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Application of ionic liquids and enzymes for the removal of proteinaceous layers from polychrome of works of art and evaluation of the cleaning effectiveness

Dissertação Apresentada na Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa para obtenção do grau de Mestre em Ciências da Conservação, especialização em Pintura.

Orientador: Doutora Irina Sandu, Investigadora da FCT-UNL
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Co-orientador: Doutor Tito Busani, Investigador do CENIMAT da FCT-UNL

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Presidente: Doutor Fernando Jorge Pina, Professor Catedrático da FCT-UNL
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Dezembro de 2012

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Very little in the field of conservation generates as much
controversy as the cleaning of surfaces
Narayan Khandekar

Resumo:

Este trabalho apresenta a utilização inovadora de líquidos iónicos como solventes alternativos para enzimas em tratamentos de limpeza e remoção de materiais proteicos presentes em superfícies policromadas ou douradas. Os líquidos iónicos são solventes potencialmente verdes, e apresentados como alternativos para tratamentos de restauro. São também chamados “designer solvents”, pois as suas características podem ser ajustadas através da seleção de diferentes combinações entre catião e anião.
Dois líquidos iónicos foram selecionados: IL1) Tetrafluoroborato de 1-butil-3-metilimidazólio ($[BMIM][BF_4]$) e IL2) etil sulfato de 1-etil-3-metilimidazólio ($[EMIM][EtSO_4]$). As formulações foram preparadas com estes líquidos iónicos e duas proteases diferentes: uma ácida (pepsina) e uma alcalina (de Aspergillus sojae), adicionalmente formulações aquosas em gel foram preparadas com estas enzimas para fins de referência. Uma terceira enzima fornecida pelo Departamento Bromotolagia da Faculdade de Farmácia da Universidade do Porto foi testada apenas na formulação em gel, a fim de avaliar a sua potencial utilização em tratamentos de limpeza.

Para compreender a atividade enzimática destas formulações e prever a sua capacidade como agentes de limpeza foram realizadas análises por espetroscopia no ultravioleta/visível (UV-Vis), e cromatografia líquida de alta eficiência (HPLC). Isto numa fase preliminar e após a limpeza realizaram-se ainda testes com espetrometria de massas por dessorção/ionização a laser assistida por matriz com analisador de tempo-de-voo (MALDI-TOF-MS).
Estas formulações foram testadas em reconstruções de superfícies, de pintura a têmpera e óleo e de douramento, preparadas de acordo com fontes históricas das técnicas artísticas.
Foi também desenvolvido, um protocolo de análise de superfície não invasivo, não destrutivo e a multi-escala para avaliação da eficácia da limpeza e caracterização de superfície antes e após o tratamento. As diferentes técnicas complementares de análise adotadas foram: a estereomicroscopia, microscopia ótica (OM) com a luz visível e de fluorescência, microscopia de força atómica (AFM), microscopia eletrónica de varrimento (SEM) e colorimetria (CIE L*a*b*).

Este protocolo de análise de superfície mostrou-se adequado, não apenas, para monitorizar o processo de limpeza, mas também para a caracterização completa da superfície, antes e após o tratamento, incluindo informação sobre a presença de resíduos e possível deterioração da superfície.
Também se comprovou que as formulações de líquidos iónicos podem ser usadas com sucesso para a remoção de material proteico, como alternativa às formulações em gel.

Estudos futuros deverão ser realizados no sentido de determinar qual o líquido iónico ou grupo de líquidos iónicos mais adequado, as principais preocupações deverão focar-se em melhorar aspetos como a compatibilidade com os outros materiais presentes na superfície, e possíveis efeitos a longo prazo de resíduos após limpeza.

Palavras-chave: Líquido iónico, enzima, proteína, verniz, conservação e restauração, tratamento de limpeza, pintura, douramento, protocolo multi-escala não invasivo.

Abstract:

A novel use of ionic liquids as alternative solvents for enzymes in cleaning treatments for the removal of proteinaceous materials from painted or gilded surfaces is presented. The ionic liquids are potentially green solvents to be applied in restoration treatments being also called designer solvents, because of their peculiar properties which can be adjusted by selecting different cation-anion combinations.

Two ionic liquids were selected: IL1)1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) and IL2) 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM][$EtSO_4$]). Formulations were prepared with these ionic liquids and two different proteases: one acid (pepsin) and one alkaline (from Aspergillus sojae). Additionally aqueous gel formulations were prepared with these enzymes for reference purpose. A third enzyme provided by the Bromatology Department at the Faculty of Pharmacy from the Porto University was tested only in gel formulation in order to assess its potential use in cleaning treatments.

To understand the enzyme activity of these formulations and predict their ability as cleaning agents, analyses were performed with ultraviolet–visible (UV-Vis) spectroscopy and high-performance liquid chromatography (HPLC) prior cleaning; and with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) after cleaning.

These formulations were tested on mock-up samples prepared in accordance with documented and historical sources of artistic techniques of egg tempera and oil painting, and gilding.

A non-invasive non-destructive multi-scale analytical protocol was carried out for cleaning effectiveness evaluation and surface characterization before and after treatment. Different surface analytical techniques were adopted to this purpose: stereomicroscopy (SM), optical microscopy (OM) with visible and fluorescence light, atomic force microscopy (AFM), scanning electron microscopy (SEM) and electron dispersive spectroscopy (EDS) and colorimetry (CIE L*a*b* system).

The surface analytical protocol proved to be adequate, not only, for monitoring the cleaning process but also for complete characterization of the surface, before and after treatment, including information on the presence of residues and possible surface deterioration.

It was also proved that the formulations of enzymes combined with ILs can be used successfully for the removal of proteinaceous material as alternatives to gel formulations.

More studies should be conducted to determine the most suitable IL or group of ILs, the main concern should focus on improving aspects such as compatibility with other surface materials, and possible long-term effects of residues after cleaning.

Keywords: Ionic liquid, enzyme, protein, varnish, conservation and restoration, cleaning treatment, painting, gilding, non-invasive and multi-scale protocol.

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Abbreviations list:

IL – Ionic liquids

IL1 – [BMIM][BF4] 1-butyl-3-methylimidazolium tetrafluoroborate

IL2 – [EMIM][EtSO4] 1-ethyl-3-methylimidazolium ethylsulfate

RTIL – Room temperature ionic liquids

E.C. – Enzyme classification

E1 – Acidic protease

E2 – Basic protease

E3 – Yeast extract

EG – Egg tempera

OG – Oil paint

GG – Gold leafing - gilding

S – Sinopia

YO – Yellow ochre

EW – Egg white varnish

CP – Fish glue varnish

Ca – Casein varnish

CA – Animal glue varnish

G – Aqueous cellulose based buffered gel

UV-Vis – Ultraviolet–visible spectroscopy

HPLC – High-performance liquid chromatography

MALDI-TOF-MS – Matrix-assisted laser desorption/ionization time of flight mass spectrometry

SM – Stereomicroscopy

OM – Optical microscopy

AFM – Atomic force microscopy

SEM – Scanning electron microscopy

EDS – Electron dispersive spectroscopy

EDX –Dispersive X-ray spectroscopy

1.Introduction:

Although cleaning is a frequent treatment in the restoration field, and it is mostly agreed on why should be performed, it is never fully accepted without controversy on how, as it goes against the major ethical principles of conservation: it is completely irreversible and causes the biggest visible alteration to the object. A way to justify the need of such treatment is accepting its benefits and choosing the material and techniques that are most compatible, with the lowest risk for the object and no long term consequences.

There is always a search for new methods as none of the known ones are suitable for every situation. The use of enzymes was a breakthrough in the cleaning process as they are highly selective to the material to be removed not affecting surrounding materials of different nature [1, 2]. Usually they are used with an aqueous system but [1], this cannot be applied for every situation. An innovative solution is related with the possible use of ionic liquids (ILs) as alternative solvent systems.

Similar problems and considerations that are usually addressed in any cleaning treatment as summarized by Khandekar [2], on the subject of aqueous gel cleaning on painted and varnished surfaces, should be considered also for the use of ionic liquids. Firstly the efficiency of the treatment: what is being cleaned and the active components, the clearance process, residues (of the varnish and cleaning solution) associated problems of compatibility and long term effects. Secondly, as stated by Khandekar, there is a need to develop 'an adequate experimental methodology'.

The experimental project developed here has the main objective of giving a scientific groundwork for the novel use of combined ionic liquids and enzyme formulations for the cleaning and removal of proteinaceous material (varnishes) in conservation and restoration surface treatments.

Another objective is to establish valid treatment monitoring and effectiveness assessment protocol, using non-invasive surface analytical techniques. The experimental protocol should be also suitable for other surface treatments and surface characterization on cultural objects.

The experimental work was mainly divided in four steps:

a) ***Formulation selection and establishment of the cleaning protocol***. Two proteases (appendixes I and II), commercially available for restoration treatments, were acquired; and a third one was provided by the Bromatology Department of the Faculty of Pharmacy from the Porto University. Several ionic liquids, selected from those available and recommended by relevant bibliography, were tested in preliminary trials for evaluation of enzymatic activity by ultraviolet–visible (UV-Vis) spectroscopy and high-performance liquid chromatography (HPLC); from these two ionic liquids were selected. Finally seven enzyme formulations were prepared with the two ionic liquids or gel and the three enzymes. The preliminary tests also helped to establish the cleaning procedures.

b) ***Complete surface characterization*** Mock-up samples were prepared as documented reconstructions of oil and tempera painting and gilding, with four proteinaceous varnishes (egg white, isinglass, animal glue and casein). The second step was a complete surface characterization of these samples by different analytical microscopy techniques, namely Stereomicroscopy (SM), Optical Microscopy (OM), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM); and also Colorimetry (CIE L*a*b*), in order to describe the surface and conservation state before the treatment.

c) ***Experimental cleaning tests*** on the mock up samples with the enzyme formulations prepared with both ionic liquids and, for reference, with gel.

d) ***Evaluation of the effectiveness assessment***, by surface characterization analysis with the techniques mentioned in step b); in addition, residue identification measurements were done with Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS).

1.1. Concepts review:

The documented reconstructions (mock-up samples) are prepared duplicating the stratigraphy (structure and materials) of artistic techniques as described in relevant literature, such as historical art treatises or manuals and other sources[1]. For this project oil (OG) and egg tempera (EG) painting and gilding (GG) were prepared following the structure represented in Figure 1.1.

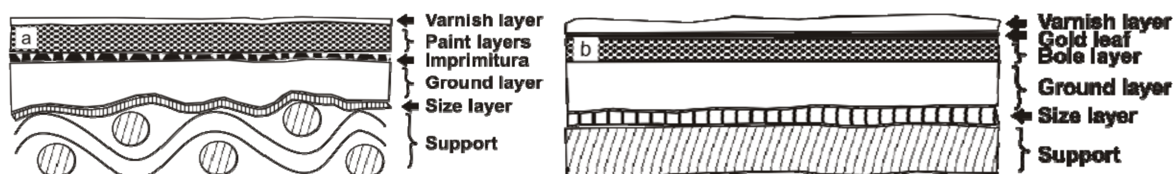

Figure 1.1: Examples of documented reconstructions as it would be observed in a cross-section, with the identification of the different types of layers; a) painting on canvas support, b) gilding on wood support.

The varnish, usually the top layer of such composites, provides protection of the surface against foreign degradation elements, and also enhances optical properties of the polychrome surface such as glow and color saturation. So it is expected of the material to be the more transparent, elastic and unaltered as possible with time[2] [1]. Different materials have been used through time for the preparations of varnishes: natural resins, proteins, even oil and more recently synthetic materials.

Painting materials and particularly varnishes suffer deterioration due to ageing (natural material degradation) enhanced by external factors (e.g.: light, humidity, microbial degradation, deposition of pollutants and others). When the deterioration reaches the point where the varnish no longer fulfills its use i.e., does not protect the surface and/or alters its perception, needs to be removed

[1] There are several ancient treatises and more recent studies and compilations on the artistic materials and techniques, here more than a specific replica the intended was a generic painting structure.

[2] Even though ancient art treatises advice the use of clear varnishes it is known that artists used colored varnishes and would add pigments and other material to achieve special effects/alter its properties. But this issue will not be addressed at this time.

and replaced. Here it will be addressed the problem of the removal of four protein varnishes as they become highly insoluble with time and can hardly be removed. The varnishes studied in this project are: egg white (EW), animal glue (CA), isinglass (fish glue, CP) and casein (Ca).

The preparation of these proteins as filmogenic materials to be applied as varnish is described in many ancient recipes and art treatises [3, 4].

Cennino Cennini[3] advises the use of animal glue as a pre-varnish for the pigment terre-verte; and egg white[4] as "a sort of varnish" to make the work seem "stronger"[5]; or for wood and stone sculptures to be applied over the skin areas of hands and face [3]. Other authors advise the use of egg white as a temporary protection varnish, Palomino[6] in the eighteen century already focusing on the conservation of paintings, points out the advantage of this varnish as a sacrificial layer because it can easily be removed with sponge and water when its dirty and again replaced with new one [4]. Other protein varnishes are found in painting surfaces as original varnish or in later additions such as in conservation treatments. For instance on tempera paintings, sometimes done without varnish layer, fish glue varnish was used as a "reviving" technique popularized by De Mayerne[7] in the seventeen century; according to him the tempera paintings would, "look as if they were painted in oil" [1].

Proteins are bio-functional[8] polymeric chains composed of amino acids, amphoteric compounds[9], containing both functional carboxylic acid group (-COOH) and basic amine (-NH_2) and a side chain (-R) that is specific to each amino acid (Figure 1.2a) [5].

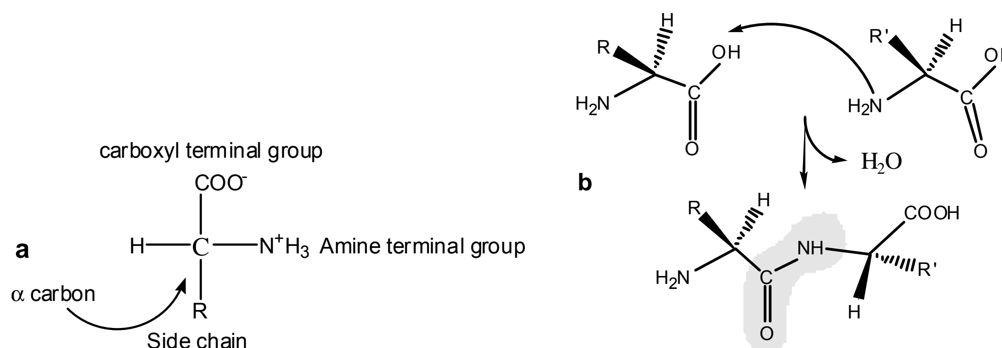

Figure 1.2: a) General structure of an amino acid; b) Peptide bond between amino acids.

There are twenty naturally occurring amino acids and a protein can have chains in the order of the thousands amino acids linked together by –NHCO– the peptide bond (Figure 1.2b). The number of amino acids and the order in which they are aligned in the chain identifies and gives specific structure and function to the proteins. Amino acids aligned themselves in chains, the primary

[3] Cennino d'Andrea Cennini (c. 1370 – c. 1440), Italian painter and author of the "Il libro dell'arte".
[4] Also historically referred as glair.
[5] These were pre-varnishes as the final varnish should be applied only after one year of the painting execution [1].
[6] Acislo Antonio Palomino de Castro y Velasco (1653 – 1726), Spanish painter and author of the "El Museo Pictórico y Escala Óptica".
[7] Théodore Turquet de Mayerne (1573 – c.1655) was a Swiss physician and author.
[8] Compounds that participate in reactions inside living cell, or have structural and mechanical function in the living tissues.
[9] Capable of reacting chemically either as an acid or as a base substance.

structure of the protein. A spatial arrangement of the chain as helixes, sheets and turns allows for interactions between non sequenced amino acids stabilized by hydrogen bonds (secondary structure). The third order of structure for the proteins is the functional shape of the secondary structure, polypeptides within the protein folds in a unique three-dimensional way [6]. With time denaturation occurs, i.e. unfolding of the natural three-dimensional configuration of the protein chain, this is what makes the surface of the protein varnish to become more opaque, hardened and brittle and the protein less soluble. Considerable more stable to oxidation processes than other materials of the painted surface, proteins are however very susceptible to humidity, being this the main factor for their deterioration [7]. The other important factor is related with their biological degradation as proteins are a food source to microorganisms.

Common proteins used as artistic and restoration materials, and considered in this study, are:

Egg white, contains about 87% water, 2% of other materials and 12% of protein mixture, of the latter about 65% is albumin, a class of proteins that are present both in egg white and egg yolk and other materials. The alteration in the egg white varnish properties was already recognized in the nineteen century, and its use inadvisable in conservation treatments since no satisfying effectiveness of the removal of these varnishes was achieved by conventional methods [8].

Gelatins are the group of proteins found in biological tissues and main constituent of animal and fish glues, the purity of the glue is related with the quality and amount of other materials. The fish and animal glue are differentiated by the specific amino acid proportions; in general the fish glues are also more pure which results in a clearer and stable glue.

Caseins are proteins obtained from precipitation of milk and may contain different impurities such us lactose, acids and butter. The casein is differentiated from the other proteins because it belongs to the phosphoproteins group, which are proteins that are physically bond to substances containing phosphoric acid [6, 9]. For this they are insoluble in water and more susceptible to deterioration by oxidation processes. The conservation practice reported some difficulty in removing the proteinaceous materials [7, 8,] and therefore, required the use of more aggressive treatments, usually toxic for the conservator, that endangered the other layers. The ideal was to find a specific treatment to proteins that did not affect the rest of the materials. This would be only achieved with the introduction of enzymes in the cleaning process [1, 2] a major breakthrough as enzymes are catalysts very specific to substrates and can be used in moderate conditions. Early works of the mid-twentieth century already reports the use of enzymes [10] albeit with doubtful approaches, and in the late 1960s Banks [11] refers the use of collagenase[10], to remove glues, with some success and conscious of the needed cares and possibilities

Enzymes are themselves proteins with specific amino acids sequences and unique three-dimensional configuration that allows the interactions with very specific materials in certain active areas, that have been described many times as a lock and key system [6, 12] Figure 1.3.

[10] The enzymes are named by adding the suffix -ase to the end of the that is to be converted to the desired product. e.g. peptidase that converts peptides to smaller molecules such as amino acids.

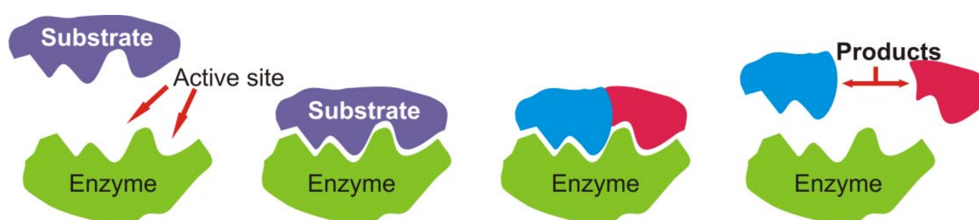

Figure 1.3 – Diagram of the "lock and key" hypothesis of enzyme action.

Enzymes of interest to conservators are those that facilitate degradation processes acting as catalyst[11] in hydrolytic reactions[12], in this case the hydrolysis of proteinaceous material specifically known as proteases or peptidases. In the international enzyme classifications it is the group E.C.3.4.[13], where E.C. stands for enzyme classification; 3. for hydrolysis and 4. for the affected substrate: peptides or proteins.

As early as 1977 Hatton [13] proposes the use of the enzymes as cleaning agent in cellulose based gel for the first time. More recently Wolbers [14] popularized the use of aqueous cleaning systems for conservation purpose, as a viable, less toxic alternative to common free organic solvents, including the preparation of enzyme in gel mixtures. Another author that disseminated the use of enzymes in conservation treatments was Cremonesi [15].

But there are situations where these aqueous systems do not give the best results. The works mentioned [14, 15] show recipes of complex mixtures with over 4 components, this high number makes difficult to fully predict the interaction of the mixture, its interaction with the treated surface and possible long term deterioration problems.

The use of organic solvents that are compatible with the enzymes is not advised as they are usually highly toxic and strong solvents in themselves establishing interactions with the other materials of the painted surface. A possible alternative could be related with the use of "green solvents", mainly based on ionic liquids.

Ionic liquids (ILs) can be defined as organic salts with a melting point below 100ºC, or more interesting as liquids at room temperature (room temperature ionic liquids; RTIL). In the last 20 years the interest from both academia and industry has gained significant impact mainly due to their environmental friendly characteristics as green[14] alternative for traditional volatile organic solvents (VOCs) and because of their wide range of physicochemical properties. There are a large number of organic cations and anions that can be combined to form different ionic liquids, and this is one of the most important features of these compounds.

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[11] A catalyst is a substance that without being consumed, or change the reaction equilibrium, accelerates its process and/or allows it to occur in more desirable conditions, such as lower temperature.

[12] Includes lipase, amylase and peptidase enzymes that catalyses the hydrolytic breakdown of lipids, sugars and proteins respectively.

[13] Enzyme classification as recommended by the International Union of Biochemistry and Molecular Biology and available at http://www.chem.qmul.ac.uk/iubmb/.

[14] The negligible vapor pressure is one of the main reasons to distinguish ionic liquids as green solvents [16], as possible release to the environment can be controlled, allows safer handling for the user and do not have the risk of fire or explosions. The green solvent status of the ionic liquids has been discussed as some of the compounds are proved to have some toxicity and the means to obtain them are sometimes also toxic [17-19]. Despite this, new productions methods are being developed, and the toxic effect could be an advantageous characteristic for controlling of microbiologic contaminations.

ILs have been also described with some peculiar properties such as their high conductivity, variable range of density and viscosity values, tunable polarity and solubility as well as their high thermal and chemical stability. Overall, the number of possible ionic liquids is estimated around the ten to the eighteenth (10^{18}), whereas the number of traditional solvents widely used in industry accounts for a few hundred.

Ionic liquids are also called “designer solvents” because, by changing cation-anion combinations [16], they can be adjusted to specific situations or requirements such as stability, solubility, viscosity, improvement of enzyme cleaning effectiveness, surface compatibility, and lower toxicity behavior [20]. The previous work of Pacheco [21] reports on the use of IL as a potential new material for heritage conservation treatments; it is focused on the use of the IL as alternative solvents and cleaning agents for resinous material, natural and synthetic, with some encouraging results.

The use of enzymes in IL media it is not an old science, and is still an unstudied area in conservation science with a large number of possibilities. The first report of the use of IL for biocatalysis with enzymes has just a little more than 10 years [22] but, rapidly started to be a major interest area of research. Kragl & coworkers [23] and Roosen & coworkers [24] show the production of articles registered on the web of science on the subject of biocatalysis using ILs in the past years. Fred van Rantwijk & coworkers [25] and Moniruzzaman & coworkers [26] reported reviews about biocatalysis using ionic liquids.

In the present work, the ILs will be addressed from the point of view of compatibility in restoration treatments when combined with enzymes, as an alternative to traditional gel formulation.

1.2. Surface characterization and treatment monitoring

The characterization of the materials present in a cultural heritage object is the first step before any conservation or restoration procedure [1, 27]. In a painted surface besides the rheological and other physical-chemical properties of paint materials, the applications method, number of paint and varnish layers and their thickness are important issues to consider.

To fully understand the object’s surface characteristics and its state of conservation analytical tools are needed and in some cases sampling of the objects is required. The sampling is always a problem to consider as it means the loss of material, even if the information retrieved is considered most valuable and the sample is minute in size. But in the case of surface treatments such as the removal of varnish layers this problem can be overcome by applying noninvasive and nondestructive surface techniques.

Surface properties of cultural heritage artifacts have been approached in recent years by conservation and material scientists and physicists who applied non-invasive analytical tools to describe the behavior of the polychrome and gilding surfaces, according to the optical, mechanical, physical and chemical characteristics [28-31]. The analytical data obtained from a multiscale and multiple technique approach is useful for the practitioner (conservator, curator or art historian) to assess the composition, conservation state and eventually provenance and authenticity, as well as

to predict future changes of the surface with regard to the choice of best preservation conditions or of the right restoration treatment (for example: cleaning, consolidation or varnishing) [5, 29, 32].

The techniques chosen herein have been previously reported for their usefulness: stereomicroscopy (SM), optical microscopy (OM), scanning electron microscopy (SEM-EDX), atomic force microscopy (AFM), colorimetry (CIE L*a*b*) [28, 29, 31, 33-36]. AFM was recently reported in researches relating to the study of the surfaces of gilding layers and the nano-scale effectiveness assessment of cleaning treatments [31, 37-39]. This technique has a great potential for monitoring cleaning interventions on paintings or polychromed surfaces of works of art, and could bring an important contribution in assessing the presence of residues of the cleaning formulation at a nano-scale.

It will be demonstrated that the use and combination of different surface analytical techniques returns an extensive and complete set of information that fully characterize the surface at different scales (from macro- through micro- to nano-scale) in order to assist the proposed treatment.

The evaluation of the formulations behavior, specifically the activity of the enzymes, was monitored before and after its use in the cleaning process. The analytical techniques used are those commonly reported in proteomic science: UV-Vis spectroscopy [40], HPLC [41] and MALDI-TOF-MS [42, 43].

2. Materials and methods

2.1. Materials

Most of the reagents used in this work were provided by Sigma-Aldrich. Acetonitrile and trifluoroacetic acid were supplied by Merck. Antares supplied two enzymes as commercial ready to use gel formulations (G+E1, pepsin; and G+E2, from Aspergillus sojae), also Tylose powder, White Spirit, Shelsol, cotton and proteinaceous glues. The sequencing grade modified trypsin, was provided by Promega; The ZipTips, pipette tips for sample preparation were supplied by Millipore. Ionic liquids were provided by Solchemar; embedding polyester resin for cross-sections is distributed by MR Dinis dos Santos.

2.2. Mock up samples from documented reconstructions

To better understand the possible use and effectiveness of the formulations suggested here, tests were done on documented reconstructions of polychromed surfaces prepared during 2009 and March of 2011[15].

Mock-up samples of tempera (EG) and oil (OG) painting on canvas and wood supports and also traditional water gilding (GG) over bole ground were replicated in accordance with historical and artistic sources [3, 44, 45] following the general scheme from figure 1.

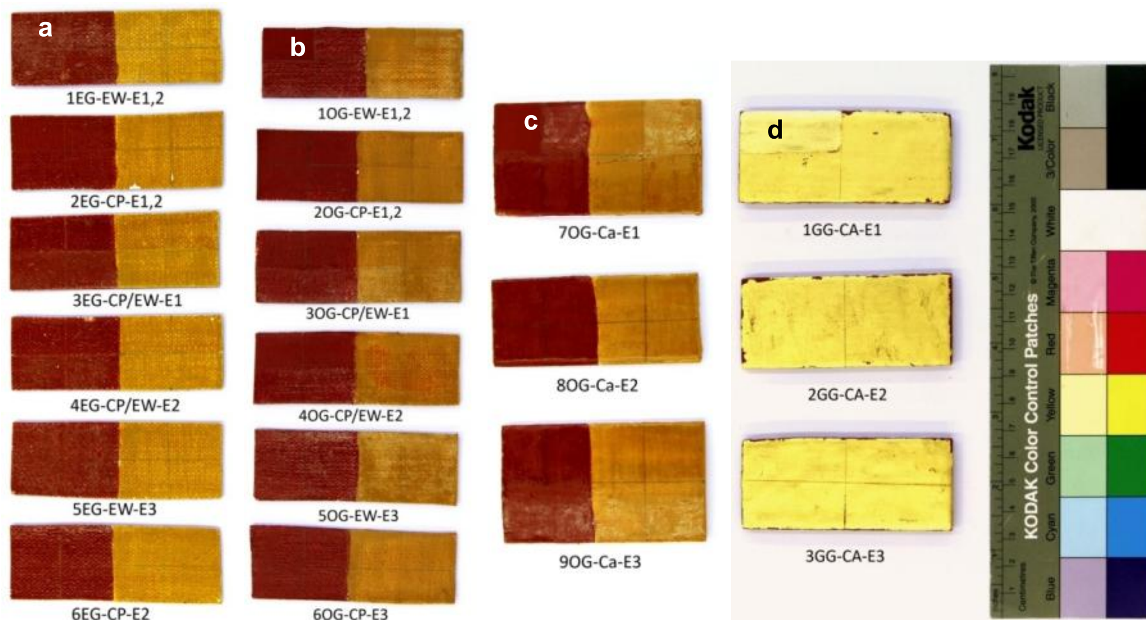

Figure 2.1 – Overall view of the initial condition of the samples used in this project, Grouped vertically: a) Tempera painting with egg white and fish glue varnishes; b) Oil painting with egg white and fish glue varnishes; c) Oil painting with casein varnish; d) Gilding with animal glue varnish; a & b prepared on canvas mounted on glass slides and c & d prepared on wood.

[15] Except for the gilded samples all of the others were previously prepared. The choice of using these samples was their availability and a way to have a less recent support as it would not be done any sort of artificial ageing at this point.

The Figure 2.1 shows the initial aspect of the prepared mock up samples. For each technique a type of protein vrnish – egg white (EW), fish glue (CP), animal glue (CA) and casein (Ca) – was selected and applied at the end of 2010 and in April 2011[16]. Except for the gilded surfaces all samples were duplicated in two different colors of iron oxide based pigments – yellow ochre and sinopia – to control possible darkening or blanching[17] of the surface after treatment. A small area of the surface in each artistic technique was preserved in order to be tested with every formula (44 areas for testing the four new IL+Enzyme formulations and other 33 for reference gel formulations); also other areas were left without varnish to serve as reference surfaces.

Although mock-up samples are not a real situation of an ancient paint or polychrome, their use allows the test of the cleaning formulations in a setting where multiple factors influence the removal process but, the precise composition is known. As pointed out by Phenix & Sutherland [46] this can be accepted as a stepping stone and a good compromise when developing new methods.

Preliminary cleaning tests were developed to establish the cleaning protocol and IL selection for this, thin layers of the same four varnishes were prepared over microscope glass slides.

2.3. Cleaning formulations

Two commercially available enzymes, used in conservation for the removal of proteinaceous materials, were acquired: an acid enzyme (E1), pepsin (appendix I) a protease of the pig's stomach mucosa and an alkaline enzyme (E2) from *Aspergillus sojae* (appendix II). A third enzyme (E3) is an extract from yeast [40] provided by the Bromatology department of the Faculty of Pharmacy from the University of Porto, to be tested as a new alternative enzymatic agent, active at pH 7, and potentially less expensive. Because E3 has not only one active agent but, is an extract with three different enzymes [40] it is expected a potentially more effective performance with a higher range of catalytic interactions. However, because is not in a purified form at this moment; the enzyme activity may vary from batch to batch.

For E1 and E2 the supplier provides also ready-to-use cellulose based buffered gel to the specific conditions of each enzyme. For the third enzyme however a gel was prepared following Cremonesi [15] instructions.[18] The enzymes were chosen according to optimal performance in the different pH ranges. Depending on the desired work conditions in a restoration treatment a basic or an alkaline or a neutral enzyme can thus be selected.

The *imidazolium* based ILs (figure) are by far the most common ILs used in enzymatic reactions [20, 24, 47-49]. But the ILs general properties, although follow some pattern, varies substantially with specific anion- cation- combinations [17, 49, 50]. Park [20] also makes notice that although polar solvents inactivate the enzymes, ionic liquids does not.

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[16] We would like to acknowledge at this point the collaboration of Michaela Crhová who helped with the preparation of the samples.

[17] Blanching – A local, opaque, whitish discoloration on the surface of a painting. Blanching occurs because of a loss of or an alteration in the binding medium in an oil film, or because of the partial dissolution of a varnish. In accordance with the definition at the Canadian Conservation Institute - CCI Notes 10/11 [http://www.cci-icc.gc.ca/publications/notes/10-11-eng.aspx].

[18] Paolo Cremonesi. L'uso degli enzimi nella pulitura di opera policrome. Padova : Il Prato Editore, 2001. page 48.

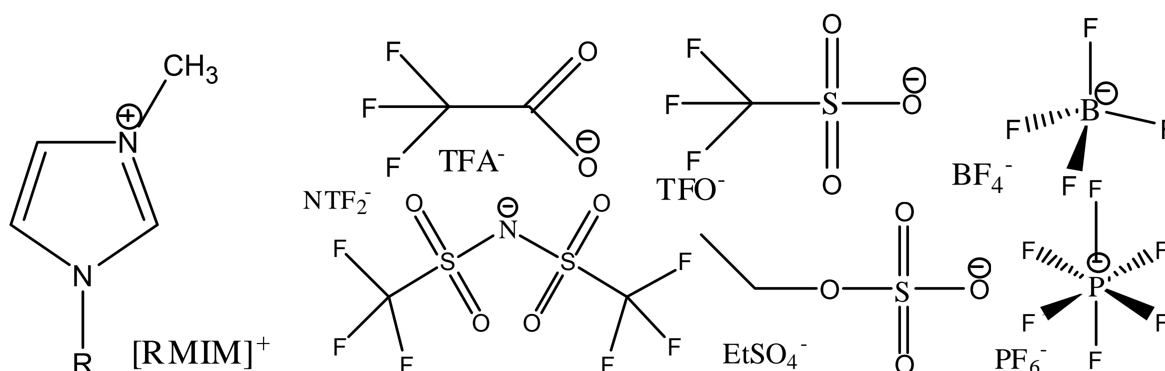

Figure 2.2: methylimidazolium cation [R-MIM]$^+$ and some examples of anions in ionic liquids: Trifluoroacetate [TFA]$^-$, Trifluoromethanesulfonate [TFO]$^-$, Tetrafluoroborate [BF_4]$^-$, bis(trifluoromethylsulfonyl)imide [NTF_2]$^-$, Ethylsulfate [$EtSO_4$]$^-$, Hexafluorophosphate [PF_6]$^-$.

In order to select among the numerous IL possibilities preliminary tests for enzyme activity and cleaning capability were done with ten ionic liquids, Figure 2.1 shows common possible anions. The different ILs were chosen in accordance with literature review for bio-compatibility, availability at the lab and predicted compatibility with the painted surfaces [21]. From the ten ionic liquids two with the best results in the preliminary tests were chosen and labeled as IL1 and IL2 (appendix III): 1-butyl-3-methylimidazolium tetrafluoroborate (IL1 – [BMIM][BF_4]) and 1-ethyl-3-methylimidazolium ethylsulfate (IL2 – [EMIM][$EtSO_4$]).

Seven formulations were prepared: four innovative formulations using two commercial proteases (E1, E2) and two ionic liquids (IL1, IL2) and three reference formulations with the three enzymes (E1, E2 and E3) in the traditional cellulose gel mixture (table 2.1).

Table 2.1 – Final formulations composition summary.

Formulation		Enzyme	Solvent		pH
E1+G	Enzyme + solvent (≅ 1g / 100ml)	Pepsin	3% Tylose / 7pH phosphate buffer	Commercial cellulose based gel	5
E2+G		from Aspergillus sojae		Commercial cellulose based gel	8.4
E3+G		Yeast extract		Prepared cellulose based gel	7
E1+IL1		Pepsin	20% water v/v	[BMIM] [BF_4]	-
E2+IL1		from Aspergillus sojae		[BMIM] [BF_4]	-
E1+IL2		Pepsin		[EMIM] [$EtSO_4$]	-
E2+IL2		from Aspergillus sojae		[EMIM] [$EtSO_4$]	-

E1 = acidic protease; E2 = basic protease; E3 = yeast protease (Porto);
G =gel; IL1 = [BMIM] [BF_4]; IL2 = [EMIM] [$EtSO_4$].

2.4. Cleaning protocol

For the new formulations an optimized cleaning protocol needed to be developed. The cleaning process is in itself a complex issue. There are a great number of variables to be considered in particular: type of substrate that is to be removed, its nature and state of conservation. The materials present in the formulation, including their proportions and possible interactions; the needed conditions to activate the formulation, mixing, temperature, pH, to name a few; and the cleaning, namely the manner and time of application, as well as the removal and clearance steps.

For an innovative method such as the one proposed here it was necessary to simplify whenever possible. Basically the protocol would follow the indications of use for the enzymes with gel formulations as described by Wolbers [14], Cremonesi [15] and in the suppliers' material data sheet (appendix I and II). The enzyme is added to the solvent in accordance with the supplier indications in a proportion of approximately 1g of the enzyme to 100ml of the proper gel (buffered at the ideal pH for each enzyme) and the same proportions were used for the ionic liquids. The mixture, after stirring, is left to stand for 20 minutes in a double-boiler set at 37°C, this step allows for some of the air introduced during the mixing to be released and to activate the enzyme. The mixture is then applied with a soft brush and let it be for different amounts of time. As this step depends upon the type of material present, the preliminary tests helped to establish the contact time for each formulation and varnish. Following, the cleaning material is removed with dry cotton swabs and in sequence, allow drying in between, two damp cotton swabs and one with white spirit[19] was used. This is the clearance phase, the first swabs are to remove all residual products and cleaning material and the last one is to inactivate possible enzyme residues on the surface, as recommended by the supplier instructions (appendix I and II) and bibliography [14, 15].

Following this cleaning protocol, different times of contact with the varnish surface (from 30 seconds to 35 minutes) were considered and tested for each enzyme + gel formulation and each protein (egg white, isinglass, animal glue and casein) on the prepared glass slides. When necessary, in order to avoid drying of the gel on the surface, the area tested was covered with a transparent polyester film.

With the application times established another round of test were done, then using different ionic liquids to choose the ones that presented, at least, similar behavior as the gel formulation, i e, that needed the same amount of time to obtain observed similar cleaning efficiency.

[19] White-spirit is a petroleum base distillate also called mineral spirit.

3. Analytical protocol and Instruments:

3.1. Enzymatic activity measurements

For the measurements of enzymatic activity the Kunitz method was followed for liquid solutions as described by Whitaker [51] and also adapted for gel solutions. Initially E1 and E2 were tested, as recommended, in buffered solutions. Stock solutions of acetate buffer (pH=5) and Tris HCL buffer (pH=8.4) were prepared as described by Gomori [52]. The enzymatic activity is obtained by quantifying the products, specifically the aromatic amino acids, in solution after a certain amount of time with UV absorbance spectroscopy and comparing these values to reference blank solutions.

It was verified that the enzyme activity protocol can be used in a similar approach with the gel formulations for the three enzymes; the same was done for the ILs with E1 and E2. This test excluded all the ILs that showed enzyme inactivation, i.e., not compatible for these enzymes.

Two UV-Vis spectrometers (Shimadzu UV-1800 UV-VIS Spectrophotometer and Varian Cary 100 Bio UV-Visible Spectrophotometer) were used for the absorption studies.

As the specific enzyme activity was not the purpose of these tests no comparison was done between the obtained values and known standards[20].

3.2. High-performance liquid chromatography (HPLC)

The HPLC at the Bromatology Department of the Faculty of Pharmacy from the Porto University was used to study some of the formulations for enzymatic activity and formulations shelf-life; these tests were done following the protocol as described by Mota & coworkers [41]. The enzymatic activity was followed by HPLC spectra with the indication of peptide separation different from the blank samples and by comparison of the peak areas. For measuring shelf-life the test was done at time intervals of the same preparation and peaks area was compared for interpretation of the possible loss of enzyme activity. Due to work constrictions HPLC was not used extensively, on all samples.

3.3. Cross-sections and staining test

Although in the present work the varnishes in the samples are well known it is suggested the preparation of cross-sections for the identification of the painting structures and generic qualitative protein identification/mapping by means of staining tests[21]. Cross-sections were prepared from minute fragments from a set of representative samples with and without varnish and before and after treatment. These fragments are imbedded in a polyester resin, followed by polishing with successive finer grain of micro-mesh paper. Images were obtained by optical microscopy (OM) technique with visible and fluorescent-light sources. For the identification of the protein material

[20] The specific enzyme activity for E1 and E2 is included in the product data sheet in appendixes I and II. For E3 further studies are to be carried out at University of Porto (Faculty of Pharmacy), but reference values are described by Teixeira [40].

[21] In real cultural objects this can be done by retrieving samples from lacunar areas, borders or material loosen during the deterioration process if the origin can be identified on the object surface.

and its location in the surface structure a spot test was done using Sypro® Ruby Protein Stain dye, this identifies and maps the distribution of the proteinaceous material by an orange color observed under green emission of fluorescence[22].

3.4. Surface characterization, assessment and control of the cleaning process

To monitor and assess effectiveness of the treatment an analytical protocol was established and used before and after the removal of the protein-based varnish. This protocol is based on the complementary use of different microscopy techniques, at a multi-scale, non-invasive and with little or no sample preparation: the stereomicroscope (SM) at 10x magnifications, then the optical microscope (OM) 50x to 200x magnifications, and scanning electron microscope (SEM) on uncoated samples allowing good resolution imaging up to 3000x magnification. The atomic force microscopy (AFM) was introduced at an image size of 5 micrometers.
Also measurements of colorimetric values (CIE L*a*b*) were performed. The comparison of the images and values obtained before and after treatment of the same surface allowed the surface characterization, but also, to gather information about cleaning effectiveness, possible residues, and other features in useful time.

3.4.1. Stereomicroscopy and Optical Microscopy

An Olympus stereomicroscope system SZX12, mounted on an extendable arm SZ-STU2 with a digital camera DP-12 and an independent light source HighLight 3100 was used to record the sample areas at 10x, 32x and 90x magnifications. Then Axioplan Zeiss 2 imaging binocular microscope (at 50x, 100x and 200x), with both visible (dark field) and fluorescent radiation, coupled to a Nikon DXM1200F digital camera was used to image the surfaces, before and after the removal of the varnish layers. The blocks of filters used for observing the fluorescence were: BP 300-400, FT 395 and LP 420 (filter 8); BP 450-490, FT 510 and LP 515 (filter 6).

3.4.2. Scanning Electron Microscopy

SEM images were acquired from backscattered electrons and low vacuum secondary electrons with an Auriga CrossBeam Workstation (SEM-FIB) operating at 1-5 kV, with a typical working distance (WD) 4.5-9.6 mm, aperture size 30µm, magnifications: from 200x up to 3000x. Due to work constrictions SEM was not used extensively, on all samples.
With similar operating conditions Electron Scanning Microscopy with Energy Dispersive X-ray Spectroscopy (SEM/EDX), was also conducted to selected samples for the purpose image processing and elemental mapping of the surfaces after treatment.

[22] Mean excitation wavelength: 302, 470nm / Mean emission wavelength (fluorescence) 610 nm. (From product data sheet available at Sigma-Aldrich: http://www.sigmaaldrich.com/.

3.4.3. Atomic Force Microscopy

MFP-3D Asylum instrument was used in non contact imaging mode (AC mode) using Si probes Tap300Al from Budget Sensors (cantilevers with 300 KHz resonance frequency and 0.2 N/m spring constant). The scan size was 5 μm^2 with a resolution of 512 by 512 lines. Before AFM measurements, the samples were cleaned with a soft N_2 flow to remove free dust contaminations. The images record the height mapping, this results in 2D images, that throughout this work are, shown in a gray scale, where brighter areas represent higher areas, from these 3D images are easily obtained using Igor Pro 6.2 Software. Images were recorded at 3 different locations, for each investigated area, in order to verify the repeatability and statistical significance of the observed features. Measurements of surface roughness, the average value of the three scanned areas, were obtained with the software. Other images were also recorded: amplitude images, which registered the tip amplitude changes at the given frequency, and phase images, which measure the dissipative forces between the tip and the samples.
In a non contact mode, the amplitude is set at constant oscillation amplitude maintained by feedback loop. When there is a height variation, in order to maintain the average tip-to-sample distance the oscillation amplitude is adjusted. The amplitude images are the result of the amplitude oscillation adjustments of the cantilever.
The phase is a qualitative measurement of the interaction forces between the tip and the sample. Even if, no amplitude changes are monitored, the phase can show regions where the sample tends to interact with the tip in a non-conservative regime: when it shows high contrast between the white areas (positive) due to attractive interactions and dark areas (negative) due to repulsive interactions. This interaction with the tip can be particularly relevant indicating, for example, the presence of different materials or just the same material with different molecular arrangement (amorphous/crystalline), for example. Notice that these are false colors and can be adjusted to different hue/tones with different software settings, in this project it was set to a gray scale.

3.4.4. Colorimetry

For the colorimetric measurements a Datacolor International Microflash colorimeter, with a Xe lamp was used. The measuring area has a diameter of 18mm (22mm illuminated area), the geometry was 0°/0° and the light source D65.
The colorimetric characterization for each investigated area was performed through the measurement of L*a*b* parameters (using CIE L*a*b* 1976 system) [34]. Calibration was carried out before each set of measurements using a pure white standard (calibration values: L= 96.43, a=-0.33, b= 1.27). Understanding possible variations of each component is important: L* value measures “lightness” from 0 to 100 corresponding to a variation from black to white. If variation of L* (ΔL*) is positive this means that the sample is lighter after the treatment than before, and when the value is negative indicates an increase in darkness. a* values ranging from positive to negative represent color changes from magenta to green, while b* variation indicates changes from yellow to blue, which means an actual change in color. For each value three sets of data were acquired

and the average values were calculated together with the standard deviation from the mean. Delta E was finally calculated from the L*, a*, b* values before and after the removal of the coating layer and compared with reference samples.

3.5. Other techniques - MALDI-TOF Mass Spectrometry

Following the cleaning procedures each third cotton swab was analyzed for the presence of peptides, by MALDI-TOF mass spectrometry in accordance with the protocol described by Kučková [42]. This allowed for the identification of peptides, demonstrating and comparing the activity of the enzymes in the different formulations, and some interpretation could be made with respect to presence of residues from the varnish material.

4. Results

Table 4.1 shows the results summary of the preliminary tests done for the two commercial enzymes (E1 and E2) and E3 in the gel formulation to establish contact time.

From the available ILs, ten structures based on *imidazolium* cation family were selected and tested in the same way. Appendix III summarizes the main characteristics of the ILs and preliminary tests observations.

Table 4.1 – Results of the removal tests obtained for different periods of time for the four proteinaceous varnishes with the three enzyme+gel formulations.

Cleaning formulation	Effectiveness/time of application			
	EW	CP	CA	Ca
E1+gel	++/3 min	++/3-9 min	++/9 min	+/35 min
(pH = 5)	+/30 seconds	+/30 seconds	-/30 seconds	- -/30 seconds
E2+gel	++/30 seconds	++/30 seconds	++/3-9 min	++/27 min
(pH = 8.4)	-	-	+/30 seconds	- -/30 seconds
E3+gel	++/3 min	++/3 min	++/9 min	+/35 min
(pH = 7)	+/30 seconds	+/30 seconds	-/30 seconds	- -/30 seconds

Legend:
++ Easy removal (the varnish layer is easily removed with the removal of excess formula);
+ **Removal** (after gel application the varnish is swollen and this allow a mechanical removal);
- **Difficult removal** (after gel application the varnish is swollen and this allows a mechanical removal, leaving residues);
- - **No removal** (the varnish could not be visibly removed).

Simultaneously, enzyme activity tests with the UV spectrometer technique[23] were done for the formulations, once more using enzymes E1 and E2 as reference, first just in buffered aqueous solution[24] (table 4.2), then in gel and finally with the most promising ILs after preliminary cleaning.

Table 4.2 – UV Spectrometry absorbance for enzyme activity:
Measurements for casein hydrolysis in buffered aqueous solutions.

Test ID	Protein	pH	Enzyme	Abs. at 280 nm
B5E1	Casein solution 50mg/ml	pH5 Acetate Buffer	E1	0.598
B8E2		pH8,4 Tris HCL Buffer	E2	1.33
B7E3		pH7 Phosphate Buffer	E3	0.530

Procedure notes: Adaptation of Whitaker's procedures [51], according with the work done by Teixeira [41]

Absorbance at 280nm represents one of the standard measurements in order to indicate enzyme activity, the results were obtained for the buffer aqueous solutions relates with concentration of aromatic amino acids present in solution which can be converted to enzyme activity units by

[23] Procedure notes: 3 tubes, one blank and 2 for testing enzymatic activity; the catalysis occurred during 30min in a double-boiler at 37ºC; after that it was added 1ml of triichloroacetic acid (TCA) to stop the enzymatic activity (to the blank tube, 1ml TCA was added before the addition of the enzyme solution); waited 30min; after centrifuge at 10000xg for 10min the supernatant was measure for absorption considering the absorption of the blank as zero [51].
[24] E3 was also analyzed at this point, with the same conditions.

comparison with standard solutions with known concentrations[25], this however was not the objective at this point. Firstly because E1 and E2 have known activity values from the supplier[26], and for E3, as it is an extract that varies from batch to batch[27]; and secondly because when changing the conditions, changing the solvent, the results are no longer comparable. However this is important in order to understand if the enzyme is active in the solution (when the conditions are similar the results can be compared).

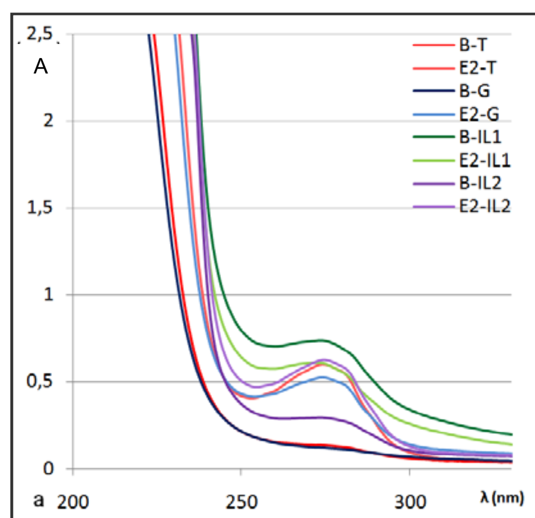

Figure 4.1 - UV spectrometry compared results with reference blank solutions (B) for the alkaline enzyme (E2) with every formulation buffer (T), gel (G) and ionic liquids (IL1 & IL2).

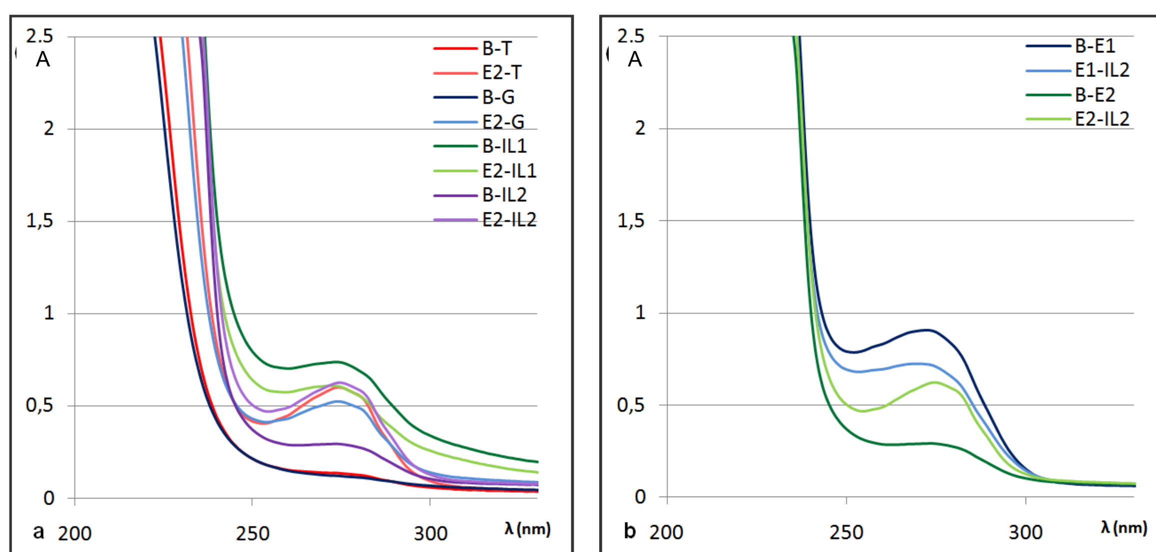

Figure 4.2 – UV spectrometry compared results: a) for the alkaline enzyme (E2) with every formulation buffer (T), gel (G) and ionic liquids (IL1 & IL2); b) tests for IL2 with enzymes E1 & E2.

25 “One unit of is defined as the amount of enzyme which yields absorbance of 1mmole of tyrosine per minute under the conditions used” [51]

26 See appendix 1 and 2 for material characteristics data sheets.

27 Complete study of its characteristics is being done at Bromatology Department of the Porto University (Faculty of Pharmacy), and results cannot be presented here.

Enzymes need a minimum amount of available water to be active [20, 53-55]. Additional UV absorbance tests were performed at different water/IL (v/v) proportions in order to identify the minimum amount of water required, that was 20%, this high amount could be related to the water affinity of the IL itself, leaving less available water for the enzymes [53, 54].
Figure 4.1 shows the complete set of results for E2; it was detected that in the IL tests the blank solution (B), measurements before enzyme catalysis show some absorbance. This observation could be due to the fact that ILs have higher viscosity and for its characteristics able to retain some amount of protein after centrifugation.
Figure 4.2 shows results for IL2 with the enzymes E1 and E2. No control of pH was done because during the preliminary cleaning tests both enzymes were active with both selected ILs. However, for the enzyme activity tests an associated problem was identified related with the preparation of protein solution with casein in alkaline medium. For the IL and E1 with affinity to acid medium, the absorption curve shows anomalies, as the blank solution (B) presented higher absorbance (Figure 4.2).
From these tests, the precise compositions of the formulations to be tested were set and also established the application times for each protein: approximately 30seconds to 1minute for fish glue and egg white varnish; 3minutes for animal glue varnish and 30minutes for casein varnish. In total seven formulations were prepared to be experimented at 1g/100ml (enzyme/solution), and for E3 1ml/100ml (extract/solution); for the IL, the formulation has 20% v/v of water content: IL1E1, IL1E2, IL2E1, IL2E2, GE1, GE2 and GE3[28] (table 2.1).

For the HPLC the same catalytic protocol[29] was followed as it was done for the UV spectrometer technique and as described by Mota [41]. Due to time restrictions, a complete study was done only for formulation IL1+E2. Figure 4.2 shows that the enzyme is fully active in the IL formulation, although the peptide distribution is different. The elution of peptides is done by their polarity and this is related with size of molecular chain; first to be eluted are the most polar components (the IL is the first component to be separated), and last the proteins[30], as polarity decreases with increasing size and secondary interaction of non-sequenced amino acids by hydrogen bonds [12]. The peak area relates with the quantity of peptides, therefore adding all peak areas gives us the amount of eluted peptides.
When comparing the three groups of peptides, for the different formulations (figure 4.3) it is observed that the cleavage in the IL, for the same amount of time results in predominantly bigger sized peptides.

[28] E1 = acidic protease; E2 = basic protease; E3 = neutral, yeast protease (Porto Faculty of Pharmacy); G =gel; IL1 and 2 = the selected ionic liquids.
[29] See footnote 23.
[30] The casein in protein form does not appear as a single peak since the casein is not a protein but, the designation for a set of proteins.

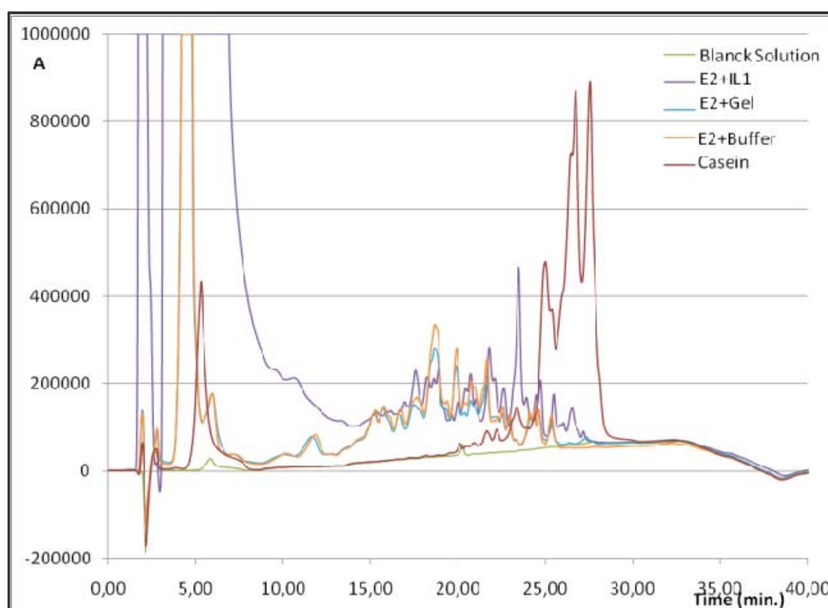

Figure 4.3 – HPLC chromatograms, for casein catalysis with E2 in different solutions: Tris HCL 8.4pH buffer (T), 8.4pH gel (G) and ionic liquid IL1.

For the same formulation a shelf-life test was done, i e, determining how long the formulation would be active after mixing all the ingredients[31]. For gel formulations it is common practice to use the same formula for just one working week, this is because the cellulose based gels are susceptible to contamination by bacterial growth and inactivation of the enzyme with time. This is less likely with IL because of toxicity levels[32], but inactivation can occur naturally, most commonly due to self cleavage processes and possible external contaminations. Tests show (table 5.1) that after one week there was still 85% activity measured in amount of peptides. The anomaly observed, for the second day measurements, is because it was not observed the waiting time[33] required for enzyme activation before adding the protein.

Enzymes need a medium that allows them to have the three-dimensional configuration required to establish an interaction with the substrate. This occurs in the presence of water; as the water molecules interacts with the enzyme maintaining the tertiary structure The protein is breakdown in peptides through the rupture of the peptide bond consuming water, which means that a small amount of water should always be added to the solution. Preliminary cleaning tests and enzyme activity tests showed that at least 20% v/v (water/IL) was needed. Such high amount can be explained because the ILs selected are hydrophilic and can strip the enzymes of the water needed for the reaction to occur and for the maintenance of the tertiary structure [20, 53].

A set of images was acquired for each sample area with all the surface analytical techniques[34], these studies gave us a surface characterization for each artistic technique and materials; appendix IV show representative examples of all the samples, varnished and unvarnished.

[31] Formulation kept in a refrigerated environment, at 4°C.
[32] The toxicity in the imidazolium ionic liquids family is related to the ring in the structure and tends to increase with the increase in the alkyl chain length [17].
[33] After refrigeration it is recommended by the supplier to wait at room temperature at least 15min.
[34] For comparisons purpose these images are presented together in appendix IV.

The SM registers the surface close to the human eye perception, this is important especially for documentation purposes of the surface before treatment. Both SM and OM make record of the surface in terms of color (the perceived hue and tone). The OM goes further registering the general appearance of the varnish layer, aspects such as, glossiness and transparency or smoothness; also relative size and color of the pigment grains. Surface defects, such as gaps or fissures, can become apparent at this scale. Some OM images were obtained with an ultraviolet (UV) light source which added information when fluorescence occurred; showing the overall blue appearance (OM-UV image, appendix IV) characteristic of the protein material of the varnish [55].
The set of images is completed with SEM and AFM scans for a micro and nano-scale. For AFM also quantitative data was gathered, measurements of roughness and profiles can give information on size and height of surface features.

If this was a situation where the samples were unknown, observation of a sample in cross-section would be of major importance. Cross-section analysis gives us the structure of the surface and the distribution of the materials used; it can also be used to perform spot tests. For the identification of proteins the Sypro ® Ruby stain was applied, the result is orange hue fluorescence under green emission (mean emission wavelength 610 nm)[35] when proteins are present.

For specific color characterization the colorimetric values (CIE L*a*b*) were acquired in three spots so to have statistical significance (appendix V).
The cleaning protocol was done for every formulation of enzyme, with both gel and IL solvent. To ascertain the effectiveness of the treatment a second set of images with the mentioned techniques was acquired, and also measurements of roughness with AFM.
AFM measurements after cleaning should be in the range of the average value for the measurement done for unvarnished reference samples (appendix VI). These values could give some indications on the cleaning effectiveness, much lower values them the ones for the reference sample could refer to surfaces with varnish residues. And on the contrary, when values are much higher than expected this could mean some deterioration of the surface.

MALDI analysis was done to the third cotton swab of the cleaning procedures (first damp swab), following Kučková protocol [42]. MALDI is a very sensitive technique with great potential for conservation science, since this requires capable techniques with unambiguous results [43]. Initially it was hoped that analysis with MALDI could give information on residues of the treatment, but limitations verified to the selected protocol only allowed analysis to the third cotton swab; it would have been needed a larger amount of samples, in order to have a statistical analysis of the complete cleaning surface to a given protein and formulation. Nevertheless the results obtained are encouraging for the use of this technique for the purpose of residues analysis. The analysis performed allow to the separation of peptides attesting to the catalysis by the enzymes on the sample varnishes.

[35] *Idem* footnote 22.

5. Discussion

Khandekar [2] discusses some of the potential problems when using Enzyme+Gel mixtures[36], namely relating to the cleaning mechanism, the precise role of each component in the formulations; the residue question, their long-tem effect on the surface and possible interaction with other materials; the same reasoning can and should be applied to E+ILs formulations.

5.1. Enzyme activity

It has been reported that the enzymes remain stable and active in the ILs although the reason for this stability is not fully understood, literary sources also report that the enzymes in IL follow the same catalytic mechanism [20, 56-59], i e, the enzyme is active in the same places, the catalysis follow the same cleavage process and the same resulting peptides. While no quantification was done, the tests performed here with UV-Vis spectrometry and HPLC showed that the enzymes were active in the formulations; also MALDI analysis, without further catalysis by trypsin[37], showed separation of peptides with the tested enzymes after hydrolysis of the varnish layers (see section 5.4).

UV-spectroscopy proved to be a good tool to verify the formulation activity[38], in the sense that gives immediate results and valid data for real time adjustments to the protocol. Although the absorptions between different formulations cannot be compared, it was used to: select potentially compatible ILs, measure the needed amount of water in the formulation and verify the enzyme activity. Some limitations were found in the protocol for the acid enzyme E1 because of the strong alkalinity of the substrate (casein in a NaOH solution), that could inactivate the enzyme.

Also when comparing protocols, adjustments were done for the ILs; it was noted the need of longer times of centrifugation, this can be related to the ILs viscosity, but also some possible capability of the ILs, by molecular interaction, to trap the proteins and peptides; was also verified by HPLC analysis. Table 5.1 show that for the IL formulations 20% of protein was still present in solution after 20 minutes the centrifugation[39], while for the aqueous systems does not reach the 9% after only 10 minutes centrifugation.

With similar protocol to UV/Vis spectroscopy, HPLC gave more information about the formulations. There are some concerns when analyzing IL with HPLC; the IL is the first to be separated and the most polar components would be masked by it, also there is a dragging effect for a short period of time (figure 4.2). This can be compensated adjusting elution gradient sequence and time.

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[36] To have in consideration when studying new cleaning methods and/or formulations: what is being cleaned and the active components, the clearance process, residues (of the varnish and cleaning solution) associated problems of compatibility and long term effects; an adequate experimental methodology [2].

[37] MALDI analytical protocol includes enzymatic digestion of the samples with sequencing grade trypsin, but in this work measurements were done also simply dissolving the sample in water and direct MALDI analysis with DHB protocol for peptides [42].

[38] In reference to the potential action of the enzyme, this is the active component of the formulation.

[39] Because the presence of protein material was still evident in the mixture after 10 minutes centrifugation cycle, a second cycle was done for the formulations with ILs.

From figure 4.2 the resulting peptides are comparable, divided in three groups; it is possible to see that for ILs there is a predominance of peptides for the pep1 group: i e, larger, less polar peptides (the first group of peptides is not comparable to the aqueous formulations because it can be affected by the dragging effect of the IL). This however, is not relevant for the cleaning process, since successful removal is less dependent on the size of the peptides, as verified in the preliminary tests.

Table 5.1 – HPLC analysis - Peptide and protein quantification for casein catalysis with enzyme E2: in Tris HCL 8.4pH buffer; 8.4pH gel and ionic liquid IL1; E2+IL1 casein catalysis for 1, 2, 4 & 8 days old solution.

Peak group Time range	PEP3 [13.9-21.3]	PEP2 [21.3-22.8]	PEP1 [22.74-23.9]	PROT1 [23.9-29.4]	Peptide Total Area %	Protein Total Area %
Buffer+E2	2.27×10^{7}	$4{,}10 \times 10^{7}$	$6{,}87 \times 10^{6}$	$7{,}05 \times 10^{6}$	93,21%	7,38%
Gel+E2	$2{,}43 \times 10^{7}$	$3{,}94 \times 10^{7}$	$7{,}04 \times 10^{6}$	$8{,}23 \times 10^{7}$	93,48%	8,62%
IL1+E2 1st Day	4.09×10^{7}	1.22×10^{7}	2.25×10^{7}	1.64×10^{7}	100.00%	17.16%
IL1+E2 2nd Day	2.88×10^{7}	6.89×10^{6}	1.59×10^{7}	1.96×10^{7}	68.18%	20.54%
IL1+E2 4rd Day	3.24×10^{7}	9.06×10^{6}	1.61×10^{7}	1.08×10^{7}	76.04%	11.29%
IL1+E2 1 week	3.74×10^{7}	1.21×10^{7}	1.50×10^{7}	1.26×10^{7}	85.25%	13.18%
Reference IL1 + E2	9.74×10^{5}	1.09×10^{5}	1.90×10^{4}	3.21×10^{6}	1.46%	3.36%
Reference IL1 + Ca	9.51×10^{4}	2.05×10^{6}	1.75×10^{6}	9.54×10^{7}	5.15%	100.00%

In accordance with most of the literature available, and in every referenced bibliography throughout this text, the IL formulation enhances enzyme stability; here measured by shelf-life tests (table 5.1). These gave optimistic results as a good enzyme activity (85%) was still observed even after a week.

5.2. Other considerations on the new formulations

Some authors who addressed the use of enzymes [14, 15] suggest different formulations in accordance with the practical needs, making mixtures with various components, most of the times more than four elements (the enzyme, buffered solution, thickener, surfactant among others). But, it can be difficult to predict the behavior of each component, unwanted interactions between them and with the surface, and also the degradation process and the long-term interaction with the surface.

With three-component formulations, as proposed here (enzyme + IL + water), it will be easier to test the different interactions and this is an advantage of these new formulations.

Some information is already available on the subject. As described above, the water is needed for the catalytic process as a reagent but at the same time as plasticizer helping the enzymes keep their tertiary structure (functional structure) [56]. The enzyme activity is therefore dependent on water availability at a minimum critical level [22]. Finding the appropriate level of water to be added to the formulation is also an important step. The hydrophilic ILs will affect this level as they will strip the enzyme of the needed water [60], which explains the high amount (20%) verified in this situation for both [BMIM][BF_4] and [EMIM][$EtSO_4$]. It is possible to predict that less polar IL (for

instance with longer alkyl chains) will be less likely to remove the available water for the enzymes, and for the hydrophobic IL it can be expected a low minimum water needed, that authors predict under 5% [48, 53, 60]. Noritomi [56] also points out that, with hydrophobic ILs, there can be the advantage of a fine dispersion of the enzyme and water, affecting positively on the enzyme availability in the formulation.

Nevertheless, the choice of experimenting with two hydrophilic ILs fell on the premise of choosing between those available and because the tested hydrophobic ILs did not show the best results (appendix III).

Going for a “green solvent” solution does not or should not depend only on the negligible vapor pressure, it is important to verify what other characteristics can be environmental friendly. The re-use of the ILs is one way to minimize its impact on the environment; in addition this helps to reduce their potential cost. The recycling process of ILs was not considered in this work because of the small amounts of IL used, but the referred bibliography [47, 56, 58] express optimistic remarks of its possibilities. Products and enzymes can be recovered by processes of filtration or centrifugation; and if the IL is hydrophobic, washing the IL with water can be a simple alternative.

Most if not all ILs in the imidazolium cation family show some level of toxicity [17-19], this however, is not considered a disadvantage, on the contrary. Knowing that bio-contamination is one of the factors that inhibit enzyme activity, the toxicity levels of these ILs will work in favor of prolonging the formulation shelf-life[40] by preventing bacterial growth.

The praised negligible vapor pressure poses however an important problem about the possible residues on the surface after cleaning. The long-term effects that these residues might have on the surface are an important issue that cannot be predicted with the limited time frame of this work; nevertheless some consideration can be brought forward. As designer solvents the prevention might be the key, choosing the cation-anion combinations that have expected non-reactive degradation products [17, 49]. For example the $[BF_4]^-$ residues will be affected by atmospheric moisture, and possible degradation products can be the hydrofluoric acid with potentially dangerous effects on the painted layers. As explained before however, in this work this anion was chosen as it was important to have a well known compatible IL for enzyme testing. But possibly, more suited ILs can be found on the non-halide ILs group. Other prevention measures may include adjusting the cleaning protocol, using for example, only the minimum amount of formulation needed for a given area, and optimizing the clearance steps.

The viscosity is another property that distinguishes the ILs from the traditional organic solvents. Even though literature addresses concerns on this point, for surface cleaning this is an advantage; ILs´ high viscosity can correspond to the gel function as solution thickener, this is important because reduces penetration to other layers, limiting formulation action to the desired top layer. A way to adjust viscosity in ILs is in the alkyl groups, a longer alkyl chains usually translates into more viscous ILs [20, 48].

[40] Shelf life is understood as the recommended time that products can be stored, during which its qualities remain acceptable for its use under the advised storage conditions.

5.3. Surface monitoring protocol

For surface analysis each technique contributed with a set of important data (appendix IV), but is the cross correlation of all data that completely characterizes the surface.
Due to the complexity of the painted structure there are multiple factors that influence the surface. Each of the proteinaceous varnish has a very different behavior as film-forming material. Egg white will form a dry film in short time that becomes insoluble with ageing, and it loses its flexibility; the casein after drying becomes promptly hard and an insoluble material. For glues, the temperature at the moment of application is determinant, as a higher temperature means a less viscous liquid and results in a more homogeneous and flat surface. Therefore the results obtained should be interpreted as features of the studied samples and not as general characteristics of the mentioned paint materials.

5.3.1. Surfaces Characterization of the varnished

In a situation where the paint or polychrome composite structure is unknown the cross-section observation gives important information, including the varnish layer properties. In the case of the samples prepared here the structure and the materials are well known and this step was done for reference purpose. Figures 5.1 and 5.2 show selected examples for two different artistic techniques with two different varnishes and reference unvarnished samples, prepared in cross-section.

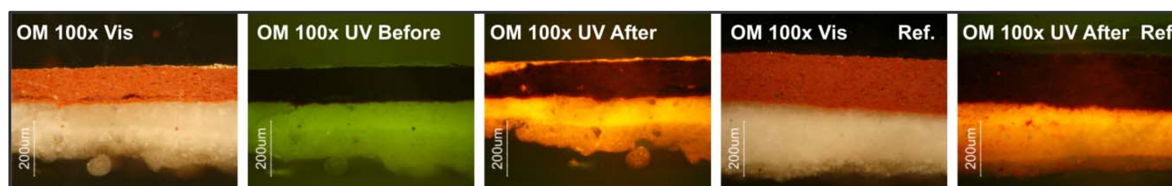

Figure 5.1 – Cross-section images of the sample GG-CA with OM at 100x with visible light and with UV light before and after staining with Sypro® Ruby. And reference unvarnished samples (ref.).

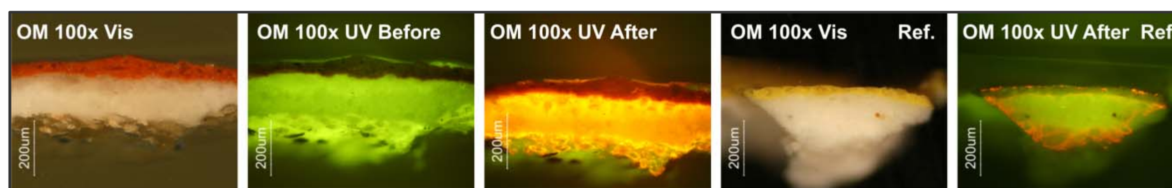

Figure 5.2 – Cross-section images of the sample OG-Ca with OM at 100x with visible light and with UV light before and after staining with Sypro® Ruby. And reference unvarnished samples (ref.).

The fluorescent staining tests with Sypro® Ruby make evident the presence of proteins with orange color. It is possible to indentify and map the protein based varnish layer at the top of the samples. As a comparison, the correspondent reference unvarnished samples show no fluorescence at the top layers. Also, it is possible to conclude that the ground layer was prepared with proteinaceous material because of the visible orange hue (the ground layer was prepared for all samples with animal glue as binding agent.).

Colorimetric values (CIE L*a*b*) obtained for each surface showed the alteration introduced by the varnish to the perceived color [34], this is important for surface characterization because it gives a quantitative color interpretation. Appendix V table1 shows the expected *E* variation (ΔE) by comparison of the average values of the varnished and unvarnished surfaces. Except for some of the fish glue varnished samples, all of them have ΔE>1, this corresponds to an important change introduced by the varnish that is visible to the human eye. One possible explanation is that these samples have a thinner varnish layer.

Each cleaning treatment introduces a big change to the surface so it is normal that the measurements reveal considerable high ΔE values. Appendix V table 2 presents the collected date for all samples end the calculated ΔE. One could reason that the ΔE if it is near to the reference values (table1) that would mean a good cleaning, going over or under this value would mean respectively surface deterioration or presence of residues. However these can be misleading, because of the unknown number of factors that influence the colorimetric values. For the present work this tool is only important for surface characterization and does not give relevant information about the treatment. Regarding the color: the pigments present in the model samples (Sinopia and Yellow Ochre) have a similar chemical composition and apart the difference in color (one is red, another one is yellow) they did not display other relevant differences.

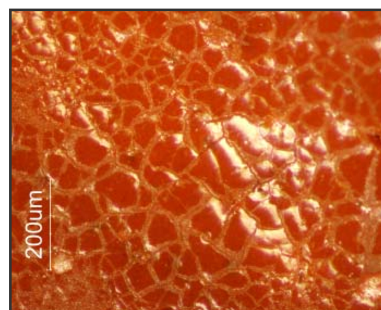

Figure 5.3 - 100x OM image of the sample EG-S-EW with visible light, showing craquelure pattern.

Observations with SM, aside from making the record of the surface, were important for treatment monitoring. Due to its portability it is possible when in doubt, or for sensitive areas, to perform the cleaning protocol under SM observation.

The images obtained by means of the OM gave qualitative information about the surface (perceived texture, color, glossiness) and some aspects of the state of conservation such as the presence of gaps or fissures. Figure 5.3 shows a sample with egg white varnish, it is easily identified a pattern of craquelure [41] on the varnish layer. It is also visible that the cracks do not prolong to the inferior layers. It is possible to conclude that they are not the result of ageing of the sample but, formed with little time after varnish application. The alteration that the varnish introduces to the surface is visible in figure 5.4 of oil painting samples on wood at 100x magnification, when comparing figure 5.4a and 5.4b: a) shows a surface without varnish, and b) shows casein varnish, the colors are stronger and brighter; and it is perceived a general glow over the surface.

[41] Craquelure on a painting is the network, or pattern, of cracks developed across the surface.

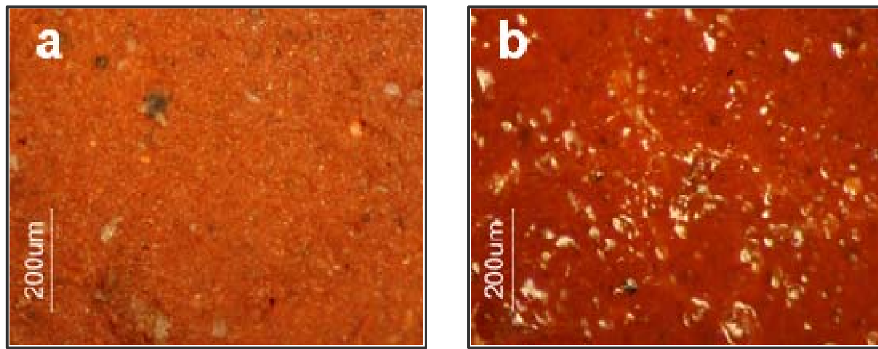

Figure 5.4 - 100x OM images with visible light, the effect of varnish: a) OG-S, unvarnished surface; b) OG-S-Ca, sample with casein varnish.

Observation under fluorescent light (UV) was used to study the auto-fluorescence of proteins varnishes present on each area, this occurs mainly due to the presence of the aromatic amino acids, such as: tryptophan and tyrosine, which results in a blue fluorescence Figure 5.5. Also, the binding medium (protein or oil, Figure 5.5b and 5.5c) displays a stronger or lighter blue-green fluorescence according to its chemical nature, deterioration, ageing and added pigments [55, 61]. Hence, unvarnished tempera painting should present a blue fluorescence as it is similar in nature to the varnishes used. The fluorescence observed in the unvarnished oil painting is associated to the autoxidation process and consequent presence of unsaturated acids. Although these were not aged samples, they were stored in the dark and this might explain the premature fluorescence of the oil [62]. The gilded surface is not fluorescent so there is a visible difference between varnished and unvarnished surfaces Figure 5.5d and 5.5e.

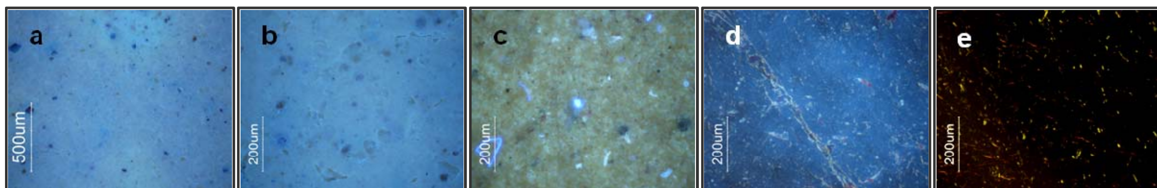

Figure 5.5 - 100x OM UV fluorescence images of the different materials used in the samples: a) EG-YO-EW Egg white varnish over a egg tempera paint sample; b) EG-YO unvarnished egg tempera paint sample; c) OG-YO unvarnished oil paint sample; d) GG-CA animal glue varnish over a gilded surface; e) GG unvarnished gilded sample.

The pigments used due to their nature can have a small contribution in diminishing the oil fluorescence [61, 62], but the fluorescence pattern is not diagnostic of the material; i e, is not possible to identify which pigment or protein is present from the observed fluorescence. In a general situation the fluorescence microscopy is a good tool to analyze and identify what is to be removed, setting the first assessment needed when choosing a cleaning method [2].

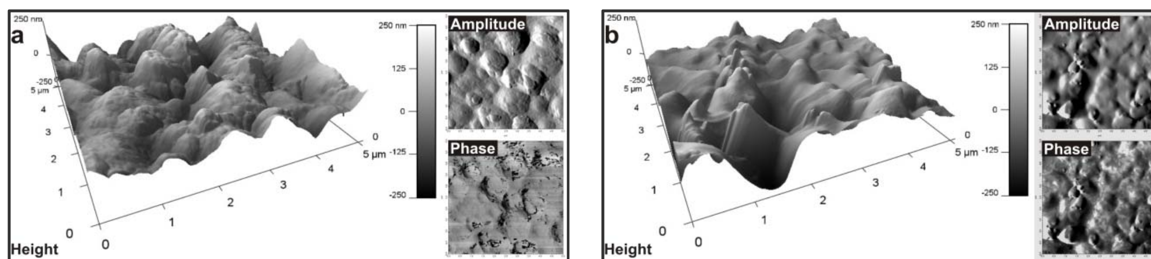

Figure 5.6 - 2D and 3D rendering of the AFM image set of height/topography, amplitude and phase (a) Unvarnished reference sample of tempera painting; (b) Unvarnished reference sample of oil painting.

AFM gives the nano-scale evaluation of the surface; at this scale a new level of features comes into evidence. To achieve nano scale details of the material in order to qualitative indentify the material and to map the topographical features of the surface, phase, amplitude and height scanning were correlated. The amplitude registers the tip alteration and in a false way replicates the surface features, where light parts are relates to movement of the tip up and what seems like shadows is when the tip adjusts down. The phase image relates with interactions to the surface material.

For example, the observation of the surface with AFM reveals the mapping of the binding material and pigment. For the tempera sample (figure 5.6a) a number of spots are visible in high contrast (black/white high phase and low phase compare to 90 deg.), this suggests the presence of different materials at the surface, and possible relates to surfacing of pigment grains that are uncovered by binding media. For the oil sample (figure 5.6b), however, the material seems more homogeneous because the phase image follows a similar pattern of the amplitude image; pigment grains appear more undifferentiated and overall the surface seems smoother.

Indeed the oil painting has a higher proportion of binding medium (oil) and pigment grains stays more embedded in the paint.

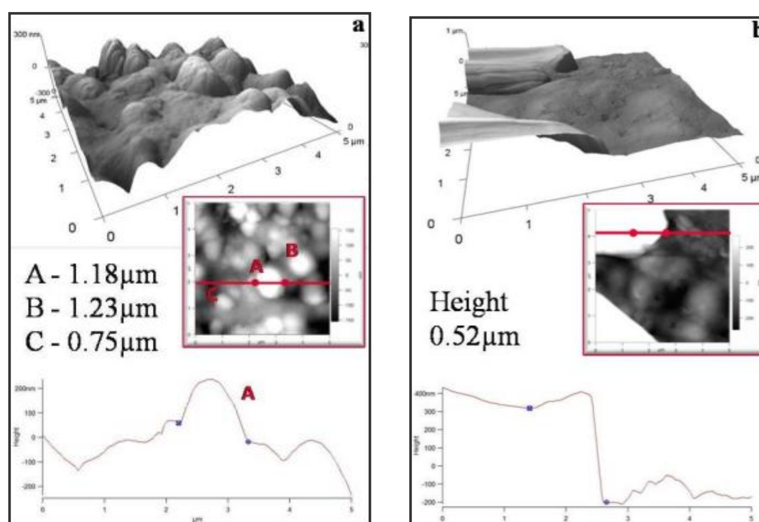

Figure 5.7 - 3D and 2D AFM height images, surface features measured on the obtained section and: a) tempera painting without varnish, pigment grain size measurements of. b) tempera painting e with egg white varnish, measurement of the height of the varnish layer in a gap area.

2D profiles can be then extrapolated using software tools. This information in a real sample is important when characterizing the surface of the sample, studying date or the provenance of the material, if natural or artificial. For example, in figure 5.7a, pigment grains identified as individual spherical features can be measured, the small uniform round grains suggests an artificially or industrialized prepared pigment. In gap areas it was possible to measure the height of the thinner layers (figure 5.7b) and observe features of other paint layers.
Appendix VI shows roughness measurements (R) done for each sample surface and standard reference samples.

Appendix IV, figures 1-4, show the most representative images of the four varnishes studied here on different surfaces, analyzed by SM, OM, AFM and for the sample prepared on canvas also some SEM images. Interesting features observed at nano-scale can be related to macro-and micro-scale properties and artistic technique. For example: glair (egg white varnish) samples show a wrinkle pattern (figure 1, appendix IV), comparable to what is observed at macro scale for varnishes applied in layers, when the top layer is applied over another that is not completely dried. The samples with animal glue are the ones with less homogeneous surface (figure3, appendix IV) and this can be related with the temperature at which the glue was applied. As it cools down, the glue ceases to melt and when applied is not a completely fluid mixture resulting in a non uniform surface. The casein is not soluble in water, for preparing the varnish it has to be mixed in an aqueous solution with sodium hydroxide (NaOH), and heated for long time; in the lower right side of the figure IV.4 (appendix IV) what is observed is probably one grain of casein that was not completely dissolved.

The comparison of the varnished areas average values with the reference unvarnished samples allows to interpret the alteration introduced by the varnish layer on the surface (appendix IV, figures 5-7). For example, all the surfaces, except the gilded ones, show lower roughness values after varnishing. This can be easily related with the change in the optical properties, as the varnish smoothed the surface, changing the refractive index and increasing specular reflection.
The gilded surfaces are the exception, and this is related with the purpose of the varnish. The animal glue is applied for lowering the glossiness, and this can be corroborated with the AFM measurements, showing in turn an increase of the surface roughness (appendix VI).
SEM gave similar information of the surface as the AFM; images show an apparent three-dimensional view of the surface, and surface features can be measured for length and area. However, is limited for the measuring of height, which is only possible with AFM profiles. Nevertheless this technique was chosen because it is already a common tool in conservation science, so there would be comparable data for AFM interpretation; and in some situations can be helpful for elemental mapping analysis.
SEM images are acquired in vacuum and can be hard to collect if the samples are non conductive. Charges effects on the surface will not allow a proper focusing impairing the quality of the pictures. A strategy around it is to coat the samples with some gold or carbon. This means contaminating

the sample. Furthermore, coating the sample would have most likely picture the coated surface instead of the original one. The wood samples were prepared with not aged common wood, which is more reactive to differences in air humidity. Left in an uncontrolled room environment, the paint and varnish layers when drying became less adhered to the support, because they do not follow the movements of wood. For these reasons, the samples prepared for casein and animal glue varnishes (on wood support) could not be analyzed by SEM. Indeed, when the wood gilded samples were introduced in the vacuum chamber, the gold started to foil out.

5.3.2. Cleaning effectiveness assessment

Cleaning effectiveness assessment means comparing the before treatment with the after treatment data and also with data obtained for reference unvarnished samples.

During surface characterization, every technique show important data that can be read separately (for some examples see appendix IV figures 8-14). However, cleaning effectiveness assessment is only possible when cross-correlating all of the mentioned surface characterization analytical techniques.

The interpretation of data for the successful cleaning is simple and immediate: images after treatment should be similar to the sample prior varnishing or reference unvarnished samples.

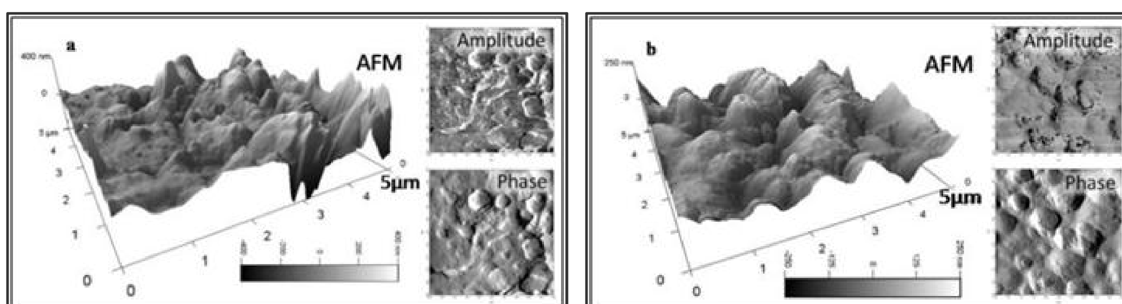

Figure 5.8 2D and 3D rendering of the AFM image set of topography, amplitude and phase (a) Tempera painting sample after cleaning; (b) Unvarnished reference sample of tempera painting.

Figure 5.8 shows a tempera painting sample after treatment with [EMIM][$EtSO_4$] and the alkaline enzyme (E2) for the removal of egg white (EW) varnish. In this case the three AFM images helped to establish that this as a successful cleaning as the surface features repeat themselves and are similar to the unvarnished reference sample (figure 5.8b).

Figure 5.9 shows the surface of a gilded sample before and after treatment for the removal of animal glue coating. The OM image obtained under UV light source show total loss in fluorescence after treatment, ascertaining the absence of proteinaceous material (figure 5.9b), as the gold leaf has no fluorescence under UV light. Therefore it can be assessed that the cleaning treatment was effective, For the figure 5.9b, however, the topographic image obtained with AFM shows surface degradation and the presence of grooves; suggesting the effect of scratching left by the cotton swabs, maybe because of excessive pressure when cleaning.

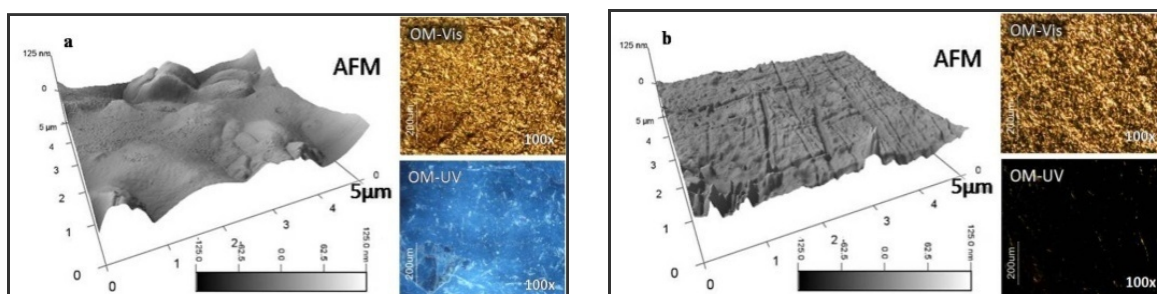

Figure 5.9 - 3D rendering of the AFM topographic image and OM with visible light and UV light, images set of animal glue coating from a gilded surface sample (a) Before cleaning
(b) After cleaning with E3+Gel.

In restoration treatments some amount of residues is acceptable in conservation [1], a way to evaluate cleaning effectiveness is also to identify the presence of varnish residues after treatment. For the assessment of varnish residues after cleaning the next two examples (figure 5.10 and 5.11) show two different situations: a) OM, SEM and AFM all revealed the presence of varnish residue and b) the residue is only registered by the AFM

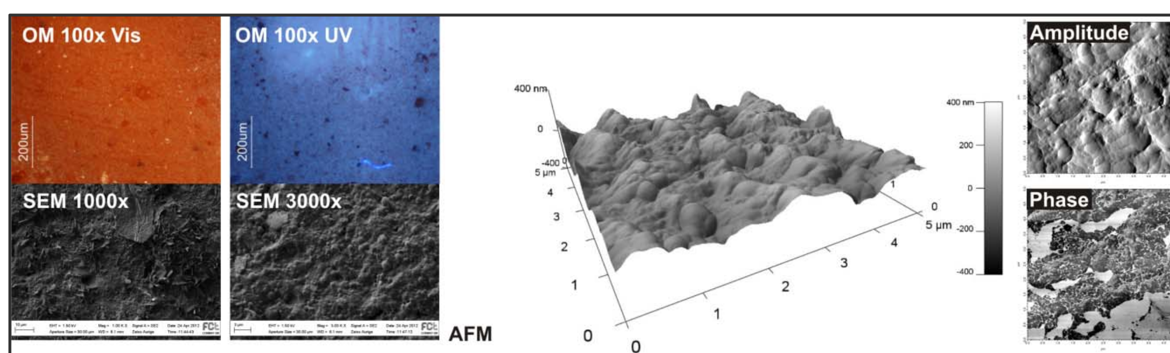

Figure 5.10 – EG-S-CP; images set after removal of isinglass varnish of a egg tempera painting sample with IL1+E1: OM images with visible light and UV light 100x; SEM at 1000x and 3000x and 2D and 3D rendering of the AFM images, , and.

Figure 5.10 shows a sample cleaned with the alkaline enzyme with IL [BMIM][BF_4] (E2+IL1) for the removal of isinglass varnish of a tempera painting surface. The image obtained with the OM, under UV made possible to registered protein residue as it gives a different fluorescence pattern on the surface. The difference in fluorescence although not enough to positively identify the material present, allows identification of the indicated areas as varnish residue, and therefore, this suggests an incomplete cleaning; in this case the conservator could choose to repeat the treatment. In the AFM phase image (figure 5.10b), a different interaction of the tip with the surface is observed resulting in areas with higher contrast. The white areas are similar in texture to the surrounding material, implying a change in the nature of the material. This reinforces the conclusion retrieved for the OM. Thus, it can be strongly concluded that the surface is not homogeneous and most likely composed by two different materials.

The same was not observed on the correspondent image of another sample with isinglass varnish on oil paint surface (figure 5.11); but, at a nano-scale with AFM both samples registered residues in this sample the residue is visible as a flat plate between crests.

The residue areas can be measured by the AFM using software tools and extrapolated to a larger scale; this gives answers on how much of the varnish is still on the surface after the treatment. This is useful information for the art restorator.

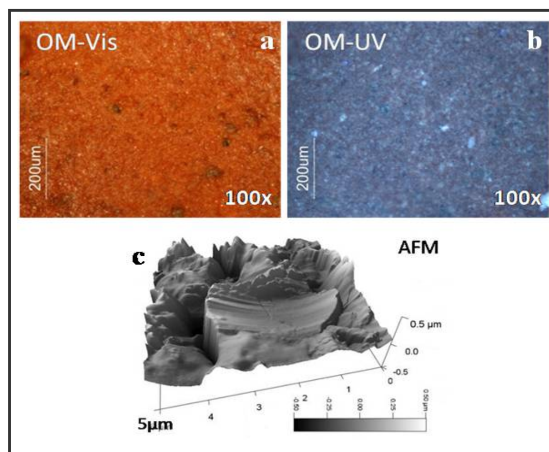

Figure 5.11 – OG-S-CP sample: OM images with visible light and UV light after removal with IL1+E1, and 3D rendering of the AFM image and, after removal of isinglass varnish.

Although enough statistical information was gathered for quantification of the amount of residue present on the surface, this is not addressed here due to space limitations. But it is important to leave this note to refer this yet another potential use for AFM.

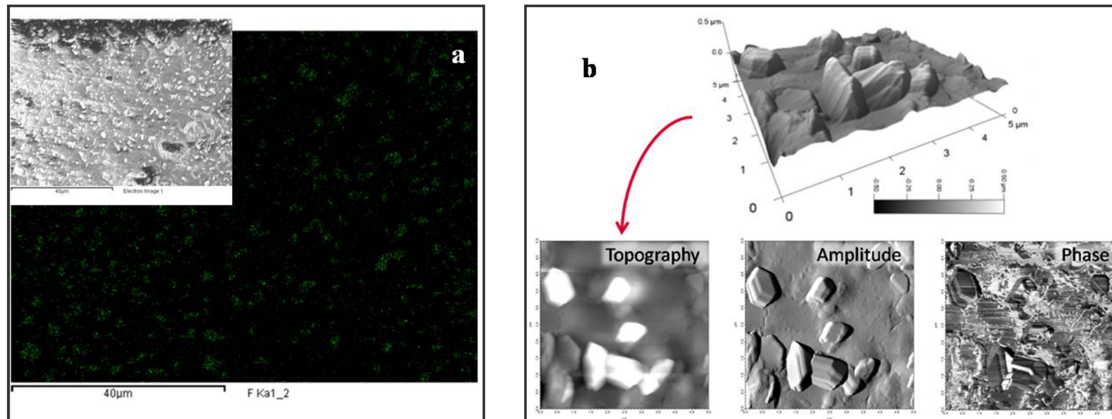

Figure 5.12 – EG-S-EW. Tempera painting sample after removal of egg white varnish with cleaning formulation: IL1+E1 (a) Elemental mapping with SEM/EDX, (b) 2D and 3D rendering of the AFM image set on the same area.

Another important aspect in evaluating the cleaning effectiveness is the ionic liquid residue. A small set of the tempera painting samples cleaned with [BMIM][BF_4] shows polyhedric crystals on the surface, visualized with both SEM and AFM methods (figure 5.12). These structures were not found before cleaning in any of the studied samples; therefore their presence is the result of the cleaning treatment. All the materials used on the samples are organic therefore, mainly composed by C chains.

The Energy-dispersive X-ray spectroscopy (EDX) is not able to differentiate between materials, since it only detects atoms and not molecules; and as to be used at low intensities, because the samples are not coated. But, the ***Fluorine*** of the [BMIM] [BF_4] can be easily mapped using the EDX. Figure 5.10a shows the elemental mapping of Fluorine on the surface as green spots, identifying the observed crystal as being in fact IL crystals. Because this only happened in a set of 4 samples, all done at the same time, and because this was not observed on other samples where the same IL was used, it is concluded that this was a flaw in the cleaning protocol.

Figure 5.13 – Cotton swab collection after cleaning of two samples.

The images obtained for each microscopic technique for the perceived cleanness, the presence of residue, and possible damage of the surface; allows a global evaluation of the cleaning effectiveness for each tested area.
For the purpose of assessing the cleaning effectiveness with the two enzyme formulations, it became evident the advantage of using the AFM comparison with OM or SEM, and the examples above mentioned demonstrate the complementary information that can be obtained using cross-correlation multi level investigation from micro to nano-scale.
Some of the samples showed some level of deterioration of the paint layer after cleaning; it was concluded that this was not caused by the cleaning agent, the enzymes, as they are highly selective to the varnish material. The cotton swabs (figure 5.13) show that the deterioration occurred with the clearance process and most probably because they are un-aged samples; the surfaces still maintains some solubility to water (used for the 3rd cotton swab) used for the removal of the gel, varnish and protein residues. This problem can be overcome by adjusting the cleaning protocol, changing either the materials used for the clearance phase or in the application procedures.

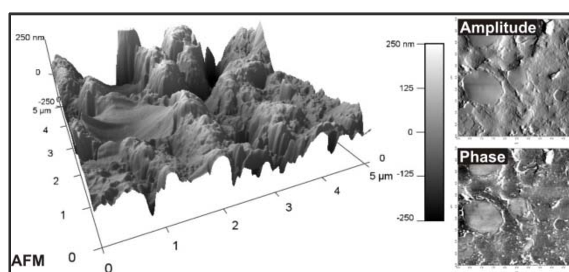

Figure 5.14 – AFM images for topography, amplitude and phase of a reference tempera painting surface with fish glue varnish sample cleaned with IL2 [EMIM][$EtSO_4$].

The global evaluation of the cleaning tests showed that all enzymes formulations presented a potentially[42] good removal capability, although enzyme activity favored the formulations with IL1

[42] Here is not taken in to account the wear of the treatment observed in some samples mostly caused, as explained by the mechanical action and clearance steps.

([BMIM][BF_2]). The justifications could be on some cleaning capability of the IL2 ([EMIM][$EtSO_4$]) itself as observed in the AFM (Figure 5.14; also appendix IV figure15).

For the E3 the surface analytical techniques showed that albeit less enzyme activity was measured, the samples showed an overall good removal of the varnish with less deterioration of the surface, this can be observed also in the roughness measurements (appendix VI).

5.4. Peptide analysis - MALDI-TOF-MS

Analysis of the cotton swabs after cleaning could show if the enzymes were in fact, active in the different formulations during the cleaning process, due to sensitivity limitations this could not be performed by HPLC technique. The technique selected was MALDI-TOF-MS.

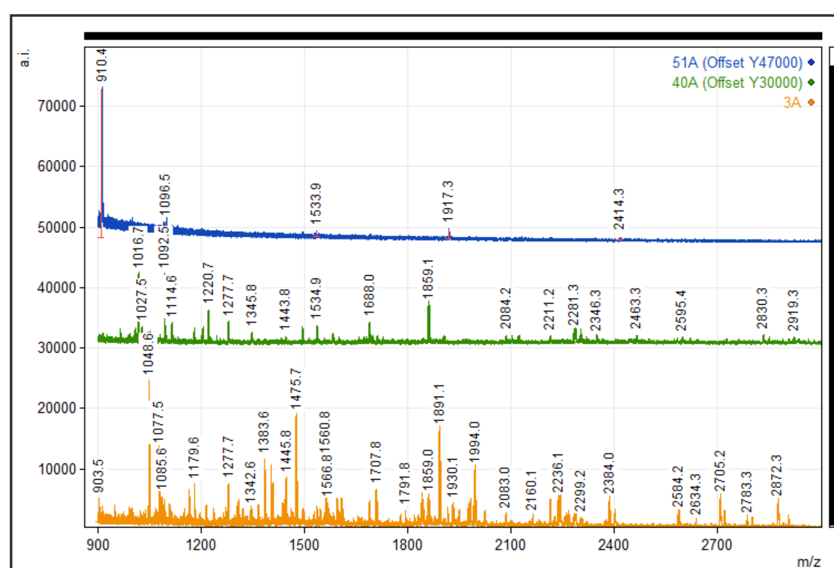

Figure 5.15 – MALDI-TOF-MS analysis of the material retrieved from the cotton swabs used on samples of egg white varnish on oil painting (OG-EW) samples, with E1 formulations (3A-Gel, 40A-IL1 and 51A-IL2).

The indication of peptides size and amino acid sequence are obtained after enzyme hydrolysis[43], this is dependent upon the specific hydrolytic process of each enzyme. Even though the limited time of action (except for casein samples, where the formulation was left on the surface for approximately 30minutes), there were some promising results, as there was peak separation in the samples measured, with no further catalysis by trypsin[44]; this proves that in fact, the catalysis of the proteinaceous varnishes occurred. Each peak corresponds to a peptide and time of separation relates to mass, smaller peptides would be the first in the obtained spectra.

Comparison of mass spectra of samples A3, A40 and A51 (figure 5.15) shows that a greater number of peptides was removed in sample A3. Using the databases, it was confirmed that pepsin was most successful in cleaving the egg white varnish in samples A3.

Figure 5.16 shows the comparison of mass spectra between the three enzymes in gel formulations.

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[43] Protein and peptide identification is done by peptide analysis after catalysis with trypsin in comparison with known databases.

[44] *Idem*, footnote 37.

A greater number of peptides was obtained in the sample A4 containing the material retrieved from the cotton swabs after cleaning with E1+Gel formulation (90 peptides). The second most successful enzyme was E2 (40 peptides) and the last was e3 (30 peptides).

The whole proteins from egg white were removed and confirmed using trypsin digestion and comparison with a database containing tryptic peptides of reference materials. In the samples A3 and A4, 25 peptides were found originating from the whole proteins.

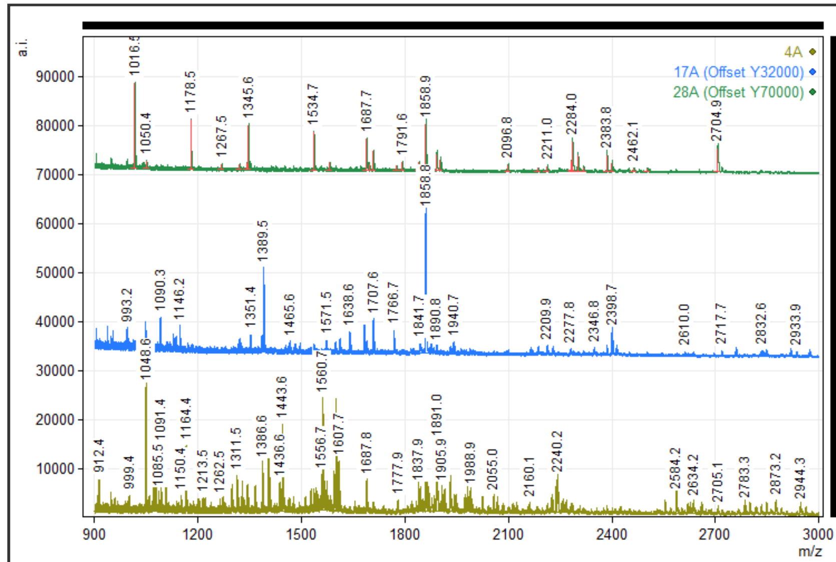

Figure 5.16 – MALDI-TOF-MS analysis of the material retrieved from the cotton swabs used on samples of egg white varnish on oil painting (OG-EW) samples, with Gel formulations (4A-E1, 17A-E2 and 28A-E3).

When peak separation is observed this proves effectiveness of the formulations, i.e., that enzyme catalysis occurred with the tested formulations on the varnished surfaces. The short period of contact of the formulation with the varnished surface explains the presence of bigger peptide/proteins (figure 5.17a). There is no complete cleavage of the protein, as there is still considerable number of peaks in the high molecular weight interval. These were most likely removed mechanically, and not in solution with the cleaning formulation.

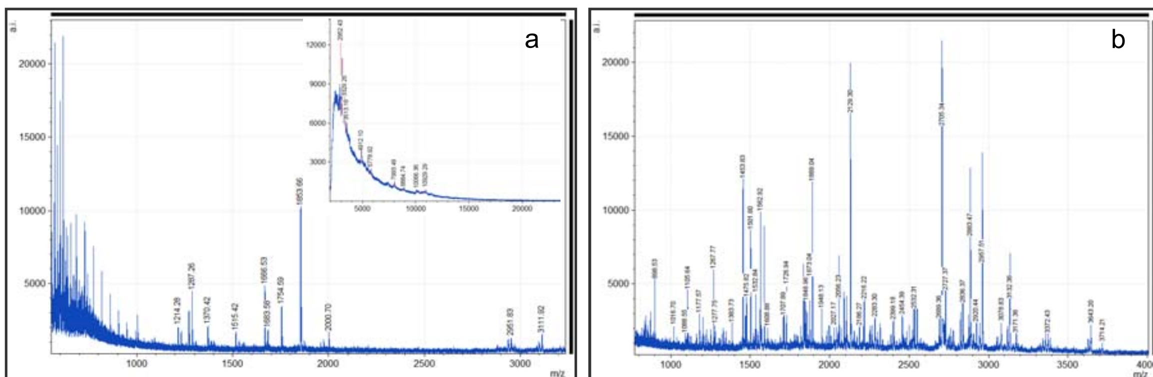

Figure 5.17 – MALDI-TOF MS analysis of material retrieved from the cotton swabs used: a) fish glue varnish sample cleaned with E1+IL2 formulation. Peptide analysis up to 3500 N/z and large peptides/proteins analysis with more than 4000 m/z b) [40], b) Animal glue varnish sample, after treatment with just IL2; measurements done with standard trypsin cleavage.

MALDI analysis of the cotton swabs with IL2 did not show relevant peak separation but, there was removal of the varnish layer. Figure 5.17b confirms the indication by the AFM (example on figure 5.11), that the ionic liquids have some cleaning capability; and was able to remove the varnish without the need of enzyme cleavage.

Some samples did not show peptide separation; this could be due to a limitation of the protocol since only the third cotton swab (of the cleaning protocol sequence) was analyzed.

6. Conclusion

Whenever a novel and alternative solvent is introduced in a cleaning formulation, it is important to evaluate its major advantages and drawbacks compared to other media. For IL the same concerns apply: efficiency, safety, handling, interaction with other layers and presence of residues, among others. Due to limitations of time and the introductory nature of this work some of them were only addressed theoretically.

The main purposes of the work were to show that the chosen enzymes were active with new formulations made of enzymes and ionic liquids, and that the protein layer could be successfully removed in a cleaning protocol with these formulations; the results obtained from testing the solutions show a promising starting point in these aspects. UV-Vis, HPLC and MALDI showed that the catalysis occurs in the selected IL and with comparable results to the gel, reference formulations.

The enzyme combination with IL1 was overall more successful, and even though there is some reservation to the use of this IL, namely due to possible long term degradation problems, it is a promising start and could be considered as reference for future studies.

The multiscale monitoring protocol of painted/gilded surface was effective for their characterization and assessment of cleaning effectiveness including the residue analysis.

An important advantage of the analytical techniques used resides in the real time monitoring of the cleaning process: comparative study of unvarnished and varnished surfaces allowed to prove their usefulness in the assessment of the removal effectiveness; and this can further help to adjust the cleaning protocol.

Going to a nano-scale with the use of AFM proved to be of major importance to achieve a further level of information on the studied surfaces; at the same time the conclusions were easily extrapolated to a larger scale. Not only AFM qualitative information was obtained but also quantitative, when measuring surface roughness and sections profiles, before and after the treatment on the same area, enabling the assessment of cleaning effectiveness and deterioration at nano-scale.

Residues could be evaluated in a qualitative approach with OM, SEM and also quantitatively using AFM.

Although further research is needed to confirm these experimental findings, these preliminary results are promising; this work gave thus an important contribution to the study of surface cleaning treatments using new formulations based on enzymes and ionic liquids; and surface analysis.

7. Future trends

This is an unstudied area and this proposal is just an introduction to the subject but it hopefully shows that entails great potential on the research of alternative cleaning formulations.

The formulation can be improved by exploring other ILs families changing anion-cation combinations. Adjustments can be done to the cleaning protocol for better answering to specific situations dependent on the surface materials and their state of conservation.

Further studies should be done for comparison between fresh materials and aged ones, and also for improving the residue analysis: the ILs degradation process, interaction with other materials present in the system and long term effects should be also studied.

The analytical multiscale protocol for surface cleaning assessment proved to be a useful tool for this type of treatment but it could also be considered for monitoring other surface treatments or used as a tool to gather better and more comprehensive knowledge about the materials present in the surface layers of cultural artifacts

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Appendix

Appendix I - Material Data Sheet: Acid enzyme - Pepsin

Appendix II - Material Data Sheet: Alkaline enzyme – Aspergillus sojae

Appendix III - Tested ionic liquids, and main characteristics

Appendix IV - Surface characterization by Stereomicroscopy (SM), Optical Microscopy (OM), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM)

Appendix V - Colorimetry analysis

Appendix VI - AFM analysis

Appendix I   Material Data Sheet: Acid enzyme - Pepsin

*A*N*alisi Tecnologie e* *A*rticoli per il *RES*tauro
AN T A RES srl via Aldo Moro, 24/a 40068 San Lazzaro di Savena (BO)
Tel.051 6259816-fax 051 6259807- e-mail: info@antaresrestauro.it
Sito: www.antaresrestauro.it

SCHEDA TECNICA
Aggiornamento Maggio 2004

PROTEASI ACIDA

Caratteristiche chimico fisiche

Composizione :	pepsina (porcine stomach mucosa)
Stato fisico:	polvere da miscelare al gel
Colore:	biancastro trasparente
Supportante gel	Gel tamponato a pH: 5 a base acquosa
Attività enzimatica:	463 units/mg solido
Solubilità:	solubile in acqua

Usi
La proteasi acida esercita un'azione enzimatica specifica di rimozione di materiali proteici quali caseina, albumine e uovo intero, collagene (colle animali). Al momento dell'utilizzo lasciare la confezione a temperatura ambiente per circa 15 minuti, aggiungere quindi l'enzima in polvere al supportante gel e mescolare fino ad omogeneità con bacchetta di vetro o plastica. Chiudere il contenitore e mantenerlo in bagno d'acqua a circa 35° C. Lasciar riposare per circa 20 minuti. Applicare a tampone lasciando agire o lavorandolo a pennello morbido. Si consiglia di effettuare prove per determinare i tempi di applicazione, partendo da tempi di 1-2 minuti. Evitare l'essiccazione del gel sulla superficie e rimuovere con tampone asciutto. Lavare la superficie prima con tampone acquoso quindi con essenza di petrolio

Stabilità
Evitare di scaldare oltre 50°C. Evitare di agitare con oggetti metallici. Evitare di mescolare a sostanze alcaline che potrebbero diminuirne l'attività. Conservare in frigorifero a 0-5° C.
I componenti non miscelati hanno una validità di 8-9 mesi. Una volta miscelati hanno una durata di 15-20 giorni in frigorifero.

Precauzioni
Polvere: Prodotto nocivo e irritante. Durante la manipolazione della polvere proteggere gli occhi ,l'apparato respiratorio e le mani con adeguati sistemi protettivi

Taglie
100 ml

Le istruzioni e le informazioni sopra riportate sono dettate da una lunga esperienza di laboratorio e di impiego e sono quindi accurate e pertinenti. Poiché le reali condizioni di utilizzo da parte degli utenti non sono da noi controllabili, esse vengono fornite da parte nostre senza alcuna
responsabilità o garanzia, implicita od esplicita.

Appendix II Material Data Sheet: Alkaline enzyme – Aspergillus sojae

*AN*alisi *T*ecnologie e *A*rticoli per I *RES*tauro
AN.T.A.RES srl via Aldo Moro, 24/a 40068 San Lazzaro di Savena (BO)
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SCHEDA TECNICA
Aggiornamento Settembre 2004

PROTEASI ALCALINA

Caratteristiche chimico fisiche

Composizione:	proteinasi di origine fungina (da Aspergillus sojae)
Supportante gel:	Gel a pH tamponato alcalino 8,4
Stato fisico:	polvere da miscelare al gel
Attività enzimatica:	>30 U/MG
Solubilità:	solubile in acqua

Usi

La proteasi alcalina esercita un'azione enzimatica specifica di rimozione di materiali proteici quali caseina, albumine e uovo intero, collagene (cole animali). Al momento dell'utilizzo lasciare la confezione a temperatura ambiente per circa 15 minuti, aggiungere quindi l'enzima in polvere al supportante gel e mescolare fino ad omogeneità con bacchetta di vetro o plastica. Chiudere il contenitore e mantenerlo in bagno d'acqua a circa 35° C. Lasciar riposare per circa 20 minuti. Applicare a tampone lasciando agire o lavorandolo a pennello morbido. Si consiglia di effettuare prove per determinare i tempi di applicazione, partendo da tempi di 1-2 minuti. Evitare l'essiccazione del gel sulla superficie e rimuovere con tampone asciutto. Lavare la superficie prima con tampone acquoso quindi con essenza di petrolio

Stabilità

Evitare di scaldare oltre 50°C. Evitare di agitare con oggetti metallici. Evitare di mescolare a sostanze acide che potrebbero diminuirne l'attività. Conservare in frigorifero a 0-5° C.
I componenti non miscelati hanno una validità di 8-9 mesi. Una volta miscelati hanno una durata di 15-20 giorni sempre mantenuti in frigorifero

Precauzioni

Bustina proteasi: prodotto nocivo. Per la manipolazione utilizzare guanti ed occhiali. Può provocare sensibilizzazione.Per la manipolazione della soluzione utilizzare le normali precauzioni: guanti e occhiali.

Taglie

100 ml

Le istruzioni e le informazioni sopra riportate sono dettate da una lunga esperienza di laboratorio e di impiego e sono quindi accurate e pertinenti. Poiché le reali condizioni di utilizzo da parte degli utenti non sono da noi controllabili, esse vengono fornite da parte nostre senza alcuna
responsabilità o garanzia, implicita od esplicita.

Appendix III Tested ionic liquids, and main characteristics

Table 1 – List of the ionic liquids tested, some of its characteristics and result of the preliminary cleaning tests.

Name	Cation	Anion	CAS number	melting point	ρ (g/cm^3)	Viscosity (cP at RT)	Miscibility with Water	Observations preliminar cleaning tests
1-Butyl-3-methylimidazolium Tetrafluoroborate	*$[BMIM]^+$*	*$[BF_4]^-$*	*174501-65-6*	*<-50*	*1.21*	*120.00*	*totally miscible*	***Similar results to those with gel formulation for both enzymes.***
1-Butyl-3-methylimidazolium Trifluoromethanesulfonate	$[BMIM]^+$	$[TFO]^-$	174899-66-2	17	1.30	88.60	totally miscible	Whitening of the surface bu after clearance there is no visible alteration
1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	$[BMIM]^+$	$[NTF_2]^-$	174899-83-3	2	1.44	69	immiscible	Some visible alteration at 3min but going until 9min does not seem to improve the effect.
1-Butyl-3-methylimidazolium Trifluoroacetate	$[BMIM]^+$	$[TFA]^-$	174899-94-6	<-50	1.22	76.78	totally miscible	Enzyme precipitation
1-Ethyl-3-methylimidazolium Ethylsulfate	*$[EMIM]^+$*	*$[ETSO_4]^-$*	*342573-75-5*	*-65*	*1.24*	*101.83*	*miscible*	***at 20% water results are similar to gel formulation.***
1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	$[EMIM]^+$	$[NTF_2]^-$	174899-82-2	-15	1.53	26.13	immiscible	Cleaning with visible residues at 3min but, no improvement after 9 min.
1-Methyl-3-octylimidazolium Hexafluorophosphate	$[OMIM]^+$	$[PF_6]^-$	304680-36-2	-82	1.24	-	immiscible	Similar results to those with gel formulation for both enzymes, at 25% water
1-Methyl-3-octylimidazolium bis(trifluoromethylsulfonyl)imide	$[OMIM]^+$	$[NTF_2]^-$	178631-04-4	-	1.32	-	immiscible	No visible changes after 9 min.
1-(2-Hydroxyethyl)-3-methylimidazolium Tetrafluoroborate	$[C_2OHMIM]^+$	$[BF_4]^-$	374564-83-7	-84	1.33	-	-	Enzyme precipitation
1-(2-methoxyethyl)-3-methylimidazolium Trifluoromethanesulfonate	$[C_3OMIM]^+$	$[TFO]^-$	-	-	1.30	-	-	Enzyme precipitation

Source: physical characteristics according with http://www.aails.com/

Appendix IV Surface characterization by Stereomicroscopy (SM), Optical Microscopy (OM), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM)

Sample surface analysis before treatment with stereomicroscope (SM) 10x, 32x and 90x, optical microscope (OM) Vis. 100x, 200x and UV 200x, scanning electron microscope (SEM) 200x, 1000x and 3000x and atomic force microscopy (AFM) 5µm x 5µm for varnished samples of egg white (EW), Isinglass (fish glue, CP), animal glue (CA) and casein (Ca); and unvirnished samples of tempera (EG) and oil (OG) painting grounds and gilding grounds (GG).

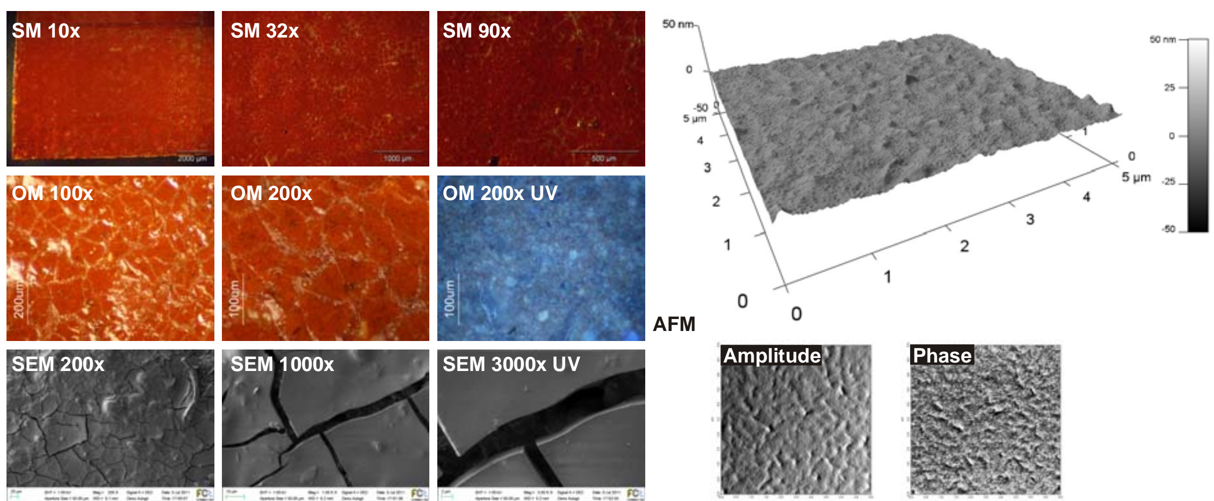

Figure 1 – Surface analysis set for sample OG-EW, oil painting with egg white varnish.

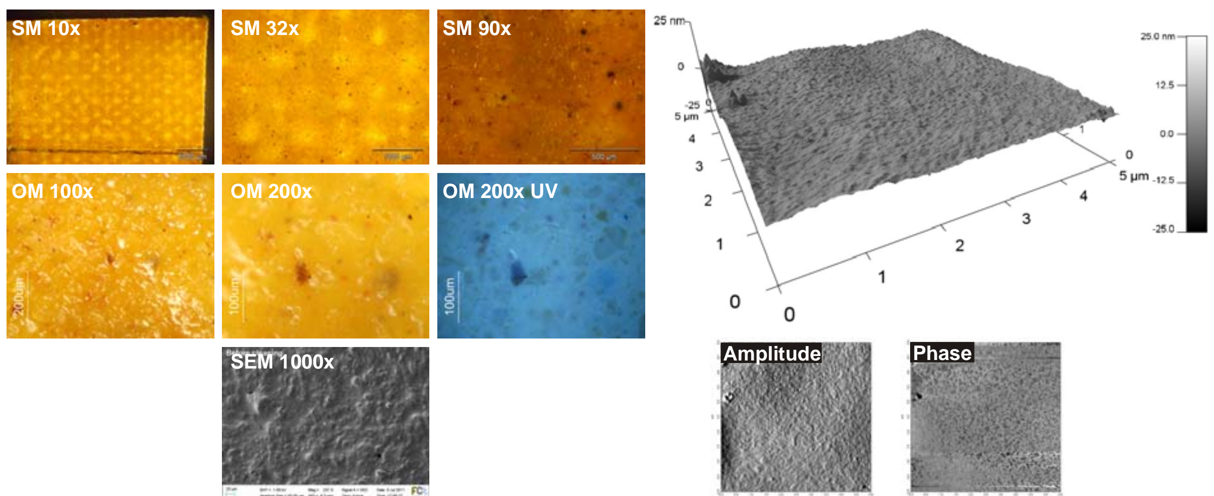

Figure 2 – Surface analysis set for sample EG-CP, egg tempera painting with isinglass varnish.

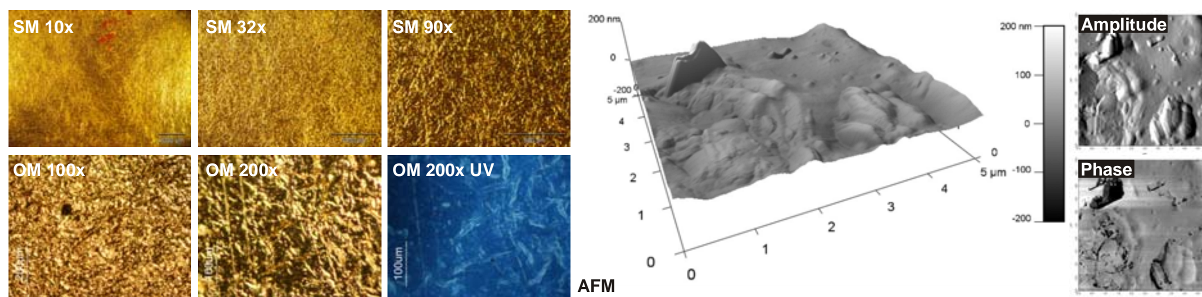

Figure 3 – Surface analysis set for sample GG-CA, gilding with animal glue varnish.

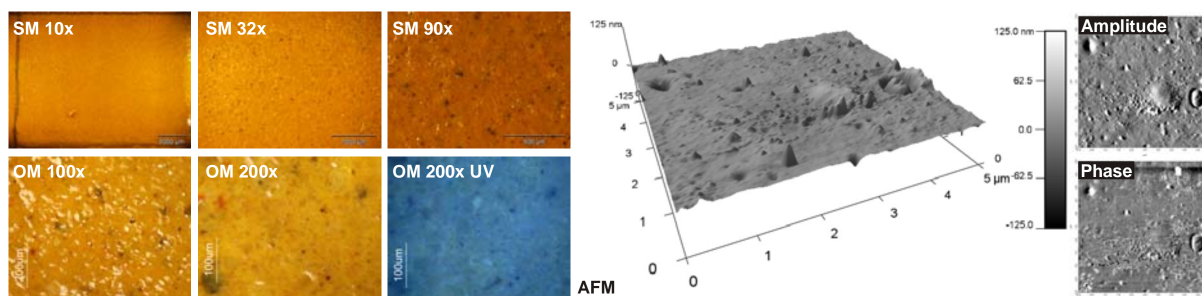

Figure 4 – Surface analysis set for sample OG-Ca, oil painting with casein varnish

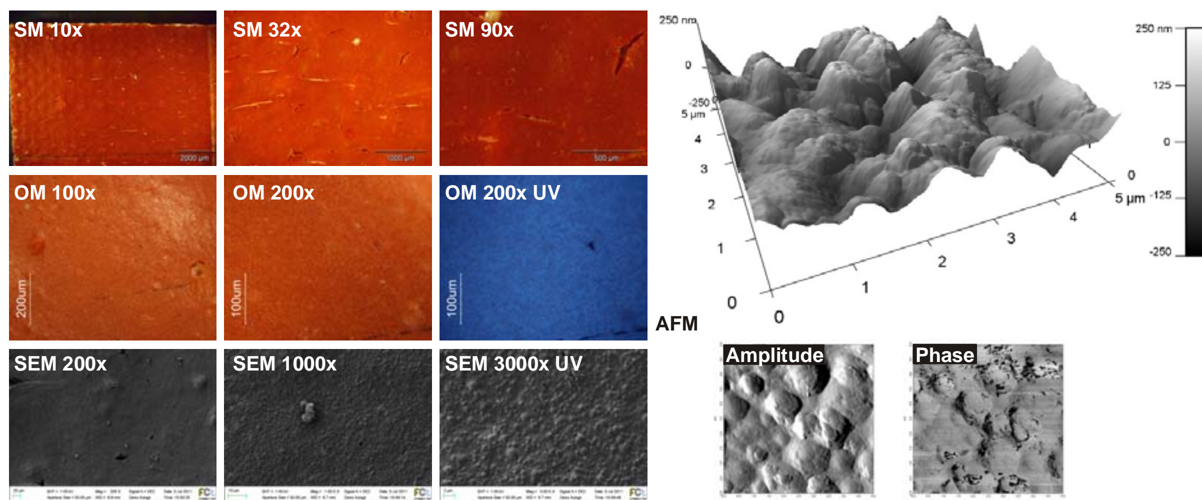

Figure 5 – Surface analysis set for sample EG egg tempera painting unvarnished reference sample.

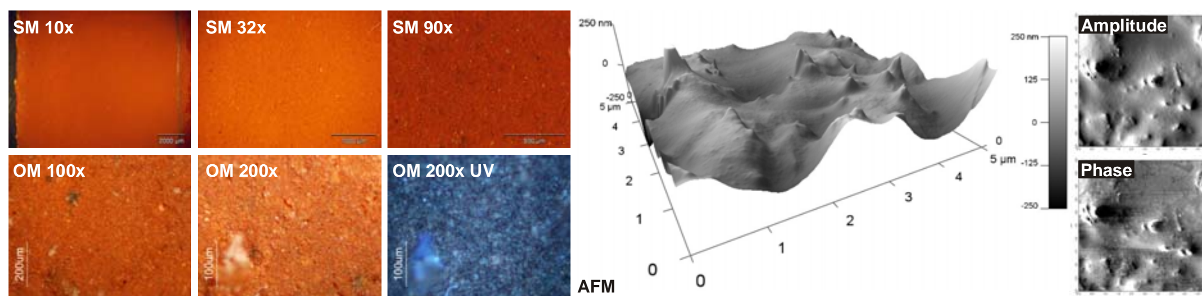

Figure 6 – Surface analysis set for sample OG oil painting unvarnished reference sample.

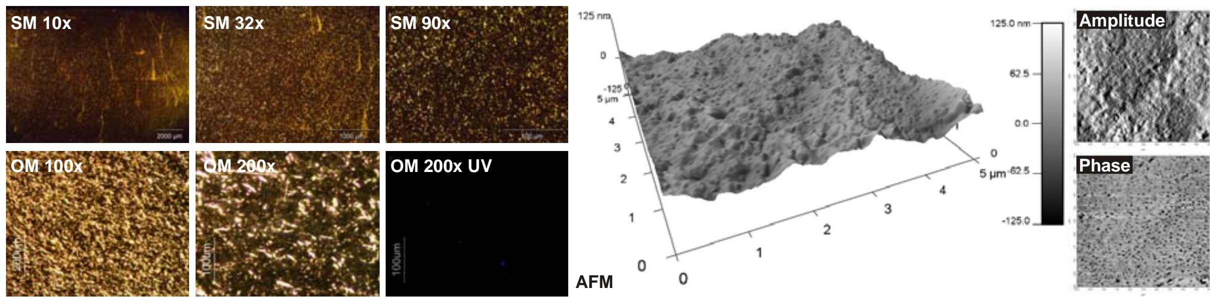

Figure 7 – Surface analysis set for sample GG, gilding unvarnished reference sample.

Sample surface analysis after treatment with the same techniques as before, an example for each cleaning formulation: Pepsin + Gel; *Aspergillus sojae* enzyme + Gel; Yeast extract + Gel; Pepsin + [BMIM] [BF_4]; Pepsin + [EMIM] [$EtSO_4$]; *Aspergillus sojae* enzyme + [BMIM] [BF_4]; *Aspergillus sojae* enzyme + [EMIM] [$EtSO_4$].

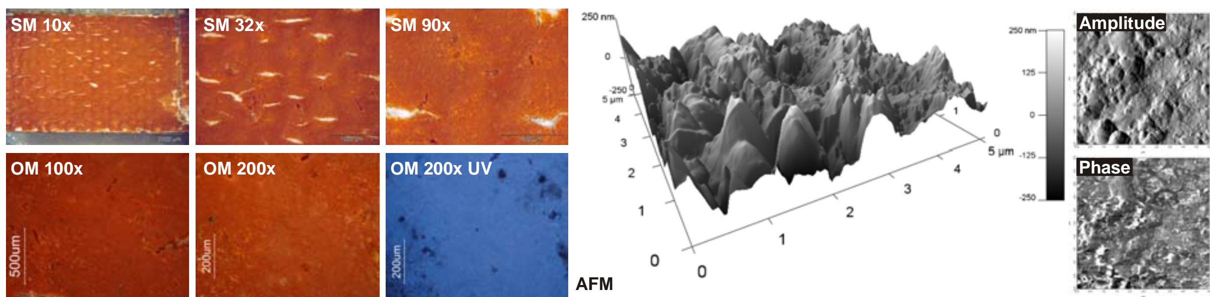

Figure 8 – Surface analysis set for sample EG-EW, tempera painting with egg white varnish, after cleaning with E1+G, Pepsin + Gel.

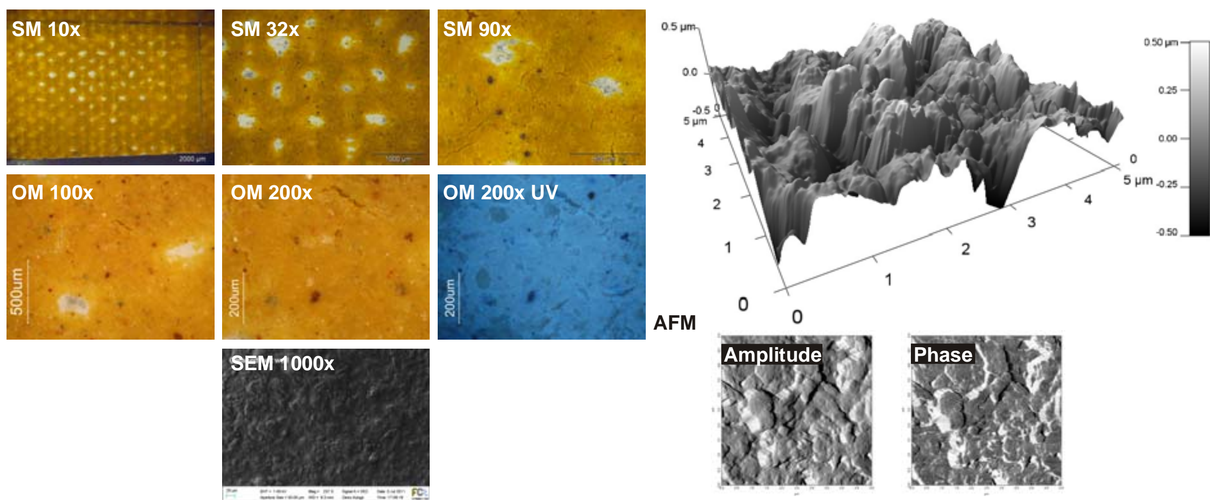

Figure 9 – Surface analysis set for sample EG-CP, tempera painting with isinglass varnish, after cleaning with E2+G, *Aspergillus sojae* enzyme + Gel.

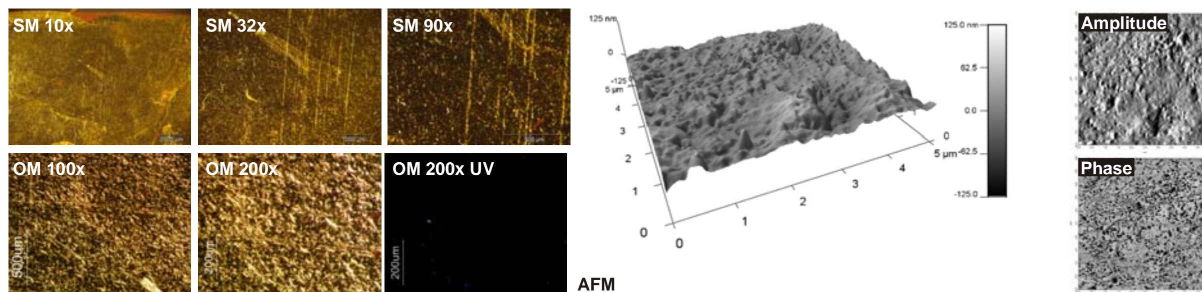

Figure 10 – Surface analysis set for sample GG-CA, gilding with animal glue varnish, after cleaning with E3+G, Yeast extract + Gel.

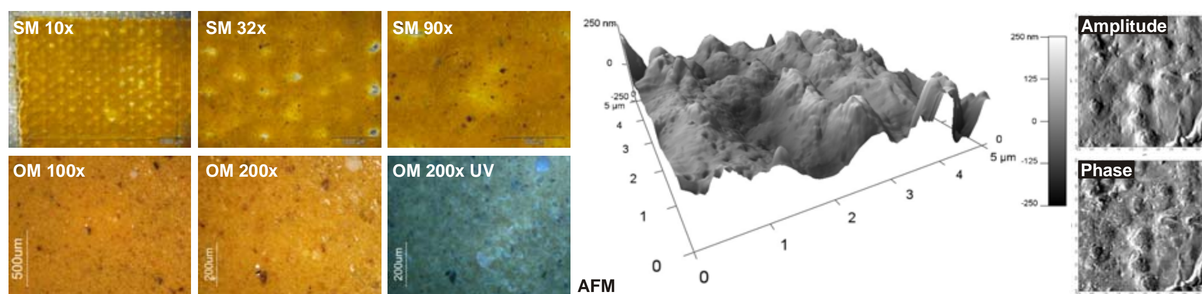

Figure 11 – Surface analysis set for sample EG-CP, tempera painting with isinglass varnish, after cleaning with E1+IL1G, Pepsin + [BMIM] [BF_4].

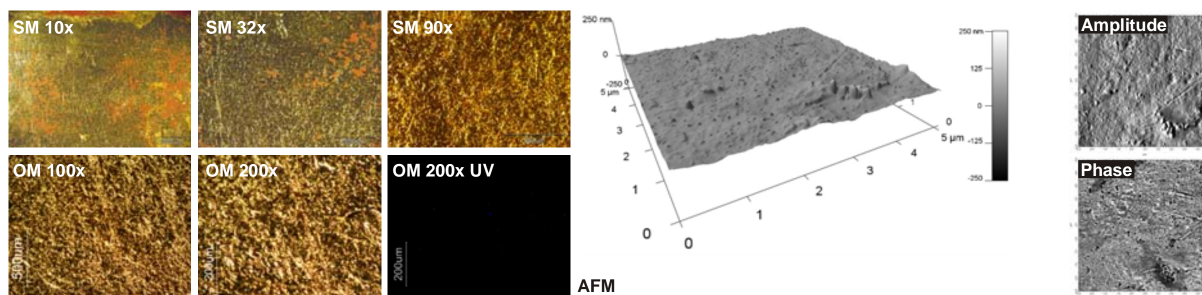

Figure 12 – Surface analysis set for sample GG-CA, gilding with animal glue varnish, after cleaning with E1+IL2, Pepsin + [EMIM] [$EtSO_4$].

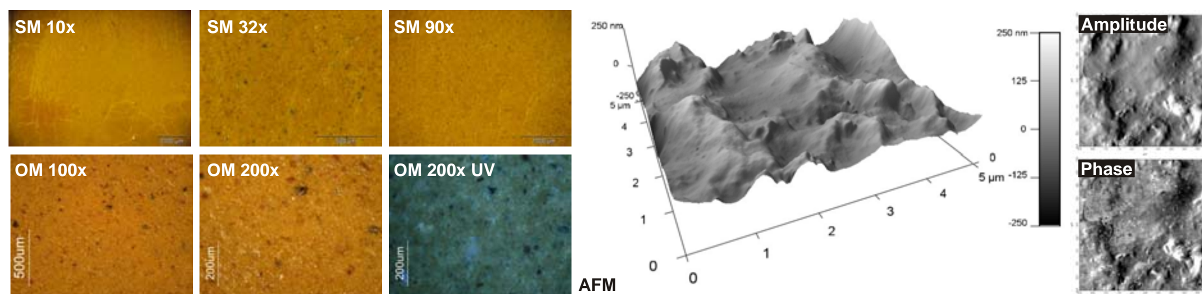

Figure 13 – Surface analysis set for sample OG-Ca, oil painting with casein varnish, after cleaning with E2+IL1, *Aspergillus sojae* enzyme + [BMIM] [BF_4].

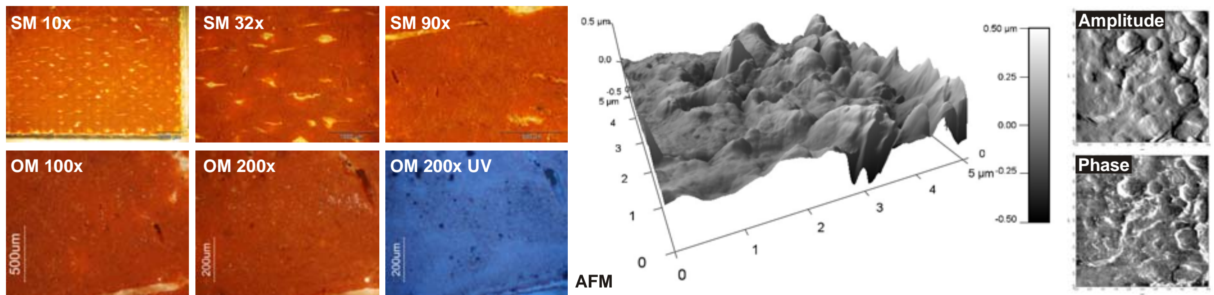

Figure 14 – Surface analysis set for sample EG-EW, tempera painting with egg white varnish, after cleaning with E2+IL2, *Aspergillus sojae* enzyme + [EMIM] [$EtSO_4$].

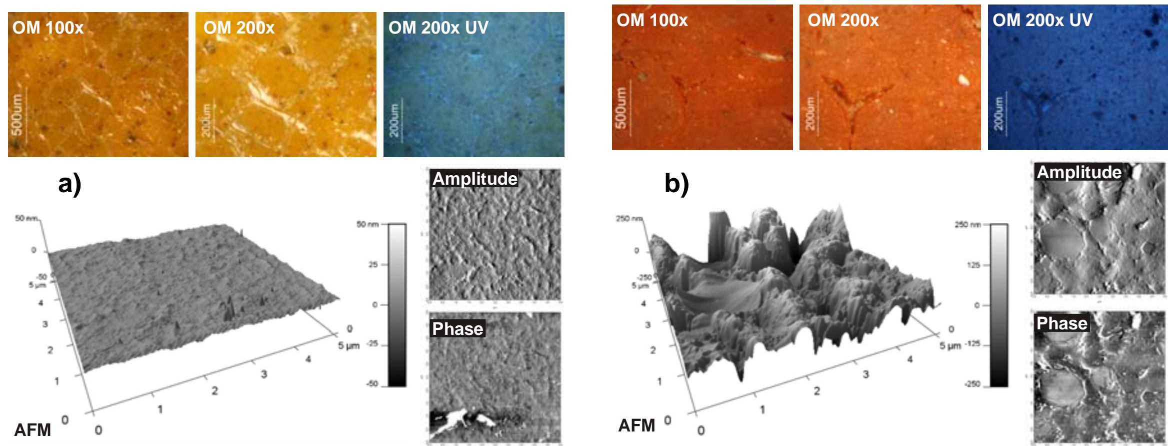

Figure 15 – Surface analysis set for reference samples cleaned only with ionic liquid. a) Left the EG-CP sample after cleaning with IL1, [BMIM] [BF_4]; b) Right the OG-EW sample after cleaning with IL2, [EMIM] [$EtSO_4$].

Appendix V Colorimetry analysis

Table 1 – Average CIE L*a*b* values for all varnish and un-varnished samples, compared values for the expected (ΔE) variation after cleaning treatment, by comparison with unvarnished reference samples.

Unvarnished Samples	L Avrg	L StDv	a Avrg	a StDv	b Avrg	b StDv	expected ΔE
2 EG-S-a	37.50	0.2159	25.44	0.2829	19.51	0.3005	-
1 OG-S-a	35.91	0.0473	23.76	0.2888	19.47	0.3889	-
7 OG-S-a	36.55	0.0643	24.06	0.1484	19.98	0.1510	-
2 EG-YO-a	59.19	0.9602	19.56	0.5093	46.23	0.9965	-
1 OG-YO-a	53.82	0.5624	18.89	0.5326	41.14	0.5907	-
7 OG-YO-a	54.71	0.3272	20.69	0.2778	43.40	0.7319	-
1 GG-a	85.22	0.4661	6.41	0.1007	36.63	0.1270	-
Varnished Samples	L Avrg	L StDv	a Avrg	a StDv	b Avrg	b StDv	-
EG-S-EW	42.16	1.0928	18.11	1.9360	14.45	1.2962	10.05
EG-S-CP	36.97	1.2079	24.86	0.9472	19.06	0.9518	0.91
OG-S-EW	38.23	1.0959	20.55	0.8945	14.50	0.8881	6.35
OG-S-CP	36.32	0.8013	22.10	0.3614	16.06	0.3909	3.81
OG-S-Ca	36.43	0.4609	22.30	0.2182	16.25	0.2500	4.13
EG-YO-EW	61.59	1.9892	16.07	2.8030	41.30	2.0451	6.50
EG-YO-CP	58.92	1.2373	19.24	0.5628	44.79	1.4788	1.50
OG-YO-EW	54.49	1.7775	15.31	1.2828	32.79	2.1961	9.11
OG-YO-CP	52.45	0.7765	16.94	0.7843	37.47	1.5336	4.37
OG-YO-Ca	53.45	1.2133	18.14	1.4112	38.26	1.8490	5.87
GG-CA	82.89	0.4388	7.76	0.2987	42.16	0.3317	6.15

Table 2 – Colorimetric measurements and ΔE values after cleaning treatment.

Sample	Formulation	Avg L before	Avg L after	Δ L	Avg a before	Avg a after	Δ a	Avg b before	Avg b after	Δ b	Δ E
1 EG-S-EWb		41,59	39,19	-2,40	15,03	23,44	8,41	16,47	19,09	2,62	9,13
1 EG-YO-EWb		64,52	63,55	-0,97	11,20	18,32	7,12	44,74	47,00	2,26	7,53
1 OG-S-EWc		37,65	37,93	0,28	21,70	17,37	-4,32	15,59	19,54	3,95	5,87
1 OG-YO-EWb		56,32	58,42	2,10	17,03	12,70	-4,33	34,22	48,24	14,02	14,83
2 EG-S-CPb		37,55	38,26	0,71	24,27	25,09	0,82	18,41	20,54	2,13	2,39
2 EG-YO-CPb	E1 + G	60,35	64,05	3,70	20,09	17,49	-2,60	47,78	45,13	-2,66	5,24
2 OG-S-CPb		36,83	37,35	0,52	22,19	17,75	-4,44	16,11	21,21	5,10	6,78
2 OG-YO-CPb		52,21	54,44	2,23	16,26	11,01	-5,25	35,88	42,47	6,59	8,72
7 OG-S-Cab		36,60	35,39	-1,21	22,22	22,58	0,36	16,14	17,10	0,96	1,59
7 OG-YO-Cab		51,77	53,27	1,50	15,88	16,68	0,80	35,79	36,76	0,97	1,96
1 GG-CAb		82,86	83,64	0,78	7,92	4,93	-2,99	42,60	43,87	1,27	3,34
1 EG-S-EWc		43,05	39,44	-3,61	14,70	24,01	9,32	16,08	18,66	2,58	10,32
1 EG-YO-EWc		63,98	63,27	-0,71	12,00	18,26	6,26	50,22	46,06	-4,16	7,55
2 EG-S-CPc		38,08	37,71	-0,37	23,66	25,36	1,70	17,93	20,42	2,49	3,04
2 EG-YO-CPc		59,24	63,68	4,44	19,47	16,60	-2,87	45,99	41,30	-4,69	7,06
1 OG-S-EWd		38,15	37,99	-0,16	20,68	19,21	-1,47	14,64	19,30	4,66	4,89
1 OG-YO-EWc	E2 + G	52,81	55,45	2,64	16,04	11,09	-4,96	35,40	43,60	8,20	9,94
2 OG-S-CPc		36,72	37,45	0,73	22,38	17,44	-4,94	16,45	20,29	3,84	6,30
2 OG-YO-CPc		52,82	56,18	3,35	17,03	11,61	-5,42	36,72	45,63	8,90	10,95
8 OG-S-Cab		36,55	36,77	0,22	21,99	22,70	0,72	16,04	16,94	0,90	1,17
8 OG-YO-Cab		53,88	54,66	0,77	18,27	18,02	-0,25	38,45	38,93	0,48	0,94
2 GG-CAb		83,18	83,13	-0,05	7,68	8,16	0,48	41,87	40,38	-1,49	1,57
5 EG-S-EWb		42,44	41,03	-1,40	18,51	17,18	-1,34	13,02	20,09	7,07	7,33
5 EG-YO-EWb		59,64	65,06	5,41	18,41	11,79	-6,61	41,85	51,50	9,65	12,89
6 EG-S-CPb		35,52	39,70	4,19	25,30	18,69	-6,61	19,88	22,49	2,61	8,25
6 EG-YO-CPb		58,00	66,33	8,33	19,38	10,34	-9,04	44,77	45,76	0,99	12,33
5 OG-S-EWb		36,66	40,05	3,39	21,39	16,14	-5,25	15,62	17,16	1,53	6,43
5 OG-YO-EWb	E3 + G	53,45	56,45	3,00	14,40	9,94	-4,47	31,23	39,22	7,99	9,63
6 OG-S-CPb		34,97	38,17	3,20	22,05	16,47	-5,58	16,21	17,93	1,72	6,66
6 OG-YO-CPb		53,40	57,01	3,61	17,98	11,65	-6,33	38,61	43,81	5,20	8,96
9 OG-S-Cab		35,74	36,85	1,10	22,29	23,21	0,92	16,36	17,68	1,32	1,95
9 OG-YO-Cab		54,29	55,27	0,98	19,69	18,66	-1,04	40,50	38,71	-1,79	2,29
3 GG-CAb		83,04	84,09	1,05	7,28	7,37	0,09	42,01	39,21	-2,80	2,99

Table 2 – (cont.) Colorimetric measurements and ΔE values after cleaning treatment.

Sample	Formulation	Avg L before	Avg L after	Δ L	Avg a before	Avg a after	Δ a	Avg b before	Avg b after	Δ b	Δ E
3 EG-S-CPa	E1 + IL1	34,81	39,01	4,19	26,33	24,17	-2,16	21,26	18,26	-3,00	5,59
3 EG-YO-CPa		56,92	60,55	3,63	18,86	17,65	-1,21	43,75	41,92	-1,84	4,25
3 EG-S-EWb		40,02	38,11	-1,91	20,77	23,99	3,21	14,90	18,64	3,75	5,29
3 EG-YO-EWb		59,66	61,45	1,79	17,31	16,52	-0,79	38,97	40,22	1,25	2,32
3 OG-S-CPa		37,14	38,28	1,14	22,04	16,62	-5,42	15,97	18,02	2,05	5,91
3 OG-YO-CPa		53,20	52,53	-0,67	16,63	16,76	0,13	36,67	37,13	0,46	0,83
3 OG-S-EWb		38,89	38,49	-0,41	20,80	16,67	-4,14	14,97	18,30	3,34	5,33
3 OG-YO-EWb		56,48	53,80	-2,68	14,38	17,92	3,54	30,42	38,96	8,54	9,62
7 OG-S-Cac		36,77	37,06	0,29	22,40	22,15	-0,25	16,35	16,25	-0,10	0,40
7 OG-YO-Cac		52,99	52,17	-0,82	18,04	17,46	-0,58	37,08	38,05	0,98	1,40
1 GG-CAc		83,41	83,80	0,39	7,49	4,76	-2,73	42,01	44,24	2,23	3,55
4 EG-S-CPa	E1 + IL2	38,22	37,82	-0,41	24,24	26,00	1,76	18,08	20,61	2,53	3,10
4 EG-YO-CPa		59,32	61,95	2,63	19,30	17,61	-1,69	45,61	44,26	-1,34	3,40
4 EG-S-EWb		43,17	38,70	-4,47	19,01	24,93	5,92	13,27	19,72	6,46	9,83
4 EG-YO-EWb		61,01	62,46	1,45	17,59	17,66	0,06	39,23	44,68	5,45	5,64
4 OG-S-CPa		36,42	36,74	0,32	22,21	18,09	-4,12	16,34	21,24	4,90	6,41
4 OG-YO-CPa		53,12	55,00	1,88	20,11	13,58	-6,53	41,03	47,11	6,08	9,12
4 OG-S-EWb		38,22	37,39	-0,83	20,46	16,79	-3,68	14,65	18,47	3,81	5,36
4 OG-YO-EWb		53,28	57,02	3,74	18,74	12,38	-6,36	39,72	47,54	7,82	10,75
8 OG-S-Cac		36,59	37,49	0,89	22,33	23,90	1,57	16,23	18,72	2,49	3,07
8 OG-YO-Cac		53,00	53,49	0,49	17,61	17,69	0,08	37,53	38,63	1,10	1,21
2 GG-CAc		82,15	83,73	1,58	8,07	6,91	-1,16	42,15	36,52	-5,63	5,97
3 EG-S-CPc	E2 + IL1	35,06	39,00	3,94	25,82	24,23	-1,59	20,26	18,99	-1,27	4,44
3 EG-YO-CPc		60,86	65,04	4,18	19,92	16,39	-3,53	49,48	40,22	-9,26	10,76
3 EG-S-EWd		40,62	37,84	-2,78	21,11	25,23	4,12	15,28	20,39	5,11	7,13
3 EG-YO-EWd		60,52	63,95	3,43	17,84	16,59	-1,26	39,66	41,35	1,69	4,02
3 OG-S-CPc		36,82	38,10	1,28	21,94	16,72	-5,21	15,84	18,52	2,69	6,00
3 OG-YO-CPc		51,09	52,03	0,94	15,84	15,93	0,09	36,14	36,01	-0,13	0,95
3 OG-S-EWd		41,71	37,85	-3,87	18,94	17,00	-1,94	13,03	18,79	5,76	7,20
3 OG-YO-EWd		52,08	52,35	0,27	14,76	16,49	1,73	32,27	37,01	4,74	5,05
7 OG-S-Cad		36,60	36,80	0,20	22,37	22,03	-0,34	16,43	16,62	0,19	0,44
7 OG-YO-Cad		51,74	51,20	-0,53	16,19	15,68	-0,52	35,70	36,15	0,44	0,86
1 GG-CAd		83,13	87,07	3,94	8,05	4,89	-3,17	42,72	43,47	0,75	5,11
4 EG-S-CPc	E2 + IL2	38,32	39,99	1,67	23,34	22,12	-1,22	17,51	16,90	-0,61	2,16
4 EG-YO-CPc		58,39	64,48	6,09	19,08	16,63	-2,45	44,39	41,06	-3,32	7,36
4 EG-S-EWd		43,12	39,81	-3,31	19,99	25,31	5,33	13,75	20,73	6,99	9,39
4 EG-YO-EWd		61,90	67,74	5,84	18,34	14,27	-4,07	41,33	37,75	-3,58	7,96
4 OG-S-CPc		36,87	36,82	-0,05	21,71	17,71	-4,00	15,58	20,75	5,17	6,54
4 OG-YO-CPc		52,49	54,66	2,17	18,07	11,77	-6,30	38,45	44,69	6,24	9,13
4 OG-S-EWd		40,03	38,66	-1,37	19,65	16,65	-3,00	13,75	18,04	4,30	5,42
4 OG-YO-EWd		54,36	56,76	2,40	19,22	13,39	-5,82	38,68	48,04	9,36	11,28
8 OG-S-Cad		36,30	37,71	1,41	22,55	21,88	-0,68	16,54	16,50	-0,04	1,56
8 OG-YO-Cad		52,28	53,00	0,72	16,71	17,09	0,39	36,68	37,24	0,56	0,99
2 GG-CAd		83,16	83,13	-0,02	7,60	7,32	-0,28	42,09	37,00	-5,08	5,09

Appendix VI AFM analysis

Table 1 - Roughness values of the studied samples and reference (not varnished) samples

Samples		Before		After	
		Surface roughness (R)	Dispersion (%)	Surface roughness (R)	Dispersion (%)
Reference unvarnished surfaces					
Canvas	EG			59.42	2.76
	OG			61.20	14.49
Wood	OG			73.91	7.55
	GG			11.31	2.26
Average reference values for varnished surfaces					
Tempera + Egg White				6.57	3.04
Tempera + Isinglass				39.93	6.06
Oil + Egg White				2.37	9.05
Oil + Isinglass				34.84	7.08
Oil + Casein				4.54	6.59
Gold Gilding + Animal Glue				14.68	0.83
OG-S-EWb	Gel +E1	2.53	2.29	96.02	1.11
OG-YO-EWb		3.60	11.67	80.44	3.19
EG-YO-CPb		8.01	3.20	102.92	5.16
OG-YO-CPb		30.26	4.82	54.68	0.96
OG-YO-Cab		5.15	19.65	57.17	4.80
GG-CAb		16.03	2.29	12.04	9.58
EG-S-EWc	Gel + E2	1.87	0.04	63.40	11.89
EG-YO-EWc		6.65	0.37	50.77	0.38
EG-YO-CPc		15.41	23.30	96.22	6.80
OG-S-EWc		1.31	2.20	64.20	2.32
OG-YO-EWc		2.49	3.33	48.61	2.87
OG-YO-CPc		35.82	7.19	44.24	3.56
OG-YO-Cab		5.50	2.10	85.60	8.19
GG-CAb		14.80	13.04	18.81	1.42
EG-S-EWb	Gel +E3	5.43	2.15	63.63	1.09
EG-YO-EWb		2.58	2.08	63.98	7.31
EG-YO-CPb		7.20	1.66	47.44	1.69
OG-YO-Cab		6.24	3.86	38.70	1.41
GG-CAb		19.19	2.19	11.10	2.59
EG-S-CPa	IL1+E1	5.47	1.85	76.61	3.33
EG-YO-CPa		6.34	2.78	69.05	2.35
EG-S-EWb		4.15	3.81	75.49	0.37
EG-YO-EWb		1.87	2.44	73.00	7.06
OG-YO-Cac		5.71	3.11	56.88	0.54
GG-CAc		14.03	2.77	12.52	4.79
EG-S-CPa	IL2+E1	25.50	5.61	71.64	5.79
EG-YO-CPa		5.18	4.95	137.95	2.65
EG-S-EWb		2.09	3.52	124.01	3.16
EG-YO-EWb		2.80	0.32	71.55	9.53
OG-YO-Cac		5.23	6.27	75.57	2.34
GG-CAc		13.42	4.83	10.49	11.03
EG-S-CPc	IL1+E2	5.29	0.44	74.62	3.78
EG-YO-CPc		5.79	8.53	72.06	8.81
EG-YO-EWd		5.86	16.84	62.53	6.60
OG-YO-Cad		5.70	8.85	49.80	5.13
GG-CAd		13.18	3.46	56.15	25.06
EG-S-CPc	IL2+E2	21.43	3.67	115.25	2.67
EG-YO-CPc		23.86	1.31	74.08	5.60
EG-S-EWd		4.28	20.52	112.67	6.14
EG-YO-EWd		2.54	7.96	93.88	6.07
OG-YO-Cad		5.95	0.69	28.78	2.10
GG-CAd		14.75	0.94	12.46	4.62

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