ACCOUNTS
of chemical research

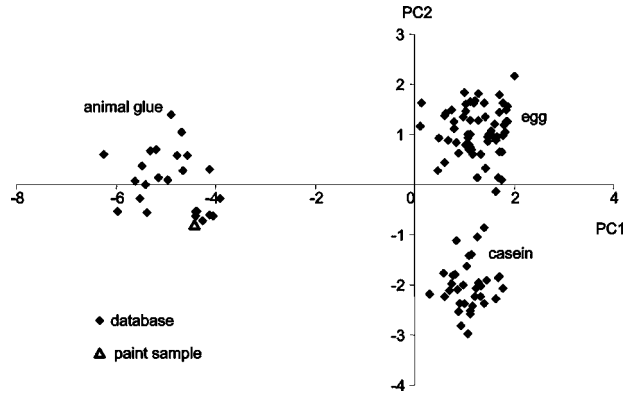
Analytical Strategies for Characterizing Organic Paint Media Using Gas Chromatography/Mass Spectrometry

MARIA PERLA COLOMBINI,* ALESSIA ANDREOTTI,† ILARIA BONADUCE,† FRANCESCA MODUGNO,† AND ERIKA RIBECHINI†

Chemical Science for the Safeguard of the Cultural Heritage Group, Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Risorgimento 35, 56126 Pisa, Italy

RECEIVED ON JUNE 27, 2009

CONSPECTUS



Throughout history, artists have experimented with a variety of organic-based natural materials, using them as paint binders, varnishes, and ingredients for mordants in gildings. Artists often use many layers of paint to produce particular effects. How we see a painting is thus the final result of how this complex, highly heterogeneous, multimaterial, and multilayered structure interacts with light.

The chemical characterization of the organic substances in paint materials is of great importance for artwork conservation because the organic components of the paint layers are particularly subject to degradation. In addition, understanding the organic content and makeup of paint materials allows us to differentiate between the painting techniques that have been used over history.

Applying gas chromatography/mass spectrometry (GC/MS) analysis to microsamples of paint layers is widely recognized as the best approach for identifying organic materials, such as proteins, drying oils, waxes, terpenic resins, and polysaccharide gums. The method provides essential information for reconstructing artistic techniques, assessing the best conditions for long-term preservation, and planning restoration.

In this Account, we summarize the more common approaches adopted in the study of the organic components of paint materials. Our progress in developing GC/MS analytical procedures in the field of cultural heritage is presented, focusing on problems that arise from (i) the presence of mixtures of many chemically complex and degraded materials, (ii) the interference of inorganic species, (iii) the small size of the samples, and (iv) the risk of contamination. We outline some critical aspects of the analytical strategy, such as the need to optimize specific wet-chemical sample pretreatments in order to separate the various components, hydrolyze macromolecular analytes, clean-up inorganic ions, and derivatize polar molecules for subsequent GC/MS separation and identification. We also discuss how to interpret the chromatographic data so as to be able to identify the materials. This identification is based on the presence of specific biomarkers (chemotaxonomy), on the evaluation of the overall chromatographic profile, or on the quantitative analysis of significant compounds.

GC/MS-based analytical procedures have for 20 years provided important contributions to conservation science, but challenges and opportunities still coexist in the field of organic-based paint materials. We give selected examples and provide case studies showing how a better understanding of the chemical composition of organic paint materials and of their degradation pathways contribute to a better knowledge our cultural heritage, and to its preservation for future generations.

Published on the Web 02/24/2010 www.pubs.acs.org/acr
10.1021/ar900185f

1. Introduction

Paints are generally made up of the same fundamental components: a pigment, which is most typically a fine powder of inorganic or organic colored material, and, with the exception of frescos, a fluid binder, which enables the pigment to be dispersed and applied with a brush. The binder may be a proteinaceous material such as egg or casein, a vegetable gum, a drying oil, a natural wax, or a mixture of two or more of these materials. After drying or curing, a solid paint film is produced. The surface on which the paint is applied may be cotton or linen canvas, a wood panel, stone, metal, glass, or plaster on a wall. Such surfaces generally need to be prepared with a ground layer. For instance, a mixture of animal glue and gypsum was used for centuries as a ground for both wooden panels and canvases. Paintings were also often coated with a varnish containing plant resins and/or oils to produce saturated, deep-toned colors and protection against environmental agents.[1]

Painters were not only artists but also "material scientists", since they had to be able to select the best paint materials, process them, and apply them in order to suit their needs and achieve the desired aesthetic results. They experimented with a wide range of natural materials[1] and often used many layers of paint to produce particular effects. To our eyes, the appearance of a painting is thus the final result of the interaction of this complex, highly heterogeneous, multimaterial, and multilayered structure with light.

This Account focuses on the chemical characterization of organic components, which is of great interest because the different organic paint materials used help us to differentiate between the various painting techniques and because the organic component of the paint layer is particularly subject to degradation. An analysis of organic paint materials is essential for their long-term preservation, to assess the best conservation and display conditions, to prevent and slow the decay processes, and to plan the best restoration. Macroscopic degradation phenomena, such as the yellowing of the varnish layers and the loss of cohesion and craquelures in the paint layers, are related to the chemical alterations of the organic media, such as depolymerization, oxidation, hydrolysis, cross-linking, and biological attack. Chemical reactions between organic materials and pigments lead to discoloration or color alteration.

Organic paint constituents are very challenging from an analytical point of view, and the following critical factors always need to be considered when planning analytical procedures:

- several organic natural and synthetic substances are often simultaneously present in the layered structure;
- nonoriginal compounds, formed as a result of aging or introduced by restoration treatments and pollution, are generally also present;
- a very low amount of organic matter (a few percentage points in the overall weight or even lower) is generally encountered in a minute heterogeneous paint sample ($\ll$1 mg).

For a complete understanding of the composition of paint layers, several techniques need to be used, including scanning electron microscopy/energy dispersive X-ray (SEM-EDX), X-ray diffraction (XRD), micro-Fourier transform infrared (micro-FTIR), micro-Raman, secondary ion mass spectrometry (SIMS), and many others.[2] Nevertheless, at present, the coupling of gas chromatography with mass spectrometry (GC/MS) is the preferred analytical approach to characterize organic paint materials. The versatility of GC in the investigation of a very broad set of natural organic materials that can be found in artwork was pioneered by Mills and White[1] and confirmed by a number of successful applications and case studies (refs 3 and 4 and references therein). The choice of GC is driven by the fact that natural organic substances are complex mixtures of many chemical species that are very similar to each other; the resolution and determination of the molecular profile is essential in order to identify the materials present and the aging pathways. Consequently, in this specific field, the coupling of GC with mass spectrometry is necessary due to the high number of compounds with similar retention times. In addition, because the most significant compounds are not available as commercial standards, identification cannot be based only on retention times and requires the confirmation by mass spectra.

This Account focuses on the critical evaluation and troubleshooting in the various steps of the analytical procedures used in our laboratory for the GC/MS analysis of organic paint media, including the choice of analytical strategies and reference materials, sample pretreatment and purification to avoid possible interferences, data interpretation, and the use of reference materials and databases. Possible future developments are also discussed.

2. Analytical Strategies

Most organic materials in a painting are macromolecular.[1] In some cases, they are natural polymers, such as proteins or plant gums. Others undergo polymerization or cross-linking as an effect of exposure to light and air, such as natural resins

and drying oils. For this reason, it is not possible to analyze them directly by GC/MS, and a preliminary step to reduce the macromolecular analytes to low molecular weight molecules is needed. This can be achieved by coupling analytical pyrolysis with gas chromatography/mass spectrometry (Py-GC/MS) or by a wet-chemical treatment of the samples prior to GC/MS.[3]

When Py-GC/MS is used, the chemical composition of the sample is reconstructed on the basis of the interpretation of the molecular profile of the thermal degradation products of the original components. The technique involves minimal sample manipulation and no sample pretreatment, thus reducing the problems of contamination and sample loss associated with wet-chemical procedures. With the exception of some synthetic polymers (such as acrylic polymers), paint materials under pyrolysis produce polar, low volatile molecules, which need thermally assisted in situ derivatization, with either silylating or methylating agents, in order for them to be efficiently revealed through GC/MS (refs 5 and 6 and references therein).

Even though Py-GC/MS is a fast and efficient technique to obtain the fingerprint of the organic materials in paint samples, GC/MS after wet chemical sample pretreatment is unsurpassed in its capacity to unravel the composition of the samples at a molecular level. The wet chemical pretreatment often includes chemolyses and derivatization reactions. The first step is needed in order to free small molecules from macromolecules or polymers (such as amino acids from proteins, fatty acids from triglycerides, or sugars from polysaccharides) thus providing molecules that are suitable for GC/MS analysis. Derivatization reactions transform molecules containing polar functional groups, such as carboxylic or alcoholic moieties, into less polar compounds, such as the corresponding silyl esters or ethers, thus increasing their volatility. As a result, in the case of heterogeneous multimaterial paint samples, when more than one class of compound, with very different chemical properties, need to be investigated, the complexity of the GC/MS procedure is increased by the necessity to separate and specifically treat the various types of analytes:[4,7]

- Proteins as polysaccharides need to undergo acidic hydrolysis in order to free the amino acids and sugars, respectively. However, to ensure the completeness of the reaction, by minimizing any loss of the most labile components, hydrolysis conditions must be specifically optimized, that is, milder for polysaccharides and harsher for proteins.
- Glycerolipids and natural waxes require alkaline hydrolysis, but the complete hydrolysis of wax esters is much more difficult than that for glycerides.

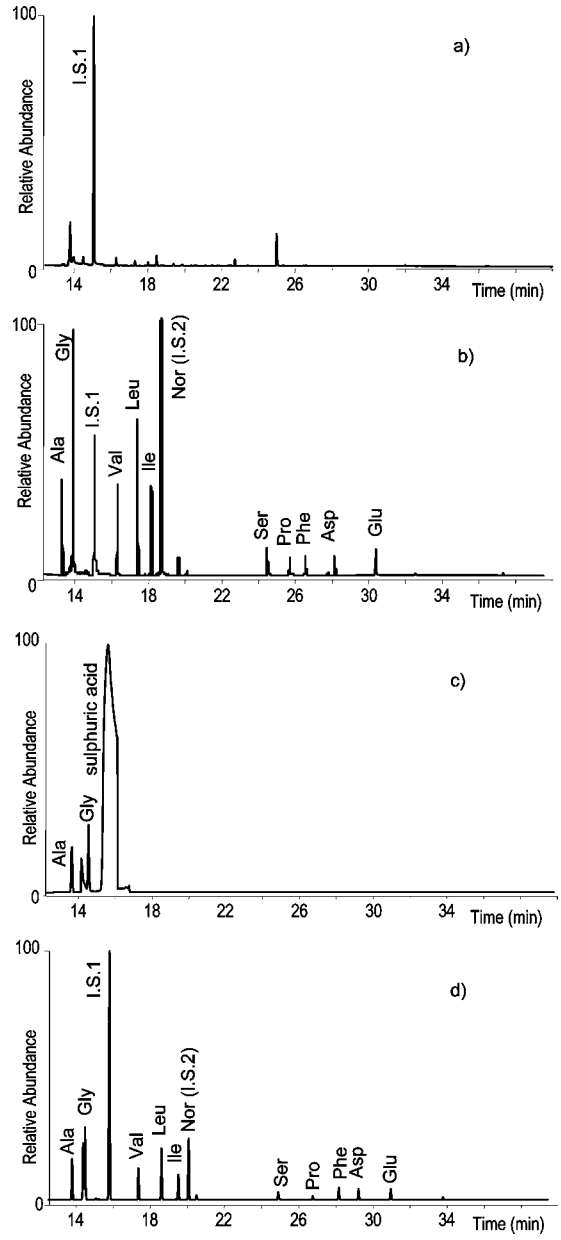


FIGURE 1. Chromatograms obtained by GC/MS analysis of amino acids after acidic hydrolysis followed by silylation of (a) a sample from an Italian wall painting from the 13th century (Crypt of the Cathedral of Siena) containing high amounts of azurite (a copper-containing blue pigment),[8] (b) the same sample after the cleanup of the inorganic species, (c) a sample from an Italian panel painting from the 14th century containing high amounts gypsum in the preparation layer, and (d) the same sample after the clean-up of inorganic species.

- Polyesters of shellac components require alkaline hydrolysis before subsequent GC/MS analysis, but the choice of reagents affects the resulting molecular profile.

In addition, the analysis of some paint binders may involve some interference in terms of the presence of inorganic compounds deriving from the support or from pigments: the characterization of proteinaceous and polysaccharide materials can

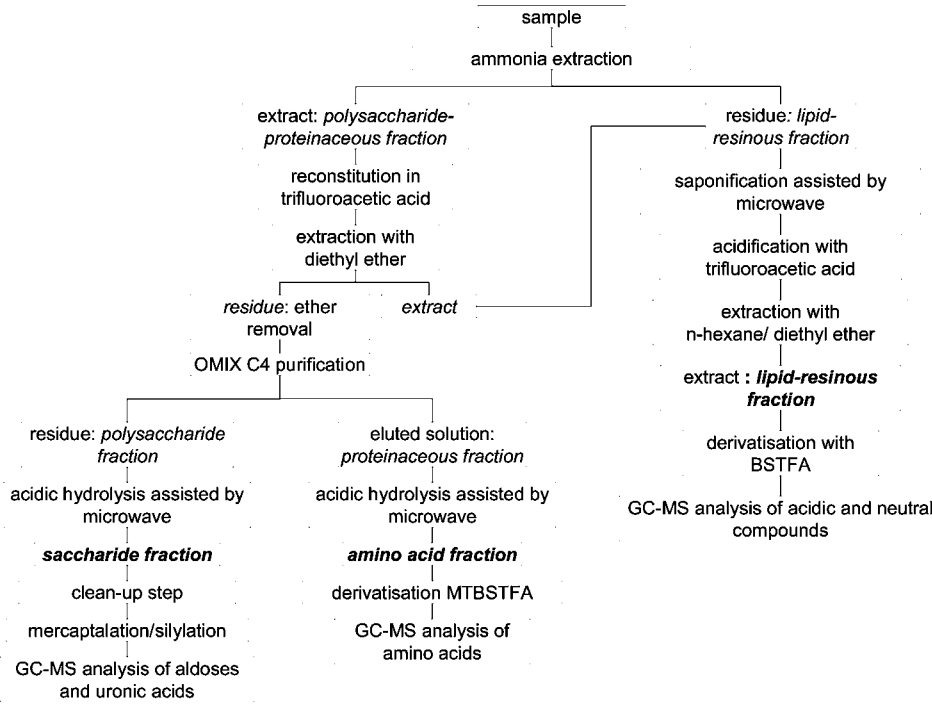


FIGURE 2. Flowchart showing a combined analytical procedure for the GC/MS analysis of glycerolipid, waxy, resinous, proteinaceous, and polysaccharide materials in one individual paint microsample.[7]

be affected by the occurrence of high amounts of inorganic species. Metal cations, including Hg^{2+}, Fe^{3+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, and Ca^{2+}, and anions give rise to analytical interferences in the characterization of proteinaceous materials.[8] To overcome these problems, a purification step based on the purification of the extract using a miniaturized sorbent tip (C18 or C4 stationary phase) can be included in the analytical procedure.[7−9]

As an example, Figure 1 shows the results of the amino acid analysis of two paint samples, one from the wall paintings in the Crypt of the Cathedral of Siena (Italy, 13th century) and the other from an Italian panel painting from the 14th century, before and after a cleanup aimed at eliminating cations and anions.[8] GC/MS analysis was performed by hydrolysis and silylation of the amino acids. The chromatogram in Figure 1a refers to a wall paint sample containing high amounts of azurite (a copper-containing pigment) analyzed without the cleanup step. It clearly shows the hexadecane peak (I.S.1, the injection internal standard), but the peaks relative to amino acids, including norleucine (I.S.2, the derivatization internal standard) are absent. This is due to the analytical interference caused by the Cu^{2+} cations from azurite, which form complexes with amino acids, thus removing them from the silylation reaction. Applying a cleanup procedure to the same sample to remove inorganic species (chromatogram in Figure 1b) solves the problem and reveals the presence of a proteinaceous binder, which in this case was recognized as egg.[8]

High amounts of sulfates (the major components of gypsum paint grounds) can also be very problematic, as highlighted in Figure 1c, relating to a sample collected from an Italian painting from the 14th century containing a portion of the gypsum ground layer. In this case, in addition to the hexadecane peak, there is a huge peak relative to the trimethylsilyl-ester of sulfuric acid. The alanine and glycine peaks are visible, but there are no other amino acids with a higher retention time than sulfuric acid, thus highlighting the chromatographic contamination caused by the sulfates. Once again, purification using a C4 tip (chromatogram in Figure 1d) removed the interference, and the proteinaceous binder could thus be identified as egg.

Taking into account the issues mentioned above and the necessity to identify as many materials as possible in just one paint microsample, a combined analytical procedure was developed for the simultaneous characterization of proteinaceous, polysaccharide, lipid, and resinous paint materials.[7] The procedure (Figure 2) is based on a multistep chemical treatment of the sample, where solvent and solid phase extractions are used to separate materials with different physicochemical properties, thus obtaining three purified fractions for GC/MS analysis: a lipid−resinous, an amino acidic,

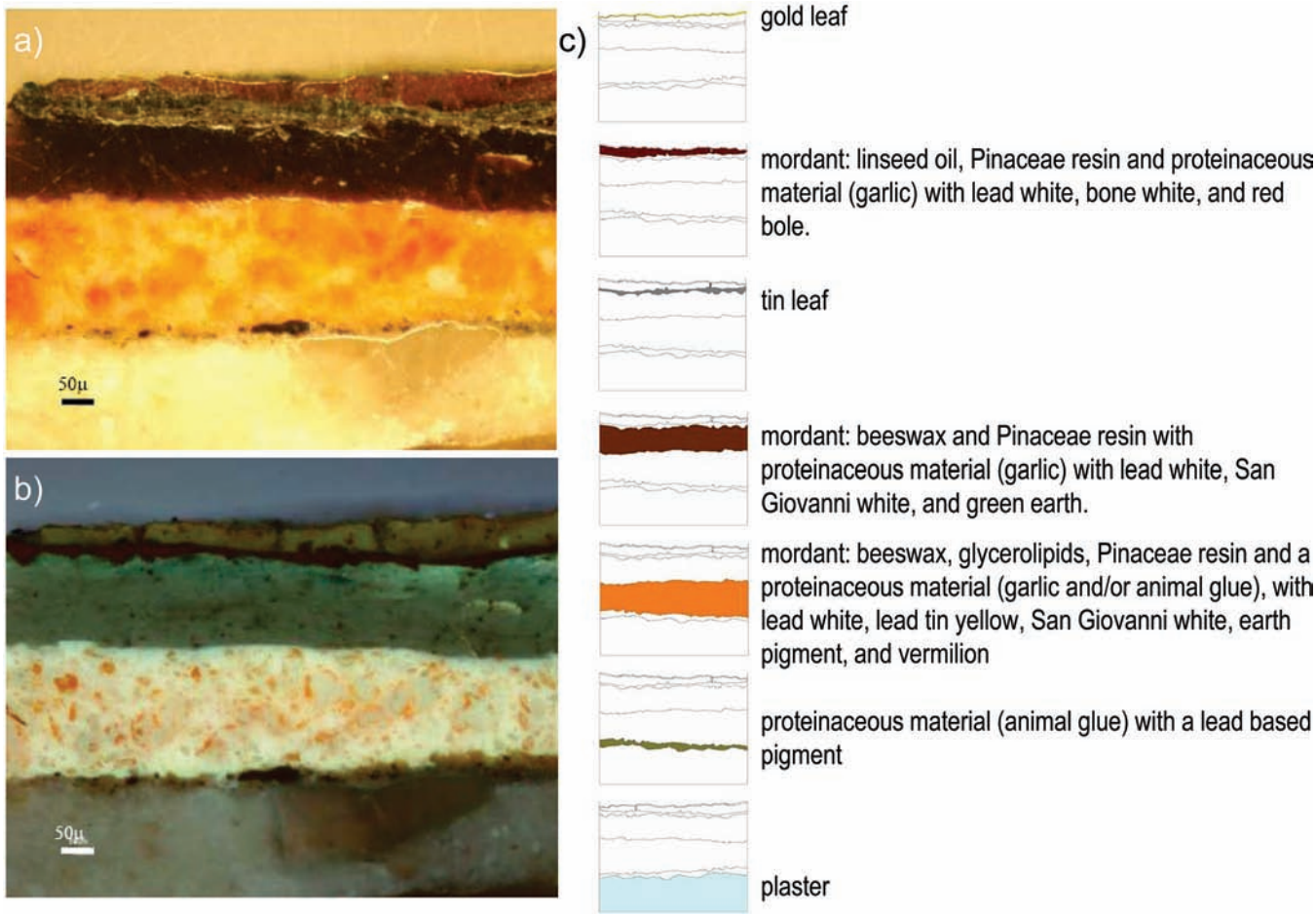


FIGURE 3. Sample collected from the gilding of the Orvetari Chapel: (a) cross-sectional image in dark field (white light); (b) same image in vis-fluorescence (UV light A filter); (c) sample composition, layer by layer.[10]

and a saccharide fraction. The method is microinvasive and can also be used to characterize very small heterogeneous samples whose organic content is only a few tenths of micrograms.

The GC/MS analysis of a paint sample as a whole allows us to identify the organic materials contained in it but gives no information on their spatial distribution in the various layers that make up the paint. Assessing the composition of each layer is essential in order to fully understand how the artist worked. One approach is to try to physically separate the layers under an optical microscope, obtaining subsamples to be separately analyzed by GC/MS. Another possibility is to combine the results of the GC/MS analysis of the whole sample with the information obtained applying imaging techniques to the cross section of a duplicate sample by specular reflection FTIR, SIMS, and SEM-EDX. This approach was used in the characterization of the gilding technique in a sample collected from a fragment of the mural paintings in the Orvetari Chapel (15th century) that remained after the collapse as a result of the bombing in 1944 of the Eremitani Church in Padua, Italy.[10] The results are summarized in Figure 3. A double metallic leaf was revealed, made up of a gold leaf and a tin leaf glued together with an oily mordant. Double metallic leaf decorations were prepared by the artist in advance, cut into the desired shape and then applied onto the wall: a mordant layer was applied on the tin side of the decoration, and then pressed onto a colored mordant previously positioned on the wall. The thin layer of proteinaceous material directly applied on the plaster support guaranteed adhesion between the plaster and the superimposed layers.

3. Data Interpretation

In works of art, the identification of organic materials characterized by GC/MS techniques is generally based on three main principles: chemotaxonomy, that is, the identification of the presence of one or more specific compounds (biomarkers), evaluation of the chromatographic pattern, and quantitative analysis.

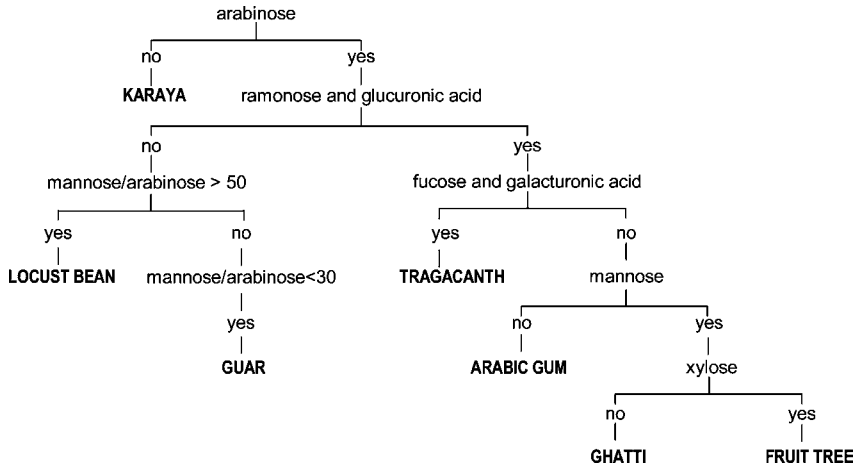


FIGURE 4. Decisional scheme for the identification of polysaccharide gums.

Chemotaxonomy. Biomarkers are considered as stable and diagnostic molecules present in a material, which have either been preserved intact or formed over the centuries due to aging. Identifying the composition at a molecular level and thus the presence of biomarkers is crucial in order to obtain information on the kinds of organic substances that were originally present in the sample and to understand the natural or anthropogenic alteration processes that have modified the original composition of the samples. Biomarkers are mainly related to the source (animal/vegetable or botanical origin) from which the material was obtained. For example, cholesterol is the most abundant sterol in animal fats, while phytosterols, mainly sitosterol, indicate a vegetable origin. Biomarkers can also shed light on the degradation processes undergone by the material, since they can be linked to the transformations caused by environmental factors and aging. The assessment of the presence and of the origin of terpenic resins, in both plants and animals, is based on the recognition of molecular biomarkers. Sandarac, colophony, dammar, and mastic resins are the most common natural resins found in paint samples, mainly as varnishes or as gilding mordants. The biomarkers used for the recognition of resins have been reported in the literature.[1,4,6,11,12]

Evaluation of the Chromatographic Pattern. In some cases, identification is not based on a few very specific biomarkers but on the simultaneous presence in the chromatogram of a series of significant molecules. These are nonspecific if considered alone but become extremely indicative when the overall chromatographic pattern is evaluated such as the entire series of alkanes, alcohols, or carboxylic acids.

Natural waxes are typically identified on the basis of their molecular profile: beeswax, after hydrolysis, is characterized by the presence of long chain fatty acids with an even number of carbons (from palmitic to dotriacontanoic acid), (ω-1)-hydroxy acids with an even number of carbons (from 15-hydroxyhexadecanoic acid to 23-hydroxytetracosanoic acid), long chain linear alcohols with an even number of carbons (from tetracosanol to tetratriacontanol), long chain (α,ω-1)-diols with an even number of carbons (from 1,23-tetracosandiol to 1,27-octacosandiol), and long chain linear saturated hydrocarbons with the prevalence of an odd number of carbons (from tricosane to tritriacontane).[1,4]

The characterization of plant gums, which are naturally occurring polysaccharides exuded by several species of plants or extracted from some seeds, is also based on the evaluation of molecular profiles. In the Mediterranean basin, the gums traditionally used were Arabic gum, tragacanth gum, fruit tree gum, and locust bean. Ghatti and karaya gum were important materials in the Indian subcontinent. They are high molecular weight polymers consisting of aldopentoses, aldohexoses, and uronic acids joined together by a glycoside bond. Their identification by GC/MS requires hydrolysis in order to free sugars, followed by derivatization.[4] Since each gum shows a different composition, their identification is based on the use of a decisional scheme,[13] as reported in Figure 4.

The presence of mixtures of saccharide materials and the effect of aging and biological attack make it difficult to identify a plant gum in a paint sample. Figure 5 shows the chromatograms of six samples collected from mural paintings of Macedonian tombs (4th to 3rd centuries B.C.; AGAT and AZ

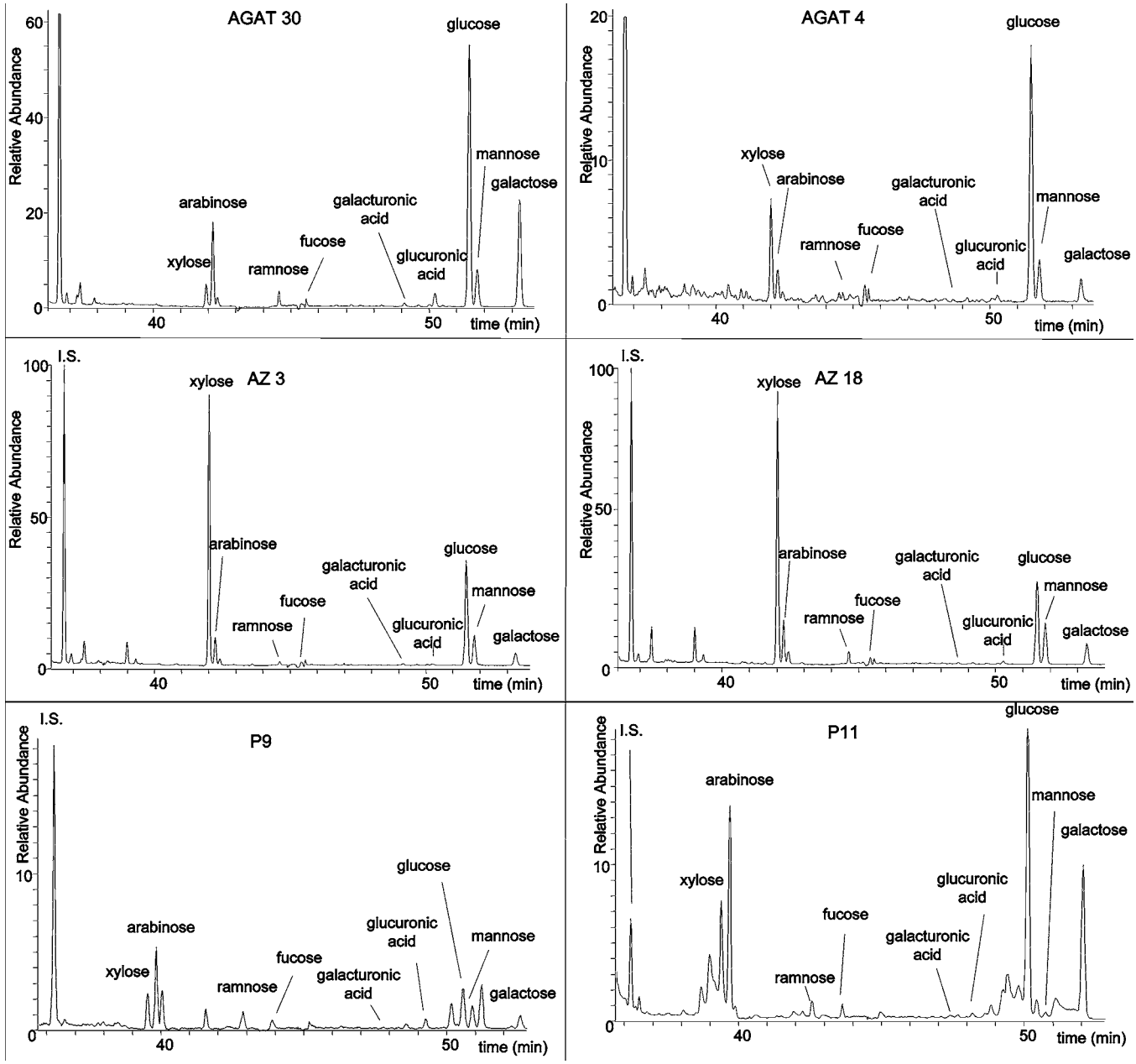


FIGURE 5. Chromatograms of six samples collected from the mural paintings of Macedonian tombs (4th to 3rd centuries B.C.; AGAT and AZ samples) and from the Nestor palace (13th century B.C.; P sample), Greece.

samples) and from the Mycenaean Nestor palace (13th century B.C.; P samples), Greece.

All samples showed the presence of saccharide materials: sample AGAT 30 contained fruit tree gum, samples 4 P9 and 17 P11 contained a mixture of tragacanth and fruit tree gums, but samples AZ 3, AZ 18, and AGAT 4 were not classified. It is fundamental to stress that most of the chromatographic profiles obtained, from a quantitative point of view, do not correspond to any of the reference gums analyzed.[2,13] The reason for these quantitative modifications of the chromatographic profiles of plant gums observed in paint samples still needs to be fully understood.

Quantitative Analysis. The specific identification of paint binders entails a quantitative determination using GC/MS of the following compounds:

- amino acids, for proteinaceous binders (egg, animal glue, casein, or milk);
- monosaccharides and uronic acids for polysaccharide gums;

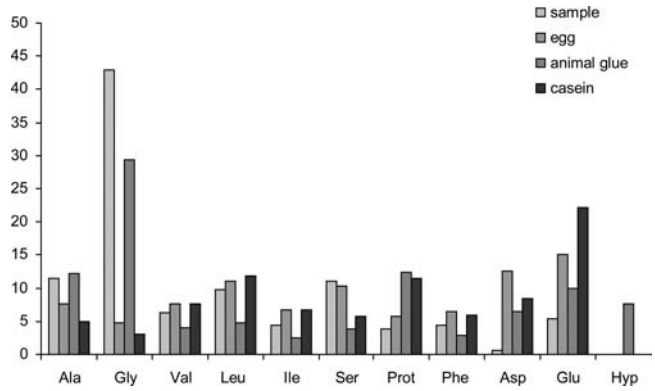


FIGURE 6. Amino acid percentage content of a sample collected from an Italian mural painting from the 16th century attacked by fungi and of reference casein, egg, and collagen samples.

- monocarboxylic and α,ω-dicarboxylic acids for drying oils (linseed, walnut, poppy seed, or tung oil).

Proteins used as paint media are essentially limited to three kinds, egg, casein, and collagen, so they can be distinguished on the basis of their different amino acid profiles.[1,3] A comparison of the amino acidic profile of an unknown sample with reference materials can be efficiently and rapidly achieved by principal component analysis (PCA). The main limitation of an analytical approach to proteinaceous paint media identification based on amino acid quantitative determination is the presence of mixtures of different proteins in the same sample. Moreover, if unexpected proteinaceous materials are present, such as blood (used as binder in African wooden sculptures[14]) or garlic (an ingredient in adhesives for the application of gilding on paintings[15]), they will give rise to a different amino acid profile.

One important aspect that must be considered when evaluating the protein content in a paint sample is the effect of biological agents such as fungi or bacteria, which can induce changes in the resulting amino acidic profile: glycine is increased, since it is the metabolism product of many bacteria and fungi, and some other amino acids are significantly decreased. Figure 6 shows the amino acid percentage content of a sample from an Italian mural painting by Niccolò D'Abbate (16th century) attacked by fungi, compared with egg, collagen, and casein reference samples. The glycine content is quite high, but because hydroxyproline is absent, the presence of collagen can be ruled out. Moreover, the contents of aspartic acid and glutamic acid are very low, revealing a profile that cannot be ascribed to egg or to casein.

With the increased availability of advanced instrumentation, proteomics may soon become the preferred approach for protein determination in paintings. Peptide mass mapping by MALDI-TOF and HPLC-MS/MS, when optimized and validated, will provide not only an identification of the kinds of proteinaceous binders but also additional information that is not accessible by amino acid analysis: for example, the distinction between egg yolk and egg glair, and the determination of the animal species from which collagen or casein have been obtained.[16]

A quantitation of monosaccharides and uronic acids must also be performed. Since saccharides are common environmental contaminants, an analysis of polysaccharide binders requires the quantitative evaluation of each sugar in order to assess whether it is present above or below the detection limit and can or cannot thus be used to identify the gum. Blank evaluations will be discussed later in this section. Moreover the identification of polysaccharide gums can be supported by the multivariate pattern analysis.[13]

The identification of lipids is based on the quantitation of mono- and dicarboxylic acids after saponification. Polyunsaturated acylic chains contained in fresh, nonaged drying oils (linseed oil, walnut oil, poppy seed oil, or tung oil) are not observed in aged oil paint layers because polyunsaturated acylic chains are subject to oxidative cleavage thus forming α,ω-dicarboxylic fatty acids, such as pimelic acid, suberic acid, sebacic acid, and azelaic acid being the most abundant, which can then be considered as markers for the presence of an aged drying oil. The amount of dicarboxylic acids formed in the curing and aging of egg lipids is substantially smaller than that in drying oils.[17] Therefore the ratio between the amounts of azelaic and palmitic acid (A/P) is taken as a parameter for differentiating drying oils from egg lipids in paint samples (values of A/P > 1 indicate a drying oil, values of A/P < 0.3 are typical of egg lipids, while intermediate A/P ratio values are observed for "tempera grassa" in which egg and a drying oil are mixed).[1] The presence of egg must be confirmed by amino acid analysis of the proteinaceous matter. Obviously the amount of dicarboxylic acids formed (sum of the percentage content of dicarboxylic acids, $\sum D$) is strictly dependent on the degree of oxidation, so the values observed may vary considerably and may be influenced by many factors such as the preheating of the oil media before use, the age of the paint, the conservation environment, and the effects of radical reactions initiated by pigments.

The ratio between the amounts of palmitic and stearic acids (P/S) has been proposed as a possible index for differentiating between drying oils.[1] The ratio is considered constant over time since these two saturated monocarboxylic acids are less subject to degradation during curing and aging. Typical P/S ratio values are reported as 1.4−2.4 for linseed oil, 2−4.5 for

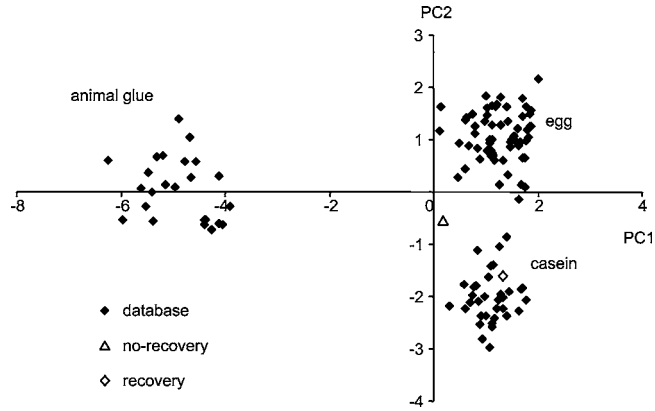


FIGURE 7. PCA score plot of a paint sample collected from an Afghan clay sculpture of the 6th century, obtained with and without performing the correction for the daily recovery.

walnut oil, 3−8 poppy seed oil, and 2.5−3.5 for egg. However, evaluating this parameter is particularly delicate because of the possible presence of mixtures of different lipid materials, as in "tempera grassa", and the contribution of fatty acids from other sources, such as natural waxes. When ready-made oil colors arrived on the market, several vegetable oils were introduced into commercial formulations, to obtain some specific physical properties of the binder or as cheap adulterating agents. One of these oils, for example, is castor oil, a common ingredient in alkyd paint media. Very little is known about modern oily media, and a systematic investigation is needed. Moreover, fatty acids, and especially palmitic and stearic acids, are abundant in the environment and may contaminate the paint layer (hand contact, residues of burning vegetable oils and animal fats for lighting, etc.). Microorganisms can also alter the P/S values of those expected for pure materials. Another important aspect is that fatty acids react with metal cations in paint films thus forming metal soaps (ref 18 and references therein). Different reactivity, migration

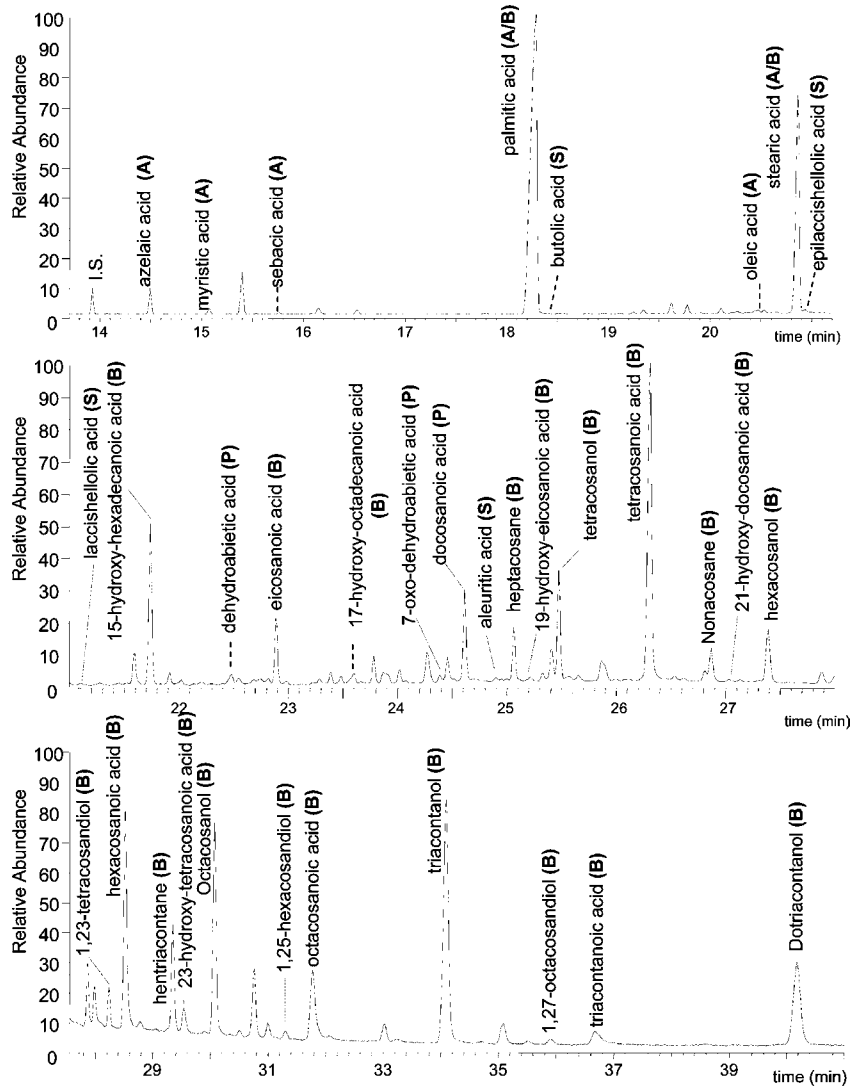


FIGURE 8. Sample collected from Greek Byzantine icon from the 15th century: (a) TIC chromatogram relative to the lipid−resinous fraction where the compounds deriving from an acyl-lipid material (A), shellac (S), a *Pinaceae* resin (P), and beeswax (B) are indicated.

speed, and solubility of the metal carboxylates in the paint media and in the cleaning solvents could alter the amounts of acids that are recovered in the analysis. Lastly, it is known that fatty acids can sublimate over time, altering the ratios between the saturated acids with a different number of carbons, that is, palmitic and stearic acids.[19] As a result of all these factors, the widely used P/S parameter should be very carefully considered, and in our opinion, further research is needed to define the criteria for reliably identifying the botanical source of a drying oil in a paint sample.

A quantitative analysis of compounds that have been previously subjected to derivatization should take several factors into account. The first is the fundamental need to use a derivatization internal standard and calibration curves. Modifications of GC/MS performances will affect compounds with different physical−chemical properties in different ways over time. Running daily standards enables us to evaluate the daily response of each analyte. Figure 7 explains the effect due to an uncalibrated response. It shows the PCA score plot of a paint sample collected from an Afghan clay sculpture from the 6th century: the sample not corrected for the daily recovery is located as an outlier, and its profile remains unassigned. After the correction, the sample is located perfectly in the casein cluster, thus indicating that this was the binder used.

Lastly, dealing with proteins, lipids, and saccharides could involve a high level of environmental contamination. Blanks must thus be run frequently and the corresponding LOD (limit of detection) and LOQ (limit of quantitation) calculated, in order to avoid any misleading interpretation when the amount of analytes determined is not significant. For example, a painting sample taken from a Chinese clay sculpture from the 16th century showed the following amino acidic profile: Ala 6.7; Gly 12.6; Val 6.8; Leu 11.1; Ile 6.0; Ser 6.2; Pro 8.4; Phe 3.9; Asp 18.7; Glu 19.6; Hyp 0.0. This profile is similar to that of egg, and in fact, when this sample is processed with the PCA method, it falls very close to the egg cluster. In actual fact, the protein content, evaluated as the sum of the quantified amino acids, was 0.29 μg, and the LOD (limit of detection) was 0.30 μg; this indicated that the sample did not contain significant level of proteinaceous binder, and the amino acids detected were likely due to environmental contamination and not to the presence of egg.

Case Study. Figure 8 shows three portions of the TIC chromatogram relating to the lipid−resinous fraction of a paint sample collected from a Greek Byzantine icon from the 15th century,[9] in order to show how the marker recognition, evaluation of the chromatographic pattern, and quantitative analysis can be used to identify the organic materials present. The

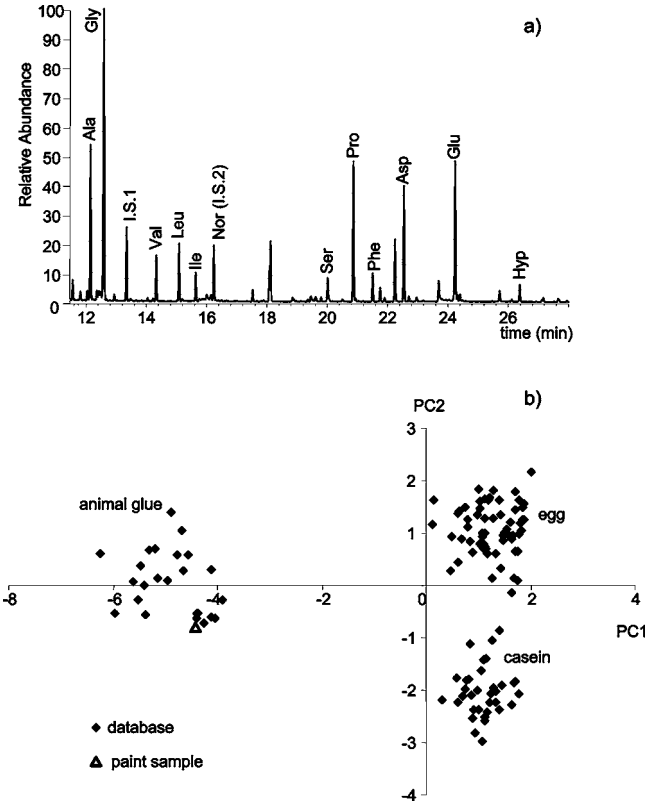


FIGURE 9. Sample collected from Greek Byzantine icon from the 15th century: (a) TIC chromatogram of the amino acid fraction; (b) PCA score plot.

characteristic biomarkers highlight the presence of shellac and pine resin. The profile of fatty acids, hydroxy acids, alcohols, diols, and alkanes points to beeswax and another acyl-lipid material.

Figure 9a shows the TIC chromatogram of the amino acid fraction, and Figure 9b shows the PCA score plot obtained by comparing the amino acid profile with reference tempera paint layers, thus indicating that the sample contained animal glue.

4. Reference Materials and Databases

Reference materials play an important role in analytical chemistry. Their basic purpose is to calibrate instruments and validate analytical procedures. In Conservation Science, they are an essential tool for laboratories involved in the development of analytical strategies for the analysis and characterization of materials from works of art. They can provide corroborating evidence for the presence of specific materials, allowing us to build up databases of chromatographic profiles and to expand mass spectral libraries. This aspect is particularly important in the case of natural organic materials. In fact, only a few compounds present both in fresh and in aged natural materials are commercially available as standards, and the analysis of ref-

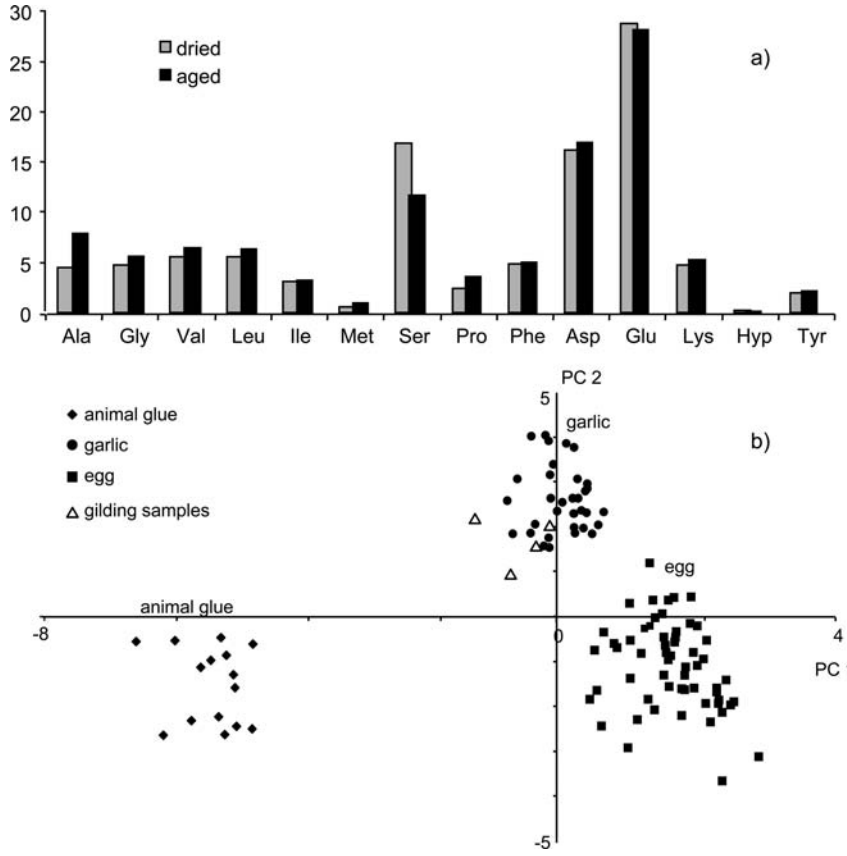


FIGURE 10. (a) Average amino acidic composition of reference samples of garlic, dry and artificially aged. (b) PCA score plot of gilding and reference samples.

erence materials of a known origin is an essential step in the set up of analytical procedures and to support data interpretation. Reference materials can be divided into different kinds: raw materials and painting replicas of a known composition made according to recipes reported in historical treatises.

Degradation due to aging from environmental parameters leads to changes in the composition of the original materials. Thus the study of painting replicas submitted to artificial aging treatments (using UV radiation, temperature, moisture, and environmental contaminants such as SO_3, NOx, or O_3) plays an important role by simulating the degradation processes and providing similar reference materials to naturally aged paintings.[4] Even though there is currently no internationally accepted artificial aging protocol, it is clear that aging tests and the use of raw materials and replicas are the only possible approaches to study and understand degradation processes. Over the last 20 years, several collections of raw materials and painting replicas have been set up whose preparation has been made according to documented recipes, and there is an increasing interest on historical documentary source research and reconstruction on art materials and techniques.[20]

Studying old technical treatises is a very useful tool for the selection and collection of reference materials and to shed light on old recipes. For example, the study of technical treatises from the 8th to the 15th century highlighted that garlic was commonly used as an ingredient in the manufacture of adhesives in gilding. The garlic protein content was then determined in several fresh, dry, and thermally aged replicas,[15] and the average amino acidic composition obtained is reported in Figure 10a. Sixteen gilding samples collected from Italian mural paintings (14−17th century) were analyzed, and garlic was identified in four of these samples. Figure 10b shows the score plot obtained.

An artist may also have used a very unusual material, and the reference materials may not yet be available or studied. The corresponding molecular markers are thus unknown. In these cases, analytical data may suggest that an unexpected material is present, and the study of selected reference materials, together with the interpretation of historical documents, may enable us to confirm this initial hypothesis and finally to improve the databases and mass spectral libraries.

5. Conclusions

For the last 20 years, developing procedures based on GC/MS for analyzing organic paint materials has been a fundamen-

tal field of research in Conservation Science. Such procedures have enabled us to unravel these complex, structured mixtures of aged natural materials that constitute paint layers, to expand our knowledge of artists' techniques, and to contribute to the preservation of paintings.

Research on organic paint materials is still an open issue, where challenges and opportunities coexist. Our knowledge of the degradation processes during aging is still far from complete; much effort needs to be made to model and understand the relations between the macroscopic alteration and degradation of the paint layers, the chemical modification undergone by the constituent materials, and the influence of the interaction with the environment. Understanding these processes and interactions is fundamental for planning an efficient conservation and to preserve Cultural Heritage for future generations. These two aims can only be achieved if model paint replicas are used correctly, appropriate artificial aging protocols are developed, and collaborations continue to be set up between research groups in order to share reference materials and databases and to perform interlaboratory exercises and round-robin analyses on shared samples so as to validate and compare analytical procedures.

BIOGRAPHICAL INFORMATION

Maria Perla Colombini currently holds the post of Full Professor of Analytical Chemistry in the Department of Chemistry (Faculty of Science) at the University of Pisa. She holds courses on Analytical Chemistry and the Chemistry of Cultural Heritage. She is Director of the Masters Course on "Materials and Diagnostic Techniques in the Cultural Heritage field". Her research work includes developing analytical procedures based on spectroscopic and chromatographic techniques for characterizing micropollutants in the environment and, especially, organic materials and their degradation products in works of art and archaeological objects. She is head of the Chemical Sciences for the Safeguard of Cultural Heritage research group and specializes in the characterization of binders, organic dyes, and resins using chromatographic and mass-spectrometric techniques.

Alessia Andreotti graduated in Chemistry in 2002 at the University of Pisa with a thesis on laser cleaning applied to the restoration of paintings. Since 2004, she has been working as a technician at the Department of Chemistry and Industrial Chemistry in the technical-scientific and data evaluation areas. Her research focuses on the characterization of natural and synthetic organic materials collected from samples in the field of Cultural Heritage using instrumental analytical techniques such as HPLC, GC/MS, Py-GC/MS, and direct exposure mass spectrometry (DE-MS). She also specializes in using lasers and other state-of-the-art techniques for cleaning of easel paintings, mural paintings, and other artifacts.

Ilaria Bonaduce is a lecturer and permanent researcher in the Department of Chemistry and Industrial Chemistry at the University of Pisa; she received her Ph.D. in Chemical Science from the University of Pisa, Italy, in 2006. Her research focuses on the characterization of natural and synthetic organic materials used in works of art and the study of how they degrade during aging. Another major research interest is the development of analytical procedures for the identification of organic materials in paint samples, using mass spectrometric techniques, such as GC/MS, Py-GC/MS, and DE-MS.

Francesca Modugno received a Ph.D. in Analytical Chemistry in 2002 from the University of Pisa (Italy) and is currently a lecturer and permanent researcher in the Department of Chemistry and Industrial Chemistry at the University of Pisa. Her research deals with using analytical techniques such as GC/MS and PY-GC/MS in the diagnosis and conservation of historical, artistic, and archaeological objects. Her research involves studying organic materials, such as terpenic resins, protein, lipids, and wood, and their degradation processes.

Erika Ribechini received a Ph.D. in Chemical Sciences in 2006 from the University of Pisa. Since January 2009, she is a lecturer and permanent researcher in Analytical Chemistry in the Department of Chemistry and Industrial Chemistry at the University of Pisa. Her research mainly involves the development of analytical procedures based on chromatography and mass spectrometry for the study and characterization of organic natural substances from archaeological findings and works of art, with specific focus on vegetable oils, plant resins, and natural waxes. Her current research also involves the chemical characterization of archaeological waterlogged wooden artifacts using mass spectrometric methods.

FOOTNOTES

*To whom correspondence should be addressed. E-mail: perla@dcci.unipi.it. Fax: 00390502219260. Phone: 00390502219305.

†For A.A., e-mail: alessia@dcci.unipi.it, fax: 00390502219260, phone: 003905-02219252. For I.B., e-mail: ilaria@dcci.unipi.it, fax: 00390502219260, phone: 00390502219252. For F.M., e-mail: frances@dcci.unipi.it, fax: 00390502219260, phone: 00390502219405. For E.R., e-mail: erika@dcci.unipi.it, fax: 00390502219260, phone: 00390502219312.

REFERENCES

1 Mills, J. S.; White, R. *The Organic Chemistry of Museum Objects*; Butterworth Heinemann: Oxford, U.K., 1994.

2 *Scientific Examination for the Investigation of Paintings. A Handbook for Conservator-restorers*; Pinna, D., Galeotti, M., Mazzeo, R., Eds.; Centro Di della Edifimi srl: Firenze, Italy, 2009.

3 *Organic Mass Spectrometry in Art and Archaeology*; Colombini, M. P., Modugno; F., Eds.; John Wiley & Sons: Chichester, U.K., 2009.

4 Andreotti, A.; Bonaduce, I.; Colombini, M. P.; Modugno, F.; Ribechini, E. Organic paint materials and their characterization by GC-MS analytical procedures. In *New Trends in Analytical, Environmental and Cultural Heritage Chemistry*; Tassi, L., Colombini, M. P., Eds.; Transworld Research Network: Kerala, India, 2008, pp 389−423.

5 Bonaduce, I.; Andreotti, A. Py-GC/MS of Organic Paint Binders. In *Organic Mass Spectrometry in Art and Archaeology*; Colombini, M. P., Modugno, F., Eds.; John Wiley & Sons: Chichester, U.K., 2009; pp 303−325.

6 Scalarone, D.; Chiantore, O. Py-GC/MS of Natural and Synthetic Resins. In *Organic Mass Spectrometry in Art and Archaeology*; Colombini, M. P., Modugno, F., Eds.; John Wiley & Sons: Chichester, U.K., 2009; pp 327−361.

7 Lluveras, A.; Bonaduce, I.; Andreotti, A.; Colombini, M. P. A GC/MS analytical procedure for the characterization of glycerolipids, natural waxes, terpenoid resins,

proteinaceous and polysaccharide materials in the same paint micro sample avoiding interferences from inorganic media. *Anal. Chem.* **2010**, *82*, 376–386.

8 Gautier, G.; Colombini, M. P. GC-MS identification of proteins in wall painting samples: A Fast clean-up procedure to remove copper-based pigment interferences. *Talanta* **2007**, *73*, 95–102.

9 Bonaduce, I.; Cito, M.; Colombini, M. P. The development of a gas chromatographic-mass spectrometric analytical procedure for the determination of lipids, proteins and resins in the same paint micro-sample avoiding interferences from inorganic media. *J. Chromatogr. A* **2009**, *1216*, 5931–5939.

10 Bonaduce I.; Boon, J. J. An integrated mass spectrometric and molecular imaging analytical approach to identify and localise constituents in paintings applied to gilded multilayer structures from 14th to 16th C works of art. In *New Trends in Analytical, Environmental and Cultural Heritage Chemistry*; Tassi, L., Colombini, M. P., Eds.; Transworld Research Network: Kerala, India, 2008; pp 345−388.

11 van der Doelen, G. A. Molecular studies of fresh and aged triterpenoid varnishes, PhD thesis, University of Amsterdam, 1999.

12 De la Rie, E. R. Old master paintings: A study of the varnish problem. *Anal. Chem.* **1989**, *61*, 1228A−1233A, 1237A−1240A.

13 Bonaduce, I.; Brecoulaki, H.; Colombini, M. P.; Lluveras, A.; Restivo, V.; Ribechini, E. Gas chromatographic-mass spectrometric characterisation of plant gums in samples from painted works of art. *J. Chromatogr. A* **2007**, *1175*, 275–282.

14 Mazel, V.; Richardin, P.; Debois, D.; Touboul, D.; Cotte, M.; Brunelle, A.; Walter, P.; Lapre'vote, O. Identification of ritual blood in African artifacts using ToF-SIMS and synchrotron radiation microspectroscopies. *Anal. Chem.* **2007**, *79*, 9253–9260.

15 Bonaduce, I.; Colombini, M. P.; Diring, S. Identification of garlic in old gildings by gas chromatography-mass spectrometry. *J. Chrom. A* **2006**, *1107*, 226–232.

16 Kuckova, S.; Hynek R.; Kodicek, M. Matrix-assisted Laser Desorption Ionisation Mass Spectrometry, Applied to the Analysis of Protein Paint Binders. In *Organic Mass Spectrometry in Art and Archaeology*; Colombini, M. P., Modugno, F., Eds.; John Wiley & Sons: Chichester, U.K., 2009; pp 165−187.

17 van den Berg, J. D. J.; van den Berg, K. J.; Boon, J. J. Identification of non cross-linked compounds in methanolic extracts of cured and aged linseed oil-based paint films using gas chromatography-mass spectrometry. *J. Chromatogr. A* **2002**, *950*, 195–211.

18 Keune, K. Binding media, pigments and metal soaps characterised and localised in paint cross-sections, Ph.D. thesis, University of Amsterdam, 2005.

19 Schilling, M.; Carson, D.; Khanjian. H. Gas chromatographic determination of the fatty acid and glycerol content of lipids IV. Evaporation of fatty acids and the formation of ghost images by framed oil paintings. Bridgland, J.; Brown, J. In *Preprints of 12th Triennial Meeting of ICOM Committee for Conservation*, James and James: London, 1999. LYON, pp 242−247.

20 www.clericus.org/astr/index.htm.