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^{1}H NMR, ^{1}H–^{1}H 2D TOCSY and GC-MS analyses for the identification of olive oil in Early Bronze Age pottery from Castelluccio (Noto, Italy)

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The development of analytical research in recent decades, at the edge between analytical chemistry and archaeology, provides new methods for the study of organic residues that are usually highly sensitive to natural decay. Using Nuclear Magnetic Resonance (NMR) and Gas Chromatography-Mass Spectrometry (GC-MS), it is now possible to chemically identify a series of natural substances preserved in archaeological environments. This paper presents a protocol to detect natural compounds, such as olive oil, from amorphous organic residues discovered inside the pores of prehistoric pottery from the Early Bronze Age settlement of Castelluccio (Noto, Italy), and dated to the end of the 3rd and the beginning of the 2nd millennium Before the Common Era (BCE).

1. Introduction

In recent decades, chemical residue analysis has proven to be very important in answering archaeological questions.[1,2] Very different disciplines have joined together to form new branches of research such as biomolecular archaeology. Many aspects of daily life in the past that remain unknown can now be investigated using advanced analytical methods. Archaeological ceramics occupy a place of absolute importance in the artifacts that have come down to us since prehistoric times.

The analysis of organic residues on ceramics employs analytical organic chemical techniques to identify the nature and origins of remains that cannot be characterized using traditional techniques of archaeological investigation (because they are either amorphous or not visible). This field is based upon the principle that the biomolecular or biochemical components of organic materials associated with human activity survive in a wide variety of locations and archaeological deposits. The archaeological information contained in organic residues is represented by the biomolecular components of natural products that contribute to the formation of a given residue.

Among the most important prototypical Mediterranean products, vegetable oil can readily be distinguished by analysis of lipid traces.[1] This kind of analysis is very useful to detect many different traces of lipids and it is possible to identify the original source, such as animal fat[3] or fish remains, even if it is much more complicated due to polyunsaturated fatty acids being extremely susceptible to deterioration. Some authors[4] employed different monounsaturated fatty acids like hexadecenoic acid ($C_{16:1}$), eicosenoic acid ($C_{20:1}$) and docosenoic acid ($C_{22:1}$) as biomarkers for cod and herring, but at least hexadecenoic acid is found in a large variety of animal and vegetable fats, implying that unambiguous identification in mixtures is problematic in many cases. In contrast, the search for wine residue markers offers specific molecules such as tartaric acid, syringic acid and polyphenols that allow us to establish with high confidence the presence of drinks from grapes or fermented fruits. This issue has been widely studied and many studies have been conducted to refine existing analytical techniques[5–14] and to introduce new or unused methodologies.[3,15] Experimental testing using replica vessels or replication of the hypothesized processes provides the necessary means to understand the problems related to the interpretation of chemical signatures in pottery, both in terms of distribution of organic compounds in the ceramic matrix and the diagenetic degradation of those compounds and of principles of porous absorption or interaction with environmental molecules in long time processes.[16–21] For this reason, the use of replicas as an internal standard is crucial for a better interpretation of the data. In an experiment involving cooking vessels[16] lipid accumulation was observed at the rim due to lipid flotation during boiling. In confirmation of this, in another study[19] a similar pattern was noted with gas chromatography coupled

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to mass spectroscopy (GC-MS) when roasting meat in a cooking jar and a similar pattern was found with ^{1}H NMR analysis using pork meat roasted in ceramics.[3] Finally, valuable information concerning fatty acid concentration gradients across potsherds was revealed.[20] In this study we applied residue analyses to study the content of some ceramic vessels in order to ascertain whether they were truly used to store vegetable oil, to process food with such oils, or to press olives and separate them from the aqueous component as archaeologists have traditionally inferred just on the basis of the formal typology of the vessels.

With regards to the prehistory of the Italian peninsula, it is emblematic to observe how such an analytical approach is largely underestimated if not neglected. In the recent *50th Riunione Scientifica dell'Istituto Italiano di Preistoria e Protostoria*, the most important Italian conference on prehistory, entitled *Preistoria del cibo. L'alimentazione nella preistoria e nella protostoria* (Roma, 5–9 October 2015), aimed to showcase the state-of-the-art in the field of study of ancient dietary habits, but hardly any presentations dealt with chemical analysis of organic residues in pottery. Prehistoric Sicily as a case study does not represent an exception.

In this perspective, this paper represents the first attempt to test the potential of this approach and to explore the possibilities that this method can offer through the study of a group of materials from Castelluccio (Noto, Italy), the type-site for the Sicilian Early Bronze Age.

2. Materials

2.1 The Early Bronze Age settlement of Castelluccio

The prehistoric settlement of Castelluccio is located on a plateau bordered by the valley of Cava della Signora in the hilly region between Noto and Palazzolo Acreide. The site, well known in the archaeological literature for being the type-site for the Sicilian Bronze Age, was excavated at the end of the 19th century by Paolo Orsi,[22,23] who explored a large cemetery of rock-cut tombs. At the end of the 1980s, some illegal excavations prompted the resumption of fieldwork with a systematic investigation of the site which, through various campaigns directed by Giuseppe Voza, ended in 1997. The excavation focused on two areas, one named Piano Sella and located right above the cemetery, and another on the northwestern rocky ridge where there are the ruins of the medieval Castelluccio, dominating the valley of the Tellaro River below. In the latter area, 12 huts were identified[24] and two contexts (Hut 2 and Hut 8) were also recently radiocarbon dated.[25,26]

2.2 Ceramics from Hut 8

Hut 8 is certainly the most relevant edifice uncovered in terms of dimensional scale and architectural features. It has a North-East/South-West orientation and an elliptical plan (18 × 6.50 m) with a perimeter structure partly dug in the bedrock and partly built with rubble masonry integrated with wooden logs (Fig. 1). According to radiometric analyses it was abandoned in 3610 ± 23 BP (MAMS-22285: 1σ cal BCE 2018–1937; 2σ cal BCE 2028–1904) and 3586 ± 23 BP (MAMS-22286: 1σ cal BCE 1959–1897, 2σ cal BCE 2018–1886).[25,26] The exceptional dimensions and plan of Hut 8 in comparison with the others suggest that it may have had a special function.

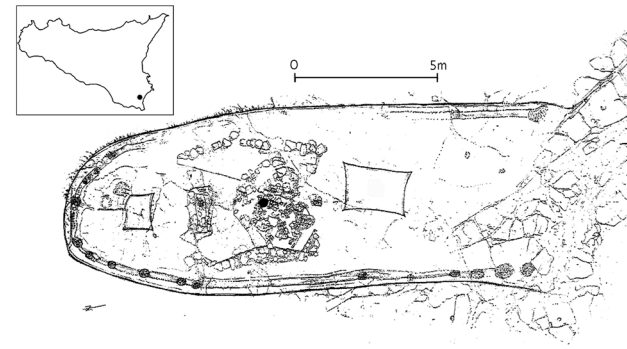

Fig. 1 Plan of Hut 8 of the settlement at Castelluccio with the find spot of the pithos in US 75.[27]

In 2015–2016, the reappraisal of a large amount of ceramics and findings recovered during the excavation began with a particular focus on the materials from stratigraphic units 42A-B and 75, which represented the main phase of use of the hut. At the center of the hut, in US 75, over 400 sherds of a *pithos* decorated with rope bands were found by a large terracotta cooking plate. Once restored, the ovoid *pithos* was 104 cm tall with two sets of three vertical handles and its peculiar decoration was identified as a typological *unicum* of the Early Bronze Age pottery repertoire[27] (Fig. 2). Nearby, in US 42A-B, two fragmented basins with an internal septum were also found, further examples of another very peculiar shape, designed to keep multiple substances together but separated[26] (Fig. 3 and 4).

Due to the particularly interesting context and uniqueness of the three artefacts, it was decided to carry out chemical analyses on organic residues in order to identify their contents and use that result to ascertain their function. A sample called 'US 75' was taken from the pithos found in US 75 and samples 'US 42A' and 'US 42B' were taken from the basins found in US 42A and in US 42B, respectively.

3. Methods

3.1 Analytical techniques

The analytical techniques chosen to investigate samples taken from the three artifacts were Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR), two techniques traditionally and successfully used on archaeological pottery.[3,19,29–32]

3.2 Sample preparation and analysis

Pottery sherd samples from US 42A and US 42B were taken from the inner surface of the sherd and ground into a fine powder using a methanol cleaned drill. The residue found on US 75A was ground into a powder using a methanol cleaned mortar and pestle. For GC-MS analysis, about 0.5 g of powdered sample was weighed and inserted into a 10 ml VOA vial, then 10 μg of *n*-tetratriacontane (Sigma Aldrich) was added as an internal

Fig. 2 Pithos from Hut 8, US 75.[28]

Fig. 3 Basin with a central septum, US 42A.[26]

standard with $CHCl_3$: MeOH in a 2 : 1 ratio (Fischer Scientific). The samples were sonicated for 30 minutes in a water bath and then centrifuged at 2500 g for 25 minutes. The organic layer (TLE) was removed, and the solvent was evaporated with a gentle stream of nitrogen and placed in a vacuum desiccator

Fig. 4 Basin with a central septum, US 42B (photo by authors).

overnight to dry the sample. Dried TLE samples were derivatized with 0.5 mL *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (ThermoFisher) and heated to 40 °C in an oil bath for fifteen minutes. The temperature was then dropped to room temperature and left overnight. The silylated samples were analyzed on a Shimadzu GCMS-TQ8040 in Q3 scan mode on a 30 m Rxi-5 Sil MS (Restek) capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm. 1.5 μL of the sample was injected in the split mode at a temperature of 250 °C. The oven temperature was held at 50 °C for 2 min, increased to 320 °C at 10 °C min^{-1} followed by an isothermal 20 min hold at 320 °C. The mass spectrometer was held at a temperature of 320 °C. The spectra were recorded between 40 and 800 m/z at 0.15 s per scan.

Lipid extraction for NMR analysis was performed using 80 mg of the powdered pottery sherd and with powdered standards of powdered pig bone, olive oil, and shortening. The 80 mg samples were suspended in a mixture of $CDCl_3$: CD_3OD : D_2O in a 1 : 1 : 0.9 ratio and sonicated in a water bath for 1 hour. The samples were then transferred into an Eppendorf tube and briefly centrifuged at high speed to separate the layers. The lower chloroform layer was removed and used for NMR analysis. All the NMR spectra were acquired at 300 K with a direct-drive Agilent NMR spectrometer operating at a proton frequency of 600.13 MHz. The mono-dimensional (1D) ^{1}H NMR spectra were recorded for the samples of olive oil, US 42B, US 42A, and US 75 using a pre-saturation method to suppress the water signal. For each sample 512 scans were collected for the ^{1}H spectrum with a 90° pulse length of 10.5, 10.2, 9.8, and 10.2 μs, respectively, and a 2 s interscan recycle delay. The resultant spectra acquired with a time domain of 320k were zero filled, and Fourier transformed with 64k data points. Only for the sample US 75, a ^{1}H–^{1}H two dimensional (2D) total correlation spectroscopy (TOCSY) experiment was performed with 2k and 2k points in $F1$ and $F2$ dimensions, respectively, using 32 scans with a ^{1}H spectrum 90° pulse length of 10 μs and a 1 s interscan recycle delay. Both 1D and 2D NMR spectra were processed using either VnmrJ, Topspin 3.2 and NMRPipe. The spectra were analyzed using Topspin 3.2 and Sparky software.

4. Results and discussion

Lipids and fatty acids in particular constitute the most common class of biomarkers utilized to reveal the function of archeological artifacts. This is due to their favorable chemical stability over other organic residues as well as their relatively high abundance within the walls of archaeological artifacts. To test whether the three vessels carry useful lipid residues we first used simple 1D ^{1}H NMR. Brief sonication of potsherd powder in a $CDCl_3$: CD_3OD : D_2O (chloroform-d : methanol-d4 : deuterium oxide) solvent system, followed by NMR analysis of the $CDCl_3$ layer, provides a rapid assessment of the amount of extracted lipids as well as a preliminary profiling of their composition.

Indeed, the ^{1}H NMR spectra of US 42A, US 42B and US 75 (Fig. 5) all show typical patterns of lipid-containing samples. Two intense multiplet signals appear in the aliphatic region at 0.78 ppm and 1.90 ppm, corresponding to the $-(CH_2)_n$, and $-(CH_2)_n-CH_3$ protons, respectively, as well as several other signals that can be attributed to free fatty acids and lipid fragments. In addition, a set of two signals, one at 5.25 ppm and one at 1.9 ppm, corresponding to $-CH{=}CH-$ and $-CH_2-CH{=}CH-$ protons, respectively, indicate the presence of unsaturated carbon chains. However, as evident from their relative intensity, the abundance of unsaturated chain fragments is significantly lower as compared to saturated chain fragments. To verify that the $-CH{=}CH-$ functional groups are parts of a larger aliphatic chain we ran ^{1}H–^{1}H 2D TOCSY on the sample of US 75. These

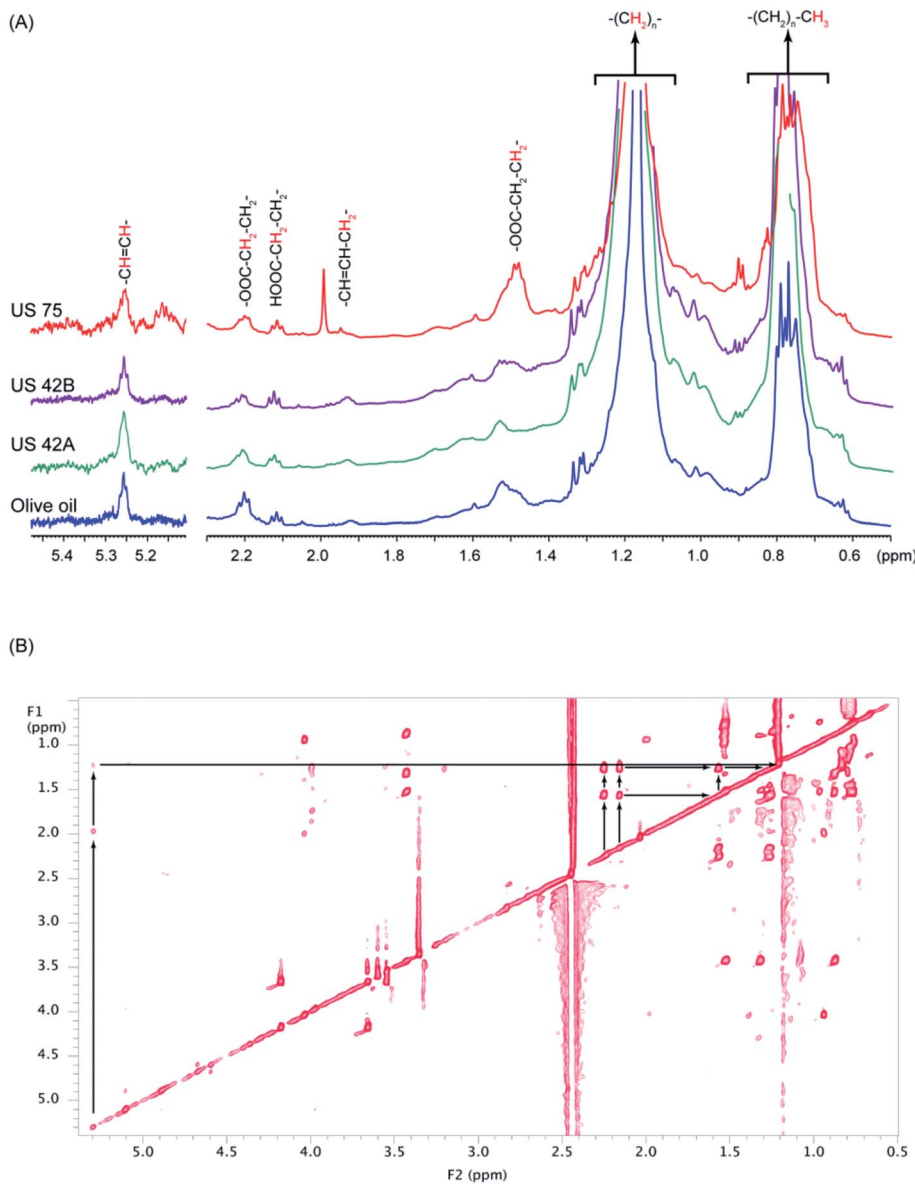

Fig. 5 NMR analysis of total lipid extracts. (A) Comparison of two selected regions of the ^{1}H 1D NMR spectra of US 75, US 42A and US 42B with the spectrum of a standard sample derived from a pot that contained olive oil. Assignments of different groups that are found in saturated and unsaturated fatty acids are provided on the top. (B) Expanded region of the ^{1}H–^{1}H 2D TOCSY spectrum of US 75, showing connectivities between different spin systems, including those of unsaturated and saturated fatty acids.

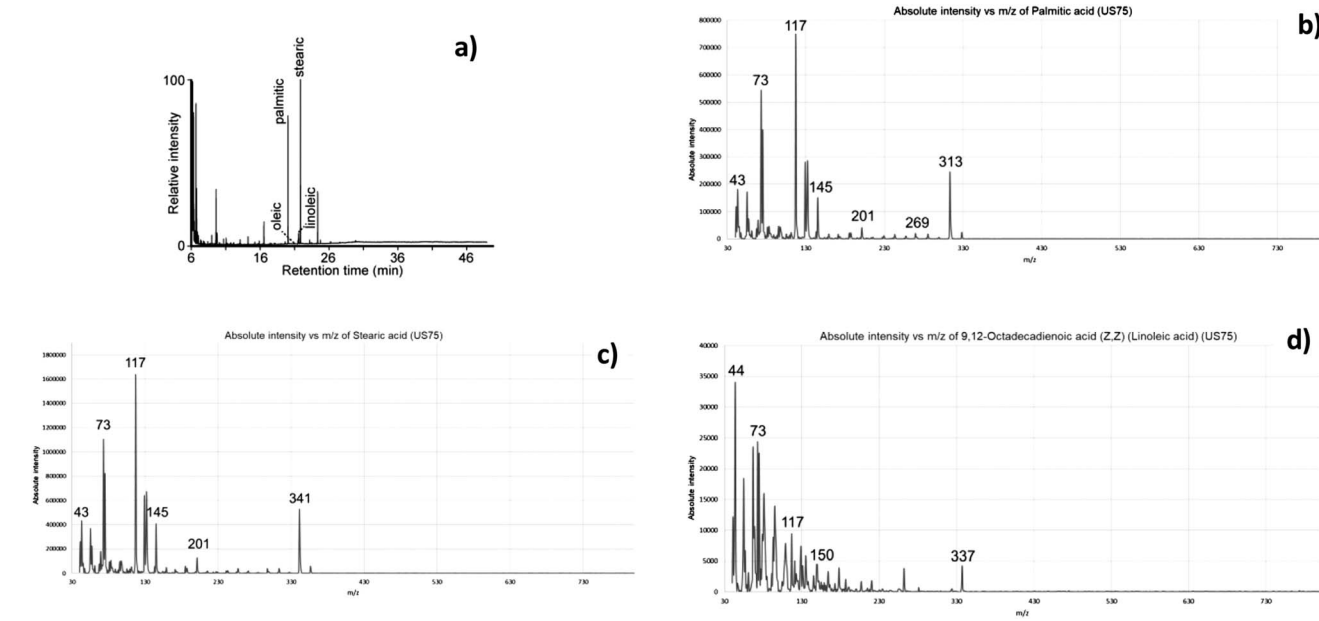

Fig. 6 (a) GC-MS analysis of the sample US 75 showing the positions of palmitic, stearic, and linoleic acids and absolute intensity *vs. m*/*z* of (b) palmitic acid, (c) stearic acid, and (d) linoleic acid for the same sample.

types of spectra allow establishing scalar connectivities of proton sites that are separated by multiple covalent bonds and assist in unambiguously identifying functional groups within molecular fragments. Evidently, such cross peaks are observed (Fig. 5) between the –C*H*═C*H*– signals and the –CH_2–CH═CH– and –(CH_2)$_n$ group, reinforcing the notion that unsaturated chains comprise part of the total lipid extract of the three samples.

Next, we sought to characterize the origin of the lipids identified. Based on the shape of the *pithos* from which sample US 75 was prepared and the fact that it was found by a terracotta cooking plate, we reasoned that it functioned as a liquid container, possibly for the storage of oil, as this is the traditional interpretation for *pithoi* of this size and type in the Sicilian Bronze Age. Similarly, the two basins from which US 42A and US 42B samples were prepared could have served as food processing tools. To test the traditional hypothesis, we

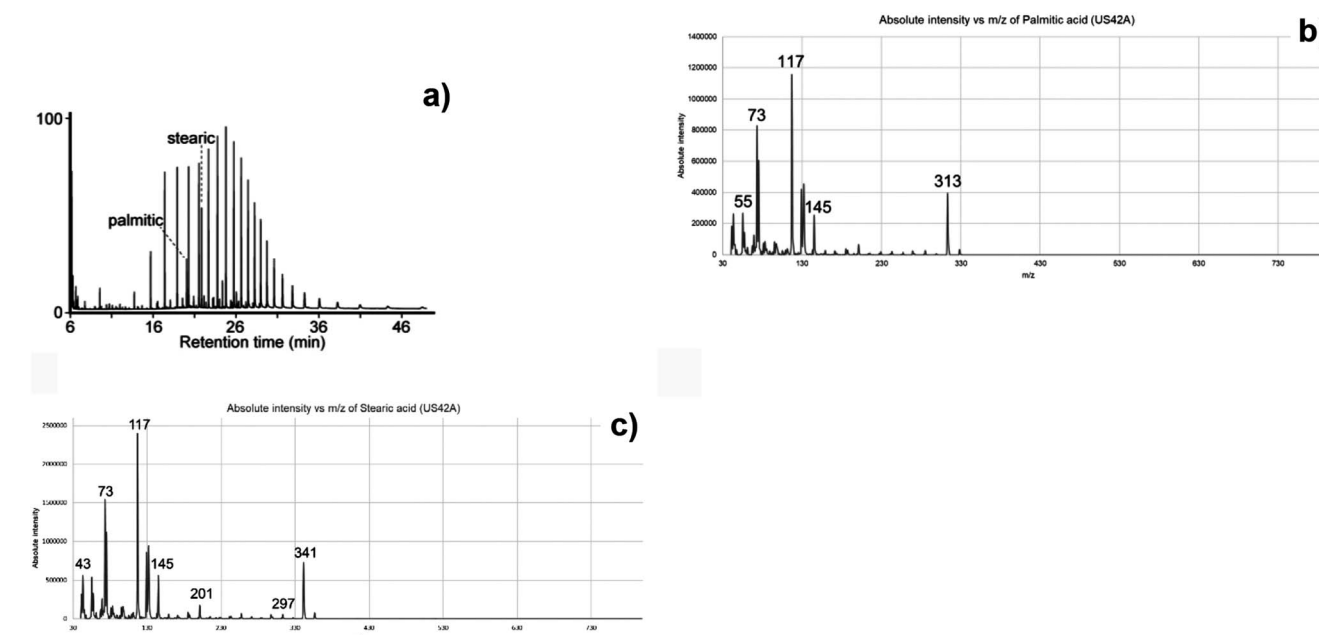

Fig. 7 (a) GC-MS analysis of the sample US 42A showing the positions of palmitic and stearic acids and absolute intensity *vs. m*/*z* of (b) palmitic acid and (c) stearic acid for the same sample.

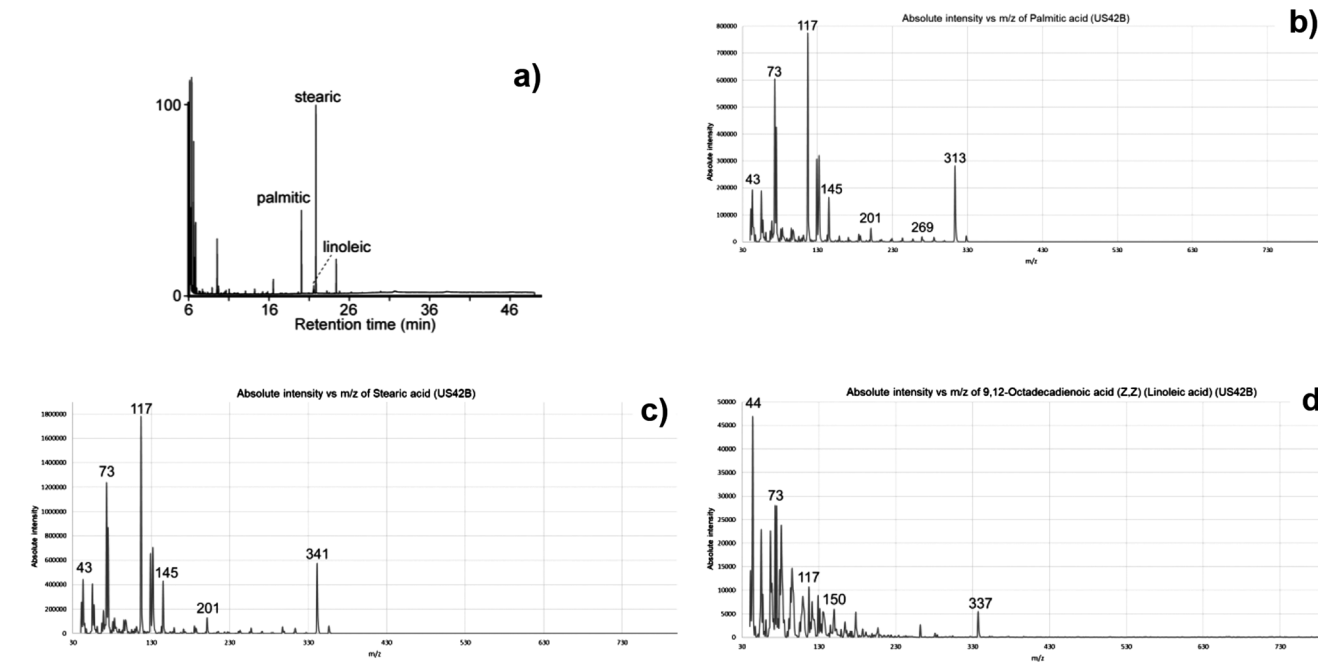

Fig. 8 (a) GC-MS analysis of the sample US 42B showing the positions of palmitic, stearic, and linoleic acids and absolute intensity *vs.* *m*/*z* of (b) palmitic acid, (c) stearic acid, and (d) linoleic acid for the same sample.

compared the ^{1}H 1D spectra of the three samples, to the ^{1}H 1D spectrum of a control sample prepared from a pot that contained olive oil. The lipid composition of olive oil is overall similar to the composition of other vegetable oils, and we would expect that this comparison yields an indication of origin. As shown in Fig. 5, there is an excellent correspondence between the spectra of the three samples and the control olive oil sample both in terms of chemical shift, *i.e.* the position of each signal in the spectrum, and in terms of multiplicity, *i.e.* the splitting of each of the signals. Thus, it is evident that all three potsherds contain organic residues that have the same spectroscopic signature as vegetable oils, if not olive oil.

Nevertheless, the severe signal overlap observed in the ^{1}H 1D spectra of the total lipid extract of US 42A, US 42B and US 75 diminishes any resolving power of the technique, since it does not permit identification of isolated fatty acids or other signature compounds and therefore further evaluation of the origin of the residues. To circumvent this problem we performed a similar extraction protocol, using $CHCl_3$: MeOH in a 2 : 1 ratio as a solvent system, and employed GC-MS as the analytical tool for identification of TMS derivatives. For all three samples, the most abundant fatty acids were palmitic ($C_{16:0}$) and stearic acids ($C_{18:0}$) (Fig. 6–8), while for US 42A a set of large signals originating from siloxanes and *n*-alkanes were also observed, but were omitted from the study. Since palmitic and stearic acid are ubiquitously detected in lipids of both animals and vegetables, they cannot be used as appropriate biomarkers to assign a particular origin to the organic residues. However, in addition to these two fatty acids, US 75 contains the unsaturated, oleic acid ($C_{18:1}$) and the polyunsaturated linoleic acid ($C_{18:2}$), and US 42B contains linoleic acid. Although the relative amounts of oleic and linoleic acids detected in US 75 and US 42B are significantly lower as compared to those of $C_{16:0}$ and $C_{18:0}$, unsaturated fatty acids are far more susceptible to decomposition and conversion to saturated fatty acids. Both oleic and linoleic acids form the major constituents of vegetable oils at different proportions, and therefore it is very likely that the residues identified in US 75 and US 42B originate from vegetable oil and most likely olive oil. Finally, the similarity of US 42B and US 42A in terms of the shape of the corresponding pottery suggests that the organic residue identified in the latter is also of vegetable oil origin, despite the fact that oleic acid was not detected in US 42A, possibly due to siloxane and *n*-alkane contamination.

5. Conclusions

The comparison of 1D ^{1}H NMR spectra of the samples US 42A, US 42B and US 75 overlap very well with the 1D ^{1}H NMR spectrum acquired for an olive oil standard sample. In particular, both the chemical shift and multiplicity of signals in the region between 2.5 and 0.5 ppm indicate a high compatibility with the olive oil standard sample. The use of ^{1}H–^{1}H 2D TOCSY allowed us to unambiguously assign the signal of an unsaturated double bond (–CH═CH–) at 5.3 ppm as a functional group of a larger fragment that contains multiple –CH_2– groups, which is a strong indication of the presence of unsaturated positions in the fatty acid chains. This was further confirmed by the use of GC-MS and the identification of oleic and linoleic acids, in addition to palmitic and stearic acids. In summary, the combined information obtained from NMR and GC-MS allows us to confirm the presence of olive oil in all the three samples.

The identification *via* chemical analyses of olive oil on Sicilian tableware dated to the end of the 3rd and beginning of the 2nd millennium BCE represents a very remarkable discovery.

The earliest olive cultivation and olive oil production in the Mediterranean, dating back to the Copper Age for some case studies in Israel, are usually well documented just from archaeological (mills and olive pressing vessels) and archaeobotanical perspectives (pollen, olives, wood, and leaves).[33] The first chemical signature of olive oil has been identified on samples from Minoan Crete: Aphrodite's Kephali (Early Minoan I *ca.* 3200–2700 BCE),[34] Chrysokamino (Early Minoan III-Middle Minoan IA 2300–1900 BCE),[35] and Tourloti (Late Minoan IIIC 1200/1190–1070 BCE).[36]

With regards to the prehistory of Italy, the only cases known of identification of chemical signatures of olive oil are those of Broglio di Trebisacce (Cosenza) and Roca Vecchia (Lecce) where large storage jars (*dolia*) dated to the local Late Bronze Age (12^{th}–11^{th} century BCE) tested positive.[37–39]

In this perspective, the results obtained with the three samples from Castelluccio become the first chemical evidence of the oldest olive oil in Italian prehistory, pushing back the hands of the clock for systematic olive oil production by at least 700 years.

Besides the important historical and archaeological implications that this discovery has for the interpretation of the function of Hut 8 and its role in the ancient prehistoric community of Castelluccio, which will be the subject of another specific work, this research has once again confirmed that 1D ^{1}H NMR and ^{1}H–^{1}H 2D TOCSY are viable approaches for analyzing prehistoric artifacts. Furthermore, the combined use of these methods and GC-MS enhances by far the chance to unambiguously assign different samples in ^{1}H NMR spectra. In fact, the intrinsic ability of GC-MS to filter each sample based on their retention time and associated MS spectra (charge-to-mass ratio) helps identify or screen different types of molecules present in the archaeological materials subject to analysis.

Conflicts of interest

There are no conflicts to declare.

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