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Investigating the structural differences of hides, skins, and leather throughout the different processing stages

With an increasing demand for more sustainable leather processes, work to develop enzyme-alternatives to conventional process has become a significant area of interest.

In this work, Dr Mehta reports on the utilisation of complementary, non-invasive/ non-destructive spectroscopic techniques to investigate the effects of chemical/ enzymatic processing on the structural properties of hides and skins during conversion to leather. Both techniques are rapid, require minimal sample preparation, and have high specificity and sensitivity.

The enzyme utilised in this study was sourced from a bacterium isolated from a waste stream of a NZ leather facility, selected based on its strong proteolytic activity. While we can observe the bulk effects of enzyme and chemical processing in the drum until now very little information about the changes to hide's structure has been reported on.

I would like to thank the selection committee of the 2020 IULTCS/IUR Young Leather Scientist Grant for giving Dr Mehta the opportunity to carry out this important study. This investigation has revealed some significant findings.

Firstly, as far as we are aware, this is the first in-depth study utilising ratiometric and chemometric analysis to investigate the structural variations at each major process stage (raw, limed/enzymatically treated, pickled, chrome tanned, retanned, fatliquored and after drying) of the leather process. These powerful techniques have been demonstrated to readily quantify the changes that occur during processing which impact on leather quality and strength.

Secondly, utilising the chosen enzyme in this study, we have shown there is the potential for greater fibre opening-up during enzyme processes than conventionally liming, with retention of more of the collagen protein and less alteration to the hide structure.

Yours sincerely

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2020 IULTCS/IUR Young Leather Scientist Grant

Investigating the structural differences of hides, skins, and leather throughout the different processing stages

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1. 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. Each chemical treatment alters the composition of the original skin either by initially extracting the components or adding cross-linking agents in the later stages of processing to ensure stability and strength to leather. It brings changes in the chemistry of collagen, which is well known, but very little information is available about analysing these processed effects on the collagen structure.

The "fresh green" is the skin acquired after expulsion from the carcass. Skins are regularly salted to safeguard the skin prior to tanning briefly, and it caused some drying out of the skins. The following stage of the leather making process is to rehydrate the skins by dousing and washing followed by a salt treatment using sodium sulfide ("liming") joined with suitable chemical or microbial enzymes ("bating"), which break-down and eliminate a portion of the nonfibrous proteins, glycosaminoglycans, and other unwanted components. It assists with opening up the leather structure, allowing better penetration of tanning chemicals in ensuing stages. After treating with alkaline chemicals, the skin is adjusted back to a lower pH and is treated with sulfuric acid and sodium chloride. The skin obtained after this stage is referred as "pickled". The pickled skin can be preserved for a year and two. A synthetic cross-linking agent is often added after pickling, which helps with the following chrome tanning stage. After the natural fats are taken out, pelts are tanned using chromium sulfate. After chromium tanning, the skin is classified as "wet blue." The colour and surface of the leather changes at this stage and will, in general, be excessively inflexible for most applications. Thus, there is a subsequent tanning stage using vegetable or synthetic tannins to make the final leather softer and fuller. After this tanning stage, the skin is classified as "retanned." At this stage, colours, fat liquors, and altered fats or oils are added to finish the leather's look 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 an offensive odour and create sludge with high alkalinity, and hazardous to the environment[3]. These chemicals get accumulated in the tannery and pose a disposal problem. The leather industry waste generally leads to severe environmental pollution and health hazards[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 utilised 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 are 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 characterise the samples obtained at each stage of leather making process. The aim was to analyse 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 rapid, require minimal sample preparation, and have high specificity and sensitivity[10]. Raman spectroscopy has the advantage of an extremely weak water signal so negligible interference from water present in biological samples[11], without causing any damage to the sample[12] and allowing in-situ detection using optical microscopes. If there is a decrease in the water signal, then the advantage of IR is its sensitivity to vibrations associated with the amide bonds in proteins. The secondary structures of proteins impact the shape of the amide bands, and IR spectroscopy gives valuable data about protein structure. We have used an ATR-FTIR spectrometer using a curve fitting method to analyse 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 the discrimination according to biochemical components in samples.

2. EXPERIMENTAL

2.1. Materials

New Zealand Leather and Shoe Research Association (LASRA) obtained raw cattle hide for processing inhouse. 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 S1). 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 analysed for FTIR scans.

2.2. 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 analysed 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 20,000g 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 a cowhide sample (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 S1), sectioned, and prepared for Raman and ATR-FTIR analysis.

2.3. Data acquisition and spectral processing

The samples were analysed using a custom-built Raman microscope based on an inverted IX71 Olympus microscope A. 532 nm excitation laser (with 10 mW laser power) was focused onto the sample with a spot size diameter of 1-2 µm using 40 × magnification and 0.65 NA objective. A Raman edge filter (12^o incident angle) (Iridian Spectral Technologies, Ontario, Canada) directed the excitation into the sample and rejected the Rayleigh scattered light. An additional Raman edge filter (normal incidence) was used to remove any residual Rayleigh scattering immediately before entering the spectrometer. The Raman scattered light was focused onto a 50-micron entrance slit of a Teledyne-Princeton Instruments FERGIE spectrometer [17,26].

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). In total, 30 spectra were recorded for each leather-making stage. A Thermo Scientific™ iD5 Nicolet™ iS™5 Attenuated Total Reflectance - Fourier Transform InfraRed (ATR-FTIR) spectrometer was used to collect ATR-FTIR spectra from the same samples. Spectra were recorded by attenuated total reflection (ATR) on a diamond crystal and 16 scans were collected from each of the triplicate samples[17].

The collected spectra were preprocessed with an algorithm written using the SciKit Learn package[13] in Python 3.7. Baseline correction, background subtraction and average spectra were obtained using the python algorithm. The spectral data was smoothed using the five-point Savitzky-Golay smoothing function to smooth out spectral noise and normalization was done by dividing each point by the norm of the whole spectrum using Origin 2021b. Curve-fitting by sums-of-Gaussians was used to determine band areas, which were subsequently used to calculate area ratios of the peaks of interest[17,26]. The most pronounced changes observed in the amide I region was shown with Gaussian overlapping band by deconvoluting the 1646 cm^{-1} (amide I) band. Principal component analysis (PCA) was applied on the preprocessed Raman and FTIR spectra to extract the significant variation and separation of the samples.

3. RESULTS AND DISCUSSION

Lime serves to open-up the fibre 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 analyse the structural variations happening throughout the leather-making stages due to change in initial pre-tanning method and monitor the effect on the grain quality using Raman and ATR-FTIR spectroscopy.

3.1. 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 (Fig. 1). The bands were assigned based on already published literature and listed in detail in Table 1[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. 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.

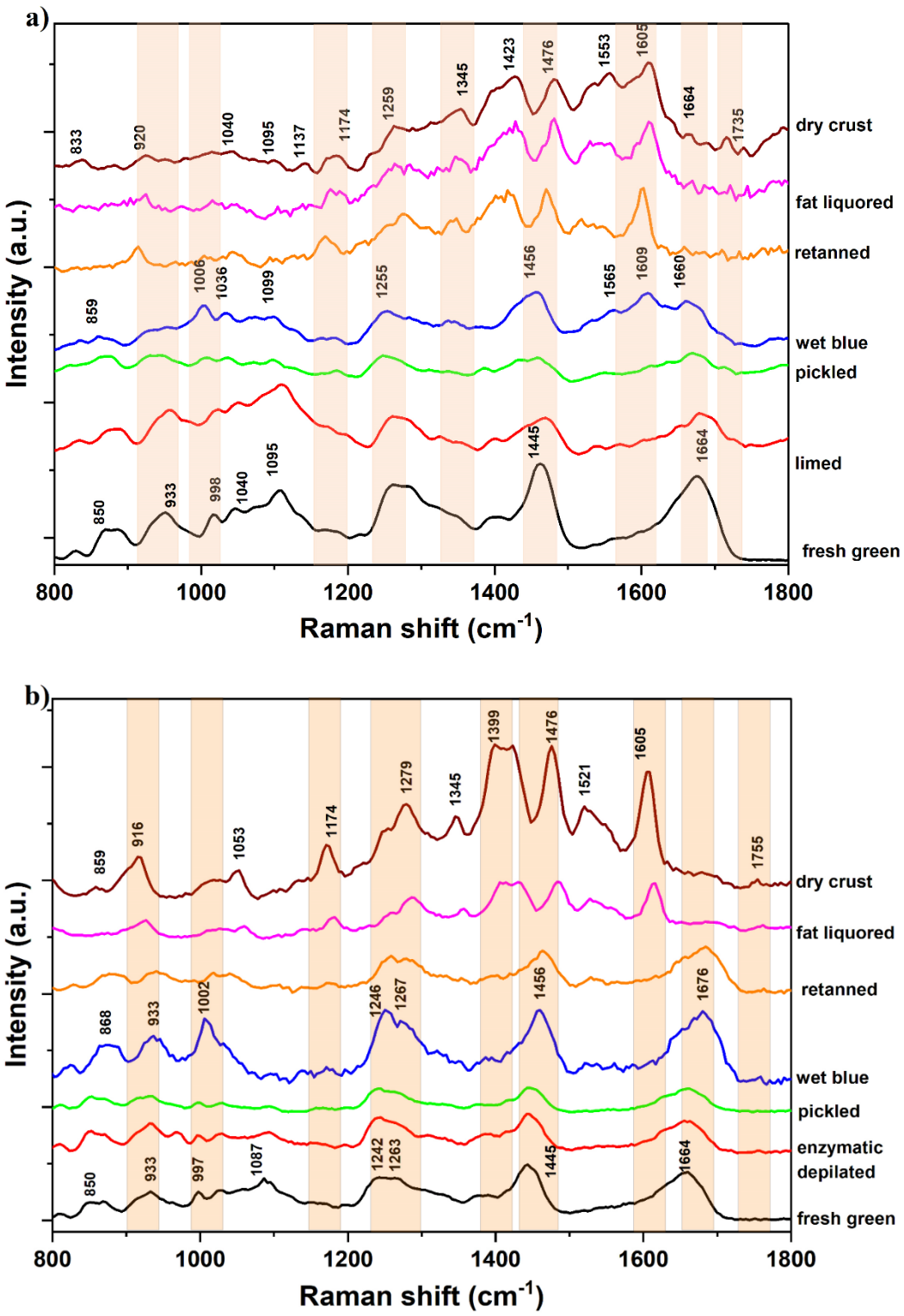

Fig 1. Raman spectra of different stages of leather-making using a) standard and b) enzymatic method.

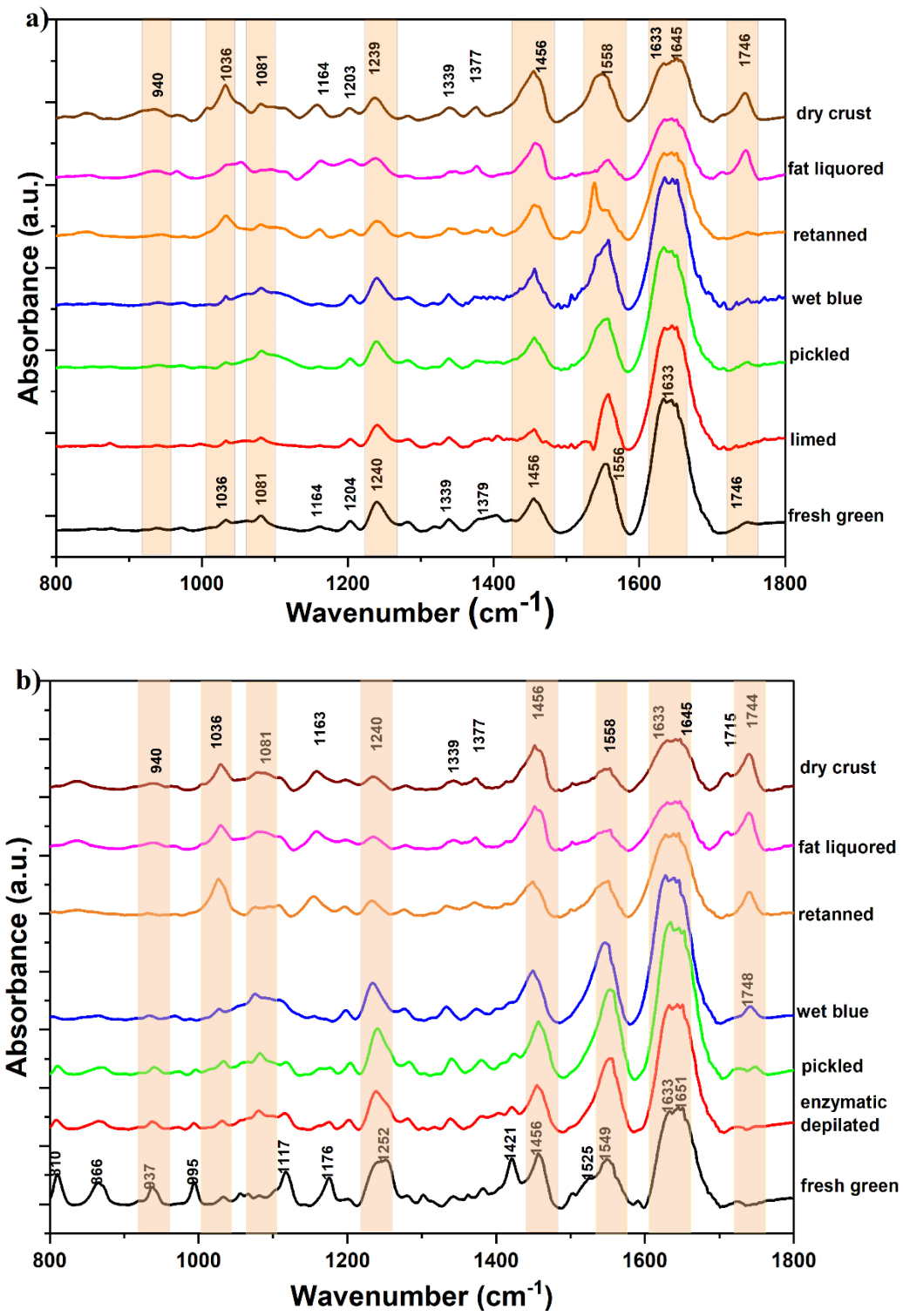

Fig 2. ATR-FTIR spectra of different stages of leather-making using a) standard and b) enzymatic method.

Table 1. 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

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 observes 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 with addition of chemicals at every stage to remove non-collagenous protein, provide strength to leather, and alters skin's 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 fibres

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 non-appearance of the amide I band in Raman spectra at final dry crust stage of standard and enzymatic method correlates with the presence of a 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} (Fig. 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, is 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 treatment 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 the stability of collagen structure, which was regained after wet blue stage with addition of few cross-links. By visual inspection, it was found that there are variations in the collagen region (1002-1680 cm^{-1}). A careful assessment of the spectra demonstrated shifting of few peaks due to the intricacy of biochemical components in leather samples which may occur due to alterations in secondary structures – α helix, β sheet, random coils or immature cross links[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 not 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, which includes band intensities, area ratios and intensity ratio calculations for the interpretation of spectra[25,26]. This ratiometric analysis was carried out for qualitative classification to reveal peak ratios which demonstrate spectral variations, quantified and represented as bar plots in Fig. 3. For quantification, area under the peaks was considered. Ratiometric analysis can resolve variations due to sample thickness and morphology, background scattering fluctuations 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].

3.2. 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 Raman signals at 920/1476 cm^{-1} represent the ratio of C-C stretch of proline and CH_2 deformation of proteins or lipids and show a significant increase in enzymatic method due to 15 times increase in proline content with a decrease in deformation of proteins and lipids compared to standard method.

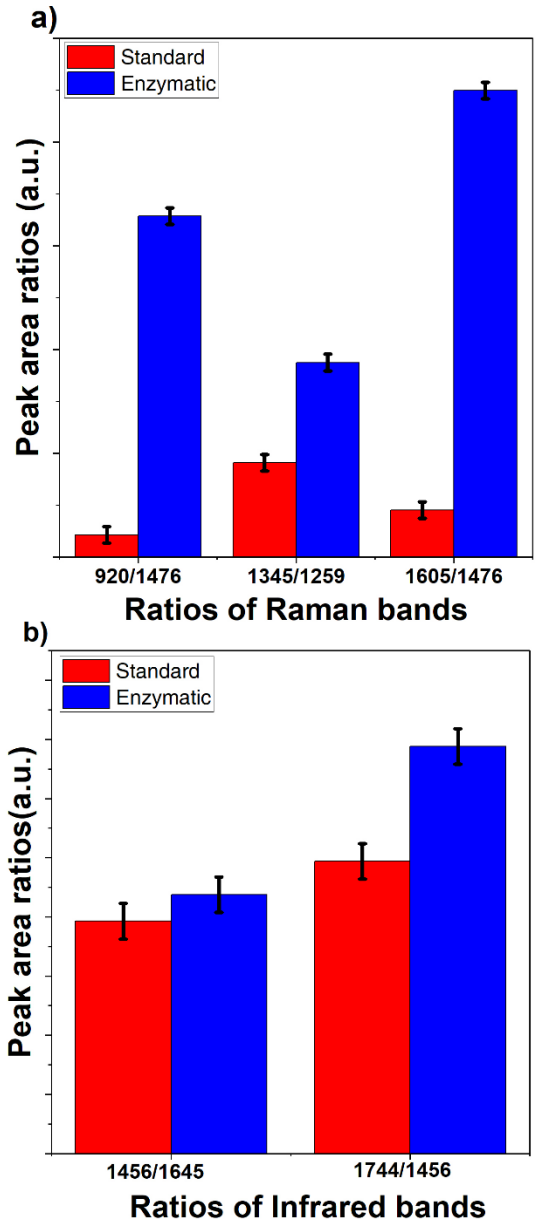

Fig 3. Peak area ratios of a) Raman and b) ATR-FTIR bands for standard and enzymatic method of leather making.

1345/1259 cm^{-1}, peak area ratio of CH_3CH_2 twisting of proteins and nucleic acids and amide III, increase with the increase in alterations of proteins and nucleic acid after complex formation with chromium during tanning stage. This leads to the more secondary structure at amide III band with a decrease in the peak area of the specific Raman band at 1259 cm^{-1}. Another peak area ratio of 1605/1476 cm^{-1} was considered to investigate the collagen protein with CH_2 deformation of proteins and lipids. The collagen protein is more significant in enzymatic method compared to standard with methyl group vibration mode of collagen. From FTIR active bands, 1456/1655 cm^{-1} (lipids/amide I proteins) peak area ratio of these bands is calculated yielding lipid to protein content information and the enzymatic method showed a marginal increase than the standard method. The other prominent FTIR marker used was 1744/1456 cm^{-1} (esters/CH_2 deformation of lipids), which shows an increase in lipid oxidation with the increase in 1744 cm^{-1} band after the addition of fat liquors. Enzymatic has higher lipid oxidation compared to standard method with the appearance of shoulder band at 1715 cm^{-1}. Therefore, Raman

and FTIR ratiometric analysis supported the hypothesis that leather processing using enzymes can be considered an effective and reliable method than harsh chemicals toxic to human health and the environment.

3.3. 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 Fig. 4.

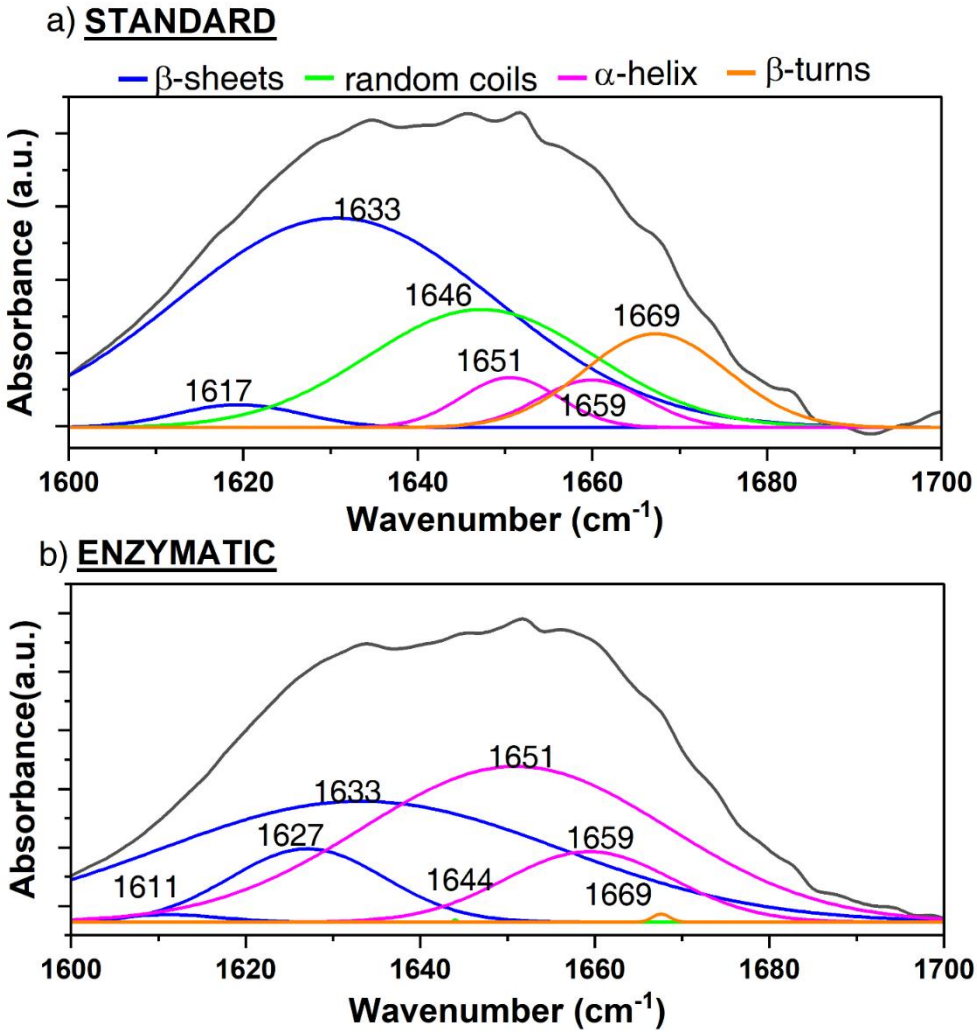

Fig 4 . Deconvoluted ATR-FTIR amide I region of standard and enzymatic method of leather making with the resolved underlying bands.

The amide region of proteins is overlapped by many underlying bands (Fig. 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 collagen's secondary 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 centred 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 2.

Table 2. 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

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 protein's 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 peptide linkage. It can form a hydrogen bond with the NH groups of another chain's 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 amide's 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.

3.4. Multivariate analysis

Multivariate analysis, statistical analytical technique, is used to find the trend and pattern in spectral data present in a large data set. An unsupervised method, Principal Components Analysis (PCA), is used to classify the data set without any assumptions of the number of classes (Mehta et al. 2020). The classes are determined by changing the dataset to a new description using variables (principal components) that maximise the separation between samples. A scores plot shows the samples plotted using the principal components. If distinct clusters of samples are observed in the scores plot, then classes exist in the dataset [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 analysing the different leather processing stages. The PCA score plot of Raman spectra (Fig. 5a, b) demonstrates the clustering of samples from each stage, highlighting specific variations in the structural profile of hide. This could be due to the opening of fibre at the liming stage and cross-linking during chrome tanning. There is a clear distinction between pre-tanning

stages separated 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 behaviour was observed with FTIR results (Fig. 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 fibre 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. Fig. 5c, d shows 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 (Fig. 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.

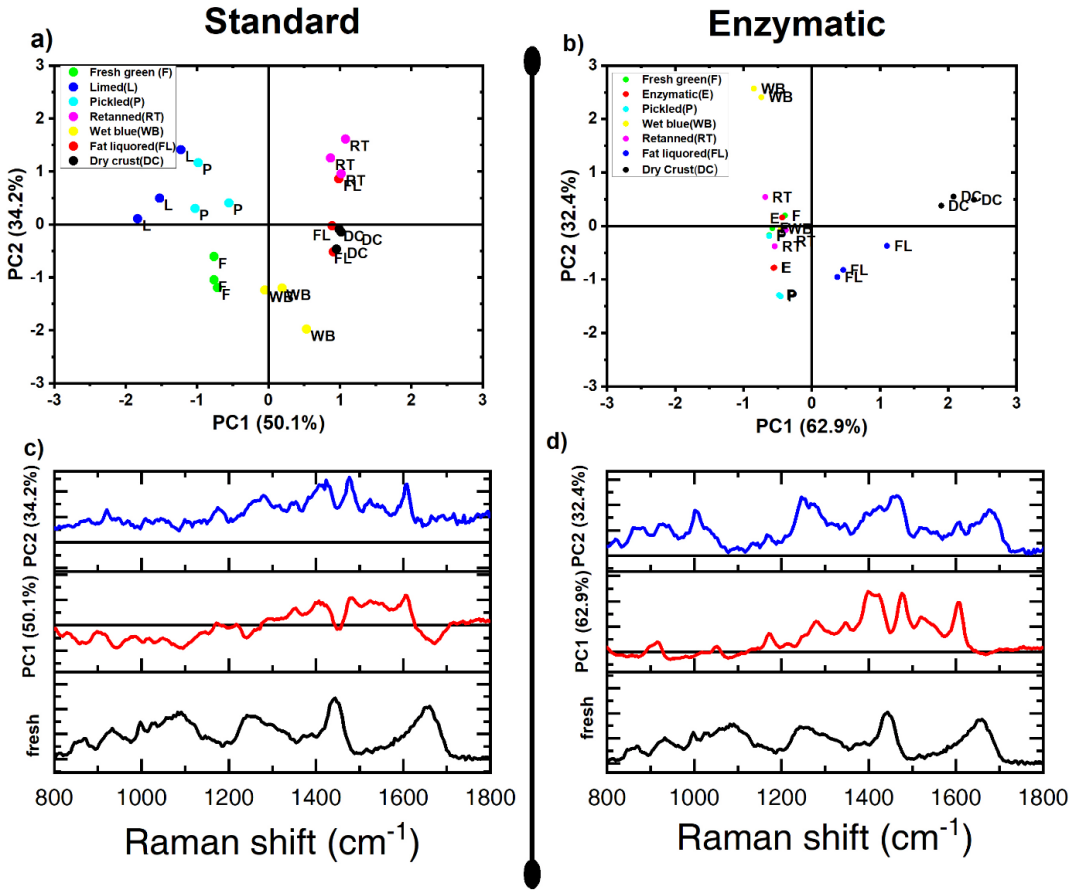

Fig 5. PCA score plots (a, b) and loadings plots (c, d) of Raman spectra of leather-making stages using standard and enzymatic method.

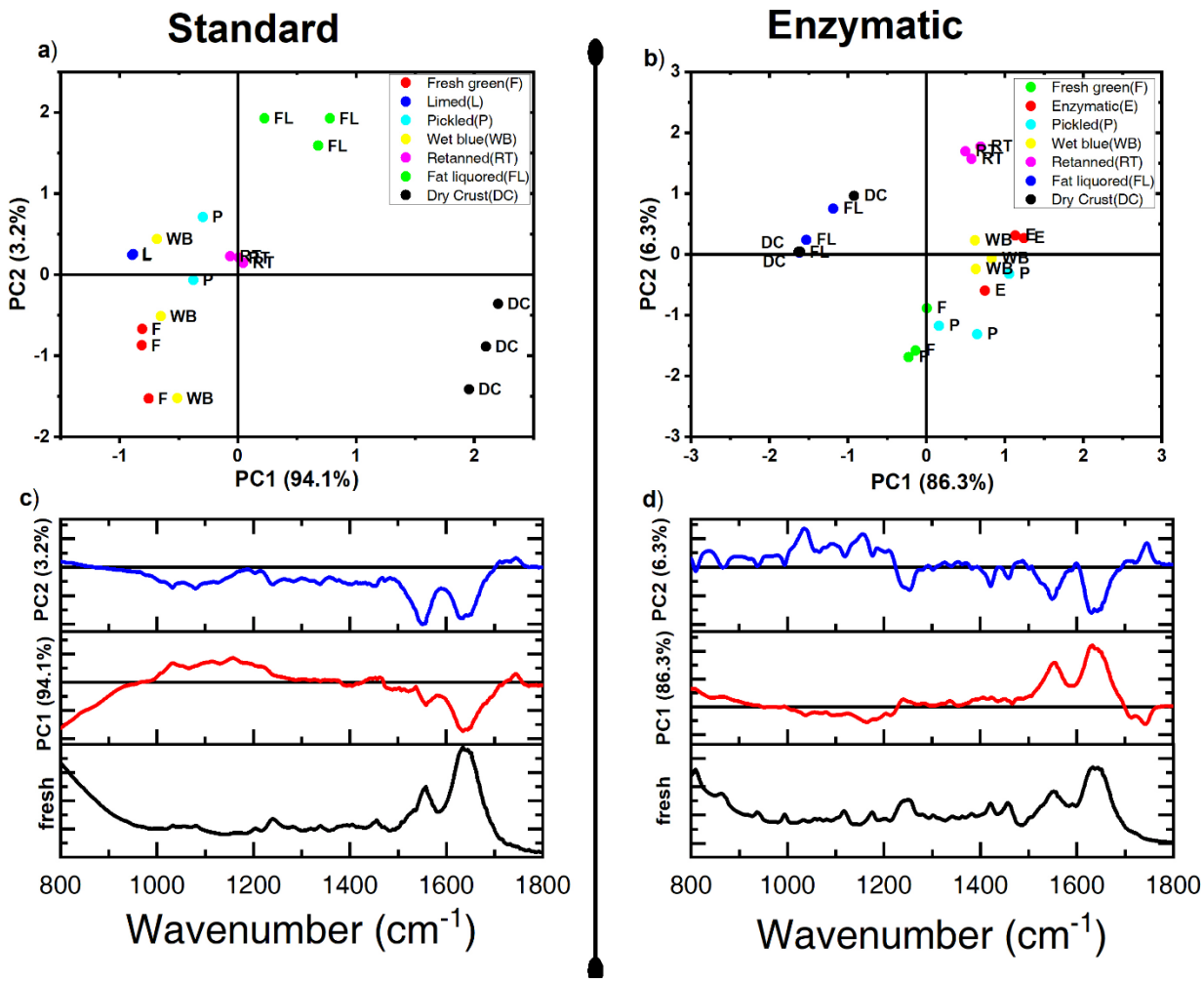

Fig 6. PCA score plots (a, b) and loadings plots(c, d) of ATR-FTIR spectra of leather-making stages using standard and enzymatic method.

4. 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 fibre 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. 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 enzyme's potential for eco-friendly dehairing of animal skins in the leather industry.

Acknowledgements

I would like to thank the IULTCS/IUR for funding the research work through the Young Leather Scientist Grant 2019.

REFERENCES

1. Ramasami, T. 1999, 'Beam house and tanning operations: process chemistry revisited', *J. Soc. Leather Tech. Chem.*, vol. 83, pp. 39-45.
2. Sizeland, K.H., Edmonds, R.L., Basil-Jones, M.M., Kirby, N., Hawley, A., Mudie, S. & Haverkamp, R.G. 2015, 'Changes to collagen structure during leather processing', *J. Agric. Food Chem*., vol. 63, no. 9, pp. 2499-505.
3. Zambare, V., Nilegaonkar, S. & Kanekar, P. 2007, 'Production of an alkaline protease by Bacillus cereus MCM B-326 and its application as a dehairing agent', *World J. Microbiol. Biotechnol.*, vol. 23, no. 11, pp. 1569-74.
4. Malathi, S. & Dhar, S. 1987, 'Production of extracellular protease by an Aspergillus flavus isolate and its application in the depilation of skins', *Leather sci*, vol. 34, pp. 67-76.
5. Aly A, Moustafa A, Hebeish A (2004) Bio-technological treatment of cellulosic textiles *J. Clean. Prod.* 12:697-705
6. Zhu, Y., Huang, W. & Ni, J. 2010, 'A promising clean process for production of diosgenin from Dioscorea zingiberensis CH Wright', *J. of Cleaner Prod.*, vol. 18, no. 3, pp. 242-7.
7. Mahmoodi, N.M., Arami, M., Mazaheri, F. & Rahimi, S. 2010, 'Degradation of sericin (degumming) of Persian silk by ultrasound and enzymes as a cleaner and environmentally friendly process', *J.of Clean. Prod.*, vol. 18, no. 2, pp. 146-51.
8. Valeika, V., Beleška, K., Valeikienė, V. & Kolodzeiskis, V. 2009, 'An approach to cleaner production: from hair burning to hair saving using a lime-free unhairing system', *J.of Clean. Prod.*, vol. 17, no. 2, pp. 214-21.
9. Ranjithkumar, A., Durga, J., Ramesh, R., Rose, C. & Muralidharan, C. 2017, 'Cleaner processing: a sulphide—free approach for depilation of skins', *Environ. Sci. Pollut. Res.*, vol. 24, no. 1, pp. 180-8.
10. Larkin, P. 2017, *Infrared and Raman spectroscopy: principles and spectral interpretation*, Elsevier.
11. Chen, Y., Dai, J., Zhou, X., Liu, Y., Zhang, W. & Peng, G. 2014, 'Raman spectroscopy analysis of the biochemical characteristics of molecules associated with the malignant transformation of gastric mucosa', *PLoS One*, vol. 9, no. 4, p. e93906.
12. Suci, P.A., Vrany, J.D. & Mittelman, M.W. 1998, 'Investigation of interactions between antimicrobial agents and bacterial biofilms using attenuated total reflection Fourier transform infrared spectroscopy', *Biomaterials*, vol. 19, no. 4-5, pp. 327-39.
13. Buitinck, L., Louppe, G., Blondel, M., Pedregosa, F., Mueller, A., Grisel, O., Niculae, V., Prettenhofer, P., Gramfort, A. & Grobler, J. 2013, 'API design for machine learning software: experiences from the scikit-learn project', *arXiv preprint arXiv:1309.0238*.
14. Bhavan, S., Rao, J.R. & Nair, B.U. 2008, 'A potential new commercial method for processing leather to reduce environmental impact', *Environ. Sci. Pollut. Res -International*, vol. 15, no. 4, pp. 293-5.
15. Kumar, S., Verma, T., Mukherjee, R., Ariese, F., Somasundaram, K. & Umapathy, S. 2016, 'Raman and infra-red microspectroscopy: towards quantitative evaluation for clinical research by ratiometric analysis', *Chem. Soc. Rev.*, vol. 45, no. 7, pp. 1879-900.
16. Eberhardt, K., Matthäus, C., Marthandan, S., Diekmann, S. & Popp, J. 2018, 'Raman and infrared spectroscopy reveal that proliferating and quiescent human fibroblast cells age by biochemically similar but not identical processes', *PloS one*, vol. 13, no. 12, p. e0207380.
17. Mehta, M., Naffa, R., Maidment, C., Holmes, G. & Waterland, M. 2020, 'Raman and ATR-FTIR spectroscopy towards classification of wet blue bovine leather using ratiometric and chemometric analysis', *Journal of Leather Science and Engineering*, vol. 2, no. 1, p. 3.
18. Movasaghi, Z., Rehman, S. & Rehman, I.U. 2007, 'Raman spectroscopy of biological tissues', *Appl. Spectrosc. Rev.*, vol. 42, no. 5, pp. 493-541.
19. Valeika, V., Beleska, K. & Sirvaityte, J. 2012, 'Alkali-free method of hide preparation for tanning', *Brazilian Journal of Chemical Engineering*, vol. 29, no. 2, pp. 315-23.

20. Kurouski, D., Postiglione, T., Deckert-Gaudig, T., Deckert, V. & Lednev, I.K. 2013, 'Amide I vibrational mode suppression in surface (SERS) and tip (TERS) enhanced Raman spectra of protein specimens', *Analyst*, vol. 138, no. 6, pp. 1665-73.
21. Susi, H. & Byler, D.M. 1983, 'Protein structure by Fourier transform infrared spectroscopy: second derivative spectra', *Biochem. Biophys. Res. Commun.*, vol. 115, no. 1, pp. 391-7.
22. Jiang, Y., Li, C., Nguyen, X., Muzammil, S., Towers, E., Gabrielson, J. & Narhi, L. 2011, 'Qualification of FTIR spectroscopic method for protein secondary structural analysis', *J. Pharm. Sci.*, vol. 100, no. 11, pp. 4631-41.
23. Khalid, M., Bora, T., Al Ghaithi, A., Thukral, S. & Dutta, J. 2018, 'Raman spectroscopy detects changes in bone mineral quality and collagen cross-linkage in staphylococcus infected human bone', *Scientific reports*, vol. 8, no. 1, pp. 1-9.
24. Payne, K. & Veis, A. 1988, 'Fourier transform IR spectroscopy of collagen and gelatin solutions: deconvolution of the amide I band for conformational studies', *Biopolymers: Original Research on Biomolecules*, vol. 27, no. 11, pp. 1749-60.
25. Jamieson, L.E., Li, A., Faulds, K. & Graham, D. 2018, 'Ratiometric analysis using Raman spectroscopy as a powerful predictor of structural properties of fatty acids', *R. Soc. OpenSci.*, vol. 5, no. 12, p. 181483.
26. Mehta, M., Liu, Y., Waterland, M. & Holmes, G. 2020, 'Characterization of the Degradation of Sheepskin by Monitoring Cytochrome c of Bacteria by Raman Spectroscopy', *Anal. Lett.*, pp. 1-18.
27. Balan, V., Mihai, C.-T., Cojocaru, F.-D., Uritu, C.-M., Dodi, G., Botezat, D. & Gardikiotis, I. 2019, 'Vibrational spectroscopy fingerprinting in medicine: from molecular to clinical practice', *Materials*, vol. 12, no. 18, p. 2884.
28. Fratzl, P. 2008, 'Collagen: structure and mechanics, an introduction', *Collagen*, Springer, pp. 1-13.
29. Surewicz, W.K. & Mantsch, H.H. 1988, 'New insight into protein secondary structure from resolution-enhanced infrared spectra', Biochim. Biophys. Acta*(BBA)-Protein Structure and Molecular Enzymology*, vol. 952, pp. 115-30.
30. Wei, W., Hu, W., Zhang, X., Y., Zhang, F.-P., Sun, S.-Q., Liu, Y. & Xu, C.-H. 2018, 'Analysis of protein structure changes and quality regulation of surimi during gelation based on infrared spectroscopy and microscopic imaging', *Scientific reports*, vol. 8, no. 1, pp. 1-8.
31. Gullekson, C., Lucas, L., Hewitt, K. & Kreplak, L. 2011, 'Surface-sensitive Raman spectroscopy of collagen I fibrils', *Biophys. J.*, vol. 100, no. 7, pp. 1837-45.
32. Das, G., Gentile, F., Coluccio, M., Perri, A., Nicastri, A., Mecarini, F., Cojoc, G., Candeloro, P., Liberale, C. & De Angelis, F. 2011, 'Principal component analysis based methodology to distinguish protein SERS spectra', *J. Mol. Struct.*, vol. 993, no. 1-3, pp. 500-5.