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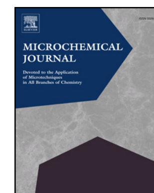

Investigation of proteins in samples of a mid-18th century colonial mural painting by MALDI-TOF/MS and LC-ESI/MS (Orbitrap)

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ABSTRACT

In this paper, we report the study of proteinaceous binders in samples from a South American Colonial 18th century painting using two complementary mass spectrometry techniques. Before restoration, seven micro-samples were extracted from representative colors of one of the mural paintings located inside the church of Our Lady of Copacabana de Andamarca built in 1723 in Bolivia. Previous analysis by gas chromatography (GC) of the amino acid derivatives of the extracted protein fraction of three of the samples, suggested the presence of animal glue and egg. In this work, we introduce a methodology that combines a protein extraction procedure with a typical treatment for analysis of proteins as tryptic peptides by mass spectrometry. For this study, we applied in the first stage, MALDI-TOF/MS and then, LC-ESI/MS (Orbitrap), an ultrahigh resolution mass spectrometry, to achieve more reliability in the identification of the protein binders.

By LC-ESI/MS (Orbitrap), we detected several peptides from egg white proteins, particularly ovalbumin, ovotransferrin and lysozyme, and egg yolk proteins, vitellogenin-2 and apolipoprotein B, with high confidence peptides each. In all the samples, the presence of collagen from animal provenance was established by MALDI-TOF/MS and LC-ESI/MS (Orbitrap).

To our knowledge, this is the first time that protein binders in Andean paintings are identified without ambiguity by MALDI-TOF/MS and LC-ESI/MS (Orbitrap) mass spectrometry using a proteomic approach.

1. Introduction

Through history, artists have used various proteinaceous binders of animal origin, such as glue, egg, and milk. Glues obtained by hydrolysis of collagen extracted from animal skin, bones, and tendons were used as pigment binders and also as adhesives, additives for plasters, and for gilding. Egg has been used as a pigment binder in traditional *tempera* painting and in mixtures with siccative oils in the *tempera grassa* technique [1]. Identification of proteinaceous binders may provide valuable information on art techniques, as well as new insights into painting styles. At the same time, this knowledge is essential in the design of appropriate cleaning, conservation, and restoration processes of artworks.

Characterization of proteinaceous materials in paintings is a challenging task due to the very low amount of organic binder, about 10% distributed in pictorial and ground layers in micro-samples excised from artworks. Moreover, ageing and degradation processes may negatively influence the identification of the binder. Therefore, protein characterization in artworks requires highly sensitive analytical techniques. Gas chromatography (GC) coupled to mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) are approaches based on the determination of the amino acid composition after protein hydrolysis followed by derivatization, while pyrolysis (Py)-GC–MS is based on the detection of specific markers formed during the pyrolytic

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step [2]. However, the presence of contaminants, similar amino acid composition of proteinaceous binders, modifications of the residues because of ageing, and changes in amino acid residues during sample pretreatment, among other factors, can affect these analyses [3]. Proteomic-based methodologies and related mass spectrometry-based approaches have been successfully applied to the identification of proteins in works of art and archaeological samples [2–15]. In a typical proteomic protocol (bottom-up approach), the peptide mixture originated by trypsin digestion from each protein occurring in the binders, constitutes a "fingerprint" indeed more specific than the amino acids profile. The peptide mixture can be analyzed by different mass spectrometric procedures. MALDI-TOF/MS analysis generates a peptide mass fingerprint that often enables a good identification by comparison with databases of reference proteinaceous binders [2,5–10,13,14]. In addition, separation of tryptic peptides by nano-HPLC and detection with analyzers in tandem (TOF/TOF or Q/TOF) allows determination of peptide sequences [2,3,10–15]. Nowadays, Orbitrap based instruments, coupled to nano-HPLC, can achieve the analysis of complex peptides mixtures, with very high mass resolution and consequently is being increasingly used in different areas. Very recently, Tripković et al. identified animal glue and egg yolk in nineteenth-century Orthodox icons by ESI-LTQ-Orbitrap [15]. A very complete review of protocols and analytical procedures for the analysis of proteins, including new analytical approaches, has been published recently by Dallongeville et al. [2]. Most procedures comprise the direct tryptic cleavage of the artwork samples [4–9,11,13,15] but some authors have explored different protein extraction conditions from binders in micro-samples. Tokarski et al. developed an efficient protocol for protein extraction using an aqueous solution acidified with 1% trifluoroacetic acid [10,12]. Leo et al. [3] have explored the alkaline extraction of proteins, a methodology successfully applied for the analysis of lipidic and proteinaceous binders in artwork micro-samples [16]. A simple protocol based on Bligh-Dyer extraction of lipids and proteins followed by MALDI-TOF/MS analysis allowed the detection of various lipid classes and protein identification by peptide mass fingerprint [14].

In this work we present a methodology based on alkaline extraction followed by MALDI-TOF/MS and LC-ESI/MS (Orbitrap) analysis for the identification of proteinaceous binders in mural painting micro-samples. First, the methodology was evaluated on painting models of animal glue, yolk, white egg, and whole egg on a gypsum support. Then, it was applied to the analysis of protein binders in micro-samples from a mural painting of the Andean Colonial church of Our Lady of Copacabana de Andamarca. By Colonial period we understand the conquest, government and evangelization of American territories under the domain of the Spanish and Portuguese crowns, beginning in the 15th century, and ending in the 19th-century with the wars of Independence.

The church of Our Lady of Copacabana, built in 1723, is located at 3800 masl in the department of Oruro, Bolivia, near the so-called Silver Route that connected the Imperial city of Potosí (Bolivia) with the port city of San Marcos de Arica (Chile). The inside walls of the church were decorated with paintings dated from mid-18th century that depict religious images that were used for the Catholic evangelization in that region. Between 2005 and 2009, the mural paintings were restored due to deterioration processes, such as flaking and detachment of the painted areas. In order to investigate pigment and plaster materials as well as the pictorial technique, micro-fragments of pigment samples were collected before the restoration of the mural paintings. In a recent work, we have characterized the chromatic palette and investigated the painting technique from micro-samples obtained from one of the mural paintings that depicts the figure of the Leviathan, together with decorations with vases of flowers, trees, and birds (Fig. 1) [17]. In this study, gypsum was identified as the ground layer on which the pigments were applied using a *secco* technique, which requires a binding medium to attach the pigments on dry gypsum. Analysis by GC–MS of the fatty acid methyl esters together with the identification of cholesterol revealed the use of a mixture of linseed oil and egg as binder. Further investigation of the proteinaceous fraction by GC analysis of amino acid derivatives and comparison with model samples suggested the presence of animal glue and egg. The identification of animal glue was based on the high proline relative content, which is characteristic of collagen proteins, while the presence of egg was inferred from high concentrations of alanine, valine, and serine [18].

Our present work proposes a methodology for the reliable identification of proteinaceous binders in painting samples through protein extraction and a proteomic-based strategy by MALDI-TOF/MS and LC-ESI/MS (Orbitrap). This methodology is being applied to identify the proteins used in binders in Colonial artworks from South America that have been scarcely studied and never addressed before by a proteomic-based approach.

2. Experimental

2.1. Chemical and reagents

Ultrapure water (Milli-Q®) was used throughout. All the solvents used were Baker HPLC grade. For the reduction, alkylation and protease digestion of the protein extract, ammonium hydrogen carbonate NH_4HCO_3 (Merck), dithiothreitol (Sigma), iodoacetamide (Sigma) and sequencing grade modified trypsin (V511 Promega) were used. Formic acid was purchased from Merck and urea from Sigma. HCCA (alpha-cyano-4-hydroxycinnamic acid) (Fluka) was used as MALDI matrix.

2.2. Painting model preparation

Mural painting models were prepared by applying proteinaceous binders on dry gypsum to obtain patterns of tryptic peptides. With this aim, solutions/emulsions of the binders were extended over gypsum supports (25 mm × 25 mm). The gypsum support was prepared by dissolving 1 g of gypsum in 0.6 ml of water following the methodology described in [17]. The binders selected were yolk, white egg, and whole egg from chicken and animal glue from Dekora®, and a mixture 1:1 (v/v) of whole egg and Dekora® animal glue.

The preparation of animal glue solution was based on a traditional recipe [1]. Solid commercial animal glue was dissolved in water at 50 °C under magnetic stirring, until total dissolution to obtain a final protein concentration of 7 mg/ml. White egg and yolk binders were prepared by separating yolk from white egg manually and whisking to obtain homogeneous emulsions.

All the painting models were prepared by applying three layers of the selected binder with a paint brush on the gypsum support, waiting for dryness between layers. After drying at room temperature to constant weight, micro-samples were scraped off the painting models for further MS analysis.

2.3. Historical painting samples

Before the restoration, micro-samples were extracted with a scalpel from colored areas of a wall painting from the church of Copacabana de Andamarca in Bolivia (Fig. 1). Six samples were selected under the microscope for protein analysis. The description of their color, location, and pigment composition is shown in Table 1.

2.4. Sample treatment prior to MS analysis

Samples of painting models (1 mg) and historical micro-samples (area < 0.04 mm^2) were placed in a reaction vial and grinded manually with spatula. Then, 200 µl of 2.5 M NH_3 aqueous solution were added and the mixture was extracted for 2 h at 60 °C in an ultrasonic bath. Then, 400 µl of $CHCl_3$ (×3) were added in order to separate the neutral lipids (acylglycerides and sterols) from the sample. After each $CHCl_3$ addition, centrifugation was performed to facilitate phase separation

Fig. 1. Mural painting from the church of Copacabana de Andamarca in Bolivia and indication of the location of samples AND1-AND7 and their optical images.

(5 min at 3200 rpm). The organic phase was separated and the combined $CHCl_3$ extracts were evaporated to dryness under a stream of nitrogen. The aqueous ammonia phase was centrifuged to separate insoluble pigments and gypsum, and then lyophilized. The solid residue containing the protein fraction was reconstituted by adding 100 μl of 1% TFA and extracted with 200 μl of diethyl ether (×3), followed by centrifugation (5 min at 3200 rpm). The ethereal extract, containing free fatty acids extracted together with the proteins by ammonia, was combined with the chloroform extract for further lipid analysis by GC/MS. The aqueous phase was lyophilized and the residue, containing the proteins, was re-suspended in 10 μl of water. Then, 3 μl of 500 mM NH_4HCO_3 buffer (pH 8) were added, followed by the addition of 1.5 μl of 1 M dithiothreitol in NH_4HCO_3 buffer, for the reduction of disulfide bonds (45 min on a stirrer at 56 °C). Alkylation of reduced cysteines by carbamidomethylation was performed by adding 1.5 μl of 550 mM iodoacetamide in 50 mM NH_4HCO_3 (45 min in the dark at room temperature). Enzymatic hydrolysis was performed by adding 1 μl of trypsin from a stock solution 0.5 μg/μl, as recommended by the supplier. Trypsin hydrolyses proteins at the COOH terminal side of lysine and arginine, except where these amino acids are followed by proline. The mixture (total volume of 30 μl, adjusted with water) was incubated overnight at 37 °C under stirring. Finally, samples were desalted and contaminants were removed using reverse-phase C18 Zip Tip pipette tips (Millipore). The methodology is summarized in Fig. 2.

In addition, painting models of whole egg and animal glue on gypsum were extracted with water under sonication for 2 h in order to test the influence of the ammonia extraction on the stability of collagen and egg proteins.

2.5. MS analysis

Tryptic digests were analyzed using an Ultraflex II Bruker Daltonics UV-MALDI-TOF-TOF mass spectrometer, equipped with a Nd:YAG laser (λ_{em} 355 nm). Spectra were obtained in positive reflectron mode, within a mass range of 800–4000 *m/z*. A saturated solution of HCCA in 0.1% TFA 30:70 water:acetonitrile, was used as matrix. The positive-ion mass spectra were externally calibrated using the Bruker commercial peptide mix standard (Peptide Calibration Standard II). Flex Control Interactive calibration method in Quadratic mode with a peak assignment tolerance of 500 ppm was used. Spectra were visualized and compared using flexAnalysis 3.3 software.

For the analyses by LC-ESI/MS (Orbitrap), a Q Exactive (Thermo Scientific) mass spectrometer was used, coupled with a nano liquid chromatograph Easy-nLC 1000 (Thermo Scientific). The chromatographic conditions used were the following: commercial reverse-phase C18 column (2.6 μm, 150 Å, 75 μm × 150 mm, Thermo Scientific EASY-Spray Accucore (P/N ES801)), temperature 35 °C, flux 300 nl/min, linear gradient of solvent A (water with 0.1% formic acid) and solvent B

Table 1
Identification and description of the seven micro-samples extracted from representative colors of the mural painting.

Sample	Color and location	Main pigment (summarized from results published in ref. [17])
AND1	Yellow in the flame	Yellow ochre probably from hydrated iron oxide, goethite (α-FeOOH), and clay minerals.
AND2	Green in the demon's head	Brochantite ($Cu_4SO_4(OH)_6$) and antlerite ($Cu_3SO_4(OH)_4$)
AND3	Black in the Leviathan's head near the mouth	Wood charcoal
AND4	Red in the flame inside the mouth of the Leviathan	Hematite (α-Fe_2O_3) and clay minerals
AND5[a]	Green in the demon's leg	Brochantite ($Cu_4SO_4(OH)_6$) and antlerite ($Cu_3SO_4(OH)_4$)
AND6	Blue handle of the flower vase	Indigo
AND7	Orange in the flower decoration	Hematite (α-Fe_2O_3) and clay minerals

[a] Since AND2 and AND5 share the same green pigment [17], AND5 was chosen as a representative sample for the analyses.

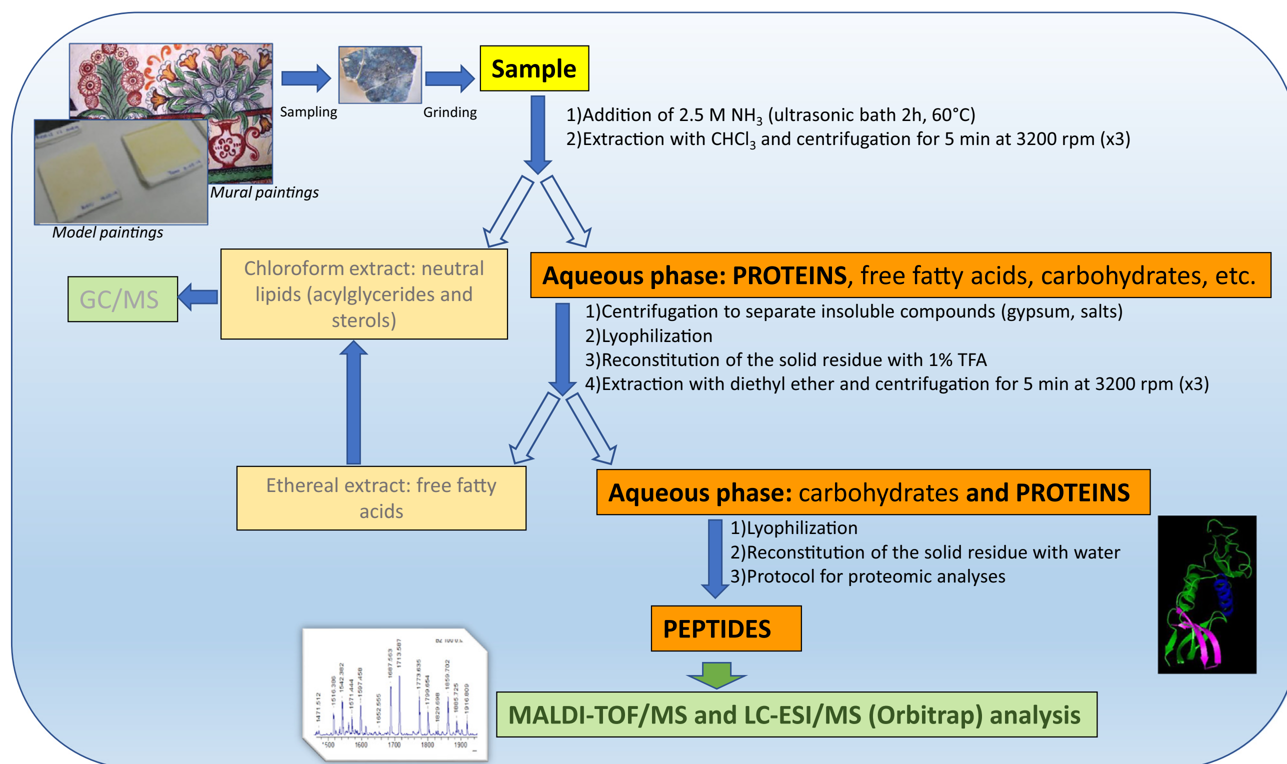

Fig. 2. Methodology for the analysis of proteinaceous binders in micro-samples from the mural painting.

(acetonitrile with 0.1% formic acid) with an increase of solvent B from 5% to 30%, run time 120 min. ESI general conditions: voltage spray 3 kV.

MS analysis conditions were the following: resolution 70,000, AGC (automatic gain control) 3 10^6, maximum IT (injection time) 200 ms, scan range 400 to 2000 *m/z*. MS/MS conditions were: resolution 17,500, AGC 1 10^5, maximum IT 50 ms, loop count 15, scan range 200–2000 *m/z*, dynamic exclusion 15 s.

Q Exactive instrument was calibrated for mass accuracy according to manufacturer instruction. For positive mode a mixture of caffeine, MRFA (Met-Arg-Phe-Ala), Ultramark 1621 (a commercially available mixture of fluorinated phosphazines), and *n*-butylamine was used for a daily calibration of the equipment. MS data was acquired until mass calibration tests were passed.

2.6. Database search strategy

The protein binders expected in these art work samples were collagen from mammalian origin and egg proteins [2,17]. A strict proteomic approach requires that the MS and MS/MS data should be searched against a database comprising the possible organisms present in the sample. We therefore decided to combine the proteome databases of *Bos taurus* and *Gallus gallus* to perform the searches. Since collagen isoforms are highly conserved in sequence in mammals, we chose *Bos taurus* database since its genome is completely sequenced. *Gallus gallus* was selected as an oviparous bird with a complete genome sequence. Taking into account the artistic background in this field [2,15] and to make the results more clear to view and compare with the literature, Tables 2 and 4 (see Section 3.3) show a filtered view of the top hit results obtained during the search, in which only collagen isoforms hits from *Bos taurus* are shown (see Section 3.3, Table 4) and only hits corresponding to egg yolk and white proteins from *Gallus gallus* are displayed (see Section 3.3).

2.7. Software and database search

MALDI-TOF/MS raw mass spectra were processed using Bruker Daltonics flexAnalysis Software. Uniprot databases [19] were used to find sequence files of the searched proteins to digest *in silico* by ProteinProspector [20].

LC-ESI/MS (Orbitrap) raw mass spectra were generated using Q Exactive Thermo Fisher Xcalibur Software. Searches were performed using Proteome Discoverer 2.1. Standard settings were as follows: precursor mass tolerance was set to 10 ppm and product ion tolerance to 0.05 Da. Static modification was set to carbamidomethylation of cysteine, and dynamic modifications were set to oxidation of methionine and N-terminal acetylation. When searching for collagen, proline oxidation was included as dynamic modification. Databases used for the identifications were downloaded from Uniprot repository (www.uniprot.org): *Bos taurus* (bovine) (UP000009136) and *Gallus gallus* (chicken) (UP000000539). Results were filtered using the Proteome Discoverer 2.1 software, using a reverse database strategy [21] and a FDR (false discovery rate) $< 1\%$ for a peptide to be qualified as “high” and 1–5% FDR for a “medium” qualification.

3. Results and discussion

3.1. Protein extraction of painting models and analysis by MALDI-TOF/MS and LC-ESI/MS (Orbitrap)

The ammonia extraction of proteins from art samples followed by hydrolysis to amino acids and further analysis by GC–MS of amino acid derivatives allows the identification of proteinaceous binders based on the comparison of the relative amino acid compositions with those of reference samples [16,17]. In this work we coupled the extracted protein fraction with the mass spectrometric analysis of the tryptic peptides derived from the protein.

Prior to MS analysis, samples of painting models were subjected to a protein extraction procedure with ammonia 2.5 M and subsequent

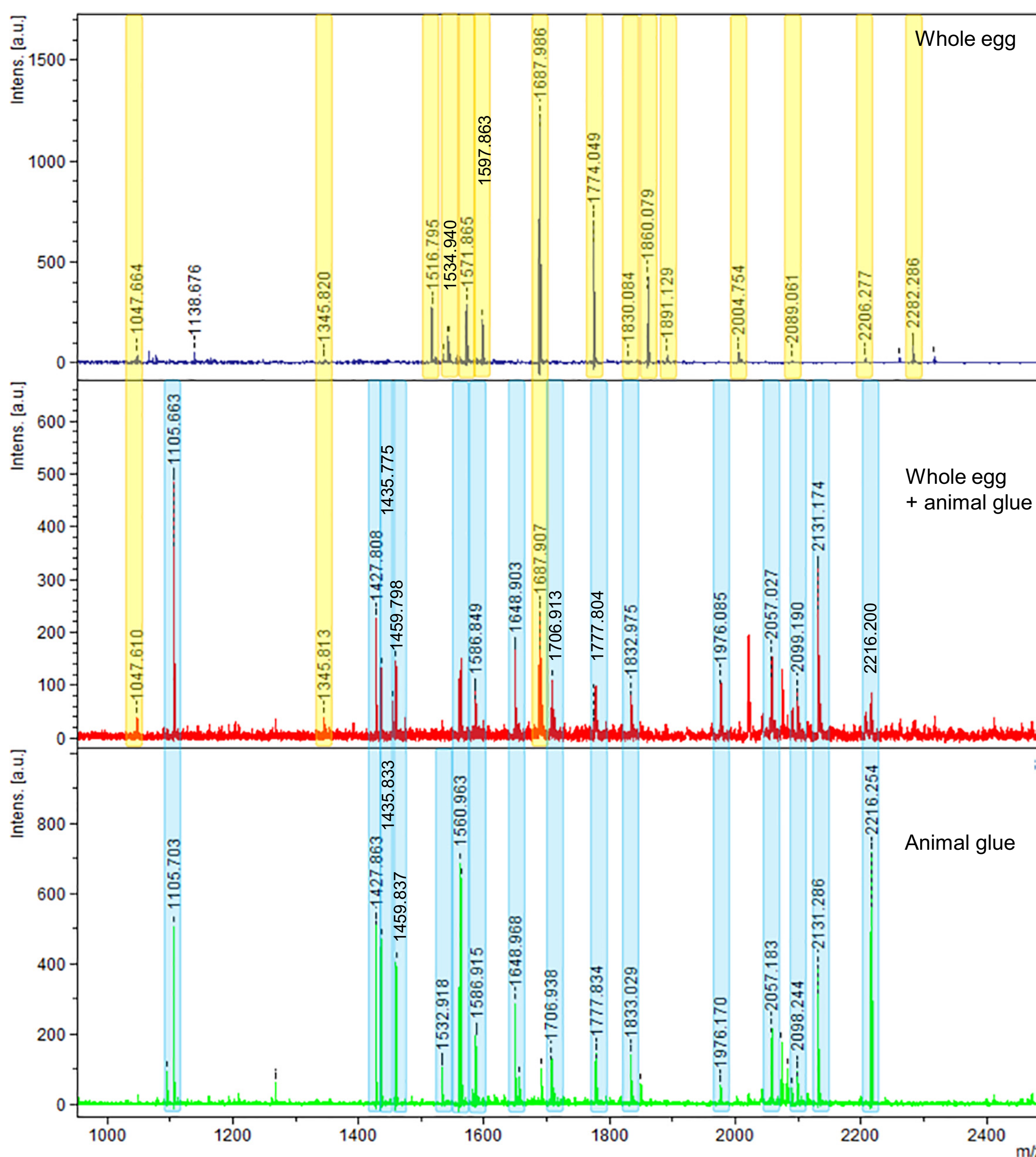

Fig. 3. MALDI-TOF/MS spectra for references: whole egg, whole egg plus animal glue, animal glue. Vertical yellow bars correspond to whole egg peptides. Vertical blue bars correspond to collagen peptides in glue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extraction with chloroform for recovery of the neutral lipids (acylglycerides and sterols) from the gypsum support (Fig. 2). This sample treatment is a modification of the one reported in the literature [16], particularly concerning the extraction of the lipid fraction.

The presence of proteins in the ammonia phase was analyzed by submitting tryptic digests derived from the model painting samples of whole egg, whole egg plus animal glue, and animal glue on gypsum through MALDI-TOF/MS (Fig. 3). The spectrum of the model painting sample with whole egg showed tryptic peptides of egg yolk and egg white. Most of the peptide signals correspond to those predicted by *in silico* digestion of ovalbumin, the main protein in white egg (ovalbumin constitutes 55% of the proteins of egg white) [22]. A few peptides could be attributed to apolipoprotein B and vitellogenin-2 from egg yolk. Peptide signals in the model sample of animal glue matched with peptides predicted by *in silico* digestion to belong to different types of collagen proteins as collagen alpha-1(I), collagen alpha-2(I), and collagen alpha-1(III) from bovine origin. The spectrum of the model sample of whole egg plus animal glue showed tryptic peptides for both binders (Fig. 3).

Further identification of model samples of egg white and egg yolk was performed by LC-ESI/MS (Orbitrap). Appendix Tables A1 and A2 show the results of the analysis against *Gallus gallus* database, in which the top 10 hits of egg white (Table A1) and the top 4 hits for egg yolk (Table A2) proteins, with the larger number of identified peptides, are summarized. The experimental results correlate with those expected for the known relative abundance of proteins in eggs, described in the literature [22]. The most relevant proteins detected in egg white were ovalbumin, ovotransferrin, alpha-1 acid glycoprotein, and lysozyme C, while in egg yolk the most abundant proteins were apolipoprotein B, vitellogenin-2 and vitellogenin-1. Appendix Table A3 includes the results of the analysis of the model sample of glue, in which the top 3 hits of collagen isoforms, with the larger number of peptides, are shown.

Although it is known that the peptide bond is hydrolyzed very slowly under low and high pH conditions [23], we still verified whether the proteins were not significantly degraded after the harsh alkaline extraction with 2.5 M NH_3 using the following strategy. Equal aliquots of the model painting samples of whole egg and animal glue applied on gypsum, prepared as described under Experimental, were extracted with water alone or submitted to the whole extraction procedure described in Fig. 2. Proteinaceous extracts obtained from both procedures were digested with trypsin, as described, and analyzed by LC-ESI/MS (Orbitrap). Three different settings were used in the search of the raw data against either *Bos taurus* (for the collagen reference) or *Gallus gallus* (for the egg reference): the first used the standard search for tryptic peptides; in the second approach semi-tryptic peptides were added to the search conditions (tryptic plus semi-tryptic), and in the third, the criterion "no enzyme" (peptides with non-tryptic ends) was included (tryptic, plus semi-tryptic, plus no enzyme). The rationale for this approach was the following: trypsin is a very specific enzyme; however, it is common to observe, when analyzing complex mixtures, that tryptic peptides can be cleaved chemically in their amino- or carboxy-side during the ionization, generating the so called semi tryptic peptides [24]. Therefore, a search for semi-tryptic peptides usually yields more identified peptides for a protein than when using only tryptic peptides as the search condition. If the proteins were significantly hydrolyzed by NH_3, peptides with no tryptic ends would be generated by the hydrolysis, and when adding the criterion "no enzyme" to the search, a higher number of peptides would appear corresponding to the protein hit, as a result of the sum of tryptic, semi-tryptic plus "no enzyme" peptides. The results are shown as bar graphs in the Appendix Fig. A1. Fig. A1 summarizes the results obtained for three collagen proteins, two egg yolk proteins, and four egg white proteins after each extraction procedure using water or ammonia. The results show that under both extraction conditions, the number of total peptides detected when using the "no enzyme" criterion is almost identical to the number of peptides using the semi-tryptic criterion, and that no significant number of "no enzyme" peptides was added to the tryptic plus semi-tryptic peptides.

From the experiments described in this section, as a whole, we can conclude that peptides corresponding to model samples from glue and egg can be detected by both MALDI-TOF/MS and LC-ESI/MS (Orbitrap) and that the alkaline extraction conditions did not significantly degrade the protein during the short time of the extraction procedure.

3.2. Analysis of mural painting samples by MALDI-TOF/MS

The presence of collagen in the mural painting samples was strongly suggested in our previous work [17] due to the high proline content in the total amino acid analysis in three of the investigated samples (AND3, AND6, and AND7). With the aim to clearly identify the presence of this proteinaceous binder in the artistic samples, tryptic peptides from six samples from the colonial mural painting were analyzed by MALDI-TOF/MS and compared to the pattern obtained for the painting model of animal glue (Fig. 4).

The MALDI–TOF/MS spectrum of the mural painting samples (Fig. 4a and b) showed remarkable signals similar to those of the reference animal glue at *m/z* (monoisotopic mass/charge) = 1105.4, 1586.5, 1648.5, 1427.5, 1435.4, and 1459.4. In particular, the last three *m/z* reflected the same relative pattern of intensities as the one of the standard glue. In this figure the most important signals are indicated with grey bars for better visualization. It can be noted the high similarity of the spectra of the model sample of animal glue and samples AND1, AND3, AND4 (Fig. 4b), and AND7 (Fig. 4a). Sample AND6 shows the same signals as the collagen reference, although much weaker than the rest. Several additional signals are shown in this sample, corresponding to characteristic peptides derived from common contaminant keratins. In the case of AND5 (data not shown) the pattern of signals was extremely weak. This comparative analysis, together with the fact that these signals match with those predicted *in silico* tools for collagen proteins [19,20], as well as with reported signals [4,14,25], allowed us to confirm the presence of collagen from animal glue.

As it is known, pigments were mixed with egg and vegetable oils to paint murals and other artworks, a technique named *tempera grassa* [26]. This fact, together with the amino acid relative composition and the presence of cholesterol in two of the three samples analyzed by GC and GC/MS in our previous work on the mural painting [17], suggested the use of egg as a binder. With the same approach used for collagen, an attempt to detect egg peptides was performed by MALDI-TOF/MS, based on the comparison with the MALDI-TOF/MS patterns displayed by whole egg reference and whole egg plus animal glue reference patterns (Fig. 3), as well as with peptides predicted by informatic tools for egg white and egg yolk proteins [19,20], and data from the literature [4,14,25]. However, no clear signals in the spectra of the mural painting samples, indicative of egg proteins, could be detected by MALDI-TOF/MS. For this reason, we decided to analyze the presence of protein binders by LC-ESI/MS (Orbitrap), a technique with high accuracy and sensibility for complex samples.

3.3. Analysis of mural painting samples by LC-ESI/MS (Orbitrap)

In order to obtain more information regarding the proteins, the mural painting samples were analyzed by LC-ESI/MS (Orbitrap).

Analysis of the mural samples by LC-ESI/MS (Orbitrap) confirmed the presence of egg proteins in some of the micro-samples. Table 2 shows that several white egg proteins were detected in sample AND5 with several peptides of high confidence; namely ovalbumin, ovotransferrin, two ovalbumin related proteins (Y and X), lysozyme, and alpha 1-acid glycoprotein. Two yolk proteins, vitellogenin-2 and apolipoprotein B, were identified in sample AND3. Sample AND6 showed a peptide predicted to be identical to the one of AND3, QQLTLVEVR, indicative of vitellogenin-2, although with medium confidence (FDR 1–5%). A close manual inspection of the MS/MS spectrum from the peptide from sample AND6, in comparison with the one of AND3, shown in Fig. 5 and Table 3, indicated that this spectrum can be trusted as a *bona fide* spectrum for the sequence QQLTLVEVR, since the *y* series of ions is almost complete. The MS/MS spectra for two other medium confidence peptides, EETEIVVGR (AND1) and LPLSLPVGPR (AND3), shown in the Appendix (Figs. A2 and A3), with > 95% confidence can be ascribed to vitellogenin-2. The fact that all the samples were collected from the same painting (Fig. 1) strongly supports the finding that they share the same egg proteins used as binders.

Apolipoprotein B, a marker of egg yolk detected in the egg yolk reference with almost the same abundance as vitellogenin-2, was only detected in AND3 with one high confidence peptide. It is noteworthy that in sample AND3, as well as in AND1 and AND6, only proteins belonging to egg yolk were detected, suggesting that yolk had been used as binder. Otherwise, proteins from egg white, such as ovalbumin and ovotransferrin, the more abundant proteins in egg, should have been detected (Table 2).

In order to investigate whether chicken was the source of the egg binder, we analyzed which of the peptides detected in the samples was unique for this species. The few peptides detected for egg yolk proteins could not identify its origin since in a Blast search (homology comparison) we could observe that these peptides were highly conserved among different species. However, the search was clearer with egg white proteins. All the peptides highlighted in bold in Table 2 are unique for *Gallus gallus* as concluded from a Blast search against all sequenced organisms. In the Appendix Fig. A4 a multialignment of ovalbumin from 4 members of the *Phasianidae* family comprising chicken (*Gallus gallus*), common turkey (*Meleagris gallopavo*), common quail (*Coturnix coturnix*), and Japanese quail (*Coturnix japonica*) is shown. This alignment, in which the unique peptides of chicken ovalbumin are highlighted, indicates that the sequence conservation is very high. In spite of this identity the underlined peptides are unique for

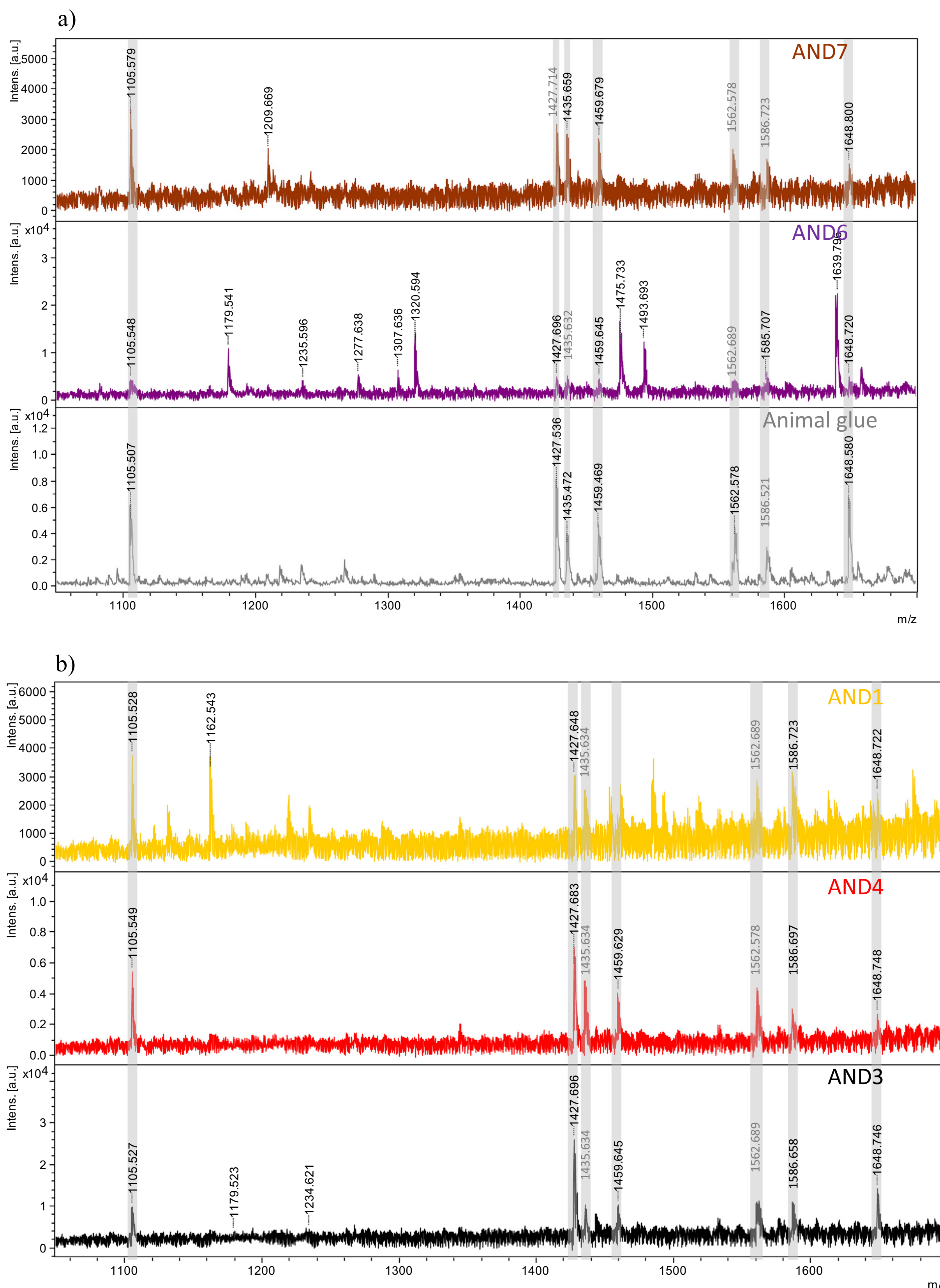

Fig. 4. a) MALDI-TOF/MS spectra of animal glue Dekora® used as reference and samples AND6 and AND7 from the colonial mural. b) MALDI-TOF/MS spectra of samples AND3, AND4, and AND1 from the colonial mural. Grey bars indicate the most important signals present in the standard and in the art-samples.

Gallus gallus. A close family, the one of *Anatidae*, with the example of *Anas platyrhynchos* (duck), a possible domestic species from which eggs could be derived, diverged more from chicken ovalbumin than the members of the *Phasianidae* family. From the fact that the highlighted peptides in ovalbumin are unique to *Gallus gallus* we can conclude that the egg used as binder in the mural samples belongs to *Gallus gallus* or

Table 2
Egg proteins (*Gallus gallus*) detected in the samples. Peptides detected which are unique of *Gallus gallus* are indicated in bold.

Sample	Entry	Protein name	Peptides[a] (modifications are indicated)	Peptide PSM	Protein location	
					Egg white	Egg yolk
AND1	F1NFL6	Vitellogenin-2	EETEIVVGR (medium)	1		X
AND3	F1NFL6	Vitellogenin-2	QQLTLVEVR	4		X
			LPLSLPVGPR (medium)	2		
	F1NV02	Apolipoprotein B	ALADLSVVDTAIR	1		X
AND5	P01012	Ovalbumin	**EVVGSAEAGVDAASVSEEFR**	14	X	
			ISQAVHAAHAEINEAGR	7		
			DILNQITKPNDVYSFSLASR	3		
			ELINSWVESQTNGIIR	3		
			LYAEERYPILPEYLQCVK (carbamidomethyl [C16])	3		
			VTEQESKPVQMMYQIGLFR (2xOx [M11; M12])	7		
			GGLEPINFQTAADQAR	14		
			LTEWTSSNVMEER	5		
			YPILPEYLQCVK (carbamidomethyl [C10])	6		
			VTEQESKPVQMMYQIGLFR (Ox [M])	4		
			LTEWTSSNVMEER (Ox [M10])	7		
			NVLQPSSVDSQTAMVLVNAIVFK (Ox [M14])	5		
			AFKDEDTQAMPFR	3		
			AFKDEDTQAMPFR (Ox [M10])	3		
			ADHPFLFCIK (carbamidomethyl [C8])	2		
			ELYRGGLEPINFQTAADQAR	2		
			DEDTQAMPFR (Ox[M7])	1		
	E1BQC2	Ovotransferrin	**GAIEWEGIESGSVEQAVAK**	7	X	
			TDERPASYFAVAVAR	6		
			DDNKVEDIWSFLSK	4		
			SDFHLFGPPGK	2		
			NLQMDDFELLCTDGR (Ox [M4]; carbamidomethyl [C11])	1		
			GDVAFVQHSTVEENTGGK	2		
			AQSDFGVDTK	2		
			YFGYTGALR	2		
			DQLTPSPR	1		
	E1BTF4	Ovalbumin related protein Y	HSLELEEFR	2	X	
			TFSVLPEYLSCAR (carbamidomethyl [C11])	2		
			YNPTNAILFFGR	1		
			FCFDVFNEMK (Ox[M9]; carbamidomethyl [C2])	1		
			IAFNTEDTR	1		
	P00698	Lysozyme C	NTDGSTDYGILQINSR	1	X	
			FESNFNTQATNR	1		
			GTDVQAWIR	2		
			GYSLGNWVCAAK (Carbamidomethyl [C9])	2		
	A0A1D5P531	Ovalbumin related protein X	**ALHFDSIAGLGGSTQTK**	1	X	
			ELLSDITASK	3		
	A0A1D5P474	Ovoglobulin	ASDLFLGSMEPSR (Ox [M9])	3	X	
	Q8JIG5	Alpha-1-acid glycoprotein	TAEVTKEQLEEFEAQLR	1	X	
			EQLEEFEAQLR	2		
			LEEAFITSPK	1		
AND6	F1NFL6	Vitellogenin-2	QQLTLVEVR (medium)	2		X

[a] The confidence of all the peptides was high (FDR < 1%) except for the three peptides indicated as medium (FDR between 1 and 5%). PSM: peptide spectrum matches.

to a very highly related non-sequenced species.

As described above, the presence of collagen in the art samples was established, as a first approach, with a rapid screening by MALDI-TOF/MS showing characteristic peaks conserved when compared to the reference animal glue. A more detailed study was performed analyzing each sample by LC-ESI/MS (Orbitrap) (Table 4). Only hits displaying at

Table 3
Predicted MS/MS fragments for peptide QQLTLVEVR from vitellogenin-2 in samples AND3 and AND6. Fragments in bold and italic font are the ones observed experimentally.

Fragments y and b of the peptide QQLTLVEVR in AND3					Fragments y and b of the peptide QQLTLVEVR in AND6				
b^{+}	b^{2+}	Seq.	y^{+}	y^{2+}	b^{+}	b^{2+}	Seq.	y^{+}	y^{2+}
129.06585	65.03657	Q			***129.06585***	65.03657	Q		
257.12443	***129.06585***	Q	***957.57276***	479.29002	257.12443	***129.06585***	Q	957.57276	479.29002
370.20850	185.60789	L	***829.51418***	415.26073	370.20850	185.60789	L	***829.51418***	415.26073
471.25617	236.13173	T	***716.43012***	358.71870	471.25617	236.13173	T	***716.43012***	358.71870
584.34024	292.67376	L	***615.38244***	***308.19486***	584.34024	292.67376	L	***615.38244***	308.19486
683.40865	342.20796	V	***502.29837***	251.65282	683.40865	342.20796	V	***502.29837***	251.65282
812.45124	406.72926	E	***403.22996***	202.11862	812.45124	406.72926	E	403.22996	202.11862
911.51966	456.26347	V	***274.18737***	137.59732	911.51966	456.26347	V	***274.18737***	137.59732
		R	***175.11895***	88.06311			R	***175.11895***	88.06311

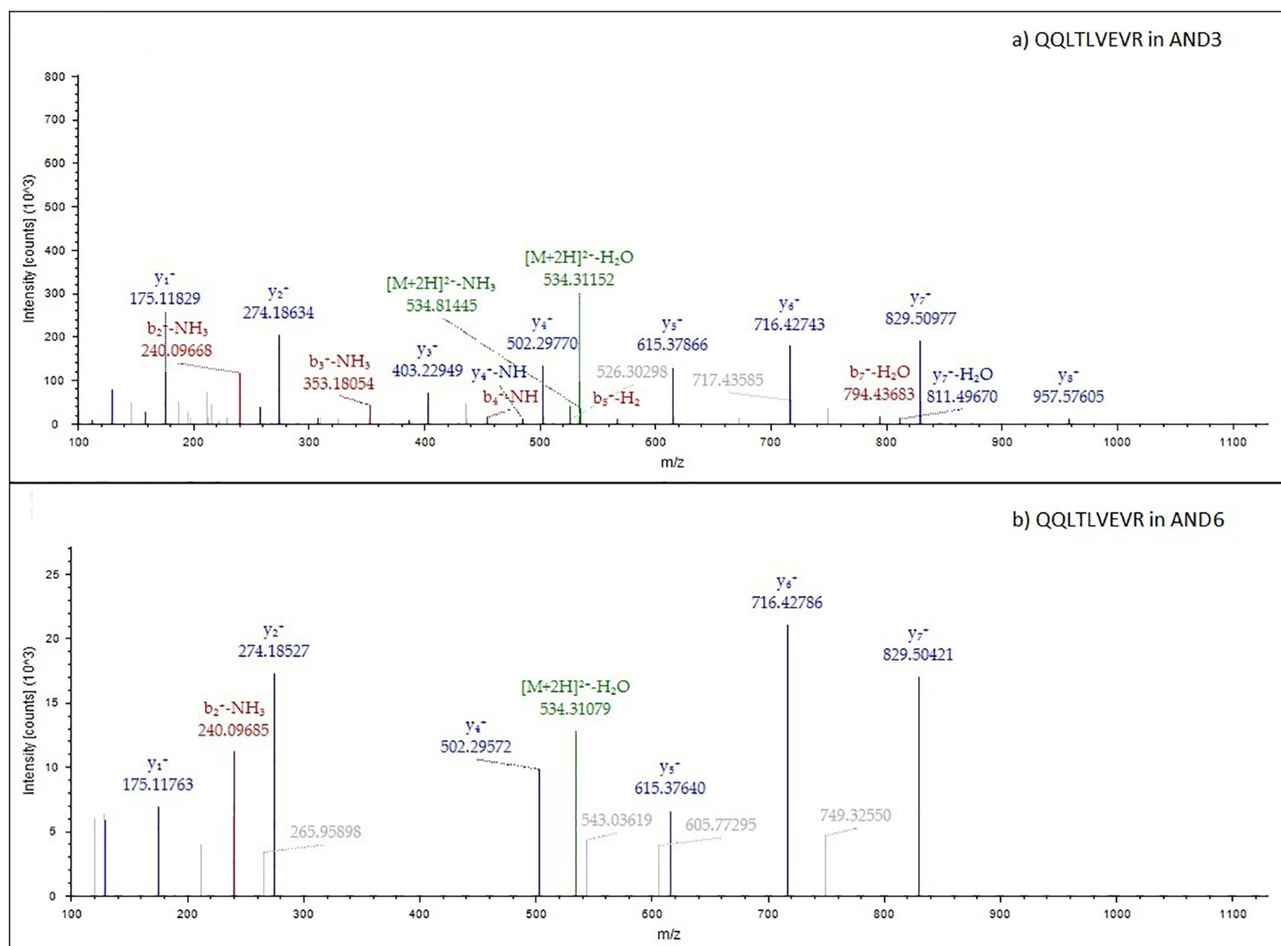

Fig. 5. MS/MS for peptide QQLTLVEVR from vitellogenin-2, a characteristic egg yolk protein: a) sample AND3; b) sample AND6.

least two peptides of high quality are shown. Different types of collagens could be identified in all the samples, although in lower amount in samples AND5 and AND6. These were the same samples that in the comparison by MALDI-TOF/MS with the animal glue reference gave the weaker signals (AND6, Fig. 4a) or no signal at all (AND5). The peptides sustaining these collagen hits correspond to those shown in Appendix Table A3 for the animal glue model sample, in which the characteristic high proline hydroxylation is observed [27].

The provenance of collagen in art samples could not be established, mainly because of the high conservation of the primary sequence of several isoforms among mammals. As an example, collagen alpha-1 (I) chain from *Bos taurus* is 99% identical to the following sequenced

Table 4
Collagen proteins (*Bos taurus*) detected in the samples.

Sample	Accession	Description	Coverage	# peptides[a]	# PSMs
AND1	P02453	Collagen alpha-1(I) chain	26.4	28	95
	P02465	Collagen alpha-2(I) chain	13.3	13	39
	F1MXS8	Collagen alpha-1(III) chain	12.1	13	28
	P02459	Collagen alpha-1(II) chain	3.5	3	7
AND3	P02465	Collagen alpha-2(I) chain	20.4	18	56
	P02453	Collagen alpha-1(I) chain	17.6	17	40
	F1MXS8	Collagen alpha-1(III) chain	6.7	5	21
AND4	P02453	Collagen alpha-1(I) chain	40.6	48	278
	P02465	Collagen alpha-2(I) chain	31.1	31	83
	F1MXS8	Collagen alpha-1(III) chain	22.1	23	66
	P02459	Collagen alpha-1(II) chain	5.1	4	11
AND5	P02453	Collagen alpha-1(I) chain	6.8	7	10
	P02465	Collagen alpha-2(I) chain	3.2	3	4
	F1MXS8	Collagen alpha-1(III) chain	1.6	2	2
AND6	P02465	Collagen alpha-2(I) chain	5.6	5	8
	P02453	Collagen alpha-1(I) chain	4.6	5	7
	F1MXS8	Collagen alpha-1(III) chain	1.8	2	2
AND7	P02453	Collagen alpha-1(I) chain	15.6	14	31
	P02465	Collagen alpha-2(I) chain	15.3	15	25
	F1MXS8	Collagen alpha-1(III) chain	8.1	8	12

[a] The confidence of all the peptides was high. PSM: peptide spectrum match.

orthologs: *Bubalis bubalus* (water buffalo), *Bos mutus* (yak), *Capra hircus* (goat), *Odocoileus virginianus* (deer), *Pantholops hodgsonii* (antelope), *Ovis aries* (sheep), and 90% identical to another 100 species, according to Uniprot [19].

4. Conclusions

In this investigation, we present the first study of proteinaceous binders in mid-18th century art samples from South America by a proteomic approach. The methodology comprised the extraction and separation of proteins from lipid components before analysis, coupled with a typical proteomic protocol. The stability of collagen and egg proteins was not affected by the ammonia used for the extraction procedure.

MALDI-TOF/MS was used as a first step for protein screening and comparison between mural painting samples and references, in order to validate the methodology and for a rapid analysis of art samples that can be selected for further identification by LC-ESI/MS (Orbitrap). This mass spectrometry technique, based on protein identification through the analysis of MS/MS spectra is more suitable for complex artwork samples.

By LC-ESI/MS (Orbitrap) we proved the presence of egg proteins in some of the mural painting samples. One sample yielded several peptides from seven egg white proteins, particularly ovalbumin, ovotransferrin, and lysozyme. Two egg yolk proteins, vitellogenin-2 and apolipoprotein B, were detected in another sample with one high confidence peptide each. These results confirm those of our own former predictions [17] establishing that egg was used as the pigment binder for an egg *tempera* technique. The identified egg white proteins allow us to conclude that the egg used as binder belongs to *Gallus gallus* or a very highly related non-sequenced species.

In all the samples, the presence of collagen from animal origin was established by MALDI-TOF/MS and LC-ESI/MS (Orbitrap). The provenance of the source of collagen is unclearly defined due to the high identity of collagen between mammals. In conclusion, our approach proved to be efficient for the study of binders in mural paintings from the Colonial period, providing an alternative methodology for simultaneous lipid and protein separation coupled with MALDI-TOF/MS and LC-ESI/MS (Orbitrap) for the identification of proteins.

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