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# Mass spectrometric methods prove the use of beeswax and ruminant fat in late Roman cooking pots

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**Abstract**

Lipid extracts of sherds of archaeological late Roman cooking pots were analysed using high temperature–gas chromatography coupled to a mass spectrometer and liquid chromatography with atmospheric pressure chemical ionization mass spectrometer detection (LC–APCI-MS). With these advanced techniques the use of beeswax was shown through identification of the constituting alkanes, mono and diesters. The detection of high amounts of saturated triacylglycerols (TAGs) further indicated that animal fat was processed in these pots. Part of the animal fat was characterised as originating from ruminants due to the presence of *trans*-fatty acids. The distribution of saturated TAGs and the higher concentration of stearic acid compared to palmitic acid in the transesterified lipid extract indicated that this was sheep fat. The results illustrate how complex mixtures can be unravelled and original contents of ancient ceramic vessels can be determined using specialised analytical equipment.


*Keywords:* Beeswax; Ruminant fat

## 1. Introduction

The coupling of chromatographic techniques (gas chromatography (GC) and liquid chromatography (LC)) to mass spectrometry (MS) allows separation of the individual molecular species with on-line mass spectrometric analysis of the eluting components. These techniques have become indispensable for the analysis of complicated lipid mixtures. The lipid extract of archaeological pottery is an example of such a highly complex mixture, as the retrieved lipids are a result of the—often multiple—use of the pot for feeding or technological purposes followed by degradation and alteration processes during burial [1]. Nevertheless important dietary or functional information can be obtained by the analysis of included organic residues and in particular the lipid fraction of the ceramics abundantly found on most archaeological sites [2]. With the use of GC–MS a wide range of products were identified including several resins [3,4], epicuticular leaf waxes of cabbage [5] and beeswax [6,7]. These commodities were identified with the help of ‘‘biomarkers’’, i.e. lipid molecules that are characteristic for plant or animal species and which remain practically unaltered dur-

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ing burial. While most fats and oils differ in fatty acid composition to a certain extent [8], fatty acid ratios of archaeological lipid extracts should be interpreted with caution as the original triacylglycerols are degraded due to oxidation processes [9], hydrolysis and microbial alterations [10]. Nevertheless it was shown that carbon isotope ratios of individual fatty acids measured with on-line gas chromatography–combustion-isotope ratio mass spectrometry (GC–C-IRMS) are preserved during decay [11]. Using this technique several animal fats could be discerned [11] and the presence of milk was established [12].

Recently, LC with atmospheric pressure chemical ionization (APCI) MS was used to identify triacylglycerols (TAGs) from fats and oils used as a fuel in antique oil lamps [13]. The TAGs are original components of the used products, because these have not undergone any hydrolysis. Moreover, contamination with TAGs from the burial matrix or microorganisms is minimal [14]. Nevertheless the unsaturated TAGs which are more susceptible to oxidation [15] are depleted.

In this paper LC–APCI-MS in combination with GC–MS is used for the identification of authentic beeswax and traces left in two late Roman cooking pots. While beeswax was extensively characterised by Aicholz and Lorbeer [16,17] with a high temperature gas chromatograph coupled to electron impact and chemical ionization mass spectrometry, this study is the first to show the use of LC–APCI-MS to identify wax esters. In addition to beeswax the presence of ruminant fat was also shown in these pots. The results indicated that cooking pots were used for processing beeswax in late Roman Turkey.

## 2. Experimental

### 2.1. Samples

The sherds with sample codes SA-01-KK-21 and SA-01-KK-76 were found inside a large urban villa occupied from mid-Imperial to early Byzantine times (2nd–7th century AD) from the archaeological site of Sagalassos, southwestern Turkey. The samples belonged to a storage room in use during the late Roman period (5th–6th century AD). However during the second half of the 6th and the first half of the 7th century AD, this room was abandoned for storage and used as a dump for food and kitchen refuse, including broken pottery. The sampled sherds belonged to this waste material. The whole room was buried after destruction by an earthquake around the middle of the 7th century AD. Based on size and frequency of inclusions, hardness, feel, colour of the matrix and fracture in addition to the heavy sooting pattern on the exterior, the samples were estimated to stem from cooking pots.

### 2.2. Extraction-derivatization

A 5-g sample of the sample was extracted with 101 μg of *n*-heptadecane (99%, Janssen Chimica, Geel, Belgium) added as an internal standard with a soxtec apparatus according to the procedure described by Kimpe et al. [13]. The extraction procedure was applied to two samples. The two lipid extracts were dried under vacuum. One sample was transesterified and analysed on a polar phase GC. Methylesters were prepared by stirring the lipid extract in 100 μl of 5% methanolic potassium hydroxide (Merck, Darmstadt, Germany), then by adding 200 μl of bidistilled water and extracting the fatty acid methyl esters (FAMEs) with 1 ml of cyclohexane. A second lipid extract, redissolved in toluene (50 μl), was trimethylsilylated using 50 μl of *N*-methyl-*N*(trimethylsilyl)trifluoroacetamide (98%, Avocado Research Chemicals, Rhodes, France) at 60 °C for 1 h. This extract was used for analysis with HT-GC and GC–MS. Part of this lipid extract or a third extract was injected into the LC–MS. Commercial samples of lamb, chicken, pork and beef meat were also treated. A beeswax sample was melted and dissolved in toluene or isopropanol for analysis with HT-GC–MS and LC–MS, respectively.

### 2.3. Gas chromatography and gas chromatography–mass spectrometry

Gas chromatographic analysis of the silylated total lipid extract was performed on a Hewlett-Packard 5890 high temperature gas chromatograph equipped with FID detector. Samples were introduced by on-column injection into a 15 m×0.32 mm I.D. fused-silica capillary, coated with CP Sil-8 stationary phase

with 0.25-μm film thickness from SGE using nitrogen as a carrier gas. The temperature program consisted of a 5-min isothermal hold at 60 °C followed by ramping from 60 to 340 °C at 10 °C/min. The temperature was then kept at 340 °C for 15 min.

The transesterified samples were separated on a second Hewlett-Packard 5890 gas chromatograph with FID detector using nitrogen as carrier gas. The sample was injected on-column into a 60 m×0.32 mm I.D. fused-silica capillary column from SGE, coated with a 0.25-μm film of BPX70 as stationary phase. The analysis was done isothermally at 180 °C.

GC–MS analyses were performed using a quadrupole type MD 800 MS directly coupled to a 8000 Fisons GC. The GC was operated using the same high-temperature column as stated above. The thermal program consisted of a hold of 1 min at 140 °C, followed by a temperature increase of 15 °C/min and a 5-min hold at 340 °C. Helium was used as carrier gas. The MS was operated as follow: ion source, 200 °C; emission current, 400 μA and electron energy, 70 eV. The GC–MS capillary interface was maintained at a temperature of 250 °C. Spectra were recorded every 500 ms from 40 until 850 *m*/*z*.

### 2.4. LC–APCI-MS

The analyses were performed on a SpectraSystem HPLC system, coupled to a Finnigan LCQ DECA mass spectrometer. The samples were analyzed using a reversed-phase column of the type Supelcosil LC-18 (25 cm×4.6 mm, Supelco) with a gradient of methanol and isopropanol as mobile phase at a flow-rate of 0.8 ml/min. The operation conditions were as follows: vaporizer temperature, 500 °C, capillary temperature, 225 °C and corona current, 5 μA. Spectra were obtained in the range of 200–1200 *m*/*z*, with a scan time of 0.2 s.

### 2.5. Statistics

Statistics were carried out using the SAS statistical programme. Statistical significance between the two obtained datasets (oil lamps and cooking pots) was determined at $P > 0.05$ according to Duncan's mean separation test. Statistical significance between the obtained datasets and the animal fat or olive oil was determined using a one-side *t*-test.

## 3. Results and discussion

### 3.1. GC–MS

The two cooking pot sherds SA-01-KK-21 and SA-01-KK-76 were characterised by high contents of *n*-alkanes (20 and 50 μg, respectively, on a total amount of 200–500 μg lipids per gram sherd), long chain alcohols and long chain fatty acids identified with GC–MS. In order to quantify the distribution of the hydrocarbons present in the lipid samples, the non-polar phase was isolated by extraction with hexane. Beeswax typically contains high amounts of hydrocarbons (20% of total lipids). A strong resemblance between the distribution of *n*-alkanes and *n*-alkenes in the cooking pots and beeswax could be noted, with heptacosane being the most important hydrocarbon followed by nonacosane and hentriacontane. The concentrations of the *n*-alkenes were somewhat depleted most probably due to oxidation reactions. The cooking pots contained higher amounts of long chain alcohols compared to beeswax, which was attributed to the presence of plant derived lipids. In correspondence with this assumption triterpenyl alcohols (*m*/*z* 189–203–393–483–496) and the major plant sterol β-sitosterol were recognized. There was also an indication for the presence of animal fat, as cholesterol was identified in both cooking pots.

### 3.2. LC–APCI-MS

The chromatogram obtained for authentic beeswax is shown in Fig. 1. Several monoesters, diesters, hydroxymonoesters and hydroxydiesters were identified. The saturated monoesters are the most important compounds of beeswax lipids and consist of long chain alcohols (C24–C36) esterified with palmitic acid. Not all components were clearly separated using a gradient of methanol and isopropanol. The hydroxymonoesters co-eluted with monoesters of similar carbon number and the monoesters with larger carbon number (C46–C50) co-eluted with hydroxydiesters. It was previously noted [13] that

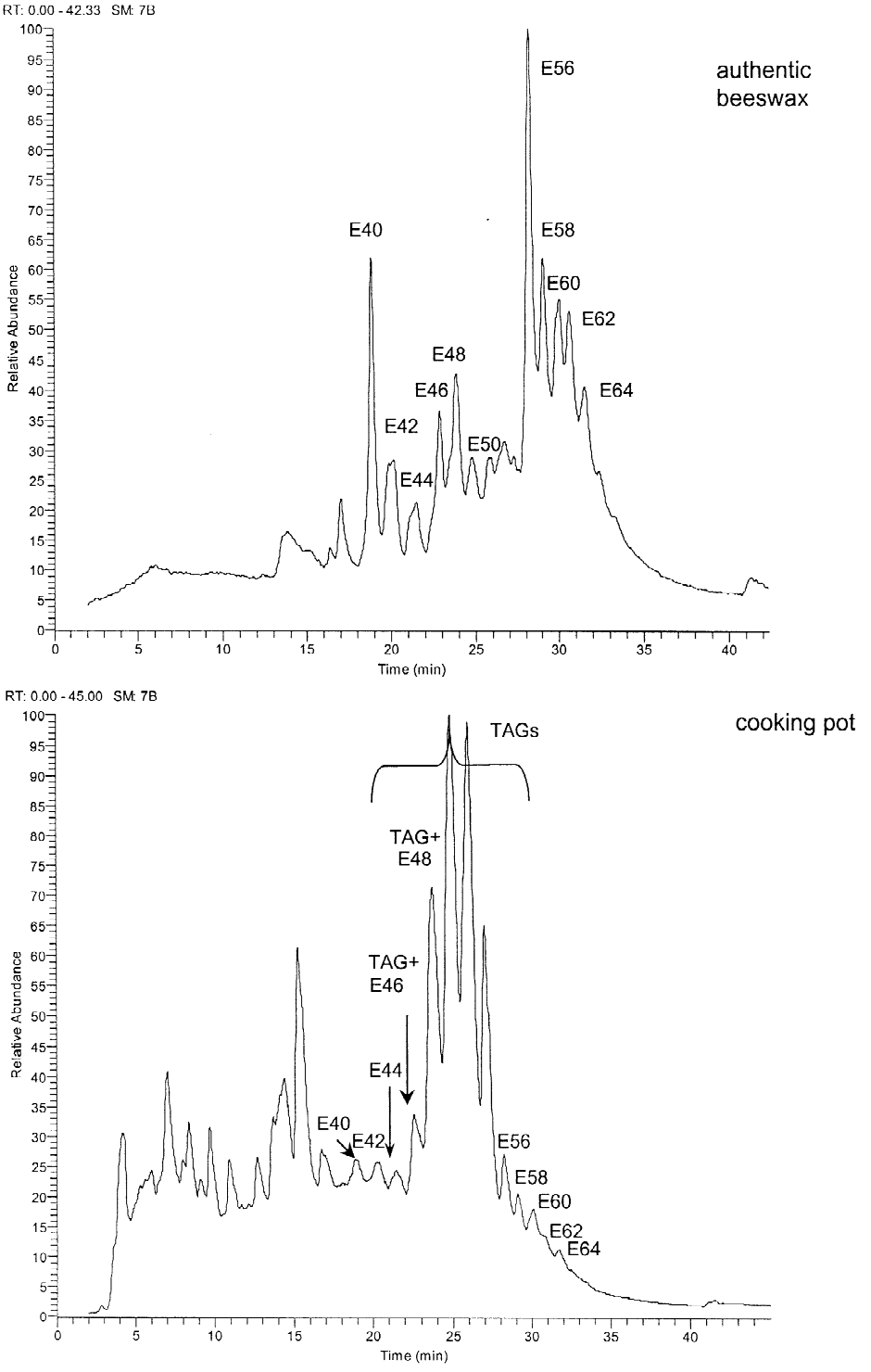


Fig. 1. HPLC–APCI-MS profile of authentic beeswax and archaeological cooking pot. The monoesters with 40–50 and diesters with 56–64 carbon atoms are indicated by E40–E50 and E56–E64, respectively.

addition of acetonitrile improved the separation, but had a strongly negative effect on the detection with APCI-MS. A better detection was preferred due to the small amount of lipids retrieved from the archaeological pots. The most important fragment ions of the identified molecules can be seen in Table 1. As was described by Aicholz and Lorbeer [17] the saturated monoesters (alkyl palmitates, C40–C52) are characterised by an intense hydride-extracted molecular ion $[M–H]^+$ and $[C_{15}H_{31}COOH–H]^+$ ion formed by the loss of the fatty acid. Also the acylium ion $[C_{15}H_{31}COO–H_2O]^+$ ($m/z$ 237) was detected. No distinction could be made between the mass spectrum of saturated and unsaturated monoesters as unsaturated monoesters form abundant $[M+H]^+$ ions [17]; in order to enhance the separation of saturated and unsaturated esters, silver ion HPLC should be used [18]. Hydroxy monoesters eluted just after the saturated monoesters and showed hydride-extracted molecular ions $[M–H]^+$ and the hydroxy-palmitic acid $[C_{15}H_{30}OHCOOH–H]^+$ ion formed by the loss of the fatty acid. The diesters (C56–C64) showed an abundant $[MH]^+$ ion and intensive fragment ions caused by the loss of palmitic acid $[MH–C_{15}H_{31}COOH]^+$. Also hydroxydiesters were detected; these showed $[MH]^+$ ion together with an $[MH–C_{15}H_{30}OHCOOH]^+$ ion formed by the loss of hydroxy palmitic acid. These hydroxy compounds were first described by Aicholz and Lorbeer [17]. In Fig. 2 an ion chromatogram is shown over $m/z$ 820–1100 where the peak patterns of diesters and hydroxyesters can be clearly seen.

In the archaeological cooking pots, mostly monoesters (C40–C50) and diesters (C56–C64) were found next to several TAGs as can seen in Fig. 1. Some of the monoesters (C46–C50) coeluted with

Table 1
Observed mass spectra of wax esters in authentic beeswax

| Retention time (min) | | Mass spectra | | |
|---|---|---|---|---|
| | Monoesters | $[M–H]^+$ | $[RCOOH–H]^+$ | $[RCOO–H_2O]^+$ |
| 18.82 | C40:0 | 591 | 255 | 237 |
| 20.14 | C42:0 | 619 | 255 | 237 |
| 21.44 | C44:0 | 647 | 255 | 237 |
| 22.76 | C46:0 | 675 | 255 | 237 |
| 23.8 | C48:0 | 703 | 255 | 237 |
| 25.18 | C50:0 | 731 | 255 | 237 |
| | Hydroxymonoesters | $[M–H]^+$ | $[ROHCOOH–H]^+$ | |
| 19.92 | C40 | 607 | 271 | |
| 21.06 | C42 | 635 | 271 | |
| 22.25 | C44 | 663 | 271 | |
| 23.48 | C46 | 691 | 271 | |
| 24.9 | C48 | 719 | 271 | |
| 25.4 | C50 | 747 | 271 | |
| | Diesters | $[MH]^+$ | $[MH–RCOOH]^+$ | $[RCOOH–H]^+$ |
| 28.11 | C56 | 847 | 591 | 255 |
| 29 | C58 | 875 | 619 | 255 |
| 29.8 | C60 | 903 | 647 | 255 |
| 30.53 | C62 | 931 | 675 | 255 |
| 31.43 | C64 | 959 | 703 | 255 |
| 32.41 | C66 | 987 | 731 | 255 |
| | Hydroxydiesters | $[MH]^+$ | $[MH–RCOOH]^+$ | |
| 22.9 | C56 | 863 | 591 | |
| 23.86 | C58 | 891 | 619 | |
| 24.8 | C60 | 919 | 647 | |
| 25.71 | C62 | 947 | 675 | |
| 26.7 | C64 | 975 | 703 | |

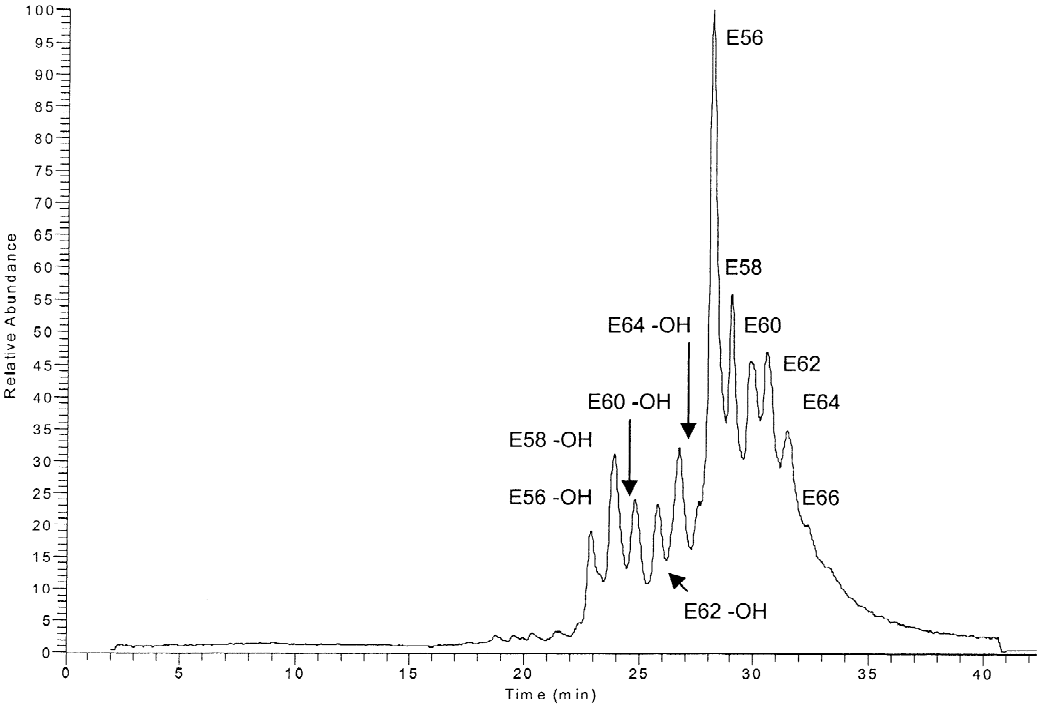


Fig. 2. Ion chromatogram over $m/z$ 820–1100 showing diesters and hydroxydiesters. E54–E64 indicate diesters with 56–66 carbon atoms, respectively. E56-OH to E64-OH are hydroxydiesters bearing 56–64 carbon atoms, respectively.

TAGs. No hydroxy esters were retrieved. The latter constituents are present in minor quantities in authentic beeswax and are more soluble in water, which explains the loss of hydroxy compounds. The close similarity between the distribution of monoesters and diesters in authentic beeswax and the lipid extracts of the cooking pots is shown in Figs. 1 and 3. Beeswax was already found in some other archaeological vessels [6,7,19]. The presence of beeswax in these pots was mainly attributed to use as a sealant or lamp fuel. These two uses of beeswax seem strange for Sagalassos as pine resin was commonly used to seal pots (results not shown) and in the analysed oil lamps no traces of beeswax have been found so far [13]. Here the presence of beeswax is shown in cooking pots, which is an indication that beeswax was processed in them for candle making. The use of cooking pots for purifying beeswax to make candles in antiquity was described by Columella [20].

Next to these wax esters, several TAG and diacylglycerol (DAG) molecules were found. A large proportion of these TAGs was saturated indicating that the TAGs were derived from animal fat. The identified TAGs are listed in Table 2. Triacylglycerol and diacylglycerol molecules were showing next to $[MH]^+$ and $[MH–RCOOH]^+$ ions, $[MH–2RCOOH+18]^+$ acylium ions. It was possible to determine for some TAGs the positions of the fatty acids on the triacylglycerol based on the intensities of the $[MH–RCOOH]^+$ ions. As the fatty acids in the *sn*-2 position form less abundant ions than in the *sn*-1/3 position, the regioisomeric composition of the triacylglycerol can be estimated [21]. However the regioisomeric positions of the constituting fatty acids could not be unambiguously determined for all triacylglycerols present as some triacylglycerols co-eluted (Table 2).

The estimated relative concentrations of the saturated TAGs in the cooking pots and in pieces of meat from lamb, pork, beef and chicken, calculated using the intensities of the diacyl ions, are shown in Fig. 4.

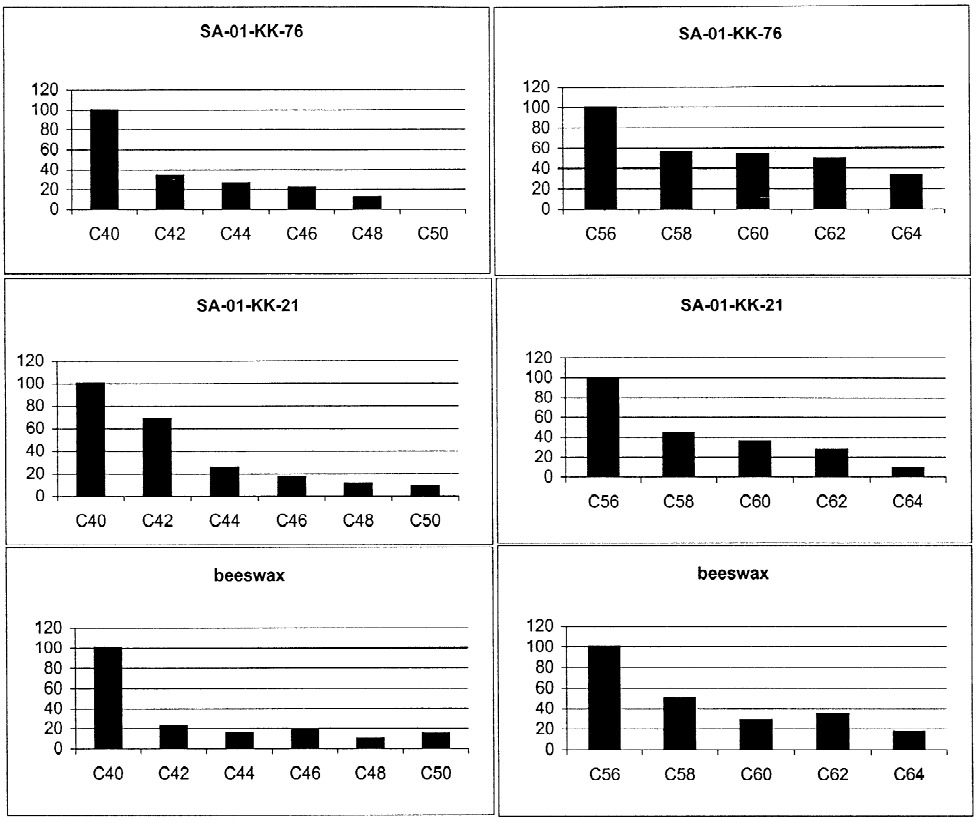


Fig. 3. Distribution of monoesters with 40–50 carbon atoms and diesters with 54–62 carbon atoms in two archaeological cooking pots and authentic beeswax.

The saturated TAGs are less susceptible to oxidation [15] and therefore this distribution can be an indication for the original fat used. From Fig. 4 it is seen that ovine fat is especially characterised by high relative concentrations of tristearin (SSS), while lard and poultry fat contain relatively high concentrations of tripalmitin (PPP). Both archaeological cooking pots contain a high concentration of distearyl-palmitoyl-glycerol (PSS) and tristearin. These high concentrations of tristearin are an indication for the presence of ovine fat, certainly in the case of SA-01-KK-21. If the animal fat originated from pork this would also be recognized by high amounts of palmitic acid in the *sn*-2 position. However palmitic acid was not present in the *sn*-2 position in *rac*-LOP as would be the case for lard [22], which again excludes this source of animal fat.

### *3.3. Polar phase-GC*

In both cooking pot samples *trans*-vaccenate, C18:1(11*tr*), was seen. *Trans*-fatty acids are in nature predominantly formed by ruminants and as such are biomarkers for ruminant fat [23]. In cooking pot SA-01-KK-21 stearic acid was present in larger concentration than palmitic acid indicating that sheep fat was processed in this pot, as this is the only source of ruminant fat that contains such high concentrations of stearic acid [24]. Notwithstanding the caution that is needed to compare or produce statistics on fatty acid distributions of archaeological fats and recent animal fats or olive oil, the ratio of C16:0 to C18:0 of the cooking pots was compared with recent sheep fat. These long chain saturated fatty acids are more stable to degradation in com-

Table 2
Observed mass spectra of triacylglycerols in cooking pot sherds

| Retention time (min) | Mass spectra | | | | Identification | 21[a] | 76[a] |
|---|---|---|---|---|---|---|---|
| | [MH−R1COOH]+ | [MH−R2COOH]+ | [MH−R3COOH]+ | [MH]+ | | | |
| 21,63 | 495 | 523 | | | *rac*-MMP | – | |
| 22,67 | 523 | 551 | | | *rac*-MPP | – | – |
| | 549 | 575 | 577 | 831 | PoPO[a] | – | |
| | 575 | 577 | 601 | 857 | *rac*-LOP | – | |
| | 601 | 603 | | 883 | *rac*-OOL | – | |
| 23,72 | 551 | 577 | | 833 | PPO[a] | – | – |
| | 577 | 603 | | 859 | OOP[a] | – | – |
| | 603 | | | 885 | OOO | – | – |
| | 551 | | | | PPP | – | – |
| 24,82 | 577 | 579 | 605 | 861 | *rac*-OPS | – | OPS[b] |
| | 551 | 579 | | | *rac*-PPS | – | – |
| 26 | 605 | 607 | | 889 | *rac*-OSS | – | |
| | 579 | 607 | | | *rac*-PSS | – | – |
| | 607 | | | | SSS | – | – |
| – | Present | | | | | | |

| Abbreviation | Trivial name | Carbon number degree of unsaturation |
|---|---|---|
| M | Myristic | 14:0 |
| P | Palmitic | 16:0 |
| Po | Palmitoleic | 16:1 |
| O | Oleic | 18:1 |
| S | Stearic | 18:0 |
| L | Linoleic | 18:2 |

[a] Sample number SA-01-KK.
[b] The positional distribution of the fatty acids was not determined; therefore the listing of fatty acids is random.

parison with unsaturated fatty acids. On the other hand it was shown that palmitic acid can be formed from oleic acid through microbial decay in anaerobic circumstances [25]. Therefore the results of the ratio of stearic to palmitic acid need to be interpreted with caution. However in the case of Sagalassos, the sherds were not buried in an anaerobic environment. Further, it was reported by Dudd et al. [26] that the relative proportions of the major fatty acids (C16:0 and C18:0) did not significantly change during oxic decay of sherds impregnated with milk fat. It can thus be reasoned that the ratio of C16:0 divided by C18:0 will reflect the ratio from the lipid source originally used. The results of statistical comparison of the cooking pots and sheep fat are shown in Table 3. The ratios of C16:0 to C18:0 from the oil lamps were also incorporated [13] to check once again the contents of these artefacts. The statistical test showed that the lipids in the cooking pots resembled sheep tallow. These results fit nicely with the other indications of the use of ovine fat such as the distribution of saturated triacylglycerols obtained with the LC–APCI-MS.

The ratio of saturated fatty acids from the oil lamps differed from the cooking pots and from olive oil. It was smaller than that of olive oil, which indicates mixing with animal fat or other vegetable oils with a smaller ratio of C16:0 to C18:0 as was also illustrated by the distributions of triacylglycerols retrieved in these oil lamps [13].

## 4. Conclusion

GC–MS and LC–APCI-MS were used for the analysis of lipid extracts from archaeological cooking pots. Beeswax was recognized in both cooking pots by identification of alkanes, mono- and diesters.

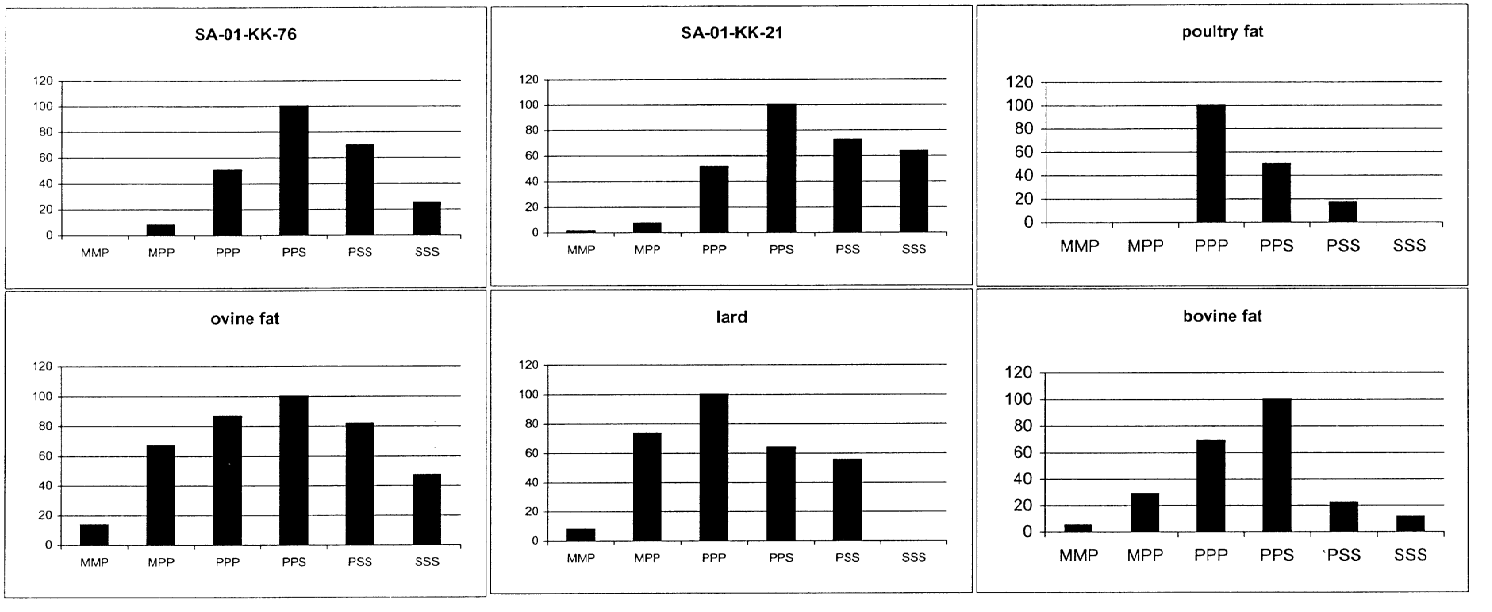


Fig. 4. Distribution of saturated triacylglycerols in archaeological cooking pots and reference materials. For abbreviations, see Table 2.

Table 3
Average ratio of C16:0/C18:0 of cooking pots containing beeswax and oil lamps from the archaeological site Sagalassos, fresh olive oil and sheep tallow

| | C16:0/C18:0 |
|---|---|
| Cooking pots ($n=2$) | $1\pm0.1^{c}$ |
| Sheep tallow | $0.8^{c}$ |
| Oil lamps | $3\pm0.7^{b}$ |
| Olive oil ($n=4$) | $4.6^{a}$ |

*n*, number of samples analysed. Values followed by the same letter do not differ significantly as regards relative proportion of the ratio C16:0/C18:0. Statistical significance determined at $P>0.05$ according to a one-side *t*-test for comparison of the means of oil lamps and cooking pots with sheep tallow and olive oil. Statistical significance determined at $P>0.05$ according to Duncan's mean separation test for comparison between cooking pots and oil lamps.

Next to beeswax, the presence of animal fat was shown by the presence of high quantities of saturated TAGs. This fat was determined to be ruminant fat due to the recognition of trans-fatty acids. The ratio of palmitic to stearic acid then further identified the animal fat as sheep tallow.

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