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PROTEOMICS TOOLS FOR THE CONTEMPORARY IDENTIFICATION OF PROTEINACEOUS BINDERS IN GILDED SAMPLES

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Abstract

This paper deals with the characterization of several gilded and polychrome samples from the carved wood Portuguese heritage using a novel proteomics approach aimed to identify and map the presence of different proteinaceous binders. Eight samples originating from two altarpieces (Main altarpiece from Miranda de Douro Cathedral and Main altarpiece of the Cathedral in Funchal, Madeira) and three polychrome sculptures (St. Joseph from Aveiro Museum and two sculptures by Machado de Castro) were taken and analysed both as fragments/separated layers (e.g. ground layer separated from the gilded/polychrome layers) and cross-sections. The methodology proposed here makes use of proteomics techniques such as ELISA, MALDI-TOF-MS, nano-LC-ESI-Q-TOF and optical microscopy coupled with a fluorescent staining test (Sypro Ruby) on samples and cross-sections and is part of the analytical task of the Gilt-Teller project (www.gilt-teller.pt). ELISA, and both mass spectrometric techniques MALDI-TOF and nano-LC-ESI-Q-TOF showed a good detection of collagens on whole fragments or separated layers and also on cross-sections, while the mass spectrometric techniques were less sensitive for egg proteins. Nevertheless, the combined use of them together with the mapping on cross-sections using the fluorescent stain made the simultaneous identification and location of these binder materials in the stratigraphy of some samples possible.

Keywords: *Proteomics; ELISA; LC-MS/MS; MALDI-TOF MS; proteinaceous binders; gilded carved wood*

Introduction

The simultaneous localisation and identification of proteinaceous binders is highly challenging for conservation scientists up to the present, because most of analytical methods (e.g. GC-MS, Py-GC-MS, HPLC), which are used for the identification of protein material in works of art, allow to identify and distinguish at least egg, animal glue and milk proteins [1–5]. Moreover, these techniques are still used for the identification of proteins mainly from fragments. However, the more challenging task is the possibility of simultaneous identification and localisation of proteins in the cross-sections of works of art [6–8]. It was specifically tested

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by coupling spectrofluorimetric measurements with fluorescent staining [9], by surface-enhanced Raman scattering (SERS) nanotags complexed to secondary antibodies in conjunction with primary antibodies to determine egg proteins (ovalbumin) and casein [10], by multiplexed chemiluminescent immunochemical imaging technique used also for detection of casein and ovalbumin [11], and by applying ELISA (enzyme linked immunosorbent assays) together with immunofluorescence microscopy (IFM) for egg white protein (ovalbumin) [12, 13]. In particular it is worthy to mention the potential of microspectrofluorometry to discriminate among different protein sources (as between egg white, egg yolk, and glues) within paint layers when used in conjunction with a dedicated fluorescence dye, Sypro Ruby, and suitable deconvolution [9]. However all these techniques are very new and need to be verified or adapted for all types of materials that have been used in paintings and polychrome sculptures.

In this paper the combination of optical microscopy (OM) together with Sypro Ruby (SR) stain [14–15] , indirect ELISA [16] and two mass spectrometric methods (MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) and nano-LC-ESI-Q-TOF (nanoscale liquid chromatography coupled to electrospray ionization quadrupole time-of-flight tandem mass spectrometry) [17–19] were applied to identification of proteins in fragments and also in cross-sections, and consequently compared.

The indirect ELISA involves two binding process of primary antibodies and labelled secondary antibodies. The primary antibodies are incubated with the antigen (certain protein from proteinaceous binder, e.g. ovalbumin from egg white) followed by the incubation with the secondary antibodies that are labelled with an enzyme. When the substrate for the enzyme is added, as a positive response the solution changes its colour or provide a fluorescent signal by excitation under UV.

On the other hand, 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 mass spectrometer and then compared and assigned to binder from reference database of protein binders [20]. 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 demands two peptides for a reliable determination of individual protein.

In this work the proteinaceous binders of the paint layers coming from the Portugal wood carved and gilded objects are analysed using a complementary protocol of investigation, based on the use of optical microscopy (OM) with Sypro Ruby, ELISA, nano-LC-ESI-Q-TOF and MALDI-TOF mass spectrometry on fragments and also cross-sections.

Experimental

Reagents and materials

2,5-dihydroxybenzoic acid (DHB) and formic acid (LC-MS grade) were purchased from Sigma (Steinheim, Germany). Trypsin (TPCK) comes from Promega (Medison, WI, USA). Acetonitrile (LC-MS grade) was purchased from Applichem (Darmstadt, Germany). Reverse phase ZipTip C18 was purchased from Millipore (Billerica, MA USA). Pepmix comes from Bruker (Bremen, Germany). Water used for analyses was purified by Milli-Q Direct Water system.

Sypro Ruby was purchased as ready to use solution from Molecular Probes (200 mL, protein blot stain, 10-40 blots).

The following primary antibodies were used: rb-anti-ovalbumin (ab1225, Millipore, Billerica, MA USA), rb-anti-casein (RCAS-10A, ICL Inc., Newberg, OR, USA), rb-anti-collagen (ab34710, Abcam, Cambridge, MA, USA). Horseradish peroxidase conjugated

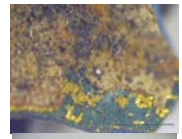
secondary antibodies were purchased from Southern Biotech (gt-anti-rabbit 4050-05, Birmingham, AL, USA). Sodium carbonate, sodium bicarbonate and PBS come from Carl Roth GmbH (Karlsruhe, Germany), sodium azide from Sigma Aldrich (St. Louis, MO, USA) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) from Southern Biotech (Birmingham, AL, USA). Hydrogen peroxide, Tween 20, citric acid, monosodium phosphate and new born calf serum (NCS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). PVC microwell plates from Becton Dickinson (#353912, Erembodegem, Belgium) were used.

Experimental conditions

Sampling

Eight samples containing polychromy and gilding were taken from five objects, two main altarpieces and three sculptures from Portugal (Table 1).

Table 1. Historical objects and analysed samples

Object	Date	Sample acronym	Sampling area description	Stereomicroscope or microscope image of the sample
Man altarpiece from the Cathedral in **Miranda de Dour**o	1633-1754	PT-AM-MD_6	Gilding from a garment	
		PT-AM-MD_16	Dark blue from a garment, including gilding layers	
		PT-AM-MD_28	Light red from a garment	
Main altarpiece from the Cathedral in Funchal (Our Lady of Assumption), Madeira	1512	PT-AM-SFU_11	Blue from the background in the central part of the altarpiece	
		PT-AM-SFU_12	Blue with gold leaf, similar area as the previous sample	
Polychrome sculpture of St. Joseph, **Aveiro** Museum	16th century	PT-PS-SSJ-MA_5	Red – "estofado" decoration from the mantle	
Polychrome sculpture – Deposition by Machado de Castro, **Coimbra**	18th century	PT-SNSP-MC_5	Polychromy from the garment of the Virgin with light blue color and gilding	
Polychrome sculpture – St. John the Baptist by Machado de Castro, **Coimbra**		PT-SSJE-MC_6	Flesh fragment from the big finger of a foot	

Cross-sections and staining tests

Cross-sections were obtained using a Polyester embedding resin (Mecaprex SS) with hardener (methyl ethyl ketone peroxide) dry polished with successively finer grades of Micro-mesh abrasive cloths (600, 800, 1200 and 4000 mesh). A 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.

Microscope images were taken from the cross-sections at different magnifications (from 50× to 500×) using an Axioplan Zeiss 2 imaging binocular microscope, coupled to a Nikon DXM1200F digital camera. The filter blocks used for observing the fluorescence were filter 8 (G 365, FT 395 and LP 420) and filter 6 (BP 450-490, FT 510 and LP 515). Visual light observations (illumination position for dark field observation, abbreviated as f2) were performed in reflection geometry.

The staining procedure was done by the application of the Sypro Ruby stain in order to map the proteinaceous binders. Sypro Ruby is a biomedical non-covalent stain [21-22], used for fluorescence mapping of proteins within the multi-layered samples. This fluorescent stain is generally used in the proteomics field (adopted from 1D and 2D gel electrophoresis), being preferred to other stains (Silver staining or Coomasie) because of its high nanogram sensitivity and selectivity, being available as a ready-to-use solution (1 drop is applied directly on the cross-section surface using a Pasteur pipette).

Protein digestion

The fragments in the weights of several mg or lower and cross-sections were digested in 15 µL of 50mM NH_4HCO_3 containing 10 $\mu g.mL^{-1}$ of trypsin at room temperature for two hours. After the trypsin digestion, the samples were purified on reverse phase ZipTip.

Matrix-Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry

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 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 Bruker Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with standard nitrogen laser (337 nm) in positive reflector mode. Obtained spectra contain mass peaks in the interval 900–2000 Daltons (*m/z*) and it has accuracy 0.3 Da. Resulting spectra were processed with XMASS software (Bruker) and mMass [23-24]. Peptide mixture Pepmix was used for the instrument calibration before the measurements. The obtained spectra were compared with our database of reference protein additives [17].

Liquid chromatography (nanoLC-ESI-Q-TOF MS)

Samples (fragments and cross-sections) were cleaved by trypsin for two hours, see above. The resulted peptide mixture were purified by reverse phase C18 (ZipTip), evaporated to dryness and dissolved in mixture of water : acetonitrile : formic acid (97:3:0.1%), and then loaded on trap column Acclaim PepMap 100 C18 (100 µm x 2 cm, particle size 5 µm, Dionex, Germany) with mobile phase flow rate of A (0.1% formic acid in water) 5 µL/min for 5 min. The peptides were eluted from trap column to analytical column Acclaim PepMap RSLC C18 (75 µm x 250 mm, particle size 2 µm) by mobile phase B (0.1% formic acid in acetonitrile) using 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. 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). Measurements were carried out in positive ion mode with precursor ion selection in the range of 400-2200 Da; up to ten precursor ions were selected for fragmentation from each MS spectrum. Measurements were carried out using UHPLC Dionex Ultimate3000 RSLC nano (Dionex, Germany) connected with mass spectrometer ESI-Q-TOF Maxis Impact (Bruker, Germany). 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 50 ppm in MS mode and 0.05 Da in MS/MS mode.

Enzyme-linked immunosorbent assay (ELISA)

Samples (50–200 μg) were extracted for 24 hours at 37°C in 0.05 M carbonate-bicarbonate buffer (pH 9.6) followed by centrifugation and transfer of supernatant with the extracted proteins to new vials. A volume of 50 μL of each sample extract was plated into the appropriate wells of a PVC microwell plate and incubated for 16 hours at 4°C. Any unbound antigens (proteins) were removed by washing the wells 3 times with 150 μL/well of PBS. The wells were blocked for 1 hour at room temperature with 100 μL/well of 5 % NCS in PBS. A volume of 50 μL of primary antibodies in blocking buffer were added to the appropriate wells in working dilutions of 1:1000 for ovalbumin (#ab1225) and casein (#RCAS-10A), 1:500 for collagen (ab34710) and incubated for 16 hours at 4°C. Unbound primary antibodies was removed by washing each well with 150 mL of washing buffer I (PBS with 0.05 % Tween-20) for 4 times, and 50 mL of secondary antibody (horseradish peroxidase (HRP) conjugated) in a working dilution of 1:500 in blocking buffer was added and allowed to incubate at 37°C for 90 minutes. The wells were washed with 150 mL of washing buffer II (PBS with 0.005 % Tween-20) for 4 times followed by a final wash with 150 μL/well of PBS. For colorimetric development ABTS powder was dissolved in phosphate-citrate buffer (pH 4.9) to a final concentration of 0.5 mg mL^{-1}. Right before use, 0.003 % hydrogen peroxide was added to the ABTS solution (HRP catalyses hydrogen peroxide oxidation of the substrates). 50 μL of ABTS/hydrogen peroxide solution were added per well and incubated for 10 min. at room temperature. The reaction was stopped with an equal volume of 0.5 mg mL^{-1} of sodium azide in phosphate-citrate buffer (pH 4.9). The optical density was measured with a spectrophotometer (SLT Spectra, Labinstrumente GmbH, Achterwehr, Germany) at 414 nm. The analyses were accompanied with negative and positive controls and samples as well as controls were run in duplicates. In addition all samples were run in serial dilutions.

Results and discussion

From the main altarpiece of the Cathedral from Miranda do Douro (1633–1754) three samples were analysed (Table 1). In sample PT-AM-MD-6 optical microscopy detected proteins in ground layer after the application of Sypro Ruby (SR). MALDI-TOF MS worked with a cross-section of this sample and identified animal glue as well as ELISA that analysed whole fragment containing ground, yellowish bole and gilding (Table 2).

Table 2. Summary of the results obtained by optical microscopy with using of Sypro Ruby, mass spectrometric methods (MALDI-TOF and LC-MS/MS) and ELISA. X – successfully identified protein binder

Objects	Sypro Ruby	Mass spectrometry	ELISA			
Sample ID	**Proteins**	**MALDI-TOF and LC-MS/MS**	**Separated layers for ELISA**	**Animal glue**	**Egg proteins**	**Sample description**
PT-AM-MD-6	x	Cross-section, MALDI: animal glue		X	-	whole fragment (ground, yellowish bole and gilding)
PT-AM-MD-16	x	MALDI: animal glue, LC-MS/MS: animal glue, egg proteins	a	X	-	red and white layers
			b	X	X	black, gilding and little of the red layer
PT-AM-MD-28	not applied	Cross-section, MALDI:	a	X	-	ground only
			b	-	X	red, gilding and pink layer

		animal glue, egg proteins LC-MS/MS: animal glue				(little ground)
PT-AM-SFU-11	x	-		-	-	whole fragment with blue, white and blue layer
PT-AM-SFU-12	x	-	a	X	-	blue with grey layer
			b	X	-	blue (top), brownish layer below - towards wood
PT-PS-SSJ-MA-5	not applied	MALDI: animal glue, egg proteins	a	X	-	ground only
			b	-	X	red, gilding, brown and varnish layer (bit ground)
PT-SNSP-MC-5	not applied	-	a	X	X	ground, red layer (bole) and gilding
			b	x	X	whole fragment with a lot of light blue colour
PT-SSJE-MC-6	not applied	MALDI: animal glue	a	X	X	white with red layer on the bottom
			b	-	x	white with red layer on top

In the next sample PT-AM-MD-16 the presence of proteins in cross-section using the fluorescent stain SR (Fig. 1) was also detected. MALDI-TOF MS analysed the whole fragment and in the spectrum animal glue was found, but it is not possible to exclude the presence of whole egg proteins (Fig. 1). The same sample was analysed by LC-MS/MS and the mixture of animal glue and egg proteins was confirmed (Table 3). ELISA analysed separated layers; animal glue was identified in red and white layers and mixture of animal glue and egg proteins in black, gilding and little of the red layer.

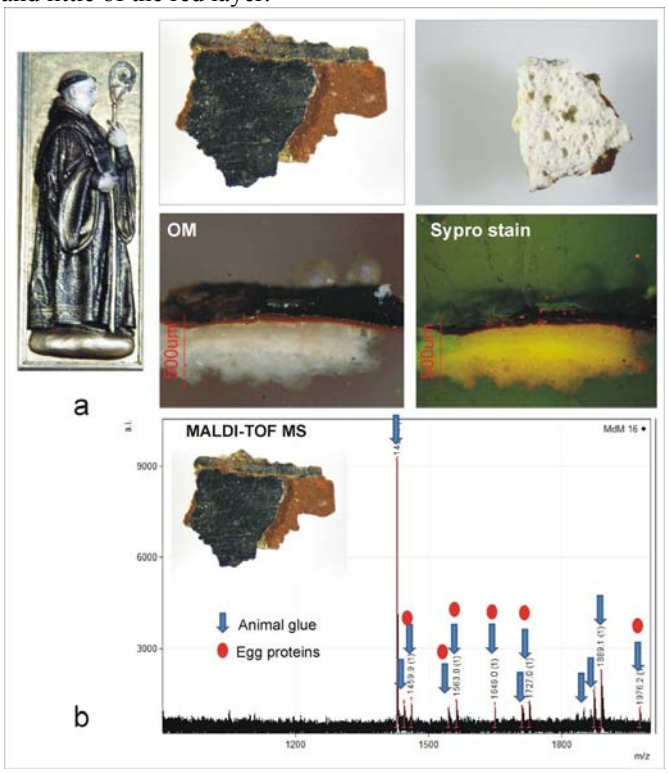


Fig. 1. Sample PT-AM-MD-16: a) localisation of sampling, photos of fragment from upper and bottom side and photos of cross-section before and after the application of the fluorescent stain Sypro Ruby; b) MALDI-TOF mass spectrum of the fragment with labelled peaks/peptides coming from animal glue and egg proteins.

Table 3. The results from LC-MS/MS analysis of sample PT-AM-MD-16 (fragment); the animal glue – collagens and egg proteins were identified.

Accession	Protein	#Peptides
CO1A1_BOVIN	Collagen alpha-1(I) chain	25
CO1A2_BOVIN	Collagen alpha-2(I) chain	28
CO3A1_BOVIN	Collagen alpha-1(III) chain	5
VIT2_CHICK	Vitellogenin-2	4
OVAL_CHICK	Ovalbumin	2
APOV1_CHICK	Apovitellenin-1	2
VIT1_CHICK	Vitellogenin-1	2
TRYP_PIG	Trypsin	3

The last sample PT-AM-MD-28 (cross-section) contained mixture of animal glue and egg proteins according MALDI-TOF MS (Fig. 2). However, LC-MS/MS identified only animal glue in the whole fragment and also in the same cross-section (after the first digestion for MALDI-TOF MS) (Table 4). ELISA detected animal glue in ground and egg proteins in red, gilding and pink layer (little ground) (Fig. 2).

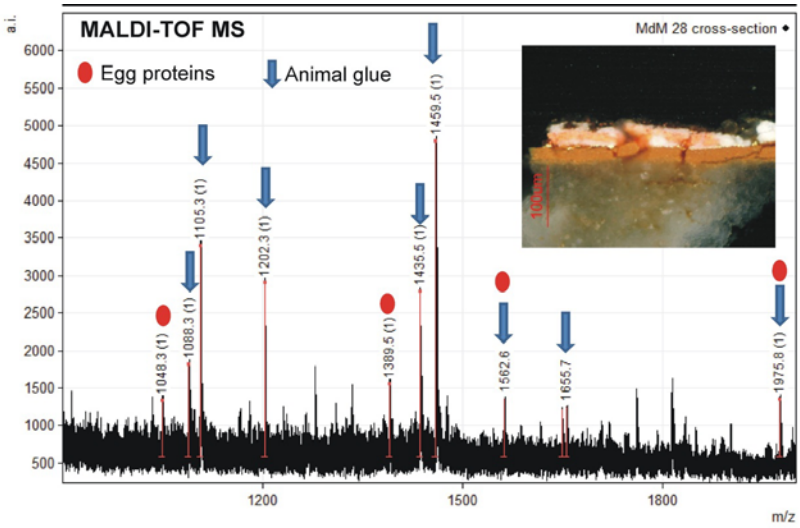


Fig. 2. MALDI-TOF mass spectrum of the cross-section of sample PT-AM-MD-28 with labelled peaks coming from animal glue and egg proteins.

Table 4. The results from LC-MS/MS analysis of sample PT-AM-MD-28 (fragment) and also its cross-section. Only the animal glue and trypsin that was used for cleavage were identified.

	Accession	Protein	#Peptides
Fragment	CO1A2_BOVIN	Collagen alpha-2(I) chain	11
	CO1A1_BOVIN	Collagen alpha-1(I) chain	9
	TRYP_PIG	Trypsin	3
Cross-section	CO1A1_BOVIN	Collagen alpha-1(I) chain	15
	CO1A2_BOVIN	Collagen alpha-2(I) chain	6
	TRYP_PIG	Trypsin	4

From the main altarpiece of the Cathedral of Our Lady of the Assumption in Funchal (Madeira) two samples containing azurite were taken (Table 1). MALDI-TOF did not find any proteinaceous binder in both samples. ELISA confirmed that the sample PT-AM-SFU-11 does not contain any proteins, even they were indicated by the SR test (Fig. 3). However, ELISA found animal glue in the second sample PT-AM-SFU-12 that was also indicated by SR (Fig. 3). The stain showed a slight coloration of the ground (where supposedly the animal glue is present) and of the upper ochre layer (bole).

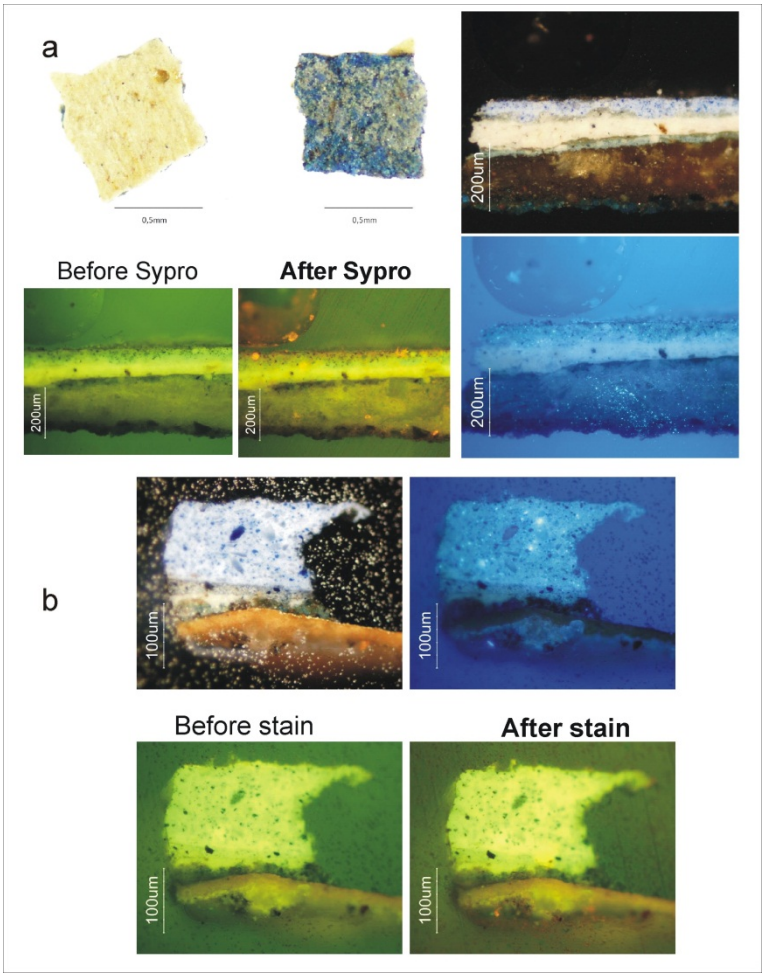


Fig. 3. Cross-sections of the samples from the main altarpiece in the Cathedral in Funchal (PT-AM-SFU): a) sample 11 – observed under Vis and UV light, before and after the application of the fluorescent stain Sypro Ruby; b) sample 12 – observed under Vis and UV light, and before and after the application of the fluorescent stain Sypro Ruby.

For the sample PT-PS-SSJ-MA-5 taken from the polychrome sculpture of Saint Joseph (16^{th} century) coming from Museum of Aveiro (Table 1, Fig. 4), MALDI-TOF MS and ELISA obtained the same results; they identified animal glue together with egg proteins (Table 2). ELISA specified the presence of egg proteins in red, gilding, brown and varnish layer (bit ground) and the presence of animal glue in ground.

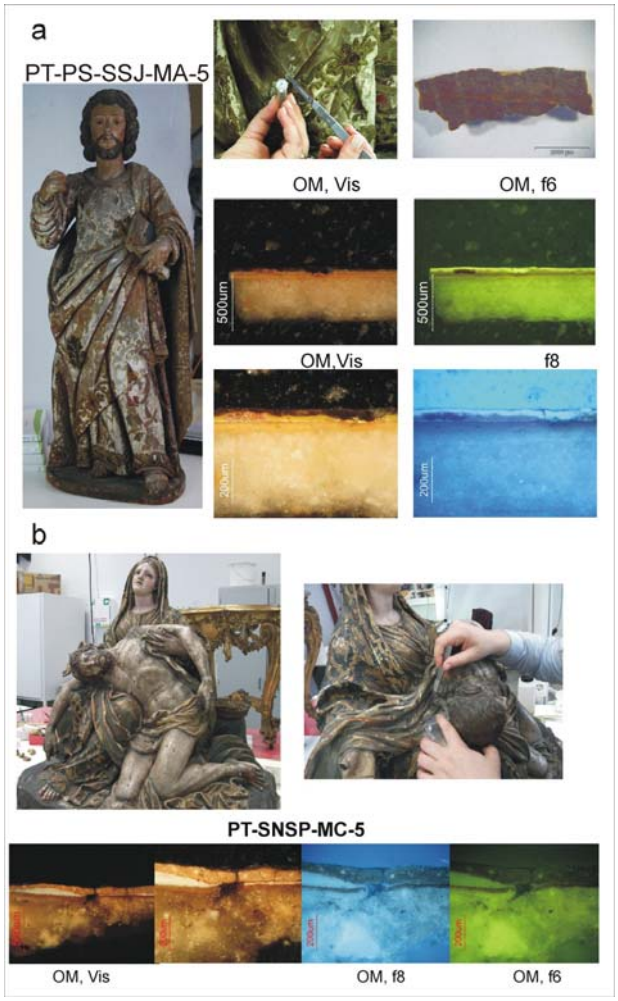


Fig. 4. Cross-sections from the two samples taken from two sculptures: a) PT-PS-SSJ-MA-5 – localisation of sampling, photos of fragment from upper side and photos of cross-section in Vis and UV light (f8) and under green filter (f6); b) PT-SNSP-MC-5 – localisation of sampling, photos of cross-section in Vis and UV light (f8), and under green filter (f6).

In sample PT-SNSP-MC-5 from the sculpture representing the Deposition of Christ, made by Machado de Castro in 18th century (Fig. 4) no proteins were identified by MALDI-TOF MS. However, ELISA found mixtures of animal glue and egg proteins in ground, red layer (bole) and gilding, and also the same mixture in the whole fragment with a lot of light blue coloured pigment.

From the polychrome sculpture of Saint John the Baptist also made by Machado de Castro, one sample (PT-SSJE-MC-6) was analysed (Table 1) and MALDI-TOF unambiguously confirmed the presence of animal glue even the spectrum contains the common peptides for egg proteins (Fig. 5). By ELISA separated layers were analysed and animal glue was thus detected in white with red layer on top and mixture of animal glue and egg proteins in white with red layer on the bottom part of sample.

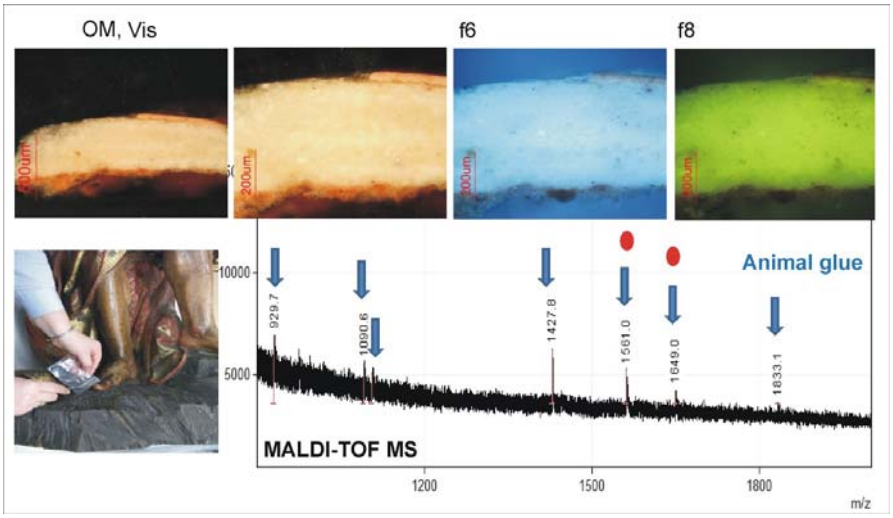


Fig. 5. Detection of animal glue using MALDI-TOF MS on sample PT-SNSP-MC-5 from St. John sculpture by Machado de Castro: localisation of sampling, photos of cross-section under Vis and UV light (f8), and under green emission fluorescence (f6).

From the obtained data it was shown that MALDI-TOF MS has difficulties with the protein identification in samples that contain the blue pigment – azurite. The presence of copper and their complexes with proteins negatively influence the analyses. However, the ELISA protocol was probably not affected by this pigment (PT-AM-SFU-11?) or just in lower level that still enables the protein identification. It is known that copper acts as a trypsin inhibitor [25], but probably we have to count also with the strong interaction between copper and proteins from binder that prevent the release of peptides to the digestion solution.

In several cases it was not possible to confirm the presence of egg proteins using MALDI-TOF MS, because they provide peptides with similar masses as those of the animal glue. Nevertheless, all the methods were tested on historical samples, where we do not know their original protein composition and therefore we cannot be sure, if some of the tested methods did not provide false positive or false negative response. Moreover, the tested samples were taken from the same objects, but they could contain different binding media, therefore the microfragments used for analysis could be made of different amounts of organic materials from the individual layers and this could also influence the final results. The test of the used methods on replica samples should be the next topic of these complementary tests.

Conclusions

In this paper four proteomic methods were used and consequently compared for identification and localization of proteins in eight samples taken from Portuguese gilded objects. On the basis of the obtained results, a combination of two or three proteomic methods together with optical microscopy can be used for the simultaneous identification and mapping on the same sample, e.g. ELISA + MALDI-TOF MS, OM + ELISA, and OM + ELISA + LC-MS/MS.

Within this work, the negative influence of the presence of copper based pigments (azurite) on the MS techniques was observed. However, ELISA was not influenced to such extent by this pigment. MALDI-TOF MS also shown to have probably lower sensitivity to egg proteins. This point is necessary to be confirmed by next tests, because the lower sensitivity could be caused by providing of similar peptides (*m/z* values of peptides) for animal glue and

egg proteins. But even then it could be caused by the nature and inhomogeneity of the samples that were divided for the individual methods that ELISA confirmed the presence of egg proteins. Moreover, the comparison of methods was done on real historical samples, without previous tests on reference samples of proteinaceous binders, and therefore it is not possible to guarantee, which of the used methods provides the correct results and not false positive or false negative responses.

While ELISA required the least amount of sample, the mass spectrometric methods (MALDI-TOF and LC-MS/MS) can be used for analyses of fragments and also cross-sections of paints that can be further studied by other analytical methods.

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