MALDI-FT-ICR-MS for archaeological lipid residue analysis

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Abstract
Soft-ionization methods are currently at the forefront of developing novel methods for analysing degraded archaeological organic residues. Here, we present little-used soft ionization method of matrix assisted laser desorption/ionization-Fourier transform-ion cyclotron resonance-mass spectrometry (MALDI-FT-ICR-MS) for the identification of archaeological lipid residues. It is a high-resolution and sensitive method with low limits of detection capable of identifying lipid compounds in small concentrations, thus providing a highly potential new technique for the analysis of degraded lipid components. A thorough methodology development for analysing cooked and degraded food remains from ceramic vessels was carried out, and the most efficient sample preparation protocol is described. The identified components, also controlled by independent parallel analysis by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), demonstrate its capability of identifying very different food residues including dairy, adipose fats as well as lipids of aquatic origin. The results obtained from experimentally cooked and original archaeological samples prove the suitability of MALDI-FT-ICR-MS for analysing archaeological organic residues. Sample preparation protocol and identification of compounds provide future reference for analysing various aged and degraded lipid residues in different organic and mineral matrices.

KEYWORDS
ancient food, lipids, MALDI-FT-ICR-MS, organic residue analyses, soft ionization

1 INTRODUCTION

Organic residue analysis has become one of the major and rapidly developing fields in archaeological sciences. The analyses of lipid residues have produced several fruitful studies allowing a better understanding of past life-ways and economy, pottery function, agricultural practices, etc.1–5 Current routine analysis evolves around gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS),6,7 sometimes combined with bulk isotopic analysis with elemental analyser isotope ratio mass spectrometry (EA-IRMS) of food crusts. These methods imply rather complicated and time-consuming sample preparation,8,9 which, depending on the extraction protocol, also include derivatization and/or breaking down complex lipid molecules. For example, acylglycerols do not preserve in the case of acid extraction and are broken into free fatty acids, whilst they can be detected in derivatized forms using solvent extraction protocol. However, data obtained on original and intact lipid molecules, ie, triacylglycerols (TAGs) highly depends on overall molecule preservation and is often not available. The bulk isotope analysis, simpler in terms of sample preparation, provides only limited information about the origin of lipids relating to the larger food groups (plants, aquatic, and terrestrial animals), trophic levels, and wider metabolic systems of the organisms analysed.

One potentially fruitful yet under-utilized analytical method for the identification of archaeological lipid residues is matrix assisted laser desorption/ionization-Fourier transform-ion cyclotron resonance-mass spectrometry (MALDI-FT-ICR-MS). MALDI is a soft ionization method which can produce ions directly from the solid state. Ionization of the analyte by the laser beam is assisted by matrix material which absorbs radiation at the laser wavelength, is in large excess compared with the substance analysed and helps to separate analyte molecules. Laser energy is absorbed by the matrix (not the analyte), and as a result the whole analyte molecules (not fragments) are ionized by adding a proton (from the matrix) or a single positively charged metal ion (available in all biological samples by nature), resulting mostly in H+ or Na+ adducts, although some other cations are possible as well.10,11

MALDI has been mainly coupled with time-of-flight mass spectrometer (TOF-MS)12–16 or Fourier transform mass spectrometer (FTMS).17,18 The combination with FT-ICR-MS makes it a sensitive method with low limit of detection. It affords high resolution (reaching hundreds of...
thousands) and high m/z accuracy (m/z error down to less than 1 ppm) which help to distinguish components with the same nominal molecular mass. This combination has been previously used in omics-related biochemical analysis, including, eg, metabolomics and with low molecular weight compounds. Although, the applications of soft ionization methods like MALDI-TOF-MS, atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) have become more widespread in the studies of historical, archaeological and heritage objects, the combination of MALDI-FT-ICR-MS which covers soft ionization and very high resolution mass analyser is still very limited.

Analysis of archaeological lipids involves several complications. We are studying unknown substances with (numerous) impurities which affect limit of detection (LoD) of the instrument. Degradation, eg, oxidation and hydration, of original lipids in the burial environment and as a result of food processing (mainly cooking) in the past are also raising issues for identification. The problem of preservation is particularly evident when targeting the whole molecules of TAGs. They are informative for identifying the initial substances but unstable and tend to fragment resulting in smaller concentrations of these important molecules in archaeological samples. Fragments themselves, although identifiable, are less informative: they are degradation products of initial substances, but one degradation product can correspond to different initial substances being therefore less diagnostic.

Considering these peculiarities relating to archaeological samples MALDI-FT-ICR-MS has several advantages compared with the more traditional methods used in archaeological lipid residue analysis. First, it allows the collection of very detailed mass spectrometric data and thus more precise identification of compounds. It can detect larger molecules, eg, TAGs with acyl carbon number over 54, which is rarely available with traditional GC-MS methods used in archaeological organic residue analysis. Being a soft ionization method, samples are analysed intact (without fragmentation, derivatization or methylation), as a result of which the original lipid mass spectra covering diacylglycerol and triacylglycerols (DAGs and TAGs) as well as other lipid compounds are obtained. In comparison with currently used traditional methods (eg, GC-MS), it is also capable of providing more detailed structural information on TAGs, eg, unsaturations and oxidations of TAGs, but can also separate different molecular mass TAGs efficiently to compare their abundances. Comparing acid and solvent extraction sample preparation procedures used for GC-MS analysis, the latter is simpler and less time consuming, especially when the derivatization steps can be omitted, as it is the case with the MALDI-MS measurements reported here. Furthermore, in principle, it is even possible to obtain results from direct samples without any specific extraction protocols (see below), including only sample solution and mixing with internal calibrants.

In this work, we present an efficient protocol for analysing archaeological food residues with MALDI-FT-ICR-MS. We exemplify the suitability of MALDI-FT-ICR-MS for archaeological organic residue analysis with 4 different archaeological samples and highlight its capability of identifying different, including degraded and oxidized TAGs from food residues. The material covers samples of both food-crust and ceramic powder from vessels discovered from the 12th to 13th century AD cemetery at Kukruse, NE Estonia, complemented with samples obtained through cooking experiments and previously published results.

2 | MATERIALS AND METHODS

2.1 | Archaeological pots

At the 12th to 13th century AD inhumation cemetery at Kukruse, NE Estonia, pots were buried as grave goods with the deceased. Pots were sampled using clean equipment: scalpels for removing food-crust and clean drill bits to sample ceramic powder avoiding areas with clear glue remains. Samples of food-crusts and ceramic powder from 4 different vessels (Table 1) were analysed in order to test the suitability of MALDI-FT-ICR-MS for archaeological lipid residue analysis.

2.2 | Experimental pots

Untreated (eg, uncooked) and pure foodstuffs cannot be used as direct reference material for studying archaeological lipids, because various alteration processes can happen due to environmental conditions and thermal processing of substances via roasting, boiling, etc. To obtain compatible study material for developing suitable analytical protocol and create a reference collection for identifying unknown archaeological substances several cooking experiments were conducted. A set of different experimental pots made from local clay with authentic moulding and burning techniques was prepared in which a selection of different food substances were later cooked. Those experiments mimicked historic cooking practices and included foodstuffs expected to be in use at a specific archaeological period based on previous botanical and zooarchaeological analysis. Cooking ingredients were attained from local small farmers, preferring producers with organic farming practices (also with controlled fodder of C3 plants), and fish obtained from local fish markets. As a result, a variety of reference material usable for archaeological sample identification and suitable for developing analytical protocol for archaeological lipid analysis with MALDI-FT-ICR-MS was created.

2.3 | GC-C-IRMS and GC-MS analysis

In order to control the results obtained with MALDI-FT-ICR-MS, all the samples reported here were also measured with gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) and gas chromatography-mass spectrometry (GC-MS). Solvent extraction method is widely employed in archaeological food residue analysis, but acid extraction sample preparation method is becoming also popular, as more efficient (yielding higher concentration of lipids) and more suitable for older and smaller samples. Both methods have their limitations but also advantages, and the preference of their use depends on specific study material and research questions. Because our aim was also to test and compare whether different sample preparations influence the data obtained and interpretation of results for identifying initial food residues in the pots, we decided to apply...
acid extraction sample preparation protocols for the samples analysed with GC-MS. Samples (1 g of ceramic powder or ca 20 mg of food crust) were prepared following acid catalysed lipid extraction method and methylation with MeOH (70 °C, 4 hours).8,9 After heating lipids were extracted with n-hexane (3 × 2 mL) and dried under the gentle stream of nitrogen at 37.5 °C. For GC-MS, samples were dissolved in 100 mL of n-hexane with the addition of 10 mg of internal standard of C36:0 (n-hexatriacontane). The same samples were later on used for GC-C-IRMS, for which toluene was used as a solvent.

The GC-C-IRMS analyses were conducted using a Trace GC equipped with a DB5 capillary column (60 m × 0.32 mm × 0.25 μm) and coupled with a Thermo Delta V mass spectrometer. Samples were dissolved to concentrations of approximately 0.10 μg/μL of each target compound using toluene as solvent. Toluene was chosen because it is considerably less volatile than the more standard solvent n-hexane, and the lower volatility is preferable when working with small sample amounts.39 Samples were injected through a programmable temperature vaporization (PTV) injector operated in solvent split mode. The injection volume was 2 μL, the injection pressure was 70 kPa, and the initial temperature was 40 °C. The splitless time was set to 1.0 minutes, the solvent vent temperature was 100 °C, and the vent flow was 100 mL per minute. Evaporation pressure was set to 140 kPa, evaporation temperature was 40 °C, the evaporation rate was 10 °C per second, and the evaporation time was 0.16 minutes. Transfer pressure was 210 kPa, transfer temperature 300 °C, and transfer rate was 12 °C per second. The cleaning temperature was 320 °C, cleaning rate 14.5 °C per second, cleaning time 20 minutes, with a cleaning flow 100 mL per minute. The GC oven was temperature programmed with an initial isothermal of 2 minutes at 120 °C, followed by an increase of the temperature with 20 °C per minute to 200 °C, followed by a temperature ramp of 5 °C per minute to 315 °C, and held at this temperature for 7 minutes. The target fatty acid methyl esters were converted to CO2 through an IsoLink II reactor system. Pulses of reference CO2 gas were injected through a ConFlo IV unit. The CO2 pulses were calibrated against a certified fatty acid methyl ester standard of known δ13C, and this was done individually for both C16:0 and C18:0. Carbon isotopic values were expressed in per mil (‰) relative to the Pee Dee Belemnite (PDB) standard.

GC-MS analysis for detecting possible biomarkers helping to identify the original food substance was conducted with Agilent 7890A Series gas chromatography and Agilent 5975C Inert XL mass-selective detector with a DB5-MS (5%-phenyl)-methylpolysiloxane column (30 m × 0.25 mm × 0.25 μm). Injected sample size was 1 μL. The splitless injector and interface were maintained at 300 °C and 280 °C, respectively, and helium was used as the carrier gas at a constant flow. The GC column was inserted directly into the ion source.

### TABLE 1 List of archaeological samples analysed

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sample type</th>
<th>Vessel area</th>
<th>Vessel find context</th>
</tr>
</thead>
<tbody>
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<td>C.i</td>
<td>Shoulder</td>
<td>At the foot of the burial VI</td>
</tr>
<tr>
<td>Kukruse XII</td>
<td>C.i</td>
<td>Base</td>
<td>At the foot of the burial XII</td>
</tr>
<tr>
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<td>Rim</td>
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<tr>
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<td>F.i</td>
<td>Shoulder</td>
<td>At the foot of the burial XXII</td>
</tr>
</tbody>
</table>

C: drilled ceramic powder, F: charred food-crust, i: internal surface.
of the mass spectrometer. The ionization energy was 70 eV, and spectra were obtained by scanning between m/z 50 and 800 amu. The temperature program was set as follows: 50 °C for 2 minutes, thereafter a gradient of 10 °C/minute up to 325 °C for 6.5 minutes. Compounds were identified with Agilent Chemstation software using also NIST mass spectra library.

2.4 MALDI-FT-ICR-MS analysis

Sample preparation for MALDI-FT-ICR-MS includes dissolving the sample and mixing it with appropriate internal calibrants and matrix solution. However, selecting suitable volumes and components of possible mixtures requires elaborate testing.

Two different sample extraction methods were tested. First, a fraction of either food crust (6 mg) or ceramic powder (30 mg for archaeological and 15 mg for experimental samples accordingly) with absorbed lipids was directly diluted in Safe-Lock Eppendorf tubes with a suitable solvent, sonicated for 15 minutes in ultrasonic bath; an aliquot of the solution was mixed with internal calibrants and applied to a MALDI target plate. This preparation technique does not involve any separation of different constituents, and the mass spectra could in principle reflect all the different substances present in the sample. Good results were obtained with this method in the case of 1 experimental pot (SI Figure S7, Table S7).

The second approach focused on lipid extraction and purification. According to the literature the most widely applied methods for MALDI-based lipid analysis are the Bligh-Dyer41 and Folch42 extraction methods, which have provided successful results for the analysis of, eg, 15th century paintings.24 Slightly modified and simplified solvent extraction (SE) method which has been widely applied in archaeological lipid residue analysis8,24 was used in this study, although 1 usual constituent, ie, chloroform, was replaced with a less toxic and volatile dichloromethane (DCM). Sample powder (ca 20 mg of food crust or 0.8 to 1 g of ceramic powder) was mixed with a solvent mixture of DCM and methanol (MeOH) (2:1 v/v), sonicated for 15 minutes, centrifuged, and the total lipid extract (TLE) removed with a pipette. The procedure was repeated 3 times after which the TLE was dried under a stream of nitrogen until the solvent was completely removed and the TLE obtained. This method proved to be suitable for the majority of the samples covering 4 archaeological and 3 experimental pots (Figure 2, Table 4, SI Figure S1-S6, Tables S1-S6) presented in this study.

2.5 Solvents and sample dilution

The solvent has to dissolve the analyte (lipids and their degradation products), mix and recrystallize with the matrix solution, and enable ionization of analyte with the matrix. The most frequently used solvents in MALDI-based lipid analysis have been either chloroform or chloroform-methanol solutions. Because those studies mainly deal with unprocessed and fresh lipid samples, different solvents and solvent mixtures were tested in order to find the most suitable combinations for thermally processed and degraded lipid samples in both organic (food-crust) and mineral (pottery, clay) matrices.

The solvents tested and their suitability are provided in Table 2. The best results were obtained using the least polar solvents like chloroform or DCM. The inclusion of more polar solvent of MeOH provided very poor results in terms of TAG detection (almost no TAGs detected), and it is possible that the inclusion of MeOH in the solvent diminished the overall evaporation of TAGs. Because the results were equally good for both apolar solvents, DCM was preferred for future work due to its lower toxicity.

Dilution of lipids is crucial part of the sample preparation protocol, because the signals tend to be suppressed at higher lipid concentrations.13 Very different sample concentrations have been reported ranging from 0.1 to 1 mg/mL13,43,44 but also an example of 6 mg/200 μL solution in art-related substance of dammar resin19 can be found.

Different TLE sample dilutions extracted from 0.8 to 1 g of ceramic powder from experimental pots were tested. It became evident that in the case of modern reference samples, too high concentrations with 250 and 500 μL of DCM did not promote co-crystallization with the matrix solution, and no informative spectra were obtained. The best results with modern solvent extracted lipids were obtained with double dilution: TLE was first mixed with 50 to 100 μL DCM (depending on the expected lipid preservation based on visible lipid residue attached to the walls of the vials after the extraction) from which an aliquot of 25 μL of sample solution was sub-diluted with
additional 200 µL of DCM. From this, an aliquot of 30 to 40 µL of sample was mixed with 0.2 µL of lower calibrants with an addition of 5 µL of higher Angiotensin II calibrant (Table 3).

With the archaeological pots, however, one has to consider that the overall preservation of lipids is much lower, and too high lipid concentration, which might be a problem for modern samples, is generally not an issue. The solvent extraction of archaeological samples was based on 0.8 to 1 g of ceramic matrix or ca 20 mg of food crust. The obtained TLE was then further dissolved in 50 µL DCM from which an aliquot of 30 µL sample was mixed with calibrants: 0.2 µL of lower calibrant mixture and 4 µL of 2.5 nmol Angiotensin II calibrant. Interpretable results were obtained for both food crusts and ceramic matrix samples.

### 2.6 Calibration

External calibration of the instrument was conducted prior to each measurement session using commercial ProteoMass Normal Mass Calibration Mix (ProteoMass™ MALDI Calibration Kit for LTQ XL™ and LTQ Hybrids, Sigma-Aldrich) mixtures for m/z range 524.26496-1758.93260 complemented by 2.5-dihydroxybenzoic acid (DHB, Sigma-Aldrich, purity 98%) peaks of m/z 137.02332 and 273.03936.

All obtained mass spectra were further internally calibrated using peaks of the added internal calibrants and specific DHB peaks. The addition of internal calibrants is necessary to control the mass axis and adjust the measurements of unknown substances accordingly. Calibrants were chosen based on the range of m/z values for the lipids: fatty acids up to ca 400; DAGs in the range of 550 to 650; and TAGs in the range of 700 to 1000. An emphasis was put on the higher m/z ratios, i.e., for the range of TAGs as the most characteristic molecules enabling the identification of the original substance cooked.

The list of internal calibrants is presented in Table 3. The majority of calibrants are different phosphazene bases thoroughly tested in our work group⁴⁵ with an addition of some higher and lower mass calibrants. Suitable calibration mixtures were prepared beforehand, and different volumes were tested. For commercial Angiotensin II (so-called 1046 calibrant, Sigma-Aldrich), different concentrations were tested: for 2.5 nmol concentration (in Milli-Q water), a volume of 5 to 6 µL was added to the sample, and for 10 nmol, 1.5 µL was sufficient. For lower m/z ratio calibrants, the volumes remained in the range of 0.1 to 0.2 µL. The best results were obtained when internal calibrants were added directly into the sample solution. In addition to separately added internal calibrants, DHB peaks⁴⁶ were also employed for calibrating the spectra. As a minimum, 3 peaks in the range from m/z 137 to 916 or 1046 were used. The maximum m/z error observed for internal calibrant peaks was 1.2 ppm (in the majority of the cases below 1.0 ppm).

### 2.7 Matrix material

2,5-Dihydroxybenzoic acid (DHB) has been reported as the most suitable and widely used matrix for lipid analysis.¹¹,¹³,¹⁶ We tested different solvents for matrix substances. First, a solution of 150 mg of DHB in 0.5 mL acetonitrile (J.T. Baker, HPLC grade) and 0.5 mL of 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich 99 %) solution in water (Milli-Q® Advantage A 10, Millipore). The second matrix solution was DHB in ethylacetate which has provided good results especially in the case of apolar lipids.¹⁰,⁴⁷ For this solution, 150 mg of DHB in 0.5 mL ethylacetate (Sigma-Aldrich, HPLC grade) and 0.5 mL of 0.1% trifluoroacetic acid (TFA, Aldrich 99 %) solution in water (Milli-Q) were mixed. In comparison, the solution of DHB in ethylacetate gave slightly better results.

### 2.8 Sample placement

Two different dried droplet sample placement methods were employed. First, a droplet of 1 µL DHB solution was applied on the plate, dried under vacuum, and a volume of sample solution in excess (2 µL) was placed on top of the matrix and dried the same way. In the second test, sample solution in DCM and the DHB solution were mixed together in 2:1 (v/v) ratio; then, a drop of 1 µL was placed on the MALDI target plate and dried in vacuum. The mixing of sample and DHB solution in 2:1 (v/v), with DHB dissolved in sample solution and recrystallized with the sample under vacuum of the instrument’s sample introduction chamber allowed better miscibility of the two and gave better results during the analysis.

### 2.9 Instrument and data analysis

Measurements of mass spectra were carried out with a Varian 930 FT-ICR mass spectrometer equipped with a 7 T superconducting magnet in a positive single scan mode. Samples were placed on an AB 192 spot stainless steel plate. The MALDI source used a New Wave Orion 50083 Nd:YAG laser with an optical attenuator and a third harmonic generator (355 nm). Ions from 5 laser shots with the frequency of 5 Hz were collected. Laser intensity was varied with optical attenuator, but usually intensity in the range of 35 to 60 % of maximum was used. The ions formed in MALDI source were collected in a hexapole trap cell, cooled with the nitrogen gas pulse, and transferred into the FT-ICR analyzer cell (using ion guide settings optimized for transferring ions in the m/z range from 500 to 1500 amu with the highest yield). Typical pressure in the analyzer region during detection was
n x 10^{-10} Torr (1 Torr = 133 Pa). Ions were cooled in ICR cell with short pulse on N2 gas and kept in the ICR cell for 5.5 seconds, excited with arbitrary waveform (DAC rate 8000 kHz; excitation amplitude voltage 200 V), and detected with a direct (broadband) acquisition mode (ADC rate 4 MHz; transient length: 2048 K) in the m/z range from 80 to 1200 amu. This m/z range is optimal for lipid analysis as their molecular masses are typically in the range from 100 for fatty acids to max 1000 for TAGs. Instrument was operated, and mass spectra were collected and analysed with Varian OMEGA 9.1.21 software. The same software has the database of exact atomic masses and enables searching for the closest molecules at any given m/z value measured using combinations of atoms (ion formulas) with previously constrained search criteria. The criteria were based on preliminary knowledge about the expected molecules: in the case of TAGs, the choice of atoms is constrained to C, O, H as well as Na and Al (in order to enable detecting Na+ and Al+ adducts). The software provides all possible ion formulas (candidates) for any experimental m/z value and ranks them according to closeness of their m/z to the experimental m/z value. The choice between the candidates proposed by the software was based on the m/z error values of measured results preferring the smallest error values and closest matches, but also considering likelihood of the existence of proposed molecules in the sample. TAGs have specific molecular structures and characteristics expressed with decimal places of measured m/z values which allow identifying exactly these molecules among

<table>
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<th>TAG formula</th>
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<th>Exact m/z</th>
<th>Δm/z (ppm)</th>
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a TAG oxidation product.
b 13C isotope satellite.
the others. These considerations were also used. For the components detected in experimental and archaeological samples, the maximum \( m/z \) error of each compound detected was below 2.3 ppm, in the vast majority of cases below 2 ppm.

3 | RESULTS AND DISCUSSION

The mass spectra obtained from 4 different archaeological and 4 experimental pots (Figure 2, Table 4, Figures S1-S7, Tables S1-S7) show numerous TAGs (mainly in the range of \( m/z \) 700–1000). In the case of intact lipid molecules TAGs have been mainly used for identifying the origin of organic food residues in archaeology\(^{27,34,48,49}\), they are the main components of natural plant oils and fresh animal fats,\(^{50}\) whilst MAGs and DAGs are only minor components found at trace levels in fresh natural lipids. Furthermore, TAGs can decompose and lose fatty acids forming DAGs and MAGs due to various degradation, hydrolysis, and oxidation processes,\(^{18,36,37}\) which may happen due to aging or as a result of cooking and various environmental processes. For these reasons, it was decided to focus on TAGs as the major and most direct natural lipid components.

Figure 2 displays a typical TAG spectrum from an archaeological sample of Kukruse XLIV pot (see further examples and details for other samples in SI Figures S1-S7, Tables S1-S7), and Table 4 presents TAGs identified in this sample. Peaks with clear visible deformations (shoulders, high intensity splits) remained unidentified, because these deformations may distort the final peak assignment considerably. TAGs (represented by the natural isotopomers) with different number of unsaturated C=C bonds indicated by the total number of hydrogens in measured ion formulas, as well as their various oxidation products are evident. They are usually found as sodium adducts and, in rare cases, also as aluminium adducts or protonated molecules. The low differences of measured and expected \( m/z \) values of specific TAGs and their oxidation products, as well as the intensity ratios of the main peaks and \(^{13}C\) satellites, give evidence of reliability of the peak assignments. It is possible to estimate the relative abundance and overall distribution of different TAGs. Besides TAGs and their oxidation products, the spectra also display peaks of some non- lipidomic compounds. One of them is \( m/z \) 719.9995 probably corresponding to fullerenol ion of the composition \(^{12}C_{60}\)\(^{+}\). The intense peak of 721.00692 could potentially belong to a combination of the \(^{12}C\) satellite (\(^{12}C_{55}^{12}C\)) of the previous peak\(^{51}\) and some other substance or aggregate that we are currently unable to identify.

The mass spectra in principle allow further identification options regarding the number of unsaturated carbon bonds. The distribution of such TAGs can provide further information for identifying the origin of fat,\(^{29,30,52}\) and this kind of information is hardly available with traditional mass-spectrometry methods showing again the benefits of MALDI-MS. However, it is also known that these unsaturated bonds are relatively unstable and subject to alterations. They tend to degrade and leach, transfer into oxidized, hydrolysed, or saturated forms to a different degree in time, and are thus less likely to survive in old archaeological samples.

For the interpretation of the results, it is important to note, that in their original form, TAGs include 6 oxygen atoms. Such examples are clearly presented in our results, and in many of these cases, it is clearly evident that MALDI-MS does provide further structural information of these TAGs: different number of unsaturated bonds and their relative abundance ratios. However, in many cases, the ion formulas identified have higher number of oxygen. These are indicators of various oxidations within fatty acid moieties of TAGs,\(^{18}\) which probably have taken place in the locales of unsaturated carbon bonds. The exact processes of the latter are difficult to establish, because different changes may have occurred due to cooking, but also as a result of later aging, environmental conditions, and further degradation in time. These might have resulted in very different degradation products such as addition of hydroxy group(s) or formation of epoxy-products replacing unsaturated bonds, but also with oxidative cleavage of double bonds. Thus, in the case of oxidized TAGs and based on detected ion formula, we cannot determine which is the exact parent molecule and which degradation processes have taken place and when. An addition of oxygen as a result of ionization mechanisms cannot be entirely excluded either. Therefore, we cannot identify the exact initial TAG with its exact number of unsaturated bonds, and the TAG attributions presented in the tables rely on the formulas as measured (with clearly oxidized examples marked). However, it is expected that despite the oxidation processes, the overall acyl C number has remained the same. The distribution of major TAGs according to their acyl C numbers has proven to be sufficient to identify the original substance because the relative abundances of various chain-length TAGs vary according to major types of animal products. Therefore, we relied on the overall acyl carbon number of TAGs for archaeological substance identification.

In order to characterize and compare the distribution of major TAGs in each sample, the relative abundances of particular TAG signals according to the number of acyl carbon atoms (40 to 56), disregarding unsaturation, were summarized. To this also, the abundances of specific TAG’s isotope satellite peaks and relevant TAG oxidation products were added. For example, for overall relative abundance of \( C_{42} \) TAG in sample XLIV presented above (Table 4) peaks with \( m/z \) of 745.63262 (\( C_{42} \) TAG), 746.63695 (\(^{13}C\) isotope satellite of the \( C_{42} \) TAG), and 759.61126 (oxidation product of the \( C_{42} \) TAG) were pooled. To make the results of the measured archaeological samples compatible with the measurements of experimental material and also with previously published data, the obtained net signals corresponding to TAGs with the number of carbon atoms ranging from 40 to 56 were ranked by intensity using the rank function in MS Excel. The original measured summed relative abundance results are available in SI, Table S8.

The results of overall TAG distribution from 4 archaeological samples (pots from burials VI, XII, XXII, XLIV) are presented in Figure 3A-C. These were compared with data obtained from the analysis of experimental material prepared in this work as well as with previously published works covering both modern reference and archaeological samples by Mukherjee et al. 2007\(^{48}\) and Dudd & Evershed 1998.\(^{53}\) To further verify the measurements obtained with MALDI-FT-ICR-MS, the parts of the same samples were saved for parallel analysis with GC-MS and GC-C-IRMS (Figure 4).

The results from pot XLIV show wide distribution of both low (\( C_{40-44} \)) and higher C number TAGs (\( C_{48-52} \)), with domination by \( C_{50} \) and relatively high \( C_{48} \) TAGs, whilst \( C_{54} \) and \( C_{56} \) are in minority. Such a wide distribution of TAGs and markedly high abundance of TAGs
with lower acyl C number have been identified in both modern and archaeological dairy products in previous studies. The same tendencies were also evident in our experimentally cooked cow milk sample. The relative differences between the archaeological and experimental samples, especially regarding the abundance of lower C number TAGs, are most likely due to later degradation and alteration processes of the archaeological samples. Previous scholars have pointed out that lower molecular weight TAGs are more prone to degradation than medium-chain and long-chain ones. It can be concluded from these results and further comparisons that dairy products were most likely cooked in the pot concealed with burial XLIV at Kukruse cemetery. The latter is also supported by the GC-C-IRMS analysis where the sample is isotopically very depleted in C18:0 fatty acid compared with C16:0 with the $\Delta^{13}C (\delta^{13}C_{18:0} - \delta^{13}C_{16:0})$ of $-3.89 \%$ indicative of dairy products (Figure 4). No clear identification about the origin of food substance in this pot was obtained with GC-MS analysis.

In sample VI (Figure 3B, SI Figure S2, Table S2) extracted from ceramic powder again, a relatively wide distribution of TAGs is evident. However, higher C-number TAGs of C50 and C52 are both present in largest quantity: the histograms are dominated by C52 and C50 (comp.}

**FIGURE 3** Distribution of TAGs from 4 archaeological pots and 4 experimental pots analysed in this study in comparison with previously published work. (A) sample from Kukruse XLIV pot identified as dairy product; (B) sample form Kukruse VI pot identified as ruminant product; (C) samples from Kukruse XXII and XII pots identified as aquatic, most likely fish product.
domination of C_{48} and C_{50} in the case of dairy), followed by C_{48} and relatively high C_{54} (comp. low abundance in the case of dairy). The abundance of smaller C-number TAGs and C_{56} TAG is considerably lower in comparison. This sample has a relatively good match with previous archaeological ruminant samples \(^{48}\) as well as our experimental ruminant pot sample. The difference between experimental and archaeological samples in lower C-number TAGs (C_{40} and C_{42}) might be due to the same issue that lower molecular weight TAGs are more prone to degradation than medium-chain and long-chain TAGs. \(^{49}\) No clear identifications about the origin of food substance in this pot was obtained with GC-MS analysis, but GC-C-IRMS (Figure 4) plots the sample in around the ruminants, too.

Sample XXII deriving from food-crust removed on the surface of a pot is noteworthy due to its rather low concentration and abundance of TAGs (SI Figure S3, Table S3), also explaining the visibly higher abundance of internal calibrant peaks (eg, m/z 888.54551) in the spectrum. The abundance of TAGs is higher for sample XII extracted from the internal surface of the vessel; F: Food-crust sample from internal surface of the vessel [Colour figure can be viewed at wileyonlinelibrary.com]

Our measurements show the high potential of MALDI-based soft ionization technique for analysing archaeological food remains. Due to its high mass accuracy, it can identify and differentiate between components with similar nominal mass m/z separable according to their detailed decimal places. This enables to avoid confusing, eg, higher C number TAGs with heavier m/z value and oxidation products of some smaller TAGs.

MALDI-MS is also capable of providing more detailed data on possible unsaturations and oxidations of original compounds which is hardly available with more traditional methods such as high-temperature (HT)GC-MS. This information allows evaluating the general quality and preservation of aged organic residues and in principle also better identification of the compounds preserved. However, this needs further testing and elaboration in the future, especially for estimating the character of original lipid components as opposed to alterations taking place as a result of cooking or aging.

Furthermore, as previous studies have shown, traditional methods have certain limits as to how large molecules can be detected, eg, TAGs beyond C_{54} are rarely reported. MALDI-MS is capable of detecting larger molecules and thus has less constraints for identifying heavier lipid compounds in archaeological samples. MALDI-FT-ICR-MS also benefits from its capacity to detect lower concentration of lipid components. Considering that TAGs are often poorly preserved and thus less approachable with traditional GC-MS methods, the high accuracy and sensitivity of MALDI-FT-ICR-MS are certainly an asset. The identification of dairy products – something which has been mostly relying on isotopic measurements with GC-C-IRMS or HTGC-MS analysis – covering also range of short-chain TAGs is one example of its potential benefits in comparison with GC-MS. The same might apply to substances including abundance of molecules with higher number of unsaturations making them less stable (eg, in the case of fish oils). It has indeed been pointed out that TAGs from fish do not usually preserve in archaeological samples. \(^{37}\) Furthermore, due to high level of (poly)unsaturations expected in fats and oils of aquatic origin HTGC-MS is not recommended, because of partial thermal degradation and polymerization of (poly)unsaturated compounds at higher temperatures. \(^{55}\) These aspects altogether could, on the one hand, explain the low abundance of TAGs in sample XXII, on the other
hand, however, prove the suitability and capacity of MALDI-FT-ICR-MS to detect very low concentrations of TAGs and (poly)unsaturated molecules (in comparison with HTGC-MS).

One issue to note with MALDI-MS is that different molecules may have different ionization efficiencies in the MALDI source. If different matrix material and laser wavelength are used, then it is not automatically guaranteed that the same signal intensity ratios are obtained from the same sample. However, if several measurements of the same sample are made and as long as all analysed spectra are obtained with the same MS system and experimental method, this is not a major problem. Furthermore, we also aimed at gaining further confirmation and comparison to our results from previously published material analysed with HTGC-MS. Additional control of the results by another independent method— isotopic measurements of major fatty acids of C₁₆:0 and C₁₈:₀—provides further confirmation to our interpretations of MALDI-MS measurements.

The other archaeology-specific question is the preservation of lipids in archaeological samples: lipid molecules in archaeological samples are degraded or altered (and can do that to a different degree depending on both their depositional environment and age). TAGs are more prone to degradation and thus the identification of initial food residues relying on them might be complicated, especially in the case of millennia-old samples as opposed to more recent ones. However, here the high mass-accuracy and low LoD of MALDI-MS might be a solution potentially allowing detection of also lower concentrations of TAGs in older samples.

Relating to the above care also has to be exercised regarding the preferential degradation of TAGs containing short-chain fatty acids and the dissolution of the latter. This is a particular problem in the case of more degraded samples (depending on age and depositional conditions), because here the degradation of short-chain TAGs could be even more pronounced resulting in the situation where different food substances result in rather similar TAG distributions. Here, additional comparison from experimental material analysed with the same instrument and previously identified archaeological samples is necessary, in the initial stage at least. As our results and comparisons exemplify, MALDI-MS provides overlapping results on the distribution of major TAGs compatible with previously published data covering both short-chain and long-chain TAGs and is thus a reliable method which is well suited for future lipid research.

Finally, one also has to keep in mind that residues in ceramic vessels might be of considerably wider origin than only food substances, eg, remains from cooking (eg, soot, resins), later additions from burial environment, contaminations from storage and post-depositional handling, etc. This has to be considered when analysing and interpreting the results of archaeological food residues with MALDI-MS but indeed with any other method, and we are most likely facing some of those issues in the case of our unidentified peaks as well. Additionally, lower concentration components might easily be overwhelmed by major ones. However, in the case of the latter MALDI-MS with its high mass-accuracy, low LoD, and capacity to separate different components at the level of ppm is certainly advantageous.

As for future work, the extended usage of the detailed data provided by the MALDI-MS (eg, information on unsaturated bonds and oxidations) is necessary. Also, further work on degradation issues (especially related to estimating the preservation of short-chain TAGs) as well as confirmation on the suitability and additional advantages of the method comparing samples of different age and from different contexts is needed.

4 | CONCLUSION

Our initial results show that MALDI-FT-ICR-MS is a suitable and indeed a prolific method capable of identifying food residues of different origin in archaeological as well as experimental ceramic vessels. It allows high mass-accuracy and thus highly reliable determination of components, enabling to differentiate between the components at the level of ppm. It can also distinguish between the TAGs with different unsaturated C=C bonds and their oxidation products providing potentially additional details useful for identifying the original substance. Furthermore, MALDI-MS can detect low concentrations of TAGs, which is particularly helpful when working with old archaeological samples, often subject to degradation.

As to future development, the limitations and possibilities for analysing direct (unextracted) samples must be tested. Currently, the analysis of direct (unextracted) archaeological samples shows that untreated samples in such a small quantities do not allow the identification of TAGs meeting the satisfactory error range criteria. The reason is most likely that the amount of analyse (lipids) is too small in comparison with all the other substances (eg, mineral ceramic matrix, carbonized food remains), and these clearly exceed the low concentration of lipids in the sample. However, in one of the experimental samples presented, TAG profiles were obtained without employing any specific lipid extraction protocols, ie, just by mixing ceramic powder including lipids with suitable solvent. It proves the potential of MALDI-MS for lipid analysis from untreated samples in very different matrices.

This study also gives evidence that MALDI-FT-ICR-MS is suitable for identifying pure foodstuffs. An additional future research should also address its capacity and efficiency to detect and identify different components in food mixtures. The latter needs further testing with both experimental and archaeological samples.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.