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Collagen-based proteinaceous binder-pigment interaction study under UV ageing conditions by MALDI-TOF-MS and principal component analysis

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This study focuses on acquiring information on the degradation process of proteinaceous binders due to ultra violet (UV) radiation and possible interactions owing to the presence of historical mineral pigments. With this aim, three different paint model samples were prepared according to medieval recipes, using rabbit glue as proteinaceus binders. One of these model samples contained only the binder, and the other two were prepared by mixing each of the pigments (cinnabar or azurite) with the binder (glue tempera model samples). The model samples were studied by applying Principal Component Analysis (PCA) to their mass spectra obtained with Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS). The complementary use of Fourier Transform Infrared Spectroscopy to study conformational changes of secondary structure of the proteinaceous binder is also proposed. Ageing effects on the model samples after up to 3000 h of UV irradiation were periodically analyzed by the proposed approach. PCA on MS data proved capable of identifying significant changes in the model samples, and the results suggested different aging behavior based on the pigment present. This research represents the first attempt to use this approach (PCA on MALDI-TOF-MS data) in the field of Cultural Heritage and demonstrates the potential benefits in the study of proteinaceous artistic materials for purposes of conservation and restoration. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: MALDI-TOF-MS; principal component analysis; pigment-proteinaceous binder interaction; FTIR protein conformational study; collagen

INTRODUCTION

The use of organic materials in artworks is well known; for example, substances like oils, gums, proteins or waxes are traditional binders in diverse painting techniques.[1,2] In particular, proteinaceous binders have been used since antiquity, e.g. egg, glue from animal skin, or milk in so-called tempera painting.[3,4] Although some research has focused on the identification of the different types of proteins, the ageing processes of proteinaceous paint binders are little studied due to their complex composition and structure.[5–11] Moreover, few studies have examined the role of inorganic pigments in the ageing processes of these binders.[11–14] Particularly, the characterization of proteinaceous binders by GC analysis of amino acids has demonstrated changes in the amino acid compositions due to the presence of pigments.[12,13] Specifically, alkyl and imino-substituted amino acids were much less affected by pigments and ageing than other amino acids.[12] The presence of calcium carbonate caused a decrease in both amino acid asp and glu in egg and milk tempera, while no effects were observed for the glue tempera.[13]

Up to now, the study of proteinaceous binders has been mainly carried out by Liquid Chromatography (HPLC), Gas Chromatography, Fluorescence Spectroscopy, Raman Microscopy (RM) or Fourier Transform Infrared Spectroscopy (FTIR).[2,5,8,9,11,15] These chromatographic methods characterize the proteinaceous binders through the determination of the derivatized amino acids previously obtained by hydrolysis of the proteinaceous material.[4–7,12,13] Commonly, the use of FTIR to study proteins has been applied to gain molecular and conformational information via the observed shift of the amide I maximum. Hydrogen bonds linking the peptide bond NH of a glycine residue with a peptide carbonyl (C O) group in an adjacent polypeptide help hold the triple-helical structure of collagen.[16] These bands, specifically the amide I band, are conformationally sensible, and are often used to determine protein secondary structure caused by intramolecular and intermolecular hydrogen bonding of amide groups.[17–20] In particular, the amide I band (carbonyl stretching vibrations)

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gives information on α-helix (1647–1660 cm^{-1}), β-sheet (1615–1640 cm^{-1}), β-turn (1660–1680 cm^{-1}), β-antiparallel sheet contents (1681–1692 cm^{-1}) or unordered structures such as random coils (1646–1641 cm^{-1}).[18–20] Moreover, the conformational changes observed could suggest the formation of non-covalent interactions and ionic bonds.[21]

Similarly, as occurs in many scientific fields, the application of mass spectrometry (MS) plays a prominent role in the characterization of organic materials from artworks.[22] The main ionization techniques used in the study of samples from Cultural Heritage are Electron Ionization (EI), Chemical Ionization (CI), Atmospheric Pressure CI, Electrospray Ionization (ESI), Desorption ESI and Matrix-Assisted Laser Desorption Ionization (MALDI). An interesting overview of mass spectrometric based techniques applied in the Cultural Heritage field can be found elsewhere.[23]

Although EI is probably the most used ionization technique in the study of historical objects[23], MALDI coupled to Time of Flight Mass Spectrometers (MALDI-TOF-MS) has gained popularity during the last decade in the study of organic materials of artworks, mainly due to its microdestructive character, the minimum sampling required and its high identification ability. Thus, this technique has been proposed to study fresh and photo-aged di- and tri-terpenoid varnish resins.[24] MALDI-TOF-MS has demonstrated its use when investigating the lipidic materials in egg tempera paint dosimeters.[14,25] Nevertheless, the analysis of proteinaceous binders is the most important application of this technique nowadays.[26] In this context, mass spectrometric proteomic approaches have been used to characterize samples from the field of Cultural Heritage. These approaches involve the protein specifically cleavage with a protease under defined conditions, the obtaining of the mass spectrum of the peptide mixture (peptide map or peptide fingerprint) and protein identification by comparing the peptide map with suitable data bases. These studies focused on the analysis of the main proteinaceous binders used in historic paints (whole egg, egg white, egg yolk, animal glues, milk casein, etc.) by peptide mass mapping or identification of the amino acid sequences.[14,27–30] Recently, a simple protocol, based on Bligh-Dyer extraction followed by MALDI-TOF-MS analysis for fast identification of paint binders in single microsamples has been proposed.[31]Also, the identification of animal glue species in gilt sample has been proposed using FTICR-MS.[32]

Multivariate techniques such as Principal Component Analysis (PCA) are widespread in the study of materials used in the Fine Arts field and, particularly, in artistic painting samples. Techniques such as T-FTIR, Diffuse Reflectance FTIR Spectroscopy, GC−MS and HPLC[2,11,33–36] provide vast amounts of data to be investigated by multivariate analysis. Similarly, mass spectra give a great quantity of data suitable to be analyzed by these multivariate techniques. In the case of PCA performed on mass spectra, it allows investigation of trends in the studied samples, and relationships or differences between samples that can be related to the research objective.

On the other hand, it is well known in the field of Cultural Heritage that over time, historical paintings transform due to exposure to diverse environmental conditions, which usually promote reactions between painting components. Among environmental threats, daylight radiation that includes visible (vis), IR and ultra violet (UV) light is important in promoting chemical changes and color modifications of paintings that cause irreversible aesthetic damage. From all these wavelengths, the most harmful ones are those of UV. Thus, an increasing number of studies on the effects of UV radiation on art materials including painted artworks (model samples) are published in the specialized literature.[11,37,38] Indeed, there is growing scientific focus on this topic based on demand for the application of UV laser technology to the treatment (cleaning, consolidation, testing, etc.) of surface paintings and their compositional characterization (with e.g. RM that may induce the so called 'laser-induced degradation' of the irradiated painting materials).[39–41]

In this paper, an accelerated UV test was carried out on painting model samples of pure rabbit glue (PG) and on two mixtures with historical inorganic pigments (cinnabar and azurite). Animal glue has been used as a binder since ancient Egypt to elaborate historical glue tempera paintings.[3] Cinnabar was a well-known pigment to the Romans, and its use was related to the Almaden mines (Spain) which were the most important source of mercury in the world. Azurite was used especially throughout the Middle Ages and the Renaissance.[42] To study the ageing of these glue-based painting samples, we propose an innovative approach based on the application of PCA to the mass spectra obtained by MALDI-TOF-MS. The complementary use of FTIR spectra to evaluate changes in the conformational structure of the proteinaceous binders after the UV ageing tests has proved useful. The PCA corroborated the alteration process of the proteinaceous glue binder and allowed estimation of the role played by the mineral pigments when mixed with the glue binder. Additionally, the analysis of the sample FTIR spectra helped to detect conformational changes in the secondary structure of collagen during photoageing.

EXPERIMENTAL

Materials

The standard protein binder was rabbit glue (hydrolyzed collagen) purchased from *Productos de Conservación* (Madrid, Spain). Cinnabar (HgS) was acquired from *Caremi Pigmentos*, reference PR106 (Seville, Spain) with particle size $<30\,\mu m$.[40] The azurite pigment ($Cu_3(CO_3)_2(OH)_2$) was purchased from *Kremer Pigments GmbH & Co*. KG (Barcelona, Spain). Pigment reference is 10200, natural azurite (CI: PB 30.77420) with particle size 80–120 μm.

All reagents were of analytical reagent grade unless stated otherwise. Reverse-osmosis type quality water (purified with a Milli-RO plus Milli-Q station from Millipore) and HPLC quality were used throughout. Trypsin was supplied by *Promega Corporation* (UK). Acetonitrile, ammonium hydrogen carbonate, trifluoroacetic acid and 2,5-dihydroxybenzoic acid (DHB) were supplied by *Sigma* (Prague, Czech Republic).

Model samples

The painting model samples (i.e. paint reconstruction samples) were prepared according to Old Master recipes to obtain standards similar to tempera paints used by medieval artists.[3,11] The painting model sample of pure glue (PG) was prepared as follows: 10 g of rabbit glue was diluted to 10% by w/v in deionized water (MilliQ-System Millipore, Bedford, MA) by gently adding the glue to the water during 24 h, and stirring periodically. Next, the obtained blend was gradually heated in a bain-marie water bath below 50 °C to obtain a homogeneous mixture. Then, the glue was carefully spread on a glass slide with a paintbrush in six successive fine coats, each applied after the previous layer had dried to a constant weight using a gel air dryer system.

Pigment-rabbit glue mixture model samples (glue tempera samples) were prepared by blending each pigment (i.e. cinnabar, Cin-G; and azurite, Azu-G) with the glue. The particular composition of the glue tempera samples (pigment/binder) was 5.2/1 (w/v) in order to obtain the combination according to Old Recipe.[11] Once the mixtures were prepared, each was spread and dried on glass slide in five fine coats as indicated above for the PG model samples.

UV accelerated ageing test

The painting model samples were aged in a UV accelerated test chamber for 3000 h. A high-speed exposure unit SUNTEST CPS, Heraeus (Hanau, Germany), equipped with a Xenon lamp was used for the ageing test. A special glass filter was employed to limit the radiation at wavelengths greater than 295 nm. Irradiance was set at 765 $W \cdot m^{-2}$ (XenoCal sensors to measure and calibrate irradiance). The possible chemical changes of the protein fraction in the painting model samples (PG, Cin-G and Azu-G) were checked periodically at 200, 500, 800, 1500, 2500 and 3000 h via the corresponding MALDI-TOF mass and IR spectra. Prior to the UV irradiation exposure (time = 0) fresh micro-samples were removed from each painting model and stored in glass vials in darkness to be further analyzed. These were considered as references to be compared with the aged samples throughout the mass and IR spectral data registered. The environmental conditions during the UV ageing test were set at $30 \pm 5\,^{\circ}C$ and the relative humidity at $15 \pm 5\%$ to avoid mineralogical transformation of azurite into malachite. The measurement was registered by a thermohygrometer (OREGON Scientific, mod. EMR812HGN; Portland, Oregon, USA).

Trypsin digestion method and MALDI-TOF-MS analysis

The analysis of the painting model samples by MALDI-TOF-MS requires previous enzymatic digestion to fragment the glue into its characteristic peptides, and purification of the digested samples to eliminate impurities. The method described in reference 30 was used for this purpose. In brief, approximately 1 mg of the pulverized model samples was digested in 20 μl of 50 mM ammonium hydrogen carbonate containing approximately 10 μg/ml trypsin at room temperature for 2 h. After the trypsin digestion, samples were purified on reverse-phase ZipTip. The mass spectra were acquired by a Bruker-Daltonics Biflex IV MALDI-TOF mass spectrometer equipped with a standard nitrogen laser (337 nm) in positive reflectron mode. The spectra were analyzed using the XMASS software (Bruker). The peptide mixture (2 μl) was mixed with 4 μl of DHB solution (15 mg of DHB in 1 ml of mixture of acetonitrile/0.1% trifluoracetic acid (1/2, v/v)); 1.5 μl of the resulting mixture was spotted on the stainless steel MALDI target and dried in air. At least 200 laser shots were collected for each spectrum and analyzed using the XMASS software (Bruker). A mass spectrum was taken for each drop between 0 and 5000 m/z. The recalibration of the mass spectrometer with a commercial standard of peptide mixture (Pepmix, Bruker) was performed every 20 mass spectra recorded.

MS data pre-treatment and PCA

In order to apply the multivariate analysis, mass data were pre-treated using the M-mass 2.3.0[43] and Delphi 6.0 programs for conversion of mass data into ASCII file format.[44] PCA analyses were performed using the Statistical Product and Service Solutions program (for Windows ver. 15, USA). This chemometric tool has been applied for different purposes, including the interpretation of ageing process of painting model samples due to its capability to identify changes in painting materials produced by different agents. [11] In particular, PCA is a powerful data-mining technique that reduces data dimensionality to obtain a more interpretable representation of the system under investigation.[44] PCA was performed independently on three matrices within the peptide mass fingerprint interval (from 1000 to 2500 m/z). Each matrix contains information about fresh and aged painting model samples (UV exposure times 0, 200, 500, 800, 1500, 2500 and 3000 h). In the case of the pure glue data matrix, mass spectra at 800 h of ageing were discarded due to experimental errors; therefore, they were not included in the multivariate analysis. Hence, PG, Cin-G and Azu-G data matrices were constructed with 60, 70 and 70 mass spectra (10 spectra per check time), respectively. The PCs were obtained using the correlation data matrices (scaling by unit variance).

Fourier Transform Infrared Spectroscopy

The FTIR spectra were collected using a NICOLET spectrometer 20SXB working in transmission mode. The instrument was connected to a Pentium 200 and the instrument software was OMNIC v 4.1, running under Windows 2000 Professional (Microsoft Corporation, USA). The FTIR spectra were registered from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 2 cm^{-1} and 200 scans. The IR spectra were obtained from five KBr pellets that were prepared by homogeneously mixing 50 μg of sample powder removed from five random places on the glass slide. The FTIR analysis was carried out on fresh and aged PG, Cin-G and Azu-G model samples at 200, 500, 800, 1500, 2500 and 3000 h.

RESULTS AND DISCUSSION

MALDI-TOF mass spectral features

An evaluation of the paint samples' mass spectra was carried out. As expected in a MALDI-TOF-MS experiment, mass spectra did not exhibit great reproducibility in terms of the precision of peak intensity values, found to be ≤ 35%. This value was estimated from the precision of the higher peaks by means of their relative standard deviation in peak intensity and using all the spectra from the same checked time (10 mass spectra). Nevertheless, it was corroborated that the precision of the relative intensity between peaks (estimated also as relative standard deviation and using at least the 10 highest intensity peak ratios) was less than 10%. Thus, mass spectra features were used to extract information from the paint samples.

In this way, the mass spectra of fresh and aged painting model samples at 3000 h were compared for all samples. Figure 1 shows the corresponding mass spectra. Comparing the fresh painting model samples (Fig. 1 a,c,e), it can be noted that the fragmentation patterns in the mass spectra of the digested glue significantly differ from those of pure glue or that mixed with cinnabar or azurite pigments. In fact, the number of m/z peak signals was higher in the case of the glue tempera samples than in pure glue samples. Despite the fact that rabbit glue was the same in all samples, low intensity signals were obtained below 1400 m/z in all PG samples, both when fresh or aged. Thus, this suggests that it could be related to changes in the glue structure before trypsin

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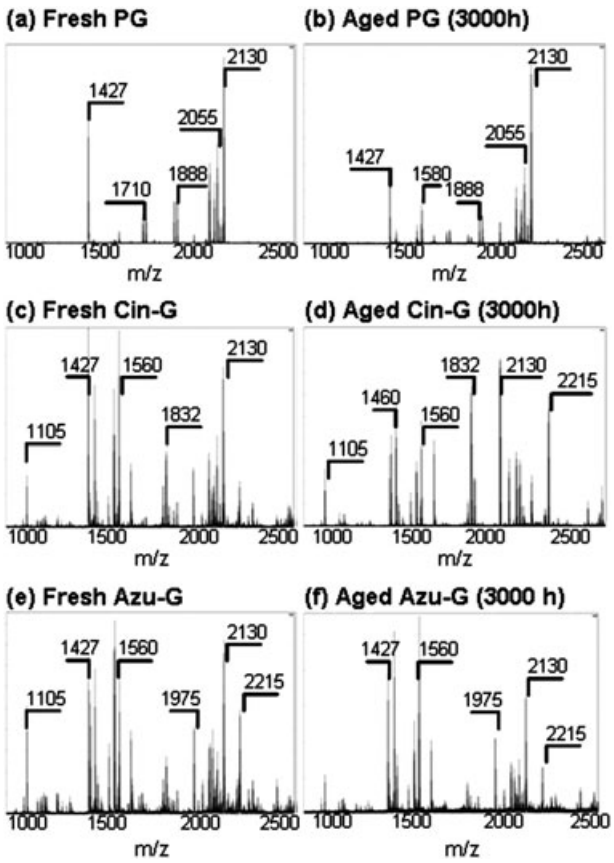

Figure 1. Mass spectra obtained by MALDI-TOF-MS of fresh and aged collagen at 3000 h of UV irradiation for: (a) (b) Pure Glue; (c) (d) Cin-G and (e) (f) Azu-G samples, respectively.

digestion due to interaction with the particular pigment. On the other hand, comparing fresh and aged painting model samples (Fig. 1) from otherwise similar samples, the m/z patterns were also different. In this case, this fact could indicate changes in the protein structure mainly due to UV ageing. Particularly, there was a new peak at 1710 m/z in aged PG sample while the peak at 1580 m/z disappeared. In the Cin-PG samples, the peak at 1427 m/z disappeared when aged and a new peak at 2215 m/z was detected. For Azu-PG samples, the peak at 1105 m/z was detected only when aged. Moreover, the mass patterns of the aged samples differ depending on their composition, i.e. pure glue or pigment based temperas (see Fig. 1 b,d,f).

Due to the complexity of the mass spectra feature, complementary strategies were needed to extract or corroborate the results. Thus, procedures such as PCA on the mass spectra were applied to highlight the changes in the mass spectra, and to look for patterns related to the interaction of the particular pigment with the proteinaceous binder, and the effect on the ageing process. Particularly, PCA was used to discriminate different sample patterns based on the mass spectra since *a priori* mass spectral differences could be attributed, for instance, to different ionization processes in the MALDI interface. Also, FTIR was used to track possible conformational changes.

PCA on MALDI-TOF-MS data

PCA was performed on the m/z data matrices of every model sample which included the m/z data at the different UV irradiation check times and also the m/z data from the fresh samples. As usual in multivariate analysis, many PCAs were performed using different m/z intervals. The m/z intervals tested by PCA were selected from those exhibiting the highest number of peaks corresponding to the most representative of the peptides from glue.[30,45] For the PG samples, the best results were obtained using the m/z interval from 1400 to 2500 m/z (900 values). In the case of both pigment-based tempera samples, two m/z intervals were selected for their satisfactory PCA results, i.e. between 1000–1760 m/z (760 values) and 1760–2500 m/z (740 values) for the Cin-G samples; and between 1090–1900 m/z (810 values) and between 1900–2500 m/z (600 values) for Azu-G samples (Table 1). The rest of the PCA results were discarded since they did not show any patterns in the samples. The PCs were obtained using both the covariance data matrices (scaling by mean-centered data) and the correlation data matrices (scaling by unit variance). Results were better when PCA was performed on correlation data matrices, so the results shown and discussed here correspond to autoscaled data. In fact, this is not surprising considering that the mass spectra reproducibility was low (low reproducibility in the intensity values of the peaks),

Table 1. PCA results

Data matrix	m/z Interval	Data number	PC	Variance explained (%)	Variance accumulated (%)
Pure Glue (PG)	1400–2500	900	PC1	91.7	91.7
			PC2	2.2	93.9
			PC3	1.6	95.6
Cinnabar-Glue (Cin-G)	1000–1760	760	PC1	80.4	80.4
			PC2	7.3	87.7
			PC3	2.5	90.2
	1760–2500	740	PC1	74.8	74.8
			PC2	10.5	85.3
			PC3	3.9	89.2
Azurite-Glue (Azu-G)	1090–1900	810	PC1	86.5	86.5
			PC2	3.1	89.6
			PC3	2.3	92.0
	1900–2500	600	PC1	87.5	87.5
			PC2	3.8	91.3
			PC3	2.1	93.5

whereas the reproducibility of the relative intensity of the peaks was high. Thus, the low intensity reproducibility was corrected by autoscaling variables (m/z) to unit variance while the overall features of the mass spectra were maintained.

The results of the multivariate analyses in terms of explained variance (%) and cumulative explained variance (%) for each model sample are shown in Table 1. From these results, it is interesting to note that in the three multivariate analyses performed, the first PC accounted for more than 74% of the total variance in the mass spectra (91.7% in the case of the PG samples, 80.4 and 74.8% in the case of the Cin-G samples, and 86.5 and 87.5% in the case of the Azu-G samples). This indicated mainly one source of variability in the spectra.

Pure glue model samples

PCA discriminated PG samples up to 1500 h of ageing. The results of the projection of the samples onto the plane of the first two PCs are shown in Fig. 2a. PC1 (accounting for 91.7% of the total variance) discriminated samples up to 500 h of ageing (negative scores) from the rest of the aged samples (positive scores). Despite explaining only 2.3% of the total variance, PC2 allowed discrimination of the initial ageing stage, i.e. fresh PG and aged PG samples at 200 h (both with positive scores) from more aged samples (with negative scores; Fig. 2a). These PCA results suggest chemical modifications of the pure glue due to the UV ageing after 200 h of exposure, as was previously demonstrated.[11] The combination of both components, PC1 and PC2 was necessary to distinguish among check times up to 1500 h of ageing. Therefore, this result evidences protein changes due to the ageing process that was detected by PCA up to 1500 h of UV irradiation. After

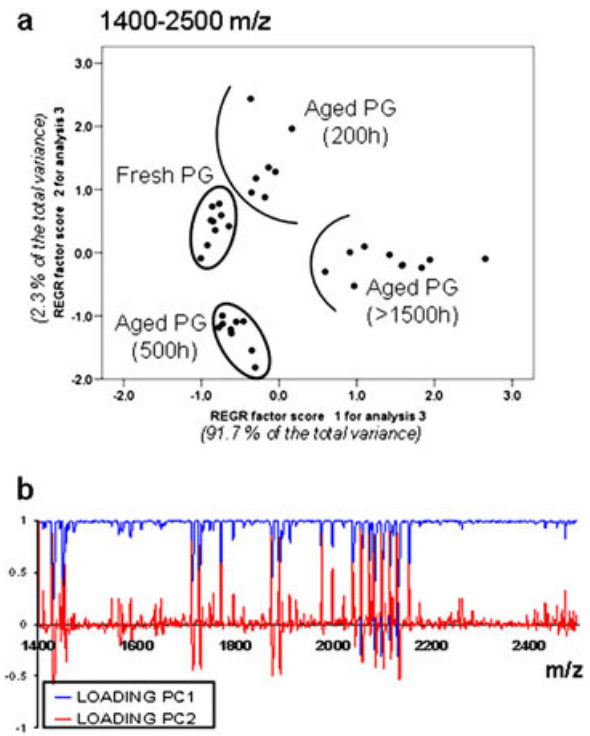

Figure 2. Score plots (a) and loading plots (b) for PC1 and PC2 of the UV ageing of pure rabbit glue (Pure Glue) in the mass interval between 1400 and 2500 m/z. Figure shows ageing at different times (Fresh, 200, 500, 1500, 2500 and 3000 h).

that time of exposure, no changes were discriminated by the multivariate analysis. It could be inferred that no relevant protein change occurred.

The loading values indicated the specific contribution of each m/z signal in the calculation of the total variance of the mass spectral data. Therefore, the loading plot helped identify those peptide ions that were important in the PCs. A closer examination of the loading plots combined with the score plots also revealed the kind of contribution associated with the PC. Since the scores of PC1 and PC2 discriminated samples aged up to 1500 h, the m/z spectral data variances due to these samples were mainly described by these PCs. The unknown peptide ions at 1872, 1888, 2055, 2081, 2097, 2113, 2128 and 2130 m/z exhibited the higher negative loading values in PC1 and the higher positive loading values in PC2. Thus, they could be related with the aged PG samples discriminated up to 200 h. Nevertheless, the number of peptides with high loading values is large. Table 2 shows all the peptide ions with loadings higher than 0.99 in PC1. This means that it needed the combination of many variables (m/z) to interpret the information explained in the PC, and this fact made it difficult to account for the results in terms of a particular peptide. There is no single set of peptides involved in the discrimination of the samples.

Additionally, the conformational structure of the proteinaceous binder (collagen) was studied from FTIR spectra of the PG, Cin-G and Azu-G samples, fresh and aged, at the checked times to track conformational changes in the collagen due to UV ageing. Figure 3 shows the most significant FTIR spectra for each kind of sample. In the case of PG samples, these spectra were those from fresh and aged samples at 200 h, 500 h and 3000 h (Fig. 3a). The IR amide bands I (carbonyl stretching vibrations) and II (NH bending vibrations) can be observed at around 1660 cm^{-1} and 1550 cm^{-1}, respectively, for fresh and aged samples as expected. The conformational study, for the three kinds of samples, was mainly based on the analysis of these proteinaceous characteristics bands. Nevertheless, it was corroborated in all the FTIR spectra (also for Cin-PG and Azu-PG samples) that the position of the amide band II was not affected by the ageing process since it was always placed at 1550 cm^{-1}. Thus, the discussion about conformational changes is based only on the amide I band features.

Given that the aged samples at 200 h for PG were well discriminated in the PCA performed on the mass data, it was inferred that the ageing process at these checked times could introduce important initial changes. Therefore, a comparative study between these fresh and aged samples was carried out tracking the modification. The IR band at 1663 cm^{-1} attributed to β-turn structure was detected in fresh samples;[19] nevertheless, the β-turn structure decreased during UV irradiation, and the amide band I shifted to an α-helix structure at 1655 cm^{-1} for samples aged 200 h.[18,20] Moreover, this fact was also evidenced for the rest of the aged samples, as can be noted in Fig. 3a for samples aged 500 h and 3000 h. Thus, this suggested that the PG samples could undergo conformational changes in the secondary structure of pure glue since slight shifts in the amide I band were detected. The β-turn structure is based on two or more polypeptide chains running alongside each other, which are linked in a regular manner by hydrogen bonds between the main chain C=O and N-H groups.[16] The FTIR results suggested that UV irradiation could break these bonds and the chains of collagen become independent turning to an α-helix structure as the basic structural unit.

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Table 2. Peptides with the highest loading values in PC1 (higher than 0.99 for PG and higher than 0.95 for Cin-G and Azu-G)

Model Samples	Mass interval (m/z)	Relevant Ion-peptides involved in PC1
P-G	1400–2500	1401, 1404, 1406, 1417, 1422, 1426, 1438–1442, 1447,1448, 1464, 1465, 1467–1469, 1477–1480, 1487, 1492, 1497, 1498, 1500, 1503, 1505, 1506, 1508, 1512–1519, 1523, 1525, 1527, 1529–1531, 1536–1538, 1541–1543, 1553–1556,1558,1559, 1578, 1579, 1592–1594, 1596–1600, 1603–1605, 1613, 1614, 1616–1629, 1631, 1635, 1641, 1643, 1644, 1646, 1647, 1661, 1663, 1665, 1667–1670, 1674–1676, 1678–1692, 1694, 1697–1707, 1716–1724. 1736–1747, 1757–1768, 1773–1777, 1780–1793, 1799, 1800, 1803–1815, 1821–1831, 1837–1847, 1851–1853, 1860–1867, 1879–1886, 1899–1909, 1914–1921, 1927–1930, 1933–1938, 1941–1952, 1956–1960, 1964–1974, 1981–1984, 1986–1994, 2001–2007, 2009–2013, 2015–2018, 2023, 2028–2036, 2062–2063, 2065, 2068–2070, 2092, 2093, 2107–2109, 2111, 2124, 2125, 2127, 2141, 2145–2147, 2149, 2157, 2160–2164, 2172, 2173, 2184, 2186–2188, 2190–2198, 2203, 2206–2208, 2211–2214, 2221–2225, 2227, 2231–2233, 2235–2237, 2239–2241, 2245, 2247, 2249–2250, 2253, 2257, 2258, 2271–2276, 2278, 2280, 2287–2300, 2302, 2304, 2307, 2311, 2313, 2315, 2316, 2322–2325, 2336, 2338, 2340, 2345, 2347, 2352, 2353, 2355, 2359, 2360, 2364, 2369, 2372, 2373, 2378–2381, 2386, 2387, 2390, 2397–2405, 2418–2429, 2434, 2437, 2439–2444, 2450, 2459–2462, 2468, 2475–2479, 2485, 2490, 2494, 2495.
Cin-G	1760–2500	**1762–1766**, **1768**, 1769, **1773–1776**, **1781**, **1786**, **1787**, **1790**, **1791**, **1792**, 1796, 1797, **1803**, **1810**,**1811**, 1843, 1844, **1861**, **1862**, 1869, **1879**, **1880**, **1883**,**1884**, **1900**, **1903**, 1913, **1914**, **1922**, **1927–1930**, 1940, **1942**, 1953, **1957**, **1964–1966**, **1969**, 1985, **1989**, **1994**, 1995, **2011–2017**, 2026, 2027, **2033–2036**, 2047, 2048, 2049, 2050, **2065**, 2066, 2080, 2105–**2107**, 2112, 2121–**2124**, **2145**, 2159, 2177, 2178, **2188–2194**, 2205–2209, **2222**, 2228, 2229, 2230, **2236**, 2244, **2245**, **2247**, **2250**, 2251, 2252, **2253**, 2254, 2255, 2256, **2257**, **2258**, **2271–2274**, **2276–2279**, **2289**, **2296**, **2297**, 2314, 2319, 2320, 2321, 2328, 2331, 2335, 2340, 2341, 2351, **2352**, **2353**, 2355, 2367, **2398**, **2400–2405**, 2406, 2417, **2418–2422**, **2424–2426**, 2433, 2448, 2449.
Azu-G	1900–2500	**1909**, 1913–**1915**, 1926–**1935**, **1937–1952**, **1955–1958**, **1963–1973**, **1981–1990**, 1995, 1996, 2000–**2002**, **2007–2013**, 2014, **2015–2018**, 2024–2027, **2028–2036**, 2041–2054, 2061, **2062**, **2063**, 2064, **2065**, 2066, 2067, **2068–2070**, 2071, 2086, 2087, 2102–2106, 2107, 2112, 2117, 2122, 2123, **2124**, **2125**, 2126, **2127**, 2139, 2140, **2141**, 2142–2145, 2166, 2167, **2172**, **2173**, 2174, 2176–2178, 2185, **2186**, **2187**, 2188, 2189, **2190**, **2191**, 2204, 2205, **2206–2209**, **2221–2227**, 2228–2230, **2234–2237**, 2238, **2241**, 2242–2244, **2245**, 2246, **2247**, 2248, **2249**, **2250**, 2251, 2252, 2254, 2255, 2256, **2257**, **2258**, 2259, 2267–2270, **2271**, **2288–2290**, **2294–2297**, **2302**, 2303, **2304**, **2305**, 2312, 2317–2320, 2333–**2336**, 2339–2342, 2349–2351, **2352**, **2353**, 2354, **2355**, 2356, 2357, 2361, 2365, 2367, 2375–2377, **2378–2381**, 2382–2384, 2391, **2397–2405**, 2406, 2414, 2415, **2418–2426**, **2434**, 2435, 2436, **2437**, 2438, **2439**–2444, 2445–2449, **2450**, 2451–2453, 2455–2458, **2459–2462**, 2463, 2469, 2474, **2475–2479**, 2488, 2489, **2490**, 2491–2493, **2494**, **2495**

In bold peptides shared with PG

Cinnabar tempera model sample

As mentioned above, PCA was performed separately in two different intervals from the mass spectra. Over the 1000–1760 m/z interval, the two first PCs accounted for 87% of the total variance in the Cin-G samples. Nevertheless, the projection of these samples onto the plane of these two components lacked of pattern of changes related to the UV test. Despite the high percentage of total variance accounted for by the first two PCs, no pattern attribution could be proposed. Hence, this interval was discarded to evaluate the alteration process of collagen. However, the PCA on the mass spectrum between 1760 and 2500 m/z allowed discrimination between different ageing check times by projecting the samples onto the three-dimensional space defined by the three first PCs as shown in Fig. 4a. The first PC (accounting for 89.2% of total variance) separated fresh Cin-G samples, with positive scores, from the rest of the samples, with negative scores; the aged Cin-G samples at 3000 h were associated with the more negative score (Fig. 4a). The aged Cin-G samples at 500 h could be discriminated (with positive score values) from the rest of the ageing check times via PC2, which accounted for 10.5% of the total variance. Finally, scores for PC3, which only accounted for 3.9% of the total variance, clearly discriminated samples aged at 3000 h (positive scores) from the rest of the samples. The loading plots of PC1 and PC2 showed high variability in the values (Fig. 4b) that made difficult their interpretation. Nevertheless, compared to the pure glue discussed above, different peptide ions were implicated when discriminating Cin-G samples, though only a few were shared. Table 2 shows all the peptide ions with loadings higher than 0.95 in PC1, indicating in bold those shared with PG. Therefore, this suggests that different collagen ageing processes depend on the presence of the pigment cinnabar.

Conformational changes were also evaluated for Cin-G samples using FTIR spectra. Prior to the ageing process, for fresh samples, the amide band I was located at 1648 cm^{-1}, which is assigned to random coils according to the consulted bibliography (Fig. 3b).[19,20] Thus, this result revealed an absence of secondary structure in the presence of cinnabar even before the UV exposure.[17–19] In fact, it is well known that the denaturation process disrupts the normal α-helix and β-sheets in a protein and uncoils it into a random shape. Therefore, in this particular case, it suggested that the denaturation effect of cinnabar on the collagen occurred prior to the ageing test. The harmful properties of mercury are well established, producing the protein degradation of tissues.[46,47] For the next state of ageing tested, i.e. at 500 h of UV exposition, a shift of the amide band I to 1627 cm^{-1} was observed, which is labelled as a β-sheet structure. This was surprising, and this shift could be related to denaturation and

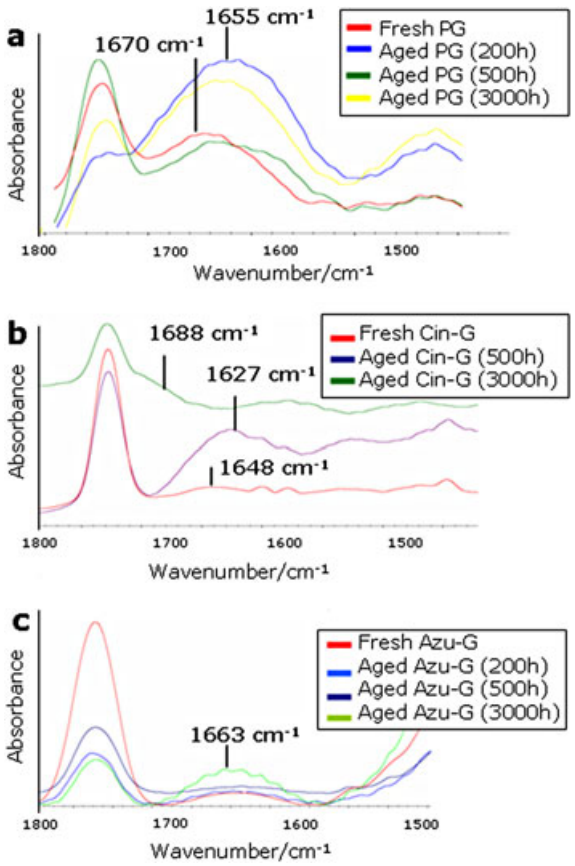

Figure 3. Infrared spectra of fresh and aged collagen in: (a) pure glue (b) Cinnabar tempera and (c) Azurite tempera model samples.

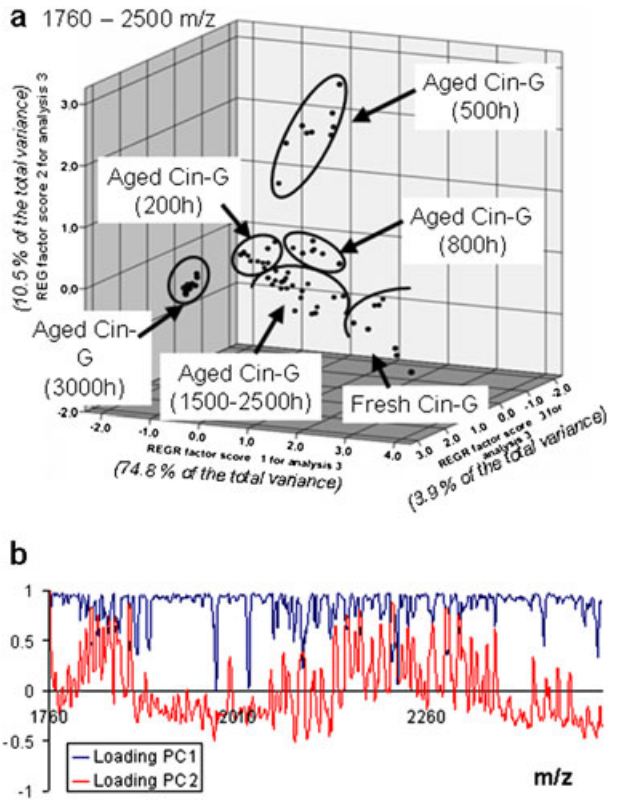

Figure 4. Score plots (a) and loading plots (b) of the Cin-G samples of PC1, PC2 and PC3 in 3-D space. PCA performed between 1760 and 2500 m/z. Figure shows ageing at different times (Fresh, 200, 500, 800, 1500, 2500 and 3000 h).

aggregation processes[19,20] more than reorganization to a β-sheet structure. Finally, the conformational study at 3000 h showed a shift to 1688 cm^{-1}. An amide band I located between 1670 and 1690 cm^{-1} is indicative of a random coil.[19] Thus, the study of the collagen conformational properties throughout the ageing test revealed the denaturation of collagen by different disordered structures, such as aggregates at 500 h and random coils for the final aged state.

Azurite tempera model samples

The best ability for discriminating among aged Azu-G samples was found in the m/z interval between 1760 and 2500 m/z (containing 600 data points). The m/z interval between 1090 and 1900 m/z (810 points) required the combination of PC1 and PC2 (89.6% of total variance) to discriminate aged Azu-G samples up to 500 h (with positive scores in both PCs) from the rest of the samples. Nevertheless, the groups were not clearly separated, and the results were not further investigated.

The projection of the scores onto the plane of the first two PCs performed on the m/z data from 1900 to 2500 m/z showed the organization of the Azu-G samples into three groups based on the ageing time. In particular, fresh samples aged at 200 h and samples aged more than 500 h were discriminated. Regarding PC1 that accounted for 87.5% of total variance, the fresh and aged Azu-G samples at 200 h (positive scores) were discriminated from the rest of the samples (negative score). Despite the fact that PC2 accounted for 3.8% of the total variance (Fig. 5a), it allowed discrimination between fresh and aged Azu-G at any

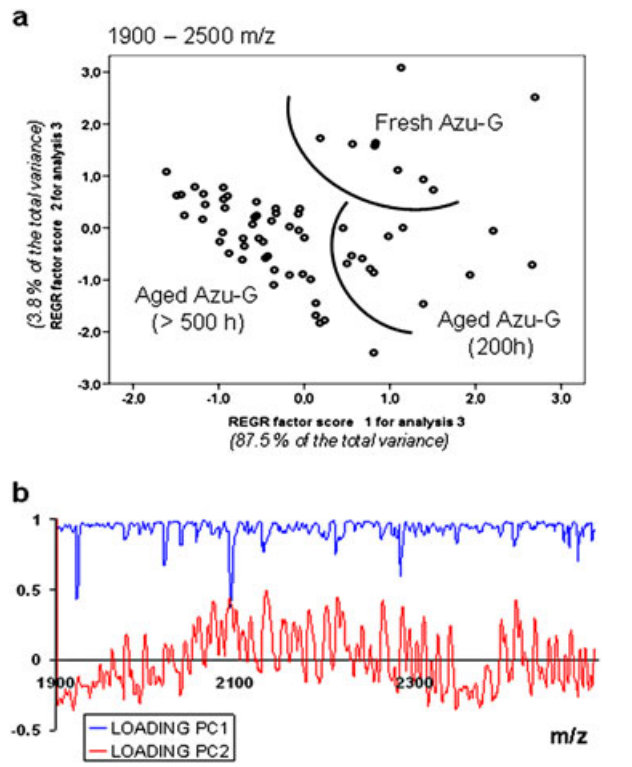

Figure 5. Score plots (a) and loading plots (b) for PC1 and PC2 of the UV ageing of the azurite tempera model samples (Azu-G) in the mass interval between 1760 and 2500 m/z. Figure shows ageing at different times (Fresh, 200, 500, 800, 1500, 2500 and 3000 h).

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UV exposure time. Again, the results demonstrated that the UV ageing process introduced changes in this tempera model samples that could be detected by PCA. Also, it suggested no changes in the Azu-G after 500 h of UV ageing that could be detected by PCA; thus, it could be inferred that these changes were minimal. Then, the modification due to UV ageing of this particular tempera containing azurite is carried out at the first stages of UV exposure (up to 200 h). After that, the tempera remains stable. Indeed, these results regarding collagen stability in the presence of azurite confirmed similar conclusions obtained elsewhere.[11,48]

In order to interpret these multivariate results for Azu-G samples, the formation of a metal complex or coordination compound could be the key to explain the photostability of collagen in the presence of azurite. In fact, the literature extensively describes the formation of chemical species of protein-copper complex even at lower pH values.[49] This chemical reaction is widely employed as method for protein detection and quantification. In particular, the Biuret Test is based on the formation of a violet-colored complex between the Biuret reactive (containing Cu^{2+} ions in alkaline solution) and the amino groups from the peptide protein bonds (–HN-CO_2-N–). Also, according to the color intensity of the complex measured by colorimetric analysis, the protein concentration is calculated.[50]

On the other hand, the study of loading plots of Azu-G samples showed different peptide ions involved in discriminating the fresh and aged samples compared to those found for PG and Cin-G samples (see the loading plot in Figs. 2, 4 and 5) with few peptides shared with PG (Table 2). It can be concluded that the ageing process of glue when mixed with azurite was different from those detected for PG or when mixed with cinnabar.

Regarding the complementary study by FTIR, the amide band I was analyzed with this technique (Fig. 3c). For fresh samples, the position of this band was at 1663 cm^{-1}, which was attributed to a β-turn structure. When aged, this amide band I remained stable at 1663 cm^{-1} for all checked times studied, i.e. 200, 500 h and 3000 h. Therefore, the β-turn structure of the glue when mixed with azurite remained stable despite exposure to UV irradiation. This suggests that no changes in the second conformational structure of collagen occurred when mixed with azurite. On the other hand, it is well known that copper ions (Cu^{2+}) bond to amide groups to form a particular protein-Cu^{2+} complex. This complex is formed by four N–H groups from two collagen chains and two groups from each chain stabilizing the β-turn structure of collagen. Thus, it could be inferred that the formation of this complex prevented conformational changes in the secondary structure of the collagen binder.

CONCLUSIONS

This work is part of an ongoing investigation on interactions between historical pigments and proteinaceous binders. The present study suggests some possible answers concerning the interaction and the role of the mineral pigments cinnabar and azurite in the UV ageing process of collagen, when present in glue tempera paintings. The combined use of analytical techniques (MALDI-TOF-MS and FTIR) and the innovative application of PCA to mass spectral data allowed discrimination of significant differences in the mass spectra of glue, and conformational changes in its structure due to UV irradiation. Results also show that chemical or structural changes occurred in different forms when these pigments are present, indicating diverse interactions between pigment and collagen that may influence the ageing process.

This new approach facilitates the analysis and interpretation of ageing processes *versus* traditional methods due to more sensitive mass data registered, whole structural information, and the small sample size needed for this specific analysis. In addition, this work proposes that the formation of a protein-copper complex could justify the additional photostabilization of the glue tempera when azurite is present, as suggested by Price (2000) and Manzano *et al.* (2010).[11,46] Moreover, this paper suggests that the alteration mechanism of collagen detected by PCA could likely be due to a secondary dimensional structure alteration.

In addition, this approach based on PCA of MALDI-TOF mass spectrometric data, with the complementary use of FTIR, is suitable to be employed for further study of protein binders affected by other degradation agents. For instance, environmental factors as such gaseous pollutants including SO_2 and NO_2 from industrial and urban emissions could be studied in depth. Also chemical modifications due to the presence of inorganic salts could be evaluated, as well as possible interactions with other organic compounds (varnishes, oils, organic pigments, etc.) which are commonly present in paintings.

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