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Studies on the effects of mixed light-thermal ageing on parchment by vibrational spectroscopy and micro hot table method

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Abstract

The damaging effects of mixed light-thermal ageing on parchment were studied by exposing mock-ups to artificial light (162 W/m² irradiance) at controlled temperature and relative humidity (52 °C, 30% RH) for up to 748 h, with testing being made during this time incrementally. The physical–chemical changes (deterioration) resulting from these conditions were analysed by applying FTIR-ATR and Raman spectroscopy, colorimetric measurements and MHT. The deterioration found includes the formation of collagen-destabilised intermediate states which then progressively convert to disorganised structures, e.g. pregelatinised collagen and gelatin. The process is characterized by molecular changes of collagen associated with its photo-oxidation. These are shown by changes in the position and intensity of the main FTIR absorption bands of collagen, namely those of Amide I, Amide II, and Amide III, as well as by the occurrence and the increase of a shoulder at 1740 cm⁻¹. The shrinking activity of the collagen fibres in the parchment mock-ups was measured by the micro-hot-table (MHT) method, which revealed the presence of multiple collagen populations with distinct thermal stabilities and confirmed the deterioration pattern suggested by the molecular changes.

Keywords: Parchment, Mixed light-thermal ageing, Deterioration, Vibrational spectroscopy, MHT method

Introduction

Parchment is one of the most important media that was used historically for recording written information. During the medieval period, manuscripts were made almost exclusively from parchment. According to Herodotus' *Terpsichore* (484–425 BC) and Pliny's *Natural History* (79 AD), the use of parchment was a common practice since the patronage of Eumenes of Pergamum, the city where parchment processing seems to have been developed [1] and from where the material's name derives. Parchment is obtained from raw animal hides through a sequence of steps during which they are thoroughly washed, soaked

in a solution containing lime, dehaired, scraped with sharp knives (to remove muscle tissue and hypodermic layers), finished (by mechanical thinning, shaving, bleaching, dying, polishing) and finally tightened on rectangular or circular frames on which they are left to dry under tension at ambient temperature [2]. The manufacturing procedure of parchment differs depending on the geographical area and the historical period, but the term “parchment” is generally associated with the “Western” (European) preparation method that uses calcium hydroxide extensively that has been carried out since the Middle Ages. Compared to the previously used papyrus, a material intrinsically brittle and sensitive to moisture, parchment is characterized by longer durability, stability, and very good optical properties [2, 3]. Its opacity allowed writing on both *grain* and *corium* sides, and its robustness led to the recycling of old classical manuscripts, a practice that became very common during the Middle Ages due to the great demand for parchment [4].

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From the chemical point of view, collagen is the main structural component of the skin, and hence of parchment. The structure of collagen gives parchment its strength and durability over time, even though the repair mechanisms which functioned in collagen *in vivo* (turnover) are obviously no longer active in a *post-mortem* tissue. Parchment deterioration generally occurs as a result of improper environmental and storage conditions or inappropriate conservation and/or restoration treatments. The process of manufacture may also alter the structure of collagen in a manner that initiates its deterioration [5, 6]. The most important physical and chemical parameters which can induce collagen deterioration are temperature, humidity, light irradiation, chemical pollutants, solvents, as well as their synergistic actions. Their effects were ascribed to three main deterioration paths: molecular denaturation, hydrolysis, and oxidation [3, 5, 6]. It is also necessary to consider that collagen deterioration generally occurs at different levels, i.e. from the macroscopic to the molecular levels, and alterations to the hierarchical structure arise as a result of molecular damage. When the collagen molecules break down, the structural hierarchy is lost, inducing irreversible damage and the weakening of the material.

Both static and dynamic accelerated ageing experiments have been performed to study these processes occurring in this material in significantly less time than under natural conditions; these consisted of modifying external parameters such as temperature, light, relative humidity and the presence of pollutants [7–17]. A dry heat treatment at 100 °C was applied by Della Gatta et al. [7] and Badea et al. [8] in order to explicitly examine heat-induced effects such as thermal destabilisation, and to quantify the distribution of collagen structures during ageing. Similar levels of deterioration and comparable damage patterns as those induced by heat were found for some historical samples that have undergone heat flattening and/or heat drying treatments [8]. By dry heating in the range of 60–170 °C for 48 h, Fessas et al. [9] found a correlation between the denaturation temperature and the water content in parchment (moisture), allowing the quantification of the degree of deterioration degree experienced by parchments. Most recently, Mühlen Axelsson et al. [10] applied a dry heat treatment of 120 °C to study the heat-induced oxidation of parchment and found significant colour changes, a decrease in hydrothermal stability, as well as changes in the amino acid composition. On the other hand, an X-ray diffraction study of parchment exposed to dry heating at 120 °C showed that this method is not appropriate to simulate some of the changes in molecular packing occurring during natural ageing [11] although dehydrothermal treatment may mimic some of the properties of historical

parchment such as oxidative damage of amino acid side chains. The synergistic effects of dry heating and visible light irradiation are also reported. Such treatments showed an intensification of the dynamics of destabilisation and denaturation of collagen with ageing time [12]. Thermal ageing below 100 °C with differing relative humidity conditions, in both static and dynamic ageing, was performed by Vest et al. [13] and Badea et al. [14] to understand how much heat and relative humidity are actually necessary to create an accelerated degradation rate. Their results showed that (i) temperature increase induces significant variations of the hydrothermal and structural parameters, whereas the influence of RH depends on predefined ratios of the ageing conditions; (ii) parchment can fairly easily withstand exposure to 40 °C when RH < 60% but; (iii) for RH between 60 and 80%, thermal denaturation will increase, which accelerates as temperature increases further. In a more complex ageing experiment that included the ageing effects of chemical pollutants (SO₂, NO_x and combined SO₂+NO_x), Budrugaec et al. [12] and Badea et al. [15] could demonstrate that pollutant attack and damage is significantly enhanced by preheating treatments. Badea et al. [14, 15] were able to establish the dynamics of deterioration by quantifying the change in collagen distribution as a function of thermal stability during the ageing treatment.

Fewer experiments focus on the artificial light-ageing of parchments. Manfredi et al. [16] reported the use of quantitative multispectral imaging for monitoring the degradation of parchment caused by light exposure at 50% RH and 30 °C, whereas Dolgin et al. [17] investigated the effect of UV radiation in order to set up a non-destructive optical method for rapid assessment of the stage of parchment deterioration. Light has a high potential for damaging collagen-based materials, the extent of which is dependent on environmental factors, e.g. the temperature and relative humidity. Consequently, investigations of the impact of light were conducted with the aim of understanding its interaction-mechanisms with collagen molecules and correlate them with the condition of collagen-based historical and archaeological objects. This issue is extremely important for the fresh (unaged) collagen-based biomaterials as well.

The main focus of this work was a systematic study of photo-oxidative deterioration of parchment provoked by sunlight irradiation. The photochemistry of pure collagen in aqueous solution, powder and film was already been extensively studied [18–28]. Photo-oxidation due to UV irradiation can cause structural damage of collagen molecules by weakening and cleaving the covalent bonds near the amino acid residues (i.e. proline) in the single chain [22, 23]. As described in 1994 by Feller [24], the chemical bonds at the C α atom are split by the free radicals formed

by the UV irradiation and thus the fragmentation of the molecule is promoted. This process is described as the auto-oxidation of organic materials, and it can be triggered by electromagnetic irradiation (photo-activation) or in some cases also by thermal activation [5, 10, 24]. In proteins, highly reactive radicals generated by radiation (high energy radiation, UV, visible light, thermal) can cause damage at both backbone sites (i.e. α -carbon) and reactive side chains [25], while oxidation of the side chains is manifested as a reduction of the number of basic amino acids such as arginine, hydroxylysine, and lysine. The oxidative breaking of the N–C covalent bonds that link neighbouring amino acid residues results in the cleavage of the polypeptide chains. It was shown that oxidative cleavage of the collagen molecules occurs preferentially at tyrosyl residues of the collagen molecule [26, 27] or in regions of charged residues [28].

The experiment in this work was carried out to study the damaging effects of mixed light-thermal ageing on collagen in the solid phase, as found in parchment. They simulated as much as possible the photo-oxidation deterioration effect by means of artificial light ageing experiments at controlled temperature and relative humidity. The monitoring and evaluation of the physical–chemical changes induced by accelerated ageing was performed by applying non-invasive infrared spectroscopy in ATR acquisition mode (FTIR-ATR), Raman spectroscopy and colorimetric measurements, together with the Micro-Hot-Table (MHT) method, a micro-invasive method widely accepted and applied in the field of conservation science. The advantage of this protocol of analysis is that it can easily be transferred to the assessment of historical parchments.

Materials and methods

Sample preparation

The experiment was carried out using calf parchment that was handmade at the Advanced Research for Cultural Heritage Laboratory (ARCH Lab) of the National Research & Development Institute for Textiles and Leather in Bucharest (INCDTP-ICPI) using technology inspired from ancient recipes. Two mock-ups sized $1 \times 3 \text{ cm}^2$ identified as PUV1 and PUV2 were taken from a whole parchment sheet and placed in a UVACUBE SOL 2/400F UV chamber (Dr. Hönle, Germany). To ensure uniform exposure of the parchment samples to the irradiation source, PUV1 was placed on the chamber plate with the grain side facing up, whereas PUV2 was placed with the corium side to the light source. The progress of molecular and structural alterations was monitored by analysing the samples before treatment and after 12, 83, 248, 392, and 748 h of exposure.

The mock-ups were exposed to artificial light irradiation using a Xenon Arc Simulator lamp with emitting radiation between 295 and 3000 nm, similar to the spectrum of solar radiation. The irradiance of the Xenon lamp was measured in both the visible and UV ranges by means of a UV-Meter Basic (Dr. Hönle, Germany). The measured irradiance value was around 162 W/m^2 , corresponding to the yearly natural outdoor conditions in Central Europe (ca. 5000 MJ/m^2 or 160 W/m^2 per year) [29–31]. Radiant exposure is defined as the time integral of irradiance ($\text{W} \times \text{s/m}^2 = \text{J/m}^2$) and the use of Xenon Arc lamp has the advantage of a very good correlation with natural sunlight in terms of spectral irradiance ($\text{W} \times \text{m}^{-2} \times \text{nm}$) [24]. Therefore, it can be assumed that the ageing performed at 162 W/m^2 is rather similar to the natural ageing caused by solar radiation [29–31]. As the temperature and relative humidity can not be customized in the chamber, a sensor AQL S500 (Aeroqual Limited, New Zealand) was used to monitor these parameters. In general, they were constant (i.e. about $52 \text{ }^\circ\text{C}$ and 30% RH) throughout the whole experiment. Under these conditions (low relative humidity and temperature) both the mechanism and the kinetics of light-induced deterioration are affected. This is not a drawback of the experiment, but rather an advantage because light never acts as a single factor, and its effect is intensified by high temperatures and low relative humidity as explained earlier [7, 8]. Recently, Carsote et al. [32] compared the behaviour of collagen in parchment and leather aged at $70 \text{ }^\circ\text{C}$ and 30% RH. They found that the simultaneous exposure to visible light irradiation induced an evident thermal stabilisation for short ageing times (i.e. up to 384 h) which then gradually declined.

It is known that the moisture content (water) in parchment changes depending on the external conditions to which it is exposed, and this also causes changes in its physical–chemical properties [33, 34]. For example, the temperature of denaturation of collagen in collagen-based materials is directly related to its moisture content [9, 15]. We can thus predict that parchment samples exposed at $50 \text{ }^\circ\text{C}$ and 30% RH will not undergo complete thermal denaturation in a short time, thus enabling a slow-ageing process more similar to the natural one. The concurrent action of high temperature, low relative humidity, and light irradiation can be expected to exacerbate the photo-oxidation process, thus these parameters were selected as an alternative to the more commonly applied thermal ageing.

Applied analytical methods

Infrared spectroscopy in attenuated total reflection mode (FTIR-ATR)

For the FTIR-ATR investigations, a Lumos FTIR-Microscope (Bruker Optics, Germany) was used. This instrument is equipped with a motorized germanium crystal

with an 8× magnification objective, an independent LED white light illumination and a high-resolution CCD camera. The ATR germanium crystal uses an internal pressure control with a resulting penetration depth of about 0.65 μm and analysed area of 100 × 100 μm².

Spectra were acquired using Opus software (Bruker Optics, Germany) in the range between 4000 and 370 cm⁻¹ by performing 64 scans with a resolution of 4 cm⁻¹. As parchment is intrinsically inhomogeneous, FTIR-ATR single-point mapping was performed by defining a grid of 9 measurement points (3×3) for both grain and corium sides.

Raman spectroscopy

Raman measurements were performed in dark room using the ProRaman-L-Dual-G of EnwaveOptronics (California, USA) which is a fully integrated and portable Raman spectrometer. As excitation source, a laser diode emitting at 785 nm (~350 mW) with a narrow linewidth of 2.0 cm⁻¹ was used. The fiber optic probe has a standard working distance of 7.5 mm and it is coupled with a Rayleigh filter. The detector is a cooled (-60 °C) CCD array detector with a spectral range in the Near-Infrared, from ~100 to 3300 cm⁻¹. The integrated microscope is equipped with a 1.3 Megapixel camera with in-line LED illumination. Six measurement points were visualized by means of a Leica 50x LWD (Long Working Distance) objective (spot size ~3.14 μm) on both corium and grain sides of the parchment samples. For a reproducible selection of the points, a self-made grid was designed, allowing measurements to be made every 4 mm. The data processing was done with Opus 7.0 software (BrukerOptics, Germany) by computing the spectral average between the acquisition points for each sample side. The obtained spectra were evaluated by comparison to a reference database of ISTA (ISTA¹ database).

Colorimetric studies

The SPM50 Gretag-Machbeth system (X-Rite Spectro Eye, Switzerland) was employed to acquire the reflection spectra of the samples in the measurement range from 380 to 730 nm. The samples were illuminated with a D65 light source for accurate colour perception and evaluation. Furthermore, a 10 Degree Supplementary Standard Observer, which is thought to be a closer approximation of human colour perception was used, and reflection was measured relative to the white standard of the instrument.

After calibrating the instrument with an internal-blank standard, 3 spots (spot diameter 1 mm) were measured on each sample side, for both, unaged and aged samples. The three-dimensional CIEL*a*b* (1994) colour space theory [35] was applied for the evaluation of the data.

Shrinkage activity by micro hot table (MHT) method

Thermal microscopy or MHT method is a combined technique based on the microscopic analysis of collagen fibres subjected to controlled heating (heating rate: 2 °C/min) in the temperature range (20–100 °C). Collagen fibres have the intrinsic property of shrinking when heated in an aqueous environment, reducing their length by up to a quarter. The hydrothermal stability and structural heterogeneity of collagen fibres is evaluated based on their shrinkage activity [36–38].

The parchment micro-samples (about 0.05–0.1 mg) were taken from both PUV1 and PUV2 samples aged for 748 h. Since artificially aged parchment generally shows homogeneous thermal behaviour, 2 fresh micro-samples were taken from the exposed surfaces: grain side of PUV1 and corium side of PUV2. The analyses were performed using MHT equipment composed of a Linkam LTS120 micro heating plate (Linkam Scientific Instruments) equipped with an automatic heating rate adjustment system and an SMZ 745 Nikon stereomicroscope. The shrinking motion was recorded by a Nikon D90 digital camera mounted on the microscope. Acquisition and processing of images were performed using the image MHT software.

Experimental, results and discussion

Infrared spectroscopy

The main FTIR-ATR absorption bands used to monitor the changes induced by the artificial, mixed-light-thermal ageing in the analysed parchment samples are listed in Table 1 which shows the three Amide bands which are most commonly used in infrared protein studies, namely Amide I, Amide II, and Amide III, and carbonyl/carboxyl band. As reported in the literature [39–43], the Amide I band found between 1600 and 1700 cm⁻¹ is indicative of the stretching vibrations of the peptide carbonyl group (C=O) coupled weakly with C–N stretching and N–H bending. It is sensitive to local order, and its exact position is determined by the backbone conformation and the hydrogen-bonding pattern within the protein molecule. The Amide II band occurs at 1500–1600 cm⁻¹ and is mainly derived from the C–N stretch along with N–H in-plane bending. Amide II is also sensitive to conformational changes. Finally, the Amide III band is found at 1200–1300 cm⁻¹. The vibrations responsible for this band are a complex mix of N–H bending and C–N stretching along with deformation vibrations of C–H and N–H.

¹ ISTA: Institute of Natural Sciences and Technology in the Arts, Academy of Fine Arts Vienna.

Table 1 List of the theoretical FTIR-ATR peak shift ranges of Amide I, Amide II, Amide III, and carbonyl/carboxyl compounds compared with the absorption maximums for the unaged parchment used in the experiments, and their relative vibrational modes

Peak shift ranges (cm ⁻¹) Literature [39–43]	Absorption peak frequency (cm ⁻¹) of unaged parchment	Vibrational mode
1600–1700	1635	Amide I (AI)
1500–1600	1552	Amide II (AII)
1200–1300	1239	Amide III (AIII)
1720–1750	1740	(C=O)

The Bruker Opus 7.0 software was utilized to compute the spectral average of the measurement points and, after normalisation and baseline correction, the area and the peak intensities of the relevant bands were calculated. The peak intensities of Amide I, Amide II, and Amide III bands were obtained by applying the “Opus J integration mode” using the highest absolute peak intensity within pre-defined ranges of interest. The “Opus D integration mode” allows the calculation of the area below the spectrum within a defined range of wavenumbers and was applied for calculating the area of the Amide I band. The obtained data together with the cumulative shift of Amide I and Amide II bands ($\Delta v_{(AI-AII)}$) are summarized in Table 2. Corresponding spectra are presented in Fig. 1.

In general, the variations of the shifts and intensities (expressed as peak height or peak area) are related to changes in the molecular structure of collagen, allowing us to identify and characterise the deterioration processes induced by mixed light-thermal ageing [44, 45]. One of the most pronounced effects of increased aging time observed in the FTIR-ATR spectra of the aged parchment was the cumulative displacement of the Amide I (AI) and Amide II (AII) bands expressed as the increase in their separation $\Delta v_{(AI-AII)}$. More precisely, for the samples PUV1 and PUV2, Amide II shifted progressively from 1552 cm⁻¹ to 1530 and 1532 cm⁻¹, respectively. It is shown in the literature that the absorbance of the Amide II peak decreased with the increase of UV radiation dose [21] and hydrogen atom transfer or structural scission of –N–H was assigned to this event [19]. The peak at 1530 cm⁻¹ represents a disordered structure of collagen, suggesting helix-random coil transformations [46]. With an increase of ageing time, Amide I shifted from 1635 cm⁻¹ to 1648 and 1646 cm⁻¹ for PUV1 and PUV 2, respectively. The prevalence of the component at 1632 cm⁻¹ was attributed by Sellaro et al. [47] to mechanical stretching. The Amide I component at around 1630 cm⁻¹ is the main component detected in spectra of fresh parchment [48]. Consequently, its shift towards 1646 cm⁻¹ can be assigned to a redistribution of Amide I components as a result of conformational rearrangements of collagen exposed to artificial ageing. This progressive separation could therefore be related to a progressive conformational re-arrangement of the

Table 2 Results of FTIR-ATR analysis for the samples exposed to artificial ageing for a maximum of 748 h: PUV1 (exposed on grain side) and PUV2 (exposed on corium side)

Exposure time (hours)	vAI (cm ⁻¹)	vAII (cm ⁻¹)	$\Delta v_{(AI-AII)}$	I(AI)	I(AII)	I(AIII)	a(AI)
PUV1 (grain side)							
0	1635	1552	83	0.27	0.206	0.093	144.97
12	1635	1552	83	0.32	0.238	0.073	149.40
83	1635	1546	89	0.09	0.066	0.057	157.96
248	1640	1546	94	0.25	0.195	0.082	169.64
392	1640	1541	99	0.14	0.103	0.057	175.83
748	1646	1530	116	0.09	0.068	0.040	185.81
PUV2 (corium side)							
0	1635	1552	83	0.313	0.227	0.089	142.86
12	1635	1549	86	0.330	0.235	0.079	148.87
83	1640	1544	96	0.270	0.182	0.071	156.60
248	1640	1540	100	0.244	0.173	0.09	168.17
392	1645	1536	109	0.228	0.152	0.076	169.96
748	1648	1532	116	0.160	0.110	0.063	180.41

The wavenumber values of Amide I (vAI) and Amide II (vAII), the cumulative peak shift of Amide I and Amide II (Δv), the intensity of Amide I, II and III bands (I(AI), I(AII), I(AIII)), as well as the area of the Amide I peak (a(AI)) are listed for each mock-up at selected times

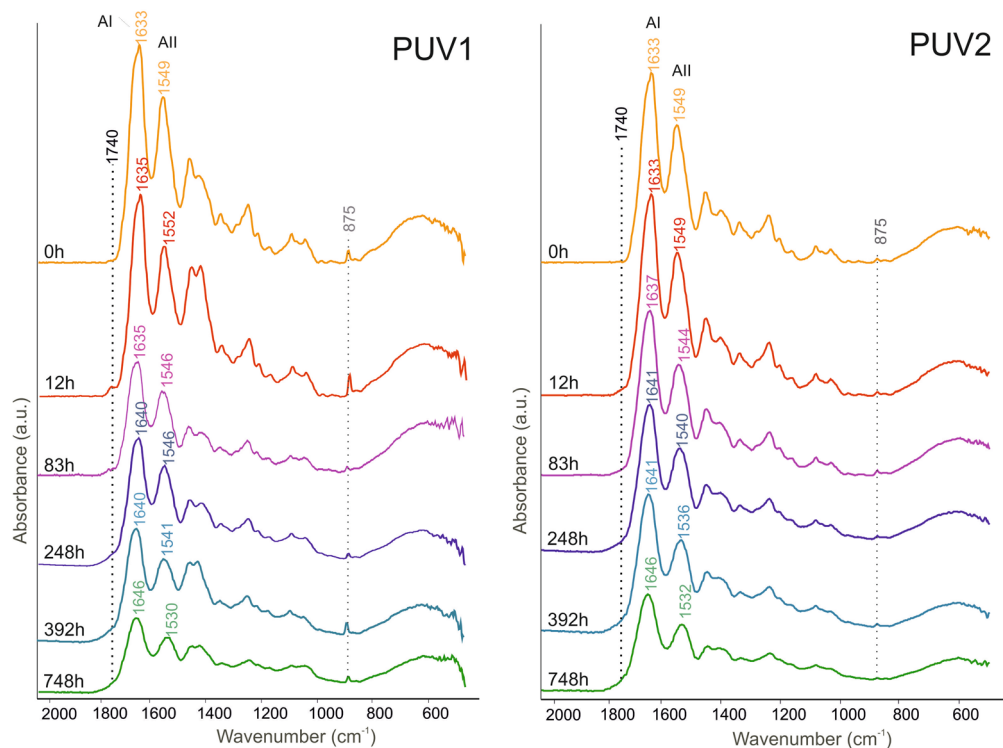


Fig. 1 FTIR-ATR spectra of PUV1 (grain side) and PUV2 (corium side) parchment samples exposed to artificial ageing for a maximum of 748 h. Amide I and Amide II appear shifted and with progressively lower absorption intensity. Amide III shows progressively lower absorption intensity as well. The shoulder-type band at 1740 cm^{-1} occurs after 12 h of artificial ageing

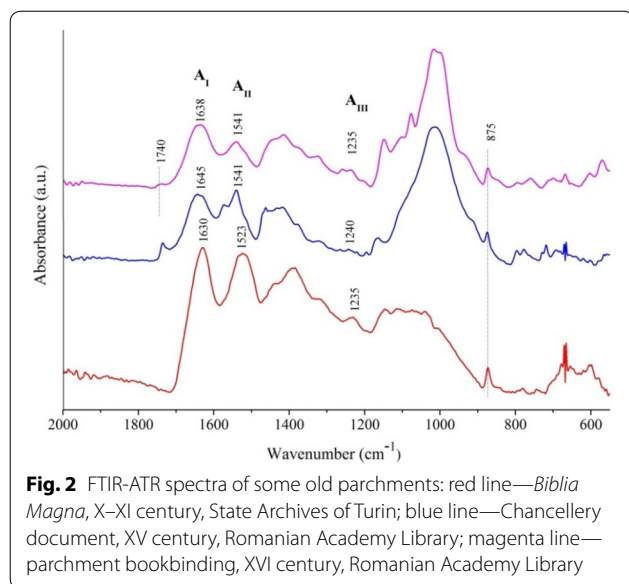
native collagen molecules due to the weakening and then cleaving of covalent bonds near the residues in the single chains damaged by photo-oxidation. The progressive micro-unfolding process leading to collagen-destabilised intermediate states maintaining the triple helical structure was reported by Miles et al. [22], during the helix-coil degradation of collagen by UV light. Such states of collagen having lower thermal stability were identified as “intermediates” in the mechanism of collagen deterioration in both parchment (chemically unmodified collagen) and leather (chemically modified collagen) [15, 32, 49]. As deterioration continues, and enough covalent bonds are cleaved, the stability between neighbouring crosslinks drops in some locations below a lower limit, and the collagen molecules become highly unstable allowing for gelatinisation and irreversible denaturation (helix-coil conversion) [22, 32, 49, 50].

Another effect of ageing was the progressive decrease in the intensity of the Amide I, Amide II, and Amide III absorption bands. This common behaviour of Amide I, Amide II, and Amide III bands confirms the progressive cleavage of peptide bonds in the helical chains of the collagen molecules with ageing [39, 44]. In addition, an absorption band in the range of $1720\text{--}1750\text{ cm}^{-1}$ ($\nu_{\text{C=O}}$)

assigned to carbonyl or carboxyl compounds and considered as a marker of oxidation of the polypeptide chains, was detected as a shoulder just to the left of the Amide I band [44, 45]. This band intensity can be related to the degree of the photo-oxidation process and was evaluated from the relative increase of the Amide I band area (a(AI)) reported in Table 2. We can thus infer that the photo-oxidation degree progressively increased with exposure time.

The presence of the band at 875 cm^{-1} is attributable to calcite derived from the liming step during the preparation of parchment. Its intensity is slightly higher on the grain side as expected.

It is also worth mentioning that similar spectral features were observed by the authors in old Italian and Romanian parchments (Fig. 2). For example, the shift of Amide II band towards 1523 cm^{-1} (Fig. 2, red line) was found when analysing the *Biblia Magna* from Novalesa Abbey (X–XI century) at the State Archives of Turin, Italy, while a cumulative shift of Amide I and Amide II bands (Fig. 2, blue line) was detected for some single sheet documents issued by the Stephen the Great Chancellery (XV century) owned by the Romanian Academy Library. Both spectra in Fig. 2 are also characterised by Amide III bands



with low absorption intensities. Additionally, almost half of the studied Chancellery documents showed a shoulder at 1740 cm^{-1} [51]. This shoulder (Fig. 2, magenta line) was also detected for some parchment book bindings (XVI century) from the Rare Books Collection of the Romanian Academy Library. All historical parchments presented the calcite absorption band as well.

To avoid possible effects due to inhomogeneity of the material and measure the strength of the linear relationship between ageing time and the spectral variables, statistical analysis of the results was performed. For this purpose, Pearson's correlation coefficient (r) was calculated for both samples PUV1 and PUV2 using the correlation formula (1) and considering as variables the

exposure time and Amide I peak area. This coefficient expresses the degree of concordance or discordance between the variables involved (X , Y), and it can range from -1.00 (perfect negative correlation) to $+1.00$ (perfect positive correlation) [52].

$$r_{XY} = \frac{\sum_{i=1}^n (X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^n (X_i - \bar{X})^2} \sqrt{\sum_{i=1}^n (Y_i - \bar{Y})^2}} \quad (1)$$

Samples PUV1 and PUV2 both displayed a positive correlation ($r_{\text{PUV1}} = +0.897$ and $r_{\text{PUV2}} = +0.905$) as well as a linear regression, with a high significance level ($p\text{-value} < 0.01$). In order to estimate the accuracy of the statistical model (also defined as significance), the ANOVA (Analysis of Variance) was also performed confirming thus the linear covariance of the two variables. As a consequence, a linear relationship between the two variables can be inferred for both samples, i.e. by increasing the exposure time (independent variable), the Amide I area (dependent variable) increases (Fig. 3) [52, 53].

Raman spectroscopy

Raman spectroscopy is a powerful and a non-invasive method for the study of changes in the secondary structure of collagen that complements the FTIR technique well. In Raman spectroscopy, the frequency of the Amide I and Amide III bands usually reveal changes in the secondary structure. This region, along with lipids and proline and hydroxyproline regions, are the three main regions frequently involved in the conformational changes of collagen structure [54, 55]. The bands in the Raman spectrum [54–57] used to monitor the artificial

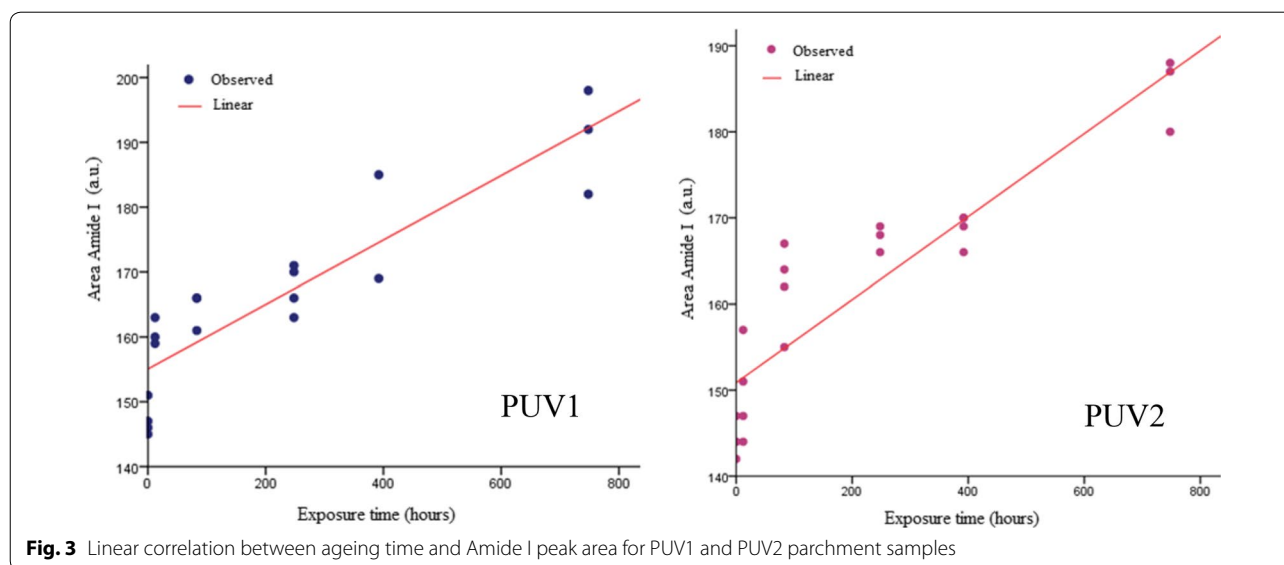


Table 3 List of the theoretical Raman shifts of collagen in parchment compared with those obtained for the unaged parchment used in the accelerated ageing experiments, and their relative vibrational modes

Raman shifts and ranges (cm ⁻¹) Literature [54–57]	Raman shift (cm ⁻¹) of unaged parchment	Vibrational mode
3030	3030	v (CH) olefinic
1660–1670	1660	v (C=O) Amide I
1550	1448	δ (CH ₂)
1230–1260	1233	Amide III
800–1000	800–1000	C–C stretching of Pro/Hydro ring C–C stretching of protein backbone

ageing-induced changes in the analysed parchment samples are listed in Table 3.

The main Raman spectral features of parchment exposed to artificial mixed light-thermal ageing are presented in Fig. 4. By comparing the spectra of PUV1 and PUV2, a slightly different ageing response was observed. In particular, for PUV1 (grain side), a spectral broadening occurred in the range from 1200 to 1700 cm⁻¹ after 748 h of ageing. A decrease of the intensity of the v(CH) olefinic band at 3300 cm⁻¹ was recorded as well. In contrast, for PUV2 (corium side), neither the spectral broadening, nor the decrease of the v(CH) olefinic band intensity were detected.

The Raman spectra of both samples PUV1 and PUV2 showed a shift of Amide I band from 1660 to 1648 cm⁻¹ and 1652 cm⁻¹, respectively, while a sharp decrease

of peak intensity was observed as the exposure time increased. In addition, the small shoulder at 1630 cm⁻¹ for PUV1 and at 1636 cm⁻¹ for PUV2 disappeared after 392 h of exposure. The position of the Amide I band at 1660 cm⁻¹ together with a shoulder at 1630 cm⁻¹ indicates the presence of the α-helix structure in the unaged material. The variations of these spectral features suggest a tendency to lose the α-helix structure during the artificial ageing [54, 55]. This is in agreement with the FTIR-ATR results and confirms the formation of collagen-destabilised intermediate states at shorter ageing time and their further conversion to less ordered structures as ageing time increases. However, it should be mentioned that a visible broadening of the Raman spectra in the Amides I, II and III range for PUV1 was registered. As described in the literature and observed by Edwards et al. [57], this behaviour could be assigned once more to the loss of the α-helix structure. This spectral aspect does not allow an objective evaluation of the Amide III band evolution during the ageing. Generally, it is known that when the Amide III band disappears, it is indicative that the hydrogen bonds that keep the α-helix stable are breaking, leading to a more disordered collagen structure.

Colorimetric measurements

The colorimetric results are given as coordinates in the three-dimensional CIEL*a*b*(1994) color space, where L* represents brightness on a scale from 0 (black) to 100 (white), a* ranges from green (– a*) to red (+ a*), and b* goes from blue (– b*) to yellow (+ b*) [35]. For each sample, the average between the acquisition points, the changes in the single-color components (Δa and Δb), in

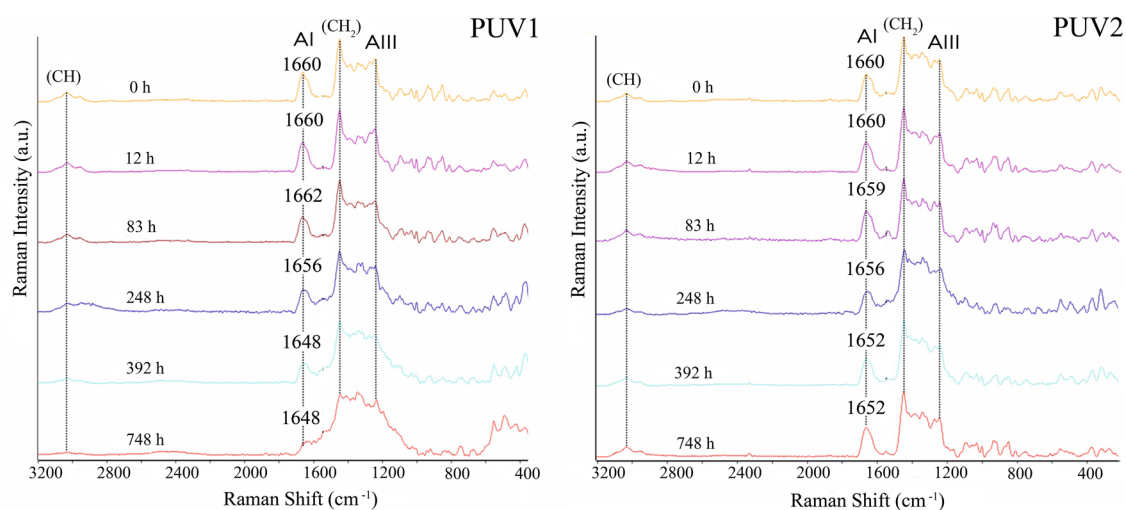


Fig. 4 Raman spectra of PUV1 (grain side) and PUV2 (corium side) parchment samples exposed to artificial ageing for a maximum of 748 h

Table 4 Overall colour variation (ΔE), changes in colour coordinates (Δa and Δb) and brightness (ΔL) for PUV1 (grain side) and PUV2 (corium side) parchment samples exposed to artificial ageing for a maximum of 748 h with respect to their unaged condition

Sample	Ageing time (h)	ΔL	Δa	Δb	ΔE
PUV1 (grain side)	12	-0.28	0.48	2.48	2.54
	83	0.08	0.23	0.93	0.97
	248	0.06	0.18	0.75	0.78
	392	-0.92	0.30	1.33	1.64
	748	-1.84	0.66	2.80	3.41
PUV2 (corium side)	12	-2.07	-0.27	0.98	2.30
	83	-1.47	-0.26	0.20	1.51
	248	-1.58	-0.35	1.01	1.91
	392	-1.88	-0.29	1.22	2.26
	748	-0.35	-0.26	2.71	2.74

brightness (ΔL), and the overall colour variation (ΔE) were calculated which is shown and summarized in Table 4.

The overall colour variation ΔE , is a metric for understanding how the human eye perceives colour difference. It can range between 0 and 100. For values ≤ 1.0 , the colour difference is defined as not perceptible by human eyes, whereas for values closed to 100 colours became the exact opposite. CIEL*a*b* (1994) calculates ΔE_{128} (polar opposites) [35].

The computed ΔE values for samples PUV1 and PUV2 show a variation during the ageing process from 0.97 to 3.41 and from 2.30 to 2.74, respectively. Such changes in the overall colour are also perceptible at a glance by human eyes. Concerning the brightness, a negligible variation (ΔL) was obtained for PUV1 (grain side) which showed a mild darkening only after 748 h of ageing. On the other hand, PUV2 (corium side) showed a decrease in brightness even after short exposure time. An overall slight decrease in brightness for parchment exposed to light (low-heat approach) ageing which was more pronounced on the grain side was also reported by Kern et al. [58]. The different values obtained for ΔE and ΔL between PUV1 and PUV2 could be related to the different responses to light-ageing of the protein constituting the grain and the corium sides of the parchment. As described by Herfeld [59], elastin and reticulin are additional components of the skin. Reticulins, in particular, show a higher concentration around the hair follicles (grain side). These proteins are less chemically stable than collagen, due to their chemical structure [60]. Therefore both photo-degradation, and the optical properties of the two sides of the

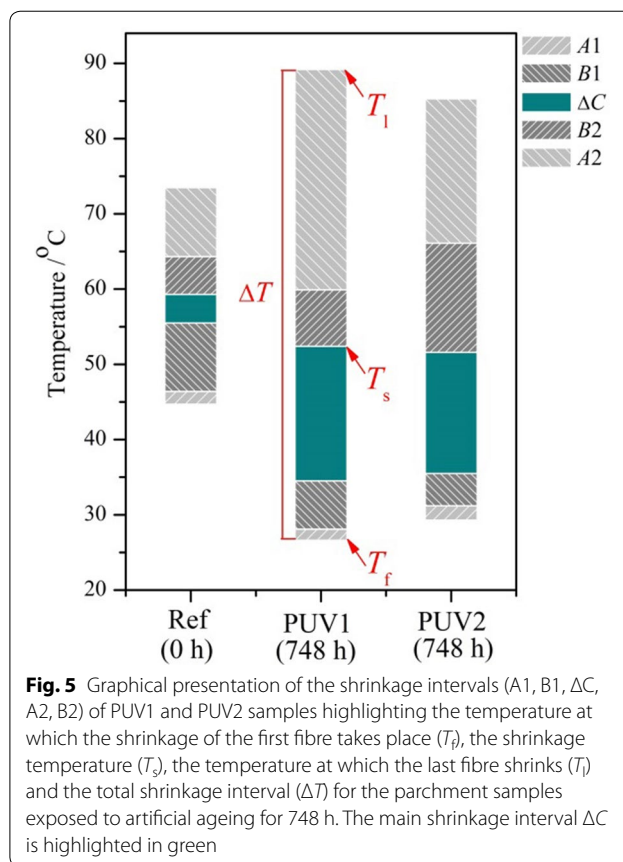


Fig. 5 Graphical presentation of the shrinkage intervals (A1, B1, ΔC , A2, B2) of PUV1 and PUV2 samples highlighting the temperature at which the shrinkage of the first fibre takes place (T_f), the shrinkage temperature (T_s), the temperature at which the last fibre shrinks (T_l) and the total shrinkage interval (ΔT) for the parchment samples exposed to artificial ageing for 748 h. The main shrinkage interval ΔC is highlighted in green

proteinaceous material are different, due to the differing concentration of these proteins in the skin matrix.

Micro hot table method

The degree of destabilisation of the organized collagen structure is well characterised by the shrinkage activity (MHT method) of collagen fibres in distinct temperature intervals. Generally, individual fibre shrinkage discretely occurs within A1, B1, B2, and A2 temperature ranges (Fig. 5), whereas the mass of fibres simultaneously shrinks within the so-called main temperature interval ΔC . The starting temperature of this interval is the shrinkage temperature, T_s . The total shrinkage interval (ΔT) is calculated as the difference between T_f (the temperature at which the very first shrinkage is observed) and T_l (the temperature of the very last observed shrinkage). The shrinkage activity levels off through B2 and A2 intervals.

New parchments are characterised by T_s values in the (55–60) °C range and a total shrinkage interval of about 10 °C [36, 37]. The shrinkage intervals and temperatures of the samples aged for 748 h show a significant departure from the corresponding values measured for the unaged

parchment (Fig. 4). For example, T_s and T_f dramatically decreased whereas ΔC and ΔT increased. T_s was about (35–36) °C while ΔT extended on a very large interval, i.e. 60 °C. Furthermore, the ΔC interval increased from 4 °C to 16–18 °C. This behaviour clearly indicates a strong thermal destabilisation and a high heterogeneity level, namely the presence of different collagen fractions with distinct thermal stabilities [7, 8, 12, 14, 15, 37], in agreement with the spectroscopic results. The very low values of T_f are due to the presence of highly unstable collagen populations, pregelatinized or even gelatinized [37]. On the other hand, the highly increased values of T_f could be assigned to a cross-linking induced by dehydration which draws the collagen molecules closer together [15, 49]. A rather similar thermal behavior was observed in some old parchment manuscripts and book-bindings. For example, a restored single sheet document from the Royal Library in Copenhagen and a bookbinding from the States Archives of Florence showed $T_s < 40$ °C, $T_f > 70$ °C and large ΔC intervals. In these samples, the majority of the collagen fibres presented various morphological alterations (i.e. frayed, unwind, flat, cracked, fragmented, shrunk, gel-like) until the total loss of fibrillar structure [37]. Another example is represented by the manuscript of the Marco Polo's will (1324, Marciana National Library, Venice), characterised by large ΔC and ΔT intervals, whereas T_s lies around 43 °C [61].

Conclusions

The FTIR-ATR and Raman techniques coupled with MHT method provided qualitative results that allowed us to characterise the ageing pattern of collagen in parchment exposed to light irradiation at 52 °C and 30% RH for increasing time. The deterioration starts with the formation of collagen-destabilised intermediate states which still maintain a prevalent helical structure. As ageing continues for longer times, these helical structures tend to progressively convert to disorganised structures, i.e. pregelatinized collagen and gelatin. The most indicative FTIR-ATR spectral changes occurring with ageing time were: (i) the increase in the separation of amide I and amide II bands, attributed to conformational rearrangements of the native collagen molecules; (ii) the decrease of peak intensities of Amide I, Amide II, and Amide III, suggesting the progressive cleavage of peptide bonds in the helical chains of the collagen molecules; (iii) the occurrence and increase of a shoulder at 1740 cm^{-1} related to carbonyl/carboxyl compounds, as a result of photo-oxidation process. The most indicative Raman spectral characteristics were represented by the Amide I shift to lower wavenumbers together with the disappearance of the shoulder at 1630 cm^{-1} , indicating a tendency to lose the α -helix structure. The shrinking activity of

collagen fibres in parchment aged for 748 h was characterised by very low values of both T_f and T_s (very low hydrothermal stability) and large ΔC (low fibre cohesivity) and ΔT intervals (very high structural heterogeneity). This behaviour reflects the presence of multiple collagen populations with distinct thermal stabilities and confirms the deterioration pattern revealed by FTIR-ATR and Raman techniques. In the FTIR-ATR results, no significant differences were observed between the corium and grain sides behaviour during artificially mixed light-thermal ageing, whereas differences in the Raman spectra were observed confirming a high degree of collagen-destabilised intermediate with loss of the α -helix conformation, especially for the corium side. Correlation of the results obtained using vibrational spectroscopies and thermal microscopy provided a reliable protocol for the evaluation of the changes induced in parchment at molecular and fibrous levels.

Abbreviations

MHT: micro hot table; FTIR-ATR: Fourier transform infrared spectroscopy in attenuated total reflection mode; T_s : shrinkage temperature; ΔC : shrinkage interval; ΔT : total shrinkage interval; UV: ultra violet; ARCH Lab: Advanced Research for Cultural Heritage Laboratory; INCDTP: National Research & Development Institute for Textiles and Leather in Bucharest; T: temperature; RH: relative humidity; LED: light-emitting diodes; CCD: charge-coupled detector; LWD: long working distance; CIE (CIE L*a*b*): International Commission on Illumination; AI: amide I; AII: amide II; AIII: amide III; Δa , Δb : single-color components; ΔL : brightness; ΔE : overall color variation; T_f : temperature at which the very first shrinkage is observed; T_l : temperature of the very last observed shrinkage.

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Authors' contributions

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Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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