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Characterization of Archaeological Beeswax by Electron Ionization and Electrospray Ionization Mass Spectrometry

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To better detect and identify beeswax in ancient organic residues from archaeological remains, we developed a new analytical methodology consisting of the analysis of (i) the trimethylsilylated organic extract by GC/MS and (ii) the crude extract by ESI-MS. Selective scanning modes, such as SIM or MRM, permit separate quantification of each chemical family (fatty acids, monoesters, monohydroxyesters, and diesters) and allow an improvement in sensitivity and selectivity, allowing the crude extract to be treated without further purification. GC/MS (SIM) was revealed to be a powerful method for the detection of components, with a detection limit down to a total lipid extract in the range of ∼50 ng in a complex matrix, such as archaeological degraded material, whereas ESI-MS/MS is instead used for the detection of nonvolatile biomarkers. Identification by GC/MS (SIM) and ESI-MS/MS (MRM) of more than 50 biomarkers of beeswax in an Etruscan cup at the parts-per-million level provides the first evidence for the use of this material by the Etruscans as fuel or as a waterproof coating for ceramics.

Among the beehive products, beeswax was widely used until the second half of the 19th century for a large variety of purposes in the fields of technology, art, and medicine and in religious rituals.[1,2] Since the beginning of the Christian era, beeswax was particularly used in lighting for candle-making before the development of the oil industry and chemistry, which brought about the replacement of beeswax by paraffin and stearin (a mixture of saturated fatty acids obtained by saponification of animal fats followed by their purification).[3]

Several archaeological clues, especially rock shelter paintings give evidence for the collection of wild beehive products since prehistoric times, even before the development of beekeeping. However, these paintings do not reveal any information about the products collected, their use, or their transformation by human beings. Recent developments in the chemistry of archaeological materials have shown that beeswax could be preserved for several millennia, especially when trapped in the porous clay matrix of ancient pottery.[2,4−7]

From a chemical standpoint, beeswax is a very complex material made of saturated and unsaturated *n*-alkanes, long-chain wax esters, fatty acids, diesters, hydroxyesters, and other minor constituents, such as flavonoids.[8−14] Some of these biomarkers were identified in ancient potsherds dating from the Neolithic to the Medieval period by several authors who performed GC/EI-MS analysis on the total lipid extract of various archaeological samples.[2,4−7] The composition of beeswax is often altered through time: the long-chain esters are partly hydrolyzed in palmitic acid and long-chain alcohols, and the alkanes may have sublimed either when preserved in a dry and warm environment as in Egypt or under the action of men who may have heated the beeswax before use.[2,4,6] Despite these chemical alterations, beeswax is generally characterized by the chromatographic pattern of *n*-alkanes and long-chain esters usually associated with their degradation products.

However, such an analytical approach is not efficient for the detection of the volatile molecular components that are known to be present in beeswax, such as diesters and hydroxyesters. It must also be noted that the analysis of archaeological beeswax by GC/MS does not always allow a suitable identification of the characteristic biomarkers, especially when it is mixed with other degraded organic materials or when it is preserved in low amount.

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Moreover, only very tiny samples are generally available for analysis, because ancient ceramic remains are usually very precious.

To increase the number of biomarkers detected in ancient samples containing beeswax and to improve the selectivity and the sensitivity of the methods allowing the detection of this natural resource in archaeological remains, we developed a methodology permitting the study of both volatile and nonvolatile molecular components. The first ones were characterized and quantified by GC/MS using both full scan and selected ion monitoring (SIM) modes in order to obtain a better sensitivity. The higher-molecular-weight compounds were identified by ESI-MS and ESI-MS/MS. To complete the semiquantification of volatile compounds performed by GC/MS using the SIM mode, total lipid extracts were analyzed by ESI-MS/MS using the multiple reaction monitoring (MRM) operation.

To obtain valuable results on archaeological samples, we first proceeded to the analysis of contemporary reference beeswax previously fractionated into its main chemical constituents and also the analysis of pure standards either commercially available or directly synthesized in our laboratory.

Using both gas chromatography coupled to mass spectrometry with electron ionization and mass spectrometry with electrospray ionization was shown to be particularly efficient to identify a large series of biomarkers of beeswax in both contemporary and archaeological samples. In particular, the selective scanning modes in mass spectrometry (SIM and MRM) allow a significant increase in sensitivity, thus making possible the study of small ceramic sherds containing beeswax (less than 400 mg of sherd). The ESI-MS analysis is complementary to the GC/MS data and allows the characterization of high-molecular-weight compounds that were never detected in any archaeological samples previously. This analytical approach, based on a significant number of biomarkers identified, allowed the unambiguous characterization of beeswax in Etruscan samples. From a historical point of view, these results provide interesting new data, completing the information published by Latin poets and agronomists about the use of honey for the aromatization or the chaptalization of the wine or game seasoning, but lacking precision about beeswax exploitation.[15]

EXPERIMENTAL SECTION

Synthesis of Monoesters. These compounds were synthesized by adapting protocols previously described.[16,17] Aliquots (100 μL, 1 μmol) of five solutions of even-numbered *n*-alcohols (C_{22}−C_{30}, Sigma-Aldrich, Milwaukee, WI) in dichloromethane (10 μmol/mL) were mixed and evaporated to dryness under a stream of nitrogen. A 50-μL portion of a 0.1 mmol/mL solution of palmitic acid (Sigma-Aldrich, Milwaukee, WI), 50 μL of a 100 μmol/mL solution of dicyclohexylcarbodiimide (DCC) in dichloromethane (5 μmol), and 5 μL of a 100 μmol/mL solution of 4-dimethylaminopyridine (DMAP) in dichloromethane were then added. The mixture was purged with nitrogen and stored at room temperature for 2 h. Approximately 1 mL of dichloromethane was added, and the organic phase was washed with saturated aqueous solutions of citric acid (3 × 1 mL) and $NaHCO_3$ (3 × 1 mL). The organic extract was dripped through an anhydrous $MgSO_4$ column to remove water and was evaporated to dryness. The residue was submitted to flash-column chromatography (SiO_2, 230−400 mesh, Sigma, Milwaukee, WI; 1 g, CH_2Cl_2) to obtain purified saturated palmitates (C_{38}−C_{46}).

The monoesters derived from palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), and oleic acid ($C_{18:1}$) were synthesized and purified by the same method.

Fractionation of Modern Beeswax Constituents. A solution of raw beeswax (1 mg/mL) from honey bee species *Apis mellifera* provided by apiarists working in the Doubs (France) was prepared by extraction with $CHCl_3$/MeOH (2:1, v/v) by ultrasonication (10 min). An aliquot of this solution (100 μL) was evaporated to dryness and dissolved in dichloromethane (25 μL). The total lipid fraction was then fractionated by anion-exchange chromatography with an aminopropyl cartridge (Varian, Palo Alto, CA) equilibrated with *n*-hexane. The neutral components were eluted by 12 mL of a *i*-PrOH/dichloromethane mixture (1:2, v/v), and the acid constituents are recovered by applying 12 mL of a solution of 5% HCO_2H in dichloromethane.

The neutral fraction (evaporated to dryness and dissolved in 50 μL of *n*-hexane) was fractionated by flash chromatography on a silicagel column (SiO_2, 230−400 mesh, Sigma, Milwaukee, WI) washed with 10 mL of *n*-hexane. *n*-Alkanes were first recovered by eluting with *n*-hexane (5 mL); diesters, by applying cyclohexane/dichloromethane (9:1, v/v, 9 mL); palmitic esters, by using *n*-hexane/dichloromethane (1:1, v/v, 9 mL); and hydroxyesters and alcohols were recovered by eluting with dichloromethane (10 mL).

Archaeological Samples. These samples consisted of 30 ceramic potsherds that lacked visible surface organic residues; consequently, this research was based on the study of the organic matter trapped in the porous inner layer of the vessel. The treatment applied to these samples was adapted from Evershed and collaborators.[5] Potsherds (0.4 to 1 g) were first cleaned by scraping with a scalpel blade to remove all surface contaminants and were then crushed using a glass mortar and pestle. After addition of an internal standard (*n*-C_{34}, 20 μL of a 0.1 mg/mL solution in dichloromethane), the powdered pieces were extracted twice by sonication for 15 min ($CHCl_3$/MeOH, 2/1, v:v, 10 mL). The organic extract was centrifuged at 4000 rpm for 20 min, and the clear solution was evaporated to dryness (nitrogen stream, 40 °C). The lipid extract was diluted in dichloromethane (500 μL) and an aliquot (100 μL) was treated further by derivatization.

Derivatization. In some instances, derivatization of compounds containing acidic hydrogen atoms was required prior to high-temperature GC (HTGC) analysis. To avoid incomplete derivatization due to the poor solubility of long-chain aliphatic lipid components in organic solvents, all of the samples were derivatized by using BSTFA (50 μL, Sigma-Aldrich, Milwaukee, WI) and pyridine (5 μL) as solvent and nucleophilic catalyzer.[13] The clear mixture was held at 80 °C for 30 min, evaporated to dryness in a flush of nitrogen (40 °C), and then dissolved by ultrasonic treatment in dichloromethane (50 μL).

Gas Chromatography. A Hewlett-Packard (Palo Alto, CA) 6890 gas chromatograph fitted with an on-column injector used

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Table 1. Abbreviated EI-MS Mass Spectra of a TMS Characteristic Compound of Each Chemical Family[a] for the Most Volatile Components of Beeswax

compound names	characteristic ions
tetracosanic acid, TMS ester	117 (30), 129 (15), 201 (25), 243 (13), 257 (13), 355 (14), 440 (63), **443** (100)
triacontanol, TMS ether	55 (26), 57 (26), 73 (40), 75 (64), 83 (23), 97 (35), 111 (27), 421 (39), 495 (4), **513** (100)
triacontanyl palmitate	55 (49), 57 (39), 67 (37), 69 (31), 81 (30), 83 (31), 97 (38), 111 (25), **256** (100), 257 (89), 420 (5), 465 (8), 676 (13)
tetracosanyl 15-hydroxypalmitate, TMS ether	55 (23), 73 (68), 117 (58), 133 (21), **301** (100), 329 (93), 331 (59), 453 (7), 591 (2), 636 (14), 665 (12), 680 (0.5)

[a] Fatty acid, alcohol, monoester, and hydroxymonoester.

in the track-oven mode and a FID detector (350 °C) was used for analysis by HTGC. The gas chromatographic separation was achieved using a CP-Sil 5 CB column (Varian, Palo Alto, CA, 15 m length, 0.32 mm internal diameter, 0.1-μm phase thickness) preceded by a 1-m precolumn. Helium was used as carrier gas with a programmed flow as follows: 2 mL/min for 17 min; 1 mL/min^2 until 4 mL/min; 4 mL/min for 4 min; 4 to 6 mL/min at a 1 mL/min^2 rate; 6 mL/min for 16 min. The oven temperature was held at 50 °C for 1 min, ramped from 50 to 350 °C at 10 °C/min and held at 350 °C for 10 min.

Gas Chromatography/Mass Spectrometry. The GC/MS analysis was performed with a ThermoFinnigan (San Jose, CA) GCQ device equipped with an ion trap analyzer linked to a Hewlett-Packard (Palo Alto, CA) 5890 chromatograph. Injection was achieved through a split/splitless injector used in the splitless mode and held at 325 °C. Modern beeswax and archaeological samples were analyzed with the same column and temperature as for HTGC analysis. Helium pressure was maintained at 16.0 psi and the GC/MS interface, at 340 °C. Mass spectra were recorded in the electron ionization mode at 70 eV, and the ion source was held at 180 °C. In full scan mode, the mass range was scanned from 50 to 800 in 0.6 s.

Electrospray-Mass Spectrometry. ESI-MS analyses were performed on a Micromass (Manchester, U.K.) Quattro II triple-stage quadrupole mass spectrometer fitted with an electrospray ion source and controlled by Mass Linx software. Synthetic monoesters and fractionated and raw modern beeswax (0.2 mg/mL) were prepared in a 0.1% TFA methanolic solution. Samples were infused (5 μL/min) into the ESI source with a Harvard (Holliston, MA) syringe pump. The electrospray interface was operated with a capillary voltage fixed at 3.3 kV at a temperature of 70 °C. The cone voltage was optimized by recording mass spectra at voltages varying between 40 and 65 V. Nitrogen was used as the nebullization and drying gas at flow rates of 15−20 and 300−400 L/hr, respectively. For ESI-MS/MS and MRM experiments, the protonated molecular cations were selected in the first quadrupole (Q1), collided with Ar (2.3 mTorr) in the RF-only second quadrupole (Q2) using a collision energy of 5−40 eV and analyzed in the third quadrupole (Q3). A solution of polypropylene glycol oligomers (mass range 30−2000 Da) was used for mass calibration.

RESULTS AND DISCUSSION

Characterization of the Molecular Constituents of Modern Beeswax by GC/MS. The mass spectra of most volatile compounds of beeswax have previously been described in the literature,[2,5,13,18−24] and Table 1 indicates the main fragments of fatty acids, *n*-alcohols, monoesters, and hydroxymonoesters. One must note that the analyses were performed with an ion trap analyzer (see Experimental Section). Consequently, the mass spectra detailed here may differ from those obtained on a quadrupole or a magnetic sector, either as a result of a discrimination against ions with m/z values below 100, as discussed elsewhere,[18] or because of secondary ion−molecule reactions occurring with residual water present in the analyzer.[19]

Free Fatty Acids.[20] The first separation on an aminopropyl SPE cartridge (Varian, Palo Alto, CA) allows a fast separation of neutral and acidic components. This procedure led to the isolation of a homologous series of saturated fatty acids ($C_{16:0}$, $C_{18:0}$, and $C_{22:0}$−$C_{34:0}$) with even carbon numbers of which tetracosanoic acid ($C_{24:0}$) is the main constituent, which is in agreement with other investigations.[14,21]

The neutral components were then fractionated by flash chromatograohy on a silica column into three different fractions: (i) hydrocarbons, (ii) monoesters, and (iii) coeluted *n*-alcohols and hydroxymonoesters.

Hydrocarbons. Alkanes and alkenes provide a typical pattern for the recognition of beeswax, extensively described by Stránský and Streibl,[21] consisting of *n*-alkanes (C_{23}−C_{33}) and *n*-alkenes (C_{27}−C_{35}) identified by their retention times by HTGC analysis when compared to those of commercial standards. The alkane/alkene ratio tends to decrease when the carbon length increases. Isolation of hydrocarbons from polar compounds, such as fatty acids and alcohols, avoids coelution phenomena and allows identification of monunsaturated alkenes with 27, 29, 31, 33, and 35 carbon atoms, present at a very low level.

Monoesters.[2,4,22] The major monoesters of beeswax contain an even number of carbon atoms and are made of even-numbered *n*-alcohols (C_{24} to C_{34}) esterified by palmitic acid.

n-Alcohols. A homologous series of *n*-alcohols with even-numbered chain length (C_{24}−C_{34}) was isolated by flash chromatography. These compounds are degradation markers of beeswax resulting from the partial hydrolysis of the palmitic esters. They

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are present in the modern material at low-parts-per-million levels but become major components in archaeological beeswax exposed to hydrolytic degradations.[2,5,6]

Hydroxymonoesters. HTGC/MS analysis allows the detection of a homologous series of monohydroxyesters resulting from the esterification of a long-chain alcohol (C_{24}−C_{34}) by 15-hydroxypalmitic acid, with triacontanyl 15-hydroxypalmitate dominating the chromatogram. These compounds provide a small molecular peak $M^{+\bullet}$ (Table 1). At proximity of the molecular ion, a weak fragment ion $[M - 15]^+$ resulting from the loss of a methyl radical from the silyloxy group and an ion $[M - 44]^{+\bullet}$ resulting from the loss of CO_2 are observed. The spectrum is dominated by ions at *m*/*z* 301 (base peak) and an intense fragment at *m*/*z* 329. This latter ion is obtained by the same pseudo-McLafferty rearrangement observed for the saturated ester on ion $[M - 15]^+$ obtained from the molecular ion after the loss of a methyl group. The former ion at *m*/*z* 301 may result from a CO loss from the ion−dipole complex between the acylium ion and the alkoxy radical, as found in methyl lactate metastable decomposition.[25]

Identification of the High-Molecular-Weight Constituents of Beeswax by ESI-MS. The most volatile biomarkers of beeswax, especially the *n*-alkanes, the fatty acids, and the long-chain alcohols, are easily detected, separated, and identified by GC and GC/MS. The long-chain monoesters and hydroxymonoesters may also be studied by this method, but their elution necessitates the use of limit conditions in GC separation, based on the use of on-column injectors,[26] short-length columns, high temperatures (350 °C at the end of the run), and important flow rates of helium. Their separation by GC/MS is usually more difficult as a result of the limit temperature of the interface; the flow rate of helium, which must be maintained at a moderate flow in order to keep an acceptable vacuum in the mass spectrometer; and the presence of the mass spectrometer following the column, which alters the separation, especially for the high-molecular-weight constituents because of the pumping system. Higher-molecular-weight components, in particular diesters, are not even detected by this method, although they are known to be present in beeswax. Analysis of diesters by HTGC and HTGC/MS requires appropriate instrumentation in order to achieve sufficient sensitivity at high elution temperatures and to avoid chromatographic loss.[14]

To elucidate more properly the structure of the higher-molecular-weight constituents of beeswax, it appeared necessary to analyze them directly from the liquid phase.[27]

General ESI-MS Profile of Raw Contemporary Beeswax. The ESI-MS profile was obtained by analyzing beeswax diluted in an acidified methanolic solution (TFA 0.1%). The mass spectrum presents three main mass zones on which major ions were detected. The low-mass range of the spectrum, below 500, is characterized by a complex mixture of components that were not studied in detail. We noted the important fragments formed by decomposition of the protonated esters in the ESI source, such as the ions at *m*/*z* 255 and 257 corresponding to the palmitoleic and palmitic protonated acids, respectively. The intermediate mass

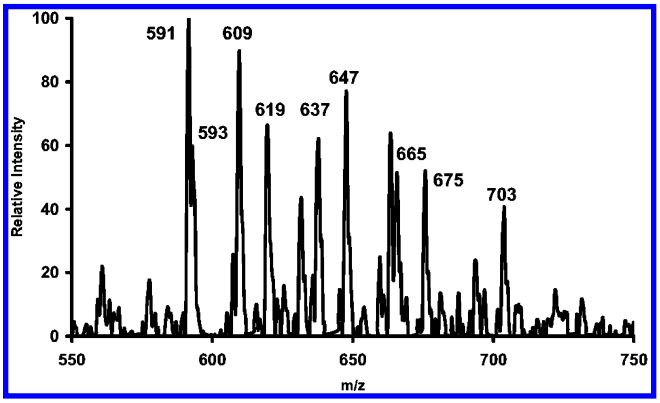

Figure 1. Profile of the monoesters of crude modern beeswax by ESI-MS (0.2 mg/mL, TFA 0.1% in MeOH, cone 55 V).

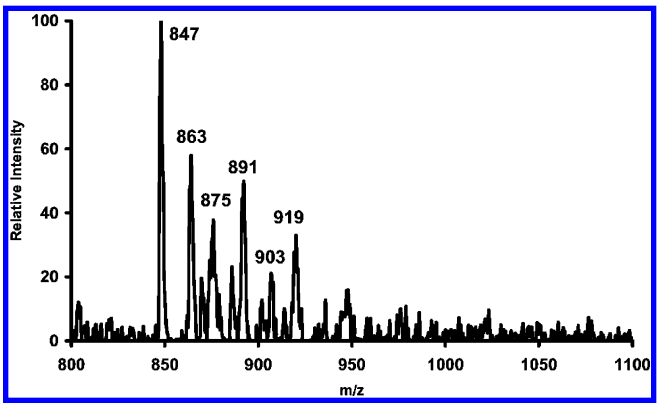

Figure 2. Profile of the diesters of crude modern beeswax by ESI-MS (0.2 mg/mL, TFA 0.1% in MeOH, cone 55 V).

range 500−900 corresponds to molecular ions of the monoesters (Figure 1), and the high-mass range above *m*/*z* 850 is characteristic of the diester species (Figure 2).

In the zone of the mass spectrum corresponding to the monoester constituents, various homologous series consisting of molecules differing by 28 amu (two carbon units) can be identified: (i) the monounsaturated esters from *m*/*z* 591 to 703, which dominate the spectrum, indicative of their easy ionization; (ii) the saturated monoesters from *m*/*z* 593 to 705; (iii) the monosaturated monohydroxylated esters from *m*/*z* 609 to 693; and (iv) the saturated monohydroxylated esters from *m*/*z* 611 to 695 (Figure 1).

Among the diester species (Figure 2), this spectrum allows distinguishing the monounsaturated (from *m*/*z* 847 to 987), the saturated (from *m*/*z* 845 to 985), and the monohydroxylated derivatives (from *m*/*z* 863 to 1003) from each other.

Comparing the HTGC/MS and the ESI-MS profiles of modern beeswax reveals the complementarity of the ESI-MS analysis, especially for polar trace constituents. Saturated esters are not easily protonated, contrary to polarizable monounsaturated and hydroxylated esters, allowing a better detection of these polar constituents.

Detailed Structure of the Fatty Acid Moieties Constitutive of the Mono- and Diester Species by ESI-MS/MS. The structures of the different mono- and diesters determined by ESI-MS/MS are detailed in Table 2. Compared to mass spectrometry analyses of beeswax with electron impact ionization, the use of ESI-MS/MS appears to be a powerful structural tool to determine the formulas

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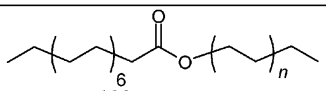
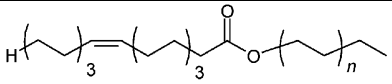
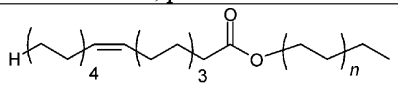
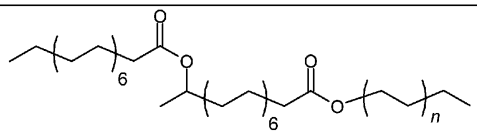
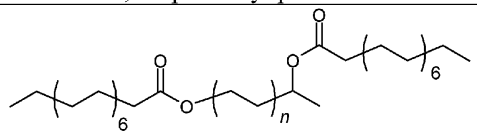
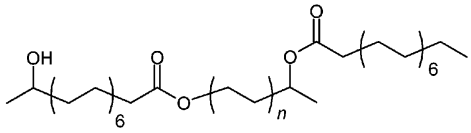
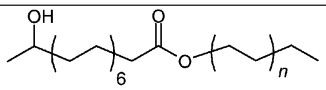
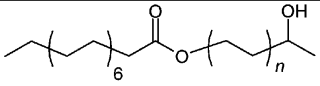
Table 2. Formulas of Mono-, Di-, and Hydroxyesters Identified by HTGC/MS or ESI-MS/MS Analysis of Crude Modern Beeswax

Monoesters	*n*
 $M^{16:0}$, palmitates	11-16
 $M^{16:1}$, palmitoleates	12-17
 $M^{18:1}$, oleates	14-15
Diesters	
 D^1, 15-palmitoyl palmitates	11-16
 D^2, 1,(ω-1)-dipalmitoyl diols	11-16
 D^3, 1-(15-hydroxy-palmitoyl), (ω-1)-palmitoyl diols	11-16
Hydroxymonoesters	
 H^1, 15-hydroxy-palmitates	11-16
 H^2, 1-palmitoyl diols	11-16

of long-chain compounds with very similar structure. GC/MS analyses allow the detection and the identification of saturated palmitate esters, whereas ESI-MS/MS analyses reveal that the monoester composition of beeswax is more complex than expected and is also made of long-chain esters containing stearic and oleic acid moieties. Similarly, the structure of the diesters, which are difficult to detect by GC/MS because of their high molecular weight and their low volatility, even when using the SIM mode, could be determined by their study in the liquid phase.

One must note that such a method, based on complementary results obtained by the analyses in the gas and liquid phases, avoids any saponification preceding the analysis, which was used by several authors and is very prejudicial to the knowledge of the complete structure of these dimers.

When infused into the ion source in an acidified methanolic solution, monoester species yield two homologous series separated by 2 amu. The first one corresponds to the monounsaturated monoesters, and the second, to the saturated palmitates. Upon CAD, MH^+ ions of monoesters species provide an abundant protonated fatty acid ion that reflects the identities of the fatty acids and *n*-alcohols. Typically, the CAD mass spectrum of protonated tetracosanyl palmitoleate synthezised in the laboratory (MH^+ at *m*/*z* 591) reflects this neutral alkene loss observed at *m*/*z* 255 corresponding to $[C_{16}H_{30}O_2 + H]^+$. The formation of the acylium cation $[C_{15}H_{29}CO]^+$ at *m*/*z* 237 is also observed along with the more surprising appearance of the radical cation corresponding to the acid side chain at *m*/*z* 382. All of the protonated saturated and monounsaturated monoesters from beeswax show strong ions at *m*/*z* 255 and *m*/*z* 257, reflecting the neutral loss of an alkene from the alcohol. Three different series of monoesters corresponding to esters of palmitic acid, palmitoleic acid, and oleic acid with long-chain alcohol ($C_nH_{2n+1}OH$, with *n* between 24 and 32) can be identified.

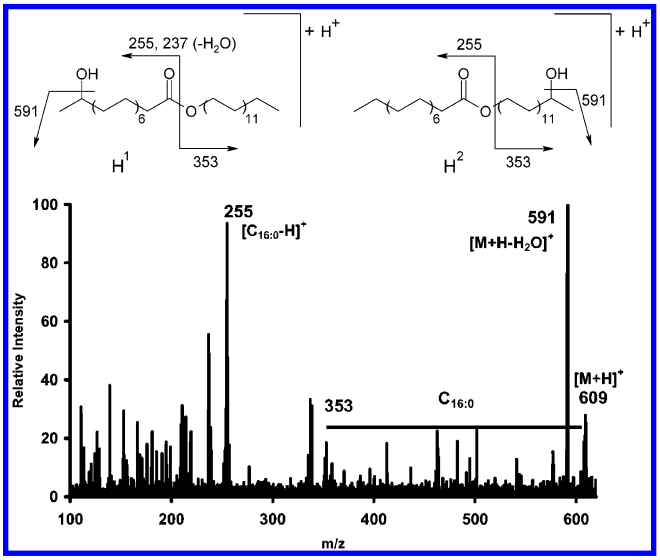


Figure 3. Tandem mass spectrum of the ion at *m*/*z* 609 corresponding to a mixture of two protonated hydroxymonoesters (cone 45 V, CID 15 eV).

Hydroxymonoesters are characterized by the typical loss of a neutral H_2O fragment from MH^+ at low collision energies (5−10 eV). The loss of a neutral hydroxy fatty acid, of an alkene from the alcohol, or of an alcohol at higher energies (15−25 eV) from the mixture of protonated hydroxypalmitoyltetracosane bearing the alcohol function on the ultimate methylene group of the acid or of the alcohol moiety (*m*/*z* 609) results in the $[M - (RCO_2H) + H]^+$, $RCO_2H_2^+$, and RCO^+ ions at *m*/*z* 339, 257 and 237, respectively (Figure 3).

Figure 2 shows the detailed ESI-MS profile of protonated diesters consisting of two homologous series of six biomarkers, each one separated from the adjacent ones by 16 amu, which corresponds to a hydroxyl function. ESI-MS/MS allows elucidation of the molecular structure of these entities resulting from the esterification of diols or hydroxyacids by fatty acids, hydroxyacids, or diacids. As for monoesters, the loss of neutral fatty acid reflects the nature of the diols or hydroxyacid substituents. All of the diesters present the same fragmentations as illustrated in Figure 4: the CAD tandem spectrum of protonated diester (16:0/22-diol/16:0) contains ions at *m*/*z* 591, 255, and 257, reflecting a neutral

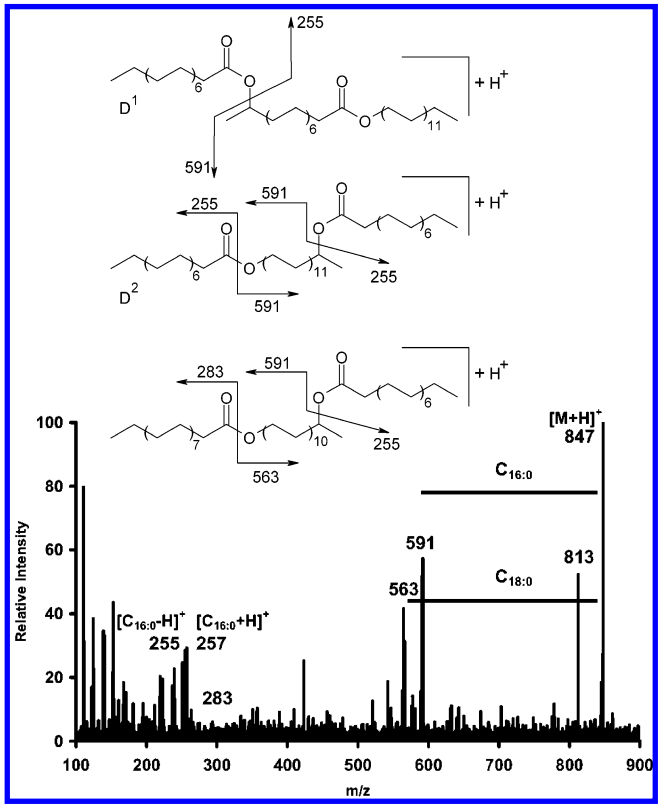


Figure 4. Tandem mass spectrum of the ion at *m*/*z* 847 reflecting a mixture of three protonated diesters (cone 45 V, CID 20 eV).

loss of palmitic acid (16:0) or of tetracosanyl palmitate, respectively, from MH^+ at *m*/*z* 847. The presence of the weak ions at *m*/*z* 563 and 283 issued from the loss of a neutral stearic acid (18:0) and a docosanyl stearate proves the existence of two isomers with the same molecular weight, resulting from the esterification of a diol by two molecules of palmitic acid for the first one, and a molecule of palmitic acid and one of stearic acid for the other one (Figure 4).

Hydroxylated diesters show as their hydroxymonoesters homologous an intense loss of neutral water at low CID energy and a loss of a neutral palmitic acid or a neutral 15-hydroxypalmitic acid from the MH^+ ion (*m*/*z* 875), providing the $[MH - (H_2O)]^+$, $[MH - (C_{16:0})]^+$, and $[MH - (HO{-}C_{16:0})]^+$ fragments at *m*/*z* 857, 619, and 601 respectively (Figure 5).

In conclusion, ESI-MS/MS analysis permits straightforward characterization of the structure of high-molecular-weight biomarkers, such as diester, avoiding any sample preparation, especially hydrolysis. The results obtained are summarized in Scheme 1. From a biosynthetic point of view, the different esters, hydroxy esters, and diesters may be considered as all derived from the palmitate esters, as presented in Scheme 1. The hydroxylation of the palmitate esters is conducive to the formation of hydroxy esters (H^1 and H^2), which are then esterified by palmitic acid (diesters D^1 and D^2). A second step of hydroxylation is responsible for the presence of diesters D^3.

SIM and MRM: Two Complementary Methods for the Semiquantification of Biomarkers by GC/MS and ESI-MS, Respectively. Each molecular family presents characteristic fragmentation of their biomarkers as $M^{+\bullet}$ in HTGC/MS analysis or as MH^+ in ESI-MS/MS sequence, as described above. The

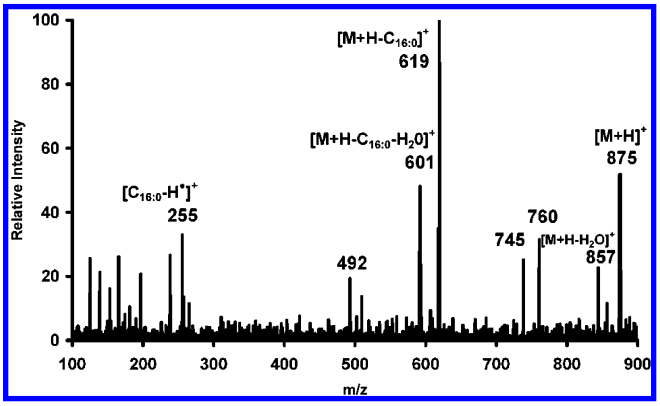
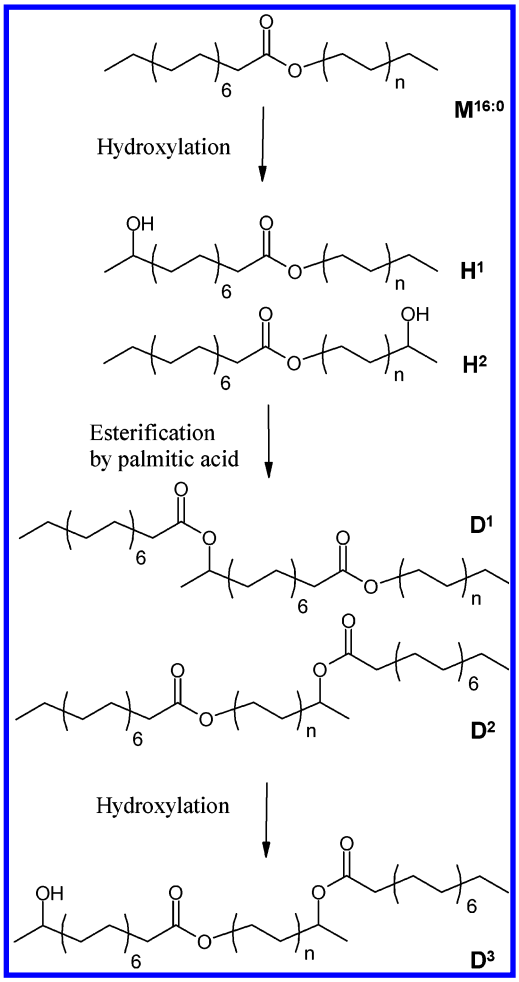


Figure 5. Tandem mass spectrum of protonated diester hexacosanediol-1,15-dipalmitoyl (cone 60 V, CID 15 eV).

Scheme 1. Relationship between the Different Homologous Ester Series Present in Beeswax.



specific fragmentation that reflects various functional groups can be exploited to enhance the selectivity and the sensitivity of the biomarker detection. In the case of HTGC/MS analysis on the ion-trap analyzer, the selected ion monitoring mode (SIM) was chosen, and the multiple reaction monitoring sequence (MRM) was exploited for the ESI-MS/MS analysis with a triple quadrupole analyzer. The scanning parameters for the SIM experiment in HTGC/MS analysis are presented in Table 3. Specific fragmentations, such as loss of a TMSOH group and the addition of an H_2O molecule for the alcohols, formation of the two intense ions at

Table 3. Scanning Parameters for the SIM Sequence in HTGC/MS Analysis

compound	SIM
n-alcohols	401, 429, 457, 485, 513, 541
saturated monoesters	256, 257
monohydroxyesters	301, 329
free fatty acids	117

Table 4. Scanning Parameters for the MRM Sequence in ESI-MS/MS Analysis of Beeswax Esters

compound	selected ion (Q1)	energy collision (Q2), eV	monitored ion (Q3)
saturated monoesters	MH^+	20	257
unsaturated monoesters	MH^+	20	255
monohydroxyesters	MH^+	5	$[MH - H_2O]^+$
diesters (D^1, D^2)	MH^+	20	$[MH - C_{16:0}]^+$
hydroxydiesters (D^3)	MH^+	5	$[MH - H_2O]^+$

m/*z* 256 and at *m*/*z* 257 for the monoesters or at *m*/*z* 301 and *m*/*z* 329 for the monohydroxyesters in the HTGC/MS procedure, or loss of a water molecule or of an alcohol from the mono- or diesters in the ESI-MS/MS analysis are exploited with the intention of improving the selectivity of the detection (Table 4). Comparison between results obtained on the monoesters and hydroxymonoesters by full scan and SIM scanning modes reflects an improvement of the ratio signal/noise by a factor of 10−50. The high selectivity of these two scanning procedures allows direct analysis of the crude lipid extract of modern or ancient material without further purification.

The amount of each molecular component was calculated by reference with the internal standard added to the solution before its analysis by HTGC/MS (see Experimental Section). This procedure allowed quantification of the different main homologous series (hydrocabons, fatty acids, *n*-alcohols, monoesters, hydroxymonoesters, and diesters) and analysis of the proportions of saturated and unsaturated components for the most volatile series, such as hydrocarbons and fatty acids (Table 5).

Moreover, in a chemical family, the ability to ionize is roughly almost constant, which permits one to calculate a relative semiquantification of each detected biomarker in each family as well as in GC/MS (SIM) and ESI-MS/MS (MRM). Consequently, we proceeded to a relative quantification in each homologous series: all molecular components are expressed as a percentage of the main one in each chemical family (Figure 6).

A comparison of the SIM and MRM showed that volatile components are more easily analyzed and quantified by SIM, whereas nonvolatile components with high molecular weight are rightly studied by MRM (Figure 6). Comparing the two analytical procedures for the saturated monoesters shows that quantification by these modes is similar but, for high dilution, MRM is more precise as a result of the low volatility of these compounds in the gas phase.

Although integration of a noisy signal in HTGC is impossible for trace biomarkers, the combination of HTGC/MS with SIM scanning mode and of ESI-MS/MS with MRM operation permits a consequent improvement of the selectivity and the sensitivity of these analyses. This new analytical protocol allows detection of traces of beeswax in a sample and is especially well-adapted to

Table 5. General Composition of the Trimethylsilylated Total Lipid Extract (TLE) of Modern Beeswax from *Apis Mellifera* Species, of an Etruscan Cup, and of Scoria from the Castellina Settlement, Determined by HTGC[a]

component	modern *A. mellifera*, %	Etruscan cup, %	archaeological scoria, %
hydrocarbons	24.6	36.8	n.d.
n-alkenes	6.8	10.3	n.d.
n-alkanes	17.8	26.5	n.d.[a]
fatty acids	3.6	11.1	5.8
unsaturated	0.4	n.d.	0.2
saturated	3.2	11.1	5.6
n-alcohols	4.3	tr[b]	1.7
monoesters	39.2	13.1	67.0
hydroxymonoesters	9.5	10.2	12.5
diesters	5.6	tr	tr
β-sitosterol	n.d.	1.5	0.7
cholesterol	n.d.	4.6	0.1
total identified components	86.8	77.3	87.8

[a] Not detected. [b] Trace.

the analysis of archaeological beeswax preserved in ancient ceramic vessels. Analysis of these ancient samples necessitates a very sensitive method in order to minimize the required sample, which has to be as low as possible (1−2 g for the domestic ceramics, 200−400 mg for the funerary or luxurious ceramics).

Identification of Low- and High-Molecular-Weight Biomarkers of Beeswax in Archaeological Samples. The archaeological samples studied in this investigation were collected during summer 1999 by one of us (N.G.) on the Etruscan settlement named La Castellina (late 8th−7th century B.C.) located on the Mediterranean cost of Italy, 70 km northwest of Rome. A series of 30 ceramic potsherds were chosen according to their size and morphology.

All of the sherds lacked visible organic residue on their surface, as has been described for other ceramic vessels.[2] However, these sherds were chemically investigated after having been ground into powder and extracted in order to analyze the organic matter potentially trapped in the porous clay matrix of the pottery.

Other samples, which were not associated with any pottery but were found as small lumps in the sediment, were also analyzed. These scoria, which look as burned dark stones, light and very porous, were discovered in the stratigraphic unit corresponding to the 8th−6th century B.C. of a supposed palatial construction of the village.[28]

Among the 30 potsherds, two-thirds present a total lipid extract (TLE) > 20 μg/g, limit value for further investigation of the organic matter by GC/MS,[29] generally found to vary between 40 and 100 μg/g in the ceramics from La Castellina. The chromatographic patterns obtained revealed the use of a wide range of natural materials which are still under investigation. In this article, we develop in detail the case of two samples, the organic content of a domestic cup and the scoria that were found to contain traces of beeswax. The pottery that yielded ancient beeswax was a

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(29) Charters, S.,; Evershed, R. P.; Goad, L. J.; Leyden, A.; Blinkhorn, P. W.; Denham, V. *J. Archaeol. Sci.* **1993**, *35*, 211−223.

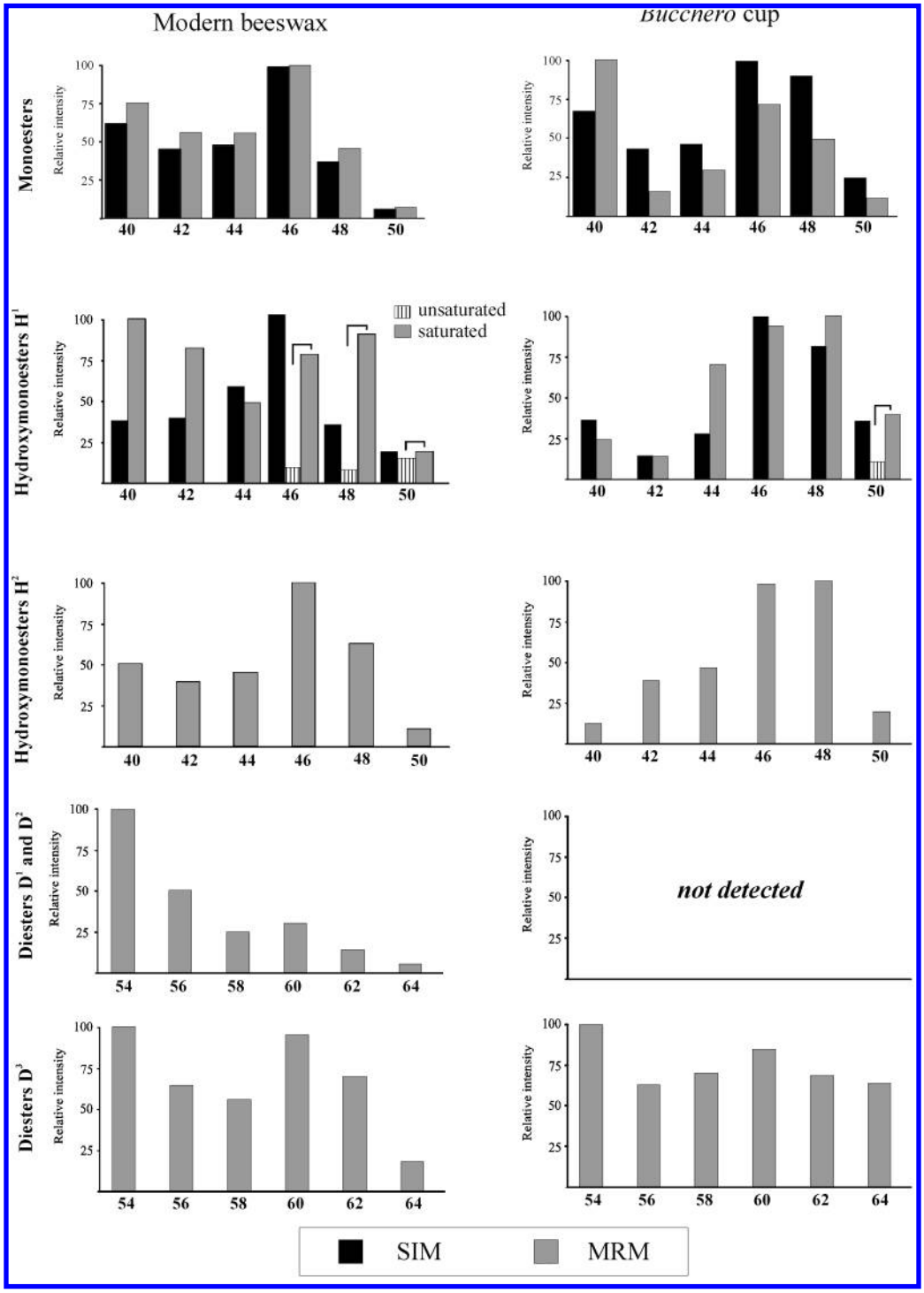

Figure 6. Semiquantifications by EI-MS (SIM mode) and ESI-MS/MS using the MRM sequence of the total lipid extracts of (a) a modern crude beeswax and (b) a bucchero Etruscan cup. Labels refer to carbon number length.

domestic cup made in bucchero, an old type of ceramic, typically from Etruria, that imitates the metal Greek vessels (Figure 7).

HTGC Analyses of Archaeological Samples. The total lipid extract from the domestic cup was of 72 μg/g of sherd. HTGC allowed detection of the major biomarkers (hydrocarbons and palmitic esters) and degradation markers (fatty acids and traces of *n*-alcohols) of beeswax in both samples (Figure 7).

The low amount of palmitic monoesters in the bucchero cup, its degraded pattern, and the *n*-alkanes profile do not permit a direct conclusion that this cup contained beeswax. HTGC/MS in full scan mode allows detection of major fatty acids (palmitic and tetracosanoic acids), degradation markers issued from the partial hydrolysis of beeswax.[2] The presence of β-sitosterol, a biomarker from plant origin, suggests that beeswax was mixed with a plant material.[30]

Contrary to the total lipid extract of the cup, the scoria are hydrocarbon-free, and we note on the HTGC-profile that mono-

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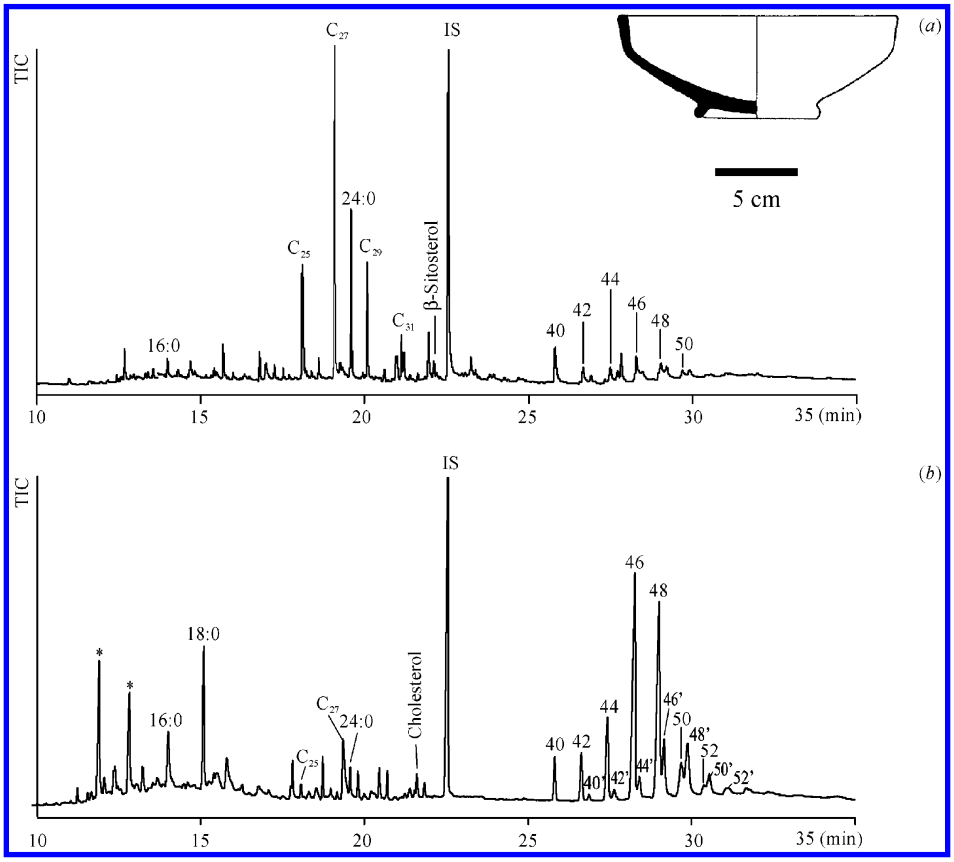

Figure 7. HTGC profile of the lipid extract of (a) an Etruscan bucchero cup and (b) scoria from the Castellina settlement (late 8th−7th century B.C.). 16:0 and 24:0 are palmitic and tetracosanoic acids, C_{25}−C_{31} are *n*-alkanes, and the saturated monoesters and hydroxymonoesters (primed numbers) are labeled by their carbon number length.

esters and monohydroxyesters are well-preserved. The presence of palmitic (16:0) and stearic (18:0) acids is certainly due to an external addition, and the association of these two saturated fatty acids with cholesterol suggests that beeswax was mixed with fats of animal origin.[31] The preservation of esters and the hydrocarbon depletion suggest that the material was not subjected to any chemical alteration during preservation in the sediment but was heated at high-temperature inducing the fast alkane vaporization.[2] The association of chemical evidence and archaeological observations (porous, heated aspect of the material) suggests that beeswax was mixed with animal fat and could have been used as a fuel.

SIM and MRM Analyses of the Archaeological Beeswax. If HTGC permits detection and quantification of only the major biomarkers, investigation of the SIM and MRM procedures allows consideration of the preservation of more than 60 beeswax biomarkers, and semiquantification of these biomarkers was made by these scanning operations (Table 5 and Figure 6). This method was essentially investigated on the bucchero cup because (i) its TLE is very low and the characterization of the preserved organic matter requires more sensitive and selective methods, and (ii) this ceramic sample is more representative of the studied material exhumed during excavations, and analysis of the organic matter preserved in the inner porous sides can bring to light the function and the use of the pottery.

To characterize in more detail the degraded beeswax, the SIM procedure was applied to the trimethylsilylated TLE and unquestionably revealed homologous series of even-numbered *n*-alcohols (C_{20} to C_{34}), of even-numbered saturated monoesters (C_{40}−C_{50} with the C_{34}−C_{38} palmitates), and of even-numbered hydroxymonoesters (C_{40}−C_{50} mentioned for the first time in archaeological samples). The alcohols, monoesters and hydroxymonoester profiles are similar to those of modern material. However an increase in the proportion of alcohols, markers of degradation by hydrolysis of esters, and relative reduction in the monoesters and diesters prove that beeswax was not subjected to any specific alteration, such as heating. The preservation of more than 50 biomarkers in the archaeological samples, which were susceptible to hydrolysis by microorganisms or bacteria present in the sediment, reflects the good preservation condition of the beeswax in the bucchero clay. Fatty acids present a more complex profile consisting of an important pattern of constituents with an even number of carbon atoms (24:0 to 34:0) corresponding to beeswax biomarkers; palmitic acid (16:0) as beeswax hydrolytic degradation marker; myristic (14:0), oleic (18:1), and stearic (18:0) acids coming from an external source of plant oil; and odd-numbered fatty acids (13:0, 15:0 and 17:0) as bacterial biomarkers.[32] By this new scanning procedure, biomarkers present at low-parts-per-million levels (1150 pg/g for C16:0, <250 pg/g for major

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(32) Dudd, S. N.; Regert, M.; Evershed, R. P. *Org. Geochem.* **1998**, *29*, 1345−1354.

monoesters, and <70 pg/g for the major hydroxymonoesters) reflect the powerful improvement in the sensitivity.

Investigating the MRM sequence to determine the molecular structure of the mono- and diesters of this archaeological beeswax, we prove the preservation of two homologous series of monoesters (saturated and unsaturated derivating from palmitic acid and from a mixture of palmitoleic and oleic acids respectively). As for the diesters, apolar biomarkers, the ionization of which requires very high cone tension (>70 eV) or very acidic strength (>0.2% in TFA) do not present a sufficient response coefficient allowing a representative semiquantification, but are detected as traces, though hydroxylated diesters (type D^3 in Table 2 and Scheme 1) on which the alcoholic function confers a greater ionizability are more easily detected and can be semiquantified. Figure 6 summarizes the different results obtained by SIM and MRM analyses of modern and archaeological beeswax preserved in the bucchero cup.

CONCLUSION

Although gas chromatographic analysis, using flame ionization detection or coupled to mass spectrometry, is the most common means for the study of the molecular composition of beeswax, the use of ESI-MS technique has significant potential for extending our knowledge of the structure of the high-molecular-weight constituents of beeswax. Working in the liquid instead of the gas phase allowed determination of the structure of several biomarkers present in beeswax, such as monounsaturated and saturated esters containing either palmitic, palmitoleic, stearic, or oleic moieties as well as hydroxyesters and diesters.

However, such analyses do not allow the detection and identification of low-molecular-weight constituents, such as fatty acids, alcohols, and *n*-alkanes. Indeed, beeswax appears to be a very complex lipidic material containing an important number of molecular constituents on a wide range of molecular weights. In consequence, further studies of this material should be based on both the use of GC/MS and ESI-MS analyses.

If such research opens new avenues for the study of contemporary beeswax, especially in the case of adulterations which can alter the quality of beehive products, they are also of considerable importance for understanding the exploitation of beeswax through time. Enhancement of the detection limits allows henceforth the study of the chemical structure of the organic matter preserved in all potsherds, even if no charred residue is visible on its surface. This more specific detection of beeswax biomarkers and degradation markers permits direct analysis of the crude or the trimethylsilylated lipid extract by ESI-MS/MS (MRM) and HTGC/MS (SIM) respectively, and avoids a long time- and solvent-consuming protocol, as described previously for the biomarkers' isolation from modern beeswax.

From the perspective of archaeology, the unambiguous identification of beeswax in ceramic vessels from the Etruscan village of La Castellina is proof of the uninterrupted use of this natural resource through time. Indeed, this material has been used in Bercy (France) since the Neolithic period, during the Egyptian period as a cosmetics or painting lacquer in the Fayum portraits or in Crete (Mochlos) as fuel, but use of beeswax in archaic Italy was not reported. The present study brings to light new elements that suggest the use of beeswax as a waterproofing agent for the porous Etruscan bucchero ceramics, or as fuel when mixed with other fats. The absence of oil lamps in meridional Etruria during the 8th−7th centuries B.C. could be explained by the use of beeswax candles for lighting.

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