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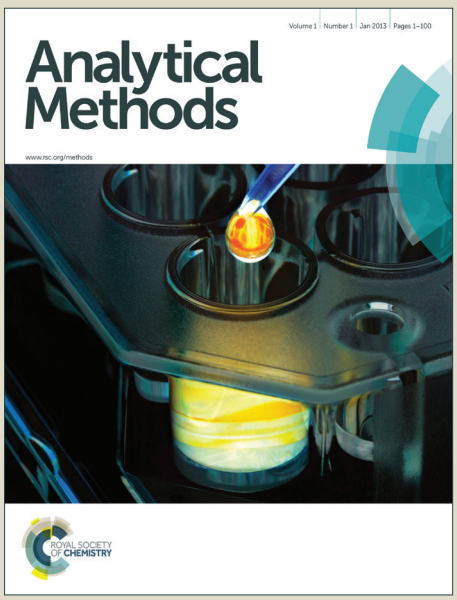

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Proteomic identification of organic additives in the mortars of ancient Chinese wooden buildings

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Abstract: Mortars are the layers paved on the surface of timber, earth or stone before painting and drawing. The analysis of their material composition and manufacture technology is necessary for revealing old technological approaches, selecting suitable technological process in restoration and protection, and guiding the development of traditional technology of Chinese painting and colored drawing. According to ancient literature, crop flour and blood have been used as binders in the mortars of Chinese wooden buildings. However, little work is published on their scientific identification and the reported methods could not figure out their precise origins, which is important to understanding ancient mortar technology. In this study, Fourier Transform Infrared Spectroscopy (FTIR), Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) and starch grain analysis were employed to analyze the three mortars taken from the Old Summer Palace (18th and early 19th century), the Eastern Royal Tombs of the Qing Dynasty (middle 17th century to early 20th century) and the Taiyuan Confucius Temple (late 19th century), respectively. FTIR analysis indicated the presence of proteins, and then different organic additives, namely wheaten flour, cattle blood and pig blood, were identified respectively in the three mortars by LC/MS/MS analysis. Starch grain analysis also confirmed the proteomic results. Thus, proteomic analysis is very effective to identify the nature and origin of organic additives in the mortars of ancient painting.

Introduction

Ancient Chinese buildings are mainly made of wooden structure and famous for their carved beams and painted rafters. Thus, painting and colored drawing is a very valuable part of ancient Chinese architectural heritage. Ancient artists used mortars as ground layers on wood in order to protect lumber and prepare for painting.[1] The analysis of historical mortars is necessary for revealing old technological approaches, understanding their unusual properties and subsequently selecting suitable technological process in restoration.

The mortars applied in ancient Chinese wooden buildings exist as a rather complex system of inorganic and organic components, including brick ash, lime, fiber, flour, blood and tung oil,[2] among which brick ash and lime have been used as filling materials, fiber as taut material, flour, blood and tung oil as binding materials.[3] The inorganic components of mortars are well studied. In several cases, different combinations of X-ray diffraction analysis, X-ray fluorescence analysis, scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) analysis, high temperature burning-acid dissolution method, and thermal analysis have been used to analyze the inorganic matters qualitatively and quantitatively.[3-5] In addition, fibers can be identified by microscopic observation according to their morphological characteristics and structural differences.[6]

As for the organic additives, pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) has been successfully employed to detect the presence of tung oil,[7] but the identification of flour and blood remains challenging because of their low contents and the susceptibility to decay during burial. Starch-iodine staining test, starch grain microscopic analysis and spectrophotometric method have been used for the qualitative and quantitative analysis of flour in ancient mortars.[8, 9] Blood stain tests in forensic science have been applied to examine the samples of short period and crude protein content could be determined by Kjeldahl nitrogen method or organic elementary analysis.[9-11] However, these analytical methods cannot determine the precise origin of these binders, which should be important to understanding mortar technology as flour and blood remarkably improve the anti-fluting property and durability of mortars.[12]

On the other hand, the binders in various archaeological contexts have been well identified by micro-chemical analysis and staining methods,[13, 14] spectroscopic techniques such as Infrared Spectroscopy and Raman Spectroscopy,[15] starch grain analysis,[16] chromatographic methods including gas-phase chromatography and liquid-phase chromatography (coupled with mass

spectrometry),[17-19] immunoassay techniques,[20] and recently developed proteomic methods.[21-23] In terms of the composition, flour and blood binders all contain certain amounts of protein components, which record abundant biological information. Since proteomic approaches can determine the precise origin of the proteins, i.e. protein species as far as they are available in the databases, even if the content is very low in ancient samples,[24] then the methods might be used to analyze the nature and origin of organic additives (flour and blood) in the mortars of ancient Chinese wooden buildings.

In this study, we proposed a multi-method approach for the identification of organic additives in three ancient mortars. Fourier Transform Infrared Spectroscopy (FTIR) was first implemented to evaluate the protein presence in the samples, and then proteomic method was employed to identify the proteins from organic additives.

Experimental section

Sample description

The three mortars were collected by Chinese Academy of Cultural Heritage. Sample A (Fig.1A) was a black brown mortar piece (~ 4 cm width) with a fibrous layer. It was taken from the peeling colored painting of the Old Summer Palace. The Old Summer Palace, known in Chinese as Yuanmingyuan, is a large royal palace with both Western and Chinese architectural styles, noted for “Garden of Gardens”. Located in the northwestern suburbs of Beijing, it was built in the 18th and early 19th century as a place where the emperors of the Qing Dynasty resided and processed government affairs. In 1860, it was destroyed and looted during the Second Opium War, and only ruins remain now.

Sample B (Fig.1B) was a brown mortar fragment (~ 5 cm width) with a fibrous layer. It was sampled from the Eastern Royal Tombs of the Qing Dynasty, located in Zunhua city (Hebei Province, China), 125 kilometers northeast of Beijing. These tombs were built since middle 17th century. It presents the most advanced technology of ancient Chinese architecture and has significant value of history, art and science. Longendian, where the archaeological sample was exactly taken, is the largest ground building of the site as the main place for ritual activities.

Sample C (Fig.1C) was a yellow mortar of granular appearance (~ 1.5 cm width). It was taken from the west wind-room of the Taiyuan Confucius Temple in Shanxi Province, China. After the original buildings were destroyed by flood in 1881, the temple was reconstructed on the

Chongshan Temple ruins the next year. During the period of the Republic of China, it was known as a place for sacrifices to Confucius. In 1919, the Museum of Educational Books in Shanxi Province was established inside the temple, which was the first museum in Taiyuan City and renamed the Shanxi Museum since 1953.

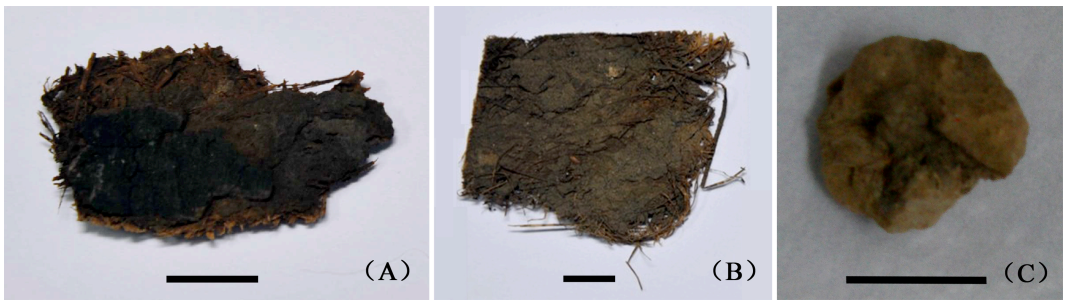

Fig.1 The three ancient mortars analyzed. (A) Sample A from the Old Summer Palace. (B) Sample B from the Eastern Royal Tombs of the Qing Dynasty. (C) Sample C from the Taiyuan Confucius Temple. Scale bars are 1 cm.

FTIR analysis

The samples were analyzed as KBr micropellets with a Nicolet 6700 (Thermo Scientific) FTIR spectrometer working in a transmission mode. Spectra were acquired over the range of 4000-400 cm^{-1} using a resolution of 4 cm^{-1}, with 32 scans per spectrum. The software OMNIC 8.0 was applied to deal with the data.

Protein extraction

The extraction procedure was modified from published references 25 and 26 and successfully carried out on modern mortars, so it was applied on ancient samples. 100 µl of the extracting solution (Tris-HCl, pH 8.0, 10 mM dithiothreitol, 10% sodium dodecylsulfate and 0.0025% bromphenol blue) was added to approximately 20 mg of ancient sample. The mixture was subjected to ultrasonic baths (3×15 min) followed by incubation for 1 h at 56 °C. Then sonicated again for 15 min and centrifuged for 15 min at 12,000 g.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein extraction was separated and purified by electrophoresis. 45 µl of the supernatant was mixed with 5 µl of glycerol, heated at 95 °C for 5 min, cooled to room temperature, and loaded onto the gel (5% stacking gel, 12% separating gel) with 25 µl of the mixture each well. The electrophoresis apparatus was connected to an 80 V power first and switched to a 120 V power

when the sample arrived at the separating gel. As the sample ran on the separating gel for approximately 3 cm, the power was turned off and the gel removed. A microwave-assisted Coomassie Blue staining protocol was followed. The gel immersed in the staining solution (0.25% Coomassie Blue w/v, 50% ethanol, 10% acetic acid) was incubated in microwave oven at medium-low heat for 45 s followed by slowly shaking for 10 min. Then the staining solution was dumped. The gel was washed with distilled water several times, immersed in the destaining solution (25% ethanol, 8% acetic acid) and slowly shaken overnight until the blue-stained protein area visible. Each sample was run on individual gel to avoid horizontal carryover of the proteins.[27]

In-gel digestion

The procedures of in-gel digestion and followed LC/MS/MS analysis were modified from published reference 28. The blue-stained protein area of the gel was cut into small particles of 1 mm^3. The gel particles were washed with distilled water three times, destained with 50% acetonitrile/25 mM NH_4HCO_3, dried with 100% acetonitrile and alkylated in the dark with 50 mM iodoacetamide at room temperature for 30 min. After the solution was removed, the gel particles were washed with 25 mM NH_4HCO_3 buffer twice, dried with 100% acetonitrile, and immersed in the trypsin solution (12.5 ng/µl trypsin in 25 mM NH_4HCO_3). To ensure the gel particles were covered with liquid. The digestion was incubated in microwave oven at 850 W for 1 min and then the peptides were extracted with 100% acetonitrile. The extraction was vacuum dried and cryopreserved for further identification by MS.

LC/MS/MS

The digested sample was re-dissolved in 0.1% formic acid (buffer A) before MS analysis. 2µl sample was injected and analyzed by RP C18 capillary LC column from Michrom Bioresources (100 µm×150 mm, 3 µm). The eluted gradient was 5-30% buffer B (0.1% formic acid, 99.9% acetonitrile; flow rate, 0.5 µl/min) for 30 min. The MS data were acquired on an LTQ Orbitrap Velos mass spectrometer using CID (sample A) or HCD (samples B and C) mode. The parameters were set as following: 20 data-dependent CID MS/MS scans per every full scan for CID mode and 10 HCD scans for HCD mode; full scans were acquired in Orbitrap at resolution 60,000; 35% normalized collision energy for CID mode and 40% for HCD mode; internal mass calibration (445.120025 ion as lock mass with a target lock mass abundance of 0%); charge state screening (excluding precursors with unknown charge state or +1 charge state) and dynamic exclusion

(exclusion size list 500, exclusion duration 30 s).

Database search

The MS/MS spectra of samples were searched against the SwissProt database using Mascot software version 2.3.02 (Matrix Science, UK). Trypsin was chosen as cleavage specificity with a maximum number of allowed missed cleavages of two. Carbamidomethylation (C) was set as a fixed modification, while deamidation (NQ) and oxidation (M) as variable modifications. The searches were performed using a peptide tolerance of 10 ppm and a product ion tolerance of 0.5 Da. The data were then filtered at a p-value < 0.05.

Starch grain analysis

Starch grain analysis was implemented to study the starch component of the flour additives in the mortars and to confirm the proteomic results. A little material was scraped into a 5 ml centrifuge tube with a scalpel and 2 ml deionized water was added. The mixture was subjected to ultrasonic baths (2×15 min) and left for several hours. After shaking, a drop of suspension was pipetted onto a slide. Before it had dried, one drop of 50:50 water/glycerin solution was added and a coverslip applied. The slide was examined with polarized and transmitted light at 500× to identify and photograph the present starch grains. 100 grains were measured to obtain data on the length of the starch grains. Since the starch grains less than 5 μm in size always show very little morphological differences and could not tell much information for identification,[29] only those exceeding 5 μm in size were counted and calculated.

Results and Discussion

FTIR characterization of the mortars

The FTIR results of the three mortars (Fig. 2) all imply the presence of proteins.[30, 31] As for samples A and B, it is possible to identify the characteristic signals of the amide group (-N(H)-C=O-). More specifically, the peaks at 3437 cm^{-1} and 3295 cm^{-1} are assigned to N-H stretching vibration region, 1636 cm^{-1} and 1650 cm^{-1} to C=O stretching vibration region, 1575 cm^{-1} and 1569 cm^{-1} to N-H bending vibration region, and 1405 cm^{-1} and 1410 cm^{-1} to C-N stretching region. The pattern of the absorption peaks is attributed to the presence of proteins in the samples. However, the spectrum of sample C is slightly different because some inorganic components (calcium carbonate) are present and interfere with the absorption peak pattern of the protein constituents.[32] The peaks at 2515 cm^{-1}, 1795 cm^{-1}, 874 cm^{-1} and 713 cm^{-1} are all

characteristic peaks of calcium carbonate. As to the peaks attributed to proteins, the peaks at 3404 cm^{-1} and 1647 cm^{-1} are assigned to N-H and C=O stretching vibration region, while 1433 cm^{-1} is considered as a combination of C-N stretching region of proteins and asymmetric stretching vibration region of carbonate. The absorption peak pattern indicates the presence of proteins and some inorganic components (calcium carbonate) in sample C.

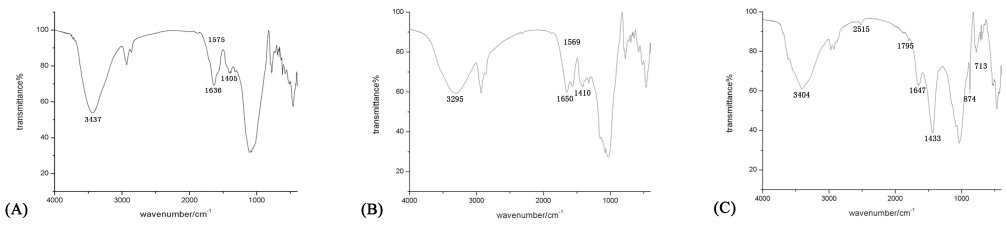

Fig.2 FTIR spectra of samples A, B and C.

Identification of flour and blood proteins

The gel figures after staining have been given as supporting information in Figs. S1-S3. As the gel figures show, proteins are separated not only in a vertical dimension, but also spread horizontally. A blue-stained protein area on the gel was shown as a whole, instead of individual protein bands, which should result from protein degradation in the sample. Considering the detection limit of Coomassie Blue staining,[33] the protein content reserved in the area is sufficient for subsequent mass spectrometry analysis.

The LC/MS/MS results displayed in Table 1 show that one protein from wheaten flour was detected in sample A, while two proteins from cattle blood and three from pig blood were identified in samples B and C respectively. At least two peptides were identified in each protein. The fragmentation of digested peptides in the collision cell mainly happens at the peptide bond position, giving rise to y and b ions. Fig. 3 shows the MS/MS spectra of two specific peptides EAVLGLWGK and VLQSFSDGLK from porcine hemoglobin subunit beta identified in sample C (Table 1), in which y and b represent the single charged mass fragments, y++ and b++ the double charged fragments, y0 and b0 the dehydrated fragments and y* and b* the deaminated fragments. The y and b ions have good continuity, suggesting the data is reliable.

Therefore it is inferred that different organic additives have been used as binders in the three mortars. In sample A, wheaten flour has been added, but the mortar could have been produced without blood material. Besides, samples B and C have used the additives of cattle blood and pig

blood respectively. As the results demonstrate, proteomic identification of organic additives has the advantage that it can not only identify specific proteins in samples, but can also accurately verify the origin of the proteins through the specific peptides.

Table 1 Proteins identified in the ancient mortars using proteomic analysis. The detailed information of each peptide is listed in Table S1.

Sample	Identified proteins	Species	Score	Sequence coverage (%)	No. Peptides (unique)	Accession number
Sample A	Alpha-amylase/trypsin inhibitor CM3	*Triticum aestivum*	74	13%	2(2)	P17314
Sample B	Serum albumin	*Bos taurus*	868	23%	16(4)	P02769
	Hemoglobin fetal subunit beta	*Bos taurus*	148	24%	3(1)	P02081
Sample C	Hemoglobin subunit beta	*Sus scrofa*	3489	83%	17(11)	P02067
	Hemoglobin subunit alpha	*Sus scrofa*	4934	75%	12(4)	P01965
	Serum albumin	*Sus scrofa*	605	13%	10(4)	P08835

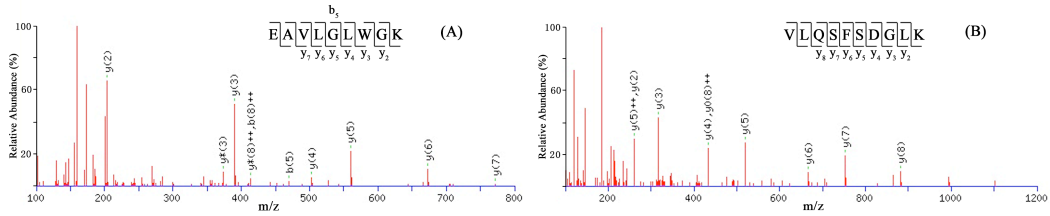

Fig.3 MS/MS spectra of two specific peptides from porcine hemoglobin subunit beta in sample C. (A) EAVLGLWGK. (B) VLQSFSDGLK (with a deamidation at Q).

Starch grain analysis VS proteomic approaches

As indicated by the proteomic results, wheaten flour has been added as organic binder in sample A. Actually starch is the main component of flour besides proteins. Thus starch grain analysis was employed in sample A to compare with and confirm the proteomic results. The starch grains found in sample A are circular, subrounded or oval in outline, with centric hilum. Some have apparent lamellae. Most of the extinction crosses are bilaterally symmetrical. When rotated to side view, they become lenticular with a longitudinal fissure (Fig. 4). The maximum length of individual grains ranges from about 10 to 40 μm (Table 2). As the characteristics and size of the present starch grains fit quite well with those of Triticeae,[34, 35] the starch grains may derive from one or

some of the plants from Triticeae, which is consistent with the proteomic results. However, because the characteristics and size of starch grains are very similar among several plants from Triticeae, as shown in Table 2, the precise origin of the flour additives (namely a wheat origin) cannot be determined by starch grain analysis but can be done by proteomic methods.

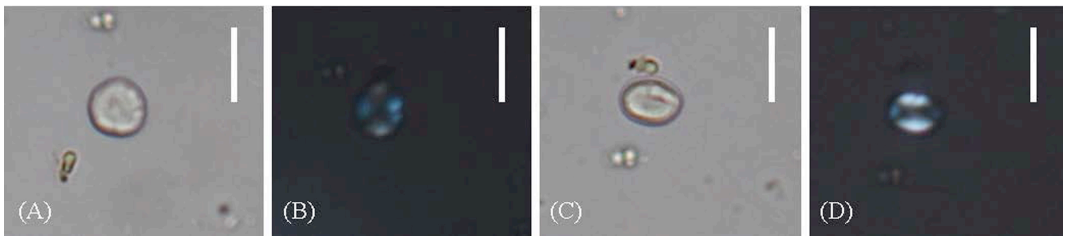

Fig.4 A typical starch grain from sample A. Four photographs were from the same starch grain. (A) and (B) Front views of the starch grain under transmitted light and polarized light respectively. (C) and (D) Side views of the starch grain under transmitted light and polarized light respectively. Scale bars are 20 μm.

Table 2 Starch grain size of modern and archaeological samples.

Material	Maximum length (μm)	Range of maximum length (μm)	Count number
Modern wheat	18.85±4.53	8.59-30.74	100
Modern barley	18.65±4.51	9.33-35.42	100
Modern naked barley	18.48±7.66	7.48-35.41	100
Sample A	18.70±5.07	8.70-31.80	100

The data of modern samples were from reference 35.

Why are different organic binders added in mortars?

Early on in ancient Egypt and Rome, different organic materials, such as proteins, lipids, saccharides, resins and so on, have been added in mortars to improve the properties.[36, 37] In China, the sticky rice-lime mortar has also been applied in the tombs dated as early as the Northern and Southern Dynasties (386-589AD)[38] and served as a representive of Chinese traditional mortars. The ancient scientific book "Tian Gong Kai Wu", which is noted for "the encyclopedia on Chinese craft in the 17th century", has recorded the sticky rice-lime mortar used in historical masonry constructions.[39] Thus, it could be deduced that ancient craftsmen, domestic and overseas, have already recognized the good mechanical properties and durability of organic/inorganic hybrid material and applied it in historical buildings.

In terms of the mortars used in ancient Chinese wooden buildings, the historical book of

"Ying Zao Fa Shi" written in the Song Dynasty (960-1279AD), which is the first official book minutely expounding constructional engineering in China, is the earliest literature referring to mortar technology and also recording wheaten additives. Up to now, the earliest discovered mortar was excavated near Chita City, dated to the Yuan Dynasty (1271-1368AD).[40] An oral source states that blood additives have been used in the mortars from the wooden structure of the Amarbayasgalant Monastry (1727-1735AD) in Mongolia.[41]

In this study, three mortars were analyzed and different organic additives were identified as binders. Wheaten flour has been added in the mortar used in colored painting of the Old Summer Palace (sample A) to improve its anti-fluting property,[12] while blood additives have been used in the other two samples to increase their durability.[12] In China, pig blood is the most widely used blood material in the mortars because of its easy accessibility and good viscosity,[3] as detected in the mortar from the Taiyuan Confucius Temple (sample C). However, in Western countries, cattle blood is preferred.[42, 43] On this account, the cattle blood identified in the mortar from the Eastern Royal Tombs of the Qing Dynasty (sample B), may indicate certain degree of cultural interchange. On the other hand, as blood is sometimes used for religious and ritual purposes[44] and Longendian, where the mortar was exactly taken, is the main place for ritual activities in the Eastern Royal Tombs of the Qing Dynasty, the addition of cattle blood may have some sort of religious significance, which needs further investigation.

Proteomics: an informative technique for the identification of organic additives in mortars

To identify the flour additives, various analytical methods and techniques could be used, including starch-iodine test,[3, 45] ordinary microscopic observation,[46] starch grain analysis,[8, 16] analysis of bran fragments,[35] infrared (IR) spectroscopy[47, 48] as well as the newly developed proteomic approaches.[49] Compared with other methods, proteomic approaches have the advantages of precisely identifying the source of flour and also determining its processing technologies through the identification of other proteins from coexisting components. In this study, proteomic technique was successfully employed to identify the flour additives in the mortars for the first time.

Moreover, the methods to characterize blood in archaeological contexts could be classified into two categories. One is aimed to identify the haemoglobin or haem moiety from blood. This category contains multiple methods, such as colourimetric test,[50] spectroscopic techniques,[51] chromatographic methods,[52] mass spectometric analysis,[44, 53] and so on. These methods can only

demonstrate the presence of blood. The other category is immunoassay technique.[54-56] Although immunoassay can figure out the origin of used blood, it is limited to specific targeted proteins. However, proteomic approaches can overcome this disadvantage and identify different origins of blood through a single run. In this study, proteomic technique was introduced to test the blood additives in the mortars and cattle blood and pig blood were successfully identified.

To summarize, proteomic approaches are of high sensitivity and can obtain abundant biological information contained in protein residues. As illustrated in this paper, proteomics is not only unique to the proteins themselves, but also can offer genus-specific or species-specific sequence information. Using this technique, the precise origin of the protein additives, namely flour and blood in the mortars, could be identified simultaneously through a single run. Thus proteomics is an informative and convenient technique for the identification of organic additives in the mortars.

However, the discussion above focuses on the qualitative identification of organic additives. Previous studies have shown that the quantification of the blood and flour additives could be determined by Kjeldahl nitrogen method, organic elementary analysis, and spectrophotometric method.[9-11] Furthermore, if two or more additives have been identified in one sample through proteomics, the relative abundance of protein groups could also be estimated by proteomics,[49] which is important to imitating ancient mortars. Thus proteomic approaches could realize the qualitative and quantitative analysis of organic additives in the mortars, which is of significant importance for unveiling old mortar technology and subsequently selecting suitable technological process in restoration.

Conclusion

More recently, proteomic approaches have been introduced to archaeology and successfully applied in the identification of the remains in archaeological pottery,[49, 57, 58] binders in artworks,[22, 59-61] protein additives in building materials,[62] and so on. Meanwhile, various kinds of protein residues have been identified, including animal proteins (meat, egg, milk, collagen from bones or skin), plant proteins (flour, seeds), and so on. The proteomic/genomic databases of protein sequences are developed and updated constantly. Even if a species is not documented and fully sequenced in the databases, the protein identification could be realized via sequence homology to phylogenetically related species.[14, 57] Furthermore, the precise origin would be determined by

species-specific markers assigned basing on mass spectrometric characterization of modern samples.[58, 63] Proteomic approaches are informative techniques for the identification of organic residues in different archaeological contexts.

In this paper, proteomic approaches have been successfully applied to approximately 20 mg of ancient sample and resulted in the identification of different protein additives (flour and blood) in the mortars. This technique not only has decided whether flour/blood had been added or not, but also for the first time identified the precise origin of the flour or blood additives in the mortars. It holds promising potential for the routine identification of organic additives in the mortars from ancient buildings.

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