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Chemical functionalization and stabilization of type I collagen with organic tanning agents

Madalina Georgiana Albu†, Viorica Deselnicu, Ioannis Ioannidis, Dana Deselnicu, and Ciprian Chelaru

INCDTP - Leather and Footwear Research Institute Bucharest, Romania
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Abstract−We investigated the interactions between selected organic tanning agents and type I fibrillar collagen as a model fibrillar substrate to enable the fast direct evaluation and validation of interpretations of tanning activity. Type I fibrillar collagen (1%) as gel was used as substrate of tanning and tannic acid, resorcinol- and melamine-formaldehyde and their combination at three concentrations as crosslinking agents (tannins). To evaluate the stability of collagen during tanning, the crosslinked gels at 2.8, 4.5 and 9.0 pHs were freeze-dried as discs which were characterized by FT-IR, shrinkage temperature, enzymatic degradation and optical microscopy, and the results were validated by statistical analyses. The best stability was given by combinations between resorcinol- and melamine-formaldehyde at isoelectric pH.

Keywords: Collagen, Organic Tanning Agents, Crosslinking, Stabilization

INTRODUCTION

Leather science has an increasing impact on the understanding of the process involved in the leather production and subsequent tanning reactions [1]. Collagen is the major connective tissue protein and principal component of dermal substance of hides, skins and leathers [2,3]. Hence, it is necessary to study the stabilization of collagen. For this reason the future technologies in leather industry could be based on simulation of interaction between collagen and tanning agents. Tannins have a long history of use in stabilizing collagen [4,5] and are known to interact extensively and stabilize proteins [6,7].

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 [8]. Effective tanning systems result in the impediment of triple helix unraveling under hydrothermal stress, due to the combination of binding defined collagen super-structural elements to surrounding water at the molecular level (functionalization) and the subsequent matrix formation around the triple helices (stabilization).

Chemical crosslinking, electrostatic interactions, hydrophobic interactions, hydrogen bonds and water activity are factors which contribute to the mechanism of collagen stabilization.

Collagen is a right handed conformation of triple helix formed by three interwoven chains (α-chains), where each chain has a left-handed poly-proline like conformation. The tight packing of the triple helix is allowed by the repetitiveness in the sequence of the chains, namely $(Gly\text{-}X\text{-}Y)_n$ where X is frequently proline and Y is hydroxyproline [9-12]. Due to basic and acidic groups of the polypeptide chains side, collagen is an amphoteric polymer. A collagen molecule contains about 240 ε-amino and guanidino groups of lysine, hydroxylisine, and arginine and 230 carboxyl groups of aspartic and glutamic acid residues [13]. In a native fibril, most of these groups interact either intra- or intermolecularly forming salt-linkages, providing significant stabilization energy to the collagen fibril [14]. Any bifunctional reagent which reacts with amino, carboxyl, and hydroxyl groups can serve as a crosslinking agent [13].

The stabilization of type I collagen using aldehydes, mineral tanning agents, and vegetable tannins, has been extensively studied in the leather industry [15]. Recent studies by Monti et al. showed interaction of collagen with chlorosulfonated paraffin tanning agents using spectroscopy and molecular dynamic simulations [16]. Moreover, to obtain collagen biomaterial, the following crosslinking agents were used: glutaraldehyde, hexamethylene-diisocyanate, polyepoxy compounds, carbodiimides, acyl azide [17], genipin [18], and polyphenols [19].

Well known and until recently employed chemical crosslinking agents, chromium, formaldehyde, glutaraldialdehyde, oxazolidine and glutardialdehyde modified products (GMPs) are highly efficient collagen stabilizers. They are easily available, inexpensive and can effectively and quickly crosslink collagenous tissues in aqueous solution, but all of them are toxic, particularly if they are released as a result of biodegradation and come in contact with the body [20-22]. Also, the use of mineral crosslinkers is retreating because of environmental legislation that becomes increasingly stringent with continuously changing requirements for leather manufacture non-renewable resources use [15]. Tannins are naturally occurring phenolic compounds that have been the subject of extensive research, leading to the development of a wide range of industrial applications [23]. Tannic acid has been shown to complex or crosslinking proteins by the formation of multiple hydrogen bonds [24]. Recently, there has been an interest on tannin-based resins due to the more widespread availability of tannins, and the lower cost of tannin-based resins [25]. Resorcinol has low toxicity and is cheap. Resor-

†To whom correspondence should be addressed.
E-mail: albu_mada@yahoo.com

cinol and melamine-formaldehyde pre-polymers can offer advantages to a lower environmental impact when applied in leather-manufacture.

In this paper, we describe an investigation of the interactions between selected organic tanning agents (tannic acid, resorcinol- and melamine-formaldehyde and their combination) 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.

MATERIALS AND METHODS

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 [26]. Tannic acid (TA) was supplied by Sigma-Aldrich, Germany. Resorcinol-formaldehyde (RF) prepolymer was obtained by in-house technology and melamin-formaldehyde (MF) prepolymer was obtained according with our patented technology [27]. 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. Collagen Discs Preparation

The collagen gel (Coll) with an initial concentration in collagen of 1.99% and pH 2.6 was adjusted at 1% collagen and three different pHs: 2.8, 4.5 and 9.0. The adjusted gels were crosslinked with different percentages of tanning agents, cast in Petri dishes (5 cm diameter) and freeze-dried with a Martin Christ Model Delta 2-24 KD lyophilizer, Germany by the program previously described [28] in order to obtain the collagen discs. The composition of the discs obtained by lyophilization is given in Table 1.

3. Methods of Analysis

3-1. Isoelectric pH Determination

The isoelectric pH was determined by titrimetric method using 0.2% collagen suspension of initial collagen gel and 1 M NaOH. The pH-meter was inoLab fitted with a special electrode sensitive to pHs in gel state.

3-2. FT-IR Analysis

FT-IR spectral measurements were recorded by spectrophotometer Jasco FT/IR-4200. All the spectra were recorded at the following parameters: spectral range 4,000-510 cm^{-1}, resolution 4 cm^{-1} with 30 acquisitions per each sample.

3-3. Thermal Analysis

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

3-4. 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 [29].

3-5. Optical Microscopy

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

3-6. Statistical Analysis

Statistical analysis was developed using the SPSS 17.0 data mining software. The variable investigated included the collagen pH, crosslinking agent type, crosslinking agent concentration, shrinkage temperature determined by MHT, and data from FT-IR spectra. The data was investigated by descriptive and inferential statistics, both to explore the statistic parameters of the investigated samples, and to determine relations between the variables under study.

RESULTS

1. Isoelectric Point Determination

The isoelectric point is an important parameter, as it controls the charge on collagen at any given pH, whereas, in general, the reactions in leather manufacture are usually charge-dependent. At the isoelectric point the following phenomena occur [30]: net charge

Table 1. Compositions of collagen discs

Discs cross-linked with TA			Discs cross-linked with RF prepolymer			Discs cross-linked with MF prepolymer			Discs cross-linked with MF+RF prepolymers			
Disc code	pH Coll	Conc. TA, %	Disc code	pH Coll	Conc. RF, %	Disc code	pH Coll	Conc. MF, %	Disc code	pH Coll	Conc. MF, %	Conc. RF, %
1-TA	2.8	1	1-RF	2.8	0.3	1-MF	2.8	0.5	1-RF-MF	2.8	0.5	0.3
2-TA	2.8	3	2-RF	2.8	0.6	2-MF	2.8	1	2-RF-MF	2.8	1	0.6
3-TA	2.8	5	3-RF	2.8	1.2	3-MF	2.8	2	3-RF-MF	2.8	2	1.2
4-TA	4.5	1	4-RF	4.5	0.3	4-MF	4.5	0.5	4-RF-MF	4.5	0.5	0.3
5-TA	4.5	3	5-RF	4.5	0.6	5-MF	4.5	1	5-RF-MF	4.5	1	0.6
6-TA	4.5	5	6-RF	4.5	1.2	6-MF	4.5	2	6-RF-MF	4.5	2	1.2
7-TA	9.0	1	7-RF	9.0	0.3	7-MF	9.0	0.5	7-RF-MF	9.0	0.5	0.3
8-TA	9.0	3	8-RF	9.0	0.6	8-MF	9.0	1	8-RF-MF	9.0	1	0.6
9-TA	9.0	5	9-RF	9.0	1.2	9-MF	9.0	2	9-RF-MF	9.0	2	1.2

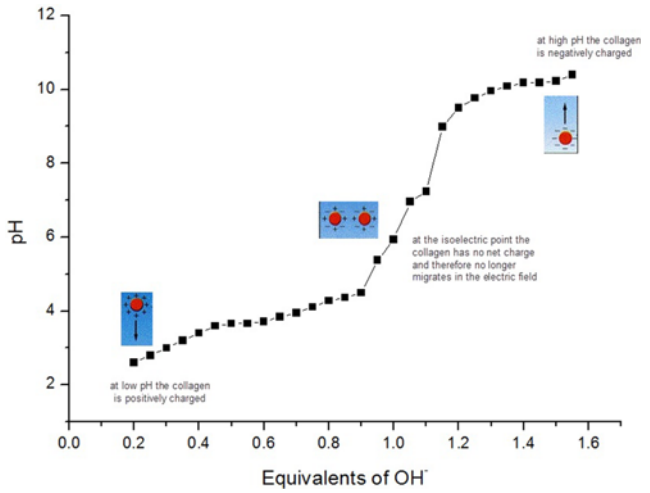

Fig. 1. The titration curve for the type I collagen gel.

is zero, content of intramolecular salt links is maximized, swelling is at minimum, and shrinkage temperature (and other aspects of hydrothermal stability) is maximum.

Fig. 1 shows the isoelectric point (IEP) for the collagen gel which we used in this study.

As one can see in Fig. 1, if the pH is higher than the IEP, the collagen is negatively charged, and if the pH is lower than the IEP, the collagen is positively charged.

The importance of this concept relates to the application of charged reagents, particularly post tanning reagents and their interaction with the charged leather substrate.

For this reason we studied the behavior of collagen in the presence of tanning agents at all the three regions of pH: 2.8 (acid), 4.5 (isoelectric) and 9.0 (alkaline) to establish its stability.

To maintain the integrity of the collagen structure, the obtained gel both crosslinked and uncrosslinked ones were preserved by lyophilization (freeze-drying) and 5 cm diameter discs were obtained.

These discs were analyzed by spectroscopic, thermal, enzymatic degradation to establish collagen stability during the tanning process.

2. FT-IR Spectroscopic Analysis

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 [31]. The amide A (AA) and B bands (AB) at 3,300 cm^{-1} and 3,070 cm^{-1}, respectively, are mainly associated with the stretching vibrations of N-H groups. The amide I band at 1,630 cm^{-1} (AI) is predominantly attributed to the stretching vibrations of peptide C=O groups. The amide II (AII) absorbance at 1,550 cm^{-1} arises from the N-H bending vibrations coupled to C-N stretching vibrations. The Amide III (AIII) centred at 1,240 cm^{-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 [32].

Fig. 2 shows the FT-IR spectrum of reference disc at isoelectric pH.

Also, with 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.

The modifications of collagen structure can be appreciated by the following semi-quantitative relations [26]:

- A_{III}/A_{1450} ratio, correlated with maintaining of integrity of triple helical structure - values higher or equal to unity indicate preservation of conformation;
- A_I/A_A ratio, correlated with the crosslinking degree: the higher the A_I/A_A ratio the more advanced the crosslinking degree;
- A_I/A_{II} ratio, correlated with the hydrolysis degree;
- difference between the frequencies of amide I and II, $\Delta\nu=(\nu_1-\nu_2)$, cm^{-1}, indicating the presence of denatured collagen: $\Delta\nu>100$ cm^{-1} indicates the presence of denatured collagen.

These semi-quantitative characteristics for the studied samples show the interaction between collagen and the tanning agents, as follows:

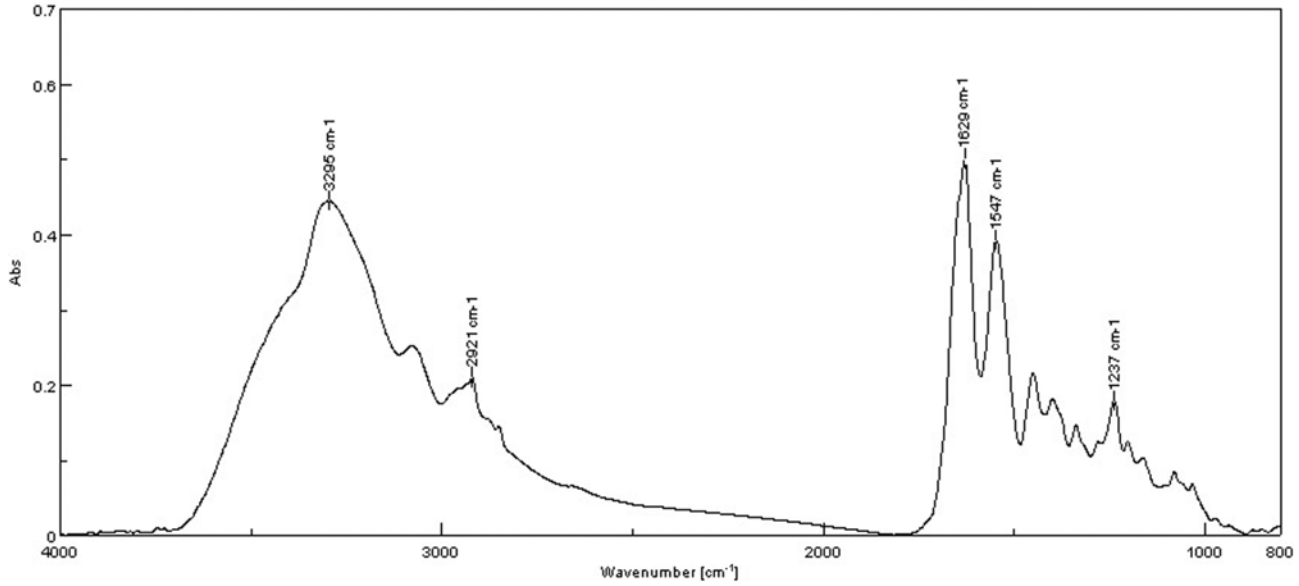

Fig. 2. FT-IR spectrum of collagen reference at pH=4.5.

- the A_{III}/A_{1450} ratio kept the values around reference collagen disc, which means that the triple helical was not destroyed during the tanning process;
- the A_I/A_A ratio is higher for all the crosslinked samples compared with the un-crosslinked ones, as we expected. The group samples obtained at 4.5 pH revealed the higher crosslinking degree.
- the $\Delta\nu=(\nu_1-\nu_2)$, cm^{-1} has values between 80 and 84 cm^{-1} which demonstrated that none of the studied samples have denatured collagen in their structure.

3. Hydrothermal Stability

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 [8]. 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 fiber to

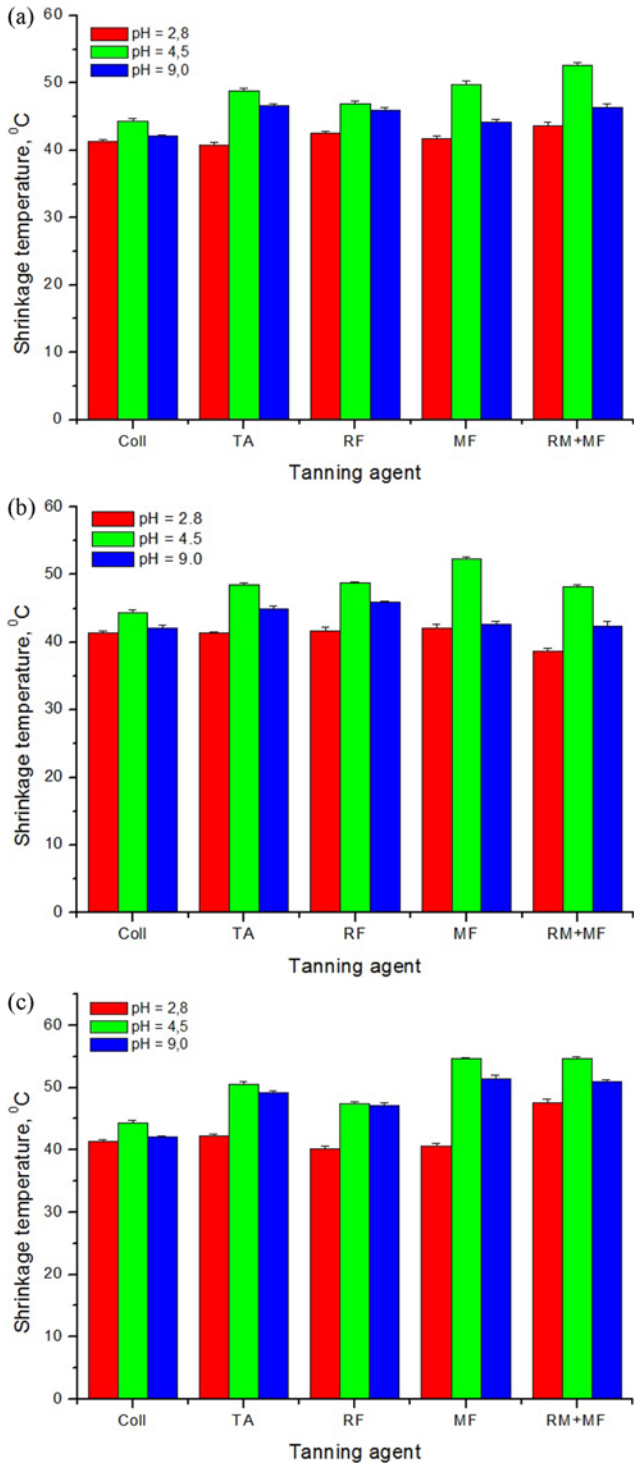

Fig. 3. Shrinkage temperature at (a) low; (b) medium, and (c) high concentration of tanning agents.

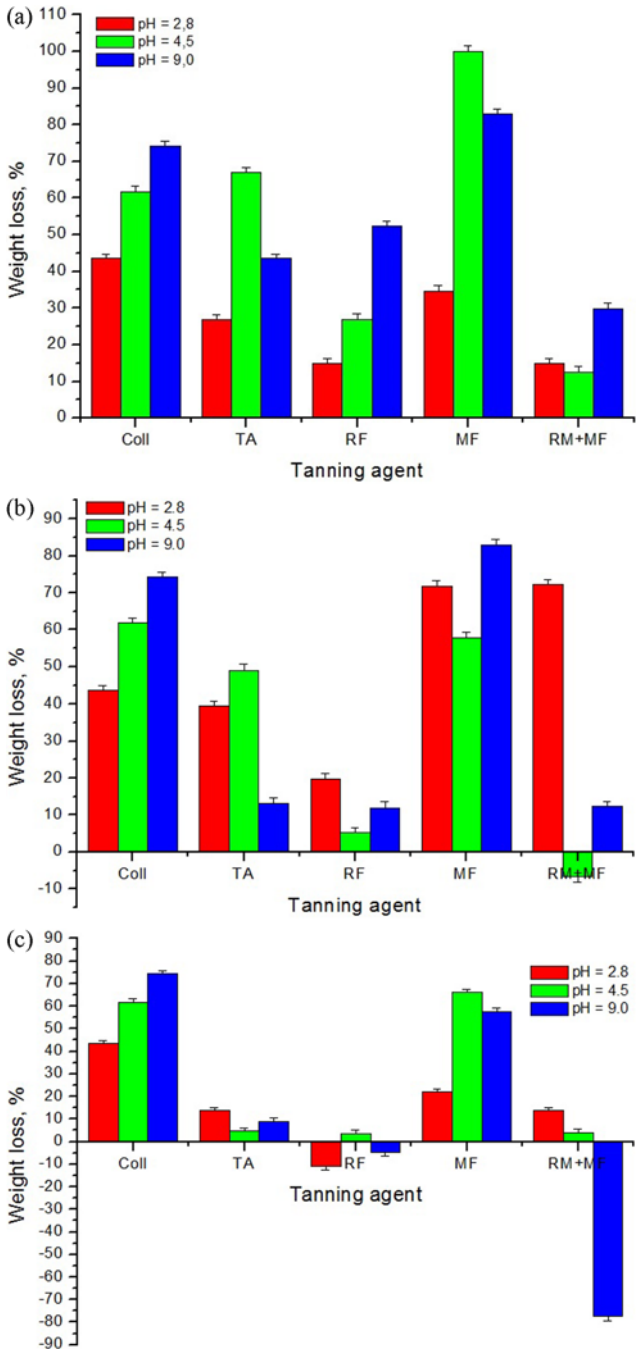

Fig. 4. Collagenase degradation (a) low; (b) medium, and (c) high concentration of tanning agents.

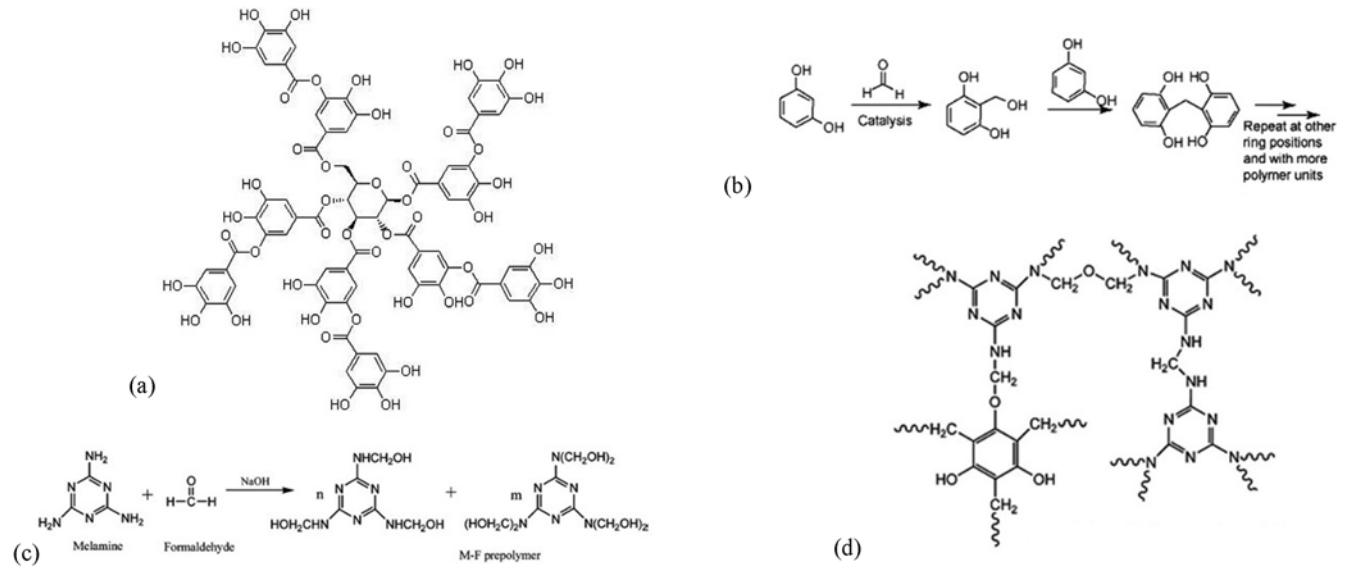

Fig. 5. Structural formula for (a) TA, (b) RF, (c) MF, and (d) RF+MF.

random coil configuration: the higher the temperature at which this transition takes place, the more crosslinks are present [24,26].

Fig. 3 shows the thermal stability for both the collagen reference samples and the crosslinked ones, with different tanning agents, at different pH values and at all offers surveyed.

As one can see in Fig. 3(a)-(c), the non-crosslinked samples had the lowest shrinkage temperature of 41.4 °C at acid pH, 44.3 °C at isoelectric pH and 42.1 °C at alkaline pH. These results show that at all pH values the triple helical structure of collagen reference discs remains intact, having values higher than 38 °C. The highest shrinkage temperature values were recorded for discs containing or not tanning agents at pH=4.5, while the lowest was measured for samples at acid pH (2.8), respectively.

4. Enzymatic Degradation Stability

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 cleaved 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 [33]. In the process of stabilization of collagen, rendering stability against collagenase is an important aspect.

For this reason we followed the interaction between collagenase and the obtained discs in order to study indirectly the degree of crosslinking.

Fig. 4 shows the enzymatic degradation during 24 hours in phosphate buffer solution at 37 °C.

As one can see in Fig. 4, the most unstable samples are the ones obtained at acidic pH. The samples which are represented as negative values are the most stable ones. They were not degraded during 24 hours; on contrary, they were swollen and took up collagenase solution without any degradation. If, now, one compared the tanning agent type used, it becomes apparent that at all pH values studied the melamine-formaldehyde pre-polymer is the most unstable.

5. Statistical Analyses

For the present study, only the correlation of the shrinkage temperature with the A_I/A_{II} ratio will be discussed. For this dependence relation, a Pearson correlation coefficient of - 0.673 was obtained, at a level of trust of 99%, indicating a medium to high intensity, negative correlation. This shows that the higher the shrinkage temperature, the higher the A_I/A_{II} ratio also. This result tentatively confirms the triple helical structure of collagen without any denaturation during tanning process and is in accordance with the thermal analyses' results.

DISCUSSION

All the studied tanning agents (crosslinkers) in this paper are of the phenolic type, both natural (tannic acid) and synthetic (resorcinol-formaldehyde and melamine-formaldehyde pre-polymers and their combinations thereof). For a better understanding Fig. 5(a)-(d) shows the structural formulation for every tanning agent.

Polyphenolic tannins are capable of crosslinking with collagen through the formation of multiple hydrogen bonds [2]. The hydroxyls are the main functional groups of interaction for these tannins, as Fig. 6 shows.

These hydroxyl groups can form a hydrogen bond with the side

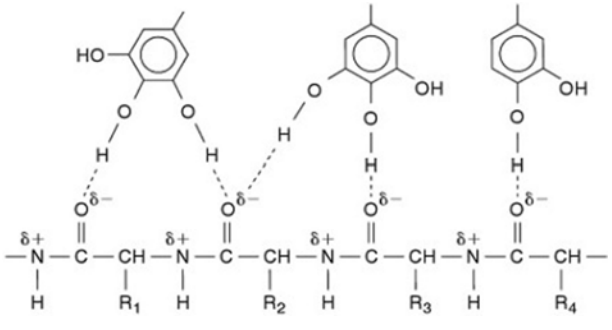

Fig. 6. Model of hydrogen bonding between polyphenol and peptide bond in collagen [1].

chain groups of polar amino acids like lysine, arginine, aspartic acid and glutamic acid. The other amino acids like serine and threonine can also involve in the hydrogen bond formation with the polyphenolic molecules [2]. All these amino acid residues can act as hydrogen bond donors and acceptors. Since these oligophenols have several hydroxyl and carboxyl groups, they can form hydrogen bonds at multiple points, imparting additional stability to the fiber matrix.

As we presented in results, the degree of crosslinking was determined by measurement of the hydrothermal shrinkage temperature, enzymatic degradation and FT-IR spectroscopy.

The higher stability was given by samples obtained at pH=4.5. Among these samples, the ones with the highest concentration of tanning agent showed the higher shrinkage temperature, the lower enzymatic degradation. The strongest hydrogen bonding was given at low intensity of AI as Fig. 7(a) showed. These samples, named in Table 1 as 6-TA, 6-RF, 6-MF and 6-RT+MF, as well as reference sample 1-C, are compared in order to establish which tanning agent is the most power one in combination with collagen. Fig. 7 shows the comparative FT-IR spectra, shrinkage temperatures and enzymatic degradation for these discs.

The results show that at optimal pH and concentration of tanning agent the stability of collagen is given in this order:

C-2<6-RF<6-TA<6-MF<6-RF-MF

Fig. 7(a) shows that the smallest recorded area for Amide A is for sample 6-RF-MF, and the highest is for collagen reference, C-2. Also, the shrinkage temperatures (Fig. 7(b)) are higher for the crosslinked samples, and weights of the enzymatic degraded samples (Fig. 7(c)) are higher for the non-crosslinked collagen. Furthermore, the collagen structure was kept intact without any degradation after crosslinking process with organic tanning agents. All the results are in strong correlation, as shown by statistical analyses. Optical images for representative samples are shown in Fig. 8.

Fig. 8 presents a denser structure for collagen crosslinked with the combination between resorcinol and melamine-formaldehyde that the reference ones, which is according to the results obtained by spectral, thermal analyses as well as enzymatic degradation.

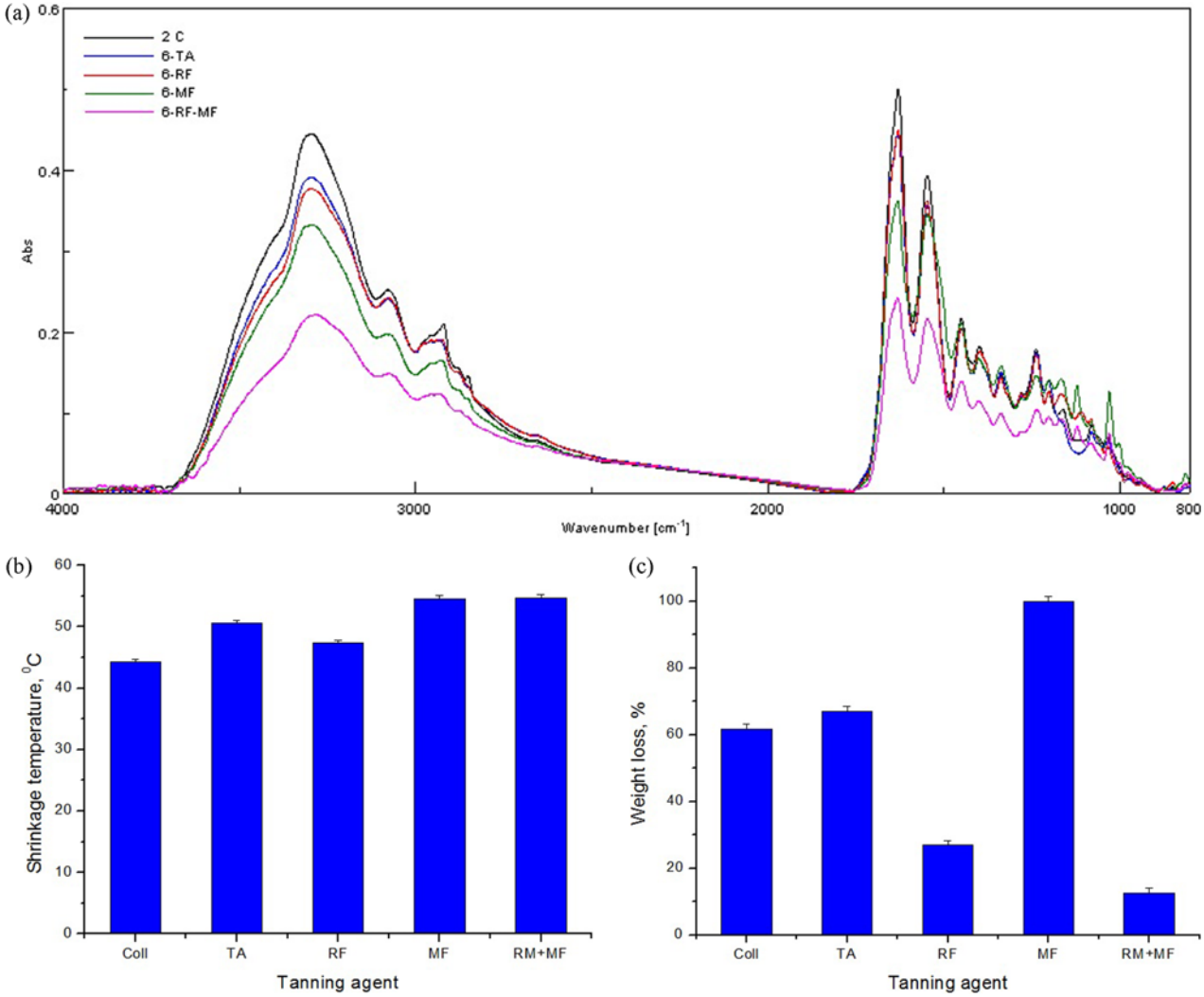

Fig. 7. Comparison between 2-C, 6-TA, 6-RF, 6-MF and 6-RT+MF: (a) FT-IR spectra, and (b) shrinkage temperatures, and (c) enzymatic degradation.

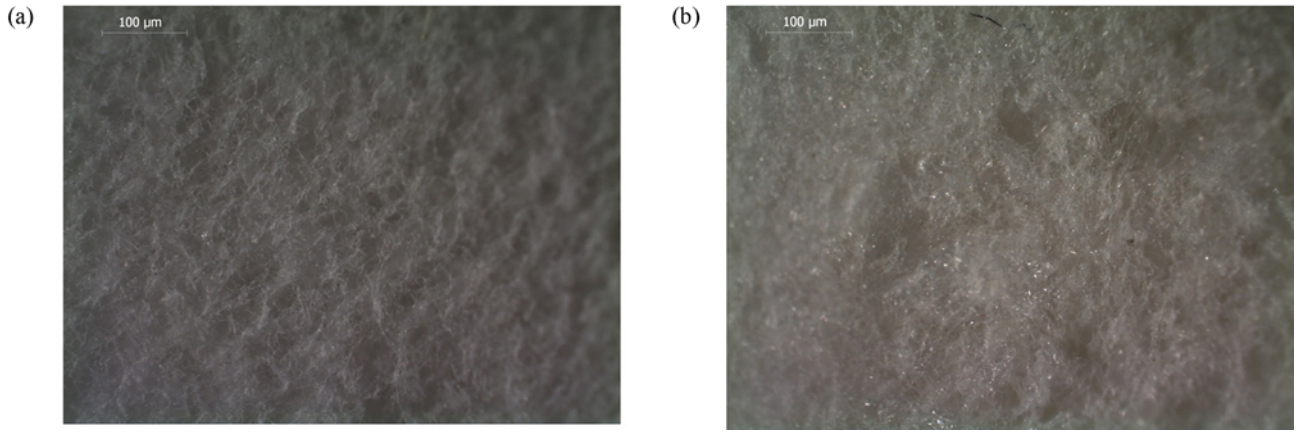

Fig. 8. The optical microscopy images for (a) C-2 and (b) 6-RF-MF.

CONCLUSIONS

Three concentrations of natural and synthetic tanning agents (crosslinkers) were used to establish the best stability of collagen at acidic, isoelectric and alkaline pHs. The best stability is given at pH=4.5 and by combinations between resorcinol-formaldehyde and melamine-formaldehyde pre-polymers. The results of this paper demonstrate that crosslinking (tanning) can be predicted using the new power bench tool described here, which, in turn will be used as a tool for the evaluation of the tanning agents, commercial and development products, applicable in leather industry.

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