The Biochemistry and Artistic studies: a novel integrated approach to the identification of proteinaceous binders in polychrome artifacts

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Abstract

The paper proposes a novel, integrated approach to the identification and quantification of the organic binders, mainly proteinaceous, as the "invisible" component of polychrome layers in artifacts belonging to movable and immovable cultural heritage. The analytical methodology discussed here will demonstrate the significance and efficiency of biochemical assays for the detection of organic binders and how these tests can improve/complement the already existing techniques, while considering also the costs and time of analysis that commonly limit their application in the current laboratory practice for artworks diagnosis.

Keywords

Biochemical analyses, organic binders, identification and quantification, works of art.

Resumo

Este artigo propõe uma abordagem nova e integrada para a identificação e quantificação aglutinantes orgânicos, na sua maioria proteicos, como componentes invisíveis das camadas policromadas dos objectos que integram o património móvel e imóvel. A discussão da metodologia analítica que aqui se faz, demonstrará a importância da eficácia dos ensaios bioquímicos na detecção de aglutinantes orgânicos e o modo como estes testes podem ser melhorados e complementar as técnicas analíticas disponíveis, tendo em conta os factores custo e tempo de realização das análises, em geral condicionantes que limitam a sua aplicação na prática corrente do trabalho laboratorial de diagnóstico em obras de arte.

Palavras-chave

Análises bioquímicas, aglutinantes orgânicos, identificação e quantificação, obras de arte.

Resumen

Este trabajo propone una novedosa aproximación para la identificación y cuantificación de los aglutinantes, fundamentalmente a base de proteínas, que forman capas "invisibles" como componentes de la policromía en objetos muebles e inmuebles del patrimonio cultural. La metodología analítica presentada demostrará la eficiencia de los ensayos bioquímicos para la detección de aglutinantes orgánicos y cómo esos tests pueden complementar las

técnicas ya existentes, incluso considerando también los costes y tiempo necesarios para los análisis que normalmente limitan aquella posible aplicación en el diagnóstico de objetos artísticos en la práctica habitual de laboratorio.

Palabras clave

Análisis bioquímicos, aglutinantes orgánicos, identificación y cuantificación, obras de arte.

Introduction

Conservation Science is a cross-bridging field, in which disciplines such as *Art History*, *Artistic Studies*, *Chemistry*, *Biochemistry*, *Physics*, contribute to further improve the knowledge and practice for the conservation of Cultural Heritage. Polychrome works of art typically present a complex history and composition, thus often requiring thorough identification of the constituent materials for the purpose of authentication [1-8] or before any conservation intervention can be carried out. The analytical effort is particularly justified when the history of the art-object is not well known, or when past restoration interventions are to be expected, as part of the anamnesis of the object. Knowledge obtained from the study of artists' materials and techniques contributes to our understanding of art history, improves the decision making process of conservators and reveals compositional changes of painting materials due to ageing, weathering and environmental factors [1-11]. In many cases, conservation scientists are asked to help with the authentication of art-objects, through the identification of the constituent materials and their degree of ageing [2-3, 6-13]. Therefore, the identification and quantification of organic binders is a major step in the study and authentication of polychrome artifacts, many times being crucial for understanding and recognizing the existing historical, artistic and technical information.

The present article proposes an innovative complementary methodology useful for the identification and quantification of proteinaceous binders and adhesives in polychrome artifacts. Considering the analytical difficulties that conservation scientists encounter when performing qualitative and quantitative analysis on organic paint materials, the paper aims to underline the potential and benefits of combining traditional and innovative techniques for an improved and accurate characterization of proteinaceous binders in polychrome art-objects from cultural heritage, showing some preliminary results obtained through the use of biochemical assays and mass-spectroscopic analyses.

1. State-of-the-Art of Proteinaceous Binders Characterization

A paint sample is a very small multilayered fragment (less than 0.5 mg), with a complex composition and structure, where the organic binder is present in quantities of 20% or less [9-11].

The identification and quantification of proteinaceous media in polychrome artworks is still considered a difficult challenge for the conservation scientist because:

- aged proteins are denaturated and scarcely soluble in water and organic solvents;
- several organic substances or pollution contaminants may be simultaneously present in the polychrome structure (especially if the objects undergone restoration interventions);
- during ageing different degradation compounds can be formed,
- a low protein content (at most 0.2 mg) is generally encountered in small heterogeneous paint samples (of 1 mg or less).

Errors or contamination in phase of sampling organic materials can lead to erroneous results or to their misinterpretation [9-12]. An important help in this preliminary phase can be received by applying non invasive techniques (UV fluorescence, IR reflectography, reflectance spectroscopy, colorimetry), in order to be able to choose the right spot where collecting materials [Fig. 1].

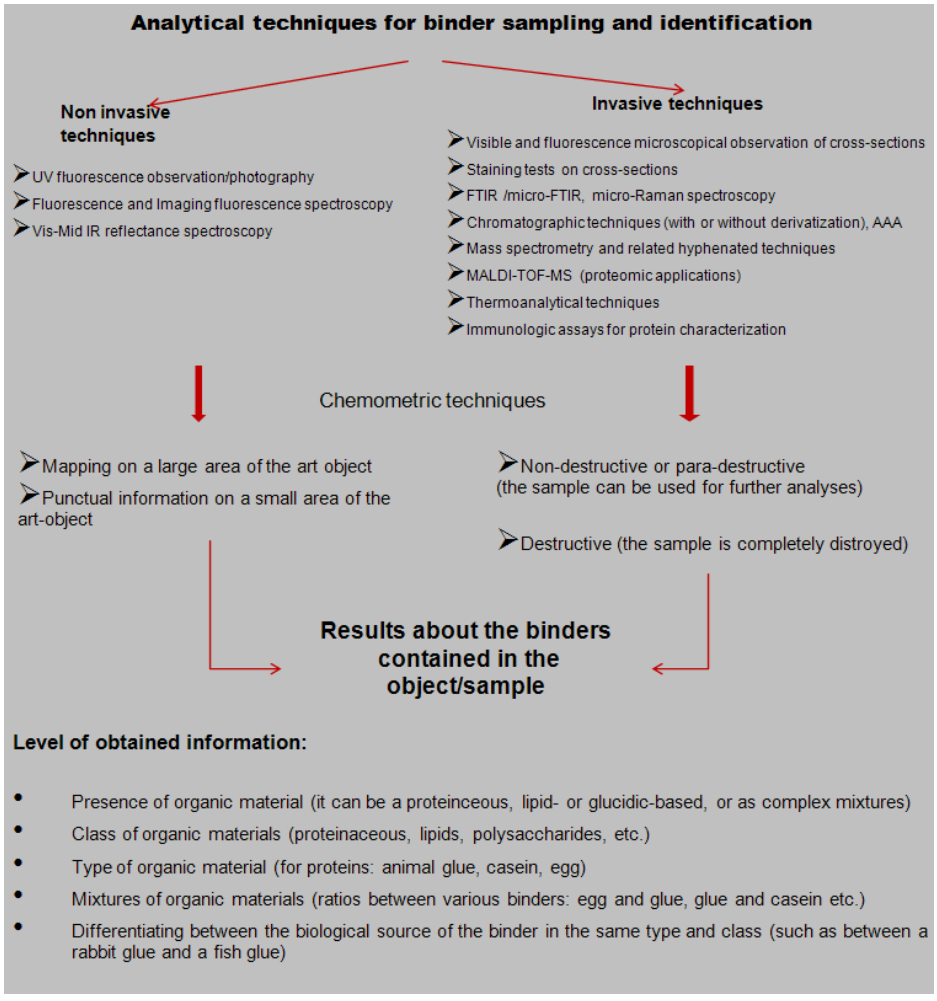

Figure 1 - Analytical techniques useful for sampling of polychrome artworks and identification of organic binders

Many techniques can be applied for the identification of paint constituents of polychrome layers. Binding media analysis can be basically performed with chromatographic and spectrometric techniques: Gas-Chromatography (GC), Pyrolysis-Gas Chromatography–Mass Spectrometry (Py-GC/MS), Liquid Chromatography (LC), High Performance Liquid Chromatography (HPLC), paper and thin layer chromatography, Size Exclusion Chromatography (SEC), Direct Temperature-Resolved Mass spectrometry (DTMS), Static Secondary Ion Mass Spectrometry (SIMS), UV-Vis and Fourier Transformed Infrared spectroscopy (FTIR), Imaging Spectroscopy (IS) etc. [13-30].

Major limitations are related with the inherently small sample size [1-2, 9-11] and quantity (up to 500 µg) [2, 6, 9-10] or with the difficulty in re-solubilizing certain binding media (such as "egg tempera") once they have dried into the paint film [2, 11, 14-18]. In addition many techniques imply sample deterioration during manipulation or preparation prior analysis [1, 9, 14-19, 23-27, 29, 32-43]; difficulties in finding appropriate extraction conditions of proteins from the binding medium without hydrolysis [9, 13-18]; and in developing analytical methods closely adapted to the small sample amount (possibly in the NANOgram order) [1, 9, 42].

As a general rule, natural organic binders are best identified by a combination of two or more different, but complementary techniques [1, 3-4, 9-11, 29, 40]. Traditional techniques (microscopy, staining tests, spectroscopy, chromatography) have been employed with good results since the '30s, but in many cases the dedicated laboratories did not develop a real methodology, with reduced costs and minimum losses of material [9-30]. Furthermore, the precise quantification of binders in complex mixtures was rarely achieved. Few innovative techniques (MALDI-TOF-MS, immune-enzymatic assays) were reported lately for identification of natural binders, but further research still has to be done [31-45].

The proteinaceous binders have a similar emission of fluorescence (around 440 nm) therefore their identification through UV fluorescence mapping on the paint surface or in cross-section is not fully and exclusively indicative; in exchange the pigments can give specific fluorescence, the pigment being able to remove the binder's fluorescence [1, 19, 24, 39].

Since the '30s, studies were developed regarding the application of **staining tests** to the identification of binders in cross-sections [1, 9, 19-24]. Microscopic staining techniques allow cross-section examination but they lack specificity, having been used to distinguish between oil and protein binders since the early part of XXth century (*Ostwald*-1935, *Plesters*-1956, *Gay*-1978, *Johnson and Packard*-1971, *Masschelein-Kleiner*-1986) [1, 19, 21, 24]. More recently, staining techniques have been proposed for detection of natural resins and carbohydrates (*Johnson and Packard*-1971, *Talbot*-1982, *Wolbers and Landrey*-1987, *Messinger*-1992) [1, 19, 22-23]. Other investigations were performed with special microscopic techniques for the examination of cross-sections [9, 20, 22-23]. A new

use of non-covalent fluorescent dyes was also reported for mapping of proteins in the field of the microscopic characterization of paint materials within multilayered samples (cross-sections) [46]. Micro-spectrofluorimetric techniques were described as successful in the identification of organic binding media and varnishes on cross-sections of paint materials [47-48], but further experiments are required for quantifying the fluorescent radiation emitted by complex mixtures of binders.

Fourier Transform Infrared (FTIR/micro-FTIR) and micro-Raman spectroscopy can also provide important information [9-10, 13, 25-30]. FTIR and micro-FTIR are commonly used to recognize the presence of proteinaceous materials in paint layers [1-4, 25-26, 29], but in case of complex mixtures the interpretation of the spectra may be extremely arduous and the distinction between egg, glue, and milk proteins can be performed only in few cases. The differentiation between "yolk tempera" and "mixed tempera" techniques is still difficult, as in the case of "tempera grassa" to be distinguished from a "egg yolk paint" as shown in Fig. 2 [3-4, 25].

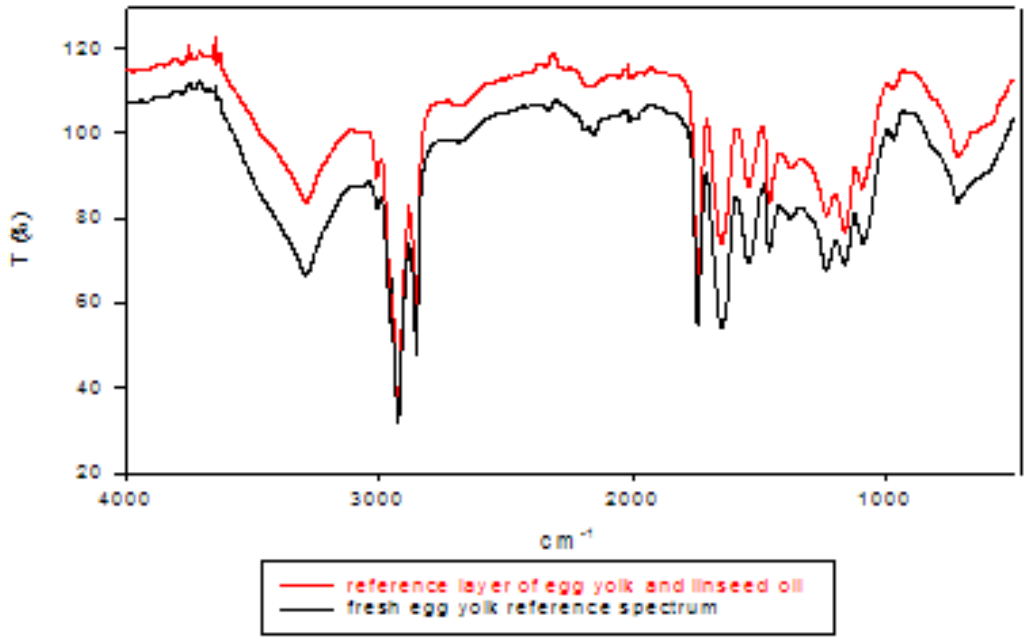

Figure 2 - Comparison of the FTIR spectra of an "egg yolk tempera" and a "mixed" technique (egg tempera and oil)

Furthermore, the presence of major proportions of inorganic matter such as paint extenders and pigments as well as organic colorants, can make (micro-)FTIR and RAMAN detection very difficult or even impossible [13, 26, 28].

The chromatographic techniques usually require time consuming sample pre-treatment but can offer good information when mixtures of oils and proteins are present, although there are several restrictions if mixtures of oil and egg are present [9, 14-17]. A protocol

for a reliable chromatographic identification of organic materials in artworks reports the application of two analytical procedures on *different aliquots* of the same micro-sample [15]. The use of PY silylation-GC/MS and GC/MS allows the recognition of markers, the quantification of some significant compounds useful for the identification of binders in samples and enables the study of degradation mechanisms of organic materials, for further evaluating the conservation state of an artwork [14, 16].

Techniques developed for **protein-peptide analysis** in biological fluids (**proteomics**) could make an important contribution to the study of proteinaceous paint media and their degradation processes [31-38]. Some papers lately reported successful attempts for analysis of protein binders using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) [34]. In this technique, the protein or peptide molecules are first ionized after irradiation with laser beam, and then analyzed in a TOF (time-of-flight) analyzer [35]. The protein samples can be analyzed in linear or reflector mode. The linear mode is preferentially used for the analysis of proteinaceous molecules having a molecular weight between 10 000 and 350 000 Da, while the reflector mode is normally used in the analysis of peptide fragments with a molecular weight up to 10 000 Da, resulting from enzymatic protein digestion [36]. Although the linear mode is more sensitive than the reflector mode due to physical constraints of the analyzer, the reflector mode provides higher resolution mass spectra. A recent paper indicates several steps of extraction of proteins from a paint sample without hydrolysis [38], while another recent article proposed a simple protocol for analysis of proteinaceous binders from 10 types of paint ground layers, using trypsin digestion [33].

MALDI-TOF-MS/MS, referred as MALDI, is a well established technique for protein identification through Peptide Mass Fingerprint (PMF) or Peptide Fragment Fingerprint (PFF). PMF combined with PFF provides a unique method for unambiguous protein identification, with some exclusive features: fast measurements, easy mass spectra interpretation and simplicity in handling [36].

Immunological techniques have also been investigated, with promising results for the identification of proteinaceous binders in polychrome samples and paint cross-sections, but for the moment their optimization is still far from being fully assessed [39-45]. Published papers report the use of immunofluorescence techniques to identify the media of XVth–XVIIIth century European paintings [43] and artificially aged collagen paints [44]. A recent paper proposes an optimized analytical protocol using Immuno-Fluorescence Microscopy (IFM) for the identification of markers of egg white and casein, respectively [39]. The radio-immunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) have been proposed as alternative methods [39, 40, 42, 45]. An ELISA protocol was developed at the Getty Conservation Institute for quick and simultaneous identification of glues, casein, plant gums etc. [39]. Antibody-based immunological approaches utilize the unique ability of antibodies (Immunoglobulins, Ig = proteins produced by all animal immune systems

in response to the presence of an antigen) to seek out and bind to specific protein-rich regions called epitopes (antigenic determinants) on the antigen's molecule (foreign body or substance that generates an immunological reaction - proteins, polysaccharides, nucleic acids). The most powerful antigens (immunogens) are proteins (lipoproteins, glycoproteins) and therefore the immunological techniques are mainly used in protein analysis.

Multivariate chemometric techniques of data handling are a precious tool to draw information from large sets of chemical data. Although in conservation science they have not yet attained diffusion comparable to other fields, a variety of multivariate criteria for analysis have been proved extremely useful in the study of cultural heritage, using pattern recognition and classification models [12-13].

2.Proposal of an Integrates Methodology for the Identification of Proteinaceous Binders

The combined use of traditional and innovative analytical techniques, such as optical microscopy, fluorescent staining tests, colorimetry, spectroscopic techniques (reflectance spectroscopy, micro-FTIR/FTIR, micro-Raman), micro-spectrofluorimetry, MALDI-TOF-MS, IFM/ELISA, will lead to a new, integrated methodology for the identification and quantification of the binders contained in polychrome layers [49-50] [Fig. 3].

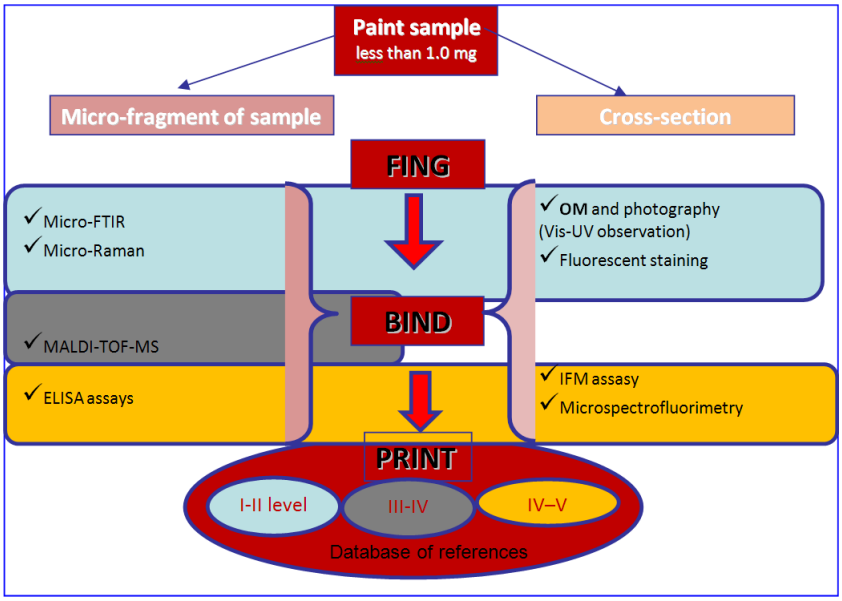

Figure 3 - Scheme of the proposed analytical protocol: FING_BIND_PRINT

A large range of samples can be analyzed: pure proteins, natural binders, bought from market (possibly bio-market) or provided by different companies (as commercial formulations: e.g. isinglass), "mock samples" containing natural binders. The later will be

made in laboratory, according to known, historical recipes and the proteinaceous binders in micro-fragments taken from them will be analyzed and quantified. Finally real samples taken from works of art (from paintings, polychrome sculptures, selectively and accurately chosen) will allow the validation of the entire experimental protocol.

The ancient treatises (as those written by Cennini, De Mayerne, Dionysos from Fourna, Filipe Nunes etc.) [51-56] offer a huge variety of traditional recipes for creating paint materials and therefore the **standard samples** (known as "mock samples") will be prepared according to the already published sources and artificially aged in conditions of fluorescent lamp exposure at a certain level of relative humidity (around 45%) and temperature (ca. 30°C), equivalent to 100 years of exposure in museum conditions [13]. The reference spectra and images obtained will lead to the creation of a database of reference materials, useful for further identification of binders in real case-studies.

Each sample will be divided in two: a part to be used in micro-destructive analytical techniques and the other fragment to be embedded in a resin (as a cross-section).

In the first phase (called FING), the application of several techniques (microscopy coupled with staining tests, colorimetry, spectroscopy) will lead to the first two levels of information:

- presence of the organic material based on the aspect, color, reflectance and specific fluorescence of each layer and on spectroscopic fingerprinting;
- primary distinction between classes of organic binder (oil, protein, resin or gum).

For example, the cross-section obtained from a "mock sample" imitating a ground layer made of calcium carbonate (or gypsum) and animal glue can be examined and photographed with an optical microscope and the distribution of the proteinaceous binder contained in it mapped through a fluorescent staining, using the filter for blue excitation (Fig. 4).

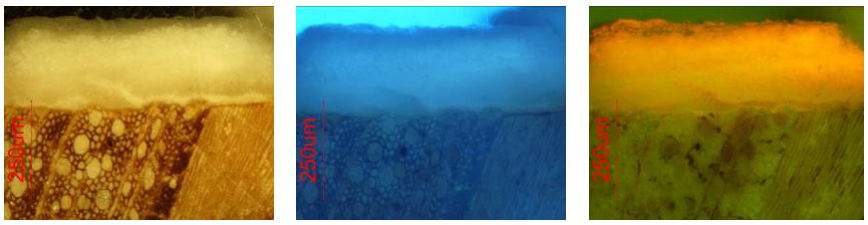

Figure 4 - Example of characteristic auto-fluorescence of an animal glue and the image of a fluorescent staining in the same layer, for mapping the distribution of the binder according the specific fluorescence ("mock sample" reproducing a ground layer made of calcium carbonate and rabbit glue, applied over a wooden support, 100x).

In a second phase (called BIND), MALDI-TOF-MS and immunological assays are useful to differentiate and quantify the proteinaceous binder in complex mixtures (as binary mixtures with another protein, with an oil or other type of natural organic material; as complex mixtures or stratifications of layers), such as in the identification between an oily

tempera ("tempera grassa") or an oil paint layer (*third level* – type of organic binder and *fourth level of information* – ratio of organic binder in a mixture). Microspectrofluorimetry will also be useful for measuring the fluorescent signal (as position, width and intensity of the emission peak/band) of the binders or of mixtures of them on cross-sections.

The MALDI-TOF preparatory step is very important to achieve reliable results, therefore a specific protocol for the preparation of protein-containing samples has to be designed and applied, trying to minimize the number of steps, for a maximum efficiency on a minimum quantity of sample. Further tests for quantification through isotopic labeling (^{18}O labeling) can be considered as a new way to many biochemical applications and as a potential substitute of ELISA [37].

As example, the same sample shown in Fig. 4 was analyzed through MALDI-TOF-MS and mass-spectra were obtained and compared for both "mock sample" fragment and another sample of only animal glue (Fig. 5).

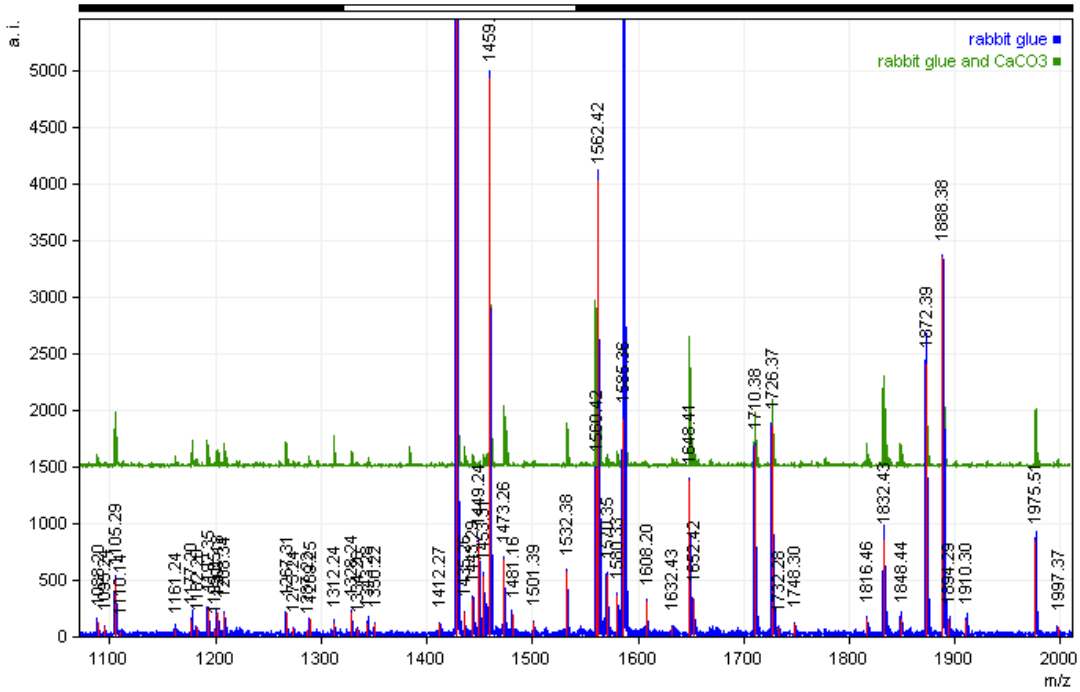

Figure 5 - Reference spectra of a calcium carbonate and animal glue ground layer and of only animal glue, obtained through MALDI-TOF-MS analysis

For obtaining the *fifth level of information* (Fig. 3), immunological essays (ELISA, IFM on cross-sections) can be applied for differentiating binders (such as between a rabbit skin glue and a fish glue/isinglass), but also as a verification of the MALDI-TOF-MS protocol of quantification.

Several samples can be analyzed at once by ELISA protocols on 96 well-microplates [Fig. 6].

The results of the immuno-enzymatic reaction can be measured quantitatively (optical density or absorbance) with a spectrophotometer or an automated plate reader. Primary antibodies specific for the proteins in the art samples can be utilized for the capture step, whereas secondary antibodies labeled with an enzyme (e.g.: Alkaline Phosphatase) can be utilized for the detection step. If both layers of antibodies are present in the microplate well, the solution will turn yellow in the presence of the enzyme's substrate (p-NPP), meaning that the antigen for the specific antibody is present [Fig. 6].

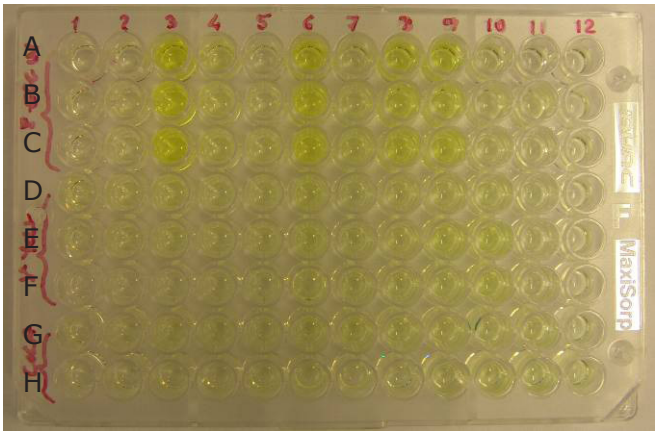

Figure 6 - Example of an ELISA plate after the addition of the substrate:
the yellow color simultaneously confirms the presence of protein-based samples as positive result for egg white (3A-C, 4A-C, 6A-C, 7A-C, 8A-C, 9A-C), egg yolk (6D-F, 7D-F, 8D-F, 9D-F, 10D-F) and animal glue samples (G7-9 and H10-12)

The third phase (called PRINT) will consist in the implementation of the designed methodology to samples taken from real works of art. Considering the complexity of real case-studies, the database of reference materials and the chemometric analysis of results will be essential for a reliable and reproducible application of the proposed analytical protocol.

3. Advantages and Disavantages of the Biochemical Approach

The method of peptide mass mapping, as MALDI-TOF-MS, applied for the identification of proteinaceous binders in polychrome artifacts, has many advantages [31-34, 50]:

- highly sensitive (detection limits in the order of femtomoles),
- small amount of sample (0.5 µg - 0.5 g),
- simultaneous analysis,
- simple and rapid (2 hours of preparation) protocol,
- may determine the individual types of proteinaceous binders in their mixtures,

but some disadvantages exist [32-33, 38]:

- sophisticated instrument set-up is needed,
- database of proteinaceous binders references has to be build-up,
- interactions with pigments can inhibit trypsin's action.

As it was previously assessed, biochemical tools as ELISA and IFM have a great potential for the use in detection of organic binders (mainly proteinaceous), constituting polychrome layers in artworks. Nevertheless, besides the advantages of the ELISA assay [40, 42, 45, 49]:

- highly sensitive (detection limits in the order of nanograms, even less than 10-9 g),
- a single extraction can be used in multiple assays, thus extending the usefulness of small sample sizes,
- simultaneous analysis of several samples,
- relatively simple (do not need sophisticated instruments) and rapid (1 to 3 days of preparation + 1 day of test) protocol,
- cost-effective (if we consider the number of samples that can be analyzed with one test),
- may determine the biological source of the protein (i.e., rabbit skin glue vs. fish glue),

there are some main disadvantages [40, 42]:

- the high degree of antibodies' specificity can also create difficulties (with both collagen and plant gums, multiple antibodies must be used to ensure detection of antigen from a broad range of possible sources);
- loss of spatial resolution (the extraction procedure effectively destroys any ability to localize a given protein to a particular layer or position in the sample);
- decrease in immunoreactivity of antigens with light and/or thermal ageing (though samples nearly 2000 years old were successfully identified);
- possible interactions with pigments where the binder may become degraded or insoluble.

Considering both the advantages and disadvantages, an IFM choice vs. ELISA will point on several characteristics that have to be considered in the analysis of real samples [39-41, 49]:

- detection of antigen by IFM is likely not to be as sensitive as ELISA;
- interfering background unspecific staining (as the bright green-yellow fluorescence of gypsum grounds), which can make the interpretation of data from real samples ambiguous;
- problem of bleaching of the fluorophore with repeated excitation, and thus the signal may degrade over repeated measurements;

- unlike with an ELISA assay, the sample remains intact and the antigen is localized to a specific region of the sample;
- it is possible to detect multiple antigens within the same sample.

Considering the above-mentioned aspects, an idea would be to use IFM in conjunction with ELISA: a small portion of the material sampled for cross-section examination can be reserved for analysis by ELISA.

When using the sample embedded as a cross-section, a pre-screening of the sample can be made to identify proteins of interest present in the cross-section and subsequent examination using IFM can then be targeted at proteins known to be present and therefore:

- this will limit the number of incubations to which a cross-section must be subject;
- will remove any ambiguity in the face of a negative staining result.

The ELISA and IFM protocols are usually expensive (antigens for specific proteins in particular), therefore using both techniques as conjugated tools together with the above-mentioned techniques (MO, fluorescent staining and micro-spectrofluorimetry, MALDI-TOF-MS, micro-FTIR/micro-Raman) will improve the detection/quantification protocol and also will reduce the costs for necessary materials.

Conclusions

The overall methodology that is proposed in the present paper creates a new and original experimental design (FING-BIND-PRINT) with several advantages: simple and fast; cost effective; requiring minimum quantity of material; easily reproducible on real samples.

From preliminary tests performed on a series of fresh (2009) and laboratory aged (7-5 years) samples of egg and glue (as film of a dried solution on a glass slide or mixed with charge or pigment) it was assessed that ELISA assay can be useful when combined with fluorescent staining microscopy [49-50]. However, optimization of blocking buffer needs to be done and further experiments will be performed for the quantification of the three binders when in mixtures between themselves or with other binding materials (oil, resins etc.). Fluorescence microscopy with a biomedical stain was useful for mapping the binder distribution in paint cross-sections of variable age (fresh "mock samples" - 2008 and naturally aged polychrome samples – XVI-XVIIIth centuries), but other mock samples with known composition and binders ratios should be tested in order to evaluate the effectiveness of the stain and also to register fluorescence spectra on each layer. From the same cross-sections thin sections should be obtained in order to be able to perform several comparative tests between classical stains, IFM and fluorescent staining and qualitatively evaluate the specific answer. An important issue to be also tested will be the reduction of the sample's amount to less than 1 mg (if possible less than 0.5 mg).

The accuracy and reproducibility of the obtained results from each technique will be evaluated based on a c*orrelation factor* (expressed as %): positive correlation (the result corresponds to the composition of the paint model), uncertain correlation (the result is uncertain as to the composition of the paint model) and negative correlation (the result does not correspond to the composition of the paint model).

Cross-referencing the results from each technique and the level of information obtained a final evaluation of the entire analytical protocol can be given for each set of paint model. The interference sources and limits will be identified and characterized for further improvements of the methodology.

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