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Complementary cross-section based protocol of investigation of polychrome samples of a 16th century Moravian Sculpture by optical, vibrational and mass spectrometric techniques

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ABSTRACT

On the occasion of its restoration treatments, the rare medieval polychrome sculpture entitled "The Mourning of Jesus Christ" (16th century, belonging to the Moravian Gallery in Brno, Czech Republic) was subject to a detailed analytical investigation in order to identify the organic and inorganic materials present in the polychrome layers and to map their distribution in the paint structure. This knowledge is extremely useful especially for art objects that suffered reconstructions and/or over paintings or other interventions and can be of help to the restorers in deciding the best conditions and materials for the treatment. In this case, the identification of all components was very important in terms of the assessment of the original painting techniques and discrimination of the presence of over painting layers, being complemented by the study of the construction process and of the history of the artwork.

Until recently, the identification of proteinaceous binders contained in small samples taken from polychrome or painted works of art was very complicated and nearly impossible in the case of cross-section analyses. Therefore, the novelty of this paper is the complementary applications of several analytical techniques, such as optical microscopy (OM), nano-LC-ESI-Q-TOF and MALDI-TOF mass spectrometry, microRaman spectroscopy and scanning electron microscopy coupled with energy dispersive X-ray analysis (SEM-EDX) directly on cross-sections. This approach is especially useful for this case as only few samples were available for binder and pigment identification and it allowed the preservation of the samples as cross-sections for further analyses.

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1. Research aims

The research results presented in this paper are part of a larger study of materials and techniques used in a Czech Medieval polychrome sculpture illustrating "The Mourning of Jesus Christ" dated around 1500 (Moravian Gallery in Brno, Czech Republic). This contribution is the second part of a paper already published by Kuckova, Sandu et al. [1], where the simultaneous identification and localization of proteinaceous binders on cross-section by MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry) was described.

The two objectives of the research presented here were:

- to compare two mass spectrometric techniques (MALDI-TOF and nano-LC-ESI-Q-TOF) applied for proteinaceous binder's identification on cross-sections;
- to identify and map the organic and inorganic components of the polychrome layers in order to characterize the polychrome materials and techniques of the sculpture and to confirm the art work provenience.

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2. Introduction

Currently, most of advanced analytical methods (e.g. GC-MS, LC-MS, optical methods), which are used for the identification of proteinaceous binders in works of art, are able to identify and distinguish at least egg, animal glue and milk proteins [4–8]. Also, though only in rare cases, some immunochemical methods (ELISA, IFM, nano-SERS antibodies, chemiluminescence) staining tests and fluorescence microscopy [9,10], and mass spectrometric methods (MALDI-TOF) [8,11–15] were reported in studies for the localization and simultaneous mapping of various protein-based materials in individual layers of samples that have been prepared to form polished cross-sections [14,15].

Both mass spectrometric methods MALDI-TOF and nano-LC-ESI-Q-TOF are working with specific peptides that are obtained after enzymatic cleavage of proteins contained in artwork's samples using trypsin. Trypsin cleaves peptide bonds only behind two basic amino acids — lysine and arginine. In the case of MALDI-TOF MS the unique peptide mixture (fingerprint of binder) is analysed by a mass spectrometer and then compared and assigned to a binder from a reference database of protein binders [16]. Nano-LC-ESI-Q-TOF mass spectrometry determines the order of amino acids in the peptides and the sequences compared to the publicly available databases of proteins. Nowadays the highly impacted proteomic journals usually demand two peptides for a reliable determination of individual proteins.

As far as the identification of inorganic pigments is concerned, there are many dedicated analytical techniques as the pigments and inert materials used in paint or polychrome layers represent their major component and their identification could serve as a tool for attribution or dating of the artwork [17,18]. The inorganic constituents are identified mainly using scanning electron microscopy coupled with energy dispersive X-ray spectrometry (SEM-EDX) [19,20], Raman spectroscopy [19], classical Fourier Transform Infrared spectroscopy (FTIR) [19–21] and infrared microspectroscopy (microFTIR) using a synchrotron source which allows the collection of high signal-to-noise ratio infrared spectra with diffraction-limited spatial resolution across the entire spectral range [22]. X-ray spectroscopy (XRF) [23] has also become established as one of the most important tools for non-destructively identifying specific elements for pigment and inert material identification. The metals could be also determined using graphite furnace atomic absorption spectroscopy (GFAAS) and inductively coupled plasma mass spectrometry (ICP-MS), where the previous extraction of pigments is necessary [24]. X-ray diffraction [21], especially X-ray micro-diffraction [25], is useful in material research of painting layers with complex stratigraphy and composition and it could distinguish inorganic phases of different natural provenances and reveal their degradation products.

In this work the inorganic and protein-based constituents of the paint layers of a 16th century polychrome sculpture are analysed using a complementary protocol of investigation, based on the use of optical microscopy (OM), nano-LC-ESI-Q-TOF and MALDI-TOF mass spectrometry, microRaman spectroscopy and scanning electron microscopy coupled with energy dispersive X-ray analysis (SEM-EDX) directly on cross-sections.

3. Experimental

3.1. Sampling

The sculpture (Fig. 1), owned by the Convent of Minorits in Brno (Czech Republic), is a complex work of figurative woodcarving (illustrating the theme of "The Mourning of Jesus Christ", comprising 11 male and female characters standing around the laid body of Christ after the Descent from the Cross) shaped in high relief (97 cm × 136 cm × 29 cm), made of four massive lime wood blocks together with several supplements dating around the year 1520. In Fig. 1 the image of the entire sculpture is given and for each statue a letter was used to label the represented character: A: Jesus Christ; B: Saint-Anna; C: Saint-John the Baptist; D: Mary Magdalene; E: Mary of Kleofas; F: Saint-Mary; G: Mary Salome; H: Joseph from Arimathea; I: Nikodem; J: member of Synedrium; K: Pharisee (Simon of Cyrene); L: member of Synedrium.

A suspicion of a possible relationship between the sculpture (Fig. 1) and a 1500 printed engraving of a woodcarving work (Fig. 2) made by Albrecht Dürer arose due to the stylistic and compositional characteristics of both [2,3].

The polychrome's stratigraphy is made of many paint layers (from the Gothic, Renaissance, Baroque and 19th and 20th centuries) applied over a white ground layer. Unfortunately, the sculpture was damaged in large areas, because of over-paintings probably made during the 19th and 20th centuries and other interventions leading to missing parts such as a finger or incarnate areas, extensive abrasions and cracks, which were appropriately used for sampling. As shown in Fig. 1, a total of 12 samples was taken from the sculpture (M1/M1a to M11) and embedded as cross-sections [1].

3.2. Materials and analytical protocol

3.2.1. Materials and reagents

The NH_4HCO_3 and acetonitrile were bought from Lachema Brno (Brno, Czech Republic). Trypsin (TPCK) comes from Promega Corporation (Madison, WI, USA). The reverse phase ZipTip was bought from Millipore Corporation (Bedford, MA, USA), and 2,5-dihydroxybenzoic acid (DHB) and trifluoracetic acid were bought from Sigma (Saint-Louis, MO, USA). Mecaprex 2S polyester embedding resin and peroxide of methyl ethyl ketone hardener come from Presi (Grenoble, France).

3.2.2. Cross-sections and OM observation

After curing of the polyester resin, the blocks were cut and polished to reveal the paint/ground composite in cross-section. The cross-sections were dry polished with successively finer grades of micromesh abrasive cloths (600, 800, 1200 and 4000 mesh). Felt was used for the final polishing. Water or other aqueous-based liquids are not used during polishing since they could dissolve the proteinaceous component in the samples. The cross-sections were observed at different magnifications (from 50× to 500×) using an Axioplan Zeiss 2 imaging binocular microscope and the images were acquired using a Nikon DXM1200F digital camera, coupled to the microscope (provided with a mercury lamp HBO100 and a halogen lamp HAL100). Visual light observations (illumination position for dark field observation, abbreviated as f2) were performed in reflection geometry. Images were captured and treated using Nikon ACT-1 software.

3.2.3. Protein digestion and purification

On the surface of the cross-sections,10 μL of 50 mM NH_4HCO_3 containing approximately 10 μg/mL of trypsin was applied and left to react at room temperature for two hours. After the trypsin digestion, the solutions were taken from the surfaces and purified on a reverse phase ZipTip. After equilibrating, binding and washing steps, target compounds were desorbed from the stationary phase [26]. The solutions were consequently used for analyses by MALDI-TOF MS and nano-LC-ESI-Q-TOF (3.2.4 and 3.2.5).

3.2.4. Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF)

An aliquot of the elution solution containing peptides (2 μL) was mixed with 4 μL of 2,5-dihydroxybenzoic acid (DHB) solution — 18 mg of DHB in 1 mL of mixture of acetonitrile/0.1% trifluoracetic acid in water (1/2 [v/v]). The part of the resulting mixture (2.8 μL) was for two times spotted on the stainless steel MALDI target and dried in air. Mass spectra were acquired by a Bruker-Daltonics Biflex IV MALDI-

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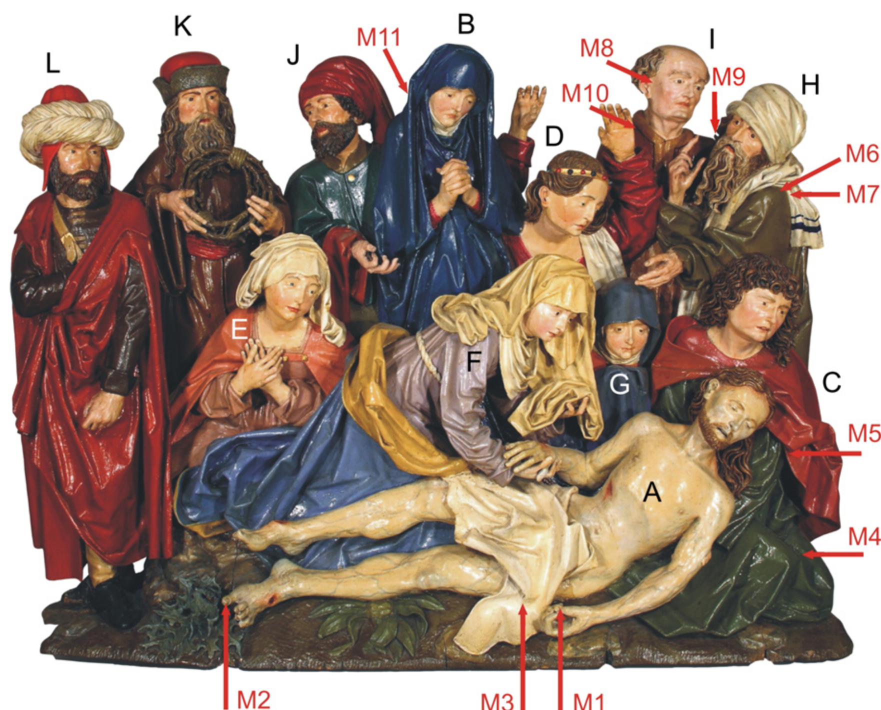

Fig. 1. Entire view of the polychrome sculpture with the sampling areas for the 12 samples.

TOF mass spectrometer equipped with a standard nitrogen laser (337 nm) in positive reflector mode with a mass accuracy of 0.2 Da; at least 200 laser shots were collected for each spectrum. The spectra were analysed using XMASS (Bruker), mMass software [27] and a homemade database of reference proteinaceous binders [28].

Fig. 2. The printed engraving by Albrecht Dürer (1500) illustrating the Mourning of Christ scene.

3.2.5. Mass spectrometry nano-LC-ESI-Q-TOF

Measurement was carried out using UHPLC Dionex Ultimate 3000 RSLC nano (Dionex, Germany) connected with a mass spectrometer ESI-Q-TOF Maxis Impact (Bruker, Germany). 10 μL of a peptide solution was previously dried and then dissolved in a 97:3:0.1% mixture of water:acetonitrile:formic acid. Consequently they were loaded on a trap column Acclaim PepMap 100 C18 (100 μm × 2 cm, size of reverse phase particles = 5 μm, Dionex, Germany) with a flow rate of mobile phase A = 5 μL/min for 5 min. The peptides were eluted from the trap column to an analytical column Acclaim PepMap RSLC C18 (75 μm × 250 mm, size of reverse phase particles = 2 μm) using the following gradient: 0 min 3% B, 5 min 3% B, 85 min 50% B, 86 min 90% B, 95 min 90% B, 96 min 3% B, 110 min 3% B. Mobile phase A was 0,1% formic acid in water and mobile phase B was 0,1% formic acid in acetonitrile. The flow rate during gradient separation was set to 0.3 μL/min. Peptides were eluted directly to the ESI source – Captive spray (Bruker Daltonics, Germany). Measurement was carried out in positive ion mode with precursor selection in the range of 400–2200 Da; from each MS spectrum, up to ten precursors were selected for fragmentation.

Peak lists were extracted from raw data by Data Analysis (Bruker Daltonics, Germany). Proteins were identified using Mascot version 2.2.04 (Matrix Science, UK) by searching protein database Uniprot version 20110-12. Parameters for database search were set as follows: Oxidation of methionine and hydroxylation of proline as variable modifications, tolerance of 50 ppm in MS mode and 0.05 Da in MS/MS mode.

3.2.6. microRaman on cross-sections

The equipment used was a Labram 300 Jobin Yvon spectrometer, equipped with a He–Ne laser of 17 mW power operating at 632.8 nm and a solid state laser operating at 532 nm. The laser beam was focused with a 50× or 100× Olympus objective lens. The laser energy was filtered up to 10% using a neutral density filter for all analyses. The

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attribution of the Raman spectra was made using databases of reference materials reported in the literature [29,30].

3.2.7. SEM-EDX on cross-sections

A VEGA II LSH scanning electron microscope (TESCAN — Czech Republic), coupled with an EDX-QUANTAX QX2 (ROENTEC Bruker — Germany) spectrometer was used. The EDX Quantax QX2 uses a detector of third generation Xflash, that does not need cooling with nitrogen and is 10 times faster than a traditional detector based on Si(Li). The SEM images and EDX spectra have been acquired in the following conditions: 20 and 30 kV voltage; 1×10^{-3} Pa; working distance of 11–20 mm (16.6 mm for EDX) and magnification: 78× to 1000×. The cross-sections were covered with a fine layer of graphite using a specific “sputter coater”.

4. Results and discussions

The results obtained from SEM-EDX and Raman spectroscopy performed on embedded fragments of polychrome layers are given in Table 1.

The mainly identified pigments were those commonly used in medieval times: lead white, lead red, vermillion, red ochre (hematite), massicot, ultramarine blue, and indigo. The SEM-EDX also identified the presence of Co in the M11 sample (probably attributed to a Co-based pigment, maybe Co blue as Al was also identified in the same area as the cobalt — Fig. 3c) and of Ba in several samples (M1, M8, M9 and M11) suggesting the use of barite (Ba sulphate) probably during the intervention of over-painting or puttying (this type of material being in use in modern times as a filler or addition to white pigments). The ground layers are made of calcium carbonate (chalk), which contains fragments of shells (coccoliths) indicating a marine sedimentary fossil origin of the calcite. The presence of coccoliths was already reported by P. Dietemann as part of the ground layers in polychrome sculptures in medieval times in Germany [31].

Fig. 3 gives a selection of 3 cross-sections on which OM, SEM-EDX and microRaman were performed. The first fragment (M1a — Fig. 3a) was taken from Jesus Christ's finger incarnate and the OM images in Vis and UV light show a series of overlapped layers over the ground (the most superficial ones could be attributed to an over-painting). The second case (Fig. 3b) shows a green area of over-painting from the knee drapery of S. John, with the optical microscopy unveiling not more than 5 overlapped layers, each of them of different colour, granulometry and fluorescence pattern. The composition and structure of the layers appear heterogeneous (big grains of different shapes, inside a more or less darker green matrix) and correlating the microRaman with the SEM-EDX results (Table 1) it seems that they contain a mixture of pigments (massicot, lead white, red ochre, a Cu-based pigment) with some clay mineral constituents (the SEM-EDX identified Fe, Al, Si). Sample M11 (Fig. 3c) is another case of an over-painted area fragment, displaying not less than seven layers of different colours, with a heterogeneous composition. Besides the ultramarine, a CO-blue pigment is also responsible for the blue colour of several layers, mixed with other pigments (red ochre, red lead) to give different tones of green, ochre and blue.

Fig. 4 shows the example of sample M5, which was taken from the welt of John's cloak. The distribution of chemical elements in the EDX mapping together with the observation of the sequence and patterns of layers under OM and the pigment identification by microRaman characterizes a complex and multi-layer structure with coccoliths in the calcitic ground and remains of gold leaf between the red upper layers of paint.

The remains of former gildings, which were used on most of the represented characters, were found in samples M5 and M10 (Fig. 5). If in the case of sample M5 an ochre bole layer is visible under the thin leaf (Fig. 5a), in the sample M10 the leaf seems to be applied directly over the white ground (Fig. 5b). The MALDI-TOF-MS detected animal glue and traces of egg proteins in both samples [1]. Unfortunately, it is nearly impossible to assign the individual binders to the ground layer or to the adhesive under-layer of the gildings, because the ground layers contain oil that could come from egg yolk or it could be added as vegetal oil and the gilding under-layer is too thin for the analyses.

The dyes Sypro Ruby and Oil red O were previously used on the polished cross-sections to detect layers containing proteins and oils [1]. The enzymatic cleavage was performed directly on the cross-sections and after the desalting the solutions containing peptides were analysed by both mass spectrometric methods MALDI-TOF and nano-LC-ESI-Q-TOF. MALDI-TOF mass spectrometry in most of the samples found mainly collagen binders and to a lesser extent, egg proteins [1]. The mass spectrum obtained from sample M10 is shown in Fig. 6.

Nano-LC-ESI-Q-TOF confirms the previous results obtained by MALDI-TOF MS and identified mainly collagens, keratin contamination (proteins derived from dermal cells) and traces of egg protein (only by one peptide) in the same samples (e.g. sample M10 — Fig. 6, Table 2). Although this technique can uniquely identify proteins and all found collagens were assigned to the *Bos taurus*, mainly in the case of collagens it could hardly identify their species specificity [32], because the collagens are very old evolutionary proteins with a highly conserved amino acid composition. The identified keratins most probably originate from surface contamination of polished sections, which may have occurred during their preparation or manipulation.

5. Conclusions

The results obtained by visible and fluorescent light OM, SEM-EDX and micro-Raman spectroscopy revealed the classical structure of a multi-layer polychromy and the composition of the materials present in this medieval polychrome statue. The ground layer is made from “marine” chalk, containing the remains of calcareous nano-fossils of eukaryote phytoplankton, known as coccoliths. The presence of these nano-fossils in the ground layer cannot add information on

Table 1
The inorganic constituents of 11 samples from “The Mourning of Jesus Christ”, identified by Raman spectroscopy and SEM-EDX.

Sample	Vermillion	Cu-based pigment	Ultramarine	Indigo	Hematite (red iron oxide)	Lead red	Lead white	Massicot	Calcite	Ba sulphate
M1	√				√		√		√	√
M1a	√			√					√	
M2	√		√				√		√	
M4		√			√		√	√		
M5	√				√		√			
M6				√			√			
M7	√						√			
M8							√		√	√
M9					√		√		√	√
M10									√	
M11			√		√	√			√	√

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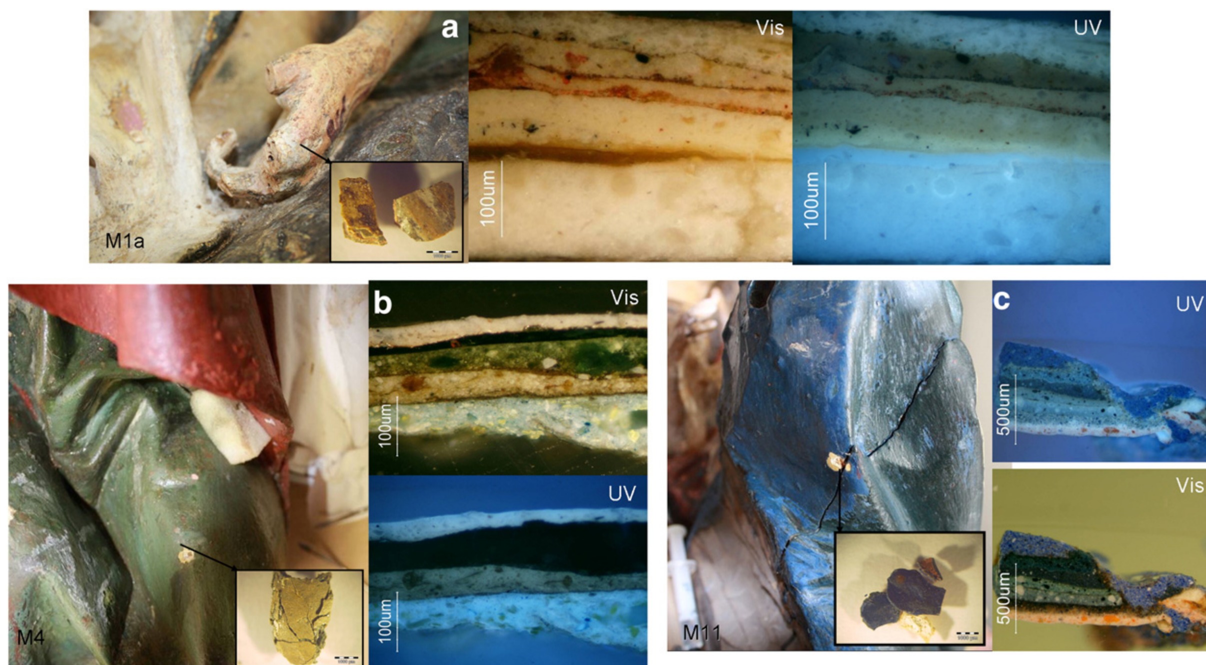

Fig. 3. Cross-section observation and related sampling areas for 3 samples: a) sample M1a; b) sample M4; c) sample M11.

the creation of the figurative woodcarving, because they are the common constituents of the calcium carbonate sediments and such material has been used all around medieval Europe [33]. Other inorganic pigments identified in the paint layers have also been commonly used since the Medieval Ages, therefore the provenience of the art work could not be securely determined based on these data. The presence of over-painting layers/materials was assessed in several samples taken from damaged areas and few pigments/fillers belonging to modern times (Co-based pigment, barite) confirmed the presence of layers of posterior intervention.

Identification of binders together with pigments and fillers in different layers of the analyzed polychrome samples allowed restorers to compositionally and stratigraphically characterize the decoration of the sculpture and to assess the presence of historically known

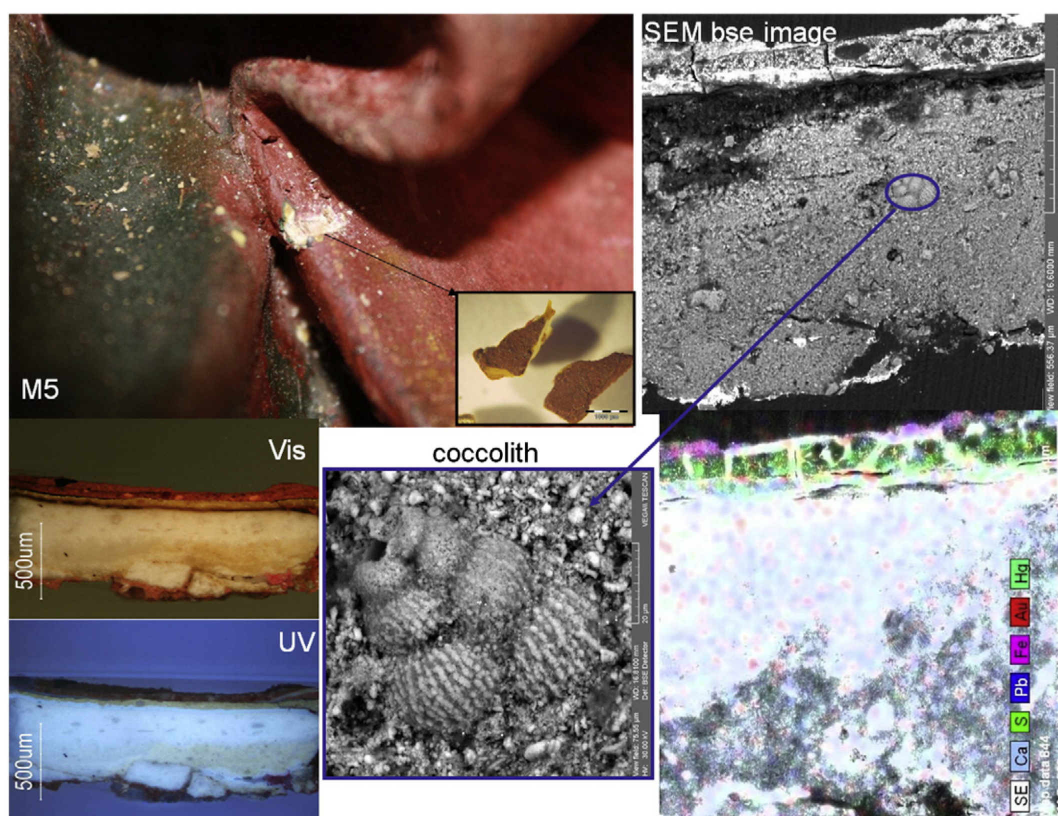

Fig. 4. Characterization of sample M5 using OM and SEM-EDX.

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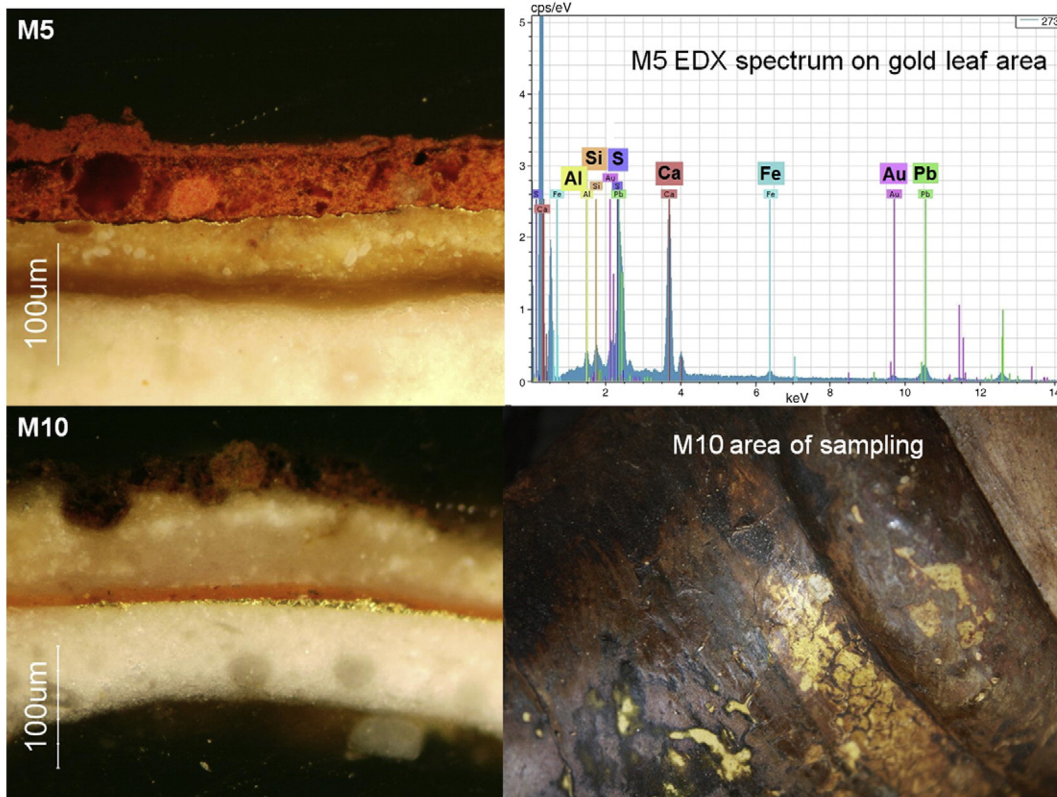

Fig. 5. Two samples with gold leaf, details of the OM Vis images and EDX spectrum for sample M5.

techniques (paint and gilding techniques) useful for further selection of the most appropriate treatment of restoration.

Based on the results of this detailed analytical protocol the wrinkled paint layers added during the 20th century, applied in very thick layers to correct defects on the heavily damaged polychromy, were removed. The early 19th century over-paints (in Renaissance, Rococo and Baroque style), were also removed. It was found that the first Gothic re-paints with artistic high quality are enough to maintain an authentic expression of the work in its entirety. The substantial parts of the older polychrome were found and many details of carving mastery of anonymous Late Gothic artists also excel.

Both mass spectrometric methods MALDI-TOF and nano-LC-MS/MS are able to identify protein binders in their mixtures, even in samples embedded as polished cross-sections. MALDI-TOF mass spectrometry

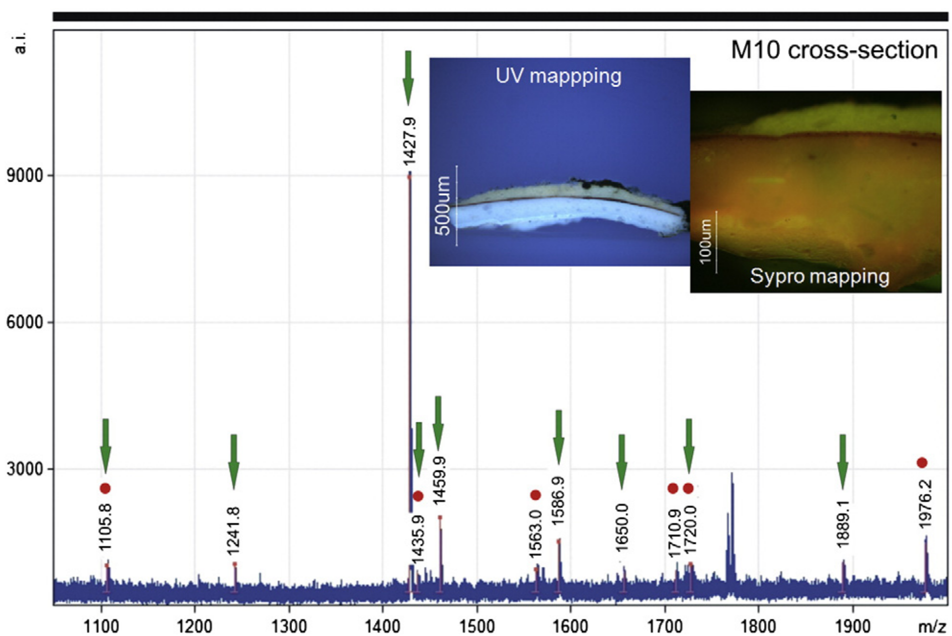

Fig. 6. Mass spectrum of a fragment from sample M10. The red circles labelled egg proteins and the green arrows animal glue.

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Table 2
The results of protein analysis of sample M10 by nano-LC-ESI-Q-TOF mass spectrometry.

Protein	No. of peptides
Collagen alpha-1(I) (*Bos taurus*)	33
Collagen alpha-2(I) (*B. taurus*)	20
Collagen alpha-1(III) (*B. taurus*)	2
Ovalbumin (*Gallus gallus*)	1
Trypsin (*Sus scrofa*)	4
Keratin, type I cytoskeletal 9 (*Homo sapiens*)	3
Keratin, type II cytoskeletal 2 epidermal (*H. sapiens*)	3

is less demanding (in terms of equipment, serviceability and financial aspects) and found more evidence for the presence of egg protein. The nano-LC-MS/MS method can clearly identify protein binders, including the identification of specific proteins from which individual peptides come from. MALDI-TOF MS is less time-consuming (tens of samples per day), while the nano-LC-MS/MS method can measure about 8 samples per day.

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