IMPLEMENTATION OF MALDI MASS FINGERPRINTING IN A MUSEUM LABORATORY FOR THE IDENTIFICATION OF PROTEINS IN WORKS OF ART

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Introduction
Proteins are present in works of art and historical artifacts as, for example, in pigment binders, adhesives and coatings. Their identification is an important component of conservation science. Methods currently available to identify proteins in museum laboratories, however, can pose challenges. (Leo 2009) In biochemical fields, peptide mass fingerprinting (PMF) has been used to identify proteins for many decades. (Henzel 2003) PMF is an analytical method that uses proteolytic enzymes to cleave proteins at specific amino acid sites thereby producing unique peptides characteristic of a protein—a peptide mass fingerprint. Recently, several research groups have shown that this method is applicable to the identification of proteins in artworks, that it has the necessary micro sample requirements, sensitivity, and specificity to accomplish that task. (Hynek 2004, Tokarski 2006, Kuckova 2007) To date, PMF results from artworks have generally come from university analytical laboratories in collaboration with museums. This paper describes the practice and application of PMF within a museum laboratory.

We have developed a simple, one-tube protein extraction and digestion protocol adapted from the literature and a procedure used at Harvard’s Mass Spectrometry and Proteomics Facility (Robinson 2009) to generate PMFs. The method is optimized for the types of samples, both size and composition, generally encountered in art conservation, and is consistent with micro destructive sampling techniques. Using this procedure with more than 100 reference samples, we tabulated a list of ions that are used to identify proteinaceous materials in artworks and have used this list to analyze more than fifty samples from works of art ranging in age from the 14th century to the present. If sample quantities permitted, we verified our PMF results with complementary techniques such as liquid chromatography tandem mass spectrometry (LCMSMS) and enzyme linked immunosorbent assay (ELISA). This paper will discuss the requirements for the routine practice of Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI) mass fingerprinting within a museum laboratory, give examples of analyses of samples from artworks, and show preliminary results for several projects we have undertaken to extend protein analysis into new areas of art conservation.

Method, materials, and equipment
The procedure described below has been used successfully with nearly 200 reference samples and samples from artworks. The PMFs obtained from reference materials provided a list of ions that were consistently observed for the various known proteins. (Table 1) Many of these ions have been tentatively identified by LCMSMS (data not shown). Knowledge of a peptide sequence from LCMSMS can be used to support the use of a particular ion as an indication of a particular protein, but it is not necessary for protein identification with PMF. This procedure is routinely used with 15-20 samples at a time; sample preparation, data collection and data analysis take approximately one and a half days. It is important to note that once samples have been dried onto the sample plate with matrix, they are stable for long periods of time and can easily be transported to available Maldi instrumentation. Instrument time for 10-12 samples is about two hours. Digests are stable for long periods of time if kept frozen.
**Procedure**

- Extract and solubilize in 30 µL trifluoroethanol, 30 µL 50 mM ammonium bicarbonate (AMBI) at 60 ºC for 30 min with intermittent vortexing/ agitation;
- Ultrasonicate for 10 min at room temperature;
- Reduce to near dryness in a vacuum centrifuge;
- Resuspend in 30 µL 50 mM AMBI;
- Reduce disulfides with 3 µL 20 mM Tris (2-carboxyethyl) phosphine hydrochloride in 50 mM AMBI at 37 ºC for 20 min;
- Endcap free sulfhydryls with 3 µL 40 mM iodoacetamide in 50 mM AMBI for 30 min at room temperature in the dark;
- Digest with 8 µL trypsin (0.02 ug/µL in 50 mM AMBI) at 37 ºC overnight;
- Terminate digestion with 1 µL formic acid.

**Maldi analysis**

- Alpha-cyano-4-hydroxycinnamic acid (αCHCA) matrix prepared as a saturated solution in 40% (v-v) acetonitrile (ACN) - 0.1% trifluoroacetic acid (TFA);
- Samples (4 µL) mixed with matrix (20 µL) in a separate tube and spotted on the Maldi plate;
- Peptide standards are used as external calibrants;
- Laser Desorption-Time of Flight Mass Spectrometer to collect positive ion spectra from 800 – 3800 Da with mass accuracy of ± 0.1 Da.

**Equipment**

The equipment listed below and shown in Figure 1 to the right includes everything that is needed to prepare samples for analysis. The total cost (excluding a Maldi instrument) is approximately $ 20,000, and all the equipment fits onto or under a 6 foot table.

- Mini microcentrifuge
- Vortex mixer
- Thermomixer
- Ultrasonic bath
- Vacuum centrifuge
- Benchtop clean hood
- Analytical balance
- Under counter freezer

Obviously, there are many options possible with the above procedure, such as the choice of solubilization solvents, proteolytic enzyme, and Maldi matrix. However, this procedure was developed and evaluated as a “turn-key” method that could be used routinely by non-experts, and, in our experience instructing first-time users, fulfills that goal. On the other hand, the method as described is open-ended such that digests can be further analyzed by, for example, LCMSMS or LCUV, etc., as desired.
Analysis of artworks

Master of the Fogg Pietà

As part of a technical study of Harvard’s Master of the Fogg Pietà, Italian (1300 – 1350), Figure 2, a sample containing both paint and ground taken from the rock in the upper right was analyzed by PMF. Figure 3 is the spectrum obtained from that sample (insert) and, with reference to the ions in Table 1, is seen to indicate the presence of animal glue, presumably from the ground, and whole egg (both yolk and white peptides are present), from the paint. LCMSMS data (not shown) from a similar sample indicated the presence of the same materials. The sample size shown in Figure 3 is typical for those we have successfully analyzed by PMF. This example also demonstrates the ability of PMF to analyze mixtures of proteins without loss of sensitivity or specificity.

David Smith, “Untitled” 1952

David Smith (1906-1965), an American abstract expressionist sculptor well known for his large metal pieces, also produced a number of abstract sketches. Based on the artist’s records, David Smith’s “Untitled” 1952 (David Smith Estate, Bolton Landing, NY, image not available, but similar to Figure 4) was assumed to consist of an egg yolk-based black ink applied over a casein-based white background, although no analysis for proteins had ever been attempted. (Mulholland 2008) We analyzed samples of the black ink as well as the white background with PMF to determine the composition of each medium. The spectrum in Figure 5 from several fibrous black pieces, as shown in the inset, contains egg yolk and casein. Ions labeled with masses in Figure 5 correspond to those in Table 1 characteristic of egg yolk. No ions characteristic of egg

Table 1. Ions used to identify proteins in artworks

<table>
<thead>
<tr>
<th>Protein</th>
<th>1048.6</th>
<th>1345.7</th>
<th>1095.6</th>
<th>830.5</th>
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<tr>
<td>Egg Yolk</td>
<td>1077.6</td>
<td>1428.6</td>
<td>1105.6</td>
<td>1251.7</td>
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<td>Egg White</td>
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<td>1555.7</td>
<td>1241.8</td>
<td>1267.7</td>
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<tr>
<td>Animal Glue</td>
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<td>1687.8</td>
<td>1267.8</td>
<td>1384.7</td>
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<tr>
<td>Casein</td>
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<td>1773.9</td>
<td>1427.7</td>
<td>1760.0</td>
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<td>2009.0</td>
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<td>1976.0</td>
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</tr>
<tr>
<td></td>
<td>2236.1</td>
<td>2703.4</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 2. Master of the Fogg Pietà.

Figure 3. PMF from sample from Master of the Fogg Pietà.

David Smith Estate, Bolton Landing, NY, image not available, but similar to Figure 4) was assumed to consist of an egg yolk-based black ink applied over a casein-based white background, although no analysis for proteins had ever been attempted. (Mulholland 2008) We analyzed samples of the black ink as well as the white background with PMF to determine the composition of each medium. The spectrum in Figure 5 from several fibrous black pieces, as shown in the inset, contains egg yolk and casein. Ions labeled with masses in Figure 5 correspond to those in Table 1 characteristic of egg yolk. No ions characteristic of egg
white are observed. Ions labeled “Cas” correspond to those in Table 1 characteristic of casein. Analysis of a sample of the white medium alone (data not shown) confirmed that that layer contained casein only. Thus, as suspected but now verified, the artist used an egg-yolk based ink on top of a casein paint layer, both choices, possibly, to obtain an overall matte finish on the sketch. Additional analyses of this sample (not shown here) further determined that the casein was from a bovine source based on the scheme devised by Chambery (2009).

Figure 4. David Smith “Untitled”, 1952.  
Figure 5. PMF of “Untitled” 1954 black media with fibers.

It should be noted that all ions listed in Table 1 might not be observed in each sample, nor might they be the only ions observed. This may be due to the non quantitative nature of MALDI. However, the presence of a large number of listed ions for a given material has provided reliable evidence for the presence of that material and has been successful for protein identification in our analyses of real artworks including, in addition to those shown above: 14th century altarpieces from Naples, Bologna, Venice, Florence and Siena; early 19th century Bucher Boxes from Pennsylvania; Mark Rothko’s Harvard Panels; a Woman’s silk cape designed by Mariano Fortuny y Madrazo; a 15th century Italian painted chest; 15th century paintings by Czech masters; a 14th century Czech altarpiece; and an 18th century Syrian reception room.

**Extending protein analysis**

*Avian species in egg tempera*

There is little in the art history literature that suggests the source of eggs used in egg tempera, nor is there any specific reason to think that hen’s eggs have been used exclusively since the advent of egg tempera painting. Thompson (1962) refers to using “fresh hen’s egg” in his recipes, but doesn’t preclude the use of eggs from other species. Cennini, in Thompson’s translation of his work (1933), refers only to “eggs” except to qualify some as “country” eggs. A more contemporary source, Porter (http://www.betsyporter.com), notes that “Eggs for egg tempera paint, just as for eating, should ideally be freshly laid by happy free-range chickens. I like the beautiful ‘extra large’ and ‘jumbo’ brown eggs from the farmers' market. If that doesn't work out, grocery store eggs are perfectly OK.” She continues: “Duck and goose eggs have thicker, yellower, more viscous yolks than chicken eggs, and are considered highly desirable for iconography if you can get them. Use a double quantity of white wine for a duck egg, triple quantity for a goose egg.” So, although factual information is scarce, there is at least some reason to think that artists may have chosen eggs other than hen’s eggs for egg tempera, although this has never been shown analytically. The goal of this project is to develop accurate species markers and apply them to real samples. As with all artists’ materials, the more knowledge one has about them, the better the artists’ work can be understood and addressed by conservators. In the case of egg tempera paintings, it may be of particular interest to art historians to know
which species of egg was preferred by different artists or schools and whether their preference was dictated by supply and demand or because of the quality of the work it produced.

For these reasons we are interested in using PMF to address the identification of species of origin of eggs in tempera, and the ready availability of PMF in our laboratory makes it possible to analyze the large number of samples involved. Films from hen eggs (gallus gallus), quail eggs (coturnix coturnix) and duck eggs (anas platyrhynchos) were homemade using standard procedures, such as described by Cennini. (Thompson 1960) Films of whole egg, yolk, and glair were air dried for three weeks or more before sampling.

Figure 6 compares the PMFs of unaged yolk-only films from three avian species: quail, duck and chicken. Additional species, such as goose and turkey, will be added in the spring when they begin laying again! It is immediately obvious from the spectra in Figure 6 that each species gives rise to very different PMFs with multiple characteristic ions. This is not altogether surprising since avian genomes “… vary pretty widely at the DNA level. Even chickens to pheasants, for example, which are about the closest relatives, are several per cent substitution.” (Sidow 2011)

Figure 6. PMFs from unaged egg yolk films from different avian species.

The spectra in Figure 7 are the PMFs from whole egg films from quail, duck and chicken, again demonstrating the large differences in the spectra which should provide a sensitive, specific means of differentiating avian species from egg tempera in artworks. It is also clear from these spectra that it will be possible to differentiate among whole egg, yolk and glair. We are aware that relative ion intensities change with both thermal and light ageing, so our films are currently being aged and will be resampled to establish markers more appropriate to real samples from artworks. Preliminary studies suggest that most peptide markers remain intact.
Identification of gums

Gum arabic (Acacia senegal, for example) is a natural resin composed of a complex mixture of polysaccharides and glycoproteins and is used in a variety of applications in artworks, especially as a water soluble medium for water color and gouache. Given the presence of protein in this class of materials, we have begun to investigate the use of PMF as a means of identifying gums in artworks. Using the procedure described above, we analyzed multiple samples of gum arabic from a variety of sources available in Harvard’s Gettens Collection of Artists’ Materials, including materials labeled as being from:

- Billings and Stover (1929)
- S. B. Penick
- Acacia Gum Powder (Florence 1920)

In addition, we have analyzed samples from Harvard’s Gettens Collection of Paint Films including:

- Gum arabic with glycerin (on glass), 12/7/1933
- Gum arabic with water, glycerin 5% and yellow ochre over gypsum glue ground, 1933/12/15
- Gum arabic with water, glycerin 5%, alizarin (Weber) over gypsum glue ground, 1933/12/15

The PMFs shown in Figure 8 are typical of those obtained with the neat materials and aged films. Based on our observations so far, there are consistent ions present in both neat and aged film standards to identify gum arabic. Ongoing work will be done with additional gums, particularly gum tragacanth and cherry gum, the most common gums used by artists, to provide a list of ions that can be used to identify gums with PMF.
Conclusion

We have described in detail the method and equipment used for protein identification via protein mass fingerprinting in a museum laboratory. Examples are shown that verify the method’s sensitivity and accuracy and demonstrate that PMF is a valuable analytical tool that can be used in conjunction with existing methods to provide an improved understanding of proteinaceous materials used by artists. Furthermore, we have also shown preliminary results using PMF to determine the species of eggs used in tempera paintings and to identify gums in paint matrices, which will be useful for expanding the art historical knowledge about the use of these materials.

With the growing availability of MALDI instrumentation and the relative ease of obtaining PMFs from artworks, it is hoped that protein analysis will be considered more frequently in technical studies of artworks in the future.

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References


Sidow, A., Stanford University, private communication, 2011.