



Rapid detection of aged collagen in archaeological residues using immunosorbent assay

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ABSTRACT

Collagen is a common component found in the degradation residues of gelatine, leather, and food. The rapid detection of collagen at archaeological sites and in residues is of significant importance to scientific archaeology. Enzyme-linked immunosorbent assay (ELISA) is a crucial method for detecting proteins in archaeological materials. In this study, three types of polyclonal antibodies were prepared. They are cost-effective, easy to use, and applicable to a wide range of purposes. These antibodies specifically target common mammalian type I collagen and are designed to recognize different immune epitopes, including the N-terminal sequence and the triple-helix region. The use of ELISA enables the detection of specific sequence fragments in aged collagen. Based on the immunological experiments, immunochromatographic strips were developed to allow rapid on-site detection of aged collagen. The practical utility of these antibodies in detecting degraded collagen was demonstrated through experiments on ancient leather and mortar samples.

1. Introduction

In archaeological excavations, organic adhesives, textiles, leather, and food remnants often exist as fragments, mud-like substances, and micro-traces, making it challenging to recognize their original appearance. Moreover, the composition of these residues is complex, with extremely low concentrations of characteristic biomarkers, posing significant challenges for separation, purification, and analysis. The protein components in residues, due to prolonged exposure to harsh conditions, undergo varying degrees of ageing and degradation. Factors such as inappropriate temperature and humidity, acidic or alkaline environments, inorganic salts in groundwater, harmful gases in the air, and microbial corrosion accelerate this process, leading to changes in the proteins' molecular weight, spatial structure, and amino acid composition. Therefore, developing methods for detecting aged proteins is of great importance to scientific archaeology.

Common methods for detecting protein components in archaeological residues include: pyrolysis gas chromatography-mass spectrometry

(Py-GC/MS) (Adamiano et al., 2013; Li et al., 2024; Stankiewicz et al., 1997) and high-performance liquid chromatography (HPLC) (Fremout et al., 2009; Kurugöl and Güleç, 2012) for amino acid analysis; immunological techniques such as crossed immunoelectrophoresis (CIEP) (Loyola et al., 2024), enzyme-linked immunosorbent assay (ELISA) (Patricia and Michael, 1990; Palmieri et al., 2011; Pavelka et al., 2011), and immunofluorescence (IF) (Hu et al., 2015; Liu et al., 2015; Wu et al., 2019) for detecting specific target proteins; mass spectrometry-based techniques like peptide mass fingerprinting (PMF and ZoomS) (Harvey, 2018; Pal Chowdhury et al., 2021) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Buckley et al., 2013; Calvano et al., 2020; Kuckova et al., 2021) for collagen analysis in zooarchaeology; as well as tandem mass spectrometry (LC-MS/MS) (Bleasdale, 2021; Elnaggar et al., 2022; Ge et al., 2023) and palaeoproteomics (Paterson et al., 2025; Warinner et al., 2022) for in-depth protein profiling. These methods can accurately identify the types of proteins and their species of origin, providing rigorous scientific support for archaeology. However, detection techniques that rely on

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large-scale instruments require sample evaluation and selection based on the specific characteristics of the equipment. During sampling and pre-treatment process, contamination from polymeric materials such as binders, resins, and sealing films, as well as proteins from latex gloves, may be introduced (Hendy, 2021; Hendy et al., 2018). These contaminants potentially affect results and mask information from archaeological samples. Before conducting destructive analysis on archaeological residue samples, it is essential to assess the preservation state of the proteins and perform a preliminary evaluation of the protein types. Therefore, a rapid testing method capable of pre-screening large quantities of samples is required. This method should not be overly demanding in terms of sample preservation conditions, nor should it involve complex pre-treatment procedures. Simplified operational steps can reduce contamination during early stages and improve testing efficiency.

ELISA is a conventional method used to identify the presence of specific target proteins. It is based on the immune reaction between specific antibodies and antigens, with a focus on the selection of immunological epitopes. Different immune epitopes can interact with peptide sequences and protein conformations, providing information about the protein type and protein degradation. Before identification using techniques such as mass spectrometry, ELISA can be used for pre-screen archaeological residues containing protein components for rapid sample selection (Child and Pollard, 1992). Collagen is the main structural protein found in the extracellular matrix of connective tissue. Type I collagen (COL1) is one of the most ubiquitous and abundant proteins found in archaeological residues. It is usually composed of two $\alpha 1$ chains and one $\alpha 2$ chain. Hydroxyproline is one of the main components of collagen (Henriksen and Karsdal, 2016; Ricard-Blum, 2011). Type I collagen is frequently present in animal bones, food remnants, leather, and binders. Additionally, collagen is the primary protein used in ZooMS for identifying animal species (Richter et al., 2022).

Based on the characteristics of collagen, we have prepared three types of polyclonal antibodies targeting aged collagen and evaluated their application in the detection of aged collagen. Combined with the spectrophotometric method for detecting hydroxyproline, we have established a rapid pre-screening method for analysing type I collagen in archaeological residues.

2. Experiments

2.1. Chemicals and instruments

Sodium citrate, n-propanol, citric acid, sodium acetate, isopropanol, dimethyl sulfoxide (DMSO), hydrogen peroxide, sulphuric acid, TRIS hydrochloride, sodium carbonate, sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium bicarbonate, copper sulphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, and potassium chloride were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chloramine T and pigskin collagen were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Perchloric acid, 4-dimethylaminobenzaldehyde, sodium tartrate, 2,2'-quinoline-4,4'-dimethanedisodium (BCA), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Aladdin Industrial Corporation (Shanghai, China). Hydroxyproline was purchased from Sigma (St. Louis, USA). Bovine serum albumin (BSA) was purchased from Phygene (Fuzhou, China). Tween 20 and Coomassie Brilliant Blue G-250 reagent were purchased from Beyotime Biotechnology (Shanghai, China). Horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (H + L) was purchased from Proteintech (Wuhan, China). Rabbit skin gelatine was purchased from Sennelier (Paris, France).

A microplate spectrophotometer (BioTek EPOCH, USA) was used to measure optical density (OD value). A simplicity water purification system (Millipore, USA), electro thermostat, benchtop high-speed centrifuge, and ultrasonic cleaner were used for sample handling.

2.2. Reagent preparation protocols

2.2.1. BCA-CuSO₄ reagent

A total of 10 g of BCA, 20 g of sodium carbonate, 1.6 g of sodium tartrate, 4 g of NaOH, and 9.5 g of sodium bicarbonate were weighed and mixed with 800 mL of water. The pH was adjusted to 11.25 by adding solid NaOH. The solution was then diluted with water to a final volume of 1 L. The solution was mixed with 4 % (w/w) copper sulphate solution in a 50:1 vol ratio to prepare the BCA-CuSO₄ reagent.

2.2.2. Citric acid-sodium acetate buffer

Citric acid monohydrate (26 g), NaOH (20 g), and sodium acetate (78 g) were weighed and mixed with 500 mL of water. To this, 250 mL of n-propanol was added, and the solution was diluted with water to a final volume of 2 L.

2.2.3. Chloramine T reagent

Chloramine T (0.705 g) was weighed and diluted with citric acid-sodium acetate buffer to a final volume of 50 mL.

2.2.4. Chromogenic reagent

4-dimethylaminobenzaldehyde (10 g) was weighed and mixed with 35 mL of 60 % perchloric acid. Then, 65 mL of isopropanol was added.

2.2.5. Coating buffer

Sodium carbonate (1.59 g) and sodium bicarbonate (2.94 g) were weighed and diluted with water to a final volume of 1 L.

2.3. Synthesis of polyclonal antibodies

The polyclonal antibodies were commercially produced by Hangzhou HuaAn Biotechnology Co., Ltd. (China), in compliance with the national ethical guidelines for animal welfare (GB/T 35892-2018).

2.3.1. Preparation of antigens

Mammalian type I collagen was selected as the target protein, with shared sequences from the N-terminal propeptide region of the type I collagen $\alpha 1$ chain (pep-C1) and the triple helix region of the type I collagen $\alpha 2$ chain (pep-C2) serving as haptens. Keyhole limpet haemocyanin (KLH) was dissolved in ethylenediaminetetraacetic acid (EDTA) solution, and Sulfo-SMCC solution was added to activate the KLH. After dialysis, the activated KLH solution was obtained. The antigen peptide solutions (pep-C1 and pep-C2) were then reacted with the activated KLH solution to form the cross-linked conjugates KLH-pep-C1 and KLH-pep-C2. After dialysis, these conjugates were used to generate polyclonal antibodies C1 and C2, respectively.

Residual hair, fat, and meat were removed from the fresh cattle hide. The hide was then cut into small pieces measuring 0.5 cm × 1 cm. These pieces were boiled in water for 6 h, followed by centrifugation to collect the supernatant. The supernatant was placed in a dialysis bag with a molecular weight cut-off of 3000 Da and dialysed for 24 h. After dialysis, the protein solution was lyophilised to obtain cattle hide collagen powder, which was used as the antigen for the preparation of polyclonal antibody C3.

2.3.2. Animal immunization and antibody purification

Healthy adult New Zealand White rabbits, approximately 2.5 kg in weight, were selected as immunised animals. The adjuvant was mixed with the antigen at a 1:1 vol ratio. The initial immunization was conducted with complete Freund's adjuvant, while the second to fourth immunizations used incomplete Freund's adjuvant, administered via multiple subcutaneous injections. After the third immunization, small serum samples were collected for testing. If the results met the desired criteria, a booster immunization was performed seven days later, and full blood collection was conducted seven days after the booster. Serum was purified using affinity chromatography, and the collected

Table 1
Extraction condition of animal hides and leather samples.

Sample Weight	Extraction Solution	Extraction Temperature	Extraction Time
50 mg	Water 0.1 mol L ⁻¹ NaOH 0.1 mol L ⁻¹ HCl 8 mol L ⁻¹ Urea	100 °C Room Temperature	6 h 2 days

Table 2
Information of animal hides, leathers and collagen products.

Sample Number	Types	Sample Information
P1	Fresh mammal skin	Pig skin
P2		Cattle skin
P3		Goat skin
G1	Tanned leather (from cattle)	Aluminium tanned cattle leather
G2		Iron tanned cattle leather
G3		Vegetable tanned cattle leather
G4	Tanned leather (from sheep)	Aluminium tanned sheep leather
G5		Iron tanned sheep leather
G6		Vegetable tanned sheep leather
J1	Commercially available collagen products	Collagen from pig skin (MW, 2000–3000)
J2		Gelatine from rabbit skin

antibodies were concentrated by ultrafiltration and dialysed to obtain the purified antibodies C1, C2, and C3.

2.4. Indirect enzyme-linked immunosorbent assay

Sample solutions were added to the wells of a microplate, at 60 µL per well with three replicates for each sample. Blank and positive control wells were also included. The plate was incubated at 37 °C for 2 h to allow coating. After coating, the wells were washed with PBS-T (phosphate-buffered saline containing 0.5 % Tween 20). Then, 80 µL of PBS (phosphate-buffered saline) with 1 % BSA was added to block non-specific binding for 1 h. Subsequently, 60 µL of primary antibody was added, and the plate was incubated at 37 °C for 1 h. The wells were then washed three times with PBS-T. Following washing, 60 µL of HRP-conjugated goat anti-rabbit IgG was added, and the plate was incubated at 37 °C for 1 h. After another three washes with PBS-T, 100 µL of TMB substrate solution (10 mg mL⁻¹ TMB in DMSO) was added to each well. After colour development, the reaction was terminated by adding 50 µL of 2 mol L⁻¹ sulphuric acid. Absorbance was measured at 450 nm and 630 nm to assess the results.

2.5. Tests of polyclonal antibodies

2.5.1. Protein extraction from animal hides and leather samples

Samples of 50 mg each were taken from three types of fresh animal hide and six types of tanned leather. Each sample was added to 1 mL of 8 mol/L urea solution and soaked at room temperature for two days to extract the proteins, resulting in a total of nine protein extraction solutions. Each sample was added to 1 mL of deionised water, 0.1 mol/L NaOH, or 0.1 mol/L HCl. The mixtures were heated in a metal bath at 100 °C for 6 h to extract the proteins, resulting in a total of 27 protein extraction solutions. The extraction methods are detailed in Table 1.

After extraction, the solutions were centrifuged at 5000 rpm for 3 min, and the supernatant was collected for protein concentration analysis. Additionally, two commercial collagen products were weighed and dissolved in coating buffer solution to be used as collagen controls in subsequent experiments. The sample information for the animal hides, leathers, and collagen products is provided in Table 2.

High-concentration urea solution (8 M urea) is a common protein denaturant that does not disrupt the amide bonds in collagen, thereby preserving its primary structure and covalent bonds. It can disrupt the triple helix structure of collagen, leading to the dissociation of the triple helix conformation (Bella, 2016; Wei et al., 2021). Heating can break hydrogen bonds within the collagen structure and transforming the triple helix into a random coil conformation (Bischof and He, 2006; Wright and Humphrey, 2002). Thermal treatment exposes the antigenic sites within the collagen structure, allowing them to bind with antibodies. The extraction of collagen using boiled water simulates the ancient method of gelatine preparation. According to the *Ben-Cao-Hui-Yan* (Collected Works of Materia Medica), gelatine is produced by boiling cattle skin to dissolve the collagen. Treatment of samples with acid or alkali at high temperature simulates collagen ageing under extreme conditions. The acid and alkaline treatments can leach out a large amount of protein and break down some covalent bonds. In general, cultural heritage materials do not undergo such extreme ageing processes. However, for collagen in archaeological residues that have been exposed to harsh preservation conditions or have been aged over long periods, this simulated experiment may provide valuable insights into their degradation. The four methods mentioned above are capable of extracting denatured collagen, gelatine, and fragmented collagen peptides from hides and leathers.

2.5.2. Collagen determination in animal hides, leathers and collagen products

A total of 36 animal leather protein extraction solutions obtained from Section 2.5.1 were measured and diluted with deionised water to fall within the suitable concentration range for the BCA assay standard curve. Then, 20 µL of each sample was mixed with 200 µL of BCA-CuSO₄ reagent and incubated at 37 °C for 60 min. Afterwards, 200 µL of the reaction mixture was transferred to a 96-well plate, and absorbance was measured at a wavelength of 562 nm using a microplate spectrophotometer. For protein quantification using the Bradford method, 10 µL of each sample was mixed with 300 µL of Coomassie Brilliant Blue G-250 reagent, and absorbance was measured at a wavelength of 595 nm. Bovine serum albumin (BSA) solution was used as the protein standard to construct the standard curve.

Samples of 50 mg each were taken from three types of fresh animal hides, six types of tanned leathers, and two commercial collagen products. These samples were treated with 0.5 mL of 6 mol/L HCl and heated at 100 °C for 6 h. After the reaction, the solution was allowed to cool to room temperature. The pH was adjusted to between 6 and 8 using 10 mol/L NaOH, and the volume was made up to 1 mL with deionised water. The samples were then centrifuged, and 60 µL of the supernatant was mixed with 60 µL of Chloramine T reagent. The mixture was left to stand at room temperature for 20 min. Following this, 60 µL of DMAB reagent and 120 µL of deionised water were added, and the mixture was thoroughly mixed. The reaction was incubated at 60 °C for 20 min, then removed and left to stand at room temperature for an additional 15 min. Finally, 200 µL of the solution was transferred to a microplate, and absorbance was measured at 560 nm. HYP aqueous solution was used as the protein standard to construct the standard curve.

2.5.3. Selection of optimal antigen

A total of 38 collagen protein solutions were prepared: 36 protein extraction solutions from nine types of animal leathers (extracted by four different methods) and two commercial collagen products dissolved in coating buffer. These solutions were diluted to 20 µg/mL and coated. The polyclonal antibodies C1, C2, and C3 were diluted in PBS as primary antibodies, and HRP-conjugated goat anti-rabbit IgG (H + L) was diluted in PBS as the secondary antibody. Indirect ELISA was used to detect the 38 collagen protein solutions, and the optimal antigen and extraction method for each antibody were selected.

Table 3
Information of archaeological samples.

Sample Number	Type	Archaeological Number	Source	Age
S1	Air-Dried Leather Piece	1415	Russian, Hasaut Valley Tomb	C.E. 829 - C.E. 879
S2	Water-Retaining Leather Piece	HWM1G-WI:2-36	China, Anhui, Wuwangdun Site	B.C.E. 476 - B.C.E. 221
S3	Tomb Soil	1415-soil	Russian, Hasaut Valley Tomb	C.E. 829 - C.E. 879
S4	Building Mortar	THD-S3	China, Beijing, Forbidden City	C.E. 1368 - C.E. 1911
S5	White Lime Surface	MZ-3-CJ	China, Shaanxi, Mizhi County	Around B.C.E. 2000

2.5.4. Detection limit and standard curve of polyclonal collagen antibodies

The optimal antigens, determined in Section 2.5.3, were diluted to concentrations ranging from 1000 to 0.001 µg/mL with coating buffer and coated onto a 96-well plate. The coating buffer was used as the blank control. The detection limit and standard curve for the three polyclonal antibodies against collagen were determined using the indirect ELISA method.

2.5.5. Specificity of polyclonal collagen antibodies

Common interfering proteins of animal origin were selected for specificity testing, including silk fibroin (from ancient clothing), keratin (from animal hair), and ovalbumin (from egg whites, commonly found in binders). These proteins were diluted to 20 µg/mL and coated onto a 96-well plate. Indirect ELISA was performed to assess the specificity of the collagen antibodies.

2.6. Immunochromatographic strips

Based on the antibody detection results in Section 2.5, antibody C1 was selected as the antigen for the preparation of immunochromatographic strips. The rapid test strip preparation process was completed by Shanghai Jieyi Biotechnology Co., Ltd.

Colloidal gold was prepared using the sodium citrate reduction

method. To begin, 100 mL of 0.01 % chloroauric acid solution was heated to boiling. Then, 1.4 mL of 1 % sodium citrate solution was rapidly added to the boiling solution. The colour of the solution changed from black to blue and eventually to deep red. The solution was boiled for a further 15 min before being allowed to cool, resulting in a colloidal gold solution.

The pH of the colloidal gold solution was adjusted to 5.0. Then, 15 mL of the colloidal gold solution was mixed with 150 µg of polyclonal antibody C1. The mixture was stirred for 1 h, followed by rapid mixing. To block unbound sites, 1.5 mL of a 1 % BSA solution was added, and the mixture was incubated at room temperature for 5 min. After centrifugation, the red, loose precipitate at the bottom was collected and washed with 0.02 mol/L PBS buffer. The washed precipitate was resuspended in 2 mL of 0.02 mol/L PBS buffer and stored at 4 °C.

The colloidal gold-conjugated antibody C1 was spray-coated onto a glass fibre membrane and dried at 37 °C. On a nitrocellulose membrane, 0.5 mg/mL of peptide antigen was coated on the test line (T line), and 1.2 mg/mL of goat anti-rabbit IgG (H + L) was coated on the control line (C line). Both lines were dried at 37 °C. The assembly of the test strip included the following components: base plate, sample pad, colloidal gold pad, nitrocellulose membrane, and absorbent pad. The strips were then cut into individual strips and packaged in plastic cards.

The antigen peptides were diluted with deionised water to concentrations ranging from 100 to 0.01 µg/mL. Protein antigens were also diluted to concentrations ranging from 100 to 0.01 mg/mL with deionised water. These various antigens were tested using the prepared rapid test strips to determine the detection limit range of the rapid test.

2.7. Archaeological samples

Five archaeological samples were selected for testing, including one air-dried leather sample, soil from the tomb containing the air-dried leather, one water-retaining leather sample, and two building mortar samples. The sample images and detailed information are provided in Table 3 and Fig. 1. The two ancient leather samples were processed using the four extraction methods described in Section 2.5.1. For the tomb soil, building mortar, and lime plaster samples, treatment procedures followed the protocols established in previous mortar testing experiments (Zhai et al., 2024). The protein content, hydroxyproline

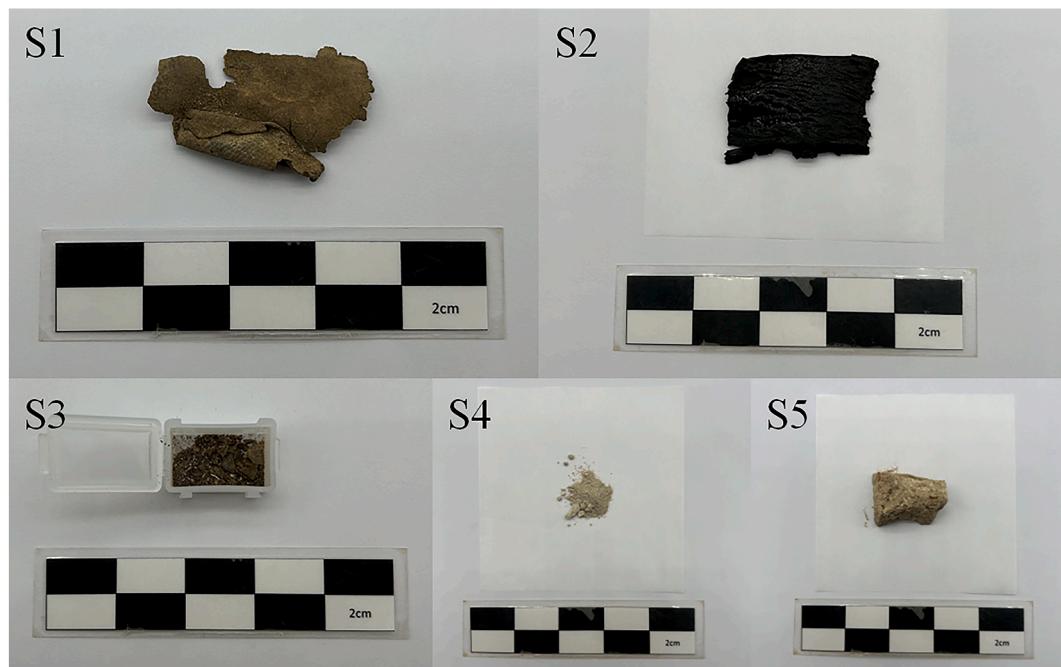


Fig. 1. Pictures of archaeological samples.

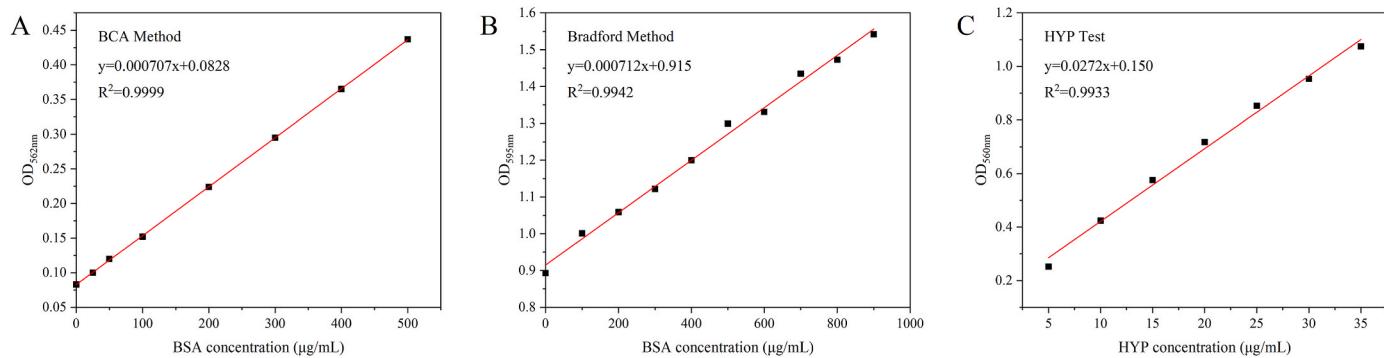


Fig. 2. Standard curves of BCA and Bradford methods.

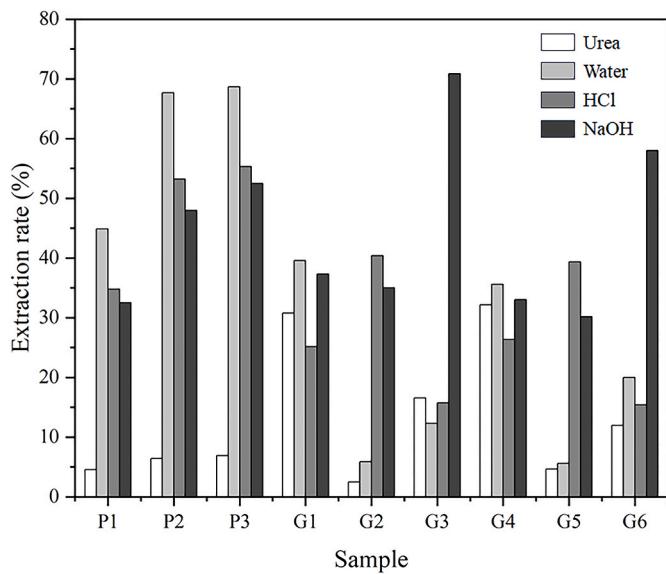


Fig. 3. Protein extraction rate of animal hides and leather samples.

content, and ELISA were performed on the extracts of the five samples.

3. Result and discussion

3.1. Extraction and Quantitative determination of collagen from animal hides and leathers

Protein quantification of the extracts obtained from the animal skin and leather samples was performed using the BCA method. During the experiment, it was observed that the tannic acid content in the vegetable-tanned leather samples (G3 and G6) was excessively high, which significantly affected the results of the BCA method. Therefore, the Bradford method was employed for protein quantification in samples G3 and G6. Fig. 2a and b presents the standard curves for protein concentration determination using the BCA and Bradford methods, respectively. The extraction efficiency was calculated as the ratio of the mass of protein extracted from the sample to the total mass of the sample used. Fig. 3 shows the protein extraction rates of the nine samples. The raw data for the protein extraction rates can be found in the Supplementary Data.

For the fresh animal skin samples P1, P2, and P3, the extraction rate using urea was the lowest (<10 %), while the highest was found with water (44 %–69 %). The extraction rate using HCl and NaOH solutions was similar (32 %–56 %). The protein extraction rate from fresh pig skin is generally lower than that from cattle skin and goat skin, possibly due to the higher fat content in pig skin. For tanned leather samples, the

Table 4
Hydroxyproline extraction rate of animal hides and leather samples.

Sample Number	Hydroxyproline Extraction Rate (%)
P1	8.7
P2	12.7
P3	11.5
G1	8.4
G2	7.0
G3	7.2
G4	7.0
G5	6.8
G6	6.5
J1	12.8
J2	13.1

appropriate extraction method varies depending on the tanning process used. The protein extraction rate of aluminium-tanned leather samples G1 and G4 is highest when using water extraction, ranging from 35 % to 40 %, with extraction rates from the other three methods varying between 25 % and 38 %. For iron-tanned leather samples G2 and G5, HCl extraction yields the highest protein content (approximately 40 %), followed by NaOH extraction (30 %–36 %), while water and urea extraction result in relatively low yields (<10 %). In the case of vegetable-tanned leather samples G3 and G6, NaOH extraction achieves the highest efficiency (58 %–71 %), with yields from the other three methods being considerably lower (12 %–20 %).

Based on a comparative analysis of the compatibility between various hides or leather types and four extraction methods, it is recommended to use water extraction for fresh hides and aluminium-tanned leathers, acid extraction for iron-tanned leathers, and alkaline extraction for vegetable-tanned leathers in order to achieve higher protein yields. The urea extraction method was found to be suboptimal for both fresh hides and tanned leathers, as it requires a relatively long processing time and results in lower extraction rates.

Hydroxyproline is a product of gelatine hydrolysis under acidic conditions and is derived from the post-translational hydroxylation of proline residues in proteins—primarily collagen. It is one of the principal components of collagen, and in animal hides and leathers, hydroxyproline is almost exclusively sourced from collagen. The hydroxyproline content of three types of fresh animal hides, six types of tanned leather, and two commercially available collagen products was determined using the spectrophotometric method. Fig. 2c displays the standard curve for hydroxyproline quantification. The extraction efficiency was determined by calculating the ratio of the mass of hydroxyproline extracted to the total mass of the sample. Table 4 presents the hydroxyproline extraction rate for the samples.

The hydroxyproline content in tanned leathers was consistently below 10 %, whereas in collagen-based products and in the raw hides of cattle and goat, the content ranged from approximately 11 %–14 %. The relatively low hydroxyproline content in pigs may be attributed to its

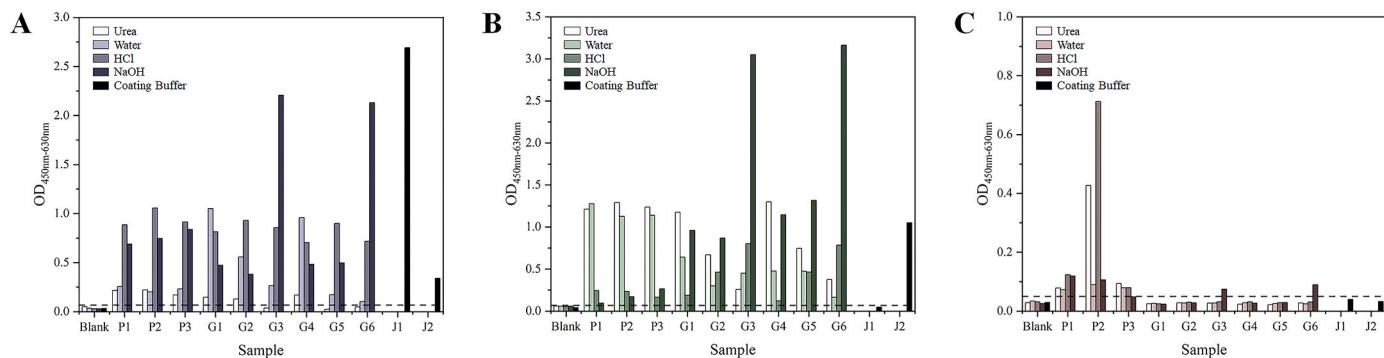


Fig. 4. Elisa results of animal hides and leather samples. A) Antibody C1; B) antibody C2; C) antibody C3.

high fat content, which similarly contributes to its lower protein extraction efficiency. In contrast, collagen products J1 and J2 exhibited slightly higher hydroxyproline contents than raw hides and tanned leathers, due to the purification of collagen during their manufacturing processes. Based on the measured hydroxyproline concentrations, it can be concluded that the majority of the extracted proteins from animal hides and leathers are collagen. These extracts are therefore directly applicable for subsequent assays involving polyclonal antibodies against collagen.

3.2. Comparison of animal hides and leather sample processing methods and selection of optimal antigen

A total of 36 extraction solutions were obtained by applying four different extraction methods to nine types of animal hides and leathers. In addition, two collagen-based commercial products, which had undergone purification during manufacturing, were dissolved in coating buffer to obtain collagen solutions J1 and J2, serving as reference controls. In total, 38 antigen solutions were prepared for detection assays.

Three collagen antibodies were used to detect the extracts from simulated samples using four different extraction methods. Two commercially available collagen products were dissolved in coating buffer for detection. Three antibodies targeted specific immunogenic epitopes: the N-terminal sequence of type I collagen (antibody C1), the triple helix sequence of type I collagen (antibody C2), and collagen extracted from cattle skin by boiled water (antibody C3). The ELISA results for the three antibodies are shown in Fig. 4. The raw data of the ELISA results can be found in the Supplementary Data. The dotted line in the figure represents the detection limit. The detection limit was defined as the mean OD value of the blank control plus three times the standard deviation. The results exhibit certain differences and patterns among fresh skins, tanned leathers, and collagen products treated by various methods.

Antibody C1 showed a positive result for all samples except for the tanned leather extracted with urea. Since antibody C1 targets an antigenic epitope located at the N-terminal sequence, its exposure may be limited due to protein conformation. As a result, water and urea extractions may not efficiently release the target fragment. In contrast, destructive HCl and NaOH extractions can break covalent bonds, exposing the antigenic site or rendering it in a free state. Therefore, the OD values of the fresh skin samples extracted with HCl and NaOH are higher than those of the samples extracted with water and urea. Antibody C1 showed higher sensitivity in detecting small-molecule collagen products, while its response to large-molecule gelatine was relatively lower. This may be due to the lime or acid process during the production of rabbit gelatine, which likely removed some of the terminal peptides, leading to the loss of antigenic sites. For tanned leather, there were also differences in the detection results depending on the tanning method. For instance, the OD values of aluminium-tanned leather extracted with water were higher than those of iron-tanned leather and vegetable-

tanned leather. The OD values of vegetable-tanned leather extracted with NaOH were significantly higher than those of leather tanned with mineral tanning agents. HCl extraction, however, yielded similar results across all types of tanned leather.

Antibody C2 showed a positive result for all samples except for the small-molecule collagen product (J1). The detection results for fresh skins and collagen products were the opposite of those observed with antibody C1. Since antibody C2 targets an antigenic site located in the collagen triple helix region, water and urea extraction cause the dissociation of the collagen triple helix structure, exposing the sequences within. In contrast, HCl and NaOH extractions likely disrupt the internal sequences of collagen. As a result, the OD values for fresh skin samples treated with water and urea were higher than those of samples extracted with HCl and NaOH. Similarly, small-molecule collagen products may have had their triple helix region sequences cleaved during the preparation process. The OD values of vegetable-tanned leather samples extracted with NaOH were particularly high. For samples extracted with urea, the OD values of aluminium-tanned leather were higher than those of iron-tanned leather and vegetable-tanned leather. In contrast, for HCl-extracted samples, the results were the opposite.

Antibody C3 showed positive results for all fresh skin samples, while it was generally negative for tanned leather samples. Antibody C3 targets a different antigenic site compared to antibodies C1 and C2. As it was generated using proteins rather than peptides for immunisation, the antigenic recognition sites include both the peptide sequences and the protein conformation. The tanning process, which causes structural damage to collagen, leads to the loss of these antigenic sites, resulting in a reduced or absent response to tanned leather samples. Since the antigen used for antibody C3 was derived from cattle skin, the antibody exhibits a degree of specificity for cattle. This distinct difference is particularly evident in the detection results of fresh pig, cattle, and sheep skins extracted with urea and HCl, where the response to cattle samples is notably higher compared to the other species.

Antibody C1 is well suited for the detection of aged collagen. With the exception of the urea extraction method, water, acid, and alkaline extractions under high-temperature conditions were all effective in retrieving the target antigenic fragments from hides and leathers. Based on the OD values, acid and alkaline extraction are recommended for detecting residual collagen. Antibody C2 also demonstrated good specificity for aged collagen. For fresh hides, water and urea extraction methods provided satisfactory results, while alkaline extraction yielded more stable outcomes when analysing tanned leathers, showing compatibility across a variety of tanning methods.

The epitope recognition profiles of antibodies C1 and C2 together cover a broad spectrum of ancient collagen residues, including those from tanned leathers, gelatine, and other collagen-containing archaeological materials. In practical applications, the use of acid or alkali to extract collagen components from archaeological residues, followed by immunodetection using antibodies C1 and C2, enables effective identification of collagen-containing materials. In contrast, antibody C3 is

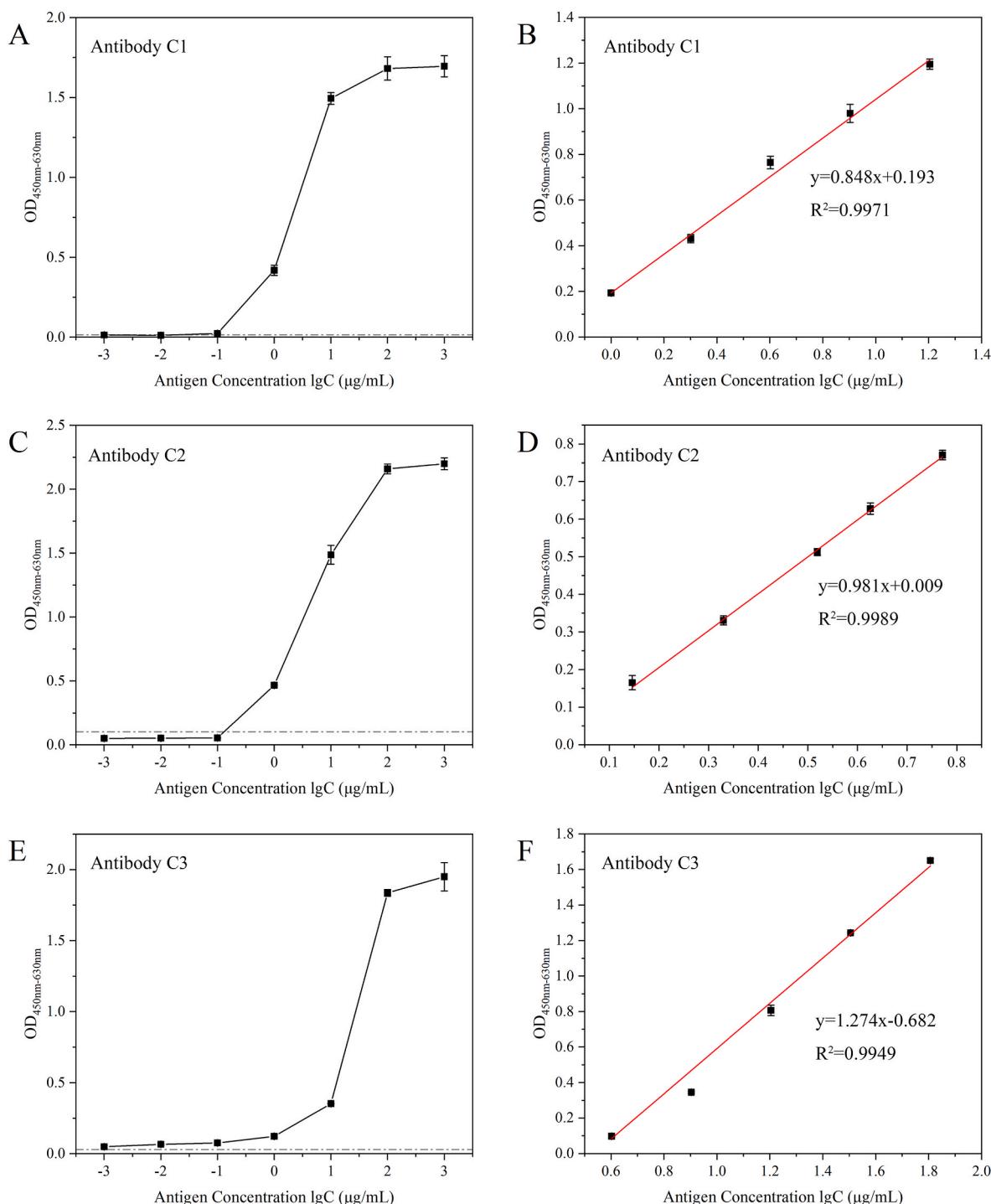


Fig. 5. The standard curves and detection limits of the antibodies.

more appropriate for the detection of fresh animal hides, particularly bovine hides, but is not suitable for the analysis of archaeological residues.

3.3. Standard curves for polyclonal antibodies and specificity analysis

Each antibody was tested by indirect ELISA after the optimal antigen for each antibody was diluted in two-fold gradients. The standard curves and detection limits are shown in Fig. 5. Antibodies C1, C2, and C3 exhibited linear relationships between the OD values and the logarithmic concentrations of the antigen within the concentration ranges of

1–16 μg/mL, 0.5–8 μg/mL, and 4–64 μg/mL, respectively. The lowest detection limits for the three antibodies were all within the ng/mL range.

To assess the specificity of the antibodies, commonly found proteins in clothing (silk fibroin and keratin) and in adhesives (ovalbumin) were selected for testing. These proteins were diluted to a concentration of 20 μg/mL and detected using the indirect ELISA method. The specificity test results are shown in Fig. 6, with the dotted line representing the detection limit. Antibodies C1, C2, and C3 all showed strong reactions with the positive control but exhibited negative reactions to silk fibroin, keratin, and ovalbumin. This demonstrates that antibodies C1, C2, and

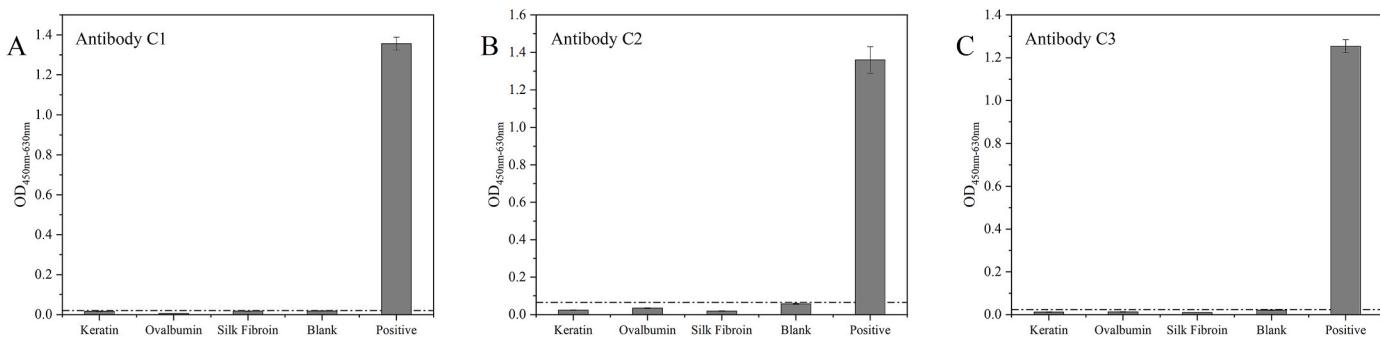


Fig. 6. The specificity detection results of the antibodies.

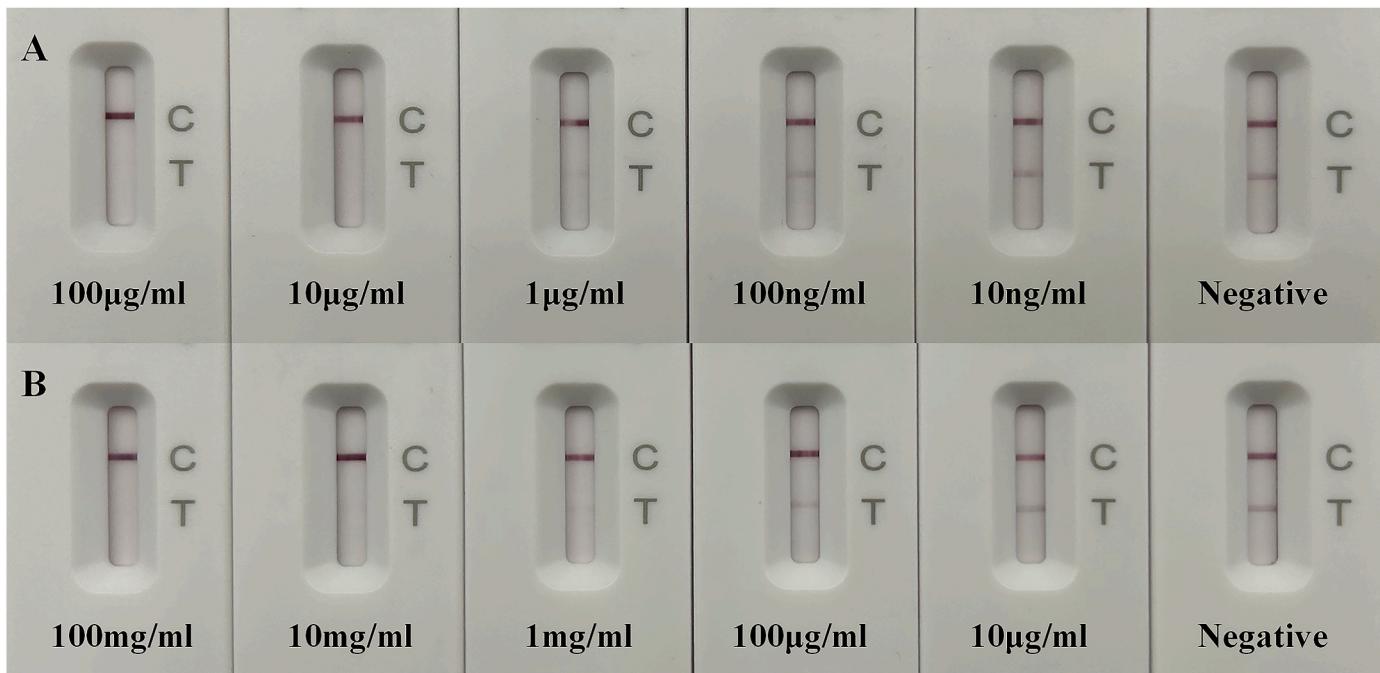


Fig. 7. Sensitivity test result for the immunochromatographic strips. A) The test results of antigen peptide; B) The test results of collagen from pig skin.

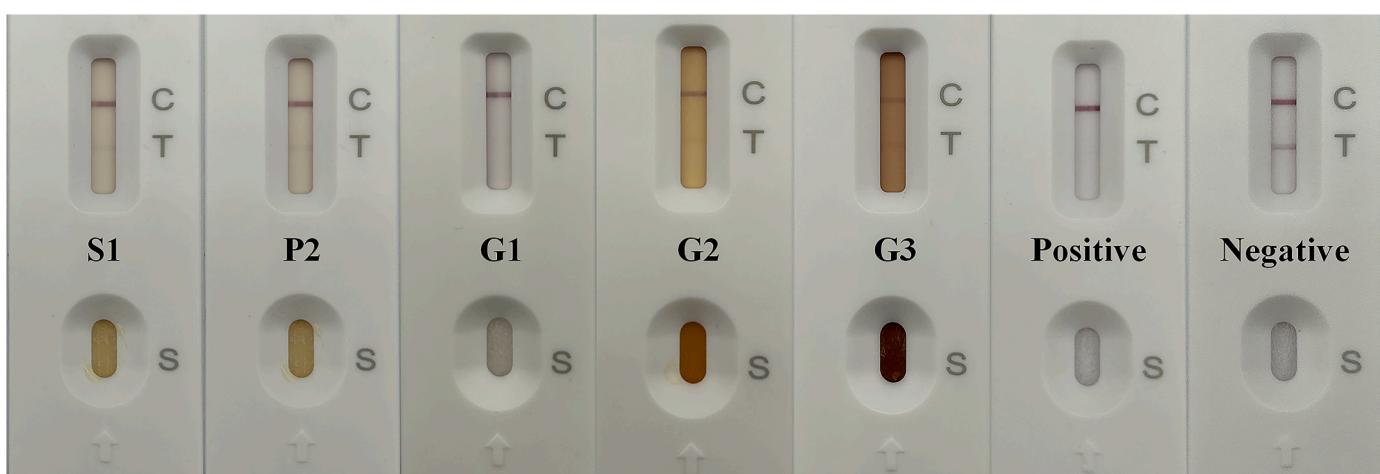


Fig. 8. Immunochromatographic test results for archaeological samples and leather samples.

Table 5
Protein and hydroxyproline extraction rate of archaeological samples.

Sample Number	Protein Extraction	Protein Extraction Rate/%	Hydroxyproline Extraction Rate/%
S1	Water	3.5	3.2
	HCl	1.1	
	NaOH	2.5	
	Urea	2.0	
S2	Water	0.64	0.49
	HCl	0.83	
	NaOH	4.9	
	Urea	1.6	
S3	Coating Buffer	8.4	0.13
S4	Coating Buffer	0.20	0.0020
S5	Coating Buffer	0.040	0.00012

C3 possess excellent specificity.

3.4. Immunochromatographic strips

The detection of collagen is based on competitive immunoreactions. In the absence of the target antigen in the test sample, red lines appear at both the test line and the control line. However, when the target antigen is present in the test sample, a red line appears only at the control line. The intensity of the red colour at the test line inversely correlates with the concentration of the target antigen in the sample. A lighter red colour at the test line indicates a higher concentration of the target antigen. The competitive immunochromatographic strip format was adopted due to the observed variations in antibody performance during preliminary evaluations. Specifically, antibodies C2 and C3 demonstrated inferior effectiveness compared to C1 in test strip preparation. Consequently, the test strip was developed using only the C1 antibody to ensure optimal performance.

Fig. 7 demonstrates the detection limit of the colloidal gold test strip. When the peptide antigen concentration is 1 µg/ml, a clear positive result is observed. When testing gradient solutions prepared using small-molecular collagen from pig skin, a distinct positive result is shown at a concentration of 1 mg/ml.

The detection limits for peptides and pig skin collagen differ due to the varying sensitivity of antibody C1 to peptides and proteins. Antibody C1 was generated through immunisation with the peptide pep-C1 conjugated to a carrier protein, resulting in a higher sensitivity to the peptide pep-C1. While collagen contains pep-C1, the immunogenic sites of pep-C1 are partially lost due to conformational changes and molecular size differences in the collagen structure, leading to a lower sensitivity of antibody C1 to collagen as compared to the peptide pep-C1.

The immunochromatographic strips were used to detect protein solutions extracted from hides and leathers (P2, G1, G2, G3) with 0.1 mol/L NaOH, as well as from archaeological samples (S1). Additionally, when protein solutions extracted from simulated and archaeological samples were tested using the colloidal gold test strip, clear positive

results were also obtained (Fig. 8). These results confirmed that the test strip is suitable for the rapid onsite qualitative detection of aged collagen.

3.5. Test results of archaeological samples

Protein and hydroxyproline were extracted and quantified from archaeological residues, and the presence of collagen residues was further confirmed through ELISA. Protein content analysis revealed that the highest amount of protein was extracted from the burial soil samples, followed by the leather samples, with the lowest amount found in mortar and lime paste samples. Hydroxyproline analysis indicated that the highest concentration of hydroxyproline was found in the leather samples, followed by burial soil, and the lowest in the mortar samples. This suggests that the burial soil contains a significant number of interfering proteins in addition to collagen. The protein and hydroxyproline extraction rates for the archaeological samples are presented in Table 5. The protein or hydroxyproline content of each sample extract was calculated using the standard curve, and the extraction efficiency was determined by the ratio of the extracted protein or hydroxyproline content to the total mass of the sample used.

Three collagen antibodies were used to detect eleven extracts from five different samples, and the results are shown in Fig. 9. The raw data of the ELISA results can be found in the Supplementary Data. Both the C1 and C2 antibodies tested positive for all eleven extracts, while the C3 antibody only showed weak positivity for six of the extracts. In the burial soil sample (S3), which contains a large number of interfering proteins, both C1 and C2 antibodies detected strong positivity for collagen. These results demonstrate that the C1 and C2 antibodies are suitable for detecting aged ancient leather samples and other archaeological residues containing collagen. It is worth noting that the specificity testing of the antibodies cannot account for all potential interfering proteins present in the residue samples. Therefore, the polyclonal antibody-based ELISA serves primarily as a screening and preliminary identification method within a defined protein range. Given the complex and often unknown composition of archaeological residues, ZooMS or LC-MS/MS analysis should be incorporated to enable the precise identification of protein types within the residue materials.

4. Conclusion

To detect collagen components in archaeological residues, three polyclonal antibodies targeting distinct epitopes of type I collagen were produced. Among them, antibody C1, which recognises the N-terminal fragment, and antibody C2, which targets the triple helix region, have proven effective in detecting aged collagen fragments, even in tanned leather residues subjected to high temperatures and extreme pH conditions. Both antibodies have demonstrated their ability to identify collagen in air-dried and water-preserved ancient leather, as well as trace amounts present in lime-based mortar under strongly alkaline

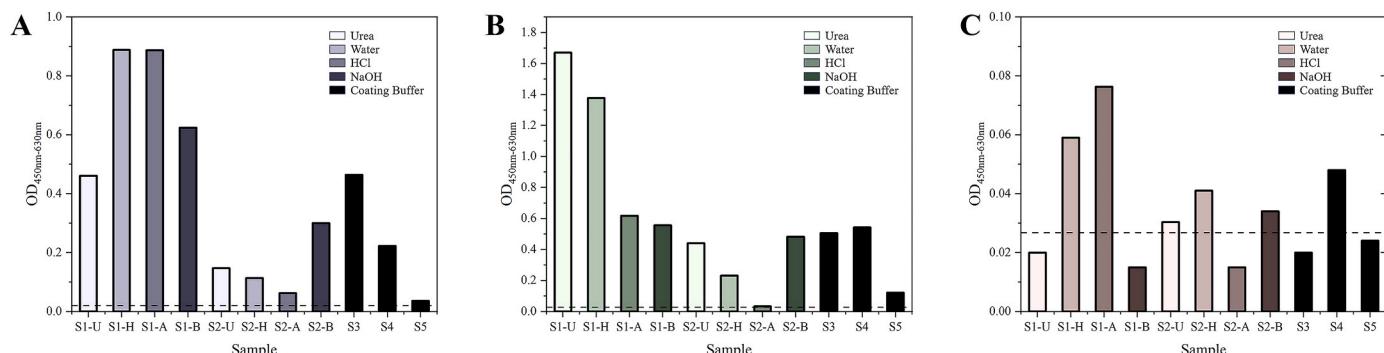


Fig. 9. ELISA results of archaeological samples A) Antibody C1; B) Antibody C2; C) Antibody C3.

conditions. Furthermore, they show excellent applicability for samples from complex environments containing numerous interfering proteins. Antibody C1 can also be employed in the development of colloidal gold test strips, facilitating rapid on-site detection during archaeological fieldwork.

CRediT authorship contribution statement

Kuanrong Zhai: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bingjian Zhang:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Longguan Zhu:** Writing – review & editing, Resources, Project administration. **Hailing Zheng:** Resources. **Jingxuan Liu:** Resources. **Di Zhang:** Investigation. **Xinrui Sun:** Resources. **Zvezdana Dode:** Resources. **Mingzhi Ma:** Resources. **Qiong Zhang:** Resources.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jas.2025.106339>.

References

- Adamiano, A., Fabbri, D., Falini, G., Giovanna Belcastro, M., 2013. A complementary approach using analytical pyrolysis to evaluate collagen degradation and mineral fossilisation in archaeological bones: the case study of Vicenne-Campochiaro necropolis (Italy). *J. Anal. Appl. Pyrolysis* 100, 173–180. <https://doi.org/10.1016/j.jaap.2012.12.014>.
- Bella, J., 2016. Collagen structure: new tricks from a very old dog. *Biochem. J.* 473, 1001–1025. <https://doi.org/10.1042/BJ20151169>.
- Bischof, J.C., He, X., 2006. Thermal stability of proteins. *Ann. N. Y. Acad. Sci.* 1066, 12–33. <https://doi.org/10.1196/annals.1363.003>.
- Bleasdale, M., 2021. Ancient proteins provide evidence of dairy consumption in eastern Africa. *Nat. Commun.* 12, 632. <https://doi.org/10.1038/s41467-020-20682-3>.
- Buckley, M., Melton, N.D., Montgomery, J., 2013. Proteomics analysis of ancient food vessel stitching reveals >4000-year-old milk protein. *Rapid Commun. Mass Spectrom.* 27, 531–538. <https://doi.org/10.1002/rcm.6481>.
- Calvano, C.D., Rigante, E., Picca, R.A., Cataldi, T.R.I., Sabbatini, L., 2020. An easily transferable protocol for in-situ quasi-non-invasive analysis of protein binders in works of art. *Talanta* 215, 120882. <https://doi.org/10.1016/j.talanta.2020.120882>.
- Child, A.M., Pollard, A.M., 1992. A review of the applications of immunochemistry to archaeological bone. *J. Archaeol. Sci.* 19, 39–47. [https://doi.org/10.1016/0305-4403\(92\)90005-N](https://doi.org/10.1016/0305-4403(92)90005-N).
- Elnaggar, A., Osama, A., Anwar, A.M., Ezzeldin, S., Abou Elhassan, S., Ebeid, H., Leona, M., Magdeldin, S., 2022. Paleoproteomic profiling for identification of animal skin species in ancient Egyptian archaeological leather using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). *Herit. Sci.* 10, 182. <https://doi.org/10.1186/s40494-022-00816-0>.
- Fremout, W., Sanyova, J., Saverwyns, S., Vandebaele, P., Moens, L., 2009. Identification of protein binders in works of art by high-performance liquid chromatography-diode array detector analysis of their tryptic digests. *Anal. Bioanal. Chem.* 393, 1991–1999 h. <https://doi.org/10.1007/s00216-009-2686-z>.
- Ge, R., Cong, L., Fu, Y., Wang, B., Shen, G., Xu, B., Hu, M., Yu, H., Zhou, J., Yang, L., 2023. Multi-faceted analysis reveals the characteristics of silk fabrics on a Liao dynasty DieXie belt. *Herit. Sci.* 11, 217. <https://doi.org/10.1186/s40494-023-01064-6>.
- Harvey, V.L., 2018. Species identification of ancient Lithuanian fish remains using collagen fingerprinting. *J. Archaeol. Sci.* 98, 102–111. <https://doi.org/10.1016/j.jas.2018.07.006>.
- Hendy, J., Welker, F., Demarchi, B., Speller, C., Warinner, C., Collins, M.J., 2018. A guide to ancient protein studies. *Nature Ecology & Evolution* 2, 791–799. <https://doi.org/10.1038/s41559-018-0510-x>.
- Hendy, J., 2021. Ancient protein analysis in archaeology. *Sci. Adv.* 7, eabb9314. <https://doi.org/10.1126/sciadv.eabb9314>.
- Henrikson, K., Karsdal, M.A., 2016. Type I collagen. In: *Biochemistry of Collagens, Laminins and Elastin*. Elsevier, pp. 1–11. <https://doi.org/10.1016/B978-0-12-809847-9.00001-5>.
- Hu, W., Zhang, H., Zhang, B., 2015. Identification of organic binders in ancient Chinese paintings by immunological techniques. *Microsc. Microanal.* 21, 1278–1287. <https://doi.org/10.1017/S1431927615015147>.
- Kuckova, S., Rambouskova, G., Junkova, P., Santrucek, J., Cejnar, P., Smirnova, T.A., Novotny, O., Hynek, R., 2021. Analysis of protein additives degradation in aged mortars using mass spectrometry and principal component analysis. *Constr. Build. Mater.* 288, 123124. <https://doi.org/10.1016/j.conbuildmat.2021.123124>.
- Kurugöl, S., Güleç, A., 2012. Physico-chemical, petrographic, and mechanical characteristics of lime mortars in historic yoros castle (Turkey). *Int. J. Architect. Herit.* 6, 322–341. <https://doi.org/10.1080/15583058.2010.540072>.
- Li, K., Han, K., Teri, G., Tian, Y., Cui, M., Qi, Y., Li, Y., 2024. A study on the materials used in ancient wooden architectural paintings at DaZhong gate in confucius temple, Qufu, Shandong, China. *Materials* 17, 2170. <https://doi.org/10.3390/ma17092170>.
- Liu, M., Xie, J., Zheng, H., Zhou, Y., Wang, B., Hu, Z., 2015. Identification of ancient silk using an enzyme-linked immunosorbent assay and immuno-Fluorescence microscopy. *Anal. Sci.* 31, 1317–1323. <https://doi.org/10.2116/ansci.31.1317>.
- Loyola, R., López-Mendoza, P., Carrasco, C., Walker, C., Fagan, J., Méndez, V., Santana-Sagredo, F., Flores, V., Soto, A., 2024. Technological and protein residue analysis on ancient stemmed projectile points of the Southern andes highlands. *Lithic Technol.* 49, 461–476. <https://doi.org/10.1080/01977261.2024.2318526>.
- Pal Chowdhury, M., Choudhury, K.D., Bouchard, G.P., Riel-Salvatore, J., Negrino, F., Benazzi, S., Slimak, L., Frasier, B., Szabo, V., Harrison, R., Hambrecht, G., Kitchener, A.C., Wogelius, R.A., Buckley, M., 2021. Machine learning ATR-FTIR spectroscopy data for the screening of collagen for ZooMS analysis and mtDNA in archaeological bone. *J. Archaeol. Sci.* 126, 105311. <https://doi.org/10.1016/j.jas.2020.105311>.
- Palmieri, M., Vagnini, M., Pitzurra, L., Rocchi, P., Brunetti, B.G., Sgamellotti, A., Cartechini, L., 2011. Development of an analytical protocol for a fast, sensitive and specific protein recognition in paintings by enzyme-linked immunosorbent assay (ELISA). *Anal. Bioanal. Chem.* 399, 3011–3023. <https://doi.org/10.1007/s00216-010-4308-1>.
- Patericia, R.S., Michael, T.W., 1990. Detection of haemoglobin in human skeletal remains by ELISA. *J. Archaeol. Sci.* 17, 255–268. [https://doi.org/10.1016/0305-4403\(90\)90023-X](https://doi.org/10.1016/0305-4403(90)90023-X).
- Paterson, R.S., Madupe, P.P., Cappellini, E., 2025. Paleoproteomics sheds light on million-year-old fossils. *Nat. Rev. Mol. Cell Biol.* 26, 1–2. <https://doi.org/10.1038/s41580-024-00803-2>.
- Pavelka, J., Kováčiková, L., Šmejdá, L., 2011. The determination of domesticated animal species from a Neolithic sample using the ELISA test. *C. R. Palevol* 10, 61–70. <https://doi.org/10.1016/j.crpv.2010.10.012>.
- Ricard-Blum, S., 2011. The collagen family. *Cold Spring Harbor Perspect. Biol.* 3. <https://doi.org/10.1101/cshperspect.a004978> a004978–a004978.
- Richter, K.K., Codlin, M.C., Seabrook, M., Warinner, C., 2022. A primer for ZooMS applications in archaeology. *Proc. Natl. Acad. Sci. USA* 119, e2109323119. <https://doi.org/10.1073/pnas.2109323119>.
- Stankiewicz, B.A., Hutchins, J.C., Thomson, R., Briggs, D.E.G., Evershed, R.P., 1997. Assessment of bog-body tissue preservation by pyrolysis-gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* 11, 1884–1890. [https://doi.org/10.1002/\(SICI\)1097-0231\(199711\)11:17<1884::AID-RCM62>3.0.CO;2-5](https://doi.org/10.1002/(SICI)1097-0231(199711)11:17<1884::AID-RCM62>3.0.CO;2-5).
- Warinner, C., Korzow Richter, K., Collins, M.J., 2022. Paleoproteomics. *Chem. Rev.* 122, 13401–13446. <https://doi.org/10.1021/acs.chemrev.1c00703>.
- Wei, X., Zhao, Y., Zheng, J., Cao, Q., Li, S., He, L., Wei, B., Zhang, J., Xu, C., Wang, H., 2021. Refolding behavior of urea-induced denaturation collagen. *Macromol. Res.* 29, 402–410. <https://doi.org/10.1007/s13233-021-9047-y>.
- Wright, N.T., Humphrey, J.D., 2002. Denaturation of collagen via heating: an irreversible rate process. *Annu. Rev. Biomed. Eng.* 4, 109–128. <https://doi.org/10.1146/annurev.bioeng.4.101001.131546>.
- Wu, M., Zou, X., Zhang, B., Zhao, F., Xie, Z., 2019. Immunological methods for the detection of binders in ancient Tibetan murals. *Microsc. Microanal.* 25, 822–829. <https://doi.org/10.1017/S1431927619000461>.
- Zhai, K., Zhu, H., Luo, L., Zhang, B., Zhu, L., Zhang, Q., Zhao, P., 2024. Exploration of the rules for the use of organic additives in the mortar of the Forbidden city. *J. Cult. Herit.* 70, 71–79. <https://doi.org/10.1016/j.culher.2024.08.017>.