ELSEVIER

Journal of Chromatography A, 767 (1997) 177–186

JOURNAL OF CHROMATOGRAPHY A

Identification of phenolic acids and inositols in balms and tissues from an Egyptian mummy

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Received 19 September 1996; revised 5 December 1996; accepted 6 December 1996

Abstract

A number of samples taken from an Egyptian mummy (ca. 100 B.C.) from the Guimet Museum in Lyon have been analyzed by GC–MS. Derivatives of aromatic acids (hydroxyhydrocinnamic, vanillic, protocatechuic and gallic acids) and inositols (non-methylated and mono-*O*-methyl) have been found among the constituents of extracts prepared by methanolysis and trimethylsilylation. From the reported electron impact mass spectra, ion sets where proposed for a sensitive and selective profiling of these selected compounds by mass fragmentometry. The source of gallic acid and inositol was found to be a vegetable tannin, an ingredient which was not previously known to be used for mummification in ancient Egypt. The nature and abundance ratios of the detected inositols also appeared to be a promising criterion to further investigate the botanical source of the tannin employed.

Keywords: Embalming mixtures; Phenolic acids; Inositols

1. Introduction

During an investigation of the nature of embalming mixtures used for mummification in ancient Egypt, samples taken from different locations on a mummy from the Guimet Museum in Lyon (France) were analyzed by gas chromatography–mass spectrometry (GC–MS). The present study follows the thorough examination practised some years ago using classical techniques in forensic medicine (radiography, histology, dental examination) [1] together with chemical analyses (heavy minerals, resins analysis by HPLC) [1,2] and geochemical analyses aimed at the characterization of the bitumen input [3,4], and other techniques in the field of anthropology and archaeology (pollen and mycological analysis, radio carbon dating...) [1]. Selected results reported in this paper focus on characteristic compounds of a vegetable tannin. The distribution of these products at various locations on the body is discussed in relation to their possible vegetable origin and the embalming process itself.

2. Experimental

2.1. Sample description

The analyzed material originated from the anonymous Egyptian mummy No. 90001255 from the Guimet Natural History Museum in Lyon. It is that of a man, about 40 years old, with arms placed straight along the body. Radiocarbon dating has been carried out from pieces of fabric not contaminated by

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PII S0021-9673(96)01067-9

balms. The results determined the absolute age of 2000±100 B.P. [1].

Twenty three samples were taken from the five canopic packages and thirteen different parts of the body. A detailed description of the samples discussed in this paper is given in Table 1. As can be seen, they were made of quite different materials such as balm, bandage soaked in balm, or a brown and fibrous substance probably consisting of desiccated human tissue infiltrated with balm. It may also be noted that two different samples were taken from the same location when possible: the first one at the surface of the embalmed body, the second at an underlying layer, i.e., closest to the body.

2.2. Materials

Materials and reagents were all of analytical grade. The trimethylsilylation reagent, Sylon-HTP, consisting of pyridine–hexamethyldisilazane–trimethylchlorosilane (9:3:1, v/v/v) was purchased from Supelco (Bellefonte, PA, USA).

2.3. Sample preparation

Bitumen, waxes, oils, resins and plant gums are known ingredients the embalmers used in ancient times [5,6]. Therefore, the molecular composition of balm samples covers a wide range of polarity and molecular mass. As we wanted to characterize potential components in a single analysis, samples were methanolysed and trimethylsilylated before GC–MS analyses. The efficiency of this sample preparation method was previously demonstrated for the characterization of vegetable tannins and all the above-mentioned natural substances, except bitumen [7,8]. Results for the characterization of lipids, waxes, resins and will be reported later [9].

Methanolysis and trimethylsilylation were carried out as previously reported [7]. Samples (typically 1.5 mg) were made up to 0.5 ml with a methanolic HCl solution prepared by adding acetyl chloride (0.4 ml) to 15 ml of methanol. Methanolysis was conducted at 80°C for 24 h. Thereafter, HCl was neutralized by adding pyridine and methanol was removed using a nitrogen stream. An excess of the trimethylsilylation agent (0.5 ml) was added to the dried material. The solutions were then heated at 80°C for 2 h. Eventually, the derivatized samples were evaporated using rotary evaporation at 50–60°C and immediately dissolved in 0.05 ml of hexane. GC–MS analyses were performed with 1 μl of this solution.

2.4. Gas chromatography–mass spectrometry

The GC–MS system consisted of a Varian Series

Table 1
Description of mummy samples and comparison of their aromatic acid and inositol content

Sample	Sample location	Sample description	Detected compounds				
			Inos	Gall	Cinn	Van	Proto
M1	Inferior side of right toe	Black piece of balm	+	+	+	+	+
M3A	External side of right knee	Black piece of balm	–	+	–	+	–
M3B	Underlying part of sample M3A	Brown and fibrous material (tissue) infiltrated with balm	+	+	+	+	+
M4	Right hip	Black piece of balm	+	+	+	+	+
M11A	Lower part of pelvis (right side)	Reddish-brown, resinous-like piece of balm	*	*	+	+	+
M11B	Underlying part of sample M11A	Brown and fibrous material (tissue) infiltrated with balm	+	+	+	+	+
M14	Canopic package containing the heart	Black piece of balm	+	+	+	+	+

Inos: non-methylated and mono-*O*-methyl inositols.
Gall: methyl gallate.
Cinn: methyl hydroxyhydrocinnamate.
Van: methyl vanillate.
Proto: methyl protocatechuate.
The * symbol denotes main constituents of the sample.

3400 gas chromatograph (Varian, Walnut Creek, CA, USA) interfaced by direct coupling to an INCOS 50 quadrupole mass spectrometer (Finnigan, San Jose, CA, USA). The gas chromatograph was equipped with a 30 m×0.25 mm I.D. fused-silica column coated with a 0.25 μm film of poly(5% phenyl, 95% methylsiloxane): DB-5 (J&W Scientific, Folsom, CA, USA). The carrier gas was helium at a flow-rate of 1.3 ml/min (measured at 40°C). Injector and transfer line temperatures were set to 300°C and 250°C, respectively. A splitless mode injection (splitless time 30 s) was followed by the oven temperature program: 40–130°C at 9°C/min, 130–290°C at 2°C/min and 290°C for 10 min.

Electron Impact (EI) mass spectra were collected in the total ion monitoring mode. Operating conditions for EI-MS were: source temperature 100°C, filament emission current 750 μA, ionizing voltage 70 eV, scan range from *m/z* 29 to *m/z* 650 with a period of 1.7 s. The other operating parameters were those set by the instrument's automatic calibration routine.

3. Results

Analysis of the methanolysed and trimethylsilylated mummy samples were performed in scan mode and allowed detection of six classes of compounds: alkanes, derivatives (TMSi ethers and/or methyl esters) of aliphatic alcohols, fatty acids, methyl glycosides of monosaccharides, inositols and aromatic acids. The occurrence of the last two classes of solutes is unusual in such a context. For this reason, we decided to concentrate so far on these well-defined classes of substances.

Most of the identification work was conducted on the balm sample taken from the lower part of the pelvis (M11A), a partial chromatogram of which is shown in Fig. 1. This sample yielded the highest aromatic acid and inositol content, thus offering a convenient situation for EI-MS identification of components of complex mixtures.

Identification of gallic acid was readily achieved from its retention time and EI mass spectrum which were controlled by comparison with those of a derivatized standard. As previously reported [7], intense diagnostic ions were observed at *m/z* 400 ($M^{\circ +}$) and *m/z* 281 ($[M^{\circ +} - CH_3^{\circ} - Si(Me)_4]^+$). Other aromatic acids were also found to be present: they were protocatechuic and vanillic acids, which are structurally related to gallic acid, and an hydroxylated derivative of hydrocinnamic acid. The base peaks of their respective EI mass spectrum (Fig. 2), were observed at *m/z* 179, *m/z* 224 and *m/z* 193 and were used for mass fragmentometry detection in mummy samples.

Identification of the second main peak in the chromatogram of the M11A sample was conducted by comparison of its EI mass spectrum with the one of persilylated *meso*-inositol (Fig. 3). Many peaks are common to both spectra, indicating a closely related structure. Additional peaks are observed in the unknown spectrum. The most intense occur at *m/z* 89, *m/z* 159, *m/z* 207, *m/z* 247 and *m/z* 260 owing to a 58 u shift of analog fragments in the spectrum in Fig. 3a or Fig. 3b. Such differences are accounted for by the presence of a methoxyl substituted carbocyclic atom instead of a trimethylsilyloxyl substituted one. The molecular peak detected at *m/z* 554 corresponds to a 58 u shift from that of *meso*-inositol standard (*m/z* 612). From this, we have identified the unknown as a mono-*O*-methyl inositol. Further ions, arising from consecutive losses of CH_3°, TMSiOH, CH_3OH, TMSiO° and CH_3O° from $M^{\circ +}$ allowed us to confirm the molecular weight of the molecule. We propose in Fig. 4 a scheme that accounts for these and other high mass ions in the spectrum in Fig. 3a. For this purpose, breakdown pathways reported in a previous EI-MS investigation of TMSi inositols [10] have been included.

Most remaining peaks in the inositols mass spectra are commonly obtained during EI fragmentation of the TMSi derivatives of sugars and polyhydroxylated compounds [11]. Structure assignments could be readily achieved according to published high resolution and isotopic labelling studies [12], and will not be discussed further.

EI mass spectra of inositols provide interesting features which enable their identification in complex mixtures by mass fragmentometry. In producing mass fragmentograms, ions of *m/z* ratios below 225 must be disregarded to avoid interference from other classes of TMSi derivatives, and especially those of sugars. Despite a lower intensity, more characteristic fragment peaks are found in the high mass range.

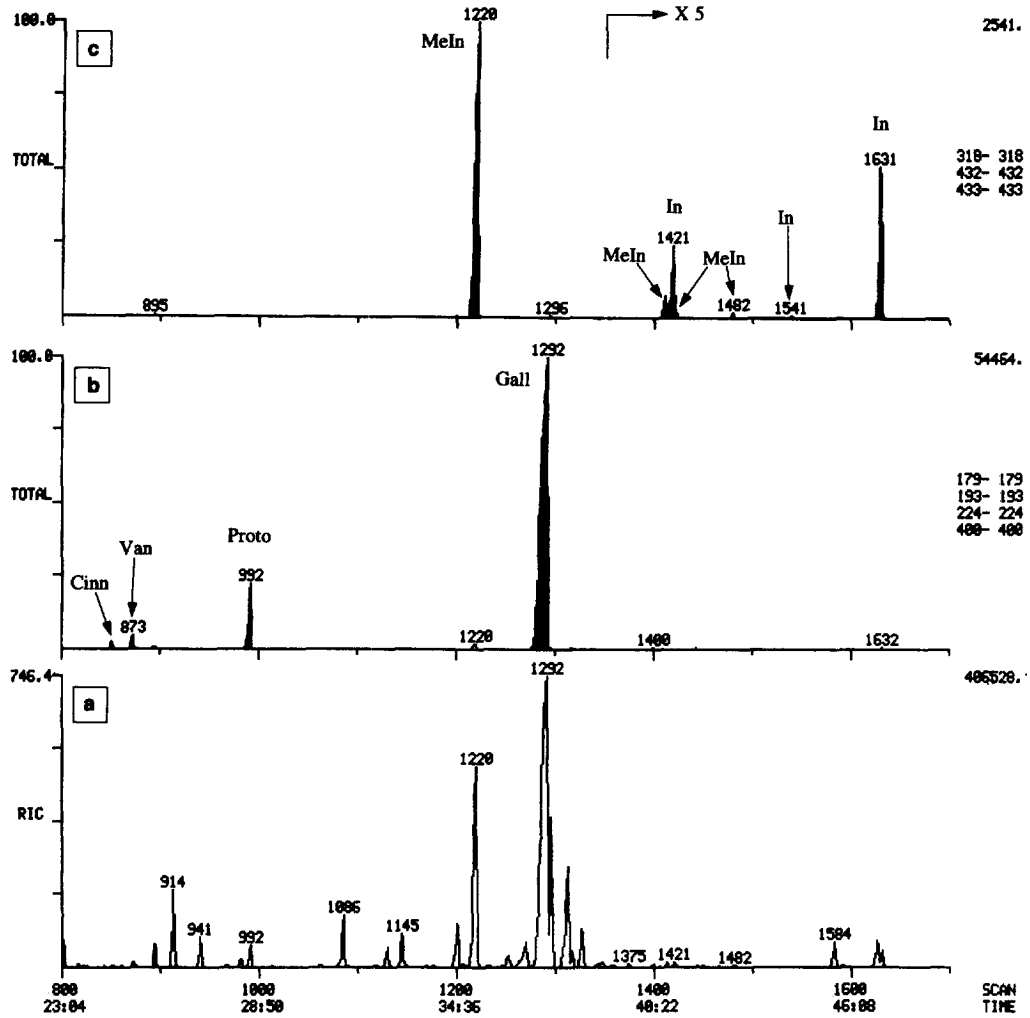

Fig. 1. Partial chromatograms of sample M11B. (a) TIC chromatogram; (b) summed *m/z* 179, *m/z* 193, *m/z* 224, *m/z* 400 mass fragmentograms; (c) summed *m/z* 318, *m/z* 432, *m/z* 433 mass fragmentograms. Identified compounds are mono-*O*-methyl inositols (MeIn), inositols (In), methyl hydroxyhydrocinnamate (Cinn), methyl vanillate (Van), methyl protocatechuate (Proto), methyl gallate (Gall).

During this work, peaks at *m/z* 318, *m/z* 432 and *m/z* 433 were selected for a systematic screening of inositols in all mummy samples. This ion set offers a sensitive and specific method of detection, as shown, for example, in Fig. 1. Hence, the mass fragmentogram revealed trace amounts of three mono-*O*-methyl and non-methylated inositol isomers in addition to the large peak eluted before methyl gallate.

Each sample was evaluated for the presence of aromatic acids and inositols by mass fragmentometry at the above-specified *m/z* ratios. Positive results obtained for seven samples among the 23 investigated are reported in a simplified form in Table 1.

Gallic acid was found in various amounts in different locations of the mummified corpse. Near the lower part of the pelvis was the only place where a corresponding predominant peak was detected. This is in contrast with the results gained at other locations: a significant peak was barely discernible on the TIC trace, but trace amounts of this compound have been efficiently revealed among coeluted solutes owing to the *m/z* 400 mass fragmentogram.

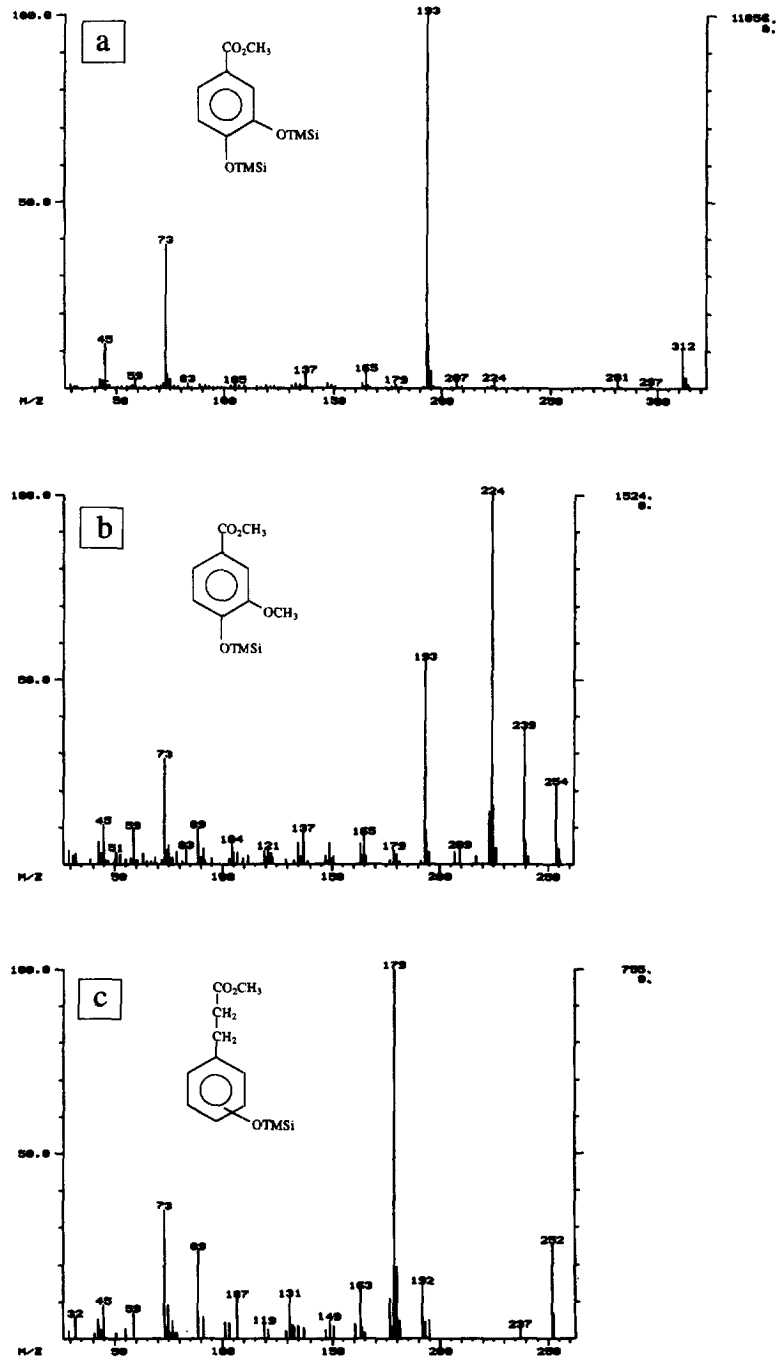

Fig. 2. EI mass spectra of aromatic acids detected in mummy samples: derivatives of (a) protocatechuic acid, (b) vanillic acid and (c) hydroxyhydrocinnamic acid.

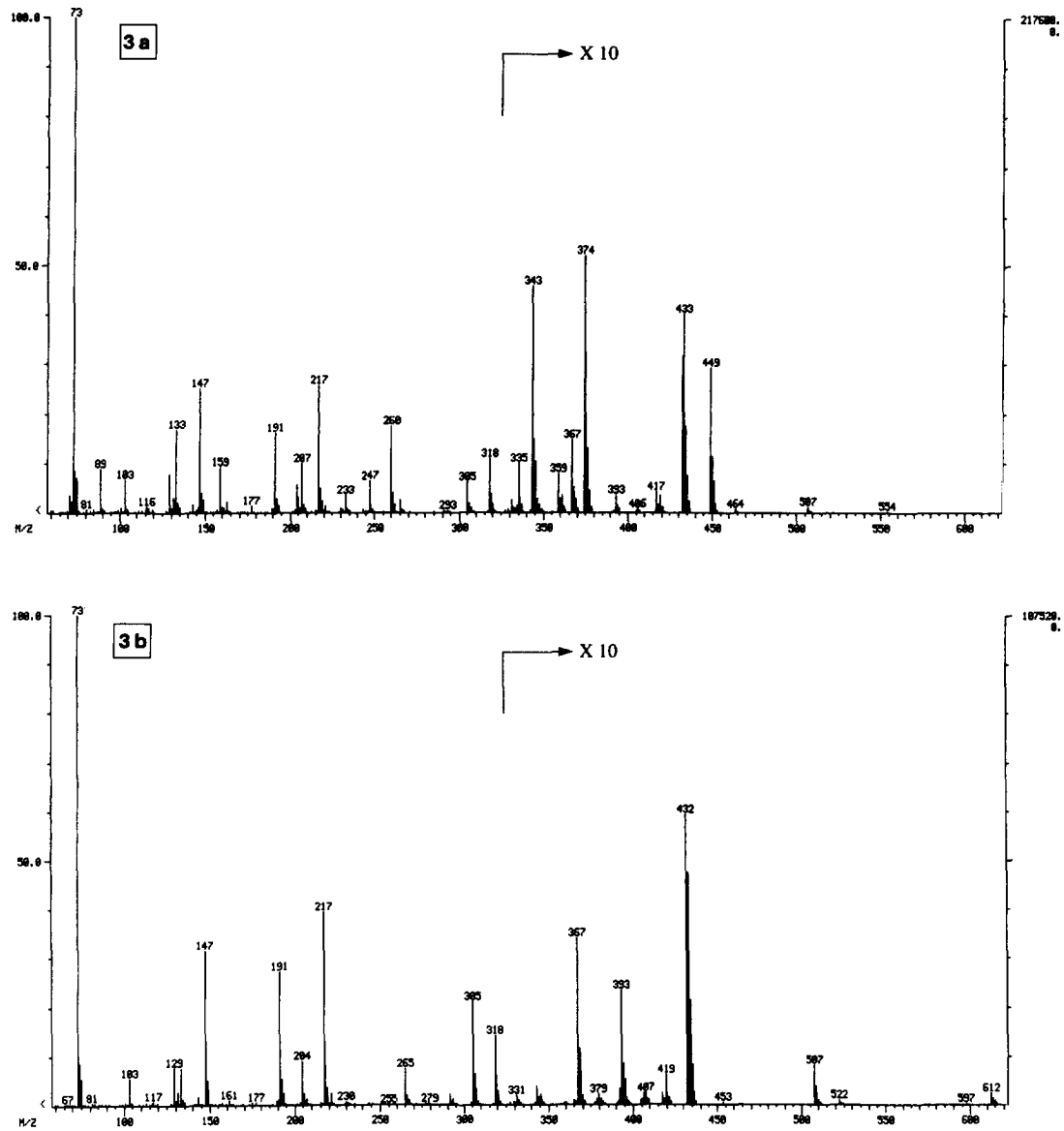

Fig. 3. EI mass spectra of TMSi derivatives of the main mono-*O*-methyl inositol detected in sample M11B (3a) and *meso*-inositol standard (3b).

Gallic acid was associated with inositols in six instances. From one sample to another, an almost regular distribution of inositols isomers was observed. It consisted of a prominent peak of a mono-*O*-methyl inositol and minor mono- or non-methylated diastereoisomers. The M11A sample yielded the highest inositol content, and also the most complete series of inositols isomers (Fig. 1). In other samples, most minor members within the inositol group were not found in sufficient amounts to give a detectable signal on the *m/z* 318 mass fragmentogram. Variations in the relative amounts of inositols and gallic acid were established by comparing peak intensities in the selected ion traces. In this

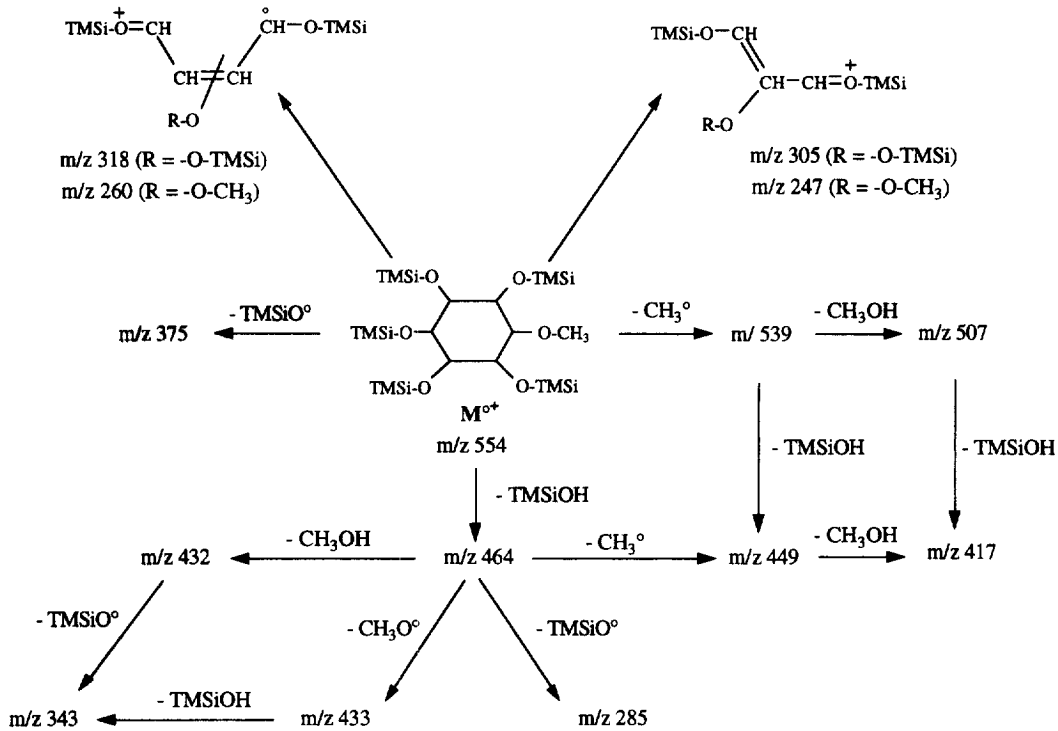

Fig. 4. EI-MS fragmentations of mono-*O*-methyl inositols.

respect, the sample M11A chromatogram must be once again underlined, due to the presence of a major peak of methyl gallate. A different situation was noted for the six remaining samples dealt with in Table 1, since the main inositol peak always appeared to be more intense than the methyl gallate peak.

The distribution of methyl vanillate, methyl hydroxy hydrocinnamate and methyl protocatechuate was difficult to correlate with the inositol or the methyl gallate content. Methyl vanillate occurred in all samples except M3A instances, together with methyl protocatechuate. The only thing we can say is that a higher amount of methyl protocatechuate, relative to that of methyl vanillate and methyl hydroxy hydrocinnamate seemed to be associated with a higher content of methyl gallate. This observation can be exemplified by comparing the mass chromatograms reported in Fig. 1b and Fig. 5.

Near the right knee, there was a hole in the balm where different layers were observed. The outer and inner part of the balm could be taken and analyzed separately (samples M3A and M3B). Comparison of the resulting mass chromatograms with respect to inositols and aromatic acids revealed quite different pictures (Fig. 5). Distinguishing features in the layer taken near the body (M3B) were an increased amount of gallic acid, presence of methyl hydroxy-hydrocinnamate and a series of inositols. A similar comparison was carried out from samples taken from the lower part of the pelvis (samples M11A and M11B). A quite different situation was observed in this case. A complete series of aromatic acids and an almost complete series of inositols were detected in both instances, but in far greater amounts in the upper balm layer rather than in the underlying human tissue.

4. Discussion

Methanolysis of mummy samples yield methyl gallate, a well-known component of numerous vegetable tannins, in particular the so-called 'hydrolys-

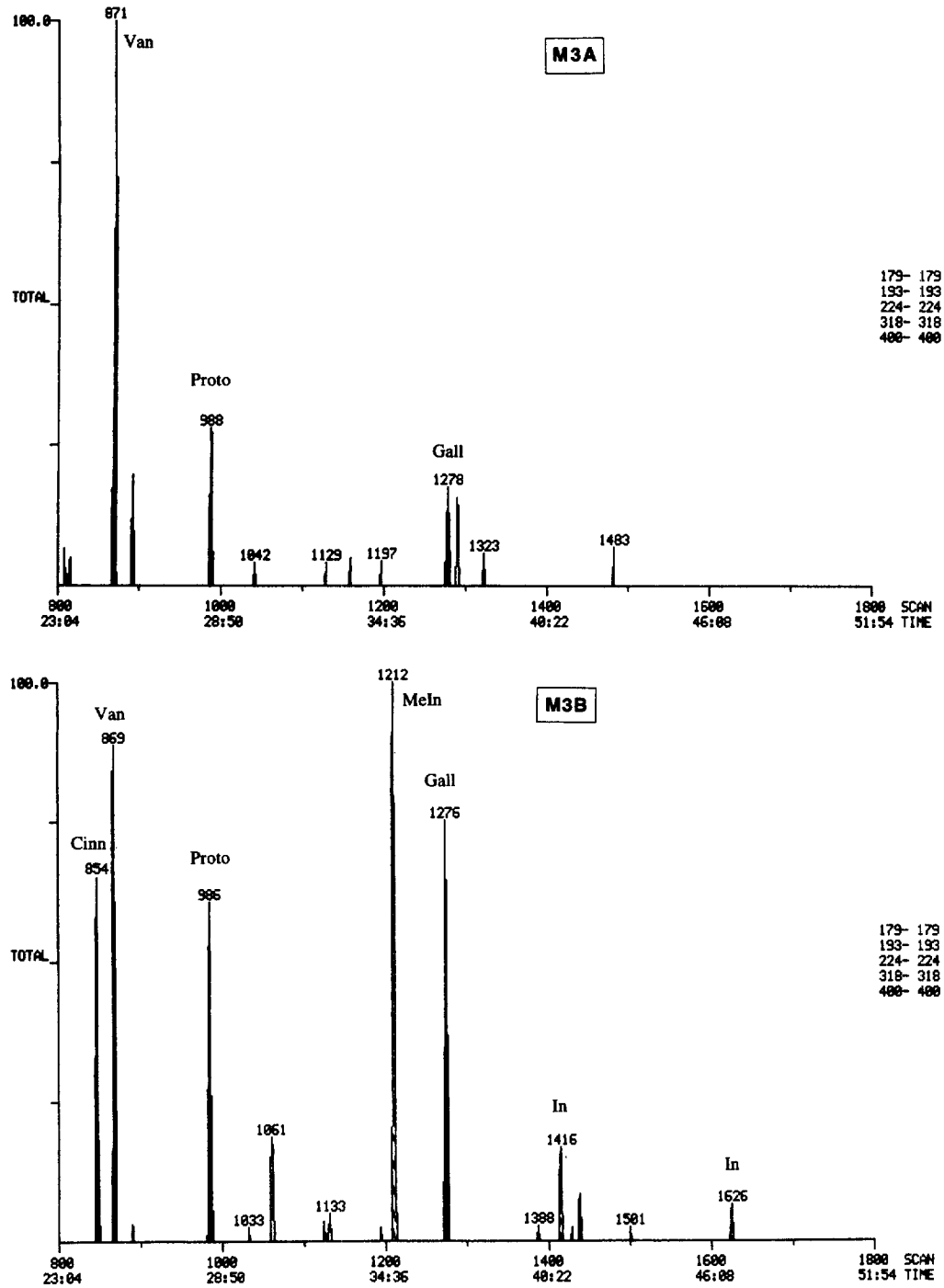

Fig. 5. Partial mass fragmentograms of samples M3A and M3B. The recorded ions m/z 179, m/z 193, m/z 224, m/z 400 and m/z 318 were selected to detect derivatives of methyl hydroxyhydrocinnamate (Cin), methyl protocatechuate (Proto), methyl vanillate (Van), methyl gallate (Gall), and inositols (In) or mono-*O*-methyl inositols (MeIn).

able tannins'. Various workers have used this marker as evidence of the presence of tannins in different matrices, e.g., in biological fluids (for clinical studies on the dietary effects of tannin-containing feedstuffs) [13], in raw plant materials [14], in metallogallic inks [15] or in archaeological leathers [16,17].

A simultaneous occurrence of inositols was encountered in five of the seven samples where this aromatic acid could be detected. This first observation led us to suppose a similar vegetable origin. That the highest content of these solutes occurred in the same sample (M11A) is an additional argument to support this assumption.

Identification of methyl gallate and inositols in different parts of the body, and also in the canopic package containing the heart, indicate the general use of a tannic substance to preserve the body from decay.

When one asked the question of the manner in which the tannic substance was used, two explanations had to be considered, i.e., its application within a mixture with other ingredients of the balm, versus a preliminary application on the desiccated body practised before the embalming. To assess these options, we compared the inositol and methyl gallate content of the outer and underlying part of the balm. Significant differences were effectively observed. However, the discrepancy between the results gained near the right knee and near the lower part of the pelvis prevented us from giving any reliable conclusion. The visual aspects of samples M11A and M3A (Table 1) led us to propose the following explanations: (i) some heterogeneity of the embalming mixture when applied, or (ii) different compositions of embalming mixture according to the part of the body treated.

The overall result was surprising for us. To our knowledge, gallic acid and other compounds associated in vegetable tannins (flavonoids, for instance), have never been reported as occurring in mummy samples. Moreover, we have not found in Egyptologic literature any argument indicating the use of a tannic substance for mummification.

Another problem to be tackled is the actual botanical source of the tannin. Pods, leaves or barks of local trees such as *Acacia nilotica* were proposed by Lucas [5] for the tanning of animals skins. So far, we are still lacking information concerning the chemical composition of tanning extracts prepared from trees or plants from the Nile area. However, a previous work of Richardin et al. [16] shed new light on the present results. The authors reported GC–MS characterization of reference tannins and archaeological leathers following optimized solvent-extraction procedures. In particular, the extract from the bark of mimosa (a tree which is botanically closely related to the *Acacia* genus) was shown to be composed of inositols and *O*-methyl inositols as major constituents, associated with lesser amounts of gallic acid. This result raises the interest of an analogous systematic study (but involving methanolysis and trimethylsilylation as the sample preparation step) aimed at obtaining a firm basis to interpret the present analytical data.

Besides gallic acid, three other aromatic acids were present but their origin is as yet uncertain. Protocatechuic acid is known as a biosynthetic precursor of tannic acids [18], and may therefore have the same origin as gallic acid and inositols. Cinnamic and benzoic acids, as well as their hydroxylated and/or methoxylated derivatives occur in several substances which could have been used by Egyptians. According to Lucas [5], barks of *Cinnamonum zeilanicum* (cinnamu tree), *Cinnamun cassia* and many resinous secretions (e.g., styrax and benzoin) are possible sources. They could also be due to the presence of propolis, a mixture with beeswax that we have effectively found in all analyzed samples.

5. Conclusions

The combination of methanolysis followed by trimethylsilylation and GC–MS analysis of derivatized samples was applied to the analysis of mummified material. The present investigation led to the finding that vegetable tannins were used during mummification in ancient Egypt, though it cannot be proved at present that it was practised throughout the Egyptian civilization.

The adopted analytical methodology has already been positively evaluated for other classes of natural substances (plant gums, oils, waxes and resins). Therefore, this appears to be a promising means of assessing the range of vegetable substances used as

preservatives and fragrances by the Egyptian embalmers.

Determination of the actual botanical source of the tannin employed must be further investigated. Without a doubt, the occurrence of a series of inositols must be considered as an attractive criterion for this task. With this objective in mind, identification of methylated and non-methylated inositols stereoisomers must be carried out to begin with. The corresponding GC–MS reference data will then allow a detailed analysis of inositols distribution in mummy samples as well as in tannins extracted from significant indigenous plants.

Acknowledgments

The authors gratefully acknowledge conservator R. Mourer for helpful discussions and for allowing us free access to the mummified body of 'l'Inconnu de Lyon'. We also thank J. Ogden for his help in preparing this manuscript.

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