

Development of an analytical protocol for a fast, sensitive and specific protein recognition in paintings by enzyme-linked immunosorbent assay (ELISA)

M. Palmieri · Manuela Vagnini · L. Pitzurra ·
P. Rocchi · B. G. Brunetti · A. Sgamellotti ·
L. Cartechini

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Abstract Enzyme-linked immunosorbent assay (ELISA) analysis of proteins offers a particularly promising approach for investigations in cultural heritage on account of its appreciated properties of being highly specific, sensitive, relatively fast, and cost-affordable with respect to other conventional techniques. In spite of that, it has never been fully exploited for routine analyses of painting materials in consideration of several analytical issues that inhibited its diffusion in conservation science: limited sample dimensions, decrease of binder solubility and reduced availability of antibody bonding sites occurring with protein degradation. In this study, an ELISA analytical protocol suited for the identification of aged denatured proteins in ancient painting micro-samples has been developed. We focused on the detection of bovine β -casein and chicken ovalbumin as markers of bovine milk (or casein) and chicken albumen, respectively. A systematic experimentation of the ELISA

protocol has been carried out on mock-ups of mural and easel painting prepared with 13 different pigments to assess limits and strengths of the method when applied for the identification of proteins in presence of a predominant inorganic matrix. The analytical procedure has been optimized with respect to protein extraction, antibodies' concentrations, incubation time and temperature; it allows the detection of the investigated proteins with sensitivity down to nanograms. The optimized protocol was then tested on artificially aged painting models. Analytical results were very encouraging and demonstrated that ELISA allows for protein analysis also in degraded painting samples. To address the feasibility of the developed ELISA methodology, we positively investigated real painting samples and results have been cross-validated by gas chromatography–mass spectrometry.

Keywords Immunoassays/ELISA · Painting binders · Casein · Ovalbumin

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M. Palmieri · M. Vagnini (✉) · B. G. Brunetti · A. Sgamellotti
Centro SMAArt, Dipartimento di Chimica, Università di Perugia,
Via Elce di Sotto 8,
06123 Perugia, Italy
e-mail: manuela@thch.unipg.it

L. Pitzurra
Dipartimento di Medicina Sperimentale e Scienze Biochimiche,
Università di Perugia,
Via del Giochetto,
06126 Perugia, Italy

P. Rocchi · L. Cartechini
Istituto di Scienze e Tecnologie Molecolari—CNR,
via Elce di Sotto 8,
06123 Perugia, Italy

Introduction

Immunological techniques exploit the high specificity of the antigen–antibody interaction for analytical purposes [1]. Antibodies found in nature are usually directed against proteins; however immunization procedures allow producing antibodies for a variety of molecules (polysaccharides, viruses, hormones, drugs, etc.), so that specific antibodies can be raised against a target antigen in animal immunization/vaccination models and harvested for virtually any use.

Immunological techniques are not new to conservation scientists [2–6]. Despite that, they have never been fully exploited for routine analyses of painting materials and

only recently have they started to be systematically assayed for this application [7–11]

In this paper, we propose an analytical protocol based on enzyme-linked immunosorbent assay (ELISA) for the ready and specific identification of proteinaceous components in paints. We focused on the detection of bovine β -casein and chicken ovalbumin as marker of bovine milk/casein and chicken albumen, respectively. These proteins belong to the class of proteinaceous materials (mainly represented by hen's egg, milk/casein and animal glue) commonly used by painters as binding media, but also as adhesives and protective coatings thanks to their capability of forming adherent and elastic films of long durability [12, 13].

Due to the relevance of proteins as painting materials, their recognition is of great interest to characterize the artistic technique and for conservation/restoration purposes; unfortunately degradation of the original materials, co-presence of different proteins, environmental contamination and precedent addition of restoring materials make this task particularly difficult to be accomplished. To this purpose, we believe that ELISA offers unique advantages over other analytical approaches used in conservation science and mainly based on chromatography (usually combined with mass-spectrometric detection) or proteomic techniques [14–18]. In fact, ELISA (a) requires minimal sample treatment, (b) is fast and cost-affordable, (c) is very sensitive and specific being selective with respect to the biological source, (d) is capable of resolving complex mixture of proteins tagged with different antibodies. On the other hand, only proteins which antibodies are raised against can be detected.

With the aim of exploring analytical potentials of ELISA in conservation science, we started a systematic experimentation of this technique for protein recognition in paintings, also in consideration of the future perspective of extending this analytical approach to the analysis of other classes of organic molecules, like carbohydrates as recently demonstrated by Mazurek et al. [10].

ELISA allows the detection of the target antigen present in painting samples after solvent extraction. The antigen is adsorbed on the plastic surface of a well plate and is detected by its specific antibody which is, in turn, conjugated to an enzyme that can activate a chromogenic or fluorogenic substrate producing an optical signal. To increase specificity and sensibility of the immunological assay, we used indirect ELISA where the antibody raised against the antigen (called primary or capture antibody) is not labeled; it is, instead, recognized by a second enzyme-conjugated antibody, named secondary or detection antibody, that binds in a very specific way to the primary one. Furthermore, in this study, we employed monoclonal antibodies instead of polyclonal ones [19]. Monoclonal antibodies recognize only one epitope within the antigen, thus they react much more specifically.

In this study ELISA was, firstly, tested on fresh and aged laboratory model samples, simulating easel and mural paintings. The study was aimed at investigating the occurrence of possible analytical interferences from inorganic components and aging effects. The analytical protocol has been optimized with respect to protein extraction conditions, antibodies' concentrations, incubation time and temperature.

As bench-mark of the developed analytical method, the optimized ELISA protocol has been applied to analyze historical samples from thirteenth century mural paintings. Gas chromatography–mass spectrometry (GC-MS) analysis was used as auxiliary methodology to support ELISA results.

Experimental

Reagents

The used primary monoclonal antibodies (MAb) were:

1. Mouse monoclonal antibody to ovalbumin (cod. ab17293, ABCAM plc, Cambridge, UK). It has been produced to react with ovalbumin in denatured and modified forms.
2. Mouse anti-bovine- β -casein, α -AH4 [20]. The antibody reacts with native β -casein and has been tailored to find food adulteration.

The secondary antibody goat anti-mouse IgG (whole molecule) conjugated enzyme alkaline phosphatase (AP-MAb) was used for colorimetric detection by enzymatic reaction in presence of *para*-nitro-phenyl-phosphate (*p*-NPP) as substrate for the enzyme. Both the secondary antibody and *p*-NPP were purchased by Sigma-Aldrich.

Phosphate-buffered saline solution (PBS, 150 mM NaCl, 5.2 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4, 0.2% Tween 20) was used to dilute antibodies and for washing steps. Carbonate/bicarbonate buffer at pH 9.6 was used to dilute reference proteins for ELISA assays. Goat serum 0.1% in PBS (Sigma-Aldrich) was used as a blocking solution.

Fresh hen's egg and whole bovine milk were purchased at the local market. Animal glue (Zecchi, Florence, Italy) and bovine serum albumin (BSA, from Sigma-Aldrich) were used as unspecific controls. ELISA antibody titrations have been carried out by dilution of an initial solution of 1 mg of fresh binder in 1 ml of a carbonate/bicarbonate buffer at pH 9.6.

Standard amino acids solution in 0.1 N HCl containing 2.5 μ mol/mL of proline (Pro), aspartic acid (Asp), glutamic acid (Glu), alanine (Ala), arginine, cysteine, phenylalanine (Phe), glycine (Gly), hydroxyproline (Hyp), hydroxylysine, isoleucine (Ile), histidine, leucine (Leu), lysine (Lys),

methionine (Met), serine (Ser), tyrosine (Tyr), threonine (Threo), and valine (Val) was purchased from Sigma-Aldrich (Germany).

A standard solution of lauric, suberic, azelaic, myristic, sebacic, palmitic, oleic, and stearic acids was prepared in acetone using single standards purchased from Sigma-Aldrich (Germany).

Calibration curves for amino acids and fatty acids were derived from the standard solutions in the range of 1–15 $\mu\text{g/g}$. We used norvaline and heptadecanoic acid (Sigma-Aldrich, Germany) as internal standards respectively for the two classes of compounds. Hexadecane (Fluka, Switzerland) was used as injection standard.

All the solvents were HPLC-grade and used without any further purification. Derivatization reagents *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) were purchased from Sigma-Aldrich (Germany).

Paint models

Paint models were prepared using hen's egg, whole bovine milk and animal glue as binders, mixed with different pigments (listed in Table 1) in a pigment to binder weight ratio of about 3:1, suitable to obtain a workable paint. Single paint layers of variable thickness (50–150 μm , depending on pigment granulometry) were applied on prepared wood panels and on dried carbonated plaster supports to reproduce easel and mural paintings, respectively. The wood supports were coated with a gypsum ground in animal glue. The plaster supports were obtained by carbonation of a mixture of lime and sand with a weight ratio of about 1:3. All the samples were analyzed after 6 months of natural aging at least. A limited number of

Table 1 Pigments (Zecchi, Florence, Italy) used to prepare the paint models

Pigment	Composition
Azurite	$2 \text{CuCO}_3 \cdot \text{Cu(OH)}_2$
Smalt	K–Co–Al silicates
Lapis lazuli	$\text{Na}_{8-10}\text{Al}_6\text{Si}_6\text{O}_{24}\text{Sn}$
Minium	Pb_3O_4
Lead white	$2\text{PbCO}_3 \cdot \text{Pb(OH)}_2$
Hematite	Fe_2O_3
Cinnabar	HgS
Giallorino	Pb_3SnO_4
Verdaccio (green earth)	Fe–Mg–K aluminosilicates
Mn black	MnO_2
Umber	Fe–Mn–Al oxides
Malachite	$\text{CuCO}_3 \cdot \text{Cu(OH)}_2$
Zinc white	ZnO

easel painting models were artificially aged by exposure to 85% RH at 40–50 $^\circ\text{C}$ for 3 months. Layers of the pure binders without pigment on the prepared wood support were included in the aging protocol.

Real samples

Three painting micro-samples (labeled CA13, CA19, GA04) from the cycle of frescoes attributed to Giotto and Cimabue decorating the transept vaults of the upper church of the Basilica of St. Francis in Assisi (Italy) have been investigated. The micro-samples (weights of about 1 mg) were collected on few painting fragments (Fig. 1) recovered during the restoration campaign after the earthquake of 1997 that brought down part of the vaults. These fragments have been made available for scientific studies [8, 15]. The aim of the present investigation was to verify the use of “a secco” painting technique in which pigments are applied on dried plaster by employment of proteinaceous binders.

Instrumentation

ELISA

ELISA tests were performed in 96 wells polycarbonate microtiter plates (CELLSTAR, Greiner bio-one). Sample absorbance at $\lambda=405 \text{ nm}$, expressed as optical density ($\text{OD}_{405\text{nm}}$), was measured by using a GDV programmable MPT reader mod. DV 990BV6.

GC-MS

A 6890N gas-chromatograph (Agilent Technologies) with split/splitless injector, interfaced to a model 5973 quadropole mass spectrometer was used. Injector worked in splitless mode at a temperature of 230 $^\circ\text{C}$, while transfer line was kept at 280 $^\circ\text{C}$. Temperatures of the ion source and of the mass quadropole were 230 $^\circ\text{C}$ and 150 $^\circ\text{C}$, respectively. The mass spectrometer operated in the EI mode at 70 eV. Helium was used as gas carrier at a constant flow of 1 mlmin^{-1} . The gas-chromatograph is equipped

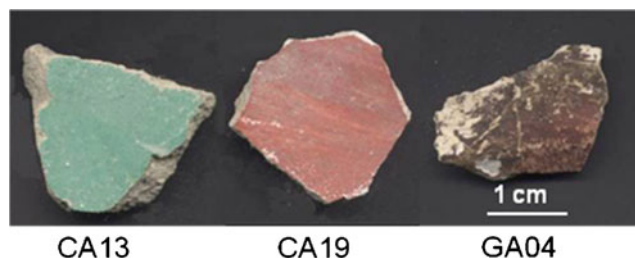


Fig. 1 Investigated fragments from the painting cycle attributed to Giotto and Cimabue decorating the transept vaults of the upper church of the Basilica of St. Francis in Assisi (Italy)

with a fused silica capillary column, model HP-5MS, with a 5% diphenyl/95% dimethyl-polysiloxane stationary phase, of 30 m length with internal diameter of 0.25 mm and 0.25 μm film thickness (J&W Scientific, Agilent Technologies, Palo Alto, CA).

During amino acids analysis, the temperature program of the oven was: initial temperature 80 °C, held for 1 min and then ramped at 8 °C/min to 280 °C where it was held for 2 min. The analysis of the glycerolipid fraction was carried out using an oven temperature program with an initial temperature of 80 °C, which was held for 2 min and then ramped at 10 °C/min to 200 °C, which was held for 2 min and then ramped at 6 °C/min to 280 °C, which was held for 1 min.

Chromatograms have been acquired in both single ion monitoring (SIM) and total ion current modes. The SIM acquisition mode was used for quantitative analysis of amino and fatty acids.

X-ray fluorescence

X-ray fluorescence (XRF) spectra were collected using a portable equipment made of an X-ray generator (EIS, mod. P/N 9910) with a tungsten source and a Peltier-cooled silicon drift detector having a resolution of about 150 eV at 5.9 keV [21]. All the measurements have been carried out at the X-ray tube operative conditions of 38 kV and 0.02 mA.

ELISA protocol

ELISA immunological tests have been carried out on paint micro-samples of about 1 mg scrapped off as fine powder from the painting surface with a micro-scalpel. The protocol for both bovine β -casein and chicken ovalbumin can be summarized as follows:

- Step 1 One hundred microliters (300 μL for real samples) of PBS (elution buffer) are added to the painting micro-sample in a 1.5 ml Eppendorf tube; proteins are extracted by sonication (2 h for non-aged samples, 4 h for artificially aged and real samples) followed by incubation at room temperature overnight.
- Step 2 Seventy microliters of the supernatant solution are placed into an ELISA well plate, covered with a special cap and incubated for 1 h at 37 °C to permit binding of the proteins to the surface of the microtiter wells.
- Step 3 One hundred microliters of the blocking solution (goat serum 0.1% in PBS) are added to the wells and incubated 30 min at 37 °C. The blocking solution is used to saturate the unspecific sites in order to avoid false positive results.

- Step 4 One hundred microliters of primary antibody are added to the wells and incubated for 1 h at 37 °C to allow binding between the antibody and the antigen.
- Step 5 One hundred microliters of alkaline phosphatase conjugated secondary antibody are added and incubated for 1 h at 37 °C to permit binding between the primary and the secondary antibody.
- Step 6 One hundred microliters of colorless *p*-NPP are added to the wells and allowed to react for 15 min at room temperature (25 °C) in a closed air-conditioned environment. In the presence of alkaline phosphatase, *p*-NPP is converted into the yellow dye *p*-nitrophenol by enzymatic reaction.
- Step 7 Fifty microliters of NaOH 2 N are added to the wells to stop the coloring enzymatic reaction after 30 min. This step is necessary to achieve quantitative comparable results since coloring depends also on the time for which the enzyme is left to be active.

Between each step, wells are thoroughly rinsed with 200 μL of PBS, repeated at least five times, in order to remove any unbound reagent (blocking solution, antibodies, and substrate).

Finally, the ELISA plate is put in the spectrophotometer to measure the optical density at 405 nm.

All the ELISA tests were performed at least three times on each sample. Measured optical densities are reported together with the standard deviation.

GC-MS protocol

Proteinaceous and glycerolipid materials present in the ancient mural painting have been characterized by GC-MS analysis of micro-samples of about 1 mg weight following a multi-step sample pre-treatment [22, 23]. The analytical protocol is based on the extraction from the micro-samples of an aqueous and an organic fraction containing the two different classes of compounds prior GC-MS analysis.

The adopted procedure consists of the following steps:

1. Proteins are extracted from the sample by sonication in 300 μL of a 2.5-M aqueous ammonia solution at 30 °C for 60 min for two times. The extracted solution is evaporated to dryness under a gentle stream of nitrogen and dissolved in 150 μL of TFA.
2. The free organic acids extracted together with proteins in step 1 are recovered from the acidic TFA solution with diethyl ether (200 μL , three times) and left for step 6.
3. The TFA acidic solution containing proteins, peptides and soluble inorganic salts, after elimination of the residual ether by nitrogen stream, is then applied onto

an Omix C4 tip to purify proteinaceous material [23] according to the protocol described by Lluveras et al. [24]. Formic acid (0.1%)/MeOH (75%)/H₂O (25%) is used as eluting solution (100 μ L, twice).

- The eluted solution is evaporated to dryness under a stream of nitrogen and then subjected to acidic hydrolysis in 300 μ L of HCl 6 N at 100 °C for 24 h in N₂ atmosphere. At the end, pure water is added to the acidic hydrolysate.
- An aliquot of the aqueous hydrolysate solution containing amino acids is evaporated to dryness under a stream of nitrogen and then subjected to derivatization by addition of 50 μ L of MTBSTFA at 60 °C for 60 min in presence of 10 μ L of DMF. Norvaline is added in this step as internal standard (final concentration 3.8 μ g/g). After silylation, 240 μ L of isooctane containing hexadecane are added and 1 μ L of the final solution is then injected for the chromatographic run. The analytical protocol allows for the quantitative determination of 14 amino acids (Pro, Hpro, Asp, Glu, Ala, Phe, Gly, Ile, Leu, Met, Ser, Tyr, Thr, and Val).
- The residue of step 1 combined with the dried ether extract of step 2 is subjected to saponification (2 h at 80 °C) with ethanolic KOH (10% KOH in EtOH);
- After saponification 100 μ L of H₂O are added and the hydroalcoholic solution is acidified with HCl. The unsaponifiable fraction is extracted with 200 μ L of *n*-hexane for three times (neutral fraction); the acidic fraction is extracted with 200 μ L of diethyl ether for three times to recover fatty and resin acids.
- The acidic and neutral fractions are joined and spiked with heptadecanoic acid (final concentration 5.0 μ g/g), evaporated to dryness under nitrogen stream and, then, subjected to silylation for GC-MS analysis with 50 μ L of BSTFA at 60 °C for 30 min. After the addition of 250 μ L of hexadecane in isooctane solution, 1 μ L of the final solution is analyzed by GC-MS.

Results

Laboratory standards

Optimum antibody dilutions (*v/v*) corresponding to the best specificity and sensitivity of the method were obtained for bovine β -casein and chicken ovalbumin by antibody panel titrations of solutions of the fresh binder (see the “Experimental” section) using PBS as blank control. Primary antibody specificity (possible presence of false positives) was tested applying the ELISA protocol for bovine β -casein to chicken albumen and BSA proteins, used as negative controls; by contrast the ELISA protocol

for chicken albumen was tested on bovine milk and on egg albumen of different birds (these were tested only at the optimized antibody dilutions). Milk from different biological sources was not tested considering the α -AH4 MAb has been produced for the high specific recognition of bovine β -casein in inspection programs of frauds in cheese production [20]. ELISA antibody titration results are reported in Tables 2 and 3.

By comparing the measured optical densities of the bovine milk-positive controls, the antibody dilutions of 1:250 for the α -AH4 MAb and of 1:2,500 for the AP-MAb gave the best compromise between test sensitivity and antibody concentration. In fact, to avoid false positives and to maintain a low background, the lowest antibody concentrations giving a satisfactory OD_{405nm} response were chosen. At these antibody concentrations, milk-positive controls at the dilutions of 1:100 and 1:10,000 showed mean OD_{405nm} values of 2.2 and 1.6, respectively, compared to an average optical density of the PBS blank control of 0.27 (OD_{405nm} values in bold font in Table 2).

In the case of chicken albumen, the optimal antibody dilutions were 1:4,000 for the chicken ovalbumin Mab and 1:1,000 for the AP-MAb. At these antibody concentrations, the albumen positive controls at the dilutions of 1:10, 1:100, and 1:1,000 showed mean OD_{405nm} values of 2.1, 1.1, and 0.4, respectively, compared to an average optical density of the PBS blank control of 0.24 (OD_{405nm} values in bold font in Table 3).

Table 2 Bovine β -casein ELISA antibody titration results for the fresh binder

Sample (dilution, <i>v/v</i>)	AP-MAb dilution (<i>v/v</i>)	OD _{405nm} ±SD		
		α -AH4 MAb dilution (<i>v/v</i>)		
		1:250	1:500	1:1,000
Bovine milk (10 ⁻²)	1:1,000	1.8±0.5	1.8±0.6	1.3±0.3
	1:2,500	2.2±0.4	1.3±0.3	0.83±0.08
	1:5,000	1.2±0.3	1.0±0.2	0.8±0.2
Bovine milk (10 ⁻⁴)	1:1,000	2.4±0.4	1.8±0.3	1.2±0.2
	1:2,500	1.6±0.1	1.3±0.2	0.9±0.2
	1:5,000	1.3±0.4	1.0±0.3	0.7±0.1
PBS	1:1,000	0.4±0.2	0.3±0.1	0.24±0.09
	1:2,500	0.27±0.02	0.21±0.08	0.19±0.09
	1:5,000	0.22±0.09	0.2±0.2	0.16±0.07
Chicken albumen (10 ⁻²)	1:1,000	0.12±0.01	0.110±0.003	0.11±0.01
	1:2,500	0.10±0.05	0.11±0.03	0.11±0.01
	1:5,000	0.10±0.01	0.11±0.02	0.12±0.02
BSA (10 ⁻²)	1:1,000	0.11±0.02	0.10±0.01	0.14±0.05
	1:2,500	0.11±0.01	0.090±0.003	0.08±0.01
	1:5,000	0.10±0.01	0.09±0.01	0.140±0.004

Table 3 Chicken ovalbumin ELISA antibody titration results for the fresh binder

Sample (dilution, v/v)	AP-MAb dilution (v/v)	OD _{405nm} ±SD		
		MAb dilution (v/v)		
		1:1,000	1:4,000	1:10,000
Chicken albumen (10 ⁻¹)	1:1,000	2±1	2.1±0.4	1.3±0.3
	1:4,000	2.2±0.5	1.6±0.3	0.8±0.2
	1:10,000	0.16±0.05	1.18±0.05	0.58±0.07
Chicken albumen (10 ⁻²)	1:1,000	1.3±0.3	1.1±0.5	0.6±0.2
	1:4,000	1.4±0.5	0.84±0.6	0.4±0.3
	1:10,000	0.18±0.04	0.4±0.3	0.25±0.06
Chicken albumen (10 ⁻³)	1:1,000	0.6±0.3	0.4±0.3	0.22±0.08
	1:4,000	0.4±0.4	0.3±0.3	0.17±0.09
	1:10,000	0.19±0.03	0.22±0.09	0.13±0.06
PBS	1:1,000	0.31±0.08	0.24±0.06	0.2±0.2
	1:4,000	0.22±0.05	0.16±0.06	0.2±0.2
	1:10,000	0.18±0.03	0.16±0.02	0.09±0.02
Bovine milk (10 ⁻²)	1:1,000	0.14±0.04	0.16±0.01	0.13±0.02
	1:4,000	0.13±0.03	0.20±0.09	0.11±0.01
	1:10,000	0.16±0.01	0.18±0.01	0.05±0.01
Duck albumen (10 ⁻²) (10 ⁻³)	1:1,000		0.078±0.006	
			0.077±0.004	
Goose albumen (10 ⁻²) (10 ⁻³)	1:1,000		0.072±0.002	
			0.071±0.005	
Pigeon albumen (10 ⁻²) (10 ⁻³)	1:1,000		0.082±0.005	
			0.068±0.003	
Turkey albumen (10 ⁻²) (10 ⁻³)	1:1,000		0.078±0.001	
			0.085±0.009	

From the results of the analysis of multiple PBS blanks, the limit of detection was fixed at OD_{405nm}=0.30, obtained as the blank average OD_{405nm} plus three times the corresponding standard deviation. This means that samples with OD_{405nm}>0.30 were considered positive to the immunological test, while those with OD_{405nm}≤0.30 were considered negative. In the ELISA titration panels for both β-casein and chicken ovalbumin, all the negative controls containing BSA, egg and milk at all the dilutions showed OD_{405nm} values below 0.30.

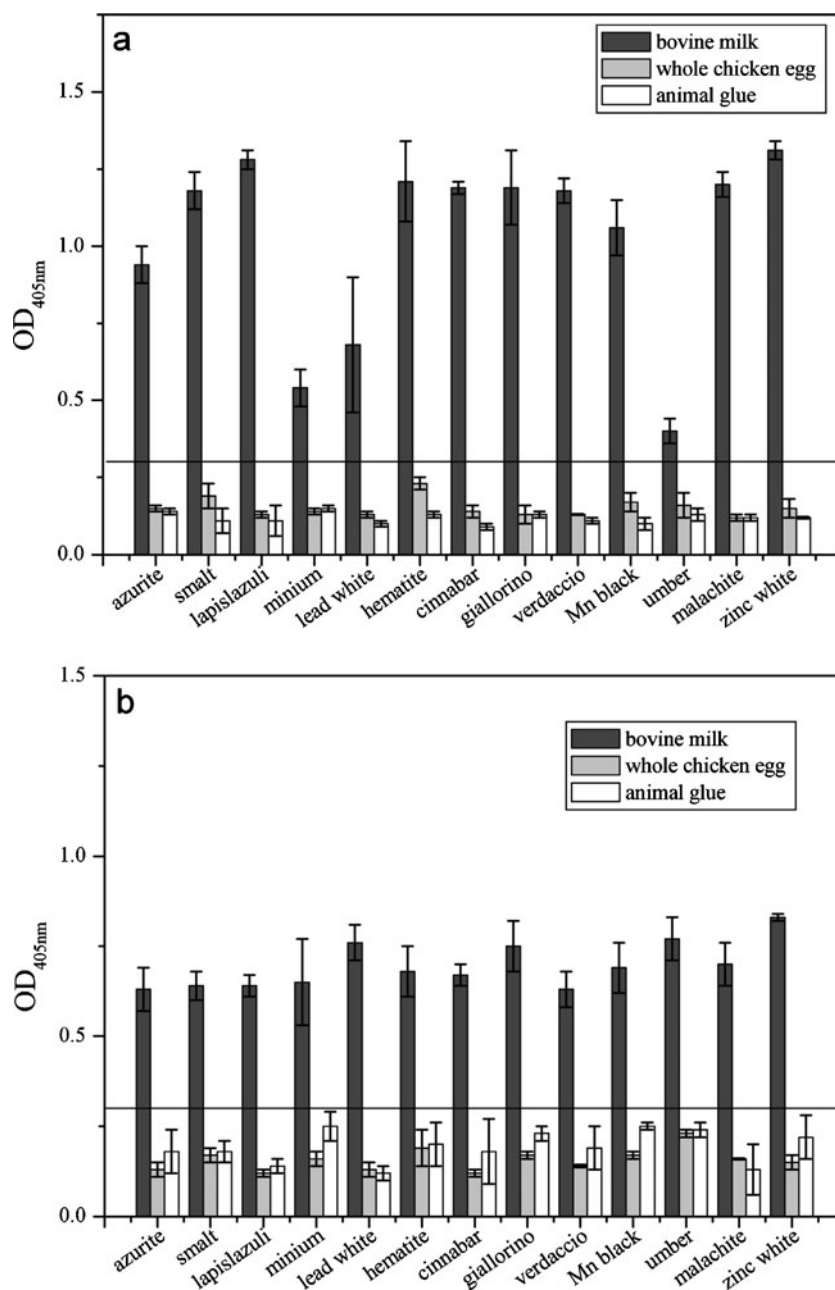
Once the ELISA protocols have been optimized for the pure binders, they were applied to the non-aged easel and mural painting models to assess whether analytical interferences from the inorganic matrix occur in protein recognition. In consideration of the relatively low background measured in the titration panels, we simplified the analytical procedure skipping the addition of the blocking solution (step 3 of the protocol). Results are reported in Figs. 2 and 3. They were obtained by using PBS as extraction solvent since it showed the best protein extraction efficiency with respect to other tested solutions (carbonate/bicarbonate buffer solution, 10 mM Tris-HCl-1 mM EDTA (tris-HCl-EDTA) buffer solution, tris-HCl-

EDTA+1% sodium dodecyl sulfate (SDS) solution, and tris-HCl-EDTA+1% SDS+6M urea solution). To test the specificity of the immunological assays, the optimized protocols were performed also including as negative controls the painting models made with animal glue as the binder. β-casein has been correctly identified in both easel and mural painting models made with milk (Fig. 2a and b, respectively); samples containing egg or glue showed negative as expected. All the samples containing whole egg as the binder showed positive to chicken ovalbumin in both easel and mural painting models (Fig. 3a and b, respectively); conversely, those samples containing a binding media different from egg showed negative.

The ELISA protocol was, then, tested on artificially aged easel painting models.

As it concerns bovine β-casein, aged samples showed many false negatives with optical densities below the limit of detection for both the pigmented and non-pigmented binder. In order to increase sensitivity of the ELISA assay, firstly antibody titration was performed on the fresh binder highly diluted (1:1,000 and 1:1,000,000 dilutions) using increased primary and secondary antibody concentrations (dilutions varied from 1:25 to 1:1,500), as shown in Table 4.

Fig. 2 ELISA optical densities obtained for the **a** easel and **b** mural painting samples treated with β -Casein MAb. Results for bovine milk (*dark gray*), whole chicken egg (*light gray*) and animal glue (*white*) and the corresponding SD are reported for the tested pigments showed on the *X* axis

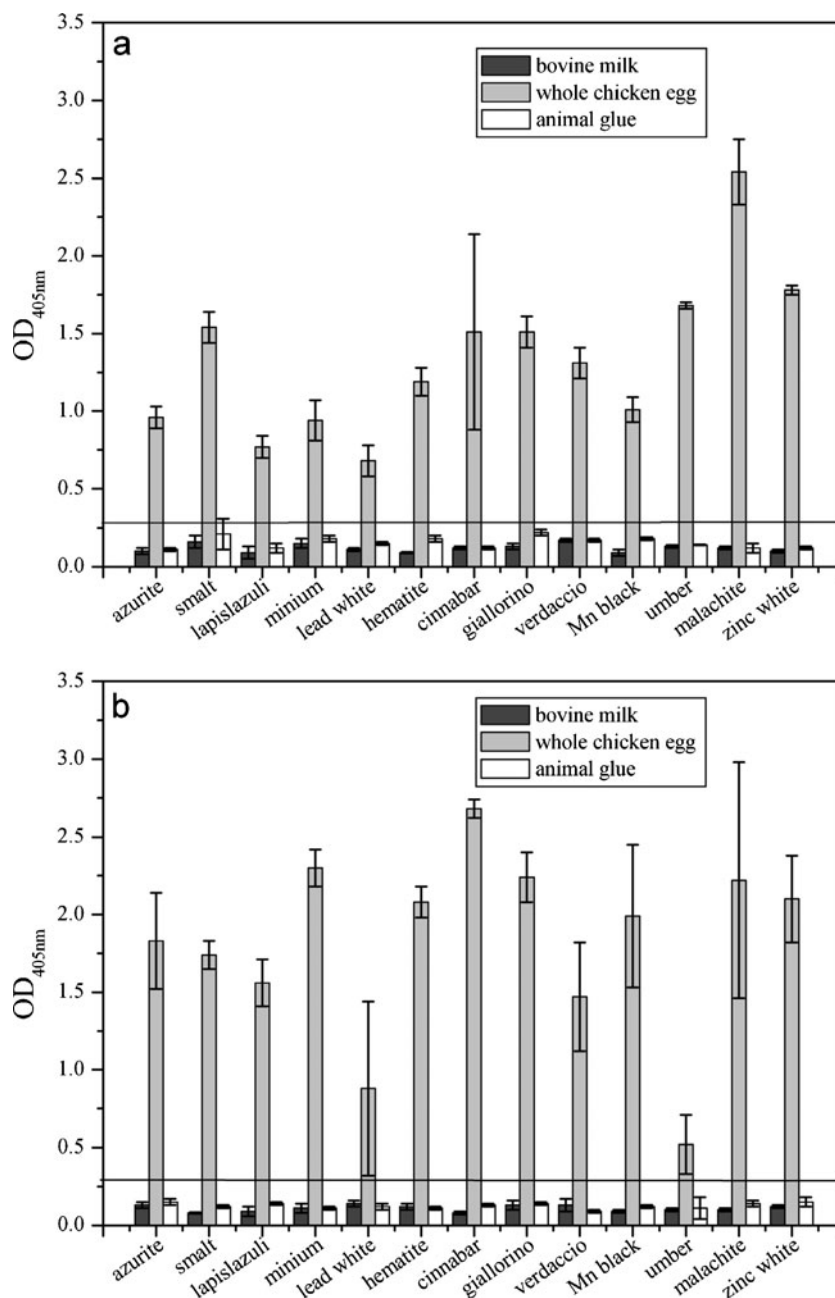


Furthermore, the background level was drastically reduced by addition of the blocking solution; thus its systematic use in the final ELISA protocol is strongly recommended. The highest test sensitivity was obtained at the antibody dilutions of 1:50 for the α -AH4 MAb and of 1:500 for the AP-MAb, for which optical density values of 1.0 and 0.3 were obtained for the milk-positive controls at the binder dilutions of 1:1,000 and 1:1,000,000, respectively. From the analysis of many PBS blank controls the limit of detection was fixed at $OD_{405nm}=0.20$. Negative controls containing chicken albumen and BSA proteins resulted below the limit of detection at all the tested antibody dilutions.

The ELISA results obtained on the artificially aged easel painting models by using the modified protocol for bovine β -casein are shown in Fig. 4 along with the negative controls containing egg and animal glue. All the samples containing bovine milk showed positive for all the pigments, while all the negative controls gave optical density below the limit of detection.

In the case of chicken ovalbumin, the antibody dilutions optimized for the analysis of non-aged samples allowed for the identification of the binder in the majority of the aged samples. On the average, a slight decrease in OD_{405nm} values has been observed but with preservation of a low background level. The results are illustrated in Fig. 5 where

Fig. 3 ELISA optical densities obtained for the **a** easel and **b** mural painting samples treated with ovalbumin MAb. Results for bovine milk (*dark gray*), whole chicken egg (*light gray*) and animal glue (*white*) and the corresponding SD are reported for the tested pigments showed on the *X* axis



the optical densities obtained for the negative controls containing milk and animal glue are, also, presented, showing OD_{405nm} values below the limit of detection (0.30). Only one false negative was obtained for lead white. To improve ELISA sensitivity for ovalbumin detection in this test sample, a higher concentration of the primary antibody solution was used (MAb dilution of 1:500) obtaining an OD_{405nm} value of 0.8 ± 0.2 . By applying the same MAb dilution to the other aged pigmented samples, a marked increase of the background level was observed without a significant improvement of the specific absorbance.

Discussion

The results obtained from the ELISA experimentation on the painting models evidenced that the optimized protocols allow to properly identify bovine β -casein and chicken ovalbumin in all the records, while no evidence of false positive was found. This means that the immunological detection of these proteins by ELISA is highly specific with sensitivity that can be estimated from the calibration curves (Figs. 4 and 5) to be about 15 μ g of milk and about 7 ng of albumen for the dried binders.

Table 4 β -Casein ELISA antibody titration results for the analysis of aged samples

Sample (dilution, v/v)	AP-MAb dilution (v/v)	OD _{405nm} ±SD	
		MAb dilution (v/v)	
		1:25	1:50
Milk 10 ⁻³	1:500	0.9±0.3	1.0±0.2
	1:1,000	0.9±0.2	0.7±0.1
	1:1,500	0.80±0.07	0.81±0.07
Milk 10 ⁻⁶	1:500	0.12±0.01	0.3±0.1
	1:1,000	0.13±0.02	0.16±0.05
	1:1,500	0.19±0.02	0.14±0.06
PBS	1:500	0.17±0.03	0.16±0.01
	1:1,000	0.20±0.02	0.13±0.02
	1:1,500	0.16±0.01	0.11±0.02
Chicken albumen 10 ⁻³	1:500	0.14±0.01	0.11±0.01
	1:1,000	0.13±0.01	0.10±0.01
	1:1,500	0.12±0.01	0.10±0.01
BSA 10 ⁻³	1:500	0.09±0.01	0.07±0.01
	1:1,000	0.08±0.01	0.07±0.01
	1:1,500	0.07±0.01	0.08±0.01

More in details, immunoassays on the non-aged easel and mural painting models showed no analytical interferences from both the substrates (gypsum/glue preparation or dried plaster) and the tested pigments (Figs. 2 and 3).

Due to practical reasons, artificial aging was applied to a restricted number of the easel painting models (tested

pigments: smalt, minium, lead white, hematite, cinabbar, giallorino, Mn black, malachite) and excluding those simulating mural paintings. Considering the absence of observable interferences from the inorganic preparation substrate in non-aged samples, the results obtained for the aged ones were significant to study the effects of possible protein-pigment interactions occurring with accelerated aging.

From literature [25–28], deamidation, oxidation, and hydrolysis are the main degradation processes affecting proteinaceous binders. These processes can be catalyzed by light [29, 30] or by metal ions [31]; they are influenced by pH and can be favored by the interaction with other reactive organic components (as, for example, lipids and carbohydrates) [27]. Degradation phenomena result in the loss of the most labile amino acids (mainly those containing hetero atoms) as well as in cross-linking and condensation reactions that decrease aqueous solubility.

In the present study, the adopted aging protocol was focused on the effects induced by high humidity conditions, kinetically accelerated by temperature. Humidity mainly promotes hydrolysis reactions that may lead to the cleavage of peptide bonds; in solid samples, water can also act as solvent/plasticizer increasing molecular mobility, thus chemical reactivity [27]. Furthermore, the exposure of the paint layers to air and the simultaneous presence of inorganic pigments favor the oxidative decay of the organic component.

The aim of the experiment was to verify whether the induced degradation phenomena could hinder the identification of proteins by ELISA in ancient materials. This may

Fig. 4 ELISA optical densities obtained for the artificially aged easel painting samples treated with bovine β -casein MAb. Results for bovine milk (*dark gray*), whole chicken egg (*light gray*) and animal glue (*white*) and the corresponding SD are reported for the pure aged binders and the tested pigments (showed on the X axis). The bovine β -casein ELISA calibration curve is also reported

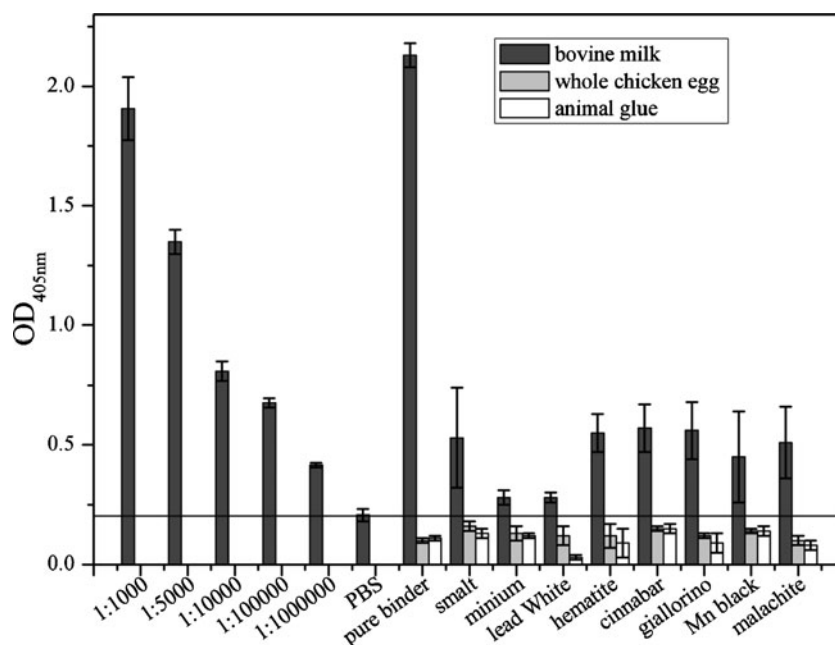
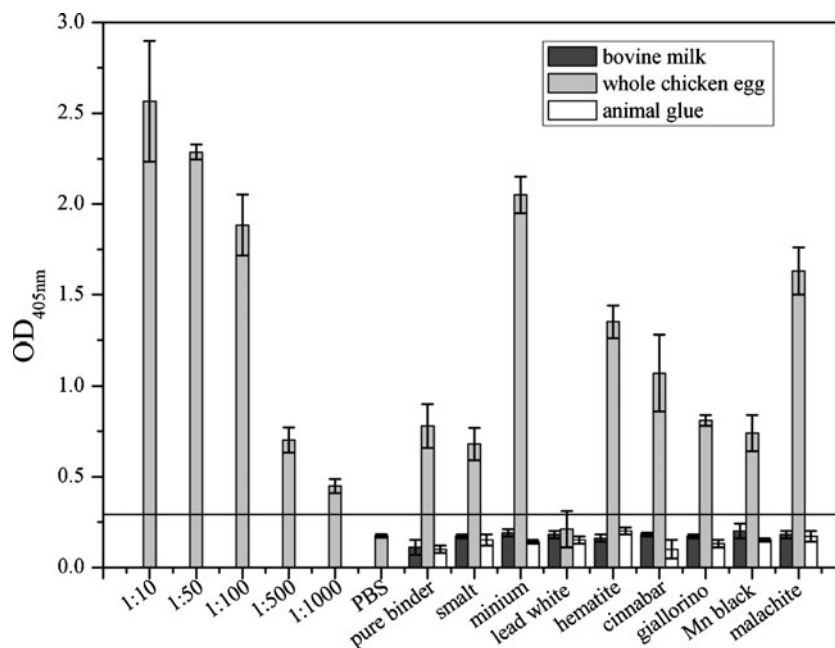


Fig. 5 ELISA optical densities obtained for the artificially aged easel painting samples treated with chicken ovalbumin Mab. Results for bovine milk (*dark gray*), whole chicken egg (*light gray*) and animal glue (*white*) and the corresponding SD are reported for the pure aged binders and the tested pigments (showed on the *X* axis). The chicken ovalbumin ELISA calibration curve is also reported



happen according to two main reasons: decrease of the binder solubility and reduced availability of the antibody bonding sites due to protein degradation.

The obtained results (Figs. 4 and 5) evidenced the need of increasing ELISA sensitivity to detect β -casein in the aged samples; conversely, the analytical response of ovalbumin remained unchanged for un-aged and aged ones, but showing a general variability of the OD_{405nm} values as a function of the different pigments.

Attempts of improving β -casein extraction by using different elution buffers and by prolonging sonication time were unsuccessful. This suggests that β -casein is probably affected by degradation processes that involve the epitope regions used for immuno-detection; furthermore the higher response obtained for the aged pure binder with respect to the pigmented samples points to a catalytic role of the pigments in protein decay.

In the case of ovalbumin, contrary to β -casein, the adopted primary MAb has been produced specifically for denatured proteins, thus explaining the good immunochemical response observed for the aged binder. However, one false negative was obtained for lead white. This can be explained, probably, by a different availability of the protein epitopes to the antibody due to a remarkable degradation of the site catalyzed by the pigment. It is worth to note that the model painting layers were prepared using whole egg which contains a significant amount of lipids. It is demonstrated that metal salts in egg-tempera paint lead to metal-catalyzed oxidation of unsaturated lipids [32] that was clearly observed in presence of lead white and azurite [33]. The

formed oxidation products easily react with the egg proteinaceous fraction, thus conditioning the chemistry of the binder in response to environmental conditions. This effect is expected to vary with pigments [14] and could particularly affect ELISA response observed for the egg-tempera painting samples. In order to study this issue, further investigations are needed (a) using different antibodies to investigate degradation process of various epitopes in the same protein after aging (b) considering different aging protocols for more pigment/binder combinations, and (c) performing a detailed characterization of the aged samples by exploitation of other analytical techniques to study protein chemical modifications.

Real samples

The ELISA protocols optimized for the identification of bovine β -casein and chicken ovalbumin in the aged laboratory models have been applied for the research of proteinaceous binders in the ancient painting samples. The study was undertaken after preliminary measurements by non-destructive fiber optic reflectance FTIR spectroscopy [21, 34] that clearly evidenced the generic presence of proteins on the mural painting fragments (data not shown). Elemental compositions of the pigments and of the carbonated substrate were characterized by XRF and results showed the presence of earth- or copper-based pigments.

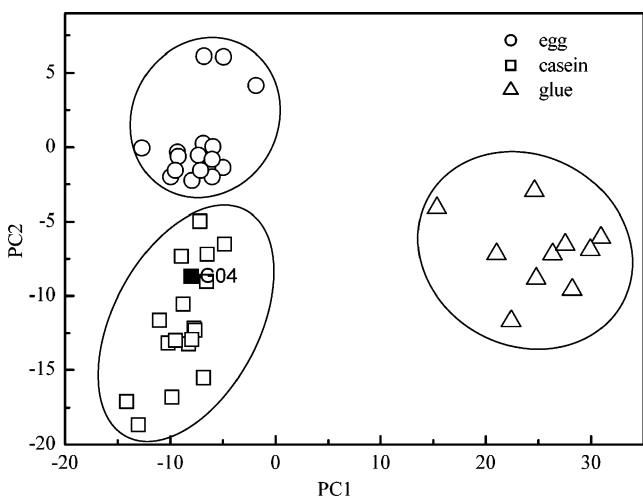
XRF and ELISA results are summarized in Table 5. ELISA measurements found samples GA04 and CA13 positive to β -casein and sample CA19 positive to chicken ovalbumin; this

Table 5 XRF elemental compositions of the ancient painting samples (minor elements are reported in parentheses) and corresponding optical densities by ELISA

Sample	Color	Elements	OD _{405nm} ±SD	
			β-casein MAb	Ovalbumin MAb
Substrate	–	Ca, Fe (K, Ti, Mn, Sr)	–	–
CA13	Green	Cu, Ca Fe (As, Sr)	0.40±0.05	0.13±0.04
CA19	Red	Ca, Fe (S, Cl, Ti, Zn, Sr)	0.15±0.05	0.5±0.1
GA04	Dark brown	Ca, Fe (S, K,Ti, Mn, Sr)	0.5±0.1	0.16±0.02

suggests that both milk/casein and egg were used as the binders. Analogous conclusions have been achieved from the analysis of similar samples, collected from fragments of the same collection that have been studied by proteomic techniques [15]. Unfortunately, the uncertain provenance of the fragments, not representative of the whole painting surface, prevented us from inferring any art-historical conclusion on the stylistic choices of the artists.

GC-MS analysis was used to support ELISA results obtained for the historical samples. The chromatographic analysis of the samples CA13 and CA19 was unable to find any organic binder probably because of the strong analytical interference of the inorganic matrix [14] combined with a lower sensitivity of the technique with respect to ELISA. Only sample GA04 showed the presence of a proteinaceous component that, in agreement with ELISA results, has been identified to be casein by principal component analysis (PCA) of the amino acids weight percentage composition [14]. PCA results are shown in Fig. 6 where the score plot obtained for the first two principal components is reported. The multivariate analysis was carried out by using as reference data set about 30 standard samples made with three of the main proteinaceous media: egg, glue, and casein. GC-MS analysis of the

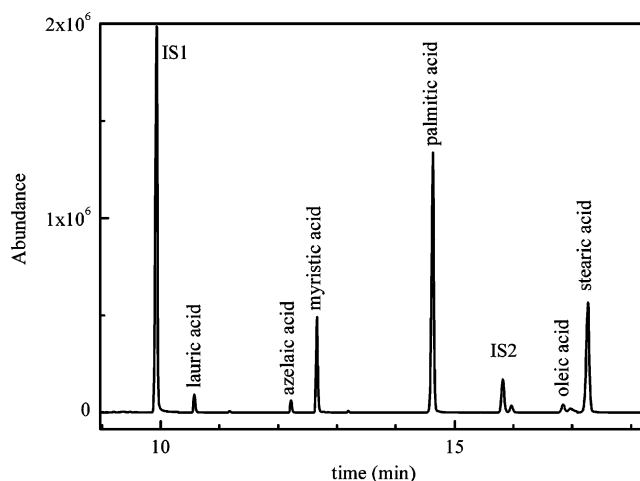
**Fig. 6** PCA score plot of the amino acid relative wt.% content of sample GA04. The first two principal components, PC1 and PC2, account for 82% of the variance of the data

sample GA04 also evidenced the presence of a lipid component by the detection of fatty acids in the chromatogram of the organic fraction of the sample extract shown in Fig. 7. The fatty acid compositional profile shows a minor relative concentration of di-carboxylic fatty acids compared to the amount of mono-carboxylic fatty acids (about 90 wt. % of the total acidic compounds). This result rules out the presence of a siccative oil while it is consistent with the possible use of milk as the binder [13].

Conclusions

The use of immuno-detection in conservation science is not new but it has been investigated only sporadically in this field, although the analytical potentials of the method are widely exploited in clinical- and bio-chemical research.

In the present work, the application of ELISA assays to the investigation of ancient proteinaceous binders has been evaluated and positively experimented on laboratory models of easel and mural paintings as well as on ancient painting samples. More in details, ELISA protocols for the recognition of bovine β-casein and chicken ovalbumin as markers of bovine milk (or casein) and chicken albumen, respectively, have been systematically tested and optimized.

**Fig. 7** Total ion chromatogram of the joint neutral and acidic fractions of the organic extract from the GA04 sample

The method works properly on fresh painting samples without interferences from the most common pigments and substrates (carbonated plaster or gypsum–glue preparation). Artificial aging of the laboratory mock-ups showed a partial decrease of the method sensitivity ascribable to a reduction of binder solubility and/or to a minor availability of the antibody bonding sites occurred with protein degradation. Our results also suggest that the use of different pigments modifies immunochemical reactivity of the binder during aging but no conclusion on the possible occurring degradation mechanisms could be inferred. The use of multiple monoclonal antibodies for different epitopes of the same protein would help to evaluate differences in epitope degradation rates and to have a new insight into protein decay processes in painting materials.

The developed protocols have been tested on historical mural painting samples from thirteenth century and results have been compared with those obtained by GC-MS analysis. Chromatographic investigations were limited by analytical interferences from the prevailing sample inorganic matrix and by materials degradation occurred with aging. A proteinaceous binder has been identified only in one sample and confirmed recognition of casein obtained by ELISA.

In the light of the obtained results, we propose ELISA as a valuable diagnostic tool for routine laboratory analysis of proteins in artistic materials. To this purpose, the analytical methodology needs much more extensive experimentation in order (a) to approach the analysis of a larger number of proteins (research for immuno-detection of glue by ELISA is, currently, in progress); (b) to evaluate the best choice of antibodies in consideration of sample degradation/alteration phenomena occurring with aging; (c) to extend the methodology also to the recognition of other natural biopolymers used in art materials as for example vegetable gums [10]. This latter task is reasonably accomplishable in consideration of the current widespread diffusion of immunological techniques in many scientific disciplines that has made commercial antibodies ready for applications in conservation science. Furthermore, manufactured antibodies can be produced on custom demand to respond to specific needs.

Limits of ELISA are its micro-destructivity, the fact that it allows only for sample bulk analysis and antigen recognition is restricted only to those antigens that the antibody is raised against. On the other hand, ELISA exhibits unquestionable analytical values: it provides fast results, requires minimal sample manipulation, and is cost effective. It is highly sensitive to small amounts of the antigen allowing for the analysis of ancient degraded micro-samples. A single sample extract can be divided into multiple aliquots and distributed among plates with more wells in order to be tested with different antibodies for multiple protein recognition. ELISA is, in fact, character-

ized by high specificity that allows for resolving complex mixture of proteins distinguishing their biological source. Such information could not be achieved by conventional chromatographic techniques otherwise. Only proteomics [15–18] equals specificity of immuno-detection but at the expense of analytical times and costs.

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References

1. Wilson K, Walker JM (1994) Principles and techniques of practical biochemistry, 4th edn. Cambridge University Press, Cambridge
2. Scott DA, Newman M, Schilling M, Derrick MR, Khanjian HP (1996) *Archaeometry* 38:695–705
3. Cattaneo C, Gelsthorpe K, Phillips P, Sokol RJ (1995) *J Archaeol Sci* 22:271–276
4. Kockaert L, Gausset P, Dubi-Rucquoy M (1989) *Stud Conserv* 34:183–188
5. Ramirez-Barat B, de la Viña S (2001) *Stud Conserv* 46:282–288
6. Heginbotham A, Millay V, Quick M (2006) *J Am Inst Cons* 45:89–106
7. Vagnini M, Pitzurra L, Cartechini L, Miliani C, Brunetti BG, Sgamellotti A (2008) *Anal Bioanal Chem* 392:57–64
8. Cartechini L, Vagnini M, Palmieri M, Pitzurra L, Mello T, Mazurek J, Chiari G (2010) *Acc Chem Res* 43:867–876
9. Scott DA, Warmländer S, Mazurek J, Quirke S (2009) *J Archaeol Sci* 36:923–932
10. Mazurek J, Heginbotham A, Schilling M, Chiari G (2008) *ICOM Committee Conserv* 2:678–685
11. Dolci LS, Scitutto G, Guardigli M, Rizzoli M, Prati S, Mazzeo R, Roda A (2008) *Anal Bioanal Chem* 392:29–35
12. Cennini C (XIV sec.) (1982) *Il Libro dell'Arte*. Neri Pozza Ed., Vicenza
13. Mills JS, White R (1994) *The organic chemistry of museum objects*, 2nd edn. Butterworths-Heinemann, London
14. Colombini MP, Modugno F (2004) *J Sep Sci* 27:147–160
15. Leo G, Cartechini L, Pucci P, Sgamellotti A, Marino G, Birolo L (2009) *Anal Bioanal Chem* 395:2269–2280
16. Kuckova S, Hynek R, Kodicek M (2009) MALDI-MS applied to the analysis of protein paint binders. In: Colombini MP, Modugno F (eds) *Organic mass spectrometry in art and archaeology*, Chapter 6. Wiley, UK
17. Tokarski C, Martin E, Rolando C, Cren-Olivè C (2006) *Anal Chem* 78:1494–1502
18. Solazzo C, Fitzhugh WW, Rolando C, Tokarski C (2008) *Anal Chem* 80:4590–4597
19. Nelson DL, Cox MM (2000) *Lehninger principles of biochemistry*, 3rd edn. Worth, New York
20. Aguita G, Martin R, Garcia T, Morales P, Haza A, Gonzalez I, Sanz B, Hernandez PE (1995) *J Dairy Res* 62:655–659
21. Miliani C, Rosi F, Brunetti BG, Sgamellotti A (2010) *Acc Chem Res* 43:728–738
22. Vagnini M, Miliani C, Cartechini L, Rocchi P, Brunetti BG, Sgamellotti A (2009) *Anal Bioanal Chem* 395:2107–2118
23. Bonaduce I, Cito M, Colombini MP (2009) *J Chromatogr A* 1216:5931–5939

24. Lluveras A, Bonaduce I, Andreotti A, Colombini MP (2010) *Anal Chem* 82:376–386
25. Karpowicz A (1981) *Stud Conserv* 26:153–160
26. van Boekel MAJS (1999) *Int Dairy J* 9:237–241
27. Lai MC, Topp EM (1999) *J Pharm Sci* 88:489–500
28. Stadtman ER (2006) *Free Radic Res* 40:1250–1258
29. Garrison WM (1987) *Chem Rev* 87:381–398
30. Kato Y, Uchida K, Kawakishi S (1992) *J Agric Food Chem* 40:373–379
31. Grant KB, Kassai M (2006) *Curr Org Chem* 10:1035–1049
32. Boon JJ, Peulvé SL, van den Brink OF, Duursma MC, Rainford D (1997) In early Italian painting technique. In: Bakkenist T, Hoppenbrouwers R, Dubois H (eds) *Limburg Conservation Institute: Maastricht*, pp 32–47
33. van den Brink OF, Boon JJ, O'Connor PB, Duursma MC, Heeren RMA (2001) *J Mass Spectrom* 36:479–492
34. Miliani C, Rosi F, Borgia I, Benedetti P, Brunetti BG, Sgamellotti A (2007) *Appl Spectrosc* 61:293–299