Analyst

PAPER

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2018, **143**, 4503

Untargeted metabolomics-like screening approach for chemical characterization and differentiation of canopic jar and mummy samples from Ancient Egypt using GC-high resolution MS†

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In Ancient Egypt it was common practice to embalm corpses and specific internal organs to ensure eternal life. The exact nature of the employed embalming fluids, particularly for organ preservation within the canopic jars, is debated. Therefore, the aim of the current study, was to chemically characterize and differentiate canopic jars (n = 28) and mummies (n = 6) using gas chromatography – high resolution mass spectrometry (GC-HR MS) with a new untargeted metabolomics-like screening approach; as part of a larger minimal-invasive transdisciplinary study on Ancient Egyptian human tissues. Post-analytical data processing included deconvolution, screening against the NIST 14 spectral database as well as a high resolution metabolomics library, and positive peak evaluation. In the majority of samples the presence of a coniferous resin was indicated by the detection of longiborneol in combination with abietadiene acid derivatives and guajacol. Beeswax, proposedly used for symbolic reasons and/or as a binding agent, was detected in 10 samples. Previously not mentioned in the literature, but identified in the current sample set, were medical-related substances like aniseed constituents, salicylic acid, chamazulene and jacobine. By applying an untargeted metabolomics-like approach to archaeological samples for the first time, extensive statistical analysis was made possible (using both identified and non-identified features; adding up to 4381 features), which showed significant differences in the overall chemical composition of canopic jar and mummy samples using principle component analysis (PCA) and partial least square-discriminant analysis (PLS-DA). This emphasizes the necessity for more extensive canopic jar studies in the future in order to interpret findings correctly.

Received 13th July 2018,
Accepted 24th August 2018

DOI: 10.1039/c8an01288a

rsc.li/analyst

Introduction

As early as 4000 BC, Egyptians purposefully preserved their corpses in order to ensure eternal life.[1,2] The resulting mummies have been under research for many years, however, very little work has been carried out to study their accompanying canopic jars; the containers that were sometimes used to hold the mummified viscera. As part of an interdisciplinary project this gap in knowledge should be filled by studying canopic jars on a genetic, medical, egyptological and chemical level.[3] Traditionally, there were four canopic jars associated with one mummy holding the liver, the lungs, the stomach and the intestines, while the other organs remained *in situ* or were removed and disposed of.[4] One of the central points during the mummification process was the treatment of the corpse and its organs with a variety of organic embalming agents. However, the exact nature of these, is still unclear, because the Ancient Egyptians left no written record of the process; only secondary textual evidence by *e.g.* Herodotus and Diodorus Siculus is available.[5–7] With the help of organic residue analysis, scientists started in the middle of the 20th century to try to decode the composition of such embalming fluids on a chemical level. In the beginning, the available analytical techniques limited such efforts

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†Electronic supplementary information (ESI) available: Table ESI 1 and Fig. ESI 1. See DOI: 10.1039/c8an01288a
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to identify very abundant organic components based on the instruments' sensitivities. Major advances in chromatographic and mass spectrometric instrumentation in recent years lead to increased sensitivities and an improved capacity to resolve the broad spectrum of biomolecular components included in the ancient embalming fluids.[8] The most common technique for the characterization of embalming fluids/materials employed in the current literature is the coupling of a gas chromatograph (GC) to a mass spectrometer (MS) or a tandem mass spectrometer (MS/MS), respectively.[9–14] The use of more specialized forms of this hyphenated technique is also described; *e.g.* the usage of sequential thermal desorption-GC-MS or pyrolysis-GC-MS.[5,15,16] Generally, the main components of balms previously identified by these means are mixture of oils, fats, waxes, resins, gums, salts, bitumen and various barks and spices.[17] One of the latest advancements in GC-MS/(MS) technology is high resolution mass spectrometry. In comparison to conventional GC-MS/(MS) methods, a greater sensitivity and selectivity and a more robust identification can be achieved due to the possibility of utilizing the accurate mass for compound identification. To the best of our knowledge, the current study is the first one to use a high resolution instrument to chemically characterize residues of canopic jar and mummy samples from Ancient Egypt. This should lead to the potential identification of new chemical components that can be associated with the embalming process. Additionally, the aim was to study the chemical contents of the analyzed canopic jars, thus filling the current knowledge gap in the literature and to support the validity of the current literature on mummy organic residue analysis. For the first time, the strategy of analysis for archaeological samples was adapted from metabolomics workflows. The key concept in metabolomics is the qualitative and quantitative characterization of small (endogenous) molecules (<1500 Da) with subsequent extensive multivariate statistical evaluation to distinguish between two sample classes.[18] By applying such a metabolomics-like approach, the aim was further, to utilize the entire chemical composition data (positive hits and non-identified features) for the differentiation of canopic jar from mummy samples with the view to establish potential similarities and differences between their treatments.

Materials and methods

Chemicals and reagents

Methoxyamine hydrochloride in powdered form was sourced from Sigma-Aldrich (Steinheim, Germany) and 1 mL solutions of *N*-Methyl-*N*-trimethylsilyl trifluoro acetamide (MSTFA) from Macherey-Nagel (Düren, Germany). Deuterated internal standards (IS) of hippuric acid-^{15}N and testosterone-d_2 were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and methanolic solution of trimipramine-d_3 (0.1 mg mL^{-1}) was acquired from Cerilliant (delivered by Sigma-Aldrich, Buchs, Switzerland). Methanol, pyridine and ethyl acetate of high-performance liquid chromatography (HPLC) grade were from Merck (Zug, Switzerland).

Sample collection

Samples used for the current study were obtained by museums in Leiden, Turin and Boston;[19–21] based on sufficient sample volume availability to the interdisciplinary canopic jar project.[3] Details regarding the sample catalog/exhibit number, origin and classification into mummy and canopic jar samples can be found in Table 1. The archaeological information and categorization into different ancient periods was based on stylistic information, radiocarbon dating (details are to be published elsewhere) and inscription and style following the classification of Sethe.[22–24] For chemical analysis, aliquots of the archaeological samples were taken from the outer core, resembling the embalming layer with presumably little organic contribution from the embalmed body or organs.

Sample preparation and GC-HR MS analysis

All samples detailed above were extracted and analyzed on the same day, within the same batch. The following extraction and sample preparation workflow was carried out. Approximately 20 mg of homogenized sample material was weighed into a 1.5 mL Eppendorf tube (Schoenenbuch, Switzerland) and spiked with 20 μL of an internal standard mixture containing testosterone-d_2 (10 μg mL^{-1}) and hippuric acid-^{15}N (100 μg mL^{-1}). Extraction was performed with the addition of 300 μL methanol. Samples were shaken (10 min at 1400 rpm) and centrifuged (5 min at 10 000 rpm) before 200 μL of the supernatant was transferred into a GC-autosampler vial with a 250 μL insert and evaporated to dryness under a gentle stream of nitrogen at 40 °C. Subsequently, the samples were reconstituted in 50 μL methoxyamine HCl in pyridine (20 μg mL^{-1}) and vortexed for 15 s. After heating of the samples at 80 °C for 15 min for successful methoximation (to stabilize keto–enol tautomerisms and therefore increase sensitivity), they were cooled down and again evaporated to dryness under nitrogen at 40 °C (schematic representation of the derivatization procedure exemplified for the used internal standards see ESI Fig. 1†). The dried samples were crimp-capped and transferred onto a GC-autosampler (Gerstel MultiPurposeSampler MPS (Gerstel, Mühlheim, Germany)). Further derivatization was performed on-line, controlled by Maestro® software (Version 1.4.40.1; Gerstel, Mühlheim, Germany), so each sample was prepared individually and directly prior to injection. 50 μL of MSTFA were automatically added to each sample vial and vortexed for 30 s. Incubation for successful silylation (to increase compound volatility) was carried out at 80 °C for 15 min with continuous shaking. After a 5 min cooling down period, 20 μL trimipramine-d_3 (100 μg mL^{-1}) were added to the samples and shaken vigorously to ensure homogenization. Subsequently, 1 μL of sample material was injected into the GC with a 5 : 1 split ratio. The same sample preparation was performed for a methanol blank sample spiked with the internal standards.

Table 1 Summary of the available canopic jar and mummy samples including their origin and further archaeological information (based on stylistic information and radiocarbon dating)

Index	Internal exhibit number/name	Origin (museum)	Classification	Archaeological information
1	F 2004 12.2	Leiden	Mummy	Ptolemaic (Greek) period*
2	Ar 35	Leiden	Mummy	Late ptolemaic to early Roman period*
3	AMM 27b	Leiden	Mummy	Late 3rd intermediate period*
4	EG ZM 62	Leiden	Mummy	Late ptolemaic to early Roman period*
5	F 1986/31	Leiden	Mummy	Late period*
6	H.III.P 2	Leiden	Mummy	Early late period*
7	Burgdorf (pilot study)	Burgdorf	Canopic jar	Late period+
8	Munich 2 (pilot study)	Munich	Canopic jar	Late period+
9	AR 14	Leiden	Canopic jar (Iroeroe)	Late period□
10	AT 1c	Leiden	Canopic jar (Neferamun)	New kingdom*□
11	CI 275	Leiden	Canopic jar (Wahibre)	Late period□
12	MFA 29.1133a	Boston	Canopic jar (Horemakhet)	Late period□
13	MFA 29.1134a	Boston	Canopic jar (Horemarkhet)	Late period□
14	MFA 29.1135a	Boston	Canopic jar (Horemarkhet)	Late period□
15	MFA 29.1136a	Boston	Canopic jar (Horemarkeht)	Late period□
16	H.III.SS 13	Leiden	Canopic jar (Pashedu)	New kingdom□
17	H.III.SS 12	Leiden	Canopic jar (Pashedu)	New kingdom□
18	N. 19035 (Cat. C3212)	Turin	Canopic jar	Late period□
19	N. 19022 (Cat. C3214/1)	Turin	Canopic jar	3rd Intermediate period□
20	Cat. C3217/1	Turin	Canopic jar	Late period+
21	RCGE 27042 (Cat. C3308)	Turin	Canopic jar	Late period+
22	Cat. C3471/2	Turin	Canopic jar (Amenemheb)	New kingdom□
23	N. 19043 (Suppl. S4306)	Turin	Canopic jar	Early middle kingdom+
24	N. 19044 (Suppl. S4307)	Turin	Canopic jar	Early middle kingdom+
25	N. 19045 (Suppl. S4308)	Turin	Canopic jar	Early middle kingdom+
26	AAL 1c	Leiden	Canopic jar (Irtu)	Late period□
27	AAL 1d	Leiden	Canopic jar (Iteroe)	Late period□
28	AAL 1e	Leiden	Canopic jar (Iteroe)	Late period□
29	AAL 3a	Leiden	Canopic jar	Late old kingdom*
30	AAL 3b	Leiden	Canopic jar	Late old kingdom+ (set with AAL 3a)
31	AAL 3e	Leiden	Canopic jar	Late old kingdom+ (set with AAL 3a)
32	AAL 8	Leiden	Canopic jar (oval box)	Early old kingdom*
33	AAL 9a	Leiden	Canopic jar (oval box)	Early old kingdom+ (set with AAL 9b)
34	AAL 9b	Leiden	Canopic jar (oval box)	Early old kingdom*

Museums are abbreviated by cities: Leiden = Rijksmuseum van Oudheden; Burgdorf = Museum für Völkerkunde; Munich = Staatliches Museum Ägyptische Kunst; Boston = Museum of Fine Arts; Turin = Museo Egizio; categorization into ancient periods based on radiocarbon dating (*), stylistic dating (+) and inscription and style following the classification of Sethe (□).[24]

For analysis, a TRACE 1300 GC system (Thermo Scientific, Bremen, Germany) was used, coupled to a Q Exactive GC Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The GC settings were as follows: TraceGOLD TG-5SilMS 30 m × 0.25 mm I.D. × 0.25 μm film capillary column (Thermo Scientific, Bremen, Germany); inlet temperature 250 °C; constant helium flow of 1 mL min^{-1} as carrier gas; gradient elution controlled by an oven temperature program, start temperature of 70 °C hold for 4 min, followed by a 20 °C min^{-1} rise up to 320 °C, which was hold for 8 min, resulting in a total run-time of 24.5 min. The MS was operated in electron ionization positive mode (70 eV) at an ion source temperature of 230 °C. Acquisition was carried out in full-scan mode within a mass range of 50–650 Da at a resolving power of 60 000 (measured as full width at half maximum at m/z 200). Lockmass-correction for continuous real-time recalibration of potentially occurring m/z shifts was performed with masses from the column bleed (m/z 207.03240, m/z 225.04290 and m/z 381.05110). The MS was controlled by Xcalibur® software (Version 4.0; Thermo Scientific, Bremen, Germany).

Data processing and analysis

Data was processed using TraceFinder™ 4.1 software (Thermo Scientific, Bremen, Germany). Initially, lockmass- and background-corrected full-scan data was deconvoluted and retention time aligned using the incorporated deconvolution plugin and then screened against the NIST 14 spectral database (contains electron impact MS data of 242 466 compounds; National Institute of Standards and Technology, Gaithersburg, MD, USA) and a high resolution metabolomics library (contains high resolution electron impact MS data of >800 primary and secondary metabolites; Thermo Scientific, Bremen, Germany). The returned list with tentative hits was manually evaluated for positive hits. The decision on whether or not a hit was a true analytical positive result was based on the search index score and the high resolution filtering value as well as on visual comparison of the acquired spectrum with the suggested library spectrum. Within this process, up to five top library hits were also considered where relevant. The acquired peak area data was neither sample weight-corrected nor corrected by an internal standard as no quantification was aimed

for. However, within the two internal standards spiked to the samples prior to the extraction procedure, hippuric acid-^{15}N was used to check for successful MSTFA derivatization and testosterone-d_2 was utilized to control successful methoximation and silylation procedure. Trimipramine-d_3 was used to check column and instrument performance. Upon failure of any of the three checks, samples would have been excluded from the evaluation.

Statistical data analysis was carried out using MetaboAnalyst 4.0, a web-based open-source tool for statistical interpretations.[25] Utilizing a user-friendly R-script, the following statistical tests were performed after filtering, transformation and scaling of the data. Principle component analysis (PCA) was used as a visualization tool to detect general trends of chemical composition differences between sample classes. Partial least-square discriminant analysis (PLS-DA) was performed for class discrimination, to investigate whether or not mummy and canopic jar samples varied significantly within their chemical composition. To validate that class separation within the PLS-DA model was not obtained by chance, cross model validation and permutation testing was carried out. Acceptance criteria are normally $Q_2 > 0.50$ and $p \leq 0.05$ according to Szymańska *et al.*[26]

Results and discussion

Across all 34 analyzed samples, a total of 4381 features was detected using the aforementioned deconvolution workflow (lockmass- and background corrected); no sample failed the internal quality check, based on the detection of the internal standards. Upon screening against the NIST 14 spectral database, supportive manual evaluation and cross-checking against the extracted blank positive hits, 754 positive hits were returned (detailed list of substances see ESI Table 1†). The sample positive hits predominantly included terpenoids, fatty acids, and many other potentially plant derived components. Screening against the high resolution metabolomics library gave 134 positive hits; mainly terpenoids and fatty acids. It was found that the metabolomics library did not add new identifications to the 754 positive hits obtained with the NIST 14 spectral database, but supported the finding of this more extensive library. It was therefore decided to focus the following evaluation and interpretation of the data on the hits returned by the NIST 14 database.

With this, a variety of breakdown products of the aforementioned compound classes were also detected. In this context, it is crucial to consider that although organic residues were found to be considerably stable over a broad timescale, their survival from ancient times until today cannot be taken for granted.[27] Therefore, the credo "absence of evidence is not evidence of absence" should always be taken into account when interpreting archaeological organic residue analyses.[8] Within the course of the archaeological timescale, particularly environmental degradation and decay or bacterial metabolism can lead to the detection of altered rather than native structures. In addition, materials can also experience alteration by processing. The use of heat, ancient and/or modern, can for example lead to the presence of oxidized and dehydrogenated products.[11] Generally, the aim is to trace back the detected altered structures to their native counterparts mainly based on their carbon atom skeleton. A prominent example, well described in literature, is the detection of fatty acids in archaeological samples, which are most likely originally derived from triglycerides, the main constituents of vegetable and body fat (animals and humans).[27] Within the analyzed sample cohort, all saturated fatty acids in the range between C6:0 and C18:0 were detected, with the exception of undecanoic acid (C11:0) and heptadecanoic acid (C17:0). The most prominent saturated fatty acid identified was hexanoic acid (C6:0) (n = 25), followed by pentadecanoic acid (C15:0) and octanoic acid (C8:0) (n = 23 each), tetradecanoic acid (C14:0) (n = 22), and nonanoic acid (C9:0) (n = 20). The other saturated fatty acids were detected with the following frequencies: heptanoic acid (C7:0) (n = 16), decanoic acid (C10:0) (n = 12), dodecanoic acid (C12:0) (n = 1), tridecanoic acid (C13:0) (n = 4), hexadecanoic acid (C16:0) (n = 7) and octadecanoic acid (C18:0) (n = 16). A predominance of even-chain length fatty acids in combination with a high abundance of C16:0 compared to the abundance of C18:0 are often taken as indications for a plant origin.[5] In contrast to this, it is well recognized in the literature that a sample is thought to contain fats from a mammalian origin, if the ratio of C16:0 to C18:0 is below 1.[28] In the current sample set, 6 canopic jar samples were found to contain both hexa- and octadecanoic acid simultaneously, with a C16:0 to C18:0 ratio of well below 1, indicative of a mammalian origin. This is also supported by the fact that a variety of odd-chain length fatty acids were detected in the analyzed mummy and canopic jar samples along with a cholesterol derivative. All but two canopic jar samples contained odd-chain length fatty acids and/or cholesta-4,6-dien-3-ol; previously described as a degradation product of cholesterol, detected in 9 of the currently analyzed samples.[17] This suggests that mammalian fats were present in most samples. However, it cannot be distinguished by these means, whether they were derived from an ingredient of animal origin specifically added during the mummification process or represent a contamination of the samples with lipid tissues of the mummified bodies.[12,29] Although various authors stated that particularly the presence of C15:0 and C17:0 suggest a ruminant origin (*e.g.* sheep, cattle and goats), it cannot be ruled out that those odd-chain length fatty acids originate from human lipids that underwent bacterial degradation.[12,30,31] A clear differentiation between ruminant and non-ruminant fats or between dairy fats and carcass fats can only be achieved by isotopic analyses of extracted fatty acids, which was not part of the current study.[8]

Alongside fatty acids, chemically also characterized as monocarboxylic acids, dicarboxylic acids were detected. At first sight, their occurrence is not expected in mummy or canopic jar samples, as dicarboxylic acids do not naturally occur in waxes, oils or fats. However, they can be formed during degradative oxidation over time. In particular nonanedioic acid

(azelaic acid; diC9) has been previously identified in archaeological samples.[12,32] Seven of the analyzed samples were found to contain azelaic acid, which indicates a previous oxidation reaction in position 9 of a Δ-9 unsaturated fatty acid, namely palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2) or linolenic (C18:3) acid.[17,32,33] Further detected dicarboxylic acids that can be formed analogous were octanedioic acid (suberic acid; diC8; detected in 1 sample), heptanedioic acid (pimelic acid; diC7; detected in 2 samples), hexanedioic acid (adipic acid; diC6; detected in 1 sample), pentanedioic acid (glutaric acid; diC5; detected in 2 samples) and butanedioic acid (succinic acid; diC4; detected in 1 sample). Similarly, hexanal, a six-carbon straight-chain aldehyde ($C_6H_{12}O$), detected in 1 mummy sample can be derived from unsaturated fatty acids by environmental oxidation. Oleanitrile, specifically derived from oleic acid by replacement of a carboxylic- with a nitrile group, was also identified in 1 mummy sample. As no unique native substance was identified in most cases and unsaturated fatty acids generally occur in various animal and vegetable fats and oils, a precise origin of the detected dicarboxylic acids and their derivatives could not be proposed.

Widely reported in the literature is the application of beeswax in the embalming process for symbolic reasons and as a binding agent.[5,11,13] Chemically, beeswax is characterized by *n*-alkanes in the range between C23 and C33, along with wax esters (C40–C50) and hydroxyl wax esters (C42–C54).[5] Within the current sample set, 9 canopic jar samples and 1 mummy sample were found to contain beeswax. This was identified based on the characteristic *n*-alkane pattern (extraction of full scan data for *m*/*z* 57 and 71) with the C27-alkane being most abundant. Wax esters and hydroxyl wax esters were not detected, due to the limited scan range of the utilized analytical method. The proposition of Buckley and Evershed[5] that beeswax was only started to be used for mummification in later dynasties can be supported, as of the beeswax-positive samples, 9 were categorized into the late period and one into the 3rd intermediate period.

Another compound class with a high frequency of occurrence within the analyzed sample cohort is the class of terpenoids. These are a major component of resins, produced by a variety of plants. In an archaeological context monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids were previously detected. This subgroup specification is based on the number of isoprene units present in a chemical structure, with 2 (10 carbon atoms), 3 (15 carbon atoms), 4 (20 carbon atoms) or 6 isoprene units (30 carbon atoms), respectively. A variety of mono- and sesquiterpenoids, were identified in the current sample set, which included borneol (n = 9), camphor (n = 9), carene (n = 2), cymene (n = 27), limonene (n = 1) and thymol (n = 6) as well as calacorene (n = 1), cuparene (n = 18), germacrene D (n = 5), longiborneol (n = 1), thujopsene (n = 2), valencene (n = 10), himachalene- (n = 33) and longifolene derivatives (n = 18). Most of these were also detected by previous organic residue analyses of archaeological samples, but due to their highly variable occurrence in many different plant extracts or essential oils, they cannot be regarded as unique markers for specific botanical species.[9,12] Additionally, terpenoids are highly volatile compounds, a property which leads to a great degree of ageing and chemical alterations within the archaeological timeframe. However, attempts have still been made to trace back identified sesquiterpenoids to their native origin. Brettell *et al.* for example reported, that cuparene and calamenene are constituents of the resin, wood and wood extracts of *Cupressaceae* and *Pinaceae*.[9] Following this, it would indicate that 18 of the 36 analyzed samples might contain a resin of these two plant families. Also well described in the literature is the occurrence of longiborneol, equivalently referred to as juniperol, as a natural constituent of cedar, juniper and pine resins.[10,34–36] This makes it highly probable that the canopic jar sample, in which longiborneol was detected, indeed contained a coniferous resin that is likely to originate from the embalming process. Another proposed marker for the use of coniferous resins, predominantly pine and/or abies resins, within the ancient embalming fluids, is the occurrence of a specific diterpenoid. Modern coniferous resins were found to be well characterized by the abundant presence of abietadiene acids, particularly abietic acid. Upon analysis of an ancient 2nd century pine resin, however, this native structure was not detected but instead products of the abietadiene degradation.[11] Dehydroabietic acid, formed during the oxidative dehydrogenation of abietic acid, was identified in 7 samples (3 canopic jar and 4 mummy samples) of the current sample set. Together with 7-oxo-dehydroabietic acid, a product of the atmospheric oxidation of dehydroabietic acid, and 1-methyl-10,18-bisnorabieta-8,11,13-triene as a thermal degradation product of abietic acid detected in 1 canopic jar sample each, this is indicative for the use of a pine and/or abies resin during mummification and embalming in a high number of analyzed samples. In contrast, the use of a sandarac resin seems more unlikely, as no pimaradiene-based compounds were identified in the current sample cohort.[5] According to studies by Buckley and Evershed, the earliest samples of embalming materials that presumably contained coniferous resins date back to the VI dynasty.[5] This is in accordance with the current results, where radiocarbon-dating revealed that the earliest samples that were found to contain an abietadiene acid derivative were native in the late Old Kingdom. Their observation, that the use of coniferous resins became more apparent in later periods (*e.g.* roman period), can neither be supported nor refuted with the current results. No compound quantitation was aimed for, so a statement whether or not the concentration of abietadiene acid derivatives increased over the dynasties cannot be made. Generally however, it seems possible that embalmers over time might have become aware of the ability of coniferous resins to inhibit microbial degradation, which could have led to an increased use within the embalming process.[5] A secondary plant product found in wood smoke and associated with the production of tar oil from cedar wood is guajacol. It was detected in 2 canopic jar and 2 mummy samples, which therefore indicates the use of coniferous wood-tar oil during the embalming of these 4 species. Most likely not known in ancient times, but a poten-

tial reason for preferential use of coniferous wood-tar oil, is the powerful anti-bacterial and anti-fungicidal activity of guajacol that is very effective in preserving alkaline phosphatase activity.[37] Next to mono-, sesqui- and diterpenoids, triterpenoids were previously discussed in the literature in the context of organic residue analysis of ancient embalming fluids. *Pistacia* resins for example can be chemically characterized by the presence of masticadienonic, isomasticadienonic, moronic and oleanonic acids.[5,38] None of these triterpenoids were detected in the current sample set, even after manual search, although their known TMS-derivatives created during the derivatization step theoretically lay within the used scan range of 50 to 650 Da. This suggests non- or very low abundant-presence of triterpenoids, although it cannot be excluded that the analytical setup prevented detection (*e.g.* limited run time). Following this, it seems unlikely that *Pistacia* resins were used in high quantities for embalming of the analyzed finds.

Another compound previously associated with ancient mummification is bitumen. Modernly, the term refers to a specific naturally occurring petroleum product (= asphalt) that has lost its volatile hydrocarbon components *via* biodegradation and/or evaporation. This leaves a black, semi-viscous or solid material. Originally, the black surface color of mummies was thought to originate from bitumen use during the mummification process, however no confirming evidence could be found for this thesis.[39,40] Rather, Clark *et al.* used organic residue analysis to find no detectable bitumen use before the new kingdom, so it is more likely that the black discoloration of mummies originates from degradation and/or burning processes.[41] Commonly accepted biomarkers for bitumen are sterane (*m*/*z* 217) and hopane (*m*/*z* 191) structures. These characteristic compounds were not detected in the current sample set. However, it has to be noted that most studies that are focusing on bitumen detection utilized chloroform or dichloromethane (DCM) for sample extraction in conjunction with selected ion monitoring (SIM) mode for analysis. Chloroform and DCM have a significant greater strength in dissolving organic molecules, while co-extracting a high amount of matrix contaminants, compared to methanol that was used in the current study (*e.g.* soil components). So it is possible that bitumen, even if present in any of the samples, was not extracted from the solid sample and thus not detected upon analysis. A contributing factor might have also been the untargeted full scan approach that was used to cover as many analytes as possible in one run with the potential to identify new constituents of embalming fluids. SIM measurement would have led to a higher sensitivity for pre-defined target compounds (*e.g.* bitumen constituents) as only target *m*/*z* values are filtered out from a complex sample matrix, however, no simultaneous untargeted analysis would have been possible. The decision for a broad analysis mode had the potential disadvantage that the low abundant bitumen biomarkers were not detected in the current mummy samples. Alternatively, Charrié-Duhaut *et al.*, the only group that previously performed organic residue analysis on canopic jar samples, did not detect bitumen in their vessels.[10] This could also suggest that bitumen was not frequently used in the process of creating ancient canopic jars.

The data evaluation so far was mainly based on the literature that is available for organic residue analysis of mummification embalming fluids. However, the current data set includes a variety of canopic jar samples and the findings by Charrié-Duhaut *et al.* highlight that a differentiation between the treatment of mummies and viscera stored in canopic jars might be necessary for the correct interpretation of certain detected compounds.[10] Extensive chemical analysis seems particularly crucial in order to verify proposed uses and constituents of canopic jars. It is very interesting that on the one hand fatty acids and terpenoids seem to occur uniformly in both sample classes. On the other hand, beeswax was only detected in 13% of the mummy samples but in 32% of the canopic jar samples, indicating a more widespread use for the embalming of ancient viscera.

A compound not previously discussed in the context of embalming fluid analysis that was detected in a canopic jar sample of the current study was mequinol. Also referred to as 4-methoxyphenol, it is an active ingredient of aniseed. It's cultivation in ancient Egypt is well documented and at that time aniseed had a significant medical status used to treat alleviating pains and stomach disturbances.[42,43] With this historic information, the detection of mequinol along with further aniseed associated compounds (*e.g.* creosol and 4-methoxyacetophenone), is not surprising and is likely to indicate aniseed use within the embalming fluids. Another substance used for medical purposes since ancient times is salicylic acid. The naturally occurring ancestor of modern aspirin can for example be found in high amounts in the bark of the willow tree and was already utilized for pain relief back then.[44] Salicylic acid also acts as a plant hormone found in small amounts in various species. The detection of salicylic acid in the analyzed sample set might indicate the use of willow bark during the preparation of embalming fluids, which seems plausible; other sources, however, cannot be excluded. Ten samples were also found to contain chamazulene derivatives; a substance that is associated with the occurrence in plants of the *Asteraceae* family. Chamazulene is highly linked to the anti-inflammatory action of chamomile, which has already been well known as an herbal remedy to treat erythema and xerosis in Ancient Egypt.[45,46] Following this, it indicates the use of another medical-related substance within the embalming fluids (*e.g.* to ensure healthy organs for the eternal life). Although, as expected, no human metabolites of the aforementioned substances were detected, it has to be noted that their occurrence might also coincide with the medical treatment immediately prior to death, so that medical related substances might have been transferred into the canopic jars by antemortem accumulation within the viscera. Another possibility might be the use of *e.g.* chamomile poultices to wrap the organs as part of the embalming process. A pyrrolizidine alkaloid only found in the leaves of the plant species *Senecio Jacobaea*, better known as ragwort, is jacobine.[47,48] The chemical compound was unexpectedly detected in a canopic jar

sample. The ancient occurrence of ragwort is not documented in the literature, but its modern native environment lets it appear possible. *S. Jacobaea* grows on sand dune communities as well as woodland and grassland communities, which are conditions that were common in the ancient Egyptian area.[49] Thus, a usage of ragwort in the preparation of embalming fluids seems possible, although a modern contamination cannot be excluded. The fact that it can only be speculated about the use of certain compounds, stresses that little reliable information is present on the exact process of carrying out the ancient embalming and mummification. Historical sources state that during the mummification process, hot melted resin was likely to be poured into the body's chest and abdominal cavities.[11] Colombini *et al.* proposed that such a thermal treatment could be assumed if oxidized and dehydrogenated products of native substances used during the embalming can be detected with organic residue analysis.[11] Although particular oxidized species can be produced upon natural degradation, the occurrence of other specific substance classes might still indicate thermal treatment of the bodies. Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that are predominantly generated during the incomplete combustion of organic material (*e.g.* coal, oil, petrol and wood).[50] During the analysis of the current sample set various PAHs were detected such as thioxanthene, pyrene, 3,7-dimethyldibenzothiophene, 2-naphthalenol, 1-methylnaphthalene, fluoranthene, 9-methylene-fluorene and 3,9-dimethylbenz[*a*]anthracene. On the one hand it is possible that these were produced as pyrogenic PAHs during the embalming process or while preparing ingredients for the embalming fluids. Possible examples would be the use of coal tar pitch or wood burning to extract active ingredients. The latter hypothesis is supported by the detection of guajacol, as detailed above. Additionally, levoglucosenone and levoglucosan, compounds formed from pyrolysis of carbohydrates such as starch and cellulose, were identified in 6 samples, which again indicate thermal degradation.[51] On the other hand it cannot be excluded that the detected PAHs, which are known to ubiquitously occur in the environment, were environmental contaminants. PAHs can be found in the gas phase of the ambient air and biological PAHs can be synthesized by certain plants and bacteria or formed during the degradation of vegetative matter, independently of a pyrolysis or combustion source.[50]

With the unique metabolomics-like approach that was used during the current study, untargeted full scan data was obtained as detailed above. In contrast to classical SIM experiments, this open screening analysis allows for unknown identification and extensive statistical evaluation. Including all features for statistical analysis, positive hits and non-identified peaks, has the potential advantage that classification into sample groups is not limited to chemical components modernly known (and included in the NIST 14 spectral database), but also takes into account potentially unknown substances anciently used for embalming, which might not be included in a modern database. Whether or not the chemical composition of mummy and canopic jar samples differed significantly was not yet investigated by another study. In general, a differentiation would not be surprising as it is plausible that mummies were more heavily embalmed and preserved compared to the viscera that were stored within the canopic jars. Indeed, PCA revealed a trend for class separation as shown in Fig. 1. While a canopic jar usually contained a single organ or, depending on the jar size, only a fraction of it (liver, lung, stomach and intestine respectively), a much greater body mass and -surface was embalmed when preparing a mummy of a full body.[4,52] This discrepancy in body mass and -surface could have also lead to differences in decomposition and degradation, resulting in a varied organic residue profile of the two sample classes. In order to determine if class separation based on their chemical composition was significant, a PLS-DA plot was utilized (Fig. 2). Leave-one-out cross validation (LOOCV) and permutation testing was performed to determine whether or not the clearly observed separation of the mummy and canopic jar group was significant. Q_2 as an estimate of the predictive ability of the PLS-DA model was 0.63 for two components, which is accepted as a marker for good discrimination power between classes.[26] Testing the hypothesis of no effect with 2000 permutations gave an empirical *p*-value of 0.58, thus statistically not significant. However, it has to be noted that a relative large sample size is required in order to reliably estimate the empirical *p*-value. For the current sample set only 6 mummy samples were available for chemical analysis, but power analysis showed that more than 1000 samples per group should be analyzed for high statistical power and a

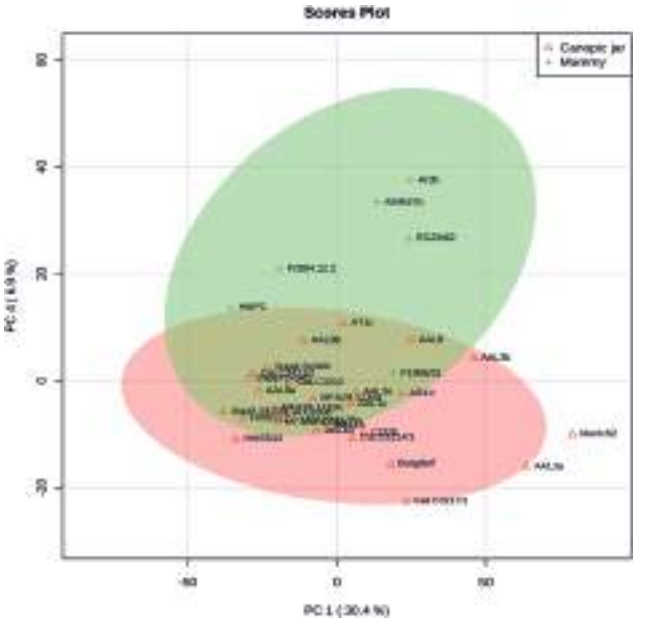

Fig. 1 Score plot of the principle component analysis (PCA) between 6 mummy (green plus) and 28 canopic jar samples (red triangle) based on the raw peak intensity data obtained during high resolution gas chromatography-mass spectrometry organic residue analysis; features were filtered based on the relative standard deviation with subsequent cube root data transformation and auto scaling to achieve a near-normal distribution; principal components 1 and 4 were visualized within the plot.

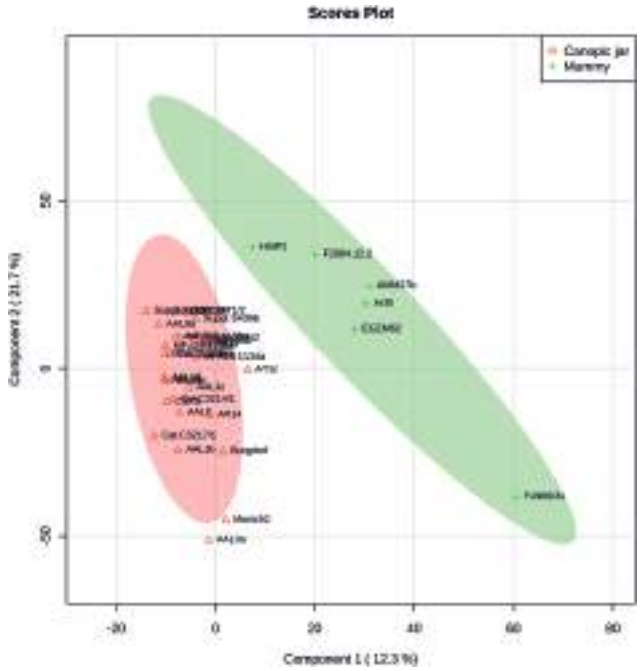

Fig. 2 Score plot of the partial least square-discriminant analysis between 6 mummy (green plus) and 28 canopic jar samples (red triangle) based on the raw peak intensity data obtained during high resolution gas chromatography-mass spectrometry organic residue analysis; features were filtered based on the relative standard deviation with subsequent cube root data transformation and auto scaling to achieve a near-normal distribution; components 1 and 2 were visualized within the plot.

reliable *p*-value estimate (based on a specified false discovery rate of 0.1). Following this, the non-significance of the obtained *p*-value can be regarded as negligible when assessing the used model. As the Q_2 value clearly indicates good class separation without overfitting of the PLS-DA model, it can be concluded that the observed differentiation between mummy and canopic jar samples was based on true differences in their chemical composition. This emphasizes that canopic jars are a unique set of samples in comparison to the well-studied mummy samples. It also stresses that care has to be taken, when interpreting canopic jar-composition based on comparison of previously obtained mummy sample-compositions, so more extensive canopic jar organic residue analyses should be carried out in the future.

Limitations of the study

As with most archaeological exhibits, their handling over the millennia is hard to trace back and modern contamination can never be ruled out in the course of chemical analysis, particularly for non-sealed jars even using most sensitive analytical instrumentation. A number of endogenous compounds were detected in the course of the analysis. These included for example urea, 1-methylhistamine, glycerol, nicotinic acid, gamma-butyrolactam, methylmalonic acid, creatinine, alanine and thymine. As contamination with endogenous compounds and/or modern contaminants cannot be excluded during sample collection and preparation, these compounds were excluded for the evaluation and data interpretation. The sample aliquots available for analysis likely originated from the outer core of the ancient samples, presumably the embalming layer, however this cannot be taken for granted. The analytical decision for an untargeted metabolomics-like approach, optimizing the scan range and run time for the detection of small molecules and applying a generic extraction solvent, meant potential non-detection of big, long-chain and/or strongly lipophilic compounds. However, the accordance of the current results with the available literature, the detection of new chemical components associated with the embalming process and the ability to perform multivariate analyses for cluster and classification determination, highly justifies the applied approach. And finally, sample number was low (*e.g.* 6 mummy samples), however, more samples had not been available for this study and results still are convincing.

Conclusion

The metabolomics-like approach that was used for the chemical characterization and differentiation of archaeological samples for the first time successfully identified several compounds/compound classes that have previously been associated with embalming fluids in Ancient Egypt (*e.g.* specific fatty acid patterns, beeswax, terpenoids and constituents of coniferous resin). Additionally, it allowed for the detection and identification of aniseed ingredients like mequinol, salicylic acid as a marker for the use of willow bark and jacobine, a specific component of ragwort (*S. jacobaea*); substances that have not been discussed in the literature so far as constituents of canopic jar and/or mummy samples. Besides these conventional analytical results, the employed metabolomics-like strategy of analysis made extensive multivariate statistical evaluation possible for the first time. Comparison between mummy and canopic jar samples revealed that their overall chemical composition is significantly different, highlighting the necessity for more extensive canopic jar content data in the future in order to interpret their chemical constitution correctly.

Compliance with ethical standards

N/A.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors express their gratitude to Emma Louise Kessler, MD for her generous legacy that she donated to the Institute

of Forensic Medicine at the University of Zurich, Switzerland for research purposes (L. B. and T. K.). The authors would like to thank the scientists from the Department of Forensic Pharmacology and Toxicology, ZIFM, for helpful discussions. This work was financially supported by the Swiss National Science Foundation (grant no. 162803 – "The Canopic Jar Project"; awarded to F. J. R.), the Mäxi Foundation (Zurich, Switzerland; awarded to F. J. R.), the Athenaeum Foundation (Heidelberg, Germany; awarded to F. J. R. and M. H.) and the Cogito Foundation (Wollerau, Switzerland; awarded to F. J. R. and M. H.).

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