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A new power tool for the evaluation of collagen type-I and chemical stabilisation agents; Part II: Mineral tanning agents

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1. Introduction

The future technologies in the tanning industry could be based on prediction of tanning agent efficiency based on simulation results of interaction between collagen model substrates and tanning agents.

Tannins have a long history of use in stabilizing collagen in leather industry [1,2] and are known to interact extensively and stabilise proteins [3,4]. The role of collagen modification during the tanning process has received an increased interest, especially after the postulation of the «link-lock» general mechanistic model [5].

Chemical cross-linking, electrostatic interactions, hydrophobic interactions, hydrogen bonds and water activity are factors which contribute to the mechanism of collagen stabilization. The stabilization of type I collagen using aldehydes, mineral tanning agents, and vegetable tannins, has been extensively studied [6].

Chrome tanning is the most efficient and common tanning method and results in the softest, most stretchable leather. Among all mineral tanning agents, four of them (Chromium (III), Aluminum (III), Titanium (IV) and Zirconium (IV)) play a significant role in the tanning modern industry [7].

Although Chromium (III) was named ‘‘perfect’’ tanning agent [8] in terms of versatility and leather performance, there are negative features which have to be taken into account: it is a limited natural resource, its safety record is cautionary and uncontrolled emission can have serious environmental impact.

In this paper, we describe the investigation of the interactions between selected mineral tanning agents, others than Chromium (III), and type I fibrillar collagen using a new bench tool, namely a model fibrillar substrate and a dedicated instrumental analytical regime in an attempt to enable the fast direct evaluation and validation of mechanistic model interpretations of tanning activity and, thereof, stir tailored reactive tanning agents accelerated and eco-sustainable development, as well as tanning and post-tanning reactions physical conditions optimization.

2. Materials and methods

2.1. Materials

Type I fibrillar collagen was obtained in gel state from bovine hides by in-house techniques developed at INCDTP – ICPI Division using the protocol that has been previously described [9]. As mineral tanning agents we used a typical Chromium (III) tanning agent and new tanning agents, obtained according with our technology, which are based on Titanium, Aluminum, Titanyl amonium sulphate, as well as combinations of Titanium-Zirconium and Titanium-Aluminum salts. Sodium hydroxide and phosphate buffer solution, PBS, (pH = 7.4) were of analytical grade. Collagenase type I, *C. histolyticum,* was purchased from Sigma-Aldrich (USA).

2.2. Collagen matrices obtaining

The collagen gel (Coll) with an initial concentration in collagen of 1.99% and pH 2.6 was adjusted at 1% collagen and there different pHs such as 2.8 and 4.5. The adjusted gels were cross-linked with different percentages of tanning agents, cast in Petri dishes (5 cm diameter) and freeze-drying with a Martin Christ Model Delta 2-24 KD lyophilizer, Germany by the program previously described [10] in order to obtain the collagen matrices. The composition of the matrices obtained by lyophilisation is given in Table 1.

Table 1. Compositions of collagen matrices

Collagen tanned with Titanium (C-T)		Collagen tanned with Aluminum (C-A)		Collagen tanned with Titanyl Ammonium Sulphate (C-TAS)		Collagen tanned with Titanium-Zirconium (C-TZ)		Collagen tanned with Titanium-Aluminum (C-TA)		Collagen tanned with Chromium (III) (C-Cr)	
Code	Conc, TA %	Code	Conc, TA %	Code	Conc, TA %	Code	Conc, TA %	Code	Conc, TA %	Code	Conc, TA %
pH = 2.8											
1C-T	0.6	1C-A	0.2	1C-TAS	2.5	1C-TZ	1.25	1C-TA	2	1C-Cr	0.125
2C-T	1.2	2C-A	0.4	2C-TAS	5	2C-TZ	2.5	2C-TA	4	2C-Cr	0.25
3C-T	2.4	3C-A	0.8	3C-TAS	10	3C-TZ	5	3C-TA	8	3C-Cr	0.5
pH = 4.5											
4C-T	0.6	4C-A	0.2	4C-TAS	2.5	4C-TZ	1.25	4C-TA	2	4C-Cr	0.125
5C-T	1.2	5C-A	0.4	5C-TAS	5	5C-TZ	2.5	5C-TA	4	5C-Cr	0.25
6C-T	2.4	6C-A	0.8	6C-TAS	10	6C-TZ	5	6C-TA	8	6C-Cr	0.5

TA - tanning agent

2.3. Methods of analysis

2.3.1. ATR FT-IR spectroscopy

ATR FT-IR spectroscopic measurements were recorded by spectrophotometer Jasco FT/IR-4200. All the spectra were recorded at the following parameters: spectral range 4000-510 cm^{-1}, resolution 4 cm^{-1} with 30 acquisitions per each sample.

2.3.2. Thermal analysis

Shrinkage temperature measurements were carried out within the range 22-to-100°C at a heating rate of 2°C/min using the micro-hot-table technique with a Caloris Micro Hot Table in co-work with a Leica Stereomicroscope.

2.3.3. Enzymatic degradation

Enzymatic degradation of collagen discs was investigated by monitoring the weight loss depending on exposure time to collagenase solution in PBS over a 24-hour period, using the method previously described [11].

2.3.4. Optical microscopy

All images were captured with a Leica Stereomicroscope model S8AP0, 20-160x magnification capacity. For better evaluation of the samples, it was use a 20x magnification and incident external cold light.

3. Results and discussion

The stability of intact native collagen can be attributed at first instance to its hierarchical structural organization. Collagen has a unique triple helical conformation which is characterized by its amide bands featured in IR spectra [12]. The amide A and B bands at 3300 cm^{-1} and 3070 cm^{-1},

respectively, are mainly associated with the stretching vibrations of N–H groups. The amide I band at 1630 cm^{-1} is dominantly attributed to the stretching vibrations of peptide C=O groups. The amide II absorbance at 1550cm^{-1} arises from the N–H bending vibrations coupled to C–N stretching vibrations. The Amide III centred at 1240cm^{-1} is assigned to the C–N stretching and N–H bending vibrations from amide linkages, as well as wagging vibrations of CH_2 groups in the glycine backbone and proline side chains [13].

Figure 1 shows the ATR FT-IR spectrum of reference matrix at 4.5 pH.

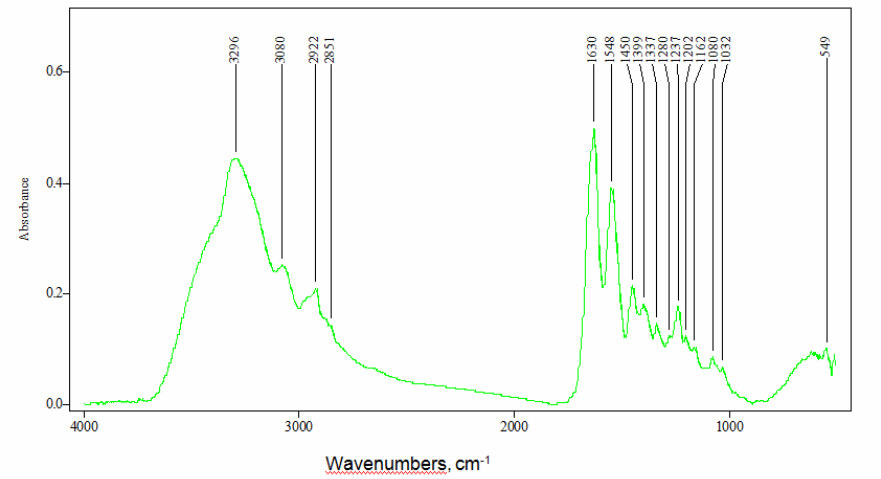

Figure 1. ATR FT-IR spectrum of collagen reference at pH = 4.5

Also, with ATR FT-IR spectroscopy analysis it has been possible to observe triple helical structure stabilization or hydrolysis denaturation, as a result of the interaction of the compounds tested and the model collagenic substrate.

These semi-quantitative characteristics for the studied samples show the interaction between collagen and the tanning agents, as following:
- the A_I/A_A ratio which indicates the cross-linking degree (the higher the A_I/A_A ratio the more advanced the cross-linking degree) of is higher for tanned samples compared with the references ones, as we expected. As we can see in Figure 2, the concentrations of tanning agents also modified the cross-linking degree.

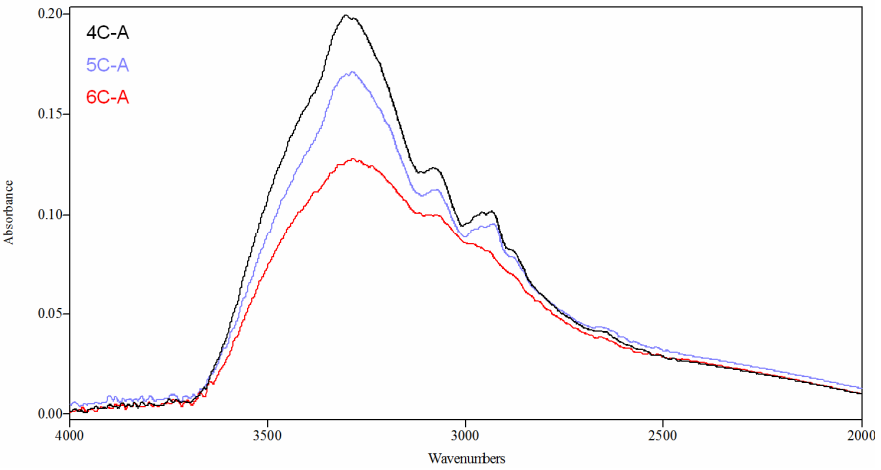

Figure 2. ATR FT-IR spectra for collagen matrices tanned with different concentration of Aluminum tanning agent

Figure 2 shows that the smallest recorded area for Amide A is for sample with maximum (0.8%) concentration of tanning agent and the highest is for the one tanned with 0.2% of aluminum tanning agents. This demonstrated that the cross-linking degree increase with mineral tanning agent concentrations.
- the A_{III}/A_{1450} ratio (which is correlated with maintaining of integrity of triple helical structure) kept the values around reference collagen matrix.
- the $\Delta\nu = (\nu_1 - \nu_2)$, cm^{-1} (which indicates the presence of denatured collagen: $\Delta\nu > 100$ cm^{-1} indicates the presence of denatured collagen) has values between 80 and 84 cm^{-1} which demonstrated that none of the studied samples have denatured collagen in their structure.

Due to the highest cross-linking degree was registered for samples tanned with the highest tanning agent concentration, Figure 3 presents comparatively Amide A for samples at 4.5 pH and maximum concentrations.

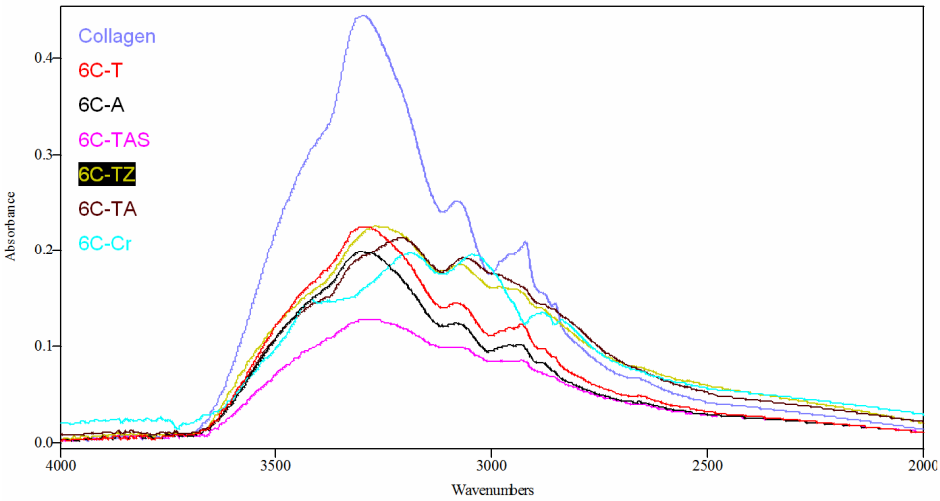

Figure 3. ATR FT-IR spectra of tanned collagen samples. Amides A

As we can see more evident in the figure 3, the area of Amide A for collagen sample is the highest and the smallest (8 times less that collagen) is for the sample tanned with Chromium(III).

The hydrothermal stability of collagen can be altered by many different chemical reactions, well known in the fields of histology, leather tanning and other industrial applications of collagen [5]. This stability is usually quantified and expressed as shrinkage temperature, also named denaturation temperature, which, in turn, signifies the transition of the collagen molecules from stretched fibre to random coil configuration: the higher the temperature at which this transition takes place, the more cross-links are present [9, 14].

Figure 4 shows the thermal stability for both the collagen reference samples and the tanned ones, with different tanning agents, at different 4.5 pH and maximum concentrations.

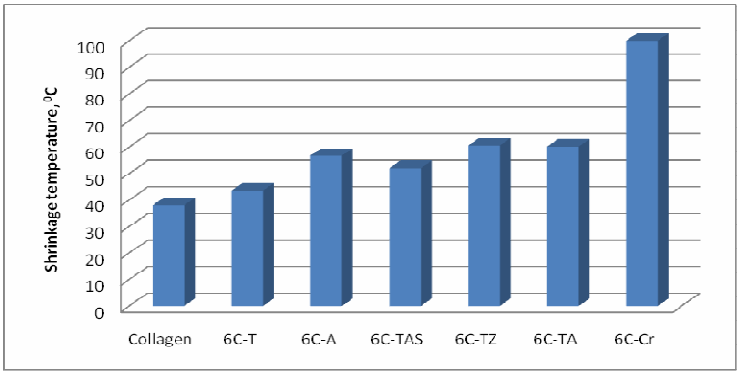

Figure 4. Shrinkage temperature for collagen matrices

As we can see in Figure 4, collagen sample had the lowest shrinkage temperature of 38.0 ^{0}C. The shrinkage temperature increased when the samples were tanned. The highest shrinkage temperature value was recorded for matrices containing tanning agent based on Chromium (III).

Native collagen is susceptible to attack only by collagenase at physiological pH, temperature and ionic strength. Bacterial collagenase, which was employed in this study exhibited less specificity and cleaves collagen predominantly at the Y-Gly bond in the sequences of type -Pro-Y-Gly-Pro-, where Y is most frequently a neutral amino acid [15]. In the process of stabilization of collagen, rendering stability against collagenase is an important aspect.

For this reason we followed in this study the interaction between collagenase and the obtained matrices in order to study indirectly the degree of cross-linking. Collagen samples were degraded in 2 hours. The collagen samples tanned with different mineral agents were completely degraded as following: collagen tanned with titanium after 2 days, collagen tanned with aluminum after 7 days, collagen tanned with Titanyl ammonium sulphate after 3 days, collagen tanned with a combination Titanium-Aluminum tanning system after 9 days, collagen tanned with combination Zirconium-Titanium system after 14 days. Collagen tanned with Chromium(III) were degraded only by 51% during a 14 days period.

Figure 5 shows the enzymatic degradation of collagen at pH=4.5, tanned with the highest mineral tanning agent offer during 48 hours in a phosphate buffer solution at 37^{0}C.

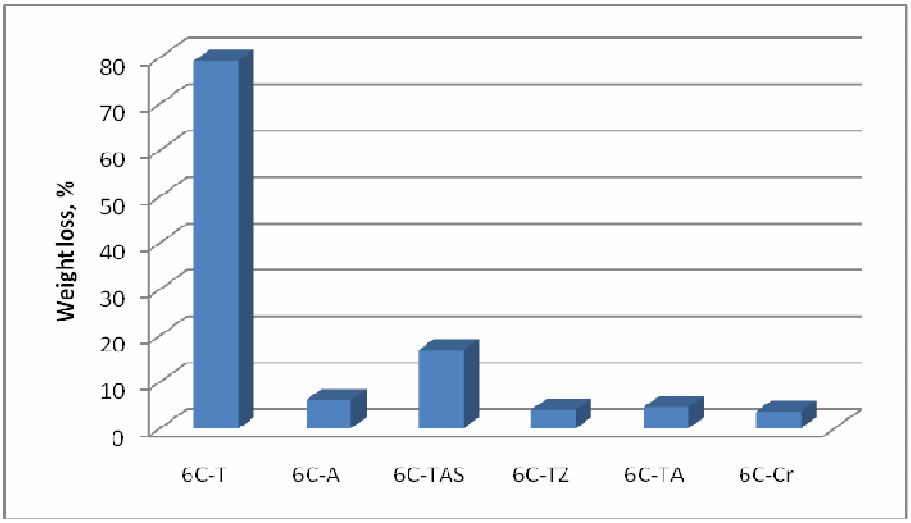

Figure 5.Collagenase degradation of collagen matrices

As we can see in Figure 5 the most unstable sample is collagen tanned with titanium. The tanning with combination of titanium with aluminum or zirconium offer high stability to collagen. The most stable specimen against enzymatic degradation was collagen tanned with Chromium(III)-tanning agents.

The optic microscopic images are in correlation with the ATR FT-IR, thermal and enzymatic degradation results. In Figure 6 are presented optic images of collagen matrices.

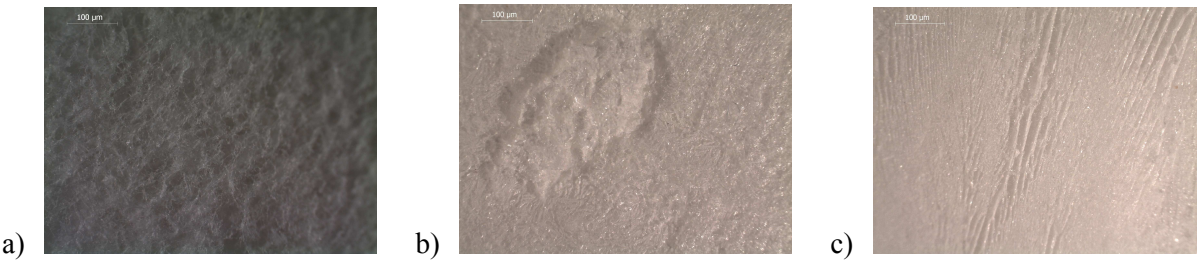

Figure 6. The optical microscopy images for: a) Collagen; b) 6C-TAS and c) 6C-TA

The results show different structural organization of collagen, depending upon the tanning agent.

The stability of treated collagen in ascending order is as follows:

Collagen < 6C-T < 6C-TAS < 6C-A < 6C-TA < 6C-TZ < 6C-Cr

These results are confirmed by ATR FT-IR spectroscopy, shrinkage temperatures and enzymatic degradation. Furthermore, collagen structure was kept intact without any degradation evident after cross-linking process with mineral tanning agents.

4. Conclusions

The results show unique hydrothermal stability, enzymatic degradation patterns and reactivity to water depending on the agent and physical conditions applied. In particular, ATR FT-IR fingerprinting spectroscopic analysis has showed that all of the mineral tanning agents surveyed kept the triple helical superstructure intact during the tanning process. The cross-linking degree was indirectly demonstrated by the micro-hot table based shrinkage temperature measurements and the collagenase degradation assay. The most stable samples were the ones tanned by Chromium based tanning agent – as expected - and the least stable were the ones tanned by Titanium (IV). The

stability of samples tanned with Titanium (IV) was improved by adding of Zirconium and Aluminum based tanning agents.

It has been possible a new model collagenic substrate and a dedicated analytical regime for the quick and reliable evaluation of mineral tanning agents, components of known and innovative solo tanning, tawing and combination systems, as well as for the definition of optimum physical conditions and tanning reaction stoichiometry. This, in turn, will serve as a power tool for the foundation of novel targeted tanning chemistry formulation and the mechanistic understanding of known tanning systems.

5. References

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