Studies of organic residues from ancient Egyptian mummies using high temperature-gas chromatography-mass spectrometry and sequential thermal desorption-gas chromatography-mass spectrometry and pyrolysis-gas chromatography-mass spectrometry

The Analyst

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The techniques of gas chromatography-mass spectrometry (GC-MS) and sequential thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) and pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) have been utilised to characterise the constituents of tissue-derived or applied organic material from two Pharaonic Egyptian mummies with a view to identifying embalming practices/substances. The results obtained using TD-GC-MS revealed a series of monocarboxylic acids with the $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ components dominating in both mummies. The thermal desorption products related to cholesterol, *i.e.*, cholesta-3,5,7-triene and cholesta-3,5-diene (only in Khnum Nakht), were detected in both mummies. Khnum Nakht also contained a number of straight chain alkyl amides (C_{16}–C_{18}) and an alkyl nitrile (C_{18}). Other products included the 2,5-diketopiperazine derivative (DKP) of proline–glycine (pro–gly) which was a major component (7.9%) in Khnum Nakht but only a very minor component in Horemkenesi. Py-GC-MS of samples of both specimens yielded a series of alkene/alkane doublets (Horemkenesi C_6–C_{18}, Khnum Nakht C_6-C_{24}) which dominated their chromatograms. Series of methyl ketones in the C_9–C_{19} chain length range were also present, with C_5–C_7 cyclic ketones occurring in Horemkenesi only. These ketones are indicative of covalent bond cleavage, probably of polymerised acyl lipids. Nitrogenous products included nitriles (C_9–C_{18}) which were significant in both samples, and amides which were only detected in Khnum Nakht. Also present amongst the pyrolysis products were three steroidal hydrocarbons, cholest-(?)-ene, cholesta-3,5,7-triene and cholesta-3,5-diene. High temperature-GC-MS of trimethylsilylated lipid extracts yielded similar monocarboxylic acids to that obtained using TD-GC-MS, while a series of α,ω-dicarboxylic acids and a number of mono- and di-hydroxy carboxylic acids not seen in the thermal desorption or pyrolysis GC-MS analyses were significant constituents in both mummy samples. Overall, the use of GC-MS and sequential TD-GC-MS and Py-GC-MS has demonstrated in both mummies the presence of a complex suite of lipids and proteinaceous components whose compositions indicates extensive alteration *via* oxidative and hydrolytic processes during long-term interment. None of the classical embalming resins was detected but an exogenous origin for at least a proportion of these components cannot be discounted since fats, oils and gelatin have been proposed as embalming agents in mummification. The combined approach of sequential TD- and Py-GC-MS has potential for application to the characterisation of embalming materials in mummies. Most importantly these techniques virtually eliminate any destruction of the mummified bodies thereby allowing the scope of investigations of ancient Egyptian funerary practices to be significantly extended.

Introduction

People have long been fascinated by the ancient Egyptian 'art' of mummification, a practice carried out in Egypt from *ca.* 2600 BC.[1] Despite this interest however, surprisingly little is known about the practice, particularly the use of organic preservatives or 'embalming resins'. Though the ancient Egyptians left no written record of embalming technology, direct evidence can be derived from the types of preservatives that have survived in association with the bodies. Proposed organic preservatives include bitumen, beeswax, true resins [*e.g.*, coniferous (diterpenoid) and non-coniferous (Pistacia)], and gum resins (*e.g.*, myrrh and frankincense).[2]

The few chemical studies performed on Egyptian mummies have used a variety of analytical techniques to determine the nature and origin of 'embalming resins' based on the presence of specific biological marker compounds. Techniques employed to date include gas chromatography-mass spectrometry (GC-MS),[3–6] pyrolysis-mass spectrometry (Py-MS),[7] high performance liquid chromatography (HPLC)[8] and fast atom bombardment tandem mass spectrometry.[9] However, many questions still remain unanswered and a great deal more research is needed before we can claim to have a reasonable understanding of the technology of embalming during the 3000 years in which mummification was practised in Egypt.

When deciding on an appropriate analytical approach it is necessary to consider both the valuable nature of the specimens from which the samples are to be taken, and the specific nature of those samples (*i.e.*, aged organic materials of uncertain origin). Due to the irreplaceable nature of the samples (*i.e.*, mummies) the ability to accommodate very small sample sizes is an important consideration, particularly since a large number of mummies need to be studied to provide a comprehensive and meaningful picture of the embalming materials employed. In addition, the approach adopted must recognise the nature of the samples analysed, taking account of the fact that the compositions of the organic materials utilised in the mummification process are likely to have changed substantially over time as a

result of natural degradative processes. These processes will include oxidation, reduction, hydrolysis, aromatisation and polymerisation. However, organic components which are resistant to chemical and biological degradation, and are characteristic of the original 'embalming resins', can be expected to survive for very long periods of time and may be recognisable in a relatively unchanged state. These specific compounds represent the biomarker components that will be used to identify the ancient 'resins'. The possibility of encountering both free and polymerised biomarker compounds must be considered when deciding upon the analytical approach.

Solvent extraction followed by GC-MS is often utilised as an appropriate approach for the characterisation and identification of a wide variety of organic components of biological tissues. This approach can provide a great deal of valuable information about the nature of aged organic residues.[3–6,10,11] After suitable derivatisation even polar polyfunctional compounds, commonly present, are amenable to GC-MS.[4,11–14] Sample preparation is however relatively time consuming and sample losses, especially of volatile components which may be trapped within the sample matrix,[15] can be problematical.

The technique of thermal desorption coupled with gas chromatography-mass spectrometry has proven to be a rapid and direct method for the identification of free biomarkers in a broad range of organic materials.[16–21] Thermal desorption is time efficient since the extraction step is effectively instantaneous. Furthermore the technique involves minimal sample manipulation which reduces the problems of contamination, sample loss and other experimental errors inherent with 'wet chemical' procedures. The very small sample sizes necessary (< 0.1 mg) allow the virtually non-destructive analysis of precious specimens, such as mummies. Importantly, TD can be conveniently combined sequentially with Py-GC-MS.

Presented herein are the results of a combined sequential TD-GC-MS and Py-GC-MS approach to study both 'free' and 'bound' biomarkers thought to be present in heterogeneous organic matter from two ancient Egyptian mummies, at least one of which had been described as embalming resin.[22] The samples were also analysed by solvent extraction then GC-MS following separation of the acidic and neutral components. The TD and solvent extraction approaches were assessed and their advantages and disadvantages discussed.

Experimental

Samples description and preparation

Samples from two Egyptian mummies were investigated. Resin-like material was taken from the left hip/spine region of Horemkenesi (*ca*. 1000 BC), Ha 7386/948 (Bristol Museum), and bandage/resin/tissue from Khnum Nakht (*ca*. 2000 BC) (Manchester Museum). Samples were ground (under liquid nitrogen) using a mortar and pestle prior to chemical analysis.

Thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) and pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS)

A CDS 1000 Pyroprobe (Chemical Data System, Oxford, PA, USA) unit fitted with a platinum coil probe was used for the thermal extraction of free biomarker compounds and the pyrolysis of bound/polymerised components from the ground samples (< 0.1 mg). The samples were loaded into quartz tubes plugged with solvent extracted glass wool. The quartz tubes were then inserted into the platinum coil of the probe which was inserted into the heated injector of a GC interfaced to the mass spectrometer. The quartz tubes were pre-cleaned by heating them in a furnace at 600 °C for 24 h. They were also heated to 1000 °C in the platinum coil probe prior to use. The TD/Py temperature was held for 10 s. TD-GC-MS and Py-GC-MS were carried out on a Carlo Erba (Milan, Italy) 4130 gas chromatograph fitted with a fused silica capillary column (50 m $\times$ 0.32 mm id) coated with a dimethyl polysiloxane bonded stationary phase (CP Sil-5 CB, 0.4 μm film thickness; oven temperature programme, 35 °C (5 min) to 320 °C (15 min) at a rate of 4 °C min^{-1}) interfaced to a Finnigan (Sunnyvale, CA, USA) 4500 mass spectrometer operated in full scan mode (40–650 Da, 1 scan sec^{-1}; electron energy, 70 eV; filament current, 350 μA; source temperature, 170 °C). The Pyroprobe interface temperature was 280 °C and the transfer line temperature from the GC to the mass spectrometer was 290 °C. Helium was used as carrier gas. Peaks were identified based on both their mass spectra (NIST/EPA/NIH Mass Spectral Database) and retention times.

TD- and Py-GC-MS

The optimum TD temperature of the Pyroprobe coil, was determined using the following sequential TD-GC-MS analysis of the ground mummy resin-like material. An aliquot of each of the two samples was heated in a stepwise fashion at 290, 310, 330, 350, 360 and 400 °C. Pyrolysis of the 'bound' components of the two thermally desorbed samples was then carried out using a temperature of 610 °C.

Sample preparation for GC-MS

The samples were ultrasonically extracted with chloroform–methanol (2 + 1 v/v; 3 $\times$ 60 min). The extracts were combined and the solvent reduced by rotary evaporation. Following transfer to a screw-capped vial samples were then evaporated under nitrogen at 40 °C. Aliquots of each of the combined extracts [dispersed in dichloromethane–propan-2-ol (DCM/IPA)] were separated into acid and neutral fractions by solid phase extraction (SPE) using a Bond Elut NH_2 aminopropyl column (500 mg per 2.8 ml) (Phenomenex Ltd, Macclesfield, Cheshire, UK). The neutral components were eluted with dichloromethane–propan-2-ol (2 + 1 v/v; 20 ml), prior to the elution of the acids with 5% acetic acid in ether (20 ml). The majority of the solvent for each fraction was then removed (rotary evaporator), and evaporated under nitrogen at 40 °C. Both fractions were trimethylsilylated at 70 °C for 1h with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% of trimethylchlorosilane (Sigma Chemical Co., St Louis, MO, USA) and analysed by GC and GC-MS.

High temperature (HT)-GC-MS

GC-MS was carried out on a Carlo Erba (Milan, Italy) 5160 gas chromatograph fitted with a fused silica capillary column (15 m $\times$ 0.32 mm id) coated with a dimethylpolysiloxane bonded stationary phase (DB1, 0.1 μm film thickness; oven temperature programme, 50 °C (2 min) to 350 °C (10 min) at a rate of 10 °C min^{-1}) interfaced to a Finnigan (Sunnyvale, CA, USA) 4500 mass spectrometer operated in full scan mode (40–650 Da, 1 scan s^{-1}; electron energy, 70 eV; filament current, 350 μA; source temperature 170 °C). Helium was used as carrier gas. Peaks were identified based on both their mass spectra (NIST/EPA/NIH Mass Spectral Database) and retention times.

Results

Analytical approach

The nature of the proposed embalming materials suggests that both free and polymerised compounds are likely to be present. Sequential TD-GC-MS and Py-GC-MS offers a particularly appropriate and convenient approach to the study of such materials for the following reasons: (1) small sample sizes are required (< 0.1 mg) thereby making it a virtually non-destructive means of studying historically valuable museum specimens such as mummies, (2) it is a rapid technique requiring minimal sample preparation, thus allowing high sample throughput, and (3) it minimises problems of contamination and sample losses by reducing sample handling and the use of 'wet' chemical treatments. The utility of this approach is demonstrated below through the analysis of two Pharaonic Egyptian mummies. The results of thermal desorption and pyrolysis will be presented first, followed by the results obtained from more conventional analyses using solvent extraction, fractionation and GC-MS. A discussion will then be given of the relative merits of the two approaches.

Optimum thermal desorption temperature for TD-GC-MS

In order to determine the most suitable thermal desorption temperature for the 'free' biomarkers (as opposed to pyrolysis which involves the cleavage of covalent bonds) the samples were heated, each one sequentially, at increasingly higher probe temperatures. The components released from each thermal desorption step were monitored. At 290 °C (see Fig. 1) a range of compounds were observed, including the pyrolysis product proline–glycine (DKP). At 310, 330, 350 and 360 °C there was no significant release of volatile components (see Fig. 1). At 400 °C GC small peaks were seen in both samples at short retention time corresponding to the generation of carbon dioxide, however, no other volatile species were observed. A probe temperature of 610 °C produced a complex series of pyrolysis products from both specimens, although these were far less abundant in Horemkenesi than in Khnum Nakht. These latter pyrolysis products are presumed to result from covalent bond cleavage in the 'bound' material, *i.e.*, polymeric and/or functionalised components. Thus, 290 °C was chosen for TD since it was as equally effective as somewhat higher temperatures, *i.e.*, up to 400 °C, whilst having the additional advantage of limiting the extent of molecular rearrangements, *e.g.*, dehydration, dehydrogenation, transmethylation, *etc*., which could take place at more elevated TD temperatures. The temperature of 610 °C was chosen for pyrolysis studies aimed at characterising polymerised components.

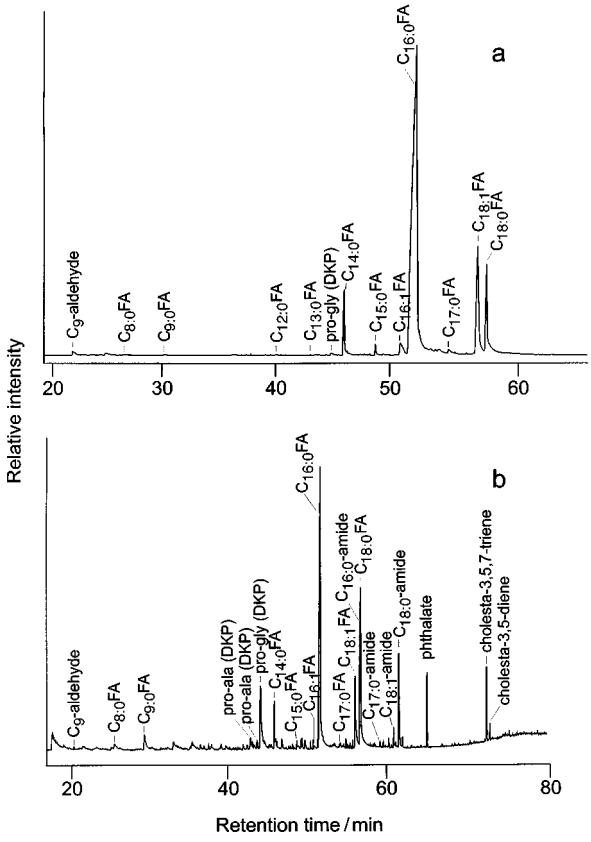

Fig. 1 Reconstructed total ion chromatograms of the thermal desorption profiles (290 °C for 10 s) of (a) Horemkenesi, resin-like materia and (b) Khnum Nakht, bandage/resin/tissue. Pro–gly (DKP) = 2,5-diketopiperazine of proline and glycine; pro–ala (DKP) = 2,5-diketopiperazine of proline and alanine. Cholesta-3,5,7-triene in Horemkenesi is not shown, only being present as a trace component.

Thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS)

The results of the TD-GC-MS analysis of resin-like material taken from the left hip/spine of Horemkenesi are shown in Fig. 1a. A range of compounds were seen which were identified by their mass spectra (see Table 1). These included a series of free fatty acids (C_6 to C_{18}). The major fatty acid was $C_{16:0}$ ($M^{+\cdot}$ 256), with $C_{18:0}$ ($M^{+\cdot}$ 284), $C_{18:1}$ ($M^{+\cdot}$ 282) and $C_{14:0}$ ($M^{+\cdot}$ 228) also present in significant amounts. These four fatty acids were the dominant components in the chromatogram. Eluting significantly later than the fatty acids, the steroidal compound cholesta-3,5,7-triene ($M^{+\cdot}$ 366) was detected albeit as a very minor component. In addition, the aldehyde *n*-nonanal ($[M-18]^{+\cdot}$ 124) was also found to be present. No other significant components were observed. At a thermal desorption temperature of 290 °C none of the characteristic alkene/alkane doublets was seen which were detected in pyrolysates of acyl lipids at higher temperatures. This indicates that the free fatty acids are present in, and desorbed from, the matrix during the thermal evaporation stage and probably do not arise as a result of covalent bond cleavage. The presence of *n*-nonanal is presumed to arise through the oxidation of the double bond in oleic acid which is present at high abundance in human tissues as well as many other animal and plant sources.[23,24]

The results of the TD-GC-MS analysis of bandage/resin/tissue from Khnum Nakht are shown in Fig. 1b. The compounds observed were again identified by their mass spectra (see Table 1). The fatty acid profile was similar to that of the Horemkenesi sample (carbon number range C_7 to C_{18}), with the major components being $C_{16:0}$, $C_{18:1}$, $C_{18:0}$ and $C_{14:0}$ in decreasing order of abundance. However, in contrast to Horemkenesi, two steroidal compounds were observed as major components in Khnum Nakht, eluting at later retention times (72.4 and 72.9 min). The earliest eluting of these was characterised by the presence of *m/z* 141, 143, 247, 253 and $M^{+\cdot}$ 366 identifying it as cholesta-3,5,7-diene (see Fig. 2a). The later eluting peak was characterised by the presence of *m/z* 145, 147, 247, 255, 260 and $M^{+\cdot}$ 368, identified as cholesta-3,5-diene (see Fig. 2b). Also present in significant amounts (again unlike Horemkenesi) were a number of compounds of proteinaceous origin. The 2,5-diketopiperazine derivative, pro–gly (ions *m/z* 70, 83, 111, 154) was identified as a major component eluting just prior to the $C_{14:0}$ fatty acid. Two 2,5-diketopiperazine derivatives of proline–alanine (pro–ala) (ions *m/z* 70, 97, 125, 168) were also identified, as were other diketopiperazine derivatives at lower abundances. Other compounds noted were octadecanenitrile ($[M-15]^{+\cdot}$ 250) characterised by ions of *m/z* 110 and 124, and $C_{16:0}$ ($M^{+\cdot}$ 255), $C_{17:0}$ ($M^{+\cdot}$ 269), $C_{18:1}$ ($M^{+\cdot}$ 281) and $C_{18:0}$ ($M^{+\cdot}$ 283) amides characterised by ions of *m/z* 59 and 72. *n*-

Nonanal was again present as a minor component. As before, typical lipid pyrolysis products (*e.g.*, alkenes, alkanes, ketones, *etc.*) were absent using a TD temperature of 290 °C. Interestingly, however, products normally associated with the pyrolysis of proteins[25–27] were seen, suggesting that these may be formed at relatively low temperatures during the TD at 290 °C. Their absence from solvent extracts suggests that they are probably not present in their free form in the ancient tissues.

Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS)

The pyrogram obtained for Horemkenesi (Fig. 3a) is much more complex and of very different character compared with the thermal desorption profile. The pyrolysis products identified by their mass spectra are listed in Table 2. Dominating the chromatogram were a series of alkene/alkane doublets (*m/z* 55/57) (C_6 to C_{18} maximising at C_6), characteristic of covalent bond cleavage of the bound polymeric constituents of the mummy tissue induced by the higher pyrolysis temperature. Methyl ketones (C_9 to C_{11} , C_{15} to C_{17} , $C_{19:0}$ and $C_{19:1}$; see Table 2), characterised by mass chromatograms *m/z* 58 and 71, were also present in significant abundance. More unusually the 6-methyl-3,5-dien-2-ones (*m/z* 81, 109 and 124; C_{17} and C_{18}; see Table 2) were observed. In addition, saturated (*m/z* 110 and 124) and unsaturated (*m/z* 108 and 122) nitriles [$C_{9:0}$ and $C_{10:0}$, $C_{16:0}$, $C_{16:1}$, $C_{16:2}$, $C_{18:0}$, $C_{18:1}$ (two isomers)] were present in appreciable amounts. The aromatic components benzene, toluene, styrene, ethyl benzene and *o*-xylene, the cyclic ketones cyclopentanone, cyclohexanone and cycloheptanone/methylcyclohexanone, and the alicyclic hydrocarbons dimethylcyclopentane and methylcyclopenta-1,3-diene (two isomers) were also observed (see Table 2). Although Py-GC-MS provided some interesting and valuable data the pyrolysate represented a minor proportion of the whole sample (*i.e.*, *c.f.* TD) which may suggest that it was less extensively polymerised compared with Khnum Nakht (see below).

The Py-GC-MS results obtained for Khnum Nakht are shown in Fig. 3b. The pyrogram is dominated by a wide range of compounds, identified by their mass spectra (see Table 2). Alkene/alkane doublets (C_6 to C_{24}) dominated the chromatogram, although the profile is quite different, with the *n*-C_{15} homologue the most abundant. Methyl ketones (C_{10}, C_{11} and C_{16} to C_{19}) and, more unusually, ethyl (3-) ketones (*m/z* 57 and 72; C_{18} and C_{19}) were identified. In addition, saturated ($C_{14:0}$ to $C_{18:0}$) and unsaturated ($C_{18:1}$) nitriles were present as major components. The $C_{16:0}$ and $C_{18:0}$ alkyl amides were present as minor components (these were absent from Horemkenesi). The profile of aromatic and alicyclic components was similar to that obtained from Horemkenesi. Cholest-(?)-ene ($M^{+\cdot}$ 370), cholesta-3,5,7-triene and cholesta-3,5-diene were also observed, as was a pyrrole. In contrast to the sample from Horemkenesi, the pyrolysate constitutes a significant portion of the sample (*i.e.*, *cf.* TD), indicating the presence of abundant bound biomarkers and pointing to a greater extent of polymerisation.

Table 1 Composition of thermal extracts (290 °C) from thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). Values in bold indicate base peaks. Masses underlined indicate molecular ions ($M^{+\cdot}$). Other masses are characteristic fragment ions

Compound name	Mass spectral data *m/z*	Relative abundance (%)[a] Horemkenesi, resin-like material	Relative abundance (%)[a] Khnum Nakht, bandage/resin/tissue
Carboxylic acids—	Saturated Unsaturated		
Monocarboxylic acids	43,57,73,129 41,55,69,123		
Octanoic acid[b,d]	—	0.2	1.1
Nonanoic acid[b,d]	<u>158</u>	0.2	3.0
Dodecanoic acid[d]	<u>200</u>	0.1	—
Tetradecanoic acid[d]	<u>228</u>	5.0	2.8
Pentadecanoic acid[d]	<u>242</u>	0.6	0.4
Hexadecenoic acid[d]	<u>254</u>	1.8	0.4
Hexadecanoic acid[d]	<u>256</u>	71.6	36.8
i+*ai*-Heptadecanoic acid[d]	<u>270</u>	Tr (< 0.1)	0.3
Heptadecanoic acid[d]	<u>270</u>	0.3	0.6
Octadecenoic acid (×2)[d]	<u>282</u>	12.2	8.0
Octadecanoic acid[d]	<u>284</u>	6.6	12.8
Aldehydes—			
Nonanal[b,d]	**57**, 70, 98, 114, 124	0.8	0.2
Amides—			
Hexadecanamide[c]	<u>255</u>, **59**, 72, 86, 128	—	8.5
Heptadecanamide[c]	<u>269</u>, **59**, 72, 86, 128	—	0.3
Octadecenamide[c]	<u>281</u>, **59**, 72, 81, 126	—	1.0
Octadecanamide[c]	<u>283</u>, **59**, 72, 86, 128	—	7.2
Nitriles—			
Octadecanenitrile[c]	**43**, 41, 97, 110, 124	—	0.4
Steroids—			
Cholesta-3,5,7-triene[c]	<u>366</u>, **43**, 135, 141, 143, 247, 351	Tr (< 0.1)	4.6
Cholesta-3,5-diene[c]	<u>368</u>, **43**, 145, 147, 247, 353	—	1.1
2,5-Diketo-piperazines—			
Pro–gly[c]	<u>154</u>, **83**, 70, 98, 111	0.4	7.9
Pro–ala (×2)[c]	<u>168</u>, **70**, 97, 125	—	0.8

[a] Relative abundances of compounds are determined from areas under peaks in TIC profiles. [b] Highly volatile components which would be expected to be reduced in abundance during sampling handling in 'wet' chemical analyses. [c] Components formed thermolytically during TD. [d] Components in their original form.

High temperature-gas chromatography-mass spectrometry (HT-GC-MS)

The results for the acid fraction of Horemkenesi obtained by solvent extraction and SPE (see Experimental) are shown in Fig. 4a. A range of carboxylic acids were detected and identified by their mass spectra (see Table 3). The chromatogram was dominated by a series of monocarboxylic acids, including *n*-alkanoic acids in the C_9 to C_{24} carbon number range (with the exception of C_{11} , C_{21} and C_{23}), the saturated *iso*- and *anteiso*-methyl branched C_{15} and C_{17} and $C_{16:1}$, $C_{18:1}$ and $C_{24:1}$ unsaturated components. The distribution of monocarboxylic acids was similar to that obtained using TD (see Figs. 1 and 4), with $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ as the major components. Also present in significant abundance were a series of α,ω-dicarboxylic acids from C_6 to C_{12}, with the C_8 and C_9 components predominating. In addition, relatively large amounts of monohydroxy and dihydroxy carboxylic acids were present with the *erythro* and *threo* isomers of both 9,10-dihydroxyhexadecanoic acid and 9,10-dihydroxyoctadecanoic acid predominating. These polar hydroxy (mono- and di-) and diacids were not seen using TD presumably since these more highly functionalised compounds are not amenable to TD-GC-MS in their underivatised form. These polar acids are not unimportant since they may well indicate post-mortem transformations of the fatty acids originally present and may give clues relating to the method of preservation and the general environmental conditions to which the body was exposed.[4,28]

The results for the neutral fraction of Horemkenesi obtained after solvent extraction and SPE are shown in Fig. 5a. The components present included methyl esters, monoalkylglyceryl ethers, monoacylglycerols and small amounts of steroidal components (see Table 4). The major neutral compound was the bis-TMS ether $C_{18:1}$ 1-monoacylglycerol, with the 1-monoacylglycerols containing $C_{14:0}$, $C_{16:0}$ and $C_{16:1}$ fatty acyl moieties present as major components. Interestingly, the $C_{18:0}$ 1-monoacylglycerol was only a relatively minor component. Observed in almost equal abundance to the major 1-monoacylglycerols were the C_{16} and C_{18} 1-*O*-monoalkylglyceryl ethers (as their bis-TMS ethers). Also dominant in the neutral fraction were the methyl esters of the carboxylic acids $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and the $C_{18:0}$ 9,10-dihydroxy compound. Cholesterol and cholesta-3,5-dien-7-one were present in low abundance. The neutral fraction of Horemkenesi represents an almost insignificant proportion of the total extractable lipid. However, the relatively high abundance of the 1-monoacylglycerols compared to the relatively small amounts of cholesterol and the cholesta-3,5-dien-7-one agrees well with the results of the TD analyses, confirming the virtual absence of steroidal compounds in the sample.

The GC profile for the acid fraction of Khnum Nakht obtained by solvent extraction and SPE is shown in Fig. 4b. A range of carboxylic acids was present and identified by their mass spectra (see Table 3). The chromatogram was dominated by a series of monocarboxylic acids. This included saturated straight chain acids in the C_{12} to C_{24} carbon number range (with the exception of C_{21}), the saturated *iso*- and *anteiso*-methyl branched acids in the C_{15} to C_{17} range, and unsaturated acids including $C_{16:1}$, $C_{18:1}$, $C_{20:1}$ and $C_{24:1}$. The profile of the monocarboxylic acids, dominated by the $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ components, was comparable with the TD profile obtained for this sample. A series of α,ω-dicarboxylic acids in the C_7 to C_{11} carbon number range were present as relatively minor components together with moderate amounts of monohydroxy and dihydroxy carboxylic acids. Although a number of the polar acids found in Horemkenesi were also detected in the Khnum Nakht acid fraction they were not nearly as abundant, suggesting that the sample from Khnum Nakht is in a far less advanced state of oxidisation.

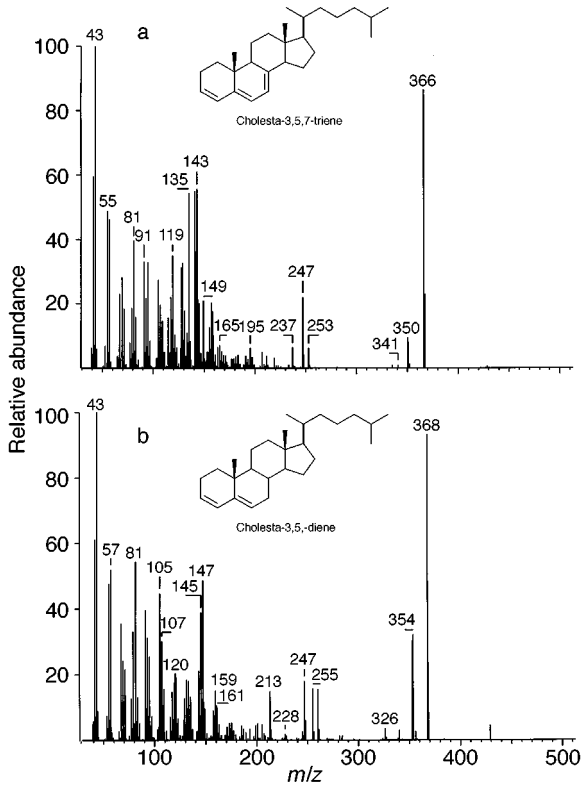

Fig. 2 Mass spectra of the two steroidal compounds detected in Khnum Nakht, bandage/resin/tissue after thermal desorption at 290 °C for 10 s; (a) cholesta-3,5,7-triene and (b) cholesta-3,5-diene.

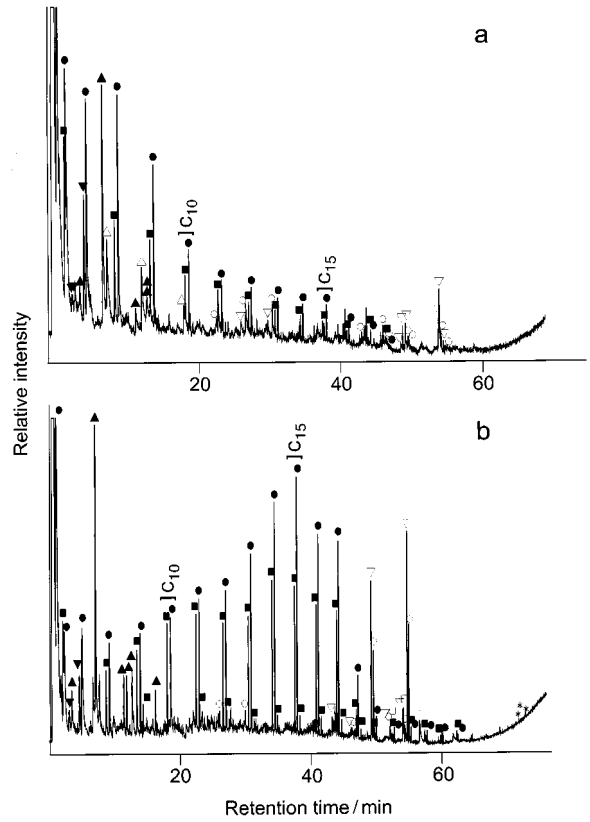

Fig. 3 Reconstructed total ion chromatogram of the pyrolysis profiles (610 °C for 10 s) of (a) Horemkenesi, resin-like material and (b) Khnum Nakht, bandage/resin/tissue, after thermal desorption (290 °C for 10 s). Note: ■ = alkenes; ● = alkanes; ▼ = alicyclic hydrocarbons; ▲ = aromatic hydrocarbons; ○ = 2-alkanones; □ = 3-alkanones; △ = cyclic ketones; ▽ = nitriles; ◇ = amides; ∗ = steroids. C_{10} and C_{15} refer to the carbon numbers of the alkenes and alkanes within the retention time windows.

Table 2 Pyrolysis products (610 °C) from pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS). Values in bold indicate base peaks. Masses underlined indicate molecular ions ($M^{+\cdot}$). Other masses are characteristic fragment ions

Compound name[a]	Mass spectral data *m/z*	Compounds present (✓)	
		Horemkenesi, resin-like material	Khnum Nakht, bandage/resin/tissue
n-*Alkenes*/n-*Alkanes*—			
C_5 (alkane)		✓	✓
C_6	Alkanes: **57**, 71, 85, 99	✓	✓
↓	Alkenes: **55**, 69,83, 97		
C_{18}		✓	✓
C_{19}		—	✓
↓			
C_{24}		—	✓
Alicyclics—			
Dimethylcyclopentane	98, **41**, 56, 70,	✓	✓
Methylcyclopenta-1,3-diene (×2)	**79**	✓	✓
2-Alkanones—			
C_9	**43**, 58, 71	✓	—
C_{10}	**43**, 58, 71	✓	✓
C_{11}	**43**, 58, 71	✓	✓
C_{15}	**43**, 58,71	✓	—
i-C_{16}	**43**, 57, 71	✓	—
n-C_{16}	**58**, 43, 71	—	✓
C_{17}	254, **43**, 58, 71	✓	✓
C_{18}	**43**, 58, 71	—	✓
C_{19}	282, **43**, 58, 71	✓	✓
2-Alkenones—			
C_{19} (×2)	**43**, 55, 58, 71	✓	✓
2-Alka-3,5-dienones—			
6-Methylhexadeca-3,5-dien-2-one	**109**, 43, 81, 124	✓	—
6-Methylheptadeca-3,5-dien-2-one	**109**, 43, 81, 124	✓	—
3-Alkanones—			
C_{18}	**57**, 43, 72, 239	—	✓
Cyclic ketones—			
C_5	84, **55**, 42	✓	—
C_6	98, **55**, 42, 69	✓	—
C_7	112, **55**, 43, 68	✓	—
Aromatics—			
Benzene	**78**	✓	✓
Toluene	**91**, 65	✓	✓
Ethylbenzene	106, **91**, 65	✓	✓
m/*p*-Xylenes	106, **91**, 65	—	✓
o-Xylene	106, **91**, 65	✓	—
Styrene	**104**, 78, 63	✓	✓
Trimethylbenzene	120, **105**, 91, 65	—	✓
Nitriles (saturated)—			
Nonanenitrile	**41**, 43, 97,110, 124	✓	—
Decanenitrile	**55**, 41, 43, 97, 110, 124	✓	—
Tetradecanenitrile	**41**, 43, 97, 110, 124	—	✓
Pentadecanenitrile	**41**, 43, 97, 110, 124	—	✓
Hexadecanenitrile	**43**, 41, 97, 110, 124	✓	✓
Heptadecanenitrile	**43**, 41, 97, 110, 124	—	✓
Octadecanenitrile	**43**, 41, 97, 110, 124	✓	✓
Nitriles (unsaturated)—			
Hexadecenenitrile	**41**, 43, 97, 108, 122, 136	✓	—
Octadecenenitrile (×2)	**41**, 43, 97, 108, 122, 136	✓	✓
Nitriles (diunsaturated)—			
Hexadecadienenitrile	**40**, 44, 105, 120, 134	✓	—
Amides—			
Hexadecanamide	**59**, 72, 86, 128	—	✓
Octadecanamide	283, **59**, 72, 86, 128	—	✓
Steroids—			
Cholest-(?)-ene	370, **355**, 43, 108, 135,147	—	✓
Cholesta-3,5,7-triene (?)	366, **43**, 135, 141, 143	—	✓
Cholesta-3,5-diene	368, **43**, 145, 147, 247, 353	—	✓

[a] None of the components of the pyrograms is detectable in the solvent extracts and thus are presumed to have derived through thermolysis of the largely heteropolymeric resin-like material.

The GC profile for the neutral fraction of Khnum Nakht after solvent extraction and SPE is shown in Fig. 5b. The neutral fraction was (unlike the Horemkenesi neutral fraction) dominated by cholesteryl esters and several related steroidal compounds, with monoglyceryl ethers making a significant, albeit smaller, contribution (see Table 4). The major free steroid identified was cholesta-3,5-dien-7-one, with cholesterol and 3-hydroxycholest-5-en-7-one (7-ketocholesterol) also abundant. At longer retention times (26.5 and 27.4 min) two cholesteryl esters were present, again as major components; these would have been too involatile to successfully elute from the column utilised in the TD-GC-MS, though it is anticipated that they would undergo a 1,2-elimination upon TD at 290 °C to give the fatty acid and cholesta-3,5-diene (with a lesser amount of cholesta-3,5,7-triene, *via* dehydrogenation). Also significant were the C_{16} and C_{18} 1-*O*-monoalkylglyceryl ethers, although in contrast to Horemkenesi, $C_{18:0}$ and $C_{18:1}$ 1-monoacylglycerols were detected only as minor components and other 1-monoacylglycerols were absent, indicating almost complete hydrolysis of the original acyl lipids that would have dominated the tissues at the time of death (if indeed they do originate from the body). Unlike Horemkenesi, the neutral fraction constitutes a much more significant proportion of the total lipid extracts of the sample. The steroids and sterol esters are present in similar abundance to the $C_{15:0}$ and $C_{17:0}$ fatty acids, although the $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ components constituted the bulk of the extractable lipid present in the sample.

Notably, the C_{16} and C_{18} 1-*O*-monoalkylglyceryl ethers were not observed in the analysis of the same sample with TD, thus demonstrating the problems associated with the analysis of polyfunctional compounds using this latter approach.

Discussion

When analysing amorphous organic materials from aged human remains it is important to consider the chemical composition of original tissues in order to be able to distinguish between human body lipids, proteins, *etc*., and components which may originate from any applied embalming materials. Adipose tissue, skin and muscle compose the three major sources of lipids in humans. Although the composition of human adipose tissue can be influenced by dietary input[29–32] it is usually reasonably consistent.[29,33,34] The tissue is largely composed of triacylglycerols (> 90% of the lipids), phospholipids (0.2–4%), glycerol ethers (≈ 1%), non-saponifiable fat (0.2–1.3%), squalene (trace amounts), proteins (2–3%) and water (5–30%). Cholesterol constitutes 0.03–0.3% and cholesterol fatty acyl esters ≈ 0.1%. The fatty acid composition of the adipose tissue is dominated (> 90%) by myristic, palmitic, palmitoleic, stearic, oleic and linoleic acids, typically 2–5, 18–25, 5–9, 3–7, 40–62 and 6–16%, respectively, of total fatty acids (free fatty acids are only minor components in adipose tissue). Human skin lipids differ markedly in composition to those found in adipose tissue.[35–40] They are dominated by wax esters (20–22%), triacylglycerols (29–32%) and free fatty acids (30–33%). Notably, the acid chain length of the wax esters is C_{16}–C_{24} (C_{24} dominant). Squalene is a major component (11–13%), with smaller amounts of free sterols (1–2%) and sterol esters (2–3%). Collagen, an important constituent of skin, is composed of polypeptide chains, the major amino acids being glycine (≈ 33%), alanine (≈ 11%), proline (≈ 13%) and hydroxyproline (≈ 10%).[41] Muscle is composed predominantly of proteins (*e.g*., myosin and actin) consisting of amino acids, of which glutamic acid (≈ 11%), leucine (≈ 10%), cysteine (≈ 10%), lysine (≈ 8%), alanine (≈ 7%), isoleucine (≈ 6%), arginine (≈ 6%), threonine (≈ 6%) and aspartic acid (≈ 5%) are the major components.[42] Of the lipids present in muscle, phospholipids dominate (> 60% of total lipids). The triacylglycerols (> 80%) and cholesterol (> 15%) constitute the majority of the neutral lipids.[10]

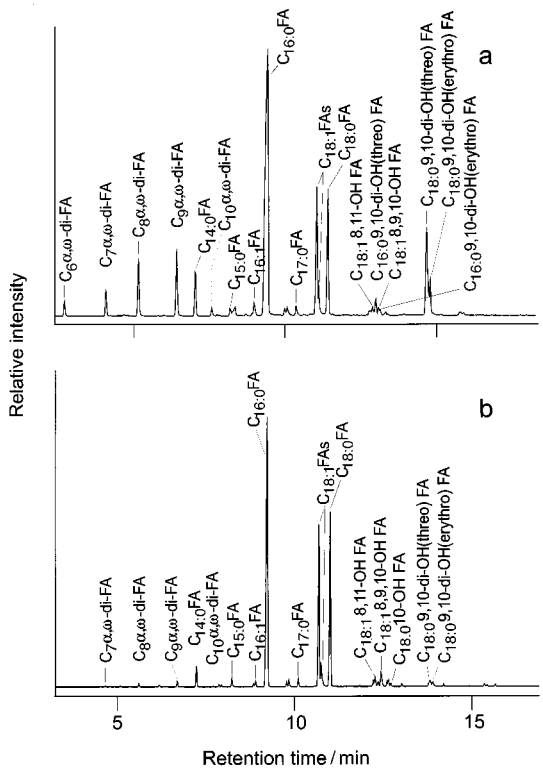

Fig. 4 Reconstructed total ion chromatograms from the GC-MS of the acid fraction (after solvent extraction and fractionation) of (a) Horemkenesi, resin-like material and (b) Khnum Nakht, bandage/resin/tissue. (Acids are present as their TMS derivatives.)

The oxidised lipids found in the total lipid extracts of the mummies studied herein are not usually present in mammalian tissues. However, previous studies carried out on aged human remains which have been naturally mummified have revealed significant amounts of oxidised lipids.[4] The relative proportion of saturated to unsaturated fatty acids (particularly $C_{16:0}$ to $C_{18:1}$) has generally been found to increase over time, partially due to the oxidation of the double bond in the unsaturated acids resulting in short chain α,ω-C_6-C_{12} diacids. These α,ω-diacids were observed by Gülaçar *et al.*[4] and their presence here is not inconsistent with an aged fat from a relatively dry aerobic burial environment. Notably, however, significant amounts of unsaturated fatty acids, including $C_{18:1}$ hydroxy fatty acids, were found in both samples. Gulaçar *et al*. observed an absence of unsaturated monocarboxylic acids and detected only saturated hydroxy fatty acids. The hydroxy and dihydroxy carboxylic acids can derive from varying degrees of oxidation of the original unsaturated $C_{16:1}$, $C_{18:1}$, $C_{18:2}$ fatty acids. However, it should be noted that since these mummies (certainly Horemkenesi) come from periods when embalming was extensively practised this cannot be assumed. Unsaturated and saturated monohydroxy carboxylic acids are found in plant waxes[23] which could have been utilised. They could also originate from the oxidation of plant or animal derived oleic/linoleic acids applied during mummification. The branched chain (*iso* and *anteiso*) $C_{15:0}$ and $C_{17:0}$, fatty acids which are present in significant amounts in bacteria, also occur in living human adipose tissue as minor components (typically 0.3% and 1.0%, respectively[29]). The quantities found in the samples are consistent with this and so do not suggest they are of bacterial origin. The longer chain C_{22} and C_{24} fatty acids are known to occur in skin lipids in significant amounts, hence, their presence in the samples in low abundance could be endogenous, rather than being microbially derived. Although phospholipids are susceptible to degradation and as such would not be expected to survive intact, the presence of the amides and proportion of the

fatty acids seen in the aged tissues of Khnum Nakht may reflect the presence of phospholipids. Since these amides were not seen in the total lipid extracts they are presumed to derive thermolytically. The steroidal compounds in Khnum Nakht indicate an animal fat, possibly human origin.

The results obtained above show how the combined approach of conventional GC-MS and sequential TD-GC-MS and Py-GC-MS has allowed the characterisation of the two mummy samples. They were both identified as being composed of degraded acyl lipids having undergone almost complete hydrolysis, resulting in the dominance of the fatty acids in both samples. However, the techniques employed revealed notable differences in the nature of the samples. The Horemkenesi sample had undergone a significant degree of oxidation and was essentially a mixture of free fatty acids having very little polymerised material present. In contrast, Khnum Nakht although hydrolysed was far less oxidised and contained appreciable amounts of steroidal compounds including cholesterol (effectively absent in Horemkenesi). Khnum Nakht was also highly polymerised in nature as indicated by the characteristic alkene/alkane doublets seen in the Py-GC-MS analyses. The large pro–gly peak present in the TD of Khnum Nakht points to a proteinaceous origin, though its formation at ≈ 300 °C is interesting since it is generally observed as a pyrolysis product at higher temperatures (*e.g.* 610 °C/750 °C[25–27]). Clearly it is being formed in the probe at relatively low temperatures.

It is significant that the samples do not appear to have been treated with any di- or triterpenoid resins traditionally associated with the embalming process in ancient Egypt.[2] Diterpenoids have been identified in a later mummy currently being investigated in our laboratory.[43] The absence of terpenoid resins in Horemkenesi is particularly surprising since he was mummified when the embalming process was at its peak. The sample analysed was considered, and in fact identified, in previous studies[22] to be a material applied extensively to the body as part of the embalming process and not endogenous to the body itself. This may serve to illustrate the need for a fairly comprehensive

Table 3 Composition of acid fraction from gas chromatography-mass spectrometry (GC-MS) following solvent extraction procedures (all compounds determined as their trimethylsilyl derivatives). Values in bold indicate base peaks. Masses underlined indicate molecular ions ($M^{+\cdot}$). Other masses are characteristic fragment ions

		Relative abundance (%)[a]	
Compound name	Mass spectral data *m/z*	Horemkenesi, resin-like material	Khnum Nakht, bandage/resin/tissue
Monocarboxylic acids—	Saturated **73**, 117, 132, 145 Unsaturated **73**, 117, 129, 145		
Dodecanoic acid	257 ($M^{+\cdot} - 15$)	Tr (< 0.1)	0.1
Tridecanoic acid	271	Tr (< 0.1)	0.2
Tetradecanoic acid	285	3.4	2.4
i-Pentadecanoic acid	299	Tr (< 0.1)	0.3
ai-Pentadecanoic acid	299	Tr (< 0.1)	0.2
Pentadecanoic acid	299	0.4	1.0
i-Hexadecanoic acid	313	Tr (< 0.1)	0.4
Hexadecenoic acid	311	1.0	0.6
Hexadecanoic acid	313	43.9	40.5
i-Heptadecanoic acid	327	0.5	0.5
ai-Heptadecanoic acid	327	0.8	0.7
Heptadecanoic acid	327	0.8	0.8
Octadecenoic acid (×2)	339	11.7	22.8
Octadecanoic acid	341	9.3	20.2
Nonadecanoic acid	355	Tr (< 0.1)	0.1
Eicosenoic acid	367	—	0.5
Eicosanoic acid	369	0.2	0.8
Docosanoic acid	397	Tr (< 0.1)	0.2
Tetracosenoic acid	423	Tr (< 0.1)	0.2
Tetracosanoic acid	425	Tr (< 0.1)	0.3
α,ω-Dicarboxylic acids—	**73**, 117, 129, 149		
Hexanedioic acid	159, 275 ($M^{+\cdot}$-15)	1.1	—
Heptanedioic acid	173, 289	2.1	0.1
Octanedioic acid	187, 303	4.4	0.3
Nonanedioic acid	201, 317	5.0	0.6
Decanedioic acid	215, 331	0.6	Tr (< 0.1)
Undecanedioic acid	229, 345	0.2	Tr (< 0.1)
Dodecanedioic acid	243, 359	Tr (< 0.1)	—
Monohydroxy-carboxylic acids—	**73**, 117, 129, 149		
8-Hydroxy- and	241	0.3	1.6
11-Hydroxy-octadecenoic acids	343		
8-Hydroxy-,	241		
9-Hydroxy- and	227	0.2	2.1
10-Hydroxy-octadecenoic acids	329		
10-Hydroxy-octadecanoic acid	215, 331	Tr (< 0.1)	0.3
Dihydroxycarboxylic acids—	**73**, 117, 129, 147		
9,10-Dihydroxyhexadecanoic acid (t)[b]	187, 317	1.3	—
9,10-Dihydroxyhexadecanoic acid (e)[c]	187, 317	0.1	—
9,10-Dihydroxyheptadecanoic acid (t)[b]	201, 317	Tr (< 0.1)	—
9,10-Dihydroxyoctadecanoic acid (t)[b]	215, 317	9.7	1.3
9,10-Dihydroxyoctadecanoic acid (e)[c]	215, 317	2.7	0.6

[a] Relative abundances of compounds determined from areas under peaks in TIC profiles. [b] Threo isomers. [c] Erythro isomers.

study before we can claim to have anything like a thorough understanding of the process of mummification.

It should be noted here that although derivatisation of the polar oxidised fatty acids, *e.g.*, methylation with tetramethylammonium hydroxide (TMAH) may provide a way forward, by increasing their volatility and thus allowing them to elute satisfactorily from the column, there are significant problems with this approach.[44] Hydroxy and dihydroxy, as well as keto acids can undergo undesirable side reactions with the methylating reagent. This leads to the formation of artifact compounds, several nitrogen containing species being produced for each oxidised acid.[44] The elucidation of these species is therefore problematic and would necessitate further work to identify the original oxidised fatty acids from which they derive. Such artifacts could also mask other components present which would otherwise be detected. With this in mind, and with convenience and ease of analysis being a major consideration, such a derivatisation step was omitted.

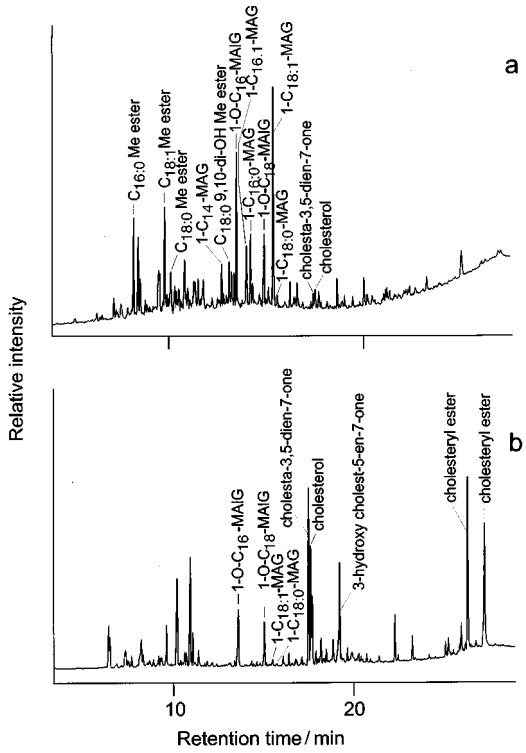

Fig. 5 Reconstructed total ion chromatogram from the GC-MS of the neutral fraction (after solvent extraction and fractionation) of (a) Horemkenesi, resin-like material and (b) Khnum Nakht, bandage/resin/tissue. MAG, monoacylglycerols; MAlG, monoalkylglycerols. (Neutrals are present as the free compounds or their TMS derivatives.)

Conclusions

In summary, the use of TD-GC-MS, Py-GC-MS and GC-MS has demonstrated in both mummies the presence of a complex suite of lipids and proteinaceous components whose compositions indicates extensive alteration *via* oxidative and hydrolytic processes during long-term interment. The findings demonstrate that though highly polar compounds are lost TD is useful as a 'fingerprint' technique for identification of the major classes of lipids in mummies. In addition, it has been shown that sequential TD-GC-MS and Py-GC-MS facilitates the very rapid screening of complex organic materials with sub-milligram sample sizes to reveal 'free' and 'bound' (possibly polymerised) components. However, polyfunctional polar compounds revealed in conventional GC-MS analyses are not seen when employing the TD- or Py-GC-MS approaches since they are either structurally altered or prevented from eluting from the GC column in their underivatised form due to their high polarities. The results obtained show that those compounds containing two carboxyl groups, a carboxyl group and one or more hydroxyl groups, and two hydroxyl groups are insufficiently volatile to be amenable to TD-GC-MS. In particular the series of aliphatic products revealed by Py-GC-MS (following TD-GC-MS) indicates the likely presence of polymerised lipid. Moreover, the use of sequential TD- and Py-GC-

Table 4 Composition of neutral fraction from gas chromatography/mass spectrometry (GC-MS) following solvent extraction procedures (all compounds determined as the free compounds or as their trimethylsilyl derivatives). Values in bold indicate base peaks. Masses underlined indicate molecular ions ($M^{+\cdot}$). Other masses are characteristic fragment ions

		Relative abundance (%)[a]	
Compound name	Mass spectral data *m/z*	Horemkenesi, resin-like material	Khnum Nakht, bandage/resin/tissue
Methyl esters—	**74**, 129, 143; **55**, 69, 123; **73**, 129, 147		—
Methyl hexadecanoate	270	9.2	—
Methyl octadecenoate	296	9.6	—
Methyl octadecanoate	298	5.7	—
Methyl 9,10-dihydroxyoctadecanoate	215, 259	4.0	
			—
1-Monoacylglycerols—	**73**, 103, 129, 147	4.0	—
$C_{14:0}$	211, 285, 343, 431	7.3	—
$C_{16:1}$	237, 311, 369, 382, 457	7.2	0.8
$C_{16:0}$	239, 313, 371, 459	24.7	0.3
$C_{18:1}$	265, 339, 397,410, 485	1.2	
$C_{18:0}$	267, 341, 399		8.2
		14.5	5.2
1-O-Alkylglycerols—	**205**, 73, 103, 117, 133, 147	9.3	
C_{16}	313, 357, 370, 445		20.7
C_{18}	341, 385, 398, 473		13.8
		1.2	9.1
Steroidal compounds—	382, 174, 161, 187, 269, 367	2.0	21.5
Cholesta-3,5-dien-7-one	458, 129, 247, 329, 353, 368, 443	—	20.3
Cholesterol	472, 73, 129, 174, 367, 382, 457	—	
3-Hydroxycholest-5-en-7-one (7-ketocholesterol)	**368**, 145, 147, 247, 260, 353	—	
A cholesteryl ester	**368**, 145, 147, 247, 260, 353		
A cholesteryl ester			

[a] Relative abundances determined from areas under peaks in TIC profiles.

MS: (i) serves to minimise contamination and sample losses; (ii) has the advantage of requiring very small sample sizes, essentially eliminating any destruction of the mummified bodies, and (iii) potentially allows the rapid screening of large numbers of samples. Together these advantages serve to aid the chemical investigation of historically valuable museum specimens of this nature. Most importantly, the use of these techniques virtually eliminates any destruction of the mummified bodies thereby allowing us to extend our studies of ancient Egyptian funerary practices. However, ultimately a combination of conventional GC-MS (of extractable components), TD- and Py-GC-MS approaches is essential in order ensure that the polyfunctionalised compounds are identified since these may provide valuable compositional information which would otherwise be overlooked, particularly important when studying aged organic residues of uncertain origin.

Turning to the embalming agents themselves, none of the classical embalming 'resins' was detected, however, an exogenous origin for at least a proportion of the lipids and proteinaceous components cannot be discounted since fats, oils and gelatin have been proposed as embalming agents in mummification.[45,46] Further investigations in this laboratory of another nine samples from various parts of the body of Horemkenesi have confirmed the presence of only lipid and proteinaceous constituents. In addition, analyses of similarly small samples of resin-like organic matter taken from several mummies of varying age have shown that these techniques have the capacity to reveal the presence of the more classical embalming substances, *e.g*., beeswax and tree resins.[43]

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