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Deamidation at Asparagine and Glutamine As a Major Modification upon Deterioration/Aging of Proteinaceous Binders in Mural Paintings

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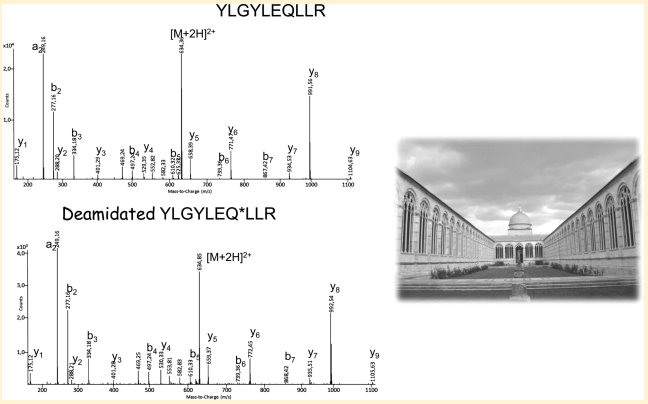
Supporting Information

ABSTRACT: Proteomic strategies are herein proved to be a complementary approach to the well established amino acid composition analysis for the characterization of the aging and deterioration phenomena occurring to proteinaceous materials in works-of-art. Amino acid analyses on several samples demonstrated that proteins in the frescoes from the Camposanto Monumentale in Pisa are deteriorated as revealed by the decrease in Met, Lys, and Tyr content and by the presence in all the samples of amino malonic acid as a result of Ser, Phe, and Cys oxidation. Proteomic analysis identified deamidation at Asn and Gln as a further major event occurred. This work paves the way to the exploitation of proteomic strategies for the investigation of the molecular effects of aging and deterioration in historical objects. Results show that proteomic searches for deamidation by liquid chromatography−tandem mass spectrometry (LC−MS/MS) could constitute a routine analysis for paintings or any artistic and historic objects where proteins are present. Peptides that can be used as molecular markers when casein is present were identified.



Safeguarding our cultural heritage is an important duty to ensure that future generations will have the opportunity to appreciate art masterpieces. Conservation must therefore be seen as one of the most exciting new fields and science can provide tools for the chemical, physical, and structural characterization of the materials used to create a work-of-art, and on the basis of this knowledge, ongoing degradation processes can be highlighted.

Among the materials composing the art object, organic materials used as binding media or protective coatings are the most subject to degradation phenomena or aging. Traditionally, a number of naturally occurring substances have been used in artworks given their ability to form homogeneous and flexible thin films when mixed with pigments and coloring materials on the appropriate supports.[1] Inevitably, these materials degrade with age, with the result that some features of the artist's work are more difficult to appreciate. Identification of the organic binders is of primary importance, and characterizing degradation products of the organic material and identifying specific markers which describe the degradation and aging processes undergone by the work-of-art is fundamental to better understand the history of a painting and to design appropriate restoration processes. The knowledge of original materials present in a work of art can in fact influence its cleaning, treatment, and storage and may also assist in the attribution of a painting to a particular workshop or artist.

The physicochemical analysis of paintings is particularly challenging due to the vast range of inorganic and organic materials which often constitute a work-of-art: canvas fibers, wooden supports and inorganic fillers, mixtures of binding media and pigments, metal decorations, varnishes, modern polymer treatments, and protective layers. The most common and often reliable analytical approaches for the analysis of organic painting materials are based on gas chromatography/mass spectrometry (GC/MS) and pyrolysis (Py)-GC/MS[2−4] analyses. However, although the paint media can be often reliably identified, changes in the composition, degradation phenomena, formation of minor components, and molecular modifications that have occurred cannot be completely understood by using only these approaches. The application of proteomics strategy to the characterization of proteinaceous binders in paintings has been recently reported in the literature.[5−8] So far, these studies were dedicated to the identification of proteinaceous paint media,

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rather than understanding modifications and degradation phenomena of the binders themselves.

Very little is known about aging and modifications induced in the proteinaceous binders. Proteinaceous materials loose water as soon as they are applied as a thin film in a paint layer. The most likely modification event of proteinaceous material is then oxidation. Aminomalonic aldehyde and acid are considered oxidation products of Ser, Phe, and Cys.[9] The oxidation of other amino acids was observed by fluorescence and Raman spectroscopy, suggesting that photooxidation of paint films can produce several fluorescent compounds, such as dityrosine, N Formil kyneurine (NFK), and 3,4-dihydroxy-phenyl alanine (DOPA), also depending on the simultaneous occurrence of pigments.[10] Moreover, it has been shown that the solubility of proteinaceous materials decreases with time and that some cations can give rise to analytical interferences, suggesting the formation of strong complexes with proteins.[4]

This paper presents some significant results obtained from the systematic characterization of proteinaceous binders aimed at identifying molecular markers to be used in detecting aged and degraded proteinaceous binders by liquid chromatography−tandem mass spectrometry (LC−MS/MS). LC−MS/MS analysis has the potential to reveal chemical modifications undergone by proteinaceous materials in a painting at a molecular level, which cannot be achieved with any other analytical technique.

In this respect deamidation of asparagine and glutamine was detected as a major modification undergone by milk and animal glue in a sample collected from the Camposanto Monumentale in Pisa. Deamidation is a spontaneous nonenzymatic postsynthetic modification of proteins. It plays an important role in protein degradation and is postulated to function as a timer in aging.[11−14] Deamidation introduces negative charges and results in a +0.984 Da mass shift, which can be detected by MS techniques,[15−18] and MS/MS methods can also provide very specific information on deamidation sites. In the contest of the samples herein characterized, deamidation could be related to the reactions taking place between the proteinaceous binder and the other materials used in the past restoration as well as to the aging of the proteinaceous binders.

EXPERIMENTAL SECTION

Materials. Ammonium hydrogen carbonate (AMBIC), ethylenediaminetetraacetic acid (EDTA), and iodoacetamide were purchased from Fluka; tri(hydroxymethyl)aminomethane (TRIS), urea, dithiothreitol (DTT), TPCK-treated trypsin, and $H_2{}^{18}O$ (95% ^{18}O) were from Sigma; formic acid was from Baker; and acetonitrile (ACN) was from Romil. Deionized water was obtained with Millipore cartridge equipment.

Painting Samples. Replicas were prepared using whole milk, fresh whole egg, and bovine skin glue. Animal glue was dissolved in warm water; whole egg was slightly beaten to obtain a fluid homogeneous medium; whole milk was used without any treatment. The pigments (Prussian blue $Fe_4[Fe(CN)_6]_3$, verdigris $Cu(CH_3COO)_22Cu(OH)_2$, minium Pb_3O_4, cadmium yellow CdS + ZnO] were mixed with the fluid binder in ratios ranging approximately between 1 part pigment and 2−3 parts fluid binder. The paint was then applied with a brush on tiles covered by a layer of plaster, which were prepared in the summer of 2003. The paint replicas were prepared in spring 2008 and stored at ambient temperature in the dark since then. All samples of painting layers were available as few milligrams fragments (1−3 mg).

Sample Treatment. Samples and a blank reference sample were digested in a heterogeneous phase by incubating for 4 or 16 h, approximately 1−3 mg of solid sample, in ammonium bicarbonate 10 mM pH 7.5, containing trypsin 0.16 μM. The supernatant was then recovered by centrifugation, dried in vacuum, resuspended in 10 μL in 0.1% formic acid, and the peptide mixture analyzed by LC−MS/MS. Control experiments with $H_2{}^{18}O$ were done by using the same procedure as described above, except that $H_2{}^{18}O$ was used in place of $H_2{}^{16}O$.

LC−MS/MS Analysis. The peptide mixtures were analyzed as previously reported,[7] using a CHIP MS 6520 QTOF equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). Raw data from nano LC−MS/MS analyses were employed to query nonredundant protein databases (UniprotSprot with taxonomy restriction to *Chordata*) using in house MASCOT software (Matrix Science, Boston, MA). Additional search parameters were a peptide mass tolerance set at 20 ppm and a fragment mass tolerance of 0.4 Da. Ions score is $-10\log(P)$, where P is the probability that the observed match is a random event. The threshold above which the individual ions score indicates identity or extensive homology ($p < 0.05$) can vary from search to search. In our searches, on average, individual ion scores >30 indicated identity or extensive homology ($p < 0.05$). A further ions score cutoff of 20 was set as the minimum acceptable score for the discussion of modified peptides. Protein scores are derived from ions scores as a nonprobabilistic basis for ranking protein hits (http://www.matrixscience.com/help/interpretation_help.html).

RESULTS

The Camposanto Monumentale, a 13th century cemetery located alongside the Leaning Tower in Pisa (Italy), was decorated with important frescoes painted by Benozzo Gozzoli, Taddeo Gaddi, Spinello Aretino, and Buonamico Buffalmacco, among others. During the Second World War, an incendiary bomb exploded in the cemetery, burning the wood and melting the lead of the roof. During the restoration following the Second World War, the paintings (i.e., paint layer and plaster) were detached from the wall using animal glue and were subsequently glued onto another canvas using non water-soluble glues. At this stage, in most cases, casein was used, but its quality was very poor and, with the passing of time, it has slowly lost its adhesive properties.[19] In addition to prevent it from rotting quickly during the restorations, formaldehyde was often added: unfortunately, in some cases this gave rise to cross-linking reactions, transforming all the paint layers into a unique insoluble and waterproof block.[20] Slaked lime was generally mixed with the casein in order to increase the strength of the glue after carbonation of the calcium hydroxide, but it has since been proven that the carbonation was still not at all complete even 20−30 years later.

GC/MS Analyses of the Paint Samples. Samples from the mural paintings of the Camposanto Monumentale were all collected from the "Giudizio Universale" by Bonamico Buffalmacco (1336−1341). In particular samples GUsp1, GUsp4 were collected from Scenes 6−7, Edge 6A and samples Ab, CS G16, A6S1a, and CSG-C bis, were all collected from the Panel A6S1. In these samples, in the course of a previous analytical campaign in the course of several years, the proteinaceous content was analyzed by the GC/MS procedure based on the acidic

Table 1. Amino Acid Relative Percentage Content of the Samples Collected from the Mural Paintings of the Camposanto Monumentale

Aminoacid / Sample	Ala	Gly	Val	Leu	Ile	Met	Ser	Pro	Phe	Asp	Glu	Lys	Hyp	Tyr
GUsp1	6.8	17.8	6.0	8.4	4.8	0.0	3.6	10.6	4.1	8.1	17.3	2.0	10.3	0.0
GUsp4	7.4	12.4	11.1	14.8	8.4	0.0	4.4	16.4	6.5	4.3	10.6	0.7	2.6	0.3
Ab	3.7	6.3	7.1	10.0	4.9	0.0	5.1	14.3	2.1	10.3	27.3	0.2	8.3	0.4
CSG16	6.6	14.5	6.8	8.9	5.1	2.4	2.9	12.6	2.4	9.0	28.8	0.0	0.0	0.0
A6S1a	5.2	6.0	8.1	13.8	7.9	4.8	3.6	6.5	6.4	9.1	28.5	0.0	0.0	0.0
CSG-C bis	8.2	5.1	15.5	18.3	12.7	7.5	1.7	14.0	10.5	3.8	1.8	0.0	0.0	0.0
Glue	14.3	23.1	5.0	6.0	2.4	1.3	5.1	12.6	3.1	5.3	7.8	3.7	9.2	1.2
Casein	3.7	1.9	6.6	11.5	7.0	3.5	7.1	11.7	6.9	8.8	25.7	2.0	0.0	3.6
Egg	8.6	4.7	10.1	14.7	8.6	6.0	7.6	4.4	8.7	11.8	10.3	3.7	0.0	0.8

hydrolysis of the samples assisted by microwave, followed by derivatization with a silylating agent.[3] Purification from inorganic materials was also performed when necessary.[3] This procedure permits one to evaluate the quantitative amino acidic content of 14 amino acids. Animal glue was used to detach the paintings, and casein was used as glue to the new support. As a result, one of these materials, or both of them, are always found in the samples collected from these paintings. Animal glue can be easily identified due to the presence of Hyp. Moreover it is characterized by high contents of Gly. Casein is characterized by a high content of Glu and a relatively high content of Pro. To identify a proteinaceous binder by GC/MS, the relative amino acid percentage content of a sample is compared to a data set of reference samples by means of principal component analysis (PCA). Table 1 reports an example of the amino acid relative percentage content observed in some samples of the mural paintings compared to the average profiles of reference samples of animal glue, egg, and casein obtained with the same analytical procedure.

Samples GUsp1, GUsp4, and Ab show the occurrence of animal glue, as indicated by the presence of Hyp. With the samples processed with PCA (score plot in Figure 1), it is possible to evaluate that all samples contain casein as well, as indicated by their position in the score plot. Finally it is important to stress that some samples do not show any Met (GUsp1, GUsp4, and Ab), some any Tyr (GUsp1, CSG16, A6S1a, and CSG-C bis), and some others any Lys (CSG16, A6S1a, and CSG-C bis). Ser and Glu are also extremely low in CSG-C bis. Finally, in all samples, amino malonic acid, which is considered to be the degradation product of Ser, Phe, and Cys,[21] was identified.

The situation shown here with few examples is typical of the samples collected from the mural paintings of the Camposanto Monumentale in the course of the years. Although in most cases identification of the proteinaceous binder is still possible, most part of the samples analyzed show modified profiles. But there is no information about how and where these modifications occur in the protein sequence and which can thus be the consequences from point of view of the stability of the materials and the conservation of the work of art itself.

Sample Ab, that was recently collected, was also subjected to the proteomics investigation.

Proteins Identification in Painting Samples by Proteomic Strategies. The identification of the proteinaceous material in the painting sample from the Camposanto Monumentale of Pisa was carried out following the minimally invasive proteomic analytical procedure previously described[7] and reported in the Experimental Section.

In the Supporting Information, Table S1 reports the results of the MS/MS ion search carried out with the MASCOT software in the UniprotSprot database on the sample from the Camposanto Monumentale of Pisa, with oxidation of methionine, hydroxylation of proline and lysine, and pyro-Glu formation from Gln at the N-terminus of peptides as variable modifications. The identification of six proteins from bovine milk and two bovine collagens confirmed the use of casein and bovine animal glue as natural adhesives.[20,22] Insertion of hydroxylation on proline and lysine residues, a post-translational modification extensively found in collagen chains, resulted in a much higher confidence in the identification of proteins from the animal glue component. As an example, addition of hydroxylations as variable modifications increased the identification score from 204 to 780 for collagen α-1 (I) chain (P02453, entry in the UniprotSprot Database) and the number of identified peptides increased from 5 to 30.

Similar analyses were carried out on reference materials (i.e., dried skimmed milk, animal glue, model samples prepared with caseins, and animal glue as binders), and database searches were performed in the same conditions as above, providing similar results, in agreement with the screening and statistical analysis carried out by Fremout and colleagues on the most common identified proteins in paint replicas.[8]

Identification of Modification Events in Proteins in Painting Samples. Database searches were successively carried out, on the sample from the Camposanto Monumentale in comparison to reference materials, with the insertion of several additional variable chemical modifications, one or two at a time, because of search algorithm optimization. Kynurenine, formylkynurenine, hydroxykynurenine, oxidation of His, Trp, Arg, Lys, nitration of Tyr, β elimination of Ser and Thr, oxidation of Cys to cysteic acid, phosphorylation on Ser, Thr, or Tyr, and formation of amino malonic acid from Ser, Phe, and Cys were all tested as possible modifications of the proteins present in the samples. The best search results were obtained by trial and error. As expected, and in agreement with the results obtained by amino acid analysis, formation of amino malonic acid from Ser (predominantly) and Phe was observed in two peptides from bovine β-casein (P02666), 1 peptide from α-S1-casein

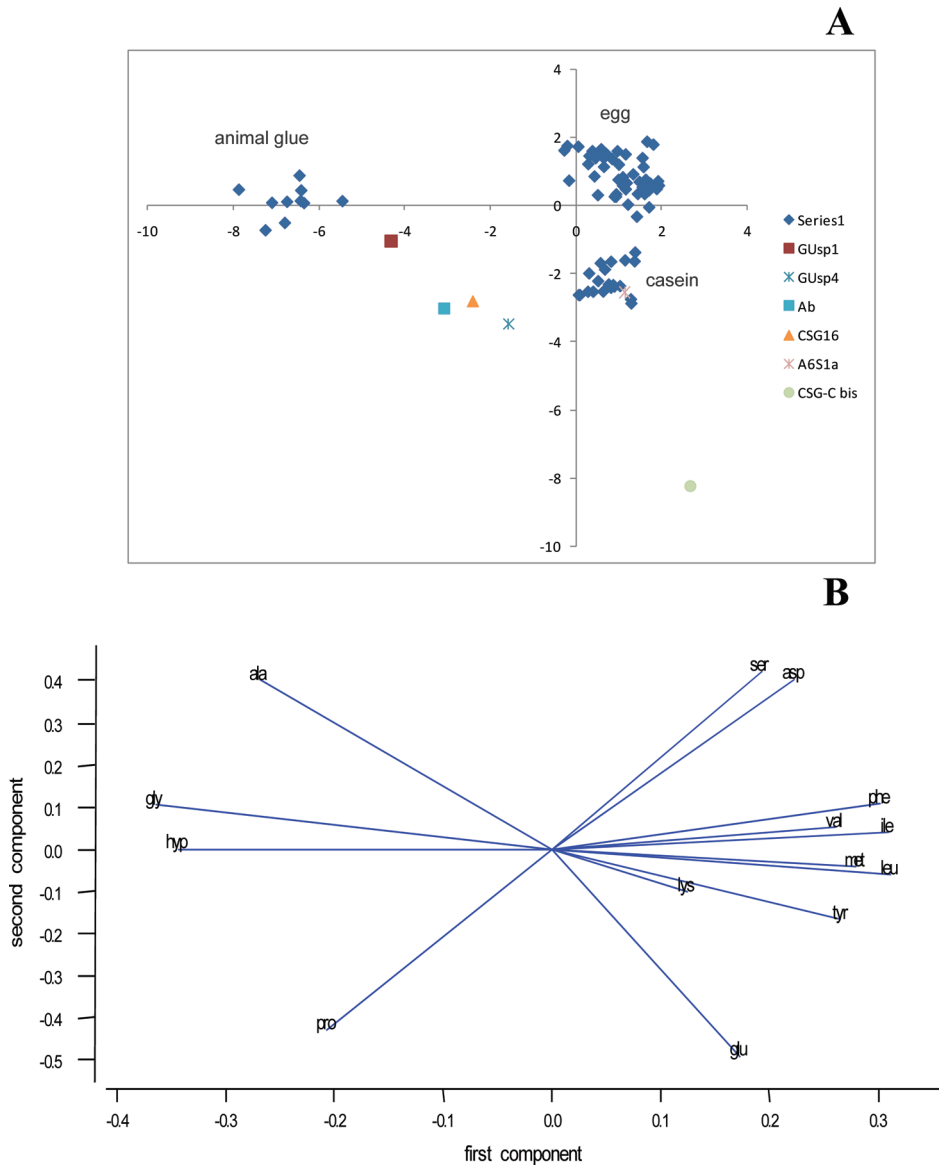

Figure 1. Score plot obtained by comparing the reference samples of animal glue, casein, and egg with the samples from the Camposanto Monumentale.

(P02662), and 1 peptide from collagen α-1(I) chain (P02453). On the contrary, oxidation of sulfur containing amino acids or modifications of tryptophan was not significantly detected.

When deamidation was considered as a variable modification, most of the Gln and Asn containing peptides that had been identified in the sample from the Camposanto Monumentale were also found as deamidated peptides (Table 2). As an example, 6 of the 10 peptides belonging to the bovine β-casein were also detected in the deamidated form. Similarly, 9 of the 15 peptides mapping the α-S1-casein were also found as deamidated peptides. We were therefore prompted to investigate the occurrence of deamidation as a marker for deterioration and aging of the materials. It is worth mentioning that deamidation escapes detection in amino acids analysis because the extreme acidic conditions in the hydrolysis of the proteins systematically transform all the Gln and Asn into Glu and Asp, respectively.

Systematic database searches were therefore eventually repeated including deamidation on glutamine and asparagine as variable modification. Table 2 reports the number of deamidated peptides observed in the single proteins identified in the sample from the Camposanto Monumentale, in a sample of skimmed milk and in the paint replicas.

Assessment of Deamidation. Deamidation introduces a small (+0.984 Da) mass shift that overlaps with the isotopic pattern. Moreover, nondeamidated and deamidated peptides behave quite similarly during reverse-phase chromatography, with deamidated peptides eluting at a moderately higher concentration of acetonitrile than nondeamidated ones.

Peptide fragmentation does facilitate detection of deamidation and provides very specific information on deamidation sites. We manually inspected several MS and MS/MS spectra originating from deamidated peptides as indicated by Mascot software in order to confirm software assignments.

However, as has been shown by a large number of authors (e.g., ref 23), many postsynthetic modifications including deamidation can occur *in vitro* during proteomics sample handling.

As Li and co-workers demonstrated,[30] artificial deamidation occurring during the tryptic digestion can be reduced with faster

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Table 2. Identification of the Proteins in Painting Samples by Trypsin Digestion in Heterogeneous Phase[a]

		overnight trypsin digestion		4 h trypsin digestion	
sample	identified protein (accession number)	no. of identified peptides[c]	no. of deamidated peptides[d]	no. of identified peptides	no. of deamidated peptides
sample from Cimitero Monumentale of Pisa[b]	β-casein1 (P02666)	9	6	8	5
	α-S1-casein (P02662)	15	9	11	6
	collagen α-2 (I) chain (P02465)	26	7	33	8
	collagen α-1 (I) chain (P02453)	35	9	39	13
	κ-casein (P02668)	4	2	5	2
	α-S2-casein (P02663)	11	6	9	4
	β-lactoglobulin (P02754)	7	0	6	0
	lactadherin (Q95114)	3	0		
model on intonaco with casein	β-casein (P02666)	6	2	7	3
	α-S1-casein (P02662)	8	3	9	3
	β-lactoglobulin (P02754)	4	1	7	1
	α-S2-casein (P02663)	5	2	6	2
	κ-casein (P02668)	2	0	2	0
	serum albumin (P02769)	4	0	2	0
	polymeric immunoglobulin receptor (P81265)	2	0		
model on intonaco with animal glue	collagen α-2 (I) chain (P02465)	26	11	35	12
	collagen α-1 (I) chain (P02453)	34	13	34	14
	collagen α-1(III) chain (P04258)	8	3	12	3
skimmed milk	α-S1-casein (P02662)	9	1	8	2
	serum albumin (P02769)	12	0	6	0
	β-lactoglobulin (P02754)	6	1	9	1
	κ-casein (P02668)	3	1	4	0
	β-casein (P02666)	4	1	4	1
	lactotransferrin (P24627)	4	0	3	0
	α-S2-casein (P02663)	4	1	6	1
	butyrophilin subfamily 1 member A1 (P18892)	2	0	1	
	glycosylation-dependent cell adhesion molecule I (P80195)	3	0	3	0
model on glass with casein	α-S1-casein (P02662)	16	7	9	2
	β-casein (P02666)	11	3	5	1
	β-lactoglobulin (P02754)	7	1		
	α-S2-casein (P02663)	14	3	7	0
	κ-casein precursor (P02668)	4	0	3	1
model on glass with animal glue	collagen α-2 (I) chain (P02465)	52	25	35	8
	collagen α-1 (III) chain (P04258)	26	11	8	3
	collagen α-1 (I) chain (P02453)	48	11	47	13
animal glue	collagen α-2 (I) chain (P02465)	33	16	16	4
	collagen α-1 (I) chain (P02453)	36	7	25	3
	collagen α-1 (III) chain (P04258)	5	1	3	0

[a] Proteins were identified searching UniprotSprot database with MSMS Ion search Mascot software (Matrix Science). *Chordata* was used as taxonomy restriction, deamidation on Gln and Asn, oxidation on Met, hydroxylation on Lys and Pro as variable modifications. Only proteins identified with at least two peptides were considered as significant. Peptides with individual ion scores >10 were considered for identification purposes, while for computing the number of deamidated peptides only those with individual ion scores >20 were considered. [b] An extended version of the table, with the sequence of the identified peptides, is reported in the Supporting Information, Table S1. [c] The number of peptides was calculated regardless of the presence of modifications, i.e., for this purpose, if a peptide was observed both as modified and not modified, herein it is considered as a single peptide. [d] The number of deamidated peptides was herein calculated regardless of the number of deamidations observed on the peptide, i.e., for this purpose, if a peptide was observed as a singly or multiply deamidated, it is herein considered as a single modification.

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trypsin digestion: digestion carried out for up to 4 h generally would not introduce additional detectable deamidations, even for rapidly deamidating peptides. To reduce the incidence of artifactual deamidation, we therefore subjected the samples to a short trypsin digestion (~4 h) as a systematic strategy. Despite the shorter hydrolysis time, proteins could be confidently identified in all the samples (Table 2).

Identification of Peptides to Be Considered As Aging/Deterioration Signatures. Deamidation was found widespread over most of the identified proteins, both from the old sample and the reference materials, (Table 2). A systematic analysis was carried out to individuate peptides that could be sorted out as aging/deterioration signatures for further investigation.

We designed specific criteria for the identification of candidate peptides as follows: (1) Peptides should have been identified in all the samples analyzed. (2) Individual ion scores should be higher than that indicated by the Mascot program as indicative of identity or extensive homology (in our searches this limit resulted to be 30, on average); (3) when deamidation was detected, individual ion scores for the deamidated peptide should be higher than that indicated by the Mascot program as indicative of identity or extensive homology. These restrictions impose to consider only fragmentation spectra of very good quality that could be easily manually inspected in order to avoid as much as possible the occurrence of false positives.

The limited variability of the proteins identified in the different samples (Table 2) (i.e., as highlighted above, the same proteins were identified in the sample from the Camposanto Monumentale of Pisa as well as in the reference samples) was instrumental in our search for biomarkers of the material deterioration. We could screen a large panel of peptides common to all samples, with a wide range of physicochemical characteristics, looking for candidates that do satisfy the prerequisites.

Table 3 lists all the glutamine or asparagine containing peptides that had been detected in the sample from the Camposanto Monumentale of Pisa, and for each of them, the occurrence of deamidation is indicated and compared to the other samples. Table 3A,B lists the peptides of the milk derived proteins and in the animal glue component, respectively.

Several deamidated peptides detected in the sample from the Camposanto Monumentale of Pisa were not deamidated either in the paint replicas or in the reference milk or animal glue samples (Table 3A,B). Moreover, peptides with multiple deamidations were detected in the sample from the Camposanto Monumentale of Pisa, whereas only singly or no deamidation in model samples or in the reference milk or animal glue samples.

A preliminary analysis of the peptides identified in the different samples clearly indicates that peptides from caseins are better candidates than collagen fragments. The copresence of a variable extent of hydroxylations on almost all of the collagen peptides enormously increases the heterogeneity of these fragments decreasing the relative abundance of the single species and thus making the confident assignment of deamidation of these peptides more challenging.

Peptide 106-YLGYLEQLLR-115 from α-S1-casein (P02662) was considered a good candidate for a number of considerations. This peptide is present in all the casein containing samples, it contains only one deamidation site, it was found to be deamidated only in the sample from the Camposanto Monumentale of Pisa, and deamidation could be easily visualized in the corresponding fragmentation spectra.

Figure 2 (panel A) shows the MS/MS spectra of the doubly charged ion at 634.36 m/z of the YLGYLEQLLR peptide from α-S1-casein (P02662), in the sample from Camposanto Monumentale of Pisa. Panel B shows the MS/MS of the doubly charged ion at 634.85 m/z corresponding to the deamidated form of the same peptide. Deamidation at Gln113 can be readily assessed since all the signals from the y series containing the Gln residue are shifted by one unit in the fragmentation spectra of the deamidated form, with deviation of the experimental data from the expected values within the 1−21 ppm range (Table S2, Supporting Information).

A second candidate peptide was defined as the 189-FALPQYLK-196 peptide from α-S2-casein (P02663) on the basis of the same considerations as discussed above. Deamidation could be readily assessed since all the signals from the y series containing the Gln residue are shifted by one unit in the fragmentation spectra (Figure S1, Supporting Information), with deviation of the experimental data from the expected values within the 1−30 ppm range (Table S2, Supporting Information).

As a further check to ensure that the observed deamidation had occurred before the trypsin digestion, we performed a parallel digestion with trypsin in $H_2{}^{18}O$, as reported by Li and co-workers.[30] Hydrolysis in $H_2{}^{18}O$ determines a mass shift of +2 Da per ^{18}O substitution because of the up to two ^{18}O atoms incorporation into the peptide C-terminus. If deamidation occurrs during trypsin digestion, an ^{18}O atom will also be introduced on the newly formed carboxylate moiety.

The observation of deamidated peptide with ^{18}O atoms incorporation at the C-terminus of the peptide and not at the deamidation site (Figure S2, Supporting Information) confirmed that deamidation was already there, before trypsin digestion, i.e., it is not an artifact due to sample manipulation.

Some more candidates were identified in our survey analysis, namely, peptide 185-VLPVPQK-191 from bovine β-casein (P02666), peptide 46-YIPIQYVLSR-55 from bovine κ-casein (P02668), and 129-RNAVPITPTLNR-140 from α-S2-casein (P02663), thus widening the range of possible markers that can be simultaneously used for diagnostic purposes and whose fragmentation spectra for the differently deamidated forms are reported in the Supporting Information (Figure S3 and S4). It is worth noting that the panel of selected peptides covers 4 of the 6 different milk proteins that have been identified in the sample from Camposanto Monumentale of Pisa and that are among the most abundant proteins in the casein fraction.

DISCUSSION

Only in recent years proteomic approaches have been proposed as valuable tool for the identification of the proteinaceous constituents of artistic and historic objects. Herein, these procedures proved to be extremely promising also for the characterization of the natural and artificial aging and deterioration products in the proteins in masterpieces.

We worked on the 14th century frescoes from the Camposanto Monumentale in Pisa that were detached in 1945 from the wall and relocated onto asbestos cement supports using a glue based on a mixture of casein, animal glue, and calcium hydroxide. Amino acid analyses on several samples demonstrated that proteins in the frescoes were deteriorated as revealed by the decrease in Met, Lys, and Tyr and by the presence of amino malonic acid as the results of oxidation of Ser, Phe, and Cys in all of the samples.

Table 3. Peptides Identified As Deamidated in the Sample from the Camposanto Monumentale of Pisa[a]

A

Peptide (n° deamidation sites)	Sample from Cimitero Monumentale				Skimmed milk				Model on intonaco with casein				Model on glass with casein			
	No deam	+1 deam	+2 deam	>2 deam	No deam	+1 deam	+2 deam	>2 deam	No deam	+1 deam	+2 deam	>2 deam	No deam	+1 deam	+2 deam	>2 deam
Casein alpha-S1 (P02662)																
EKVNELSK (1)	X	X			-	-			-	-			X	-		
VPQLEIVPNSAEER (2)	X	X	-		X	-	-		X	-	-		-	-	-	
YLGYLEQLLR (1)	X	X			X	-			X	-			X	-		
HQGLPQEVLNENLLR (4)	X	X	X	-	X	X	-	-	X	X	-	-	X	-	-	-
HIQKEDVPSER (1)	X	-			-	-			-	-			-	-		
YKVPQLEIVPNSAEER (2)	X	X	-		X	-	-		-	-	-		-	-	-	
HPIKHQGLPQEVLNENLLR (4)	X	X	-	-	X	X	-	-	-	-	-	-	-	-	-	-
Kappa-casein precursor (P02668)																
YIPIQYVLSR (1)	X	X			X	-	-		X	-			X	-		
SPAQILQWQVLSNTVPAK (4)	X	X	-	X	X	-	-	-	X	-	-	-	X	X	-	-
Casein alpha-S2 (P02663)																
LNFLKK (1)	X	-			-	-			-	-			-	-		
FALPQYLK (1)	X	X			X	-			X	-			X	-		
AMKPWIQPK (1)	X	X			X	-			-	-			X	-		
NAVPITPTLNR (2)	X	X	X		X	X	-		X	X	-		X	-	-	
RNAVPITPTLNR (2)	X	X	X		X	-	-		X	-	-		X	X	-	
ALNEINQFYQK (4)	X	X	-	-	X	-	-	-	X	-	-	-	-	-	-	-
Beta-casein precursor (P02666)																
VLPVPQK (1)	X	X			X	-			X	-			X	-		
AVPYPQR (1)	X	X			-	-			X	-			X	-		
VLPVPQKAVPYPQR (2)	X	-	-		-	-	-		X	-	-		X	-	-	
DMPIQAFLLYQEPVLGPVR (2)	X	X	X		X	X	-		X	X	-		X	X	-	
AVPYPQRDMPIQAFLLYQEPVLGPVR (3)	-	X	X	-	-	-	-	-	-	-	-	-	-	-	-	-
FQSEEQQQTEDELQDK (5)	-	X	-	-	X	-	-	-	-	-	-	-	-	-	-	-
Beta-lactoglobulin (P02754)																
IDALNENK (2)	X	-	-		-	-	-		X	-	-		-	-	-	
VYVEELKPTPEGDLEILLQK (1)	X	-			X	X			X	-			X	-		

B

Peptide (n° deamidation sites)	Sample from Cimitero Monumentale				Animal glue				Model on intonaco with animal glue				Model on glass with animal glue			
	No deam	+1 deam	+2 deam	>2 deam	No deam	+1 deam	+2 deam	>2 deam	No deam	+1 deam	+2 deam	>2 deam	No deam	+1 deam	+2 deam	>2 deam
Collagen alpha-1 (I) chain (P02453)																
GVVGLPGQR (1)	X	-			X	-			X	X			X	X		
GVQGPPGPAGPR (1)	X	X			X	-			X	X			X	X		
GQAGVMGFPGPK (1)	X	-			X	-			X	X			X	X		
GPSGPQGPSGPPGPK (1)	X	-			-	-			X	-			X	X		
DGLNGLPGPIGPPGPR (1)	-	X			-	-			-	X			-	-		
GANGAPGIAGAPGFPGAR (1)	-	X			-	X			-	X			-	X		
VGPPGPSGNAGPPGPPGPAGK (1)	X	X			-	X			X	X			X	X		
GEPGPTGIQGPPGPAGEEGKR (1)	X	X			X	-			X	X			X	X		
TGPPGPAGQDGRPGPPGPPGAR (1)	X	-			X	-			X	X			X	X		
GDAGPPGPAGPAGPPGPIGNVGAPGPK (1)	X	-			-	-			-	-			-	-		
GPPGSAGSPGKDGLNGLPGPIGPPGPR (1)	-	X			-	X			-	X			-	X		
GAPGADGPAGAPGTPGPQGIAGQR (2)	X	X	-		X	-	-		X	X	X		X	X	-	
GFSGLQGPPGPPGSPGEQGPSGASGPAGPR (2)	X	X	-		-	-	-		X	-	-		-	-	-	
GNDGATGAAGPPGPTGPAGPPGFPGAVGAK (1)	-	X			-	-			-	-			-	-		
GAPGDRGEPGPPGPAGFAGPPGADGQPGAK (1)	X	X			X	-			X	X			X	X		
GEPGPPGPAGFAGPPGADGQPGAK (1)	-	X			-	-			-	X			-	-		
GVPGPPGAVGPAGKDGEAGAQGPPGPAGPAGER (1)	X	X			X	-			X	X			X	-		
GLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPR (2)	X	-			X	-	-		-	-	-		-	-	-	
GEQGPAGSPGFQGLPGPAGPPGEAGKPGEQGVPGDLGA PGPSGAR (3)	X	X	-	-	-	-	-	-	-	X	-	-	-	-	-	-
Collagen alpha-2(I) chain (P02465)																
GDQGPVGR (1)	X	-			-	-			-	-			X	X		
GVVGPQGAR (1)	X	X			X	-			X	X			X	X		
IGQPGAVGPAGIR (1)	X	-			X	-			X	X			X	X		
GFPGSPGNIGPAGK (1)	X	X			X	X			X	X			X	X		
GEPGNIGFPGPK (1)	X	X			-	X			X	X			X			
GELGPVGNPGPAGPAGPR (1)	X	-			-	-			X	-			-	-		
GPNGDSGRPGEPGLMGPR (1)	-	X			-	-			X	X			-	-		
HGNRGEPGPAGAVGPAGAVGPR (1)	-	X			X	-			-	-			X	X		
GYPGNAGPVGAAGAPGPQGPVGPVGK (2)	X	X	-		X	X	-		-	-	-		X	X	X	
GPSGPPGPDGNKGEPGVVGAPGTAGPSGPSGLPGER (1)	X	X			X	-			-	-			-	-		
GAPGAIGAPGPAGANGDRGEAGPAGPAGPGPR (1)	-	X			-	X			-	X			-	X		
GPPGASGAPGPQGFQGPPGEPGEPGQTGPAGAR (3)	X	X	X	-	-	-	-	-	-	X	-	-	-	-	-	-

[a] Analysis of the peptides observed as deamidated in the proteins identified in the sample from the Camposanto Monumentale of Pisa digested with trypsin in heterogenous phase for 4 h (see Table 2 for the details of the protein identification). UniprotSprot Database search was carried out as with MSMS Ion search Mascot software (Matrix Science), and *Chordata* was used as taxonomy restriction. Detection of peptides with single, double, and >2 deamidations are indicated with X. Only peptides identified with individual ion scores >20 were considered as deamidated. Part A reports the analysis for the casein containing samples, and part B for the animal glue containing samples.

Proteomic analyses confirmed the presence of amino malonic acid and identified deamidation at Asn and Gln of proteinaceous binders as a major deterioration event. Deamidation is a spontaneous nonenzymatic postsynthetic modification of proteins and cannot be assessed by amino acid analysis because of the extreme conditions of the acidic hydrolysis that convert all

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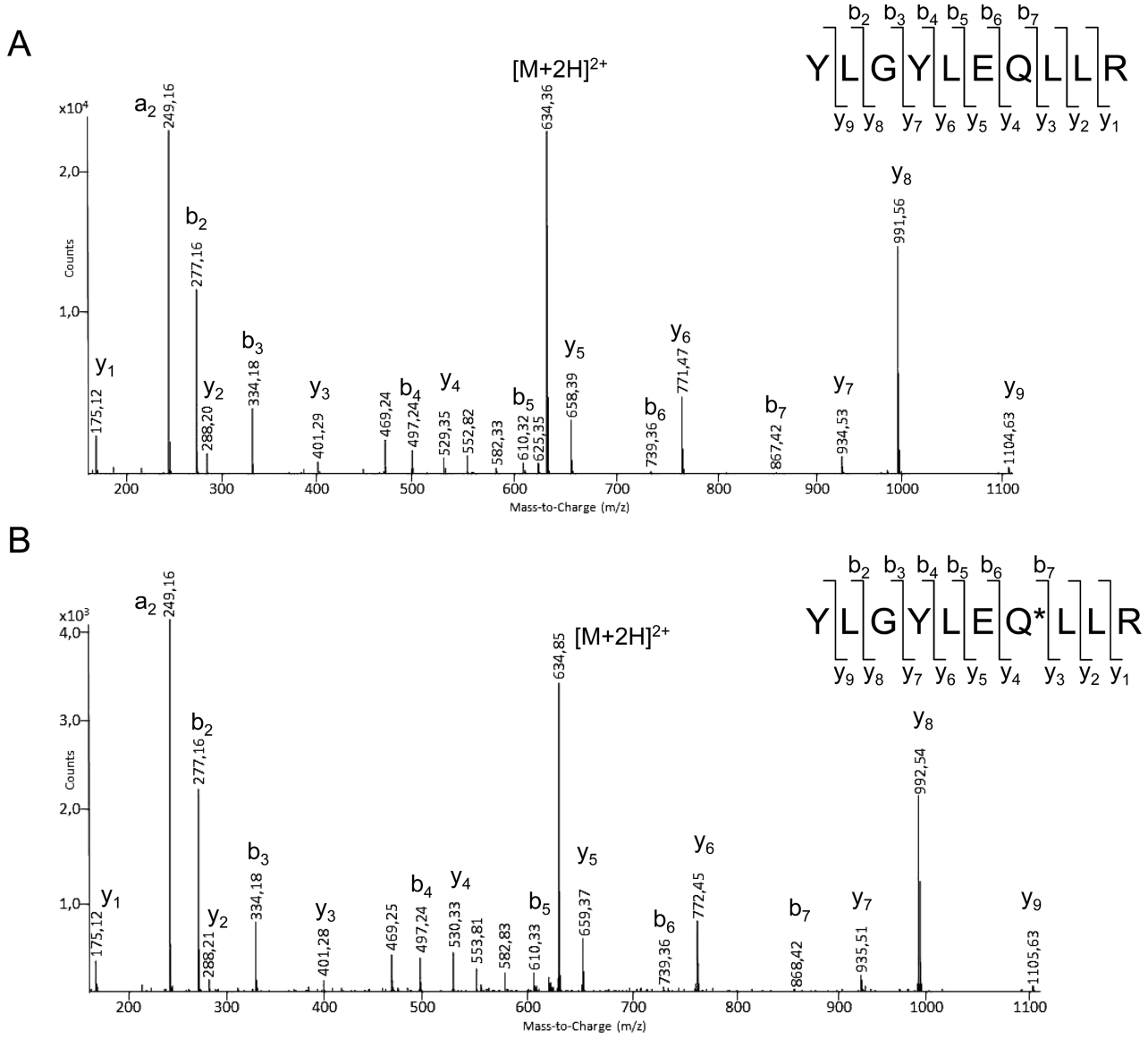

Figure 2. MS/MS spectra of the doubly charged ions at *m/z* 634.36 (A) and 634.85 (B) of the peptide 106-YLGYYLEQLLR-115 from bovine α-S1-casein (P02662) and its deamidated form, respectively, identified in the analysis of the sample from Camposanto Monumentale of Pisa. The product ions are indicated with the observed masses.

the amide moieties into the corresponding acids. It plays an important role in protein degradation and is postulated to function as a timer in aging.[11−14]

Deamidation at Asn residues takes place much more rapidly than at Gln (up to 10 times faster), and the reaction mechanisms of nonenzymatic deamidation have been extensively studied. Asn deamidation proceeds through the formation of a succinimide ring, which in turn opens up to give a mixture of aspartic and iso-aspartic residue.[11] Gln deamidation, similarly, can proceed through formation of a glutarimide moiety, but since this reaction is relatively slow, direct hydrolysis also contributes significantly.[11] The systematic analysis of the samples herein reported indicates extensive deamidation occurring on most of the peptides identified, suggesting that the miniature molecular clock, as Robinson and Robinson defined any amide residue present in peptides or proteins,[11] might well be used as a molecular marker in artworks.

However, many factors can influence deamidation, such as protein sequence,[14,24,25] secondary structure,[26] local three-dimensional structure,[27] pH, temperature, ionic strength, buffer ions, turnover of the protein, and other solution properties.[28,29] Moreover, in this respect, it must be kept in mind that the sample taken into consideration was collected from a work of art. Milk is an emulsion and is already in the liquid form. To use casein, which is sold as a powder, as an art material, a fluid must be obtained, which can be achieved by using an alkaline solution as a solvent. In the case of the Camposanto Monumentale, it is known that slaked lime was added to casein, to solubilize it and to obtain a stronger glue, after the carbonation of the lime took place. Slaked lime is extremely alkaline, and it must be expected that this has reacted with casein during years, although a reduced reaction rate must be expected when the glue was dried and water thus evaporated.

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It is therefore clear that the modifications detected here have to be considered as the results of the work of art preparation, the treatment itself (i.e., the presence of slaked lime) and the actual aging, without any possibility, at this stage, of distinguishing the different phenomena. Our data indicate that whenever milk or casein fraction is present, several peptides can constitute the molecular signatures of degradation, and we selected possible candidates from different proteins that could be used individually or simultaneously as markers.

Interestingly, most of the selected peptides present Gln as the deamidation site. We suggest that Gln deamidation, intrinsically slower than deamidation at a Asn site, might reveal to be more useful in the quantitative perspective on historical objects: Asn deamidation might occur too fast to be used for the actual evaluation of the aging of the object itself.

Awareness and measurement of deamidation could constitute a routine analysis for paintings or any artistic and historic object where proteins are present. However, a contribution in the level of deamidation due to artwork preparation itself and deamidation artifacts produced during the analysis must be carefully guarded against and considered. Future works will be devoted to the quantitative analysis of deamidation as a function of aging necessary to deeply investigate and possibly separate the contributions of actual aging and/or the preparation and treatment of the work-of art.

In conclusion, our data identified deamidation as an extensive deterioration process occurring in pictorial objects and pave the way to the identification of molecular signatures that can be useful in characterizing artworks that contain proteins and suggest further exploitation of proteomic strategies for the investigation of aging molecular effects.

■ ASSOCIATED CONTENT

Supporting Information. Full list of the peptides and the corresponding proteins identified in the sample of the Camposanto Monumentale of Pisa. MS/MS spectra of selected peptides and of peptides after digestion in ^{18}O. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Mills, J. S.; White, R. *The Organic Chemistry of the Museum Objects*, 2nd ed.; Butterworth Heinemann Ltd.: Oxford, U.K., 1994.
(2) Colombini, M. P.; Andreotti, A.; Bonaduce, I.; Modugno, F.; Ribechini, E. *Acc. Chem. Res.* **2010**, *395*, 715–727.
(3) Bonaduce, I.; Andreotti, A. In *Organic Mass Spectrometry in Art and Archaeology*; Colombini, M. P., Modugno, F., Eds.; John Wiley & Sons Ltd.: Chichester, U.K., 2009; pp 303−326.
(4) Andreotti, A.; Bonaduce, I.; Colombini, M. P.; Modugno, F.; Ribechini, E. In *New Trends in Analytical, Environmental and Cultural Heritage Chemistry*; Tassi, L., Colombini, M. P., Eds.; Transworld Research Network: Kerala, India, 2008; pp 389−423.
(5) Tokarski, C.; Martin, E.; Rolando, C.; Cren-Olive, C. *Anal. Chem.* **2006**, *78*, 1494–1502.
(6) Domènech-Carbò, M. T. *Anal. Chim. Acta* **2008**, *621*, 109–139.
(7) Leo, G.; Cartechini, L.; Pucci, P.; Sgamellotti, A.; Marino, G.; Birolo, L. *Anal. Bioanal. Chem.* **2009**, *395*, 2269–2280.
(8) Fremout, W.; Dhaenens, M.; Saverwyns, S.; Sanyvova, J.; Vandenabeele, P.; Deforce, D.; Moens, L. *Anal. Chim. Acta* **2010**, *658*, 156–162.
(9) Boon, J. J.; Peulvè, S. L.; van der Brink, O. F.; Duursma, M. C.; Rainford, D. In *Proceedings of Early Italian Paintings Techniques and Analysis Symposium*; Bakkenist, T., Hoppenbrouwers, R., Dubois, H., Eds.; Limburg Conservation Institute: Maastricht, The Netherlands, 1996; pp 35−56.
(10) Nevin, A.; Anglos, D.; Cather, S.; Burnstock, A. *Appl. Phys. A: Mater. Sci. Process.* **2008**, *92*, 69–72.
(11) Robinson, N. E.; Robinson, A. B. *Molecular Clocks: Deamidation of Asparaginyl and Glutaminyl Residues in Peptides and Proteins*; Althouse Press: Cave Junction, OR, 2004.
(12) Robinson, N. E. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5283–5288.
(13) Robinson, N. E.; Robinson, A. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 944–949.
(14) Bischoff, R.; Kolbe, H. V. J. *J. Chromatogr., B: Biomed. Appl.* **1994**, *662*, 261–278.
(15) Robinson, N. E.; Zabrouskov, V.; Zhang, J.; Lampi, K. J.; Robinson, A. B. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 3535–3541.
(16) Robinson, N. E.; Lampi, K. J.; Speir, J. P.; Kruppa, G.; Easterling, M.; Robinson, A. B. *Mol. Vision* **2006**, *12*, 704–711.
(17) Robinson, N. E.; Lampi, K. J.; McIver, R. T.; Williams, R. H.; Muster, W. C.; Kruppa, G.; Robinson, A. B. *Mol. Vision* **2005**, *11*, 1211–1219.
(18) Schmid, D. G.; von der Mulbe, F.; Fleckenstein, B.; Weinschenk, T.; Jung, G. *Anal. Chem.* **2001**, *73*, 6008–6013.
(19) Colombini, M. P.; Fuoco, R.; Giacomelli, A.; Moscatello, B.; Baracchini, C.; Caponi, G. In *Scienza e Beni Culturali: "La pulitura delle superfici dell'architettura*; Libreria Progetto: Padova, Italy, 1995; pp 227−236.
(20) Bonaduce, I.; Colombini, M. P. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2523–2527.
(21) van den Brink, O. F.; Boon, J. J.; O'Connor, P. B.; Duursma, M. C.; Heeren, R. M. *J. Mass Spectrom.* **2001**, *36*, 479–492.
(22) Colombini, M. P.; Modugno, F.; Giacomelli, A. *J. Chromatogr., A* **1999**, *846*, 101–111.
(23) Xing, G.; Zhang, J.; Chen, Y.; Zhao, Y. *J. Proteome Res.* **2008**, *7*, 4603–4608.
(24) Capasso, S. J. *Pept. Res.* **2000**, *55*, 224–229.
(25) Geiger, T.; Clarke, S. *J. Biol. Chem.* **1987**, *262*, 785–794.
(26) Xie, M. L.; Schowen, R. L. *J. Pharm. Sci.* **1999**, *88*, 8–13.
(27) Robinson, N. E.; Robinson, A. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4367–4372.
(28) Tylercross, R.; Schirch, V. *J. Biol. Chem.* **1991**, *266* 22549–22556.
(29) Wright, H. T. *Crit. Rev. Biochem. Mol. Biol.* **1991**, *26*, 1–52.
(30) Li, X.; Cournoyer, J. J.; Lin, C.; O'Connor, P. B. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 855–864.