Supplementary issue paper

Searching for blood in Chinese lacquerware: zhū xiě huī 豬 血 灰

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The study gives an overview of the tests and analyses undertaken in the past 20 years to establish the presence of blood in the foundation layers of Chinese lacquer artefacts and also shows the development of analytical methods over that period. When undertaking the conservation of lacquer objects it is crucial to know the type of binding medium as this influences the selection of any consolidants that may be required in the treatment. Microchemical tests to identify blood using benzidine and luminol, various chromatographic and mass spectrometric techniques and DNA analyses were assessed on selected Chinese lacquer objects, and the results gained are summarized.

Keywords: Chinese lacquerware, Blood, Ground layer, Benzidine and luminol test, THM-Py-GC-MS, Nano-LC-MS/MS, DNA analyses

Introduction

For more than 2000 years blood from pigs or cattle was used as a binding medium in the layers that were used to prepare the surface of lacquerware in China. According to Hsu (2014) early evidence for the use in China of a ground layer containing pig’s blood can be seen on lacquerware from the Warring States period that dates from approximately the fifth to third century BCE. When mixed with lime and other filler materials such as brick powder it made an appropriate binder when preparing a ground layer to receive lacquer, due to its adhesive properties and good water resistance.

The use of pig’s blood in the ground layers of Chinese lacquerware is described in various historical Chinese sources: ‘Before applying such varnish over wood, the Chinese used — but not always — to give it a bed or primer, as painters usually do before painting, in the following way. Take the blood of a pig [...] and mix it with powdered quicklime, and coat the wood with this mixture [...] once it is dry, smooth it down with pumice stone or something similar’ (Bonanni, 2009, p. 23).

There are few detailed descriptions of the manufacture of ground layers based on pig’s blood for lacquer, which is known in Chinese as zhū xiě huī (豬 血 灰). The same name is also used in Chinese architectural decoration, which has been more thoroughly studied (Chang, 2014, personal communication). There is a long tradition of pig’s blood-lime mortars in Chinese architecture, which dates back at least to Xianyang Palace — the royal palace of the state of Qin (Qin dynasty, 221–206 BCE) — in modern day Shaanxi province (Zhao et al., 2014).

Blood, in the form of ox blood, can be found as a binding medium in Europe. It is mentioned in old recipes for waterproof putties to be applied to wooden surfaces and buildings, sometimes referred to as ‘Chinese blood-putty’ (Lehner, 1877), or in

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recipes for wall and wood paints. There are no records of the use of blood in Japan, except in lacquerware from Okinawa Island, which is subject to a strong Chinese influence as a result of its proximity (Hsu, 2014, p. 5).

In 1994 two Chinese lacquer artists, Wu Guofen and Wu Xi were invited by the Schönbrunn Palace in Vienna to demonstrate the build-up of Chinese lacquerware with the aim of developing, together with conservators from the palace, a conservation plan for the so-called Vieux-Laque room. In the course of this workshop sample boards were produced (Fig. 1) that gave important practical insights into the preparation of the surface to receive lacquer:

- the multiple uses to which blood was put, as sealing medium for the wood, as glue for the paper layer and as binding medium for the ground layer;
- the colour change of the blood from red to a greenish shade as soon as it is mixed with lime; and
- the use of tung oil as an intermediate layer before the lacquer layer is applied.

Wu Xi and Wu Guofen had brought a readymade lime-blood mixture with them and during the three-day journey from China this had developed a noticeable smell, which points to the need to use freshly made putty and not to keep it for long.

The normal procedure can be described as follows:

After collecting, the fresh blood is filtered as it soon starts to clot; the filtration is carried out by kneading the blood with the hands and forcing it through a sieve. A simple mixture of the filtered blood with lime is used to seal the surface (Hsu, 2014, personal communication), but for the ground layers further fillers are added to this mixture. In certain traditions straw is added when filtering the blood and the blood is warmed slightly, although never in excess of 70°C (Wu, 2014, personal communication); other materials added to the mixture include tung oil (Chang, 2014, personal communication).

The recipes for paints used in Europe suggest that the serum and the blood cells were separated, but this does not seem to be the case for putties, either in Europe or China. As mentioned above, blood was probably used as a binding medium because it imparted good adhesive and waterproofing properties, but was also a cheap alternative to other materials, including the lacquer itself. For the conservation of lacquer objects, it is important to know which type of binding medium has been used in the preparatory layers, as it influences the selection of any consolidants that may be required in the treatment.

This contribution presents an overview of the tests and analyses undertaken between 1995 and 2015 to investigate the presence of blood in lacquer objects. It sets out to address two questions — is blood present and, if so, from which species does it originate — and to give some information about the methods of analysis that have developed during this time. This development in approaches to analysis is well exemplified by the investigations conducted on the Chinese lacquer panels of around 1720 that decorate the Vieux-Laque room at the Schönbrunn Palace (1767–1770: Fig. 2) (Miklin-Kniefacz et al., 2010).

The first attempts to look for blood in the ground layers were undertaken in 1995 at the Institute of Legal Medicine, University of Vienna, using benzidine and luminol tests. The results were not convincing because positive results were obtained from samples of lacquer that lacked a ground layer, while other samples comprising the ground layer alone gave negative results. Almost 10 years later, in 2006, Simone

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2 Two recipes for oxblood paint are given by Kremer Pigmente. Oxblood paint: 100 l of cow’s blood is left to stand three days yielding serum that can skimmed off. To 30 l of this serum 250 ml of clove oil is added to avoid spoiling, along with 26 kg slaked lime, 5 kg natural iron oxide, 3 l of limewater and 10% linseed oil; see http://kremer-pigmente.de/Texte/oxblood-paint.pdf (accessed 23 December 2015).
Mueller at the Institute of Veterinary Medicine, University of Vienna, applied a newly developed DNA test, finding both pig and cattle DNA by real-time polymerase chain reaction (real-time PCR).

Advances made by Michael Schilling in characterizing Asian lacquers and European lacquers at the Getty Conservation Institute by means of thermally assisted hydrolysis and methylation pyrolysis-gas chromatography/mass spectrometry (THM-Py-GC/MS), allowed markers for blood to be detected (Schilling, 2013).

Within the scope of a research project funded by a grant from the Austrian Science Fund (2013–2016), it was possible to test again the possibilities and benefits of DNA analyses at the Austrian Central DNA laboratory of the Institute of Legal Medicine in Innsbruck, where special primers were developed and pig DNA could be detected.

Finally, the most recent tests were undertaken at the University of Chemistry and Technology in Prague in 2014, where pig’s blood and cattle blood could be detected in a single measurement procedure using nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LC–MS–MS).

**Different methods for blood identification in detail**

**Benzidine and luminol**

Ten samples of ground and lacquer layers from the *Vieux-Laque* room (sample size c.5 × 3 mm) were tested with hydrogen peroxide (H₂O₂) and a solution of benzidine in glacial acetic acid. If blood is present an enzymatic reaction causes the benzidine to change colour to a dark blue (Odegaard et al., 2005).

In addition, a test using luminol was used, following the procedure outlined by Weber: in a darkened space samples were treated with a mixture of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and hydrogen peroxide. Three samples, which had previously given positive results with the benzidine test, emitted blue light when treated with the luminol reagent, indicating the presence of blood. Seven other samples that gave a negative result when tested with benzidine also gave a negative result with luminol. The results were ambiguous, as some of the samples from lacquer layers that lacked a ground layer (and should therefore have contained no blood) gave positive reactions while other samples, which comprised solely the ground layer, tested negative.

Further investigations were conducted to investigate the selectivity of the test methods; the benzidine test gave negative results when exposed to other proteins, such as fish glue, casein or egg, but positive results when applied to rabbit skin glue, which implies that it is not entirely diagnostic of the presence of blood.

**Benzidine and GC–MS**

While it does not give incontrovertible proof of the presence of blood, the benzidine test provides a good initial screening method and can be applied in combination with other methods such as with gas chromatography–mass spectrometry analysis (GC–MS), which can provide an amino acid profile of the sample that can be matched to those of reference samples of...
blood. In 2011, a sample from the ground layer of a Chinese lacquer tea box (Canton, around 1820–1850, private collection) was analysed in the Conservation Science Department of the Kunsthistorisches Museum Vienna (KHM) using this technique. The sample was first treated with hydrogen peroxide and benzidine, with the resulting blue colouration indicative of the presence of blood. Subsequently, GC–MS analysis was carried out to confirm the results suggested by the chemical test. Comparison of the chromatographic profile of the amino acids present in the ground sample (after acid hydrolysis with 6 M HCl at 105 °C for 24 hours) with that of a reference sample of blood showed the ground to contain blood (Fig. 3). In this case the GC–MS analyses were performed using a 6890N gas chromatograph connected to a quadrupole MS (model 5973N, Agilent Technologies); the sample size was ∼1 mm² and yielded 0.5–1.0 mg.

**DNA analysis**

At the beginning of 2014 the question of whether pig blood had been used as a key material in the Chinese cabinets was addressed by the Institute of Legal Medicine at the Medical University of Innsbruck. The analysis of the nucleotide sequence of the mitochondrial cytochrome b gene is an established method to determine from which animal species a sample derives and has been used in a wide variety of forensic genetic cases (Parson et al., 2000). Although the method is sensitive and can be applied to very small amounts of source material, it is also sensitive to any external contamination that might be present. When the sample is of putative animal origin but has been handled by humans without proper care taken to avoid contamination, then the test results are expected to indicate a mixture of the animal and the human sources. Such mixtures are generally difficult to interpret based on the nucleotide sequence data.

The DNA was extracted from a set of samples from the ground layers of different lacquer panels from the Chinese cabinets from the Schönbrunn Palace using the EZ1 Advanced instrument (Qiagen, Hilden, Germany) and following the manufacturer’s protocols. The cytochrome b gene was sequenced in the same manner as in previous experiments (Parson et al., 2000), and the resulting consensus sequences were matched using GenBank (National Center for Biotechnology Information, US National Library of Medicine). In three samples of ground layers from the reference sample board prepared by Wu Xi a consensus sequence was found that matched GenBank entries of *Sus scrofa* (wild pig). Two other panels showed human cyt b sequences, most likely explained by human contamination that masked the presence of other species (Fig. 4).

In a second set of experiments new primers specifically designed for identification of DNA from the *Sus* genus detected sequences corresponding to *Sus scrofa* in these two panels, with some contamination from the human cyt b gene sequence.

The authors believe that modern human contamination in the laboratory can be excluded as a source for the human sequences as the samples were taken and handled with great care, explicitly following the guidelines for forensic mtDNA testing (Carracedo et al., 2000; Tully et al., 2001; Parson et al., 2014). However, earlier human contamination cannot be excluded and may derive from the steps taken to prepare the ground layers, such as kneading the blood with bare hands.

In a third set of experiments alternative cytochrome b gen primers were employed (F15416-RMMd16) that confirmed the results of the second analysis. The sample size was about 1 cm² on average (Fig. 5).

The samples on which species identification was attempted were very small for DNA analysis, and spongy. Such samples are difficult to work with in the lab and are likely to contain severely degraded DNA because of their age. The material was used as received in the laboratory; it was impossible to dissect the different layers and the samples were instead broken into smaller pieces to maximize DNA...
extraction efficacy. The small sample sizes meant that other tests, such as chemical methods of detecting the presence of blood, were not performed, as the risk of losing the samples or introducing contamination would have been too high.

**THM-Py-GC–MS**

Pyrolysis enables direct analysis of solid blood samples without time-consuming pre-treatments. The sample size can be very small, less than 0.5 mg, and the pyrolysis products are then subsequently separated and identified by GC–MS. The procedure was developed within the framework of a research project on the characterization of Asian and European lacquers by Michael Schilling and Arlen Heginbotham and is described in detail elsewhere in this publication.

The proteinaceous binder found in the ground layer sample from one of the Chinese lacquer panels from the *Vieux-Laque* room was examined by THM-Py-GC–MS at the Getty Conservation Institute. Markers characteristic for both animal glue and blood were successfully detected (Schilling, 2013).

**Nano-LC–MS–MS**

Nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LC–MS–MS) is used in the field of biochemistry, called proteomics, as a fundamental tool for the identification and relative quantification of proteins. The method does not analyse whole proteins, but is based on the detection of shorter components termed peptides. The peptides are created from the proteins by the action of enzymes, which cleave the peptide bonds between amino acids in proteins. The mode of digestion is specific as particular enzymes cause cleavage only at certain peptide bonds. One of the most specific enzymes, which is commonly used in proteomic laboratories, is trypsin, which cleaves only the bonds behind arginine and lysine. The resulting peptide mixture is analysed by LC–MC–MS and the results compared to those in publicly available databases.

Until recently, biochemists have worked only with proteins in solution, but the use of trypsin has allowed proteins that are embedded in solid phases, such as those taken from works of art, to be analysed. When trypsin cleaves whole or partial proteins on the...
surface of a sample the resultant peptides are transferred into solution and subsequently analysed by mass spectrometry. Nano-LC–MS–MS analysis determines the molecular weight and the sequence of amino acids in the peptides, with identification of the unknown proteins based on comparison of these detected sequences with the sequences of proteins stored in references libraries. The advantage of the use of combined enzymatic digestion and mass spectrometric methods such as LC–MS–MS and MALDI-TOF-MS in the field of cultural heritage is to allow mass mapping of peptides that leads to unambiguous identification of the type of protein binder from very small (microgram) fragments or even in cross-sections made from samples from works of art (Kuckova et al., 2013; Sandu et al., 2013). In many cases the analyses could be considered as non-destructive, because after the enzymatic digestion it is frequently possible to use the sample in a subsequent analytical procedure. Moreover, the presence of more than one type of protein binder in a single sample does not impair the analyses as, although each binder comprises a number of individual proteins, LC–MS–MS is able to identify many hundreds of peptides in a single analysis. The major drawback of mass spectrometric methods — and of many other commonly used analytical procedures used for protein identification — is that they are not able to determine from which of the layers in the sample the proteins that have been identified originate.

Initial tests of the method were carried out as part of the investigation of particles in the ground layers of different lacquer objects, such as a Chinese carved lacquer screen from the Weltmuseum Wien (Pitthard et al., 2016). During this investigation in Prague, very tiny samples (<0.5 mg) were cleaved with a trypsin solution to yield a peptide mixture that was purified and then redissolved for analysis. Measurements were carried out using a UHPLC Dionex Ultimate3000 RSLC nano-LC connected to an ESI-Q-TOF Maxis Impact mass spectrometer (Bruker, Germany). The sample solution was loaded on a trap column (Acclaim PepMap 100 C18) and the peptides were eluted to an analytical column (Acclaim PepMap RSLC C18) and then directly to the ESI source (Captive spray, Bruker Daltonics, Germany). The mass spectrometer was set up in the positive ion mode with precursor selection in the range of 400–2200 Da, and up to ten precursors from each mass spectrum were selected for the fragmentation. The constituent proteins were identified using the Uniprot database (version 2010–12). The analyses confirmed the presence of pig’s blood in the sample from the ground layers of the Chinese screen at the Weltmuseum Wien (Fig. 6 and Table 1).

### Table 1

| Accession  | Protein Description          | Number of peptides |
|------------|-----------------------------|--------------------|
| HBB_PIG    | Hemoglobin subunit beta     | 5                  |
| HBA_PIG    | Hemoglobin subunit alpha    | 4                  |
| TRYP_PIG   | Trypsin                     | 2                  |
| HBB_TARBA  | Hemoglobin subunit beta     | 2                  |

Figure 6 Ground layers of the Chinese carved lacquer screen from the Weltmuseum Wien (No. 71.233).

**Conclusion**

Analysis using GC–MS and THM-Py-GC–MS are commonly used and practicable methods to detect blood as a binding medium in lacquer ground layers, although it necessary to have good reference samples available when GC–MS is employed.

DNA analysis can also be applied and has the potential advantage of allowing species identification. As samples from historic objects are particularly prone to degradation, comparatively large samples are needed in order to perform such analyses, which
might be problematic when the samples must be removed from a significant and valuable object.

The use of nano-LC–MS–MS allows both blood and the species from which it derives to be determined from very small samples, although the species identification should not be regarded as definitive.

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