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Research Article

Characterization of pigments and ligands in a wall painting fragment from Liternum archaeological park (Italy)

Spectroscopic and MS techniques were used to characterize the pigments and the composition of polar and nonpolar binders of a stray wall painting fragment from Liternum (Italy) archaeological excavation. X-ray fluorescence and diffraction analysis of the decorations indicated mainly the presence of calcite, quartz, hematite, cinnabar, and cuprorivaite. Infrared spectroscopy, GC coupled to flame-ionization detector, and MS analysis of the polar and nonpolar components extracted from paint layers from three different color regions revealed the presence of free amino acids, sugars, and fatty acids. Interestingly, LC-MS shotgun analysis of the red painting region showed the presence of αS1-casein of buffalo origin. Compared to our previous results from Pompeii's wall paintings, even though the Liternum painting mixture contained also binders of animal origin, the data strongly suggest that in both cases a tempera painting technique was utilized.

Keywords: Cultural heritage / Gas chromatography-mass spectrometry / Infrared spectroscopy / Liquid chromatography-electrospray ionization-mass spectrometry / Wall painting
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1 Introduction

The city of Liternum was one of the most ancient Roman colonies founded in 194 B.C. on the banks of the Literna palus (the Clanis river formed the Literna palus, today Patria lake), situated near the Domitiana road that connected it to Rome and to the Phlegrean area. Following a prospering period in the Augustan age, a gradual decline of the city occurred in the fifth century A.C., due to the transformation of the area into a marsh. The land was colonized again during Middle Ages by the Benedictines of "San Lorenzo di Aversa". Only in 1932, the excavations, conducted under the direction of Amedeo Maiuri and carried out by Giacomo Chianese, allowed the discovery of many archaeological finds, including the remains of the forum with its capitolium, the basilica and theatre, dwellings and roads, an artisanal area and commercial environments, the remains of a portico of a thermal complex, and an amphitheater [1]. Subsequent excavations unearthed the remains of living quarters and some stretches of urban roads and, more recently, other structures in the south west of the forum and, in the north, some commercial environments facing a perpendicular axis to the Domitiana road [2]. This Campanian archaeological site, as well as Pompeii one, is of utmost importance for the study of ancient remains of great historical and scientific relevance [3]. Nevertheless, very few studies have been performed on the constituent materials of ancient paintings whose characterization is fundamental for the understanding of the artists painting techniques. In addition, these analyses may provide useful suggestions for the selection of suitable conservation treatments to assure their preservation and transfer to future generations. Due to the complexity of their molecular components, the analysis of aged and degraded painting samples is a very challenging task that requires the knowledge of complementary disciplines, including chemistry, biochemistry,

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Abbreviations: **BSTFA**, *N,O*-bis(trimethylsilyl)trifluoroacetamide; **FID**, flame-ionization detector; **FT-IR**, Fourier transform infrared; **PLGS**, Protein Lynx Global Server software; **TMS**, trimethylsilyl; **TPCK**, L-1-Tosylamide-2-phenylethyl chloromethyl ketone; **XRD**, X-ray diffraction; **XRF**, X-ray fluorescence

as well as historical and conservative competences [4, 5]. In this framework, it is of interest to study wall painting samples from different archaeological areas with the aim to compare the binders composition used in different geographical areas.

Among ancient artworks, Pompeian wall paintings have been extensively studied mainly with regard to their pigment composition by applying conventional and innovative chemical analyses [6, 7]. Here, we applied an experimental strategy for the analysis of pigments and binders (polar and nonpolar) of a stray piece of mural painting collected in the archaeological area of Liternum. A comparison of the Liternum painting materials with those observed within stray wall painting fragments collected in Pompeii from "Villa Imperiale, Insula Occidentalis" [7] is also discussed.

2 Materials and methods

2.1 Reagents and standard solutions

The analytical HPLC solvents, ACN, formic acid, methanol, ethanol, dichloromethane, chloroform, and pyridine were from JT Baker (Deventer, The Netherlands). KOH was purchased from Merck (Merck KGaA, Darmstadt, Germany). Purified water was prepared with Milli-Q Reagent Water System (Millipore Corporation, Bedford, MA, USA). The solution of HCl/butanol 3N was purchased from Regis Technologies (Morton Grove, IL, USA). All other reagents were of analytical grade. Labeled amino acid standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). A methanol solution of labeled internal standards, containing 25 μM of ^{15}N;2-^{13}C-glycine, and 5 μM of $^{2}H_4$-alanine, $^{2}H_8$-valine-, $^{2}H_3$-methionine, $^{13}C_6$-phenylalanine-, $^{13}C_6$-tyrosine, $^{2}H_3$-aspartate, $^{2}H_3$-glutamate, $^{2}H_2$-ornithine, $^{2}H_3$-citrulline, $^{2}H_4$;5-^{13}C-arginine, and $^{2}H_3$-leucine, was prepared and used for quantitative analysis. The 5-α-cholestane (Sigma, St. Louis, MO, USA) was dissolved in chloroform/methanol (2:1, v/v) at a final concentration of 1 mg/mL. The D-ribose (Carlo Erba Reagents, Italy) was dissolved in distilled water to a final concentration of 1 mg/mL. The bis(trimethylsilyl)trifluoroacetamide was purchased from Sigma. The acetyl chloride was from Carlo Erba Reagents.

2.2 Wall painting sample

The painting sample was collected within a still not identified domus situated in an area of recent excavation of the Archeological Park of Liternum (Campania region, Italy). It was dated approximately to the first century A.D. by P.G., Archaeologist of the "Soprintendenza per i beni archeologici di Napoli e Caserta" on the basis of its technical aspects and from the stratigraphic contest of the archaeological area. The specimen size was approximately 5 × 3 × 2 cm (Fig. 1) and appeared in a good state of conservation. The fragment was selected for the study since showed the presence of a good color variety (i.e. black, azure, red, white, and pink).

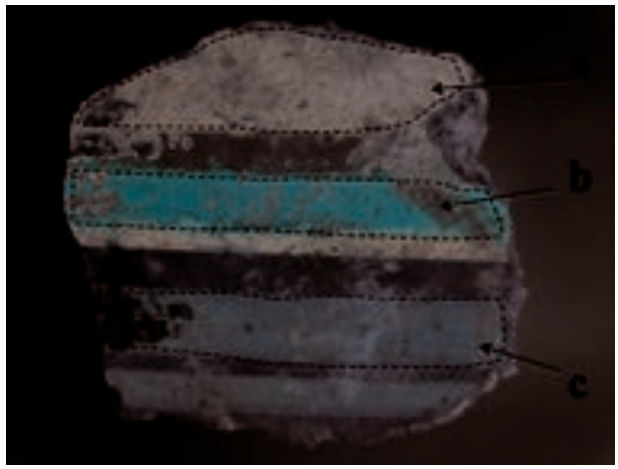

Figure 1. Picture of the Liternum's wall painting sample showing the different color decorations. The black (a), red (b), and pink (c) sampling area for chemical analysis is delimited by a dashed line.

2.3 XRF and XRD analysis

X-ray fluorescence (XRF) analyses of painted sample were carried out using a portable XRF spectrometer Assing-Q (Assing, Rome), whereas the X-ray diffraction (XRD) analyses were made with a Rigaku Miniflex diffractometer (The Woodlands, TX, USA).

2.4 Sample preparation

For chemical analysis, equal amounts (~50 mg) of powder were carefully scraped with a scalpel from the wall painting surface. A combined extraction of polar and nonpolar compounds was carried out according to the Standard Metabolic Reporting Structure working group [8]. Briefly, 100 μg of D-ribose was added to the powder as internal standard for the sugar analysis. Following the addition of methanol (8 mL/g of powder) and water (1.70 mL/g of powder), samples were vortexed for 3 min. Then, chloroform (4 mL/g of powder) was added and the vortexed samples were incubated on ice for 10 min. Finally, chloroform (4 mL/g of powder) and water (4 mL/g of powder) were added and, following a brief vortexing for 3 min, samples were centrifuged at 12 000 × *g* for 15 min at 4°C. The upper layer phase (polar phase; about 0.7 mL) and the lower layer phase (lipophilic phase; about 0.4 mL) were dried under N_2 stream and redissolved in water (50 μL) and chloroform/methanol (2:1, v/v; 50 μL), respectively.

2.5 Fourier transform infrared (FT-IR) spectromicroscopy

Aliquots of 5 μL of polar extract and nonpolar extracts were separately layered on 3-mm ZnS window, dried under a white lamp (60 W), and analyzed with a Nicolet 5700 equipped with a microscope Continuμm (Thermo, West Palm Beach, FL,

USA). For each sample, transmission spectra (200 acquisitions) were collected using the microscope focusing windows set at 50 × 50 μm. Spectra were analyzed by using the Omnic software. Peak assignment was further evaluated on the basis of data library [9].

2.6 Shotgun analysis by LC-MS for protein analysis

For protein analysis, red, pink, and black powder samples (about 8–10 mg) were suspended in 100 μL of 50 mM NH_4HCO_3, sonicated and incubated in 5.8 mM dithiothreitol (DTT) for 5 min at 95°C for disulfide bridges reduction. Cysteine residues were alkylated by incubating samples with 11.7 mM iodoacetamide for 15 min in the dark at room temperature. For enzymatic digestion (16 h at 37°C), 10 ng of TPCK-trypsin (where TPCK is L-1-tosylamide-2-phenylethyl chloromethyl ketone) was added per milligram of samples.

The tryptic digest was dried, resuspended in 20 μL of 0.1% formic acid in water, and analyzed by ESI-LC-MS/MS by using a quadrupole time of flight mass spectrometer equipped with an ESI source (ESI Q-TOF MS, Waters, Milford, MA, USA). Tryptic peptides were separated by means of a modular CapLC system (Waters) as reported elsewhere [10]. Samples were loaded onto a C18 precolumn (5 mm × 300 μm ID) at a flow rate of 20 μL/min and desalted for 5 min with a solution of 0.1% formic acid. Peptides were then directed onto a symmetry-C18 analytical column (10 cm × 300 μm ID) using 5% CH_3CN, containing 0.1% formic acid at a flow rate of 5 μL/min. The elution was obtained by increasing the CH_3CN/0.1% formic acid concentration from 5 to 55% over 60 min. The precursor ion masses and associated fragment ion spectra of the tryptic peptides were mass measured with the mass spectrometer directly coupled to the chromatographic system. The TOF analyzer of the mass spectrometer was externally calibrated with a multipoint calibration using selected fragment ions that resulted from the CID of human [Glu1]-fibrinopeptide B [500 fmol/μL in CH_3CN/H_2O [50:50], 0.1% formic acid] at an infusion rate of 5 μL/min in the TOF MS/MS mode. The instrument resolution in MS/MS mode for the [Glu1]-fibrinopeptide B fragment ion at *m/z* 684.3469 was found to be above 5000 FWHM (full width at half-maximum).

Electrospray mass spectra and MS/MS data were acquired on the Q-TOF mass spectrometer operating in the positive-ion mode with a source temperature of 80°C and with a potential of 3500 V applied to the capillary probe. MS/MS data on tryptic peptides were acquired in the data-directed analysis (DDA) MS/MS mode, automatically switching between MS and MS/MS acquisition for the three most abundant ion peaks per MS spectrum. Charge state recognition was used to select doubly and triply charged precursor ions for the MS/MS experiments, which also includes the automated selection of the collision energy based on both charge and mass. A maximum of three precursor masses were defined for concurrent MS/MS acquisition from a single MS survey scan. MS/MS fragmentation spectra were collected from *m/z* 50 to 1600. For spectra processing, raw data were centroided, deisotoped, and charge-state-reduced to produce a single, accurately measured monoisotopic mass for each peptide and the associated fragment ions by using the Protein Lynx Global server software (PLGS; Waters Corp.). For protein identifications, processed peak lists were searched against the uniprot_sprot database without taxonomy restrictions (release 2011_02; 20254 entries). Trypsin endoprotease was selected for cleavage specificity allowing one missed cleavage site. *S*-Carbamidomethyl derivative of cysteine and oxidation of methionine were specified in Mascot as fixed and variable modifications, respectively. Database search was performed using a mass tolerance of 100 ppm and a fragment ion tolerance of 0.6 Da. Identifications were accepted as positive when probability scores were significant at $P < 0.05$.

2.7 Amino acid analysis by MS/MS

For the amino acids analysis, 10 μL of each polar extract solution (red, black, and pink) was added to a stoppered microvial containing 100 μL of internal standard amino acid solution. The samples were vigorously mixed and placed on an orbital shaker for 30 min at room temperature. Then, samples were evaporated under nitrogen flow at 40°C and derivatized by adding 60 μL of butanolic HCl to dry residue for 20 min at 65°C. Following derivatization, samples were further evaporated under nitrogen flow at 40°C and redissolved in 100 μL of mobile phase (ACN/water, 50:50, containing 0.1% formic acid). Aliquots of solution (10 μL) were analyzed by LC-ESI-MS/MS.

The amino acids were analyzed by MS/MS using a triple quadrupole mass spectrometer (Micromass Quattro-Micro, Waters) equipped with an electrospray source (ESI), operating in positive-ion mode and controlled by a workstation equipped with MassLynx 4.1 software. The samples were introduced into the mass spectrometer source using a 2695 Alliance HPLC pump. Internal standards were used for of amino acids quantification by evaluating the relative signals ratios. When the specific stable isotope was not available, the following ratios were used for calculation: proline/$^2H_8$-valine, lysine/$^2H_2$-ornithine, threonine/$^2H_8$-valine, 5-oxoproline/$^2H_8$-valine, serine/$^2H_4$-alanine. The mass spectrometer was tuned by using a working solution of Val, Leu, Met, Phe, and Tyr at equimolar concentration of 50 μM in water. A flow injection analysis was performed by pumping the mobile phase (ACN/water, 50:50, + 0.1% formic acid) at a flow rate of 0.08 mL/min. Samples (10 μL) were directly injected into the ESI source and the run time was of 2.5 min. Mass spectrometer capillary voltage was 3.30 kV, source and desolvation gas temperature, desolvation gas flow, and cone gas flow were set to 100 and 250°C, 450 and 40 L/h, respectively; gas cell pirani was 2×10^{-3} mbar using argon as collision gas. Specific neutral loss settings were used for the amino acid analysis as follows: neutral loss scan of *m/z* 102 was used for neutral amino acids (mass range:

140–270, collision energy: 20 eV, cone: 20 V), neutral loss of *m/z* 119 was used for lysine, ornitine + asparagine, and citrulline (mass range: 170–240, collision energy: 20 eV, cone: 25 V), neutral loss of *m/z* 161 for arginine (mass range: 225–240, collision energy: 25 eV, cone: 30 V), and neutral loss of *m/z* 56 for glycine (mass range: 120–160, collision energy: 10 eV, cone: 20 V).

2.8 GC (GC-FID and GC-MS)

The sugar analysis was performed according to Ha and Thomas [11]. Briefly, 35 μL of the polar extract solution was dried under nitrogen stream and resuspended in 0.5 mL of a methanolic HCl solution prepared by adding acetyl chloride (0.4 mL) to 15 mL of methanol. Methanolysis was conducted at 80°C for 24 h. Thereafter, the solvent was removed using a nitrogen stream, the residue was derivatized using a mixture of pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (0.2 mL, 3:7), and the solution was heated at 80°C for 30 min. The derivatized sample was dried under nitrogen stream and the residue was dissolved in 50 μL of CH_2Cl_2. Aliquots of samples (1 μL) were analyzed by GC-FID (where FID is flame-ionization detector) and GC-MS.

For lipid analysis, the chloroform extract solution (45 μL) was dried under nitrogen stream and resuspended in 1 mL of BF_3/methanol (10%, w/w). Methylation was performed at 60°C for 10 min. Then, the sample was mixed with 1 mL of distilled water and the lipids were extracted two times with 1 mL of hexane. The upper layers (lipophilic phases) were pooled and 10 μg of methyl heptadecanoate and 10 μg of 5-α-cholestane were added as external standards. Samples were dried under a gentle stream of nitrogen and the residue was dissolved in 50 μL of CH_2Cl_2. Aliquots of samples (1 μL) were analyzed by GC-FID and GC-MS.

Both sugars and lipids analysis were performed with an equipped FID system (GC-FID, HP-5890, Agilent Laboratories, CA, USA) and a Fisons apparatus (GC-MS model GC 8000/MD800, Fisons Instrumentations, Ipswhich, UK) controlled by a workstation equipped with the MassLab 3.4 software. GC was performed by using a SAC-5 capillary column (30-m length, 0.25-mm ID, 0.25-μm film thickness; Supelco/Sigma-Aldrich, Munich, Germany). The linear velocity of carrier gas (helium or nitrogen) was 45 cm/s. The head column gas pressure was set to 19.5 psi. The injector and detector temperatures were fixed at 300°C.

For sugar analysis, the initial temperature of the oven was 100°C; it was then increased to 280°C with a rate of 6°C/min. For fatty acid analysis, the initial temperature of the oven was 105°C; it was increased to 280°C with a rate of 7°C/min and maintained at this temperature for 15 min. For sterol analysis, the oven temperature was fixed at 280°C and maintained at this temperature for 25 min. Qualitative analysis was performed by GC-MS by scanning the mass range from *m/z* 50 to 550 and by comparing the compound mass spectra with those reported into mass spectra libraries (Wiley and National Institute of Standards and Technology). Quantitative analysis was performed using GC-FID, and compounds identification was obtained by the relative retention time (Rf = Rt analite/Rt IS). The Rf was determined by using single standard compound. Sugar and lipid concentrations were calculated from the ratio of peak area compared to D-ribose and methyl heptadecanoate values, respectively.

3 Results and discussion

3.1 Pigment analysis by XRF and XRD

To assess the nature of pigments, the paint surface layer was subjected to XRF and XRD analysis. To this aim, samples of surface areas corresponding to the red, azure, pink, and black colors were separately analyzed. Our results revealed that the red color contained a cinnabar-based (HgS) pigment while in the azure region a copper-based pigment was revealed. However, cinnabar traces were also detected in the azure sample likely due to a repainting on the red layer. The pink color was likely an Fe pigment based and the black was probably of organic origin because the XRF spectrum showed only the presence of Ca (Supporting Information Fig. S1). The XRD analysis, performed on the azure and pink pigments showed mainly the presence of calcite, quartz, hematite, cinnabar and calcite, quartz, cuprorivaite, respectively (Supporting Information Fig. S2).

Since natural binders are mainly composed of polysaccharides, proteinaceous media, oils, waxes, and resins [12–14], a combined strategy based on spectroscopy, GC, and MS analysis of polar and nonpolar sample extracts was applied in order to define the molecular composition of the organic binders.

3.2 FT-IR spectroscopy profile analysis

The red, black, and pink powders of Liternum's samples were analyzed by FT-IR spectroscopy, a technique largely recognized as a rapid method for the identification of a wide range of materials. The FT-IR spectra profile is characteristic to each paint sample and this remark is consistent with the different sugar, protein, and lipid content [15]. The FT-IR spectroscopy analysis of the water/methanol (polar) fraction showed the presence of several major bands that were indicative of the presence of organic materials (Supporting Information Fig. S3). In particular, all samples contained characteristic peaks most likely corresponding to N-H stretching region (3600–3000 cm^{-1}), although this region was also indicative of the presence of H-bonded OH [9]. The presence of the C-H stretching region (3100–2800 cm^{-1}), indicative for the presence of carbohydrates components, was also revealed. In addition, other bands, characteristic of hydrocarbon chains, were present at 1383–1350 cm^{-1}. Regions corresponding to amide I and II (1648 and 1595 cm^{-1}, respectively) indicated the presence of proteinaceous materials. The intense absorption band around 1045 cm^{-1} suggested the presence of

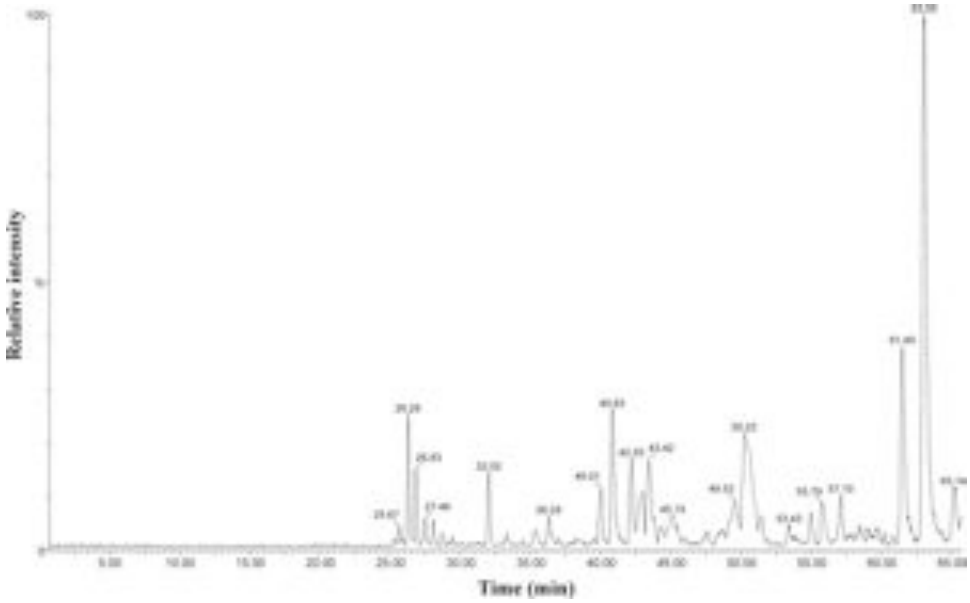

Figure 2. Chromatographic profile from LC-ESI-MS/MS analysis of red powder tryptic digest.

carbohydrates side group (COH) [16]. However, FT-IR spectrum of polysaccharides usually overlapped with the carboxyl and carboxylate vibrations around 1730 and 1600 cm^{-1} [17]. Bands mostly indicative of calcite (around 2512, 1741, 1456, 1086, 871, 717 cm^{-1}) were also observed [16]. However, the hydroxyl group stretching (3500–3700 cm^{-1}), SiO stretching modes (950–1100 cm^{-1}), and OH bending modes at 824 and 677 cm^{-1}, indicative of glauconite or celadonite, often observed in Roman-aged wall paintings [12, 18], cannot be excluded. In addition, a band appearing around 1595 cm^{-1} can be also ascribed to the presence of metal amino acid complexes [19].

Within the nonpolar fraction (Supporting Information Fig. S4), the presence of the C-H stretching region (3100–2800 and 1576–1380 cm^{-1}) typical of lipid components was detected. In addition, a strong absorption band at 1735 cm^{-1}, usually associated with the nonhydrogen-bonded ester carbonyl C=O stretching mode, was also observed. Bands appearing around 1660 cm^{-1} could be ascribed to metal–fatty acid complexes [19]. The band at 717 cm^{-1} could be characteristic of $\delta(CH_2)_n$ plane rotation of linear long carbon chain, which is common to all long-chain fatty acids, *n*-alkanes, and esters present in beeswax. All the major bands observed in the spectra together with peak assignments are reported in Supporting Information Table S1.

3.3 Shotgun analysis by LC-MS/MS

In order to characterize the protein fraction of the Liternum samples, a shotgun LC-MS/MS analysis was performed on the tryptic digest of the red, pink, and black powder samples as described in Section 2. A representative LC-MS chromatographic profile of tryptic digest of the red powder sample is reported in Fig. 2. The fragmentation spectra of peptides were processed using the PLGS 2.0 and the resulting peak lists were used for protein identification by searching the Swiss-Prot database using Mascot software.

Three peptides, assigned to the αS1-casein, have been de novo sequenced with a good level of significance (Table 1, Mascot Score 151). By applying the same approach, the presence of ion signals related to protein material was not detected in the pink and black samples, likely due to the lower amounts of proteins in these samples. To gain information on the species related to the identified αS1-casein, a multiple alignment of sequences of αS1-casein of milk from goat,

Table 1. Peptides identified by LC-MS/MS analysis

Accession number	Peptide sequence	Mw exp[a)]	Theoretical Mw[b)]	Experimental Mw[c)]	*z*	Δ(Da)	Ion score
P02662	R.YLGYLEQLLR.L	634.59	1266.70	1267.17	2	370	74
P02662	R.FFVAPFPEVFGK.E	693.12	1383.72	1384.22	2	357	45
P02662	K.EGIHAQQKEPMIGV NQELAYFYPQLFR.Q	802.68	3205.60	3206.70	2	342	32

a) Mw exp, molecular mass of the revealed ionic signal.
b) Theoretical molecular mass calculated from peptide formula.
c) Experimental molecular mass calculated from ionic signal.

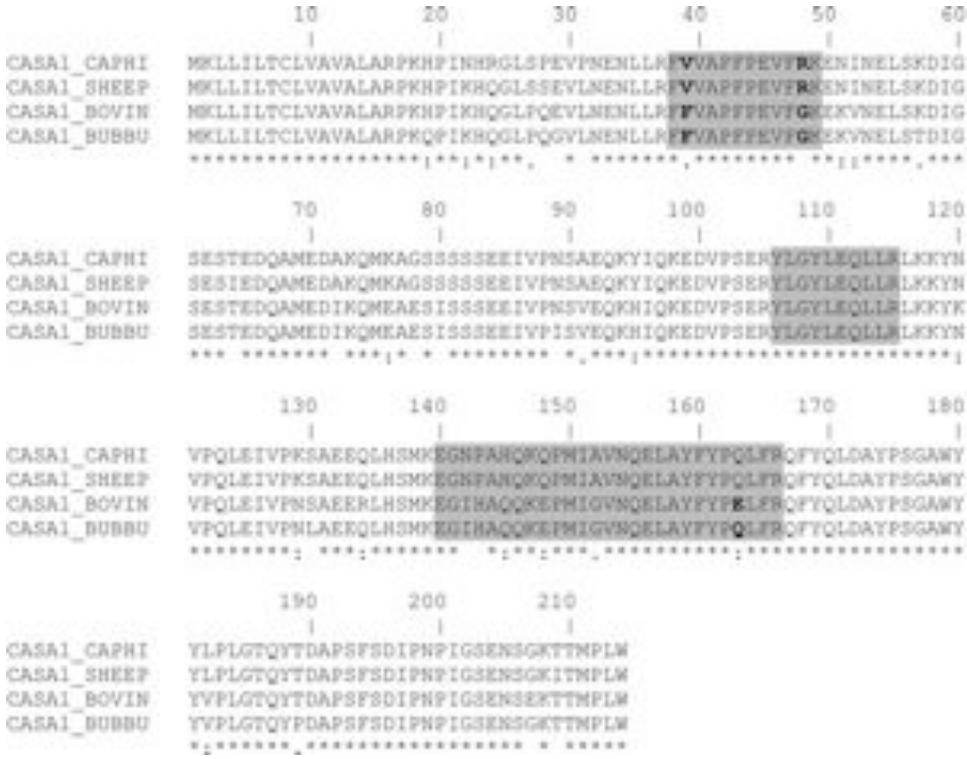

Figure 3. Sequence alignment of αS1-casein. CAPHI, goat (AC: P18626); SHEEP, sheep (AC: P04653); BOVIN, bovin (AC: P02662); BUBBU, buffalo (AC: O62823). Amino acid substitutions indicative of species origin are reported in bold. The matched peptides by LC-ESI MS/MS are gray shaded. *Note*. "*" identical, ":" conserved, and "." semiconserved residues.

sheep, cattle, and buffalo using the software Clustal W was performed (Fig. 3). Given the high percentage of identity of αS1-casein sequences, many tryptic peptides are common to many species. However, two of the identified peptides (position 38–49 and 140–166 sequence) contained amino acid substitutions that allowed the origin species discrimination. Indeed, peptide 38–49 showed a valine at position 39 in casein sequences from sheep and goats, which is substituted by a phenylalanine residue in the caseins of buffalo and bovin species. Similarly, at position 48, the arginine residue in caseins from sheep and goats is substituted by a glycine in buffalo and bovin species (residues in bold in Fig. 3). The peptide 38–49 is therefore proteotypic of buffalo and/or bovin species. Furthermore, the presence of a glutamine residue at position 163 was indicative of the use of buffalo milk, based on the substitution Q163E in the bovine αS1-casein.

3.4 Amino acid analysis by MS/MS

The polar fractions extracted from Liternum painting powders were analyzed by MS/MS in order to detect the presence of free amino acids. This technique is largely recognized as highly specific for the identification of amino acids in a wide range of materials [20]. The amounts (mg/kg of powder) and the percentages of the 19 amino acids identified from the sample mixture are reported in Table 2. The most abundant amino acids (>6%) were found to be Met, Ile + Leu, Val, Pro, 5-Oxo-Pro, Ser, and Gly. Compared to the analysis performed on wall painting samples from Pompeii [7], although some differences were observed for single amino acid percentages,

Table 2. Amino acid profile in Liternum and Pompeii paintings

Amino acid	Liternum		Pompeii	
	mg/kg	%	mg/kg	%
Arg	0.22	0.8	0.69	2.2
Cit	0.39	1.4	1.60	5.1
Lys	0.51	1.8	2.32	7.3
Orn + Asn	1.06	3.7	1.12	3.6
Glu	0.85	3.0	1.94	6.1
Asp	1.10	3.8	1.90	6.0
Tyr	0.41	1.4	0.54	1.7
Phe	0.45	1.6	0.39	1.2
Met	2.19	7.7	0.33	1.0
Ile + Leu	1.70	6.0	1.26	4.0
Val	4.18	14.6	2.96	9.4
Pro	7.68	26.9	4.18	13.2
Thr	0.83	2.9	0.97	3.1
5-Oxo-Pro	1.97	6.9	6.12	19.4
Ala	1.58	5.5	1.44	4.5
Ser	1.71	6.0	1.57	5.0
Gly	1.74	6.1	2.24	7.1
Total	28.56	100.0	31.57	100.0

the total content of free amino acid in Liternum and Pompeii samples was similar. The observed free amino acid profile was found to be also similar to those observed in different cereals [21], wheat [22] and gums of vegetable origin [23], even though the last two works referred to the amino acids analysis following acid hydrolysis. These findings suggested that the

Table 3. Sugar profile in Liternum and Pompeii paintings

Sugar	Liternum			Liternum[a)]	Pompeii[b)]	Pompeii[c)]
	Black mg/kg	Red mg/kg	Pink mg/kg	%	%	%
Arabinose	-	-	-	-	7.5	18.6
Xylose	-	-	-	-	5.9	14.7
Mannose	-	-	-	-	2.7	6.6
Galactose	3.6	3.1	2.5	5.8	2.6	6.3
Glucose	66.5	34.6	41.7	84.6	21.7	53.8
Galacturonic acid	-	-	17.8	9.6	-	-
Myo-inositol	-	-	-	-	59.6	-
Total	70.1	37.7	62.0	100.0	100.0	100.0

a) Mean percentage of sugar content in black, red, and pink samples.
b) Sugar percentages including myo-inositol contribution.
c) Sugar percentages excluding myo-inositol contribution.

Liternum's wall painting sample was probably made using pigments in a liquid medium and organic binders containing free amino acids likely of wheat origin. An underestimation of the whole components should be taken into account considering the potential degradation of the materials through time and/or the different composition of the ancient wheat flour compared to the contemporary one.

3.5 Sugars and lipids analyses by GC-FID and GC-MS

The analysis of sugars in the polar fraction was performed after methanolysis and trimethylsilyl (TMS) derivatization of samples using GC-FID followed by GC-MS (Supporting Information Fig. S5). The percentages of the sugars content are reported in Table 3. In the Liternum's samples, only glucose, galactose, and galacturonic acid were revealed. In particular, the high glucose content suggested that the binders used might contained polysaccharides of both vegetable (wheat) [24] and animal (milk) origin. This finding is not correlable with the sugar profile determined in the Pompeii painting, where the proteinaceus material was not detected [7]. In addition, the absence of other sugars (arabinose, xylose, mannose, mostly deriving from gums) suggested that gums based binders were not used within Liternum painting. Moreover, the presence of galacturonic acid, at least in the pink powder, suggested a possible derivation from the oxidation of galactose or from sugar contained in beet or juices [25]. Even considering the accuracy and sensitivity of the GC-MS analysis, the presence of other oxidized sugars was not revealed. Overall, these findings underline the difficulties in determining the exact composition of polysaccharide in ancient paint samples, since changes occurring with ageing by the action of external factors (e.g. fungi and/or bacteria) are largely unknown. However, the absence of myo-inositol in the Liternum's sample suggested its preservation from bacterial or fungal contamination [26].

Table 4. Lipid profile in Liternum and Pompeii paintings

Carbon length	Liternum			Liternum[a)]	Pompeii
	Black mg/kg	Red mg/kg	Pink mg/kg	%	%
C16:1	9.1	3.2	2.0	2.3	-
C16:0	133.4	72.1	29.6	37.7	33.5
C18:2	26.1	22.8	5.4	8.5	16.1
C18:1	32.1	25.9	6.8	10.2	20.9
C18:0	139.6	94.5	28.7	41.3	29.5
Total	340.3	218.5	72.5	100.0	100.0

a) Mean percentage of lipid content in black, red, and pink samples.

The analysis of the nonpolar fraction was performed after trans-esterification using GC-FID followed by GC-MS (Supporting Information Fig. S6). The mean percentages of the fatty acids determined in the Liternum's sample ranged from 2.3% of C16:1 to 41.3% of C18:0 (Table 4). Less-abundant peaks, relative to compounds with variable carbon length (i.e. from C6 to C14 and over C20), were also detected but their mass spectra did not accurately match with those reported in the MS libraries. The percentage values determined in the three samples (black, red, and pink) were quite variable, thus suggesting different lipid content in the preparation of the painting colors. Compared to Pompeii samples, the lipid profile of Liternum samples mainly showed the presence of saturated fatty acids (C16:0 and C18:0). This finding suggested that the Liternum painting was likely prepared using a mixture of different oils mostly of animal origin. However, it cannot be excluded that the observed profile originated from the deterioration of the paint layer due to the environmental context during aging [27]. Due to the absence, in all GC-MS analysis, of very long fatty acid or alkanes and odd numbered linear hydrocarbons, it is unlike that the profile could resemble that of waxes. However, our results could suffer of the degradation process probably occurred during the centuries on the original compounds used (e.g. wax, beeswax, and other lipid compounds) and the presence of wax in the original samples cannot be excluded [27].

4 Concluding remarks

In this study, the presence of proteins, polysaccharides, free amino acids, and fatty acids in a stray wall painting fragment from Liternum archaeological area was identified. Our results suggested the use of tempera painting, likely composed of finely ground pigments mixed to organic compounds. Although our results do not clearly identify the origin of sugars, the lipid profile indicated that they may derive from a mixture of vegetable and animal matrices. The possible use of binders of animal origin was also assessed by the presence of buffalo αS1-casein. The capability to discriminate milk origin on the basis of specific alpha-casein proteotypic peptides diagnostic

of a given species is also important and might indicate the presence of buffalo in Liternum colony during Roman age.

In conclusion, the Liternum binders composition, compared to Pompeii, differs for the higher percentage of saturated fatty acids, for sugars and proteins of milk origins, and for the absence of gums based binders. These findings might enlarge the actual knowledge on the matrices utilized for the preparation of ancient paintings and will open new perspectives in archaeological fields for the study of past human habits and diets.

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