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Identification of protein binders in artworks by MALDI-TOF/TOF tandem mass spectrometry

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ABSTRACT

Aim of this work is to propose an analytical protocol for proteinaceous binder identification in paintings using tryptic peptide analysis and MALDI-TOF mass spectrometry strengthened with MALDI-TOF/TOF tandem mass spectrometry (LIFT method). Proteinaceous binders are enzymatically digested with trypsin. From each individual protein frequently occurring in binders, a specific set of peptides is releasing during enzymatic digestion giving a peptide mass fingerprint (PMF) of that particular protein. The most intensive peptide peaks in PMF were determined and annotated with their corresponding amino acid sequence by MALDI-TOF/TOF analysis and subsequent database search. Before analyzing historical painting samples, procedure was tested and optimized on several painting model samples for a reliable and efficient identification of proteinaceous materials. The method is avoiding sample manipulation as much as possible in order to reduce sample loss. Since the applied procedures led to protein identification of binding media in model samples, MALDI-TOF/TOF was for the first time applied for analysis of proteinaceous binders in old painting samples.

1. Introduction

From the ancient times artists have used various proteinaceous binders in artworks for paints, ground layers, plasters and mortars. The choice of the binding medium was dependent of painting technique and availability in artist's surrounding. Through history, different recipes, including milk, lime, and pigments were tried as well as olive oil, linseed oil, eggs, animal glue or waxes, producing varying results in terms of durability. Protein binders were the most common type of painting medium before the expansion of siccative oils. Proteinaceous substances were used not only as binders for pigments in tempera, but also as adhesives, additives for plasters and mortars and for gilding. Oil replaced tempera as the principal medium in artworks during the 15th century but proteinaceous binders remained the main medium used for panel painting and illuminated manuscripts in the South East Europe (Byzantine world) and continues to be used in Greece and Russia for Orthodox icons [1].

Knowledge about original materials present in an art object is essential for cleaning, treatment, restoration and storage as well as attribution of work of art. Various spectroscopic techniques can be used for characterization of binding media [2]. Characterization by mass spectrometry is well-suited approach because of its sensitivity and ability for coupling with separation techniques, as liquid or gas chromatography. Gas chromatography-mass spectrometry (GC-MS) was one of the first mass spectrometry based techniques applied for identification of protein binders in works of art and historical monuments. Amino acids were determined by GC-MS after complete hydrolysis of the proteins and their ratio or content used for protein identification in wall [3–8], easel and panel paintings [9–11] and sculptures [12–14]. Methods based on HPLC-FD (fluorescence detection) [14,15] and HPLC-DAD (UV diode array detection) [16] were also applied to analyze amino acid composition of proteins present in samples of this type. Pyrolysis GC-MS was used for identification of characteristic pyrolytic markers for different binders [17,18] and applied on determination of animal glue and casein in wall paintings [19]. Curie point pyrolysis with GC-MS has also been employed for characterization of proteinaceous media used in the artworks [20].

In recent years, proteomic approach proved to be successful in the identification of the proteinaceous components in art objects. Analysis of tryptic peptides, by bottom-up approach, is a powerful technique in conservation science for the identification of protein binders and characterization of their aging and deterioration products. HPLC-DAD is applied for analysis of tryptic peptides obtained by enzymatic hydrolysis of painting samples [21] but recently analysis were achieved using LC-MS/MS experiments [22–25]. This later approach allows undoubted identification of

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peptides from given proteins, not only by their retention times and m/z values, but also by peptide fragmentation patterns and amino acid sequence. It is possible to identify PTMs (post translational modifications) and their location in peptide sequence [26]. Recently, high resolution mass spectrometry (FT-ICR MS) was applied in examination of protein residues on archeological findings [27] as well as origin differentiation of animal glues for gilding [28].

One of the first proteomic approaches was developed on MALDI-TOF instrumentation for identification of protein binders by peptide mass fingerprint (PMF) [29]. Peptides obtained by enzymatic digestion are analyzed by MALDI-TOF mass spectrometry and obtained mass spectra compared through database search. MALDI-TOF MS is highly applicable because of its speed, ease of use, high sensitivity and wide mass range detection. It is tolerant on small amounts of contaminants, salts, and surfactants, therefore meeting requirements for analysis of samples with minimal pretreatment. MALDI-TOF MS is applied with success for determination of the proteinaceous binders in different model samples and historical samples. Kuckova et al. [29–31] published protein binder identification in paintings by various artists using MALDI-TOF mass spectrometry. They established their own PMF database of proteinaceous binders for comparison with spectra of real samples. The same group analyzed milk proteins in historical mortars [32] and few peptide sequences *de novo* assigned by mass spectrometry. Recently, statistical classification methods are employed in order to distinguish proteinaceous binders based on PMF approach [33].

In the present work, we attempted to identify natural proteinaceous binders usually found in artworks by using MALDI-TOF MS peptide mass fingerprint approach with complementary MS/MS experiments (TOF/TOF analysis) of selected peptides. Sample preparation was not extensive in order to minimize sample losses. PMF approach involve database search for comparing the resulting mass spectra with calculated peptide masses for protein cleavage *in silico*. These studies, sometimes gave scarce or inconclusive results for paint binders, due to complexity of samples. Peptide sequences obtained from MALDI-TOF/TOF experiments confirmed presence of certain proteins in model samples as well as historical painting samples.

2. Materials and methods

2.1. Chemicals and reagents

All solutions were prepared with ultrapure water (water purification system Milli-Q, Millipore, Bedford, MA, USA; specific resistance ≥10 MΩ/cm, 25°C). The Trypsin Singles, Proteomics Grade Kit (T7575, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) are used for hydrolysis of proteins. All peptides for external calibration, 2,5-Dihydroxybenzoic acid (DHB), ammonium hydrogen carbonate (AMBIC), dithiothreitol (DTT) and iodoacetic acid (IAA) were also from Sigma. Acetonitrile was purchased from VWR International (Fontenay sous Bois, France), trifluoroacetic acid (TFA) from Acros Organics (Noisy-Le-Grand, France) and urea from Biorad (Marnes la Coquette, France). Pigments French ochre (Fe_2O_3, Product No. 40020) and zinc white (ZnO, Product No. 46300) were from Kremer (Aichstetten, Germany).

2.2. Samples of proteinaceous binders

As reference materials we used proteinaceous binders from egg (egg yolk and white), milk (quark cheese and casein powder) and different collagens (rabbit, bovine, porcine and sturgeon). Bovine bone glue (Product No. 63000) and hide glue (Product No. 63010), rabbit skin glue, refined (Product No. 63025) and raw (Product No. 63028), Salianski isinglass (Product No. 63110), technical gelatin from pig skin (Product No. 63040) and casein powder (Product No. 63200) are purchased from Kremer (Aichstetten, Germany). Eggs and quark cheese were obtained from the local market.

2.3. Proteinaceous binders preparation

Animal glues are dissolved in warm deionized water during several hours at a final 8% (w/v) solution concentration. Egg white is divided from egg yolk and slightly beaten to make foam; after a while, liquid part is separated and used. Egg yolk membrane was cut and liquid part of yolk diluted with deionized water to obtain a fluid homogeneous medium. Cheese and casein binders are prepared with addition of borax, as reported by Fremout [21].

2.4. Model samples preparation

Painting models are prepared using binders named above mixed with pigments French ochre and zinc white. The pigments are mixed with the fluid binder in ratio 1:1 (w:w), mixtures spread as thin film on microscope glass slides and stored at ambient temperature in the dark for several months. Prior to tryptic hydrolysis, the surface of model samples is scraped with a spatula and weighted.

2.5. Historical paintings samples

Painting samples are collected on few different icons from the iconostasis of the church of the Holy Virgin in Barič, created between 1834 and 1838 year by three Serbian painters Konstantin Lekić, Janja Stergević Moler and Dimitrije Jakšić. One sample is obtained from the iconostasis of the church Annunciation of The Holy Mother of God in Idvor, painted by Stevan Todorović, also Serbian artist of the 19th century. Small chips of painting layer are gently scraped with a scalpel before conservation treatments and preserved for analysis. Samples were too small to be weighted.

2.6. Enzymatic hydrolysis

Two protocols for enzymatic hydrolysis are implemented. First hydrolysis procedure is applied for reference materials, preceded by cysteine reduction step with DTT in presence of urea (8 M) in AMBIC buffer (40 mM, pH 8.2) and carboxymethylation with IAA. Starting protein concentration was 1 mg/mL. After urea dilution with AMBIC buffer to maximal concentration of 2 M before trypsin addition (to avoid denaturation of trypsin that occurs in the presence of urea in high concentrations) final protein concentration was still sufficient for MALDI TOF analysis. In aliquot of 100 μL trypsin was added (10 μg/mL), samples incubated at 37 °C for 16 h and acidified with TFA to adjust pH to about 2. After desalting step samples are mixed with matrix solution of DHB in volume ratio 1:2 and analyzed.

Because of extensive dilution due to urea and low concentration of proteinaceous materials in samples, application of described procedure for hydrolysis of historical paintings samples was inadequate. To avoid sample manipulation and dilution, shortened and simplified procedure similar as described elsewhere [22,23,26,29–33] was tested on model samples and applied on historic painting samples afterwards. Procedure is based on digestion in a heterogeneous phase by incubating approximately 1 mg of solid model sample at 37 °C for 16 h, in 100 μL of AMBIC buffer (40 mM, pH 8.2), containing trypsin 10 μg/mL. After digestion samples were acidified with TFA to adjust pH and supernatant recovered after centrifugation. All preparation steps are done in

the same vial thus reducing the risk of sample losses. Real samples were too small to be weighted prior the hydrolysis.

2.7. Peptide desalting

For the digest peptide desalting and contaminants removal ZipTip C_{18} tips (Millipore, Bedford, MA, USA) are used. This simple and rapid method improved mass spectra quality and enriched trace peptides. Use of ZipTip C_{18} tips packed with RP chromatographic material was like follows. After wetting ZipTip with acetonitrile and conditioning with water, samples were loaded on the micro-column. Subsequently they were washed to remove salts with 0.1% TFA and peptides were eluted in two steps with mixture of 0.1% TFA and acetonitrile (1:1) and pure acetonitrile. Extracts were collected into the same vial, aliquot mixed with matrix solution in volume ratio 1:2 and applied on stainless steel MALDI target (Bruker Daltonik GmbH, Bremen, Germany). Matrix was solution of DHB (15 mg/mL in mixture of ACN and 0.1% TFA (1:2 v/v)).

2.8. Sample analysis

Tryptic digests were analyzed by Autoflex III Smartbeam MALDI-TOF mass spectrometer (Bruker Daltonics, Germany), operated by FlexControl v3.3 and equipped with a 355-nm nitrogen laser. All spectra were obtained with the delayed extraction technology in positive reflectron mode and averaged from 200 laser shots to improve the signal-to-noise (S/N) ratio. The voltage parameters were set at IS1 19 kV, IS2 16.7 kV, lens 8.5 kV, reflector 1 21.0 kV, and reflector 2 9.7 kV. The delay time was 10 ns, and the acquisition mass-to-charge range was 500–4000 Th. External high precision calibration (HPC) was performed on daily basis using a peptide mixture containing bradykinin (757.39916 residues 1–7 $[M\text{-}H]^+$ mono), angiotensin II (1046.54180 $[M\text{-}H]^+$ mono), P14R (1533.85765 $[M\text{-}H]^+$ mono), ACTH (2465.19830 clip 18–39 $[M\text{-}H]^+$ mono), and insulin (oxidized chain B 3494.65130 $[M\text{-}H]^+$ mono).

Samples analyzed by PMF were additionally analyzed using LIFT MS/MS from the same target. The most abundant ions per sample are chosen for MS/MS analysis. Analyses are performed in positive LIFT reflectron mode. Precursor Ion Selector (PCIS) range was 0.65% of parent ion mass. The voltage parameters were set at IS1 6 kV, IS2 5.3 kV, lens 3.00 kV, reflector 1 27.0 kV, reflector 2 11.45 kV, LIFT 1 19 kV and LIFT 2 4.40 kV. For the precursor ion 200 laser shots were accumulated and 500 shots for fragmentation.

2.9. Database search

The raw mass spectra are processed using the FlexAnalysis v 3.3 Software (Bruker Daltonik GmbH, Bremen, Germany). TOF/TOF tandem mass spectra were smoothed and baseline-subtracted and the sophisticated numerical annotation procedure (SNAP) algorithm is used to for detection the monoisotopic peak values, with a quality factor threshold of 30. The S/N threshold was set at 6 for MS analysis and at 3 for MS/MS analysis. BioTools 3.2 software (Bruker Daltonik GmbH) connected to the Mascot search engine [34] is used for SwissProt database (SwissProt_2012_03.fasta) search of datasets. PMFs are searched independently, and together with complementary LIFT-MS/MS spectra. Search parameters are optimized by iteration. Mass tolerance of 50–150 ppm for PMF and tolerance of 50–150 ppm and 0.6–0.8 Da for MS/MS search were allowed, depending of data set accuracy. All MS/MS data were searched using the ion series set for MALDI-TOF/TOF in Mascot (a, a-17, a-18, b, b-17, b-18, y, y-17, y-18, immonium and internal yb and ya ions below 700 Da). Ion charge state was set to +1, the species are restricted to *Metazoa* and no restrictions were placed on isoelectric points and mass of the proteins. Number of miscleavages was set to 1 in error tolerant mode, and oxidation of methionine, lysine and proline, N-terminal acetylation, phosphorylation of serine and threonine and deamidation of asparagine and glutamine were considered as variable modifications. Carboxymethylation of cysteine is considered as fixed modification in reference materials datasets. The Mowse probability score (that the observed match between the experimental data set and each sequence database entry is a chance event, $p < 0.05$) [35] was used as criterion for correct identification. Scores are reported as −10Log10(P), where P is the probability. The match with the lowest probability, i.e. the highest score is reported as the best match. Identity threshold is typically a score about 70 for PMF and 30–40 for MS/MS search. Whether the best match will be a significant depends on data quality and the size of the database. Ideally, the correct match is the best and a significant match [34].

3. Results and discussion

There are two approaches for protein identification: peptide mass fingerprint when measured peptide masses are compared to calculated masses of peptides from protein hydrolisis and fragment ion mass searches. PMF approach implies that each protein subjected to triptic hidrolysis give a unique set of peptides which represent fingerprint of the protein. In this research peptide mass fingerprints of studied binding media are established by MALDI-TOF mass spectrometry. Detected peptide masses in PMF spectra are searched via Mascot search engine against SwissProt database and resulting matches are scored accordingly. Besides mass values, peak intensities are used, in order to improve the specificity of the identifications. The peak lists were limited to the range 800–3000 Da, since many peaks of salts and the DHB matrix clusters are present in low mass region, while peaks in the higher region are typical for miscleavages.

Although PMF is a simple and fast method, it has some shortcomings. Peptides are only characterized by mass and peak intensity and analysis relies on their comparison with theoretical peptide masses. The best results PMF gives for single proteins or simple mixtures, but complex and contaminated samples, such as proteinaceous binders may impose the problem. PMF analysis of some samples in this study has poor performance and in that case MS/MS approach was extremely helpful. The MALDI-TOF/TOF allows acquiring MS/MS data on the same spot that was used for MALDI PMF. MS/MS increase the amount of information obtained for peptide ions. They are containing fragmentation pattern of peptides which helps revealing the exact peptide sequences. Fragment ions produced from the singly charged peptide ions in the MALDI source are a mixture of y-, b- and a-ions accompanied by ions resulting from neutral loss of ammonia or water. Singly charged peptides are prone to fragmentation at specific site. Produced daughter ions have various intensities according to the ease of breaking peptide bonds and the charge location. Ions formed due to preferential cleavage are dominant in MS/MS spectra. In the MALDI source singly charged peptides show a preferred cleavage at C-terminal of aspartic and glutamic acid and N-terminal of proline [36]. Preferential cleavages are decreasing the information content of the MALDI MS/MS spectra and complete y-ion series usually are not detectable, so some sequence information can be absent. Fragment ions mass search increase the probability of protein identification with fewer detected peptides. This is especially important in proteinaceous binder determination on old paintings where processes of ageing and degradations significantly reduce chance of finding a large number of unaltered peptides after hydrolysis, which is prerequisite for successful PMF analysis. LC-MS/MS data can contain hundreds and thousands fragmentation spectra in the single file and recording time can be

very long, as well as data filtering and unwanted fragmentation spectra removal. Comparing to that, MALDI-TOF/TOF spectra are recorded as separate files that can be combined into a single file, easy for processing and database search. Fragmentation spectra of a parent ion can be recorded few times to achieve the best possible spectrum quality. Protein identity confirmation can be obtained in very short time by recording and database search fragmentation spectra of a small number of characteristic peptides for a protein indicated in PMF analysis. PMF analysis without MS/MS confirmation can be in some cases inconclusive or misleading, as can be seen in some examples in this research (full data provided in Supplementary material), as well as some publications cited above [29,31–33].

MS/MS data in this study were submitted to database search as PMF file combined with belonging LIFT-MS/MS spectra. Only fragmentation spectra of good quality were took into consideration in order to avoid false positives. Total scores of identified proteins and scores of individual ions are obtained. A single PMF or a single LIFT spectrum may lead to a Mascot score with a probability that the observed match is a random event. Results of combined spectra database search showed dramatically improved significance. Protein scores are derived from the ions scores. They are the sum of the highest ion scores for each particular sequence, excluding the scores of duplicate matches. A small correction is applied to reduce the contribution of low-scoring random matches [34]. Protein scores provide a logical order to the report. If multiple queries match to a same protein, but the individual ions scores are below threshold, the combined ions scores can still place the protein high in the report.

A criterion for the identification of candidate peptide of specific protein was individual ion score higher than threshold value given by the Mascot as indicative of identity or extensive homology (about 30–40). Not all peptide sequences identified in the reference materials are found in model samples and vice versa. Peptides may contain PTM or can be destroyed or altered by aging which can cause their absence or change of m/z value. Other observed spectral features might be background peaks or contamination (e.g. keratin).

The most frequently observed PTM in this study are oxidation (mass shift of +16 Da) in animal glues, phosphorylation (+80 Da) in the milk proteins, and N-terminus peptide acetylation. Deamidation reported by Leo et al. [26] as PTM which introduces a small (+0.984 Da) mass shift that can overlap the isotopic pattern was also found in some peptides. Unexpected or less frequent modifications are included in the Mascot search results by enabling error tolerant searches. For some PTMs, such as phosphorylation of serine and threonine observed in milk proteins, there are fewer site options and, with good data, can be revealed the exact location of the modified residues. Collagen peptides are rich in proline which is prone to hydroxylation. Several variants of these peptides, with different PTM positions, can be suggested by Mascot, but confident sequence assignment and PTMs arrangement is more difficult.

Fig. 1 is an example of a fragmentation spectrum recorded for model sample of bovine cheese binder mixed with zinc white pigment successfully matched to a peptide YLGYLEQLLR from Alpha-S1-casein.

In this spectrum the complete y-ion series is annotated, as well as b2–b9 and a1–a5 product ions. Ion score of 81 (>39) was obtained by matching 46 of 133 fragment ions using the 42 most intense peaks. This does not mean that the unmatched peaks are noise, but matching the 42 most intense peaks gave the lowest

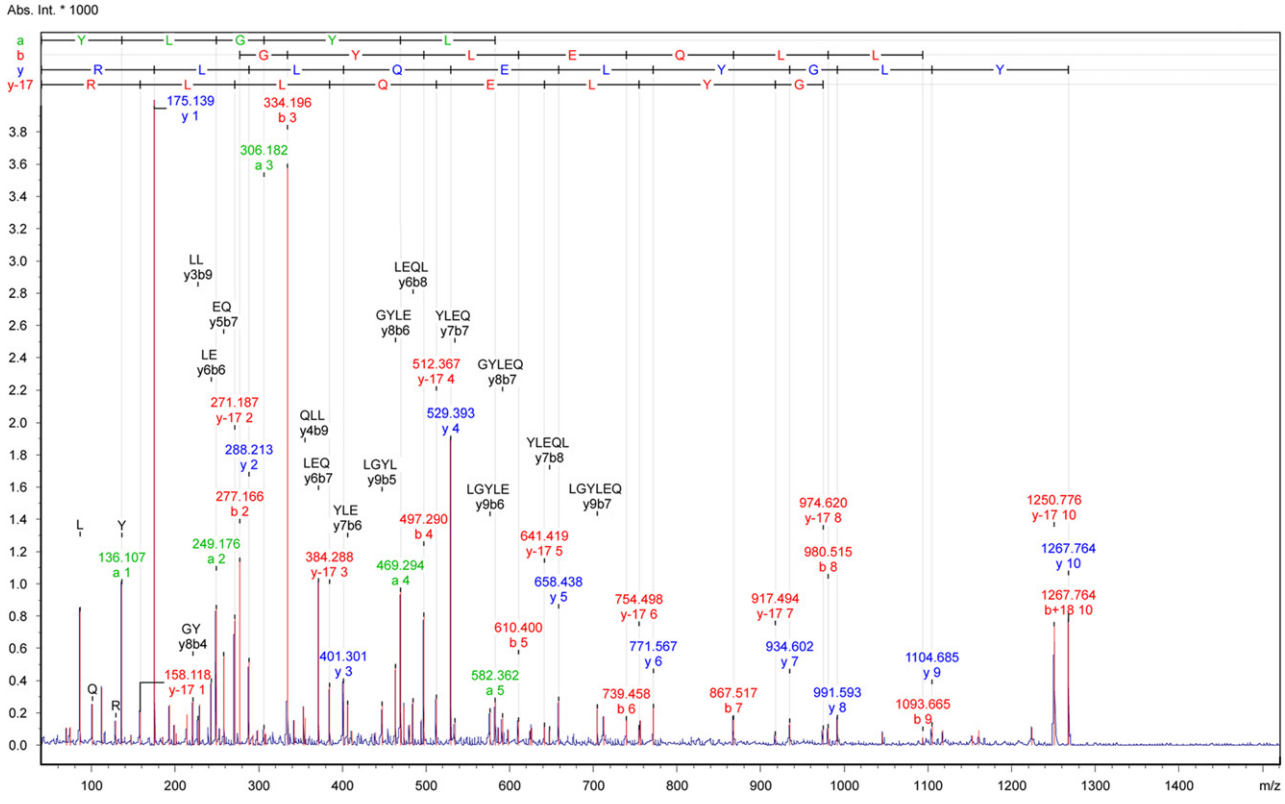

Fig. 1. MS/MS spectrum of the parent ion at m/z 1267.764, identifying peptide sequence YLGYLEQLLR from bovine Alpha-S1-casein (CASA1_BOVIN) in model sample with bovine cheese and zinc white (7M11).

Table 1
Peptides in model samples identified by MALDI-TOF/TOF tandem mass spectrometry and Mascot database search with individual ion scores bigger than indication of identity or extensive homology, $p < 0.05$ (numbers in brackets).

Model Sample	Protein identification	Total score	Number of peptides	Meas. m/z	Ion Scores	Sequence
Rabbit skin glue+Zinc white 3M11	*Collagen alpha-2*(I) *chain* *Bos taurus* CO1A2_BOVIN	144	4	1427.7	70 (38)	GIPGEFGLPGPAGAR + 2 Oxidations (P)
				2056.0	41 (38)	EGPVGLPGIDGRPGPIGPAGAR + Oxidation (P)
Rabbit skin glue+French ochre 3M21	*Collagen alpha-2*(I) *chain* *Bos taurus* CO1A2_BOVIN	163	4	1427.7	38 (37)	GIPGEFGLPGPAGAR + 2 Oxidations (P)
				2131.0	98 (37)	GLPGVAGSVGEPGPLGIAGPPGAR + 3 Oxidations (P)
	Collagen alpha-1(I) *chain* *Bos taurus* CO1A1_BOVIN	53	3	1975.9	40 (37)	SGDRGETGPAGPAGPIGPVGAR
Bovine cheese+Zinc white 7M11	*Alpha-S1-casein* *Bos taurus* CASA1_BOVIN	595	7	1267.8	81 (39)	YLGYLEQLLR
				1384.8	70 (39)	FFVAPFPEVFGK
				1660.9	80 (39)	VPQLEIVPNSAEER 10: Phospho (ST)
				1760.0	131 (39)	HQGLPQEVLNENLLR
				1952.0	66 (39)	YKVPQLEIVPNSAEER 12: Phospho (ST)
				2316.3	145 (39)	EPMIGVNQELAYFYPELFR
Bovine cheese+French ochre 7M21	*Alpha-S1-casein* *Bos taurus* CASA1_BOVIN	412	6	1267.7	83 (36)	YLGYLEQLLR
				1759.9	127 (36)	HQGLPQEVLNENLLR
				1952.0	40 (36)	YKVPQLEIVPNSAEER 12: Phospho (ST)
				2316.1	124 (36)	EPMIGVNQELAYFYPELFR
Egg glair+Zinc white 9M11	*Ovalbumin Gallus gallus* OVAL_CHICK	584	9	1345.8	101 (40)	HIATNAVLFFGR
				1555.8	70 (40)	AFKDEDTQAMPFR
				1581.8	75 (40)	LTEWTSSNVMEER
				1687.7	102 (40)	GGLEPINFQTAADQAR
				2089.0	90 (40)	EVVGSAEAGVDAASVSEEFR 5: Phospho (ST)
				2281.3	90 (40)	DILNQITKPNDVYSFSLASR
Egg glair+French ochre 9M21	*Ovalbumin Gallus gallus* OVAL_CHICK	490	9	1209.5	41 (40)	DEDTQAMPFR
				1345.8	75 (40)	HIATNAVLFFGR
				1555.7	61 (40)	AFKDEDTQAMPFR
				1581.7	78 (40)	LTEWTSSNVMEER
				1859.1	42 (40)	ELINSWVESQTNGIIR
				2089.1	108 (40)	EVVGSAEAGVDAASVSEEFR 5: Phospho (ST)
				2281.3	74 (40)	DILNQITKPNDVYSFSLASR
Egg yolk+Zinc white 10M11	*Vitellogenin-2 Gallus gallus* VIT2_CHICK	149	5	1445.8	48 (41)	VGATGEIFVVNSPR
				1560.8	53 (41)	SPQVEEYNGVWPR
	Ovalbumin Gallus gallus OVAL_CHICK	86	2	1687.9	59 (41)	GGLEPINFQTAADQAR
	Apovitellenin-1 Gallus gallus APOV1_CHICK	78	2	1891.0	50 (41)	AGQFLLDVSQTTVVSGIR
Egg yolk+French ochre 10M21	*Vitellogenin-2 Gallus gallus* VIT2_CHICK	247	10	1342.7	39 (38)	NIPFAEYPTYK
				1560.8	60 (38)	SPQVEEYNGVWPR
	Ovalbumin Gallus gallus OVAL_CHICK	91	2	1687.7	39 (38)	GGLEPINFQTAADQAR
				1860.0	52 (38)	ELINSWVESQTNGIIR 12: Deamidated (NQ)
	Apovitellenin-1 Gallus gallus APOV1_CHICK	60	2	1891.0	43 (38)	AGQFLLDVSQTTVVSGIR

probability, i.e. the highest ion score. Secondary losses can be occurred during peptide fragmentation in the MALDI source, for example loss of NH_3 or H_2O moiety [37]. In this particular case almost complete y-17 series (with an exception of y-17 9 product ion) is annotated.

3.1. Analysis of reference samples of proteinaceous binders

Results of PMF database search for egg white indicated presence of 4 proteins – Ovalbumin, Ovotransferrin, Lysozyme C and Ovalbumin-related protein Y, first two having high Mascot scores and good sequence coverage (i.e. 71%, 32%, 39% and 11% respectively for Ovalbumin and Ovotransferrin, Lysozyme C and Ovalbumin-related protein Y). MS/MS analysis confirmed 15 peptide sequences for Ovalbumin, 11 having scores above 38 (indication of identity or extensive homology, $p < 0.05$). Mascot score for Ovalbumin was 961 while in PMF analysis was 149. Presence of Ovotransferrin is confirmed by determination of 9 peptide sequences and protein score of 177 is found, however, no results from MS/MS query for Lysozyme and Ovalbumin-related protein Y are obtained, due to low sensitivity and low concentrations of these proteins in egg white.

PMF analysis of egg yolk indicated two proteins, Vitellogenin-2 and Ovalbumin (with 23% and 32% of sequence coverage), but MS/MS analysis, except the above two proteins, provided evidences for Apovitellenin-1 and Lysozyme C with lower scores and at least one significant ion score. Vitellogenin-2 is big protein (205 kDa, 1850 amino acids) and it is expected to find a high number of peptides in PMF spectra after tryptic hydrolysis, but few peaks are detected proportionally to its mass. In the best case sequence coverage was 23% for PMF analysis, while number of identified peptides by MS/MS is lower and 7 of 27 sequences are confirmed with decent ion scores. Total protein score for Vitellogenin-2 was 305, despite low ion scores. MS/MS data of peaks at m/z 1445.6 (VGATGEIFVVNSPR) and 1560.7 (SPQVEEYNGVWPR) display the highest ion scores and allow the attribution of Vitellogenin-2 without ambiguity.

In PMF spectrum of casein binder Alpha-S1-casein and Alpha-S2-casein are almost equally represented. According to MS/MS data Alpha-S1-casein is dominant with 7 resolved peptide sequences and protein score 463, while Alpha-S2-casein is represented by only one matched peptide with a modest score. Additionally, one sequence, with m/z 2186.2 with ion score of 124 pointed out the presence of Beta-casein. In quark cheese besides 3 proteins found in casein binder, PMF revealed the presence of Beta-lactoglobulin, but only Alpha-S2-casein had significant Mascot score (>63). Combined MS/MS data confirmed Alpha-S1-casein, Beta-lactoglobulin, Alpha-S2-casein and Beta-casein and noticed one peptide sequence from Kappa-casein (YIPIQYVLSR at m/z 1251.7).

By analysis of animal glue samples of different provenance, peaks at m/z 1105.6, 1427.7, 1435.7, 1453.7, 1560.8, 1586.8, 2056.1 and 2131.1 in PMF spectra are found to be indicative for the presence of collagen, but origin cannot be reliably determined even by using MS/MS experiments. The most information in the database are available for, bovine collagen, so far the most intensively studied, while for other organism amount of structural information is scarce. Therefore, the most frequent matches during database search of PMF and fragmentation spectra of animal glues in this research are bovine collagen alpha-1(I) chain (CO1A1_BOVIN) and collagen alpha-2(I) chain (CO1A2_BOVIN), even for samples of a different provenance. Bovine collagens were identified for samples of bovine (63000 and 63010) and rabbit glue (63025 and 63028). For technical gelatin from pig skin (63040), the best ion scores obtained are for two sequences matching Collagen alpha-2(I) chain (*Canis familiaris*), but for Salianski isinglass (63110) which is sturgeon air bladder, results are very different. There are matches for different collagen chains of various origins—from *Oncorhynchus mykiss* and *Cynops pyrrhogaster* to *Homo sapiens*.

Provenance determination of collagen in animal glues is difficult. The main reason is that existing libraries contain complete collagen sequences from only a limited number of species. The protein sequences of some animals, as rabbit or sturgeon are incomplete or missing in the SwissProt protein database. Also, because of the high degree of similarity between collagen chains in different species and its repetitive arrangement, database search can provide multiple possible annotations with similar scores. Buckley et al. [38,39] tried to establish collagen fingerprints of various provenances in bone and teeth remains using MALDI-TOF mass spectrometry and PMF approach. Even by high resolution techniques, determination of origin for animal glues is difficult. Dallongeville et al. [28] are suggesting that some analyzed rabbit glue samples by LC-FTICR are in fact bovine glue.

Through SwissProt database search of MS/MS data in this study few sequences imposed to be characteristic for collagen binders. GIPGEFGLPGPAGAR+32 Da (m/z 1427.6), GLPGEFGLPGPAGPR +32 Da (m/z 1453.6), EGPVGLPGIDGRPGPIGPAGAR+16 Da (m/z 2055.9), GLPGVAGSVGEPGPLGIAGPPGAR+48 Da (m/z 2131.0) are annotated peptide sequences determined in Collagen alpha-2 (I) chain of *Bos Taurus* and *Canis Familiaris*, while GPAGPQGPR (m/z 836.4) and GFSGLQGPPGPPGSPGEQGPSGASGPAGPR+48 Da (m/z 2705.3) are peptide sequences determined in Collagen alpha-1(I) chain of *Bos Taurus*.

3.2. Protein binders identification in model samples

The effect of pigments and simplified sample preparation procedure on the identification of proteinaceous binders was evaluated through the analysis of model samples prepared with considered proteinaceous materials. High concentration of inorganic pigments can interfere to the identification of the protein [40]. Recent report [41] established that pigments promote cross-linking and hydrolysis of the polypeptide chains, and to a lesser extent, oxidation of side amino acid chains. Proteins rich in methionine and cysteine are the most labile and prone to cross-linking and oxidation. Additionally, some cations, such as Hg(II), Fe(III), Cu(II), Pb(II), Cd(II), Zn(II), Ca(II), are believed to give rise to non-covalent complex species with proteins, [42].

C_{18} pipette tip purification procedure was adapted for cleaning up and concentrating peptides after tryptic hydrolysis, thus eliminating signal suppression due to salts and inorganic compounds. Although search results obtained for model samples are containing less peptide matches (Table 1) and lower Mascot scores comparing to reference materials, identification of proteins by MS/MS analysis was still successful.

Table 2 shows a comparison of protein identification results from PMF and MS/MS datasets for model samples. Data obtained by MALDI-TOF/TOF experiments enabled identification of more proteins with higher confidence then PMF analysis for all model samples.

Only for 3 proteins identified in model samples the best match obtained by Mascot search of PMF data was significant, having Mowse score above 63. Also, PMF failed to provide protein identification for 3 of 8 samples. As regards the sample hydrolysis procedure, resemblance of the results obtained for reference materials, after carboxymethylation step, and model samples hydrolyzed by shortened and simplified procedure, underline the fact that omission of cysteine derivatization in the sample preparation protocol does not have a strong impact on the ability of protein identification. In contrast, sample treatment simplification prevents sample loss and dilution. Peptides identified by MS/MS do not contain carboxymethyl group, in majority of cases, except for peaks at m/z 1248.6 and 1523.8 in Ovalbumin, 2551.9 and 2049.0 in Ovotransferrin and 1716.8 in Beta-lactoglobulin. In fact, all proteinaceous materials considered are not rich in cysteine residues. Only Vitellogenin-2 contains 36 cysteine residues [43]. However, as for Vitellogenin-2 in reference materials was identified by MS/MS a small number of peptides comparing to the size of the protein, only in 3 of them carboxymethyl group was found.

Fragmentation spectra of parent ions at m/z 1345.8 for samples of egg white with both pigments considered are reported in Fig. 2.

Both enabled identification of chicken Ovalbumin sequence HIATNAVLFFGR. The clear-cut fragmentation pattern provides an extensive y-ion series in sample with zinc white and good sequence coverage from a- and b-product ions, which allowed unambiguous sequence assignment. For example with French ochre, lower intensity of parent ion caused low signal-to-noise ratio of peaks in fragmentation spectra, but the same sequence was identified with lower ion score (75 vs. 101). That shows, for peptides ions that can be easily fragmented giving a good fragmentation pattern, even low intensity spectra can lead to reliable peptide identification.

Table 2
The comparison of protein identification results by PMF and MS/MS database search for model samples.

Model Sample	Protein identification by PMF	Number of peptides	Protein Mascot score by PMF	Protein identification by MS/MS	Number of peptides	Protein Mascot score by MS/MS
Rabbit skin glue+Zinc white **3M11**	~*			Collagen alpha-2(I) chain *Bos taurus*	4	144
				Collagen alpha-2(I) chain *Gallus gallus*	2	37
Rabbit skin glue +French ochre **3M21**	~*			Collagen alpha-2(I) chain *Bos taurus*	4	163
				Collagen alpha-1(I) chain *Bos taurus*	3	53
				Collagen alpha-2(I) chain *Gallus gallus*	2	43
Bovine cheese+Zinc white **7M11**	Alpha-S1-casein *Bos taurus*	7	68	Alpha-S1-casein *Bos taurus*	7	595
	Beta-lactoglobulin *Bos taurus*	4	34	Alpha-S2-casein *Bos taurus*	2	64
				Beta-casein *Bos taurus*	3	57
				Beta-lactoglobulin *Bos taurus*	2	39
				Kappa-casein *Bos taurus*	1	33
Bovine cheese+French ochre **7M21**	Alpha-S1-casein *Bos taurus*	5	46	Alpha-S1-casein *Bos taurus*	6	412
	Beta-lactoglobulin *Bos taurus*	4	35	Alpha-S2-casein *Bos taurus*	1	21
				Kappa-casein *Bos taurus*	1	19
Egg glair+Zinc white **9M11**	Ovalbumin *Gallus gallus*	11	72	Ovalbumin *Gallus gallus*	9	584
Egg glair+French ochre **9M21**	Ovalbumin *Gallus gallus*	12	73	Ovalbumin *Gallus gallus*	9	490
				Ovotransferrin *Gallus gallus*	2	38
				Lysozyme *Gallus gallus*	1	21
Egg yolk+Zinc white **10M11**	~*			Vitellogenin-2 *Gallus gallus*	5	149
				Ovalbumin *Gallus gallus*	2	86
				Apovitellenin-1 *Gallus gallus*	2	78
Egg yolk+French ochre **10M21**	Vitellogenin-2 *Gallus gallus*	19	59	Vitellogenin-2 *Gallus gallus*	10	247
				Ovalbumin *Gallus gallus*	2	91
				Apovitellenin-1 *Gallus gallus*	2	60

* for sample are not obtained significant matches during database search.

In MS/MS spectrum of the α-S1-casein peptide FFVAPFPEVFGK found in samples of bovine quark cheese with zinc white (Fig. 3) single charged fragments are annotated as y2–y4, y6–y10, b2-b6, b8–b12.

Furthermore, some peaks can be attributed to a-fragments (a1, a2 and a5). In this particular case the MS/MS spectrum provides a successful sequence identification with a high confidence ion score of 70, no matter that number of attributed fragment peaks was only 31 (vs. total of 73 selected peaks).

Considering model samples containing rabbit skin glue (63025) only the presence of bovine Collagen alpha-2(I) chain was confirmed with reliability. Peptides GIPGEFGLPGPAGAR+32 Da at m/z 1427.7 (Fig. 4), EGPVGLPGIDGRPGPIGPAGAR+16 Da at 2056.0 and GLPGVAGSVGEPGPLGIAGPPGAR+48 Da at 2131.0 displayed the highest ion scores. In model sample of Rabbit skin glue with French ochre bovine, besides bovine Collagen alpha-2(I) chain, Collagen alpha-1(I) chain was represented by 3 peptide sequences, one of them SGDRGETGPAGPAGPIGPVGAR at m/z 1975.9 with ion score 40.

In some cases identification only by the PMF can be misleading as can be seen from Table 3.

There are displayed differences in the peptide annotations obtained by PMF and MS/MS analysis that have emerged in this study. Likewise, peptide annotations that other authors [29–33] assigned by PMF analysis are not always consistent with our MS/MS peptide identifications. Inconsistencies can arise within peptides of a single protein, but more common are cases of m/z values attributed to a peptide of one protein while MS/MS fragmentation in our research points to peptide sequence of another protein.

3.3. Analysis of historical painting samples

Analyzing samples from icons painted by Serbian artists of 19th century by the same procedure as model samples, the collagen

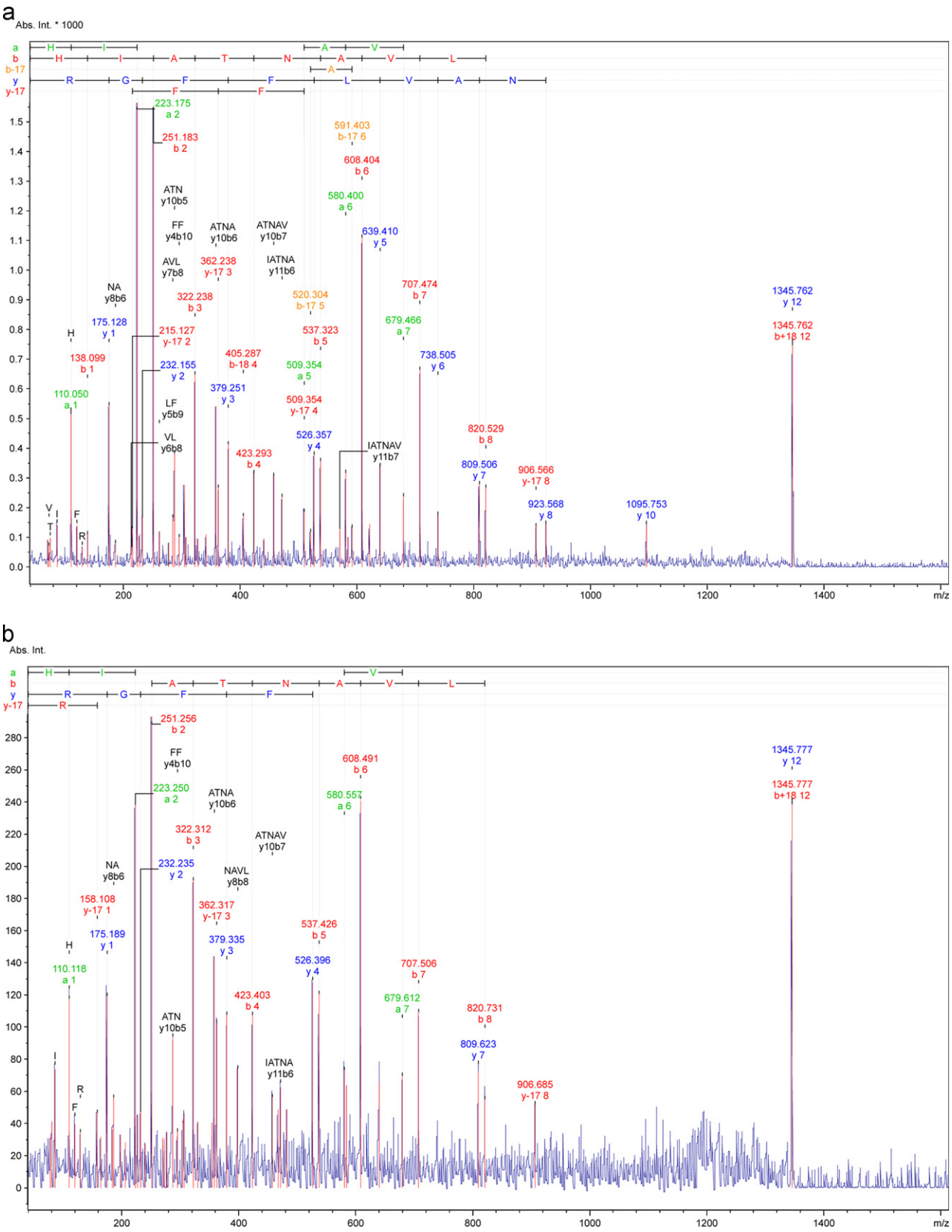

Fig. 2. MS/MS spectra of parent ions at m/z 1345.8, identifying peptide sequence HIATNAVLFFGR from chicken Ovalbumin (OVAL_CHICK) in model samples with egg glair and (a) zinc white (9M11) or (b) French ochre (9M21).

presence is determined in general. For the sample B16, PMF database search feedback is pointing to the bovine Collagen alpha-1(I) chain as the best hit with Mascot score of 70. PMF spectra of other analyzed samples are containing peaks found to be characteristic for different collagen chains (peaks at m/z 1105.6, 1427.7, 1435.7, 1459.7, 1560.8, 1586.8, 1975.9, 2056.1, 2131.1 and 2705.1), but in some cases not enough information for protein identification. In most of quarries protein identification was

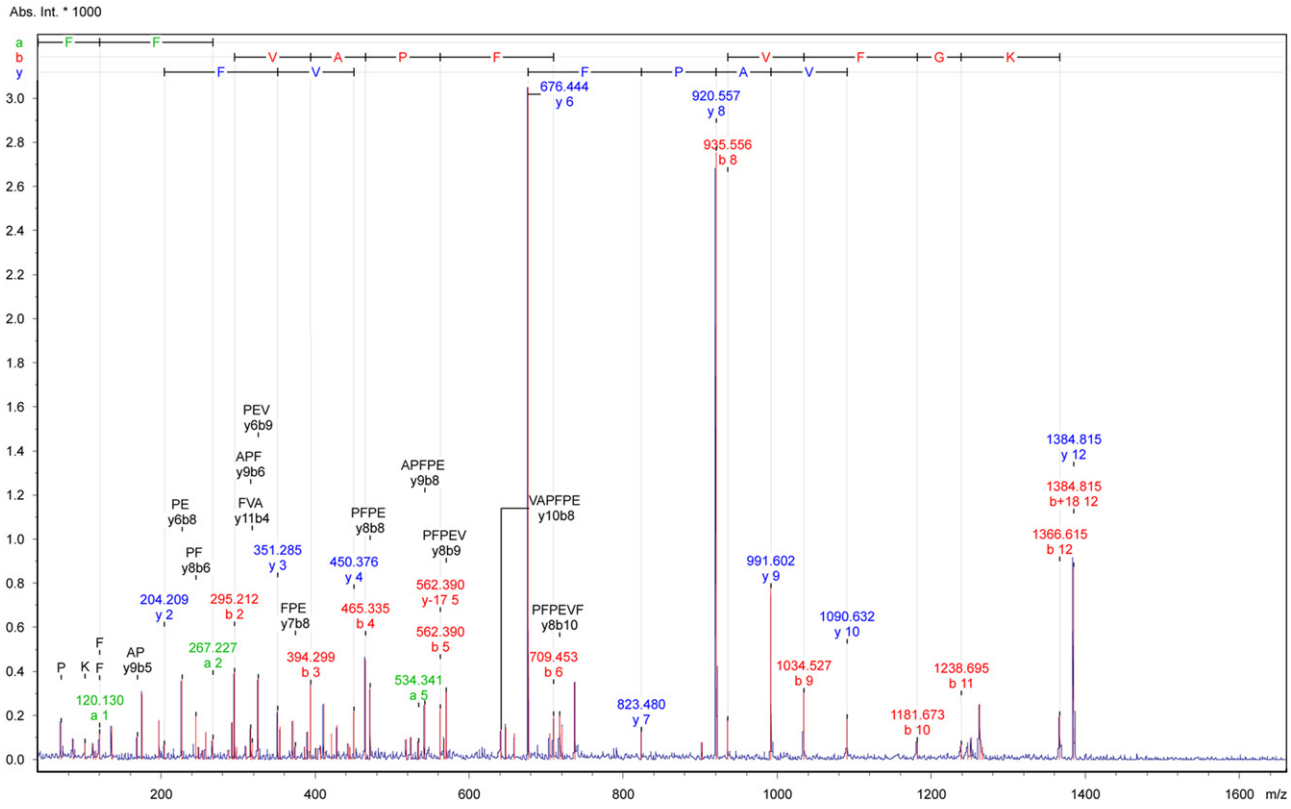

Fig. 3. MS/MS spectrum of the parent ion at m/z 1384.815, identifying peptide sequence FFVAPFPEVFGK from bovine Alpha-S1-casein (CASA1_BOVIN) in model sample with bovine cheese and zinc white (7M11).

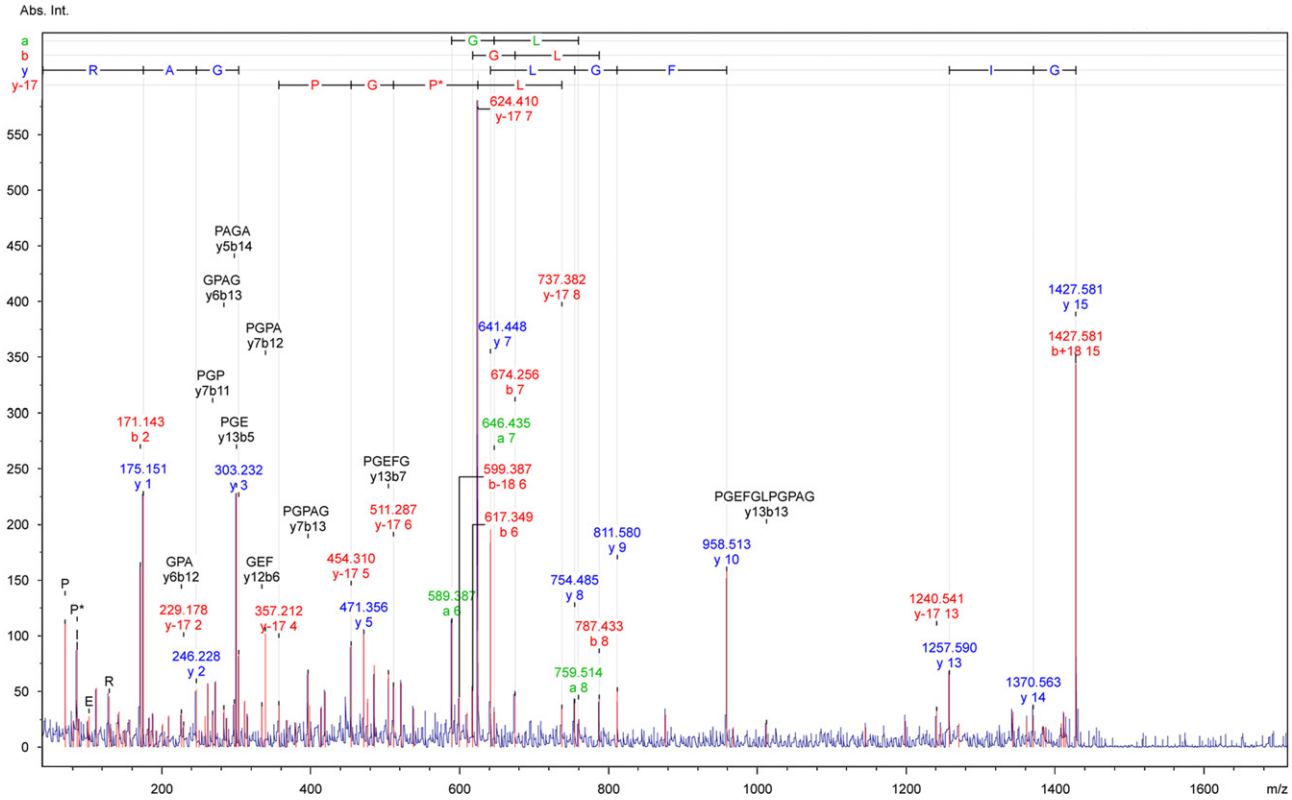

Fig. 4. MS/MS spectrum of the parent ion at m/z 1427.749, identifying peptide sequence GIPGEFGLPGPAGAR 3: Oxidation (P) 9: Oxidation (P) from bovine Collagen alpha-2 (I) chain (CO1A2_BOVIN) in model sample with rabbit skin glue and zinc white (3M11).

Table 3
Inconsistencies in the peptide annotations by PMF and MS/MS analysis that have emerged in this study.

Sample	Proteins identified	Sequences identified by PMF analysis					Sequences identified by MS/MS analysis				
		Identified peptides	Range	Calc. MH+	Meas. m/z	Modifications	Identified peptides	Range	Calc. MH+	Meas. m/z	Modifications
Egg yolk	**Vitellogenin-2** VIT2_CHICK	ADTYFDNYR	633–641	1164.496	1164.479		FLEVVQLCR	333–341	1164.608	1164.576	8: Carboxymethyl (C)
		NAVSFGHSWILEEAPCR	1740–1756	1973.917	1973.776	16: Carboxymethyl (C)	NSIAGQWTQPVWMGELR	774–790	1973.954	1973.776	6: Deamidated (NQ)
Bovine quark cheese	**Alpha-S2-casein** CASA2_BOVIN	LTEEEKNR	168–175	1098.483	1098.518	2: Phospho (ST)	AMKPWIQPK	204–212	1098.613	1098.518	
Bovine bone glue (**63000**)	**Collagen alpha-1**(**I**) **chain** CO1A1_BOVIN	GPAGPQGPR	1084–1092	836.437	836.449		GPPGPQGAR	267–275	836.437	836.449	
Bovine hide glue (**63010**)	**Collagen alpha-1**(**I**) CO1A1_BOVIN	GEGGPQGPR	352–360	886.401	886.352	2 Oxidations (P)	GSEGPQGVR	361–369	886.438	886.352	
		GAAGEPGKAGER	586–597	1099.549	1099.429		GADGAPGKDGVR	751–762	1099.549	1099.429	
Rabbit skin glue, **refined** (**63025**)	**Collagen alpha-2**(**I**) **chain** CO1A2_BOVIN	KAVILQGSNDVELVAEGNSR	1286–1305	2115.104	2115.094	Oxidation	GLPGVAGSVGEPGPLGIAGPPGAR	881–904	2115.119	2115.094	2 Oxidations (P)
Egg white+Zinc white (**9M11**)	**Ovalbumin** OVAL_CHICK	VTEQESKPVQMMYQIGLFR	201–219	2285.130	2285.087	Deamidated (NQ)	VTEQESKPVQMMYQIGLFR	201–219	2286.114	2286.242	2 Deamidated (NQ)
Egg yolk+French Ochre (**10M21**)	**Vitellogenin-2** VIT2_CHICK	TVDLNNCQEK	182–191	1164.520	1164.518	Deamidated (NQ)	ADTYFDNYR	633–641	1164.496	1164.518	
		ILGIDSMFKVANK	1083–1095	1436.782	1436.766	12: Deamidated (NQ)	DASFIQNTYLHK	1008–1019	1436.717	1436.766	
		TVDLNNCQEKVQK	182–194	1560.769	1560.791	N-Term: Acetyl	SPQVEEYNGVWPR	74–86	1560.744	1560.791	
Sample B4 from the *Icon of Jesus Christ*	**Collagen alpha-2**(**I**) **chain** CO1A2_BOVIN	GEPGAVGQPGPPGPSGEEGKR*	359–379	2055.921	2055.922	6 Oxidations (P)	EGPVGLPGIDGRPGPIGPAGAR	463–484	2056.093	2056.169	Oxidation (P)

* Sequence identified by PMF analysis in Bovine bone glue sample (63000).

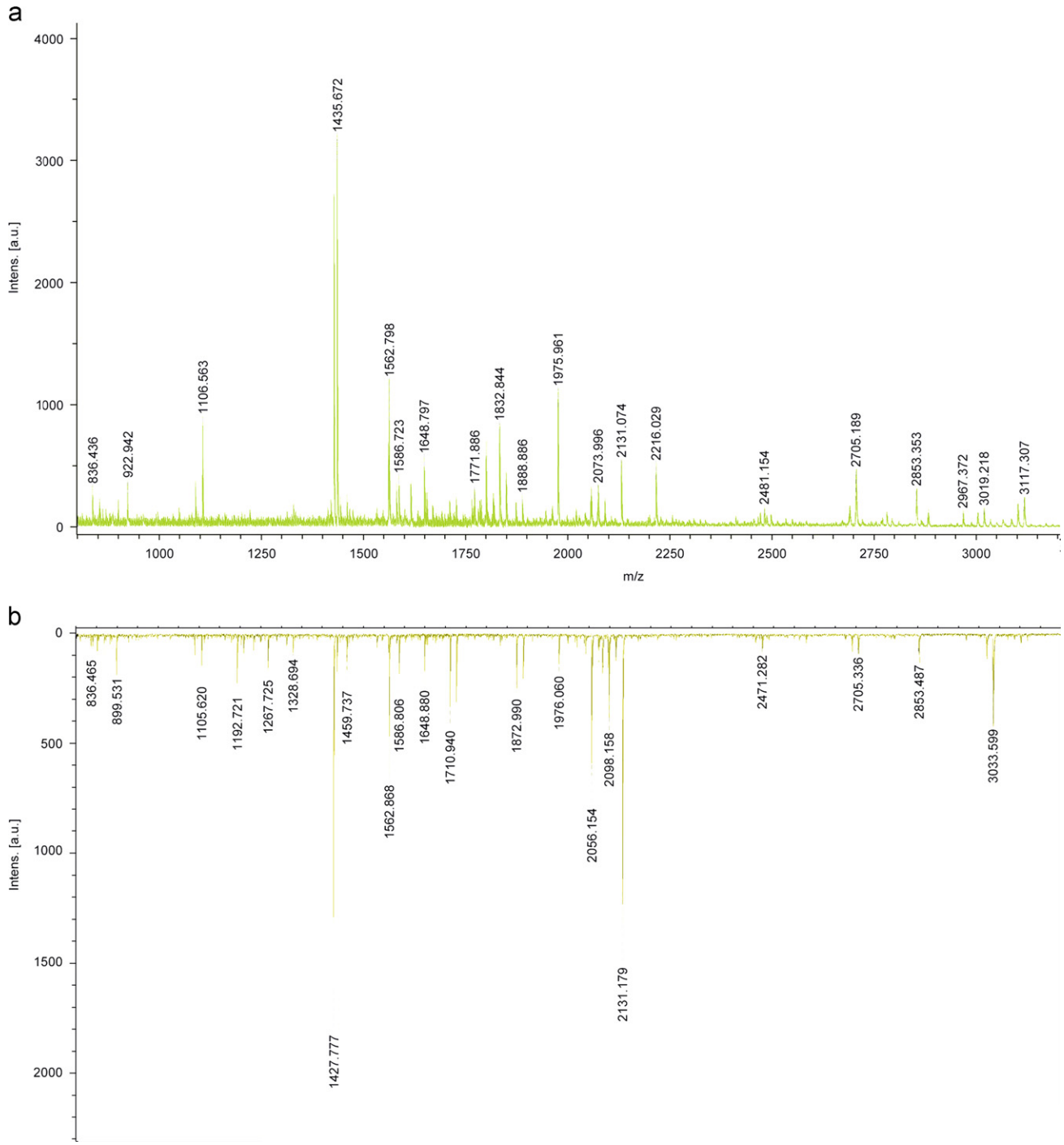

Fig. 5. Head to tail PMF MALDI-TOF spectra recorded for the sample from the iconostasis of church of the Holy Virgin in Barič B15 (a) and reference sample of rabbit glue (63025) (b) illustrates mutual similarities.

uncertain because of low Mascot scores, while in some was completely omitted. As example, the MS spectrum recorded for the painting sample B15 from the Icon of the Holy Virgin is given on Fig. 5 and compared to that of the rabbit skin glue. The best hit for sample B15 during PMF database search was the bovine Collagen alpha-1(I) chain with Mascot score 34, although many of peaks characteristic for collagens are present.

MS/MS analysis confirmed the presence of animal glues in all samples, with high or low ion scores, depending on concentration of binder and sample quantity. Some of sequences, pointed before as characteristic for collagen are found. Fig. 6 represents fragmentation spectrum obtained for the parent ion at m/z 1427.7 for the sample B4.

Identified sequence was GIPGEFGLPGPAGAR+32 Da from Collagen alpha-2(I) chain *Bos Taurus*. From the database is also proposed presence of two proline hydroxylations as PTM at position 3 and 9. Previously, the same peptide sequence was identified in model samples and reference materials. It is the one of the most commonly identified sequences in analyzed historical painting samples together with GLPGVAGSVGEPGPLGIAGPPGAR +48 Da (m/z 2131.2) from Collagen alpha-2(I) chain *Bos Taurus*, GEPGPAGLPGPPGER+48 Da (m/z 1435.7) and

Fig. 6. MS/MS spectrum of the parent ion at 1427.733 in sample B4 from the iconostasis of church of the Holy Virgin in Barič, identifying sequence GIPGEFGLPGPAGAR (containing two hydroxylations of proline at positions 3 and 9) from bovine Collagen alpha-2(I) chain (CO1A2_BOVIN).

GSAGPPGATGFPGAAGR+32 Da (m/z 1459.7) from Collagen alpha-1 (I) chain *Bos Taurus*, as well as GEPGPAGSVGPVGAVGPR (m/z 1560.8) from Collagen alpha-2(I) chain *Canis familiaris*. Full data are provided in Supplementary materials.

Many publications dealing with the identification of proteinaceous binders in painting samples [24,29,30,33] came up with similar findings. They identified animal glue in samples without reference to the possibility of presence of other binders, such as egg yolk. It is well documented that egg yolk proteins, having lower proportion of stable amino acids and higher concentrations of reactive amino acids (methionine and cysteine), are more susceptible to the effects of aging and photo-oxidation processes than animal glues [40,44,45]. Moreover, animal glue in ground layer is protected by surface painting layers and therefore is usually better preserved [39]. We found no evidence of egg proteins by this technique, but that does not mean that painting technique was distemper unambiguously. It is more likely to assume that collagen is present in primers, because rabbit skin glue, has been widely used in the iconography for the preparation of ground layers [1].

4. Conclusion

Although the power of mass spectrometry methods for protein identification is undoubted, the presence of large amounts of contaminants in complex samples, such as samples from paintings, can seriously prevent the obtaining of clear-cut results. The proteomic analysis proposed here was the softest treatment that could be applied and integrity of the protein molecule is not required for reliable results. Just a few peptides can be extracted from the material by the trypsin hydrolysis, and their identification is sufficient for successful protein identification.

Overall, tandem mass spectrometry and peptide mass fingerprint data, allowed, in all model samples, successful identification of the proteinaceous binders, regardless the pigment used. The number of peptide sequences enough for protein identification is not well defined, but suggestions are that a reliable identification of a protein requires at least two peptides with a reasonable score.

Comparing the results obtained by the PMF approach and MS/MS analysis, reduced number of identified peptides is evident. Regardless of that, protein identification is more reliable. MS/MS approach increases the chances of identifying protein binders in contrast to PMF method in the case of model and real samples. PMF method using Mascot database search is dependent upon a peak list length. The consequence is that when number of peptides is reduced, MS/MS identification is more trustworthy. Peptide qualification using additional information contained in fragmentation spectra besides m/z value increases the chance of protein identification.

In historical painting samples, PMF analysis supported with MS/MS analysis, despite low concentration of proteinaceous materials, changes due to aging processes and high concentrations of pigments, succeeded in recognition of animal glue as binder. Several peptide sequences from different collagen chains are found. It was enough to conclude that samples are containing animal glue, probably in ground layer, but precise origin of collagen was not determined. The major causes which prevented provenance determination of found animal glues were lack of detailed information about collagens of various animal origins in SwissProt database and extensive homology between animal glues because of very small evolutionary differences in collagen structure.

The results of the research reported here illustrate that proteins present as binding media may be successfully identified by applied technique and, to our knowledge, this is the first time that protein binders in old paintings are identified by MALDI-TOF mass

spectrometry supported by MALDI-TOF/TOF tandem mass spectrometry using a proteomic approach. The aim of this work was not to confirm results obtained by PMF approach, but to accentuate the difference between PMF and TOF/TOF results. It is shown that by MS/MS fragmentation of few peptides in degraded binder, protein identification can be achieved in contrast to PMF approach where higher number of peptides is necessary. MS/MS approach increases the chances of appropriate protein binder identification by using additional information contained in fragmentation spectra.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.03.071.

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