Case Report: Museum

The Lady of Pavia: microbiological essays and qualitative GC/MS characterization of embalming materials

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Abstract: The application of molecular characterization techniques to an Egyptian mummy preserved in the Archaeological Museum of the University of Pavia, as part of a wide research project conducted by the Mummy Project Research, described the embalming technique adopted. The number of analytical methods applied has also made it possible to identify the presence of some molecules attributable to plants, not included among those commonly reported in similar cases. Before the study, a microbiological screening of ten zones on the surface of the mummy revealed the effective antiseptic properties of the substances present. The chemical composition of the balms was then investigated using quadrupole Gas Chromatography/Mass Spectrometry (GC/MS), testing the effects of different extraction solvents and the use of a derivatizing agent, which allowed to improve the detection of some compounds. Overall, the compounds identified are linked to an embalming technique that involves the use of bitumen, oils, and resins, common in the Late and Ptolemaic Periods. Some analytical methods have also made it possible to detect traces of compounds possibly attributable to some genera of plants used by the Egyptians, which grow on the Mediterranean coast of North Africa or in southern tropical regions. The work, still ongoing, highlights the importance of using different analytical methods to detect even minor amounts of compounds difficult to identify. This approach aims at the improvement of the understanding of medical and embalming techniques used in ancient Egypt.

Keywords: mummification, embalming materials, microbiological essays, GC/MS, terpenoids

Introduction

Organic embalming materials

The ancient Egyptians made extensive use of organic substances of plant, animal, and mineral origin in their medical and funerary practices. In mummification processes, they were used in large amounts after the body had been dehydrated with salts such as natron (Abdel-Maksoud & El-Amin, 2011). Being generically defined as balms, their use has also given the name to the embalming process itself. The organic substances were applied directly to the skin, the inner parts of the body and also between bandages. Their use was mainly linked to the effectiveness they demonstrated in stopping or slowing down the organic decay (Abdel-Maksoud & El-Amin, 2011).

Compounds of mineral origin were essentially bitumen, asphalt and natural pitch, which were generally found in natural oil outcrops. The most used came from the Dead Sea area and were known as bitumen of Judea or pitch of Judea. Starting from the New Kingdom, they were used for embalming and found increasing popularity so that a study has assessed that in the Ptolemaic and Roman periods, bitumen was present in 87% of the cases taken into account (Clark et al., 2016).

Among the substances coming directly from living organisms, those of animal origin have been used to some extent in embalming techniques e.g. animal fat, beeswax ,and likely propolis (Moser & Nicola 2017; Mezzatesta et al., 2020). However, the number of substances of vegetabl origin, used in the form of resins, oils and extracts, was by far larger (David, 1992).

A wide class of vegetable oleoresins historically used for embalming derived from the shrubs of the *Pistacia* genus, which are widespread in the Mediterranean basin and include species such as lentisk, terebinth and pistachio. These once precious substances are chemically similar and are now difficult to distinguish from each other (De Vartavan, 2007). They are often referred to as mastic resin or Chios mastic, from the name of the Greek island on which a particularly valuable quality is produced from lentisk (Assimopoulou & Papageorgiou, 2005).

In addition to these, also resins and oils from coniferous, especially cedar, juniper and pine are of primary importance (Abdel-Maksoud & El-Amin, 2011). Although coniferous oils and resins have been used since the VI dynasty, their use becomes common only in periods that are more recent. Analysis of Roman mummies, for example, revealed a very widespread use of them indicating a content of up to 37% inside the organic tissues and bandages of the mummies (Buckley & Evershed, 2001). The progressive emergence of the use of coniferous oils and resins indicates an evolution of the embalming technique and is probably linked to their excellent antiseptic properties which are also used by plants themselves with a protective function from parasites, fungi, and bacteria (Guimarães et al., 2019).

The use of numerous other plants in funerary rites and embalming techniques is reported in international literature and in ancient sources without, however, always having adequate support from direct chemical analyses. These include as instance myrrh, frankincense, cassia, cinnamon, onion, henna, and lichens (Abdel-Maksoud & El-Amin, 2011).

The reason why direct evidence is often poor or lacking can be found in several factors. It may be for instance because many substances are labile and degrade over time. In this case, they leave traces difficult or impossible to detect or in the best cases their components get oxidized at various extent and became difficult to identify (Mezzatesta et al., 2020). Furthermore, often a specific substance is difficult to recognize lacking a unique marker that allows to distinguish each plant (Ménager et al., 2014). Finally, the issues of the identification may be due to an insufficient sensitivity or specificity of the analytical tools used. As will be illustrated in the following, these limits can be partially overcome using a combination of multiple gas chromatographic methods and using new generation instrumentation capable of detecting substances present even in very limited amounts (Łucejko et al., 2017).

1.2 The mummy of the Lady of Pavia and the Mummy Project

The subject of this work is a series of laboratory analyses carried out on the mummy of the Lady of Pavia, as part of a wide research project conducted by the Mummy Project Research team to reconstruct the profile and the story of the individual embalmed and to organize a new Egyptian Section in the Archaeological Museum. She is unwrapped, without any funerary equipment and preserved in a black wooden container from the nineteenth century, which resembled a real coffin. In the inner part of the lid there is the inscription: "Egyptian Mummy. 810 BC., a donation of Dr. Stefano Giorgiani of Cairo, 1824". In 2012, during a survey of the Egyptian human remains in the Lombardia Region, the mummy was inserted in the list of the Mummy Project, to be studied. The Lady of Pavia stayed in the black coffin until 2015 when she was studied by the Mummy Project.

The mummy was donated by Dr. Stefano Giorgiani, Professor of Medicine at the University of Bologna, to the Human Anatomy Laboratory around 1824/1825 and was later transferred to the Museum of Natural History of Pavia University (February 24, 1933). In the early 1960s the mummy was placed in a tower of Visconti's Castle, wherein the collections of the Natural History Museum were kept. Then, in February 2012, she was moved to the Archaeological Museum of the University of Pavia. In March 2017, as a part of the Mummy Project, the mummy has been finally moved in the new Egyptian Section of the Museum, entitled "Egyptian corner" where it is currently kept.

The mummy is completely unwrapped and, like many other mummies, the skin has a blackish tone. The body is that of a young woman who died at an age between 20 and 22 and lived in the 3rd or the 4th century BCE (De Pietri, 2018; Malgora et al., 2017). The provenance is unknown; however, according to the inscription on the lid of the black container, Thebes could be considered. The arms are crossed in the attitude of the god Osiris, typical of Late Period mummies (Malgora, 2013; Malgora, 2014; Malgora & Elias, 2014). Small screws driven into the heels caused a detachment of the tissue and some crumbling occurred in the upper left leg and in the pelvis area. The right parietal bone of the skull was cut long after her death, for an inspection and then restored, like the abdomen that shows a wide horizontal incision. The mummy, in fact, was autopsied at the School of Medicine in the 19th century (De Pietri 2018). In 2016, she was in an endangered state of conservation and thus the Mummy Project has been actively involved in an action aimed at preserving it. Art and archaeology conservators Gian Luigi Nicola and Alessandro Nicola performed the required operations. In this circumstance a screening was performed on the surface of the mummy by means of microbiological investigations involving 10 areas that were visually compromised (e.g. white spots, dusty or crumbling surface, etc.). The intent was to assess the presence of in-progress decomposition phenomena, sustained by deteriorative microorganisms. Some micro-samples were also taken to conduct chemical analyses in laboratory and were analyzed with Gas Chromatography/Mass Spectrometry GC/MS. The results are presented in this paper.

The analytical campaign has been part of a wider research project. The aim was to achieve a stronger awareness about the mummy, granting a wide availability of new data. During the project several activities have been developed: investigations, medical and anthropological analyses, including the CT scan, to get a full descriptive picture of the finding from the Egyptological, human, and pathological point of view. Other activities comprised: 3D forensic facial reconstruction, C14 dating, microscopic and molecular analysis of the tissues, and DNA analysis to support the anthropological investigations and to study possible pathologies. However, the greatest focus has been to ensure the best preservation of the mummy. One of the main preservation actions has been the construction of a special glass-case for the mummy, in conformity with the European regulation, providing full visibility. This "casket" takes into consideration not only preservation needs but also fulfils people's ethics and sensibility. Further aims have also been to offer new teaching methods and assure understanding by people with special needs implementing a 3D full scale copy of the mummy, realized by the team of Spazio Geco of Pavia. This special "tool" allows a deeper study also by the sense of touch and it is precious, above all, for the blind or visually handicapped. The 3D forensic facial reconstruction can give back a sort of "living" picture and somehow allows to understand, that this finding, is not just there to evoke history, but it is also a human body, valuable and worthy of our respect.

Materials and Methods

Archeological Samples

Samples were taken from the loose material that could be observed in several places around the mummy, prior to undertake the conservative intervention. In particular, the four samples subjected to GC/MS analysis derive from the area of the lower abdomen and of the right shoulder of the mummy. In both areas, a conspicuous amount of detached powdery material and fragments were visible (see Fig. 1). The samples are listed in the following Table.

Solvents and Reagents

All solvents were of the highest purity grade (mass grade). Dichloromethane, cyclohexane, hexane, toluene, acetic acid buffer, ammonium buffer, and sodium methoxide were all supplied by Sigma–Aldrich.

Extractions

METHOD 1 and 2 (Derivatization and extraction respectively with cyclohexane and dichloromethane).

Each sample, before being extracted, is subjected to derivatization with sodium methoxide. After derivatization, the sample is introduced into a tube with a screw cap, and an extraction is carried out for 1 hour by introducing it into a sonicator at 35-40 °C. The extract is completely collected from the screw tube and filtered on a membrane at 0.22 microns in a drop flask. The

Figure 1. Location of sampling areas.

tube is then washed twice with 1-2 ml of solvent to collect all the extract. Filtration aims to eliminate any solid residues that could damage the subsequent chromatographic process. The drop flask is finally placed in a rotating vacuum evaporator programmed at 65 °C and the solvent is eliminated by distillation. The residue obtained is cooled, taken up with cyclohexane or dichloromethane and set to volume. Finally, the desired dilutions are made using the same solvent.

METHOD 3 (Direct multiple extractions).

The sample is homogenized with blender apparatus and two distinct aliquots subjected to solid/liquid extraction respectively with toluene and hexane. Subsequently, after centrifugation at 5000RPM for 10', the supernatant is treated with a basic aqueous solution (water + ammonia buffer) or acid aqueous solution (water + acetic acid buffer). This last step has been followed by a second extraction in liquid/liquid solvent to bring the unknown analytes back into the organic phase useful for splitless injection in GC/MS.

Table 1.

Name	Position	Aspect
LP0	Lower Abdomen	Fragment
LP1	Lower Abdomen	Fragment
LP2	Lower Abdomen	Powder
LP3	Right Shoulder	Powder

GC/MS conditions

A Shimadzu GC-2010 gas chromatography system was used consisting of a Shimadzu AOC-201 automatic sampler coupled with a Shimadzu GCMS-QP2010 Plus mass spectrometer. The samples obtained after each type of extraction in the various dilutions were processed as follows. Injection into gas chromatograph with medium polarity separation column and quadrupole mass detector that allows the identification of a wide spectrum of mainly lipophilic molecules such as terpenes, hydrocarbons with various carbon content, either cyclic, linear or branched and other chemical species of origin vegetable.

The identification obtained in GC/MS was performed with the SCAN analysis followed SIM mode and the environmental conditions required for the execution of the test are those between18-30 °C temperature and 25-50% RH. The injection volume is 1μl. The identification of the analytes is based on: a) Retention time of the analyte (window ± 5%) and/or Kovats index; b) three SIM transitions.

The interpretation was conducted with the NIST08 library for the qualitative identification of the compounds. Confirmation of the identification of the analyte occurs directly to the moment of integration of the chromatographic peak, having primarily optimized the masses of the target ions and for some analytes evaluated about the currents of the mass spectrum. The method has been tested on some samples of essential oils balm (thymol) suitably diluted before the test on the samples from the mummy.

Microbiological essays

Ten zones of the mummy surface were investigated processing the materials and swabs using dextrose agar Sabouraud medium.

Each swab has been sown in culture on Sabouraud Agar incubated in a climatic chamber at 25 °C and controlled humidity for the detection of molds and yeasts. Furthermore, the testing materials were also seeded on Agar P.C.A. incubated in a thermostat at 32 °C for 5 days, for the search for heterotrophic deteriogenic bacteria.

Results and Discussion

The microbiological survey of the mummy was performed to check for molds, yeasts and heterotrophic bacteria that can cause deterioration. All the samples show no microbial growth after five days of incubation at 32 °C confirming the high antiseptic activity of the substrate. Although some forms of decay on the mummy (see for example the whitish point-like areas in Fig. 2) were initially indicated as being of possible microbiological origin, the analyses carried out suggest to find the causes elsewhere. In particular, in the light of the results, it is possible to hypnotize that similarly to what has been observed in other cases such signs of degradation (i.e. whitish dots, spots, etc.) could be actually attributable to accumulations of substances of e.g. waxy derivation possibly due to recrystallization following cyclic changes in microclimatic conditions (Goyon & Vergnieux 1987; Tchapla et al., 2004).

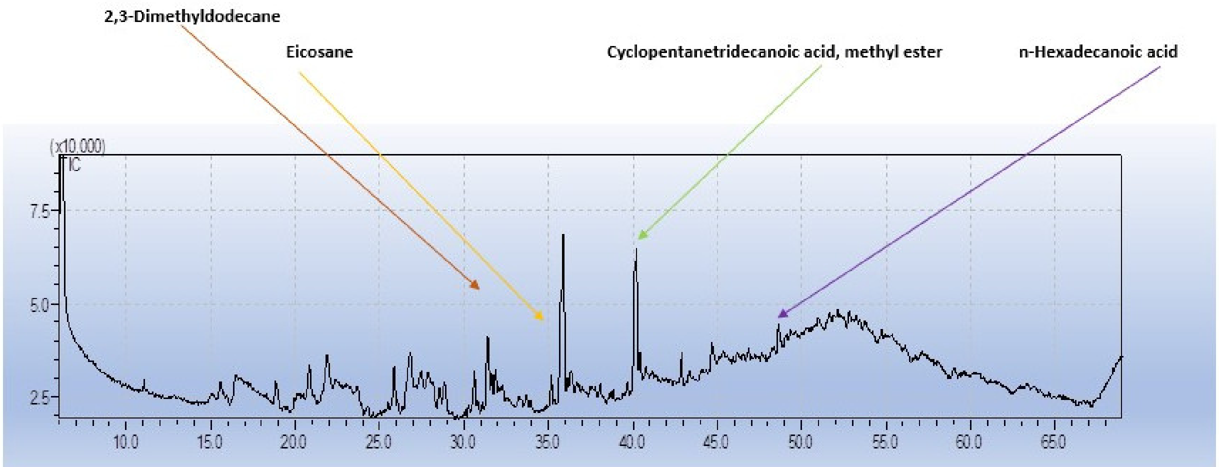
The GC/MS analyzes performed to investigate the chemical composition of the embalming materials reveal the presence of a large number of organic substances.

Method 1 and 2 were used to study LP1, LP2 and LP3.

In a first attempt, LP1 was analyzed individually but the limited amount of sample available and a certain degree of heterogeneity within the sample itself reduced somehow the amount of information obtainable. For this reason, a more homogeneous and abundant sample (i.e. LPA) has been obtained grinding the residual part of LP1 together with LP2 and LP3. In this way the sample LPA results as representative of the balms and other substances present in the mummy. The analyses performed on LP1 highlighted the presence of a specific marker i.e. the methyl ester of 15-hydroxydehydroabietic acid. It is a diterpenic substance that analogously to many other dehydroabietic acid derivatives, is linked to the presence of aged coniferous resins (Tchapla et al., 2004).

The Method 1 (i.e. derivatization and extraction with cyclohexane) applied to the sample LPA (see Fig. 3) highlights the presence of alkanes and of long-chain fatty acids and their esters (particularly the derivatives of the fatty acids containing 16 and 18 carbon atoms). Like many other compounds identified the latter are often oxidized or recombined at various extent likely due to the long time that has elapsed since their use. The presence of high amounts of Cyclic Fatty Acid Monomers (CFAM) such as cyclopentanetridecanoic acid, methyl ester can be also attributable to an heat treatment that maybe was part of the processes used in ancient times to extract or apply some oils or lipidic substances (Sebedio et al., 1987). The presence of alkanes can be due both to the use of bitumen

Figure 2. An example of whitish point-like areas on the mummy's surface.



	RT	AREA(A)	A%	Height(H)	H%	Name
1	20.842	61895	1.59	7110	1.48	Octane, 2,3,3-trimethyl-
	21.892	74297	1.91	6679	1.39	2-propyl tridecyl ester
	30.617	51454	1.32	8664	1.80	Tridecanol, 2-ethyl-2-methyl-
2	31.403	205118	5.27	18522	3.85	2,3-Dimethyldodecane
	31.641	55143	1.42	7218	1.50	Oxalic acid, 6-ethyloct-3-yl ethyl ester
	31.870	46270	1.19	6765	1.41	Sulfurous acid, hexyl octyl ester
3	35.159	42891	1.10	7409	1.54	Eicosane
	36.118	14315	0.37	3186	0.66	Sulfurous acid, octadecyl 2-propyl ester
	40.110	277196	7.12	33880	7.04	Hexadecanoic acid, 15-methyl-, methyl ester
4	40.207	225591	5.79	36732	7.63	Cyclopentanetridecanoic acid, methyl ester
	40.408	61741	1.59	8130	1.69	Eicosane, 1-iodo-
5	48.620	39936	1.03	6490	1.35	n-Hexadecanoic acid

Figure 3. The chromatogram obtained on the sample LPA using Method 1.

or to the presence of natural waxes such as beeswax (Ménager et al., 2014). A more specific distinction between bitumen and beeswax was not possible due to the lack of appreciable peaks referable to specific GC/MS markers such as steranes and hopanes for bitumen and long chain fatty alcohols for beeswax (Régert et al., 2001; Clark et al., 2016). However, the presence of trace amounts of some polycyclic aromatic hydrocarbon (PAH) and some sterane derivatives is likely a hint for the presence of bitumen while the high amount of palmitic acid and its derivatives suggest also the presence of beeswax. Nevertheless, it is not impossible that the fatty acids come also from fats and oils of different kinds such for instance castor oil (Tchapla et al., 2004). There is a limited presence of short and/or odd number fatty acid esters and significative amounts of eicosane and its derivatives that possibly come from arachidonic acid. All these kinds of fatty acids are generally present in animal fats and oils and so it is possible that these substances were among the minor components of the embalming materials. Their presence could also be due to the decay of fat from the body of the mummy (Tchapla et al., 2004).

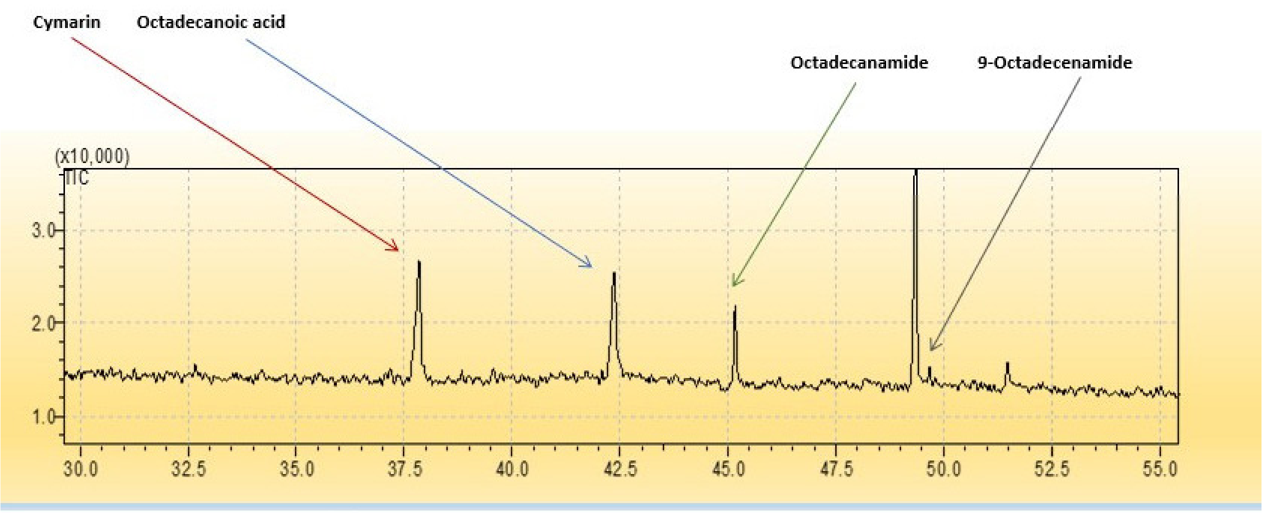
All the materials identified so far are common among the ones that were used in the embalming techniques performed during the reference period (3rd-4th century BCE) and can be found in many of the cases presented in international literature (e.g. Colombini et al., 2000; Colombini et al., 2004; Tchapla et al., 2004; Colombini et al., 2007; Ménager et al., 2014; Mezzatesta et al., 2020). However, Method 2 (i.e. derivatization and extraction with dichloromethane) applied to sample LPA and Method 3 (i.e. direct multiple extractions) applied to LP0 allow to detect a significative amount of molecules referable also to plants whose identification is uncommon or has never been reported.

This is the case of cymarin which is a cardiac glycoside and an anti-arrhythmia and cardiotonic. It is found in plants of the genus *Apocynum*, including

Apocynum cannabinum and *Apocynum venetum*. A significative signal linked to the presence of this molecule has been detected in the chromatogram obtained from LPA using Method 2 (see Fig. 4). Cymarin is a compound found in some plants of the genus *Strophanthus* (Knittel et al., 2016). The plant is still used in traditional medicine by the populations living in tropical regions (Ofori–Baah & Borquaye, 2019). In Sudan, for instance, a plant of this genus it is used as an antidote for the bite of black-necked cobra and to heal wounds and disease (Agyare et al., 2013). It has also been commonly used since ancient times by many African populations to poison arrows (Fraser & Mackenzie, 1910).

Among the many molecules found in minor amounts, a mention deserves scillarenine. It is a cardiac glycoside peculiar of *Drimia maritima* (Knittel et al., 2015) and it has been detected on LP1. *Drimia maritima* is a plant commonly known as sea squill or sea onion and was used in medicine since very ancient times being also among the ones identified in ancient sources such as the Ebers papyrus (Greeff & Schadewaldt, 1981). It was used for many purposes for instance to improve diuresis or as a remedy for cough, asthma, indigestion, alopecia, pain, and lice (Mahboubi et al., 2019). It was considered also by ancient scholars such as Hippocrates, Theophrastus, Dioscorides, Pliny and Pseudo-Apuleius (Bozorgi et al., 2017).

Method 3 (multiple direct extractions) has currently been applied only to LP0 which is an anomalous detached fragment coming probably from a decayed zone in the lower abdomen. The direct selective extractions performed reveal the presence of significative amounts of terpenoids referable to some plants of a great interest regarding their possible use as embalming materials or as medical treatments. Among them there is Bisabolol (see Fig. 5). Bisabolol is a natural alcohol commonly found in the oil of chamomile and oils of plants of the genus *Teucrium* (e.g. *Teucrium chamedrys*, *Teucrium flavum* and *Teucrium polium*) growing in the coastal area of Egypt and in Middle East (Al-Qudah et al., 2011; Sadeghi et al., 2014). *Teucrium* has been commonly used in medical practices to treat for instance diabetes while chamomile had many uses and was also among the materials found on the mummy of Pharaoh Ramses II (Desroches-Noblecourt, 1997; Buckland & Panagiotakopulu, 2001; Afifi et al., 2005). Other interesting molecules detected in this way are neomyrrhaol – a triterpene found in organic extracts of myrrh – and camphene – a minor component of many natural oils and resins. The analysis also demonstrated



RT	Area(A)	A%	Height(H)	H%	Name
37.925	64255	0.12	10256	0.25	Cymarin
42.368	58666	0.10	9145	0.23	Octadecanoic acid
45.164	29677	0.05	7799	0.20	Octadecanamide
49.343	130411	0.22	28811	0.73	9-Octadecenamide

Figure 4. Detail of the chromatogram obtained on the sample LPA using Method 2. The peak of cymarin is highlighted.

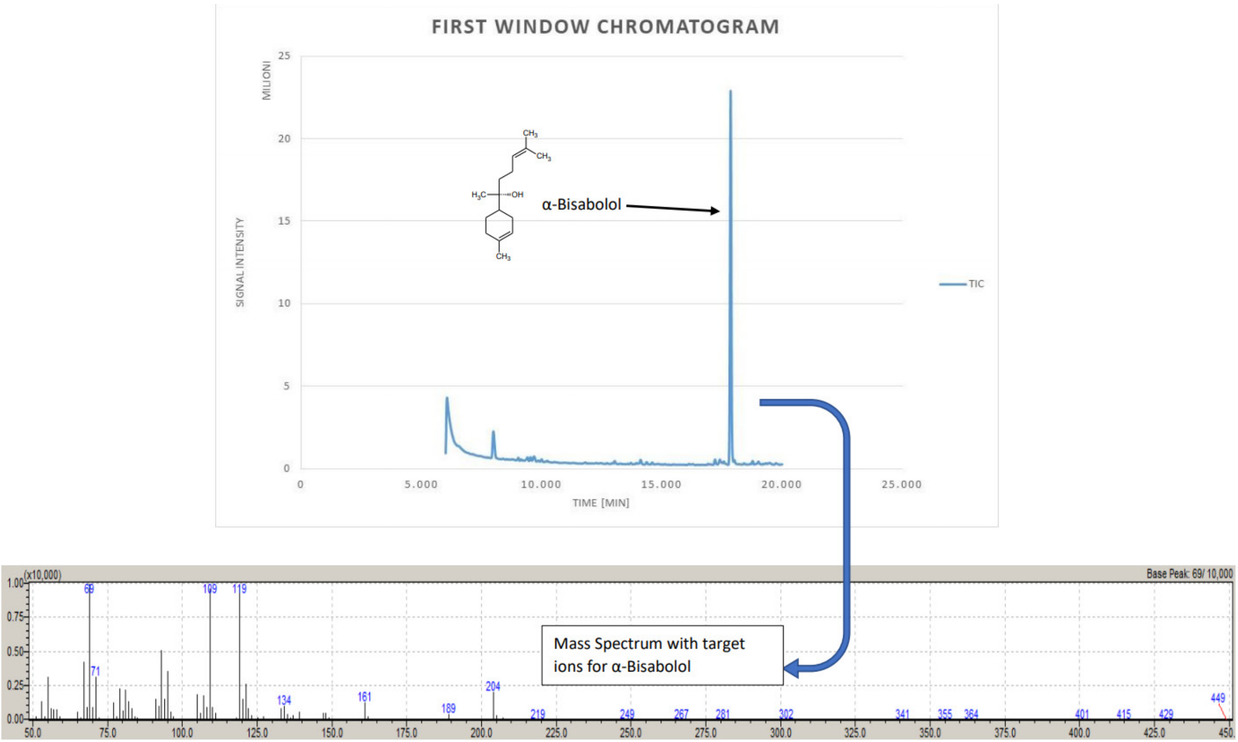


Figure 5. Exemplification of GC-MS analysis. The high panel represents first window chromatogram. The low panel represents the mass spectrum with target ions for the identification of α-Bisabolol in SCAN and SIM analysis.

the presence of guaiacol and its derivatives. This kind of compounds has antifungal and antimicrobial activity and can be found in wood smoke, wood-pitch, and wood tar oil used in mummification (Baumann, 1960; Abdel-Maksoud & El-Amin, 2011). It is also possible that they have been added in XIX-XX century CE with preservatives such as wood-creosote. The detection of cresols and esters of salicylic acid is also referable to such kind of preservation treatments (Nicola et al., 2008).

Conclusions

The ongoing analytical study involving the mummy of Pavia has already made it possible to obtain important results. The microbiological essays indicated that the main cause of decay should not be found in microbial attacks but rather in the environmental stress. The results enforced the decision to improve the climate control using a better showcase to contain the mummy. The preliminary GC/MS analyses confirmed the presence of many compounds with antimicrobial and antiseptic properties such as coniferous resins, and probably bitumen and/or beeswax. Besides of these substances that are commonly found in many mummies, the GC/MS methods used allowed to find some very interesting molecules that can be associated with plants used in embalming techniques or in medical practices. They could come from plants of the genus *Strophantus*, *Drimia*, *Teucrium* and from chamomile and myrrh. Although their use has been suggested their presence has however to be confirmed with further more in-deep and systematic analyzes on more specific samples to avoid any misinterpretation or overinterpretation (Buckland & Panagiotakopulu, 2001). Indeed, also different approaches could be used if possible, e.g. characterization with Nucleic Magnetic Resonance of some specific extracts, palynologic and paleobotanic studies, or the search for ancient DNA from parts of plants leaves or seeds that could luckily be present among the embalming materials. However, it has to be taken into account the fact that little is known of the mummy about the period that goes from its discovery until its arrival in Pavia. Furthermore, some unknown treatments could have also been used during the stay of the mummy in the Gabinetto di Anatomia so any result have to take into account these possible sources of contaminants. In any case, it cannot be denied that the study carried out and the approach adopted are an interesting starting point. They represent a possible model for future studies aimed at improving not only the conservation of the finds but also the knowledge about them. In particular, the final goal is to help to obtain results that in the near future will dramatically improve the knowledge of embalming techniques used by ancient Egyptians and even of their medical practices and of forensic aspects.

Acknowledgements
We are thankful to the University of Pavia for authorizing technical analyses and to the Mummy Project Research team. We are also grateful for the technical assistance of Alida Dell'Anna in preliminary sampling, Elisa Ariotti in microbiological essays and sampling and Alessandro Marello in GC/MS analyses.
Microbiological essays and GC/MS analyses has been funded by LAV srl and are part of the 2016-2019 Internal Research Project on Analysis of Biodeteriogens and Materials in Cultural Heritage.

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