A simple and reliable methodology to detect egg white in art samples

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A protocol for a simple and reliable dot-blot immunoassay was developed and optimized to test work of art samples for the presence of specific proteinaceus material (i.e. ovalbumin-based). The analytical protocol has been extensively set up with respect, among the other, to protein extraction conditions, to densitometric analysis and to the colorimetric reaction conditions. Feasibility evaluation demonstrated that a commercial scanner and a free image analysis software can be used for the data acquisition and elaboration, thus facilitating the application of the proposed protocol to commonly equipped laboratories and to laboratories of museums and conservation centres. The introduction of method of standard additions in the analysis of fresh and artificially aged laboratory-prepared samples, containing egg white and various pigments, allowed us to evaluate the matrix effect and the effect of sample aging and to generate threshold density values useful for the detection of ovalbumin in samples from ancient works of art. The efficacy of the developed dot-blot immunoassay was proved testing microsamples from 13th–16th century mural paintings of Saint Francesco Church in Lodi (Italy). Despite the aging, the altered conditions of conservation, the complex matrix, and the micro-size of samples, the presence of ovalbumin was detected in all those mural painting samples where mass-spectrometry-based proteomic analysis unambiguously detected ovalbumin peptides.

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1. Introduction

The correct identification of materials used by artists is of paramount importance for conservators in revealing working practices, authenticating and dating the artworks (Leo *et al.* 2011). Different substances, including oils, gums and proteins (mainly hen's egg, milk or casein, and animal glue), have been used as paint binders in mural and easel paintings. In particular, protein identification is a challenging problem to solve (Cartechini *et al.* 2010), given the low protein content of paint samples (Colombini *et al.* 2010) and the degradation undergone by these materials in the course of aging (Bonaduce *et al.* 2012; Duce *et al.* 2012).

Protein identification in art objects is often performed through Fourier transform infrared (FTIR) spectroscopy that, in imaging mode, allows the stratigraphic localization of proteinaceous material in paintings (Van Der Weerd *et al.* 2002; Bonaduce and Boon 2008; Cotte *et al.* 2008; Cotte

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et al. 2009). Unfortunately, often FTIR spectroscopy techniques lack the required specificity and the presence of different proteins may return arduous signals to interpret (Colombini and Modugno 2004; Dolci *et al.* 2008; Vagnini *et al.* 2008), often masked in ancient paint samples by the massive simultaneous presence of other organic and inorganic components (Arslanoglu *et al.* 2010). Other techniques used in this field are gas chromatography coupled to mass spectrometric analysis detection (GC-MS) and to flame ionization detection (GC-FID), and high-performance liquid chromatography (HPLC) coupled to mass spectrometric or spectroscopic detection (UV-Vis absorption or fluorescence) (Gimeno-Adelantado *et al.* 2002; Rampazzi *et al.* 2004; Bonaduce *et al.* 2009; Lluveras *et al.* 2010; Sciutto *et al.* 2011). Nevertheless, several doubts were advanced because of the susceptibility to matrix interference and degradation process of the relative abundance of quantified amino acids (Colombini and Modugno 2004). Recently, since the first reports on the use of proteomic strategies by Hynek *et al.* (2004) and Tokarski *et al.* (2006), to identify rabbit glue in a sample from a 19th century painting and egg proteins in samples from a Renaissance painting, respectively, several papers reported the successful application of advanced mass spectrometric analyses of enzymatically hydrolysed samples of different origin, to identify proteinaceous material (milk, animal glue, egg) in artworks (Kuckova *et al.* 2007; Leo *et al.* 2009; Fremout *et al.* 2010; Dallongeville *et al.* 2011; Leo *et al.* 2011; Rasmussen *et al.* 2012; Toniolo *et al.* 2012). The approach was revealed extremely successful in unambiguously determining the protein nature in artworks, as also demonstrated by the development of a library specifically dedicated to various sources of proteins in works of art (Fremout *et al.* 2012). However, the amino acid approach suffers complex sample pre-treatment by using multistep procedures (i.e. protein extraction, hydrolysis, sample clean-up, derivatization), which increase the risk of analyte loss and contamination. In lucky cases, this pre-treatment can be avoided in the proteomic approach (Leo *et al.* 2009), since an enzymatic hydrolysis in heterogeneous phase can be carried out directly on the sample. In addition, although these methods have proven to be highly successful and reliable, they are not usual in conservation laboratories because of the expensive equipments and the dedicated skills needed (Arslanoglu *et al.* 2010).

Immunochemical methods are widely used in clinical and bioanalytical chemistry, in various branches of medicine, pharmaceutical and food industries, and in environmental monitoring (Morozova *et al.* 2005; Schubert-Ullrich *et al.* 2009). Based on the high specificity and sensitivity of the antigen antibody interaction, immunochemical methods are very precise in protein identification, distinguishing among different proteins (Tijssen 1985; Dolci *et al.* 2008). The identification of proteins is possible due to the high specificity of antibody–antigen binding (Vagnini *et al.* 2008), detecting protein presence at very low quantities, as often happens in mural samples (Colombini and Modugno 2004). Furthermore, immunochemical methods are rapid, applicable to routine analysis, simple in the sample preparation and do not require expensive instrumentation (Tijssen 1985; Morozova *et al.* 2005). Despite that, in the last 20 years relatively few works have exploited immunochemical methods in the field of conservation science for the identification of proteins in artworks, mostly based on immunochemical imaging methodology (Kockaert *et al.* 1989; Ramirez-Barat and De La Vina 2001; Heginbotham *et al.* 2006; Dolci *et al.* 2008; Vagnini *et al.* 2008; Cartechini *et al.* 2010; Sciutto *et al.* 2011). Other studies have been based on the enzyme-linked immunosorbent assay (ELISA) methodology (Heginbotham *et al.* 2006; Zevgiti *et al.* 2007; Scott *et al.* 2009; Cartechini *et al.* 2010; Arslanoglu *et al.* 2010; Palmieri *et al.* 2011).

Dot-blot immunoassay was developed in 1982 as a screening method for protein markers in bacteriological, epidemiological and immunological studies (Hawkes *et al.* 1982). This technique summarizes principal advantages both from ELISA, more commonly used for protein quantification, and from Western blot, where the immunodetection is usually achieved on protein samples subjected to electrophoresis under denaturing conditions. Respect to ELISA, dot-blot immunoassay methodology better fits with the need to detect analytes in samples treated with denaturing agents to ease protein solubilisation (Gil *et al.* 2003; Yamada *et al.* 2004; Guillemin *et al.* 2009). Moreover dot-blot immunoassay is very simple and affordable, and its principle is similar to that of other immunochemical assay formats amenable also for a not-specialized laboratory, known as strip-based immunoassays (Matsumoto *et al.* 1997; Blazkova *et al.* 2009). A revised procedure for a dot-blot immunoassay able to detect ovalbumin in wall paintings was recently reported (Potenza *et al.* 2012), but to our knowledge the effect of pigments and sample aging has never been explored in the development of dot-blot immunoassay as an analytical tool for the analysis of samples from works of art.

In this work, we present the development of an analytical method based on the use of non-competitive dot-blot immunoassay to detect ovalbumin in paint samples from works of art, and to be addressed to museum and conservation centre laboratories. Ovalbumin is the most abundant protein present in the egg albumen (Phenix 1997), and its detection allows the identification of egg white or whole egg in a paint sample. The study was performed on laboratory-prepared samples of egg white paint layers, with and without various pigments, using denaturing sample-extraction conditions. Fresh and artificially aged pigmented laboratory-prepared samples were analysed to yield threshold response values that were defined by the evaluation of matrix and sample aging effects. The developed analytical methodology has

been then applied to the analysis of gilding samples collected from 13th–16th century mural paintings, and results were compared with the unambiguous identification of the protein component in the sample obtained by MS-based proteomic analyses.

2. Materials and methods

2.1 *Reagents*

Ovalbumin (chicken egg albumin; A5378), fish gelatine (G7765), anti-ovalbumin antibody (rabbit anti-chicken egg albumin whole antiserum; C6534), secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase; A3687), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (SigmaFast BCIP/NBT; B5655), and bovine serum albumin (A2153) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Egg white powder, casein and rabbit glue were purchased from Bresciani S.r.l. (Milano, Italy).

2.2 *Paint laboratory samples*

The laboratory samples were prepared by applying to glass slides a mixture of a water solution of dry egg white and the following pigments: azurite ($Cu_3(CO_3)_2(OH)_2$), red ochre (Fe_2O_3), minium (Pb_3O_4), cinnabar (HgS) and calcite ($CaCO_3$). Binder and pigments were mixed until a fluid paintable consistence was reached. Painted glass slides were left to dry at room temperature. One set was then analysed by dot-blot immunoassay, and another one set was artificially aged in an aging chamber Solarbox 1500e RH (Erichsen, Germany). Samples were exposed for 720 h at 25°C and 40% relative humidity with a soda-lime glass UV filter, in order to simulate indoor exposure, according to the normative UNI 10925:2001 (UNI 2001). For protein extraction, the painted layer was scraped from the paint laboratory samples by a scalpel to obtain a powder.

2.3 *Gilding samples from works of art*

The choice of the samples from works of art was driven by the fact that egg was often used as binder in mural paintings (Cennini 1954), but it is also a proteinaceous material that can be found in mordants for gilding (Bonaduce *et al.* 2006). Gilding samples (0.399–1.021 mg) from mural paintings from Saint Francesco Church in Lodi (Italy) were thus selected to be tested, after a short-timed pre-treatment, for the presence of ovalbumin with dot-blot immunoassay. The sample description is reported in supplementary table 1.

2.4 *Sample pre-treatment*

Sample powder (5 mg; i.e. scraped paint layer of laboratory samples) was homogenised by pestle and mortar in 1 mL of 6 M urea for 2 min. Gilding samples were shortly crushed against the Eppi tube walls by a spatula and suspended to 1 mg/mL in 6 M urea. The suspension was mixed 15 min by vortex, and centrifuged for 15 min, 11000*g*, room temperature. Where it is stated, the vortex step was replaced by a 15 min ultrasound treatment in water bath (Elma Transsonic T700, Singen, Germany), or homogenisation step was not carried out. For the laboratory samples, the supernatant was diluted to 0.05 mg mL^{-1} with 6 M urea prior the dot-blot immunoassay.

2.5 *Dot-blot immunoassay analysis*

The MiniFold 1 System dot-blotting apparatus (Whatman) was assembled according to the manufacturer instructions with 3MM (Whatman) blotting paper (2 sheets) and 0.45 µm nitrocellulose membrane (N9763–5EA, Sigma-Aldrich, St. Louis, MO, USA) pre-wetted in 0.1 M Tris-HCl pH 7.4, 0.5 M NaCl (TBS). 140 µL of sample (laboratory samples: 0.21–3500 ng; gilding samples: 0.002–100 µg), ovalbumin (0.21–3500 ng), or sample spiked with known amount of ovalbumin, in 6 M urea was pipetted in each well of the mounted dot-blotting apparatus, let stand for 20 min and then adsorbed by gentle vacuum application. Wells were washed four times with TBS (200 µL/well). The first two times, the excess buffer solution was pipetted up, and twice it was removed by gentle vacuum application. The dot-blotting apparatus was dismounted and the membrane was washed once with 0.05% Tween 20 in TBS (TBS-T), and blocked with 2% fish gelatine in TBS by a 2 h incubation at room temperature under constant agitation. Blocking solution was well drained away and the membrane was incubated with anti-ovalbumin antibody diluted 1:2000 in TBS containing 1% fish gelatine (TBS-G) for 3 h at room temperature under constant agitation. After 4 washes with TBS-T (5 min each), membrane was incubated with secondary antibody at a concentration of 1:3000 in TBS and 1% fish gelatine for 2 h at room temperature. Both incubations and washes were carried out under constant agitation. Secondary-antibody excess was removed by 4 washes with TBS-T and a final one with TBS (5 min each). The membrane was incubated in 12 mL of the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chromogenic substrate (1 tablet) in deionized water without agitation. The reaction was stopped by water dilution after 4 min and the membrane was dried on filter paper. The image of the developed membrane was digitized using Expression 1680PRO scanner (Epson) or, where it is stated, using a consumer use scanner (SX115, Epson). All

scanner automatisms were removed and the scanner cover was used as white reference for the white point correction. Densitometric analyses were performed using ImageMaster 1D Elite software (Nonlinear Dynamics Ltd/Amersham Pharmacia Biotech, Cologno Monzese, Italy) or, where it is stated, using the freely downloadable software ImageJ (Abramoff *et al.* 2004) equipped with the Dot Blot Analyzer tool (Gilles Carpentier, Faculté des Sciences et Technologies, Université Paris, France). In the densitometric analyses, to minimize the effect of variability in the definition of well area, density values were obtained subtracting the ratio between volume (sum of intensities of every pixel within the defined area) and the defined area (pixel number) with the density of the background (6 M urea in the absence of sample or ovalbumin). A nonlinear regression function (logistic, 4 parameters) was fitted to the data of the density values (Motulsky and Brown 2006) in order to generate dose–response curve.

The method of standard additions (MSA) (Howey *et al.* 1987) was applied to the dot-blot immunoassay (MSA-DBA) in order to assess the presence and quantify ovalbumin in samples in the presence of matrix interference (see supplementary material section 2).

Considering the average values and variability of both density at the lower limit of detection, and matrix effect obtained by MSA-DBAs of paint laboratory samples (fresh and artificially-aged), threshold density values were calculated to decide about the presence of ovalbumin in work of art samples. In every membrane, a set of ovalbumin amounts in 6 M urea (0.21–3500 ng) was always included and the highest response density value was used as normalizator. The same normalization procedure was applied in the calculation of threshold density values. According to the normalized values of the response densities, work of art samples were considered for ovalbumin presence as: negative when the normalized value was <9%; suspect positive, or false positive due to the matrix interference contribute, when the normalized value was comprised between 9% and 29%; positive when the normalized value was >29%.

2.6 *Proteomic analyses*

The identification of the proteinaceous material was carried out following the same proteomic analytical procedure described previously (Leo *et al.* 2009), except for a preliminary incubation of the sample in strongly protein denaturing conditions (6 M urea) that were introduced in order to favour the exposure of the proteinaceous material to the action of proteases.

Microsamples (~100–500 μg) were digested in an enzymatic reaction by proteomics-grade trypsin. A pre-treatment of the solid samples with 6 M urea was carried out by incubation for 1 h in 20 μL followed by sonication for 30 min at room temperature. The samples were then 6-fold diluted with 10 mM ammonium bicarbonate pH 7.5 and enzymatic digestion carried out by addition of 1 μg of trypsin at 37°C for 16 h. The supernatants were then recovered by centrifugation and the peptide mixtures concentrated and purified using reverse-phase C18 Zip Tip pipette tips (Millipore). Peptides were eluted with 20 μL of a solution made of 50% acetonitrile, 0.1% formic acid in Milli-Q water and analysed by LC-MS/MS.

The peptide mixtures were analysed using a CHIP MS 6520 QTOF mass spectrometer equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, Ca). After loading, the peptide mixture (8 μL in 0.1% formic acid) was first concentrated and washed at 4 μL min^{-1} in 40 nL enrichment column (Agilent Technologies chip), with 0.2% formic acid in 2% acetonitrile as eluent. The sample was then fractionated on a C18 reverse-phase capillary column (75 μm×43 mm in the Agilent Technologies chip) at flow rate of 400 nL min^{-1} with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 7% to 60% in 50 min.

Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 400 to 2000 *m/z*) followed by MS/MS scans of the three most abundant ions in each MS scan. Spectra from doubly and triply charged peptides were acquired on a *m/z* 400–2000 range and the MS/MS spectra were acquired on the three most abundant ions in each MS scan, using the sequent empirical equations for the calculation of the best collision energy collision energy (CE) applied during fragmentation: CE=4 V/100 Da–2.4 V. Raw data from nanoLC-MS/MS were converted in mzData (.XML) file format using Qualitative Analysis software (Agilent MassHunter Workstation Software, version B.02.00) and MS/MS spectra were searched against non-redundant protein databases UniprotSprot (Sprot_40.21.fasta, 533049 sequences; 189064225 residues), without any restriction in the taxonomy, using a licensed MASCOT software (*www.matrixscience.com*) version: 2.1.04. The Mascot search parameters were: 'trypsin' as enzyme allowing up to 2 missed cleavages, carbamidomethyl on cysteine residues as fixed modification, oxidation of methionine and formation of pyroGlu N-term on glutamine were selected as variable modifications, 20 ppm MS/MS tolerance and 0.6 Da peptide tolerance.

2.7 *Other analyses*

2.7.1 *Thermogravimetry (TG):* The protein content of the laboratory samples was determined on an aliquot of the paint layer analysed by thermogravimetric analysis (TGA) (see supplementary material section 2). Since

cinnabar has no residual mass at 800°C, we could not determine the protein content in the laboratory samples with this pigment.

2.7.2 *Colorimetric method for protein quantification:* Protein concentration in 6 M urea extracted samples was determined by the Bradford assay (Bradford 1976) using bovine serum albumin in 6 M urea as standard.

2.7.3 *Denaturing gel electrophoresis:* Denaturing gel electrophoresis (SDS-PAGE) was run under reducing conditions (0.175 M 2-mercaptoethanol) according to Laemmli (1970) by using 6 M urea samples (0.3–3 µg protein) properly diluted in Laemmli sample buffer. Western blot analyses were carried out by a standard protocol using anti-ovalbumin antibody.

2.8 *Statistical analysis*

Statistically significant differences in pairwise comparisons were determined by Student's *t*-test analysis ($p<0.05$).

Analysis of variance (ANOVA) via a software run in MATLAB environment (Version 7.0, The MathWorks Inc, Natick, USA) was applied to statistically evaluate any significant differences among more than two samples. Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Statistically significant results were depicted by p-values<0.05.

3. Results and discussion

3.1 *Development of dot-blot immunoassay for ovalbumin detection*

Considering altogether both the denaturing conditions needed for the sample preparation and the conditions optimized for immunochemical reactions, a dot-blot based immunoassay procedure was developed using a commercially available anti-ovalbumin antibody. Briefly, the immunochemical assay consisted of (i) coating the sample protein material to a solid-membrane support in a 96-spot format, (ii) saturating the solid-membrane support with an antibody-unrecognized protein, (iii) immunorecognition of the analyte (i.e. ovalbumin) by incubation with the primary antibody, (iv) immunorecognition of bound primary antibody by incubation with an enzyme-conjugated secondary antibody, (v) colorimetric reaction driven by the enzyme conjugated to the secondary antibody, and finally, (vi) densitometric analysis of the digitized image of the developed solid-membrane support. Some basic parameters were optimized preliminarily, facing the problems of sample leakage, background evaluation and cross-contamination. During the coating step, sample-leakage from the spot was avoided by inserting two sheets of blotting paper to increase the tightness of the system. Nitrocellulose was chosen as solid-membrane support because it generated a lighter background than polyvinylidene difluoride. Taking into account that proteins of samples from works of art are aged for years in complex matrices, the coating solution was chosen accordingly to the need of efficiently extracting such degraded materials, and keeping it solubilized during the whole coating process. As coating solution, 6 M urea in the presence of 1% SDS (Heginbotham *et al.* 2006) and 6 M urea alone were compared. The 6 M urea coating solution was preferred because SDS caused foaming during the coating step, leading to cross-contaminated spots, and because it gave rise to signals of a lower intensity after the colorimetric reaction. Using the established coating solution, 6 M urea, the incubation times (coating, blocking, primary antibody, and secondary antibody steps), and the concentrations of the saturating agent (i.e. fish gelatin), primary antibody, and secondary antibody were defined by using different ovalbumin concentrations in 6 M urea, and choosing the conditions (described in the paragraph 'Dot-blot immunoassay analysis...' of the 'Materials and methods' section) that allowed to obtain highest signal above background responses (data not shown).

The conditions for the colorimetric step and the densitometric analysis were also evaluated. Different times of incubation (1–6 min) of the colorimetric reaction were carried out and the background incidence was measured as the percent ratio between the response of 6 M urea in the absence of ovalbumin (negative control) and the response in the presence of 3500 ng ovalbumin in 6 M urea. Beyond background incidence, the ovalbumin detectability (ED_{50}) was measured as the concentration that yielded half of the maximum response. The background incidence (±SD) ranged from 22.3 (±9.2) to 31.1 (±0.5)% in the time window of 3 to 5 min incubation of the colorimetric reaction, and was lower than that observed at 2 and 6 min (40.3 (±10.0) and 38.9 (±9.8)%, respectively). Although the lowest background incidence (16 (±5%)) was reported at 1 min of incubation time, the ED_{50} value was 2.3 times higher than the lowest ED_{50} value that was observed at 4 min incubation. Therefore, the incubation time of 4 min was chosen for the colorimetric reaction in the conditions of the optimized immunochemical assay. Probably because of the choice of different extracting solutions, the background incidence in dot-blot immunoassay is higher than that reported for the ELISA assay for ovalbumin detection in paintings (Palmieri *et al.* 2011). However, since samples from artworks are complex, 6 M urea, which ensures an efficient extraction of the proteins, was preferred to the phosphate buffer saline solution used for ELISA, despite the higher background observed.

To verify the contribute of the densitometric analysis to the response variability, 4 independent densitometric analyses were carried out on the same membrane containing 3 replicates

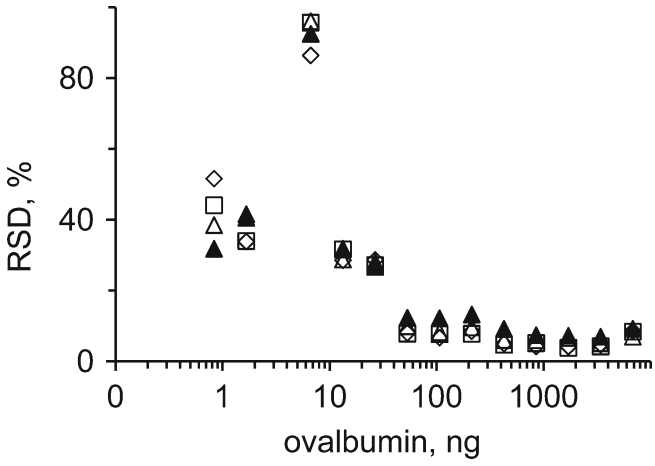

Figure 1. Intra-assay precision profile of response of dot-blot immunoassay at different ovalbumin amounts. Response variability (RSD%) of 4 independent densitometric analyses (rhomb, square, full triangle, empty triangle) carried out on the same membrane containing 3 replicates of each set of the indicated ovalbumin amounts in 6 M urea is reported.

of each set of different ovalbumin amounts (0.1–7000 ng) in 6 M urea. The precision profile of the response (figure 1) shows that intra-assay response variability (RSD%) was above 30% only at ovalbumin amounts below 10 ng, and that response variability is thus not linked to the digitization step and data elaboration protocol. This indicates that densitometric analysis contributes very little to the response variability, which must mainly be ascribed to one or more steps taking place before the colorimetric reaction. As immunochemical reaction steps are by now highly standardized leading to minimization of the response variability (Tijssen 1985), we can suppose that the sample coating was an important source of the response variability observed at the low ovalbumin amounts.

Different tools for the densitometric analysis of dot-blot immunoassay of ovalbumin in 6 M urea were employed. In particular, densitometric analysis performed using a purchased scientific software (ImageMaster 1D Elite) on images digitized by a professional scanner (Expression 1680PRO) was compared with that carried out by a freely downloadable scientific software (ImageJ) on images digitized by a consumer-use scanner (Epson Stylus SX115). The elaborated results showed no difference by Student's *t*-test analysis ($p<0.05$; data not shown), indicating that the densitometric analysis does not require dedicated professional equipment, thus being feasible also to commonly equipped labs.

A typical dose–response plot generated by the optimized dot-blot immunoassay (DBA) of 15 different ovalbumin amounts (0.2–3500 ng) in 6 M urea is shown in supplementary figure 1. The lower limit of detectability (LOD), i.e. the analyte amount giving a response two times the standard deviation calculated on background noise, and the upper limit of detectability (UOD), i.e. the analyte amount giving a response equal to density of maximum value minus two times its standard deviation, were calculated on the fitted nonlinear regression. The LOD and UOD values were used to delimitate the response linearity range showing that it encompassed over at least 1 order of magnitude of the ovalbumin amounts (i.e. 4.9–228.8 ng) with an ED_{50} (±SD) value of 41.2±2.9 ng.

Immunospecificity of the anti-ovalbumin antibody was investigated by carrying out DBA of different amounts of other proteinaceous binders and conservation materials: egg white, casein and rabbit glue, as well as bovine serum albumin and fish gelatine. Only egg white showed a response different from the background over all the tested amounts (figure 2), and similar to the response yielded by ovalbumin, showing that the developed DBA should discriminate ovalbumin-derived proteinaceus material from the other kinds of proteinaceus materials commonly present in samples from works of art.

3.2 *Detection of ovalbumin in the laboratory samples*

Painting laboratory samples were prepared by painting microscope glass slides with a mixture of egg white and various pigments. Pigments were chosen among the most used in all the art history (i.e. azurite, red ochre, minium, cinnabar and calcite) (Matteini and Moles 2003). Our preliminary investigations by DBA indicated that a lower concentration of immunodetected protein was present in calcite painting laboratory samples respect to the painting laboratory samples with the other pigments. Therefore, different procedures for sample preparation from calcite painting laboratory samples were

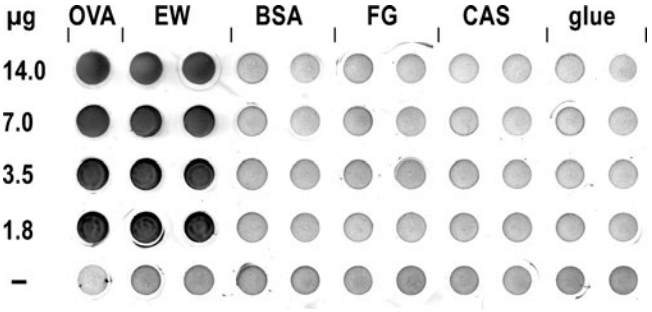

Figure 2. Immunospecificity of the anti-ovalbumin antibody. Developed membrane of dot-blot immunoassay of high amounts of proteinaceous binders and conservation materials for artworks (egg white, EW; casein, CAS; rabbit glue, glue), and bovine serum albumin (BSA), fish gelatine (FG) and ovalbumin (OVA) in 6 M urea. Sample amounts are indicated on the left. The line with negative controls (6 M urea in the absence of sample) is indicated (−).

considered and compared for the response to the DBA of different amounts of extracted material. When homogenization, combined with a vortexing step, was included in the 6 M urea extraction of the powder scraped from calcite painting laboratory samples, the ED_{50} value was 7.0-fold lower than that obtained by the omission of the homogenization step or 4.0-fold lower than that obtained by replacing vortexing step with an ultrasound treatment. This means that homogenisation improves 6 M urea extraction of immunodetectable material especially when it is combined with a vortexing step. The entire optimized extraction procedure took only ~35 min.

DBAs of egg white painting laboratory samples containing as pigments azurite, red ochre, minium, cinnabar and calcite were performed. The amounts of ovalbumin specifically immunodetected in these samples are reported in table 1 and are compared with the amounts of total protein detected by both a colorimetric method and a thermogravimetric method. Immunodetected ovalbumin was at least 2.4-fold overestimated respect to the protein amount. The azurite painting laboratory samples gave the highest overestimation (4.0-fold) of the immunodetected ovalbumin. Western blot analyses of ovalbumin and egg white were carried out using the anti-ovalbumin antibody to assess its specificity toward the protein components of egg white. The immunodetected profile of egg white was similar to that of ovalbumin (data not shown), indicating that the bias in the ovalbumin detection must be due to matrix effects and not to different immunoreactivity properties of ovalbumin and egg white. It is recognized that the analysis of proteinaceous material can be affected by the presence of mineral pigments (Bonaduce *et al.* 2009), and this aspect was till now poorly explored in the immunoassay protocols proposed for cultural heritage analyses.

Table 1. Ovalbumin quantified by dot-blot immunoassay of pigmented painting laboratory samples (protein amounts detected in the same samples by colorimetric and thermogravimetric methods are also reported)

	Protein amount (% ± SD)[a]		
Sample pigment	Colorimetric method	TGA method	Immunodetected ovalbumin (% ± SD)[a]
Azurite	52.2±3.3	67.0±3.4	308.8±22.0
Red ochre	54.5±0.3	64.4±3.2	142.0±48.8
Minium	37.4±3.7	51.0±2.6	139.5±10.8
Cinnabar	38.2±0.4	n.d.[b]	90.7±14.7
Calcite	22.0±1.4	23.4±1.2	69.8±25.3

[a] Data are reported as percent of weighted sample material ± SD (n=3).

[b] n.d., not detectable (see the paragraph on Thermogravimetry (TG) in the 'Materials and methods' section).

The method of standard additions (MSA) (Howey *et al.* 1987) was used to solve the matrix effect problem. The pigmented painting laboratory samples were spiked with known amounts of ovalbumin. The amount of sample to be spiked was defined analyzing detectability in dose–response curves generated by preliminary DBAs of different amounts of unspiked samples. This value was 6.8 ng for all samples with the exception of calcite painting laboratory samples (13.7 ng). The amounts of immunodetected ovalbumin by applying the method of standard addition to the dot-blot immunoassay (MSA-DBA) of the pigmented egg white painting laboratory samples were not significantly different from the protein amount determined by the thermogravimetric method (TGA; figure 3). The protein amount detected in the same samples by the colorimetric method resulted to be lower than that determined by TGA method in azurite and minium painting laboratory samples (Student's *t*-test; $p<0.05$). This can be explained hypothesising that the dye used has a lower reactivity towards ovalbumin than to the protein used for calibration (bovine serum albumin) (Read and Northcote 1981).

Artificially aged laboratory samples were also analysed. The amounts of ovalbumin quantified by MSA-DBA in the aged samples were statistically similar to those revealed in the fresh samples (table 2). This result is extremely important as it indicates that the set up method for the MSA-DBA quantification of ovalbumin is not affected by the tested aging conditions.

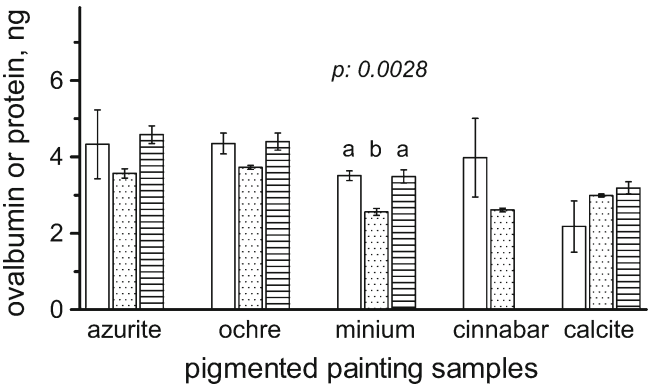

Figure 3. Quantification of ovalbumin in egg white painting laboratory samples by the application of method of standard additions to dot-blot immunoassay (MSA-DBA). Values reported (white bars) represent the amount of ovalbumin immunodetected in 6.8 ng of sample in 6 M urea with the exception of calcite painting laboratory samples (13.7 ng). Quantification of protein in the same samples by colorimetric (pointed bar) and thermogravimetric (dashed bars) methods are also reported. Error bars represent ±SD from the mean of three independent measurements. The *p*-values obtained by ANOVA analysis were all greater than 0.05, except for minium (0.0028) for which a post-hoc analysis (Tukey's HSD, $p<0.05$) was performed: means sharing the same letter are not significantly different from each other.

Table 2. Ovalbumin quantified by applying method of standard additions to the dot-blot immunoassay (MSA-DBA) of fresh and aged pigmented painting laboratory samples

	Immunodetected ovalbumin[a]			
	ng		RSD%[b]	
Sample pigment	Fresh	Aged	Fresh	Aged
Azurite	4.3	4.6	20.9	19.6
Red ochre	4.4	4.3	6.8	7.0
Minium	3.5	3.6	2.9	13.9
Cinnabar	4.0	2.8	25.0	14.3
Calcite	2.2	2.0	31.8	25.0

[a] Values reported represent the amount of ovalbumin immunodetected in 6.8 ng of painting laboratory sample, with the exception of calcite (13.7 ng). No statistically significant differences between fresh and aged painting laboratory samples were determined by Student's *t*-test analysis ($p<0.05$).

[b] n=3.

Once the quantification of ovalbumin by the developed MSA-DBA was showed to be unaffected by the presence of pigments and the sample-aging, the results of many MSA-DBAs of ovalbumin in fresh and artificially aged pigmented laboratory samples were analysed using a normalization routine in order to face the challenge of the typical micro-size and low-number features of samples from works of art. The variability and the matrix effect components of density values were evaluated and threshold density values were defined (see 'Dot-blot immunoassay analysis...' paragraph of the 'Materials and methods' section) as a decisional tool for detection of ovalbumin by DBA of samples from works of art.

3.3 *Detection of ovalbumin in gilding samples from works of art*

The developed DBA was used to detect ovalbumin in gilding samples collected from 13th–16th century mural paintings (figure 4). The obtained response densities were compared with the threshold density values defined by MSA-DBAs of laboratory samples (see above) in order to decide about positivity of the ancient gilding samples for the presence of ovalbumin. These samples have been categorized as reported in table 3 according to the results of DBA of 100 µg sample amount per assay. Among these samples, differences in the detection of ovalbumin with DBA could be noticed and were underlined with a semi-quantitative scale based on the recurrence in the threshold density category. Samples 13, 19, 22 and 25 resulted positive for ovalbumin presence. Sample 18 was classified as negative, while samples 20 and 28 were classified as maybe positive because their response densities were not lower than the threshold density for negativity in all

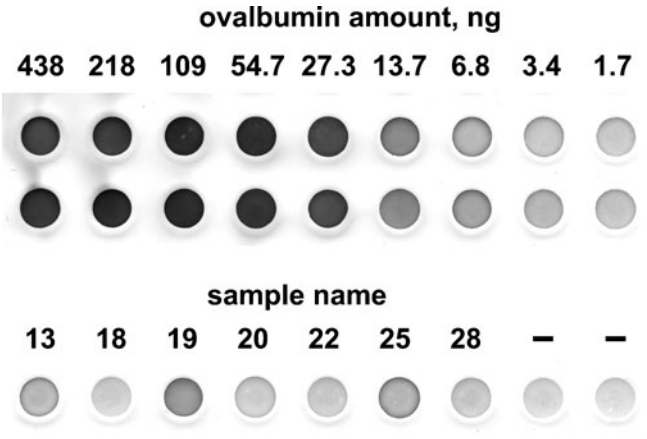

Figure 4. Example image of a dot-blot immunoassay analysis of real samples after the colorimetric reaction step. The signal responses of different amounts (1.7–438 ng, as indicated) of ovalbumin (upper part) and the gilding samples (100 µg; lower part) from Saint Francesco Church in Lodi (Italy) assayed in the same membrane are shown. Spots with negative controls (6 M urea in the absence of sample) are indicated (−).

the DBA assays. Sample 19 was suspected to be positive also when the amount of sample tested by DBA was lowered to 30 µg. Considering both the amounts of tested samples and the LOD for ovalbumin, detected ovalbumin could be estimated to be at least 5 ng in 100 µg of the work of art samples classified as positive.

In order to evaluate the performances of the developed DBA method, the results described above on the analysis of gilding samples were compared to the results obtained by MS-based proteomics on the same samples. Briefly, the proteomic approach relies on matching experimental mass spectra of digested proteins to the virtual ones calculated for the proteins that are present in sequence databases. Following trypsin digestion of the gilding samples that had

Table 3. Ancient samples from Saint Francesco Church in Lodi (Italy): dot-blot immunoassay analysis for ovalbumin

Sample name	Ovalbumin presence[a]
13	++
18	−
19	+++
20	+/−
22	+
25	++
28	+/−

[a] Evaluation of ovalbumin presence based on the frequency distribution of the sample density from four dot-blot immunoassay replicates in the classes delimitated by the calculated threshold density values.

been previously treated with 6 M urea, peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 400 to 2000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Raw data from nano-LC–MS/MS analyses were used to query non-redundant protein databases (NCBI, on all entries or with the taxonomy restriction to Chordata) using in-house MASCOT software (Matrix Science, Boston, USA), without the insertion of any fixed chemical modification but the possible oxidation of methionine and the formation of pyroglutamic acid from glutamine residues at the N-terminal position of peptides. In four samples, proteins from chicken egg were identified with reasonable confidence. Table 4 reports the identification of the proteins in the samples after trypsin digestion and LC-MS/MS analysis, with the sequences of the peptides that allowed protein identification. Interestingly, in all the samples the identification of chicken egg as the proteinaceous binder present in the painting gilding samples was allowed by the detection of ovalbumin (only in the case of sample 25, vitellogenin-2 was also identified); even more worthwhile noting, in all the cases the peptide 128–143 (GGLEPINFQTAADQAR) has been detected and its fragmentation spectrum scored very high, thus providing the identification with high confidence.

Table 4. Identification of proteins in gilding samples by proteomic strategies

Sample	Protein[a] (accession number)	Matched sequence[b]
13	Ovalbumin (P01012)	^{106}LYAEER^{111}
		^{128}GGLEPINFQTAADQAR^{143}
18	n.d.	n.d.
19	Ovalbumin (P01012)	^{220}VASMASEK^{227}
		^{265}LTEWTSSNVMEER^{277}
		^{128}GGLEPINFQTAADQAR^{143}
		^{324}ISQAVHAAHAEINEAGR^{340}
20	n.d.	n.d.
22	Ovalbumin (P01012)	^{128}GGLEPINFQTAADQAR^{143}
25	Ovalbumin (P01012)	^{220}VASMASEK^{227}
		^{128}GGLEPINFQTAADQAR^{143}
		^{324}ISQAVHAAHAEINEAGR^{340}
		^{144}ELINSWVESQTNGIIR^{159}
	Vitellogenin-2 (P02845)	^{1515}MVVALTSPR^{1523}+ Oxidation (M)
		^{260}QQLTLVEVR^{268}
28	n.d.	n.d.

[a] n.d., not detectable.

[b] Oxidation of methionine, and pyro-Glu formation at Gln at the N-terminus of peptides were inserted as variable modifications in the MS/MS Ion Search Program.

All samples were collected from gildings on mural paintings from the 13th–16th centuries, and their protein content had thus undergone severe aging conditions, compared to the artificial aging performed in this study on the laboratory samples. Despite the scarcity of sample together with the very low amount of ovalbumin, the proposed DBA has been able to detect ovalbumin in such complex samples, and the results are highly reliable given the comparison with the unambiguous identifications performed by MS-based proteomics. Moreover, the tested laboratory samples were valuable for estimating the threshold density values useful in the interpretation of the results of DBAs of the gilding samples. The presence of ovalbumin in these gilding samples is in agreement with the fact that proteinaceous materials were commonly used to apply metallic leaves ensuring the cohesion between the mordant and the preparatory layers (Bonaduce *et al.* 2006; Bonaduce and Boon 2008).

4. Conclusions

The need to use harsh conditions (e.g. 6 M urea) to efficiently extract proteinaceous material from a complex and aged matrix led us to develop a fast and reliable dot-blot immunoassay (DBA) for ovalbumin detection in paint laboratory samples and in work of art samples (i.e. gildings on mural paintings) of 13th–16th century. The inclusion of the method of standard additions in the dot-blot immunoassay procedure (MSA-DBA) allowed the quantification of ovalbumin in fresh and artificially aged paint laboratory samples composed of egg white binder and different pigments. Moreover, the extensive analysis of differently pigmented paint laboratory samples by MSA-DBA allowed the evaluation of the matrix effect to define threshold density values that were used as decisional parameters in the detection of ovalbumin by dot-blot immunoassay of the work of art samples.

To our knowledge, for the first time, results of an immunochemical assay (i.e. dot-blot immunoassay) of work of art samples were compared with those obtained by a MS-based proteomic approach. Despite the aging, the altered conditions of conservation and the complex matrix, it was possible to detect by DBA the presence of ovalbumin in all the work of art samples in which ovalbumin peptides were unambiguously identified by the proteomic approach, thus validating the DBA for the detection of ovalbumin in works of art. Considering the specificity of the anti-ovalbumin antibody, the DBA developed in this work can be used to assess the presence of albumen within the sample. This is an important information when deepen knowledge of the artistic techniques used in a work of art is requested, and is an advantage of DBA over the other techniques (e.g. GC-MS) that cannot discriminate between egg albumen and egg yolk.

Moreover feasibility evaluation demonstrated that a consumer-use scanner and a freely downloadable image analysis software can be used for the data acquisition and elaboration. This aspect, together with the relatively easy procedure and its low costs, makes the developed dot-blot immunoassay methodology a useful tool which could be adopted by commonly-equipped laboratories and by museum and conservation centre laboratories facing the identification of binding media in paintings. The procedure in fact allows a reliable identification of specific proteins (ovalbumin in this case) in works of art, helping in understanding either the painting technique of the artist or the presence of restoration layers, or defining and planning the best conservation treatments, all information essential to museum managers, art historians, conservators, restaurateurs, and stakeholders in general (Appolonia 1995; Heginbotham *et al.* 2006).

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