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# **An innovative multi-analytical strategy to assess the presence of fossil hydrocarbons in a mummification balm**

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## **Abstract:**

This research work focuses on the detection of radiocarbon contamination, for instance bitumen, in a mummification balm prior to its radiocarbon dating. A new analytical methodology combining ATR-FT-IR (*Attenuated Total Reflexion - Fourier Transform Infrared Spectroscopy*), TLC (*Thin Layer Chromatography*), is developed in order to allow a straightforward identification of contaminants directly from the excavation site and monitor the sampling prior to radiocarbon dating. The mummy of Padiimenipet, currently held at the Louvre Museum, was chosen to build the methodology as previous work relying on GC-MS (*Gas Chromatography – Mass Spectrometry*) identified bitumen in this balm. Radiocarbon dating of multiple aliquots of the sample by AMS (*Accelerator Mass Spectrometry*) confirmed the presence of fossil compounds and their important heterogeneity. ATR-FTIR approaches allowed a preliminary characterization, with identification of the main chemical families from the balm and notably hydrocarbons. TLC, used in normal phase and using methanol as eluent, confirmed in a very efficient way the presence of hydrocarbons in the sample and such technique could easily be used *in situ* to select samples devoid of these compounds, possibly fossil. GC-MS analysis requiring specific SPE (*solid phase extraction*) concentration confirmed the presence of bitumen but is not designed to offer an easily implemented diagnosis. . Consequently, the combined use of ATR-FTIR and TLC could be used in the future as a new on-site methodology to perform a screening of organic samples before radiocarbon dating and identify fossil contamination.

**Keywords:** TLC, FTIR, GC-MS, mummification balm, radiocarbon dating, Egyptian mummy, bitumen

## 1. Introduction

Egyptian mummification procedures involve various organic products (oils, plant resins, waxes, tars and petroleum bitumen), referred to as “balm” when found impregnated on mummies [1,2]. These practices are documented to evolve through time, from Early Dynastic period to the end of Roman period [3]. Some of these products can influence the radiocarbon dating of the sample. Bitumen, a degradation product of biomass depleted in  $^{14}\text{C}$ , has been repeatedly identified in balms [4–11]. Simultaneously, bitumen has been reported to create artificial ageing of the sample if not identified and extracted prior to radiocarbon dating [12,13]. Its detection using physico-chemical techniques is thus very important prior to sampling.

Extensive work has documented the presence and detection of bitumen in Egyptian balms. The first identification of this product was documented in the early 1990's using a combination of Gas Chromatography coupled with Mass Spectrometry (GC-MS) and of additional mass spectrometric approaches [5]. Later on, several laboratories have developed protocols to improve the detection of bitumen in mummification balm thanks to GC-MS [7,8,10,14–16]. Recently, a new approach has been developed using Solid Phase Extraction (SPE) to improve the detection of bitumen by fractionation before GC-MS [17]. Nevertheless, the detection of bitumen in balms is not straightforward and requires specific approaches.

However powerful these techniques based on GC-MS may be, they require laboratory sophisticated devices, high vacuum ( $10^{-7}$ - $10^{-9}$  mbar) and multi-step time consuming analyses. For all these reasons, GC-MS related approaches are not suitable for a straightforward on-site diagnosis regarding the presence or absence of bitumen which will guide the sampling for radiocarbon dating. Moreover, approaches that could be implemented outside a specialised laboratory using Thin Layer Chromatography (TLC) have been proposed to study the compositions of medieval mummy balms [18]. Different chromatographic scenarios are then required depending on the targeted molecules: resins, sugars, proteins, waxes.

In this paper, we present a multidisciplinary strategy applicable on-site for diagnosis of hydrocarbons in mummification balms, prior to radiocarbon dating, relying on Attenuated Total Reflexion - Fourier Transform Infrared Spectroscopy ATR-FTIR as well as Thin Layer Chromatography (TLC). Several samples are investigated: a fragment of balm sampled from a Graeco-Roman mummy, a fragment of unidentified organic material suspected not to contain hydrocarbons, and modern samples used as references for the various chemical families studied. Further analyses are performed in the lab: GC-MS for extensive identification of contaminants when dealing with a sample contaminated, and radiocarbon dating to assess the presence of fossil compounds. This approach will help monitor on-site sampling in the future as it will allow distinction between samples containing hydrocarbons such as bitumen, which will require complex cleaning protocol, and other samples, with a classic pre-treatment only prior to radiocarbon dating.

## 2. Research aim

The objective of this study is to develop an analytical strategy for routine *in situ* diagnosis to assess the presence of fossil hydrocarbons in a complex organic sample prior to its radiocarbon dating. This is achieved by combining ATR-IR and TLC, while GC-MS is used as a benchmark. The balm of an Egyptian mummy is thus characterized and dated by carbon 14 ( $^{14}\text{C}$ ) to highlight the issue of the ageing effects induced by the presence of fossil carbon as well as the heterogeneities of its formulation. The proposed mobile analytical approaches therefore prove to be efficient to screen sample on site and detect the presence of fossil carbon.

## 3. Materials and methods

### 3.1. Samples

Two different archaeological samples were studied. The first one is a fragment of mummification balm already studied. Previous analyses [15] have identified diterpenic resin thermally treated, beeswax and bitumen in the composition of this balm.

The presence of bitumen notably made it an ideal sample to develop diagnosis methodology. The balm was collected from the crotch area of a mummy, the mummy of Padiimenipet, a young man who lived in Thebes under Trajan and died at 21 in 116 CE as mentioned by a carved inscription on the funerary ensemble [19–21]. His family is referenced as *the Soter Group*, gathering men and women from Graeco-Roman and Egyptian origins around the figure of the patriarch Soter. The family was buried collectively in a reused pharaonic tomb in Cheikh abd el-Gurna (TT32), which was rediscovered in 1820 [22]. The finds were purchased by adventurers and art collectors and found their way to the main European museums. They gained considerable attention, especially after Champollion himself recognized their importance as examples of the production from Roman Egypt, whose features gather Egyptian traditions and Graeco-Roman influences [23]. Padiimenipet's ensemble was acquired by the French explorer Frédéric Cailliaud and sent to the French National Library (Bibliothèque Nationale de France). Thanks to deposits and transfers between collections, it is now kept in the Louvre Museum (département des Antiquités égyptiennes), except for one papyrus still at the Bibliothèque Nationale de France. The Louvre Museum currently displays Padiimenipet's wooden coffin (E 13048, E 13016), painted shroud (E 13382), gilded funerary crown (E 13417) and the metallic eyes and tongue that covered the face of the mummy (BN 2738, 2739 et 2740). The mummy itself (BN 1810) is in storage. It was unwrapped by Cailliaud himself in 1823, during one of the sensational "scientific sessions" dear to 19<sup>th</sup> century Egyptology [24]. It shows the extremely well-preserved corpse of the young man whose flesh was covered with black balm and gold leaves, a ritual practice from Roman Egypt.

The second sample N°372660 is a fragment of an archaeological organic material originating from Ancient Egypt and more precisely from the Graeco-Roman period (~300 BCE - 400 CE). It was found associated with funerary wrappings and kept in the Louvre museum (N° 372660). Its colour (orange and transparent) allows the absence of bitumen to be suspected. As a resinous material is usually used for embalming practices, this second sample will be compared with a modern plant resin and, if consistent, it will allow its chemical behaviour to be evaluated. It will also help compare the physico-chemical responses to the proposed analytical protocols when bitumen is present in a balm with when it is absent. Optical microscopy photos of both samples are shown in Figure 1.

### 3.2. Reagents and reference materials

Chloroform (CHCl<sub>3</sub>) was provided by Carlo Erba. Methanol (MeOH), n-hexane, toluene, acetone and diethylether (DEE) were purchased from VWR. Dichloromethane, THF and ethanol were provided by Merck. Derivatizing agent for GC-MS *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from Merck. Acetic acid (AcOH) was purchased from Acros Organics.

Several reference organic products were used to compare both of the archaeological samples with known materials. These organic products are chosen as representative of the main chemical families documented for embalming in Ancient Egypt. Colophony (diterpenic resin) was provided by Laverdure in Paris, France. Mastic (triterpenic resin), bitumen and beeswax were purchased from Sennelier (Paris, France). Castor oil was purchased from Aromazone (Paris, France).

### 3.3. Analytical procedures

#### 3.3.1. Radiocarbon dating

Preparations and measurements by *Accelerator mass spectrometry (AMS)* were performed at the Artemis facility (LMC14/LSCE-IPSL, Saclay, France). To assess the homogeneity of the radiocarbon dating of the balm, three aliquots of balm were sampled from a loose fragment originating from the mummy crotch area. The pre-treatment of the samples consisted of one acidic wash (HCl, 0.5 N during one hour at 80°C) in order to remove any post depositional carbonate left. Only the acidic wash was achieved instead of the routine Acid-Base-Acid pre-treatment for radiocarbon dating of organics because of the very low amount of material available, each cleaning step causing an unavoidable loss of material, and as the contamination by humic and fulvic acids was quite unlikely due to the conservation of the mummy in museum collection as well as before

entering the collection (Cheikh abd el-Gurna Theban necropolis). After pre-treatment, masses of respectively 1.5, 2.5 and 2.6 mg were gathered to replicate the measurement for radiocarbon dating of pre-treated balm.

An additional aliquot of balm was also dated, without any pre-treatment to evaluate the possible influence of pre-treatment on contamination concentration. The archaeological organic sample N°372660 was dated following the classic acidic pre-treatment. The four aliquots of balm and the fragment of archaeological sample N°372660 were then burned with approximately 500 mg of copper oxide and a silver strand during 5h at 835°C. The CO<sub>2</sub> produced by combustion was catalytically reduced by Fe powder at 600°C in Hydrogen excess (ratio H<sub>2</sub>/CO<sub>2</sub> of approximately 2.5) to form graphite. The iron and graphite powders were then pressed in aluminium pellets for measurement [25].

The carbon isotopic ratios were measured with the AMS and compared to the isotopic ratios of the international reference material, oxalic acid II [26]. A correction for isotopic fractionation was applied thanks to the  $\delta^{13}\text{C}$  value measured on-line by the AMS. A process blank determined from <sup>14</sup>C free samples was prepared and analysed with dated samples [27]. The data were calibrated using OxCal 4.4 software [28] using the INTCAL20 calibration curve [29].

### 3.3.2. FTIR measurements

FTIR analyses were achieved at the MONARIS lab (Paris, France). FTIR spectra of the bulk balm and on the archaeological resinous sample were obtained using the ATR-diamond module of the Alpha spectrometer (Bruker). Absorbance was measured between 4000 and 400 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> and 128 scans were accumulated for each spectrum.

The balm was measured as is but also after two extraction protocols relying on solvents of different polarities that have been used to separate the chemical families present. The first one, designed as P1, relies on two successive extractions using first 1 mL of n-hexane, then 1 mL of chloroform, during 15 minutes in an ultrasonic bath (Thermo Fisher Scientific) with a power of 90 W. The second protocol, referred to as P2, relies on two successive extractions using first 1 mL of methanol, then 1 mL of chloroform in the same conditions as P1. Each organic fraction is kept for FTIR analyses.

FTIR spectra on organic fractions were measured as follows: a few droplets of organic solvent are deposited on the diamond crystal of the spectrometer and allowed to dry. The chemicals solubilised in the solvent concentrate at the outer rim of the droplet. Absorbance was measured between 4000 and 400 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> and 128 scans were accumulated for each spectrum.

### 3.3.3. TLC (Thin Layer Chromatography) analyses

TLC analyses were achieved at the MONARIS lab (Paris, France).

Both the organic balm and the archaeological resinous sample were analysed by TLC. Two solutions of 100 mg/mL of organic material in CHCl<sub>3</sub> were prepared for each sample. Separation was achieved on a 5x10 cm TLC aluminium plate coated with silica gel (provided by Merck, reference number 16835), with a pore size of 60 Å, a particle size between 9.5 and 11.5 µm and a thickness between 165 and 235 µm. The elution was achieved using MeOH. The visualization was done by lighting the plate at a wavelength of 312 nm. A lamp at 254 nm was also tested and yielded similar revelation.

### 3.3.4. GC-MS (Gas Chromatography Coupled with Mass Spectrometry) analyses

GC-MS analyses were achieved at the IMBE lab (Avignon, France)

*First protocol:* global extraction with dichloromethane

10 mg of sample were dissolved by 3 x 1mL of dichloromethane (DCM, CH<sub>2</sub>Cl<sub>2</sub>) using an ultrasonic bath during 5 minutes then centrifuged at 6000 rpm during 5 minutes. After each solubilisation step, the supernatant was collected. The three supernatants were then evaporated under a gentle flow of N<sub>2</sub>, derivatized using 200 µL of BSTFA/TMCS and heated 30 minutes at 70°C. The trimethylsilylated extracts were then dried under a stream of N<sub>2</sub> then dissolved in 0.2 to 1mL of hexane/DCM (1/1, v/v) before filtration on PTFE filters 0.45 µm before injection in GC-MS.

#### *Second protocol: concentration by SPE (solid phase extraction)*

This protocol proposed by Mezzatesta *et al.* [17] is herein summarized and aimed to concentrate the sample hydrocarbon fraction to enhance its detection. 10 mg of sample were dissolved by 3 x 1 mL of a mixture hexane/tetrahydrofuran (THF) (1/1, v:v) using sonication during 5 minutes then centrifuged at 6000 rpm during 5 minutes. After each extraction, the supernatant was collected. The fractions were evaporated under a stream of N<sub>2</sub> then dissolved in 500 µL of hexane/THF (1/1, v/v). This mixture will be called “the charge”.

A SPE cartridge strata NH<sub>2</sub> 200 mg/3 mL (Phenomenex) was conditioned in a SPE vacuum manifold with 4 mL of hexane at a flow of about 1.2 mL/min. After that, the charge was deposited on the cartridge and eluted with 4 mL of hexane, then with 4 mL of ethanol, and finally with 4 mL of DEE containing 2% AcOH. Three fractions were thus eluted and collected in separate vials. Each fraction was then dried under a gentle stream of N<sub>2</sub>, derivatized using 200 µL of BSTFA/TMCS and heated 30 minutes at 70°C. The trimethylsilylated extracts were then dried under a stream of N<sub>2</sub>. Fractions 1 (hexane) and 2 (ethanol) were dissolved in 60 µL hexane/DCM (2/1, v/v) whereas fraction 3 (DEE with 2% AcOH) was dissolved in with 1.5 mL hexane/DCM (2/1, v/v) before injection in GC-MS.

The chromatographic system was composed of a Thermo Scientific AI 3000 auto-sampler coupled with an ITQ 700 ion trap mass spectrometer. The GC column used was a fused silica capillary column trace GOLD TG-5MS (5% diphenyl / 95% dimethylpolysiloxane, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness) provided by Thermo Fisher Scientific. The carrier gas was helium and the flow was set at 1 mL/min. A splitless injection of 1 µL of each sample was done. The temperature of the injector was set at 250°C. The ionization mode used for the recording of mass spectra was electron impact (EI) mode with an electron ionization voltage of 70 eV, an ionization time of 25 ms and a mass range of 40-650 *m/z*. The temperatures of the transfer line, ion trap and manifold were respectively set at 300°C, 200°C and 50°C. The identification of detected compounds was achieved using the NIST database as well as published articles [11,17,30–33]. The heating program of the oven was set as follows: initial temperature of 50°C, isothermal for two minutes, then increased by 10 °C.min<sup>-1</sup> up to 200 °C, followed by a second increase of 2.5 °C.min<sup>-1</sup> up to 310°C, followed by a final increase of 8°C.min<sup>-1</sup> up to 320°C, when the temperature was held for six minutes. The total analysis time was sixty-nine minutes.

## **4. Results**

### **4.1. Radiocarbon dating**

The radiocarbon results obtained for the four aliquots of the balm of Padiimenipet and the archaeological resinous sample N°372660 are displayed in **Appendix S-1**. They were calibrated with the Oxcal program ver. 4.4 [28] using InCal20 calibration curve [29] and the distribution ranges are displayed in Figure 2 A and Figure 2 B. The range obtained for fragment 1 was composed of two statistical distributions with a probability of 18.8% associated to the interval 5,617-5,587 BCE, and a probability of 76.7% associated to the interval 5,566-5,477 BCE. The range obtained for fragment 2 was composed of two statistical distributions with a probability of 93.9% associated to the interval 4,345-4,240 BCE and a probability of 1.6% associated to the interval 4,186-4,177 BCE. The range obtained for fragment 3 was composed of three statistical distributions with a probability of 1.1% associated to the interval 4,652-4,640 BCE, a probability of 93.6% associated to the interval 4,614-4,446 BCE and a probability of 0.8% associated to the interval 4,417-4,407 BCE. For all these three samples, the time intervals are rather scattered and much older than the known age of the mummy, 116 CE. The range obtained for the fragment of balm not pre-treated was composed of three statistical distributions with a probability of 24.6% associated to the interval 4,239-4,186 BCE, a probability of 66.2% associated to the interval 4,176-4,042 BCE and a probability of 4.7% associated to the interval 4,017-3,995 BCE. The comparison of these distributions with the calendar age known for Padiimenipet, displayed in Figure 2, indicates a significant ageing of the balm due to the presence of fossil contaminants.

The dating of sample n°372660 yielded, after calibration, the four following distributions: 259-280 CE with a probability of 6.6%, 336-440 CE with a probability of 75.2%, 454-479 CE with a probability of 4.7% and 495-479 CE with a probability of

8.9% [Figure 2 A]. These distributions are consistent with the time period associated to the sample (Graeco-Roman period) and thus confirm the absence of fossil compounds which was suspected from the visual observation of the archaeological sample n°372660. If traces of a fossil compound were present, the age obtained would be significantly older than this.

## 4.2. ATR-FTIR

### 4.2.1. Bulk balm and archaeological resin

The ATR-IR analyses of the bulk balm and of the archaeological resinous sample gave information about the chemical composition of both samples. The obtained spectra were compared with reference colophony and reference bitumen (Figure 3). ATR-IR spectra of six reference organic compounds usually used in mummification balms are presented in supplementary materials (**Appendix S-2**).

The spectrum of the archaeological resinous sample indicated a composition very similar to that of modern colophony: shoulder at  $3070\text{ cm}^{-1}$  associated with the vibration of the CH bond in a vinyl group  $\text{CH}_2=\text{CRR}'$  and present only in diterpenic compounds [34,35], band at  $2650\text{ cm}^{-1}$  and carbonyl band centred at  $1693\text{ cm}^{-1}$ . This spectroscopic result, combined with microscopic observation and radiocarbon dating information, led us to design this sample as archaeological resin.

The spectrum of the bulk mummy balm showed the presence of an organic mixture. The presence of a diterpenic resin could be assessed thanks to characteristic bands ( $3070\text{ cm}^{-1}$ ,  $2650\text{ cm}^{-1}$  and  $1693\text{ cm}^{-1}$ , the latter is shifted at  $1705\text{ cm}^{-1}$  in triterpenic resins). However, when comparing the spectrum of the bulk mummy balm with reference colophony and bitumen, it can be observed that the presence of bitumen in the balm cannot be assessed based on bulk FTIR. This is notably due to the lack of discriminating bands in bitumen [Figure 4].

In an attempt to simplify the FTIR signature of the mummy balm and to identify compounds present in low quantities or difficult to detect, two extraction protocols were used prior FTIR analyses to separate the compounds present in the balm according to their polarity

### 4.2.2. ATR-FTIR on extracted fractions

The fractions extracted from the balm in both protocols were gathered in vials (Figure 4). The visual differences observed on both fractions (hexane and  $\text{CHCl}_3$  for P1; MeOH and  $\text{CHCl}_3$  for P2) indicate different chemical affinities between the balm and the organic solvents. The ATR-FTIR spectra obtained from each of the developed protocols (P1 and P2) are presented in Figure 5.

After extraction of the balm using protocol P1, two fractions were collected with respectively n-hexane and  $\text{CHCl}_3$ . The obtained spectra indicated no significant differences for the C-H bands ( $3000\text{--}2800\text{ cm}^{-1}$ ) however the C=O bands between  $1800$  and  $1500\text{ cm}^{-1}$  showed some evolution. The hexane fraction has retained compounds ranging from  $1700$  to  $1730\text{ cm}^{-1}$ , suggesting the presence of esters, possibly coming from waxy components. The intense band at  $756\text{ cm}^{-1}$  in the chloroform fraction is not taken into account as it corresponds to the remaining amount of solvent.

After extraction of the balm using protocol P2, two fractions were collected with respectively MeOH and  $\text{CHCl}_3$ . Protocol P2 provided good separation between the different organic constituents, especially hydrocarbons, avoiding the overlapping of bands in the area between  $1730$  and  $1600\text{ cm}^{-1}$ . The resin materials are mostly retained in the methanol fraction, as indicated by the intense band at  $1693\text{ cm}^{-1}$  present in this fraction. The hydrocarbon materials are gathered in the chloroform fraction, as indicated by intense C-H bands at  $2924$  and  $2855\text{ cm}^{-1}$  and the shoulder at  $1610\text{ cm}^{-1}$  due to C=C vibration of aromatic compounds. However, the origin of these hydrocarbons cannot be deduced from FTIR only, they could originate from bitumen but also pitch or tar. Still in the chloroform fraction, the small and bulky band around  $1708\text{ cm}^{-1}$  is possibly due to a mixture of wax esters and a small amount of remaining resin. The intense band at  $756\text{ cm}^{-1}$  in this fraction is not taken into account as it corresponds to the remaining amount of solvent.

### 4.3. Thin Layer Chromatography

The results obtained after TLC analyses of the archaeological resin and the balm of Padiimenipet are presented in Figure 6. To assess the reliability of this result, two other TLC plates have been eluted in identical conditions. They are displayed in supplementary materials (**Appendix S-3**). After revelation, the TLC spots have been surrounded by pencil to highlight the shapes observed under the UV light.

The chromatogram of Figure 6-A) illustrates the behaviour of the archaeological resin N°372660 when eluted by MeOH on a silica plate. Compared with colophony and mastic (diterpenic and triterpenic resins) and after revelation at 312 nm, the archaeological resin can be identified as diterpenic resin, probably degraded.

The chromatogram of Figure 6-B) corresponds to the analysis of the balm of Padiimenipet. The elution of the balm with MeOH on a silica plate and the comparison with colophony (diterpenic resin) and bitumen provided the straightforward substitute separation of the balm in two constitutive elements. By comparison with 6-A, it can be observed that the balm of Padiimenipet is composed of a compound that gives a wide elution spot, very similar to that of archaeological resin and probably due to the complex and degraded mixture of compounds present in the balm, but also of a very dark spot that does not elute because due to the presence of very apolar compounds. By comparison with the elution of bitumen, the latter spot can be identified as hydrocarbons.

### 4.4. Gas chromatography coupled with Mass Spectrometry

#### 4.4.1. Global extraction prior to GC-MS

Global extraction of the sample using DCM provided complementary information regarding the chemical composition of the organic balm (Figure 7). Several monocarboxylic acids (MC) and dicarboxylic acids (DC) were identified, as well as unsaturated compounds (MC C<sub>16:1</sub> and C<sub>18:1</sub>). They indicate the presence of fats, of undetermined origin and in a degraded state as diacids are obtained after cleavage of a long-chain carboxylic acid. Here, the two diacids identified (DC C<sub>8</sub> and C<sub>9</sub>) correspond to the degradation of the most abundant fatty acids in both plant oils and animal fats, palmitic (MC C<sub>16:0</sub>) and stearic acids (MC C<sub>18:0</sub>).

Global extraction (Figure 7) also allowed the identification of diterpenoids to be performed such as several pimarane acids (pimaric, sandaracopimaric and isopimaric acids) as well as abietane ones (dehydroabietic acid (DHA), 7-hydroxy-DHA, 7-oxo-DHA) characteristic of mildly degraded diterpenic resin of Pinaceae [37,38].

#### 4.4.2. SPE protocol prior to GC-MS

The three fractions obtained thanks to SPE pre-fractionation were analysed using GC-MS and provided complementary information to the global extraction (Figure 8).

The first fraction, composed of hexane-soluble compounds, contained many hydrocarbons (alkanes) as well as DHAM (*dehydroabietic acid methyl ester*) and 7-oxo-DHAM, which are methylated derivatives of diterpenic resins suggesting a heating treatment of the resin resulting in a wood tar. Indeed, DHAM and its derivatives are produced during the distillation of pine wood, as methanol obtained from wood pyrolysis can react with abietane acids [11,36]. Such assignments give precious information about how diterpenic resin was thermally treated before being used to embalm Padiimenipet. Additionally, traces of hopane, characteristic of bitumen, have been found in this fraction. The extracted ion chromatogram m/z 191, necessary to assess the presence of hopanes, is presented in supplementary materials (**Appendix S-4**). The second fraction, composed of ethanol-soluble compounds, shows traces of fatty acids (MC C<sub>9</sub> and C<sub>16:0</sub>), DHAM and important amount of 7-oxo-DHA were identified. The last fraction, eluted with DEE and 2% AcOH, contained various saturated and unsaturated fatty acids (MC<sub>14:0</sub>, MC<sub>16:0</sub>, MC<sub>18:1</sub> and MC<sub>18:0</sub>) as well as diterpenic acids (isopimaric acid and DHA).



## 5. Discussion

### 5.1. Radiocarbon dating of the balm and aging effect from fossil carbon

As already mentioned, previous analyses of the Padiimenipet balm (Connan, 2005) have highlighted the presence of bitumen. The literature regarding the evolution of embalming procedures through Ancient Egypt provides some chronological information [3]. A mummy from Roman period, with a routine embalming procedure evolved from the Ptolemaic period and an abundant use of bitumen, can hardly be mistaken with the first attempts at body preservation from Predynastic Egypt [39]. At the end of Neolithic Egypt, the funerary practices are characterized by foetal position of the dead body and natural mummification caused by the dryness of Egypt. Sometimes, the corpses are impregnated with various organics (such as plant wax, fats or oils, pine resin [40]) but more for ritual purposes than for embalming itself according to a strategy [41,42]. So far and to the best of our knowledge, no use of bitumen was reported from these early times. Moreover, the mummy of Padiimenipet shows standardized features typical from the Ptolemaic period, such as left side incision to remove the viscera, the blackened aspect to impersonate Osiris or the use of gold leaf to cover some areas of the body, such as the face [20]. The latter specificity was also noted during the analysis of the golden mummy from Dunkirk, dated between the 3<sup>rd</sup> and the 4<sup>th</sup> centuries CE [43]. Even using with care the archaeological data about the death of the individual, the characteristics of the Padiimenipet mummy are consistent with a death around 116 CE.

The repeatability of the sample preparation and AMS measurements was first tested by dating 3 aliquots of the balm. The calibrated ages obtained from these three aliquots yielded a broad time range (1400 years between aliquot 1 and 2) and exhibit a significant ageing with respect to the age of the mummy.

Next, the influence of pre-treatment on the radiocarbon dating of the balm was addressed. It has been supposed that the acid wash of the sample prior to dating could eliminate part of the resinic material and thus concentrate the fossil hydrocarbons present in the balm. The results displayed in Figure 2 indicate that in the absence of conventional pre-treatment of the balm, a slightly younger Gaussian distribution was obtained. This result indicates the influence of acidic treatment on the balm and confirms the partial dissolution of resin materials as the oldest dates obtained for the balm all correspond to pretreated aliquots. However, this observation needs to be nuanced as the balm is highly heterogeneous and the aliquots could be polluted by some remaining younger post-depositional contamination of the sample, not eliminated because of the absence of pre-treatment.

Two important pieces of information could be drawn from the dates obtained. The first one is that the dates of the three aliquots treated in the same way are very different from each other, which highlights the wide heterogeneity of the balm. Although the different aliquots come from the “same sampling”, the balm is far from being a homogeneous preparation. The second is that among the several organic products composing the balm, at least one of them is highly depleted in <sup>14</sup>C leading to radiocarbon ages significantly older than the mummy's age. Furthermore, pre-treatment of the sample with acid does not significantly affect the dating result. The very important gap between the oldest and the youngest aliquot certainly excludes the possible re-use of a product “stored” by the previous generations for the preparation of the balm and points to the use of a radiocarbon-dead based compound consistent with the documented presence of bitumen in the balm.

These dating results highlight the importance of the issue of dead carbon contamination of complex organic mixtures. Thanks to the OxCal program, a simulation was achieved to obtain the radiocarbon age of the mummy based on the calendar known age: 116 CE, yielding a median age of 1941 BP after 20 simulations. The four radiocarbon-dated aliquots indicated an ageing in radiocarbon age of the balm compared to the documented age of the mummy of 4645, 3485, 3754 and 3350 years for respectively fragments 1, 2, 3 and not pre-treated. Previous studies by Georgette Delibrias [44] draw a correspondence between the ageing caused by a fossil carbon contamination and the approximate percentage (w/w) of such contamination in the sample dated (Figure 9). In the case of Padiimenipet, the ageing range observed between the balm and the documented age is between 4645 and 3350 BP, which would imply an amount of fossil carbon of approximately 35-45% (w/w) in the dated sample (Figure

9). However, it is difficult to deduce a reliable quantification of the composition of the balm because, as was observed when assessing the reproducibility of the radiocarbon dating of the balm, the mixture is very heterogeneous and these proportions vary in an important manner depending on the fragment investigated.

Measurements of Padiimenipet balm by GC-MS after SPE extraction highlighted, as expected, the presence of bitumen chemical markers (Figure 8). This approach is not quantitative, but beyond the analytical criteria, the heterogeneity of the chemical composition of the balsam highlighted by the radiocarbon dates underlines the difficulty of tackling this question, especially with small amounts of sample available. Beyond the question of identifying the presence of bitumen by elaborate laboratory protocols, the *a priori* assessment of its impact on the radiocarbon age is tricky to address. As a very strong heterogeneity of the balm has been underlined, the question in relation to the relevance of radiocarbon dating is more to identify its presence before the measurements than to try to quantify it. Assessing its presence, even heterogeneous, is of capital importance because even traces of fossil hydrocarbons will induce a bias in the radiocarbon age.

To be informative, the radiocarbon dating of Egyptian mummies requires an accuracy of at most a hundred years. Below that accuracy, stylistic criteria are sufficient to date approximately a mummy. Fossil contaminants such as bitumen need to be characterized and avoided to allow a precise measurement. Assess quantitatively the presence of fossil compounds is hardly possible, because of the very heterogeneous nature of the organic balms used to preserve the corpses. The type of sample chosen for radiocarbon dating (textile, flesh or muscle) and the way it retains organic balms will influence the role of contaminants on the radiocarbon ages. Consequently, a much documented sampling with thorough chemical characterization to identify impregnated materials is of prime importance when trying to date a mummy with radiocarbon.

## 5.2. Characterization of fossil compounds

Previous analyses already documented extensively the identification of the organic compounds used to embalm Padiimenipet [15]. They reported the presence of oxidized beeswax, of fatty acids probably originating from animals, of bitumen and of pine pitch, identified thanks to the significant presence of the methylated ester of dehydroabiatic acid. These results relied on specific samples preparations targeting chemical of very different natures [45]. The global characterization of the balm was thus not the goal of our study but rather to investigate ways to separate compounds according to their chemical families and to detect bitumen contamination prior to radiocarbon dating using analytical techniques easy to transfer from the lab to the excavation or conservation site.

The Figure 10 outlines the analytical techniques implemented in this study in order to assess their ability, prior to radiocarbon dating, to identify the different chemical families of organic compounds (particularly the fossil ones) and to be suitable for on-site or laboratory deployment.

### 5.2.1. Strategies involving infrared spectroscopy

Identification of fossil compounds such as bitumen using bulk ATR-FTIR analysis turned out to be inefficient due to important overlapping of the bands indicating hydrocarbons materials. A new strategy was implemented to separate the constituents from the balm according to their polarity with various organic solvents, following two different protocols. In both protocols, the second step (chloroform extract) solubilised totally the balm, but the first step (hexane in P1 and methanol in P2) solubilised specific compounds having chemical affinity with the solvent. Efficient separation has been observed, notably regarding the protocol designed as P2 : after collection of all polar components (resins, fats) in the methanolic fraction, the hydrocarbon material has been clearly isolated in the chloroform fraction (Figure 5). This strategy performed well to enhance ATR-IR potential to identify hydrocarbons from a complex mixture.

Currently, miniaturised infrared spectrometers allow ATR measurements to be achieved in the field. Pre-fractionation of the sample using solvents and simple glassware allows the separation of the different chemical families of the mixture prior to ATR-FTIR analysis. This “simplification” of the mixtures could be used in a systematic way to provide preliminary detection of the hydrocarbons materials in a complex organic sample. Such diagnosis could be achieved directly during the sampling on site to help choose uncontaminated fragments prior to radiocarbon dating.

#### 5.2.2. *Strategy involving a portable chromatographic technique*

In order to obtain analytical strategies applicable on site, we evaluated the possibility of using Thin Layer Chromatography (TLC) to detect bitumen in complex mixtures. Indeed, on site it is quite possible to use chromatographic plates, some solvents, a selected set of reference materials and a miniature UV lamp. TLC analyses of both archaeological samples yielded complementary information concerning their chemical composition. Figure 6 A) identified the pure archaeological resin as a diterpenic resin. The differences observed between the elution of the archaeological resin and the modern colophony can be due to the degradation of the archaeological sample. A more comprehensive TLC study, comparing various certified resin derivatives, could improve the quality of information that can be drawn from these analyses. Figure 6 B) presents the unambiguous identification of bitumen hydrocarbons in the balm of Padiimenipet. The TLC analysis of the mummification balm identified the presence of two main compounds: hydrocarbons from bitumen and a mixture composed in particular of diterpenic resin, very similar to the pure archaeological resin N°372660 analysed in Figure 6 A). It is important to indicate that, even though TLC approach requires sampling and destruction by solubilisation, only a micro sample of about 50 to 100 mg of organic material was here used to perform the analysis. Additionally, experiments conducted during the development of TLC procedure led us to think that a concentration of about 10 mg/mL of balm in chloroform would be enough to identify the presence of bitumen, allowing even smaller sampling in the future.

This separation technique can therefore effectively identify the presence of bitumen in a complex mixture. The screening of balms can thus be carried out by comparing chromatographic TLC between the sample of interest, reference bitumen and a few reference products representing the main chemical families, such as a few resins, a wax, a sugar, a fat. Later on, this integrated methodology could be pursued with a more comprehensive set of organic materials, originating from well documented museum collections, analyzed using ATR-FTIR, TLC and GC-MS to eventually allow an efficient diagnosis *in situ* based only on ATR-FTIR and TLC.

## 6. Conclusion

This study provided new insight into the diagnosis of the presence of bitumen as a prerequisite for sampling for radiocarbon dating with an original angle aimed at on-site implementation. This is to target or avoid taking samples that would be contaminated with fossil carbon, which induces significant ageing of radiocarbon ages, with the goal to open a dialogue between chemical characterization and radiocarbon dating. An embalming material from a Greco-Roman Egyptian mummy allowed the development and validation of the proposed approach. Previous analyses already documented extensively the chemical composition of the organics used for the balm of the Padiimenipet mummy [15], including the presence of bitumen.

The radiocarbon dating of four aliquots of the Padiimenipet balm (including one without conventional acid pre-treatment) highlighted significant aging due to bitumen and high heterogeneity of results. The issue of the relevance of the results in the presence of bitumen is thus underlined and the heterogeneity of the balm emphasises the need for a drastic selection of samples for dating purposes.

Laboratory approaches based on GC-MS analyses have, as expected, shown their ability to detect the presence of bitumen. However, they cannot be performed at the time of sampling at the archaeological site or museum. We therefore propose to use protocols that can be implemented on site based on infrared spectroscopy and/or Thin Layer Chromatography analyses.

ATR-FTIR analyses on the raw balm do not allow minor compounds to be identified in such a complex organic mixture, in particular those with infrared signatures exhibiting few specific features (as bitumen). We then proposed to use solvents to separate the different chemical families according to their polarity and thus improve the detection of minor components by ATR-FTIR, such as hydrocarbons which reveal the possible presence of bitumen. A complementary approach using Thin Layer Chromatography was developed on reference mixtures and validated on Padiimenipet balm. For Padiimenipet balm these TLC analyses provided an efficient identification of resin (diterpenic resin as opposed to tripenic resin) as well as the detection of hydrocarbons originating from bitumen. The TLC approach proved very promising as it could be used directly from the excavation site to control sampling for radiocarbon dating.

When hydrocarbons in general and bitumen in particular are detected in an organic material, sampling for radiocarbon dating shall be avoided, or specific organic pre-treatment to extract bitumen will be necessary prior to radiocarbon measurement. Routine radiocarbon pre-treatment (Acid-Base-Acid washes) fails to eliminate this organic contamination and although attempts were made to use organic solvent cleaning, so far no efficient organic cleaning method has been documented [12,13]. In order to minimise sampling of archaeological artefacts and to obtain consistent radiocarbon dates, screening must be carried out on site during sampling, or at least in the lab before the radiocarbon measurement. The approach combining ATR-IR and TLC could be implemented on site or quite simply in radiocarbon laboratories as a screening step to assess the presence of organic contamination on a mummy or textile sample prior to the measurement. The combination of these two techniques proposes an efficient and an easy analytical strategy to diagnosis the presence of bitumen in complex organic mixtures and strongly enhances the possibility to better select mummy samples for  $^{14}\text{C}$  dating on archaeological site.

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Figure captions:

Figure 1: Photo of the two archaeological samples by optical microscopy (x 5); A) Fragment of the balm (N°E25491-BN1810); B) Archaeological resinous sample (N°372660)

Figure 2: Radiocarbon data; A) Calibrated ranges of the radiocarbon dates obtained for the archaeological resinous sample n°372660. The blue shape indicates approximatively the Graeco-roman period; B) Calibrated ranges of the radiocarbon dates obtained for four fragments of balm, compared with the calendar age known for Padiimenipet.

Figure 3: ATR-IR spectra of reference colophony, of the archaeological sample N°372660, of the bulk Padiimenipet balm and of reference bitumen

Figure 4: Photos of the different fractions from protocol P1, balm extracted with hexane then  $\text{CHCl}_3$  (A) and from protocol P2, balm extracted with MeOH then  $\text{CHCl}_3$  (B)

Figure 5: ATR-IR spectra of the bulk balm of the fractions obtained from protocol P1 and the fractions obtained from protocol P2. The crosses indicate bands due to chloroform residues.

Figure 6: TLC chromatograms, normal phase, elution with MeOH, revealed at 312 nm then surrounded by pencil; A): Archaeological resin N°372660 compared to references colophony and mastic; B): Balm of the mummy compared to references colophony and bitumen. The shapes visible on each plate correspond to the organic material eluted. The dark spots still present on the deposit line on B) are due to not-eluted apolar molecules. Figure 7: TIC (Total Ion Chromatogram) obtained by GC-MS for the Padiimenipet balm - global extract with DCM

Figure 7. TIC (Total Ion Chromatogram) obtained by GC-MS for the Padiimenipet balm - global extract with DCM.

Figure 8: TIC (Total Ion Chromatogram) obtained by GC-MS for the 3 fractions of the Padiimenipet balm obtained after SPE pre-fractionation (F1: Hexane; F2: EtOH; F3: DEE+2%AcOH)

Figure 9: Curve describing the ageing of a sample caused by fossil contamination (adapted from Delibrias, 1985) on which the age range obtained on the Padiimenipet balm is plotted

Figure 10: Outline of the combinaison between sample preparation and analytical techniques, as implemented in this study to identify hydrocarbons, with products identified after each protocol (in *italic*).

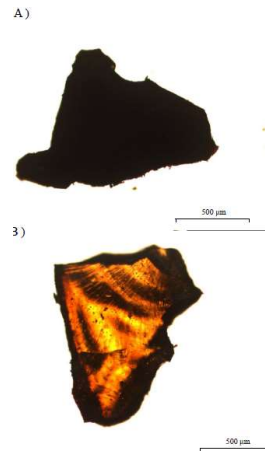


Figure 2: Photo of the two archaeological samples by optical microscopy (x 5); A) Fragment of the balm (N°E25491-BN1810); B) Archaeological resinous sample (N°372660)

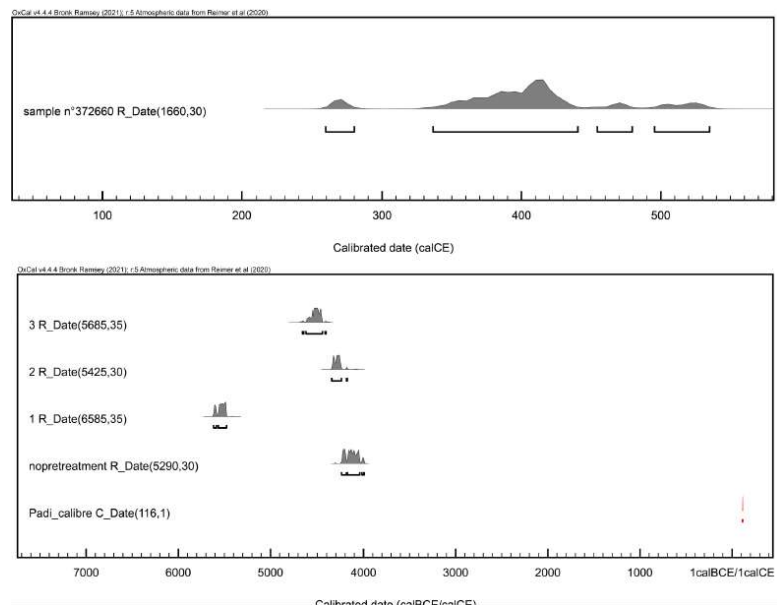


Figure 2: Radiocarbon data; A) Calibrated ranges of the radiocarbon dates obtained for the archaeological resinous sample n°372660. The blue shape indicates approximatively the Graeco-roman period; B) Calibrated ranges of the radiocarbon dates obtained for four fragments of balm, compared with the calendar age known for Padiimenipet.



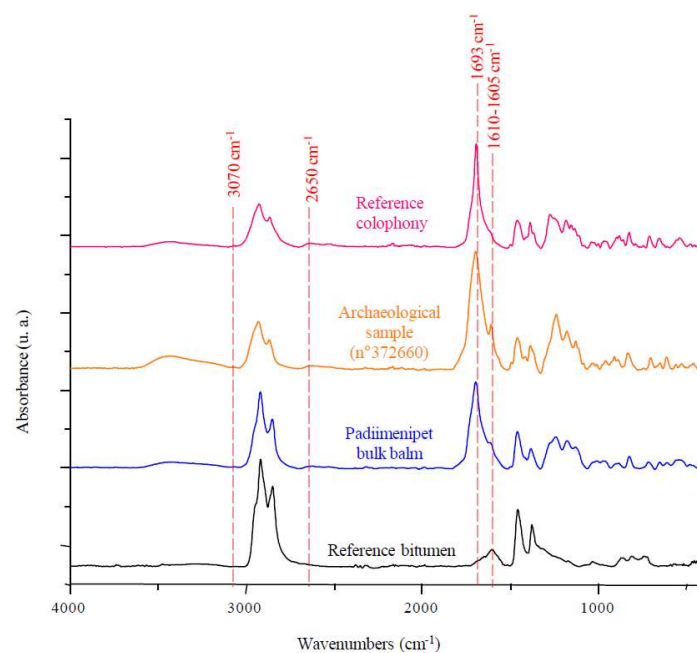


Figure 3: ATR-IR spectra of reference colophony, of the archaeological sample N°372660, of the bulk Padiimenipet balm and of reference bitumen

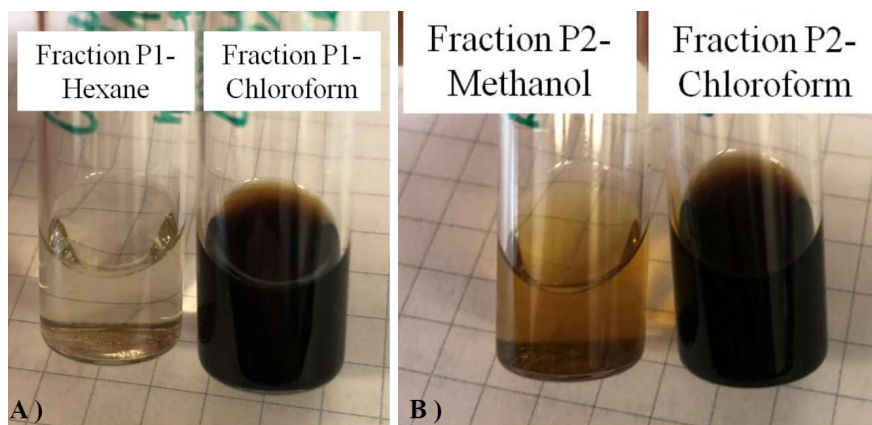


Figure 4: Photos of the different fractions from protocol P1, balm extracted with hexane then  $\text{CHCl}_3$  (A) and from protocol P2, balm extracted with MeOH then  $\text{CHCl}_3$  (B)

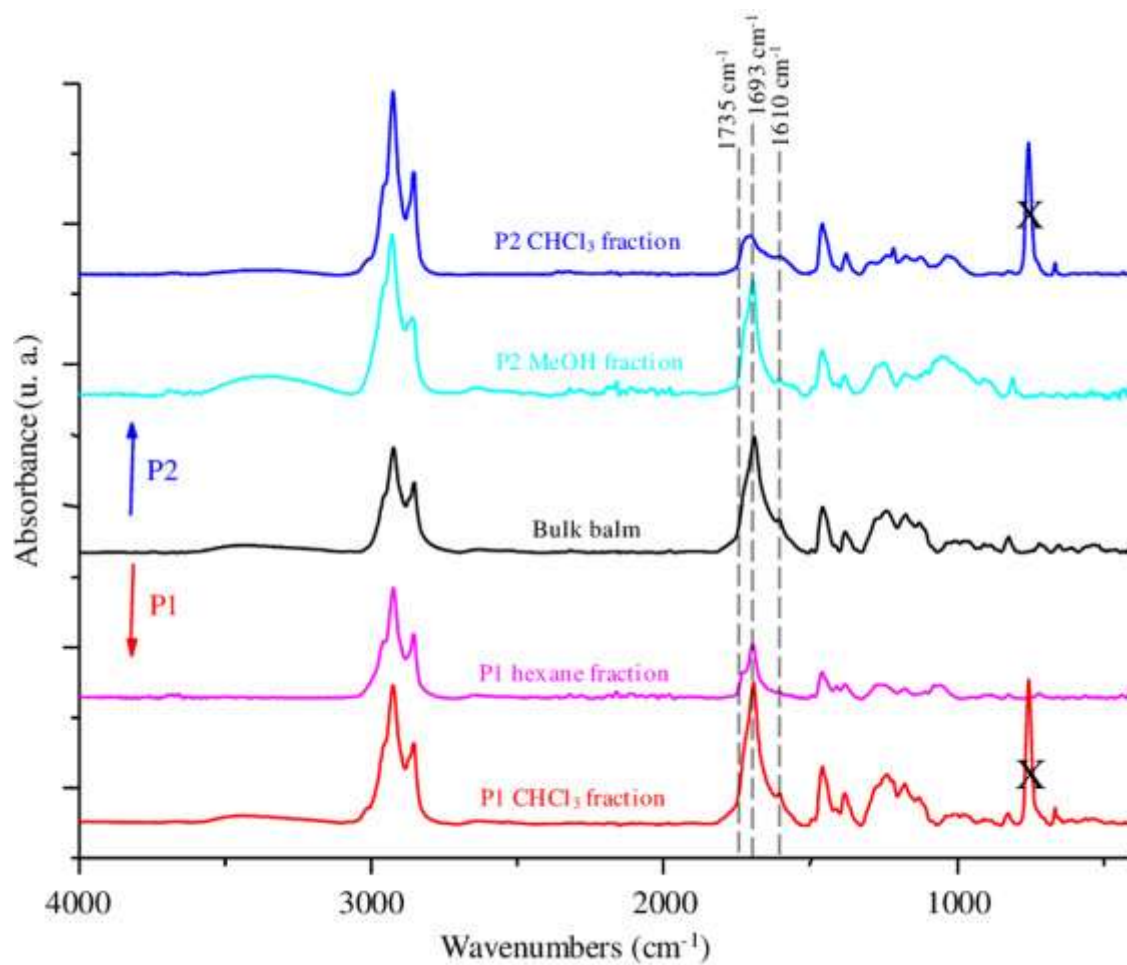


Figure 5: ATR-IR spectra of the bulk balm of the fractions obtained from protocol P1 and the fractions obtained from protocol P2. The crosses indicate bands due to chloroform residues.

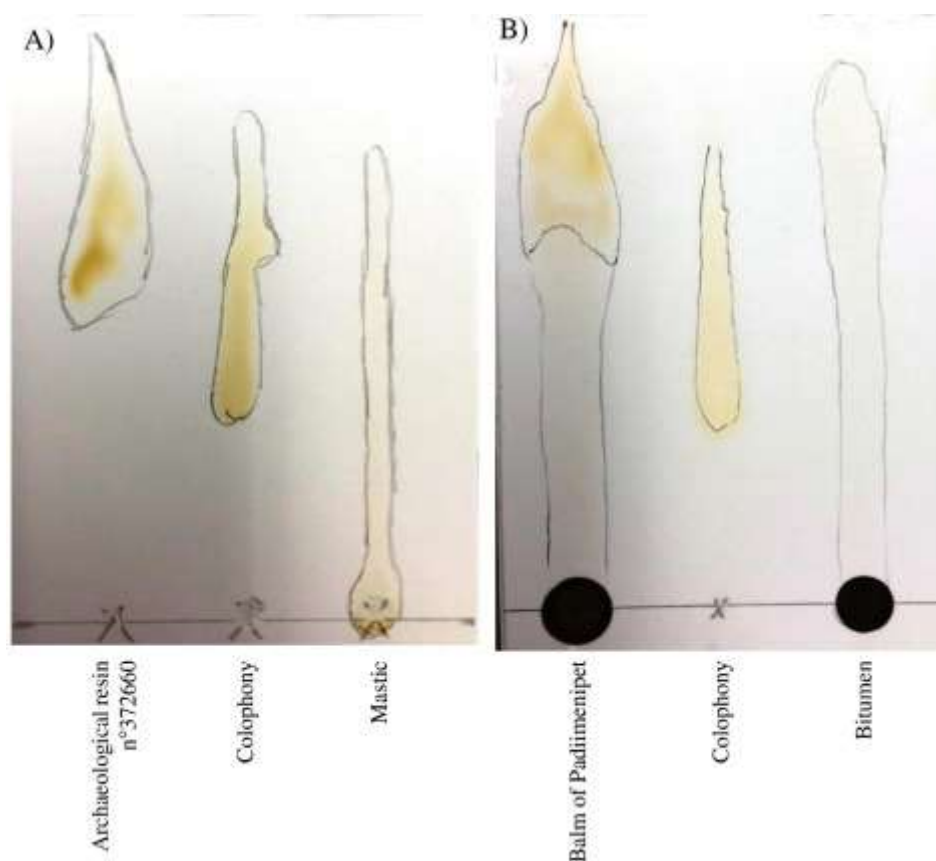


Figure 6: TLC chromatograms, normal phase, elution with MeOH, revealed at 312 nm then surrounded by pencil; A): Archaeological resin N°372660 compared to references colophony and mastic; B): Balm of the mummy compared to references colophony and bitumen. The shapes visible on each plate correspond to the organic material eluted. The dark spots still present on the deposit line on B) are due to not-eluted apolar molecules. Figure 7: TIC (Total Ion Chromatogram) obtained by GC-MS for the Padiimenipet balm - global extract with DCM

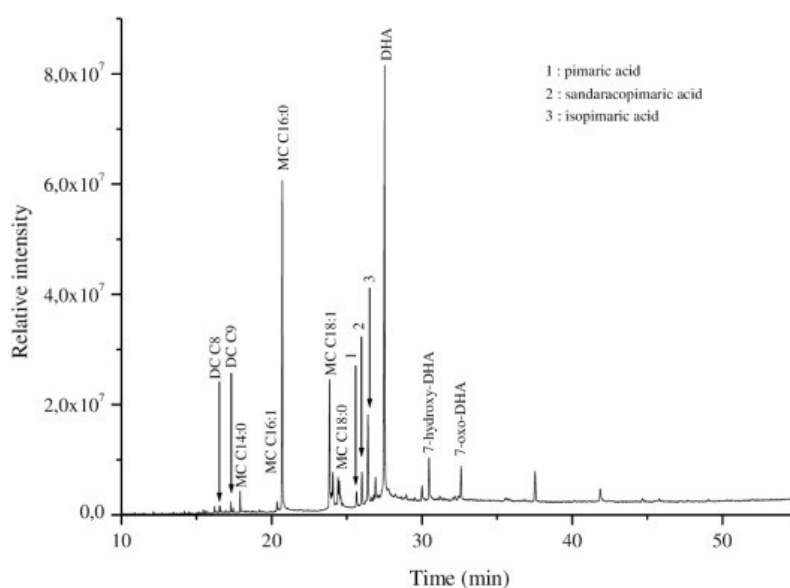


Figure 7. TIC (Total Ion Chromatogram) obtained by GC-MS for the Padiimenipet balm - global extract with DCM.

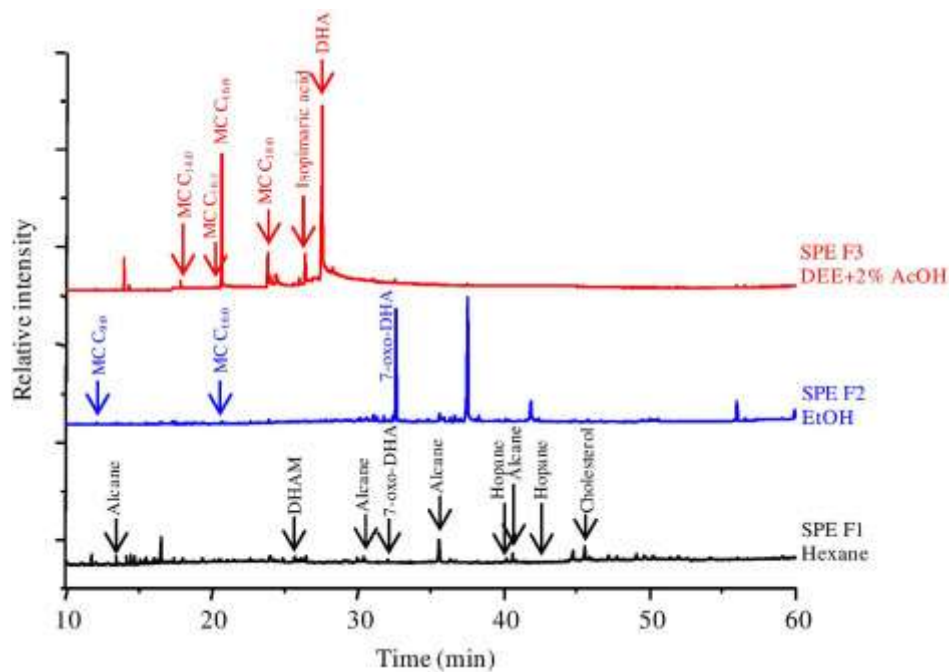


Figure 8: TIC (Total Ion Chromatogram) obtained by GC-MS for the 3 fractions of the Padiimenipet balm obtained after SPE pre-fractionation (F1: Hexane; F2: EtOH; F3: DEE+2%AcOH)

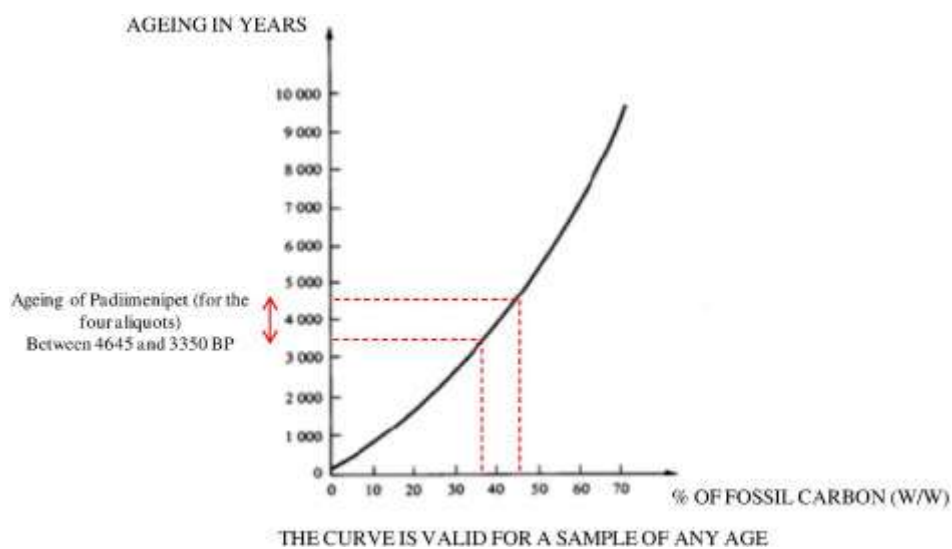


Figure 9: Curve describing the ageing of a sample caused by fossil contamination (adapted from Delibrias, 1985) on which the age range obtained on the Padiimenipet balm is plotted

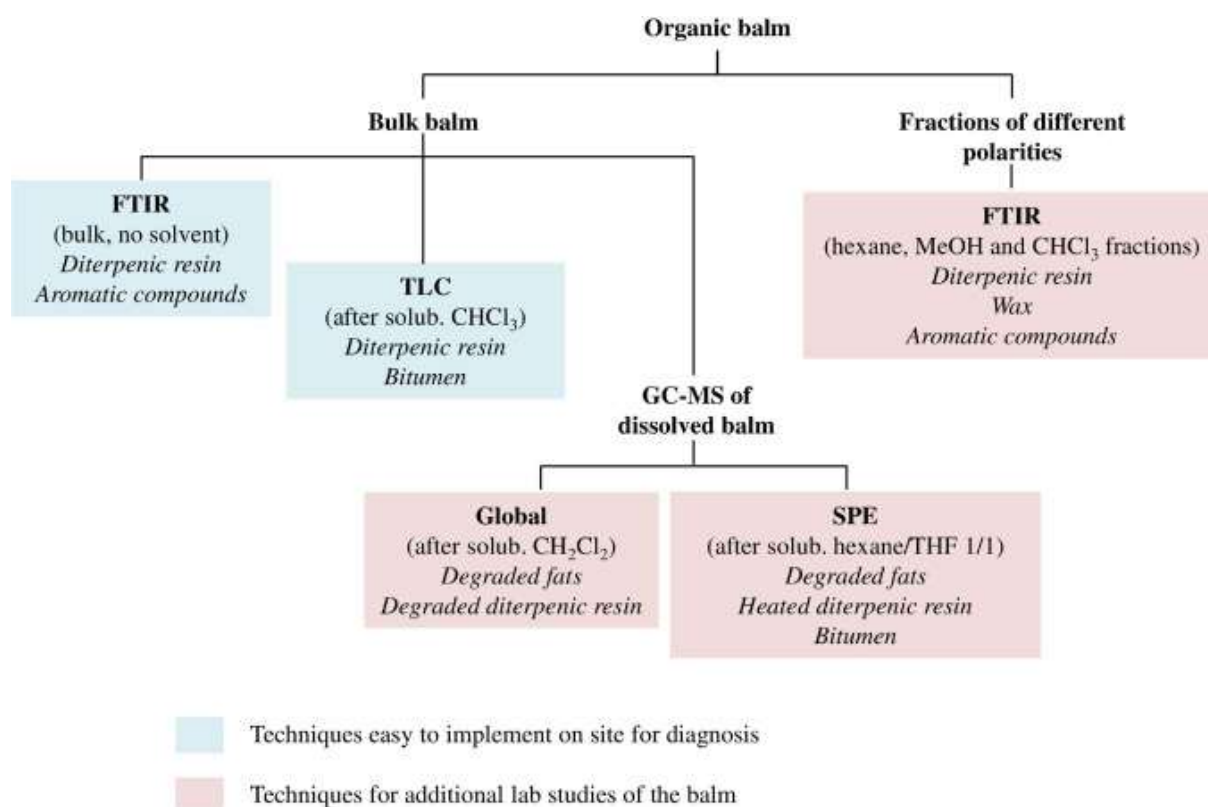


Figure 10: Outline of the combinaison between sample preparation and analytical techniques, as implemented in this study to identify hydrocarbons, with products identified after each protocol (in italic).