REVISTA

DE PIELĂRIE ÎNCĂLŢĂMINTE

Leather and Footwear Journal

December / Decembrie 2017
Volume / Volumul 17
No / Numărul 4

INCDTP - SUCURSALA INSTITUTUL DE CERCETĂRI PIELĂRIE ÎNCĂLȚĂMINTE
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, Research and Innovation of Romania, National Authority for Scientific 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, 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 100 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 – Dr. Viorica Deselnicu

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

REVISTA DE PIELĂRIE ÎNCĂLŢĂMINTE LEATHER AND FOOTWEAR JOURNAL

ISSN: 1583-4433
Volume 17, No. 4, December 2017
https://doi.org/10.24264/lfj.17.4

POLYCARBODIIMIDE AND POLYURETHANE CROSS-LINKERS FOR LEATHER FINISHING

Sholingur C. RAMKUMAR, Adhigan MURALI, Govindarajan PREETHI, Bangaru CHANDRASEKARAN, Palanivel SARAVANAN, Sellamuthu N. JAISANKAR*

CSIR-Central Leather Research Institute (CLRI), Adyar, Chennai-600 020, Tamil Nadu, India

Received: 24.03.2017 Accepted: 13.07.2017 https://doi.org/10.24264/lfj.17.4.1

POLYCARBODIIMIDE AND POLYURETHANE CROSS-LINKERS FOR LEATHER FINISHING

ABSTRACT. This review focuses on polyurethane and carboxylic acid-carbodiimide cross-linked systems for leather finishes. Recently, these cross-links have become very popular in leather finishing. The cross-links in leather finishes improve the strength properties of the finished leather, the excess of cross-links causes brittleness. The multifunctional carbodiimide cross-linkers are useful for leather surface properties improvement. The carbodiimide groups are sensitive to hydrolysis; life time of this functional group during polymerization is enhanced. Water dispersible isocyanates, polyurethane copolymers and polyisocyanate cross-links have applications in new technologies are discussed in this review.

KEY WORDS: cross-linker, polycarbodiimide, diisocyanate, leather, polyurethane

AGENŢI DE RETICULARE PE BAZĂ DE POLICARBODIIMIDĂ ŞI POLIEURETAN PENTRU FINISAREA PIELII

REZUMAT. Acest articol se concentrează asupra sistemelor de reticulare pe bază de poliuretan şi acid carboxilic-carbodiimidă pentru finisarea pielii. Recent, aceste tipuri de reticulări au devenit foarte populare în finisarea pieilor. Reticularea pielii la finisare îmbunătăţeşte proprietăţile de rezistenţă ale pieii finite, în timp ce excesul de legături încrucişate determină fragilitate. Agenţii de reticulare multifuncţionali carbodiimidici sunt utili pentru îmbunătăţirea proprietăţilor de suprafaţă ale pielii. Grupările carbodiimidice sunt sensibile la hidroliză; durata de viaţă a acestui grup funcţional în timpul polimerizării este sporită. Reticulările cu izocianaţii dispersabili în apă, copolimerii poliuretanici şi poliizocianaţi discutate în această lucrare au aplicaţii în tehnologii noi.

CUVINTE CHEIE: agent de reticulare, policarbodiimidă, diizocianat, piele, poliuretan

AGENTS DE RÉTICULATION POLYCARBODIIMIDE ET POLYURÉTHANE POUR LA FINITION DU CUIR

RÉSUMÉ. Cet article met l'accent sur les systèmes de réticulation à base de polyuréthane et de l'acide carboxylique-carbodiimide pour la finition du cuir. Récemment, ces types de réticulations sont devenus très populaires dans la finition du cuir. La réticulation du cuir dans l'opération de finition améliore les propriétés de résistance du cuir fini, tandis que la réticulation excessive entraîne une fragilité. Les agents de réticulation multifonctionnels de carbodiimide sont utiles pour améliorer les propriétés de surface du cuir. Les groupes carbodiimide sont sensibles à l'hydrolyse; la durée de vie de ce groupe fonctionnel pendant la polymérisation est augmentée. Les réticulations avec des isocyanates dispersables dans l'eau, des copolymères de polyuréthane et des polyisocyanates décrits dans cet article ont des applications dans de nouvelles technologies.

MOTS CLÉS: agent de réticulation, polycarbodiimide, diisocyanate, cuir, polyuréthane

** Correspondence to: Sellamuthu N. JAISANKAR, CSIR-Central Leather Research Institute (CLRI), Adyar, Chennai-600 020, Tamil Nadu, India, Tel: +91 44 24422059, Fax No: +91 44 24911589, e-mail: snjsankar@clri.res.in, snjaio@yahoo.com*

INTRODUCTION

In leather industry majority of the finishing systems are waterborne and are most often based on aqueous polyurethane dispersions (PUDs) or acrylic as principal binders. Often these two binders are used in combination also. The PUDs are best replacement of solvent based cross-linker and exhibit high flexibility and resistance up to -65°C. Polyurethanes (PU) are good cross-linking binders, which create a bond, that is resistant to oil, fat, heat and different organic solvents [1].

PU binders are formed by the reaction of isocyanates (R-N=C=O) with hydroxyl group [2]. The benefits of polyurethane binders are improved toughness and cohesive strength with low tack. The basic chemical products, in PU systems are diisocyanate, polyols (polyester or polyether), solvent (organic or aqueous), chain extender, viscosity modifier and catalyst (see Figure 1). Outstanding properties of PU are extensibility, elasticity, abrasion resistance combined with mirror like gloss and clarity. The PU dispersions are miscible with almost all chemicals, have good solvent resistance, excellent low temperature properties and good weather resistance. However PU dispersions are quite expensive compared to acrylics and have poor product stability (shelf life).

For many high performance applications

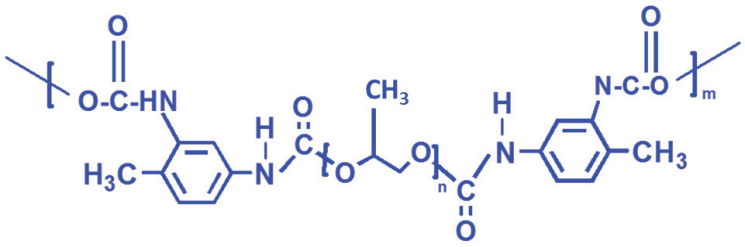


Figure 1. Polyurethane structure [3]

such as upholstery leather finishing, dress lining, cross-linking is essential and more commonly encountered cross-linkers are medium oligomeric molecular weight water dispersible isocyanates prepolymers. They are available in solvent medium at 50-60 weight % solids or as solvent free viscous liquids. Cross-linking is employed to improve performance related to water resistance, solvent (straining) of toughness to improve mechanical film properties like hardness, toughness, abrasion resistance and rose flux. In leather industry the most prevalent cross linking involves the use of dispersed oligometric isocyanates. The polymeric binders in such aqueous coatings are either polyurethane dispersion in water or acrylic latexes or two combinations [4]. The carbodiimide could act as excellent cross-linkers and provide better platform to the leather, plastic and footwear industries. This review will be useful to design new water based cross-linking applications.

CHEMISTRY OF CROSS-LINKING

Cross-linking is the process of chemically joining two or more molecules by a covalent bond and formation of three dimensional networks. The cross-linking agents are molecules that contain two or more reaction ends capable of chemically attaching to specific functional groups like primary amine on protein or other trifunctional amines or alcohols. When combined with different sizes and types of chemical backbones (called spacer arms because they define the distance between respective reaction ends) the numbers of cross-linking compounds are enormous.

Selection of Cross-Linkers

Cross-linkers are selected on the basis of their chemical reactivity (i.e. specially for particular functional groups) and other chemical properties, that affect their behaviour in different applications (see Table 1). Chemical specificity refers to the reactive targets of the cross-linkers reactive ends. A general consideration is whether the reagent has the same or different reactive groups carbodiimide (R-N=C=N-R), isocyanates (-NCO) aziridine (-CH2-NH-CH2) at either end, mono or bifunctional cross-linkers have identical reaction groups, they must be used in one step reaction procedures to randomly “fix” or polymerise the molecules.

Table 1: Materials and physical properties

Materials		
	Isocynates	Carbodiimides
1)	Oligomeric	Oligomeric
2)	Aliphatic	Aliphatic
3)	Functionality (f=>2, averages<4)	Functionality (f=>2, averages<4)
4)	Avail in PMAc,* EEP#	Avail in PMAc @50%
5)	Solids: 50 up to 100%	Or 40 % aq. (zero VOC)
6)	Use: hotpotted or in-line	Use: hotpotted or in-line
7)	Geometry: branched	Geometry: linear [2].

*PMAc: Propylene glycol methyl ether acetate, #EEP: Ethyl 3-Ethoxypropionate

These reagents allow single conjunction of molecules that have the respective target functional groups and also allow for sequential conjugations that minimise undesirable polymerisation.

TYPES OF CROSS-LINKERS

Isocyanates

The cross-linkers based on diisocyanate chemistry have high end applications in leather finishing particularly for automotive applications. This is due to an excellent property profile with regard to processability and quality of the resulting leather. The finish does not suffer from thermally or UV induced yellowing and other severe ageing systems. The key advantage of isocyanate is its flexibility with respect to the property profile (Figure 2). This is due to the broad variety of isocyanates and polyols (see Figure 3), which are available for combination

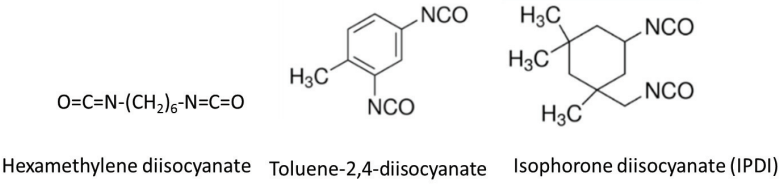


Figure 2. Different isocyanates for PU synthesis

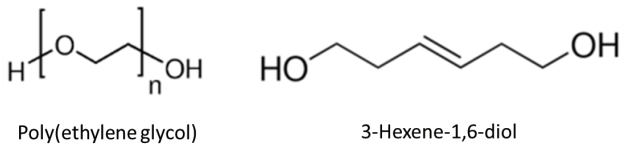


Figure 3. Different types of polyols used for polyurethane synthesis

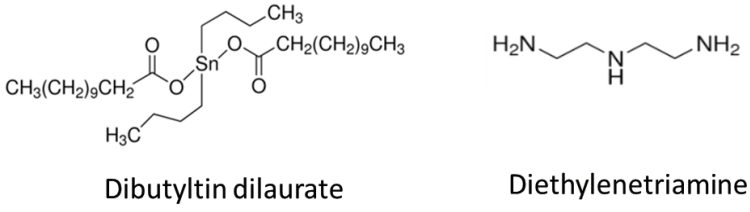


Figure 4. Different types of catalysts used for polyurethane synthesis

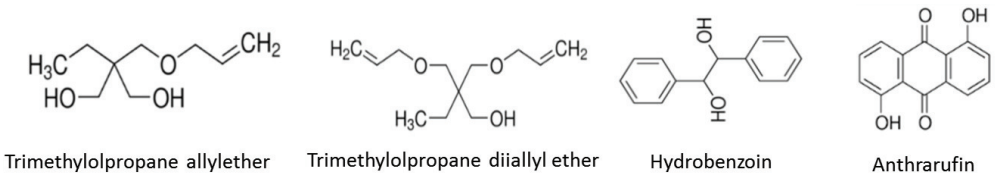


Figure 5. Different types of cross-linkers

to form polyurethanes in polyaddition reaction [5]. Different types of catalysts and cross-linkers are used for polyurethane synthesis as shown in Figures 4 and 5.

Polycarbodiimides

In leather finishing most of the prevalent cross-linking system involves the use of water dispersible oligomeric polyisocyanates. In many industrial coating applications, aqueous carboxyl acrylics act as main binders and were cured with cross-linkers at high temperature. However, it had concerns about toxicity, handling and even yellowing on ageing. Hence better way to cure carboxyl polymers in water with carbodiimide and their properties. The carbodiimide has excellent chemical resistances and very low VOC compared with other isocyanates. It can be easily reacted with –COOH functional groups to provide better hardness. Unfortunately, carbodiimide will be hydrolyzed at pH <11, since it will prevent –COOH from forming. Moreover, polycarbodiimide could be a suitable alternative to isocyanates for preparation of PU [6].

A dispersion containing particles of one type with carbodiimide (-N=C=N-), the other containing carboxylic acid groups (–COOH), and cross-linking occurs to form or N-acylurea in reaction-diffusion process (Figure 6), to low volatile emission during processing for the environmental protection and work hygiene [7, 8]. A hybrid of polyisocyanate (a dynamic helical polymer) and isocyanate prepolymers (a static helical polymer) is the backbone of polycarbodiimide [9].

Carbodiimides are much more stable in water than isocyanates, water borne polycarbodiimides have useful pot life of at least several days to a week or more. Polycarbodiimides can be viewed as a hybrid between the static polyisocyanate and the dynamic polyisocyanate helical polymers [10].

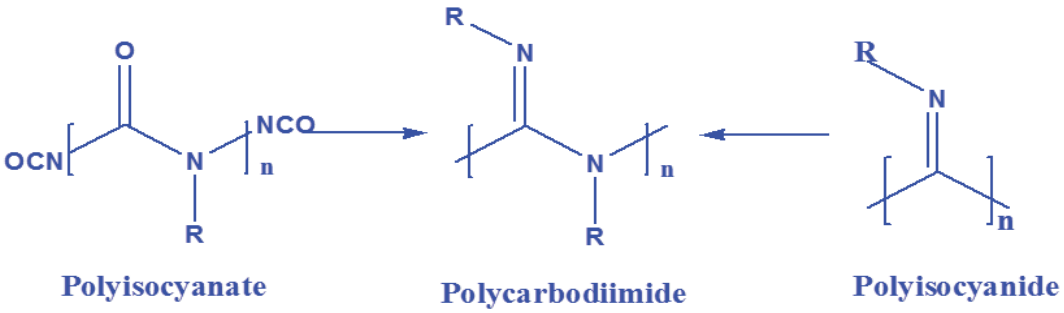
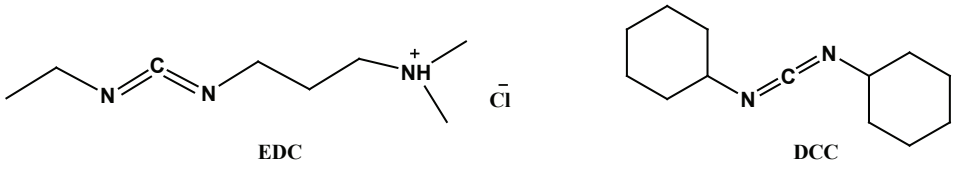


Figure 6. Structure of various cross-linkers

High performance multifunctional polycarbodiimides offer maximum resistance in wet abrasion, hydrolysis, sweat, chemical resistance and UV durability. The advantage of aqueous polycarbodiimide can be used in line, top coats (7-10 weight %) to achieve better performances. Polycarbodiimide and polyisocyanate cross-linkers can be used together to achieve high reactivity of the polycarbodiimide and carboxylic acid promotes an early and fast curing. The better performance originates from the formation of a continuous network, which may be formed by reaction of cross-linkers with the binder. For the latter route reactive groups on the polymer chain are needed [9]. One of the most profile properties of polycarbodiimides is their asymmetric back bone which allows the formation of excess helical sense and/

or chiral polycarbodiimides. EDC (l-ethyl-3-(3-dimethylaminopropyl)carbodiimide) hydrochloride cross-linking reactions must be performed in conditions devoid of extraneous carboxyl and amines (see Figure 7). Acidic (pH of 4.5 to 5.8) MES (2-[morpholino]ethanesulfonic acid) buffer is most effective. Polycarbodiimide can be effective, environmentally friendly, long pot-life cross-linking agents. MES buffer solution is most suitable for carbodiimide reaction system. MES (other non-amine or non-carboxylate buffer) can be used at neutral pH to increase their efficiency.



EDC **DCC**

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide . HCl **Dicyclohexylcarbodiimide**

MW 191.70
Spacer Arm 0.0 A^0

MW 206.33
Spacer Arm 0.0 A^0

Figure 7. Carboxyl reactive cross-linker

Though carbodiimides react with carboxylic acids, it is not the only path freely available to carbodiimide for most ambient curing aqueous coating chemistry. High performance multifunctional water dispersible polycarbodiimides have also come out in the systems.

CROSS-LINKING SYSTEM IN CARBODIIMIDES

Carboxyl reactive chemicals in biomolecular probes for labelling and cross-linking carboxylic acids to primary amines include carbodiimides compounds EDC and N-N'-dicyclohexyl carbodiimide (DCC) (see Table 1). Very few types of groups are known to provide specific conjugation to carboxylic acids (-COOH) such as in proteins and various other biomolecules. Carbodiimides work through activating carboxyl groups for direct reaction along with primary amines via amide bond formation [7].

Table 2: Properties of EDC [11]

Molecular formula	$C_8H_{17}N_3$. HCl
Molecular weight	191.7 kDA
Storage conditions	-20 ˚C, protect from moisture, use only fresh solutions
Reactive group	Carbodiimide
Column of property	Carboxyl-reactive at pH 4.7-6.0, intermediate then reacts with amines

EDC Reaction Chemistry

EDC is most common used carbodiimide and it has water soluble materials. The toxicity of carbodiimide can be low, when EDC transformed into non-toxic urea derivatives in the coupling reactions. EDC reacts with carboxylic acid groups to form an active o-acylic urea intermediate that is easily displaced by nucleophilic attack to form primary amino groups, in the reaction process (see Figure 8). The primary amine forms an amide bond with the carboxylic group and an EDC by product released as a soluble urea derivative. The o-cyclic urea intermediate is unstable in aqueous solutions, failure to react with an amine results in hydrolysis of the intermediary, regeneration of the carboxylic group and the release of an unsubstituted urea.

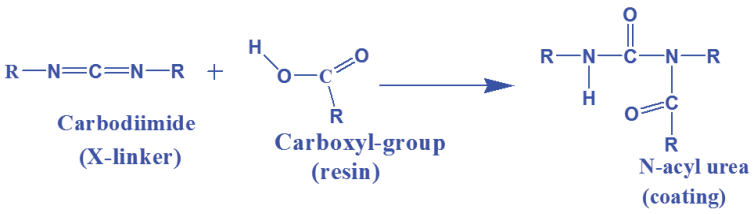


Figure 8. Carbodiimide cross linking mechanism [12]

EDC cross-linking is most effective in acidic (pH 4.5) conditions and must be performed in buffers, devoid of extraneous carboxyl and amines. MES is a suitable buffer, phosphate buffers and neutral (up to pH 7.5) conditions are compatible. Polycarbodiimides are very effective cross-linking agents for carboxylic group containing polymers in water such as acrylic acid containing polymers, polyacrylates latexes (see Figures 7, 9).

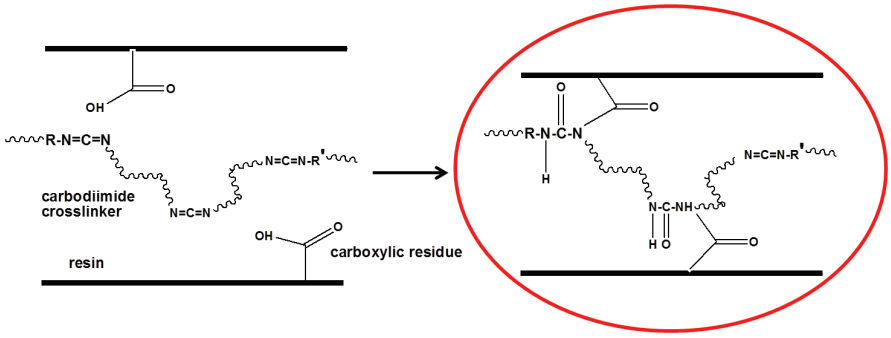


Figure 9. Generic carbodiimide cross-linking reaction [12]

Polymers with carbodiimide groups form additional interpenetrating networks (IPN). The network formation has the potential to reduce elongation and to increase tensile strength and hardness. The water dispersibility of carbodiimide is achieved by incorporating some hydrophilic components into the cross-linkers [13]. This was done by adding an external surfactant or by having an internal surfactant (polyethylene oxides PEO or salts) built into carbodiimide itself [14]. In most leather finishes there is no significant amount of hydroxyl or other reactive functionality accomplished of reacting with isocyanates (see Figure 10). The polyisocyanates and polycarbodiimide are used in leather finishing based on aliphatic back bones. Both types of the cross-linkers have an average functionality per cross-linker molecule of greater than 2.

These cross-linkers can be mixed into aqueous finishing before being applied. The cross linking will generally reduce ultimate % of elongation at break [6].

APPLICATIONS OF CARBODIIMIDES

Carbodiimide mediated inter or intra molecular cross-link of protein is highly useful [15-21]. The availability of functional groups, such as carboxyl, amino, thiol and imidazols in protein and enzymes allow interaction with carbodiimides [22-25]. The reaction of carbodiimide alone with a protein can lead to deactivation, but since o-acylureas are liable to hydrolysis reaction occurs in presence of water. Intramolecular cross-linking is often used to study the folding of proteins. Film forming carbodiimide homo and copolymers are used in amino encapsulation techniques for pressure sensitive adhesives. In dying, treatment of wool or hair with carbodiimide improves the wash fastness of applied dyes. A variety of carbodiimides are commonly used (see Figure 7). However EDAC or EDC as it is known is particularly useful in aqueous reactions [7].

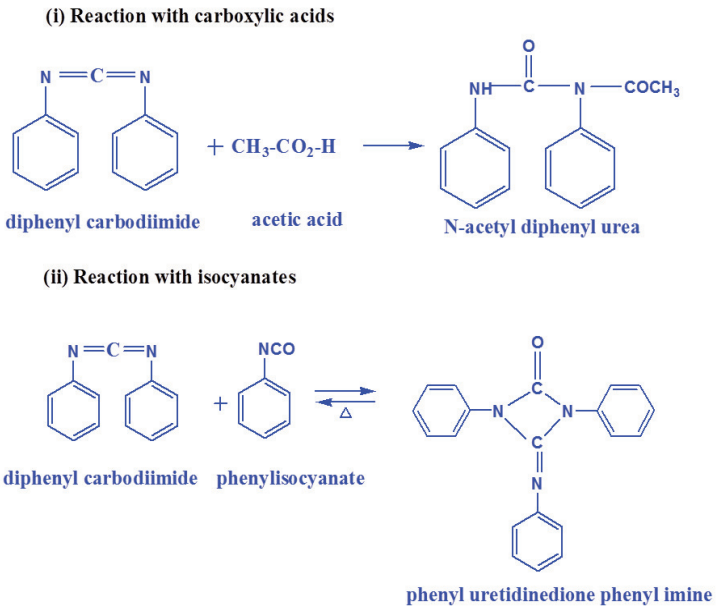


Figure 10. Carbodiimides and isocyanates reactions [6]

Pot-life

The carbodiimide cross-linkers [26-33] exhibit good pot-life in aqueous systems. Multifunctional water dispersible polycarbodiimides display a higher performance, when used as a cross-linker. Water dispersibility of carbodiimide is obtained with some hydrophilic components in the cross-linker, by the addition of an external surfactant or internal surfactant. The cross-linking capability in leather finish can be tested by Veslic tests [8, 34] differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA). In DMA the tan δ peak value can be identified as a glass transition temperature, can be useful for identifying the processing of the cross-linking applications [8]. The carbodiimide cross-linking is calculated based on the amount of carbodiimide required for polymeric acid. The less stoichiometric carbodiimide is used to react with all of the carboxylic acid present. There is a possibility that in some instances rapid loss of carbodiimide functionality in excess may be due to self-condensation, similar in the case of polyisocyanates [35].

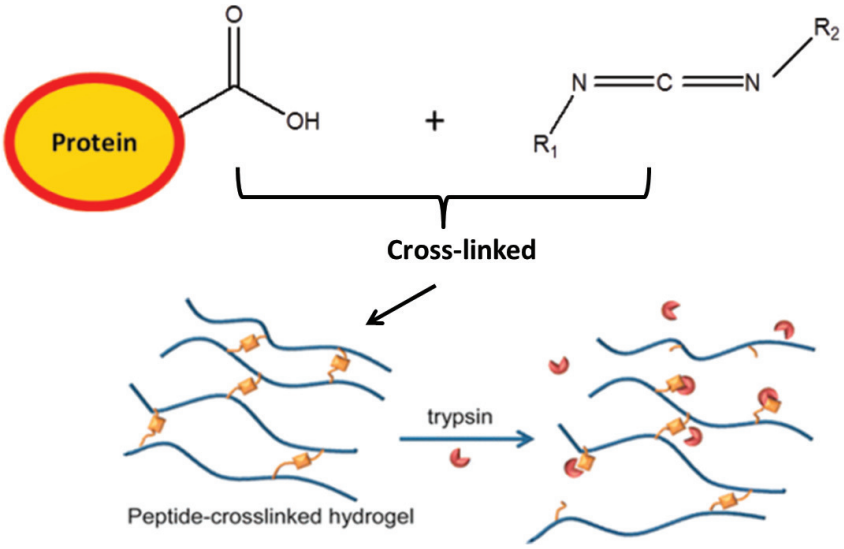


Figure 11. EDC cross-linker with protein. Reprinted with permission from [36], Knipe *et al.*, *Biomacromolecules*; 16, 962 (2015), © 2015, American Chemical Society

Polycarbodiimides have practical application advantage in base coat level of 2-4% as it improves stacking due to low tack. On top coat to achieve desired wear properties, carbodiimides provide an "economical way to achieve performance inputs" [34]. Carbodiimide compounds provide the most popular and versatile method for labelling or cross-linking to carboxylic acids. Xiaoyan and Zhiwen [37] have synthesized a new cross-linker for microcapsule by interfacial polymerization using hydrophobic carbodiimide cross-linker (Figure 11) for high tensile strength [35]. The reaction between –COOH and –NCN- groups forms an N-acylurea which is less polar as shown in Figure 12. Both carboxylated latex and carbodiimide containing latex films rate of diffusion was much slower than the cross-linked latex [38].

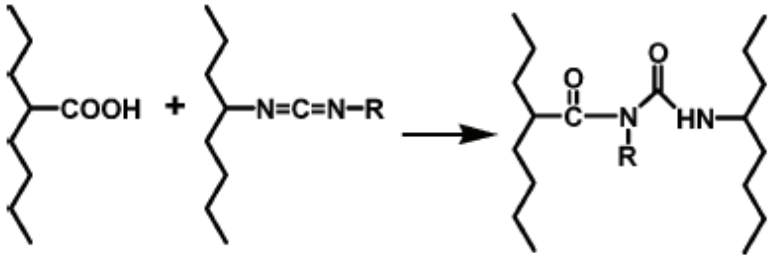


Figure 12. Chart for –COOH, -NCN- contains latex films. Reprinted with permission from [38], Pham, *et al*., *Macromolecules*, 39, 4, 1425 (2006). © 2006, American Chemical Society.

Benefits Offered by Carbodiimides

i. Low VOC, zero alcohol non-polluting properties, fast drying in line.
ii. Low odour, non-flammability, no toxicity.
iii. Clarity of water when dry.
iv. Significant rub, mark and block resistance.
v. Grease, alcohol, alkali and improved moisture resistance.
vi. Use of carbodiimides can impart greater strength and flexibility, heat resistance, increased hardness and rub resistance, upgrade adhesion.
vii. Shift in application technology "hot pot" mixing to "in line" activation which implies, automatically controlled, ideally continuous mixing of the coating components.

CROSS-LINKED POLYURETHANE FOR LEATHER PROCESSING

To improve the leather properties [39-44], photoactive agent such as benzophenone and rose bengal were mixed into polyurethane matrix [45-47]. These mixed polyurethane solutions were applied on leather surface by painting method for effective anti-microbial leather coating [48]. Moreover, polyurethanes are interesting block copolymers, mostly it exists in hard and soft segments. Both these segmentation is responsible for the better mechanical properties. However, the hard segment built from diisocyanate, short chain diol such as butane diol, and ethylene diols. The soft segments are developed from polypropylene glycol (PPG), polycarbonate and polytetrahydrofuran [15].

Xu *et al*. stated that water borne polyurethane cross linked acrylate composite was synthesized via *in-situ* method using different cross-link agents. The obtained composite materials could have been used in coatings, leather finishing, adhesives, sealants and also plastic coatings [16, 17]. Keywani [18] reported that acrylic resin based water borne polyurethanes [19] could enhance water resistance, chemical resistance also used in coating, print, ink and leather adhesive [49-51]. We developed polyurethane emulsion, dispersion for leather applications [2, 5].

CONCLUSIONS

This review describes that carbodiimide cross-linking and polyurethane latexes dispersions with improved properties of cross-linked films. The aqueous cross-linkers are environmentally friendly because they reduce VOC emission, are easy to handle and have

longer pot-life. The carbodiimides are effective cross-linking agents providing better chemical resistance, higher hardness and more density to coating. The carbodiimide is an alternative to other cross-linking agents with right combination of resin and cross-linkers can be used. The high reactivity with carboxylic acid promotes an early cure enhancing property development. The dosage of carbodiimide is important and can contribute to additional property development, like mechanical film performances and they can build properties upwards from base coat.

Acknowledgements

The authors acknowledge to ZERIS, CSC 0103 WP-13&21 for funding.

REFERENCES

1. Lowe, A.B., McCormick, C.L., Synthesis and Solution Properties of Zwitterionic Polymers, *Chem Rev*, **2002**, 102, 4177-4190.
2. Jaisankar, S.N., Lakshminarayana, Y., Radhakrishnan, G., Ramasami, T., Modified Castor Oil Based Polyurethane Elastomers for One-Shot Process, *Polym Plast Technol Eng*, **1996**, 35, 781-789.
3. Murali, A., Gurusamy-Thangavelu, S.A., Jaisankar, S.N., Mandal, A.B., Enhancement of Physicochemical Properties of Polyurethane-Perovskite Nanocomposite via Addition of Nickel Titanate Nanoparticles, *RSC Adv*, **2015**, 5, 102488-102494, https://doi.org/10.1039/C5RA17922J.
4. Wang, C., Chu, F., Graillat, C., Guyot, A., Gauthier, C., Chapel, J.P., Hybrid Polymer Latexes: Acrylics–Polyurethane from Miniemulsion Polymerization: Properties of Hybrid Latexes versus Blends, *Polymer*, **2005**, 46, 1113-1124.
5. Mohamed, M., Vuppalapati, R.R., Bheemreddy, V., Chandrashekhara. K., Schuman. T., Characterization of Polyurethane Composites Manufactured Using Vacuum Assisted Resin Transfer Molding, *Adv Comp Mater*, **2015**, 24, 13-31, https://doi.org/10.1080/09243046.2014.909975
6. Neumann, W., Fisher, P., The Preparation of Carbodiimides from Isocyantes, *Angew Chem*, **1962**, 12, 621-625.
7. Stloukal, P., Jandíková, G., Koutny, M., Sedlarik, V., Carbodiimide Additive to Control Hydrolytic Stability and Biodegradability of PLA, *Polym Test*, **2016**, 54, 19-28.
8. Hesselmans, L.C.J., Derksen, A.J., Van Den Goorbergh J.A.M., Polycarbodiimide Crosslinkers, *Prog Org Coat*, **2006**, 55, 142-148.
9. Sakurai, S.I., Ohsawa, S., Nagai, K., Okoshi, K., Kumaki, J., Yashima, E., Two-Dimensional Helix-Bundle Formation of a Dynamic Helical Poly (phenylacetylene) with Achiral Pendant Groups on Graphite, *Angew Chem*, **2007**, 119, 7749-7752.
10. Kennemur, J.G., Novak, B.M., Advances in Polycarbodiimide Chemistry, *Polymer*, **2011**, 52, 1693-1710.
11. Kołodziejska, I., Piotrowska, B., The Water Vapour Permeability, Mechanical Properties and Solubility of Fish Gelatin-Chitosan Films Modified with Transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl) Carbodiimide (EDC) and Plasticized with glycerol, *Food Chem*, **2007**, 103, 295-300, https://doi.org/10.1016/j.foodchem.2006.07.049.
12. Posthumus, W., Derksen, A.J., Van den Goorbergh J.A.M., Hesselmans, L.C.J., Crosslinking by Polycarbodiimides, *Prog Org Coat*, **2007**, 58, 231-236.
13. Damink, L.O., Dijkstra, P.J., Van Luyn, M.J.A., Van Wachem, P.B., Nieuwenhuis, P., Feijen, J., Cross-Linking of Dermal Sheep Collagen Using a Water-Soluble Carbodiimide, *Biomaterials*, **1972**, 17, 765-773.
14. Sundar, S., Vijayalakshmi, N., Gupta, S., Rajaram, R., Ganga, R., Aqueous Dispersions of Polyurethane–Polyvinyl Pyridine Cationomers and Their Application as Binder in Base Coat for Leather Finishing, *Prog Org Coat*, **2006**, 56, 178-184, https://doi.org/10.1016/j.porgcoat.2006.04.001

15. Fillingame, R.H., Purification of the Carbodiimide-Reactive Protein Component of the ATP Energy-Transducing System of *Escherichia coli*, *J Biol Chem*, **1976**, 251, 6630-6637.
16. Staros, J.V., Wright, R.W., Swingle, D.M., Enhancement by N-hydroxysulfosuccinimide of Water-Soluble Carbodiimide-Mediated Coupling Reactions, *Anal Biochem*, **1986,** 156, 220-222.
17. Bogdanov, A.A., Klibanov, A.L., Torchilin, V.P., Protein Immobilization on the Surface of Liposomes via Carbodiimide Activation in the Presence of N-hydroxysulfosuccinimide, *FEBS Lett*, **1988**, 231, 381-384, https://doi.org/10.1016/0014-5793(88)80854-8.
18. Mehta, R.V., Upadhyay, R.V., Charles, S.W., Ramchan, C.N., Direct Binding of Protein to Magnetic Particles, *Biotechnol Tech*, **1997**, 11, 493-496.
19. Odom W.R., Bricker, T.M., Interaction of CPa-1 with the Manganese-Stabilizing Protein of Photosystem II: Identification of Domains Cross-Linked by 1-ethyl-3-[3-dimethylamino) propyl] Carbodiimide, *Biochem,* **1992**, 31, 5616-5620.
20. Timkovich, R., Polymerization Side Reactions during Protein Modifications with Carbodiimide, *Biochem Biophys Res Commun*, **1977**, 74, 1463-1468.
21. Buechler, J.A., Taylor, S.S., Differential Labeling of the Catalytic Subunit of cAMP-dependent Protein Kinase with a Water-Soluble Carbodiimide. Identification of Carboxyl Groups Protected by Magnesium ATP and Inhibitor Peptides, *Biochem*, **1990**, 29, 1937-1943.
22. Scouten, W.H., Luong, J.H.T., Brown, S.R., Enzyme or Protein Immobilization Techniques for Applications in Biosensor Design, *Trends Biotechnol*, 13, **1995**, 178-185.
23. Yamada, H., Ryota, K., Miyuki, H., Taiji, I., Intramolecular Crosslinkage of Lysozyme. Imidazole Catalysis of the Formation of the Crosslink between Lysine-13 (. epsilon.-amino) and Leucine-129 (Alpha-Carboxyl) by Carbodiimide Reaction, *Biochem*, **1983**, 4551-4556.
24. Elia, G., Biotinylation Reagents for the Study of Cell Surface Proteins, *Proteomics*, **2008**, 8, 4012-4024.
25. Berthelot, T., Alexandre, G., Le, X.T., El Morsli, J., Jégou, P., Palacin, S., Viel, P., "Versatile Toolset" for DNA or Protein Immobilization: Toward a Single-Step Chemistry, *Appl Surface Sci*, **2011**, 257, 3538-3546, https://doi.org/10.1016/j.apsusc.2010.11.071.
26. Sung, H.W., Chang, W.H., Ma, C.Y., Lee, M.H., Crosslinking of Biological Tissues Using Genipin and/or Carbodiimide, *J Biomed Mater Res Part A*, **2003**, 64, 427-438, https://doi.org/10.1002/jbm.a.10346
27. Lee, J.M., Edwards, H.H.L., Pereira, C.A., Samii, S.I., Crosslinking of Tissue-Derived Biomaterials in 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *J Mater Sci Mater Med,* **1996**, 7, 531-541.
28. Tomihata, K., Yoshito I., Crosslinking of Hyaluronic Acid with Water-Soluble Carbodiimide, *J Biomed Mater Res Part A,* **1997**, 37, 243-251.
29. Ahn, J.I., Kuffova, L., Merrett, K., Mitra, D., Forrester, J.V., Li, F., Griffith, M., Crosslinked Collagen Hydrogels as Corneal Implants: Effects of Sterically Bulky vs. Non-Bulky Carbodiimides as Crosslinkers, *Acta Biomater*, **2013**, 9, 7796-7805, https://doi.org/10.1016/j.actbio.2013.04.014.
30. Billiar, K.L., Murray, J., Laude, D., Abraham, G., Bachrach, N., Effects of Carbodiimide Crosslinking Conditions on the Physical Properties of Laminated Intestinal Submucosa, *J Biomed Mater Res Part A*, **2001**, 56, 101-108.
31. Coogan, R.G., Post-Crosslinking of Water-Borne Urethanes, *Prog Org Coat*, **1997**, 32, 51-63.

32. Sannino, A., Pappada, S., Madaghiele, M., Maffezzoli, A., Ambrosio, L., Nicolais, L., Crosslinking of Cellulose Derivatives and Hyaluronic Acid with Water-Soluble Carbodiimide, *Polymer,* **2005**, 46, 11206-11212.
33. Osborne, C.S., Barbenel, J.C., Smith, D., Savakis, M., Grant, M.H., Investigation into the Tensile Properties of Collagen/ Chondroitin-6-sulphate Gels: The Effect of Crosslinking Agents and Diamines, *Med Biol Eng Comp*, **1998**, 36, 129-134.
34. Mikoz, X., lajczyk, M., Kiez, X.L., Recent Developments in the Carbodiimide Chemistry, *Tetrahedron*, **1981**, 37, 233-284.
35. Choinopoulos, I., Koinis, S., Pitsikalis, M., Synthesis and Characterization of Chiral Poly (l-lactide-b-hexyl isocyanate) Macromonomers with Norbornenyl End Groups and Their Homopolymerization through Ring Opening Metathesis Polymerization to Afford Polymer Brushes, *J Polym Sci A Polym Chem*, **2017**, 55, 1102-1112, https://doi.org/10.1002/pola.28471.
36. Knipe, J.M., Chen, F., Peppas, N.A., Enzymatic Biodegradation of Hydrogels for Protein Delivery Targeted to the Small Intestine, *Biomacromolecules*, **2015**, 16, 962-972, https://doi.org/10.1021/bm501871a.
37. Xiaoyan, P., Zhiwen, D., Synthesis and Application Characteristics of Carbodiimide Crosslinking Agent, *China Leather*, **2007**, 23, 011.
38. Dafei, Z., Wie, X., Junjie, B., Xu, G., Preparation and Characterization of Anti-Microbial Waterborne Polyurethane, *Paint Coat Ind*, **2007**, 9, 014.
39. Bajza, Z., Ivana, V.V., Fatliquoring Agent and Drying Temperature Effects on Leather Properties, *J Mater Sci*, **2001**, 36, 5265-5270.
40. Huang, X., Hsieh, F.H., Physical Properties, Sensory Attributes, and Consumer Preference of Pear Fruit Leather, *J Food Sci*, **2005**, 70, 10.1111/j.1365-2621.2005.tb07133.x.
41. Liu, C-K., Latona, N.P., Cooke, P., Effects of Stretching and Drying Rate on the Mechanical Properties of Chrome-Free Leather, *J Am Leather Chem As*, **2006**, 101, 330-335.
42. Kaya, S., Kahyaoglu, T., Thermodynamic Properties and Sorption Equilibrium of Pestil (Grape Leather), *J Food Eng*, **2005**, 71, 200-207, https://doi.org/10.1016/j.jfoodeng.2004.10.034.
43. Liu, C.K., Latona, N., Ashby, R., Ding, K., Environmental Effects on Chrome-Free Leather, *J Am Leather Chem As*, **2006**, 101, 10, 368-375.
44. Kanagaraj, J., Velappan, K.C., Chandra Babu, N.K., Sadulla, S., Solid Wastes Generation in the Leather Industry and its Utilization for Cleaner Environment-A Review, *J Sci Ind Res (India)*, **2006**, 65, 541-548.
45. Hong, K.H, Gang, S., Photoactive Antimicrobial Agents/Polyurethane Finished Leather, *J Appl Polym Sci,* **2010**, 115, 1138-1144, https://doi.org/10.1002/app.31221.
46. Lacombe, S., Thierry P., New Materials for Sensitized Photo-Oxygenation, *Photochem*, **2010**, 38, 307-329, https://doi.org/10.1039/9781849730860-00307.
47. Bunning, T.J., Lalgudi, N.V., Vincent, T.P., Sutherland, R.L., Holographic Polymer-Dispersed Liquid Crystals (H-PDLCs), *Annu Rev Mater Sci*, **2000**, 30, 83-115.
48. Overbeek, A., Polymer Heterogeneity in Waterborne Coatings, *J Coat Tech Res*, **2010**, 7, 1-21.
49. Shono, M., Kazuhiko, E., Masa-Aki, K., Yokhiaki, I., Shigeo, K., Akitada, N., Allergic Contact Dermatitis from Para-Tertiary-Butylphenol-Formaldehyde Resin (PTBP-FR) in Athletic Tape and Leather Adhesive, *Contact Dermatitis*, **1991**, 24, 281-288.
50. Vélez-Pagés, T., Martín-Martínez, J.M., Application of One-Component Primer to Avoid the Roughening of Leather and Increase its Adhesion to Polyurethane Adhesive, *Int J*

Adhes Adhes, **2005**, 25, 320-328, https://doi.org/10.1016%2Fj.ijadhadh.2004.11.001.

51. Kronthal, L., Levinson, J., Dignard, C., Chao, E., Down, J., Beva 371 and its Use as an Adhesive for Skin and Leather Repairs: Background and a Review of Treatments, *J Am Inst Conserv*, **2003**, 42, 341-362, https://doi.org/10.1179/019713603806112796.

DIFFERENCES IN PLANTAR PRESSURE BETWEEN THE DIABETIC AND HEALTHY SUBJECTS

Rongtian LIU[1], Mingyu HU[1], Yiming GU[2], Hong LIN[2], Bo XU[1,3], Jiuping XU[3], Jin ZHOU[1,4*]

[1]National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, P.R.China, e-mail: zj_scu@qq.com

[2]Guangdong Huizhou Quality & Measuring Supervision Testing Institute, Huizhou 516003, China

[3]School of Business, Sichuan University, Chengdu 610065, P.R. China

[4]Chengdu Anland Co., LTD, Chengdu 610065, P.R. China

Received: 19.03.2017 Accepted: 12.06.2017 https://doi.org/10.24264/lfj.17.4.2

DIFFERENCES IN PLANTAR PRESSURE BETWEEN THE DIABETIC AND HEALTHY SUBJECTS

ABSTRACT. Since varied thresholds were reported in the literature and in what range of peak pressure is safe was not answered, the aim of this study was to compare the plantar pressure between the diabetic and healthy subjects and then to find the risk threshold of pressure for diabetic patients. 177 volunteers (83 diabetic patients and 94 control ones) were recruited and their plantar pressure were measured by the Emed pressure system. Plantar region was divided into seven regions: big toe, MTH1-5 and MF, under which mean pressure (kPa) was calculated. The results show that major variations existed and mean pressure of the patients was 2.5% lower than that of healthy counterparts; however, the law of differences between the diabetic patients and healthy ones in 3%, 50% and 96% percentile level could not be found. Overall, attention should be paid to changes in feet of diabetic patients, who must receive appropriate treatment when their mean pressure exceeds 100kPa (about 10N/cm^2).

KEY WORDS: diabetic feet, foot ulcers, mean pressure, diabetic peripheral neuropathy

DIFERENŢE DE PRESIUNE PLANTARĂ ÎNTRE SUBIECŢII DIABETICI ŞI CEI SĂNĂTOŞI

REZUMAT. Întrucât s-au raportat praguri variate în literatură şi nu există un consens pentru intervalul de siguranţă al presiunii maxime, scopul acestui studiu a fost de a compara presiunea plantară între subiecţii diabetici şi cei sănătoşi şi apoi de a stabili pragul de risc al presiunii pentru pacienţii diabetici. Au fost recrutaţi 177 de voluntari (83 de pacienţi cu diabet zaharat şi 94 de pacienţi în grupul martor), iar presiunea lor plantară a fost măsurată utilizând sistemul de presiune Emed. Regiunea plantară a fost împărţită în şapte regiuni: degetul mare, MTH1-5 şi MF, în care s-a calculat presiunea medie (kPa). Rezultatele arată că au existat variaţii majore, iar presiunea medie a pacienţilor diabetici a fost cu 2,5% mai mică decât cea a subiecţilor sănătoşi; cu toate acestea, nu am putut stabili o lege a diferenţelor dintre subiecţii diabetici şi cei sănătoşi la niveluri de 3%, 50% şi 96%. În general, trebuie să se acorde atenţie modificărilor la nivelul picioarelor pacienţilor cu diabet zaharat, iar aceştia ar trebui să beneficieze de un tratament adecvat atunci când presiunea medie depăşeşte 100 kPa (aproximativ 10 N/cm^2).

CUVINTE CHEIE: picior diabetic, ulceraţii la nivelul piciorului, presiune medie, neuropatie periferică diabetică

DIFFÉRENCES DE LA PRESSION PLANTAIRE ENTRE LES SUJETS DIABÉTIQUES ET CEUX EN BONNE SANTÉ

RÉSUMÉ. Étant donné que des seuils variés ont été signalés dans la littérature et qu'il n'y a pas de consensus sur la gamme de pression maximale de sécurité, le but de cette étude a été de comparer la pression plantaire entre les sujets diabétiques et les sujets sains, puis de trouver le seuil de risque de pression pour les patients diabétiques. 177 bénévoles (83 patients diabétiques et 94 témoins) ont été recrutés et leur pression plantaire a été mesurée par le système de pression Emed. La région plantaire a été divisée en sept régions: le gros orteil, MTH1-5 et MF, sous lesquelles la pression moyenne (kPa) a été calculée. Les résultats montrent qu'il y a des variations majeures et que la pression moyenne des patients a été inférieure de 2,5% à celle des homologues sains ; cependant, nous n'avons pas pu trouver la loi des différences entre les patients diabétiques et les personnes en bonne santé dans un niveau de 3%, 50% et 96%. Généralement, il faut accorder une attention particulière aux changements de pieds chez les patients diabétiques et ils doivent recevoir un traitement approprié lorsque leur pression moyenne dépasse 100 kPa (environ 10 N/cm^2).

MOTS CLÉS: pieds diabétiques, ulcères du pied, pression moyenne, neuropathie périphérique diabétique

** Correspondence to: Jin ZHOU, National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, P.R.China, e-mail: zj_scu@scu.edu.cn*

INTRODUCTION

Diabetes Mellitus is a serious chronic disease caused by both environmental and genetic factors [1] and it was reported that by 2014, the number of people worldwide with diabetes was over 422 million [2]. Major challenges for diabetes mellitus patients are the diabetic complications, which would induce ulceration, amputation, and even the death. Those complications absolutely could heavy the financial burden of patients, as a number of lower distal amputations and ulcers are repeatedly occurring in the diabetic feet [1]. Although deformities, trauma and peripheral neuropathy in feet are the most important factors responsible for feet ulcers, high peak pressure is the consequence of the above factors and is the direct cause of feet ulcers [1, 3-7]. Its mechanism has three steps. In the first stage, the sense of feeling is attenuated in the diabetic patient with peripheral neuropathy; meanwhile the strength of muscle and tendon is also reduced, so as to cause the feet deformities, such as the collapse of the foot arches. In the second stage, as the foot structure changed, plantar pressure would concentrate in a specific area and cause the inner tissue lesion of the feet. In the third stage, accompanied by infection, the ulcer develops in the feet [8].

Current literature reports varied ways of pressure distribution in diabetic population. Mueller [9], Delbridge [10], Murray [3] showed that excessive callus and limited joint activity increase the risk of feet ulcers. Pitei [11] and Young [12] found that the callus increased the peak pressure, while by removing callus, a 25-32% peak pressure reduction can be achieved. Boyko [13] and Ahroni [14] systematically reviewed the risk factors for diabetic feet ulcer and they concluded that feet deformities significantly increased plantar pressure and risk of diabetic ulcer; for instance, hallux valgus changed the pressure distribution in the forefoot and increased the pressure value at medial-lateral forefoot. Similarly, Ledoux [4] demonstrated that claw toes and hammer toes were strongly correlated with the high pressure and the occurrence of foot ulcers. Liu *et al*. [15] focused on the Chinese diabetic population and studied the correlation between the plantar pressure and occurrence of ulcers. Their outcomes implied that 69% of increased plantar pressure was found in diabetic group. However, varied thresholds in peak pressure were reported in the above literature, but variables of mean pressure which would be more helpful for indicating the foot ulcers were ignored.

Therefore, the aim of this study was to compare the mean pressure between the Chinese diabetic and healthy subject groups.

METHODS

Subjects

In total, 177 volunteers [94 healthy people (47 normal male/47 normal female) and 83 diabetic patients (male 27/female 56)] were recruited in this study. Patients without definite diabetes mellitus diagnosis, with history of ulceration or amputation, with neuropathy disease were excluded. The aim and method of this study was explained to each patient, and their agreements were received. The whole procedure was supervised by the Ethical Committee of University and the protocol followed the principles of Helsinki Declaration.

Plantar Pressure Measurements

The distribution of plantar pressure was obtained by the Emed pressure system (0.5m, Novel, Germany). Emed pressure measurement system has been confirmed its reliability [16], and the system has been widely used in scientific research. A two-step initial protocol [17, 18] was performed by the subjects and they were guided to walk with their selected speed across the pressure plate, which was embedded in the middle of a six-meter track. Before each measurement, the system was calibrated; and then a three- to five-minute warm up period was provided. At least three successful measures in each side of the foot were required in this study.

The plantar region was divided by Automask software (Novel Automask software, Novel gmbh, Munich) into seven regions [19, 20] (Figure 1): big toe, the first to fifth metatarsal head (MH1-5) and Midfoot (MF), under which mean pressure (kPa) was calculated. Since the ulcer was usually found at the mid and forefoot, those regions were more critical rather than the hind foot. Thereby the pressure distribution at hind foot was not included in this study.

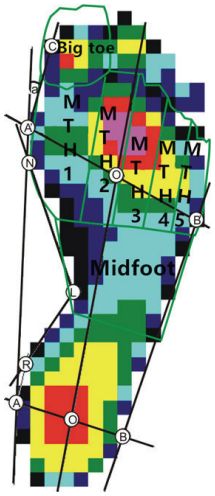


Figure 1. 7 masks for plantar area division

Data Processing and Statistical Analysis

First, inter subjects data of three measures were first averaged, and then the normal distribution was approved by the One-sample K-S test; further, independent T test showed that no significant differences existed between left and right feet and they were joined together for further analysis. Both mean and percentiles value in 3%, 50% and 95% were calculated and contrasted by independent T test between diabetes and control groups. All the above analyses were based on SPSS (V16.0, SPSS Inc., Chicago) with a significant level of 0.05 and a confidence interval of 95%.

RESULTS

Mean age of diabetic subjects is 63.8 ± 9.0 years, mean height is 160.9 ± 7.2cm, mean body weight 57.9 ± 7.6Kg and mean BMI is 22.4 ± 2.5; while mean age of their counterparts is 64.0 ± 7.4 years, mean height is 157.0 ± 7.4cm, body weight is 59.5 ± 9.6Kg and mean BMI is 24.0 ± 3.0.

Table 1: Comparison of the pressure of diabetic patients and healthy ones in each region (kPa)

Regions	Control	Diabetic	Differences (control-diabetic)	Significant value
mean_pressure_bigtoe	130.2±70.3	123.1±42.0	7.1	0.249
mean_pressure_MH1	91.9±63.5	119.2±47.0	-27.3*	0.000
mean_pressure_MH2	135.0±68.5	97.9±36.8	37.1*	0.000
mean_pressure_MH3	132.0±56.1	107.3±40.2	24.6*	0.000
mean_pressure_MH4	90.0±29.2	90.3±33.6	-0.3	0.920
mean_pressure_MH5	102.1±59.7	105.8±46.0	-3.7	0.514
mean_pressure_MF	43.5±16.7	53.0±21.0	-9.5*	0.000
mean_of_all_regions	103.8±27.4	99.6±33.0	4.2*	0.029

* significant of differences <0.05

As shown in Table 1, the mean pressure in the control group was 4.2% higher than that of diabetic group ($p=0.029<0.05$); moreover, MH1,2,3 and MF were also recorded with significant differences between the two groups, where MH1 and MF of diabetic were significantly higher than that of their counterparts ($p=0.000<0.05$ for all variables). 3% of the data represented lower bound of all the subjects' pressure distribution, mean pressure of diabetic patients in the toe region and MTH1 were 27.8% and 20.0% higher than those of their counterparts; while, those at 2-3 MTH were 19.9% and 21.5% lower (Figure 1A). In terms of 50% of the data which indicated the common pressure value of all the subjects, mean pressure of diabetic patients at MTH1 and MTH5 were 50.0% and 22.6% larger than those of control subjects, with the exception that pressure at MTH2 of diabetic patients was 23.9% lower (Figure 1B). In terms of 95% of data, overall, the healthy subject showed a larger pressure distribution in major areas (31.6% for hallux, 33.4% for MTH1, 21.8% for MTH2, 24.2% for MTH3 and 6.6% for MTH5 higher than those of the diabetic patients) (Figure 1C).

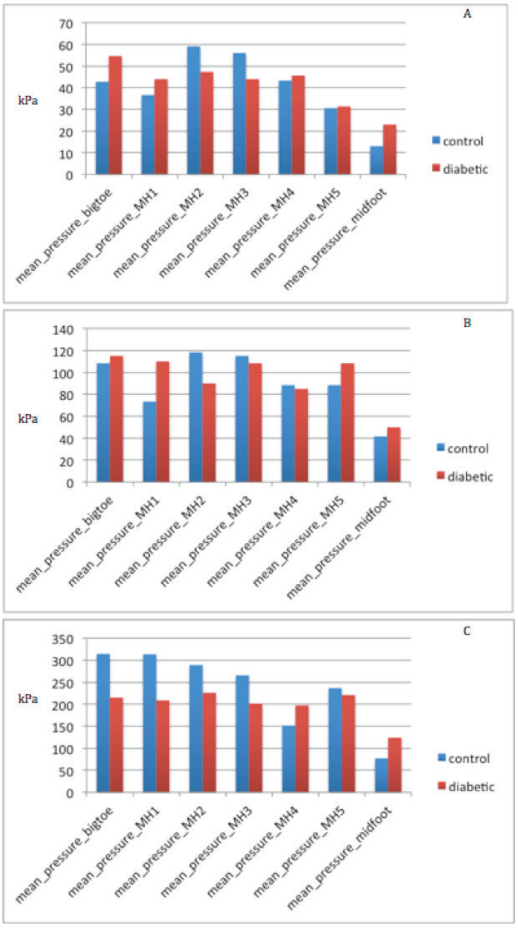


Figure 2. Comparison of 3%, 50% and 96% of peak pressure between the diabetic and control group (A: percentile data of 3%; B: percentile data of 50%; C: percentile data of 96%)

DISCUSSION

In this study, foot pressure data was measured from 83 diabetics and 94 control subjects, and their data were compared in 3%, 50% and 95% percentile level. The results show that major variations existed and mean pressure of the patient was 4.2% lower than that of their healthy counterparts; however, the rules of differences between the diabetic patients and healthy ones in 3%, 50% and 96% percentile level could not be found.

The results showed that the pressure values of diabetic subjects were similar or lower than that of healthy ones and this finding was quite different from current literature. The risk range of pressure for diabetic patients was recorded by a 30 months prospective study of 86 diabetics and Veves summarized risk pressure value from patients whose high foot pressure had foot ulcers that was ≥ 10 kg/cm^2 (about 1000kPa) which was considered as the risk threshold for occurrence of foot ulcers [6]. Meanwhile, Bus [5] found a peak pressure of diabetic feet was more than 600kPa in a specific MTH area. In another study concerning therapeutic footwear for diabetic patients which was prescribed to reduce the risk of ulceration [21], regions of interest with peak pressure 200 kPa were selected as target for pressure relieving. Hence Bus concluded that 200 kPa of peak pressure or 100 kPa of mean pressure was in risk situation. Similarly, Boyko [13] demonstrated a higher risk of foot ulcer among patients with a peak plantar pressure of 12.3 kg/cm^2 (about 1230 kPa); further Veves [6] suggested that this value was the threshold between the normal and abnormal pressure distribution.

The authors postulated that the differences might be due to the variables selected. As

most of the reports used the peak pressure to describe the plantar pressure distribution and it indicated the instance value when loading; while those of the mean pressure were constant and represented a relative force applying in a specific region. So the authors assumed that the mean pressure was more effective to indicate the ulcer, but there are few studies that discuss mean pressure and ulceration.

Additionally, the results also showed that the highest mean pressure of diabetic patients occurred at the MF and MTH1, which were 29.6% and 18.4% higher than that of healthy control group (Table 1); the authors suggested that tissues under these two sites became stiff and thin, which were also observed in our other study [15], where the subcutaneous tissue of diabetic patients was thinner than that of healthy counterparts. Moreover, no severe foot deformities were found in our diabetic group, thereby no significant differences between the two groups were obtained. Further, no rules of differences between the diabetic patients and healthy ones in 3%, 50% and 96% percentile level were found and it implied that the pressure distribution of diabetic patients varied in each other.

CONCLUSION

Overall, although no rules were found between the diabetic and healthy population in terms of mean pressure distribution and mean pressure exceeded 100 kPa in some regions, the authors recommended that patients even with the mean pressure higher than 100 kPa shall take care and caution; and more important, cushion footwear should be prescribed and used by them while walking.

Acknowledgement

The authors thank the financial support from China Postdoctoral Science Foundation (2015M571896) and project from Chengdu Science and Technology Administration (2015-HM01-00039-SF).

REFERENCES

1. International Diabetes Foot Working Group, Beijing: People's Military Medical Publishing House **2003**.
2. WHO, http://www.presstv.ir/Detail/2016/04/06/459437/diabetes-WHO-study-Lancet.
3. Murray, H.J., Young, M.J., Hollis, S. *et al*., *Diabet Med*, **1996**, 13, 979–982.
4. Ledoux, W.R., Shofer, J.B., Smith, D.G. *et al*., *J Rehabil Res Dev*, **2005**, 42, 665–672.
5. Bus, S.A., Maas, M., de Lange, A. *et al*., *J Biomech*, **2005**, 38, 1918–1925.
6. Veves, A., Murray, H.J., Young, M.J., Boulton, A.J., *Diabetologia*, **1992**, 35, 660–663
7. Fernando, D.J., Masson, E.A., Veves, A. *et al*., *Diabetes Care*, **1991**, 14, 8–11.
8. Abbott, C.A., Vileikyte, L., Williamson, S. *et al*., *Diabetes Care*, **1998**, 21, 7, 1071
9. Mueller, M.J., Diamond, J.E., Delitto, A. *et al*., *Phys Ther*, **1989**, 9, 453–462.
10. Delbridge, L., Perry, P., Marr, S. *et al*., *Diabet Med*, **1988**, 5, 333–337.
11. Pitei, D.L., Foster, A., Edmonds, M., *J Foot Ankle Surg*, **1999**, 38, 251–255.
12. Young, M.J., Cavanagh, P.R., Thomas, G. *et al*., *Diabet Med*, **1992**, 9, 55–57.
13. Boyko, E.J., Ahroni, J.H., Stensel, V. *et al*., *Diabetes Care*, **1999**, 22, 1036–1042.
14. Ahroni, J.H., Boyko, E.J., Forsberg, R.C., *Diabetes Care*, **1999**, 22, 965–972.
15. Yaoxia, L., Bo, X., Jin, Z. *et al*., *Chinese Journal of Diabetes*, **2011**, 3, 5, 371-375.
16. Maetzler, M., Bochdansky, T., Abboud, R.J., *Gait Posture*, **2010**, 32, 391–394.
17. Bus, S.A., de Lange, A., *Clin Biomech*, **2005**, 20, 892–899.
18. Nicholson, D.E., Armstrong, P.F., MacWilliams, B.A. *et al*., *Gait Posture*, **1998**, 7, 146.
19. Ute, W., *Foot Ankle Surg*, **2004**, 10, 121–124.
20. Kernozek, T.W., Elfessi, A., Sterriker, S., *J Am Podiatr Med Assoc*, **2003**, 93, 97–103.

21. Bus, S.A., Haspels, R., Buschwestbroek, T.E., *Diabetes Care*, **2011**, 34, 7, 1595.

22. Veves, A., Sarnow, M.R., Giurini, J.M. *et al*., *Diabet Med*, **1995**, 12, 585–589.

STATISTICAL-MATHEMATICAL PROCESSING OF ANTHROPOMETRIC FOOT PARAMETERS AND ESTABLISHING SIMPLE AND MULTIPLE CORRELATIONS. PART 1: STATISTICAL ANALYSIS OF FOOT SIZE PARAMETERS

Mirela PANTAZI[1*], Ana Maria VASILESCU[1], Aura MIHAI[2], Dana GURĂU[1]

[1]INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu, 031215 Bucharest, Romania, pantazimirela@yahoo.com

[2]Gheorghe Asachi Technical University of Iasi, 67 Dimitrie Mangeron Blvd., Iasi, Romania, amihai@tex.tuiasi.ro

Received: 19.09.2017 Accepted: 20.11.2017 https://doi.org/10.24264/lfj.17.4.3

STATISTICAL-MATHEMATICAL PROCESSING OF ANTHROPOMETRIC FOOT PARAMETERS AND ESTABLISHING SIMPLE AND MULTIPLE CORRELATIONS. PART 1: STATISTICAL ANALYSIS OF FOOT SIZE PARAMETERS

ABSTRACT. The purpose of this study is to evaluate anthropometric parameters resulting from measurements by their statistical and mathematical processing. The study was conducted on a sample of 300 male subjects from three geographic regions of Romania: South (100 subjects), East (100 subjects) and Centre and West (100 subjects). The anthropometric data collected by 3D scanning of the left and right foot were grouped into four samples (South, East, Centre-West and Total) and the statistical indicators of characterization (arithmetic mean and standard deviation) and statistical variables (the minimum and maximum values, the amplitude and the coefficient of variation) were calcuated for each variational sequence of the 20 anthropometric parameters studied. To verify the working hypothesis that there were no significant differences between left and right foot, Student's t-test and the Fisher (F) test were used to verify the variants. Both tests have shown that there are no significant differences between the left foot and the right foot for most anthropometric parameters. Samples for the four groups studied were based on the average of anthropometric parameters of the right and left foot. One-dimensional normal-size distribution for Foot Length (Lp) and Toe Girth (Pd) anthropometric parameters was verified. In this respect, the limits of the frequency classes, the centre of the class and the relative and absolute frequencies were established. This distribution is found in the commissioning of the shoe size series. For an optimal fit of the shoe, it is necessary to divide the size series into length and width classes.
KEY WORDS: anthropometric parameters, foot, statistical indicators

PRELUCRAREA STATISTICO-MATEMATICĂ A PARAMETRILOR ANTROPOMETRICI AI PICIORULUI ŞI STABILIREA CORELAŢIILOR SIMPLE ŞI MULTIPLE. PARTEA 1: ANALIZA STATISTICĂ A PARAMETRILOR DIMENSIONALI AI PICIORULUI

REZUMAT. Scopul acestui studiu constă în evaluarea parametrilor antropometrici rezultaţi din măsurători, prin prelucrarea statistico-matematică a acestora. Studiul a fost efectuat pe un eşantion format dintr-un număr de 300 subiecţi, sex masculin, din trei regiuni geografice ale României: Sud (100 subiecţi), Est (100 subiecţi) şi Centru şi Vest (100 subiecţi). Au fost grupate datele antropometrice prelevate prin scanarea 3D a piciorului stâng, respectiv drept, în patru eşantioane (Sud, Est, Centru-Vest şi Total) şi au fost calculaţi indicatorii statistici de caracterizare (media aritmetică şi abaterea standard) şi indicatorii statistici de variaţie (valoarea minimă şi cea maximă, amplitudinea şi coeficientul de variaţie) pentru fiecare şir variational ai celor 20 de parametri antropometrici studiaţi. Pentru verificarea ipotezei de lucru conform căreia nu există diferenţe semnificative între piciorul stâng şi piciorul drept, s-a utilizat testul t Student, iar pentru verificarea variantelor s-a utilizat testul Fisher (F). Ambele teste au demonstrat că nu există diferenţe semnificative între piciorul stâng şi piciorul drept pentru majoritatea parametrilor antropometrici. S-au constituit eşantione pentru cele patru grupuri studiate în baza mediilor parametrului antropometric pentru piciorul drept şi piciorul stâng. A fost verificată repartiţia unidimensională de tip normal pentru parametrii antropometrici Lungimea piciorului (Lp) şi Perimetrul la degete (Pd). În acest sens, au fost stabilite limitele claselor de frecvenţă, centrul clasei şi frecvenţele relativă şi absolută. Această repartiţie se regăseşte în formarea comisioanelor la stabilirea seriei de mărimi a încălţămintei. Pentru o potrivire optimă a încălţămintei este necesar ca seria de mărimi să fie împărţită pe clase de lungimi şi lărgimi.
CUVINTE CHEIE: parametri antropometrici, picior, indicatori statistici

LE TRAITEMENT STATISTIQUE-MATHÉMATIQUE DES PARAMÈTRES ANTROPOMÉTRIQUES DU PIED ET LA DÉTERMINATION DES CORRÉLATIONS SIMPLES ET MULTIPLES. PREMIÈRE PARTIE : L'ANALYSE STATISTIQUE DES PARAMÈTRES DIMENSIONNELS DU PIED

RÉSUMÉ. Le but de cette étude est d'évaluer les paramètres anthropométriques résultant des mesures, par leur traitement statistique et mathématique. L'étude a été réalisée sur un échantillon de 300 sujets masculins de trois régions géographiques de la Roumanie: Sud (100 sujets), Est (100 sujets) et Centre et Ouest (100 sujets). Les données anthropométriques recueillies par le balayage 3D du pied gauche et du pied droit ont été regroupées en quatre échantillons (Sud, Est, Centre-Ouest et Total) et les indicateurs statistiques de caractérisation (moyenne arithmétique et écart-type) et les variables statistiques (les valeurs minimale et maximale, l'amplitude et le coefficient de variation) ont été calculés pour chaque séquence variationnelle des 20 paramètres anthropométriques étudiés. Pour vérifier l'hypothèse de travail selon laquelle il n'y avait pas de différence significative entre le pied gauche et le pied droit, le test-t de Student et le test de Fisher (F) ont été utilisés pour vérifier les variantes. Les deux tests ont montré qu'il n'y avait pas de différence significative entre le pied gauche et le pied droit pour la plupart des paramètres anthropométriques. Les échantillons pour les quatre groupes étudiés ont été basés sur la moyenne anthropométrique du pied droit et du pied gauche. La distribution de taille normale unidimensionnelle pour les paramètres anthropométriques de longueur du pied (Lp) et de périmètre aux orteils (Pd) a été vérifiée. À cet égard, les limites des classes de fréquence, du centre de la classe et des fréquences relative et absolue ont été établies. Cette distribution se trouve dans la mise en service de la série de chaussures. Pour un ajustement optimal de la chaussure, il est nécessaire de diviser les séries de tailles en classes de longueur et de largeur.
MOTS CLÉS : paramètres anthropométriques, pied, indicateurs statistiques

* *Correspondence to: Mirela PANTAZI, INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu, 031215 Bucharest, Romania, pantazimirela@yahoo.com*

INTRODUCTION

Manufacturing comfortable footwear relies on anthropometric research, which determines the morphological character of the foot, the behavior of the foot-footwear system and the results of the morpho-functional optimization of the shape of the product.

The natural anatomic-morphological construction of the foot, its correct static and dynamic functioning is ensured by a rational inner shape of the shoe, the shape which is determined in turn by the construction of the last.

The construction of the last, the establishment of the dimensions required to meet the comfort requirements of a larger proportion of consumers with minimal production costs, must be based on the knowledge and the most accurate characterization of the anatomic-morphological differences of the types of foot encountered within that population of consumers and the frequency of these types within the population.

For this purpose, it is periodically necessary to perform anthropometric studies on the population differentiated according to certain criteria (sex, age, geographical region, etc.) in order to obtain information about the dimensional particularities of the average representative foot for that population, the laws of distribution of anthropometric parameters that characterize the representative average foot of the population of the respective country.

All this information will be used directly for the rational dimensioning of lasts to solve two important aspects: on the one hand the standardization of the shape and the dimensions of the last, i.e. finding the optimal relationship between the dimensions of the separate parts based on the construction of real biometric models of the wearer's foot and the variation of its dimensional characteristics within the population in order to define the particular foot sizes of the population, and on the other hand, the calculation of the percentage distribution of the footwear corresponding to a particular size of the wearer's foot (defining tallies of sizes and widths).

Tallies of serial production must match the shape and the internal dimensions of the shoe given by the last and the shape and dimensions of the foot of the majority of wearers.

The values of the anthropometric parameters reveal the dimensional changes of the foot depending on gender, age, geographical conditions.

Anthropometric data sampling can be done using two measurement methods:

- by direct contact with the foot to be measured, where the values are measured and recorded manually;
- by collecting anthropometric data without direct contact, in which case the foot conformation and dimensions are obtained through 3D scanning systems that allow storage and processing of data with specialized software.

The correlation of the inner dimensions of the shoe with the anthropometric dimensions of the foot is particularly important in order to meet the comfort conditions, the most representative being: toe width, toe height, heel breadth, and foot length.

The purpose of this study is to evaluate anthropometric parameters resulting from the measurements by means of their statistical and mathematical processing.

EXPERIMENTAL

Materials and Methods

The 3D foot shape based on which anthropometric parameters are measured was scanned using the INFOOT USB system (Figure 1) made up of the 3D scanner and the dedicated MEASURE 2.8 software. The system enables foot shape scanning and can automatically recognize and place up to 20 anatomical measurement points.

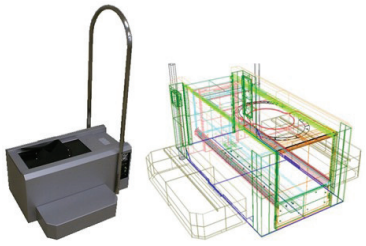


Figure 1. INFOOT USB 3D Scanner

Foot shape scanning and placement of anatomical points on the surface of the scanned foot shape enabled determination of values for a set of 20 anthropometric parameters, lengths, widths, girths and angles (Table 1) [1].

Table 1: Analysed anthropometric foot parameters

1.	Foot length	Lp	(mm)
2.	Ball girth circumference	Pd	(mm)
3.	Foot breadth	Id	(mm)
4.	Instep circumference	Pr	(mm)
5.	Heel breadth	Ic	(mm)
6.	Instep length	Lr	(mm)
7.	Toe height	Hd	(mm)
8.	Instep height	Hr	(mm)
9.	Toe 1 angle	Ud1	(°)
10.	Toe 5 angle	Ud5	(°)
11.	Toe 1 height	Hd1	(mm)
12.	Toe 5 height	Hd5	(mm)
13.	Height of navicular	Hn	(mm)
14.	Height of Sphyrion fibulare	Hsf	(mm)
15.	Height of Sphyrion	Hs	(mm)
16.	Height of the most lateral point of lateral malleolus	Hme	(mm)
17.	Height of the most medial point of medial malleolus	Hmi	(mm)
18.	Heel angle	Uc	(°)
19.	Heel girth	Pc	(mm)
20.	Ankle girth	Pg	(mm)

Anthropometric footprints obtained using the INFOOT USB system were statistically analyzed using the SPSS software package, which is a package dedicated to statistical data processing, making it easy to obtain the desired results quickly.

Subjects

Anthropometric studies were conducted on a sample of 300 male subjects from three geographic regions of Romania: South (100 subjects), East (100 subjects), and Centre and West (100 subjects). Subjects with particular anthropometric features, including deformities and structural abnormalities of the foot, were excluded.

RESULTS AND DISCUSSIONS

Verifying the Statistical Significance of Differences between Anthropometrical Parameters of the Left and Right Foot Using Student Test and Fisher Test

Student's t-test was used to test the working hypothesis that there are no significant differences between left and right foot. The test determines the associated probability to determine if two samples are likely to come from the same two basic populations that have the same mean. The research hypothesis in the case of the group of subjects is represented by the statistical significance of the recorded values for the left and right foot compared to the mean values for the left and right foot. The T test confirms whether there is a significant difference between these values. This will allow the use of mean values in establishing correlations and variation laws by building samples based on the mean value of the antropometric parameter associated to the left foot and the one associated to the right foot.

To determine whether the results obtained are statistically significant, the calculated values are compared to p = 0.05 for a 95% probability. To confirm the null hypothesis, the p values must be greater than p = 0.05.

The results of the Student's Test are presented in Table 2. T test values lower than the significance threshold of 0.05 are indicated for the following anthropometric parameters: instep height (Hr), toe 1 height (Hd1), height of navicular (Hn), height of Sphyrion fibulare (Hsf), height of the most lateral point of lateral malleolus (Hme), heel angle (Uc), and ankle girth (Pg). For the other anthropometric parameters, the null hypothesis is confirmed, according to which there are no statistically significant differences between the means of the studied parameters.

The Fisher test was used to verify that variants in the matrix of anthropometric parameters for the left foot did not differ significantly from the variants of the right foot matrix values. The values obtained for the F test are shown in Table 2.

The analysis of the values obtained by applying the two types of tests results in the rejection of the null hypothesis, and consequently the existence of significant differences between the right and the left foot in the case of one anthropometric parameter, namely the height of Sphyrion fibulare (Hsf).

Table 2: T (student) test and F (Fisher) test

	Anthropometric parameters		T test	F test
1.	Foot length	Lp	0.317589	0.564553
2.	Ball girth circumference	Pd	0.338263	0.483241
3.	Foot breadth	Id	0.101553	0.319551
4.	Instep circumference	Pr	0.298322	0.63871
5.	Heel breadth	Ic	0.416227	0.680185
6.	Instep length	Lr	0.005163	0.259382
7.	Toe height	Hd	0.381454	0.83237
8.	Instep height	Hr	4.07E-05	0.830783
9.	Toe 1 angle	Ud1	0.067916	0.116667
10.	Toe 5 angle	Ud5	0.083654	2.4E-274
11.	Toe 1 height	Hd1	0.02601	0.730987
12.	Toe 5 height	Hd5	0.145579	0.058903
13.	Height of navicular	Hn	8.66E-23	0.643273
14.	Height of Sphyrion fibulare	Hsf	0.001586	0.009536
15.	Height of Sphyrion	Hs	0.059013	0.660882
16.	Height of the most lateral point of lateral malleolus	Hme	0.001909	0.102374
17.	Height of the most medial point of medial malleolus	Hmi	0.262626	0.800037
18.	Heel angle	Uc	2.22E-07	0.398818
19.	Heel girth	Pc	0.114933	0.863741
20.	Ankle girth	Pg	0.020876	0.475921

Statistical Indicators of Characterization and Variation of Anthropometric Parameters

The main calculated statistical indicators are arithmetic mean ($\bar{x}\bar{x}$), standard deviation (S), sample dispersion (S^2), minimum, maximum, variance amplitude (A) and coefficient of variation (CV). The statistical indicators for characterization and variation of the calculated anthropometric parameters are presented in Table 3.

Arithmetic mean ($\bar{x}\bar{x}$) represents the central trend of statistical selection, it can be strongly influenced by extreme values and reflects the values of the entire variational sequence. The arithmetic mean ($\bar{x}$), for the unmatched data (simple series) [2, 3, 4] is calculated with the relation:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \quad (1)$$

where: $\sum_{i=1}^{n} x_i \sum_{i=1}^{n} x_i$ = sum of scores; nn = total number of scores.

Dispersion is how the values in the series revolve around the series. This is calculated as an arithmetic mean of squares of deviations from their arithmetic mean [5, 6]. For simple series:

$$S^2 = \frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n} \quad (2)$$

The standard deviation represents the degree of scattering of the series values compared to the mean. This is the square root of the dispersion value, which indicates how much the values of the series deviate from their arithmetic mean [7, 8, 9]. For simple series:

$$S = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n}} \quad (3)$$

The amplitude of the variation is the difference between the maximum value and the minimum value of a distribution and indicates the value range of the distribution. The amplitude is obtained by the difference between the maximum value and the minimum value of the series values and is calculated by the formula [10, 11, 12]:

$$A = x_{max} - x_{min} \quad (4)$$

Coefficient of variation (CV%) - refers to the degree of scattering of values in a series around the mean. It is defined as the ratio of

the standard deviation of a distribution and its arithmetic mean [13]. It is used to analyze the degree of scattering of a distribution.

$$CV = \frac{S}{\bar{x}} * 100\ (\%) \quad (5)$$

- If CV<15% then the scattering is small - the case of most anthropometric parameters;
- If 15%<CV<30%, scattering is medium - these situations are highlighted in yellow in Table 3;
- If CV>30% then scattering is high - these situations are highlighted in red in Table 3.

The statistical indicators for characterization and variation of the calculated anthropometric parameters are presented in Table 3.

Table 3: Statistical indicators of characterization of anthropometric parameters

No.	Anthropometric parameters	Group	No. of subjects	$\bar{x}$ (mm)	S (mm)	S^2 (mm^2)	Min (mm)	Max (mm)	A (mm)	CV (%)
1	Lp	South	100	259.70	21.28	453.01	244.65	274.75	30.10	8.20
		East	100	263.25	17.96	322.58	250.55	275.95	25.40	6.82
		Centre and West	100	280.78	6.82	46.56	275.95	285.60	9.65	2.43
		Total	300	267.43	13.15	172.81	229.05	302.50	73.45	4.92
2	Pd	South	100	275.13	11.14	124.03	267.25	283.00	15.75	4.05
		East	100	258.95	4.74	22.45	255.60	262.30	6.70	1.83
		Centre and West	100	271.45	12.94	167.45	262.30	280.60	18.30	4.77
		Total	300	261.09	13.28	176.49	217.60	297.35	79.75	5.09
3	Id	South	100	111.45	4.60	21.13	108.20	114.70	6.50	4.12
		East	100	105.98	0.18	0.03	105.85	102.50	0.25	0.17
		Centre and West	100	110.88	7.11	50.50	105.85	115.90	10.05	6.41
		Total	300	106.89	5.73	32.78	87.35	122.00	34.65	5.36
4	Pr	South	100	311.95	52.11	2715.85	275.10	348.80	73.70	16.71
		East	100	264.03	4.42	19.53	260.90	267.15	6.25	1.67
		Centre and West	100	268.08	1.31	1.71	267.15	269.00	1.85	0.49
		Total	300	263.31	15.00	225.02	224.75	348.80	124.05	5.70
5	lc	South	100	72.13	1.73	3.00	70.90	73.35	2.45	2.40
		East	100	68.23	2.72	7.41	66.30	70.15	3.85	3.99
		Centre and West	100	71.90	2.47	6.13	70.15	73.65	3.50	3.44
		Total	300	69.98	3.96	15.70	59.95	84.60	24.65	5.66
6	Lr	South	100	186.63	13.19	173.91	177.30	195.95	18.65	7.07
		East	100	191.40	14.07	198.01	181.45	201.35	19.90	7.35
		Centre and West	100	202.73	1.94	3.78	201.35	204.10	2.75	0.96
		Total	300	191.86	10.02	100.31	157.25	218.10	60.85	5.22
7	Hd	South	100	163.33	13.19	173.91	154.00	172.65	18.65	8.07
		East	100	168.80	10.96	120.13	161.05	176.55	15.50	6.49
		Centre and West	100	180.18	5.13	26.28	176.55	183.80	7.25	2.85
		Total	300	169.03	8.46	71.54	142.30	188.80	46.50	5.00

8	Hr	South	100	51.95	0.00	0.00	51.95	51.95	0.00	0.00
		East	100	44.75	3.25	10.58	42.45	47.05	4.60	7.27
		Centre and West	100	47.23	0.25	0.06	47.05	47.40	0.35	0.52
		Total	300	46.11	3.55	12.64	36.60	58.50	21.90	7.71
9	Ud1	South	100	5.20	3.25	10.58	2.90	7.50	4.60	62.55
		East	100	7.33	7.81	61.05	1.80	12.85	11.05	106.67
		Centre and West	100	3.28	2.09	4.35	1.80	4.75	2.95	63.69
		Total	300	6.14	4.41	19.48	-5.45	17.50	22.95	71.90
10	Ud5	South	100	18.25	1.56	2.42	17.15	19.35	2.20	8.52
		East	100	13.63	4.91	24.15	10.15	17.10	6.95	36.07
		Centre and West	100	12.65	3.54	12.50	10.15	15.15	5.00	27.95
		Total	300	14.64	4.05	16.37	0.60	24.85	24.25	27.64
11	Hd1	South	100	27.28	3.43	11.76	24.85	29.70	4.85	12.57
		East	100	24.78	4.56	20.80	21.55	28.00	6.45	18.41
		Centre and West	100	25.83	3.08	9.46	23.65	28.00	4.35	11.91
		Total	300	24.56	2.21	4.89	19.75	34.60	14.85	9.00
12	Hd5	South	100	22.50	0.85	0.72	21.90	23.10	1.20	3.77
		East	100	20.60	2.26	5.12	19.00	22.20	3.20	10.98
		Centre and West	100	22.43	0.32	22.20	22.20	22.65	0.45	1.42
		Total	300	20.99	2.49	6.21	11.75	31.30	19.55	11.87
13	Hn	South	100	38.38	0.25	0.06	38.20	38.55	0.35	0.64
		East	100	37.88	0.32	0.10	37.65	38.10	0.45	0.84
		Centre and West	100	38.55	0.64	0.41	38.10	39.00	1.65	1.65
		Total	300	38.09	0.86	0.74	35.80	40.80	5.00	2.25
14	Hsf	South	100	56.23	10.36	107.31	48.90	63.55	14.65	18.42
		East	100	57.08	8.73	76.26	50.90	63.25	12.35	15.30
		Centre and West	100	62.65	0.85	0.72	62.05	63.25	1.20	1.35
		Total	300	58.23	4.51	20.38	42.90	69.65	26.75	7.75
15	Hs	South	100	57.45	13.44	180.50	47.95	66.95	19.00	23.39
		East	100	65.23	4.84	23.46	61.80	68.65	6.85	7.43
		Centre and West	100	68.45	0.28	0.08	68.25	68.65	0.40	0.41
		Total	300	66.43	5.65	31.89	45.35	80.85	35.50	8.50
16	Hme	South	100	74.83	11.99	143.65	66.35	83.30	16.95	16.02
		East	100	75.15	12.23	149.64	66.50	83.80	17.30	16.28
		Centre and West	100	83.30	0.71	0.50	82.80	83.80	1.00	0.85
		Total	300	77.84	5.24	27.42	63.85	92.95	29.10	6.73
17	Hmi	South	100	76.45	14.71	216.32	66.05	86.85	20.80	19.24
		East	100	85.45	6.51	42.32	80.85	90.05	9.20	7.61
		Centre and West	100	90.48	0.60	0.36	90.05	90.90	0.85	0.66
		Total	300	87.22	6.46	41.71	64.60	103.15	38.55	7.40
18	Uc	South	100	2.30	2.33	5.45	0.65	3.95	3.30	101.45
		East	100	3.00	0.49	0.25	2.65	3.35	0.70	16.50
		Centre and West	100	1.88	2.09	4.35	0.40	3.35	2.95	111.25
		Total	300	3.42	2.47	6.09	-3.75	9.60	13.35	72.16
19	Pc	South	100	358.03	17.57	308.76	345.60	370.45	24.85	4.91
		East	100	338.70	16.97	288.00	326.70	350.70	24.00	5.01
		Centre and West	100	360.05	13.22	174.85	350.70	369.40	18.70	3.67
		Total	300	347.09	16.24	263.58	298.85	392.55	93.70	4.68

20	Pg	South	100	286.05	16.19	262.21	274.60	297.50	22.90	5.66
		East	100	272.88	12.48	155.76	264.05	281.70	17.65	4.57
		Centre and West	100	290.83	12.90	166.53	281.70	299.95	18.25	4.44
		Total	300	277.79	15.15	229.43	230.95	335.85	104.90	5.45

Figures 2-5 graphically represent coeffi-cients of variation of studied anthropometric parameters.

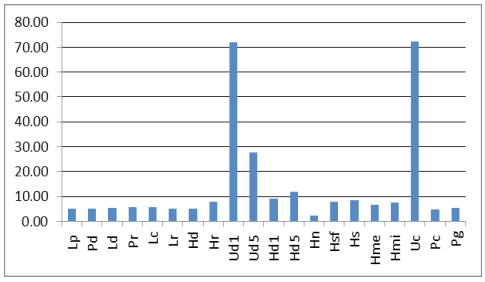


Figure 2. Distribution of coefficient of variation for TOTAL group

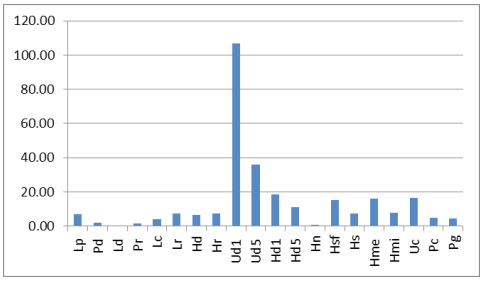


Figure 4. Distribution of coefficient of variation for EAST group

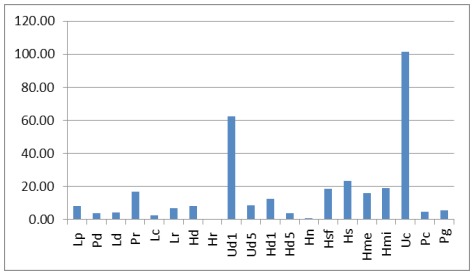


Figure 3. Distribution of coefficient of variation for SOUTH group

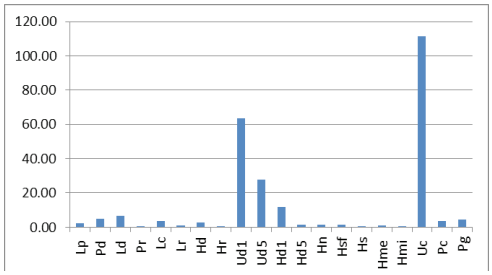


Figure 5. Distribution of coefficient of variation for WEST & CENTRE group

One-dimensional Distribution

The analytical form of distribution is [3, 13]:

$$f(x,\mu,\sigma)=\frac{1}{\sigma\sqrt{2\pi}}e^{-\frac{(x_i-\mu)^2}{2\sigma^2}} \quad (6)$$

where:

f(x,μ,σ) – the frequency of finding particular values x_i of variable x within the selection;

μ_x – arithmetic mean of variable x;

σ^2,σ – scattering and standard deviation of variable x;

e=2.71828 – the basis of the natural logarithm.

Variational Sequence of Lp Variable (Foot Length)

For the group of subjects, consisting of n = 300, the distribution of the foot length (Lp) variable is presented in Table 4. The values of the variational sequence are grouped into classes; for each class the absolute frequencies of the Lp variable and the relative frequency of the selection volume were set.

Table 4: Distibution of Lp variable (foot length)

Class interval (mm)	Class centre	Absolute frequency	Relative frequency (%)
<=242		10	3.33
242 - 247	245	2	0.67
247 - 252	250	26	8.67
252 - 257	255	24	8.00
257 - 262	260	42	14.00
262 - 267	265	40	13.33
267 - 272	270	48	16.00
272 - 277	275	38	12.67
277 - 282	280	34	11.33
282 - 287	285	20	6.67
287 - 292	290	8	2.67
292 - 297	295	4	1.33
297- 302	300	2	0.67
>=302		2	0.67
Total		300	100

Figure 6 presents the histogram of absolute frequencies of Lp variable and the trend curve approximating the histogram.

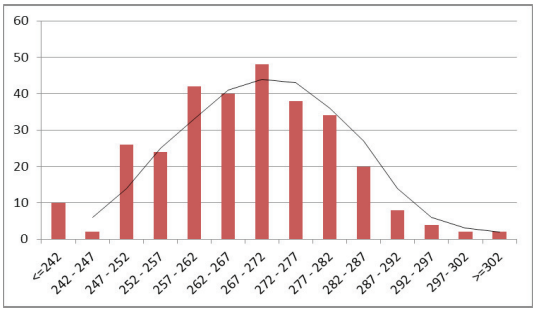


Figure 6. Distribution of absolute frequencies of Lp variable (foot length) and trend curve on class intervals for TOTAL group

Variational Sequence of Pd variable (Toe Girth)

Table 5 presents intervals of classes and absolute frequencies of Pd variable as well as the relative frequencies to the selection volume.

Table 5: Distribution of Pd parameter (toe girth)

Class interval (mm)	Class centre	Absolute frequency	Relative frequency (%)
< 222		2	0.67
222 - 227	225	0	0.00
227 - 232	230	2	0.67
232 - 237	235	6	2.00
237 - 242	240	12	4.00
242 - 247	245	18	6.00
247 - 252	250	38	12.67
252 - 257	255	40	13.33
257 - 262	260	56	18.67
262 - 267	265	28	9.33
267 - 272	270	32	10.67
272 - 277	275	32	10.67
277 - 282	280	18	6.00
282 - 287	285	8	2.67
287 - 292	290	4	1.33
>292		4	1.33
Total		300	100.0

Figure 7 presents the histogram of absolute frequencies of Pd variable and the trend curve approximating the histogram.

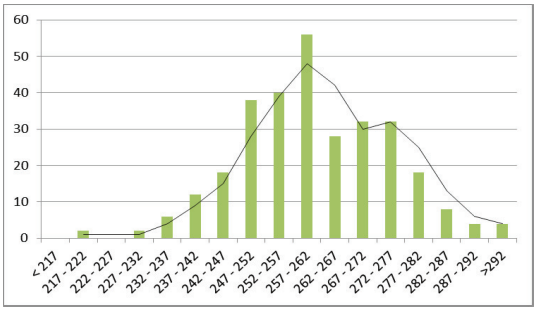


Figure 7. Distribution of absolute frequencies of Pd variable (toe girth) and trend curve on class intervals for TOTAL group

CONCLUSIONS

➢ In order to verify the working hypothesis according to which there are no significant differences between the left foot and the right foot, Student's t-test and Fisher (F) test were used to check the variants. Both tests have shown that there are no significant differences between the left foot and the right foot for most anthropometric parameters. Furthermore, samples were made for the four groups studied based on the mean of anthropometric parameters of the right foot and the left foot.

➢ Foot length (Lp) per total recorded an average of 267.43 mm, with a coefficient of variation of 4.92%. In the South and East, the average value is lower than the national average, while in the Centre-West region the average is significantly higher (280.78 mm). This implies that in the size series formation, in the South and East regions the frequency of the first series sizes is higher, while in the Centre-West region the frequency of the second series is higher.

➢ Nationwide, toe girth has an average of 262.09 mm. A value higher by 14.4 mm is recorded for the South region and 10.36 mm for the Centre-West region. In the Eastern region there is an average Pd value close to the national average (258.95 mm). The same situation is recorded for the foot breadth (Id). Since these two anthropometric parameters give the shoe width, it can be concluded that footwear for the

South and Centre-West regions has to be made in larger sizes than the Eastern region.

➢ Instep girth (Pr) varies widely for the South region, where an average of 311.95 mm is recorded, well above the national average of 263.31 mm. This entails restrictions on footwear models for this region that should be designed with adjustable straps to accommodate this large variation of the instep girth.

➢ The heel width (Ic) shows coefficients of variation below 6% both at national level and at the level of each region. This justifies the standardization of the rear area of the last and the shoe components that structure this area (counter, heel, shank), thus reducing the cost of these components.

➢ Instep length (Lr) has average values higher than the national average for the Centre region (202.73 mm versus 191.86 mm). Also, instep height (Hr) records the same trend.

➢ Toe height (Hd) is higher than the national average for the South region (51.96 mm vs. 46.11 mm). This situation also occurs in the case of toe 1 height (Hd1) and toe 5 height (Hd5), respectively. This justifies the design of the last and the shoe so as to allow for a larger space and therefore a looseness in the toe area in the case of footwear made for the South region.

➢ Anthropometric parameters of angle characterization (Ud1 - toe angle 1, Ud5 - toe angle 5, and Uc - heel angle) have a very large scattering (the coefficient of variation is over 30%). This demonstrates that very many subjects have deviations from the normal anatomical position of toes 1 and 5, and deviations of the heel, respectively. Most of the time, wearing a shoe improperly sized in the toe area (narrower or tighter) causes this situation. The worst situation is found in the eastern region where the coefficient of variation is very high, even compared to the national average. This is corroborated with the conclusion from the previous paragraph.

➢ As far as the ankle girth (Pg) and the heel girth (Pc) are concerned, it is found that in the South and Centre-West regions the average values are higher than the national average, while in the Eastern region these parameters have smaller average values.

➢ The normal one-dimensional distribution for anthropometric parameters such as foot length (Lp) and toe girth (Pd) was verified. In this respect, the limits of the frequency classes, the centre of the class and the relative and absolute frequencies were established. This distribution is found in the commissioning of the shoe size series. For an optimal fit of footwear, it is necessary to divide the size series into length and width classes.

Acknowledgements

This work was financed by the Romanian Ministry of Research and Innovation in the framework of Nucleu Program Inova-Tex-Pel, 2016-2017, project code PN 16 34 04 01.

REFERENCES

1. Pantazi, M., Vasilescu, A.M., 3D Imaging Capture of the Foot and Data Processing for a Database of Anthropometric Parameters, Proceedings of The 6th International Conference on Advanced Materials and Systems - ICAMS 2016, ISSN: 2068-0783, CERTEX Press, Session 3 - Innovative Technologies, **2016**, 387-392.

2. Avadanei, M., Theoretical and Experimental Contributions on the Use of Antropometric Data in Designing Clothing Items (in Romanian), PhD Thesis, "Gh. Asachi" Technical University of Iasi, **2000**.

3. Gheorghiu, D., Statistics Applied in Psychology (in Romanian), Bucharest, "Titu Maiorescu" University Press, ISBN 973-86202-2-8, **2003**.

4. Radu, E., Adam, M., Gaghes, R., Anthropological Indicators of Medical Relevance, *Studies and Research in Anthropology* (in Romanian), vol. 24, **1987**, 19-30.

5. Erdemir, A. *et al*., Local Plantar Pressure Relief in Therapeutic Footwear: Design Guidelines from Finite Element Models, *Journal*

of Biomechanics, **2005**, 38, 9, 1798–806, https://doi.org/10.1016/j.jbiomech.2004.09.009.

6. Patterson, R.P., Shoes and the Army, statement issued June 4, 1945, *The Quartermaster Review*, July-August **1945**.

7. Raulea, C., Psychological Statistics and Computerized Data Processing - Introductive Course (in Romanian), **2010**.

8. Taloi, D., Optimization of Metallurgic Processes (in Romanian), Didactic and Pedagogic Press, Bucharest, **1983**.

9. Ujevic, D., Theoretical Aspects and Application of Croatian Anthropometric System (CASE), University of Zagreb, Faculty of Textile Technology, Croatia, ISBN 978-9537-105-28-0, **2010**.

10. Mochimaru, M., Kouchi, M., Dohni, M., Analysis of 3D Human Foot Forms Using the FFD Method and its Application in Grading Shoe Last, *Ergonomics*, **2000**, 43, 9, 1301-1313.

11. Nácher, B., Alemany, S., González, J., Alcántara, E., A Footwear Fit Classification Model Based on Anthropometric Data, SAE Technical Paper, 2006-01-2356, **2006**.

12. Ujevic, D., K. Doležal, R., Hrženjak, B., Croatian Anthropometric System, TEDI Vol 1, **2011**, 40-48.

13. Gheorghiu, D., Psychological Statistics and Computerized Data Processing (in Romanian), Bucharest, **2005**.

A NOVEL EXTRACELLULAR KERATINASE FROM EXIGUOBACTERIUM SP. DG1: ENZYME PRODUCTION AND DEHAIRING APPLICATION

Jajang GUMILAR[1*], Lies Mira YUSIATI[2], Ambar PERTIWININGRUM[2], Tomoyuki NAKAGAWA[3], Suharjono TRIATMOJO[2]

[1]Faculty of Animal Husbandry, Padjadjaran University, Bandung, Indonesia

[2]Faculty ofAnimal Science, Gadjah Mada University, Yogyakarta, Indonesia

[3]United Graduate School of Agricultural Science, Gifu University, Gifu, Japan

Received: 01.08.2017 Accepted: 02.11.2017 https://doi.org/10.24264/lfj.17.4.4

A NOVEL EXTRACELLULAR KERATINASE FROM EXIGUOBACTERIUM SP. DG1: ENZYME PRODUCTION AND DEHAIRING APPLICATION

ABSTRACT. In this research, we determined the optimum conditions for production of extracellular keratinase in *Exiguobacterium* sp DG1, and showed the possibility of application of the keratinase for sheep skin dehairing process. Strain DG1 showed the highest productivity of the keratinase activity during the growth in sheep hair meal (SHM) medium (pH 8) containing 10 g/L of SHM with aerobic incubation at 27°C for 48 hours. Moreover, dehairing process using an extracellular keratinase from strain DG1 decreased several index parameters in the treatment wastes. For example, in the treatment wastes using keratinase from strain DG1, COD and BOD values decreased drastically (82.1% and 73.7% respectively), compared to chemical dehairing process, and sulfide content was very low (less than 0.01 mg/L). These facts indicate that the extracellular keratinase from strain DG1 possesses a great potential of application for green leather tanning industry.

KEY WORDS: keratinase, enzyme production, *Exiguobacterium* sp. DG1, dehairing

O NOUĂ KERATINAZĂ EXTRACELULARĂ DIN EXIGUOBACTERIUM SP. DG1: OBȚINEREA ENZIMEI ȘI APLICAREA ACESTEIA LA ÎNDEPĂRTAREA PĂRULUI

REZUMAT. În acest studiu s-au determinat condiţiile optime pentru producerea keratinazei extracelulare din *Exiguobacterium* sp. DG1 şi s-a demonstrat posibilitatea aplicării keratinazei în procesul de îndepărtare a părului de pe pielea de ovină. Tulpina DG1 a prezentat cea mai mare productivitate a activităţii keratinazei în timpul creşterii în mediu cu blană de ovine (SHM) conţinând 10 g/l SHM (pH 8) cu incubare aerobă la 27°C timp de 48 de ore. Mai mult decât atât, procesul de îndepărtare a părului utilizând o keratinază extracelulară din tulpina DG1 a condus la scăderea mai multor parametri la tratarea deşeurilor. De exemplu, în cazul tratării deşeurilor care utilizează keratinaza din tulpina DG1, valorile COD şi BOD au scăzut drastic (82,1% şi respectiv 73,7%), comparativ cu procesul de îndepărtare chimică a părului, iar conţinutul de sulfuri a fost foarte scăzut (sub 0,01 mg/l). Aceste valori indică faptul că keratinaza extracelulară din tulpina DG1 are un mare potenţial de aplicare în industria de tăbăcire ecologică a pielii.

CUVINTE CHEIE: keratinază, producerea de enzime, *Exiguobacterium* sp. DG1, depărare

UNE NOUVELLE KÉRATINASE EXTRACELLULAIRE D'EXIGUOBACTERIUM SP. DG1: LA PRODUCTION DE L'ENZYME ET SON APPLICATION DANS L'ÉPILAGE

RÉSUMÉ. Dans cette recherche, on a déterminé les conditions optimales pour la production de kératinase extracellulaire d'*Exiguobacterium* sp. DG1 et on a montré une possibilité d'application de la kératinase dans le processus d'épilage de la peau de mouton. La souche DG1 a montré la plus grande productivité de l'activité de la kératinase au cours de la croissance en milieu (pH 8) de poils de moutons (SHM) contenant 10 g/L de SHM à l'incubation aérobie à 27°C pendant 48 heures. De plus, le procédé d'épilage utilisant une kératinase extracellulaire provenant de la souche DG1 a diminué les différents paramètres d'indexation dans les déchets de traitement. Par exemple, dans le cas des déchets de traitement utilisant la kératinase à partir de la souche DG1, les valeurs de DCO et de DBO ont diminué drastiquement (82,1% et 73,7%, respectivement) par rapport au processus d'épilage chimique, et la teneur en sulfure a été très faible (moins de 0,01 mg/L). Ces valeurs montrent que la kératinase extracellulaire de la souche DG1 possède un grand potentiel d'application pour l'industrie du tannage écologique du cuir.

MOTS CLÉS: kératinase, production d'enzymes, *Exiguobacterium* sp. DG1, épilage

[*]*Correspondence to: Jajang GUMILAR, Faculty of Animal Husbandry, Padjadjaran University, Bandung, Indonesia, email: j.gumilar@unpad.ac.id*

INTRODUCTION

Keratins are structural proteins located in skin and its appendages such as hair, nails, feathers and also in epithelial tissues within the body, and most of them are insoluble in water and hardly biodegradable [1]. The stability of keratin depends on tightly packed in the α-helix (α-keratin) or β-sheet (β-keratin) into a supercoiled polypeptide chain and its degree of cross linkages by disulfide and hydrogen bonds [2-3]. Keratins are grouped into soft keratin and hard keratin according to the sulfur content. Soft keratins are found in skins and callus; they have a low content of disulfide bonds and are more pliable. On the other hand, hard keratins are found in appendages such as hair, hooves, nails, and feathers, and they have high sulfur content and are tough also inextensible [4].

Generally, keratins are known to be a stable protein, which is resistant to decomposition, and in leather processing industry, hair, which consists of keratins mainly, should be removed from the leather. Leather processing involves four distinct sets of unit operations, i.e. pre tanning, tanning, dyeing, and finishing. During the conventional leather processing by chemical treatment, every unit operations dump specific pollutions. For example, in pre tanning operation especially in conventional dehairing process, they use high proportions of lime and sulfide, these materials are a source of pollution generating large quantities of solid waste [5]. As a result, the waste contains large amounts of chemical oxygen demand (COD), and biological oxygen demand (BOD), it also leads to production of toxic gases, such as hydrogen sulfide [6]. From these backgrounds, various efforts have been made to attempt to reduce and replace the use of $Ca(OH)_2$ and Na_2S, which are the main chemical ingredients in conventional dehairing process. Hence, several researchers in the leather industry have been actively involved in the development of enzyme base for dehairing, defleshing, and fiber opening processes to replace conventional process [7-8].

The keratinases (EC 3.4.99.11) belong to the group of hydrolases, and are large serine or metalloproteases which are capable of degrading the structure of keratinous proteins [9]. The keratinases are not normal proteases such as subtilisin, papain, and pepsin [10], and they degrade keratin without damaging other structural proteins such as collagen that are potentially used in the leather industry [11]. Therefore, the keratinases from microorganisms have several purposes especially in the green leather industry, and they are one of the important industrial enzymes, because the enzymes are utilized for hydrolyzing feather, hair, wool, and other components. From these facts, several researchers have tried to find the keratinases from microorganisms, such as several bacteria, actinomycetes and fungi. However, only the keratinase from *Bacillus licheniformis* has reached commercial level so far [12]. On the other hand, we had newly screened keratinase producing bacteria, *Exiguobacterium* sp. DG1. The strain was isolated from soil containing sheep hair, obtained at traditional Garut sheep farm in Garut District, West Java Province, Indonesia. There have been no reports that genus *Exiguobacterium* produces keratinase except for our work, and strain DG1 had enough keratinolytic and low collagenase activity [13]. Therefore, we think that keratinase from strain DG1 has a great potential to be used as an enzyme in dehairing process.

In this research, we aimed to show optimum condition for the keratinase production using keratinase producing bacteria, *Exiguobacterium* sp. DG1, and to indicate the possibility of application of the keratinase to the enzymatic leather processing.

EXPERIMENTAL

Material and Method

Bacterial Strain, Media, and Cultivation

Exiguobacterium sp. DG1 was used for the keratinase production test in this work.

For enzyme production, strain DG1 cultivated in SHM medium contained 1% sheep hair meal; as the sole carbon and nitrogen source with minimal mineral containing 0.03% K_2HPO_4; 0.04% KH_2PO_4, 0.05% NaCl, pH7 [11-13]. The keratinolytic enzyme was produced in 250 ml Erlenmeyer flasks containing 50 ml culture medium, maintained for 48 h at 25°C and 180 rpm.

Enzyme Assay

The culture medium was filtered and centrifuged for 15 min at 10.000 rpm and 4°C. The supernatant was used as crude keratinase enzyme for further analysis. The keratinolytic

activity was determined by using keratin azure (Sigma-Aldrich, St. Louis, USA) as the insoluble substrate. The enzyme samples (500 µL) were incubated in a solution of 5 mg of keratin azure in 500 µL of 50 mM sodium phosphate buffer (pH 7.5) [14], for 30 min at 180 rpm and 30°C. The absorbance of the supernatant was measured at 595 nm. Control samples were prepared in a similar manner except the enzyme was replaced by sodium phosphate buffer. The assays were conducted in four replicates. One unit of keratinase was defined as the amount of enzyme causing an increase of 0.01 absorbance between the sample and control at 595 nm in 30 min under the given condition.

Effect of Culture Condition on Keratinase Production

For optimization of keratinase production by *Exiguobacterium* sp. DG1, sheep hair meal substrate was used with the following parameters: cultivation times (12, 24, 36, 48, 60 hours), amount of substrate (5, 10, 15, 20, and 25 g/L), pH value (8, 9, 10, 11, and 12), temperature (23, 25, 27, 29, and 31°C). The treatments were replicated four times.

Dehairing Application

Dehairing application studies were carried out on Garut Sheep skin salted at the tannery facility of PT. Elco Indonesia Sejahtera (Garut district, West Java province, Indonesia). This study used a combination of 0.25% Na2S with 1% enzyme [5] compared that of commercial formulations in dehairing process which used 2.5% Na_2S. The enzyme was crude enzyme produced by *Exiguobacterium* sp. DG1.

The skin for histological preparations taken after the dehairing process. The method modified from [15]. The skin was cut 1.5 × 1.0 cm with a length parallel to the line of the back, then it was put in a fixative liquid with formaldehyde concentrations of 10% (w/v). The skin was dehydrated using alcohol 80, 95, and 100% (v/v) then it was put in the xylene. Skin was put in paraffin and then was cut crosswise using the microtome. Preparations were fixed with thymol and stained with Mallory method. Furthermore they were observed microscopically to identify: (a) epidermis, (b) hair follicle, and (c) corium / dermis.

The content of COD was analyzed using the method of American Public Health Association (APHA) (2005) section 5220-B and BOD content was tested by APHA (2005) section 5210-B. The sulfide content was analyzed using Standard Methods For The Examination of Water and Wastewater 2012 (SMEWW-4500-S-D).

Statistic Analysis

Data were analyzed as completely randomized design. Effects of treatments on enzyme activity were performed using analysis of variance and the difference between treatments was analyzed using Duncan's Multiple Range Test. Every treatment was replicated four times.

RESULTS AND DISCUSSION

Effect of Culture Condition on Keratinase Production of the Strain DG1

Cultivation Times

Exiguobacterium sp. DG1 produces an extracellular keratinase enzyme bearing an ability to degrade sheep hair meal, where the levels of expressed enzyme varied during incubation period of growth. Keratinase enzyme production was observed in the fermentation broth every twelve hours. Bacterial growth increased up to 48 hours incubation time (OD 0.56), after that the growth was relatively decreasing. This is because of the continuing depletion of nutrients and buildup of metabolic wastes resulting in death of the cells [16]. Keratinase enzyme production of the strain also increased up to 48 hours, but after that time, the production was decreased (Fig. 1). Incubation time as long as 48 hours had the highest keratinase production (4.20 U/ml), and it was significantly different ($P<0.05$) compared to other incubation times. Optimum keratinase production with incubation time 48 hours in line with keratinase that was produced by *B. halodurans* strain PPKS-2 [16], *B. licheniformis* ER-15 [17], and *Chryseobacterium* sp. Kr6 [18]. Generally, the maximum keratinase production was generated during the exponential growth phase or at the end of the stationary phase [19].

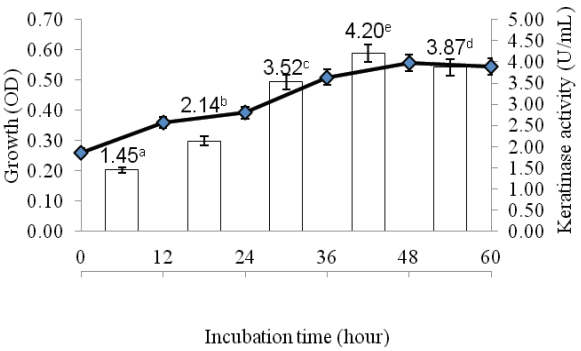


Figure 1. Effect of incubation times on bacterial growth and keratinase enzyme production.
a, b, c, d, e shows significant differences (P<0.05) among treatments.

Substrate Concentration

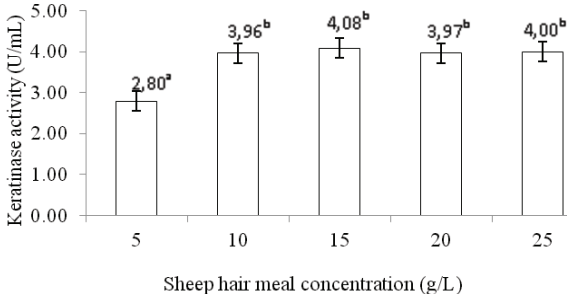


Figure 2. Effect of substrate concentrations on keratinase enzyme production.
a, b shows significant differences (P<0.05) among treatments.

Induction levels of keratinase activity during growth of strain DG1 on SHM medium depended on substrate concentrations and cultivation conditions. When substrate concentration was 5 g/L, the strain showed the smallest keratinase activity (2.80 U/mL). According to statistic analysis using Duncan Multiple Range Test, the significant difference was lower (P<0.05) than the other concentrations. On the other hand, when substrate concentration was over 10g/L, keratinase induction levels did not show any significant difference (Fig. 2). Based on this finding, we could use substrate as much as 10 g/L to 25 g/L. This result was similar with *B. subtilis* KD-N2 that produced highest keratinase activity at 16 g/L human hair as a substrate [20] and *Bacillus* sp. produced the highest keratinase activity in 2% feather meal as a substrate [21].

Sheep hair meal substrates as carbon, nitrogen, as well as mineral sources were added to the fermentation medium used by *Exiguobacterium* sp. DG1 as a source of nutrients. Living organisms need nutrients in a certain amount for growth and production. If the sources of nutrients are fewer, then the growth will be disturbed and will not have an optimal production. Giving too much substrate will cause a decrease in the production of keratinase, because the use of the substrate has a limitation. If too much substrate is added, it will cause inhibition and repression on keratinase production. The higher the concentration of the substrate, the higher the viscosity of the medium, so that the mixture of components and air circulation in the medium are hampered by increasing the medium viscosity [22]. Such conditions are very unfavorable for an aerobic bacteria growth.

pH

Many factors affecting to the enzyme productions such as incubation time, pH, temperature, and substrate concentration. This study showed that fermentation condition at pH 8 induced the highest keratinase activity (4.06 U/mL). The keratinase activity from cells grown at pH 8 showed a higher significant difference (P<0.05) than that from other pH grown cells. Otherwise the cells grown at pH 12 showed the lowest keratinase production, then it was lower significant difference (P<0.05) than other pH grown cells (Fig. 3).

pH value changes not only affect the nature of the media and substrates, but it also affects bacteria growth and enzyme production. pH affects several enzymes transport across the cell membrane and reduced access to the substrate, so that the growth in unsuitable conditions limit the speed of growth and enzyme production. The rate of microbial growth fall at a pH lower or higher than the optimum pH is due to a change in the three-dimensional proteins structure. Changes in pH affect the ionization of the R groups of amino acids in the active site or in other parts of the enzyme, so that the enzyme changes shape and it decreases the enzyme catalytic ability. Changes in the pH cause denaturation of proteins that decrease the enzyme catalytic activity in the cell. The optimal keratinase enzyme production from bacteria observed at a wide range of pH started from pH 4.5 until 10 [19]. In this study, the optimal keratinase production was pH 8. This result is

similar with keratinase produced by *Pseudomonas stutzeri* [23], *Bacillus megaterium* F7-1 [22], and *Chryseobacterium* sp. kr6 [18].

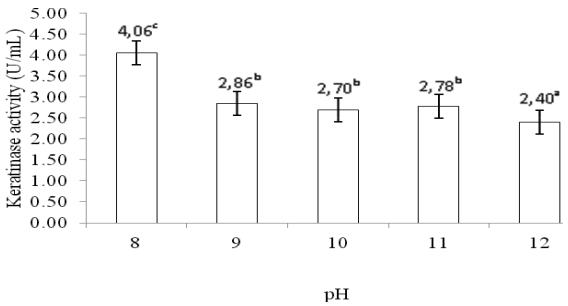


Figure 3. Effect of pH concentrations on keratinase enzyme production.
a, b, c shows significant differences (P<0.05) among treatments.

Temperature

Referring to the Fig. 4, we can see that the highest production of keratinase enzyme (4.25 U/mL) was produced at 27°C incubation temperature. It was a higher significant difference (P<0.05) than the other treatments. This *Exiguobacterium* sp. DG1 is a mesophilic bacteria exhibit the optimal enzyme production and activity ranging from 20 to 30°C [24]. Incubation temperature is a characteristic of an organism and profoundly affects the enzyme yield and duration of enzyme synthesis phase. This finding was similar with the optimum temperature of *B. subtilis* KD-N2 [20] and *Penicillium* spp. [25].

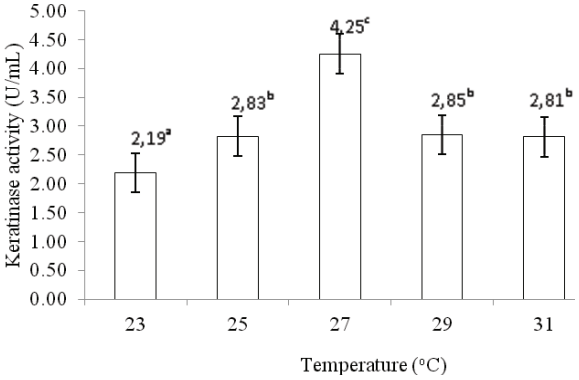


Figure 4. Effect of temperatures on keratinase enzyme production.
a, b, c shows significant differences (P<0.05) among treatments.

Dehairing Application

This study showed that the sheep skin with enzymatic dehairing used keratinase from *Exiguobacterium* sp. DG 1 were cleaner, whiter, and brighter, compared to the chemically dehaired skin (Fig 5, A1). This study was in line with the study conducted by [26] that used enzyme from *P. aeruginosa* in dehairing of buffalo hide. The sheep skin's color changed becoming blue as a result of interaction between Na_2S and skin, in chemical dehairing process (Fig 5, A2).

Microscopic analysis of the keratinase treated in dehairing process showed that the epidermis was digested and the hair follicle

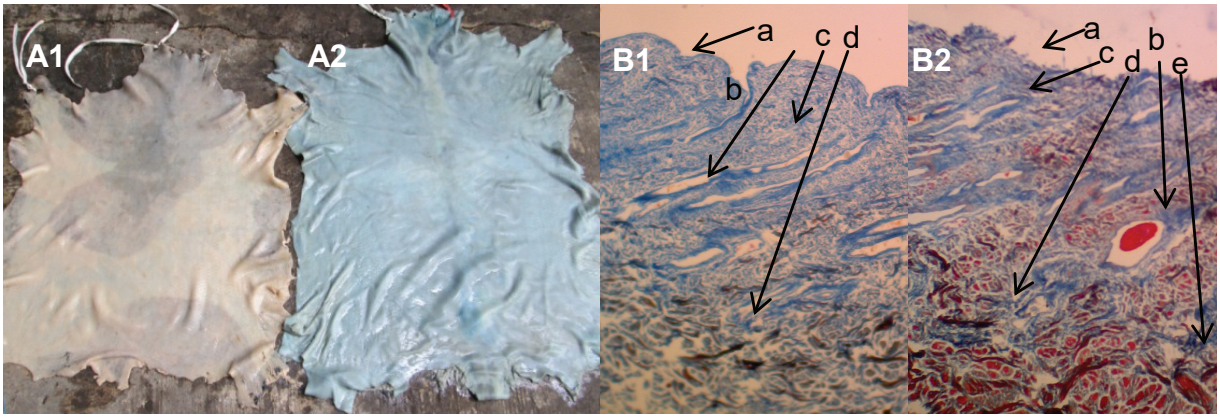


Figure 5. Sheep skin produced by keratinase enzyme dehairing process (A1), and chemical dehairing process (A2). Microscope examination of sheep skin produced by keratinase enzyme dehairing process (B1), and chemical dehairing process (B2). Epidermis (a), folicle (b), collagen / blue (c), elastic tissue / dark brown (d), muscle tissue / orange red (e).

is empty, it indicates that the hair is loose completely, also the muscle can already be digested (Fig. 5, B1). The use of keratinase in dehairing process has been able to break the soft keratin in the hair follicle so the hair can be detached from the skin. While the use of 2.5% Na_2S treatment showed that the epidermis still remains, appearing rough and not compact, leaving the rest of hair in the follicles root, and the muscle still intact (Fig. 5, B2). Collagen was not damaged, its structure appears to be not modified, and also the elastic tissue may become disorganized, but was still present and was not removed in either the enzymatic dehairing or the chemical dehairing process.

This study showed that the use of 1% keratinase could already remove the hair perfectly without damaging the collagen network. The use of 1% crude enzyme from *B. cereus* MCM B-326 on cow hide enzymatic dehairing also showed good dehairing activity without damaging the collagen and the fibers in the skin were still intact [27]. Enzymatic dehairing on goat skin using enzymes from *B. subtilis* P13 could remove the epidermis and hair in the follicle perfectly [28].

The hair resulting from this treatment was also different; the hair produced by enzymatic process was still intact, and however the hair produced by a chemical process turned into pulp. Chemical dehairing attacks the hair and reduces it to pulp, meanwhile enzymatic dehairing destroys or modifies the epidermal tissue surrounding the hair bulb, so that the hair is loosened and can be removed mechanically [28].

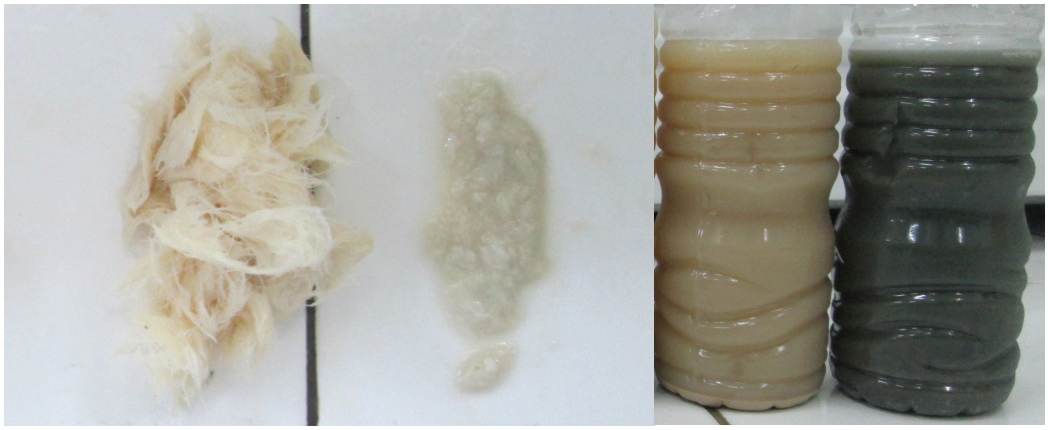


Figure 6. Hair waste produced by keratinase enzyme dehairing process (A1) and chemical dehairing process (A2). Wastewater produced by keratinase enzyme dehairing process (B1) and chemical dehairing process (B2)

Wastewater quality from these treatments had different characteristics. The spent liquor from chemical dehairing and enzymatic dehairing process was collected separately and analyzed for pollution parameter such as COD, BOD, and sulfide content. As compared to the chemical dehairing, the enzymatic dehairing showed reduction of COD from 20.16 to 3.61 g/L, that is 82.09%; and also of BOD from 4.75 to 1.25g/L, that is 73.68%. The keratinase enzyme destroys or modifies the epidermal tissues surrounding the hair bulb, so the hair is loose [28]. Enzymatic dehairing process allows to separate the hair and avoiding the huge semi gelatinous content and high level of organic matter produced by chemical dehairing process in the wastewater [11].

Sulfide content in wastewater generated from the enzymatic dehairing processes was less than 0.01 mg/L, whereas the control that used chemical dehairing process produced an average sulfide waste of 494.25 mg/L. All sulfide in the waste comes from Na_2S that used to destroy the hair and breakdown the elements containing sulfides from the dehairing process. Paper [5] stated that almost all the content of sulfide in wastewater was generated in the dehairing process, also [29] stated that the traditional methods of dehairing process using Na_2S contribute 100% of the sulfide waste in leather tanning process.

CONCLUSIONS

In this work, we showed that *Exiguobacterium* sp. DG1 is capable of producing

keratinase enzyme during incubation on the SHM medium (pH 8) with 10g/L sheep hair meal at 27°C for 48h. Keratinase produced by *Exiguobacterium* sp. DG1 could be used in dehairing process and could lead to a reduction of wastewater as much as 82.09% in COD, 73.68% in BOD, and less sulfide content (0.01 mg/L), compared to the chemical dehairing process using natrium sulfide. From these results, it was suggested that keratinase from *Exiguobacterium* sp. DG1 possesses a great potential of application in the leather tanning industry.

Acknowledgments

This research was funded by Padjadjaran University and Directorate of Higher Education at The Ministry of Technology, Research, and Higher Education Republic of Indonesia in the program of improving the quality of international publications.

REFERENCES

1. Friedrich, J., Gradisar, H., Vrecl, M., Pogacnik, A., *In vitro* degradation of porcine skin epidermis by a fungal keratinase of *Doratomyces microsporus*, *Enzyme Microb Technol*, **2005**, 36, 455–460, https://doi.org/10.1016/j.enzmictec.2004.09.015.

2. Xie, F., Chao, Y., Yang, X., Yang, J., Xue, Z., Luo, Y., Qian, S., Purification and characterization of four keratinases produced by *Streptomyces* sp. Strain 16 in native human foot skin medium, *Bioresource Technol*, **2010**, 101, 344–350.

3. Rahayu, S., Syah, D., Suhartono, M.T., Degradation of keratin by keratinase and disulfide reductase from *Bacillus* sp. MTS of Indonesian origin, *Biocatal Agric Biotechnol*, **2012**, 1, 152–158, https://doi.org/10.1016/j.bcab.2012.02.001.

4. Gupta, R., Ramnani, P., Microba keratinases and their prospective applications: an overview, *Appl Microbiol Biotechnol*, **2006**, 70, 21–33, https://doi.org/10.1007/s00253-005-0239-8.

5. Thanikaivelan, P., Rao, J.R., Nair, B.U., Ramasami, T., Progress and recent trends in biotechnological methods for leather, *Trends Biotechnol*, **2004**, 22, 4, 181-188, https://doi.org/10.1016/j.tibtech.2004.02.008.

6. Jaouadi, B., Chaabouni, S.E., Ali, M.B., Messaoud, E.B., Naili, B., Dhouib, A., Bejar, S., Excellent laundry detergent compatibility and high dehairing ability of the *Bacillus pumilus* cbs alkaline proteinase (SAPB), *Biotechnol Bioprocess Eng*, **2009**, 14, 503–512, https://doi.org/10.1007/s12257-008-0244-8.

7. Thanikaivelan, P., Rao, J.R., Nair, B.U., Ramasami, T., Recent trends in leather making: Processes, problems, and pathways, *Environ Sci Tech*, **2005**, 35, 37-79, https://doi.org/10.1080/10643380590521436.

8. Sundararajan, S., Kannan, C.N., Chittibabu, S., Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent, *J Biosci Bioeng*, **2011**, 111, 2, 128–133, https://doi.org/10.1016/j.jbiosc.2010.09.009.

9. Agrahari, S., Wadhwa, N., Isolation and characterization of feather degrading enzymes from *Bacillus megaterium* SN1 isolated from Ghazipur poultry waste site, *Appl Biochem Microbiol*, **2012**, 48, 2, 175–181, https://doi.org/10.1134/S0003683812020020.

10. Yamamura, S., Morita, Y., Hasan, Q., Yokoyama, K., Tamiya, E., Keratin degradation: a cooperative action of two enzymes from *Stenotrophomonas* sp., *Biochem Biophysic Research Com*, **2002**, 294, 1138–1143, https://doi.org/10.1016/S0006-291X(02)00580-6.

11. Macedo, A.J., Beys Da Silva, W.O., Gava, R., Driemier, D., Henriques, J.A.P., Termigonu, C., Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities, *Appl Environ Microbiol*, **2005**, 71, 594–596.

12. Gupta, R., Rajput, R., Sharma, R., Gupta, N., Biotechnological applications and prospective market of microbial keratinases, *Appl Microbiol Biotechnol*, **2013**, 97, 9931-9940, https://doi.org/10.1007/s00253-013-5292-0.

13. Gumilar, J., Triatmojo, S., Yusiati, L.M., Pertiwiningrum, A.,. Isolation, identification and dehairing activity of Indonesian native keratinolytic bacteria *Exiguobacterium* sp. DG1, *Pak J Biotechnol*, **2015**, 12, 1, 41-48.

14. Wang, S., Hsu, W., Liang, T., Yen, Y., Wang, C., Purification and characterization of three novel keratinolytic metaloproteases produced by *Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium, *Bioresource*

Technol, **2008**, 99, 5679–5686, https://doi.org/10.1016/j.biortech.2007.10.024.

15. Zambare, V.P., Nilegaonkar, S.S., Kanekar, P.P., Production of an alkaline protease by *Bacillus cereus* MCM B-326 and its application as a dehairing agent, *World J Microbiol Biotechnol*, **2007**, 23, 1569–1574, https://doi.org/10.1007/s11274-007-9402-y.

16. Prakash, P., Jayalakshmi, S.K., Sreeramulu, K., Production of keratinase by free and immobilized cells of *Bacillus halodurans* strain ppks-2: partial characterization and its application in feather degradation and dehairing of the goat skin, *Appl Biochem Biotechnol*, **2010**, 160, 1909–1920, https://doi.org/10.1007/s12010-009-8702-0.

17. Tiwary, E., Gupta, R., Medium optimization for a novel 58 kDa dimeric keratinase from *Bacillus licheniformis* ER-15: Biochemical characterization and application in feather degradation and dehairing of hides, *Bioresourece Technol*, **2010**, 101, 6103–6110, https://doi.org/10.1016/j.biortech.2010.02.090.

18. Riffel, A., Lucas, F., Heeb, P., Brandelli, A., Characterization of a new keratinolytic bacterium that completely degrades native feather keratin, *Arch Microbiol*, **2003**, 179, 258–265, https://doi.org/10.1007/s00203-003-0525-8.

19. Brandelli, A., Daroit, D.J., Riffel, A., Biochemical features of mikroba keratinases and their production and applications, *Appl Microbiol Biotechnol*, **2010**, 85, 1735-1750, https://doi.org/10.1007/s00253-009-2398-5.

20. Cai, C.G., Zheng, X.D., Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology, *J Ind Microbiol Biotech*, **2009**, 36, 875–883, https://doi.org/10.1007/s10295-009-0565-4.

21. Pandian, S., Sundaram, J., Panchatcharam, P., Isolation, identification and characterization of feather degrading bacteria, *Euro J Exp Biol*, **2012**, 2, 1, 274–282.

22. Park, G.T., Son, H.J., Keratinolytic activity of *Bacillus megaterium* F7-1, a feather degrading mesophilic bacterium, *Microbiological Res*, **2009**, 164, 478–485, https://doi.org/10.1016/j.micres.2007.02.004.

23. Chaturvedi, V., Bhange, K., Bhatt, R., Verma, P., Production of kertinases using chicken feathers as substrate by a novel multifunctional strain of *Pseudomonas stutzeri* and its dehairing application, *Biocatal Agric Biotechnol*, **2014**, 3, 2, 167–174, https://doi.org/10.1016/j.bcab.2013.08.005.

24. Allpress, J.D., Mountain, G., Gowland, P.C., Production, purification, and characterization of an extracellular keratinase from Lysobacter NCIMB 9497, *Lett Appl Microbiol*, **2002**, 34, 337–342.

25. El-Gendi, M.M., Keratinase production by endophytic *Penicillium* spp. Morsy 1 under solid-state fermentation using rice straw, *Appl Biochem Biotechnol*, **2009**, 162, 780–794, https://doi.org/10.1007/s12010-009-8802-x.

26. Zambare, V.P., Nilegaonkar, S.S., Kanekar, P.P., Production optimization and purification of a novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327, *New Biotechnol*, **2011**, 28, 2, 173-181.

27. Nilegaonkar, S.S., Zambare, V.P., Kanekar, P.P., Dhakephalkar, P.K., Sarnaik, S.S., Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326, *Biores Tech*, **2007**, 98, 1238–1245, https://doi.org/10.1016/j.biortech.2006.05.003.

28. Pillai, P., Mandge, S., Archana, G., Statistical optimization of production and tannery applications of a keratinolytic serine protease from *Bacillus subtilis* P13, *Proc Biochemis*, **2011**, 46, 1110–1117, https://doi.org/10.1016/j.procbio.2011.01.030.

29. Srinivas, D., Naik, G.R., Characterization of alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity, *Intr Biodeter Biodegr*, **2011**, 65, 29–35, https://doi.org/10.1016/j.ibiod.2010.04.013.

PRESCRIPTION VARIABLES FOR THE MEDICAL FOOTWEAR

Daniel PETCU*

INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu St., Bucharest, Romania,

email: crispinian.ro@gmail.com

Received: 08.09.2017 Accepted: 29.11.2017 https://doi.org/10.24264/lfj.17.4.5

PRESCRIPTION VARIABLES FOR THE MEDICAL FOOTWEAR

ABSTRACT. Medical footwear is a medical device used in the conservative treatment of foot and lower limb pathomechanics. The standard EN ISO 9999:2016 - "Assistive products for person with disability-Classification and terminology", includes orthopedic shoes in the category of foot orthoses which are medical devices that encompass the whole or part of the foot, being divided in two categories: prefabricated or custom fabricated. According to the legal requirements, a custom fabricated device is based on a medical prescription containing the specific design characteristics as established by the medical practitioner. In many situations from clinical practice, the medical prescription does not contain the specific design characteristics but only some generic data such as the name and type of the orthopedic footwear. The purpose of this paper is to review the basic prescription variables of the medical footwear according to the experience from developed foot care systems.
KEY WORDS: medical footwear, prescription variable

VARIABILE DE PRESCRIPȚIE PENTRU ÎNCĂLȚĂMINTEA MEDICALĂ

REZUMAT. Încălțămintea medicală este un dispozitiv medical utilizat în tratamentul conservator al patologiilor de natură mecanică ale piciorului și ale membrelor inferioare. Standardul EN ISO 9999: 2016 - „Produse de asistență pentru persoane cu dizabilități - clasificare și terminologie" include încălțămintea ortopedică în categoria ortezelor pentru picior care sunt dispozitive medicale ce cuprind întregul picior sau o parte a acestuia, fiind împărțite în două categorii: prefabricate sau personalizate. Conform cerințelor legale, un dispozitiv special fabricat se bazează pe o rețetă medicală care conține caracteristicile specifice de proiectare stabilite de către medic. În multe situații din practica clinică, prescripția medicală nu conține caracteristicile specifice de proiectare, ci doar câteva date generice precum denumirea și tipul încălțămintei ortopedice. Scopul acestei lucrări este de a revizui variabilele de bază ale prescripției pentru încălțămintea medicală, în conformitate cu experiența din sistemele dezvoltate de îngrijire a piciorului.
CUVINTE CHEIE: încălțăminte medicală, variabile de prescripție

VARIABLES DE PRESCRIPTION POUR LES CHAUSSURES MÉDICALES

RÉSUMÉ. Les chaussures médicales sont un dispositif médical utilisé dans le traitement conservateur de la pathomécanique du pied et du membre inférieur. La norme EN ISO 9999: 2016 - "Produits d'assistance pour personnes avec handicap - Classification et terminologie", inclut les chaussures orthopédiques dans la catégorie des orthèses pour les pieds qui sont des dispositifs médicaux qui englobent tout ou partie du pied, étant divisées en deux catégories: préfabriqués ou fabriqués sur mesure. Selon les exigences légales, un dispositif fabriqué sur mesure est basé sur une prescription médicale contenant les caractéristiques de conception spécifiques établies par le médecin. Dans de nombreuses situations de la pratique clinique, la prescription médicale ne contient pas les caractéristiques de conception spécifiques, mais seulement quelques données génériques comme le nom et le type de chaussure orthopédique. Le but de cet article est d'examiner les variables de prescription de base pour les chaussures médicales en fonction de l'expérience des systèmes développés de soin du pied.
MOTS CLÉS: chaussures médicales, variable de prescription

** Correspondence to: Daniel PETCU, INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu St., Bucharest, Romania, email: crispinian.ro@gmail.com*

INTRODUCTION

The orthopedic footwear is a foot orthosis, part of the lower limb orthoses category, defined as being "designed to modify the structural and functional characteristics of the neuro-musculoskeletal systems of the lower limb" [1]. The literature dealing with medical footwear uses different terms to name it: "orthopaedic" [1], "therapeutic"-"diabetic"-"neuropathic" [2], "custom-moulded shoes" [3], "medical grade footwear" [4]. Despite all these confusing names, the importance of using the footwear as a medical device in the prevention of injuries, improving the performance or treatment of the foot and lower limb pathomechanics, is highlighted and documented on different levels of medical evidence by the large number of scientific literature dealing with the kinematic and kinetic effects of the footwear in different pathological or non-pathological conditions [5-7]. There are a lot of design characteristics considered starting from simple wedges in the studies related to medial knee osteoarthritis treatment [8], to complex designs as in those involved in the manipulation of the center of pressure used for training purposes [9]. In the case of custom fabricated medical devices the legal requirements ask for a medical prescription which is the responsibility of "a duly qualified medical practitioner" or "other person authorized by virtue of his professional qualification to do so" [10]. The problem of the lack of prescription variables of medical devices included in the medical prescription has been treated in the medical literature from an advanced system such as that of the USA pertaining to orthotics [11] or footwear [12]. It is notable that in the field of footwear, quite recently in 2013, Dennis Janisse - a renowned USA Cped, has given a "measure" of the lack of prescription variables, stating that "for every prescription we receive that reads, "Extra-depth shoes with Velcro closures, heel-to-toe rocker soles, extended steel shanks, custom Plastazote foot orthotics with MTH offloading, and partial foot filler on L," we get ten that read, "Shoes and inserts"" [12]. A similar point of view regarding the increasing expertise of the orthotist while a physician's training decline is noticed in the field of orthotics prosthetics from USA was recently expressed [13]. The huge difference between the developments of the prescription form of the footwear between different countries could be seen by reviewing the models of prescription forms provided by the state insurance systems. For example, in Australian New South Wales state, the "Equipment Request Form" is 3 pages long asking very clear information related to footwear characteristics and regarding recommendation, justification or evaluation planning of the medical device [14]. In opposition with this situation, in Romania there is no specific template for the medical prescription of a custom fabricated medical device but a general one which only asks for the name and the type of the recommended medical device as information which can be categorized as specific design characteristics [15]. At the same time, there is no orthopedic footwear producer who would provide an online prescription form, this in opposition with the practice from USA where numerous companies are providing online prescription form for medical footwear. Moreover, in Romania the only book describing in detail the footwear characteristics in each chapter related to a foot pathology dates from 1964 [16]. The prescription definition and its implication especially in the countries where specific professions such as podiatry or pedorthics are not developed was extensively treated in a previous article [17]. As in the podiatric literature the foot orthotics are seen as "in-shoe" medical devices, numerous papers dealing with their prescription variables [18-20], the purpose of this paper was to

focus on the specific prescription variables of the medical footwear, part of the medical prescription, without considering those of the "in-shoe" medical devices. These prescription variables could be part of the "technical prescription" elaborated by the physician or by orthotist/pedorthist according to their expertise highlighted by literature [11, 13, 17].

BASIC PRESCRIPTION VARIABLES

The medical footwear is a complex product which is characterized by a multitude of design/manufacture characteristics. It is obvious that not any design characteristic should be a prescription variable to be included in the medical prescription. This is the reason for which, according to the purpose of this paper, the design/manufacture variables should be divided into two categories:

- prescription variables: those design characteristics which are essential for the medical footwear in order to achieve the medical objectives and in agreement with the functional description established by the medical practitioner;

- technical variables: those design characteristics that are not essential for the medical footwear in order to achieve the conservative treatment objectives established by the physician. These are established by the technical specialist (pedorthist, orthopedic shoemaster, orthotist). We consider that, even if they are essential, the technical variables related to the achievement of a good fitting of the medical footwear should be in the area of the technical specialist competences.

According to the legal rules, the prefabricated devices which are modified for therapeutic purposes are not considered custom-made devices. Even in this condition as they are a therapeutic option for the conservative treatment of foot pathomechanics, they contain specific design characteristics which makes them suitable for this purpose. This is the reason for which the analysis of the prescription variables of the medical footwear is valid also for the prefabricated (or mass-produced) devices. It is not the intent of the present paper to describe in detail the characteristics of the prescription variables. The main objective of this paper is to present a list of basic prescription variables which can increase the awareness regarding the potential use of the footwear in the conservative treatment of foot pathomechanics.

The basic prescription variables for each important section are presented below.

Footwear's Last and Foot's Cast Prescription Variables

- **Foot casting / scanning position**. Generally, the semi-weight bearing casting position could be considered the reference position for casting but in special cases, casting in the position of function (for example in standing position as for the severe rigid deformities) or under partial pressure with slight manipulation or correction is recommended [21-25].

- **Cast modifications**. These are very well documented in the case of functional foot orthotics or lower limb orthoses and prostheses [20, 26]. The most encountered modifications in the case of orthopedic footwear are related to cast balancing, creating a toe rise, adding a toe box and adding material in order to accommodate the sensitive areas [21-25].

- **Type**: curved / semicurved / straight should be indicated in agreement with foot shape [24]. This prescription variable should be indicated as in many cases the final last is obtained through modification of an existing straight last which will not give adequate results for C-shaped feet.

- **Sagittal profile**. The sagittal profile of the bottom part of the last has the potential to influence the subject's posture through a more correct weight distribution between rearfoot and forefoot [27]. It is important also for in-shoe

medical devices stability inside the footwear.

- **Last's heel height or elevation** is an important prescription variable related to footwear balance in the sagittal plane [24, 25].

- **Depth / extra depth**. It will create the necessary allowances for accommodating in-shoe medical devices or feet deformities. In the case of lower limb discrepancy, when a tapered raise is prescribed, the measurement under heel and metatarsophalangeal area should be provided [28]. An adequate space inside the footwear should be provided if foot or ankle foot orthoses which encompass the lateral or dorsal areas of the foot are prescribed [29].

- **Girth last allowance** is defined as a decreasing of the last's girth relative to the foot's girth in different sections of the midfoot or forefoot [30]. Together with the shoe's upper material's stiffness it will influence the pressure and friction at the foot-upper interface. Generally, mass produced footwear lasts are made with a smaller ball girth than foot ball girth.

- **Bottom curvature** in the metatarso-phalangeal area (marked with "A" in Figure 1). This curvature is a characteristic of the mass produced lasts. Together with the girth's last allowance it has two main roles: a functional one (to allow the stability of the foot inside the footwear through the reduction of the frictional forces between foot and shoe uppers) and a design role (to create the appearance of a "slim" foot in its largest width area). The curvature of the bottom part of the last has the potential to place the first metatarso-phalangeal joint in a dorsiflexed position related to the second to forth metatarsal joint. The negative effects of this positioning of the metatarsal joints could be the increasing of pressure ~~in~~ under the central metatarsal joint [31] and the facilitation in time of the development of the functional hallux limitus.

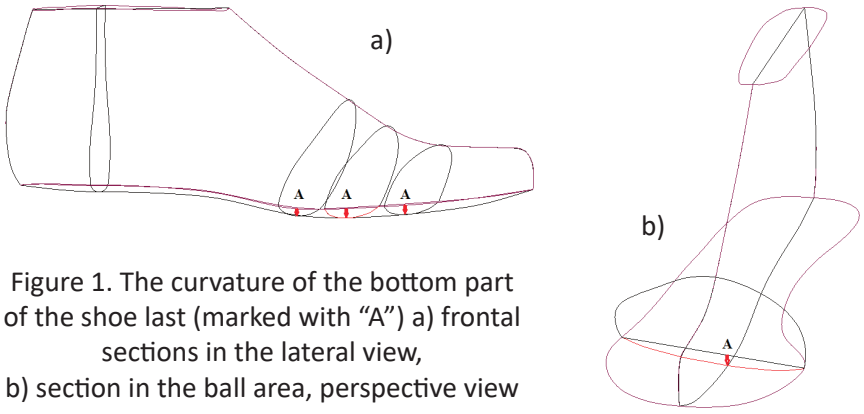


Figure 1. The curvature of the bottom part of the shoe last (marked with "A") a) frontal sections in the lateral view, b) section in the ball area, perspective view

- **3-point force system**. The application of a 3 point force system is mentioned as a potential method to modify the forefoot-rearfoot transversal plane relation [32] but specific data about how a last should be designed are not available. A good reference point could be the experience gained based on the use of the two-piece adjustable orthosis initially set for 20-25° outflare in the case of flexible or rigid metatarsus adductovarus and allowing the manipulation of the position of the forefoot relative to the rearfoot in the transversal plane [33, 34]. Important to be mentioned is that the outflare angle (from 20-25° initially to 45° after 4-6 weeks) and time of wearing (from 21-24 hours/day to 16-18 hours/day) are subjects of prescription [34].

Shoe Sole's Prescription Variables

- **Wedges** are built in the structure of the sole as varus or valgus wedges and placed in the rearfoot or forefoot area. The main purpose is

to modify the position of the center of pressure which is the point of application of ground reaction force [24, 35].

- **Flares** are built on the medial, lateral or posterior walls of the sole allowing more center of pressure displacement with a potential to influence the pronator or supinator moments through modifying the moment arm of the ground reaction vector around various lower extremity joints axis.

- **Wide base** is practically a medial, lateral and posterior flare built on the same sole.

- **Lifts** are used in the case of lower limb discrepancies when this cannot be accommodated only with in-shoe heel lift. The length of the heel lift should be indicated. In the case of a full length lift when a tapered raise is prescribed, the measurement under heel and metatarsophalangeal area should be provided [28].

- **Variable stiffness shoes soles** are built with different stiffness values of the medial and lateral part, with the stiffer lateral one creating a valgus wedge effect in dynamics [36, 37].

- **Rocker soles** are utilized to facilitate the forward movement around the heel, ankle or metatarso-phalangeal joints as pivoting centers or to decrease the plantar pressure under the metatarso-phalangeal joint [6]. As a function of the placement of the rocker point relative to the joint of interest, there are different types: negative heel, double or forefoot rocker. Modifying the angle rocker influences the value of the pressure under metatarso-phalangeal joints [38]. Standard types could be defined according to the joint of interest (ankle joint rocker, Lisfranc rocker, heel rocker, etc.). Materials with different density or stiffness used in different areas of the sole as in the case of MBT shoes have for example the purpose to create instability as the main action of a training device [39]. Changing the rocker sole profile's orientation relative to the line of progression has the potential to influence the ankle joint's range of motion [40].

- **S.A.C.H heel** (Solid Ankle Cushion Heel) – is prescribed for shock absorption purposes being built as an wedge in the proximal area of the sole, from a material with lower stiffness compared with the rest of the sole's material [41]. The same principle behind the S.A.C.H could be applied to prescribe dynamic inverted or everted heels [24, 25].

- **Heel modifications** are known as Thomas heel, having the medial part extended distal or reverse Thomas having the lateral part extended distal. The main purpose is to increase the capacity of body's weight support offered by the footwear and to modify the pronator or supinator moments around foot's joint axes.

- **Bars** are sole modifications with the purpose of redistributing pressure on metatarsal heads (metatarsal bars), supporting the midfoot (Thomas bar) or facilitating the forward movement around the metatarsophalangeal joints as pivoting centers (rockers bar).

- **Sole stiffener** in the form of steel shank or carbon fiber plate has the role to stiffen the sole in order to prevent motions in the different joints of the foot as in the case of the rocker soles [35].

- **Sole material's** stiffness/hardness should be indicated based on range of values (for example 50-60 Shore A) or based on a qualificative (for example: high stiffness, medium stiffness or low stiffness). It has to be indicated when the equilibrium between pronator and supinator moments around subtalar joint can be influenced in a negative way (for example when a foot orthotics is prescribed in order to increase the supinator moments but this effect is canceled or diminished by a low stiffness sole).

- **Medial stabilizer for midfoot area** (buttress) has the role of increasing the supinator moments around subtalar and midtarsal joint through a support placed outside of the shoe in the medial area of the longitudinal arch.

- **Heel height** will give the shoe's final heel height. As described above, in the case of lower limb discrepancy, when a tapered raise is prescribed,

the measurement of the heel height and under metatarsophalangeal area should be provided [28].

- **Shoe's toe rise** represents the height of the distal point of the bottom of the shoe sole. Similar with rocker's angle, its purpose is to facilitate the forward movement around the third pivoting center which are the metatarso-phalangeal joints. It should be mentioned that the shoe toe rise is not to be confused with the last toe rise.

- **Foot drop** is mentioned as a sport footwear characteristic having the potential to influence biomechanics of sport activities [42, 43]. It is defined as the difference between heel and forefoot heights.

Shoe Uppers' Prescription Variables

- **Shoe style** (for example: Derby, Oxford, Mary Jane, sport, etc.) [44]. The upper's prescription variables are essential in the context of the importance of the shoe design and its influence on the patient adherence to the conservative treatment [45]. For this reason, prescription variables which are related to the footwear design should be included in the medical prescription. The decision regarding the shoe style should be taken in agreement with patient's expectation with a positive effect on the footwear acceptance as a treatment option.

- **Shoe type**: high-low quarters (high - for partial foot amputee) [46].

- **Heel stabilizers / counter reinforcement** – in order to increase the pronator or supinator moments around subtalar joint [46, 57].

- **Closing/closure system** (lacing or velcro) – in order to facilitate donning/doffing.

- **Seamless lining** - to protect the dorsal surface of the foot especially in the forefoot area of the diabetic foot.

- **Toe filler** is used to balance the lever arm propulsion in the case of the shorter foot length-equinus or in the case of partial foot-amputations.

- **Flexibile materials** for upper parts - to accommodate local foot deformities (e.g.: a balloon patch).

- **Valgus / varus strap** are used to increase the supinator or pronator moments through a medial or lateral directed force at the level of uppers [47].

- **Padded tongue**, rim/minimal toe puff in order to protect the dorsal surface of the forefoot [47].

Shoe uppers have an increasing potential to alter foot biomechanics [48, 49] even if it is difficult to define clear prescription variables.

PRESCRIPTION FORM AS A COMMUNICATION TOOL BETWEEN FOOT CARE SPECIALISTS

Next to the legal responsibilities related to the content of the medical prescription, it is also an important tool for communication between the foot care team members. Taking into account the experience from the podiatry where the advances in the field of the theoretical models on foot function have led to debates [50] which raise questions regarding the way in which the new concepts are transposed into the practical description of the prescription variables, a simple way of the indication of the design characteristics is proposed:

- the medical footwear manufacturer is proposing his own template with standard definitions of the prescription variables. For example, a standard lateral flare could mean: maximum width of 6 mm in the cuboid area, starting and ending points from the most proximal point to the most distal point of the last bottom center line. A visual representation (sketch or technical drawing) of the manufacturer's standard definition of the prescription variable is recommended;

- when the medical practitioner is prescribing a different geometrical characteristic of a prescription variable, a specific blank template provided by the manufacturer will be used. On this template, the practitioner will provide all the necessary elements for those characteristics. Both the practitioner and the manufacturer should establish a communication protocol which will ensure that the manufacturer has well

understood what the practitioner wants. Such a protocol could include a validation of the virtual design of the custom-made medical footwear prior to entering the manufacturing stage.

It is obvious that the prescriber shall think about and indicate any prescription variables which are considered essential for the medical footwear to achieve its medical objectives even if this variable is not clearly indicated in a prescription form. With the advance of the 3D CAD-CAM technologies, the problem is not whether a specific design could be manufactured but to think about how the functional description of the medical device is translated into prescription variables/design characteristics having the same signification for both the prescriber and the manufacturer.

CONCLUSIONS

This paper has presented a list of basic prescription variables of the medical footwear described in the medical literature and clinical practice. A list of 33 basic prescription variables which define the complexity of the footwear as medical device were identified. In order to avoid confusions in terminology, the term "medical footwear" is proposed as an unifying term for the footwear used as medical device in the conservative treatment of the foot and lower limb pathomechanics. Some of the prescription variables described are difficult to be managed in practice in a prescription form. However, the prescriptioner should be aware of any prescription variable which can influence the effectiveness of the medical footwear. It is the medical practitioner's responsibility to use his knowledge about foot pathomechanics and foot functioning models in order to establish the specific prescription variables of the medical footwear through a medical prescription.

REFERENCES

1. Assistive products for persons with disability - Classification and terminology, EN ISO 9999:**2016**, https://www.iso.org.
2. World Health Organisation, WHO, Priority Assistive Products List, **2016**, http://www.who.int.
3. Healthcare Common Procedure Coding System, HCPCS Code A5501, Diabetic custom molded shoe, https://hcpcs.codes/a-codes/A5501/.
4. Medical-grade Footwear – Prescribing medical aids and equipment, Queensland Government, Australia, https://www.health.qld.gov.au/mass/prescribe/footwear.
5. Frecklington, M., Dalbeth, N., McNair, P., Gow, P., Williams, A., Carroll, M., Rome, K., Footwear interventions for foot pain, function, impairment and disability for people with foot and ankle arthritis: A literature review, Seminars in Arthritis and Rheumatism, Available online 3 November **2017** In Press, Accepted Manuscript, https://doi.org/10.1016/j.semarthrit. 2017.10.017.
6. Hutchins, S., Bowker, P., Geary, N., Richards, J., The Biomechanics and Clinical Efficacy of Footwear Adapted with Rocker Profiles-Evidence in the Literature, *The Foot*, **2009**, 19, 3, 165-70, https://doi.org/10.1016/j.foot.2009.01.001.
7. Hatton, A.L., Rome. K., Dixon, J., Martin, D.J., McKeon, P.O., Footwear Interventions A Review of Their Sensorimotor and Mechanical Effects on Balance Performance and Gait in Older Adults, *J Am Podiatr Med Assoc*, **2013**, Nov-Dec, 103, 6, 516-33.
8. Fisher, D.S., Dyrby, C.O., Mündermann, A., Morag, E., Andriacchi, T.P., In Healthy Subjects without Knee Osteoarthritis, the Peak Knee Adduction Moment Influences the Acute Effect of Shoe Interventions Designed to Reduce Medial Compartment Knee Load, *J Orthop Res*, **2007**, 25, 4, 540-6, https://doi.org/10.1002/jor.20157.

9. Khoury, M., Wolf, A., Debbi, E.M., Herman, A., Haim, A., Foot center of pressure trajectory alteration by biomechanical manipulation of shoe design, *Foot Ankle Int*, **2013**, 34, 4, 593-8, https://doi.org/10.1177/1071100713477613.
10. Directive 2007/47/EC of the European Parliament and of the Council of 5 September 2007, Official Journal of the European Union, 21.9.**2007**, http://eur-lex.europa.eu.
11. Henderson, W.H., Lamoreux, L.W., The Orthotic Prescription Derived from a Concept of Basic Orthotic Functions, *Bull Prosthet Res*, **1969**, Spring, 89-96.
12. Janisse, D., Working with physicians: Footwear Prescriptions, OPEDGE, September **2013**.
13. Fisk, J.R., DeMuth, S., Campbell, J., DiBello, T., Esquenazi, A., Lin, R.S., Malas, B., McGuigan, F.X., Fise, T.F., Suggested Guidelines for the Prescription of Orthotic Services, Device Delivery, Education, and Follow-up Care: A Multidisciplinary White Paper, *Mil Med*, **2016**, Feb, 181 (2 Suppl), 11-7, https://doi.org/10.7205/MILMED-D-15-00542.
14. Prescribers, Enable NSW Health Support Services, New South Wales Government, Australia, http://www.enable.health.nsw.gov.au/home/prescribers.
15. Medical prescription-recommendation on granting medical devices for the rehabilitation of some organic or functional impairments, Annex 39D from the Order no. 763/2016, http://www.cnas.ro.
16. Denischi, A., Medrea, O., Popovici, N., Diseases of the foot (in Romanian), **1964**, Ed. Medicala, Bucharest.
17. Petcu, D., Rosculet, V., Regulation for the prescription of footwear as medical device, *Leather and Footwear Journal*, **2017**, 17, 1, 59-66, https://doi.org/10.24264/lfj.17.1.8.
18. Banwell, H.A., Mackintosh, S., Thewlis, D., Landorf, K.B., Consensus-based Recommendations of Australian Podiatrists for the Prescription of Foot Orthoses for Symptomatic Flexible Pes Planus in Adults, *J Foot Ankle Res*, **2014**, 7, 49, https://doi.org/10.1186/s13047-014-0049-2.
19. Landorf, K., Keenan, A.M., Rushworth, R.L., Foot Orthosis Prescription Habits of Australian and New Zealand Podiatric Physicians, *Am Podiatr Med Assoc*, **2001**, 91, 4, 174-183.
20. Menz, H.B., Allan, J.J., Bonanno, D.R., Landorf, K.B., Murley, G.S., Custom-made foot orthoses: an analysis of prescription characteristics from an Australian commercial orthotic laboratory, *J Foot Ankle Res*, **2017**, 10, 23, https://doi.org/10.1186/s13047-017-0204-7.
21. Kennedy, S., Custom Shoes: The Importance of the Cast, The O&P EDGE, November **2009**.
22. Kramer, C., Treatment of talipes equini: correction options in functional and structural talipes equini, *Foot & Shoe*, **2015**, 1, 26-31.
23. Maurer, G., Case Report - Custom-made footwear after prior trauma, *Foot & Shoe*, **2014**, 2, 20-26.
24. Edwards, C.A., Orthopedic Shoe Technology for the Orthopedic Shoe Technician, Precision Printing Co, 1st edition, **1981**.
25. Edwards, C.A., Orthopedic Shoe Technology Clinical Conditions Requiring Orthopedic Footgear, Precision Printing Co, 1st edition, **1985**.
26. Kapp, S., Cummings, D., Transtibial Amputation: Prosthetic Management, in Atlas of Limb Prosthetics: Surgical, Prosthetic, and Rehabilitation Principles, Rosemont, IL, American Academy of Orthopedic Surgeons, edition 2, **1992**, reprinted 2002.
27. Dananberg, H., Comfortable High Heel Shoe, US Patent US5782015, July, 21, **1998**.
28. Tyrrell, W., Carter, G., Treating patients with complex pathologies in Therapeutic Footwear: A Comprehensive Guide, Elsevier Health Sciences, **2009**, 196-223.
29. Roniger, L., When the shoe doesn't fit: footwear in Down syndrome, *Lower Extremity Review*, May, **2016**.

30. Chen, R., An investigation into shoe last design in relation to foot measurement and shoe fitting for orthopaedic footwear, PhD Thesis, University of London, June **1993**.
31. Rossi, W.A., Why Shoes Make "Normal" Gait Impossible, Podiatry Management, March **1999**.
32. Maas, J.C., Dallmeijer, A.J., Huijing, P.A., Brunstrom-Hernandez, J.E., van Kampen, P.J., Jaspers, R.T., Becher, J.G., Splint: the efficacy of orthotic management in rest to prevent equinus in children with cerebral palsy, a randomised controlled trial, *BMC Pediatr*, **2012**, 12, 38, https://doi.org/10.1186/1471-2431-12-38.
33. Chen, W., Pu, F., Yang, Y., Yao, J., Wang, L., Liu, H., Fan, Y., Correcting Congenital Talipes Equinovarus in Children Using Three Different Corrective Methods A Consort Study, *Medicine* (Baltimore), **2015**, Jul, 94, 28, e1004, https://doi.org/10.1097/MD.0000000000001004.
34. Allen, W.D., Weiner, D.S., Riley, P.M., The Treatment of Rigid Metatarsus Adductovarus with the Use of a New Hinged Adjustable Shoe Orthosis, *Foot Ankle*, **1993**, Oct, 14, 8, 450-4.
35. Kennedy, S., If the Shoe Fits… Pedorthic Modifications for Optimal Function - The O&P EDGE, April, **2006**.
36. Bennell, K.L., Kean, C.O., Wrigley, T.V., Hinman, R.S., Effects of a Modified Shoe on Knee Load in People with and Those without Knee Osteoarthritis, *Arthritis & Rheumatism*, **2013**, 65, 3, 701–9, https://doi.org/10.1002/art.37788.
37. Kean, C.O., Bennell, K.L., Wrigley, T.V., Hinman, R.S., Modified Walking Shoes for Knee Osteoarthritis: Mechanisms for Reductions in the Knee Adduction Moment, *J Biomech*, **2013**, 46, 12, 2060–66, https://doi.org/10.1016/j.jbiomech.2013.05.011.
38. Preece, S.J., Chapman, J.D., Braunstein, B., Brüggemann, G.P., Nester, C.J., Optimisation of rocker sole footwear for prevention of first plantar ulcer: comparison of group-optimised and individually-selected footwear designs, *J Foot Ankle Res*, **2017**, 10, 27, https://doi.org/10.1186/s13047-017-0208-3.
39. Nigg, B., Federolf, P.A., von Tscharner, V., Nigg, S., Unstable shoes: functional concepts and scientific evidence, *Footwear Sci*, **2012**, 4, 2, 73-82.
40. Petcu, D., Bucur, D., Karavana, H.A., Nistor, M., Pantazi, M., Georgescu, M., The influence of the rocker sole profile's orientation on the sagittal ankle range of motion, The 4th IEEE International Conference on E-Health and Bioengineering - EHB 2013, Iasi, Romania, November 21-23, **2013**.
41. Wu, W.L., Rosenbaum, D., Su, F.C., The Effects of Rocker Sole and SACH Heel on Kinematics in Gait, *Med Eng Phys*, **2004**, 26, 8, 639–46, https://doi.org/10.1016/j.medengphy.2004.05.003.
42. Herbaut, A., Simoneau, E., Barbier, F., Roux, M., Guéguen, N., Chavet, P., Lower shoe drop can reduce impact forces experienced by junior tennis players performing an open-stance forehand, *Footwear Sci*, **2015**, 7, sup1, S112-S113, https://doi.org/10.1080/19424280.2015.1038638.
43. Besson, T., Morio, C., Rossi, J., Effects of shoe drop on running mechanics in women, *Comput Methods Biomech Biomed Engin*, **2017**, 20, sup. 1, 19-20, https://doi.org/10.1080/10255842.2017.1382840.
44. Tyrrell, W., Carter, G., It's not 'just a pair of shoes' in Therapeutic Footwear A Comprehensive Guide, Elsevier Health Sciences, **2009**, 1-18.
45. Boulton, A.J., Jude, E.B., Therapeutic footwear in diabetes: the good, the bad, and the ugly? *Diabetes Care*, **2004**, Jul, 27, 7, 1832-3.
46. Kirby, K.A., Shoes and therapy in the treatment of posterior tibial dysfunction, *Precision Intricast Newsletter*, Precision Intricast Inc., Payson, Arizona, May, **2000**, 105-106.
47. Zamosky, I., Shoe modifications in lower-extremity orthotics, *Bull Prosthet Res*, **1964**, 10, 2, 54–95.

48. Nigg, B., Biomechanics of Sport Shoes, Edited by Benno Nigg, Printed in Canada by Topline Printing Inc. Calgary, Alberta, **2010**, ISBN: 978-0-9867421-0-1.
49. Villwock, M.R., Meyer, E.G., Powell, J.W., Fouty, A.J., Haut, R.C., Football Playing Surface and Shoe Design Affect Rotational Traction, *Am J Sports Med*, **2009**, Mar, 37, 3, 518-25, https://doi.org/10.1177/0363546508328108, Epub 2009 Jan 23.
50. Groner, C., Study challenging Root concepts reignites debate, *Lower Extremity Review*, June **2017**.

THE EFFECT OF FOOT TYPE ON THE FOOT MORPHOLOGY AND PLANTAR PRESSURE DISTRIBUTIONS OF OBESE CHILDREN

Shiyang YAN[1], Yue WANG[2], Ruoyi LI[1], Linshan ZHANG[2], Wuyong CHEN[1], Luming YANG[1]

[1]Key Laboratory of Leather Chemistry and Engineering (Sichuan University), Ministry of Education, Chengdu, China

[2]National Engineering Laboratory for Clean Technology of Leather Manufacture, Chengdu, China

Received: 03.08.2017 Accepted: 16.11.2017 https://doi.org/10.24264/lfj.17.4.6

THE EFFECT OF FOOT TYPE ON THE FOOT MORPHOLOGY AND PLANTAR PRESSURE DISTRIBUTIONS OF OBESE CHILDREN

ABSTRACT. Obese children with flatfoot tolerate not only the excessive load, but also the foot structure deformity. The purpose of this study is to investigate the effect of different foot types on the foot morphology and plantar pressure distributions of obese children. Relative data of 30 obese children with flatfoot and another matched 30 obese children with normal arch foot were recorded. When the foot measures were normalized to the length of the foot, significantly lower instep height and longer instep circumference were found in obese children with flatfoot. Obese children with flatfoot displayed significantly greater pressure rate and force-time integral beneath the midfoot region, while displayed decreased load pattern beneath the 5th metatarsal region. Small variations in foot morphology must be incorporated to the last design to meet the comfort and functionality requirements of specialized shoe for obese children with flatfoot. Obese children with flatfoot could be at an increased risk for midfoot injuries such as stress fractures.

KEY WORDS: flatfoot, obese children, foot morphology, dynamic plantar pressure distribution

INFLUENȚA TIPULUI DE PICIOR ASUPRA MORFOLOGIEI PICIORULUI ȘI DISTRIBUȚIEI PRESIUNII PLANTARE LA COPIII OBEZI

REZUMAT. Copiii obezi cu picior plat tolerează nu numai sarcina excesivă, ci și deformarea structurii piciorului. Scopul acestui studiu este de a investiga influența diferitelor tipuri de picior asupra morfologiei piciorului și distribuției presiunii plantare la copiii obezi. Au fost înregistrate datele a 30 de copii obezi cu picior plat și 30 de copii obezi cu boltă plantară normală. După normalizarea măsurătorilor privind lungimea piciorului, la copiii obezi cu picior plat s-a constatat înălțimea semnificativ mai mică a căputei și circumferința mai mare a căputei. Copiii obezi cu picior plat au prezentat o rată de presiune și o integrală forță-timp semnificativ mai mari în regiunea mediană a piciorului, în timp ce sarcina a fost redusă în regiunea celui de-al 5-lea metatarsian. Micile variațiuni în morfologia piciorului trebuie să fie încorporate în designul calapodului pentru a satisface cerințele de confort și de funcționalitate ale pantofilor specializați pentru copiii obezi cu picior plat. Copiii obezi cu picior plat ar putea prezenta un risc crescut de leziuni în regiunea mediană a piciorului, cum ar fi fracturile de stres.

CUVINTE CHEIE: picior plat, copii obezi, morfologia piciorului, distribuția presiunii plantare în dinamică

L'EFFET DU TYPE DE PIED SUR LA MORPHOLOGIE DU PIED ET LA DISTRIBUTION DE LA PRESSION PLANTAIRE CHEZ LES ENFANTS OBÉSES

RÉSUMÉ. Les enfants obèses avec le pied plat tolèrent non seulement la charge excessive, mais aussi la déformation de la structure du pied. Le but de cette étude a été d›étudier l›effet de différents types de pieds sur la morphologie du pied et la répartition de la pression plantaire chez les enfants obèses. Les données relatives à 30 enfants obèses avec le pied plat et 30 autres enfants obèses avec une voûte plantaire normale ont été enregistrées. Les mesures ont été normalisées à la longueur du pied et chez les enfants obèses avec le pied plat ont a trouvé la hauteur de la cambrure significativement inférieure et la circonférence de la cambrure plus longue. Les enfants obèses avec le pied plat ont montré un taux de pression et une force-temps significativement plus importants sous la région du milieu du pied, tandis que la charge a été réduite sous la région du 5ème métatarse. Les petites variations dans la morphologie du pied doivent être intégrées à la conception des formes pour les chaussures pour répondre aux exigences de confort et de fonctionnalité de la chaussure spécialisée pour les enfants obèses avec le pied plat. Les enfants obèses avec le pied plat pourraient être plus à risque de subir des blessures au mi-pied comme des fractures de stress.

MOTS CLÉS : pied plat, enfants obèses, morphologie du pied, répartition dynamique de la pression plantaire

** Correspondence to: Luming YANG, Key Laboratory of Leather Chemistry and Engineering (Sichuan University), Ministry of Education, Sichuan University, No.24 in South pare of First ring road, Wuhou district, Chengdu, Sichuan, China, e-mail: ylmll1982@126.com*

INTRODUCTION

Childhood obesity is perceived as a great possibility of becoming adult obesity. Obese children's implication in orthopaedic problems is mainly associated with excessive and repetitive load. Particularly, the association of foot structure, especially of flatfoot, and children with obesity has been widely researched [1-5]. Sadeghi-Demneh *et al*. [4] and Pfeiffer *et al*. [6] have demonstrated the significant association of body mass with flatfoot in school- and preschool-aged children. Numerous studies have acknowledged that with increasing the body mass, the proportion of children with flatfoot increased [6-9].

Therefore, flatfoot presents a high incidence in obese children. Obese children with flatfoot suffer not only greatly from the excessive body mass, but also deformity in the foot structure. It has been found that individuals with flatfoot were at a high risk for the development of lower limbs injuries, such as metatarsal stress fractures, iliotibial band syndrome, and patellofemoral pain syndrome [10]. Besides, previous literature has indicated that individuals with flatfoot could be at an increased risk for the development of medial and lateral midfoot injuries [11]. However, Chuckpaiwong *et al*. [12] focused on altered plantar loading patterns as it is related to foot type and reported that individuals with flatfoot were at a lower risk for lateral column metatarsal stress fractures.

Flatfoot, as a typical consequence of foot deformity for obese children, should be further investigated in the future study. However, to date, the variations in foot morphology and dynamic plantar pressure distribution of obese children with flatfoot have not been discussed in previous study. This study was designed to investigate the variations in foot morphology and plantar pressure of obese children with flatfoot relative to the obese children with normal arch foot. The purpose of this pilot study was to investigate the effect of foot types (lower arch type and normal arch type) on the foot morphology and dynamic plantar pressure distribution of obese children. Are obese children with flatfoot at a high risk for foot deformity and foot injuries?

METHODS

Participants

All the participants were selected from a foot morphology database of totally 551 children, including 280 boys and 271 girls, aged 7-16 years. All the participants were recruited from consenting primary schools and middle schools, Chengdu and Yantai, China. The arch structure was calculated simultaneously by Foot Angle (FA) and Chippaux-Smirak Index (CSI). Footprints of each subject were classified into high, normal, intermediary, lower and flat arch to characterize the surface area of each child's foot in contact with the ground, following the protocol of Forriol and Pascual [13].

Totally 30 obese children with flatfoot (OFF group) aged 7-14 years (9 girls and 21 boys) without other foot diseases and health problems were selected from this database. Another 30 obese children with normal arch foot (ONAF group) (9 girls and 21 boys), matched to their counterparts for age, gender and body mass index (BMI), were also collected from the same database. Obesity was defined by the body mass index (BMI) reference norm which was established by Group of China Obesity Task Force (GCOTF) [14]. BMI was calculated by the following formula: BMI = weight/height^2 (kg/m^2). Descriptive characteristics were shown in Table 1.

Table 1: Descriptive characteristics[#] of participants

	Group 1	Group 2
Age (year)	9.1 ± 1.9	9.1 ± 1.9
Height (cm)	143.8 ± 10.8	142.0 ± 10.9
Body mass (kg)	53.1 ± 14.9	52.6 ± 15.1
BMI (kg/m^2)	25.2 ± 3.3	25.5 ± 3.6

[#]Values are the mean ± SD.

Group 1: obese children with normal arch foot;
Group 2: obese children with flatfoot.

Experimental Procedures

Each participant's height and body mass were measured respectively to the nearest 0.1 cm and 0.1 kg barefoot by using a calibrated height and weight scale (Omron Corporation, Japan). All the anthropometric measurements were measured by a fixed experimenter.

A three-dimensional scanning system

(Infoot, I-Ware Laboratory, Japan) was used to scan and measure the external foot morphology. The participant was under the weight-bearing condition of half body-weight (HWB) [15], and the external shape of each foot was laser scanned respectively and captured by eight cameras. In the HWB condition, the participant was in a balance standing posture with barefoot and eyes focused straight ahead for equal loads on both foot. Once the experimental device is moved, alignment is performed to the system before a new test. Both feet of each participant were tested only once respectively due to the high precision and high reliability of the device.

A footscan® plantar pressure plate system (RSscan International) was used to test the plantar pressure distribution during walking. This plate system included a 40 cm × 100 cm platform with 4 sensors per square centimetres. A high sample frequency of 253 Hz was selected to record the data of dynamic plantar pressure. This plate was mounted on a smooth and level ground. Each participant was tested using a two-step gait initiation protocol [16]. After several practice runs under the experimenter's guidance, participants were then required to walk barefoot three times or more across the plate at self-preferred speed to complete the test procedure. Participants stood on one side of the walkway and about a meter away from the plate, then went across it to the other side of the walkway. Three trials of a participant were selected as valid by the following criteria: a whole gait cycle on the plate during naturally walking but not aiming at.

Footprints of each participant were taken by using a footprint plate (Bauerfeind AG, Germany). The underside of the membrane was inked and re-inked to keep a clear image before a new record. One piece of pedograph paper was placed beneath the membrane and mounted to the fixed position. The participant put one foot on the surface of the membrane and stood in a HWB condition, then the distinct footprint outline was taken by the experimenter with a special-purpose pen.

Plantar Regions and External Foot Morphology

Three-dimensional foot shape was measured by Delcam SoleEngineer 13.0 Software. Totally 38 foot dimensions were selected, including data of lengths, widths, heights, girths and hallux valgus angle. Totally 10 observed values which are necessary for shoe last design of obese children with flatfoot that are supposed to be valid in this study. Approach to measure the foot morphology is described in Table 2.

Table 2: Approach to measure the foot morphology

Foot Measure	Code	Description
Foot Length	FL	Distance from the heel to the anterior point of the most protruding toe
Medial Ball Length	MBL	Distance from the heel to the 1st MTP* MTP joint protrusion
Lateral Ball Length	LBL	Distance from the heel to the 5st MTP joint protrusion
Forefoot Width	FW	Distance across the 1st MTP joint to 5st MTP joint protrusion on the y-axis
Ball of Foot Circumference	BFC	Circumference over the 1st MTP joint and 5st MTP joint protrusion
Instep Circumference	IC	Circumference over the tarsal protrusion and 5st metatarsal tuberosity point
Navicular Circumference	NC	Circumference over the navicular point to the heel protrusion
Instep Height	IH	Distance from the prominent tarsal point to the ground plane
Navicular Height	NH	Distance from the navicular point to the ground plane
Hallux Valgus Angle	HVA	Angle between the tangent of medial forefoot and hallux and medial tangent of forefoot and heel

Ten plantar anatomical regions were defined by footscan 7 gait 2nd generation (V7. 97) (Figure 1). Biomechanical parameters of peak pressure, contact area, pressure rate, pressure-time integral and force-time integral were extracted from the system for statistical analysis.

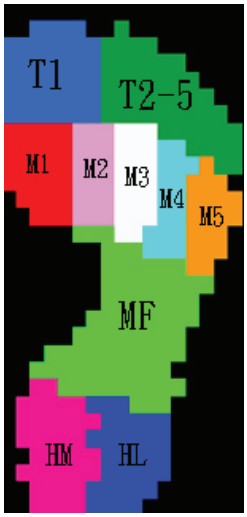


Figure 1. Ten anatomical regions of foot. T1: hallux, T2-5: 2nd-5th toes, M1: 1st metatarsal, M2: 2nd metatarsal, M3: 3rd metatarsal, M4: 4th metatarsal, M5: 5th metatarsal, MF: midfoot, HM: heel medial, HL: heel lateral.

Reliability and Explore Analysis of the Variables

All the foot dimensions were measured manually, so it is necessary to practice a test-retest pilot study before the statistical analyses in this study. Totally 10 children (aged: 7-14, 5 boys and 5 girls) who were unrelated to the main subject cohorts were selected to perform the reliability of the procedures. Intra-class correlation coefficient (ICC) of the 10 anthropometric measurements, excluding hallux valgus angle, ranged from R = 0.939 to 0.943. Therefore, the procedures were considered highly reliable and reproducible.

Explore analysis was conducted to screen the data preliminarily before testing. The outliners and extreme values which were out of the range between 25th and 75th percentiles were eliminated. All the absolute foot measurements were normalized to the respective foot length to eliminate the impact of foot length on other foot measurements.

Statistical Analysis

Hylton [17] has suggested that incorrect results from paired data can be avoided by only selecting one foot. In addition, no significant difference was found between left and right foot for obese children with flatfoot and obese children with normal arch foot respectively in the present study. The author hereby chose right foot (the dominant foot) to perform the statistical analyses. One-simple Kolmogorov-Smirnov test was used to perform all data for normality. The result shows all data comply with normal distribution. Independent t test was then used to perform the statistical analyses. p values less than 0.05 were perceived as significant in all statistical description. SPSS software (version 17.0 for Windows; SPSS Corp., Chicago, IL) was conducted to perform the analysis procedures.

RESULTS

External Foot Morphology

Comparison by arch height with absolute and normalized foot measures, significant differences of foot morphology between the OFF group and the ONAF group are presented in Table 3. In absolute terms, the OFF group displayed significantly lower instep height and navicular height than the ONAF group ($p < 0.01$). When the foot measurements were normalized to the length of the respective foot, statistical analyses indicated that difference of navicular height disappeared, and significantly lower instep height and longer instep circumference were found in the OFF group ($p < 0.001$).

Table 3: Values of absolute and normalized foot measures of ONAF group and OFF group

	ONAF	OFF	*t*-value	*p*-value
Absolute Measures (mm)				
FL	223.9 ± 17.1	220.8 ± 16.3	0.72	0.48
IH	60.1 ± 6.2	55.9 ± 5.1	2.85	< 0.01
NH	73.0 ± 7.0	68.3 ± 6.1	2.80	< 0.01

Foot Measures (% of foot length)				
MBL	75.0 ± 1.3	75.2 ± 1.1	- 0.61	0.54
LBL	65.6 ± 2.2	64.7 ± 1.9	1.67	0.10
FW	39.5 ± 1.1	40.2 ± 1.8	-1.95	0.06
BFC	99.8 ± 3.1	101.0 ± 4.1	- 1.31	0.20
IC	102.2 ± 3.0	105.7 ± 4.0	- 3.75	< 0.001
NC	133.9 ± 13.3	133.6 ± 13.8	0.08	0.94
IH	26.8 ± 1.7	25.4 ± 1.8	3.26	< 0.01
NH	32.8 ± 4.2	31.1 ± 3.5	1.77	0.08

#Values: mean ± SD.
Group 1: obese children with normal arch foot; Group 2: obese children with flatfoot.

Dynamic Plantar Pressure Distribution

Descriptive data of the plantar pressure is presented in Tables 4-5. Statistical analyses indicated that no significant difference for peak pressure was found beneath all plantar regions between the OFF group and the ONAF group, except the elevated peak pressure of the OFF group beneath the 2nd-5th toes region (p = 0.026). Significantly greater pressure rate and larger contact area of the OFF group were found beneath the midfoot region compared to the ONAF group (p = 0.002, p < 0.001).

Table 4: Descriptive statistics# of peak pressure, contact area and pressure rate for ONAF group and OFF group

	Peak Pressure (N/cm²)		Contact Area (cm²)		Pressure Rate (N/cm². ms)	
Regions	ONAF	OFF	ONAF	OFF	ONAF	OFF
T1	4.64 ± 2.21	5.61 ± 2.04	14.37 ± 2.12	14.67 ± 2.31	0.02 ± 0.01	0.02 ± 0.01
T2-5	1.08 ± 0.54	1.50 ± 0.84*	17.85 ± 3.87	19.25 ± 3.81	0.01 ± 0.01	0.02 ± 0
M1	5.05 ± 1.91	5.11 ± 1.84	15.84 ± 2.85	15.94 ± 2.38	0.02 ± 0.01	0.02 ± 0.01
M2	10.87 ± 3.94	10.94 ± 3.38	9.31 ± 1.64	9.32 ± 1.83	0.03 ± 0.02	0.03 ± 0.01
M3	12.28 ± 3.97	12.49 ± 3.94	8.67 ± 1.60	8.60 ± 1.31	0.03 ± 0.02	0.03 ± 0.01
M4	10.46 ± 3.54	9.60 ± 3.26	8.27 ± 1.58	8.36 ± 1.31	0.03 ± 0.01	0.03 ± 0.02
M5	5.73 ± 2.35	4.71 ± 2.08	9.46 ± 1.91	9.48 ± 1.22	0.02 ± 0.01	0.02 ± 0.01
MF	3.42 ± 1.19	3.96 ± 1.05	36.16 ± 7.72	43.80 ± 7.23**	0.02 ± 0.01	0.04 ± 0.02**
HM	9.48 ± 2.48	9.15 ± 1.79	17.17 ± 2.82	16.83 ± 2.91	0.18 ± 0.15	0.22 ± 0.19
HL	9.05 ± 2.04	8.58 ± 1.60	14.80 ± 2.47	14.28 ± 2.42	0.25 ± 0.22	0.34 ± 0.31

#Descriptive statistics: mean ± SD.
**Significant difference (P < 0.01), *Significant difference (P < 0.05).

Significantly greater pressure-time integral of the OFF group was found beneath the 2nd-5th toe region (p = 0.024). Increased force-time integral of the OFF group was found in the 2nd-5th toe (p = 0.014) and midfoot regions (p = 0.034). While significantly greater pressure-time integral and force-time integral were found for the ONAF group beneath the 5th metatarsal region.

Table 5: Descriptive statistics# of pressure-time integral and force-time integral for ONAF group and OFF group

	Pressure-Time Integral (N/cm². s)		Force-Time Integral (N· s)	
Regions	ONAF	OFF	ONAF	OFF
T1	1.37 **± 0.80**	1.74 **± 0.82**	20.20 **± 11.64**	26.28 **± 13.59**
T2-5	0.25 **± 0.14**	0.37 **± 0.25***	4.91 **± 3.28**	7.73 **± 5.11***
M1	1.70 **± 0.76**	1.79 **± 0.88**	28.02 **± 12.82**	30.04 **± 14.63**

M2	3.84 ± **1.57**	3.78 ± **1.44**	37.71 ± **18.63**	36.82 ± **16.29**
M3	4.44 ± **1.58**	4.25 ± **1.29**	41.20 ± **20.20**	38.29 ± **14.17**
M4	3.86 ± **1.28**	3.41 ± **1.01**	34.05 ± **14.16**	29.76 ± **10.86**
M5	1.95 ± **0.90**	1.51 ± **0.60***	19.71 ± **10.44**	15.10 ± **6.97***
MF	1.22 ± **0.57**	1.38 ± **0.48**	47.48 ± **29.85**	64.0 ± **28.99***
HM	3.15 ± **0.94**	2.77 ± **0.97**	56.58 ± **21.50**	49.88 ± **25.01**
HL	2.95 ± **0.90**	2.51 ± **0.96**	45.44 ± **16.95**	38.25 ± **19.69**

[#]Descriptive statistics: mean ± SD.

[**]Significant difference ($P < 0.01$), [*]Significant difference ($P < 0.05$).

DISCUSSION

This study, to our knowledge, is the first study to explain the effect of different foot type (flat or normal) on the foot morphology and dynamic plantar pressure distributions of obese aged 7-14 years. The results of the study indicated that different foot type can influence the foot morphology and foot loading patterns in the specific foot regions.

From the results of absolute foot measurements, ONAF group displayed greater instep height and navicular height. However, when the foot measurements were normalized to the foot length, the significant difference of navicular height disappeared. Furthermore, another significantly longer instep circumference of the OFF group showed up. It is speculated that lower instep height for OFF group might be attributable to the deformity of arch structure. Mickle et al. [18] measured the foot anthropometry and arch index of 19 preschool children with overweight/obesity, their findings confirmed that the flatter foot of children with overweight/obesity was caused by the arch structure change in the midfoot region. The result of the study also showed that OFF group displayed longer instep circumference. It is speculated that as a crucial foot anthropometry, the increase of longer instep circumference can result in the increase of midfoot width. A detailed knowledge involved in matching the foot to the construction of the shoe is of prime importance, it is perceived as a prerequisite for functional design of footwear [19]. It is known to all that the foot is very sensitive to the tiny changes in the shoe's structure. Obese children with flatfoot tolerate heavier loading and suffers more pain beneath their foot, so it is necessary to design specific shoe lasts for them. However, to our knowledge, only foot measurements in length and in width were considered into the shoe last design in the traditional way. In this study, it is suggested that foot measurements in girth and in height should be incorporated into shoe last design to further improve the shoe last for obese children with flatfoot. Small variations in foot morphology must be considered into the shoe last design to match the foot shape in order to protect the foot well.

Several studies have indicated the effect of foot type on development of lower extremity overuse injuries [10, 20, 21]. However, no consensus has been reached in the previous studies with respect to the effect of foot type on the risk of foot and ankle injuries. The greater peak pressure was found for the OFF group beneath the 2nd-5th toe region compared to their counterparts, while Chuckpaiwong et al. [12] and Queen et al. [11] indicated that no significant shift in lesser toes loading between the participants with different foot type. Due to the different BMI reference norm, it is speculated that testing conditions such as racial background and environmental factors, rather than BMI alone, can probably explain different foot loading pattern among different population.

The significantly greater pressure-time integral and force-time integral were found for the OFF group beneath the 2nd-5th toe region, while a decrease was found beneath the 5th metatarsal region. These results were in line with the previous studies which indicated that individuals with a flat foot could be at a lower risk for lateral column metatarsal stress fractures [12]. The results of this study indicated a greater force-time integral beneath the midfoot region of the OFF group, which implied a potential higher

risk for midfoot injuries than the ONAF group. A previous study has indicated that increased force-time integral can imply the potential damage to the foot structure [25]. Therefore, collapse of arch structure can deteriorate the plantar load condition of obese children with flatfoot during walking. Pressure rate is an relative parameter of plantar pressure used to describe the rate of pressure changes per millisecond and assess cushioning function of the foot [26]. Significantly increased pressure rate found for the OFF group beneath the midfoot region further confirms that midfoot has a high risk of arch collapse and stress fractures.

CONCLUSIONS

This study investigated the effects of foot type on foot morphology and dynamic plantar pressure distributions of obese children. From the results, we can conclude that small variations in foot morphology must be considered into the orthopaedic shoe last design to assist the treatment of flatfoot. Moreover, obese children with flatfoot more likely suffer from pain in the midfoot region during walking, because they not only have to tolerate excessive body mass, but also be at a high risk of foot pathology. Therefore, obese children with flatfoot could be at an increased risk for midfoot injuries such as stress fractures which can adversely affect the foot development of children.

Acknowledgments

The present study obtained financial support from National Natural Science Foundation of China, grant number: 11502154. The authors would like to acknowledge all the experimenters for subject recruitment and data collection, and to acknowledge all the participants who made this study possible. No writing assistance we had in this paper.

REFERENCES

1. Chen, K.C., Yeh, C.J., Tung, L.C., Yang, J.F., Yang, S.F., Wang, C.H., Relevant factors influencing flatfoot in preschool-aged children, *Eur J Pediatr*, **2011**, 170, 7, 931-6, https://doi.org/10.1007/s00431-010-1380-7.
2. El, O., Akcali, O., Kosay, C., Kaner, B., Arslan, Y., Sagol, E. *et al*., Flexible flatfoot and related factors in primary school children: a report of a screening study, *Rheumatol Int*, **2006**, 26, 11, 1050-3, https://doi.org/10.1007/s00296-006-0128-1.
3. Evans, A.M., The paediatric flat foot and general anthropometry in 140 Australian school children aged 7 - 10 years, *J Foot Ankle Res*, **2011**, 4, 1, 12, https://doi.org/10.1186/1757-1146-4-12.
4. Sadeghi-Demneh, E., Jafarian, F., Melvin, J.M., Azadinia, F., Shamsi, F., Jafarpishe, M., Flatfoot in school-age children: prevalence and associated factors, *Foot Ankle Spec*, **2015**, 8, 3, 186-93, https://doi.org/10.1177/1938640015578520.
5. Villarroya, M.A., Esquivel, J.M., Tomas, C., Moreno, L.A., Buenafe, A., Bueno, G., Assessment of the medial longitudinal arch in children and adolescents with obesity: footprints and radiographic study, *Eur J Pediatr*, **2009**, 168, 5, 559-67, https://doi.org/10.1007/s00431-008-0789-8.
6. Pfeiffer, M., Kotz, R., Ledl, T., Hauser, G., Sluga, M., Prevalence of flat foot in preschool-aged children, *Pediatrics*, **2006**, 118, 634-639, https://doi.org/10.1186/1757-1146-2-25.
7. Chang, J.H., Wang, S.H., Kuo, C.L., Shen, H.C., Hong, Y.W., Lin, L.C., Prevalence of flexible flatfoot in Taiwanese school-aged children in relation to obesity, gender, and age, *Eur J Pediatr*, **2010**, 169, 4, 447-52, https://doi.org/10.1007/s00431-009-1050-9.
8. Mauch, M., Grau, S., Krauss, I., Maiwald, C., Horstmann, T., Foot morphology of normal, underweight and overweight children, *Int J Obes (Lond)*, **2008**, 32, 7, 1068-75, https://doi.org/10.1038/ijo.2008.52.
9. Chen, J.P., Chung, M.J., Wang, M.J., Flat foot prevalence and foot dimensions of 5- and 13-year-old children in Taiwan, *Foot Ankle Int*, **2009**, 30, 326-332, https://doi.org/10.3113/FAI.2009.0326.

10. Kaufman, K.R., Brodine, S.K., Shaffer, R.A., Johnson, C.W., Cullison, T.R., The effect of foot structure and range of motion on musculoskeletal overuse injuries, *Am J Sports Med*, **1999**, 27, 5, 585-93, https://doi.org/10.1177/03635465990270050701.
11. Queen, R.M., Mall, N.A., Nunley, J.A., Chuckpaiwong, B., Differences in plantar loading between flat and normal feet during different athletic tasks, *Gait Posture*, **2009**, 29, 4, 582-6, https://doi.org/10.1016/j.gaitpost.2008.12.010.
12. Chuckpaiwong, B., Nunley, J.A., Mall, N.A., Queen, R.M., The effect of foot type on in-shoe plantar pressure during walking and running, *Gait Posture*, **2008**, 28, 3, 405-11, https://doi.org/10.1016/j.gaitpost.2008.01.012.
13. Forriol, F., Pascual, J., Footprint analysis between three and seventeen years of age, *Foot Ankle Int*, **1990**, 11, 101-104, https://doi.org/10.1177/107110079001100208.
14. Force GoCOT, Body mass index reference norm for screening overweight and obesity in Chinese children and adolescents, *Chin J Epidemiol*, **2004**, 25, 97-102.
15. Xiong, S., Goonetilleke, R.S., Zhao, J., Li, W., Witana, C.P., Foot deformations under different load-bearing conditions and their relationships to stature and body weight, *Anthropol Sci*, **2009**, 117, 2, 77-88, https://doi.org/10.1537/ase.070915.
16. Hallemans, A., De Clercq, D., Van Dongen, S., Aerts, P., Changes in foot-function parameters during the first 5 months after the onset of independent walking: a longitudinal follow-up study, *Gait Posture*, **2006**, 23, 2, 142-8, https://doi.org/10.1016/j.gaitpost.2005.01.003.
17. Menz, H.B., Two feet, or one person? Problems associated with statistical analysis of paired data in foot and ankle medicine, *The Foot*, **2004**, 14, 1, 2-5, https://doi.org/10.1016/S0958-2592(03)00047-6.
18. Mickle, K.J., Steele, J.R., Munro, B.J., The Feet of Overweight and Obese Young Children: Are They Flat or Fat?, *Obesity*, **2006**, 14, 1945-1953, https://doi.org/10.1038/oby.2006.227.
19. Hawes, M.R., Sovak, D., Quantitative morphology of the human foot in a North American population, *Ergonomics*, **1994**, 37, 7, 1213-26, https://doi.org/10.1080/00140139408964899.
20. Kanatli, U., Yetkin, H., Yalcin, N., The relationship between accessory navicular and medial longitudinal arch: evaluation with plantar pressure distribution measurement system, *Foot Ankle Int*, **2003**, 24, 6, 486-489, https://doi.org/10.1177/107110070302400606.
21. Ledoux, W.R., Hillstrom, H.J., The distributed plantar vertical force of neutrally aligned and pes planus feet, *Gait Posture*, **2002**, 15, 1, 1-9.
22. Kim, H.W., Weinstein, S.L., Flatfoot in children: Differential diagnosis and management, *Current Orthopaedics*, **2000**, 14, 6, 441-447, https://doi.org/10.1054/cuor.2000.0156.
23. Kanatli, U., Yetkin, H., Cila, E., Footprint and radiographic analysis of the feet, *J Pediatr Orthop*, **2001**, 21, 2, 225-8.
24. Harris, E.J., Vanore, J.V., Thomas, J.L., Kravitz, S.R., Mendelson, S.A., Mendicino, R.W. *et al*., Diagnosis and treatment of pediatric flatfoot, *J Foot Ankle Surg*, **2004**, 43, 6, 341-73, https://doi.org/10.1053/j.jfas.2004.09.013.
25. Filippin, N., Barbasa, V., Sacco, I., Lobo da Costa, P., Effect of obesity on plantar pressure distribution in children, *Braz J Phys Ther*, **2007**, 11, 495-501.
26. Yan, S.H., Zhang, K., Tan, G.Q., Yang, J., Liu, Z.C., Effects of obesity on dynamic plantar pressure distribution in Chinese prepubescent children during walking, *Gait Posture*, **2013**, 37, 1, 37-42, https://doi.org/10.1016/j.gaitpost.2012.05.018.

THERMALLY RESISTANT POLYMER COMPOSITES REINFORCED WITH FIBREGLASS

Mihai GEORGESCU*, Maria SÖNMEZ, Mihaela NIŢUICĂ, Laura ALEXANDRESCU, Dana GURĂU

INCDTP – Division: Leather and Footwear Research Institute, 93 Ion Minulescu, Bucharest, Romania,
mihai.georgescu@icpi.ro, mihai.georgesku@yahoo.com

Received: 29.05.2017 Accepted: 30.11.2017 https://doi.org/10.24264/lfj.17.4.7

THERMALLY RESISTANT POLYMER COMPOSITES REINFORCED WITH FIBREGLASS

ABSTRACT. The aim of this paper was to obtain and characterize polymeric composites based on polyamide (PA) and polycarbonate (PC), reinforced with chemically activated surface fibreglass (FG), with advanced properties and temperature resistance. Polyamide was employed as the polymeric matrix, and the polycarbonate as disperse phase. As PA and PC are immiscible due to the polarity differences, a polyoxazoline compatibiliser was used. Afterwards, fibreglass was introduced in this composite. However, in order to obtain these properties, many variations of recipes had to be tested, with different concentrations of the components. The starting point was a mixture of 70% PA and 30% PC. The amount of compatibiliser varied up to 5%, and the FG amount, up to 30%. The characteristics of these polymeric composites with compatibiliser and simple and treated fibreglass were studied.
KEY WORDS: polymeric composites, polyamide, polyethylene, fibreglass

COMPOZITE POLIMERICE TERMOREZISTENTE RANFORSATE CU FIBRĂ DE STICLĂ

REZUMAT. Scopul acestui articol a fost realizarea şi caracterizarea compozitelor polimerice pe bază de poliamidă (PA) şi policarbonat (PC), armate cu fibre de sticlă cu suprafeţe activate chimic (FG), cu caracteristici performante şi rezistente la temperatură. A fost utilizată ca matrice polimerică poliamida, iar faza dispersă a fost policarbonatul. PA şi PC fiind nemiscibile datorită diferenţelor de polaritate, a fost utilizat un compatibilizator pe bază de polioxazolină. În acest compozit s-au introdus, apoi, fibre de sticlă. Totuşi, în vederea obţinerii acestor proprietăţi au trebuit testate mai multe variante de reţete cu diferite concentraţii ale componenţilor. Astfel s-a pornit de la un amestec de 70% PA şi 30% PC. Cantitatea de compatibilizator a variat până la 5%, iar cea de FG până la 30%. Au fost studiate caracteristicile acestor compozite polimerice cu compatibilizator precum şi cu fibre de sticlă funcţionalizate şi nefuncţionalizate.
CUVINTE CHEIE: compozite polimerice, poliamidă, polietilenă, fibră de sticlă

COMPOSITES POLYMÈRES THERMOSTABLES RENFORCÉS AUX FIBRES DE VERRE

RÉSUMÉ. Le but de cet article était de concevoir et de caractériser des composites polymères à base de polyamide (PA) et polycarbonate (PC), renforcés aux fibres de verre activées chimiquement (FG) avec des caractéristiques de haute performance et de résistance à la température. La polyamide a été utilisée comme matrice polymère et la phase dispersée était le polycarbonate. PA et PC étant non miscibles en raison des différences de polarité, un agent de compatibilité à base de polyoxazoline a été utilisé. La fibre de verre a ensuite été introduite dans ce composite. Cependant, afin d'obtenir ces propriétés, plusieurs variantes de recettes avec différentes concentrations des composants ont dû être testées. On a parti d'un mélange de 70% PA et 30% PC. La quantité d'agent de compatibilité a varié jusqu'à 5% et la quantité de fibres de verre jusqu'à 30%. Les caractéristiques de ces composites polymères avec l'agent de compatibilité ainsi qu'aux fibres de verre fonctionnalisées et non-fonctionnalisées ont été étudiées.
MOTS CLÉS : composites polymères, polyamide, polyéthylène, fibre de verre

* *Correspondence to: Mihai GEORGESCU, INCDTP – Division: Leather and Footwear Research Institute, 93 Ion Minulescu, Bucharest, Romania, mihai.georgescu@icpi.ro, mihai.georgesku@yahoo.com*

INTRODUCTION

Composites can be defined as materials that consist of two or more chemically and physically different phases separated by a distinct interface. The different systems are combined judiciously to achieve a system with more useful structural or functional properties non-attainable by any of the constituents alone. Composites are becoming an essential part of today's materials due to the advantages such as low weight, corrosion resistance, high fatigue strength, and faster assembly. They are extensively used as materials in producing advanced structures [1, 2].

Composites are combinations of materials differing in composition, where the individual constituents retain their separate identities. These separate constituents act together to give the necessary mechanical strength or stiffness to the composite part. A composite is a material composed of two or more distinct phases (matrix phase and dispersed phase) and having bulk properties significantly different from those of any of the constituents. Matrix phase is the primary phase having a continuous character. Matrix is usually more ductile, a softer phase. It holds the dispersed phase and shares a load with it. Dispersed (reinforcing) phase is embedded in the matrix in a discontinuous form [1].

In this paper polymer blends based on polyamide (PA) and polycarbonate (PC) were obtained. PA is a widespread polymer in the industry with a low processing cost, and was mainly employed due to its high temperature resistance.

Polyamides have good dimensional stability, high rigidity (especially when PA is reinforced with fibreglass), good resistance to compression, wear, shocks and vibrations; they are hard materials, and maintain their hardness and tenacity at high temperatures, with no visible transformations up to 80-90°C [3-9]. PAs are semitransparent in moulded parts with thin wall and opaque in moulded parts with thick wall. Reinforcing polyamides results in improved properties of tensile strength, bending resistance and higher values for elastic modulus and hardness. Applications include mechanical engineering (friction parts, gears, wheel bands), automotive parts (housings, fans, parts with complex forms, fuel tanks, bushings, flexible cabling, brake fluid reservoirs), electrical and electronics parts, household items (fruit juicers, kitchen appliances, handles of tableware), sport and tourism equipment (ski boots, roller skates, tents, climbing ropes and cords, protective helmets, anti-drop system for bicycle chain) [10].

Due to the fibreglass strength it is possible to provide polymer composites with new properties. Fibreglass advantages include effective reinforcing, with minimal impact resistance loss, thermal stability and resistance, improved resistance.

Designing and development of multiphasic polymeric composites are strongly dependent on two major parameters: interface and morphology control. Generally, the term "morphology" refers to the shape and organization at a higher level than the atomic one (e.g. the arrangement of the elastomer molecules in the crystalline or amorphous regions) and how molecules are organized into more complex units. Thus, the morphology of polymeric composites indicates the size, shape and spatial distribution of the component phases. It is known that most of the mechanical, optical, rheological, physical-chemical and dielectric properties of polymer composites are strongly influenced by the type and finesse of phase structure.

PA and PC are immiscible due to differences in polarity, processing temperature and solubility. These factors lead to poor dispersion of PC in the PA matrix. It is necessary that the blend be compatibilised. The compatibilisation must accomplish: a) optimize interfacial tension, b) stabilize the morphology against high stress during forming, and c) enhance adhesion between the phases in the solid state [11, 12].

Grafted copolymers are used on a large scale as compatibilisation agents in blends with other plastic materials. These are, generally, obtained by grafting the free radicals in melt on the main chain. Most commonly used monomers are maleic anhydride, glycidyl methacrylate, vinyl and acrylic monomers and containing reactive functionalized groups. In this paper oxazoline (2-ethyl-2-oxazoline) was used as compatibiliser.

Polymer composites based on reinforced polymer blends are currently considered a new group of materials, required for high-performance applications. In this group of materials, required properties are obtained mainly by selecting the type of polymeric

components, composition, adding fibreglass and processing conditions leading to optimal physical and mechanical properties.

In the last two decades a multitude of new multicomponent polymeric materials have been developed. Multiphase polymer polymeric composites have been identified as the most versatile method to produce new economic thermally resistant polymers that are able to meet the complex requirements of performance.

This paper was aimed at obtaining and characterization of polymeric composites with advanced features resistant to temperature, based on polyamide (PA) and polycarbonate (PC) reinforced with chemically activated surface fibreglass.

MATERIALS AND METHODS

Materials

Materials used were:

- Polyamide - standard PA for injection - Sebamid 6 s3c (Basplast),
- Polycarbonate - CALIBRETM polycarbonate resin, density 1.2g/cm^3 (Trinseo Gmb),
- 2-ethyl-2-oxazoline – Mw-500.000, density: 1.14g/cm^3 (lit) (Sigma-Aldrich Chemistry, USA),
- Simple fibreglass - BMC3 4.5 mm,
- Organosilane - 3-Aminopropyl) trimethoxysilane, Mw-179.29 g/mol, bp: 91-92°C (lit) (Sigma-Aldrich Chemistry, USA).

Method

Formulation of polymeric composites with resistance to high temperature, flame, and impact, a processing technology and formulations for these materials were designed. In order to make the comparison between composites possible, only the type and amount of fibreglass were varied. The ratio between the components of the polymeric composites is as follows: 70% PA, 30% PC, 5% compatibiliser and 10-30% simple and treated fibreglass. In order to select the optimal variants, these new materials were characterized according to the rubber and plastic specific standards.

Table 1: Formulations of polymer composites based on PA/PC/FG

Sample	UM	P_9	P_{10}	P_{11}	P_{12}	P_{13}
Polyamide	%	70	70	70	70	70
Polycarbonate	%	30	30	30	30	30
Oxazoline	%	5	5	5	5	5
Fibreglass (APTMS)	%	-	-	10	-	30
Fibreglass (simple)	%	-	10	-	30	-

The laboratory-scale technological process for polymeric composites reinforced with simple and treated (APTMS) fibreglass is detailed in Figure 1.

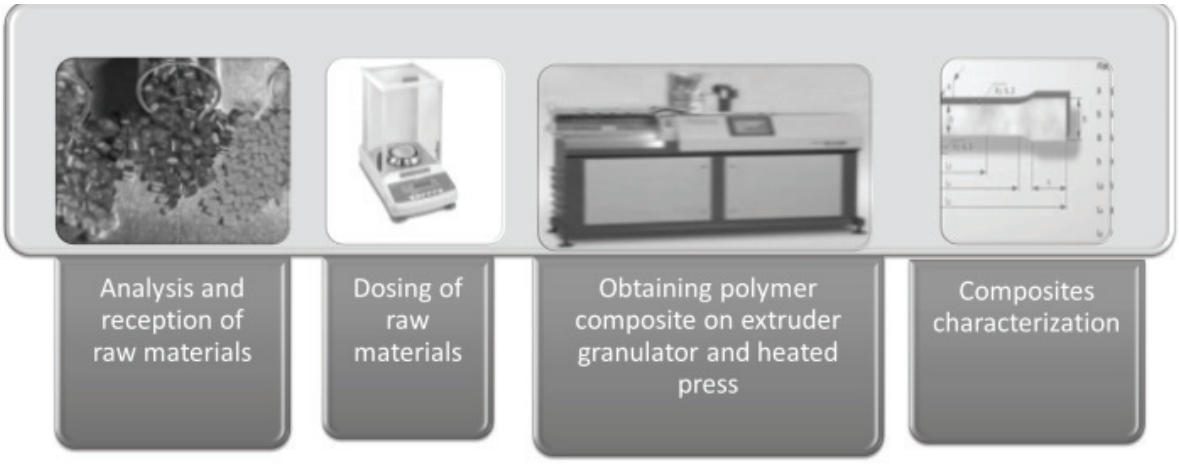


Figure 1. Stages for processing and characterization of the polymeric composites

The formulations in Tables 1 were processed in a twin screw extruder. The method for achieving multiphase polymer composites is as follows:

- Weighing the raw materials, according to the formulations;
- Base components, PA and PC, along with the compatibiliser (oxazoline) are mixed together;
- Temperatures were set according to the Table 2;
- The previously obtained mix is introduced in the mixing chamber, and depending on the formulation, the fibreglass is added.

Processing parameters of the extruder-granulator are shown in Table 2. They were set so as to obtain a homogeneous material, and the size of the composite granules was 3 x 3 mm.

Table 2: Processing parameters

No.	Processing parameters	M.U.	Values
1	Temperature in:	°C	
	Zone I		180
	Zone II		190
	Zone III		200
	Zone IV		210
	Zone V		220
	Zone VI		220
	Zone VII		210
	Zone VIII		200
	Zone IX		200
2	Extruder's twin-screws speed (frequency converter)	Hz	14
3	Extruder's feeding screws speed (frequency converter)	Hz	5.3
4	Cutting wheel speed	RPM	200
5	Running Current Intensity	A	24

RESULTS AND DISCUSSION

Characterisation of Composites

Optical Microscopy Characterisation

Functionalization plays an important role in the fibreglass final properties and implicitly on the polymeric composite embedding them, creating bonds with the polymeric materials used in the composite. Thus, the functioning of the glass fibers used in this paper was performed with 3-aminopropyltriethoxysilane (5%) in ethyl alcohol medium.

To control the functioning of the glass fibers, they were observed microscopically. The microscope used for this purpose was the Leica CME Microscope, with magnification between 40X and 1000X and warm incident light. The images (Figure 2) show the deposition of the functional agent (APTMS) on the fibreglass.

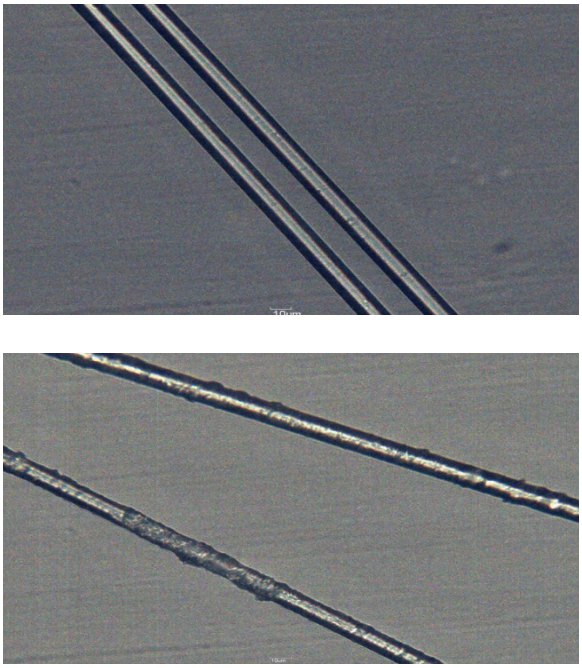


Figure 2. Microscopic images of simple and functionalized glass fibres (400X)

Physical-Mechanical Characterisation

The results of the physical-mechanical properties of the samples obtained for the polymer composites based on PA, compatibiliser, PC, and reinforced with fibreglass, are shown in Table 3.

Physical-mechanical characterization was carried out according to standards in force. Following the basic composites characterization, P9 sample (70% PA / 30% PC / 5% compatibiliser), samples with simple and treated fibreglass (10-30%) were tested.

Table 3: Physical-mechanical characterization of the polymeric composites

Characteristic / Sample	P9	P10	P11	P12	P13
Hardness °Sh D SR ISO 7619-1:2011	80	82	80	81	79
Tensile strength, N/mm^2, (SR ISO 37:2012)	23.8	31.7	40	38.6	47

Hardness

When adding simple fibreglass, hardness increases but using the treated fibreglass instead, the hardness decreases by 1-2°Sh D. Thus, for the polymeric composites containing compatibiliser and treated fibreglass this property decreases by 2-3°Sh D.

Tensile Strength

Tensile strength decreases when mixing the plastomers, PA and PC, based on the ratio used between them. The addition of compatibiliser improves the tensile strength. With the addition of simple fibreglass tensile strength increases compared to P4 formulations. When using treated fibreglass with the compatibiliser, the value of tensile strength greatly improves. In that sense the composites P11 and P13 with 30% fibreglass show the best values of the tensile strength, of 40 N/mm^2 and 47 N/mm^2, respectively.

Melt Flow Index

The materials used have different characteristics and properties, such as colour, density, hardness, different processing temperature, etc. Thus, the melt flow index was determined at the same temperature, of 230°C, and a pressure force of 5 Kg. The values of the melt flow index obtained for the polymeric composites processed in the Brabender mixer are shown in Figure 3.

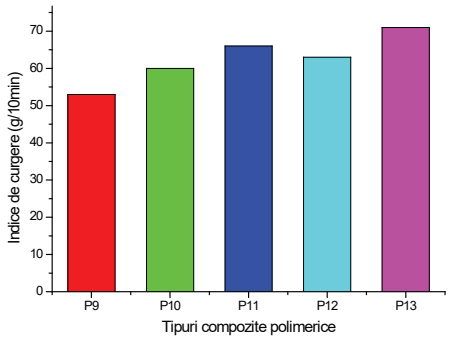


Figure 3. Melt flow index for polymeric composites

It is noted that there are considerable differences between the viscosities of the two base polymers (PA and PC) which affects the flow of material. Thus, the values obtained for PA were 60g/10 min and for PC, 11.7g/10 min. Melt flow index for the obtained composite materials varies widely between these two extremes, due to the base polymer ratio, compatibiliser, and the type of fibreglass. The addition of the treated fibreglass in a proportion of 10% increases the melt index value from 53 to 60g/10min. When using 30% APTMS functionalized fibreglass the melt index value increase to 71g/10min.

This characteristic is important in establishing the processing parameters of the polymeric composites into finished products, on the industrial equipment.

CONCLUSIONS

This paper presents new polyamide (PA) and polycarbonate (PC) thermally resistant polymers with high performance, reinforced glass fibres with chemically activated surfaces to meet the current quality requirements for the automotive industry.

After testing the composite samples the following were found:

- The compounds based on PA, PC thermoplastic polymers, compatibiliser and treated fibreglass have been selected because they will take advantage of the synergy of the two polymers such as chemical resistance, low water permeability, high temperature, flame, and impact resistance;
- The composite testing specimens were obtained in an electrical heated press, by means of compression, between its plates, at a temperature of 220°C and at a pressure of 300 kN;
- Developed formulations for polymer compounds based on 70% PA/ 5% compatibiliser/ 30% PC/ 10-30% simple and treated (APTMS) fibreglass;
- Hardness decreases when adding the compatibiliser, and for the polymeric composites containing compatibiliser and treated fibreglass this property decreases by 2-3°Sh D.
- Tensile strength decreases when mixing the plastomers, PA and PC, based on the ratio used between them. The addition of compatibiliser improves the tensile strength of the composites. The composites P11 and P13 with 5% compatibiliser and

30% treated fibreglass show the best values of the tensile strength, of 40 N/mm^2 and 47 N/mm^2, respectively.

- Melt flow index ranges vary widely for the obtained polymeric composites. Thus, the flow index of composites P9-P13 decreases with increasing percentage of PC. Using an amount of 5% of oxazoline compatibiliser the melt index value increases to 53g/10min. The addition of the treated fibreglass in a proportion of 10% the melt index value increases from 53 to 60g/10min. When using 30% APTMS functionalized fibreglass the melt index value increases to 71g/10min.

The data presented show that the fibreglass functionalized with APTMS favourably influences physical-mechanical and processing properties compared to the simple fibreglass.

Acknowledgements

This research was financed by ANCSI through NUCLEU Program 2016-2017, project code PN16340109: "New Types of Polycarbonate/Polyamide/Fibreglass Compounds Resistant to Impact, Used in Automotive and Electrical Insulators Industries", contract no. 26N/2016.

REFERENCES

1. Jones, R.O., Ballone, P.A., A Combined Density Functional and Monte Carlo Study of Polycarbonate, *Molecules*, **2010**, 15, 3680.
2. Kricheldorf, H., Luebbers, D., Polymers of Carbonic Acid. 3. Thermotropic Polycarbonates Derived from 4,4`-dihydroxybiphenyl and Various Diphenols, *Macromolecules*, **1990**, 23, 10, 2656-2662, https://doi.org/10.1021/ma00212a010.
3. Liaw, D.J., Chang, P., Synthesis and Characterization of Aromatic and Brominated Aromatic Polycarbonates by Two-Phase-Transfer-Catalyzed Polycondensation of Bisphenols with Trichloromethyl Chloroformate, *J Appl Polym Sci*, **1997**, 63, 195-204.
4. Liu, Z., Cunha, A.M., Yi, X.S., Bernardo, A.C., Key Properties to Understand the Performance of Polycarbonate Reprocessed by Injection Molding, *J Appl Polym Sci*, **2000**, 77, 1393-1400.
5. Park, J.H., Hyun, J.C., Kim, W.N., Kim, S.R., Ryu, S.C., Extensional and Complex Viscosities of Linear and Branched Polycarbonates, *Macromol Res*, **2002**, 10, 135-139.
6. Pokharkar, V., Sivaram, S., Poly(alkylene carbonate)s by the Carbonate Interchange Reaction of Aliphatic Diols with Dimethyl Carbonate: Synthesis and Characterization, *Polymer*, **1995**, 36, 4851-4854, https://doi.org/10.1016/0032-3861(95)99302-B.
7. Lee, J., Song, C., Kim, J.I., Kim, J.H., Preparation of Aromatic Polycarbonates Nanoparticles Using Supercritical Carbon Dioxide, *J Nanopart Res*, **2002**, 4, 53-59.
8. Sweileh, B.A., Al-Hiari, Y.M., Kailani, M.H., Mohammad, H.A., Synthesis and Characterization of Polycarbonates by Melt Phase Interchange Reactions of Alkylene and Arylene Diacetates with Alkylene and Arylene Diphenyl Dicarbonates, *Molecules*, **2010**, 15, 5, 3661-3682, https://doi.org/10.3390/molecules15053661.
9. Sathyanarayana, M.N., Yaseen, M., Role of Promoters in Improving Adhesion of Organic Coatings to a Substrate, *Prog Org Coat*, **1995**, 26, 275-313, https://doi.org/10.1016/0300-9440(95)00572-2.
10. Loewenstein, K.L., The Manufacturing Technology of Continuous Glass Fibers, 3rd revised ed., Elsevier, **1993**.
11. Drown, E.K., Moussawi, H.A., Drzal, L., Glass Fiber Sizings and Their Role in Fiber-Matrix Adhesion, Silanes and Other Coupling Agents, Ed. K.L. Mittal, VSP Utrecht, Netherlands, 513-529, **1992**.
12. Sautrot, M., Abel, M.-L., Watts, J.F., Powell, J., Incorporation of an Adhesion Promoter in a Structural Adhesive: Aspects of Durability and Interface Chemistry, *J Adhesion*, **2005**, 81, 2, 163-187, https://doi.org/10.1080/00218460590921995.

METHOD FOR DETERMINATION OF AMINO ACID CONTENT IN PROTEIN PRODUCTS FOR MEDICAL USE

Gabriela MACOVESCU*, Ciprian CHELARU, Dana GURĂU

INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu St., Bucharest, Romania, email: icpi@icpi.ro

Received: 25.10.2017 Accepted: 28.11.2017 https://doi.org/10.24264/lfj.17.4.8

METHOD FOR DETERMINATION OF AMINO ACID CONTENT IN PROTEIN PRODUCTS FOR MEDICAL USE

ABSTRACT. The paper presents a method for determination of amino acid content in collagen biomaterials for medical use produced in the Collagen Department of INCDTP - Division ICPI and its validation. The method has three stages: the first consists in hydrolysing the collagen biomaterial sample down to amino acids; the second step refers to derivatization of amino acids with N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) which is a silylation reagent; derivatized amino acids are detected using a mass spectrometer after gas chromatography. The method was validated to establish performance parameters and to check compliance with the intended purpose.

KEY WORDS: collagen, amino acids, biomaterials

METODĂ PENTRU DETERMINAREA CONŢINUTULUI DE AMINOACIZI DIN PRODUSELE PROTEICE PENTRU UZ MEDICAL

REZUMAT. Se prezintă o metodă de determinare a conţinutului de aminoacizi din biomaterialele colagenice pentru uz medical produse în Departamentul Colagen al INCDTP – Sucursala ICPI şi validarea ei. Metoda are trei etape: prima constă în hidroliza probei de biomaterial colagenic până la stadiul de aminoacizi; a doua etapă se referă la derivatizarea aminoacizilor cu N, O-bis (trimetilsilil) trifluoracetamidă (BSTFA) care este un reactiv de sililare; aminoacizii derivatizaţi sunt detectaţi pe un spectrometru de masă după gaz cromatografie. Metoda a fost validată pentru a se stabili parametrii de performanţă şi pentru verificarea conformării cu scopul propus.

CUVINTE CHEIE: colagen, aminoacizi, biomateriale

PROCÉDÉ DE DÉTERMINATION DE LA TENEUR EN ACIDES AMINÉS DANS DES PRODUITS PROTÉIQUES À USAGE MÉDICAL

RÉSUMÉ. On présente une méthode de détermination de la teneur en acides aminés de biomatériaux de collagène à usage médical fabriqués dans le Département Collagène de INCDTP-ICPI et sa validation. La méthode comporte trois étapes : la première consiste en l'hydrolyse de l'échantillon de biomatériau de collagène au stade des acides aminés ; la deuxième étape se rapporte à la dérivatisation d'acides aminés avec du N, O-bis (triméthylsilyl) trifluoroacétamide (BSTFA) qui est un réactif de silylation ; les acides aminés dérivés sont détectés sur un spectromètre de masse après chromatographie en phase gazeuse. La méthode a été validée pour établir les paramètres de performance et vérifier la conformité avec l'objectif prévu.

MOTS CLÉS : collagène, acides aminés, biomatériaux

** Correspondence to: Gabriela MACOVESCU, INCDTP - Division: Leather and Footwear Research Institute, 93 Ion Minulescu St., Bucharest, Romania, email: icpi@icpi.ro*

INTRODUCTION

Collagen is the main structural protein in soft, lax, semirigid and rigid conjunctive tissues (skin, bones, tendons, basal membranes, etc.), that primarily provides structural integrity to tissues, but also plays an important role in determining the cell phenotype and in cell adhesion.

Due to its excellent biocompatibility and biodegradability, well-defined structure, biological characteristics and to the way it interacts with the body, collagen is always one of the most widely used biomaterials. Extracted as aqueous solution or gel, type I fibrillar collagen may be moulded into various forms: medical devices, artificial implants, drug delivery systems and scaffolds for tissue regeneration, with an important role in today's medicine [1-5].

As a natural protein, collagen cannot heal by itself the infected tissue because bacteria may use it as a substrate. In severe wound infections, systemic drug delivery may lead to an insufficient drug concentration in the infected area or to side effects associated with the drug and/or to systemic toxicity. This deficiency was successfully solved by local drug delivery, developing drug delivery systems with collagen as a substrate and an antibiotic/antiseptic as a drug for infection control [2, 4, 6, 7].

The primary structure of collagen is determined by the sequence of the 20 amino acids that "build" first α-helix polypeptide chains (secondary structure) by peptide bonds, and then the tertiary and quaternary macromolecular superstructure is formed by means of α-helixes.

Amino acids are particularly important for the human body. They are basic structural elements of proteins, compounds with a remarkable biological importance. Amino acids may be obtained from proteins by acid, basic or enzymatic hydrolysis of peptide bonds.

The following amino acids are included in the collagen composition: glycine (1/3), alanine, proline, hydroxyproline (1/3) and other amino acids (1/3). Amino acids are found in a triple helix conformation: two polypeptide chains are identical, and the third slightly differs in the amino acid composition [8-10].

In the polypeptide chain the amino acids form peptide bonds by coupling the carboxyl group to an amino group; once bound in the protein chain, the amino acid "turns" into a "residual" amino acid, and the carbon, nitrogen, hydrogen and oxygen atoms involved in the bonds form the "skeleton" of the protein. As a result, it is necessary to know the amino acid composition of the products for medical purposes when establishing links between the affected tissues and the products used in the treatments.

As a result, a study was conducted on methods of analysis of amino acid content in protein products, biological fluids, foodstuffs and fodders, in order to develop a qualitative and quantitative method for the identification of amino acids in collagen-based medical materials, for better knowledge of product structure, reproducibility of batches and for improving their quality.

The proposed method was validated in order to establish performance parameters and to check compliance to the intended purpose by determining: detection limit, quantification limit, selectiveness, sensitivity, robustness, accuracy and reliability of the method.

The method of amino acid analysis in collagen-based materials for medical use was verified in order to ensure reproducibility and is supported by determinations carried out on samples from the Collagen Department of ICPI.

MATERIALS AND METHOD

Method Principle

Determination of amino acids from collagen materials for medical use is carried out based on the following three basic stages:

a) Hydrolysis of collagen materials for medical use, with 6M hydrochloric acid, for 24 hours at 1000C;

b) The resulting amino acids are evaporated to dryness to remove moisture and resuspended with acetonitrile to derivatize with N, O-bis (trimethylsilyl) trifluoracetamide (BSTFA) which is a silylation reagent;

c) Derivatized amino acids are detected using a mass spectrometer after gas chromatography.

Reagents

Only reagents of known analytical grade and distilled water or demineralized water or

equivalent purity water are used.

- L-alanine (Ala), L-glycine (Gly), L-proline (Pro), L-aspartic acid (Asp), L-glutamic acid (Glu), all these amino acid standards were purchased from Sigma-Aldrich;
- Derivatizing agent N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) purchased from Merck;
- Hydrochloric acid (HCl) 6 M was used as hydrolysis agent (HCl) 0,1 M;
- Acetonitrile, purchased from Merck.

Equipment

- NANOCOLOR VARIO C2 thermoblock for hydrolysis of collagen materials;
- TurboVap II equipment for concentration of samples using nitrogen gas;
- FOCUS GC Gas chromatograph coupled with DSQ II MS mass spectrometer, AutoSampler TriPlus, flame ionization detector, Split/Split less injector, XCalibur Thermo Scientific software, equipped with a non-polar capillary column;
- capillary column: TR 5MS: 5% phenyl – 95% dimethylpolysiloxane, length: 60 m, inner diameter: 0,32 mm, film thickness: 0,25 µm.

WORK METHOD

Sample Preparation

- About 5 mg solid samples were weighed with an accuracy of 0.01 mg and approximately 100 mg liquid samples were weighed with an accuracy of 0.01 mg;
- 1 ml of 6 M hydrochloric acid solution as hydrolysis agent was added, the tube was covered and placed in the aluminium thermoblock at 100°C±20°C for 24 hours for hydrolysis;
- Using a pipette, a volume of 100 µl of hydrolysed is introduced in a vial placed in TurboVap to remove moisture with nitrogen gas;
- The dried amino acid residues were dissolved in a volume of 100 µl of acetonitrile;
- They are derivatized with a volume of 100 µl of N, O-bis (trimethylsilyl) trifluoroacetamide;
- The sealed vial is subjected to ultrasound for 1 minute;
- The vial is placed in the thermoblock at 100°C+2°C for 30 min. to complete the derivatization reaction;
- The vial is placed in the gas chromatograph sample stand;
- 10 injections of 1 µl per sample are performed.

Standard Stock Solutions

Standard amino acid stock solutions were prepared by dissolving each amino acid in 0.1 M HCl down to a concentration of 5 µmol/ml. The solutions were stored at 4°C until analysis. The calibration standards at 4 different concentrations (0.625-5 µmol/ml were prepared using standard amino acid stock solutions.

Standard amino acid samples were processed by dissolving about 0.1 mg of each amino acid in 1 ml of 0.1 M hydrochloric acid. A 100 µl volume was dried under a constant nitrogen stream.

The remaining amino acid was dissolved in a volume of 100 µl acetonitrile and derivatised with a volume of 100 µl of N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and analyzed by GC/MS.

RESULTS AND DISCUSSIONS

Since collagen materials for medical purposes in the form of sponges, gels, atomized or liquid hydrolysates are stabilized for good preservation over time, acid hydrolysis has been chosen until the matrix is destroyed and individual amino acids are released.

Hydrochloric acid has been chosen because it has been found to be the most widely used, with the advantage that it is not an oxidizing acid and can be removed quickly from the system.

Several variants of hydrolysis have been attempted, ranging from 2 hours to 24 hours, and it has been observed that a good separation of the amino acids from the samples requires a longer time, therefore a 24-hour hydrolysis at 100°C was opted for.

N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) (Figure 1) is a common silylation reagent that replaces acidic protons of amino acids (e.g. SH, OH, NH and COOH) with nonpolar trimethylsilane groups (TMS).

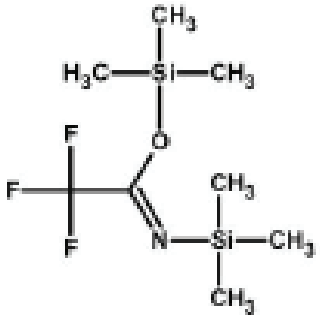
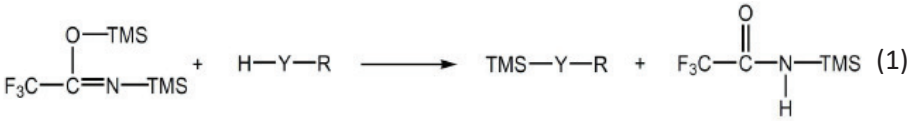


Figure 1. Chemical formula of N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA)

According to some studies, by replacing acidic protons with nonpolar TMS groups, polarity of an amino acid is reduced and its volatility is increased [11-17] which is an advantage in gas chromatographic analysis.



(1)

Equation (1) represents derivatization (silylation reaction) of amino acids in which TMS=Si(CH3)3, Y=O, S, NH, COO and R= alkyl or aryl radical.

The advantage of derivatization with TMS is that it requires a single step, while other derivatization methods usually have two or more reaction steps.

The major drawbacks of TMS derivatives are the sensitivity to moisture and the derivatization of Gly and Glu, which are affected by the polarity of the solvent [18-20]. For example, both the di-trimethylsilyl derivative and the trimethylsilyl derivatives of glycine are obtained after derivatization using acetonitrile or acetone as solvents [18].

Amino acids derivatized with TMS are typically analyzed using gas chromatography coupled with mass spectrometry (GC / MS) and identified according to a combination of retention times and mass spectra [21, 22].

GC / MS is a powerful tool for separating and identifying components in complex mixtures and plays a central role in amino acid analysis, presenting the advantages of improved resolution, sensitivity and quantification of smaller amounts in the sample as well as rapid analysis.

The literature reports contradictory results with regard to optimal amino acid derivatization conditions using BSTFA. These results may be related to several limitations that were observed in those studies, such as the limited number of iterations for each of the derivatization conditions [22-24], using either peak height or peak area without evaluating the calibration linearity, sensitivity, or the limit of detection (LOD), using either SCAN mode or SIM mode, or using relatively long GC temperature programs (e.g. 54 min [25], 62 min [26], and 90 min [27]).

The derivatized amino acids were analyzed with a FOCUS GC gas chromatograph coupled with a DSQ II MS mass spectrometer, TriPlus AutoSampler, flame ionization detector, Split/Split less injector, XCalibur Thermo Scientific software equipped with a nonpolar capillary column: TR 5MS: 5% phenyl – 95% dimethylpolysiloxane, length: 60 m, inner diameter: 0.32 mm, film thickness: 0.25 µm; ultra-high purity helium was used as carrier gas at a constant flow rate of 20 ml/min. The column transfer flux was 1.0 ml/min.

The column temperature was programmed to increase from 700°C to 1700°C at a rate of 100°C/min, and then was raised to 280°C at a rate of 30°C/min, the temperature at which the elution took place for 8 min. Total running time was 21mins 66sec.

The sample was injected at 280°C in split injection mode (ratio 1:20) using an injection volume of 1 µl.

For each sample, triple injections were performed and the results were averages of the three determinations. Acetonitrile was also used as a control solvent before each injection. Three solvent ampoules, namely acetonitrile, methanol, acetone, were used successively as cleaning solvents for the autosampler injection syringe.

Amino acids were detected with a mass spectrometer in automatic scanning mode (SCAN), and the individual identification was performed using the specific retention times of the reference materials and the spectra library of the device. In Selected Ion Monitoring Mode (SIM), identification was made based on specific fragments of maximum intensity.

The mass spectrometer operated in full scan mode (SCAN) at m/z of 50 to m/z of 650, with a scan time of 0.3 s and in the selected ion monitoring mode (SIM) with a scanning time of 0.2 s.

VALIDATION OF THE METHOD

The validation method and the analysis procedure of the amino acid content were performed according to validation guides for EURACHEM analytical methods.

The concentration range is the interval between the lower and upper concentration of the analyte in the assay for which it has been demonstrated that the procedure has an appropriate level of precision, accuracy and linearity.

Linearity is the ability of an analytical method to yield results proportional to the concentration of the analyte in the sample.

As a result, these parameters have been studied for the four amino acid standards which have an important share in collagen hydrolysates, namely:

- Glycine;
- Proline;
- Glutamic acid;
- Aspartic acid.

To evaluate the linearity and sensitivity of the signal in relation to the concentration, eight linear calibrations were generated for each amino acid.

The calibration curves of each amino acid were plotted in the 0.625-5 µmol/ml range, and the linearity range for which the correlation coefficient that characterizes the regression line R2 was obtained, was examined visually.

The mass spectrometer operated in automatic scanning mode (SCAN) and selected ion monitoring mode (SIM). The mass spectrometer operated in full scan mode (SCAN) at m/z of 50 to m/z of 500, with a scan time of 0.3 s and in Selected Ion Monitoring Mode (SIM) with a scan time of 0.2 s.

The performance parameters of the reference amino acid method, concentrations, limit of detection (LOD), limit of quantification (LOQ) and calibration curves were statistically calculated using Excel 2010 and are shown in Table 1. All statistical tests were performed at a confidence level of 95% and k = 2.

Table 1: Performance parameters of the amino acid determination method

Performance parameters / Amino acid	Correlation coefficient R^2	Limit of detection LOD **µmol/ml**	Limit of quantification LOQ **µmol/ml**	Retention time
Glycine SCAN	0,9999	0.004365	0.014549	8,98
Glycine SIM	0,9996	0.01817	0.060565	8,98
Proline SCAN	0,9998	0.010724	0.035745	11,40
Proline SIM	0,9999	0.002622	0.00874	11,40
Glutamic acid SCAN	0,9995	0.001592	0.005308	13,59
Glutamic acid SIM	0,9999	0.003978	0.013261	13,60
Aspartic acid SCAN	0,9999	0.005337	0.01779	13,37
Aspartic acid SIM	0,9998	0.096521	0.321737	13,33

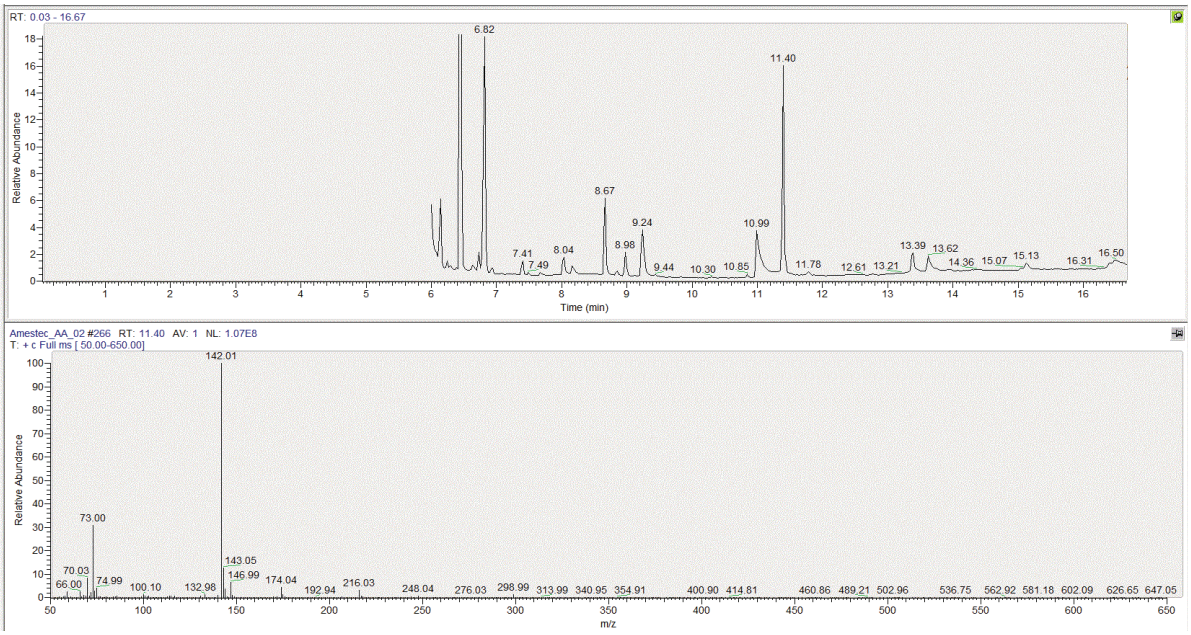


Figure 2. GC-MS chromatogram of standard amino acids in the mixture

ANALYSES OF BIOMATERIALS FOR MEDICAL USE

Seven samples of collagen materials for medical use were studied and physically and chemically characterized, the results are presented in the table below:

Table 2: Characterization of collagen products for medical use

Characteristics / Product	Dry substance, %	Ash*, %	Total nitrogen*, %	Appearance
Pancol L1	85,79	1,64	15,78	White spongy foil
Pancol L2	87,43	1,96	15,19	White spongy foil
Gevicol G1	87,57	2,72	16,45	Violet spongy foil
Gevicol G2	86,52	1,46	14,80	Violet spongy foil
Gel Zetta Skin 5	2,10	0,95	17,62	Transparent gel
Collagen hydrolysate HO 8 (Liquid)	28,76	0,56	17,62	Yellow liquid
Collagen hydrolysate HO8 (Atomized)	95,64	0,45	17,70	Yellowish powder

*values are recalculated free of volatile matter

The samples were hydrolyzed with 6M hydrochloric acid at 100°C±2°C for 24 hours, and the aliquot of the hydrolyzed sample was dried with nitrogen gas.

The derivatization was performed with N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA), and subsequently the derivatized amino acids were analyzed with a FOCUS GC gas chromatograph coupled with a DSQ II MS mass spectrometer, TriPlus AutoSampler, flame ionization detector, Split/Split less injector, XCalibur Thermo Scientific software, equipped with a non-polar capillary column.

Samples of collagen materials for medical use, hydrolysed, derivatized and analyzed by the mass spectrometric gas chromatographic method are shown in Figures 3-14, and the amino acid composition in Tables 3-9.

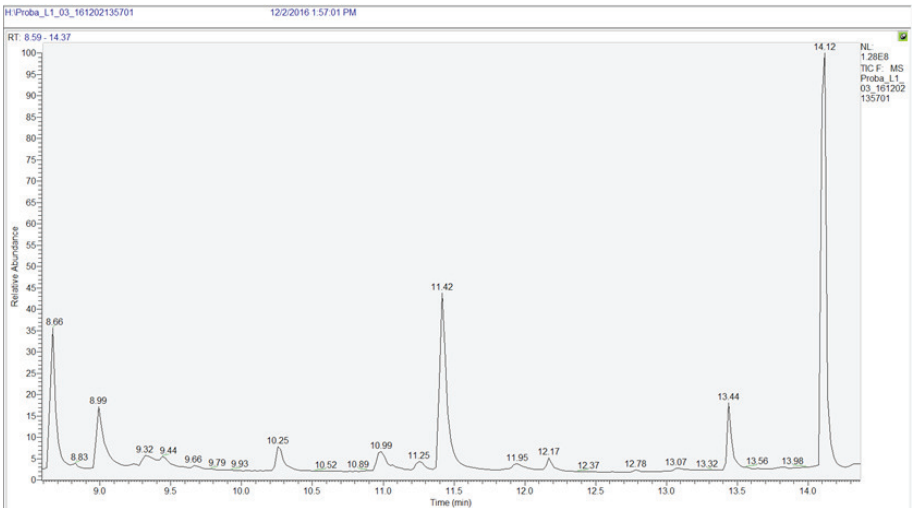


Figure 3. GC-MS profile of amino acids in Pancol L1

The amino acid composition of the Pancol L1 sample and the retention times obtained are shown in Table 3.

Table 3: Amino acid composition of Pancol L1 sample

SAMPLE L1_pancol				
No.	Retention time	Compound	Majority fragment	%
1	8.66	Alanine	116	13.20
2	8.99	Glycine	102	8.40
3	10.25	Valine	144	3.16
4	10.99	Leucine	158	3.85
5	11.25	Isoleucine	158	1.21
6	11.42	Proline	142	22.94
7	11.95	Serine	204	0.92
8	12.17	Threonine	218	1.15
9	13.44	Hydroxyproline	230	5.33
10	14.12	Tartaric acid	147	39.85

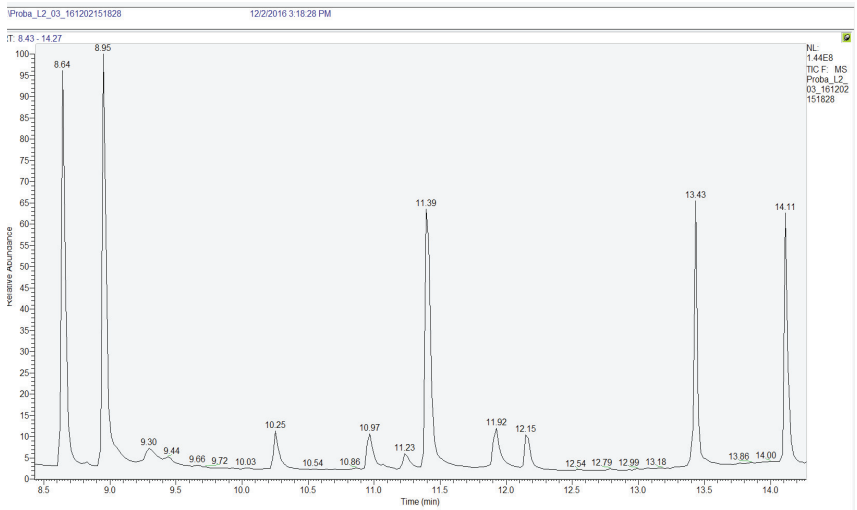


Figure 4. GC-MS profile of amino acids in Pancol L2

Table 4: Amino acid composition of Pancol L2 sample

SAMPLE L2_pancol				
No.	Retention time	Compound	Majority fragment	%
1	8.64	Alanine	116	20.79
2	8.95	Glycine	102	24.56
3	10.25	Valine	144	2.36
4	10.97	Leucine	158	3.14
5	11.23	Isoleucine	158	1.26
6	11.39	Proline	142	18.72
7	11.92	Serine	204	2.89
8	12.15	Threonine	218	2.19
9	13.43	Hydroxyproline	230	11.47
10	14.11	Tartaric acid	147	12.62

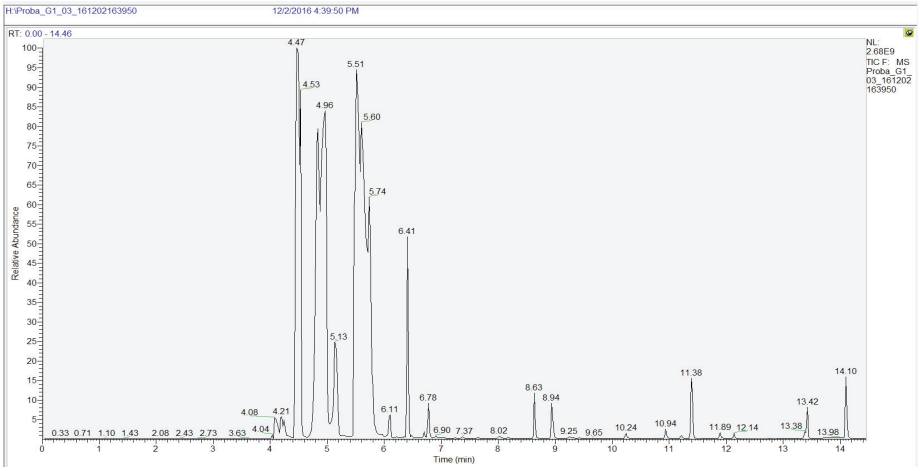


Figure 5. GC-MS chromatogram of Gevicol G1 sample

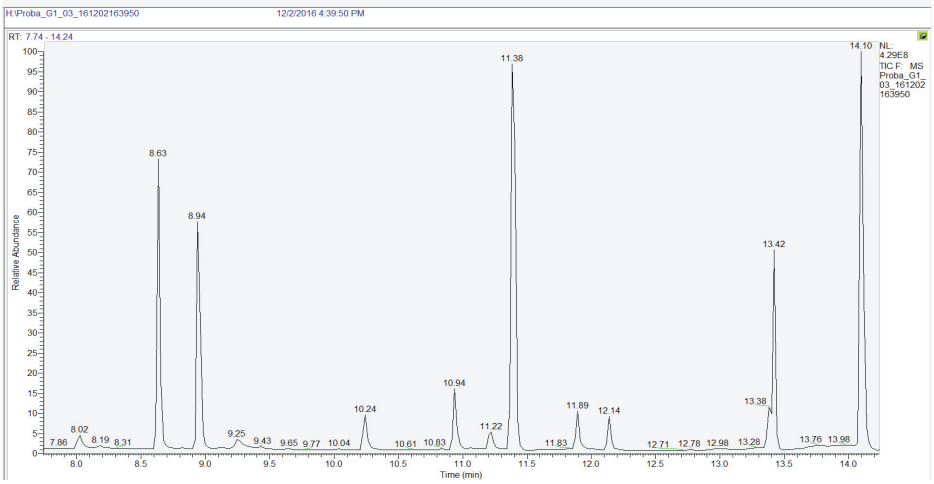


Figure 6. GC-MS profile of amino acids in Gevicol G1

Table 5: Amino acid composition of Gevicol G1 sample

SAMPLE G1_gevicol				5.83 mg
No.	Retention time	Compound	Majority fragment	%
1	8.02	Lactic acid	147	1.11
2	8.63	Alanine	116	13.70
3	8.94	Glycine	102	13.69
4	10.24	Valine	144	2.35
5	10.94	Leucine	158	3.54
6	11.22	Isoleucine	158	1.27
7	11.38	Proline	142	27.33
8	11.89	Serine	204	2.19
9	12.14	Threonine	218	1.74
10	13.38	Acid Aspartic	232	2.37
11	13.42	Hydroxyproline	230	8.09
12	14.1	Tartaric acid	147	22.61

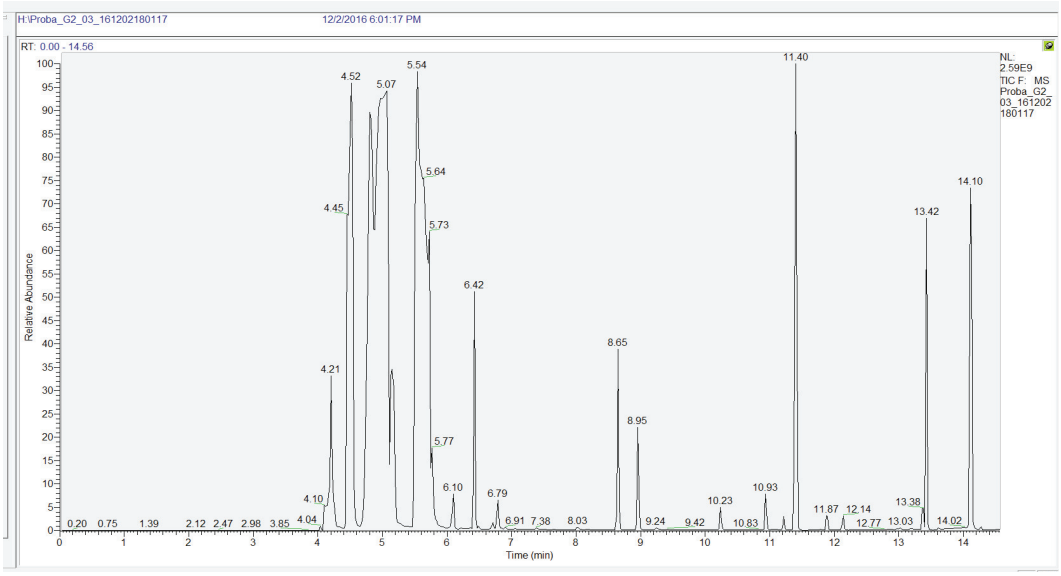


Figure 7. GC-MS chromatogram of Gevicol G 2 sample

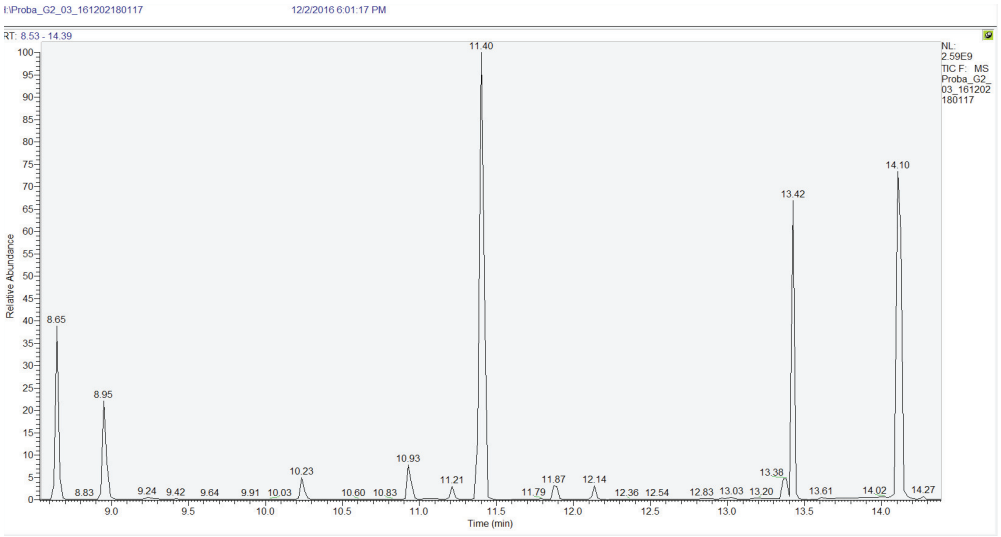


Figure 8. GC-MS profile of amino acids in Gevicol G2

Table 6: Amino acid composition of Gevicol G2 sample

SAMPLE G2_gevicol				5.13 mg
No.	Retention time	Compound	Majority fragment	%
1	8.65	Alanine	116	9.41
2	8.95	Glycine	102	6.50
3	10.23	Valine	144	1.39
4	10.93	Leucine	158	2.23
5	11.21	Isoleucine	158	0.72
6	11.4	Proline	142	34.69
7	11.87	Serine	204	1.16
8	12.14	Threonine	218	0.76
9	13.36	Aspartic acid	232	1.75
10	13.42	Hydroxyproline	230	14.20
11	14.1	Tartaric acid	147	27.18

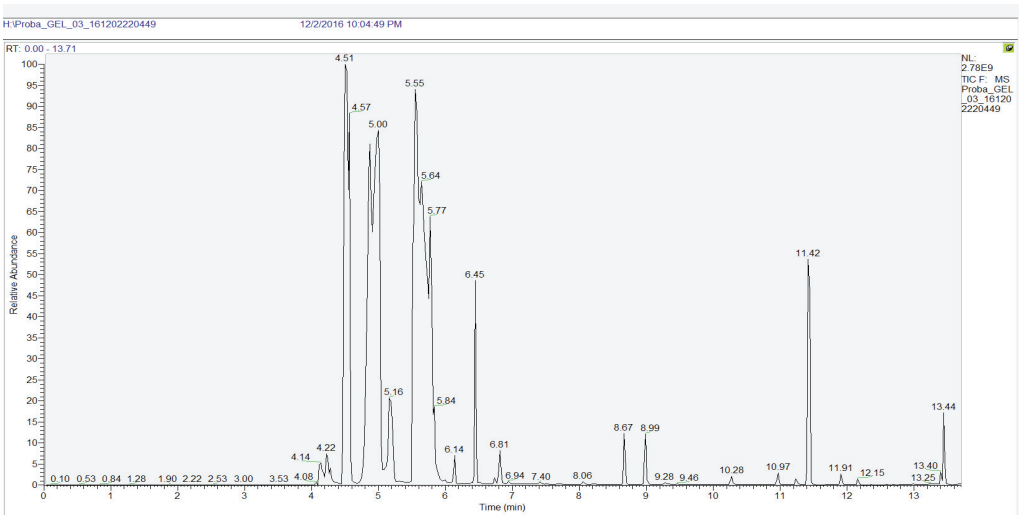


Figure 9. GC-MS chromatogram of Gel zeta sample

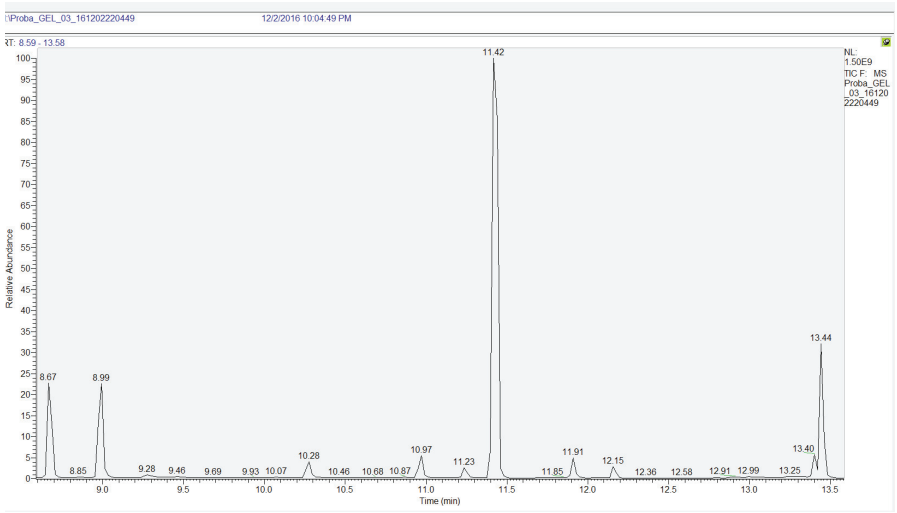


Figure 10. GC-MS profile of amino acids in Gel zeta

Table 7: Amino acid composition of Gel zeta sample

SAMPLE GEL_zeta				
No.	Retention time	Compound	Majority fragment	%
1	8.67	Alanine	116	10.13
2	8.99	Glycine	102	10.65
3	10.28	Valine	144	1.91
4	10.97	Leucine	158	2.22
5	11.23	Isoleucine	158	1.01
6	11.42	Proline	142	57.25
7	11.91	Serine	204	1.67
8	12.15	Threonine	218	1.07
9	13.4	Acid Aspartic	232	1.67
10	13.44	Hydroxyproline	230	11.19
11	14.12	Tartaric acid	147	0.43
12	14.32	Phenylalanine	218	0.32
13	15.77	Lysine	174	0.49

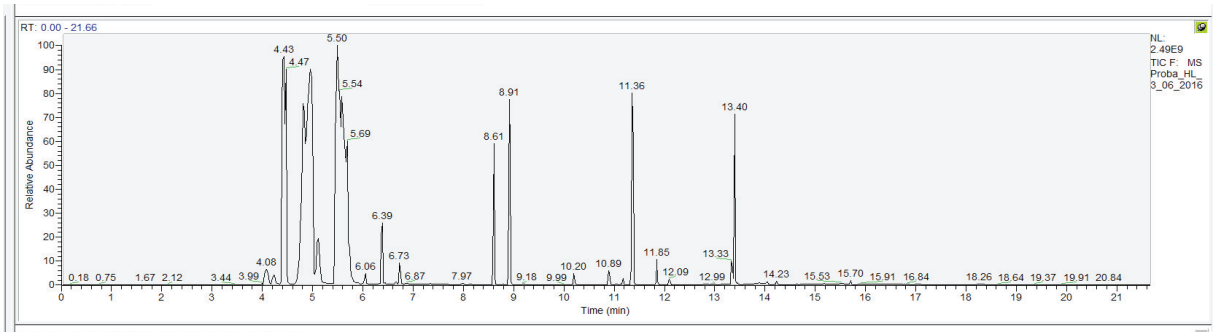


Figure 11. GC-MS chromatogram of liquid HO8 sample

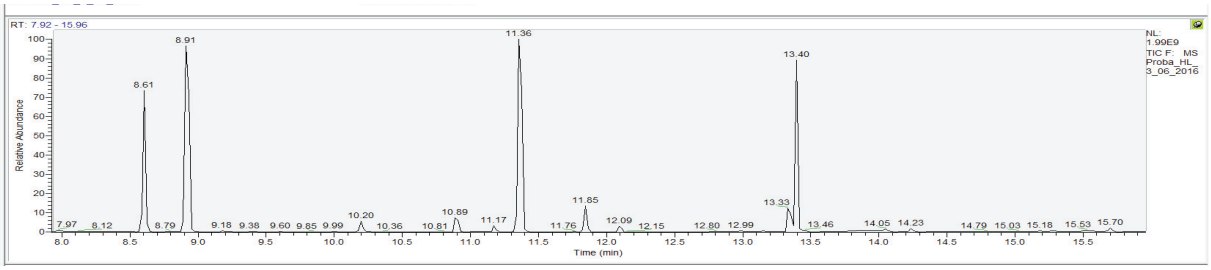


Figure 12. GC-MS profile of amino acids in liquid HO8

Table 8: Amino acid composition of liquid HO8 sample

Sample: Liquid hydrolysate HO8				
No.	Compound	Majority fragment	Retention time	%
1	Alanine	116	8.61	14.36
2	Glycine	102	8.91	28.65
3	Valine	144	10.2	1.30
4	Leucine	158	10.89	2.26
5	Isoleucine	158	11.17	0.66
6	Proline	142	11.36	30.04
7	Serine	204	11.85	2.63
8	Threonine	218	12.09	0.82
9	Aspartic acid	232	13.33	2.96
10	Hydroxyproline	230	13.4	14.98
11	Glutamic acid	156	14.05	0.51
12	Phenylalanine	218	14.23	0.38
13	Lysine	174	15.7	0.47

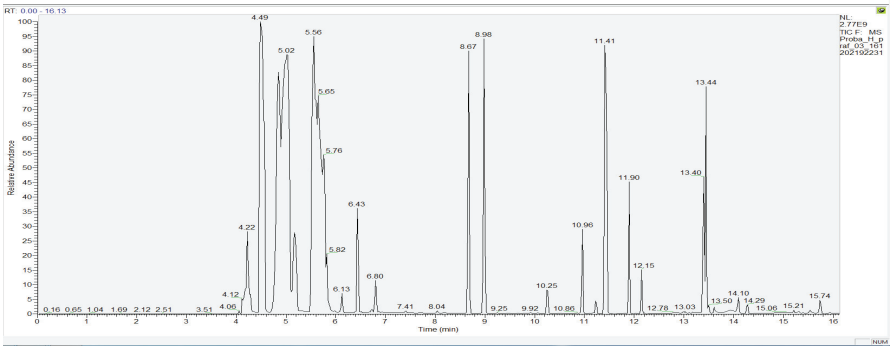


Figure 13. GC-MS chromatogram of atomized HO8 sample

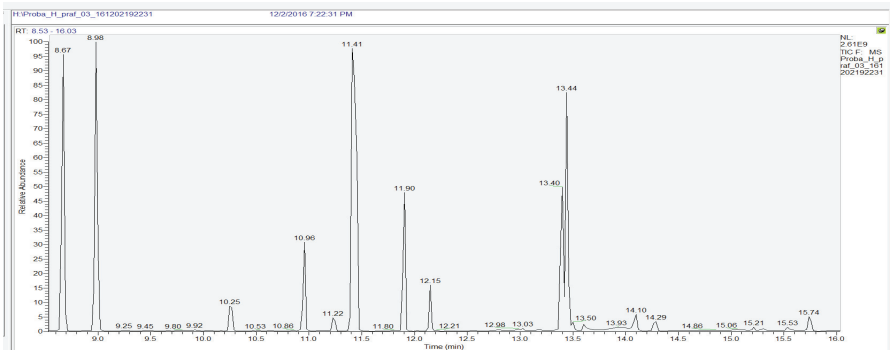


Figure 14. GC-MS profile of amino acids in atomized HO8

Table 9: Amino acid composition of atomized HO8 sample

SAMPLE HO8 atomized hydrolysate				
No.	Retention time	Compound	Majority fragment	%
1	8.67	Alanine	116	14.96
2	8.98	Glycine	102	16.92
3	10.25	Valine	144	1.90
4	10.87	Glycerol	147	0.03
5	10.96	Leucine	158	4.18
6	11.22	Isoleucine	158	0.87
7	11.41	Proline	142	29.67
8	11.9	Serine	204	6.98
9	12.15	Threonine	218	1.95
10	13.4	Acid Aspartic	232	8.98
11	13.44	Hydroxyproline	230	11.02
12	14.1	Tartaric acid	147	0.96
13	14.29	Phenylalanine	218	0.66
14	15.74	Lysine	174	0.91

CONCLUSIONS

Validation of the method included assessment of independent acid hydrolysis procedures, amino acid derivatization and GC/MS analysis.

To fully evaluate the performance of these procedures, a standard amino acid mixture allowed the GC/MS method to be verified, including derivatization and effects of acid hydrolysis on amino acids.

The validated analytical method for determining the amino acid content of collagen biomaterials for medical purposes fulfills all the conditions necessary for use and application for the intended purposes.

It was shown that this method can be used both for collagen hydrolysates and gels as well as for spongy collagen matrices because it is repeatable and reproducible.

Acknowledgment

This study was funded by MCI in the frame of Nucleu program 2016-2017, project code PN 16 34 04 04, contract 26N/2016.

REFERENCES

1. Albu, M.G., Titorencu, I., Chelaru, C., The stability of some collagen hydrohels, Leather and Footwear Journal, 2011, 11, 1, 11-20.
2. Albu, M.G., Titorencu, I., Biocompatibility study of collagen nerve conductors, Leather and Footwear Journal, 2011, 11, 4, 329-340.
3. Albu, M.G., Ghica, M.V., Tang, K., Liu, J., Coara, Gh., Rheological behavior of some collagen extracts, Leather and Footwear Journal, 2012, 12, 3, 193-200.
4. Vranceanu, M.D., Saban, R., Albu, M.G., Antoniac, I., Preparation and characterization of collagen: amorphous calcium phosphate composites, Leather and Footwear Journal, 2012, 12, 3, 215-222.
5. Albu, M.G., Leca, M., Trandafir, V., Rheological Behavior of Some Collagen Creams, Leather and Footwear Journal, 2012, 12, 4, 257-270.
6. Albu, M.G., Trandafir, V., Leca, M., Carsote, C., Characterization of collagen-gentamicin systems used in controlled release of drugs, Leather and Footwear Journal, 2007, 4, 3-9.
7. Albu, M.G., Ficai, A., Lungu, A., Preparation and characterization of collagen matrices obtained at different freezing temperatures, Leather and Footwear Journal, 2010, 10, 3, 39-50.
8. Shoulders, M.D., Raines, R.T., Collagen structure and stability, Annu Rev Biochem, 2009, 78, 929–958, https://doi.org/10.1146/annurev.biochem.77.032207.120833.
9. Bhattacharjee, A., Bansal, M., Collagen Structure: The Madras Triple Helix and the Current Scenario, Life, 2005, 57, 3, 161–172, https://doi.org/10.1080/15216540500090710.
10. Macovescu, G., Chelaru, C., Albu Kaya, M.G., Albu, L., Determination of Hydroxyproline in Collagen Biomaterials for Medical Use and

Validation of Method, Leather and Footwear Journal, 2016, 16, 2, 147-162, https://doi.org/10.24264/lfj.16.2.5.
11. Zumwalt, R.D., Kuo, K.C.T., Gehrke, C.W., Eds., Amino Acid Analysis by Gas Chromatography, vols. I–III, CRC Press, Boca Raton, Florida, 1987.
12. Molnar-Perl, I., Katona, Z.F., GC-MS of Amino Acids as their Trimethylsilyl/t-butyldimethylsilyl Derivatives: in Model Solutions III, Chromatographia, 2000, 51, 1, S228-S236.
13. Kaspar, H., Dettmer, K., Gronwald, W., Oefner, P.J., Automated GC-MS Analysis of Free Amino Acids in Biological Fluids, J Chromatogr B Analyt Technol Biomed Life Sci, 2008, 870, 222-232, https://doi.org/10.1016/j.jchromb.2008.06.018.
14. Miller, J., Chromatography: Concepts and Contrasts, John Wiley & Sons Inc., Hoboken, NJ, 2005.
15. Gehrke, C.W., Nakamato, H., Zumwait, R.W., Gas-Liquid Chromatography of Protein Amino Acids Trimethylsilyl Derivatives, J Chromatogr, 1969, 45, 24-51, https://doi.org/10.1016/S0021-9673(01)86179-3.
16. Kühnel, E., Laffan, D.D., Lloyd-Jones, G.C., Martínez, C.T., Shepperson, I.R. etal., Mechanism of Methyl Esterification of Carboxylic Acids by Trimethylsilyldiazomethane, Angew Chem Int Ed Engl, 2007, 46, 7075-7078, https://doi.org/10.1002/anie. 200702131.
17. Gehrke, C.W., Leimer, K., Trimethylsilylation of amino acids. Effect of solvents on derivatization using bis(trimethylsilyl)trifluoroacetamide, J Chromatogr, 1970, 53, 201-208, https://doi.org/10.1016/S0021-9673(01)98459-6.
18. Berezin, V.G., Capillary Gas-Solid Chromatography, Usp Khim, 1996, 65, 991-1012.
19. Robards, K., Haddad, P.R., Jackson, P.E., Principles and Practice of Modern Chromatographic Methods, Academic Press, San Diego, CA, 1994.
20. Shen, X., Deng, C., Wang, B., Dong, L., Quantification of Trimethylsilyl Derivatives of Amino Acid Disease Biomarkers in Neonatal Blood Samples by Gas Chromatography-Mass Spectrometry, Anal Bioanal Chem, 2006, 384, 931-938, https://doi.org/10.1007/s00216-005-0241-0.
21. An, Y., Schwartz, Z., Jackson, G.P, δ13C Analysis of Amino Acids in Human Hair Using Trimethylsilyl Derivatives and Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry, Rapid Commun Mass Spectrom, 2013, 27, 1481-1489.
22. Bruckner, H., Lupke, M., Use of chromogenic and fluorescent oxy-carbonyl chlorides as reagents for amino acid analysis by high-performance liquid chromatography, J Chromatogr, 1995, A 697, 1-2, 295-307, https://doi.org/10.1016/0021-9673(94)00938-6.
23. Bank, R.A., Jansen, E.J., Beekman, B., te Koppele, J.M., Amino acid analysis by reverse-phase high-performance liquid chromatography: improved derivatization and detection conditions with 9-fluorenylmethyl chloroformate, Anal Biochem, 1996, 240, 2, 167-76, https://doi.org/10.1006/abio.1996.0346.
24. Bjørklund, J., Einarsson, S., Engstrøm, A., Grzegorczykd, A., Beckere, H.D., Josefssona, B., Automated amino acid determination by high-performance liquid chromatography with 2-(9-anthryl)ethyl chloroformate as precolumn reagent, J Chromatogr, 1998, A 798, 1-2, 1-8, https://doi.org/10.1016/s0021-9673(97)00987-4.
25. Chaimbault, P., Petritis, K., Elfakir, C., Dreux, M., Ion-pair chromatography on a porous graphitic carbon stationary phase for the analysis of twenty underivatized protein amino acids, J Chromatogr, 2000, A 870, 245-254, https://doi.org/10.1016/S0021-9673(99)00863-8.
26. Haynes, P.A., Sheumack, D., Greig, L.G., Kibby, J., Redmond, J.W., Applications of automated amino acid analysis using 9-fluorenylmethyl chloroformate, J Chromatogr, 1991, 588, 1-2, 107-114, https://doi.org/10.1016/0021-9673 (91)85012-5.
27. Šimek, P., Heydová, A., Jegorov, A., High resolution capillary gas chromatography and gas chromatography - mass spectrometry of protein and non-protein amino acids, amino alcohols, and hydroxycarboxylic acids as their tert-butyldimethylsilyl derivatives, J High Resolut Chromatogr, 1994, 17, 145-152, https://doi.org/10.1002/jhrc.1240170305.

INVESTIGATION OF EXTRACELLULAR PROTEASE ACTIVITY OF TWO DIFFERENT EXTREMELY HALOPHILIC ARCHAEA ISOLATED FROM RAW HIDE

Fevziye Işıl KESBİÇ[1], Binnur MERİÇLİ YAPICI[2*]

[1]Kastamonu University, Central Research Laboratory, Kastamonu-37100,Turkey, ikesbic@kastamonu.edu.tr

[2]Çanakkale Onsekiz Mart University, Faculty of Arts and Sciences, Department of Biology,

Çanakkale-17020, Turkey, byapici@comu.edu.tr

Received: 23.10.2017 Accepted: 16.11.2017 https://doi.org/10.24264/lfj.17.4.9

INVESTIGATION OF EXTRACELLULAR PROTEASE ACTIVITY OF TWO DIFFERENT EXTREMELY HALOPHILIC ARCHAEA ISOLATED FROM RAW HIDE

ABSTRACT. In this study, it was aimed to investigate extracellular protease activity of two different extremely halophilic archaea and to identify them by using phenotypic and molecular methods. The haloarchaea were isolated from salted raw hide. The isolates were screened for production of protease enzyme and two isolates which have extracellular protease activity were selected. To identify the halophilic archaea, Gram staining procedure, antibiotic sensitivity tests and molecular methods were used. According to the 16S r-RNA molecular identification results, isolate 1 identified as *Halococcus morrhuae* JCM 8876 with % 99 sequence similarities and isolate 2 identified as *Natrinema pallidum* 153 with % 99 sequence similarities. Azocasein was used as a substrate for measuring their proteases activities daily and their optimum activities were determined. *H. morrhuae* and *N. pallidum* showed daily maximum proteases activity on 6 and 4 days, respectively. According to the results of the study, it was found that proteases activity was related with different reaction conditions. These conditions were detected as different pH (4-11), temperature (4-70°C) and NaCl concentration (1-5 M) values. *H. morrhuae* showed the maximum activity at pH 9.5, 2M NaCl concentration and 50°C temperature and *N. pallidum* displayed the optimum activity at pH 9.0, 2M NaCl concentration and 40°C temperature.

KEY WORDS: salted hide, haloarchaea, extracellular protease enzyme, 16S r-RNA sequence analysis.

INVESTIGAREA ACTIVITĂȚII PROTEAZICE EXTRACELULARE A DOUĂ ARCHAEA EXTREM HALOFILE DIFERITE IZOLATE DE PE PIELEA CRUDĂ

REZUMAT. În acest studiu, s-a urmărit cercetarea activității proteazice extracelulare a două archaea extrem halofile diferite și identificarea acestora utilizând metode fenotipice și moleculare. Haloarchaea au fost izolate de pe pielea crudă sărată. Izolatele au fost evaluate în funcție de capacitatea de producere a enzimelor de tip protează și s-au selectat două izolate care au activitate proteazică extracelulară. Pentru a identifica archaea halofile, s-au utilizat proceduri de colorare Gram, teste de sensibilitate la antibiotice și metode moleculare. Conform rezultatelor identificării moleculare ARNr 16S, izolatul 1 a fost identificat ca *Halococcus morrhuae* JCM 8876 cu asemănări de secvență de 99%, iar izolatul 2 a fost identificat ca *Natrinema pallidum* 153 cu asemănări de secvență de 99%. S-a utilizat azocaseina ca substrat pentru măsurarea zilnică a activității proteazice și s-a determinat activitatea optimă a acestora. *H. morrhuae* și *N. pallidum* au prezentat activitate proteazică maximă zilnică la 6, respectiv 4 zile. Conform rezultatelor studiului, s-a constatat că activitatea proteazică a fost corelată cu diferite condiții de reacție. Aceste condiții au constat în valori diferite ale pH-ului (4-11), ale temperaturii (4-70°C) și ale concentrației de NaCl (1-5 M). *H. morrhuae* a prezentat activitate maximă la pH 9,5, la concentrația de NaCl de 2M și la temperatura de 50°C, iar *N. pallidum* a prezentat activitate optimă la pH 9,0, concentrația de NaCl de 2M și temperatura de 40°C.

CUVINTE CHEIE: piele sărată, haloarchaea, enzimă protează extracelulară, analiză de secvență ARNr 16S.

ENQUÊTE SUR L'ACTIVITÉ PROTÉASE EXTRACELLULAIRE DE DEUX ARCHÉES DIFFÉRENTES EXTRÊMEMENT HALOPHILES ISOLÉES DE LA PEAU BRUTE

RÉSUMÉ. Dans cette étude, l'objectif a été d'étudier l'activité protéase extracellulaire de deux archées différentes extrêmement halophiles et de les identifier en utilisant des méthodes phénotypiques et moléculaires. Les haloarchées ont été isolées de la peau brute salée. Les isolats ont été évalués selon la production d'enzyme protéase et deux isolats avec une activité protease extracellulaire ont été sélectionnés. Pour identifier les archées halophiles, une procédure de coloration de Gram, des tests de sensibilité aux antibiotiques et des méthodes moléculaires ont été utilisés. Selon les résultats d'identification moléculaire d'ARNr 16S, l'isolat 1 a été identifié comme *Halococcus morrhuae* JCM 8876 avec des similitudes de séquence de 99% et l'isolat 2 a été identifié comme *Natrinema pallidum* 153 avec des similitudes de séquence de 99%. L'azocaséine a été utilisée comme substrat pour mesurer quotidiennement leurs activités protéases et leurs activités optimales ont été déterminées. *H. morrhuae* et *N. pallidum* ont montré une activité protéase maximale quotidienne à 6 et 4 jours, respectivement. Selon les résultats de l'étude, on a constaté que l'activité protéase a été liée à différentes conditions de réaction. Ces conditions ont été détectées avec différentes valeurs de pH (4-11), de température (4-70°C) et de concentration de NaCl (1-5 M). *H. morrhuae* a montré une activité maximale à pH 9,5, une concentration de NaCl de 2M et une température de 50°C et *N.* pallidum a montré une activité optimale à pH 9,0, une concentration de NaCl de 2M et une température de 40°C.

MOTS CLÉS: peau salée, haloarchées, enzyme protéase extracellulaire, analyse des séquences d'ARN-r 16S.

* *Correspondence to: Binnur MERİÇLİ YAPICI, Department of Biology, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, 17020, Çanakkale, Turkey, byapici@comu.edu.tr*

INTRODUCTION

Microbial cells live in association with other cells in nature and populations of cell interact with other populations in microbial communities. The abundance and diversity of any microbial community is controlled by the resources available and conditions such as temperature, pH, presence or absence of oxygen that prevail in that community [1]. A phylogenetic analysis based upon ribosomal RNA sequence characterization reveals that living systems represent three domains: the eubacteria, eukarya and archaea [2]. Archaea are the main group to thrive in extreme environments in terms of salinity, pH and temperature [3]. Halophilic archaea require 1.5-4 M NaCl for optimal growth and most of halophilic archaea can thrive up to the saturation point of NaCl around 5.5 M [4]. Haloarcheae are found in habitats with high salinity such as salt and soda lakes, salted hides and fish [5]. Adaptations to extreme environments provide to serve in several of biotechnological and industrial applications [6]. Halophiles produce several biotechnological products. The most important of them are biopolymers, bacterio- and halorhodopsins, biosurfactants and enzymes [7, 8]. Proteases are one of the most important groups of enzymes. Annual usage values of microbial proteases are determined as 25% for alkaline protease and 21% for other proteases. Halophilic proteases are used in the production of food, leather and detergent industry in particular [9]. Therefore, the aim of the present study was to investigate extracellular proteolytic activities of different halophilic archaea isolated from salted raw hide, to identify them phylogenetically and determine optimum and variable conditions such as pH, concentration of NaCl and temperature.

MATERIALS AND METHODS

Two halophilic archaea which were isolated from salted raw hide were identified using Gram staining procedure, antibiotic sensitivity tests and 16S r-RNA sequence analysis. SW 25 medium was used for culture isolation, activation, growth and phenotypic tests. This medium was composed of 833.4 ml/l SW 30 solution, 5g/l yeast extract, and 20 g/l agar. SW 30 solution contained 234 g/l NaCl, 39 g/l $MgCl_2$, 61 g/l $MgSO_4$,1 g/l $CaCl_2$, 6 g/l KCl, 0.25 g/l $NaHCO_3$ and 0.7g/l NaBr. Media were adjusted to pH 7.5 with 1N NaOH. To detect Gram reactions and cell morphology of the isolates Gram staining was performed [10]. The disc diffusion method was used to determine antibiotic susceptibilities of the isolates with the following antibiotics: Novobiosin 30 µg (NV30), Penicillin G 10 U (P10), Streptomycin 10 µg (S10), Ampicillin 10 µg (A10), Bacitracin 10 µg (B10), Erythromycin 15 µg (E15), Tetracycline 30 µg (T30), Vancomycin 30 µg (VAN30), Chloramphenicol 30 µg (C30), and Cefotaxime 30 µg (CTX30).

For genomic DNA isolation of isolates, commercial Bacterial DNA isolation kit was used and Dyall-Smith [11, 12] method was modified. The cultures grown in 250 ml Erlenmeyer flasks containing 50 ml SW-25 liquid medium were incubated at 40°C for 5 days at 150 rpm. 1.5 ml of samples (OD_{600}~ 1) were taken and centrifuged at 10.000 g for 10 minutes at +4°C. The supernatants of the samples were removed and 400 µl of sterile cold ultra pure water was added to the tubes to disintegrate the cells. The samples were centrifuged at 10,000 g for 3 minutes at +4°C to remove supernatants and genomic DNA isolation was performed according to the instructions of the DNA isolation kit. Genomic DNAs were measured spectrophotometrically at absorbance values of 260 nm (A_{260}) and 280 nm (A_{280}). The purity grades of DNA were determined by A_{260} / A_{280} ratio and those between 1.5-1.8 were used for Polymerase Chain Reaction (PCR). To control the DNA bands, 0.8% agarose gel was prepared with 1X TAE. Syber® Green I and 6X gel loading dye were used for loading the samples into the agarose gel.

A commercial Taq DNA Polymerase kit was used for PCR setup protocol. The reaction was prepared in sterile 0.2 ml PCR tubes and the tubes were placed in the Thermal Cycler after brief centrifugation. At the end of the reaction, the PCR products were controlled

on an 1.7% agarose gel. Chromatograms of sequence analyses were edited via Chromas and forward-reverse sequences were aligned on NCBI's (National Center of Biotechnology Information) website. The 16S r-RNA gene regions of the isolates were analyzed by the nucleotide BLAST (Basic Local Alignment Search Tool) and compared with the 16S r-DNA sequences present in the GenBank database. A phylogenetic tree was constructed using the Mega program to establish the relationship between the isolates and their phylogenetically similar species.

For the determination of the protease production, skimmed-milk was added into the SW 25 medium. Clear zone observed around the colony was considered as positive protease activity. To product protease enzyme in shake flasks, 1 ml of a week old culture of isolates was inoculated into a sterilized 250 mL Erlenmeyer flask containing 50 mL of the SW 25 medium and incubated at 40°C in an incubator shaker at 150 rpm. 1 mL of culture was taken on a daily basis and the contents were centrifuged at 10.000 g for 10 minutes to remove cells and insoluble materials. The cell free supernatant was used as the source of the crude enzyme [13, 14].

Proteolytic activity was determined using Azocasein solution as a substrate. The solution was composed 0.4% Azocasein, 2M NaCl, 50mM Tris-HCl at pH 7.0. 500 µl of Azocasein substrate was incubated at 40°C. After 10 minutes 100µl enzyme was added to the reaction mixture and incubated at 40°C for 30 minutes. The reaction was stopped by the addition of 250 µl of %20 TCA solution. The reaction mixture was centrifuged at 10.000 g for 10 minutes. 100 µl of supernatant was taken and mixed with 900µl 2 M NaOH solutions. The enzyme activity was determined by measuring the absorbance at 440 nm. The absorbance increase of 0.01 units at 440 nm in reaction conditions was determined as 1 unit (U) activity [15, 16]. Bacterial biomass was measured with VIS spectrophotometer (Mecasys Optizen Pop QX UV\VIS, Korea) at 600 nm [17, 14]. In the study the enzyme activities were measured daily.

The extracellular protease activities of two different archaea were monitored at different pH values (pH 4.0-11.0) at Azocasein substrate solutions with NaOH, sodium acetate, and Tris- HCl buffer. The effect of temperature on the protease production was studied at different reaction temperature (4-70°C). The other parameters were kept constant. The different NaCl concentrations (1.0-5.0M) were used in the Azocasein substrate to determine the optimum salinity.

RESULTS AND DISCUSSION

The haloarchaea isolated from salted raw hide were screened for production of protease enzyme. Two different isolates which have extracellular protease activity were selected. According to the gene bank database, it was found that isolate 1 was similar to *Halococcus morrhuae* JCM 8876 as 99% and isolate 2 was similar to *Natrinema pallidum* 153 as 99%, too. Phylogenetic tree based on 16S-rRNA sequence data indicating the relationship of isolates is shown in Figure 1.

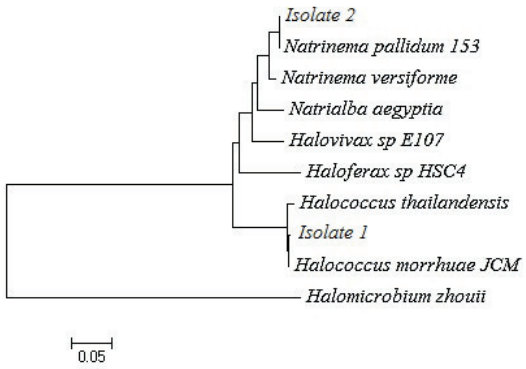


Figure 1. Phylogenetic tree based on 16S r-RNA sequence data indicating the relationship of isolates

Protease activities of isolates were

measured daily. While the highest protease activity of *Halococcus morrhuae* was determined on the sixth day of growth (Figure 2), the maximum protease activity of *Natrinema pallidum* was observed on the fourth day (Figure 3).

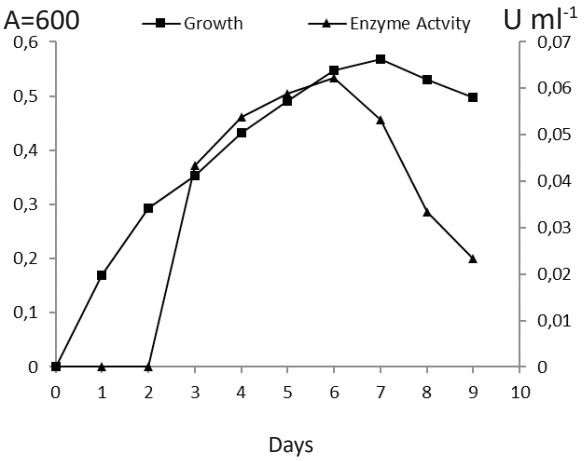


Figure 2. Maximum protease activity of *H. morrhuae* on the sixth day of growth

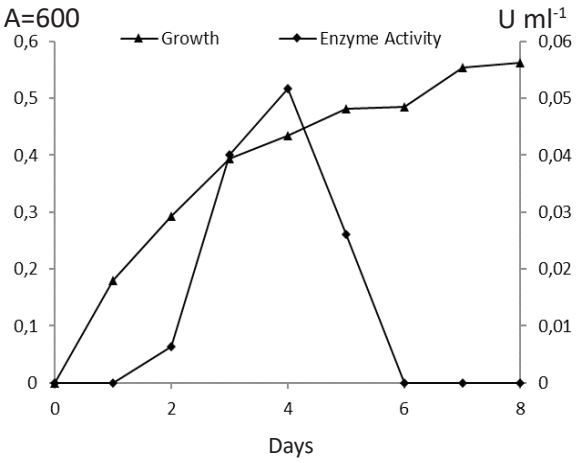


Figure 3. Maximum protease activity of *N. pallidum* on the fourth day of growth

The effect of pH on protease activity of *Halococcus morrhuae* and *Natrinema pallidum* was determined in the presence of 2 M NaCl. *Halococcus morrhuae* enzyme exhibited significant activity in the pH range of 5.5-9.5 and the maximum activity value at pH 9.5 was defined as 0.075 U/ml. No activity could be determined at pH 5.0 and 10.5 (Figure 4). The enzyme of *Natrinema pallidum* showed considerable activity in the pH range of 5.0-10.0 and reached the highest value at pH 9.0 (0,063 U\mL). The activity was observed at pH 10.5 as 0.023 U\mL but no activity was observed at pH 11.0 (Figure 5).

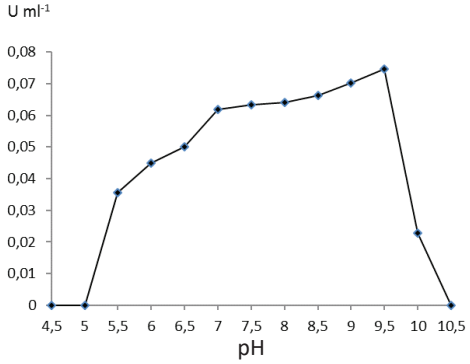


Figure 4. Effect of pH on extracellular protease activity of H. *morrhuae*

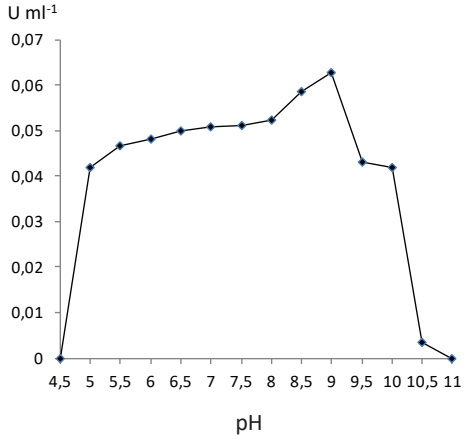


Figure 5. Effect of pH on extracellular protease activity of N. *pallidum*

For both isolates, it was found that 2M NaCl was optimum concentration for the highest enzyme activity. However *H. morrhuae* and *N. pallidum* isolates were found to exhibit protease activity at 1.0-4.5M NaCl and 1.0-3.0M NaCl, respectively (Figures 6 and 7).

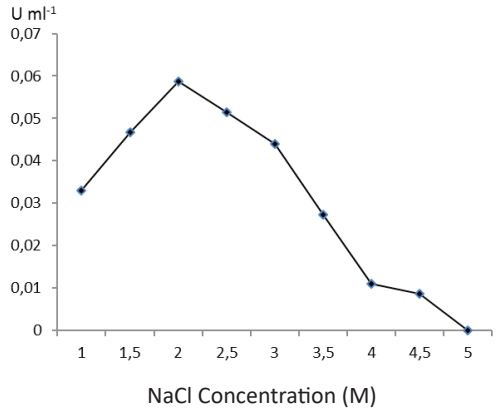


Figure 6. Effect of NaCl concentration on extracellular protease activity of H. *morrhuae*

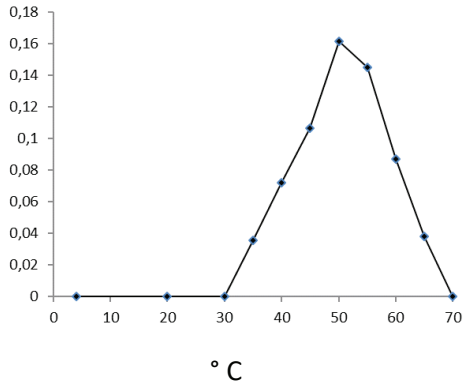


Figure 8. Effect of temperature on extracellular protease activity of H. *morrhuae*

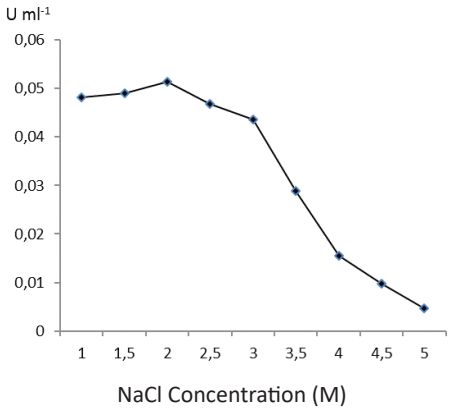


Figure 7. Effect of NaCl concentration on extracellular protease activity of *N. pallidum*

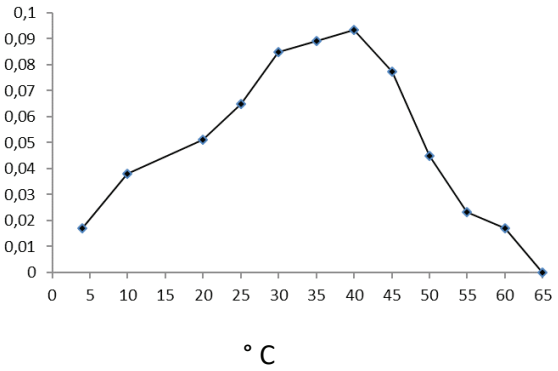


Figure 9. Effect of temperature on extracellular protease activity of N. *pallidum*

The effect of temperature on the protease activity of *H. morrhuae* and *N. pallidum* isolates was shown in Figures 8 and 9 respectively. While the optimum temperature for the enzymatic activity of *H. morrhuae* was found to be 50°C, the optimum temperature for enzyme activity of *N. pallidum* was determined as 40°C. On the other hand, it was found that protease activity was to be at a wide temperature range of 30-70°C for *H. morrhuae,* 4-75°C for *N. pallidum* (Figures 8 and 9).

Accordingly, the obtained values for maximum extracellular protease activity of *H. morrhuae* and *N. pallidum* were determined as 50°C, 2M NaCl, pH 9.5 and 40°C, 2M NaCl, pH 9.0, respectively.

Capiralla *et al*. [18] reported that optimum pH value for extracellular serine protease enzyme of *Halobacterium halobium* S9 was 8.0. Gimenez *et al*. [19] showed that the extracellular protease enzyme which isolated from *Natrialba magadii* exhibited an optimum activity between pH 8.0-10.0. We can say that our findings are similar to the findings obtained by the researchers in terms of pH value. Vijayanand *et al*. [14] reported that protease activity did not occur at a value below pH 5.0 by *Halobacterium sp*. Js1. However, *N. pallidum* 153 exhibited protease activity between

pH 4.5 -5 in our study. Kamekura *et al*. [20, 21] studied about *Natrialba asiatica* and their extracellular serine alkaline protease enzyme activity. Their results showed that maximum enzymatic reaction was at 1.3 M NaCl concentration. On the contrary, for both isolates, it was found that 2M NaCl was optimum concentration for the maximum enzyme activity, in our study. Temperature is one of the most important parameters for enzyme activities. Studies to determine the optimum temperature revealed that the protease showed a maximum activity at 40 and 50°C. It could be said that our research findings in terms of temperature values were similar with the findings of these investigations. On the other hand, present studies show that most halophilic microorganisms did not exhibit enzyme activity at low temperature [22, 23, 15]. In contrast, it was found that protease activity was to be at a wide temperature range of 30-70°C for *H. morrhuae*, 4-75°C for *N. pallidum*, in our study.

CONCLUSIONS

In the study, firstly the haloarchaea were isolated from salted raw hide. The isolates were screened for production of protease enzyme and two isolates which have extracellular protease activity were selected. The halophilic archaea isolates were identified by using Gram staining method, antibiotic sensitivity tests and 16S rRNA sequence analysis. It was determined that both isolates were Gram negative and susceptible to novobiocin and bacitracin antibiotics. According to the 16S rRNA molecular identification results, isolate 1 and isolate 2 identified as *Halococcus morrhuae* JCM 8876 and *Natrinema pallidum* 153 with 99% sequence similarities, respectively. Secondly, protease activity conditions of the two extreme halophilic archaea were quantitatively determined. According to the research findings, the enzyme of *Halococcus morrhuae* and *Natrinema pallidum* exhibited activity in the pH range of 5.5-9.5 and 5.0-10.0, at 1.0-4.5M NaCl and 1.0-3.0M NaCl concentrations, at a wide temperature range 30-70°C and 4-75°C respectively. Extremely halophilic archaea with proteolytic enzyme activities may cause major quality problems not only on the raw material but also the final leather. If archaea causing damage to the hide and skin by proteolytic activities were identified, target-specific antiarchaeal substances can be used to control them. Thus, the problems on the salted raw hide and skin can be solved. On the other hand, the obtained values for maximum extracellular protease activity of *H. morrhuae* and *N. pallidum* were determined as 50°C, 2M NaCl, pH 9.5 and 40°C, 2M NaCl, pH 9.0, respectively. Finally, this study also showed that two different archaea isolated from salted raw hide can be evaluated in a wide variety of areas of the industry by further investigations.

REFERENCES

1. Madigan, M.T., Martinko, J.M., Brock Biology of Microorganisms, 11th Ed., Prentice Hall, International Inc, San Francisco, **2010**, 5.
2. Woese, C.R., Fox, G.E., Phylogenetic Structure of The Prokaryotic Domain: The Primary Kingdoms. Proceedings of the National Academy of Sciences, **1977**, 74, 11, 5088-5090.
3. Rampelotto, P.H., Extremophiles and Extreme Environments, *Life*, **2013**, 3, 482-485, https://doi.org/10.3390/life3030482.
4. DasSarma, S., Extreme Halophiles Are Models for Astrobiology, *Microbe*, **2006**, 1, 3, 120-126, https://doi.org/10.1128/microbe.1.120.1.
5. Akolkar, A.V., Deshpande, G.M., Raval, K.N., Durai, D., Nerurkar, A.S., Desai, A.J., Organic Solvent Tolerance of Halobacterium sp. sp1(1) and Its Extracellular Protease, *J Basic Microbiol*,

2008, 48, 1-5, https://doi.org/10.1002/jobm.200800012.

6. Margesin, R., Schinner, F., Potential of Halotolerant and Halophilic Microorganisms for Biotechnology, *Extremophiles*, **2001**, 5, 73– 83.
7. Adams, M.W.W., Kelly, R.M., Enzymes in Extreme Environments, *Chem Eng News*, **1995**, 73, 32-42, https://doi.org/10.1021/cen-v073n051.p032.
8. Banat, I.M., Makkar R,.S., Cameotra, S.S., Potential Commercial Applications of Microbial Surfactans, *Appl Microbiol Biotechnol*, **2000**, 53, 495-508.
9. Rao, M.B., Tanksale, A.M., Gathe, M.S., Deshpande, V.V., Molecular and Biotechnological Aspects of Microbial Proteases, *Microbiol Mol Biol Rev*, **1998**, 62, 3, 597-635.
10. Dussault, H.P., An Improved Technique for Staining Red Halophilic Bacteria, *J Bacteriol*, **1955**, 70, 484-485.
11. Dyall-Smith, M., The Halohandbook - Protocols for Haloarchaeal Genetics, Version 7, **2008**, 7, 144.
12. Bilgi, S.T., Meriçli Yapici, B., Karaboz, İ., Determination of Hydrolytic Enzyme Capabilities of Halophilic Archaea Isolated from Hides and Skins and Their Phenotypic and Phylogenetic Identification, *J Am Leather Chem As*, **2015**, 110, 2, 33-42.
13. Ibrahim, A.S.S, Al-Salamah, A.A., Optimization of Media and Cultivation Conditions for Alkaline Protease Production by Alkaliphilic Bacillus Halodurans, *Res J Microbiol*, **2009**, 4, 7, 251-259.
14. Vijayanand, S., Hemapriya, J., Selvin, J., Kiran, S., Production and Optimization of Haloalkaliphilic Protease by an Extremophile-Halobacterium sp. js1, Isolated from Thalassohaline Environment, *Global J Biotechnol Biochem*, **2010**, 5, 1, 44-49.
15. Karadzic, I., Masui, A., Fujiwara, N., Purification and Characterization of a Protease from *Pseudomonas aeruginosa* Grown in Cutting Oil, *J Biosci Bioeng*, **2004**, 98, 3, 145-152, https://doi.org/10.1016/S1389-1723(04)00258-0.
16. Uratani, J.M., Kumaraswamy, R., Rodríguez, J., A Systematic Strain Selection Aproach for Halotolerant and Halophilic Bioprocess Development: A Review, *Extremophiles*, **2014**, 18, 4, 629-639, https://doi.org/10.1007/s00792-014-0659-4.
17. Henroette, C., Zinebi, S., Aumaitre, M.F., Petitdemange, E., Petitdemange, H., Protease and Lipase Production by a Strain of *Serratia marcescens*, *J Ind Microbiol*, **1993**, 12, 129-135.
18. Capiralla, H., Hiroi, T., Hirokawa, T., Maeda, S., Purification and Characterization of a Hydrophobic Amino Acid Specific Endopeptidase From *Halobacterium halobium* S9 with Potential Application in Debittering of Protein Hydrolysates, *Process Biochem*, **2002**, 38, 4, 571-579, https://doi.org/10.1016/S0032-9592(02)00180-2.
19. Gimenez, M.I., Studdert, C.A., Sanchez, J.J. De Castro R.E., Extracellular Protease of *Natrialba magadii*; Purification and Biochemical Characterization, *Extremophiles*, **2000**, 4, 181–188.
20. Kamekura, M., Seno, Y.A., Halophilic Extracellular Protease from a Halophilic Archaebacterium 172 P1, *Biochem Cell Biol*, **1990**, 68, 352–359, https://doi.org/10.1139/o90-048.
21. Kamekura, M., Dyall-Smith, M.L., Taxonomy of the Family Halobacteriaceae and the Description of Two New Genera *Halorubrobacterium* and *Natrialba*, *J Gen Appl Microbiol*, **1995**, 41, 333–350.
22. Huang, Q., Peng, Y., Li, X., Wang, H., Zhang, Y., Purification and Characterization of

an Extracellular Alkaline Serine Protease with Dehairing Function from *Bacillus pumilus*, *Curr Microbiol*, **2003**, 46, 169-173, https://doi.org/10.1007/s00284-002-3850-2.

23. Gomes, J., Stetiner, W., The Biocatalytic Potential of Extremophiles and Extremozymes, *Food Technol Biotechnol*, **2004**, 42, 223-235.

NATIONAL AND INTERNATIONAL EVENTS

SIMAC TANNING TECH
20th-22nd FEBRUARY, 2018, MILAN, ITALY

Simac Tanning Tech is the international event with the most qualified offer of machinery and technologies for the footwear, leather goods and tanning industries, which takes place in Milan, concurrently with Lineapelle.

There are many services dedicated exclusively to the visitor, allowing greater enjoyment range of merchandise, ease in the movements, better planning of the stay during the exhibition and a wider range of entertainment in the city.

Gabriella Marchioni Bocca *states that "This event is a unique and unmissable opportunity for encounters, casting the spotlight on a* ***dynamic market****. A dynamism expressed by companies via the offer of* ***ever more efficient and eco-friendly technologies****: now key factors in remaining competitive on the international scene and that will be more visible and expressed than ever at the next Simac Tanning Tech."*

The forthcoming Simac Tanning Tech will be held **from the 20th to 22nd February 2018** at Fiera Milano Rho.

More information: www.simactanningtech.it

20th UITIC CONGRESS
16th-18th MAY, 2018, PORTO, PORTUGAL

The 20th congress of The International Union of Shoe Industry Technicians (UITIC) will take place on May 16th to 18th, 2018, in Porto, Portugal. This congress will bring together experts and decision makers who all play an important role in the worldwide footwear business, such as brands, retailers, footwear manufacturers, that present their vision to the future.

The networking opportunities and the visits to some of the most technological advanced companies can bring innovative solutions to cope with the future consumers' needs and prepare what will become the new technological age.

Participants registering until the 10th of February 2018 will benefit from a discount.

More information: www.porto2018.uitic.org

3rd SOUTH EAST EUROPEAN CONFERENCE ON SUSTAINABLE DEVELOPMENT OF ENERGY, WATER AND ENVIRONMENT SYSTEMS (SDEWES SEE2018 NOVI SAD) JUNE 30th-JULY 3rd, 2018, NOVI SAD, SERBIA

The main challenge for South East Europe (SEE) economies is to commit to, and sustain the implementation of, long-term reforms aimed at increasing competitiveness and promoting sustainable, inclusive and balanced development, as well as better integration between the EU Member States, candidate and potential candidate countries and neighbouring countries. An adequate response to this challenge will certainly require using the best available scientific knowledge and constant re-evaluation of the development process in light of the scientific findings. Therefore, it will be essential to enhance the scientific understanding, improve the long-term scientific assessments, strengthen the scientific capacities and ensure that the sciences are responsive to the emerging needs.

Along this line, a regional series of biannual Sustainable Development of Energy Water and Environment Systems (SDEWES) conferences have been initiated to provide a venue for the researchers from the SEE region, but also for world-wide researchers and specialists and those interested in learning about the sustainability of development, to present research progress and to discuss the state of the art, the future directions and priorities in the various areas of sustainable development and regional integration.

The 3rd edition of the conference is going to be held in the European Youth Capital 2018 city of Novi Sad, Serbia from June 30-July 3, 2018.

More information: http://www.novisad2018.sdewes.org

THE 8th INTERNATIONAL ADVANCES IN APPLIED PHYSICS AND MATERIALS SCIENCE CONGRESS & EXHIBITION APRIL 24th-30th, 2018, OLUDENIZ, TURKEY

The 8th International Advances in Applied Physics and Materials Science Congress & Exhibition will be held on April 24-30, 2018 in the stunning Convention Centre of one of the largest international resort in Turkey right in the heart of Blue Lagoon (Oludeniz) area.

APMAS 2018 intends to be a global forum for researchers and engineers to present and discuss recent innovations and new techniques in Applied Physics and Material Science. In

addition to scientific seminars, a wide range of social programs including boat cruises and visits to historical places will be available.

The Organizing Committee also encourages companies and institutions to showcase their modern products and equipment in the conference area.

More information: http://www.apmascongress.org

5th INTERNATIONAL CONGRESS ON TECHNOLOGY - ENGINEERING & SCIENCE FEBRUARY 1st-2nd, 2018, KUALA LUMPUR, MALAYSIA

The 5th International Congress on Technology - Engineering & Science will take place on February 1st to 2nd, 2018 in Kuala Lumpur, Malaysia.

The congress will provide a platform to bring together academician, researchers, industrialists, practitioners and other related experts from home and abroad to share knowledge and experiences, which will promote intellectual and practical development in the all field of Technology, Engineering and Science. The congress will offer technical activities, including research/technical sessions, poster sessions, exhibitions, an industry track, a developers track etc.

The main objectives of the congress is to promote information of research and development on related topics and interchange updated scientific information among the researchers, developers, engineers, students and practitioners working worldwide.

More information: www.icontes.org

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.

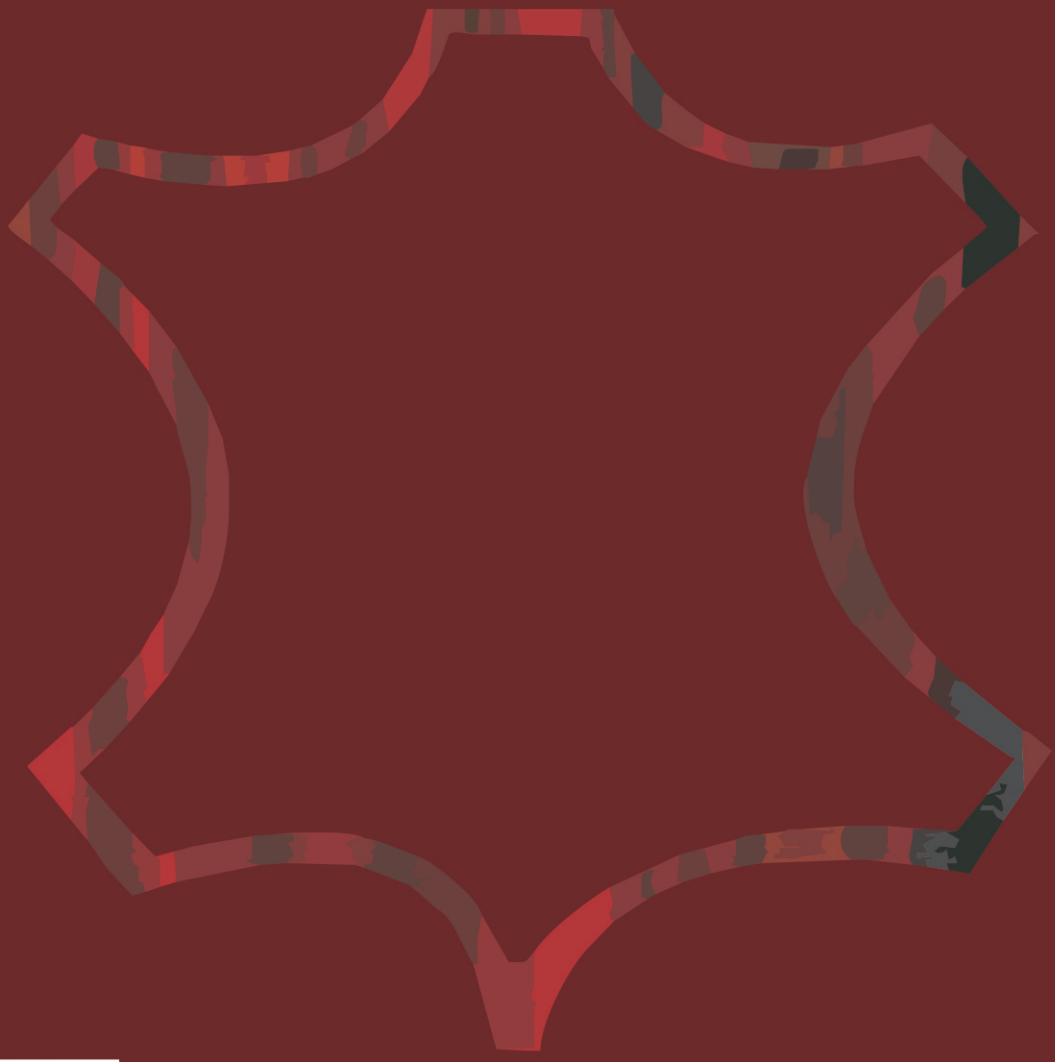
Paper template is available for download on the journal's website, http://www.revistapielarieincaltaminte.ro.

Manuscript Submission

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

Dr. Viorica DESELNICU, 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 CERCETĂRII ŞI INOVĂRII