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In-situ hydrogel extraction with dual-enzyme digestion of proteinaceous binders: the key for reliable mass spectrometry investigations of artworks

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Abstract

A novel strategy based on *in-situ* dual-enzyme digestions of paint layers proteinaceous binders is introduced for faster and more confident identification, ensuing a bottom-up proteomics approach by MALDI-TOF mass spectrometry (MS). *In-situ* sampling/extraction of proteinaceous binders using small pieces of a hydrophilic gel, previously loaded with trypsin and chymotrypsin proteolytic enzymes, was successfully exploited. Along with minimal invasiveness, the synergy of both enzymes was very useful to increase the number of annotated peptide peaks with their corresponding amino acid sequence by database search and subsequent MALDI-TOF/TOF analysis. The protocol was initially aimed at enhancing the identification of egg-based binders and then validated on fresh and aged model pictorial layers; an increased protein coverage was significantly attained regardless of the used painting binders. Optical microscope images and spectrophotocolorimetry analysis evidenced that the painting layers were not damaged or altered at all because of contact/sampling without leaving hydrogel residues. The proposed protocol was successfully applied on a painted altarpiece "Assumption of the Virgin" dated XVI century and on an angel statue of the Nativity crib dated XII century, both from the Altamura's Cathedral (Apulia, Italy). The occurrence of various protein binders of animal origin was easily and reliably ascertained.

1. Introduction

Identification of organic binders in works of art is noteworthy to characterize the artist's preferences, to explore the painting framework, and, most importantly, to choose the best conservation and restoration practices [1–3]. The widely used in cultural heritage are proteinaceous binders of animal origin such as eggs, milk, skin, bones, offal, etc. Non-invasive methods are, without doubts, preferred to examine precious and exclusive artworks. Indeed, infrared (IR) spectroscopy coupled to principal component analysis has been commonly used to investigate cultural heritage samples and to classify binding media[4,5]. Diffuse reflectance spectrophotometry[6] and laser ablation surface-enhanced Raman micro-spectroscopy[7,8] have been applied as well. Despite the great performance of novel portable instruments, the analysis is still challenging, and only preliminary or indicative results are currently described[6]. Accurate identification of proteinaceous binders in artwork samples can be achieved by mass spectrometry (MS)[1,9] especially coupled to soft ionization techniques such as electrospray (ESI) and matrix-assisted laser desorption/ionization (MALDI)[10] following classical bottom-up proteomics approaches [11–14]. For a better understanding of human history or more, proteomics methods have been successfully employed for the identification of protein binders in historical[9,15–17], Renaissance[18–20] and mural paintings [21], polychrome pottery [22], parchment documents [23,24,25], collagen species discrimination [26–28,29], or even from vessels [30,31]. To ensure artwork preservation and avoid required micro-sampling, the recent investigation was focused on minimally invasive sampling protocols[2,32] preliminary to MS analysis[17,33–35]. Interesting approaches have been recently suggested based on the use of various films which can be functionalized with strong cation/anion exchange resins [32,36] or with fungal proteins *Vmh2* hydrophobin for trypsin immobilization [37] and directly applied onto the surface of artworks for *in-situ* protein digestion. Very recently, a simplified protocol of *in-situ* sampling/extraction and

protein digestion has been developed from our group by using a hydrophilic gel named pHEMA/PVP (i.e., poly(2-hydroxyethyl methacrylate)/poly(vinylpyrrolidone) loaded with trypsin[38]. The sampled area was greatly minimized (i.e., < 10 mm^2) and protein digestion was performed in less than thirty minutes. We are aware that this user-friendly protocol is willing to further improvements, especially for the identification of egg-containing binders that were not conceivable by PMF but by recurring to reversed-phase liquid chromatography ESI-MS/MS, so lengthening the global time of analysis. In fact, the extraction/digestion of proteinaceous egg binders is particularly challenging due to polymerization/degradation processes occurring during ageing[39], post-translational modifications [40], and intrinsic inhibition action towards trypsin [41] due to the long hydrophobic regions without the cleavage sites of arginine or lysine. Thus, the number of trypsin digested peptides was relatively low, greatly reducing the protein coverage, which remains a first demand for a reliable identification. A likelihood to maximize sequence coverage can be the use of multi-enzyme strategies combining proteases in parallel or in sequence as recently proposed for other samples[42,43]. While multi-enzyme systems were initially applied to characterize hydrophobic proteins in membranes[44,45] and non-alkylated proteins in barley malt[46], the parallel combined use of multi-enzymes was successfully employed by Nardiello et al.[47,48] as a direct route to improve protein identifications of food frauds. Here, a hydrophilic gel loaded with a couple of proteolytic enzymes for *in-situ* digestion of painting binding media in artworks is proposed. The simultaneous gel-immobilization of both trypsin and chymotrypsin is an efficient step forward toward a minimally invasive and sustainable tool in the field of cultural heritage diagnostics and is highly preferable to the single enzyme protocol since it assures higher sequence coverages with a very confident proteinaceous binder identification.

2. MATERIALS AND METHODS

2.1. Protocol on pictorial and historical samples. The used chemicals are reported in Supporting Information. Paint replicas were made by dispersing inorganic pigments in milk casein, egg yolk, bovine, and rabbit collagen binders; four years aged replicas were also examined. The wet hydrogel pHEMA/PVP [49,50] was initially cut in small pieces (3 mm x3 mm) which were weighed and left to dry at air ambient until they have lost at least 20% in weight. Later, each small piece of the hydrophilic gel was immersed in a water solution made up of 50 µL of trypsin (20 pmol/mL) and 50 µL of chymotrypsin (20 pmol/mL) for 30 minutes to allow the enzymes to soak into the hydrogel. In-situ multi-enzyme digestion of protein binders was carried out by putting the loaded hydrogel pieces onto the replica surface for 30 min. Upon removal, each hydrogel was immersed in 100 µL of a solution of 70:30 % ACN: H_2O with 0.1% TFA and then left for 15 min in an ultrasonic bath to allow the quantitative release of peptides. The resulting solution was vacuum dried to preconcentrate the sample and then analysed by MALDI MS(/MS). For in-situ analyses of real samples, the dried gel was kept in a vial containing both enzyme's solution and transported the to the site of sampling. If the sampling requires longer time, it is recommended to leave the dried gel and the enzyme solution apart and to wet the gel just half an hour before the application on the painted surface. MALDI instrumentation, microscopy, and colorimetric measurements are described in the Supporting information.

2.3. Database searching. The peptide mass fingerprinting (PMF) obtained by MALDI MS was processed with the Protein prospector MS-Fit tool (Regents of the University of California). Proteins were identified using the SwissProt database with *Bos taurus, Oryctolagus cuniculus,* or *Gallus gallus* as taxonomy restriction. Search parameters for MS analysis were the following: peptide mass tolerance 100 ppm, enzyme slymotrypsinFYWKR, allowed missed

cleavages up to 3. No fixed chemical modification was inserted, but oxidation of Met residues, hydroxylation of Pro, and phosphorylation of Ser, Thr, and Tyr were considered as variable modifications. The acquired MS/MS data set was processed by mMass™ 5.5.0 using the MS-Tag tool; a tolerance of 0.5 Da was set for the precursor and fragment ions.

3. RESULTS AND DISCUSSION

3.1 Method development on a standard protein sample

The sampling performance of a dual enzyme-loaded hydrophilic gel was evaluated by digesting a standard solution of bovine serum albumin (BSA), chosen as a model protein binder and comparing results by those obtained by a trypsin-loaded gel. A glass slide was prepared by drop-casting a BSA solution (10 μg/mL) with and without mixing calcium carbonate as a pigment. In parallel, both trypsin- and trypsin/chymotrypsin-loaded hydrogels were placed onto the dried surface. Upon completing the protocol, the resulting peptide mixtures were explored by MALDI-TOF MS (Fig. S1). In both cases, BSA was confidently identified as first output: the *in-situ* dual-enzyme digestion exhibited a sequence coverage of 42.3% being almost twice that 21.1% obtained by the trypsin-loaded hydrogel (Table S1). This simplified model sample demonstrated that the combination of trypsin with chymotrypsin guarantees a greater peptide coverage, most likely because both these endopeptidases work in synergy, thus increasing the cleavage of peptide bonds at the C-terminal of Lys and Arg along with Tyr, Phe, and Trp.

3.2 Dual-enzyme protocol application to fresh and aged paint models

The ensuing step was to apply the *in-situ* dual-enzyme digestion and sampling protocol to paint models blended with various pigments. Figure 1 displays typical MALDI-TOF spectra

obtained by *in-situ* hydrophilic gel dual-enzyme digestion of paint replicas composed of caseins (A), rabbit collagen (B), chicken egg yolk (C) mixed with calcium carbonate. For comparison, the same samples were tested with both hydrophilic gels trypsin-loaded and bare as well; as expected, no MALDI MS signals were detected in the latter case. While the tryptic digestion of caseins provides coverages of 43% for α-s1-casein, 30.2% for α-s2-casein, 32.1% for β-casein and 14.7% for k-casein, the proposed dual-enzyme remarkably increased these data up to 52.3%, 69.8%, 59.4%, 35.8 %, respectively, and 21.4% for casein kinase isoform δ not at all detected by the trypsin digestion (see Table 1 and Table S2). Interestingly, peak signals arising from mono- and multi-phosphorylated peptides were even evidenced in both digested samples. Apparently, this may be due to an enrichment process of polar molecules, namely phosphopeptides, by the hydrophilic gel due to its binding ability. As an example, Figure S2 shows the MS/MS spectrum of the *m/z* 2061.67 from a casein-based paint sample that was annotated as the phosphopeptide (K)FQSEEQQQTEDELQDK(I) of milk β-casein. In the case of rabbit collagen, the coverages obtained either by single or dual-enzyme approaches were comparable, perhaps because of the prevailing presence of Pro, Gly, and Ala that do not undergo specific tryptic or chymotryptic cleavage. However, the *in situ* dual-enzyme protocol unveiled spectra with a systematically improved S/N ratio (*vide infra*). Furthermore, the great difference in protein identification was experienced for egg-based binders where coverages were increased up to three-fold using the *in-situ* dual-enzyme digestion protocol.

A critical matter to be challenged in ancient samples is represented by a drastically reduced proteome identification due to ageing processes, microbial and chemical degradation,[51,3,39] often accelerated by pigments. The *in-situ* trypsin/chymotrypsin-loaded hydrogel was tested on painting models (rabbit collagen+vermillion, whole egg+vermillion, and caseins+white zinc) aged up to four years under natural light and moisture (Figure S3).

Compared to fresh samples, the coverages were considerably lowered mainly in the case of trypsin loaded hydrogels (See Table 1); this outcome may be due to crosslinked egg white proteins, resistant to trypsin digestion. Conversely, the *in-situ* dual-enzyme protocol of aged samples provided higher protein coverages, namely double or triple compared to trypsin alone. Some selected peptides were subjected to tandem MS analysis for confirmation, and fragmentation spectra were searched by on online databases and manually validated (See for instance, Fig. S4-S6). Another inspected issue of the present protocol was to prove the dose of invasiveness. To this aim, replicas were analyzed by optical microscopy and colorimetry; the relatively short time of contact between the hydrogel and the sampled surface ensured the present protocol as very effective in preserving both the painting surface and the pigments as well (*vide infra*, and see Fig. S7, S8). Nonetheless, the absence of any minimal surface damaging of paint layers and pigment modification caused by the hydrogel sampling contact was validated.

3.3 Historical samples

The protocol was then applied to historical samples represented by a painted altarpiece of the XVI century (size 4.9 m x 3.36 m) attributed to Leonardo Castellano and an angel statue of a Nativity both exposed in the Cathedral Church of Altamura (Apulia, Italy). Figure S9 and S10 report the images of artworks with historical details and sampled points labelled as A, B, C, D, E.

After the *in-situ* dual-enzyme digestion, MALDI spectra were registered (Figure 2) and the *m/z* values were inserted in the database for PMF search. The spectra were also manually compared to those of the replicas and some selected peptides were subjected to MS/MS analysis (See Figures S11, S12) as a confirmation. For example, the ion at *m/z* 1267.7 could be

formed both from the collagen-α-2(I) chain or α-s1-casein; MALDI-TOF/TOF spectrum (see Figure S11) and manual validation permitted to identify the peptide YLGYLEQLLR of α-s1-casein. As far as the peak signal at *m/z* 1105.6 (Figure S12), it was annotated as peptide GVQGPP(Oxidation)GPAGPR from bovine collagen α-1(I)-chain. The detailed outcomes of the assigned peptides are listed in Tables S3, S4. As can be seen, bovine glue, egg, and casein were reliably identified as binders in the altarpiece sample and the angel statue. The *in-situ* hydrogel sampling with dual-enzyme digestion was very useful to identify peptides from collagen α-1 (I), collagen α-2 (I), collagen α-1 (II), α-S1-casein, α-S2-casein, k-casein, beta-casein, vitellogenin-1, vitellogenin-2, vitellogenin-3, ovalbumin, and ovotransferrin. Note that three different points were investigated in parallel on both the Nativity statue and altarpiece painting by using trypsin-loaded hydrogel. As expected, most peptides from collagen and casein binding media were easily recognized while a very few peptides from egg yolk were barely detected. These findings on real samples confirm the difficult task in digesting egg proteins by trypsin without using at least a micro-sampling.

Conclusions

A simple non-invasive protocol for *in-situ* digestion of proteinaceous paint binders using a hydrophilic gel soaked with a dual-enzyme is proposed. Minimal or even absent invasiveness is guaranteed by the very limited dimension (less than 10 mm^2) of the hydrophilic gel used for the sampling/digestion step and from the short time of contact between gel and sample surface. All these inherent advantages were successfully demonstrated. In our view, the in-situ digestion of binding media using a hydrophilic-loaded gel with a dual-enzyme will be very useful to examine a great deal of worldwide artworks. Due to the overall simplicity, the sampling step could be carried out even by restores or other personnel without on-topic

222 professional ability.

223

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231

232 **Conflict-of-interest**

233 The authors declare no conflict-of-interest.

234

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331 **Table 1.** Identified proteins and sequence coverage results for fresh and aged paint replicas by
332 using single or dual-enzyme digestions. Calcium carbonate as a pigment was employed in
333 freshly prepared paint replicas. Aged replicas are casein (aged 2 years) mixed with white zinc,
334 rabbit glue (aged 4 years) and whole egg (aged 4 years) each mixed with vermillion.
335

Sample	Identified Proteins	Coverage (%)			
		Trypsin	Trypsin & Chymotrypsin	Trypsin	Trypsin & Chymotrypsin
		Fresh		Aged	
Casein	Alpha-S1-casein	43.0	52.3	25.2	43.9
	Alpha-S2-casein	30.2	69.8	19.4	55.9
	Beta-casein	32.1	59.4	17.9	53.6
	Kappa-casein	14.7	35.8	-	47.4
	Casein Kinase I, δ	-	21.4	-	15.2
Rabbit glue	Collagen alpha-1(I)	23.5	27.5	11.5	11.9
	Collagen alpha-1(II)	13.9	20.7	11.6	11.8
	Collagen alpha-1(III)	21.9	28.5	8.6	8.8
	Collagen alpha-2(I)	20.9	27.9	9.6	10.1
Egg[1]	Vitellogenin-1	5.2	28.7	6.3	27.6
	Vitellogenin-2	8.6	36.8	4.9	27.4
	Vitellogenin-3	-	42.9	-	17.9
	Apovitellenin-1	25.2	97.2	-	-
	Apolipoprotein A-I	36.4	47.0	-	48.9
	Apolipoprotein B[2]	17.3	54.0	-	41.8
	Ovotransferrin[3]	-	-	-	37.3
	Ovomucoid[3]	-	-	-	53.8
	Ovalbumin[3]	-	-	-	30.6

336 [1] Egg yolk in fresh samples and whole egg in aged samples; [2] Fragment of Apolipoprotein B; [3] Proteins present
337 only in the egg white.
338

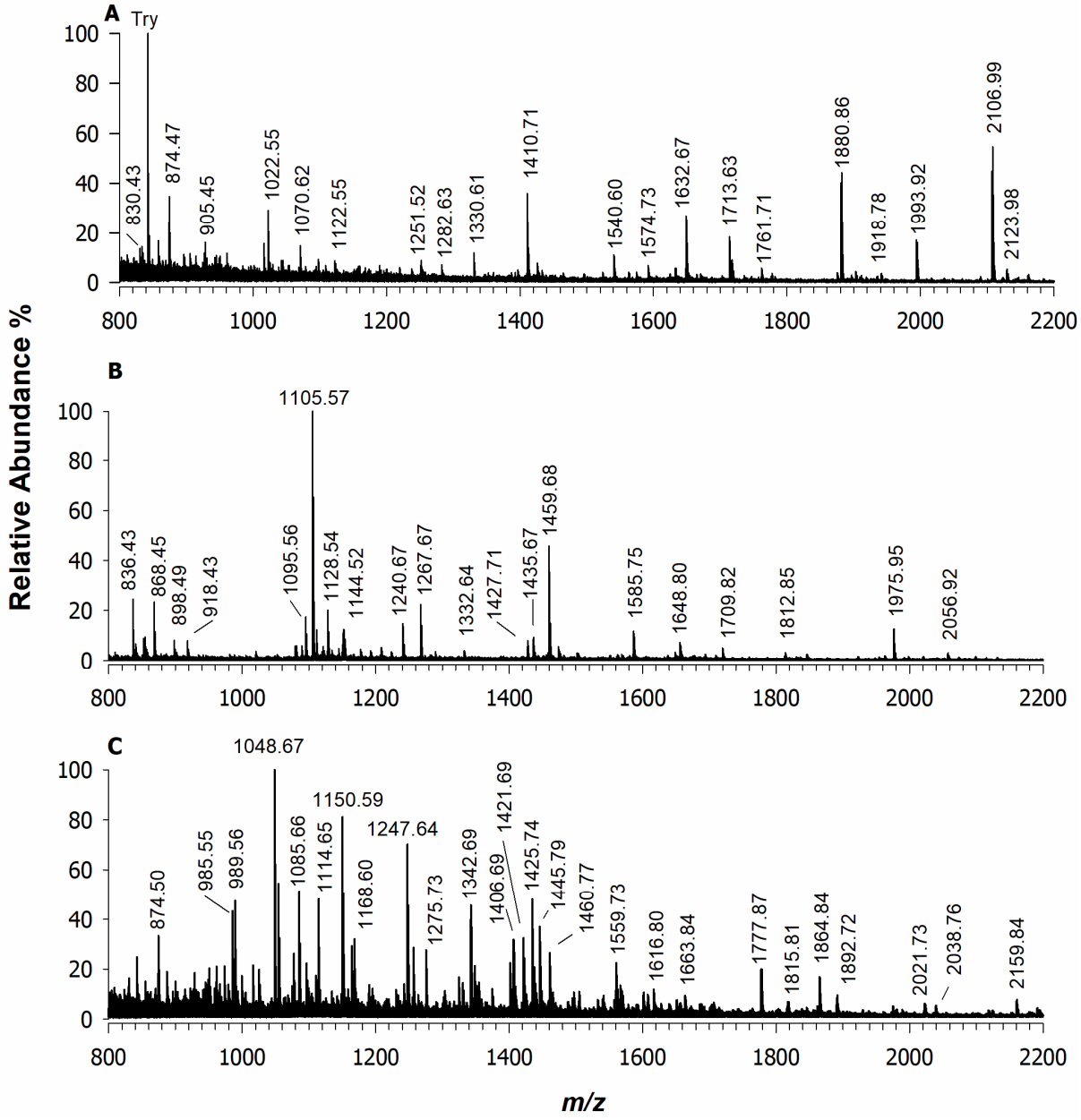

Figure 1. MALDI-ToF mass spectra of an *in-situ* chymotrypsin/trypsin-loaded hydrogel digestion of paint replicas from caseins (A), rabbit collagen (B) and egg yolk (C) and calcium carbonate as a pigment. All main peaks of spectra are listed in Table S2.

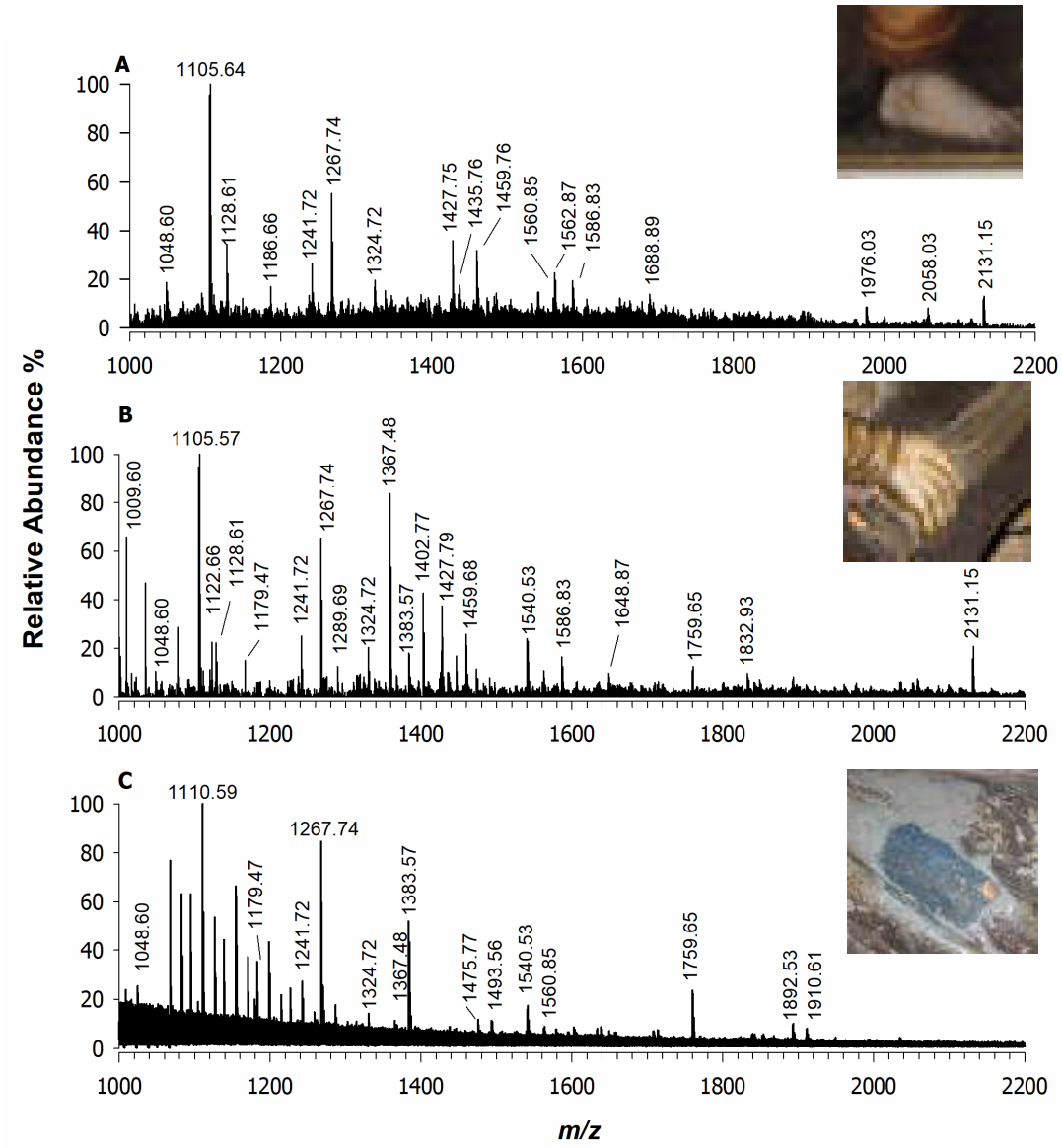

Figure 2. MALDI-ToF mass spectra of *in-situ* chymotrypsin/trypsin-loaded hydrogel digestion performed on historical samples on points B (plot A), C (plot B) and D (plot C). Identified binders are reported in Table S3 while all main peaks of spectra are detailed in Table S4.