Microchemical Journal 144 (2019) 319–328

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Identification of proteinaceous binders in paintings: A targeted proteomic approach for cultural heritage

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ARTICLE INFO

Keywords:
Multiple Reaction Monitoring (MRM)
Proteomics
Ancient proteins
Cultural heritage
Paintings
LC-MSMS

ABSTRACT

Identification of proteins in paintings and polychrome objects is a challenge, which requires the development of tailored analytical approaches. In the present study, a targeted proteomics approach was developed for discriminating among the three most common proteinaceous materials used as paint binders, i.e. milk, egg, and animal glue. In this study a specific database of peptides was created based on tandem MS analyses of tryptic digests of several paint samples collected from a variety of art objects of different ages and conservation conditions. Specific peptide markers of each protein were then selected and monitored by LC-MSMS in Multiple Reaction Monitoring (MRM) ion mode, together with their specific precursor ion-product ion transitions, as defined by their unique amino acid sequence. The developed method enabled a sensitive and reliable detection of the target peptides in a selection of case studies, leading to the unambiguous identification of the proteins used as paint binders. The method showed greatly increased sensitivity compared to currently available strategies.

1. Introduction

The inherently multidisciplinary nature of proteomics has recently led to its application to assorted areas that include the field of cultural heritage [1] for the identification of protein-based materials in artworks and polychromies, archaeological remains, and paleontological objects [2].

The most up-to-date approaches for the characterization of proteinaceous materials in artworks and polychromies, and in general when dealing with ancient proteins, rely on mass spectrometry technologies that are able to identify proteins from the MS analysis of their digested peptides [2,3] (and references therein). Typically, MS-based proteomics pipelines aimed at identifying proteins in ancient samples follow the classical bottom up approach, where samples are enzymatically digested to peptides, which are either analyzed by simple MS, or separated by liquid chromatography and eventually analyzed by tandem mass spectrometry. Peptides and thereof proteins are identified by correlating experimental spectra to virtual ones in a protein sequence database [4]. In the classical full scan operating mode (typical of LC-MSMS analysis in discovery proteomics or shotgun proteomics, otherwise named global profiling proteomics or untargeted proteomics), the mass spectrometer continuously repeats the full scan mass spectrum and selects and fragments the n (typically $n = 1–10$) most abundant ions (information-dependent acquisition, IDA).

So far, the applications to the cultural heritage field are untargeted, discovery proteomics based experiments. The fascinating potential of this operating mode lies in the possibility of uncovering proteins without any "a priori" knowledge or hypothesis. It is therefore compatible with both well and poorly characterized systems in cultural heritage, the only basic requirements being the actual presence of proteins in the sample and the presence of the protein sequences or homologous sequences in a properly selected database [2,3].

Since the first report of LC-MSMS in 2006 [5], despite the tremendous success of these approaches in a wide range of works of art

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https://doi.org/10.1016/j.microc.2018.09.021
Received 18 June 2018; Received in revised form 7 September 2018; Accepted 18 September 2018
Available online 19 September 2018
0026-265X/

and archaeological remains [5–24] standard based proteomics strategies keep being developed to address the field-specific analytical challenges in samples from cultural heritage, by improving the steps in the standard proteomics protocols, from the solubilization step on [20,21,25–28]. So far, the greatest attention was addressed to the preparation of the sample, correctly considering the unusual nature/state of the sample itself as the biggest challenge in the analysis.

However, as far as the mass spectrometric analysis itself is concerned, some of the limits in the detection in a LC-MSMS analysis can also arise from the automated peak selection and the linked instrument's bias towards repeatedly selecting and fragmenting the species with the most intense MS signals. The complexity of these unusual samples in works of art and archaeological remains, in terms of chemical composition, the unavoidable presence of contaminating protein-based materials (even coming from the environmental dust), as well as the high levels of molecular damage found in ancient samples, can overwhelm even the most modern mass spectrometer [3,21,25], sometime making the identification of protein components in these samples extremely challenging. This because the mass spectrometer might miss signals coming from important, relevant proteins, but instead might “waste its time” on signals from uninformative contaminants, for instance. Searching for significative signals in a multitude of untargeted signals might then become like looking for a needle in a haystack with the naked eye.

There are cases, however, such as the identification of the protein-based binders in paintings, where, in principle, the search could be basically simplified to a discrimination between the most commonly used materials, i.e., animal glues, egg, and milk casein. Therefore, the analysis can be “targeted” towards a search for the protein components of these materials, within a limited range of possibilities, in an alternative, focused approach that by selectively searching for a restricted set of marker proteins, can eventually guarantee a higher sensitivity and reduce the interference from contaminations as lowest as possible.

In these cases, a significant advance in the solution of the analytical challenge can be the multiple reaction monitoring (MRM) ion mode, an alternative mass spectrometric strategy, the actual gold standard in mass spectrometric technique for selectivity and high signal-to-noise ratio that selectively and non-redundantly search for specific ions of one or, more desirably, a few peptides that will unequivocally constitute protein markers to be identified as unique signatures for each binder. The mass spectrometer will therefore focus on selected signals, getting rid of the “noise” of the other ions from the background, thus sensibly increasing sensitivity. Quoting the commentary on Nature Methods on 2013 [29], MRM “can be thought of as the mass spectrometrist's ELISA. It targets proteins using a predetermined assay with high sensitivity and selectivity [30]: molecular ions of a target peptide are selected in the first mass analyzer, fragmented in the collision cell and one or several of the fragment ions uniquely derived from the target peptide are measured by the second analyzer. MRM is referred to as targeted approach as only predetermined ions are measured, and mass spectrometer acts as a mass filter selectively monitoring a specific analyte molecular ion and one or several fragment ions generated from the analyte. MRM has been first developed and applied for decades in the pharmaceutical industry to quantify small molecules [31] and evolved to be applied in the field of proteomics, to detect low abundant proteins in complex matrices, to verify biomarker candidates, and has proved to be successful in clinical settings [32–42].

Mass spectrometry-based strategies that search for specific peptides as markers in the analyses of protein-based binders have been reported [20,43–46]. However, in none of them, to the best of our knowledge, has ever been used to discriminate between collagen-, milk- and egg-based binders. We propose this technique as a probe to specifically identify peptide markers of proteinaceous materials in paintings. Herein, we developed and applied a method based on a targeted proteomic approach that makes use of tandem mass spectrometry in multiple reaction monitoring (MRM) ion mode to selectively monitor a number of specific peptides belonging to proteins markers of individual proteinaceous binders in paintings. This alternative strategy, by selectively and non-redundantly searching for specific signatures of the proteinaceous binders, could represent a significative advance in the solution of the analytical challenge set by ancient samples. Differently from previously reported methods [20,43,44,46] that also search for specific peptides but in untargeted, typical shotgun runs, where relative abundance of the single peptide ions can still strongly affect the results, MRM by its two-level mass filtering instrumental operating mode results in an increase of selectivity, and an incredibly high signal-to- noise ratio for the target analytes, getting rid of most of the nuisances arising from abundant contaminating proteins in the samples that can unavoidably affect, for instance, mass fingerprinting methods but also standard LC-MSMS approach.

In this work, the most prominent and specific protein markers for protein-based binder were chosen by screening a collection of LC-MSMS analyses of a panel of paintings/test samples that had been carried out during the years. Samples had different origin, had been prepared with different procedures, with different pigments, in order to be as general as possible in the selection of the target peptides, and provide a versatile tool that could be applied to any painting sample. Specific peptide markers of each protein were then selected on the basis of their frequency of detection in the set of samples and of their mass spectrometric behavior together with their specific precursor ion-product ion transitions as defined by their unique amino acid sequence. Then, a single MRM method was constructed to detect all the target peptides in a single analysis with high sensitivity, selectivity and accuracy, leading to the unambiguous discrimination among the different proteinaceous binders. The method was tested on a panel of samples of known composition and then successfully applied to a set of paint samples of unknown composition.

Results presented here demonstrate the unique ability of MRM method to selectively assess the nature of proteinaceous binders in paintings, and its successful application to samples where standard LC-MSMS analysis had failed, or to disclose previously undetected components.

2. Materials and methods

2.1. Reagents

Ammonium hydrogen carbonate (AMBIC), Ethylenediaminetetraacetic acid (EDTA); Tri(hydroxymethyl)aminomethane (TRIS), and TPCK-treated trypsin were from Sigma; recombinant Peptide N-Glycosidase F (PNGaseF) was from Roche. Formic acid and Acetonitrile (ACN) were purchased from Baker. Deionized water was obtained from Millipore cartridge equipment.

2.2. Painting samples

The list and the characteristics of paint replicas and historical samples used to build the database of selected peptides are reported in Table S1. Historical samples were from collections that have been published elsewhere [26,47–50], and criteria for sampling and description of the single cases are therein given.

Samples were motley analyzed in the course of the years, and were generally treated in heterogeneous phase with trypsin with variations in respect to the minimally [21] invasive protocol as reported in Table S1, and the resulting peptide mixtures were analyzed by LC-MSMS, as detailed in the therein indicated references [21,25,26,47–50]. For those samples that were used in the setup of the database that have not been published previously, digestion with trypsin, LC-MSMS analysis and data analysis were carried out as described in detail in the Supplementary information.

In order to build up the database, the proteins and the peptides most frequently detected in standard global profiling experiments were screened regardless the treatment procedures they had undergone and

the mass spectrometer that was used in the specific analysis. A relational database was designed on PivotTables (https://support.office.com) to simplify the data processing.

2.3. LC-MSMS analysis in multiple reaction monitoring ion mode

In MRM mode, two steps of mass filtering are used on a triple quad mass spectrometer. The technique is more sensitive than full scan MSMS because both Q1 and Q3 are parked on a single *m/z* ion, with both mass analyzers dwelling on a single ion.

In a typical MRM workflow, the targets must be selected. MRM-based assay usually starts with the selection of a signal providing optimal signal intensity and discriminating the target peptide from the other species. The predicted best transitions and collision energy to generate the maximal fragment intensities are then provided by in silico analysis by using Skyline software [51]. These data are then used for MRM method construction. The detailed information of the herein used MRM method is reported as follows.

Peptide mixtures were analyzed by LC-MSMS analysis using a Xevo TQ-S (Waters) with an UPLC Microflow Source coupled to an UPLC Acquity System (Waters) using an IonKey (Advion) device. For each run, 1 µl peptide mixture was injected and separated on a TS3 1.0 mm × 150 mm analytical RP column (Waters, Milford, MA, USA) at 60 °C with flow rate of 3 µl/min. Peptides were eluted (starting 1 min after injection) with a linear gradient of eluent B (0.1% Formic acid in 100% ACN) in A (0.1% Formic acid in water (LC–MS grade)) from 7% to 95% in 55 min. The column was re-equilibrated at initial conditions for 4 min with eluent A. MRM mass spectrometry analyses were performed in positive ion mode. Skyline Software drew the choice for the selection of virtual best transitions and collision energy calculated to generate maximal fragmentation intensities for each analyte. The developed MRM method was used with MRM detection window set to 0.5–1.6 min per peptide, the duty cycle was set to automatic and dwell times were minimal 5 ms. Cone voltage was set to 35 V. The mass spectral MRM parameters are reported in Table S2.

3. Results

3.1. Database setup and selection of the binder specific proteins

Results from LC-MSMS analyses of a collection of 105 samples from artistic and archaeological samples (Table S1), which had showed to contain milk, animal glue and egg, were screened to identify specific protein biomarker candidates for each material. Milk proteins were found in 43 samples, animal glue in 29 samples and egg in 43 samples. Table S3 shows the details of the identifications for all the samples that were inserted in of the database that had not been published elsewhere.

Fig. 1 reports the summary of the results relative to the samples that contained milk. Whenever milk was detected, alpha S1 casein was always identified, while beta casein was identified in 32 samples out of 43, and alpha S2 casein observed in 30 samples (Details in Table S4). Similarly, in the animal glue containing samples, as expected, two collagen proteins were detected in almost all samples, collagen alpha-1(I) identified in all samples and collagen alpha-2(I) in 28 out of 29 samples (Fig. S1, and details in Table S5).

The case of egg binder was more complex since artists could alternatively use either albumen or yolk or whole egg. Moreover, pure albumen can be quite easily obtained from a whole egg whereas yolk is very likely contaminated by egg white. Therefore, the results obtained with the 43 egg containing samples were evaluated by dividing them into two subgroups of samples, i.e. those containing only albumen (27 samples) and those containing yolk plus albumen (16 samples). No sample was considered as a pure yolk. As expected (Fig. S2, and details in Table S6), chicken ovalbumin, the main protein of egg white, was identified in all samples containing only albumen (27 out of 27) and in 38 out of the total 43 samples including those containing also egg yolk.

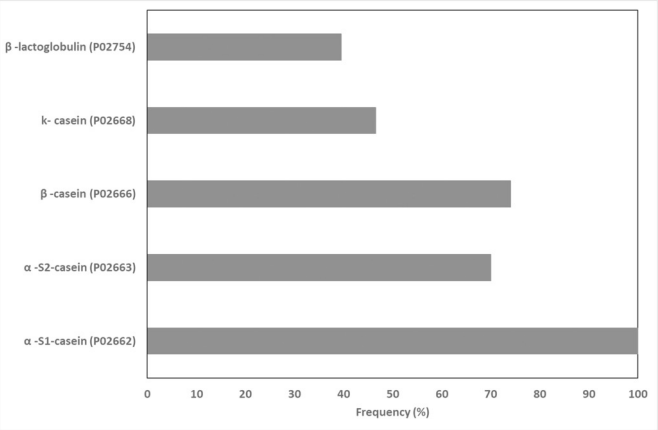

Fig. 1. Graphical representation of proteins occurrence in 43 milk containing samples. The occurrence has been reported as percentage of the analyzed samples. The UniProt code is reported in brackets. Details of the proteins occurrence are shown in Table S4.

Similarly, ovotransferrin was observed in 21 out of 27 albumen only samples and in 8 of the samples containing yolk plus albumen, thus accounting for 29 out of 43 total samples. Vitellogenin-1 and vitellogenin-2 were the most frequently identified proteins from yolk, observed in 12 and 14, out of 16 samples containing egg yolk.

From the results obtained, alpha S1 casein, alpha S2 casein and beta casein were selected for the subsequent selection of the peptides to be used for the identification of milk, collagen alpha-1(I) collagen alpha-2(I) for animal glue, ovalbumin and ovotranferrin as generic representative of egg containing binders and vitellogenin-1 and vitellogenin-2 specifically for yolk containing binders.

3.2. Selection of the target peptides

Unique peptides belonging to the target proteins characteristic of each proteinaceous binder were thus selected to develop the MRM assay. Specific peptides were chosen on the basis of their frequency of observation and quality of fragmentation spectra in the LC-MSMS analyses. As an example, Fig. 2 reports the frequency of observation of the alpha S1 casein peptides in the milk containing samples. The peptide YLGYLEQLLR encompassing the 106–115 region of the alpha S1 casein was observed in 77% of cases while the peptide FFVAPFPEVFGK, encompassing the 38–49 sequence was identified in 74% of cases and the peptide HQGLPQEVLNENLLR, position 23–37, in 53%. These three peptides were recognised as the most recurrent in milk binder and were thus selected as suitable targets for the development of the MRM method. Similarly, four peptides from alpha S2 casein and two peptides from beta casein were selected as the most frequently detected in the LC-MSMS analyses (Fig. S3 and S4, in the Supplementary material).

These peptides were also considered to investigate whether they could be used to discriminate among the different milk sources i.e. either bovine or ovine milk. The biological origin of proteins in samples from cultural heritage can be ascertained by identifying species-specific peptides, and is an attractive and still challenging problem [6,17,19,20,47] that can in principle be tackled by MRM approach. Two of the selected peptides from alpha S1 casein and three peptides from alpha S2 casein were demonstrated to be able to discriminate bovine and ovine milk [47]. In particular, FFVAPFPEVFGK and HQGLPQEVLNENLLR are specific of bovine alpha S1 casein, and FALPQYLK, AMKPWIQPK and NAVPITPTLNR of bovine alpha S2 casein, respectively. Similarly, the orthologues peptides FVVAPFPEVFR and NAGPFTPTVNR that are specific to goat alpha S1 casein and goat alpha S2 casein, respectively, were also inserted in the MRM method (Table S2).

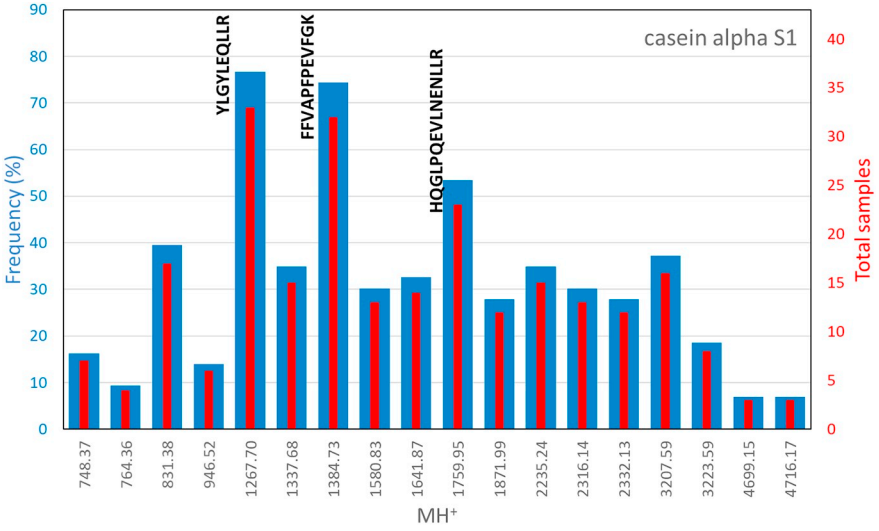

Fig. 2. Frequency of alpha S1 casein detected peptides in milk containing samples. On the main vertical axis the percentage is reported as calculated in respect to the number of samples in the milk containing samples database The secondary vertical axis reports the actual number of observations. In the statistical analysis, detected peptides have been considered only once, regardless possible deamidation detection, while oxidated peptides were considered separately. The sequence of the selected peptides is also reported.

Table 1
Selected peptides: peptides selected upon the analysis of the occurrence in group of samples. The number of times the peptide has been observed and the corresponding percentage in the group of samples is reported. Position in the protein sequence is given as flanking apexes. For peptides from vitellogenin 1 and vitellogenin 2, the percentage in the set of yolk and albumen samples is also shown.

Sample group (number of samples)	**Protein** (UniProt entry)	**Peptide**	**MH+**	**No.of observations**	**%**	**% in the yolk and albumen samples**
Milk containing samples (43)	**Alpha S1 casein** (P02662)	^{106}YLGYLEQLLR^{115}	1267.70	33	77	
		^{38}FFVAPFPEVFGK^{49}	1384.73	32	74	
		^{23}HQGLPQEVLNENLLR^{37}	1759.95	23	53	
	Alpha S2 casein (P02663)	^{189}FALPQYLK^{196}	979.56	24	56	
		^{130}NAVPITPTLNR^{140}	1195.68	22	51	
		^{204}AMKPWIQPK^{212}	1098.61	15	35	
		^{96}ALNEINQFYQK^{106}	1367.7	19	44	
	Beta casein (P02666)	^{192}AVPYPQR^{198}	830.45	30	69	
		^{199}DMPIQAFLLYQEPVLGPVR^{217}	2186.17	19	44	
		^{199}DMPIQAFLLYQEPVLGPVR^{217} + Oxi (M)	2202.16	23	39	
Animal glue containing samples (29)	**Collagen alpha-1(I)** (P02453)	^{1062}SGDRGETGPAGPAGPIGPVGAR^{1083}	1975.99	23	79	
		^{1084}GPAGPQGPR^{1092}	836.44	17	59	
		^{958}GVVGLPGQR^{966} + Hydroxy (P)	898.51	21	72	
	Collagen alpha-2(I) (P02465)	^{326}GIPGPVGAAGATGAR^{340} + Hydroxy (P)	1267.67	16	55	
		^{572}GIPGEFGLPGPAGAR^{586} + 2 Hydroxy (P)	1427.73	19	65.5	
		^{1066}IGQPGAVGPAGIR^{1078}	1192.68	19	65.5	
Albumen containing samples (43)	**Ovalbumin** (P01012)	^{265}LTEWTSSNVMEER^{277}	1581.72	23	53	
		^{128}GGLEPINFQTAADQAR^{143}	1687.87	32	74	
		^{324}ISQAVHAAHAEINEAGR^{340}	1773.90	30	70	
		^{144}ELINSWVESQTNGIIR^{159}	1858.97	32	74	
	Ovotransferrin (P02789)	^{595}ANVMDYR^{601}	868.40	21	49	
		^{120}GTEFTVNDLQGK^{131}	1308.64	23	53	
		^{289}AQSDFGVDTK^{298}	1067.50	23	53	
		^{275}DDNKVEDIWSFLSK^{288}	1695.82	26	60	
		^{155}GAIEWEGIESGSVEQAVAK^{173}	1959.96	26	60	
		^{141}SAGWNIPIGTLLHR^{154}	1534.85	25	58	
Yolkc ontaining samples (15)	**Vitellogenin-1** (P87498)	^{831}LTELLNSNVR^{840}	1158.65	4	9	25
		^{472}SNIEEVLLALK^{482}	1228.71	4	9	25
		^{315}LQDLVETTYEQLPSDAPAK^{333}	2118.06	4	9	25
		^{813}VAGNVQAQITPSPR^{826}	1437.78	6	14	37.5
	Vitellogenin-2 (P02845)	^{1515}MVVALTSPR^{1523}	973.55	10	23	62.5
		^{456}EALQPIHDLADEAISR^{471}	1777.91	10	23	62.5
		^{260}QQLTLVEVR^{268}	1085.63	13	30	81
		^{642}VGATGEIFVVNSPR^{655}	1445.77	12	28	75
		^{226}QSDSGTLITDVSSR^{239}	1465.71	11	26	69
		^{240}QVYQISPFNEPTGVAVMEAR^{259}	2236.11	11	26	69
		^{919}NIGELGVEKR^{928}	1114.62	7	16	44
		^{1543}LPLSLPVGPR^{1552}	1048.65	8	19	50

The analysis of peptides from animal glue samples lead to the selection of three peptides for the definition of collagen alpha-1(I) and three peptides for collagen alpha-2(I). Non-hydroxylated or unambiguously singly hydroxylated peptides were preferred, since hydroxylation in different position within the same peptide leads to different ions upon fragmentation; however, a peptide of collagen alpha-2(I) with multiple hydroxylation sites was also considered as a test, and the transitions for the different possibilities were separately considered (Tables 1 and S2).

Peptides identified in egg binder showed a larger distribution of observation frequency, making the selection of the marker peptides more complex. A higher number of peptides was therefore selected for ovoalbumin, ovotransferrin, vitellogenin-1 and vitellogenin-2 (four, six, four and eight peptides respectively), in order to maximise our probability of detecting egg in the MRM analysis of samples of unknown composition.

Table 1 reports the ultimate list of the selected peptides with the frequency of observation in the set of paintings/test samples analyzed. To design the MRM method, the selected peptides were thus analyzed in silico using the Skyline software to determine the predicted best transitions and collision energy to generate maximal fragmentation intensities. Table S2 reports the full list of defined peptides for each single target protein of the various proteinaceous binders, with the specific transitions and collision energy that have been used to develop the MRM method.

3.3. MRM analysis

A single MRM method able to detect in a single run the presence of egg, animal glue and milk proteins in samples from cultural heritage was thus developed. This method contains all the precursor ion-daughter ions transitions associated with the selected target peptides constituting the protein signature of each individual proteinaceous binder (see Table S2).

As a preliminary check of the developed assay, some samples with known composition, (containing either milk, animal glue or egg as single proteinaceous component, as well as some combinations of them), were used as test and were analyzed by using the newly set-up MRM method. As an example, Fig. 3 shows the MRM TIC chromatograms for the transitions selected for the peptide 106–115 of bovine alpha S1 casein. All the signals were recorded at the same retention time, thus confirming that the transitions were originated by the same peptide. Table S7 reports the transitions detected in the MRM analyses of the test samples with known composition. The method showed the

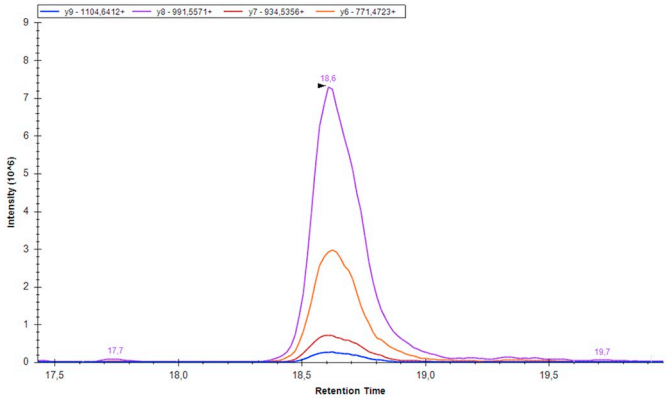

Fig. 3. Test specimen MRM analysis. MRM TIC chromatograms for the best four transitions (634.35 > 1104.64, 634.35 > 991.56, 634.35 > 934.53, 634.35 > 771.47) related to ^{106}YLGYLEQLLR^{115} peptide belonging to alpha S1 casein from *Bos taurus*. The perfect co-elution of the monitored transitions for the selected peptide, at 18.6 confirmed the presence of milk in the analyzed sample and the high selectivity and specificity of the developed method.

transitions associated to the target peptides leading to the unambiguous identification of the specific protein and thus to the assessment of the individual proteinaceous binder. Moreover, in the test sample E17 four peptides from collagen proteins and in the test sample G9 three peptides from vitellogenin-2 could be clearly detected indicating the presence of contaminating material besides the expected binders, contamination possibly occurring during the preparation of the painting tests themselves.

3.4. Analysis of historical samples

The developed MRM approach was thus applied to a sample from the paintings of the Monumental Cemetery of Pisa that had previously been examined by a standard proteomic approach [48]. Several transitions that were ascribed to peptides from caseins and collagens could be detected, thus confirming the occurrence of milk and animal glue (Table S8). Moreover, most interestingly, MRM transitions of peptides from ovalbumin, ovotransferrin and vitellogenin 2 could also be clearly observed indicating the presence of egg within the sample (Fig. 4). It should be underlined that no egg proteins were identified in previous experiments carried out by the standard LC-MSMS procedure [48], thus confirming the higher sensitivity achieved with the MRM assay. Furthermore, when the MRM analysis was carried out on a second sample from the Monumental Cemetery painting that had been previously submitted to a deglycosylating pretreatment with N-Glycosidase F, an even higher number of peptides from egg proteins were detected (Table S8), accordingly with the observation that such a step improves accessibility to protease of the polypeptidic chain of egg proteins [26]. It is worth mentioning, however, that even after the deglycosylation step, no egg proteins were detected in untargeted LC-MSMS analysis carried out on another aliquot of the same sample, possibly because of the overwhelming presence of milk and animal glue proteins. The presence of egg in these samples is not surprising, as it has been previously shown, by means of GC/MS based approaches, that egg was often present in samples collected from the Monumental Cemetery paintings, as it was used as paint binder in other selected areas of the mural paintings [52–54].

Paint samples from the polychromies of the giant Buddha statues from the Baymian valley in Afghanistan that had already been analyzed by standard LC-MSMS procedure [47] were also subject to the developed MRM assay. The detection of several mass transitions associated to the specific peptides from caseins confirmed the presence of milk in the binder, as already assessed by previous analyses [47] (Table S8). The greatly improved performances of MRM procedure were demonstrated by the analysis of some samples from the Buddha statues for which untargeted LC-MSMS analysis provided no results. Fig. S3 shows the MRM analysis of the fragments from the Western Buddha 22-1 [47] clearly displaying the occurrence of several transitions associated to two peptides from bovine alpha S1 and one from alpha S2 caseins, thus confirming the presence of milk also in this sample. Similarly, the MRM approach was able to detect and identify the binder in other three Buddha samples that were negative in the standard LC-MSMS analyses [47] (Table S8). Moreover, since we have previously demonstrated that a mixture of bovine and ovine milks was used as binder in the decoration of the Buddha statues [47], we took advantage of this observation to test the capability of our MRM methods to distinguish highly similar protein sequences even in ancient samples, and eventually provide a tool that could be useful in the challenging problem of identifying organism species. We therefore introduced in the MRM method the transitions for two peptides from alpha S1 and alpha S2 casein from *Capra hircus*, that are homologous to two peptides in the corresponding alpha S1 and alpha S2 casein from *Bos taurus* (Table S2). The concomitant detection of the peptides from ovine and bovine caseins in the MRM analyses confirmed that a mixture of bovine and ovine milks was used as binder [47]. Accordingly, the MRM chromatograms of the sample 214-2 [47] (Fig. 5) clearly displayed the specific

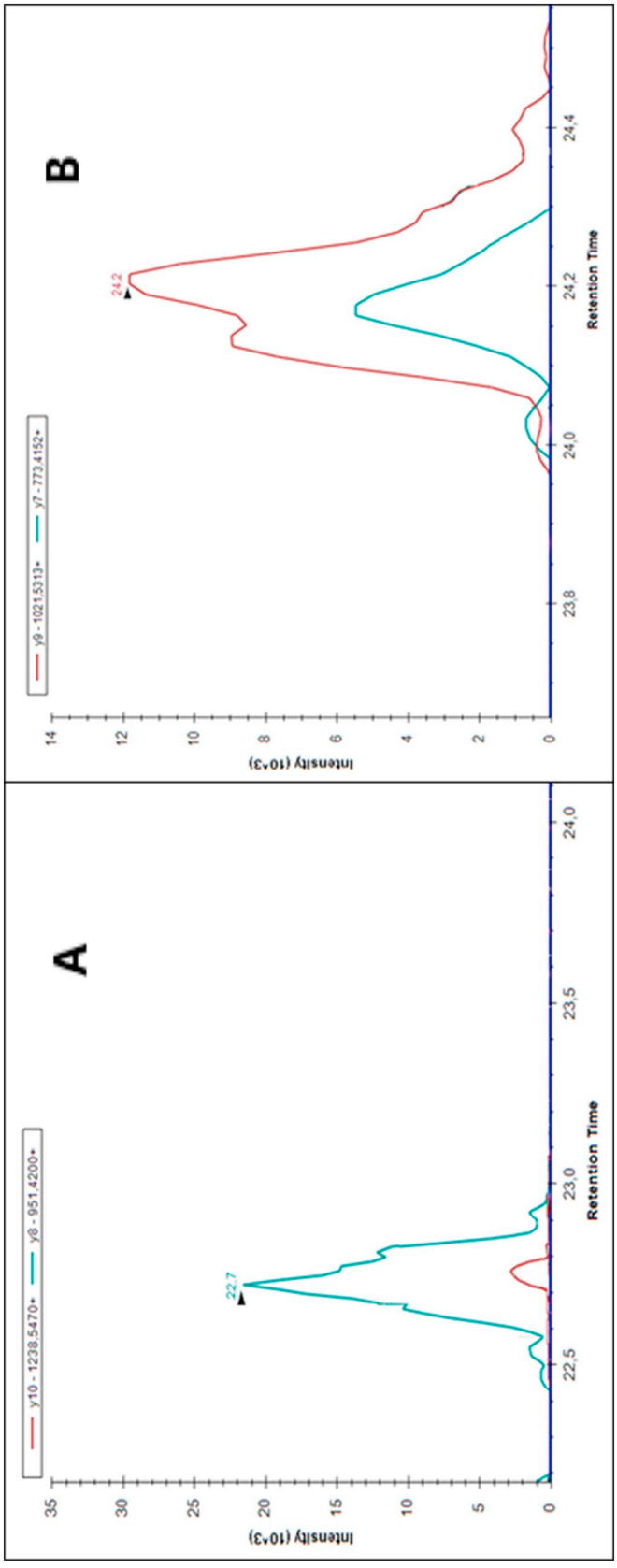

Fig. 4. Monumental Cemetery sample. Panel A: the MRM analysis on Monumental Cemetery sample showed the presence of unidentified egg's proteins from previous LC-MSMS analyses. This data demonstrates the higher sensitivity of the MRM approach and in particular in Panel A representatives MRM TIC chromatograms of monitored transitions 791.36 > 1238.59 and 791.36 > 951.42 for the ^{265}LTEWTSSNVMEER^{277} peptide of Ovalbumin are shown. In Panel B, representatives MRM TIC chromatograms of monitored transitions 654.83 > 1021.53 and 654.83 > 773.41 for ^{120}GTEFTVNDLQGK^{131} peptide of Ovotransferrin are reported.

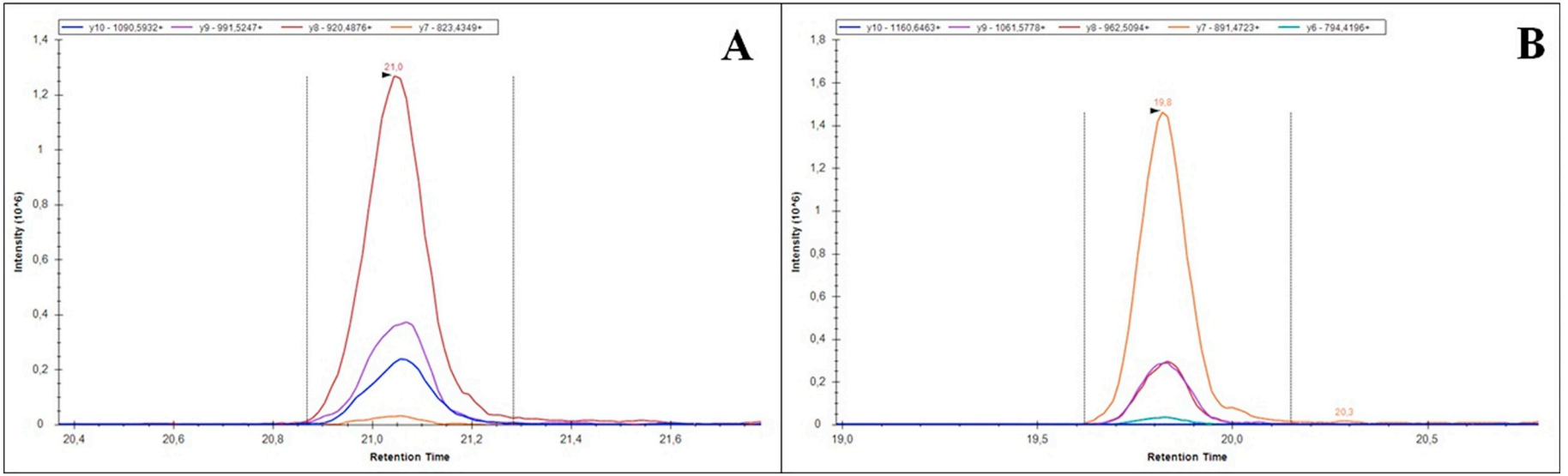

Fig. 5. Buddha sample 214 Clay. Panel A shows representatives MRM TIC chromatograms related to 692.86 > 1237,66, 692.86 > 1090.59, 692.86 > 991.52, 692.86 > 920.49, 692.86 > 823.43 transitions monitored for ^{38}FFVAPFPEVFGK^{49} peptide belonging to alpha S1 casein of *Bos taurus*. Panel B shows representatives MRM TIC chromatograms related to the five monitored transitions (654.35 > 1160.64, 654.35 > 1061.57, 654.35 > 962.50, 654.35 > 891.47, 654.35 > 794.42) for ^{38}FVVAPFPEVFR^{48} peptide for alpha S1 casein of *Capra hircus*. These reported data unambiguously confirmed the presence of bovine and ovine milk in the Buddha 241 Clay sample.

mass transitions for the FFVAPFPEVFGK (Fig. 5A) and FVVAPFPEVFR (Fig. 5B) peptides from bovine and ovine caseins, respectively.

These results clearly demonstrated that the targeted proteomic approach was able to identify proteins that were not detected with a standard proteomics approach, that is capable of identifying proteins in complex mixtures, and distinguish homologous peptides.

4. Discussion

The field of diagnosis in cultural heritage is continuously in search for more and more reliable and alternative methods to identify the materials used in a work of art while preserving as much as possible the artistic object itself [55]. While fully noninvasive techniques will always be preferred as first choice, the development of very sensitive methodologies will greatly reduce the impact of minimal invasive procedures. Identification of proteinaceous binders in paintings was successfully addressed by proteomics approaches providing informative results on precious and irreplaceable samples ([2] and references therein). This paper reports an evolution of this process turning from an untargeted to a targeted proteomics strategy in which only specific peptides representative of selected, relevant proteins, are searched to discriminate between the three most common typologies of protein-based binders in paintings.

Once defined the list of specific proteins most commonly detected in each binder-milk, animal glue and egg based- the selection of diagnostic peptides and the corresponding set of suitable mass transitions needed to develop the MRM method constitutes the critical step. Predictive models based on experimental data [56] have proven to be extremely powerful in the selection of proteotypic peptides in targeted proteomics, and several software packages exist that facilitate this step of the assay development [57]. However, among the factors that could affect the effective detection of peptides in this specific application, the peculiar, solid and aged nature of the samples was considered, and a more empirical approach to the selection of target peptides was herein used.

As a large cohort of painting samples had already been examined during the years in our laboratory by standard LC-MSMS analyses, we screened the results in search for peptides most frequently detected in these untargeted experiments for the three classes of protein-based binders. Discriminative peptides of caseins and collagen that could be considered as representatives of milk and animal glue binders were easily selected. Moreover, an appropriate selection of casein peptides was also used as test to distinguish the animal origin of milk, a specific request in case of milk binders.

Selection of peptides representative of egg binders was more challenging for a number of reasons. First, as stated above, artists used either albumen or yolk or the whole egg and often yolk was contaminated by albumen. Moreover, it has been demonstrated that the extensive glycosylation of egg proteins results in a poor production of suitable peptides and that the confidence of identification can be improved by the introduction of a deglycosylation step before trypsin digestion [26]. The molecular signature of egg binders was eventually defined by selecting ovoalbumin and ovotransferrin as generic representative of the presence of egg and vitellogenin 1 and vitellogenin 2 as specific markers of yolk binders.

The method was validated by analyzing model samples of known composition and was then used to characterize samples from ancient paintings and polychromies: the Monumental Cemetery in Pisa and the Giant Buddha statues in the Baymian valley in Afghanistan. These samples had already been analyzed by standard LC-MSMS procedure, and MRM analyses confirmed previous positive results, and provided successful identification in cases where untargeted procedures had failed, demonstrating the higher sensitivity and selectivity of this targeted approach. As an example, the overabundant milk and animal glue used in the restorations of the mural paintings of the Monumental Cemetery in Pisa prevented the identification of egg in standard LC-MSMS analyses [48], while the MRM analysis unambiguously revealed the presence of egg-derived peptides, possibly revealing the original binder, covered then by overabundant layers of materials used in restorations.

The higher sensitivity of the MRM procedure also allowed the identification of traces of milk proteins in some of the samples from the Giant Buddha statues for which the untargeted approach had failed [47]. Moreover, the specific case of the Giant Buddha statues was the test case to investigate the potentiality of MRM to distinguish highly similar protein sequences even in the case of ancient samples. This positive result can be considered as the preliminary test paving the way to the exploitation of the MRM assay in the challenging field of species identification. The biological origin of proteins by identification of species-specific peptides is an extremely challenging problem in several fields of the cultural heritage analysis [6,17,19,20,47] that could thus be faced by MRM approach, as we demonstrated by the specific case of distinguishing the animal origin of the milk.

In conclusion, we demonstrated that a targeted, MRM analysis can be successful in samples such as those from ancient objects. Searching for specific signals in a multitude of untargeted signals might be like looking for a needle in a haystack with the naked eye, while mass spectrometer working in a targeted mode could be considered as the use of a magnet in this search.

Acknowledgments

LB and AA conceived the project; RV constructed the databases, selected proteins and peptides for milk and egg containing samples, ADC the database and selected proteins and peptides for animal glue containing samples. AI and AC selected the transitions, developed the method and performed the MRM analyses. ALT prepared test samples. LB, AA, IB, PP and GM contributed to the paper elaboration and writing. Authors are indebted to Catharina Blaensdorf and Erwin Emmerling, Technische Universitaet Muenchen, Deutschland, for the samples from Buddha's statua, and to POR, Parco Archeologico Urbano di Napoli (PAUN) for funding.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2018.09.021.

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