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Review

Research on the Organic Binders in Archaeological Wall Paintings

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Abstract: Wall painting realized using organic binders is the oldest form of parietal painting and precedes the birth of the *affresco* by about 20,000 years. This paper reports the results obtained from the main studies in the field of archaeological wall paintings. The attention was paid to the study of organic binders used for the application of the color, as well as on the instrumental techniques chosen to obtain such information. Different techniques can be used for the study of organic material in archeological paintings: non-destructive techniques, which can be applied directly in situ without sampling, and laboratory micro-invasive techniques for a more in-depth characterization. Among these, the chromatographic techniques represent a potential tool to acquire as much information as possible about chemical composition of binders.

Keywords: wall paintings; organic binder; characterization; non-invasive techniques; micro-invasive techniques; GC-MS

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1. Introduction

Wall painting realized using binders is the oldest form of parietal painting and precedes the birth of the *affresco* by about 20,000 years. The first testimonies of parietal paintings made with organic binder date back, in fact, to the Upper Paleolithic: between 40,000 and 10,000 years ahead of Christ. These are cave paintings found in different geographical sites: in Africa, Europe and Asia. They were made using egg binders, blood, blood serum, urine, animal fat, animal marrow and milk. Throughout history the use of organic binders on the wall had a widespread diffusion, at all times, often combined with *affresco* in a mixed technique [1].

Between 18,000 and 10,000 years ago the rock paintings of Altamira (discovered in 1879) and Lascaux (discovered in 1940) were produced. In this case, however, it is not a dry technique with organic binders: the pigments were applied pure on the wall, naturally rich in calcium hydroxide, the surface of which was impregnated by moisture condensation. Thus, a carbonation process took place that incorporated the pigment (lying in compact form) made the rock painting very resistant and durable. It is the same chemical reaction that occurs in the *affresco* technique, but it was developed in later times.

The early wall paintings were prepared by using simple procedures. As evidenced by studies carried out on ancient murals in different cultures paintings, plant tempera, using binders of a specifically vegetable nature, was among the first to be used [2]. It was obtained by "softening" pigments, usually of mineral nature, into binders of a polysaccharide nature (plant gums mostly). In Ancient Egypt dry paintings were made on the wall, with organic binders that were preserved well thanks to the very dry climate. The preparatory layer was based on gypsum, with additions of glue, or based on clay obtained from the Nile slusion (it naturally contains calcium carbonate and mixed with chopped straw to maintain moisture) or clay obtained from the base of the mountain reliefs. The organic binder in which the pigment was tempered could be tragacanth gum, acacia gum, egg, fish glue with additions of starch made from cereals [1,3].

The investigation of the wall paintings in the tomb of Nefertari, the wife of Rameses II, recognized a gum Arabic, without rhamnose which could have come from a local acacia species still growing in the Luxor region. Later, vegetable tempera painting was replaced by animal tempera, characterized mainly by the presence of proteins [4].

Another example of the use of organic binders on the wall are the African cave paintings in Algeria and Libya, dating back to the early Bronze Age. It is dry painting with the use of milk and casein as binders (probably since they were peoples of sheep breeders), application carried out not with hands, but with brush prodrome tools [1].

In Iran, between the 16th and 18th centuries B.C., dry wall paintings were made on cooked and raw brick walls covered with a clay-based preparatory layer and an additional layer of chalk. The pictorial layer is obtained by protein tempera, probably egg [1,3].

The study of documentary sources (recipe book, deeds of expenditure, contracts, inventories, epistolary collections, regulatory documents, treaties and other archival material) can be a good starting point to trace the type of organic binder used [3].

Among documentary sources, spending records are those that provide fairly accurate information about the materials used by the artist, however often the expenses of economic material, such as less valuable pigments or organic binders, are often not marked.

However, the sources do not cover exhaustively and continuously every period and every place, for this reason reconstructing precisely the types of binders used in history is very complex. It should also be borne in mind that these documents offer an often-inaccurate knowledge of the materials used: the use of jargon from compilers not experts in the technique is wide, they can be lacking and lack some parts [5].

The information that can be drawn from these documents does not provide a certain tool of knowledge, the technical terms cannot be interpreted in a single way, they are imprecise. It should also be borne in mind that in the past knowledge was traditionally transmitted orally, so many notions have been lost.

Pliny in Historia Naturalis [6] and Vitruvius in De Architectura [7] talk about Roman painting. Vitruvius refers to the *affresco*: << *Colores autem, udo tectorio cum diligenter sunt inducti, ideo non remittunt sed sunt perpetuo permanentes* >> (However, when the colors are laid out on damp plaster, they do not loosen but remain permanent). Vitruvius also talks about arid painting, that is, *a secco* painting, but there is no chapter in which he specifically delves into the binders, it is logical to think that the wall paintings were executed affrescoed.

The study of organic binders in Roman painting is an open question: the chemical-physical approach has produced vague and partial data, often discordant [8]. A "*affresco* theory" has been formulated in this regard, carried out by Mora and Philippot [1]. He claims that Roman wall painting was exclusively affrescoed. According to this theory, the final aesthetic effect is similar to that of dry technique thanks to the final polishing. However, pigments have been detected that do not tolerate the alkality of lime, also suggested by Pliny [6] and Vitruvius [7] in their writings, therefore not suitable for *affresco* technique.

The Mappae clavicula is a medieval Latin document containing recipes for materials of art and craftsmanship [9].

A literary source regarding the use of organic binders around 1000 D.C. is represented by the book De Diversis Artibus by Theophilus Monaco [10].

The only preserved text that refers to the technique of wall painting in the Upper Middle Age is a passage from the 8th century manuscript of Lucca, whose author seems to be a Greek settled in Italy: the pigments were laid out without organic binder, therefore with the *affresco* or lime technique [1].

The 14th century is a time of great novelty with regard to wall painting. The wall painting of 1300 finds in Giotto its greatest exponent: in the *affresco* technique the use of sinopia is introduced. The preliminary drawing for affresco l, named for the reddish-brown pigment traditionally used to draw or transfer it. In tempera the egg is preferred as a binder, but also milk and skin glue. The oil painting technique on the wall is introduced, linseed oil is widely used [2].

Cennino Cennini tells us about the technique of wall painting used around 1300 in The Book of Art, written at the beginning of the 15th century, but which reports the technique in use since the 14th century [11]. It is the first Italian treatise to deal with oil painting on a wall to which he devotes ample space. Some chapters are also dedicated to the tempera on the wall. For egg-based tempera there are three types of preparation suggested by Cennini:

- Whole egg mixed with fig milk. Dry plaster should be impregnated with water-tempered egg.
- Egg yolk.
- Egg yolk mixed with animal glue.

To understand the pictorial technique, a valuable help is provided by the scientific approach of the diagnostics of pictorial works, which allows the identification of the organic binder. In some cases, chemical-physical analyses, performed on wall paintings, revealed the presence of organic material used as binder for the application of pigments on the surface of dried plaster, as reported by different studies, some of which are chosen to be described in this paper.

Casadio et al. carried out an extensive review of organic materials in mural paintings, examining historical literature and scientific research [12]. In particular, they focused their attention on the paintings in the period between the Middle Ages and the Baroque in Europe, period of great changes, with the aim to collect references about the type of organic material present in paintings. This examination is the result of a basic research for the Organic Materials in Wall Painting project, coordinated by the Getty Conservation Institute, Los Angeles. The acquisition of such data revealed very important firstly for the knowledge of organic materials used in wall paintings and then for the development of analytical procedures for their characterization, in a perspective of in-depth knowledge of painting techniques for art history studies and to identify suitable methods for conservation interventions [13,14].

In this review, various articles have been considered, choosing the wall paintings of the archaeological period. It began by describing the applications of non-destructive techniques to identify organic binders in wall paintings and continued by considering destructive techniques, with particular emphasis on gas chromatographic techniques, being those that have provided the most numerous and important results.

2. The Binders in the Wall Paintings

For the realization of wall paintings and, in later times, for their preservation, different materials with functions of binders, adhesives, paints, protective and consolidating were and are still necessary. There is a very large class of products which, as far as I know, can have both constitutive functions but also a function of conservation and restoration. Until the mid-1900s, for one or the other destination, the main source of supply of these products was the natural one, without prejudice to a quantity of manipulations to extract, purify, slightly modify them, etc.

Binding media are complex materials, employed to allow pigment grains to adhere to each other and to the surface of the support, through the formation of a coherent and homogeneous film.

The function of the binder consists, therefore, in keeping the pigment particles firmly together and at the same time adhering them in the form of a coherent thin film to the surface of the support. The binder must obviously be in the fluid state, in order to form with the pigments a stable, homogeneous, stretchy, and viscous dough.

The choice of the type of medium used to paint the wall depends on various factors, such as the type of pigment chosen, the historical period and the geographical location.

Specific chemical, physical and optical properties are required for the binder, in order to fully perform its function and they can be summarized as follows:

- Cohesive and adhesive: to allow the cohesion of pigment grains and their adhesion to the support in the form of a thin film, adherent and well consistent to the surface. For this purpose, the painting medium must therefore possess filmogenic properties, in

order to create with pigments a homogeneous fluid, with a right degree of viscosity, in such a way as to be laid easily; after application it should have the property of drying in a solid, resistant, non-sticky and elastic layer, so that it can last over time. The stability of this suspension should also be left to completed and dried paint.
- Chemicals: the binder must not react chemically with the pigment, nor solubilize it; it must also have a high resistance to photochemical reactions, atmospheric chemicals and all those substances that could cause its degradation.
- Protective: to isolate pigment particles from the atmosphere preventing their alteration.
- Optics: transparency and absence of color are the main qualities that a good binder should possess, so as not to significantly alter the optical characteristics of the pigment.

The principal materials used as binder in archeological wall paintings were natural, based on proteinaceous, lipidic, glucidic or waxy material.

The proteinaceous materials most used in the artistic field as pictorial binders in tempera technique, as well as protective, adhesive and consolidating, are obtained from the following materials:

egg, which can be used either whole, or separately yolk (red) or egg white (white);

animal glues, extracted from scraps of animal skins and other cartilage parts (skin glue), mammalian bones (bone glue) and various parts of fish (fish glue);

milk and casein.

The most widely used polysaccharidic materials in the artistic field refer to sugary substances deriving from vegetable gum exudates, honey, steeds and starches contained in cereal seeds, tubers and other parts of green plants. The types of vegetal gums most used historically were: arabic gum, tragacanth gum and gum from fruit plants. In this regard, Pliny the Elder reports "... it also indicates rubber glue and for Egypt's best tragacanth rubber; then the one extracted from almonds, cherries, plums" [6]. Glycerolipids, oils and fats, are a heterogeneous class of natural organic compounds of both plant and animal origin. The term "oils" refers to liquid materials at room temperature; the term "fat" to solid or semi-solid ones. Among all the substances with filmogenic characteristics are of particular importance in the field of pictorial techniques and they mainly contain triglycerides, that is, esters of glycerin with saturated and unsaturated fatty acids in long linear chains. The principal lipid binders used in painting are characterized by the presence of drying oils, i.e., linseed oil, walnut oil and poppy oil. The whole egg and yolk can also be considered partially lipid binders since besides to the protein part they contain a high amount of lipid material.

A possible alternative material used in wall paintings was natural waxes, which could derive from animals (beeswax, Chinese wax, lanolin, spermaceti wax), vegetables (carnauba wax, candelilla wax) or mineral (paraffin, mountain wax, ceresin). Waxes are solid substances that easily merge. The natural waxes are composed of a mixture of fatty acids, free alcohols, hydrocarbons with long chains and fatty acids esterified with long-chain alcohols [15].

Different factors can affect the choice of the binder to use in wall paintings, i.e., the presence of pigments, which can be more or less stable in specific conditions, the exposure of the work and the knowledge in the time. Moreover, the information about the type of materials present in wall paintings and the knowledge of their chemical-physical properties results very important for the conservators in case of restoration interventions.

3. Techniques and Procedures to Characterize the Organic Binders in Wall Paintings

The characterization of organic materials in the wall paintings and their chemical-physical properties is fundamental for the knowledge of ancient paint techniques and for development of suitable conservation procedures.

The Getty Conservation Institute (Los Angeles) developed an international data collection project (Organic Materials in Wall Paintings, OMWP) for the characterization of organic materials in wall paintings, by means of either non-invasive or micro-invasive techniques. The study was based on the analyses of known composition replicas deriving from Affresco Workshop of Vainella (Centro Tintori, Prato, Italy), in which instrumental potentiality and limits in the characterization of organic materials in wall paintings were compared [14,16].

Firstly, non-invasive techniques, which require the use of portable instruments for a general analysis in situ without sampling, can give information about the distribution of organic materials on the wall painting especially in the surface. Non-invasive methods represent a speed procedure to obtain general information about the composition of paintings, since they allow to acquire repeated measures in different points, by also identifying eventual regions contaminated by materials used in past restoration interventions. Moreover, the identification of the most significant regions can be helped by the use of imaging techniques and scan of the surface. In this way, it is possible also to select the most suitable points for sampling when a more in-depth investigation is required.

Interpretative caution is always required in their use, as there is a risk of incomplete or partially distorted reading of data for certain component substances. In recent years, the various non-invasive techniques have been used in combination with each other to obtain a wide complete knowledge of the analyzed pictorial artifact. The characterization and the mapping of organic compounds is a challenge for the non-invasive technique of wall paintings.

A wide range of non-invasive techniques are available, some of which can be summarized as follows [17]:

- UV-induced fluorescence. Ultraviolet-induced fluorescence is an imaging technique that records visible light emitted from certain materials in the form of fluorescence when these materials are exposed to UV radiation. This technique allows to obtain information about the presence of fluorescent materials, i.e., colorants, varnishes, binders based on egg or oils, restoration products and any material which shows fluorescence after UV light excitation [18,19]. Moreover, it is possible to use also portable multispectral imaging systems, which can identify the presence of fluorescent materials in wall paintings in relation to their multispectral properties [20].
- Time-resolved laser-induced fluorescence spectroscopy (TR-LIF) and fluorescence lifetime imaging (FLIM). These two methods allow to detect the presence of organic fluorescent materials and their distribution in the wall paintings [21].
- Mid-Fourier transform infrared spectroscopy (FTIR) fiber reflectance spectroscopy. The FTIR spectroscopy by portable instruments is commonly used to obtain information about the presence of both organic and inorganic material [22–24].
- Hyperspectral imaging in the NIR and mid-IR region. Present scientific attention is focused on the progress of mapping/imaging multi-/hyperspectral methods, since area examination naturally meets the demands of a holistic art approach by revealing not only the chemical composition of painting materials but also their semi quantitative spatial distribution with respect to what is visible to the naked eye.
- These methods have been assessed and provided promising results in other types of paintings (with a clearly higher binder to pigment ratio) but possibly further developments will allow their successful application for the screening and mapping of also in wall paintings [25–27].
- Raman spectroscopy. It has been used for many years in the study of cultural heritage, especially for the examination of materials of inorganic nature, but considered potentially usable for the study of organic media, as exposed by some papers carried out on pure materials and pigment/binder mixtures [28]. Some authors consider Raman microscopy the ideal technique for the investigation of materials used on works of art because it is very sensitive, reliable, specific, nondestructive, with high spatial resolution (typically ranging between 1 and 10 μm) and can be used in situ, therefore avoiding any sampling and consequently any damage to the item under investigation [29].

- Integrated multispectral imaging systems. This type of imaging system is based on the selection of specific wavelengths and the acquisition of black and white images, in order to underline the presence of non-homogeneous regions in the surface This technique provides non-invasive mapping and classification of materials with different chemical and optical properties. It is mentioned here, because it is very interesting, but there are no known applications that have allowed to characterize organic binders [30].
- Fiber optic reflectance spectroscopy (FORS). The FORS technique gives information about materials by analyzing their optical properties [31].

Very often to complete the study it is necessary to obtain specific information about chemical composition, the presence of degradation products or information about the stratigraphic distribution of organic binding media in the wall paintings. For this reason, after a general investigation performed by non-invasive methods, the taking of samples is necessary, in order to carry out a detailed characterization of analytes.

For this reason, when we decide to arrive at an in-depth characterization of the organic binder, it is necessary to resort to laboratory investigation techniques on pictorial material, thus having to provide for a sampling of material from wall painting.

When dealing with sampling of the paint fragments you need to be aware of important issues.

A relevant limit in the study of organic materials in wall paintings is represented by the low quantity of these, compared to inorganic materials (mortar and pigments).

Since not more than 1 mg of sample is generally obtained from sampling and being the organic fraction lower than the inorganic one (about 10% w/w), sometimes the quantification of analytes results very difficult, by obtaining a low signal to noise ratio and signals lower than the instrumental detection limit. Moreover, the possibility that organic components could be present in mixture and that they could be subjected to degradation phenomena in relation to their stability in time, their detection results very complex, above all if only some traces are present.

Finally, the presence of materials used in past conservative interventions, i.e., fixatives based on egg, can alter the original composition of the paintings, and makes the identification of original organic materials more difficult.

The selection of the most suitable regions where to take the samples requires a lot of attention and should be performed after careful observation of the surface, in relation to the information to be obtained.

This is why the use of several complementary analytic techniques may be necessary.

Further and more in- depth characterization of organic fraction contained in samples can be obtained by different invasive-techniques, some of which can be summarized as follows:

- Fourier transform infrared spectroscopy (FTIR). The FTIR spectroscopy is a simple and fast method to obtain information about the organic components (by identifying the class of materials present) and the inorganic materials, through the interpretation of the characteristic vibrational modes of the functional groups when they interact with NIR light [32,33]. Such technique can be used in transmission or attenuated total feflectance (ATR) mode, in relation to the physical morphological properties of samples. The possibility to couple the ATR-FTIR to a microscope allows to perform a punctual analysis of the sample surface and evaluate the distribution of materials [34,35].
- Thin-layer chromatography (TLC). It is a chromatographic technique of simple preparation and rapid execution; this makes it particularly suitable for carrying out qualitative or semi-quantitative evaluations of organic materials. The main advantages of TLC are its low cost and the relative speed of analysis [36].
- High performance liquid chromatography (HPLC). A review shows the application of HPLC for the characterization of organic materials in historical objects. The aim of the document is to compare different separation methods and detectors, taking into account the selectivity and sensitivity of the analysis, and to present future prospects for the application of high-performance separation techniques in archaeometry [37].

- Gas chromatography-mass spectrometry (GC-MS): it provides qualitative and quantitative determination of different analytes, such as amino acids, fatty acids, sugars, compounds constituents of the wax [38,39].
- Pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC-MS). It is a potent technique for the identification of organic materials present in painting. The thermal degradation of macromolecules (i.e., oils, proteins, polysaccharides, etc.) using heat (thermal energy) generates smaller pyrolysis products, which are easier to identify and study. Some of these pyrolysis products are molecular markers consenting the identification of a specific [40,41].
- Organic mass spectrometry. An important text on the application of organic mass spectrometry offers an overview of the examination of art and archaeological materials using techniques based on mass spectrometry [42].
- Time of flight secondary ion mass spectrometry (ToF-SIMS). It is a leading technique to obtain molecule-specific chemical information from surfaces with molecular layer surface sensitivity [43].
- Enzyme-linked immunosorbent assay (ELISA). ELISA is an antibody-based technique generally used in biological examination to exactly recognize small samples of protein or other biological macromolecules using a reliable colorimetric test [44].

4. Exploring Research Results

Scientific investigations carried out in recent decades on a number of wall paintings have revealed some important information about the techniques and the use of different materials, in relation to the geographical sites where the works of art are located.

It was decided not to illustrate the research in chronological order, but to start with those that predicted the use of non-invasive techniques, and then move on to those conducted on samples taken from wall paintings to carry out laboratory investigations.

4.1. Non-Invasive Techniques

Rosi et al. (2009) have proposed a study with the aim is to develop a method for the not invasive and in situ identification of organic binders in wall paintings by fiber optic fiber optic reflectance infrared spectroscopy [22]. As part of the OMWP project, wall painting replicas of known composition from the collection of the Tintori Center in Prato have been employed to classify and interpret reflectance mid-FTIR spectra [13].

Striova et al. have proposed a recent interesting review on the use of reflectance imaging spectroscopy in heritage science, with a focus on painting materials, pigments, binders in wall paintings. The authors illustrate, very accurately, the state of the art of the synergistic application of reflectance imaging spectroscopy in the UV-VIS-NIR-midIR region and other non-destructive techniques for the analysis of works of art [45].

Moreover, within the OMWP project, studies were carried out using time-resolved fluorescence spectroscopy and fluorescence lifetime imaging for the purpose of detecting organic materials in wall painting Leonetto Tintori replicas. The authors pointed out that the fluorescence has high sensitivity, but that many open answers remain to investigate organic binders in ancient wall paintings [46].

Formerly, Comelli et al. proposed the portable fluorescence multispectral imaging system for the analysis of heterogeneous surfaces associated with wall paintings, carrying out a specific study on Masolino wall paintings. They had the results to illustrate the original technique employed for finishing decorations [19].

Researchers from the University of Perugia (Italy) carried out interesting work using non-invasive techniques. They used in situ investigations a simply manageable fiber-optic Fourier transform mid-infrared (mid-FT-IR) reflectance spectrophotometer to study the Renaissance mural painting by Pietro Vannucci, called il Perugino, located in the church of Santa Maria delle Lacrime (1521), Trevi, Perugia, Italy. They have defined the organic binders for the class to which they belong [23].

Vandenabeele et al. (2000) have proposed the use of micro-Raman spectroscopy for the identification of binding media in paintings. This non-destructive and micro-analytical technique has been used to obtain a database of 26 spectra of organic binders and varnishes. These painting materials have been classified, according to their chemical, into four main groups. The spectra obtained establish clear distinctions allowing a fast and non-destructive identification of the medium present in the work of art [25,47].

A paper by Nevin et al. (2007) presents Raman spectra acquired from reference materials obtained by thin films of proteinaceous materials, which are frequently used as binding media in painted works of art. The aim of this work was to propose a new and nondestructive alternative based on Raman spectroscopy to other techniques usually used for the investigation of proteinaceous materials [48].

Casadio et al. (2018) present an interesting overview on Raman spectroscopy applied to cultural heritage. They considered applications, new frontiers in instrumentation, sampling modalities and data processing [49].

4.2. Invasive Techniques

One of the very first articles on the characterization of binding medium, conducted on samples taken from the wall painting, was carried out by De Silva [50]. In this work it has been proposed the use of thin layer chromatography for a preliminary recognition of unknown compounds, using standard reference substances.

Masschelein-Kleiner and Tricot-Marckx have conducted studies on wall paintings paintings in Pompeii, taking samples, analyzed by means of preliminary FTIR spectroscopy analysis and TLC. They identified the presence of gums [51].

A stratigraphic evaluation of wall paintings could be achieved by preparing cross sections of the sample in resin and exposing them to UV-VIS investigation, before and after the application of specific stain examinations. In this way it is possible to identify the presence of organic constituents (specific classes of organic materials, i.e., proteins, lipids and gums) and their distribution in the layers [52,53].

Sotiropoulou et al. (2016) have carried out a study, using Fourier transform infrared spectroscopy on thin sections of samples taken from prehistoric, Roman, Hellenistic and post-Byzantine wall paintings, in order to study the aging and the decay of organic binders and to understand the link with the formation of metal oxalates/carboxylates [54].

Birstein studied the organic components of grounds and pictorial layers in samples from central Asian and Crimean wall paintings. A first result allowed to see the presence of gelatin in mansur-depe wall paintings. In other 8th century wall paintings, the use of the plant has been found, by investigation with infrared spectroscopy, polysaccharides attributed to the Prunoideae sub-family [55,56].

Dneprovskaya studied the XII-XIII century wall paintings of David-Garedji, in Georgia, dating to the 10th and 11th centuries. She found that gypsum plaster has animal glue and that pigments are laid out using egg yolk mixed with animal glue, using TLC, UV spectroscopy and electrophoresis to analyze samples [57].

The analytical research techniques that are presently most common in characterization of the organic media in mural paintings are infrared spectroscopy (FTIR), gas chromatography (GC-FID, GC-MS), pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS), and high-performance liquid chromatography (HPLC).

Interesting papers present overviews of the literature highlights that the analytical approach for use in GC-MS analysis of organic media in cultural heritage samples is strongly dependent on the specific problematic posed by conservators and art historians. The complex mixtures of molecular species present in organic materials, the requests to be answered, and consequently the analytes to be examined for, determine the choice of analytical approach, especially concerning sample pretreatment [58,59].

A problem may arise when the extraction method used is not satisfactory for an ancient binding medium, then part of the binder may not be extracted, producing an incomplete characterization of its composition [8].

A very recent article addresses the problem of extraction of collagen-based binders (animal glue) in mural paintings. This work considered non-aged and aged samples and presents a comparative study on the extraction effects of six agents, used to extract the residual proteins. The protein extraction efficiencies of the selected agents were quantitatively determined by bicinchoninic acid method, and then processed by multivariate analysis of variance. The authors claim that, for both non-aged and aged samples, the extraction efficiency of 2 M guanidine hydrochloride was significantly higher than the other five agents, with less damage to the protein structure during the extraction procedure [60].

A study looked at an extensive series of standard compounds, reference pure materials, and reference paint materials prepared at Opificio delle Pietre Dure in Florence (Italian Ministry of the Cultural Heritage) Italy, simulating ancient painting techniques, using GC-MS to determine amino acids and fatty acids, and submitting the results obtained to statistical analysis [61–63].

An analytical procedure carried out on reference paint materials for the simultaneous characterization of proteinaceous binders, drying oils, natural waxes, plant and animal terpenoid resins on the same microsample using GC/MS has been proposed [39].

Another paper takes it into consideration again the reference paint materials prepared at Opificio delle Pietre Dure in Florence, Italy: the fatty acid distribution for lipid binders and the amino acid content for proteinaceous media were determined by GC-MS before and after being artificially aged through exposure to UV light, under defined conditions. The Authors noted that UV aging processes do not significantly affect protein degradation binders, while influencing that of lipid binders to some extent [3].

A mini review on analytical pyrolysis to investigate organic substances on wall paintings has been illustrated. A micro-sample (50–100 µg) is destroyed during the analysis, but the absence of sample preparation makes Py-GC-MS a very attractive technique with a much-reduced analytical time and cost compared to other chromatographic [64].

Research of Rampazzi et al. was carried out on Neolithic wall paintings by Sos Furrighesos necropolis, a very important funerary monument in Sardinia, Italy. The use of GC-MS revealed egg in the samples taken, applied directly to the stone surface of the graves. It was believed that for the first time the use of egg binder in Neolithic mural paintings was found [65].

Another work by Rampazzi et al. examined a Neolithic hypogeum site located in Sardinia, decorated with red and black wall paintings. This is Domus de Janas (Home of the Fairies, or Home of the Witches). Again, the goal of the work was to understand the pictorial technique. The result obtained, analyzing samples from different graves, confirmed the data found in the previous study, namely the use of egg, as a binder to lay out the color [66].

Brecoulaki et al. examined Mycenaean wall paintings in the "Palace of Nestor" in Pylos (Western Messenia, Greece), dated from the Late Bronze Age (ca. 1200 BC), for the purpose to establish the first comprehensive analytical documentation for the study of the painting techniques practiced during the Late Bronze Age. The samples studied using GC-MS and Py-GC-MS showed egg, animal glue and plant gums. The authors believe that, for the first time, tempera technique has been identified for such an early period in the history of ancient Greek mural painting [67].

A study has been carried out on the polychromy of some fragmented architectural reliefs from the Palace of Apries in Memphis, Egypt (26th Dynasty, ca. 589–568 BCE The samples taken were studied using FTIR to identify the class of organic substances and GC-MS. Polysaccharides have been found, identified as plant gums, used as binding media, in some samples. Other fragments showed animal glue presumably mixed with egg. Another interesting information concerning the presence of synthetic wax (applied during ancient conservation treatments) and as beeswax, on the surface of the paintings [68].

A paper of Colombini et al. shows the analysis of a wide range of raw plant gums, watercolors, and naturally aged reference painting specimens with the aim to optimize the analytical procedure, but also to collect data for constructing a data set, which is useful for binder identification when a chemometric method such as principal component analysis (PCA) is applied. The analytical methodology was applied to the study of wall paintings (4th–3rd centuries B.C) of Macedonia [69].

A study on the wall paintings of the Abu El Leaf Monastery, also known as Deir Abu Lifa, is considered one of the most important and famous monasteries in Fayoum Oasis, Egypt, was carried out. Arabic Gum as organic binding medium suggests that tempera technique was used in The Abu El Leaf Monastery in Fayoum Oases [70].

A study conducted by Guasch-Ferré et al. (2019) considered samples of Maya wall paintings of ten archaeological sites situated in Campeche and Yucatan regions. This work, carried out by GC-MS, defined the presence, in significant amounts, of a series of monosaccharides, being glucose and mannose between the most abundantly found. This detection opens the question of the deliberate employ of these organic polymers as additives to improve workability and mechanical properties in the preparation layer mortar and to confer cohesion to the pigments in the paint layer. Unfortunately, the small sample size and sensitivity of the analytical technique prevent the identification of the vegetal gum used. Identification is further complicated if it is assumed that the samples from wall paintings are composed of mixtures of polysaccharide materials from bark and various parts of plants, which can be easily extracted in water [71].

Pallecchi et al. conducted an extensive study on the pictorial technique used in the Etruscan tomb "Tomba della Quadriga Infernale" in Sarteano (Siena, Italy). The numerous pigments used have been identified and the presence of egg has been found, by GC-MS, as a binder for the drafting of polychromy [72].

Still talking about Etruscan painting mural, a study in ancient city of Caere near Rome, Italy, identified the limited pigments used and did not find the presence of an organic binder, suggesting a lime water binder [73].

A study of Corso et al. has employed spectroscopic, techniques and GC coupled to flame-ionization detector to study binders of a mural painting fragment from Liternum (Italy) archaeological excavation [74]. The results obtained were compared with those obtained from the examination of collected samples in Pompeii from "Imperial Villa, Insula Occidentalis" suggesting that in both cases tempera painting with binders of animal origin had been used [75,76].

A paper by Corso et al. (2012) considered Pompeii wall paintings samples of different age. FT-IR spectra obtained from the Pompeii samples of different age showed almost overlapping patterns with very few differences thus suggesting a quite similar chemical composition among the samples, therefore not showing variations in the historical periods considered [77].

Several other studies have been conducted by Corso et al. on Pompeian wall paintings. The authors, after extraction of the pulverized paint powder with polar and nonpolar solvents, used liquid chromatography, gas chromatography with flame ionization detection, and gas chromatography-mass spectrometry to estimate the free amino acids, and fatty acids profiles [74,78].

Research carried out by Duran et al. has employed several analytical techniques for study Roman mural paintings from House of the Golden Bracelet in Pompeii and the Villa of Papyri in Herculaneum. Organic binders have been sought using infrared spectroscopy and Py-GC-MS. The results of FTIR spectroscopy indicate the presence of some glue or gum compound type. The completion of the study showed vegetable wax or beeswax and some types of protein and gum compounds, thus highlighting the presence of dry paint [79].

Research on Insula del Centenario in Pompeii was conducted. The objectives of this study are two: to locate the presence of protein and lipid materials to spread pigments and identify organic matter in painting materials due to previous restoration work. Small amounts of sample (0.1–0.8 mg) were taken by scraping the surfaces of the paintings with a bistoury (Figure 1). Samples collected from wall paintings from different rooms were studied by Fourier Transform Infrared Spectroscopy and GC-MS. Analytical results show that these Roman wall paintings were made without the use of lipid and protein materials, presumably in the *affresco* technique. In addition, wax, eggs and animal glue have been identified, the presence of which is due to previous restoration work [80].

Figure 1. The picture of Season in room 42 (the antechamber of the so-called erotic cubiculum) of *Insula* del Centenario, Pompeii, Italy, where sample 42/2 was collected.

A paper has been published on Villa of the Papyri in Herculaneum, in order to know the presence of an organic binder. The authors found egg, by means of GC-MS, in some samples, where the pigment required *a secco* technique. They also investigated the presence of wax employed as superficial treatments of the wall painting. The fact that they didn't detect beeswax in all the analyzed samples give further information that the encaustic technique was not used for the realization of the wall paintings of the Vesuvian area [81].

An interesting work examined the wooden roof and ceiling of the House of the Telephus Relief in Herculaneum. The goal of this study was to assess the techniques used to decorate the wooden ceiling, using a multianalytical approach (SEM/EDX, FT-IR spectroscopy, GC/MS and Py-GC/MS). The results showed that a tempera painting has been chosen. Egg was employed as binding medium, as confirmed by the analysis of the proteinaceous fraction and by the presence of cholesterol. In only one sample, casein was determined, probably employed as an adhesive. The paint layer was directly applied the wooden ceiling, without a preparatory stratum. A motivating aspect of this paper is that the authors argue that, based on chemical investigations, this is the first time it has been possible to prove that egg tempera has been used to decorate wooden architectural elements [82].

Cuní highlights the fact that investigations of ancient Roman wall painting show the difficulty of efficiently extracting organic binders. The author considers that sometimes it can be considered an affresco painting, due to the lack of results obtained, when the organic binder may be present, but not identified for purely analytical problems. The use of inappropriate extraction methods for ancient paint supports can cause the transformation of some components of the binder, producing an incomplete characterization of the composition He pointed out that chemical analyses of Roman wall painting have identified other types of paint binders and that there is a clear difference between the results of one research group and the other. These factors could be related to the difficulty of extracting ancient painting binders and, in addition, the influence of microbiological contamination. The absence of data in most studies on the efficiency of extraction actions used in antique samples and the effect of microbial contamination may give rise to doubts about some of the results obtained [8].

Cuní et al. (2012) studied eight samples of Roman wall paintings from three archaeological sites in Spain using full attenuated reflectance Fourier transform infrared spectroscopy and GC-MS, in order to recognize organic binder. It was seen the samples featured beeswax and soap. These results show for the first time that Roman artists used in mural paintings a water-soluble encaustic paint of beeswax and soap. This research highlights that wax and soap encausto could be a common pictorial technique among Roman artists. The authors believe that the composition found may correspond to an ancient encaustic formulation searched for the last five centuries by many artists [83].

Birolo et al. (2017) present a multi- methodological investigation of mural paintings from Cuma, a Roman archaeological. They report the results on mortars, pigments and binding media, focusing on differences between an indoor (*domus*) and outdoor fabrication (a temple, *Tempio con Portico*). As for the organic binders, the investigations carried out with chromatographic techniques, have shown differences between the wall paintings of the domus and the Temple. Protein-based binders have been found in *domus* (possibly egg and animal glue), whereas in the temple, the use of drying vegetable oil is proposed [84].

Sr Daniilia et al. (2007) have carried out a study on the wall paintings in the Protaton Church (1295) on Mount Athos, Greece. The purpose of this work was to understand whether these paintings were made according to the traditional technique of the traditionally defined "Byzantine affresco", with lime water as the only binder. Instead, they demonstrated, using gas chromatography/mass spectrometry, that these mural paintings were realized using both *affresco* and a *secco* technique. For the a *secco* technique had been used egg together with a modest quantity of animal glue [85].

Multidisciplinary research was conducted on the first cycle of paintings of the church of Santa Maria delle Cerrate, an italo-greek monastery, most likely built in the 12th century. Microscopic, spectrophotometric and chromatographic techniques were used. The pigments were identified, and it was seen that they were applied both *affresco* and with egg-based tempera [86].

A study was carried out during the restoration of the medieval wall paintings of the Dome of the Baptistery of Parma, which for the type of degradation presented particular lifts of the pictorial film. By GC/MS, milk has been identified as the binder of pigments [38].

In a study of the wall paintings of the Cathedral of Cremona, innovative guidelines and a flow chart were provided for the identification of binding materials of a protein character [87]. Reference proteinaceous binding media of the kinds historically used in painting (specially prepared for this study) and samples collected from the wall paintings of Cremona Cathedral were analyzed by GC-MS. The data have been processed by several multivariate chemometric techniques, such as cluster analysis, principal component analysis, factor analysis and SIMCA technique in order to classify the binding media of the ancient works of art on the basis of the proteinaceous material used. The description of ancient samples was possible by the joint use of SIMCA technique and factor analysis [88].

An overview of application of chemometrics for the description of cultural heritage has been provided [89]. The principal component analysis of painted plasters acid soluble components and aggregate granulometric distributions and multivariate characterization of the mass spectral and of the amino acid 'fingerprints' of proteinaceous binding media in mural paintings of the Cupola of Florence Cathedral points out the differences in both plasters and binders used by Vasari from those used by Zuccari, evidencing the peculiarities in the painting technique adopted by each artist [90].

Ling et al. (2007) examined various Chinese art artifacts, using Py-GC-MS technique. Among the various materials identified Tung oil, also called "Chinese wood oil" has been identified as binding medium of wall ancient Chinese palace architecture paintings [91].

A paper by Maa et al. (2016) have studied black residues of supposed binding media in a tomb archaeological site of the Tang dynasty (618–907AD), which was one of the most prosperous periods in the ancient Chinese history. The study was conducted using FTIR and GC-FID. The results of the analysis indicated that the black residue was indeed the binding medium, consisting of a mixture of animal glue and egg [92].

Zhang et al. in a recent article, illustrate the characterization of organic materials used in the gilding decoration of wall paintings in Kizil Grottes, China, using Py-GC-MS technique. They saw that drying oil was used as the mordant for the adhesion of tin foils, while lac resin was employed for gold foils [93].

In recent years, proteomics procedures have become increasingly popular for the characterization of proteinaceous materials in ancient samples of several cultural heritage objects. A simplified protocol of in-situ protein digestion was proposed. They demonstrated the possibility of using a very small piece of hydrophilic gel loaded with trypsin to digest proteins directly in-situ on works of art with good efficacy. The strategy was developed on fresh and aged paint replicas, allowing the recognition of the usually employed organic binder based on egg, collagen, and casein [94].

A proteomic approach, spectrometry using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to study the degradation of proteins present in the wall paintings of the Monumental Cemetery in Pisa, Italy. The authors noted that the deamidation of some amino acids present in the proteinaceous binder causes a process of deterioration in works of art, including wall paintings [95].

Calvano et al. (2020) has proposed a quasi-non-invasive analysis of paint medium, based on the in situ digestion of the proteins, using a hydrogel previously loaded with trypsin, and applied onto the objects surface. The digested peptides were examined by MALDI-ToF-MS and/or LC-ESI-MS/MS [96].

In the article, the authors report the results of the investigations, through ToF-SIMS and HPLC, of samples taken from the wall painting "Madonna and Child enthroned with saints" (1335–1338) by Ambrogio Lorenzetti. The results of this study show that the ToF-SIMS technique is able to identify protein-based binding supports in the samples considered. In the secco-applied layers, markers for the components of egg (cholesterol and phosphocholine) were observed in the ToF-SIMS spectra obtained [43].

Chambery et al. (2009) have developed a procedure for identifying protein binders for molecular characterization of samples from the mural painting of St. Dimitar Cathedral in Vidin, Bulgaria, using LC-ESI/Q-q-TOF tandem mass spectrometry. The use of the proteomic survey made it possible to identify both egg yolk proteins and egg white proteins [97].

Always linked to the decay of the organic binder in ancient wall paintings, a recent article addressed the problem of blurring of the paint layer. Based on the experimental results obtained, the authors believe the decay of the organic binder can cause the blurring of wall paintings while keeping the pigment unchanged. Studies have been conducted on simulated wall paintings and on a fragment of the blurred ancient tomb mural painting Zang Huailiang Tomb, AD 730, Tang Dynasty, Sanyuan, Shaanxi, Chin [98].

5. Concluding Remarks

In this paper, it was chosen to examine the organic binders present in the color in the archaeological wall paintings.

Considering the wall paintings, the characterization of organic compounds is a very valuable source of information for the knowledge of the materials used, which allows you to know the paint technique of an artist. Understanding the composition of paint materials allows us to distinguish between painting techniques that have been used over the centuries. The knowledge of organic substances in wall painting materials is of great importance for the preservation of works of art, because the organic components of the paint layers are particularly subject to alterations and degradation and from this knowledge it is therefore possible to propose adapted storage conditions and better conservation/restoration methodologies.

Research on organic paint materials in wall paintings is still an open issue, where challenges and opportunities coexist. Organic compounds are included in a complex matrix. They are submitted to degradation, environmental stress, interference of inorganic species, denaturation and ageing for years. In this paper, non-destructive analytical techniques, to be used in situ, and laboratory techniques, after sampling pictorial material, are described. Particular space is due to the chromatographic techniques used in different works illustrated.

When choosing to sample pictorial material to obtain a precise characterization of the organic material, the identification of organic binders is subject to several analytical problems, such as: the low amount of sample available (usually less than 1 mg) as there is a tendency to preserve as much as possible the integrity of the wall painting, the reduced organic binder content present in the sample (about 10% *w*/*w*), the presence of an inorganic component due to the pigments. In addition, a problem may arise when the extraction method used is not satisfactory for an ancient binding medium, then part of the binder may not be extracted, producing an incomplete characterization of its composition.

Because of the very low sample amounts available for analysis, it is essential to use sensitive and specific diagnostic methodologies.

My work experience and the information obtained from many scientific articles has allowed me to understand how often the careful vision, made by an expert, of the work of art is not enough to understand the artistic technique of a painter and that only investigations on small fragments of pictorial material can define the artist's choices. Although, one is aware that several open questions and research challenges remain in the analysis of organic binders in wall paintings and the probability of definitive answers through currently available analytical methodologies maintains a certain degree of uncertainty, which leaves room and the motivation for further analytical approaches and developments.

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