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CIM® monolith chromatography-enhanced ELISA detection of proteins in artists' paints: Ovalbumin as a case study

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ABSTRACT

ELISA (Enzyme-linked ImmunoSorbent Assay), as one possible application of antibody-based methods, has proved to be a good candidate for the determination of proteins in works of art due to the low limit of detection, high sensitivity (in the nanogram range), specificity and micro invasiveness of required sampling. Despite the advantages of this method, interference by metal ions present in pigments, lipids, saccharides and in other components of paints, grounds, and varnishes may cause several problems for incontestable identification of proteins. To overcome the drawbacks of ELISA, a novel way to purify the extracted mixtures to predominantly protein fractions, by the DEAE-modified (weak anion exchange) CIM® (Convective Interaction Media) chromatographic monoliths was introduced. Utilisation of monolithic chromatographic supports greatly enhanced the ELISA detection of ovalbumin in the model paint samples (aged and non-aged), for at least one tenfold dilution of a sample. In several cases the purification enabled detection of the target protein, otherwise not possible with non-purified samples. For the first time in the field of Cultural Heritage, the combined approach of ELISA and CIM® monolith-supported extraction was successfully tested also for the detection of protein in real-case paint layers.

1. Introduction

Among materials present in art objects, polymer organic compounds (e.g. proteins) were used as adhesives, protective coatings, and especially as binding media for colouring materials [1,2]. An investigation of proteins used for such purpose can be challenging due to their low concentration present in solid matrix (paints), co-presence of different organic substances (complex mixtures, derived from different animal/plant sources), pre-existing conservation treatments, etc. Furthermore, their propensity to undergo alterations during ageing (fragmentation, alteration of amino acids such as oxidation, deamination, hydrolysis, and biodeterioration) represents additional difficulty in their specific identification in different works of art. These processes can be accelerated by environmental conditions (heat, excessive light exposure, variations in humidity, etc.) in which particular cultural heritage objects can be stored [3–7].

When investigating proteins, such as casein (milk), ovalbumin (egg white), or collagen (bone, skin), which are the most abundant in the art materials, classical instrumental methods, such as FTIR, py-GC, GC–MS, HPLC [8–15] can therefore stand out not to be enough specific or can give ambiguous results due to the sample size, the sample mixture, protein degradation, as well as the influence of omnipresent microorganisms.

In scientific analysis of works of art, there is constant focus on getting as much information as possible either non-invasively or by taking the smallest possible representative sample, therefore the analytical techniques advance in both sensitivity and specificity. For that reason, the implementation of immunological analytical techniques, such as ELISA [16–22] or Immunofluorescence Microscopy (IFM) [16,23–25] shows the potential to become essential in the determination of proteins in works of art. They offer high specificity of the interaction between the antibody and antigen, which enables distinction between different types of proteins, as well as lowering the limit of detection, and increasing its sensitivity and accuracy. In addition, immunological methods are faster, easier, and do not require large amounts of sample and expensive instrumentation.

However, there are still several issues with the ELISA identification of proteins in art and cultural heritage objects. The obstacles, challenges, and some of the possible avenues of improvement have been extensively

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and comprehensively reviewed by the group from the Metropolitan Museum of Art [17]. Protein modifications such as cross linking, hydrolysis and oxidation can be catalysed by metal ions present in some pigments [21]. These ions can also change the protein structure and affect the recognition of a target protein by an antibody. Additionally, interference by lipids and saccharides, present as components of paints, grounds and varnishes may cause several problems for incontestable identification of proteins. Several improvements in the technique (extraction reagents, micro-titre plates, and reporting enzyme selection optimization) were proposed and implemented with various degrees of success; however the influence of above-mentioned impurities remains a factor of concern [18–21]. To improve ELISA-based detection of proteins in heterogeneous matrices, such as paint layers, purification by chromatography was proposed as one of the possible alternatives.

In recent years, porous monoliths were used as sorbents in sample preparation and provided impressive results [26]. Convective Interaction Media CIM® chromatographic monoliths are porous methacrylate-based polymers composed of highly interconnected channels, and further modification of the monolith matrix surface enables to produce monoliths with different functional groups. The structure of CIM® chromatographic monoliths enables fast and flow independent operation, high chromatographic resolution, as well as high dynamic binding capacities for macromolecules [27–29]. Moreover, because of straightforward preparation of the monolith in any size or shape (in-mould polymerisation) this support is very convenient for preparation of microscale devices, such as pipette tips or similar. That makes CIM® chromatographic monoliths a preferred choice for solid phase extraction and purification of binder proteins from milligramme-range amounts of samples taken from art objects.

For the first time in the field of Cultural Heritage, advantages of both, ELISA and CIM® monolith-supported extraction were synergistically employed for significantly improved detection of ovalbumin in artists' paints in this investigation of model and real-case paint layers.

2. Materials and methods

2.1. Chemicals

The extraction of protein fraction from bulk samples was performed by using *Tris*(hydroxymethyl)aminomethane–HCl (Tris–HCl) (Fluka), EDTA (Sigma-Aldrich), urea (Sigma-Aldrich), sodium dodecyl sulphate (SDS) (Sigma-Aldrich) and $NaHCO_3$ (Merck, p.a.). The samples purified by CIM® chromatographic monoliths were extracted in $NaHCO_3$ (Merck) and chromatographically purified using Tris–HCl (Sigma-Aldrich) and $(NH_4)HCO_3$ (Alfa Aesar) buffers.

Anti-chicken egg albumin antibody produced in rabbit (Sigma-Aldrich, C6534) as primary polyclonal antibody and anti-rabbit IgG (whole molecule) alkaline phosphatase-conjugated antibody produced in goat (Sigma-Aldrich, A0418) as secondary antibody were used in the ELISA procedure. Alkaline phosphatase yellow p-NPP (Sigma-Aldrich, P7998) was employed as the reporting dye for colorimetrical detection (O.D. at 405 nm) of the immune-complex. Antibodies were diluted in 5% new born calf serum (Sigma-Aldrich), which served also as a blocking reagent. During the ELISA procedure, several washing steps were performed, using 0.05% Tween-20 in phosphate buffer saline (PBS), both purchased from Sigma-Aldrich. Calibration curve of standard reference protein (ovalbumin) was prepared with albumin, from chicken egg white (Sigma-Aldrich, A5503-1G). All listed chemicals were used without further purification.

2.2. Model painting preparation

Canvas (30 × 30 cm of effective surface area) was stretched to a wooden sub-frame and treated with a hot rabbit glue solution. Subsequently, white gesso ground was applied, dried, and the surface was polished (this application procedure was repeated three times).

For the preparation of paint layers on model paintings, egg tempera was employed in separate squares (6 × 6 cm) on the gesso ground in two different variations regarding the binders used. For the first mock-up series, the selected pigments were bound in egg yolk exclusively, while for the second the same pigments and egg white binder were used. Egg yolk tempera was derived from whole hen's egg (purchased at the local market) which was mixed with the same volume of water, and a drop of apple cider vinegar. Egg white binder was prepared the same way as glair (see below). Each square (paint layer) was divided into three sections, depending on the used finishing protective layer/varnish. First division was left uncoated, the second was coated by glair only, and the third by a combination of glair and mastic. A schematic diagram of paint and varnish layers is provided in Fig. 1. To prepare glair varnish, the egg white was separated from the yolk and beaten, until foam was formed. After overnight incubation (2–8 °C) in the fridge, the foam was separated from the liquid part, to which 2 g of sugar per one egg was added [2]. Gummi Mastix was purchased from Kremer Chemicals Nr. 60050 and mixed with Fir turpentine, purchased from Kremer Chemicals Nr. 70010 (1:3 ratio).

2.3. Ageing protocol

One set of the prepared model paintings was exposed to accelerated, artificial ageing in climatic chambers with well-defined and controlled temperature, relative humidity, and light conditions. Lightfastness was carried out according to ASTM D 4303-03 standard: "Test Methods for Lightfastness of Colorants Used in Artists Materials", with appropriate modifications. For the simulation of UV–Vis radiation, metal halide lamp (irradiance 100 W/m^2) was used, equipped with window glass filters, which is a good simulation of the sunlight. 30 days of light exposure was followed by another 30 days exposure in climatic chamber, with oscillations of temperature and relative humidity (from 0 °C to 50 °C and from 20% to 90%, respectively). One set of model painting was left non-aged and served as a control.

2.4. Real samples

In order to test the reliability of the novel purification system and its further application to the ELISA method, two micro-samples (approximately 1 mg, extracted by scalpel) from real artworks were investigated: a paint layer from 16th Century painting of Vittore Carpaccio, Presentation at the Temple (Fig. 10 — left), and a paint layer of the 17th century painting from Pietro Liberi, Saint Nicholas between Saint Hermagoras and Saint Fortunatus (Fig. 10 — right).

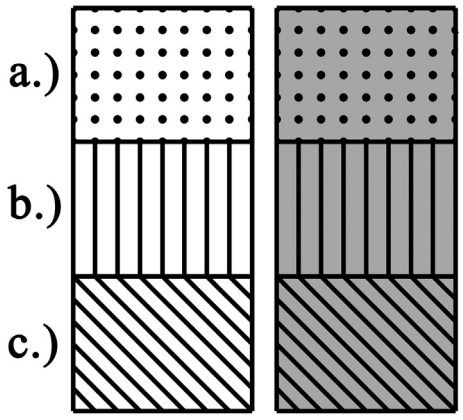

Fig. 1. Schematic presentation of the model paint layers, prepared in left: egg yolk binder; right: egg white binder. Both paint layers were a.) not coated or coated with b.) egg white, c.) egg white and mastic. This pattern was repeated for all five selected pigment-binder systems.

2.5. Instrumentation

2.5.1. ELISA

ELISA test was performed using micro-titre plates (NUNC-Immuno™ 96 MicroWell™ Plates, MaxiSorp™; Thermo Scientific). Sample absorbance at $\lambda = 405$ nm, expressed as optical density ($OD_{405\ nm}$), was measured by Synergy H1, BioTek Instruments, USA.

2.5.2. FTIR

FTIR analyses of samples were carried out with a Perkin Elmer Spectrum 100 FTIR spectrophotometer coupled to a Spotlight FTIR microscope equipped with nitrogen-cooled mercury-cadmium telluride (MCT) detector. The spectra were collected in a transmission mode as 64 scans in the range between 4000 and 600 cm^{-1}, at 4 cm^{-1} spectral resolution using a diamond anvil cell.

2.5.3. Monolith columns

A single use CIM® diethylaminoethyl (DEAE) weak anion exchange custom prepared 4 μL monolithic columns packed into plastic micropipette tips (monolith tip) were obtained from BIA Separations (Ajdovščina, Slovenia). Compared to C18 hydrophobic columns that are being used for peptide (shorter fragments of proteins) extraction, and a research done by Lee et al. [22] where C4 hydrophobic column was used for depletion of metal ions from extracted materials, DEAE column not only allows usage of completely inorganic solvent/buffer components, but also enables to purify very large peptides and intact proteins, which are necessary for successful determination of target proteins by ELISA. Additionally, monoliths yield great recovery of large biomolecules, which makes them exceptionally suitable for analysis of sub-milligramme amounts of samples [30,31], and can bind multifold higher amounts of extracted protein (80 μg of protein per monolith tip used in this study). Furthermore, to enable greater experimental flexibility, solvent buffer system in this study was chosen to match the requirements of both, ELISA and LC–MS/MS techniques simultaneously, without any sample post-processing. Lastly, monoliths are highly porous materials with rigid structure and well defined pore radius (monoliths with 1.5 μm pore diameter were used in this study) so they can serve as filters, which minimises sample preparation and handling.

2.6. Protocols

2.6.1. Sample protein extraction

Each of the binders was mixed with 5 different pigments and investigated in different combinations of varnish, ageing (see Section 2.2 and Fig. 1) and purification pre-analysis, for a total of 24 combinations for each paint layer and 120 in total.

Approximately 1 mg samples of each of the 24 model paints/varnish/ageing combinations were collected from the canvas and, before the application onto ELISA microtitre plate, extracted by the following steps:

·For the direct determination of proteins by ELISA (12 of the 24 samples), the extraction was done according to slightly modified procedure by Heginbotham et al. [16] 1 mg of the sample taken from canvas painting was diluted in a 3.33 ml buffer (10 mM Tris–HCl, 1 mM EDTA, 6 M urea, 0,1% SDS), mixed well on a vortex and incubated at 37 °C for 5 h. Afterwards, 6.67 ml of 100 mM $NaHCO_3$, pH 9.6, was added to the extraction buffer, maintaining 2:1 ratio.

·For the series of samples (the other 12 of 24), which were chromatographically purified and concentrated before ELISA procedure, 1 mg of model paint each was suspended in 100 μL of 0.1 M $NaHCO_3$. The suspension was alternately treated by ultrasonic bath to aid dissolution and vigorously shaken on a vortex mixer for 1 h. The suspension was centrifuged 1 min at 11,000 *g* to remove any possible undissolved material, and approximately 90 μL of supernatant was loaded onto previously equilibrated monolith-equipped tip (Fig. 2).

Fig. 2. Monolith support inside plastic pipette tip.

2.6.2. Purification by CIM® chromatographic monoliths

CIM® chromatographic monolith tips were equilibrated by washing with 100 μL deionised water followed by washing with 50 μL of 20 mM Tris buffer, pH 7.4, 50 μL of 20 mM Tris + 2 M ammonium carbonate buffer, pH 7.4, and finally again with 50 μL of 20 mM Tris buffer, pH 7.4. The 90 μL of sample (Section 2.6.1) was loaded onto the tip, · washed with 50 μL of 20 mM Tris buffer, pH 7.4, and the proteins were finally eluted with 50 μL of 20 mM TRIS + 2 M ammonium carbonate buffer, pH 7.4. Each washing/loading/eluting step required 10 min of centrifugation at 1700 *g*.

2.6.3. ELISA protocol

The ELISA protocol was also adopted from Heginbotham et al. [16], and used with minor modification.

The purified samples (Section 2.6.1, followed by Section 2.6.2) were re-suspended with bicarbonate buffer (100 mM $NaHCO_3$, pH 9.6) to achieve the same theoretical concentration of the total protein in purified and non-purified samples (Section 2.6.1, first bullet point). The Bradford Ultra protein assay [32] was performed to establish a "perceived" actual total protein concentration of each sample (purified and non-purified). As this and other protein quantification tests are influenced by impurities that are regularly found in paint layers (model or real), this test was not used as an absolute measure in this study (therefore not reported and data not shown), but more as a guide or check-up tool to verify that the total protein concentrations were within the same order of magnitude for all purified and non-purified samples' pairs. Each of the samples was further diluted in four steps (10-fold dilutions), all the dilutions were applied onto microtitre plates (each dilution into three wells (triplicates); application in triplicates was used also for the calibration curve, and all the control samples), and incubated overnight (2–8 °C). Afterwards, 300 μl of 5% calf serum albumin (CSA) blocking solution was added to prevent unspecific binding of antibodies in the further steps of the procedure. The indirect ELISA method was performed by using rabbit anti-ovalbumin primary polyclonal antibodies (100 μl, 1 h incubation at room temperature), followed by the addition of alkaline phosphatase-conjugated (AP) goat-anti-rabbit secondary antibody (100 μl, 1 h incubation at room temperature). To obtain enzyme-substrate reaction, 100 μl of colourless p-NPP was added to the wells and incubated for 30 min at the room temperature. The indicator was, in the case of a positive reaction, converted into a coloured product by the reporting enzyme. Finally, the results were read by a spectrophotometer (optical density at 405 nm). Between each step, the wells were washed twice using 300 μl of 0.02% Tween-20 in PBS, and twice with PBS only. After 30 min, the chromogenic enzymatic reaction was stopped by adding 50 μL of 2 M NaOH.

3. Results and discussion

3.1. Model paintings

The main aim of the study was to investigate the effects of pre-purification of the samples by CIM® chromatographic monoliths on the enhancement of the ELISA detection of ovalbumin used as a binder or finishing protective layer in model and real paintings. To that end, a wide variety of samples were prepared, a half of them artificially aged. The selection of binders was intended to provide different quantities

Table 1
Pigments used for the preparation of the model paintings.

Pigment	Chemical formula	Product no.
Verdigris	$(Cu(CH_3COO)_2.[Cu(OH)_2]_3.2H_2O$	Kremer 44450
Dragon's blood	$C_{18}H_{18}O_4$	Kremer 37000
Malachite	$Cu_2CO_3(OH)_2$	Kremer 10300
Lead white	$Pb(CO_3)_2 \cdot Pb(OH)_2$	Kremer 46000
Green earth	Al-, K-, Mg-, Ca-, Fe-silicates	Schminke 18519

of ovalbumin in the samples. Whereas there is very little ovalbumin to be expected in the egg yolk tempera (in theory, ovalbumin is present only in egg white) [33], an abundance of the protein is expected in egg white tempera. Moreover, to study the influence of the different concentration of the protein and the presence of other organic macromolecules, two finishing protective layers (glair and mastic) were applied. Pigments used (Table 1) were selected with the expectation of different influence on the integrity of ovalbumin (conformation change due to binding of metal ions to the protein, its degradation catalysed by these metals before or during ageing, etc.) [34] and/or their direct effect on the ELISA detection system [35]. Indeed, it is known from the conservation practice (real paintings) that paint layers containing verdigris are often severely degraded and that lead white-containing layers can exhibit instability. Conversely, good stability is reported with green earth and malachite (although, as verdigris, it is a copper-based pigment) paint layers. As a contrast to these metal-based, dragon's blood was used as an organic pigment, and was expected to show the least interference either with the binder or the ELISA detection system. Therefore, the effect of binder-pigment interaction, ageing, and combination of the two on the ELISA detection was also studied in this work.

Indeed, a visual inspection of the aged model paintings, compared to the non-aged revealed striking differences between the two. The most affected paint layers were those containing verdigris (striking colour change from almost turquoise blue to black) and dragon's blood (very strong fading of the red colour and/or pronounced browning thereof). Lead white and malachite coloured surfaces showed moderate fading and varnish darkening. The least prominent visual change was observed in the green earth paint layers.

In preliminary tests, a series of step-wise tenfold dilutions (starting with the 0.1 mg/ml of initial dried ovalbumin standard) was used as a template to ascertain the optimal primary and secondary antibody dilutions. Recommendations of the antibody manufacturers of 1:500 and 1:600, respectively, were confirmed as optimal, and the mentioned step-wise tenfold dilutions range was used as the calibration curve for ovalbumin concentration, as well as a positive control in all subsequent ELISA assays. Furthermore, primary antibody specificity/possible cross-reactivity (false positive results) was controlled by tests against collagen (several terrestrial and fish species), casein, and several other binders commonly used in cultural heritage, all in 0.1 mg/ml standard solutions. Additionally, to evaluate possible false positive results, different types of negative controls were made (combinations of buffers and antibodies used in the assay, in the absence of ovalbumin) on every assay plate. The highest result of their respective means plus three times their standard deviation (SD) was assumed as limit of detection (LOD).

To illustrate how the results of all the 120 combinations of pigment, binders, protective coatings, and ageing and purification procedures are presented, always relative to the standard calibration curve of ovalbumin and taking into consideration OD values of the negative controls, only one investigated paint layer (dragon's blood prepared in egg yolk and coated with glair; reference (non-aged), non-purified/purified) (Fig. 3) is presented in detail. The horizontal line ($OD_{405\ nm} = 0.1$) indicates the limit of positive detection in that assay. The columns that extend above this threshold present positive detection of ovalbumin in that sample dilution. Numbers 1, 10, 100, and 1000 on the horizontal axis (above paint samples) present the step-wise tenfold dilutions steps of the initial (1 mg/ml) sample solutions. To enable comparison, results of the same, but *aged*, paint layer (non-purified/purified) are presented in Fig. 4 in the same manner.

Positive detection of ovalbumin was possible in both, non-purified and purified reference (non-aged) samples, but for the latter, the positive response was obtained in the 100 times more diluted sample (100 times lower limit of detection) (Fig. 3). For the non-purified aged samples, the positive identification of ovalbumin was possible only in the most concentrated solution. In comparison, in the according purified sample, the signal (OD reading) was much higher. Even more importantly, the identification was positive also in the ten-fold diluted sample (Fig. 4).

Due to the large number of investigated model paint samples, the rest of the results are not presented in such a detailed manner. However, the same rigorous procedure of results evaluation (calibration curve/positive control, negative controls, LOD determination) as described in detail above (also Figs. 3 and 4) was used for all of the tested samples' combinations. The results are summarised in charts, separately for each of the studied pigments (Figs. 5-9). In these charts, the pairs of bars representing the ELISA response of the non-aged samples are situated on the left side of each section of a diagram, and of the aged on the right. It should be noted that darker columns in the pairs represent response of the ELISA test of the purified samples. Each bar in Figs. 5–9 therefore represents a series of tenfold dilutions of one

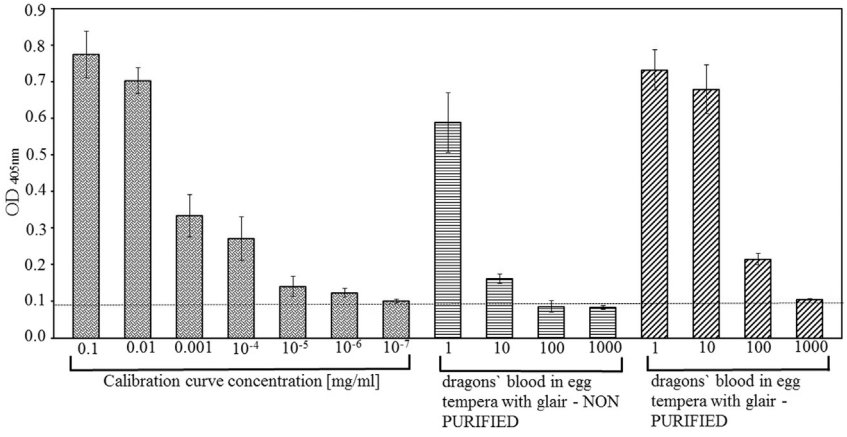

Fig. 3. ELISA optical densities obtained for (from left to right): Calibration curve concentration of standard ovalbumin solution (0.1 mg/ml of initial dried ovalbumin standard), followed by non-aged paint layers of dragons' blood in egg tempera with glair — non purified and purified. The horizontal line indicates limit of detection (LOD). The error bars represent standard deviation.

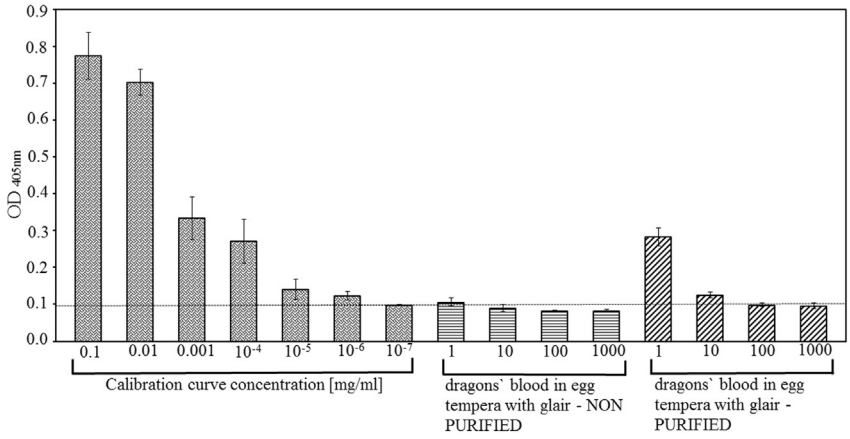

Fig. 4. ELISA optical densities obtained for (from left to right): Calibration curve concentration of standard ovalbumin solution (0.1 mg/ml of initial dried ovalbumin standard) followed by aged paint layers of dragons' blood in egg tempera with glair — non purified and purified. The horizontal line indicates limit of detection (LOD). The error bars represent standard deviation.

binder/pigment/ageing/purification sample combinations (one four-bar series presented in detail in Figs. 3 and 4). The results are given as the number of step-wise tenfold dilutions for which a positive reaction was obtained. Therefore the higher the bar, the more diluted sample was positively identified containing ovalbumin. In other words, the higher the bar, the lower concentration of ovalbumin was detected, the lower amount of initial paint layer sample would have been needed for positive identification of the said binder.

In all investigated non-aged paint layers of dragons' blood, it was evident that the purification enhanced the ELISA response for at least one tenfold dilution (Fig. 5). Interestingly, the detection of ovalbumin was possible even on the samples prepared with egg yolk, although it is known that ovalbumin is present there in a very low concentration. [33] The ageing processes clearly affected the protein in the paint layer, which was noticed as the decrease in ELISA response. However, this unfavourable effect was at least partially offset by the purification of samples, as it was possible to detect the ovalbumin in these parallels for up to one tenfold dilution higher than the respective non-purified (Fig. 5).

For the paint layers prepared with green earth (Fig. 6), the detection of ovalbumin was not possible where only egg yolk was used as a binder, probably due to the low concentration of ovalbumin in egg yolk. [33] However, when the samples contained also glair (mostly egg white, abundant in ovalbumin — see Section 2.2) surface coating, it is evident, that even thin layer of varnish, contained enough ovalbumin for "classical" ELISA detection, although having an almost negligible mass share in the entire paint layer sample. Purification enhanced the ELISA response in all cases, but especially so on aged samples of coatings (glair or mastic) on pre-prepared egg tempera paint layer. In these samples, the detection became possible only after the purification, no response was obtained with the "traditional" ELISA. The enhancement of ELISA response was obtained also for the purified paint layers prepared in egg white, egg white with glair or egg white with two coatings, but it was less pronounced (one tenfold dilution further) (Fig. 6).

In the case of malachite paint layers (Fig. 7) prepared only in egg yolk tempera, the ovalbumin was detected on both, purified and non-purified samples (the same ELISA response), but after ageing the detection of the ovalbumin was impossible even in the purified samples. Due to the higher amount of the ovalbumin present in the egg white, [33] the ELISA response is generally higher on the samples which contains glair, especially on the purified ones. As in the case of the two previously described binder/pigment systems, artificial ageing negatively influenced the ELISA detection across the board. However, in both aged and non-aged samples, the purification enhanced the ELISA response,

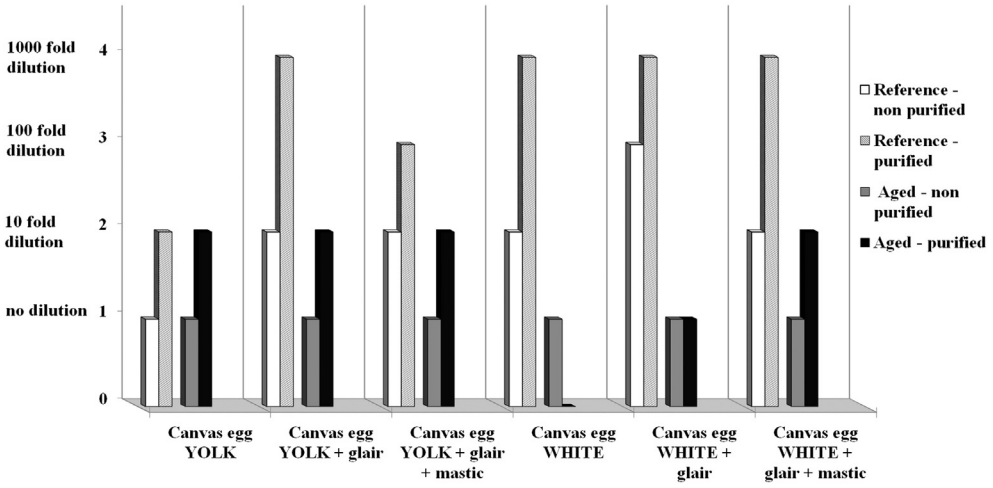

Fig. 5. The summarised ELISA response results (expressed in the number of step-wise tenfold dilutions for which a positive reaction was obtained) of variously prepared dragons' blood-containing samples. Combinations of two binders and three protective layers (along the x-axis), subjected to ageing and purification procedures (reference — non purified, reference — purified, aged — non purified, aged — purified) were assayed.

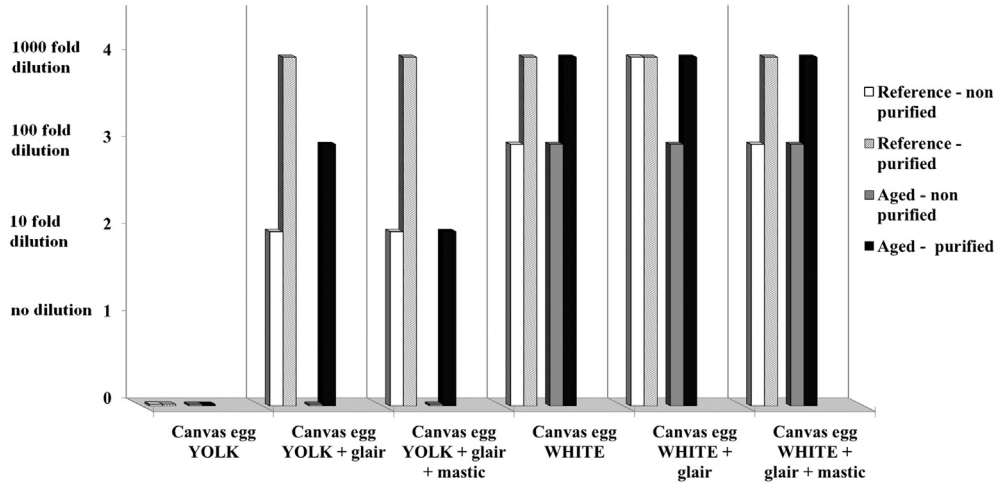

Fig. 6. The summarised ELISA response results (expressed in the number of step-wise tenfold dilutions for which a positive reaction was obtained) of variously prepared green earth-containing samples. Combinations of two binders and three protective layers (along the x-axis), subjected to ageing and purification procedures (reference — non purified, reference — purified, aged — non purified, aged — purified) were assayed.

except for aged paint layer prepared with egg white only and the non-aged sample of egg white binder and glair varnish, where it was equal for purified and non purified samples (Fig. 7).

On the lead white paint layers (Fig. 8), the influence of the pigment on the detection of protein (ovalbumin) was more prominent, regardless whether ageing procedure was applied. According to the results of the ELISA test, the general response to ovalbumin was lower in comparison to above-described paint layers (dragon's blood, malachite, and green earth). None of the more than 100-fold diluted samples yielded a positive ELISA response. Moreover, a positive response for 100-fold dilutions, and a general improvement of detection by the CIM® chromatographic monolith purification was obtained only for the samples prepared in egg white binder (higher amount of the initial target protein) (Fig. 8) [33].

The most varied results were obtained when verdigris was used as the pigment for the preparation of paint layers (Fig. 9). There were two sets of samples, reference egg tempera and aged egg white tempera, where ovalbumin could not be detected with ELISA. Conversely to the other four tested pigment/binder systems, there was also no generally consistent trend (either positive or negative) observed on either the influence of ageing, or monolith purification on the ability to detect ovalbumin by ELISA. Such a result was not completely unforeseen, as it is well-documented that verdigris paint layers (blue-green copper acetate) can undergo alteration, mostly browning, which causes irreversible, non-esthetical degradation of the paintings. This phenomenon has been subject of much research in the last decade, but the mechanism details are not yet completely understood (Fig. 9) [36–39].

It is also known that some metal ions (especially copper) can influence the catalytic activity of enzymes used in ELISA and thusly interfere with the detection of binding media. Copper ions are also a well-known catalyst in Fenton-like chemical reactions, which yield oxygen-based radicals (and other oxygen reactive species). [40–42] These can, in turn, damage the protein structure in several ways, including causing unspecific oxidation, and depolymerisation. Moreover, these ions can also change the structure (folding) of proteins by linking to the cysteine amino acid residues [43] that can therefore possibly affect its ability to bind to the microtitre plate walls and/or hinder the recognition of a target protein by an antibody, thusly interfering with ELISA on another level. As discussed above, the effect of metal ions in extracted solution was successfully eliminated with other paint samples by using the purification with CIM® chromatographic monoliths. However, in the case of

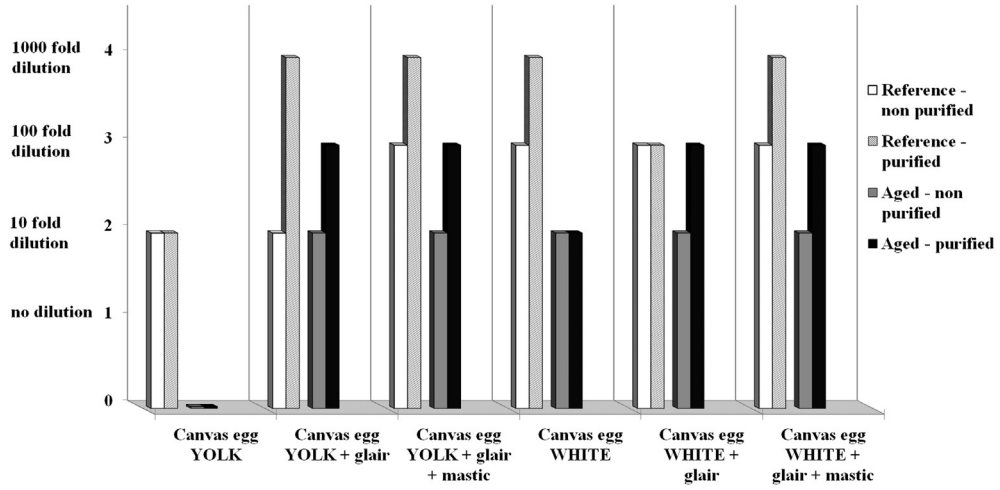

Fig. 7. The summarised ELISA response results (expressed in the number of step-wise tenfold dilutions for which a positive reaction was obtained) of variously prepared malachite-containing samples. Combinations of two binders and three protective layers (along the x-axis), subjected to ageing and purification procedures (reference — non purified, reference — purified, aged — non purified, aged — purified) were assayed.

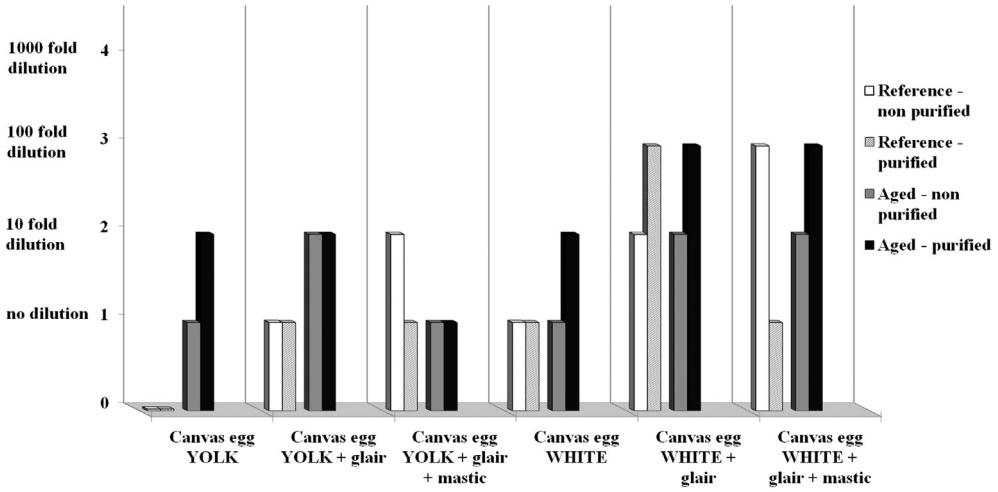

Fig. 8. The summarised ELISA response results (expressed in the number of step-wise tenfold dilutions for which a positive reaction was obtained) of variously prepared lead white-containing samples. Combinations of two binders and three protective layers (along the x-axis), subjected to ageing and purification procedures (reference — non purified, reference — purified, aged — non purified, aged — purified) were assayed.

verdigris paint layers, the problem of protein–pigment interaction could not be completely eliminated, most likely because of considerably higher solubility of verdigris in water-based buffers, and consequently higher concentration of Cu^{2+} in samples being analysed. One of the indicators that this is the case is an observation that the monolith columns were coloured blue (the general colour of diluted verdigris) after the extraction mixture was loaded onto them. They remained blue even after elution of proteins from the CIM® monolith chromatographic tip using high salt concentration buffer. Such an effect was not so pronounced when purifying paints which contained other pigments. Moreover, also the eluent from the chromatographic tip, which was supposed to contain the purified ovalbumin, was coloured lightly blue. Due to the combined effect of relatively high solubility of verdigris and good chelating capability of proteins, probably a good portion of copper remains attached onto the protein even after purification (with the undesired consequences towards the ELISA effectiveness and reliability described in this paragraph above). In order to overcome this problem, further research will be focused on the implementation of various extraction and elution buffers, with the goal to eliminate copper ions from the protein solution (extract, during chromatography, or eluent).

Overall, the amount of ovalbumin, theoretically present in the studied samples, was a significant factor in obtaining a positive ELISA response. Generally, a better response was obtained for the samples that were intended to contain a higher amount of this protein (egg white tempera, and especially all samples containing glair). Nevertheless, that ovalbumin was detected in samples containing egg yolk tempera testifies to the strength and robustness of the ELISA method, to its ability to detect even extremely low concentrations of targeted molecules. Namely, any ovalbumin present in such samples is considered to be an impurity, and a consequence of "contamination" from egg white during the manual separation of the two during binder preparation.

According to the above-presented results purification enhances the detection of the ovalbumin by ELISA in majority of the investigated paint layers, most likely due to the removal of the impurities. Moreover, since the purification improves detection, it is possible to successfully detect also aged proteins that cannot be detected by conventional methods. This finding is significant while the samples that are taken from the real works of art are mostly deteriorated and the detection of the proteins can be very difficult.

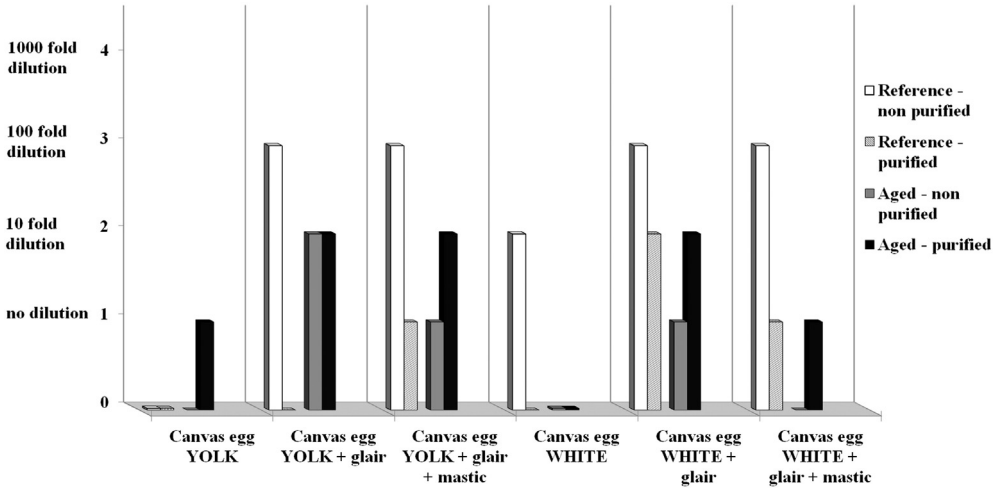

Fig. 9. The summarised ELISA response results (expressed in the number of step-wise tenfold dilutions for which a positive reaction was obtained) of variously prepared verdigris-containing samples. Combinations of two binders and three protective layers (along the x-axis), subjected to ageing and purification procedures (reference — non purified, reference — purified, aged — non purified, aged — purified) were assayed.

Fig. 10. (Left) Vittore Carpaccio, The Presentation at the Temple, 16th Century; 1 indicates area of sampling. (right) Pietro Liberi, Saint Nicholas between Saint Hermagoras and Saint Fortunatus, 17th Century; 2 indicates area of sampling.

To our best knowledge, the only similar research on the pre-purification of the protein containing paint samples that were further tested by ELISA was done by Lee et al. [22]. The authors observed that purification step using C4 columns did not have an effect on ELISA detection. Since proteins express great tendency towards formation of stable metalloprotein complexes [44], extraction of proteins from C4 support using 0.1% TFA in 50% acetonitrile probably does not disturb metalloprotein complexes sufficiently, which leads to incomplete removal of metal ions and consequently does not improve ELISA sensitivity. On the other hand, Tris, which is present in both, washing and elution buffers, has strong complexing affinity towards metal ions [45]. During the sample preparation procedure, monolith tips were washed with 25 column volumes of buffers containing Tris (0.25 mg of Tris, compared to approximately 1 mg of paint sample, from which the majority of the material remained undissolved). Therefore at least a part of metal ions were extracted from proteins to Tris buffers, which may have lead to an increase of detection sensitivity of ELISA. Additionally, preliminary SDS-PAGE investigation (data not shown) could not show consistently the amount of protein loss in the purification step, probably due to interference with metal ions which were present with non-purified samples. Even with purified samples, it was apparent that the binders mixed with lead white, and particularly so with verdigris, were either still complexed with the metals (not fully solubilised) or a fraction of them was strongly degraded, or combination thereof, as some of their band lines were also smeared. Therefore any eventual loss of investigated protein was not due to the lack of binding capacity of the monolith (Section 2.5.3) or recovery yield, which is reported to be high in the

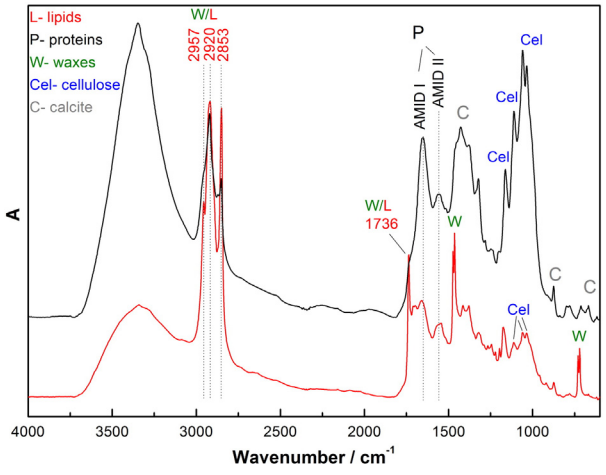

Fig. 11. FTIR spectra of the Sample 1 (Vittore Carpaccio, The Presentation at the Temple). Identified compounds are marked in the spectra.

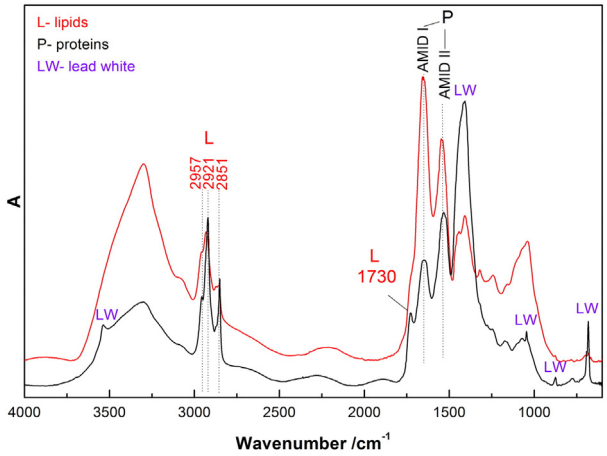

Fig. 12. FTIR spectra of the Sample 2 (Pietro Liberi, Saint Nicholas between Saint Hermagoras and Saint Fortunatus). Identified compounds are marked in the spectra.

monoliths for large biomolecules [30,31]. Inconsistencies in the enhancement of the ELISA response in some cases of lead white, and more generally in the case of verdigris-containing paints are therefore likely due to enhanced degradation of the binder influenced by the metals contained in the said pigments and not due to possible sample loss in the purification step.

3.2. Paints in art works

After successfully testing the coupling of the two powerful techniques, ELISA and CIM® monolith-supported extraction, and proving that their combination significantly improves the ability to detect ovalbumin in the model paint samples, further tests were made to validate this procedure on real works of art. In order to transfer the novel purification system and its further application to ELISA method into conservation science analytical practice, the micro-samples (approximately 1 mg) collected from the real artworks were investigated. Two art samples were analysed; from a paint layer from 16th Century painting of Vittore Carpaccio, Presentation at the Temple (Sample 1, Fig. 10 — left), followed by the paint layer of the 17th Century painting from Pietro Liberi, Saint Nicholas between Saint Hermagoras and Saint Fortunatus (Sample 2, Fig. 10 — right).

To obtain the initial, general information on the materials' composition present in the paint layers, a FTIR analysis was done on the both collected samples. According to the analysis in the Sample 1, lipids, [46] waxes, [47] cellulose (present in the textile support/canvas), [48] calcite, [49] and proteins [46] were present (Fig. 11), while in the Sample 2 lipids, proteins, and lead white [50] were determined (Fig. 12). In the both cases of the investigated bulk samples, proteins were detected (Figs. 11 and 12; marked as P), but due to the unspecific character of this method, it was impossible to determine the protein type. To overcome this challenge and to determine the source of protein found in these paintings, the ELISA test was performed, synergistically with purification by CIM® chromatographic monoliths. Since the use of egg tempera (as binder) or egg white (as finishing protective layer) had been frequently used as the masters` technique in the 16th and 17th century, the presence of protein ovalbumin (present in egg) [33] was suspected.

In the investigated paint samples, the suspected source of ovalbumin can be either egg tempera or glair. [2,33] Therefore it was expected that the relative share of ovalbumin in the mass of the removed sample would be very small, almost negligible. Moreover, the paints were naturally aged for centuries and a mixture of compounds was found in the investigated samples (see FTIR analysis), which can influence the ELISA test. However, the presented results confirm the sensitivity of the ELISA method, especially when using CIM® chromatographic monoliths purification. Positive results for the ovalbumin can be obtained even in the 10 fold dilution of the initial sample solutions (1 mg/ml), relative to the standard ovalbumin calibration curve (Fig. 13).

Finally, with the analytical results obtained by ELISA on model paints as well as on the authentic samples of V. Carpaccio (The Presentation at The Temple) and Pietro Liberi (Saint Nicholas between Saint Hermagoras and Saint Fortunatus) it was demonstrated that the synergistical employment of CIM® chromatographic monolith-supported extraction and ELISA is very effective for the detection of ovalbumin in paints.

4. Conclusions

Outstanding characteristic of the ELISA method (low limit of detection, differentiation between types of proteins, high specificity, sensitivity, accuracy and cost-efficiency) is paving the way for it to become a very important tool for the detection of proteins in different works of art. Nevertheless, the specific recognition of a target protein by an antibody (a prerequisite for a successful and most of all correct ELISA result) can be severely compromised, mostly due to the complexity of the materials present in the samples taken from the objects of Cultural Heritage and effects of ageing thereof.

To overcome these issues, chromatographic purification using CIM® chromatographic monoliths was tested as a complement to ELISA in this work, and broad synergistic action of the two powerful techniques was clearly demonstrated on a comprehensive set of 120 investigated samples. Moreover, the joint CIM® monolith chromatography/ELISA approach was successfully validated by the investigation of real works of art.

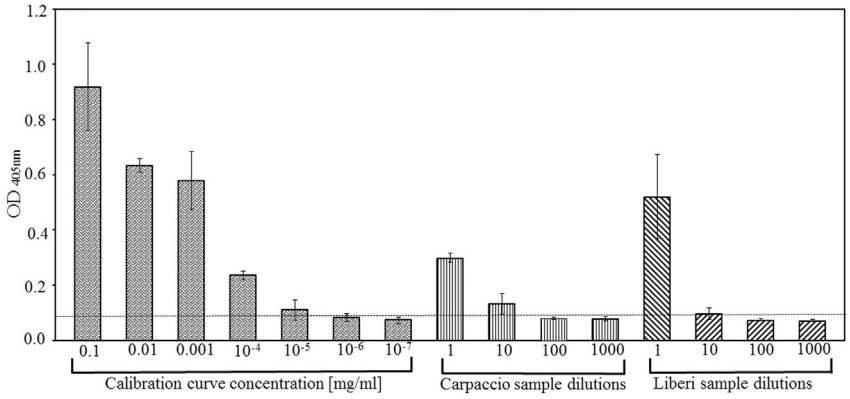

Fig. 13. ELISA optical densities obtained for (from left to right): Calibration curve concentration of standard ovalbumin solution (0.1 mg/ml of initial dried ovalbumin standard) followed by paint samples of V. Carpaccio and P. Liberi. The horizontal line indicates limit of detection (LOD). The error bar represents standard deviation.

The results also confirmed that ageing of proteins in cultural heritage binders bears a negative impact on their ability to bind antibodies raised against their recently isolated counterparts. However, since the purification improves detection, it is possible to successfully detect also aged proteins that cannot be detected by conventional methods. In practical terms that means that purification by CIM® chromatographic monoliths improves/in some cases enables the detection of aged samples. Therefore, it is strongly believed that simple prepurification and concentration of protein samples using CIM® monolith chromatography can enhance the detection also for other immunology-based (protein) identification and characterisation techniques (other types of ELISA).

The presented work is a significant breakthrough in antibody-based identification of cultural heritage material components (robustness, reliability, limit of detection). With further optimisation of the CIM® monolith chromatography purification and its integration with various types of immunology-based techniques, an expansion is foreseen for the identification of not only proteins, but also other organic components extracted from works of art.

Acknowledgements

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