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A medium throughput approach for improved taxonomic identification of lipids preserved in ancient pottery

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Keywords

Organic residue analysis; Triacylglycerols; MALDI; Medieval Sicily; Dairy products; Plant oils

Abstract

Organic residue analysis (ORA) is a valuable tool for the study of ancient diets, but conventional methods remain limited in terms of taxonomic identification or to resolve mixtures. Here, we propose a method to further explore a class of compounds, triacylglycerols (TAGs), using high-resolution mass spectrometry (MS) to overcome these limitations in an attempt to better characterise culinary practices. Over 70 medieval Sicilian pots and a wide range of authentic fresh products were studied by matrix-assisted laser desorption/ionisation-mass spectrometry (MALDI-MS and MALDI-MS/MS). MALDI-MS analysis can distinguish fresh foodstuffs, but provides little additional information regarding the contents of archaeological pottery compared to conventional ORA methods. In contrast, product ion analyses were able to deconvolute a range of animal carcass fat mixtures. In addition, detailed analysis of the composition of saturated T_{44} and unsaturated T_{50}-T_{54} TAGs was able to provide greater taxonomic resolution regarding dairy products and plant oils.

Introduction

Cuisine is a vehicle of cultural and social identity. Cuisines often define cultural groups and distinguish sub-groups within these, for example by social status. The choice of products for consumption, the method of transformation (e.g. boiling, roasting, steaming, fermenting), how foods are mixed to create recipes and how they are served constitute the fundamental elements of culinary traditions. Before written records, i.e. in prehistoric and protohistoric contexts, culinary traditions are hard to decipher, and in later periods (historic contexts) written records tend to focus on the upper echelons of society and places of power. Faunal and botanical remains offer insight into the availability of resources and various cooking utensils (pots of specific shapes, oven, etc.) provide some information into how these might have been processed. Organic residue analysis (ORA) of artefacts, on the other hand, potentially offers a more direct approach to study ancient cuisines. It provides a method of identifying which commodities have been processed with which device. However, conventional ORA methods rarely provide a detailed picture of the natural origin of the products and are often unable to adequately characterise mixtures.

One of the main methods of foodstuff identification in ORA is the characterisation of stable carbon isotopes of palmitic and stearic acids (Evershed et al. 1994; Regert, 2011). However, the determination of animal fats is limited to the distinction between ruminant and non-ruminant carcass fats, dairy products and marine and freshwater oils and does not provide information on plant products (Steele et al. 2010), except for the distinction between C_3 and C_4 plants. Isotopes therefore only offer a general characterisation of the type of food with limited taxonomic resolution.

In addition, mixtures of foodstuffs can lead to false interpretation of stable carbon isotope values. For example, a 50:50 mixture of non-ruminant carcass fat and dairy fat can be mistaken for ruminant carcass fat (Copley et al. 2005; Evans et al. 2023), the addition of marine oil to a terrestrial fat can lead to the erroneous identification of dairy products (Courel et al. 2020), and mixing millet with non-ruminant fats can produce values consistent with ruminant carcass fat (Taché et al. 2021). Isotope models offer a way of deconvoluting simple mixtures of two commodities when endpoints for pure references are known (Fernandes et al. 2017). However, difficulties remain regarding complex mixtures (more than two products), unknown endpoints, the impact of fatty acid concentration in mixtures and the criteria to use when marine and C_4 plants are mixed.

Proteins preserved in archaeological pottery can provide precise information about the taxonomic origin of the products contained in the pots, but they seem to be preserved only in very specific contexts (e.g. Craig et al. 2000; Evans et al. 2023; Hendy et al. 2018).

When the acyl lipids are preserved, an alternative approach is to study triacylglycerols (TAGs), usually by high temperature gas chromatography (HT-GC), sometimes coupled to mass spectrometry (GC-MS). From these data, the TAG profile of archaeological samples is usually compared with those of modern authentic products (e.g. Dudd et al. 1999; Evershed et al. 1997; Kimpe et al. 2002). Ruminant and non-ruminant carcass fats and dairy products can thus theoretically be distinguished, but in practice the comparison between archaeological extracts and modern product profiles is not straightforward. This is due, on the one hand, to the potential mixing of products and, on the other hand, to natural degradation mechanisms that alter the profiles by preferentially degrading the most unsaturated and shortest TAGs (Dudd et al. 1998; Dudd and Evershed, 1998; Nawar, 1969).

In addition, the degree of unsaturation is generally not considered because two TAGs with the same number of carbon atoms that differ by one unsaturation are too close in polarity to be adequately separated on apolar columns used in HT-GC, which considerably limits the identification of plant oils.

The study of the content of pottery from early medieval Sicily (Drieu et al. 2021; Lundy et al. 2021, 2023) is a typical example of the challenges encountered in interpreting ORA data to study food and foodways. Compound-specific stable carbon isotope values and molecular distributions suggest a widespread mixing of contents. The information from compound-specific stable carbon isotope analyses and TAG profiles is also contradictory in some cases, probably due to the presence of complex mixtures that are difficult to deconvolute. In addition, some samples display characteristic patterns of plant oil or dairy products, but it is not possible to determine the species that produced them. Despite major political and religious transitions between the 6th and 13th centuries in Sicily, conventional ORA methods identified only tenuous changes in culinary practices. Whilst these results suggest that local culinary practices remained consistent throughout these transitions, it is possible that more nuanced differences are hidden.

For example, the consumption of dairy products appears to be heterogeneous across Sicily, depending on the type of site (rural/urban) and the chronological period. This picture of the culinary use of milk in medieval Sicily lacks information on the species exploited for their milk, while this involves very different economic strategies, and can have implications for cooking and consumption (fat content,

lactose content, etc.). Were the same species milked at all sites and at all periods? Were certain milks favoured for the manufacture of certain culinary products?

As for plant oils, archaeobotanical studies suggest that the degree of olive cultivation and consumption in Sicily varied between the 6th and 13th centuries (Chowaniec et al. 2021; Fiorentino et al. 2022, 2024; Michelangeli et al. 2022; Stella and Fiorentino 2022; Tinner et al. 2009). Did this have an impact on trade and foodways? Were other plant oils used instead of olive oil, such as linseed and cotton, cultivated in Sicily in Late Antiquity and the Middle Ages? While the excellent state of preservation of the charred cotton seeds found in medieval Sicily sites rules out their use for oil production, the cultivation of a specific type of flax, *Linum usitatissimum* cf. convar. *mediterraneum*, with large grains particularly suited to oil production (Grasso et al. 2021), could have offered an alternative to olive oil, particularly for illumination (Fiorentino et al. in press).

Detailed TAG compositions can be highly specific to a taxon and it is this proxy that is often used in food authentication studies to address questions of adulteration (e.g. Blasi et al. 2008; Cossignani et al. 2019; Indelicato et al. 2017; Parcerisa et al. 2000; Smiddy et al. 2012). In general, the variability in fatty acid composition of TAGs between species is greater than the variation within a species, as shown in milk (e.g. Blasi et al. 2008; Mirabaud 2007, p.254-255; Smiddy et al. 2012) or plant oils (e.g. Parcerisa et al. 2000; Wiesman & Chapagain, 2009). Some work has attempted to extract precise taxonomic information from the TAGs preserved in archaeological vessels using liquid chromatography-mass spectrometry (Kimpe et al. 2002, 2001; McGovern et al. 1999; Romanus et al. 2009, 2007; Saliu et al. 2014, 2011) and mass spectrometric analyses with different ionisation sources: electrospray ionisation (ESI and nano-ESI ; Garnier et al. 2009; Mirabaud et al. 2007) and matrix-assisted laser desorption/ionisation (MALDI; Oras et al. 2017; Smith 2013a, b, 2014). These studies demonstrated that it is possible to improve the analysis of archaeological TAGs, both in terms of detection limits and accuracy in identifying certain fatty foodstuffs. Precise taxonomic information, that could not be obtained with HT-GC, was obtained for specific samples, in particular using tandem mass spectrometry, such as the identification of olive oil and for distinguishing goat's milk and cows' milk.

These studies are generally based on a small number of archaeological samples and/or fresh authentic products, which allows for a one-to-one comparison of archaeological and modern samples for identification of the commodities. However, one-to-one comparison limits the range of foodstuffs that can be screened and the number of archaeological samples that can be studied. It is therefore not possible to assess the relative importance of a specific commodity to past populations, nor to carry out studies on a large geographical or chronological scale, as permitted by compound-specific stable carbon isotopes analyses. This probably explains why the various TAG-based approaches, although they have proved valuable in identifying natural substances, have not since been routinely used in archaeology.

The aim of the present study was to implement a routine method for a thorough investigation of TAGs in archaeological ceramics. In order to keep sample preparation and analysis time to a minimum and ensure a quick and detailed analysis of the fatty acid composition of TAGs, we chose to use MALDI-MS and MALDI-MS/MS analysis (Asbury et al. 1999; Oras et al. 2017; Smith 2013a, b, 2014). MALDI was chosen as the ionisation method for its speed of analysis, ease of use, good sample throughput, and ability to generate reliable signals from even very small amounts of sample. The implementation of the MALDI analyses on a Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) brought the additional significant advantages of this instrument's excellent mass accuracy (allowing confident assignment of elemental compositions), outstanding mass resolution (which makes it possible to distinguish even low intensity TAG signals from chemical background), and the ability to increase the signal-to-noise ratio of low intensity signals by trapping the ions for longer if necessary to

optimise detection of low intensity components. In addition, it was necessary to set up a method for processing a large number of archaeological samples, comparing them with a wide range of modern foodstuffs, and ensuring adequate identification. The developed method was then used to identify, in detail, the lipid content of Sicilian pottery dating from the 6th to the 13th century AD, both in terms of taxonomic precision and mixtures.

Material and methods

Archaeological samples

Within a corpus of 296 samples (Drieu et al. 2021; Lundy et al. 2023, 2021 and unpublished data) analysed to study culinary practices in medieval Sicily, 71 samples yielded TAGs, some in very small amounts (Table S1). These originate from eight sites: Castello San Pietro (CSP), Gancia church (GA), Palazzo Bonagia (PB), Casale San Pietro (CLESP), Monte Jato (MI), Mazara del Vallo (MZ), Valle dei Templi, Quartiere Ellenistico (QER), and Rocchicella di Mineo-Paliké (RCL). Details of the ceramic samples are available in Supplementary Table 1.

The methods of extraction of lipids from archaeological samples with dichloromethane/methanol (DCM/MeOH, 2:1, v/v) and hexane after acid-catalysed methyl transesterification have been described elsewhere (Drieu et al. 2021; Lundy et al. 2023, 2021). Briefly, the potsherds were drilled after removal of the inner surface and ~2 g of powder were obtained. The samples were divided in two and an internal standard was added to each subsample (*n*-C_{34}, 10 µL, 1 mg mL^{-1}). Lipids from the first subsample were extracted three times with DCM/MeOH, concentrated under a gentle nitrogen flow and derivatised with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The second sub-sample was sonicated in methanol, before addition of sulfuric acid and heating to 70°C for 4 h. The lipids were extracted three times in hexane and concentrated. A second internal standard (n-C_{36}, 10 µL, 1 mg mL^{-1}) was added to all samples before analysis.

Samples extracted with DCM/MeOH were analysed by high temperature gas chromatography (HT-GC) to determine the concentration of preserved lipids in the potsherd extracts, to identify samples in which TAGs were preserved, and to plot the TAG profiles. Analyses were carried out using an Agilent 7890A Series gas chromatograph equipped with a DB1-High Temperature apolar column (15 m × 0.32 mm × 0.1 µm) and fitted with a flame ionisation detector (FID). The samples were injected in splitless mode, with helium as the carrier gas, and the temperature programme was as follows: 50°C for 2 min, then a rise to 375°C at a rate of 10°C min^{-1} and a temperature plateau at 375°C for 10 min. When the amounts of lipids resulting from acidic methanol treatment were sufficient (> 5 µg g^{-1}), measurements of the stable carbon isotopes of palmitic and stearic acids ($\delta^{13}C_{C16:0}$ and $\delta^{13}C_{C18:0}$, respectively) were performed using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to obtain information on the natural origin of the products. $\delta^{13}C_{C16:0}$ and $\Delta^{13}C$ ($\delta^{13}C_{C18:0}$ - $\delta^{13}C_{C16:0}$) were used to distinguish between dairy products ($\Delta^{13}C$ < -3.3‰), ruminant carcass fats (-1.1‰ < $\Delta^{13}C$ < -3.3‰), suidae carcass fats and marine oils ($\Delta^{13}C$ > -1.1‰ and $\delta^{13}C_{C16:0}$ > -27‰) (Craig et al. 2007; Dudd and Evershed, 1998; Mukherjee et al. 2007). The HT-GC and GC-C-IRMS analytical conditions and results have been described in detail elsewhere (Drieu et al. 2021; Lundy et al. 2023, 2021).

All archaeological samples in which TAGs were detected by HT-GC (n=71) were analysed by MALDI-MS and MALDI-MS/MS. Methodological studies have shown that lipid extracts in hexane obtained after solvent extraction can be directly used for MALDI-MS and MALDI-MS/MS analysis and that no purification step is necessary (Smith, 2015).

Modern authentic products

Forty-five authentic modern samples (Supplementary Table 1) were obtained directly from producers (oil, nuts, seeds, milk), local traders (butchers, cheesemongers) and supermarkets. A total of 10 samples of animal carcass fat were analysed: 3 samples of pig carcass fat and 7 samples of ruminant carcass fat (3 beef, 2 lamb, 1 veal and 1 goat). Sixteen samples of milk products were analysed: 6 samples of sheep dairy products, 5 goat dairy products and 5 cow dairy products. These included milk (2 cow's milk and 1 goat's milk), cheese (2 cow's cheese, 4 goat's cheese and 6 goat's cheese) and butter (1 cow's butter). Seven types of oil plants available in Sicily in the early Middle Ages were selected: olive, grape seed, linseed, pistachio, hazelnut, almond and walnut. For each of them, three samples of different origin and form (oil, seed/nut) were analysed (total number of plant oil samples = 20).

Plant oils, in liquid form, were directly diluted in hexane. Solid (lard, beef tallow, butter) or liquid (milk) animal fats were dissolved in DCM. Animal fats, cheeses and ground seeds and nuts were ultrasonically extracted in DCM (between 100 and 300 mg in 5 mL DCM). An aliquot of the fats and oils dissolved in DCM was taken, dried under a nitrogen stream and redissolved in hexane for analysis.

Sample spotting for MALDI-MS

Extracts from modern authentic samples and archaeological pottery were analysed using the same procedure. Two MALDI matrices were used. Following methodological developments (Smith, 2015, pp. 113-116), the first matrix was prepared from a solution of 2,5-dihydroxybenzoic acid (DHB, 10 mg mL^{-1}) in acetonitrile, which showed good results for the analysis of TAGs with various solvents (Asbury et al. 1999; Oras et al. 2017; Picariello et al. 2007). 1 µL of this matrix solution was deposited on the spots of a Bruker AnchorChip™ MALDI target plate and allowed to air dry. The matrix was recrystallised by depositing 1 µL of acetone on top of each spot. After drying, 1 or 2 µL of archaeological or modern lipid extract in hexane was deposited on top and allowed to dry. The loss of laser power between two batches of analysis required the use of a matrix that gave sufficient signal intensity with lower laser power for the second batch of samples analysed. The second matrix was prepared by mixing in equal proportions a solution of *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB; 100 mg mL^{-1} in tetrahydrofuran) and a solution of sodium trifluoroacetate (NaTFA; 1 mg/mL in tetrahydrofuran). 0.5 µL of this matrix solution was deposited on an AnchorChip™ MALDI target plate and allowed to dry. After drying, 0.5 µL of archaeological or modern lipid extract in hexane were deposited on top and allowed to dry. A series of samples analysed in the first batch with the DHB matrix was re-analysed with the DCTB matrix to ensure the reproducibility of the results from one matrix to the other.

MALDI-MS analytical settings

Samples were analysed in the positive ion mode using a Bruker solariX XR 9.4T (Fourier-transform ion cyclotron resonance, FT-ICR, mass spectrometer) with a smartbeam™ Nd:YAG laser (355 nm). Mass spectra were recorded over the *m/z* range 200 – 2000, with a transient length of 0.73 s to give a resolution of 130000 at *m/z* 400. Eight spectra were summed in steps of 800 shots which were combined into one spectrum and the laser power was set to between 40% and 60%.

MALDI-MS/MS analytical settings

The analysis conditions using the collision cell were the same as for the MS analyses for all samples, except that the laser power was set to between 60% and 80%.

Because it is not possible to select a precursor window that is narrow enough to transmit only a single TAG species at a time, two fragmentation protocols were used:

- in order to fragment only the saturated TAGs, the precursor *m/z* was selected so that only the saturated TAGs were transmitted (Table 1a).
- in order to transmit and fragment all unsaturated TAGs simultaneously, the *m/z* window was centred on the value for the diunsaturated TAG (Table 1b). This does not allow the fatty acid (FA) composition of the individual TAGs to be detailed, as each peak is due to several $[TAG\text{-}FA+Na]^+$ combinations, but to create a fingerprint for each sample that can be compared to those of others.

	Target TAG to be fragmented	Target $[M+Na]^+$ ion to be fragmented	Selected precursor *m/z* value	Range transmitted
a	$T_{54:0}$	913.81946	915	913-917
	$T_{52:0}$	885.78816	887	885-889
	$T_{50:0}$	857.75686	859	857-861
	$T_{48:0}$	829.72556	831	829-833
	$T_{46:0}$	801.69426	803	801-805
	$T_{44:0}$	773.66296	775	773-777
b	$T_{54:3}$, $T_{54:2}$, $T_{54:1}$	907.77251, 909.78816, 911.80381	909	907-911
	$T_{52:3}$, $T_{52:2}$, $T_{52:1}$	879.74121, 881.75686, 883.77251	881	879-883
	$T_{50:3}$, $T_{50:2}$, $T_{50:1}$	851.70991, 853.72556, 855.74121	853	851-855

Table 1: Masses selected for fragmentation and corresponding TAGs

Data analysis

The intensity of the ions produced from each TAG was calculated relative to the total TAG ion intensity. The variability of these relative intensities in the samples was analysed using principal component analysis (PCA) in R software (factoextra package), without scaling (variance-covariance matrix).

Results and discussion

MALDI-MS analysis

Identifying fresh authentic products with MALDI-MS

As observed in previous studies (Oras et al. 2017; Smith, 2015, pp. 119–121), the ionisation produced mainly sodiated TAGs ($[M+Na]^+$), and very few potassiated species ($[M+K]^+$), probably due to a higher amount of sodium than potassium in the extracts and the solvents, the main sources of cations in our analyses.

The range of carbon numbers in TAGs detected was very variable from one foodstuff to another (Supplementary Table 1). Most dairy products had a very wide range (T_{26}-T_{54}). The values for ruminant carcass fats were more variable according to species and age: T_{46}-T_{54} for beef and lamb, T_{42}-T_{54} for veal and goat (Figure 1). The range was narrower for pig fats (T_{46}-T_{54}), as well as for vegetable oils (T_{50}-T_{54}). The mass spectra were mainly composed of ions for unsaturated TAGs (1 to 6 unsaturations). Saturated TAGs were a minor component in the spectra of extracts from animal products and none were detected in those of plant oils (Figure 1).

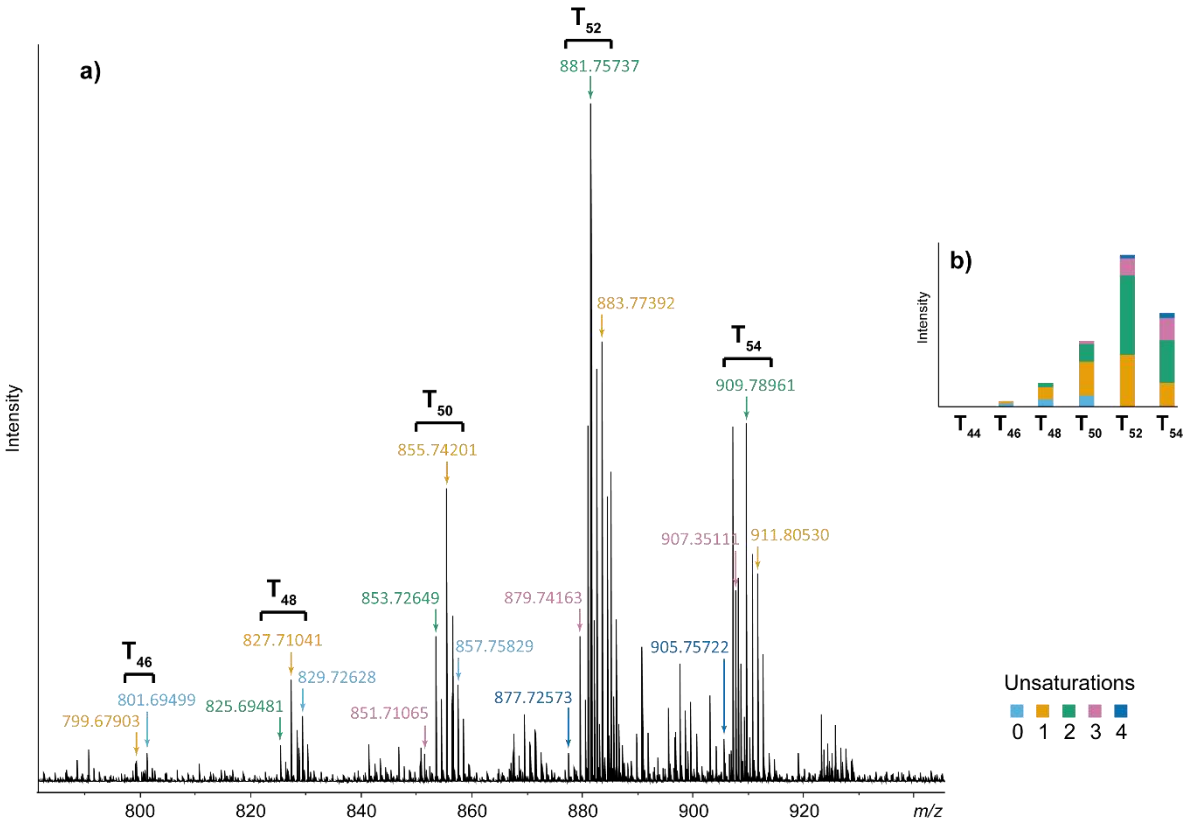

Figure 1: a) Mass spectrum obtained from the analysis of beef tallow; b) bar chart constructed based on the relative intensities of the individual signals in the mass spectrum

In order to compare a large number of archaeological samples and modern references and the relative intensity of the TAG peaks in each sample, we used principal component analysis (PCA). The PCA showed a clear distinction between plant and animal products, with the main separation being in principal component 1, which separates long-chain highly unsaturated TAGs ($T_{54:3}$, $T_{52:3}$ and $T_{52:2}$) from plant oils and other TAGs, mostly from animal products (Figure 2). Some of the longest and most unsaturated TAGs, such as $T_{52:3}$ and to a lesser extent $T_{50:2}$ and $T_{50:3}$, can be used to differentiate pig carcass fats (as they are abundant in these products) from ruminant fats, based on principal component 2. This result is in agreement with data previously obtained by gas chromatography, showing the very clear dominance of TAGs T_{50} and T_{52} in pork carcass fat, compared to ruminant fat, which has a more diverse TAG distribution (Mukherjee et al. 2007). These data are also consistent with the results of liquid phase chromatography mass spectrometry and tandem mass spectrometry analyses that detected higher amounts of $T_{52:3}$ and $T_{50:2}$ in pork carcass fats than in beef and sheep carcass fats, mainly $C_{16:0}$-$C_{18:1}$-$C_{18:2}$ (POL) and $C_{18:2}$-$C_{16:0}$-$C_{16:0}$ (LPP) (Garnier et al. 2009; Romanus et al. 2007; Saliu et al. 2011, 2014).

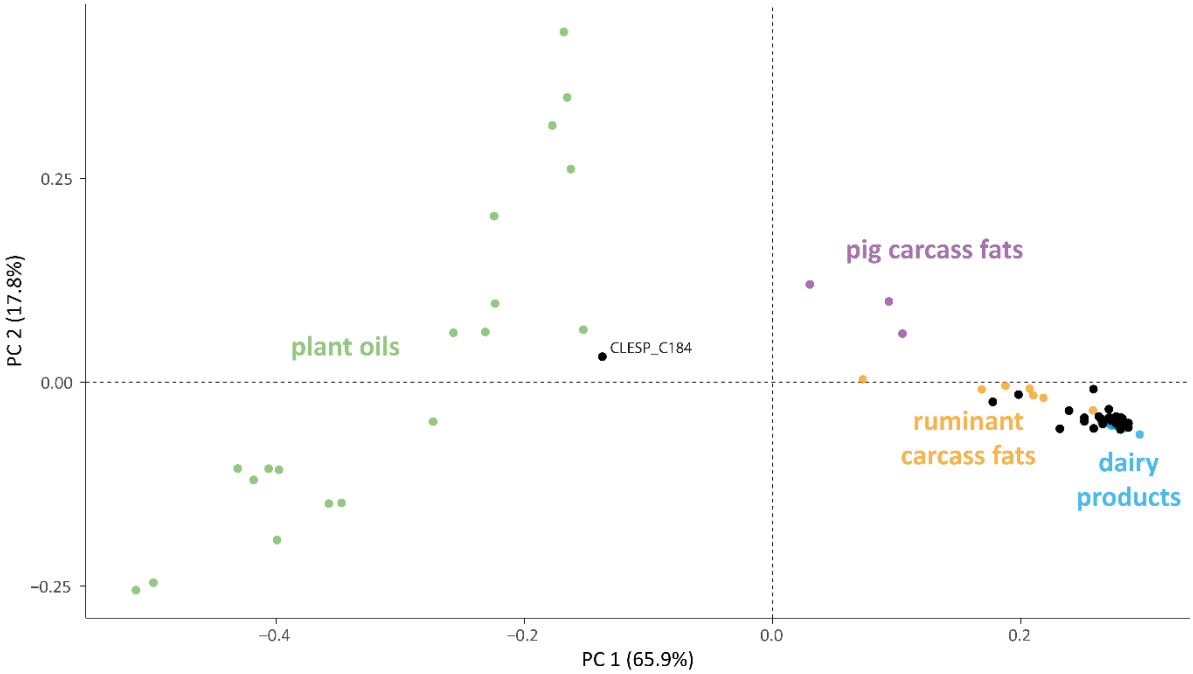

Figure 2: PCA of MALDI-MS data from authentic fresh products (coloured circles), with projection of archaeological samples (black circles).

Identifying commodities in archaeological pottery with MALDI-MS

In 19 samples, 1 to 4 additional TAGs were detected with MALDI-MS compared to HT-GC, in 21 samples as many TAGs were detected with MALDI-MS as with GC, and in 26 samples fewer TAGs were detected with MALDI-MS than with GC (between 1 and 3 fewer TAGs). In some cases, a clear TAG profile was obtained using MALDI-MS while only traces were available in GC (e.g. CLESP_C12, CLESP_C183). There were significant differences between the data from samples from different sites (Supplementary Table 1): MZ samples had much narrower distribution and less unsaturated profiles than CLESP and CSP samples (triunsaturated TAGs were preserved in half of CLESP and CSP samples), probably due to differential degradation processes. Many hypotheses could explain this differential preservation. For example, it could be related to the type and pH of the soil (Drieu 2020). Very acidic soil pH levels are highly favourable for lipid preservation, in particular acylglycerols, as they limit the activity of micro-organisms (DeLaune et al. 1981; Moucawi et al. 1981). The clay deposits of CLESP, most likely acidic, could be responsible for the better preservation of organic matter compared to the calcareous coastal sediments and calcarenites of the MZ and MI contexts. The more significant degradation of the TAGs at MZ than at CLESP could also be linked to the date of the excavations, which were very recent at CLESP (2014-2019) and more than twenty years old at MZ (1997), since TAGs have been shown to degrade in unburied pottery (TAG hydrolysis and fatty acid auto-oxidation by atmospheric oxygen), including ethnographic pots or archaeological potsherds after excavation (Drieu 2020; Dudd 1999, pp. 252).

Based on the data from fresh authentic products, we identified dairy products in samples where the TAG profiles ranged from at least T_{42}-T_{54}, a criterion also used in GC analysis (Dudd and Evershed, 1998). Among the seven samples attributed to dairy products by compound-specific stable carbon isotopes, four also complied with this TAG length criterion, two other samples had narrower profiles (suggesting degradation of the TAGs), and one gave too weak a signal to allow conclusions to be drawn. A very wide TAG profile, typical of dairy, was detected using MALDI-MS in four samples identified as non-dairy commodities by compound-specific stable carbon isotope analysis, suggesting mixtures

(Supplementary Table 1). For two of these, the wide range of TAGs had not been detected using HT-GC.

The projection of the archaeological sample data onto the PCA plot based on the data from the fresh authentic products (Figure 2) showed that the archaeological extracts were mainly composed of saturated TAGs, presumably due to the preferential degradation of unsaturated TAGs (Nawar, 1969). One archaeological sample (CLESP_C184) was distinct from the others because of its high amount of unsaturated TAGs, suggesting the presence of plant oil. This result could only be obtained by MALDI-MS, as the HT-GC analysis showed very low levels of TAGs in CLESP_C184, making it impossible to determine a specific distribution.

MALDI-MS/MS analysis

Identifying fresh authentic products with MALDI-MS/MS

The fragmentation to yield an ion corresponding to a fatty acid sodium salt was found to be minimal under the analytical conditions applied. The intensity of $[TAG\text{-}FA+Na]^+$ ions was therefore measured (Figure S1).

For animal products (carcass fats and dairy fats), the study focused on the fatty acid composition of saturated TAGs, in order to limit bias linked to degradation when compared to archaeological samples. Although the mechanisms of TAG degradation by hydrolysis modify the overall TAG profiles obtained in HT-GC (Dudd et al. 1998; Dudd and Evershed, 1998; Nawar, 1969), previous high-resolution mass spectrometric analyses have shown that these mechanisms led to only minor variations in fatty acid composition of saturated TAGs compared to the interspecies variability (Mirabaud 2007, p. 266; Saliu et al. 2011). Saturated TAGs T_{48}, T_{50} and T_{52} were consistently found in animal products. While the fragmentation profile of $T_{50:0}$ appeared to be very similar for all products, distinctive patterns were seen between ruminant carcass fats, dairy products and pig carcass fats for $T_{48:0}$ and $T_{52:0}$ (Figure 3a). $T_{48:0}$ was composed of a wide variety of fatty acids in dairy products and ruminant carcass fats, with an equally high intensity of $C_{16:0}$ and $C_{18:0}$ loss peaks, whereas in pig carcass fats the intensity of the $C_{16:0}$ loss peak was much higher than that of the other fatty acids. $T_{52:0}$ was very largely composed of $C_{18:0}$ in pig carcass fats, but had a higher proportion of $C_{16:0}$ in ruminant products (carcass fats and dairy products; Figure 3a). This result can, in part, be explained by the higher content of $C_{16:0}$-$C_{18:0}$-$C_{18:0}$ (PSS) in ruminant carcass fats (sheep and cattle) than in pig carcass fats (Mottram et al. 2001; Romanus et al. 2007).

This one-to-one comparison of product ion profiles was suitable for a small number of samples. However, our goal was to compare dozens of archaeological samples with the product ion profiles from the authentic food products. The one-to-one comparison was time-consuming and could not provide a comprehensive overview of the samples from a specific period or region. To facilitate comparison with a large number of archaeological samples, ion intensity ratios were calculated between ions resulting from the loss of different fatty acids. The intensity ratios of ions resulting from the loss of $C_{18:0}$ and $C_{16:0}$ in $T_{48:0}$ and $T_{52:0}$ proved to be the most appropriate for separating the different types of animal products (Figure 3b).

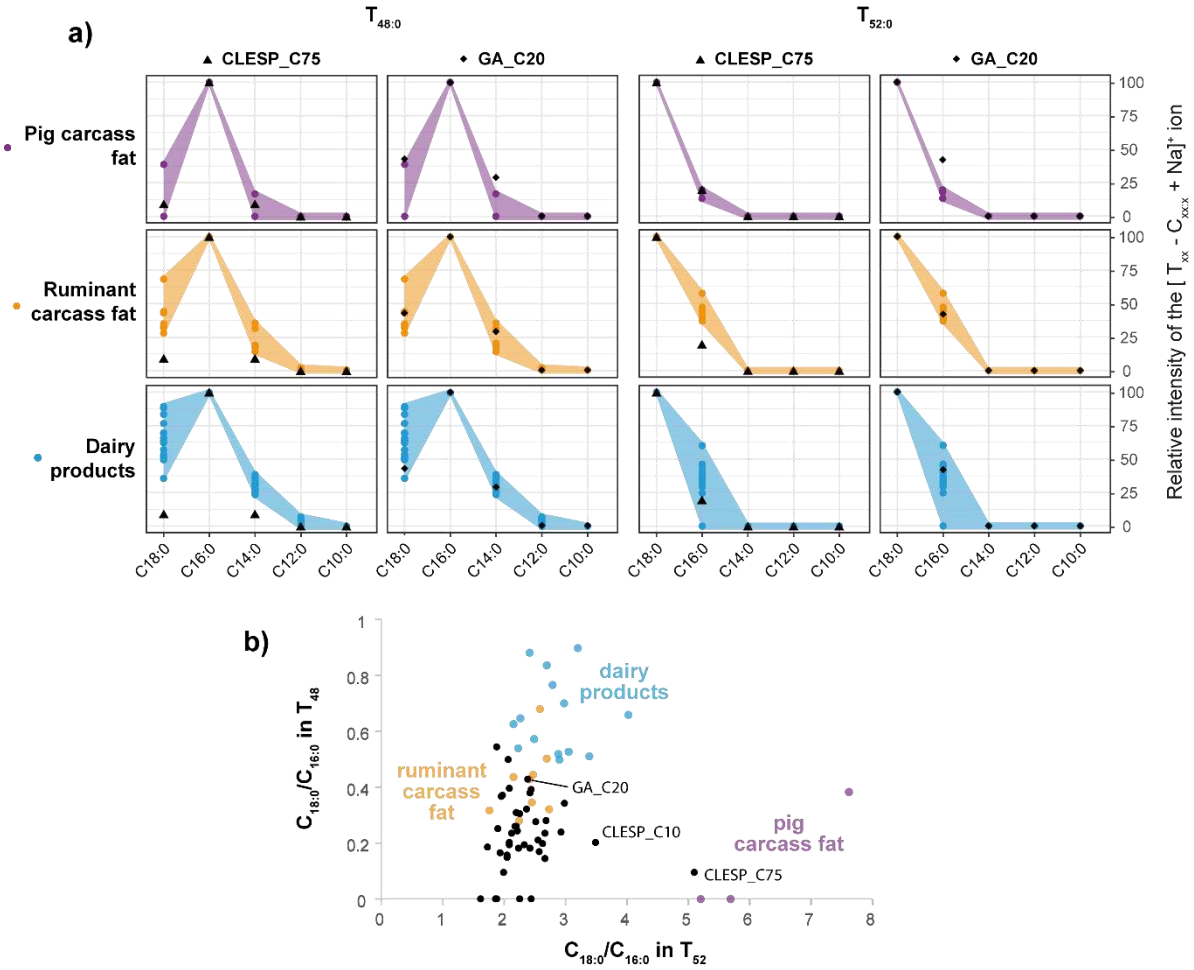

Figure 3: Fatty acid composition of $T_{48:0}$ and $T_{52:0}$ in extracts of animal products (coloured circles) and archaeological samples (black dots). (a) Product ion profiles. Each coloured circle represents the value obtained from the analysis of a sample of authentic animal product. The black dots show the product ion profile of an archaeological sample, superimposed on each authentic animal product profile. The coloured areas cover the minimum and maximum relative peak intensities obtained for each type of animal product (between 3 and 16 samples for each type of animal product); (b) Scatterplot representing the ratio of the intensity of the $C_{18:0}$ to $C_{16:0}$ loss peaks from $T_{48:0}$ against $T_{52:0}$.

Among the shortest saturated TAGs, $T_{44:0}$ was only detected and fragmented with MS/MS in the dairy samples and one lamb and one veal carcass fat samples. Three different patterns, associated with cow, sheep and goat dairy products were identified by fragmentation of $T_{44:0}$ (Figure 4a). While all fragmentation profiles were dominated by the loss of $C_{16:0}$, the intensity of ions resulting from the loss of $C_{18:0}$, $C_{14:0}$, $C_{12:0}$ and $C_{10:0}$ varies between species (Figure 4a). A similar distinction between cow and goat milk was obtained by ESI-MS/MS analysis (Mirabaud et al. 2007). The ion intensity ratios resulting from the loss of $C_{16:0}$, $C_{14:0}$ and $C_{12:0}$ proved to be the most diagnostic for differentiating between cow, goat and sheep milk and dairy products (Figure 4b). The difference between the product ion profiles of goat dairy products on the one hand and cow and sheep dairy products on the other, which are richer in $C_{14:0}$, is probably due to the high amounts of $C_{14:0}$-$C_{14:0}$-$C_{16:0}$ (MMP) in cow's and sheep's milk (Gresti et al. 1993; Nájera et al. 1999), which is not detected in goat's milk (Gastaldi et al. 2011). Cow's milk and sheep's milk are both rich in $C_{12:0}$-$C_{16:0}$-$C_{16:0}$ (LaPP; Gresti et al. 1993; Nájera et al. 1999), but the presence of $C_{12:0}$-$C_{14:0}$-$C_{18:0}$ (LaMS) in cow's milk probably explains why the ratio of $C_{16:0}$ loss to $C_{12:0}$ loss ions is lower in the data from cow dairy products than in those of sheep dairy products. However, the type of dairy product (milk, butter, cheese) did not seem to have a major effect on the composition of TAGs. This result is in agreement with food science studies that show no change in fatty acid composition during cheese making (Raynal-Ljutovac et al. 2008). The carcass fat profiles of the veal

and lamb samples were very different from the dairy samples, which ensures that they cannot be mistaken. The veal profile consisted only of the ions resulting from the loss of $C_{16:0}$ and $C_{14:0}$, the latter being the dominant ion, while the $T_{44:0}$ fragmentation of lamb fat resulted mainly in the loss of $C_{16:0}$ and $C_{14:0}$ in similar amounts and very little $C_{12:0}$.

These preliminary results, based on a small number of authentic samples, need to be confirmed on a larger scale by analysing samples from meat- and milk-producing animals with varied diets and obtaining products in different seasons, as these factors may affect their molecular composition. However, for the distinction of dairy species based on $T_{44:0}$, the impact of seasonality and diet is most likely negligible because the variability of this specific TAG as a function of season has been shown not to be significant (Pacheco-Pappenheim et al. 2021).

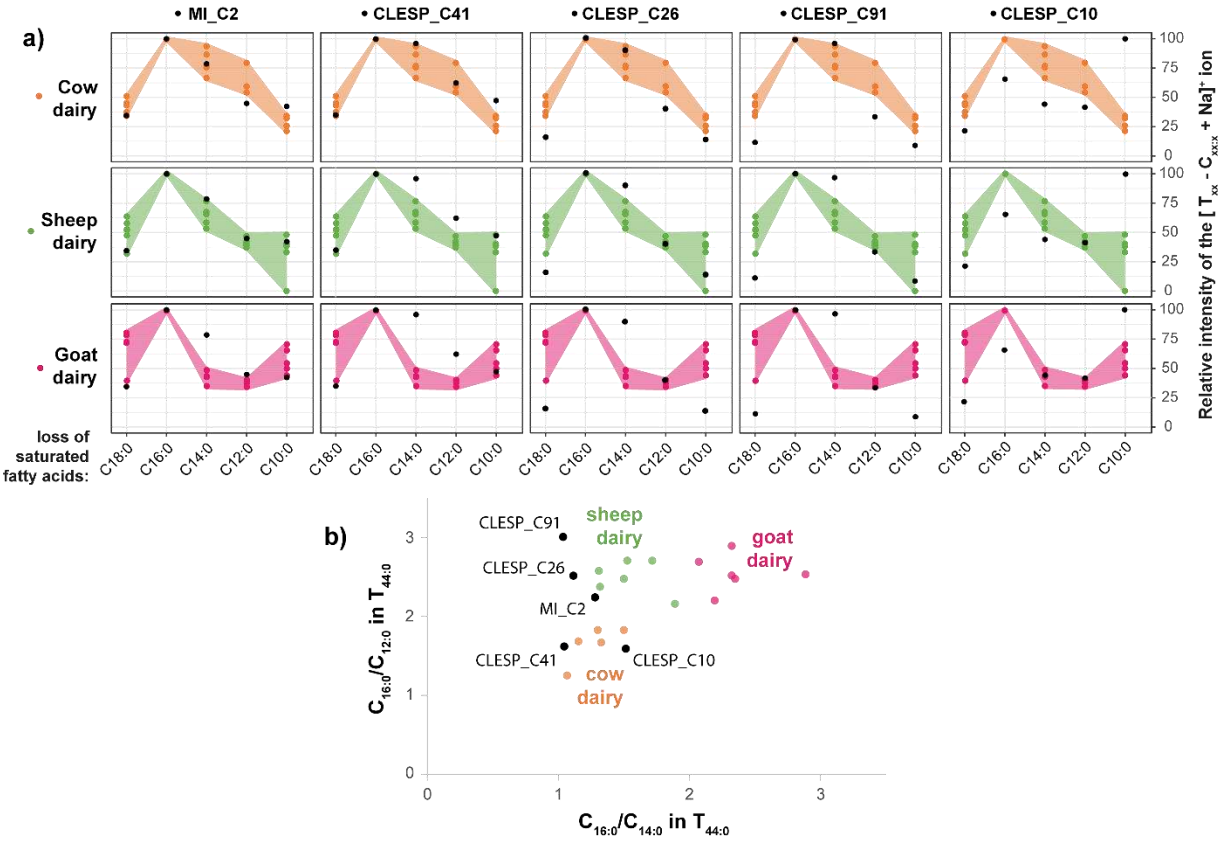

Figure 4: Fatty acid composition of $T_{44:0}$ in extracts of dairy products (coloured circles) and archaeological samples from Casale San Pietro and Monte Iato (black cicles). (a) Product ion profiles. Each coloured circle represents the value obtained from the analysis of a sample of authentic dairy product. The black circles show the product ion profile of an archaeological sample, superimposed on each authentic dairy product profile. The coloured areas cover the minimum and maximum relative peak intensities obtained for each ruminant species (between 5 and 6 samples for each ruminant species). (b) Scatterplot representing the ratio of the intensity of the $C_{16:0}$ to $C_{14:0}$ against the ratio of $C_{16:0}$ to $C_{12:0}$ loss peaks in $T_{44:0}$.

The fatty acid composition of unsaturated TAGs had to be used as a proxy for plant oil identification, as no saturated TAGs were detected in modern plant oils. As the precursor ion selection window for unsaturated TAGs covered a range of TAG species, the intensity of the fragmentation peaks reflected the sum of ions from different fatty acid losses from different TAGs (Figure S2). Figure 5 shows product ion profiles from unsaturated TAGs T_{50}, T_{52} and T_{54} in extracts of modern plant oils and one archaeological sample. The different types of fingerprints obtained by simultaneous fragmentation of all unsaturated TAGs with the same number of carbon atoms are due to differences in the composition of individual TAGs, but it is not possible (or necessary) to precisely deconvolute these fingerprints in

order to obtain useful information. The fragmentation profiles of T_{54} were not helpful in distinguishing vegetable oils, as they are almost exclusively composed of C_{18} fatty acids. However, plant oils can be grouped based on similar fingerprints from unsaturated T_{52} fragmentation: almond-pistachio, hazelnut-olive, grape seed-walnut, linseed (Figure 5). These groups are consistent with the detailed composition of individual T_{52} species from the literature. The specific fragmentation profile of unsaturated T_{52} from linseed oil is probably due to its unique composition of $C_{18:3}$-$C_{18:3}$-$C_{16:0}$ (LnLnP), $C_{18:3}$-$C_{18:2}$-$C_{16:0}$ (LnLP) and $C_{18:1}$-$C_{18:3}$-$C_{16:0}$ (OLnP; Holčapek et al. 2003; Lísa & Holčapek, 2008). The other oils are probably separated according to their respective relative amounts of $C_{18:1}$-$C_{18:2}$-$C_{16:0}$ (OLP), $C_{18:2}$-$C_{18:2}$-$C_{16:0}$ (LLP) and $C_{18:1}$-$C_{18:1}$-$C_{16:0}$ (OOP; Bail et al. 2008, 2009; Holčapek et al. 2003; Lísa & Holčapek, 2008; Wiesman & Chapagain, 2009). The unsaturated T_{52} in almond and pistachio oils are mainly OLP and to a lesser extent LLP and OOP. Those of hazelnut and olive oils are mainly OOP and to a lesser extent OLP and LLP. Those of grape seed and walnut oils are mainly LLP and to a lesser extent OOP.

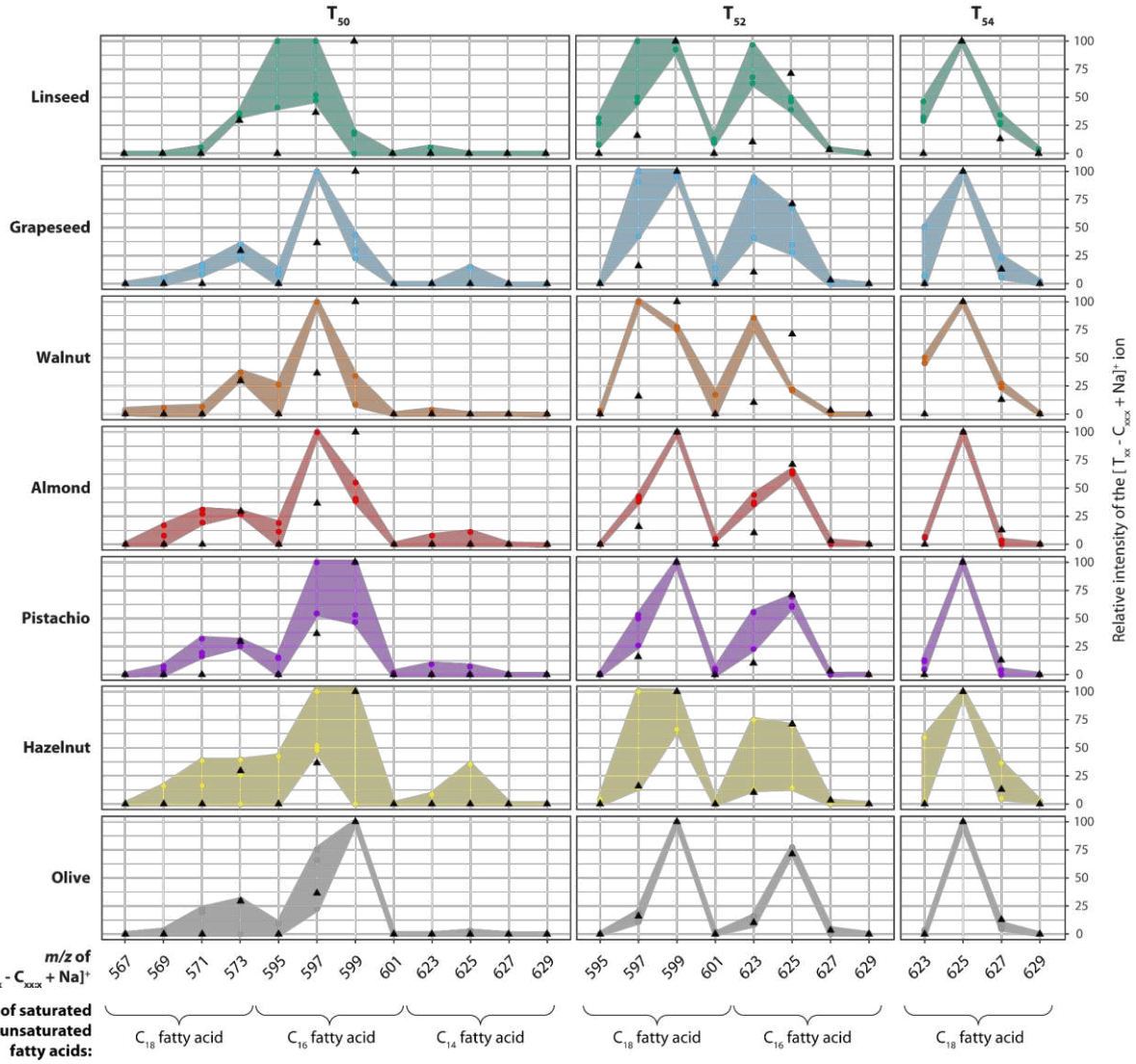

Figure 5: Product ion profiles obtained from unsaturated TAGs T_{50}, T_{52} and T_{54} in extracts of seven plant oils and one archaeological sample from Casale San Pietro (CLESP_C184). Each coloured spot represents the value obtained from the analysis of a sample of authentic plant oil. The black triangles show the product ion profile of archaeological sample CLESP_C184, superimposed on each authentic oil profile. The coloured areas cover the minimum and maximum relative peak intensities obtained for each oil (between 2 and 3 samples for each oil).

Identifying products in archaeological samples using MALDI-MS/MS

Most archaeological samples had a fatty acid composition in $T_{48:0}$ and $T_{52:0}$ close to that of ruminant fats (Figure 3). Samples with fatty acid stable carbon isotope ratios ($\Delta^{13}C < -3.3‰$) or broad TAG profiles (at least T_{42}-T_{54}) indicating dairy products did not always have TAG fatty acid compositions consistent with dairy products ($C_{18:0}/C_{16:0}$ in $T_{48} > 0.5$; Figure 6), although there is a weak correlation between $\Delta^{13}C$ and $C_{18:0}/C_{16:0}$ in $T_{48:0}$ for these samples ($R^2 = 0.2673$). This suggests that different ruminant products (carcass fats and dairy products) were processed in the same pots, a hypothesis already proposed to explain the differences in interpretation between the isotopic data and the TAG profiles obtained using HT-GC (Lundy et al. 2021). In addition, as TAGs are readily degraded by heating (Nawar, 1969), those preserved are more likely to preferentially represent the last uses of the vessel. Fatty acids, on the other hand, are more likely to be integrated from foodstuffs processed in the pot over its use-history, especially bound fatty acids that were released by acid-methanol extraction for GC-C-IRMS. Therefore, in cases where the MALDI-MS/MS of TAGs and the GC-C-IRMS of fatty acids do converge, it is likely that the vessel had a restricted range of uses.

For medieval Sicily, which saw a succession of political and religious regimes, the question of pork consumption is fundamental to the study of culinary practices. Compound-specific isotopic data have suggested the consumption of non-ruminants at Casale San Pietro, but such values may also be due to plant products (Lundy et al. 2021; Lundy et al. 2023). The very high intensity of $C_{18:0}$ loss relative to that of $C_{16:0}$ in $T_{52:0}$ of sample CLESP_C75 ($C_{18:0}/C_{16:0}$ in $T_{52} = 5.08$) suggested that this vessel primarily contained non-ruminant fat, possibly porcine fat, a hypothesis consistent with the ^{13}C values measured on this sample ($\Delta^{13}C = 0.38‰$; Figure 6). Two other samples, CLESP_C10 and MZ_C134, appeared to have a non-ruminant component, with a value of $C_{18:0}/C_{16:0}$ in $T_{52:0}$ slightly higher than the ruminant fat range ($C_{18:0}/C_{16:0}$ in $T_{52:0} = 3.47$ and 3.85), suggesting a mixture of fats. This result is consistent with the consumption of non-ruminants in CLESP between the 10th and 12th centuries, including during the Islamic period, as indicated by suid remains (Aniceti and Albarella, 2022b). In Mazara, the pot containing non-ruminant fat dates from the 13th century, under Swabian rule, in line with the increase in the proportion of suids in the faunal assemblage after the Islamic period (Aniceti and Albarella, 2022b). These results could not be obtained solely on the basis of HT-GC data, due to the difficulty of reliably distinguishing ruminant and non-ruminant TAG patterns using this method of analysis, and mixing in certain samples. Expanding the range of authentic non-ruminant fats with additional product ion analyses would be necessary to determine if the signal in the pot is from pork or other non-ruminants (e.g. poultry).

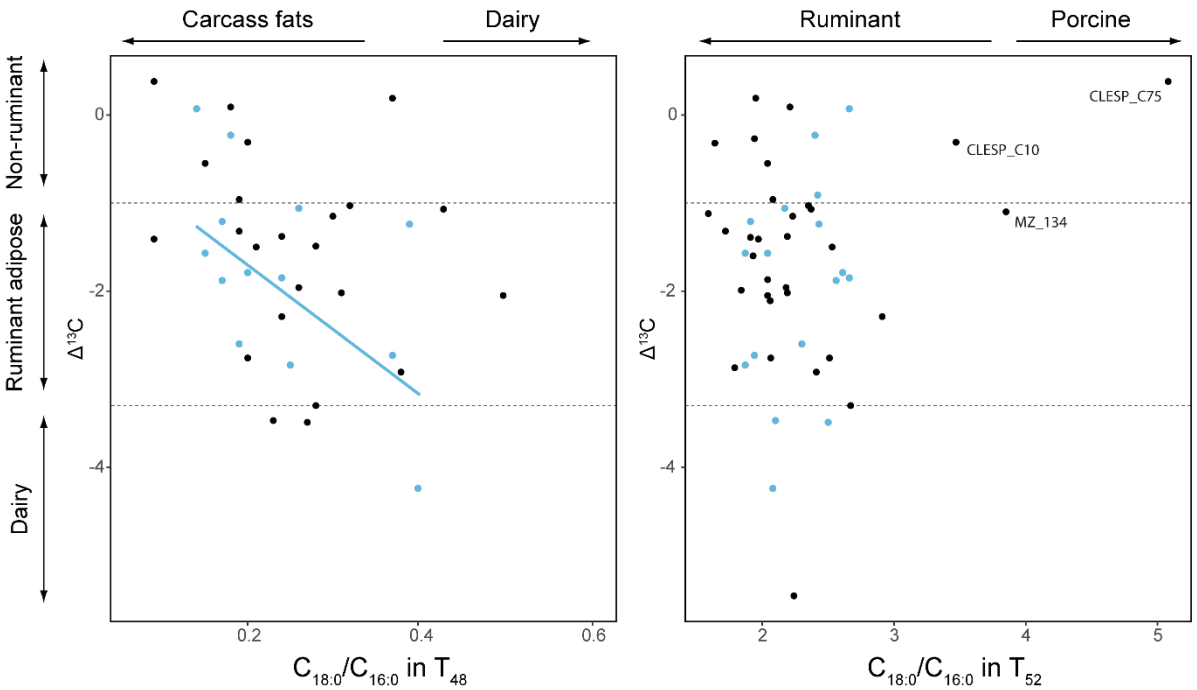

Figure 6: $\Delta^{13}C$ ($\delta^{13}C_{18:0}$ - $\delta^{13}C_{16:0}$ values) values of archaeological samples plotted against ion intensity ratios associated with the loss of different fatty acids in T_{48} and T_{52}. The blue circles indicate samples with a wide range of TAGs (T_{42}-T_{54}) detected by HT-GC indicative of dairy products (a linear trend line is shown for these samples).

Among the samples with broad TAG profiles detected using MALDI-MS (at least T_{42}-T_{54}) and interpreted as dairy products, the fragmentation pattern of $T_{44:0}$ in the extracts of two pots was almost identical to that of authentic dairy products, from sheep (MI_C2) and cow (CLESP_C41) respectively (Figure 4a). For two other pots (CLESP_C26 and CLEPS_C91), the fragmentation pattern was close to that of sheep's milk, but cow's milk could not be excluded (Figure 4a). The fragmentation pattern of $T_{44:0}$ in CLESP_C10 extract was more difficult to interpret due to the high intensity of ions resulting from the loss of $C_{10:0}$. This feature made it appear similar to goat's milk, although the relative proportions of ions associated with the loss of $C_{16:0}$, $C_{14:0}$ and $C_{12:0}$ were closer to the pattern in cow's milk (Figure 4a). The use of ion intensity ratios associated with the loss of different fatty acids helped to confirm or clarify certain interpretations (Figure 4b). The dairy products in MI_C2 and CLESP_C41 were confirmed to be from sheep and cow respectively and in CLESP_C91 and CLESP_C26 dairy products appeared to be mainly from sheep. In CLESP_C10, the main dairy product appeared to be from cows, but contributions from goats' milk could not be excluded because of a lower intensity of ions associated with the loss of $C_{14:0}$ than in most cow's milk products. These results are consistent with the archaeozoological data: both cattle and caprine animals were exploited for their milk during the Arab and Norman periods in Sicily (Aniceti and Albarella, 2022a). The minimal presence of goat's milk in pottery is in line with the predominance of sheep over goats in medieval Sicily (Aniceti and Albarella, 2022a).

Only dairy products from sheep's milk were identified in amphorae used for transport or storage (CLESP_91 and MI_C2). This may be due to the high quality of sheep's milk compared to goat's and cow's milk for the production of long-life dairy products such as cheese (Bittante et al. 2022). Malic acid, a plant (fruit) marker, was also detected in similar amounts in these two amphorae (Drieu et al. 2021). Although successive uses with different contents cannot be ruled out, it is possible that plants were added to sheep milk to curdle it, flavour it or help preserve it (Drieu et al. 2020).

The cooking vessels in which dairy products from different species have been identified are also different in terms of their method of manufacture and origin: CLESP_41 and CLESP_10 which contain

cow dairy are both wheel-thrown pots likely imported from Palermo, whereas CLESP_26 which contains sheep dairy is a handmade slow-thrown cooking pot made in Western Sicily (Lundy et al. 2021). New data will be needed to confirm the use of milk from different species in different types of pots, in order to determine whether each type of container had a specific purpose.

One sample, amphora CLESP_C184, showed a sufficiently well-preserved unsaturated TAG profile (the numerous product ions suggest a good preservation of various unsaturated TAG precursors) to be compared with those of authentic oils (Figure 5). Its fragmentation profile was very similar to that of olive (*Olea europaea*) and hazelnut (*Corylus avellana*) oils. The similarity in the composition of these oils has been noted previously; the TAGs that reliably distinguish them are present in low concentrations ($C_{18:1}$-$C_{18:3}$-$C_{18:1}$=OLnO, $C_{18:3}$-$C_{18:1}$-$C_{16:0}$=LnOP, $C_{18:3}$-$C_{18:2}$-$C_{18:1}$=LnLO; Parcerisa et al. 2000). Separative analyses coupled with high resolution mass spectrometry would be required to determine the exact origin of the oil in sample CLESP_C184. From an archaeobotanical, archaeological and historical perspective, both oil plants are suitable candidates. Olive oil is one of the main oils produced and traded in the Mediterranean since classical Antiquity (e.g. Bevan, 2014; Brun, 2020). In Sicily, olive growing is attested by charcoals, pollens, and fruit remains (including at MZ and CLESP; Fiorentino et al. 2022), even though it seems to have declined during Late Antiquity and the Islamic period, before rising again in the 12th century (Chowaniec et al. 2021; Fiorentino et al. 2022; Michelangeli et al. 2022; Stella and Fiorentino 2022; Tinner et al. 2009). The presence of the genus *Corylus* in Sicilian vegetation is widely supported by pollen diagrams (Michelangeli et al. 2022) and charred hazelnut fragments were found at some Sicilian medieval sites, for example in MZ (Fiorentino et al. 2022). Textual sources attest to the trade of hazelnuts in the medieval Mediterranean, for example from southern Italy (Citarella, 1968), although of much less importance than the olive oil trade in terms of volume. Whole hazelnuts are unlikely to have left traces of lipids in the walls of a container, but it is possible that hazelnuts were exploited for their oil. These data from extracts from a late antique imported amphora found in central Sicily suggest the trade in olive or hazelnut oil on the island to be used for culinary, medicinal/cosmetic or as a fuel for lighting purposes (Amouretti, 1986, p.181-190; Mataix and Barbancho, 2006).

The study of experimental samples and/or ethnographic vessels should be undertaken in the future to verify the exact impact of degradation mechanisms on the fatty acid composition of unsaturated TAGs in plant oils, and to check that the degradation of a polyunsaturated oil (e.g. linseed oil) cannot produce a profile similar to a low-unsaturated oil (e.g. olive or hazelnut oil).

Conclusion

This study demonstrated on a large scale the potential of the detailed study of TAGs for the more precise identification of animal and plant taxa consumed in pottery, taking into account intraspecies molecular variability. The short analysis time of MALDI-MS and MALDI-MS/MS made it possible to analyse a large number of archaeological samples and, for the first time in a study of archaeological TAGs, to account for intraspecies molecular variability by analysing several modern samples of the same product type. Using this technique, non-ruminant carcass fat and dairy products were identified, including in samples with mixed products. For the best preserved dairy samples, it was possible to trace the species that produced the milk, as was done in a previous ESI-MS/MS study (Mirabaud et al. 2007). Finally, the exceptional context of CLESP preserved traces of very slightly degraded vegetable oils that were identified as olive or hazelnut oil.

This method is not intended to replace conventional GC and GC-MS analyses after DCM/MeOH extraction, which remains an effective way to assess overall lipid preservation and detect a wide range of compounds (alkanes, alkanols, wax esters, etc.). However, it can be carried out directly as a second

analytical step on a large number of samples with preserved TAGs, without any additional preparation steps. In the case of samples with good TAG preservation, such as at the site of CLESP, this technique can be implemented routinely to refine interpretations of the type of products consumed and to help deconvolute commodity mixtures.

For further investigation, TAG isomers could be analysed independently, for example after separation using liquid chromatography coupled to tandem mass spectrometry, to deconvolute the overall fingerprints provided by MALDI-MS and MALDI-MS/MS. Studies could then focus on the TAG isomers most resistant to degradation, perhaps increasing the number of samples yielding interpretable results. The coupling of liquid chromatography to tandem mass spectrometry could also help to further refine the accuracy of taxonomic identification, for example to distinguish between olive and hazelnut oils or between different ruminant carcass fats, by separating and studying each TAG individually.

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Supplementary Information

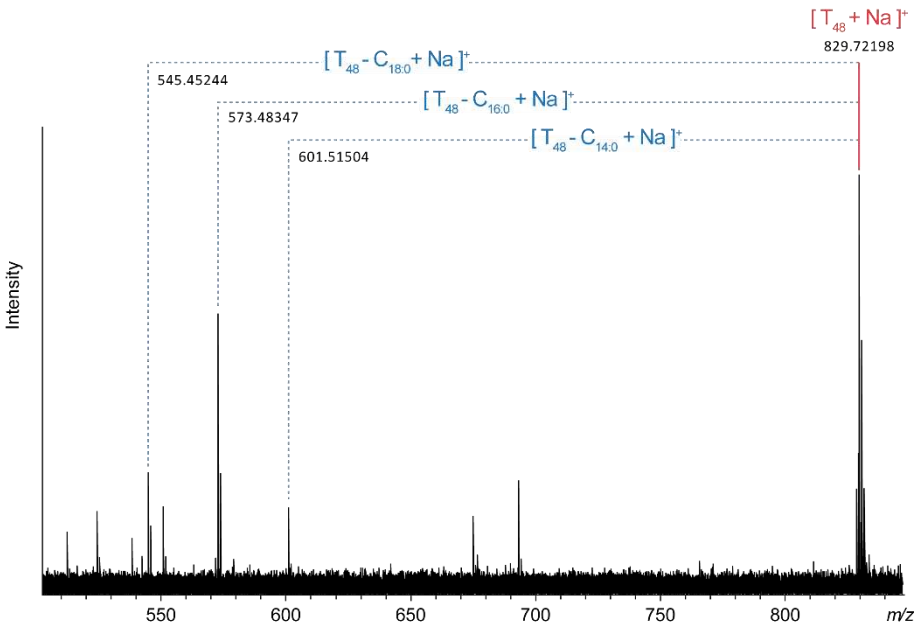

Figure S1: Product ion spectrum obtained from saturated T_{48} by loss of fatty acids from TAG in modern beef tallow.

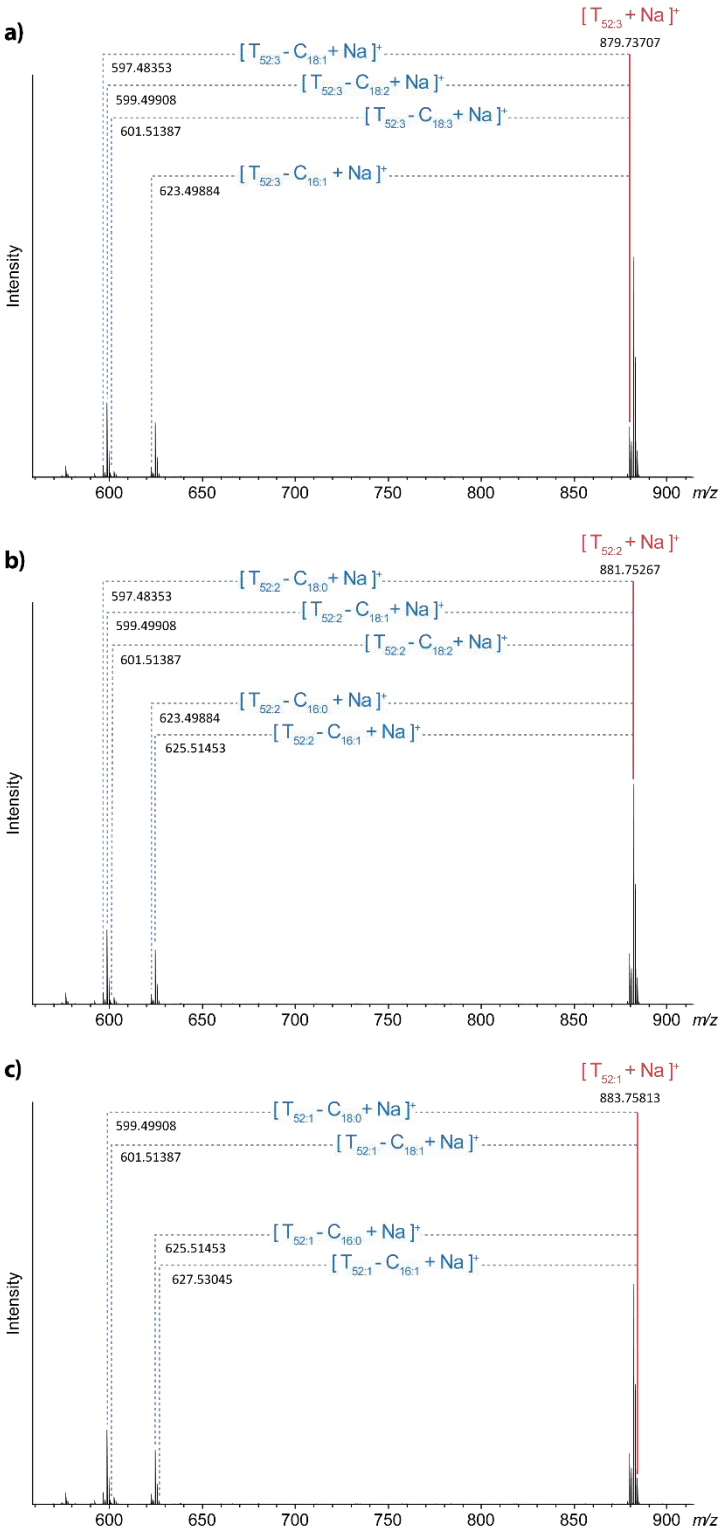

Figure S2: Fragmentation of unsaturated T_{52} by loss of fatty acids from TAGs in modern olive oil, demonstrating how the spectrum results from fragmentation of the sum of several precursors. a) fragmentation of $T_{52:3}$; b) fragmentation of $T_{52:2}$; c) fragmentation of $T_{52:1}$.