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A MULTIDISCIPLINARY ASSESSMENT TO INVESTIGATE A XXII DYNASTY WOODEN COFFIN

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Abstract

The characterization of historical artefacts at a molecular level is becoming an increasingly important aspect for cultural heritage. All the developments in extraction, separation and analytical methodologies can be helpful for challenging tasks, such as the identification of chemical compounds (proteinaceous binders, oils, varnishes) used by artists. Any information thus obtained, is fundamental to investigate the executive techniques and the constitutive materials, at the same time they can also be used to develop protocols for conservation treatment. In this paper, we present the molecular characterization of the organic components extracted from the preparation layers and the pictorial surface of an Egyptian wooden coffin. The artefact is part of the Egyptian Collection held at the Archeological National Museum of Naples (IT) and belongs to a specific type known with the name of "yellow coffin", dated at the beginning of the XXII Dynasty. Following the identification of the wood used to build the coffin, we performed on each of them an extraction of the organic components, which, according to differences in chemical-physical properties, were subsequently divided into three categories (monosaccharides, lipids and proteins) and separately analyzed. Pigments are not the subject of the current study. The ingenuity of our methodology relies on the use of powerful analytical methodologies (i.e. high-resolution MS) which led to the unambiguous identification of heterogeneous molecules.

Keywords*: Preparation layers; Painting technique; Wood identification; Proteins; Lipids; Polysaccharides; Mass spectrometry; Egyptian coffin*

Introduction

The characterization of the materials used for historical artworks at a molecular level is becoming an increasingly important aspect for cultural heritage [1]. Nowadays all the developments in extraction, separation and analytical methodologies can be helpful for challenging tasks, such as the reconstruction of the "chemical palette" used by artists [2-4].

Information on the manufacturing technique and on the materials (which is the aim of this investigation) are a great source of knowledge useful also for conservation treatments. However, there are several difficulties to be overcome. Primarily, in order to preserve the

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integrity of the artefact, the amount (size, weight, etc.) of samples to take for the analysis must be as small as possible, thus requiring the use of a very sensitive analytical methodology. Additionally, ageing, storing conditions and previous conservation treatment attempts can affect the intrinsic nature of the original compounds thus introducing further heterogeneity in the molecules to be identified [5, 6]; therefore the analytical methodology must be able to deal with very complex matrices.

Because of these difficulties, a widescreen procedure able to collect as much information as possible, avoiding numerous sampling procedures, is rather desirable. To this aim, a multi-analytical approach can represent the best choice.

According to literature data, numerous approaches for the characterization of the organic components of artefacts are focused on compounds with the same (or similar) chemical and physical properties [7], while some others [8] suggest the possibility of using a single generic procedure capable of extracting analytes with diverse properties, followed by different analytical strategies. Our approach is based on the latter assumption; the novelty of our methodology relies on the combination of analytical methodologies, which led to the simultaneous and unambiguous identification of a large number of different analytes on the same sample, thus greatly improving the quality and reliability of the results.

Artefacts from ancient Egypt represent an interesting case of study; as it is in fact well-known, natural compounds were used for the realization of utensils, sacred objects, artworks, etc. For example, animal proteins (glue, albumen), polysaccharides elements (resin, gum) and fatty acids esters/oils (beeswax) were used (among the others) as adhesives [9]; similarly, gelatin, glue, gum, albumen and beeswax were mixed as binders with pigments [9]. All listed natural compounds are characterized by significative differences in both chemical and physical properties and can be grouped into three main categories: proteins, polysaccharides and fatty acids.

Our protocol was applied to the characterization of the organic materials extracted from the preparation and paint layers of the lid of an Egyptian wooden coffin (Fig. 1).

Fig. 1. The Egyptian wooden sarcophagus under investigation. Sampling areas are highlighted

A cross-section (Fig. 2) image of a fragment taken from the lid of the coffin clearly shows the presence of four layers above the wooden support, therefore in order to collect informations on all the different layers, three micro samples on the surface of the lid were selected from exposed parts, as shown in figure 1.

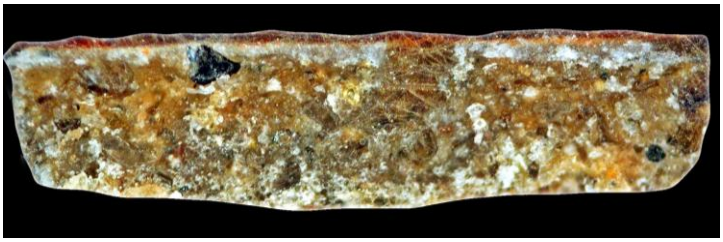

Fig. 2. Cross section of a fragment taken from the lid of the sarcophagus observed with an optical microscope (100X).

After the recognition of the wood used to build the coffin, the first step of our workflow was the extraction of the organic molecules. Several extraction procedures have been tested by different groups [10-13], we selected the one based on ammonia solutions that seemed to return the best results [8, 14].

The second step of our protocol was the fractionation (based on the solubility in different organic solvents) of analytes into the three categories (polysaccharides, lipids and proteins) previously indicated.

The third step of our workflow (aimed at the identification of extracted analytes) was based on mass spectrometric analysis by GC-MS (for monosaccharides and lipids) and LC-MSMS (for proteins), preceded by appropriate derivatization and/or digestion procedures depending on the molecules to be identified. As nowadays widely accepted, MS represents one of the most established methodologies in the diagnosis in conservation and fine arts, because of its sensibility as well as its ability to analyze complex matrices.

Coffin description

The coffin herein analyzed belongs to the Drosso-Picchianti Collection, sold to the Real Museum of Naples on February 27th 1828, as attested by a document reporting of a "mummy of a priest" purchased for the price of 240 ducats (Archivio Storico, Soprintendenza ai Beni Archeologici delle province di Napoli e Caserta, IV B 10, 27/02/1828). The artefact is an inner anthropoid coffin and belongs to a specific type known in Egyptology as “yellow coffin” characterized by a rich decoration painted on a yellow priming layer, from which the name derives. According to its iconography and inscriptions, the coffin is datable towards the end of the XXI and the beginning of the XXII Dynasty (959-889 BC ca.) [15-17].

The coffin was made using different wooden planks, joined along the edges with circular dowels or tenon and mortise joints, finally adding on the surface separately carved parts (the face and the hands). Both lid and base show three different stages of preparation (Fig. 2). Immediately on the last layer, there is a red drawing used to outline the figures initially, which was then filled with vivid colors for the final rendering of the shapes.

Materials and Methods

Chemicals

Urea, ammonium bicarbonate (AMBIC), Trypsin TPCK-treated and of N,O-bis-(trimethylsilyl)-acetamide (TMS) from bovine pancreas were from Sigma-Aldrich. Acetonitrile, formic acid, chloroform and methanol were purchased from Baker. Acetone, n-hexane, and chloroform were supplied by Sigma-Aldrich; acetyl chloride, acetonitrile (ACN), were provided by Fluka; formic acid and methyl alcohol anhydrous were supplied by Carlo Erba; bidistilled water (MilliQ) was provided by Millipore. All other reagents and solvents were of the highest purity available from Carlo Erba, Rodano, Italy.

Sample description

For the characterization of the constituent materials, four micro-samples were collected from parts that were exposed because of some lacks.

Three samples (A, B and C) were collected as follow:

- Sample A, white preparation layer (Fig. 1);
- Sample B, red paint layer/drawing (Fig. 1);
- Sample C, yellow priming layer (Fig. 1).

For the identification of the wood, two samples were collected as follow:

- Sample 1, wood from the lid of the coffin (Fig. 1);
- Sample 2, wood from the base of the coffin (Fig. 1).

The dimension of each sample was considerably small: about 1 mm^3volume and about 1 mg weight.

Wood identification

Light microscopy and scanning electron microscopy (SEM) observations of wood section features were performed for the identification of the woods. We referred to analytical keys texts [18, 19]. Terminology according to Schweingruber (1990) was adopted for the descriptions of characters useful for wood identification.

For light microscopy, transverse and longitudinal (tangential and radial) handmade sections of sampled woods were observed with a Zeiss Axiolab microscope (Zeiss, Jena, Germany) and photographed with a Nikon Digital Sight DS-L1 (Nikon, Tokyo, Japan). For SEM observations, wood samples were coated with gold to about 30nm. The samples were observed under a VEGA3 TESCAN environmental scanning electron microscope at an accelerating voltage of 20kV (fig. 3).

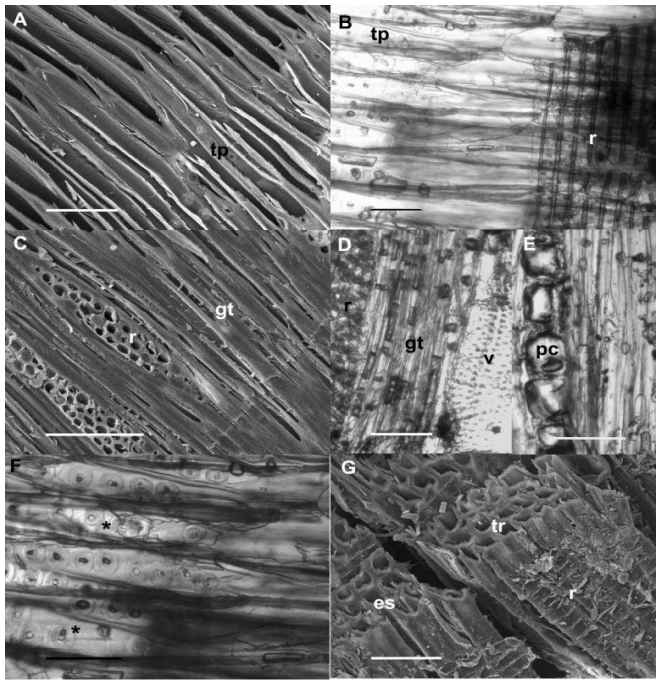

Fig. 3. Cupressus sempervirens wood:
A. wood surface showing tracheids with uniseriate pits (tp);
B. radial section showing tracheids with uniseriate pits (tp), and a ray (r);
C. wood surface showing ground tissue (gt) and rays (r);
D. tangential section showing a ray (r), ground tissue (gt), and a vessel (v);
E. tangential section showing prismayic crystals (pc);
F: bordered pit erosion (asterisks);
G: tracheids (tr) with middle lamella erosion (es).
Notes: C-D. Acacia sp. Wood, F-G bacterial and fungi erosion on cypress wood,
Bar: A, C and G = 100µm, B, D and F = 50µm

Cross sections optical microscopy

Observations of cross sections were carried out on a Nikon Eclipse L150 microscope [20].

Extraction of the organic components

In order to extract the organic fraction from each sample, according to literature data [8, 14], we used an ammonia solution-based procedure summarized in figure 4. More analytically, 400µL of 2.5M NH_3 were added to each sample; the extraction was performed in an ultrasonic bath at room temperature for 30min and then for 120min at room temperature. The procedure was repeated twice. The extracts were evaporated to dryness under vacuum and then suspended in 100 µL of tri-fluoro-acetic acid (TFA) 1.0%; to separate polar compounds (proteins and polysaccharides) from the non-polar ones (lipids) we performed a liquid-liquid extraction by the addition of 200µL of diethyl ether (three times). Subsequently, proteins were separated from the polysaccharides by precipitation by the addition of 200µL Methanol in the in -20ºC for 120min. All the fractions were analysed separately.

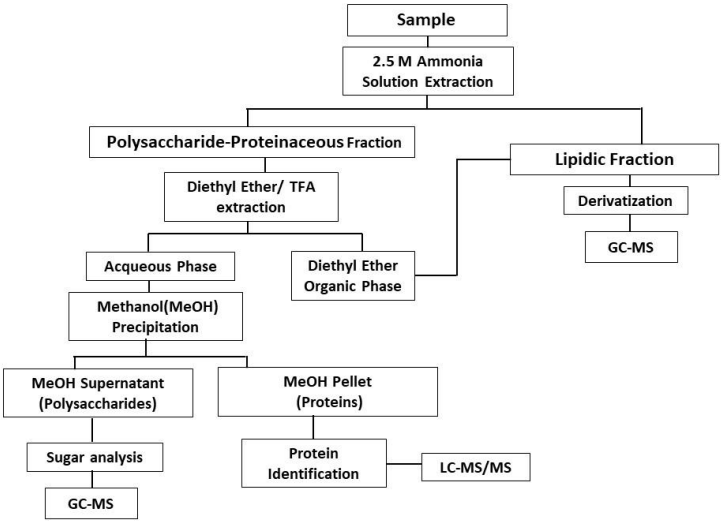

Fig. 4. Flow chart describing the principal steps of the protocol used to extract analytes

Polysaccharides fraction

Aliquots (100µL) of the polysaccharides containing fraction were dried under vacuum and submitted to methanolysis. The reaction was performed by adding 500µL of methanolic HCl (125µL of acetyl chloride were added to 2,5mL of methyl alcohol anhydrous) to the samples; the re-N-acetylation of the monosaccharides mixture was performed by adding 500µL of methanol, 10µL of pyridine and 50µL of acetic anhydride at room temperature for 15min. Sugars were finally trimethylsilylated in 200µL of N,O-bis-(trimethylsilyl)-acetamide (TMS) at 70°C for 15min. Each sample was dried down under nitrogen, dissolved in 50µL of hexane and centrifuged to remove the excess of solid reagents. The hexane supernatant (1/60) was used for the GC-MS analysis.

Lipids fraction

After liquid-liquid extraction (Fig. 4) lipids were analysed as Fatty Acids Methyl Esters (FAME). Briefly, lipid containing fractions were dried under vacuum. Transesterification was performed by adding 150µL of sulphuric acid to 850µL methanol, 95°C for 16h. Subsequently, the pH was corrected to neutrality using 2mL of a solution of ammonium bicarbonate 100mg/mL. The excess of salts was then removed by water. The resulting fatty acids methyl

esters were dried under nitrogen flow and then suspended in 1.0mL of n-hexane. 1.0µL of the sample was analyzed by GC-MS.

GC-MS analysis

GC-MS analyses were performed on an ISQ-QD quadrupole mass spectrometer (Thermo Fisher scientific) equipped with a TRACE™ 1300 Gas Chromatograph using a Zebron ZB-5HT Inferno (5%-Phenyl-95%-Dimethylpolysiloxane) fused silica capillary column (Column 30×0.32×0.10µm) from phenomenex.

The injection temperature was 250°C, the oven temperature was held at 70°C for 2min and then increased to 230°C at 20°C/min, increasing to 240°C at 20°C/min and finally to 270°C at 20°C/min and held for 3min. Electron Ionization mass spectra were recorded by continuous quadrupole scanning at 70eV ionization energy, in the mass range of m/z 30-800. Mass spectra assignment was generally based on the direct match with the spectra of NIST library, if the correlation match index was higher than 95%, the identification was considered reliable. Each analysis was repeated in triplicate.

Proteins fraction

The proteins containing fractions were evaporated under vacuum and the pellet suspended in 10µL Urea 6M. Disulfide bonds were reduced and alkylated. Reduction was performed by using a 10:1 (DTT: cysteins) molar ratio. After 2h incubation at 37°C, iodoacetamide was added to perform carboxyamidomethylation using an excess of alkylating agent of 5:1 to the moles of thiolic groups. The mixture was then incubated in the dark at room temperature for 30min. The alkylation reaction was stopped by addition of formic acid to an acidic pH. Samples were 6-fold diluted with ammonium bicarbonate 10mM pH = 7.5 and enzymatic digestion carried out by the addition of 1µg of trypsin at 37°C for 16h. The supernatants were then recovered by centrifugation, filtered on 0.22µm PVDF membrane (Millipore), and peptides were desalted and concentrated by in-house C18 extraction stage tips as described by *Cappellini* [21]. Peptides were eluted by 20µL of 50% Acetonitrile, 0.1% Formic acid solution and analyzed by LC-MS/MS on an LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany).

LC-MS/MS

C-18 reverse phase capillary column 75µm×10cm (Thermo Fisher Scientific), was used using at a flow rate of 300nL/min, with a gradient from eluent A (0.2% formic acid in 2% acetonitrile) to eluent B (0.2% formic acid in 95% acetonitrile). The following gradient conditions were used: t = 0min, 5% solvent B; t = 10min, 5% solvent B; t = 90min, 50% solvent B; t = 100min, 80% solvent B; t = 105min, 100% solvent B; t = 115min, 100% solvent B; t = 120min; 5% solvent B. Peptides analysis was performed using data-dependent acquisition of one MS scan followed by CID fragmentation of the five most abundant ions. For the MS/MS experiment, we selected the three most abundant precursors and subjected them to sequential CID-MS/MS acquisitions. For the MS scans, the scan range was set to 400–1800m/z at a resolution of 60,000, and the automatic gain control (AGC) target was set to 1 × 106. For the MS/MS scans, the resolution was set to 15,000, the AGC target was set to 1 × 105, the precursor isolation width was 2Da, and the maximum injection time was set to 500ms. The CID normalized collision energy was 35%; AGC target was set to 1 × 105. Data were acquired by Xcalibur™ software (Thermo Fisher Scientific). Each analysis was repeated in triplicate.

MASCOT identification

The acquired MS/MS spectra were transformed in Mascot Generic files (.mgf) format and used to query the SwissProt database 2015_04 (548, 208 sequences; 195, 282, 524 residues), with Chordata as taxonomy restriction for protein identification. A licensed version of Mascot software (www.matrixscience.com) version 2.4.0 was used with trypsin as enzyme; 3, as allowed number of missed cleavage; 10ppm MS tolerance and 0.6Da MS/MS tolerance; peptide charge from +2 to +3. No fixed chemical modification was inserted, but possible oxidation of methionine, deamidation of asparagine and glutamine were considered as variable

modifications. When collagen proteins were identified, a further identification search was carried out, with the insertion of hydroxylation on lysine and proline as variable modifications, since more confident identifications are commonly obtained for these proteins, considering the extensive post-translational modifications. Only proteins presenting two or more peptides were considered as positively identified. Individual ion score threshold provided by Mascot software to evaluate the quality of matches in MS/MS data was applied and the protein with scores lower that this were rejected.

Results and Discussion

Wood identification

In order to identify the wood species of the lid and the base of the coffin, wood samples were collected as shown in figure 1 (samples 1 and 2).

The following histological characteristics led to the identification of the wood samples:

Sample 1 (lid sample): *Acacia* sp. Rays generally 3 to 4 seriate, average height 10 to 20 cells. Rays homogeneous to slightly heterogeneous. Perforation plates simple, no spiral thickenings of the vessel wall. Prismatic crystals in long chains in the axial parenchyma (Fig. 3C and D).

The principal trees growing in Egypt during dynastic times and whose timber was employed by carpenters and joiners were Acacia, Sycamore, Fig and Tamarisk. Acacia wood has been identified in many artefacts (coffins, boxes etc.) produced from the Predynastic Period up to the Late 5th century A.D. [9]. Moreover, Acacia is one of the few trees depicted on the walls of tomb and temple that can be recognized with certainty [9]. Nile Acacia is native to Egypt, growing on the Nile banks, and is seldom straight growing, of great length or girth, although it is very hard, heavy and durable [22].

The wooden sample 1 is from a species belonging to the genus *Acacia* (Fig. 3C and D). The correspondence of the characteristic anatomical features with those detected in the wood of *Acacia nilotica*, identified by *Abdrabou et al*, [22] in a Saqqara coffin dated Late Period (712-332 BC), would suggest that *Acacia nilotica* wood was used as the support of the coffin under investigation.

Sample 2 (base sample): *Cupressus sempervirens*. Resin canals absent in observed samples. Rays homocellular. Average height of rays 10 to 15 cells. Tracheid pits uniseriate (Fig. 3A and B). This sample has been taken from a wooden element probably added during a XIX Century conservation treatment. The wood sample show bacterial degradation of bordered pits (Fig. 3 F) with border erosion (asterisk) characteristic of type 3 bacterial attack [23] and erosion of the compound middle lamella (Fig. 3G) usually displayed by white rot fungus attack on wood previously degraded by bacterial erosion [24]. The greater resistance of the oldest acacia wood to the attack of fungi and bacteria is consistent with the high resistance of archaeological woods to such types of attack reported in the work of *Björdal and Nilsson* [24].

Layers characterization

The cross section image (Fig. 2) shows the red layer on the top used as drawing, immediately below, there is a yellow priming layer (the last layer of the preparation, used also as color), that is characteristic of coffins belonging to the "yellow coffin" typology [25]; finally, there is a white preparation layer. X-ray diffraction analysis (XRD) of the white and yellow layers (data not shown) clearly shows the presence of anhydrite (anhydrous calcium sulfate). The first layer of preparation called "*mouna*" (not under investigation in this study), is a material of mineral origin with a large granulometry composed of clay, stone fragments and plant fibers, directly in contact with the wooden support.

We then addressed our attention to the characterization of the organic components in the white preparation layer, in the yellow priming layer and in the paint layer of the coffin (respectively samples Fig. 1A, B and C).

To investigate the nature of the organic materials possibly present in the samples, we applied a multistep extraction procedure (described in Materials and Methods section and summarized in figure 4) to separate the three main classes of organic molecules: polysaccharides, lipids and proteins. We used different extraction solvents, taking advantage of the different physical and chemical properties of the extracted molecules.

In order to identify putative binders constituted by polisaccharidic polymers, Methanol supernatant fractions (Fig. 4) were subjected to a sugar analysis based on methanolysis, TMS derivatization and GC-MS analysis to investigate the presence of the monosaccharides thus produced. Figure 5 shows the chromatogram obtained by the analysis of sample A, similar chromatograms were obtained by the other two samples (data not shown).

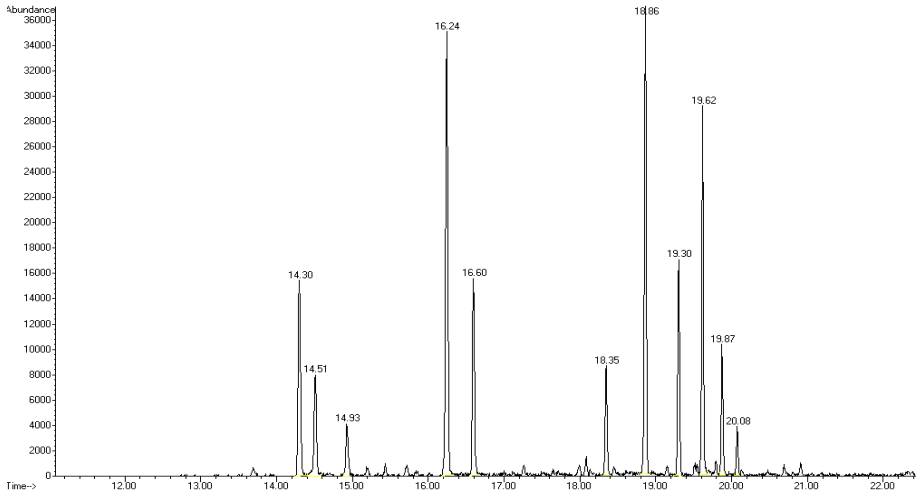

Fig. 5. TIC showing the profile of extracted and TMS derivatized monosaccharides extracted from sample A

Taking advantage of the fragmentation spectra and of the retention times (compared with the ones of standard mixtures) we could identify the analytes. As reported in Table 1 we could detect Galactose, Glucuronic Acid, Ramnose and Arabinose in all the samples. In particular, the co-presence in samples A and C (as long as the reproducibility of the relative ratios) of all of them is strongly indicative of the presence of Arabic gum since these sugars are its main constituents [26, 27]. Arabic gum is one of the most common binders used by ancient Egyptians [9, 28] and our data suggest its presence. Sample B (the paint layer) show some differences compared to the internal ones (samples A and C), probably for this sample (being the external one) some degradation events occurred related to ageing, to conservation conditions and, more generally, to interactions with the external environment.

Fig. 6 shows the chromatogram of GC-MS analysis of FAMEs extracted from sample A; also in this case, the other two chromatograms (samples B and C) were similar. Results are summarized in Table 2. Palmitic and stearic acid (saturated fatty acids) and oleic acid (monounsaturated fatty acid) were identified as the most abundant components; though, these FAMEs cannot be used to univocally identify a specific material, since they are among the most common molecules that can be found in many different biological matrices. Their co-presence, as long as the relative quantities, however, is compatible with the presence of biological matrices such as egg [29] likely used in painting techniques.

Moreover, the identification of linoleic acid in the paint layer (sample B) is very interesting since this fatty acid is one of the most abundant components of natural oils, used as drying oils.

Table 1. TMS Monosaccharides profile obtained by GC-MS analysis

Sample	RT(min)	Monosaccharide	Abbreviation	%
A	14.30	Rhamnose	Rha	5.2
	16.25	Arabinose	Ara	25.3
	18.34	Galactose	Gal	43.8
	22.03	Glucuronic acid	GlcA	25.7
B	13.49	Rhamnose	Rha	2.3
	15.71	Arabinose	Ara	29.5
	18.46	Galactose	Gal	68.2
C	14.3	Rhamnose	Rha	6.2
	16.24	Arabinose	Ara	28.1
	18.35	Galactose	Gal	41.9
	22.07	Glucuronic acid	GlcA	23.8

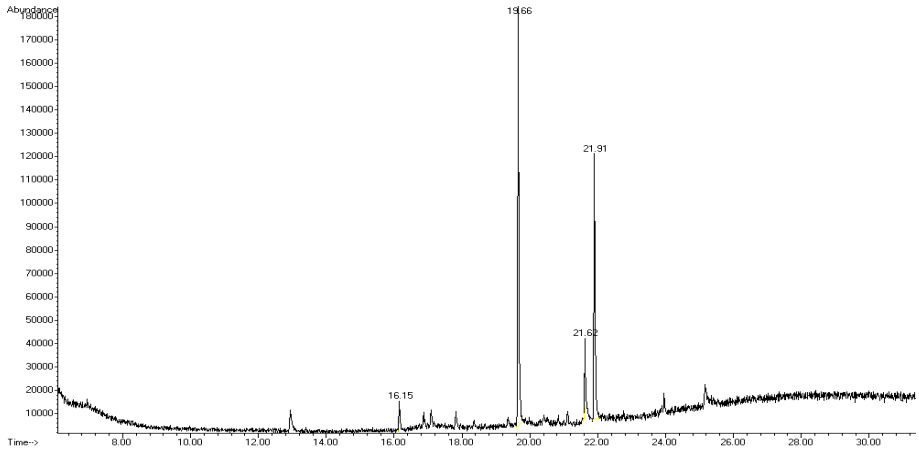

Fig. 6. TIC showing the profile of extracted lipids (FAME) from sample A

Table 2. Fatty Acids Methyl Ester profile obtained for each sample by GC-MS analysis.
*(C:N) indicates the number of carbon atoms (C) and double bonds (N) in the fatty acid side chains.

Sample	RT(min)	Fatty acids methyl esters	C:N*	%
A	19.21	Myristic acid	14:0	1.7
	22.12	Palmitic acid	16:0	48.3
	24.12	Oleic acid	18:1	8.0
	24.4	Stearic acid	18:0	41.9
B	19.65	Palmitic acid	C16:0	24.0
	21.53	Linoleic acid	C18:2	1.8
	21.62	Oleic acid	C18:1	26.3
	21.9	Stearic acid	C18:0	45.9
C	19.65	Palmitic acid	16:0	44.6
	21.61	Oleic acid	18:1	6.9
	21.89	Stearic acid	18:0	48.5

Methanol pellets from the three samples (refer to Fig. 4) were submitted to a MS based proteomics protocol to identify proteins eventually present, according to the analytical procedure reported in Materials and Methods. As detailed in Table 3, collagen was unambiguously identified in the preparation layers (samples A and C), suggesting the use of animal glue in the manufacture of the coffin itself. Moreover, in the external layer (sample B) we could identify lysozyme, one of the most abundant proteins in albumen, suggesting that egg was used in the painting of the external layers of the coffin. Egg yolk was commonly used in tempera painting technique, though in some cases the whole egg was used, as in this case the identification of lysozyme (one of the most abundant protein in albumen) suggests [30].

Table 3. Identification of the proteins in the samples from Sarcophagus by overnight trypsin digestion.

Sample	Identified protein (Accession number)	Score	Sequence coverage (%)	Matched sequence (Oxidation of methionine, hydroxylation of proline and lysine, deamidation Gln and Asn were inserted as variable modifications in the MSMS Ion Search Program).
A	Lysozyme (P00700)	100	24	-.KVFGR.C R.HGLDNYR.G R.CELAAAMKR.H + Oxidation (M) R.CELAAAMKR.H R.CKGTDVQAWIR.G
	Collagen alpha-1(I) (P02453)	89	11	R.GVVGLPGQR.G + Hydroxylation (P) R.GVQGPPGPAGPR.G + Deamidated (NQ); Hydroxylation (P) R.GEPGPAGLPGPPGER.G + 3 Hydroxylation (P)
	Collagen alpha-2(I) (P85154)	72	4	R.GEAGPAGPAGPAGPR.G R.GAPGAIGAPGPAGANGDR.G + Deamidated (NQ); 2 Hyroxylation (P)
B	Lysozyme (P00700)	68	17	-.KVFGR.C R.HGLDNYR.G
C	Collagen alpha-1(I) (P02453)	764	41	R.GVVGLPGQR.G + Hydroxylation (P) R.GRPGAPGPAGAR.G + 2 Hydroxylation (P) R.GVQGPPGPAGPR.G + Deamidated (NQ); Hydroxylation (P) R.GQAGVMGFPGPK.G + Deamidated (NQ); Oxidation (M); Hydroxylation (P) R.GPSGPQGPSGPPGPK.G + Hydroxylation (K) R.GSAGPPGATGFPGAAGR.V + 2 Hydroxylation (P) R.GETGPAGPAGPIGPVGAR.G R.GLTGPIGPPGPAGAPGDK.G + 2 Hydroxylation (P) K.DGEAGAQGPPGPAGPAGER.G + Hydroxylation (P) R.VGPPGPSGNAGPPGPPGPAGK.E + Deamidated (NQ); 3 Hydroxylation (P) R.VGPPGPSGNAGPPGPPGPAGK.E + Deamidated (NQ); 3 Hydroxylation (P) R.GPPGPMGPPGLAGPPGESGR.E + Oxidation (M); 2 Hydroxylation (P) K.GEPGPTGIQGPPGPAGEEGK.R + 2 Hydroxylation (P) K.TGPPGPAGQDGRPGPPGPPGAR.G + Deamidated (NQ); 4 Hydroxylation (P)
	Collagen alpha-2(I) (P02465)	419	24	R.GEPGNIGFPGPK.G + Deamidated (NQ); Hydroxylation (K) (K); Hydroxylation (P) R.IGQPGAVGPAGIR.G + Hydroxylation (P) R.GEAGPAGPAGPAGPR.G R.GIPGPVGAAGATGAR.G + Hydroxylation (P) R.GAPGAIGAPGPAGANGDR.G + Deamidated (NQ); 2 Hydroxylation (P) K.GAAGLPGVAGAPGLPGPR.G + 3 Hydroxylation (P) R.GPPGESGAAGPTGPIGSR.G + Hydroxylation (P) R.GSTGEIGPAGPPGPPGLR.G + 2 Hydroxylation (P) K.GEPGAPGENGTPGQTGAR.G + 2 Deamidated (NQ); 2 Hydroxylation (P) R.GPNGDSGRPGEPGLMGPR.G + Deamidated (NQ); 3 Hydroxylation (P) R.GPPGNVGNPGVNGAPGEAGR.D + 3 Deamidated (NQ); 3 Hydroxylation (P) R.TGPPGPSGISGPPGPPGPAGK.E + Hydroxylation (K) (K); 3 Hydroxylation (P)
	Collagen alpha-1(III) (P02458)	278	24	R.GPAGANGLPGEK.G + Deamidated (NQ); Hydroxylation (P) K.GESGAPGVPGIAGPR.G + 2 Hydroxylation (P) R.GPPGPPGTNGVPGQR.G + 2 Deamidated (NQ); 3 Hydroxylation (P) K.DGASGHPGPIGPPGPR.G + 2 Hydroxylation (P) R.GETGPAGPSGAPGPAGSR.G + Hydroxylation (P) R.GPTGPIGPPGPAGQPGDK.G + Deamidated (NQ); 2 Hydroxylation (P) R.GVAGEPGRNGLPGGPGLR.G + Deamidated (NQ); 3 Hydroxylation (P) R.GGPGERGEQGPPGPAGFPGAPGQNGEPGAKGERGAPGEK.G + Deamidated (NQ); Hydroxylation (K); 4 Hydroxylation (P) R.GENGSPGAPGAPGHPGPPGPVGPAGK.S + Deamidated (NQ); 4 Hydroxylation (P)
	Lysozyme (P00700)	120	24	-.KVFGR.C R.HGLDNYR.G R.CELAAAMKR.H R.CKGTDVQAWIR.G

Proteins were identified searching Uniprot database, with all entries as taxonomy restriction, with MS/MS Ion search Mascot software (Matrix Science). Only identification of proteins with at least two peptides with individual ion score above the significance threshold (>34), were considered as significative.

Conclusions

In ancient Egypt, the design and structure of coffins are strongly connected to the historical periods as well as to the social position of the dead. Coffins of royals and nobles were made of precious imported wood, whereas coffins of people from middle or lower classes were generally made of local wood, which was of lower quality and properties and cheaper [22, 31].

The characterization of artefacts materials, at a molecular level, is more and more gaining momentum. Data collected can be useful not only to increase historical knowledge, but also can contribute in the selection of the most suitable conservation methodology and materials to be used. Several protocols have been proposed in the last few years to gain a comprehensive picture of the chemical nature of binders, preparation layers, painting techniques etc.; in this perspective, by using a multistep protocol, we aimed at obtaining an unambiguous identification of different classes of heterogeneous molecules in a single campaign.

In the preparation layers, we could detect anhydrite (data not shown) mixed with an rganic matrix composed by Arabic gum. Anhydrite was widely used by Egyptians in the preparation layers of coffins, as already demonstrated in Sekhemkhet pyramid in Saqqara [9, 32-39]. This identification can explain the significative cracks on the lid and in the base, as well as the widespread leaching of the paint film on the lid. In fact, high humidity can cause the partial dissolution of the calcium sulphate and its continuous recrystallization resulting in to the deterioration of the paint film (swellings, falls, etc.).

Arabic gum, a widely used binder in Egyptian tempera painting, was identified in the preparation layers (samples A and C, and some monosaccharides ascribable to the same polysaccharide also detected in the decorative layer, sample B). Arabic Gum is made from Acacia exudate and forms a transparent, slightly viscous film soluble in water, the Egyptians used to dilute the pigments in water and then add the gum in order to give body and robustness to the colour backgrounds.

The lipids we identified are widespread in many biological matrices and their detection cannot be conclusive in the classification of the biological matrix present; their presence could be also attributed to secondary applications (detergents, waxes, etc.) occurred over the centuries. However, their identification can be very interesting, especially if considered in combination to other data. In this respect, the simultaneous detection of lysozyme, one of the most abundant proteins in albumen definitively points towards the use of chicken egg in the decoration of coffin. In the other way around, the identified lipids are compatible with the detected protein in the decorative layers (sample B). It is worth mentioning that the decorative layers are the external ones, they interact with the environment making proteins identification extremely challenging because of extensive degradation.

Nevertheless, we were able to identify proteins in all the layers analysed. The presence of collagen in the preparation layers, suggest that animal glue was used in the construction of the coffin.

As a whole our campaign, shows an interesting characterization of the materials used for the realization of an Egyptian wooden yellow coffin. We identified of the original wood used to build the coffin and we could identify some parts added more recently thus suggesting some restoration attempts. At the same time, thanks to the organic component's characterization, we could identify the materials used to obtain the coffin in its final aspect. After the identification of the Arabic gum, we could unambiguously identify the proteins extracted from the layers, thus being able to identify the painting technique and the composition of the preparation layers.

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References

[1] R. Vinciguerra, *Proteomics and Cultural Heritage*, PhD Thesis, Università degli Studi di Napoli Federico II, 2016.

[2] V. Pauk, P. Barták, K. Lemr, *Characterization of natural organic colorants in historical and art objects by high-performance liquid chromatography***, Journal of Separation Science, 37,** 2014, pp. 3393-3410.

[3] J. Romero-Noguera, I. Martín-Sánchez, M.M. López-Miras, J.M. Ramos-López, F. Bolívar-Galiano, *Biodeterioration patterns found in dammar resin used as art material*, **Electronic Journal of Biotechnology, 13**(3)**,** 2010, pp. 1-8, DOI: 10.2225/vol13-issue3-fulltext-7.

[4] S. Rinaldi et al, **La fabbrica dei colori: pigmenti e coloranti nella pittura e nella tintoria,** Roma: II Bagatto, 1986.

[5] G. Leo, I. Bonaduce, A. Andreotti, G. Marino, P. Pucci, M.P. Colombini, L. Birolo, *Deamidation at asparagine and glutamine as a major modification upon deterioration/aging of proteinaceous binders in mural paintings*, **Analytical Chemistry, 83,** 2011, pp. 2056-2064.

[6] A. Lluveras-Tenorio, R. Vinciguerra, E. Galano, C. Blaensdorf, E. Emmerling, M.P. Colombini, L. Birolo, I. Bonaduce, *GC/MS and proteomics to unravel the painting history of the lost Giant Buddhas of Bāmiyān (Afghanistan),* **Plos One, 12,** 2017, https://doi.org/10.1371/journal.pone.0172990

[7] W. Chen, W. Li-Qin, Y. Lu, Z.-Z. Ma, *Application of gas chromatography-mass spectrometry for the identification of organic compounds in cultural relics*, **Chinese Journal of Analytical Chemistry, 41,** 2013, pp. 1773-1779.

[8] A. Lluveras, I. Bonaduce, A. Andreotti, M.P. Colombini, *GC/MS analytical procedure for the characterization of glycerolipids, natural waxes, terpenoid resins, proteinaceous and polysaccharide materials in the same paint microsample avoiding interferences from inorganic media*, **Analytical Chemistry, 82,** 2009, pp. 376-386, https://doi.org/10.1021/ac902141m

[9] A. Lucas, J. Harris, **Ancient Egyptian Materials and Industries**, Courier Corporation, 2012.

[10] M.P. Colombini, R. Fuoco, A. Giacomelli, B. Muscatello, *Characterization of proteinaceous binders in wall painting samples by microwave-assisted acid hydrolysis and GC-MS determination of amino acids*, **Studies in Conservation, 43**(1)**,** 1998, pp. 33-41.

[11] R. Vinciguerra, A. De Chiaro, P. Pucci, G. Marino, L. Birolo, *Proteomic strategies for cultural heritage: from bones to paintings*, **Microchemical Journal, 126,** 2016, pp. 341-348, https://doi.org/10.1016/j.microc.2015.12.024.

[12] C. Tokarski, E. Martin, C. Rolando, C. Cren-Olivé, *Identification of proteins in renaissance paintings by proteomics*, **Analytical Chemistry, 78**(5)**,** 2006, pp. 1494-1502, https://doi.org/10.1021/ac051181w

[13] W. Fremout, S. Kuckova, M. Crhova, J. Sanyova, S. Saverwyns, R. Hynek, M. Kodicek, P. Vandenabeele, L. Moens, *Classification of protein binders in artist's paints by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: an*

evaluation of principal component analysis (PCA) and soft independent modelling of class analogy (SIMCA), **Rapid Communications in Mass Spectrometry, 25,** 2011, pp. 1631-1640.

[14] G. Gautier, M.P. Colombini, *GC–MS identification of proteins in wall painting samples: a fast clean-up procedure to remove copper-based pigment interferences*, **Talanta, 73,** 2007, pp. 95-102, doi: 10.1016/j.talanta.2007.03.008

[15] G.L. Nicola, *I sarcofagi della XXI dinastia nel Museo Egizio di Torino*, **Sarcofagi della XXI dinastia (CGT 10101-10122)**, Ministero per i Beni e le Attività Culturali – Soprintendenza al Museo delle Antichità Egizie, Torino, 2004.

[16] R. Cantilena, P. Rubino, *La Collezione egiziana del museo archeologico nazionale di Napoli*, Published by Arte Tipografica, Napoli, Italy, 1989.

[17] * * *, **Guida alla collezione egizia del MANN**, 2016, ISBN 9788891811240

[18] F.H. Schweingruber, **Anatomie europäischer Hölzer. Ein Atlas zur Bestimmung europäischer Baum, Strauch- und Zwergstrauchhölzer**, 800 S., 3473 SW-Fotos. Verlag Paul Haupt, Bern und Stuttgart, 1990, ISBN 3-258-04258-6.

[19] S.L.S. Berti, N. Macchioni, L. Sozzi, **Progetto Xyloteca, Banca dati Legnami e software di riconoscimento specie per chiavi dicotomiche, versione per Sistemi operativi MS-Windows. Operating systems** (in Italian). I.Va.L.S.A. G. L. Vottero, Ecodata, Italy, 2002

[20] Archaeometric and Chemometric Studies Involved in the Authentication of Old Heritage Artefacts II. Old linden and poplar wood put into work

[21] Spiridon, P., Sandu, I.C.A., Nica, L., Iurcovschi, C.T., Colbu, D.E., Negru, I.C., Vasilache, V., Cristache, R.A., Sandu, I., **Revista de Chimie, 68**(10), 2017, pp. 2422-2430.

[22] E. Cappellini, L.J. Jensen, D. Szklarczyk, A. Ginolhac, R.A. da Fonseca, T.W. Stafford Jr, S.R. Holen, M.J. Collins, L. Orlando, E. Willerslev, *Proteomic analysis of a pleistocene mammoth femur reveals more than one hundred ancient bone proteins*, **Journal of Proteome Research, 11,** 2011, pp. 917-926, https://doi.org/ 10.1021/pr200721u

[23] A. Abdrabou, M. Abdallah, I.A. Shaheen, H.M. Kamal, *The application of multispectral imaging and reflectance transformation imaging to an ancient Egyptian polychrome wooden stela,* **The 18th Triennial Conference of ICOM-CC, Copenhagen, Denmark**, 2017, pp. 4-8, DOI:10.13140/ RG.2.2.27181.18404

[24] H. Greaves, *Micromorphology of the bacterial attack of wood,* **Wood Science and Technology, 3,** 1969, pp. 150-166.

[25] C. Björdal, T. Nilsson, *Waterlogged archaeological wood—a substrate for white rot fungi during drainage of wetlands,* **International Biodeterioration & Biodegradation, 50**(1)**,** 2002, pp 17-23.

[26] R. Salem, A. Owais, R. Salama, *The Conservation of an egyptian polychrome wood coffin from late period,* **Egyptian Journal of Archaeological and Restoration Studies, 6,** 2016, p. 23-37

[27] M.E. Osman, P.A. Williams, A.R. Menzies, G.O. Phillips, *Characterization of commercial samples of gum Arabic*, **Journal of Agricultural and Food Chemistry, 41,** 1993, pp. 71-77, https://doi.org/10.1021/jf00025a016

[28] A. Gallina, E. Fiorese, P. Pastore, F. Magno, *Identification and semi-quantitative determination of gum arabic in wines by GC-MS and size exclusion chromatography*, **Annali di Chimica: Journal of Analytical, Environmental and Cultural Heritage Chemistry, 94,** 2004, pp. 177-184.

[29] P.T. Nicholson, I. Shaw, **Ancient Egyptian materials and technology**, 2000, ISBN-13: 978-0521120982

[30] S. Kuckova, R. Hynek, M. Kodicek, *MALDI-MS applied to the analysis of protein paint binders,* **Organic Mass Spectrometry in Art and Archaeology,** 2009, pp. 165-187, DOI: 10.1002/9780470741917.ch6
[31] M.P. Colombini, G. Gautier, **GC/MS in the Characterisation of Protein Paint Binders,** John Wiley & Sons: Chichester, United Kingdom, 2009.
[32] P.T. Nicholson, I. Shaw, **Ancient Egyptian Materials and Technology,** Cambridge University Press, 2000
[33] Sandu, I.C.A., Roque, A.C.A., Matteini, P., Schafer, S., Agati, G., Correia, C.R., Viana, J.F.F.P., *Fluorescence recognition of proteinaceous binders in works of art by a novel integrated system of investigation,* **Microscopy Research and Technique, 75**(3), 2012, pp. 316-324. DOI: 10.1002/jemt.21060.
[34] Kuckova, S.H., Sandu, I.C.A., Crhova, M., Hynek, R., Muralha, S.V., Fogas, I., Schafer, S., *Protein identification and localization using mass spectrometry and staining tests in cross-sections of polychrome samples,* **Journal of Cultural Heritage, 14**(1), 2013, pp. 31-37. DOI: 10.1016/j.culher.2012.03.004.
[35] Sandu, I.C.A., Schafer, S., Magrini, D., Bracci, S., Roque, A.C.A., *Cross-Section and Staining-Based Techniques for Investigating Organic Materials in Painted and Polychrome Works of Art: A Review,* **Microscopy and Microanalysis, 18**(4), 2012, pp. 860-875. DOI: 10.1017/S1431927612000554.
[36] P. Hatchfield, R. Newman, *Ancient Egyptian gilding methods*, **Gilded Wood**, Sound View Press, 1991, pp.27-48
[37] D. Bigelow, **Gilded Wood: Conservation and History**, Madison, Conn.: Sound View Press, 1991.
[38] Kuckova, S.H., Sandu, I.C.A., Crhova, M.K., Coufalova, L., Krizova, I., Hynek, R., Muralha, S.V., Schafer, S., Fogas, I., *Material Research of Gilding on Medieval Polychrome Sculpture*, **Chemicke Listy, 109**(11), 2015, pp. 864-869.
[39] Kuckova, S.H., Schultz, J., Veiga, R., Murta, E., Sandu, I.C.A., *Proteomics tools for the contemporary identification of proteinaceous binders in gilded samples,* **International Journal of Conservation Science, 6**(SI), 2015, pp. 507-518 Special Issue: SI.

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