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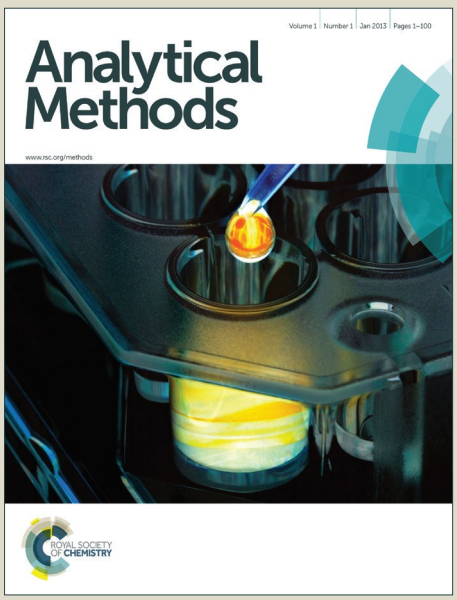

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ARTICLE

Degradation By-Products of Ancient Paper Leaves from Wash Waters

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Water cleaning of paper leaves is a preliminary step in many customary deacidificaction treatments of ancient books in conservation workshops. Usually, the washing solutions are considered "waste waters" and they are thrown away. In this work, an analytical protocol for the analysis of wash waters for the assessment of the conservation state of ancient books is proposed. Wash waters of leaves in different conservation conditions of a 16th-century-printed book were investigated using UV-VIS, IR, Raman, NMR, EPR, XRF, ESI-MS, ICP-MS, CE-PDA and elemental microanalysis. Analysis of the wash water extracts allowed one to identify a large number of degradation by-products, ranging from low-molecular mass organic acids up to cellulose oligomers. Reasons for the very different conservation conditions of the leaves emerged, indicating that wash waters analysis provides a deep insight into the conservation state and composition of leaves, as well as into degradation reactions occurred on them.

Introduction

The relevance of paper as a material can be hardly overestimated, since it has been for centuries the support for delivering human knowledge.[1] Although nowadays the digital storage of information is ever growing, it is widely recognized that a proper knowledge of the degradation reactions is essential in designing an efficient conservation treatment of paper-based documents.

As it is well known, paper is a material prone to degradation: the synergistic action of the hydrolytic scissions and the oxidative reactions of cellulose chains in the amorphous domains can deeply affect the integrity of the writing support over the time.[2-4] For this reason, most aged papers show the typical yellowing, brittleness and foxing stains, which are expected in the pages of the old books. On the other hand, some aged papers are found to be stable, remaining relatively white and strong over time.

As a matter of fact, historical paper is a complex material, where cellulose and sizing substances (like gelatin, alum and mineral fillers) interact in a complicated fashion: the variable quality of the raw materials as well as the evolution in the techniques of paper production over the centuries explain the reasons for the observed different conservation conditions. Great attention has been put on certain factors, such as gelatin content and trace metals content, which can play an important role in the paper conservation.[5] Gelatin was commonly used as a sizing material between 1400 and 1800 to prevent the bleeding of the inks and to improve the resistance of a sheet of paper to the sorption of water. Gelatin has been derived from the larger protein collagen, which is a major constituent of connective tissues in animals. Historically, the gelatin size was prepared by boiling animal tissues such as hide, skin and bones in water:[6] in the treatment, peptide bonds are broken randomly giving rise to shorter amino acids chains. Despite their length, all the polypeptides and oligopeptides obtained by gelatin hydrolysis show a characteristic high amount of hydroxyproline bound in the numerous amino acid triplets glycine-X-Y, where X and Y are most often proline and hydroxyproline, respectively.

The role of gelatin in the paper durability has been investigated just in the last thirty years. It has been proposed that gelatin may act as an acid acceptor, meaning that the component amino acids act as a buffer.[7-9] Studying the relationship between the actual condition of the historical papers and the amount of gelatin present, [9-11] Barrett showed that increasing amounts of gelatin were related to historical samples in better conditions, in terms of improved endurance and reduced discoloration. In 2003, Dupont proposed that gelatin is likely to behave as a sacrificial component in paper due to preferential hydrolysis of the protein molecules over those of the cellulose.[14,15] On the contrary, it is known that iron and copper salts cause damage to paper, depolymerising cellulose chains and increasing the discoloration of paper.[15-23] Metal ions such as Al(III), Cu(II), Fe(II), Fe(III) are commonly found in paper and are powerful catalysts for hydrolysis and oxidation. Particularly in the Fenton reaction, the redox couple Fe(III)/Fe(II) gives rise to very reactive hydroxyl radicals, which are seen as one of the most important causes of the oxidative degradation of cellulose.[24,25] Because of these reasons, their occurrence in paper (as traces left by the production processes or as complexes in iron/iron-copper-

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based inks applied on the sheets) is regarded as a warning for possible active degradation reactions in paper. This affirmation is confirmed by EPR studies, performed on ancient paper, which have demonstrated that very badly deteriorated sheets of paper often show strong Fe(III) and in some cases also Cu(II) signals.[26-29] The intricacy of paper degradation prompted numerous studies, most of them on modern cellulose samples, artificially degraded and analyzed usually with a limited range of techniques. Although these studies are very relevant, a comparison with naturally aged paper leaves is essential in designing improved artificial ageing methods and in evaluating their capacity of mimic natural ageing, especially over long ageing periods. On the other hand, very old paper documents are few and valuable, and usually a limited range of non-destructive techniques can be applied.

In this work, we show that the analysis of waters used to wash ancient book leaves could be an efficient way to characterize the "health" condition of an ancient book: washing the leaves with water is a common deacidification method, often carried out routinely in paper conservation ateliers, and wash waters are considered "waste waters". Through the application of a very broad multi-technique approach (IR, NMR, Raman, EPR, XRF, CHNS microanalysis, ICP-MS, CE-PDA, ESI-MS), we show that these waters are actually rich of paper degradation by-products.

Specifically, we characterized the water extracts obtained by washing the different leaves of a 16th century book, "De Divina Providentia", during a deacidification treatment.[30] According to simple visual inspection, the sheets showed different conservation conditions, ranging from very degraded to not degraded, and were distributed in a random way in the book. This is noteworthy because the sheets, being bound in the same volume, have been exposed to the same environmental conditions over the centuries. Therefore, this is a very rare case where a comparative study can be performed on naturally aged paper sharing the same environment over a long period of time, focusing in this way the attention on composition differences among the book leaves. The study shows that the analysis of the water extracts is able to explain the different degradation of the sheets, therefore indicating that the collection of wash waters in deacidification treatments of ancient documents could be a powerful way to get information about the "health" condition of the book, as well as general information about the natural ageing of paper.

Experimental

De Divina Providentia

"De Divina Providentia" is a printed book, written by Domanino Lattanzio, a Mantuan Carmelite friar, and published by the editor Francesco Osanna in 1592. The book belongs to the Mantua Biblioteca Comunale Teresiana, but other copies of it are conserved in many Italian libraries. The ink used for the printed book is lamp black.

Samples

Aqueous extracts were obtained by washing very degraded (1), slightly degraded (2) and not degraded (3) leaves of "De Divina Providentia". Leaf degradation was just qualitatevely assessed by means of visual inspection, taking the paper yellowing and simultaneous brittleness as main parameters for the evaluation of paper condition. Usually, higher levels of yellowing are related to lower DP in ancient paper.[11-13]

Each leaf was separately immersed in bidistilled water (250 mL) at 50°C and left to soak until the water temperature decreased to room temperature. This allowed extracting the water-soluble low molecular mass fraction formed in the leaves as a result of degradation reactions on cellulose and other paper components and induced by natural ageing. All the waters obtained from leaves with the same degradation condition were collected in a single batch (1 L for each type: very degraded, slightly degraded or not degraded), which was splitted for practical purposes in 50 mL aliquots. Each aliquot was labelled according to the level of degradation of the leaves (Table 1) and stored in the freezer (-20 °C) until use. For the analyses, small frozen amounts were melted and then quickly freeze-dried to obtain powders (numbered 1,2,3 for the respective extracts). It has been estimated that around 25 mg of powder can be obtained from 1 g of paper for the all three sample solutions.

Methods

The pH of three extracts (Table 1) was measured by using a standard pH meter (Inolab, Sigma Precision S.r.l.).

Table 1. General properties of the water Extracts

	Origin	pH	Colour
Ext 1	Very degraded leaves	4.64	Very brown
Ext 2	Slightly degraded leaves	5.92	Light brown
Ext 3	Not degraded leaves	7.67	Yellowish

The FT-IR spectra were collected on a Bruker Equinox 55 spectrometer, equipped with an ATR Sampling Accessory (diamond cell) and a MCT detector. A small amount of powder of each sample was deposited uniformly on the ATR plate. Each IR spectrum was the result of 64 scans with a 4 cm^{-1} resolution. In the figures, each IR spectrum has been normalized with respect to the maximum absorbance. The reference sample of Whatman paper was a piece 0.5 x 0.5 cm of Whatman filter paper n.44 (ashless, <0.01% ash). The UV-Vis spectra were acquired on a Cary 5 double beam UV-Vis-NIR spectrophotometer. The measurements were made on dilute solutions of all samples (20 µg of powder in 2 mL of bidistilled H_2O). Standard cuvettes with 1 cm pathlength were used. The ^{1}H NMR spectra and the ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectra were obtained on a Bruker Advance DMX-600 spectrometer, equipped with a three-axis gradient TXI probehead. The spectra were obtained at room temperature on solutions of the three samples in D_2O. A Renishaw in Via Raman microscope (633 nm exciting line) was used to perform measurements on the powders of all the samples. The Raman measurements of the Extract 1 were carried out with a 5x objective, 100% laser power (15 mW), 5 accumulations and 15 s scan time. The spectra of all samples show a strong fluorescence band from polyelectronic compounds formed over the degradation.[31] Fluorescence was quenched using the SERS technique to obtain the Raman spectrum. SERS signals were recorded in presence of naked

gold nanoparticles of about 20 nm obtained by laser ablation synthesis in solution (LASiS).[32,33] To the purpose of the present analysis, 40 mL of the gold nanoparticle solution (3 nM) were mixed with 10 mL of sample water solution and the obtained mixture was left to evaporate on a slide. The spectra were acquired directly on various parts of the stain using a 50x objective, 5% laser power (0.75 mW), 5 accumulations and 30 s scan time. The samples did not show variations before and after the laser application.

The continuous-wave EPR spectra have been acquired on a Bruker ER200 instrument, equipped with a TE102 probehead. Microwave frequency was about 9.5 GHz, modulation amplitude 0.2 mT and microwave power 2 mW.

XRF measurements were carried out on a XRF system constituted by an X-rays tube with Mb anode (operated at 20 kV and 1.2 mA in helium stream). The measurement time was set at 300 s.

Thermo Scientific Flash 2000 was used for CHNS microanalysis: it was constituted by a primary combustion column at 950°C, where few mg of sample were introduced in a separation column for the combustion gases and a TCD detector.

Calculations and analytical procedures were carried out using a specific software tool (Flash 2000 Eager Xperience), which provides for the use of K-Factor as a method of calibration and of BBOT2,5-Bis-(5-tert-butyl-benzoxazol-2-yl)-thiophen as standard.

ICP analyses were performed on an ICP-MS Agilent 7700, equipped with a fragmentation chamber in a helium stream and a signal correction system based on the use of internal standards.

Mass spectra (electrospray ionization source, ESI-ToF) of the Extract 3 were performed by operating in positive-ion mode by means of PerSeptiveBiosystem Mariner instrument (Framingham, MA). The sample was prepared by dissolving 0.2 mg of the Extract 3 powder in 250 µL of a solution 1:1 acetonitrile-water. The mobile phase used was a 1:1 acetonitrile-water solution added with 0.1% formic acid.

The mass spectra were run in two different mass ranges, from 100 to 4000 Da and from 100 to 1000 Da. As most of the peaks fell in the lowest mass range, only the lower mass range was deeply studied.

The investigation and the quantification of the low molecular mass organic acids (LMMOA) was performed by means of CE-PDA (capillary electrophoresis with photodiode array detection), using a method previously developed and applied on aqueous extracts obtained from artificially aged papers and 18th and 19th century books of various pulp composition.[34,35] Electrophoretic separations were performed by means of P/ACE MDQ capillary electrophoresis instrument (Beckman Coulter). The system operation and the data acquisition were performed using 32 Karat 5.0 software (Beckman Coulter). A bare fused silica capillary of 75 µm internal diameter and 65 cm length was used (55 cm effective length to the detector window). Prior to the sample injection, the capillary was rinsed by flushing 2 min with NaOH 0.1 M and 2 min with deionised water. The third step involves the conditioning of the capillary for 3 min with the electrolyte (buffer). The buffer was prepared with PDC 5 mM as background electrolyte (BGE) and CTAB 0.5 mM, at pH 5.6, adjusted with NaOH 1 and 0.1 M. The injection was made in the hydrodynamic mode by applying a pressure of 0.7 psi for 4.5 s. A separation voltage of -25 kV was applied to the anodic end. The resulting current was 20.1-20.5 µA. Indirect UV detection at 350 nm was used with reference at 200 nm and a band width of 20 nm with a photodiode array. More details on the methods can be found elsewhere.[33]

Before analysis, all the solutions used in the measurements (distilled water, 0.1 M NaOH solution, conditioning and separation buffer solutions and sample solutions), were filtered using a PTFE syringe filter (0.45 µm; Millipore).

The CE-PDA method was applied on the aqueous extracts of three leaf types; among other yet unknown peaks, all electropherograms showed the full range of organic acids used as models (Table 2).

Peak attributions in the electropherograms were based at first on the migration time (t_m). In order to double-check for the presence of the different acids, the model organic acids were added one at a time to the samples solutions (spiking) and the samples re-analyzed. Even in the case of slight variations in t_m, the differences in the intensity of a specific peak after the addition of the corresponding standard acid helped in the identification.

It was not possible to assign the peaks just by the simple comparison of the electropherograms of the samples and the standard acids, as a matrix effect affected the migration time of the different LMMOA in the samples.

The quantification of the individual acids in the three samples was carried out by extrapolation from the respective calibration curves, which were built using six solutions (5, 10, 20, 50, 75 and 100 ppm) of each model LMMOA (Table 2) with six levels of concentration produced using one individual stock solution. Each calibration point corresponds to three injections.

Results and discussion

The three water sample solutions respectively obtained by very degraded, slightly degraded and not degraded leaves of “De Divina Providentia” showed an intense color, indicating the high capacity of water to extract degradation by-products from the paper leaves. Every single solution has been analyzed by means of a wide range of spectroscopic techniques.

Extract 1

The UV-Visible spectrum of the Extract 1 (Figure 1, trace *a*) is characterized by an intense absorption in the ultraviolet range and a slow decrease in the visible range up to 700 nm: the broad absorption between 200 – 300 nm is normally attributed to n-π* and π-π* transitions, which are typical of unsaturated and possibly conjugated groups.[28, 34-37]

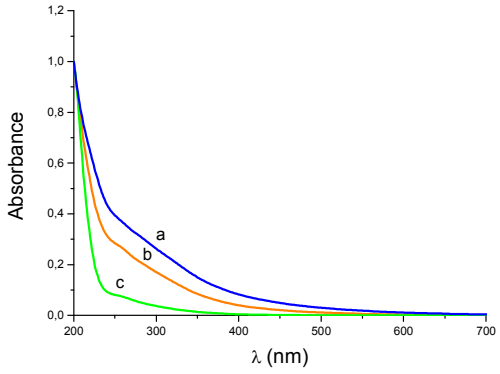

Figure 1. UV-Vis spectra of the Extract 1 (a), the Extract 2 (b) and the Extract 3 (c).

A shoulder at 280 nm, related to carbonylic and carboxylic n-π* transitions, can be also observed. The appearance of the long tail in the visible range suggests the presence of conjugated systems in the degradation products dissolved in the Extracts.[30,40]

The smooth profile of the FTIR spectrum (Figure 2, upper panel, trace *a*), as compared with the rugged profile of monomers, suggests the presence of cellulose oligomers composed of about eight to thirteen units, which represents the upper limit of their water solubility. This assumption has been confirmed by the preliminary HPLC-MS analysis.[30]

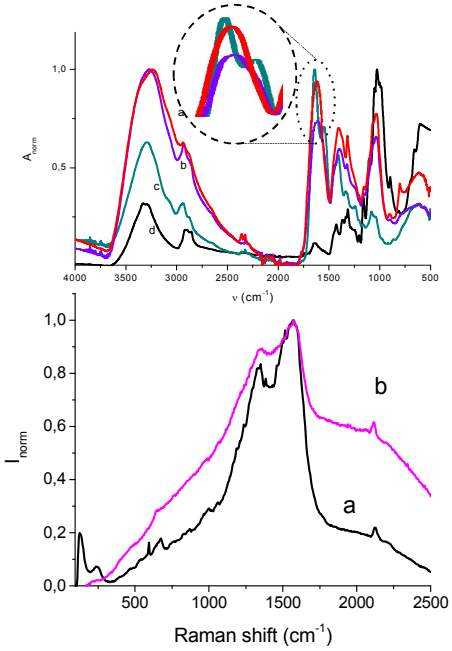

Figure 2. Upper panel: IR spectra of the Extract 1 (a, red), Extract 2 (b, purple), Extract 3 (c,green), Whatman paper (d). The region around 1600 cm^{-1} is magnified to show the doublet of the green trace c. Lower panel: Raman spectra of the Extract 1 (a) and Extract 2 (b).

The FTIR spectrum is mainly characterized by an intense band at 1630 cm^{-1}: in the literature,[30,38,39] this signal has been typically found in IR spectra of fully degraded papers and it has been identified as an envelope of vibrational modes assigned to various functional groups due to oxidation and dehydration reactions, like carbonyl and carboxyl groups (1700-1730 cm^{-1}),[41,42] carboxylates (1550-1610 cm^{-1}),[36,41] α-diketones (frequently present in their enol form) and/or α,β-unsaturated carbonyl and carboxyl groups (about 1660 cm^{-1}),[36-38, 43] C=C double bond (about 1640 cm^{-1})[36,43,44] and adsorbed water (about 1635 cm^{-1}) bound to carbohydrates through hydrogen bonds. The Raman spectrum of the Extract 1 shows at 1575 cm^{-1} the peak, recognized in literature as a "marker" for oxidative processes occurred on paper (Figure 2, lower panel, trace *a*). This signal is normally assigned to C=C symmetric stretching and O=C-O asymmetric stretching.[30,31] The presence of the carboxylate groups, identified in the FTIR and Raman spectra, is in agreement with the relatively high pH value of the Extract 1 (Table 1): it indicates that the carboxylic groups are mainly present in their deprotonated form, accounting for the high solubility of the Extract 1 only in a polar solvent like water.[45] Carboxylate species arise from low molecular mass organic acids, whose study in ancient and historical papers has been widespread in literature, and/or from oxidized glycosidic units of cellulose chain or, as in this case, oligomers (see below).

The HMQC spectrum (Figure 3, trace *a*) shows signals at 3.5-77 ppm and 4.1-71 ppm, which can be attributed respectively to the H_3-C_3 and H_5-C_5 spin systems of glucuronic acid or its salt glucuronate,[46-48] verifying the formation of oxidized glucose derivatives in the cellulose oligomers. This latter signal (4.1-71 ppm) can be due also to the H_2-C_2 spin system of gluconate.[49]

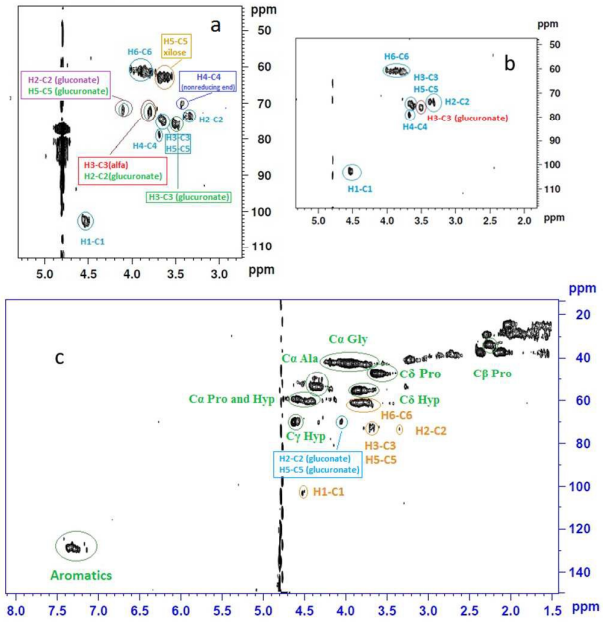

Figure 3. HMQC spectra of the Extract 1 (a), Extract 2 (b) and Extract 3 (c).

The cellulose oligomer degradation seems to proceed to the further step of decarboxylation, as pointed out by the peak at 3.42–62 ppm attributed to xylosic units (H_5-C_5). The decarboxylation processes, giving rise to xylosic units, are commonly observed in carbohydrate samples upon ozone treatment and for this reason they represent a reliable proof of oxidative paths occurring on cellulose.[50,51] In this case, it is unlikely that the xylosic rings can be attributed to residual hemicellulose, as the paper sheets, from which the sample was extracted, are composed by pure linen, a pure cellulose material usually without hemicellulose.

Finally, the correlation peak at 3.8–73 ppm is assigned to α-glucose (H_3-C_3(alfa)): this species occurs only if single molecules of glucose are present in the degradation by-products. Its detection provided a further proof of the occurred acidic hydrolysis on the cellulose chains of the book leaves. This datum has been also confirmed by the preliminary HPLC-MS.[30]

Low molecular mass organic acids (LMMOA), such as glycolic, formic, succinic, lactic and acetic acid, were investigated. Their identification and quantification were performed by means of a developed and largely tested protocol based on capillary electrophoretic analysis, CE-PDA: their concentration in each Extract is reported in Table 2.

A deep study of the metal content of the sample has been performed as well: a strong effort has been dedicated on the detection and quantification of metal ions, with a particular attention on Fe(II)/Fe(III) and Cu(II) species.

Table 2. Concentration (ppm) of acetic (A), formic (F), succinic (S), glycolic (G) and lactic (L) acid measured by CE-PDA.

	A (ppm)	F (ppm)	S (ppm)	G (ppm)	L (ppm)
Ext 1	4.5508	2.5864	7.4143	0.8176	3.1986
Ext 2	4.1926	2.2248	5.2780	0.6413	2.9259
Ext 3	6.3475	4.0322	8.1977	1.6300	4.6359

The species can originate from the metal-based ink applied on paper and/or from the water and the metallic tools used in paper manufacturing.

Concerning "De Divina Providentia", the painting medium used in the text is lamp black, ink composed by fine soot collected from incompletely burned carbonaceousmaterials. This indication implies that the possibly detected metal ions in our samples were directly present in the paper matrix. EPR, ICP-MS and XRF analysis were carried out on the Extract 1 in order to probe the type, the coordination and the amount of metal ions.

The EPR spectrum of the Extract 1 (Figure 4, trace *a*) is characterized by a sharp line around 150 mT and by a weaker broad line, between 300-350 mT, resolved in six hyperfine lines.

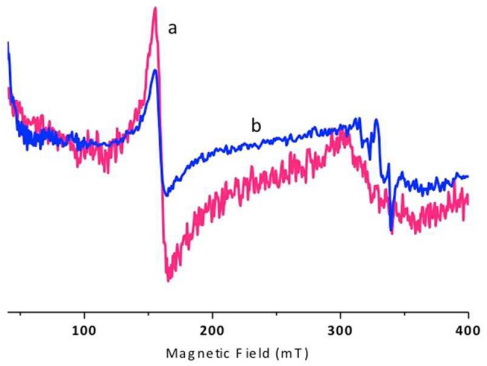

Figure 4. EPR spectra of the Extract 1 (a) and Extract 2 (b)

The sharp line is due to Fe(III) complexes far from the cubic symmetry ("rhombic site"), attributed generally to Fe(III) strongly bound. A second broad line around 350 mT is generally observed in ancient papers, due to Fe(III) in quasi-cubic symmetry and ascribed to hydrated Fe(III) or Fe(III) weakly bound to cellulose. This line is lacking in our samples. The six lines between 300-350 mT are due to Mn(II).[26-29] No Cu(II) signals can be observed.

The significant presence of Fe was also confirmed by XRF (Table 3) and ICP –MS (Table 4), which, unlike EPR, allowed also the detection of Al species. The presence of Al(III) ions in the wash waters could indicate the use of alum during the sizing procedure of the sheets, from which the Extract 1 was obtained. Alum is recognized as a paper degradation agent, due to its catalytic action on hydrolysis, and its presence could explain the higher degradation level of these leaves.

However, the absence of detectable sulphur signal through XRF and the very low amount of sulphur according to elemental analysis (Table 5) suggest that Al might derive from alternative sources, e.g. from rags maceration. If Al derived from rock alum $KAl(SO_4)\bullet 12H_2O$ (which was the alum used before the 19th c.) a comparable amount of Al and S would be obtained, which is not the case. On the other hand, Al could be released easily from the silicate stones of the tub used during alkaline leaching (with lye) of rags, a step often used in rags maceration.

Table 3. Results of XRF analysis (elements found)

Ext 1	Al	Si	P	Cl	K	Ca	Cr	Mn	Fe	Cu
Ext 2			P	Cl	K	Ca			Fe	Cu
Ext 3			P	Cl	K	Ca			Fe	

Table 4. Results of ICP-MS analysis

	Al (ppm)	Fe (ppm)	Zn (ppm)
Ext 1	66.6 (1.7)	8.8 (2.5)	74 (24)
Ext 2	24.0 (1.7)	5.3 (2.5)	8 (24)
Ext 3	37.1 (1.7)	2.9 (2.5)	47 (24)

Table 5. Results of elemental analysis

	Extract 1	Extract 2	Extract 3
C	38.14%	37.67%	38.97%
H	5.08%	5.19%	5.76%
N	4.53%	3.93%	10.19%
S	0.16%	0.26%	0.34%

Extract 2

The Extract 2 shows a similar composition of the Extract 1. However, the lower intensity of the IR signal around 1600 cm^{-1} (Figure 2, upper panel, trace *b*), the lack of NMR peaks attributed to α-glucose and to xylose (Figure 3, panel *b*), the lower intensity of the band at 280 nm in the UV-VIS spectra with a shorter tail in the visible range of the absorption (Figure 1, trace *b*) and the slightly lower amount of LMMOA (Table 1) indicate that the Extract 2. underwent milder hydrolysis and oxidation reactions than the Extract 1. This assumption has been also confirmed by the elemental analysis results (Table 5). According to these, the formulas of compounds in the Extracts 1 and 2 are respectively: $C_{3.18}H_{5.09}N_{0.32}O_{3.26}$ and $C_{3.13}H_{5.19}N_{0.28}O_{3.31}$. In a previous work,[30] analyses demonstrated that the Extract 1 was mainly constituted by cellulose oligomers.

The similar spectroscopic features of the Extract 2 suggest that an analogue situation holds. Therefore, we can consider sugars and glucose-derivates as main components of the two samples, neglecting the N content, and we can round the two empirical

formulas to the closest integers, as $C_{64}H_{102}O_{65}$ for the Extract 1 and $C_{63}H_{104}O_{66}$ for the Extract 2. These latter formulas correspond approximately to a ten-ring chain in a cellulose polymer. The calculated degree of unsaturation for this ten-ring chain is 14 for the Extract 1, while it is 12 for the Extract 2. Taking into account that a ten-ring cellulose-type chain corresponds to 10 unsaturations, and assuming that the other possible unsaturations are only due to double bonds (C=C and C=O), we estimate every ten rings an average of 4 double bonds for the Extract 1 and 2 double bonds for the Extract 2.

The compounds in the Extract 1 show one unsaturation every 2.5 pyranose rings, while in the Extract 2 they bear one unsaturation every 5 rings. This result is in agreement with the spectroscopical data, indicating that the Extract 2 arises from paper with a minor extent of degradation than the Extract 1.

Extract 3

The UV-Vis spectrum of the Extract 3 (Figure 1, trace *c*) does not show the long tail in the visible range, and its carbonyl and carboxyl band is less intense. The FTIR spectrum of the Extract 3 (Figure 2, upper panel, trace *c*) presents an intense doublet at 1630 cm^{-1} and 1540 cm^{-1} (see the magnified inset in Figure 2, upper panel), which is typical of "amide I" and "amide II" vibrational modes, and it is indicative of the presence of peptides, most likely coming from the gelatin used for sizing.

These results are supported by the HMQC data (Figure 3, panel *c*): the NMR spectrum is characterized by a large amount of peaks, mostly assigned in the literature to the amino acids typically present in the collagen structure, i.e. glycine (33% in animal gelatin), alanine (10%), proline (12%) and hydroxyproline (10%.) residues.[52,53] Some cellulose signals are present, as well, while the correlation peak at 7.3-129 ppm could indicate the presence of aromatic species, probably phenylalanine (~ 1.4%) and tyrosine (~ 0.5%).

An ESI-MS characterization study was performed in order to investigate more deeply the nature of the oligopeptides present in the Extract 3. As the spectroscopic results have shown the presence of protein in the Extract 3 and,in particular, they have identified hydrolyzed gelatin (collagen) as the main component of the sample, all the ESI-MS m/z values, reported in Table 6, were attributed considering the collagen structure and amino acids composition.

The elemental analysis (Table 5) shows that the Extract 3 has the highest N ratio among the three samples. The N ratio calculated from the molecular formula of collagen is about 15%. The N ratio of the Extract 3 is lower (10%) because of the presence of a small amount of cellulose oligomers, as suggested by the NMR analysis, but confirms the high content of protein in this sample.

Therefore, the spectroscopic and chemical analyses indicate that the cellulose structure of the leaves, from which the Extract 3 was obtained, is almost undamaged, as cellulose degradation by-products were not the main components in the wash water of this leaves. The EPR spectrum of the Extract 3 (not reported) does not present signals assignable to Fe(III) or Cu(II). Accordingly, the ICP-MS values (Table 4) show that the Extract 3 presents the lowest iron content, and the content of aluminum, a metal active in catalyzing hydrolysis, is lower in the Extracts 2 and 3 than in 1.

Generally, the different amount and type of metal ions detected in the Extract 1 and 2 can explain the different degradation level of the leaves, from which the Extracts were collected. On the other hand, the Al content of the Extract 3 is very close to the one detected in the Extract 2, while the Fe amount is slightly lower (mostly present as Fe(II) or iron oxides, as no isolated Fe(III) EPR signal was detected). As the metal amount in the Extract 3 is very close to the Extract 2 one, the highly persevered state found in the leaves, from which the Extract 3 was obtained, is mainly due to the high amount of gelatin used in the leave sizing, confirming in this way the literature data.[9-11,14,15] The data, as a whole, suggest that gelatin, by permeating the paper fibers, is more directly in contact with air and polluting agents, and thus can somewhat protect the fibers from oxidation and acidic hydrolysis by acting as a sink for the reactants. It is also possible to deduce that these leaves belonged to a different paper ream from that of the other leaves, from which the Extracts 1 and 2 were obtained.

Table 6. Results of ESI-MS in positive mode of the Extract 3

Observed Mass	Type	Calculated Mass	Hypothetic Composition
173.0939	$[M+H]^+$	173.0999	1 G, 1 P
203.5651	$[M+H+Na]^{2+}$	203.0941	3 G, 2 P
211.5511	$[M+H+Na]^{2+}$	211.0916	3 G, 1 P, 1 Hyp
224.0763	$[M+2H]^{2+}$	224.1144	3 G, 1 A, 1 W
232.0645	$[M+H]^+$	232.1406	3 A
270.1667	$[M+H]^+$	270.1562	1 G, 2 P
317.2212	$[M+H]^+$	317.1606	2 G, 1 A, 1 Hyp
354.1328	$[M+Na]^+$	354.1571	2 G, 3 A
362.1012	$[M+2H]^{2+}$	362.1933	6 G, 1 A, 3 P
365.1322	$[M+Na]^+$	365.1582	2 G, 1 A, 1 P
391.2826	$[M+H]^+$	391.1763	2 G, 1 P, 1 F
401.2001	$[M+H]^+$	401.2002	5 G, 1 P
489.6904	$[M+H]^+$	489.2315	4 G, 1 P, 1 F
533.2515	$[M+Na]^+$	533.2553	2 G, 2 A, 1 P, 1 Hyp
570.1923	$[M+H]^+$	570.2777	4 G, 1 P, 2 Hyp
618.2118	$[M+H]^+$	618.27775	6 G, 1 A, 1 W
698.2758	$[M+H]^+$	698.3435	2 G, 5 A, 1 P, 1 Hyp
748.4337	$[M+Na]^+$	748.3568	3 G, 3 A, 1 P, 2 Hyp

A further hypothesis could suggest that the gelatin has protected the cellulose chain from the oxidation,[11] avoiding the formation of carboxylate groups: indeed, these latter are able to form stable distorted complexes with Fe(III), allowing it to act as a catalyst of the degradation reactions.

As well, these results are consistent with the measured pH values (Table 1), as the most degraded leaves, showing a more acidic pH, dissolve in the extract solution a larger amount of metal ions than the least degraded leaves.

The difference in pH among the three samples also explains the upstream results of the PDA-CE analysis: the quantification results (Table 2) demonstrated that the aqueous extracts obtained from the book sheets in good conservation condition (Extract 3) had the highest yield in total LMMOA degradation products. These unexpected results could be due to the different pH of the three

samples: given the pKa of the LMMOA, it is likely that in the paper the equilibrium was shifted to the deprotonated form in the Extract 3 more than in the other two extracts. Carboxylates are largely nonvolatile as opposed to carboxylic acids. Acids in the Extracts 1 and 2 may have off-gassed continuously from the leaves upon their production even before the paper was washed, whereas in Extract 3, being the carboxylate ions predominant, they could stay *in situ* and could thus be extracted during the washing.

Conclusions

In this work, it has been demonstrated that wash waters used in common deacidification treatments of books in restoration workshops are rich in degradation by-products. The study has also shown that the analysis of these by-products could provide useful indications on the "health" of an ancient book. However, we must point out that a full validation of the method will require to relate the type of byproducts in the water extracts with composition and degradation level of the leaves: the level of degradation in the leaves should be reliably assessed through quantifiable parameters (DP, yellowing index, etc.), which was unfortunately impossible in the present case. Also, compositional parameters (like gelatin content, metal ions, etc.) are relevant, and should be assessed on the leaves before and after the treatement. E.g., the presence of gelatine in the less degraded leaves was guessed on the basis of the by-products from the water extracts, but its actual presence on these leaves should be confirmed by direct examination, as well as its absence on the most degraded. Finally, the study should be performed on a statistically significant number of ancient books. Once validated, the proposed protocol could be very useful, since it perfectly integrates into the book conservation workshop, which acquires in this way an added value, as it slows down the book degradation but, at the same time, recovers waste material which can give precious information about the degradation pathways responsible for book deterioration.

Since many restoration workshops are present in Italy and in Europe, and a large amount of leaves from ancient books are treated in this way, a planned collection of wash waters from the different ateliers could provide a large database of degradation by-products.

The main by-products formed on the most damaged leaves of the book "De Divina Providentia" are strongly oxidized and unsaturated cellulose oligomers, while the analyses performed on the water extracts of the not degraded sheets have further confirmed and highlighted the protective role of the gelatin towards the paper over the centuries. The type and amount of metal ions detected in the Extract 1 and 2 reflects the high degradation level and dark color shown by the leaves, from which they were extracted. However, the amount of metal ions detected in the Extract 3 is quite high, once correlated to the good conservation state of its leaves, underlining the benefices of a proper sizing toward the preservation of ancient paper. In general, the differences in composition between the most degraded samples and the not degraded one suggests that the sheets, from which the analyzed solutions were extracted, could belong to different reams. This is not the only possible explanation, but it is sensible, as generally several reams of paper were used for the assembly of a single book.

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