

# Analysis of egg-based model wall paintings by use of an innovative combined dot-ELISA and UPLC-based approach

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**Abstract** The chemical analysis of egg-based wall paintings—the *mezzo fresco* technique—is an interesting topic in the characterisation of organic binders. A revised procedure for a dot-enzyme-linked immunosorbent assay (dot-ELISA) able to detect protein components of egg-based wall paintings is reported. In the new dot-ELISA procedure we succeeded in maximizing the staining colour by adjusting the temperature during the staining reaction. Quantification of the colour intensity by visible reflectance spectroscopy

resulted in a straight line plot of protein concentration against reflectance in the wavelength range 380–780 nm. The modified dot-ELISA procedure is proposed as a semi-quantitative analytical method for characterisation of protein binders in egg-based paintings. To evaluate its performance, the method was first applied to standard samples (ovalbumin, whole egg, egg white), then to model specimens, and finally to real samples (Giotto's wall paintings). Moreover, amino acid analysis performed by innovative ultra-performance liquid

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chromatography was applied both to standards and to model samples and the results were compared with those from the dot-ELISA tests. In particular, after protein hydrolysis (24 h, 114 °C, 6 mol L<sup>-1</sup> HCl) of the samples, amino acid derivatization by use of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate enabled reproducible analysis of amino acids. This UPLC amino acid analysis was rapid and reproducible and was applied for the first time to egg-based paintings. Because the painting technique involved the use of egg-based tempera on fresh lime-based mortar, the study enabled investigation of the effect of the alkaline environment on egg-protein detection by both methods.

**Keywords** UPLC-based amino acid analysis · dot-ELISA · Egg-based wall paintings · Cultural heritage conservation

## Introduction

Many analytical techniques have been developed for characterisation of protein-based painting binders in cultural heritage conservation. As an example, spectroscopic techniques, for example Fourier-transform infrared (FTIR) spectroscopy [1, 2], near-infrared spectroscopy (NIRS) [3], and micro-Raman ( $\mu$ Raman) spectroscopy [4–6] give information about the presence of protein-based binders. Unfortunately these techniques suffer from severe interferences from other organic molecules present in the materials [2]. Moreover, depending on the techniques used for spectra collection and sample preparation (e.g. KBr pellet or diamond ATR device), several reactivity and/or reflection phenomena can often affect the final quality and resolution of the spectra.

In contrast, it is well known that chromatographic techniques, e.g., gas chromatography coupled to mass spectrometry (GC–MS) [7–15] and high-performance liquid chromatography coupled to fluorescence (HPLC–FD) [7, 16, 17] or UV–visible (HPLC–PDA) [18–20] detection are regarded as most effective analytical techniques for accurate characterisation of protein binders. In particular, identification of protein binders is based on the relative abundance of the protein, peptide, and amino acid components determined [16–19]. The main disadvantages of GC–MS techniques are long analysis times, volatilization of the sample, and preliminary extraction of the proteins, which can involve loss of material. Sometimes pyrolysis is used to fragment proteins into their amino acid components; these must then be derivatized, separated, and finally detected by GC–MS (Py–GC–MS). The disadvantage of this technique is the complexity of the pyrograms, which are very often extremely difficult to interpret. Nevertheless, in some cases the presence of characteristic fragments, which can be regarded as markers [21, 22], enables characterisation of specific protein components.

In this paper a liquid chromatographic technique, ultra performance liquid chromatography (UPLC) with UV detection coupled with pre-column derivatization with AccQ•Tag Ultra methodology is proposed. In comparison with conventional HPLC instruments, UPLC technology has the advantages of greater sensitivity and resolution, reduced analysis time and solvent consumption, because of the smaller diameter of the packing particles (1.7  $\mu$ m) and the high pressure in the columns [23]. Use of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), which reacts with primary and secondary amino acids to yield fluorescent derivatives readily detectable by UV-absorbance, fluorescence, electrochemical, and/or mass spectrometric detection, enabling detection of amino acids at sub-picomolar concentrations, overcomes many of the disadvantages of other derivatizing reagents, for example phenylisothiocyanate (PITC), which yields derivatives detectable by UV, and *o*-phthalaldehyde (OPA), dimethylaminonaphthalensulfonyl chloride (Dansyl-Cl), and 9-fluorenylmethyl chloroformate (FMOC), which yield fluorescent derivatives. Instead, AQC has many advantages: derivatives are formed rapidly, excess reagent is rapidly hydrolyzed ( $t_{1/2} < 15$  s) to yield 6-aminoquinoline, *N*-hydroxysuccinimide, and carbon dioxide, and after 1 min no further derivatization can occur (Electronic Supplementary Material, Fig. S1); moreover salts and detergents do not interfere with the reaction [24–26].

The purpose of this study was to emphasize the potential of two different techniques, UPLC and modified dot-ELISA, for analysis of protein-based painting binders. dot-ELISA is a dot immunoblotting assay that uses a highly specific immunological antigen–antibody reaction to detect the presence of the antigen, in which the antigen is adsorbed on the surface of a membrane. There has recently been renewed interest in immunological techniques for characterisation of protein binders. In particular, immuno-fluorescence microscopy (IFM) [27–30], immuno-chemiluminescence microscopy [31], and enzyme-linked immunosorbent assays (ELISA) [29, 30, 32, 33] have been used to obtain interesting results, mainly highly specific and sensitive identification of proteins in micro-samples of art and archaeological materials. Despite these recent results from application of immunoassay-based techniques to cultural heritage conservation, the literature is still lacking comparative studies between these immunochemical techniques and spectroscopic and chromatographic techniques.

In particular, to develop a method for semi-quantitative analysis by use of modified dot-ELISA tests, one purpose of this study was to achieve calibration by using different concentrations of pure ovalbumin, whole egg, and egg white as standard analytes. The dot-ELISA tests were then applied to both model samples and real samples from Giotto's wall paintings in the Santa Croce Basilica in Florence. The specificity of the method for yolk-based binders was also

investigated. Determination of colour staining was performed by use of a visible light spectrophotometer with integrating sphere on the dried solid support, with the objective of developing a rapid, easy to perform and interpret, relatively inexpensive technique requiring minimum sample handling.

UPLC analysis was performed on known concentrations of hydrolysates of ovalbumin, lyophilized egg white, and whole egg standards, and furnished amino acid profiles characteristic of the protein components. A comparative study of the experimental data obtained by analysis of lyophilized egg white, whole egg, and model standards with those obtained from pure ovalbumin is also reported. The main purpose of using modified dot-ELISA was to analyse the efficiency of the method and to study the benefits of this technique when applied to studies of cultural heritage. Finally, because the model samples of egg-tempera were prepared by painting on to not set lime-based mortars, the effect of the alkaline environment on the detection capability of the two techniques was also investigated.

## Materials and methods

### Binder samples

For both analytical techniques, standard ovalbumin (Sigma-Aldrich, USA, 98 % purity) was used. Egg white, yolk, and whole egg for standard samples were obtained from eggs acquired commercially; egg white and yolk were separated conventionally; egg white and whole egg were lyophilized. Model painting layer samples were prepared in accordance with traditional painting procedures. A layer of tempera prepared from different types of protein material and red ochre pigment was applied to a mortar surface (surface of a lime and sand, 1:3 v/v) one day after the mortar preparation, i.e. when the extent of drying and carbonation were both very low. The natural binders selected to prepare these samples were whole egg, milk, and rabbit glue, pure or mixed as whole egg–glue binder. The only pigment analysed was red ochre, to minimise the effects of interference from mineral pigments: indeed, red ochre is a mineral pigment with little effect on binder analysis [30]. On the other hand, it is known that inorganic pigments can affect dot-ELISA test results and a recent review [30] stated that “the different role played by different pigments is still not clear” [7, 9]. Trying other pigments would have inserted other complex variables making it difficult to study the effect of the alkaline environment of the *mezzo fresco* technique. The ratio of binding medium to pigment was 1:2 (v/v) for whole-egg tempera, 1:2 (v/v) for milk tempera (the milk used had previously been diluted 1:1 (v/v) with water), and 1:2 (v/v) for glue tempera (the glue was a sol of rabbit glue dispersed

in water 1:10 w/v). Indeed, the egg–glue tempera specimen was a double layer prepared by applying, first, egg tempera and then glue tempera. The decision to apply the tempera layers when the mortar surface was at the beginning of the setting process (the so-called *mezzo fresco* technique) was intentional; the purpose was to investigate how the painting technique could affect subsequent analysis of protein species, because of the high pH encountered by the binder when the painting layer was applied to the mortar surface. The red painted layer obtained was subjected to three years of natural aging in the laboratory atmosphere. No artificial aging was conducted, because the main purpose of this work, apart from combining the two techniques (dot-ELISA and UPLC), was to study the effect of the alkaline environment on the protein binders; to simplify the set of the conditions affecting the chemical behaviour of the protein binders, artificial aging was, therefore, not performed. Micro-samples only from the painting layers were collected by means of a small scalpel; the mass of powder taken from the mortar surface was in the range 1.5–2.0 mg, and it was finely ground before analysis.

Real samples were from Giotto’s wall painting “The Stigmata of St. Francis” in the Basilica of Santa Croce in Florence. The samples—only micro-fragments of painting layers—were collected by the conservators of the Opificio delle Pietre Dure, Florence, Italy. Sample A was taken from the upper frame region; sample B was taken in the proximity of the bottom-left part of the painting; sample C was from the vest of the Saint. All samples were less than 1 mg in mass.

### Modified dot-ELISA

Elution buffer was prepared by mixing 25 mmol L<sup>-1</sup> tris (hydroxymethyl)aminomethane (TRIS), 6 mol L<sup>-1</sup> urea, 191 mmol L<sup>-1</sup> glycine, and 868 mmol L<sup>-1</sup> sodium dodecyl-sulfate (SDS). Phosphate saline buffer (PBS) was prepared by mixing 137 mmol L<sup>-1</sup> NaCl, 2.7 mmol L<sup>-1</sup> KCl, 10 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, and 1.4 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>. Blocking–washing buffer (B buffer) was prepared by mixing 10 % (v/v) PBS, 90 % (v/v) H<sub>2</sub>O, 1 % (v/v) polyoxyethylene (20) sorbitan monolaurate (TWEEN 20), and 5 % (w/v) non-fat dry milk (Oxoid, UK). This was used as protein source for blocking non-specific reactions, as an incubation medium, and for subsequent washing to remove unreacted reagents [30]. The buffers were freshly prepared. All the reagents were analysis-grade (>99.5 % purity) and were purchased from Merck (Darmstadt, Germany). Colour development substrate in tablet FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium), anti-ovalbumin IgG (anti-chicken egg albumin, developed in rabbit), and anti-rabbit IgG alkaline phosphatase conjugate (developed in goat) antibodies were purchased from Sigma–Aldrich.

Methanol (GC purity >99.5 %) was supplied by Merck. Spectrophotometric measurements were obtained by use of a Perkin–Elmer Lambda 35 UV–visible spectrophotometer with double beam and double light sources (deuterium lamp for UV spectra and tungsten lamp for visible wavelengths), and equipped with an internal integration sphere for diffuse reflectance spectra. Analyses were performed in the range 780–380 nm.

#### Amino acid analysis to obtain characteristic protein fingerprints

AccQ•Tag Ultra derivatization kit (borate buffer and reagent), AccQ•Tag Ultra eluents A and B, HPLC-grade acetonitrile, and amino acid standard solution were purchased from Waters (USA). Phenol, sodium hydroxide, and constant-boiling hydrochloric acid solution was supplied by Merck. Norvaline (Nval) was purchased from Sigma–Aldrich. Water was purified with a Millipore (USA) Milli-Q system. Chromatography was performed with a Waters (USA) Acquity UPLC instrument equipped with a BEH C18 column (2.1 × 100 mm, 1.7 μm particle size) and a UV detector (detection wavelength 260 nm). Two different eluents were used: A (AccQ•Tag Ultra eluent A, diluted in the ratio 1:20 with Milli-Q water) and B (AccQ•Tag Ultra eluent B). The gradient elution program was: 0.54 min, 0.1 % B; 5.74 min, 9.1 % B; 7.74 min, 21.2 % B; 8.04 min, 59.6 % B; 8.05 min, 90 % B; 8.73 min, 0.1 % B; 9.50 min, 0.1 % B. The column temperature was 55 °C, the flow-rate was of 0.7 mL min<sup>-1</sup>, and the injection volume was 1 μL (partial loop).

#### Procedure for modified dot-ELISA

Lyophilized egg (whole egg and egg white) and ovalbumin samples were dispersed in the elution buffer in the concentration range 0.1–10 μg mL<sup>-1</sup>. Model samples were ground and dispersed in the elution buffer (100 μL). All the dispersions were centrifuged at 4,500 rpm for 10 min, to deposit the insoluble fraction. The solution (5 μL) was then deposited on poly(vinylidene fluoride) (PVDF) strips (Millipore, USA) which had previously been soaked in methanol and placed on Petri plates with 5 mL B buffer. Elution buffer (5 μL) was used as a blank control and 5 μL of a solution of milk and glue (1:10 in elution buffer) was used as negative control. The plates were incubated at 4 °C overnight. Primary anti-ovalbumin antibody (1 mL), diluted 1:1,000 (v/v) in PBS, was applied to the plates with B buffer (5 mL) and incubated for 60 min at 37 °C. After washing the plates (2 × 10 min) with B buffer (10 mL), the secondary antibody, anti-rabbit IgG developed in goat, alkaline phosphatase conjugate, diluted 1:5,000 (v/v) in PBS (5 mL) was introduced. Plates were incubated for 60 min at 37 °C and then washed

with B buffer (10 mL, 1 × 10 min) and with PBS (10 mL, 1 × 10 min). Finally, the colour development fresh solution was prepared (1 tablet Sigma FAST in 10 mL Milli-Q water) and applied (5 mL). The substrate solution contained BCIP (0.15 mg mL<sup>-1</sup>), NBT (0.30 mg mL<sup>-1</sup>), TRIS buffer (100 mmol L<sup>-1</sup>), and MgCl<sub>2</sub> (5 mmol L<sup>-1</sup>). After 2 h at 37 °C the staining reaction occurred and the plates were washed with Milli-Q water: this last procedure was the result of a series of tests at different temperatures (4, 15, 25, and 37 °C) with the purpose of optimization of the appearance of the colour. For each sample seven replicates were performed to check reproducibility. Photographic documentation was performed 1 day after colour development on the strips and again 3–4 days later. Once dried, the strips were analyzed by use of the spectrophotometer.

#### Procedure for amino acid analysis

Solutions of lyophilized egg white, whole egg, and pure ovalbumin were prepared in water in the concentration range 100–5 μg mL<sup>-1</sup>, placed in a glass tube, and lyophilized. The lyophilized material was then subjected to hydrolysis in vapour phase in a nitrogen atmosphere at 114 °C for 24 h with 200 μL 6 mol L<sup>-1</sup> HCl solution with 0.1 % phenol to prevent halogenation of Tyr. The samples were then re-suspended in 50 μL 0.1 mol L<sup>-1</sup> HCl and subjected to the derivatization process. Internal standard (25 μL Nval 5 mmol L<sup>-1</sup>) and NaOH (393 μL 0.5 mol L<sup>-1</sup>) was introduced into AccQ•Tag Ultra borate buffer. For each sample, 10 μL of solution was collected and placed in a glass vial insert with 70 μL of a solution of 0.0417 mmol L<sup>-1</sup> Nval/0.0655 mmol L<sup>-1</sup> NaOH in AccQ•Tag borate buffer and 20 μL AccQ•Tag Ultra reagent. For each cycle of analysis a calibration standard (CS) containing 17 amino acids and Nval at a concentration of 10 pmol μL<sup>-1</sup>, except Cys, present at a concentration of 5 pmol μL<sup>-1</sup>, was used. This solution was prepared with 40 μL 2.5 mmol L<sup>-1</sup> amino acid standard solution and 20 μL 5 mmol L<sup>-1</sup> Nval in 940 μL Milli-Q water. CS (10 μL) was placed in the glass insert with 70 μL borate buffer and 20 μL AccQ•Tag Ultra reagent. All samples were mixed, heated to 55 °C for 10 min, and injected for UPLC analysis.

Samples from painted mortar were collected by means of a scalpel, ground, weighed, and subjected to hydrolysis under the same experimental conditions as for the standards described above. For the analysis, approximately 1.5 mg paint layer was used. To avoid loss of protein material, neither extraction nor pre-treatment of the model samples was conducted and the samples were subjected to hydrolysis immediately after sampling. After hydrolysis, HCl (50 μL, 0.1 mol L<sup>-1</sup>) was added and the samples were vortex mixed. Buffer solution containing 25 μL Nval 5 mmol L<sup>-1</sup> and a double amount of 0.5 mol L<sup>-1</sup> NaOH (786 μL in 3 mL



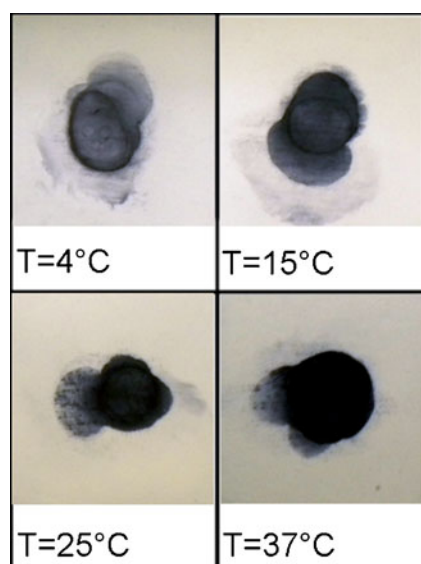
AccQ•Tag Ultra borate buffer) was prepared. Because the amount of protein in model samples was much lower than in standard samples, 30  $\mu\text{L}$  rather than 10  $\mu\text{L}$  solution was subjected to derivatization and 50  $\mu\text{L}$  rather than 70  $\mu\text{L}$  borate buffer was added; therefore, the concentration of Nval was 20.7  $\text{pmol } \mu\text{L}^{-1}$ . The suspension was centrifuged to separate the insoluble fraction and 60  $\mu\text{L}$  solution was collected from each sample, placed in a vial, and heated to 55  $^{\circ}\text{C}$  for 10 min. Each sample was then analysed twice by UPLC; the injection volume was always 1  $\mu\text{L}$ .

## Results and discussion

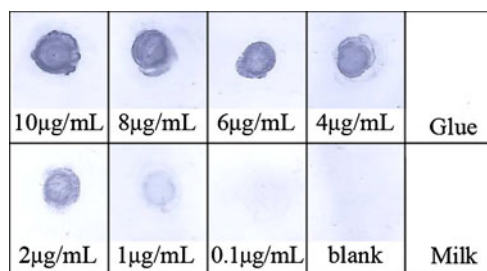
### Modified dot-ELISA

The first objective of this part of the study was to optimise the staining conditions to maximise the stain-to-background ratio. Previous tests had revealed the need to adjust the temperature during development of the stain, because of the drastic effect of temperature on enzyme activity. The staining reaction was performed for 2 h at four different temperatures: 4, 15, 25 and 37  $^{\circ}\text{C}$ . The results reported in Fig. 1 show that the temperature maximising enzyme activity, and therefore blot-to-background ratio was 37  $^{\circ}\text{C}$ . No particular effects of evaporation rate were observed during stain development.

To study the detection limit, the concentrations investigated were from 10  $\mu\text{g mL}^{-1}$  to 0.1  $\mu\text{g mL}^{-1}$ , corresponding to 50–0.5 ng per spot (Fig. 2). The results obtained with samples of pure ovalbumin (Fig. 2), showed that at a concentration of 1  $\mu\text{g mL}^{-1}$  (5 ng per spot), clearly visible

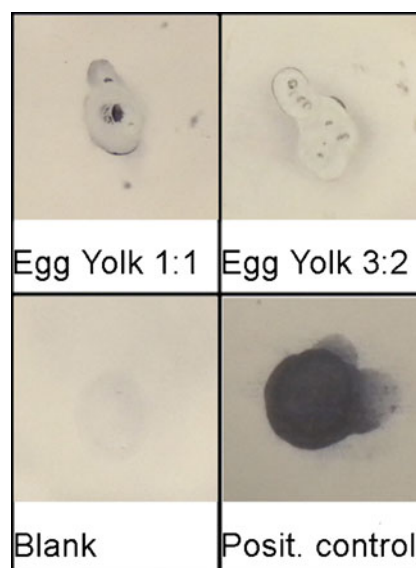


**Fig. 1** Colour staining for dot-ELISA tests of ovalbumin samples as a function of temperature

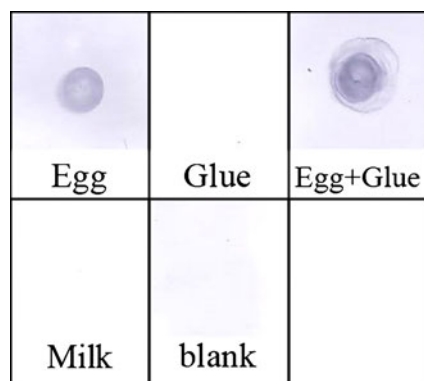


**Fig. 2** dot-ELISA results for ovalbumin/blank/negative controls (milk and glue) samples at different concentrations

staining of the strip could be observed, whereas no stain was detectable at lower protein concentration. It was interesting to notice that the minimum detectable amount of standard protein material was approximately 5 ng, which was a good improvement on the 7 ng of recent results [32], but the limit remained worse than that which can be achieved by the ultrasensitive chemiluminescent technique [31] or immuno-fluorescence microscopy [30]. For all seven replicates the detection limit was that reported above. For egg white samples the detection limit was lower than for ovalbumin, reaching 0.5  $\mu\text{g mL}^{-1}$  (2.5 ng per spot), whereas for whole egg the lower intensity of staining of the sample at a concentration of 1  $\mu\text{g mL}^{-1}$  compared with pure ovalbumin was attributed to the smaller amount of ovalbumin present in whole egg (Electronic Supplementary Material Figs. S2 and S3). To test the specificity of interaction of the antibody with the ovalbumin protein, egg yolk samples were submitted to dot-ELISA. The egg yolk was accurately separated from the white to minimise the presence of residues of white egg protein. The negative results (Fig. 3) demonstrated the ability of the immunoenzymatic reaction of the



**Fig. 3** dot-ELISA results for egg yolk with blank and positive control; yolk was 1:1 (v/v) and 3.2 (v/v) in elution buffer

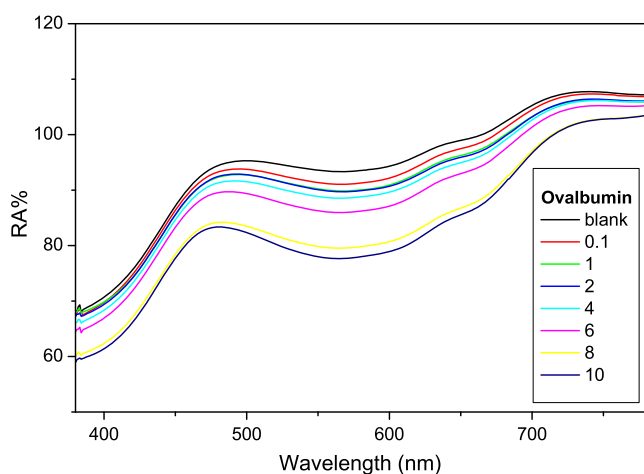


**Fig. 4** dot-ELISA results for model samples, blanks, and negative controls. Negative controls were milk and glue tempera samples

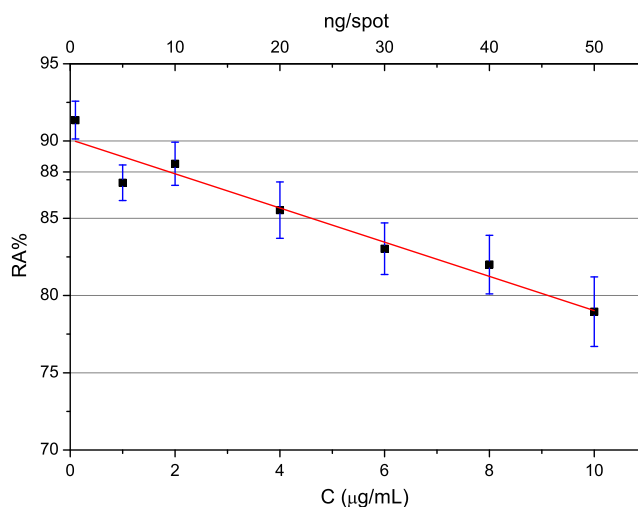
dot-ELISA analysis to distinguish egg yolk from egg white protein. dot-ELISA of model samples (Fig. 4) confirmed the expectation of obtaining a positive response for samples composed of egg tempera and egg–glue tempera. In contrast, for samples consisting of milk and glue tempera, the assay was negative, as expected. For egg–glue tempera, spot intensity was greater than that for egg tempera; this result was particularly interesting and deserving of deep interpretation (vide infra).

As can be deduced from the figures reporting the dot-ELISA results, all the measurements were conducted simultaneously, with background and control experiments that enabled exclusion of spurious effects.

To attempt semi-quantitative dot-ELISA determination, the samples were analyzed using a spectrophotometer with integrating sphere in the visible wavelength range (380–780 nm). Use of the integrating sphere enabled collection of light scattered by the whole sample surface which is always larger than the staining spot size: this procedure always enabled

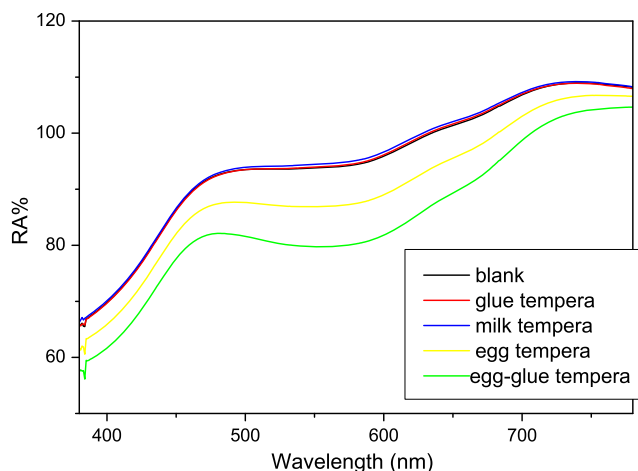


**Fig. 5** Reflectance curves for PVDF strips relating to dot-ELISA performed on blank samples and on ovalbumin at different concentrations ( $0.1\text{--}10\ \mu\text{g mL}^{-1}$ ), expressed as reflectance RA% in the wavelength range 380–780 nm



**Fig. 6** Reflectance values (RA% at 570 nm) for samples of ovalbumin as a function of concentration ( $0.1\text{--}10\ \mu\text{g mL}^{-1}$ ) and of the amount of protein per spot (ng). The linear fit with standard error bars is reported. At RA% = 88 the cut off line is reported

collection of light scattered from the entire spot. Moreover, the power of the light source and angle of analysis were kept constant to avoid effects of these conditions on reflectance values. As for ELISA tests read by optical density measurements [33], spectrophotometric measurements are known to be reliable for semi-quantitative analysis being wide enough to detect the so-called “blank average” [33] (vide infra). Figure 5 shows the reflectance curves obtained from the ovalbumin samples. As expected, a high reflectance value corresponded to the response for a slightly coloured dot-ELISA strip whereas a low value corresponded to the response for a sample giving an intense colour, because the reflectance is strongly determined by the white background and is depressed by colour development. The spectra were very similar and had three reflectance bands with maxima at



**Fig. 7** Reflectance curves for PVDF strips relating to dot-ELISA performed on model samples, expressed as reflectance RA% in the wavelength range 380–780 nm

approximately 470, 640, and 720 nm and a minimum at approximately 570 nm, which became more pronounced for curves corresponding to samples of higher concentration (darker spots). In an attempt to perform semi-quantitative determination of the colour response of the test, the reflectance values for the samples of ovalbumin at the three maxima and at the minimum were plotted as a function of ovalbumin concentration. The best linear fit was obtained for  $\lambda=570$  (the minimum) and therefore we used these data for fitting, and obtained the equation:

$$RA\% = -(1.1 \pm 0.5)C + (90.1 \pm 2.1) (r^2 = 0.884) \quad (1)$$

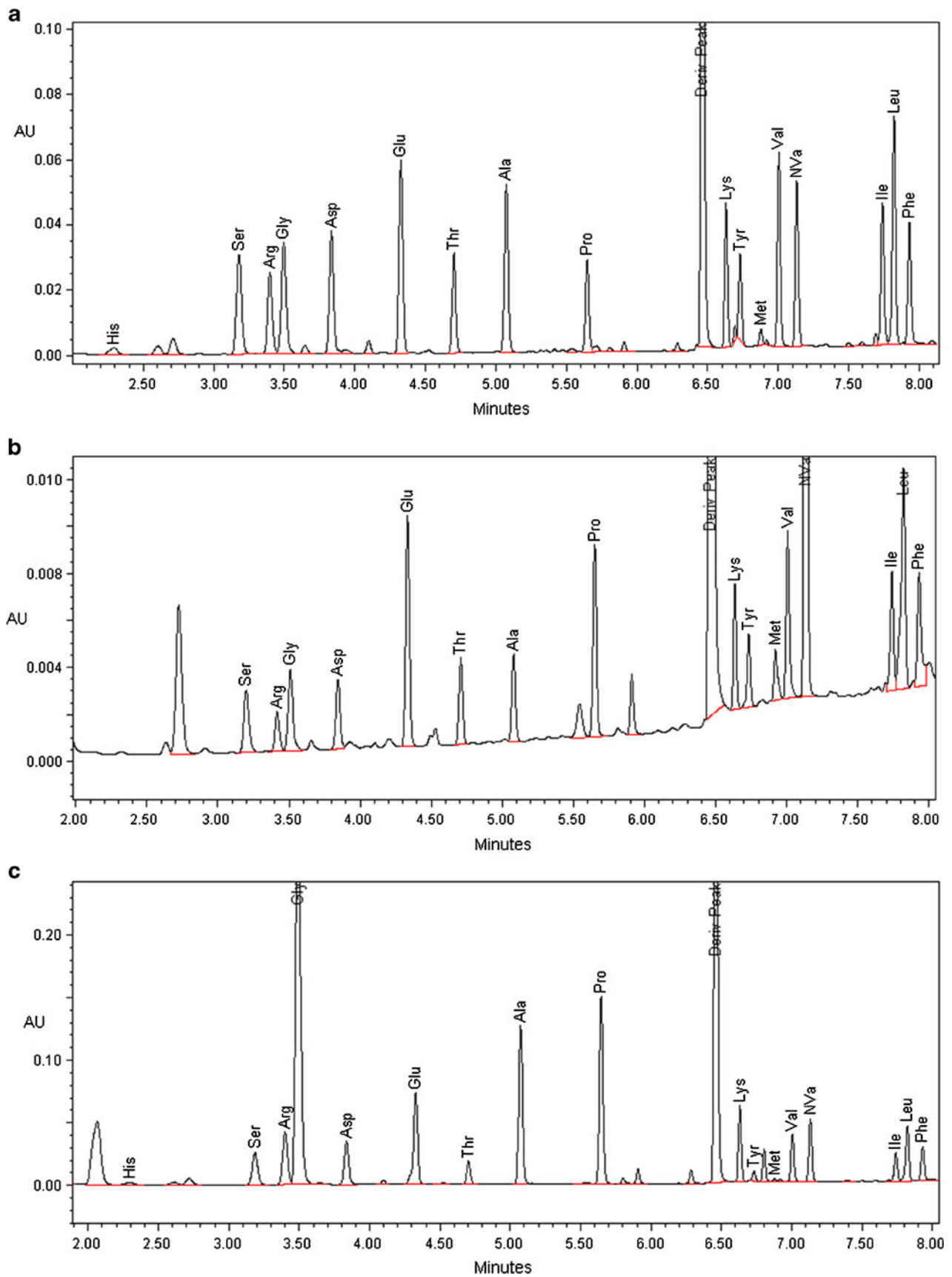
where  $C$  is the concentration of the analyte in  $\mu\text{g mL}^{-1}$ . Indeed, the integral of the curves was also plotted against ovalbumin concentration and the results were comparable with those of the RA% at 570 nm. The detection limit of the assay, estimated as the concentration of the protein giving an RA% value at 570 nm lower than the average of the RA% values at 570 nm of the background samples minus three times its standard deviation [33], was  $1.91 \mu\text{g mL}^{-1}$ , corresponding to 9.55 ng per spot. Analogous results were obtained for samples containing whole-egg and egg-white (Fig. 6).

Moreover, the model samples were subjected to the same spectrophotometric analysis. The corresponding reflectance curves, reported in Fig. 7, clearly showed that for samples containing milk and glue the curves almost overlapped the background curves, whereas for curves for samples containing egg or mixed egg-glue binders the reflectance was lower depending on development of the staining. Applying the above equation to the results obtained with the egg-based model samples, the concentration of the ovalbumin in the samples was calculated. For egg tempera, the reflectance value at 570 nm was

87.11, which is above the detection limit calculated above: the corresponding amount of ovalbumin in the sample was  $2.72 \mu\text{g mL}^{-1}$ . Considering the known composition of the painting layer and the amount of material sampled it was possible to conclude that the yield of ovalbumin (by weight) detection was approximately 5%. For the egg-glue tempera sample the same reflectance value was 79.90, leading to an amount of ovalbumin in the sample of  $9.27 \mu\text{g mL}^{-1}$ , which is approximately 3.5 times higher than the value determined for pure egg samples, leading to a yield of approximately 18%. These very low yields could be ascribed to severe degradation of the protein by the alkaline environment rather than to aging (only natural for three years). Another factor affecting protein deterioration could be the presence, together with high pH during painting, of  $\text{Ca}^{2+}$  cations: whereas some authors report this effect in the presence of chalk [30], other studies [31, 33] do not consider the effect of  $\text{Ca}^{2+}$  cations particularly critical. In particular, in recent work [33] the Conclusions state that the “method (dot-ELISA test) works properly without interferences from the most common pigments and substrates (carbonated plaster)”. Indeed, some tests were conducted in which samples of egg-based tempera were applied to well dried plaster at neutral pH but in the presence of  $\text{Ca}^{2+}$  and the response was always much more pronounced than that for samples for which the pH was strongly alkaline. It was surprising to notice that for the mixed binders sample the yield in ovalbumin was greater, suggesting that the glue layer, added some time after the first deposition of the egg layer, acted as a protective layer inhibiting deterioration of ovalbumin by alkalinity. From this perspective the glue could be regarded as a type of sacrificial layer for the original egg-based painting.

**Table 1** Comparison of ovalbumin, egg white, whole egg, and model samples, expressed in  $\text{ng } \mu\text{L}^{-1}$  and % (w/w) amino acid

Amino acid	Standard samples at a concentration of $1,000 \mu\text{g mL}^{-1}$						Model samples							
	Ovalbumin		Egg white		Whole egg		Egg tempera		Milk tempera		Glue tempera		Egg-glue tempera	
	% (w/w)	$\text{ng } \mu\text{L}^{-1}$	% (w/w)	$\text{ng } \mu\text{L}^{-1}$	% (w/w)	$\text{ng } \mu\text{L}^{-1}$	% (w/w)	$\text{ng } \mu\text{L}^{-1}$	% (w/w)	$\text{ng } \mu\text{L}^{-1}$	% (w/w)	$\text{ng } \mu\text{L}^{-1}$	% (w/w)	$\text{ng } \mu\text{L}^{-1}$
His	2.33	3.34	2.44	3.31	2.27	1.42	0.84	0.36	0.00	0.00	0.52	0.37	1.47	0.11
Ser	7.39	10.60	7.02	9.53	7.20	4.51	5.97	2.57	3.91	0.25	3.20	2.24	5.08	0.38
Arg	5.75	8.25	6.14	8.33	6.04	3.78	7.39	3.19	3.53	0.23	8.09	5.68	5.54	0.42
Gly	3.51	5.03	3.92	5.32	3.53	2.21	4.26	1.84	3.24	0.21	25.22	17.71	3.38	0.25
Asp	9.68	13.87	9.87	13.40	10.59	6.62	8.89	3.83	5.15	0.33	5.15	3.62	9.83	0.74
Glu	15.01	21.52	14.05	19.07	15.07	9.42	13.89	5.99	17.30	1.12	11.80	8.29	14.47	1.09
Thr	4.19	6.01	4.46	6.06	4.85	3.03	4.80	2.07	4.34	0.28	1.79	1.26	3.37	0.25
Ala	6.17	8.85	5.97	8.11	6.04	3.78	6.94	2.99	3.72	0.24	10.61	7.45	6.56	0.50
Pro	4.12	5.91	4.28	5.81	4.11	2.57	4.71	2.03	10.33	0.67	15.59	10.95	6.94	0.52
Cys	1.02	1.46	1.22	1.65	0.94	0.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lys	5.94	8.51	6.10	8.27	6.89	4.31	4.77	2.06	4.46	0.29	4.07	2.86	4.01	0.30
Tyr	3.77	5.41	4.08	5.54	3.90	2.44	4.39	1.89	4.44	0.29	0.85	0.59	4.34	0.33
Met	4.38	6.28	3.88	5.27	3.42	2.14	0.68	0.30	3.12	0.20	0.18	0.12	8.63	0.65
Val	5.95	8.54	5.95	8.08	5.82	3.64	7.77	3.35	7.57	0.49	3.08	2.16	5.70	0.44
Ile	5.07	7.26	4.80	6.51	4.64	2.90	6.70	2.89	6.28	0.41	2.20	1.54	4.93	0.37
Leu	8.71	12.49	8.87	12.04	8.77	5.48	11.09	4.78	14.64	0.95	4.57	3.21	8.43	0.63
Phe	7.01	10.06	6.95	9.43	5.92	3.70	6.62	2.86	7.98	0.52	3.09	2.17	7.31	0.55
	100.00	143.40	100.00	135.76	100.00	62.55	100.00	43.13	100.00	6.49	100.00	70.22	100.00	7.54



**Fig. 8** Chromatograms obtained from model samples: (a) egg tempera; (b) milk tempera; (c) glue tempera



## Amino acid analysis

Amino acid analysis by UPLC was performed after hydrolysis in the vapour phase, to minimise contamination, because the acid solution was not in contact with the samples. To reduce other sources of contamination and loss of material, neither extraction nor purification was performed on the samples, especially in analysis of model samples. This was particularly important to reduce both analysis time and the amount of sample required.

First, standard ovalbumin samples of concentration 100, 50, 10, and 5 ng  $\mu\text{L}^{-1}$  were analysed (Electronic Supplementary Material Table S1). When recovery of the ovalbumin in the samples was determined, the yield was approximately 60–70 % (Electronic Supplementary Material Table S1).

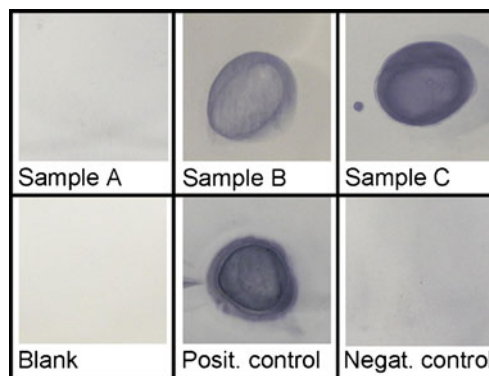
Table 1 shows the agreement of the results obtained for the egg tempera model sample, the ovalbumin standard, egg white, and whole egg (for whole egg, milk, and glue tempera samples the corresponding chromatograms are reported in Fig. 8). For egg white the amino acid percentage values obtained were almost the same as for pure ovalbumin, as expected. Some slight differences that are apparent from Table 1 are because of the presence of the other egg white proteins, for example ovotransferrin and lysozyme, containing more Arg (lysozyme) and more Lys (ovotransferrin) than pure ovalbumin. In contrast, for whole egg the amino acid percentage values were quite different from those for pure ovalbumin. For whole egg the contribution of ovalbumin is lower, because of the presence of a large amount of lipids in yolk and because of the presence of other types of protein, e.g., vitellin, lipovitellin, and phosvitin. Yolk protein content is richer in Thr and Lys and less rich in Phe than ovalbumin. In Table 1 the blue colour indicates the percentages of the amino acids (Glu, Asp, Ala, and Leu) that are characteristic of egg. Yellow indicates the percentages of amino acids that undergo changes depending on the presence or absence of other proteins. For example, the supply of Phe and Ser is more important in ovalbumin than in yolk; also the slightly higher content of Arg in egg white is because of the presence of lysozyme.

In the egg tempera model sample, high Glu, Asp, Leu, Arg, Ala, Phe, and Ser content was observed. These results were compared with those for milk, glue, and egg–glue binder. The milk tempera sample contained a higher percentage of Glu, Pro, and Leu (red colour in Table 1) and a lower percentage of Ala and Asp than in the egg tempera sample. For the glue tempera sample, the very high percentage of Gly and Pro is representative of this medium (green colour in Table 1). Also, the glue tempera contained a large amount of Hyp. In fact, a broad peak observed in Fig. 8c ( $R_T=2.05$ ), corresponding to a polar amino acid, can be attributed to Hyp. In egg–glue tempera the presence of a large amount of Glu, Asp, Leu, and Ala, which are

indicative of egg-based binders, was observed. In contrast, amounts of some amino acids characteristic of glue-based tempera, for example Gly and Pro, were very low and Hyp was not detected. With regard to this last result for model samples (egg-based or mixed egg–glue) it was interesting to note that the yield of the egg-based model sample was good compared with that of whole egg (Table 1; recovery 43.13 % compared with 62.55 %), whereas it was not for the mixed sample (Table 1; recovery 7.54 % compared with 62.55 %). It was also interesting to observe that for the mixed model sample, as emphasised above, no glue was observed. Combining these results with those obtained for the same two model samples (egg and egg–glue based) by use of dot-ELISA enabled the following conclusion. In the presence of a strongly alkaline environment amino acid analysis is more powerful than dot-ELISA for the egg-based model sample, because of the high probability that pH affected the protein sites of the dot-ELISA reaction rather than the final amino acid composition of the sample. On the other hand, application of a glue red ochre layer to a pre-existing egg-based layer results in some protection of the ovalbumin, by the glue, against the alkaline environment, enabling increased dot-ELISA sensitivity (see above); at the same time, however, amino acid analysis did not succeed in detecting glue and lost sensitivity for egg protein components. The reason for this behaviour should be the subject of further investigations; nevertheless in the Electronic Supplementary Material a tentative hypothetical explanation is given (Electronic Supplementary Material Fig. S4).

## Case study

The dot-ELISA method was applied to three samples (A, B, C) taken from the Giotto “St. Francis receiving the Stigmata” wall paintings in the Basilica of Santa Croce in Florence. The issue to be investigated was the presence of a layer of unknown organic material, previously applied as consolidating/



**Fig. 9** Results from dot-ELISA of Giotto’s “S. Francis receiving the Stigmata” samples with blank, negative, and positive controls. The meaning of A, B, and C is explained in the text

protective agent. For the three samples the dot-ELISA method was applied. However, 10  $\mu\text{L}$  instead of 5  $\mu\text{L}$  of sample protein solution were used to react with the primary antibody, to enhance the response because of the possibly small amount of protein expected. The dot-ELISA results for identification of ovalbumin (Fig. 9) showed the presence of a large amount of the protein in samples B and C and a negative result for sample A. Because only the regions where samples B and C were taken belonged to the painting, region A being on the frame around the painting, this result clearly showed that the protein was only on the painting portions and not outside. This result was in agreement with the hypothesis of the conservation team, who suspected the presence of white egg-based varnishes on the painting only as result of past conservation treatment probably at the beginning of 20th century. Therefore, a century of natural aging did not inhibit the response of the dot-ELISA tests.

## Conclusions

The development of a modified dot-ELISA as an analytical method for egg-based paintings is reported. The results showed that temperature critically affected the development of staining, so the procedure was adjusted to enhance the response of the test. In particular, the best results were obtained by incubating the samples for 2 h at 37 °C during the staining reaction. By use of this procedure it was possible to reduce the detection limit and to perform semi-quantitative analysis by plotting reflectance in the range  $\lambda=380\text{--}760$  nm against amount of the ovalbumin, or lyophilized white/whole egg. The straight-line calibration plot obtained confirmed the reliability of analysis by the developed modified dot-ELISA. This semi-quantitative approach enabled determination of the amount of ovalbumin in both egg-based painting layers and mixed egg–glue tempera. The sensitivity was shown to be better than for other recent results, but worse than for the ultrasensitive chemiluminescence technique or immunofluorescence microscopy. It was interesting to note that the alkaline environment typical of the painting technique adopted did not inhibit the potential of the dot-ELISA test, even though it strongly depressed the yield, possibly because of degradation of a large amount of active sites.

Complete hydrolysis of the proteins, as required by UPLC, and subsequent analysis of the amino acid composition instead of the sensing of the active site of the protein as for the dot-ELISA test, was a powerful system for enhancing the yield. UPLC, a new and reliable method for amino acid analysis, was used as an analytical technique for investigating the different protein components of egg-based wall paintings for cultural heritage conservation. The results obtained confirmed the reliability of those obtained by use

of the modified dot-ELISA method and demonstrated that the UPLC method could be a powerful technique for analysis of protein binders in paintings conservation.

The study showed that protein binders applied to an almost fresh high-pH mortar surface did not affect the possibility of detecting the proteins with both techniques used.

The two analytical techniques were tested on model samples that were neither pre-treated nor submitted to extraction processes, showing that good results can be achieved after very little sample manipulation, a great advantage when dealing with analysis of artefacts.

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## References

- Carbo MTD, Reig FB, Adelantado JVG, Martinez VP (1996) *Anal Chim Acta* 330:207–215
- Meilunas RJ, Bentsen JG, Steinberg A (1990) *Stud Conserv* 35:33–51
- Jurado-López A, Luque de Castro MD (2004) *Anal Bioanal Chem* 380:706–711
- Vandenabeele P, Wehling B, Moens L, Edwards H, De Reu M, Van Hooydonk G (2000) *Anal Chim Acta* 407:261–274
- Nevin A, Osticioli I, Anglos D, Burnstock A, Cather S, Castellucci E (2007) *Anal Chem* 79:6143–6151
- Nevin A, Osticioli I, Anglos D, Burnstock A, Cather S, Castellucci E (2008) *J Raman Spectrosc* 39:993–1000
- Colombini MP, Modugno F (2004) *J Sep Sci* 27:147–160
- Schilling MR, Khanjian HP, Souza LAC (1996) *J Am Inst Conserv* 35:45–59
- Schilling MR, Khanjian HP (1996) *J Am Inst Conserv* 35:123–144
- Casoli A, Musini PC, Palla G (1996) *J Chromatogr A* 731:237–246
- Colombini MP, Modugno F, Giacomelli M, Francesconi S (1999) *J Chromatogr A* 846:113–124
- Colombini MP, Carmignani A, Modugno F, Frezzato F, Olchini A, Brecolaki H, Vassilopoulou V, Karkanis P (2004) *Talanta* 63:839–848
- Andreotti A, Bonaduce I, Colombini MP, Gautier G, Modugno F, Ribechini E (2006) *Anal Chem* 78:4490–4500
- Prikryl P, Havlíková L, Pacáková V, Hradilová J, Tulík K, Hofta P (2006) *J Sep Sci* 29:2653–2666
- Gautier G, Colombini MP (2007) *Talanta* 73:95–102
- Wouters J, Van Bos M, Lamens K (2000) *Stud Conserv* 45:106–116
- Peris-Vicente J, Gimeno Adelantado JV, Doménech Carbó MT, Mateo Castro R, Bosch Reig F (2006) *Talanta* 68:648–654
- Halpine SM (1992) *Stud Conserv* 37:22–38
- Ronca F (1994) *Stud Conserv* 39:107–120
- Checa-Moreno R, Manzano E, Mirón G, Capitan-Vallevey LF (2008) *Talanta* 75:697–704

21. Carbini M, Stevanato R, Rovea M, Traldi P, Favretto D (1996) *Rapid Commun Mass Spectrom* 10:1240–1242
22. Chiavari G, Gandini N, Russo P, Fabbri D (2001) *Chromatographia* 53:311–314
23. Nováková L, Matysová L, Solich P (2006) *Talanta* 68:908–918
24. Boogers I, Plugge W, Stokkermans YQ, Duchateau ALL (2008) *J Chromatogr A* 1189:406–409
25. Fiechter G, Mayer HK (2011) *J Chromatogr B* 879:1353–1360
26. Pappa-Louisi A, Nikitas P, Agrafiotou P, Papageorgiou A (2007) *Anal Chim Acta* 593:92–97
27. Ramirez-Barat B, de la Vinia S (2001) *Stud Conserv* 46:282–288
28. Kockaert L, Gausset P, Dubi-Rucquoy M (1989) *Stud Conserv* 34:183–188
29. Heginbotham A, Millay V, Quick M (2006) *JAIC* 45:89–105
30. Cartechini L, Vagnini M, Palmieri M, Pitzurra L, Mello T, Mazurek J, Chiari G (2010) *Acc Chem Res* 43(6):867–876
31. Dolci LS, Sciutto G, Guardigli M, Rizzoli M, Prati S, Mazzeo R, Roda A (2008) *Anal Bioanal Chem* 392:29–35
32. Arslanoglu J, Schultz J, Loike J, Peterson K (2010) *J Biosci* 35(1):3–10
33. Palmieri M, Vagnini M, Pitzurra L, Rocchi P, Brunetti BG, Sgamellotti A, Cartechini L (2010) *Anal Bioanal Chem* 399:3011–3023