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Identification of Proteins in Renaissance Paintings by Proteomics

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The presented work proposes a new methodology based on proteomics techniques to identify proteins in old art paintings. The main challenging tasks of this work were (i) to find appropriate conditions for extracting proteins from the binding media without protein hydrolysis in amino acids and (ii) to develop analytical methods adapted to the small sample quantity available. Starting from microsamples of painting models (ovalbumin-based, which is the major egg white protein, and egg-based paintings), multiple extraction solutions (HCl, HCOOH, NH_3, NaOH) and conditions (ultrasonic bath, mortar and pestle, grinding resin) were evaluated. The best results were obtained using a commercial kit including a synthetic resin, mortar and pestle to grind the sample in an aqueous solution acidified with trifluoroacetic acid at 1% with additional multiple steps of ultrasonic baths. The resulting supernatant was analyzed by MALDI-TOF in linear mode to verify the efficiency of the extraction solution. An enzymatic hydrolysis step was also performed for protein identification; the peptide mixture was analyzed by nanoLC/nanoESI/Q-q-TOF MS/MS with an adapted chromatographic run for the low sample quantity. Finally, the developed methodology was successfully applied to Renaissance art painting microsamples of ∼10 μg from Benedetto Bonfigli's triptych, The Virgin and Child, St. John the Baptist, St. Sebastian (XVth century), and Niccolo di Pietro Gerini's painting, The Virgin and Child (XIVth century), identifying, for the first time and without ambiguity, the presence of whole egg proteins (egg yolk and egg white) in a painting binder.

Art paintings have traveled through the ages despite their composition made of easily damaged organic compounds. All the paints are composed of the same basic components: pigments and binders.[1−3] The component we perceive as color is pigment, which is mostly typically a fine powder of inorganic material made from ground stone or synthetic materials. The pigment is dispersed in a binder, which allows it to be spread out and which binds the individual powder granules together and to the surface to which the paint is applied. Traditionally, multiple coats of 10−50 μm compose the old paintings. A preparation coat is spread on the support in order to smooth it over, a white subcoat is placed over it for the artists' drafts, and finally, the pictural coat is located at the top, covered up by the varnish. Binders, present in the pictural coat, are the major organic compounds used. Over centuries, artists have evaluated many of them like eggs, milk, or glue. These binders were used alone, mixed together, but also mixed with oils or with other organic materials. Due to the sample amount and complexity, the identification of the binder proteins, representing fundamental information for art historians and restorers, is defying analytical techniques.[4,5] Different methods have been proposed in the literature. The first set of techniques is based on protein detection[6,7] with organic dyes as the Naphthol Blue Black-based method[8,9] or with an immunodetection-based method, but so far, this last method was rarely employed on old paintings.[10,11] Another set of techniques employed is based on chromatography and, more precisely, on the determination of amino acid composition, which results from the hydrolysis of the proteins present in the sample. Amino acids have been identified in the past using thin-layer chromatography,[12−14] but it does not

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offer the specificity required to monitor the amino acids needed for proper identification of proteinaceous material. One of the first analytical technique used for amino acid quantitative analysis was gas chromatography,[15−21] which remains the most used technique to determine proteins in painting binding media. However, a study proposed by Colombini and co-workers[15] highlights the need to use aged painting models for comparative studies. Other studies have demonstrated the potentiality of pyrolysis gas chromatography coupled to mass spectrometry[22−24] to characterize proteinaceous binding media, but the comparison of the analyzed sample spectra versus painting model spectra is generally a difficult task due to high sample deterioration during the pyrolysis. Finally, a high-performance liquid chromatography system coupled with UV−visible spectrophotometry[25−28] has been used for quantitative amino acid analysis in art objects but remains highly dependent on the sample amount. The last set of techniques used for binder analysis are infrared[29−31] and Raman spectroscopy[32−34] providing some encouraging data. All these cited analytical methods are not destructive but give few quantitative and structural information and are not sufficiently distinctive to allow precise protein identification. Recently, mass spectrometry, mainly used for biological sample analysis,[35,36] was introduced for painting analysis generating promising results.[5,37−42]

This work presents a new methodology based on nanochromatography and tandem mass spectrometry to identify without ambiguity proteins in art painting samples. The first part of the described work presents the research of appropriate conditions for protein extraction from paint binding media without protein hydrolysis in amino acids. To experiment with the various extracting solutions, we have included a formulation work to obtain painting models containing (i) one egg protein and (ii) the whole egg proteins. The efficiency of the extraction procedures was evaluated by MALDI-TOF mass spectrometry. The next step was to identify the extracted proteins; consequently, we decided to optimize and to adapt the various steps of the proteomics analysis (enzymatic hydrolysis, peptide purification, tandem mass spectrometry analysis, protein databank interrogation) to the very low amount of material to analyze (~10 μg of sample). Finally, the methodology was successfully applied to two Renaissance art paintings: the Benedetto Bonfigli triptych, The Virgin and Child, St. John the Baptist, St. Sebastian (Italian School, XVth century), and the Niccolo di Pietro Gerini painting, The Virgin and Child (Italian School, XIVth century).

EXPERIMENTAL SECTION

Reagents. Ovalbumin (hen egg albumin, grade V) and all other chemicals used (lead white $2PbCO_3$, $Pb(OH)_2$; linseed oil; tris(2-amino-2-(hydroxymethyl)-1,3-propanediol); guanidine hydrochloride; dithiothreitol; iodoacetamide; hydrogen chloride; trifluoroacetic acid; ammonia; sodium hydroxide) were obtained from Sigma (l'Isle d'Abeau Chesnes, France). Trypsin was purchased from Promega (Charbonnières, France). Microdialyzer units were supplied by Pierce (Rockford, IL). Deionized water was obtained from Millipore cartridge equipment (Bedford, MA). The molecular grinding resin was obtained from Genotech (St Louis, MO). The fresh hen eggs were purchased at the market.

Renaissance Art Paintings. Renaissance art painting samples were obtained from the Laboratoire des Musées de France (Louvre, Paris). The Benedetto Bonfigli triptych, The Virgin and Child, St. John the Baptist, St. Sebastian (Italian School, XVth century), is shown in the Petit Palais Museum in Avignon, France. The microsample was excised from the St. Sebastian left arm for proteomic analysis. The Niccolo di Pietro Gerini painting, The Virgin and Child (Italian School, XIVth century), is shown in the Petit Palais Museum in Avignon, France. The microsample was excised from the red dress of The Virgin. The samples were too small and too precious to be weighed; consequently, their weights were estimated by measuring their size using a microscope and assuming a density of 1.

Formulation of Paint Media Emulsions. (1) Painting Model Using a Single Protein. Ovalbumin (10% w/v) and lead white (50% w/v) were dissolved in water. Oil emulsion was obtained by agitation of linseed oil (20% v/v of the final volume) with the mixture containing the protein and the pigment. The emulsion was spread as a thin film on several microscope glass slides and evaporated at 40 °C. The samples were conserved at room atmosphere without special precautions.

(2) Egg Painting Model. Lead white (50% w/v) was dissolved in water. Egg/oil emulsion was obtained by agitation of linseed

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oil (20% v/v of the final volume) with egg white (8% v/v of the final volume) and egg yolk (8% v/v of the final volume). The aqueous solution was added to the egg/oil emulsion. The emulsion was spread as thin film on several microscope glass slides and evaporated at 40 °C. Samples were naturally aged by keeping them at room light, ambient temperature and humidity.

Sample Preparation. (1) Extraction. A microsample of 200 μg was excised from the model painting and crushed with a single-use synthetic resin (guaranteed free of proteins, RNA, and DNA) in a 50-μL solution containing 1% trifluoroacetic acid. This extract was dialyzed against deionized water in order to separate the proteins from the inorganic pigments. The mixture (solution and painting pieces) was subjected to ultrasonic baths (3 × 15 min). The supernatant was obtained after centrifugation of the mixture (2 min at 2000*g*).

(2) Enzymatic Hydrolysis. The extracted proteins were denatured[37] in 80 μL of 100 mM Tris-HCl, pH 7.25 by guanidine hydrochloride at 6 M final concentration. The sample was flushed with nitrogen and incubated for 30 min at 50 °C. Then it was reduced with 12 mM dithiothreitol (final concentration) and incubated at 50 °C for 4 h. The alkylation[37] was realized with iodoacetamide at 300 mM final concentration, and the reaction was carried out in the dark at room temperature for 30 min. This sample was dialyzed against deionized water overnight. Trypsin was added in excess (1 ng in 50 μL/10 μg of microsample) in the ovalbumin-based, egg-based, and old paintings protein extracts. The mixture was incubated at 37 °C for one night.

Sample Analysis and Database Search. The protein spectra and the peptide mass fingerprint spectra were obtained using a MALDI-TOF Voyager DE-STR mass spectrometer (Perseptive Biosystems, Framingham, MA) equipped with a 337-nm nitrogen laser. All spectra were obtained with the delayed extraction technology, linear mode for proteins analysis and reflector mode for peptides analysis. Proteins were analyzed using a matrix containing 5-methoxysalicylic acid and 2,5-dihydroxybenzoic acid (1:9) (10 mg/mL of methanol/water (v/v)); the acceleration voltage was fixed at 20 kV, the grid potential at 93%, and the extraction delay at 1200 ns. Peptides were analyzed using a solution of 2,5-dihydroxybenzoic acid (10 mg/mL of methanol/water (v/v)); the acceleration voltage was fixed at 20 kV, the grid potential at 66%, and the extraction delay at 200 ns. A total of 300 laser shots were accumulated for protein spectra and 200 for peptide spectra. For protein spectra, external calibration was used (α-lactalbumin, bovine, 16 247 Da; glutamate dehydrogenase, bovine, 61 512 Da; transferrin, human, 84 901 Da). Peptide spectra were calibrated using tryptic hydrolysis ion peaks as internal standards (842.5100; 1045.5642; 2211.1046). All searches using peptide mass fingerprint spectra were carried out using the MS-FIT program of Protein Prospector (http://prospector.ucsf.edu/) and the MASCOT program (http://www.matrixscience.com) with the NCBI database. The species was restricted to *Gallus gallus* for peptide mass fingerprint experiments. No restrictions were placed on isoelectric points and mass of the proteins; the tolerance was set to 50 ppm.

The peptide sequence spectra were obtained using nanochromatography (LC-Packings, Dionex) on-line with a Q-STAR Pulsar mass spectrometer (Perseptive Biosystems). A LC Packings Famos autosampler aspirated 1 μL of the peptide solution into a 10-μL sample loop using 98:2 water/acetonitrile, 0.1% formic acid as the transfer reagent. A Switchos pump was used to concentrate and desalt the sample on a C18 nanoprecolumn. The precolumn is connected with a C18 Pepmap column, and peptides were eluted at 200 nL/min using an Ultimate LC system. The elution was performed using a gradient of solvent A (95% H_2O, 5% ACN, 0.1% HCOOH) and solvent B (95% ACN, 5% H_2O, 0.1% HCOOH): solvent B content was increased from 5 to 95% in 60 min, was kept for 20 min at 95%, and finally was decreased to 5% in 1 min. The column was allowed to reequilibrate for ~20 min before another run. The LC system is on-line with the ESI-Qq-TOF mass spectrometer, which was equipped with the nanoelectrospray source. Detection was carried out in positive mode with a 2.2−2.5 kV HV, and the spectra were acquired on a m/z 300−2000 range, the nanoLC setup being coupled to the mass spectrometer. The data acquisition was controlled by software Bioanalyst (Applied Biosystems) in IDA mode with a detection cycle time of 10 s. Fragmentation was triggered for any doubly, triply, and four charged species reaching an intensity threshold of 30 counts with a relative collision energy calculated by taking account of the selected species charge state. Once selected and fragmented, eluted species were excluded from the MS/MS selection (±0.3 Da mass window exclusion around the ion m/z value) for 10 s. Tryptic autolysis ion peaks were used as internal standards for the spectra calibration. The spectra corresponding to peptide sequences were submitted to the ProID software (Applied Biosystem), which contains local NCBI protein database for protein identification. The protein identifications were realized using second software, MASCOT (http://www.matrixscience.com), to confirm the results. No restrictions were placed on species or mass for the MS/MS experiments. The mass tolerance of precursor ions and fragment ions was set at 0.1 Da. All MS/MS spectra leading to protein identification were manually checked to verify sequence assignments.

RESULTS AND DISCUSSION

Methodological Development. Considering that proteins are in a complex medium and highly denaturated for years, the two major difficulties of this work were (i) to find appropriate extracting conditions of proteins from the binding medium without hydrolysis in amino acids and (ii) to develop an analytical method closely adapted to the small sample amount available.

An approach based on painting models was investigated to evaluate the different steps of the analysis: protein extraction, protein extract hydrolysis, peptide analysis, and protein identification. Painting models were obtained by an extensive formulation optimization using the major components of old painting: pigments and binders. Egg and linseed oil largely influenced the different painting techniques until the development of synthetic media, so, the model painting formulation was based on these two components. Two painting models were formulated, one based on the major egg white protein, ovalbumin, to facilitate the evaluation of the efficiency of the various protein extraction solution, and the second based on whole egg, to evaluate the efficiency of the proteomics approach, i.e., the identification of proteins extracted from a complex medium. The painting models are composed of lead white (50% w/v) as pigment, linseed oil (20% v/v), and ovalbumin (10% w/v) or egg (8% v/v egg white and 8% v/v egg yolk) as binders.

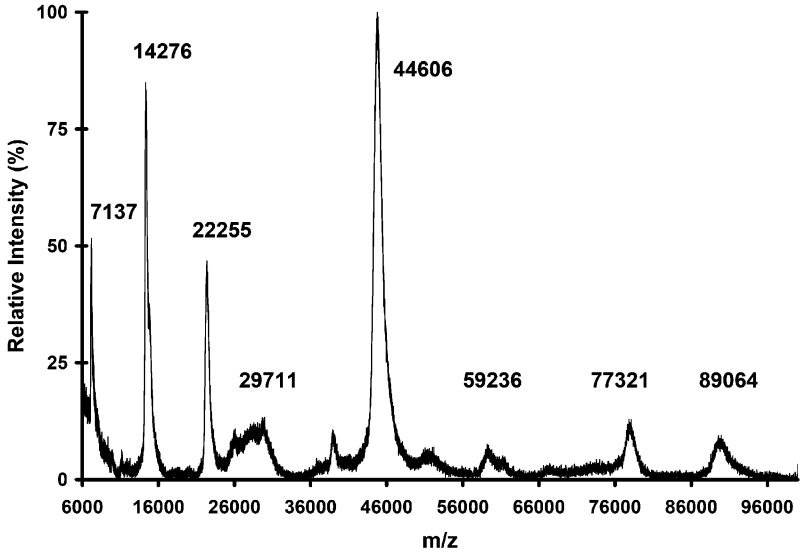

Figure 1. MALDI-TOF MS spectrum in linear mode of commercial protein ovalbumin presenting its monocharged ion (44 606 Da), its doubly charged ion (22 255 Da) and its dimer (89 064 Da), but also presenting the monocharged ion (29 771 Da) and the dimer (59 236 Da) of ovomucoid, the monocharged (14 276 Da) and the doubly charged ion (7137 Da) of lysozyme, and the monocharged ion (77 321 Da) of conalbumin (also named ovotransferrin).

Different protein extraction methods[16,19,28] using concentrated HCl, NaOH, or NH_3 have already been proposed, but they often lead to selective degradation of amino acids, which affects the protein identification, so a softer technique was required. Multiple conditions were evaluated using ultrasonic bath, mortar, and pestle, and numerous extraction solution have been tested, acidic ones (HCl, HCOOH) and alkaline ones (NH_3, NaOH) at different concentrations (from 0.1 to 1.5 M) and temperatures (from 25 to 100 °C). However, the only protein spectra obtained result from the extraction with a homemade solution composed of an aqueous solution acidified with 1% of trifluoroacetic acid of samples grinded with single use synthetic resin, mortar, and pestle using a commercial kit dedicated to extract DNA in micro-samples. The resin is particularly significant in this step of the protocol because it allows a fine sample grinding, and it minimizes the sample loss by keeping the processing in a single tube. After grinding, the mixture was submitted to ultrasonic baths (3 serial baths, 15 min each). The supernatant was obtained after centrifugation of the mixture. The resulting solution was analyzed with a MALDI-TOF mass spectrometer in order to check the efficiency of the extraction. Figure 1 shows the MALDI-TOF spectrum (linear mode) of the commercial ovalbumin before its inclusion in the painting. The main peak at 44 606 Da can be attributed to ovalbumin, the peaks at 22 255 and 89 064 Da correspond respectively to its doubly charged ion and its dimer. Nevertheless, we can observe on this spectrum the presence of other proteins. The large detected peak at 29 771 Da (dimer at 59 236 Da) can be attributed to a minor egg white protein, the ovomucoid. Three other peaks can be attributed to two other egg white proteins, the lysozyme at 14 276 Da (and its doubly charged ion at 7137 Da) and the ovotransferrin (conalbumin) at 77 321 Da. So the ovalbumin sample, in contradiction with its commercial specification, is not free from other egg white proteins. Figure 2 presents the MALDI-TOF spectrum corresponding to the protein extract from the painting model. Despite the harsh treatment applied to proteins during the elaboration of the painting media, the MALDI-TOF spectrum shows some peaks that can be attributed to proteins, demonstrating that the extraction procedure from the microsample is efficient. The generated peaks may correspond to ovalbumin at 44 298 Da, ovomucoid at 29 376 Da, and lysozyme at 15 568 Da (and probably its doubly charged ions at 7137 Da) as previously briefly reported. However, the peaks can be differently attributed, for example, to modified forms of lysozyme. Consequently, an approach based on peptide mass fingerprinting for the robust and reliable identification of the proteins was investigated.

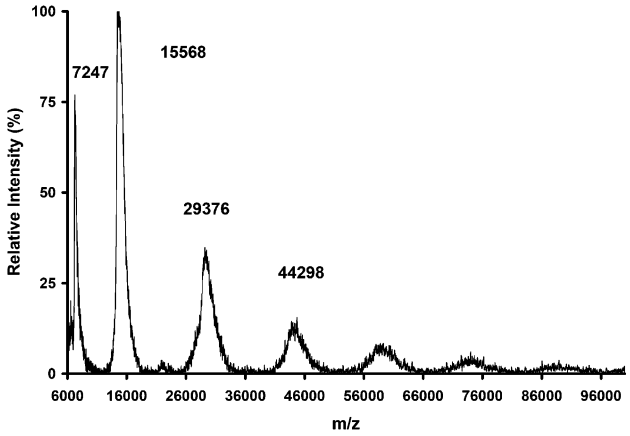

Figure 2. MALDI-TOF MS spectrum in linear mode of the protein extract from the egg-based model painting made with the commercial protein ovalbumin. The generated peaks can be attributed to ovalbumin monocharged ion (44 298 Da) but also to the ovomucoid monocharged ion (29376 Da), the lysozyme monocharged (15 568 Da), and the doubly charged (7247 Da) ions.

We evaluated the peptide mass fingerprinting method on a painting model made with fresh egg to improve the complexity of the model and to be closer to old painting formulation. This model was submitted to the extraction step and to an enzymatic hydrolysis with trypsin prior to mass spectrometric analysis. Figure 3 presents the generated peptides obtained by MALDI-

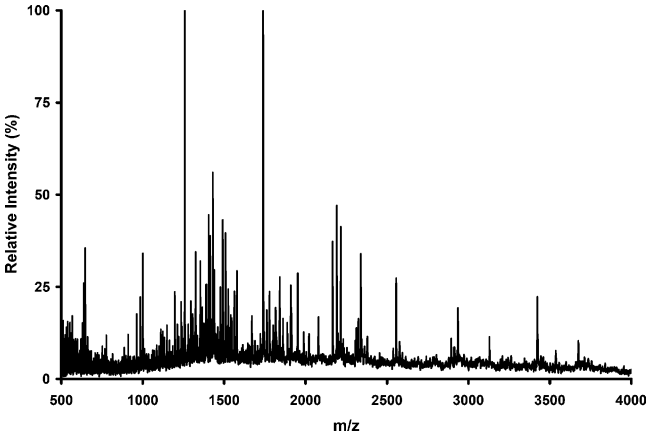

Figure 3. MALDI-TOF MS spectrum in reflector mode of the hydrolyzed protein extract from the egg-based painting. Each monoisotopic peak of each detected peptide is used for protein identification by the peptide mass fingerprint method.

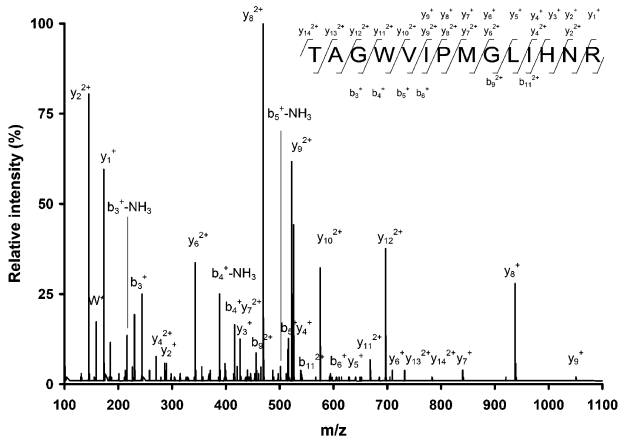

Figure 4. MS/MS spectrum of the triply charged ion at *m*/*z* 522.28, from the hydrolyzed extract of the egg-based painting, presenting the y and b fragments of the ovotransferrin peptide 480−493 (TAGWVIP-MGLIHNR). W* corresponds to the tryptophan immonium ion.

TOF analysis. The data obtained from the peptide mass fingerprint (Table 1) display proteins with matched peptide numbers varying between 6 and 13. This table presents both egg white protein (ovotransferrin (rank 1 in Table 1), ovomucoid (rank 3), ovalbumin (rank 20)) and egg yolk proteins (vitellogenin (rank 6)). This result proves that proteins modified during the elaboration of the painting and modified by oxidative changes[43] can be extracted from the media and analyzed by mass spectrometry, and protein identification can be completed without major difficulties.

To verify the protein identification, and keeping in mind that the methodology has to be applied to highly denatured proteins, we investigated a deeper structural approach by tandem mass spectrometry. Peptides generated by protein hydrolysis were then separated by nanochromatography and on-line analyzed with a Q-q-TOF mass spectrometer. This integrated system performs ion MS/MS experiments of the peptides beforehand, separated in function of their hydrophobicity through a C18 column of 75-μm inner diameter. The chromatographic gradient was adapted to the low material quantity; it was extended to 100 min (see details in the Experimental Section) to obtain the highest quantity of information from a single run. Figure 4 presents the MS/MS spectrum of a triply charged ion at *m*/*z* 522.28 from the extract of egg painting. The interpretation of this spectrum leads to the identification of the ovotransferrin peptide 480−493 (TAGWVIP-MGLIHNR) by its y (y_2^{2+}, y_4^{2+}, y_6^{2+}−y_{14}^{2+}, y_1^{+}−y_9^{+}) and b fragments (b_9^{2+}, b_{11}^{2+}, b_3^{+}−b_6^{+}), which is a constitutive egg white protein. Globally, seven ovotransferrin peptides and five ovalbumin peptides (Table 2) were identified confirming the presence of egg white as binder. Additionally, two vitellogenin peptides were identified (Table 2) confirming the presence of egg yolk. Because the developed method leads for the first time to protein identification in painting binding media, we decided to apply it to the study of old art painting samples.

Application of the Developed Methodology to Renaissance Art Painting. The developed methodology was applied to two Renaissance art paitings. The first one is the Benedetto Bonfigli's triptych, The Virgin and Child, St. John the Baptist, St. Sebastian, exhibited in the Petit Palais museum (Avignon, France) (Figure 5). This painting belongs to the Italian School of the XVth century. A 10-μg microsample was obtained from the left elbow of St. Sebastian. The microsample was submitted to protein extraction with 1% trifluoroacetic acid solution using the synthetic resin for crushing it, and to protein enzymatic hydrolysis without any physical coats separation prior the nanoLC/nano-ESI/Q-q-TOF MS/MS analysis. Figure 6 shows the MS/MS spectrum of the triply charged ion at *m*/*z* 551.61, which allows identifying the ovotransferrin *m*/*z* 443−457 peptide TDERPASYFAVAVAR by its y (y_5^{2+}−y_8^{2+}, y_1^{+}−y_8^{+}) and b fragments (b_8^{2+}−b_{11}^{2+}, b_4^{+}, b_6^{+}−b_{10}^{+}). Three other sequences have been attributed to this egg white protein (Table 2) including two sequences already identified in egg-based painting: SAGWNIPIGTLLHR and AQSDFGVDTK. Another egg white protein, not identified in the model painting,

Table 1. Proteins Identified in Hydrolyzed Protein Extract (from Egg-Based Painting) by Peptide Mass Fingerprint Method Presenting Both Egg White (Ovotransferrin, Ovomucoid, Ovalbumin) and Egg Yolk (Vitellogenin) Proteins

rank	MOWSE score	masses matched, %	coverage, %	mean error, ppm	MS-digest index no.	protein, MW/pI	accession no.	protein name
1	6.97×10^8	13 (9)	17	1.44	576223	77777/6.8	1351295 M	ovotransferrin precursor (conalbumin) (Gal d III)
2	1.35×10^8	12 (9)	17	2.35	765389	77894/6.5	757851	(X02009) ovotransferrin
3	9.48×10^7	11 (8)	19	−1.6	398420	20198/4.8	223464	ovomucoid
4	8.47×10^7	10 (7)	19	0.27	352004	22592/4.8	124757 M	ovomucoid precursor (allergen Gal d 1)
6	1.14×10^7	6 (4)	7	2.64	827585	204810/9.2	138595 M	vitellogenin II precursor [contains: lipovitellin I (LVI); phosvitin (PV); lipovitellin II (LVII); YGP40]
20	9.69×10^4	8 (6)	10	−2.89	324332	42750/5.2	223299	ovalbumin

Table 2. Proteins Identified in Hydrolyzed Protein Extract from Egg-Based Painting, Benedetto Bonfigli's Triptych and Niccolo Di Pietro Gerini's Painting by the Developed Methodology Based on Tandem Mass Spectrometry

protein identified	matched sequence	sequence position	retention time, min	precursor ion mass/charge	
		Egg-Based Painting Model			
ovotransferrin	TAGWVIPMGLIHNR	480−493	47.325	522.28	3+
			48.965	782.92	2+
	SAGWNIPIGTLLHR	141−154	45.312	512.28	3+
			45.324	767.92	2+
	KGTEFTVNDLQGK	119−131	41.793	479.58	3+
	FYTVISSLK	680−688	50.533	529.29	2+
	NKADWAK	572−578	51.542	416.71	2+
	AQSDFGVDTK	289−298	38.854	534.25	2+
	YFGYTGALR	540−548	49.023	524.26	2+
ovalbumin	AFKDEDTQAMPFR	187−199	44.104	519.24	3+
			44.532	778.36	2+
	HIATNAVLFFGR	370−381	50.675	449.24	3+
	GGLEPINFQTAADQAR	127−142	50.826	563.28	3+
			50.952	844.42	2+
	ISQAVHAAHAEINEAGR	323−339	46.432	887.45	2+
			46.298	591.96	3+
	VYLPR	280−284	32.679	324.19	2+
vitellogenin II	EDLR	974−977	35.576	532.27	1+
	QEFPK	1337−1341	40.354	324.66	2+
		Benedetto Bonfigli's Triptych			
ovotransferrin	TDERPASYFAVAVAR	443−457	47.532	551.61	3+
	SAGWNIPIGTLLHR	141−154	45.201	512.27	3+
	KDSNVNWNNLK	458−468	30.116	444.55	3+
	AQSDFGVDTK	289−298	37.384	534.25	2+
ovalbumin	AFKDEDTQAMPFR	187−199	44.015	519.24	3+
	LTEWTSSNVMEER	264−276	45.985	791.35	2+
	VYLPR	280−284	31.547	324.20	2+
vitellogenin II	AGVR	50−53	35.562	402.28	1+
lysozyme	FESNFNTQATNR	52−63	41.351	714.82	2+
		Niccolo di Pietro Gerini's Painting			
ovotransferrin	HTTVNENAPDQK	229−240	38.409	451.88	3+
	GTEFTVNDLQGK	120−131	45.637	654.82	2+
	YFGYTGALR	540−548	47.568	524.26	2+
ovalbumin	AFKDEDTQAMPFR	187−199	44.513	519.23	3+
	ISQAVHAAHAEINEAGR	323−339	46.436	887.45	2+
vitellogenin II	EDLR	974−977	35.562	532.27	1+

was identified in the triptych; its MS/MS spectrum is presented Figure 7. This figure shows a sequence of y^+ fragments (y_1^+−y_{10}^+) obtained from the dicharged ion at m/z 714.82 identifying the lysozyme peptide 52−63 (FESNFNTQATNR). Furthermore, the complete ion sequence (AGVR) of y^+ fragments (monocharged ion m/z 402.28, Figure 8) and the b sequence from b_2^+ to b_4^+ allow the identification of the vitellogenin II peptide m/z 50−53, an egg yolk protein. The vitellogenin II (1−1850), the most abundant of the three described vitellogenins (I, II, and III), is present in the database as precursor protein including the chains lipovitellin I (1−1111) and II (1329−1566), phosvitin (1112−1328) and YGP40 (1567−1850). The identified sequence AGVR is a peptide of the lipovitellin I chain. Globally, the structural information, obtained by tandem mass spectrometry, allows us to identify eight egg white peptides (4 ovotransferrin peptides, 1 lysozyme peptide, 3 ovalbumin peptides; see Table 2 for the details) and one egg yolk peptide (vitellogenin). The low vitellogenin peptide number found in the tryptich (and in the egg-based painting) is probably due to the hydrophobic nature of vitellogenin as the aqueous extraction procedure used is not very useful for the extraction of such hydrophobic proteins. In fact, in contrast to egg white proteins, egg yolk proteins are in general more hydrophobic; because the egg yolk contains 35% lipids[44] (against 0.01% for egg white), these proteins are adapted to a lipidic matrix, presenting themselves with some strong lipidic properties. As an example, the vitellogenin peptide identified in the triptych contains, of a total of four amino acids (AGVR), two very hydrophobic ones (A, hydrophobicity score[45] 1.8; V, hydrophobicity score[45] 4.2; the hydrophobicity range varying between − 4.7 for hydrophilic proteins and 4.7 for hydrophobic proteins). To conclude analysis, the developed methodology allows identifying precisely egg white and egg yolk as binder in the Bonfigli triptych.

This methodology was applied to a second art painting from the XIVth century, Niccolo di Pietro Gerini's painting The Virgin and Child; the microsample was obtained from the dress of the Virgin. For example, Figure 9 presents the MS/MS spectrum of the triply charged ion at m/z 519.23. This spectrum allows identifying the ovalbumin peptide 187−199 (AFKDEDTQAMPFR, already identified in the egg-based painting and in the triptych) by its y (y_3^{2+}−y_7^{2+}, y_{11}^{2+}, y_1^+−y_6^+) and b fragments (b_6^{2+}−b_9^{2+}, b_1^+−b_3^+, b_5^+−b_9^+). This peptide presents a tryptic missed cleav-

(43) Boon, J. J.; Peulve, S. L.; Van den Brink, O. F.; Duursma, M. C.; Rainford, D. **1997**, 35−56.
(44) Sugino, H.; Nitoda, T.; Juneja, L. R. In *Hen Eggs: Basic and Applied Science*; Yamamoto, T., Juneja, L. R., Hatta, H., Kim, M., Eds.; CRC Press: Boca Raton, FL, 1997; pp 13−24.
(45) Kyte, J.; Doolittle, R. F. *J. Mol. Biol.* **1982**, *157*, 105−132.

Figure 5. Benedetto Bonfigli's triptych, The Virgin and Child. St. John the Baptist. St. Sebastian (Italian School, XVth century), Petit Palais museum, Avignon, France. ©CRRMF.

age, which is probably due to the presence of an acid amino acid (aspartic acid) next to the cleaving site (lysine). This fact was observed as well in the other studied paintings (as, for example, the ovotransferrin peptide 458−468 KDSNVNWNNLK identified in the Benedetto Bonfigli triptych). In this Renaissance painting analysis, one other ovalbumin peptide was identified (ISQAVHAAHAEINEAGR, see Table 2; it was identified in the egg-based model painting as well). The number of identified ovalbumin peptides is relatively low in this art painting, as in the triptych and egg-based painting, considering that it is the major egg white protein[44] (50% of the egg white proteins). This fact was observed earlier in the MALDI-TOF spectrum (Figure 2) of the egg-based painting protein extract, which shows a higher number of peaks for ovomucoid (11% of egg white proteins)[44] and lysozyme (3% of egg white proteins)[44] than for ovalbumin. Additionally, the enzymatically hydrolyzed protein extract from the egg-based painting (Figure 3) results in the identification of ovalbumin in the twentieth position (Table 1). We have no explanation for the low ovalbumin peptide/protein presence in our results; however, this can be rationalized by the fact that this protein can be highly involved in the polymerization process.[46] Globally, the number of peptides identified in the Niccolo di Pietro Gerini painting is less important (6 peptides, see Table 2) than those identified from the Benedetto Bonfigli triptych (9 peptides for the triptych); this is probably due to the smaller amount of sample obtained (less than 10 μg). However, the analysis of this Renaissance painting results in the identification of both egg white proteins (2 ovalbumin peptides, 3 ovotransferrin peptides) and egg yolk protein (1 vitellogenin peptide already identified in the egg-based painting model, both having approximately the same retention time on the nanoLC column, 35.576 min for the model painting and 35.562 min for the old painting). It should be noted that all the peptides

(46) Shirai, N.; Tani, F.; Higasa, T.; Yasumoto, K. *J. Biochem. (Tokyo)* **1997**, *121*, 787−797.

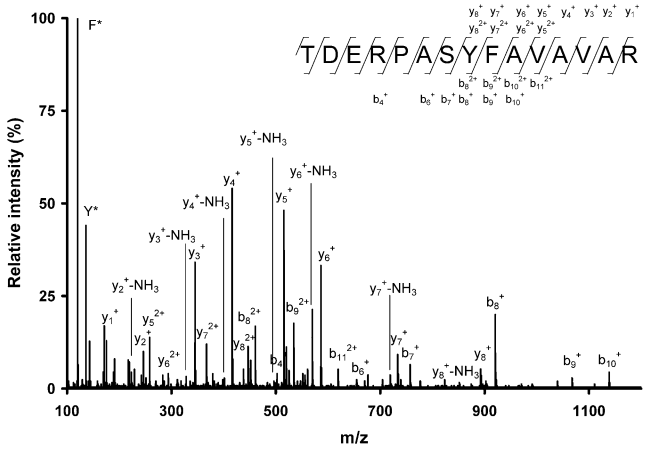

Figure 6. MS/MS spectrum of the triply charged ion at *m*/*z* 551.61, from the hydrolyzed extract of Benedetto Bonfigli's triptych, presenting the y and b fragments of the ovotransferrin peptide 443−457 (TDERPASYFAVAVAR). F* and Y* correspond respectively to the phenylalanine and tyrosine immonium ions.

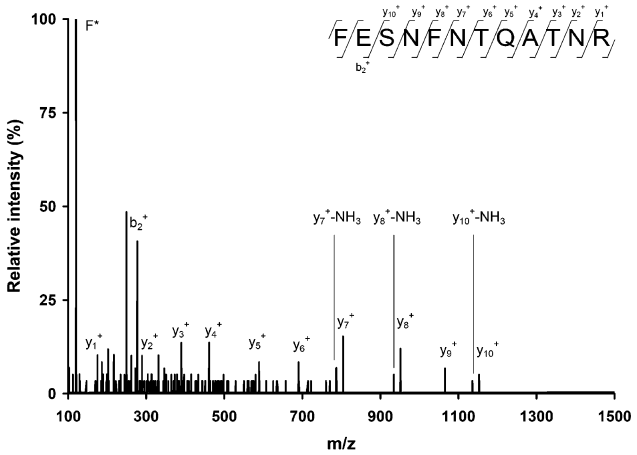

Figure 7. MS/MS spectrum of the doubly charged ion at *m*/*z* 714.82, from the hydrolyzed extract of Benedetto Bonfigli's triptych, presenting the y and b fragments of the lysozyme peptide 52−63 (FESNFNTQATNR). F* corresponds to the phenylalanine immonium ion.

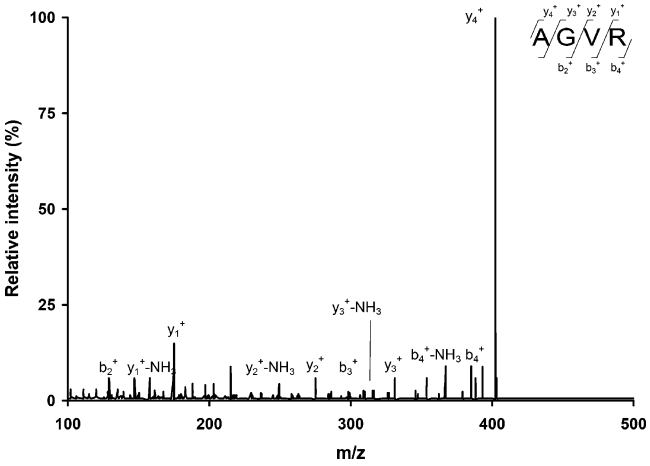

Figure 8. MS/MS spectrum of the monocharged ion at *m*/*z* 402.28, from the hydrolyzed extract of Benedetto Bonfigli's triptych, presenting the y and b fragments of the vitellogenin II peptide 50−53 (AGVR).

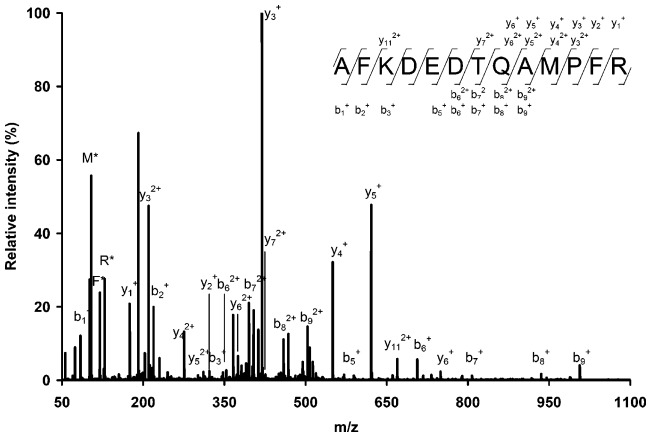

Figure 9. MS/MS spectrum of the triply charged ion at *m*/*z* 519.23, from the hydrolyzed extract of Niccolo di Pietro Gerini's painting, presenting the y and b fragments of the ovalbumin peptide 187−199 (AFKDEDTQAMPFR). M*, F*, and R* correspond respectively to the methionine, phenylalanine, and arginine immonium ions.

that have been detected come from specific cleavage by trypsin and that no peptides corresponding to protein unspecific hydrolysis were found.

So, these results allow identification of binders'proteins for the first time and agree to the presence of egg white and egg yolk as binders in the two studied Renaissance art paintings. Even if these constituents correspond to traditional recipes used in Italy during this period, the presence of egg white is particularly interesting not only because it is rarely reported in old manuscripts but also because, in this case, preliminary histochemical and heating tests have shown the presence of oil. So the binder used by Benedetto Bonfigli and Niccolo di Pietro Gerini was more complex than a simple "tempera" (containing only egg yolk), since it is a mixture of egg white, yolk, and oil. These artists chose an emulsion of oil and whole egg, often called "tempera grassa", in order to obtain particular chromatic effects such as bright and shimmering effects.

CONCLUSION

The developed proteomics procedure allows us for the first time to identify proteins in binding media starting from low amounts of sample. Different protein extraction conditions and procedures were evaluated on ovalbumin-based and egg-based model paintings. An efficient procedure was found using a commercial resin to grind the microsample using acidified water for the protein extraction; its efficiency was measured by MALDI-TOF mass spectrometry. A hydrolysis step was added for protein identification; the extracts were submitted to nano-LC/nanoESI/Q-q-TOF MS/MS analysis using optimized parameters (chromatographic gradient, etc.). The methodology was applied to Renaissance art painting microsamples from Benedetto Bonfigli's triptych, The Virgin and Child, St. John the Baptist, St. Sebastian (XVth century), and from Niccolo di Pietro Gerini's painting, The Virgin and Child (XIVth century). The developed method allows for the first time the identification of binder proteins. We characterized here egg white proteins and egg yolk proteins for the two Renaissance art paintings. So, combining a classic proteomics strategy and the use of the most powerful mass spectrometers, we succeed in identifying proteins encased for years in a complex solid medium. This strategy will open new research fields in art and archaeological samples enabling us, for example, to study the human diet through the ages and understand the origin of dietary

habits using proteins trapped in the clay of archaeological potteries.

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