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TECHNICAL NOTE

Polar and non-polar organic binder characterization in Pompeian wall paintings: comparison to a simulated painting mimicking an "a secco" technique

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Abstract The use of Fourier transform infrared spectromicroscopy and mass spectrometry (MS) allowed us to characterize the composition of polar and non-polar binders present in sporadic wall paint fragments taken from Pompeii's archaeological excavation. The analyses of the polar and non-polar binder components extracted from paint powder layer showed the presence of amino acids, sugars, and fatty acids but the absence of proteinaceous material. These results are consistent with a water tempera painting mixture composed of pigments, flours, gums, and oils and are in agreement with those obtained from a simulated wall paint sample made for mimicking an ancient "a secco" technique. Notably, for the first time, we report the capability to discriminate by tandem MS the presence of free amino acids in the paint layer.

Keywords Pompeii's wall painting · Cultural heritage · GC-MS · LC-ESI/MS/MS · FT-IR spectromicroscopy

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Introduction

During the last years, there has been increasing interest toward the study of the molecular properties of ancient paintings due to their great historic relevance [1], and efforts are aimed at the improvement of analytical procedures focused on the development of rapid, sensitive, and accurate methods for dissecting the molecular components of paint layers in wall paintings.

Organic residue analysis utilizes analytical chemical techniques to identify the nature and origins of organic remains that cannot be characterized using traditional techniques of archaeological investigation. The archaeological information contained in organic residues is represented by the biomolecular components of the natural products that contribute to the formation of a given residue. By applying appropriate separation (chromatographic) and identification (mass spectrometric) techniques, the preserved and altered biomolecular components of such residues can be revealed [2].

In this framework, several analytical techniques for the molecular identification of organic materials in ancient works

of art have been developed. These include techniques such Raman and Fourier transform infrared (FT-IR) spectroscopy, immunodetection-based methods, and gas chromatography-mass spectrometry (GC-MS) [3]. Proteomic techniques have been also recently introduced for painting analysis, generating promising results for the identification of proteinaceous components within the samples with a high level of sensitivity and accuracy by the optimization of the amount of sample and the number of analytical steps required for the analysis [4–8]. However, organic binders are less stable and deteriorate faster than the inorganic components. Therefore, only traces remain in old murals, making these organic components difficult to be identified. In addition, the potential contamination coming from restoration intervention performed with organic matrices, as adhesives or consolidators, further increase the complexity of samples.

Among ancient artworks analyzed, Pompeian paints, mainly wall paintings, have been extensively studied especially with regard to their pigment composition by conventional chemical analyses [9]. Recently, analyses of wall decorations from Pompeii made by FT-IR spectroscopy and mass spectrometry showed the presence of high amounts of hydrocarbons and fatty acids that lead to suppose the presence of vegetable compounds and wax in the Pompeian paintings under investigation [10].

In this work, sensitive and accurate procedures have been employed for the unambiguous identification of polar and non-polar organic binders in pigments collected from sporadic wall painting fragments from Pompeii. The aim of the work was focused to clarify the origin of materials employed as binders in Pompeii's wall artworks. To this purpose, we also analyzed a homemade sample of mural painting prepared for mimicking an "a secco" painting technique.

Experimental

Mural painting samples

Two sporadic samples of a wall decoration were collected from "Villa Imperiale, Insula Occidentalis" in Pompeii excavations. They dated approximately to the first century AD, appeared roughly in a good state of conservation, and have not been subjected to any restoration. The surface of the samples was around 3×2 cm (Fig. 1).

Simulated mural painting sample

A simulated sample of mural painting was prepared in our laboratory as follows: Two grams of wheat flour were dissolved in 50 ml of water, and the solution was boiled for 3 min. Then, 0.5 g of tragacanth gum and 0.5 ml of olive oil were added to the mixture. Finally, mercury sulfide powder (about 2 mg/10 ml) was added to the suspension to obtain the desired red tonality. The simulated painting mixture was applied on a compact wall piece of calcite (3×2 cm), and a uniform painting layer was obtained (Fig. 1c). The simulated wall was obtained layering on a flat stone fresh lime (CaO in water, about 3–4 mm), and after its complete solidification and desiccation (about 1 month), it was used for painting. Wheat flour, tragacanth gum, and olive oil, which were used for the preparation of the simulated painting mixture, were also analyzed following the procedure described for organic compound analysis of painting powder.

Samples analyses

Equal amounts (50 mg) of wall painting powder from both Pompeii and simulated samples were carefully scraped from surface and used for chemical analysis. Combined extraction of polar and non-polar compounds from painting powder was carried out by using the protocol suggested by the Standard Metabolic Reporting Structure working group [11]. An aliquot of polar extract solution, corresponding to 15 mg of powder scraped, was employed for the analysis of free amino acids using tandem mass spectrometry by a triple quadrupole. The sugar analysis was performed on 30 mg of powder according to the protocol described by Ha and Thomas [12]. For lipid analysis, the chloroform extract solution, corresponding to 45 mg of powder, was employed for the analysis of fatty acids after a transesterification step using BF_3-methanol. Both sugars and lipids were analyzed

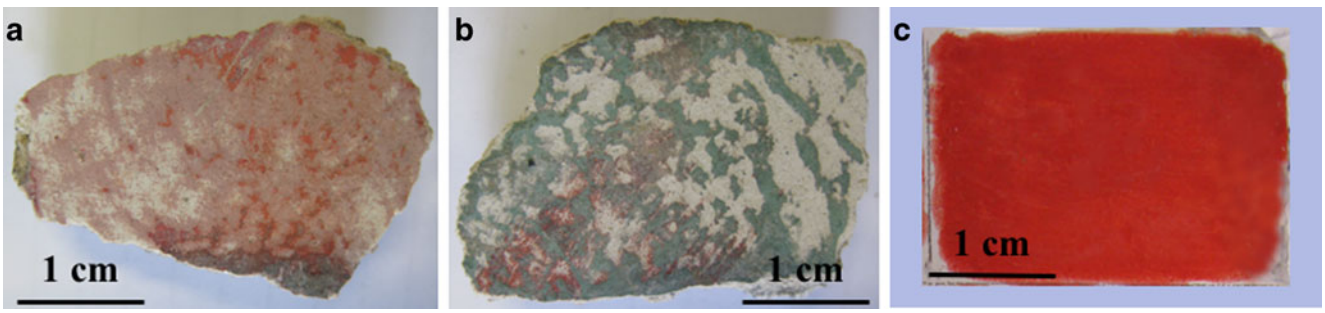

Fig. 1 Photograph of the Pompeii's and simulated wall painting samples. **a** Red, **b** Green, **c** simulated sample

by gas chromatography and detected by flame ionization detector (GC-FID) and/or mass spectrometry (GC-MS). The remaining aliquots of polar and non-polar extracts, corresponding to 5 mg of powder, were used to acquire the spectra by FT-IR spectroscopy.

For the analysis of proteins, an additional aliquot of powder was scraped and resuspended in ammonium bicarbonate buffer; the liquid chromatography mass spectrometry (LC-MS) analysis was performed by electrospray ionization–tandem quadrupole-time-of-flight (ESI–Q-q-TOF) MS according to the procedure reported by Chambery et al. [7].

Detailed experimental procedures are reported in Electronic supplementary material.

Results and discussion

Our investigation was focused on the nature of the organic binders employed in the chromatic layer of decorative ancient paintings from Pompeii. Firstly, powder samples were preliminary analyzed by LC-tandem mass spectrometry (MS/MS) according to a previously reported procedure for shotgun protein analysis [7]. In this instance, no proteinaceous material was detected by ESI–Q-q-TOF analysis. This result was also confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis followed by Blue Coomassie staining (data not shown). Beyond proteinaceous media, natural binders are mainly derived from oils, waxes, and resins and from polysaccharide of plant origin [13]. Therefore, we decided to apply a different strategy based on spectroscopy, gas chromatography, and tandem mass spectrometry analysis of polar and non-polar sample extracts, in order to define the composition of the organic binders. A comparative analysis by using the same procedures was also performed on a homemade sample with the aim to reproduce an ancient a secco painting technique.

FT-IR spectromicroscopy

The water/methanol-soluble and chloroform-soluble compounds extracted from paint powder of both Pompeii's and homemade samples were analyzed by FT-IR spectroscopy. The FT-IR spectromicroscopy analysis showed the presence in the polar and non-polar extracts of several major bands that were indicative of the presence of organic materials (Electronic supplementary material Fig. S1). In particular, the polar fraction (Electronic supplementary material Fig. S1a) contained characteristic peaks most likely corresponding to N–H stretching region (3,600–3,000 cm^{-1}), although this region is also indicative of the presence of H-bonded OH. The presence of the C–H stretching region (3,100–2,800 cm^{-1}) that can be assigned to carbohydrates components was also clearly detected. Other bands characteristic of hydrocarbon chains were present at 1,576; 1,565; and 1,492–1,343 cm^{-1}. In addition, bands appearing at 1,576 and 1,575 cm^{-1} can be also ascribed to metal amino acid complexes [14]. The amide I and II regions (1,654 and 1,540 cm^{-1}, respectively), indicating the presence of proteinaceous materials, were not observed, thus confirming the ESI–Q-q-TOF MS results. The intense absorption band around 1,037 cm^{-1} suggested the presence of carbohydrates side group (COH), although FT-IR spectrum of polysaccharides is usually overlapped by the carboxyl and carboxylate vibrations at around 1,730 and 1,600 cm^{-1}. The band at 710 cm^{-1} potentially characteristic of $\delta(CH_2)_n$ plane rotation of linear long carbon chain, is common to all long-chain fatty acids, *n*-alkanes.

The non-polar fraction (Electronic supplementary material Fig. S1b) showed mainly the presence of the C–H stretching region (3,100–2,800 and 1,576–1,380 cm^{-1}) that can be assigned to the presence of lipid components. In addition, a strong absorption band at 1,740 cm^{-1}, usually associated with the non-hydrogen bonded ester carbonyl C=O stretching mode, was also observed. Bands appearing at 1,600–1,400 cm^{-1} could be ascribed to metal–fatty acid complexes [14].

We also analyzed the samples after removal of the painting layer (Electronic supplementary material Fig. S1c). The white plaster showed the presence of bands that were mostly indicative of calcite (around 2,516; 1,793; 1,425; 1,080; 874; 710 cm^{-1}). However, the hydroxyl group stretching (3,500–3,700 cm^{-1}), SiO stretching modes (950–1,100 cm^{-1}), and OH bending modes at 796 and 677 cm^{-1}, indicative of glauconite or celadonite, usually found in Roman aged wall paints [11] cannot be excluded. Similar FT-IR spectra were observed in the homemade polar and non-polar extracts and lime. All the major bands observed in the spectra together with peak assignments are reported in Table S1 of the Electronic supplementary material.

Tandem mass spectrometry

The polar fraction was analyzed by MS/MS to detect the presence of free amino acids using a mass spectrometry technique largely employed to analyze the profiles of amino acids and other compounds from complex matrices [15].

As reported in Electronic supplementary material Fig. S2, the MS/MS analysis showed a significant presence of 17 signals (*m/z*) in Pompeii's sample. Electronic supplementary material Fig. S2a reports a typical MS/MS spectrum of neutral loss of *m/z* 102 that is specific for Ala, Pro, Val, Leu/Ile, Met, Phe, Tyr, Asp, and Glu. Electronic supplementary material Fig. S2b shows the spectrum of neutral loss of *m/z* 56, specific for Gly and the neutral loss spectra of *m/z* 161 and 119 specific for basic amino acids, Arg (Electronic supplementary material Fig. S2c) and Orn/Asn, Lys, and Cit (Electronic supplementary material Fig. S2d), respectively. All recorded signals were integrated, and their concentrations, expressed as milligrams per kilogram of powder

Table 1 Amino acid profiles in Pompeii's and simulated paintings

Amino acids	Pompeii painting		Simulated painting	
	mg/kg	%	mg/kg	%
Arginine	0.41	1.1	1.32	3.4
Citrulline	2.23	5.8	1.23	3.2
Lysine	1.65	4.3	3.34	8.6
Ornithine + asparagine	0.55	1.4	1.18	3.1
Glutamate	1.54	4.0	1.53	3.9
Aspartate	1.31	3.4	1.87	4.8
Tyrosine	0.45	1.2	1.52	3.9
Phenylalanine	0.37	1.0	0.68	1.8
Methionine	0.36	0.9	0.52	1.3
Isoleucine + leucine	1.09	2.8	1.32	3.4
Valine	2.95	7.6	5.64	14.6
Proline	3.94	10.2	23.07	59.6
Threonine	0.81	2.1	1.37	3.5
5-Oxo-Proline	8.22	21.2	10.03	25.9
Alanine	1.08	2.8	1.24	3.2
Serine	1.03	2.6	2.03	5.2
Glycine	0.93	2.4	0.47	1.2
Total	28.91	100	58.36	100

scraped, were calculated using the abundances of labeled internal standards. Table 1 reports the concentration and percentage of amino acids found in Pompeii's and simulated samples showing in both of them the presence of 19 amino acids. The most abundant amino acids present in both samples (>3%) were Cit, Lys, Glu, Asp, Val, Pro, and OxoPro. The free amino acid profile resulted proportionally similar to that found in different cereals [16]. It should be noted that, although in the simulated painting the total amount of free amino acids was twofold higher than the Pompeii's sample, their percentage composition was quite closer. This finding suggested that Pompeii's wall paint was probably made of pigments dissolved in a liquid medium and organic binders, such as wheat flour. However, our results can be underestimated by not considering degradation processes of these materials through time.

Gas chromatography-mass spectrometry

Analysis of sugars in the polar fraction was performed after methanolysis and trimethylsilyl derivatization of samples by using GC-FID followed by GC-MS. As reported in Electronic supplementary material Fig. S3a, the GC profile of Pompeii's sample showed the presence of several peaks identified as sugars. Compared with the simulated sample (Electronic supplementary material Fig. S3b), the intensities of peaks in Pompeii's sample resulted much lower. However, the percentage of sugar content in both samples (Table 2) is quite closer. In the Pompeii paint sample, the sum of arabinose, xylose, and glucose accounted for more than 85%, excluding the contribution of myo-inositol, likely due to bacterial or fungal contamination, the sugar composition was in agreement to that reported in other wall painting samples [17]. Table 2 also reports the percentages of sugars present in wheat flour and tragacanth gum employed to prepare the simulated painting. These results suggested that, in this instance, the binders also contained organic material of vegetable origin, likely polysaccharides. In particular, the higher amount of arabinose and glucose suggested the use in the paint mixture analyzed of gums from fruit trees or tragacanth [18].

The analysis of the non-polar fraction was performed after transesterification using GC-FID followed by GC-MS. As depicted in Fig. 2, the presence of four fatty acids was revealed in the GC-FID profile of both Pompeii's (Fig. 2a) and simulated (Fig. 2b) paintings. These components were identified by mass spectrometry as C16:0, C18:2, C18:1, and C18:0. Table 3 reports the percentage of the fatty acids found in both samples. The values from Pompeii's sample ranged from

Table 2 Percentage of sugars content in Pompeii's and simulated paintings

Sugars	Pompeii[a] painting	Pompeii[b] painting	Simulated[c] painting	Wheat flour	Tragacanth gum
Arabinose	7.5	18.6	24.4	13.4	44.5
Rhamnose	–	–	1.4	–	4.1
Fucose	–	–	0.4	–	3.7
Xylose	5.9	14.7	6.8	6.8	17.1
Mannose	2.7	6.6	0.4	1.1	–
Galactose	2.5	6.3	6.9	14.4	11.6
Glucose	21.7	53.8	57.9	64.3	12.4
Galacturonic acid	–	–	1.9	–	6.3
Glucuronic acid	–	–	–	–	0.3
Myo-inositol	59.6	–	–	–	–

[a]Sugar percentages including myo-inositol contribution

[b]Sugar percentages excluding myo-inositol contribution

[c]Painting containing wheat flour and tragacanth gum (4:1)

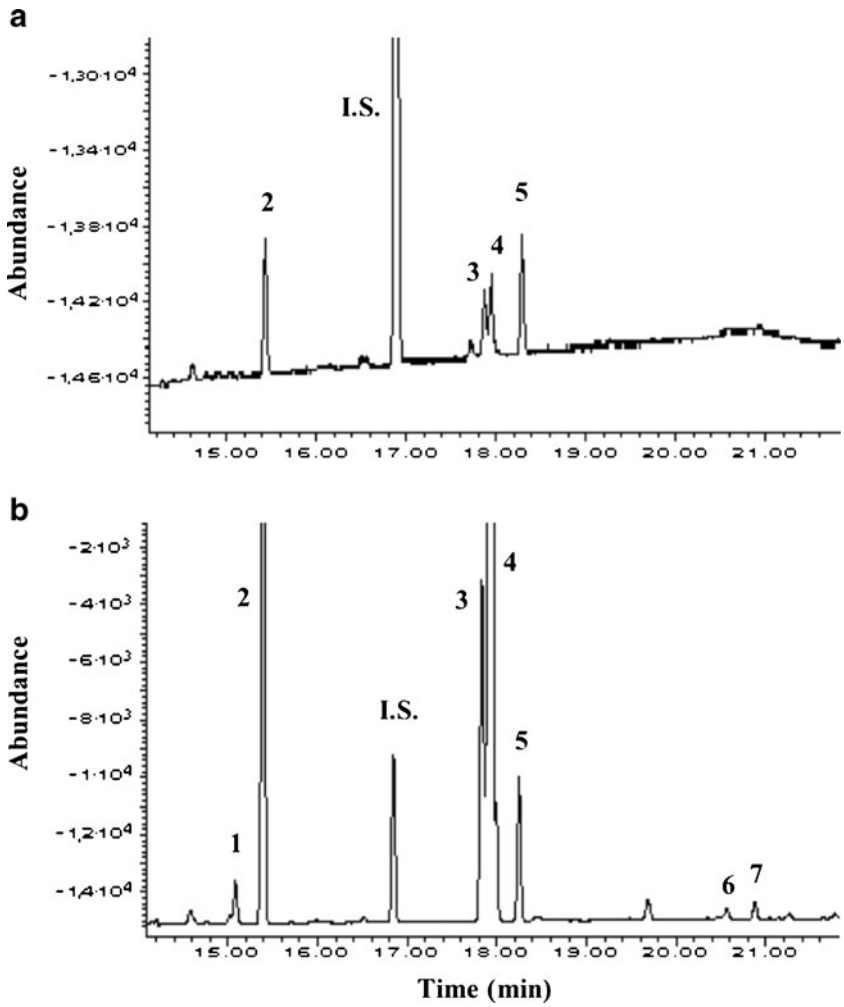

Fig. 2 GC-FID analysis of methylated fatty acids from **a** Pompeii painting and **b** simulated painting lipophilic extract solutions. Peaks: *1*, C16:1; *2*, C16:0; *3*, C18:2; *4*, C18:1; *5*, C18:0; *6*, C20:1; *7*, C20:0; *I.S.* (internal standard) methyl heptadecanoate

16.1% to 33.5%. Other peaks were attributed to contaminants (e.g., phtalate) or unidentified compounds and were not considered for calculations. The percentages of fatty acids content of both samples (Table 3) were indicative of a different composition of non-polar components in Pompeii's sample compared with the simulated sample. Table 3 also shows the percentages of fatty acid composition of the olive oil used to prepare the simulated painting compared with the olive oil and palm oil analyzed by Kurata et al. [18]. The comparison suggested that the fatty acids found in Pompeii's sample probably derived by a mixture of different vegetable oils, such as palm oil, olive oil, and other oils. However, it cannot be excluded that the observed profile may be partially derived from the deterioration of the paint layer due to the environmental context [19]. Our results are partially in agreement with those reported by Duran et al. [10] that reports the identification of non-polar organic material derived from oils or waxes in Pompeian paintings studied. In our samples, the

Table 3 Percentage of fatty acid content in Pompeii's and simulated painting

Fatty acids	Pompeii painting	Simulated painting	Olive oil[a]	Olive oil[b]	Palm oil[b]
C16:1	–	1.3	1.2	0.7	0.2
C16:0	33.5	17.7	12.8	11.1	43.1
C17:0	–	0.2	0.2	0.1	–
C18:2	16.1	9.2	6.6	7.4	9.0
C18:1	20.9	67.1	76.4	76.0	40.3
C18:0	29.5	3.5	2.2	3.2	3.0
C20:1	–	0.5	0.3	0.3	–
C20:0	–	0.5	0.3	0.4	–
Other	–	–	–	0.8	4.4

Comparison to common oils content

[a]Our results

[b]Results reported by Kurata et al. (2005) [18]

presence of waxes in the binder was not considered since any very long fatty acid or alkanes (neither odd-numbered linear hydrocarbons) were revealed in all GC-MS analysis performed on the Pompeian samples. However, the presence of wax in the original samples cannot be excluded since degradation processes due to ageing and/or artificial degradation of beeswax by temperature [19] may have been occurred.

Conclusions

In this work, the presence of polysaccharides, free amino acids, and fatty acids was detected in sporadic Pompeii's wall painting fragments by means of accurate and sensitive analytical techniques. These findings appear to be consistent with a water tempera composed of finely ground pigments probably mixed to wheat, gums, and oils. Our results have been compared with those obtained from the analysis of a simulated painting whose composition mimicked an ancient wall painting made with the a secco technique. Notably, for the first time, we report the capability to discriminate by MS/MS the presence of free amino acids in the colored powder scraped from wall painting specimens.

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