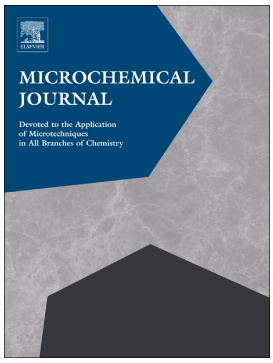

Sidestepping the challenge of casein quantification in ancient paintings by dot-blot immunoassay

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Title

Sidestepping the challenge of casein quantification in ancient paintings by dot-blot immunoassay

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Abstract

A straightforward procedure based on dot-blot immunoassay is proposed as an effective diagnostic tool suitable for detecting and quantifying milk casein in cultural heritage samples. A polyclonal primary antibody, denaturing conditions and the standard addition method were used to overcome barriers common to the traditional analysis of protein-based artistic materials, providing the possibility of achieving specific and detailed results in an easy and cost effective way. The optimized procedure detected, and successfully quantified, casein in both freshly dried and artificially aged model samples prepared with milk casein and various pigments (azurite, calcite, cinnabar, minium and red ochre). Moreover, the experiments revealed that pigments as well as artificial ageing did not differently influence the dot-blot response. Thermogravimetric analysis confirmed the obtained results. The detection and quantification of casein applied to canvas 70-years ago for conservation purposes provided final proof of the feasibility of the methodology. Despite the ageing, the complex matrix and the micro-size of the sample, it was possible to detect and quantify casein by dot-blot immunoassay. The specific and unambiguous result makes the proposed protocol a suitable procedure to recognize aged proteins with a degraded amino acid pattern.

Keywords

Dot-blot immunoassay, casein, protein quantification, cultural heritage, pigment

1. Introduction

The determination of the chemical composition of paint samples is a useful tool in helping conservators to prevent deterioration and plan conservation treatments. Moreover, an accurate knowledge of the painting materials is extremely interesting from the art history point of view, and also helps specialists assess the authenticity of artistic work [1].

In recent decades, researchers have put a lot of effort into the development of analytical methods suitable for the detection and characterization of the chemical components found in the complex structure of

paintings [2, 3]. Protein recognition, especially, is a source of great interest as artists have been using proteinaceous substances as raw material since ancient times [4]. However, the detection and identification of proteinaceous materials is challenging [5]. Indeed, the uniqueness of a sample and its dimensions, the simultaneous occurrence of organic and inorganic materials, the chemical modifications undergone by these materials over time (ageing), and the presence of non-original restoration materials all give rise to analytical problems that make the reliable identification of the proteinaceus material a complex task [1, 5, 6]. The most employed techniques for the analysis of the protein components of paintings have been, to date, chromatographic and spectroscopic techniques, due to their great versatility in obtaining analytical information from both inorganic and organic materials [1, 3, 5]. However, despite their marked contribution to organic binder studies in art work, these techniques require complex sample pre-treatment, expensive equipment and a level of knowledge and competence not common in conservation laboratories [7-9]. Moreover, most of these analytical methods give little quantitative and structural information, and proteinaceous materials are detected without the identification of their biological origin [10].

On the other hand, immunochemical techniques represent a promising alternative tool to efficiently and selectively detect proteinaceous materials as they are sensitive to nanogram quantities of protein, and are based on the highly specific antigen-antibody reaction [9].

The present work proposes a procedure based on the dot-blot immunoassay as a simple and inexpensive method to identify proteinaceous binders in samples from works of art. The dot-blot immunoassay offers the advantage of analysing, with minimal sample pre-treatment, complex samples containing a protein mixture, facing the outstanding problem of structural alterations in aged proteinaceous material, and providing unambiguous significant and detailed results of easy interpretation [11]. Recently, the dot-blot immunoassay was successfully employed for the detection and quantification of egg white in samples from works of art [12]. To the best of our knowledge, no dot-blot immunoassay protocol has been reported for the detection of other proteinaceous materials in works of art.

Among proteinaceous materials, milk and casein were a valid alternative to egg tempera for artists in the past. Though less popular than egg tempera, milk and casein have been used as paint binders [13]

especially for mural painting and polychrome objects as well as a conservation material [8, 14-17]. In this work, a dot-blot immunoassay protocol for casein detection has been set up. The protocol was optimized on pigmented model samples, analyzed both freshly dried and artificially aged, and then applied to the characterization of a naturally aged sample collected from a canvas used to detach a mural painting decorating the Monumental Cemetery walls in Pisa (Italy). Certainly the availability of an easy technique to detect and quantify casein in art work offers a significant advance in discerning casein as a major or minor organic component in artwork, helping conservators to better define deterioration processes undergone by art materials, and to choose the best and most suitable conservation treatment.

2. Materials and Methods

2.1 Reagents

Primary affinity-purified rabbit polyclonal anti-bovine casein antibody (1 mg/mL) (RCAS-10A) was purchased from the Immunology Consultants Laboratory (Portland, OR, USA). According to the manufacturer, the antibody, raised using highly purified bovine casein from milk as immunogen, does not react with the serum proteins α-lactalbumin, whey, β-lactoglobulin, or lactoferrin. Secondary goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (A3687), fish gelatine (G7765), casein from bovine milk (C7078), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (SigmaFast BCIP/NBT, B5655), chicken egg albumin (A5378), bovine milk α-lactalbumin (L6010), bovine milk β-lactoglobulin (L0130), bovine serum albumin (A2153) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calf skin collagen (234112) was purchased from Calbiochem (Merk KGaA, Darmstadt, Germany). Skimmed fresh and powdered bovine milk, as well fresh goat milk, were bought from a local market. Milk casein for conservation was obtained from the restoration product supplier Bresciani (Milano, Italy).

2.2 Samples

2.2.1 Pigmented model samples.

The optimized protocol was tested on pigmented model samples prepared according to Gambino et al. [12]. Briefly, two sets of glass slides were set up with a painted layer of a water mixture composed of milk casein for conservation and azurite ($Cu_3(CO_3)_2(OH)_2$), calcite ($CaCO_3$), cinnabar (HgS), minium (Pb_3O_4) and red ochre (Fe_2O_3), according to traditional painting techniques [18]. One set of painted glass slides was used as freshly dried samples, the other set was artificially aged in an ageing chamber Solarbox 1500e RH (Erichsen Instrumentation, Hemer, Germany). Samples were exposed for 720 h at 25 °C and 40% relative humidity with a Soda-lime glass UV filter to simulate indoor exposure, according to the normative UNI 10925: 2001 [19].

2.2.2 Sample from the Monumental Cemetery of Pisa.

The naturally aged sample was a fragment of canvas used in 1945 to detach a mural painting, and glue it onto an asbestos cement support during a conservation treatment carried out soon after the Second World War [15]. The mural painting belongs to a cycle decorating the Monumental Cemetery walls in Pisa (Italy). Previous studies carried out on samples collected from these paintings, including the paint surface itself, the layer between the canvas and the support structure and the asbestos support, always showed the presence of animal glue and casein [15, 16, 20].

2.3 Protein extraction

One mg of each sample (freshly dried, artificially aged and naturally aged) was scraped from the surface and ground to a fine powder with pestle and mortar. The powder was suspended in 1 mL of 6 M urea prepared in 100 mM Tris-HCl pH 8.0 (6 M urea-Tris). The suspension was vortexed for 15 min, centrifuged for 15 min at 11000 g at room temperature and submitted to dot-blot immunoassay. Supernatants from freshly dried and artificially aged model samples were 20-fold diluted with 6 M urea-Tris prior the dot-blot immunoassay, while supernatant from the naturally aged sample was analysed directly.

Standard proteins used in the optimization of the experimental conditions, and in the antibody specificity assay, were suspended in 6 M urea-Tris and submitted to the same extractive procedures.

2.4 Dot-blot immunoassay

2.4.1 Experimental procedure.

The dot-blot immunoassay was performed according to Gambino et al. [12] with some modifications. Briefly, a MiniFold 1 Systems dot-blotting apparatus (Whatman) was assembled according to manufacturer instructions and samples were spotted onto nitrocellulose membrane (N9763–5EA, Sigma-Aldrich, St. Louis, MO, USA), let stand for 20 min and then adsorbed by gentle vacuum application. After washing steps (four times) with Tris-buffered saline (TBS; 0.1 M Tris-HCl pH 7.4, 5 M NaCl), the dot-blotting apparatus was dismantled and the membrane was: i) blocked with 2% fish gelatine in TBS for 12 h at 4 °C; ii) incubated with the primary anti-bovine casein antibody diluted 1:8000 in TBS and 1% fish gelatine for 3 h at room temperature; iii) washed four times with 0.05% Tween 20 in TBS (TBS-T) at room temperature (4 min each washing); iv) incubated with the secondary antibody diluted 1:3000 in TBS and 1% fish gelatine for 2 h at room temperature; v) washed three times with TBS-T and once with TBS at room temperature (4 min each washing). The membrane was finally incubated with the SigmaFast BCIP/NBT chromogenic substrate dissolved in 12 mL of deionised water (1 tablet, the resulting solution contained 0.12 mg/mL BCIP, 0.25 mg/mL NBT, 83 mM Tris buffer and 4.16 mM $MgCl_2$, pH 9.25-9.75) for 4 min and stopped by dilution with 50 mL of water.

After washing three times with water, the membrane was dried on filter paper and the image was digitized using Expression 1680PRO scanner (Seiko Epson Corporation, Amsterdam, Netherlands), removing all scanner automatisms and using the scanner cover 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). 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 background density.

2.4.2 Interval of detection.

The interval of detection was established for each single assay. Three analytical replicates of 15 two-fold serial dilutions (0.2-3500 ng) of standard bovine casein in 6 M urea-Tris were spotted onto the same membrane of the sample to be analysed. Background density was obtained by spotting 3 replicates of 6M tris-urea only. Density values were plotted against standard casein quantity, and a calibration dose-response four-parameter logistic curve was obtained via GraphPad Prism (GraphPad Software, San Diego, CA, USA) providing, along with others, the bottom density and the top density value parameters. The lower limit of detection (LOD) was estimated as the amount of standard bovine casein providing a density signal equal to the bottom density value plus two times the background standard deviation [21]. Any value below this threshold was considered as not indicative of the presence of the proteinaceous binder in the sample. The upper limit of detection (UOD) was calculated as the amount of protein giving a density signal corresponding to the top density value minus two times the background standard deviation. No values above this threshold could be quantified because of the absence of linear correlation between dose and response.

2.4.3 Detection of casein.

To detect casein, three analytical replicates of 15 two-fold serial dilutions of pigmented model (0.2-3500 ng) or naturally aged (9-150000 ng) samples in 6 M urea-Tris were spotted onto nitrocellulose membrane. On the same membrane three analytical replicates of 15 two-fold serial dilutions (0.2-3500 ng) of standard casein in 6 M urea-Tris were also spotted and the interval of detection was defined. The presence of casein was confirmed when sample spots showed densitometric values over the LOD. For each pigmented model and naturally aged sample, the concentration immediately above the LOD was chosen to be used for casein quantification by the standard addition method (SAM).

2.4.4 Standard addition method.

To quantify casein in pigmented model and the naturally aged samples the SAM was applied [22-24]. The dilution of each pigmented model and naturally aged sample immediately above the LOD was spotted onto nitrocellulose membrane with the addition of three different known amounts of standard casein (1.5-15 ng). On the same membrane three analytical replicates of 15 two-fold serial dilutions (0.2-3500 ng) of standard casein in 6 M urea-Tris were also spotted to define the interval of detection. Sample density values inside the interval of detection were plotted by the added amount of standard casein, and the regression line determined. The line intercepts with both the x and y axis were calculated. The negative x-intercept represents the amount of casein in the sample while the y-intercept is the sample density in the absence of added standard casein.

Sample densities (y-intercepts) were normalised to the density of membrane co-loaded standard casein (5 ng), and the immunodetection response of casein in the presence of each pigment was calculated by dividing the normalised density response of each pigmented model sample with the respective ng of detected casein (x-intercept).

2.5 Other analyses

2.5.1 SDS-PAGE and western blot.

Denaturing gel electrophoresis (SDS-PAGE) was run under reducing conditions according to Laemmli [25], using samples in 6 M urea-Tris (5 µg of sample) properly diluted in Laemmli sample buffer. Western blot analyses were carried out by a standard protocol using the anti-casein antibody.

2.5.2 Thermogravimetric analysis (TGA).

TGA was used to determine the protein content in paint layer aliquots of the model samples as previously reported [26, 27]. The weight losses of pigment with protein (1), pigment alone (2) and protein (3) were recorded. The protein concentration was calculated from the following formula: $C=(R_2-R_1)/(R_2-R_3)$, where R is the percentage of residual mass after the heating treatment at 800°C leading to complete protein

decomposition [26]. Since cinnabar has no residual mass at 800°C, the protein content in the laboratory samples with this pigment was not determined.

2.5.3 Colorimetric method for protein quantification.

The quantitative determination of protein was performed by the Bradford colorimetric assay [28]. The analyses were carried out on both freshly dried and artificially aged model samples using a sample dilution of 0.05 mg/mL. A calibration line with bovine serum albumin in 6 M urea-Tris as a standard was used to quantify the casein content.

2.6 Statistical analysis

Each experiment was repeated at least four times and three technical replicates were performed for each experiment. Values are reported as the mean of these data. Two-tailed ANOVA and Student's *t*-test analysis via a software run in MATLAB environment (Version 7.0, The MathWorks Inc, Natick, USA) were applied to statistically evaluate any significant differences among the samples and concentrations. The ANOVA and Student's *t*-test analyses were carried out after verifying data independence (Pearson's Chi-square test), normal distribution (D'Agostino-Pearson normality test) and homogeneity of variances (Bartlett's test). 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

3.1 Optimization of experimental conditions

To set up the dot-blot immunoassay procedure for casein detection and quantification preliminary experimental optimization was carried out, taking into account protein extraction and primary antibody operating titer and specificity.

3.1.1 Sample protein extraction.

To find the best extraction procedure, bovine casein (10 mg) was solubilised into the same volume (1 mL) of i) water, ii) 6 M urea, iii) 6 M urea pH 8.2 (NaOH), iv) 6 M urea-Tris, and v) 100 mM Tris-HCl pH 8.0, and submitted to the extractive steps reported in the 'Materials and Methods' section. The amount of solubilised protein was quantified by Bradford assay, and its percentage with respect to the amount of initially suspended bovine casein was calculated. The results revealed that water, 6 M urea and 6 M urea pH 8.2 poorly solubilise bovine casein (dissolved casein: water: 17.5 ± 2.3%, 6 M urea: 49.5 ± 3.7%, 6 M urea pH 8.2: 36.9 ± 2.4%), whereas 100 mM Tris-HCl pH 8.0 and 6 M urea-Tris were significantly more effective in dissolving casein (dissolved casein: 100 mM Tris-HCl pH 8.0: 90.4 ± 2.9%; 6 M urea-Tris: 85.3 ± 4.7%; statistically comparable values). Indeed, of all the tested extraction solutions, 6 M urea-Tris was selected as the best compromise for the study; it guaranteed both the strong denaturing conditions (due to the presence of urea) needed to detach the protein component from the sample matrix, and an alkaline pH to keep the casein in solution, thus providing conditions suitable for a quick and easy extraction procedure.

3.1.2 Primary antibody titration.

To ensure the best performance of the dot-blot immunoassay, an optimum primary antibody working concentration was determined. Replicates of 400 ng of bovine casein were spotted onto the nitrocellulose membrane and, after the blocking step, membrane strips were incubated with different two-fold serial dilutions (from 1:1000 to 1:2049000) of the primary antibody, followed by incubation with a single secondary-antibody dilution. The responses were plotted in a titration curve fitted to a four-parameter logistic function. The antibody concentration that gave a response equal to 70% of the maximum response was chosen as the optimal primary antibody working dilution, because it was considered the best compromise, between the highest analyte detection under saturating conditions and the lowest background, to favour analytic sensitivity. According to this criterion, the optimal primary antibody working dilution was established to be 1:8000 (0.125 µg/mL).

3.1.3 Antibody specificity.

The primary antibody specificity was tested applying the casein-optimized dot-blot immunoassay protocol to different amounts (from 200 to 7000 ng) of standard bovine casein, chicken egg albumin, calf skin collagen and common milk proteins such as α-lactalbumin, β-lactoglobulin and bovine serum albumin (BSA), as well as milk casein sold as conservation material. Model samples prepared with egg white, milk casein and animal glue as binders were also included in this analysis. Bovine casein and model samples prepared with milk casein showed an evident positive response. On the contrary, α-lactalbumin, β-lactoglobulin, BSA, chicken egg albumin, calf skin collagen and model samples containing egg or animal glue showed negative responses with density values below the LOD (Figure 1A).

The antibody specificity was also checked by applying the Western-blot to α-lactalbumin, β-lactoglobulin and BSA commonly found in milk, and to goat and bovine skimmed fresh milk as well as powdered bovine milk. The presence of casein was observable. The antibody recognized casein in both bovine milk and goat milk, but not α-lactalbumin, β-lactoglobulin and BSA (Figure 1B).

Given the compliance between the Western-blot and dot-blot immunoassay results, the specificity of the primary antibody in the casein detection was confirmed.

3.2 Application of dot-blot immunoassay to pigmented model and naturally aged samples

3.2.1 Interval of detection.

The interval of detection was calculated for each dot-blot immunoassay. The average LOD, corresponding to the minimal average amount of standard bovine casein detectable by the dot-blot immunoassay, resulted 2.88 ± 0.04 ng, while the average UOD, corresponding to the maximum average amount of standard bovine casein detectable by the dot-blot immunoassay, was found to be 493.43 ± 53.17 ng.

3.2.2 Pigmented model samples.

Proteins extracted from both freshly dried and artificially aged model samples were tested by dot-blot immunoassay, and the standard addition method (SAM) was used to sidestep the matrix effect of the inorganic materials on the quantitative determination of milk casein. For both freshly dried and artificially aged pigmented model samples, the dilution of extracted proteins corresponding to 6.8 ng of sample gave back a casein immunodetection response immediately above the LOD. This dilution was chosen to be used in the SAM. Table 1 shows the amount of bovine casein specifically immunodetected by applying the SAM, in both freshly dried and artificially aged samples, compared to the amount of total protein detected by the Bradford assay. To further validate the methods, the dot-blot immunoassay results were also compared to those obtained on the same sample with TGA. No statistically significant differences were observed between the casein quantified by dot-blot immunoassay and the protein amount obtained by TGA in both freshly dried and artificially aged samples. Moreover, the casein amount detected by dot-blot immunoassay was statistically higher than that detected by the Bradford assay, except for the calcite freshly dried sample where the Bradford assay revealed a protein amount no different from that found by TGA and dot-blot immunoassay.

The immunodetection responses from the different pigmented model samples were also compared at fixed casein concentrations (Fig. 2). The results demonstrated that neither pigments, nor natural and artificial ageing gave rise to significant differences in the immunodetection responses. Therefore, no differences were detected between the freshly dried and the corresponding artificially aged pigmented model samples.

3.2.3 Naturally aged sample.

To evaluate the procedure's performance on naturally aged samples, a sample taken from a canvas used in a post-World War II restoration to detach a mural painting from the Monumental Cemetery in Pisa, (Italy), was analysed for casein content. Standard bovine casein was also analysed and used to define the interval of detection. First, 15 two-fold serial dilutions of the real sample extract were processed. Spots between 4.7 and 150 μg showed densitometric values over that of the lower limit of detection, confirming the

presence of milk casein in the naturally aged sample (Figure 3A and 3B). Therefore, dot-blot immunoassay with SAM was applied to quantify the amount of casein present, overcoming the interference effect of the inorganic matrix. As the proteins extracted from 4.7 µg of scraped material gave a milk casein density response immediately above that of the LOD they were spotted on nitrocellulose membrane with the addition of 1.5, 7 and 15 ng of standard bovine casein (Figure 3C and 3D). SAM revealed 15 ± 1 ng of milk casein in the canvas, corresponding to 0.32% of the weight.

4. Discussion

In previous work published by our research group, a systematic study was carried out to establish the best appropriate experimental conditions for the detection and quantification of egg white in artworks by dot-blot immunoassay [12]. Although in the past work the basic parameters were already set up, i.e. sample leakage, background evaluation, cross-contamination, colorimetric reaction and densitometric analysis, we further optimized other experimental conditions in this work in order to apply the previous protocol to the detection of milk casein: sample protein extraction as well as primary antibody and titration antibody specificity were set up.

Indeed, protein extraction in the field of art is recognised to be a complex task. The complexity of matrices and the poor solubility of aged proteins challenge the separation of the protein fraction from the inorganic part [7]. Complexation with pigments, aggregation as well as cross-linking phenomena, further affects protein solubility, thus affecting the amount of retrievable protein [4, 29-33]. Moreover, casein is characterized by a high level of hydrophobicity, a relatively high charge, a limited α-helix and β-sheet secondary structure, and its solubility is strongly pH dependent (minimal solubility at pH 4.5 with a gradual increase with alkalinization) [34, 35]. Considering these problems, in the present study different options for protein extraction were tested to find the most efficient extraction method able to solubilise the greatest amount of protein in only one step, and, at the same time, to limit the risks of sample loss and sample damage. The 6 M urea-Tris solution (6 M urea in Tris buffer at pH 8.0; see ‘Materials and Methods’) was chosen from among the tested extracting solutions, as urea has a strong denaturing power while the

alkaline pH is able to solubilise casein. Colombini et al. [20] reported that ammonia is a valuable alternative to extract proteinaceus binding media from not-aged painting samples, giving a quantitative recovery for casein. In addition, the pH used for extraction limits the solubility of several inorganic salts deriving from pigments and paint supports, reducing their interference with the analytical procedure [20].

In order to produce a methodology that can be easily put into practice by commonly-equipped museum and conservation laboratories, a commercially available primary antibody was chosen. Thus the selected primary antibody had been used by Cartechini et al. [9] and Arslanoglu et al. [36] to successfully detect casein samples in cultural heritage. Indeed, the choice of a polyclonal antibody enhances the chances of identifying degraded proteins as multiple epitopes can be probed [37]. This is very important in our case as antigens degrade over time and potential epitopes might be lost. Moreover, the use of an enzyme-conjugated secondary antibody amplifies, by several orders of magnitude, the immunoreaction of casein. Previous studies have adopted the same antibody in the Enzyme Linked Immunosorbent Assay (ELISA) and SERS Nanoplex biotags techniques [9, 36]. Thus specific conditions to use this primary antibody in the dot-blot immunoassay were set-up in order to achieve the best performance with the minimum background. Indeed, taking into account that the analytical response is highly dependent on the amount of primary antibody able to specifically bind protein, the primary antibody titer was evaluated. Moreover, in order to avoid false positive results, its specificity was tested. The results demonstrated that the antibody is highly specific as it reacted only with standard bovine casein or samples containing casein, there being no response in the case of negative controls, in accordance with data reported in the literature [9, 36].

The optimized dot-blot immunoassay protocol was applied to model paints, which were analysed before and after artificial light-ageing in indoor conditions. It has been reported that ageing strongly affects casein structure in the presence of pigments, leading to the formation of aggregates, with the complete disappearance of intermolecular β-sheets and an increase in intramolecular β-sheets and random coils [4]. Moreover, the tertiary and quaternary structures of proteins change due to cross-linking reactions among the functional groups of the protein chains [4, 32]. The promising results obtained with the artificially aged samples suggested that the primary anti-casein antibody was able to quantitatively detect proteins

undergoing degradation processes, without losing sensitivity. Previous work based on the same antibody revealed a reduction in casein detectability when pigmented samples were submitted to artificial ageing [36]. Although this needs further investigation, a possible explanation for this different behaviour could be ascribable, at least in part, to the fact that in our case the samples were prepared and submitted to the dot-blot immunoassay under denaturing conditions.

In this research, the standard addition method (SAM) was successfully proposed to quantify the proteins present in each sample, overcoming the matrix effect due to pigments. An overview of the literature on the study and characterization of proteinaceous materials in painting samples indicates that inorganic compounds deriving from the support or the pigments could interfere with the analysis [38, 39]. Ren et al. [32] showed that pigments containing copper, e.g. azurite, accelerate protein degradation significantly while Colombini et al. [20] found that pigments containing iron (e.g. red ochre) and calcium (e.g. calcite) interfere drastically in analytical procedures. Detailed research using gas chromatographic-mass spectrometric analytical methods has reported that metal cations, including Hg^{2+}, Fe^{3+}, Cu^{2+}, $Pb^{2+,}$ Cd^{2+}, Zn^{2+}, and Ca^{2+}, and anions from pigments can act directly on protein structure stability because of their interaction with amino acid functional groups, or indirectly, promoting oxidative stress. In turn, oxidative stress can induce global unfolding rearrangements in proteins, which hampers the analytical identification of the proteinaceous binder [6, 38].

Gambino et al. [12] found that dot-blot immunodetected ovalbumin in pigmented model samples was significantly overestimated with respect to the protein amount. In line with previous literature, the preliminary experiments (see supplementary material S1) performed with the cinnabar freshly dried model sample showed that immunodetection of casein was biased by the occurrence of a matrix effect. Indeed, the amount of casein in the sample were overestimated by 32.1 ± 13.8 % compared to that obtained by applying SAM. According to IUPAC recommendations [24], a verified or expected matrix effect should be matched against calibration samples or, if these are unavailable as in the case of heterogeneous artworks samples, SAM should be used for quantification. SAM applied to dot-blot immunoassay of our samples displayed results in lines with those achieved by TGA (Table 1). On the other hand, the Bradford assay gave

back a lower amount of casein than SAM. However, it is possible to ascribe these differences to a previously reported underestimation problem in the Bradford assay mainly due to a lower reactivity of the dye toward casein than to the protein used for calibration (BSA) [40, 41]. Consequently, SAM applied to dot-blot immunoassay could be considered a correct method to be applied to artwork samples, which are heterogeneous and have complex, diversified, unknown or scarcely characterized matrices.

The analysis of the immunodetection response at a fixed casein concentration revealed no statistical differences among samples, suggesting that, under the denaturing conditions chosen for the protein extraction, pigments, even aged, do not show a different matrix effect.

Finally, our methodology was used to investigate the presence of casein in a naturally aged sample collected from the Monumental Cemetery in Pisa (Italy). During the Second World War, an incendiary bomb exploded in the cemetery, burning the wood and melting the lead of the roof. During the conservation treatment carried out soon after the Second World War, the paintings were detached from the walls using animal glue, and were subsequently glued onto canvas using a mixture of casein and calcium hydroxide. The canvases were later relocated onto asbestos cement supports [15, 16]. Previous research demonstrated that the samples collected from the Monumental Cemetery in Pisa are characterized by widespread degradation derived from the peculiar environmental conservation conditions [15]. Colombini et al. [20] reported that proteins in these samples are drastically altered and Leo et al. [16] showed, in all their samples, the widespread presence of malonic acid, a consequence of serine, phenylalanine and cysteine oxidation. Deamidation of asparagine and glutamine residues as a molecular signature of ageing and alkaline treatment of the casein was also observed.

In our research, 1 mg of a naturally aged sample was sufficient to quantify the amount of casein and to confirm obtained values through four replicates. Dot-blot immunoassay by SAM successfully detected a casein amount of 15 ± 1 ng, corresponding to 0.32% of the sample weight. In our opinion, in a condition of protein structure degradation, the detection and quantification of casein using a commercial antibody raised against not-aged protein can be considered an interesting result.

5. Conclusion

In this study a simple and straightforward procedure based on dot-blot immunoassay was validated as an effective diagnostic tool, suitable for detecting and quantifying milk casein in cultural heritage. Sensitive to nanograms of protein, this procedure addresses conservators' needs to obtain unambiguous, significant and detailed results of easy interpretation in a rapid, easy and inexpensive way. Moreover, compared to traditional analytical methods, the proposed dot-blot immunoassay method has the advantage of resolving complex protein mixtures, also discriminating their biological sources, allowing the opportunity to overcome common barriers found in the traditional analysis of protein-based materials used by artists, i.e. the challenge of matrix interference. Finally, the availability of commercially manufactured reagents and cheap equipment would give even simple laboratories easy access to the methodology.

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Table

Sample pigment	Freshly dried samples			Artificially aged samples		
	Protein amount (ng)			Protein amount (ng)		
	Bradford	TGA	Immunodetected bovine casein (ng)	Bradford	TGA	Immunodetected bovine casein (ng)
Azurite	2.3±0.06 [a]	3.1± 0.2 [b]	3.4±0.1 [b]	0.6±0.01 [a]	2.3±0.1 [b]	2.5±0.3 [b]
Calcite	3.0 ±0.03 [a]	3.5 ±0.2 [a]	3.3±0.5 [a]	1.7±0.04 [a]	3.6±0.2 [b]	3.5±0.3 [b]
Cinnabar	3.2 ±0.05 [a]	n.d	3.9± 0.3 [b]	3.0±0.01 [a]	n.d.	3.4±0.2 [b]
Minium	1.1±0.00 [a]	1.8±0.1 [b]	1.7±0.1 [b]	1.5±0.03 [a]	2.0±0.1 [b]	2.1±0.3 [b]
Red ochre	3.7±0.07 [a]	4.7±0.2 [b]	4.7±0.5 [b]	2.6±0.04 [a]	3.9±0.2 [b]	3.9±0.3 [b]

Table 1. Protein and bovine casein quantification in freshly dried and artificially aged samples. Data represent the mean ± standard deviation (n=4) of the amount of protein quantified by a colorimetric method (Bradford) and TGA, and the amount of immunodetected bovine casein by applying the SAM to the dot-blot immunoassay, in pigmented model samples (6.8 ng). Different superscript letters indicate significant differences (Tukey's HSD, $p \leq 0.05$) between the three methods. n.d., not detectable.

Figure

Figure 1. Immunospecificity of the anti-casein antibody. Representative dot-blot immunoassay (panel A) and Western Blot analysis (panel B) of different amounts of proteinaceus material. In the panel B, SDS-PAGE (left) of the corresponding Western Blot membrane (right) is reported. Standard bovine casein (CA), milk casein for conservation (CA-R), model painting sample prepared with casein (CA-P), chicken egg albumin (AL), model painting sample prepared with egg white (AL-P), calf skin collagen (COL), model painting sample prepared with animal glue (COL-P), standard α-lactalbumin (LA), standard β-lactoglobulin (LG), bovine serum albumin (BSA), powder bovine milk (BM-P), skim fresh bovine milk (BM-F) and fresh goat milk (GM-F). The negative controls (6 M urea-Tris in the absence of sample) is indicated as 0.

Figure 2. Normalised immune-detection response of each pigmented model sample. Data represent the mean ± standard deviation (n=4).

Figure 3. Dot-blot immunoassay of a naturally aged sample from a 70-years old canvas. panel A: Representative dot-blot immunoassay membrane loaded with 15 two fold serial dilutions of the casein extracted from the naturally aged sample (from 150000 ng to 9.2 ng, respectively from spot 1 to 15) and of the standard bovine casein (from 3500 ng to 0.2 ng respectively from spot 1 to 15), used to define the interval of detection. Spot 0 refers to the negative control, loaded with 6 M urea-Tris only. panel B: Density value of standard bovine casein (circle) and naturally aged sample (triangle) against the sample quantity. Data are reported as means ± standard deviation of at least four independent replicates. The logistic dose-response curve of standard bovine casein and the interval of detection are reported (UOD, upper limit of detection; LOD, lower limit of detection). panel C: Representative dot-blot immunoassay membrane obtained by applying the SAM. Spots correspond to four replicates of the naturally aged sample dilution immediately above the LOD with the addition of 1.5, 7 and 15 ng of standard bovine casein. The negative control loaded with 6 M urea-Tris in the absence of the sample is indicated as (0). panel D: SAM regression line obtained by plotting the density value of the naturally aged sample against the amount of the added

standard bovine casein. The line equation, the goodness of fit (R^2) and the x-intercept are reported. Negative value of x-intercept corresponds to the amount of casein in the sample.

Sidestepping the challenge of casein quantification in ancient paintings by dot-blot immunoassay

Cristina Cattò, Michela Gambino, Francesca Cappitelli, Celia Duce, Ilaria Bonaduce, Fabio Forlani

SUPPLEMENTARY MATERIAL

Fifteen two-fold serial dilutions of a cinnabar freshly dried model (0.2-3500 ng) sample in 6 M urea-Tris and 15 two-fold serial dilutions (0.2-3500 ng) of standard casein in 6 M urea-Tris were spotted onto nitrocellulose membrane, and submitted to dot-blot immunoassay analysis, as reported in the '2.4.1 Experimental procedure' section of the main manuscript. Three analytical replicates of each dilution were spotted. A calibration dose-response four-parameter logistic curve was obtained via GraphPad Prism (GraphPad Software, San Diego, CA) and the interval of detection was defined according to the '2.4.2 interval of detection section' of the main manuscript. Casein in the cinnabar freshly dried sample was quantified by interpolating the cinnabar freshly dried sample densitometric values included in the interval of detection against the standard casein calibration curve (SCC).

Casein quantification by SCC showed that the cinnabar freshly dried sample contained 75.7±7.9 % of bovine casein, significantly more than that found in the same sample by dot-blot immunoassay through standard addition method (SAM) (Figure S1, Table S1). Indeed, the amount of casein by SCC was overestimated by 32.1±13.8 %, compared to that obtained by SAM.

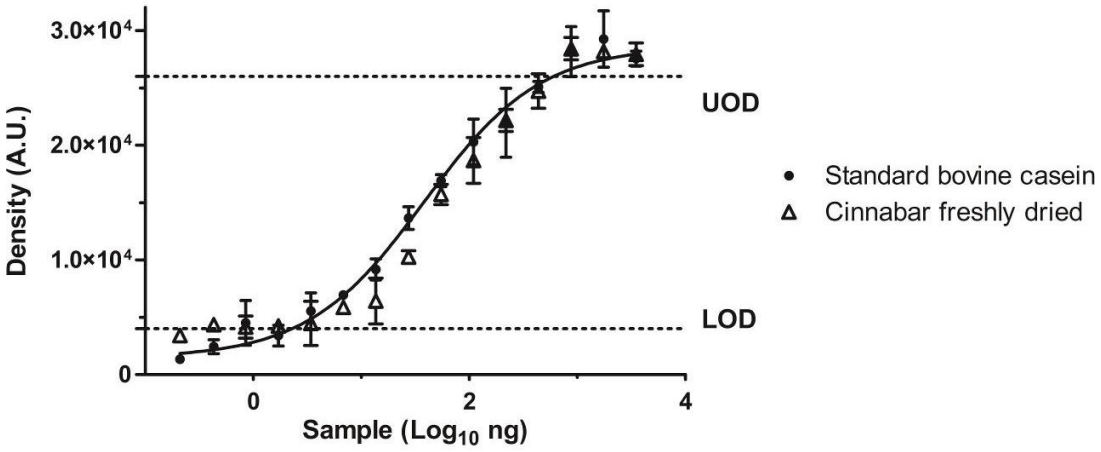

Figure S1. Bovine casein quantification by SCC in cinnabar freshly dried sample. Density value of standard bovine casein (circle) and cinnabar freshly dried (triangle) against the sample quantity. Data are reported as means ± standard deviation of at least four independent replicates. The logistic dose-response curve of standard bovine casein and the interval of detection are reported (UOD, upper limit of detection; LOD, lower limit of detection)

Immunodetected bovine casein (ng)	
SAM	SCC
3.9± 0.3 [a]	5.1±0.4 [b]

Table S1. Protein and bovine casein quantification in cinnabar freshly dried sample. Data represent the mean ± standard deviation (n=4) of the amount of immunodetected bovine casein by applying the SAM and the SCC to the dot-blot immunoassay, in pigmented model samples (6.8 ng). Different superscript letters indicate significant differences (Tukey's HSD, $p \leq 0.05$) between the methods.

Highlights

A simple and straightforward procedure based on dot-blot immunoassay was developed for detecting and quantifying milk casein in cultural heritage.
A quick and easy extraction procedure of the sample was guaranteed applying denaturing conditions.
The interval of detection ranged between 2.88 and 493.43 ng of casein.
No differences were revealed comparing dot-blot immunoassay with thermogravimetric analyses of pigmented samples.
Dot-blot immunoassay successfully detected and quantified casein in a naturally-aged sample collected from a canvas used 70-years ago to detach a mural painting decorating the Monumental Cemetery walls in Pisa (Italy).