Changes to the Collagen Structure using Vibrational Spectroscopy and Chemometrics: A Comparison between Chemical and Sulfide-Free Leather Process

by

Megha Mehta,[1*] Yang Liu,[1] Rafea Naffa,[1a] Mark Waterland[2] and Geoff Holmes[1]

[1]*Leather and Shoe Research Association of New Zealand*, P.O. Box 8094, Palmerston North 4472, New Zealand.

[2]*School of Fundamental Sciences, Massey University*, Palmerston North, New Zealand

Abstract

Chemical and physical changes take place when hides and skins are processed to leather that affect the quality and strength of the material. Understanding the structure at each leather-making stage is the basis of this study but also intend to improve the process through a biochemical approach, employing a proteolytic enzyme for processing leather more cleanly with reduced environmental impact. Raman and ATR-FTIR spectroscopy in conjunction with chemometrics was used to investigate each leather-making stage from fresh green cattle hide to dry crust leather. The changes in proteins, lipids, nucleic acids and other biomolecules with leather processing was measured and reported using three novel Raman ratiometric markers, 920/1476, 1345/1259 and 1605/1476 cm^{-1}, to discriminate the structural changes in collagen of hide using standard chemical and enzymatic method. Amide I band was deconvoluted to investigate the collagen secondary structures using curve fitting by Gaussians function. The results of Principal Component Analysis are well-corroborated with the ratiometric markers of structural changes.

In summary, the results obtained demonstrates the potential of vibrational spectroscopy (Raman and ATR-FTIR), in conjunction with multivariate analysis, in generating universal biochemical signatures representing structural changes and helps in bringing the better ecofriendly method for greener leather process.

Introduction

A leather-making process is an approach to preserve skins and hides from decomposition and to provide flexible and robust leather. The process involves a series of mechanical and chemical treatments starting from the fresh skin to the final dry crust leather. With every chemical treatment, structural properties of the original skin are altered either by isolating the essential proteins, collagen, from the raw skins or hides or adding cross-linking agents in the later stages of processing to ensure stability and strength of the leather. It modifies the chemistry of collagen, which is widely known, but very little information is available about analyzing these processing effects on the collagen structure.

The skin acquired from the animal carcass after expulsion is termed as "fresh green". Skins are regularly salted to safeguard the skin prior to tanning briefly, and it caused some drying out of the skins. The next step of the leather making process is to rehydrate the skins by dousing and washing followed by a salt treatment using sodium sulfide termed as liming combined with suitable chemical or microbial enzymes termed as "bating", which separates and eliminates unwanted components. It assists with opening up the leather structure, allowing chemical penetration in ensuing stages. After treating with alkaline chemicals, the objective is to preserve the skin from putrefaction by adjusting to low pH and then treating with extreme acidic solution of sulfuric acid and sodium chloride. The skin obtained after this stage is termed as "pickled". The pickled skin can be preserved for a year and two. After pickling, a synthetic cross-linking agent is generally added, which helps with the following chrome tanning stage. The obtained pelts were then tanned using chromium sulfate and classified as "wet blue." The color and surface of the leather changes at this stage and will, in general, be excessively inflexible for most applications. Therefore, to make leather softer and fuller, vegetable or synthetic tannins, are added and the stage is termed as "retanned". Then the desired colors and fat liquors are added to provide the final finish to the leather and mentioned as "fat liquored." The leather obtained after is dried, softened, and is designated "dry crust" leather.[1,2]

The leather industry contributes to the economy significantly and poses a risk to the environmental due to release of various chemicals. Lime and sodium sulfide used at the liming stage of leather processing for unhairing skins and hides cause environmental pollution. The disulfide bonds in the keratin of hair and epidermis are broken down by sodium sulfide allowing the removal of hair from skin keeping the dermis intact and unaffected. The byproducts obtained from lime and sulfide produces foul smell and creates highly alkaline sludge which is hazardous to the environment[3] and poses a disposal problem for the leather industry.[4]

The role of enzymes in dehairing using alkaline proteases can serve as an alternative method to chemical unhairing to gain ecological advantages. Proteolytic enzymes are the most industrially utilized

*Corresponding author email: megha.mehta@lasra.co.nz

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enzymes. Microbial proteases are more commonly used in the leather industry for being more environmentally friendly than chemical processes.[5-7] There are different classes of proteases used in leather processing, such as neutral proteases used in soaking, alkaline proteases for dehairing, and acid proteases for bating process.[8] There are several advantages of enzymatic unhairing which includes the removal of hair without affecting collagen content of dermis and no solubilizing of hair, decrease or even total disposal of sodium sulfide, great quality hair recuperation and improved working condition.[9]

In the present work, we have used vibrational spectroscopy techniques, Raman spectroscopy, and Attenuated Total Reflectance - Fourier Transform InfraRed (ATR-FTIR) to thoroughly characterize the samples obtained at each stage of leather making process. The aim was to analyze the variations in the collagen structure by comparing the samples obtained from standard leather-making protocol using hazardous chemical sodium sulfide and enzymes that are relevant for developing environmentally clean technologies. Both non-destructive techniques are fast, require minimal or no sample preparation, and are very specific and sensitive. Raman spectroscopy has the advantage of an extremely weak signal from water, so that the slight interference from water present in biological samples causes no damage to the sample and allows on-site detection with microscopes optics. If there is a decrease in the water signal, then the advantage of IR is its sensitivity to vibrations associated with amide bonds in proteins. The secondary structures of proteins influence the shape of the amide bands and IR spectroscopy provides extensive information regarding the structure of proteins. We have used an ATR-FTIR spectroscopy using a curve fitting method to analyze the secondary structures within the amide I band for collagen strength. The work is upheld with ratiometric and chemometric techniques, for example, Principal Component Analysis (PCA), which uses statistical and mathematical algorithms for quantitative data interpretation and allows for discrimination according to biochemical components in samples.

Experimental

Materials

New Zealand Leather and Shoe Research Association (LASRA) obtained raw cattle hide for processing in-house. Samples were obtained from the official sampling position (OSP) and removed from the same hide during several leather-making stages following the standard LASRA leather processing protocol as provided in the supplementary information (Table SI). These stages were termed fresh green, limed, pickled, wet blue, retanned, fat liquored, and dry crust. All samples had high moisture content, except dry crust, because they were obtained during the leather processing. The samples were sectioned using a freezing microtome (Leica CM1850 UV, Germany) to 60-µm thickness and transferred onto microscopes slides for Raman measurements. For FTIR measurements, samples were sliced into a thick piece and analyzed for FTIR scans.

Enzymatic depilation

A bacterium producing proteolytic enzyme was isolated and identified from tannery compost. Briefly, serial dilutions of the compost suspension were plated onto Luria broth agar plates with skimmed milk. After 24 hours, colonies surrounded by clearing zones were picked and cultured in LB broth at 25°C overnight. The identification of the bacteria was achieved by 16S rRNA gene sequencing, the results of which were analyzed using the Targeted Loci Nucleotide BLAST. From 16S rRNA gene sequencing and phylogenetic analysis, the proteolytic bacterium was identified to be *Vibrio metschnikovii*.

V. metschnikovii cryopreserved with glycerol at -80°C was activated by inoculation in 5 mL, 1:1000 ratio of LB broth and cultured overnight at 25°C on a shaking incubator. The activated culture was used to inoculate 3 L of freshly prepared fermentation media, which was then cultured for a further 48 hours. The crude enzyme extract was collected by centrifuging the resultant culture at 8000 g relative centrifugal force (rcf) for 20 min at 4°C. Ammonium sulphate precipitation was carried out to separate the total proteins from the crude protease extract, followed by centrifugation at 20,000 g for 30 min at 4°C.

The partially purified protease was rehydrated using 100 mL PBS and enzymatic depilation paint was prepared by mixing the enzyme solution with Solvitose (50 g/L) as a thickener. The resultant enzyme painting paste was applied on the flesh side of cowhide samples (20×20 cm^2), which were then kept at 25°C. Complete depilation was achieved after 24 hours of incubation. The depilated slats were processed into crust leather following standard LASRA protocol (Table SI), sectioned, and prepared for Raman and ATR-FTIR analysis.

Data acquisition and spectral processing

Raman spectra were acquired from samples under ambient conditions using a custom-built Raman microscope. 532 nm excitation laser (Laser Quantum Torus) was focused onto the sample using 0.65 NA and 40 magnification microscope objective. The laser power at the sample was around 10 mW. 532 nm Raman edge filter (12° incident angle) (Iridian Spectral Technologies, Canada) was focused onto a 50-micron entrance slit of a Teledyne-Princeton Instruments Isoplane 81 (FERGIE) spectrometer[17,26] and rejected the Rayleigh scattered light. Lightfield 6.1 software was used for the acquisition of spectra. Triplicate samples from each stage were prepared, and Raman spectra were acquired with an exposure time of 5 seconds per frame over 10 frames (each frame was saved separately) for data analysis. In total, 30 spectra were recorded for each leather-making stage.

ATR-FTIR spectra was recorded by attenuated total reflection (ATR) on a diamond crystal with 16 scans from each of the triplicate samples[17] using a Thermo Scientific™ iD5 Nicolet™ iS™5 Attenuated Total Reflectance - Fourier Transform InfraRed (ATR-FTIR) spectrometer.

The recorded spectra were preprocessed using the SciKit Learn package[13] algorithm written in Python 3.7. Baseline correction

using asymmetric least-squares method was performed on average spectra using the python algorithm. Smoothing using the five-point Savitzky-Golay smoothing function to remove any spectral noise and normalization was carried out by dividing each point by the norm of the whole spectrum using Origin 2021b. Band areas of peaks of interest were determined by deconvoluting the peaks using curve-fitting function.[17,26] The most dominant changes observed in the amide I region was shown by deconvoluting the 1646 cm^{-1} (amide I) band. To account for the variability between the datasets of different leather stage samples, principal component analysis (PCA) was performed to extract meaningful comparison with significant variation of samples.

Results and Discussion

Lime serves to open-up the fiber bundles of hide matrix to the desired degree and helps to remove the hair completely. In the present method, alkaline proteolytic enzyme lime has been replaced to depilate the skin with good grain quality.[14] Hence, it is necessary to analyze the structural variations happening throughout the leather-making stages due to change in initial pre-tanning method and monitor the effect at the molecular level using Raman and ATR-FTIR spectroscopy.

Fundamental characteristic of Raman spectra of standard and enzymatic processed hide

The Raman spectra obtained from different leather-making stages are highly complex, consisting of a superposition of Raman scattering peaks from many biochemical constituents. Spectral variations were observed in the peaks highlighted (Figure 1). The bands were assigned based on already published literature and listed in detail in Table SII.[15-17] Peaks at 833-859,868 and 920 cm^{-1} are assigned to the collagen tyrosine, proline and hydroxyproline matrix, C-C skeletal of collagen backbone and the peak at 1002 - 1028 cm^{-1} is characteristic of phenylalanine. Other distinct peaks were observed at 1259 cm^{-1} (amide III ν(C-N) and δ(N-H) of proteins), 1345 cm^{-1} (CH_3CH_2 twisting of proteins and nucleic acids), 1445 cm^{-1} ($\delta(CH_2)$ deformation of proteins and lipids), 1553 (ν(C = C) of amide III), 1605cm^{-1} (δ(C = C) of phenylalanine) and 1655 cm^{-1} (amide I ν(C = O) of lipids). The Raman spectra of limed skin shows an overall increase in the intensity of all peaks with the maximum increase observed at 1095 cm^{-1} band and was about 3 times higher than for fresh skin. This band is attributed to lipids,[18] which might be released due to the breakdown of keratin and swelling of the skin producing sulfur compounds in conjunction with lime.

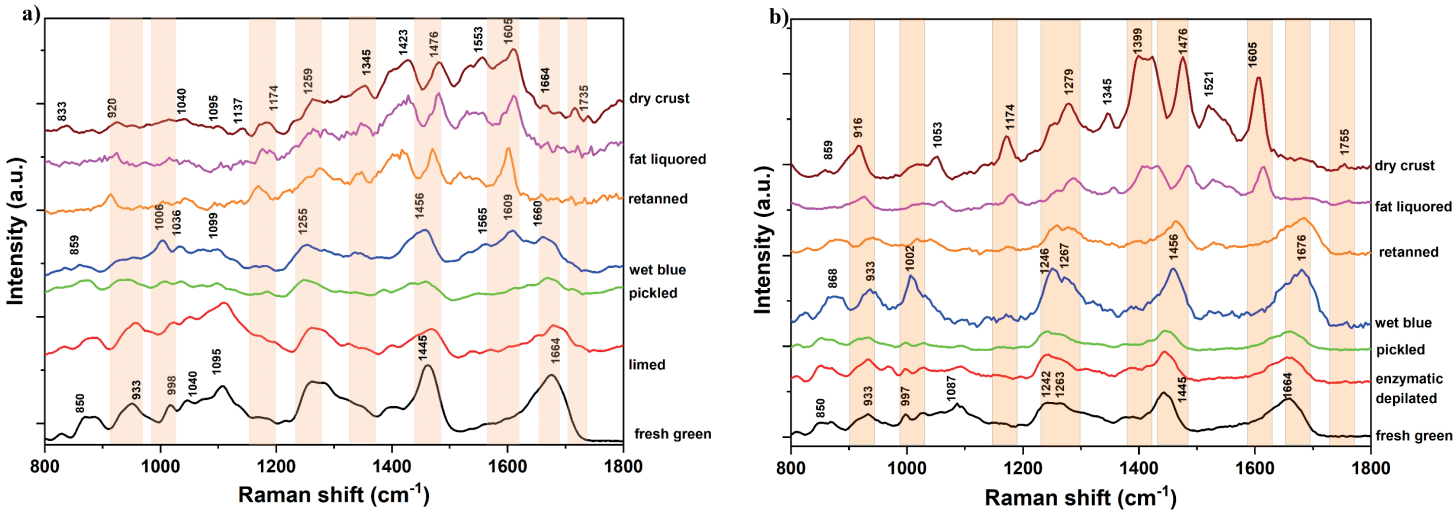

Figure 1. Raman spectra of different stages of leather-making using **a)** standard and **b)** enzymatic method.

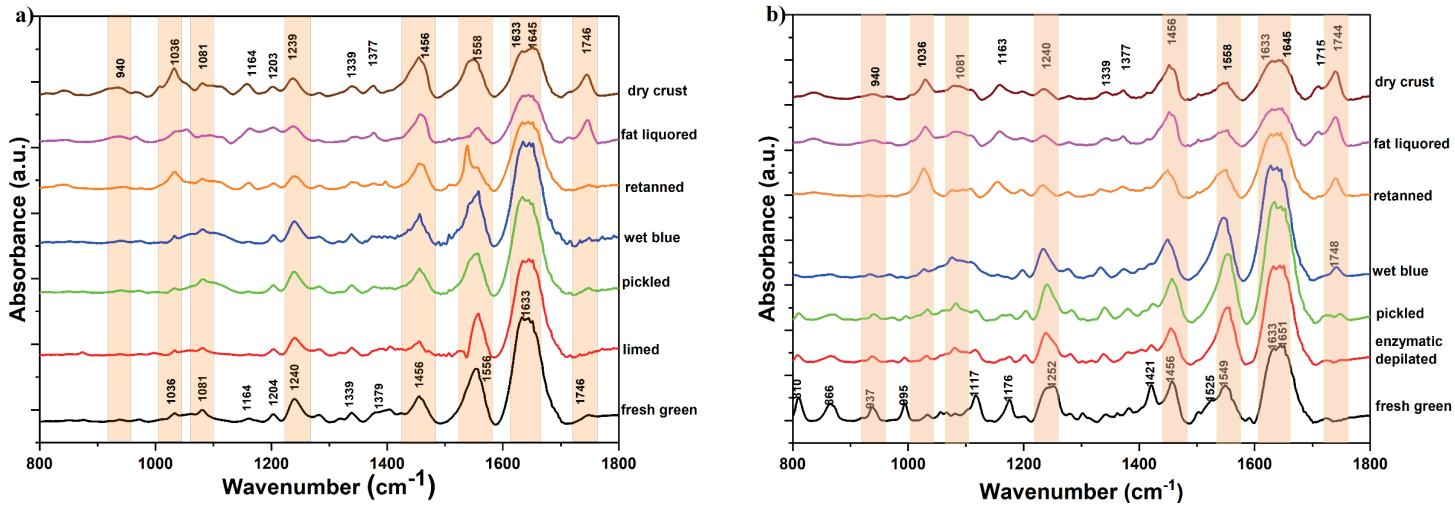

Figure 2. ATR-FTIR spectra of different stages of leather-making using **a)** standard and **b)** enzymatic method.

ATR-FTIR standard results also show two bands at 1036 and 1081 cm^{-1}, which can be attributed to sulfo groups due to the sodium sulfide[19] and lime used during liming. Raman spectra of enzymatic-depilated skin show a decrease in intensity than fresh skin. This could be due to an effective opening of the derma structure using the enzyme solution that degrades soft keratin, which is present in the roots of the hair, and amino acids during hide processing.

The band around 1745 cm^{-1} and the band at 1399 cm^{-1} can be attributed to ν(C=O) modes of lipids due to the addition of fatty acids in final stages of leather processing. There is a change in protein observed at every stage with a slight shift in Raman bands. Phenylalanine band at 1002 cm^{-1} shows a significant increase in intensity at the wet blue stage and almost disappears when it reaches the dry crust stage. This indicates that the addition of chemicals at every stage to remove non-collagenous protein, provide strength to leather, and alters skins biochemical structures. The Raman band at 1176 cm^{-1} appears at wet blue stage and becomes intense in later stages which might be due to interaction of chromium (Cr) with hide protein and addition of fatty acids forms complexes with carboxylate groups making the bond much stronger. This is quite evident at dry crust stage of enzymatic method with the onset of 1399 cm^{-1} band which attributes to C=O symmetric stretch and not observed in standard (liming) process. A change in the amide I position around 1664 cm^{-1} corresponds to collagen fibers explaining the force involved for reorganization of shape associated with the stretching and straightening of twists and turns. Such variations occur due to changes in the H-bonded network or other structural reorganizations in the collagen structure. The Raman spectra of each leather processing stage adds to change in protein vibration bands arising from polypeptide backbone (amide bands), intra- and intermolecular hydrogen bonds, and side chain groups. The disappearance of the amide I band in Raman spectra at final dry crust stage of standard and enzymatic method correlates with the presence of bulky side chains, such as, tyrosine and phenylalanine that would be relied upon to display spectra with a suppressed or absent amide I band.[20] It was assumed that the silence of amide I bands in Raman spectra results from the separating of peptide bonds from the metal (chromium)-protein surface after tanning stage. The closeness could depend on the length (bulkiness) of the amino acid side chains, which act as spacers between the fatty acid particles and the peptide bonds. The addition of fatty acids causes a change in the relative position of the domains, but complex formation does not change their conformational structure.

The characteristic bands obtained by ATR-FTIR results of the standard and enzymatic leather process method do not show any major shifts and can be visualized easily for slight changes. Raman band centered at 1664 cm^{-1} (Figure 2) represents the amide I of collagen, which usually consists of several secondary structures of amide I band (1600–1700 cm^{-1}), corresponds to C=O stretching vibrations, and the most sensitive part of the protein when determining the secondary structure.[21] The bond explains the backbone conformations and different type of secondary structure due to different C=O stretching. Therefore, ATR-FTIR spectroscopy is an effective tool for assessing the secondary structure of the protein. The amide I band is mainly used to quantify the secondary structure and conformational changes of proteins and polypeptides.[22] Fourier self-deconvolution (FSD) method, mathematical approach was used to isolate highly overlapping components of amide I, which originate from different secondary structural elements. While investigating the proteins, the amount of carboxyl groups reflects the ongoing situation. The IR results of fat liquored and dry crust (staked) showed that peak areas in enzymatic hide spectra at 1746 cm^{-1} emerged suddenly due to the increase in the amount of carboxyl groups with low hide pH of 3.6. The IR spectra of standard method showed characteristic absorption bands at 1748 cm^{-1} in pickled hide which disappear in chromated sample (wet blue) while two bands arising at 1717 and 1746 cm^{-1} at dry crust stage. The first disappearance and appearance of 1745 cm^{-1} band with shoulder band at 1717 cm^{-1} may be referred to the chromium complex while the band at 1745cm^{-1} may be referred to the free carboxylic groups of oligomers which might be created from fragmentation of peptide chains. Likewise, enzymatic method shows consistent appearance with increasing intensity of 1748 cm^{-1} band with fragmentation into two bands getting to the final stage. Each of the chemical treatments modifies the composition of collagen proteins and can be observed at peak 1664 cm^{-1}. With liming, deliming several non-fibrous proteins break down which can be seen in several secondary structures at pickled stage. It indicates the loss of the stability of collagen structure, which was regained after wet blue stage with addition of a few cross-links. After careful assessment, spectral variations were observed in the collagen region (1002-1680 cm^{-1}) indicating few shifts in peaks which could be due to intricacy of different biochemical components at each leather processing stage which create alterations in secondary structures leading to α helix, β sheet, cross links or random coils.[23,24]

Two spectral analysis techniques were employed to investigate the biochemical changes in the structural transformation from hide to leather. Few spectral differences were visible with naked eye but can be visualized using statistical techniques to identify a clear difference between the chemical and enzymatic processes.

- A univariate statistical method uses a single variable to investigates peak shifts, peak intensities, or peak area by calculating the ratios of peaks of interest for the interpretation of spectra.[25,26] This ratiometric analysis is a parameter for quantification and help the spectroscopists to understand spectral variations, and represent using statistical plots, such as bar plots in Figure 3. It can resolve variations due to sample morphology and other instrumental effects.[15]
- A multivariate statistical method - Principal Component Analysis, an unsupervised method that extracts the basic features from the full spectrum, based on the analysis of variance characteristics. Multivariate analysis does not make any priori assumptions about selecting the best variables for classification.[17,27]

Ratiometric analysis

Ratiometric analysis was performed using three novel Raman ratiometric markers, 920/1476, 1345/1259 and 1605/1476 cm^{-1}, to discriminate the structural changes in collagen of hide using standard chemical and enzymatic method. The final dry crust spectra were used for Raman and ATR-FTIR ratiometric analysis.

The amino acid contents (hydroxyproline and proline) are very important constituents in collagen because they play an important role in stabilizing the triple helical structure. From the ratio 920/1476 cm^{-1}, proline content (peak at 920 cm^{-1}) in collagen from enzymatic method was slightly higher than that in standard method, which suggests that collagen from enzymatic method, may have a more complex structure than that from standard method which determines higher collagen strength. Reduction of pH values at dry crust stage cause the decrease in intensity of the band located at 1476 cm^{-1}, which can be explained by H^+ ion binding to nitrogen bond of side chain amino acids which helps in stabilizing the collagen structure. 1345 cm^{-1} is an indicator of protein alpha-helix orientation, the intensity of which is highly sensitive to molecular orientation. Increase in ratio of 1345/1259 cm^{-1} explains about structural changes in lipids and proteins due to interaction of chromium (Cr) with hide protein and addition of fatty acids makes it much stronger after interaction with carboxylate groups to form complexes. Such complexation enhances the quality of finished leather which is observed higher in enzymatic method. 1605 cm^{-1} contributed to phenylalanine (collagen proteins) and 1645 cm^{-1} to the amide I mode and may indicate a combined effect from changes of protein secondary structure.

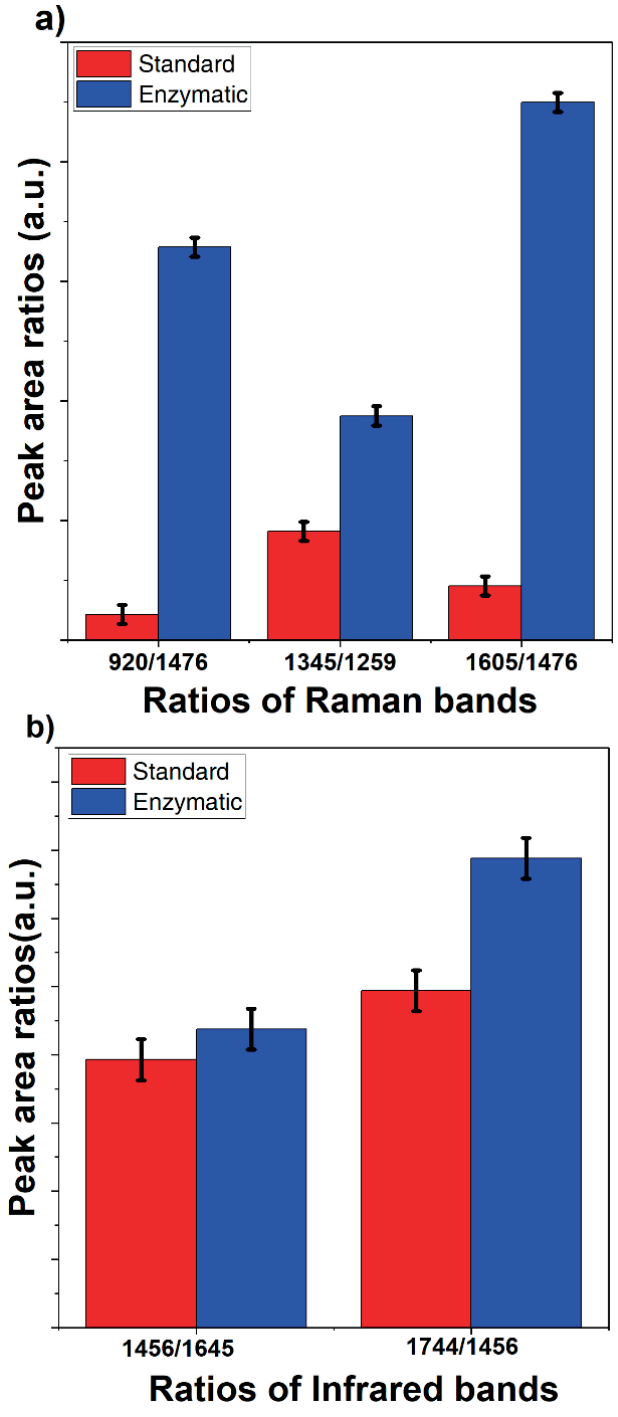

Figure 3. Peak area ratios of **a)** Raman and **b)** ATR-FTIR bands for standard and enzymatic method of leather making.

The other prominent FTIR marker used was 1744/1456 cm^{-1} (esters/CH_2 deformation of lipids). 1720–1750 cm^{-1} (νC=O) assigned to carbonyl or carboxyl compounds and considered as the evidence of hydrolysable tannins. It represents the absorption of the C= O bonds of the ester groups which shows an increase in lipid oxidation with the increase in 1744 cm^{-1} band after the addition of fat liquors. Standard method has higher lipid oxidation compared to enzymatic method. Such peaks highlight the changes in spectra for identifying carboxylic acids in fat liquors. These chemicals are responsible to cause chromium III (CrIII) to oxidize towards CrVI. These fragments can generate reactive oxygen species (ROS), resulting from lipid oxidation. The defined ratios indicate higher protein rearrangement and modifications using the enzymatic processing method, by opening-up of the fiber network without damaging the fibrous collagen to maintain collagen fibril strength. Therefore, Raman and FTIR ratiometric analysis supported the hypothesis that leather processing using enzymes can be considered as an effective and reliable method rather than using harsh chemicals toxic to human health and environment.

Alterations in collagen network

Raman spectra show disappearance of amide I band during the retanning state, which allowed investigating FTIR spectra for amide, I region sensitive to changes in the protein secondary structure. The amide I band of final dry crust leather has been picked out to compare standard and enzymatic method and are magnified in Figure 4.

The amide region of proteins is overlapped by many underlying bands (Figure 4). Deconvolution of hidden peaks using curve-fitting was carried out on FTIR data for components in the 1600 – 1700 cm^{-1} region to investigate the spectral changes in collagens secondary

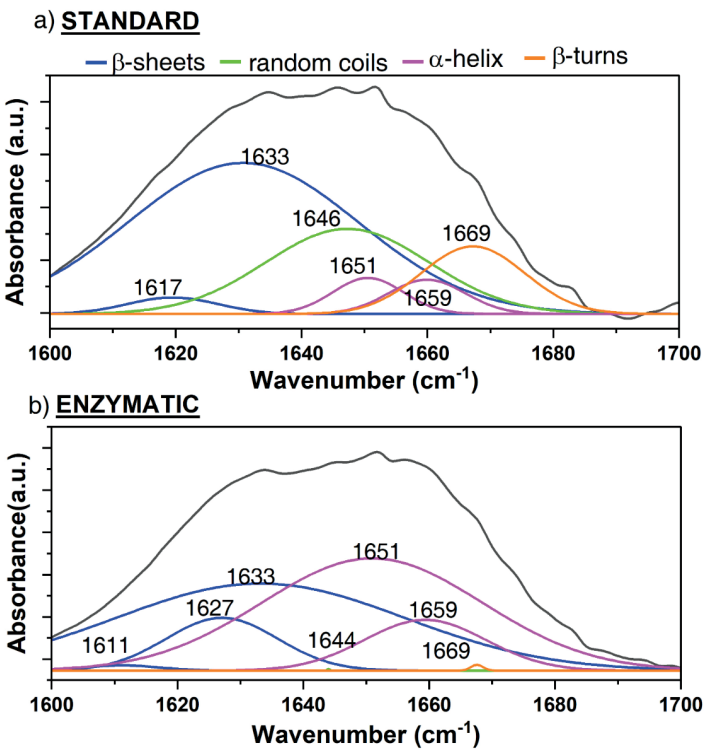

Figure 4 . Deconvoluted ATR-FTIR amide I region of standard and enzymatic method of leather making with the resolved underlying bands.

structures upon chemical treatment through different stages to final leather. Quantitative peak-fitting analysis of amide I band, as applied in this investigation, has been demonstrated helpful in studying the nature and the degree of protein conformation changes.[21,22]

Amide I band is the main contributor of protein in the skin.[28] Generally, proteins with α-helical content show an amide I band centered around 1650–1659 cm^{-1}, while those with predominantly β-sheets structures show the band at 1611-1635 cm^{-1}, a proportion of random coil or disordered structure attributes to proteins with an amide I band centered at 1642–1647 cm^{-1} and 1660~1700 cm^{-1} for β-turn.[29,30] The peak areas are calculated to identify the types of secondary structures in different samples, the component peaks, their assignment and percentage content of secondary structures is listed in Table I.

Due to variations in hydrogen bonding of polypeptide bond of α-helix, β-sheet, or disordered structures, there is a correlation between the FTIR band frequency and the proteins secondary structure. ATR-FTIR bands related to the peptide linkage (O––C–N– H) are designated as the amide bands. The conformationally sensitive amide I band is contributed mainly by the C=O stretching mode of the collagen. It can form a hydrogen bond with the NH groups of another chains peptide bond (interchain) or of the same chain at different sequence positions (intra-chain).[31] If the hydrogen bonds are formed between the C=O and NH on the same chain, the polypeptide backbone is in α-helix, and the amide I band occurs at 1655 cm^{-1}. Deconvolution of amide I band spectral analysis reveals that α-helical content was highest in the enzymatic method whereas β-sheet structure was more significant in standard method. Significant shape changes in the amides complex peak area can be observed because of the peak change occurring at both the α-helical conformation and the β-sheet conformation. α-helix is the most intense component in providing the stability of collagen structure at the finished leather stage, which supports the enzymatic method as an effective treatment for improving the leather quality.

Multivariate analysis

Multivariate analysis, a statistical analytical technique, is used to find the trend and pattern in spectral data present in a large data set. Principal Components Analysis (PCA) is an unsupervised multivariate method used to reduce the dimensionality of large dataset into smaller dataset without any assumptions (Mehta et al. 2020). The grouped datasets of similar variability are ordered in accordance with highest importance as PC1(principal component 1), PC2(principal component 2), PC3(principal component 3) and so on. A score plot is used to demonstrate the discrimination among different datasets observed in the form of distinct clusters of samples.[32] For each unique variable, PCA creates coefficients that describe how much that variable adds to the basic functions.

PCA analysis was performed on the Raman and FTIR spectra obtained after analyzing the different leather processing stages. The PCA score plot of Raman spectra (Figure 5a, b) demonstrate the clustering of samples from each stage, highlighting specific variations in the structural profile of hide. This could be due to the opening of fibers at the liming stage and cross-linking during chrome tanning. There is a clear distinction between pre-tanning stages separated

Table I

ATR-FTIR Amide I band assignments for protein secondary structures

Amide I (cm^{-1})	Assignment	Average absorbance (%)	
		STANDARD	Enzymatic
1650 to 1659	α-helix	6	24
1611 to 1635	β-sheet	28	16
1669	β-turn	12	1
1642 to 1657	random coils	15	2

along PC2 and post tanning stages of retanning, fat-liquored and dry crust along PC1 are clustered together. PCA score plot of the enzymatic method shows not much discrimination during the pre-tanning process, but later stages of fat liquored and dry crust leather are very well separated along the PC1. Similar behavior was observed with FTIR results (Figure 6a, b), showing not much separation within samples obtained from the enzymatic method. This suggests that the enzymatic treatment is significantly dehairing the hide keeping the collagen intact without much alteration in the collagen network. PCA results well supports the findings obtained after ratiometric analysis, which represents that collagen content is high in the enzymatic method compared to the standard method. Also, well corroborated with the deconvolution of peaks analysis where the enzymatic method has higher α-helical content, which provides collagen strength. The standard method has a lime presence that opens-up the collagen fiber network to affect the penetration of further chemical treatments and provide a clear distinction between stages, whereas this was not observed in the enzymatic method. Figure 5c, d shows

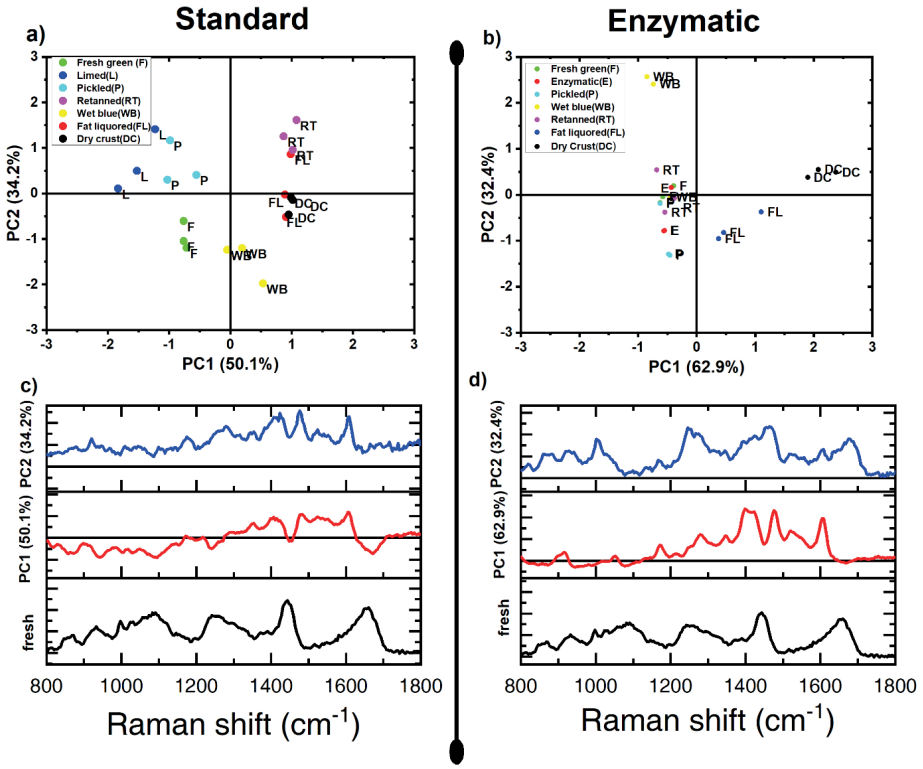

Figure 5. PCA score plots **(a, b)** and loadings plots **(c, d)** of Raman spectra of leather-making stages using standard and enzymatic method.

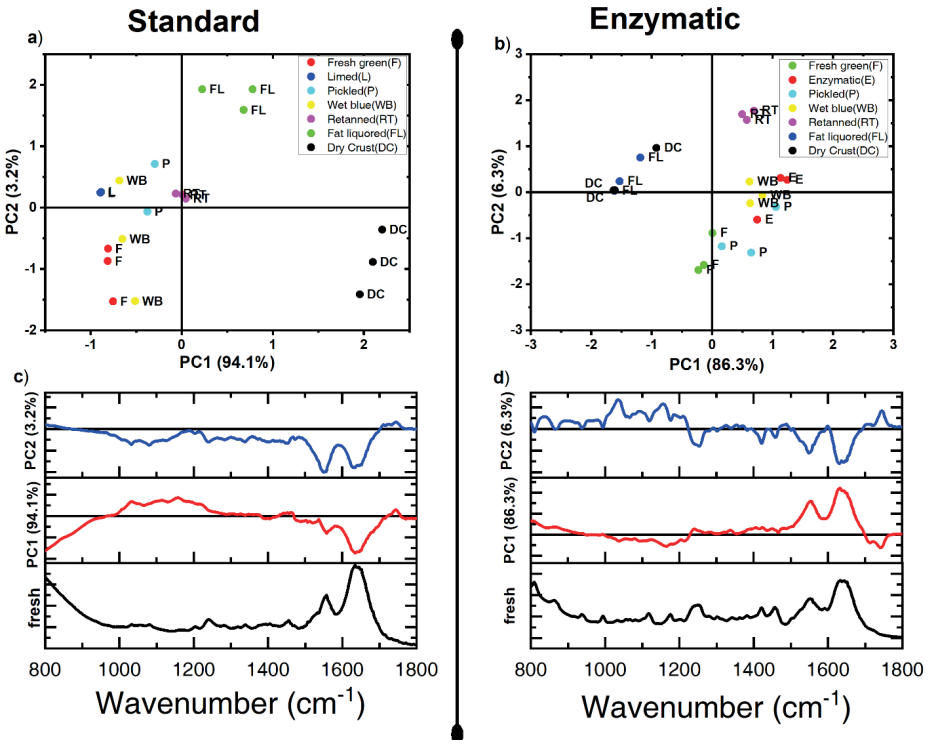

Figure 6. PCA score plots **(a, b)** and loadings plots **(c, d)** of ATR-FTIR spectra of leather-making stages using standard and enzymatic method.

the loading plots of the first two principal components of Raman spectra for standard and enzymatic methods. PC1 explains 50.1% of the standard and 62.9% for enzymatic data, while PC2 explains 34.2%, for standard and 32.4% for enzymatic method. The loading plots indicate which spectral bands contribute most to the variance described by the principal component. The fresh rawhide average spectrum is used as a reference for comparison of loadings. This gives an understanding of the origin of differences between the samples corresponding to spectral variations. The strong contribution in PC1 and PC2 is from the amide I stretch in the region of 1630 - 1690 cm^{-1}, mainly collagen proteins. Another contribution is from the 1456 cm^{-1} band, which is due to CH_3CH_2 deformation of proteins and lipids. PC2 also has spectral contributions from the amide I band, two significant peaks in the region of 1400-1500 cm^{-1}, which contributes to the deformation of proteins and lipids with C=N stretching from side-chain amino acids. Enzymatic method loading plot also has a similar contribution but with higher intensity with PC2 showing significant contribution around 1003 cm^{-1}, which is phenylalanine of collagen. ATR-FTIR loading plots (Figure 6c, d) for standard have a significant contribution from amide I band, but the enzymatic method has several minor contributions from collagen proteins in the lower wavenumber region.

Conclusion

The present work replaces the lime and sulfide with an alkaline proteolytic enzyme produced from bacteria *Vibrio metschnikovii* in leather processing. Raman and ATR-FTIR analysis was employed for comparing both chemical (lime and sulfide) and enzymatic methods. The results demonstrate that the extent of fiber opening using enzyme is better to conventionally lime and sulfide processed leathers by efficiently dehairing the process with retention of collagen protein. To the best of our knowledge, this is the first study done in depth using ratiometric and chemometric analysis to investigate the structural variations happening at every stage of leather process. Unsupervised multivariate analysis is well-corroborated with ratiometric results and provided an explicit discrimination in identifying subtle differences. Raman and ATR-FTIR, both vibrational spectroscopy techniques, can easily quantify the changes in biomolecules that impact leather quality, strength, and sustainability. This process also exhibits a significant reduction in pollution loads and eliminates the formation of H_2S gas and lime-bearing sludge, which are a significant concern for the environment. Therefore, these techniques can be used to monitor and evaluate any modification made to improve leather processing and paves the way to reduce environmental impact. In addition, results demonstrate better physicochemical properties of dried crusts with reduced pollution load, further confirms this enzymes potential for eco-friendly dehairing of animal skins in the leather industry.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author (Megha Mehta) on reasonable request.

Acknowledgements

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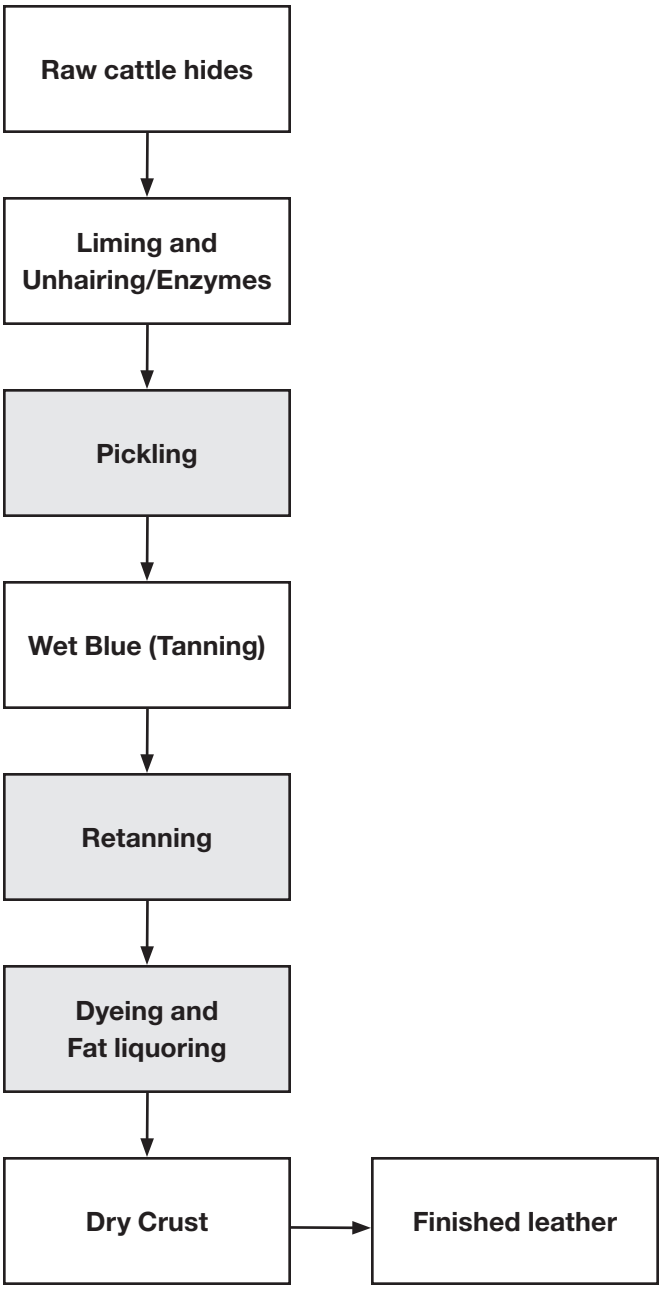

Figure S1. Main stages of leather processing

Table SI

Standard Leather Processing Protocol

Process	Amount (%)	Chemical	Temperature (°C)	Time (min)
Soaking	200	WATER	28	120
	0.5	Tetrapol LTN		
	0.05	TCMTB (30%)		overnight
Unhairing (Liming)	100	Water		
	1.5	Sodium hydrosulphide		
	2.5	Sodium sulphide		
	1.5	Lime		180 (overnight)
Washing	100	Water	28	
	100	Water		
	500	Water		
	0.5	Ammonium chloride		
Deliming	35	Water	30	
	0.5	Hydrogen peroxide		15
	2.5	Ammonium chloride		
	0.2	Tetrapol LTN		90
Pickled	0.04	Tanzyme		
	100	Water	35	20
	100	Water (Running wash)	20	20
	60	Water	20	
	8	Salt		10
	1.2	Sulphuric acide		
	10	Water	20	180
Tanning (Wet Blue)	6	Chrome powder		
	0.18	Fungicide		180 or O/N
Basification	0.3	MgO	40	O/N or until exhaustion
Neutralising	100	Water	35	60
	1	Tannigan Pak-N		
	1	Sodium formate		
	0.15	Sodium bicarbonate		overnight
Retanning	100	Water	35	10
	2	Tanicor		10
	3	Mimosa		15
	1	Dye		10
	50	Water		
Fat liquoring	1	Synthetic oil	50	
	1.5	Sulphirol		
	3.5	Polyol AK(Neatsfoot replacement)		45
Fixing	0.5	Formic acid (85%)	30	30
Dry Crust			15	

Table SII

Raman and ATR-FTIR observed bands for standard and enzymatic leather-making process.[15-17]

Peak position (cm^{-1})	Biochemical Assignments	Observable
833	Out of plane ring breathing tyrosine	Raman
859	Tyrosine/collagen	Raman
868	Proline	Raman
920-940	C–C stretching mode of proline and valine and protein backbone (α-helix conformation)	Raman & ATR-FTIR
1002 -1040	C-C phenylalanine of collagen	Raman & ATR-FTIR
1079-1099	S-O antisymmetric stretch; PO^{2-} stretch; C–C stretch of lipids,	Raman & ATR-FTIR
1053	C-O stretching, C-N stretching (protein)	Raman
1087-1099	Lipid, n(C-N), Phosphodioxy group in nucleic acids	Raman
1137	Fatty acids	Raman
1160-1179	C–H in-plane bending mode of tyrosine and phenylalanine (proteins); Cytosine and Guanine	Raman & ATR-FTIR
1203	Nucleic acids and phosphates	ATR-FTIR
1235-1300	Amide III of proteins (N-H bend in plane and C-N stretch) with significant mixing with CH_2 wagging vibration from the glycine backbone and proline sidechain	Raman & ATR-FTIR
1339-1345	CH_3 CH_2 side chain vibrations of collagen	Raman & ATR-FTIR
1370-1380	Lipids.	Raman & ATR-FTIR
1399	C=O symmetric stretch; fatty acids	Raman
1403-1457	CH_2/CH_3 deformation of proteins/lipids	Raman & ATR-FTIR
1476	C=N stretching	Raman
1521	-C=C- carotenoid	Raman
1532 -1575	Amide II (protein N–H bend in plane and C–N stretch) FTIR	Raman & ATR-FTIR
1600-1610	Phenylalanine, tyrosine, C=C (protein)Cytosine (NH2) protein assignment	Raman
1634-1695	Collagen Amide I (protein C=O stretch)	Raman & ATR-FTIR
1735-1755	CO–O–C ester carbonyl stretching vibration lipids	Raman & ATR-FTIR

Table SIII

Average Peak area ratios of Raman and ATR-FTIR spectra of standard and enzymatic method of leather processing.

Spectra	Average		
	Ratios	Standard	Enzymatic
RAMAN	920/1476	21.346	328.34
	1345/1259	90.65	187.34
	1605/1476	45.07	449.55
ATR-FTIR	1457/1645	39.266	43.74
	1744/1457	49.36	68.81