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**DEVELOPMENT OF INNOVATIVE
ANALYTICAL TECHNIQUES FOR THE
CHARACTERIZATION OF PROTEIN
MATERIALS IN ART, ARCHITECTURE
AND ARCHAEOLOGY**

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Abstract

Abstract

The scientific investigation applied to art and archaeology artifacts is not only useful for improving our knowledge of techniques and materials used by the artists, but also to understand the degradation processes affecting works of art, and to choose suitable conservation treatments for their preservation. The project, carried out during the PhD program, concerned the development of innovative techniques for the characterization of proteinaceous material in the field of conservation of cultural heritage. As it is known, the organic compound and in particular the protein material in a work of art is susceptible of a strong decay process due to the inherent characteristics of these materials. Their recognition is still difficult because the degradation of original material due to ageing and pollution consequence, the very small amount of precious material available and the possible simultaneous presence of different organic compounds always bound to inorganic ones. This work aims is to investigate two research techniques for the characterization of proteins materials: the first, based on an immunological approach, with the development and assessment of a Dot-ELISA methodology and the second based on a chromatographic technique with the evaluation of the effectiveness of a UPLC with AccQ•Tag Ultra detecting method. Concerning the immunological approach, the set up of the method was performed and its effectiveness in the protein characterization was assessed. Moreover an artificial ageing process has been performed on laboratory-made specimens for the study of the capability of the technique in recognition of degraded compounds. Thanks to the *TemArt* project, a study on samples coming from the Giotto's wall painting "The Stigmata of St. Francis" and from the "Maestro di Figline" wall painting "Assunzione" in the Basilica of Santa Croce in Florence was performed. In this way the technique was tested for real samples. Also for the AccQ•Tag Ultra UPLC methodology the assessment of the effectiveness of the technique was performed with reference protein standards, laboratory-made specimens and finally samples from

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original panel painting, thanks to the S.I.C.A.M.O.R. Project.

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PART I

INTRODUCTION

CHAPTER 1.

THE CHARACTERIZATION OF PROTEINS IN CULTURAL HERITAGE: STATE OF ART AND AIM OF THE WORK.

Scientific investigation applied to cultural heritage is not only useful for improving our knowledge of techniques and materials used by the artists, but also to understand the degradation processes affecting works of art, and to choose suitable conservation treatments for their preservation. An analytical approach to the art and archaeology began in the last years of the 18th century has grown steadily, particularly in the second half of the 20th century, when a wide range of techniques and methodologies, in the service of diagnostics for art have been developed (Doménech-Carbó M.T. 2008).

Among the materials composing the art objects, organic compounds, used as protective coatings and binding media, drew the attention of conservators, due their susceptibility to phisico-chemical changes caused by ageing. The organic component, compared to the inorganic one, tends to undergo more degradation processes and this event complicates their recognition. In protein binders, degradation processes consist, for example, in the loss of adhesion, due to phenomena of swelling and shrinkage, resulting from changes in humidity and

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temperature, and in the development of moulds and microorganism. Other decay events can be attributed to photo-oxidation phenomena induced by UV and visible radiation and to interactions with the pigments or binders of different nature.

Although the diagnostics offers several techniques and methodologies to study proteins from works of art, their identification is still a problematic issue due to: (i) the possible simultaneous presence of different binders; (ii) the degradation of the original materials as a result of ageing and pollution; (iii) the very small amount of precious material available.

This work aims is to investigate two research techniques for the characterization of proteins materials. The first based on an immunological approach and the second based on a chromatographic technique.

1.1 Protein materials in cultural heritage

The set of properties required for a material, to be used as paint binder, has limited, historically, the choice of possible media. This choice is derived from a natural evolution, which is the result of centuries of experimentation. Depending on the binder used for the realization of a painting, different performing techniques are recognized, which have also characterized the different periods: tempera, oil, mixed, modern techniques. The classical protein tempera, used in the Medieval and Renaissance painting on panels or in polychrome sculptures, was based on the use of the egg and often the egg yolk. In the decoration of miniature paint manuscripts it was used, preferably, animal glues and

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egg white, as well as vegetable gums (Matteini M. *et al.*). With the advent of the use of drying oils, protein-based tempera, first, was used in mixture with these and then it was gradually abandoned. The use of protein tempera, in particular the egg-based tempera, is found in the wall paintings in the “secco” technique. The original recipe of egg tempera has been handed down from *Cennino Cennini* in his "Il Libro dell'Arte", written in the late fourteenth century. Traditional recipes predicted the use of both the whole egg and the yolk, to dilute the pigments; sometimes, also, adding the fig milk or vinegar was suggested as preserving agents (Cennini C.).

Egg yolk is a fat-in-water emulsion with about 50% dry weight. It consists of 65% lipids, 31% protein and 4% carbohydrates, vitamins and minerals. The main proteins components of egg yolk are 68% of LDL (low density lipoproteins, lipovitellenin), 16% HDL (high density lipoproteins, lipovitellins), 10% livetins and 4% phosvitins. The lipids of the yolk are mixtures of glycerides and phosphatides: the latter representing approximately 16% ether extract total. The predominant fatty acids are oleic, palmitic, linoleic, stearic and linolenic acids. The fatty acids fraction is composed by 31% from saturated fatty acids; unsaturated part is respectively composed by 50% of oleic acid, 20% of linoleic acid and 3% of linolenic acid. The lecithins, which contain arachidonic acid, are present in the amount of about 8% (Belitz H.-D. *et al.*).

The egg albumen is an aqueous solution of various proteins, in which the ovalbumin is the most abundant (54% of the total protein). This, as several others albumen proteins, is a glyco-phospho protein with 3.2% carbohydrates (Tab. 1.1).

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Table 1.1: Proteins of egg white (Belitz *et al.*)

Proteins	% of the total proteins	Molecular weight (KDa)
Ovalbumin	54.0	44.5
Conalbumin ¹	12.0	76
Ovomucoid	11.0	28
Ovomucin	3.5	5.5-8.3x10 ⁶
Lysozyme ²	3.4	14.3
Ovoglobulin G2	4.0	30-45
Ovoglobulin G3	4.0	
Flavoprotein	0.8	32
Ovoglycoprotein	1.0	24
Ovomacroglobulin	0.5	760-900
Ovoihibitor	1.5	49
Avidin	tr	68.3
Cystatin	tr	12.7

In addition to the widespread egg tempera, a good fortune had been the milk tempera, which produces a visual effect similar to that of egg tempera. There isn't a lot of literature about the milk painting, but it seems that milk has been widely used both during the realization and the restoration of works of art. In fact, the milk was used as a fixative for paints and as an additive. Milk is an emulsion (or colloid) of

¹ Ovotransferrin

² Ovoglobulin G1

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butterfat globules within a water-based fluid that contains dissolved carbohydrates and protein aggregates with minerals. The lipid fraction of the milk is similar to those of egg. The milk lipids consist mainly of triglycerides (96%) from diglycerides, phospholipids, glycosphingo lipids and sterols. Approximately 70% of the composition in triglycerides, is composed of saturated fatty acids, among which the most abundant are: palmitic acid, oleic acid, stearic acid and myristic acid (Belitz H.-D. *et al.*). Among the milk proteins (Tab. 1.2), the most abundant is casein, which is a phosphoprotein obtained for flocculation of the milk, used also as protein binder. This was used in the form of calcium caseinate, as tenacious adhesive, and in the form of ammonium caseinate, as binder. The casein tempera are characterized by an opaque film, which tends to become brittle and cracked with ageing. After the nineteenth century, the casein has been used for the preparation of the canvases, for the relining of the paintings, as consolidant/protective coating and for the operations of restoration, such as pictorial retouching (Matteini M. *et al.*).

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Table 1.2: Proteins of milk (Belitz H.-D. *et al.*)

Fraction	Portion³	Mol. Weight (KDa)
<i>Caseins</i>	80	
α_{s1} casein	34	23.6
α_{s2} casein	8	25.2
κ casein	9	19
β casein	25	24
γ casein	4	12-21
		20.5
γ_1 casein		11.8
γ_2 casein		11.6
γ_3 casein		
<i>Whey proteins</i>	20	
β -Lactoglobulin	9	18.3
α -Lactalbumin	4	14.2
Serum albumin	1	66.3
Immunoglobulins	2	

Animal glues are natural polymers derived from mammalian or fish collagen. Collagen is the most abundant structural protein of various connective tissues, making up from 25% to 35% of the whole-body protein content. It is mostly found in fibrous tissues such as tendons, ligaments and skin, and is also abundant in corneas, cartilage, bones and blood vessels. Collagen molecular structure consists of three

³ As % of skim milk total protein.

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polypeptide α chains, twinned together to form triple helix. Each α chain composes of repeated sequence of triple (Gly-X-Y)_n where X and Y are often proline (Pro) and hydroxyproline (HyP). Hyp, with hydroxyllysine (HyL) are the amino acid markers for this protein; other frequently occurring amino acids include Ala, Lys, Arg, Leu, Val, Phe and Glu (Zevgiti S. *et al.*). The tertiary structure of collagen is a triple helix, which consists of three left-handed polyproline II-like chains intertwined to form a right-handed triple helix. The constitutional unit of collagen is the tropocollagen or "collagen molecule", which is a subunit of larger collagen aggregates such as fibril. The fibroblast is the most common cell which creates collagen.

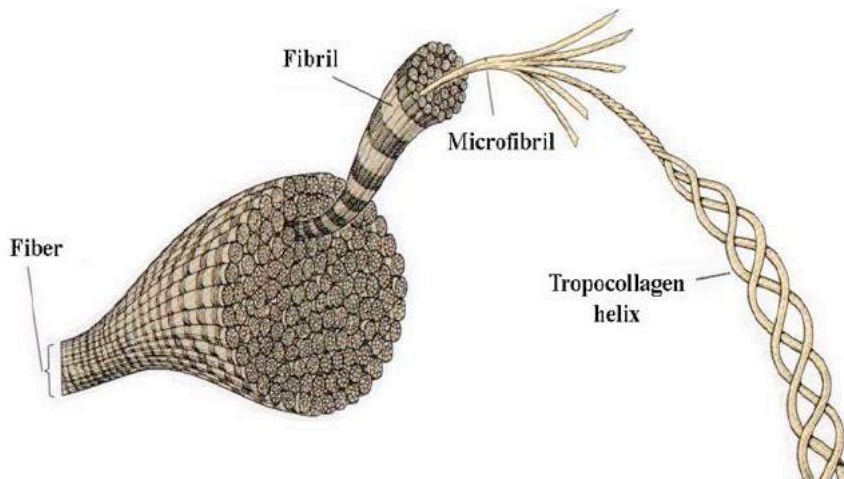


Figure 1.1: structure of a collagen fibre.

The animal glues may exhibit varied physical, chemical and mechanical properties, depending on their origin and method of preparation. The glues are derived from animal skins and other parts of cartilage (hide glue), bones of mammals (bone glue) and also from fish (fish glue). Depending on the quality and purity, the glues are divided

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into low purity grade glues, called “colle forti”, and gelatines (or “collette”), consisting almost exclusively of collagen. High purity grades glues were reserved for use as a paint binder or in the application of gold layers. The less pure glues, instead, have been used mostly as tenacious adhesives. Furthermore, animal glues have been widely used for the preparation of the primer of panels and canvases, mixed with gypsum and often, also, with other inert materials (Matteini M. *et al.*).

In this initial digression on proteinaceous materials commonly used in Italian painting, other types of protein material, less common and therefore less analysed, have been omitted; for example, all those materials usually considered from diagnostics for archaeology, such as bone fragments, blood traces, traces of remains of food, have not been listed.

1.2 The analysis of proteins in cultural heritage: state of art

The use of scientific techniques in materials conservation has notably expanded in recent decades. Beyond the interest in knowing the original techniques and the materials used by artists in centuries, also the understanding of the degradation phenomena, that occur in a work of art, has promoted the development of these technique. An understanding of the state of materials at a molecular level can provide valuable information for conservators, enabling them to decide on a conservation procedure. Also, these techniques are very useful in authentication and dating purposes. No general analytical techniques

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exist to identify a protein media in a paint or a finishes, because material used as binding media are high complex organic substances, formed by the same common elements. Moreover, these materials undergo considerable deterioration over long periods of time, which results in difficulties of identification. In addition, analysis of these materials is hard, due to the number and the size of the samples imposed by the their unique and inimitable character of work of art. Usually non-invasive/non-destructive techniques are preferred; although, at the present, the most useful analysis is obtained from high sensitive techniques that requires sub-milligram samples (Doménech-Carbó M.T. 2008). Moreover, in the last years, a multi-technique approach is preferred, because it provides complementary information from different techniques and methodologies.

With the aim of identifying only the protein material in a complex sample, such as those coming from the field of art, the techniques elemental show their limits, due to the repetitive atomic composition of the proteins. Among the various analytical techniques available, some studies have been performed by means of thermal analysis (Odlyha M. 1995), microbeam analysis (Keune K. *et al.*), but the techniques commonly used for the analysis of proteins are the spectroscopic, chromatographic, microchemical and immunochemical techniques. Spectroscopic techniques, as Fourier-Transform InfraRed spectroscopy (FTIR) (Doménech-Carbó M.T. *et al.* 1996), Near-InfraRed Spectroscopy (NIRS) (Jurado-López A. *et al.*), and micro-Raman spectroscopy (μ Raman) (Vandenabeele P. *et al.*, Nevin A. *et al.* 2007, Nevin A. *et al.* 2008), are useful to obtain information about inorganic and organic material, thus proteins too, in a single analysis; moreover,

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they requires a minimum sample treatment and are widely used in laboratories. Unfortunately these techniques can suffer of severe interferences of other organic molecules present in materials and, depending on the technique to acquire the spectra and sample preparation (i.e. KBr pellet or diamond ATR device), poor quality of the spectra can be obtained due to phenomena of reactiveness and/or reflections.

On the other hand, it is well known that chromatographic techniques, as Gas-Chromatography coupled to a Mass Spectrometry (GC-MS) (Colombini M.P. *et al.* 1998, 1999a, 1999b, 2000, 2004, Schilling M.R. *et al.* 1996a, 1996b, Casoli A. *et al.*, 1995, 1996, Aruga R. *et al.*, Bonaduce I. *et al.*, Andreotti A. *et al.*, Přikril P. *et al.*, Gautier G. *et al.*, Cruz-Cañizares (de la) J. *et al.*) and High Performance Liquid Chromatography coupled to Fluorescence Detector (FD-HPLC) (Wouters, J. *et al.* 2000a, 2000b, Peris-Vicente J. *et al.*, Přikril P. *et al.*, Grzywacz C.M.), UV/vis detector (PDA-HPLC) (Halpine S.M. 1992, 1996, Ronca F., Checa-Moreno R. *et al.*), or MS spectrometer (Zangrando R. *et al.*, Chambery A. *et al.*, Tokarsky C. *et al.*, Fremout W. *et al.* 2010) are considered up to now the most effective diagnostics tools for the accurate determination of protein binders. The identification of proteins was performed by determining the presence and the relative abundance of protein/peptide/amino acids components (Schilling M.R. *et al.* 1996a, 1996b, Wouters, J. *et al.* 2000a, 2000b., Halpine S.M. 1992, 1996, Ronca F.) and/or using chemometric approaches, such as the Principal Component Analysis (PCA) (Casoli A. *et al.* 1995, Andreotti A. *et al.*, Přikril P. *et al.*, Gautier G. *et al.*, Peris-Vicente J. *et al.*), factor analysis (Aruga R. *et al.*), SIMCA

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(Checa-Moreno R. *et al.*). Sometimes, pyrolysis is used to produce protein fragmentation obtaining the amino acid components to be derivatized, separated and detected by GC-MS (Py-GC-MS) (Carbini M. *et al.*, Chiavari G. *et al.*). The disadvantages of this technique is associated to very complex pyrograms that are very often extremely difficult to be interpreted (Doménech-Carbó M.T. 2008). Nevertheless, in some cases the presence of characteristic fragments that can be considered as markers (Carbini M. *et al.*, Chiavari G. *et al.*) allows the identification of the specific protein components of the material.

Proteomics is the most up-to-date approach developed in the last years to precisely characterize protein primary structures (amino acid sequence) (Tokarski C. *et al.*, Hynek R. *et al.*, Kuckova S. *et al.* 2005, 2007, 2008, 2009, Fremout W. *et al.* 2009, 2010, Stevens S.M. *et al.*, Leo G. *et al.*). This methodology involves several steps to be performed sequentially: i) an enzymatic pre-treatment of the sample to obtain proteins fragmentation into peptides; ii) the characterization by mass spectrometry after direct injection or coupled to chromatographic separation. Assignment of peptide fragments is made according to the evaluation and comparison of the profile with reference patterns (peptide mass fingerprint) (Hynek R. *et al.*, Kuckova S. *et al.* 2005, 2007, 2008, 2009) or by tandem mass spectrometry to determine the amino acid sequence (Tokarski C. *et al.*, Fremout W. *et al.* 2010, Leo G. *et al.*). This method (based on characterization of protein components by peptide amino acid sequences) allows to identify unambiguously the protein to which they belong thanks to protein databases (i.e., SWISS-PROT, Trembl, UniProt, etc.). In this context also some methods for the identification of proteinaceous binders in

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artwork based on MALDI-TOF mass spectrometry analysis of peptide mixture after tryptic cleavage have been proposed (Hynek R. *et al.*, Kuckova S. *et al.* 2005, 2007, 2008, 2009).

While the evolution of technology is reflected in the development of innovative, effective and sensitive techniques in the detection of proteins, on the other hand, those used in the past are revalued, adjusted to today's level of technology and re-proposed. It is the case of microchemical tests, which originate from the histology practice. Microchemical tests have been widely used for identifying binding media in the past (Johnson M. *et al.*, Martin E.), and are still used in laboratories (Sandu I.C.A. *et al.*, Kuckova S. *et al.* 2013) in combination with instrumental techniques. Their main advantage is their simplicity and their capability of informing on the spatial distribution of organic compounds (stain reaction in cross-section). Although, they exhibit a lack in specificity and, for this reason, have been replaced progressively from more sensitive and specific techniques. Immunological techniques have been proposed as an alternative of classical histological methodologies. They are based on the specific reaction between an antigen and an antibody. First, immunofluorescence microscopy (IFM) was proposed by Kockaert L. *et al.* in 1989, followed by Ramirez-Barat B. *et al.* in 2001. Then, Heginbotham A. *et al.* in 2006 proposed a comparison between the IFM technique and an Enzyme-Linked ImmunoSorbent Assay (ELISA) for cultural heritage. In the last years, an increased production of publications about these immunologic techniques, occurred.

Beyond IFM works (Vagnini M. *et al.*, Cartechini L. *et al.*) and ELISAs-based works (Palmieri M *et al.* 2010, 2013, Arsanoglu J. *et*

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al.), Immuno-Chemiluminescence microscopy (Dolci L.S. *et al.*, Sciutto G. *et al.* 2011, 2013) and immunoblot (Gambino M. *et al.*) techniques were developed. The advantage of these techniques is the high specificity and sensitivity. It is obvious that they are useful only for proteins detection and not for the rest of organic compounds.

1.3 Aim of the work

The project, carried out during the PhD program, concerned the development of innovative techniques for the characterization of proteinaceous material in the field of conservation of cultural heritage. The study was divided into two distinct directions: an immunological approach, with the development and assessment of a Dot-ELISA methodology, and a chromatographic approach with the evaluation of the effectiveness of a UPLC with AccQ•Tag Ultra detecting method. The text will be divided in these two sections.

For the immunological part, has been taken into account a Dot-ELISA protocol, already in use. Dot-ELISA is an enzyme-linked immunoassay performed on solid membrane, such as nitrocellulose or Polyvinylidene fluoride (PVDF). This has been modified according to the needs of diagnostic art, in order to present a standard procedure for identification of any type of protein. In fact, first, it was assessed the capability of the assay in detection of egg albumin. Then, other proteins were submitted to the assay and when it was been necessary, the protocol was adjusted to requirements. A study about the capability of the techniques in detect aged proteins was conducted. An artificial ageing was performed on laboratory-made mock-ups, prepared with

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the three usually used protein binders (egg, milk and glue), mixed with different pigments. The results obtained were interesting. As proof of these results, another technique was used, the western blotting. This technique is based on a prior electrophoretic separation and on a subsequent immunological detection of the proteinaceous sample. From the results obtained, we were expecting a further explanation about the effects that ageing had produced on proteins. During the study, it has been possible analyzing real samples from works of art. In this way, it was possible to verify and evaluate the performances of the technique.

Regarding the chromatographic approach, in this work, the AccQ•Tag Ultra methodology for the amino acids analysis was assessed for the protein binder characterization. The method is based on the chromatographic separation of hydrolyzed proteins by means of an UPLC instruments, equipped with a TUV detector. The method was born for protein hydrolysates of native proteins, but it was not yet proved on samples coming from works of art. The method involves the complete hydrolysis of proteins in the vapour phase and a pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). First, the protein reference samples and then the laboratory-made specimens were analysed. It will be shown, by means of the assessment of the amino acid content, what are the damages that a protein subjected to artificial ageing reports.

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CHAPTER 2.

ANALYSIS OF PROTEIN-BASED PAINTINGS BY DOT-ELISAS

The Dot-ELISA is a solid phase immunoblotting assay based on the highly specific immunologic reaction antigen-antibody to detect the presence of the antigen or the antibody dotted onto the surface of a membrane. In particular, the technique is based on the indirect detection through the use of a specific primary antibody for the antigen and an enzyme conjugate secondary antibody specific for the primary one. The technique has been widely studied and used in the 1980's for easy and quick detection of viruses (Mancianti F. *et al.*), but no application to the cultural heritage has ever been done. Because its ease and rapidity of execution, today this is a laboratory technique used for routine analysis.

In order to develop a solid and efficient method for the determination of the protein material in art and archaeological artifacts, in this work a Dot-ELISA protocol was improved and submitted to evaluation.

Recently a renewed interest in the immunological techniques for the characterization of protein binders has been shown. In particular, Immuno-Fluorescence Microscopy (IFM) (Ramirez-Barat B. *et al* 2001; Kockaert L. *et al* 1989; Heginbotham A. *et al* 2006; Cartechini

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L. *et al* , Vagnini M. *et al.*), Immuno-Chemiluminescence Microscopy (Dolci L.S. *et al*, Sciutto G. *et al.* 2011, 2013), Enzyme-Linked ImmunoSorbent Assays (ELISA) (Heginbotham A. *et al*; Cartechini L. *et al* , Arsanoglu J. *et al.*, Palmieri M. *et al* 2010, 2013) and finally immunoblot (Gambino M. *et al.*) have shown interesting results mainly for the high specific and sensitive identification of proteins in micro-samples of art and archaeological materials.

As previously seen, the protein material in cultural heritage has been widely used for different scopes. The aim of this work is to create a valid method for the identification of protein material in all fields of art and archaeology. For this reason, starting to apply a standard Dot-ELISA protocol to egg albumin standards samples and then to laboratory-made specimens, it has been possible to optimize the experimental conditions for the best performances for all the proteins analysed.

In particular, first, the Dot-ELISA method was applied to standard ovalbumin samples at different dilution to assess the sensitivity and to determine the minimum quantification limit and the minimum detection limit. The determination of the colour staining was performed by a visible light spectrophotometer with integrating sphere on the dried solid support. Then the procedure was applied to identification of milk and glue proteins. In this case some modifications were necessary to the protocol to avoid dangerous interferences between antibodies and blocking buffer. During the study, thanks to the opportunity offered by two different projects (TemArt and S.I.C.A.M.O.R.- see the Acknowledgements section) it

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was possible to evaluate the efficacy of detection of the method also in case studies with original real samples (Ch. 5).

2.1 Identification of ovalbumin by Dot-ELISA

In this work, a semi-quantitative determination of ovalbumin in laboratory-made specimens was performed, starting from a calibration curve of known dilutions of standard ovalbumin. Previously it was observed that the intensity of the stain was proportional to the amount of protein dotted onto the membranes. For this reason, after membranes dried, they were submitted to spectrophotometric analysis. The stain intensity evaluation was made plotting the values at a selected wavelength to obtain a linear fit. The equation of the fit was used to quantify the concentration of protein in specimen samples.

2.1.1 Materials and methods

Reagents

Elution (or reduction) buffer was prepared by mixing 25 mM tris (hydroxymethyl)aminomethane (TRIS), 6 M urea, 191 mM glycine, and 868 mM sodium dodecylsulfate (SDS). Phosphate saline buffer (PBS) was prepared by mixing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.4 mM KH₂PO₄. Blocking–washing buffer (B. buffer) was prepared by mixing 10 % (v/v) PBS, 90 % (v/v) H₂O, 1 % (v/v) polyoxyethylene (20) sorbitan monolaurate (TWEEN 20), and 5 % (w/v) non-fat dry milk (Oxoid, UK). This was used as protein source for blocking non-specific reactions, as an incubation medium, and for subsequent washing to remove unreacted reagents (Johnson D.A. *et*

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al.). The buffers were freshly prepared. All the reagents were analysis-grade (>99.5% purity) and were purchased from Merck (Darmstadt, Germany). Color development substrate in tablet FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium), anti-ovalbumin IgG (anti-chicken egg albumin, developed in rabbit), and anti-rabbit IgG alkaline phosphatase conjugate (developed in goat) antibodies were purchased from Sigma–Aldrich. Methanol (GC purity>99.5%) was supplied by Merck. Spectrophotometric measurements were obtained by use of a Perkin–Elmer Lambda 35 UV–visible spectrophotometer with double beam and double light sources (deuterium lamp for UV spectra and tungsten lamp for visible wavelengths), and equipped with an internal integration sphere for diffuse reflectance spectra. Analyses were performed in the range 780–380 nm.

Samples

Standard ovalbumin was purchased from Sigma–Aldrich (USA, 98 % purity). Egg white, yolk, and whole egg for standard samples were acquired from a local market; egg white and yolk were separated conventionally; egg white and whole egg were freeze-dried. Model painting layer samples were prepared in accordance with traditional painting procedures (Cennini C.). A layer of tempera prepared from different types of protein material and red ochre pigment was applied to a mortar surface (surface of a lime and sand, 1:3 v/v) one day after the mortar preparation, i.e. when the extent of drying and carbonation were both very low. The natural binders selected to prepare these samples were whole egg, milk, and animal glue, pure or mixed as whole egg–glue binder. The only pigment analyzed was red ochre, to

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minimize the effects of interference from mineral pigments: indeed, red ochre is a mineral pigment with little effect on binder analysis (Cartechini L. *et al.*). On the other hand, it is known that inorganic pigments can affect dot- ELISA test results and a recent review (Cartechini L. *et al.*), stated that “the different role played by different pigments is still not clear” (Colombini M.P. *et al.*, Schilling M.R. *et al.*). Trying other pigments would have inserted other complex variables making it difficult to study the effect of the alkaline environment of the mezzo fresco technique; the study of the effects of pigments on protein binder will be treated in a next section (Ch. 3). The ratio of binding medium to pigment was 1:2 (v/v) for whole egg tempera, 1:2 (v/v) for milk tempera (the milk used had previously been diluted 1:1 (v/v) with water), and 1:2 (v/v) for glue tempera (the glue was a sol of animal glue dispersed in water 1:10 w/v). Indeed, the egg–glue tempera specimen was a double layer prepared by applying, first, egg tempera and then glue tempera. The choice to apply the tempera layers when the mortar surface was at the beginning of the setting process (the so-called mezzo fresco technique) was intentional; the purpose was to investigate how the painting technique could affect subsequent analysis of protein species, because of the high pH encountered by the binder when the painting layer was applied to the mortar surface. The red painted layer obtained was subjected to three years of natural aging in the laboratory atmosphere. On these samples no artificial aging was conducted, because one of the purpose of this work was to study the effect of the alkaline environment on the protein binders; the effects of artificial aging will be studied in a second series of hand-made specimens. Micro-samples only from the painting layers were collected by means of a small scalpel; the mass of powder taken

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from the mortar surface was around .5 mg, and it was finely ground before analysis.

Procedure for Dot-ELISA

Freeze-dried egg (whole egg and egg white) and ovalbumin samples were dispersed in the elution buffer in the concentration range 0.1–10 µg/mL. Model samples were ground and dispersed in the elution buffer (100 µL). All the dispersions were centrifuged at 4,500 rpm for 10 min, to deposit the insoluble fraction. An aliquot of these solutions was then deposited on poly-vinylidene fluoride (PVDF) membranes (Millipore, USA), previously soaked in methanol and placed on Petri plates. The samples were blocked with 5 mL B. buffer. Elution buffer was used as a blank control and a solution of milk and glue (1:10 in elution buffer) was used as negative control. The plates were incubated at 4°C overnight. Primary anti-chicken egg albumin antibody (1 mL), diluted 1:1,000 (v/v) in PBS, was introduced into the plates with B. buffer and incubated for 60 min at 37°C. After washing the plates (2 x 10 min) with B. buffer, the secondary antibody, anti-rabbit IgG developed in goat, alkaline phosphatase conjugate, diluted 1:5,000 (v/v) in PBS (5 mL) was introduced. Plates were incubated for 60 min at 37 °C and then washed with B. buffer (1x10 min) and with PBS (1x 10 min). Finally, the color development fresh solution was prepared (1 tablet Sigma FAST in 10 mL Milli-Q water) and applied (5 mL) for each plates. The substrate solution contained BCIP (0.15 mg/mL), NBT (0.30 mg/mL), TRIS buffer (100 mM), and MgCl₂ (5 mM). After 2 h at 37°C the staining reaction occurred and the plates were washed with Milli-Q water: this last procedure was the result of a series of tests at different temperatures (4, 15, 25, and 37°C) with the purpose of

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optimization of the appearance of the color. For each sample seven replicates were performed to check reproducibility. Photographic documentation was performed when strips were dry. Once dried, the strips were analyzed by use of the spectrophotometer.

2.1.2 Results and discussion

2.1.2.1 Optimization of the experimental conditions

Staining temperature

The first objective of this part of the study was to optimize the staining conditions to maximize the stain-to-background ratio. Previous tests had revealed the need to adjust the temperature during development of the stain, because of the drastic effect of temperature on enzyme activity. The staining reaction was performed for 2 h at four different temperatures: 4, 15, 25 and 37°C. The results reported in Fig. 2.1 show that no visible differences were between 15, 25 and 37°C dot color intensity and the blot-to-background ratio was the same, but it was chosen to maintain the temperature at 37°C for analytical reasons. No particular effects of evaporation rate were observed during stain development.

Selectivity

To understand if the antibody chosen was selective with the ovalbumin protein or if it had some cross-reactivity phenomena with other egg proteins, we submit the anti-chicken egg albumin to the Dot-ELISA test with positive standard of egg yolk. The egg yolk was accurately separated from the white to minimize the presence of residues of white

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egg protein and two samples were prepared in different dilution with the elution buffer. The negative results (Fig. 2.2) demonstrated the high selectivity of the antibody that leaves unbound the antigen.

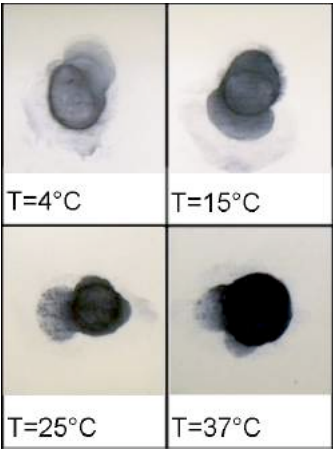


Figure 2.1: Control of the Dot-ELISA assay on ovalbumin samples at different staining temperatures.

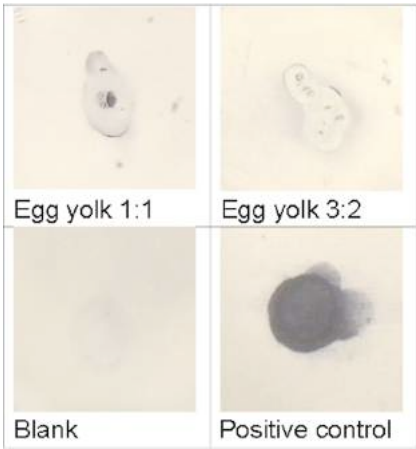


Figure 2.2: Selectivity test on egg yolk samples submitted to Dot-ELISA for ovalbumin search.

Sensitivity

To study the detection limit, the ovalbumin concentrations investigated were from 10 $\mu\text{g/mL}$ to 0.1 $\mu\text{g/mL}$, corresponding to 50–0.5 ng per spot. The results obtained with samples of pure ovalbumin (Fig. 2.3), showed that at a concentration of 1 $\mu\text{g/mL}$ (5 ng per spot), clearly visible staining of the strip could be observed, whereas no stain was detectable at lower protein concentration. It was interesting to notice that the minimum detectable amount of standard protein material was approximately 5 ng, which was a good improvement on the 7 ng of recent results (Arslanoglu J. *et al.*), but the limit remained worse than that which can be achieved by the ultrasensitive CL technique (Dolci L.S. *et al.* 2008) or IFM (Cartechini L. *et al.*). For all seven replicates

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the detection limit was that reported above. For egg white samples the detection limit was lower than for ovalbumin, reaching 0.5 $\mu\text{g/mL}$ (2.5 ng per spot), whereas for whole egg the lower intensity of staining of the sample at a concentration of 1 $\mu\text{g/mL}$ compared with pure ovalbumin was attributed to the smaller amount of ovalbumin present in whole egg (Figs 2.4 and 2.5).

				
10 $\mu\text{g/mL}$	8 $\mu\text{g/mL}$	6 $\mu\text{g/mL}$	4 $\mu\text{g/mL}$	Glue
				
2 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	0.1 $\mu\text{g/mL}$	blank	Milk

Figure 2.3: Dot-ELISA results for ovalbumin/blank/negative controls (milk and glue) samples at different concentrations.

			
10 $\mu\text{g/mL}$	5 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	Glue
			
0.5 $\mu\text{g/mL}$	0.1 $\mu\text{g/mL}$	blank	Milk

Figure 2.4: Dot-ELISA results of egg white/blank/negative controls (milk and glue) samples at different concentrations.

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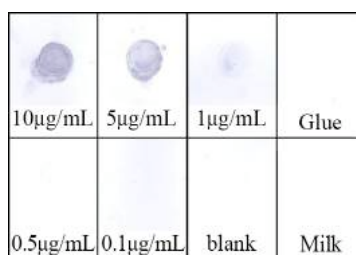


Figure 2.5: Dot-ELISA results of whole egg/blank/negative controls (milk and glue) samples at different concentrations.

2.1.2.2 Semi-quantitative determination of standard and model samples

Dot-ELISA of model samples (Fig. 2.6) confirmed the expectation of obtaining a positive response for samples composed of egg tempera and egg–glue tempera. In contrast, for samples consisting of milk and glue tempera, the assay was negative, as expected. For egg–glue tempera, spot intensity was greater than that for egg tempera; this result was particularly interesting and deserving of deep interpretation (vide infra). As can be deduced from the figures reporting the dot-ELISA results, all the measurements were conducted simultaneously, with background and control experiments that enabled exclusion of spurious effects.

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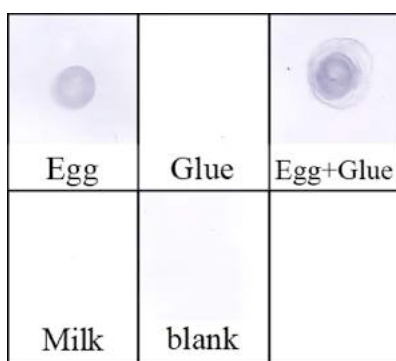


Figure 2.6: Dot-ELISA results of model samples/blank/negative controls.

To attempt semi-quantitative Dot-ELISA determination, the samples were analyzed using a spectrophotometer with integrating sphere in the visible wavelength range (380–780 nm). Use of the integrating sphere enabled collection of light scattered by the whole sample surface, which is always larger than the staining spot size. Moreover, the power of the light source and angle of analysis were kept constant to avoid effects of these conditions on reflectance values. As for ELISA tests read by OD measurements (Palmieri M. *et al.* 2010), spectrophotometric measurements are known to be reliable for semi-quantitative analysis being wide enough to detect the so-called “blank average” (Palmieri M. *et al.* 2010) (vide infra). Figure 2.7 shows the reflectance curves obtained from the ovalbumin samples. As expected, a high reflectance values corresponded to the response for a slightly colored Dot-ELISA strip whereas a low values corresponded to the response for a sample giving an intense color, because the reflectance is strongly determined by the white background and is depressed by color development. The spectra were very similar and had three reflectance bands with maxima at around 470, 640, and 720 nm and a relative minimum at approximately 570 nm, which became more

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pronounced for curves corresponding to samples of higher concentration (darker spots).

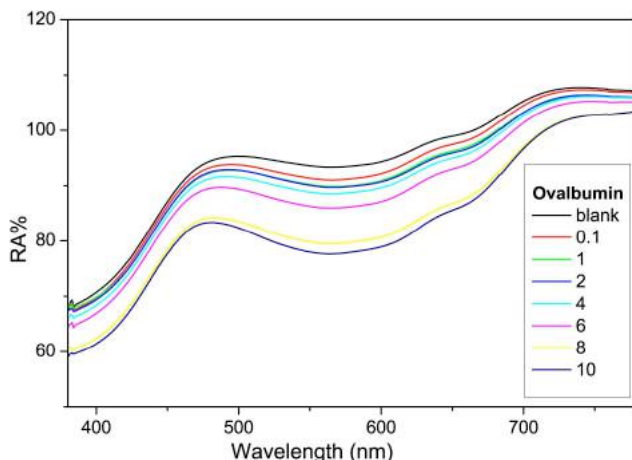


Figure 2.7: Reflectance curves of PVDF strips relating to Dot-ELISA performed on ovalbumin/blank samples at different concentrations (0.1-10 µg/mL), expressed as reflectance RA% in the wavelength range 380-780 nm.

In an attempt to perform semi-quantitative determination of the color response of the test, the reflectance values for the samples of ovalbumin at the three maxima and at the minimum were plotted as a function of ovalbumin concentration. The best linear fit was obtained for RA% 570 (the minimum) and therefore we used these data for fitting, and obtained the equation:

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

where C is the concentration of the analyte in µg/mL. Indeed, the integral of the curves was also plotted against ovalbumin concentration and the results were comparable with those of the RA% at 570 nm. The detection limit of the assay, estimated as the concentration of the protein giving an RA% value at 570 nm lower than the average of the

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RA% values at 570 nm of the background samples minus three times its standard deviation (Palmieri M. *et al.* 2010), was 1.91 $\mu\text{g/mL}$, corresponding to 9.55 ng per spot (Fig. 2.8). Analogous results were obtained for samples containing whole egg and egg white.

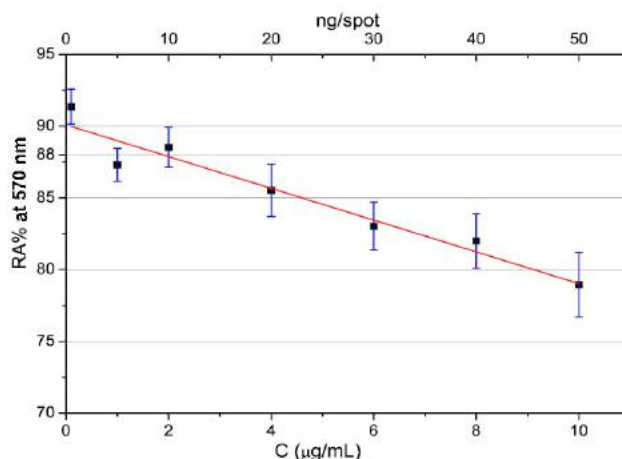


Figure 2.8: Reflectance values (RA% at 570 nm) of samples of ovalbumin as a function of different concentration (0.1-10) in $\mu\text{g/mL}$ and the relative amount of reacted protein in ng; the linear fit with standard error bars and the cut-off are reported.

Moreover, the model samples were subjected to the same spectrophotometric analysis. The corresponding reflectance curves, reported in Fig. 2.9, clearly showed that for samples containing milk and glue the curves almost overlapped the background curves, whereas for curves for samples containing egg or mixed egg-glue binders the reflectance was lower depending on development of the staining.

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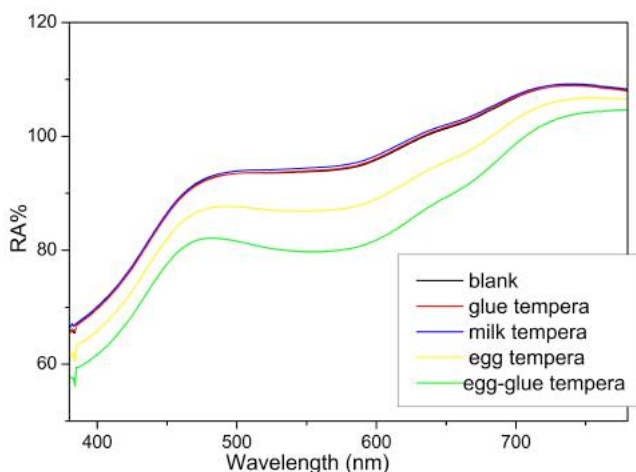


Figure 2.9: Reflectance curves of PVDF strips relating to Dot-ELISA performed on model samples, expressed as reflectance RA% on wavelength range of 380-780 nm.

Applying the above equation to the results obtained with the egg-based model samples, the concentration of the ovalbumin in the samples was calculated. For egg tempera, the reflectance value at 570 nm was 87.11, which is above the detection limit calculated above: the corresponding amount of ovalbumin in the sample was 2.72 $\mu\text{g/mL}$. Considering the known composition of the painting layer and the amount of material sampled it was possible to conclude that the yield of ovalbumin (by weight) detection was approximately 5% (Fig.2.10). For the egg-glue tempera sample the same reflectance value was 79.90, leading to an amount of ovalbumin in the sample of 9.27 $\mu\text{g/mL}$, which is approximately 3.5 times higher than the value determined for pure egg samples, leading to a yield of approximately 18% (Fig.2.10). These very low yields could be ascribed to degradation of the protein by the alkaline environment rather than to aging (only natural for three years). Another factor affecting protein

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deterioration could be the presence, together with high pH during painting, of Ca^{2+} cations: whereas some authors report this effect in the presence of chalk (Cartechini L. *et al.*), other studies (Dolci L.S. *et al.*, Palmieri M. *et al* 2010) do not consider the effect of Ca^{2+} cations particularly critical. In particular, in recent work (Palmieri M. *et al.* 2010) the “Conclusions” state that the “method (ELISA test) works properly without interferences from the most common pigments and substrates (carbonated plaster)”. Indeed, some tests were conducted in which samples of egg-based tempera were applied to well dry plaster at neutral pH but in the presence of Ca^{2+} and the response was always much more pronounced than that for samples for which the pH was strongly alkaline. This issue will be treated in ch.3, in which the effects of the pigments will be analyzed. It was surprising to notice that for the mixed binders sample the yield in ovalbumin was greater, suggesting that the glue layer, added some time after the first deposition of the egg layer, acted as a protective layer inhibiting deterioration of ovalbumin by alkalinity.

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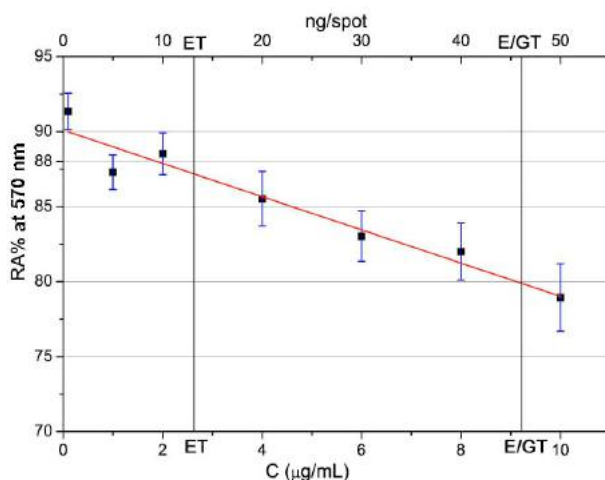


Figure 2.10: Reflectance values (RA% at 570 nm) of samples of ovalbumin as a function of different concentration (0.1-10) in $\mu\text{g/mL}$ and the relative amount of reacted protein in ng; it is reported the calculated concentrations of egg tempera (ET) and egg-glue tempera (E/GT); the linear fit with standard error bars are reported.

2.2 Identification of milk and glue-based binders

The method developed for the identification of egg albumin in pictorial samples was optimized for the identification of milk and animal glue binders.

2.2.1 Materials and methods

Reagents

Elution buffer, PBS, blocking-washing buffer and enzyme development substrate FAST BCIP/NBT were prepared as previous assays (see par. 2.1.1). All the reagents were analysis-grade (>99.5 % purity). For blocking-washing buffer it was used NFDM (Oxoid), gelatin and bovine serum albumin (Sigma Aldrich) and soybean milk

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(purchased from a local market). The antibodies used were: anti-bovine whole serum (B9398), anti-bovine whey proteins (delipidized whole antiserum- W3501), anti-goat whole serum (G5018) and anti-sheep whole serum (S4265) developed in rabbit and anti-rabbit IgG (R1131) developed in goat. The AP conjugate secondary antibodies used were: anti-rabbit IgG developed in goat (A3687) and anti-goat IgG developed in rabbit (A4187). All antibodies were purchased from Sigma Aldrich.

Samples

To evaluate the assay response with different type of proteins, positive standards control of bovine milk (from a local market) and bovine casein (Sigma Aldrich) were purchased. Also, standard references of several kind of glues were purchased: rabbit glue (one from Zecchi, one from CTS and one from an unknown source), bone glue (Zecchi), animal glue (one in pearl and one in tablet), a pure hide glue and an hide glue and a commercial gelatin (porcine source) were purchased. The different glues were prepared according the traditional recipes, leaving to swell the solid glue in water overnight. Then the solution was slightly heated until 60°C to yield a liquid gelatin. Aliquots of 200 µL were collected and dispersed in elution buffer in a ratio 1:5.

2.2.2 Results and discussion

The protocol developed for the identification of egg albumin was followed for these proteins, too. The results (Fig. 2.11) show the capability of the anti-bovine whey protein to recognize the casein and

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the milk proteins. These proteins were recognized also from anti-goat and anti-sheep, but they produce a high background in anti-rabbit IgG assays, due to the cross-reactivity between the secondary antibody (anti-goat) and the non fat dry milk-based blocking buffer. This will be the purpose of the study, in order to optimize the assay for the detection of any kind of protein, without changing the composition of the blocking and thus without cross-reactivity phenomena. Indeed, it's theoretically incorrect to use a NFDM-based blocking buffer in assays for detection of caseins or milk proteins. In the same way it is wrong to use a gelatin-based blocking agent in assays to detect collagen.

Among the glues, bone glue, rabbit glue (Zecchi) and animal glue (in pearl) were submitted to assay. Results show that only the bone glue was recognized from the anti-bovine serum, anti-whey protein and anti-goat (Fig. 2.11). From these results, the bone glue composition was mainly bovine bones-based. The composition of animal and rabbit glue was unknown, because first the anti-rabbit and then the other antibodies didn't recognize the antigens. A part of the study will cover this issue.

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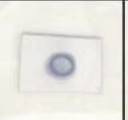
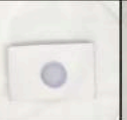
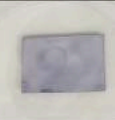
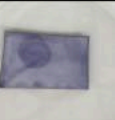
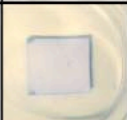
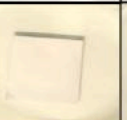
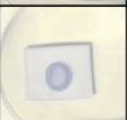
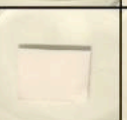
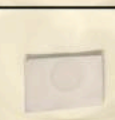
	Anti-bovine whole serum	Anti-bovine whey protein	Anti-goat whole serum	Anti-sheep whole serum	Anti-rabbit IgG
Bovine milk					
Casein					
Rabbit glue					
Bones glue					
Animal glue					

Figure 2.11: Dot-ELISA results of different protein materials (bovine milk, bovine casein, rabbit glue, bone glue and animal glue) submitted to assay with anti-bovine whey protein, anti-rabbit IgG and anti-bovine, anti-goat and anti-sheep sera.

2.2.2.1 Optimization of the blocking buffer

In order to set up a valid assay for every kind of protein (milk proteins, egg proteins and glues) different blocking solution were tested. In particular T-PBS (1%Tween20 in PBS 1X), BSA 3% in T-PBS , gelatin 3% in T-PBS , NFDM 5% in T-PBS and finally soybean milk 3% in water were tested. The results with the soymilk-based buffer are interesting because they don't show any cross-reactivity phenomena with any antibody, therefore it was chosen soybean milk buffer to meet

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the requirement. Also, the we have chosen to use the T-PBS solution as washing buffer, because the presence of a blocking protein during some steps of the protocol (washing step before the introduction of the secondary antibody) can inhibit the bound between the ligands.

2.2.2.2 Detection of glues

The problem of detection of animal glues and in particular of the rabbit glue, was very troublesome. The first results on rabbit glue tested for the anti-rabbit detection with Dot-ELISA were negative. The first problem was the high intensity of the background stain. This problem was overcome by using a different blocking/washing buffer. The presence of milk protein in the buffer produced cross-reactivity phenomena with the anti-goat developed in rabbit, the secondary antibody for the primary anti-rabbit developed in goat. Moreover, in all cases, the stain spot was not detected and frequently it was present several unknown not-specificity effects. In a second time, the antibody was changed and two types of anti-collagen (anti-collagen type I and anti-bovine collagen type I) were chosen and utilized for the detection of different kind of glues: hide glue, bone glue and two different rabbit glues (Zecchi and unknow source). The results (data not shown) were positive but not completely fulfilling; comparing the results obtained for the same sample with the different antibodies, it was clearly that the problem was in the different glues used as positive standards. Finally, all reference standards of glues were tested with the anti-rabbit IgG and the anti-bovine whole serum (Fig. 2.12). Despite the intensity of the color stain is evident only in the case of animal glue in tablet, tested for anti-rabbit IgG, these results were considered encouraging.

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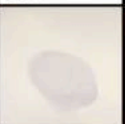
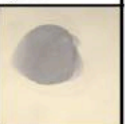
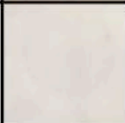
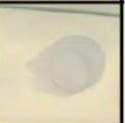
	Hide glue	Rabbit glue1	Rabbit glue 2	Animal glue
Anti-rabbit IgG				
Anti-bovine whole serum				

Figure 2.12: Dot-ELISA assays for rabbit IgG and bovine serum detection in several glue samples: hide glue, rabbit glue from Zecchi, rabbit glue from unknown source and animal glue in tablet.

On this way, other reference standard glues have been submitted to Dot-ELISA with different antibodies, to identify their animal origin (Fig 2.13).

From Hodgins and Hedges (1999), we know that the problem of detection of the glues is not new. In order to evaluate species-specific antibodies with various types of glues to identify their origin, Hodgins and Hedges have found, by means of ELISAs, that not always the commercial reference corresponds animal origin. In the case of glues and adhesives, differently from archaeological bones, identification is more complex due to the absence of morphological cues in glues and because of greater exposure to degradation due to the absence of a protective structure as in the bones. Moreover, they stated that anti-collagen antibodies were not strictly species-specific, because the collagens of related species is similar and because cross-reactivity phenomena occur between more far related species.

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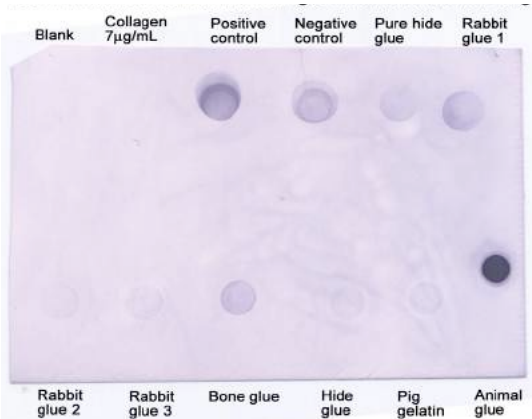


Figure 2.13: Dot-ELISA results of anti-rabbit IgG search in different type of glues. A positive control of animal glue, a negative control of ovalbumin and a blank were also submitted to analysis.

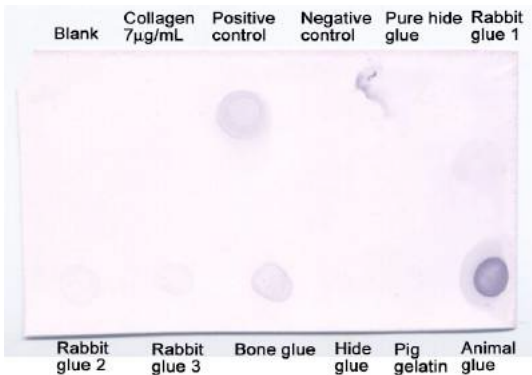


Figure 2.14: Dot-ELISA results of anti-bovine whole serum search in different type of glues. A positive control of animal glue, a negative control of ovalbumin and a blank were also submitted to analysis.

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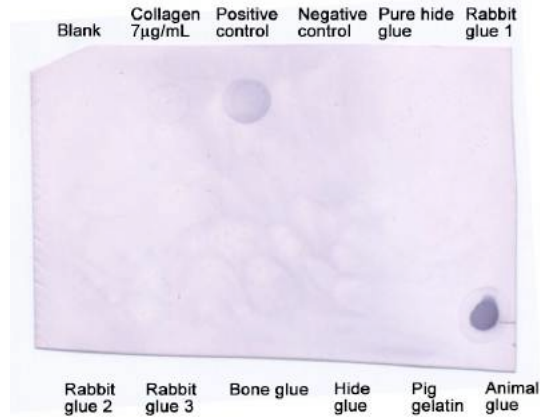


Figure 2.15: Dot-ELISA results of anti-bovine collagen type I search in different type of glues. A positive control of animal glue, a negative control of ovalbumin and a blank were also submitted to analysis.

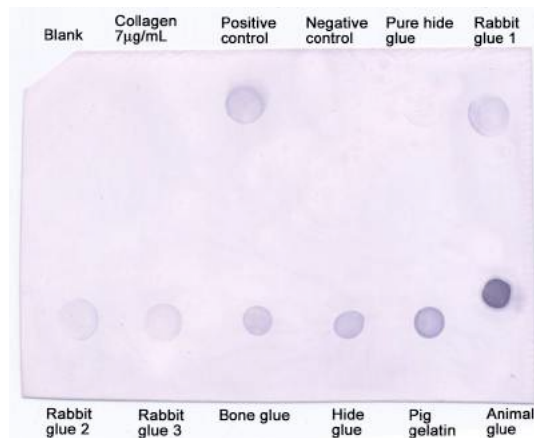


Figure 2.16: Dot-ELISA results of anti-pig whole serum search in different type of glues. A positive control of animal glue, a negative control of ovalbumin and a blank were also submitted to analysis.

From the results, it can be seen as the anti-bovine collagen type I recognizes the animal glue and the positive control (Fig. 2.15). The anti-bovine whole serum identifies the usual bone glue, animal glue and the positive control (Fig. 2.14). The anti-rabbit IgG recognizes the

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presence of the immunoglobulins of the rabbit in a variety of samples including the usual animal glue in the tablet, the positive control, but also the bone glue and rabbit glue from Zecchi (Fig. 2.13). The anti-pig serum identifies well the pork edible gelatin (derived from pig waste) and also recognizes the animal glue in tablet, the hide glue and the bone glue (Fig. 2.16). Moreover, it must be highlighted that there are other positive but weak signals especially for the rabbit glues. These results are very interesting, because they confirm the hypothesis that modern commercial glues for artworks, especially rabbit glues, are not prepared according to traditional recipes from rabbit waste, but from various animals. These findings would deserve further investigation. In this context, the use of Dot-ELISA, thus a immune-enzymatic assay, was crucial to understand this issue.

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CHAPTER 3.

STUDY OF THE EFFECTS OF PIGMENTS AND AGEING

3.1. Introduction

In order to study the effects of the presence of inorganic pigments and the effects of ageing on protein binders, mock-ups were prepared and successively submitted to artificial ageing. The aim is to verify the reliability of the Dot-ELISA in presence of pigments and understand their influence on the detection of protein, also after artificial ageing. It is well known that, at first, the drying process of a paint layer induces a series of modifications in the protein structure, caused by the rearrangement of the structure for loss of water (Karpowicz A.). The empty spaces, produced by decrease of water content, are filled by the re-organization of the peptide chain, which lose its ordered status to become uncoiled and unfolded. The side chain of functional groups, usually faced in the inner side of the chain in the native state of protein, become available for external molecules interactions; this process increase the chemical reactivity of the protein. Often the denaturation of proteins starts during the preparation of media, such as in alkaline precipitation of casein. Some authors (Davies K.J.A. *et al*), consider the denaturation, thus the unfolding of the protein, responsible of the following degradation occurrences (fragmentation and aggregation).

During the natural ageing, a series of chemical changes occurs: oxidation and cross-linking of the organic matter, interactions of pigments with binders and with environment. Long time, the most

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studied degradation process involved the oil-based paintings. Indeed, the cations in some pigments (zinc, copper and lead) react with free fatty acids to form insoluble metal soaps. Metal soaps are carboxylates formed from metals and long chain fatty acids such as palmitic and stearic acid (rarely azelaic acid, product from the degradation of polyunsaturated C18 fatty acids in the oil matrix). These can aggregate on the paint surface and produce inclusions and protrusions (Plater M.J. *et al.*), which usually damage the appearance of the paint. For example, verdigris, a Cu-based acetate or carbonate pigment (according to the production technique) darkens in old paints, because of the Cu soap formation between the copper and the fatty acids arising from the hydrolysis of glycerolipids. (Kuhn H.)

Also for protein degradation, metal cations from pigments, such as Cu^{2+} , Hg^{2+} , Fe^{3+} , Pb^{2+} , Zn^{2+} , Ca^{2+} and Cd^{2+} , interact with the protein forming strong complexes, which interfere in GC-MS analysis (Shilling M.R. *et al.*, Colombini M.P. *et al.*, Wouters J. *et al.*). Duce C. *et al.* (2012) shows how the cinnabar interacts with proteins; in particular it forms stable mercury-protein complexes and promote the hydrolysis and the intermolecular cross-linking in casein and mainly oxidation in egg albumin. These results were confirmed in Khandekhar N. *et al.*: the cinnabar/egg-based tempera induces the cross-linking in which high molecular weight molecules were found, in aged samples the cinnabar induces a fragmentation of protein chain into lower molecular weight species. Other works, dealing the protein-pigments degradation mechanisms, focused on azurite, smalt and lead pigments (Odlyha M. *et al.* 2000a and b, Cohen N.S. *et al.*), demonstrated that several degradation effects occur in egg temperas before and after

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ageing. In the case of smalt-based tempera, the cobalt ions promote the formation of cross-linked aggregates of lecithins, which form a network structure, increasing the mechanical properties of binder. In the same series of works, Odlyha M and Cohen N.S. analyse the behavior of lead pigments and azurite in egg tempera: the first promote the formation of metal soaps from free fatty acids coming from the oxidation of cholesterol and glycerolipids and the second promote copper catalysed phenomena of autoxidation during the curing process. In a recent study (Duce C. *et al*, 2013), these results were confirmed, both for azurite and also for calcium carbonate and for red lead pigments. The decrease in thermal stability is due to direct interaction of pigments with the functional group of amino acids or indirect promotion of oxidative stress, which induce unfolding and rearrangement of protein. This decrease in thermal stability can be expressed in an increase in proteolytic susceptibility, which can be expressed as the resistance of protein to enzymatic digestion. To this end, it is known that the proteolytic susceptibility of a protein increases in oxidative conditions (Davies K.J.A. *et al*), thus in the presence of radical species such as hydroxyl radical or hydroperoxides, usually due to photo-oxidation (Karpowicz A.). On the other hand, it is well known that the main event that occurs in an organic film exposed to UV-vis irradiation, is the formation of free radicals by autoxidation of unsaturated compounds. To induce oxidation in proteins the presence of photosensitizers is needed, which catalyse the production of hydroperoxides when excited. In the presence of water, the damage on proteins is due to effect of splitting the water molecule into OH and H radicals and hydrated free electrons (Karpowicz A.). The exposure to the hydroxyl radical generates phenomena of fragmentation and/or

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aggregation of the protein structures (depending on the presence or absence of free oxygen) and changes in the net charge; it also involves changes in the amino acid sequence with the loss of the Trp and the production of dityrosine biphenol (Davies K.J.A. *et al*). Dityrosine is a covalently bound biphenol, produced by reaction of two tyrosyl radicals or a tyrosyl radical with a molecule of tyrosine; it is responsible of the aggregation of proteins molecules due to its feature to form intermolecular bonding. Besides the dityrosine formation, the process of aggregation can be due also to the formation of disulphide bridges (Duce C. *et al*, 2013) and to the intermolecular hydrophobic interactions. Moreover, intermolecular interactions can be established between amino acid radicals and with amino acid molecules (Davies K.J.A. *et al*). The protein aggregation, thus the presence of molecular species with a higher molecular weight, induces a more thermal stability in pure aged proteins. Because of the low content in S-H groups and Tyr in its primary structure, the casein shows a simple aggregation, but not cross-linking formation. The photo-oxidation most susceptible amino acid are His, Trp, Tyr, Met and Cys. Two -SH groups of cysteine, in oxidation process, can bind through a disulfide bridge (-SS-) losing a molecule of water and forming cystine. Met oxides creating sulphoxides and disulphoxides (Karpowicz A., Leo G. *et al*.) demonstrate that, besides the presence of amino malonic acid as oxidation product of Ser, Cys and Phe, the deamidation of Asn and Gln can be considered the major deterioration in aged proteins. This occurrence could be due to several factors, including a variation of pH. Indeed, the high basic environment during the preparation of casein solution for consolidating purpose in the cited work, caused by the addition of slaked lime, could have affected the degradation process.

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Studies on the ageing/pigments effects in the detection of protein by means of Dot-ELISA have not yet been published. The only study in the field of diagnostics for cultural heritage, which use an immunological technique to identify proteins, refer to the ELISA (Palmieri M. *et al.*, 2010 and 2013). In the first work, Palmieri *et al.* (2010) submitted pigmented aged samples to ELISA assays, obtaining differences in results in protein detection only for aged samples. The decrease of the optical density is justified as lower solubility of proteins and a less availability of antigenic sites, caused by the degradation factors such as deamidation, hydrolysis and oxidation. The decrease in assay sensitivity was reported also in the second work of Palmieri M. *et al.* (2013), in which it is stated that the variability in OD measures of the ELISA results was due to the non homogeneous used micro-sample.

The aim of this part of work is to evaluate as the artificial ageing and the pigment presence can affect the protein detection by Dot-ELISA. Paint mock-ups were prepared using different pigments with whole egg, bovine milk and animal glue and submitted to light and thermally ageing.

3.2. Materials and methods

Samples

Paint mock-ups were prepared according to traditional painting procedures (Cennini C.). A layer of tempera was painted on the specimen surface, which is prepared with CaCO_3 and sand (Sigma-Aldrich) (1:3 v/v). The pigments (all from Zecchi, Florence) selected were: S. Giovanni white (BSG), azurite (BC), smalt (BS), yellow ochre (OG), hematite (EMA), cinnabar (CIN), minium (MIN) and verdigris (VRG). To compare the effects of only ageing a layer of only binders was applied on the plaster ground surface (ST). The binder used were: whole egg, bovine milk and animal glue (from Zecchi) in ratio 1:2 (v/v) with pigments. The animal glue were prepared according to traditional uses: it was leave to swell in water and then slightly heated at 60°C to yield a liquid gelatin. The sampling on non-aged and aged mock-ups, was performed by means of a scalpel scraping off only the painted layer and not the plaster ground. For all samples around 1 mg of paint was taken. Two type of ageing were performed: a light ageing for 400h and a thermal ageing for 600h. Light ageing was conducted in a Solarbox CO.FO.MEGRA model 3000-e equipped with a Xenon-arc lamp with an outdoor-type UV filter (cut-off < 290 nm, Irradiance= 550 W/m² and BST=65°C). Thermal ageing was performed at 80°C at RH=75%.

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Table 3.1: Denomination of each specimen according to binder and pigment used.

	Egg binder	Milk binder	Animal glue binder
BSG	BSG-U	BSG-L	BSG-C
BC	BC-U	BC-L	BC-C
BS	BS-U	BS-L	BS-C
OG	OG-U	OG-L	OG-C
EMA	EMA-U	EMA-L	EMA-C
CIN	CIN-U	CIN-L	CIN-C
MIN	MIN-U	MIN-L	MIN-C
VRG	VRG-U	VRG-L	VRG-C
ST	ST-U	ST-L	ST-C

Materials

PBS, Sigma FAST and elution buffer were prepared as in par. 2.1.1; blocking buffer was soybean milk-based and washing buffer was T-PBS (0.2% Tween 20 in PBS 1X) (see par.2.2.2). Primary antibodies used were: anti-chicken egg albumin for egg temperas, anti-bovine whey protein for milk temperas and anti-rabbit IgG, anti-pig (whole serum) and anti-bovine (whole serum) for glue temperas. Secondary antibody was anti-rabbit IgG, AP conjugate and anti-goat IgG AP conjugate. All antibodies were purchased from Sigma Aldrich. All others materials used were the same that in par. 2.1.1.

Procedure

The Dot-ELISA procedure was the same previously described (see par. 2.1.1). The antibodies dilution was 1:6,000 (final concentration) for the primary one and 1:5,000 for the secondary one. The introduced volume

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of sample solution was 2 μL for each one. For the assessment of staining intensity it was used a flat-bed commercial scanner and the image analysis was performed using the ImageJ software (<http://rsb.info.nih.gov/ij>). First, the dry membranes were scanned; to the resulting images (.jpg), the background contribute was subtracted (25 ball radius), the inversion process were performed and all the spots were measured by means of a circular standard area. Results of color intensity were reported as “integrated density”.

3.3 Results and discussion

The choice to submit the mock-ups to a strong artificial ageing by high temperature and high relative humidity percentage, has been taken to promote the degradation processes discussed in the Introduction paragraph. In particular, the samples were first subjected to aging to encourage the processes of activation of free radicals by autoxidation, which would have triggered the fragmentation and aggregation of proteins. From previous test, it was found that the thermal aging of the casein at higher temperatures produced a strong increase of fluorescence intensity ($T=80^{\circ}\text{C}$ vs $T=60^{\circ}\text{C}$) (Fig. 3.1). On the other hand, it doesn't exist an generally accepted protocol for the ageing of protein material. The results on aged and non-aged egg temperas, tested for the egg albumin research, were reported in Fig.3.2. The non-aged samples did not undergo the influence of the pigment, even if, as was previously discussed, some pigments, such as azurite, verdigris and cinnabar are quite reactive already from the drying process. The aged samples, instead, show a disappearance of the colored spot: the

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antibody was not able to recognize the epitope of the egg protein. This finding confirm that of Palmieri M. *et al.* (2010), in which ELISA tests were performed on artificial aged and non aged pigmented samples. They found that the only pigmented mock-ups, submitted to ELISA tests, did not show any clear differences in measured optical density (OD) compared to the un-pigmented sample. The aged samples, instead, show a decrease in OD values, caused by the degradation processes catalyzed by pigments. Cartechini L. *et al.*, also, stated that the presence of pigments induces a modification in the OD values after artificial ageing.



Figure 3.1: Different temperatures in artificial ageing treatments produce a different fluorescence response in samples of casein spotted onto glass slides

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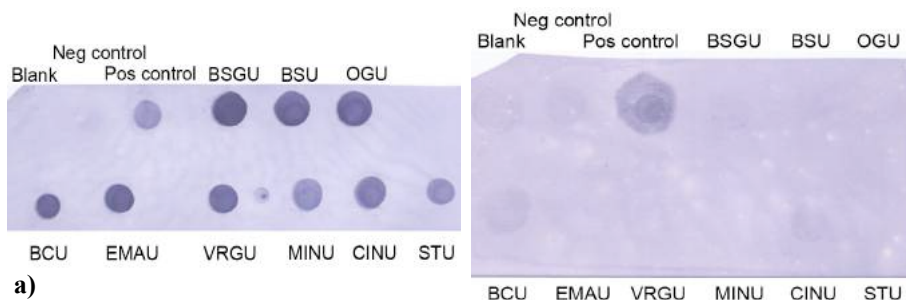


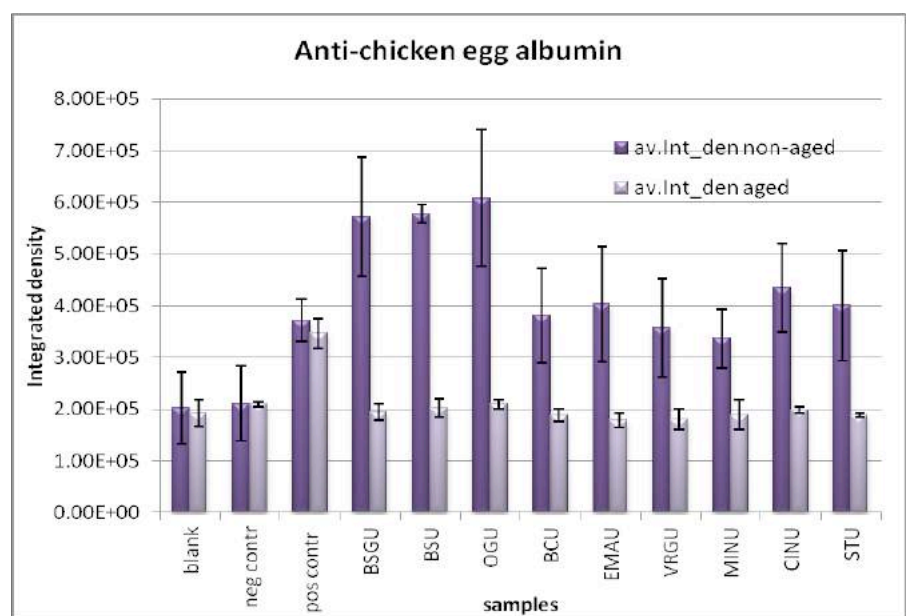
Figure 3.2. Dot-ELISA result of non-aged (a) and aged (b) egg tempera mock-ups, submitted to assay for detection of anti-chicken egg albumin.

By means of the image analysis with ImageJ, it was performed an evaluation of the decrease of the spot signal. The integrated densities, obtained for each spot, were plotted in a bar graph (Gr. 3.1). It is clear that the integrated density values of the aged samples are comparable with those of blanks. For only pigmented samples some differences are visible: in S. Giovanni white (BSG-U), smalt (BS-U) and yellow ochre (OG-U) egg tempera samples, the protein didn't seem to suffer the influence of pigments and the Dot-ELISA test is able to recognize the epitope. These findings could be due to non-homogeneity of the samples, but literature confirm that this pigments are very little reactive with protein before ageing. The width of the standard deviation bars of non-aged samples is wide and variable, except in the case of smalt tempera and positive control, for which the ratio between its standard deviations and that of blank is minor of 1 (Tab. 3.2). From the literature, Odlyha M. *et al.* highlighted that the smalt produces a reinforcement in protein structure and a increased adherence to substrate; moreover, the presence of lecithins in egg temperas induces the formation of a network structure composed by cross-linking aggregations, promoted form Co^{2+} ions. After ageing, instead, the color

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intensity of the spot decreases, demonstrating that ovalbumin undergoes a strong degradation. As for CaCO_3 influence on binders, Duce C. *et al* (2013) stated that the presence of calcium carbonate-based pigments increases the thermal stability after ageing, because the CaCO_3 establishes a strong interaction with carboxyl group of the peptide bond, but at the same time, it promotes hydrolysis phenomena. Azurite, hematite, verdigris, red lead and cinnabar temperas show the same behaviour as the unpigmented specimen. The azurite is a widely used and studied pigment, because of the high reactivity of the cation Cu^{2+} , which is involved in coordination metal-protein with the nitrogen atoms the side chain of various amino acids, such as Lys and Arg; Pb^{2+} in red lead, has the same behaviour with the oxygen atoms of the amino acids. These pigments induce modifications of the protein secondary structure, enhanced after aging by producing a decrease in thermal stability, hydrolysis and aggregation to higher molecular weights due to the weakening of the intermolecular interactions (Duce C. *et al.*, Khandekar N. *et al.*., Odlyha M. *et al.*). Regarding to cinnabar tempera, the presence of the pigment produces degradation effects of hydrolysis and molecular aggregation starting from the drying process (Duce C. *et al.* 2012, Romero-Pastor J. *et al.*, Khandekar N. *et al.*). In our case the color intensity of the dot is not depressed, but in aged samples, the enhanced effects of the degradation induce the signal disappearance. According to these findings, Cartechini L. *et al.* in ELISA tests highlighted the decrease in OD values more in glue tempera but also in casein.

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Graph 3.1: Image analysis performed on Dot-ELISA for detection of anti-chicken egg albumin on egg tempera-based specimens. Results are expressed as average of measured Integrated density for each aged and non-aged samples: the darken violet bars identify the non-aged samples and the lighter violet bars identify the aged samples.

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Table 3.2: Average of Integrated density (av_ID) values with its standard deviations (SD) and the % of the SD values respect to the blank value were reported for each aged and non-aged egg-based tempera samples obtained by means of image analysis.

Non-aged			Aged	
sample	av_ID $\pm$ SD	% SD on blank	av_ID $\pm$ SD	% SD on blank
blank	202,849 $\pm$ 70,031	1.00	191,828 $\pm$ 25,993	1.00
-ve contr	210,931 $\pm$ 72,332	1.03	209,855 $\pm$ 5,438	0.21
+ve contr	372,176 $\pm$ 40,347	0.58	346,807 $\pm$ 28,777	1.11
BSGU	571,731 $\pm$ 115,307	1.65	194,391 $\pm$ 16,398	0.63
BSU	577,723 $\pm$ 18,180	0.26	201,957 $\pm$ 17,515	0.67
OGU	608,380 $\pm$ 131,608	1.88	209,886 $\pm$ 9,241	0.36
BCU	380,459 $\pm$ 91,351	1.30	188,081 $\pm$ 11,467	0.44
EMAU	403,187 $\pm$ 112,140	1.60	178,772 $\pm$ 13,758	0.53
VRGU	357,403 $\pm$ 95,285	1.36	180,520 $\pm$ 19,048	0.73
MINU	336,786 $\pm$ 57,266	0.82	189,066 $\pm$ 28,946	1.11
CINU	435,165 $\pm$ 85,290	1.22	198,647 $\pm$ 5,796	0.22
STU	400,659 $\pm$ 105,912	1.51	188,470 $\pm$ 3,281	0.13

The similar results were obtained for milk protein research on aged and non-aged milk temperas (Gr. 3.2). In this case, the red lead, cinnabar and non-pigmented specimen show a decrease in the color intensity of the stain, confirming what previously stated about the effects of the degradation of cinnabar and red lead on proteins. In addition, the result of the non-pigmented sample also confirms that the preparation conditions can affect the preservation of the protein. In this case, as the milk was applied on a not completely carbonated plaster and therefore rich in calcium hydroxide, the environment conditions may have produced a protein denaturation and a basic hydrolysis. Also in anti-

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bovine whey protein Dot-ELISAs, the hematite and the s. giovanni white milk-based temperas show low reactivity with the protein media.

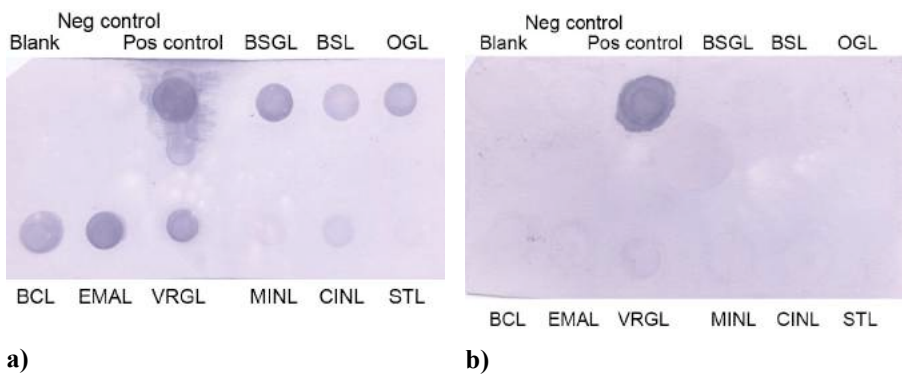
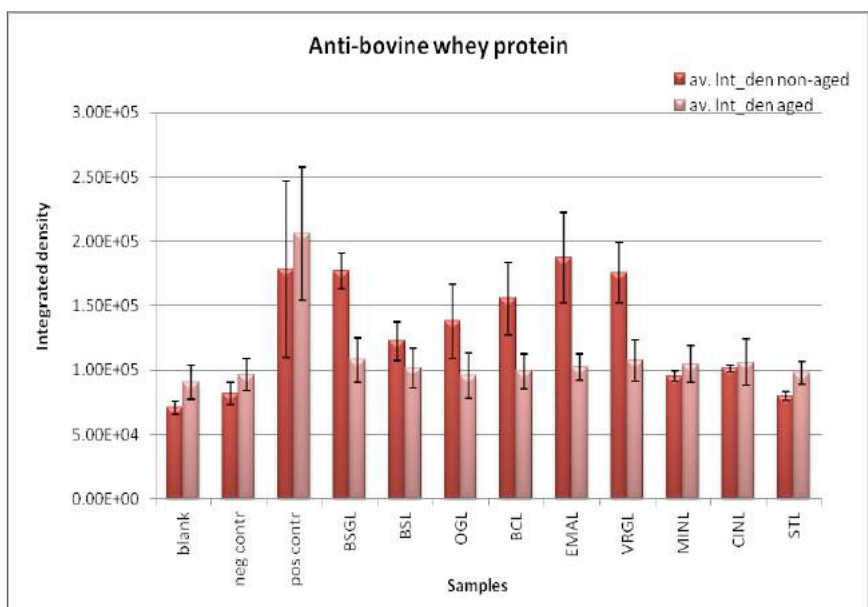


Figure 3.3: Dot-ELISA result of non-aged (a) and aged (b) milk tempera mock-ups, submitted to assay for detection of anti-bovine whey protein.



Graph 3.2: Image analysis performed on Dot-ELISA for detection of anti-bovine whey protein on milk tempera-based specimens. Results are expressed as average of measured Integrated density for each aged and non-aged samples: the darken red bars identify the non-aged samples and the lighter red bars identify the aged samples.

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Table 3.3: Average of Integrated density (av_ID) values with its standard deviations (SD) and the % of the SD values respect to the blank value were reported for each aged and non-aged egg-based tempera samples obtained by means of image analysis.

sample	Non-aged		Aged	
	av_ID $\pm$ SD	% SD on blank	av_ID $\pm$ SD	% SD on blank
blank	71,022 $\pm$ 5,098	1.00	90,573 $\pm$ 12,881	1.00
-ve contr	81,903 $\pm$ 8,929	1.75	96,357 $\pm$ 12,356	0.96
+ve contr	178,197 $\pm$ 68,615	13.46	206,070 $\pm$ 51,729	4.02
BSGL	177,104 $\pm$ 13,888	2.72	108,148 $\pm$ 17,243	1.34
BSL	122,650 $\pm$ 15,183	2.98	101,482 $\pm$ 15,200	1.18
OGL	138,050 $\pm$ 28,996	5.69	95,751 $\pm$ 17,744	1.38
BCL	155,662 $\pm$ 28,085	5.51	99,228 $\pm$ 13,514	1.05
EMAL	187,222 $\pm$ 34,924	6.85	102,364 $\pm$ 10,142	0.79
VRGL	175,432 $\pm$ 23,283	4.57	107,469 $\pm$ 16,153	1.25
MINL	95,320 $\pm$ 3,876	0.76	104,750 $\pm$ 14,287	1.11
CINL	101,320 $\pm$ 2,515	0.49	106,138 $\pm$ 17,854	1.39
STL	79,801 $\pm$ 3,337	0.65	98,010 $\pm$ 8,830	0.69

For animal glue-based temperas, different antibodies were used to detect protein material in aged and non-aged samples. The detection of glues has been treated in the Ch. 2.2.1. In this case, a commercial reference rabbit glue was used to paint the mock-ups. The first antibody used to detect the protein material was anti-rabbit IgG. This was chosen because it showed good results in detection of historical samples (data not shown). The results of Dot-ELISA were surprising because both aged and non aged samples were not identified (Fig 3.4). This result could be inferred to various reasons. First the presence of pigments (previously discussed) could affect the protein integrity also in non-aged samples. Cartechini L. *et al.* demonstrate, with ELISA test

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on painted and aged samples, that the animal glue is the most affected protein material by pigments influence, so much that the OD of the cinnabar-based aged samples disappear. Also Manzano E. *et al.*, in a study about the effects of UV irradiation on rabbit glue binder samples painted with a blue-copper-based pigments, demonstrate that the glue undergoes to strong degradation after artificial ageing. Secondly, the small amount of material collected and analyzed: because of its low viscosity, glue is adsorbed in the porous ground and forms a very thin film, difficult to remove. Finally the initial degradation of the glue caused by industrial production processes (temperature, pH, solvents) could affect the protein causing its denaturation and degradation. As previously stated, the real animal origin of glue is not predicted from the commercial denomination. For this reason, also anti-bovine and anti-pig serum were tested with these samples. The results are shown in Figs 3.5 and 3.6.

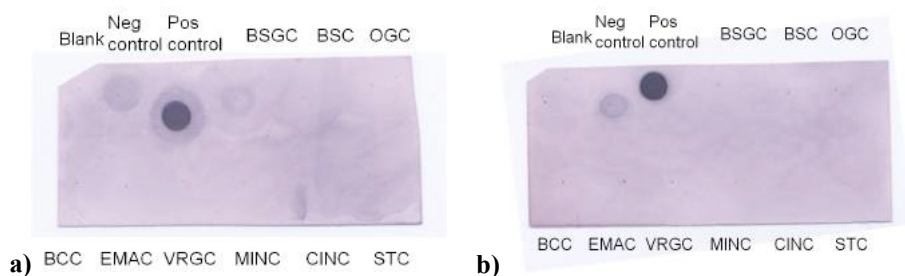


Figure 3.4: Dot-ELISA result of non-aged (a) and aged (b) glue tempera mock-ups, submitted to assay for detection of anti-rabbit IgG.

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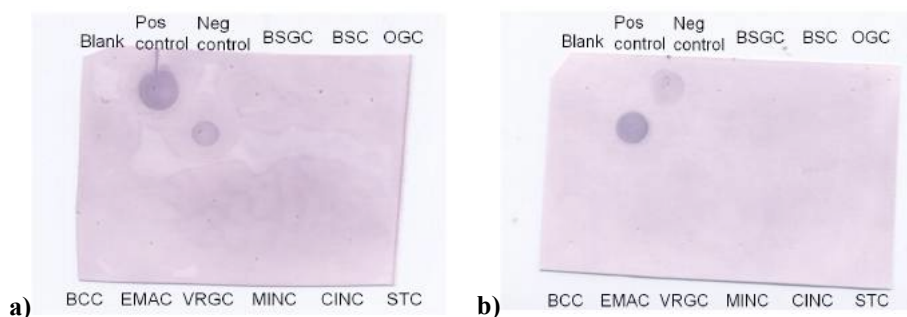


Figure 3.5: Dot-ELISA result of non-aged (a) and aged (b) glue tempera mock-ups, submitted to assay for detection of anti-bovine serum.

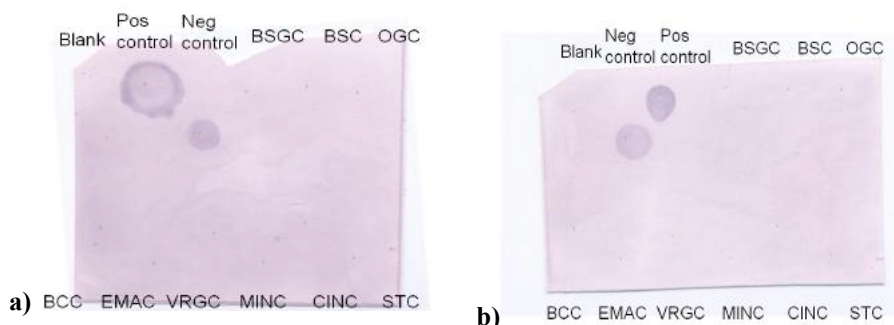
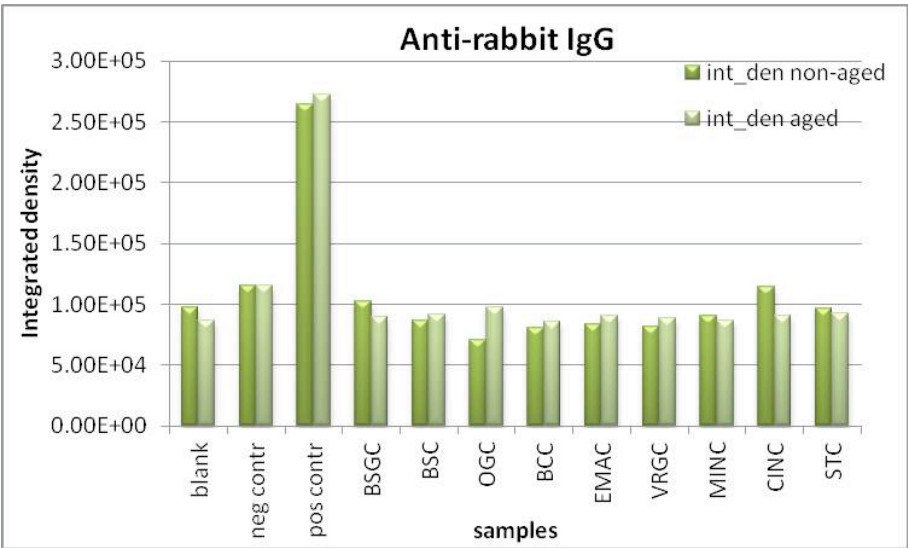


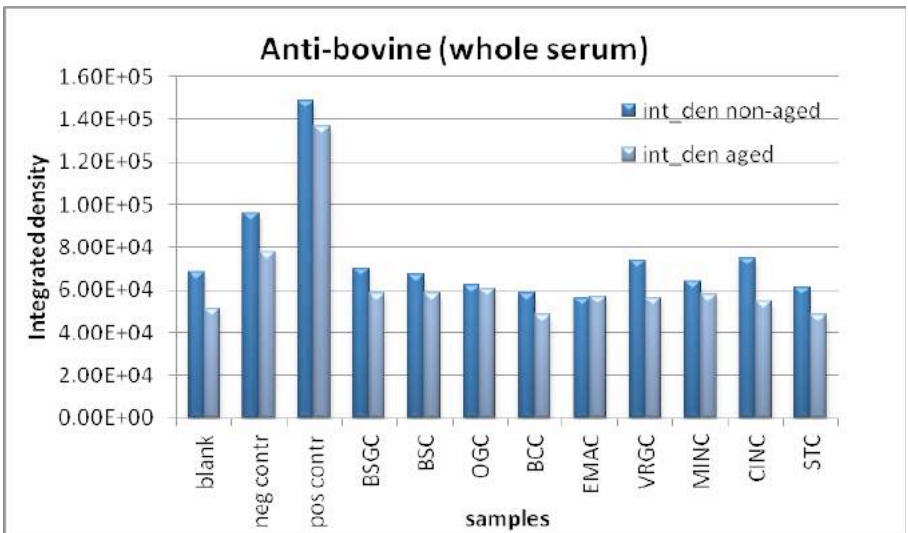
Figure 3.6: Dot-ELISA result of non-aged (a) and aged (b) glue tempera mock-ups, submitted to assay for detection of anti-pig serum.

Also in these results the coloured spots were not visible in aged and in non-aged samples. In case of anti-pig serum the positive and the negative controls have similar intensities highlighting that cross-reactivity phenomena occurs between egg albumin and the anti-pig antiserum. In graphs 3.3-3.5, plotted integrated densities of spots for each samples were shown. In all graphs the sample related ID values are lower than those of negative control and blank control. In all cases no relevant differences are noticeable between the aged and non-aged samples.

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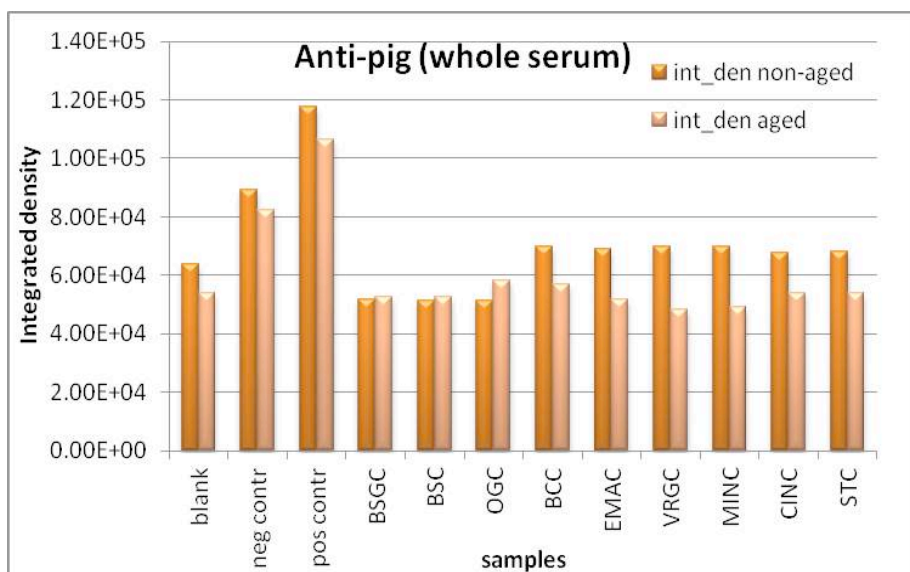


Graph 3.3: Image analysis performed on Dot-ELISA for detection of anti-rabbit IgG on glue tempera-based specimens. Results are expressed as average of measured Integrated density for each aged and non-aged samples: the darkened green bars identify the non-aged samples and the lighter green bars identify the aged samples.



Graph 3.4: Image analysis performed on Dot-ELISA for detection of anti-bovine serum on glue tempera-based specimens. Results are expressed as average of measured Integrated density for each aged and non-aged samples: the darkened blue bars identify the non-aged samples and the lighter blue bars identify the aged samples.

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Graph 3.5: Image analysis performed on Dot-ELISA for detection of anti-pig serum on glue tempera-based specimens. Results are expressed as average of measured Integrated density for each aged and non-aged samples: the dark orange bars identify the non-aged samples and the lighter orange bars identify the aged samples.

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CHAPTER 4.

ANALYSIS BY MEANS OF WESTERN BLOT

Western Blot is a widely used technique in cell and molecular biology, able to identify a specific protein in a complex mixture of protein material from cell extracts or tissue homogenates. The principles of Western Blot is to use three elements to accomplish this task: 1) separation by size, 2) transfer onto a solid support and 3) immunologic detection with a proper antibody. The advantage of the technique, compared to traditional ELISA, is the separation of mixture of protein based on molecular weight, by means gel electrophoresis. The obtained bands are then transferred to a membrane by means of an electrical current that induces the proteins to migrate from the gel to membrane. After the transfer the proteins are immobilized on membranes and then revealed by immunologic detection.

4.1 The Western Blot technique

The history of technique is curious and the authorship attribution is not clearly defined. In 1979, several research groups were working on the blotting of proteins. The first publication that contains a passive transfer of membrane proteins is that of J. Renart & J. Reiser of the George Stark's research group at Stanford University. After a few months, the research team of H. Towbin in Basel, Switzerland, with J. Gordon (at Friedrich Miescher Institut) and T. Staehelin (at Hoffman-La Roche), also publish a procedure similar to what is now meant by Western Blotting. They developed the method of electrophoretic

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transfer of proteins to membranes (instead of passive transfer like Stark) and the now-ubiquitous immunoblotting sandwich using electric current to transfer proteins from the SDS-PAGE gel to the membrane. Their procedure also used secondary antibodies for detection. In the same year also W.N. Burnette, working in Robert Nowinski's lab at the Fred Hutchinson Cancer Research Center in Seattle, tried to publish his work but is rejected. Although he uses the electrophoretic separation of proteins, followed by transfer procedure of the membrane. He deserves the credit for having given a name to the technique, calling western, referring to both the southern technique, invented by Edwin Southern at University of Edinburgh in 1975 and northern blotting, invented by George Stark's Stanford group in 1977, and because his lab was on west coast.

For the traditional use of this technique, the proteins were obtained by means of lysis treatment of cells and tissues. In the case of paint media proteins this step is not required, but a sample pre-treatment is suggested. After extraction, it is important to determine the protein concentration with a Bradford, Lowry or BCA assay, to balance the mass of sample to load in the wells.

Antibodies typically recognize a small portion of the protein of interest (referred to as the epitope) and this domain may reside within the 3D conformation of the protein. To enable access of the antibody to this portion it is necessary to unfold the protein, i.e. denature it. To denature the protein, usually it is used a loading buffer with the anionic denaturing detergent sodium dodecyl sulfate (SDS), and boiled the mixture at 95-100°C for 5 min. The standard loading buffer is called 2 X Laemmli buffer, first described in 1970 (Laemmli U.K.). When SDS

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is used with proteins, all of the proteins become negatively charged by their attachment to the SDS anions. SDS denatures proteins by “wrapping around” the polypeptide backbone. SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length i.e. the denatured polypeptides become “rods” of negatively charged clouds with equal charge or charge densities per unit length. In denaturing SDS-PAGE separations, therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. Moreover, it is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size by adding β -mercaptoethanol or dithiothreitol (DTT). Glycerol is added to the loading buffer to increase the density of the sample to be loaded and hence maintain the sample at the bottom of the well, restricting overflow and uneven gel loading. To enable visualization of the migration of proteins it is common to include a small anionic dye molecule (e.g. bromophenol blue) in the loading buffer. The dye will migrate the fastest of any component in the mixture to be separated and provide a migration front to monitor the separation progress.

Alternatively, an antibody may recognize an epitope made up of non-contiguous amino acids in their native conformation. Although the amino acids of the epitope are separated from one another in the primary sequence, they can be closer to each other in the three-dimensional structure of the protein. The antibody will only recognize the epitope as it exists on the surface of the folded structure. It is imperative in these circumstances to run a Western Blot in non-

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denaturing conditions. In general, a non-denaturing condition simply means leaving SDS out of the sample and migration buffers and not heating the samples. Certain antibodies only recognize protein in its non-reduced form *i.e.* in an oxidized form (particularly on cysteine residues) and the reducing agents β -mercaptoethanol and DTT must be left out of the loading buffer and migration buffer (non reducing conditions).

The electrophoresis is a technique used in biochemistry and molecular biology to separate, and sometimes to purify, macromolecules such as proteins and nucleic acids. It is based on the principle that, when these charged molecules are subjected to an electric field, they migrate toward the opposite pole. In gel electrophoresis, the gel acts as a molecular sieve to separate proteins with same charge, depending on the size. When separated on a polyacrylamide gel, the procedure is abbreviated as SDS-PAGE (for Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis). The technique is a standard means for separating proteins according to their molecular weight. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N-methylenebis-acrylamide (Bis). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate (APS), along with either 4-Dimethylaminopyridine (DMAP) or Tetramethylethylenediamine (TEMED). The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons crosslinked by methylene groups.

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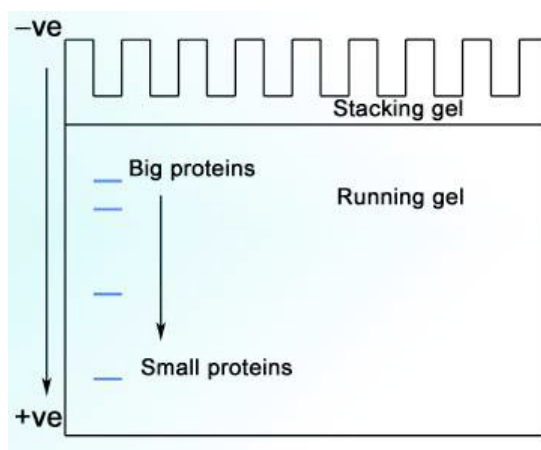


Figure 4.1: SDS-PAGE system

The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors: the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the percentage of acrylamide increases, the pore size decreases. Gels are designated as percent solutions and will have two necessary parameters. The total acrylamide is given as a percentage (w/v) of the acrylamide plus the bis-acrylamide. A 4% stacking gel (slightly acidic) is a porous gel, which separates protein poorly, but it allows them to form thin and sharply defined bands. A 12% resolving gel (or separating gel) has higher polyacrilamide content, making the gel's pores narrower. In this way, the proteins are separated according to their size, so smaller proteins run more easily and more rapidly than large ones. In the resulting gel, we will find proteins with lower molecular weight in the lower region of the gel. Since the proteins are loaded in the wells with a negative charge, they move toward the positive electrode when the voltage is applied (Fig. 4.1).

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In SDS-PAGE, for the sole separative purpose, the visualization of proteins is performed by a staining. The first, but also the least sensitive of the solutions, is the Coomassie staining. A good alternative and more sensitive is the silver stain, or the copper stain, which is also reversible, therefore useful in the Western Blot.

The principle for transfer is the migration of proteins upon application of an electrical charge from the gel onto a membrane (Fig. 4.2). Transfer can be done in wet or semi-dry conditions. Semi-dry transfer is generally faster but wet transfer is less prone to failure due to drying of the membrane and is especially recommended for large proteins, >100 kDa. For both kinds of transfer the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and clamped between solid supports to maintain tight contact between the gel and membrane. The negatively-charged proteins travel towards the positively-charged electrode, but the membrane stops them, binds them, and prevents them from continuing on.

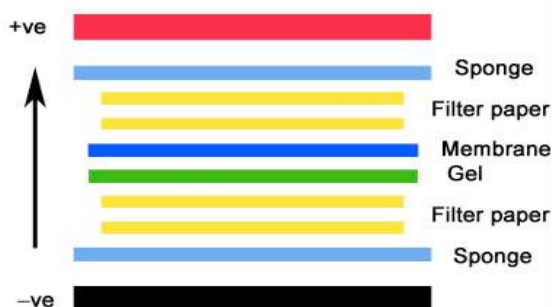


Figure 4.2: pattern of transfer step in Western Blot technique

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Usually, the membranes used for Western Blot are nitrocellulose and PVDF. Nitrocellulose has less binding capacity than PVDF, but it has the advantage of low background. On the other hand, PVDF has greater solvent resistance and physical strength.

After the transfer procedure, the membranes are ready to blocking step and the following immunodetection. For this purpose, the previous developed Dot-ELISA protocol was optimized.

In the field of cultural heritage, the use of Western Blot technique for the characterization of protein material was reserved only for archeological purposes (Child A.M. *et al.*, Grupe G. *et al.*, Newman M.E. *et al.*, Schmidt-Schultz T.H. *et al.*, Schweitzer M.H. *et al.*). A similar technique, that combine the principle of electrophoretic separation with immunological detection, are the crossover (or counter) immunoelectrophoresis (Seeman M.F. *et al.*, Downs E.F. *et al.*, Shanks O.C. *et al.*). The use in detection of proteins in paint media has not yet been found.

The aim of our work is assess the efficacy of the immuno-enzymatic protocol of Dot-ELISA in Western Blot technique. This technique is a valid tool, also, to understand how the artificial ageing has acted on protein material in laboratory-made specimens. The hypothesis of a protein fragmentation or aggregation, due to degradation processes occurred on samples, could be confirmed by means of this technique. Indeed, as the SDS-PAGE is skilled in separate proteins according to their molecular weights, a composite sample of peptide fragments and protein aggregates, will produce a series of defined bands related to the respective molecular weights. Then, by submitting to immuno-enzymatic assay the peptide fragments, transferred from the gel to

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PVDF membrane, it will be possible to determine which of fragments are immunologically active.

First, an optimization step was performed with ovalbumin and egg materials (yolk, white egg and whole egg); then, reference protein materials, whole egg and casein, were submitted to Western Blot. Finally the laboratory-made specimens were submitted to Western Blot and some conclusions were drawn.

4.2 Materials and Methods

Samples

The aged and non-aged samples (<1mg) were taken from the mock-up surface by scraping with a scalpel and collecting only the granules relative to the paint layer (thus excluding the layer of plaster below). These were weighed and solubilised in 100 μ L of 1X PBS (the weight is between 0.5 and 1 mg). The samples obtained were placed in glass tube and sonicated (ELMA S30H) at 50-60 Hz and at T=30-45°C for 60 minutes. Reference sample whole egg was prepared in 10% v/v in 1X PBS and the casein sample was prepared in a 100 μ g/mL concentration; these were placed in glass tubes and sonicated as paint samples. Ovalbumin (Sigma), egg white and yolk (from a local market) were solubilized in water and directly subjected to the sample treatment (see *Procedure* par.).

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Reagents and materials

The vertical gel electrophoresis system for 0.75mm gels, the sandwich cassette for transfer and the power supply were Bio-Rad. The 12% resolving gel (for 10-70 kDa proteins) and the 4% stacking gel were performed as in Tab. 4.1. The sample buffer (or loading dye) was made up to 60 mM TRIS-HCl, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.001% bromophenol blue (pH=6.8). The running buffer was 1X TGS from dilution of 10X TGS (25 mM TRIS base, 190 mM glycine, 0.1% SDS, pH=8.3, from BioRad). The Transfer buffer was prepared by 80% TG buffer (25 mM TRIS base, 190 mM glycine, from BioRad) and 20% methanol. Staining solution was prepared with 40% methanol, 10% acetic acid, 50% Milli-Q water and 0.1% Coomassie Brilliant Blue (Sigma Aldrich B0770); destaining solution was made up of 50% methanol, 10% acetic acid, 40% Milli-Q water. All reagents, (where not specified) were purchased from Sigma Aldrich. The PVDF membranes (Immobilion P) were purchased from Millipore. PBS 1X, blocking buffer, washing buffer were prepared as in par 2.1.1. The staining solution for visualization of protein was prepared with Sigma FAST (BCIP/NBT).

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Table 4.4: preparation of running and stacking gels

	12% resolving gel	4% stacking gel
H₂O	32.5%	60%
Acrylamide 30%	40%	13.5%
TRIS-HCl	25% (1.5M a pH=8.8)	25% (0.5M pH=6.8)
SDS 10%	1%	1%
APS 10%	1%	1%
TEMED	0.1%	0.1%

Procedure

Sample preparation and SDS-PAGE

The samples were centrifuged briefly to deposit the insoluble part and mixed with the loading dye. The diluted samples must be kept in a bathroom for about 10 minutes at 100°C. In order to settle to the bottom the insoluble material or the vapor drops, samples were spun down for a few minutes and then an aliquot (10-15µL) of sample solution was introduced to each well. For each gel, a molecular weights marker (Page Ruler Prestained Protein, BioRad), a positive and a negative control, for the next immune-enzymatic step, were introduced. Two identical gels were prepared, loaded with the sample and subjected to electrophoresis. The gel was run for 30-45 min at 60mA in a 1X TGS buffer. After the run one of the gels was stained with the Coomassie blue solution (30-60 min) and the second was submitted to transfer procedure. After the staining step, a destaining solution was used (60-120 min) to wash out the excess of colour stain

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Transfer

The transfer procedure used was the wet transfer. The gel and the membrane are sandwiched between sponge and paper (sponge/paper/gel/membrane/paper/sponge) and all are clamped tightly together after ensuring no air bubbles have formed between the gel and membrane. The sandwich is submerged in transfer buffer to which 220 mA were applied for 120 min. The running cassette must be kept in ice bath to avoid overheating and protein and reagents degradation.

Immuno-enzymatic procedure

After the run the membrane was blocked with a soybean milk-based buffer (see par 2.2.2.1) overnight at 4°C. At this stage of the procedure, the previously developed Dot-ELISA protocol was followed. For the first detection of standard ovalbumin, egg white and yolk it was used a nitrocellulose membrane. For whole egg and casein temperas, instead, it was used the PVDF membrane. For detection of protein in whole egg-based temperas and the whole egg reference sample, the anti-chicken egg albumin antibody in 1:6,000 (v/v) dilution (final concentration⁴) was used. The secondary antibody, the anti-rabbit AP conjugate, in 1:5,000 (v/v) dilution was used. The usual washing steps were performed with the T-PBS solution (0.05% Tween 20 in 1X PBS) and with only 1X PBS (before the II antibody and the staining step). The staining was performed with the 20 mL of Sigma Fast (Sigma Aldrich).

⁴ The antibody volume was dispersed in 1X PBS and added to 25 mL of blocking buffer.

4.3 Results and discussion

The preliminary tests were conducted on ovalbumin standards at known concentration (Fig.4.3). First of all, these tests confirmed that the immunological detection is much more sensitive than Coomassie blue and that the detection limit for ovalbumin samples analyzed with the protocol used on a nitrocellulose membrane, was 75ng. In ovalbumin samples there are visible the Coomassie stained bands at 42.7 KDa and 44.3 KDa, relative to the ovalbumin molecular weight, without and with the carbohydrate and phosphate portion⁵. In immunological detection one band is recognized from anti-chicken egg albumin antibody. Then white egg and yolk were submitted to Western Blot in same condition as previous proofs. Egg white was well detected and yolk was not recognized from immunological treatment, as expected (Fig.4.4).

5 From the amino acid sequence, the peptide portion of the molecule consists of 385 residues and has a molecular weight of 42.7 kDa. The carbohydrate and phosphate portions account for an additional 1428 and 160 grams per mole respectively, giving a total molecular weight of 44.3 kDa (Nisbet A.D. *et al.*)

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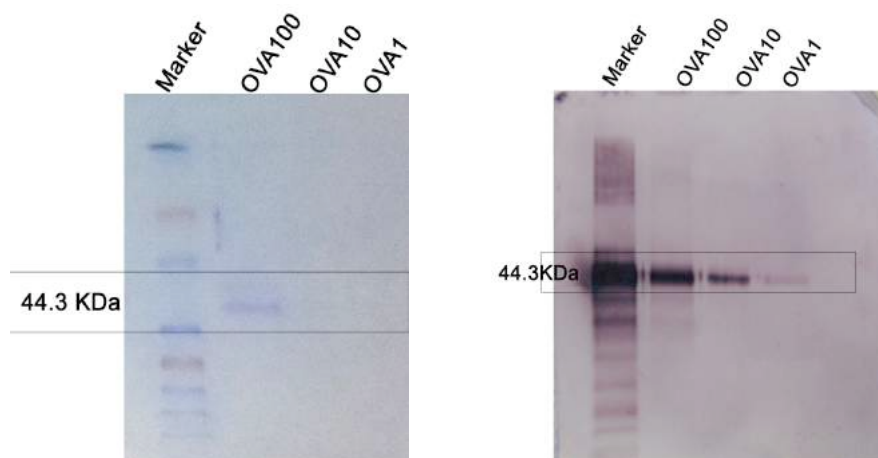


Figure 4.3: The gel stained with Coomassie blue (left) and the immuno-enzymatic detection of the membrane obtained by transfer from the gel (right). The same ovalbumin standards at known concentrations were separated and analyzed. OVA 100 is ovalbumin sample at 100 μ g/mL.

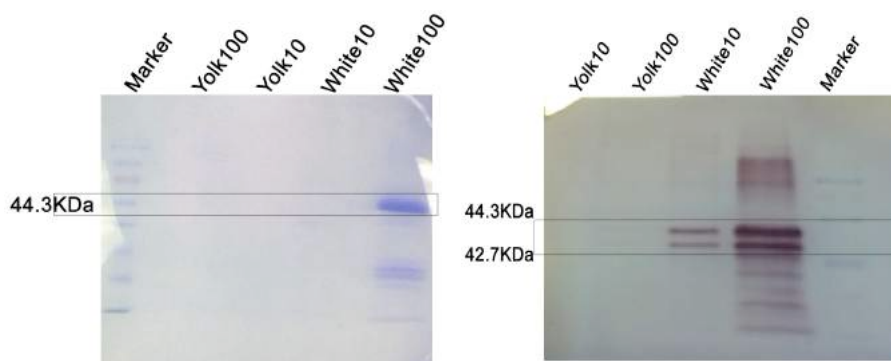


Figure 4.4: The gel stained with Coomassie blue (left) and the immuno-enzymatic detection of the membrane obtained by transfer from the gel (right). Yolk and egg white samples at two different concentrations were analyzed. In gel, the molecular weight bands are visible for ovalbumin and for ovoglobulins only in 100 μ g/mL sample; in membrane the two antibody's recognized bands are highlighted.

In order to establish a standard procedure for the analysis of protein material in samples of pictorial tempera both aged and not, the

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previous procedure was adopted, with the only modification of the PVDF use, instead of nitrocellulose. This is because PVDF is more sensitive than nitrocellulose, although it need a treatment in methanol before use. First a sample of whole egg and one of casein at known concentration were run and stained with the Coomassie blue (Fig. 4.5). In Fig 4.5, the molecular weight relative bands of the whole egg and casein proteins were shown. The amount of whole egg reference sample was too high; thus a dilution 1:10 of these was done and used for the later tests.

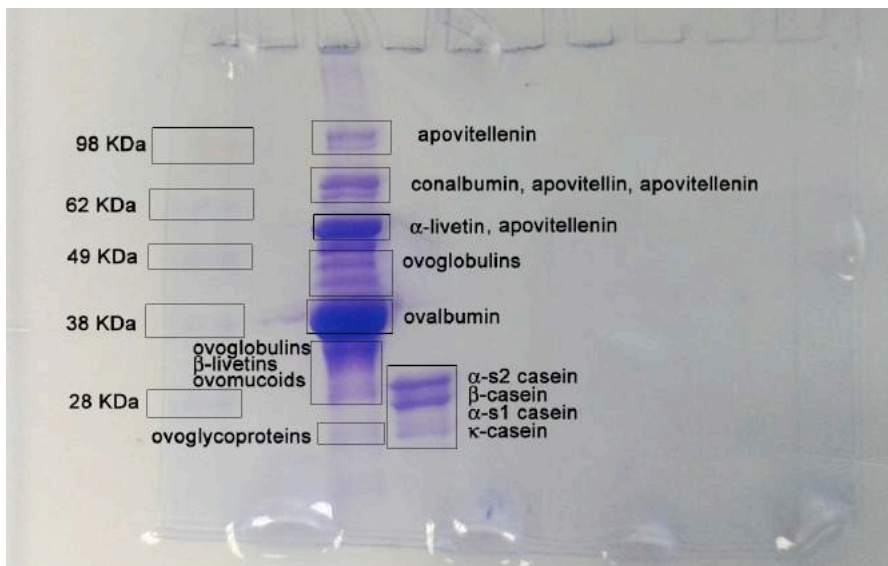


Figure 4.5: Coomassie blue stained gel of whole egg and casein samples: the molecular weight bands of several egg and casein proteins were highlighted.

The first tests carried out on samples of egg-based tempera, both artificially aged and non-aged, show a too low concentration of ovalbumin which can't be revealed with Coomassie blue (Fig. 4.6). In

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this first proof, the electrophoretic run time was too long: in fact the ovalbumin band is in the lower part of the gel, but it doesn't affect the readability of the result. The immunological detection shows no defined bands corresponding to the molecular weight of the protein, but an uneven staining along the well (Fig. 4.7). It is important to note that this phenomenon is related only to non-aged samples. The aged samples relative wells, were not recognized from antibody: in fact the wells are clear. This could mean that the effects of fragmentation and protein aggregation due to aging, which is discussed previously (Ch.3), produced protein species that have not been properly separated in the gel and consequently have not been recognized by antibody. In fact, the lower molecular weight peptide fragments might not be retained by the gel; then may have been eluted from the gel and not transferred on the membrane. Large proteins tend to precipitate in the gel, hindering the transfer; moreover, the useful percentage for large proteins is 8% or less but for the analysis it was used a 12% resolving gel and maybe the protein aggregates might not have been separated in the gel. Since the transfer efficiency depends on protein size, gel percentage, SDS and methanol balance, the size of protein aggregates could have affected the transfer and the final immunologic detection. Indeed, for large proteins, both the transfer than the electrophoretic run, may be very slow and the operating time used for the aged samples might have been not adjusted.

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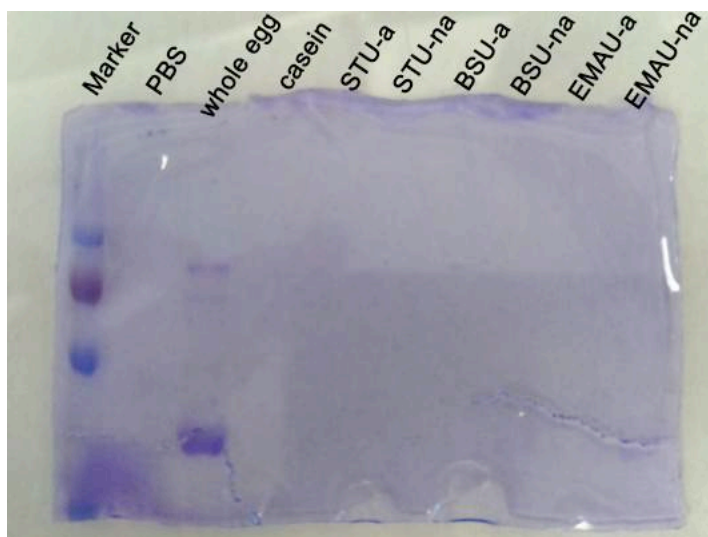


Figure 4.6: Coomassie blue stained gel of aged and non-aged samples from laboratory-made specimens. A positive control of whole egg and a negative control of casein were introduced. The run time has been too long and some protein band (caseins) was lost.

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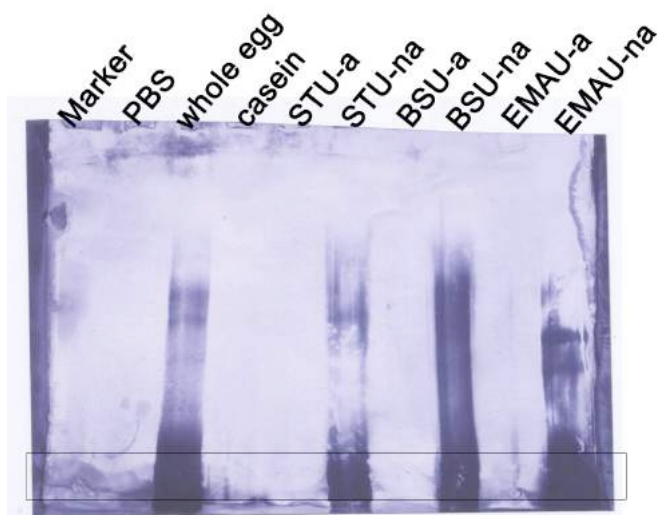


Figure 4.7: Immuno-enzymatic detection of transferred membrane of aged and non-aged samples with the anti-chicken egg albumin antibody; a blank, a positive (whole egg) and a negative (casein) controls were introduced.

In the reported second test, the Coomassie stained gel shows only the marker, the whole egg positive and the casein-based negative control (Fig.4.8). The immuno-enzymatic stained band relative to ovalbumin is well visible in whole egg and non-aged samples (Fig.4.9). As in previous results, also in this membrane the aged samples were not recognizable from antibody. In this case, moreover, the non-aged verdigris-based tempera doesn't present the ovalbumin band in the stained membrane and only a slightly patchy coloration of well is visible: this result confirm the high degradation power of verdigris pigment, which induces a protein fragmentation (Khandekar N. *et al.*).

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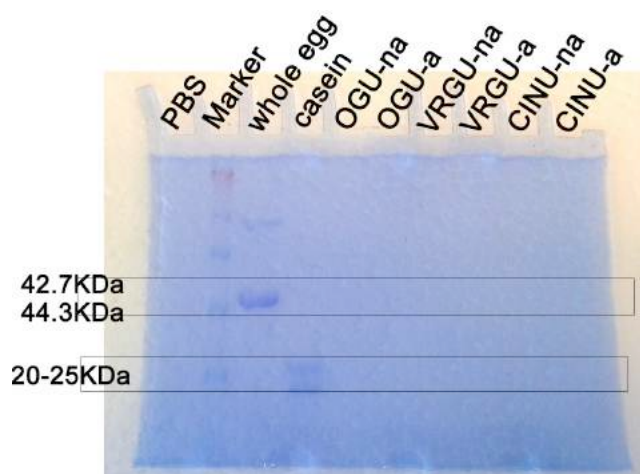


Figure 4.8: Coomassie blue stained gel of aged and non-aged samples from laboratory-made specimens. A positive control of whole egg and a negative control of casein were introduced.

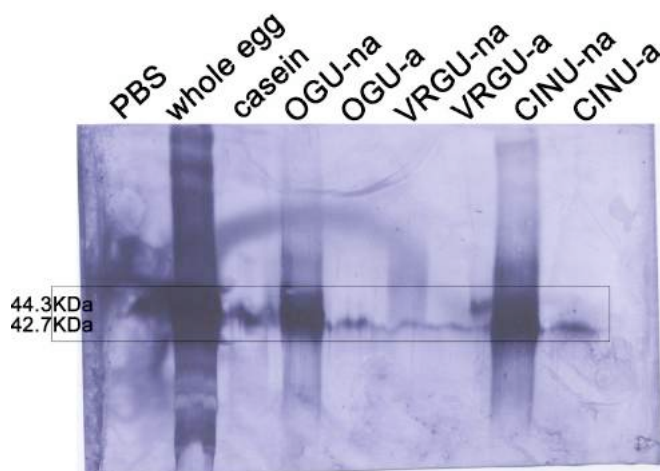


Figure 4.9: Immuno-enzymatic detection of transferred membrane of aged and non-aged samples with the anti-chicken egg albumin antibody; a blank, a positive (whole egg) and a negative (casein) controls were introduced.

The encouraging results obtained for anti-chicken egg albumin detection on aged and non-aged samples of egg tempera, prompted us to try to detect the milk proteins in milk-based tempera samples (Fig.

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4.10). Also in this case, a reference casein-based standard sample was injected and the whole egg sample was used as negative control. As expected, the whole egg was not recognized from antibody, but the high concentration produce some cross-reactivity interference. The casein is recognized from antibody in several bands and the phenomena previously described for aged and non-aged samples occur also in this proofs.

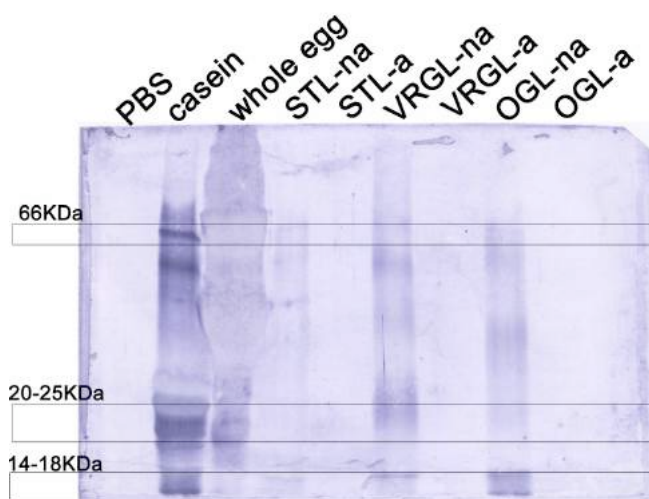


Figure 4.10: Immuno-enzymatic detection of transferred membrane of aged and non-aged samples with the anti-bovine whey proteins antibody; a blank, a positive (whole egg) and a negative (casein) controls were introduced.

The results obtained with the Western Blot technique are certainly incomplete and require further studies. First, it needs to understand whether the disappearance of protein bands in the aged samples is due to the effects of degradation caused by artificial aging, as presumed. It is important to understand if it is necessary a pre-treatment of the sample, such as a purification from salts and pigments. The multiple

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bands could be due to the contaminants effects. Also it would be desirable to optimize the solutions and staining times both for gel and membrane. For example, to increase the detection sensitivity in stained gels, one might use the silver staining. Once the method was optimized, it is possible to think to reveal more proteins on the same membrane using electrophoretic separation and revealing the different proteins with a multiplexed immunoassay method. This is based on the combination of antibodies for the recognition of different proteins in a single step or in subsequent steps and in a simultaneous detection of antigens, bound to the antibodies. This could be a future perspective. It is noted that the collagen-based binders, i.e. glues, can't be analyzed simultaneously with the milk and egg-based binders. This is because the collagen, in native form, has a high molecular weight, which is not resolved by the gel used for the milk and egg proteins. Moreover, even for the degraded proteins may be necessary to use a different acrylamide percentage gel, due to all the problems of fragmentation and aggregation discussed.

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CHAPTER 5.

CASE STUDIES

5.1 Analysis of “The Stigmata of S. Francis” wall painting in Santa Croce’s Basilica in Florence

Original samples were from Giotto’s wall painting “The Stigmata of St. Francis” in the Basilica of Santa Croce in Florence. The aim of the study was to characterize the upper brown “patina”. First, it was investigated the protein nature of the patina in order to understand if this is the product of aging of some repainting operation and/or the product of protein-based mixtures, called “beveroni”, used in previous restorations (early 900) as consolidating agents. The analysis was performed in the scope of the *TemArt* project, which aims were the development and the application of new technologies for the conservation and the promotion of cultural heritage.

The samples -only micro-fragments of painting layers- were collected by the conservators of the Opificio delle Pietre Dure, Florence, Italy. Sample A was taken from the upper frame region; sample B was taken in the proximity of the bottom-left part of the painting; sample C was from the habit of the Saint in a repainting zone (Fig 5.1). All samples were less than 1 mg in mass. More also the cleaning water of the surface of the painting were collected, filtered (three different aliquots were separated at different degree of purity) and finally submitted to Dot-ELISA.

In a second session of analysis a unique sample (sample 1) was

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collected and submitted to Dot-ELISA. This was taken from the lower left part of the Giotto's wall paint. In this area a first cleaning operation was carried on only with water to remove the upper layer. The underlying layer requires a slightly basic solution to be removed. The issue to be investigated in this second sampling was the nature of this second layer.



Figure 5.1: a) Giotto's wall painting "The Stigmata of S. Francis" with sampling points; b) detail of the sampling point of the A sample; c) detail of the sampling point of the C sample; d) and e) details of the sampling point of the sample 1.

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5.1.1 Materials and methods

For the four micro-fragments of pictorial layer the Dot-ELISA method was applied. 10 μ L of sample protein solution were used to react with the primary antibody to enhance the response because of the possibly small amount of protein expected. The procedure was the same of the previous ovalbumin assays. The antibodies, used in concentration 1:1,000 (v/v) in PBS with an NFDm-based blocking buffer (1:6,000 final concentration), were shown in Tab. 5.1. The concentration of the AP conjugate secondary antibody was 1:5,000 (v/v) in PBS for all in exclusion of the anti-chicken IgY that is already enzyme conjugate. For the cleaning water solution, a procedure of separation by filtration and a freeze-drying step was managed to obtain finally three different samples in elution buffer. These were submitted to the Dot-ELISA assays.

Table 5.1: list of the antibodies used for Dot-ELISAs and the relative target protein.

	Anti-chicken egg albumin	Anti-chicken IgY	Anti-bovine whey protein	Anti-goat whole serum	Anti-bovine whole serum
Target protein	Egg albumin	Yolk protein	Bovine milk protein	Goat proteins (milk and serum)	Bovine proteins (milk and serum)

5.1.2 Results and discussion

The Dot-ELISA results for identification of ovalbumin (Fig. 5.2) show the presence of a large amount of the protein in samples B and more in C and a negative result for sample A. The sample A was taken from

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the upper frame, out of the main scene of the paint, whereas the sample C was taken in a repainted area in the main scene (habit of the Saint): probably this result could mean that the Giotto's painted scene have been painted with the "secco" technique with a egg-based binder o that it was subjected to a restoration with an egg-based protective mixture. The test results for the yolk protein research explicate that no yolk proteins were found. The absence of these proteins could suggest the real composition of the painting but it could be due to the degradation of these proteins because of the presence of lipids. The whey protein assays were positive for all samples, but the anti-goat and the anti-bovine serum assays produce negative or dubious signals. For the sample 1 the aim of the investigation was to understand if the second layer which required a basic cleaning agent, was done with casein. The results show that egg white and milk protein were found. In tab 5.2 all the results obtained were shown.

5.1.3 Conclusions

From the results we can conclude that there was a milk-based "patina" (probably casein) over the painting, used as protective/consolidating treatment; indeed the whey proteins were found in all samples. The Giotto's painted area, instead, was prepared with an egg-based binder or it was subjected to a restoring intervention with an egg white-based preparation. This results was in agreement with the hypothesis of the conservation team, who suspected the presence of calcium caseinate layer on the painting as result of past conservation treatment probably at the beginning of 20th century. Probably during the sampling also the under pictorial layer was taken and this can explain the presence of egg

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white protein. Therefore, a century of natural aging did not inhibit the response of the Dot-ELISA tests.

Table 5.2: results of every sample of the Dot-ELISA assays for each antibody used.

	Anti-chicken egg albumin	Anti- chicken IgY	Anti-bovine whey protein	Anti-goat whole serum	Anti-bovine whole serum
A	–	–	+	–	–
B	+	–	+	–	?
C	++	–	++	?	?
I	+	nt	+	?	–

+ = positive result; ++ = clearly positive result – = negative result; ? = undefined result; nt = not tested

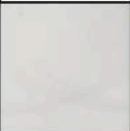
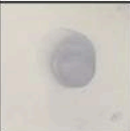
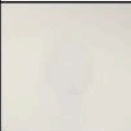
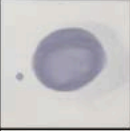
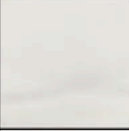
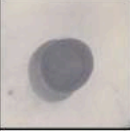
	Anti-chicken egg albumin	Anti-chicken IgY	Anti-bovine whey protein	Anti-goat- whole serum	Anti-bovine- whole serum
Sample A					
Sample B					
Sample C					
Sample 1					

Figure 5.2: Dot-ELISA results; the violet dot suggests the positive outcome.

5.2 Analysis of samples from the “Assunzione” painting of the Maestro di Figline in the Basilica of Santa Croce in Florence

The aim of the work was the diagnostics of the proteinaceous materials. These are placed in layers, related to several restorations occurred over time, of which there are no documented reports. The work was sponsored by the Tuscany Region in the project *TemArt*, whose purpose was to promote the development of new technologies for the study and valorization of the artistic heritage. In the project, a collaboration with the “Opificio delle Pietre Dure” of Florence and with the CNR-IFAC, was established. From this collaboration and from the synergistic work, a publication was born (Progetto *TemArt*, I dipinti murali: Nanotecnologie e applicazioni, Nardini editore, *in press*). The issue to be investigate was the protein composition of the stratification and the nature of the binders of some repainted areas.



Figure 5.3: The “Assunzione” wall painting of Maestro di Figline in Basilica of Santa Croce in Florence.

5.2.1 Materials and methods

The materials used for Dot-ELISA analysis and the procedure were the same used in Ch. 2.2.1.

Samples

A total of 23 samples were collected from several zones of the painting in two sampling campaigns. Two types of samples were taken: the classical superficial powder or micro-fragments and the residue of the material ejected during the laser ablation LQS and deposited on a slide. In Tab. 5.3 a description of samples was reported.

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Table 5.3: description of each sample collected

Sample	Description
1	Remaking at the bottom right corner of the painting-repainting-cleaned area
2	Remaking at the bottom right corner of the painting-repainting-not cleaned area
3	Remaking on the right side of the painting -repainting- not cleaned area
4	laser cleaning grid- not cleaned area
5	LQS 1064 nm laser cleaning test- cleaned area (yellowish repainting)
6	QS 532 nm laser cleaning test- cleaned area
7	intense blue repaintings
8	green drippings
9	Madonna's Mandorla ⁶ - yellow ochre repainting
10	Madonna's Mandorla - light yellow ochre
11	Madonna's Mandorla - yellow ochre- not cleaned zone
12	Laser ablation- remaking at the bottom right corner of the painting
13	Laser ablation- blue background
14	Laser ablation (I) - Madonna's Mandorla
15	Laser ablation (II) - Madonna's Mandorla
16	Laser ablation (III) - Madonna's Mandorla
17	Patches of intense blue color on a blue background; repainting nineteenth-twentieth century cleaned with laser
18	Background of blue-gray - cleaned area with QS laser
19	Chemically cleaned background; presence of a yellowish halo (absence of pigment)

⁶ In painting or sculpture the term is used to describe the almond-shaped enclosure which is sometimes depicted around Christ or the Virgin Mary. Mandorlas are most often depicted in the context of Christ's Ascension and the Virgin's Assumption.

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21	Blue background
21	Blue background for FTIR analysis
22	Repainting at the bottom right corner of the painting – not cleaned zone
23	Repainting at the bottom right corner of the painting – cleaned zone



a) Samples: 1, 2, 12, 22, 23



b) Samples: 1, 2, 12, 22, 23



c) Sample 8

Figure 5.4: the bottom right corner of the painting; the remaking part is visible in a) and b); in c) the green drippings are visible. For each picture, the samples taken in the relative painting area were reported.

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a) Samples 4, 5, 6, 7



b) Sample 7



c) Samples 4, 5, 6, 7



d) Samples 9, 10, 11



e) Samples 9, 10, 11

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f) Sample 13



g) Samples 14, 15, 16

Figure 5.5: the central part of the wall painting; a), b) and c): the laser cleaning tests grid; d) and e): Mandorla of the Madonna. For each picture, the samples taken in the relative painting area were reported.

5.2.2 Results and discussion

The results obtained for the samples analysed were shown in Tab. 5.4. and in Fig. 5.6 (only the first 11 samples are reported).

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Table 5.4: Summary of the results obtained for each used antibody; + means a positive result, - means a negative result, ? means an uncertain result.

Sample	Anti-chicken egg albumin	Anti-bovine whey proteins	Anti-chicken IgY
1	—	?	—
2	—	?	—
3	—	+	—
4	+	+	—
5	?	+	—
6	+	+	—
7	?	?	—
8	?	+	—
9	+	?	—
10	?	+	—
11	++	+	—
12	—	—	?
13	+	—	+
14	+	—	?
15	+	—	—
16	+	—	—
17	—	—	+
18	—	—	?
19	—	—	—
20	+	+	+
22	—	—	+
23	—	—	—

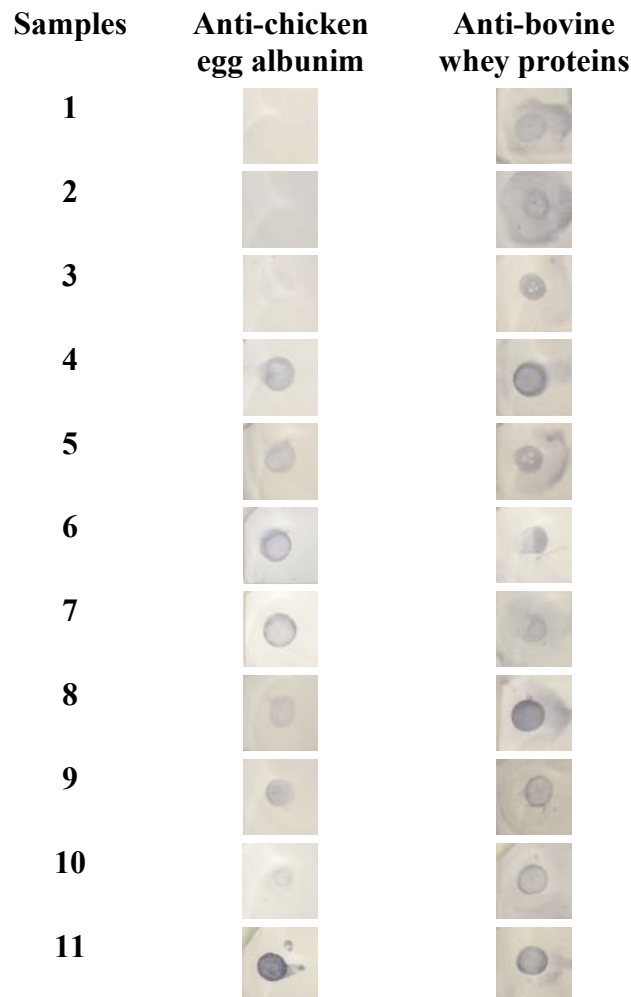


Figure 5.6: Results of Dot-ELISA for the search of chicken egg albumin and bovine whey proteins of the samples 1-11.

5.2.3 Conclusions

The diagnostic analysis carried out by means of the Dot-ELISA technique for the identification of protein material in the painting,

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revealed a complex situation. The cause may be sought in the overlapping of original and restoration layers, which are not easily discriminable with the Dot-ELISA technique without a selective sampling and because the strong degradation of the proteinaceous material can compromise the identification. However, combining the results of the first and second series of tests it is possible to draw the conclusions. First, three different parts of the painting were selected for the sampling, in which it is possible to recognize the various issues to be investigated: the area of the Mandorla of the Madonna, the nineteenth-century reconstructions and the background. Several tests of laser cleaning were performed on the painting background: a grid with different degree of cleaning was performed. The aim was not only the identification of the protein material, but we want to understand where and how these was used. The characterization of the binder in pictorial layer and in the restoration's overlying layer were the objectives. Confirming the assumptions made by the Scientific Laboratory of the OPD, the background might have been painted by means of the “fresco” technique (the so-called "Veneda") on which a “secco” layer might have been painted. The Dot-ELISA analysis, conducted on a sample of the blue background, taken in an area not cleaned (Sample 20), result to be positive both to egg protein (albumin and yolk proteins) than casein. The hypothesis is that the binder is based on egg and it is present a superficial casein-based layer. This hypothesis was supported from the results obtained on samples submitted to laser cleaning: the samples taken from non-cleaned or slightly cleaned parts show the presence of albumin and milk protein (samples 4, 5, 6). The results of the analysis on sample 7, taken from a cleaned area of the laser cleaning grid (probably composed only from a

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pictorial layer), reported the presence of albumin. Even the sample 17, relative to a light blue finishing of a nineteenth century repainting, has reported the presence of albumin and milk proteins. The samples taken by the remaking and the repainting of nineteenth century (samples 1, 2, 3, 12, 17, 22) are always positive to cow's milk protein; it is possible to confirm their casein-based composition. The Mandorla of the Madonna showed several different colored areas. This could mean that in this part of the paint several restoring intervention were performed longtime. The different layers were probably composed by different protein materials. The dark yellow ochre layer, relative to a repainting work, resulted to be composed by albumin, while the lighter layer was positive for albumin and milk proteins. The sample from a non-cleaned area of the Mandorla show the presence of albumin and milk protein; the analysis of the material from the laser ablation (stuck on the glass slide) reported the presence of egg albumin in all three ablations.

Finally, we can affirm that the binder of the drippings of verdigris is casein.

5.3 Detection of collagen by means of Dot-ELISA technique for the study of the aragonite crystal growth in archaeological bones

The aim of the work was to study the carbonation process occurring between nanostructured calcium hydroxide and atmospheric carbon dioxide in the presence of promoting agents for the growth of the meta-stable polymorph aragonite. In particular, it was interesting to verify the conditions under which the formation of the aragonite carbonated phase, which is known to possess excellent mechanical properties, were promoted. Many authors indicate the possibility to modulate the crystallization of calcium carbonate polymorphs in presence of metal ions (Reddy M. M. *et al.*) as well as using collagenous matrices in association or not with magnesium ions (Jiao Y. *et al.*, Falini G. *et al.*) Then, the goal was to enhance the formation of the meta-stable aragonite during Ca(OH)_2 carbonation by atmospheric CO_2 by exploiting the properties of collagen coating the deteriorated bones as aragonite-promoter.

5.3.1 Materials and Methods

Samples.

Bone fragments from the San Clemente's relics (Late Middle Ages) are owned by the parish church of “San Vittore Martire” (Linate, Milan, Italy), and there conserved. They were made available in the frame of a scientific cooperation. After a collapse of the wooden structure onto which the shrine was placed, it was decided to perform a complete restoration of the whole complex, including garments and the shrine.

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Many decay processes were affecting S. Clemente's relics, but the priority objective was to recover the hardness and the cohesion of the bones remains: fragility and de-cohesion were indeed the main damage effects they were presenting and a consolidation work was needed.

Bone treatment

The skeleton from which the fragments were taken had been cleaned using acetone and disinfected. The various parts of the skeleton were immersed in Nanorestore® diluted in 2-propanol/water mixtures (90/10 in v/v) in different ratio depending on the necessity of each area to be treated, in order to ensure a complete penetration of the dispersion in the whole volume of bones. The operation was repeated 10 times; each of them was performed after the solvent was completely evaporated. All these operations were performed inside a church with climate parameters similar to the ones of the church in which San Clemente's relics were kept, in order to maintain constant the environmental conditions and facilitate the carbonation process thanks to high values of relative humidity (around 75 %). After three months sampling of the fragments used for this study was carried out. The complete carbonation of calcium hydroxide nanoparticles was ascertained on all the fragments by FT-IR measurements.

Dot-ELISA.

Elution buffer and PBS was prepared as in previous recipe (see Ch. 2.1.1). Blocking buffer was prepared using 0.2% (v/v) TWEEN 20, 5% (w/v) nonfat dry milk in PBS. The buffers were freshly prepared. All the reagents were analysis grade (> 99.5% purity) and were purchased from Merck (Germany). Enzyme substrate in tablet SIGMA FAST™ BCIP/NBT and rabbit anti-goat IgG alkaline phosphatase conjugate

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(1:5,000 final concentration) antibody were purchased from Sigma-Aldrich. Goat anti-collagen type I (1:2,000 final concentration) was purchased from AbCam (Cambridge, UK). Methanol (GC purity >99.5%) was supplied by Merck. The procedure used was the same that the previous assays.

5.3.2 Results and discussion

Ca(OH)₂ nanoparticles dispersed in 2-propanol (Nanorestore®), specifically developed to consolidate carbonate matrices (i.e. wall paintings, carbonate stones) (Dei L. *et al.*, Ambrosi M. *et al.*) and for the deacidification of cellulose based materials (Giorgi R. *et al.*, Poggi G. *et al.*) were used in this study for the consolidation of Late Middle Ages relics made of bone fragments. Once applied, Ca(OH)₂ nanoparticles react with atmospheric CO₂ to give CaCO₃: the new crystalline carbonate matrix formed into the original weakened porous substrate, acts both as binder and as a filler for areas affected by flaking, cracking phenomena, as well as by detachment of the surface. Then, this innovative nanostructured Ca(OH)₂-based method allows satisfying the following requirements: (i) capability to reinforce and harden fragile bones, stopping flaking and powdering; (ii) good physical-chemical compatibility with the porous substrate; (iii) cost effectiveness; (iv) user-friendly employment. Moreover, the alkaline local character of Ca(OH)₂ nanoparticles dispersed in 2-propanol enhance the anti-microbiological activity and avoid all the undesired effects associated to the use of water based systems. It is known that auxiliary molecules may promote or inhibit crystal nucleation depending on their composition: the effect of proteins on the

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morphology of crystal growth has been described in terms of activation energy and variation in complementarity of various crystal faces and the organic substrate (Weissbuch I. *et al.*) In the presence of an organic matrix the nucleation of inorganic non-specific polymorph is controlled through the interaction with it and activation energy can be influenced in three different ways involving thermodynamic and kinetic aspects (Mann S. *et al.*). Some authors have investigated both in nature and in laboratory the controlled phase transition between aragonite and calcite in nacre with the use of soluble polyanionic proteins (Belcher A.M. *et al.*, Thomposon J.B. *et al.*); in this context, some works highlighted the possibility to enhance the crystallization of calcium carbonate in aragonite phase by tuning the concentration of many different organic compounds such as Poly-L-Aspartate and gelatin (Falini G. *et al.*) as well as collagen (Shen F.H. *et al.*) in association or not with metal ions like Mg^{2+} (Jiao Y. *et al.*) As a consequence of above, $Ca(OH)_2$ nanoparticles (Nanorestore ®) appeared to be a possible precursor of aragonite calcium carbonate if associated with a protein containing matrix. Being collagen the main protein constituting bone tissues, the presence of such protein within the bone fragments was expected and this should enhance the subsequent formation of aragonite after $Ca(OH)_2$ carbonation.

It is in this context that the Dot-ELISA technique was used to confirm the presence of collagen in bones samples. The results were compared with those obtained with GC-MS, performed the Scientific Dept of the Getty Conservation Institute.

Before the treatment by nanolime, some fragments of bone from the femur were collected as reference samples (NT) representative of the

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state of preservation of the skeleton before the treatment by $\text{Ca}(\text{OH})_2$ nanomaterial. The same fragments characterized before the treatment were subjected to $\text{Ca}(\text{OH})_2$ application according to the procedure reported in Materials and methods. Both not treated (NT) and treated samples (T) were studied by means of several techniques in order to verify if aragonite phase was actually crystallized conferring hardness to demineralized bone remains. Dot-ELISA test verified the presence of collagen: in the not treated (NT) sample was clearly evident the stain dot, whereas in the treated sample the staining was less intense (Fig. 5.3.1). This was due to the interaction between the collagen and the strong local alkalinity surrounding the $\text{Ca}(\text{OH})_2$ nanoparticles possibly inducing partial denaturation of the protein. The amino acids analysis performed with GC-MS confirm this finding. The result of amino acid composition of the protein contained in the two bone fragments treated (T) and not treated (NT) are in agreement with the presence of collagen within the samples (data not shown).

The carbonation process occurred and a controlled crystallization into aragonite phase was observed by means of Scanning Electron Microscopy and Fourier Transformed Infrared Spectroscopy (data not shown). Needle-shaped crystals were shown to crystallize onto bones surfaces, especially onto the back side (*spongiosa*); the comparison between spectra of not treated sample (NT) and treated sample (T) indicated the presence of aragonite as a newly formed mineralogical phase by the distinctive peak at 855 cm^{-1} (bending O-C-O of aragonite calcium carbonate) (data not shown).

The effectiveness in consolidating bones was evaluated by crossing results obtained with microtomography images, helium porosimetry,

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Vickers microhardness test and Atomic Force Images. Flaking phenomena and powdering were not detectable after the carbonation of nanolime, leading to a more compact and smooth surface. Moreover, the data suggest that the crystallization occurred not only on the bones surface (where a smooth film of aragonite was identified) but also inside pores, reducing porosity values and conferring hardness to the bone tissue.

5.3.3 Conclusions

In conclusion from the results obtained it is possible to confirm that a collagen-based matrix positively affects the formation of aragonite crystals in the presence of a source of calcium, as in the case of Nanorestore ®. In this case the use of the technique Dot-ELISA was secondary but useful for the purpose. Furthermore we must not underestimate that the technique can be used for the detection of collagen not only in the research phase, but also and above all in the diagnostic phase. The implications of the case study presented concerning diagnostics and new bone remediation method in archaeological and paleontological fields.

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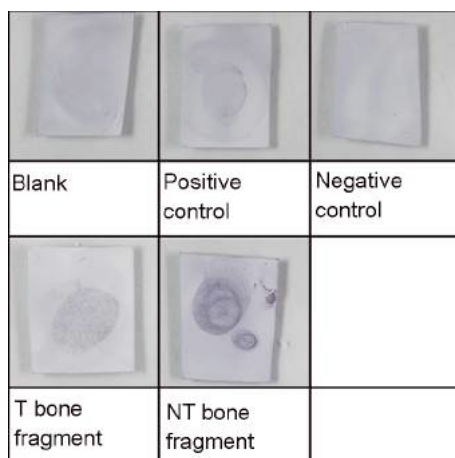


Figure 5.7: Dot-ELISA results for non-treated (NT) and treated (T) samples of bone; a blank, a positive and a negative control were reported.

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CHAPTER 6.

CONCLUSIONS

In this part of the work it has been presented an immunological approach for the detection of protein in works of art. First the Dot-ELISA method was developed, adjusting the procedure steps, the used reagents and the chosen antibodies, to the needs of diagnostics in cultural heritage. The first protein on which the attention was centered has been the chicken egg albumin for the egg tempera characterization. As the outcome of the assay, meaning as the intensity of the stain dot, is proportional to the antigen dose, a semi-quantitative determination was proposed. Ovalbumin, albumen and whole egg reference samples were submitted to assay at different concentration and analysed with a spectrophotometer to measure the reflectance values of the stain dots. Assured that these values were proportional to antigen dose, a linear fitting was drawn with the ovalbumin standards at different concentration. To support the proposal, some laboratory-made specimens were subjected to Dot-ELISA and, by means of the obtained curves, the concentration of ovalbumin in the sample was found. In order to develop a diagnostic method to detect the proteins in the cultural heritage field, the method needed to be proved with different types of proteinaceous material. For this reason also milk and glue-based reference standard were submitted to the assay. It has been necessary to modify some steps of the procedure and some reagent, especially, as the blocking buffer. In fact, differently from classical uses of the technique, in our case the normally used blocking agents

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(due to their low cost) can not be used because they interfere with the antigen searched and with the antibody used. A soybean milk-based blocking buffer was proved and adopted for the protocol. During the set up of the method, it was possible to test the technique with original samples from works of art (see the case studies, Ch.5). In the most cases the technique has produced reliable results; in some cases, indeed, the Dot-ELISA failed to give a precise unambiguous response. This is due probably to degradation effects induced by ageing and environmental conditions. For this reason, a study of detection effectiveness of the technique on damaged proteins has been addressed. An artificial light and thermal ageing was conducted on laboratory-made mock-ups, prepared by painting plaster supports with three types of tempera (egg, milk and animal glue) with different pigments. The choice of the terms and conditions of aging was based on existing literature. There are no standard rules for the artificial aging of proteinaceous material. The literature reports various protocols, among which someone induces significant changes in the protein structure. It has been chosen to stress very hard the protein-based samples by subjecting them first to a light aging and then to a strong thermal aging with a high relative humidity. The result of this strong ageing has been the completely disappearance of the stain dot on aged samples. This decreasing in color intensity was measured by means of an image analysis with a free software ImageJ, expressed as integrated density. The disappearance of the stained spot was explained as effect of oxidation phenomena which induces fragmentation and molecular aggregation. By using another technique, the western blot, we have tried to explain this event. The technique uses the advantages of the SDS-PAGE separation of proteins and an

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immunological protocol for the detection of specific antigens. Through the electrophoretic separation, it was tried to separate the peptide fragments and to transfer these on the membrane, which, later, was submitted to immuno-enzymatic detection. The immunologic detection results of non-aged samples don't show a defined band corresponding to the molecular weight of protein searched, but a diffuse staining of the well. Probably, the antibody recognizes the protein fragments, which were previously separated on the gel according to their molecular weights and transferred on the membrane, producing this stain. In the case of aged samples, the results were different: the immuno-enzymatic detection didn't show no signal. Probably the protein fragments or the molecular aggregates were lost during the electrophoretic run or during the sample preparation. It is clear that, as future perspectives, the study of the ageing protein by means of western blot can be suggested. Maybe also the protocol will require some modifications to improve the damaged protein detection. It is important to highlight that the immuno-enzymatic procedure in western blot was that of Dot-ELISA.

In conclusion we can affirm that Dot-ELISA is a valid tool for identification of the protein material in the field of conservation of works of art. It is a simple and low-cost technique, useful for a fast and easy characterization of protein in artistic and archaeological samples. It is clear that the method can be improved further with the use of monoclonal or specifically produced antibodies, but this work demonstrate that, also with anti-sera, we have reached good results.

As future perspective, the study of a multiplexed procedure for the identification of different protein in a same sample, might be an

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interesting issue. This is based on a double immuno-detection with two different antibodies in a single or consecutive steps. The western blot technique could be used as a separating tool in order to visualize the different protein bands on the membrane. Some proof has been done, but with poor results. The aim will be reduce drastically the analysis time.

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CHAPTER 7.

ANALYSIS OF PROTEIN-BASED PAINTINGS BY ACCQ•TAG™ ULTRA UPLC

7.1 Introduction

In this work a liquid chromatographic technique, the Ultra Performance Liquid Chromatography (UPLC) with UV detection, coupled with a pre-column derivatization with the AccQ•Tag™ Ultra methodology, was proposed. In comparison to the conventional HPLC instruments, UPLC technology has the advantage of increasing sensitivity and resolution, reducing analysis time and solvents consumption, due to the smaller diameter of the packing particles (1.7 μm) and the high pressure in the columns (Nováková L. *et al.*). The AccQ•Tag™ Ultra method uses the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), as derivatizing agent, allowing amino acid detection at under-picomolar levels. This reacts with primary and secondary amino acids to yield fluorescent derivatives, readily detectable by UV-absorbance, fluorescence, electrochemical, and/or mass spectrometric detection.

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The use of AQC, overcomes many of the drawbacks associated with the other derivatizing reagents, such as phenylisothiocyanate (PITC), which yields derivatives detectable by UV, and *o*-phthaldialdehyde (OPA), dimethylaminonaphthalensulphonyl chloride (Dansyl-Cl) and 9-fluorenylmethyl-chloroformate (FMOc), which yield fluorescent derivatives. In particular, PITC needs a long derivatization time (20 min) and the removal of any excess reagent by drying is mandatory; OPA and its analogues fail to react with secondary amino acids and the derivatives are often unstable; FMOc yields multiple derivatives and significant interference due to the excess of reagent; Dansyl-Cl needs a long derivatization time (30 min) and derivatives are often unstable. Moreover, FMOc and PITC exhibit decreased derivatization efficiency in the presence of common buffer salts or detergents. Despite this, the above-mentioned methods have been used often for the HPLC analysis in the field of diagnostics applied to the study of the cultural heritage, and in particular of the binders media. PITC was used in Halpine S.M. studies in 1992 and in Ronca F. work in 1994, but the procedure was affected by the presence of pigments, such as copper carbonate and calcium carbonate; FMOc was used by Grzywacz C.M. in 1994.

The AQC has many advantages: derivatives are quickly formed, the excess of the reagent is rapidly hydrolyzed ($t_{1/2} < 15$ sec) to yield 6-aminoquinoline, N-hydroxy succinimide, and carbon dioxide, and after 1 min no further derivatization can take place (Fig 7.1); moreover salts and detergents do not interfere with the reaction (Boogers I. *et al.*, Fiechter G. *et al.*, Pappa-Louisi A. *et al.*). The AccQ•Tag™ Ultra method, also, have been used for protein analysis in cultural heritage.

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Wouters J. *et al.* in 2000 used the method, but with fluorescence detection, for analyses of stuccoes samples.

In this work we propose the UPLC-based amino acid analysis as diagnostic technique for not pre-treated or submitted to extraction processes samples, showing that good results can be achieved with very scarce sample manipulation and great advantage. We applied the amino acids analysis carried out by the AccQ•Tag™ Ultra UPLC first to the standard protein material and then to model (laboratory made specimen).

The results obtained confirmed the reliability of the data achieved and demonstrated that the AccQ•Tag™ Ultra UPLC method could be a powerful technique to be applied to the relevant field of protein binders diagnostics for paintings conservation.

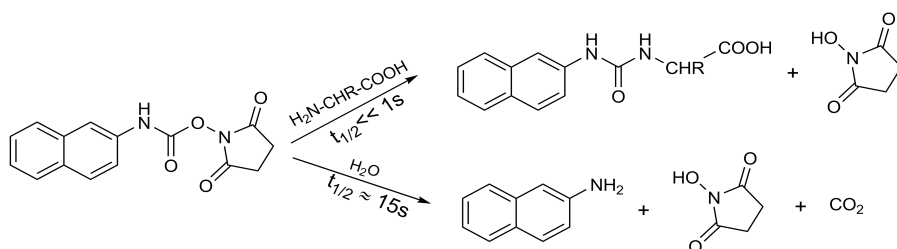


Figure 7.1: derivatization reaction of a generic amino acid with AQC: the amino-derivative is quickly formed with N-hydroxy succinimide; the excess of the reagent is rapidly hydrolyzed ($t_{1/2} < 15$ sec) to yield 6-aminoquinoline (AMQ), N-hydroxy succinimide, and carbon dioxide, and after 1 min no further derivatization can take place.

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7.2 Materials and methods

Apparatus and chromatographic conditions

Chromatographic measurements were performed using an Acquity UPLC instrument with a BEH C18 column (Waters, USA) (2.1mm x 100mm, 1.7µm particle size), equipped with an UV detector (detection wavelength 260 nm). Column temperature was 55 °C, the flow-rate was of 0.7 mL/min and the injection volume was 1µL (partial loop). Two different eluents were used: A (AccQ•Tag™ Ultra eluent A ratio of 1:20 with water Milli-Q) and B (AccQ•Tag™ Ultra eluent B). The elution program was as follows: 0.54 min, 0.1% B; 5.74 min, 9.1% B; 7.74 min, 21.2% B; 8.04 min, 59.6% B; 8.05 min, 90% B; 8.73 min, 0.1 % B; 9.50 min, 0.1 % B. The software used was Empower 3.

Chemicals and Reagents

AccQ•Tag™ Ultra derivatization kit (borate buffer and derivatizing reagent), AccQ•Tag™ Ultra eluent A and B, HPLC-grade acetonitrile and amino acid standard solution were purchased from Waters (USA). Phenol, sodium hydroxide, and constant boiling hydrochloric acid solution were supplied by Merck; Norvaline (Nval) was purchased from Sigma-Aldrich. Water was purified with a Millipore (USA) Milli-Q system.

Reference material

For this study a collection of different type of proteinaceous material were used. Chicken ovalbumin and bovine casein were purchased from Sigma-Aldrich (USA). Eggs and milk came from local markets, while animal glue was purchased from Zecchi (Florence).

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Model samples

Model painting layers samples were prepared according to the traditional painting recipes. A layer of tempera constituted of different types of protein material and ochre red pigment, was applied on a mortar surface (upper part of a square made of lime and sand in the 1:3 by vol.), one day after the mortar preparation, i.e. with very low degree of both drying and carbonation. The natural binders selected to prepare these samples were whole egg, yolk, milk, Barium caseinate and rabbit glue, pure or mixed. The pigment used was only red ochre in order to minimize the effects of the interference due to some pigments: indeed, red ochre is one of the most harmless mineral pigments for affecting the binder analysis. The obtained red painting layer was subjected to three years of natural aging in the laboratory atmosphere. Micro-samples only from the painting layers were collected by means of a little scalpel: the weight of the powder taken away from the mortar surface was around 1 mg and it was finely ground before submitting to the hydrolysis.

Analytical procedure

Solutions of reference material in different concentration were prepared and freeze-dried. .

Hydrolysis was carried out in vapor phase in nitrogen atmosphere at 114°C for 24 hours with 200 μ L of 6 M HCl solution with 0.1% phenol (out of glasstubes) to prevent halogenations of Tyr.

After hydrolysis the samples were solved in different volumes of 0.1M HCl (it depends on the amount and the property of the obtained protein material) to submit them to derivatization process. For each sample, 10 μ L of solution were collected and placed into a glass vial insert with 70

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μL of a solution 0.0417 mM of Nval/0.0655 mM of NaOH in AccQ•Tag™ borate buffer and 20 μL of AccQ•Tag™ Ultra Reagent. For each cycle of analysis a calibration standard (CS) containing 18 amino acids and Nval in concentration of 10 pmol/μL, except Cys present in concentration 5 pmol/μL, was used. The CS was prepared with 40 μL of amino acid standard solution 2.5 mM and 20 μL of Nval 5 mM in 940 μL of Milli-Q water. 10 μL of CS were placed in the glass insert with 70 μL of AccQ•Tag™ borate buffer and 20 μL of AccQ•Tag™ Ultra Reagent. All samples were mixed, heated to 55 °C for 10 min and injected into the UPLC instrument. Model and real samples were ground, weighed, and subjected to hydrolysis and derivatization process under the same experimental conditions as for the standards above described and the final solution was centrifuged to separate the insoluble material. In all cases 1 μL of injection volume was used.

7.3 Results and discussion

Amino acid analysis represents a suitable tool for the precise determination of protein content and relative characteristic amino acid fingerprint. The acid hydrolysis technique can contribute to a variation of the amino acid content, due to a complete or partial decomposition of some amino acids as well to well-known side-reactions. In particular, Trp is completely decomposed; Gln and Asn are converted into Glu and Asp respectively; Ser, Tyr, and Thr are partially decomposed; Met can be subjected to oxidation. Besides, Cys is typically recovered as cystine, but this is usually in low quantity because of a partial decomposition or the possible reduction to Cys.

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Moreover, peptide bonds involving Val and Ile are partially cleaved. The loss of Trp, Gln and Asn (both converted into Glu e Asp) (Colombini M.P. *et al.*) during hydrolysis, limited the detection to 17 of the 20 principal amino acids. Possible contaminations of the samples can cause mistakes in the assessment of the amino acid content, due to the sample manipulation or contact with degraded solvents. In the vapour phase hydrolysis, this contamination is minimized because the acid solution is not in contact with the samples. To reduce other sources of contamination and loss of material, no extraction and purification process were performed on the samples, in particular in model samples analysis. This was particularly important to reduce both the time analysis and the required sample amount.

First of all, standard ovalbumin reference samples in concentration 100 - 50 - 10 - 5 ng/ μ L were analysed and the results reported in Tab. 6.1. A high content of Glu, Asp, Leu, Ala, Val and Phe, that are characteristic of ovalbumin (Schilling M.R. *et al.* 1996a and b, Prikryl P. *et al.*, Wouters J. *et al.* 2000a and b) was observed. It was important to notice that Glu, that is present in high concentration, is also present in casein but in higher concentration than in ovalbumin. Other parameters to distinguish ovalbumin, by amino acid profile, are its higher rates of Ala and low Pro content than casein (Prikryl P. *et al.*, Ronca F., Keck S. *et al.*). As for Gly and Ser, it is worthwhile to specify that the sample handling and contamination of the environment can influence the analysis performance and overestimate the concentration. It was also possible that the yield of some amino acids could be distorted because of incomplete rupture of the bonds during hydrolysis, as in the case of Val and Ile, or because of complete or partial destruction of acid sensitive amino acids such as in Cys, Thr,

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Ser, and Met. Despite these considerations, since the conditions of hydrolysis, derivatization, and separation were the same, it was concluded that from the AccQ•Tag™ Ultra method a good match between the amount of the various amino acids detected at the different concentration of the standard ovalbumin samples. Concerning the recovery value of the ovalbumin , the yield was around 60-70% (Tab. 7.1).

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Table 7.1: Results of amino acid analysis of the reference samples of standard ovalbumin in 100-50-10-5 $\mu\text{g/mL}$ concentration: for each amino acid, the amount expressed in $\text{pg}/\mu\text{L}$ and in $\text{pmol}/\mu\text{L}$ and their relative weight expressed in % (%w) are reported.

	R T	Ovalbumin											
		100 $\mu\text{g/mL}$ (100 ng/ μL)			50 $\mu\text{g/mL}$ (50 ng/ μL)			10 $\mu\text{g/mL}$ (10 ng/ μL)			5 $\mu\text{g/mL}$ (5 ng/ μL)		
		pmol/ μL	pg/ μL	%w	pmol/ μL	pg/ μL	%w	pmol/ μL	pg/ μL	%w	pmol/ μL	pg/ μL	%w
His	2.38	10.37	1606.92	2.68	5.52	856.19	2.53	0.45	69.19	1.18	0.00	0.00	0.00
Ser	3.23	26.82	2816.06	4.71	15.95	1674.50	4.20	4.27	448.64	7.02	5.52	579.72	16.41
Arg	3.44	21.10	3670.90	6.14	11.20	1948.60	5.58	1.86	323.58	5.22	0.49	84.98	2.25
Gly	3.45	23.08	1730.86	2.88	13.35	1001.43	3.50	4.99	374.58	6.03	4.89	367.03	10.23
Asp	3.88	43.20	5745.45	9.61	28.75	3823.29	10.72	4.72	627.33	10.14	2.31	307.87	8.67
Glu	4.37	66.85	9827.44	16.44	42.12	6191.59	17.67	7.34	1078.37	17.41	3.76	552.66	15.53
Thr	4.74	22.21	2643.50	4.43	12.09	1438.45	4.09	2.04	243.25	3.93	1.04	123.38	3.47
Ala	5.11	41.43	3686.94	6.17	24.68	2196.42	6.35	4.32	384.13	6.21	2.15	191.48	5.37
Pro	5.67	19.54	2247.61	3.76	11.13	1280.46	3.90	2.02	232.48	3.74	1.03	118.80	3.32
Cys	6.57	0.66	79.88	0.14	0.33	39.72	0.06	0.00	0.00	0.00	0.00	0.00	0.00
Lys	6.63	23.62	3447.79	5.79	14.50	2116.35	6.02	2.63	383.69	6.20	1.35	197.65	5.55
Tyr	6.74	12.60	2280.15	3.80	6.54	1183.89	3.52	1.23	223.09	3.59	0.64	116.60	3.27
Met	6.88	13.62	2028.71	3.43	8.81	1312.41	2.87	0.45	67.33	1.14	0.00	0.00	0.00
Val	7.01	36.48	4267.70	7.14	20.51	2399.64	7.01	3.70	433.07	6.99	1.98	231.40	6.49
Ile	7.73	26.68	3495.69	5.85	14.82	1941.01	5.68	2.64	345.94	5.58	1.37	179.14	5.02
Leu	7.82	41.55	5443.54	9.11	22.96	3007.34	8.84	4.07	533.55	8.61	2.08	272.20	7.62
Phe	7.92	28.81	4754.47	7.92	14.62	2413.05	7.45	2.64	435.89	7.00	1.47	243.35	6.79
		458.62	59773.61	100.00	267.87	34824.35	100.00	49.38	6204.11	100.00	30.10	3566.28	100.00

In order to avoid loss of protein material, no process of extraction and pre-treatment of the model samples were carried out and the samples were subjected to hydrolysis immediately after sampling. Since the amount of protein in model samples was much lower than in the standard samples, 20 instead of 10 μL of solution were subjected to derivatization and 60 μL instead of 70 μL of borate buffer were added; therefore, the concentration of Nval was 21 $\text{pmol}/\mu\text{L}$. After the derivatization process, each sample was centrifuged to separate the supernatant from the insoluble fraction; then, they were introduced into

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the UPLC and analyzed twice. In data processing the average of the results were performed and reported. Table 7.2 (for the whole egg, the milk and the glue tempera samples the corresponding chromatograms are reported in Fig. 7.2) shows the concordance of the results obtained for egg tempera model sample and the standard ovalbumin, egg white and whole egg ones. Egg white presents almost the same amino acids % values as pure ovalbumin, as expected. Some slight differences that can be detected from Table 7.2 are due to the presence of the other egg white proteins, as ovotransferrin and lysozyme, containing more Arg (lysozyme) and more Lys (ovotransferrin) with respect to pure ovalbumin. On the contrary, for whole egg the amino acids % values were quite different with respect to pure ovalbumin. In whole egg the contribution of ovalbumin is lower, due to the presence of a large amount of lipids in yolk and due to the presence of other type of proteins, i.e., vitellin, lipovitellin, and phosvitin. Yolk protein content is richer in Thr and Lys and less in Phe than ovalbumin. In Tab. 7.2, the blue colour highlights the percentage of the amino acids (Glu, Asp, Ala, and Leu) that are characteristic of egg. In yellow the percentages of amino acids that undergo changes depending on the presence or absence of other proteins are highlighted. For example, the supply of Phe and Ser is more important in ovalbumin than in yolk; also the slightly higher content in Arg in egg white is due to the presence of lysozyme.

In the egg tempera model sample a high content of Glu, Asp, Leu, Arg, Ala, Phe, and Ser was observed. These results are compared with those of milk, glue and egg-glue binder: milk tempera sample contained a higher percentage of Glu, Pro, and Leu (red colour in the Tab. 7.2) and a percentage of Ala and Asp lower than egg tempera sample. For the

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glue tempera sample, the very high percentage of Gly and Pro is representative of this medium (green colour in the Tab. 7.2). Also the glue tempera contained a large amount of Hyp. In fact, a broad peak observed in Fig. 7.2c) ($R_T = 2.05$), corresponding to a polar amino acid, can be attributed to Hyp. In egg/glue tempera the presence of a large amount of Glu, Asp, Leu and Ala, that are representative of egg-based binders, was observed. On the other hand the amount of some characteristic amino acids of glue-based tempera, such as Gly, and Pro, is very low and HyP was not detected.

Concerning the recovery of all samples reported in the Table 7.2, as expected the values for ovalbumin and white egg is around 70%, while for whole egg is circa 30%; this is for the presence of other materials (lipids) in the total amount of the sample. For the recovery of model samples, different results were obtained: for the egg and the glue tempera, the yields were in agreement with the expecting results, while for the milk and the composed egg-glue tempera the recovery was very low. This might be ascribed to the non-homogeneous material collected.

It was also interesting to observe that for the composed egg-glue model sample, no glue was observed. The hydroxyproline peak was not detected and the amino acid composition was comparable with those of the egg tempera. This sample was performed by painting first the egg tempera and then the glue-based tempera. Combining these results with those obtained on the same two model samples (egg and egg-glue based) by Dot-ELISA allowed to draw the following picture. In the presence of strong alkaline environment amino acid analysis revealed itself more powerful as Dot-ELISA for the egg-based model sample,

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due to higher probability that pH affected protein sites of Dot-ELISA reaction rather than the final amino acid composition of the sample. On the other hand, when a glue red ochre layer is added onto a pre-existing egg-based one, there is a kind of protection of the ovalbumin by the alkaline environment by glue enabling to increase the Dot-ELISA sensitivity (see above), but at the same time amino acid analysis did not succeed in detecting glue and lost sensitivity against egg protein components. Due to the much higher fluidity of the red ochre glue mixture with respect to the same egg-based, the glue diffuses into the inner part of the pre-existing egg layer, somehow isolating it from the alkalinity underneath of the setting mortar and this accounts for the best conservation of egg in mixed sample. Simultaneously this glue segregation did not allow to recover amino acids typical of glue, when sampling the external layer of the red ochre painting (see results reported in Table 7.2, column egg-glue tempera). The migration of the glue during brushing towards the inner layers caused also a consequent diffusion of the egg proteins from the external layer leading to a lower concentration of these proteins in the sampled material and this could explain the lower yield of the amino acid analysis for egg proteins in mixed tempera with respect to pure egg-based one.

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Table 7.2: Comparison between ovalbumin, egg white, whole egg and model samples of egg, milk, glue and egg-glue tempera; the amino acid content is expressed as %w and ng/ μ L. In blue color, the characteristic amino acids for egg proteins, in yellow the variable amino acids (depending on the presence of other proteins); in red the characteristic amino acids of milk and in green the marker of glues (hydroxyproline is not considered here). The egg-glue tempera shows a characteristic pattern of egg-based tempera.

	Ovalbumin		Egg white		Whole egg		Egg tempera		Milk tempera		Glue tempera		Egg-Glue tempera	
aa	w %	ng/ μ L	w %	ng/ μ L	w %	ng/ μ L	w %	ng/ μ L	w %	ng/ μ L	w %	ng/ μ L	w %	ng/ μ L
His	2.33	3.34	2.44	3.31	2.27	1.42	0.84	0.36	0.00	0.00	0.52	0.37	1.47	0.11
Ser	7.39	10.60	7.02	9.53	7.20	4.51	5.97	2.57	3.91	0.25	3.20	2.24	5.08	0.38
Arg	5.75	8.25	6.14	8.33	6.04	3.78	7.39	3.19	3.53	0.23	8.09	5.68	5.54	0.42
Gly	3.51	5.03	3.92	5.32	3.53	2.21	4.26	1.84	3.24	0.21	25.22	17.71	3.38	0.25
Asp	9.68	13.87	9.87	13.40	10.59	6.62	8.89	3.83	5.15	0.33	5.15	3.62	9.83	0.74
Glu	15.01	21.52	14.05	19.07	15.07	9.42	13.89	5.99	17.30	1.12	11.80	8.29	14.47	1.09
Thr	4.19	6.01	4.46	6.06	4.85	3.03	4.80	2.07	4.34	0.28	1.79	1.26	3.37	0.25
Ala	6.17	8.85	5.97	8.11	6.04	3.78	6.94	2.99	3.72	0.24	10.61	7.45	6.56	0.50
Pro	4.12	5.91	4.28	5.81	4.11	2.57	4.71	2.03	10.33	0.67	15.59	10.95	6.94	0.52
Cys	1.02	1.46	1.22	1.65	0.94	0.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lys	5.94	8.51	6.10	8.27	6.89	4.31	4.77	2.06	4.46	0.29	4.07	2.86	4.01	0.30
Tyr	3.77	5.41	4.08	5.54	3.90	2.44	4.39	1.89	4.44	0.29	0.85	0.59	4.34	0.33
Met	4.38	6.28	3.88	5.27	3.42	2.14	0.68	0.30	3.12	0.20	0.18	0.12	8.63	0.65
Val	5.95	8.54	5.95	8.08	5.82	3.64	7.77	3.35	7.57	0.49	3.08	2.16	5.70	0.44
Ile	5.07	7.26	4.80	6.51	4.64	2.90	6.70	2.89	6.28	0.41	2.20	1.54	4.93	0.37
Leu	8.71	12.49	8.87	12.04	8.77	5.48	11.09	4.78	14.64	0.95	4.57	3.21	8.43	0.63
Phe	7.01	10.06	6.95	9.43	5.92	3.70	6.62	2.86	7.98	0.52	3.09	2.17	7.31	0.55
	100.00	143.40	100.00	135.76	100.00	62.55	100.00	43.13	100.00	6.49	100.00	70.22	100	7.54

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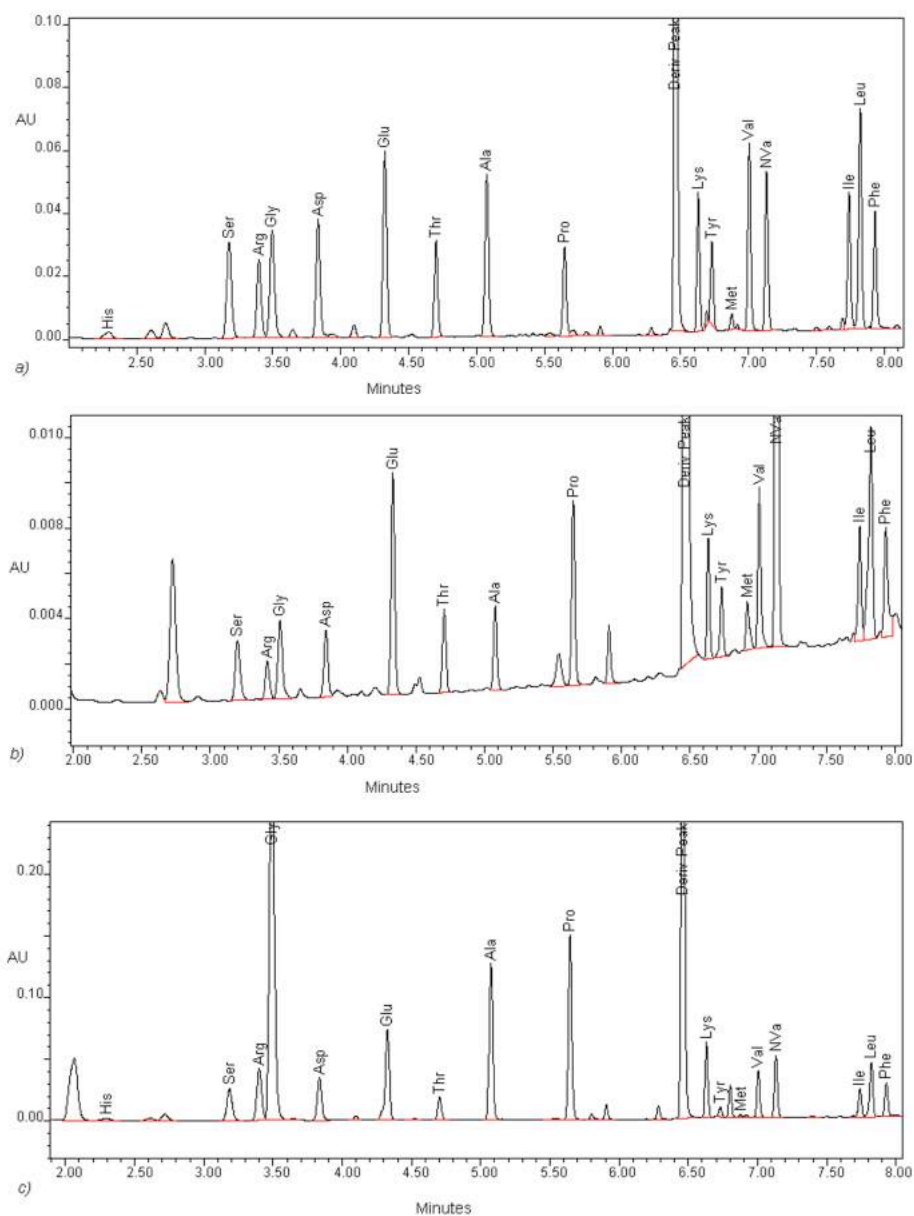


Figure 7.2: Chromatograms of model samples: a) egg tempera, b) milk tempera, c) glue tempera (the first non identified peak is related to HyP).

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CHAPTER 8.

STUDY OF THE EFFECTS OF PIGMENTS AND AGEING BY MEANS OF UPLC TECHNIQUE.

As for the Dot-ELISA technique, also with the AccQ•Tag Ultra method, a study of the effects of several pigments and artificial ageing on proteins has been performed.

It is well known, and the above-discussed Dot-ELISA study has proved it, that the proteins subjected to ageing suffer the modification in structure and function. As above discussed, the presence of pigments might trigger a series of modifications in the properties of a protein promoting degradation phenomena either before the ageing (e.g. in the case of cinnabar), or after the ageing. In the cultural heritage field, the nature of the protein-based samples entails the presence of inorganic elements from pigments and grounds. In fact, both in samples of binders or protective/consolidating in pictorial art, both in the field of archeology, the proteins are always intimately associated to inorganic components. This also happens to other types of biological samples, but in the field of diagnostics for cultural heritage, the discriminating factors are the ageing and the low amount of sample available.

As stated in Karpowicz A., proteins start to decay during the drying process, because of the loss of water, but often the degradation starts during the preparation of pictorial binders, as in the case of casein. In a recent publication (Leo G. *et al.*) by means of the amino acid analysis, an assessment of the decay state of the samples was carried out. The presence of decreased amount of Met, Lys and Tyr and the presence of

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amino malonic acid as result of Ser, Phe and Cys oxidation, are clear clues of the degradation of proteins. Moreover, by means of proteomic analysis, the deamidation of Asn and Gln is identified as marker of the decay. This event cannot be identified in classical HPLC analysis preceded by the hydrolysis, because of the strong acidic conditions lead the conversion of Asn and Gln in their respective acids. Many factors can influence deamidation, such as protein sequence, secondary and tertiary structure, but also pH, temperature, buffer ions, ionic strength and others. In the case of Leo G. *et al.*, the deamidation was addressed to the preparation mode of the casein used. In fact the slaked lime was added to casein to solubilize it and to obtain a strong glue, after the carbonation of the lime took place.

In addition to the phenomena just described, other known phenomena that affect the primary structure of proteins occur. First, it is worth mentioning the formation of disulfide bonds of cysteine under the effect of oxidation to form cystine; also the formation of sulfoxides and disulphoxides caused by the methionine oxidation (Karpowicz A.). The reactions of proteins with lipid molecules, triggered by the formation of free radicals, are held responsible for the polymerization of the proteins and the formation of imines by reaction of the side chain of lysine with hydroperoxides or ketones species. The formation of imines is responsible for the browning of proteins. The condensation reactions between proteins or amino acids and carbohydrates, known as the Maillard reaction, is responsible for the formation of two amino acid species: furosine and pyridosine. The exposure to alkali treatment leads to the formation of dehydroalanine, which, in turn, form crosslinks with lysine residues. By acid hydrolysis of subjected to alkaline treatment proteins, new products have been found: lysino-

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alanine, ornithino-alanine, lanthionine and β -aminoalanine (Karpowicz A.).

Finally, the exposure to the hydroxyl radical generates phenomena of fragmentation and/or aggregation of the protein structures (depending on the presence or absence of free oxygen) and changes in the amino acid sequence with the loss of the Trp and the production of dityrosine biphenol (Davies K.J.A. *et al.*) (see Ch.3). Dityrosine is responsible of the aggregation of proteins molecules due to its feature to form intermolecular bonding (Davies K.J.A. *et al.*). On the other hand, it must be highlighted that, apart from the amino malonic acid, none of these new products has been detected in the aged samples (Colombini M.P. *et al.* 2004). Certainly, the ageing process produces a reduction of the solubility and a decrease in the recovery of all the amino acids and, in particular, some of these are most affected, such as lysine and tyrosine (Colombini M.P. *et al.* 2004).

It is also clear that the hydrolysis or extraction treatments of proteins, can affect the yields of amino acids. The presence of metal ions from pigments and grounds in the hydrolyzates, indeed, can catalyze degradation phenomena under the strong acidic condition of hydrolysis and interfere in the derivatization procedure, forming stable complexes with the amino acids. In fact, the presence of oxygen, heavy metal and carbohydrates are the main responsible of the loss of 10% for Phe, His and Arg; 20% for Thr, Tyr and Ser; up to 60% for Cys and Met. (Colombini M.P. *et al.* 2004)

The use of pre-treatments for the suppression of interferences is usually suggested in HPLC analysis. These methods could be performed before the hydrolysis, such as the basic or acidic extraction

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of the proteinaceous matter, or after the hydrolysis, such as a clean-up with an ion exchange resin (Colombini M.P. *et al.* 1999) and complexation with EDTA (De la Cruz-Canizares J. *et al.*). The basic extraction with NaOH or NH₃ has been very successful in the past and has produced good results, especially on proteins as well. Aged proteins, however, have a different behavior, due to several factors, and this could influence the amount of extracted material, that may decrease dramatically.

Complexation of the metal ions with a strong chelating agent, such as EDTA has been proposed with satisfactory results in the final discrimination between the different proteinaceous temperas (Grzywacz C.M., De la Cruz-Canizares J. *et al.*).

The cation exchange resins have been used for the clean-up of the samples, followed by a basic elution. This procedure, although has produced good results, involves a prolongation of the analysis time and an increase in sample handling. This last issue leads to an increased risk of loss and contamination of the sample.

In order to avoid losses and dangerous handling of the sample, the AccQ•Tag Ultra method was adjusted and performed without preliminary extraction and clean-up procedures. On the other hands, the detection and the identification of model samples, performed in the Ch. 6, didn't suffer of the issues above discussed. The pigment used for preparation of those model samples was red ochre, which contains Fe³⁺. As the results were very interesting and didn't show a negative effect ascribable to the presence of pigments, this strategy was also kept for the study of the aged and non-aged laboratory-made protein temperas.

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As for the Dot-ELISA study, paint mock-ups were prepared using different pigments with whole egg, bovine milk and animal glue and submitted to light and thermally ageing.

8.1 Materials and methods

The apparatus conditions, the chemicals and the reagents used are the same of those cited in the Ch. 3.2. The reference standard protein materials used are: ovalbumin and casein purchased from Sigma; eggs, milk and porcine gelatin from a local market and the rabbit and animal glue purchased from Zecchi (Firenze).

The preparation of the paint samples and the ageing procedures were the same as in the Ch. 3.2 (Samples).

Also the derivatization procedure for the standard and the aged and non-aged samples was the same as previous analysis. The only modification regards the hydrolysis time, which was prolonged until 48 h for laboratory-made specimens. For these samples, after the derivatization procedure a centrifugation step was performed.

8.2 Results and discussion

First of all, the reference standard proteins were subjected to analysis. In particular, in addition to the previously studied egg, samples of milk, casein, food grade gelatin from porcine source and animal glue at different concentrations were analysed (Tab. 8.1 and Fig. 8.1)

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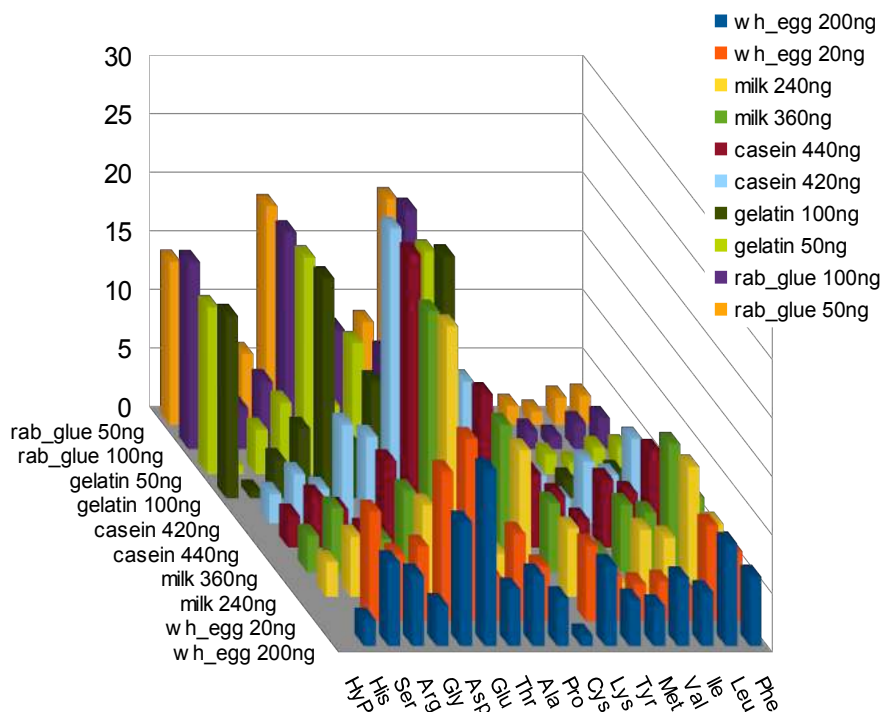


Figure 8.1: bar graph of reference protein standards

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Table 8.1: amino acids content of the reference protein standards expressed in percentages in weight (%w/w); the ng indicate the amount of the injected material

	whole egg		milk		casein		porcine gelatine		animal glue	
	200ng	20ng	240ng	360ng	440ng	420ng	100ng	50ng	100ng	50ng
HyP	0.00	0.00	0.00	0.00	0.00	0.00	15.60	14.24	15.99	13.92
His	2.27	0.00	2.98	3.14	2.73	2.51	0.63	0.76	0.54	0.50
Ser	7.20	9.40	5.15	5.49	4.38	4.19	3.29	3.79	3.54	4.80
Arg	6.04	5.70	2.09	2.38	3.31	2.93	5.99	6.10	5.83	6.05
Gly	3.53	6.40	2.52	2.81	1.53	8.41	18.81	18.50	18.54	18.68
Asp	10.59	12.80	7.83	6.96	7.40	7.40	4.89	5.83	4.94	5.38
Glu	15.07	15.60	23.11	22.28	25.09	25.16	9.94	11.17	10.21	10.62
Thr	4.85	3.90	3.65	3.87	3.49	3.30	1.16	1.32	1.19	1.30
Ala	6.04	7.50	3.70	3.44	2.91	2.74	7.94	8.62	8.24	8.78
Pro	4.11	4.70	12.55	12.61	13.10	12.11	20.69	19.01	20.46	19.27
Cys	0.94	0.00	0.00	0.03	0.03	0.00	0.00	0.00	0.00	0.00
Lys	6.89	6.50	5.88	5.87	6.33	5.17	2.41	2.88	2.21	2.57
Tyr	3.90	3.90	1.14	1.25	4.48	4.07	0.73	0.59	0.65	0.59
Met	3.42	3.20	1.20	1.71	2.42	2.12	0.30	0.00	0.00	0.00
Val	5.82	3.40	5.74	5.76	5.83	5.25	1.68	1.66	1.61	1.61
Ile	4.64	2.80	5.07	5.07	4.69	4.09	0.97	0.93	1.12	1.12
Leu	8.77	8.20	11.14	10.96	8.27	7.20	2.30	2.24	2.28	2.31
Phe	5.92	6.00	6.25	6.38	4.00	3.37	2.68	2.36	2.67	2.49
tot	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

8.2.1 Egg-based temperas

As previously discussed, the presence of pigments induces some slightly modifications in primary structure of a protein, involving also its final properties and functions. In the Figs. 8.2 and 8.3, the bar graphs are reported in which the weight percentages of each amino acid for the non-aged egg-based tempera samples analysed are compared. In particular in Fig. 8.2 the reference whole egg sample, the un-pigmented egg-based tempera and the first four pigmented samples (S.Giovanni white, yellow ochre, minium and verdigris) are shown. In the Fig. 8.3 the same reference whole egg and un-pigmented samples were shown and compared with the remaining samples (azurite, smalt, cinnabar and ematite). In the graphs the cysteine values are not reported: this choice is justified by the fact that it is not a diagnostic

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peak and the yield is very low, because it undergoes a strong degradation during the hydrolysis process. The values were obtained from the average values of the percentages by weight of 4 injections of two samples. The percentages by weight were obtained from the values expressed in pmol/ μ L, calculated by the software.

The peaks that undergo changes in recovered percentage are Tyr and Met. In the specific case of egg tempera, HyP is not contained and therefore it should not to be found. In the results, the peak of HyP is detected in almost all the samples, even in STU (unpigmented), except in BSGU, CINU and MINU. This peak is probably related to an amino acid form resulting from the degradation of the protein which has been in contact with a strongly basic matrix, as the calcium carbonate-based plaster support. When the binder layer was spread, in fact, the support was not yet fully carbonated and this event may have led to a degradation of the protein. In addition, can not be excluded that the presence of residues of calcium carbonate plaster, induced strong modifications to the amino acids during the hydrolysis process. Certainly it can not be said that the peak is related to the HyP.

From the results, it is visible that the reference whole egg samples and the STU show a low variability, only for HyP, His, Met and Val values. The sample with high variability with respect to the STU is the VRGU sample, more in Glu, Asp and Tyr peaks.

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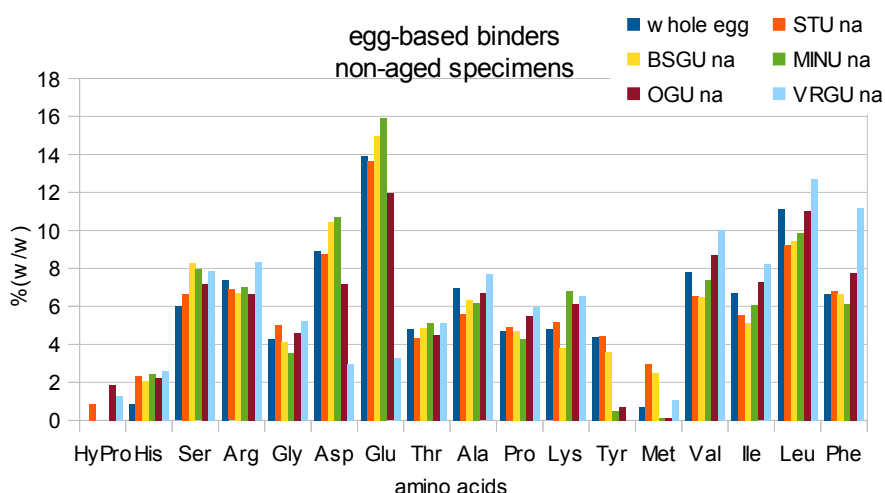


Figure 8.2: bar graph of non-aged egg-based temperas. The weight percentage is reported for each amino acid except for cysteine. The whole egg reference sample and the unpigmented sample were compared with pigmented samples.

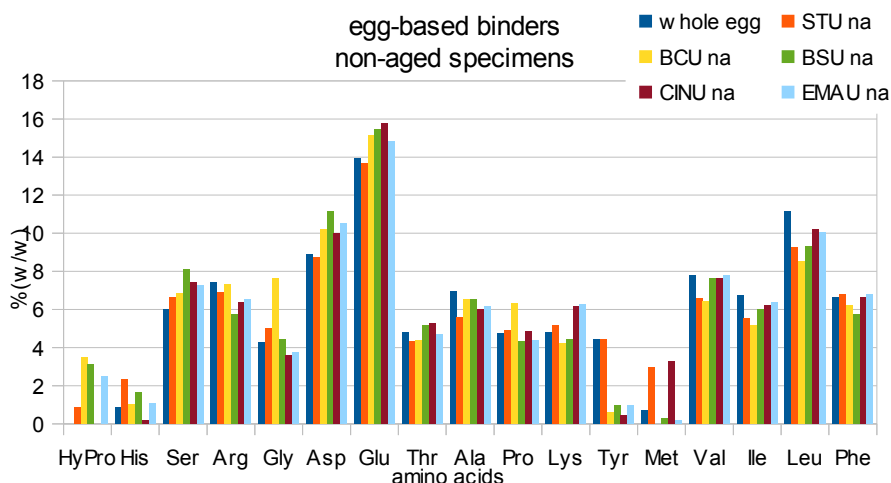


Figure 8.3: bar graph of non-aged egg-based temperas. The weight percentage is reported for each amino acid except for cysteine. The whole egg reference sample and the unpigmented sample were compared with pigmented samples.

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The verdigris, a copper acetate-based pigment, results as the pigment that induces greater variations in the content of amino acids; in fact, as stated in Khandekar N. *et al.*, the verdigris cause fragmentation of proteins. This phenomenon express itself with a loss of adhesion of the paint layer with the support and in a loss of cohesion of the pigment particles. Cinnabar, that forms stable mercury-protein complexes of different molecular weights (Duce C. *et al.*), promoting protein cross-linking, hydrolysis and oxidation, however do not show high reactivity, such as to induce strong changes in yields of individual amino acid.

The results of aged samples (Figs 8.4 and 8.5) show a very different situation. In fact, differently from the results on non-aged samples, in this case, there is a high variability between the values of the different specimens, relating to the same amino acid. The aged unpigmented sample also presents significant differences with respect to the non-aged one. Although, the only presence of the calcium carbonate-based support and the composition of the binder undergo strong degradation under the ageing conditions imposed.

It must be pointed out that several unknown peaks are eluted from the column and detected from the TUV detector, but the software was not able to identify them. Moreover, often the retention time of some peaks was shifted and a manual identification was be done. Probably some different species were eluted and mistakenly identified with an amino acid. This could be overtaken studying the retention times of the above discussed secondary products of decay, such as lysino-alanine, ornithino-alanine, lanthionine, β -aminoalanine, dityrosine and malonic acid.

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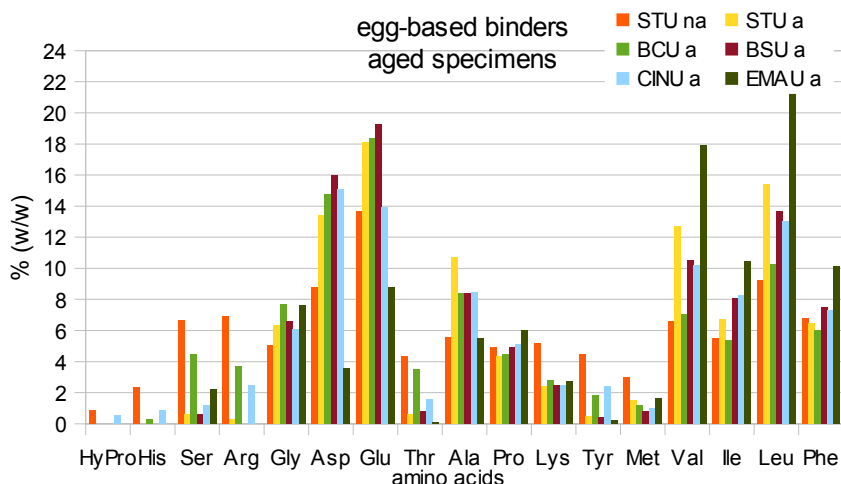


Figure 8.4: bar graph of aged egg-based temperas. The weight percentage is reported for each amino acid except for cysteine. The non-aged (STU na) and the aged (STU a) unpigmented samples were compared with pigmented samples.

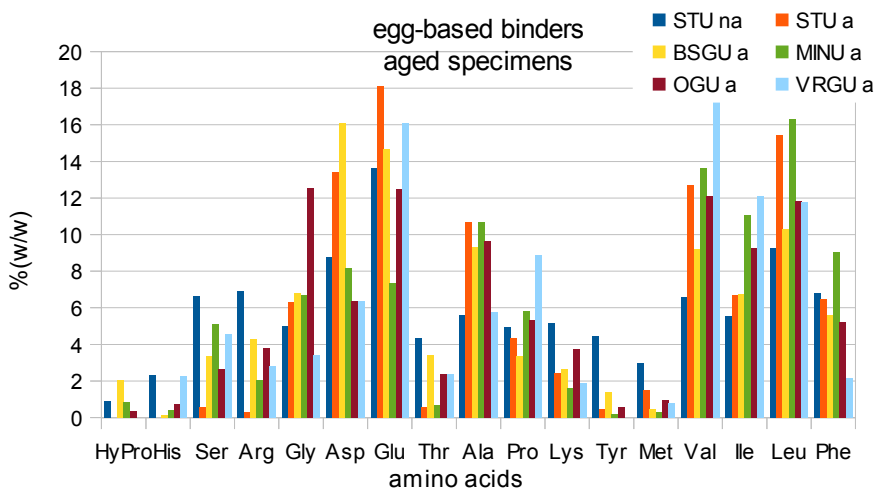


Figure 8.5: bar graph of aged egg-based temperas. The weight percentage is reported for each amino acid except for cysteine. The non-aged (STU na) and the aged (STU a) unpigmented samples were compared with pigmented samples.

8.2.2 Milk-based temperas

As for the results of egg temperas, the amino acids that undergo to strong decrease are Tyr and Met (Figs. 8.6 and 7.7). Also for the milk temperas, the unexpected HyP peak appears in some samples, but it could be addressed to degradation effects of the presence of Ca^{2+} cations from plaster support. In the case of milk temperas, the Glu peak is highly variable for the samples analysed. Moreover, in Fig. 8.7 the EMAL sample and, less the BSL sample, have high percentage of Gly, which is commonly related to contamination effects. Also in this case the VRG sample has the most variability in the amino acid content respect to the reference milk sample and the unpigmented milk tempera.

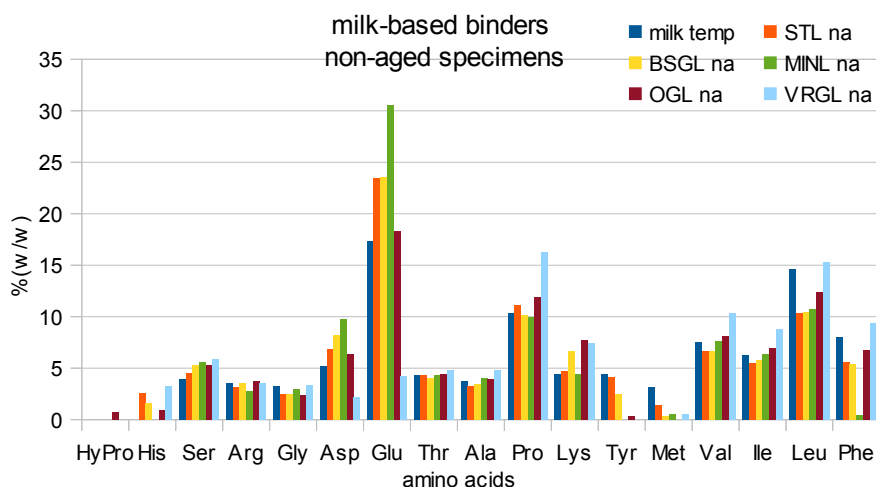


Figure 8.6: bar graph of non-aged milk-based temperas. The weight percentage is reported for each amino acid except for cysteine. The milk reference sample and the unpigmented sample were compared with pigmented samples.

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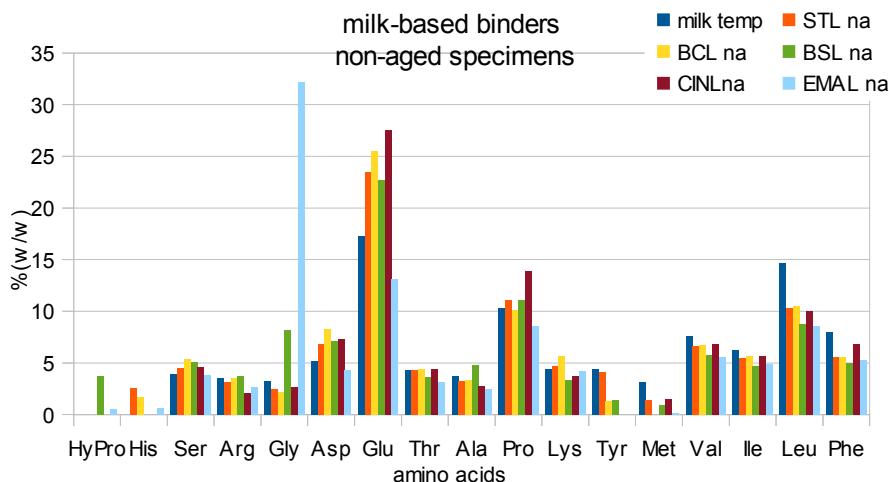


Figure 8.7: bar graph of non-aged milk-based temperas. The weight percentage is reported for each amino acid except for cysteine. The milk reference sample and the unpigmented sample were compared with pigmented samples.

As for the egg temperas, also in this case, the ageing procedure caused an increase of the variability in the weight percentage for each amino acids, even if in a lesser extent than the tempera egg. It is interesting to notice that, in aged milk-based specimens, the HyP peak has not been detected in any sample (Figs. 8.8 and 8.9). Among the unpigmented tempera, a remarkable difference between the aged and non-aged samples occurs in the individual amino acids recovery, except for Ile, Leu, Phe, Ala and Glu.

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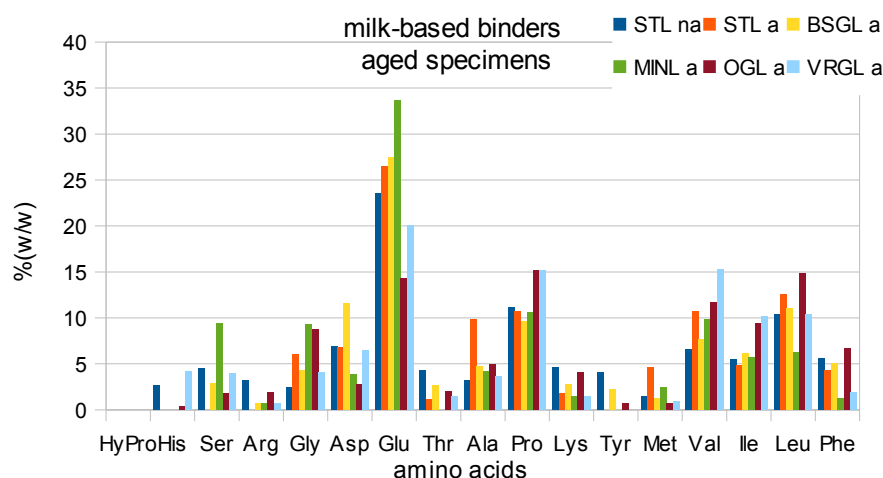


Figure 8.8: bar graph of aged milk-based temperas. The weight percentage is reported for each amino acid except for cysteine. The non-aged (STL na) and the aged (STL a) unpigmented samples were compared with pigmented samples.

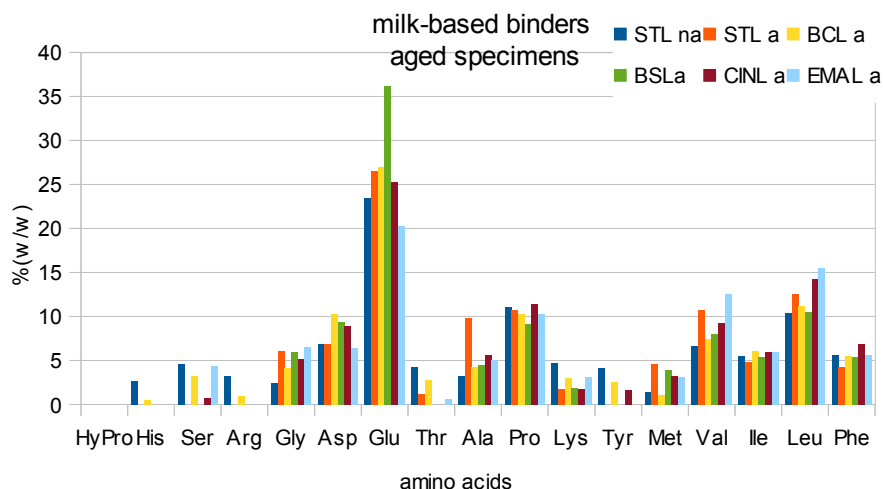


Figure 8.9: bar graph of aged milk-based temperas. The weight percentage is reported for each amino acid except for cysteine. The non-aged (STL na) and the aged (STL a) unpigmented samples were compared with pigmented samples.

8.2.3 Glue-based temperas

Apart from the high percentage of Pro in the glue reference sample, that is different from the other samples, not in the non-pigmented specimen, and apart from the drastic decrease of Tyr and Met values, the percentages of the individual amino acid are comparable in all samples (Figs. 8.10 and 8.11). The effects of denaturation of collagen at the hands of cinnabar, stated by Romero-Pastor J. *et al.*, do not affect the recovery of amino acids; therefore they don't produce modifications in the primary structure of collagen. In the above-mentioned work, it is stated that these effects are already observed in non-aged samples. In the same work, it was proposed, as primary reaction between collagen and azurite, the formation of protein-copper complex that induces a photostabilization to the UV irradiation. This complex formation has the role of protector for the UV-induced modifications. In the results of non-aged samples, this phenomenon of complex formation does not involve any change in the recovery of amino acids, compared to non-pigmented specimen.

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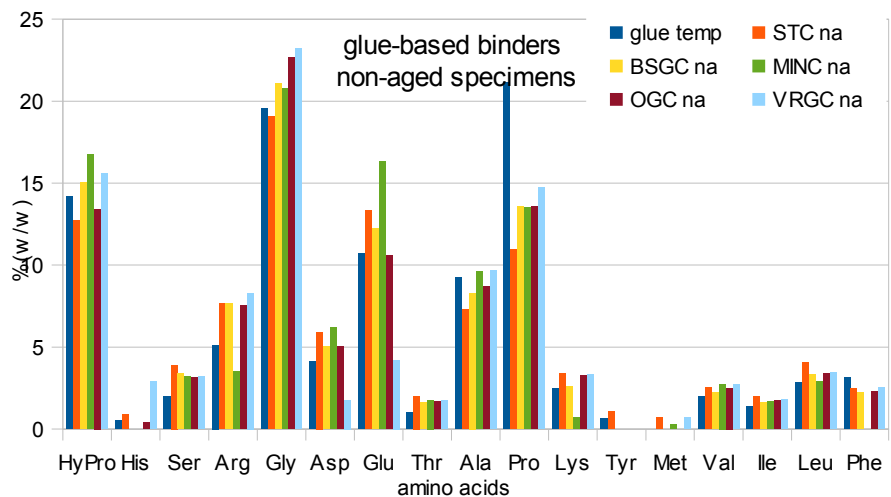


Figure 8.10: bar graph of non-aged glue-based temperas. The weight percentage is reported for each amino acid except for cysteine. The animal glue reference sample and the unpigmented sample were compared with pigmented samples.

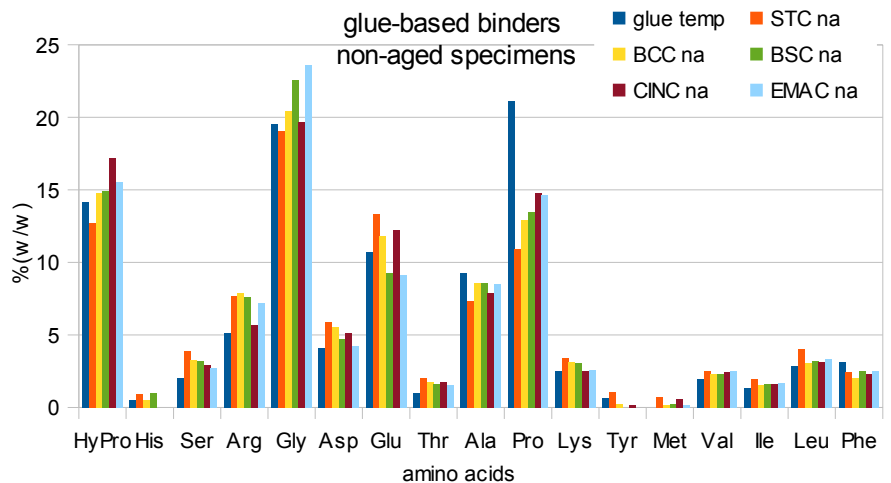


Figure 8.11: bar graph of non-aged milk-based temperas. The weight percentage is reported for each amino acid except for cysteine. The animal glue reference sample and the unpigmented sample were compared with pigmented samples.

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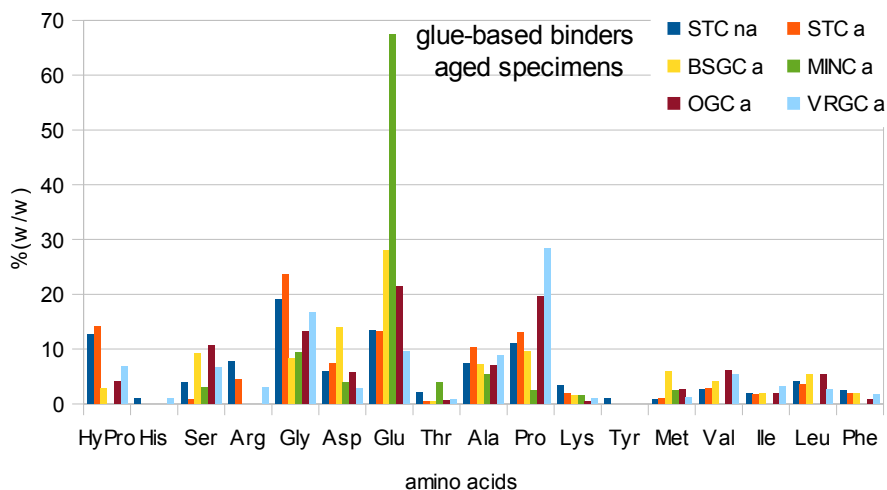


Figure 8.12: bar graph of aged glue-based temperas. The weight percentage is reported for each amino acid except for cysteine. The non-aged (STC na) and the aged (STC a) unpigmented samples were compared with pigmented samples.

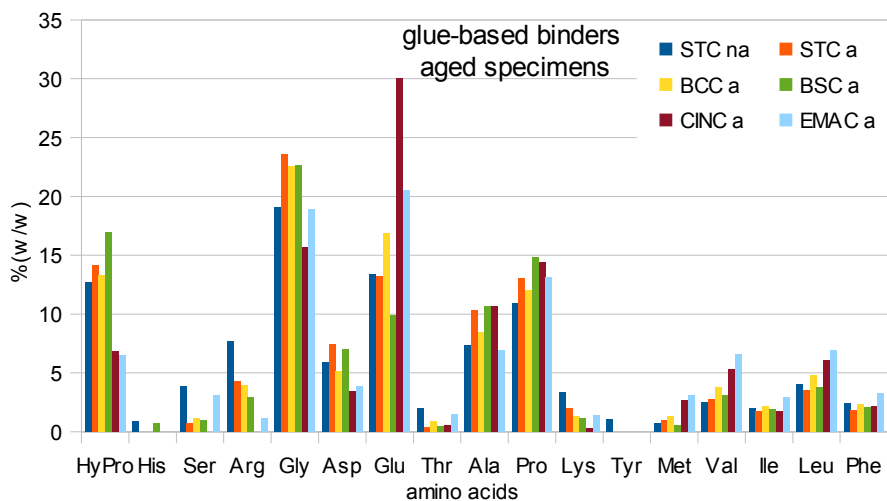


Figure 8.13: bar graph of aged glue-based temperas. The weight percentage is reported for each amino acid except for cysteine. The non-aged (STC na) and the aged (STC a) unpigmented samples were compared with pigmented samples.

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As in the previous case, also for animal glue temperas the variability between the amino acids weight percentages increases in aged specimens. In Fig. 8.12, the very high content of Glu in MINC sample is a clue of this phenomenon; the other visible effect is the disappearance of HyP peak. In Figs 8.12 and 8.13, the comparison between the STC na and STC a samples reveals that differences are visible only for some amino acids, such as His, Ser, Thr, Tyr. The ageing decay has produced slight modifications in the primary structure of the collagen. For CINC sample, instead, the degradation effects produced by the presence of Hg^{2+} are visible in the variations in amino acid content, such as the decrease of HyP, the increase of Glu, the disappearance of His, Ser, Arg, Lys and Tyr.

In conclusion, it is possible to state that the artificial ageing resulted in many changes of the protein structure. In fact in all cases large variations between aged samples and non-aged ones are found. In many cases the resulting chromatographic patterns don't allow the identification of the protein in the analysed specimen.

Regarding to the recovery values, the non-aged samples report higher values than the aged samples (Tab.8.2). These are in the range of 0.09-10% of the total material submitted to hydrolysis. This high difference is due likely to the non-homogeneity of the starting material, which could have been involved also from the carbonate plaster particles.

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Table 8.2: recovery values expressed in ng/μL.

	recovery in ng/μL					
	Non-aged			aged		
	egg U	milk L	glue C	egg U	milk L	glue C
BS	74.66	11.56	69.52	20.96	3.10	41.75
BSG	24.61	21.99	11.25	29.57	11.63	3.26
BC	41.93	17.30	60.94	22.93	9.37	8.99
EMA	39.83	91.99	51.47	13.37	3.17	4.12
MIN	54.22	12.35	45.71	37.78	3.35	1.79
CIN	217.36	15.54	53.49	45.52	7.69	6.44
OG	106.51	52.17	40.69	34.57	19.00	5.53
VRG	56.61	50.92	27.66	31.70	8.32	27.90
ST	31.28	15.75	35.22	14.85	2.70	11.41

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CHAPTER 9.

COMPARATIVE STUDIES

9.1 Identification of protein binder in Andrea di Niccolò's panel "Madonna col Bambino tra i Santi Caterina d'Alessandria, Agostino, Monica e Sebastiano" by means of Dot-ELISAs and determination of amino acid content by AccQ•Tag Ultra UPLC.

The analyzed samples came from the XV century's panel "Madonna col Bambino tra i Santi Caterina d'Alessandria, Agostino, Monica e Sebastiano" of Andrea di Niccolò, from the Pinacoteca Nazionale in Siena. The aim of the investigation was the identification of the protein binder by means of Dot-ELISA and by the determination of the amino acid content by means of the AccQ•Tag Ultra UPLC technique. Samples were provided in the SICAMOR project (S.I.C.A.M.O.R. - Sviluppo di Indagini Chimiche Applicate al Mantenimento delle Opere e al Restauro, PAR FAS Regione Toscana, Linea di Azione 1.1.a.3 (Decr. n. 155 del 13/01/10), which aim was the enhancement and the promotion of cultural heritage by means of the development of innovative analytical techniques.

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9.1.1 Materials and methods

Samples

Three samples were taken from the easel painting and they were weighed and listed (Tab.9.1). From the same sample two micro-samples were obtained for the two analysis. For the samples saved for Dot-ELISAs, the painted layer was selected and submitted to dilution in extraction buffer.

Table 9.1: list and description of the samples and the relative weight in mg.

Sample	Description	Weight in mg
4	Greenish micro-fragment taken from the lapel of the Madonna's mantle	1.0
5	Brown micro-fragment taken from the vessel	3.3
6	Greenish micro-fragment taken from the lapel of the throne's drape	1.4

a)

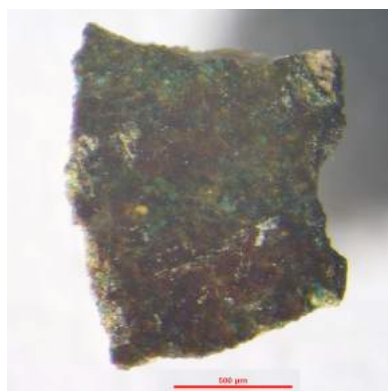


Figure 9.1a: image of the sample 4 at the OM; the red bar is for 500 µm

b)

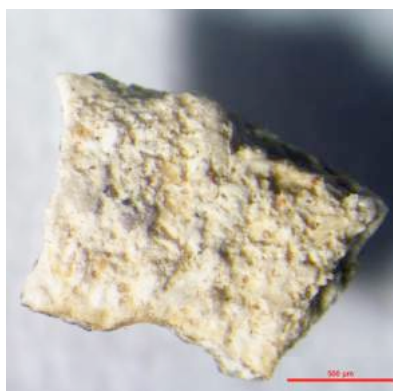


Figure 9.1b: image of the back side of the sample 4 at the OM; the red bar is for 500 µm

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a)



Figure 9.2a: image of the sample 5 at the OM; the red bar is for 500 μm

b)

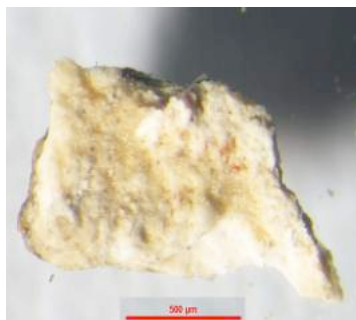


Figure 9.2b: image of the back side of the sample 5 at the OM; the red bar is for 500 μm

a)

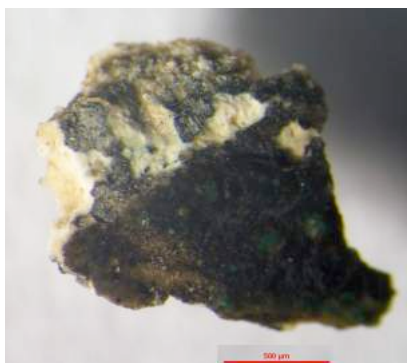


Figure 9.3a: image of the first fragment of the sample 6 at the OM; the red bar is for 500 μm.

b)



Figure 9.3b: image of the back side of the first fragment of the sample 6 at the OM; the red bar is for 500 μm.

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c)

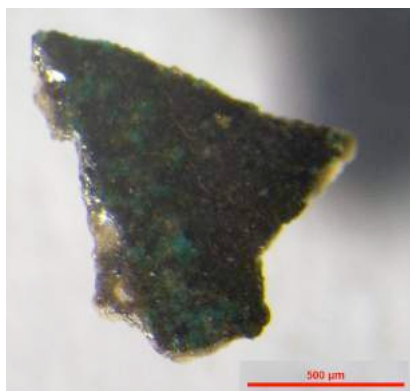


Figure 9.3c: image of the second fragment of the sample 6 at the OM; the red bar is for 500 μm.

d)

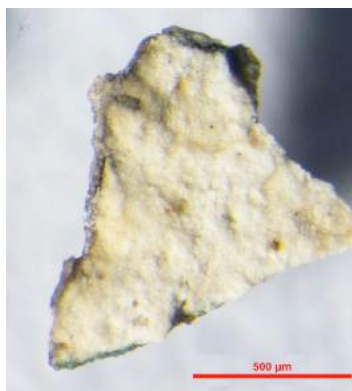


Figure 9.3d: image of the back side of the second fragment of the sample 6 at the OM; the red bar is for 500 μm.

Dot-ELISA

Dot-ELISA was performed with the same procedure adopted in previous case studies. The antibodies used were: Anti-chicken egg albumin, Anti-chicken IgY, Anti-bovine whey protein, Anti-bovine whole serum and Anti-bovine collagen type I; their dilution was 1:2,000 (v/v) ratio (final concentration) for anti-bovine collagen type I and 1:6,000(v/v) for the others antibodies. Also for the secondary antibodies, the ratio used was the same of the previous assays: 1:5,000 (v/v) ratio (final concentration) for both anti-rabbit and anti-goat. The blocking agent used was the soybean milk. The aliquot introduced on the membrane was 5 μL.

Amino acid analysis by AccQTag Ultra UPLC

Samples were ground and submitted to hydrolysis in nitrogen atmosphere in HCl 6M + 0.1% phenol vapors at 114°C for 48h. The procedure for derivatization by AccQ•Tag Ultra method, the eluents,

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the column and the instrument settings were the same that in Ch. 6. The injection volume was always 1 μ L.

9.1.2 Results and discussion

Tab 9.2: Dot-ELISA results for the samples 4, 5 and 6, submitted to the assay for the respective albumin, yolk, bovine milk and bovine glue search.

	Samples		
	4	5	6
Anti-chicken egg albumin	+	+	+
Anti-chicken IgY	-	-	-
Anti-bovine whey protein	+	+	+
Anti-bovine, whole serum	-	-	+
Anti-bovine collagen type I	-	-	-

Table 9.2 shows the results of Dot-ELISA assays. For the egg protein, the results show the presence of ovalbumin, but yolk proteins are absent. This result can be explained in two ways: it could be the real composition of only egg glair-based binder but also it could be due to the stronger decay of the yolk proteins due to the presence of lipids. The results show also that the test for the search of bovine whey proteins was positive.

Even though only the pictorial layer was selected, the search of glue was performed. For the presence of animal glue, only the bovine glue

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was searched. Samples 4 and 5 present negative results both for bovine collagen than for whole bovine serum; sample 6, instead, shows a result not clear for the whole bovine serum, since a negative result for the bovine collagen. This last event could be attributed to a cross reactivity phenomena with the whey protein. As expected, the search of glues reported negative results: the sampling was well done.

Since the detection of proteins by the antibody is based on the reaction between antibody and antigenic determinant of the protein, in the case of a protein strongly degraded and hydrolyzed, it is possible that the antibody is not able to recognize the epitope. Thus, we deepened these findings with the amino acid analysis with UPLC method using AccQ•Tag Ultra.

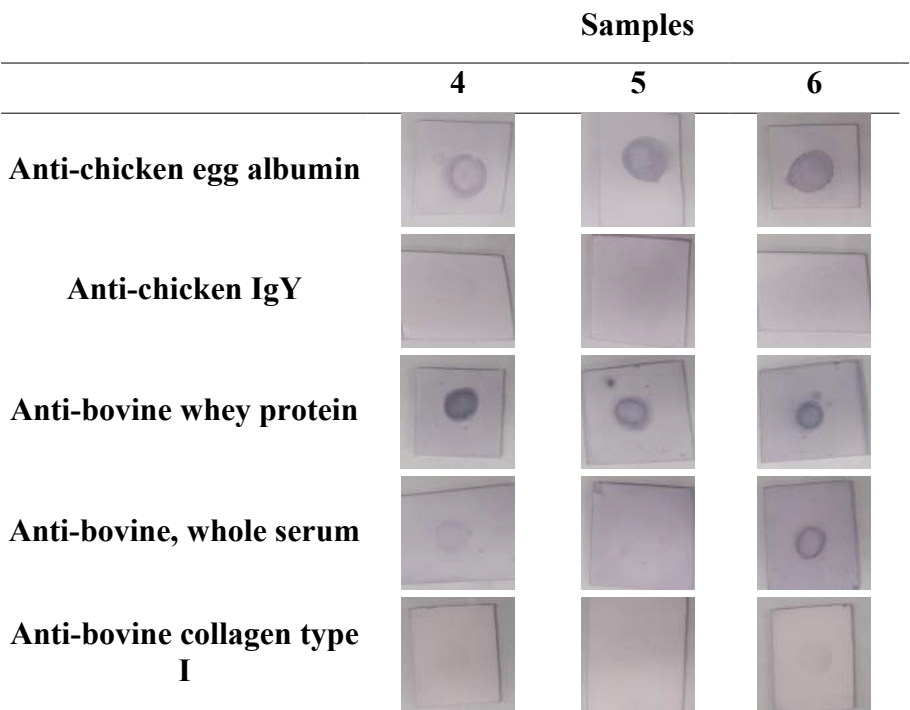


Figure 9.4: Dot-ELISA results of samples 4, 5 and 6 for each antibodies tested.

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The amino acid analysis was performed on the whole samples, including the preparation layer. The results (Tab. 9.3) were calculated starting from the instrument estimates, which were obtained according to the ratio between the amino acid peak area and the Norvaline peak area, used as internal standard. From these values, expressed in pmol/ μ L, the weight concentration in pg/ μ L and finally as weight percentage, were obtained. In the Tab. 9.3, the weight percentages of the analyzed samples were reported, compared with the standard egg, milk and glue tempera values.

The results show the strong presence of the glue, from the preparation layer; in fact, the wide peak of hydroxyproline and then the high percentage of proline and glycine are clear evidence of the collagen presence. However, the high content of aspartic acid and glutamic acid and their ratio suggest the presence of another binder, very likely an egg-based binder, relative to the painted layer.

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Table 9.3: Results expressed in percentages in weight, % (w/w), of each amino acid detected for the samples 4, 5 and 6, compared with reference samples of whole egg, milk and animal glue.

a.a	4NA	5NA	6NA	Egg-ST	Milk-ST	Glue-ST
HvP	11.73	14.44	8.06	0.88	0.00	12.72
His	3.14	1.00	2.44	2.33	2.62	0.91
Ser	3.95	3.32	5.52	6.62	4.54	3.91
Arg	4.40	4.75	4.91	6.91	3.19	7.66
Glv	19.83	20.46	17.46	5.00	2.46	19.05
Asp	7.46	8.73	10.11	8.75	6.88	5.92
Glu	10.09	13.19	12.19	13.66	23.49	13.36
Thr	2.79	2.20	3.72	4.35	4.27	2.02
Ala	11.35	9.42	8.79	5.58	3.24	7.31
Pro	10.16	10.71	6.26	4.91	11.12	10.93
Cvs	0.00	0.00	0.00	0.33	0.00	0.00
Lvs	2.98	1.97	2.44	5.17	4.67	3.38
Tvr	0.13	0.15	0.56	4.44	4.10	1.06
Met	0.38	0.52	0.71	2.97	1.44	0.73
Val	3.75	2.76	4.82	6.55	6.58	2.53
Ile	2.51	1.71	3.45	5.52	5.45	1.98
Leu	3.93	3.15	5.51	9.23	10.36	4.06
Phe	1.40	1.52	3.04	6.80	5.60	2.46
	100.00	100.00	100.00	100.00	100.00	100.00

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9.1.3 Conclusions

In conclusion, the findings of the Dot-ELISA results and of the amino acid content assessment, infer that the composition of the wooden panel preparation is a classical glue-based compound. In the painting layer both egg and milk were found; this suggest a milk-egg composed tempera or a sequence of different layers. Probably a milk-based restoration work was performed to protect the original egg tempera painting.

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CHAPTER 10.

CONCLUSIONS

In this part of the work a well known method for the analysis of proteins was proposed for diagnostics purposes in the field of cultural heritage. In particular, the AccQ•Tag Ultra UPLC methodology was used for the identification of protein materials. The aim of the work was to adjust a well established method for the analysis of protein hydrolysates to samples coming from works of art.

First, the AccQ•Tag Ultra method was tested with the egg-based temperas: model painting samples were subjected to analysis and the results were compared with those of reference standard egg proteins. This procedure was carried out for the three types of protein materials used as binders for pictorial layers. Then, also, some unknown samples were analyzed with the method (see the Ch. 9-Comparative studies).

It is important to note that the samples submitted to analysis didn't undergo to pre-treatments procedures of extraction or clean-up to remove the inorganic components and/or the salts. Since the results were fulfilling and did not seem to suffer the negative effects of the presence of salts and cations, this policy was followed for every analyzed sample.

As for the Dot-ELISA technique, also in this case a study of the aging effects on laboratory protein-based specimens was performed. The aging procedure was the same as for Dot-ELISA analysis: a light aging in Solarbox and a thermal aging at 80°C and RH=75%. As the previous results have demonstrated, this artificial aging has had a strong impact on the protein detection, caused by the modifications

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induced on the primary structure. In many cases, after the artificial aging the protein component of the specimen analyzed was not longer identifiable. In this context, the suggested clean-up or extraction methods for removing cations from the sample solution, could be in service to the detection of these aged proteins. A future study on this topic is suggested.

PART IV

FINAL REMARKS

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion of this project, aimed at the development of innovative analytical techniques for the characterization of proteinaceous materials in works of art samples, it can be said that the objectives have been achieved.

Regarding the immunological approach, the Dot-ELISA technique was developed and adjusted to the purposes and needs of diagnostics for art and archaeology. Moreover, at the same time and thanks to the advances made with the Dot-ELISA, it was possible to use the Western Blot technique. It has opened up new possibilities in the detection of proteins. Future prospects are related precisely to the use of this technique with a multiplexed detection step: exploiting the electrophoretic run for the separation and detection of the protein bands in a single phase enzyme immunoassay using a cocktail of antibodies, a reduction in time and materials is to be expected.

Concerning the chromatographic approach, the methodology AccQ•Tag Ultra UPLC has proved to be very useful for the characterization of protein in cultural heritage conservation. The good performance of the chromatography UPLC were improved with the method AccQ•Tag Ultra, which presents itself as a quick and easy method for protein analysis. Moreover, the elimination of the

PART IV. FINAL REMARKS

extraction and/or cleaning steps of the sample has made the analysis even faster. The results on aged proteins, however, were probably influenced by this choice. In the future it may be useful to study the same materials by introducing a pre-treatment step for the sample purification. In addition to the obtained data, it is suggested to structure it with a chemometric analysis, such as PCA, SIMCA and/or others.

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List of abbreviations

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Gly	Glycine
Gln	Glutamine
Glu	Glutamic acid
His	Histidine
HyL	Hydroxylisine
HyP	Hydroxiprolin
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
NVal	Norvaline
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
Trp	Tryptophan
Val	Valine

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Proceedings

Potenza M., Sabatino G., Giambi F., Rosi L., Papini A.M. and Dei L., *Analysis of egg-based model wall paintings using an innovative combined Dot-ELISA and UPLC-based approach*, XXIII Congresso Nazionale della Divisione di Chimica Analitica della Società Chimica Italiana, Isola d'Elba, 16-20 Settembre 2012, Abstract P152, p.298.

Colombini M.P, Dei L., Degano I., Modugno F., Ghelardi E., **Potenza M.**, *Analysis of contemporary art materials: synthetic pigments and binders of "Ri de pomme" by Julian Schnabel at the Pecci Museum, Prato*, XXIII Congresso Nazionale della Divisione di Chimica Analitica della Società Chimica Italiana, Isola d'Elba, 16-20 Settembre 2012, p.287.

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Sabatino G., Di Pisa F., **Potenza M.**, Rosi L., Dei L. and Papini A.M. *Statistical analysis of amino acid fingerprint to characterize protein binders in works of art*, Peptides 2012, in press.

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