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Journal of Chromatography A, 676 (1994) 177–183

JOURNAL OF CHROMATOGRAPHY A

# Identification of proteinaceous binding media in paintings by amino acid analysis using 9-fluorenylmethyl chloroformate derivatization and reversed-phase high-performance liquid chromatography

Cecily M. Grzywacz

*The Getty Conservation Institute, 4503 Glencoe Avenue, Marina del Rey, CA 90292, USA*

**Abstract**

Identification of binding medium, the vehicle which adheres pigment particles to each other and to a backing or substrate, is important to both art conservators and curators. Proteinaceous binders such as egg, glue and milk casein have been widely used by artists. A protocol for the identification of different proteinaceous binding media in paintings was developed using HPLC analysis with fluorescent detection of 9-fluorenylmethyl chloroformate (FMOC-Cl) derivatives of amino acids.

A scheme based on peak area ratios of FMOC-amino acid derivatives was developed and successfully used on museum samples. The sample preparation techniques, identification scheme and museum applications are discussed.

## 1. Introduction

Quantitative amino acid analysis has been used to distinguish proteinaceous binding media in cultural property since the late 1960s [1,2]. However, the quantity of sample required, 1–4 mg, frequently prohibited sample collection; imagine the visible damage to a painting if 1–4 mg were scraped from its surface. Typical sample sizes currently routinely taken from art objects are only a few micrograms to at most one-half a milligram. Recent advances in HPLC detectors and columns have led to increased sensitivity and a drastic reduction in required sample size [3] increasing the feasibility of amino acid analysis of paint layers.

The Waters Pico-Tag method (Millipore, Milford, MA, USA) has been used to identify binding media [4,5]. However, there have been literature reports of variable recoveries of lysine, aspartic acid and glutamic acid due to matrix interferences from the presence of salts and/or metal ions in the samples [6]. Accurate quantification of these amino acids is critical to identification of proteinaceous binding media.

9-Fluorenylmethyl chloroformate (FMOC-Cl) precolumn derivatization was selected because of its high sensitivity, good derivative stability and

0021-9673/94/$07.00 
*SSDI* 0021-9673(94)00421-5

low potential for interferences from sample matrices, such as pigments, substrates, ground layers, varnishes, glazes, etc. [7–10].

## 2. Background

Popular forms of cultural objects are painted objects such as paintings, polychrome sculptures and wall painting fragments. Museum visitors enjoy painted artifacts aesthetically, admiring shape, colors and imagery. Conservation scientists scrutinized art objects more closely, investigating the specific materials used to create the painting, including but not limited to pigments and binding media.

The function of binding media is fourfold: (1) a binder coats each pigment particle and holds the pigment particles in suspension, (2) adheres the paint layer to a substrate, (3) imparts optical properties that intensify the natural color of pigments and (4) protects pigment particles from the potentially damaging effects of the environment. Identification of binding medium can assist curators and art historians in the provenance of painted objects. Throughout the ages artists have been experimenting with different binders. A timetable of binding media use can be created and used to confirm the period of the artifact. Binding media is important in the study of artists' techniques as well. Artists have used a variety of binder recipes depending upon how they wished to modify the handling and/or optical properties of paint. Many choices of binding media have led to non-durable binders which has resulted in early degradation of the pigment layer requiring conservation. By understanding the composition of the binder in paint layers, conservation scientists are better able to offer advice for restoration and preservation of painted objects.

Casein, egg and collagen are proteinaceous materials used as binding media. Animal and fish collagen products are used as a strong adhesive for wood, as a paint binding medium and most often as a binder in ground layers of paintings. Ground layers are a paste of fine chalk or gypsum and an adhesive which is applied over a canvas fabric or wood panel to create a smooth surface before the application of paint layers. The painting technique tempera uses whole egg or egg yolk as binders; egg yolk provides a richer binding medium. Glair is the use of only egg white, and it is used as a temporary varnish or as a sealant between ground layers and paint layers [1,11,12]. Milk curds, whey or whole milk have been identified in binders based on the presence of milk protein, casein [1,13].

## 3. Experimental

### 3.1. Sample weighing and transfer techniques

Samples removed from museum objects typically weighed 10–200 $\mu$g. Sample particles were transferred with a dissecting needle using static electricity attractive forces. A particle was carefully transported by gently touching it with a dissecting needle. Extreme care was used throughout this procedure as too much pressure on the particles would cause them to flip away and be lost. Particles were weighed and transferred to a 1.0-ml vacuum hydrolysis tube (Pierce, Rockford, IL, USA) by depositing them in the tip of a 9-in. (1 in. = 2.54 cm) pre-cleaned Pasteur pipette with the dissecting needle. The pipette was inserted into the hydrolysis tube and the sample was delivered to the bottom. A predetermined amount of norleucine as an internal standard (Sigma, St. Louis, MO, USA) was delivered to the tip of a 9-in. Pasteur pipette. The tip of the pipette was suspended 2 cm above the bottom of the hydrolysis tube and the norleucine was flushed into the tube by adding 300–400 $\mu$l of 6 *M* constant-boiling sequanal-grade HCl (Pierce) to the top opening of the Pasteur pipette. This method of transferring samples and adding small volumes of reagents was necessary to guarantee successful sample preparation of the $\mu$g samples.

### 3.2. Hydrolysis

The hydrolysis step was critical due to the small sample size. Samples of 10–200 $\mu$g were

transferred to the 1.0-ml hydrolysis vacuum tubes, 1 μg norleucine per 10 μg sample was added as an internal standard and 300 μl of constant-boiling 6 *M* HCl was added to the tube. Tubes were placed in a Reacti-Therm module (Pierce) adjusted to 110°C for 20–24 h. The samples were cooled and heated to dryness under a nitrogen stream. The dried samples were taken up in 50–300 μl borate buffer at pH 8.5.

### 3.3. FMOC Derivatization

A 2-μl volume of a collagen hydrolysate standard (Sigma A-9531) or 5–50 μl of a hydrolyzed protein sample in borate buffer was diluted with borate buffer to 50 μl total volume in a 300-μl glass vial. Samples were derivatized using the method of Haynes et al. [7].

### 3.4. HPLC Analysis

The analytical system consisted of two Waters (Millipore) 510 pumps controlled by a DEC Pro 380 mini-computer using Waters Expert software version 6.21. Samples were introduced to the system via a Waters WISP 712 autosampler. The syringe motor rate was reduced to 1.85 μl $s^{-1}$ to improve reproducibility. The analytical column was a Phenomenex (Torrance, CA, USA) Spherex 3 μm $C_{18}$ (ODS) 150 × 4.6 mm column, with a 2-μm precolumn filter and a Spherex 3 μm $C_{18}$ (ODS) 50 × 4.6 mm guard column. A Hewlett-Packard (Wilmington, DE, USA) 1046A detector was set for fluorescence with the photomultiplier tube detector gain setting at 10 and adjusted as needed based on the sample concentration. The excitation wavelength was 265 nm with emission detection at 315 nm. The column temperature was maintained at 43°C.

The FMOC-amino acid derivatives were separated with binary gradient elution based on the method developed by Haynes et al. [7] The gradient profile is graphically displayed in Fig. 1. The two eluents, (A) 50 m*M* sodium acetate and 7 m*M* triethylammonium acetate (TEAA) with 10% acetonitrile adjusted to pH 6.5 with acetic acid and (B) acetonitrile–water (90:10, v/v), were filtered and degassed under a helium sparge. The flow-rate was 1 ml $min^{-1}$. Injection volumes ranged from 2–20 μl depending upon sample concentration. A more concentrated sample was prepared if 20 μl did not give sufficient detector response. The total analysis time was 45 min.

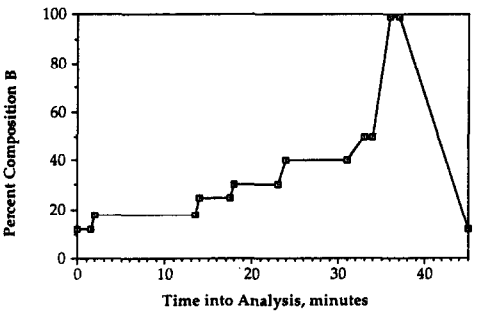


Fig. 1. Gradient profile: sodium acetate–acetonitrile–TEAA buffer system. (A) 50 m*M* Sodium acetate and 7 m*M* TEAA with 10% acetonitrile adjusted to pH 6.5 with acetic acid and (B) acetonitrile–water (90:10, v/v) at 1 ml $min^{-1}$.

## 4. Results and discussion

### 4.1. Analysis of standard binding media materials

Known binding media standard materials from The Getty Conservation Institute's Binding Media Reference Collection were analyzed to validate the identification protocol. Samples of rabbit skin glue, casein, whole egg, egg white and egg yolk were hydrolyzed, derivatized with FMOC-Cl, and analyzed to determine which ratios of amino acids were best suited for distinguishing between proteinaceous binding media. Chromatograms for glue, casein and egg materials are shown in Fig. 2. Each material had a distinct amino acid profile. A scheme was developed to differentiate proteinaceous binders based on FMOC-amino acid peak area ratios. Identification was based on the following distinctions: hydroxyproline was only present in collagen-based glues which also had a very large glycine peak; aspartic acid and glutamic acid peaks in collagen glues were large and nearly equal; casein had a very large glutamic acid peak

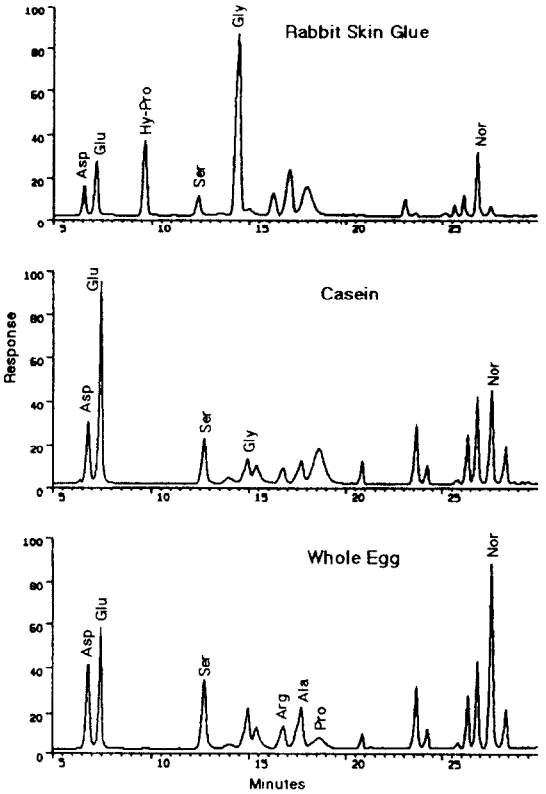


Fig. 2. Amino acid analysis of different proteinaceous binding media: rabbit skin glue, casein and egg.

which was two to three times greater than the aspartic acid peak; and egg components had nearly equal amounts of glycine, aspartic acid and glutamic acid. The differences between egg components were more subtle and peak areas differed by only a few percent. For very important, critical samples, it was necessary to use confirmation techniques. For example, the presence of phosphorus confirmed casein identification and cholesterol differentiated egg yolk from egg white or glair. Fatty acid profiles can also be used to distinguish between the various egg components [1].

### *4.2. Analytical strategy for identification of proteinaceous binding media*

Amino acid analysis applied by conservation scientists to identify protein binding media use molar percent ratios of all amino acids present [1–3,5,14–16]. This technique is feasible when larger sample sizes are available and quantification of all amino acids present is guaranteed. However, when dealing with microgram quantities, not all of the amino acids may be detected due to amino acid degradation as well as isolation difficulties or incomplete binding-medium extraction from the paint layer. These factors make it hard to correlate contemporary egg, casein and collagen standards with older, aged paint layer samples.

The continuous degradation of the $C_{18}$ column from the repeated exposure to basic FMOC-amino acid samples affected the ability to identify all amino acids in proteinaceous binding media. The gradient was adjusted to accommodate changes in column resolution. Also, by using ratios of peak areas rather than percent molar quantities, the small losses in resolution were not critical. Minor peaks which were not well resolved were eliminated in the identification scheme.

A number of critical amino acid ratios were chosen for identification of protein binding media (Table 1). The detection of hydroxyproline distinguished collagen-based glues from the other proteinaceous binders. A 3:1 ratio of glycine to hydroxyproline was an important secondary confirmation. Casein binders were determined by a 3:1 ratio of glutamic acid to aspartic acid, other protein binders had a glutamic acid:aspartic acid ratio of less than 2. Egg binders were identified by the absence of hydroxyproline and the presence of equal amounts of glycine, glutamic acid and aspartic acid. To distinguish egg yolk from egg white from whole egg, it was necessary to use more than one amino acid peak area ratio as the compositions were similar. The ratios to distinguish between the egg components were alanine:proline, glutamic acid:serine, proline:aspartic acid and aspartic acid:serine. To distinguish whole egg from egg white, the ratios of glutamic acid:serine and aspartic acid:serine were used. The systematic identification scheme for proteinaceous binders developed is shown in Fig. 3. To corroborate the identity of binding

Table 1
FMOC-Amino acid peak area ratios for the identification of proteinaceous binding media (single-letter codes for amino acids)

| Amino acid ratio | Glues | Casein | Whole egg | Egg white | Egg yolk |
|---|---|---|---|---|---|
| Hydroxyproline:serine | >1.5 | <1.5 | <1.5 | <1.5 | <1.5 |
| Hydroxyproline:glycine | >0.2 | <0.2 | <0.2 | <0.2 | <0.2 |
| Glutamic acid:aspartic acid | <2.0 | >2.0 | <2.0 | <2.0 | <2.0 |
| Glutamic acid:glycine | <1.0 | >3.0 | 1.0 < (E/D) < 3.0 | 1.0 < (E/D) < 3.0 | 1.0 < (E/D) < 3.0 |
| Glutamic acid:alanine | <0.8 | >3.0 | 1.0 < (D/A) < 3.0 | 1.0 < (D/A) < 3.0 | 1.0 < (D/A) < 3.0 |
| Alanine:proline | 0.5 < (A/P) < 1.5 | <0.5 | >1.8 | >1.8 | <1.8 |
| Glutamic acid:serine | 1.5 < (E/S) < 2.5 | >2.5 | 1.0 < (E/S) < 1.2 | >1.2 | <1.0 |
| Valine:glutamic acid | <0.5 | <0.5 | >0.5 | >0.5 | >0.5 |
| Aspartic acid:serine | >1.0 | >0.8 | <1.0 | >1.0 | <0.9 |
| Aspartic acid:threonine | >2.0 | <2.0 | >1.5 | >1.9 | <1.9 |
| Serine:tyrosine | >1.7 | <1.7 | >1.7 | >1.7 | >1.7 |
| Methionine:threonine | >1.0 | <1.0 | 0.8 < (M/Y) < 1.5 | >1.5 | <1.0 |
| Proline:aspartic acid | >2.0 | >1.5 | <0.5 | <0.5 | >0.5 |
| Proline:alanine | 0.5 < (P/A) < 1.5 | >2.0 | >0.5 | >0.5 | >0.5 |
| Leucine:alanine | <0.5 | >1.5 | 0.8 < (L/A) < 1.0 | <1.0 | >1.0 |

media in paint samples, the scheme in Fig. 3 should be used in conjunction with the confirmation ratios presented in Table 1.

### 4.3. Analysis of museum samples

The analysis scheme and confirmation FMOC-amino acid peak area ratios were applied to samples of unknown binding media obtained from cultural properties. For several samples the HPLC identifications were confirmed with other techniques. Five analysis results are described.

*Yungang grotto Chinese wall painting*

The Getty Conservation Institute (GCI) and China, with assistance from UNESCO, are col-

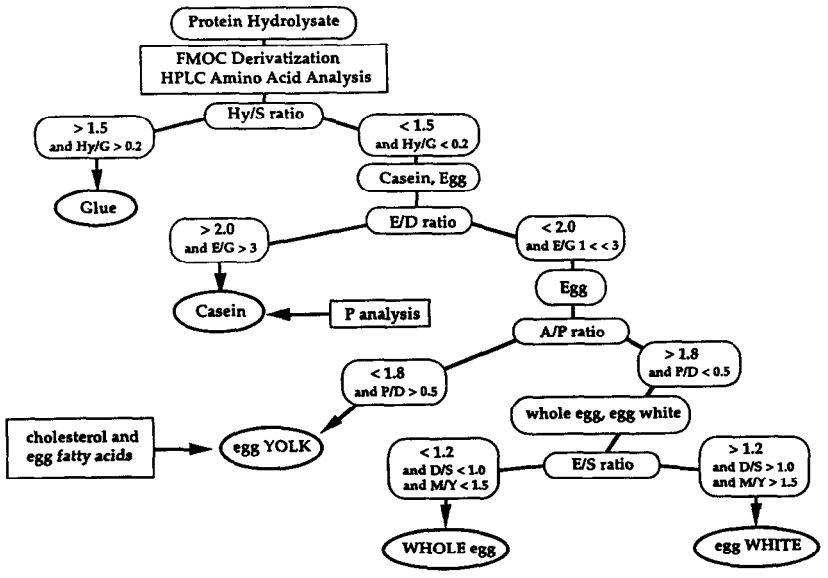


Fig. 3. Flowchart diagram for the identification of binding media. Single-letter codes for amino acids; Hy = hydroxyproline and P analysis = phosphorus analysis.

laborating on the conservation of two of China's most important cultural sites, the ancient rock temples of the Mogao grottoes located near Dunhuang on the edge of the Gobi Desert and the Yungang grottoes near Datong, located approximately 270 km due west of Beijing. The Dunhuang site is inscribed in the World Heritage List; the Yungang caves are in the process of inscription. These two great ancient sites of mankind record the patterns of interchange of civilization, trade, and Buddhist art along the Silk Road into China. Both sites are national treasures [17].

A mg sample was removed from an existing crack in the restored plaster of a historic wall painting at one of the Yungang grottoes and analyzed. From the chromatogram in Fig. 4 and the scheme in Fig. 3, it was determined that the binder was an animal glue based on the relative amounts of hydroxyproline and glycine which are indicative of collagen animal glue binders.

*Rembrandt's "The Raising of Lazarus"*

The curator of the Paintings Department at the Los Angeles County Museum of Art was interested in identifying the binder in the various layers of Rembrandt's "The Raising of Lazarus". A cross-section was carefully removed and examined by a number of analytical techniques. Infrared microspectroscopy indicated that the binding medium in both the ground and paint layers contained oil, natural resin and protein. GC–MS analysis determined that the paint layer binding medium was linseed oil mixed with whole egg or egg yolk; the ground layer contained linseed oil with no indication of egg. A paint layer and ground layer sample were prepared for HPLC analysis to confirm the proteinaceous binders.

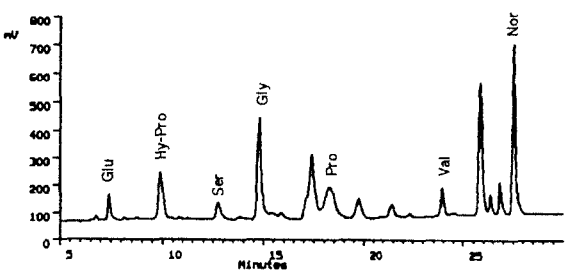


Fig. 4. Amino acid analysis chromatogram of Yungang Chinese wall painting glue sample.

The binder in the ground layer was a mixture of proteinaceous binding media based on the ratios of FMOC-amino acid derivatives. One component was identified as glue because of the significant amount of hydroxyproline. The GC–MS analysis did not find evidence of egg yolk; thus, the second binder was egg white. The binder in the pink paint layer was determined to be egg yolk, based on the ratios of alanine, proline and lysine to each other.

*Mantegna's "Presentation in the Temple"*

Samples of Andrea Mantegna's "Presentation in the Temple" from the Staatliches Museum in Berlin were received for analysis. Protein was identified in all of the samples by IR spectroscopy. GC–MS cholesterol analysis indicated that egg yolk was not present in concentrations greater than 1% (w/w) of the paint sample. Scanning electron microprobe analysis indicated that some phosphorus was present in one sample while another sample showed no indications of phosphorus. The HPLC analysis of both samples indicated that glue was the binding medium because of the presence of hydroxyproline and high amounts of glycine. This is in agreement with the techniques known to have been used by Andrea Mantegna.

*Mantegna's "Cardinal Trevisano"*

A paint sample from "Cardinal Trevisano" painted by Andrea Mantegna was analyzed by HPLC. From the large amounts of hydroxyproline and glycine seen in Fig. 5, the binder was determined to contain collagen-based glue.

*Czechoslovakian panel painting, "Master of Vysebrotsky Altar"*

Samples from the green ground layer of a Czechoslovakian panel painting, "Master of Vysebrotsky Altar", were obtained for HPLC analysis. Hydroxyproline and a large amount of glycine indicated that a collagen-based glue was used.

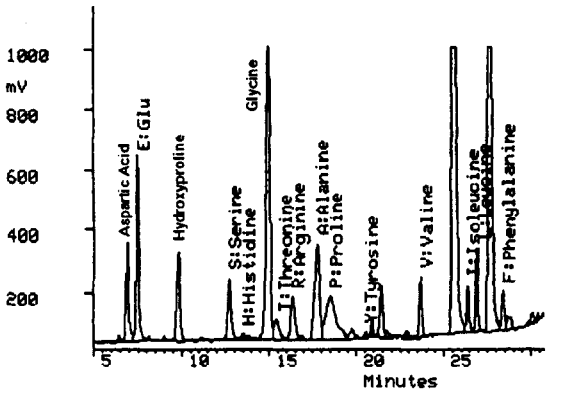


Fig. 5. Amino acid analysis of Mantegna's "Cardinal Trevisano".

## 5. Conclusions

The use of FMOC-amino acid peak area ratios proved to be the best method to distinguish between proteinaceous binding media. By using peak areas rather than molar percent compositions of amino acids, small losses of resolution due to column degradation were not critical. The analytical scheme presented in Fig. 3, along with the use of confirmation peak area ratios listed in Table 1, successfully identified binding media in museum samples.

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