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Molecular insights into tempera art: an approach to phosphorylated proteins profiling

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The protein-based binders used in tempera painting techniques are complex natural matrices, typically from animal sources such as egg yolk or milk. These proteins exhibit numerous post-translational modifications (PTMs), particularly phosphorylation, which significantly influence their chemical and physical properties. The formation of three-dimensional networks through protein cross-linking underpins the creation of the pictorial layers. It is therefore reasonable to hypothesize a correlation between the stability of these layers and the structural properties of the constituent proteins. This study proposes an analytical methodology for the extraction and characterization of phosphoproteins. The procedure involves enrichment protocols and advanced biomolecular mass spectrometry techniques, initially optimized using pictorial specimens and subsequently applied to historical samples. The results demonstrate the potential for comprehensive characterization of the biomolecules under investigation, revealing considerable variability—particularly in historical samples, which are more susceptible to degradation and environmental exposure. The data are available through ProteomeXchange with the identifier PXD053038.

Cultural heritage artefacts hold a unique and irreplaceable value for society, often regarded as *"unica"*. Their protection and preservation are vital for both current and future generations. These objects are continually exposed to environmental factors—such as light, heat, humidity, and microbial activity—that can alter the structural and chemical composition of their organic and inorganic components, ultimately compromising their integrity[1,2]. Consequently, the scientific community is increasingly focused on developing non-invasive or minimally invasive analytical methods to achieve precise molecular characterization of artistic materials and their degradation products[3]. Understanding how the constituents of artworks evolve due to aging and degradation is essential for assessing their conservation status. Moreover, elucidating potential degradation processes is crucial for refining conservation strategies and implementing effective restoration measures[4].

Among cultural heritage artefacts, painting techniques represent a particularly compelling area of study. Over centuries, different methodologies have emerged, each employing distinct natural materials. Typically, a painting comprises multiple layers: a priming layer, a colored layer, and a protective varnish, often derived from plant resin[5]. The binders employed in the pictorial layer are primarily sourced from animals' sources (e.g., milk, eggs) or plants (e.g., resins, oils).

The tempera technique, as described by Cennino Cennini in his 15th-century treatise "Il Libro dell'Arte", involves mixing powdered pigments with a binder in an aqueous emulsion. The most common form combines finely ground pigments with egg yolk[6,7]. Depending on the binder, tempera can be classified into subtypes such as egg tempera, casein tempera, and gum tempera, further categorized as "lean" or "fat". Lean tempera is diluted with water, while fat tempera—enriched with oily-resinous components—requires solvents such as turpentine spirit.

Protein-based binders, extracted from natural matrices like milk, casein, and egg components[8], are favored in tempera for their ability to form durable, elastic, and adherent three-dimensional films. These matrices consist of complex, heterogeneous mixtures of biomolecules—including lipids, oligosaccharides, proteins, and organic acids—posing significant challenges for identification and characterization[9]. Notably, the proteins in these binders undergo extensive post-translational modifications (PTMs), with phosphorylation being particularly prevalent. This PTM involves the addition of a phosphate group to an amino acid residue, profoundly influencing a protein's structure, function, and molecular interactions[10,11].

In egg proteins—such as ovalbumin, ovotransferrin (phosphoglycoproteins), ovomucoid and ovomucin (N-linked glycoproteins), ovalbumin-related protein Y, riboflavin-binding protein (phosphoproteins), and

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Fig. 1 | "*Battesimo di Cristo*", wooden panel painting (Cesare Turco, 16th century). The area highlighted in the image and zoom on the side shows the section of the painting from which the pictorial film was extracted for analysis.

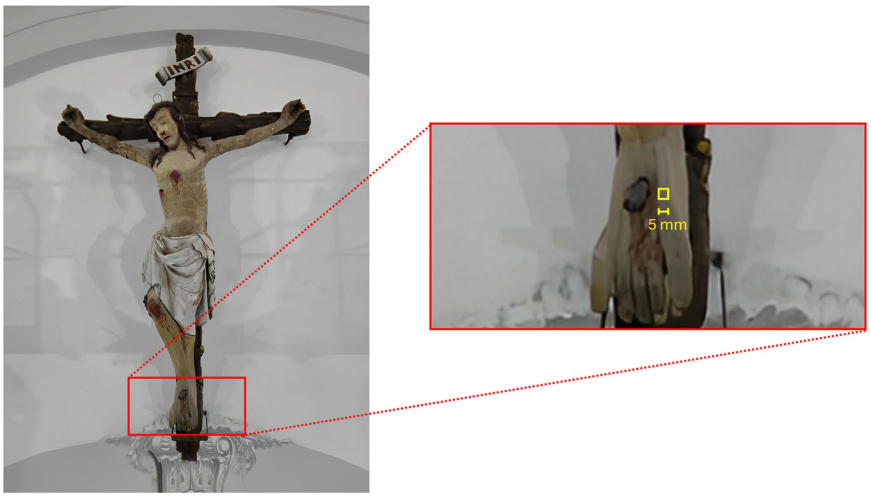

Fig. 2 | The "Great Polychrome Crucifix from the 13th century". The area highlighted in the image and zoom on the side shows the section of the painting from which the pictorial film was extracted for analysis.

lysozyme—PTMs have been widely studied due to their abundance[12]. Similarly, bovine milk proteins (e.g., αS1-casein, αS2-casein, β-casein, and κ-casein) are predominantly phosphorylated (expect κ-casein)[13]. Phosphorylation is critical for maintaining milk's micellar structure and calcium-binding capacity, stabilizing its colloidal system.

This paper presents an optimized analytical method for the extraction and characterization of phosphorylated proteins in tempera binders. While existing protocols for protein analysis in artworks (via LC-MS/MS)[14,15] typically involve extraction and enzymatic hydrolysis without specific enrichment of phosphorylated peptides, we introduced an additional enrichment step to improve detection. Given the high phosphorylation levels in egg, animal glue, and casein proteins, this refinement enhances specificity.

The primary aim of the current study was to optimize an analytical procedure for the identification of phosphopeptides in both unaged and artificially aged proteinaceous paint specimens, and to compare these results with data from authentic historical samples. The protocol was optimized using specimens prepared according to historical recipes[16], then subjected to artificial aging to mimic natural paint degradation. Yellow ochre was selected as the pigment due to its historical prevalence, chemical stability, and minimal protein interaction, reducing variability and facilitating a more streamlined and reliable analysis. The same methodology was applied to a 16th-century painting, "*Battesimo di Cristo*" (Fig. 1), and a 13th-century wooden sculpture, "*The Great Polychrome Crucifix*" (Fig. 2).

Comparative analysis between aged/unaged specimens and historical samples highlighted differences in PTM profiles. Samples were collected via minimal surface scraping, followed by a workflow comprising: protein extraction, enzymatic hydrolysis, phosphopeptide enrichment, andadvanced biomolecular mass spectrometry.

Custom data analysis parameters were applied to identify PTMs (see Methods Section). The results provide a molecular characterization of tempera-painted surfaces, deepening insights into these artefacts. While this study does not yet directly inform restoration methodologies, it established a foundation for advanced analytical techniques in art conservation. Future work could explore diverse paint formulations and aging conditions, but these findings already pioneer a new approach to molecular-level characterization—particularly regarding protein-pigment interactions.

Methods

Fresh pictorial specimens

Six painting specimens were prepared using poplar wood panels as supports, since poplar is the most historically used material in panel paintings within the Italian artistic tradition[17]. Hide glue from Bresciani was employed both as a primer and as a binder for the ground layer. Granular hide glue was

Table 1 | Detailed Tempera recipes chosen for pictorial specimens' preparation

Tempera	*Binder*					
	Egg yolk	*Egg white*	*Milk*	*Casein*	*Water*	*Vinegar*
Egg yolk tempera	12 g	-	-	-	12 g	0.07 g
Egg white tempera	-	12 g	-	-	3 g	0.07 g
Whole egg tempera	12 g	12 g	-	-	15 g	0.14 g
Casein tempera	-	-	-	2 g	3 g	-
Casein and yolk tempera	12 g	-	-	1 g	11 g	0.07 g
Milk and yolk tempera	12 g	-	12 g	-	-	0.07 g

mixed with water at a 1:8 volume ratio and left to swell for 24 h. It was then heated to 60 °C in a water bath and applied to the wooden panels as a primer. The same hide glue mixture was combined with sifted Bologna gypsum from Giosi S.r.l. and brushed onto the primed panels, applying a total of eight layers. After air-drying for 48 h, the ground layer was sanded to ensure a smooth surface. To further isolate the ground layer before applying the tempera, two layers of a 1:12 hide glue mixture were brushed on. The tempera was then prepared following historical recipes sourced from the literature[16] by mixing different types of binders (egg white, yolk, whole egg, casein, casein and yolk, milk and yolk) with a pigment (yellow ochre, iron (III) hydroxide, from Maimeri)[18] (Table 1).

The specimens were stored at room temperature for one week until fully dried and classified as "fresh specimens" since they had not undergone any form of aging. Before analysis, the tempera was mechanically sampled using a scalpel from an area of approximately 5 mm × 5 mm.

Historical samples

To validate the extraction and characterization methods proposed in this study, two ancient works of art from the 16th and 13th centuries were investigated.

The first sample is a wooden panel painting depicting the "*Battesimo di Cristo*" (Fig. 1), attributed to the painter Cesare Turco[19]. This painting belongs to the Church of *S. Maria delle Grazie* in Caponapoli (Naples, Italy), and is kept in the deposits of the Superintendence of Archaeology, Fine Arts, and Landscape for the municipality of Naples. The paint layer was carefully scraped off with a scalpel by restorers from a 5 mm × 5 mm area of fragments deemed unsuitable for restoration.

The second sample investigated is collected from the original color layer of a wooden sculpture ("the Great Polychrome Crucifix from the 13th century"), belonging to the property of the Church of Santa Maria Assunta and San Giovanni Battista, located in Lettere (Naples, Italy) (Fig. 2). The paint layer was scraped with a scalpel from a 5 mm × 5 mm area. Since the analyses are not quantitative, small areas with the least possible visual impact were chosen for both works of art to collect the pictorial films.

Artificial aging of specimens

Photooxidative aging was conducted as described by Melchiorre et al. (2023)[20], under mild humidity conditions (RH 50%) and 40 °C, using a forced-air environmental chamber (Angelantoni SU250) equipped with a borosilicate glass-filtered low-pressure mercury UV lamp. The use of the filter excluded wavelengths below 300 nm, preventing non-representative photochemical degradation. The samples were exposed for 7 days to controlled UV radiation before being subjected to further analysis.

Proteins extraction

For each sample, proteins were extracted from the pictorial film using 100 µL of *denaturing buffer* containing 8 M guanidine hydrochloride, 1 mM EDTA, and 30 mM Tris HCl (pH 9). All samples were vortexed, sonicated for 20 min, and centrifuged at 12,000 rpm for 15 min[21]. The supernatant was recovered for subsequent hydrolysis.

Reduction, carbamidomethylation and enzymatic digestion

Disulfide bond reduction was carried out by adding 20 µL of 100 mM dithiothreitol (DTT) to the supernatants obtained from the extraction, and incubating for 60 min at 60 °C. The samples were then alkylated by adding 40 µL of 100 mM iodoacetamide in the dark for 1 h at room temperature. The protein mixture was purified by precipitation in chloroform/methanol/water[22]. The supernatant was removed, and the pellet was dried. Enzymatic digestion of the protein mixture was then carried out in 10 mM ammonium bicarbonate (Ambic) using trypsin as a proteolytic enzyme, through incubation at 37 °C form 16–18 h. The reaction was stopped by adding 10 µL of 10% formic acid (HCOOH).

Phosphopeptides analysis

To address the challenges of identifying phosphopeptides by LC-MS/MS, due to their low ionization efficiency in the presence of non-phosphorylated peptides, specific strategies were employed. The peptide mixture obtained from enzymatic hydrolysis was subjected to a phosphopeptide enrichment protocol using titanium dioxide (TiO_2, from Sigma-Aldrich) powder packed into a tube. After digestion, tryptic peptides were dissolved in a solution of 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 80% acetonitrile (ACN) and 2% HCOOH (*loading buffer*). The TiO_2 resin was activated with 200 µL of ACN under continuous mixing for 20 min at room temperature. Phosphopeptide enrichment was carried out according to the method described by Larsen et al.[23].

Once the supernatant containing the enriched phosphopeptides was recovered, it was dried down and subjected to desalination by bench-top reverse phase chromatography (C18 - Stage Tip)[24]. After the elution, phosphopeptides were dried under vacuum before nanoLC-MS/MS analysis.

nanoLC-MS/MS analysis

Enriched digested phosphopeptides, suspended in 10 µL of 0.5% formic acid (HCOOH), were analyzed using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with nano-HPLC. After loading, the peptide mixture was first concentrated and desalted on a precolumn (C18 EasyColumn L = 2 cm, ID = 100 µm, Thermo Fisher Scientific). Each sample was then fractionated on a reverse-phase C18 capillary column (C18 EasyColumn L = 10 cm, ID = 75 µm, particle size = 3 µm, Thermo Fisher Scientific) operating at a flow rate of 300 nL/min, with an eluent gradient B (0.2% formic acid, 2% acetonitrile, LC-MS Grade). Peptide analysis was performed using data-dependent acquisition (DDA) with an MS scan (mass range of 300 to 1800 m/z) followed by an MS/MS scan of the five most abundant ions in each MS scan. The mobile phase consisted of H_2O with 0.1% formic acid (buffer A) and 100% acetonitrile with 0.1% formic acid (buffer B). The gradient ranged from 5% to 30% buffer B over 95 min, followed by 30% to 60% B over 15 min, a step gradient to 85% B for 5 min, and a return to the initial conditions of 5% B, with a flow rate of 0.42 µL/min.

Data handling and interpretation

The acquired MS/MS spectra were converted into Mascot Generic Files (.mgf) format. MS/MS analysis data were used to identify proteins using the MS/MS Ion Search Program in Mascot software version 2.4.0 (http://www.matrixscience.com). The "Swissprot" database, with all entries as taxonomic restrictions, was used to identify peptides from pictorial specimens and artwork. Standard parameters used in the searches included trypsin as the enzyme, allowing for up to 3 missed cleavages, a 10 ppm MS tolerance, and a 0.6 Da MS/MS tolerance, with peptide charges ranging from +2 to +4. Carbamidomethylation of cysteines was set as the sole fixed chemical modification, while possible oxidation of methionines, formation of pyroglutamic acid from glutamine residues at the N-terminal position of peptides deamidation at asparagine and glutamine residues and phosphorylation of serine, threonine, and tyrosine were considered as variable modifications.

Results

The development of innovative diagnostic and restoration methodologies for artistic artefacts relies on a deep molecular understanding of works of art.

Table 2 | LC-MS/MS results obtained for the identification of phosphoproteins in the six tempera pictorial specimens

Phosphoprotein	Egg Tempera	White Tempera	Yolk Tempera	Casein Tempera	Yolk+Casein Tempera	Yolk+Milk Tempera
Vitellogenin-1	x		x		x	x
Vitellogenin-2	x		x		x	x
Vitellogenin-3	x				x	x
Ovalbumin	x	x	x			x
Ovotransferrin	x	x	x		x	x
Ovalbumin-related protein X	x	x				
Ovalbumin-related protein Y	x	x				x
Alpha-S2 casein				x	x	x
Alpha-S1 casein				x	x	x
Beta-casein				x	x	x
Beta-caseinA1				x		
Serum albumin				x		x
Kappa-casein				x	x	
Lactoperoxidase				x		

Due to the high historical significance of such artefacts, molecular analysis requires minimally invasive, sensitive, and reproducible methodologies to ensure the preservation of the work of art[25]. These methodologies are usually refined and validated on specimens created using popular historical recipes[8], minimizing the need for sampling from the artefacts under study, which are often of inestimable value. Once the reliability of the best protocol is established, it can be applied to the analysis of authentic historical samples.

Fresh pictorial specimens

To achieve molecular characterization focused on identifying protein phosphorylation in the pictorial binders of the two artefacts under investigation, experimental procedures for phosphopeptides enrichment and LC-MS/MS analysis were first tested using fresh paint samples prepared according to various traditional tempera recipes (as detailed in the Experimental Section). Specifically, six different binders were prepared using natural matrices like whole egg, yolk, albumen, casein, yolk mixed with casein, and yolk mixed with milk. Following specimen preparation, a minimal quantity of each sample was processed according to the hydrolysis and purification protocol and analyzed by LC-MS/MS as described in the Experimental Section.

Results of identified proteins and phosphoproteins with relative phosphorylation sites from each specimen analyzed are reported in Supplementary materials (Tables S1–S12).

Overall, several typical egg phosphoproteins were identified in the enriched fraction of egg tempera, including vitellogenins 1, 2, and 3, ovotransferrin, ovalbumin and ovalbumin-related proteins X and Y[26], along with their relative phosphopeptides. These results are corroborated by the identification of the same phosphoproteins separately in the egg white and yolk-based specimen. Specifically, typical albumen phosphoproteins such as ovotransferrin, ovalbumin and ovalbumin-related protein X and Y[27] were identified in the egg white tempera, while vitellogenin proteins were absent (Table S3), being more prevalent in the yolk specimen sample[11] (Tables S5 and S6). In egg white tempera sample, only ovalbumin phosphopeptides were identified (Table S4), probably due to its naturally abundant phosphorylation[28] compared to ovotransferrin both contained in albumen.

As expected, a plethora of phosphoproteins were identified in the casein tempera specimen (Tables S7 and S8). In particular, the presence of the major phosphoproteins of the caseins (α-S1, α-S2 and β-casein)[29], with the related enriched phosphopeptides, validates the effectiveness of the phosphopeptides enrichment protocol used.

In specimens with mixed matrices (yolk with casein, and yolk with milk), the same proteins from both egg yolk and casein samples were identified, namely α-S1, α-S2, and β-caseins, as well as vitellogenin 1, 2, and 3 (Tables S9 and S11). The analysis of phosphopeptides (Tables S10 and S12) showed that it is not possible to specifically discriminate between the two binder mixtures.

As detailed in Table 2, major phosphoproteins from different biological matrices were identified. Furthermore, the efficient enrichment and identification of phosphorylated proteins enables discrimination between milk-based and egg-based tempera.

Aged pictorial specimens

Following the artificial aging protocol detailed in the Methods section, all six tempera variants underwent identical processing to their fresh counterparts, including: protein extraction, tryptic hydrolysis, phospopeptides enrichment, and LC-MS/MS analyses. Complete dataset for identified proteins and phosphoproteins with relative phosphorylation sites, are provided in Supplementary materials (Tables S13 – S23).

In the aged egg tempera, phosphoprotein profiles remained consistent with fresh sample, although their scores were significantly lower (Table S13). A marked decrease in phosphopeptide detection was noted: while fresh egg tempera contained multiple vitellogenin phosphopeptides, aged samples revealed only a single ovalbumin phosphopeptide (Table S14 and S16). These results are consistent across all aged specimens, as shown in Table 3.

Specifically, ovalbumin, ovotransferrin, and ovalbumin-related proteins X/Y were identified in both fresh and aged egg white tempera (Table S15). However, in the case of aged egg white tempera, only a single phosphopeptide related to ovalbumin was identified (Table S16).

Aged yolk tempera, on the other hand, showed detectable phosphoproteins (vitellogenin-1, -2, -3, ovotransferrin, ovalbumin) but no identifiable phosphopeptides (Table S17).

Casein-based tempera demonstrated greater resilience to the effects of aging. Although protein scores were notably lower compared to the fresh sample (Table S18), this specimen yielded more phosphopeptides than other aged samples (Table S19), likely due to the naturally high phosphoprotein state of the casein.

Mixed-matrix specimens, containing both egg and casein phosphoproteins (Tables S20 and S22), showed a predominance of casein-derived phosphopeptides (Tables S21 and S23), reflecting casein's superior ability to preserve phosphorylation sites.

Comparative analysis highlights that artificial aging substantially affects phosphopeptide detection. Casein fractions exhibit greater analytical stability, while mixed specimens show intermediate characteristics. These findings suggest distinct degradation pathways for different binder types. This systematic comparison between fresh and aged specimens provides crucial insights into the molecular changes occurring during

Table 3 | LC-MS/MS results obtained for the identification of phosphoproteins in the six aged pictorial specimens

Number of identified phosphopeptides						
Phosphoprotein	**Egg Tempera**	**White Tempera**	**Yolk Tempera**	**Casein Tempera**	**Yolk+Casein Tempera**	**Yolk+Milk Tempera**
Vitellogenin-1	-		-		1	-
Vitellogenin-2	-		-		-	-
Vitellogenin-3			-		-	
Ovalbumin	1	1	-			
Ovotransferrin	-	-	-		-	
Ovalbumin-related protein X	-	-				
Ovalbumin-related protein Y	-	-				
Alpha-S2 casein				2	-	
Alpha-S1 casein				5	3	2
Beta-casein				-	1	1
Beta-caseinA1				2		
Serum albumin				2		
Kappa-casein				-	-	
Lactoperoxidase				1		

The number indicates the amount of phosphopeptides found, whereas the dash suggests the identification of the protein in the matrix and the absence of related phosphopeptides.

paint aging, serving as an important reference for interpreting historical sample analyses.

Historical samples

To validate our experimental approach, we applied the established phosphoprotein characterization protocol to two significant cultural heritage objects: a 16-th century wood panel painting ("*Battesimo di Cristo*") and a 13th-century polychrome wooden crucifix. This comparative analysis served to evaluate the method's effectiveness on authentic historical materials.

LC-MS/MS analysis, supported by specific bioinformatic processing, successfully identified phosphorylated proteins from *Gallus gallus* species in both artefacts.

In the wood panel painting "*Battesimo di Cristo*", three characteristic peptides were detected: one ovalbumin-derived peptide, one vitellogenin-2 phosphopeptide, and one phosphopeptide from a minor egg protein component (Tables S24, S25).

In the second historical sample, the "*Great Polychrome Crucifix from the 13th century*", multiple phosphoproteins belonging to the *Gallus gallus* species were identified, including vitellogenin-2, ovalbumin, ovotransferrin, vitellogenin-1, and ovalbumin-related protein Y (Table S26). Notably, only two specific phosphopeptides were detectable despite the broader protein identification (Table S27).

Discussion

The intrinsic complexity of pictorial artefacts stems from the diverse biomolecules within their constituent natural matrices, particularly protein-based binders. Precise molecular characterization of these components is critical for advancing ongoing research in art conservation and restoration[30].

This study presents a novel strategy for profiling phosphorylation in tempera binders, a modification likely instrumental in forming the three-dimensional networks that underpin paint layers. Phosphorylation influences protein-protein interaction dynamics, potentially enhancing cross-linking and thereby reinforcing the paint matrix's structural integrity over time[31,32]. Moreover, phosphorylated proteins in historical paintings may exhibit heightened resistance to environmental stressors, contributing to long-term preservation. Our approach combines phosphopeptide enrichment with LC-MS/MS analysis. These techniques were optimized using six model tempera specimens before application to two historical artifacts

The titanium dioxide enrichment protocol successfully isolated phosphopeptides from fresh tempera specimens. In casein-based tempera, we detected phosphopeptides from α-S1, α-S2, and β-casein, while egg-based fresh tempera yielded phosphopeptides from ovalbumin and vitellogenins. Although the same protocol enabled phosphoprotein identification in artificially aged tempera specimens, the identification scores were significantly lower than those obtained from fresh samples. The most notable difference emerged in phosphopeptide detection. Aged tempera specimens - particularly those with egg-based binders - showed substantially fewer identifiable phosphopeptides. This observation can be attributed to the increased complexity of the aged matrix, resulting from photooxidative reactions induced by the artificial aging chamber. Importantly, this artificial aging process did not account for microbial degradation, which often occurs in genuine historical samples. Applying the same analytical protocol to historical artefacts, LC-MS/MS analysis identified several phosphoproteins and their associated phosphopeptides. The "Battesimo di Cristo" wooden panel painting revealed three phosphopeptides from Gallus gallus proteins (ovalbumin and vitellogenin-2). The polychrome crucifix sample demonstrated a more extensive range of phosphoproteins (vitellogenin-1, vitellogenin-2, ovalbumin, ovotransferrin, and ovalbumin-related protein Y), yet yielded fewer detectable phosphopeptides. These findings illustrate both the increased molecular complexity and advanced degradation state of historical protein-based binders compared to fresh specimens[33]. The results highlight the analytical challenges posed by natural aging processes while simultaneously demonstrating the potential for methodological refinements to improve the study of ancient artefacts. The results of this study demonstrate the efficacy of our analytical approaches for extracting and characterizing post-translationally modified proteins from both freshly prepared specimens and historical samples.

The significant variation in phosphopeptide identification between fresh and aged samples underscores the substantial impact of degradation processes resulting from both natural aging and environmental exposure. Several mechanistic factors may contribute to these observed differences: i) alterations in phosphopeptide ionization efficiency during LC-MS/MS analysis, potentially caused by matrix modifications in aged samples[34]; ii) progressive dephosphorylation through oxidative pathways[35].

A promising direction for future research could involve investigating how aging degradation impacts the morphology of painter layer surfaces, offering further insights into the conservation and restoration of ancient artefacts. Building on these findings, we identify two paths for further investigation: i) systematic examination of how aging-induced degradation affects paint layer morphology at the microstructural level; ii) development of correlative approaches linking molecular-level changes to macroscopic paint properties. Such studies would provide valuable insights for advancing

conservation strategies and informing restoration protocols for cultural heritage objects. The integration of material characterization with surface analysis techniques could prove particularly fruitful for understanding long-term degradation pathways in protein-based paint systems. By elucidating the role of phosphorylation in paint binder stability, we move closer to understanding and preserving the material legacy of cultural heritage.

Supporting information

The Supporting Information is available free of charge on the ACS Publications website. LC-MS/MS results for the phosphoproteins and their related phosphopeptides identification in pictorial specimens (Tables S1–S12); in aged pictorial specimens (Tables S13–S23); in wood panel painting (Tables S24, S25); and in crucifix sculpture (Tables S26, S27) (PDF).

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[36] partner repository with the dataset identifier PXD053038.

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References

1. Janas, A. et al. Mechanical properties and moisture-related dimensional change of canvas paintings–canvas and glue sizing. *Herit. Sci.* **10**, 160 (2022).
2. Hogan, C. & Pieve, F. D. Colour degradation of artworks: an ab initio approach to X-ray, electronic and optical spectroscopy analyses of vermilion photodarkening. *J. Anal. At. Spectrom.* **30**, 588–598 (2015).
3. Rizzo, E., Morelli, M., Melchiorre, C., Naso, A. & Amoresano, A. A. Carpentieri, The scent of an archaic wreck: minimally invasive characterisation of archaeological finds. *Rend. Fis. Acc. Lincei* **35**, 719–724 (2024).
4. Gerony, F. et al, Monitoring the aging process in egg-tempera paint films. *Spectrochim. Acta Part A: Mol. Biomol. Spectrosc*. 125371. https://doi.org/10.1016/j.saa.2024.125371 (2024).
5. Gavrilov, D., Maev, R. G. & Almond, D. P. A review of imaging methods in analysis of works of art: Thermographic imaging method in art analysis. *Can. J. Phys.* **92**, 341–364 (2014).
6. Bottari, F., Oliveri, P. & Ugo, P. Electrochemical immunosensor based on ensemble of nanoelectrodes for immunoglobulin IgY detection: Application to identify hen's egg yolk in tempera paintings. *Biosens. Bioelectron.* **52**, 403–410 (2014).
7. Sugino, H., Nitoda, T., Juneja, L. R., General Chemical Composition of Hen Eggs, In: Hen Eggs, C. R. C. Press (1996).
8. Gettens, R. J., Stout, G. L. Painting Materials: A Short Encyclopedia, Courier Corporation (2012).
9. Cipolletta, B. et al. Molecular Characterization of Adhesives (Glue Lining Pastes) Used in Restoration. *Anal. Chem.* **96**, 16551–16560 (2024).
10. Rieloff, E. & Skepö, M. Phosphorylation of a Disordered Peptide—Structural Effects and Force Field Inconsistencies. *J. Chem. Theory Comput.* **16**, 1924–1935 (2020).
11. Wang, H. et al. Quantitative Comparative Integrated Proteomic and Phosphoproteomic Analysis of Chicken Egg Yolk Proteins under Diverse Storage Temperatures. *J. Agric. Food Chem.* **68**, 1157–1167 (2020).
12. Mann, K. The chicken egg white proteome. *PROTEOMICS* **7**, 3558–3568 (2007).
13. West, D. W. Structure and function of the phosphorylated residues of casein. *J. Dairy Res.* **53**, 333–352 (1986).
14. Kuckova, S. Cejnar, P., Santrucek, J., Hynek, R. Characterization of proteins in cultural heritage using MALDI–TOF and LC–MS/MS mass spectrometric techniques. *Phys. Sci. Rev.* **4**, https://doi.org/10.1515/psr-2018-0011 (2019).
15. Ntasi, G. et al. Proteomic Characterization of Collagen-Based Animal Glues for Restoration. *J. Proteome Res.* **21**, 2173–2184 (2022).
16. Thompson, D. V., The Practice of Tempera Painting, Courier Corporation (1962).
17. Cennini, C., Il libro dell'arte: o, Trattato della pittura, F. LeMonnier, (1859).
18. Mastrotheodoros, G. P. & Beltsios, K. G. Pigments—Iron-based red, yellow, and brown ochres. *Archaeol. Anthropol. Sci.* **14**, 35 (2022).
19. Moioli, P. & Seccaroni, C. *Tecniche radiografiche applicate ai beni culturali* (2004).
20. Melchiorre, C. et al. Green solvents and restoration: Application of biomass-derived solvents in cleaning procedures. *J. Cultural Herit.* **62**, 3–12 (2023).
21. Secondulfo, A. P. et al. Molecular characterisation of the tempera painting on canvas "The Martyrdom of Gorcum"., In: (2022).
22. Melchiorre, C. et al. Identification and Relative Quantification of hFSH Glycoforms in Women's Sera via MS–PRM-Based Approach. *Pharmaceutics* **13**, 798 (2021).
23. Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P. & Jørgensen, T. J. D. Highly Selective Enrichment of Phosphorylated Peptides from Peptide Mixtures Using Titanium Dioxide Microcolumns. *Mol. Cell. Proteom.* **4**, 873–886 (2005).
24. Illiano, A. et al. Determination of Fertility Hormones and Adipokines by LC-MRM/MS Analysis. *ACS Omega* **9**, 35482–35489 (2024).
25. Vinciguerra, R., De Chiaro, A., Pucci, P., Marino, G. & Birolo, L. Proteomic strategies for cultural heritage: From bones to paintings. *Microchemical J.* **126**, 341–348 (2016).
26. Sun, H. et al. Comparative Quantitative Phosphoproteomic Analysis of the Chicken Egg during Incubation Based on Tandem Mass Tag Labeling. *J. Agric. Food Chem.* **67**, 13353–13361 (2019).
27. Sun, Y., Jin, H., Sun, H. & Sheng, L. A Comprehensive Identification of Chicken Egg White Phosphoproteomics Based on a Novel Digestion Approach. *J. Agric. Food Chem.* **68**, 9213–9222 (2020).
28. Abeyrathne, E. D. N. S., Lee, H. Y. & Ahn, D. U. Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents—A review. *Poult. Sci.* **92**, 3292–3299 (2013).
29. Eigel, W. N. et al. Nomenclature of Proteins of Cow's Milk: Fifth Revision1. *J. Dairy Sci.* **67**, 1599–1631 (1984).
30. Rigante, E. C. L., Calvano, C. D., Sabbatini, L. & Cataldi, T. R. I. Historical proteinaceous-based pictorial binders' analysis: a novel proteomic minimally invasive approach. *J. Phys.: Conf. Ser.* **2204**, 012084 (2022).
31. Nishi, H., Shaytan, A. & Panchenko, A. R. Physicochemical mechanisms of protein regulation by phosphorylation. *Front. Genet.* **5**, 270 (2014).
32. Volkov, V. & Cavaco-Paulo, A. In vitro phosphorylation as tool for modification of silk and keratin fibrous materials. *Appl Microbiol Biotechnol.* **100**, 4337–4345 (2016).
33. Leo, G. et al. Deamidation at Asparagine and Glutamine As a Major Modification upon Deterioration/Aging of Proteinaceous Binders in Mural Paintings. *Anal. Chem.* **83**, 2056–2064 (2011).
34. Maes, E. et al. The use of elemental mass spectrometry in phosphoproteomic applications. *Mass Spectrom. Rev.* **35**, 350–360 (2016).
35. Rosenstein, R. W. & Taborsky, G. Mechanism of the oxidative dephosphorylation of the phosphoprotein phosvitin. *Biochemistry* **9**, 649–657 (1970).
36. Perez-Riverol, Y. et al. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res.* **50**, D543–D552 (2022).

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Author contributions

A.C. conceived the project, reviewed, and edited the manuscript. A.C. and G.M. coordinated and supervised the activities. M.M. provided the pictorial samples and the restorer's point of view. E.R., M.N., and C.M. carried out the experimental activities. E.R. analyzed and interpreted experimental data and drafted the manuscript. A.A. provided the MS setup. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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