

## Molecular data: clues to trace the origin of archaeological organic materials

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**Abstract:** Molecular studies of archaeological remains using a fractionation scheme of organic extracts and mass spectrometric techniques (GC-MS) allow identification of specific biomarkers. Such components are likely to provide information about the type of organic substances present and, more extensively on their history. In this paper, special emphasis has been put on the necessity for precise structural identification to distinguish beeswax and cuticular waxes. The results of the analysis of two unknown residues are also reported: the content of a pilgrim flask (Egypt, XXVI<sup>th</sup> dynasty, 664-525 BC) and the wall coating of Middle Age undergrounds (Corrèze, France).

**Keywords:** Biomarkers, GC-MS, beeswax, cuticular wax, honey, pitch, vegetal tar, charcoal .

**Résumé:** Les études moléculaires des résidus organiques retrouvés en contexte archéologique, basées sur le fractionnement rigoureux d'un extrait organique par classes de polarité et sur des techniques de spectrométrie de masse (GC-MS), permettent l'identification de biomarqueurs spécifiques. Ces composés sont susceptibles de fournir des informations sur la nature des substances organiques rencontrées et, de façon plus générale, sur leur histoire. Pour illustrer l'utilité des biomarqueurs ainsi que la nécessité d'une identification structurale précise, l'accent a été mis sur les esters linéaires à longues chaînes appelés « wax esters » présents dans les cires d'abeilles et les cires cuticulaires des végétaux. L'analyse de deux résidus inconnus montrera les contraintes liées au contexte archéologique : le contenu d'une gourde dite de pèlerin (Egypte, XXVI<sup>th</sup> dynasty ; 664-525 BC) et l'enduit des murs de souterrains du Moyen-Age (Corrèze, France).

**Mots clés:** Biomarqueurs, GC-MS, cires d'abeilles, cires cuticulaires, miel, poix, goudron végétal, charbon de bois.

### Introduction

Amorphous organic materials found in archaeological remains and artwork, precious remains of past history, can derive from various classes of raw organic substances: bitumen, animal fat, beeswax, resin, vegetal tar, etc. The most widely adopted analytical approach to assess their composition, their degree of

alteration and to get some insight into their history is their molecular and isotopic study (e.g. R. P. Evershed, 2008; A. Macke et al., 2002; J.S. Mills & R. White, 1994; K.E. Peters et al., 2005; A.M. Pollard & C. Heron, 1996; M. Regert & C. Rolando, 1996). The chemical investigation of natural substances, complex mixtures covering wide ranges of molecular weight, polarity and

volatility, is a real analytical challenge. It is even more complicated because of the wide range of materials used (nature of components and proportions in mixtures), the various preparation techniques and the alteration processes linked to both human handling (e.g. heating) and natural ageing (photochemistry, oxidation, biodegradation).

As in bioorganic geochemistry, chromatographic techniques in tandem with mass spectrometry (GC/MS, LC/MS, GC-C-IRMS) are methods of separation and characterization that really suit to the study of complex organic mixture. They enable the identification of a large range of molecular tools called biomarkers used to establish composition and origin of archaeological organic materials and to follow the molecular transformations induced by alteration processes. In the same way that a person has a unique genetic fingerprint, a product derived from naturally occurring organic substances is characterized by a specific molecular fingerprint which consists of an association of biomarkers. Typically, biomarkers are compounds which have a carbon skeleton specific enough to be related to their biological lipid precursors, to natural decay derivatives and to anthropogenic byproducts thus enabling the identification of the natural substances initially used and of their alteration processes. Gas chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) which provides  $\delta^{13}\text{C}$  values of individual compounds is a valuable complementary technique to identify sources of archaeological organic matter (D. Damiani et al., 2003; J.A. Tripp, R.E.M. Hedges, 2004; B. Stern et al., 2008).

To illustrate the importance of chemical molecular data in archaeometry, this paper describes a specific class of components with a powerful biomarker potential: wax esters of different origins (beeswax, cuticular wax). This is exemplified by the study of wax detected in a coating made of alveola discovered in an Egyptian child coffin from the XVIII<sup>th</sup> dynasty and in organic remains stored in Egyptian containers from the Deir el-Médineh necropolis (XVIII<sup>th</sup> dynasty). Moreover, the molecular characterization of organic residues present on art and archaeological items is generally carried out on raw samples without or with limited pretreatment (G.A. Van der Doelen et al., 1998;

M.P. Colombini et al., 2000a; D. Scalarone, 2003). We will therefore show how extraction and fractionation can be of great help for a more in-depth material characterization especially by concentration of compounds often not detected by bulk techniques. Such minor compounds may have a key value to characterize a sample's origin or a degree of alteration (not discussed here). Moreover, this fractionation simplifies the complex molecular fingerprints and makes the identification of components easier.

Lot of works on archaeological materials show positive results thanks to biomarkers and underline the importance of precise structural identification of biomarkers to give *in fine* details about the history of these materials. However, in a second part, we will show that chemical analysis must be adapted according to the specific archaeological context. The molecular analysis of two unknown residues is discussed: the organic content of a pilgrim flask (Egypt, XXVI<sup>th</sup> dynasty, 664-525 BC, Fig. 1c) and a black coating spread on the walls of Middle Age undergrounds (Corrèze, France, Fig. 1d).

## Experimental

### Samples

#### Reference material

In each case, there was no limitation in quantity.

Beeswax was provided by beekeepers after consolidation at moderate temperature without purification. They come from Witzhausen (Deutschland, 2004) and Alsace (France, 2008). The  $\delta^{13}\text{C}$  value of the bulk samples is  $-28\text{‰}$ PDB.

Commercial honeys (acacia, blossom, mandarin honeys), mead, figs, dates, pomegranate juice and vinegar were purchased to be submitted to the same analytical procedure as the archaeological samples.

Extracts of two charcoals are also isolated. Charcoals came from a production site in Auriac (commune de Rilhac Xaintrie, 45°13'08.24N, 2°11'00.34"E, # 2641, flotation sieving to give particles greater than 1 mm, Corrèze, France) and from a pitch kiln at La Font de Mars (Sainte Anne-du-Castellet, Var, France; (A. Gensel, 2005; J. Connan & M. Tengberg 2006)). The sample #2641 from Auriac, obtained by flotation sieving, is composed of particles of wood from beech and deciduous oak (Dr. A.

Durand, personal communication). The sample #2028 from La Font de Mars is made of *Pinus halepensis* wood.

### Archaeological material

*Egyptian coating made of alveoli from the XVIII<sup>th</sup> dynasty*: When opening an Egyptian child coffin made in a tree trunk and dated from the XVIII<sup>th</sup> dynasty, archaeologists discovered a coating made of hundreds of fragile alveoli which were interpreted as a beehive remain (Fig. 1a) (F. Janot, 2002). The  $\delta^{13}\text{C}$  value of the bulk sample, without any acid treatment to remove possible contribution of carbonates, is  $-17\text{‰}$ /PDB. The radiocarbon age determination of the coating gave:  $890 \pm 50$  yr cal B.C., i.e. the Third Intermediate Period (1070-712 BC). The organic substance is thus more recent than the coffin dated 1504-1450 BC which age has previously been estimated by typological observation of the terracotta jar lying close to it. Surprisingly no pollen was found in this sample though to be ancient beeswax. 100 mg of alveoli were used to prepare the organic extract.

*Epicuticular waxes in Deir el-Médineh containers* : Egyptian organic remains stored in an exceptional set of containers unearthed from the Deir el-Médineh cemetery (XVIII<sup>th</sup> dynasty) and put at disposal by the Department of Egyptian Antiquities of the Louvre Museum were analyzed as part of a PhD project (Fig. 1b). The samples were taken from twenty containers under the control of the curator G. Pierrat-Bonnefois (Louvre Museum). The substances were pasty to sticky, orange to brown. Approximately 1mg of sample was used for methanolysis and 30 mg for organic extract and fractionation.

*Pilgrim flask*: The seepage through the wall of a pilgrim flask (Egypt, XXVI<sup>th</sup> dynasty) was taken off by the curator G. Pierrat-Bonnefois (Louvre Museum). It represents only 5 mg of the whole material.

*Coating spread on the walls of Middle Age undergrounds*: Investigations were carried out on four black coatings (references LB01, LB02, LB03, LC01) scratched from the walls of undergrounds likely used as cellar during the Middle Age (La Borde, area of Saint-Cirgues-La Loutre,  $45^{\circ}04'49.88''\text{N}, 2^{\circ}06'06.08''\text{E}$ , La Combe,  $45^{\circ}04'42.94''\text{N}, 2^{\circ}03'18.59''\text{E}$ , area of Saint Geniez ô Merle, Corrèze, France; Fig. 1d) (H. Pigeyre, 2008). 1g of the raw material was

used to prepare an organic extract (0.7 to 3 % of organic extract depending on sample).

### Extraction and fractionation

The analytical procedure (Fig. 2) is directly adapted from the methodology used in petroleum geochemistry to analyze bitumen, source rocks and oils (J. Connan, 2002). All solvents were distilled in glassware before use, to avoid any contamination. The organic samples were extracted ultrasonically three times for five minutes with dichloromethane/methanol (60/40 v/v). The combined solvent extracts, forming the total organic extract, were concentrated by evaporation. The analyses were performed using GC-MS after splitting the total organic extract into fractions F1, F2 and F3, according to different polarities by  $\text{SiO}_2$  column liquid chromatography, fractions F1 and F2 being further split by thin layer chromatography (A. Charrié-Duhaut et al., 2007; A. Charrié-Duhaut et al., 2009a). This scheme allowed us to isolate aliphatic and aromatic hydrocarbons, esters, ketones, alcohols and acids.

### Methanolysis

The crushed sample was taken up in 0.5 ml of methanolic HCl solution (J. Bleton et al., 1996). Methanolysis was conducted at  $80^{\circ}\text{C}$  for 24 h. Thereafter, methanol and HCl were removed using a nitrogen stream. The extract was then acetylated by a mixture  $\text{Ac}_2\text{O}$ /Pyridine.

### GC-MS

Compounds with hydroxyl and/or carboxyl groups have to be derivatised before being analyzed by GC-MS to improve their chromatographic behaviour on the apolar GC-column used in the laboratory.

### Acetylation

500  $\mu\text{l}$  pyridine and 500  $\mu\text{l}$  acetic anhydride were added to the dry alcohol and polyfunctionalised compounds fractions (about 2 mg) for two hours, at  $60^{\circ}\text{C}$  (H.E. Innes et al., 1997). The fractions were then evaporated to dryness under a gentle stream of nitrogen. The alcohol fraction was purified by column liquid chromatography over silica before GC-MS analysis, to eliminate any trace of reagents.



Figure 1 - Photographs of a) nest cells from Egypt, b) containers E16432, E14013, E16446 from Deir el-Médineh (Louvre Museum), c) pilgrim flask, d) Wall of the La Borde underground (Middle Age, © Henri Pigeyre).

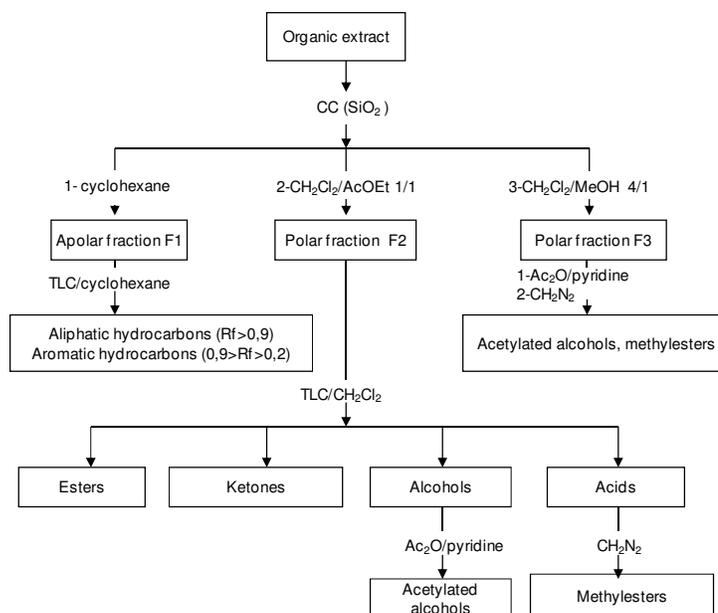


Fig. 2

Figure 2 - Fractionation scheme used for organic samples (CC: column liquid chromatography; TLC: thin layer chromatography).

### **Methylation**

1 ml of ethereal solution of diazomethane, prepared using *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) as precursor (Technical Information Bulletin N°AL-180, Aldrich Chemical) (H.M. Fales et al., 1973; F. Ngan & M. Toofan, 1991) was added for two hours at room temperature to the dry acid fraction. The reaction mixture was then evaporated to dryness under a gentle stream of nitrogen.

### **GC-MS**

GC-MS analyses were carried out on a triple quadrupole ThermoFisher TSQ Quantum spectrometer connected to a Trace GC Ultra gas chromatograph (PTV –on column mode- injector, injector initial temperature : 35 °C, HP-5 MS column, 30 m x 0.32 mm i.d., 0.25 µm film thickness; temperature program a: 40 °C (1 min), 40-100 °C (10 °C/min), 100-300 °C (4 °C/min), isothermal 300 °C, temperature program b used for fractions containing wax esters: 40 °C (1 min), 40-100 °C (10 °C/min), 100-320 °C (4 °C/min), isothermal 320 °C). Helium was used as carrier gas (1.7 ml/min). Mass spectra were produced at 70 eV, source 200 °C, in full detection mode over 50-800 amu.

### **Elemental composition**

The  $\delta^{13}\text{C}$ , TOC (Total organic carbon) measurements and elemental analyses (C, H, N, S) were performed at the Service Central d'Analyse of the C.N.R.S. (Vernaison, France).

## **Results**

### **Discrimination between wax esters of different origin**

#### ***Beeswax***

Beeswax is a natural resource exploited as early as the end of the Ice Age which occurs in a wide variety of archaeological artifacts, such as Neolithic potteries (C. Heron, 1994), medieval ceramic vessels (S. Charters et al., 1995), Egyptian mummification balms (A. Charrié-Duhaut et al., 2009; J. Connan, 1999; M.P. Colombini et al., 2000b; S.A. Buckley & R.P. Evershed, 2001; J. Maurer et al., 2002), Minoan lamps (R.P. Evershed et al., 1997). Its specific molecular fingerprint is well established and recognizable when mixed with other natural

substances or even after alteration. It includes  $\text{C}_{21}\text{-C}_{33}$  odd-carbon-numbered *n*-alkanes, long-chain ( $\text{C}_{24}\text{-C}_{32}$ ) alcohols, even-numbered free fatty acids ( $\text{C}_{22}\text{-C}_{30}$ ), aliphatic monoesters derived from palmitic acid ( $\text{C}_{40}\text{-C}_{52}$  with an even over odd predominance) and hydroxyl wax esters ( $\text{C}_{42}\text{-C}_{54}$ ) (A.P. Tulloch, 1973; P.E. Kolattukudy, 1976). Although alteration processes lead to the depletion of some of these biomarkers like *n*-alkanes, palmitic wax esters can still be identified (M. Regert et al., 2001) by its typical and specific distribution. This characteristic fingerprint was found in two present-day European beeswaxes used as reference for this work (Fig. 3a) and in other archaeological Egyptian materials like the coating of Osiris statuettes (A. Charrié-Duhaut et al., 2009a).

#### ***Egyptian coating made of alveoli from the XVIII<sup>th</sup> dynasty***

At the time of the opening of an Egyptian child coffin (XVIII<sup>th</sup> dynasty), archaeologists discovered a coating made of hundreds of fragile hexagonal alveoli (Fig. 1a). They visually identified it as a beehive (F. Janot, 2002). Consequently we expected to find similarities with beeswax previously described.

Preliminary information about the nature of the coating is given by its elemental composition (carbon 21.4 %, hydrogen 3.0 %, nitrogen 4.9 %, sulfur 0.6 %) and by the low organic extract yield (22 %). These data differ from reference beeswax which are totally soluble in organic solvents and where the percentage of nitrogen is almost zero.

The aliphatic monoesters derived from palmitic acid, ranging from  $\text{C}_{40}$  to  $\text{C}_{52}$  with an even over odd predominance described in beeswax are present in the analysed nest cells but occur with other esters: odd components corresponding to wax esters, long chain aliphatic monoesters derived from other fatty acids (stearic or arachidic), mixed monoesters between cholesterol derived components and the two major fatty acids (palmitic and stearic, Fig. 3b). The ketone fraction of alveoli is dominated by steroidal compounds derived from both cholesterol and stigmaterol in same proportion (Fig. 4a). These features are not found in beeswax. All these data point out that the origin of this enigmatic coating is not a hive from bees.

Discarding hive raised wasp nest as a possibility. Wasp habitat is mainly a paper nest and therefore may be identified by searching for cellulose degradation products namely sugars. After methanolysis and acetylation of the raw sample (J. Bleton et al., 1996), several sugar classes were indeed identified (Fig. 4b): glucopyranoses, galactopyranoses, galactofuranose s. Indicator of non-extractable polysaccharides, their presence explains at least in part the low yield of organic extract. Saccharides are not detected in beeswax. Several natural substances are likely to contribute to this mixture: fruits, chitine (identification of O-methoxy-2-acetamidogalactopyranoses), and cellulose. Some specific insects are able to produce building material after chewing a cellulose source and mixing it with their own excretes. This assumption was confirmed by Claire Villement (Museum of Natural History in Paris) who identified the insects on the basis of their alveoli. Alveoli are typical of a nest of social Hymenoptera. As cells are hexagonal and organised like honeycomb, it cannot be mason bees or wasps which build mud's nests. The only wasp species able to build paper nests is *Vespula Germanica*, the common wasp (Fabricius, 1793).

In conclusion, the molecular study of this specific coating leads to a conclusion completely different to the one based only on visual interpretation. Beeswax is present but admixed with wasp paper nest.

#### ***Epicuticular waxes in Deir el-Médineh containers***

Organic remains found in an exceptional set of containers from Deir el-Médineh (XVIII<sup>th</sup> dynasty) were analyzed as part of this work (Fig. 1b). These containers, still full, were found in a necropolis located on the east side of the Valley of the Kings. The identification of the complex organic mixtures, via the biomarker analysis, may help to have some information about the population of the necropolis. The global molecular analysis indicates only the presence of a lipid base (animal fat or vegetable oil), less or more degraded. Half of the twenty samples analyzed still contained about 40 % of triglycerides. Other biomarkers appear only after fractionation. They correspond to odd numbered *n*-alkanes, phytosterols and co-elutions of wax

esters indicating complex mixtures with flowers or leaves cuticular waxes (E. Ribechini, 2008). Epicuticular wax occurs in almost all vascular plants and acts as a protective coating (J.H. Langenheim, 2003). Unlike beeswax whose biomarkers distribution remains relatively stable, the chemical composition of cuticular wax may differ with plant species, plant part (leaves, aerial parts, etc), plant history (age, season, local conditions, way of incorporation in the archaeological sample through extraction, heating,...). However, in each case, the major components are long-chain odd-carbon-numbered *n*-alkanes, long-chain alcohols, even-numbered free fatty acids and alcoholic esters of fatty acids (C<sub>40</sub>-C<sub>52</sub>). Other compounds such as sterols, terpenoids and saccharides may also occur (G. Rieley et al., 1991; G.A. Logan, 1995). This composition is relatively close to the beeswaxes one. Figure 5 shows the distribution of wax esters in actual beeswax and in an organic mixture from one of the Deir el-Médineh containers. The similarity may be misleading: strong even predominance from C<sub>40</sub> to C<sub>50</sub>. Not only their presence but also their contribution pattern is important for interpretation. In beeswax, wax esters are exclusively alcoholic esters of palmitic acids characterized by a specific fragment at *m/z* 257 (figure 6a). In epicuticular wax, several fragments are detected: *m/z* 257 and 285 and also *m/z* 313, 341 (28 u.m.a. offset one relative to another, figure 6b). Depending on plants and where esters are synthesized, the acidic counterpart may vary: palmitic acid for *m/z* 257, stearic acid for *m/z* 285. Accordingly, in epicuticular waxes, it is possible to have coelution between esters of the same molecular weight but with different structures (M. Regert, 2005). As no diagnostic terpenic structures were detected thus excluding the use of natural resins, cuticular waxes are the only remaining indicators of a plant product input.

#### **Molecular studies of unknown residues**

##### ***Pilgrim flask***

The terms "pilgrim flask" refer to faience containers with a round base and a neck-shaped umbels of papyrus, very popular in Egypt at the Saite period (XXVI<sup>th</sup> dynasty, 664-525 BC).

Molecular data: clues to trace the origin of archaeological organic materials

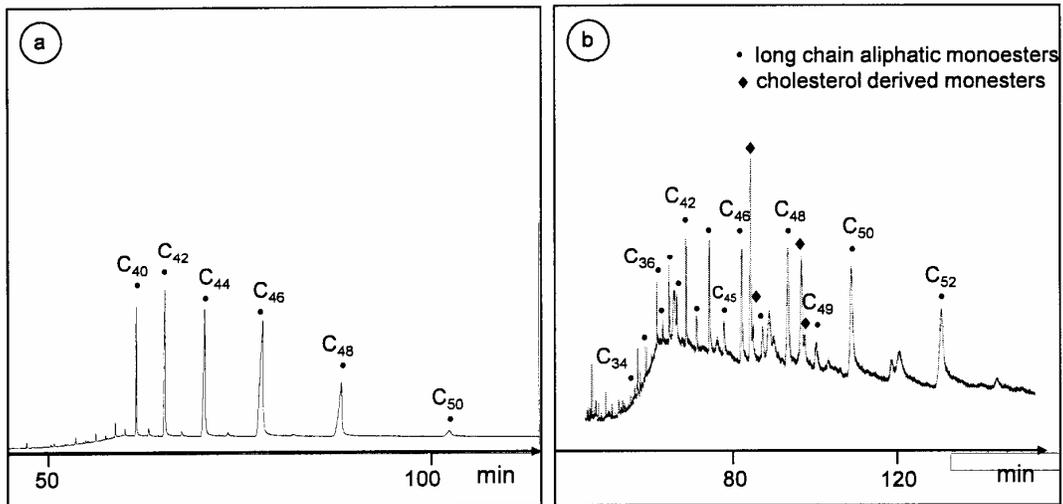


Figure 3 - RIC (GC-MS) of the ester fractions corresponding to beeswax (a) and to the analyzed nest cells (b).

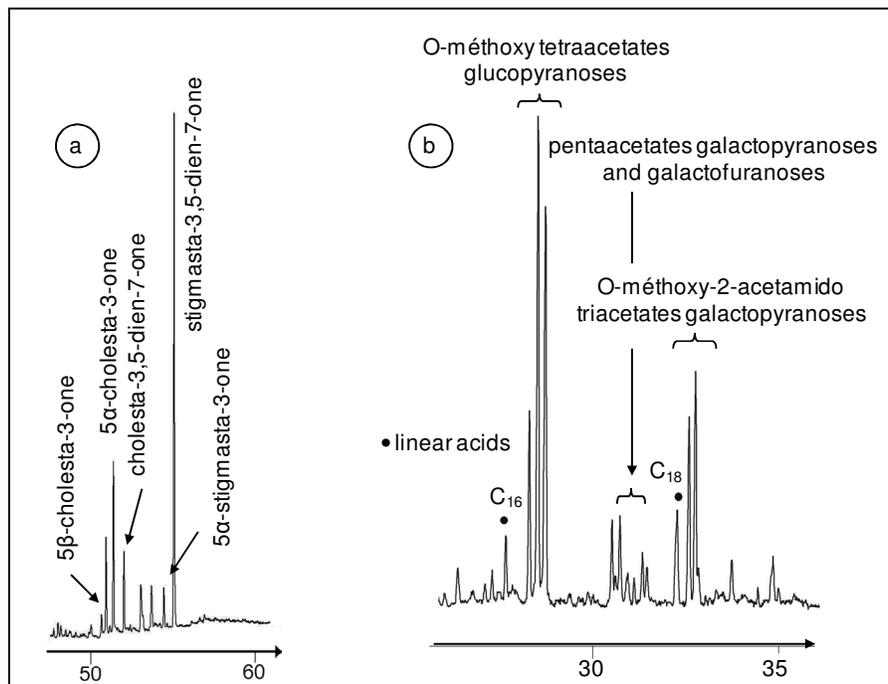


Figure 4 - RIC (GC-MS) of the ketone fraction (a) and of the fraction obtained after methanolysis and acetylation (b) corresponding to the analyzed nest cells.

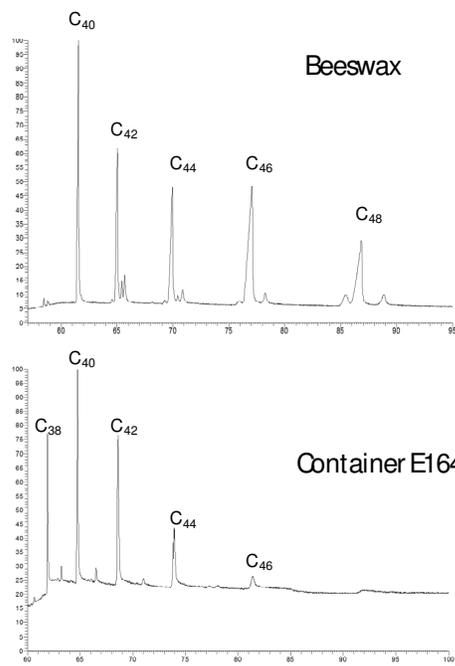


Figure 5 - RIC (GC-MS) of the ester fraction showing the distribution of wax esters in actual beeswax and in an organic remain from the Deir el-Médineh containers.

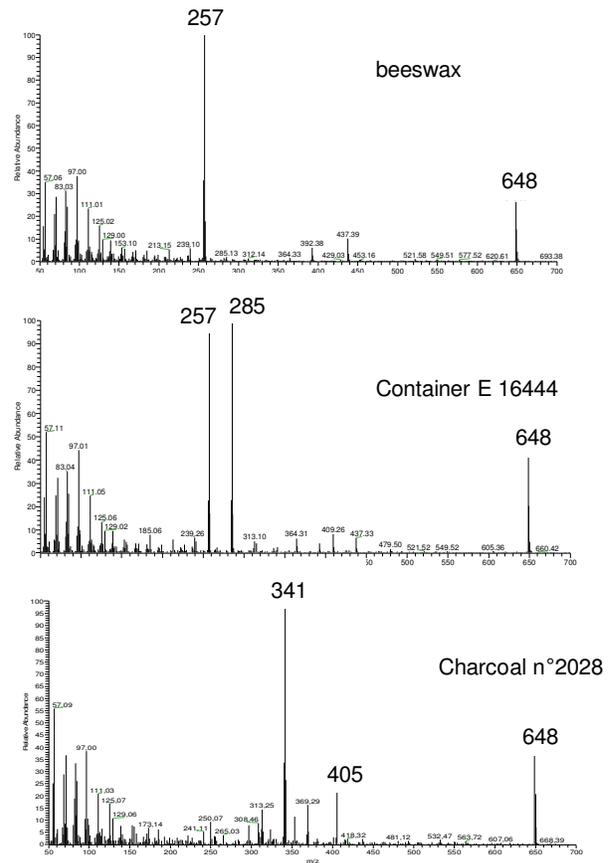


Figure 6 - Mass spectra (EI, 70 eV) of the ester (molecular weight 648, C<sub>44</sub>) detected in modern beeswax, in an organic remain from the Deir el-Médineh containers and in conifer charcoal #2028.

## Molecular data: clues to trace the origin of archaeological organic materials

They are rightly known as “flasks of the New Year” due to the presence of a form of greetings engraved on most of them (S. Guichard & G. Pierrat-Bonnefois, 2005). Present in many museums, they remain mysterious: what was their content? Why they became so widespread around the Mediterranean Basin? The assumption most often given is that pilgrim flasks, filled with water of the Nile, were exchanged as present on New Year’s Day. Exceptionally, one flask belonging to the collection of the Louvre Museum reveals a content which seeps through the wall (Fig. 1c). Hermetically sealed, it cannot be open without damage. Therefore only the seepage, presents as small patches, could be scratched. Molecular study of this residue was used to get some insight into the story of these pilgrim flasks.

Quantitative data, obtained by splitting the organic extract according to classes of polarity, already indicate a particular sample: organic extract representing 80% of the whole sample with a very low content (<5 %) of aliphatic and aromatic hydrocarbons, esters, ketones, alcohols but a high content of components with a polarity corresponding to acids (30 %). Indeed, the complete dissolution of the sample in water could be explained by the presence of sugar at the polarity of alcohols and acids. Sugars are abundant in gums, fruit juices and honey. Given the syrupy aspect of the product which seeps through the wall, the assumption of gums occurrence was less favored than fruit juices and honey. Different modern reference products (juices from figs, dates, pomegranates, vinegar of pomegranates, honey from several sources, mead) were analyzed to compare their molecular composition to the archaeological sample’s one. Each of these products is likely to have been used at the time of the flask. In the residue from the pilgrim flask, no terpenic biomarker was detected as in honey and mead, unlike fruit juices and gums (arabic for instance). The fingerprint of sugars present in the alcohol and acid fractions from the archaeological residue is different from the ones corresponding to fruit juices and mead but the similarity with the sugars distribution in honey is striking (fig. 7). This similarity is really noteworthy when one considers the fact that the comparison is made between a residue exposed to the atmosphere and necessarily altered and a

honey bought at random. However the similarity found for sugars does not extend to other molecular classes: oleic acid (18:1) is predominant in pilgrim flask while it is only weakly detected in current honey, perhaps because actual honey is heated. Is this linked to different botanical sources pollinated by bees and /or to treatments implemented on honey after harvest? To complete the data, methanolysis experiments were undertaken in order to identify sugar classes. The fingerprints of sugars in honey and fruit juices are similar. They differ from those of mead where some specific biomarkers were identified: glycerol, citric acid. The residue from pilgrim flask was not submitted to methanolysis due to the lack of material. Identification at several polarities of compounds derived from the stigmaterol points out a vegetable origin of the residue. Distribution of *n*-alkanes (C<sub>16</sub> to C<sub>25</sub>) without odd or even predominance, and detection of traces of pristane and phytane, hopanes and steranes are indicative of the presence of bitumen. However, the fact that bitumen appears only in traces in a single fraction is in favor of an environmental contamination of the sample exposed to a City atmosphere for almost a century without specific care rather than bitumen as historic component deliberately added. This contamination was already recognized in other samples stored in the Louvre Museum. As only small amounts of aromatics structures (alkylbenzenes, alkylphenanthrenes, etc) were identified, this contamination is low.

Chemical data reveal the presence of a sweet herbal substance. If presumptions in favor of honey are high, the study should be considered as not completed but the lack of material did not allow other further analysis like methanolysis, datation, pollen analysis. However, honey is a flagship product of ancient Egyptians. It is one of the most cited products in the medical Ebers papyrus (T. Bardinet, 1995): 248 times which corresponds to 23.5 % of the corpus, just before animal fats.

### *Coating spread on the walls of Middle Age undergrounds*

Investigations were carried out on four residues (references LB01, LB02, LB03, LC01) taken from the black coating spread on the walls of two undergrounds likely used as cellar during

the Middle Age (H. Pigeyre, 2008). This coating, particularly resistant to humidity present in the undergrounds, is a “soft and creamy” material, very slightly soluble in water and absorbs significantly organic solvents (experimental observations). The Total Organic Carbon (TOC) values are very low (1.8 to 2.3 %), as well as the percentages of organic extract (<3 %). The Rock-Eval pyrolysis (J. Espitalie, 1993), a classical screening tool used for the characterization of organic matter in petroleum source rocks (Tab.1), indicates the predominance of a highly carbonized organic matter (soot? or charred material? with HI between 85 and 88 mg HC/g of TOC), a very low S<sub>1</sub> (0.07-0.11 mg HC/g of TOC) and a very high Tmax (461-493 °C). In addition, the oxygen index (OI) is extremely high, due to the intense oxidation of the organic matter or to decomposition of mineral constituents; The X-Ray diffraction analysis (Tab. 1) did not show any carbonates therefore the hypothesis of oxidized organic matter is very likely. Petrographic analysis (Dr. F. Laggoun-Defarge, personal communication) confirms the absence of wood structures and the presence of a high reflectance maceral without any fluorescence in 99 % of particles. This maceral is similar to pyrobitumen. These samples are well differentiated from a chimney soot or charcoals from Auriac where classical particle of high reflecting particles of pyrofusinite are identified with remains of wood cells. Consequently archaeological samples from La Borde and La Combe are mainly composed of pyrobitumen-type materials with minor amounts of extractable organic matter.

In an attempt to find the source of these samples, their organic extracts were compared to vegetal tars made from the heating of conifer wood, i.e. pitch (A. Charrié-Duhaut et al., 2009b; J. Connan & A. Charrié-Duhaut, 2010) and to extracts of charcoals, one from the residue from a production site at Auriac (# 2641, mixture of wood from beech and deciduous oak) and the other from *Pinus halepensis* wood used to produce pitch (# 2028).

In samples of the La Borde underground (# LB01 and LB03) and Auriac (# 2641), the GC-MS traces of aliphatic hydrocarbons are dominated by *n*-alkanes with a strong odd predominance in the high molecular weight range (C<sub>29</sub> predominant, Fig. 8). Such distributions are

characteristic of a higher land plants and wood contribution. No terpenic structure was detected except in sample #2028 where traces of nor-abietane, a diterpenoid characteristic of Conifer species were detected.

Unlike samples LB01 and LC01 whose aromatic fractions are below detection limits, GC-MS of the aromatic hydrocarbons from LB03 shows the presence of abietic acid related to diterpenoid structures (Fig. 9). The occurrence of this molecular family suggests that the sample contain pitch, i.e; a tar derived from conifer wood. Its distribution is indeed partly similar to that recorded in pitch (A. Charrié-Duhaut et al., 2009b; J. Connan & A. Charrié-Duhaut, 2010) and in charcoals (#2028) of a pitch kiln (Fig. 9). These diterpenoid structures were not identified in sample #2641 which does not contain coniferous wood but beech and oak. Polyaromatic structures were also detected in the three fractions: T-alkylbenzenes, naphthalenes, phenanthrenes, etc. The distribution of the naphthalene bulk with a molecular weight of 212 is the same in LB03 and Auriac samples. Polyaromatic components are usually not present in pitch and would rather characterize tars obtained at high temperature. However, samples #2028 and 2641 show a bulk of compounds corresponding to sterols and triterpenoids of plant origin, which is not detected in LB03. One should also notice the occurrence of fluoranthene, pyrene and benzanthracene, chrysene in the Auriac sample which are classical pyrolytic products, abundant in coal tars. To summarize, the aromatic fraction of LB03 sample suggests that the sample shows a dual contribution reflected by affinity with pitch, represented by *Pinus Halepensis* charcoal, but also with tar from other woods exemplified by the Auriac charcoal.

In the three archaeological samples, wax esters were present. Their occurrence had been revealed during a preliminary survey of the underground samples in another laboratory which led to suggest the possible presence of beeswax. This preliminary assumption turned out to be false as the wax esters identified herein correspond to those of cuticular waxes as presented in the part 2 of this paper. The distribution extends from C<sub>40</sub> to C<sub>48</sub> with a strong even predominance but odd esters are also present (Fig. 10).

**Molecular data: clues to trace the origin of archaeological organic materials**

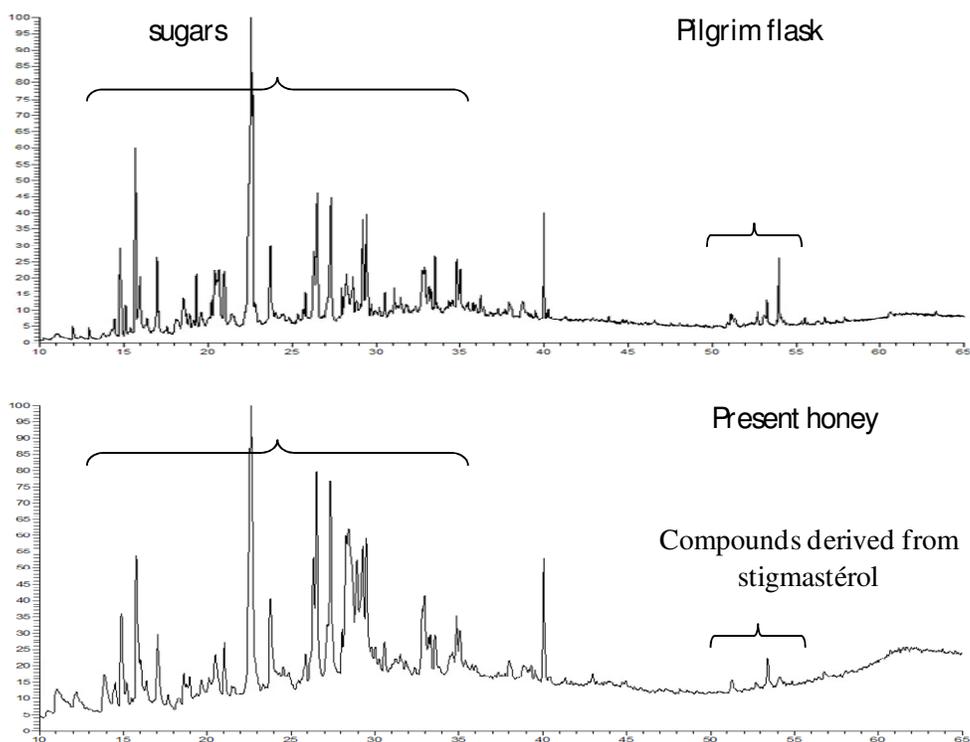


Figure 7 - RIC (GC-MS) of the alcohol fractions corresponding to the residue of pilgrim flask (a) and to actual honey (b).

sample number	location	reference	TOC (% by weight of sample)	S <sub>1</sub> (mg HC / g of sample)	S <sub>2</sub> (mg HC / g of sample)	S <sub>3</sub> (mg CO <sub>2</sub> / g of sample)	Tmax (°C)	HI (mg HC / g TOC)	OI (mg CO <sub>2</sub> / g TOC)	Mineralogy (RX analysis)
2527	La Borde	LB01	1.8	0.07	1.58	9.71	493	88	539	feldspaths, clay minerals (illite, muscovite?)
2528		LB02	2.0	0.12	1.77	10.49	461	87	514	
2529		LB03	2.3	0.11	1.98	11.96	475	85	516	quartz, feldspaths, clay minerals (illite, muscovite?, serpentine?)

Table 1- Data from Rock-Eval pyrolysis and RX analysis (HC: hydrocarbons, TOC: total organic carbon, HI: hydrogen index, OI: oxygen index, Tmax: temperature of S<sub>2</sub> peak maximum).

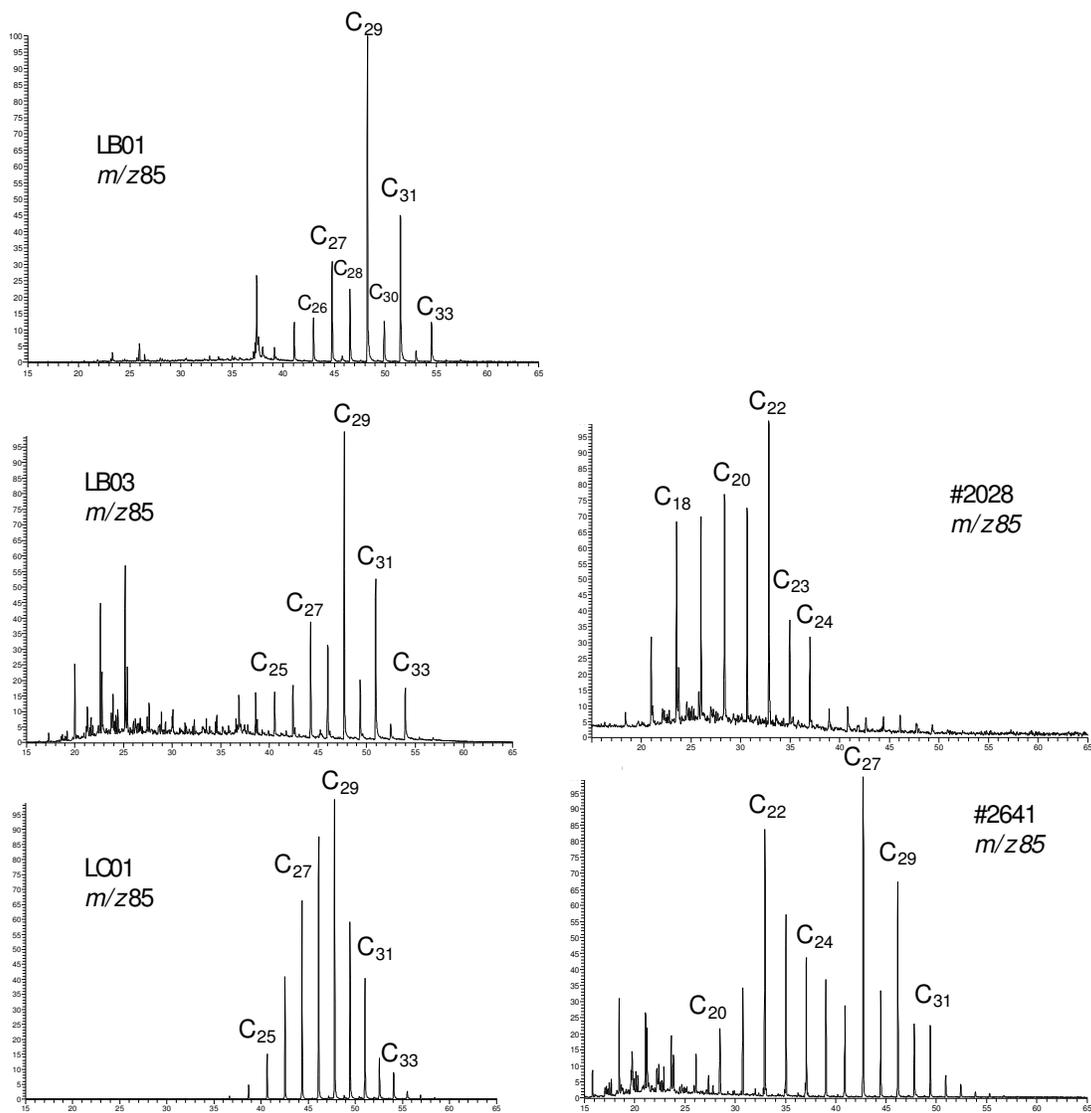


Figure 8 - Mass fragmentogram  $m/z$  85 showing the distribution of  $n$ -alkanes in LB01, LB03, LC01, # 2028, and # 2641.

**Molecular data: clues to trace the origin of archaeological organic materials**

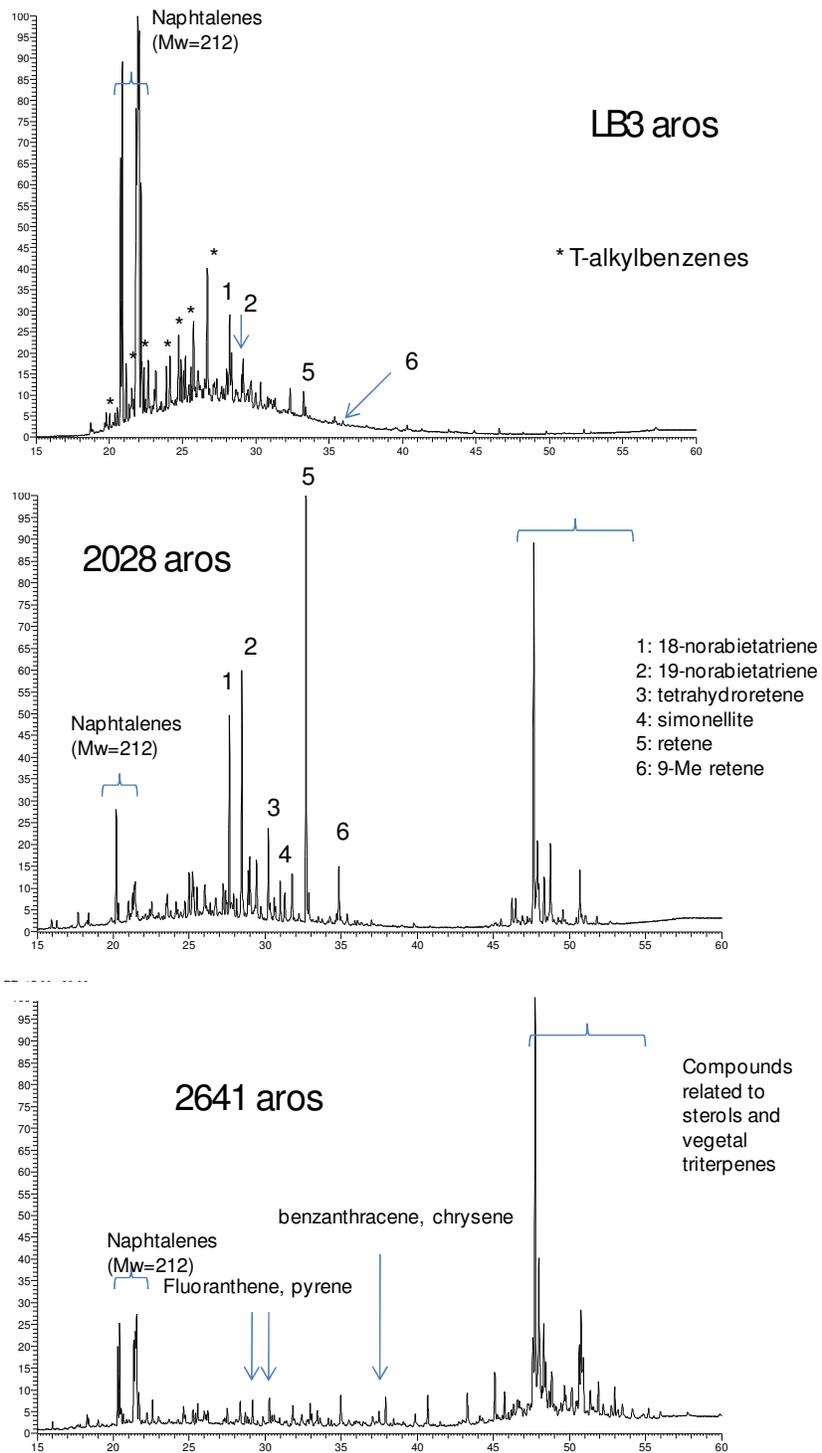


Figure 9 - RIC (GC-MS) of aromatic fractions from LB03, # 2028, and # 2641.

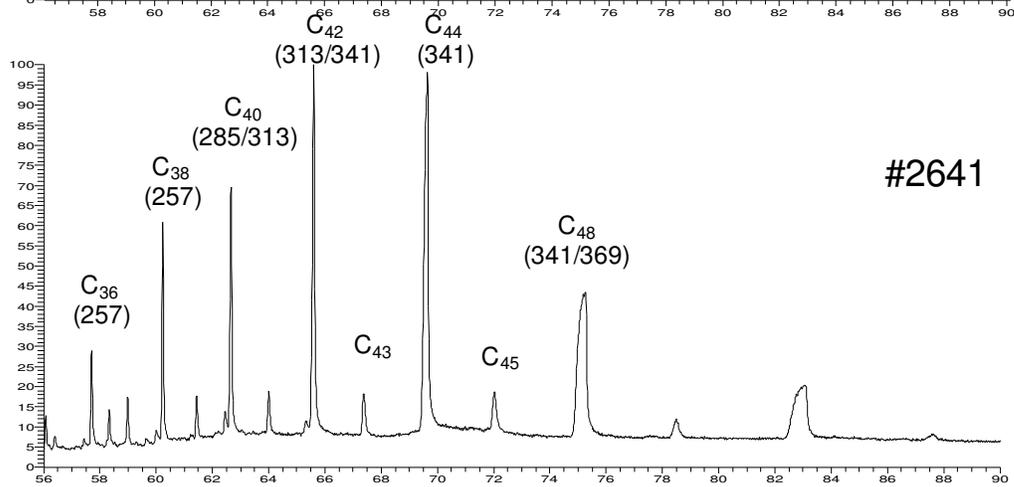
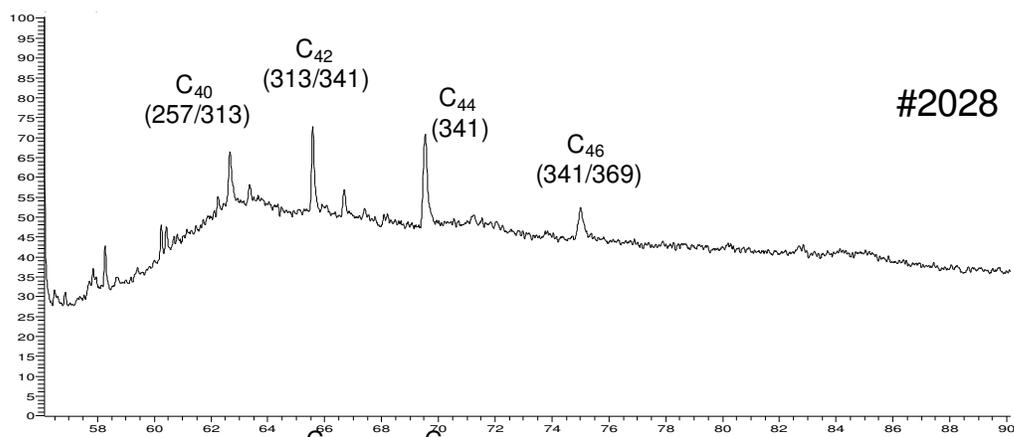
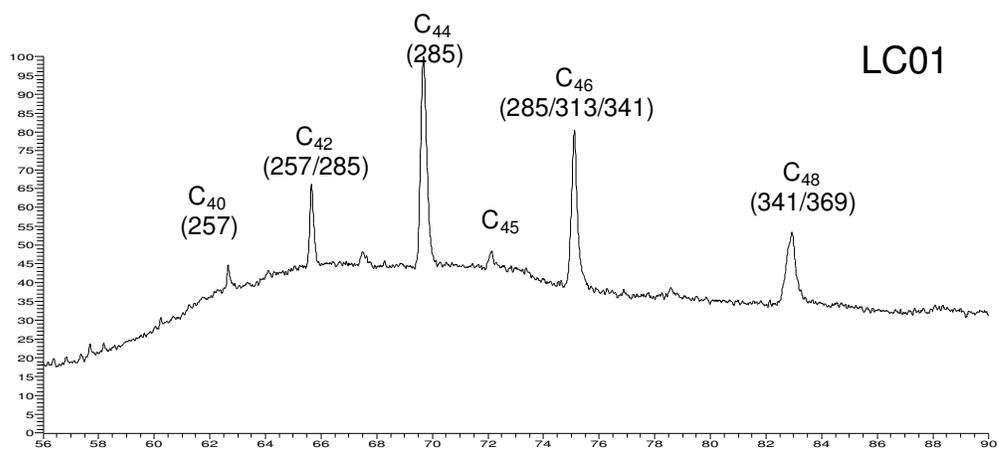


Figure 10 - RIC (GC-MS) of wax esters from LC01, # 2028, and #2641. Major fragments are indicated in brackets.

## Molecular data: clues to trace the origin of archaeological organic materials

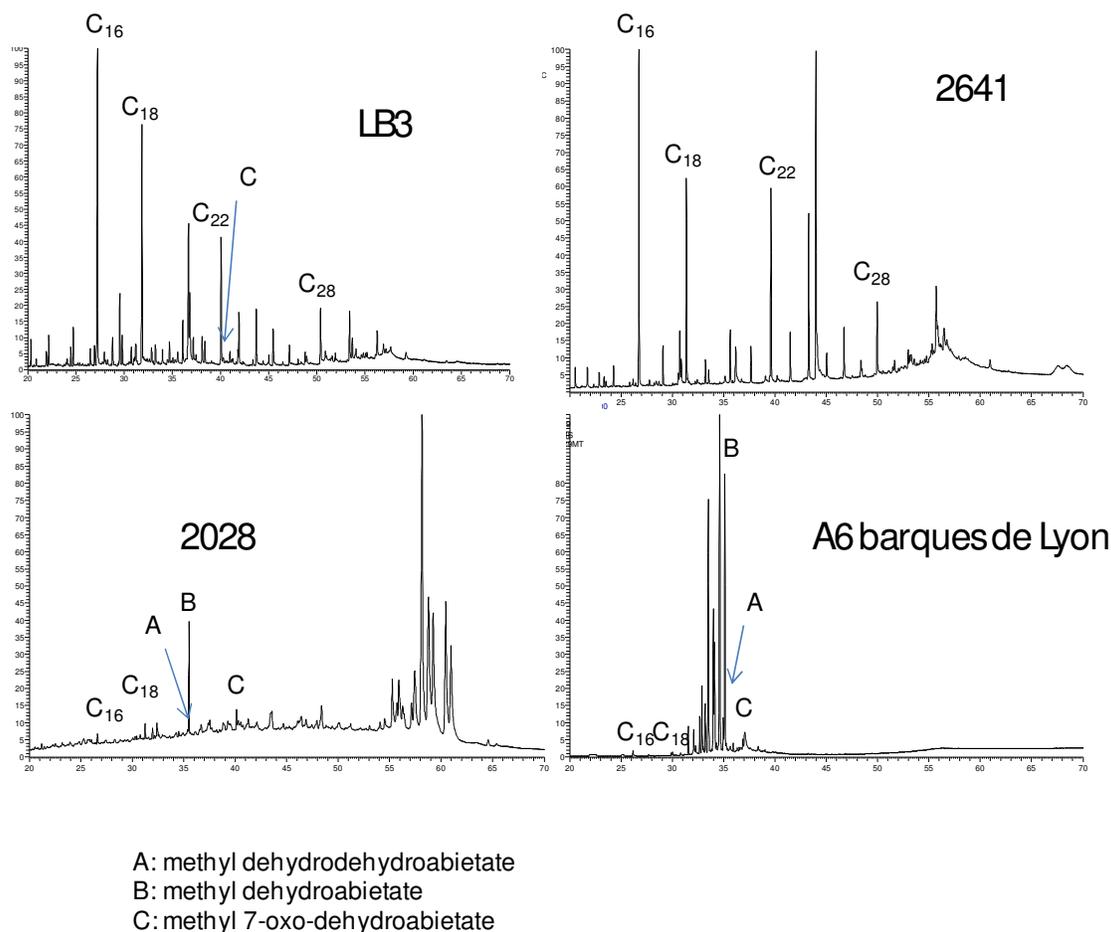


Figure 11 - (GC-MS) of acid fractions from LB03, # 2028, # 2641 and of a classical pitch (Connan & Charrié-Duhaut, 2010).

The spectra show several specific fragments ( $m/z$  257, 285, 313, 341) as previously underlined. Such waxes were identified in samples #2028 and #2641 with distribution that looks similar but beware the spectra of each compound are different.

As in the Auriac sample (#2641), the polar fractions of the three archaeological samples are dominated by linear structures, for instance linear alcohols ( $C_{16}$ - $C_{28}$ , even predominance), and linear acids ( $C_{14}$ - $C_{32}$ , even predominance) (Fig. 11). This is not the case for the Font de Mars sample (#2048) or for pitch exemplified by a sample of the Roman boat of Lyon (J. Connan, A. Charrié-Duhaut, 2010) (Fig.

11). In each sample, compounds characteristic of biomass pyrolysis were detected: components related to sugars, and lignin pyrolysis products (e.g. B.R.T. Simoneit et al., 1993; C. Saiz-Jimenez, J.W. De Leeuw, 1986). In pitch, components related to the family of abietic acid are mostly detected even in degraded material, especially in the acid fraction. In the acid fraction from sample #2028, they exhibit important proportions but are only detected only as traces in the three archaeological samples.

In summary the product coating the walls of the La Borde undergrounds still remains rather enigmatic. This product is composed of a dominant highly carbonized pyrobitumen phase

in which wood cells were not found. This homogeneous phase with a high reflectance has an unknown origin and is not clearly understood. Why this insoluble phase was incorporated if it was deliberately added? The most likely explanation is that the product used to cover the walls was a byproduct from the charcoal production which reached high temperatures leading to the production of mainly thermally degraded insoluble phase called pyrobitumen. The minor tar phase, extractable in organic solvents and associated with pyrobitumen, indicates that the whole product has been generated from a mixture of wood in which Conifer was present. The other woods were not identified.

### Conclusions

Molecular analysis, based on precise structural elucidation of biomarkers, often provides answers where archaeologists and curators can only make assumptions. This approach gives access to different information like identification of natural substances (pure or composite material), indication on manufacturing processes (e.g. heat treatment). The first part of this paper has been focused on identifying the well-known "wax esters". Used currently as biomarkers of beeswax, they are characterized by a specific fragment  $m/z$  257. The study of antique insects nest cells, of organic material present in jars from Deir el-Médineh and of a coating from Middle Age undergrounds reveal a different source of the ester distribution namely the cuticular waxes from plants. They are characterized by coelution of esters presenting the same molecular weight but with different structures entailing specific fragments  $m/z$  257, 285, 313 and 341.

Characterization of a wide range of unknown materials is enabled by prior fractionation allowing simplification of molecular mixtures and concentration of minor compounds. In the collection of residues present in jars from Deir el-Médineh, bulk analysis only shows the presence of fatty substances. Fractionation allows to identify the biological origin of the fat (Bastien, PhD thesis in prep) and to highlight the presence of an admixed plant component. The extreme miniaturization of sample quantities (less than 1 mg) will not give so extensive information. This is especially true

in cases where the advanced state of degradation reduces the amount of organic extract. However, it should be born in mind that the samples from artworks and archaeological contexts are unique, precious and very valuable. The analytical approach depends on objectives of the study and final conclusions are not necessarily achieved. Thus the most likely hypothesis for the content of the pilgrim flask (Egypt, XXVI<sup>th</sup> dynasty) is honey. The black coating spread on the walls of Middle Age undergrounds (Corrèze, France) would be a highly carbonized tar, byproduct of the charcoal production from a wood mixture in which conifer timbers are present.

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### References

- Bardinet T, 1995, *Papyrus médicaux de l'Égypte pharaonique*, Editions Fayard.
- Bleton J, Mejanelle P, Sansoulet J, Goursault S, Tchaplal A, 1996, *Characterization of neutral sugars and uronic acids after methanolysis and trimethylsilylation for recognition of plant gums*, Journal of Chromatography A, 720, p. 27-49.
- Buckley SA, Evershed RP, 2001, *Organic chemistry of embalming agents in*

## Molecular data: clues to trace the origin of archaeological organic materials

- Pharaonic and Graeco-Roman mummies*, Nature, 413, p. 837-841.
- Charrié-Duhaut A, Connan J, Rouquette N, Adam P, Barbotin C, de Rozières M-F, Tchaplà A, Albrecht P, 2007, *The canopic jars of Rameses II: real use revealed by molecular study of organic residues*, Journal of Archaeological Science, 34, p. 957-967.
- Charrié-Duhaut A, Burger P, Maurer J, Connan J, Albrecht P, 2009a, C R Chimie 12:1140-1153.
- Charrié-Duhaut A, Connan J, Darnell M, Spangenberg J, Szymczyk E, Bissada A, Albrecht P, 2009b, *Molecular and isotopic characterization of organic samples from the wreck of the Saint-Etienne merchant ship (XVIIIth century): Identification of pitch, fat, hair and sulfur*, Organic Geochemistry, 40, p. 647-665.
- Charters S, Evershed RP, Blinkhorn PW, Denham V, 1995, *Evidence for the mixing of fats and waxes in archaeological ceramics*, Archaeometry, 37, p. 113-127.
- Colombini MP, Modugno F, Giannarelli S, Fuoco R, Matteini M, 2000a. *GC-MS characterization of paint varnishes*, Microchemical Journal, 67 p. 385-396.
- Colombini M. P, Modugno C, Silvano F, Onor M, 2000b, *Characterization of the balm of an Egyptian mummy from the seventh century B.C.*, Studies in Conservation, 45, p. 9-29.
- Connan J, 1999, *Use and trade of bitumen in antiquity and prehistory: molecular archaeology reveals secrets of past civilizations*, Philosophical Transactions of the Royal Society, biological Sciences, 354, p. 33-50.
- Connan J, 2002, *Géologie de la préhistoire: méthodes, techniques, applications*, Miskovsky J. C (Ed.), Association pour l'étude de l'environnement géologique de la préhistoire, Maison de la géologie, Paris.
- Connan J, Tengberg M, 2006, Identification de pin d'Alep et de poix dans les échantillons du four à poix de la Font de Mars (Sainte Anne du Castellet-Var). Unpublished report.
- Connan J, Charrié-Duhaut A, 2010, *Etude géochimique d'enduits de surface, de tissus de calfatage, de blocs de poix et de bitume des épaves gallo-romaines du Parc Saint-Georges de Lyon*, Archaeonautica, 16, p. 147-172.
- Damiani D, Gliozzo E, Memmi Turbanti I, Spangenberg JE, 2003, *Pigments and plasters discovered in the House of Diana (Cosa, Grosseto, Italy): an integrated study between art history, archaeology and scientific analyses*, Archaeometry, 45, p. 341-354.
- Espitalie J, 1993, *Applied Petroleum Geochemistry*, Bordenave ML (Ed.) Technip, Paris, pp. 237-261.
- Evershed R. P, Vaughan S. J, Dudd S. N, Soles J. S, 1997, *Fuel for thought? Beeswax in lamps and conical cups from Late Minoan Crete*, Antiquity, 71, p. 979-985.
- Evershed R. P, 2008, *Organic residue analysis in archaeology: the archaeological biomarker revolution*, Archaeometry, 50, p. 895-924.
- Fales H. M, Jaouni T. M, Babashak J. F, 1973, *Simple device for preparing ethereal diazomethane without resorting to codistillation*, Analytical Chemistry, 45, p. 2302-2303.
- Gensel A., 2005, Restauration du four à poix de la Font de Mars au Castellet (Var). Unpublished report.
- Guichard S, Pierrat-Bonnefois G, 2005, *Le mystère des gourdes du Novel-An*, Les dossiers d'archéologie, 304, p. 46-51.
- Heron C, Nemcek N, Bonfield KM, Dixon D, Ottaway BS, 1994, *The chemistry of Neolithic beeswax*, Naturwissenschaften, 81, p. 266-269.
- Hovaneissian M, Archier P, Mathe C, Vieillescazes C, 2006, *Contribution de la chimie analytique à l'étude des exsudats végétaux styrax, storax et benjoin*, Comptes Rendus Chimie, 9, p. 1192-1202.
- Innes HE, Bishop AN, Head IM, Farrimond P, 1997, *Preservation and diagenesis of hopanoids in recent lacustrine sediments of Priest Pot*, England, Organic Geochemistry, 26, p. 565-576.
- Janot F., 2002, Memnonia XII/XIII:73-81.
- Kolattukudy P. E, 1976, *Chemistry and biochemistry of natural waxes*, Elsevier, Amsterdam.
- Langenheim J. H, 2003, *Plant resins: chemistry, evolution, ecology and ethnobotany*, Timber Press, Portland, Cambridge.
- Logan G. A, Smiley C. J, Eglinton G, 1995, *Preservation of fossil leaf waxes in association with their source tissues, Clarkia*,

- Northern Idaho, USA*, *Geochimica et Cosmochimica Acta*, 59, p. 751-763.
- Macke A, Macke-Ribet C, Connan J, 2002, *Ta Set Neferou. Une Nécropole de Thèbes-Ouest et son Histoire: momification, chimie des baumes, anthropologie, paléopathologie*, vol. 5, Dar Namatallah Press, Giza, Le Caire, République Arabe d'Égypte.
- Maurer J, Möhring T, Rullkötter J, Nissenbaum A, 2002, *Plant lipids and fossil hydrocarbons in embalming material of Roman Period mummies from the Dakhleh Oasis, Western Desert, Egypt*, *Journal of Archaeological Science*, 29, p. 751-762.
- Mills J. S, White R, 1994, *The Organic Chemistry of Museum Objects*, 2<sup>nd</sup> Edition, Butterworth Heinemann Ltd, Oxford.
- Ngan F, Toofan M, 1991, *Modification of preparation of diazomethane for methyl esterification of environmental samples analysis by gas chromatography*, *Journal of Chromatographic Science*, 29, p. 8-10.
- Peters K. E, Walters C. C, Moldowan JM, 2005, In: *The Biomarker Guide: Biomarkers and Isotopes in the Environment and Human History*, vol. 1, Cambridge University Press, New York.
- Pigeyre H, 2008, Rapport de fin de sondage sur le souterrain de La Borde (Saint Cirgues La Loure, Corrèze). Service Régional de l'Archéologie du Limousin. Unpublished report.
- Pollard AM, Heron C, 1996, *Archaeological Chemistry*, Royal Society of Chemistry Paperbacks, Cambridge, UK.
- Regert M, Rolando C, 1996, *Archéologie des résidus organiques. De la chimie analytique à l'archéologie: un état de la question*, *Techné*, 3, p. 118-128.
- Regert M, Colinart S, Degrand L, Decavallas O, 2001, *Chemical alteration and use of beeswax through time: accelerated ageing tests and analysis of archaeological samples from various environmental contexts*, *Archaeometry*, 43, p. 549-569.
- Regert M., Langlois J., Colinart S., 2005, *Characterisation of wax works of art by gas chromatographic procedures*, *Journal of Chromatography A*, 1091, p. 124-136.
- Ribechini E., Modugno F., Colombini M.-P., Evershed R. P., 2008, *Gas chromatographic and mass spectrometric investigations of organic residues from Roman glass unguentaria*, *Journal of Chromatography A*, 1183, p. 158-169.
- Rielley G, Collier R. J, Jones D. M, Eglinton G, 1991, *The biogeochemistry of Ellesmere Lake, UK--I: Source correlation of leaf wax inputs to the sedimentary lipid record*, *Organic Geochemistry*, 17, p. 901-912.
- Saiz-Jimenez C, De Leeuw J. W, 1986, *Lignin pyrolysis products: their structures and their significance as biomarkers*, *Produits de pyrolyse de la lignine: leur structure et signification comme biomarqueurs*, *Organic Geochemistry*, 10, p. 869-876.
- Scalarone D, van der Horst J, Boon J. J, Chiantore O, 2003, *Direct temperature mass spectrometric detection of volatile terpenoids and natural terpenoid polymers in fresh and artificially aged resins*, *Journal of Mass Spectrometry*, 38, p. 607-617.
- Simoneit B. R. T, Rogge W. F, Mazurek M. A., Standley LJ, Hildemann LM, Cass GR, 1993, *Lignin pyrolysis products, lignans, and resin acids as specific tracers of plant classes in emissions from biomass combustion*, *Environmental Science and Technology*, 27, p. 2533-2541.
- Stern B, Lampert Moore C. D, Heron C, Pollard A. M, 2008, *Bulk stable light isotopic ratios in recent and archaeological resins: towards detecting the transport of resins in antiquity?*, *Archaeometry*, 50, p. 351-370.
- Tripp JA, Hedges R. E. M, 2004, *Single-compound isotopic analysis of organic materials in archaeology*, *LC-GC Europe*, 17, p. 358-364.
- Tulloch A. P, 1973, *Comparison of some commercial waxes by gas liquid chromatography*, *Journal of the American Oil Chemists' Society*, 50, p. 367-371.
- Van der Doelen G. A, van den Berg K. J, Boon J. J, Shibayama N, Rene de la Rie E, Genuit W. J. L, 1998, *Analysis of fresh triterpenoid resins and aged triterpenoid varnishes by high-performance liquid chromatography-atmospheric pressure chemical ionisation (tandem) mass spectrometry*, *Journal of Chromatography A*, 809, p. 21-37.