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## Contents



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# Studies on the Correlation between Surface and Sewability Properties of Crust Leather

by

Niklesh C.,[1,4] Jayakumar G.C.[1,4] and Phebe Aaron K*[2,3,4]

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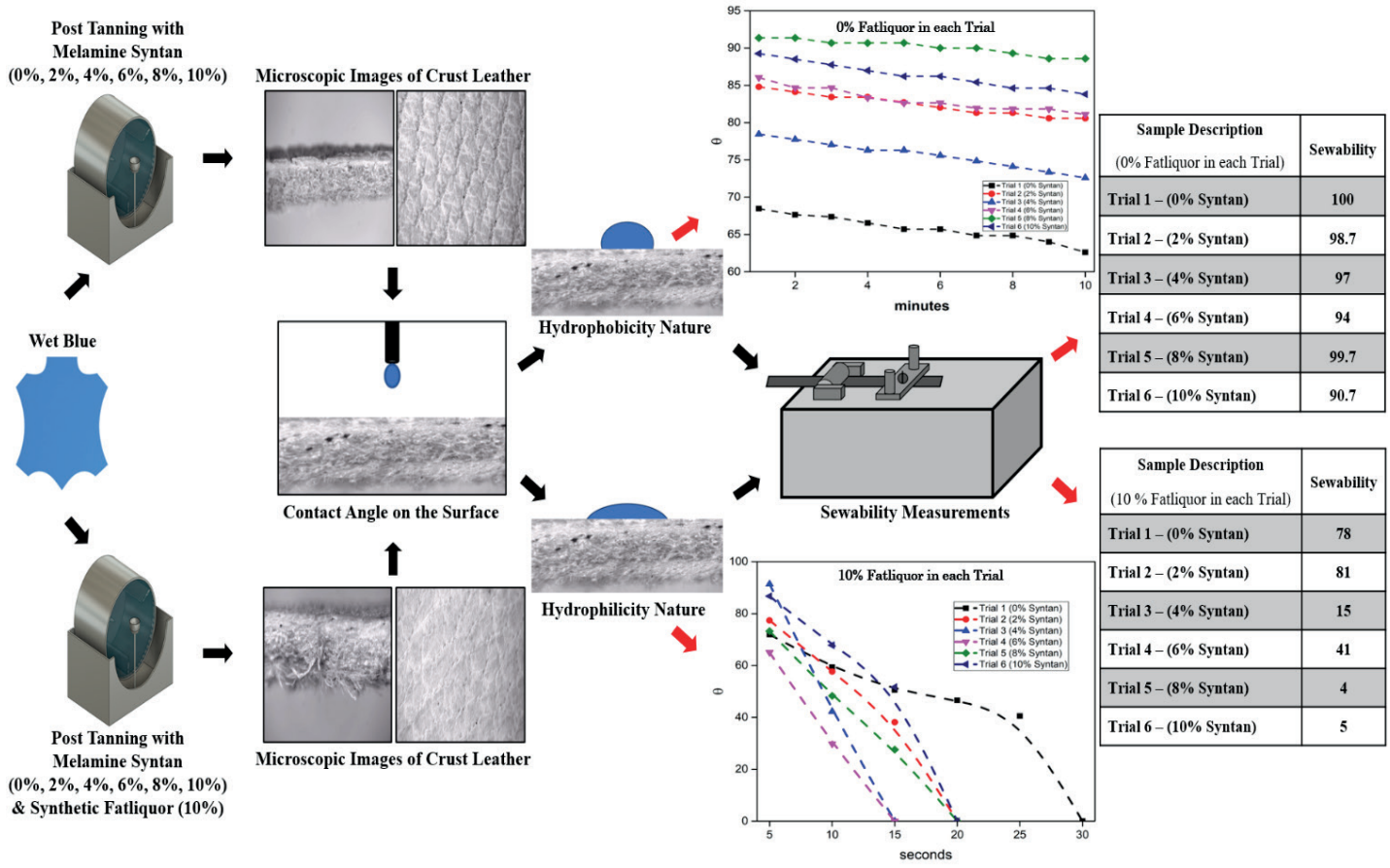
## Graphical Abstract




| Sample Description (0% Fatliquor in each Trial) | Sewability |
|---|---|
| Trial 1 – (0% Syntan) | 100 |
| Trial 2 – (2% Syntan) | 98.7 |
| Trial 3 – (4% Syntan) | 97 |
| Trial 4 – (6% Syntan) | 94 |
| Trial 5 – (8% Syntan) | 99.7 |
| Trial 6 – (10% Syntan) | 90.7 |

| Sample Description (10 % Fatliquor in each Trial) | Sewability |
|---|---|
| Trial 1 – (0% Syntan) | 78 |
| Trial 2 – (2% Syntan) | 81 |
| Trial 3 – (4% Syntan) | 15 |
| Trial 4 – (6% Syntan) | 41 |
| Trial 5 – (8% Syntan) | 4 |
| Trial 6 – (10% Syntan) | 5 |

## Abstract

The surface properties of leather are critical factors that influence the aesthetic quality of the leather. Commonly, organoleptic properties are evaluated for leathers to ascertain the bulk properties such as compactness, smoothness, softness and general grain appearance. Though these parameters are very subjective, still these properties are essential for leather product manufacture. In the current research, we discuss the correlation between the surface parameters and sewability of the leathers. In our earlier studies, we discussed the influence of syntans and fatliquors on sewability. In aligning to a similar subject, the impact of surface properties has been evaluated through contact angle measurements and correlated with the sewability properties of the leather. From the spread ability measurements, it can be inferred that the sewability properties are better towards the surface of the leather, which is more hydrophilic. This result agrees that the fatliquoring of leathers has enhanced the stitch ability; moreover, the studies provide biophysical parameter importance for qualifying leather sewability.

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## Introduction

Leather goods and products gain importance due to their unique properties, such as breathability and comfort.[1-3] During leather manufacture, the post tanning and finishing processes are crucial in imparting bulk and surface properties. In post tanning; retanning and fat liquoring chemicals are used to achieve performance properties such as softness, roundness, pliable, grain compactness, etc.[4,5] Physical characteristics such as tensile, tear, grain crack, fastness, sewability, drape are also crucial properties achieved during the post tanning process. In our earlier studies, we have studied the influence of synthetic fatliquor, melamine and phenolic syntan on the sewability properties of the leather.[6,7] Our research group has been involved in understanding the individual post tanning chemicals to attain better sewability properties for the leathers, an inevitable parameter in manufacturing most leather goods and garments. From the sewability studies, it is understood that melamine syntan behaves similar to phenolic syntan, and the presence of fatliquor is significant to better sewing the leather.[7] However, the surface properties of the leathers also play a significant role in sewing. The needle penetration on the leather is usually disoriented depending on the surface properties.[8]

In our recent research, we have established the correlation between performance chemicals and physical strength characteristics. In continuation with the study, a post-tanning experiment was carried out without offering the fatliquor while just varying the melamine syntan percentage. The experimental leathers were further evaluated for their sewability, grain crack, tensile strength and microscopic images. Also, the coherent relationship between the surface sewability properties of the melamine retanned leathers has been studied. The wettability of the melamine crust leathers has been determined using a contact angle meter. Further, gloss values at 20°, 60° and 85° have also been determined for the crust leather to establish the correlation between the organoleptic properties such as roundness, softness, grain compactness and smoothness. The surface property and physical strength characteristics have been correlated to establish the impact of sewability of the melamine crust leathers.

## Materials and Methods

Chrome-tanned goat leather was used as a raw material for the study and commercial-grade chemicals were used for the post-tanning process. Experiments with different concentrations of melamine syntan with 0% fatliquor process are given in Table I and methodology adopted for the study is shown in Figure 1.

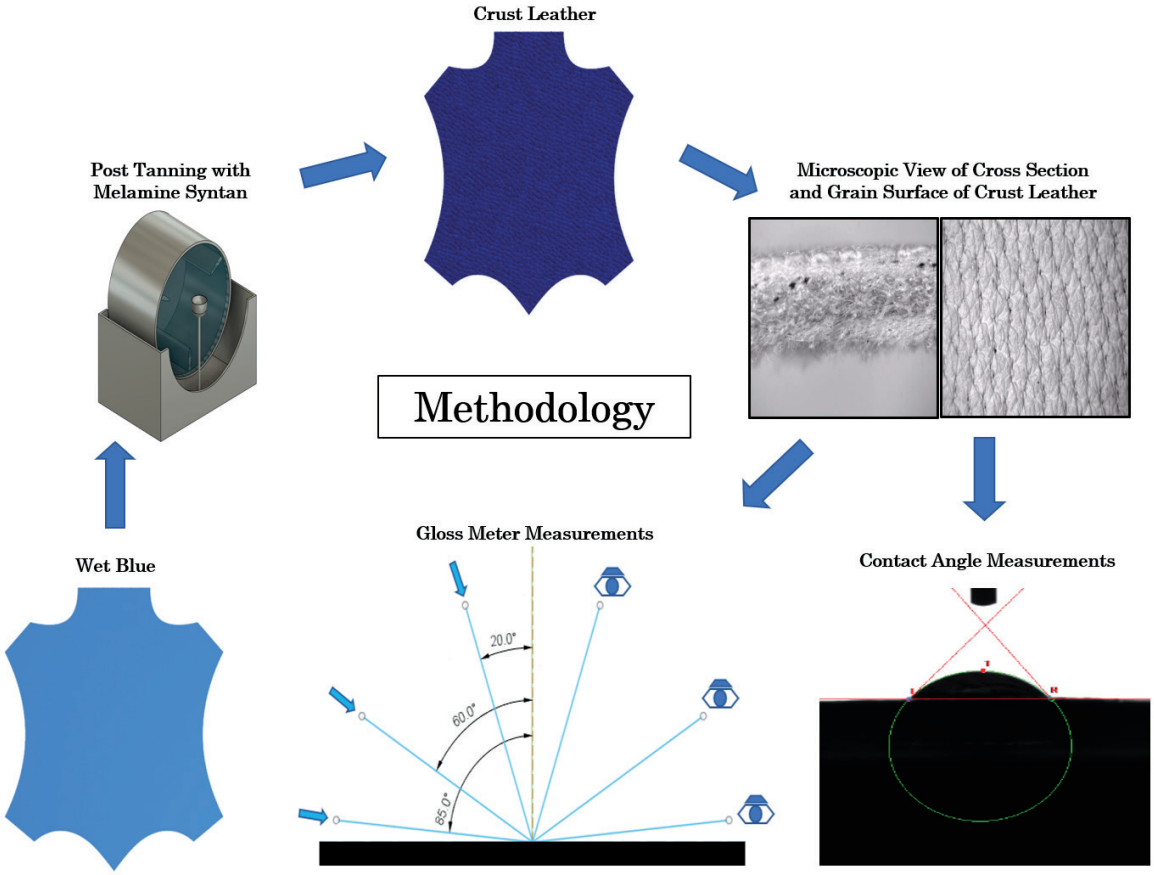



**Figure 1.** Process Methodology of the present study

**Table I**

**Post tanning process**

| Process | Percentage | Durations |
|---|---|---|
| **Neutralization** | | |
| Water | 150% | 60 min |
| Nutrigan | 0.50% | (pH 5.0 to 5.5) |
| Drain & Wash | | |
| **Retanning** | | |
| Water | 100% | |
| **Syntan Base:** | Melamine Base (FB6) | |
| Trial 1 | 0% | |
| Trial 2 | 2% | |
| Trial 3 | 4% | 30 min (Each Trial) |
| Trial 4 | 6% | |
| Trial 5 | 8% | |
| Trial 6 | 10% | |
| **Fixing** | | |
| Formic Acid | 0.50% | 30 min (pH 4.0) |
| Drain & Wash | | |

**Contact Angle Analysis**

The contact angle was measured using a drop deposition method wherein a drop of liquid was placed on the crust leather surface and later the same was analyzed using software.[9,10] This experiment was carried out using Apex contact angle meter (ACAM Series – ACAM NSC 17, Contact Angle measurement 0°-180° with an Accuracy of ±0.05°).

**Gloss Meter Analysis**

A beam of light was projected over the surface of the crust leather and the reflection of that light was measured at an equal but opposite angle.[11,12] This experiment was carried out using 3nh Gloss Meter (YG268 Triangle Gloss Meter, Measuring Angle: 20°, 60°, 85°).

**Sewability Analysis**

The crust leather was cut into the required dimensions (30 × 350 mm) and then used to determine the sewability values. This experiment was carried out using an L&M tester machine (penetration rate – 100/min, needle number – 90, needle system – 34LR).[7] The sewability property efficiency is determined by the percentage values. The values ranging between 0 to 10% are recommended to have better stitching properties.[13,14]

**Physical Testing of the Sample**

The control and experimental crust leathers are characterized by their physical strength. Before testing, the leathers were conditioned and tested as per standard norms of Tensile (IUP 6, 2000) and grain crack (IUP 9, 1996).[15,16]

**Optical Microscopic Studies**

The microscopic images of the control and experimental leathers were captured using an optical microscope connected to a camera.

## Results and Discussion

The functional and performance properties of the leather are greatly influenced by choice of post-tanning chemicals. During the post-tanning process, the physical strength characteristics such as tensile, tear, grain crack, fastness and sewability depend on the type and concentration of retanning and fatliquoring chemicals.[17] Our research group focuses on the ongoing evidence-based symbiotic relationship between syntan and fatliquor for the sewability properties of leather. Phenolic and melamine syntans behave similarly on the sewing properties.[6,7] In the present study, the surface properties of the melamine syntan retanned crust leathers have been evaluated for their hydrophobic-hydrophilic nature and gloss values.

Table II shows the sewability measurements of the melamine retanned crust leather without fatliquor trials. By observing the trend of the obtained sewability values, it is clearly understandable that the values obtained in each trial are very high (above 90%), which makes it unsuitable for stitching. The grain crack values

**Table II**

**Sewability Measurements**

| Sample Description | Standard Threshold | Sewability (High) |
|---|---|---|
| TRIAL 1 – (0% Fatliquor; 0% Syntan) | 417 | 100.0 |
| TRIAL 2 – (0% Fatliquor; 2% Syntan) | 450 | 98.7 |
| TRIAL 3 – (0% Fatliquor; 4% Syntan) | 400 | 97.0 |
| TRIAL 4 – (0% Fatliquor; 6% Syntan) | 450 | 94.0 |
| TRIAL 5 – (0% Fatliquor; 8% Syntan) | 450 | 99.7 |
| TRIAL 6 – (0% Fatliquor; 10% Syntan) | 425 | 90.7 |

**Table III**

**Lastometer Ball Burst Test - Grain Crack**

| Sample Description | Measurement | Value |
|---|---|---|
| TRIAL 1 (0% Fat-Liquor; 0% Syntan) | Load at Grain Crack (Kg) | 25.5 |
| | Distention at Grain Crack (mm) | 9.2 |
| TRIAL 2 (0% Fat-Liquor; 2% Syntan) | Load at Grain Crack (Kg) | 26.5 |
| | Distention at Grain Crack (mm) | 9.2 |
| TRIAL 3 (0% Fat-Liquor; 4% Syntan) | Load at Grain Crack (Kg) | 27.3 |
| | Distention at Grain Crack (mm) | 9.2 |
| TRIAL 4 (0% Fat-Liquor; 6% Syntan) | Load at Grain Crack (Kg) | 24.8 |
| | Distention at Grain Crack (mm) | 9.1 |
| TRIAL 5 (0% Fat-Liquor; 8% Syntan) | Load at Grain Crack (Kg) | 23.2 |
| | Distention at Grain Crack (mm) | 9.2 |
| TRIAL 6 (0% Fat-Liquor; 10% Syntan) | Load at Grain Crack (Kg) | 23.5 |
| | Distention at Grain Crack (mm) | 9.1 |

**Table IV**

**Tensile Strength and Elongation Measurements**

| Sample Description | Direction | Tensile Strength (MPa) | Elongation at Break % |
|---|---|---|---|
| TRIAL 1 (0% Fat-Liquor; 0% Syntan) | 1 | 13.2 | 77.3 |
| | 2 | 23.7 | 33.8 |
| TRIAL 2 (0% Fat-Liquor; 2% Syntan) | 1 | 12.2 | 73.0 |
| | 2 | 21.3 | 34.1 |
| TRIAL 3 (0% Fat-Liquor; 4% Syntan) | 1 | 14.9 | 80.4 |
| | 2 | 26.6 | 34.3 |
| TRIAL 4 (0% Fat-Liquor; 6% Syntan) | 1 | 10.7 | 94.0 |
| | 2 | 24.8 | 35.0 |
| TRIAL 5 (0% Fat-Liquor; 8% Syntan) | 1 | 12.7 | 68.1 |
| | 2 | 20.5 | 33.0 |
| TRIAL 6 (0% Fat-Liquor; 10% Syntan) | 1 | 12.5 | 85.2 |
| | 2 | 24.2 | 34.6 |

of experimental leathers without fatliquor are given in Table III, which are found to be comparatively lesser than those offered with 10% fatliquor. A similar trend is inferred in Table IV for tensile strength and elongation measurements. The values obtained for the experimental melamine retanned leathers with the offer of 10% fatliquor have been found to improve strength properties, as reported in our previous study.[7]

All these experiments (Tables II, III & IV) were carried out to support our earlier reported experiments on melamine retanned leather with 10% fatliquor trials. In the previous work, we reported that the crust leathers with 8% melamine syntan and 10% fatliquor exhibited better sewability properties.[7] Similar trend in the properties has been achieved for the grain crack and tensile strength properties. The present study summarizes a holistic understanding of the symbiotic relationship between surface and sewability properties.

**Contact Angle Evaluation**

From the sewability studies, 0% fatliquor with various concentrations of syntan, showed higher sewability values, indicating that the leathers were stiffer. The contact angle measurement results inferred that the control leather trials are more toward hydrophobicity in nature. The trend of contact angle measurements of various concentrations of syntan with 0% fatliquor retanned crust leather is shown in Figure 2. Also, the contact angle values of leathers retanned with 10% fatliquor and various concentrations of melamine syntan are shown in Figure 3. The results show that these leathers are found to be more hydrophilic, and the sewability value is found to be higher. This is in accordance with the organoleptic property of the 0% fatliquor and 10% fatliquor offering with melamine syntan leather (Figure 2 and 3).

**Significance of Hydrophobicity - Hydrophilicity on Sewability Property**

Hydrophobicity – hydrophilicity nature of the leather is conventionally tunable using performance chemicals during the post-tanning and finishing steps.[18] Fatliquoring process is the significant step which provides hydrophobic nature to the leathers.[19-22] However, when fatliquor is less and dried, leathers tend to show a hydrophobicity nature. This is primarily due to the coalescence of collagen fibers and the removal of water molecules from the leathers. The softness of the leather depends on the fatliquoring, which provides pliability

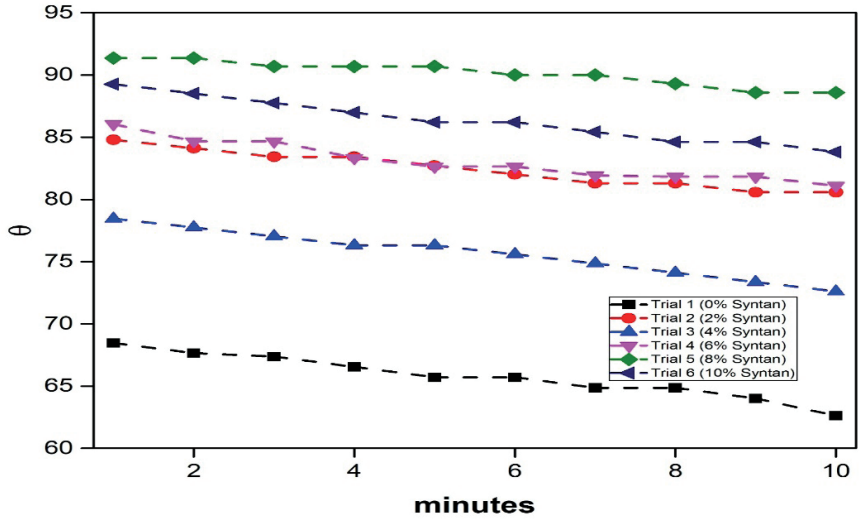



**Figure 2.** Graph for Contact Angle Values of Leathers with 0% Fatliquor

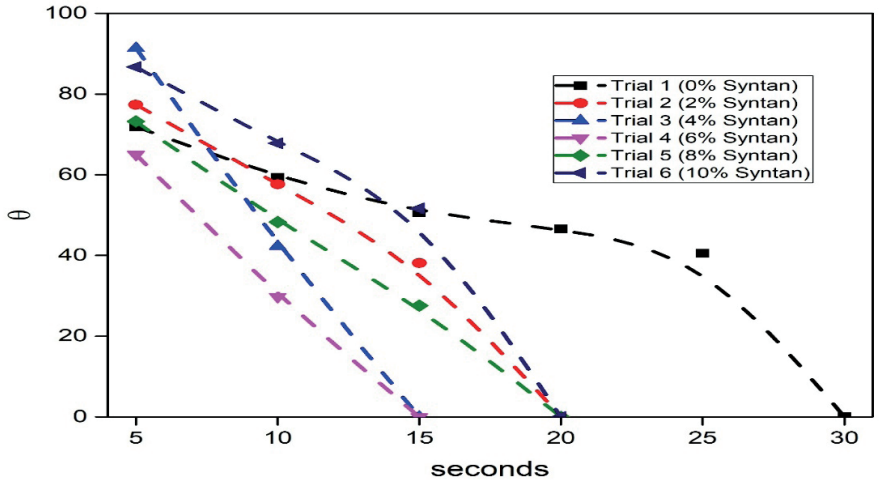



**Figure 3.** Graph for Contact Angle Values of Leathers with 10% Fatliquor

and eases the stitching properties. From our earlier studies, it can be inferred that the leathers' stiffness is higher for the leathers with less offer of fatliquor, which shows that the stiffness property is inversely proportional to the sewability properties.[6,7] In the present study, the similar synergistic effect of fatliquor on spread ability values has been determined using a contact angle meter. It was found that the leathers retanned without the fatliquor showed more hydrophobicity which results in less sewability property. Sewability values imply the importance of fatliquoring for the stitching of leathers. Moreover, the studies also establish that the absence of fatliquor leads to higher leather stiffness and a reduced sewability property.

**Gloss Meter Evaluation**

The glossiness of leather is one of the significant properties of leather products. The glossiness of the leather surface is influenced by choice of post-tanning chemical and finishing operations.[12] The glossiness effect on the crust leather has been studied using a gloss meter. The gloss values of control and experimental leathers are shown in Figures 4 and 5. The gloss values at 20° for both types of leathers are found to be similar, which is between 0.5 to 1.0. The difference in gloss values is probably due to no significant glossiness on the leather. However, the gloss value at 60° and 85° varied for the control and experimental leathers. The experimental leathers showed higher glossiness compared to control leathers which are post-tanned without the offer of fatliquor. The presence of fatliquor has enhanced the glossiness property of the leather. From the gloss values, it can be inferred that the high glossiness of the crust leather is attributed to better sewability of the leather, which is similar to the contact angle measurement observations.

**Microscopic Evaluation**

The optical images of the leathers are shown in Figures 6 and 7 where it can be observed that the leathers are stiff in the case of 0% fatliquor offered compared to 10% fatliquor offered melamine retanned leathers.

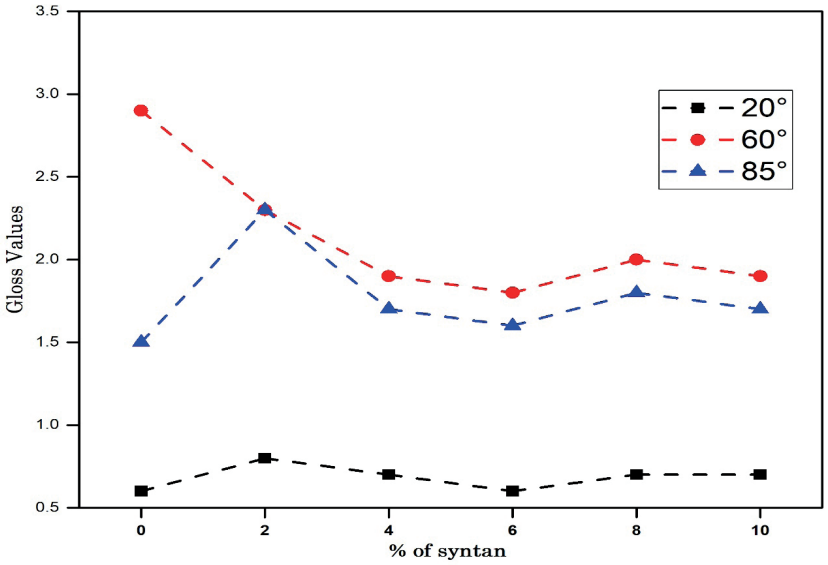



**Figure 4.** Graph for Gloss Values of Leathers with 0% Fatliquor

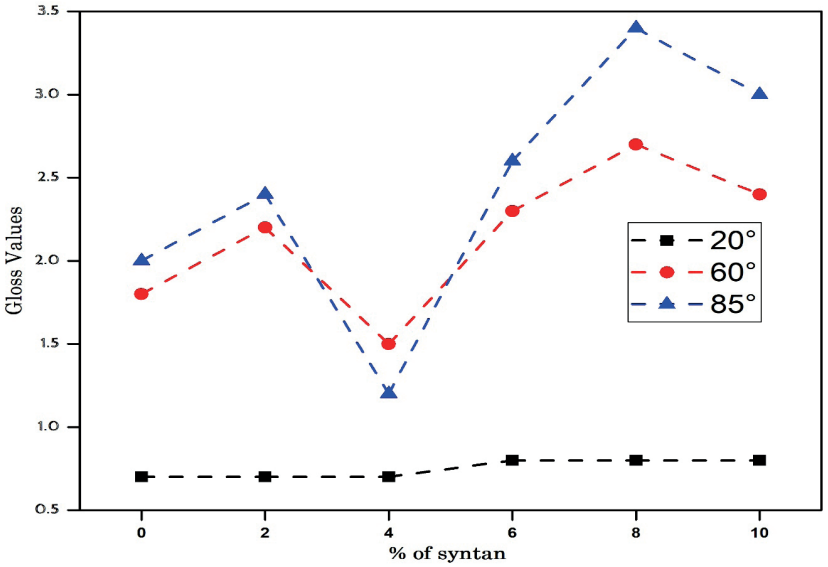
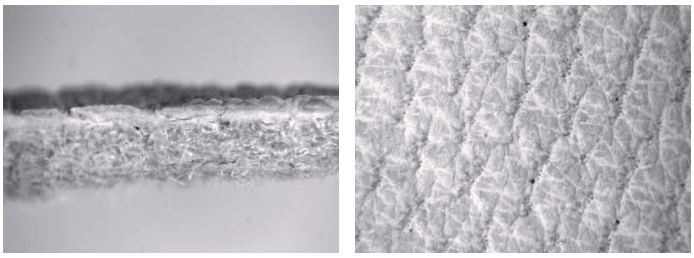
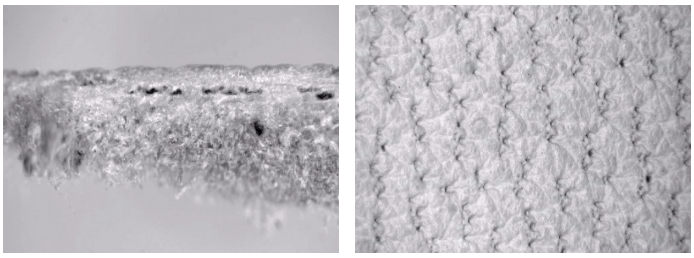


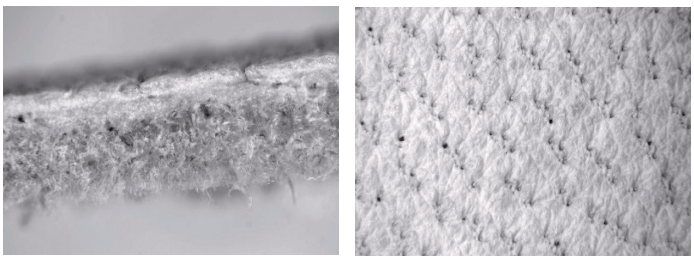

**Figure 5.** Graph for Gloss Values of Leathers with 10% Fatliquor



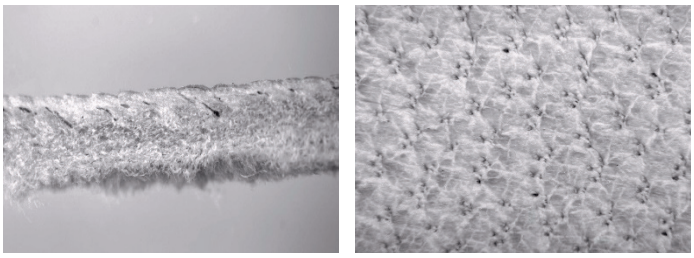
**(a)** (0% Syntan) Cross Section and Grain Surface



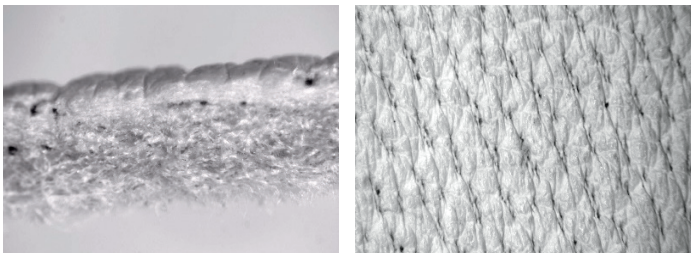
**(b)** (2% Syntan) Cross Section and Grain Surface



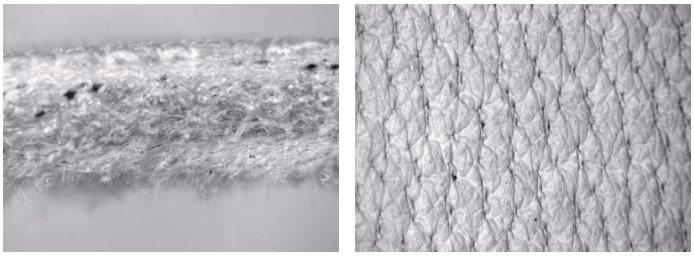
**(c)** (4% Syntan) Cross Section and Grain Surface



**(d)** (6% Syntan) Cross Section and Grain Surface

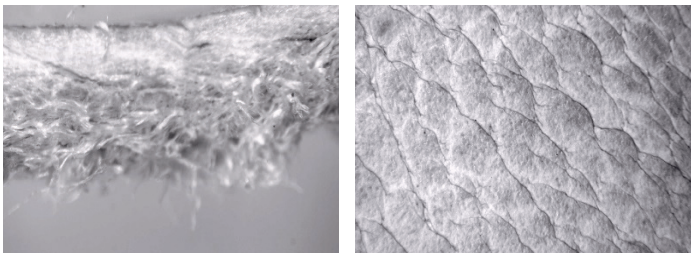


**(e)** (8% Syntan) Cross Section and Grain Surface

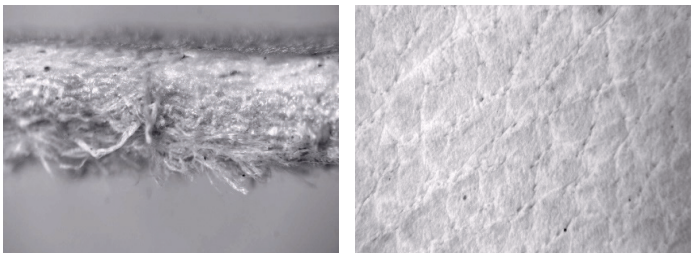


**(f)** (10% Syntan) Cross Section and Grain Surface

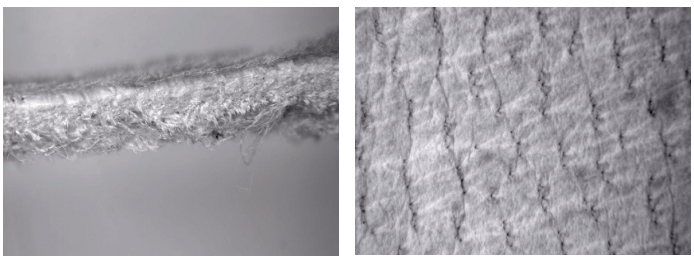
**Figure 6.** Optical images of leathers with 0% of fatliquor (cross section and grain surface)



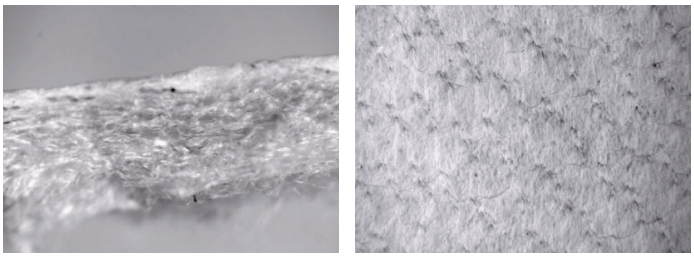
**(a)** (0% Syntan) Cross Section and Grain Surface



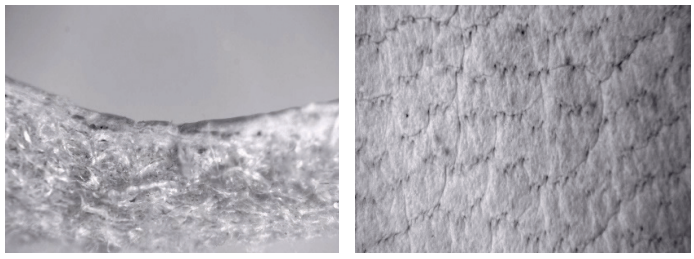
**(b)** (2% Syntan) Cross Section and Grain Surface



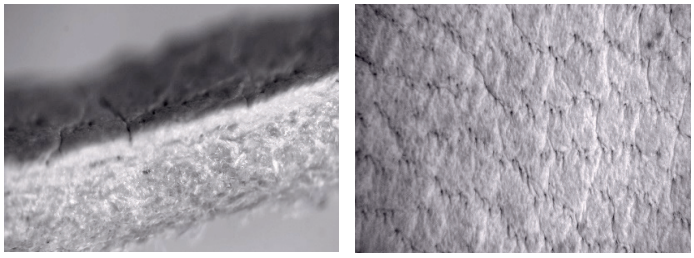
**(c)** (4% Syntan) Cross Section and Grain Surface



**(d)** (6% Syntan) Cross Section and Grain Surface



**(e)** (8% Syntan) Cross Section and Grain Surface



**(f)** (10% Syntan) Cross Section and Grain Surface

**Figure 7.** Optical images of leathers with 10% of fatliquor (cross section and grain surface)

## Conclusion

The chemical and physical characteristics of the leathers are an essential standard norm for varied applications. The choice of leather products desires the specificity of the post-tanning and finishing processes. Stitching is an inevitable mechanical operation required for assembling goods, garment manufacturing, and other leather products through sewing. From our earlier studies, our research group has attempted to cognize the relationship between the sewing operation and post-tanning chemicals. The impact of phenolic and melamine syntans on leather sewability has been studied. In persistence to the recently published research article on *"Physico-Insight on Sewability Properties of Crust Leathers using Melamine Syntan and Synthetic Fatliquor"*, the current article establishes the influence of hydrophilicity-hydrophobicity behavior of leather on sewability. The surface behavior of the leather has been determined using contact angle meter and gloss value measurements and correlated with the sewing values. The θ values obtained for the leathers treated only with varied offers of melamine syntan have shown less sewing performance compared to the leather treated with 10% fatliquor and varied offers of melamine syntan. The disparity in sewing values is mainly attributed to the fatliquoring of leather. Moreover, the leather, which has adequately lubricated fibers, limits the coalescence of collagen fibers, easing the leather's stitchability.

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## References

1. Virginija Jankauskaitė, Indira Jiyembetova, Ada Gulbinienė, Justa Širvaitytė, Kęstutis Beleška, Virginijus Urbelis; Comparable Evaluation of Leather Waterproofing Behaviour upon Hide Quality. I. Influence of Retanning and Fatliqouring Agents on Leather Structure and Properties. *Materials Science*, Vol. **18**, No. 2. 2012.
2. Krishnaraj, K., Thanikaivelan, P., Chandrasekaran, B.; Relation between drape and mechanical properties of goat suede garment leathers. *J. Soc. Lea. Tech. Chem*. **93**, 1-7, 2000.
3. Phebe, K., Chandrasekaran, B., Mandal, A.B.; Sewability of sheep nappa garment leather. *Res. J. Textile. Apparel*, **18** (2), 49-55, 2014.
4. Kallen Mulilo Nalyanya, Ronald K. Rop, Arthur Onyuka, Zephania Birech, Alvin Sasia; Effect of crusting operations on the physical properties of leather. *Leather and Footwear Journal*, **18**, 4, 2018.
5. Anthony D. Covington, William R. Wise; Current trends in leather science. *Journal of Leather Science and Engineering,* **2**:28, 2020.
6. Jayakumar GC, Phebe Aaron, K., Krishnaraj, K.; A comprehensive understanding on sewability of natural biomaterial: an insight on process optimization during leather manufacture. *JALCA* **116**, 233-238, 2021.
7. Jayakumar GC, Niklesh C, Jeyas Kandhan S, Phebe Aaron K, Krishnaraj K: Physico-Insight on Sewability Properties of Crust Leathers Using Melamine Syntan and Synthetic Fatliquor. *JALCA* **117**(10), 2022.
8. Katarina Zelova, Viera Glombikova, Antonin Havelka; Problems with perfect-looking two-needle decorative seams in the manufacture of leather car seat covers. *Textile Research Journal,* Volume: **90**, Issue: 1, Page(s): 49-62, January 1, 2020.
9. C.A. Papakonstantinou, H. Chen, V. Bertola, A. Amirfazli; Effect of condensation on surface contact angle, *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* Volume **632**, 2 January 2022, 127739.
10. Mamoni Probha Borah, Binita Baishya Kalita; Functional Finishing of eri silk and its union fabrics for hydrophobicity. *International Journal for Modern Trends in Science and Technology*, **6**(9S): 175-182, 2020.
11. M.P.Cook, K.Thomas; Evaluation of gloss meters for measurement of moulded plastics, *Polymer Testing,* Volume **9**, Issue 4, 1990, Pages 233-244.
12. M. Khabir Uddin, Mubarak A. Khan, K. M. Idriss Ali; Development of Polymer Films and Its Application on Leather Surfaces, *Journal of Applied Polymer Science*, Vol. **60**, 887-895 (1996).
13. Gararda, A.; Investigation of the seam performance of pet/nylon-elastane woven fabrics. *Textile Research J*., **78** (21), 21-27, 2008.
14. Phebe, K., Krishnaraj, K., Chandrasekaran, B.; Evaluation of sewability of nappa leathers. *J. Fashion Technol. Textile Eng.*, **4**:2, 2016.
15. IUP 6, Measurement of tensile strength and percentage elongation, *J. Soc. Lea. Technol. Chem*., **84**, 317-321, 2000.
16. IUP 9, Measurement of distension and strength of grain by the ball burst test. Official methods of analysis, *J. Soc. Leather Tech. Chem*., 1996.
17. Peter Maina, Moses A Ollengo and Esther W Nthiga; Trends in leather processing: A Review. *International Journal of Scientific and Research Publications*, Volume 9, Issue 12, ISSN 2250-3153, December 2019
18. Danish, P., Kneip, M.; Modern hydrophobic systems: new water repellents and retannages for shoe uppers, *Journal of the American Leather Chemists Association*, 91, 120-125, 1996.
19. Ya-e Feng, Xuepin Liao, Ya-nan Wang, Bi Shi; Improvement in Leather Surface Hydrophobicity through Low-pressure Cold Plasma Polymerization, *Journal of the American Leather Chemists Association*, March 2014.
20. Burçak Karagüzel Kayao lu, Emre Öztürk; Imparting Hydrophobicity to Natural Leather through Plasma Polymerization for Easy Care Effect, *Fibers and Polymers,* 2013, Vol.14, No.10, 1706-1713.
21. Carlos Ruzafa Silvestre, María Pilar Carbonell Blasco, Saray Ricote López, Henoc Pérez Aguilar, María Ángeles Pérez Limiñana, Elena Bañón Gil, Elena Orgilés Calpena and Francisca Arán Ais; Hydrophobic Leather Coating for Footwear Applications by a Low-Pressure Plasma Polymerisation Process, *Polymers*, 2021, 13, 3549.
22. Olga Serenko, Zarina Nizamova, Mikhail Kalinin, Yuri Ostrovsky, Ludmila Polukhina, Aziz Muzafarov; Effect of the Morphology of Leather Surface on the Hydrophobic-Hydrophilic Properties, *Advances in Materials Physics and Chemistry*, 2014, 4, 13-19

# A Rapid Quantification of Hydroxyproline in Leather using High-Performance Liquid Chromatography - Fluorescence Detection (HPLC-FLD) Method

by

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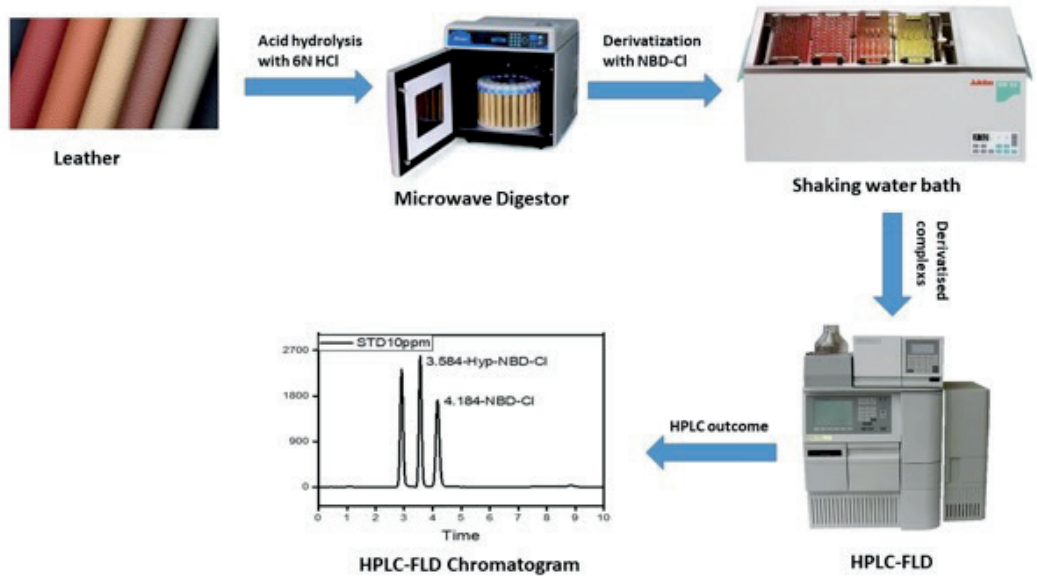
## Abstract

In the area of leather development research, determining collagen content through measurement of 4-Hydroxyproline (Hyp) has been major challenging task due to the interference of various leather-processing chemicals. To overcome this problem, for the first time we have used a High-Performance Liquid Chromatography coupled with Fluorescence Detection (HPLC-FLD) method for accurate determination of Hyp in leather samples through chemical derivatization of Hyp with 7-chloro 4 nitro benzofuran (NBD-Cl) reagent. The HPLC-FLD analysis was performed on a PICO TAG column with an isocratic mobile phase (80 % of 0.1M, pH 7.2 sodium acetate and 20% of acetonitrile, v/v) at a flow rate of 0.7mL/min. The detection was carried out at an excitation wavelength of 465 nm and its emission at 535nm. The retention time of Hyp was found to be ~3.5 minutes and the total run time was about 10 minutes. The method validation indicated that this analytical method is precise (3-12%RSD), accurate (90-100%), the limit of detection 0.01µg/ml, the limit of quantification - 0.03 µg/ml and linear ($R^2$ -0.9995) over the concentration range of 0.1 -2.0 µg/ml. The obtained result indicated that the assay linear range was acceptable for repeated analysis and suitable for the complete range of hydroxyproline levels present in leather samples. Compared to the traditional method (IUC 17:1980), this analytical method demonstrates higher simplicity, specificity, reproducibility, and it could be useful for certifying leather products as well as inspecting international trade in leather and hides.

## Introduction

Leather is made from animal skin and chemical tanning agents such as synthetic or natural chemicals.[1,2] By cross-linking collagen molecules, a main structural protein found in the skin, these chemical tanning techniques improve the mechanical strength and stability of leather. Hydroxylated amino acids like 4-Hydroxyproline (Hyp) of about 12-14% are seen in long fibrous proteins namely collagen.[3,4] For synthesis and maintenance of collagen's triple-helical structure, amino acids are vital. Several colorimetric methods for estimating collagen content of various samples by measuring Hyp have been developed over the years.[5-7] The leather matrix includes a high concentration of salt due to its complicated nature. These salts, which are like chemical tanning agents, amino acids, fatty acids, and binders may generate considerable interferences and compromise

## Graphical Abstract

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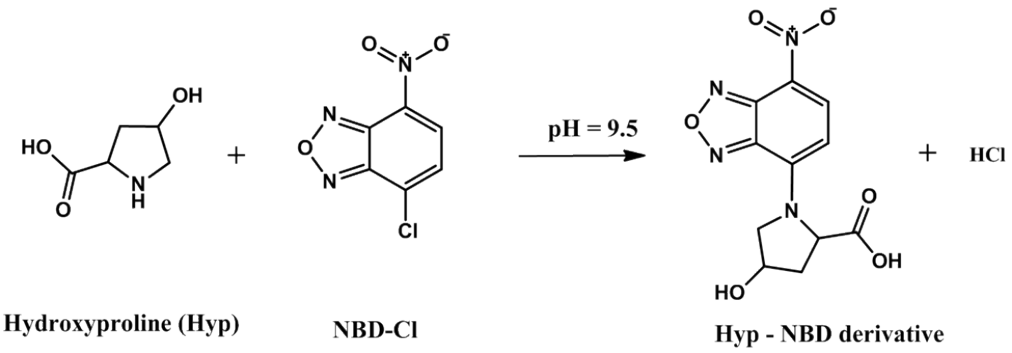


**Figure 1.** The general reaction between Hydroxyproline and NBD-Cl reagent.

the precise determination of Hyp. These calorimetric procedures are only applicable to pure collagen materials and not to leather samples. As a result, developing a precise method for quantifying Hyp in leather samples is crucial for the development of leather products.

Recently, numerous chromatography techniques have been used to measure Hyp from collagen content, which include high-performance liquid chromatography (HPLC), gas chromatography (GC), and micellar electrokinetic chromatography (MEKC).[8-11] The HPLC method is a simple, accurate, and cost-effective method for detecting Hyp in collagen-based products.[9] The low sensitivity and selectivity, as well as the lengthier time required for sample preparation, limit the usefulness of HPLC method. Different pre-column derivatizing chemicals have been used in the HPLC process to improve sensitivity and selectivity.[12-14] The following pre-column derivatizing agents are used such as phenylthiocarbamide (PTC), phenylisothiocyanate (PITC), 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl), O-phthalaldehyde (OPA), and 4-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS).[8,12,15-19] These derivatized materials were analyzed via HPLC using various detectors like UV-visible absorption, fluorescence, electrochemical detection, and mass spectrometry.[20-24] The most widely used derivatizing agent for quantifying Hyp is NBD-CI. This is due to various biological samples that show excellent sensitivity, selectivity, and reactivity towards primary and secondary amines.[18, 25] Furthermore, the NBD-Cl has a significant fluorescence property, which increases the capacity to detect the analyte even at low concentrations.[9] However, because the fluorescent derivative based on NBD-Cl–Hyp is particularly light sensitive, it requires a longer chromatographic separation time of roughly 50 minutes.[26-28] As a result, NBD-Cl is currently being investigated as a derivatizing reagent in an HPLC approach with a short chromatographic separation time.

To date the traditional colorimetric method (IUC17:1980) was the only method available to estimate the Hyp in leather.[29] In this method, Hyp was derivatized with p-Dimethylaminobenzaldehyde (DMAB) and oxidized to pyrrole. These traditional methods provide better sensitivity and selectivity for determination of Hyp in pure hide and untanned leather material. However, this method is not suitable for tanned leather material due to requiring longer time for hydrolysis and involving a complicated oxidation reaction. This observation can be ascribed to the interference of numerous chemicals, which were used during the leather tanning process.

In this study hydrolysis time of leather into hydroxylates was reduced to 120 minutes and the proposed pre-column derivatization method for hydroxyproline quantification in leather, using NBD-Cl as a fluorescent labelling reagent for HPLC with fluorescence detection (FLD) was established. In accordance with CITAC Eura Chem requirement this method was validated.[30] The derivatization of hydroxyproline with NBD-Cl is depicted schematically in (Figure 1).

## Experimental

### Materials and Methods

#### Chemicals

Hydroxyproline (purity > 98%) and 7-chloro-4-nitrobenzol-2-oxa-1,3-diazole or NBD-Cl were purchased from Sigma-Aldrich Corporation, St. Louis MO, USA. NBD-Cl reagent (3000 µg/ml) was prepared in Methanol. Acetonitrile, Methanol, Dichloromethane and Tetrahydrofuran were of HPLC grade and procured from Merck Corporation Germany. Leathers of cow, buffalo, sheep and goat origin were collected from the pilot tannery of the institute. Hide powder was prepared as per standard procedure and characterized to meet international standards. All other chemicals used were of analytical grade without further purification. Water used for mobile phase and application was produced in the laboratory using Type I water purifier model Flex 3 of ELGA lab water UK.

#### Chromatographic conditions

A Waters e2695 HPLC-FLD system (Waters Instruments Corporation, Milford, USA) equipped with an alliance pump (2695 model), an autosampler and a multi λ fluorescence detector (2475 model) was used to analyze the hydroxyproline. The separation was performed on a PICO TAG column (3.9 x 300 mm 60A, 4µm) with an isocratic mobile phase comprising 80% of 0.1M sodium acetate and 20% of acetonitrile. The flow rate was fixed at 0.7 mL/min and 10µL of the sample was injected through Waters autosampler at

the temperature of 25°C. The fluorescence intensities of eluate were observed with a Waters fluorescence spectrophotometer (Model 2475) using an excitation wavelength of 465 nm and an emission wavelength in the range of 470 -800 nm respectively. Empower software, Version 2.0 (Waters Instruments Corporation, Milford, USA) was used to record and process the observed result. The recovery of hydroxyproline from the sample was calculated from the corresponding chromatogram peak area.

**Preparation of Standard Hydroxyproline Solution**

A stock solution of hydroxyproline (7.62 mM) was prepared by dissolving 100 mg of Hyp using acetonitrile (ACN) and water (H2O) (1:1) mixture in 100 ml of standard flask. Standard solutions (5, 10, 25, and 50 µg/ml) were prepared by serial dilution of stock solution with ACN: $H_2O$ mixture and used for the preparation of the calibration curve for the sample analysis.

**Preparation of Sample and derivatization**

Leather (2 g, width 4mm × height 4mm) was taken in a 250 mL amber bottle and 50 mL of dichloromethane (DCM) and tetrahydrofuran (THF) solvents mixture (1:1 ratio) was added for the leather degreasing process. Subsequently, these solutions were ultrasonicated for 30 minutes at room temperature to remove the fat and top finishing chemicals present in leather. This degreased leather was taken out from an amber bottle and dried in a hot air oven at 50°C for 6 h to remove the adsorbed solvents. The dried leather sample was placed in a 100 mL amber bottle and 15 mL of Type-1 water was added and kept in a shaker for 30 minutes at 60 rpm to remove the soluble metals in the degreased leather. Following this, the leather was dried in a hot air oven at 120°C for 10 minutes and kept in a desiccator till further work. From the desiccator, 50 mg of degreased leather sample was taken in a glass vessel employed with microwave digester CEM Corporation model MARS (USA) and 10 ml of hydrochloric acid solution (6 N) was added for the hydrolysis process. The microwave-based hydrolysis reaction was performed with 100 psi at 120°C for 120 minutes. After this, the hydrolysate, which was obtained, was made up to 50mL by using Type-1 water. Ten mL of diluted hydrolysate was pipetted into a China dish and dried completely over the water bath at 50°C for 60 minutes. The obtained yellowish residue was re-dissolved and made up to 10mL by using 0.4M borate buffer and stored in the refrigerator at 4°C until use.

For the derivatization procedure, an aliquot of the hydrolyzed sample (100 µL) or standard stock solution (50-500 µL) was taken in a 10mL standard flask and 0.5mL of NBD-Cl reagent was added (3000 µg/ml in methanol) along with 1 mL of 0.4N borate buffer (pH = 9.5) and was made up to completion by adding methanol. The resulting mixture was transferred to a 25mL polypropylene centrifuge tube and placed in a shaking water bath at 60°C for 5 minutes. To this, 100 µL of 1N HCl solution was slowly added and cooled to 0°C for 30min. The obtained final solution was filtered through Whatman filter paper number 42 and further filtered with 0.2 µm Millipore hydrophilic syringe filter paper, injected into HPLC-FLD. The obtained results from the leather samples were compared against standard collagen and hide powder.

**Method Validation**

The proposed chromatographic method was validated according to EuraChem CITAC guidelines determining linearity, limits of detection (LOD), limit of quantification (LOQ), Precision, Accuracy and Robustness.

**Linearity and concentration range**

The linearity of the chromatographic method was tested for Hyp-NBD derivatives. The linearity was studied within the concentration range of 0.1-2.0 µg/mL for Hyp. The calibration curves were constructed by plotting fluorescent peak area against concentrations of Hyp. The obtained data were statistically treated using the linear regression analysis and the analytical parameters were calculated.

**Limit of detection and quantification**

The limits of detection (LOD) and quantification (LOQ) of Hyp were determined at respective signal-to-noise (S/N) ratios of 3 and 5 by repeated analysis (n=10) of the same concentration of Hyp solution. LOD and LOQ were calculated as 3 × sA/B and 10 × sA/B respectively, where sA is the standard deviation of response and B is the slope of the linear equation used for calibration.

**Accuracy**

Accuracy of the chromatographic method was determined by spike recovery studies at three different concentration levels (30%, 100% and 200% is concentration level reported in percentage) of Hyp within the LOQ range. Three samples from each concentration level were analyzed and the average result is presented as a percentage of relative spike recovery.

**Precision**

Precision (Inter-day) of the chromatographic method was assessed by six repeated analyses of three different concentration levels (30%, 100% and 200%) of Hyp within the LOQ range. The obtained precision result is represented as a percentage of relative standard deviation.

**Robustness**

The robustness of the proposed method was assessed by evaluating the influence of six different analysts and obtained results are expressed in relative standard deviation.

**Estimation of Fibrous protein in the tanned leather sample**

The fibrous protein (collagen) content in leather samples were estimated from the hydroxyproline content of leather hydrolysate. The mass ratio of collagen to hydroxyproline in leather sample was calculated using a factor ranging from 7.14-7.69. The percentage of

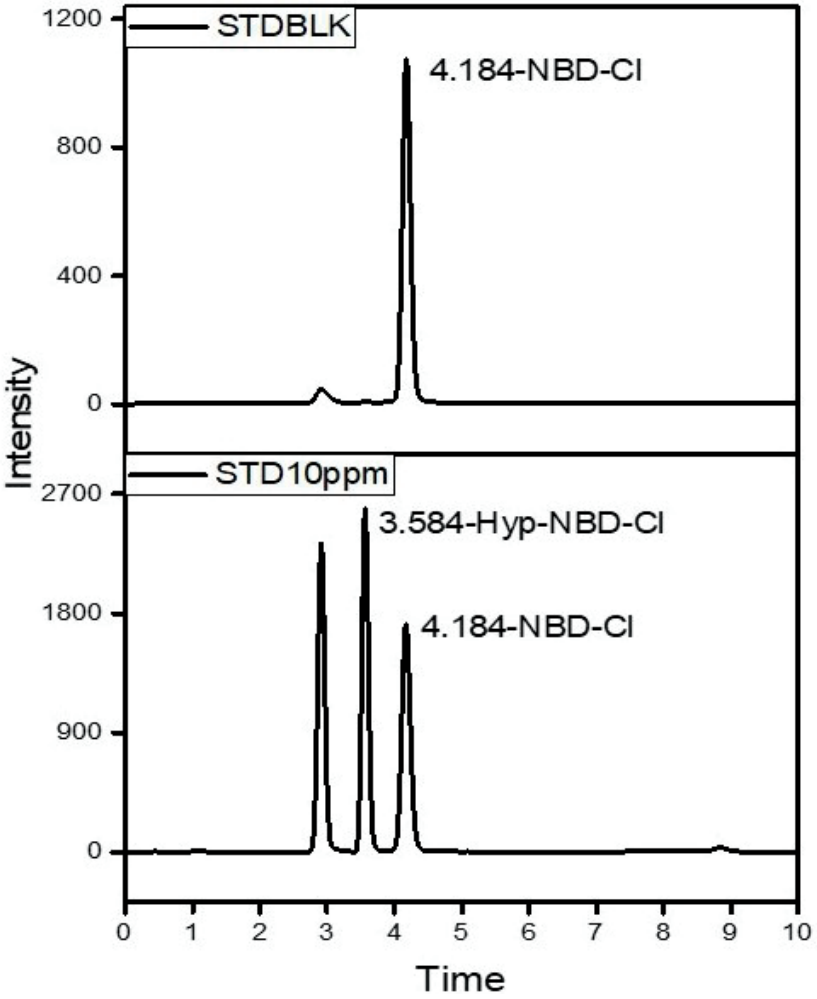


**Figure 2.** HPLC chromatogram of Blank and Standard Hydroxyproline.

fibrous protein in leather sample can be calculated through using equation 1.[31, 32]

$$\frac{\textit{Micrograms hydroxyproline in 1 mL hydrolysate}}{\textit{Micrograms leather represent in 1 mL hydrolysate}} \; x\,7.46\,x\,100 \qquad (1)$$

## Results and Discussion

**Method development and optimization**

The prepared derivatized sample (standard Hyp-NBD) was injected in the HPLC-FLD system and isocratic mobile phase separation was detected in fluorescence and chromatogram system (Figure 2). In fluorescence spectrum, the appearance of broad emission at 535nm indicated that NBD-Cl was successfully derivatized with Hyp. This observation was further confirmed by the HPLC chromatogram. The derivatized hydroxyproline exhibited several elution peaks with higher symmetry and resolution. The elution peaks appeared at a retention time of 2.913, 3.584 and 4.194 minutes corresponding to blank, Hyp-NBD-Cl and un-reacted NBD-Cl respectively. The absence of co-elution along with Hyp peak suggested that this developed method has higher specificity in the determination of hydroxyproline. To find the suitable derivative reaction and separation condition for the HPLC-FLD method, various experimental parameters including column type, mobile phase composition, pH of the buffer, the concentration of NBD-Cl, reaction temperature and time of the derivative reaction were investigated.

Derivatization of standard hydroxyproline with NBD-Cl was performed in acidic and basic pH conditions. The results of the experiment indicated that Hyp-NBD based fluorescent emission peak appeared only at pH above 9.5, whereas no emission peak was observed in acidic (pH 2.2) conditions. This result clearly suggested that derivatization specifically occurred in Hyp at basic pH conditions through SN2 nucleophilic reaction between the Hyp and NBD-Cl reagent.[28, 30]

Three column materials, viz., Gemini (18) (5µ), Chromolith RP-18e and PICO TAG were used to separate the Hyp-NBD derivatives. The obtained chromatogram indicated that the PICO TAG column showed better separation compared to the other two columns.

The role of the mobile phase was investigated by varying the composition. Sodium acetate and acetonitrile were used as mobile phases at 60:40, 70:30 and 80:20 v/v. A better separation

**Table I**

**Optimization of chromatographic method through changing the various parameters.**

| Columns used | Mobile Phase | Flowrate (mL/min) | Observation | Result |
|---|---|---|---|---|
| Gemini (18) (5μ)) (110A°) 150 × 4.60mm | (Sodium Acetate: Acetonitrile)<br>60:40<br>70:30<br>80:20 | 0.7 - 1.0 | Not Proper Separation | Method Rejected |
| Chromolith® RP-18e 2 μM, 130 Å | (Sodium Acetate: Acetonitrile)<br>60:40<br>70:30<br>80:20 | 0.7 - 1.0 | Not Proper Separation and variable RT | Method Rejected |
| PICO TAG, 3.9 × 300mm 60A, 4μm | (Sodium Acetate: Acetonitrile)<br>60:40<br>70:30<br>80:20 | 0.7 | Proper Separation | Method Accepted |

of hydroxyproline was observed at sodium acetate to acetonitrile ratio of 80:20 v/v.

The time and temperature of microwave hydrolysis play an important role in the recovery of Hyp. Initially, the microwave hydrolysis was carried out for 30 minutes at 110°C and the Hyp recovery was around 3-5% in hide powder. When microwave hydrolysis was extended for 60 minutes at 115°C, Hyp recovery increased to 8-10%. Further extension of microwave hydrolysis to 120 min at 120°C leads to 12-13% of Hyp recovery.

The influence of NBD-Cl volume (5 μL, 10 μL and 100 μL i.e, 7.62 μM, 38.1 μM and 76.2 μM) in the recovery of Hyp was analyzed. Hyp recovery was around 5, 25 and 95% with 5, 10 and 100 μL of NBD-Cl solution respectively. This result indicated that the reaction of NBD-Cl with Hyp is mostly completed when the concentration of NBD-Cl is 150 times that of the Hydroxyproline.

The optimum conditions were defined as: pH of borate buffer 9.5, NBD-Cl of volume -100μL, mobile phase containing 80:20 v/v sodium acetate: acetonitrile, flowrate 0.7mL/min, reaction temperature and time of 120°C and 120 minutes respectively. The characteristic retention time was around ~3.5 minutes for the Hyp-NBD derivative. The result of the method optimization is summarized in Table I.

## Method validation

### Linearity

The obtained calibration curve is presented in (Figure 3). The results from the linearity study indicated a linear relationship in the concentration range of 0.1-2.0 μg/ml for the Hyp-NBD derivative. From the regression analysis, a linear equation y = 3.00 × 1007(x) -1.66x 1006, where y is the fluorescent peak area and x

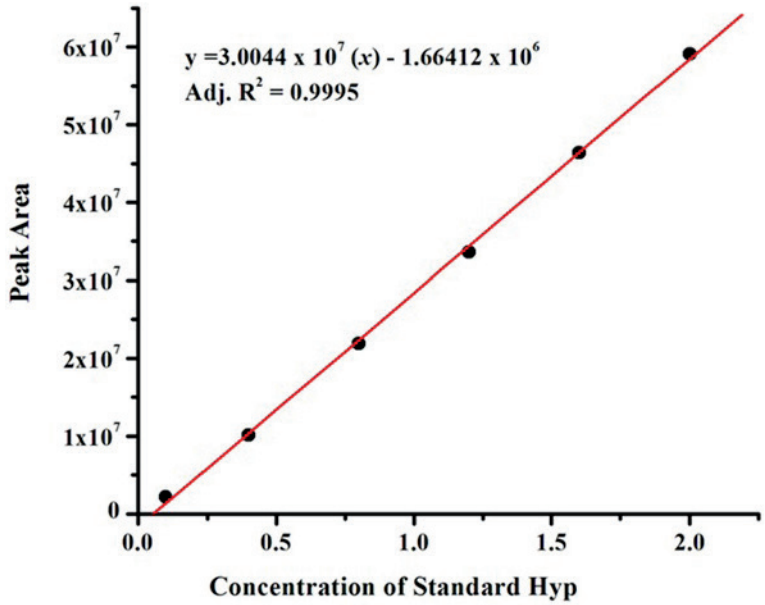


**Figure 3.** Linearity curve of standard Hyp-NBD-Cl.

**Table II**

**Summary of parameters in Method Validation**

| Parameter | | | Hydroxyproline | Acceptance Criterion |
|---|---|---|---|---|
| LOD | | µg/ml | 0.01 | - |
| LOQ | | µg/ml | 0.03 | - |
| Linearity | | $R^2$ | 0.9995 | $R^2$ value is ≥0.99 |
| Accuracy | 30% LOQ | Average Recovery (%) | 96.3 | Values found to be within the range of 70-120% |
| | 100% LOQ | | 99.9 | |
| | 200% LOQ | | 113.6 | |
| Method Precision | 30% LOQ | RSD | 9.4 | RSD value was found to be ≤20% |
| | 100% LOQ | | 7.0 | |
| | 200% LOQ | | 8.1 | |
| Robustness | | RSD | 7.0 | RSD value was found to be ≤20% |

is the concentration of Hyp has been established. The correlation coefficient ($R^2$) was found to be 0.9998, indicating the excellent linearity of the proposed method.

**LOD and LOQ**

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were assessed based on the respective signal to noise ratios of the standard Hyp (Table III). The LOD and LOQ were found to be 0.01 and 0.03 µg/mL respectively. The observed LOD value was much lower than the previously reported method (LOD <1 µg/ml and LOQ 5.5 µg/ml).[33] The LOQ of Hyp-NBD-Cl obtained by the proposed system was compared with the previous reports and the results clearly indicated a higher sensitivity for Hyp analysis.

**Table III**

**Limits of detection (LOD) and quantitation (LOQ) of proposed method**

| Number of Injections | Area |
|---|---|
| 1 | 570745 |
| 2 | 401692 |
| 3 | 432223 |
| 4 | 659130 |
| 5 | 519687 |
| 6 | 400695 |
| 7 | 691522 |
| 8 | 573333 |
| 9 | 506866 |
| 10 | 512869 |
| Mean | 526876.2 |
| St.dev(sA) | 99934.6 |
| B(Slope) | 30044194 |
| LOD=3X(sA)/B | 0.01 |
| LOQ=10X(sA)/B | 0.03 |

## Accuracy

The accuracy was determined based on the recovery of Hyp and the results were expressed as the relative spike recovery for three different percentage solutions (Table IV). The obtained results showed that spike recovery ranges from 99.89% to 113.61%. The obtained results showed good agreement between the measured and actual value (70-120%) indicating the high accuracy of the proposed method.

## Precision

Inter-day precision was examined at three different concentration levels and the obtained results are summarized in Table V. The measured relative standard deviations were below 20% indicating an excellent precision of the proposed method.

**Robustness**

The results of robustness analysis indicated that a change of analyst did not significantly affect the percentage of RSD values. The robustness results are summarized in Table VI. The obtained percentage of RSD values were within the acceptable limits (RSD< 20 %) which clearly suggested that the proposed chromatographic method is highly reliable under the optimized condition. Summarization of the parameter in method validation is given in Table II.

**Table IV**

**Precision of the proposed method**

| Analyte | % Of LOQ (Number of Injections) | Injection values (Area) | Mean | Standard deviation | RSD |
|---|---|---|---|---|---|
| Hyp | 30% of LOQ | | | | |
| | 1 | 137739 | 143875 | 13515 | 9.4 |
| | 2 | 148667 | | | |
| | 3 | 132508 | | | |
| | 4 | 161223 | | | |
| | 5 | 155906 | | | |
| | 6 | 127209 | | | |
| | 100% of LOQ | | | | |
| | 1 | 570745 | 529029 | 37251 | 7.0 |
| | 2 | 551692 | | | |
| | 3 | 492223 | | | |
| | 4 | 559130 | | | |
| | 5 | 519687 | | | |
| | 6 | 480695 | | | |
| | 200% of LOQ | | | | |
| | 1 | 779530 | 724377 | 58830 | 8.1 |
| | 2 | 688695 | | | |
| | 3 | 793335 | | | |
| | 4 | 662538 | | | |
| | 5 | 756118 | | | |
| | 6 | 666043 | | | |

**Table V**

**Accuracy of the proposed method**

| Analyte | % Of LOQ | Spike Conc. (µg/ml) | Sample Conc. (µg/ml) | Actual Conc. (µg/ml) | Recovery | Average (%) |
|---|---|---|---|---|---|---|
| Hyp | 30% of LOQ | 0.01 | 0 | 0.008 | 80.0 | 96.3 |
| | | 0.01 | 0 | 0.0114 | 114.0 | |
| | | 0.01 | 0 | 0.0095 | 95.0 | |
| | 100% of LOQ | 0.03 | 0 | 0.0295 | 98.3 | 99.9 |
| | | 0.03 | 0 | 0.0323 | 107.7 | |
| | | 0.03 | 0 | 0.0281 | 93.7 | |
| | 200% of LOQ | 0.06 | 0 | 0.0853 | 108.8 | 113.6 |
| | | 0.06 | 0 | 0.0707 | 117.8 | |
| | | 0.06 | 0 | 0.0885 | 114.2 | |

**Table VI**

**Robustness of the proposed method**

| Analyte | 100 % Of LOQ (Number of Injections) | Injection values (Area) | Mean | Standard deviation (sA) | RSD |
|---|---|---|---|---|---|
| Hyp | 1 | 570745 | 529029 | 37251 | 7.0 |
| | 2 | 551692 | | | |
| | 3 | 492223 | | | |
| | 4 | 559130 | | | |
| | 5 | 519687 | | | |
| | 6 | 480695 | | | |

**Tanned leather sample**

The optimized chromatographic condition was applied to the various animal origin leather samples to determine the hydroxyproline content in the leather. The obtained results (Figure 4) indicated that the retention time of Hyp was around 3.5 minutes for all leather samples. This observation clearly suggested that the retention time of hydroxyproline in leather samples was not significantly changed in the presence of various chemical interferences when compared to pure collagen samples. The quantity of Hyp and collagen content in leather samples is presented in Table VII. In addition, the quantified Hyp content in the leather sample matched with pure collagen and hide powder (Hyp, 12-14%).[34] The obtained result indicated that there is an insignificant difference in the Hyp content based on the animal origin. Therefore, future work will focus on the differentiation of leather samples through the measurement of hydroxyproline content in advanced spectral techniques.

## Conclusion

In this study, we have demonstrated the applicability of the HPLC-FLD method to accurately determine the hydroxyproline in leather samples. The developed method was validated according to EuraChem CITAC guidelines. The main feature of the developed method is the short retention time of around 3.5 minutes. This method is simple, sensitive, linear, precise, accurate, reproducible and readily applicable to leather from different animal origins. This method is versatile in quantification of animal protein in leather

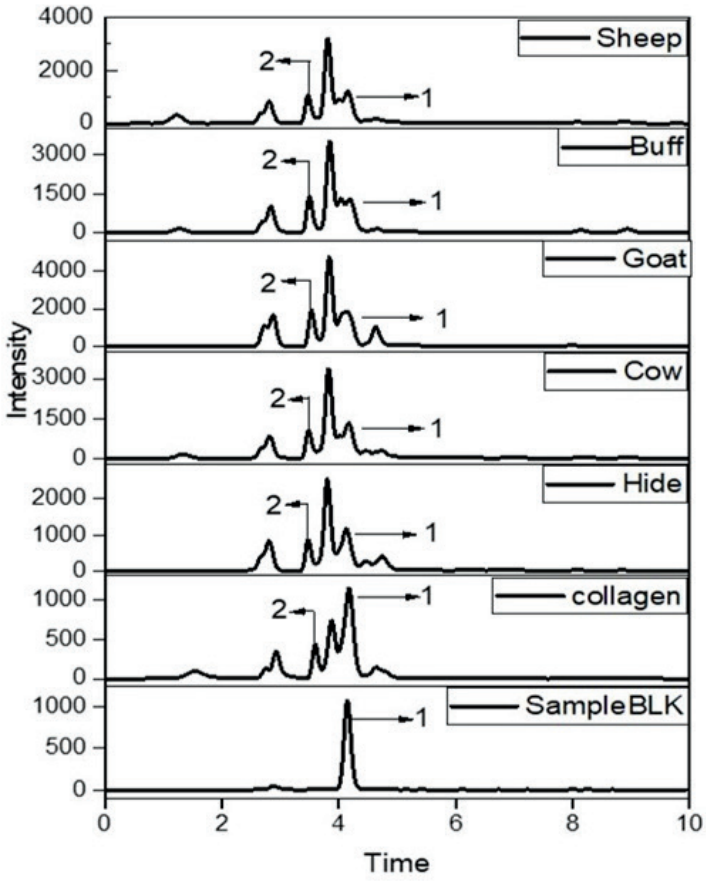


**Figure 4.** HPLC chromatogram of collagen, Hide and different origin leather samples, Where 1 denotes the retention time of NBD-Cl and 2 denotes the retention time of Hyp-NBD-Cl complex.

**Table VII**

**Total collagen content calculated using the equation**

| S.No. | Specimen | Hydroxyproline Content | Total collagen Content |
|---|---|---|---|
| 1. | Collagen-Type-I | 0.136 | 97.92 |
| 2. | Hide Powder | 0.132 | 95.04 |
| 3. | Buff leather | 0.092 | 66.24 |
| 4. | Cow leather | 0.097 | 69.84 |
| 5. | Sheep leather | 0.098 | 70.56 |
| 6. | Goat leather | 0.097 | 69.84 |

using hydroxyproline estimation. This method can be used to distinguish leather from vegan based leather-like materials such as those from cactus, pineapple etc., and leather like material based on the polymeric material. This study can establish the genuineness of the leather product.

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**Declaration of interest statement**

Priya Narayanan, Suresh Sethurajan, Mohan Vedhanayagam, Kalarical Janardhanan Sreeram all the authors are full time employees of CSIR-Central Leather Research Institute. None of the authors have conflicts to declare.

## Author contribution

Priya Narayanan: Conceptualization, Methodology, Validation, and Writing - Original Draft, Suresh Sethurajan: Data Curation, Software, Vedhanayagam: Writing - Review & Editing Kalarical Janardhanan Sreeram: Visualization, Investigation, Supervision.

## References

1. Pinto, P. C.; Sousa, G.,Crispim, F., Silvestre, A. J., Neto, C. P.; Eucalyptus globulus bark as source of tannin extracts for application in leather industry. *ACS Sustain. Chem. Eng.***1**, 950-955, 2013.
2. Vedhanayagam, M., Teddy, T.,Sreeram, K.J., Rao, J. R., Nair, B. U.; Value added leather auxiliaries from paper and pulp industry waste., *JALCA* **110**, 295-301, 2015.
3. Sun, X., Jin, Y., Lai, S., Pan, J., Du, W., Shi, L.; Desirable retanning system for aldehyde-tanned leather to reduce the formaldehyde content and improve the physical-mechanical properties. *J. Clean. Prod*., **175**, 199-206, 2015.
4. Lyu, B., Chang, R., Gao, D., Ma, J.; Chromium footprint reduction: nanocomposites as efficient pretanning agents for cowhide shoe upper leather. *ACS Sustain. Chem. Eng.*, **6**, 5413-54235, 2018.
5. Miyada, D., Tappel, A.; Colorimetric determination of hydroxyproline. *Anal. Chem*., **28**, 909-910, 1956.
6. Kivirikko, K., Liesmaa, M.; A colorimetric method for determination of hydroxyproline in tissue hydrolysates. *Scand. J. Clin. Lab.*, **11**, 128-1337, 1959.
7. Kolar, K.; Colorimetric determination of hydroxyproline as measure of collagen content in meat and meat products: NMKL collaborative study. *JALCA* **73**, 54-57, 1990.
8. Dunphy, M., Bhide, M., Smith, D.; Determination of hydroxyproline in tissue collagen hydrolysate by derivatization and isocratic reversed-phase high-performance liquid chromatography. *J. Chromatogr. B Biomed. Appl.*, **420**, 394-397, 1987.
9. VazquezaOrtaz, F., Morana, Fuenmayor, O., Gonzaleza, mandez, N.; Hydroxyproline measurement by HPLC: Improved method of total collagen determination in meat samples., *J.Liq.Chromatogr. Relat*., **27**, 2771-2780, 2004.
10. Colgrave, M. L., Allingham, P. G., Jones, A.; Hydroxyproline quantification for the estimation of collagen in tissue using multiple reaction monitoring mass spectrometry. *J. Chromatogr. A*.**1212**, 150-153, 2008.
11. Dong, Y.-l., Yan, N., Li, X., Zhou, X.-m., Zhou, L., Zhang, H.-j., Chen, X.-g.; Rapid and sensitive determination of hydroxyproline in dairy products using micellar electrokinetic chromatography with laser-induced fluorescence detection. *J. Chromatogr. A*., **1233**, 156-160, 2012.
12. Teerlink, T., Tavenier, P., Netelenbos, J. C.; Selective determination of hydroxyproline in urine by high-performance liquid chromatography using precolumn derivatization. *Clin. Chim. Acta*.,**183**, 309-315, 1989.
13. Kakinuma, M., Watanabe, Y., Hori, Y., Oh-i, T., Tsuboi, R.; Quantification of hydroxyproline in small amounts of skin tissue using isocratic high-performance liquid chromatography with NBD-F as fluorogenic reagent. *J. Chromatogr. B*. **824**, 161-165, 2005.
14. Ren, Y., Zhao, J., Shi, Y., Chen, C., Chen, X., Lv, C.; Simple determination of L-hydroxyproline in idiopathic pulmonary fibrosis lung tissues of rats using non-extractive high-performance liquid chromatography coupled with fluorescence detection after pre-column derivatization with novel synthetic 9-acetylimidazol-carbazole. *J. Pharm. Biomed. Anal*. **664**, 435-439, 2017.
15. Sormiachi, K., Ikeda, M., Akimoto, K., Niwa, A.; Rapid determination of dabsylatedhydroxuproline from cultured cells by reversed-phase high-performance liquid chromatography. *J. Chromatogr. B Biomed. Appl*.**664**, 435-439, 1995.
16. Heinrikson, R. L., Meredith, S. C.; Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal. Biochem*, **136**, 65-74, 1984.
17. Dai, Z.; Wu, Z.; Jia, S.; Wu, G.; Analysis of amino acid composition in proteins of animal tissues and foods as pre-column o-phthaldialdehyde derivatives by HPLC with fluorescence detection. *J. Chromatogr. B*. **964**, 116-127, 2014.
18. Teng, Z., Lv, H., Zhang, Y., Zhang, Y., Guan, J., Chengyin, W.; Development of a fluorescent labeling reagent for determination of proline and hydroxyproline in myeloma patient plasma by HPLC-FLD. *J. Chromatogr., Sci*. **54**, 1743-1751, 2016.
19. Ahnoff, M., Grundevik, I., Arfwidsson, A., Fonselius, J., Persson, B.-A.; Derivatization with 4-chloro-7-nitrobenzofurazan for liquid chromatographic determination of hydroxyproline in collagen hydrolysate. *Anal. Chem*. **53**, 485-488, 1981.
20. Ignataeva, N. Y., Danilov, N., Averkiev, S., Obrezkova, M., Lunin, V.; Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. *J. Anal. Chem*.**62**, 51-57, 2007.
21. Inoue, H., Iguchi, H., Kono, A., Tsuruta, Y.; Highly sensitive determination of N-terminal prolyl dipeptides, proline and hydroxyproline in urine by high-performance liquid

chromatography using a new fluorescent labelling reagent, 4-(5, 6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride. *J. Chromatogr. B Biomed. Appl.*,**757**, 369-373, 2001.

22. Kaspar, H., Dettmer, K., Gronwald, W., Oefner, P. J.; Automated GCMS analysis of free amino acids in biological fluids. *J. Chromatogr. B*., **870**, 222-232, 2008.
23. Conventz, A., Musiol, A., Brodowsky, C., Maller-Lux, A., Dewes, P., Kraus, T., Schettgen, T.; Simultaneous determination of 3-nitrotyrosine, tyrosine, hydroxyproline and proline in exhaled breath condensate by hydrophilic interaction liquid chromatography/electrospray ionization tandem mass spectrometry. *J. Chromatogr.B*., **860**, 78-85, 2007.
24. Ikehara, T., Habu, N.,Nishino, I., Kamimori, H.; Determination of hydroxyproline in rat urine by high-performance liquid chromatography with electrogenerated chemiluminescence detection using tris (2, 2-bipyridyl) ruthenium (II). *Anal. Chim. Acta*., **536**, 129-133, 2005.
25. Aly, H., El-Shafie, A. S., El-Azazy, M.; Utilization of 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) for spectrochemical determination of L-ornithine: A multivariate optimization-assisted approach. *RSC Adv.,* **9**, 22106-22115, 2019.
26. Hughes, H., Hagen, L., Sutton, R.; Liquid-chromatographic determination of 4-hydroxyproline in urine. *Clin. Chem*., **32**, 1002-1004, 1986.
27. Codini, M., Palmerini, C., Fini, C., Lucarelli, C., Floridi, A.; High-performance liquid chromatographic method for the determination of prolyl peptides in urine. *J.Chromatogr. A*. **536**, 337-341, 1991.
28. Lindblad, W. J., Diegelmann, R. F.; Quantitation of hydroxyproline isomers in acid hydrolysates by high-performance liquid chromatography, *Anal. Biochem*, **138**, 390-395, 1984.
29. IUC 17 - Determination of hydroxyproline in materials containing collagen, 1980.
30. Eurachem/CITAC -Guide-the Fitness for Purpose of Analytical Methods - A Laboratory Guide to Method Validation and Related Topics, Second Edition, 2014.
31. Colgrave, Michelle L., Peter G. Allingham, and Alun Jones; Hydroxyproline quantification for the estimation of collagen in tissue using multiple reaction monitoring mass spectrometry. *J. Chromatogr. A,* **1212**.1-2, 150-153, 2008.
32. Robert E. Neuman, Milan A. Logan; The determination of collagen and elastin in tissues. *JBC*, **186**, 549-556, 1950.
33. Kakinuma M, Watanabe Y, Hori Y, Oh-i T, Tsuboi R.; Quantification of hydroxyproline in small amounts of skin tissue using isocratic high-performance liquid chromatography with NBD-F as fluorogenic reagent. *J. Chromatogr. B,* **824**, 161-162, 2005.
34. Etherington, D.J, Sims, T.J.; Detection and estimation of collagen. *J. Sci. Food Agric*., **32**, 539-546, 1981.

# Study on the Crosslinking Modification of Collagen-Based Materials by DMTMM

by
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## Abstract

In order to explore the mechanism of interaction between DMTMM and collagen-based materials, gelatin was used as a skin simulant and prepare DMTMM crosslinked modified gelatin materials. FT-IR and Zeta potential measurements were used to analyze and characterize DMTMM crosslinking modified gelatin materials; revealing the mechanism of interaction between DMTMM and collagen-based materials. Firstly, the "zero-length" crosslinking agent DMTMM activated the carboxyl groups on the collagen-based materials and promoted the formation of amide bonds between the collagen-based materials. At the same time, the hydrogen bond formed between the hydroxyl group and its active group causes zero-length crosslinking modification of collagen-based material under the joint action of these two kinds of bonding. DSC, TG, swelling properties, SEM and other analytical methods were used to test the thermal stability and microstructure of DMTMM crosslinked modified gelatin materials. It was found that DMTMM can effectively improve the stability of collagen-based materials, increase the thermal denaturation temperature and humidity and reduce the decomposition rate.

## Introduction

The reagent 4-(4, 6-dimethoxy-1, 3, 5-triazine-2-group) -4-methylmorpholine chloride (DMTMM) can activate carboxyl group in liquid or solid phase peptide synthesis. Regardless of whether the solvent medium is alcohol or aqueous solution, it can effectively promote carboxyl group activation and react with amino group to form amide bond.[1] Moreover, DMTMM will not be embedded into the final product during the reaction process, and there is no linker or spacer between the bonding molecules directly, so DMTMM can be called a "zero-length" crosslinking agent.[2] The amide bond derived from the activation of carboxyl group is the structural basis of protein. However, since carboxyl hydroxyl group in carboxyl group is difficult to cleave, we tried to introduce an activating group with good cleaving performance to replace carboxyl hydroxyl group to form acyl compounds, so as to facilitate the subsequent conversion reaction.

The cross-linking modification of collagen-based materials is the basis of leather tanning, and it is also a necessary way to expand the application of collagen-based biomaterials to improve their stability.[3,4] Based on the above background, in this paper, DMTMM has the property of efficiently activating carboxyl group to form acyl compounds, and the cross-linking modification effect of DMTMM on rubber base materials will be explored. However, due to the complex composition and structure of rawhide, it is not to explore the interaction mechanism between DMTMM and collagen, as well as the changes in the morphology and properties of finished products. Under the action of hydrolysis and other external factors, collagen can destroy its three-strand helical structure and partially bond and break, thus forming a denatured product, that is gelatin. Therefore, the amino acid composition and chemical properties of gelatin are similar to collagen.[5] In order to better explore the mechanism of action between DMTMM and dermal collagen and the effect of better reaction on various properties, gelatin with homology can be selected as the simulation of rawhide.

## Experiment

### Experimental materials and instruments

Main instruments and models: Nano particle size and potential analyzer, Nano S90 (Malvern, Germany); Vacuum Freeze-dryer, Scientz-10N (Ningbo Xinzhi Biotechnology Co., LTD.); Fourier Transform Infrared Spectrometer, Nicolet iS 10 (Thermo Scientific); Scanning electron microscope, JSM-7500LV (JEOL, Japan); Thermogravimetric Analyzer, TG209F1 (NETZSCH GMBH); Differential scanning calorimeter, DSC200PC (NETZSCH, Germany).

Main materials: gelatin (Chengdu Jinshan Chemical Reagent Co., LTD.); DMTMM (Shanghai Aladdin Biochemical Co., LTD.).

### Process flow

A certain mass of gelatin was dissolved in deionized water and adjusted with sodium bicarbonate ($NaHCO_3$) solution to the desired pH value to make a 10 wt.% gelatin solution. Subsequently, the DMTMM was dissolved into a small amount of deionized water, at

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**Table I**

**Preparation process of DMTMM crosslinking modified gelatin material**

| Processes | Material | Dosage | Remarks |
|---|---|---|---|
| Prepare gelatin solution | $NaHCO_3$ | x | A gelatin solution with a concentration of 10 wt.% and pH=10.0 was prepared |
| | deionized water | 100 ml | |
| | gelatin | 10.0 g | |
| Crosslinking modification | Gelatin solution | 100 wt.% | |
| | DMTMM | 5 wt.% | 35°C, the reaction in the oscillator for 6 h |

35°C, added to the gelatin solution, placed in the oscillator to mix it fully, and reacted for the corresponding time length, and the crosslinked modified composite was freeze-dried to be used. Except that no DMTMM is added, the other steps are the same as the above method. This sample is recorded as a gelatin blank sample. The specific reaction process is shown in Table I.

### Testing and characterization

*Fourier Transform Infrared Spectroscopy (FT-IR)*

The infrared spectrum was measured by potassium bromide tablet method.

*Isoelectric point measurement*

The samples were prepared with deionized water to a solution at a concentration of 0.1 g / L, and their isoelectric points were determined using a Zeta potentiometer.

*Thermogravimetric Analysis (TG)*

The weight loss of the sample with temperature was measured and recorded using the TG209F1 model TG instrument.

*Thermal denaturation temperature (DSC)*

The thermal denaturation temperature of the DMTMM cross-linked modified gelatin material was determined using the DSC200PC model DSC instrument.

*Swelling test*

Take the right amount of freeze-dried collagen-based material, soak it in the buffer solution, remove it and wipe it after a period of time, and weigh it. Repeat this process until the weight almost does not change.

*Micro-morphology test*

A scanning electron microscope (SEM) was used to observe the cross section and analyze the micromorphology of the samples.

## Results and discussion

### Mechanism of action between DMTMM and gelatin

Under the action of crosslinker DMTMM, whether gelatin crosslink modification is produced can analyze the infrared spectral absorption peak of DMTMM crosslink modified gelatin material using FT-IR and judge the bonding situation according to the characteristic absorption peak of the functional group.

The FT-IR profiles of the sample are shown in Figure 1. Among them, the 1 SPs are the C=O bond, N-H bond and N-H bond at 1643 $cm^{-1}$,1548 $cm^{-1}$ and 1447 $cm^{-1}$, respectively.[6] Compared with the gelatin blank samples without cross-linked modification, the characteristic absorption peaks of DMTMM at 1643 $cm^{-1}$,1548 $cm^{-1}$and 1447 $cm^{-1}$[7]. The absorption peak around 1100 $cm^{-1}$ moves toward a low wavenumber, usually due to the hydrogen bonding between the hydroxyl group and the active group in the gelatin.[8] At the same time, because the strength and position of the expansion vibration peak at the amide I band did not change greatly, it indicates that the three-dimensional spiral knot of collagen was not destroyed after the modification by DMTMM crosslinking.[9] Moreover, there are

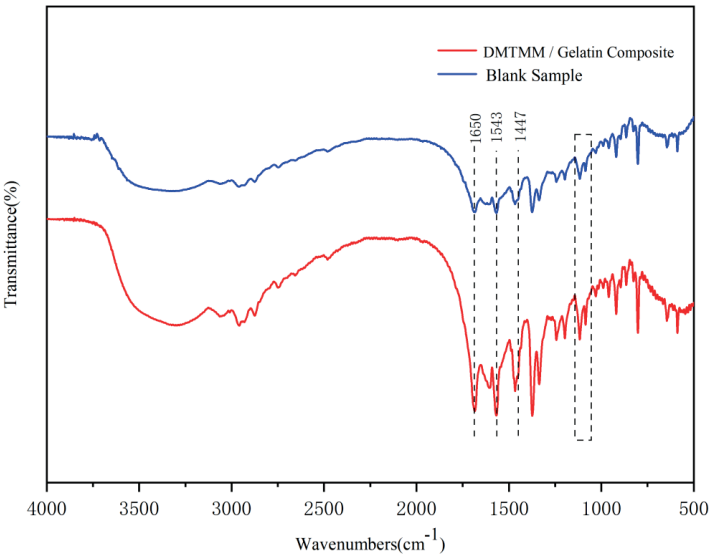


**Figure 1.** FT-IR spectra of collagen-based materials

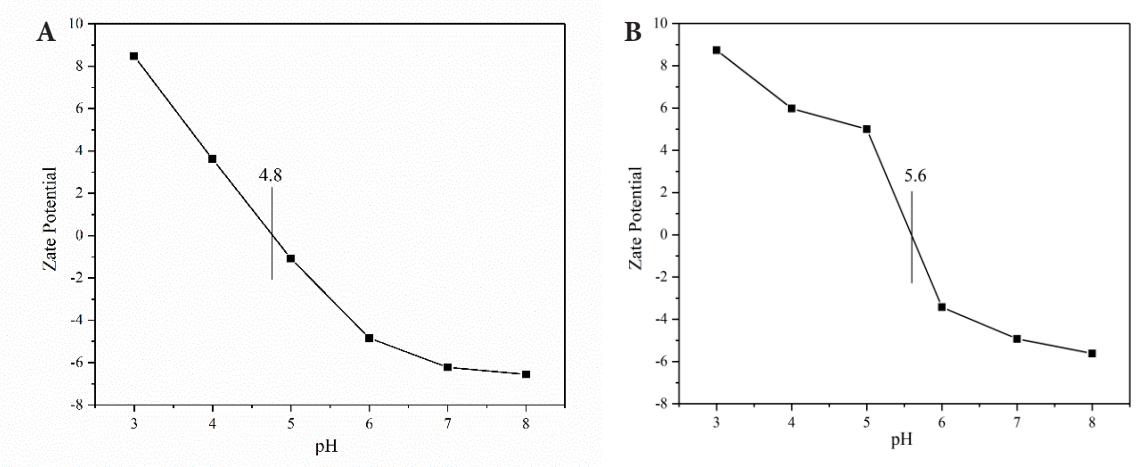


**Figure 2.** The isoelectric point titration graph of DMTMM crosslinked gelatin
**A**—Gelatin(Control), **B**—DMTMM crosslinked gelatin

that the gelatin inter-molecules were not introduced, forming a "zero-length" cross-link.

**Isoelectric point determination and analysis of gelatin material modified by DMTMM crosslinking**

Zeta potential is a description of the charge properties of the particle surface.10 The height of the isoelectric points in the gelatin solution is determined by the degree of functional group hydrolysis of the side chain. In Figure 2, the isoelectric point determination curve of the gelatin blank sample is shown, and the isoelectric point is 4.8, while the isoelectric point determination curve of the DMTMM crosslinked modified gelatin material rises to 5.6, which is probably due to the reaction of the charged groups inside the gelatin under the action of the crosslinker DMTMM. Therefore, it can be speculated that the carboxyl group in the gelatin will be activated by DMTMM, and then react with the amino group to form an amide bond. Therefore, the relative negative charge of the gelatin particle is reduced, and the hydrolysis of the amide bond is weakly alkaline, making the isoelectric point increase.[11]

**Effect of DMTMM on gelatin thermal stability**

The thermal denaturation temperature and thermal degradation stability of DMTMM crosslinked modified gelatin materials were tested by DSC and TG, respectively, to explore the effect of DMTMM on the thermal stability of collagen-based materials.[12,13] The results are shown in Figure 3. As can be seen from the figure, the peak of gelatin heat absorption increased significantly after DMTMM crosslinking modification, and the thermal denaturation temperature of DMTMM crosslinking modified gelatin material increased from 75.6°C to 87.8°C, increasing by 16.2%. The temperature of DMTMM modified gelatin material at 5%, 10% and 50% and the mass residue at 700°C are significantly improved compared with the gelatin blank sample, and its thermal stability increases in the 200~350°C, which shows that the gelatin can effectively improve its thermal stability[14] after DMTMM modification. As can be seen in the DTG graph, the maximum thermal weight loss temperature of DMTMM crosslinked modified gelatin material has been increased from 295.3°C to 396.7°C, which means that the weight loss rate corresponding to the maximum thermal weight loss temperature after gelatin crosslinked modification by DMTMM has decreased, slowing down the decomposition rate.[15]

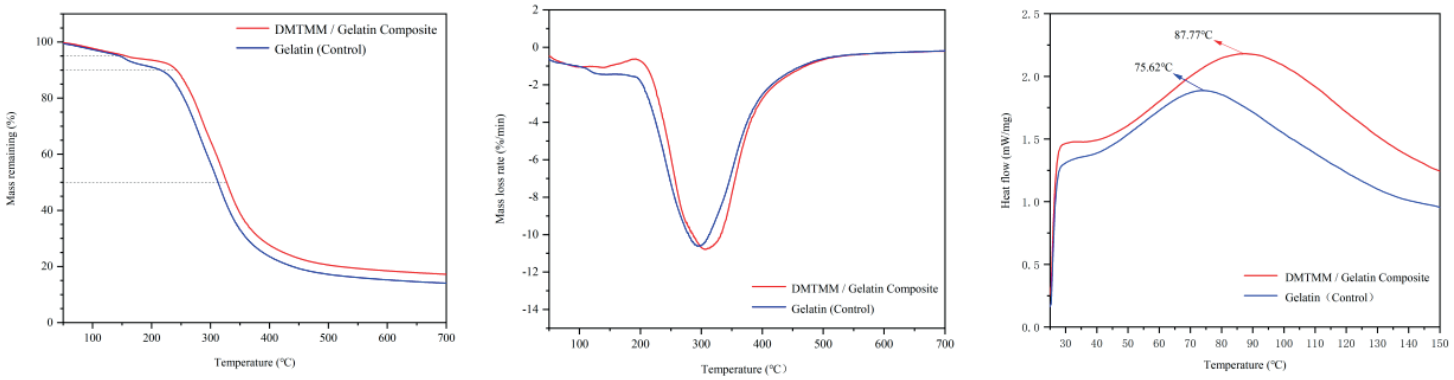


**Figure 3.** The TGA, DTG and DSC curves of collagen-based materials

**Table II**
**The swelling of collagen-based materials**

| Sample | initial mass /g | final mass / g | Balance swelling/% |
|---|---|---|---|
| Control | 0.308 ± 0.013 | 0.445 ± 0.002 | 44.5 ± 1.5 |
| DMTMM /Gelatin Composite | 0.299 ± 0.002 | 0.430 ± 0.004 | 43.8 ± 2.1 |

**Effect of DMTMM on gelatin swelling performance**

The swelling performance characterizes the morphological stability of the collagen-based material in the solution. Then the collagen-based material changes its size, mass and volume after absorbing the liquid. This process is called swelling. When the dry collagen-based material absorption solution reaches saturation, its various physical quantities will not change, which is now called the swelling equilibrium. The size of the swelling equilibrium[16] can indirectly reflect the crosslinking degree of the collagen-based materials. When the swelling equilibrium is smaller, the degree of the crosslinking degree is better. Table II shows the swelling degree of the gelatin blank sample and the DMTMM crosslinking-modified gelatin material and Figure 4 shows the swelling curve. From the above experimental results, both swelling increased significantly in the first 80 min, then flattened out, and both became close to swelling equilibrium at around 80 min. However, it is obvious from the swelling curve that the swelling growth rate of gelatin blank sample is much faster than that of DMTMM, and the final equilibrium swelling (44.5±1.3%) is greater than that of DMTMM (43.8±1.3%). It can be explained that the collagen-based material can effectively limit its intermolecular motion, limit the change of its physical size, and increase its stability.

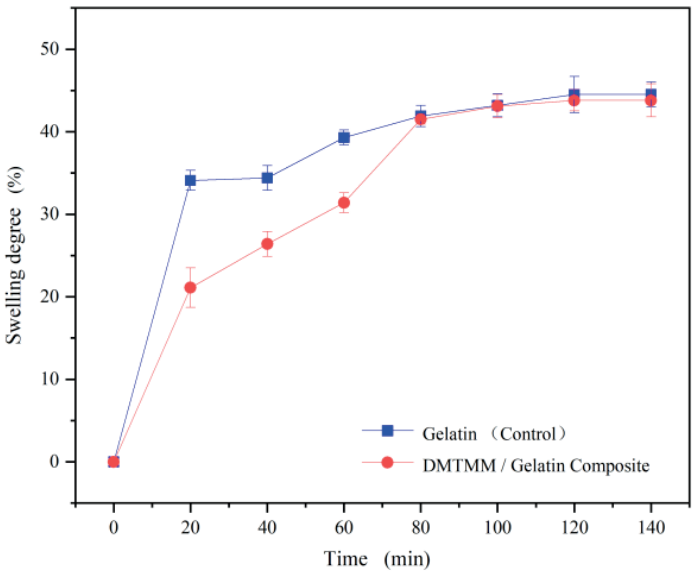


**Figure 4.** The swelling curves of collagen-based materials

**Effect of DMTMM on gelatin micromorphology**

The micromorphology of the gelatin blank sample (A / a) and DMTMM modified gelatin material (B / b) was observed by SEM,

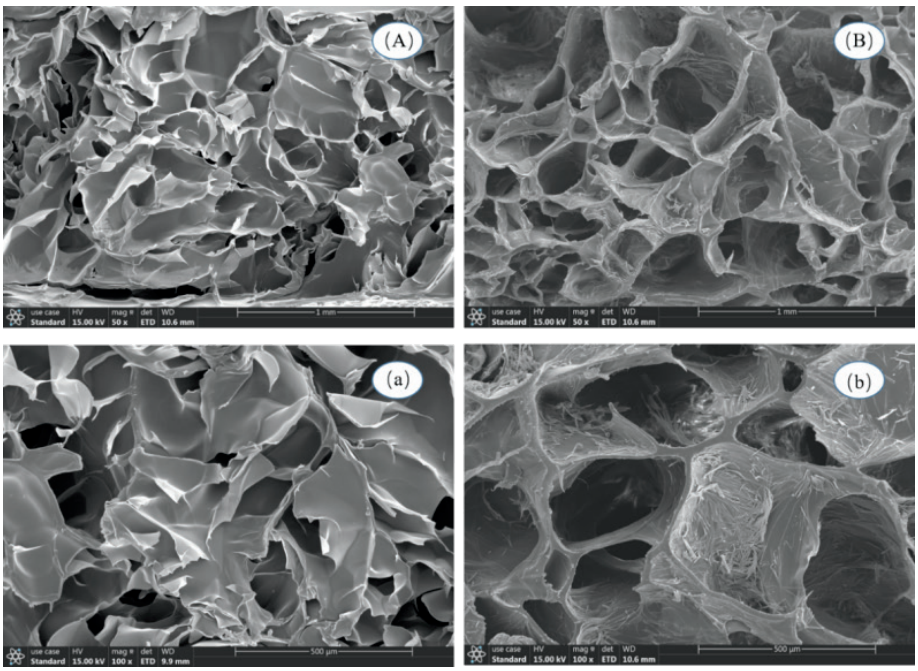


**Figure 5.** SEM photo of gelatin sample magnified 50/100 times **(A/a)**
SEM photo of DMTMM crosslinking modified gelatin material magnified 50/100 times **(B/b)**

and the cross section of the sample was enlarged by 50 and 100 times, respectively, as shown in Figure 5.

The cross section of gelatin blank samples and DMTMM lyophilized samples have porous structure, but they have obviously different characteristics. The gelatin blank sample pores are irregular with more membrane, membrane wall surface is smooth and flat and relatively thin, by the film accumulation form larger and relatively loose holes and the preparation of DMTMM crosslinking modified gelatin material holes is three-dimensional, loose porous structure, wall surface is rough and has a certain thickness, and the wall surface has more obvious fiber. By comparing the micromorphology of different samples, the holes of DMTMM crosslinking are dense, orderly, connected and arranged in parallel; the walls are significantly thickened and rough. This is because under the action of DMTMM cross-linking modification, the molecules in the gelatin matrix produce covalent crosslinking, so that the composite forms a more dense, orderly porous structure.[17]

## Conclusion

Based on the above experimental results, the following conclusions can be obtained:

1. The amide bond can be formed directly between the amino and carboxyl groups of gelatin molecules when catalyzed by DMTMM. At the same time, the hydrogen bond was also generated between the hydroxyl group and the active group. As a result, "zero length" crosslinking between the active groups of gelatin molecules was established without the use of an additional crosslinking agent.
2. The crosslinking degree of gelatin was improved after catalyzed by DMTMM, therefore, the thermal and structural stability of collagen-based material was improved.
3. The multipoint crosslinking between collagen-based material molecules can be generated by the modification of DMTMM. And the related properties of the materials were improved with better dispersion of collagen.

In conclusion, under the effects of DMTMM crosslinking modification, the amide bond and hydrogen bonds are formed to generate the "zero length" crosslinking modification. After the cross-linking modification, the thermal stability of the collagen-based materials is significantly improved, and the decomposition rate is significantly reduced.

## Acknowledgements

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## References

1. Munetaka, Kunishima, et al.; Synthesis and characterization of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, *Tetrahedron Letters*, 1999, **40**(29):5327-5330.
2. Beghetto V, Agostinis L, Gatto V, et al.; Sustainable use of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl) -4-methylmorpholinium chloride as metal free tanning agent, *Journal of Cleaner Production*, 2019, **220**(MAY 20):864-872.
3. Xiao Y, Reis L A, Zhao Y M, et al.; Modifications of collagen-based biomaterials with immobilized growth factors or peptides, *Methods*, 2015, 84.
4. Ouyang M, Hu K H, Jiang Q W, et al.; An approach on chromium discharge reduction: Effect and mechanism of ketone carboxylic acid as high exhaustion chrome tanning agent, *Journal of Cleaner Production*, 2022, 367
5. Duconseille A, Astruc T, Quintana N, et al.; Gelatin structure and composition linked to hard capsule dissolution: A review, *Food Hydrocolloids*, 2015, **43**:360-376.
6. Liu Y, Song B, Zhang J, et al.; Aluminum tanning of hide powder and skin pieces under microwave irradiation, *Journal of Leather Science and Engineering*, 2020, **2**(1): 23.
7. Yu J, Xiang Q, Zhou M.; Preparation, characterization and visible-light-driven photocatalytic activity of Fe-doped titania nanorods and first-principles study for electronic structures, *Applied Catalysis B:Environmental*, 2009, **90**(3-4): 595-602.
8. Waidhas F, Haschke S, Khanipour P, et al.; Secondary Alcohols as Rechargeable Electrofuels: Electrooxidation of Isopropyl Alcohol at Pt Electrodes, *ACS Catalysis*, 2020, **10**(12): 6831-6842.
9. Zhang Z T, Liu J, Gao W W, et al.; Action of silicic acid derived from sodium silicate precursor toward improving performances of porous gelatin membrane, *Journal of Applied Polymer Science*, 2020, **137**(30): 48912.
10. Fraenkel C, The Chemistry and Reactivity of Collagen, *Journal of the American Chemical Society*, 1956, **78**(22):5960-5960.
11. Cheng H M, Chen M, Li Z Q.; Review on Characterization Methods of Isoelectric Point of Collagen and Leather, *Leather Science and Engineering*, 2012, **22**(04): 20-24.
12. Zhang J, Chen W, Gai D C.; Influence of microwave on chromium complex composition in tanning liquor, *Journal of Leather Science and Engineering*, 2020, **2**(1): 10.

13. Zhang X, Xu S, Shen L, et al.; Factors affecting thermal stability of collagen from the aspects of extraction, processing and modification, *Journal of Leather Science and Engineering*, 2020, **2**(1): 19.
14. Wang Y T, Zhao H B, et al.; Green Approach to Improving the Strength and Flame Retardancy of Poly(vinyl alcohol)/ Clay Aerogels: Incorporating Biobased Gelatin, *ACS Applied Materials & Interfaces*, 2017, **9**(48): 42258-42265.
15. Qi Z, Zhang W, He X, et al.; High-efficiency flame retardancy of epoxy resin composites with perfect T8 caged phosphorus containing polyhedral oligomeric silsesquioxanes (P-POSSs), *Composites Science and Technology*, 2016, **127**: 8-19.
16. Yang S Q, Li H C.; Study on Properties of Chitin Gelatin Composite Film, *Food Research and Development*, 2019, **40**(18): 44-51.
17. Chen M J, Zhang Y H, Xie Q, et al.; Long-Term Bone Regeneration Enabled by a Polyhedral Oligomeric Silsesquioxane (POSS)-Enhanced Biodegradable Hydrogel, *ACS Biomaterials Science & Engineering*, 2019, **5**(9): 4612-4623

# A Novel and Efficient Collagen Extraction Method Assisted by Microwave Irradiation

by

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## Abstract

A new and efficient microwave heating method was developed to extract intact Type I collagen from bovine limed splits. Orthogonal experiment was conducted to optimize the parameters of extraction, and the extraction yield was measured by ultraviolet spectra (UV). The hierarchical structures of the obtained collagen were determined by amino acid analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), UV, Fourier transform infrared spectra (FT-IR), circular dichroism spectra (CD), fluorescence spectra (FL) and ultra-sensitive differential scanning calorimetry (US-DSC). The results indicated the optimal conditions for this microwave assisted extraction were 37°C for 7 h by using solid to liquid ratio of 1:45. The resultant yield of collagen was 14.35% under the optimal conditions, which is 1.5 times higher than that obtained by the control water bath-heating method. Amino acid analysis, UV and SDS-PAGE revealed that the primary structure of collagen extracted with microwave assistance was type I collagen. Although the extraction yield was promoted drastically with microwave assisting, the secondary structure of the collagen involving triple helix still maintained according to FT-IR, CD, FL and VP-DSC results. In brief, microwave irradiation could be a new routine for collagen extraction with advantages of fast and effective. Moreover, this study might offer a potential choice for utilizing hide or skin limed split wastes.

## Introduction

Statistically, more than 6.9 million tons of wet salted hides and skins are converted into leather worldwide annually.[1] Solid waste generated simultaneously from tannery processes is estimated at approximately 0.8 kg per kg of raw hides/ skin,[2-4] of which about one-third is limed split wastes. These wastes can be utilized by microbiological methods as material for biodiesel or for composting.[5-8] Particularly, limed split wastes have been used for the production of high value-added collagen as they are less contaminated by chemicals.[9,10]

Type I collagen is the dominant protein in mammals, accounting for 25-30% of the total protein weight.[11] This fibrous structural protein consists of three left-handed spiral peptide chains with a right-handed supercoil structure named triple helix.[12] It is widely used in biomedical, food, cosmetics and so on.[13,14] Therefore, the development of collagen extraction remaining natural structure will bring momentous scientific significance and application value. At present, hides and skins are important resources for collagen extraction. The methods for preparing collagen mainly include acid extraction, enzymatic extraction, alkali extraction and neutral salt extraction.[15-17] Among these methods, the collagen extracted by acid method sustains complete structure with high purity, but the extraction yield is lower.[18]

Since microwaves were used to treat nuclear waste in Harwell Laboratory at 1970, they have been widely used in various chemical fields as a transmission medium or heating energy source.[19,20] Now, microwave irradiation has become an important technology to accelerate chemical reactions.[21, 22] Moreover, previous studies proved that microwave-assisted methods could shorten the time and increase the extraction yield of collagen, gelatin and collagen hydrolysates.[23-26] Unfortunately, the application of microwave in collagen extraction was limited to short-time pretreatment, and especially remaining collagen triple helix structure, has not been reported.

This study explores a new extraction method by microwave irradiation assistance, so as to prepare structurally intact Type I collagen more effectively. In this work, collagen was extracted by an acid method, and microwave irradiation was used as a heating source in the whole extraction process. A conventional heating process was used as a control, and the extraction yield was optimized by an orthogonal method and calculated based on UV testing. Structure and properties of the obtained collagen were characterized from its primary and secondary structures. This work would be a novel method for more efficient collagen extraction and provide scientific guidance for utilizing bovine limed split wastes effectively.

## Experimental

### Materials

Salted bovine hide was bought from local slaughterhouse in Chengdu, Sichuan province, China. Standard type I collagen was purchased from Sigma (USA) and without any further purification. Acetic acid was provided by Chengdu Kelong Chemical Co. LTD.

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All chemicals used for preparing limed pelt were industrial reagents, and the chemicals for collagen extraction and determination were commercially available of analytical grade.

**Sample preparation**

*Pretreatment of pelt*

Limed pelt from salted bovine hide was prepared by a conventional beamhouse process. After splitting, the samples with the size of 100 × 80 cm were taken symmetrically along the backbone and weighed. Then the samples were delimed with 2% ammonium chloride and 0.5% hydrochloric acid for 1 h to adjust pH between 6.0 and 7.0, and the cross section of pelt was colorless by phenolphthalein indicator. The samples, afterwards, were rinsed with distilled water for 30 min and cut into fragments of 0.5 × 0.5 cm. Component analysis of the fragments was carried out according to the method of AOAC.[27] The main constituents of the raw material were proteins and water accounting for 17.1% and 72.2% respectively. The raw material was lyophilized and stored in sealing bag at 4°C.

*Collagen extraction process*

The 10 g dried limed pelt fragments were extracted with 0.5 mol/L acetic acid solution under 35°C at a solid to liquid ratio of 1:40 (w/v) for 8 h. The extraction with microwave-assistance was carried out in MCR-3C microwave reactor (Xi'an Yuhui instrument Ltd.), and the control was heated by using DF-101S water bath heater (Wuhan Ke'er instrument Company) at corresponding conditions. After extraction, the solution was filtered with 200 mesh nylon filter cloth to obtain crude extract. The crude extract was centrifuged at 8000 r/min for 10 min by using TG-20 high speed centrifuge (Sichuan Shuke instrument Co., Ltd; China) and supernatant was collected. Sodium chloride was added to the supernatant and until the concentration was 3 mol/L and precipitation appeared. Subsequently, the precipitation was collected by centrifugation again and dissolved in 0.5 mol/L acetic acid solution. The solution was dialyzed with distilled water for 3 days and lyophilized. The prepared collagen was stored in a dryer.

*Extraction optimization*

On the basis of single factor experiment, orthogonal experiments of three factors and three levels were carried out on temperature (33, 35, 37 °C), solid to liquid ratio (1:35, 1:40, 1:45) and extraction time (6, 7, 8 h). The extraction temperature was settled below the critical denaturation temperature of collagen (37°C).

**Testing methods**

*Collagen extraction yield*

With acetic acid solution of 0.5 mol/L as reference, the absorbance of collagen solution was recorded over the range of 200-400 nm at the scanning speed of 400 nm/min by UV1900 (Shanghai Flying Art instrument Co., Ltd.; China). Standard type I collagen solutions of 0, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/L were prepared with 0.5 mol/L acetic acid solution. According to absorbance of these solutions at the characteristic absorption peak, standard curve used to calculate collagen concentration of the crude extract was made. Confirmed collagen concentration and absorbance to Lambert-Beer's law, the linear relationship was y=0.5799x+0.0307, besides, the linear relationship variance was 0.9955. The yield of collagen was calculated according to following formula based on UV testing:

$$Y = (C \times V)/ (M \times F) \times 100\%$$

Y – The extraction rate of collagen (%);

C – Collagen concentration in crude extract (mg/L);

V – Volume of crude extract (L);

M – the weight of raw material (mg);

F – percentage of protein in raw material skin (%).

*Amino acid analysis*

About 0.1 g collagen was completely hydrolyzed by 6 mol/L hydrochloric acid at 120°C for 24 h in the absence of oxygen. Hydrolyzed solution was filtered and diluted to 50 mL. An aliquot of 0.1 mL was applied to L-8900 analyzer (Hitachi Co., Ltd.; Japan).

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)**

Collagen was analyzed by SDS-PAGE gel electrophoresis with 8% separation gel and 5% concentrated gel system respectively. The collagen was dissolved in 0.5 mol/L acetic acid to obtain a final concentration 5 mg/mL collagen solution, then mixed with the sample buffer (0.0625 mol/L Tris-HCl, pH 6.8, containing 2.3% SDS,10% glycerol and 5% mercaptoethanol) at a ratio of 1:1(v/v). The mixture was boiled for 5 min. Electrophoresis was performed by an electrophoresis instrument (Min-PROTENIN3, Bio-Rad Laboratories, Inc. USA) at a constant current of 60 V through the concentrated glue and then 120 V the separating glue. The protein markers with high molecular weight (Sigma Chemical Co., USA) were loaded alongside the collagen samples at a loading of 10 μL. The gels were stained by 0.25% Coomassie Brilliant Blue R-250 for 1h and eluted until the background color was colorless in 10% methanol and glacial acetic acid. Ultimately, the gray level of the gels was analyzed by software, Image J 1.48 v, to calculate the molecular weight distribution.

**Fourier transform spectroscopy measurement (FT-IR)**

Collagen was mixed with potassium bromide (1:100), ground and pressed evenly for spectrum recording. Subsequently, it was scanned by Nicolet iS10 Fourier transform infrared spectrometer (Seymour Technology Co., Ltd.; America) for 32 times with a wave number range of 400-4000 $cm^{-1}$ at room temperature.

**Circular dichroism spectroscopy measurement (CD)**

Collagen was dissolved in 0.5 mol/L acetic acid to prepare 0.05 mg/mL collagen solution, and the solution was monitored by CD using Jasco J-1500 spectrometer (Jasco Corporation, Japan). The container for the determination was a rectangular quartz cell with a path length of 1 cm. Spectrum was recorded from 250 to 190 nm with standard sensitivity under nitrogen atmosphere. The wavelength, scanning speed, band width, response and data pitch were set at 50 nm/min, 2 nm, 1 s and 0.5 nm, respectively.

**Fluorescence Spectroscopy measurement (FL)**

The fluorescence was measured under 25°C. Before the measurement, collagen concentration of 0.5 mg/mL in 0.5 mol/L acetic acid solution was equilibrated for 3 min in a fluorescence spectrophotometer (CaryEclipse, Agilent, America). The excitation wavelength was fixed at 275 nm, and the emission spectrum was recorded in the range of 280-400 nm under 25°C at scan rate of 120 nm/min. Fluorescence excitation and emission slits were 5 nm.

Synchronous fluorescence spectrum was scanned between wavelength 200 and 400 nm at the excitation wavelength of 275 nm. Both the excitation and the emission slits were 5 nm. Wavelength shift (Δλ) between excitation and emission was 15 nm. And the scanning temperature was 25°C with a scanning speed of 120nm/min.

**Ultra-sensitive differential scanning calorimetry (US-DSC)**

0.5 mg/mL collagen was prepared by dissolving collagen in 0.5 mol/L acetic acid and degassed in the instrument for 30 min. Then it was tested under the temperature range 20 to 60 °C by VP-DSC hypersensitive differential scanning calorimetry (Microcal; USA) with the increasing rate of 1 °C / min.

## Results and discussion

**Optimization of microwave-assisting collagen extraction**

According to the analysis of the orthogonal experiment (Table I), the highest extraction yield was 11.65%, and the corresponding extraction conditions were 35°C, solid to liquid ratio 1:35 and 7 h (Number 4). In the table, S and R were the variance and range of $K_1$, $K_2$ and $K_3$, respectively. The larger the variance and the range difference were, the more significant the influence of this factor was on the extraction yield, illustrating that the order of the influence on the extraction yield was as follows: irradiation temperature, irradiation time, solid to liquid ratio.

Under the optimal condition (extraction temperature 37°C, solid to liquid ratio 1:45 and irradiation time 7 h) from orthogonal experiment, the verification experiment showed that the collagen extraction yield could reach to 14.35%, while the yield under corresponding condition with water bath heating was only to 9.30%, showing microwave irradiation could improve the extraction yield significantly.

**Table I**

**Orthogonal test results of collagen extraction by microwave irradiation**

| Number | Irradiation temperature /°C | solid-liquid ratio | Irradiation time /h | Extraction yield/% |
|---|---|---|---|---|
| 1 | 33 | 1:35 | 6 | 5.98 |
| 2 | 33 | 1:40 | 7 | 10.77 |
| 3 | 33 | 1:45 | 8 | 8.59 |
| 4 | 35 | 1:35 | 7 | 11.65 |
| 5 | 35 | 1:40 | 8 | 7.78 |
| 6 | 35 | 1:45 | 6 | 10.38 |
| 7 | 37 | 1:35 | 8 | 11.48 |
| 8 | 37 | 1:40 | 6 | 10.80 |
| 9 | 37 | 1:45 | 7 | 11.49 |
| $K_1$ | 25.34 | 29.11 | 27.16 | |
| $K_2$ | 29.81 | 29.36 | 33.91 | |
| $K_3$ | 33.77 | 30.46 | 27.85 | |
| R | 8.42 | 1.36 | 6.75 | |
| S | 11.84 | 0.35 | 9.19 | |

**Table II**

**Amino acid composition of different kinds of collagen**

| Amino acid | Molar percentage /% | | |
|---|---|---|---|
| | Experimental product | Control product | Type I collagen |
| **Asp** | 4.41 | 4.55 | 4.68 |
| **Thr** | 1.52 | 1.72 | 1.81 |
| **Ser** | 3.77 | 3.73 | 3.66 |
| **Glu** | 7.72 | 7.60 | 7.52 |
| **Gly** | 33.90 | 34.38 | 33.95 |
| **Ala** | 10.54 | 10.17 | 10.48 |
| **Val** | 1.32 | 0.22 | 1.21 |
| **Met** | 0.63 | 2.53 | 1.47 |
| **Ile** | 1.41 | 1.33 | 1.24 |
| **Leu** | 3.10 | 3.04 | 2.94 |
| **Tyr** | 0.22 | 0.43 | 0.41 |
| **Phe** | 1.89 | 1.96 | 1.41 |
| **Hylys** | 0.76 | 0.77 | 0.86 |
| **His** | 0.38 | 0.31 | 0.52 |
| **Lys** | 2.94 | 2.86 | 3.17 |
| **Arg** | 5.48 | 5.35 | 5.65 |
| **Hypro** | 7.40 | 7.42 | 7.81 |
| **Pro** | 12.60 | 11.64 | 11.21 |

During acid extraction, the structure of salt bond and Schiff base between collagen molecules was destroyed by acid and resulted in collagen dissolving.[28] Acetic acid solution, as an extracting agent, was chemically simple containing molecules of water and acetic acid only. The electromagnetic field generated by microwave changed the ionization of acetic acid in water. Consequently, the extraction system contained more acid with microwave assisting compared with water bath heating, and the higher acidity weakened the inter-molecule hydrogen bonds of collagen in pelt.[29] On the other hand, polar molecules in solution and pelt would have more complicated and extra oscillation with the change of microwave electromagnetic field, it benefit for molecular collision and chemical bond breaking. Just as microwave could accelerate and promote other chemical reactions, the more efficient collagen extraction was obtained under microwave irradiation with higher extraction yield.

**Primary structure of collagen extracted by microwave assisted**

***Amino acid composition of collagen***

The amino acid composition of collagen extracted under microwave irradiation (the experimental product) and water bath heating (control product) as well as standard type I collagen were shown in Table II. Compared with standard type I collagen and bovine collagen,[30] the two products contained about one-third glycine, 11% alanine and without tryptophan, meaning the extracted collagen had the characteristics amino acid of type I collagen. The ratio of hydroxyproline to proline of the experimental product was 0.59, similar to that of the control product (0.63), indicating that the stability of the two products was comparable.[31]

**Molecular weight of collagen**

The electropherogram of three kinds of collagen was shown in Figure 1. It was clear that there were three distinct electrophoretic bands. In addition to the cross-linked β chain, the samples included at least

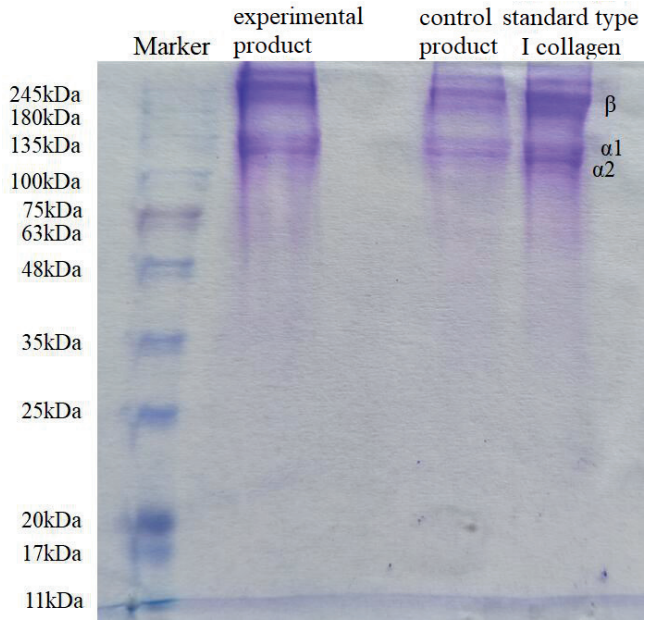


**Figure 1.** Electropherogram of different kinds of collagen

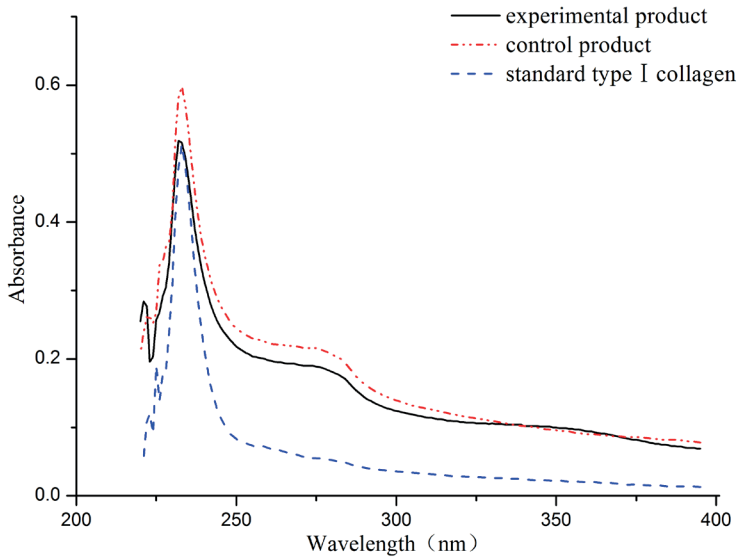


**Figure 2.** UV-Vis spectra of different kinds of collagen

two α chains ($\alpha_1$ and $\alpha_2$) with molecular weight larger than 100 kDa, conforming to the characteristic of type I collagen.[32,33] According to the gray level measurement, the molecular weight proportion of experimental product above 100 kDa was 89.4%, indicating its higher purity. Furthermore, it was basically the same as that of the standard type I collagen and was not significantly different from that of control product.[34,35]

**UV-Vis Spectra of collagen**

The ultraviolet spectra of standard type I collagen and the extracted products were shown in Figure 2. There was a maximum absorption peak of the products at 234 nm, and no significant difference between three samples could be observed, showing that microwave irradiation would not impair the primary structure of collagen.

**Secondary structure of collagen extracted by microwave assisted**

***FT-IR spectra of collagen***

FT-IR spectrum of different kinds of collagen was shown in Figure 3. There were five amide bands. Amide A band was caused by the stretching vibration of N-H at 3400-3440 $cm^{-1}$. Amide B band at 2800-3000 $cm^{-1}$ was a stretching vibration peak generated by the asymmetric $CH_2$. Amide I band absorption peak between 1640 and 1660 $cm^{-1}$, the main characteristic absorption peak of collagen infrared spectrum, was attributed to the vibration peak of the C=O group, and it was often used for the analysis of secondary structure of collagen.[36, 37] Since the absorption peaks of the samples were almost at the same position, it inferred that the microwave irradiation extraction technique did not destroy the conformation of the product. Due to the C-N stretching vibration and N-H bending

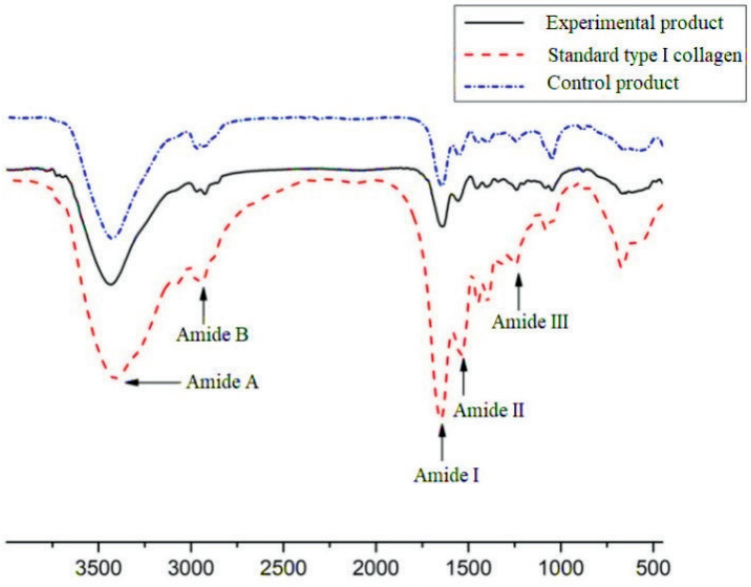


**Figure 3.** FT-IR spectra of different kinds of collagen

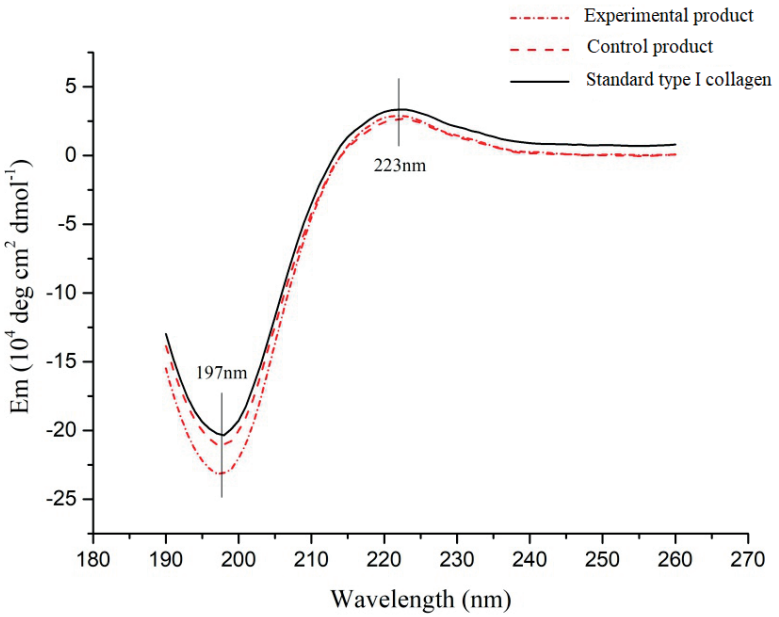


**Figure 4.** CD spectra of different kinds of collagen

vibration of collagen, the absorption peak of the amide II band appeared at 1500-1600 cm$^{-1}$, and the absorption peak of the amide III band was produced between 1200 and 1360 cm$^{-1}$. Therefore, it could be speculated that the products possess a complete triple helix structure.[38]

**CD spectra of collagen**

Circular dichroic spectrum of the samples was shown in Figure 4.The circular dichroic spectrum of collagen was characterized by a positive absorption peak around 225 nm and a negative absorption peak near 197 nm.[39] The collagen became denatured gradually, and the molar ellipticity of the absorption peak near 225nm decreased while that near 197nm increased.[40] There was a significant negative absorption peak at 197 nm and a weak positive absorption peak at 223 nm in the circular dichroic spectrum of samples. These three kinds of collagen had almost same circular dichroic spectrum, indicating that the collagen under microwave irradiation still has a complete spatial configuration of the polypeptide chain.

**The intrinsic fluorescence analysis of collagen**

The fluorescence emission spectra of the samples is shown in Figure 5. Phenylalanine and tyrosine could be used as internal probes to measure intrinsic fluorescence.[41] Collagen contained a high content of tyrosine, the main source of endogenous fluorescence. Tyrosine residues mainly exhibited the emission peak of 310 nm, and its fluorescence intensity increased with the rise of collagen

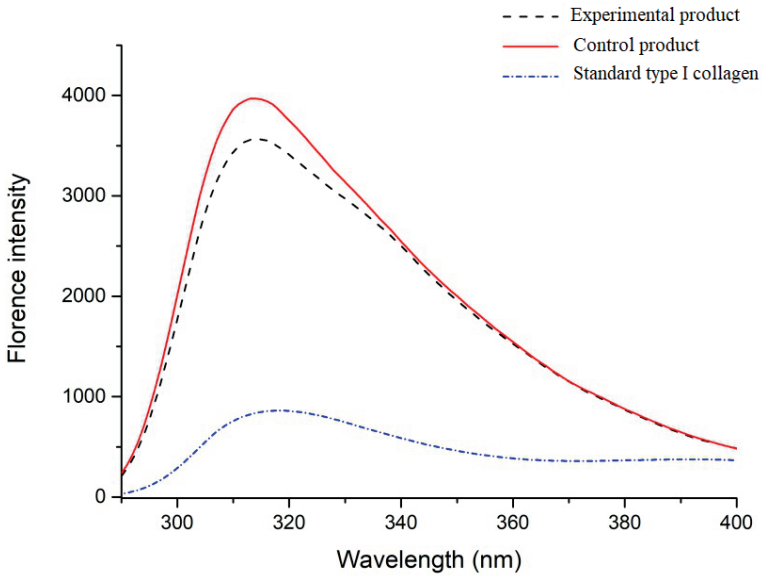


**Figure 5.** Fluorescence emission spectra of different kinds of collagen

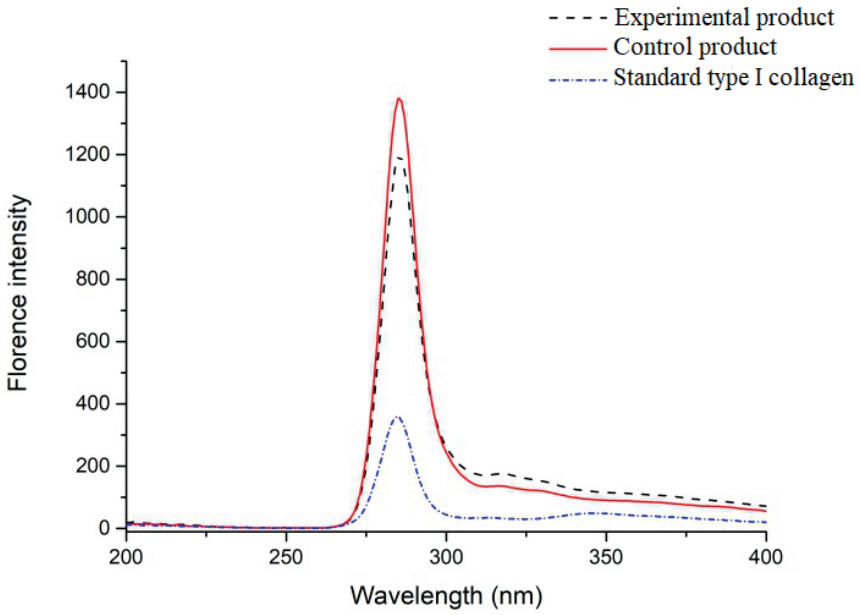


**Figure 6.** Synchronous fluorescence spectra of different kinds of collagen

concentration.[42] Although the fluorescence intensities of the samples were different, there was a significant fluorescence emission peak in the range of 300-400 nm and the fluorescence spectrum curve didn't change. The same peak meant that the chemical condition of tyrosine in the experimental sample and the control was identical. It was evidence that there was no negative effect on collagen structure during microwave extraction process.

**The synchronous fluorescence analysis of collagen**

Figure 6 is a synchronous fluorescence spectrum of the products and the standard type I collagen. The spectrum not only maintained the sensitivity of general fluorescence correlation, but also possessed the characteristics of narrowing the spectral band, simplifying the emission spectrum and narrowing the spectral range.[43] It could reflect the effect of extraction methods on the endogenous fluorescence of collagen. The picture revealed the larger fluorescence intensity of the control product. This was because a conjugated rigid structure could increase the fluorescence intensity,[44] but under the action of microwave field, the movement of collagen molecules was more intense, and it wasn't conducive to molecular aggregation and conjugate formation. In addition, the characteristic peaks of synchronous fluorescence of the samples were mainly absorbed by tyrosine residues, thus, a significant absorption peak appeared at 284 nm, indicating that the process of microwave irradiation extraction of collagen didn't damage the structure of collagen.

**Denaturation temperature of collagen**

Table III shows the thermal denaturation temperature of collagen obtained with different extraction methods. There were two endothermic peaks on the typical collagen VP-DSC curve, corresponding to the transition temperatures $Tm_1$ and $Tm_2$, respectively. Among them, $Tm_2$ was an important indicator of the thermal stability of collagen, and the value and the stability of collagen were positively correlated.[41] The thermal denaturation temperature of experimental product was 38.82°C, which was consistent with the literature.[18] Compared with the control product, the extraction under microwave irradiation had little effect on thermal stability of collagen.

To sum up, the collagen extracted under microwave irradiation still had a complete spatial configuration of the polypeptide chain. This extraction method could prepare structurally intact collagen more effectively.

**Table III**

**Thermal denaturation temperatures of different kinds of collagen**

| Sample | Experimental product | Control product | Standard type I collagen |
|---|---|---|---|
| Denaturation temperature/°C | 38.82 | 38.91 | 39.09 |

## Conclusions

Type I collagen was extracted from limed splits by using microwave irradiation as heating source. The yield of structurally intact Type I collagen was 14.35% at an optimum process temperature of 37°C, a solid to liquid ratio 1:45 and an irradiation time of 7 h. By comparison, the extraction yield was 9.30% by using the conventional water bath heating under corresponding conditions. The new microwave assisted extraction method was effective in preparing structurally intact Type I collagen, and the extracted collagen retained a complete primary structure and secondary structure of the collagen involving triple helix. This new method increased the extraction yield of collagen and thus would be potential way for limed splits utilization.

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## References

1. Puhazhselvan, P., Pandi, A., Sujiritha, P. B., *et al.;* Recycling of tannery fleshing waste by a two step process for preparation of retanning agent. *Process Safety and Environmental Protection* **157**, DOI10.1016/j.psep.2021.11.003, 2021.
2. Hu, J., Xiao Z. B., Zhou R. J., *et al.*; Ecological utilization of leather tannery waste with circular economy model. *Journal of Cleaner Production* **19**, 221-228, 2011.
3. Zhang, Z. K., Li, G. Y., Shi, B.; Physicochemical properties of collagen gelatin and collagen hydrolysate derived from bovine limed split wastes. *Journal of the Society of Leather technologists and Chemists* **90**, 23-28, 2006.
4. Kanagaraj, J., Panda, R. C., Vinodh, K. M.; Trends and advancements in sustainable leather processing: Future directions and challenges—A review. *Journal of Environmental Chemical Engineering* **8**, doi.org/10.1016/j.jece.2020.104379, 2020.
5. Colak, S., Zengin G., Oezguenay H., et al.; Utilization of leather industry pre-fleshings in biodiesel production. *JALCA* **100**, 137-141, 2005.
6. Collivignarelli, C., Barducci G.; Waste recovery from the tanning industry. *Waste Management & Research* **2**, 265-278, 1984.
7. Sandhya, K.V., Abinandan S., Vedaraman N., *et al.*; Extraction of fleshing oil from waste limed fleshings and biodiesel production. *Waste Management* **48**, 638-643, 2016.
8. Vasudevan, N., Ravindran A.D.; Biotechnological process for the treatment of fleshing from tannery industries for methane generation. *Current Science* **93**, 1492-1494, 2007.
9. Chen, Y. H., Li G. Y., Jia Y.; Rheological behaviour of alkali solubilized collagen from limed bovine split wastes. *Journal of the Society of Leather technologists and Chemists* **97**, 195-199, 2013.
10. Li, D., Li G.Y., Jiang D. Y., *et al.*; Extracting of native collagen from bovine limed split wastes:pretreatment and extraction. *China Leather*, **37**, 8-11 2008.
11. Zhang, X. X., Xu, S. C., Shen L. R., *et al.*; Factors affecting thermal stability of collagen from the aspects of extraction, processing and modification. *Journal of Leather Science and Engineering* **2**, https://doi.org/10.1186/s42825-020-00033-0, 2020.
12. Shoulders, M.D., Raines R.T.; Collagen structure and stability. *Annual Review of Biochemistry* **78**, 929-958, 2010.
13. Adamiak, K., Sionkowska A.; Current methods of collagen cross-linking: review. *International Journal of Biological Macromolecules* **161**, 550-560, 2020.
14. Nagaraj, A., Etxeberria, A. E., Naffa, R., *et al.*; 3D-Printed Hybrid Collagen/GelMA Hydrogels for Tissue Engineering Applications. *Biology-Basel* **11**, DOI10.3390/biology11111561, 2022.
15. Schmidt, M.M., Dornelles, R. C. P., Mello R.O., *et al.*; Collagen extraction process. *International Food Research Journal* **23**, 913-922, 2016.
16. Hervas, F. F., Celma, P., Punti, I., *et al.*; The enzyme activity of trypsin on sheepskin trimmings in a two-step collagen extraction process. *JALCA* **102**, 1-9, 2007
17. Matinong, A. M. E., Chisti, Y., Pickering, K. L., *et al.*; Collagen extraction from animal skin. *Biology-Basel* **11**, DOI10.3390/biology11060905, 2022.
18. Shen, J. Q., Tang, J., Shen, Y. L., *et al.*; Study on extraction of bovine collagen by acid method and its physicochemical property and structure. *The Food Industry* **3**, 43-46, 2009.
19. Jolly, J.; Microwave assisted reactions in organic chemistry: a review of recent advances. *International Journal of Chemistry* **4**, 29-43, 2012.
20. Nozariasbmarz, A., Dsouza K., Vashaee D.; Field induced decrystallization of silicon: evidence of a microwave non-thermal effect. *Applied Physics Letters* **112**, DOI10.1063/1.5020192 , 2018.
21. Diaz-Ortiz, A., Prieto P., de la Hoz A.; A Critical overview on the effect of microwave irradiation in organic synthesis. *Chemical Record* **19**, 85-97, 2019.
22. Zhang, J. W., Chen W. Y.; A faster and more effective chrome tanning process assisted by microwave. *RSC Advances* **10**, 23503-23509, 2020.
23. Mirzapour-Kouhdasht, A., Sabzipour F., Taghizadeh M.S., *et al.*; Physicochemical, rheological, and molecular characterization of colloidal gelatin produced from Common carp by-products using microwave and ultrasound-assisted extraction. *Journal of Texture Studies* **50**, 416-425, 2019.
24. Park, J. H., Choe J. H., Kim H. W., *et al.*; Effects of various extraction methods on quality characteristics of duck feet gelatin. *Korean Journal for Food Science of Animal Resources* **33**, 162-169, 2013.
25. Li, Y., Li J., Lin S.J., *et al.*; Preparation of antioxidant peptide by microwave- assisted hydrolysis of collagen and its protective effect against H2O2-induced damage of RAW264.7 Cells. *Marine Drugs* **17**, DOI10.3390/md17110642, 2019.
26. Li, X, W., Li H. J.; Optimization of microwave-assisted extraction process for collagen from pigskin. *Food Science* **6**, 19-22, 2012.
27. AOAC, Official methods of analysis. *Association of official analytical chemist Inc*, Arlington, 2000.
28. Gaar, J., Naffa, R., Brimble, M.; Enzymatic and non-enzymatic crosslinks found in collagen and elastin and their chemical synthesis. *Organic Chemistry Frontiers*, **7**, 2789-2814, 2020

29. Yang, H., Xu S. C., Shen L. R., *et al*.; Changes in aggregation behavior of collagen molecules in solution with varying concentrations of acetic acid. *International Journal of Biological Macromolecules* **92**, 581-586, 2016.
30. Li, H., Liu B.L., Gao L.Z., *et al*.; Studies on bullfrog skin collagen. *Food Chemistry* **84**, 65-69, 2004.
31. Josse, J., Harrington W.F.; Role of pyrrolidine residues in the structure and stabilization of collagen. *Journal of Molecular Biology* **9**, 269-287.1964
32. Ilyas, K., Qureshi S.W., Afzal S., *et al*.; Microwave-assisted synthesis and evaluation of type 1 collagen-apatite composites for dental tissue regeneration. *Journal of Biomaterials Applications* **33**, 103-115, 2018.
33. Ali, A. M. M., Benjakul, S., Prodpran, T., *et al*.; Extraction and characterisation of collagen from the skin of Golden Carp (Probarbus Jullieni), a processing by-product. *Waste and Biomass Valorization* **9**, 783-791, 2017.
34. Wu, J. M., Li, Z. H., Yuan X. Y., *et al*.; Extraction and isolation of type I, III and V collagens and their SDS-PAGE analyses. *Transactions of Tianjin University* **17**, 111-117, 2011.
35. Lin, Y. K., Liu, D. C.; Comparison of physical-chemical properties of type I collagen from different species. *Food Chemistry* **99**, 244-251, 2006.
36. Kittiphattanabawon, P., Benjakul S., Visessanguan W., *et al*.; Isolation and Characterization of collagen from the skin of brownbanded bamboo shark (Chiloscyllium punctatum). *Food Chemistry* **119**, 1519-1526, 2010.
37. Mehta, M., Naffa, R., Maidment, C., *et al*.; Raman and ATR-FTIR spectroscopy towards classification of wet blue bovine leather using ratiometric and chemometric analysis. *Journal of Leather Science and Engineering*, **2**, https://doi.org/10.1186/s42825-019-0017-5, 2020.
38. Liu, S. C., Liu W. T., Li G. Y.; Effect of ionic liquids pretreatment on the extraction of collagen from calf skin. *JALCA* **114**, 391-399, 2019.
39. Akita, M., Nishikawa, Y., Shigenobu, Y., *et al*.; Correlation of proline, hydroxyproline and serine content, denaturation temperature and circular dichroism analysis of type I collagen with the physiological temperature of marine teleosts. *Food Chemistry* **329**, DOI: 10.1016/j.foodchem.2020.126775 , 2020.
40. Ikoma, T., Kobayashi, H., Tanaka J., *et al*.; Physical properties of type I collagen extracted from fish scales of Pagrus major and Oreochromis niloticas. *International Journal of Biological Macromolecules* **32**, 199-204, 2003.
41. Ding, C. C., Zhang M., Wu K., *et al*.; The response of collagen molecules in acid solution to temperature. *Polymer* **55**, 5751-5759, 2014.
42. Duan, L., Yuan, J. J, Yang, X., *et al*.; Interaction study of collagen and sericin in blending solution. *International Journal of Biological Macromolecules* **93**, 468-475, 2016.
43. Cui, F.L., Wang J.L., Cui Y.R., *et al*.; Fluorescent investigation of the interactions between N-(p-chlorophenyl)-N'-(1-naphthyl) thiourea and serum albumin: Synchronous fluorescence determination of serum albumin. *Analytica Chimica Acta* **571**, 175-183, 2006.
44. Ding, C. C., Zhang, M., Li G. Y.; Fluorescence study on the aggregation of collagen molecules in acid solution influenced by hydroxypropyl methylcellulose. *Carbohydrate Polymers* **136**, 224-231, 2016.

# COUNCIL CONFERENCE CALL MINUTES
# AMERICAN LEATHER CHEMISTS ASSOCIATION
# FALL COUNCIL MEETING

December 15, 2022

**PRESENT**

| | |
|---|---|
| Officers: | Joe Hoefler, John Rodden |
| Council Members: | Shawn Brown, Myron Hooks, Steve Lange, Roger Pinto |
| Executive Secretary: | Carol Adcock |

1. **WELCOME**. The meeting was called to order. It was determined that a quorum was present.

2. **Minutes.** All previous minutes have been approved by Council.

3. **2023 Update of Annual Convention.** Sarah Drayna, 2023 Convention Chair, was absent so Mrs. Adcock gave the following report regarding the 2023 Annual Convention preparations:

   **Contract Provisions**
   The 2023 site will be Grand Geneva Resort & Spa, Lake Geneva, WI, June 20-23. A deposit of $2,500 is on file with Grand Geneva with a final payment of $2,500 being due 2/15/2023.

   The rates will be as follows:

| | |
|---|---|
| Single | $440 per night, inclusive |
| Double | $610 per night for two, inclusive |

   Rates quoted are per night and include lodging, breakfast, lunch, dinner, all taxes and amenity fee. Package begins with lunch on day of arrival and ending with breakfast on day of departure. Deadline for reservations is Monday, May 22, 2023.

   The rates for pre/post arrival are as follows:

| | |
|---|---|
| Single/Double | $229 per room plus taxes and resort fee |

   Rates for early arrival include accommodation only.

   **Schedule**
   The tentative schedule for the convention was previously emailed to Council. It is similar to the 2010 convention at Grand Geneva.

   **Entertainment**
   Tentatively a boat cruise is planned for Wednesday evening after dinner at the hotel. We will have to get transportation to take people from the hotel to the boat and back. We will have drinks on the boat and maybe dessert. Plans are still being formulated. After reviewing the cost of the boat cruise, it was suggested that each person attending should be given a certain number of drink tickets and then it would be a cash bar after that. Mrs. Adcock will check with Mrs. Drayna to see if that is possible.

**Sponsorship Campaign –**
The sponsorship campaign for 2022 was used to make one for 2023. It was sent to Council prior to the meeting. Discussion followed if this should be the same or changed for 2023. Discussion followed about raising or changing the rates. Mrs. Adcock will work with President Hoefler and Vice President Rodden to consolidate some of the categories and make changes to the rates for each category. Donis Bosworth as Convention Vice Chair will start the campaign in early 2023.

Attendance at the convention was discussed. There was concern that attendance might not be as good as earlier anticipated due to the current economic situation.

The role of the LHCA as far as sponsorship of the convention was discussed. Mr. Lange will speak to Stephen Sothmann about their potential sponsorship of the convention in one of the new categories.

**Sports and Social Coordinator**
Mrs. Adcock reported there was no need for a Social Coordinator for 2023, but a Sports Coordinator for the golf tournament will be obtained. Discussion followed about the problems with the golf tournament this year, and people not be charged for their clubs.

It was suggested that the fee for golf for 2023 should be $150 as the Highlands Course is much better than other courses we have recently played. Councilor Shawn Brown volunteered to be the Sports Coordinator for 2023.

**Proposed Budget**
A proposed budget for the 2023 convention was emailed to Council for their review. It was reviewed during the call. Discussion followed regarding the registration fee. Motion was made, seconded and unanimously carried to charge $400 for the convention registration fee beginning in 2023 with an early registration fee rate of $350 for those registering 60 days in advance of the opening day of the convention. It was felt that the sponsorship campaign needed to be revised and some other items checked on before approving a budget for the 2023 convention. It will be addressed again in January.

**AV Person**
Ms. Drayna will find an AV Coordinator for 2023.

**Technical Program** – John Rodden, Chair
Mr. Rodden already has some commitment from 2022 presenters to return in 2023. Mr. Brown had some ideas for industry updates and will send names of potential speakers to Mr. Rodden. Mr. Rodden discussed his conversation with Stephen Sothmann of the LHCA and is looking forward to receiving names of potential speakers from him. The aim is to have papers on industry updates and regulations mainly on the second day of the convention when more LHCA people might attend. Mr. Rodden asked for help from everyone to obtain speakers and hopes to have the program together early in 2023.

**Wilson Lecture** – Jeff Miller, Chair
Mike Redwood with Leather Naturally will present the 2023 Wilson Lecture. The title will be Retelling "Viewing Leather Through the Eyes of Science" A Century On. Mr. Redwood has already booked his flight for the convention and will have his abstract ready early next year.

**Alsop Award** – Joseph Hoefler, Chair
It was decided that since the approved recipient of the ALSOP Award for 2022 was unable to attend the convention last year, he would be asked to attend the 2023 convention to receive the award.

**O'Flaherty Service Award** – Sarah Drayna, Chair
A name for the 2023 recipient of the O'Flaherty Service Award has not been submitted to Council.

**2024 Convention Site**
This item was tabled until January.

**4. REPLACEMENT OF EXECUTIVE SECRETARY**

Mr. Lange reported that he has a job description ready and would like to post it in January. It was suggested that the Association explore keeping the office in Lubbock, TX due to the lower cost of living and Mrs. Adcock being physically located there and able to train a new person. Mrs. Adcock has volunteered to continue to come by the office after a new person is hired to make sure things are moving smoothly if that option is considered. The committee will meet later this month and discuss the various options.

**5. FINANCIAL REPORTS – Carol Adcock**

Year to Date Financial Reports

Council reviewed the Profit and Loss Statement and Balance Sheet through November 30, 2022. Mrs. Adcock pointed out that the amount for printing the journal was skewed as she did not receive invoices for the last quarter of 2021 until 2022. Therefore 15 issues were paid for in 2023 instead of the usual 12.

The Membership Breakdown as well as a dues and subscriptions breakdown for 2023 was emailed to Council prior to the meeting and reflected the following:

119 Active, 45 Active Life, 4 Active Life Mutual, 21 Active Life Retired, 15 Active Mutual, 43 Active Retired, 1 Student, and 28 SLTC along with 3 SLTC Students, for a total of 279 members. Out of the above membership that is anticipated for 2023, dues will be collected from 136 paying members, excluding the dues that will be collected from the SLTC members. The list of canceled memberships was reviewed. There are 49 subscriptions that have been invoiced for renewal for 2023.

Mrs. Adcock also noted that 4 advertisers have committed for 2023, with one being a new one. Two other 2022 advertisers are still awaiting approval from their company.

Motion was made, seconded and passed to accept the Financial Reports as submitted.

A rough draft of the 2023 Association Budget was sent to Council prior to the meeting. Discussion was tabled until January so the 2023 Convention Budget could be approved and other matters concerning the replacement of the Executive Secretary and expenses involved with that.

**5. EDITOR'S WRITTEN REPORT – Steve Lange**

After the meeting, the Editor submitted the following report which was emailed to Council.

December 15, 2022

Dear ALCA Officers and Councilors:

**Editor's Report**

We have published 51 papers in 2022. Confirmed manuscripts are scheduled through May 2023. Seven papers have been rejected in 2022. Submissions seemed to have slowed in recent months. (Received 5 submissions in September but only 2 in November.)

Publication cost ranged from $1934.00 to $2304.00 per issue. Biggest variable seems to be the typesetting cost. I don't think UC uses the same designer for each issue, and they don't set the cost.

Respectfully submitted,

Steven D. Lange, *Journal* Editor

Council voted to accept the Editor's Report as written via email after the meeting.

**6. WAYS AND MEANS COMMITTEE REPORT – Shawn Brown and Steve Schroeder**

Due to the short notice of this meeting, Mr. Brown will submit his report at the beginning of 2023 showing how the investment portfolio did for 2022.

**7. TECHNICAL COMMITTEE REPORT**

Mrs. Adcock reported that the Education Subcommittee has two new students both from S.B. Foot Tanning Company.

**8. NOMINATING COMMITTEE REPORT – Mike Bley, Chair**

The 2023 Nominating Committee submits the following names for the 2023 Slate of Candidates for Councilor:

Goetz Hagen
Janio Rocha
Todd Salzman
Eric Webb

Motion was made, seconded and unanimously carried to approve the 2023 Nominating Committee's Slate of Candidates for Councilor. The Nominating Committee will determine that each of these candidates are still willing to run and then obtain bios from each.

**9. OLD BUSINESS**

This item was tabled until January.

**10. NEW BUSINESS**

This item was tabled until January.

**11. LOCATION AND DATE OF NEXT COUNCIL MEETING**

Next Council conference call will be in January. A date will be circulated closer to that date.

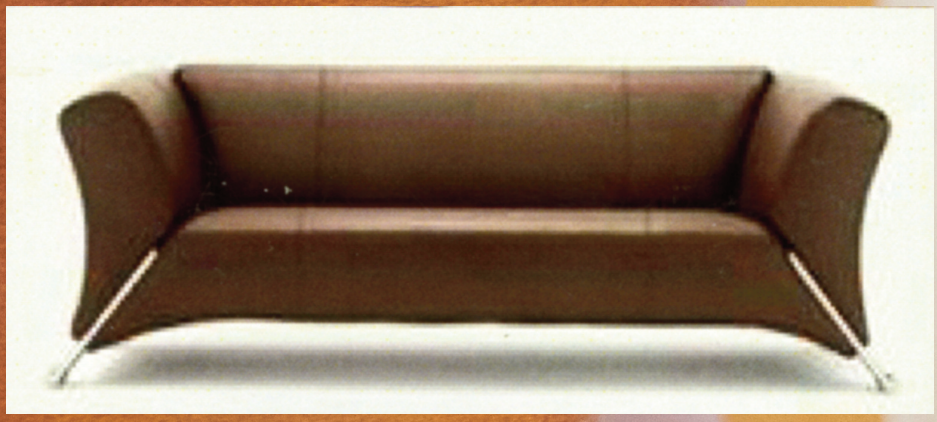
There being no further business before Council, the meeting was adjourned.

Respectfully submitted,
Carol Adcock, Executive Secretary

LEATHER

AVELLISYNCO

# Selected Dyestuffs



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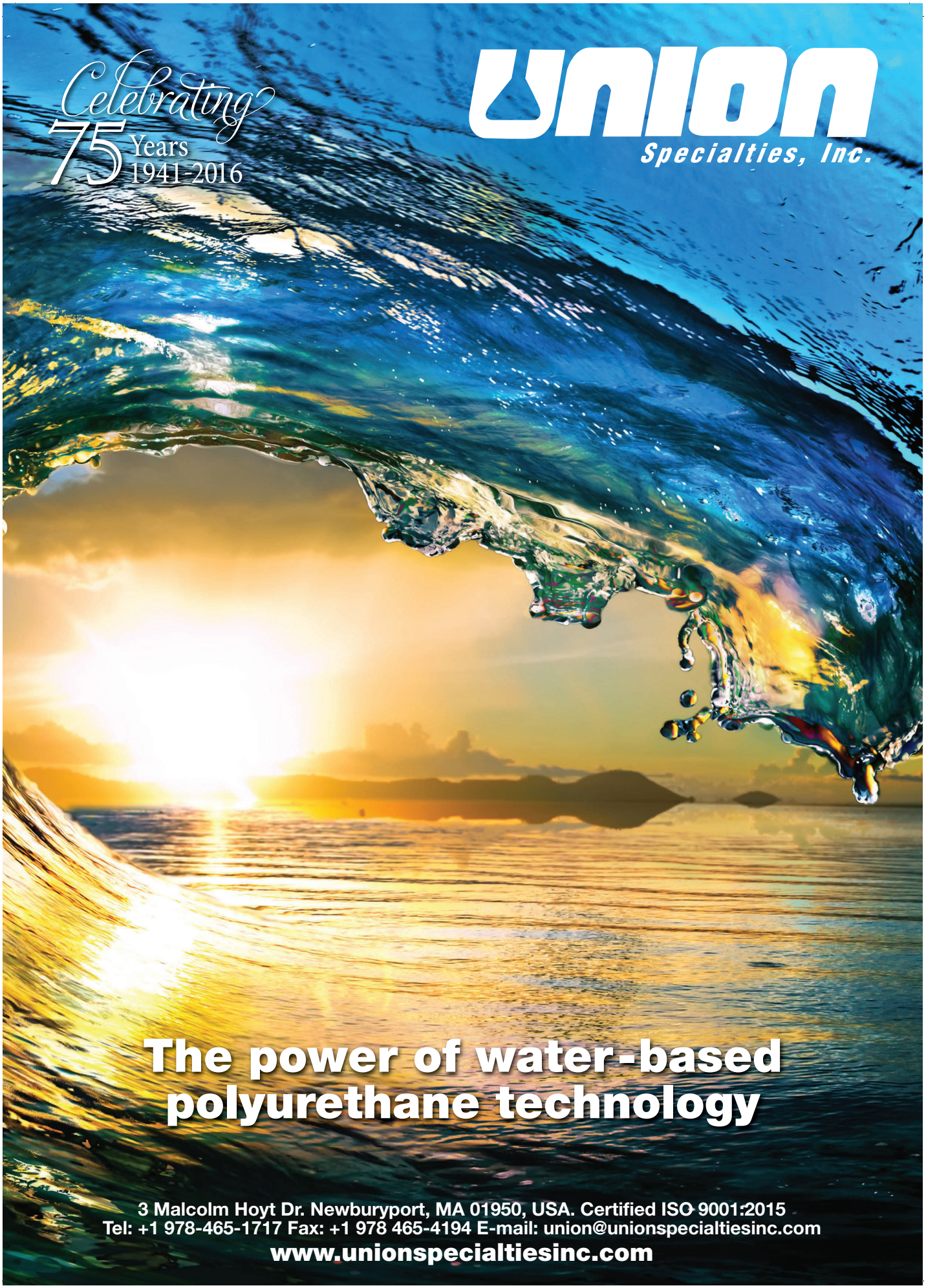
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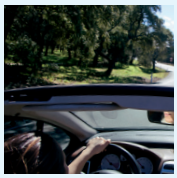
# Stahl's innovations driven by sustainability

With the rise of both electric and self-driving, cars are becoming quieter and anti-squeak and rattle materials are becoming increasingly important. At the same time, improved anti-stain performance is required, because of the current trend for pale-colored car seats. Therefore, we have developed Stay Clean. This low-VOC coating technology protects pale-colored leather and vinyl surfaces against common stains, such as dye from jeans, spilled coffee and dirt. Our solution also makes surfaces low-squeak, which is a great asset as global research has shown that a squeaking car interior is one of the biggest annoyances among car owners. Another trend in car interior is the popularity of matt surfaces. Therefore, we have developed PolyMatte®. This non-squeaking solution provides a luxurious feel to the finished article in combination with flexibility and scratch and abrasion resistance. Our portfolio contains many products, varying from beamhouse products, tanning systems to finishes, duller concentrates, crosslinkers and thickeners to leveling agents, defoamers, colorants and hand modifiers. Our most sustainable option is Green PolyMatte®, which is based on rapeseed oil (20%) instead of crude oil-derived intermediates. If you would like to know what our Stahl solutions for automotive can do for your business, please visit **www.stahl.com** or contact us at: **alexander.campbell@us.stahl.com**.

If it can be imagined, it can be created

Stay Clean



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# ALCA News

## Executive Secretary Retiring

In 1999, after being housed at the University of Cincinnati for over 50 years, the ALCA found out they were losing their offices and would need to find a new home. The Leather Research Institute under Director Dr. Dennis Shelly became the official sponsor of the ALCA on the Texas Tech Campus in Lubbock, TX. A search was then underway to find a new Executive Secretary. Dr. Shelly posted the job opening and received several candidates; however, he personally asked a friend of his family, Carol Adcock, to consider applying for the position. Dr. Shelly submitted all applications to the then Council consisting of Robert White, President, Elton Hurlow, Vice President, Maryann Taylor, Vice President-Elect, and Council members Chris Black, Nicholas J. Cory, Ricardo E. Lopez, Frank Metsaars, David G. Ouellette and David T. Radwell. Council approved hiring Carol. Her first duty was to attend a Council Meeting in Chicago, IL on October 1. From there she flew to the ALCA offices at the Tanners' Council Research Laboratory at the University of Cincinnati to oversee the moving of the offices and attend the leather course offered by the Leather Research Laboratory.

Since the previous June, the Association did not have an Executive Secretary, and things were not in the best of shape. The boxes arrived at the Texas Tech campus and were put in an unused lab in the Chemistry Building which was the initial location for the ALCA office and library. In August of 2000, the offices of the LRI and the ALCA were moved to the SW Campus of Texas Tech University on Quaker Avenue right inside Loop 289 around Lubbock. The ALCA thrived there for almost three and a half years until again they were asked to find another location. Dennis and Carol looked at other University properties and then started looking outside of the University. On February 1, 2004 the ALCA and LRI moved to the ALCA's current office at 1314 50th Street, Suite 103, Lubbock, TX.

Carol has worked as the only paid employee of the ALCA for over 23 years, maintaining and keeping the books, orchestrating the annual convention, and many, many other duties that the position required. She feels it has been a very rewarding job due to the variety of duties but mainly due to the great people in the leather industry. Working with 20 different presidents, namely Robert F. White, Elton L. Hurlow, Maryann M. Taylor, Jerome F. Levy, Dean T. Didato Rodney Hammond, Doulas G. Morrison, W. Nathan Mullinix, Dennis C. Shelly, William N. Marmer, Stephen S. Yanek, David LeBlanc, Craig Glover Keyser, Andreas Rhein, Steven Gilberg, Steven D. Lange, Sarah Drayna, David Peters, Mike Bley and Joseph Hoefler and three different editors, namely, Kenneth A. Boni, Robert F. White and Steven D. Lange, has been a real pleasure.

Carol has been married to her husband Woody for 45 years. They have three children who have given them one grandson each. Carol's son Paul Lackey lives in Dallas, TX, and her grandson Bran is completing his first year of college. Carol's daughter Holly Lackey lives in Lubbock with her son Charlie Max Hooda who is in the sixth grade. Carol's daughter Julie Bessemer also lives in Lubbock with her son Dane Ryan Bessemer who is in the eighth grade.

Carol is very grateful to everyone for their support during her tenure with the ALCA and wishes the ALCA another 120 years of prosperity. She is hoping for one of the biggest crowds ever at her last ALCA Annual Convention, June 20-23, at the Grand Geneva Resort at Lake Geneva, Wisconsin.

## Peabody Leather Workers' Museum and George Peabody House

After many years of volunteer work, the Grand Re-opening of the Peabody Leather Workers' Museum and George Peabody House will take place May 6, 2023, from 10:00 am to 3:00 pm. (Rain date: May 13) All are welcome to attend and discover the pivotal role Peabody played in pioneering the World's leather industry. Featured speakers during the celebration will be the current and former Mayors of Peabody, Ted Bettencourt and Michael Bonfanti respectively along with key representatives of the leather industry. Address: 205 Washington Street, Peabody, MA 01960, telephone: 978-278-5133.

# Lifelines

**Niklesh C.** see *JALCA* **117**, 451, 2022

**Jayakumar G.C.** see *JALCA* **117**, 451, 2022

**Phebe Aaron K** see *JALCA* **117**, 451, 2022

**N. Priya** is a postgraduate in Chemistry with over 15 years of experience in Analytical chemistry of Leather and in particular specialized in the area of Hazardous Chemicals analyses. An expert in various Chromatography and Spectroscopic techniques.

**S. Suresh** is a postgraduate in Chemistry with over 15 years of experience in Analytical chemistry of Leather and in particular specialized in the area of Hazardous Chemicals analyses. An expert in various Chromatography and Spectroscopic techniques.

**Mohan Vedhanayagam** received his B.Sc. (2008), M.Sc. (2010) and Ph.D. (2019) in Chemistry from University of Madras Chennai, India. He is currently working as a CSIR –Research Associate at Central Leather Research Institute, Chennai in India. His current research interests include Polymer, Nanomaterial, Sensor, Tissue engineering, Drug delivery, Bio-Imaging, Cancer Theranostics and Smart Leather. He has published around 15 research papers in international journals, 01 patent (India) and 01 book chapter. He has passion for teaching and research.

**K. J. Sreeram** see *JALCA*, **109**, 135, 2014

**Wang Yingxuan** received her master's degree from Sichuan University in 2022. She is currently working at Sichuan Institute of Fine Chemical Research & Design.

**Zhang Zongcai** received his Doctor's degree in leather chemistry and engineering from Sichuan University in 2005. His research mainly focuses on the cleaner technology and development of fine chemicals for leather and fur.

**Zhao Qilong** is Director of Technical Development Department in Sichuan Institute of Fine Chemical Research & Design.

**Jinwei Zhang**, engineer in major of leather manufacturing and cleaner leather making technology, obtained his Ph.D. degree in 2018. He is working at the College of Biomass Science and Engineering, Sichuan University, China. His research interests focus on new and cleaner leather making technology, chrome-free tanning material and method, pickle-free tanning method and leather byproduct utilization. He also helps leather chemical company and tannery apply and develop new material and leather product.

**Yulin Cheng**, obtained a Master's Degree in Light Industry Technology and Engineering from Sichuan University in 2022. Now, she is an engineer in a logistics company in charge of leather goods transportation.

**Jiacheng Wu,** obtained a Ph.D. degree in Biomass Chemistry and Engineering from Sichuan University in 2019. Now he is a teacher in University of Electronic Science and Technology of China. He aims to help students study abroad in major of new and functional materials.

**Wuyong Chen**, see JALCA 112, 250, 2017.

# 117th ALCA ANNUAL CONVENTION June 20-23, 2023 Grand Geneve Resort & Spa Lake Geneva, Wisconsin

***Featuring the 62nd John Arthur Wilson Memorial Lecture***

***Retelling "Viewing Leather Through the Eyes of Science"***

***A Century On***

***By Mike Redwood, Leather Naturally,***
***Teacher at University of Bath School of Management,***
***and Trustee of the U.K. Leather Conservation Centre***

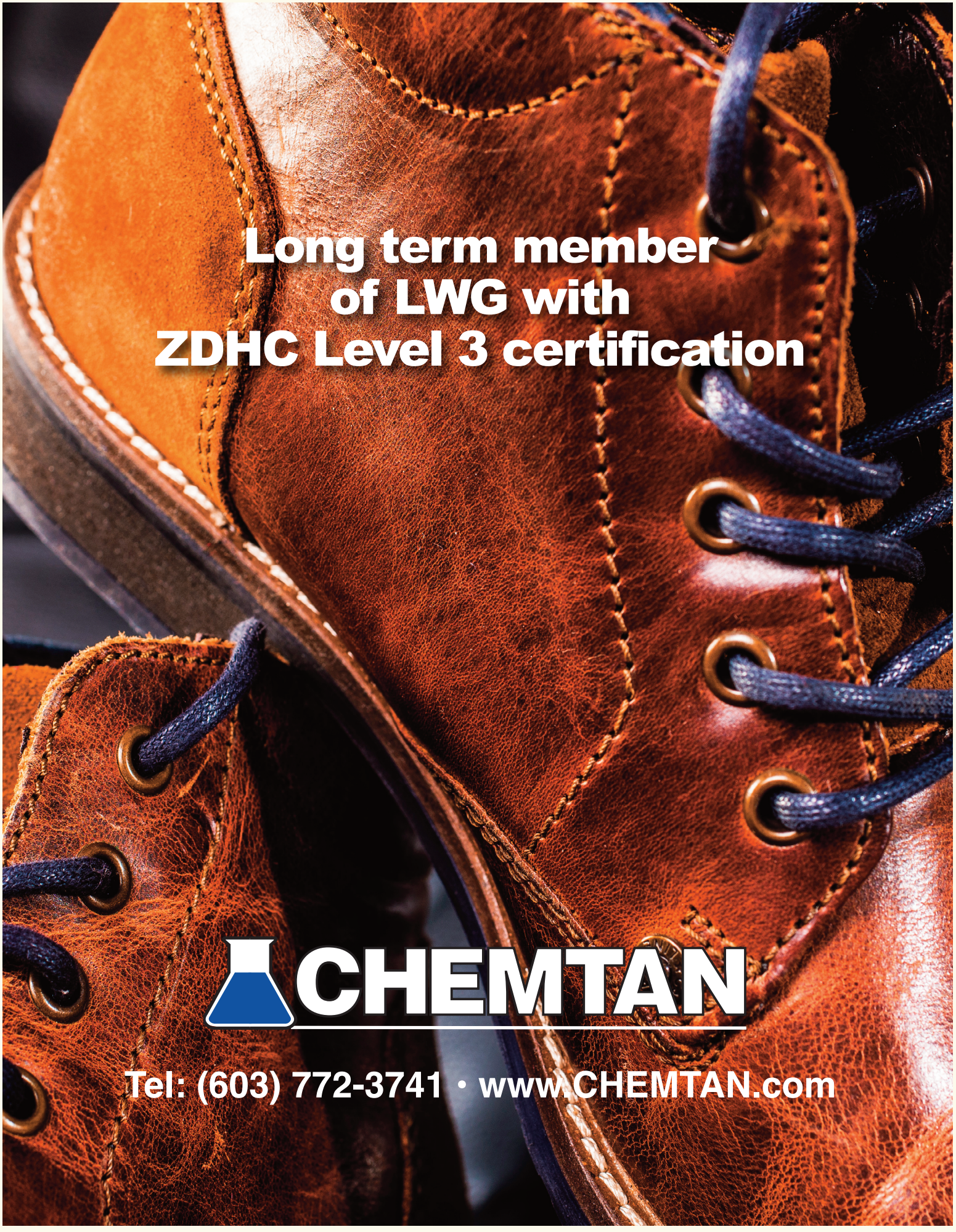
**Tentative Schedule**

**Tuesday, June 20**
***Golf Tournament, Opening Reception and Dinner***

**Wednesday, June 21**
***John Arthur Wilson Memorial Lecture***
***All Day Technical Sessions, Fun Run, Dinner***
***Boat Cruise***

**Thursday, June 22**
***All Day Technical Sessions, Annual Business Meeting***
***Activities Awards Luncheon***
***Social Hour, Awards Banquet***

***Visit us at www.leatherchemists.org for full details***
***under Annual Convention as they become available***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