

Identification of animal glue and hen-egg yolk in paintings by use of enzyme-linked immunosorbent assay (ELISA)

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Abstract We report the development of an indirect ELISA procedure for specific identification of chicken-egg yolk and animal glues in painting micro-samples. The results presented integrate previously published work on ELISA recognition of bovine β -casein and chicken ovalbumin in painting materials. The integrated final ELISA procedure—optimised for protein extraction, immuno-reagent concentrations, blocking solution, incubation time, and temperature—enables multiplex identification, in single samples, of proteinaceous materials, i.e. chicken-egg yolk and albumen, animal glues, and bovine milk and/or casein, mainly used by painters in the past. The procedure has been systematically tested on laboratory models of mural and easel paintings, both naturally and artificially aged, to assess possible inhibitory effects on the immuno-reaction caused by inorganic painting materials (pigments and substrates) and by protein degradation resulting from aging processes. Real samples from case studies, which had previously been investigated and characterised by spectroscopy and chromatography, were successfully studied by use of the developed ELISA procedure. The commercial availability

of all the immuno-reagents used, the affordable analytical equipment, and the specificity, sensitivity, and rapidity of ELISA make this method very attractive to diagnostic laboratories in the field of cultural heritage science. Possible further developments to the analytical potential of this technique include improvement of antibody performance and inclusion of other classes of bio-molecules as analytical targets.

Keywords ELISA · Paintings · Proteinaceous binder · Animal glue · Egg yolk

Introduction

Challenges to analytical investigation of organic components in painting materials [1] are necessary for the optimisation and integration of analytical methods already established in this field [1–6], as well as for the experimentation with new diagnostic approaches developed in other branches of science [2, 7–9].

In recent years immunological techniques have attracted increased attention from those working in cultural heritage, because they have the potential to enable sensitive and specific identification and localisation of proteins in historic art materials [10–15]. These techniques exploit the high specificity of the interactions between antibodies and their related antigens which are at the basis of the immunoreponse of living organisms [16]. To increase the use of these techniques for investigation of binders and adhesives in paintings, ongoing scientific research includes:

1. testing antibodies' specificity for target antigens of different species origin [11, 17];
2. widening analysis use to include different classes of biomolecule (for example vegetable gums) [17, 18];
3. developing analytical methods to reduce false responses of immunoassays used with complex matrixes [19, 20]; and

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4. solving the challenging problem of multiplex imaging for simultaneous detection of proteins in painting stratigraphies [12, 21].

In previous work [20] we successfully used enzyme-linked immunosorbent assay (ELISA) to identify bovine β -casein and chicken-egg albumin, both on laboratory painting models and on samples from historical paintings. ELISA has the advantages of being affordable, relatively simple, and fast, with limited sample manipulation, and is capable of multiple antigen recognition. These features make ELISA a promising technique for routine analysis of proteinaceous materials and biopolymers in artwork-conservation laboratories.

In this study we report extension of the ELISA procedure, previously used to identify bovine β -casein and chicken-egg albumin [20], to identification of chicken-egg yolk and animal glues. The literature discussing detection of collagen in painting materials by use of ELISA is sparse [11, 13, 14, 22–24], and immunological detection of egg yolk has, as far as we are aware, never been reported.

The objective of the research is to provide an effective tool for full detection of the most widely used proteinaceous materials in historical artworks [1], i.e. animal glues, egg (distinguishing yolk and albumen), and milk (or its by-product casein). This is achieved by dividing each sample extract into aliquots, enabling testing for different antibodies and thus recognition of more proteins.

To guarantee greater sensitivity and specificity an indirect approach was chosen in which the primary antibody (or capture antibody) is targeted by enzyme-conjugated secondary antibodies specific to the primary antibody class [11, 16, 20]. The standard ELISA method, processed in solution by use of micro-plates, was preferred to the simpler and faster dot-blot methods [15, 25], which are solid-phase immunoassays. In these methods protein detection is achieved by means of colorimetric measurements in reflectance rather than in transmittance [15] or by densitometric analysis by image processing [25]; as a consequence, quantitative analysis is less reliable.

Because of their increased specificity, monoclonal primary antibodies are generally preferable; unfortunately, for animal glues only polyclonal antibodies were found to be commercially available and hence only these could be used in this research. This increased the probability of antibody cross-reactions with collagen from different species [16]. Because this structural protein from different biological sources is very similar, and because of the degradation processes that can alter the chemical and physical properties of the molecule, species identification of ancient collagen is generally a very challenging task [23, 26, 27]. With the exception of immunological studies using in-house production of antibodies against collagen of different biological origins [23], only proteomics [28] and DNA analysis [29]

are currently known to be able to distinguish animal glue species in historical painting materials.

The proposed ELISA method has been tested on easel and mural-painting replicas that have undergone natural and artificial aging. The procedure was then used to analyse two micro-samples from historical paintings. The results obtained have been cross-validated by use of mid-FTIR spectroscopy and gas chromatography–mass spectrometry.

Experimental

Reagents

Primary antibodies investigated for use in detection of collagen and chicken-egg yolk were, respectively, rabbit polyclonal antibody to type I collagen (Rockland/BioTrend Chemikalien, Germany) and mouse monoclonal antibody to chicken-egg yolk immunoglobulin (IgY) (BioTrend Chemikalien).

Goat anti-mouse IgG (whole molecule)-conjugated enzyme alkaline phosphatase was used as the secondary antibody. Immuno-complex colorimetric detection was performed by use of enzymatic reaction, with *para*-nitrophenyl phosphate (p-NPP) present as the enzyme substrate. Sample absorbance was measured at $\lambda=405$ nm, and is expressed as optical density (OD_{405nm}). Both the secondary antibody and p-NPP were purchased from Sigma–Aldrich. Antibodies were diluted in phosphate-buffered saline solution (PBS, 150 mmol L⁻¹ NaCl, 5.2 mmol L⁻¹ Na₂HPO₄, 1.7 mmol L⁻¹ KH₂PO₄, pH 7.4, 0.2 % Tween 20). PBS was also used for washing after each step of the ELISA procedure. Standard solutions of reference proteins for calibration curves were prepared from 1 mg dried egg yolk (Sigma–Aldrich) and dried bovine (Kremer, Aichstetten, Germany) and rabbit (Bresciani, Milano, Italy) glues in 1 mL carbonate–bicarbonate buffer at pH 9.6. Goat serum 0.1 % in PBS (Sigma–Aldrich) was used as blocking solution.

Paint models

Paint models were prepared by applying single paint layers to two different supports: primed wood panels and dried carbonated plaster. The wood supports were coated with a ground mixture of gypsum and animal glue of unknown composition (Zecchi, Florence, Italy). Dried plaster was obtained by carbonation of a mixture of lime and sand in a weight ratio of approximately 1:3. Fresh hen's egg (whole egg and yolk), whole bovine milk (both purchased at the local market), and bovine (Kremer) and rabbit (Bresciani) glues were used as the binders. The following binders were prepared in accordance with old recipes:

- 3 g whole egg+2 g deionised water+0.5 g vinegar
- 2 g yolk+3 g deionised water+0.5 g vinegar

- 10 g bovine milk+1 drop NH_3 2.5 mol L^{-1}
- bovine glue+deionised water (heated at 40 °C until dissolution)
- rabbit glue+deionised water (heated at 40 °C until dissolution)

A pigment-to-binder weight ratio of approximately 3:1 was used to obtain a mixture that could be applied as paint.

The pigments zinc white (ZnO), lead white ($2\text{PbCO}_3\cdot\text{Pb}(\text{OH})_2$), giallorino ($\text{Pb}_3(\text{SnO}_4)$), malachite ($\text{CuCO}_3\cdot\text{Cu}(\text{OH})_2$), smalt (K–Co–Al silicates), azurite ($2\text{CuCO}_3\cdot\text{Cu}(\text{OH})_2$), lapis lazuli ($\text{Na}_8(10\text{Al}_6\text{Si}_6\text{O}_2)4\text{S}_2$), hematite (Fe_2O_3), cinnabar (HgS), minium (Pb_3O_4), verdaccio (Fe–Mg–K aluminosilicates), umber (Fe–Mn–Al oxides), and Mn black (MnO_2) were selected for this study. All pigments were purchased from Zecchi except lead white and minium, which were supplied by Sigma–Aldrich.

Before analysis, painting samples were naturally aged for five years for whole egg (13 samples), two years for animal glue (26 samples), and at least six months for egg yolk (13 samples). Accelerated aging was performed on a limited number of easel-painting models (made with lead white, giallorino, malachite, smalt, minium, cinnabar, hematite, and Mn black) by means of exposure to 85 % RH at 45 °C for three months. The pure binders applied to the prepared wood support also underwent this aging procedure.

ELISA procedure

Sample proteins were extracted and analysed by use of the procedure optimised in our previous research [16]. Briefly, a painting micro-sample (approx. 1 mg) is carefully scraped from the painting surface by use of a scalpel, avoiding removal of material from the substrate underneath (preparation layer or carbonated plaster). PBS (300 μL) is then added to the painting micro-sample in a 1.5-mL Eppendorf tube, and proteins are extracted by sonication for 4 h followed by incubation at room temperature for 48 h. The extraction solution (70 μL) is placed in an ELISA polycarbonate microtiter well plate (Cellstar, Greiner bio-one), and incubated for 1 h at 37 °C to enable proteins to bind to the plastic surface. Blocking solution (100 μL) is then added to the wells and incubation is performed for 30 min at 37 °C. Binding of the first and the second antibodies is performed sequentially, by incubating 100 μL antibody solution for 1 h at 37 °C. Enzyme–substrate reaction is then performed by adding 100 μL colourless p-NPP to the wells and incubating for 20 min at room temperature (25 °C) in a closed, air-conditioned, and dark environment. The chromogenic enzymatic reaction is stopped by adding 50 μL 2 mol L^{-1} NaOH. Finally, the $\text{OD}_{405\text{nm}}$ of the ELISA plate is read by use of a spectrophotometer (GDV programmable MPT reader mod. DV 990BV6). Between each step, the wells are thoroughly rinsed at least five times with 200 μL PBS.

Each analysis was repeated at least three times for all samples, and the $\text{OD}_{405\text{nm}}$ readings are reported with the corresponding standard deviations (SD).

Results and discussion

Laboratory standards

Investigation of the ELISA procedure for recognition of animal glues and chicken-egg yolk began with antibody panel titration of standard solutions of the binders, using PBS as control. This enabled us to establish optimum dilutions for the primary and secondary antibody solutions, at which immunoassay sensitivity was maximised and background minimised. Primary antibody specificity was tested by checking cross-reactivity with animal glue, chicken albumen, and bovine milk. Replicate blank measurements enabled us to fix the detection limits (expressed as the blank $\text{OD}_{405\text{nm}}$ average plus three times the corresponding standard deviation) at $\text{OD}_{405\text{nm}}=0.30$ for animal glues and $\text{OD}_{405\text{nm}}=0.35$ for egg yolk. Calibration curves were then constructed for the optimum antibody dilutions (Fig. 1). Immunodetection of bovine and rabbit glues was performed for the optimised primary and secondary antibody dilutions of 1:250 and 1:500, respectively. At these antibody concentrations sensitivity of immunoassays was similar for the two species (Fig. 1 calibration curves). This is the result of the high sequence homology of collagen from different biological sources, and removes any possibility of discrimination between them. However, this lack of discrimination does increase the likelihood of successful collagen detection in real samples. In fact, historical animal glue preparations used several sources of collagen; therefore the final product was often a mixture of materials of different biological origin.

Optimum antibody dilutions for chicken-egg yolk were 1:50 and 1:500 for the primary and the secondary antibodies, respectively. The optimised antibody solutions were successfully checked for absence of cross-reactions of the primary antibody with yolk from other poultry species (Fig. 1). For this binder a reduction in OD response was observed as the primary antibody aged after thawing, despite the fact that the antibody was always correctly stored at 4 °C. To overcome this problem micro-aliquots of the original antibody solution were stored separately at -80 °C, so that one aliquot could be thawed at a time for immediate use.

Comparison of the calibration curves for collagen and chicken-egg yolk (Fig. 1) shows the response of the immunoassay for the two binders is very different: the detection limit is reached at maximum binder dilutions of 1:20,000 for collagen and of 1:100 for egg yolk. This is because of an unfavourable weight ratio of detected antigen (IgY immunoglobulins) to total egg yolk (less than 3 % w/w of the

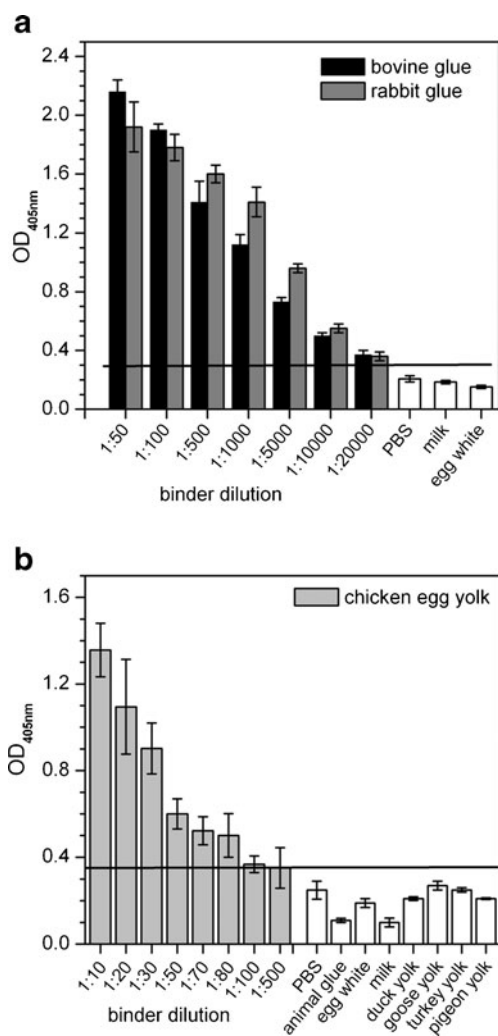


Fig. 1 ELISA calibration curves obtained for (a) bovine (black) and rabbit (grey) glues and (b) chicken-egg yolk (light grey) at the optimised primary and secondary antibody dilutions of 1:250 and 1:500 for (a) and of 1:50 and 1:500 for (b). Standard solutions of the reference proteins were prepared by progressive dilution of 1 mg dried binder in 1 mL carbonate-bicarbonate buffer at pH 9.6. OD_{405nm} responses for PBS (blank control) and negative controls are also shown (white)

dried binder [30]). The estimated minimum amount of dried binder detectable by use of the developed method is 5 ng for animal glue and 1 µg for egg yolk.

The optimised ELISA procedure has been tested on samples from laboratory paint models (see “Experimental” section). The large number of assays performed for different combinations of binders and pigments enabled us to investigate possible inhibitory effects of inorganic painting materials on immunological reagent function. Because of the poor antigen detection limit for egg yolk, these ELISA assays were performed on painting layers which used only yolk as binder.

ELISA results for the naturally aged easel and mural-painting models are reported in Figs. 2 and 3 for animal glue

and egg yolk, respectively. The results support the following conclusions:

1. no interference from interactions of the system with the inorganic components is observed;
2. average optical densities are similar for the same binder in the presence of different pigments and supports; variations in OD intensity may be related to the heterogeneous microscale composition of the painting layer, and to the small size of the samples analysed;
3. the antibody to rabbit and bovine type 1 collagen is not specific for the two species; and
4. the antibody to egg yolk is effective in the presence of a prevailing inorganic matrix, despite the unfavourable antigen concentration.

Analysis of the easel-painting models after accelerated aging validated the optimised ELISA procedure. Results are reported in Fig. 4. The experiment’s purpose was to investigate possible adverse effects of protein degradation on the immunoassay results. It has been established that modifications of proteinaceous binders during aging can be quite complex, because of interactions with light, oxygen,

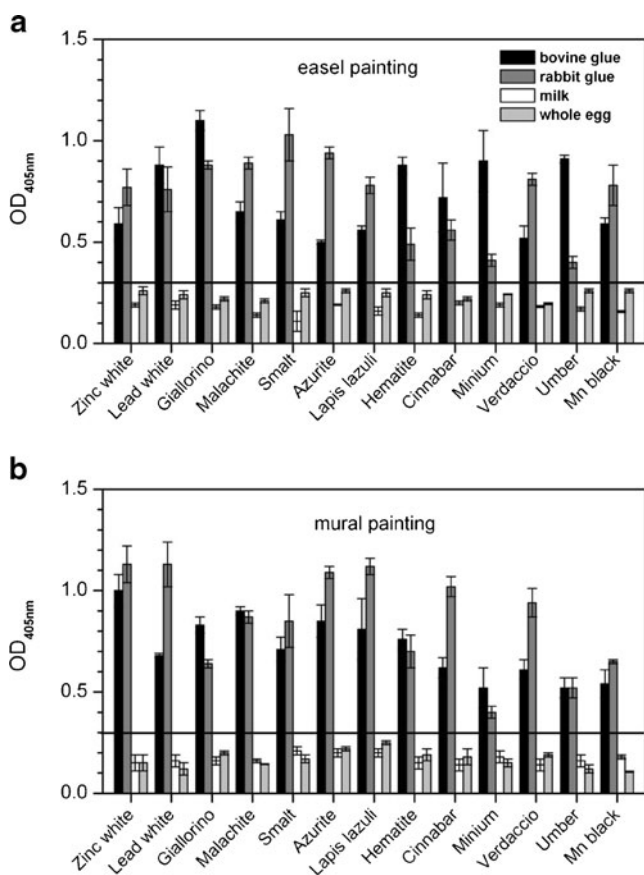


Fig. 2 ELISA optical densities obtained for (a) easel and (b) mural-painting samples treated with polyclonal antibody to type I collagen. Results for different binders and the corresponding SD are reported for the tested pigments shown on the x-axis

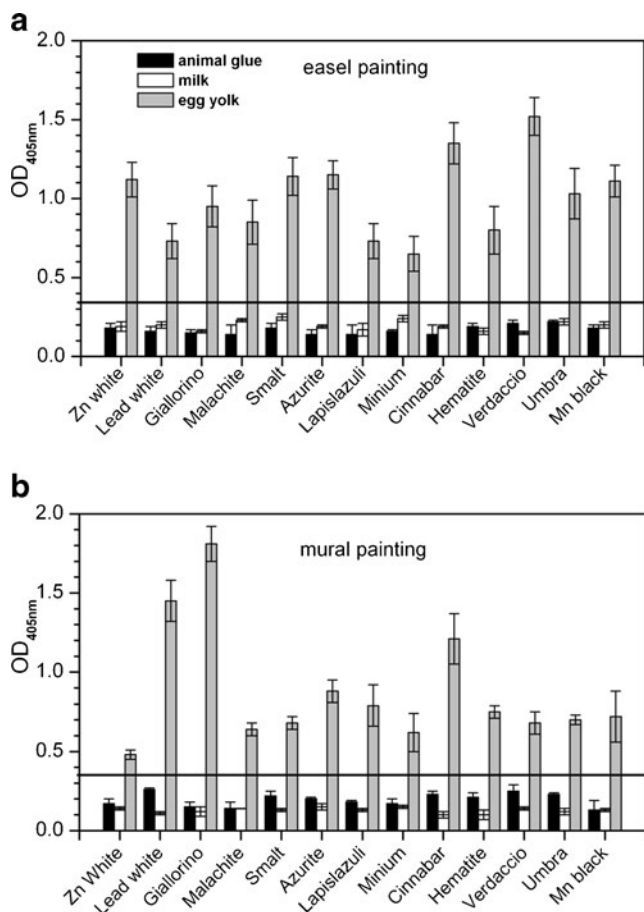


Fig. 3 ELISA optical densities obtained for (a) easel and (b) mural-painting samples treated with monoclonal antibody to chicken-egg yolk IgY immunoglobulins. Results for different binders and the corresponding SD are reported for the tested pigments shown on the x-axis

humidity, metal cations, and other organic components possibly present in the painting layer. Hydrolysis, aggregation, and oxidation reactions may occur [31–33], leading to reduced protein solubility and loss of active epitopes [34]. However, the results in Fig. 4 show that the response of the aged samples remained, on average, comparable with that of the unaged ones. A slight reduction in OD can be observed for both binders, but an exact estimate of the effect of aging on ELISA response is complicated by the microscopic compositional variability of the samples and by the different granulometry of the pigments, which leads to local variations of the pigment-to-binder ratio.

Further investigation is needed to evaluate the effect of protein degradation induced by light exposure and pH on the ELISA results with regard to preservation of the active epitopes. UV light is not present in light exposure under indoor conditions, but has been occasionally used for accelerated aging of proteinaceous binders. Recent analytical work, based on complementary use of MALDI-TOF-MS with PCA statistical data analysis and mid-infrared spectroscopy, found evidence of the effect of a pigment on the extent of UV-light-induced conformational

changes to the secondary structure of the collagen binder [35]. However, GC-MS investigations found that the compositional profile of stable amino acids used in multivariate analysis of GC-MS data remained substantially unchanged after UV exposure [36]. UV-filtered light is more commonly used in procedures for aging painting materials, often in combination with other environmental variables (RH and T) [31, 33, 37, 38]. Such procedures have been used to study aging by use of different analytical techniques, all of which agree on the effect of pigments on aging. For the painting technique called “mezzo fresco”, in which pigments are applied, with a binder, to a partially carbonated plaster support, the effect of pH can be important. A recent combined ELISA and ultra-performance liquid chromatography study found evidence of severe degradation of ovalbumin resulting from the alkaline environment [15].

Real samples

Two micro-samples (each approximately 1 mg) from historical artworks were tested for ELISA recognition of bovine

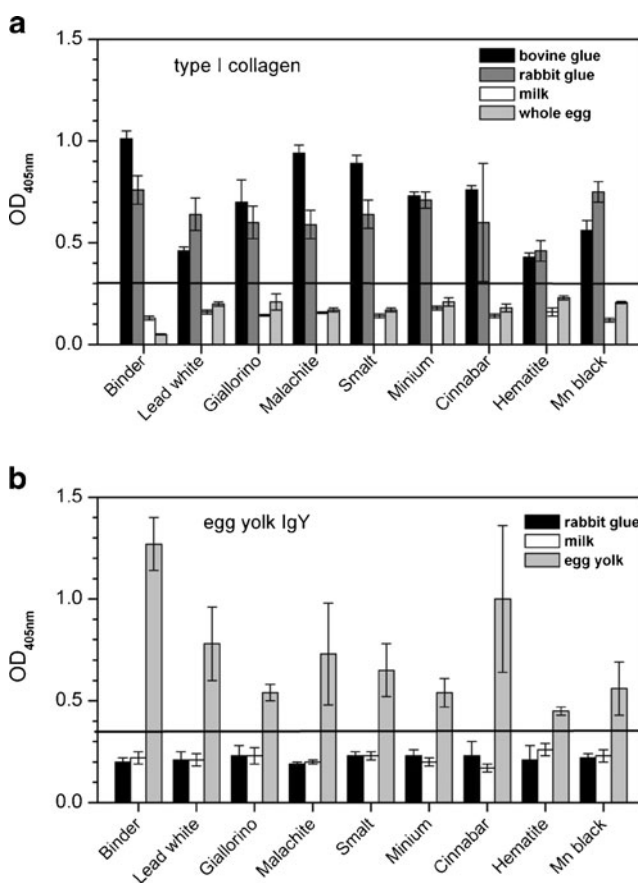


Fig. 4 ELISA optical densities obtained for samples from artificially aged easel-painting models treated with (a) antibody to type I collagen and (b) antibody to chicken-egg yolk IgY immunoglobulins. Results for different binders and the corresponding SD are reported for the tested pigments shown on the x-axis

Table 1 ELISA optical densities for historical painting samples treated with different antibodies. Sample A is from “*Pala Albergotti*” by G. Vasari and sample B is from “*Polittico di S’Angelo*” by N. Liberatore. The detection limit was fixed at $OD_{405nm}=0.35$ for chicken-egg yolk and at $OD_{405nm}=0.30$ for animal glues, chicken egg albumen, and bovine β -casein

Sample	$OD_{405nm} \pm SD$			
	Collagen type 1	Egg yolk IgY	β -casein	Ovalbumin
A	0.78 ± 0.03	n.t.	0.22 ± 0.03	0.20 ± 0.02
B	n.t.	0.44 ± 0.02	0.26 ± 0.02	0.20 ± 0.08

n.t.= not determined (see “[Results and discussion](#)”)

β -casein, animal glue, chicken-egg yolk, and ovalbumin, by use of analytical procedures developed in our laboratory. The first micro-sample (sample A) belonged to the preparation layer of the XVI century “*Pala Albergotti*” by G. Vasari, preserved in the “*Badia delle Sante Flora e Lucilla*” in Arezzo. The immunological assay was performed after preliminary measurements by use of non-destructive fibre-optic reflectance FTIR spectroscopy, which clearly showed the generic presence of proteins on the painting and the absence of a lipid component [39]. This result was confirmed by GC–MS analysis of micro-samples, which also established the presence of glue [39].

The second micro-sample (sample B) was from “*Polittico di S’Angelo*” (1499) by the Umbrian painter Niccolò Liberatore, called “*l’Alunno*”. Before restoration, this painting was the subject of an extensive investigation that used complementary analytical techniques (X-ray fluorescence spectroscopy, micro-FTIR spectroscopy, scanning electron microscopy, EDX micro-analysis, GC–MS, and X-ray absorption spectroscopy) with the purpose of identifying painting materials and evaluating their state of preservation [40]. The spectroscopy results showed that the binder used was “tempera”; both proteinaceous and lipid fractions were detected, the latter being shown, by use of chromatography, to have a fatty acid profile different from that of a siccative oil (ratio of azelaic acid to palmitic acid was <0.3).

On the basis of results from non-invasive mid-FTIR spectroscopy and GC–MS analysis, sample A was tested by use of ELISA for animal glue and other proteinaceous binders not containing lipids, i.e. casein and egg albumen. Sample B was tested for bovine milk, chicken-egg yolk (both having a lipid component), and ovalbumin; the latter was tested to distinguish whole egg from egg yolk. This enabled us to reduce the number of sample aliquots to be tested with different antibodies, increasing the concentration of proteins for each aliquot and thus increasing the chance of their successful detection.

Table 1 shows the ELISA results for the historical samples. In the ELISA analysis of sample A, animal glue is

clearly identified, and the presence of bovine casein and of hen albumen can be excluded. For sample B, ELISA results show the presence of egg yolk but not of albumen, and milk (the other binder possibly consistent with the GC–MS and mid-FTIR findings) was not detected.

Conclusions

This study focused on selective identification, by use of ELISA, of chicken-egg yolk and animal collagen in painting micro-samples. The work is the extension of a previous method (sample preparation and immunology procedure) developed for identification of bovine β -casein and chicken albumin [20] in painting materials. Integration of these analytical results with those reported previously provides a fast and simple analytical tool for specific and sensitive multiplex-detection of the four proteinaceous materials most used by painters in the past: chicken egg (distinguishing yolk from albumen), bovine milk, and animal glues. These materials were commonly used as binding media, and as adhesives and protective coatings. Conservation scientists therefore frequently encounter their simultaneous presence in painting layers. Analytical identification of proteins in painting materials is a very challenging task because of:

1. the complex chemistry of the painting matrix (organic components mixed with inorganic pigments and substrates at the micrometric scale);
2. alteration of the proteins which may have occurred; and
3. the limited amount of material available for laboratory analysis (usually micro-samples weighing <1 mg).

The proposed ELISA method has been shown to enable accurate detection of target proteins, both in tempera painting models, in the presence of different pigments and substrates, and in degraded pictorial materials which have undergone prolonged exposure to high RH at 45 °C. The final integrated procedure for identification of animal collagen, bovine β -casein, and chicken-egg yolk and albumen has been successfully used for investigation of historical samples, and results have been validated by spectroscopic and chromatographic analysis of the materials. A systematic investigation of ELISA multiplex detection of proteinaceous binders will be reported in a later publication.

Among the analytical techniques capable of providing detailed information on organic components in painting materials, ELISA is notable for its high sensitivity and specificity in multiple-protein identification, with the added advantage of being relatively fast and affordable. However, it is worth noting that ELISA is complementary to other analytical techniques, which also contribute to the ultimate objective of fully identifying organic substances in painting materials. The main disadvantage of the technique is its insensitivity to antigens

other than those recognised by the antibodies used. However, its specificity is helpful in improving our understanding of painting materials, both in terms of production techniques and for authentication purposes.

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