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Guest editors:
Klaas Jan van den Berg, Ilaria Bonaduce, Ester Ferreira, Ken Sutherland, David Peggie and Chris Maines

1. Getty Conservation Institute, 1200 Getty Center Drive, Suite 700, Los Angeles, CA 90049, USA

2. The J. Paul Getty Museum, 17985 Pacific Coast Highway, Pacific Palisades, CA 90272, USA

3. Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

4. Nara Women's University, Kita-Uoya-Nishi Machi, Nara, Nara 630-8506, Japan

5. University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan

corresponding author:
jmazurek@getty.edu

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CHARACTERIZATION OF BINDING MEDIA IN EGYPTIAN ROMANO PORTRAITS USING ENZYME-LINKED IMMUNOSORBANT ASSAY AND MASS SPECTROMETRY

Joy Mazurek[1], Marie Svoboda[2], Jeffrey Maish[2], Kazuki Kawahara[3], Shunsuke Fukakusa[4], Takashi Nakazawa[4], Yoko Taniguchi[5]

Abstract

Romano-Egyptian panels in the collections of the J. Paul Getty Museum dating to 180-200 A.D. were found to contain proteinaceous paint media. Animal glue was detected in the ground layers of all three panels using Enzyme-linked immunosorbent assay (ELISA) and mass spectrometry. Results were verified with gas chromatography/mass spectrometry (GC/MS) by the identification of 4-hydroxyproline, the major amino acid that occurs in animal glue collagen but not egg albumin. The animal species used to make the glue was identified as cow (*Bos taurus*) by using nano-liquid chromatography-electrospray ionization-tandem MS (nanoLC-ESI-MS/MS). A paint fragment from the *Bearded Man* contained tryptic peptides, type III collagen α1 chain, which is specifically expressed in skin suggesting that the animal glue was derived from cow hide. ELISA and ultraviolet (UV) fluorescence were used to isolate egg on the surface of the three paintings, and may be an artifact due to past restoration of the portraits.

1 Introduction

The study centered on three panels acquired by the Getty as a group in 1974 (Figure 1). The panels may have been displayed as a folding shrine or "klappbilder" and may present the earliest painted triptych in Western art of which all panels survive, and thus may represent the precursor to the Christian altarpiece[1]. The central portrait was created in the Romano-Egyptian funerary tradition and the three panels had been previously attributed to one artist. However, the central portrait of the *Bearded Man,* is stylistically different as compared to the more three dimensional *Isis* and *Serapis* and could perhaps have been painted by a different artist[2]. The wood on all three panels is Sycamore Fig and the unpainted edges on all four sides of the bearded man indicates it was framed at one point in time and perhaps displayed by

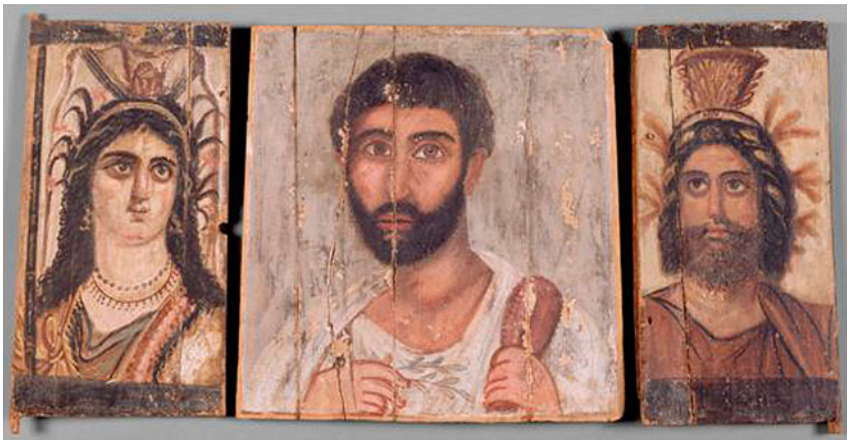

Figure 1: Unknown. Romano-Egyptian, Egypt, about A.D. 180 - 200. Tempera on wood. *Isis* 15 3/4 x 7 1/2 x 1/2 in. 74.AP.22; *Bearded Man* 14 3/16 x 14 3/4 x 1/8 in. 74.AP.20; *Serapis* 15 3/8 x 7 1/2 x 5/8 in. 74.AP.21

relatives in a form of ancestor worship. Most painted surfaces on wood have deteriorated over an extended time period, but Egypt's desert climate has preserved hundreds of these portraits for millennia in remarkably good condition.

1.1 Binding Media of Ancient Egypt

Two main classes of binding media from ancient Egypt have been described; "tempera" is a water soluble binding agent that is most frequently an animal glue or plant gum, and "encaustic" is paint made with beeswax[3-7]. Previous publications show the variety of different examples of portraits from Roman Egypt, however binding media results are often based on visual examination due to the reluctance to sample[3]. However, some results have been reported in the literature; animal glue was identified as the medium of a Fayum-region mummy portrait, as well as many other objects from Egypt[4]. Egg tempera was identified in a portrait from the Petrie museum, but only by the presence of non-drying lipids and solubility tests[5]. Plant gum has been identified in many Egyptian wall paintings, including in the tomb of Nefertari[6]. Animal glue was identified in an Egyptian painting on linen, 3rd to 4th C. A.D. by an amino acid analyzer[7]. Pyrolysis-GC/MS identified wax based and animal binders on Egyptian wooden sarcophagus and cartonnage (664-524 B.C.)[8]. Recently, we analyzed cartonnage samples from the Petrie museum and identified egg, animal glue, and plant gum using ELISA and GC/MS[9]. The majority of Egyptian portraits appear to have been painted in wax (based on visual examination) that originated from the Greek tradition (Encausto), but very few papers have been published on binding media of ancient panel and canvas paintings[10].

1.2 Identification of Protein Binding Media

Protein based binding media are almost always identified by gas chromatography/mass spectrometry (GC/MS) due to the relative ease of sample preparation and access to instrumentation in most conservation labs. Drawbacks of this technique are that it cannot identify the animal species used to prepare the glue, milk casein, or egg albumin, the protein must be hydrolyzed into the amino acids for identification, and difficulties occur with mixtures of proteins. Enzyme-linked immunosorbent assay (ELISA) may be able to identify the animal species but it requires expensive specialized antibodies and the similarities in the collagen molecule in mammals may produce false positives. In contrast, proteomics methodologies such as Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can identify and characterize species of proteins in works of art[12-19]. The sample preparation involves protease digestion to cleave specific peptide bonds, thus producing peptides with amino acid sequences characteristic of a protein. One critical issue of proteomics is the lack of amino acid sequences in the databases; due to difficulties finding representative reference samples[11].

MALDI-TOF MS has been used to discriminate between egg yolk, egg white, casein, milk, curd, whey, gelatin, and various animal glues in a painting by Edvard Munch[20]. It was used to identify proteins and lipids in a late-15th century Italian panel painting [21], animal glue in Tang Dynasty polychrome pottery[22], proteinaceous binders in cross-sections of a Czech medieval polychrome sculpture[23], and the collagen in a 5300-year-old Tyrolean mummy[24]. Egg white and egg yolk were identified as binders in two renaissance paintings using MALDI-TOF MS and nano LC/nanoESI/Q-q TOF MS/MS[25]. Mitochondrial DNA (mtDNA) has been used to identify the biological origin of proteinaceous binding media in combination with DNA amplification techniques utilizing polymerase chain reaction (PCR). By using ancient DNA analysis, the biological origin of the binding media used to make a polychrome terracotta Madonna of Citerna by Donatello (1415–1420) was identified as cow (*Bos tarus)*[26].

2 Materials and Methods

We utilized a combination of techniques to study the binding media in this study; enzyme-linked immunosorbent assay (ELISA), gas chromatography / mass spectrometry (GC/MS), and nano-liquid chromatography-electrospray ionization-tandem MS (nanoLC-ESI-MS/MS). The combination of the three techniques is complimentary; ELISA positively identified mixtures of proteins, GC/MS provided quantitative amino acid results, and nanoLC-ESI-MS/MS proteomics gave species level identification.

2.1 nanoLC-ESI-MS/MS

Samples were ground in a mortar with a single-use synthetic pestle, and mixed with 500 µL of 0.1 M NH_4HCO_3 to extract proteins. After filtering through a membrane (Whatman/GDX-PTEE), heated to 60 °C for 30 min to denature the extracted proteins, and dialyzed five times against 25 mM NH_4HCO_3 solution using Amicon Ultra centrifugal filter units with a 3,000 Da cut off membrane (Millipore). Add sequence grade trypsin (10 µg/mL 0.1 M NH_4HCO_3) and incubate at 37 °C for 24 hr. The solution containing tryptic peptides are loaded onto the equilibrated ZipTip C18 Pipette Tip (Millipore), and eluted with 50% (v/v) aqueous acetonitrile (AcCN) containing 0.1% (v/v) trifluoroacetic acid (TFA).

For protein identification, we used a ZAPLOUS HPLC-MS/MS System (AMR Inc.) composed of an ADVANCE UHPLC dual solvent delivery device (Michrom BioResources) and a Finnigan LTQ linear ion-trap mass spectrometer (Thermo Fischer Scientific) equipped with an XYZ nanoelectrospray ionization source (AMR Inc.). Prior to the analysis, the peptide solution was evaporated and re-dissolved in MS-grade water (20 µl) containing 0.1% TFA and 2% AcCN. 1 µL aliquots were loaded with an HTC PAL autosampler (CTC Analytics) onto a short trap column (L-Column Micro, 0.3 x 5 mm, Chemical Evaluation Research Institute) for desalting and concentrating the peptides. Transfer to a capillary reverse phase column (L-column, 0.1 x 150 mm, Chemical Evaluation Research Institute) by washing the trap column with 0.1% aqueous TFA containing 2% AcCN. The flow rate was ~500 nL min^{-1}. Concentration gradient of AcCN: 5% to 30% in 0.1% aqueous HCOOH for 40 min, 95% for 1 min, constant at 95% for 3 min. The column temperature was 60 °C. Effluents from the separation column were introduced into the mass spectrometer via a FortisTip nanoelectrospray ionization needle (outer/inner diameter of

150/20 in µm each; OmniSeparo-TJ, Inc.). The ESI voltage was 1.6 kV and the temperature of transfer capillary at the LTQ inlet was 200 °C. The subsequent MS and MS/MS analysis in the order of output from the nanoLC unit were performed by automatic data acquisition system operated with Xcalibur software (Thermo Fischer Scientific). MS survey scans were performed in a mass range of m/z 450–1800 and helium gas was used for collision-induced dissociation in MS/MS analysis.

2.2 Protein Database Search

All the MS/MS spectral data obtained from nanoLC-ESI-MS/MS analysis were searched with Mascot search engine (version 2.1.04; Matrix Science, London, UK) against the SwissProt database (July 2013, 540,546 entries) with a peptide mass tolerance of 2.0 Da and fragment mass tolerance of 0.8 Da. In order to reduce the possibility of missing modified peptides, we allowed for increments of 16 Da for the oxidation of Met and Pro, 1 Da for the deamidation of Asn and Gln as well as maximally 2 miss-cleavage sites in trypsin digestion. In the database search, the taxonomy was filtered to 'bony vertebrates' entry, which includes 83,286 sequences. All the peptide sequences acquired from nanoLC-ESI-MS/MS analysis coupled with the Mascot database search were further checked by manual inspection to verify the sequence assignments.

2.3 GC/MS: Proteins

Proteins were hydrolyzed with 6 M HCl (100 µL) in sealed vials at 105 °C for 24 h. After evaporating the residue was dissolved in 25 mM HCl to a final volume of 60 µL, mixed with ethanol (32 µL), pyridine (8 µL), and 5 µL of ethyl chloroformate (ECF). The resulting solution was shaken for 5 sec, and added 1% chloroform solution of ECF (100 µL). The chloroform layer was transferred into a vial and extracted with a pipette, and repeated once. The chloroform layers were dried with anhydrous sodium sulfate, concentrated to 50 µL, and injected into the GC/MS. An INNOWAX (25 m x 0.2 mm x 0.2µm) capillary column was used for the separation. Helium carrier gas was set to a linear velocity of 38.8 cm/s, at the flow rate of 1 mL/min. Splitless injection was used with a 60 s purge off time, and was set to 240 °C. The temperature of MS transfer line was set to 240 °C. The GC oven temperature program was: 70 °C for 1 min; 20 °C/min to 250 °C; isothermal for 3.5 min. Total run time was 12 min.

2.4 GC/MS: Oils, waxes, resins

100 µL solution of Meth Prep II (m-trifluoromethylphenyl trimethylammonium hydroxide) in toluene 1:2 was added to the vials. The vials were warmed on a hotplate at 60 °C for 1 h. After cooling, the vials were centrifuged, and the contents were injected into the GC/MS. An INNOWAX (25 m x 0.2 mm x 0.2µm) capillary column was used for the separation. Helium carrier gas was set to a linear velocity of 44 cm/s. Splitless injection was used with a 60 s purge off time, and was set to 260 °C. The MS transfer line was set to 260 °C. The GC oven temperature program was: 80 °C for 2 min; 10 °C/min to 260 °C; isothermal for 15 min; 20 °C/min to 260 °C; isothermal for 2 min. Total run time is 38 min[27].

2.5 ELISA

ELISA identifies plant gum, egg, animal glue and casein in one paint sample. The sample data was interpreted as positive (+) when absorbance readings above 0.3 OD at 405 nm. ELISA will give false negatives when the antibodies do not recognize proteins that are denatured by pigments or aging. The procedure has been described in previous publications[8, 28,29] and is briefly described here.

Add 100 to 500 µg of each sample into 2-mL micro-centrifuge tubes. Standards of each paint reference material: egg white, animal glue (typically bovine), cow's milk (casein), and gum Arabic were placed into separate micro-centrifuge tubes. 20 µL of elution buffer was added to each tube, and also to a sterile "blank" tube (containing no protein) and left for 1–2 days at room temperature. 200 µL of 100 mM sodium bicarbonate was added to each tube, agitated, and left for 10 minutes. Multiple dilutions for each sample are used to verify the results of the assay. The plates were covered with Parafilm and put in a refrigerator, 4 °C for 24 hours. Each well was washed using a multi-channel pipette with 300 µL of phosphate buffered saline (10x PBS). Add 300 µL Sea BlockTM Buffer (diluted 1:10 v/v in 10x PBS) and sit for 60 minutes at room temperature. Empty the wells, add 80 µL of the diluted primary antibody listed in Table 1, and let sit 2 hours at room temperature. Rinse 3 times with 300 µL of 10x PBS and add 80 µL of secondary antibody to each row of wells and let sit 2 hours at room temperature. Rinse the plates 3 times with 10x PBS (crucial to remove any unbound secondary antibody). Add 80 µL of p-nitrophenyl phosphate (pNPP) and measure at 405 nm using an automated plate reader, up to 1 hour. If desired, the reaction can be stopped by adding 80 µL 0.75 M NaOH. In the case of strong responses, the results can be read qualitatively by eye.

Primary Antibody Dilution Used	Secondary Antibody Dilution Used
Egg Ovalbumin # AB1225 800 (5 µL > 4 mL)	Rabbit IgG #AP132A 500 (30 µL > 15 mL)
Collagen # AB6577 200 (10 µL > 2 mL)	Rabbit IgG #AP132A 500 (30 µL > 15 mL)
Collagen #AB19811 400 (5 µL > 2 mL)	Goat IgG #AB6742 400 (5 µL > 2 mL)
Casein, #RCAS-10A 800 (5 µL > 4 mL)	Rabbit IgG #AP132A 500 (30 µL > 15 mL)
Plant gum #JIM 13 50 (40 µL > 2 mL)	Anti-Rat IgG #A8438 400 (5 µL > 2 mL)

Table 1: Primary and corresponding secondary antibodies that were used in the ELISA tests. Antibodies were diluted in Sea Block Solution. The primary antibodies are listed with the catalog number, and the secondary antibody is shown that corresponds to the primary antibody.

3 Results and Discussion

3.1 GC/MS and ELISA

Figure 2 shows the locations of the very small samples (100 to 200 micrograms) that were sampled as close to the edge of the triptych paintings as possible, or in already damaged areas. The samples contained ground material and/or several layers of paint, it was difficult to separate the thin paint samples as they easily fragmented and multiple layers were often analyzed together in bulk.

Table 2 reports the amino acids found in the samples (reported as parts per million). The paint samples were weighed and analyzed by GC/MS for drying oils, waxes and tree resins, and the subsequently the same sample was analyzed for proteins. Fatty acids from drying oils or lipids, natural tree resins, and beeswax were not detected. High molecular weight hydrocarbons (*m/z* 57) similar to paraffin wax were identified in *Serapis* and *Bearded Man* (results not shown). Table 2 shows that the samples contain variable amounts of amino acids that best correlate to animal glue, the correlation coefficients are between 0.96 and 0.99. Egg albumin and animal glue collagen are distinguished by comparing the amino acid compositions to known reference materials. If a material contains albumin or collagen exclusively, the composition should be identical with that of the respective standard sample, giving a perfect match with a correlation coefficient of 1.0 or a practically acceptable match of 0.98. However, the coefficient is greatly reduced if a sample contains several proteins or is contaminated with a protein artifact[30].

Figure 2: Sample locations. *Isis* #5 (yellow over black with ground) and #6 (ground below #5); *Bearded Man* # 1 (red and ground), #3 (white with ground), #5 (red and ground), #7 (grey and ground), #8 (grey), and #9 (white); *Serapis* #2 (thin black with ground), #3 (ground below #2), and #4 (black).

Sample	Description	Sample, µg	Alanine	Glycine	Valine	Leucine	Isoleucine	Proline	Serine	Threonine	Phenylalanine	Aspartic	Hydroxyproline
Isis #5	Yellow, over black	136	17	17	33	7.0	13	5.0	16	13	6.5	7.8	9.2
Isis #6	Ground (below #5)	76	7	6.9	18	2.5	4.2	1.7	8.0	3.2	2.3	2.6	5.5
Bearded Man #1	Red and ground	20	1.9	4.0	0.9	1.6	0.7	1.9	1.2	0.8	1.1	1.3	1.2
Bearded Man #3	White with grey ground	100	7.4	16	2.8	4.4	1.8	8.6	2.9	2.0	2.7	2.1	5.4
Bearded Man #5	Red and ground	92	13	21	6.9	11	5.0	11	9.1	5.0	6.7	6.9	5.3
Bearded Man #7	Grey and ground	60	0.9	2.0	0.4	0.7	0.3	0.9	0.5	0.6	0.5	0.3	0.6
Bearded Man #8	Grey	87	6.8	15	2.8	4.6	2.0	7.5	2.7	2.0	2.9	4.1	4.7
Bearded Man #9	White	93	4.7	8.2	2.3	3.9	1.5	4.0	2.5	1.9	2.5	1.7	2.5
Serapis #2	Black thin w/ ground	56	13	22	6.6	10	4.5	10	6.3	4.7	6.6	8.9	7.2
Serapis #3	Ground (below #2)	31	2.0	3.9	1.3	1.8	0.8	2.7	1.7	1.4	1.2	2.7	2.9
Serapis #4	Black paint	41	1.0	2.4	0.3	0.5	0.2	0.9	0.5	0.9	0.3	0.1	1.0

Table 2: Summary of Amino Acid composition by GC/MS (parts per million).

Sample	Description	ELISA	GC/MS % Amino Acids	Animal Glue (corr. coeff.)
Isis #5	Yellow, over black	Egg	3.1	0.99
Isis #6	Ground (below #5)	ND	2.4	0.99
Beard Man #1	Red and ground	Egg, Animal glue	2.5	0.98
Beard Man #3	White with grey ground	Animal glue	1.7	0.99
Beard Man #5	Red and ground	Egg, Animal glue	3.3	0.96
Beard Man #7	Grey and ground	ND	0.4	0.99
Beard Man #8	Grey	ND	1.9	0.99
Beard Man #9	White	ND	1.1	0.97
Serapis #2	Black thin w/ ground	Egg, Animal glue	5.4	0.97
Serapis #3	Ground (below #2)	Animal glue	2.1	0.99
Serapis #4	Black paint	ND	0.6	0.99

Table 3: Summary of ELISA and GC-MS Results. ELISA results are positive if above 0.3 OD, ND is < 0.3 OD. Correlation coefficient (corr. coeff.) to animal glue, and % amino acids is (w/w) based on paint sample weight are also reported.

Table 3 shows that *Serapis* contained between 0.6 to 5.4% amino acids, *Bearded Man* 0.4 to 3.3%, and *Isis* 2.4 to 3.1%. ELISA detected a mixture of egg and animal glue in *Serapis* #2 and *Bearded Man #1 and #5,* while GC/MS identified only animal glue. This is because hydroxyproline is present in animal glue and not egg, making the correlation coefficient produced by the mixtures of these proteins difficult to interpret. ELISA detected egg albumin in *Serapis* #2, *Bearded Man* #1 and #5, and *Isis* #6, but GC/MS did not detect fatty acids in these samples. Since fatty acids are only present in egg yolk and not egg white, this supports the use of egg white in these samples. ELISA did not detect any protein in *Isis* #6, *Bearded Man* #7~9, and *Serapis* #4 because the target protein was most likely denatured by pigments, UV, and aging[31].

3.2 ELISA and Ultraviolet (UV) Imaging

In order to isolate possible locations of egg white on the paintings, ultraviolet (UV) imaging was employed (Figure 3). *Serapis* fluoresced blue, especially around the beard and eyes, indicating a recent restoration. The black band on *Isis* fluoresced white and egg was previously detected in this area. A wet swab was gently applied to the surface of the black band on the lower left side and it tested positive for egg with ELISA.

UV microscopy and ELISA were employed on two red paint samples that contain egg (*Isis* #4B and *Serapis* #7B) and two that did not have egg (*Bearded Man* #8B and *Isis* #5B**).** Half the sample was mounted in cross sec-

Figure 3: Photographs of *Serapis* and *Isis* by Marie Svoboda. UV imaging (left) and white light (right). Area of swab sample *Isis* is indicated with a white arrow.

tion and the other half was analyzed by ELISA. Figure 4 shows a cross section of a red paint sample from *Isis* #4b and *Bearded Man* #8b under UV light. *Isis* has a multi layered stratigraphy, with a thick layer fluorescing white on the surface. UV light excites organic materials and they will fluoresce white to light-blue[32]. *Isis* #4B and *Serapis* #7B fluoresced white on its surface and tested positive for egg. Conversely, *Bearded Man* #8B and *Isis* #5B did not fluoresce on its surface and tested negative for egg. Thus, egg was applied on the surface of the painting, visible as a white surface layer with UV microscopy and tested positive for egg by ELISA.

3.3 nanoLC/ESI-MS/MS

The detection of amino acids by GC/MS in the grey background of the *Bearded Man* prompted us to perform further investigation using nanoLC/ESI-MS/MS. We tested a few hundred micrograms of the remaining grey background of the *Bearded Man #8*, composed of calcium, iron, lead, and copper[33]. In conjunction with

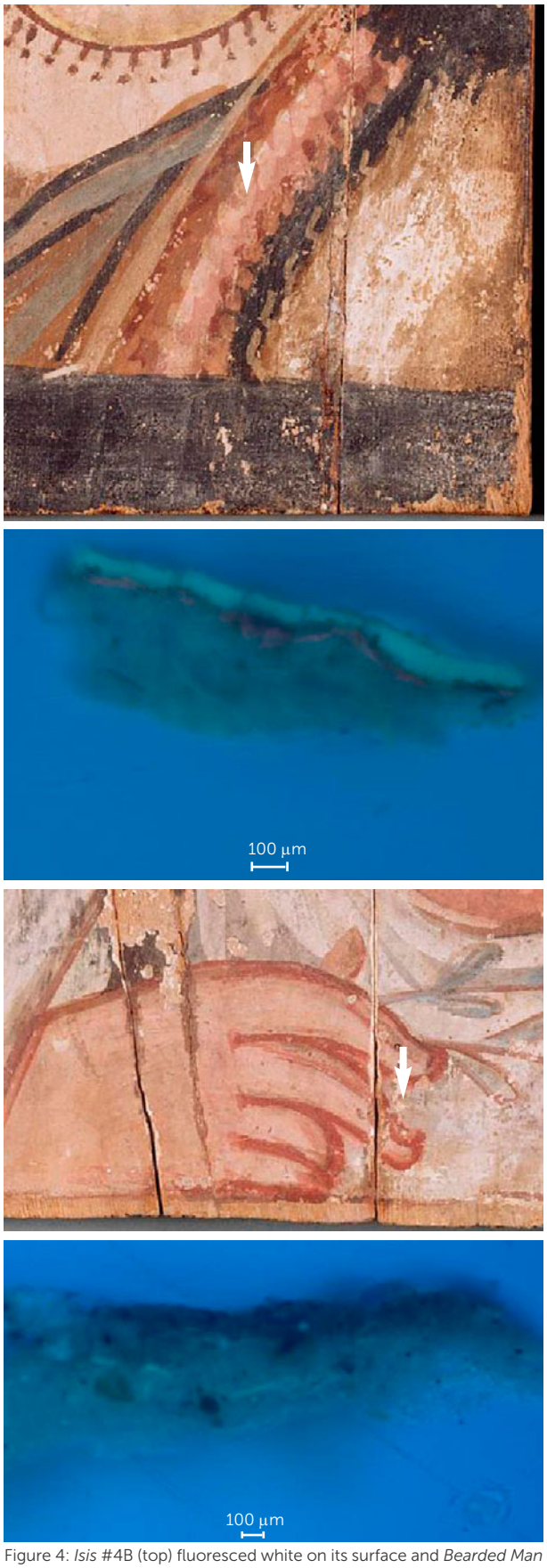

Figure 4: *Isis* #4B (top) fluoresced white on its surface and *Bearded Man* #8B (bottom) did not fluoresce on its surface. Sample locations are noted with a white arrow.

protein database search using Mascot database search engine, important peptides derived from several proteins remained in the grey background paint (*Bearded Man* #8) were successfully identified (Figure 4). Among the various contaminants such as human keratins and microbial peptides as well as synthetic polymers such as polyethylene glycol (PEG), sample separation technique using nanoLC allowed us to detect tryptic peptides from collagen molecules. A total of 41 collagen peptides derived from cow (*Bos taurus*) with characteristic X-Y-Gly sequence repeats were detected with sufficient accuracy (Table 4).

Peptide sequence	Position	m/z	Charge	Mascot score	Number of Oxidation (n)[a]	Number of Deamidation (n)[a]
List of peptides from cow Type I collagen α1 chain identified in the sample (Bearded Man #8)						
GEPGSPGENGAPGQMGPR	286-303	881.00	2	55	4	2
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK	322-351	850.50	3	96	3	1
GANGAPGIAGAPGFPGAR	397-414	794.25	2	64	3	1
GPSGPQGPSGPPGPK	415-429	666.90	2	79	1	1
GEPGPTGIQGPPGPAGEEGK	448-467	932.85	2	75	2	1
GEPGPAGLPGPPGER	472-486	718.71	2	78	3	0
GFPGADGVAGPK	493-504	544.79	2	72	1	0
GSPGEAGRPGEAGLPGAK	520-537	828.99	2	45	3	0
TGPPGPAGQDGRPGPPGPPGAR	552-573	686.89	3	55	4	1
GVPGPPGAVGPAGKDGEAGAQGPPGPAGPAGER	598-630	961.04	3	79	3	1
GEQGPAGSPGFQGLPGPAGPPGEAGKPGEQGVPGDLGAPGPSGAR	631-675	1372.69	3	45	6	2
GVQGPPGPAGPR	685-696	553.88	2	57	1	1
GADGAPGKDGVR	751-762	550.52	2	53	0	0
GLTGPIGPPGPAGAPGDKGEAGPSGPAGPTGAR	763-795	951.86	3	68	2	0
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	796-825	901.85	3	54	4	0
GSAGPPGATGFPGAAGR	865-881	730.74	2	102	2	0
GFPGLPGPSGEPGK	970-983	672.92	2	51	3	0
GPPGPMGPPGLAGPPGESGR	994-1013	917.42	2	39	3	0
GETGPAGPPGAPGAPGAPGPVGPAGK	1036-1061	1093.55	2	74	4	0
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR	1111-1140	897.89	3	68	2	1
GPPGSAGSPGKDGLNGLPGPIGPPGPR	1141-1167	825.06	3	59	4	1
List of peptides from cow Type I collagen α2 chain identified in the sample (*Bearded Man* #8).						
GAAGLPGVAGAPGLPGPR	308-325	782.16	2	45	3	0
GIPGPVGAAGATGAR	326-340	634.30	2	91	1	0
GSTGEIGPAGPPGPPGLR	380-397	825.26	2	65	2	0
GIPGEFGLPGPAGAR	572-586	714.79	2	67	2	0
GPSGPPGPDGNKGEPGVVGAPGTAGPSGPSGLPGER	608-643	1072.15	3	64	3	1
GAPGAIGAPGPAGANGDRGEAGPAGPAGPAGPR	674-706	927.77	3	78	2	1
GDGGPPGATGFPGAAGR	776-792	737.41	2	73	2	0
GLPGVAGSVGEPGPLGIAGPPGAR	881-904	1065.99	2	140	3	0
GYPGNAGPVGAAGAPGPQGPVGPVGK	947-972	755.30	3	80	2	2
GEPGPAGAVGPAGAVGPR	977-994	766.98	2	83	1	0
IGQPGAVGPAGIR	1066-1078	605.74	2	66	1	1
List of peptides from cow Type III collagen α1 chain identified in the sample (*Bearded Man* #8)						
GSDGQPGPPGPPGTAGFPGSPGAK	171-194	1085.76	2	45	5	0
GEVGPAGSPGSSGAPGQR	195-212	800.65	2	82	2	0
GEMGPAGIPGAPGLIGAR	240-257	827.26	2	40	2	0
GPPGPPGTNGVPGQR	258-272	719.78	2	49	3	2
GPAGANGLPGEK	336-347	543.09	2	61	1	1
GVAGEPGRNGLPGGPGLR	363-380	570.86	3	49	3	1
GGAGPPGPEGGK	540-551	499.06	2	42	1	0
GPTGPIGPPGPAGQPGDKGESGAPGVPGIAGPR	606-638	990.25	3	54	4	1
GSPGGPGAAGFPGGR	708-722	645.47	2	83	3	0

[a]Number of oxidation (P) and deamidation of amino acids (N and Q) in the peptide was determined by MS/MS fragmentation pattern obtained by nanoLC-ESI-MS/MS.

Table 4: List of peptides from cow Type I collagen α1, α2, α3 chain identified in the sample (Bearded Man #8)

For example, as shown in Figure 6, the fragment pattern observed in the MS/MS spectrum of triply charged ion at *m/z* 951.86 clearly assigned to the sequence of GLTGPIGPPGOAGAOGDKGEAGPSGPAGPTGAR, in which two Pro residues are post-translationally modified to hydroxyproline (O). This 33-residue sequence corresponds to the sequence present in position 763-795 of the type I collagen α1 chain of cow (*Bos taurus*). It should be also noted that amino acid deamidation, by which Gln and Asn are converted to Glu and Asp respectively, occurred in half of the detected peptides due to the degradation and/or aging of the painting samples.

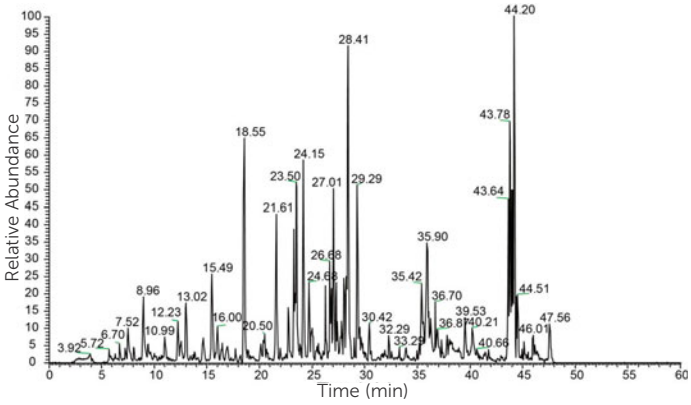

Figure 5: Base peak chromatogram obtained from nanoLC-ESI-MS/MS of the tryptic digest of proteins extracted from the sample (*Bearded Man* #8).

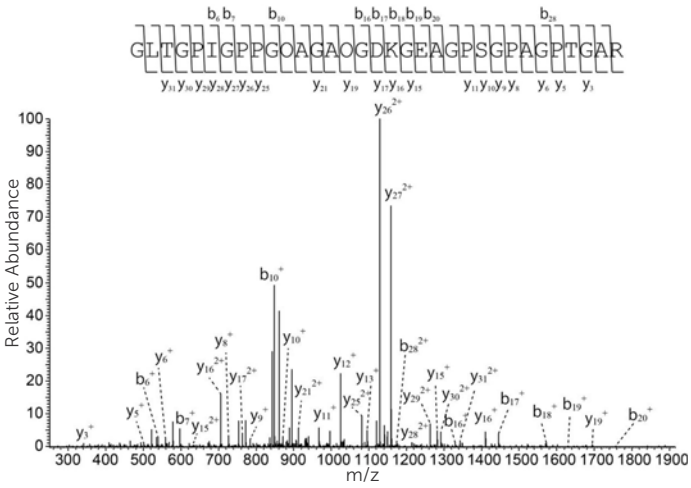

Figure 6: MS/MS spectrum of the triply charged ion at *m/z* 951.86, resulting in the sequence, GLTGPIGPPGOAGAOGDKGEAGPSGPAGPTGAR.

4 Conclusion

The proteomics approach and protein database search, using the amino acid sequences available in the current SwissProt database, identified collagen peptides found in cow (*Bos taurus*) in the grey background from the *Bearded Man* #8. Tryptic peptides of type III collagen α1 chain were found that are specific to skin. These results strongly indicate that the animal glues used to make these archaeological materials were prepared by cow hide. Mixtures of egg albumin and animal glue were detected by ELISA, and GC/MS quantified the percent amino acids in the paint samples. Fatty acids were not detected in the samples with egg albumin, indicating that egg white or glair was applied to the paintings. The location of the egg is on the surface of the paint cross-sections as the surface fluoresces white under UV microscopy. ELISA detected egg albumin on a wet swab gently applied to a black band that fluoresced white on *Isis,* providing further evidence that egg was applied on the surface, perhaps from restoration rather than as an original binder. The determination of binding media in ancient paintings is especially difficult because of small sample sizes, variable aging, pigment interactions, and contamination due to past restorations. The preparation of cross sections and the utilization of multiple techniques in this study enabled the identification of cow hide glue and showed that selected areas contain egg glair.

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