clienților, utilizând cultura locală ca principal element de inovare.

CUVINTE CHEIE: inovație, design de încălțăminte, metoda Modified Design Thinking, cultură locală

DÉVELOPPER DES CONCEPTIONS DE CHAUSSURES INNOVANTES : PREUVE EMPIRIQUE DE L'INDONÉSIE

RÉSUMÉ. Cette recherche vise à développer des conceptions de chaussures innovantes en utilisant le Design Thinking modifié. Cette recherche est une recherche qualitative avec Focus Group Discussion (FGD) comme source de données. De plus, le Design Thinking modifié a été utilisé pour analyser les données de recherche. Les résultats de la recherche renforcent l'utilisation de la pensée de conception modifiée dans le développement de modèles de chaussures innovants en identifiant les besoins des clients potentiels. En outre, la culture locale est considérée comme le facteur le plus influent dans la conception des chaussures. En tant que tel, le processus d'innovation en Indonésie est unique car il intègre la culture locale dans la création de la conception de chaussures. Cette recherche enrichit l'innovation de conception de chaussures existante du point de vue des clients en utilisant la culture locale comme principal élément d'innovation.

MOTS CLÉS : innovation, conception de chaussures, Design Thinking modifié, culture locale

* *Correspondence to: Tina MELINDA, Universitas Ciputra, Citraland – Surabaya, Indonesia, tina.melinda@ciputra.ac.id*

INTRODUCTION

The national industrial development policy has set "manufacturing industry base" as a priority industry in the formation of linkages in footwear industries [1]. The purpose of this policy is to strengthen the structure of the footwear industry by expanding the raw material supply industry, as well as other relevant supporting industries. The rapid changes in the footwear industry, paired with the globalization effects from the ASEAN Economic Community and Asian Free Trade Agreement, have significantly influenced the level of competitiveness. As a result, industries need to increase the competency of their labour force in order to compete in the global market.

Based on the data from the Department of Industry, there were as many as 398 SMEs in Mojokerto in 2015. These enterprises were located in three villages, namely Miji, Surodinawan, and Prajurit Kulon. Among the footwear manufacturers across the three villages, 19% were shoes manufacturers; 49% were slippers manufacturers; 29% were shoes and sandals manufacturers; and 3% were shoe last makers. Overall, four major problems were encountered by the majority of these SMEs [2].

First, footwear SMEs had difficulty obtaining quality raw materials. Most enterprises were highly dependent on imported raw materials. In addition, they were occasionally forced to accept lower-quality raw materials due to the unavailability of imported materials that matched the desired specifications. They purchased raw materials directly from the stores, sales agents, distributors, or other sources such as factories, grocery stores, or malls.

Second, the business management and production system were still traditional. The business owners handled all issues personally. Furthermore, documentation system and technology utilization in the production process were very limited. Equally limited was the number of experts in the field of footwear industry.

Third, the footwear sales were based on B2B demands from other factories or wholesalers, or B2C demands from individual retailers. And fourth, the coordination and synergy among stakeholders to plan and implement activities for the development of the SMEs needed improvement. Nonetheless, the footwear makers only produced by order. They were not confident enough to create their own design in fear of rejection. Hence, the purpose of this research is to develop innovative fashion footwear designs that would cater to the customers' needs.

LITERATURE REVIEW

Innovation Product Development

Innovation is considered as the success factor in a business, because the aspect allows business owners to understand customer needs and generate customer satisfaction [3]. It is also believed to be the driving force behind many contemporary successful companies [4]. Angelmar [5] defined innovative product as a new or improved product, while Reguia added that innovation process involved new techniques and means in production methods. Additionally, product innovation reflects the firm's image. In fact, the firm's success depends on the product's ability to fulfil consumers' needs and desires, from which the information will be used to develop new products [3].

Most theories distinguish innovation, innovation product, and product development as separate theories. Unfortunately, there were very few definitions on innovation product development in the scientific articles reviewed. Product development is defined as "the set of activities beginning with the perception of a market opportunity and ending with the production, sale and delivery of a product" [6]. In brief, product development could be defined as the "process of eliminating the uncertainty about the product".

Bilgili, Erciş, & Ünal [7] argued that the need to develop new products was precipitated by the rapid growth and development of technology, expansion of marketing environment and competition, and the limited life-span of products in the market. Balachandra & Friar [8] added that the New Product Development (NPD) and Research and Development (R&D) projects have three dimensions: innovation (incremental-radical), technology (low-high), and market (new-existing). A fourth dimension, the nature of the

industry, was also mentioned [8]. Several other studies suggested a different kind of conceptual model for the new product development process, which includes idea screening, commercial launching for preliminary market, and business or technical assessments [9].

The focus of the next stage of new product development is to design and develop the product. To do this, a large amount of information and knowledge is needed [9]. Su, Chen, & Sha [10] insisted that knowledge is an asset in today's digital economy, and that managerial knowledge helps organizations develop innovative products and make strategic decisions. Additionally, technological competence and customer needs are closely related in relation to product innovation, because they provide the 'know-how' in the process of securing market acceptance [10]. Moreover, Danneels [11] proposed that knowledge management is crucial in the development of innovative products. Product and service innovation require managerial knowledge as a means to create competitive advantages for the company.

The market competition has grown rapidly in recent years. Consequently, small and medium-sized enterprises (SMEs) must survive and defend their positions through new product developments (NPDs) [12]. NPD is the strongest weapon against the growing competition [7]. As a matter of fact, many experts believed that innovative products should not be taken for granted, because the organization and its members must also be fully committed to the purpose. Therefore, SMEs employees must be equipped with the necessary skills to improve the company's product development capability as a whole.

Most of the articles reviewed in this paper discussed innovation product development (IPD) in the context of advance technology. Additionally, no universal definition could be found regarding IPD. The majority of the literature discussed innovation and product development separately, as opposed to innovation product development as a singular concept. It appears that the term 'IPD' was more frequently used in newer studies such as the ones conducted by [7] and [12].

The term New Product Development (NPD) was also used in newer articles, such as the study of [12]. However, NPD is not the same as IPD, as new products are not always innovation products. Hence, researchers must not confuse the two terms. Based on the theory reviewed in this paper, IPD is the creation of new or improved product which may include new production techniques, as well as uncertainty in the decision-making process due to the lack of information concerning the product's development [3, 5, 6]. The next section will discuss about the use of Modified Design Thinking to identify the needs of potential customers in the fashion footwear industry.

Modified Design Thinking

Pink [13] argued that the current era of creativity demanded a different set of abilities from its players. As a result, there is a tendency to incorporate multiple disciplines to form a scientific viewpoint, particularly in the last two decades. In design perspective, conceptions of design thinking were introduced. The approach used by the new conceptions is quite the opposite of the traditional approach, which was problem solving.

One of the most important abilities in the creativity era is design ability. Several interpretations were made on design ability. For instance, [14] considered it as design attitude. Meanwhile, Brown [15] perceived it as design thinking. Design thinking is a series of divergent and convergent thinking processes. The essence of the divergent process is to create choices, while the essence of the convergent process is to make choices [15]. This suggestion is in line with the opinion of [13] who believed that design is a unique ability of whole thought – a combination of usefulness and meaning. In other words, design thinking optimizes the combined capacities of the left and right brains.

Design thinking is an approach that revolutionizes our thinking patterns in various areas of life and problem-solving perspectives. Therefore, design thinking often starts with the question 'What problem is being encountered?' followed by 'What is the cause of the problem?' Design thinking uses the designer's sensibility and methods in creating something valuable for consumers based on opportunities that are in accordance with desirability, technological feasibility and business strategy viability to

foster sustainable innovation [15]. The mission of design thinking is to translate observation to inspiration. Inspiration then leads to "the creation of products and services" that will increase the quality of life. This method inspires individuals to have a broad spectrum of innovative activities using human-based design ethos [15, 16]. In other words, design thinking is acting out the innovation process. This method will help reveal hidden needs and opportunities, as well as create new solutions.

However, in reality, the tendency of "satisficing" must be taken into consideration [17]. The satisficing theory states that human beings are constrained by bounded rationality when making decisions and solving problems. As a result, they are not able to maximize the outcome [17, 18]. Hence, when developing a product concept, the best option from a set of alternatives cannot be determined, because there are no alternatives. Therefore, there is the need to choose an alternative that best meets the design criteria. This is the essence of design thinking (D-Thinking) in producing something.

Design thinking focuses on innovative solutions. The data collection process is aimed at obtaining inspiration through benchmarks and trend observation. This search includes consumer needs and desires, technological capabilities, communities, and financial availability. It requires awareness of consumers' needs and desires and the ability to build people's expectation of a better life. The discovery of technological and community capabilities encourages the community to make changes. On the other hand, financial availability is a limitation that must be overcome by innovative solutions. Focusing on strength will lead to the creation of innovative solutions that will provide more satisfying answers to the existing challenges.

Design thinking allows the culprit to initiate activities with ignorance. In order to unravel new opportunities, design thinking is systematized under the following steps: defining opportunities, presenting and evaluating alternative solutions, making prototypes, testing the market, revising and developing superior alternatives, and choosing and executing the best alternative. For example, in new product development activity, defining opportunities can be done by adopting a famous phrase from Peter Drucker which says, "Converting need into demand." Without diminishing the importance of more conventional approaches such as focus group discussions and surveys to capture consumer voices, design thinking employs a more holistic and integrated approach involving consumers, designers, and the management [19].

The main mission of design thinking is the ability to translate observation to a whole new insight on new opportunities up to the point that it becomes goods and services that consumers really need. To achieve this, empathy is needed. Empathy involves observing and understanding what other say, do, or think. It is a success determinant for solving others' problems and a source of inspiration to find unarticulated needs. In other words, we need to know and care about others in order to come up with meaningful innovations for them.

It will not come as a surprise to see skeptical views on design thinking in the beginning. Using only observation, it will be difficult to use design thinking to invent groundbreaking products, because the products offered are the results of extrapolation from the habits of the user's community up to date. The articulation of opportunities and point of view is done by combining three elements, namely user, need, and insight. User refers to the identity of the user, while need is defined using verbs inspired by the insights.

After successfully defining the opportunities, extra precaution must be taken in the next phase as it may be ambiguous. Unless carefully followed, the stages of developing a concept may turn out to be misleading. One solution is to generate possibilities to reduce the ambiguity. However, the ideation process must be broad enough for various solutions, while at the same time, narrow enough to generate specific solutions. Once the ideation process is complete, a rapid prototype can be made to improve and test understanding [15]. We must bear in mind that the design is made to think and not the other way around.

When using design thinking, decision makers should not follow the rigid pattern of accepting or rejecting a solution. Rather, they must be open to the possibility of using a combination of several alternatives. Decision making, in essence, is an art. Design thinking

becomes meaningful because problem solvers have limitations. By nature, we tend to satisfy, rather than maximize. The next section will discuss the methods used to develop innovative footwear designs for this research.

RESEARCH METHODS

This research is a qualitative research. The research informants consist of six footwear customers, four manufacturers, four government officers, and an academician. Snowball sampling was used to select the fifteen informants. Two separate Focus Group Discussions (FGDs) were used to collect the primary research data. Additionally, observation and documentation were used as secondary data. The data analysis was performed within the Modified Design Thinking framework, and could be formulated as such:

Step 1: The use of customer insight to define opportunities

Step 2: The presentation and evaluation of alternative solutions

Step 3: Prototype creation

Step 4: Market testing

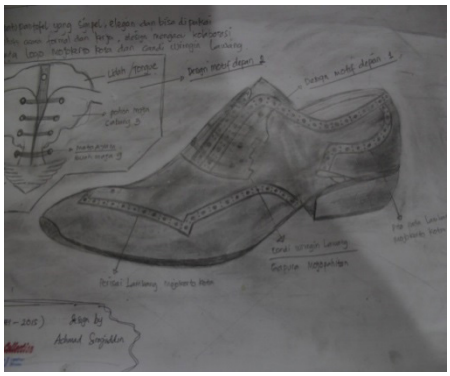
Step 5: Standardization of footwear quality

DATA ANALYSIS AND DISCUSSION

The key points of the focus group discussion can be summed up into the following steps:

Step 1: The Use of Customer Insight to Define Opportunities

The footwear development model for Mojokerto was based on the customer insights into the modern market. To learn more about consumer profiles and design trends, several visits were made to a number of shopping centres around Surabaya, including Surabaya Plaza, Tunjungan Plaza, and Ciputra World Mall. The visit to Surabaya Plaza was conducted to get an overview of the footwear design trends favoured by middle-class consumers. Meanwhile, the Tunjungan Plaza and Ciputra World Mall visits were used to obtain similar data from top-middle-class consumers. Based on the customer insights and footwear design trends, a blend of modern and traditional styles was used to design the footwear shown in Figure 1 below.



1. Majapahit Oxford Model

2. Heel Boot Model

3. High Heel Model

Figure 1. Fashion Footwear Designs Based on Customer Insights

Figure 1 illustrates the designs for the Majapahit Oxford, heel boot, and high heel models. The tongue-shaped design of the Majapahit Oxford model represents the maja tree, maja fruit, and the Archway of Wringin Lawang, which serves as a symbol for the fourteenth-century Majapahit kingdom gate. In addition, the shield symbol of Mojokerto is branded on the top part of the shoes. In general, the Majapahit Oxford model is simple, elegant, and suitable for both formal and casual occasions.

Figure 2. The Archway of Wringin Lawang as a Symbol of the Majapahit Kingdom

The heel boot design also incorporates the Wringin Lawang gate. This model is intended for dinner events and is designed to look anti-mainstream, attractive, beautiful, and elegant. At the same time, it creates simple, casual, and cool look, particularly for women with smaller body size than the average women. In general, heel boots give the illusion of added height and are perfect for people with long legs. Meanwhile, the high heel model is designed as a closed toe heel with Batik pattern, regardless of the suggestion that most women prefer open toe heels because they are more common and fashionable.

Step 2: The Presentation and Evaluation of Alternative Solutions

The three aforementioned designs were presented and evaluated at the focus group discussion. Some informants approved the designs and recognized the symbols of Mojokerto. The footwear designs, the heel boots in particular, were believed to be in accordance with the youth and young-adult market segments which include people between the ages of fifteen and thirty. The potential of this market reaches up to fifty percent of the national footwear market.

One suggestion from the focus group discussion was to develop the Majapahit Oxford model first before developing the other two models. Participants believed that the other two models had limited usage and appeal, as they were not in line with the current market trends. They also recommended expanding the utility of the heel boots to more than just dinner events. As for the high heels, open toe heels were preferred by the participants because they looked more fashionable.

One participant mentioned that Mojokerto had many artefacts which represented the city's identity, such as Majapahit sun and the maja tree. These ornaments are the symbols of the Majapahit kingdom. Once the design concept was agreed, participants selected the materials for the footwear prototype at PT. Karya Mitra. Leather and accessories were eventually chosen as the materials. However, these materials are very difficult to obtain in retail.

Step 3: Prototype Creation

The prototype creation began with the computer design, then followed by the top-part process by making the main form. The process is then followed by outsole process, assembling, and finishing. The computer design is shown in Figure 3 below.

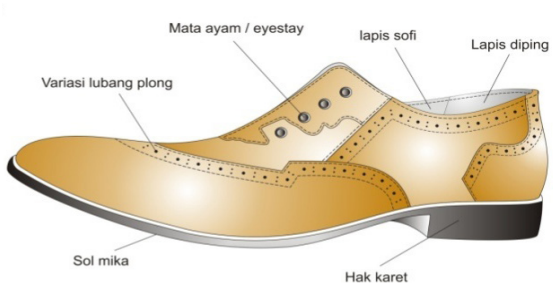


Figure 3. The Computer Design of the Majapahit Oxford Model

Figure 3 describes the material specifications of the top-part, insole, and outsole. The top-part materials consist of full-grain or brush-off leather and lax with the mixed colour of olive tan. By default, the materials create a shiny surface, and are easy to clean. Even without regular polishing, the surface of

the skin pores will still look natural. The top part also uses an inner layer called lining. The lining materials are a mixture of combed cotton, *sofi* imitation, and dipping material imitation.

Meanwhile, the insole material uses two layers called the top and bottom parts. The top part uses *Texon* coated with combed cotton. Combed cotton is chosen because it uses cool and limp fabrics, and has finer fibre than carded cotton in general. The bottom part, on the other hand, uses TW to maximize strength. Finally, the outsole material is made from a mix of *Javarino* and rubber soles. Rubber sole is used because it is relatively lighter, less slippery, and more flexible in movement.

The top-part process involves creating a main form for the design pattern, drawing patterns on the leather's surface, cutting and skiving, and folding the leather's edge after it is glued with latex, as shown in Figure 4 below.

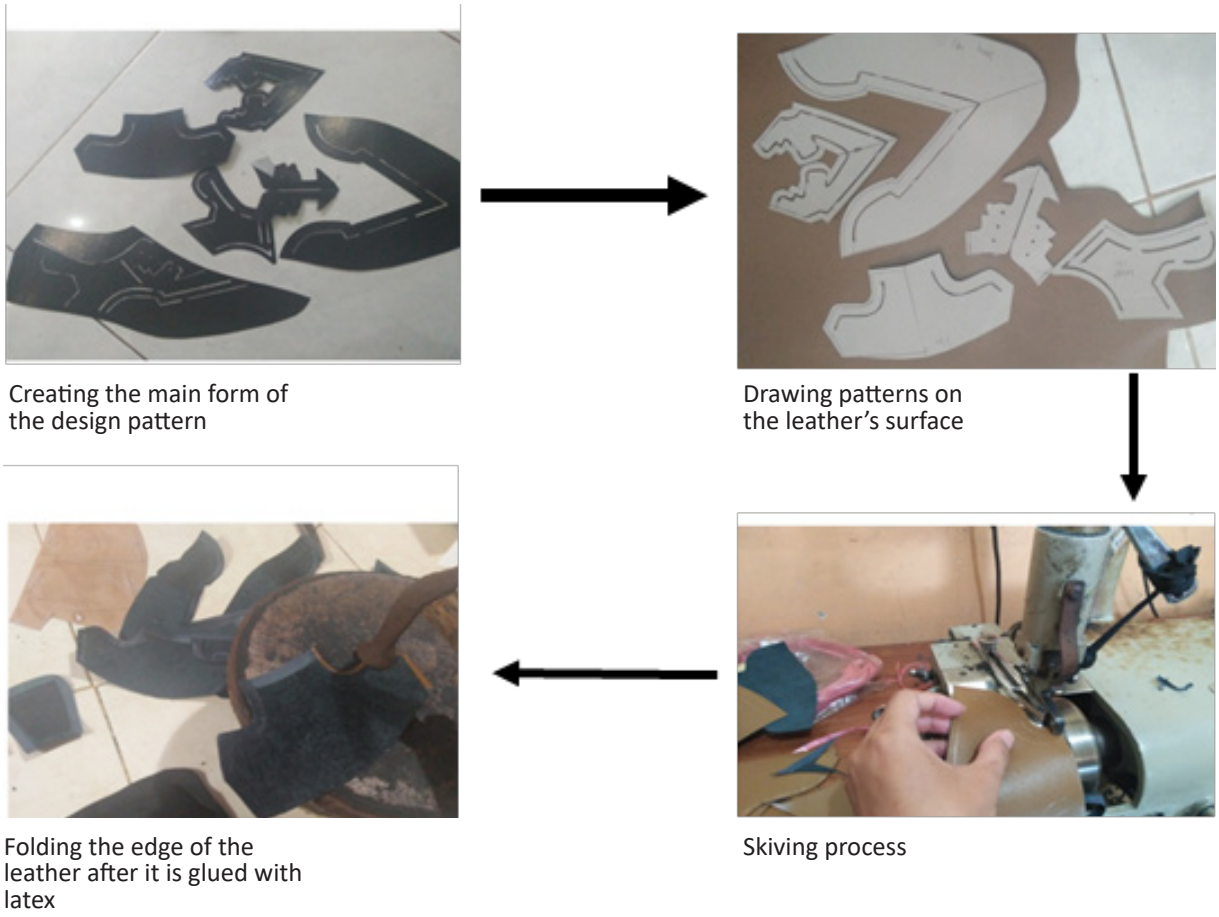


Figure 4. The Top-part Process (Step 1)

The cutting process is the process of cutting raw materials before they are shaped into the top part of the shoes. The leather is cut to form cardboard patterns according to the sample pattern prepared in advance. After the raw material is cut based on the pattern, it is sewn part-by-part onto the top part using the stick cotton technique to make the seams invisible, as shown in Figure 5 below.

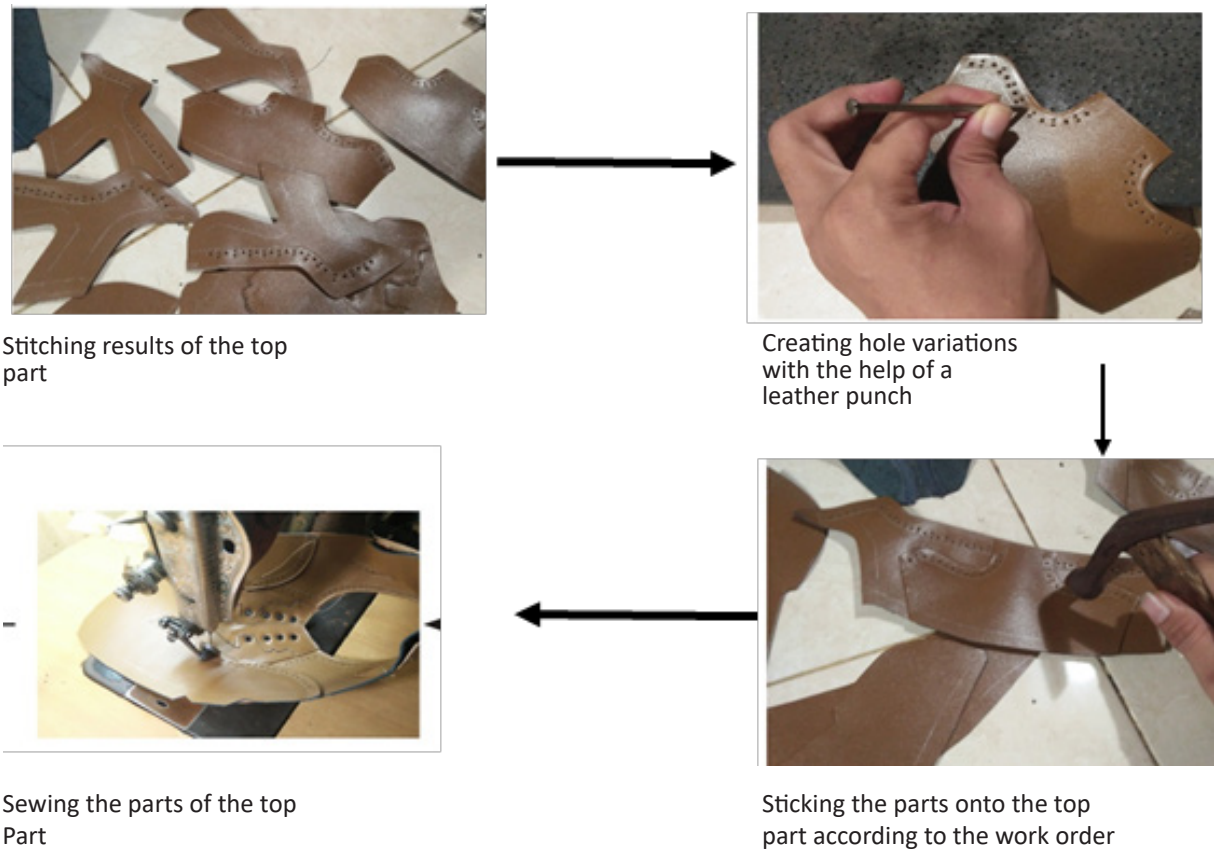


Figure 5. The Top-part Process (Step 2)

After the top-part process is completed, the next step is initiating the bottom part process. This process is divided into two parts: outsole production and insole production. Outsole is the bottom part of a shoe which makes a direct contact with the ground. The characteristics of a good outsole are, among others, durable, water-resistant, and firm grip. Meanwhile, insole is the inner part of a shoe which makes direct contact with the sole of the feet. The insole material will determine the comfort level of the shoe.

Once the top and bottom parts are completed, the next process is attaching the top part to the bottom part. This process is known as assembling, as shown in Figure 6. It starts by cementing the bottom part of the top side using special glue. During the assembling process, the top and bottom parts come in pairs with predetermined size. In order for the shoe form to match the foot contour, *Laste* is used. And finally, compressor press is performed to provide maximum pressure.

Figure 6. Assembling Process

The next stage of the prototype creation is the finishing process, as shown in Figure 7 below.

Figure 7. Finishing Process

This stage involves cleaning the glue and ink residue with gasoline or liquid F4, painting the sides of the sole with black car paint, putting lining under the insole, spraying paint with clear gloss paint, and attaching a shoelace and other accessories.

Step 4: Market Testing

The completion of the prototype is followed by market and shoe-quality tests. The purpose of the market test is to determine the market's response to the design and quality of the footwear prototype. Meanwhile, the durability test was conducted by the Indonesian Footwear Industry Development Center (BPIPI). The durability of the footwear was marked as 2.8 out of 3, which was the passing standard. This result indicates the use of low-quality or insufficient amount of adhesive.

Figure 8. First Prototype

Figure 9. Improved Prototype

The market tests were conducted twice at the Fashion and Leather Shoe Exhibition (SKF) in Jakarta and Crafina Exhibition in Surabaya. The percentage of positive comments ('good' or 'very good') during the first market test at SKF was shown at 47.73%. This means that the design might not fit the current market trends and preferences.

Based on the feedback, improvements were made on the first prototype shown in Figure 8. Brighter colour and thinner shoelaces were used, while the toe cap was made pointier. Overall, the shoe looked more symmetrical and pleasant-looking, as shown in Figure 9. When the second market test was conducted at the Crafina Exhibition, the percentage of favourable opinion increased to 75.88%. The number confirmed that the improved prototype was more in line with the current market trends and preferences.

Step 5: Standardization of Footwear Quality

Standardization is needed to certify the production quality of the footwear. The three aspects of standardization are technical, non-technical, and consumer perception aspects. The complete standardization guideline is as follows:

1. The technical aspects include durability, raw materials, comfort, and progressive designs
2. The non-technical aspects include convenience, adjustment to trend, footwear designs, and stitching quality
3. The consumer perception standards on footwear quality
   a. Convenience: 60% of respondents mentioned 'Comfortable' and opinion assessment team.
   b. Adjustment to trend: 60% of respondents mentioned 'Trendy' and opinion assessment team.
   c. Footwear design: 60% of respondents mentioned 'Good design' and opinion assessment team.
   d. Product uniqueness: 60% of respondents mentioned 'Unique product' and opinion assessment team.
   e. Raw materials quality: 60% of respondents mentioned 'Good raw material quality' and opinion assessment team.
   f. Stitching quality: 60% of respondents mentioned 'Good stitching quality' and opinion assessment team.

IMPLICATIONS

Modified Design Thinking can be used to find innovative design inspiration based on current market trends and preferences. Since the products generated by the innovation process incorporate local culture in their design, they tend to fit the market's preferences. Additionally, prototypes can be repaired to prevent greater loss in mass production with the help of market testing as a part of the continuous innovation process.

CONCLUSION

The two main problems encountered by many small and medium-sized enterprises

in Mojokerto are monotonous design and dependence on imported raw materials. Furthermore, the production process was carried out in a traditional fashion, while the market development was very limited. By implementing Modified Design Thinking, this study concludes that the innovation process in Indonesia is unique as it incorporates local culture into the process. The first market testing at SKF indicated that 47.73% of the public's opinion leaned towards 'Good' and 'Very good'. Additionally, the percentage of the favourable comments was shown at 75.88% after the second market testing was conducted.

REFERENCES

1. Pusat Komunikasi Publik Kementerian Perindustrian, Rencana Induk Pembangunan Industri Nasional 2015 – 2035, **2015**, retrieved August 23, 2018, from http://www.kemenperin.go.id/ripin.pdf.
2. Dishubkominfo. Sepatu Mojokerto Siap Memasuki Pasar MEA: Focus Group Discussion (FGD) Penyusunan Standarisasi Produk Alas Kaki UKM, **2016**, retrieved August 23, 2018, from http://new.mojokertokota.go.id/home/readmore_berita/nw2016031513231327.
3. Reguia, C., Product innovation and the competitive advantage, *Eur Sci J*, **2014**, 10, 10.
4. Dereli, D.D., Innovation Management in Global Competition and Competitive Advantage, *Procedia Soc Behav Sci*, **2015**, 195, 1365-1370, https://doi.org/10.1016/j.sbspro.2015.06.323.
5. Angelmar, R., Product Innovation: A Tool for Competitive Advantage, *Eur J Oper Res*, **1990**, 47, 2, 182-189, https://doi.org/10.1016/0377-2217(90)90277-I.
6. Beauregard, Y., Polotski, V., Bhuiyan, N., Thomson, V., Optimal Utilisation Level for Lean Product Development in a Multitasking Context, *Int J Prod Res*, **2016**, 55, 3, 795-818, https://doi.org/10.1080/00207543.2016.1213448.
7. Bilgili, B., Erciş, A., *Ünal*, S., Kano Model Application in New Product Development and Customer Satisfaction (Adaptation of Traditional Art of Tile Making to Jewelries), *Procedia Soc Behav Sci*, **2011**, 24, 829-846, https://doi.org/10.1016/j.sbspro.2011.09.058.
8. Balachandra, R., Friar, J.H., Factors for Success in R&D Projects and New Product Innovation: A Contextual Framework, IEEE Xplore, **1997**, https://doi.org/10.1109/17.618169.
9. Hu, Y., Aziz, E.S.S., Chassapis, C., Creativity-Based Design Innovation Environment in Support of Robust Product Development, *Int J Interact Des Manuf*, **2016**, 10, 4, 335-353, https://doi.org/10.1007/s12008-014-0242-3.
10. Su, C.T., Chen, Y.H., Sha, D.Y., Linking innovative product development with customer knowledge: A data-mining approach, *Technovation*, **2006**, 26, 7, 784-795, https://doi.org/10.1016/j.technovation.2005.05.005.
11. Danneels, E., The Dynamics of Product Innovation and Firm Competences, *Strateg Manag J*, **2002**, 23, 12, 1095-1121, https://doi.org/10.1002/smj.275.
12. Woschke, T., Haase, H., Enhancing New Product Development Capabilities of Small- and Medium-Sized Enterprises through Managerial Innovations, *J High Technol Manag Res*, **2016**, 27, 1, 53-64, https://doi.org/10.1016/j.hitech.2016.04.005.
13. Pink, D., A Whole New Mind, **2005**, USA: Penguin Group.
14. Avital, M., Boland, R.J., Design with a Positive Lens: An Affirmative Approach to Designing Information and Organizations, *ICIS 2008 Proceedings*, https://doi.org/10.1016/S1475-9152(07)00201-3.
15. Brown, T., Design Thinking, Harvard Business Review, **2008**, 86, 6, 84-92.
16. Brown, T., Change by Design: How Design Thinking Transforms Organizations and Inspires Innovation, **2009**, New York, NY.: HarperCollins Publishers.

17. Simon, H.A., Models of Man: Social and Rational, **1957**, England: Wiley.
18. Simon, H.A., A Behavioral Model of Rational Choice, The Quarterly Journal of Economics, **1955**, 69, 1, 99 – 118, https://doi.org/10.2307/1884852.
19. Lockwood, T., The Expanding Influence of Design Management, *Design Management Journal*, **2010**, 5, 1, 3, https://doi.org/10.1111/j.1948-7177.2010.00017.x.

THE INFLUENCE OF CHITOSAN TOWARDS ANTIBACTERIAL PROPERTIES IN NATURAL LEATHER

Mustafidah UDKHIYATI[1*], Nur Mutia ROSIATI[1], Fitrilia SILVIANTI[2]

[1]Department of Leather Processing Technology, Politeknik ATK Yogyakarta, Sewon, Bantul, 55188, Yogyakarta, Indonesia, ukyfida@gmail.com

[2]Department of Rubber and Plastic Processing Technology, Politeknik ATK Yogyakarta, Sewon, Bantul, 55188, Yogyakarta, Indonesia

Received: 10.02.2020 Accepted: 03.12.2020 https://doi.org/10.24264/lfj.20.4.8

THE INFLUENCE OF CHITOSAN TOWARDS ANTIBACTERIAL PROPERTIES IN NATURAL LEATHER

ABSTRACT. The abundant hydroxyl groups (-OH) of vegetable tanned leather enhances the bacterial growth possibility. In this work, antibacterial vegetable tanned leather was developed taking advantage of chitosan antibacterial activity. Different chitosan utilization method was conducted, namely drum impregnation and spraying. Antibacterial testing was carried out using agar diffusion method to identify the effect of chitosan against Gram-positive bacteria (*Staphylococcus aureus*). The results showed that the use of 1% (w/v) chitosan by spraying method tends to be more effective in improving the antibacterial properties of vegetable tanned leather. While, the leather sample of drum impregnation at initial pH 4 has the lowest antibacterial activity among others. The results of FTIR analysis showed that there was no significant difference between control and chitosan modified tanned leather, confirming the similar functional groups between it.
KEY WORDS: leather, antibacterial, chitosan

INFLUENȚA CHITOSANULUI ASUPRA PROPRIETĂȚILOR ANTIBACTERIENE ALE PIELII NATURALE

REZUMAT. Grupările hidroxilice (-OH) care se găsesc din abundență în pielea tăbăcită vegetal favorizează creșterea bacteriană. În această lucrare s-a tratat pielea tăbăcită vegetal cu chitosan pentru a-i oferi proprietăți antibacteriene. Chitosanul a fost aplicat prin impregnare în tambur și prin pulverizare. Testarea antibacteriană a fost efectuată folosind metoda de difuzie în agar pentru a identifica efectul chitosanului asupra bacteriilor Gram-pozitive (*Staphylococcus aureus*). Rezultatele au arătat că utilizarea a 1% chitosan (m/v) prin metoda pulverizării tinde să fie mai eficientă în îmbunătățirea proprietăților antibacteriene ale pielii tăbăcite vegetal. Proba de piele impregnată cu chitosan în tambur la pH-ul inițial 4 are cea mai mică activitate antibacteriană, printre altele. Rezultatele analizei FTIR au arătat că nu a existat nicio diferență semnificativă între proba martor și pielea tăbăcită tratată cu chitosan, confirmând grupările funcționale similare ale acestora.
CUVINTE CHEIE: piele, antibacterian, chitosan

L'INFLUENCE DU CHITOSANE SUR LES PROPRIÉTÉS ANTIBACTÉRIENNES DU CUIR NATUREL

RÉSUMÉ. Les groupes hydroxyle (-OH) que l'on trouve en abondance dans le cuir à tannage végétal favorisent la croissance bactérienne. Dans cet article, la peau tannée végétale a été traitée avec du chitosane pour lui conférer des propriétés antibactériennes. Le chitosane a été appliqué par imprégnation dans le tambour et par pulvérisation. Des tests antibactériens ont été réalisés en utilisant la méthode de diffusion sur gélose pour identifier l'effet du chitosane sur les bactéries Gram-positives (*Staphylococcus aureus*). Les résultats ont montré que l'utilisation de 1% de chitosane (m/v) par la méthode de pulvérisation tendent à être plus efficace pour améliorer les propriétés antibactériennes du cuir à tannage végétal. L'échantillon de cuir imprégné de chitosane dans le tambour au pH initial de 4 a, entre autres, l'activité antibactérienne la plus faible. Les résultats de l'analyse FTIR ont montré qu'il n'y avait pas de différence significative entre l'échantillon témoin et le cuir traité au chitosane, confirmant leurs groupes fonctionnels similaires.
MOTS CLÉS : cuir, antibactérien, chitosane

** Correspondence to: Mustafidah UDKHIYATI, Department of Leather Processing Technology, Politeknik ATK Yogyakarta, Sewon, Bantul, 55188, Yogyakarta, Indonesia, ukyfida@gmail.com*

INTRODUCTION

The challenges of the global industry are improving dynamically following various pressures including globalization of world trade, standardization, and also government regulations. If in the past the operating system focused on low costs, in the present era it is expected that the operating system is also considerate to environmental problems. Especially in leather section, the establishment of the ASEAN Free Trade Area (AFTA) since 2015, increasing competition in the global leather market [1]. Therefore, alternative tanning processes that are more environmentally friendly are needed, especially considering the leather tanning industry which has been known as a waste contributor industry. The use of chrome as a main tanning agent is known to be a health and environmental risk. Alternative to environmentally friendly tanning processes can be considered with the utilization of natural tanning agent. However, the durability problem is still the limitation of natural leather.

The vegetable tanning agent (tannin) contains abundant hydroxyl groups (-OH), thus vegetable tanned leather has the potential to be a good bacterial growth medium [2]. Coating process is one of the efforts that can be done to increase the leather antibacterial properties [3]. Various antibacterial agents have been developed to produce antibacterial properties in a material. Some organic materials that have been widely developed as antibacterial agents are Eucalyptus, Lavender [4], *Origanum minutiflorum* [5], and chitosan [3, 6]. Chitosan is an organic material that has high potential to be developed as an antibacterial agent. Chitosan (α- (1-4) -2-amino-2-deoxy-β-D-glucan) is a chitin derivative that is abundant, non-toxic, and easily degraded in nature [7]. The antibacterial ability of chitosan to microorganisms such as bacteria, molds and fungi has become an interesting study in various fields. Some research to confer antimicrobial capacity in the leather industry have been reported as a result of the use of a methacrylic acid–acrylamide–chitosan copolymer [8], development of chitosan-based antimicrobial leather coatings in footwear material made from wet blue (chrome tanning) [3], and initial study of chitosan utilization in natural leather [9]. However, those previous studies have not observed yet the effect of different concentration and the utilization method of chitosan towards the antibacterial properties of natural leather. It is expected that through the experimental application with several method variations, the optimal conditions for the use of chitosan as an antibacterial agent on natural skin is obtained.

EXPERIMENTAL

Materials and Methods

Materials

Chitosan (Pharmaceutical Grade) corresponding to flakes with size 149 µm, deacetylation degree 94%, and dynamic viscosity of 100 mPa.s purchased from CV. ChiMultiguna was used. Chitosan solutions were prepared by mixing the desired amount of chitosan in formic acid aqueous solutions (2%, v/v), with stirring overnight (200 rpm at 50ºC) [3]. Formic acid was chosen since it is the most referred in the literature and due to its use in the tanning industry (Tannin and Dye Fixation Stage).

Instrumentations

Characterization using Fourier Transform InfraRed (FTIR) (Perkin-Elmer) was carried out to determine functional group of leather sample.

Leather Samples Coating Processes

Sheep pickled skins were processed by vegetable tanning method. Leather samples were collected after fatliquoring fixation stage (Figure 1). Different approaches for coating were tested at a pilot scale, they were:

a) Impregnation using drum: impregnations were carried out at different initial pH (3 and 4), using 1.0% (chitosan/leather

ratio, w/w), prepared in 2% (v/v) formic acid solutions. The initial pH variation of impregnation was conducted to identify the effect of pH on the antibacterial activity of chitosan. The absorption of chitosan to the leather was analyzed by comparing the initial concentration of chitosan with the concentration of chitosan after the process is finished (residual).

b) Spraying: chitosan solution (1%, w/v, formic acid) was applied as a finishing step using the spray-gun placed at a distance of 14 cm from the leather sample. For control purposes, leather samples without chitosan coating were prepared.

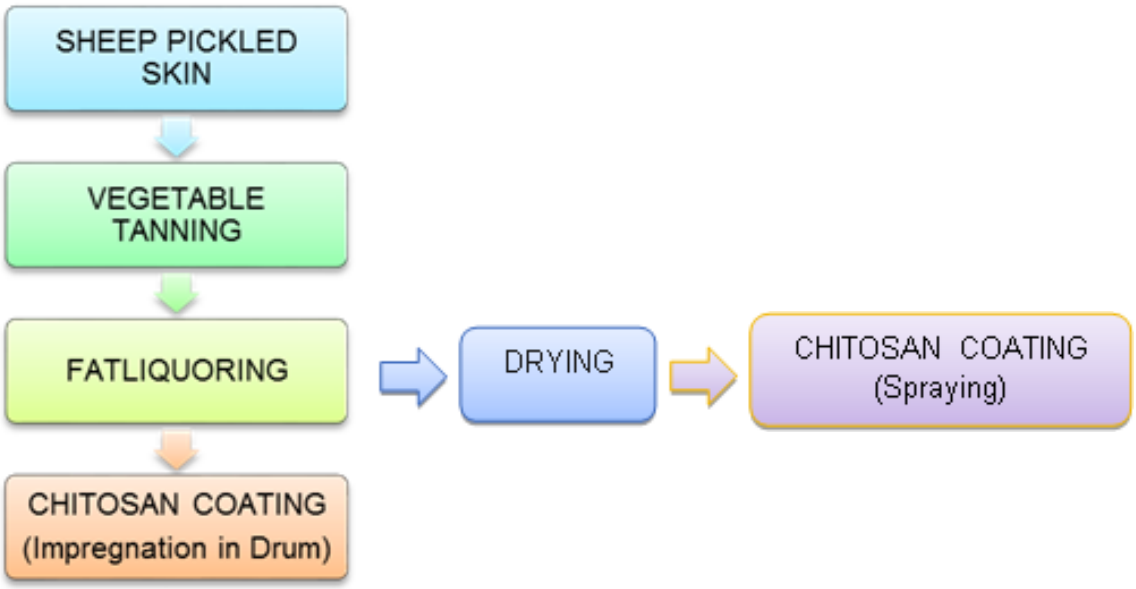


Figure 1. Procedure Scheme in Experiment

Antibacterial Activity Assays

Antibacterial testing was carried out using agar diffusion method to identify the effect of chitosan against Gram-positive bacteria (*Staphylococcus aureus*). Briefly, the bacteria inoculum was prepared by aseptically transferring 4 isolated colonies to nutrient broth, which was then incubated during 24 h at 37±1ºC. The inoculum was diluted to 0.5 McFarland turbidity standard (corresponding to a concentration of 1.5–3.0×108 CFU/mL). Antibacterial activity test was conducted by placing the leather sample (2cm×2cm) on a petri dish containing 0.5mL of bacterial inoculant in 20mL nutrient agar. The Petri dish was then incubated for 24 hours at 37±1ºC. Bacterial growth inhibition was observed by measuring the clear area at the edge of the leather sample in Petri dish.

RESULTS AND DISCUSSIONS

FTIR Analysis

The results of the infrared absorption of leather control were shown in Figure 2. The stretching vibration of O-H and it of N-H from collagen were proven by the appearance of broad absorption bands due to the formation of hydrogen bonds in the region 3402 cm^{-1}. The band at 2924 cm^{-1} was resulted from the stretching vibration of the C-H bond [10]. Amide I absorption was observed at wave number of 1651 cm^{-1} which was the stretching vibration of the collagen carbonyl group. This absorption band was overlapping with absorbance of -OH bending vibration. Amide II characteristic absorption shown at 1543 cm^{-1} due to C-N stretching vibration overlapping with

N-H bending vibration [11-14]. The stretching vibration of C-N was also observed at 1450 cm^{-1}. The absorption band at 1234 cm^{-1} was attributed to the presence of amide III of leather [12]. Meanwhile, the absorption of the methylene group appeared at the 1342 cm^{-1}, and the stretching vibrations of C-O-C were seen in the region of 1034-1165 cm^{-1} [10].

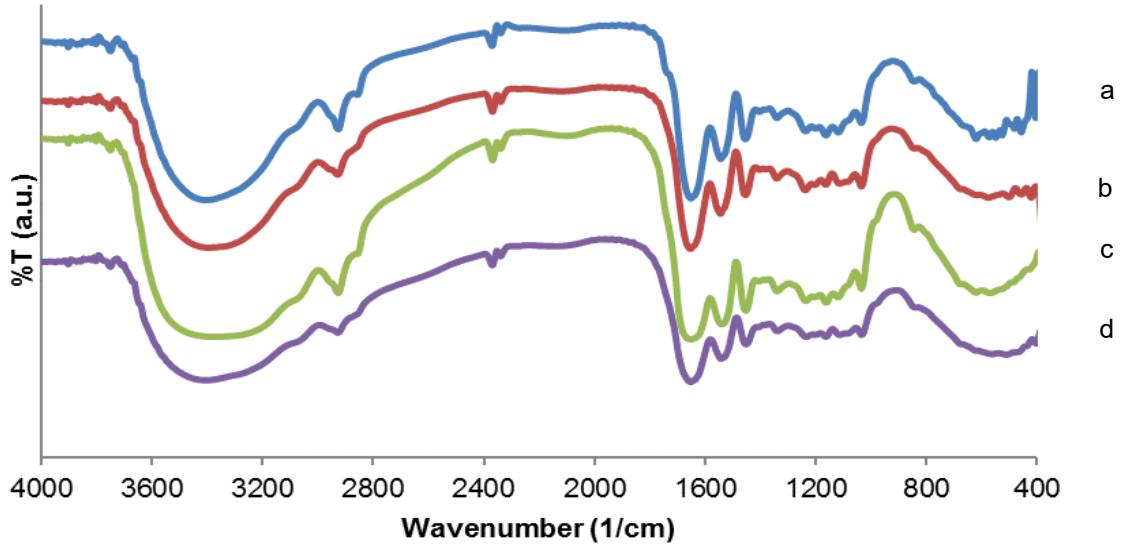


Figure 2. FTIR spectra. Control (a), impregnation in drum at initial pH 3 (b), pH 4 (c), spraying (d)

FTIR spectra showed no significant alteration between the infrared absorption of control leather with chitosan modified leather. This happened because the functional groups in chitosan were the same as the functional groups in the control leather, containing methylene, amine and carbonyl groups. The broad absorption band in the region of 3380-3400 cm^{-1} and 555-617 cm^{-1} showed the existence of O-H and N-H stretching vibrations from collagen and chitosan [10, 14]. The absorption band was observed to be broader after being modified with chitosan. This phenomenon proved that more hydrogen bonds were formed due to chitosan modification in the leather. The presence of a methylene group was characterized by the visible absorption at 2924-2932 cm^{-1} as asymmetrical stretching vibrations and 1335-1342 cm^{-1} as bending vibrations [10, 15, 16]. Based on Figure 5, there was no wavenumber shift of amide groups I, II, and III. It showed that there were no alteration bonds due to the modification chitosan in the leather.

Effect of Initial pH and Different Type of Coating on Antibacterial Activity of Chitosan

By visually observing the inhibition zones displayed around the samples coated, the spraying sample treatment showed the largest inhibition zone among the treatments (Figure 3). The difference is increasingly visible in the measurement results of bacterial inhibition area. The result showed that though was not statistically significant, the highest value of bacterial inhibition area was shown in the spraying sample group with a value about 582.67 mm^2 (Table 1). Meanwhile, the tendency of antibacterial activity of the drum method at pH 3 and 4 were less effective as the lower value of inhibition area than the control. Based on the results obtained there is a tendency that the use of chitosan as an antibacterial on vegetable tanned leather was more effective in the spraying method compared to the impregnation method in the drum.

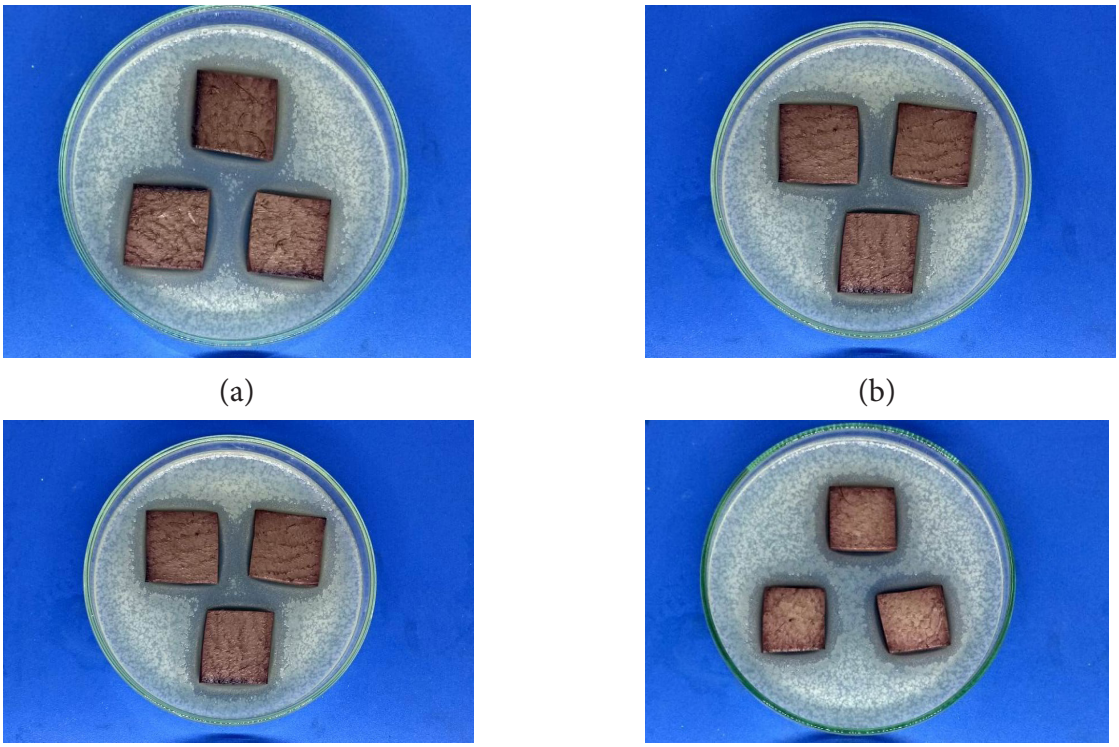


Figure 3. Measurement of antibacterial activity using agar diffusion method: a) control; b) spraying; c) impregnation in drum at initial pH 3; d) pH 4

Table 1: Bacterial inhibition area

No	Leather Samples	Inhibition Area (mm^2)			Average
		1	2	3	
1	Control	500	500	561	520,33[b]
2	Spraying	500	624	624	582,67[b]
3	pH 3 (1%)	500	500	500	500,00[b]
4	pH 4 (1%)	384	276	276	312[a]

[a,b)] Different superscripts in the same line showed the significant difference among the treatments

This phenomenon can be explained that basically in addition to chitosan, antibacterial activity was also influenced by the interaction of vegetable tanning agent (tannin) against the leather. Vegetable tanning agent that used in this study was mimosa, which was the type of vegetable condensed tannin. This type of vegetable tanning agent consists of flavonoid structures, some of them were in the form of gallocatechin and epigallocatechin [17]. Flavonoids have specific antibacterial mechanism depending on the type of flavonoid. For example, epigallocatechin has antibacterial mechanism by inhibiting the function of cytoplasmic membrane [18].

In the case of spraying leather, the surface of vegetable tanned leather is rich of polyphenol groups thus had high possibility to react with chitosan in several possible bond types. Including covalent bonding (Figure 4) as well as the hydrogen bond between -OH of the polyphenol group and amine of chitosan [19]. The interactions formed by chitosan and polyphenols on the surface of the vegetable tanned leather affect the enhancement of the antibacterial properties of leather. Chitosan antibacterial mechanism can be affected by the interaction between the positively charged chitosan and the negatively charged microbial cell wall leads to the leakage of the intracellular constituents. The binding of chitosan with DNA and the inhibition of mRNA synthesis occurs via the penetration of chitosan into the nuclei of the microorganisms and interfering with the synthesis of mRNA and proteins [20].

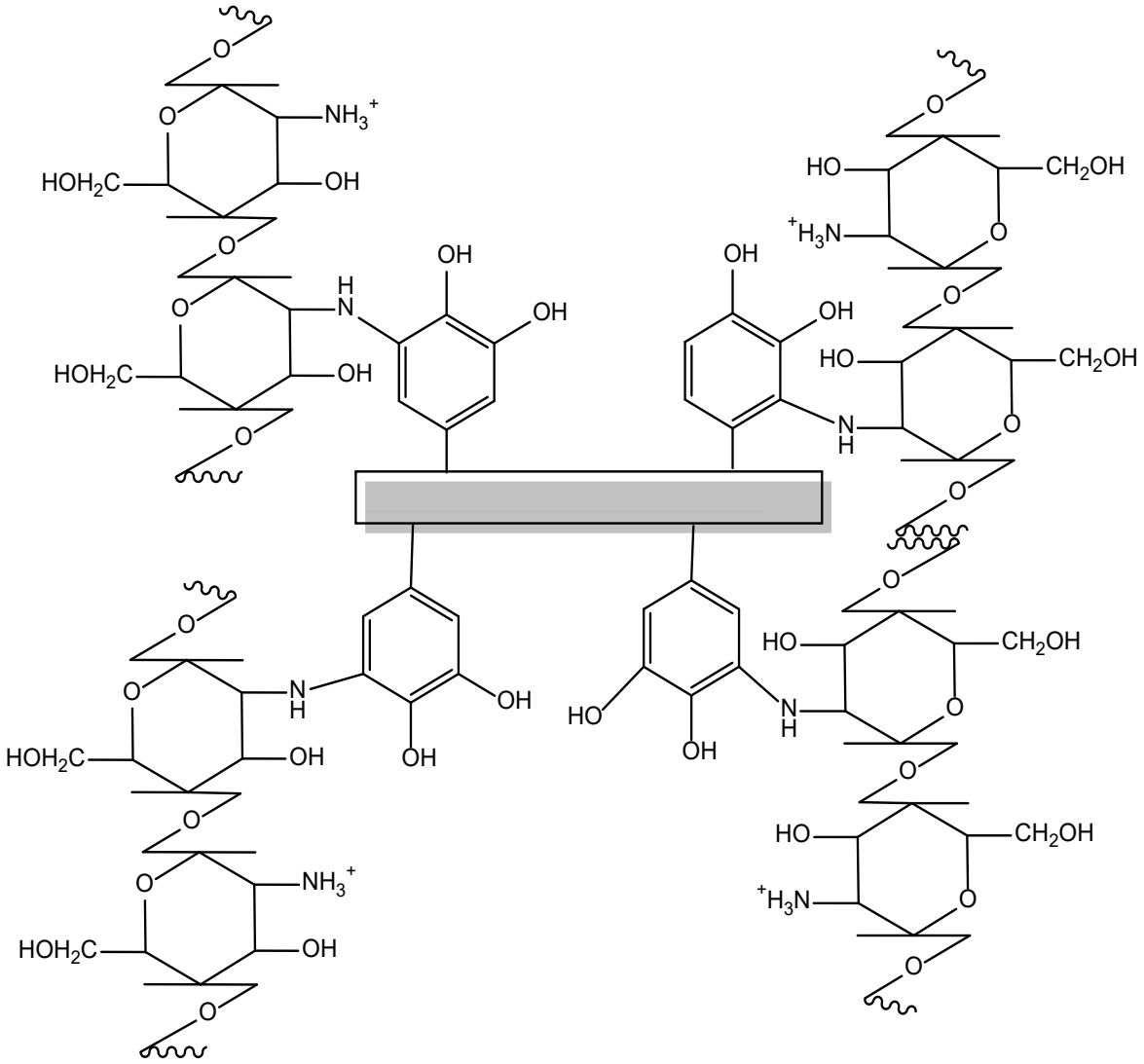


Figure 4. Interaction model of chitosan bonding on the natural leather surface

Meanwhile, the different possible interaction of chitosan in the drum method leather affect the antibacterial properties of leather. The possible interactions are hydrogen bonding between chitosan and collagen (Figure 5) and covalent bonding between chitosan and polyphenol on the leather surface as occurred in the spraying leather (Figure 4). The presence of amine groups in chitosan initiates the formation of strong hydrogen bonds between phenolic groups and amine groups in chitosan [19]. The direct interaction of chitosan and collagen allows the reduction of interaction between tannins and leather, thereby exerting an influence on the antibacterial effect produced by tannin. This phenomenon might explain the low antibacterial properties of the drum impregnation method group. Antibacterial properties of leather group with chitosan impregnation treatment at initial pH 4 showed the lowest significantly among the other three groups. This is thought to be influenced also by the differences in levels of chitosan that is absorbed into the leather.

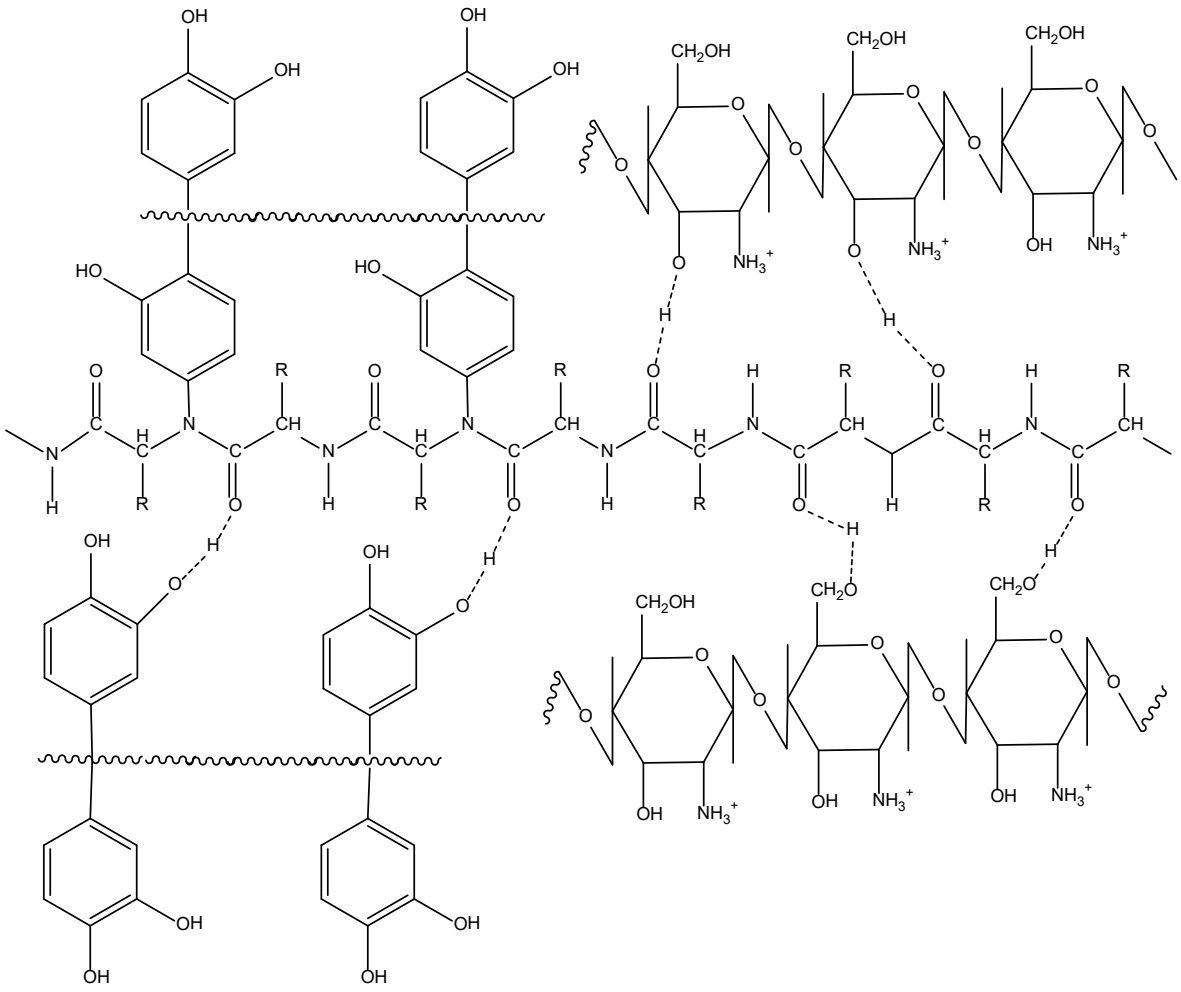


Figure 5. Interaction model of the hydrogen bonding between chitosan and collagen

Based on the results obtained, chitosan residual amount in waste of the drum method group with initial pH 4 higher than the pH 3 (Table 2). It showed that the amount of chitosan absorbed into the leather on pH 3 higher than pH 4 group. The higher amount of chitosan absorbed in the leather has been clearly proven to increase the antibacterial properties of the leather. The antibacterial ability of chitosan is strongly influenced by pH. This is related to the level of solubility of chitosan in an acidic atmosphere, and the change in the chitosan molecule to polycationic as the pH decreases below pKa (6.3-6.5) [21, 22]. The polycationic structure of chitosan is a prerequisite for antibacterial activity. As environmental pH is below the pKa of chitosan and its derivatives, electrostatic interaction between the polycationic structure and the predominantly anionic components of the microorganisms' surface [22]. Although according to previous studies it was mentioned that the antibacterial properties of chitosan decreased below pKa (6.3-6.5), but in this experiment at pH 4 a decrease in the antibacterial ability of chitosan has begun.

Table 2: Chitosan residual determination

No	Sample	Weight of chitosan residual deposition in waste (gram)		Average
		1	2	
1	pH 3 (1%)	0.035	0.033	0.034
2	pH 4 (1%)	0.072	0.037	0.055

CONCLUSIONS

The evaluation of antibacterial activity of chitosan against Gram-positive bacteria (*Staphylococcus aureus*) based on different method confirmed that the impregnation in drum method sample have lower antibacterial activity than spraying. There is a tendency that the use of chitosan as an antibacterial on vegetable tanned leather was more effective in the spraying method compared to the impregnation method in the drum, showed by the highest value of bacterial inhibition area about 582.67 mm^2. The leather sample of drum impregnation at initial pH 4 has the lowest antibacterial activity among others. Meanwhile, based on FTIR spectra there was no alteration absorption bands due to the modification chitosan in the leather.

Acknowledgements

This work was funded by Politeknik ATK Yogyakarta through research grant of Riset Pembinaan, contract number 178/SK/BPSDMI/ATK/XII/2019.

REFERENCES

1. Purba, F., Green Productivity of Indonesia Leather Industry (Case Study in Bogor and Magetan District) (Produktivitas Hijau Industri Kulit Samak Indonesia (Studi Kasus di Bogor dan Magetan)), Bogor: Sekolah Pascasarjana, Institut Pertanian Bogor, **2018**.
2. Orlita, A., Microbial biodeterioration of leather and its control: a review, *Int Biodeterior. Biodegrad*, **2004**, 53, 157–163, https://doi.org/10.1016/S0964-8305(03)00089-1.
3. Fernandes, I.P., Amaral, J.S., Pinto, V., Ferreira, M.J., Barreiro, M.F., Development of chitosan-based antimicrobial leather coatings, *Carbohydr Polym*, **2013**, 98, 1229–1235, https://doi.org/10.1016/j.carbpol.2013.07.030.
4. Sirvaityte, J., Siugzdaite, J., Valeika, V., Application of Commercial Essential Oils of Eucalyptus and Lavender as Natural Preservative for Leather Tanning Industry, *Rev Chim*, **2011**, 10, https://doi.org/10.3176/proc.2012.3.12.
5. Bayramoglu, E.E.E., Natural and Environment-friendly New Bactericide for Leather Industry: Essential Oil of *Origanum minutiflorum*, *J Biol Sci*, **2005**, 5, 455–457, https://doi.org/10.3923/jbs.2005.455.457.
6. Zhang, Z., Chen, L., Ji, J., Huang, Y., Chen, D., Antibacterial Properties of Cotton Fabrics Treated with Chitosan, *Text Res J*, **2003**, 73, 1103–1106, https://doi.org/10.1177/004051750307301213.
7. Panos, I., Acosta, N., Heras, A., New Drug Delivery Systems Based on Chitosan, *Curr Drug Discov Technol*, **2008**, 5, 333–341, https://doi.org/10.2174/157016308786733528.
8. Lv, S.H., Yan, X.L., Gao, R.J., Preparation and Properties of Copolymer of Methacrylic Acid and Acrylamide onto Degraded Chitosan Initiated by HRP/H2O2/ACAC, *Appl Mech Mater*, **2011**, 80–81, 396–399, https://doi.

org/10.4028/www.scientific.net/AMM.80-81.396.

9. Udkhiyati, M., Silvianti, F., The Utilization of Chitosan as Natural Antibacterial for Vegetable Tanned Leather, *Mater Sci Forum*, **2019**, 948, 212–216, https://doi.org/10.4028/www.scientific.net/MSF.948.212.
10. Corazzari, I., Nisticò, R., Turci, F., Faga, M.G., Franzoso, F., Tabasso, S., Magnacca, G., Advanced physico-chemical characterization of chitosan by means of TGA coupled on-line with FTIR and GCMS: Thermal degradation and water adsorption capacity, *Polym Degrad Stab*, **2015**, 112, 1–9, https://doi.org/10.1016/j.polymdegradstab.2014.12.006.
11. Bekale, L., Agudelo, D., Tajmir-Riahi, H.A., Effect of polymer molecular weight on chitosan–protein interaction, *Colloids Surf B Biointerfaces*, **2015**, 125, 309–317, https://doi.org/10.1016/j.colsurfb.2014.11.037.
12. Lu, Z., Xiao, J., Wang, Y., Meng, M., In situ synthesis of silver nanoparticles uniformly distributed on polydopamine-coated silk fibers for antibacterial application, *J Colloid Interface Sci*, **2015**, 452, 8–14, https://doi.org/10.1016/j.jcis.2015.04.015.
13. Ramalingam, S., Sreeram, K.J., Rao, J.R., Nair, B.U., Organic Nano-colourants: A self-fixed, optothermal resistive silica supported dyes for sustainable dyeing of leather, *ACS Sustain Chem Eng*, **2016**, 4, 5, 2706–2714, https://doi.org/10.1021/acssuschemeng.6b00218.
14. Djobo, Y.J.N., Elimbi, A., Dika Manga, J., Djon Li Ndjock, I.B., Partial replacement of volcanic ash by bauxite and calcined oyster shell in the synthesis of volcanic ash-based geopolymers, *Constr Build Mater*, **2016**, 113, 673–681, https://doi.org/10.1016/j.conbuildmat.2016.03.104.
15. Pan, H., Li, G.-L., Liu, R.-Q., Wang, S.-X., Wang, X.-D., Preparation, characterization and application of dispersible and spherical Nano-SiO2 @Copolymer nanocomposite in leather tanning, *Appl Surf Sci*, **2017**, 426, 376–385, https://doi.org/10.1016/j.apsusc.2017.07.106.
16. Nuryono, N., Rosiati, N.M., Rettob, A.L., Suyanta, S., Arryanto, Y., Coating of 2-Aminobenzimidiazole and 1-(o-Tolyl) biguanide Functionalized Silicas on Iron Sand Magnetic Material for Sorption of [AuCl4]–, *Indones J Chem*, **2019**, 19, 395, https://doi.org/10.22146/ijc.34653.
17. Covington, A.D., Tanning chemistry: the science of leather, Cambridge, UK: Royal Society of Chemistry, **2009**.
18. Cushnie, T.P.T., Lamb, A.J., Antimicrobial activity of flavonoids, *Int J Antimicrob Agents*, **2005**, 26, 343–356, https://doi.org/10.1016/j.ijantimicag.2005.09.002.
19. Girardi, L.G.J., Morsch, M., Cechinel-Filho, V., Meyre-Silva, C., Rodrigues, C.A., Isolation of flavonoids from Aleurites moluccana using chitosan modified with benzaldehyde (CH-Bz) as chromatographic support, *Die Pharmazie*, **2003**, 58, 9, available at: https://www.ingentaconnect.com/content/govi/pharmaz/2003/00000058/00000009/art00006# [Accessed October 9, 2019].
20. Banerjee, M., Mallick, S., Paul, A., Chattopadhyay, A., Ghosh, S.S., Heightened Reactive Oxygen Species Generation in the Antimicrobial Activity of a Three Component Iodinated Chitosan−Silver Nanoparticle Composite, *Langmuir*, **2010**, 26, 8, 5901–5908, https://doi.org/10.1021/la9038528.
21. Lim, S.-H., Hudson, S.M., Application of a fiber-reactive chitosan derivative to cotton fabric as an antimicrobial textile finish, *Carbohydr Polym*, **2004**, 56, 227–234, https://doi.org/10.1016/j.carbpol.2004.02.005.

22. Kong, M., Chen, X.G., Xing, K., Park, H.J., Antimicrobial properties of chitosan and mode of action: A state of the art review, *Int J Food Microbiol*, **2010**, 144, 51–63, https://doi.org/10.1016/j.ijfoodmicro.2010.09.012.

EUROPEAN RESEARCH AREA

COTANCE NEWSLETTERS

Starting with January 2019, the COTANCE Council will issue a monthly ***COTANCE Newsletter*** with the purpose of **promoting an improved image of leather** to relevant decision makers and domestic stakeholders including Members of the European and National Parliament, Governmental authorities, Ministerial officers, Customers of the leather industry, Brands, Retail chains, Relevant NGOs, Designers, etc. The monthly newsletters present topics that tell the truth about a controversial aspect or a fact that is not well known by the general public to bring about a better understanding of leather and the European leather industry, as well as a positive predisposition to legislate in favor of the leather industry. The newsletters are available in seven languages at https://www.euroleather.com/index.php/newsletter, and were also published in the 2019 issues as well as the first 2020 issues of *Leather and Footwear Journal*. Newsletter 8 is given below.

NEWS 8/2020

A reality check

That leather is an animal product can be a difficult issue for some consumers. There are **misconceptions about animals being reared solely to make leather**, that animals are abused or that leather production is driving the destruction of the rain forests. Such fears have seen companies like the Prada group ban kangaroo leather and Calvin Klein, Tommy Hilfiger and Mulberry drop exotic leathers from their collections. Agenda groups constantly push the narrative that, to save the lives of animals, we must stop buying leather.

The reality is very different. The vast majority of hides and skins are recovered from animals reared and slaughtered for meat, milk and wool, from which the hide or skin is a waste. The kangaroo skins banned by Prada are recovered from animals culled to protect endangered grasslands and wildlife. A very small number of animals, the so-called 'exotics', are reared for leather but even here, things are not black and white, as demand for this leather drives conservation of species, habitats and cultures, while creating jobs and income for local populations.

The reality is that stopping using leather would not prevent the slaughter of any of these animals. Meat consumption has continued to rise with two of the largest producers, Brazil and the USA, reporting their best and second best years, respectively, for meat exports in 2019. At the same time, due to falling demand for leather, **nearly 20% of the hides that they produced were simply thrown away**.

The kangaroo culls in Australia are not motivated by demand for leather and will continue even if there is no market for the skins. For some exotic species, where demand for their skins has added value to conserving them and their habitats, a halt in leather production could see them under threat as they switch from valuable product to undesirable pests. The crocodiles of the Northern Territories of Australia were almost entirely culled before value was added to their skins and habitats. Removing that value could see them endangered again and is why the IUCN spoke out against Chanel, when it removed reptile skin leather from its collections.

The reality is that avoiding leather would not prevent the slaughter of animals, but it would see manufacturers replacing it with other materials. These would inevitably be plastics, derived from fossil fuels, for short-lived products that, when thrown away, would linger and pollute the planet. While plant-based alternatives to leather have received massive publicity, the truth is that none of them are available in the quantities required to replace leather. Furthermore, nearly all are held together with plastic, making them a flawed solution at best. In contrast, leather is made from a renewable, sustainable raw material and will make long-lasting products which will degrade when disposed of.

The reality is that, at time when most people still eat meat, resulting in millions of tonnes of hides and skins, and when the terrible impact of alternative materials is becoming more apparent, **the only logical, ethical, sustainable choice is to choose leather**.

edited by

in collaboration with

leather UK

INSTRUCTIONS FOR AUTHORS

Publication Ethics and Malpractice Statement

Revista de Pielarie Incaltaminte publishes peer-reviewed articles. It is necessary to agree upon. The Publication Ethics and Malpractice Statement for *Revista de Pielarie Incaltaminte*, based on COPE's Best Practice Guidelines for Journal Editors, clearly outlines standards of expected ethical behavior for all parties involved in the act of publishing (the author, the journal editor(s), the peer reviewer and the publisher) and is available on the journal's website, http://www.revistapielarieincaltaminte.ro.

Open Access Statement

Revista de Pielarie Incaltaminte is a peer reviewed, open access journal. All articles published open access will be immediately and permanently free for everyone to read, download, copy and distribute, under the provisions of a Creative Commons Attribution (CC BY) which lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Open Access Publication Fee

Revista de Pielarie Incaltaminte requires article processing charges of 100 EURO per article, for accepted manuscripts, payable by the author to cover the costs associated with publication. There are no submission charges.

Author Rights

The copyright for all articles published in *Revista de Pielarie Incaltaminte* shall remain the property of the author(s). The copyright on the layout and final design of the articles published in *Revista de Pielarie Incaltaminte* belongs to INCDTP – Division: Leather and Footwear Research Institute and cannot be used in other publications.

Presentation of Papers

The scientific papers should be presented for publishing in English only. The text of the article should be clear and precise, as short as possible to make it understandable. As a rule, the paper should not exceed fifteen pages, including figures, drawings and tables. The paper should be divided into heads and chapters in a logical sequence. Manuscripts must meet high scientific and technical standards. All manuscripts must be typewritten using MS Office facilities, single spaced on white A4 standard paper (210 x 297 mm) in 11-point Times New Roman (TNR) font.

Paper Format

Title. Title (Centered, 12 pt. TNR font) should be short and informative. It should describe the contents fully but concisely without the use of abbreviations.

Authors. The complete, unabbreviated names should be given (Centered, 10 pt. TNR font), along with the affiliation (institution), city, country and email address (Centered, 9 pt. TNR font). The author to whom the correspondence should be addressed should be indicated, as well as email and full postal address.

Abstract: A short abstract in a single paragraph of no more than 200-250 words must accompany each manuscript (8 pt. TNR font). The abstract should briefly describe the content and results of the paper and should not contain any references.

Keywords. Authors should give 3-5 keywords.

Text

Introduction. Should include the aims of the study and results from previous notable studies.

Materials and Methods. Experimental methods should be described clearly and briefly.

Results and Discussions. This section may be separated into two parts. Unnecessary repetition should be avoided.

Conclusions. The general results of the research are discussed in this section.

Acknowledgements. Should be as short as possible.

References. Must be numbered in the paper, and listed in the order in which they appear.

Diagrams, Figures and Photographs should be constructed so as to be easy to understand and should be named "Figures"; their titles should be given below the Figure itself. The figures should be placed immediately near (after or before) the reference that is being made to them in the text. Figures should be referred to by numbers, and not by the expressions “below” or “above”. The number of figures should be kept to minimum (maximum 10 figures per paper).

Tables. Should be numbered consecutively throughout the paper. Their titles must be centered at the top of the tables (12 pt. TNR font). The tables text should be 9 pt. TNR font. Their dimensions should correspond to the format of the Journal page. Tables will hold only the horizontal lines defining the row heading and the final table line. The tables should be placed immediately near (after or before) the reference that is being made to them in the text. Tables should be referred to by numbers, and not by the expressions "below" or "above". The measure units (expressed in International Measuring Systems) must be explicitly presented.

Formulas, Equations and Chemical Reactions should be numbered by Arabic numbers in round brackets, in order of appearance, and should be centered. The literal part of formulas should be in Italics. Formulas should be referred to by Arabic numbers in round brackets.

Nomenclature. Should be adequate and consistent throughout the paper, should conform as much as possible to the rules for Chemistry nomenclature. It is preferable to use the name of the substances instead of the chemical formulas in the text.

References should be numbered consecutively throughout the paper in order of citation in square brackets; the references should list recent literature also. Footnotes are not allowed. If the cited literature is in other language than English, the English translation of the title should be provided, followed by the original language in round brackets. Example: Handbook of Chemical Engineer (in Romanian), vol. 2, Technical Press, Bucharest, 1951, 87.

We strongly recommend that authors cite references using DOIs where possible. DOIs are persistent links to an object/entity and can be used to cite and link to any article existing online, even if full citation information is not yet available. DOIs should always be displayed as full links. Example: Onem, E., Cin, G., Alankus, A., Pehlivan, H., Mutlu, M.M., Utilization of Chestnut Shell Wastes as a Dyeing Agent for Leather Industry, *Revista de Pielarie Incaltaminte* (*Leather and Footwear Journal*), **2016**, 16, 4, 257-264, https://doi.org/10.24264/lfj.16.4.1.

Citation of Journal Articles: all authors' names (surname, name initials), abbreviated journal title, year, volume number, issue number, full page reference, e.g.: Helissey, P., Giorgi–Renault, S., Renault, J., *Chem Pharm Bull*, **1989**, 37, 9, 2413-2425.

In case the reference is not cited in original, the author(s) should also list the original paper that has been consulted.

Citation of Books: authors' full name and name (initials), title of the book, issue number in Arabic numbers, publishing house, editors' names (if present), city where the book has been published, year of publication, the page(s) containing the text that has been cited.

Citation of Patents: all authors' names (surname, name initials), or company's name, country and patent number, date of issuance.

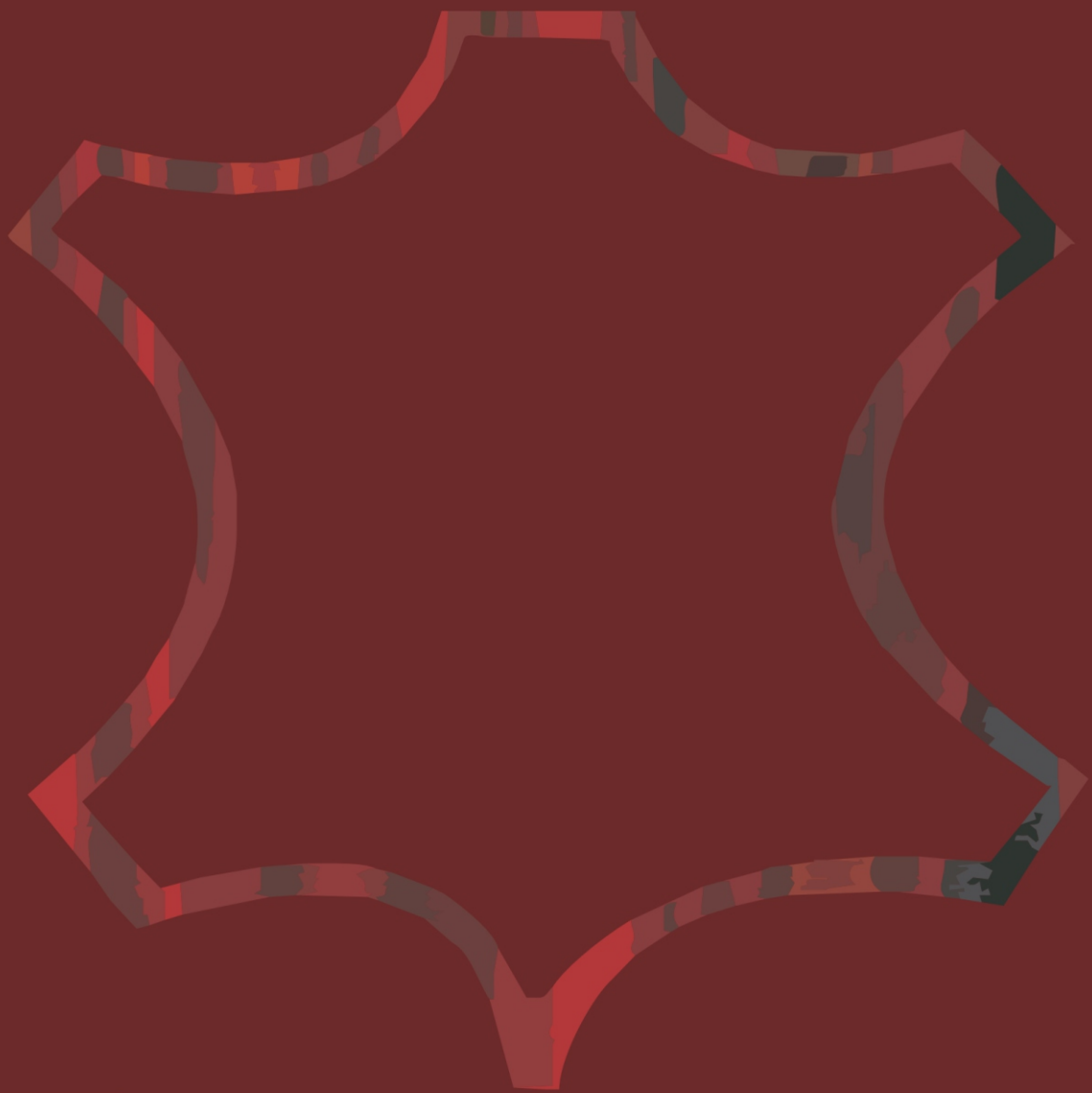
Paper template is available for download on the journal's website, http://www.revistapielarieincaltaminte.ro.

Manuscript Submission

Manuscripts should be submitted in electronic format by email to the following address:

Dana GURĂU, Editor-in-chief
INCDTP - Leather and Footwear Research Institute (ICPI)
93 Ion Minulescu St., code 030215, Bucharest, Romania
Phone: +4021-323.50.60; Fax: +4021-323.52.80.
E-mail: jlfjournal@gmail.com

INCDTP - SUCURSALA INSTITUTUL DE CERCETĂRI PIELĂRIE ÎNCĂLȚĂMINTE



ISSN 1583-4433

LUCRARE EDITATĂ CU SPRIJINUL MINISTERULUI EDUCAȚIEI ȘI CERCETĂRII