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Development and application of innovative chemical methodologies for diagnostics in cultural heritage

PhD candidate

Brunella Cipolletta

Tutor

Prof. L. Birolo

Supervisor

Prof. A. Amoresano

Coordinator

Prof. A. Napolitano

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A handwritten signature in black ink, which appears to read 'Alessandra Napolitano', is written below the printed name.

Table of Contents

1.

Table of Contents.....	3
Abbreviations.....	x
Acknowledgments	xii
Abstract.....	1
References	3
1. Organic Molecules Analysis In The Cultural Heritage: Focus On Mass Spectrometry-Based Methods	5
1.1 Chemistry and cultural heritage.....	5
1.2 Organic compounds in artistic and archaeological objects	5
1.3 Organic molecules analysis in cultural heritage materials	9
2. Protein Investigation In The Cultural Heritage Field: Focus On Mass Spectrometry-Based Proteomics	11
2.1 Proteins in cultural heritage artifacts	11
2.3 Protein investigation in cultural heritage.....	12
2.3 Mass spectrometry (MS)-based proteomics.....	15
2.4 Bottom-Up Proteomics Workflow.....	16
2.5 Mass Spectrometry Imaging (MSI)	27
2.6 GC-MS analysis	38
References	40
3. Aims of the research	45
References	48

4. Analytical strategies for the identification of organic molecules in cultural heritage materials	51
Manuscripts	53
Research Projects	53
4.1 Molecular characterization of adhesives (glue lining pastes) used in restoration	54
4.2 The challenge of organic content of potteries: multistrategic approaches	Erreur ! Signet non défini.
Case study 1: Archaeological potteries from Pani Loriga	Erreur ! Signet non défini.
Case study 2: Amphorae from the Ancient Harbor of Neapolis	Erreur ! Signet non défini.
Case study 3: Funeral Equipment from A Hellenistic Young Woman Tomb	Erreur ! Signet non défini.
4.3 Mixed carbon-iron gall ink used in Vanvitelli's letters	Erreur ! Signet non défini.
4.4 Proteomic analysis of ancient silk fibers from Pompeii	Erreur ! Signet non défini.
4.5 Towards minimally invasive protein analysis workflows	Erreur ! Signet non défini.
Minimally invasive Protein Identification on a XIV century painting by Colantonio	Erreur ! Signet non défini.
Immobilized Trypsin – Enhanced MALDI-Imaging analysis of surfaces and cross-sections of works of art	Erreur ! Signet non défini.

5.	Development of new proteomics workflow for the chemical characterization of proteinaceous materials	73
	Post-translational and diagenetic modifications in ancient proteins	73
	Degradation pathways in collagen-based materials.....	76
	Interactions between proteins and inorganic pigments in paintings	76
	Analytical challenges and bioinformatic approaches in paleoproteomics	77
	A comparative and structural approach to protein degradation studies ..	78
	Manuscripts.....	80
	References	81
	5.1 Molecular details of the interactions between inorganic pigments and casein-based glue in reference paint reconstructions . Erreur ! Signet non défini.	
	5.2 Characterization of the secondary structure, renaturation and physical ageing of gelatine adhesives	83
	5.3 From waste to function: valorization of collagen-based wastes with Natural Deep Eutectic Solvents for bioadhesive applications.....	111
	5.4 Multi-tasking collagen: one molecule, different outfits and uses Erreur ! Signet non défini.	
6.	Final Conclusions	151
	References	156
	PhD Activities Summary	159
	Attended Courses	159
	Attended Congresses	159
	Attended Schools.....	160

Presented Posters	160
Presented Oral Presentations	161
Awards	161
Attended Seminars	161
Period Abroad.....	162
Support For Teaching Activity	162
Publications	163

*“There is an art to science, and a science in art;
the two are not enemies,
but different aspects of the whole.”*

*Isaac Asimov
from *The Roving Mind* (1983).*

Abbreviations

ACN: Acetonitrile

AMBIC: Ammonium bicarbonate

BSTFA: N,O-

Bis(trimethylsilyl)trifluoroacetamide

CH: Cultural heritage

CHCA: α -cyano-4-hydroxycinnamic acid

DDA: Data-dependent acquisition (DDA)

DESS: deep eutectic solvents

DTT: Dithiothreitol

EI: Electron Impact (EI) source

FT-IR: Fourier-transform infrared

FTICR: Fourier-transform ion cyclotron resonance

GC-MS: Gas Chromatography-Mass Spectrometry

HCOOH: Formic acid

HPLC-ESI-Q-TOF: High-Performance Liquid Chromatography with Electrospray Ionization Quadrupole Time-of-Flight mass spectrometry

IAM: Iodoacetamide

LCA: Lowest common ancestor

LC-MS/MS: Liquid Chromatography coupled to Tandem Mass Spectrometry

LiP-MS: Limited Proteolysis coupled to Mass Spectrometry

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

MQ: MaxQuant

MRM: Multiple Reaction Monitoring

MS: Mass Spectrometry

MS/MS: Tandem mass spectrometry

m/z: Mass-to-charge ratio

nanoESI: Nano-Electrospray Ionization

OM: Optical microscopy

ORA: Organic residue analysis

PMF: Peptide mass fingerprinting

PNGase F: Peptide-N-Glycosidase F

PSMs: Peptide Spectrum Matches

PTMs: Post-translational modifications

SEM-EDS: Scanning Electron Microscopy with Energy Dispersive Spectroscopy

SEM-EDX: Energy-dispersive X-ray spectroscopy

SIM: Selected Ions Monitoring

TFA: Trifluoroacetic acid

TMS: Trimethylsilylation

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Abstract

The chemical investigation of cultural heritage (CH) materials represents one of the most challenging and interdisciplinary areas within analytical sciences.¹ Works of art and archaeological artefacts are complex, multicomponent systems composed of organic and inorganic substances that have interacted and transformed over centuries. Understanding their molecular composition and degradation pathways is fundamental to reconstruct manufacturing and artistic techniques and to develop scientifically informed conservation strategies.^{2,3} However, CH materials pose major analytical challenges due to their heterogeneity, the extremely low concentration of target analytes, pervasive contamination and degradation, and strict sampling limitations imposed by the uniqueness and fragility of the artefacts.^{2,4}

The overarching aim of this doctoral research was the development and application of innovative mass spectrometry (MS)–based analytical strategies for CH diagnostics, with particular emphasis on organic materials. The project was structured around two complementary objectives: (1) the identification of ancient proteins and organic molecules in historical and archaeological samples through highly sensitive, selective, and minimally invasive workflows, and (2) the chemical characterization of proteinaceous materials to elucidate degradation mechanisms, pigment–binder interactions, and the effects of manufacturing and environmental factors on long-term material evolution.

In the first research line, several MS-based workflows were developed and applied to diverse case studies, including archaeological pottery residues, historical iron-gall inks, restoration adhesives⁵, and ancient proteinaceous fibers. These studies combined gas chromatography–mass spectrometry (GC–MS), liquid chromatography–tandem mass spectrometry (LC–MS/MS) proteomics, and complementary spectroscopic and microscopic techniques, demonstrating that multi-analytical strategies enable comprehensive molecular fingerprinting of complex historical matrices while maintaining minimal invasiveness. Tailored extraction and solubilization protocols were established for highly degraded and carbonized materials, enabling, for example, the molecular identification of ancient silk proteins from Vesuvian archaeological textiles.

The research further advanced minimally invasive proteomics through the development of a novel workflow integrating minimally-invasive sampling using a trypsin-functionalized cellulose acetate sheet^{6,7} with direct MALDI–mass spectrometry imaging (MALDI–MSI).

This approach established a new paradigm for non-destructive, spatially resolved proteomic analysis of surfaces and cross-sections of works of art.

The second research line focused on the chemical characterization of proteinaceous materials to elucidate their degradation and ageing behavior. An analytical and computational pipeline was implemented to semi-quantitatively assess post-translational and diagenetic modifications in proteins and to visualize the distribution of chemical changes along their sequences. This approach supported the first proteomic investigation of pigment–protein binder interactions in casein-based paint reconstructions, revealing pigment-specific effects on casein degradation pathways. A limited proteolysis mass spectrometry (LiP–MS) method was further proposed to probe pigment-induced structural alterations, extending proteomic analysis toward conformational and reactivity studies in complex heritage materials.

Complementary studies on collagen-based materials—including historical glues and novel bioadhesives—demonstrated that chemical treatments leave diagnostic molecular fingerprints detectable through MS-based proteomics. Comparative analyses linked specific modification patterns (deamidation, oxidation, backbone cleavage) to manufacturing chemistry and degradation conditions, providing a molecular framework for evaluating collagen’s adhesive performance and stability.⁸

Overall, this doctoral research advanced the analytical and interpretative framework for the molecular investigation of organic CH materials. By integrating multi-analytical approaches, minimally invasive proteomics, and advanced computational pipelines, it achieved significant progress in adapting MS methodologies to the unique constraints of heritage science.

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2. Organic Molecules Analysis In The Cultural Heritage: Focus On Mass Spectrometry-Based Methods

1.1 Chemistry and cultural heritage

Cultural heritage (CH) encompasses both tangible expressions—such as artworks, historical monuments, architectural structures, and archaeological sites—and intangible elements, including traditions, rituals, and oral practices that embody a society's collective identity. The conservation and restoration of this heritage are fundamental for preserving history and ensuring its transmission to future generations, a mission reinforced by international initiatives such as the UNESCO conventions established since 1954. The safeguarding of cultural heritage is an inherently interdisciplinary endeavor, requiring collaboration among conservator–restorers, archaeologists, art historians, architects, and, increasingly, chemists.

Chemistry plays a pivotal role in this field, as it enables the identification, characterization, and preservation of heritage materials through analytical and diagnostic techniques.¹ Analytical chemistry for CH focuses on determining the identity of materials, studying ancient production techniques, understanding mechanisms of degradation, and evaluating restoration treatments. Furthermore, it supports archaeological and historical interpretations through archaeometric analyses aimed at revealing how ancient materials were produced, their provenance, usage, authenticity, and dating.¹ From the molecular examination of pigments, binders, and varnishes in paintings to the study of corrosion and aging processes, to the identification of organic residues in archaeological remains, chemistry provides a bridge between science and the humanities—unveiling hidden information, guiding conservation strategies, and deepening our understanding of human creativity through time.

1.2 Organic compounds in artistic and archaeological objects

Organic materials are among the most chemically and structurally diverse substances employed or encountered in CH objects. They originate from a broad spectrum of biological sources—animal, vegetal, and microbial—and have been used by humans for millennia as binding media, adhesives, coatings, colorants, supports, and embalming substances.

Beyond their intentional application, organic matter may also occur as residues absorbed into archaeological matrices or as degradation products resulting from the long-term transformation of original materials.²

From a chemical perspective, these substances constitute complex multicomponent systems composed primarily of biopolymers (proteins, polysaccharides, and lipids) and a range of low-molecular-weight compounds, including terpenoids, alkaloids, phenolics, and wax esters. Their composition is further complicated by the coexistence and interaction of multiple organic classes within a single artefact and by their intimate association with inorganic substrates such as mineral pigments, ceramics, metals, and geological matrices. These hybrid systems are inherently unstable: they evolve over time through oxidation, hydrolysis, cross-linking, microbial decay, and photoinduced reactions, resulting in profound molecular alterations and the formation of secondary products.³ Understanding this chemical complexity is fundamental to reconstructing historical technologies, assessing authenticity, and developing effective conservation strategies.

In paintings and polychrome sculptures, organic materials primarily function as binding media, serving to suspend and fix pigments onto a substrate.^{3,4} These binders are broadly categorized into proteinaceous, lipidic, and carbohydrate-based classes.

Proteinaceous binders include animal glues (derived from collagen hydrolysates), casein (from milk), and egg-based media (egg yolk and egg white).⁵ Chemically, these materials consist of polypeptide chains characterized by specific amino acid sequences and stabilized by hydrogen bonding and, in some cases, disulfide linkages. Their degradation involves peptide bond hydrolysis, amino acid oxidation, and cross-linking reactions, which alter solubility, flexibility, and overall mechanical behavior.³

Lipidic binders, such as drying oils from linseed, walnut, or poppyseed, are composed mainly of triacylglycerols rich in unsaturated fatty acids (oleic, linoleic, and linolenic acids).⁶ Upon exposure to oxygen and light, these oils undergo autoxidation and polymerization, forming complex three-dimensional networks of oxidized and cross-linked species.⁷ The generation of hydroperoxides, dicarboxylic acids (e.g., azelaic acid), and metal carboxylates (“metal soaps”) are characteristic aging phenomena in such systems.⁸

Carbohydrate-based binders, notably plant gums (arabic, tragacanth) and starch pastes, are composed of polysaccharides such as arabinose, galactose, and glucose linked through glycosidic bonds.^{9,10} Hydrolysis of these bonds under humid conditions leads to

depolymerization, while oxidative reactions introduce carbonyl and carboxyl functionalities, modifying rheological behavior and adhesion.

Varnishes and waxes serve as protective or aesthetic coatings. Natural resins such as dammar, mastic, copal, and shellac are composed of terpenoid and resin acid derivatives, often containing small quantities of fatty materials.¹¹ Over time, oxidation and polymerization of these components result in yellowing, embrittlement, and loss of transparency. Beeswax—a complex mixture of long-chain esters, fatty acids, and alcohols—has been used both as a painting medium (in the encaustic technique) and as a surface finish.¹¹ Its aging involves ester hydrolysis and oxidation of unsaturated constituents, leading to loss of gloss and mechanical weakening.

Paint layers are chemically and physically heterogeneous systems in which organic and inorganic phases coexist and interact dynamically.^{12–15} Metal pigments catalyze lipid oxidation, while carboxylic acids released from oil degradation react with metal ions to form metal soaps⁸, often visible as protrusions or efflorescences on painted surfaces. Environmental factors—pollutants, light, and humidity—further accelerate these degradation pathways. The investigation of these processes, drawing upon polymer chemistry, surface science, and analytical chemistry, is essential to understanding the long-term evolution of artistic materials.

Many paleontological and archaeological artefacts contain proteins as inherent organic components. In bone, ivory, dentin, and teeth, collagen constitutes the principal organic phase, accounting for up to 30% of the dry weight of bone. Collagen is a fibrous protein organized in a triple-helical structure composed of repeating sequences of glycine, proline, and hydroxyproline.¹⁶ In archaeological contexts, collagen provides critical information on diagenetic processes, burial environments, and paleodiets.¹⁷ However, it is highly susceptible to hydrolysis and oxidative cleavage, resulting in chain scission, denaturation of the triple helix, and conversion into gelatin.¹⁶ Additional cross-linking reactions—through Maillard-type condensation or oxidative coupling—can enhance brittleness and reduce solubility.

Animal-derived fibers such as wool and hair consist primarily of keratin, a sulfur-rich protein stabilized by disulfide bonds.^{18,19} Its deterioration involves cleavage of these bonds, oxidation of cysteine to cysteic acid, and hydrolytic loss of peptide integrity. Silk, composed mainly of fibroin (β -sheet-rich) and sericin (amorphous), undergoes degradation through peptide hydrolysis and conformational changes in its crystalline regions.^{18,19}

Plant-based textiles such as linen and cotton are dominated by cellulose, a $\beta(1\rightarrow4)$ -linked D-glucose polymer. Degradation proceeds via acid-catalyzed hydrolysis of glycosidic bonds and oxidation leading to the formation of carbonyl and carboxyl groups, processes often accelerated by metal contaminants and light exposure. The interplay of these chemical transformations governs the mechanical stability and aesthetic preservation of ancient fibers and fabrics.

Porous archaeological ceramics frequently retain lipid residues derived from ancient foodstuffs absorbed into their matrix.² The molecular composition of these residues reflects their original use—animal fats, dairy products, or plant oils. Diagnostic markers include saturated and unsaturated fatty acids, ω -hydroxyacids, and dicarboxylic acids formed through oxidation. The C_{16:0}/C_{18:0} fatty acid ratio and the presence of short-chain acids are used to distinguish dairy products from adipose fats. Prolonged burial results in triacylglycerol hydrolysis, decarboxylation, and oxidative polymerization, yielding complex assemblages of polar and nonpolar degradation products.

Organic residues associated with funerary and ritual practices often contain mixtures of beeswax, plant oils, animal fats, conifer resins, and bitumen.² Terpenoid compounds (diterpenes and triterpenes) act as molecular fingerprints for botanical sources. With aging, these compounds undergo oxidation, dehydrogenation, and rearrangement, producing a suite of oxidized terpenoids and aromatic derivatives²⁰. Molecular characterization of such mixtures provides valuable insights into ancient trade networks, resource procurement, and technological practices.

Although less stable than lipids, proteins and carbohydrates can persist in ceramics^{21–23}, lithics, or metal artefacts under favorable preservation conditions. Advanced analytical techniques such as LC–MS/MS proteomics enable the species-level identification of ancient proteins (e.g., milk caseins, blood albumins), revealing dietary and ritual practices.^{21,22,24} Similarly, monosaccharide profiling after acid hydrolysis can detect plant gums, starch residues, and honey traces, contributing to the molecular reconstruction of organic residues in archaeological contexts.

Since the 19th century, the conservation of artworks has introduced numerous synthetic polymers, including cellulose nitrate, polyvinyl acetate, acrylic resins, and epoxy adhesives.²⁵ Initially considered chemically stable, these materials are now known to undergo degradation via chain scission, plasticizer migration, oxidation, and yellowing. Their interaction with original materials can generate additional layers of complexity,

particularly when degradation products migrate, react, or catalyze further deterioration. A detailed understanding of these mechanisms at the molecular level is crucial for developing sustainable and reversible conservation treatments.

Organic materials in CH objects constitute an extraordinarily intricate chemical domain, where natural biopolymers, synthetic polymers, and small organic molecules coexist and transform continuously over time. Their study demands an inherently interdisciplinary approach, integrating organic chemistry, analytical science, materials research, and archaeology.¹ Molecular-level investigations not only elucidate degradation mechanisms but also allow the reconstruction of ancient technologies and inform the design of more effective and sustainable conservation practices. Far from being static relics, organic CH materials are dynamic chemical systems, recording within their molecular architecture the intertwined history of their creation, use, alteration, and preservation.

1.3 Organic molecules analysis in cultural heritage materials

Given the chemical complexity of CH samples, the identification and characterization of organic molecules rely on a combination of complementary analytical techniques. A variety of analytical methods, both non-invasive and micro-invasive, have been developed for the study of organic materials in CH samples. Spectroscopic techniques, such as Fourier Transform Infrared (FTIR) and Raman spectroscopy, have been extensively applied for the identification of both inorganic and organic components in works of art.^{26,27} These non-invasive techniques allow for functional group identification and mapping organic–inorganic interfaces.

Mass spectrometry (MS) represents a powerful analytical tool in investigations of CH objects due to its high sensitivity and capability to analyze complex mixtures when coupled to high-resolution chromatographic technique.^{28,29} Chromatographic methods, particularly gas chromatography coupled to mass spectrometry (GC–MS)^{30,31} and pyrolysis-GC–MS³², represent key analytical approaches for organic components identification in works of art and historical object. Advances in sample pretreatment have enabled simultaneous or sequential determination of multiple analyte classes (e.g., proteins, glycerolipids, waxes, terpenoid resins, and polysaccharides) from a single micro-sample.^{20,30,31}

Over the past two decades, proteomics combined with tandem MS (LC–MS/MS) has emerged as a successful tool for the reliable species identification and characterization of proteins in archaeological and artistic objects.^{29,33,34} MS-based proteomics allows both

species-level protein identification and the detailed characterization of protein modifications³ and interactions for a more-in depth knowledge of degradation processes related to ageing and specific conditions (storage, environmental factors, pollution, restoration treatments, interaction with inorganic materials).

Increasingly, micro-sampling and non-destructive analytical strategies are prioritized to minimize material loss and preserve the integrity of irreplaceable artefacts.³⁵ Ongoing methodological advances now permit analyses from few micrograms of sample, often approaching non-invasive sampling^{36,37}, as well as to transcend the protein identification, pursuing a higher understanding of protein degradation processes.³

Despite the wide range of analytical techniques applied to CH samples, this doctoral study focuses specifically on MS-based methodologies.

The investigation of proteins will be extensively discussed in **Chapter 2**, with particular attention to proteomic strategies combined with MS analyses, namely bottom-up proteomics and spatially resolved proteomics.

Chapter 3 provides a concise description of the gas chromatography–mass spectrometry (GC-MS) technique, which was employed in this research for the identification of both polar and nonpolar organic compounds in a variety of CH materials. Special emphasis is placed on the sample preparation workflows and the instrumental setup.

3. Protein Investigation In The Cultural Heritage Field: Focus On Mass Spectrometry-Based Proteomics

Proteins represent a crucial source of information in the analysis of artistic, archaeological, and paleontological objects. Their study and characterization allow for a deeper understanding of the materials involved, offering insights into the artist's manufacturing techniques as well as the conservation state of the object.^{29,33}

A concise review of the principal analytical techniques—both non-invasive and micro-invasive—employed in the investigation of proteins within CH is presented. Particular attention is given to the proteomic strategies combined with mass spectrometry (MS) analyses applied throughout this doctoral research, namely bottom-up proteomics and spatially resolved proteomics.

Bottom-up proteomics, has recently emerged as a powerful tool for the in-depth study of ancient works of art and archaeological remains, enabling accurate protein identification and reliable species determination. This chapter also highlights the potential of bottom-up proteomics workflows for the comprehensive characterization of cultural heritage materials, with special emphasis on their ability to reveal chemical modifications related to protein degradation and ageing processes.^{3,16,17}

Finally, mass spectrometry imaging (MSI) is presented as an innovative technique that allows spatially resolved protein identification directly on the surface of works of art.^{38,39}

2.1 Proteins in cultural heritage artifacts

Proteins are key molecules in the studies of art, archaeological and paleontological artefacts. In fossils, ancient bones, and other ancient material such as dental calculus, proteins offer crucial information on ancient and extinct species, human evolution, past migrations and phylogenetic trees.^{33,40} Proteins constitute or are trapped in ancient objects of daily-life activities, including clothing, tools, cosmetics or medicines, ceramic vessels or other containers.² Their analyses can reveal valuable information on socio-cultural aspects, manufactory, diet, and ancient trade in past populations.

Protein-based materials were also extensively used in artistic production: e.g., egg, casein, animal glue binders used in wall and easel paintings or applied to stuccoes and

sculptures, adhesive for gildings, writing supports (like parchment-based manuscripts), textiles (wool, silk), drawing sizing, but also adhesives, consolidants or waterproof coatings used for preservation.

The study and characterisation of these molecules provide accurate knowledge of the material, inform the artist's manufacturing technique, and state the object's conservation. The characterisation of the deterioration products may help in studying the deterioration causes and may assist the definition of adequate preservation strategies (i.e., restoration treatments, control of exposure and environmental parameters).

The characterisation of proteinaceous material is an analytical challenge for various reasons: the analyte is often embedded in a complex and heterogeneous matrix consisting of organic and inorganic substances (pigments, binders and other substances contaminants) that interact with each other. Furthermore, proteins are subject to ageing and to degradation processes related to their storage conditions, environmental factors³ (e.g., temperature, relative humidity, light exposure), interactions with other compounds^{12–14,41} (external or internal to the object), and invasive or unsuitable conservation conditions and restoration treatments. Another critical issue in the study is the low amount of material available for the scientific analysis to guarantee the artefact's minimal invasiveness.³⁵

2.3 Protein investigation in cultural heritage

A variety of analytical methods, both non-invasive and micro-invasive, have been developed for the study of proteins in cultural heritage materials. Non-invasive spectroscopic techniques, in particular, have been extensively applied for the identification of both inorganic and organic components in works of art.

Vibrational spectroscopies, such as Fourier-transform infrared (FT-IR) and Raman spectroscopy, have long been employed for the rapid recognition of proteinaceous materials by detecting characteristic functional groups (e.g., amide bands).^{12,42–44} In recent years, progress in instrumentation—such as portable devices, imaging and mapping microscopes, and the introduction of surface-enhanced Raman scattering (SERS) – has considerably expanded their applicability. Furthermore, external reflection FT-IR (ER-FTIR) has proven useful for distinguishing proteinaceous from polysaccharide-based binders⁴⁵, while laser-induced fluorescence (LIF) exploits the intrinsic fluorescence of aromatic amino acids (Phe, Tyr, Trp) to detect proteins and monitor degradation products or amino acid cross-linking reactions. The non-destructive nature of LIF enables material identification without

sampling or transportation. The integration of multi-spectroscopic mobile systems combining LIF, laser-induced breakdown spectroscopy (LIBS), and portable Raman has further enhanced in-situ analyses of binding media.⁴³

Chromatographic methods, particularly gas chromatography coupled to mass spectrometry (GC–MS) and pyrolysis-GC–MS, represent another key analytical approach for organic components in artworks.^{20,30,31,46} After protein hydrolysis and amino acid derivatization, the analysis and relative distribution of amino acids allow the identification of proteinaceous binders and their degradation products. Advances in sample pretreatment have enabled simultaneous or sequential determination of multiple analyte classes (e.g., proteins, glycerolipids, waxes, terpenoid resins, and polysaccharides) from a single micro-sample.^{20,30,31} More recently, pyrolysis-evolved gas analysis mass spectrometry (EGA/MS) has been optimized to study aged and degraded proteinaceous materials characterized by high thermal stability and low solubility, enabling rapid evaluation of aggregated or cross-linked proteins without prior extraction.

Similarly to GC-MS, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) enables protein identification through amino acid fragment detection and localization within paint cross-sections. This spatial capability provides insights into the painter's technique and facilitates the discrimination between original and restored layers.

Immunological techniques have also gained increasing relevance in cultural heritage studies. Methods such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence surface-enhanced Raman scattering (immune-SERS) combine high specificity with sensitivity, allowing detection, discrimination, and mapping of target proteins in complex matrices through antigen–antibody interactions. The development of portable immunoassay devices, including chemiluminescent (CL) systems coupled with lateral flow immunoassays (LFIA), has further enabled the in-situ detection of multiple proteins—such as ovalbumin and collagen—directly on artifacts.⁴⁷

Over the past two decades, proteomics combined with mass spectrometry (MS) has emerged as a as a successful tool for the reliable species identification and characterization of proteins in archaeological and artistic objects. MS-based proteomics allows both species-level protein identification and the detailed characterization of protein modifications and interactions for a more-in depth knowledge of degradation processes related to ageing and specific conditions (storage, environmental factors, pollution, restoration treatments, interaction with inorganic materials).^{3,29,33,34,48} Ongoing methodological advances now

permit analyses from few micrograms of sample, often approaching non-invasive sampling³⁵, as well as to transcend the protein identification, pursuing a higher understanding of protein degradation processes.^{3,17}

Within this framework, the Limited Proteolysis coupled to Mass Spectrometry (LiP-MS) approach⁴⁹ has been explored in this doctoral study to investigate whether chemical interactions with intrinsic components of artistic materials induce structural alterations in proteins. LiP-MS is based on the principle that conformational changes influence protease accessibility; hence, differential proteolysis patterns reflect structural modifications. Originally developed for proteins in solution and in biological contexts, this approach has been adapted here to the challenging case of dry, solid-state proteins embedded in paint layers.

The growing demand for spatially resolved molecular information has also stimulated the development of mass spectrometry imaging (MSI) techniques.³⁸ Among these, matrix-assisted laser desorption/ionization (MALDI)-MSI has emerged as particularly promising for high-throughput, multiplexed molecular screening.³⁹ MSI extends conventional mass spectrometry by enabling the visualization of molecular distributions directly from the sample surface, without homogenization or extraction. Recent technological advances have led to improved spatial resolution and acquisition speed, broadening MSI applications from biomedical and forensic analyses to cultural heritage research.

In contrast to microscopy or spectroscopic imaging—such as Raman or scanning electron microscopy-energy dispersive X-ray spectroscopy, which provide molecular bond or elemental information—MSI delivers label-free, chemically specific mapping of analytes based on their mass-to-charge (m/z) ratios. This capability allows simultaneous localization of proteins, peptides, lipids, metabolites, and inorganic species (such as pigments). Understanding the composition of pigments and binders within individual paint layers is fundamental to reconstructing artistic techniques and material histories. MALDI-MSI uniquely offers the possibility to map both organic and inorganic compounds within paint cross-sections or across surfaces at micrometer resolution (5 μm), providing unparalleled insight into the molecular composition of works of art.

2.3 Mass spectrometry (MS)-based proteomics

The term proteome, first introduced in 1994, refers to the complete set of proteins expressed by the genome of a cell, tissue, or organism at a specific moment and under defined environmental conditions.⁵⁰ Unlike the genome, which remains relatively constant within an organism, the proteome is dynamic. The identity, abundance, structure, and localization of proteins within a cell vary according to developmental stage, physiological state, and external stimuli.

Proteomics is the discipline dedicated to the large-scale study of the proteome, aiming to identify, characterize, and quantify the full complement of proteins present in a given system. Beyond protein identification, proteomics also seeks to elucidate the diversity of protein isoforms, post-translational modifications (PTMs), protein–protein interactions, higher-order structural organizations, and the functional complexes in which proteins participate.⁵⁰

In proteomics, MS allows the determination of the exact molecular weight of proteins or their proteolytic fragmentation products (peptides) by measuring the mass-to-charge ratio (m/z) of produced gas-phase ions.

In a conventional proteomic workflow, proteins are first extracted using mild conditions that prevent hydrolysis into amino acids. When necessary, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or similar techniques may be employed to separate complex protein mixtures. Prior to MS analysis, proteins can be enzymatically or chemically cleaved into peptides (for peptide-based analyses) or examined as intact macromolecules.

Depending on the analytical strategy, the resulting analyte solution may be introduced directly into the mass spectrometer—such as in matrix-assisted laser desorption/ionization (MALDI) systems—or first separated by liquid chromatography in LC–MS workflows. Following MS spectra acquisition, bioinformatics tools integrating proteomic and genomic databases are used to match experimental spectra to specific protein sequences, thereby enabling reliable protein identification.

Mass spectrometric measurements can be conducted in single-stage MS mode, which records the masses of intact ions, or through tandem mass spectrometry (MS/MS), where precursor ions are further fragmented to generate sequence-specific product ions.

The latter approach underpins two principal proteomic strategies: the bottom-up approach, which investigates peptides derived from proteolytic digestion, and the top-down approach, which analyzes intact proteins directly. These complementary methodologies collectively form the analytical foundation of modern proteomics.^{51,52}

2.4 Bottom-Up Proteomics Workflow

In cultural heritage investigations, bottom-up proteomics is the mainstream strategy for protein identification and characterization. Since pioneering results in the 2000s⁵³, this strategy based on protein digestion has been greatly improved, leading to a more in-depth insight into artistic and historic samples.

The bottom-up proteomics approach, also called “shotgun proteomics”, when performed on a proteinaceous mixture^{51,52}, is based on the enzymatic or chemical digestion of these macromolecules into peptides before the MS analysis. The wide implementation of this approach principally relies on easier separation, ionization and fragmentation of peptides compared to intact proteins.

Following the protein digestion, the MS analysis can be performed using two different approaches. The first strategy, peptide mass fingerprinting (PMF), consists of the acquisition of intact peptide masses, matched with theoretical diagnostic peptides from the protein public repository. The second approach, peptide sequencing (MS/MS or MS2), involves the isolation and fragmentation of specific peptides in the source of the mass spectrometer's analyzer, followed by the reconstruction of the amino acid sequence using bioinformatic tools and protein database.

Proteins of species with unsequenced genomes (therefore not present in the database) can be characterized via de novo sequencing based on the direct interpretation of the amino acid sequence using the MS/MS spectrum. A cross-species homology search can follow to identify the peptide sequences.

PMF has been widely implemented as a routine methodology to shed light on the species of origin of the proteinaceous material in artistic and archaeological objects. The strategy ZooMS (Zooarchaeology by Mass Spectrometry)^{54,55} has emerged as a fast and straightforward method for the characterisation of collagen-based objects using PMF of Type I collagen via MALDI-TOF. During the last years, the significant expansion of this technique leads to the construction of a comprehensive database, including collagens peptides diagnostic for different animal breeds, crucial for biological species identifications.

The first studies on cultural heritage objects implementing peptide sequencing also date back to the 2000s. The strategy, applied using high-performance liquid chromatography coupled with nanoESI-based MS, provides a confident and accurate protein identification in significantly heterogeneous and degraded samples.^{34,48}

In the last years, numerous developments were achieved on sample preparation, instrument performance, and data processing, enhancing ancient proteins' characterisation. Along with the progress on protein identification, increasing attention has been addressed to understand sample ageing and degradation processes through the identification and localization of PTMs and/or chemical modifications such as, for example, deamidation or oxidation.^{3,16,17,23,56}

The proteomic analysis through bottom-up principally consists of four main steps: (i) protein extraction, (ii) digestion into peptides, (iii) analysis and sequencing via LC/MS-MS and (iv) data interpretation assisted by bioinformatics tools.

The general lines of the bottom-up proteomic workflow applied to materials from CH are shared with the classical protocol for modern biological samples. Nonetheless, tailored approaches at each step are essential to ensure the investigation of proteins extracted from a complex and often degraded matrix in limited amount of priceless and unique samples.

Sampling (Toward Minimal Invasiveness)

Sampling represents one of the most delicate steps in the analytical workflow. The careful selection of the sampling area is crucial not only for preserving the physical integrity of the CH object but also for ensuring the reliability and representativeness of the subsequent chemical analyses.³⁵

According to the UNI EN 16085 standard, approved by the European Committee for Standardization (CEN) in 2012, sampling should be carried out only for justified analytical purposes and always in close collaboration between the custodians of the object and the analysts. The regulation underscores the need to safeguard the artwork's integrity and to approach sampling within a well-defined ethical and scientific framework.³⁵ Consultation among conservators, art historians, and scientists is essential to determine whether the required information can be obtained through non-invasive methods. When invasive or micro-invasive sampling becomes necessary, it should be conducted in a manner that minimizes visible damage while ensuring that the selected area is representative of the investigated material or condition. The number and size of samples must be kept to the

absolute minimum required, considering the nature of the object, the type of material to be analyzed, and the sensitivity of the analytical instrumentation.

In proteomic investigations, standard microsampling typically involves the removal of a few micrograms of material from the object. However, in some cases—such as the analysis of organic residues in archaeological ceramics—the proteinaceous fraction is extremely limited and often entrapped within a complex mineral matrix. In such situations, the extraction of a few grams of material, usually scraped from the inner surface of a vessel, may be required to obtain a sufficient amount of protein for reliable identification.

In recent years, considerable efforts have been directed toward developing minimally invasive or non-destructive sampling strategies capable of collecting multiple micro-samples without compromising the object's integrity.³⁵ Several innovative approaches have been proposed, often exploiting physical or mechanical extraction mechanisms. One example is the triboelectric method, which relies on the gentle rubbing of the surface with polyvinyl chloride (PVC) polymers to induce electrostatic extraction of proteins. Initially applied to parchment⁵⁷, this method was later extended to other materials such as leather⁵⁸ and bones^{59,60}. More recent developments include the introduction of PVC-free erasers and the use of micro-abrasion techniques employing fine polishing films (1–30 μm particles, 14,000–600 grit) for surface material collection.^{37,61,62}

In parallel, biochemical approaches have been proposed to collect surface-bound proteins through in situ enzymatic digestion. These include trypsin-functionalized cellulose acetate sheets modified with the fungal hydrophobin Vmh2^{36,63}, and hydrophilic hydrogels based on poly(2-hydroxyethyl methacrylate)/poly(vinylpyrrolidone) (pHEMA/PVP)⁶⁴ both pre-loaded with trypsin solutions. Other hydrogel-based systems, such as those using ethyl-vinyl acetate (EVA) matrices, have also been developed for similar purposes.^{65,66}

The underlying principle of all these methodologies relies on the high sensitivity of proteomic analyses, which allows the identification of proteinaceous materials from only a few diagnostic peptides. Consequently, even the small quantities of proteins collected from the surface of an artifact are often sufficient for accurate and confident material identification.

Sample preparation

Once the sample is collected, proteinaceous substances must be extracted from the complex matrices in which they are embedded and subsequently solubilized, primarily through the disruption of intramolecular interactions (denaturation).

Various extraction and solubilization strategies have been proposed^{33,34}, employing different buffering conditions: acidic (e.g., 1% TFA), slightly basic (e.g., 50 mM ammonium bicarbonate, AMBIC), and alkaline (e.g., 2.5 M ammonia). Chaotropic or denaturing agents such as urea, guanidinium hydrochloride, and trifluoroethanol are commonly used to promote protein unfolding, often combined with detergents like SDS or CHAPS to improve the solubilization of membrane-associated proteins. Heat-assisted denaturation and ultrasonic baths are also frequently applied, particularly in the analysis of artistic and archaeological samples, to enhance protein extraction efficiency.

After solubilization, the reduction of disulfide bonds with agents such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) is typically performed to increase protease accessibility. This is followed by an alkylation step, commonly using iodoacetamide (IAM), to prevent the reformation of disulfide bonds. When proteins are mixed with other organic components in complex CH matrices, multistep extraction protocols may be necessary to ensure selective recovery of proteinaceous materials. Prior to enzymatic digestion, the analyte solution is often desalted or diluted to remove solubilization reagents (e.g., urea) that may inhibit protease activity and to restore optimal conditions for digestion.

Sample preparation represents one of the main challenges in the detection and identification of proteinaceous materials within CH objects. Protocols therefore must be adapted to address the unique characteristics of ancient, degraded proteins—often present in low amounts, exhibiting reduced solubility due to aggregation and cross-link formation during aging, and embedded in heterogeneous matrices⁶⁷.

Several studies have focused on facilitating protease access to particularly challenging target proteins. For example, a deglycosylation step using Peptide-N-Glycosidase F (PNGase F) has been shown to enhance the analysis of egg-based binders.^{36,68}

Furthermore, homogenization has been used to improve ancient protein recovery and solubilization, especially when proteins are embedded in rigid or fibrous matrices such as bone, wool and silk.

Among the recent innovations, Filter-Aided Sample Preparation (FASP) protocol and S-Trap™ protocols have gained particular relevance⁶⁹. FASP uses a high-molecular-weight cut-off filter to retain proteins while efficiently removing salts, detergents, and other low-molecular-weight contaminants—especially SDS—through urea-based buffers. Following reduction, alkylation, and on-filter trypsin digestion, peptides are eluted in a clean solution suitable for MS analysis, with the filter acting as a miniature reactor. Similarly, the S-Trap™ method integrates protein binding, washing, and on-filter digestion into a single tube, minimizing losses and further increasing peptide recovery. Both FASP and S-Trap™ thus represent powerful, low-loss workflows that have significantly improved the analytical potential of proteomic investigations in cultural heritage research.

Protein digestion

In bottom-up proteomics, proteins are enzymatically cleaved into smaller peptide fragments prior to MS analysis, typically using proteases with defined cleavage specificities. Among them, trypsin is the most widely employed enzyme due to its high specificity, which results in cleavage at the carboxy-terminal side of lysine and arginine residues (except when followed by proline). This predictable cleavage pattern produces peptides of optimal length for sequencing, yielding high-quality MS/MS spectra and enabling confident protein identification. To improve sequence coverage and enhance protein identification, multi-enzyme digestion strategies are often applied, combining trypsin with other proteases such as Lys-C, which acts at the same cleavage sites, or enzymes with different specificities, including chymotrypsin and Glu-C. The choice of enzyme or enzyme combination depends on the protein's sequence and structural properties; for instance, silk fibroin may require alternative or sequential proteolysis protocols to achieve effective digestion.⁷⁰

Following enzymatic digestion, peptide clean-up is a crucial step to remove salts, detergents, and other interfering substances that can suppress ionization during MS analysis. Various strategies have been developed for this purpose.

Reverse-phase C18 materials, implemented in ZipTips or StageTips, are commonly used to desalt and concentrate peptides prior to LC-MS/MS injection.⁴⁸ These solid-phase extraction devices rely on hydrophobic interactions between peptides and the stationary phase, allowing contaminants to be washed away before elution in MS-compatible solvents. For larger sample volumes, Sep-Pak cartridges or integrated cleanup-digestion systems like the S-Trap™ can be used to streamline preparation and minimize sample loss.³⁰ Together,

these digestion and cleanup strategies ensure high peptide quality and reproducibility, which are essential for robust LC-MS/MS-based proteomic analyses of cultural heritage materials.

MALDI-TOF

MALDI-TOF analysis is often included in a classical bottom-up proteomics workflow as it serves as a preliminary check of sample quality and cleanliness before LC-MS/MS analysis.

MALDI stands for matrix-assisted laser desorption ionization, which requires the dissolution of the sample in a suitable solvent together with an appropriate matrix, whose nature depends on the analytes of interest. The MALDI analysis of peptides mixtures is usually performed on 0.5-1 μ L of sample with equal volume of α -cyano-4-hydroxycinnamic acid (CHCA, 10 mg/mL in 70% acetonitrile and 30% of 50 mM citric acid) as matrix.

Sample and matrix are spotted on a metal plate and after a short period of air-drying they co-crystallize. The plate is then located into the source, which works under vacuum, and the solid sample is irradiated by a laser beam, whose energy is initially absorbed by the matrix. The excited matrix molecules cease their energy to the analytes in the sample leading to their desorption and ionization.

The Time of Flight (TOF) mass analyzer consists of a flight tube held in vacuum without electric fields. Ions are provided with the same initial acceleration at the beginning of the tube, so that they will cross the analyzer and reach the detector at different times according to their m/z ratio. In fact, ions with a greater mass will travel at a lower speed than ions with lower mass.

TOF has the advantage of being able to detect a very wide mass range. The tube linearity of a conventional analyzer can influence its resolving power because ions entering the TOF carry different kinetic energies, and this subsequently will affect resolution and molecular ion measurements. However, this drawback was overcome by the development of the reflectron (electrostatic ion mirror), which is an ion optic device that changes the path of the ions within the TOF. Ions with higher kinetic energy will penetrate deeper into the ion mirror and hence ions will be gradually repelled, improving the resolution of the TOF spectrum. Another factor is the tube length; because ions are traveling a longer path, better resolution as well as accurate mass measurements can be obtained due to the increase in the flight time.

LC-MS/MS

The peptides mixtures are analyzed by nano-liquid chromatography (nanoLC), directly coupled with tandem mass spectrometry (nanoLC-MS/MS).

Samples are separated by nano-flow reversed-phase HPLC (nano-RP-HPLC), typically interfaced with a mass spectrometer via an electrospray ionization (ESI) source. Chromatographic separation simplifies complex mixtures, while MS/MS fragmentation provides structural information on the analytes.

Due to the limited number of peptides that can be analyzed simultaneously, efficient chromatographic separation is critical for maximizing information and dynamic range. Low-pH reversed-phase liquid chromatography (RP) implementing a non-polar sorbent material as the stationary phase is used to separate the molecules according to their hydrophobic interactions with the support of a mobile phase. Stationary phases such as C18 columns are the most used for the separation of complex mixtures of peptides. Peptides are separated by hydrophobicity using a gradient of increasing organic content (often acetonitrile). Peptide elution is monitored via retention time (RT) in the total ion chromatogram.

NanoLC and nanoESI source are particularly effective for analyzing complex protein mixtures from a minimal starting amount of sample. In ESI, peptides and proteins are ionized from a dilute solution sprayed through a capillary under high voltage, producing charged droplets. As solvent evaporates, analytes acquire multiple protons, forming multiply charged ions (e.g., $[M+H]^+$, $[M+2H]^{2+}$) which are introduced into the mass spectrometer. Nanoscale flow rates enhance ESI performance by improving ionization efficiency, reducing sample consumption, and extending analysis time. As droplets shrink, increasing charge density leads to ion release via Coulombic repulsion. Peptides are typically dissolved in water-organic solvent mixtures (e.g., acetonitrile with up to a few percent by volume of formic, acetic acid or other volatile acid) to enhance ionization.

In bottom-up proteomics applied to cultural heritage, CID and HCD are the predominant fragmentation methods. Although ETD can provide enhanced PTM analysis, its use is limited since it principally detects high charge states, missing several doubly charged ions. In CID, precursor ions collide with inert gas molecules (e.g., helium or argon), generating fragment (daughter) ions. The resulting m/z values enable determination of peptide sequences and PTM localization.

NanoLC-MS/MS analyses are often performed using an LTQ Orbitrap XL mass spectrometer. This instrument couples high-resolution, high-accuracy Orbitrap analysis with rapid CID fragmentation in a linear ion trap.

In the Orbitrap analyzer, ions oscillate within an electrostatic field between central and outer electrodes, generating a current detected and processed via Fourier transform to produce the mass spectrum. Resolution can reach up to 10^5 with mass accuracy below 10 ppm. Typically, full MS scans are acquired in the Orbitrap, while the most intense precursor ions are fragmented by CID and analyzed in the trap for rapid data acquisition. The choice of this configuration depends on the need to analyze fragments quickly.

Data acquisition

The acquisition modes of LC-MS/MS mostly implemented are two: data-dependent acquisition (DDA) and data-independent acquisition (DIA). The first one (DDA) is the most used in proteomic studies of cultural heritage in the so-called “discovery-oriented study”: the MS instrument firstly scans all precursors eluted in the entire m/z range (full scan) then it selects precursor ions for fragmentation based on their intensity. This technique allows the observation of peptides without knowledge a priori of the sample composition, but it may prevent the fragmentation of low-intensity ions. In DIA mode, instead, the instrument selects and fragments all precursors in a specific m/z range avoiding the detection and the selection of individual precursor ions during the analysis.

Data analysis

The interpretation of MS and MS/MS spectra for peptide and protein identification relies fundamentally on bioinformatics tools that search against comprehensive protein databases. The primary repositories include UniProtKB, which encompasses two complementary components: Swiss-Prot, featuring manually annotated and peer-reviewed entries with high-quality functional annotation⁷¹, and TrEMBL, containing computationally annotated sequences derived from genome databases that await manual verification. The NCBI database, assembled from multiple heterogeneous sources including GenBank translations and RefSeq, provides an alternative or complementary resource.

Various sophisticated algorithms have been developed to perform *in silico* digestion of protein sequences present in databases, define theoretical fragmentation patterns, and match these predictions with experimentally acquired MS/MS spectra. Prominent search

engines include Sequest (one of the earliest algorithms, using cross-correlation scoring), Mascot (employing probability-based scoring derived from MOWSE)⁷², Andromeda (the MaxQuant-integrated engine optimizing for high-resolution data)^{73,74}, and Byonic (incorporating sophisticated glycan and modification searches). Each algorithm employs distinct scoring metrics and statistical frameworks, which can lead to complementary identification results.

The most widely adopted software platforms in paleoproteomics studies are MASCOT, valued for its user-friendly interface and well-established scoring system; MaxQuant, particularly powerful for label-free quantification and featuring the integrated Andromeda search engine optimized for high-resolution Orbitrap data; PEAKS Studio, offering integrated de novo sequencing and the possibility to expand the search to all PTMs gathered in Unimod database⁷⁵; and Proteome Discoverer, providing a flexible node-based workflow architecture suitable for complex experimental designs.⁷⁶

Regardless of the chosen platform, critical parameters must be defined prior to analysis. These include the proteolytic enzyme specificity (typically trypsin for bottom-up proteomics, though paleoproteomics may also consider non-specific cleavage due to diagenetic protein degradation), variable modifications (PTMs or diagenetically induced) and fixed modifications (e.g., carbamidomethylation of cysteine), the number of permitted missed cleavages (often increased in ancient samples due to incomplete digestion or backbone cleavage), the target database and taxonomy, mass tolerance windows for both precursor and fragment ions (specified in ppm or Da depending on instrument resolution), and fragmentation method (CID, HCD, ETD).

Results are reported with probability-based scores that evaluate the quality of peptide-spectrum matches (PSMs), with statistical validation typically achieved through false discovery rate (FDR) estimation using the target-decoy approach or alternative methods. The standard FDR threshold in proteomics is 1% at the PSM level.

Sequence-based identification remains the gold standard in proteomics; however, such identification is critically influenced by database selection and the predefined modification search space, creating a fundamental limitation: only peptides that are explicitly sought can be identified. This constraint is particularly significant in paleoproteomics where unexpected diagenetic modifications or sequence variants may be prevalent.

A critical challenge in ancient proteins identification is the significant taxonomic bias in database coverage. While model organisms and species of medical or agricultural

importance are extensively represented, many archaeological and paleontological specimens derive from extinct species, geographically isolated populations, or organisms that remain poorly characterized at the genomic level. Many species relevant to protein identification in most samples from cultural heritage often lack direct sequence representation in available databases. This paucity necessitates the use of phylogenetically related reference proteomes, introducing potential sequence mismatches that can reduce identification sensitivity and specificity.

To address database limitations and ensure taxonomic specificity, peptide identifications are typically subjected to manual validation.

The use of BLASTp is central to this process, as it enables peptide sequences to be matched against the entire NCBI non-redundant (nr) protein database identifying any taxonomically informative peptides. The BLASTp search allows peptide identification based on sequence homology and returns a list of matching proteins along with their associated taxonomic information, ranked by percent identity.⁷⁷

When *species-specific peptides* are identified—i.e., peptides that exhibit 100% sequence identity with only one species in the database—precise, high-confidence taxonomic assignments can be made. However, such matches are not always found. Peptides that match multiple species necessitate careful evaluation, considering preliminary identifications and incorporating contextual data—such as the sample’s origin, its artistic/historical/geographical context—to guide taxonomic interpretation.

When multiple matches occur and attribution to a single species is not possible, the *lowest common ancestor* (LCA) can be assigned, which is the most specific taxonomic level shared by all matching species. Unipept, a freely available online tool⁷⁸, provides complementary validation by enabling rapid taxonomic attribution of peptide sequences against the Swiss-Prot database, yielding the LCA assignments that can clarify taxonomic resolution.

In cases of unknown or poorly characterized samples, an iterative searching strategy is advisable: beginning with a broad taxonomic scope (e.g., Chordata, Mammalia) before narrowing to candidate families or genera based on initial findings and contextual evidence. Using multiple databases in parallel—such as UniProtKB, NCBI, and specialized or in-house databases—can further enhance coverage, improve identification rates, and strengthen confidence in taxonomic assignments.

Contemporary software increasingly integrates de novo sequencing capabilities, machine learning algorithms, and hybrid workflows that reduce database dependence and enable characterization of novel or variant sequences. De novo sequencing represents a particularly valuable alternative approach for paleoproteomics, proving especially effective when analyzing organisms lacking genomic representation. This strategy derives peptide sequences through direct interpretation of experimental MS/MS spectra without database consultation, relying instead on ion mass differences to infer amino acid composition and sequence.

PEAKS Studio exemplifies sophisticated implementation of de novo sequencing, incorporating several powerful modules: PEAKS DB for traditional database searching, PEAKS PTM for unbiased post-translational modification discovery across the entire Unimod database (enabling detection of unexpected diagenetic modifications without prior specification), and SPIDER for identifying sequence variants and mutations through hybrid database-de novo approaches. This integrated capability to identify unsequenced species, discover unanticipated modifications, and investigate amino acid substitutions makes PEAKS particularly valuable for studying ancient proteinaceous materials where degradation, diagenesis, and taxonomic uncertainty converge.⁷⁵

Other tools such as Novor and DeepNovo (employing deep learning) offer alternative de novo sequencing implementations, while software like MetaMorpheus combines traditional database searching with open-modification searches to discover unexpected mass shifts.

Bioinformatic software also enable semi-quantitative evaluation of identified peptides and proteins, enabling indirect assessment of diagenetic chemical modifications. Spectral counting approach, where peptide abundance is estimated by counting the number of MS/MS spectra (PSMs) assigned to each peptide, have recently emerged as the method of choice for ancient protein analysis, despite the predominance of intensity-based methods in contemporary proteomics.⁷⁹

This preference reflects the unique characteristics of paleoproteomics datasets. Ancient samples typically yield highly redundant, overlapping peptides from heavily degraded proteins, yet with relatively limited total PSM numbers compared to modern samples. Intensity-based quantification, which compares precursor ion intensities or integrates peak areas, requires pairwise detection of peptides across samples and is severely limited by missing values in sparse datasets. Furthermore, reliable intensity-based comparison of modified versus unmodified peptides—essential for modification extent assessment—

demands simultaneous detection of both forms, which is frequently impossible in extensively degraded samples where modifications may be nearly complete.

Spectral counting circumvents these limitations by leveraging the total PSM count. To assess modification extent, the number of PSMs containing a specific modification on a given amino acid (as provided in search engine outputs) is normalized against the total number of PSMs containing that amino acid, regardless of modification state.⁷⁹ This normalized value represents the degree of modification in a sample and enables meaningful comparison across samples. While less precise than intensity-based methods for modern samples, spectral counting provides robust semi-quantitative data suitable for the challenges inherent in ancient protein analysis.⁷⁹

With mass spectrometry becoming a routinely used tool in analysis of CH samples, the questions asked have often moved beyond the basic identification of compositions or attribution of species of origin. With more intricate questions, new mass spectrometry tools have to be adapted from classical proteomics field.

2.5 Mass Spectrometry Imaging (MSI)

Over the last two decades, mass spectrometry imaging (MSI) has become a widely used analytical technique for spatially mapping and identifying molecular species directly from sample surfaces.^{80,81} In the context of cultural heritage (CH), most existing imaging approaches rely on microscopy and spectroscopy techniques. Techniques such as Raman spectroscopy and scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX) provide insights into molecular bonds and elemental composition, respectively. However, they often lack the chemical specificity afforded by the mass-to-charge (m/z) ratio measurements characteristic of MSI.

In contrast, MSI enables simultaneous, label-free mapping of diverse analytes, including proteins, lipids, metabolites, and inorganic compounds such as pigments.^{4,39} By building on conventional mass spectrometry, MSI allows for the direct visualization of molecular distributions across a sample without the need for homogenization or extraction. Each mass spectrum is acquired at defined spatial coordinates, generating detailed molecular maps. Advances in instrumentation have led to improved spatial resolution and faster acquisition speeds, significantly broadening MSI's applicability—from biomedical and pharmaceutical research to forensic science—and more recently, to the analysis of CH materials.

Secondary Ion Mass Spectrometry (SIMS) was the earliest MSI technique developed for spatial molecular analysis. It provides exceptionally high spatial resolution but is typically limited to the detection of small molecules. In SIMS, a high-energy primary ion beam bombards the sample surface, causing the ejection of atoms and molecules as secondary ions (SI), which are then analyzed by a mass analyzer—most commonly time-of-flight (TOF) in TOF-SIMS.⁴

TOF-SIMS can achieve spatial resolutions in the range of tens to hundreds of nanometers, making it especially useful for analyzing samples like paint cross-sections or gilding, where layer thicknesses often fall within that range. Additionally, TOF-SIMS provides depth profiling, enabling three-dimensional molecular imaging. However, the technique faces notable challenges: the high-energy (keV) ion beam induces extensive fragmentation by breaking chemical bonds, which often precludes the identification of larger biomolecules. For example, in typical TOF-SIMS experiments, the presence of proteins is inferred by detecting amino acid fragments, but information about the specific protein identity or structure is lost.

Nevertheless, TOF-SIMS has been applied successfully to CH studies, including investigations of pigment degradation and organic/inorganic composition of paint layers.⁴ In a study of paintings by Nicolas Poussin, Noun et al. demonstrated how TOF-SIMS could identify artist materials and their degradation products, showcasing the potential of MSI tools in heritage science.⁸²

A major breakthrough in MSI came with the development of Matrix-Assisted Laser Desorption/Ionization (MALDI), which enabled the analysis of larger biomolecules, such as peptides and intact proteins, directly from surfaces.^{38,83} MALDI involves coating the sample with a thin layer of matrix, typically a small organic molecule capable of absorbing UV or IR laser light. This matrix facilitates soft ionization, meaning that analytes are transferred into the gas phase with minimal fragmentation. Although the precise mechanism of MALDI ionization remains under debate, it is generally accepted that the matrix assists in both analyte extraction and energy absorption, improving ionization efficiency.

MALDI can be interfaced with various mass analyzers, including TOF, Fourier-transform ion cyclotron resonance (FTICR), Orbitrap, and ion trap instruments. As the laser raster-scans across the sample surface, mass spectra are collected at each position, creating individual MSI pixels. Each pixel contains a full mass spectrum encompassing signals from

multiple molecular species—ranging from small metabolites and lipids to larger macromolecules.

The resulting MSI data cube enables visualization of specific molecular distributions by extracting ion images corresponding to selected m/z values. While early MALDI-MSI experiments suffered from slow acquisition times (e.g., ~1 minute per pixel) and low spatial resolution, commercial systems now achieve resolutions down to 10 μm , with optimizations enabling 5 μm pixel sizes. These improvements have made MALDI-MSI increasingly relevant to CH applications, where sample features are often on the micron scale and composed of complex, layered structures.

Further enhancements have come from combining MALDI with high-resolution mass spectrometry (e.g., FT-MS, Orbitrap), which offers greater mass accuracy and resolving power, making it possible to analyze chemically complex matrices and resolve fine isotopic distributions.

Despite being the most widely used MSI approach in other fields, MALDI-MSI is still underutilized in CH research. While its spatial resolution is lower than that of TOF-SIMS, it is generally sufficient to address many key questions in CH studies.

MALDI-MSI's ability to ionize a broad range of chemical classes with minimal fragmentation makes it well-suited for investigating the composition and migration of materials within layered structures, such as paint cross-sections.³⁹

MALDI-MSI also offers significant multimodal imaging potential. It can be coupled with other imaging modalities—such as optical microscopy, SEM, or Raman spectroscopy—allowing spatial correlation of complementary data from a single sample.

However, the full potential of MSI in CH research remains largely untapped, due in part to challenges in sample preparation, instrumentation access, and the lack of dedicated data analysis workflows tailored to CH-specific questions. Nevertheless, retaining spatial information is essential for investigating layered systems, as material changes often occur at interfaces or surfaces rather than in the bulk. This is particularly critical for studying pigment-binder interactions, degradation mechanisms, and artistic techniques.

MALDI-MSI stands out in its ability to provide label-free, high-specificity imaging across multiple molecular classes. While an extensive literature exists on MSI of peptides, proteins, lipids, and carbohydrates in biomedical contexts, studies targeting organic binders or pigments and other CH-specific materials remain scarce.

One promising application of MALDI-MSI involved tracking spatial and temporal degradation in oil paints. This recent study demonstrated how photo-induced degradation of geranium lake pigment could be monitored by mapping the distribution of the original eosin molecule and its degradation products in cross-sections of aged oil paints using MALDI-MSI.³⁹ This approach provided direct experimental evidence of multi-step degradation processes, underscoring the technique's diagnostic power.

A more detailed overview of the MALDI-MSI workflow is presented in the following sections, as this technique forms the core of one of the experimental approach presented in this doctoral research. Emphasis is placed on sample preparation protocols, instrumentation setup, including laser and mass analyzer specifications, and data analysis workflows. These steps are discussed in light of current limitations and best practices in applying MSI to CH materials.

Sample loading on support plate

Selecting an appropriate sample preparation method based on the research question is critical to the success of a MALDI-MSI experiment, particularly because no universal protocol exists that enables the simultaneous analysis of all biomolecular classes. Each molecular target may require tailored handling to preserve both chemical integrity and spatial localization.⁸⁰

MSI sample preparation protocols must ensure that analytes are extracted, ionized, and detected without disrupting their original spatial distribution. In biological tissue analysis, for instance, samples are typically snap-frozen immediately after collection and stored at low temperatures until sectioning. Thin sections are then cut using a cryomicrotome and mounted onto aluminum, stainless steel, or gold-coated MALDI target plates, using either thaw-mounting techniques or mechanical means such as double-sided adhesive tape.

While such cryo-sectioning and thaw-mounting approaches are well established in biomedical applications, including imaging of soft tissues and bone, they are not directly transferable to the analysis of multilayered paint samples. The granular structure, heterogeneous morphology, and variable solubility of components in traditional paint formulations present unique challenges that require adapted protocols.

For example, in the preparation of paint cross-sections for MSI, samples are often embedded in materials like carboxymethylcellulose and sectioned at ~10 μm thickness using a cryomicrotome. To maintain the structural integrity of fragile paint layers during

sectioning, common stationery tape is adhered to the exposed surface of the sample block. After sectioning, the resulting cross-section—still attached to the tape—is mounted onto a glass slide, adhesive side facing up, using double-sided tape. A slightly different strategy is described by Alvarez-Martin A. et al., who opted for microtoming of non-embedded samples to prevent potential delocalization of pigment molecules during embedding.³⁹ In their approach, cracking during sectioning was mitigated by protecting the sample surface with a thin PVC sheet. This alternative approach further highlights the need for customized methods when working with historical paint samples.

Bright-field microscopy, typically using a polarized light microscope, is lastly employed to document the morphology of each section prior to MSI analysis.

On-sample digestion for proteomic analysis

Although the direct analysis of intact proteins using MALDI-MSI is theoretically possible, it presents significant practical limitations, particularly in the context of CH applications. First, intact proteins often exceed the effective mass range of many standard mass analyzers due to their large molecular size and the low charge states generated by MALDI ionization. For instance, in TOF instruments, intact protein analysis often requires linear mode operation, which significantly compromises resolution compared to reflectron mode. Second, the ionization and transmission efficiencies for large protein ions are generally low, resulting in poor detection of a substantial portion of the proteome.

To address these challenges, a bottom-up proteomics strategy can be employed using on-sample (in situ) enzymatic digestion. This approach involves digesting proteins directly on the tissue or paint matrix surface, producing peptides that are more easily ionized, detected, and spatially mapped using MALDI-MSI. Furthermore, these peptides can be correlated with conventional LC-MS/MS-based bottom-up proteomics data, enabling protein identification through established bioinformatic workflows.

However, bottom-up MSI workflows still face several limitations. Key challenges include ensuring uniform enzyme application without inducing analyte delocalization. This is the reason why there are still not established workflows for on-sample digestion applied to CH samples.

Enzymes such as trypsin can be applied by automated spraying systems that carefully control droplet size and deposition rate. For optimum enzyme activity, the tissue sample has to be relatively wet and incubated at 37 °C for a time period from one hour to overnight,

depending on the analyte. Excess liquid on the tissue surface can lead to diffusion of analytes during incubation.

Despite its promise, the application of MALDI-MSI to CH samples remains inherently invasive, as it requires microsampling and the irreversible application of matrix.

An innovative approach to reducing sampling invasiveness involves the use of trypsin-functionalized sheets^{36,63}, which are placed directly onto the surface of the original artifact to enable enzymatic digestion in situ with minimal impact.

Matrix choice and deposition

Following mounting and fixation of the sample onto the support plate, the next critical step in MALDI-MSI is the application of the matrix. Matrix choice and deposition method significantly affect both the quality of mass spectral data and the preservation of spatial localization of analytes.⁸⁰ As such, careful matrix choice and optimization of application procedure is essential for successful MSI analysis.

Matrix compounds are small, UV-absorbing organic acids that co-crystallize with analytes, aiding in the desorption and ionization process. Matrix selection should be guided by the chemical class of the target molecules and the ionization mode (positive or negative). Commonly used matrices include:

- α -cyano-4-hydroxycinnamic acid (CHCA) – widely used for low molecular weight peptides and small molecules, in positive ionization mode.
- 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid, SA) – typically used for high molecular weight proteins.
- 2,5-dihydroxybenzoic acid (DHB) – versatile and often employed for lipids and peptides.
- 9-aminoacridine (9-AA) and 1,5-diaminonaphthalene (1,5-DAN) – preferred for negative ion mode, especially for acidic compounds.

No matrix gives ideal signal sensitivity for all molecular species, and trade-offs have to be made based on the desired results (e.g., prioritizing ionization of lipids over inorganics). Development of new matrices is still ongoing in order to expand the capabilities of MALDI both focusing on boosting sensitivity and matrix stability.

Washing tissue sections prior to matrix application can enhance spectral quality. This step, however, is often incompatible with CH materials, where many target analytes are not

tightly bound within a cellular structure (as in biological tissues), but rather exist in free or weakly bound states. As such, bulk washing steps may lead to analyte migration, undermining spatial integrity and compromising the value of the MSI data.

The method of matrix application critically influences spatial resolution, crystal size, reproducibility, and ionization efficiency. Among the available techniques:

Manual aerosol spraying or airbrushing remains a common and low-cost method. When performed with care, this technique can produce fine, homogeneous matrix crystals smaller than the laser beam diameter (typically 50–100 μm), thereby preserving spatial resolution. However:

- Overspraying may cause analyte delocalization due to excessive solvent,
- Underspraying can result in insufficient analyte extraction.

Automated sprayers offer improved control and reproducibility by standardizing droplet size, solvent composition, and application parameters. However, uniform coverage of large areas often requires multiple overlapping passes, which can introduce intensity variation artifacts in the resulting ion images.

Spotting techniques, such as piezoelectric or inkjet-based deposition, deliver highly uniform and precisely placed microdroplets of matrix. Although the resulting droplet sizes are generally larger than those produced by aerosol methods, these techniques provide excellent control over matrix placement and analyte extraction efficiency, especially for proteins.

Sublimation is a solvent-free method that produces very fine matrix crystals with uniform distribution across the sample surface, resulting in high spatial resolution and minimal analyte delocalization. However, because no solvent is involved, this method offers limited analyte extraction, making it less suitable for certain molecular classes without additional rehydration or spraying steps.

Matrix solvent composition, flow rate, and application parameters can all be optimized depending on the target analytes. For example, adjusting the organic content, acidity, or spraying conditions can improve extraction and ionization for specific molecular classes. In the context of CH research, this often requires balancing spatial resolution and sensitivity while minimizing analyte migration and ensuring the sample remains as intact as possible throughout the MSI workflow.

MSI and data analysis

Advancements in mass spectrometric instrumentation have significantly enhanced the capabilities of MALDI-MSI, enabling high-throughput spatial mapping of peptides, proteins, lipids, and metabolites directly from tissue surfaces.

Initially developed using time-of-flight (TOF) analyzers, MALDI-MSI was first demonstrated by Ryan D. et al.⁸⁴ in mapping insulin distribution in rat pancreas tissue sections. Since then, the technique has expanded to incorporate high-resolution mass analyzers such as Fourier transform ion cyclotron resonance (FT-ICR), ion trap/Orbitrap hybrids, and quadrupole-Orbitrap systems. These configurations offer superior mass resolution and accuracy, which are crucial for precise analyte annotation and protein identification.

In this doctoral study, MALDI-MSI was performed using an atmospheric pressure scanning microprobe MALDI (AP-SMALDI, TransMIT GmbH, Giessen) ion source, coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific GmbH, Bremen). This instrumental set-up is characterized by a resolving power of 70,000 (at m/z 200) with a pixel size down to 5 μm .

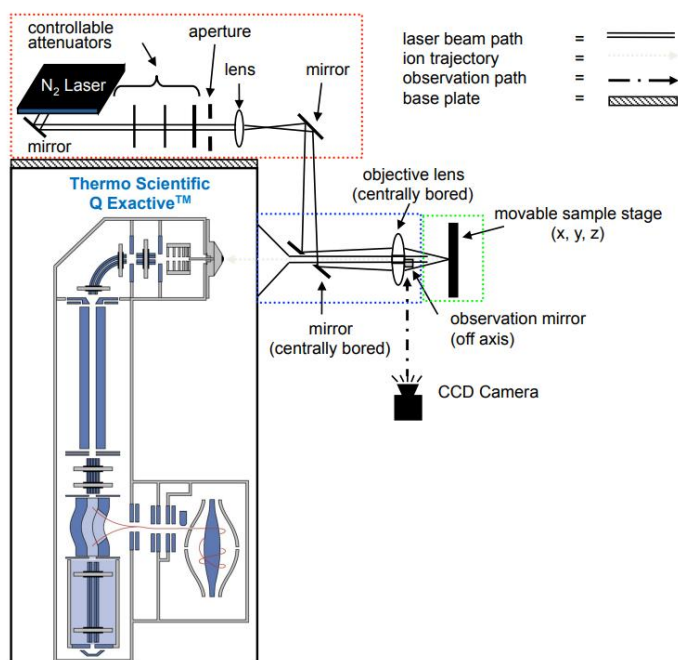


Figure 1. Schematic representation of an Atmospheric Pressure MALDI (TransMIT, Germany) source coupled to an Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, Germany).

The AP-SMALDI⁵ AF system employs a tightly focused pulsed laser beam that is guided through a series of optical components, including focusing lenses and mirrors, and directed orthogonally onto the sample surface. Sample visualization and positioning are facilitated by an integrated off-axis camera, while autofocus ensures consistent laser focus across heterogeneous sample surfaces.

The AP-SMALDI⁵ AF source can operate in two primary imaging acquisition modes via the SMALDIControl software:

High-resolution imaging (2D Pixel Mode): Pixel size of $5\ \mu\text{m} \times 5\ \mu\text{m}$, using a laser spot size of $5\ \mu\text{m}$. Each pixel is irradiated with a single or few laser pulses centered on its coordinates, allowing for detailed spatial resolution.

High-sensitivity imaging (Full Pixel Mode): Pixel sizes $\geq 25\ \mu\text{m} \times 25\ \mu\text{m}$ with a $5\ \mu\text{m}$ laser spot, scanned across the pixel area in a meandering pattern. This approach improves ion yield, increasing sensitivity at the expense of spatial resolution.

Several key parameters of both the AP-SMALDI⁵ AF ion source and the Q Exactive mass spectrometer must be carefully optimized to achieve high-quality MSI data. For the AP-SMALDI source, critical parameters include:

Laser energy and attenuator settings: Directly influence desorption/ionization efficiency and the size of the ablation spot.

Z-axis positioning: Determines the laser focus on the sample surface and affects spatial resolution. This can be adjusted manually or automatically via the autofocus system.

Laser repetition rate and pulse count: Can be adjusted based on pixel size and acquisition mode, impacting both acquisition time and ion signal intensity.

Spot overlap and raster pattern: Affect the consistency and completeness of ablation across the sample

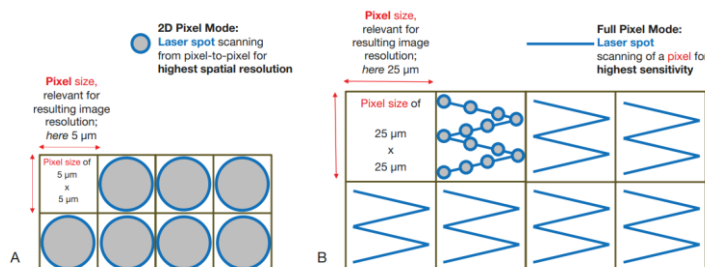


Figure 2. Various acquisition modes of the AP-SMALDI⁵ AF ion source are accessible; a selection of MS Imaging acquisition modes is shown here.

The Q Exactive is a hybrid mass spectrometer that combines the performance of a quadrupole for the precise selection of precursors and an orbitrap for high-resolution and accurate mass (HR/AM) detection. This set-up mainly includes an ion source, a stacked-ring ion guide (S-lens), a quadrupole mass filter, a curved linear trap (C-trap), a Higher Energy Collisional Dissociation (HCD) cell, and an Orbitrap mass analyzer.

When combined to AP-SMALDI⁵ AF ion source, the mass spectrometer Q-Exactive can be controlled by the Thermo Q Exactive Tune software. Tunable parameters in the Q Exactive Tune software include:

Mass range (m/z window): Must be selected based on the molecular targets (e.g., peptides, lipids).

Spray voltage and S-lens RF level: Influence ion transmission efficiency.

Resolving power settings: Trade-off between acquisition speed and mass resolution.

Automatic gain control (AGC) target and maximum injection time: Crucial for optimizing ion population in the Orbitrap analyzer.

Lock mass calibration: Enhances mass accuracy (≤ 1 ppm) by referencing a known matrix or contaminant peak during acquisition.

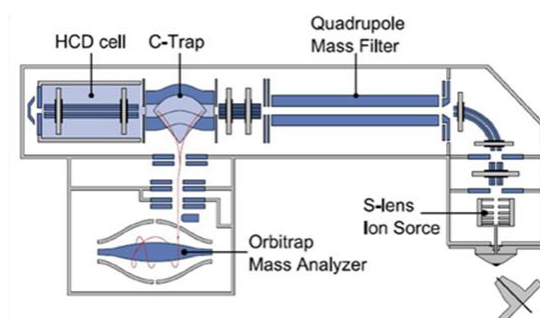


Figure 3. Schematic representation of an Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, Germany).

Prior to imaging, it is essential to acquire high-resolution optical images of the sample with a microscope, which can later be co-registered with MS images for multimodal analysis. Samples are typically mounted on glass slides using double-sided tape and inserted into the AP-SMALDI source, ensuring a flat and stable surface. The region of interest is defined in SMALDControl software, where the pixel count and scan parameters also allow for the estimation of total acquisition time. Measurement time is increasing very rapidly with higher number of pixels.

MALDI-MSI generates a mass spectrum at each discrete spatial location, or pixel, across the sample's surface, resulting in a hyperspectral data cube consisting of thousands of spectra. This spatially resolved molecular information allows for the construction of ion images representing the distribution of specific m/z features throughout the sample. However, MSI datasets are often large and complex, requiring advanced computational strategies to extract meaningful information.

Typical MSI data analysis usually involves several sequential steps: (i) baseline correction, spectral normalization, and noise reduction; (ii) peak picking or dimensionality reduction through wavelet or scale-space transforms; (iii) multivariate analysis, involving principal component analysis (PCA), spectral clustering, supervised classification, and spatial segmentation for identifying discriminative features and specific molecular patterns; (iv) post-processing, such as image magnification, contrast enhancement, and co-registration with high-resolution microscopy images for multimodal analysis.

With advances in the field of machine learning, innovative data processing methods have been used in biomedical contexts, to answer questions such discrimination between healthy and diseased tissues and could similarly be applied to samples from CH. MSI data can be successfully coupled among others with histology, fluorescence microscopy, and Raman spectroscopy, techniques which have previously all been independently used to in cultural heritage.

Database-driven annotation tools such as METASPACE²⁰²⁰ facilitate the identification of features of interest (e.g., lipids, peptides, metabolites) in a reproducible manner.⁸⁵ Commonly used databases include LipidMaps (lipids), CSDB (glycans), and UniProt (proteins). Furthermore, METASPACE allows the incorporation of custom peptide databases, enabling tailored analysis of samples from CH objects. A significant limitation, however, remains the lack of user-friendly tools to create such comprehensive peptide databases—particularly those accounting for post-translational modifications (PTMs) and diagenetically induced chemical modifications, which are critical in CH applications. Addressing this gap is essential for extending the capabilities of MSI-based molecular annotation in complex and historically relevant samples.

2.6 GC-MS analysis

In a gas chromatograph, the unknown mixture is introduced and vaporized in a high-temperature chamber. At this point, the resulting gaseous mixture passes through a chromatographic column carried by the flow of a carrier gas. The chromatographic column may consist of a very thin liquid layer or covalently bonded groups. The separation takes place taking advantage of the difference in the boiling points of the mixture components, realizing through an oven, in which the column is placed, temperature gradients. In this way, the various components of the mixture arrive at the spectrometer at different times.

GC and MS techniques were not easily coupled. Indeed, there is a switch from an instrument at atmospheric pressure (chromatograph) to one in a vacuum (spectrometer). The analytes then enter the source, where they are fragmented. The source used is the Electron Impact (EI) source, where a tungsten filament is heated, generating an electron beam, which impacts sample molecules, thus generating positive ions. The consequence of the impact is the generation of radical ions, which tend to distribute the high instability through vibrational, rotational and translational motions, thus leading to the fragmentation of the molecule into radicals and cations. The EI source is usually coupled with a quadrupole analyzer, consisting of four cylindrical metal rods arranged in parallel.

The rods are held at an oscillating electromagnetic potential. Electrons, accelerated by the accelerator plates, enter the tunnel bounded by the rods, repelled by the positive poles and attracted by the negative poles. In this way, the ions are kept at the centre of the quadrupole, since the diagonally arranged electrodes have the same potential and have to travel an oscillating trajectory because of the current alternating radio frequency. Only ions with a given mass-to-charge ratio (m/z) pass through the analyzer. This allows the selection of a particular ion, or the scanning into the mass range by varying voltages. A detector quadrupole can thus operate in two different modes: SIM (Selected Ions Monitoring) by selecting specific ions or SCAN, that is scanning a specific m/z interval. In the former case, the advantage is the lowering of the limit of detection, as the instrument has to scan fewer fragments and thus the interference of the array is lower. In the case of the SCAN mode, the sensitivity of the instrument inevitably decreases due to fewer scans per second since more fragments have to be analysed. This mode is therefore useful for the determination of an unknown mixture.

When the ions arrive at the detector, a current increase is observed and a peak. The chromatogram is presented as a function of Retention Time (RT), and each peak corresponds to a mass spectrum, thus obtaining the relative fragmentation that allows the identification of the tested substance. The identification of the analytes is carried out by comparison with spectra in the database. It is also possible to carry out a quantitative analysis, performed by employing internal or external standards. In the first scenario, a substance of known concentration is added to the mixture known with similar RTs; in the second a calibration line is constructed with external standards, so that the concentration of the analytes can be traced.

The GC-MS technique is particularly suitable for studying the complex organic mixtures commonly found in samples from cultural heritage.^{10,20,30} However, the presence of molecules bringing hydroxy and carboxylic moieties requires a derivatization step before chromatographic analysis. Derivatization reactions are required to render polar, nonvolatile and thermolabile compounds suitable for GC analysis. Methylation and trimethylsilylation (TMS) are the most common derivatization reactions for the GC/MS analyses of biomolecules. TMS reagents are able to derivatize groups with an active/polar proton (OH, NH and SH), converting them into TMS ethers and/or esters. Methylation reagents are able to convert organic acids into methyl esters.

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4. Aims of the research

The chemical investigation of cultural heritage (CH) materials represents one of the most challenging and interdisciplinary fields within analytical sciences.¹ Works of art and archaeological artefacts are complex, multicomponent systems composed of organic and inorganic substances that have interacted and transformed over time through natural aging, environmental exposure, and human intervention. Their study requires reconstructing both the original composition and the chemical transformations that have occurred during centuries of alteration. From an analytical perspective, CH materials are characterized by high heterogeneity, low concentration of target analytes, and limited sample availability due to the uniqueness and fragility of the objects.^{2,4,38} These constraints demand analytical methodologies that are highly sensitive, selective, and minimally invasive, capable of extracting molecular information from microscopic amounts of material while preserving the integrity of the artefacts.

Among the available techniques, mass spectrometry (MS) has emerged as one of the most powerful tools for the molecular investigation of CH materials. Coupled with chromatographic or imaging separation methods, MS offers unparalleled sensitivity and molecular specificity, enabling the detection and identification of a wide range of organic compounds including proteins, lipids, polysaccharides, waxes, and natural resins.^{5,27,87} The molecular information derived from MS-based analyses provides crucial insights into the materials and techniques employed by artists and artisans, the technological traditions of past cultures, and the chemical mechanisms responsible for material aging and decay.

Within this context, two key aspects can be distinguished: the identification and the chemical characterization of CH materials, which together form the analytical and conceptual foundation of this doctoral research.

The **identification** of organic substances in works of art and archaeological objects constitutes the first and most essential step in their chemical study. Determining which materials—such as binding media, varnishes, adhesives, or food and plant residues—are present in a given sample provides information not only about the technological and artistic practices involved in the object's creation, but also about its geographical, historical, and cultural context. In paintings, for example, identifying a specific proteinaceous or lipidic binder can reveal the painter's technique or workshop tradition^{10,12,55}, while in archaeology,

the identification of organic residues in ceramic vessels can illuminate aspects of diet, trade, and ritual practices of ancient populations.^{9,31} However, the analytical challenges associated with such identifications are considerable. Organic compounds in historical and archaeological samples are typically present in extremely low abundance, often chemically modified, polymerized, or crosslinked due to aging and environmental exposure.^{10,23,24} They coexist with inorganic pigments, fillers, and degradation products that interfere with extraction and detection. Furthermore, the heterogeneous and stratified nature of samples complicates both representative sampling and solubilization.

Conventional analytical workflows designed for modern or biological samples are generally unsuitable for CH materials and must be adapted through optimized sample preparation, selective extraction protocols, and high-resolution MS detection to maximize analytical performance while minimizing invasiveness. **Chapter 4** of this thesis focuses on these challenges, presenting a series of case studies that demonstrate how innovative MS-based workflows can be applied to diverse historical and archaeological materials. These include the study of restoration adhesives⁵, archaeological residues, ancient inks, and proteinaceous fibers, as well as the implementation of minimally invasive identification techniques^{6,7}. Collectively, these investigations advance the identification of organic compounds in CH materials while reducing the analytical footprint on the studied objects.

Beyond identification, the **chemical characterization** of CH materials seeks to understand how these substances have changed over time and why they behave in specific ways within their complex matrices. This aspect focuses on the study of chemical modifications and degradation processes affecting organic compounds, particularly proteinaceous materials such as collagen, casein, and egg proteins, which were widely used as binders, adhesives, and coatings. Over time, these proteins undergo chemical and structural alterations—including oxidation, deamidation, hydrolysis, and crosslinking—that profoundly influence their solubility, mechanical properties, and detectability.^{10,23,24} Furthermore, interactions between proteins and inorganic pigments can modulate degradation pathways, either catalyzing oxidative reactions or stabilizing protein structures through processes that are far from being completely understood.^{19–21,44,45}

Understanding these intricate processes requires analytical methods capable of detecting, localizing, and quantifying chemical modifications across specific protein regions, thereby linking molecular alterations to broader degradation phenomena. **Chapter 5** of this thesis addresses these issues through the development of new proteomic workflows for the

chemical characterization of proteinaceous materials. By integrating high-resolution MS with advanced bioinformatic analysis, the research investigates post-translational and diagenetic modifications in proteinaceous materials from different sources to further elucidate how chemical treatments, environmental factors and interaction with inorganic pigments influence protein degradation.

The overarching aim of this doctoral project is therefore the development and application of MS-based strategies for the molecular study of CH materials.

The research is structured around two complementary and interrelated objectives: (1) the identification of ancient proteins and organic molecules in historical and archaeological samples through innovative, highly sensitive, and minimally invasive analytical workflows; and (2) the chemical characterization of proteinaceous materials to elucidate degradation mechanisms, interactions with inorganic pigments, and the impact of manufacturing and environmental factors on long-term material evolution.

By combining these two research directions, this work seeks to advance proteomic methodologies in heritage science, improving our ability not only to identify the molecular components of complex historical materials but also to interpret the chemical and structural transformations that shape their history and preservation. Ultimately, the approaches and findings developed through this research aimed to contribute to a deeper molecular understanding of cultural heritage materials and provide a scientific basis for more informed conservation and restoration strategies.

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5. Analytical strategies for the identification of organic molecules in cultural heritage materials

In recent years, analytical chemistry techniques, especially mass spectrometry (MS), have gained increasing popularity for the identification of organic materials in ancient cultural heritage (CH) objects. Understanding the materials used in a work of art is crucial not only to provide insights into the historical and artistic context, but also to develop appropriate conservation and restoration strategies. Similarly, the identification of organic residues in archaeological samples (such as potteries) plays a vital role in reconstructing the customs, habits, and daily life of ancient populations, offering perspectives not only on dietary practices but also on rituals such as funerary ceremonies.

However, standard protocols routinely applied to modern samples must be carefully adapted when analyzing CH materials. These samples typically contain extremely low amounts of organic compounds, and are characterized by complex, heterogeneous matrices and unusual physical states. They often exhibit high levels of degradation and contamination. Additionally, the uniqueness and irreplaceable value of CH samples also necessitate the development of minimally invasive or non-invasive analytical workflows.

This chapter presents several practical case studies demonstrating the implementation through this doctoral study of innovative analytical strategies for identifying organic molecules in various historical and archaeological samples, each addressing different challenges and objectives within the CH field.

Section 4.1 presents a novel analytical protocol for the identification of organic components in a specific adhesive used in historical painting restoration, commonly known as "glue lining paste." Due to the high molecular complexity of this adhesive, a multi-step extraction protocol was applied to simultaneously recover and fractionate the three major classes of organic compounds (lipids, polysaccharides, and proteins) from a single microsample. High-performance separation techniques, coupled with high-resolution MS, were used to identify specific components in each fraction.

Section 4.2 reports a series of case studies involving organic residue analysis (ORA) for the reconstruction of archaeological pottery contents. A multi-strategic approach—combining lipid analysis, proteomics, and analysis of other organic molecules—is proposed to tackle the challenges posed by complex residue compositions. Integration with

archaeological information, such as vessel shape and excavation context, is also discussed as a tool to guide and refine the analytical strategy.

Section 4.3 describes a multi-analytical approach combining non-invasive and micro-invasive techniques (GC-MS, HPLC-ESI-Q-TOF, optical microscopy (OM), SEM-EDS, Raman spectroscopy, and FTIR-ATR) for the characterization of organic and inorganic constituents in ancient ink samples from the Royal Palace Museum of Caserta. Both original ink components and degradation products were identified.

Section 4.4 presents a compelling case study: the identification of silk fibers in an ancient gold textile excavated from the Vesuvian area. A novel integrated proteomic protocol was developed and optimized to enable the identification of silk and other proteinaceous fibers, despite the severe degradation and unknown biological origin of the samples. The protocol also addresses known difficulties related to silk protein extraction and solubilization, as well as the extremely limited sample availability.

Section 4.5 illustrates the contributions of this doctoral research to reducing the invasiveness of proteomic workflows. First, the application of a minimally invasive technique—using a trypsin-functionalized cellulose acetate sheet for in situ digestion of proteins on the surface of an artwork—is described. This approach allowed the identification of proteinaceous binders on a 14th-century painting from the Capodimonte Museum (Naples) without removing any material. Additionally, organic varnishes were identified via GC-MS. Finally, the section introduces the first application of MALDI-MSI on a trypsin-functionalized sheet, reflecting the protein content distribution of the work of art with which it has been in contact.

Manuscripts

Cipolletta, B., Morelli, M., Perlingieri, C., Somma, P. I., Amoresano, A., Marino, G., & Carpentieri, A. (2024). Molecular Characterization of Adhesives (Glue Lining Pastes) Used in Restoration. *Analytical Chemistry*, 96(42), 16551-16560. <https://doi.org/10.1021/acs.analchem.4c02138>.

Cipolletta, B., Fiore, M., Pizzimenti, S., ... & Birolo, L. (2025). The challenge of organic content of potteries: multistrategic approaches. *Manuscript in preparation*.

Alberico, M., Carpentieri, A., Rossi, M., Schisano, C., Fiore, M., Alberico, M., Cipolletta, B., ... & Vergara, A. (2025). Chemical characterization of a funeral equipment from a Hellenistic young woman tomb found in Battipaglia. *Manuscript in preparation*.

Alberico, M., Cipolletta, B., Rossi, M., Pironti, C., Tortino, ... & Vergara, A. (2025). The Vanvitelli's ink recipe. *Manuscript in preparation*.

Research Projects

Proteomic analysis of ancient silk from Pompeii: Designed and validated an integrated workflow for the identification of degraded proteinaceous fibers, successfully applied to archaeological textiles.

Minimally invasive protein identification on a XIV century painting by Colantonio: Applied a trypsin-functionalized cellulose acetate sheet to digest proteins directly on the artwork's surface without any removal of material from the artifact.

Immobilized Trypsin – Enhanced MALDI-Imaging analysis of surfaces and cross-sections of works of art: Established the first MALDI-MSI method using trypsin-functionalized films to spatially map protein content on artworks and cross-sections, optimized on aged paint replicas.

4.1 Molecular characterization of adhesives (glue lining pastes) used in restoration

B. Cipolletta, M. Morelli, C. Perlingieri, P. I. Somma, A. Amoresano, G. Marino, and A. Carpentieri.

The molecular characterization of samples from works of art can provide valuable insights into the composition of ancient restoration materials and their conservation state. Here, we present a novel analytical protocol for the molecular characterization of a specific adhesive used in historical painting restoration, known as "glue lining pastes." Due to the high molecular complexity of these adhesives, we propose a multi-step extraction protocol to simultaneously recover and fractionate from a single microsample the three main classes of biomolecules contained in glue pastes (lipids, polysaccharides, and proteins). High-performance separation coupled with high-resolution MS techniques were applied to the isolated fractions to identify specific components. The proposed method was optimized using test specimens of various traditional glue pastes applied to canvases and successfully applied to a historical glue paste sample from the 17th-century painting "La fuga in Egitto," part of the Pagliara collection at the University Suor Orsola Benincasa (Naples, Italy). The data collected in this work provide insights into the specific recipe used for adhesive preparation, supporting artistic and historical interpretations, and contributing to a broader understanding of old restoration practices. Data are available via ProteomeXchange with identifier PXD051480.

Introduction

Characterizing historical artifacts at a molecular level is increasingly important for cultural heritage (CH) research. Chemical composition information is crucial for investigating execution techniques, constitutive materials, and supporting artistic, archaeological, and historical interpretations.¹ Molecular characterization can also provide insights into the conservation state of historical objects, revealing degradation and aging processes.² This information is vital for designing appropriate restoration interventions and ensuring the preservation and accessibility of ancient artifacts.³

When analyzing a canvas painting, detailed information regarding its molecular composition can offer valuable insights into the materials present within the artwork, such as pictorial binders or varnishes^{4,5}, as well as the restoration materials utilized on the same painting.

Canvas paintings represent one of the most intriguing subjects in CH research. Over time, exposure to environmental conditions can lead to degradation and structural issues that periodically necessitate restoration efforts. These issues often involve cellulose degradation of the canvas, tears, severe deformation, color imperfections, and more.⁶ When a canvas painting loses its structural integrity, the preferred restoration method is re-lining, a process that provides new support to the artwork.⁶ The objective of re-lining is to reinforce the original canvas by affixing a new canvas to its back using an adhesive substance.⁶ The selection of the adhesive is crucial and varies based on the composition of the painting's layers. Common re-lining methods are typically categorized by the type of adhesive used, including "glue paste lining," "wax-resin lining," and "lining with synthetic materials".⁶

Dating back to the 17th century, glue paste lining emerged as the predominant re-lining technique.^{6,7} This method is distinguished by the use of a specific adhesive known as "glue paste," traditionally formulated in the Florentine tradition with ingredients including wheat flour, rye flour, linseed flour, water, Venetian turpentine, fungicide (such as atagol sodium), and strong glue.⁶ However, variations in glue paste recipes, which include ingredients and application procedures, have emerged over centuries depending on restoration laboratories and geographic regions.⁸ Despite these differences, cereal flours and animal glues remained the primary components due to their widespread availability.⁸ Regional adaptations might involve the use of different types of flours (e.g., wheat flour, whole grain rye flour, linseed meal, whole-wheat flour, etc.) or combinations thereof. Furthermore, the choice of animal glue varied between laboratories, with fish glue (sturgeon glue), rabbit glue (Lapin glue), and bovine glue being commonly cited in traditional recipes.^{8,9} Additives were frequently incorporated to improve adhesive performance or prevent mold growth.¹⁰ Plasticizers such as Venetian turpentine, linseed oil, honey, and sugar molasses were commonly used, while wetting agents like ox gall and vinegar were added to enhance mixture properties.¹¹ Preservatives such as Nipagin sodium and Atagol sodium were also employed to mitigate the risk of microbial degradation.¹¹

In summary, glue paste linings comprise complex and heterogeneous formulations containing a variety of chemical components, both organic and inorganic in nature. These components interact strongly, influencing both the adhesive's mechanical performance and the degradation processes experienced by canvases lined with them.^{10,12} Owing to the organic matrices they contain, glue pastes are susceptible to various degradation mechanisms, including oxidative processes and microbial attacks.^{12,13} Recent studies have revealed that canvas paintings re-lined with traditional glue pastes are often the most

infested and damaged.¹³ Consequently, glue pastes are no longer utilized in restoration practices but remain the subject of extensive research within the field of CH chemistry.^{10,13}

In this context, a comprehensive analysis of the molecular composition of historical glue pastes can offer valuable insights into these restoration materials and shed light on the specific mechanisms of ongoing degradation and aging.

However, there are numerous technical and methodological challenges associated with analyzing glue paste samples. These challenges primarily include: 1. Limited sample availability: Historical materials often provide only small amounts for analysis. 2. High molecular complexity: Glue paste samples exhibit intricate molecular compositions. 3. Intrinsic contamination: Environmental exposure can introduce contamination issues. 4. Aging and deterioration: Physical and microbial agents can cause modifications over time.^{2,13} These factors collectively pose significant hurdles to the molecular characterization of glue paste samples. Consequently, substantial efforts are required to develop and optimize novel analytical procedures capable of addressing the complexities inherent in these unique and valuable historical materials.

High-resolution analytical methodologies are essential for deciphering the intricate mixtures of biomolecules and achieving unambiguous identifications. Mass spectrometry (MS) coupled with appropriate chromatographic techniques stands out as the most widely adopted experimental approach for this purpose.¹⁴⁻¹⁷

Furthermore, a pretreatment step is necessary for glue paste sample analysis, with a crucial aspect being the extraction of the compounds of interest from the matrix. This is particularly critical for CH materials like glue pastes due to their unique nature and the limited organic fraction they contain.¹⁸

An innovative approach involves a multi-step extraction protocol designed to separate the sample into desired molecular fractions using minimal amounts of material.¹⁴ Recently, a GC-MS and LC-MS/MS based analytical procedure has been utilized for simultaneous identification of lipids, waxes, proteins, and resinous materials from micro-samples taken from artworks, such as an Italian easel painting on canvas from the 17th to 18th centuries.^{14,15} This procedure was also applied by Melchiorre et al. to investigate a painting from an Etruscan marble sarcophagus dating back to the 4th century BC.¹⁵

This research aims to develop and optimize a novel analytical procedure for comprehensive molecular characterization of glue lining pastes. Here, we present a multi-

step extraction protocol designed to simultaneously extract and separate the three main molecular fractions found in glue pastes (i.e., polar, nonpolar, and protein fractions), leveraging their differing chemical and physical properties, such as solubility.¹⁵ High-performance chromatographic methodologies coupled with MS techniques are then applied to identify specific components present in each fraction. Specifically, GC-MS analysis is used for the polar and nonpolar fractions, while LC-MS/MS is employed for the protein fraction.^{14,15}

Materials And Methods

Test specimens

The test specimens analyzed in this study were specifically prepared for this project by the Laboratorio di Restauro delle Tele of the University Suor Orsola Benincasa (Naples, Italy). They comprise glue lining pastes that were applied to canvases and subjected to artificial aging (refer to Supplementary material **Fig. S1**).

Different glue pastes were prepared according to seven traditional recipes documented in the literature.⁸ These recipes were selected based on their prevalence in specific geographical regions. As the sample under investigation originates from a canvas painting attributed to the 17th century Neapolitan School, three recipes commonly used in the Campania area during that period were chosen for preparing the test specimens. These recipes, sourced from the restoration laboratories of the Reggia di Caserta (B1), the Accademia di Belle Arti di Napoli (B2), and the University Suor Orsola Benincasa (E1), share similarities in terms of the flour and plasticizer employed, namely wheat flour and honey/molasses. However, they differ in the type of animal glue used and the presence of Venetian turpentine, allowing for the distinction of various formulations.

In addition to the Neapolitan recipes, traditional Florentine (C1 and C2) and French (D1 and D2) recipes⁸ were selected to prepare additional test specimens to gather further insights into the chemical composition of glue lining pastes. These recipes feature a combination of different types of flour (such as whole-wheat flour, rye flour, and linseed meal), presenting an additional analytical challenge. The inclusion of French recipes was particularly notable due to the absence of a polysaccharide matrix (honey/molasses) in their formulation.⁸ **Table 1** displays the detailed recipes selected for preparing the test specimens.^{8,19}

Ingredients	Neapolitan Methods			Florentine Methods		French Methods	
	B1	B2	E1	C1	C2	D1	D2
Wheat flour	X	X	X	X	X	X	X
Whole-wheat flour					X		
Rye flour				X		X	X
Linseed meal				X	X		
Rabbit glue		X		X			X
Fish glue	X	X	X		X	X	
Bovine glue				X			
Molasses/Honey	X	X	X	X	X		
Venetian Turpentine		X	X	X	X		X
Ox Gall	X		X				
Phenol						X	X
Alum Rock				X	X		
Linseed						X	X
Neodesogen				X	X		
Sodium Nipagin	X						
Atagol Sodium		X	X				

Table 1. Detailed glue paste recipes chosen for test specimens' preparation.

Equal quantities of each glue paste recipe were applied to a linen canvas using pressure and heat to accurately simulate the re-lining process.⁶ Furthermore, to replicate authentic aging conditions, the specimens underwent artificial aging within a controlled chamber (refer to section IIB. Artificial aging). Finally, a piece approximately 1 cm² in size was extracted from each canvas for analysis purposes.

Historical samples

To provide robust validation for the experimental approach proposed in this study, a glue paste sample from a historical canvas painting was examined for its molecular composition. Specifically, the historical glue paste sample was obtained from the 17th-century painting "La fuga in Egitto" (**Fig. 1**), which is part of the Pagliara collection at the University Suor Orsola Benincasa in Naples, Italy. This oil painting on canvas (100.8x85x2.4 cm) depicts the flight into Egypt, a frequently depicted episode from the life of Jesus in figurative arts. The representation of the "Escape to Egypt" by river, with the Holy Family awaited by a ferryman, is characteristic of the style of Luca Giordano, a prominent Neapolitan artist. The painting, attributed to the workshop of Nicola Malinconico, a fellow of Giordano, is believed to date from the late 17th century (1675-1699).

Extensive restoration work was undertaken on this painting at the Laboratorio di Restauro delle Tele due to various damages, including tears on the canvas. During the re-lining process, a micro-sample of the historical glue paste used in the 17th century was extracted from the back of the painting (refer to Supplementary material **Fig. S1**). This study aims to molecularly characterize this sample to identify the specific ingredients of the historical recipe, thus facilitating investigation into the degradation processes the canvas has undergone over the years.



Figure 1. Recto and verso of the painting "La fuga in Egitto," with the detail of the glue paste.

Artificial ageing

The test specimens underwent photooxidative aging under mild humidity conditions (RH 50%) and a temperature of 40°C in a forced air environmental chamber (Angelantoni SU250). The chamber was equipped with a borosilicate glass-filtered low-pressure mercury UV lamp to filter out radiation with wavelengths lower than 300 nm, which could cause excessive photochemical deterioration not representative of real aging. The emitted light had a power of approximately 5.5 W in the range of 300–400 nm and 7.1 W in the range of 400–615 nm.²⁰ After 7 days, aged test specimens were collected and stored in the laboratory at room temperature (RT).

Extraction of the organic fraction

A multi-step extraction protocol, as outlined in references¹⁴⁻¹⁵, was employed to recover the organic components and separate them into the three main classes of biomolecules (i.e., lipids, saccharides, and proteins). Initially, 400 μL of 2.5 M ammonia solution were added to each sample, and extraction was conducted in an ultrasonic bath at RT for 30 minutes followed by an additional 120 minutes at RT (this procedure was repeated twice). The resulting extracts were evaporated to dryness under vacuum and then suspended in 400 μL of 1.0% tri-fluoro-acetic acid (TFA) to separate polar compounds (proteins and saccharides) from the nonpolar ones. Liquid-liquid extraction was performed by adding 500 μL of diethyl ether three times. Subsequently, proteins were separated from the polar fraction by precipitation with the addition of 800 μL methanol at -20°C for 120 minutes. The three obtained fractions were then treated and analyzed separately. A schematic representation of the experimental protocol is given in **Figure 2**.

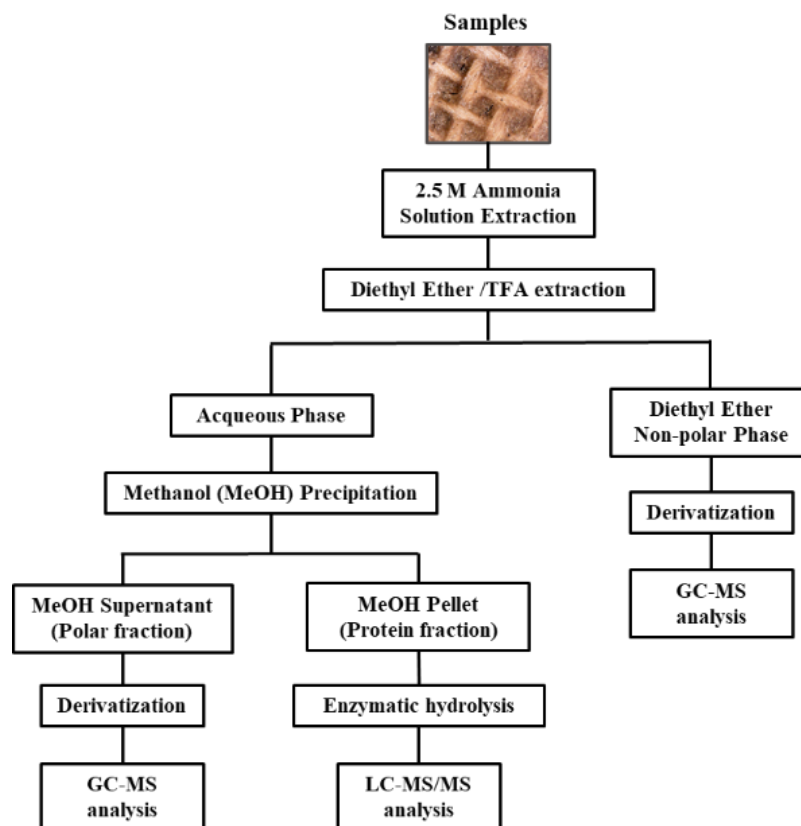


Figure 2. Schematic representation of the experimental protocol. Adapted from references¹⁴⁻¹⁵.

Nonpolar fraction

The lipids present in the isolated nonpolar fraction were analyzed as fatty acids methyl esters (FAMES) using GC-MS due to their reduced polarity. Transesterification was carried out by combining 150 μL of sulfuric acid and 850 μL of methanol with 1.0 mL of chloroform at 95°C for 16 hours. Subsequently, the pH was adjusted to neutrality by adding 2.0 mL of a 100 mg/mL ammonium bicarbonate solution. The chloroform phase containing FAMES was recovered, and excess salts were removed with water. The resulting FAMES were dried under a nitrogen flow, then suspended in 1.0 mL of n-hexane and centrifuged to remove excess solid reagents. A 1.0 μL aliquot of the hexane supernatant (diluted 1:20) was used for GC-MS analysis.

Polar fraction

The polar fraction, which contained saccharides, was dried under vacuum and subjected to methanolysis. This involved adding 1.0 mL of methanolic HCl (1:20 acetyl chloride - methyl alcohol anhydrous). Subsequently, the monosaccharide mixture was re-N-acetylated by adding 500 μL of methanol, 10 μL of pyridine, and 50 μL of acetic anhydride at RT for 30 minutes. The sugars were then trimethylsilylated in 100 μL of N, O-bis-(trimethylsilyl)-acetamide (TMS) at 70°C for 45 minutes. Each sample was dried under nitrogen, dissolved in 1.0 mL of hexane, and centrifuged to remove excess solid reagents. A 1.0 μL aliquot of the hexane supernatant (diluted 1:20) was used for GC-MS analysis.

GC-MS analysis

GC-MS analysis was conducted using an ISQ-QD quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with a TRACE™ 1300 Gas Chromatograph and a Zebron ZB-5HT Inferno (5%-Phenyl-95%-Dimethylpolysiloxane) fused silica capillary column (30 $\times$ 0.32 $\times$ 0.10 μm) from Phenomenex.

For the nonpolar fractions, Electron Ionization (EI) mass spectra were recorded by continuous quadrupole scanning at 70 eV ionization energy, with a mass range of 30 to 800 atomic mass units (u.m.a.), and a solvent delay of 6 minutes. The temperature profile started at 70°C for approximately 2 minutes, then ramped to 230°C at a rate of 20°C/min, followed by further increases to 240°C and 270°C. The maximum temperature reached by the instrument was 325°C.

For the polar fractions, EI mass spectra were recorded by continuous quadrupole scanning at 70 eV ionization energy, with a mass range of 30 to 600 u.m.a., and a solvent delay of 5.50 minutes. The temperature profile was the same as for the nonpolar fractions. Mass spectra assignment was based on direct matching with spectra from the NIST library, with identifications considered reliable if the correlation match index was higher than 95%.

Protein fraction

The protein fraction was evaporated under vacuum, and the resulting pellet was suspended in 50 μ L of a 6 M urea, 25 mM ammonium bicarbonate (AMBIC) solution. Disulfide bonds were reduced by adding 20 μ L of 100 mM dithiothreitol (DTT) and incubating at 60°C for 1 hour, followed by alkylation with 20 μ L of 120 mM iodoacetamide (IAM) for 1 hour at room temperature in the dark. Alkylation was quenched by adding 20 μ L of 100 mM DTT. The urea concentration was then reduced to 1 M by adding 190 μ L of 50 mM AMBIC. Enzymatic digestion was carried out by adding 6 μ g of trypsin and incubating at 37°C for 16-18 hours. The resulting tryptic peptide mixture was desalted and concentrated using a benchtop C18 reversed-phase liquid chromatography (Sep-Pak C18 Classic Cartridge). The resin was activated by successive additions of (i) 2 mL of methanol, (ii) 2 mL of a solution containing 80% acetonitrile (ACN) and 20% formic acid (HCOOH) (0.5%), and (iii) 2 mL of 0.5% HCOOH. Next, 1 mL of the sample suspended in 0.5% HCOOH was loaded onto the column, and the eluent was discarded. Washing was performed with 2 mL of 0.5% HCOOH, and elution was achieved with 2 mL of 80% ACN, 20% HCOOH (0.5%). The eluted peptides were dried and suspended in 50 μ L of 2% ACN, 80% formic acid (HCOOH). Finally, 1 μ L of each sample was injected for LC-MS/MS analysis.

LC-MS/MS analysis

Proteins were analyzed on a LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap MS System (Thermo Scientific, Bremen, Germany) on a C18 capillary reverse-phase column (200 mm, 75 μ m, 5 μ m) at 250 nL/min flow rate, using a step gradient of eluent B (0.2% HCOOH, 95% ACN LC-MS grade) in eluent A (0.2% HCOOH in 2% ACN) from 5 to 50% over 80 min and to 80% over 5 min. MS analyses were performed using data-dependent acquisition (DDA) mode over the 400 to 1800 m/z range, at a resolution of 60 000, and the automatic gain control (AGC) target was set to 1×10^6 , followed by acquisition in MS/MS of the five most abundant ions. For the MS/MS scans, the resolution was set to 15 000, the AGC target was set to 1×10^5 , the precursor isolation width was 2 Da, and the maximum injection time was

set to 500 ms. The CID normalized collision energy was 35%; AGC target was set to 1×10^5 . Data were acquired by Xcalibur software (Thermo Fisher Scientific).

Data handling and interpretation

The acquired MS/MS spectra were converted to Mascot Generic files (.mgf) format and subsequently queried against the NCBI nr database using a licensed version of the Mascot software (version 2.4.0) available at www.matrixscience.com for protein identification. 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 and formation of pyroglutamic acid from glutamine residues at the N-terminal position of peptides were considered as variable modifications.

An individual ion score threshold provided by the Mascot software was applied to evaluate the quality of matches in the MS/MS data, and proteins with scores lower than this threshold were rejected.

Each peptide sequence identified by the Mascot software was subjected to the BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the NCBI nr database to determine species specificity. BLAST compares the query sequence to a database of protein sequences and calculates the statistical significance of matches. Only matches with 100% Percent Identity were considered for taxonomic attribution. Peptide sequences attributed by BLAST to a single taxonomic species were considered species-specific markers of their biological origin. Peptide sequences that were not species-specific but attributable to only one of the animal or plant species possibly present in the glue paste recipes were used to recognize the presence of specific ingredients in the samples.

Results And Discussion

The molecular characterization of the glue pastes was accomplished using a multi-step extraction protocol (as detailed in the Materials and Methods section) to extract the organic components and separate them into three distinct fractions: nonpolar, polar, and protein fractions. This was achieved by employing a range of solvents designed to extract organic compounds with varied physical and chemical properties.

Test specimens

The experimental protocol was initially optimized using test specimens of glue pastes prepared according to various traditional recipes and applied to canvases (see the Materials and Methods section). Results obtained from the analysis of test specimens are presented below for each isolated molecular fraction: the nonpolar, polar, and protein fractions.

Nonpolar fraction

Lipids present in the nonpolar fractions of the test specimens were analyzed as FAMES using GC-MS. These analyses facilitated the reliable identification of specific molecular markers indicative of the presence of particular ingredients in the recipes.

As detailed in Supplementary material **Table S1**, linear fatty acids were observed in all the analyzed samples, with hexadecanoic (palmitic) acid and octadecanoic (stearic) acid being the most prominent. While fatty acids are found in many ingredients expected in glue pastes, such as cereal flour and linseed meal, their detection alone does not conclusively define the presence of a specific biological matrix.

Venetian turpentine serves as a significant additive in glue paste preparation, widely utilized by artists and restorers for its plasticizing and gloss-enhancing properties in oil colors, varnishes, mediums, and adhesives.¹¹ Derived from the conifer subfamily *Pinaceae*, particularly from *Larix decidua* trees, Venetian turpentine is classified as a diterpenic resin. Conifer resins predominantly contain diterpenoids of three primary skeletal types, namely pimarane and abietane compounds, primarily acids such as abietic, neoabietic, laevopimaric, and palustric acids, which are prevalent in *Pinaceae* resins.²¹ Abietane-type molecules, characterized by conjugated double bonds, are prone to oxidation reactions.²² The presence of Venetian turpentine in samples B2, E1, C1, C2, and D2 was confirmed by the detection of abietanic and pimaranic diterpenoids, indicating the presence of natural pine resin.²¹⁻²⁷ Conversely, diterpenoid compounds were not detected in samples B1 and D2, where Venetian turpentine is not included in the recipes.

The total ion current (TIC) obtained for the nonpolar fraction of sample C1 (refer to Supplementary material **Fig. S2**) reveals the presence of diterpenoid acids with abietane skeletons in the 20–35 min range. Among these, dehydroabietic acid (DHA) and 7-oxo-dehydroabietic acid (7-ODA) are prominent, serving as principal markers of Venetian turpentine.²⁵ The presence of 7-ODA may be attributed to the artificial aging process undergone by the samples.²⁵⁻²⁷ DHA is formed when pine resin is heated in the presence of

wood, as methanol released during wood heating reacts with diterpenic acids, primarily producing DHA. This compound is absent when the pitch is produced solely by the pyrolysis of resin.²⁴

Polar fraction

The compounds present in the polar fraction of each test specimen were analyzed by GC-MS as TMS-derivatives. Saccharide compounds detected in samples were identified by comparison with a standard mixture of known monosaccharides. Results obtained for the polar fractions of the test specimens are provided in Supplementary material **Table S2**.

A noteworthy example can be seen in the TIC obtained for sample B2 (refer to Supplementary material **Fig. S3**). The most intense peak at 19.6 min (Retention time, RT) observed in all analyzed glue pastes can be attributed to the predominant presence of glucose in the samples. Glucose is found in various biological matrices expected in glue pastes, with the most significant source being starch from flours²⁸⁻²⁹, honey, and molasses.³⁰⁻

³²

Honey and molasses are specifically used as plasticizers to enhance the plasticity and fluidity of the adhesive material.¹¹ The choice between one or the other was at the discretion of the artist or restorer and depended on their availability. These matrices primarily consist of sugars, with glucose and fructose being the most prevalent monosaccharides, along with sucrose as a disaccharide composed of α -D-glucose and β -D-fructose.³³ Other monosaccharides may also be present in honey and molasses, depending on the different fermentation pathways glucose and fructose undergo.³³⁻³⁴ Honey may also contain various sugars due to contamination by nectar and royal jelly.³⁴

Differentiating between honey and molasses was not feasible in this study, as both matrices exhibited almost identical GC-MS profiles. However, the presence of honey or molasses in addition to flour could be confirmed. In theory, the detection of fructose could potentially be utilized for this particular task, as this type of sugar is not consistently found in flour.³¹⁻³² However, practical implementation faced challenges. Despite attempts, distinguishing fructose was not achievable due to its retention time (RT) positioning with that of glucose, as indicated by data from standards. Nevertheless, other monosaccharides were successfully discerned in the polar fraction. This detection served as an indicator of the presence of honey or molasses in the sample. Specifically, the identification of xylose, mannose, and myoinositol enabled the recognition of honey/molasses in samples B1, B2,

E1, C1, and C2, aligning with the recipes outlined in **Table 1**. The presence of glucose was attributed to the presence of flour, which was consistent across all samples. The differentiation of the specific type of flour present in the samples was achievable solely through the analysis of the protein fraction.

Protein fraction

The protein fraction in glue paste samples consists of two main sources: animal glues³⁵⁻³⁶ and cereal flours.²⁸⁻²⁹

Cereal flour serves as a filler agent in these samples.¹¹ Cereal grains typically contain about 10% of their weight in protein. Among these proteins, only a small fraction, up to 0.5%, is in the form of albumins, while the majority consists of globulins. Globulins can be further classified into prolamins and glutelins. It is worth noting that some of these proteins contain significant amounts of glutamic acid residues. For instance, gliadin, found in wheat, contains approximately 40% glutamic acid.²¹

Recipes for glue paste preparation can vary regionally and may include different types of flours or combinations thereof.⁸ Wheat flour is a common ingredient across most recipes. Wheat flour is rich in starch and contains proteins specific to wheat (*Triticum aestivum*).^{28,29,37,38} It also contains small amounts of lipids and insoluble fibers such as cellulose and lignin. Additionally, some recipes may incorporate other types of flour such as rye flour³⁹ and linseed meal.⁴⁰⁻⁴³ Rye flour shares similarities with wheat flour but differs in the composition of its proteins, which are specific to rye (*Secale cereale*).³⁹ Linseed meal, on the other hand, primarily consists of α -linoleic acid and contains polysaccharides⁴⁰ as well as low molecular weight proteins specific to linseed (*Linum usitatissimum*).⁴²

Animal glue is a crucial component of glue paste recipes, providing the adhesive properties necessary for the final formulation.¹¹ Widely employed in restoration practices, animal glue serves various purposes such as adhesion, binding, coating, and consolidation for both organic and inorganic materials.^{37,44}

Typically derived from connective tissues, animal glues are primarily composed of collagen. Collagen, in its natural state, forms a triple helix protein structure characterized by a repetitive Glycine-X-Y sequence, along with a notably high content of Proline and Hydroxyproline. These distinctive features make collagen easily identifiable in the realm of proteins. Through treatment with acids or alkalis in hot water, the typically insoluble collagen undergoes a transformation into a soluble form known as gelatin, which is then

utilized as animal glue.³⁵ In the restoration field, three main types of animal glues are commonly utilized: 1. Sturgeon^{45,46} and rabbit glues⁹: These types are derived from soft connective tissues and skin. 2. Bovine glue⁹: Mainly sourced from bones. Each type of animal glue offers unique properties and is selected based on the specific requirements of the restoration project.

In this study, the protein extract underwent enzymatic hydrolysis to produce peptides, which were then analyzed using LC-MS/MS, as described in the Materials and Methods section. Initially, proteins were identified using Mascot software through interrogation of the NCBI database with spectra generated by LC-MS/MS analyses. The decision to use the NCBI database was driven by the requirement to search for unreviewed collagen sequences. It is important to note that peptides identified with a Mascot score below 50 were excluded from the analysis.

Upon thorough examination of the Mascot results obtained from the NCBI database, it was observed that numerous proteins were identified with the same set of peptides. This phenomenon was particularly prominent for collagen proteins derived from animal glues. This can be attributed to the high sequence homology that exists between collagen chains from different species. Discriminating between animal species, especially in the case of collagen, poses a significant challenge due to the reliance on the detection of a very limited number of unique peptides.⁴⁵ This challenge is exacerbated when samples contain a mixture of animal glues from two different species. In such cases, distinguishing between the species becomes even more difficult. Therefore, accurate identification and differentiation of animal species based on collagen peptides present in the samples require careful consideration and advanced analytical techniques.

To ascertain the precise biological origin of collagen proteins within glue paste samples, each identified collagen peptide was individually scrutinized against the NCBI database using the BlastP tool, available online (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE/Proteins>). BlastP compares a query peptide sequence to a database of protein sequences and evaluates the statistical significance of matches. Only matches exhibiting a 100% Percent Identity were considered for robust taxonomic attribution.

To simplify the process of species assignment, the search scope was limited to sequences of collagen type I and III. Collagen alpha-1 and -2(I) are universally present in all collagen-bearing tissues used in the preparation of animal glues.³⁵ Specifically, collagen alpha-1(III)

is prevalent in soft connective tissues and skin, while its synthesis in bones is relatively minimal.³⁵

Animal glues sourced from bovine and rabbit origins can be distinguished by detecting peptides specific to *Bos taurus* and *Oryctolagus cuniculus*, respectively. Conversely, identifying peptides specific to fish collagen chains presents a more complex challenge due to the relatively sparse sequencing data available for fish compared to mammalian species.⁴⁵ Several studies in conservation science have highlighted the ambiguity of results obtained from database interrogation when dealing with non-sequenced proteins, such as sturgeon collagen.⁴⁵ Identifying fish proteins is constrained by the data within proteomics databases, which are continuously evolving. Consequently, the identification of fish proteins typically relies on sequence homology with other fish species.⁴⁵ This approach allows researchers to infer the likely origin of fish-derived collagen based on similarities to known sequences, despite the limited direct sequencing data available for fish species.

In our study, the identification of peptides specific to collagen chains from fish allowed to confirm the presence of fish glue in samples B1, B2, C2, D1, and E1. Specifically, let us examine the case of sample B1. Through interrogation of the LC-MS/MS data against the NCBI nr database, five peptides from collagen type I alpha-1 and alpha-2 were identified, attributed to various fish species. The specificity of these peptides was verified using the BlastP tool. For instance, the sequence "GAAGEAGKPGER," initially assigned by Mascot to collagen type I alpha-1 from grass carp (*Ctenopharyngodon idella*), was found in 33 other collagen chains, all from fish sources, as detailed in Supplementary material **Table S3**. Similarly, four other peptides were found in several fish collagen proteins. Despite the relatively limited number of sequenced fish collagen proteins, this analysis facilitated the identification of five peptides originating from collagen across various fish species. This outcome strongly suggests that fish glue was used in the creation of sample B1. Comparable results were obtained for all other test specimens containing fish glue, as indicated in Supplementary material **Table S3-S7**.

On the other hand, the presence of bovine glue was confirmed in sample C1, which was the only sample containing bovine glue. This determination was based on the identification of two peptides attributed to collagen type I alpha-1 specific to *Bos taurus*. The peptides "GADGAPGKDGVR" and "GETGPAGPAGPIGPVGAR" were uniquely linked to *Bos taurus*, according to the glue paste recipes.

In the identification of rabbit glue in samples B2, C1, and D2, achieving unambiguous taxonomic attributions through Mascot software alone proved challenging. To enhance the sensitivity of the analysis and identify peptides specific to *Oryctolagus cuniculus*, a more detailed manual investigation was conducted into the LC-MS/MS spectra. MS-Digest, available at <https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest>, was utilized to perform in-silico tryptic digests of collagen alpha-1 and -2(I) chains from both *Bos taurus* and *Oryctolagus cuniculus*. This tool provided the mass of each predicted tryptic peptide, encompassing both singly and multiply charged peptides. Through sequence alignments performed between collagen chains from *Bos taurus* and *Oryctolagus cuniculus*, utilizing tools such as Uniprot Align (<https://www.uniprot.org/align>), species-specific peptides were selected for both animal species. Peptides with double and triple charges were prioritized for further investigation, as they are commonly generated by Electrospray Ionization (ESI) sources. In Supplementary material **Table S8** all species-specific peptides of collagen identified through LC-MS/MS analysis are presented for each analyzed test specimen.

In a manner analogous to the identification of species-specific peptides from collagen, the recognition of peptides from different plant species facilitated the determination of the type of flour present in each sample. The detection of peptides specific to the Poaceae family proved effective in confirming the presence of wheat flour (*Triticum aestivum*) in nearly all analyzed samples. Additionally, peptides specific to *Linum* species were identified in samples C1 and C2, indicating the presence of linseed meal. Furthermore, peptides specific to *Secale cereale* were detected in sample D1 and D2, confirming the utilization of rye flour in the French recipes. Although *Secale*-specific peptides were not identified in sample C1, certain peptides detected could still be attributed to rye flour.

The detailed list of identified peptides from flour proteins for each test specimen is provided in Supplementary material **Tables S9-S15**. Overall, these results align well with the recipes outlined in **Table 1**.

Historical sample

In this historical case study involving the glue paste from the 17th-century canvas painting "La Fuga in Egitto" from the Pagliara Collection of the University Suor Orsola Benincasa (Naples, Italy), the analytical strategy proposed in this work was applied to provide robust validation.

Firstly, the compounds present in the nonpolar fraction were analyzed as FAMES via GC-MS. As detailed in Supplementary material **Table S16**, various fatty acids were detected, with hexadecanoic acid (palmitic) and octadecanoic acid (stearic) being the most prominent. However, the absence of diterpenoid acids with an abietane skeleton indicated the absence of Venetian turpentine in the historical glue paste recipe.

Subsequently, the compounds present in the polar fraction were analyzed as TMS derivatives via GC-MS (data provided in Supplementary material **Table S16**). The detection of glucose suggested the presence of starch from flour. Additionally, the presence of xylose indicated the inclusion of a saccharide-based biological matrix, such as honey or molasses, alongside flour.

Lastly, LC-MS/MS analysis, in conjunction with specific bioinformatic tools, enabled the characterization of the protein fraction. Peptides specific to collagen from *Bos taurus* and *Oryctolagus cuniculus* were identified, indicating the utilization of a mixture of bovine and rabbit glue in the historical glue paste. Furthermore, peptides specific to *Triticum aestivum* confirmed the presence of wheat flour in the historical recipe. All species-specific peptides identified in the historical sample are detailed in Supplementary material **Tables S17**.

Conclusions

The molecular characterization of samples from works of art can provide valuable insights into the composition of ancient restoration materials and their conservation status. In this report, we present a novel analytical protocol for the molecular characterization of a specific adhesive used in the past for the restoration of paintings, known as "glue lining pastes." Due to the high molecular complexity of these adhesives, a multi-step extraction protocol is proposed to simultaneously recover and fractionate the three main classes of biomolecules contained in glue pastes, namely lipids, polysaccharides, and proteins. High-performance separation techniques coupled with high-resolution MS were applied to the three isolated fractions to identify the specific components present. The proposed method was initially optimized using test specimens of various traditional glue pastes applied to canvases.⁸ Subsequently, it was successfully applied to a historical glue paste sample obtained from a 17th-century painting titled "La fuga in Egitto" which is part of the Pagliara collection at the University Suor Orsola Benincasa in Naples, Italy.

The comprehensive data collected in this study provide valuable insights into the recipes utilized for the preparation of glue pastes, thereby contributing to a broader understanding of historical restoration practices. By applying the described procedure to test specimens of various traditional glue pastes, a rich database of information regarding the molecular composition of these adhesives was generated. The identification of reliable molecular markers has facilitated the recognition of specific ingredients used in glue paste formulations. In particular, GC-MS analysis emerged as a powerful technique for obtaining detailed information regarding the lipid and saccharide components of these pastes. This analytical method enabled the identification of biomolecular compounds specific to Venetian turpentine and honey/molasses, thereby enhancing our ability to characterize and understand the composition of these historical adhesives.

The combination of LC-MS/MS technique with appropriate bioinformatic analysis demonstrated remarkable efficiency in providing detailed information regarding the protein content of the glue pastes. This approach successfully identified numerous species-specific collagen chains derived from animal connective tissues present in the test specimens, thereby allowing for the determination of the source of the glue. Importantly, this method remained effective even when glues from different animal sources were mixed. Specifically, animal glues of bovine and rabbit origin were successfully distinguished through the detection of species-specific peptides. In the case of sturgeon glue, the identification of collagen relied on sequence homology with other fish species. Additionally, the LC-MS/MS data enabled the identification of a wide range of proteins from flour, with the detection of species-specific peptides facilitating the recognition of specific types of flour or mixtures thereof.

The analysis of a historical glue paste sample from the 17th-century canvas painting "La Fuga in Egitto" underscored the applicability of the developed method in analyzing restoration materials from works of art. The main ingredients of the historical glue paste recipe were identified as wheat flour, honey/molasses, and a mixture of bovine and rabbit glues. Based on these results, the proposed procedure proved highly successful in enabling a comprehensive identification of traditional glue paste recipes. The data collected in this work significantly contribute to a deeper understanding of ancient restoration materials, particularly focusing on their molecular composition. Such information is pivotal for designing proper restoration interventions and determining the optimal conditions for preserving and displaying historical paintings.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD051480.

Author Contribution

B.C. and M.M. equally contributed to this work. A.C. conceived the project, reviewed, and edited the manuscript. A.C., G.M. and P.I.S. coordinated and supervised the activities. C.P. provided the samples and the restorer's point of view. B.C., M.M. and C.P. carried out the experimental activities. B.C. and M.M. analyzed and interpreted experimental data and wrote and edited the manuscript. A.A. provided the MS setup. All authors read and approved the final manuscript.

References

6. Development of new proteomics workflow for the chemical characterization of proteinaceous materials

Post-translational and diagenetic modifications in ancient proteins

Proteinaceous materials such as egg, collagen, and casein have been extensively employed throughout history as paint binders and varnishes in polychrome artworks, panel paintings, and murals, as well as adhesives in gildings.¹ Collagen, in particular, is not only a major constituent of animal glues used for artistic and restoration purposes but also the intrinsic structural protein of many cultural heritage (CH) materials. Archaeological findings like bones, ivory, or teeth, as well as parchment and leather artifacts, are all largely collagen-based.² The wide presence of proteinaceous substances across artistic, paleontological and archaeological contexts makes their molecular characterization essential for understanding the degradation and ageing processes that shape their long-term preservation.³

Over time, these proteinaceous matrices undergo a variety of physicochemical changes. Such transformations can substantially alter the molecular structure of proteins, reducing their solubility and thereby affecting the efficiency of proteomic analyses that rely on protein solubilization and proteolytic digestion.⁴ Moreover, database search algorithms used for protein identification may underperform if degradation-related chemical modifications are not considered, leading to incomplete or inaccurate protein characterization. For this reason, the study of post-translational and diagenetic modifications in proteinaceous materials is central to the proteomic investigation of CH objects.

Post-translational modifications (PTMs) are chemical changes that occur to amino acid residues after protein biosynthesis. In vivo, these modifications—often enzymatically mediated—are crucial for protein function, stability, and localization. Common biological PTMs include glycosylation, phosphorylation, and hydroxylation, which, given their chemical stability, may persist in ancient protein samples and serve as markers of original biological functions. However, in the context of artworks and archaeological materials, PTMs of biological origin coexist with *diagenetic modifications*—chemical alterations introduced by environmental, chemical, or physical factors after the protein’s deposition or application.⁵

Diagenetic modifications can reveal valuable information on the degradation history of proteinaceous materials, offering molecular fingerprints of manufacturing, conservation treatments, and environmental conditions experienced by the artifact. Among these modifications, deamidation of glutamine (Gln) and asparagine (Asn) residues is the most extensively studied and the most commonly identified nonenzymatic modification in ancient proteins.^{1,6–8} This reaction, converting amide side chains to carboxylic acids, leads to the formation of glutamic acid (Glu) and aspartic acid (Asp) and involves either direct hydrolysis or formation of a cyclic intermediate, such as succinimide or glutarimide.⁹ Deamidation occurs spontaneously under physiological or post-depositional conditions, and its rate depends on multiple factors, including pH, temperature, ionic strength, and the local structural flexibility of the protein.¹⁰

The kinetics of deamidation are complex: in acidic conditions, direct hydrolysis predominates, whereas in neutral or basic environments, the cyclic intermediate pathway is favored.^{9,11,12} Asparagine residues typically deamidate up to ten times faster than glutamine^{9,13}; however, in archaeological and cultural heritage contexts, Gln deamidation is often more informative due to its slower rate and broader timescale sensitivity.^{1,6,13,14} Importantly, deamidation rates are substantially reduced in solid-state proteins, such as those constituting paints, glues, or bones, compared with proteins in solution. This property is particularly relevant for the study of cultural heritage samples, where proteins exist in aggregated, insoluble matrices and have lost their native tertiary and quaternary structures.

Oxidation is another key process contributing to the degradation of proteinaceous materials.⁵ Upon exposure to light and oxygen, proteins become susceptible to photo-oxidative reactions. Amino acid side chains containing chromophoric groups—such as tryptophan (Trp), tyrosine (Tyr), histidine (His), cysteine (Cys), methionine (Met), and phenylalanine (Phe)—are especially prone to oxidation, leading to the formation of a variety of products including di-tyrosine crosslinks, N-formylkynurenine, and 3,4-dihydroxyphenylalanine.⁵ These oxidative processes can proceed via two main pathways: direct photo-oxidation through UV excitation (Type I) or indirect oxidation mediated by singlet oxygen (Type II), where pigments and other exogenous chromophores act as sensitizers.⁵ Moreover, there is a general conviction that one of the most important mechanisms of protein oxidative damage is metal-catalyzed oxidation (MCO), resulting in modification of the amino acid side chains, cleavage of polypeptide backbone, and cross linking.¹⁵

Beyond oxidation, the irreversible process of backbone cleavage of the polypeptide chain into increasingly smaller pieces of the original protein, is among the most relevant forms of degradation and expected as a degradation feature in ancient proteins.^{5,16,17} It has been shown that aging of proteins can lead to partial hydrolysis of the polypeptide chains, aggregation, cross-linking by covalent bonds, and the formation of stable complexes with inorganic materials, such as pigments.^{18,19}

The extent and pattern of chemical modifications in proteins are shaped by a combination of environmental and anthropogenic factors. Temperature, humidity, pH, light exposure, and microbial activity all influence protein stability and degradation kinetics. Low temperatures inhibit both chemical reactions and microbial growth, promoting exceptional preservation in frozen or permafrost contexts.²⁰ Conversely, moisture accelerates hydrolysis and microbial degradation, while extreme pH values—both acidic and alkaline—promote peptide bond cleavage and side-chain modifications such as deamidation. In bone, the mineral matrix acts as a natural pH buffer and physical barrier, slowing the infiltration of water and microorganisms and thus stabilizing collagen over archaeological timescales.

Protein degradation may also begin during manufacturing processes or material preparation. The use of lime in parchment and leather production induces oxidation and deamidation even before aging occurs.²¹ The drying of a paint film, associated with water loss, introduces oxidation in proteins.²² Similarly, boiling bones and hides to prepare animal glues causes collagen hydrolysis, resulting in lower molecular weight peptides.¹⁷ During the lifetime of an object, temperature changes, mechanical stress, and exposure to environmental contaminants further promote degradation. Cooking pots subjected to temperature changes, the mixing of paints and binders, or washing textiles, are inherent sources of modifications during an object's lifetime.

Restoration interventions can also contribute to the chemical alteration of proteinaceous materials. Cleaning agents, solvents, consolidants, and adhesives may either extract proteins from the substrate or introduce new reactive species that accelerate hydrolysis and oxidation. The use of formaldehyde, for example, can cause the formation of protein crosslinks.²³ Some modifications, like oxidation and deamidation, can be also introduced during sample preparation. Although this cannot be fully prevented, it can be controlled to some extent by minimizing, if it is possible, the use of “stressful” extraction strategies as well as handling samples reproducibly. Consequently, recognizing the specific

modification patterns linked to both natural aging and anthropogenic treatments is crucial for accurate interpretation of proteomic data.

Degradation pathways in collagen-based materials

Collagen-based materials such as animal glues, leather, and parchment represent an important subset of proteinaceous cultural heritage substrates. Collagen's triple-helical structure confers exceptional mechanical and chemical stability, contributing to its long-term survival in archaeological contexts.¹⁶ However, chemical treatments such as those used for glue manufacturing and tanning methods can significantly affect its molecular integrity and degradation behavior.^{17,21,24} For example, alkaline treatments used in parchment preparation promote rapid deamidation of glutamine, while acidic tanning processes in leather lead to different deamidation kinetics and preferential modification of asparagine residues.²¹ Moreover, animal glues derived from different sources and subjected to diverse preparation protocols exhibit distinct patterns of deamidation and peptide backbone cleavage.¹⁷ The same authors further showed that collagen from bones excavated at Pompeii and Herculaneum—though coeval—displays markedly different modification profiles, attributable to the distinct thermal conditions experienced during the volcanic eruption.¹⁶ These findings highlight the strong influence of environmental factors and processing histories on the molecular evolution of proteinaceous materials.

Understanding the degradation patterns of collagen is an important step to understand the use and conservation history of archaeological and historical objects made from hide or bone materials and can be further applied to the modern leather, food and adhesive industries.²¹

Interactions between proteins and inorganic pigments in paintings

In painted artworks, the interaction between proteinaceous binders and inorganic pigments introduces an additional layer of complexity to degradation mechanisms. Pigments containing metal ions such as mercury (Hg^{2+}), copper (Cu^{2+}), lead (Pb^{2+}) or iron (Fe^{2+}) can react with protein functional groups, forming stable metal–protein complexes that modify the protein's secondary structure and thermal stability. For instance, Duce et al. (2013) reported the formation of casein–Hg complexes that significantly modified the structural organization of the binder.^{18,19} These interactions may induce conformational

changes—such as increased intramolecular β -sheet formation at the expense of intermolecular hydrogen bonding—leading to aggregation and reduced solubility.^{4,18,19}

Moreover, inorganic pigments influence the chemical microenvironment within the paint matrix, altering factors such as pH, redox potential, and light sensitivity. Metal-containing pigments may catalyze oxidative processes or act as photosensitizers, thereby accelerating or modulating photo-oxidative degradation of proteinaceous binders.¹⁹ In contrast, certain pigments may offer protective effects, stabilizing the protein matrix by scavenging reactive oxygen species or by physically hindering oxygen diffusion. Such pigment–binder interactions are therefore pivotal to understanding the chemical and structural evolution of painted layers during aging and conservation.

Despite growing evidence of these interactions, the molecular pathways underlying pigment-induced protein aging remain insufficiently understood. Few studies have rather focused on lipid degradation in egg tempera reconstructions in presence of pigments.^{25,26} This is why ongoing studies of these interactions are crucial for understanding the long-term preservation and interpretation of historical tempera paintings.

Analytical challenges and bioinformatic approaches in paleoproteomics

The proteomic characterization of artistic and archaeological materials presents unique analytical challenges. Proteins in such samples are not in solution but embedded in solid, heterogeneous matrices where they exist in aggregated or denatured forms. They have lost their native three-dimensional structures and may already contain substantial chemical modifications resulting from historical processing and curing before any long-term aging occurs. Furthermore, they coexist with diverse organic and inorganic compounds that can interfere with protein extraction and digestion, as well as with mass spectrometric detection.⁴

Chemical characterization studies by proteomics relies primarily on mass spectrometry (MS) combined with bioinformatic tools for peptide identification and chemical modifications analysis. Database search algorithms such as Mascot²⁷, Andromeda²⁸, and Sequest^{29,30} enable the detection of modified peptides by considering variable and fixed modifications during spectral matching. However, expanding the number of variable modifications increases computational complexity and can reduce confidence in peptide identifications at a fixed false discovery rate. As a result, researchers must balance sensitivity

and specificity in order to capture relevant diagenetic modifications without compromising data quality.

Quantitative approaches for assessing the extent of modifications, particularly deamidation, have been developed in recent years. Tools such as Q2E¹³, MALDIpqi¹⁴ have been designed to calculate the overall deamidation of glutamine from ZooMS spectra. DeamiDATE allow for bulk and site-specific quantification of Gln and Asn deamidation of proteins from LC-MS/MS spectra.⁶

Nevertheless, there is still no universally accepted protocol or analytical framework for the comprehensive interpretation of chemical modifications in ancient proteins. The lack of standardized procedures for sample preparation, data acquisition, and computational analysis remains one of the main challenges in paleoproteomics.

A comparative and structural approach to protein degradation studies

Given the complexity of protein degradation mechanisms and the variability introduced by matrix composition, pigment interaction, environmental conditions and artificial chemical treatments, a comparative experimental approach is essential. By analyzing samples that have undergone similar handling and analytical protocols but experienced different degradation histories, it is possible to isolate and interpret the chemical signatures associated with specific aging/degradation pathways. Combining mass spectrometry-based proteomics with semiquantitative bioinformatic analyses offers a promising strategy to unravel the molecular processes governing protein alteration in cultural heritage materials.

In this chapter, an innovative analytical pipeline is applied to evaluate the occurrence and distribution of chemical modifications in proteins, enabling a semi-quantitative assessment and visualization of their localization across specific regions of the protein sequence. Building on the conceptual framework outlined above, this work addresses two main research directions.

Section 5.1 focuses on the investigation of chemical interactions between casein and four inorganic pigments in artificially aged paint reconstructions. The extent of a wide range of chemical modifications typically encountered in historical paintings is semi-quantitatively assessed across differently pigmented samples to explore potential pigment-specific effects on casein degradation. Furthermore, the distribution of these modifications along the casein sequences is examined with the future aim of linking modification patterns

to structural susceptibility. This analysis is based on the hypothesis that protein regions more exposed in the three-dimensional structure—or directly involved in pigment interactions—are more prone to chemical alteration. To this end, a novel *limited proteolysis mass spectrometry* (LiP-MS) approach is introduced to probe structural alterations in casein potentially induced by pigment–binder interactions.

Sections 5.2 to 5.4 addresses the investigation of chemical modification patterns in collagen-based materials, ranging from traditional animal glues used in restoration practices to novel bioadhesives derived from tannery waste. By assessing deamidation and other diagenetic modifications in collagen, this study aims to elucidate how distinct preparation methods and chemical treatments influence molecular integrity and, ultimately, adhesive performance. This section includes three manuscripts, two of which have been published or submitted in collaboration with the University and the CNR Institute of Pisa (Italy). The first two manuscripts focus on the characterization of secondary structure, renaturation behavior, and physical aging in adhesives used for restoration, followed by the investigation of bioadhesives derived from collagen-based tannery wastes. The third manuscript presents a systematic comparative proteomic study of chemical modification patterns in collagen from animal glues prepared under distinct processing conditions, vegetable- and chrome-tanned leather, and reference materials including standard collagen and gelatin.

Collectively, these investigations advance the development of standardized and reproducible proteomic methodologies for assessing PTMs and diagenetic modifications, while deepening our understanding of degradation mechanisms in proteinaceous materials from our cultural heritage.

Manuscripts

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5.2 Characterization of the secondary structure, renaturation and physical ageing of gelatine adhesives

E. Pulidori, C. Duce, E. Bramanti, L. Ciccone, B. Cipolletta, G. Tani, L. Dello Ioio, L. Birolo and I. Bonaduce.

Animal glues have been used since antiquity, but their popularity decreased in the twentieth century with the rise of synthetic adhesives. Currently they are primarily used in restoration of works of art. This study focuses on animal glue samples derived from bone and hide tissues used mainly for veiling and carpentry applications, examining their secondary structure, thermal and rheological properties, to shed light into their adhesive behavior. Thermogravimetric analysis and differential scanning calorimetry highlight differences between hide and bone glues, showing that the latter are more hydrolyzed. The calorimetric curves show varying values of denaturation enthalpy thus indicating a varying degree of gelatine renaturation. Additionally, the calorimetric analysis demonstrates the physical ageing of the glue samples, which is known to play a key role in maintaining their adhesive properties under specific storage conditions. The rheological data provide fundamental information on properties which are relevant for the use of animal glues as adhesives in various applications. XRD data suggest a low structural order in the samples investigated, where the amorphous component complicates the univocal interpretation of the spectra. FTIR reveal a relatively high content of β structures in all commercial glue samples, as well as gelatine and collagen standards. Moreover, spectra of the glue films dried for 1 week show that the β -structures are recovered following dissolution in water and application. Therefore, β -structures seem crucial to explain the structure-dependent mechanical properties observed.

Introduction

Animal glues have been used for centuries as adhesives in a variety of applications^{1,2}. The earliest evidence of its use dates back to the Paleolithic period, where traces of animal glue were found in cave paintings³. In later years, the use of animal glues became an integral part of various industries and everyday life.^{4,5} In the twentieth century, the demand for animal glue declined due to the advent of synthetic adhesives and animal glue remained the adhesive of choice for traditional bookbinders, luthiers and restorers.^{6,7} For the conservation of artifacts, animal glues and synthetic glues are used alternatively based on the type of intervention to be carried out. Animal glues are mostly used in countries such as Italy or

Spain, since they are traditionally employed in their restoration processes and old recipes. Animal ones follow one of the cardinal principles of modern conservation, the compatibility of restoration materials with the constituent materials of ancient works.⁸ This characteristic is not found in synthetic glues. On the other hand, synthetic adhesives are useful in case of unfavorable thermo-hygrometric conditions of the conservation environment of the artwork, in this case it is preferable to introduce a more inert, not hygroscopic material that cannot be attacked by microorganisms.

Scientific research is trying to experiment with mixtures of both natural and synthetic glues⁹, to exploit the good characteristics of both. The glue production process from animal tissue involves the hydrolysis and denaturation of native triple helix of collagen to obtain water-soluble gelatine, mainly in a random coil conformation.^{10–14} Typically, the glue production process consists of several steps: (1) the raw material is washed with cold water to remove residual blood and other impurities; (2) the washed material is pre-treated in acids or alkalis; (3) the treated material is boiled in water at temperatures of around 60–70 °C for several cycles; (4) the “glue liquor” obtained is dried and crushed into flakes or pellets¹². Glues derived from hide are usually pre-treated with lime, an inorganic material consisting mainly of calcium oxides and hydroxides, while those derived from bone are pre-treated in dilute acids.^{10,13} The temperature of the boiling process can also vary, but in this case, it mainly depends on the age of the animal: the older the animal, the higher the temperature used.¹⁴ The amino acid composition of gelatine may differ slightly from that of collagen as the manufacturing process may result in the chemical modification of the amino acids.¹⁴ For example, the alkaline process employed for gelatine extraction results in the deamination of glutamine.¹⁵ Consequently, the primary structure of gelatine closely resembles that of collagen, with glycine, proline, and hydroxyproline as the main amino acids. However, its secondary and higher-order structures are significantly different. A partial recovery of the collagen structure occurs during cooling and drying, where the helices partially reform. This process is called renaturation or gelation process.¹⁴ The structural order achieved depends on the molecular weight of the polypeptide chains and on several other factors including their amino acidic composition (mainly Pro and Hyp content). The molecular weight distribution of the gelatine polypeptides depends on the production process, which causes molecular degradation¹⁶ as well as the type of tissue and the age of the animals. As reported by Elharfaoui et al.¹⁶, the extraction of highly cross-linked collagen from old tissue results in the formation of high molecular weight gelatine molecules. However, these molecules are highly branched making the renaturation very improbable.

Guo et al.¹⁷ reported that gelatine can be organized in different ways depending on the length of the α -chains, which also affects the stability and reversibility of these structures. Different types of reorganization at a higher structural level can occur involving one or more helices linked by hydrogen bonds¹⁷. These lead to the formation of gel junction zones which act as sites for the formation of a three-dimensional network. The structural order of the network depends on molecular weight and chemical composition (mainly Pro and Hyp content) of gelatine molecules. Long, high molecular weight chains and high Pro and Hyp content promote highly ordered network.^{18,19} There are some studies in the literature that investigate the chemical structure of glue and relate it to the mechanical and adhesive properties of animal glues²⁰⁻²², but the adhesion mechanism is far from being well understood. Literature reports that the content of native-like triple helices formed during the renaturation process is important as it affects the thermal, mechanical and adhesive properties of the glues²⁰⁻²². The degree of renaturation and the structural order achieved by the gelatine also depends on the storage conditions, in particular temperature and humidity²³⁻²⁵. Obas et al.²³ have reported that in high-solids confectionary gel made with gelatine, the amount of renatured structures is reduced when the gelatine sample is stored at or above the gelling temperature (20 °C). Furthermore, Dai et al.²⁴ have observed that the amount of structural gelatine in films is strictly dependent on the drying temperature with the degree of renaturation decreasing as the temperature increases. Recently, Mosleh et al.²⁶ have reported on the effect of hygrothermal ageing on the properties of various gelatine glues films from mammalian and fish tissues, showing how humidity and temperature affect the microstructure of the gelatine and, thus, the mechanical properties of the glue.

Generally, the presence and content of triple-helix-like structures are determined through X-ray diffraction analysis and differential scanning calorimetry as the enthalpy of denaturation is positively correlated with the amount of triple-helix-like structures^{21-23,26-28}. The few studies reported in the literature directly correlate the amount of triple helices present with the mechanical and adhesion properties. In particular, a greater quantity of these structures leads to a reduction in the degree of swelling, an increase in gel strength (higher degree of Bloom) and an increase in Young's modulus^{20,21}. β -structures have an influence on the properties of silk protein and gelatine-electrospun materials^{29,30}, but the effect of β -structures on the mechanical properties of adhesives has not been investigated. Therefore, this aspect warrants attention and should be considered in the study of gelatine-based adhesives.

Further insights can be gained from the calorimetric analysis of polymers and biopolymers. From the enthalpy relaxation superimposed on the glass transition, it is possible to obtain insights into physical ageing. Physical aging in polymers and biopolymers refers to the gradual changes in the physical properties of a polymeric material over time due to its molecular structure and the environmental conditions it is exposed to. This process is related to the relaxation of the polymer's chains toward a more thermodynamically stable state. As polymers age, they can undergo various physical changes that can impact their mechanical, thermal, and optical properties^{31,32}.

Physical ageing is commonly observed when an amorphous polymer is rapidly cooled below its glass transition temperature (T_g) and then stored at a temperature below T_g ³¹. An amorphous polymer in the glassy state is in a non-equilibrium condition, whereas in the rubbery state, above the glass transition temperature, it reaches an equilibrium state. Consequently, if the polymer is stored at a temperature below its T_g , it will gradually tend to relax towards a state of thermodynamic equilibrium. Physical ageing affects the thermodynamic and mechanical properties of the polymer³¹. The identification of this phenomenon is crucial in material studies, particularly in the pharmaceutical and food area, as it offers valuable insights into optimal storage conditions, shelf life, and how material properties may evolve over time due to factors such as temperature, humidity, and irradiation.

Therefore, although this phenomenon has primarily been studied in synthetic polymers^{31,33-37}, recent interest in examining the physical aging of biopolymers has grown. These studies have observed physical ageing in carbohydrates^{38,39} and several protein-based materials⁴⁰⁻⁴⁴. Literature reports that in synthetic glues physical ageing affects their properties causing a reduction in adhesion strength, an increase in brittleness, a loss of structural integrity, and a loss of flexibility⁴⁵⁻⁴⁷. Recently it has been reported that physical ageing can also affect the properties of gelatine glue. Enrione et al.⁴⁸ have conducted a study of physical aging of a model gelatine film, which was prepared by dissolving salmon gelatine in water and then drying it, using the same procedure applied in the production of animal glue. The results have showed that during the ageing of the model gelatine film the material changes its mechanical properties becoming stiffer. Physical ageing also causes a modification in porosity and density. All these can decrease the adhesive properties over time^{26,49}.

In this study, we have examined the structural characteristics of a subset of gelatine-based animal glues derived from hide and bone tissues, among those characterized by Ntasi et al.⁵⁰, and compared them to commercial collagen, gelatine A and gelatine B. The work of Ntasi et al.⁵⁰ focused on the determination of the animal origin, degree of deamidation of glutamine and asparagine, the molecular weights of the acid-soluble collagen fraction, backbone cleavage and the amount of 2,5-diketopiperazines produced during thermal degradation. Table 1 reports the samples investigated in this work and it also includes a summary of the main results of Ntasi et al.⁵⁰ on the same samples.

The glues analysed were divided into two main groups based on the material used in their production. The first group includes glues derived from hide, further divided into HP and HM depending on whether they come from a single animal (HP) or from multiple animals (HM). The second group includes glues derived from bones (BM), all from multiple animals. All HM and HP glues were originally labelled as “rabbit glues,” while BM glues were categorized as “strong glues.” However, this classification did not reflect the real biological origin of the materials, as pointed out in the study by Ntasi et al.⁵⁰. The labels mainly referred indeed to the adhesive properties of the glues themselves.

The “rabbit glues,” compared to other animal-based glues, are distinguished by their transparency, stability and good elasticity. Because they can be diluted in varying proportions, their adhesive strength can be adjusted, making them ideal for tasks such as veiling and small bonding applications. In addition, they can be combined with gypsum to fill any imperfections⁵¹. Generally, rabbit glues have a high bloom strength, ranging from 300 to 550 g⁵². Samples of HM and HP were used, for example, to make the plastering of a 15th-century painting on panel and plaster of Bologna (Fig. 1a, HP3 sample) and the veiling of a 17th-century painting on canvas (Fig. 1b, HM1 sample). The “strong glue,” derived primarily from the bones of large animals, offers adhesion, stiffness and heat resistance greater than rabbit glue. These adhesives are commonly used for structural bonding, often in combination with nails, joints and strong materials that require considerable holding force. Figure 1c,d reports the preparation and the use of BM5 in carpentry. Typically, strong glues are classified as low Bloom degree, with values ranging from 50 to 300 g⁵².

In this work we have used thermogravimetric analysis (TGA) to compare their thermal degradation profiles, attenuated total reflectance–Fourier transform infrared spectroscopy (ATR-FTIR) to obtain information on their secondary structures, including helix, β -structures and random coil content, and differential scanning calorimetry (DSC) to

determine temperature and denaturation enthalpy, glass transition temperature (a useful parameter for the application of protein biomaterials), and enthalpy relaxation related to physical ageing. XRD diffractograms and rheological data have been collected on a subset of samples to obtain information about the degree of crystallinity, viscosity, gel strength, shear resistance, and gelation temperature, which are fundamental parameters for the study of adhesive materials.



Figure 1. (a) Plastering with rabbit glue and Bologna gypsum, “Sacred conversation with the Madonna of the girdle”, Unknown, fifteenth century, panel painting, church of SS. Annunziata—Sorrento (NA); (b) Facing with rabbit glue, “portrait of a warrior”, Unknown, seventeenth century, canvas painting, private collection; (c) preparation of BM5 glue samples; (d) use of BM5 glue in carpentry.

Materials And Methods

The animal glue samples were provided by Museo Nacional del Prado (Madrid) and the restoration workshop of the University Suor Orsola Benincasa (Naples). Table 1 reports the samples’ list including sample name, and a summary of the main results for each sample obtained by Ntasi et al.⁵⁰, such as taxonomy, molecular weight (MW) of acid-soluble collagen (ASC) obtained by SDS-PAGE, backbone cleavage %, and the area of the pyrolytic signals related to all 2,5-diketopiperazines (DKPs) detected by Py-GC/MS. According to this, the samples were classified as Hide Pure (HP), Hide Mixed (HM) and Bone Mixed (BM). Standard gelatine A from porcine skin (G2500, MW = 50–100 kDa) and gelatine B from bovine skin (G9382, MW = 40–50 kDa) and standard collagen derived from calf skin (Bornstein and Traub Type I) were purchased from Sigma-Aldrich (Milan, Italy). These samples were characterized for their taxonomy, backbone cleavage % and deamidation

degree, according to the protocols reported in Ntasi et al.⁵⁰, except for minor modifications due to changes in the laboratory equipment and software availability (refer to the section “Materials and methods” in the supplementary material for details). A summary of the main results obtained is also given in **Table 1**.

	Sample name	Taxonomy	MW (kDa) of ACS	Backbone cleavage percent	Sum of the normalized areas of all DKPs $\times 10^{-4}$	Deamidation degree***
Standards	Collagen	<i>Bos taurus</i>	–	~ 30%	–	+
	Gelatine A	<i>Sus scrofa</i>	–	~ 35%	–	++
	Gelatine B	<i>Bos taurus</i>	–	~ 35%	–	++
Hide Pure (HP) glues	HP3* (rabbit glue)	<i>Bos taurus</i>	≥ 30	25%	144	+++
	HP4* (rabbit glue)	<i>Bos taurus</i>	≥ 40	~ 10%	146	+++
	HP6** (fish glue)	<i>Bos taurus</i>	≥ 60	25%	157	+++
	HP8** (rabbit glue)	<i>Bos taurus</i>	≥ 50	~ 15%	239	++
Hide Mixed (HM) glues	HM1* (rabbit glue)	<i>Bos taurus/Sus scrofa</i>	≥ 50 (with some fraction at lower molecular weight)	~ 35%	165	++
	HM4** (rabbit glue)	<i>Bos taurus/ Oryctolagus cuniculus</i>	≥ 50 (with traces at lower molecular weight)	~ 20%	126	++
	HM5** (rabbit glue)	<i>Oryctolagus cuniculus/ Sus scrofa</i>	≥ 70 (with traces at lower molecular weight)	~ 25%	156	+
Bone Mixed (BM) glues	BM1* (strong glue)	<i>Bos taurus/ Sus scrofa</i>	~ 25; ~ 60; ≥ 100	~ 60%	207	++
	BM2* (strong glue)	<i>Bos taurus/ Sus scrofa/ Equus asinus</i>	~ 25; ~ 60; ≥ 100	~ 70%	197	++
	BM3** (strong glue)	<i>Bos taurus/ Sus scrofa/ Equus asinus</i>	~ 25; ~ 60; ≥ 100	~ 45%	181	++
	BM4** (strong glue)	<i>Bos taurus/ Sus scrofa/ Equus asinus</i>	~ 25; ~ 60; ≥ 100	~ 30%	214	++
	BM5** (strong glue)	<i>Bos taurus/ Sus scrofa/ Ovis aries/ Equus asinus</i>	~ 25; ~ 60; ≥ 100	~ 85%	174	++

Table 1. Molecular features (taxonomy, MW (kDa) of ACS, backbone cleavage percent, area of DKPs and deamidation degree) of standard collagen and gelatine and animal glue samples. Animal glues’ data are reported from Ntasi et al.⁵⁰. The sample name column shows the abbreviation given according to the tissue of origin, and the original name is given in parentheses. *Sample provided by restoration workshop of the University Suor Orsola Benincasa (Naples). **Sample provided by Museo Nacional del Prado (Madrid). ***+++Corresponds to deamidation % of asparagine>90% and glutamine>60%;++ corresponds to deamidation % of asparagine between 50 and 90% and glutamine between 1 and 30%;+ corresponds to deamidation % of asparagine<30%.

For rheological and XRD analysis, a representative subset of glue samples was selected including HM1 and HM4 among mixed hide samples, HP6 and HP4 among pure hide samples, BM1 and BM5 among bone-derived samples. The solutions for rheological analysis were prepared by dispersing the solid in deionized water in a concentration of 6.6% m/v for 24 h and then heating at 60 °C for 30 min. Subsequently, the samples were centrifuged at 360 rpm for 30 s to eliminate the air microbubbles. This preparation methodology is inspired by the procedure traditionally used for glue preparation in handicrafts, and the choice of the 6.6% m/v concentration is according to Bloom’s degree determination^{6,53,54}.

The glue films for XRD analysis were obtained by brushing the solution (6.6% m/v aqueous solution) on a glass slide for a representative subset of glue samples (HM1, HM4, HP6, HP4, BM1, BM5). The glue films were left to dry for 1 week at room temperature before the analysis. Both the solution and film samples were also analysed by ATR-FTIR.

Thermogravimetric analysis

TG measurements were conducted on collagen, gelatine A and B and on solid glue samples using TA Instruments Thermobalance model Q5000IR, from 25 to 900 °C, at a heating rate of 10 °C/min. The thermogravimetric analysis was carried out under nitrogen flow (25 mL/min). Approximately 5 mg of sample were weighted and put in platinum crucibles. The instrument was mass calibrated using certified mass standards in the range of 0–100 mg and temperature calibrated using five reference materials (Alumel, Ni, Ni83%Co17%, Ni63%Co37%, Ni37%Co63%). TA Universal Analysis 200 ver. 4.5A was employed for data treatment.

Differential scanning calorimetry

DSC analyses were performed using a TA Instruments Discovery DSC model 250 under nitrogen flow (50 mL/min). The instrument was temperature-calibrated with indium. For each sample, about 5 mg was weighed and hermetically sealed into aluminum DSC pans. An empty pan was used as a reference. Collagen, gelatine A and B, and solid glue samples were subjected to a heating/cooling/heating cycle in the temperature range of 10–130 °C at a heating rate of 10 °C/min and a cooling rate of 50 °C/min.

The physical ageing study was carried out on glue samples consisting of one type of collagen belonging to the same animal species, *Bos taurus*, i.e., HP3, HP4, HP6, HP8 samples and on one sample derived from bone tissue, namely BM5. The procedure was the following: the samples were heated/cooled/reheated under the same conditions as described above, and after the second heating scan, the samples were cooled to 40 °C (temperature below the glass transition temperature) at 50 °C/min, held at 40 °C (annealing temperature) for up to 3 h and heated to 130 °C at 10 °C/min. The samples were then cooled to 40 °C at 50 °C/min, held at 40 °C for up to 6 h and heated to 130 °C at 10 °C/min. The sequence was repeated with the sample held at 40 °C for up to 12 h. The same procedure was repeated at an annealing temperature of 30 °C only on HP6 in order to confirm the presence of physical ageing. This procedure was inspired by Farahnaky et al.⁴⁰. For each

calorimetric method used, a baseline was recorded and subtracted from the calorimetric curve of each sample. TRIOS v5.1.1 was employed for data treatment.

Attenuated total reflectance-Fourier transform infrared spectroscopy

ATR-FTIR investigation of the protein secondary structure in the collagen, gelatine A and B and animal glue samples was performed using a Perkin-Elmer Frontier FTIR spectrophotometer, equipped with a universal Attenuated Total Reflectance (ATR) accessory and a triglycine sulphate (TGS) detector. To monitor any changes in the secondary structure during the process of glue dissolution in water and subsequent drying, spectra were recorded on: (1) all commercial glue samples as is; (2) a few microliters of 6.6% m/v solution of a representative subset of glue samples described before; and (3) a representative subset of glue films described before. For the ATR measurements of the solution, a few microliters of 6.6% m/v solution were deposited on the ATR crystal and left to dry for a few seconds. All spectra were recorded in the 4000–600 cm^{-1} range in ATR mode after background acquisition. For each sample, 128 scans were recorded, averaged, and Fourier transformed to generate a spectrum with a nominal resolution of 4 cm^{-1} . Spectrum software (Perkin-Elmer) and a written in-house LabVIEW program for peak fitting were employed to run and process spectra, respectively^{55–57}. A straight baseline passing through the ordinates at 1800 cm^{-1} and 1480 cm^{-1} was subtracted before processing the curves, and the spectra were normalized in the 1700–1600 cm^{-1} region. This approach was taken to avoid artefacts in absorptions near the limits of the region examined. The second derivatives of the amide I band of the spectra examined were then analysed to determine the initial data (number and position of the Gaussian components) required for the deconvolution procedure.

The amide I band was chosen for structural analysis because of the very low contribution of the amino acid side chain absorptions present in this region^{58,59}, and its higher intensity compared to the other amide modes (amide II at 1540 cm^{-1} and amide III between 1350 and 1190 cm^{-1}). Based on the infrared assignment of amide components, assuming that the extinction coefficient is the same for all the secondary structures, the secondary structure composition can be obtained from the FTIR spectra. The percentages of the different secondary structures were estimated by expressing the amplitude value of the bands assigned to each of these structures as a fraction of the total sum of the amplitudes of the amide I components. Although the general validity of the above assumption about extinction coefficients remains to be verified, there are studies in the literature that show good

correlation between the distribution of secondary structures obtained by FTIR and X-ray crystallography approaches, suggesting that the assumption made is reasonable⁵⁶.

X-ray diffraction

X-ray powder diffractograms were collected using a Bruker D8 Venture single-crystal diffractometer equipped with a Photon III CCD area detector, available at Center for Instrument Sharing of the University of Pisa (CISUP), simulating a Gandolfi-like geometry (microfocus CuK α radiation). The analysis was conducted on the representative sub-set of commercial glue samples and films described before to check for the presence of crystalline domains.

Rheological analysis

Rheological measurements on gelatine standards and glue solution samples described before were conducted by Thermo Scientific HAAKE Rheostress 6000 controlled stress rheometer equipped with Peltier temperature control unit. The geometry plate-plate with 35 mm diameter, and a gap of 0.3 mm was used. In steady state condition the viscosity flow curves were obtained by rotational measurements in which the shear rate ($\dot{\gamma}$) was varied in the range 1–100 s⁻¹ and the shear stress (τ , Pa) was recorded. The range of shear rate investigated was chosen considering a manual application of the glue, by brush or spatula, which is the commonly used method for applying these samples. The viscosity (η , Pa s) was obtained by the ratio between τ and $\dot{\gamma}$. Temperature sweep test was performed varying the temperature in the 15–40 °C range at shear stress of 0.1 Pa and frequency of 1 Hz (values within the linear viscoelastic range). The presence of a gel point can be determined by the presence of a cross-over point between storage modulus (G' , parameter proportional to the elastic/solid component of the material) and loss modulus (G'' , parameter proportional to the viscous/liquid component of the material). RheoWin DataManager software was employed for data treatment.

Results

Thermogravimetric analysis

The thermal degradation of collagen, gelatine standards and glue samples was investigated by TGA under nitrogen flow. Figure 2 shows thermogravimetric (TG), and differential thermogravimetric (DTG) curves obtained for all samples. All the samples exhibit the same thermal degradation profile, which primarily consists of two steps. The first mass loss (10–12% w/w) below 60 °C is due to moisture evaporation and the second (65–75% w/w) centered at 300–320 °C is due to protein pyrolysis resulting in the formation of 2,5-diketopiperazines (DKP), the most abundant of which in the glue samples are cyclo(Pro-Gly) and cyclo(Pro-HyP)₅₀ and of several aromatic and nitrogen-containing compounds, such as pyrrole, indole, phenol, and the respective alkylated compounds.^{60,61} The residue at 900 °C is in the range of 15–20% w/w, as generally reported in literature for proteins. Table S1 shows the percentage mass loss and the corresponding temperatures determined for each sample.

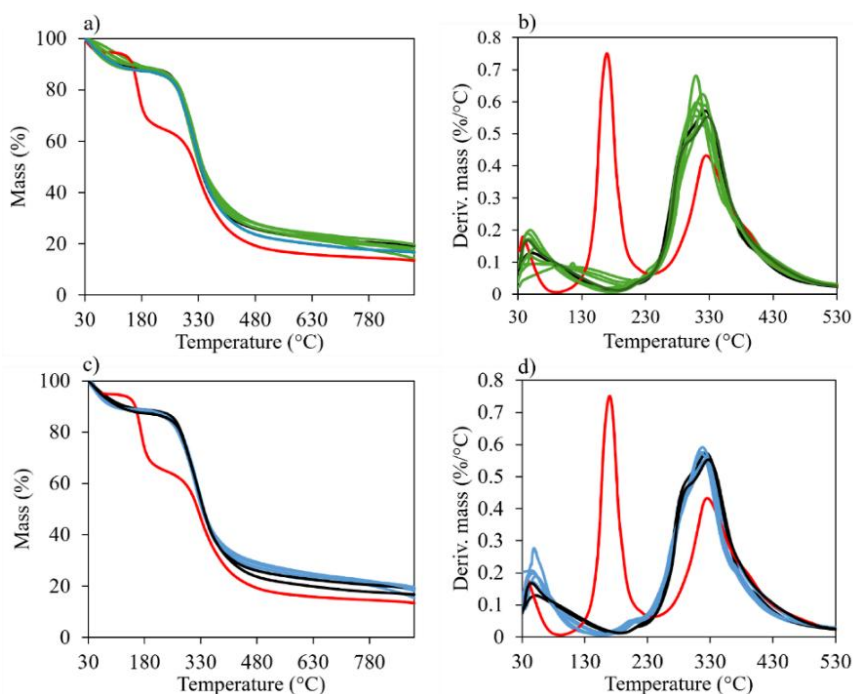


Figure 2. TG (a, c) and DTG (b, d) curves of collagen (red line), gelatine A and gelatine B (black line), and glue samples (green line for HP and HM samples, blue line for BM samples) obtained under 25 mL/min nitrogen flow and using 10 °C/min as heating rate.

Standard collagen showed an additional degradation step below 200 °C. As reported in the literature, this is due to the evaporation of the strongly H-bonded water involved in the stabilization of the triple helix⁶²⁻⁶⁴. By observing the shape of the DTG related to water loss, gelatine standards exhibited a broader signal over a wide temperature range (from 30 to 200 °C) indicating the presence of structural water. The DTG curves of glue samples suggest that HP3, HP6 and HM5 contain more structural water than the standard gelatine, while the other samples have little to none. Different degrees of hydrolysis of collagen and the different animal species can affect the thermal degradation^{50, 65-67}.

By considering the t_{endset} and t_{onset} (Table S2), a slight difference was observed for gelatine standards, as gelatine A has t_{onset} higher than gelatine B, indicating higher thermal stability, but t_{endset} lower than gelatine B, i.e., a lower amount of thermostable cross-linked/aggregated structures. This result agrees with the literature and depends on the different production processes used. Gelatine A undergoes an acid pretreatment, whereas gelatine B undergoes a basic pretreatment. This leads to gelatine with different properties and, in general, gelatine A is less covalently cross-linked than gelatine B⁶⁸, probably due to the protonation of groups involved in crosslinking formation.

A slight shift of the maximum towards lower temperatures was observed for animal glue samples, suggesting the presence of more hydrolyzed molecules compared to gelatine and collagen. Furthermore, looking at the t_{endset} in Table S4, BM samples completed their degradation at a lower temperature than the other samples. This result further suggests a higher degree of hydrolysis, a lower degree of aggregation and the presence of molecules with a lower molecular weight in BM samples, as reported by Ntasi et al.⁵⁰, who identified highly hydrolyzed collagen in BM samples through SDS-PAGE, backbone cleavage percentage and Py-GC/MS analysis (Table 1).

Another factor to consider is the percentage of deamidation. Notably, hide glues exhibit higher levels of deamidation compared to bone glues⁵⁰. This increased deamidation can result in a higher number of charges, potentially leading to the formation of more thermally stable aggregates.

Regarding the HP and HM samples, no clear difference in thermal degradation was observed. Both HP and HM samples have a t_{onset} lower than that of collagen and gelatine and a t_{endset} lower than collagen but equal to or higher than that of gelatine, indicating the presence of more hydrolyzed molecules as well as thermostable aggregates.

Differential scanning calorimetry

Calorimetric analysis was performed on glue samples to obtain information on the thermal stability of the protein structures and to compare them with those of gelatine and collagen standards. The calorimetric curves obtained are reported in Fig. 3. The glass transition temperature (t_g) and associated heat capacity change (ΔC_p) determined from the first and second heating scans, onset temperature (t_{onset}), peak temperature (t_{peak}), width signal expressed as the difference between the temperature at the start and end of the event (Δt), and enthalpy (ΔH) calculated for the two signals observed in the first heating scan only are reported in Table S3.

The calorimetric curve of the collagen standard showed in the first heating scan a broad endothermic signal between 36 and 121 °C with a maximum at 82 °C accounting for 18 J/g as enthalpy value and ascribable to collagen unfolding⁶⁹. In the second heating scan, the only signal observed was the glass transition occurring at almost 0 °C. This result is not expected, as the literature reports a glass transition temperature for tendon collagen of around 40–45 °C.^{70,71} No information is provided about the processes undergone by standard collagen, but this difference may suggest that standard collagen is not in its native form and has undergone a treatment that partially altered its structure, as also observed in the secondary structure analysis. The unfolding enthalpy of collagen reported in the literature is in the range 40–50 J/g.^{70,71} Our lower value could be due to a partial denaturation of collagen, as indicated by the β -sheets content. Considering the proximity of β -sheets and native collagen helix in the Ramachandran plot⁷², collagen is unlikely to return to its native shape, but rather it could give rise to β -sheet structures, which are notoriously very stable.

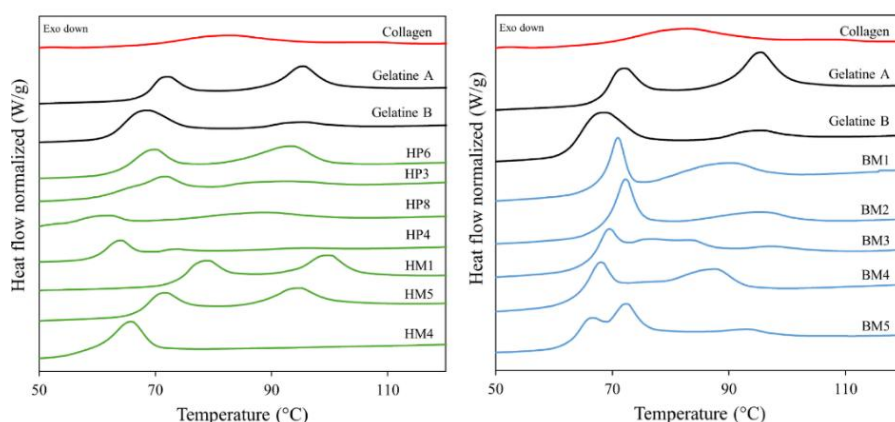


Figure 3. DSC curves obtained during the first heating scan at 10 °C/min of collagen (red line), gelatine A and B (black line), hide-derived glues (green line), bone-derived glues (blue line).

The calorimetric curve of both gelatine A and B showed two distinct endothermic signals during the first heating scan. The first signal (6 J/g and 12 J/g, respectively) at about 70 °C is an overshooting overlapped to the glass transition. The second signal (11 J/g and 3 J/g, respectively) at 95 °C corresponds to the unfolding of the gelatine structure, which is associated with the degree of renaturation of the gelatine.^{25,71} The results indicate that gelatine A exhibits a higher degree of renaturation compared to gelatine B. This difference may be attributed to variations in their covalent cross-linking levels, as well as the conditions (temperature and humidity) during the renaturation process. The presence of a higher number of covalent bonds in gelatine B may hinder the reorganization, resulting in a lower degree of renaturation. The calorimetric profiles of all glue samples in the first heating scan were similar to gelatine more than collagen, displaying two endothermic signals: the first in the temperature range 50–80 °C, overlapping the glass transition, the second, in the temperature range 80–110 °C (Fig. 3). In the second heating scan all the analysed samples exhibited only the signal related to glass transition (Figure S1 in Supplementary material). The signal superimposed to the glass transition was better investigated in order to confirm it to be associated with structural relaxation associated with physical ageing of the sample as reported in literature for synthetic polymers, some proteins and gelatines.^{25,40,73,74}

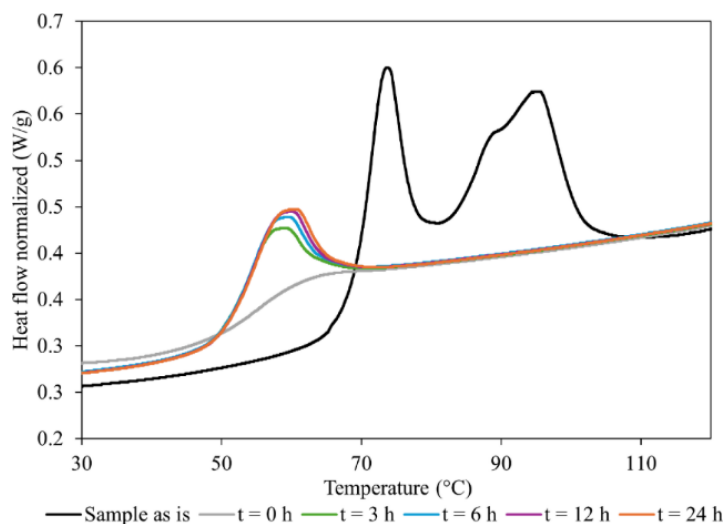


Figure 4. DSC curves of HP6 before and after physical ageing: black line: first heating at 10 °C/min from 10 to 130 °C; grey line: second heating at 10 °C/min from 10 to 130 °C; green line: third heating at 10 °C/min from 10 to 130 °C carried out after 3 h of isothermal; purple line: fifth heating at 10 °C/min from 10 to 130 °C carried out after 12 h of isothermal at 40 °C; orange line: fifth heating at 10 °C/min from 10 to 130 °C carried out after 24 h of isothermal at 40 °C.

The study of physical ageing was performed on glue samples of the same animal origin (HP3, HP4, HP6, HP8) to reduce the variability associated with different animal sources and on one bone glue sample (BM5) to confirm the presence of this phenomenon also in the bone glues. To study the physical ageing, after the first heating up to 130 °C, the samples were kept at 40 °C (temperature below the T_g) for different periods of time (3 h, 6 h, 12 h, 24 h) as specified in the experimental section and heated to 130 °C in order to observe the enthalpy relaxation signal. Since the extent of physical ageing depends on the annealing temperature, the experiment was repeated on sample HP6 also at the annealing temperature of 30 °C. Specifically, as the annealing temperature increases, both the peak temperature and the relaxation enthalpy value also increase.^{40,41,75} The values of the enthalpy relaxation and the peak temperature calculated at each time point are reported in the table S4. Figure 4 reports the DSC curves obtained for HP6 as example.

The characteristics of this relaxation peak are compatible with the physical ageing phenomenon occurring in the glues, as described in the literature⁴⁰: the signal intensity increases over time and the peak temperature increases linearly with the logarithm of the ageing time (Fig. 5). The same behavior was observed for BM5 (Table S4).

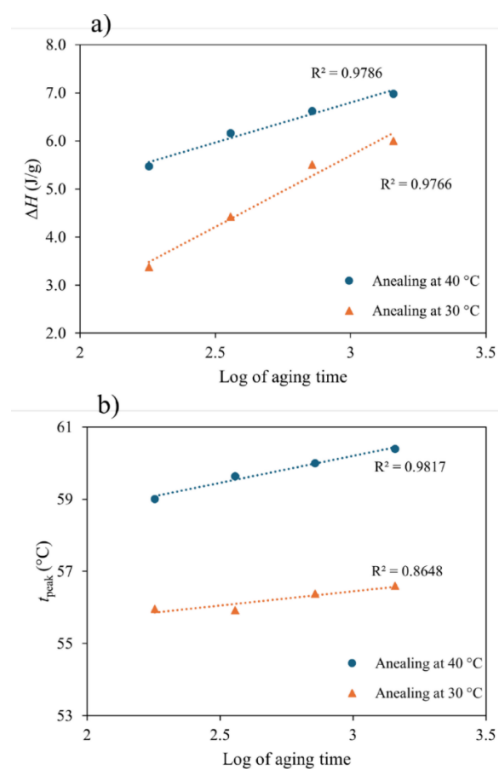


Figure 5. Dependence of enthalpy (a) and peak temperature (b) on the logarithm of the ageing time (min) for HP 6 sample aged at 30 °C (orange triangle) and 40 °C (blue circular dot).

Regarding the glass transition, when applied to proteins, this term refers to the change in the dynamic properties of proteins that takes place at a specific temperature. It has been reported in the literature that at temperatures above the glass transition the dynamics of protein molecules are dominated by large scale motions of groups of atoms, whereas at lower temperatures vibrational motions predominate. The temperature at which this change in molecular dynamics occurs is influenced by the degree of hydration of the protein and the intrinsic temperature dependence of the motions.⁷⁶ All the glue samples have the same moisture content (around 10%, as proved by TGA data reported below) and the t_g values, obtained from the first heating scan, range from 52 °C (i.e., HP8) to 73 °C. (i.e., HM1), with the gelatine A and B values almost in the middle at 62 °C and 68 °C, respectively, without any clear correlation with the animal source (Figure S1 and Table S3). In general, t_g is lower in the second heating scan compared to the first one, except for HP4 and HP8, which exhibited nearly the same temperature. As reported in ref³⁴ and by Tseretely and Smirnova⁷⁷, this is due to the release of bounded water during the denaturation process, which acts as a plasticizer by lowering the t_g . The values of the heat capacity change (ΔC_p) associated with the glass transition, are in the range 0.65–1.3 J/ Kg and 0.53–1.3 J/Kg calculated from the first and second heating scan, respectively. These values agree with the data reported in literature on amorphous gelatines and disordered crystalline gelatines⁷⁶ and they are slightly higher than those reported for other proteins.^{32,43,78} All the glues (both from hide and from bone) show the relaxation enthalpy peak superimposed to the glass transition with values varying between 5 (i.e., HP4 and HP8) and 11 J/g (i.e., HM4). The value obtained for gelatine is 6 J/g as well as HM5 (Table S3a). For the collagen standard, as well as BM3, BM4, and BM5 samples, it was not possible to determine the relaxation enthalpy associated with the glass transition due to the signal overlapping with other signals at higher temperatures. This signal is often overlooked and there are few studies in which the enthalpy relaxation has been determined in gelatinous samples. Enrione et al.⁴⁸, determined the enthalpy associated with physical ageing of fish gelatine film and obtained a value of 2.42 J/g after an ageing of 40 h at annealing temperature 5 °C lower than t_g , i.e., 29 °C. The higher enthalpy relaxation values of the glue samples obtained in this study could be due to the annealing temperature used, which was 20–30 °C lower than t_g . In these conditions, the enthalpy relaxation can be higher because the sample is far away from equilibrium state. The unfolding peak in the hide glues is well separated in temperature from the relaxation enthalpy peak with a t_g peak varying between 89 °C (i.e., HP8) and 100 °C (i.e., HM1) and enthalpy values varying between 3 (i.e., HP3) and 11 J/g (i.e., gelatine and HP6), and almost

absent in HP4 and HM4. These values are lower than those obtained for collagen, indicating a lower structural order due to the higher degree of hydrolysis and lower molecular weight of the gelatine molecules that hinder renaturation.⁷⁷ For bone glues it is not possible to determine separate values for the relaxation enthalpy and for the unfolding one, because all the DSC peaks are partially superimposed.

Secondary structure of gelatine in animal glue samples

ATR-FTIR analysis was carried out on commercial animal glue samples, collagen, gelatine A and B, solutions and films of the representative sub-set described in “Materials and Methods section”. (HM1, HM4, HP6, HP4, BM1, BM5), to investigate the protein secondary structures. Figure S2, S3 and S4 shows the FTIR spectra obtained for all commercial samples, for the sub-set solutions and for the sub-set films. Spectra display the typical features of protein materials, i.e., the amide I band in the 1700–1600 cm^{-1} region (corresponding to C=O stretching vibration), the amide II in the 1600–1480 cm^{-1} region (corresponding to out-of-phase combination of the NH in plane bend and the CN stretching vibration) and the amide III in the 1350–1190 cm^{-1} region (corresponding to in-phase combination of the NH bending and the CN stretching vibration).¹⁶ The amide I frequency is strongly affected by the protein conformation¹⁶, consequently the amide I shift observed in the animal glue samples clearly indicates a change in the protein structure. To gain a deeper understanding of the gelatine conformation, a curve-fitting method was applied to deconvolve the complex amide I band, allowing for the determination of the different percentages of secondary structures.^{55–57} Characteristic components of the Amide I band are assigned to the following secondary structures: helix (1650–1660 cm^{-1}), random (1637–1660 cm^{-1}), collagen native triple helix (1660–1666 cm^{-1}), antiparallel β sheets (two components around 1620 cm^{-1} and 1690 cm^{-1}), parallel β -sheets (one component around 1630 cm^{-1}), β -turns (1670–1685 cm^{-1})⁵⁹. Table 2, Table S5 and Table S6 show the secondary structure percentages, the wavenumber in cm^{-1} and half height bandwidth (HHBW), based on the results of the peak fitting of Amide I band, of all the commercial samples (Table 2), of the sub-set solutions (Table S5), and of the sub-set films (Table S6).

Figure 6 compares the data of secondary structure of all glue samples of the sub-set and the reference gelatine A and B. The assignment of the bands assigned to helix and random coil structures is challenging, as the bands due to these structures overlap in these kinds of samples because of the wide bandwidth of the band assigned to random structure and the

likely low percentage of helix (Table 2). Thus, it was not possible to discriminate between these two structures in all glue samples.

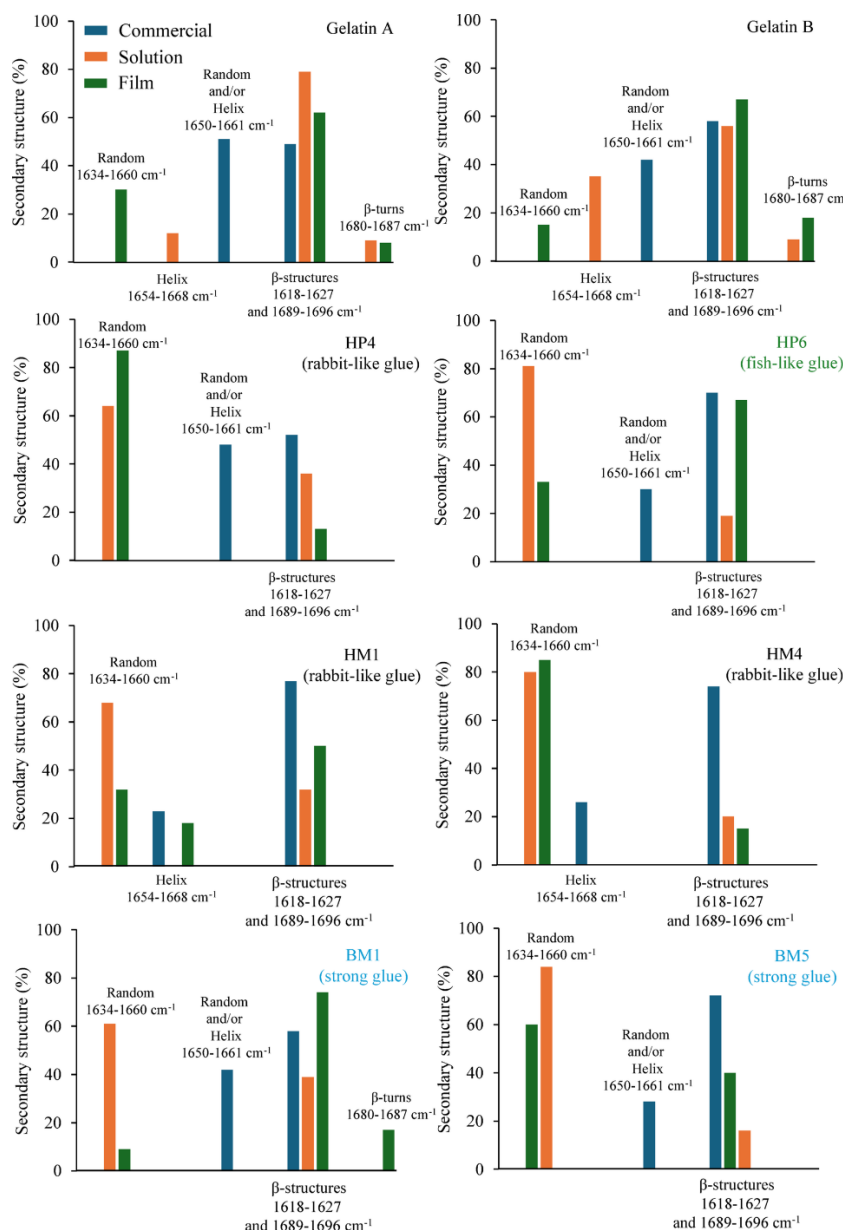


Figure 6. Secondary structures (%) of glue sub-set (gelatine A, gelatine B, HM1, HM4, HP6, HP4, BM1, BM5) of commercial glue samples (orange), solution glue samples (blue), and of film glue samples (green).

The assignment to β-sheet structures in Table 2 includes parallel and antiparallel β-sheets and β-turn.^{59,79} The results obtained on the standard of collagen indicate that the collagen is not in its native form, as a significant amount of β-structures are present. The same results can be observed for gelatine standards. Looking at the results shown in Fig. 6,

the secondary structures of gelatine A and B samples undergoes similar changes after the dissolution and 7 day drying process, and they remain predominantly random/helix and β -sheet structures. The analysis of gelatine A and B solutions on ATR crystal show a certain percentage of helix (10–35%). All solid glue samples are predominantly β -sheet and helix/random structures. Once dissolved in water, the random structure increases and their structural order decrease. After 7 days drying the “strong glues” BM1 and BM5 recover more than 50% of β -sheets respect to the initial β -sheets content (BM1=128% and BM5=56%). “Rabbit like-glues” HM1, HM4 and HP4 recover less than 60% of β -sheets, whereas “fish like-glue” HP6 recover about 100%. XRD measurements were performed on a subset of solid glues before and after dissolution in water and drying to check for the possible presence of crystalline domains (Figure S5). The XRD diffractograms are characterized by a broad characteristic peak, more or less visible, around $2\theta=8.0^\circ$ and another around 20° , 20, 26, 80, resulting in agreement with those reported in the literature for gelatins. However, these profiles are suggestive of low-order material where the amorphous component complicates the univocal interpretation of the XRD spectra.

Bond strength, viscosity and viscoelastic properties of animal glue samples

The rheological behavior of animal glue samples during the laying and application steps were studied by recording the steady-state flow curve and temperature sweep. Figure 7 and Figure S6 show the flow curves and the trend of G' and G'' as temperature changes, respectively.

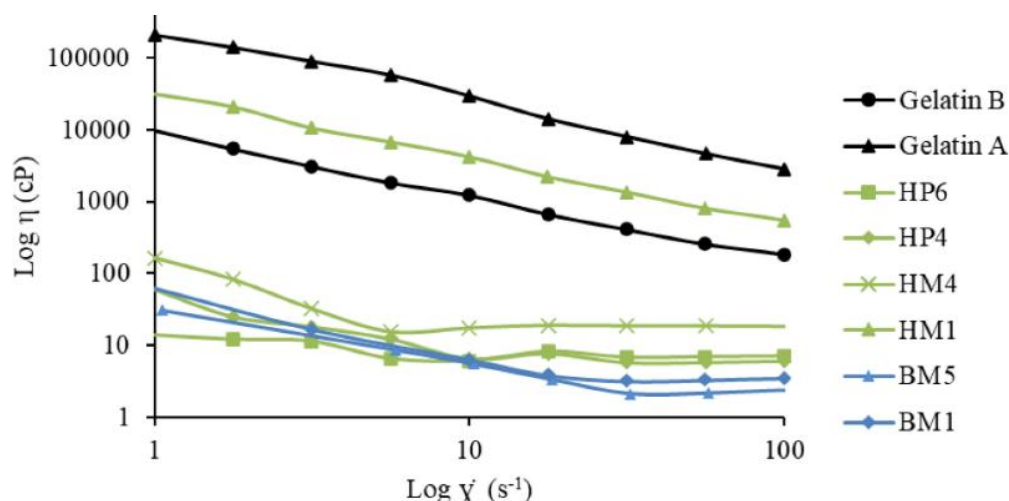


Figure 7. Flow curves of Gelatine A, Gelatine B, HP4, HP6, HM1, HM4, BM1, and BM5 obtained by rotational measurements in steady state, varying the shear rate between 1 and 100 s^{-1} . The graph is plotted on a logarithmic scale for both axes.

The results show higher viscosity and pseudoplastic behavior for both standards gelatine and HM1 glue. The other samples have viscosity lower than gelatines and HM1 and they show a decrease in viscosity in the shear rate range from 1 s^{-1} to about 10 s^{-1} . In contrast, for shear rates above 10 s^{-1} they exhibit Newtonian behavior. The different behaviors observed may be due to the difference in molecular weight of the gelatines. Higher viscosity and pseudoplastic behavior are indicative of the presence of molecules with longer chains that can form stronger and more robust polymer networks. From the curves obtained from the temperature sweep measurements (Figure S3), it results that sample BM5 behaves like a liquid over the entire temperature range investigated, as the loss modulus is always higher than the storage modulus. For the other samples, it is possible to identify the temperature at which the sol–gel transition occurs. Gelatine A and HM1 have a gelation temperature (32°C) higher than the other samples. Gelatine B gel has a gelation temperature at 29°C and following a decreasing order we find HM4, HP6, BM1 and HP4 (Table 3). Gelling temperature is a key parameter in the use of glues because it determines the transition between the liquid and gelled state of the material. This has a direct impact on viscosity, which in turn affects both ease of application and adhesive performance. The temperature at which gelation occurs depends on the molecular weight. Gelatins with higher molecular weights exhibit a higher gelation temperature. Another important parameter for evaluating the performance of a glue is the shear resistance. An indication of this parameter can be provided by observing the value of the storage modulus (G'). Specifically, considering 25°C as the reference temperature, glues with higher G' value will have higher shear resistance. The data show that gelatine A has the highest shear resistance compared to the others, followed by HM1, gelatine B, and HM4. HP4, HP6, BM1 and BM5 have a very low G' value, thus showing very low shear resistance (Table 3). Regarding bond strength, in the case of gelatine, this is referred to the Bloom's degree, which is the measure of gel strength of a 6.6% m/v solution^{6,53,54}, and is related to the length and degree of cross-linking of the chains. Generally, the higher the MW of the gelatine the higher the degree of Bloom. High Bloom degree gelatines are characterized by having a high gelation temperature, a high G' shear modulus, and higher viscosity. Therefore, considering the rheological data obtained, we can classify HM1 as a high Bloom degree glue, while all others as low Bloom degree glues.

Discussion And Conclusion

TGA data clearly indicated that glue samples are more similar to gelatine than to the reference collagen. Therefore, they do not show the degradation step at around 200 °C that is due to the evaporation of the strongly H-bonded water involved in the stabilization of the triple helix.^{62–64} In addition, they present an unfolding enthalpy that is less than half of that of collagen. HP and HM samples are less hydrolyzed than BM samples, having an average MW above 40 KDa (Table 1). HP4 and HM4 appear to be the glue samples with the lowest degree of structural order given their low enthalpy value (about 1 J/g). This is likely due to the conditions used during the drying process after extraction, the storage conditions and the possible presence of covalent cross-linking preventing renaturation. BM3, BM4, and BM5 have exhibited a calorimetric curve significantly different from the other samples. Their signal consists of several overlapping signals, likely due to the higher degree of hydrolysis in the gelatine, as it was found by Ntasi et al.⁵⁰. This suggests that a well-defined three-dimensional network is unlikely to form, but rather several aggregates with lower structural order. In contrast, BM1 and BM2 displayed a denaturation signal similar to that of HP and HM samples, indicating a higher degree of renaturation compared to the other BM samples. Rheological results obtained revealed that HM1 showed a high degree of Bloom. A glue with a high degree of Bloom is more difficult to apply uniformly, tends to form stiffer films with higher cohesion, which may improve the strength of the glue once dry, but may reduce the initial adhesiveness compared to glue with a lower degree of Bloom. HM1 is confirmed to have a “rabbit glue” behavior and it is commonly used in veiling restoration (Fig. 1b). A lower degree of Bloom leads to a less viscous and more fluid glue, which promotes better penetration into porous materials and thus more effective adhesion on some surfaces. Glues with a low degree of Bloom can form more flexible films, which is useful for applications in which the glue must retain some elasticity after drying. The BM samples with a low Bloom’s degree are “strong glue” employed in carpentry (Fig. 1c,d). Another key observation in this study is that the physical ageing characterizes all the glue samples analysed, regardless of animal origin or tissue type. As reported in the literature, the enthalpy relaxation value is influenced by storage conditions, including temperature and humidity. Since the glass transition temperature is higher than room temperature, physical aging will inevitably occur, and because it affects the mechanical properties of the material, it should be considered when animal glues are used. XRD data collected on a sub-set of glue samples investigated in this work, both before and after dissolution in water show profiles in agreement with the XRD diffractograms reported in literature for the animal glues^{20,26,80}.

These profiles are suggestive of a low-order material where the amorphous component complicates the univocal interpretation of the spectra. Thus, XRD measurements, as well as FTIR analysis, make objectively impossible to discriminate the helical component from the random one.

FTIR results revealed a relative high content of β -structures in all commercial glue samples, gelatine and collagen. This highlights that it is extremely difficult to obtain native collagen after the extraction process from tissue and it explains the low denaturation enthalpy of collagen. Moreover, spectra of the glue films dried for 1 week at room temperature have shown that the secondary structure tends to return to the structure of the solid glue before the dissolution process. It is important to highlight that, under the drying conditions used, gelatine molecules assemble into β -sheet structures rather than α -helix. Probably, under these conditions, the formation of hydrogen bonds is favored, which guide the reorganization into β -sheet. In fact, in the literature, many studies reported on the effect of the drying conditions on the structural reorganization of gelatine protein.^{23–26} Saiani et al.⁸¹ reported that the type of surface on which the peptide dries can also induce one type of secondary structure rather than another.

The percentage of β -structure recovery partially correlates with the adhesive behavior of the glues studied. In fact, “strong glues” (BM1 and BM5), characterized by a very short setting time, show a very high degree of β -structure recovery after 1 week of drying, while “rabbit-like glues” (HM1, HM4 and HP4) show a lower one, with some variability within the subset. “Fish-like glue” (HP6) recovers almost as well as a strong glue. This behavior suggests that the presence of β -structures has an influence on adhesiveness, but other factors play a role in the adhesive and mechanical behavior of a gelatine glue such as molecular heterogeneity mainly due to the processing history. The gelatine adhesives analysed are inherently polydisperse mixtures, containing a range of fragments including α -, β -, and γ -chains, as well as smaller peptides.⁴⁷ This work and the work of Ntasi et al.⁵⁰ show that these samples have different degrees of hydrolysis and different degrees of deamidation, resulting in different molecular weights and charge distributions. Thus, the structural and mechanical characterization could be specific to each gelatine depending on its manufacturing conditions.

The presence of β -sheet structures in the examined glues could contribute significantly to their mechanical properties, as it is reported in the literature that β -sheet protein structures thanks to the architecture of their hydrogen bonds give molecular assemblies

characterized by extraordinary strength and unique properties. The zig-zag pattern, stabilized by hydrogen bonds is further stabilized by hydrophobic interactions between sheets and are held together by van der Waals interactions.^{82–84} These structures are known for their high resistance to rupture and high strength (e.g., in silk exhibiting high tensile strength and in amyloid fibrils forming rigid, cohesive structures)^{85–87}. Moreover, in gelatine-electrospun materials was observed that the stiffness increases with the increasing of β -sheet content.³⁰ Although the structural analysis of gelatine in the literature is focused on the evaluation of the triple helix content using X-ray diffraction, supported by DSC results^{21,23,25,26,48,78,88} and structure property studies link the mechanical properties of glues to the triple helix content^{21,23,26}, we believe that in animal glues, it is crucial to consider not only the triple-helix-like structures but also the amount of β -sheet structures to explain the structure-dependent mechanical properties and the adhesive mechanism.

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Author Contributions

E.P., C.D. and E.B. conceptualization; E.P. investigation; E.P., G.T., L.C. data curation; E.P. Writing—Original Draft; C.D., E.B., L.B., B.C., L. D. I., G.T., L.C. and I.L. Writing—Review & Editing; C.D., E.B., and I.B. Supervision; C.D., E.B., L.B. and I.B. Funding acquisition; L.B and L. D. I., Resources. More specifically B.C. and L.B. performed the proteomics analysis.

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5.3 From waste to function: valorization of collagen-based wastes with Natural Deep Eutectic Solvents for bioadhesive applications

C. Pelosi, E. Micheli, E. Pulidori, G. Caroti, B. Cipolletta, B. Campanella, I. Corsi, S. Pizzimenti, L. Birolo, I. Bonaduce, C. Duce and E. Bramanti.

In this study, we propose a novel, environmentally, economically, and energetically sustainable approach for the valorization of vegetable-tanned leather waste, aiming at producing bio-based adhesives with antibacterial properties and promising water resistance. The treatment of leather scraps was carried out using various Natural Deep Eutectic Solvents (NADES) in mild conditions (1h, 60°C), i.e., lactic acid:urea in molar ratio 2:1, choline chloride: lactic acid in molar ratio 1:1, and choline chloride dehydrated oxalic acid in molar ratio 1:1. The resulting bioadhesives exhibited excellent binding performances, in particular on wood substrates. Structural modifications and its thermal behavior of collagen after the treatment were investigated using Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (ATR-FTIR), Thermogravimetric Analysis (TGA and TGA/FTIR), Evolved Gas Analysis–Mass Spectrometry (EGA-MS), Pyrolysis – Gas Chromatography – Mass Spectrometry (Py-GC-MS), and proteomic techniques. Overall, this approach highlights a circular and green strategy for upcycling leather industry by-products into high-performance materials, aligning with current goals in waste minimization and resource efficiency.

Introduction

Leather, traditionally produced processing animal hides, is recognized as one of the earliest materials utilized by humans.¹ However, its production (which currently has an estimated annual revenue of USD 50 billion) poses significant environmental challenges, including the management of the solid waste generated.² Collagen, a fibrillar protein abundantly present in the solid wastes,^{2,3} Collagen can be found in several forms - 29 distinct types have been identified so far. Type-I collagen (composed of three polypeptide chains with a triple helical structure) is the most abundant form. The helices are characterized by repeating Gly-X-Y sequences, where the X and Y positions are mainly proline and hydroxyproline residues, respectively.^{4,5}

Numerous studies in the literature report the valorization of collagen for applications in various fields, including food, pharmaceutical, cosmetic, or biomedical industries.^{4,6–9} Despite this potential, the recovery and reuse of collagen from the leather industry residues

is complicated by the fact that the biomacromolecule has previously undergone a tanning process, resulting in its stabilization through cross-linking. Different tanning methods are available on the market, depending on the quality of the leather required by consumers, mainly divided into mineral tanning (made with chromium salts, which is the most common, or aluminum, iron, or zinc salts), and organic tanning (including vegetable, aldehyde, or oil tanning^{10,11}). The vegetable tanning is based on the formation of multi-hydrogen links between the polyphenols extracted from different vegetable matrices (e.g., chestnut, mimosa, and quebracho tree) and collagen, making the latter non-biodegradable and resistant to high temperature.¹²

While the recovery of collagen from chromium-tanned leather is particularly challenging due to the strong coordination bonds formed with chromium(III), an interesting approach, so far unexplored in the scientific literature, is the sustainable valorization of solid wastes deriving from the vegetable-tanning industry. In this paper, we present an innovative green treatment of vegetable-tanned leather waste using three different Natural Deep Eutectic Solvents (NADESs), leading to the formation of bioadhesives.

Deep eutectic solvents (DESs) constitute a class of fluids that have garnered significant attention due to their wide-ranging potential applications in various technological fields, e.g., materials development,¹³ gas storage,^{14,15} polymer science,^{16,17} biomass extraction,^{18–20} electroplating,^{21,22} and separation techniques.²³ They are usually prepared by combining two or more components forming an eutectic mixture whose melting point is lower than what would be expected if the mixture had an ideal behavior in the liquid phase.²⁴ They are considered a platform of green solvents, which properties can be adjusted according to the specific purpose, e.g. varying the starting components or with the addition of a cosolvent.^{25,26} Natural deep eutectic solvents (NADESs) are of particular interest, as they are formulated entirely from natural, biodegradable components. These solvents offer advantages such as low toxicity and reduced production costs, making them highly suitable for implementation within the framework of sustainable chemistry.²³

In this study, we propose the use of: i) lactic acid: urea in a molar ratio of 2:1 (namely DES1); ii) choline chloride: lactic acid in a molar ratio of 1:1 (namely DES2); iii) choline chloride: dehydrated oxalic acid in a molar ratio of 1:1 (namely DES3), already classified in the literature as natural deep eutectic solvents (NADESs),^{27–30} to extract collagen from vegetable-tanned leather waste. The constituents of the NADESs are cheap, biodegradable, and non-toxic, thus considered suitable for environment-friendly processes. Moreover, they

have been recently proposed for collagen extraction from untanned fish skin, and for the solubilization of native collagen,^{31–33} making them promising for our purposes.

More in detail, Abdallah et al. have employed DESs based on urea (U) and lactic acid (LA) for the extraction of bioactive compounds from marine by-products, which are typically discarded in large quantities.³¹ Similarly, Bisht et al. have utilized an aqueous 0.75 M solution of LA and U in a 2:1 molar ratio to extract type I collagen from the skin of Atlantic cod.³⁴ The system has demonstrated high efficiency in extracting bioactive compounds from plant-based matrices under ultrasound-assisted conditions.²⁹ Hamdi et al. developed a method for isolating type I collagen from seabass fish scales, preserving the native collagen's secondary and triple helical structures, utilizing a ChCl: LA-based system-assisted ultrasonication (US) technical route.³⁵ In another study, the authors monitored collagen solubilization at different temperatures (45 °C, 70 °C, and 90 °C), followed by regeneration at 4 °C using ethanol.³² DES2 has been applied to other valuable processes, including the extraction of lignin and polyphenols.^{30,36} ChCl:AO, i.e., the DES3 proposed in this study, has been reported in the literature for a variety of applications,^{18,37–40} including the extraction of approximately 50% high-purity collagen peptides from cod skin (ChCl:AO (1:1) at 45 °C).³³

In the present paper, the treatment of vegetable tanned leather with DESs1-3 was conducted under mild experimental conditions (60 °C for 1 hour), following the main principles of green chemistry: the biomass treated was a waste material furnished by a local industry, which was fully converted into a bioadhesive using green solvents. Only the leather treated with DES1 and DES2 produced bioadhesives with good adhesive properties. The resulting materials were successfully employed as bio-based glue as-is or treated in an acidic or basic environment to explore the pH effect on their properties. The glue strength on steel-wood surfaces was also quantified by the tack-test using a rheometer.

A unique characteristic of the bioadhesives proposed in this study is that, unlike the adhesives reported in the literature,^{41–46} they inherently contain tannins that enhance their moisture resistance⁴⁷ making them particularly promising for bonding organic surfaces (such as paper-to-paper or wood-to-wood) under high-humidity conditions. Moreover, tannins are naturally occurring polyphenols that possess antimicrobial properties.^{47–49}

To understand how treatment with NADESs affected collagen at the molecular level, and how the protein chemical and structural changes influenced their adhesive properties, the biomaterials obtained were studied by Fourier-Transformed Infrared Spectroscopy (FTIR), Thermogravimetric Analysis (TGA) coupled with FTIR spectroscopy of evolved

gases, Evolved Gas Analysis with Mass spectrometry (EGA-MS), pyrolysis – Gas Chromatography – Mass Spectrometry (Py-GC-MS), and Proteomic (LC-MSMS) analysis. The collagen extracted was compared with the collagen present in the original vegetable-tanned leather to assess the molecular-level modifications induced by the NADES treatments.

Overall, the results obtained demonstrate how an innovative and green process, which employs NADESs, can valorize waste from the leather industry to produce biomaterials with adhesive properties, in line with the principles of circular economy.

Material And Methods

Materials

Vegetable-tanned calf split leather (containing tannins, e.g., mimosa, quebracho, and chestnut extract) was gently provided by *Conceria Zabri* (Fucecchio, Italy). Two commercial animal glues, BM5 and HP3 were sourced from the Museo Nacional del Prado (Madrid) and from the restoration workshop of the University Suor Orsola Benincasa (Naples). Collagen from calf skin (CAS-No: 9007-34-5, purity 99.9%) was obtained from Sigma-Aldrich. Choline chloride (CAS-No: 67-48-1, ChCl, purity 98%) was purchased by Thermo Scientific and used as a component of the eutectic solvents after drying at approximately 100 °C under vacuum for 2 hours. Urea (CAS-No: 57-13-6, U, purity 99%) was purchased by ChemCruz. Lactic acid (CAS-No: 50-21-5, LA, purity 98%) was obtained by Carlo Erba. Sodium hydroxide (CAS-No: 1310-73-2, NaOH, ACS reagent, reag. Ph. Eur., ≥98%, pellets) and Hydrochloric acid (CAS-No: 7647-01-0, HCl, ACS reagent, reag. ISO, reag. Ph. Eur., ≥ 37%) were purchased by Sigma-Aldrich. Dehydrated oxalic (CAS-No: 6153-56-6, OA. Purity 98%) acid was obtained by Materiamadre. Unless otherwise specified, chemicals were purchased and used without further purification.

Preparation of Deep Eutectic Solvents (DESs)

The DES selected for the treatment of tanned leather are described in **Table 1**.

All the systems were prepared by mixing the components at 50 °C and stirring them for 20 minutes (DES1) or 1 h (DES2 and DES3). Samples were stored at room temperature and used directly as solvents for the treatment of vegetable-tanned leather, without any further processing.

System name	Components	Molar ratios
DES1	Lactic acid: urea	2:1
DES2	Choline chloride: lactic acid	1:1
DES3	Choline chloride: dihydrated oxalic acid	1:1

Table 1. DESs systems prepared and used in the treatment of tanned leather.

Mild treatment of vegetable-tanned leather waste

For our purposes, vegetable-tanned leather was grated (**Fig. S1A-B**) using a commercial grater with an approximate pore size of 0.5 mm, and then treated with DES1, DES2, and DES3 (**Fig. 1**) using 100 mg of leather in 2 mL of DES. The mixture was kept at 60 °C for 1 hour under magnetic stirring. Subsequently, 1 mL of water was added to the system and stirred at room temperature for another 30 minutes. After this step, samples were centrifuged (~14000 rpm, at 25°C for 5 minutes) and the solid residue—namely gelatin—was collected. The obtained gelatin was washed three times with water to remove most of the residual DES. The wet gelatin sample (Gel_x) was employed both without any further treatment or after treating it with 0.1 M HCl (Gel_x_HCl) or 0.1 M NaOH (Gel_x_NaOH) to evaluate the effect of pH on the protein structure. One mL of 0.1 M HCl or 0.1 M NaOH was added to the gelatin under magnetic stirring for 1 hour at room temperature and centrifuged to remove the excess liquid.

To evaluate the recyclability of the solvents, all collected supernatants (from Gel_x tests) were left under a fume hood at room temperature for 24 hours. These conditions proved effective in allowing a substantial portion of the water to evaporate. The regenerated DES was then subjected to a subsequent cycle of leather treatment, Gel_1_II° and Gel_2_II°, respectively.

Furthermore, to better understand the role of the DES in the treatment of tanned leather, a control experiment (CE) was performed using the same procedure but replacing the DES with water. Specifically, a solid-to-liquid ratio (S/L) of 0.5:10 m/v was maintained, with the treatment conducted at 60°C for 1 hour, followed by the addition of 1 mL of water and subsequent washing steps. The sample was called Gel_blank.

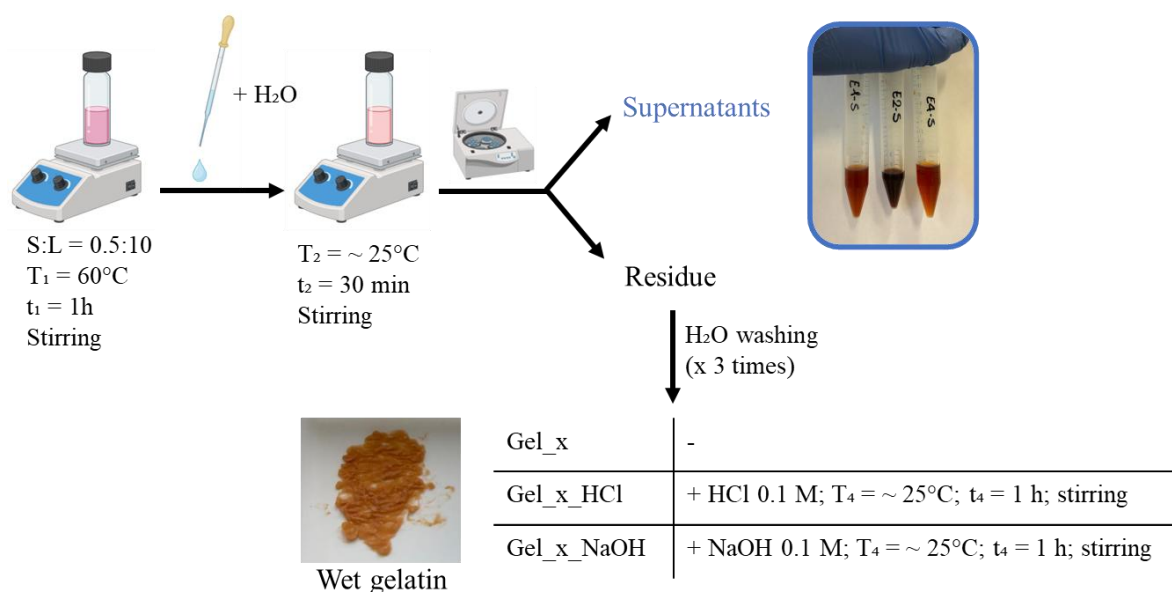


Figure 1. Scheme of the mild treatment of vegetable-tanned leather carried out with a solid-to-liquid ratio (S/L) of 0.5:10, where S refers to the vegetable-tanned leather and L to the deep eutectic solvent used (DES1, DES2, or DES3). The isolated supernatants and the gelatin obtained from the treatment (wet gelatin) are also shown.

Table 2 summarizes the list of the samples obtained from the mild treatment of tanned leather with different DESs, using the sample code Gel_x_y, where x = 1 (DES1), 2 (DES2), and 3 (DES3) and y = HCl or NaOH. More in detail, Gel_1 series gathers the samples prepared using DES1, Gel_2 series gathers the samples prepared using DES2, and Gel_3 series gathers the samples prepared using DES3.

Sample name	Mild treatment
Gel_1	DES1
Gel_1_HCl	DES1 + HCl 0.1M
Gel_1_NaOH	DES1 + NaOH 0.1M
Gel_2	DES2
Gel_2_HCl	DES2 + HCl 0.1M
Gel_2_NaOH	DES2 + NaOH 0.1M
Gel_3	DES3
Gel_3_HCl	DES3 + HCl 0.1M
Gel_3_NaOH	DES3 + NaOH 0.1M

Table 2. List of samples obtained after the mild treatment of tanned leather with different deep eutectic solvents (DESs).

Fourier transform infrared spectroscopy-attenuated total reflectance

Before analyzing them, samples were dried at room temperature for 48 hours. Infrared spectra were recorded by using a Perkin-Elmer Frontiers FTIR Spectrophotometer, equipped with a universal attenuated total reflectance (ATR) accessory and a triglycine sulfate TGS detector. Measurements on vegetable tanned leather (powder and fragment) and gelatin derived from the mild treatment method described above were performed in ATR mode, after the background acquisition. For each sample, 128 scans were collected, averaged, and processed to generate a spectrum with a nominal resolution of 4 cm^{-1} . The Perkin-Elmer software and a written-in-house Lab-VIEW program for peak fitting were employed to run and process spectra.^{50–53} Before spectral processing, a linear baseline was subtracted by drawing a straight line through the absorbance values at 1800 and 1480 cm^{-1} . Subsequently, spectra were normalized within the $1700\text{--}1600\text{ cm}^{-1}$ region to minimize potential artefacts at the spectral boundaries. The second derivative of the amide I band was then computed and analyzed to determine the number and positions of the Gaussian components required for the curve-fitting and deconvolution procedures.

The amide I region was specifically selected for secondary structure analysis due to its minimal interference from amino acid side chain vibrations⁵⁴ and its greater absorbance intensity compared to the amide II ($\sim 1540\text{ cm}^{-1}$) and amide III ($1350\text{--}1190\text{ cm}^{-1}$) regions. Based on established assignments of secondary structure components within the amide I band, and under the assumption of equal extinction coefficients for all structural elements, the relative proportions of secondary structures were quantified. This was achieved by calculating the ratio of the amplitude of each band, corresponding to a given secondary structure, to the sum of the amplitude of all deconvoluted amide I components.⁵⁰

Thermogravimetric measurements (TG and TG/FTIR)

Before analysis, the samples were dried at room temperature for 48 hours. TGA experiments were carried out on $2\text{--}5\text{ mg}$ of solid residue using a TA Instruments Thermobalance model Q5000IR at a rate of $10^\circ\text{C}/\text{min}$, from room temperature to 900°C under nitrogen flow (25 mL min^{-1}). Data processing was carried out using instrument software (TA Universal Analysis). The TG analyses were performed in triplicate. In addition to standard TGA, thermo-gravimetric analysis coupled with Fourier-transform infrared spectroscopy (TG-FTIR) was performed using a TA Instruments Q5000IR thermobalance connected to an FTIR spectrometer (Agilent Technologies). Measurements were carried out under nitrogen atmosphere (flow rate: 70 mL min^{-1}) from 25°C to 900°C , with a constant

heating rate of 20 °C min⁻¹. FTIR spectra were collected over the range of 700–4000 cm⁻¹, using a spectral resolution of 4 cm⁻¹. For each analysis, approximately 8–10 mg of sample was used. To minimize interference from ambient water vapor and carbon dioxide, the FTIR optical path was continuously purged with nitrogen. A background spectrum was recorded before each run to eliminate environmental contributions and ensure accurate detection of the evolved gases.

Evolved Gas Analysis – Mass Spectrometry (EGA-MS)

Analyses were performed with an EGA-PY/3030D microfurnace pyrolyzer (Frontier Laboratories, Japan) coupled to a 6890 Gas Chromatograph and a 5977 Mass Selective Detector single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, USA). The sample (~40 µg) was weighed into a stainless-steel cup and introduced into the furnace, and the temperature was raised from 50 to 800°C at 10°C/min. The interface temperature was kept 100°C above the furnace temperature up to a maximum of 280°C. Evolved gases were carried by a helium flow (1 mL/min) to the GC injector (280°C, split 50:1) and through a deactivated stainless-steel capillary tube (UADTM-2.5N, 3 m x 0.32 mm, Frontier Laboratories) inside GC oven kept at 300°C, reaching the MS detector (EI positive mode, m/z range 50 – 600, ion source 230°C, quadrupole 150°C). Thermograms were acquired in full scan mode using MassHunter Workstation software (version 10.0, Agilent Technologies).

Pyrolysis – Gas Chromatography – Mass Spectrometry (Py-GC-MS)

The Py-GC-MS instrumentation consisted of a Multi-Shot Pyrolyzer EGA/Py-3030D micro-furnace (Frontier Laboratories Ltd. Fukushima, JP) coupled to a 6890 Gas Chromatograph and a 5977 Mass Selective Detector single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, USA). The GC was equipped with a split/splitless injector, a pre column (2m, deactivated silica, i.d. 0.32 mm) and a capillary column HP-5MS Ultra Inert (stationary phase 5% diphenyl-95% dimethyl-polysiloxane, 30 m x 0.25 mm i.d., 0.25 µm, Agilent J&W, USA). The sample (~150 µg) was weighed into a stainless-steel cup and introduced into the furnace, which was maintained at a temperature of 550 °C. The pyrolysis products were eluted with a helium flow (purity 99.9995%) of 1.0 mL min⁻¹ and a split ratio of 1:10. The temperature program was as follows: isothermal at 50 °C for 5 min, 10 °C min⁻¹ to 310 °C, isothermal for 30 min. The GC interface and transfer line were maintained at 280 °C throughout the analysis. The mass spectrometer operated via electron ionization (EI)

at 70 eV and was equipped with a quadrupole operating in positive mode within the mass range of 35–550 m/z. The ion source temperature was set to 230 °C and the quadrupole temperature to 150 °C. Chromatograms were acquired in full scan mode using MassHunter Workstation software (version 10.0, Agilent Technologies). Peak identification was performed by comparing the mass spectra of the detected peaks with those in the NIST (National Institute of Standards and Technology, US) spectral libraries.

Proteomics

Enzymatic digestion of samples was performed with S-Trap columns according to the protocol reported in ref.⁵⁵ with slight modifications. 0.5-1 mg of sample was suspended in 25 µL of lysis buffer (10% SDS, 100 mM ammonium bicarbonate (AMBIC) and 25 µL of Milli-Q water (H₂O). Samples were incubated for 16 h at room temperature and then for 30 min in the ultrasonic bath. 8 µL of phosphoric acid (12%) was added to the mixture. The samples were then centrifuged at 6,000 rpm for 3 min to recover the supernatant. The pellets were discarded, and 348 µL of S-Trap buffer (100 mM AMBIC in 90% methanol/10% H₂O) was added to load the sample onto the S-Trap membrane. The solutions were centrifuged at 6,000 rpm for 3 min to promote protein adsorption onto the membrane. Three washes with 350 µL of S-Trap buffer were performed to clean up the protein sample, followed by centrifugation at 6,000 rpm for 3 min. For enzymatic digestion, 125 µL of trypsin (0.125 µg/µL) dissolved in 50 mM AMBIC was added to each sample. The digestion reaction was performed at 37°C overnight in the thermostatic bath. To recover the peptides after enzymatic hydrolysis, the resin was washed sequentially with 80 µL of 10 mM AMBIC in 0.2% formic acid (HCOOH), 80 µL of 0.2% HCOOH, and 80 µL of an 50% acetonitrile (ACN)/50% H₂O/0.2% HCOOH solution. Each washing step was followed by centrifugation at 6,000 rpm for 3 min. The eluate was dried using a SpeedVac concentrator and stored at – 20°C. Peptides were desalted and concentrated on in-house C18 extraction stage tips as described by (Cappellini et al., 2012).

Peptides were eluted with 10 µL of 50% ACN /0.1% HCOOH and analyzed by LC-MS/MS on a Dionex Ultimate 3000RSLCnano system coupled to an Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific). Data were acquired by Xcalibur software (Thermo Fisher Scientific) and the raw data were processed by MaxQuant (MQ) software version 2.7.3.0 for protein identification.^{56,57} Standard parameters in the MQ searches were trypsin as the enzyme (semitrypsin when searching for backbone cleavages); 3, as the allowed number of missed cleavages; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; and

peptide charge from 2+ to 3+. Minimum peptide length was set to 7; and no fixed modification was set, while oxidation of methionine, deamidation at asparagine and glutamine, hydroxylation of proline, and hydroxylation of lysine were set as variable modifications, with up to a maximum of 5 modifications per peptide. Protein identifications were supported by a false discovery rate (FDR) of 0.01 applied. To reduce the search space, searches were carried out using the UniProt reference *Bos taurus* proteome database (6,052 sequences).

The extent of chemical modifications in samples was semi-quantitatively assessed by a spectral counting approach,⁵⁸ where the number of PSMs containing a specific modification on a given amino acid, as provided in the outputs of the search engine, is normalized with respect to the total number of PSMs containing that amino acid. This semi-quantitative value represents the degree of modification in a sample and provides an adequate comparison between samples.⁵⁸ Similarly, the extent of backbone cleavage in samples was evaluated as percentage of semitryptic peptides over the total number of identified peptides, on the basis of the PSMs. The extent of modification as well as the extent of backbone cleavage in samples were calculated using home-developed command-line scripts available at: https://github.com/brunellacip-prot/proteomics-mod-analyzer_public and https://github.com/brunellacip-prot/backbone_cleav_analyzer_public. The proteomics results shown were obtained analyzing two replicates for each sample. Error bars in graphs represent the standard error of the mean value.

UV-Vis Measurements

UV-Vis measurements were performed on DESs used for multiple extraction cycles using a Jasco V550 spectrophotometer. The spectra were acquired in the range 200-900 nm, at a speed rate 400 nm/min. The samples, diluted 1:300 in water, were measured in a quartz cell with 1cm length. The baseline (acquired using an empty quartz cell) was acquired in the same conditions and subtracted to each measurement.

Adhesion tests

For the adhesion tests, gelatin was used as the adhesive immediately after the treatment (Gel_x, Gel_x_HCl, and Gel_x_NaOH), without undergoing any drying process. Two commercial glue samples commonly employed in restoration were used as reference standards for the comparison of the adhesive properties of the DES-treated samples: HP3, classified as rabbit glue, and BM5, classified as strong glue. Further details on HP3 and BM5

are reported in Ref.⁵⁹ To evaluate bonding performance, two approaches were employed: an indirect method (qualitative adhesion) and a direct method (bonding strength). In the qualitative adhesion tests, gelatin was applied (~100 mg) as a thin layer on the surface of paper, glass, and wood. A second surface of paper or wood was placed on top, gently pressed by hand, and left to dry at room temperature for 30 minutes. After this, the two surfaces were manually separated to assess whether the gelatin functioned effectively as an adhesive. Wet resistance was assessed by bonding two surfaces (paper strips, paper-to-glass, or wooden pieces) following the same procedure described for qualitative adhesion. The bonded specimens were then immersed in water at 25 °C, and at predetermined time intervals, detachment was monitored to evaluate the adhesive strength under wet conditions. Adhesive strength was measured using a stress-controlled Thermo Scientific HAAKE Rheostress 6000 rheometer equipped with Peltier temperature control unit. The geometry plate-plate with 35 mm diameter, using a steel-made upper plate and a wood-made lower plate. The procedure used followed the principles of a tack test.⁶⁰ More in detail, the upper plate was lowered until a gap of 1mm, then, after 5s of contact, normal strength of the upper plate was evaluated while increasing the gap at the constant speed rate of 0.1 mm/s. The measurement was conducted at 25°C. RheoWin DataManager software was employed for data treatment.

Results And Discussion

Characterization of vegetable-tanned leather

Before performing the treatment with DES, the leather was grated to increase the surface area and enhance the interaction with the solvent system (Fig. 1SB). The study of collagen structure after grating was evaluated by FTIR-ATR and thermogravimetric measurements (**Figure 2**).

FTIR-ATR spectra of the leather before and after the grating process were similar to those of commercial collagen and exhibited the characteristic peaks reported in the literature (**Figure 2A** and **Fig. S2**).⁶¹ In particular, the spectra show the typical protein absorption bands (amide A, amide B, amide I, amide II, and amide III): the broad peak at ~ 3300 cm⁻¹ and the shoulder at ~ 3081 cm⁻¹ correspond to the amide A and amide B bands, respectively, which are attributed to N–H stretching vibrations;^{34,62,63} the peaks at 2927 and 2855 cm⁻¹ represent the asymmetric stretching vibrations of –CH₂ and –CH₃ groups, respectively;⁶⁴ the amide I band, located at 1623 cm⁻¹, is associated with the vibration of

peptide C=O groups;⁶⁴ the amide II band at 1525 cm^{-1} corresponds to the N–H bending vibration coupled with C–N stretching;⁶⁵ the amide III band, centered at 1234 cm^{-1} , is assigned to C–N stretching and N–H bending vibrations from amide linkages.⁶⁶ To better investigate the potential changes in the protein's structure due to the leather grating, the amide I region of the FT-IR spectra of the vegetable-tanned leather, examined both as a powder and as a fragment, was analyzed using a peak fitting procedure. The peak deconvolution of the Amide I band of leathers as-is sample showed three main components at 1620 cm^{-1} and 1689 cm^{-1} ascribable to β -structures and 1660 cm^{-1} ascribable to helices.^{67,68} In the grated leather, the same components were observed, but with a slight decrease of the relative content of helix (**Table**), suggesting that the grating process induces minor alterations to the protein structure.

This observation was further supported by thermogravimetric analysis (TGA). **Fig.2B** and **Table S1** show the thermogram and the decomposition of grated leather. The process can be divided into three stages: stage I, ranging from $30\text{--}180\text{ }^{\circ}\text{C}$; stage II, from $180\text{--}600\text{ }^{\circ}\text{C}$; and the final stage III, from $600\text{--}900\text{ }^{\circ}\text{C}$. The mass loss observed in stage I is attributed to the loss of H_2O and other volatile compounds. The actual protein thermal degradation occurs in Stage II. The onset degradation temperature (T_{onset} , reported in Table S1) was used to compare the thermal stability of the samples. It can be observed that the T_{onset} of the piece of tanned leather is lower than that of collagen,⁶⁹ which may indicate that the vegetable tanning process has slightly weakened the collagen structure. T_{onset} of grated leather shows a further slight reduction, but both the leather piece and the grated leather samples exhibit a maximum decomposition rate at approximately $310\text{ }^{\circ}\text{C}$. Interestingly, this temperature is lower than that observed for collagen (326.1°C), in agreement with previously published studies.⁶⁹ Finally, the material undergoes slow decomposition until the end of the experiment. This phenomenon is likely due to processes such as the restructuring of the char, the desorption of volatile compounds previously retained within the char matrix, and similar mechanisms.^{70,71} As reported in the literature, the residue of the leather fragment and grated is around 11% higher than the collagen residue, due to the pyrolytic residue of tanning agents and collagen reticulation.⁶⁹

Figure 2. (A) ATR-FTIR spectra of the grated vegetable-tanned leather and (B) thermogravimetric curve (TG - left axis) and its derivative (DTG - right axis – red line) of the grated tanned leather under nitrogen flow at a heating rate of 10 °C/min. Peak1 and T max are reported. The sample was analyzed in triplicate.

Mild treatment of vegetable-tanned leather with DESs

The systems prepared (reported in Erreur ! Source du renvoi introuvable.) are homogeneous liquids exhibiting medium to low viscosity (around 0.5 Pa·s) and have been previously characterized and classified as Deep Eutectic Solvents in the literature.^{18,72–75} They were used to treat the grated vegetable-tanned leather under mild experimental conditions ($T_1 = 60\text{ °C}$, $t_1 = 1\text{ h}$), as reported in **Figure 1**.

The experimental conditions were set after preliminary tests. In brief, we observed that short times and low temperatures (i.e., 30 min, 25°C) were not sufficient to make the material adhesive. On the other hand, at longer times and high temperatures (24h, 60°C) DESs tends to interact very strongly with collagen, resulting in its complete solubilization: The collagen recovered after the dialysis process, though, was not showing adhesive properties either. The choice of experimental conditions therefore proved to be the best compromise for the effective promotion of adhesiveness, minimizing the amount of collagen solubilized on DES (which lowers the yield of final adhesive material obtained) and the total energy used in the process. The energy required for the treatment (a standard hotplate requires around 0.6 kW/h to maintain a temperature of 60°C for 1 hour, with an average cost of 0.15€) is 4-6 times less than that required for the preparation of commercial adhesives from animal collagen sources (which requires 4-6 cycles of boiling the tissues in water⁵⁹).

The supernatants isolated after the treatment appeared colored (**Figure 1**), indicating the presence of tannins removed from the tanned leather. The complete removal of tannins by the DES could be a promising and sustainable strategy for recovering tannins from leather processing waste and will be the focus of future investigations.

After the extraction, water was added to the residue – the DES-tanned leather system – to reduce the system's viscosity, allowing the centrifugation of the mixture and the isolation of gelatin. This observation suggests that DES not only reduces collagen–tannin interactions but also likely weakens collagen–collagen interactions, resulting in fiber swelling.³⁰

To better understand how DESs may interact with tanned leather, we refer to the existing literature on the interaction between DESs and native collagen. Both the native collagen and the DES chosen in this work contain a high density of polar functional groups ($-\text{COOH}$, $-\text{NH}_2$, $-\text{OH}$).⁴ It is reasonable to hypothesize that DESs can penetrate the leather fiber network, leading to fiber swelling.³² To the best of our knowledge, although several studies have reported the use of LA:U (DES1) systems with native collagen,^{31,34} no specific hypotheses have been proposed regarding the interaction mechanisms between DES1 and the protein. It is well established that urea acts as a protein denaturant through hydrogen bond disruption.⁷⁶ Likewise, lactic acid, due to its hydroxyl and carbonyl groups, may also compete with protein functional groups for hydrogen bonding.³⁵ These considerations support the hypothesis that DES1 could alter the protein structure through combined polar and hydrogen interactions, which may contribute to the observed effects in our treatment.

According to the literature, the interaction between DES2 and collagen is primarily governed by the disruption of inter- and intramolecular hydrogen bonds, as well as ionic interactions among collagen chains.^{32,35} The high abundance of hydrogen and oxygen atoms in the functional groups of various amino acid residues in collagen, such as hydroxyl ($-\text{OH}$), carbonyl ($\text{C}=\text{O}$), and amine ($-\text{NH}_2$) groups, provides numerous sites for hydrogen bonding with DES2 constituents. Chloride ions (from ChCl) and functional groups such as hydroxyl and carbonyl (from LA) play a key role in the formation of these interactions. Similar interactions can be assumed for collagen in tanned leather.

The interaction between DES3 and collagen is also governed by the disruption of inter- and intramolecular hydrogen bonds within collagen fibers, followed by the formation of new hydrogen bonds between collagen and DES3.³³ In our case, the gelatin obtained from treatment with DES3 proved extremely difficult to isolate, as it remained dissolved in the DES3 medium. This observation suggests a strong interaction between DES3 and collagen.

Moreover, the small amount of gelatin that could be isolated as residue did not exhibit adhesive properties, making it unsuitable for our intended applications. These limitations led us to refrain from further investigating the use of DES₃ for the mild treatment of tanned leather.

The possibility of reusing DESs for subsequent extractions was explored, aiming to make the process more attractive to industry, in line with the principles of the circular economy. More in detail, the aqueous DES solution (recovered by combining the liquid part of DES used in mild treatment and the subsequent aqueous waste obtained from the repeated washing steps) was collected. After removing the excess water (by simply evaporation under a fumehood overnight), the resulting DES was used to perform a second cycle of mild treatment on grated leather (as described in the experimental section). The procedure was successful, allowing the obtainment of new adhesive material (Gel_1_II° and Gel_2_II°). Further cycles, instead, did not prove to be functional for the formation of a bioadhesive material. It is worth noticing that in all these cycles, DES was reused in the simplest possible way, i.e., without performing any purification step for polyphenol recovery. This condition, which is the best from the point of view of a possible industrial scale-up of the process, involves the presence of tannins in the reused DES, as evidenced by UV-Vis measurements (**Fig. S4**). We may assume that after the second cycle, the DES was somehow “saturated” with polyphenols, thus it could not be used anymore to produce bioadhesives.

Finally, the gelatinous biomaterial was treated with either 0.1 M HCl or 0.1 M NaOH in the last procedure stage, with the aim of investigating potential effects of pH on the samples' properties.

Characterization of the Biomaterial

The characterization of the gelatin samples obtained after the mild treatment with DES₁ and DES₂ is reported below. **Fig. 3** shows the ATR spectra of Gel_1 and Gel_2, compared with the spectra of DES₁ and DES₂, respectively.

Figure 3. ATR-FTIR spectra of samples analyzed. (A) Dried gelatin obtained from DES1 treatment (Gel_1 - green line) compared with DES1 (red line). (B) Dried gelatin obtained from DES2 treatment (Gel_2 - green line) compared with DES2 (red line). On the right side a zoom in the fingerprint region is reported and collagen characteristic bands are highlighted.

The samples Gel_1 and Gel_2 exhibit features comparable to those of grated vegetable-tanned leather, and they display the characteristic absorption bands of gelatin.^{34,63,77} Unlike the grated leather, however, the post-treatment samples exhibit an absorption peak at 1718 cm^{-1} , attributable to the C=O stretching of LA present in DES1.³¹ This indicates that, despite the repeated washing in water, traces of DES1 are still present in the samples, likely due to formation of stable bonds with collagen fibers (**Figure 3A**). Similarly, the samples obtained from the mild treatment using DES2 also show a peak at 1730 cm^{-1} , which can be assigned to the C=O stretching of LA, confirming the presence of residual DES2 in these samples (**Figure 3B**).

Fig. S3 reports the ATR-FTIR spectra of the samples obtained under mild treatment conditions followed by the treatment with 0.1 M HCl or 0.1M NaOH. At first glance, the ATR-FTIR analysis does not reveal significant differences in the protein spectral features. To obtain more precise information on possible changes in the protein structure following

mild treatment in neutral, acidic, and basic conditions, the amide I region of the FT-IR spectra of the dried gelatins was analyzed through peak fitting. **Table 3** reports the deconvolution results of the Amide I of the spectra of leather fragment, grated leather, Gel_blank, Gel_1, Gel_1_HCl, Gel_1_NaOH, Gel_2, Gel_2_HCl, and Gel_2_NaOH. The assignment of bands corresponding to helix and random coil structures was difficult, as these bands tend to overlap due to the broad bandwidth of the random coil signal. Consequently, it was not possible to clearly distinguish between these two structures in all samples. The β -sheet assignments in **Table 3** cover both parallel (p) and antiparallel (ap) β -sheets, as well as β -turns. Gel_1, Gel_1_HCl, and Gel_1_NaOH show a higher content of random coil structures compared to grated leather, suggesting that treatment with DES1 induces significant structural changes in the protein, likely due to the presence of urea, which acts as a denaturant. Similarly, Gel_2 and Gel_2_HCl display an increased proportion of random structures relative to grated leather, indicating that DES2 treatment also destabilizes and alters the protein's secondary structure. Interestingly, when NaOH was added following the treatment, the protein appears to partially regain structural order, reaching a β -structure content of 92%. It is worth noting that even the treatment without DES (Gel_blank) is promoting the increase of the random protein structure, suggesting that these changes depend not only on DES, but also on the experimental conditions used (t: 60 min, Temperature 60°C).

Sample	Wavenumber (cm ⁻¹) (HHBW) / Secondary structure percentage	
	Random and helices*	β structures (β-sheets/ β-sheet (p+ap)/ β-turn)
Leather as-is	1660 (40) (helix) / 30%	1610 (33) / 3% 1620 (17); 1689 (18) / 63% 1672 (29) / 7%
Grated leather	1662 (30) (helix) / 21%	1618 (32); 1693 (18) / 58% 1671 (19) / 21%
Gel_blank	1652 (48) / 65%	1634 (17) / 8% 1623 (23); 1694 (22) / 27%
Gel_1	1651 (58) / 69%	1619 (32) / 31%
Gel_1_HCl	1659 (41) / 55%	1622 (27) / 13% 1610 (15) / 32%
Gel_1_NaOH	1655 (49) / 36%	1611 (25) / 58% 1622 (20) / 6%
Gel_2	1659 (33) / 41%	1610 (12) / 8% 1622 (34); 1689 (26) / 51%
Gel_2_HCl	1660 (49) / 39%	1610 (12) / 8% 1622 (34); 1689 (26) / 53%
Gel_2_NaOH	1661 (53) / 8%	1625 (40); 1689 (30) / 84% 1672 (22) / 8%

* Where not specified this band includes random and helix structures because the peaks assigned to these two components in collagen are too close (1650-1660 cm⁻¹ and 1640-1660 cm⁻¹) and they may not be separated by the peak deconvolution procedure. An ambiguous assignment to the helix is established thanks to the narrow HHBW.

Table 3. Wavenumber, half height bandwidth (HHBW), and secondary structure percentages of Amide I component of the samples. The variation coefficient for secondary structure percentage was <7% for a content of secondary structures >30% and 7-15% for a content of secondary structures < 30%.⁷⁸

In a second moment, the thermal stability of the gelatines was assessed by TGA coupled with FTIR analyses of evolved gases (**Figure S5-7**).

The thermal analysis pointed out that the treatment with water of the grated leather (forming Gel_blank) provoked only a slight thermal stabilization of the collagen degradation peak (317°C of Gel_blank respect to 310°C of grated leather observed in the mass loss derivative), but except for this change the thermogram shape remains practically identical, highlighting that water alone does not significantly alter the thermal stability of collagen. On the other hand, the TGA curves of the samples obtained with DES treatment present the features: after an initial step of humidity loss (below 50°C), all these samples present the degradation of the residue DES1 (with a maximum in DTG at around 160°C) or DES2 (with the maximum in DTG at around 240-250°C) respectively, followed by collagen thermal degradation. The presence of DES was confirmed by the FTIR spectra of gases evolved at these temperatures, which showed spectral features in agreement with the nature of the products of its thermal degradation e.g., CO₂ (2400–2300 cm⁻¹), carboxylic moieties (1800-1700 cm⁻¹), NH₃ (double peak at 965 and 930 cm⁻¹ only for DES1), and C-H stretching (2800-3100 cm⁻¹, only for DES2). The collagen thermal degradation was associated with the production of molecules characterized by similar spectral features, mainly CO₂, NH₃, and carbonylic moieties, as well as methane (3016 and 1304 cm⁻¹) and ethane (2850–3000 cm⁻¹).⁷⁹ The broad curve peak between 2600 and 3000 cm⁻¹ visible above 250°C may be due to the O-H signal of polyphenols derived from the release of the vegetable tanning. The partial concomitance of DES and collagen degradation, as well as the FTIR signals overlapping and the complexity of the overall spectra, did not permit highlighting a clear onset temperature for the collagen, useful to evaluate its degree of hydrolysis after the treatment. Anyway, aware of the possible errors, we attempted to compare the collagen signals in the different samples by looking at the maximum of DTG peak corresponding to its degradation (**Table S2**). Samples treated with DES1 show a slight decrease in the maximum temperature, while the degradation in the samples treated with DES2 occurs at temperatures even lower. This highlights that the treatment with the latter seems to have a higher impact on collagen thermal stability.

The thermal stability of the different samples was further investigated with EGA-MS, enabling molecular information to be obtained. **Fig. S8** reports the thermograms of Gel_1, Gel_1_HCl, and Gel_1_NaOH. In accordance with TGA-FTIR, we observed that Gel_1 still contains some residual DES, thermally decomposing in the zone below 250°C. After 250°C, the thermograms of Gel_1 and Gel_1_HCl are very similar, even though the maximum

temperature of the main peak of Gel_1 is slightly shifted to higher temperatures, and the mass spectra present fragment ions associated with the DES. The presence of NaOH affects mass spectrometric analysis, producing a very noisy thermogram. Curves with a similar profile were obtained for Gel_2, Gel_2_HCl, and Gel_2_NaOH. To further investigate at the molecular level the characteristics of the collagen obtained with the extraction procedures described above, we thus focused only on gels washed with HCl (Gel_1_HCl and Gel_2_HCl) and not those treated with NaOH. **Fig. S10** shows the extracted ion thermograms (EITs) of the fragment ion m/z 154 of grated vegetable-tanned leather, Gel_blank, Gel_1_HCl, and Gel_2_HCl. Relevant pyrolysis products of proteins are diketopiperazines (DKPs), whose mass spectra are dominated by m/z 70, 111, 124, and 154.⁸⁰ The peak of Gel_2_HCl is shifted by 10 °C to lower temperatures, meaning that the collagen in Gel_2_HCl presents a lower thermal stability, in accordance with the temperature reduction observed in the maximum peak in the DTG (**Table S2**).

The samples were also subjected to Py-GC-MS. The most abundant fragment ions, already identified in the EGA-MS thermograms, were selected and extracted. M/z 70, m/z 124 and m/z 154 are typical of DKPs,⁸⁰ m/z 124 is also formed from the pyrolysis of catechol-derived compounds,⁸¹ m/z 94 is the molecular ion peak in the mass spectrum of phenol,⁸² m/z 110 is the molecular ion peak in the mass spectrum of catechol,⁸¹ m/z 135 is the molecular ion peak of the mass spectrum of benzothiazole, and m/z 186 is correlated to heteroaromatic compounds (see mass spectra in Fig. S10). Extracted ion chromatograms are reported in **Fig. 4**.

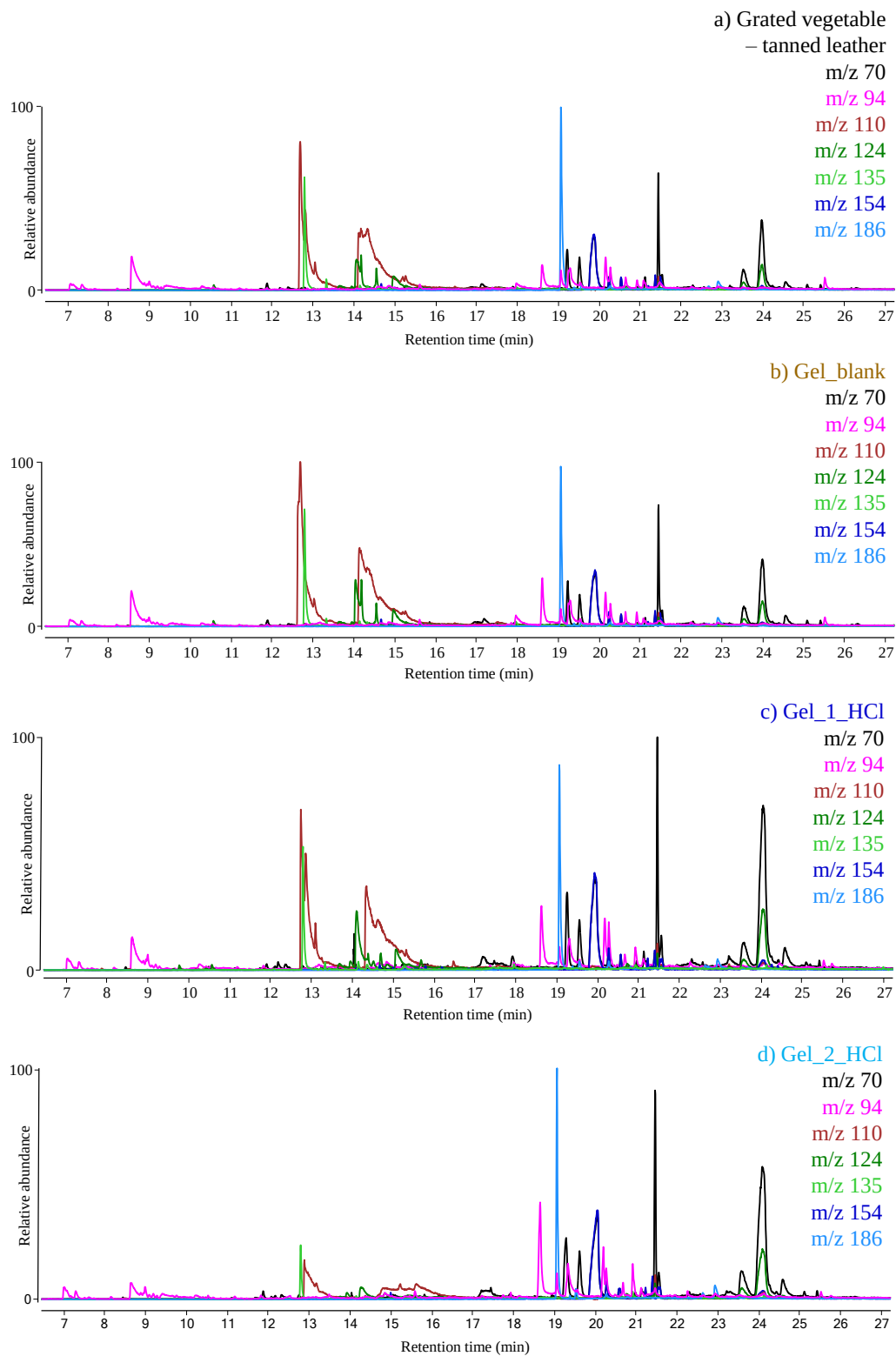


Figure 4. Extracted Ion Chromatogram (EIC) obtained with Py-GC-MS of selected ion fragments of a) grated vegetable – tanned leather, b) Gel_blank, c) Gel_1_HCl, and d) Gel_2_HCl.

EICs are divided into two regions: before and after 17 min. The first part is dominated by the peaks of phenol, catechols, benzothiazole, and catechol-derived compounds. These compounds are formed from the thermal decomposition of the most thermally stable portion of collagen. All the pyrograms present several peaks which spectra present an abundant m/z 94 fragment ion, while collagen shows only the peak of phenol with m/z 94.⁸⁰ We hypothesize that these other peaks with m/z 94 in their mass spectral features may be associated with polyphenols deriving from the leather tanning process. The second part of the EICs is dominated by the peaks of DKPs and heteroaromatic compounds: this part is characteristic of compounds formed upon thermally induced depolymerization of collagen. What stands out is that the relative intensity of the first and the second part in the EICs: in a) grated vegetable-tanned leather and b) Gel_blank the proportion is almost equal. On the contrary, the relative intensity of the second part in the EIC of Gel_1_HCl, and even more in that of Gel_2_HCl is higher. This indicates that the collagen in the extracted gelatine is less thermally stable than in leather, suggesting that partial hydrolysis has taken place.

In a second moment, glue samples were analyzed by a shotgun proteomics approach using LC-MS/MS after trypsin digestion and compared with the two samples of grated vegetable-tanned leather and Gel_blank, following the diagnostic strategy developed in Ref.⁸³ for the molecular characterization of commercial animal glues. Details of protein identifications are reported in **Table S3**.

Previous proteomics studies on collagen-based materials—including animal glues, parchments and leathers—have shown that manufacturing processes and chemical treatments leave diagnostic molecular fingerprints that can be detected by MS-based approaches. In particular, collagen extraction and glue preparation using acidic or alkaline treatments modulate specific collagen degradation pathways, often including extensive hydrolysis of the protein backbone, resulting in lower-molecular-weight peptides. By comparing collagen samples analyzed under identical conditions but derived from different extraction and post-treatment protocols, it is possible to identify molecular signatures associated with specific degradation mechanisms and to elucidate how these procedures differentially affect molecular integrity and, ultimately, adhesive performance.

Backbone cleavage or hydrolysis of the collagen polypeptide chain is a well-known feature of animal glues⁸³ and reflects degradation occurring during collagen extraction and glue preparation. The backbone cleavage can be semi-quantitatively evaluated since semi-

tryptic peptides are generated upon trypsin digestion, with trypsin cleavage site only at one end.

As shown in **Fig 5a**, Gel_1 and Gel_2 display comparable levels of backbone cleavage, slightly higher than those observed in the starting grated leather sample. This indicates that the DES-based extraction procedure induces some additional hydrolysis of the collagen backbone, in agreement with Py-GC–MS results. Notably, samples subjected to further acidic or alkaline treatments after DES extraction exhibit a markedly higher extent of backbone cleavage. Among these, the Gel_1_HCl series shows the highest values, with Gel_1_HCl reaching approximately 25%, representing an increase of about 20 percentage points relative to the starting grated leather. This pronounced effect is consistent with previous observations on animal glues, where exposure to acidic or alkaline environments during preparation has been reported to strongly promote hydrolysis of the polypeptide chain.⁸³

Detailed results of backbone cleavage data are reported in **Tables S4**.

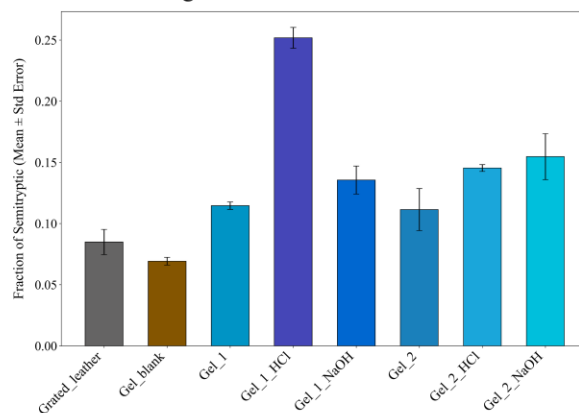
We next evaluated deamidation, a non-enzymatic modification of glutamine and asparagine residues that converts neutral amide side chains into negatively charged carboxylates, resulting in a mass shift of +0.98402 Da detectable by MS. Deamidation is particularly relevant for animal glues, as changes in charge distribution along collagen chains are known to influence physicochemical properties, like dependence of viscosity on pH.⁸⁴

As shown in **Fig. 5b**, Gel_1 and Gel_2 do not exhibit significant differences in deamidation extent. In contrast, glue samples subjected to acidic or alkaline post-treatments (Gel_x_HCl and Gel_x_NaOH) generally show slightly higher deamidation levels, approximately 5-10% greater, compared to samples treated under neutral conditions, consistent with increased hydrolysis of amide moieties at acidic and alkaline pH values.

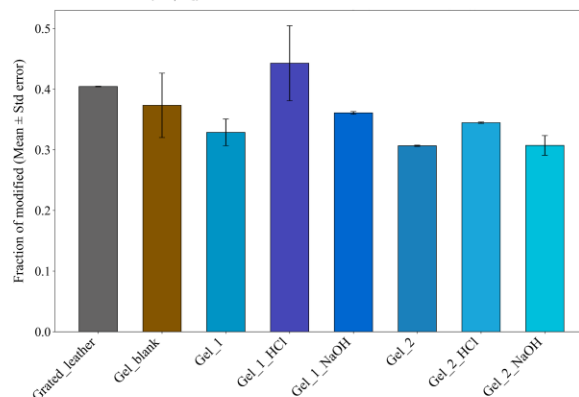
Interestingly, also the starting vegetable-tanned leather samples display a higher extent of deamidation, approximately 10% greater than that observed in neutral gel samples. This difference may reflect preferential deamidation of collagen molecules located at the surface of the grated leather, which are more exposed to environmental factors and may be preferentially sampled during collagen solubilization prior to trypsin digestion when DES extraction is not applied.

Oxidation of methionine residues was assessed as an additional marker of collagen alteration. This modification was previously reported to be significantly abundant in commercial animal glues analyzed by Ntasi et al.⁸³ In the present study, all samples, from the starting grated leather samples on, exhibit very high levels of methionine oxidation, approaching 90%. This uniformly elevated oxidation suggests that oxidative modification was already largely present in the original vegetable-tanned leather and is therefore attributable to the tanning process rather than to the DES-based extraction or subsequent chemical treatments. Detailed results of deamidation and methionine oxidation analysis are provided in **Table S5**.

A) Backbone cleavage



B) Deamidation (N|Q)



C) Oxidation (M)

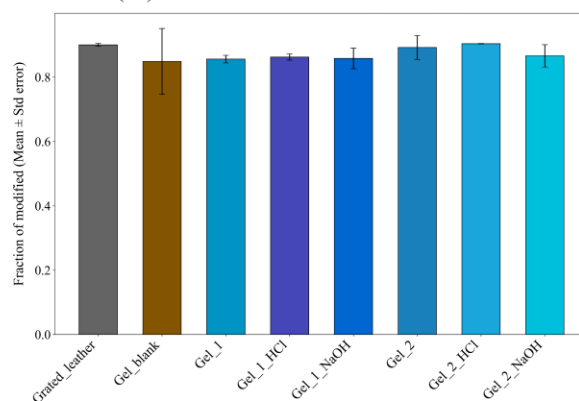


Figure 5. A) Extent of backbone cleavage, reported as fraction of semitryptic peptides over the total number of identified peptides (tryptic plus semitryptic). Table S4 reports the number of semitryptic peptides the backbone cleavage data is based on. B) Extent of deamidation (N|Q), reported as fraction of modified sites over detected (modified plus unmodified) ones. N|Q represents combined deamidation at both asparagine (N) and glutamine (Q) residues. C) Extent of methionine oxidation, reported as fraction of modified sites over detected (modified plus unmodified) ones. Table S5 reports the number of modified and detected amino acid sites the modification data is based on. The results shown were obtained averaging data from two replicates. Error bars in all graphs represent standard error of the mean value.

Adhesion Tests

The wet gelatin samples obtained immediately after the mild treatment were tested for their adhesive properties. Adhesion was evaluated on different material combinations, including paper–paper (P–P), paper–glass (P–G), wood–wood (W–W), and wood–aluminum (W–Al) (**Figure S11-12**). More in detail, paper and wood were selected as substrates because commercial animal glues are typically used with these materials. Since such glues are produced from leather and bone waste, and our adhesive is similarly derived from vegetable-tanned leather scraps, these substrates allowed us to evaluate whether the DES-treated samples exhibit comparable or improved adhesion where animal glues are normally effective. In addition, glass and steel were included as model substrates on which conventional animal glues generally show poor adhesion, enabling us to assess whether the DES treatment also enhances bonding on materials that are not usually compatible with animal-based adhesives. Moreover, steel was tested because the upper plate used in the quantitative rheological adhesion test is made of stainless steel.

As reference materials, two glues commonly used in restoration for different applications were considered. HP3, derived from bovine hide, is typically employed for plastering operations, whereas BM5, obtained from mixed animal bones, is mainly used for carpentry applications. Generally, bone-derived glues exhibit higher initial adhesion compared to hide-derived ones, which are stiffer and more difficult to spread.⁵⁹ A distinctive feature of animal glues, advantageous in restoration, where reversibility is required, but limiting in other applications, is their solubility in hot water, which allows the adhesive to be removed. Consequently, the qualitative adhesion test was also carried out by evaluating the resistance of the DES-treated samples in hot water. **Fig. 6A** shows the results of the adhesion test performed on the samples, testing the adhesion on dry surfaces (A) and after their immersion in hot water (60 °C) (B).

A

Surfaces	Gel_1	Gel_1_II°	Gel_1_HCl	Gel_1_NaOH	Gel_2	Gel_2_II°	Gel_2_HCl	Gel_2_NaOH	Gel_blank	BM5	HP3
P-P	Green	Green	Green	Green	Green	Green	Green	Green	Red	Green	Yellow
P-G	Green	Yellow	Yellow	Red	Green	Yellow	Yellow	Yellow	Red	Green	Yellow
W-W	Green	Yellow	Green	Green	Green	Yellow	Green	Green	Red	Green	Red
W-S	Green		Green	Green	Yellow		Yellow	Green		Green	Red

↓ 

B

Surfaces	Gel_1	Gel_1_II°	Gel_1_HCl	Gel_1_NaOH	Gel_2	Gel_2_II°	Gel_2_HCl	Gel_2_NaOH	Gel_blank	BM5	HP3
P-P	Red	Red	Red	Red	Red	Red	Red	Red	White	Red	Red
P-G	Red	Red	Red	White	Red	Red	Red	Red	White	Yellow	Red
W-W	Green	Red	Green	Green	Yellow	Red	Yellow	Yellow		Yellow	

Figure 6. Adhesion test results using gel_1, gel_1_II°, gel_1_HCl, gel_1_NaOH, gel_2, gel_2_II°, gel_2_HCl, gel_2_NaOH, Gel_blank, and BM5 and HP3 as references. Adhesion ability between surface pairs: paper–paper (P–P), paper–glass (P–G), wood–wood (W–W), wood–steel (W–S). A) Test made on dry surfaces. Color coding: green = strong adhesion, yellow = weak adhesion, red = no adhesion. B) Adhesion after immersion in hot water. Color coding: green = samples that remained attached after more than 3 days in water at 60°C; yellow = samples detached after approximately 1 min; red = samples detached immediately upon immersion.

The first important observation in our samples is that Gel_blank exhibits no adhesive properties, underscoring the essential role of DES in producing adhesive materials.

The other samples, instead, show medium-to-good adhesive properties for the attachment of dry surfaces, except for Gel_1_NaOH which failed to glue paper and glass. The gels recovered after the second cycle of DES reuse (Gel_1_II° and Gel_2_II°) show good adhesive properties for gluing paper-paper surfaces, and medium properties on the other surfaces, highlighting the potentialities in DES reuse for multiple cycles of treatment. The best samples (highlighted green in the table) were further tested for water resistance by immersion in hot water (Figure 6B). Notably, three samples (Gel_1, Gel_1_HCl, and Gel_1_NaOH) exhibit outstanding water resistance for gluing wood surfaces, which remained firmly adhered even after three days of immersion in hot water. According to the literature, collagen-based adhesives typically face significant challenges compared to their synthetic counterparts, particularly in terms of adhesion strength and water resistance.^{43,47} Numerous studies have focused on the improvement of the performance of collagen adhesives, adopting strategies as the incorporation of silane coupling agents and cross-

linking technologies.^{43,44,85} In contrast to these approaches, the gelatins obtained through our DES-based mild treatment were used as adhesives without any further modification. Probably, the presence of tannins in our matrix, with their well-documented ability to enhance collagen water resistance,^{41,43,47} promotes the improvement of the overall adhesive properties of the material.

The adhesive properties of the materials for gluing wood-steel surfaces were also tested through a tack test, using a rheometer.⁶⁰ To compare the tack properties of the investigated samples, the parameter ΔF (defined as the difference between F_{peak} and F_0 values) was considered, where F_0 is the initial normal force at contact and F_{peak} is the maximum detachment force (Table 4). This correction was necessary because the initial F_0 varied among samples. Such variations are not purely instrumental but reflect intrinsic differences in gel deformability and viscoelastic response: softer gels exhibit lower F_0 because they deform more easily under contact, whereas stiffer gels resist deformation and thus show higher F_0 .⁸⁶ Since F_0 directly contributes to the absolute detachment force, using uncorrected values would lead to biased conclusions. By subtracting F_0 , ΔF isolates the additional force developed during detachment, providing a more reliable comparison across formulations and treatments. The tack test curves revealed clear differences in adhesion behaviour among the samples (see Figure S12). All profiles displayed the typical viscoelastic adhesive response, with an initial increase in normal force followed by a detachment peak and decay.⁸⁷ Nevertheless, the shape and stability of the curves strongly depended on the sample formulation and chemical environment. Looking at the results, we observed that Gel_1 exhibited a sharper, more pronounced peak and higher ΔF value compared to Gel_2, suggesting a stronger cohesive network and well-defined detachment profile. Acidic treatment (HCl) weakened the adhesive response, especially for Gel_2_HCl ($\Delta F \approx 6$), where the detachment peak was smoother. Gel_1_HCl, the effect was milder ($\Delta F \approx 13$), suggesting higher resistance of Gel_1 to acid-induced destabilization. This reduction in adhesion is likely due to protonation of functional groups, which decreases electrostatic interactions and weakens the gel network. On the other hand, alkaline treatment (NaOH) produced the most distinctive profiles. In Gel_1_NaOH, detachment was accompanied by oscillations, pointing to discontinuous failure with multiple breakage; still, the corrected force was significant ($\Delta F \approx 11$). In Gel_2_NaOH, the profiles displayed sharp peaks and $\Delta F \approx 17$ value comparable to Gel_1 ($\Delta F \approx 17$). These results suggest that alkaline conditions may favour structural rearrangements, such as deprotonation and enhanced ionic interactions, which strengthen and diversify adhesive contacts.

The ΔF_n value obtained from the tack test measurements on the two commercial animal glue samples (BM5 and HP3, visible in Table 4) is significantly lower than that of the samples treated with DES. This confirms that the DES treatment modifies fundamental parameters governing the adhesion mechanism. In particular, the presence of the DES may induce a structural reorganization at the level of the tertiary structure and influence the way proteins aggregate, leading to a greater availability of functional groups capable of interacting with the substrate surface and thus further enhancing the adhesive response. These modifications affect not only the adhesive interactions with the substrate but also the cohesive forces within the material. The residual presence of tannins inside the protein matrix in the DES-treated samples can increase cohesive forces, due to the ability of tannins to form multiple hydrogen bonds and π – π interactions with polypeptide chains. This results in a more robust internal network capable of dissipating more energy during debonding.

Furthermore, the DES may induce a reorganization of the protein chains, altering their flexibility, the distribution of functional groups, and the degree of plasticization. The combination of enhanced internal cohesion and a more reactive interface toward the substrate leads to a significant increase in the measured adhesive strength.

It is also important to note that the concentration used for the commercial glues corresponds to the one typically employed in their common applications, whereas the sample obtained through the DES treatment is likely more concentrated. Therefore, this higher concentration may also contribute to the increased adhesion observed in the DES-treated samples. These considerations could certainly be further demonstrated by performing surface tension and contact angle measurements; however, at this stage such analyses are not feasible because the sample obtained after the DES treatment exhibits extremely high viscosity, which prevents reliable testing with these techniques.

Sample	Gel_1	Gel_1_ HCl	Gel_1_ NaOH	Gel_2	Gel_2_ HCl	Gel_2_ NaOH	BM5	HP3
ΔF (N)*	17	13	11	13	6	17	0.2	0.09
Adhesion test (W-Al)								

Table 4. Difference between the force at peak maximum and the initial force (ΔF) obtained in the tack test, compared with the results obtained in the adhesion tests. The results refer to adhesive properties on wood-steel surfaces. *Values reported with an error of ± 3 N for Gel_x series and ± 0.02 N for commercial glues.

These quantitative results from the tack tests are consistent with the observations from preliminary manual detachment tests, where the adhesive behaviour was assessed qualitatively. In the manual tests, it was evident that Gel_2 and Gel_2_HCl exhibited noticeably lower adhesion compared to the other samples. The ΔF measurements confirm this trend, providing a more precise and reproducible evaluation of the relative adhesive strength across all samples and treatments. Notably, the quantitative approach also reveals subtle differences that were not apparent in the manual tests, such as the enhanced adhesion of Gel_2_NaOH, which could not be captured by the qualitative assessment alone.

Differences in adhesion performance can be discussed by looking at the issue from different angles. Thermo-analytical techniques (TGA-FTIR, EGA-MS, and Py-GC-MS) and proteomic analysis highlight the collagen hydrolysis in Gel_1_x and Gel_2_x with respect to the grated leather. From this perspective, we could say that treatment with both DESs promotes the unravelling of collagen fibers through the destruction of inter- and intramolecular bonds, followed by a certain degree of hydrolysis of the polypeptide chains. In particular, samples coming from treatment with DES2 (Gel_2_x series) are more thermally destabilized than samples treated with DES1 (Gel_1_x series). Proteomic analyses demonstrated a gross similarity between Gel_1 and Gel_2 samples at the level of covalent structure. Moreover, and most importantly, no significant other chemical modification of collagen due to DES treatment was observed although the peak fitting analysis indicates that all samples are composed of random coil and β -structures, while leather before extraction presents a less random structure. Acidic and basic treatments induce a higher degree of hydrolysis than the neutral gel; however, this difference is not reflected in the adhesive properties. It is worth noting that no direct correlation can be established between the secondary structure of the protein and the adhesive performance of the DES-treated samples. The control sample (Gel_blank), treated under identical conditions but without DES, displays a secondary structure profile comparable to that of the DES-treated samples, yet shows no adhesion. This clearly indicates that secondary structure modifications alone do not govern adhesive strength, which instead likely arises from DES-mediated interactions and from the synergistic contribution of tannins, affecting cohesion and interfacial bonding. Therefore, the differences in adhesive performance observed among the samples cannot be ascribed solely to variations in secondary structure, in agreement with the findings reported by Pulidori et al.⁵⁹

A further degree of complexity comes from the change in pH, which has a significant impact on the adhesiveness of the material. This aspect, only briefly mentioned in this work, will be object of more systematic studies in the future.

Process sustainability

The sustainability of the process was evaluated using *Path2Green*, one of the most recent metrics, which gives a comprehensive method evaluation of the base of the 12 Green Chemistry principles.⁸⁸ The pictogram obtained with the mobile app proposed by the authors is reported in **Fig. 7**, and the scores used to build it are reported in **Table S6**. The pictogram displays the 12 icons relative to the 12 green principles, and highlights with a colorimetric scale the process adherence to each principle: red, yellow, and green to indicate poor, neutral, or good adherence, respectively. The final process score, calculated by weighing the score of each principle for its environmental, social, and economic impact, is reported in the center of the figure.



Figure 7. Pictogram created with the App Path2Green highlighting the sustainability of the process.

Our process displays a final score of 0.360 on a scale going from completely negative -1 and completely positive +1. Its main strength points rely mainly on the biomass selected (Principles 1-2), the treatment conditions (Principles 4, 7, 9), and the various application fields (Principle 10). More in detail, the leather pieces are waste biomasses from the tanning industry of the Zabri tannery, which is located in the Tuscany tanning district, one of the main areas for leather processing on a national and international level. The valorization of waste biomasses proposed in this work is therefore of great interest from several points of view, including environmental and economic sustainability (the energy required is around 4-6 times lower than the standard animal glue production) and the social impact of tanneries on the territory, as shown by the high score obtained from the evaluation of Principles 1 and 2. The solvents chosen for the treatment were DESs composed of

biodegradable and economic components, and this allowed us to maximize the score for Principle 4. The process required a much lower energy consumption in comparison with other procedures necessary to produce glue from animal sources,^{89,90} with a consequent good impact on environmental and economic sustainability. After the DES-based treatment, the product purification was necessary, but water was used as solvent (Principle 6), and no other post-treatment was necessary (Principle 8). The final material presented excellent qualities, providing a complete valorization of the starting biomasses (Principle 7). Moreover, considering the antimicrobial properties of tannins,⁴⁹ the biomaterial obtained has potential applications as antimicrobial glues in several fields, e.g. restoration, material science, biomedical field, or manufacturing (Principle 10). The possibility of reusing DES for consecutive treatments is reflected in the maximization of Principle 11.

The weaker points of the process are related to Principles 3, 5, 6, and 12. As a matter of fact, the biomass was subjected to physical pre-treatment (which has a slightly negative impact on the process score reflected in Principle 3), even though pretreatments with biological and hazardous chemicals were carefully avoided. Besides, the procedure was performed on a scale laboratory, therefore it was performed in batches (-1 at Principle 5), and water for purification of the material was needed (-0.5 at Principle 6). Waste management (Principle 12) is currently a negative point, but with good potential for improvement. The possibility of reusing DES for multiple leather treatment cycles and ongoing studies on the tannins' recovery from DES are the starting points for desirable waste reduction. Moreover, seeing the good premises found in this work, we believe that future optimization on a larger scale can be of interest, with a view to achieving a competitive process at an industrial level.

Conclusions

The results presented here demonstrate, for the first time, that NADES (lactic acid and urea in a molar ratio 2:1, DES1, and choline chloride: lactic acid in a molar ratio 1:1, DES2) can be employed to gently modify vegetable-tanned leather scraps (wastes of a local industry), leading to the production of a collagen-based biomaterial with adhesive properties. The process's greatest strengths lie in the use of green solvents (NADES), the complete valorization of waste biomass, and the low energy required for treatment (1 hour, 60°C).

Spectroscopic (FTIR-ATR), thermoanalytic (TGA-FTIR, EGA-MS, Py-GC-MS), and proteomic techniques gave molecular insights on changes induced in collagen by the treatment. In particular, we observed that the experimental treatment conditions induced in all the samples a reduction of helices with consequent increase of random-coil secondary structures. TGA-FTIR analyses highlight that a partial DES amount remains tied in the final gelatin samples, while EGA-MS, Py-GC-MS and proteomic analysis showed that DES presence induced a certain reduction in collagen thermal stability (higher in DES 2 respect to DES 1), likely ascribable to backbone cleavage, which, in any case, did not exceed 25%.

The DES presence was essential for the final properties of the materials, which indeed showed good adhesive performance (especially on organic surfaces such as wood or paper). The intrinsic presence of tannins in the material (which come from the vegetable tanning process carried out on the raw leather) gave it additional antibacterial and water-resistant properties, making it an attractive alternative to common commercial glues of animal origin. Preliminary tests showed that the obtainment of adhesive materials was possible also using a recycled DES.

Overall, the process described represents a first step towards a more complete recovery and reuse of waste from the tanning industry.

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Author Contribution

C.P., E.M., G.C., B.Ci., and S.P. contributed to data curation, investigation, methodology, and writing – original draft. E.P. contributed to data curation, methodology, writing – review and editing, and supervision. B.Ca. contributed to investigation and methodology. J.C., I.B., C.D., and E.B. contributed to conceptualization, funding acquisition, and writing – review and editing. L.B. contributed to conceptualization, funding acquisition, supervision, and writing – original draft. B.Ci. additionally contributed to software development and visualization.

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7. Final Conclusions

The chemical investigation of cultural heritage (CH) materials represents one of the most challenging and interdisciplinary fields within analytical sciences. Works of art and archaeological artefacts are complex, multicomponent systems composed of organic and inorganic substances that have interacted and transformed over time through natural aging, environmental exposure, and human intervention.¹ Understanding their chemical composition and degradation pathways is essential to reconstruct manufacturing techniques, artistic practices, and cultural traditions, as well as to design scientifically informed conservation strategies. The study of CH materials thus requires reconstructing both their original composition and the chemical transformations accumulated over centuries of alteration. From an analytical perspective, these materials are characterized by pronounced heterogeneity, low concentration of target analytes, and strict sampling constraints due to the uniqueness and fragility of the objects.² These factors demand highly sensitive, selective, and minimally invasive methodologies capable of extracting molecular information from microscopic samples while preserving the integrity of the artefacts.³

The overarching aim of this doctoral project was the development and application of novel mass spectrometry (MS)-based analytical strategies for CH diagnostics, with particular emphasis on organic materials. The research was structured around two complementary and interrelated objectives: (1) the identification of ancient proteins and organic molecules in historical and archaeological samples through innovative, highly sensitive, and minimally invasive analytical workflows, and (2) the chemical characterization of proteinaceous materials to elucidate degradation mechanisms, pigment–binder interactions, and the effects of manufacturing and environmental factors on long-term material evolution. Together, these directions aimed to strengthen the analytical potential of MS in heritage science by bridging molecular identification with chemical understanding.

The first research line addressed the challenges related to the identification of organic molecules in works of art and archaeological artefacts. Determining the materials present in a sample provides key information about the technological and artistic practices involved in the object's creation⁴—such as identifying specific binders or adhesives that reveal workshop traditions—and about its historical and cultural context. In archaeology, organic residue identification in ceramic vessels can illuminate dietary habits, trade networks, and ritual

practices.^{5–8} However, organic compounds in historical and archaeological samples occur in trace amounts, are embedded in complex matrices, and often exhibit high levels of degradation and contamination. These challenges, combined with the limited availability of material, necessitate the development of optimized, minimally invasive workflows involving selective extraction and high-resolution MS analysis to maximize analytical performance while minimizing sampling impact.

Within this context, several innovative MS-based workflows were developed through this doctoral study and applied across distinct case studies, including the study of restoration adhesives (glue lining pastes)⁹, organic residues in archaeological pottery, historical iron-gall inks, and ancient proteinaceous fibers, as well as the implementation of minimally invasive identification techniques. Collectively, these investigations advanced the molecular identification of organic compounds in CH materials while reducing the analytical footprint on the studied objects.

The adoption of multimethodological analytical approaches proved particularly effective for the molecular fingerprinting of complex historical materials. By combining GC–MS, bottom-up proteomics, and sometimes complementary spectroscopic and microscopic techniques, it was possible to achieve comprehensive identification of organic components in diverse contexts. This integrated strategy was first applied to organic residue analysis in archaeological pottery, where lipid and sugar analyses and proteomics jointly revealed the molecular composition of vessel contents, refined through archaeological contextual data. A similar multi-analytical workflow—combining GC–MS, HPLC–ESI–QTOF, microscopy and spectroscopic techniques—was applied to historical ink samples from the Royal Palace Museum of Caserta, identifying both original components and degradation products and reconstructing the complex ink recipe. Furthermore, a multi-step extraction protocol followed by GC–MS and proteomic analysis enabled the simultaneous recovery and identification of the diverse organic components of historical glue-lining pastes from a single microsample.⁹ These studies demonstrated that multi-analytical workflows can provide a more complete chemical picture of complex matrices while maintaining minimal invasiveness.

A second major result was the development of a tailored sample preparation protocol for the proteomic analysis of highly degraded, carbonized proteinaceous materials available in minute quantities. This approach enabled the successful identification of ancient silk fibers from a gold textile excavated in the Vesuvian area, overcoming long-standing

difficulties related to silk insolubility, degradation, and low protein content. The optimized protocol established a methodological benchmark for the molecular analysis of carbonized ancient fibers.

The research further advanced minimally invasive proteomic methodologies. The application of a trypsin-functionalized cellulose acetate sheet developed within the research group for in situ protein digestion directly on artwork surfaces^{10,11} allowed the identification of proteinaceous binders from a 14th-century painting at the Capodimonte Museum (Naples) without physical material removal. Building on this methodology, a new analytical workflow was designed, in collaboration with the University of Bordeaux, for minimally invasive spatially-resolved proteomics in heritage science, with the aim of bridging chemical identification with spatial information. For the first time, sampling using a trypsin-functionalized sheet was coupled directly with MALDI-MSI, capturing the spatial localization of the protein content while minimizing impact on the artwork. Preliminary experiments on casein-based paint replicas highlighted both the potential and the technical challenges of this strategy, particularly in maintaining spatial fidelity and minimizing peptide diffusion. Future efforts will be oriented toward overcoming these difficulties and validating the method on surfaces and cross-sections of historical artifacts.

Beyond molecular identification, the second axis of the research focused on the chemical characterization of proteinaceous materials to elucidate degradation mechanisms and long-term molecular evolution. Proteins such as collagen, casein, and egg proteins—widely used as binders, adhesives, and coatings—undergo complex chemical and structural transformations over time, including oxidation, deamidation, backbone cleavage, and crosslinking, which affect both their physical properties and their detectability by proteomic methods.^{12–16} Interactions with inorganic pigments further modulate degradation, promoting or inhibiting specific chemical reactions through metal–protein interactions that remain only partially understood.¹⁷ Because diagenetic modifications encode information on manufacturing and degradation histories of proteinaceous materials, their study is essential for interpreting protein degradation in CH contexts. Yet, no standardized protocol currently exists in this field for the systematic evaluation and interpretation of chemical modifications.

To address these challenges, an innovative analytical and computational pipeline was developed to assess and visualize chemical modification distributions along protein sequences, enabling semi-quantitative comparison of degradation patterns among samples.

Initially implemented in Excel and subsequently automatized in Python, this workflow facilitated the comparative assessment of post-translational and diagenetic modifications detected by software like MaxQuant, allowing direct interpretation of degradation pathways and structural susceptibilities. The integration of high-resolution MS with this computational framework significantly enhanced the interpretative power of MS/MS data from degraded proteinaceous materials.

This pipeline supported the first proteomic investigation of pigment–protein binder interactions, addressing a key but underexplored aspect of painting conservation. The study focused on casein-based paint reconstructions artificially aged in the presence of four inorganic pigments. The extent and distribution of chemical modifications were mapped across casein sequences, revealing pigment-specific effects on protein ageing. Azurite was shown to promote extensive oxidative damage, consistent with its known role in metal-catalyzed oxidation, while calcium carbonate displayed stabilizing interactions linked to calcium–phosphate coordination sites on the protein. These results provide molecular evidence of pigment-induced degradation patterns that had previously been inferred primarily through spectroscopic methods.^{17–22} Building on these findings, a limited proteolysis mass spectrometry (LiP–MS) approach was proposed, for the first time to CH materials, probing structural alterations in proteins induced by pigment–binder interactions. The approach paves the way for the molecular elucidation of protein structural dynamics and pigment-specific reactivity in painted systems, offering a route to connect chemical modification patterns to three-dimensional structural alterations.

In parallel, collagen-based materials, ranging from historical animal glues used in restoration practices to modern bioadhesives derived from tannery waste, were investigated, in collaboration with the University and the CNR Institute of Pisa, to evaluate how preparation methods and chemical treatments influence molecular integrity and adhesive performance.²⁰ Comparative proteomic analyses revealed distinct profiles of deamidation, oxidation, and backbone cleavage depending on processing chemistry—such as chrome or vegetable tanning, or alkaline boiling—confirming that manufacturing treatments leave diagnostic molecular fingerprints. These results, consistent with previous observations on collagen samples^{15,16,23,24}, demonstrated that even coeval materials (e.g., bone collagen from Pompeii and Herculaneum¹⁵) can exhibit markedly different modification profiles attributable to distinct thermal, chemical and environmental conditions. The findings link molecular degradation patterns to macroscopic material

behavior, providing a molecular-level framework for evaluating both historical and modern collagen-based adhesives.

Overall, this doctoral research advanced the analytical framework for the identification and chemical characterization of organic materials in CH, providing new insights into the composition and degradation mechanisms of historical organic substances. By integrating multi-analytical approaches, tailored sample preparation protocols, minimally invasive in situ proteomics, and advanced computational pipelines, this work enhanced both the sensitivity and the interpretative power of MS applied to CH, achieving significant progress in adapting MS-based strategies to the specific challenges posed by these materials. Ultimately, the knowledge gained through this research underscores that the preservation of CH is, at its core, a molecular endeavor—one that bridges the material and the intangible, the past and the future.

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PhD Activities Summary

Attended Courses

“Bioplastics” by Prof. C. V. L. Giosafatto, Dept. Chemical Sciences, 16 h, February 2023.

“Natural Phenolic Compounds: Structure, Reactivity and Applications” by Prof. L. Panzella, Dept. Chemical Sciences, 16 h, February-March 2023.

“Chemical methodologies for cultural heritage” by Prof. A. Vergara and Prof. L. Birolo, Dept. Chemical Sciences, 16 h, July 2023.

“Applications of Optical Microscopy” by Prof. M. Rossi, Dept. Chemical Sciences, 4 h, July 2023.

“Entrepreneurship And Innovation 2024” by Prof. Pierluigi Rippa, Piazzale Tecchio Room C, 12 h, June 2024. (Transversal course)

“PhD Course in the frame of the arCHIMede project of the Department of Chemical Sciences”, May 8-15-16, 2024.

“Physical chemistry I” (CHIM/02), Prof. Paduano (Module A), Dept. Chemical Sciences, October 2, 2025 / Prof. Crescenzi (Module B), Dept. Chemical Sciences, September 18, 2025.

“General and Inorganic chemistry II” (CHIM/03), Prof. Busico, Dept. Chemical Sciences, October 9, 2025.

Attended Congresses

CONGRESSO ABC – XX Congresso Nazionale della Divisione di Chimica dell'Ambiente e dei Beni Culturali, Ischia, September 28 – 1 October, 2023.

MetroArchaeo 2023 - International Conference on Metrology for Archaeology and Cultural Heritage, Rome, October 19-21, 2023.

The Omics & Heritage (O&H) Workshop Metagenomes and Microbiomes for the study of cultural heritage conservation and archaeology, Rome, May 15-16, 2024.

Il contributo dei giovani chimici in Campania, Università degli Studi della Campania Luigi Vanvitelli, Caserta, July 19, 2024.

Proteomics for the Study of the Past: From the Field to the Museum, University of Bordeaux, Bordeaux (France), December 12, 2024.

TECHNART 2025 - International Conference on Analytical Techniques for Heritage Studies and Conservation, Perugia, May 6-9, 2025.

JFSM 2025 – 41st French Days of Mass Spectrometry, Montpellier, June 30 – July 3, 2025.

Attended Schools

EuBIC-MS Winter School 2024, Winterberg, Germany, January 15-19, 2024. This winter school is an initiative of the European Proteomics Association (EuPA) for user-oriented bioinformatics for mass spectrometry-based omics. Lectures and practical workshops have covered the aspects of identification, quantification, result interpretation, and integration of MS data.

Bordeaux Summer School - Advanced mass spectrometry applied to cultural heritage, Bordeaux, France, June 16-20, 2025. This summer school is an innovative and inter / multi-disciplinary training based on advanced MS to decipher the complexity of cultural heritage material, i.e., artworks, archaeological and paleontological materials, via their organic macromolecular signatures encoding (proteins, lipids, polysaccharides).

Presented Posters

“Current analytical challenges for the identification of organic residues in archaeological pottery”, XX Congresso Nazionale della Divisione di Chimica dell'Ambiente e dei Beni Culturali, Ischia, September 28 - October 1, 2023.

“Molecular characterization of collagen-based animal glues by proteomics and spectroscopic analyses”, XX Congresso Nazionale della Divisione di Chimica dell'Ambiente e dei Beni Culturali, Ischia, September 28 - October 1, 2023.

“Proteomics and spectroscopic analyses for the molecular characterization of collagen-based animal glues”, MetroArchaeo 2023 - International Conference on Metrology for Archaeology and Cultural Heritage, Rome, October 19-21, 2023.

“The challenge of extracting proteins from potteries”, MetroArchaeo 2023 - International Conference on Metrology for Archaeology and Cultural Heritage, Rome, October 19-21, 2023.

“Molecular characterization of collagen-based animal glues by proteomics and spectroscopic analyses”, EuBIC-MS Winter School 2024, Winterberg, Germany, January 15-19, 2024.

“From leather wastes to high-value natural materials: chemical characterization of collagen from leather wastes using proteomics approaches”, XVIII Congresso Internazionale dell'Italian Proteomics Association (ItPA), Rome, November 27-29, 2024.

“Immobilized trypsin – enhanced MALDI-Imaging analysis of surfaces and cross-sections of works of art”, TECHNART 2025 - International Conference on Analytical Techniques for Heritage Studies and Conservation, Perugia, May 6-9, 2025.

“From Leather Wastes To Collagen-Based Glues: Proteomic Characterization”, JFSM 2025 – 41st French Days of Mass Spectrometry, Montpellier, June 30 – July 3, 2025.

Presented Oral Presentations

“The challenge of extracting proteins from potteries”, MetroArchaeo 2023 - International Conference on Metrology for Archaeology and Cultural Heritage, Rome, October 19-21, 2023.

“Binder-pigment interaction by proteomic approaches”, The Omics & Heritage (O&H) Workshop Metagenomes and Microbiomes for the study of cultural heritage conservation and archaeology, Rome, May 15-16, 2024.

“Binder-pigment interaction by proteomic approaches”, Il contributo dei giovani chimici in Campania, Università degli Studi della Campania Luigi Vanvitelli, Caserta, July 19, 2024.

“From Leather Wastes To Collagen-Based Glues: Proteomic Characterization”, TECHNART 2025 - International Conference on Analytical Techniques for Heritage Studies and Conservation, Perugia, May 6-9, 2025.

“Immobilized trypsin – enhanced MALDI-Imaging analysis of surfaces and cross-sections of works of art”, JFSM 2025 – 41st French Days of Mass Spectrometry, Montpellier, June 30 – July 3, 2025.

Awards

Best Poster Award, TECHNART 2025 - International Conference on Analytical Techniques for Heritage Studies and Conservation, Perugia, May 6-9, 2025.

Attended Seminars

Janeiro-na-Madeira (J-n-M) Winter Summer Event on proteomics and related topics, conference online, January 9-13, 2023.

“Science Monday Workshop, Workshop for chemistry and biology in glycoscience and related fields” by Prof. Yann Guerardel, Jesus Angulo, Angelo Fontana, Linda Cerofolini, Dept. Chemical Sciences, February 27, 2023.

“To B or not to B” in Nucleic Acids research” by Prof. Ulf Olsson from Konan University and “Quadruplexes are everywhere!” by Dr. Jean-Louis Mergny from the Institut Polytechnique de Paris, Dept. Chemical Sciences, May 17, 2023.

“On discovery and sensitivity in (photo)catalysis” by prof. Frank Glorius from Westfälische Wilhelms-Universität Münster, Complesso Universitario Monte S. Angelo, September 5, 2023.

“Il contributo della Chemiometria ai nuovi approcci multiparametrici della Chimica Analitica by Prof. S. Materazzi from La Sapienza University of Rome, Dept. Chemical Sciences, October 27, 2023.

“Novel physical and chemical methodologies for the conservation and restoration of cultural heritage”, III edition, Accademia Nazionale delle Scienze detta dei XL”, conference online, October 25, 2023.

“Single-molecule assays for point of care” by prof. Luisa Torsi from University of Bari Aldo Moro, Dept. Chemical Sciences, December 5, 2023.

"Biorefinery: Challenges on making Science and changing lives in Amazon" by Prof. Luis Adriano Santos do Nascimento dell' Universidade Federal do Pará, Dept. Chemical Sciences, February 12, 2024.

Period Abroad

Secondment of 6 months at the laboratory of the Bordeaux Proteome Platform (<https://proteome.u-bordeaux.fr/>), University of Bordeaux, Bordeaux (France), under the supervision of Prof. C. Tokarski. The research activities at the Bordeaux Proteome Platform primarily focused on developing a minimally invasive kit for the spatially resolved MALDI imaging analysis of surfaces and cross-sections of paint layers and works of art (see Section 4.4).

Support For Teaching Activity

Supervision and mentoring of 5 MSc. students in Chemical Sciences. Encouraging free expression, creativity and directly participation in the planning and the execution of the experiments, students have been introduced in the field of the chemistry of cultural heritage and achieved excellent results.

Participated to the organization of orienteering activities and events for the Dept. of Chemical Sciences.

Publications

Published

Cipolletta, B., Morelli, M., Perlingieri, C., Somma, P. I., Amoresano, A., Marino, G., & Carpentieri, A. (2024). Molecular Characterization of Adhesives (Glue Lining Pastes) Used in Restoration. *Analytical Chemistry*, 96(42), 16551-16560. <https://doi.org/10.1021/acs.analchem.4c02138>.

Pulidori, E., Duce, C., Bramanti, E., Ciccone, L., **Cipolletta, B.**, Tani, G., ... & Bonaduce, I. (2025). Characterization of the secondary structure, renaturation and physical ageing of gelatine adhesives. *Scientific Reports*, 15(1), 23022. <https://doi.org/10.1038/s41598-025-04910-8>.

Submitted

Pelosi C., Micheli, E., Pulidori, E., Caroti, G., **Cipolletta, B.**, ..., & Bonaduce, I. (2025). From waste to function: valorization of collagen-based wastes with Natural Deep Eutectic Solvents for bioadhesive applications. *Submitted*.

In preparation

Carpentieri, A., Rossi, M., Schisano, C., Fiore, M., Alberico, M., **Cipolletta, B.**, ... & Vergara, A. (2025). Chemical characterization of a funeral equipment from a Hellenistic young woman tomb found in Battipaglia. *Manuscript in preparation*.

Cipolletta, B., Aprea, C., Pizzimenti, S., Pinto, G., ... & Birolo, L. (2025). Molecular details of the interactions between inorganic pigments and casein-based glue in reference paint reconstructions. *Manuscript in preparation*.

Cipolletta, B., Aprea, C., Pizzimenti, S., ... & Birolo, L. (2025). Multi-tasking collagen: one molecule, different outfits and uses. *Manuscript in preparation*.

Cipolletta, B., Fiore, M., Pizzimenti, S., ... & Birolo, L. (2025). The challenge of organic content of potteries: multistrategic approaches. *Manuscript in preparation*.

Alberico, M., **Cipolletta, B.**, Rossi, M., Pironti, C., Tortino, ... & Vergara, A. (2025). The Vanvitelli's ink recipe. *Manuscript in preparation*.

Table of Contents

8.

Table of Contents clxiv

Section 11

Molecular characterization of adhesives (glue lining pastes) used in restoration 1

The challenge of organic content of potteries: multistrategic approaches **Erreur ! Signet non défini.**

Chemical Composition of Vanvitelli's Ink **Erreur ! Signet non défini.**

Proteomic analysis of ancient silk fibers from Pompeii **Erreur ! Signet non défini.**

Minimally invasive Protein Identification on a XIV century painting by Colantonio **Erreur ! Signet non défini.**

Immobilized Trypsin – Enhanced MALDI-Imaging analysis of surfaces and cross-sections of works of art **Erreur ! Signet non défini.**

Section 214

Molecular details of the interactions between inorganic pigments and casein-based glue in reference paint reconstructions **Erreur ! Signet non défini.**

Characterization of the secondary structure, renaturation and physical ageing of gelatine adhesives 14

From waste to function: valorization of collagen-based wastes with Natural Deep

Eutectic Solvents for bioadhesive applications 19

Multi-tasking collagen: one molecule, different outfits and uses **Erreur ! Signet non défini.**

List of Tables **Erreur ! Signet non défini.**

List of Figures **Erreur ! Signet non défini.**

Section 1

Molecular characterization of adhesives (glue lining pastes) used in restoration



Figure S1. Glue paste samples analyzed in this work. a) Test specimens analyzed in this study were specifically prepared for this project by the Laboratorio di Restauro delle Tele of the University Suor Orsola Benincasa. They comprise glue lining pastes that were applied to canvases and subjected to artificial aging. Different glue pastes were prepared according to seven traditional recipes documented in the literature. b) The historical glue paste sample was obtained from the 17th-century painting "La fuga in Egitto", which is part of the Pagliara collection at the University Suor Orsola Benincasa. During the re-lining process, a micro-sample of the historical glue paste used in the 17th century was extracted from the back of the painting.

Identified compound	RT (min)	B1	B2	E1	C1	C2	D1	D2	Biological matrix associated
Azelaic acid	11,37	X	X	X	X	X	X	X	Oxidation production of fatty acids
Myristic acid	15,23	X		X					Vegetable oils; nutmeg butter
Palmitic acid	18,8	X	X	X	X	X	X	X	Vegetable oils; ox gall; flour
Linoleic acid	20,93	X		X					Vegetable oils; linseed meal
Oleic acid	21,2	X			X				Vegetable oils; ox gall; flour
Stearic acid	21,3	X	X	X	X		X		Vegetable oils; ox gall; flour
Isopimaric acid	22,4		X	X					Pine resin
Kaur-16-en-19-oic acid	22,46				X				Vegetable resin
Podocarp-8-en-16-oic acid	22,5			X	X	X		X	Pine resin
Dehydroabietic acid (DHA)	23,5		X	X	X	X		X	Pine resin
12-beta-hydro-ketomethylpodocarpa	23,8		X	X					Vegetable resin
7-oxo-dehydroabietic acid (7-ODA)	25,6		X	X	X	X		X	Pine resin

Table S1. GC-MS results obtained for the nonpolar fraction of the test specimens.

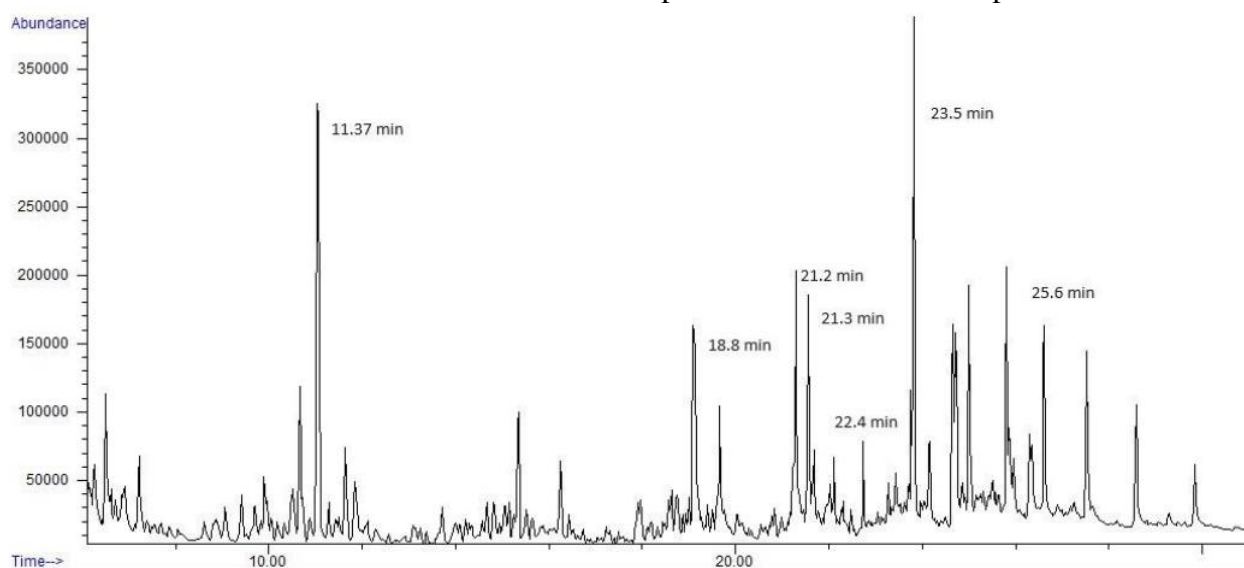


Figure S2. Total ion current (TIC) of the nonpolar fraction of the sample C1 (reported as a significant example). Diterpenoid acids with abietane skeleton are encountered in the range 20–35 min. The main one is dehydroabietic acid (DHA), accompanied by 7-oxo-dehydroabietic acid (7-ODA). DHA and 7-ODA are the principal markers of Venetian turpentine.

Identified compound	RT (min)	B1	B2	E1	C1	C2	D1	D2	Biological matrix associated
Xylose	14,8	X	X	X	X	X	X	X	Honey; Molasses
Xylose	15,2	X		X					Honey; Molasses
Xylose	15,6	X	X	X	X	X	X	X	Honey; Molasses
Mannose	17,49	X		X					Honey; Molasses
Glucose	19,04	X			X				Flour; Honey; Molasses
Glucose	19,06	X	X	X	X		X		Flour; Honey; Molasses
Myoinositol	21,7		X	X					Honey; Molasses

Table S2. GC-MS results obtained for the polar fraction of the test specimens.

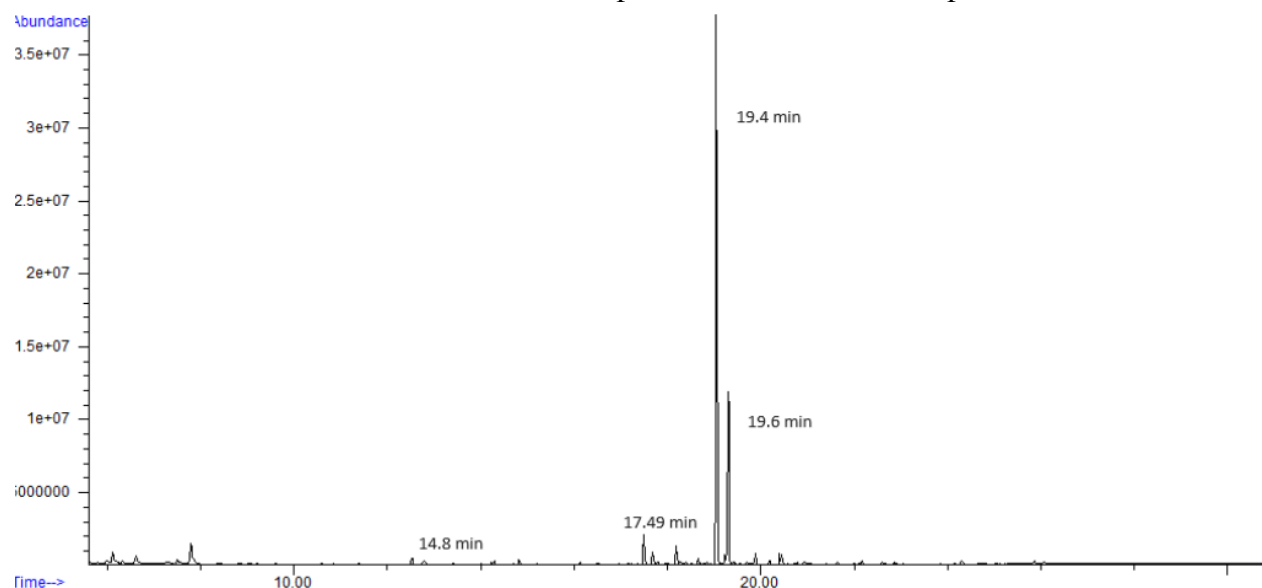


Figure S3. Total ion current (TIC) of the polar fraction of the sample B2 (reported as a significant example). The most intense peak at 19.6 min (RT), present in all the analyzed glue pastes, can be attributed to the dominant presence of glucose in samples. Glucose is contained into various biological matrices expected in the glue pastes, the most important being starch from flours, honey and molasses.

Species identification in test specimens containing fish glue

The identification of fish collagen chains was investigated by sequence homology with other fish species. Results obtained from BLASTp searches are reported in the following tables.

Fish-specific peptides of collagen chains identified in sample B1 with all possible taxonomic attributions obtained by BLASTp						
Peptide sequence identified by MS/MS	Protein assignment (BlastP results)			Attributed matrix		
	Protein name	Protein identifier (NCBI)	Taxonomy	Bovine glue	Rabbit glue	Fish glue
GAAGEAGKPGER	Collagen alpha-1(I) chain	A0A3N0XEP2	Anabartius grahami			
	Collagen, type I, alpha 1a	A0A8B9RJ30	Astyanax mexicanus			
	Collagen alpha-1(I) chain	A0A556TV12	Bagarius yarrelli			
	Collagen type I alpha 1	B5U968	Carassius auratus			
	Collagen alpha-1(I) chain Alpha-1 type I collagen	A0A6G1QBU3	Channa argus			
	Collagen alpha-1(I) chain-like	A0A8J4WXG7	Clarias magur			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen alpha-1(I) chain	A0A8D8JV18	Culex pipiens			
	Collagen alpha-1(I) chain-like	A0A3Q2CSU4	Cyprinodon variegatus			
	Collagen, type I, alpha 1a	A0A8C1T6P8	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen alpha-1(I) chain precursor	Q6U1J5	Danio rerio			
	Collagen, type I, alpha 1a	A0A553NGG9	Danionella translucida			
	Collagen type I alpha 1 chain	A0A4W4E9W2	Electrophorus electricus			
	Collagen alpha-1(I) chain	A0A3P8YD14	Esoc lucius			
	Collagen alpha-1(I) chain	A0A3Q2P8B7	Fundulus heteroclitus			
	Collagen type I alpha 1	A0A077B3P8	Hypophthalmichthys molitrix			
	Collagen type I alpha 1 chain	W5N610	Lepisosteus oculatus			
	Collagen, type I, alpha 1a	A0A3P9CTF5	Maylandia zebra			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Collagen alpha-1(I) chain-like	A0A9D2XFE3	Nothobranchius furzeri			
	Collagen, type I, alpha 1a	A0A1A8K041	Nothobranchius kuhntae			
	Collagen, type I, alpha 1a	A0A668RYQ9	Oreochromis aureus			
	Collagen, type I, alpha 1a	I3JGN1	Oreochromis niloticus			
	Collagen, type I, alpha 1a	A0A087YGY9	Poecilia formosa			
	Collagen alpha-1(I) chain-like	A0A3B3U341	Poecilia latipinna			
	Collagen type I alpha 1 chain	A0A3B3YK05	Poecilia mexicana			
	Collagen alpha-1(I) chain-like	A0A3P9NHJ7	Poecilia reticulata			
	Collagen type I alpha 1 chain	A0A3B4CGH3	Pygocentrus nattereri			
	Collagen alpha-1(I) chain-like	A0A671T011	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SC42	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWV7	Sinocyclocheilus rhinoceros			
	Collagen type I alpha 1 chain	A0A3B5QFH2	Xiphophorus maculatus			
GESGPAGPAGAAGPAGPR	Collagen alpha-1(I) chain-like	A0A6P6JU61	Carassius auratus			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen, type I, alpha 1a	A0A8C1T7W7	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Uncharacterized protein	A0A7J6DBF9	Onychostoma macrolepis			
	Collagen alpha-1(I) chain-like	A0A671LPK0	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SCV6	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWD5	Sinocyclocheilus rhinoceros			
GPAGPPGATGFPGSAGR	Type 1 collagen alpha 1	Q5NT96	Paralichthys olivaceus			
EGPAGPSGQDGR	Collagen, type I, alpha 2	A0A7N5ZT19	Anabas testudineus			
	Collagen alpha-2(I) chain Alpha-2 type I collagen	A0A6G1QXZ2	Channa argus			
	Collagen alpha-2(I) chain-like	A0A8J4U710	Clarias magur			
	Collagen, type I, alpha 2	A0A8C1WN17	Cyprinus carpio			
	Collagen, type I, alpha 2	A0A9J7Y6W6	Cyprinus carpio carpio			
	Collagen type I alpha 2 chain	A0A3Q3KIB6	Monopterus albus			
	Collagen type I alpha 2 chain	A0A5J6XX13	Nibeia coibor			
	Collagen, type I, alpha 2	A0A668V485	Oreochromis aureus			
	Collagen, type I, alpha 2	H3C5Y2	Tetraodon nigroviridis			
GAAGAQQGAR	Collagen type I alpha 2 chain	H2ZXF3	Latimeria chalumnae			

Table S3. Fish-specific peptides of collagen chains identified in sample B1 with all possible taxonomic attributions obtained by BLASTp.

Fish-specific peptides of collagen chains identified in sample B2 with all possible taxonomic attributions obtained by BlastP						
Peptide sequence identified by MS/MS	Protein assignment (BlastP results)			Attributed matrix		
	Protein name	Protein identifier (NCBI)	Taxonomy	Bovine glue	Rabbit glue	Fish glue
GAAGEAGKPGER	Collagen alpha-1(I) chain	A0A3N0XEP2	Anabailus grahami			
	Collagen, type I, alpha 1a	A0A8B9RJ30	Astyanax mexicanus			
	Collagen alpha-1(I) chain	A0A556TV12	Bagarius yarrelli			
	Collagen type I alpha 1	B5U968	Carassius auratus			
	Collagen alpha-1(I) chain Alpha-1 type I collagen	A0A6G1QBU3	Channa argus			
	Collagen alpha-1(I) chain-like	A0A8J4WXG7	Clarias magur			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen alpha-1(I) chain	A0A8D8JVI8	Culex pipiens			
	Collagen alpha-1(I) chain-like	A0A3Q2CSU4	Cyprinodon variegatus			
	Collagen, type I, alpha 1a	A0A8C1T6P8	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen alpha-1(I) chain precursor	Q6U1J5	Danio rerio			
	Collagen, type I, alpha 1a	A0A553NGG9	Danionella translucida			
	Collagen type I alpha 1 chain	A0A4W4E9W2	Electrophorus electricus			
	Collagen alpha-1(I) chain	A0A3P8YD14	Esoc lucius			
	Collagen alpha-1(I) chain	A0A3Q2P8B7	Fundulus heteroclitus			
	Collagen type I alpha 1	A0A077B3P8	Hypophthalmichthys molitrix			
	Collagen type I alpha 1 chain	W5N610	Lepidosteus oculatus			
	Collagen, type I, alpha 1a	A0A3P9CTF5	Maylandia zebra			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Collagen alpha-1(I) chain-like	A0A9D2XFE3	Nothobranchius furzeri			
	Collagen, type I, alpha 1a	A0A1A8K041	Nothobranchius kuhntae			
	Collagen, type I, alpha 1a	A0A668RYQ9	Oreochromis aureus			
	Collagen, type I, alpha 1a	I3JGN1	Oreochromis niloticus			
	Collagen, type I, alpha 1a	A0A087YGY9	Poecilia formosa			
	Collagen alpha-1(I) chain-like	A0A3B3U341	Poecilia latipinna			
	Collagen type I alpha 1 chain	A0A3B3YK05	Poecilia mexicana			
	Collagen alpha-1(I) chain-like	A0A3P9NHJ7	Poecilia reticulata			
	Collagen type I alpha 1 chain	A0A3B4CGH3	Pygocentrus nattereri			
	Collagen alpha-1(I) chain-like	A0A671T0I1	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SC42	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWV7	Sinocyclocheilus rhinoceros			
	Collagen type I alpha 1 chain	A0A3B5QFH2	Xiphophorus maculatus			
GGAGPPGATGPPGAAGR	Collagen alpha-1(I) chain	A0A556TIC5	Bagarius yarrelli			
	Collagen type I alpha 3	B5U970	Carassius auratus			
	Collagen alpha-1(II) chain	A0A8D8F409	Culex pipiens			
	Collagen, type I, alpha 1b	A0A8C1J6D1	Cyprinus carpio			
	Collagen, type I, alpha 1b	A0A9J8A1V2	Cyprinus carpio carpio			
	Collagen, type I, alpha 3	Q6PEI9	Danio rerio			
	Collagen, type I, alpha 1b	A0A2D0SA79	Ictalurus punctatus			
	Collagen alpha-1(I) chain-like protein	A0A498N2K6	Labeo rohita			
	Collagen type I alpha 1b	A0A7D4XVD3	Megalobrama amblycephala			
GESGPAGPAGAAGPAGPR	Collagen alpha-1(I) chain-like	A0A6P6JU61	Carassius auratus			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen, type I, alpha 1a	A0A8C1T7W7	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Uncharacterized protein	A0A7J6DBF9	Onychostoma macrolepis			
	Collagen alpha-1(I) chain-like	A0A671LPK0	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SCV6	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWD5	Sinocyclocheilus rhinoceros			

Table S4. Fish-specific peptides of collagen chains identified in sample B2 with all possible taxonomic attributions obtained by BLASTp.

Fish-specific peptides of collagen chains identified in sample C2 with all possible taxonomic attributions obtained by BlastP						
Peptide sequence identified by MS/MS	Protein assignment (BlastP results)			Attributed matrix		
	Protein name	Protein identifier (NCBI)	Taxonomy	Bovine glue	Rabbit glue	Fish glue
GESGPAGPAGAAGPAGPR	Collagen alpha-1(I) chain-like	A0A6P6JU61	Carassius auratus			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen, type I, alpha 1a	A0A8C1T7W7	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Uncharacterized protein	A0A7J6DBF9	Onychostoma macrolepis			
	Collagen alpha-1(I) chain-like	A0A671LPK0	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SCV6	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWD5	Sinocyclocheilus rhinoceros			
GPAGPPGATGFPGAAGR	Collagen alpha-1(I) chain-like	A0A3Q1GEL1	Acanthochromis polyacanthus			
	Collagen, type I, alpha 1a	A0A3Q0SY15	Amphilophus citrinellus			
	Collagen alpha-1(I) chain-like	A0A3Q1D4G0	Amphiprion ocellaris			
	Collagen, type I, alpha 1a	A0A3P8U044	Amphiprion percula			
	Collagen alpha-1(I) chain	A0A556TV12	Bagarius yarrelli			
	Collagen, type I, alpha 1a	A0A6P7NB9	Betta splendens			
	Collagen alpha-1(I) chain Alpha-1 type I collagen	A0A6G1QBU3	Channa argus			
	Collagen alpha-1(I) chain-like	A0A8J4WXG7	Clarias magur			
	Collagen alpha-1(I) chain	A0A8C4NXC0	Dicentrarchus labrax			
	Collagen, type I, alpha 1a	A0A8C4IG55	Dicentrarchus labrax			
	Collagen alpha-1(I) chain-like	A0A665UYW3	Echeneis naucrates			
	Collagen, type I, alpha 1a	A0A8C5CNN6	Gadus morhua			
	Collagen type I alpha 1 chain	A0A3Q2W9Y5	Haplochromis burtoni			
	Collagen type I alpha 1 chain	A0A3Q3CY74	Haplochromis burtoni			
	Collagen alpha-1(I) chain-like	A0A3Q3FGS3	Kryptolebias marmoratus			
	Collagen type I alpha 1 chain	A0A4W6CS94	Lates calcarifer			
	Collagen, type I, alpha 1a	A0A7N8WW01	Mastacembelus armatus			
	Collagen alpha-1(I) chain-like	A0A3Q3KD18	Monopterus albus			
	Collagen alpha-1(I) chain-like	A0A3Q4IH39	Neolamprologus brichardi			
	Collagen, type I, alpha 1a	A0A668S5I7	Oreochromis aureus			
	Uncharacterized protein	A0A5N5MFS7	Pangasianodon hypophthalmus			
	Collagen type I alpha 1	A0A291LSB7	Trachinotus ovatus			
VGPSGPAGAR	Collagen type I alpha 2	B5U969	Carassius auratus			
	Collagen type I alpha 2	E2IPR2	Ctenopharyngodon idella			
	Collagen, type I, alpha 2	A0A8C1WN17	Cyprinus carpio			
	Collagen, type I, alpha 2	A0A9J7Y6W6	Cyprinus carpio carpio			
	Collagen type I alpha 2	A0A2H4ZEX8	Hypophthalmichthys molitrix			
	Collagen alpha-2(I) chain-like protein	A0A498MWD6	Labeo rohita			
	Collagen type I alpha 2	A0A7D4XUU0	Megalobrama amblycephala			
	Collagen alpha-2(I) chain-like	A0A671RDX3	Sinocyclocheilus anshuiensis			
	Collagen type I alpha 2 chain	A0A672Q315	Sinocyclocheilus grahami			
	Collagen alpha-2(I) chain-like	A0A673HFN8	Sinocyclocheilus rhinoceros			
GAAGAQQAR	Collagen type I alpha 2 chain	H2ZXF3	Latimeria chalumnae			

Table S5. Fish-specific peptides of collagen chains identified in sample C2 with all possible taxonomic attributions obtained by BLASTp.

Fish-specific peptides of collagen chains identified in sample D1 with all possible taxonomic attributions obtained by BlastP						
Peptide sequence identified by MS/MS	Protein assignment (BlastP results)			Attributed matrix		
	Protein name	Protein identifier (NCBI)	Taxonomy	Bovine glue	Rabbit glue	Fish glue
GESGPAGPAGAAGPAGPR	Collagen alpha-1(I) chain-like	A0A6P6JU61	Carassius auratus			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen, type I, alpha 1a	A0A8C1T7W7	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Uncharacterized protein	A0A7J6DBF9	Onychostoma macrolepis			
	Collagen alpha-1(I) chain-like	A0A671LPK0	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SCV6	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWD5	Sinocyclocheilus rhinoceros			
TGDREGSPAGPAGAAGPAGP	Collagen alpha-1(I) chain-like	A0A6P6JU61	Carassius auratus			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen, type I, alpha 1a	A0A8C1T7W7	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Collagen alpha-1(I) chain-like	A0A671LPK0	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SCV6	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWD5	Sinocyclocheilus rhinoceros			
GPAGPPGATGFPGAAGR	Collagen alpha-1(I) chain-like	A0A3Q1GEL1	Acanthochromis polyacanthus			
	Collagen, type I, alpha 1a	A0A3Q0S5Y1	Amphilophus citrinellus			
	Collagen alpha-1(I) chain-like	A0A3Q1D4G0	Amphiprion ocellaris			
	Collagen, type I, alpha 1a	A0A3P8U044	Amphiprion percula			
	Collagen alpha-1(I) chain	A0A556TV12	Bagarius yarrelli			
	Collagen, type I, alpha 1a	A0A6P7NB9	Betta splendens			
	Collagen alpha-1(I) chain Alpha-1 type I collagen	A0A6G1QBU3	Channa argus			
	Collagen alpha-1(I) chain-like	A0A8J4WXG7	Clarias magur			
	Collagen alpha-1(I) chain	A0A8C4XNC0	Dicentrarchus labrax			
	Collagen alpha-1(I) chain-like	A0A665UYW3	Echeneis naucrates			
	Collagen, type I, alpha 1a	A0A8C5CNN6	Gadus morhua			
	Collagen type I alpha 1 chain	A0A3Q2W9Y5	Haplochromis burtoni			
	Collagen alpha-1(I) chain-like	A0A3Q3FGS3	Kryptolebias marmoratus			
	Collagen type I alpha 1 chain	A0A4W6CS94	Lates calcarifer			
	Collagen, type I, alpha 1a	A0A7N8W01	Mastacembelus armatus			
	Collagen alpha-1(I) chain-like	A0A3Q3KD18	Monopterus albus			
	Collagen alpha-1(I) chain-like	A0A3Q4IH39	Neolamprologus brichardi			
	Collagen, type I, alpha 1a	A0A668S5I7	Oreochromis aureus			
	Collagen alpha-1(I) chain-like	A0A3B4XHU9	Seriola lalandi dorsalis			
	Collagen alpha-1(I) chain-like	A0A671YMZ1	Sparus aurata			
	Collagen type I alpha 1	A0A291LSB7	Trachinotus ovatus			
	Collagen alpha-1(I) chain-like	A0A6I9N4I7	Notothenia coriiceps			
	Type 1 collagen alpha 1	Q5NT96	Paralichthys olivaceus			

Table S6. Fish-specific peptides of collagen chains identified in sample D1 with all possible taxonomic attributions obtained by BLASTp.

Fish-specific peptides of collagen chains identified in sample E1 with all possible taxonomic attributions obtained by BlastP						
Peptide sequence identified by MS/MS	Protein assignment (BlastP results)			Attributed matrix		
	Protein name	Protein identifier (NCBI)	Taxonomy	Bovine glue	Rabbit glue	Fish glue
QGPGSPSGER	Collagen alpha-1(I) chain	A0A444UK09	Acipenser ruthenus			
	Collagen alpha-1(I) chain	A0A3N0XEP2	Anabarrilus grahami			
	Collagen type I alpha 1	B5U968	Carassius auratus			
	Collagen type I alpha 1	E2GK07	Ctenopharyngodon idella			
	Collagen alpha-1(I) chain	A0A8D8JV18	Culex pipiens			
	Collagen, type I, alpha 1a	A0A8C1N292	Cyprinus carpio			
	Collagen, type I, alpha 1a	A0A9J7X041	Cyprinus carpio carpio			
	Collagen alpha-1(I) chain precursor	Q6U1J5	Danio rerio			
	Collagen type I alpha 1 chain	M3W2F5	Felis catus			
	Collagen type I alpha 1	A0A077B3P8	Hypophthalmichthys molitrix			
	Collagen type I alpha 1 chain	A0A667J4W5	Lynx canadensis			
	Collagen alpha-1 chain	A0A485NGL2	Lynx pardinus			
	Collagen type I alpha 1a	A0A7D4Y5M8	Megalobrama amblycephala			
	Collagen type I alpha 1	G9M6I5	Oreochromis niloticus			
	Collagen type I alpha 1 chain	K7FHL1	Pelodiscus sinensis			
	Collagen type I alpha 1 chain	A0A6J2M7Z2	Phyllotomus discolor			
	Collagen type I alpha 1 chain	A0A670J592	Podarcis muralis			
	Collagen alpha-1(I) chain-like	A0A671SWL5	Sinocyclocheilus anshuiensis			
	Collagen alpha-1(I) chain-like	A0A672SBR4	Sinocyclocheilus grahami			
	Collagen alpha-1(I) chain-like	A0A673GWD5	Sinocyclocheilus rhinoceros			
	Collagen type I alpha 1 chain	A0A673UBH8	Suricata suricatta			
	Collagen type I alpha 1 chain	A0A5G2QQE9	Sus scrofa			
	Collagen alpha-1(I) chain	A0A1L8ESC0	Xenopus laevis			
	Collagen, type I, alpha 1	L7N2N3	Xenopus tropicalis			

Table S7. Fish-specific peptides of collagen chains identified in sample E1 with all possible taxonomic attributions obtained by BLASTp.

Peptides identified in test specimens and attributed to a single animal/vegetal species

Samples	Peptide sequence identified by MS/MS	Protein assignment		
		Protein name	Taxonomic attribution	Attributed matrix
B1	GAAGEAGKPGER	Collagen alpha-1(I)	Neopterygii	Fish glue
	GESGPAGPAGAAGPAGPR		Neopterygii	Fish glue
	GPAGPPGATGFPGSAGR		Paralichthys olivaceus	Fish glue
	EGPAGPSGQDGR	Collagen alpha-2(I)	Neopterygii	Fish glue
	GAAGAQGAR		Latimeria chalumnae	Fish glue
B2	GAAGEAGKPGER	Collagen alpha-1(I)	Neopterygii	Fish glue
	GGAGPPGATGFPGAAGR		Neopterygii	Fish glue
	GESGPAGPAGAAGPAGPR		Cyprinoidei	Fish glue
	VGGVPGLLER		Oryctolagus cuniculus	Rabbit glue
	QTTQTETPPK		Oryctolagus cuniculus	Rabbit glue
	SGDRGETVSSR	Collagen alpha-2(I)	Oryctolagus cuniculus	Rabbit glue
	GDAGPKGADGSPGKDGVR		Oryctolagus cuniculus	Rabbit glue
	GPAGPTGPAGK		Oryctolagus cuniculus	Rabbit glue
	DGNPGSDGPPGR		Oryctolagus cuniculus	Rabbit glue
C1	GADGAPGKDGVR	Collagen alpha-1(I)	Bos taurus	Bovine glue
	GETGPAGPAGIPVGAR		Bos taurus	Bovine glue
	GEPGPTGVQPPGPAGEEGK		Oryctolagus cuniculus	Rabbit glue
	KTNEWGKTIIIEYK	Collagen alpha-2(I)	Oryctolagus cuniculus	Rabbit glue
	TNEWGKTIIIEYKTNKPSR		Oryctolagus cuniculus	Rabbit glue
C2	GESGPAGPAGAAGPAGPR	Collagen alpha-1(I)	Neopterygii	Fish glue
	GPAGPPGATGFPGAAGR	Collagen alpha-2(I)	Craniata	Fish glue
	VGPSGPAGAR		Neopterygii	Fish glue
	GAAGAQGAR		Cynipriphysae	Fish glue
D1	GESGPAGPAGAAGPAGPR	Collagen alpha-1(I)	Cyprinoidei	Fish glue
	TGDRGESGPAGPAGAAGPAGPR		Cyprinoidei	Fish glue
	GPAGPPGATGFPGAAGR		Craniata	Fish glue
	GGAGPPGATGFPGSAGR		Notothenia coriiceps	Fish glue
	GPAGPPGATGFPGSAGR		Paralichthys olivaceus	Fish glue
D2	SGDRGETVSSR	Collagen alpha-1(I)	Oryctolagus cuniculus	Rabbit glue
	ETSSTLAERVK		Oryctolagus cuniculus	Rabbit glue
	RCGSPSPWPGEDSSQCRVGR		Oryctolagus cuniculus	Rabbit glue
	ADQPRSPPSLRPK	Collagen alpha-2(I)	Oryctolagus cuniculus	Rabbit glue
E1	QGSPGSPGER	Collagen alpha-1(I)	Metazoa	Fish glue

Table S8. Species-specific peptides of collagen identified in test specimens.

Species identification of peptides from flour in test specimens

Peptide sequence identified by MS/MS	Protein name	Taxonomic attribution (Blast results)			Attributed matrix
		Triticum aestivum	Secale cereale	Linum usitatissimum	
AQQPATQLPTVCR	HMW glutenin				Wheat flour
LTAASITAVCR	Dimeric alpha-amylase inhibitor				Wheat flour/Rye flour
AQQLAAQLPAMCR	HMW glutenin subunit				Wheat flour/Rye flour
VAIMEVNPR	Globulin 3B				Wheat flour
VFLQQQCIPVAMQR	LMW glutenin				Wheat flour
VAIMEVNPR	Globulin 3C				Wheat flour
LQCVGSQVPEAVLR	Monomeric alpha-amylase inhibitor				Wheat flour
SAPEELVQQVLSAGWR	Beta-amylase				Wheat flour
RSDPNSSVLK	Alpha-amylase/trypsin inhibitor CM1				Wheat flour
APFASIVAGIGGQ	Gamma-gliadin				Wheat flour
NIVSCADILALAAR	Peroxidase 16 precursor				Wheat flour

Table S19. Taxonomic attribution of peptides of flour proteins identified in sample B1.

Peptide sequence identified by MS/MS	Protein name	Taxonomic attribution (Blast results)			Attributed matrix
		Triticum aestivum	Secale cereale	Linum usitatissimum	
AAAAMVGHPEWEFPR	Beta-amylase				Wheat flour
SGPELTIEMILQAAQPK	Beta-amylase				Wheat flour
NVGVSDDIFYTDQHGTR	Beta-amylase				Wheat flour
NIEYLTGVDQPLFHGR	Beta-amylase				Wheat flour/Rye flour
LVAVSQVVR	HMW glutenin subunit				Wheat flour
ELQESSLEACR	HMW glutenin subunit				Wheat flour/Rye flour
SVAVSQVAR	HMW glutenin subunit				Wheat flour
EALQAEVGLPVDR	Granule-bound starch synthase				Wheat flour/Rye flour
ACQQVMDQQLR	HMW glutenin subunit				Wheat flour
AQQLAAQLPAMCR	HMW glutenin subunit				Wheat flour
VAIMEVNPR	Globulin 3B				Wheat flour
LQCVGSQVPEAVLR	Monomeric alpha-amylase inhibitor				Wheat flour
QQMADAVTALYGR	rRNA N-glycosylase				Wheat flour/Rye flour
DLLGDTDKLTNVALGR	rRNA N-glycosylase				Wheat flour/Rye flour
LEGSDALSTR	HMW glutenin subunit				Wheat flour
LTAASITAVCR	Dimeric alpha-amylase inhibitor				Wheat flour/Rye flour
CQAIHNVAEAIR	Avenin-like protein				Wheat flour
EFIAGIVGR	Trypsin/alpha-amylase inhibitor				Wheat flour
LLAEALGTSGK	Triticin				Wheat flour
GAVVSCADILALAAR	Peroxidase				Wheat flour
DALLQQCSPVADMSFLR	Gamma gliadin				Wheat flour
LGGFLGIWR	Puroindoline b				Wheat flour

Table S10. Taxonomic attribution of peptides of flour proteins identified in sample B2.

Peptide sequence identified by MS/MS	Protein name	Taxonomic attribution (Blast results)			Attributed matrix
		Triticum aestivum	Secale cereale	Linum usitatissimum	
LGSLGSR	Globulin-3A				Wheat flour
VAIMEVNPR	Globulin-3A				Wheat flour
LVAVSQVVR	HMW glutenin subunit				Wheat flour
QGGQLEGGQPGGQQTR	HMW glutenin subunit				Wheat flour
SGNVGESGLIDLPQCPR	Alpha-amylase/trypsin inhibitor				Wheat flour
CGDLSSMLR	Monomeric alpha-amylase inhibitor				Wheat flour
LQCVGSQVPEAVLR	Monomeric alpha-amylase inhibitor				Wheat flour
LTAASITAVCR	Dimeric alpha-amylase inhibitor				Wheat flour/Rye flour
LQCNQSQVPEAVLR	Dimeric alpha-amylase inhibitor				Wheat flour
LPIVIDASGDGAYVCK	Dimeric alpha-amylase inhibitor				Wheat flour
AQQPATQLPTVCR	HMW glutenin subunit				Wheat flour
YDPTAYNTILR	Beta-amylase				Wheat flour/Rye flour
LSNQLVEGQNYVNFK	Beta-amylase				Wheat flour
TSPASTGLTLATR	rRNA N-glycosylase				Wheat flour/Rye flour
QQMADAVTALYGR	rRNA N-glycosylase				Wheat flour/Rye flour
CCKELYDASQHCR	Alpha amylase inhibitor CM1				Wheat flour
TGPYCYAGMGLPINPLEGCR	Alpha amylase inhibitor CM1				Wheat flour
DIGLAAGLLR	Peroxidase 1				Wheat flour
GAVVSCADILALAAR	Peroxidase 1				Wheat flour
QILEQQLTGR	Globulin 1				Wheat flour
CCQQLSVSR	Globulin 1				Wheat flour
DYEQSMPPLGEGR	Globulin 1				Wheat flour
VAYLDAAPR	Cupin type-1 domain-containing protein				Wheat flour
DVSPECQPVGGPVAR	HMW glutenin subunit				Wheat flour
LEGSDALSTRQ	HMW glutenin subunit				Wheat flour
VFLQQCSPVAMPQSLAR	LMW glutenin				Wheat flour
ACQVMDQQLR	HMW glutenin subunit				Wheat flour
LLAEALGTSRK	Cupin type-1 domain-containing protein				Wheat flour
LFLQQCSPVAMPQR	LMW glutenin				Wheat flour
TTTRVPFGVGTGVGGY	LMW glutenin subunit				Wheat flour
CQAIHNVAEIR	Avenin-like b precursor				Wheat flour
QILEQQLTGR	Globulin 1				Wheat flour
DYEQSMPPLGEGR	Globulin 1				Wheat flour
QCCQPLAQISEQAR	Gamma-hordein-3				Wheat flour
ILPTMCSVNVPLYR	LMW glutenin subunit				Wheat flour
VFLQQCSPVAMPQSLAR	LMW glutenin subunit				Wheat flour
SDCQVMQQCCQLAQIPR	Gamma-gliadin				Wheat flour
VFLQQCIPVAMQR	LMW glutenin subunit				Wheat flour
TPDYVEEAR	Oleolin				Wheat flour
MADAAAAAGHKTQQAGHAIQSR	Oleolin				Wheat flour
DALLQQCSPVADMSFLR	Gamma gliadin				Wheat flour
DVAGGGGAQQCPVETK	Puroindoline-A				Wheat flour
APFASIVAGIGGQ	Gamma gliadin				Wheat flour
SFIQPSLQQQLNPCK	Gamma gliadin				Wheat flour
QCCQPLAQISEQAR	Avenin-like a2				Wheat flour
SQVLQQSTYQLLR	Alpha-gliadin				Wheat flour
GLLLPAYSNAPK	Unnamed protein product				Linseed meal
SLEAQNLAEGR	Em protein				Wheat flour
VTNPATGAQVTAR	pathogenesis-related protein 4				Wheat flour

Table S11. Taxonomic attribution of peptides of flour proteins identified in sample C1.

Peptide sequence identified by MS/MS	Protein name	Taxonomic attribution (Blast results)			Attributed matrix
		Triticum aestivum	Secale cereale	Linum usitatissimum	
SCQQFLWEK	Conlinin				Linseed meal
GGQGGGQQQQCEK	Conlinin				Linseed meal
QDIQQGQQQEVER	Conlinin				Linseed meal
SCQQFMWEK	Conlinin				Linseed meal
SCQQFMWEKVQR	Conlinin				Linseed meal

Table S12. Taxonomic attribution of peptides of flour proteins identified in sample C2.

Peptide sequence identified by MS/MS	Protein name	Taxonomic attribution (Blast results)			Attributed matrix
		Triticum aestivum	Secale cereale	Linum usitatissimum	
VAIMEVNPR	Globulin-3A				Wheat flour
ELQESSLEACR	HMW glutenin subunit				Wheat flour/Rye flour
VQQPATQLPIMCR	HMW glutenin subunit				Wheat flour
QQPGQGQIGQGQLGQGR	HMW glutenin subunit				Wheat flour
SGNVGESGLIDLPGCPR	Alpha-amylase/trypsin inhibitor				Wheat flour
LLVAPGQCNLATIHNV	Alpha-amylase/trypsin inhibitor CM3				Wheat flour
ACQQVMDQQLR	HMW glutenin subunit				Wheat flour
NIEYLTGLGVDDQPLFHGR	Beta-amylase				Wheat flour
LVLANALYFK	Serpin-Z1B				Wheat flour
VSSVFHQAFVEVNEQGTEAAASTA	Serpin-Z1B				Wheat flour
LTAASITAVCR	Dimeric alpha-amylase inhibitor				Wheat flour
LQCNGSQVPEAVLR	Dimeric alpha-amylase inhibitor				Wheat flour
ELQESSLEACR	HMW glutenin subunit				Wheat flour/Rye flour
VAYLDAAPR	Cupin type-1 domain-containing protein				Wheat flour
EGEGVLVLLR	Cupin type-1 domain-containing protein				Wheat flour
NIEYLTGLGVDDQPLFHGR	Beta-amylase				Wheat flour/Rye flour
LEGSDALSTRQ	HMW glutenin subunit				Wheat flour
TTTRVPFGVGTGVGGY	LMW glutenin subunit				Wheat flour
VFLQQQCSPVAMPQSLAR	LMW glutenin subunit				Wheat flour
AIISIVLQEQQVVR	LMW glutenin				Wheat flour
NVLLQQCSPVALVSSVR	75k gamma secalin				Rye flour
TYVVSQICHVGPR	Trypsin inhibitor				Wheat flour/Rye flour
LLAEALGTSGK	Cupin type-1 domain-containing protein				Wheat flour
LFLQQQCSPVAMPQR	LMW glutenin subunit				Wheat flour
DLLGDTDKLTNVALGR	rRNA N-glycosylase				Wheat flour/Rye flour
EGEGVLVLLR	Cupin type-1 domain-containing protein				Wheat flour/Rye flour
LQCVGSQVPEAVLR	Monomeric alpha-amylase inhibitor				Wheat flour
LSNQLLEGQNYVNFK	Beta-amylase				Rye flour
APFASIVAGIGGQ	Gamma-gliadin				Wheat flour
DALLQQCSPVADMSFLR	Gamma-gliadin				Wheat flour
ALPDVLANAYR	Hypothetical protein BRADI_4g29131v3				Wheat flour
ALATGIFER	Xylanase inhibitor protein				Wheat flour

Table S13. Taxonomic attribution of peptides of flour proteins identified in sample D1.

Peptide sequence identified by MS/MS	Protein name	Taxonomic attribution (Blast results)			Attributed matrix
		Triticum aestivum	Secale cereale	Linum usitatissimum	
QGGQLEGGQPGGQQTR	HMW glutenin subunit				Wheat flour
VAIMEVNPR	Globulin-3A				Wheat flour
SGNVGESGLIDLPQCPR	Alpha-amylase/trypsin inhibitor CM3				Wheat flour
AIWTSIQGDLSGFK	Grain softness protein				Wheat flour
SCEEVQNQCCQQLR	Grain softness protein				Wheat flour
LEGSDALSTRQ	HMW glutenin subunit				Wheat flour
IFWGIPALLK	HMW glutenin subunit				Wheat flour
LVLANALYFK	Serpin-Z1B				Wheat flour
VAYLDAAPR	Cupin type-1 domain-containing protein				Wheat flour
EGEGLVLLR	Cupin type-1 domain-containing protein				Wheat flour
NVLLQCCSPVALVSSVR	75k gamma secalin				Rye flour
TTTRVPFGVGTGVGGY	LMW glutenin subunit				Wheat flour
LQCVGSQVPEAVLR	Monomeric alpha-amylase inhibitor				Wheat flour
ALPVDVLANAYR	Hypothetical protein BRADI_4g29131v3				Wheat flour
EFIAGIVGR	Trypsin/alpha-amylase inhibitor				Wheat flour

Table S14. Taxonomic attribution of peptides of flour proteins identified in sample D2.

Peptide sequence identified by MS/MS	Protein name	Taxonomic attribution (Blast results)			Attributed matrix
		Triticum aestivum	Secale cereale	Linum usitatissimum	
LQASTPLR	Cupin type-1 domain-containing protein				Wheat flour
LLAEALGTSGK	Cupin type-1 domain-containing protein				Wheat flour
LSVNIDPSR	Cupin type-1 domain-containing protein				Wheat flour
TNANSMVSHIAGK	Cupin type-1 domain-containing protein				Wheat flour
SQAGLTEYFDEENEQFR	Cupin type-1 domain-containing protein				Wheat flour
LASTISSNPK	Serpin-Z1B				Wheat flour
AGILQADK	Starch synthase, chloroplastic/amyloplastic				Wheat flour
LLVAPGQCNLATIHNV	Alpha-amylase/trypsin inhibitor CM3				Wheat flour
ASLNFTCAEMR	Beta-amylase				Wheat flour
SAVQMYADYMASFR	Beta-amylase				Wheat flour
LQNGSQVPEAVLR	Dimeric alpha-amylase inhibitor				Wheat flour/Rye flour
LTAASITAVCK	Dimeric alpha-amylase inhibitor				Wheat flour/Rye flour
VIACVGETLEQR	Triosephosphate isomerase				Wheat flour/Rye flour
EAGSTMAVVAEQTK	Triosephosphate isomerase				Wheat flour
ASINFTCAEMR	Beta-amylase				Wheat flour
TSPASTGLTLATR	rRNA N-glycosylase				Wheat flour/Rye flour
QQMADAVTALYGR	rRNA N-glycosylase				Wheat flour/Rye flour
APEDATYLEDGK	Protein disulfide-isomerase				Wheat flour
VVVADNVHDDVVF	Protein disulfide-isomerase				Wheat flour
DIGLAAGLLR	Peroxidase				Wheat flour
VAIMEVNPR	Globulin-3A				Wheat flour
ATITVVNR	Antifungal protein				Wheat flour

Table S15. Taxonomic attribution of peptides of flour proteins identified in sample E1.

Results obtained for the historical glue paste

Identified compound	RT (min)	Biological matrix associated
Azelaic acid	11,4	Oxidation production of fatty acids
Palmitic acid	18,9	Vegetable oils; ox gall; flour
Stearic acid	20,9	Vegetable oils; ox gall; flour
Xylose	15,2	Honey; Molasses
Xylose	15,6	Honey; Molasses
Glucose	19,4	Flour; Honey; Molasses
Glucose	19,6	Flour; Honey; Molasses

Table S16. GC-MS results obtained for the historical glue paste.

Peptide sequence identified by MS/MS	Protein assignment		
	Protein name	Taxonomic attribution	Attributed matrix
GPAGPQGPR	Collagen alpha-1(I)	Bos taurus	Bovine glue
GPAGPAGPIGPVGAR	Collagen alpha-1(I)	Bos taurus	Bovine glue
GETGPAGPAGPIGPVGAR	Collagen alpha-1(I)	Bos taurus	Bovine glue
IGQPGAVGPAGIR	Collagen alpha-2(I)	Bos taurus	Bovine glue
GPAGPSGPAGKDGR	Collagen alpha-2(I)	Bos taurus	Bovine glue
GEAGPAGPAGPAGPR	Collagen alpha-2(I)	Bos taurus	Bovine glue
GEPGPTGVQPPGPAGEEGKR	Collagen alpha-1(I)	Oryctolagus cuniculus	Rabbit glue
VGPPGPSGNAGPPGPPGPVGKEGGKGPR	Collagen alpha-1(I)	Oryctolagus cuniculus	Rabbit glue
GPAGPTGPAGKDGR	Collagen alpha-2(I)	Oryctolagus cuniculus	Rabbit glue
TNEWGKTIEYKTNKPSR	Collagen alpha-2(I)	Oryctolagus cuniculus	Rabbit glue
SVYQELGVR	Monomeric alpha-amylase inhibitor	Triticum aestivum	Wheat flour

Table S17. Specie-specific peptides identified in the historical sample.

Section 2

Characterization of the secondary structure, renaturation and physical ageing of gelatine adhesives

The complete online version of supplementary material is available at: <https://www.nature.com/articles/s41598-025-04910-8#Sec14>. Below is provided the material related to proteomics analyses, my specific contribution to this work.

Proteomic characterization of standard collagen derived from calf skin, gelatine A from porcine skin and gelatine B from bovine skin.

Samples were prepared and analysed as reported in Ntasi et al. (2022), with the exception of minor modifications due to changes in the laboratory equipment and software availability.

Briefly, 1–2 mg of each sample was resuspended in 10 μ L of 6 M urea. Samples were incubated for 20 min at room temperature and then for 30 min in the sonicator. Samples were then 6-fold diluted with 10 mM ammonium bicarbonate at pH 7.5, and enzymatic digestion was carried out by the addition of 1 μ g of trypsin at 37 °C for 16 h. The supernatants were then recovered by centrifugation and filtered on 0.22 μ m PVDF membrane (Millipore). Peptides were desalted and concentrated on commercially available ZipTip C18 pipette tips and eluted with 10 μ L of 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) in Milli-Q water. Peptides were analyzed by LC-MS/MS on a Vanquish Neo nanoLC system coupled to an Orbitrap Eclipse mass spectrometer (Thermo Fisher Scientific), using a step gradient of eluent B (0.1% formic acid, 80% acetonitrile LC/MS Optima grade Fisher chemical) in eluent A (0.1% formic acid in water LC/MS Optima grade Fisher chemical) from 3 to 50% over 54 min, increase to 90% over 1 min and hold at 90% during 5 min. The flow rate is 300 nL/min. Mass spectrometric analyses were performed in MS positive mode with the Orbitrap analyser, over the mass range 375-1500, at a resolution of 120 000, with standard automatic gain control (AGC) target and automatic maximum injection time. The MS/MS scans were acquired using data-dependent acquisition (DDA) mode with ion trap detector, fixed HCD collision energy (30%), standard AGC target, automatic maximum injection time and precursor isolation width of 1.6 Da. Data were acquired by Xcalibur 4.6 software (Thermo Fisher Scientific).

For protein identification, MS/MS spectra were analyzed by Proteome Discoverer 2.4.0 software (Thermo Fisher Scientific). Standard parameters in the searches were trypsin as the enzyme

(semitrypsin when searching for backbone cleavages); 2, as the allowed number of missed cleavages; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; and peptide charge from 2+ to 3+. Minimum peptide length was set to 7; and no fixed modification was set, while oxidation of methionine, deamidation at asparagine and glutamine, hydroxylation of proline and hydroxylation of lysine were set as variable modifications, with up to a maximum of 5 modifications per peptide. Protein identifications were supported by a false discovery rate (FDR) of 0.01 applied. Contaminant proteins were assessed using the cRAP database, which includes common laboratory contaminants. These protein hits were excluded from further analysis. To reduce the search space and recover more focused results, searches were carried out using the homemade database *COLLE* (60 sequences; 88 859 residues) that collects the sequences of collagen type I and III for all the common domesticates generally used for animal glues). Mass spectrometry data and the databases used have been deposited to Mendeley Data (Mendeley Data, V1, doi: 10.17632/24fjxr8wcx.1).

The extent of each chemical modification (deamidation, backbone cleavage) in samples was semi-quantitatively assessed by a spectral counting approach, as reported in ref. [doi: 10.1038/s41596-024-00975-3.]. The extent of deamidation is reported as percentage of modified sites compared to the total detected sites (modified plus unmodified), considering the peptide spectrum matches (PSMs). Similarly, the percent of backbone cleavage in samples was evaluated as percentage of semitryptic peptides over the total number of identified peptides, on the basis of PSMs. Results shown for deamidation in Fig. S3a,b and for backbone cleavage in Fig. S4a,b were obtained averaging data from two biological replicates.

Sample name	Identified collagen chains	Taxonomy	# Identified peptides	Sequence coverage (%)	Backbone cleavage	Deamidation degree*
Collagen	Collagen alpha-1(I)	<i>Bos taurus</i>	107	47	~30%	+
	Collagen alpha-2(I)	<i>Bos taurus</i>	90	42	~35%	+
	Collagen alpha-1(III)	<i>Bos taurus</i>	32	17	~30%	+
Gelatin A	Collagen alpha-1(I)	<i>Sus scrofa</i>	105	37	~30%	++
	Collagen alpha-2(I)	<i>Sus scrofa</i>	43	29	~35%	+
	Collagen alpha-1(III)	<i>Sus scrofa</i>	24	17	~15%	++
Gelatin B	Collagen alpha-1(I)	<i>Bos taurus</i>	139	40	~30%	++
	Collagen alpha-2(I)	<i>Bos taurus</i>	76	43	~35%	++
	Collagen alpha-1(III)	<i>Bos taurus</i>	44	23	~25%	++

Table S7. Molecular features of collagen chains identified in standard collagen, gelatine A and gelatine B: taxonomy, total number (#) of identified peptides, sequence coverage (%), backbone cleavage and deamidation extent.

*+++ corresponds to deamidation % of asparagine > 90% and glutamine > 70%; ++ corresponds to deamidation % of asparagine between 50% and 90% and glutamine between 1% and 70%; + corresponds to deamidation % of asparagine < 50% and glutamine < 40%.

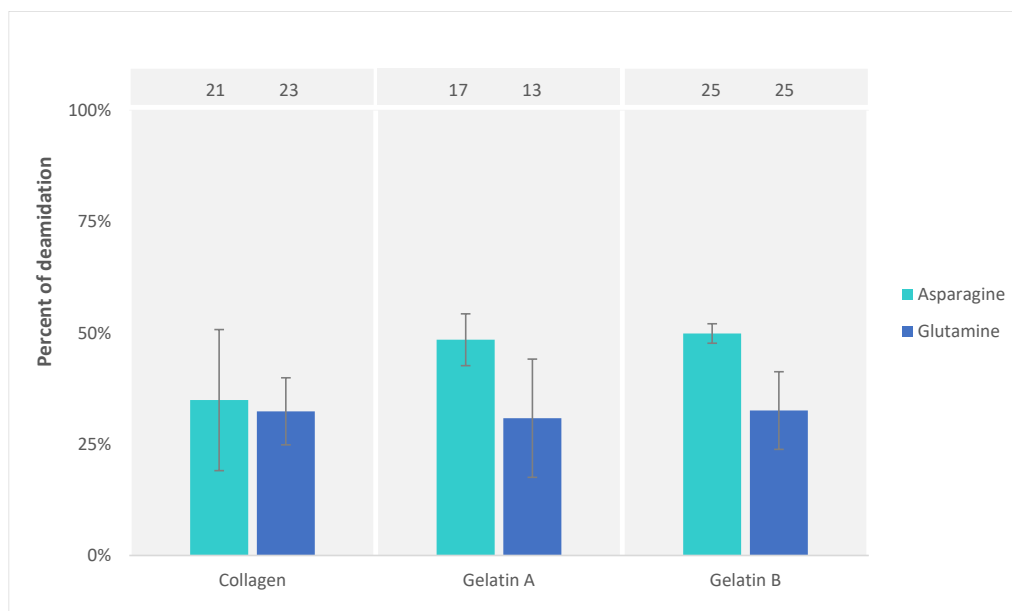


Figure S7a. Extent of deamidation for Asn (N) and Gln (Q) residues calculated for standard collagen and gelatine samples, reported as percentage of modified sites over detected (modified plus unmodified) ones. The results shown were obtained averaging data from two biological replicates. Error bars represent standard error of the mean. Numbers above each bar represent the number of modified peptides the data is based on.

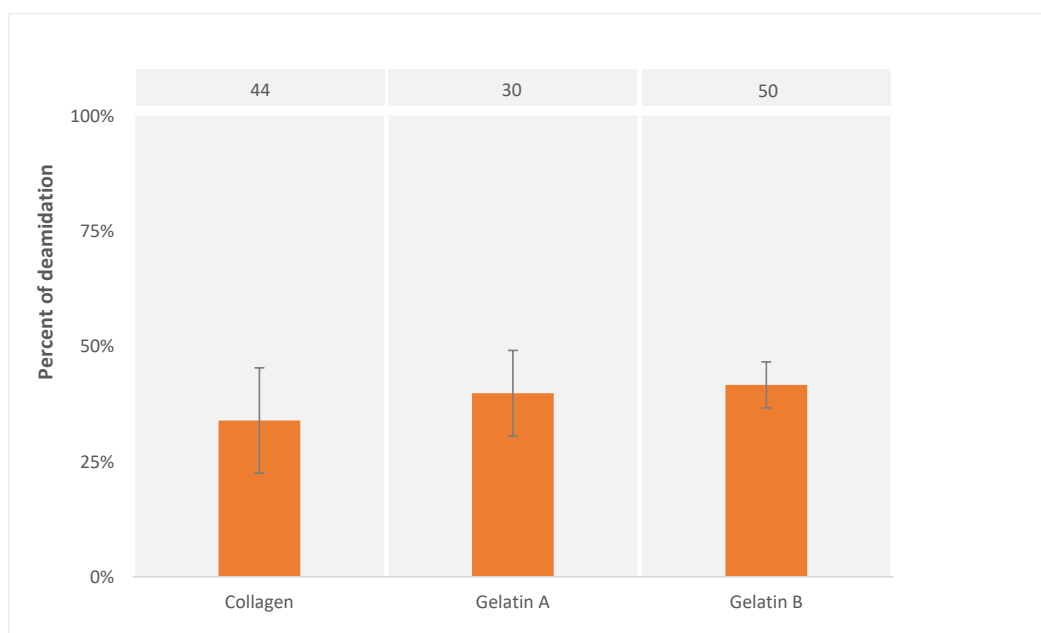


Figure S7b. Total extent of deamidation (NQ) calculated for standard collagen and gelatine samples, reported as percentage of modified sites over detected (modified plus unmodified) ones. The results shown were obtained averaging data from two biological replicates. Error bars represent standard error of the mean. Numbers above each bar represent the number of modified peptides the data is based on.

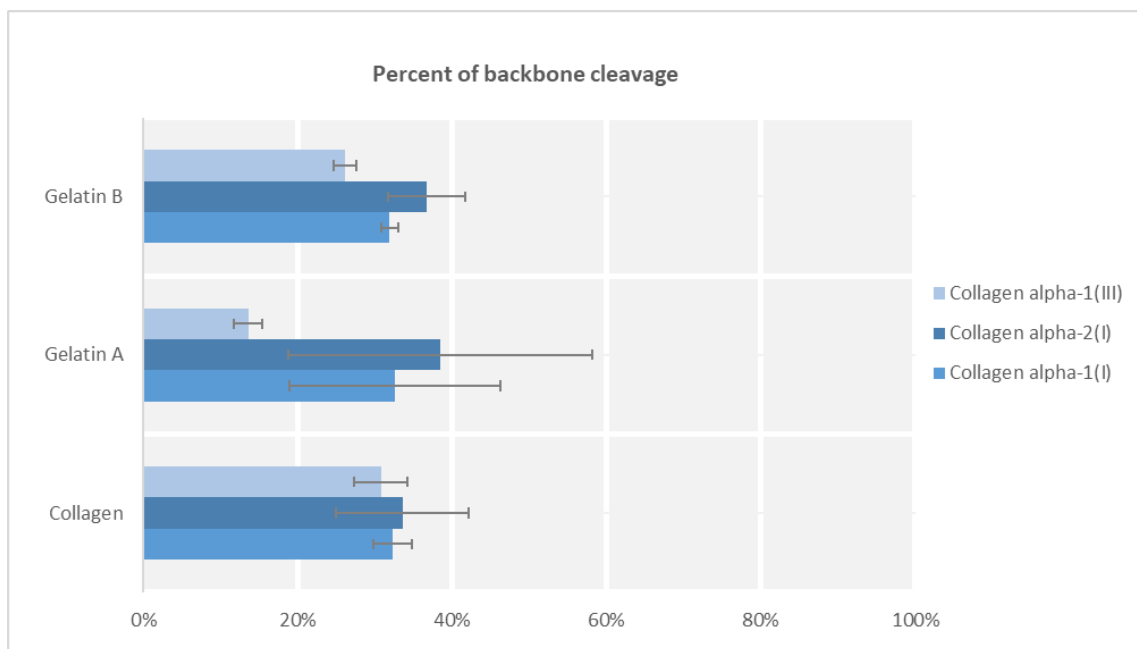


Figure S8a. Percent of backbone cleavage in standard collagen and gelatine samples, reported as percentage of semitryptic peptides over the total number of identified peptides (tryptic plus semitryptic) for each considered collagen chain. The results shown were obtained averaging data from two biological replicates. Error bars represent standard error of the mean.

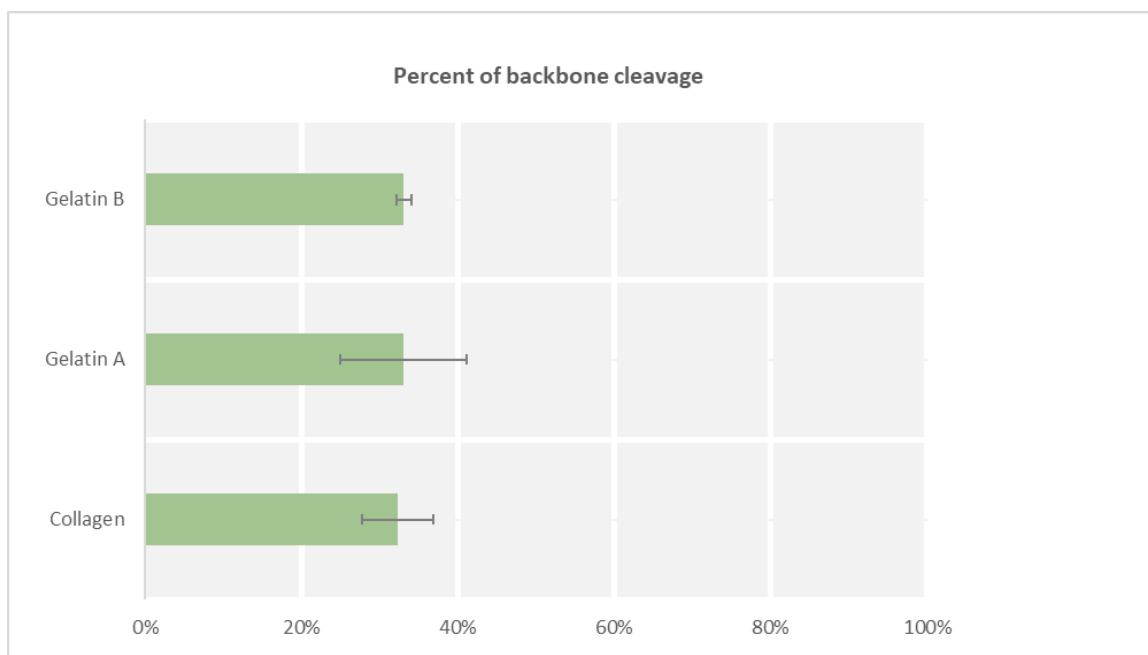


Figure S8b. Total percent of backbone cleavage in standard collagen and gelatine samples, reported as percentage of semitryptic peptides over the total number of identified peptides (tryptic plus semitryptic). The results shown were obtained averaging data from two biological replicates. Error bars represent standard error of the mean.

From waste to function: valorization of collagen-based wastes with Natural Deep Eutectic Solvents for bioadhesive applications

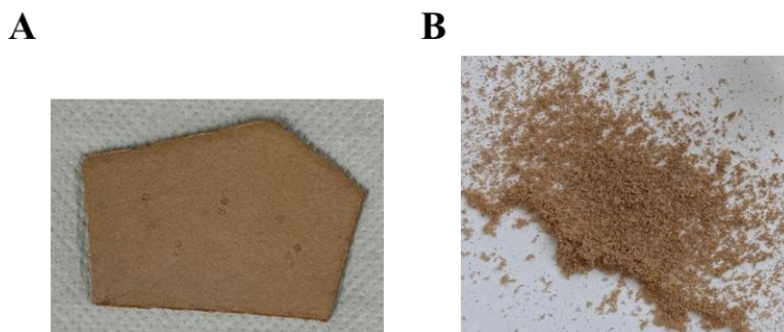


Figure S1. Vegetable-tanned leather sample as supplied by the tannery (A) and after grating into small pieces (B).

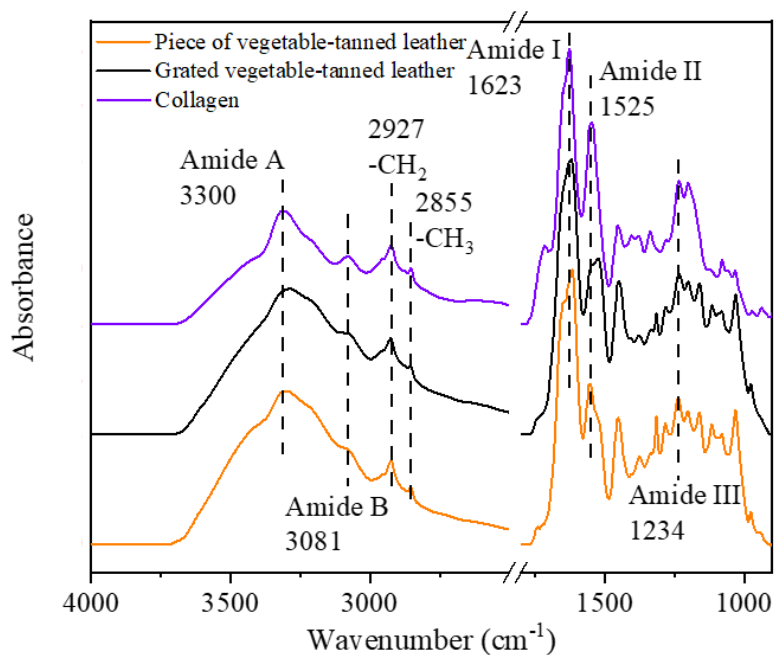


Figure S2. ATR-FTIR spectrum of the collagen (purple line), vegetable-tanned leather sample as provided by the supplier (orange line) and grated vegetable-tanned leather (black line). Characteristic bands associated with the collagen structure are highlighted.

Table S1. TGA analysis of the samples: as-received tanned leather, grated tanned leather, gel_1, gel_1_HCl, gel_1_NaOH, gel_2, gel_2_HCl, and gel_2_NaOH.*

Sample	T _{onset} (°C)	T (1° Peak) (°C)	T (2° Peak) (°C)	T max (°C)	Residue Mass % (T=900°C)	Mass loss (wt%)		
						Stage I (25-200°C)	Stage II (200-600°C)	Stage III (600-900°C)
Collagen	299.3	38.3	169.7	326.1	13	32	52	3
Leather as received	268.8	46.2		309.6	26	15	54	5
Grated leather	263.7	39.1		310.3	23	11	59	7

*Error (expressed as $\pm$ standard deviation) was evaluated by performing in triplicate representative samples. More in detail, error in the temperatures was $\pm 0.3^\circ\text{C}$, and in the mass losses was $\pm 4\%$.

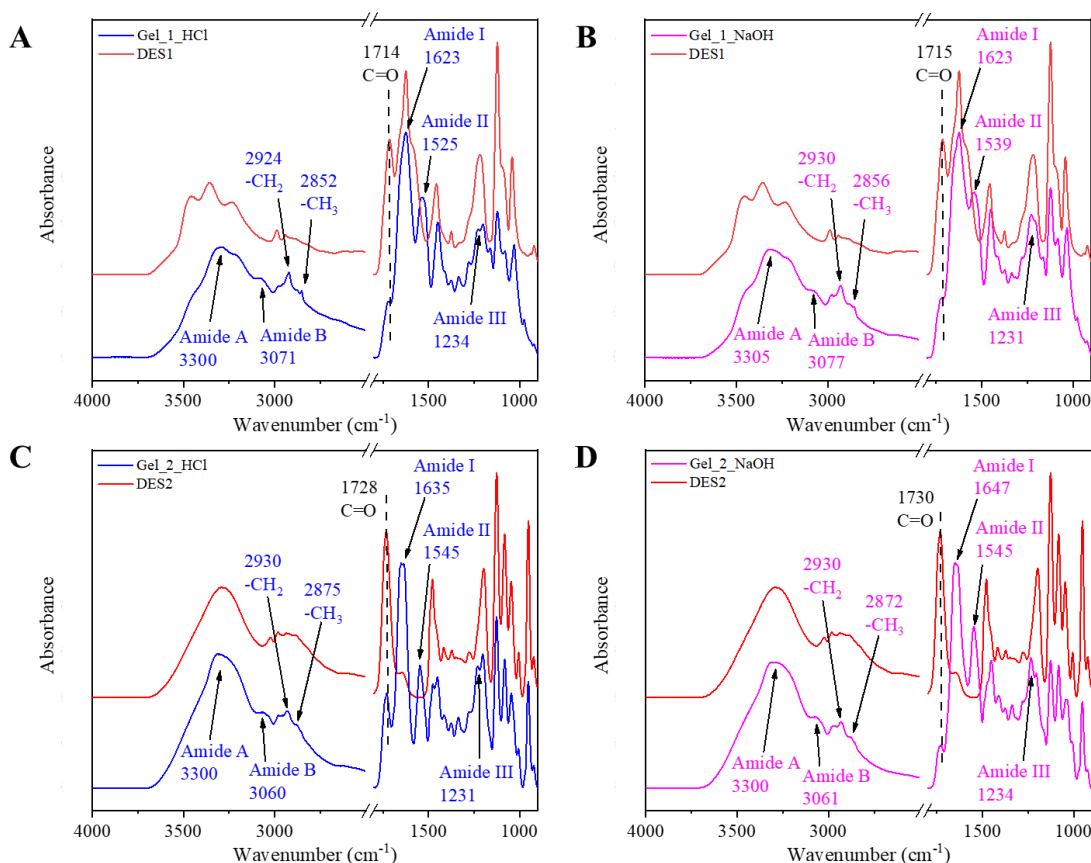


Figure S3. ATR-FTIR spectra of samples analyzed. (A) Dried gelatin obtained from DES1 + 0.1M HCl treatment (Gel_1_HCl - blue line) compared with DES1 (red line). (B) Dried gelatin obtained from DES1+ 0.1M NaOH treatment (Gel_1_NaOH - pink line) compared with DES1 (red line). (C) Dried gelatin obtained from DES2 + 0.1M HCl treatment (Gel_2_HCl - blue line) compared with DES2 (red line). (D) Dried gelatin obtained from DES2+ 0.1M NaOH treatment (Gel_2_NaOH - pink line) compared with DES2 (red line). Characteristic bands associated with the collagen structure and interactions with DES2 are highlighted.

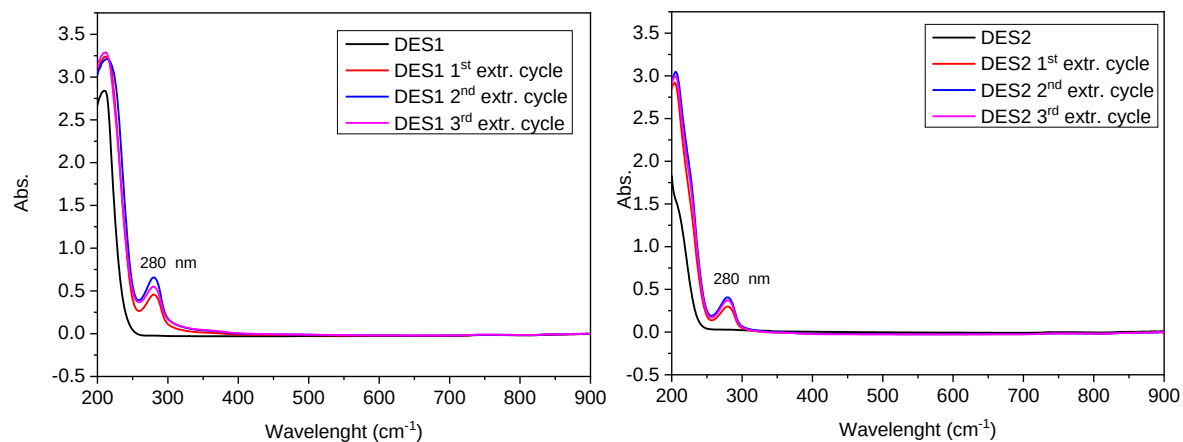


Figure S 4. UV-Vis of DES 1 (on the left) and DES 2 (on the right) used in subsequent cycles. Samples are diluted 1:300 in water. The signal at 280 nm of mimosa and quebracho extracts containing tannins are visible (DOI: <http://dx.doi.org/10.1016/j.indcrop.2016.07.022>) while the signal of chestnuts extracts, and the other signals of previous extracts are covered by the signal of DES.

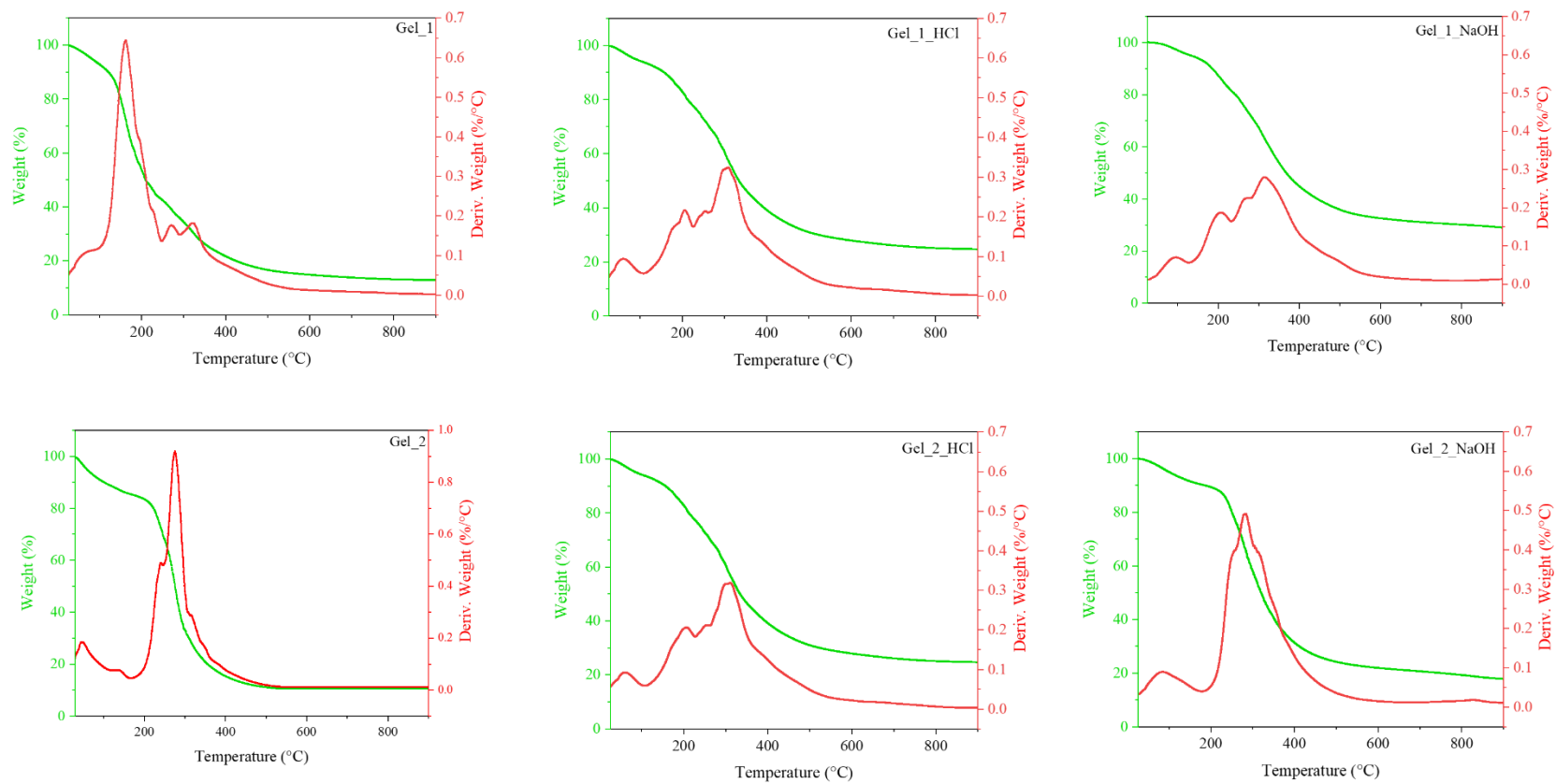


Figure S5. TGA of the samples Gel_1, Gel_1 HCl, Gel_1 NaOH, Gel_2, Gel_2 HCl, Gel_2 NaOH.

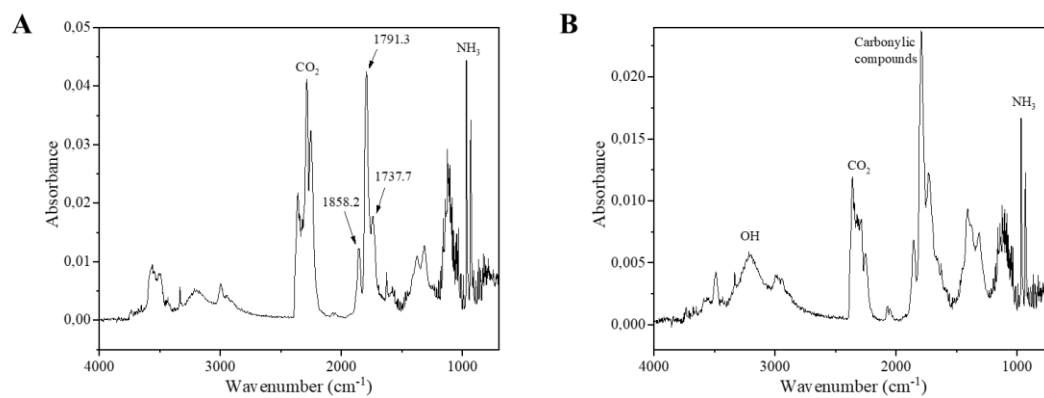


Figure S6. FTIR spectrum of the gases evolved during thermal degradation of Gel_1 at 163°C (A) and at 323°C (B), Gel_1_HCl at 210°C (C) and at 310°C (D) and Gel_1_NaOH at 210°C (E) and at 315°C (F), under nitrogen flow at a heating rate of 20 °C/min.

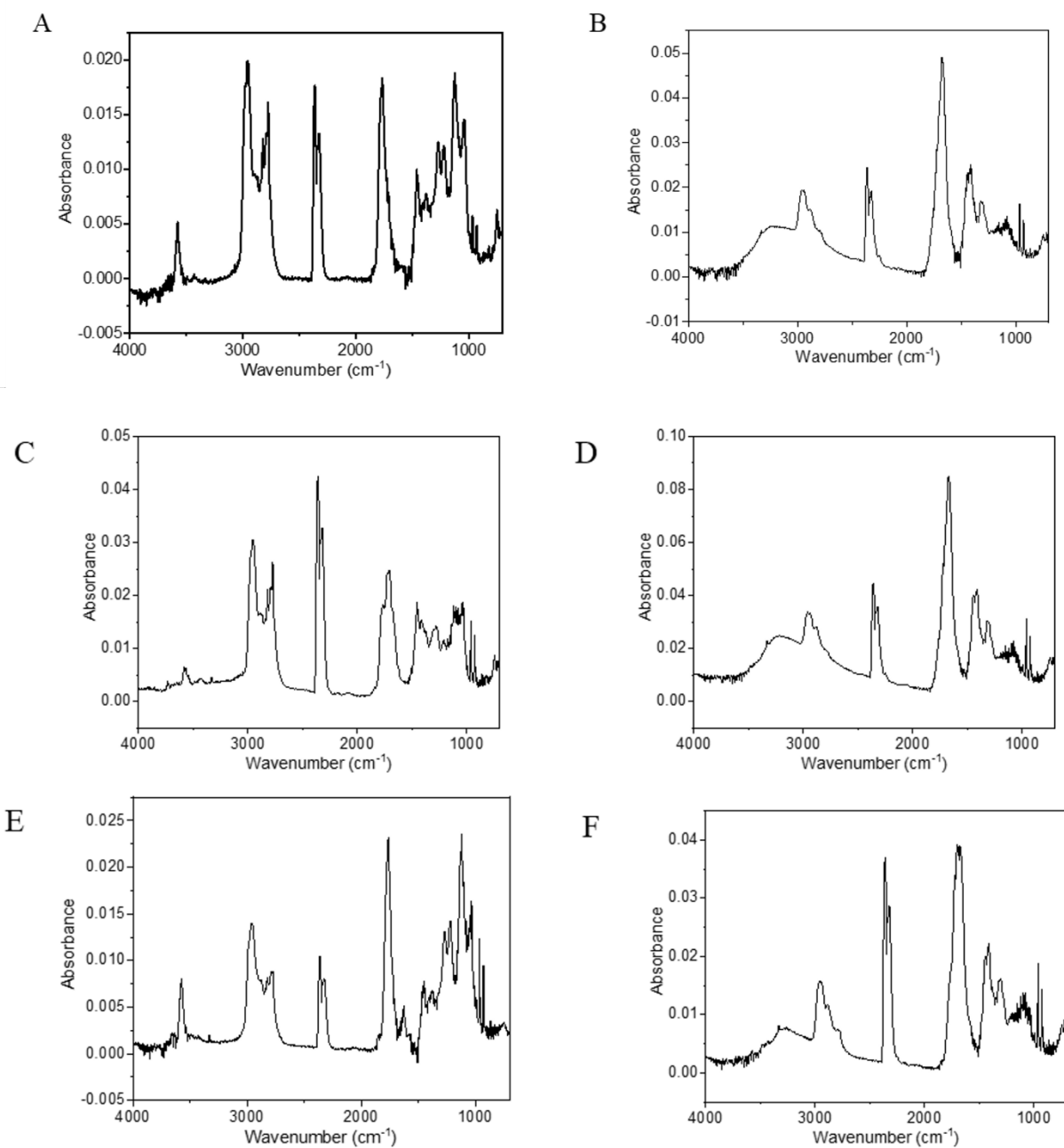


Figure S7. FTIR spectrum of the gases evolved during thermal degradation of Gel_2 at 220°C (A) and at 280°C (B), Gel_2_HCl at 220°C (C), and at 280°C (D) and Gel_2_NaOH at 220°C (E) and at 280°C (F), under nitrogen flow at a heating rate of 20 °C/min.

Table S2. Maximum temperature in DTG associated with collagen degradation.

Sample	Leather as received	Grated leather	Collagen	Gel_1	Gel_1_HCl	Gel_1_NaOH	Gel_2	Gel_2_HCl	Gel_2_NaOH
T max DTG (°C)	310	310	326	302	301	314	275	283	282

*Error ($< \pm 2^{\circ}\text{C}$, expressed as $\pm$ standard deviation) was evaluated by performing in triplicate representative samples.

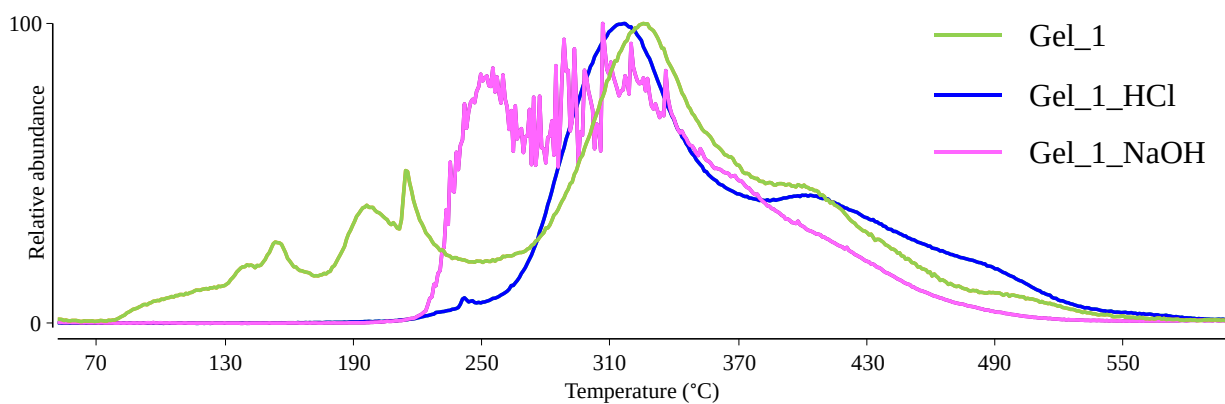


Figure S8. Total Ion Thermograms (TITs) of Gel_1 (green), Gel_1_HCl (blue) and Gel_1_NaOH (pink)

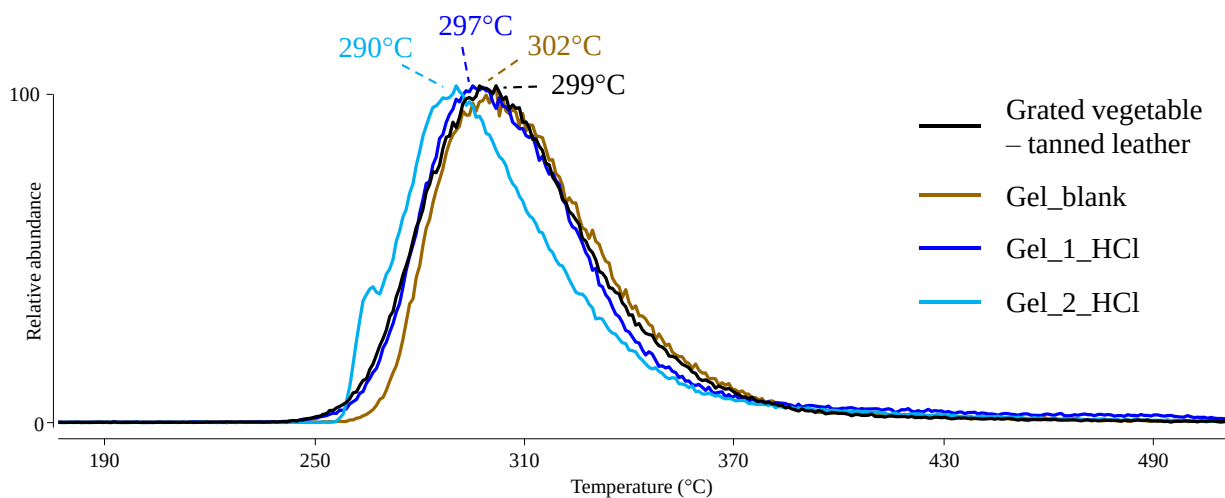


Figure S9. Extracted Ion Thermograms (EITs) of fragment ion m/z 154 of grated vegetable – tanned leather (black), Gel_blank (brown), Gel_1_HCl (blue) and Gel_2_HCl (light blue).

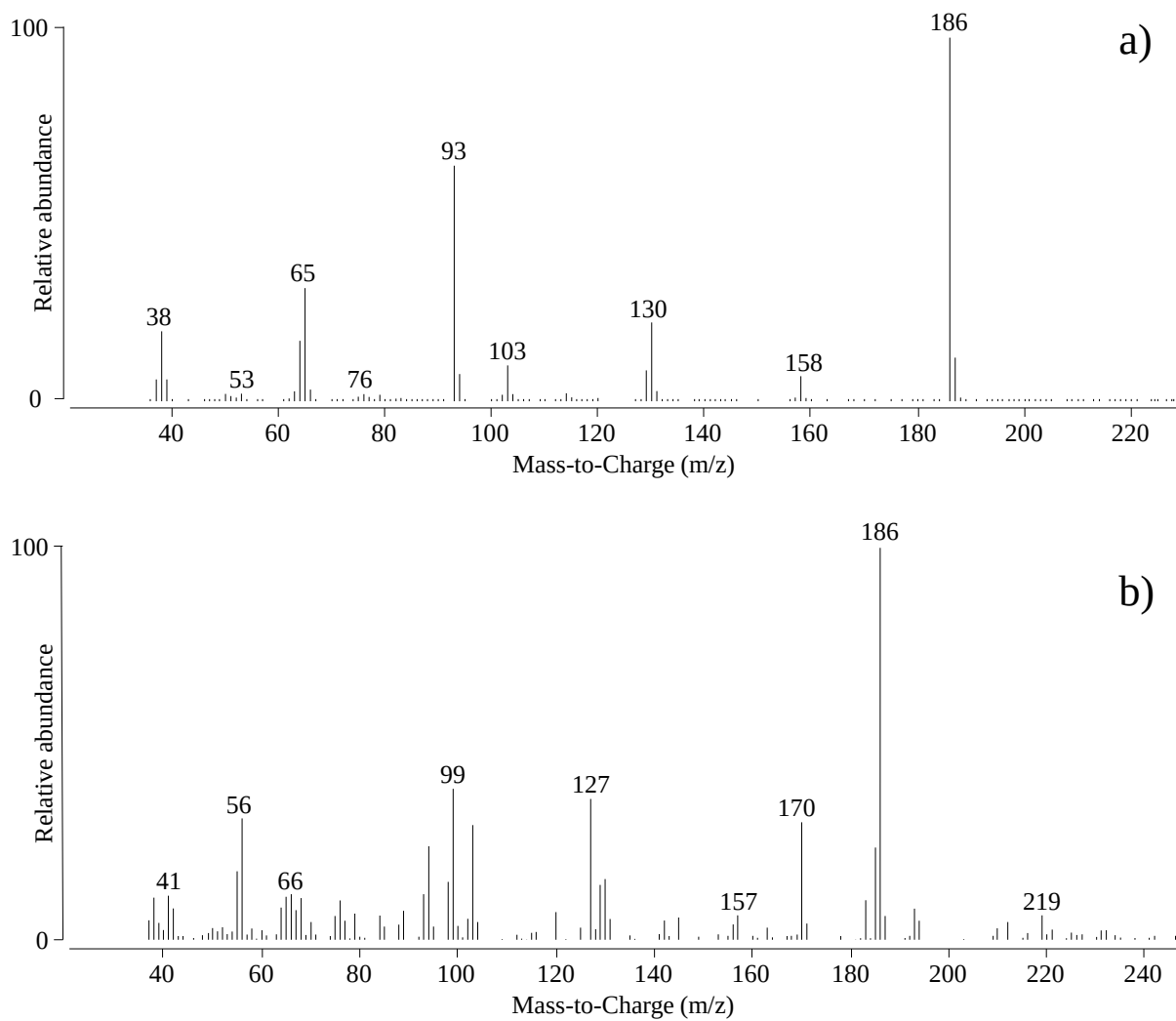


Figure S10. Mass spectra of heteroaromatic compounds from EICs in Fig 4 at a) 19.1 min and b) 22.9 min.

Table S3. Summary of protein identification results. For each protein, data show number of identified peptides, percentage of sequence coverage, number of peptide isoforms, and counts of unmodified and modified peptides. Modified peptide counts represent the number of peptide isoforms containing the specific modification used for calculating modification extent.

Sample	Protein name (Uniprot ID)	Identified peptides	Sequence coverage (%)	Peptide isoforms	Unmodified Peptides	Oxidized (M)	Deamidated (N)	Deamidated (Q)
Gel_1_REP1	Collagen alpha-1(I) chain (P02453)	77	58.8	255	31	14	24	33
Gel_1_REP2		46	42.9	145	11	16	16	15
Gel_2_REP1		74	57.4	300	42	20	23	35
Gel_2_REP2		65	54.0	227	16	21	17	25
Gel_blank_REP1		63	56.2	256	28	17	26	30
Gel_blank_REP2		46	44.4	105	14	14	9	15
Grated_leather_REP1		52	49.9	137	16	17	9	19
Grated_leather_REP2		30	31.6	52	10	11	6	4
Gel_1_REP1	Collagen alpha-2(I) chain (P02465)	53	54.9	108	8	5	28	7
Gel_1_REP2		28	33.6	56	4	0	13	4
Gel_2_REP1		55	58.3	132	10	10	36	7
Gel_2_REP2		48	51.8	109	10	8	33	6
Gel_blank_REP1		51	57.0	144	9	13	45	6
Gel_blank_REP2		33	36.6	63	4	5	19	5
Grated_leather_REP1		43	50.6	75	6	5	28	3
Grated_leather_REP2		22	26.6	33	2	1	7	2
Gel_1_REP1	Collagen alpha- 1(III) chain (P04258)	55	70.1	168	7	7	42	21
Gel_1_REP2		33	50.5	70	4	8	21	8
Gel_2_REP1		59	70.9	188	3	18	48	25
Gel_2_REP2		46	61.2	125	4	13	37	12
Gel_blank_REP1		48	62.8	150	5	10	43	16
Gel_blank_REP2		36	44.7	70	3	8	22	6
Grated_leather_REP1		37	50.1	87	3	11	27	10
Grated_leather_REP2		20	26.9	35	1	5	9	3

Table S4. Backbone cleavage per protein. Data show the mean ratio of semitryptic to total peptides for each protein across samples, with standard error and number of peptide isoforms (N) calculations are based on. Semitryptic/TOT ratios indicate the extent of protein backbone cleavage, where higher ratios reflect increased proteolytic degradation. Total counts of identified tryptic and semitryptic peptides backbone cleavage data is based on are reported for each sample replicate.

Sample	Experiment	Total Tryptic	Total Semi-tryptic	Semitryptic/TOT (Mean)	Std Error
Gel_1	Gel_1_REP1	199	25	0.11	0.00
	Gel_1_REP2	120	16		
Gel_1_HCl	Gel_1_HCl_REP1	454	146	0.25	0.01
	Gel_1_HCl_REP2	466	164		
Gel_1_NaOH	Gel_1_NaOH_REP1	480	68	0.14	0.01
	Gel_1_NaOH_REP2	470	81		
Gel_2	Gel_2_REP1	317	33	0.11	0.02
	Gel_2_REP2	305	45		
Gel_2_HCl	Gel_2_HCl_REP1	477	83	0.15	0.00
	Gel_2_HCl_REP2	517	86		
Gel_2_NaOH	Gel_2_NaOH_REP1	486	102	0.15	0.02
	Gel_2_NaOH_REP2	515	81		
Gel_blank	Gel_blank_REP1	282	22	0.07	0.00
	Gel_blank_REP2	99	7		
Grated_leather	Grated_leather_REP1	62	5	0.08	0.01
	Grated_leather_REP2	38	4		

Table S5. Analysis of protein modification extent. Mean values and standard errors for the extent of methionine (M) oxidation and deamidation (N|Q) are presented by sample, along with the number of detected and modified amino acid sites the data is based on. N|Q represents combined deamidation at both asparagine (N) and glutamine (Q) residues.

Sample	Oxidation (M)				Deamidation (N Q)			
	Detected M	Modified M	Fraction of modified/TOT	Std Error	Detected N Q	Modified N Q	Fraction of modified/TOT	Std Error
Gel_1	77	67	0.86	0.01	1094	485	0.33	0.02
Gel_1_HCl	76	65	0.86	0.01	1092	394	0.44	0.06
Gel_1_NaOH	49	42	0.86	0.03	624	198	0.36	0.00
Gel_2	99	90	0.89	0.04	1168	403	0.31	0.00
Gel_2_HCl	106	92	0.90	0.00	1085	334	0.34	0.00
Gel_2_NaOH	70	63	0.87	0.03	747	229	0.31	0.02
Gel_blank	58	48	0.85	0.10	509	177	0.37	0.05
Grated_leather	35	31	0.90	0.00	236	96	0.40	0.00

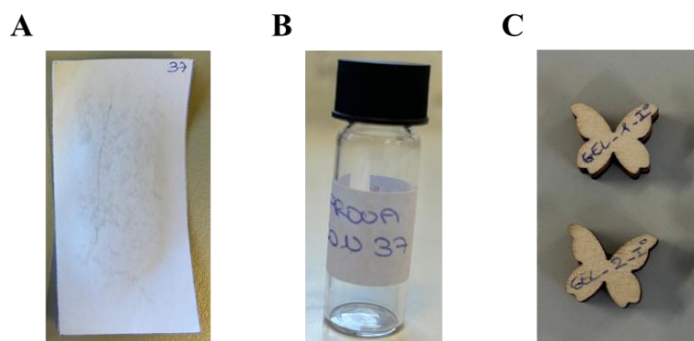


Figure S11. Samples from adhesion tests, specifically: A) paper–paper, B) paper–glass, and C) wood–wood.

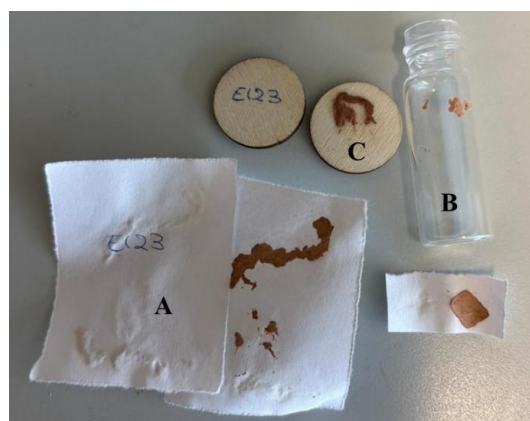


Figure S12. Samples of control experiment (Gel_blank) from adhesion tests, specifically: A) paper–paper, B) paper–glass, and C) wood–wood.

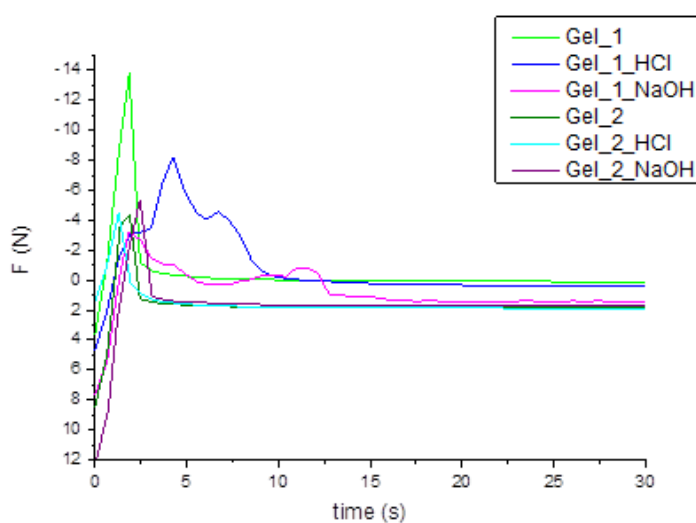


Figure S13. Stack test made on the samples to test the adhesive strength on steel–wood surfaces.

Table S6. Scores attributed to each principle to evaluate the process greenness, made according to Path2Green application.

	Principles	Score*	Motivation	Weight**
1	Biomass	+1	Waste biomasses	6
2	Transport	+0.5	40 km distance	5
3	Pre-treatment	-0.2	Physical pretreatment	2.5
4	Solvent	+1	Recommended solvents (DESS)	6
5	Scaling	-1	In batches	3
6	Purification	-0.5	Water	2.5
7	Yield	+1	Complete valorization of the biomass	4
8	Post-treat.	+1	Ready-to-use extract	2.5
9	Energy	+0.5	Low energy dependence	5
10	Application	+0.66	To be applied in four domains	4.5
11	Repurposing	+1	Non-Virgin raw materials are used	6
12	Waste	-1	Waste generated: 100% m/m	6

*Scores and weights were chosen following the recommendations of (DOI: 10.1039/d4gc02512a).

