

NON-INVASIVE CHARACTERISATION OF COLLAGEN-BASED MATERIALS BY NMR-MOUSE AND ATR-FTIR

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Collagen water interactions and its secondary structure in various collagen based-materials were non-invasively investigated by NMR-MOUSE and ATR-FTIR. NMR-MOUSE measurements showed that spin-lattice relaxation time T1 can differentiate among gelatin, parchment and vegetable leather, being indicative of the degree of ordering of water in collagen materials. The short spin-spin relaxation time T2eff-short also discriminates among leather, parchment and gelatine but does not depend on the tannin type or collagen species. The long spin-spin relaxation time T2eff-long clearly differentiates between parchment (lower values) and leather (higher values) as well as between bovine and fish gelatin. ATR-FTIR measurements have shown that the relative positions and intensities of the amide bands are indicative of the interferences in hydrogen binding caused by the various fabrication processes as well as of the collagen species.

Keywords: collagen, vegetable tanned leather, parchment, gelatin, hydrogen bonding, NMR-MOUSE, ATR-FTIR

1. Introduction

Mankind has used leather and parchment since antiquities. Parchment is a biomaterial which was mainly used as support for writing, but also for making book bindings, musical instruments, furniture accessories etc. It is obtained by processing the skins of mammals, particularly calf, goat and sheep, by scraping,

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liming and drying under tension [1]. Depending on storage conditions, parchment has proved great longevity. Some examples are the oldest parchments dating back to the 2nd century B.C. such as the scrolls of Khirbet Qumran caves (Dead Sea Scrolls), those found in Avroman (Kurdistan) and Dura-Europos (Syria) [2-4].

Vegetable leather has been used for manufacturing different objects like shoes, garments, book bindings, tools, weapons, and industrial accessories. Leather processing involves the use of tannins which permanently alter the chemical structure of collagen and impart a higher thermal stability by comparison with parchment.

Gelatin, as a product, is traditionally obtained from animal bones and skins, especially of pig and calf. It has been mainly used for adhesives fabrication, in photography and as additive for foods, drugs and cosmetics. On the other hand, sturgeon and rabbit gelatin has been used for restoration purposes. Recently, fish gelatin has received much attention [5-6] due to the growing demand in the food and biomaterials industries. Moreover, ethical and religious issues have stimulated the study of fish gelatin at the expense of the traditional pork and bovine gelatin.

Considering the uniqueness of the historical objects and artefacts as well as their specific requirement for *in situ* non-destructive and non-invasive methods, we have investigated the capability of both NMR-MOUSE and ATR-FTIR to characterise and distinguish among gelatin, parchment and vegetable leather. While Attenuated Total Reflection-Infrared Spectroscopy (ATR-FTIR) is a consolidated method for the analysis of collagenous materials such as historical and archaeological materials [7-9] as well as skin [10] and gelatin [11-12], the NMR-MOUSE (Nuclear Magnetic Resonance - Mobile Universal Surface Explorer) technique has been only recently developed [13]. Since the mid 1990's, various application of the NMR-MOUSE in the field of cultural heritage [2,14-15] and industry [16-17] has been reported. Non-destructive and non-invasive methods of investigation such as NMR-MOUSE and ATR-FTIR are fundamental in evaluating the structural and physical-chemical changes related to ageing and deterioration in unique historical materials and artefacts. Only a few pilot studies used portable NMR instruments to assess the level of deterioration of collagen in historical parchments [2,14] and characterise the effectiveness of various tanning agents [18,19], while there is not yet any study on gelatin. Recently we have reported the effects of accelerated ageing [20] and gamma irradiation [21] on vegetable leather. This is the very first study that comparatively investigates the collagen-water interaction in gelatin, parchment and vegetable tanned leather.

2. Experimental

2.1. Materials

2.1.1. Gelatin

Bovine skin with a content of volatile matter of 71.2 % was finely minced and dispersed in water at a ratio of 1:1. After adjusting the pH to 5.5-6.0, the suspension was heated under continuous stirring and gelatin was extracted in the temperature range of 70 - 90 °C according to a patent pending technology set up at INCDTP-ICPI.

Before mincing, the fish skin with a volatile matter content of 68.9 % was repeatedly washed with cold water (5 - 10 °C), treated with 0.2N NaOH solution, and then neutralized with 0.1N acetic acid solution. The suspension was then heated at (60 ± 2) °C under continuous stirring and gelatin was extracted according to a patent pending technology set up at INCDTP-ICPI.

After the quantitative filtration, gelatin solutions were cooled at 4 °C for 16 - 18 hours. Gelatin with molecular weight of 40-50 kDa was collected with no deposits and dried in hot air flow. Gelatin films with a volatile matter content of 87.0 % were obtained.

2.1.2. Parchment

The calf parchment sample used in this study was selected from a series of parchment samples investigated within the EC project *Improved Damage Assessment of Parchments* IDAP EVK4-2001-00061 supplied by the Henk De Groot workshop (NL).

2.1.3. Leather

The vegetable leather was obtained at INCDTP-ICPI from calf hides using traditional tanning methods. Two types of tannins, e.g. chestnut-wood extract (hydrolysable tannin) and quebracho-wood extract (condensed tannin), were used.

2.2. NMR-MOUSE

The NMR measurements were carried out with a portable, single-sided NMR-MOUSE bar magnet (RWTH University). This is a small and compact device designed to perform non-invasive and non-destructive analyses [22]. The equipment was connected to a Bruker Minispec spectrometer operating at a ¹H resonance frequency of 20.05 MHz. The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [23-24] was used to measure the spin-spin relaxation time $T_{2\text{eff}}$. The CPMG curves (Fig. 1) were best analyzed by a combination of two exponential functions according to the equation (1):

$$A(t) = A_{\text{short}} \exp\left(-\frac{t}{T_{2\text{eff_short}}}\right) + A_{\text{long}} \exp\left(-\frac{t}{T_{2\text{eff_long}}}\right) \quad (1)$$

where t is the time and A is the signal amplitude with $A_{\text{short}} + A_{\text{long}} = 100\%$. The spin-lattice relaxation times, T_1 , were measured with a saturation-recovery pulse sequence using a Hahn-echo with an echo time of 25 μs for detection. The analysis of the saturation-recovery data (Fig. 2) was best performed with the help of a single exponential function:

$$A(t) = A_{\text{equilibrium}} \left(1 - \exp \left(-\frac{t}{T_1} \right) \right) \quad (2)$$

where A has the same meaning as for equation (1), t is the saturation-recovery time and $A_{\text{equilibrium}}$ is the signal amplitude at saturation times much longer than T_1 .

All experiments were performed at room temperature. Moreover, both T_1 and $T_{2\text{eff}}$ were measured three times for each sample and the average values are reported with the standard errors.

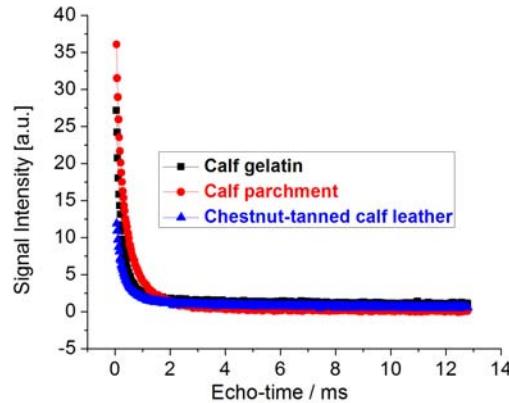


Fig. 1. Typical CPMG decay curves and the fit analysis to determine the $T_{2\text{eff}}$ spin-spin relaxation time for calf gelatin, calf parchment and chestnut-tanned calf leather.

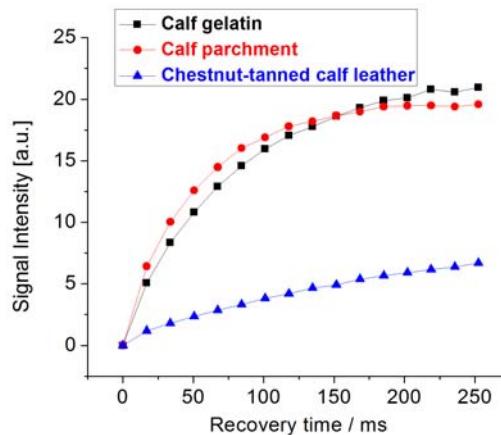


Fig. 2. Typical saturation-recovery curves and the fit analysis to determine the T_1 spin-lattice relaxation time for calf gelatin, calf parchment and chestnut-tanned calf leather.

2.3. ATR-FTIR

Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) measurements were performed with a portable Alpha (Bruker Optics) spectrometer equipped with a Platinum ATR module. During the experiments, 32 scans were co-added to achieve an acceptable signal-to-noise ratio, with wave number ranging from 4000 to 650 cm^{-1} . All the spectra were recorded at a resolution of 4 cm^{-1} and evaluated using Opus 7.0 software. Six measurements were averaged for each sample.

3. Results and discussion

3.1. Collagen-water interaction in gelatin, parchment and leather

The overall model of water hydration in collagenous tissues/materials involves the monomolecular layer in which the water molecules are H-bonded to the helical structures and several outer polymolecular layers which are considerably more mobile [25-26]. The investigated materials have different macromolecular structures, i.e. unmodified triple helix structure in parchment and chemically modified triple helix in leather through cross-links and gelatin through hydrolysis. The specific interaction between water and such structures has an important contribution to the stabilisation of the investigated materials. The structure of water surrounding the triple helix, the collagen-tannin matrix and the gelatin random coils is expected to be affected by the macromolecular structure. The values of spin-spin ($T_{2\text{eff}}$) and spin-lattice (T_1) relaxation times obtained for the investigated gelatins, parchment and leathers and listed in Table 1 show significant differences for the three materials. T_1 values increase in the following sequence: collagen-condensed tannin complex < collagen-hydrolysed tannin complex < parchment < bovine gelatin < fish gelatin. This behaviour suggests that the degree of ordering of water in collagen materials decreases following the same sequence. We can state that T_1 can differentiate among gelatin, parchment and leather, the faster decay being observed for leather and the lowest one for gelatin. The T_1 value measured for parchment is similar to that previously obtained by Badea *et. al* [3] and Della Gatta *et. al* [27] using a NMR-MOUSE equipment for a number of new parchments manufactured from various animal hides. It is worth mentioning that the animal origin and the type of preparation have no influence on the T_1 values [27]. Della Gatta *et. al* [27] inferred a correlation between higher T_1 values and peptide chain hydrolysis. If dipolar relaxation is predominant, longer T_1 values can be correlated to a weaker collagen-water interaction. In fact, the hydrolytic fragmentation of fibres disrupts the regular assembly of water molecules around the triple helix and the amount of less tightly bound water increases. As a consequence, high T_1 values can characterize the hydrolytic cleavage of the collagen macromolecules. On the other hand, the higher T_1 value obtained for fish gelatine compared to that of bovine gelatin could be ascribed to

its lower concentrations of amino acids (proline and hydroxyproline) compared to mammalian gelatins [28] which stabilize the ordered conformation when gelatin forms a gel network. It is worth mentioning that the triple-helix structure of the gelatin gel, which is critical for the gel properties, is stabilized by steric restrictions. These restrictions are imposed by both the pyrrolidine rings of the Amino acids in addition to the hydrogen bonds formed between amino acid residues.

For the two leathers, the different T_1 values confirm that this relaxation parameter is sensitive to the tannin type (e.g. condensed or hydrolysable) within the collagen-tannin complex [20-21].

By comparison with parchment, the lower T_1 values found for leather could be explained by the presence of the collagen-tannin interactions which substitute part of the collagen-water interactions.

The transverse relaxation times $T_{2\text{eff}}$ of the investigated materials splits into two components: a short relaxation time in the range of (0.16 – 0.26) ms and a long relaxation time ranging between (0.72 – 2.27) ms. For leather, $T_{2\text{eff}}$ is similar to the findings from literature [18,21], while lower values were measured for gelatine and parchment. $T_{2\text{eff-short}}$ shows thus to be sensitive to the macromolecular structure, being discriminative for the three types of collagenous materials, but does not vary depending on the tannin type or collagen species. $T_{2\text{eff-long}}$ clearly differentiates between parchment (lower values) and leather (higher values). The dispersion of the $T_{2\text{eff-long}}$ values of bovine and fish gelatins might be due to the collagen species as mentioned above [28].

Table 1
Values of spin-lattice relaxation time T_1 , and spin-spin relaxation times $T_{2\text{eff_long}}$ and $T_{2\text{eff_short}}$ in gelatin, parchment and vegetable leather

Samples	T_1 / ms	$T_{2\text{eff_long}}$ / ms	$T_{2\text{eff_short}}$ / ms
Bovine gelatin GB1S	77.09 ± 0.88	1.29 ± 0.18	0.18 ± 0.01
Fish gelatin GPCS	84.59 ± 1.54	2.27 ± 0.14	0.19 ± 0.01
Parchment	50.03 ± 0.29	0.72 ± 0.04	0.16 ± 0.01
Leather - chestnut	24.60 ± 0.15	1.86 ± 0.12	0.24 ± 0.01
Leather - quebracho	19.50 ± 0.44	1.91 ± 0.01	0.26 ± 0.01

3.2 Molecular characterisation of gelatin, parchment and leather

Infrared (IR) spectroscopic analysis of protein structure involves the use of infrared radiation to assess vibrational modes arising from atoms within protein molecules and relating this to the primary, secondary, tertiary, and quaternary structure of the protein. Infrared spectral band frequency, band intensity, and band width can be used to determine protein structure and dynamics. The vibration of the protein amide bonds are the most widely used for protein structure analysis using infrared spectroscopy [29].

Amide I and amide II bands are the two major bands of the collagen infrared spectrum. The amide I band ($\sim 1650 \text{ cm}^{-1}$) is mainly associated with the C=O stretching vibration (70-85%) and is directly related to the backbone conformation. The amide I band position is determined by the backbone conformation and the hydrogen bonding pattern. Amide II ($\sim 1550 \text{ cm}^{-1}$) results from the N-H bending vibration (40-60%) and from the C-N stretching vibration (18-40%). This band is conformationally sensitive. Amide III ($\sim 1235 \text{ cm}^{-1}$) is a very complex band depending on the nature of side chains and hydrogen bonding, being therefore only of limited use for the extraction of structural information. Amide A (about 3300 cm^{-1}), with more than 95% due to the N-H stretching vibration, does not depend on the backbone conformation but is very sensitive to the strength of hydrogen bonds.

The assignment of IR bands of amide A, amide I and amide II for the investigated materials, e.g. bovine (GB1S) and fish (GPCS) gelatin, parchment and vegetable tanned leathers, is reported in Table 2. By comparing the spectrum of native collagen from calf skin (Sigma-Aldrich) with those of gelatin and parchment, a shift of the amide A, amide I and amide II bands to lower wavenumbers is observed (Fig. 3). We can attribute the amide I band shift to either an eventual disruption of hydrogen bonding or prevalence of weaker hydrogen bonding (or long-range interactions) [29-30]. In case of vegetable tanned leathers, due to the overlapping of the vibration bands of the tanning agents (chestnut and quebracho extracts), the positions and intensities of the collagen amide bands are disturbed. After separating the tannins signals that overlap in the collagen spectrum we observed that amide I and amide II bands are shifted to higher wavenumbers, e.g. from 1630 cm^{-1} to 1654 cm^{-1} and from 1521 cm^{-1} to 1545 cm^{-1} , respectively (Fig. 4). This could be attributed to the ability of collagen-tannin complex to form stronger hydrogen bonding (and generally, short range interactions) as indicated by the T_1 values, too.

Table 2
Assignment of IR bands of amide A, amide I and amide II for the investigated materials in accordance with type I collagen spectrum

Sample	Amide A / cm^{-1}	Amide I / cm^{-1}	Amide II / cm^{-1}
Native collagen	3314	1644	1553
Bovine gelatin GB1S	3290	1628	1539
Fish gelatin GPCS	3255	1632	1530
Parchment	3290	1631	1540
Leather - chestnut	3300	1654	1545
Leather - quebracho	3303	1654	1545

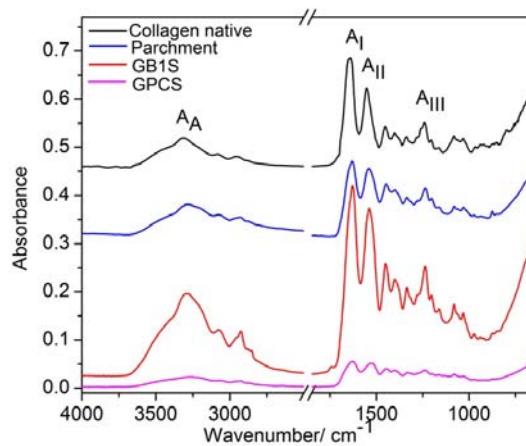


Fig. 3. ATR-FTIR spectra of parchment and gelatin compared to that of native collagen

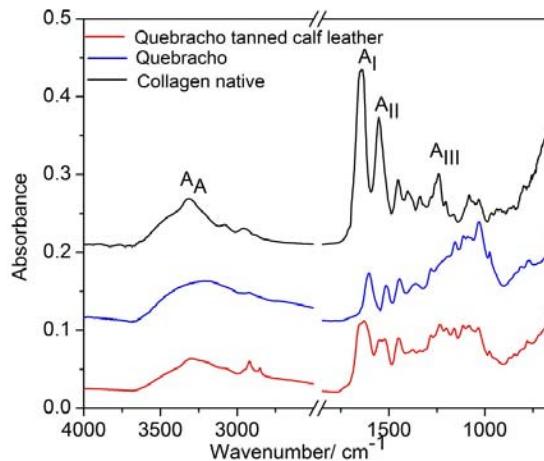


Fig. 4. ATR-FTIR spectra of quebracho-leather and quebracho extract compared to that of native collagen

It was previously reported that the relative positions and intensities of the amide I and amide II bands reflect the conformational changes of the collagen macromolecule induced by deterioration. For example, hydrolysis, which involves the peptide bonds breakdown, has been quantified by the A_I/A_{II} ratio, while the relative position ($\Delta\nu$) of amide I and amide II bands has been related to the gelatinisation process [3,31-32]. These parameters have been used for evaluating the conservation state of historical parchments. For new parchment and leather, we found that A_I/A_{II} ratio values are close to 1.1 – 1.2 and 1.25 - 1.30, respectively, and $\Delta\nu$ is close to 90 cm^{-1} . Moreover, the integrity of the collagen triple helix can be evaluated considering the peak absorbance ratio of amide III (1235 cm^{-1}) and 1450 cm^{-1} band corresponding to the stereochemistry of

pyrrolidine rings of proline and hydroxyproline residues [33], essential for the triple-helix conformation. It was shown that the collagen triple helix conformation is intact if this ratio is close to 1.0 while the ratio values for denatured collagen are around 0.5 [34].

The amide I and II bands relative intensities and positions for the investigated materials are listed in Table 2 together with the peak absorbance ratio of $1235\text{ cm}^{-1}/1450\text{ cm}^{-1}$ bands. These results show lower values of the $A_{\text{I}}/A_{\text{II}}$ ratio for collagen in gelatin, parchment and leather compared with native collagen indicating a structural destabilization that increases as follows: collagen in leather < collagen in parchment < gelatin. On the other hand Δv was significantly higher for fish gelatin and vegetable leather, while remaining unmodified for parchment (unmodified collagen). For leather, this could be indicative of the interference caused by tannins in the hydrogen bonding, while for fish gelatin it could be attributed to the lower content in amino acid residues. The A_{III}/A_{1450} ratio only slightly decreased for both gelatin and parchment indicating that the collagen triple helix is not destabilised. However, it was not possible to properly calculate this ratio for vegetable leather due to the disturbances caused by the overlapping of the tannin vibrational signals in the fingerprint area.

Table 3

Amide I and II bands relative intensities and positions as well as the peak absorbance ratio $A_{\text{III}}/1450\text{ cm}^{-1}$ for the investigated materials

Sample	$A_{\text{I}}/A_{\text{II}}$	Δv	A_{III}/A_{1450}
Native collagen	1.42 ± 0.01	91 ± 0.10	1.00 ± 0.01
Bovine gelatin GB1S	1.16 ± 0.01	89 ± 0.89	0.89 ± 0.01
Fish gelatin GPCS	1.12 ± 0.01	103 ± 1.86	0.81 ± 0.01
Parchment	1.18 ± 0.02	90 ± 1.66	0.80 ± 0.01
Leather - chestnut	1.23 ± 0.02	112 ± 0.56	-
Leather - quebracho	1.26 ± 0.06	109 ± 0.58	-

4. Conclusions

In this paper three types of collagen-based materials, i.e. gelatin, parchment and vegetable tanned leather, have been non-invasively characterised by NMR-MOUSE and ATR-FTIR. This preliminary study is the first investigation that comparatively analysis the effect of fabrication process on both the collagen-water interaction and collagen secondary structure in gelatin, parchment and vegetable tanned leather.

We have found that proton spin-lattice relaxation times T_1 values can differentiate between gelatin, parchment and leather, the shorter relaxation time being observed for leather (highest degree of ordering for water molecules) and the longest for gelatin (lowest degree of ordering for water molecule). T_1 relaxation time has also shown to be sensitive either to collagen species or tannin type. The spin-spin relaxation time short component $T_{2\text{eff-short}}$ values have proved

to be discriminative for the three types of collagenous materials, but are independent on the tannin type or collagen species. The spin-spin relaxation time long component $T_{2\text{eff-long}}$ has clearly differentiated between parchment (lower values) and leather (higher values) as well as between fish and bovine gelatine.

The ATR-FTIR investigation of gelatin, parchment and vegetable leather and the comparison with native collagen from calf skin has permitted us to evidence modifications of the collagen secondary structure due to very specific FTIR parameters such as A_I/A_{II} ratio, A_I and A_{II} relative position and A_{III}/A_{1450} ratio. These modifications have been partially related to the interferences in hydrogen binding induced by the fabrication processes (hydrolysis or tannin inclusion) and partially to collagen species (i.e. bovine or fish).

Both NMR-MOUSE and ATR-FTIR provides very effective tools for *in situ* characterisation of collagen-based materials without sampling. Furthermore, their sensitivity is essential for assisting the design of new collagen-based materials with innovative and smart functionalities. Additional investigations are ongoing in our lab.

Acknowledgments

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