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An easily transferable protocol for in-situ *quasi*-non-invasive analysis of protein binders in works of art

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Abstract

Proteomic approaches based on mass spectrometry have become increasingly popular for protein binder's identification in works of art. The identification of the binder employed may offer key information on paintings and other polychrome objects and contribute to assess their historical and technical context, also providing useful hints for a proper restoration and/or conservation treatment. Usually, the protocols employed to this purpose are invasive and at least micro sampling is required. Here, we present a simple transferrable method for a *quasi*-non-invasive analysis of binders in artworks based on the use of a very small poly(2-hydroxyethyl methacrylate)/poly(vinylpyrrolidone) hydrogel (3x3 mm) previously loaded with trypsin for the *in-situ* digestion of proteins and applied onto the objects' surface. Upon extraction of digested peptides from the hydrogel, they were examined by MALDI-TOF-MS and/or LC-ESI-MS/MS. The method was validated on fresh and aged model pictorial layers; optical microscope images, and spectrophotocolorimetry confirmed that neither damage nor color alteration of the painting layer occurred, and no hydrogel residue was left. X-ray photoelectron spectroscopy carried out on paint models confirmed that the treatment with trypsin-loaded gels did not modify the pigment composition, even on aged samples. The protocol was successfully applied to a painting on wood mockup aged thirty years, a statue dated XV century exposed in San Lorenzo church (Bisceglie, Bari, Apulia), and a liturgical scroll *Benedictio ignis et fontis (Benedizionale)* of the Museo Diocesano of Bari dated eleventh century; in all these objects the proteinaceous binder was readily identified.

1. Introduction

The identification of the medium used (i.e., proteinaceous binder) in artworks and polychromies can provide significant hints for assessing the historical context and designing the most suitable restoration and/or conservation treatment [1][2]. Protein-based media such as animal glues, casein and egg white or egg yolk are among the oldest binders used in painting. Currently, their identification is carried out by removing minute amounts of sample and proceeding with the classic bottom-up proteomic approach [1,3–8] by exploiting the use of soft ionization techniques such as electrospray (ESI) and matrix-assisted laser desorption/ionization (MALDI) coupled to powerful mass spectrometry (MS) instrumentations [9]. Thus, it has been possible to identify the presence of egg in Renaissance paintings [10–15], milk in frescos [2,16], collagen of different origins [17–20] in fragments of pottery, parchments, crucifixes [21] and golden statues [22–24].

In order to avoid the micro-sampling and to preserve the artwork content, the recent research in cultural heritage has been increasingly devoted to developing minimally invasive protocols [2,25]. Although preliminary results have been described by multivariate analysis of combined Raman and reflectance spectra [26][27], MS based techniques are still required for the unambiguous identification of proteinaceous binders, especially in complex samples [28]. Softer protein extraction methods have been proposed, based on a heterogeneous phase digestion [29–31], where the aqueous solution of protease, typically trypsin, is deposited on the surface of the artwork and then softly removed without hopefully affecting the sample itself. Recently, two novel strategies for the minimally invasive analysis of proteins have been described such as the use of ethyl-vinyl acetate film functionalized with strong cation/anion exchange and C8 resin [25,32] and the use of cellulose acetate film modified with fungal proteins *Vmh2* hydrophobin to immobilize trypsin [33]. Sheets of

modified cellulose large 2x2 cm were applied on the surface of artworks for protein digestion and the generated peptides investigated by MALDI-MS.

Here, a simplified protocol of *in-situ* protein digestion is proposed, whereby the sampled area was strongly minimized by using a hydrophilic gel (3x3 mm) based on poly(2-hydroxyethyl methacrylate)/poly(vinylpyrrolidone) (pHEMA/PVP) previously loaded by a trypsin-based solution. The protein digestion was accomplished on a very small pictorial contact area in less than thirty minutes; the simplicity of the sampling protocol makes it easy to be used even by non-specialized personnel. To investigate possible modifications occurring on the outmost layers of the samples, the effect of gel application (with or without loaded enzyme) onto paint replica surfaces was also examined by X-ray photoelectron spectroscopy (XPS). The released peptides from the pictorial layer directly on the gel were easily recovered and identified by MALDI MS. The proposed approach was successfully validated by sample extract examination also via reversed-phase liquid chromatography coupled with ESI-MS. The protocol was applied on a pictorial layer on wood aged thirty years and on two authentic artworks: a statue dated XV century and a liturgical parchment scroll dated eleventh century. Apparently, the suggested protocol represents an innovative step forward for the use of a *quasi*-non-invasive tool in the proteomics field of cultural heritage specimens without the need for sampling and avoiding the use of organic solvents or special pretreatments as well.

2. MATERIALS AND METHODS

2.1 Chemicals. Water, acetonitrile, ammonium bicarbonate, and formic acid were obtained from Sigma-Aldrich (Milan, Italy). All solvents used were LC–MS grade. Nanorestore Gel® Medium Water Retention - ("Extra Dry") is an Italian Patent from Consorzio CSGI,

University of Florence, based on poly(2-hydroxyethyl methacrylate)/poly(vinylpyrrolidone) (pHEMA/PVP) hydrogel. Fresh hen eggs and cow milk were purchased at local supermarkets. Collagen standards (bovine bone glue (63000), bovine skin glue (63010), rabbit skin glue (63025)) were obtained from Kremer Pigmente GmbH & Co (Aichstetten, Germany). Pigments (see Table 1) were purchased from Carenza (Bari, Italy) or from Kremer Pigmente GmbH & Co (Aichstetten, Germany). Calibrating solution containing caffeine, methionine-arginine-phenylalanine-alanine peptide and Ultramark, a mixture of fluorinated phosphazines were purchased from Thermo Scientific (Waltham, Massachusetts, United States).

2.2. Protocol on pictorial and historical samples. Paint replicas were made on 76×26 mm glass slides by applying dispersions of the common inorganic pigments in egg yolk, milk casein, bovine and rabbit collagen binders (Table 1). Aged replicas up to four years in presence of light were also analyzed. The transparent hydrogel based on pHEMA/PVP network is sold loaded with water [34][35]; it was cut in small 3x3 mm pieces, which were dried at air ambient until they have lost at least 20% in weight. Thus, they are immersed in 100 µL of a solution of trypsin (20 pmol/mL in ammonium bicarbonate at pH 7.0 or in water) for 30 minutes to allow the enzyme to soak into the hydrogel. In-situ digestion of protein binders was carried out by putting the trypsin loaded hydrogel pieces in intimate contact with the replica surface under investigation for 30 min. Upon removal the hydrogel was immersed in 100 µL of a solution of 70%:30 ACN:H_2O with 0.1% TFA, for 15 min in an ultrasonic bath to extract the peptides released into the gel after in-situ digestion, vacuum dried to preconcentrate the sample and then analyzed by MALDI MS(/MS) or LC-ESI-MS/MS. The same protocol was applied both on standard paint replicas and authentic works of art.

When the real samples were investigated by in-situ sampling/digestion the dried gel was kept in a vial containing the trypsin solution and transported the to the site of sampling. The gel was left wetting in the trypsin solution up to one hour. For longer time needs, such as sampling many points, reaching a far site of the artwork, etc., it is suggested to leave the dried gel and trypsin solution apart and to wet the gel just half an hour before the application. At the end of the sampling, once the gel is removed from the artwork surface, the piece of gel should be dipped in the elution solution almost for half an hour; sonication is suggested for a more efficient extraction.

2.3. MALDI-TOF/TOF MS analysis. For MALDI-MS analysis, the analyte solutions were mixed with both CHCA or CClCA (10 mg/mL in 70% acetonitrile and 0.1% TFA) as the matrix. Typically, 1 µL of matrix was applied to the metallic sample plate and 1 µL of analyte was added. All experiments were performed using a 5800 MALDI-ToF/ToF analyzer (SCIEX, Darmstadt, Germany) equipped with a neodymium-doped yttrium lithium fluoride (Nd:YLF) laser (345 nm), in a reflectron positive mode, with a mass accuracy of 10 ppm. In MS and MS/MS modes, 1000 laser shots were typically accumulated by a random rastering pattern, at laser pulse rates of 400 and 1000 Hz, respectively; each shown mass spectrum was averaged on at least five single mass spectra (1000 laser shots each). MS/MS experiments were performed setting a potential difference of 1 kV between the source and the collision cell; ambient air was used as the collision gas with a medium pressure of 10^{-6} Torr. The delayed extraction time was set at 450 ns. DataExplorer software 4.0 (Sciex) was used to control the acquisitions and to perform the initial elaboration of data, whereas SigmaPlot 11.0 was used to graph the final mass spectra.

2.4. RPLC-ESI-MS instrumentation and operating conditions. LC-ESI-MS measurements were performed using an LC-MS apparatus consisting in an Ultimate 3000 UHPLC system coupled to a Velos Pro mass spectrometer equipped with a linear ion trap analyzer (Thermo Scientific, Waltham, MA, USA). LC separation was performed at 40 °C and flow rate 0.2 mL/min on two in series Ascentis Express C18 (150 × 2.1 mm ID, 2.7 µm particle size) equipped with an Ascentis Express C18 (5 × 2.1 mm ID) security guard cartridge (Supelco, Sigma-Aldrich Chemie GmbH, Germany). Peptides released from the hydrogel upon in-situ digestion were injected into the column via a 5 µL sample loop. The following gradient elution program, based on H_2O (solvent A) and ACN (solvent B), both containing 0.1% formic acid, was adopted: 0 – 2 min at 5% solvent B; 2 – 12 min linear from 5% to 35% (v/v) of B; 12 – 17 min isocratic at 35% B; 17-35 min linear from 35% to 100% (v/v) of B; 35-37 min isocratic at 100% B; 37- 39 min linear from 100% to 5% (v/v) of B, followed by 5 min equilibration time. The column effluent was transferred into the mass spectrometer through the HESI source. The main electrospray and ion optic parameters adopted during acquisitions were the following: sheath gas flow rate, 30 (arbitrary units); auxiliary gas flow rate, 15 (arbitrary units); spray voltage, 2.5 kV; capillary temperature, 275°C; S-lens radio frequency level, 60 arbitrary units. Positive MS full-scan spectra were acquired in the *m/z* range 130–2000, while MS/MS experiments based on low-energy CID were performed at collision energy 35 % (a 400% value corresponds to a 100 V excitation voltage) using a 1 *m/z* unit wide isolation window centred on the monoisotopic *m/z* value. The control of the LC-MS instrumentation and the first processing of data were performed by the Xcalibur software 2.2 SP1.48 (Thermo Scientific). The post analyses data processing was performed by using SigmaPlot 11.0 to graph final mass spectra.

2.5. Database searching. Peptide mass fingerprint was carried out with Protein prospector (Regents of the University of California) software by employing the MS-Fit tool. Proteins were identified using SwissProt database with *Bos taurus, Oryctolagus cuniculus* or *Gallus gallus* as taxonomy restriction to cover all the possible binders as collagen, milk and egg. Search parameters for MS analysis were the following: peptide mass tolerance 100 ppm, allowed trypsin missed cleavages up to 3. No fixed chemical modification was inserted, but oxidation of methionine residues, and hydroxylation of prolines were considered as variable modifications. The acquired MS/MS data set was processed by mMass™ 5.5.0 using MASCOT search engine (Matrix Science, London, United Kingdom) and MS-Tag from Protein prospector. A tolerance of 0.5 Da was set for the precursor ion and for MS/MS fragment ions.

2.6. XPS analysis. XPS characterization was performed on paint replicas #3, #7 and #12 as listed in Table 1 using a Versaprobe II spectrometer (PHI) with monochromatized X-ray radiation (1486.6 eV, 200 µm spot size). Survey spectra were acquired with a pass energy of 117.4 eV; whereas high-resolution (HR) spectra were acquired with a pass energy of 46.95 eV. Depending on the pigment and binder combination of samples, different elements were identified and their corresponding principal photoelectronic signals registered as HR spectra. Different areas of the samples were analyzed comparing untreated parts, portions treated with bare gel, and the ones exposed to trypsin-loaded gel. Sample #15 was also characterized after four months of aging. MultiPak™ (v. 9.7.0.1, PHI-ULVAC) software was used to process the data. Binding energy (BE) scale was corrected considering aliphatic C1s component at 284.8 eV. The % of element variations were calculated on three replicate points.

2.7. Microscopy analysis. Optical analyses of samples were carried out by means of Nikon "Eclipse80i" motorized microscope for large fields or alternatively with Nikon stereomicroscope "SMZ800". The objective aperture of Nikon "Eclipse80i" was bigger compared to stereomicroscope and leads to better resolution rates for flat samples while stereomicroscope was employed for rough surfaces like parchment roll. The acquisition of images was carried out with Nikon DS-5M microscopes camera and processed by LUCIA-Nikon software. The microscopic analysis was performed on dried samples.

2.8 Colorimetric measurements. Evaluation of color changes of replica substrates was carried out by means of colorimetric measurements using a Konica-Minolta Chroma Metre (CM-2600d). The results are registered as CIE-L*a*b* system. Color changes (ΔE) were obtained by measurements before and after the gel application on dried surface. The instrument was set to automatically give the average value of the colorimetric coordinates (L*, a*, b*) of three measurements calculating the resultant chromatic change ΔE* as follows: $\Delta E^* = \sqrt{(\Delta L^{*2}} + \Delta a^{*2} + \Delta b^{*2})$.

3. RESULTS AND DISCUSSION

3.1 Method development and validation on paint models

Preliminarily, the activity of trypsin-loaded hydrogels was assessed by digesting a standard solution of bovine serum albumin (BSA) as a model protein. An aqueous solution of BSA (10 μg/mL) was properly spread out on a glass slide with and without mixing with blue ultramarine as a pigment; after sample drying, a piece of trypsin-loaded hydrogel was placed onto the surface and incubated at room temperature for 15 min. As described in the

experimental section, the released peptides were collected in an acidified solution of CH_3CN/H_2O (70:30 v/v). For comparison, BSA was also digested by using a conventional in-solution digestion, i.e. 50 mM NH_4HCO_3 buffer at 37 °C overnight. Both mixtures of digested peptides obtained by immobilized and in solution trypsin were examined by MALDI-MS and the relevant spectra are reported in plots A and B of Figure 1, respectively. In Table S1 are listed the experimental and theoretical *m/z* values of peptides along with their amino acid sequences. Interestingly, the outcome proves that in both cases BSA was confidently identified as first output. Although the processing time was shortened from about 12 h to less than one hour, the *in-situ* protocol digestion exhibited a sequence coverage of 20.6%, which is comparable with 26.0% obtained by the conventional one. Therefore, the enzyme was active in the hydrogel and its small contact area was enough to obtain reliable results also for low BSA concentrations.

To test the efficacy of trypsin loaded hydrogels, paint models composed of chicken egg yolk, different types of animal glue (collagen), and caseins from cow milk mixed with various pigments (see Table 1 and Figure S1) were examined. MALDI-TOF spectra recorded on the recovered supernatants upon carrying out the whole protocol are reported in Figure 2. It is worth noting that no MALDI-MS signals were detected when bare hydrogel pieces, not loaded with trypsin, were used. Database and literature search allowed us to successfully assign the main peptides of paint replicas to caseins (plot 2A), bovine collagen (plot B), rabbit collagen (plot C) and egg yolk (plot D); in Table 2 the number of peptides, the corresponding protein and coverage of each investigated paint replicas are summarized. Additional details are reported in Table S2, including the experimental and theoretical *m/z* ratios of peptides, their sequence, the corresponding identified protein, and the relevant coverage for all investigated samples. For instance, the peptides of caseins provide a

coverage of 43% for α-s1-casein, 30% for α-s2-casein, and 32% for β-casein. Furthermore, some peak signals attributable to the mono- and multi-phosphorylated peptides (see Table S2), which often got undetected using conventional tryptic digestion protocols due to their low content and ionization signal suppression, could also be recognized. It is suggested that the present protocol may provide an enrichment of small polar molecules as phosphopeptides most likely due to the binding ability of the hydrophilic gel. The same tryptic digests were analyzed by reversed-phase liquid chromatography (RPLC) coupled to ESI-MS and some selected peptides were examined by tandem MS; the fragmentation spectra obtained by collision-induced dissociation (CID) were interpreted by MS-Tag search engine and manually validated. As an example, Figure S2 shows the MS/MS spectra of the two doubly charged peptides at *m/z* 1031.3^{2+} (A) and *m/z* 830.9^{2+} (B), both from a casein paint sample that have been assigned by PMF to the phosphopeptide (K)FQSEEQQQTEDELQDK(I) (at *m/z* 2016.67) of β-casein and the phosphopeptide (K)VPQLEIVPNSAEER(L) (at *m/z* 1660.67) of α-s1-casein, respectively. The corresponding fragmentation pattern and matched intensity demonstrated a reliable sequence assignment. While in Figure S3A is displayed the MS/MS spectrum of one marker peptide at *m/z* 524.8^{2+} assigned to LPLSLPVGPR (vitellogenin-2) from egg paint sample together with the corresponding fragmentation pattern, Figure S3B reports the MS/MS spectrum of a marker peptide of bovine collagen at *m/z* 714.4^{2+}, corresponding to GIPGEFGLPGPAGAR with 2 hydroxyproline from collagen-α2(I). Note that rabbit and bovine collagen show a high sequence identity resulting in similar PMF. Indeed, 92% of similarity was found for collagen-α2 type I sequence in rabbit and bovine species (http://www.uniprot.org/blast/uniprot/) from BLAST comparison. Only partial sequences of rabbit type I collagen, 53 and 526 amino acids for α1(I) and α2(I) chains, respectively, are registered in the UniProt database making

difficult their recognition. In this case, species-specific peptides should be considered to discriminate between bovine and rabbit glues by comparing results with literature data [10,18,20]; for example, we observed the signals at *m/z* 1562.6 and 2097.6 corresponding to oxidized peptides GLPGVAGALGEPGPLGIAGPPGAR and GEPGPAGSIGPVGAAGPR previously reported as markers of rabbit collagen α-2(I) chain [18]. Unfortunately, these peptides were also detected in bovine collagen spectra, thus inferring that commercial glue products were not pure. This agrees with previous remarks that some skin glues sold as rabbit were in fact cattle or bovine ones [19].

By using LC-MS/MS it was possible to confidently identify the binder protein also with a few numbers of matched peptides because the chromatographic separation can be useful to discriminate isobaric peptides. For example, Figure 3A reports the extracted ion current (XIC) chromatogram of a bi-charged ion at *m/z* 634.3 from rabbit collagen (black) and casein (red) binders; this peptide is usually observed as monocharged ion in MALDI at *m/z* 1267.7 for both samples. Looking at the plot a of Figure 3 it is readily apparent that two dissimilar peptide sequences with the same *m/z* value, elute at different retention times, i.e., 10.2 and 15.2 min. Tandem MS spectra registered on each precursor ion are shown in plots B and C; database searching and manual validation allowed us to identify both peptides as $GIP_{ox}GPVGAAGATGAR$ from collagen-α-2(I) chain (plot B) and as YLGYLEQLLR from α-s1-casein (plot C).

3.2. Optical, colorimetric and X-Ray spectroscopy analysis of the surface.

To assess the protocol's impact on the painting surface of laboratory made replicas, optical microscopy and X-ray photoelectron spectroscopy (XPS) were employed. Figure S4 (see Supplementary Material) shows the enlarged images of the surfaces of paint layers made by

(A) casein mixed with calcium carbonate and (B) collagen mixed with ultramarine, before (left) and after (right) the hydrogel application. As can be seen the samples' surface was not significantly altered by the trypsin treatment and no gel residue remains on the surface. Therefore, at least at the microscope level, the protocol invasiveness is minimal. Moreover, chromatic changes were measured with spectrophotocolorimetry on two paint replicas made of collagen and calcium carbonate (sample #13) and casein mixed with ultramarine lazurite (sample #3) before and after the gel application. The measurements were registered on dried samples (around two hours later the gel removal to allow the gel to dry) exactly on the same points by using a proper mask. For the treated areas the ΔE values were below 1 for both samples which is remarkably lower than the ΔE limit value (ΔE = 5) when chromatic changes become visible by the naked eye.

XPS analysis was also particularly useful in assessing the ability to enrich the hydrogel of protein binders without affecting the pigment composition. To this aim, different paint replicas were investigated by comparing the surface chemical composition of the areas exposed to trypsin digestion with not-treated ones. Moreover, the effects of contact with bare gel were evaluated as well. Considering the chemical composition of each binder, nitrogen, oxygen and carbon contents can be used to monitor the fate of this component after protease activity. Due to the ubiquitous nature of C and O in the XPS analysis chamber, the N/C ratio was considered more informative on the surface protein content. Depending on the pigment chemical formula, the Al/Si and Ca/C ratios of ultramarine blue lazurite and calcium carbonate, respectively, were examined. The N/C ratio diminished after sample contact with the enzyme as demonstrated on all tested samples; indeed, whereas the N/C ratio of tempera binder of sample #7 reduced from 0.034±0.002 to 0.023±0.002, the Ca/C ratio remained unchanged at 0.004±0.001, thus suggesting that the protocol did not cause

significant pigment loss. Similarly, on sample #3 (casein binder) and sample #12 (rabbit skin glue binder), the N/C ratio changed from 0.07±0.01 and 0.27±0.01 to 0.03±0.01 and 0.20±0.01, respectively. Interestingly, the Al/Si ratio was found unchanged and equal to 0.27±0.03 in all the cases, thus implying that the protocol does not remove the pigment. It was also proved that the bare hydrogel did not cause any significant modification to the sample surface, as demonstrated by the typical C1s spectrum in Figure 4A, acquired on an untreated area of sample #12 (blue curve) and compared with those recorded on an area treated with either bare hydrogel (red dashed line) or trypsin-loaded hydrogel (black curve). The C1s components of proteinaceous origin falling at about 286 eV and 288 eV (Figure 4B) associated to amidic and carboxylic moieties [36] decrease significantly in trypsin-treated areas. A similar trend was observed on other binders, as shown in Figure S5A for sample #3. The latter was examined after a natural aging of four months and showed similar C1s spectra (Figure S5B). Once more, the measured N/C and Al/Si ratios on three different areas (untreated, treated with bare hydrogel, and treated with hydrogel + trypsin) agreed with those values found on freshly prepared samples.

3.3. Aged and historical samples.

A fundamental issue to be faced in the characterization of organic binders in ancient samples is represented by ageing processes naturally occurring since proteins are easily susceptible to chemical and microbial degradation [37][38][39] and the co-presence of pigments could strongly influence these processes and affect the proteomics outcomes. Before analysing works of art, the trypsin-loaded hydrogel protocol was tested on laboratory aged replicas, exposed up to four years in presence of light, under ambient conditions, prepared with well-known pigments such as vermilion and white zinc. In Figure S6 are displayed the MALDI mass

spectra of caseins (A), rabbit collagen (B) and egg binders (C) while Table S3 lists the identified peptides with the corresponding proteins. Notably, the number of peptides was reduced compared to fresh samples; indeed, several aggregation and crosslinking formation among proteins can hinder the accessibility of trypsin, while side reactions on amino acid residues such as hydroxylation, oxidation, deamidation can introduce mass shifts, thus making more difficult the identification by databases. However, we were still able to identify the binder, and we were encouraged to apply the protocol on three different aged and/or samples: a liturgical scroll *Benedictio ignis et fontis (Benedizionale)* of the Museo Diocesano of Bari, a wooden statue exposed in San Lorenzo Church (Bisceglie, Bari, Apulia) and a pictorial layer on wood aged thirty years. The liturgical scroll type of illuminated manuscripts was produced in southern Italy between the X and XIV centuries AD (sample #1 in Table 3). The only information retrieved on the painted wood statue is about its period and attribution to the Franciscan order, which experienced a period of expansion in the fifteenth century (sample #2 in Table 3). The thirty years old laboratory mockup is composed of four zones: a gold layer with no organic binder, a white sector with rabbit collagen mixed to Bologna gypsum (sample #3 in Table 3), a black zone composed of collagen and black coal (sample #4 in Table 3), and a brown region prepared as egg tempera mixed with ultramarine.

The pictures of the tested works of art (scroll, statue and painting mockup), with an indication of the sampling points as well, are available respectively in Figure 6 and in Supporting Information (Figures S7-S9). The acquired MALDI mass spectra are shown in Figure 5 respectively for scroll (A), statue (B) and painting (C). The result interpretation was carried out by comparing spectra with those of the painting tests along with PMF in the Mascot database search; the identified proteins with the detected peptides are listed in Table 3. Bovine or rabbit collagen was reliably identified in all the samples; the occurrence in

the parchment scroll of peak signals at *m/z* 2883.2 and 1196.6, recognized markers of sheepskin, corroborated our previous results obtained on other points of the same historical paint but using a conventional micro-destructive protocol [22]. In the tempera painted area of the mockup, it was possible to detect a few egg peptides (data not shown) at *m/z* 985.5, 1048.6, 1406.7, 1457.7 (see Table S2). As recently reported by Cicatiello et al. [33], the analysis of egg proteins is especially challenging due to their post-translational modifications including glycosylation [40], the limited extraction efficiency of ovalbumin due to polymerization processes occurring during ageing [41] and their intrinsic inhibition action towards trypsin proteolysis [42]. For these reasons some selected peptides extracted from this area were subjected to RPLC-ESI-MS/MS analysis. Database search and manual interpretation of the fragmented ions confirmed their assignment to peptides from egg yolk protein with many of them arising from vitellogenins (Table 4). Moreover, the parchment scroll surface (Figure 6) was investigated with optical stereomicroscope in various points before (Figure 7A) and after (Figure 7C) hydrogel digestion. Figure 7B shows also the enlarged image during gel application to give an idea that the proposed strategy is indeed quasi non-invasive. After gel application, no residue deposited on scroll was detectable (Figure 7C) and no visible change of the investigated area was appreciable.

Conclusions

Here we have demonstrated the possibility of using a very small piece of hydrophilic gel loaded with trypsin to digest proteins directly in-situ on works of art with good efficacy. The strategy was developed on fresh and aged paint replicas, allowing the recognition of the usually employed organic binder based on egg, collagen, and casein. In order to assess the ability of the protocol to digest in-situ the protein components without affecting the pigment, the sampled area was investigated for the first time by using XPS analysis, so

obtaining precious information on the outmost surface of the painting sample. Optical microscope images and photocolorimetric measurements confirmed the absence of pictorial layer damage caused by the proposed protocol, which was tested on historical samples containing collagen and egg proteins. The key advantages of this protocol rely on the almost non-invasiveness (sampled area 3x3 mm) and on the easy sampling procedure that does not require any specific technical expertise. Our future work is addressed in improving egg binder recognition by testing different proteolytic enzymes.

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Conflict-of-interest

The authors declare no conflict-of-interest.

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FIGURE CAPTIONS

Figure 1. Positive MALDI-ToF mass spectrum of a tryptic digest of BSA obtained by (A) *in-situ* loaded hydrogel and (B) overnight in solution protocol. ClCCA was used as a matrix.

Figure 2. MALDI-ToF mass spectra of an *in-situ* trypsin-loaded hydrogel digestion of paint replicas from caseins (sample #2, A), bovine collagen (sample #10, B), rabbit collagen (sample #16, C) and egg yolk (sample #6, D). The asterisked peaks are due to phosphorylated peptides. All main peaks of spectra are listed in Table S2.

Figure 3. (A) Extracted ion current (XIC) chromatogram of the bi-charged ion at *m/z* 634.3 from rabbit collagen (black) and casein (red) binders; (B) MS/MS spectrum registered on *m/z* 634.3 isolated at 10 min and identified as $GIP_{ox}GPVGAAGATGAR$ from collagen-α-2(I) chain; (C) MS/MS spectrum registered on *m/z* 634.3 isolated at 15 min and identified as YLGYLEQLLR from α-s1-casein.

Figure 4. a) Typical C1s spectra acquired on different areas of sample #12: untreated (blue curve), treated with bare gel (dashed red line), treated with trypsin-loaded hydrogel (black curve). b) C1s components identified in the C1s spectrum of untreated sample #12.

Figure 5. MALDI-ToF mass spectra of *in-situ* trypsin-loaded hydrogel digestion performed on historical samples: (A) parchment scroll dated eleventh century, (B) wooden statue dated fifteenth century and (C) a 30 years aged paint mockup (collagen mixed with white gypsum area). All main peaks of spectra are given in Table 3.

Figure 6. (A) Detail of the *Benedictio ignis et fontis on Folio* n. 2 of the *Benedizionale* and (B) zoom on the binding strip between Folio 1 and 2 (Museo Diocesano, Bari, Italy).

Figure 7. Optical stereomicroscope imagines before (A), during (B) and after (C) hydrogel treatment.

Table 1. List of the tested paint replicas.

n.	Fresh paint replicas (binder+ pigment)
#1	Casein + calcium carbonate
#2	Casein + white zinc
#3	Casein + ultramarine blue lazurite
#4	Casein + ultramarine blue
#5	Egg yolk + calcium carbonate
#6	Egg yolk + ultramarine blue
#7	Egg yolk + linseed oil + calcium carbonate
#8	Egg yolk + linseed oil + ultramarine blue
#9	Bovine skin glue (63010) + ultramarine blue
#10	Bovine skin glue (63020) + calcium carbonate
#11	Bovine skin glue (63020) + ultramarine blue lazurite
#12	Rabbit skin glue (63028) + ultramarine blue lazurite
#13	Rabbit skin glue (63028) + calcium carbonate
n.	**Aged* paint replicas (binder+ pigment)**
#14	Rabbit skin glue (63028) + vermilion
#15	Egg yolk + vermilion
#16	Casein + white zinc

* #14, #15 naturally aged four years; * #16 naturally aged two years.

Table 2. Number of identified peptides by PMF, related protein and sequence coverage for three tested paint replicas.

Sample (#)	Peptides number	Protein	Coverage (%)
Casein (#4)	11	α-s1-casein	43.2
	8	α-s2-casein	30.2
	7	β-casein	32.1
Egg Yolk (#6)	13	Vitellogenin 1	6.3
	21	Vitellogenin 2	10.9
	4	Vitellogenin 3	8.6
	7	Apolipoprotein B	13.1
	5	Apolipoprotein A-I	17.4
	5	Very low-density lipoprotein receptor	6.1
	4	Low-density lipoprotein receptor-related protein 8	4.4
Rabbit collagen (#12)	20	Collagen α-2(I) chain	20.9
	13	Collagen α-1(II) chain	13.9
	15	Collagen α-1(III) chain	21.9
Bovine collagen (#9)	17	Collagen α-1(I) chain	15.4
	13	Collagen α-2(I) chain	13.8
	8	Collagen α-1(II) chain	8.7
	16	Collagen α-1(III) chain	23.5

Table 3. List of the identified peptides by PMF in historical samples: #1= scroll, #2=statue, #3= thirty years aged mockup (collagen+gypsum), #4= thirty years aged mockup (collagen+black coal).

	Sample				Sequence	Protein
m/z	#1	#2	#3	#4		
836.43	X		X	X	GPPGPQGAR	Collagen α-1 (I) chain
840.46	X				GVVGPQGAR	Collagen α-2 (I) chain
841.45			X	X	GLPGERGR	Collagen α-1 (II) chain
					GEPGAPGLK + 1 ox	Collagen α-1 (III) chain
852.42	X			X	GPPGPQGAR + 1 ox	Collagen α-1 (I) chain
868.46	X		X	X	GPPGPQGAR + 2 ox	Collagen α-1 (I) chain
					GPSGPQGIR	Collagen α-2 (I) chain
898.48	X				GVVGLPGQR + 1 ox	Collagen α-1 (I) chain
918.43			X	X	GDKGEPGDK + 1 ox	Collagen α-2 (I) chain
946.42				X	GEKGETGLR	Collagen α-2 (I) chain
1095.55	X		X	X	GAAGLPGPKGDR	Collagen α-1 (I) chain
					GPSGPPGPDGNK + 1 ox	Collagen α-2 (I) chain
					GPRGDQGPVGR	Collagen α-2 (I) chain
					GRPGLPGAAGAR + 1 ox	Collagen α-1 (III) chain
1105.57	X	X	X	X	GVQGPPGPAGPR + 1 ox	Collagen α-1 (I) chain
1128.54	X	X	X	X	GLPGTPGTDGPK + 2 ox	Collagen α-1 (II) chain
					GLAGPPGMPGAR + 3 ox	Collagen α-1 (III) chain
1144.53	X		X	X	GLPGTPGTDGPK + 3 ox	Collagen α-1 (II) chain
					GLAGPPGMPGAR + 4 ox	Collagen α-1 (III) chain
1165.78		X			NWYISKNPK + 1 ox	Collagen α-1 (I) chain
1193.65			X	X	GQAGVMGFPGPK + 3 ox	Collagen α-1 (I) chain
1223.60			X	X	GPAGPSGPAGKDGR	Collagen α-2 (I) chain
1241.64	X	X	X	X	GSPGGPGAAGFPGGR	Collagen α-1 (III) chain
1267.67	X	X	X	X	GIPGPVGAAGATGAR + 1 ox	Collagen α-2 (I) chain
1289.60			X	X	GSPGGPGAAGFPGGR + 3 ox	Collagen α-1 (III) chain
1427.71	X	X	X	X	GSAGPPGATGFPGAAGR	Collagen α-1 (I) chain
					GIPGEFGLPGPAGAR + 2 ox	Collagen α-2 (I) chain
					ALLIQGSNDVEIR	Collagen α-1 (II) chain
1435.67	X	X	X	X	GEPGPAGLPGPPGER + 3 ox	Collagen α-1 (I) chain
					GPPGPPGTNGVPGQR + 3 ox	Collagen α-1 (III) chain
1448.92		X			GARGVAGKPGPRGQR + 1 deam	Collagen α-1 (XI) chain
1459.69	X	X	X	X	GSAGPPGATGFPGAAGR + 2 ox	Collagen α-1 (I) chain
1473.66	X	X	X	X	GDGGPPGATGFPGAAGR + 2 ox	Collagen α-2 (I) chain
1532.75			X	X	DGASGHPGPIGPPGPR + 4 ox	Collagen α-1 (III) chain
1560.77	X	X	X	X	STGISVPGPMGPSGPR + 4 ox	Collagen α-1 (I) chain
					GPAGMPGFPGMKGHR + 4 ox	Collagen α-1 (III) chain
1562.79			X	X	NWYISKNPKEKR	Collagen α-1 (I) chain
					GDKGETGEQGDRGIK + 1 ox	Collagen α-1 (I) chain
					GAAGLPGVAGAPGLPGPR + 3 ox	Collagen α-2 (I) chain
1586.73	X	X	X	X	GNSGEPGAPGSKGDTGAK	Collagen α-1 (I) chain
					GETGPAGPSGAPGPAGSR + 4 ox	Collagen α-1 (III) chain
1648.78			X	X	AGEDGHPGKPGRPGER + 2 ox	Collagen α-2 (I) chain
1655.77		X	X	X	GFPGADGVAGPKGPAGER + 1 ox	Collagen α-1 (I) chain
					GEPGSSGVDGAPGKDGPR + 1 ox	Collagen α-1 (III) chain
1693.80			X	X	LLSTEGSQNITYHCK	Collagen α-1 (II) chain
					GENGVPGEDGAPGPMGPR	Collagen α-1 (III) chain

1709.79			X	X	GENGVPGEDGAPGPMGPR + 1 ox	Collagen α-1 (III) chain
1781.85		X			TGPPGPSGISGPPGPPGPAGK	Collagen α-2 (I) chain
1813.83		X	X	X	NGETGPQGPPGPTGPSGDK + 4 ox	Collagen α-1 (III) chain
1832.83				X	GPPGPMGPPGLAGPPGESGR + 3 ox	Collagen α-1 (I) chain
1845.84		X	X	X	TGPPGPSGISGPPGPPGPAGK + 4 ox	Collagen α-2 (I) chain
					AGGAQMGVMQGPMGPMGPR + 1 ox	Collagen α-1 (II) chain
1922.88		X	X	X	GERGPPGESGAAGPTGPIGSR + 1 ox	Collagen α-2 (I) chain
					GDSGAPGERGPPGAGGPPGPR + 5 ox	Collagen α-1 (III) chain
1962.88		X	X	X	GEPGPAGLPGPPGERGGPGSR + 4 ox	Collagen α-1 (I) chain
1975.94		X	X	X	SGDRGETGPAGPAGPIGPVGAR	Collagen α-1 (I) chain
					GEPGAVGQPGPPGPSGEEGKR + 1 ox	Collagen α-2 (I) chain
					GPPGPQGARGFPGTPGLPGVK + 2 ox	Collagen α-1 (II) chain
2056.98			X	X	GAPGADGPAGAPGTPGPQGIAGQR	Collagen α-1 (I) chain
2074.93			X	X	GDIPDPGLPGDQGPPGPDGPR + 4 ox	Collagen α-4 (IV) chain
2099.01			X	X	GVQGPPGPAGPRGANGAPGNDGAK	Collagen α-1 (I) chain
2131.05	X	X	X	X	GVQGPPGPAGPRGANGAPGNDGAK + 2 ox	Collagen α-1 (I) chain
						Collagen α-2 (I) chain
					GLPGVAGSVGEPGPLGIAGPPGAR + 3 ox	Collagen α-1 (II) chain
					GMPGPQGPRGDKGETGEAGER + 3 ox	
2263.01			X	X	DGVQGPVGLPGPAGPAGSPGEDGDK + 2 ox	Collagen α-1 (XI) chain

587

588

Table 4. List of the identified peptides by LC-ESI-MS/MS in the pictorial layer of the mockup aged 30 years and based on egg yolk mixed with ultramarine.

m/z	Sequence	RT	Protein
634.3^{2+}	LRSKMSLSMAK^{+2} + 1 ox	14.33	Vitellogenin-1
659.3^{3+}	LSSKLEISGLPENAYLLK+3	10.53	Vitellogenin-2
718.3^{2+}	LSQLLESTMQIR^{+2} + 1 ox	11.87	Vitellogenin-2
780.4^{2+}	LEISGLPENAYLLK^{+2}	14.07	Vitellogenin-2
998.0^{2+}	SSSKSSSSSSSSSSSSSK^{+2} + 3 phospho	10.14	Vitellogenin-2
998.0^{3+}	GLSHPFSIDIFEDYIYGVTYINNR^{+3} + 2 phospho	10.14	Low-density lipoprotein receptor-related protein 1

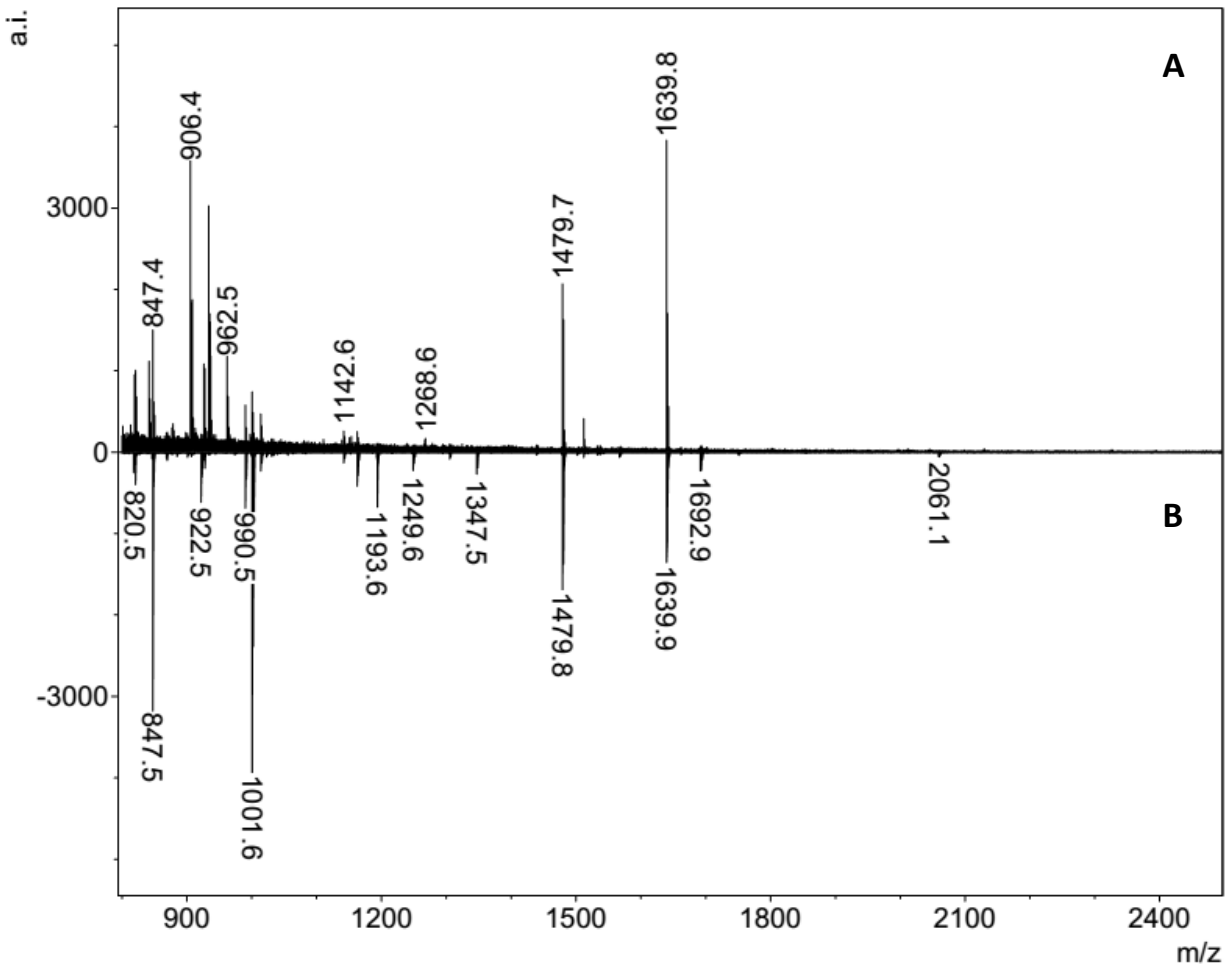
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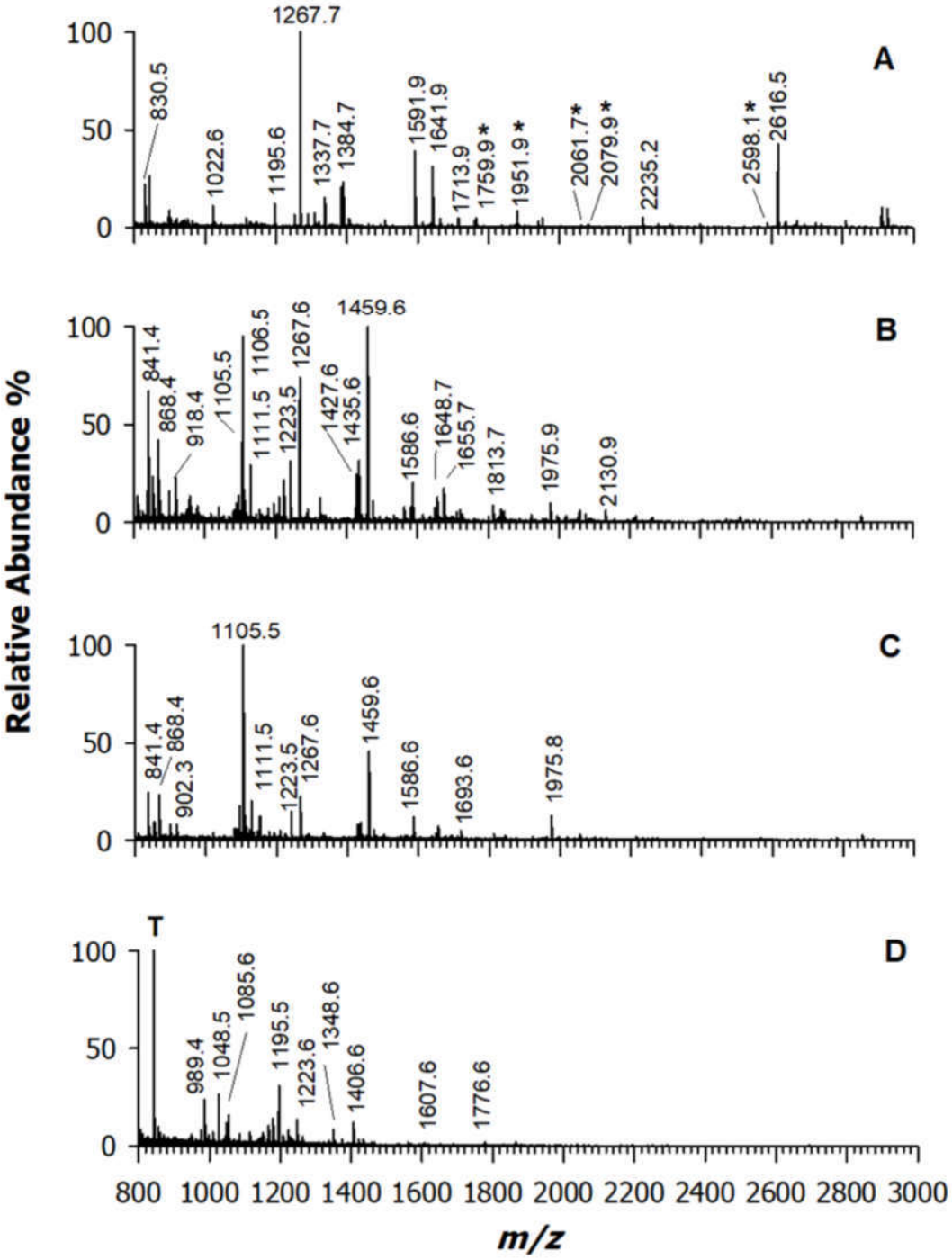
Figure 1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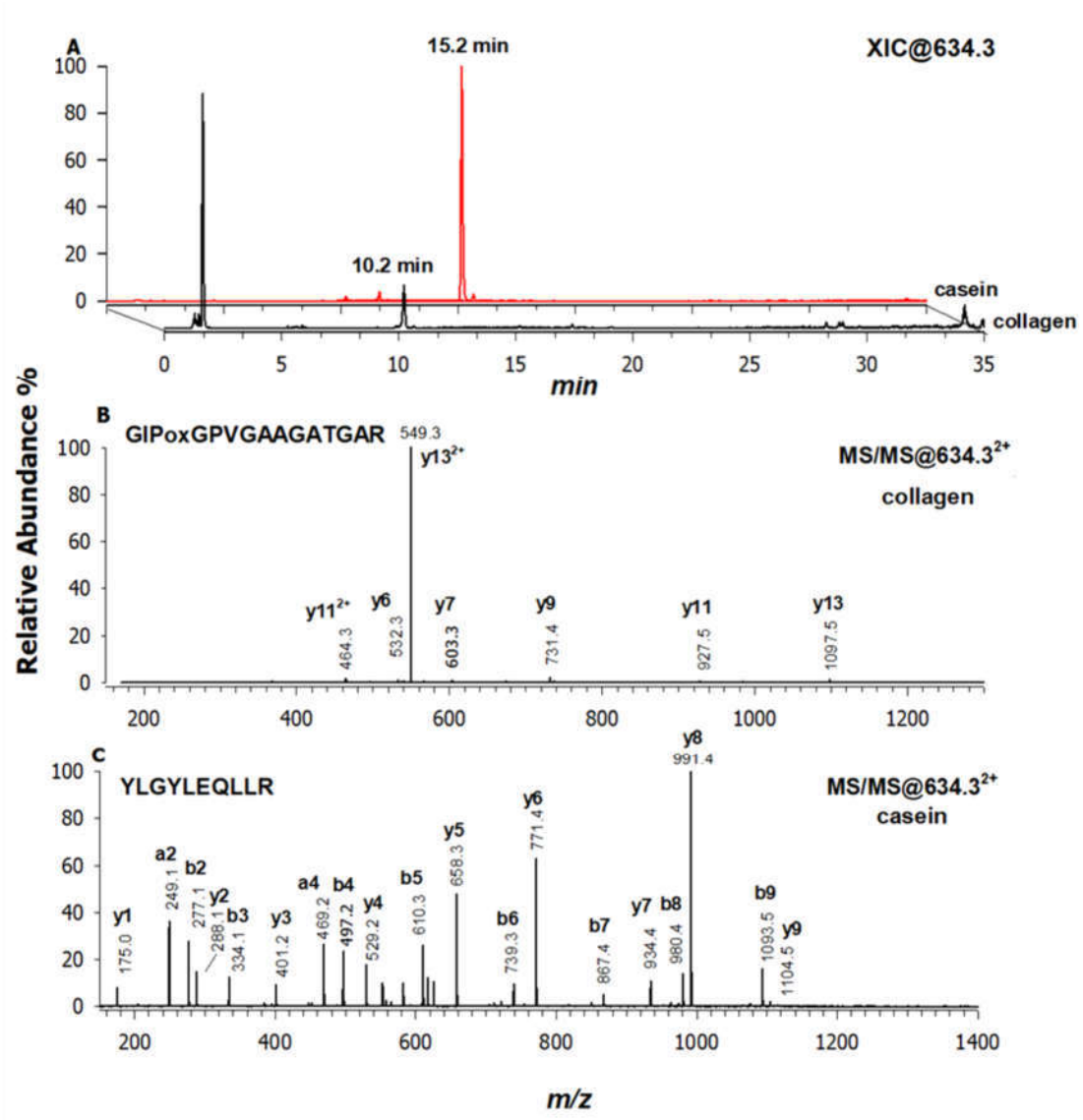
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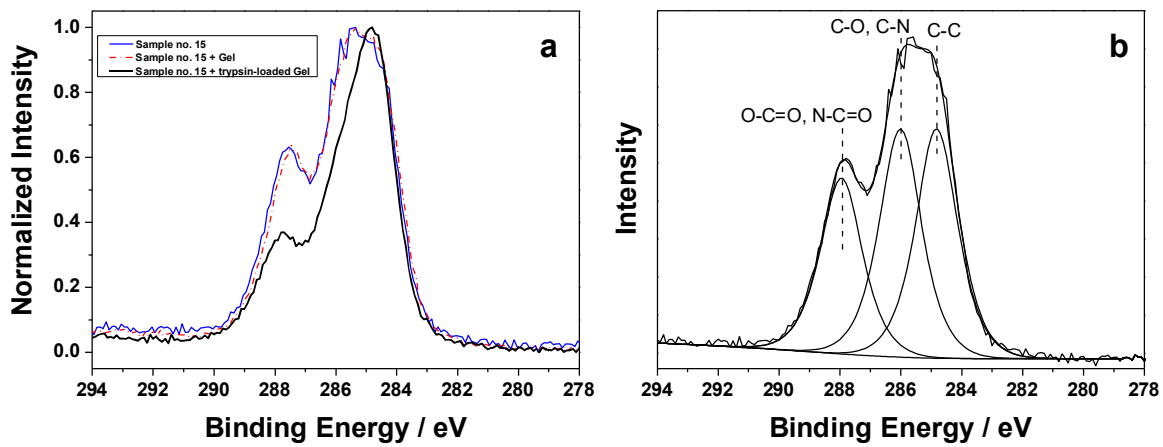
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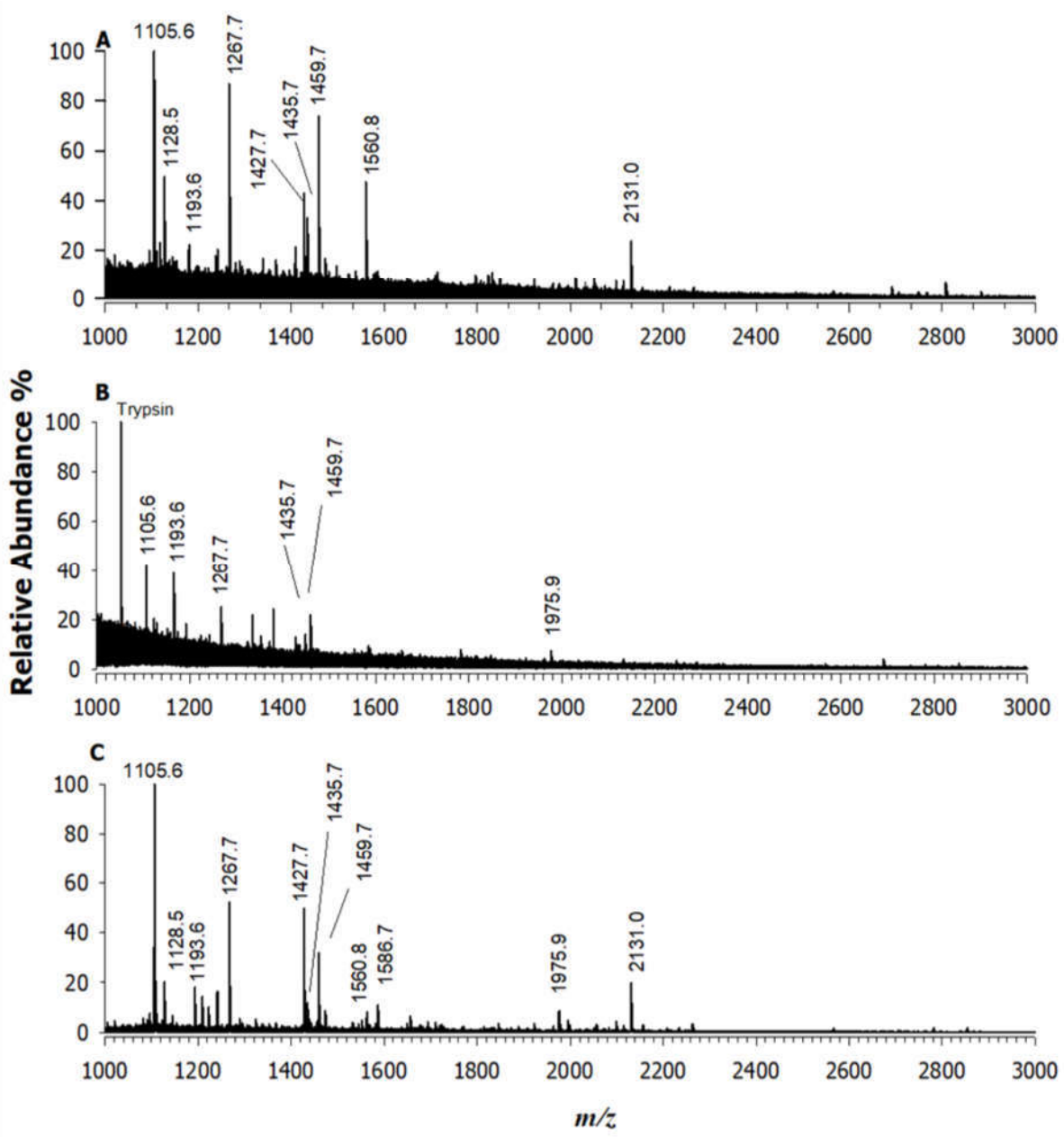
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1435.7
1459.7
1267.7
1975.9
C
1105.6
1435.7
1267.7
1427.7
1459.7
1128.5
1193.6
1560.8
1586.7
1975.9
2131.0
Relative Abundance %
m/z

Figure 5

A

B

Figure 6

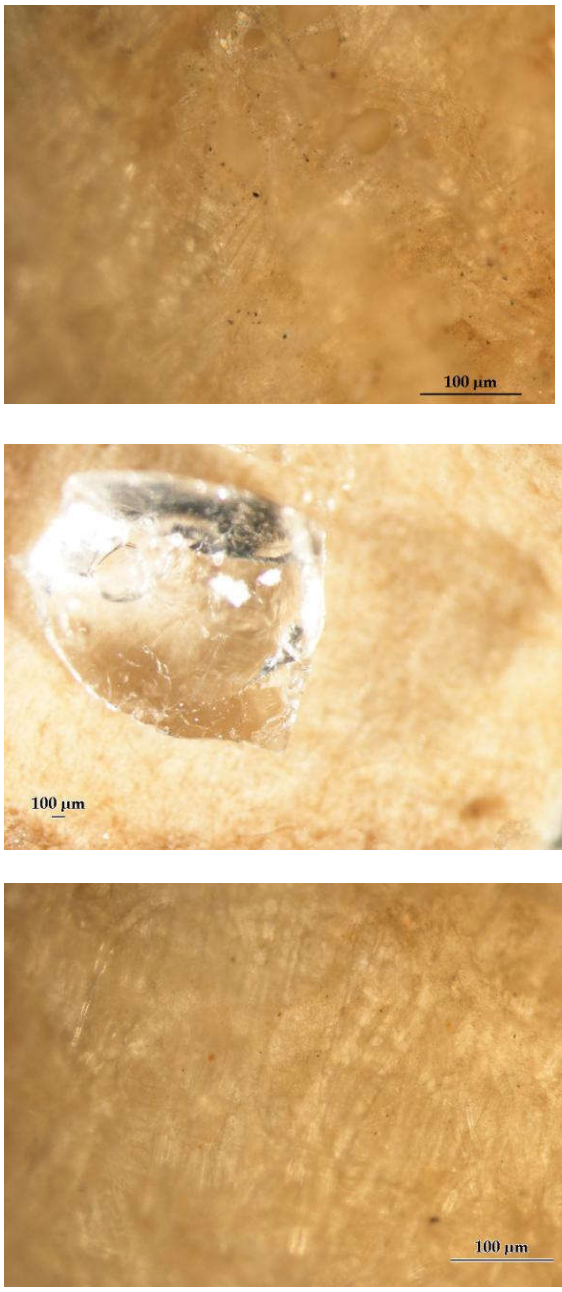

Figure 7