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Determination of animal species origin of blood in artworks by LC-MS/MS

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This study introduces an innovative approach to the long-standing challenge of determining the animal origin of blood used in artworks – an issue of central relevance to art historians aiming to understand historical techniques, symbolic meanings, and the cultural traditions associated with artistic materials. Using LC-MS/MS analysis, species-specific peptide sequences of blood proteins were identified, allowing for the discrimination of seven animal species (cat, cow, dog, goose, hen, human, and pig). This analytical approach was successfully applied to a series of model samples containing blood, confirming both the proteinaceous composition and the animal origin of the blood. Furthermore, the method enabled the identification of pig blood in historical samples taken from Japanese and Chinese lacquer artefacts dating from the 18th and 19th centuries. The novelty of this work lies in a new strategy for data evaluation that facilitates the creation of custom peptide databases tailored to distinguish specific animal species. This approach overcomes a major limitation in proteomic studies – namely, the lack of complete sequence data for many animals – by incorporating homologous sequences from closely related species. The strategy demonstrates a high degree of effectiveness when implemented within a clearly defined group of animal species, such as those historically utilised in the production of blood-based artistic materials. This approach offers a novel pathway for the molecular identification of animal origin in cultural heritage contexts and establishes a robust foundation for future interdisciplinary investigations bridging art history, conservation science, and molecular biology.

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Introduction

Characterisation of proteinaceous materials in cultural heritage is of considerable interest for conservation and restoration. One often overlooked material is blood, which has historically been employed in diverse applications, ranging from practical uses – such as glazes for painting, protective coatings for wooden structures and mill components, or preservatives applied to beams, walls, and threshing floors[1–4] – to practices that may also carry symbolic or aesthetic significance, given its origin from a living organism. The earliest known evidence of blood combined with pigments appears in Palaeolithic cave paintings,[5] while residues of blood have likewise been identified on a 90 000 year-old stone tool from the Tabun cave in Israel.[11] One of the first documented uses of blood as a proteinaceous additive in construction materials is recorded in China (221–206 BC), where it was mixed with lime or other fillers to create durable, water-resistant binders.[6] Such formulations are frequently mentioned in historical sources,[3,7,8] and their continuity is exemplified by later applications, for instance in the lacquered panels of the Vieux-laque Room at Schönbrunn Palace in Vienna.[9] Despite the historical prevalence of blood-based coatings, identification in the Czech Republic remains limited to two areas, Horní Bečva and Korouhev.[10] However, further investigation is likely to reveal additional locations, as total degradation of blood proteins occurs over time even under the high pH conditions provided by calcium hydroxide in lime.

Chemically, the combination of blood and lime is advantageous, as coatings of pure blood show poor resistance to humidity and environmental conditions and degrade over time.[1,7] Historical sources vary: some describe cow blood as the sole component in Baroque and Rococo paints, valued for ease of application and glaze effect,[1] while others include blood as part of complex paint mixtures.[7,12]

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Ex vivo, blood coagulates into a clot of solid components and a yellowish to colourless serum, providing binding properties both in fresh mixtures and after several hours of separation. This effect, enhanced by adding fresh blood, was historically used in constructions such as hermitages or "clay of wisdom" (blood and quicklime).[1] Later recipes substituted egg white or milk casein, retaining binding properties through ovalbumin or casein. Although ratios and additional ingredients vary, lime and blood remain foundational, producing colour differences depending on composition and curing time. For example, one part cow blood to twenty parts lime yields a greenish hue,[1] while Dostál achieved shades of green with a one-to-five ratio after 12 hours.[13] Blood consistently dominates the protein content, likely due to symbolic significance or practical use of slaughterhouse by-products.[14]

Only a limited number of studies have focused on the identification of blood in cultural heritage objects, and even fewer on determining its animal species of origin. Among the most frequently employed techniques are Fourier transform infrared spectroscopy (FTIR) and chromatographic methods coupled with mass spectrometry. A comprehensive set of analytical approaches – including FTIR, Raman spectroscopy, gas chromatography coupled with mass spectrometry (GC-MS), and mass spectrometric techniques such as matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) and liquid chromatography-electrospray ionisation-quadrupole-time-of-flight (LC-ESI-Q-TOF) – was applied to assess the lowest detectable concentrations of blood in model lime mortar samples.[15] Despite the strong alkaline conditions of the mortar, particularly during the first month of curing, which hydrolyse blood proteins and alter amino acids, blood could still be detected at very low levels: MALDI-TOF and LC-ESI-Q-TOF identified blood at concentrations as low as 0.01% (w/w); GC-MS detected amino acids from blood at 0.1% (w/w); while vibrational spectroscopic techniques (FTIR and Raman spectroscopy) were able to detect blood proteins at concentrations of 1% (w/w).

Other studies have demonstrated the application of pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) to identify porcine blood, animal glue and egg, all of which were commonly employed as binding media in ancient Chinese artworks and objects such as polychromy, architecture and furniture.[16] In this case, the presence of blood was confirmed by the detection of characteristic nitrogen-free pyrolysis products. A combination of FTIR and liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used for the analysis of three mortars from Chinese monuments dating from the mid-17th to the early 20th century.[17] FTIR revealed the presence of proteins, while LC-MS/MS enabled the identification of specific organic additives, including wheaten flour, cattle blood and pig blood. Similarly, blood residues were identified on the surfaces of two African sculptures using FTIR, Py-GC/MS and LC-MS/MS,[18] with the animal origin of the blood further determined through shotgun proteomics as blood from chicken and goat. In study,[19] voltammetry of microparticles (VMP) was applied to haemoglobin-containing archaeological samples from the architectural complex of the Longshan Temple in Lukang (18th century, Taiwan) to assist in dating the material. Initially, the presence of blood in the polychromed samples was confirmed using GC-MS. However, the animal species origin of blood cannot be specified by the both methods.

In our work, we are presenting a reliable and easier protocol of animal blood origin determination, by using LC-ESI-Q-TOF MS, liquid chromatography – electrospray ionization – quadrupole – time of flight mass spectrometry. This technique enables the identification of potentially characteristic peptides for the blood of individual animal species, including those that have not yet been sequenced by usage of the homologous sequences from closely related species. The amino acid sequences of these peptides are regarded as potentially characteristic as they were derived from a limited set of animal species (cat, cow, dog, goose, hen, human, and pig) and may thus serve as species-specific fingerprints. Similar fingerprinting strategies are routinely applied in MALDI-TOF MS analyses for species identification[20] and the concept of using characteristic peptides has previously been applied, for example, to distinguish between raw and cooked fish meat by LC-MS/MS.[21] Current proteomic strategies for species identification from various tissues (*e.g.*, muscle tissue, baleen) typically rely on peptide searches in publicly available databases (UniProt, NCBI) and on probabilistic assessments of species assignment.[22] The approaches are similar, but using different software evaluations. The advantage of the proposed approach lies in its potential applicability to routine practice, avoiding the need for detailed evaluation using publicly available databases (UniProt, NCBI) of each individual sample. The main limitation, however, is the restricted number of animal species currently included as potential sources of blood in cultural heritage objects. As the database of characteristic peptides expands with new species, the number of peptides considered characteristic for a given taxon may decrease. To confirm the characteristic peptides, an attempt was made to identify unique peptides using blood protein sequences from animals available in selected geographical regions (Europe and Asia). The proteins sequences were downloaded from publicly available protein databases UniProt and NCBI. The potentially characteristic and unique peptides were used to determine the animal origin of the blood used in six model blood coating samples, and finally to determine the origin of blood identified in four samples from Asian works of art dating to the 18th and 19th century.

Materials and methods

Reagents and materials

Acetonitrile (LC-MS grade), formic acid, trifluoroacetic acid (TFA) and $KAl(SO_4)_2 \cdot 12H_2O$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium hydrogen carbonate (NH_4HCO_3) was obtained from Lachema (Czech Republic). Pierce Trypsin Protease MS Grade comes from ThermoFisher Scientific (USA). The commercially available reverse phase C18 ZipTip pipette tips were obtained from Millipore Corporation (Bedford, MA, USA). The water was purified with a Milli-Q water purification system (Millipore, USA).

Reference blood samples

Blood sample providers include Veterinary clinic – Aurum Vet s.r.o. for cats and dogs, Jihočeská masna s.r.o. for domestic cows and pigs, home-made slaughter for domestic chickens (hens) and geese, and expert intravenous sampling for humans. Human blood (from a volunteer) was collected in Motol University Hospital, Prague, Czech Republic in accordance with the ethical principles of University of Chemistry and Technology in Prague (Czech Republic) (UCT Prague), established by the Code of Ethics of UCT Prague dated 1/1/2025 and with the principles of Helsinki declaration from 1964 (Ethical Principles for Medical Research Involving Human Subjects). Ethical approval was obtained from the UCT Prague ethical committee (approval no. IC: EC 10/25) and an informed consent was obtained from the human donor. No animals were slaughtered specifically for blood; samples were obtained as a by-product of routine, legally sanctioned slaughtering in compliance with Czech animal welfare regulations and legislation, ensuring no additional harm. After collection, the samples were stored in plastic tubes at the temperature of −80 °C. The animals were chosen on the basis that they were the most viable for obtaining their blood in the past.

Model blood coatings

A total of six samples were made in 2019 in the workshop of restorers Mgr. Tomas Dostal, DiS., and Ludek Dvorak in the Wallachian Open Air Museum in Roznov pod Radhostem, Czech Republic. The composition of the created coatings was inspired by historical recipes,[1] which are presented in Table 1. The goal was to create coloured cow blood coatings for proteomic analysis in which it would be possible to determine the original material composition of the samples and the animal origin of the added blood.

Artworks samples

Three samples (C1, C2, and C3) from two Chinese lacquer objects and one sample (J) from a Japanese lacquer object from the collection of the Weltmuseum Wien (Austria) were studied.

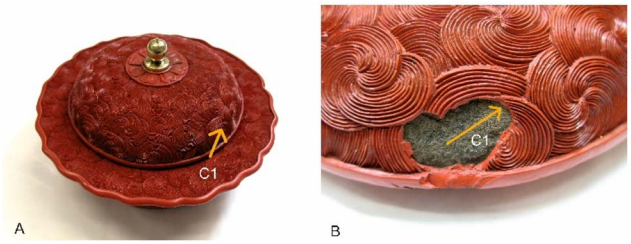

Fig. 1 Chinese lacquer spittoon (10 × 15 cm), VO_21295 (A), and the sampling spot C1 (B). ©KHM-Museumsverband, Weltmuseum Wien, Silvia Miklin-Kniefacz.

Sample C1 was taken from the lid of a small red carved lacquer box from China, Qing dynasty, Qianlong period (1736–1795) with the inventory number Inv. Nr. VO_21295 (Fig. 1A).

The wooden box is covered on the outside with numerous red lacquer layers on grey coarse ground layers. Due to some missing lacquer areas, the ground was exposed and enabled us to take a sample (Fig. 1B). The mixture of blood and animal glue had already been qualitatively detected in the ground layer by GC-MS in the Kunsthistorisches Museum Vienna.[23]

Samples C2 and C3 were obtained from the dark grey ground layer from the plinth of the 18th century Chinese lacquer screen (Inv. No. VO_71233) (Fig. 2A and B).

This three-panel wooden screen is also from the Qianlong period (1736–1795) of the Qing dynasty. With its impressive dimensions (3.30 × 2.60 m), carved lacquer with yellow, green,

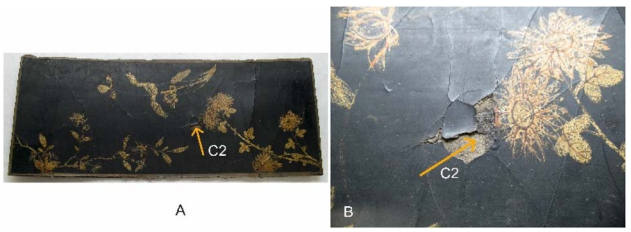

Fig. 2 Chinese lacquer screen VO_71233 (3.30 × 2.60 m) (before conservation) (A) and the sampling spot C2 (B). ©KHM-Museumsverband, Weltmuseum Wien.

Table 1 Materials used for preparation of six model samples of blood coatings

No. of sample	Used materials
1	Blood
2	Blood (400 ml) + limewater (20 ml) + bile (10 ml)
3	Blood (500 ml) + casein paste (19 ml) + limewater (25 ml) + egg white (1/3 from one egg)
4[a]	Blood + lime + aluminium potassium sulphate ($KAl(SO_4)_2 \cdot 12H_2O$) + rye flour + animal glue[b]
5	Blood (500 ml) + casein paste (20 ml) + limewater (40 ml) + egg white (from one egg) + aluminium potassium sulphate ($KAl(SO_4)_2 \cdot 12H_2O$) (5 ml)
6	Blood (500 ml) + casein paste (25 ml) + limewater (24 ml) + egg white (from one egg) + tannin (10 ml) + linseed oil (5 g) + bile (12 ml)

[a] for this recipe we do not indicate the proportion of materials for reason of use in the result of an ongoing project. [b] the animal glue solution was prepared by mixing bone glue and hide glue in a 1 : 1 ratio.

Fig. 3 Japanese lacquer tablet VO_34795 (25.5 × 10.5 cm) (A) and the sampling spot J (B). ©KHM-Museumsverband, Weltmuseum Wien, Silvia Miklin-Kniefacz.

and red layers on the front side and gold-painting on black lacquer on the back side, it was originally housed at the Imperial Hunting Palace at Nan-Haidze Park situated outside of Beijing, China. The composition of the Asian lacquer layers has been studied by Pitthard *et al.*[24] and the presence of blood in the ground layer has already been confirmed by Miklin-Kniefacz *et al.*[9]

Sample J was collected from the dark grey ground layer from the middle of the front side of a small 19th century Japanese lacquer tablet, called "Lackprobe" (lacquer sample) in the inventory, Inv. No. VO_34795 (Fig. 3A and B).

The small wooden panel with black lacquer and gold painting was collected by Heinrich Siebold in Japan, although the place of origin might be a different one. The lacquer panel dates to the Meiji Period (1868–1912), possibly before 1873. The mixture of blood and animal glue has already been qualitatively detected in the ground layer by GC-MS in the Kunsthistorisches Museum Vienna.[23] The results indicate an origin of the tablet with Chinese background (China, Okinawa?), because – as far as we know – blood was not commonly used in traditional Japanese lacquer technique.

Sample preparation

The reference samples of animal blood were prepared using the protocol in,[25] only the time of trypsin cleavage was shortened to 2 hours. These samples were analysed by LC-ESI-Q-TOF MS three times to obtain characteristic peptide profiles.

Small samples with dimensions of approx. 0.2 × 0.1 × 0.1 mm were taken from the blood coatings (Table 1) applied to the wooden sticks. These samples and similarly the small fragments of Chinese and Japanese artworks were immersed in 15 μl of trypsin solution (at the concentration of 1 mg ml^{-1} in 50 mM NH_4HCO_3) and subjected to enzymatic digestion for two hours at the temperature of 25 °C. After cleavage of proteins contained in the samples, the solutions containing peptides were purified and concentrated on the reverse phase C18. The 8 μl of the final solution was evaporated to complete dryness at room temperature and later used for the mass spectrometry analysis.

LC-ESI-Q-TOF MS conditions and data acquisition

Measurements were carried out using a UHPLC Dionex Ultimate3000 RSLC nano (Dionex, Germany) connected to an ESI-Q/TOF Maxis Impact mass spectrometer (Bruker Daltonics, Germany). Purified and evaporated samples (after trypsin digestion) were dissolved in a 97 : 3 : 0.1% mixture of water:-acetonitrile:formic acid, loaded into an Acclaim PepMap 100 C18 trap column (100 μm × 2 cm, size of reverse phase particles 5 μm; Dionex, Germany) with a flow rate of the mobile phase A of 5 μl min^{-1} for 5 minutes. The mobile phase A consisted of 0.1% formic acid in water and mobile phase B of 0.1% formic acid in acetonitrile. The peptides were then eluted from the trap column into an Acclaim PepMap RSLC C18 analytical column (75 μm × 150 mm, size of reverse phase particles 2 μm) using the following gradient: 0 min 3% B, 5 min 3% B, 30 min 40% B, 31 min 90% B, 40 min 90% B, 41 min 3% B, 55 min 3% B. The flow rate during gradient separation was set at 0.3 μl min^{-1}. The peptides were eluted directly to an SI source – Captive spray (Bruker Daltonics, Germany). Measurements were carried out in positive ion mode with a precursor selection in the range of 400–2200 Da; up to ten precursors were selected for fragmentation from each MS spectrum.

From the primary data, mass spectra were extracted by DataAnalysis 4.1 (Bruker Daltonics) and uploaded to Proteinscape version 4.2. Mascot server version 2.4.1 (Matrix Science) was used for protein identification. A single-species database was used, the proteomes of the given animals were downloaded from the UniProt website (**https://www.uniprot.org**), and the database was supplemented with common laboratory contaminants and the enzyme trypsin. The historical objects were searched against Animals (SwissProt). One allowed omitted cleavage site was set in the parameters and oxidation of methionines (and proline for the historical samples because the presence of collagens was expected) was set as a variable modification. A tolerance of 10 ppm was allowed for assigning precursors, in the case of fragments it was a tolerance of 0.05 Da.[26] Proteins were filtered with a FDR of 1%.

The proteins were accepted as relevant when there was sufficient peptide representation detected, *i.e.* at least two peptides identical to the amino acid sequence of the given protein listed in the UniProt database. The vast majority of proteins found in the sample were represented by only one peptide, so it was appropriate to exclude such a protein and deem it non credible. Proteins with the largest peptide representations were nominated in the table as potential identifiers of the original composition.

The PostgreSQL database system (2023.3.4, DatabaseSpy® 2024) was used for data processing and analysis, which was carried out using the pgAdmin tool (6.21, PostgreSQL Community Association of Canada). The characteristic values were found with the following set parameters: all duplicates of amino acid sequences were erased before comparing, the frequency of amino acid sequence occurrence was set to 3, which means that each amino acid sequence occurs only in one animal species blood and in all its three-sample repetition.

Results and discussion

Characteristic amino acid sequences obtained from the reference samples of blood

The blood of cat, cow, dog, goose, hen, human, and pig were subjected to analysis by LC-ESI-Q-TOF in three repetitions. It is

relatively common to find *i.e.* blood proteins from mammoth or frogs, because the blood proteins are highly evolutionary conservative in the results. That means that the same amino acid sequences can be found in many different animal species. However, the proteins with the same function in organisms can differ in certain amino acid at different positions in protein/peptide sequences that enable determination of the animal species origin. On this basis, we can find the characteristic amino acid sequences for the animal species (by PostgreSQL and pgAdmin) and then compare them to the results from analysed samples.

Characteristic amino acid sequences for all studied animal blood samples found by PostgreSQL are shown in SI, Tables S1–S7. The most characteristic peptides were found for pig blood 144, then for cow 127, hen 94, cat 80, human 77, dog 68, and for goose 36. The proteins whose peptides were found in large quantities in almost all samples include not only α and β chains of haemoglobin, but also complement proteins, serum albumin, α-2-macroglobulin, and apolipoproteins.

However, to confirm the specificity of the found characteristic peptides – since they were identified in only a small group of animals (from which the blood was the most often used in artworks) and may still include peptides originating from undetected proteins – unique peptides were searched for.

Unique amino acid sequences obtained from blood proteins

To verify whether the experimentally found characteristic sequences for distinguishing animal species are also unique to blood proteins of geographically available animals (within Europe and Asia). A comparison was conducted with 14 selected animal groups (cat (*Felis catus*), chicken (*Gallus gallus*), cow (*Bos taurus*), deer (*Cervus*) – Ruminantia (suborder) (*Cervus elaphus*, *Cervus nippon*, *Cervus nippon mantchuricus*, *Dama dama*, *Moschus moschiferus*, *Muntiacus muntjak*, *Muntiacus reevesi*, *Rusa unicolor swinhoei*), dog (*Canis lupus familiaris*), domestic water buffalo (*Bubalus bubalis*), duck – Anatidae (family) (*Anas falcata*, *Anas platyrhynchos*, *Anas zonorhyncha*, *Aix galericulata*, *Aythya fuligula*, *Cairina moschata*, *Histrionicus histrionicus)*, goat (*Capra hircus*), *goose* – Anatidae (genus) (*Anser anser*, *Anser brachyrhynchus*, *Anser cygnoides*, *Anser fabalis*), horse (*Equus caballus*), human (*Homo sapiens*), pig (*Sus scrofa*), rabbit (*Oryctolagus cuniculus*), roe deer (*Capreolus capreolus* – no sequences), sheep (*Ovis aries*)). The groups of deer, duck, and goose include multiple related species in order to maximize the number of available protein sequences. This was necessary due to the very limited number of protein sequences for the most common deer species (*Cervus elaphus*) and domestic species such as duck (*Anas platyrhynchos*) and goose (*Anser anser*). For all the 14 groups, protein sequences of blood proteins that were experimentally identified in the analysis of model blood coatings (Tables S1–S7) were obtained from UniProt and NCBI (downloaded on February 14–22, 2025). However, not all protein sequences were found, because they were not available for each animal species. Furthermore, a comparison using PostgreSQL was made to determine whether these protein sequences contained the experimentally identified characteristic sequences. As a result, 400 unique sequences (Table S18) were found out of the original approximately 600 characteristic sequences (Tables S1–S7). Specifically, for pig 113, human 66, hen 61, cat 61, dog 50, cow 36, and for goose blood 13 unique peptides were found.

Analyses of the model blood coatings

The LC-ESI-Q-TOF MS method revealed that proteins in individual model samples originate from different materials, as shown in the SI, Tables S8–S12. In these tables only selected proteins that confirm the used material in the model samples and those with the highest numbers of peptides are shown. Animal proteins were found to predominate, which is consistent with historical recipes, as they include plant materials only to a limited extent. The identified proteins and materials from which sample no. 3 was prepared are shown in Table 2. The table is not complete, it only shows eleven proteins selected by the authors, which are crucial for finding the original recipe. Specifically, for this sample, presence of 162 animal proteins was detected. Based on the results of sample number 3 (Table 2), the blood coatings contain blood, egg white, and milk (milk-derived material), as indicated by the presence of haemoglobin, ovalbumin, and either casein or lactoglobulin.

It is clear from Table 3 that milk proteins that were not used in the original recipe were incorrectly detected in sample no. 1. Samples 2 and 6 contained an admixture of bile, but the presence was not detected in neither of them. Identifying proteins in

Table 2 Selection of identified proteins and the protein materials used for sample no. 3 preparation

Protein ID	Protein	Score	% Coverage	No. of peptides	Material
HBB_BOVIN	Haemoglobin β	4006,6	97.2	25	Blood
HBBA_BOSJA	Haemoglobin β-A	3614.9	84.1	21	Blood
HBA_BOVIN	Haemoglobin α	2819.0	90.8	15	Blood
HBBA_CAPHI	Haemoglobin β-A	1550.6	53.8	12	Blood
HBB_SHEEP	Haemoglobin β	1110.4	37.9	8	Blood
OVAL_CHICK	Ovalbumin	261.0	22.0	5	Egg white
CASA1_BOVIN	Alpha-S1-casein	188.3	27.1	5	Milk/milk product
CASK_BOVIN	Kappa-casein	226.8	40.0	4	Milk/milk product
CASA2_BOVIN	Alfa-S2-casein	87.1	20.3	3	Milk/milk product
LACB_BOVIN	Beta-lactoglobulin	91.2	33.7	3	Milk/milk product
CASB_BOVIN	Beta-casein	115.0	8.5	2	Milk/milk product

Table 3 A summary and comparison of the identified protein-based ingredients in six model blood coatings

No. of sample	Identified composition	Found all used protein materials	Contamination
1	Blood	No	Milk/—
2	Blood	Yes	—
3	Blood, casein, egg white	Yes	—
4	Blood, rye flour, animal glue	Yes	—
5	Blood, casein, egg white	Yes	—
6	Blood, casein, egg white	Yes	—

bile using LC-MS/MS is challenging due to its complex composition, low protein concentration, and the presence of interfering substances such as bile acids, lipids, and proteases. Additionally, proteolytic degradation can further hinder protein identification, and the instability of bile requires careful sample handling and storage. Due to these factors, specialized sample

Table 4 The main proteins identified in Japanese (J) and Chinese (C1, C2, and C3) artwork samples

Sample	Protein ID	Animal	Protein	Score	% Coverage	No. of peptides
C1	HBB_PIG	*Sus scrofa* (pig)	Haemoglobin subunit beta	92.2	50.3	8
	CO1A2_ONCMY	*Oncorhynchus mykiss* (rainbow trout)	Collagen alpha-2(I) chain	829.9	4.1	7
	HBA_PIG	*Sus scrofa* (pig)	Haemoglobin subunit alpha	275.7	39.7	6
	HBBN_AMMLE	*Ammotragus lervia* (Barbary sheep)	Haemoglobin subunit beta-C(NA)	120.9	15.6	6
	K2C1_HUMAN	*Homo sapiens* (human)	Keratin, type II cytoskeletal 1	63.6	9.0	5
	CO1A1_BOVIN	*Bos taurus* (bovine)	Collagen alpha-1(I) chain	102.9	2.3	4
	HBA_ODOVI	*Odocoileus virginianus virginianus* (Virginia white-tailed deer)	Haemoglobin subunit alpha-1/2	26.0	14.9	4
	K1C9_HUMAN	*Homo sapiens* (human)	Keratin, type I cytoskeletal 9	33.0	4.0	3
	K1C10_HUMAN	*Homo sapiens* (human)	Keratin, type I cytoskeletal 10	103.4	1.2	2
	CO1A1_HUMAN	*Homo sapiens* (human)	Collagen alpha-1(I) chain	70.7	7.8	2
	TRYP_PIG	*Sus scrofa* (pig)	Trypsin	38.4	0.6	2
	CO2A1_BOVIN	*Bos taurus* (bovine)	Collagen alpha-1(II) chain	92.2	50.3	2
C2	HBA_PIG	*Sus scrofa* (pig)	Haemoglobin subunit alpha	344.4	22.0	3
	HBB_PIG	*Sus scrofa* (pig)	Haemoglobin subunit beta	197.7	22.4	2
	HBBF_BOVIN	*Bos taurus* (bovine)	Haemoglobin fetal subunit beta	249.7	14.5	2
	HBB_TARBA	*Taraba major* (great antshrike)	Haemoglobin subunit beta	60.8	15.8	2
	HBA_ODOVI	*Odocoileus virginianus virginianus* (Virginia white-tailed deer)	Haemoglobin subunit alpha-1/2	45.8	5.0	2
	CO1A1_BOVIN	*Bos taurus* (bovine)	Collagen alpha-1(I) chain	20.6	0.6	2
C3	HBA_PIG	*Sus scrofa* (pig)	Haemoglobin subunit alpha	1536.2	39.7	7
	HBB_PIG	*Sus scrofa* (pig)	Haemoglobin subunit beta	977.3	41.5	6
	CKAP5_HUMAN	*Homo sapiens* (human)	Cytoskeleton-associated protein 5	19.4	0.7	5
	HBA_ODOVI	*Odocoileus virginianus virginianus* (Virginia white-tailed deer)	Haemoglobin subunit alpha-1/2	115.9	14.9	4
	HBB_TARBA	*Taraba major* (great antshrike)	Haemoglobin subunit beta	239.5	14.5	3
	HBBN_AMMLE	*Ammotragus lervia* (Barbary sheep)	Haemoglobin subunit beta-C(NA)	111.6	15.8	3
	HBBF_BOVIN	*Bos taurus* (bovine)	Haemoglobin fetal subunit beta	38.5	7.1	3
	ZNRF3_MOUSE	*Mus musculus* (mouse)	E3 ubiquitin-protein ligase ZNRF3	20.4	1.5	2
	CO2A1_BOVIN	*Bos taurus* (bovine)	Collagen alpha-1(II) chain	17.5	0.6	2
J	CO1A1_BOVIN	*Bos taurus* (bovine)	Collagen alpha-1(I) chain	344.1	11.0	15
	HBB_PIG	*Sus scrofa* (pig)	Haemoglobin subunit beta	1477.1	71.4	12
	CO1A2_BOVIN	*Bos taurus* (bovine)	Collagen alpha-2(I) chain	155.5	7.3	11
	HBA_PIG	*Sus scrofa* (pig)	Haemoglobin subunit alpha	1349.2	55.3	8
	ALBU_PIG	*Sus scrofa* (pig)	Serum albumin	249.1	10.2	7
	HBA_ODOVI	*Odocoileus virginianus virginianus* (Virginia white-tailed deer)	Haemoglobin subunit alpha-1/2	755.9	42.5	6
	HBBN_AMMLE	*Ammotragus lervia* (Barbary sheep)	Haemoglobin subunit beta-C(NA)	571.7	15.6	6
	HBB_AILFU	*Ailurus fulgens* (Himalayan red panda)	Haemoglobin subunit beta	400.7	14.9	6
	HBB_CROCR	*Crocuta crocuta* (spotted hyena)	Haemoglobin subunit beta	646.3	31.5	5
	HBBF_BOVIN	*Bos taurus* (bovine)	Haemoglobin fetal subunit beta	424.6	22.8	4
	HBB_TARBA	*Taraba major* (great antshrike)	Haemoglobin subunit beta	526.8	15.8	3
	HBA_PERCA	*Peromyscus californicus* (California mouse)	Haemoglobin subunit alpha	109.7	14.2	3
	CO2A1_RAT	*Rattus norvegicus* (rat)	Collagen alpha-1(II) chain	23.8	1.6	3

preparation techniques, including bile salt and lipid removal, as well as protein enrichment strategies are often necessary to improve detection and identification efficiency.[27] Inorganic materials, such as lime and aluminium potassium sulphate ($KAl(SO_4)_2 \cdot 12H_2O$), influence the number of peptides identified, potentially affecting protein identification. Lime, which increases pH, can cause protein degradation.[15] Similarly, inorganic ions from aluminium potassium sulphate and lime can also contribute to the degradation of protein materials.[28]

All model samples of blood coatings were prepared from the same type of animal blood. The usage of cow blood was confirmed in sample 3. In 268 selected peptides (without amino acid sequences duplicates) coming from blood proteins (*e.g.* apolipoprotein A-I, complement C3, fibrinogen gamma-B chain, haemoglobin subunit alpha, haemoglobin subunit beta, catalase, peroxiredoxin-2, prothrombin, serum albumin, spectrin beta chain erythrocytic) in Table S13, 46 peptides were assigned to cow blood (about 17% of all peptides coming from blood). Only two amino acid sequences (R.GPLLVQDVVFTDEMAHFDR.E from catalase, R.FLDLLEPLGR.R from spectrin beta chain, erythrocytic) were assigned to pig blood and one (K.AVMDDFAAFVEK.C) belongs to human serum albumin. From the 46 characteristic peptides, 17 are unique for cow blood. No other unique peptides were found.

The most frequently occurring proteins whose peptides primarily determine the animal species origin were: alpha-2-macroglobulin, serum albumin, apolipoprotein A-I, peroxiredoxin and then haemoglobin subunit alpha and beta.

This finding proves that when determining the animal origin of various materials, it is more appropriate to deal with a comprehensive analysis of the entire protein composition of the given material than to deal selectively with only one type of protein – for example, with only haemoglobin in blood materials.

Determination of blood origin of the asian artworks

The results of the analyses of the Chinese and Japanese artworks fragments are shown in Table 4. All samples contain blood and collagen proteins. In Tables S14–S17, only the peptides originating from blood-specific proteins, excluding collagen, keratin, and other non-blood proteins are listed. The duplicate amino acid sequences were omitted from the lists.

According to Tables 4 and it is not possible to determine the animal origin of blood based on the proteins assigned to the individual animal species when the results are obtained from a comprehensive animal database (UniProt, *etc.*). For this reason, the sets of data from artworks presented in Tables S14–S17, were compared to characteristic values (amino acid sequences) of all studied animals (cat, cow, dog, goose, hen, human, and pig) in Tables S1–S7 and to the unique sequences in Table S18. The animal origin of the used blood was identified as a pig blood. No other values characteristic for the other animal species were found by neither approach. In sample C1 five characteristic peptides (four of them belong to unique) coming from pig blood were found (18 blood peptides were found in total), in C2 two characteristic peptides (one unique) from pig blood (10 in total), in C3 five characteristic (two unique) from pig blood (18 in total), and in sample J 9 characteristic (eight unique) peptides from pig (43 in total). It represents

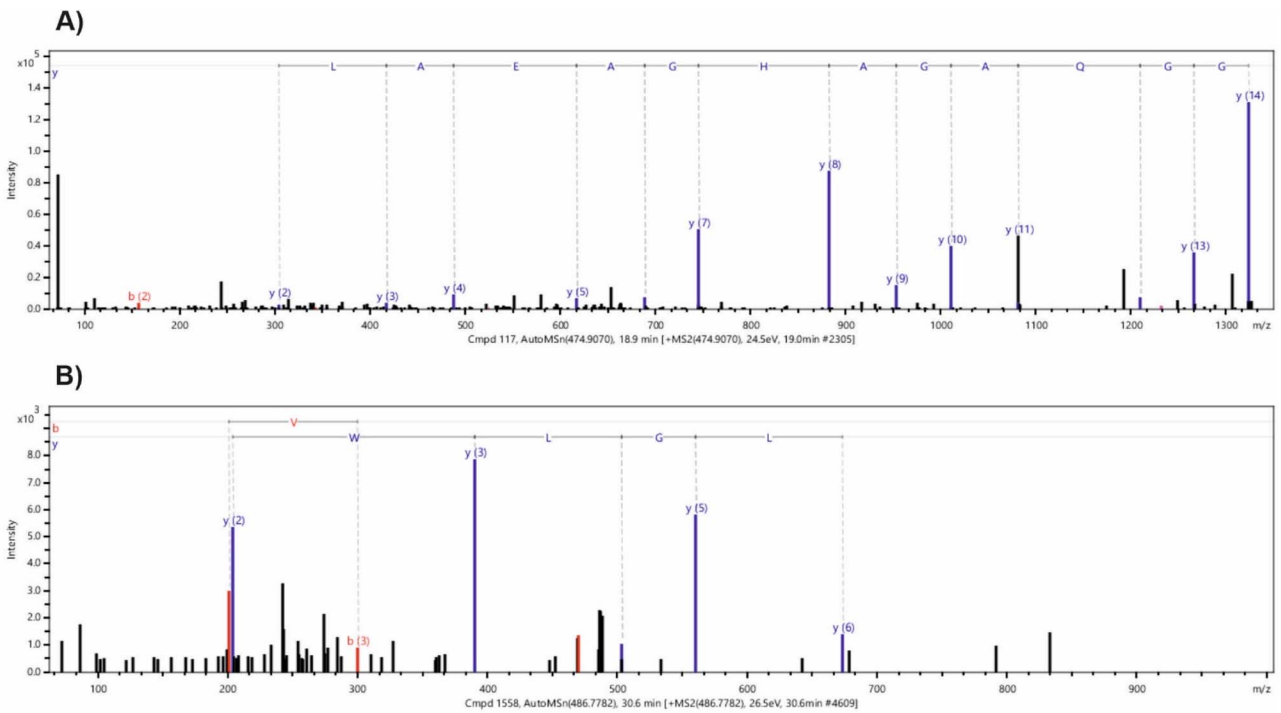

Fig. 4 (A) Peptide K.VGGQAGAHGAEALER.M in C3 (the 18th century Chinese lacquer screen), $m/z = 474.90705$, $z = 3$, error = −1.28 ppm; (B) K.EAVLGLWGK.V in sample C3, $m/z = 486.7782$, $z = 2$, error = −2.29 ppm.

28% (22%) in C1, respectively 20% (10%) in C2, 28% (11%) in C3, and 21% (19%) in J of characteristic (unique) peptides coming from pig blood. The spectra of the two most frequently observed unique peptides derived from porcine blood (SI, Tables S14–S17) – K.VGGQAGAHGAEALER.M (found in C1, C2, and C3) and K.EAVLGLWGK.V (in C1, C3, and J), originating from haemoglobin subunits alpha and beta, respectively – are shown in Fig. 4.

Conclusions

Cat, cow, dog, goose, chicken, human, and pig whole blood samples were analysed by LC-ESI-Q-TOF MS. Characteristic *m*/*z* values were experimentally found for the given animal species and assigned to individual proteins with corresponding amino acid sequences. It has been shown that not only different sequences of haemoglobin chains play an important role in distinguishing blood samples, but also differences in other blood proteins, *e.g.* alpha-2-macroglobulin, apolipoprotein, complement C3, fibrinogen gamma-B chain, serum albumin, and serum transferrin. The characteristic values were compared to theoretically found unique peptides for 14 animal groups living in Europe and Asia (possibly available for their blood usage in artworks). Comparing this theoretical approach to the experimental, it shows its limitation in the lack of protein sequences for not-yet sequenced animals in publicly available databases (UniProt, NCBI).

In six model samples of blood coatings, proteins leading to the determination of their original recipes were identified. In addition, contamination in the form of milk proteins was found in sample containing only blood. Such contamination could have occurred at any stage of the experiment, including preparation, coating, transport, or laboratory handling. According to the characteristic values, the use of cow blood was confirmed.

Results from the analysed Japanese and Chinese artworks from 18th and 19th century show that the ground layers contain collagens (animal glue) and pig blood.

The LC-ESI-Q-TOF MS proved to be a useful tool for determining the animal species origin of blood used in blood coatings. In general, the percentage representation of characteristic peptides in the model sample containing cow blood was about 17%, while in real artworks containing pig blood, it varied from 20% to 28%. Although these numbers may appear to be relatively low, it is important to note that they are representative of the characteristic peptides selected from all blood peptides for each studied animal. To enhance the identification of animal blood in artworks, further investigation should focus on peptide modifications (*e.g.*, deamidation), additional testing, the inclusion of expanded reference materials, and inter-laboratory studies. One of the other optimised parameters could be the estimation of suitable sample size. High-quality results achieved on LC-timsTOF Pro with minimal sample amounts of 20–200 ng of HeLa cells were obtained.[29] For cultural heritage samples, optimisation of sample preparation and analytical conditions is essential to ensure sufficient sensitivity and reproducibility. Considering the low protein content in paint layers (typically a few percent),[23,30] the minimal sample requirement is estimated at ~20 μg. Additional factors, such as protein degradation and the presence of inorganic matrix components, can influence the necessary sample quantity and depend on the material type, age, and long-term preservation conditions. Once sufficient results are obtained, chemometric tools can be applied to support data interpretation.

Author contributions

Stepanka Kuckova: conceptualization, funding acquisition, software, supervision, methodology, writing – original draft, data curation, project administration, resources, formal analysis. Tadeas Matecha: investigation, validation, writing – review & editing. Jiri Santrucek: data curation, investigation, writing – review & editing. Alena Meledina: formal analysis, writing – review & editing. David Straka: formal analysis, writing – review & editing. Katarina Harcarikova: formal analysis, writing – review & editing. Tomas Dostal: investigation, writing – review & editing, resources. Ludek Dvorak: investigation, writing – review & editing, resources. Vaclav Pitthard: data curation, writing – review & editing. Silvia Miklin-Kniefacz: data curation, visualization, writing – review & editing, resources.

Conflicts of interest

There are no conflicts to declare.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD065754. The data supporting this article have been included as part of the SI. Supplementary information is available. See DOI: **https://doi.org/10.1039/d5ay01156f**.

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