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ANALYTICAL STRATEGIES FOR DISCRIMINATING ARCHEOLOGICAL FATTY SUBSTANCES FROM ANIMAL ORIGIN

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Mass spectrometry (MS) is an essential tool in the field of biomolecular archeology to characterize amorphous organic residues preserved in ancient ceramic vessels. Animal fats of various nature and origin, namely subcutaneous fats of cattle, sheep, goats, pigs, horses, and also of dairy products, are those most commonly identified in organic residues in archeological pottery. Fats and oils of marine origin have also been revealed. Since the first applications of MS coupled with gas chromatography (GC) in archeology at the end of 1980s, several developments have occurred, including isotopic determinations by GC coupled to isotope ratio MS and identification of triacylglycerols (TAGs) structure by soft ionization techniques (ESI and APCI). The combination of these methods provides invaluable insights into the strategies of exploitation of animal products in prehistory. In this review, I focus on the analytical strategies based upon MS that allow elucidation of the structure of biomolecular constituents and determination of their isotopic values to identify the nature of animal fat components preserved in highly complex and degraded archeological matrices. © 2010 Wiley Periodicals, Inc., Mass Spec Rev

Keywords: archeology; animal fats; lipids; biomarkers; stable isotopes; mass spectrometry

I. INTRODUCTION

Archeology is the study of ancient human communities with a combination of methods that allow investigation of the remains of material culture preserved over time. It “is concerned with the full range of past human experience—how people organized themselves into social groups and exploited their surroundings; what they ate, made, and believed; how they communicated and why their societies changed” (Renfrew & Bahn, 1996: 17). The objects preserved in the archeological record cover a wide variety of inorganic and organic materials, including lithic and bone tools, ceramic vessels, and metal utensils. Faunal (bones, teeth, ivory and antlers) and botanical (pollens, seeds, and charcoals) remains are also commonly encountered in ancient deposits. Macro- and microscopic observations are the first steps in the study of such artifacts and ecofacts. However, in an increasing number of cases, analytical chemistry is providing vital information related to the kinds of materials used, their production methods, provenance, and degree of alteration

(Regert, Guerra, & Reiche, 2006a,b; Pollard et al., 2007). Other materials exploited by human beings for long periods of time are preserved as amorphous organic residues in various contexts. They result from the use of key substances for human life such as animal fats, dairy products, beeswax, fermented beverages, resins, and tars (Fig. 1).

All of these materials represent precious sources of information to address questions that deal with the exploitation and management of natural substances over time, including their choice and ways of procurement, their mode of storage, use or consumption, and their techniques of transformation, that might include several processes, such as heating, grinding, biochemical, or chemical modifications. Because they lack recognizable morphological attributes, the only way to determine their nature and origin relies on the development of analytical strategies that allow elucidation of their chemical composition (Evershed et al., 1992a,b; Evershed, 1993a).

Amorphous organic remains might be preserved as charred or absorbed residues in pottery vessels, as remnants of glue still adhering to lithic or bone tools, and as free accumulations or deposits in the sedimentary matrix (Regert et al., 2003a). Whatever their age or the environment in which they were preserved, they comprise complex mixtures that contain numerous molecular constituents of widely ranging volatilities, polarities and molecular weights, transformed by human activities and altered by natural decay. Their identification is thus a major analytical challenge and requires the development of innovative methodologies that mostly rely on the use of MS.

The story of the analytical study of archeological amorphous organic remains could be divided into two main stages, one before and the other after the application of MS to the analysis of ancient organic residues.

As early as the end of the 19th century and beginning of the 20th century, black organic crusts visible on the surface of ceramic vessels were thought to contain valuable archeological information. The first attempts to identify such remnants relied on wet chemistry and basic chemical experiments such as solubility or burning tests. Hypotheses on the presence of birch bark tar (an adhesive produced by heating white birch bark in controlled conditions), dairy products, and different kinds of lipids, were then proposed (Heintzel, 1880, 1881; Cotte & Cotte, 1917; Grüss, 1933). In the 1960s, infrared spectroscopy began to be used to address archeological questions (Sandermann, 1965; Funke, 1969). The first chromatographic analyses followed shortly afterwards, and were performed in the 1970s to lead to the identification of fatty materials, olive oil, and wine phenolic biomarkers (Condamin et al., 1976; Condamin & Formenti, 1978; Rottländer & Schlichtherle, 1979). GC–MS analyses

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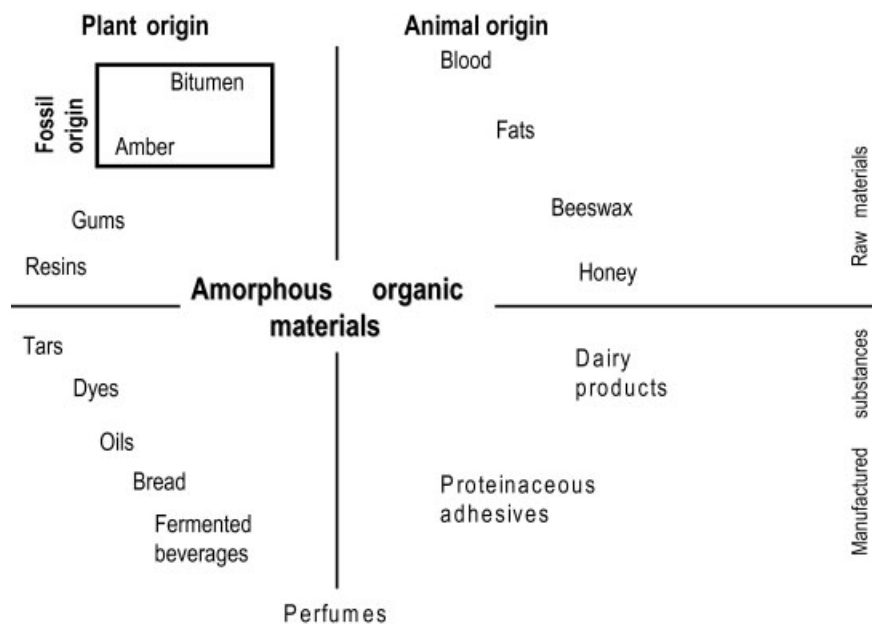


FIGURE 1. Natural substances that may have been exploited since the prehistory. Some of them are preserved as amorphous organic residues in ancient ceramic vessels. Adapted from Regert (2001) and Regert et al. (2003a).

began to be applied in the 1980s, especially to study terpenoid resins and tars (Evershed, Jerman, & Eglinton, 1985; Robinson et al., 1987). At that time, there was no systematic research development on the analysis of archeological amorphous organic remains, and the pioneer articles related to this subject usually relied on the analysis of a limited number of samples by FTIR, GC, or GC–MS (Condamin et al., 1976; Evershed, Jerman, & Eglinton, 1985; Heron & Pollard, 1988). They were, nevertheless, of great importance because they showed the considerable potential of these analytical investigations to improve our knowledge on various kinds of natural substances exploited during the past.

The situation radically changed at the beginning of the 1990s for several reasons. First of all, MS was widely adopted in research that aimed to identify archeological organic residues to provide spectra that could be related to molecular markers characteristic of specific materials. Second, systematic investigations of major series of archeological and reference samples were conducted, especially by the team lead by Richard Evershed in England (see for example Evershed et al., 1992a,b, 1999, 2002; Heron & Evershed, 1993; Evershed, 1993a, 2008a, and references therein). Last, the recent advances in MS and hyphenated techniques allowed the determination of carbon stable isotope compositions of individual compounds by GC–C-IRMS (Evershed et al., 1994; Evershed, 2009) and offered additional possibilities to elucidate molecular structures by soft ionization techniques. In particular, APCI and ESI, opened up new perspectives for the determination of natural resources processed in ancient pottery vessels (Mottram & Evershed, 1996, 2001; Kimpe, Jacobs, & Waelkens, 2001; Mottram, Crossman, & Evershed, 2001; Garnier et al., 2002; Kimpe, Jacobs, & Waelkens, 2002; Kimpe et al., 2004; Mirabaud, 2007; Mirabaud, Rolando, & Regert, 2007).

A number of various products have been chemically identified in ancient pottery vessels, including beeswax (Heron

et al., 1994; Evershed et al., 1997a, 2003; Regert et al., 2001a; Garnier et al., 2002; Regert, 2008), wine (Garnier et al., 2003; Guasch-Jané et al., 2004, 2006a,b), cacao (Henderson et al., 2007), resins, and tars (Regert & Rolando, 2002; Colombini, Modugno, & Ribechini, 2005; Regert et al., 2008a; Stern et al., 2008). However, animal fats of different origins are by far the most frequently encountered substances in archeological ceramic containers (Evershed et al., 1997b; Dudd, Evershed, & Gibson, 1999). Due to their wide distribution in the archeological record and their significant value to understand past societies, these key substances have been the focus of numerous analytical investigations performed during the last 15 years. In this review, I focus on the analytical strategies developed specifically to identify ancient animal fats in pottery containers, to assign their nature (adipose fat or dairy product) and species origin (cattle, sheep, goat, pig or other terrestrial animals, marine resources, aquatic products), and to understand their alteration pathways.

Lipid components from archeological origin are preserved in low concentrations in highly degraded and complex matrices in ceramic vessels. Their study necessitates the development of appropriate methodologies of extraction, purification and analysis of the total lipid extract (TLE) by HT GC–MS, avoiding any step of hydrolysis (Evershed et al., 1992a,b; Evershed, 1993b,c; Heron & Evershed, 1993).

Advances in gas chromatography analysis were of particular importance to allow the separation and identification of compounds of wide ranging volatility, polarity, and molecular weights, often preserved at trace concentrations (Evershed et al., 1987; Evershed & Goad, 1987; Evershed, Heron, & Goad, 1990). A substantial part of the work also focused on the modes of transformation and alteration of animal fats from replica ceramic vessels (Charters et al., 1997) and degradation experiments (Dudd, Regert, & Evershed, 1998). By detailed study of the distributions of fatty acids, sterols, MAGs, DAGs, and TAGs, it was possible to assess the presence of animal fats, to distinguish

ruminant from non-ruminant adipose fats and to detect the presence of dairy products (Charters et al., 1995; Evershed et al., 1997b; Dudd & Evershed, 1998). However, when animal fats are not well-preserved, different materials might exhibit great similarities in their chromatographic pattern.

Two different yet complementary approaches were explored to resolve this problem: (i) investigate the bulk carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope compositions of organic residues (DeNiro, 1987) and/or determine the carbon isotope compositions of individual fatty acids (Evershed et al., 1994 for the first application in archeology; Godin, Fay, & Hopfgartner, 2007; Evershed et al., 2007; Evershed, 2008a, 2009 for recent reviews in life sciences and palaeoecology); (ii) determine the fine structure of TAGs after appropriate steps of sample treatments with soft ionization techniques and tandem mass spectrometry, with or without previous liquid chromatographic separation (Mottram & Evershed, 1996, 2001; Kimpe, Jacobs, & Waelkens, 2001; Mottram, Crossman, & Evershed, 2001; Kimpe, Jacobs & Waelkens, 2002; Kimpe et al., 2004; Mirabaud, 2007; Mirabaud, Rolando, & Regert, 2007; Romanus et al., 2007). These latter methods are effective only with a good preservation of TAGs.

Beyond the essential developments in analytical chemistry, the archeological significance of the results obtained must also be carefully considered and it is important to stress the highly interdisciplinary character of such research that rely on paradigms of both analytical chemistry and archeological science.

This article is organized according to the chronological evolution of analytical advances from GC–MS analyses to GC–C-IRMS and soft ionization techniques. However, it must be kept in mind that it is often a combination of these complementary approaches, that provides the most promising results.

II. MS INVESTIGATIONS WITH ELECTRON IONIZATION

Because amorphous organic materials preserved in archeological pottery vessels are all composed of complex molecular mixtures, chromatographic techniques are the methods of choice for their analysis. The universal character of the flame ionization detector, the early development of GC–MS hyphenated techniques, and the various sample derivatization methods prior to GC or GC–MS analyses that enable analysis of a wide range of compounds in a single analysis, explain why GC–MS is extensively used to study total lipid extracts obtained from ceramic containers.

However, the development of methodologies adapted to the specific characteristics and properties of archeological samples were necessary before one could obtain accurate compositional assessments. Sampling operations, sample treatments, and analytical conditions in GC and MS had to be optimized before the study of large series of precious samples that contain low concentrations of lipids. Once the methods were adequately adapted, identification of several kinds of molecular markers followed, allowing discrimination of different types of animal fats improving our understanding of the various processes of alteration reflecting the molecular composition of the residues and context of preservation.

Methods have also been developed to obtain mass spectral “fingerprints” of micro-samples without any pre-treatment by direct inlet electron ionization MS (DI EI-MS). This approach

allows the rapid assessment of the composition of the residues, as an aid to classify them, and to guide further analyses depending on the molecular constituents preserved.

After consideration of amorphous organic remains preserved in ancient ceramic vessels, I detail the first investigations carried out with DI EI-MS and GC–MS and their most recent applications, with emphasis on the analytical developments, the biomarker approach, and degradation studies.

A. Amorphous Organic Materials in Ceramic Vessels

Amorphous organic remains associated with archeological ceramic vessels are classified into several distinctive categories (Regert, 2007a,b; Regert et al., 2008b). First of all, the permeable and porous matrix of unglazed pottery walls is able to absorb substantial amounts of organic matter, particularly lipids, during the prolonged and repeated uses of the vessels (Rice, 1987; Evershed, Heron, & Goad, 1990; Heron & Evershed, 1993; Evershed et al., 1997b). The presence of such invisible organic residues is undetectable by simple visual observation, but the lipids are readily solvent-extracted and analyzed according to the protocols detailed below. Surface deposits have also been described (Heron & Evershed, 1993; Regert et al., 2001b). Their occurrence is not so common as absorbed residues, but they are extensively recovered in vessels from anaerobic lacustrine or fluvial environments. Depending on their appearance, thickness, and distribution on the vessels, such residues could be divided into four classes (Fig. 2): (i) heterogenous, black, carbonized, and brittle residues present on the inner and outer surfaces of pottery vessels, the latter forming when the contents overflowed during vessel use; (ii) homogenous residues that strongly adhered to the surface, most commonly encountered inside the vessel, but also occurring to a lesser extent on the outer walls, that could be opaque black or brown tar-like materials or translucent plant resins; (iii) thin coatings that cover the surface of the pottery, and (iv) residues located in ancient cracks that provide evidence for repair of ceramic vessels.

All these residues are of prime importance to understand pottery production and function but also to assess the exploitation of natural substances through time. Not all residues provide the same type of information: absorbed and carbonized surface deposits have commonly been shown to be related to the processing and consumption of foodstuffs, particularly animal products, whereas the other categories indicate the presence of tarry or resinous substances either transformed and stored in the vessels, such as those used to burn incense (Regert et al., 2008a), to waterproof the permeable walls of the ceramic containers (Heron & Evershed, 1993; Regert et al., 2003b), or to repair pottery vessels (Binder et al., 1990; Charters, Evershed, & Goad, 1993; Heron & Evershed, 1993; Regert et al., 2008b).

Accumulation and preservation of organic matter in pottery vessels is influenced by several factors that include the nature and properties of biomolecular components, the burial environment, the characteristics of the vessels and the intensity, and modes of use (Evershed et al., 1992a,b; Heron & Evershed, 1993; Spangenberg, 2004; Copley et al., 2005a; Craig et al., 2007a).

Depending on their nature, concentration, visible or invisible character, and degree of preservation, organic residues preserved in pottery vessels have been investigated with a variety of analytical methodologies developed to unravel their chemical composition. I focus below on the methods dedicated to lipids of animal origin.

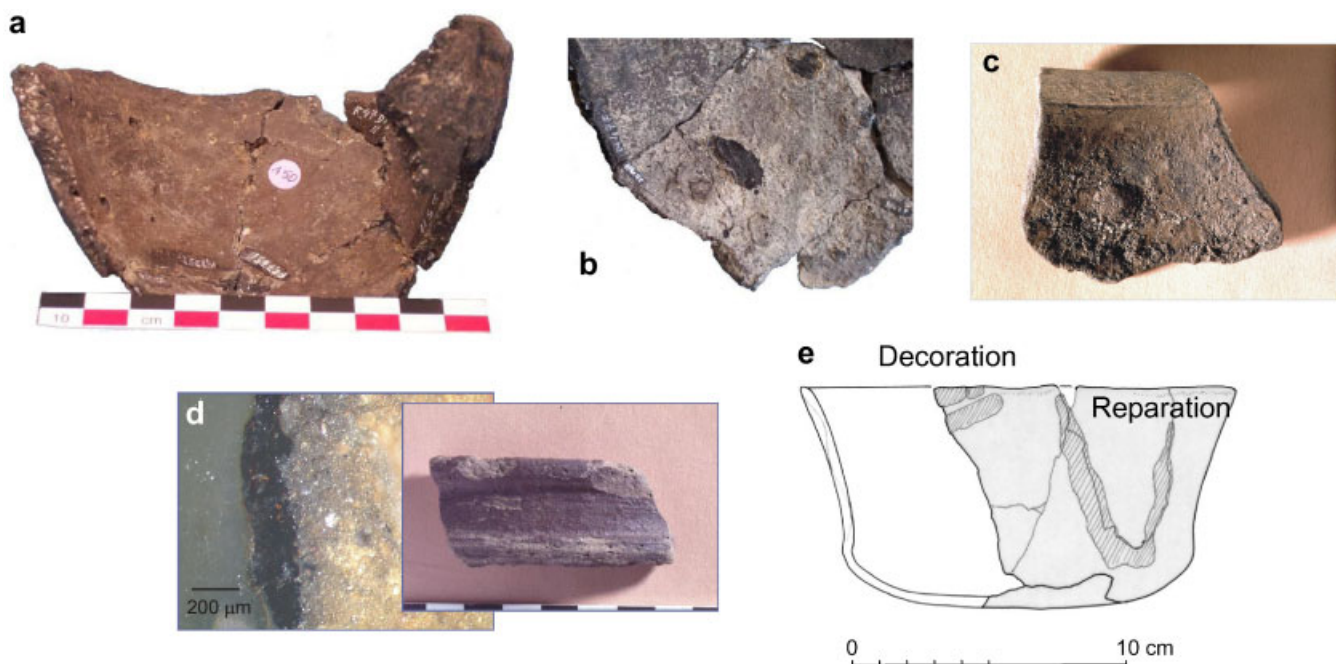


FIGURE 2. The different types of organic remains preserved in ancient pottery vessels. Adapted from Regert et al., 2008b. **a:** Photography of a sherd from the neolithic site of Clairvaux XIV, Jura, France, with no visible residues but containing organic matter absorbed in the porous pottery walls, © S. Mirabaud. **b:** Black and brittle organic residue inside a sherd from the neolithic site of Clairvaux XIV, Jura, France, © S. Mirabaud. **c:** Brown adhesive residue from the Iron Age site of Grand Aunay, Sarthe, France, © M. Regert. **d:** Sherd covered with a waterproofing organic agent from the Iron Age site of Grand Aunay, Sarthe, France, © M. Regert. **e:** Vessel with decoration and mending traces of adhesives (drawing A.-M. Pétrequin). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

B. Samples and Sampling Protocols

The first investigations of lipids preserved in various archaeological matrices focused on GC of the fatty acids released by saponification (Condamin et al., 1976; Morgan et al., 1984; Patrick, de Koning, & Smith, 1985). However, this approach has several disadvantages (Evershed, Heron, & Goad, 1990). Indeed, the process of hydrolysis or transesterification modifies the initial structures of complex lipids, for example, acylglycerols, and wax esters, resulting the loss of compositional information. In addition, multi-step wet chemical treatments increase the risks of contamination and loss of trace components. Lastly, overall the procedure is time-consuming, which is restricting to study large numbers of archeological samples.

To overcome these problems, an alternative analytical methodology that avoids any hydrolysis or transesterification step and pre-fractionation, was developed by Evershed and co-workers in the early 1990s (Evershed, Heron, & Goad, 1990; Evershed et al., 1992a,b; Charters et al., 1993, 1995). The procedure reduces to a minimum number of sample treatments and includes only solvent extraction and derivatization (Evershed, Heron, & Goad, 1990). The first applications to several hundred samples (Evershed, Heron, & Goad, 1991) showed that sufficient lipid could routinely be extracted from 2 g sherds obtained from fragments of pottery of approximately 4 cm² and a few mm of thickness. Prior to analysis, the surface of the sherds must be cleaned either by scraping with a sterile scalpel blade or with an electronic hand drill to remove soil

contamination (Heron, Evershed, & Goad, 1991; Charters et al., 1993). When visible residues are present, they are sampled and analyzed separately. The quantity of surface residue is highly dependent upon the amount present on the sherd which typically varies from 10 to several hundred milligrams. Samples are ground to powder with a clean mortar and pestle, and solvent-extracted with HPLC grade chloroform–methanol (10 mL; 2:1, v/v) with ultrasonication (2 × 15 min). The total lipid extract is dissolved in 500 µL of CHCl₃/MeOH (2:1, v/v), and an aliquot (1/5) derivatized with trimethylsilylation to decrease the polarity and increase the volatility of analytes that bear carboxy and hydroxy groups (Evershed, Heron, & Goad, 1990). The total lipid extract is analyzed with HT GC and HT GC–MS (see below). An internal standard (*n*-tetratriacontane) is added at the beginning of the procedure to quantify the different components of the extracts of the carbonized residues or the clay matrix of pottery vessels.

Using appropriate analytical conditions, it is possible, in a single analysis, to separate, detect, identify, and quantify a wide range of components including fatty acids, sterols, MAGs, DAGs, and TAGs, thereby providing an overview of the lipid composition of the archeological samples. The approach provides a straightforward screening step before focusing on specific components of taxonomic interest (Evershed, Heron, & Goad, 1990). After its development at the beginning of the 1990s by Evershed's team, this mode of sample preparation was adopted by other research groups. Although described in several articles, the most complete description of the procedure

is presented by Charters et al. (1995). It is now currently used in various laboratories, sometimes with minor modifications (Kimpe, Jacobs, & Waelkens, 2001, 2002; Regert et al., 2003a,b; Craig et al., 2005a; Mirabaud, Regert, & Rolando, 2007; Ribechini et al., 2008a).

During the initial studies of organic residues in pottery vessels, soils were also investigated with the same procedure to assess the possible contamination of the organic content of the sherds by biomolecular constituents naturally present in the surrounding burial environment. The results of these investigations clearly showed that, in most cases, the lipids identified in the vessels are the result of human activities related to the use of pottery vessels, and do not arise from migration of lipids from the sedimentary matrix (Heron, Evershed, & Goad, 1991). This matter of fact is explained by the hydrophobic properties of lipids that limit their transfer from the soil to the clay wall of the pottery and vice versa. Furthermore, the clay matrix protects the lipid residues from microbial degradation, and minimizes decay over time (Heron, Evershed, & Goad, 1991).

Before applying the procedure described above, it is essential to clearly define the series of archeological samples to be investigated. The analytical work begins at the archeological excavation, or soon after, with the study of the ceramic vessels (drying, reconstruction, description of the vessels, etc.), the detection, and description of visible charred surface residues when present. The selection of samples is of prime importance to establish relationships between vessel shape and contents (Evershed et al., 1997b; Copley et al., 2005b) and to optimize the chances to obtain high concentrations of well-preserved lipids. The analysis of the total lipid extract of sherds sampled at different locations on ancient vessels (base, body, and rim) tends to show that the best compromise is obtained from sherds from the body of the vessels (Charters et al., 1993; Charters et al., 1997; Evershed, 2008a). Lipids are indeed mainly accumulated at the top of ceramic jars, and their concentration decreases down the vessel profile from the top to bottom (Charters et al., 1993; Evershed, 2008a). However, it has been noted that, although present in significant concentrations around the rim, these lipids exhibit a higher degree of degradation, probably due to their contact with air that promotes oxidation (Regert et al., 1999). Furthermore, it has been shown that concentration gradients exist between the inner and the outer surface of the sherds (Stern et al., 2000).

Following the HT GC or HT GC–MS screening of the lipid extracts, other sample treatments are applied to underivatized or insoluble fractions, depending on the molecular markers identified.

In particular, preparation of methylated fatty acid methyl esters (FAME) is used to study in detail the distribution of these components or to determine their $\delta^{13}\text{C}$ values (Dudd, Evershed, & Gibson, 1999; Mottram et al., 1999; Copley et al., 2003; Spangenberg, 2004; Craig et al., 2005a; Spangenberg, Jacomet, & Schibler, 2006). FAME preparation is carried out on a portion of the total lipid extract, typically 1/4 (Copley et al., 2005c) beginning with hydrolysis with methanolic or ethanolic sodium or potassium hydroxide (Mottram et al., 1999; Spangenberg, Jacomet, & Schibler, 2006) to release fatty acid moieties from MAGs, DAGs, and TAGs, and from possibly other bound lipids (Regert et al., 1998). The resulting fatty acids are methylated with a boron-trifluoride methanol reagent; after neutralization and

extraction, the solution of FAMES is stored at 4°C before analysis.

When unsaturated fatty acids are preserved, the usual GC–MS analyses of the methyl or trimethylsilyl esters from archeological animal fats do not allow elucidation of their positional or geometric isomer compositions. For example, their EI mass spectra are insufficiently diagnostic to allow determination of the position of the double bonds. The formation of dimethyl disulfide adducts by addition of dimethyl disulfide (DMDS) to the carbon–carbon double bond is a convenient method to determine the position of alkenyl groups (Attygalle, Jham, & Meinwald, 1993). These derivatives are obtained by treatment of the FAMES with DMDS and iodine in diethyl ether (Mottram et al., 1999). By combining the GC elution order of the different isomers and their mass spectra, it is possible to establish the location of the double bonds and their stereochemistry (Evershed, 2000; Berstan et al., 2004). Although frequently used for the structural elucidation of a variety of compounds with alkenyl groups (Carbeilleira & Sepúlveda, 1992; Attygalle, Jham, & Meinwald, 1993; Stránský, Valterová, & Fiedler, 2001), this procedure has been rarely employed to unravel the structure of archeological lipids. Nevertheless, it allowed detailed investigation of the structures of mono-unsaturated fatty acids to discriminate ruminant from non-ruminant adipose fats (Evershed et al., 1997b; Mottram et al., 1999; Evershed, 2000; Berstan et al., 2004). The mass spectra are dominated by two fragments that arise from cleavage of the carbon–carbon bond on which the $-\text{SCH}_3$ substituents are located and present a prominent M^+ that allows the number of carbon atoms present in the fatty acid to be determined (Evershed, 2000; Fig. 3). Table 1 lists the main fragment ions of octadecenoic acids that depend on the position of the double bond (Evershed, 2000).

In the case of particularly complex lipid mixtures or when it appears necessary to focus on a specific class of compounds, it is useful to fractionate the extracts. Two protocols have been employed with this aim: one relies on solid-phase extraction with either small-scale flash columns directly prepared in the laboratory or commercial cartridges (Evershed et al., 1994; Garnier et al., 2002; Mirabaud, 2007; Mirabaud, Rolando, & Regert, 2007), and other provides acidic and neutral fractions by solvent–solvent extractions with solvents of varying polarities and pH (Dudd, Regert, & Evershed, 1998; Ribechini et al., 2008b). In this way, it is possible to analyze separately the isolated acidic and neutral components after derivatization (Dudd, Regert, & Evershed, 1998; Ribechini et al., 2008b), or to analyze separately fractions that contain only hydrocarbons, ketones, sterols, and alcohols, DAGs, and TAGs or fatty acids (Evershed et al., 1994; Mirabaud, Rolando, & Regert, 2007).

Even though all of these methods only give access to the lipid markers that are solvent-soluble, other constituents, undetectable after such sample treatments, could also be preserved. To overcome this problem and to gain insights into the other compounds that arise from the resources processed in the vessels, such as proteins, polysaccharides, polyphenols, or bound lipids, the insoluble residues are submitted to further chemical treatments. First, alkaline treatment of the insoluble fraction has been carried out to release fatty acids or other biomarkers possibly linked together with ester bonds into a polymeric organic matrix (Regert et al., 1998). This method was found to be particularly effective to reveal the presence of the oxidized fatty acids discussed further below. Alternatively, a step

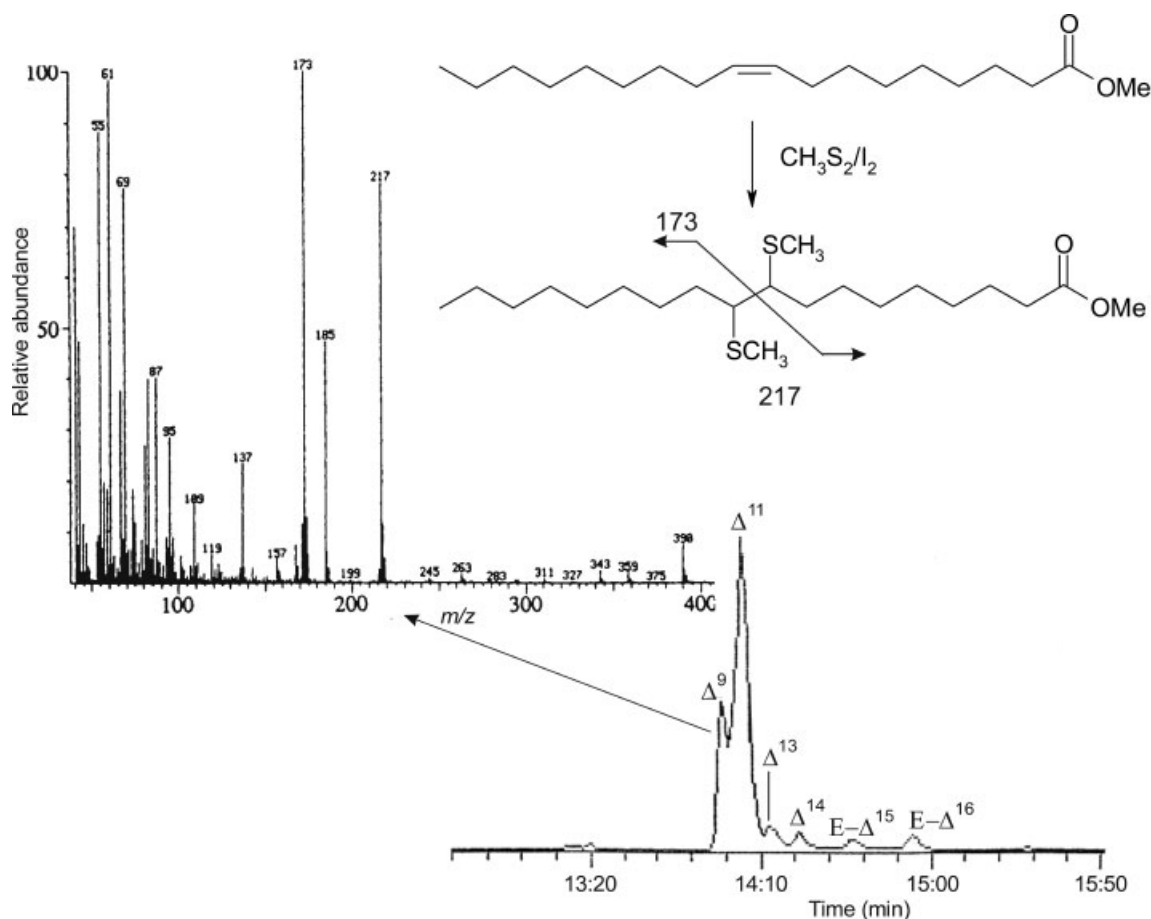


FIGURE 3. Partial ion chromatogram obtained on an archeological sample showing the distribution of unsaturated fatty acids (Mottram et al., 1999) and mass spectrum obtained by electron ionization of the dimethyldisulfide derivative of methylated oleic acid. Reproduced from Evershed, 2000 with permission from Wiley, copyright 2000. The formula of the initial methylated fatty acid and its DMDS derivative are indicated above.

of pyrolysis can be introduced before GC and GC–MS analysis to release several types of organic molecules incorporated into biopolymers (Oudemans & Boon, 1991; Regert et al., 2001b; Garnier et al., 2003). The latter can be performed with or without

in situ derivatization, for example, with tetramethylammonium hydroxide—depending on the nature of the compounds preserved. This approach could be a valuable tool to assess the presence of non-lipid compounds but it has yet to be widely applied.

The range of solvent and chemical treatments developed to extract and release components of organic residues present in ceramic vessels are summarized in Figure 4.

Those methods are the fruit of more than two decades of analytical developments that mainly focused on lipid constituents, opening the way to their analysis with GC and GC–MS methods discussed in detail below.

TABLE 1. Characteristic fragment ions from mass spectra of DMDS adducts of octadecenoic acid, depending on the location of the double bond

Location of the double bond	Fragment ions (m/z)
Δ^9	173, 217
Δ^{10}	159, 231
Δ^{11}	145, 245
Δ^{12}	131, 259
Δ^{13}	117, 273
Δ^{14}	103, 287
Δ^{15}	89, 301
Δ^{16}	75, 315

Evershed (2000).

C. Analytical Conditions

Research that dealt with the determination of the organic content of ancient pottery vessels has made impressive progress when it became possible to qualitatively and quantitatively analyze the total lipid extract and to avoid prior chemical degradation, such as saponification (Evershed, Heron, & Goad, 1990). This possibility emerged at the end of the 1980s and the beginning of 1990s based on improvements with gas chromatography conditions and the interface between the chromatograph and the mass spectrometer (Evershed & Goad, 1987). The challenge arose from the need to analyze complex mixtures that contain polar and apolar

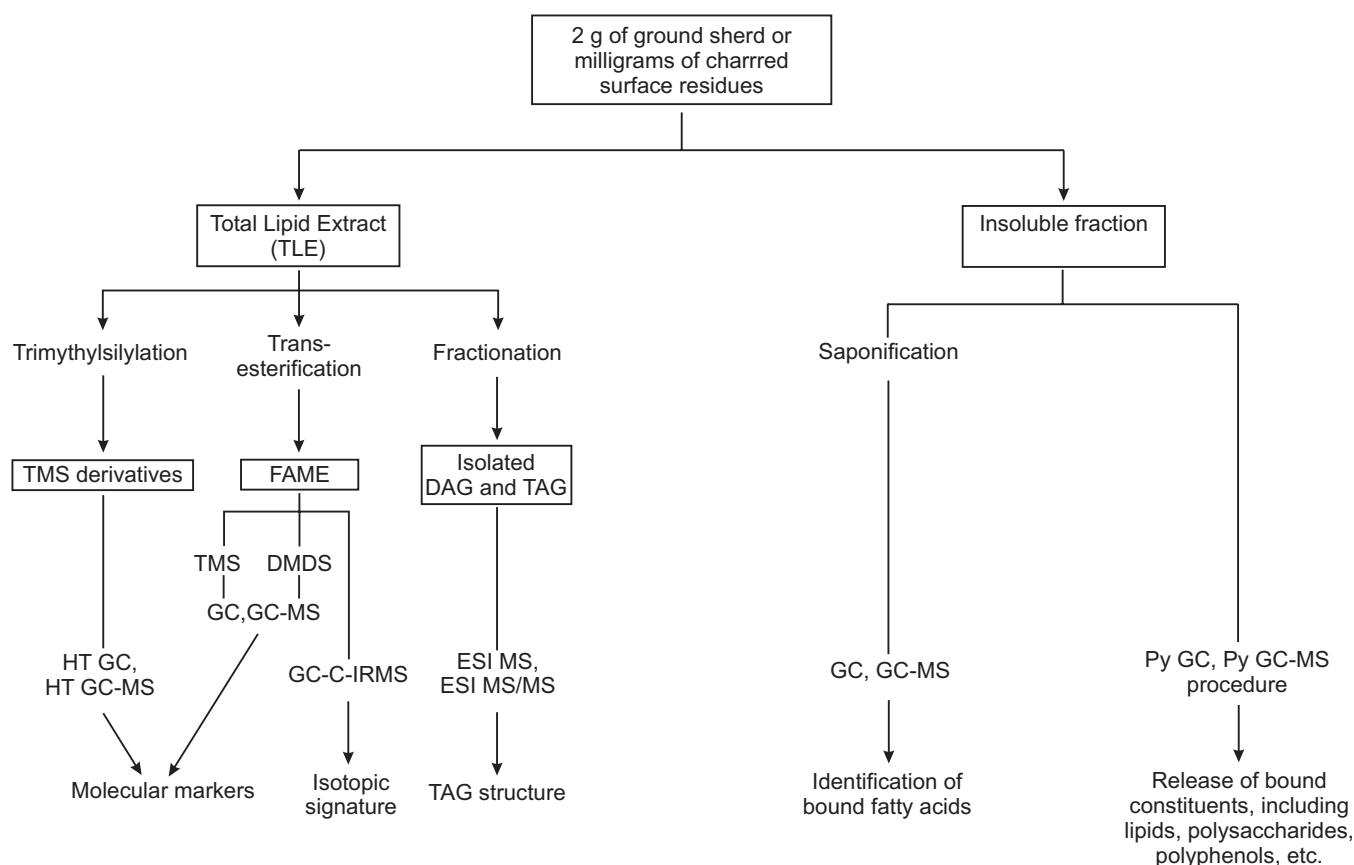


FIGURE 4. Summary of the various sample treatments explored for the analysis of lipids preserved in archeological ceramic vessels.

compounds displaying a large scale of volatilities and structural types (Evershed, Heron, & Goad, 1990). It was necessary to separate, detect, and identify, in a single analysis, with optimum sensitivity and resolution, fatty acids, sterols, high molecular weight esters, and MAGs, DAGs, and TAGs, together with other constituents formed by transformation of the initial biomarkers.

To overcome the polarity, samples are trimethylsilylated before GC analysis with an apolar column coated with a thin film (0.1 μm) of polydimethylsiloxane phase. To maximize the amount of sample transferred to the chromatographic system, on-column injection was shown to be particularly suitable. The other advantage of this injection system is the avoidance of any septum bleed, which can be particularly problematic when high temperature programming of the oven up to 350°C are used (Rood, 1998). However, the heterogeneity of the samples and the presence of micro-particles in solution analyzed might affect the efficiency of the chromatographic system. Moreover, because the sample is introduced directly onto the capillary column through the on-column injector, the analytes are dispersed by capillarity at the beginning of the column. This phenomenon can produce splitting into two peaks for the highest molecular weight constituents. To avoid these disadvantages, a deactivated fused-silica retention gap can be installed between the injector and the capillary column (Evershed, Heron, & Goad, 1990; Ogden, Pardo, & Tchaplá, 1998) to trap most of the impurities present in the solution, and can be replaced as soon as any loss of chromatographic performance is observed (changes of retention times, peak broadening or tailing, etc.). The inclusion

of retention gap results in preconcentration of all the analytes at the beginning of the capillary column and avoids peak splitting.

The optimization of chromatographic conditions led to the routine use of short capillary apolar columns (15 m or less) coated with a thin inner phase (0.1 μm) and temperature programming up to 350°C (Evershed, Heron, & Goad, 1990; Charters et al., 1995). The use of a flow program of the carrier gas from 2 to 6 mL min^{-1} further aids the analysis of mixtures containing analytes of widely ranging molecular weights (Regert, Langlois, & Colinart, 2005). Under these chromatographic conditions, the analysis of highly complex lipid mixtures that range from fatty acids with 14 carbon atoms to high carbon number constituents such as TAGs that contain up to 54 carbon atoms can be achieved in 40 min at high sensitivity and resolution, as shown in Figure 5 (Evershed, Heron, & Goad, 1990).

When HT GC is insufficient to identify the different eluted analytes, HT GC-MS must be performed. One of the difficulties in undertaking HT GC-MS is the efficiency of the GC-MS interface. If it is too low, high molecular weight analytes, such as TAGs, are condensed in the interface and thus cannot reach the MS ion source of the mass spectrometer. However, interfaces that can routinely reach 350°C are available nowadays, which was not the case 20 years ago. To overcome this problem, modifications of the interface changed the heating module to increase the temperature up to 400°C, even though 350°C is usually sufficient (Evershed & Goad, 1987). A loss of resolution is always observed between chromatograms obtained by HT GC with a flame ionization detector and those acquired by HT GC-MS. This loss

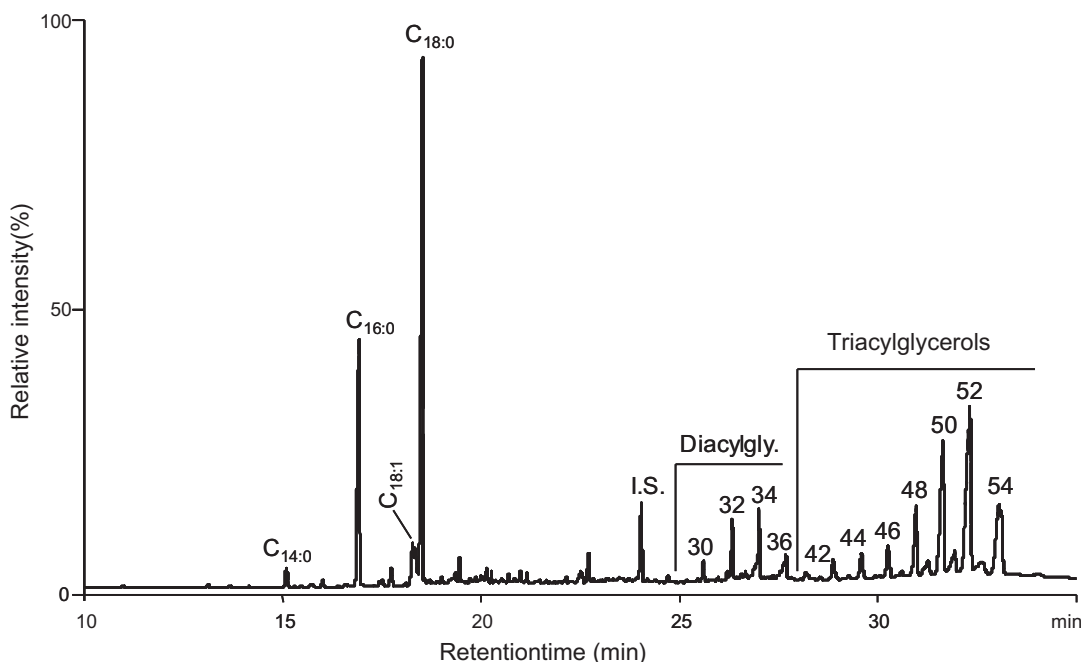


FIGURE 5. Example of a characteristic gas chromatogram of the total lipid extract obtained from an archeological pottery (neolithic site of Chalain, sample 422AB0).

might be explained by the flow rate of carrier gas that must be limited to obtain an acceptable vacuum in the MS, and by the presence of the pumping system following the GC–MS interface (Garnier et al., 2002). Nevertheless, with appropriate choices of scan range and cycle times, good quality EI mass spectra of the majority of the analytes can be obtained (Evershed, Heron, & Goad, 1990).

The above HT GC–MS conditions are particularly well-suited to the analysis of ancient degraded animal fats. However, these substances only represent one class of the commodities¹ processed, stored in, or consumed from the vessels. With the aim to “fingerprint” the composition of organic residues and to avoid sample pre-treatment, some authors have submitted micro-samples of surface residues to direct inlet or direct exposure EI MS (Regert & Rolando, 2002; Colombini, Modugno, & Ribechini, 2005; Modugno, Ribechini, & Colombini, 2006; Oudemans, Eijkel, & Boon, 2007; Regert et al., 2008a; Ribechini et al., 2008a,b; Regert, in press). This approach could be considered as a preliminary screening step as a basis to select samples for further analysis and to develop more targeted analytical protocols.

Two main systems of direct sample introduction have been used: the direct inlet probe (DIP) and the direct exposure probe (DEP). In the first mode, the sample, either in the solid or liquid state, is placed in a small crucible located at the end of the probe, which is then introduced into the MS and heated ($T \leq 450^\circ\text{C}$) to desorb organic constituents. The DEP consists of a wire filament onto which a drop of sample is deposited. Heating to $1,000^\circ\text{C}$ is optimal for the volatilization of high molecular weight components and cleavage of biopolymers. To cover the wide

range of compounds, mass spectra are first acquired over a scan range of m/z 50–900 or 1,000. If only low molecular weight components are present and if enough sample is available, a second analysis is performed scanning a narrower m/z range to improve the sensitivity of the analysis (Colombini, Modugno, & Ribechini, 2005). The source of the MS is usually maintained at a temperature above 200°C with mass spectra being acquired for a few minutes. The results comprise the desorption curve and mass spectral “fingerprints” obtained by averaging the mass spectra within the time range of interest (Ribechini et al., 2008a).

Interpretations rely on the knowledge of EI fragmentation patterns and comparisons with spectra from reference substances. Depending on the analyzer used, different mass spectra might be obtained from the same substance. Particularly, an “ion trap analyzer is noted for its discrimination against ions with m/z values below 100” (Regert & Rolando, 2002); that discrimination explains the high relative intensity of M^+ that is of particular value to interpret the spectra.

This methodology has been mainly used to study resins (Regert & Rolando, 2002; Colombini, Modugno, & Ribechini, 2005; Modugno, Ribechini, & Colombini, 2006; Regert et al., 2008a) and waxes (Regert et al., 2006c; Ribechini et al., 2008a; Regert, in press), but it has recently provided interesting data to detect the presence of fatty and waxy substances in Roman glass vessels (Fig. 6, Ribechini et al., 2008b).

D. Molecular Criteria to Identify Animal Fats

In the field of organic geochemistry, the concept of biomarkers is widely used to assess the origin of biomolecular constituents preserved in ancient sedimentary deposits. Biomarkers are defined as organic compounds preserved in the geological record, and are characterized by a specific carbon skeleton that might be related to their biological source (Philp & Oung, 1988).

¹The term commodity is used to designate all kind of raw or transformed natural substance that has been exploited by ancient communities.

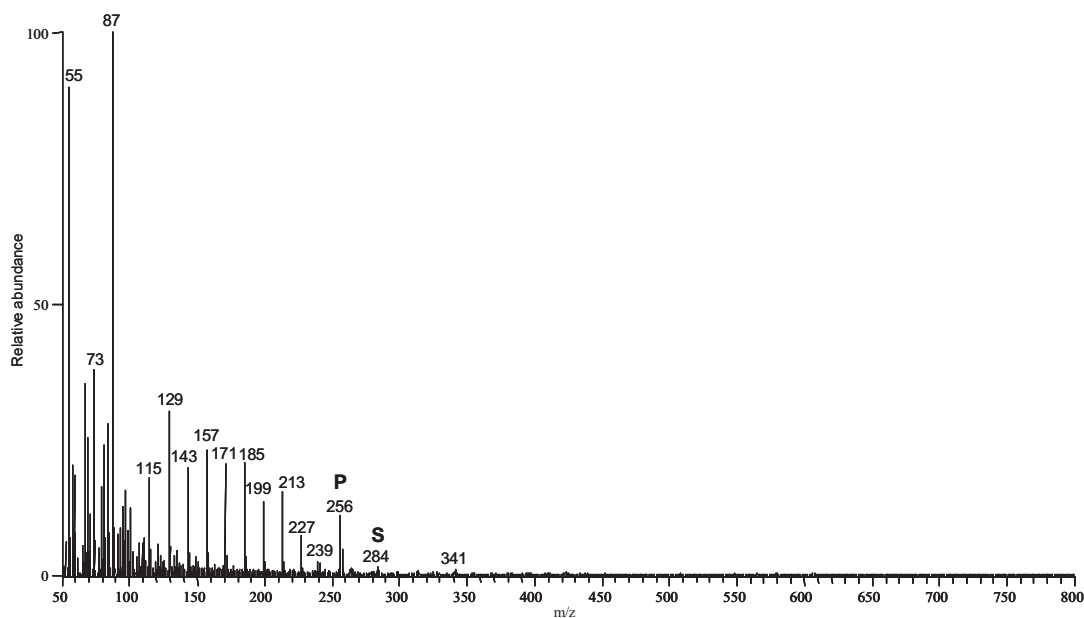


FIGURE 6. Mass spectrum obtained by DE-MS with electron ionization showing characteristic ions of palmitic and stearic acids in an archeological substance sampled in a Roman glass bottle from Pompei (Ribechni et al., 2008b). P, molecular ion of palmitic acid; S, molecular ion of stearic acid. With permission from Elsevier, copyright 2008.

Because they derive from geological sediments, they have experienced a wide range of natural degradation processes resulting in the loss of their functional groups (Eglinton & Logan, 1991). The case is slightly different for biomolecules extracted from anthropogenic sediments or archeological materials because of the shorter duration of burial of archeological compared to geological deposits. In contrast to geological materials, archeological residues result from various anthropogenic transformations that alter their initial chemical composition. For these reasons, several types of molecular markers have been defined by bioarchaeologists: (i) *biomarkers* correspond to native molecules whose association might be linked with the natural sources; (ii) *anthropogenic transformation markers* are the result of chemical transformations induced by different human activities; (iii) *natural degradation markers* are formed by natural decay of the initial biomarkers or transformation markers in the archeological deposits by chemical or biochemical processes; (iv) *migration markers*, also termed contaminants, arise from the migration of components from the sediment to the archeological organic residues (Evershed et al., 1992a,b; Evershed, 1993a, 2008a; Regert, 2001, 2007a,b).

By combining complementary information on all of these molecular markers, it is possible to detect the presence of animal fats in ancient pottery vessels and to discriminate four main types of natural resources: subcutaneous adipose fats of ruminants and non-ruminants, dairy products, and marine or aquatic resources. These discriminations are based on the study of the distribution of biomolecular components, their carbon skeletons, and the position, number, and stereochemistry of the double bonds.

Although fresh animal fats comprise ca. 95% TAGs (Gurr & Harwood, 1991), all archeological fats are damaged by hydrolysis, resulting in MAGs, DAGs, and fatty acids (Evershed et al., 1992a,b; Heron & Evershed, 1993). Sterols and their degradation derivatives might also be identified in low concen-

tration. A typical gas chromatogram of archeological-degraded animal fats extracted from a ceramic vessel is shown in Figure 5. Degradation products specific to particular treatments or native precursors were also identified in several cases (Evershed et al., 1995, 2002; Raven et al., 1997; Copley et al., 2004; Hansel et al., 2004; Craig et al., 2005a).

We discuss first the diagnostic potential of the main biomarker components of animal fats, including TAGs, fatty acids, and sterols. This is followed by an assessment of transformation and degradation markers.

The identification of TAGs with HT GC-MS is based on three criteria: (i) comparison of their retention times with those of commercial standards, (ii) elution orders of homologous series of compounds; and (iii) interpretation of their mass spectra (Evershed, Heron, & Goad, 1990).

Analyzing reference compounds, such as trimyristin, tripalmitin, and tristearin, under the same analytical conditions as those used for HT GC of total lipid extracts of archeological ceramic vessels has allowed the detection and identification of these lipid biomarkers in many samples. By considering the retention times of two homologues, it is possible to identify other TAGs present in the mixture but for which standards are not available. In the case of complex molecular mixtures, particularly when constituents other than TAGs elute within the same retention time range, such as esters of beeswax, HT GC-MS is required to discriminate the TAGs from other components. Mass spectra of TAGs are characterized by a base peak that results from the loss of an acylium group $[M-RCOO]^+$ and intense ions that are formed by the loss of a neutral RCO_2H fragment (Hites, 1975; Laakso, 1996, 2002; Ribechni et al., 2008b) as illustrated in Figure 7. Other noticeable fragment ions correspond to $[RCO]^+$ and $[RCO + 74]^+$ ions (Hites, 1975). These EI spectra are rather uninformative because they are so dominated by single major fragment ion (Mirabaud, Rolando, & Regert, 2007). However, by

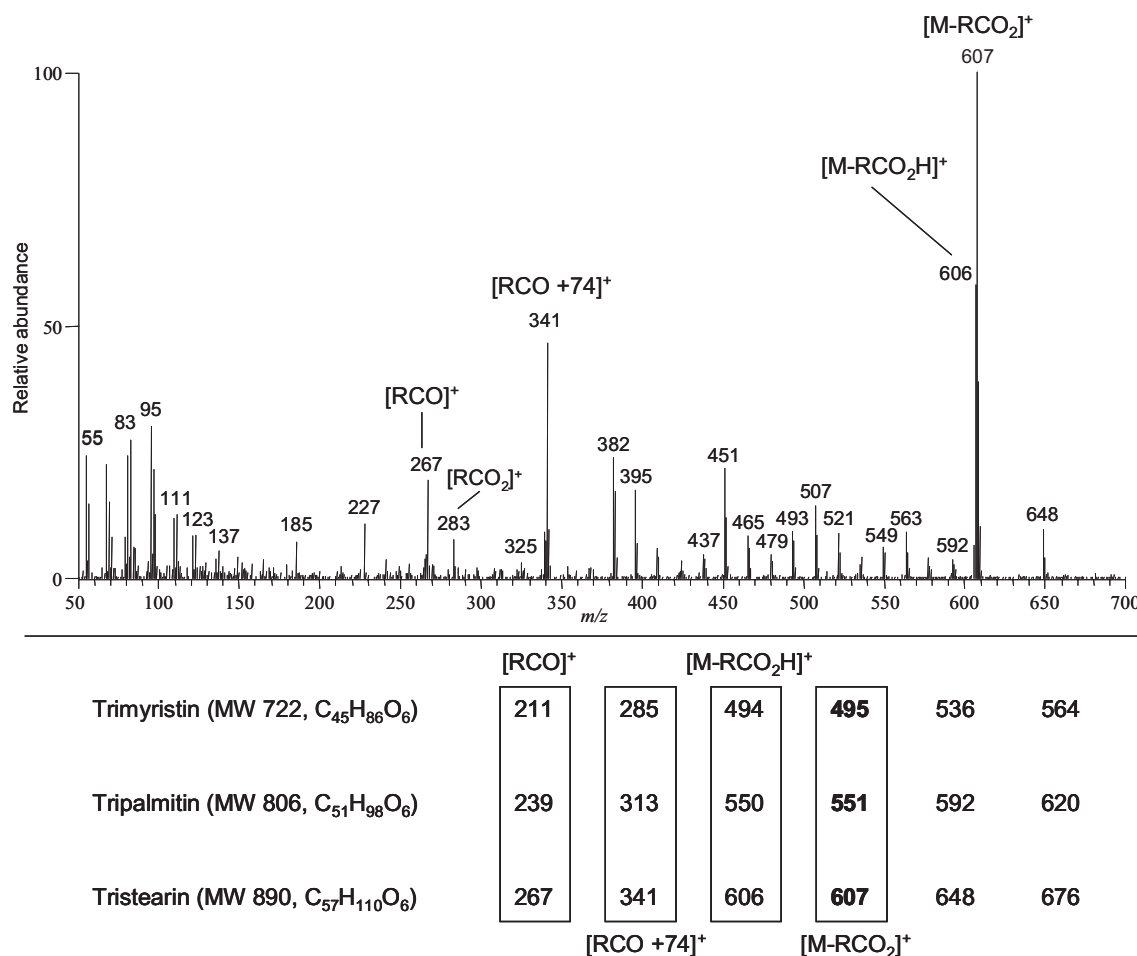


FIGURE 7. Mass spectrum of tristearin and main characteristic fragments of triacylglycerols obtained on a Polaris Q ion trap external ionisation mass spectrometer. Adapted from Ribechini et al. (2008b). With permission from Elsevier, copyright 2008.

combining mass spectral information with retention times, it is possible to interpret the distributions of TAGs which are generally characterized by an even number (40–54) of acyl carbon atoms. The analyses of contemporary reference fats by HT GC–MS has shown that ruminant (bovine and ovine) and non-ruminant (porcine) adipose fats and milk can be distinguished based on their TAG distributions (Fig. 8).

Ruminant adipose fats contain TAGs of total acyl carbon numbers that range between C₄₂ (for bovine) or C₄₄ (for ovine) and C₅₄ (Dudd, Evershed, & Gibson, 1999; Mukherjee et al., 2007). The adipose fat of non-ruminant species (porcine) presents a narrower distribution of TAGs that range from C₄₄ to C₅₄, with very low abundances of C₄₄, C₄₆, and C₄₈ (Dudd, Evershed, & Gibson, 1999; Mukherjee et al., 2007). In milk fat products, the presence of short-chain fatty acids is responsible for a broad distribution of TAGs ranging from C₂₈ to C₅₄ (Dudd & Evershed, 1998; Dudd, Regert, & Evershed, 1998; Mottram & Evershed, 2001; Copley et al., 2005a,b,c,d). Although these distributions partly persist in archeological animal fat residues, they are modified to varying extents over time (Dudd & Evershed, 1998; Dudd, Regert, & Evershed, 1998; Mukherjee et al., 2007). In particular, hydrolytic processes preferentially affect the shorter TAGs to lead to a distribution of TAGs in degraded dairy

products that range from C₄₀ to C₅₄ (Dudd & Evershed, 1998). Consequently, in archeological animal fats, two main distributions of TAGs are encountered: a narrow one, that corresponds to non-ruminant adipose fats, and a broad one, ranging from C₄₀ or C₄₂ to C₅₄, that indicates the presence of ruminant adipose or dairy fats. Where a broad TAG distribution is seen, those with a low abundance of C₄₂, C₄₄, and C₄₆ TAGs are attributed to ruminant adipose fats, whereas those with a greater abundance of the lower carbon number TAGs are characteristic of dairy products (Fig. 8; Dudd, Evershed, & Gibson, 1999). Further minor differences between bovine and ovine subcutaneous fats include the former being characterized by saturated TAGs that range from C₄₂ to C₅₄ whereas the latter only contains C₄₄ (trace) to C₅₄ TAGs (Fig. 8; Mukherjee et al., 2007).

A comparison between the different TAGs distributions is shown in Figure 8. As an aid to classify animal fats depending on their TAGs characteristics, two parameters, the average carbon number of the acyl moieties of the TAGs (average carbon number, M), and the dispersion factor (DF), have been defined (Mirabaud, Rolando, & Regert, 2007). Graphs that plot the dispersion factor versus the average carbon number allow the three main distributions of TAGs in archeological samples to be distinguished (Fig. 9).

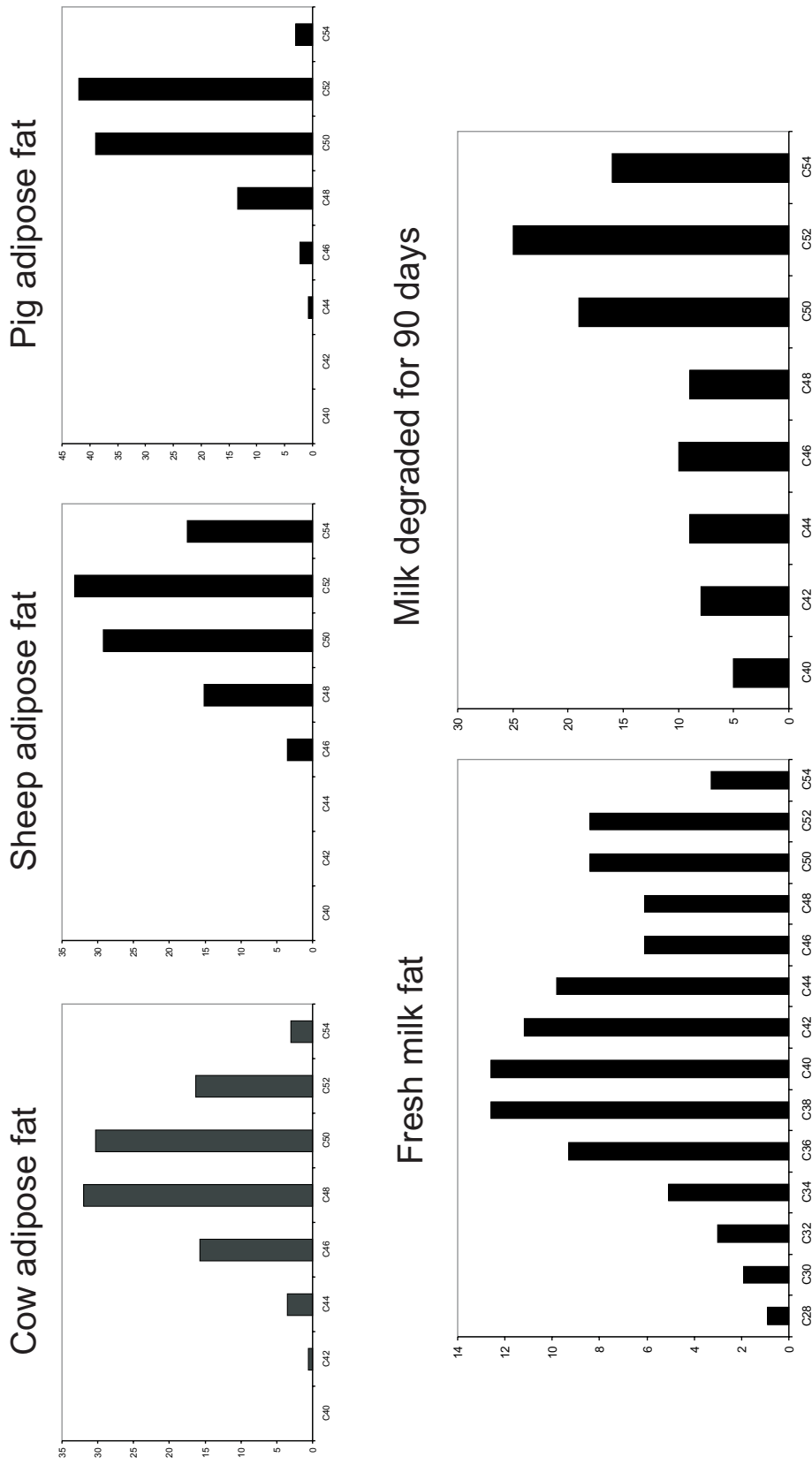


FIGURE 8. Histograms of characteristic triacylglycerols distributions of various reference contemporary animal fats. Reproduced from Mukherjee et al. (2007) with permission from Antiquity, copyright 2007.

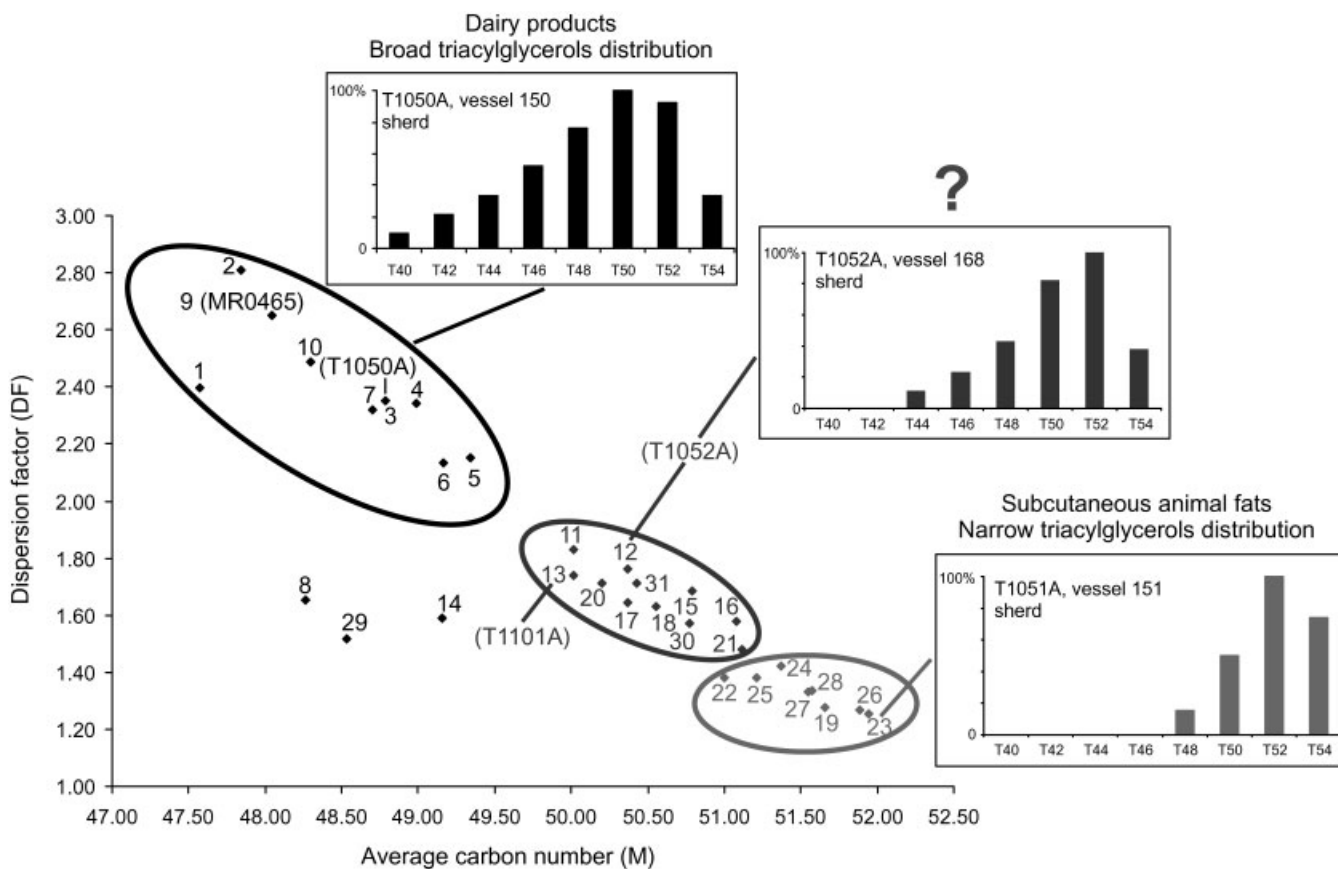


FIGURE 9. Classification of animal fats from archeological samples using average carbon number (M) and dispersion factor (DF). $M = (\sum(P_i C_i) / \sum P_i)$; $DF = (\sqrt{\sum[(C_i - M)^2 \times C_i P_i]} / \sum P_i)$; with C_i = number of carbon atoms, and P_i = relative percentage of each triacylglycerol. Adapted from Mirabaud, Rolando, and Regert (2007).

Despite the great interest of TAG distribution in reference and archeological samples, this criterion must be used cautiously. Firstly, in cases where hydrolysis is very extensive, TAGs are often undetectable and thus cannot be used. Secondly, degradation pathways are not fully understood, and might differ, depending on the burial environment. It is thus often necessary to consider TAG distributions as preliminary information that allow assessment of the degree of preservation of organic residues and which provide a criterion to detect commodities other than fats. More definitive assessments of the origins of fats rely on robust and complementary criteria based on the stable carbon isotope compositions of individual fatty acids (Evershed et al., 1994, 1997b, 2002; Copley et al., 2003, 2005a,b,c,d), or on the fine structures of the TAGs (Mirabaud, Rolando, & Regert, 2007), as discussed in sections III and IV of this review.

Fatty acids represent another class of biomolecular constituents that have valuable chemotaxonomic potential to determine the nature of lipid substances processed in ancient pottery vessels. Although they were the first compounds used to assess the origin of fats and oils present in ceramic vessels (Condamin et al., 1976; Rottländer & Schlichtherle, 1979), “their diagnostic potential is limited when considered in isolation” (Heron & Evershed, 1993) because of their wide-

spread distribution and the different degradation processes that modify their structures over time or during culinary activities. The use of the P/S ratio, largely debated in the literature, is still proposed to discriminate fatty substances (Romanus et al., 2007). P/S values in the range 4.0–9.4 for commercial olive oils, 2.9 for modern bovine milk, and 4.9 for ovine milk have been proposed, and a P/S ratio < 1.3 is considered as indicative for non-ruminant fats (Romanus et al., 2007). These data have nevertheless to be considered very cautiously because of the complexity of degraded animal fats that have been modified by alteration processes and that might result from mixtures of different substances. It is only a combination of criteria including fatty acids distribution, abundances of minor components, such as odd-carbon number branched-chain fatty acids, and positional isomers of unsaturated compounds, that enables distinctions to be made among different types of animal fats (Evershed et al., 1997b; Dudd & Evershed, 1998; Dudd, Evershed, & Gibson, 1999; Mottram et al., 1999).

The most common fatty acids preserved in total lipid extracts of organic residues from ceramic containers are even-carbon number *n*-alkanoic acids that range from $C_{14:0}$ to $C_{18:0}$, and maximize either at $C_{16:0}$ or $C_{18:0}$ (Evershed et al., 1997b; Regert et al., 2001b; Spangenberg, 2004; Craig et al., 2007a). Fatty acids are readily identified by comparison of their

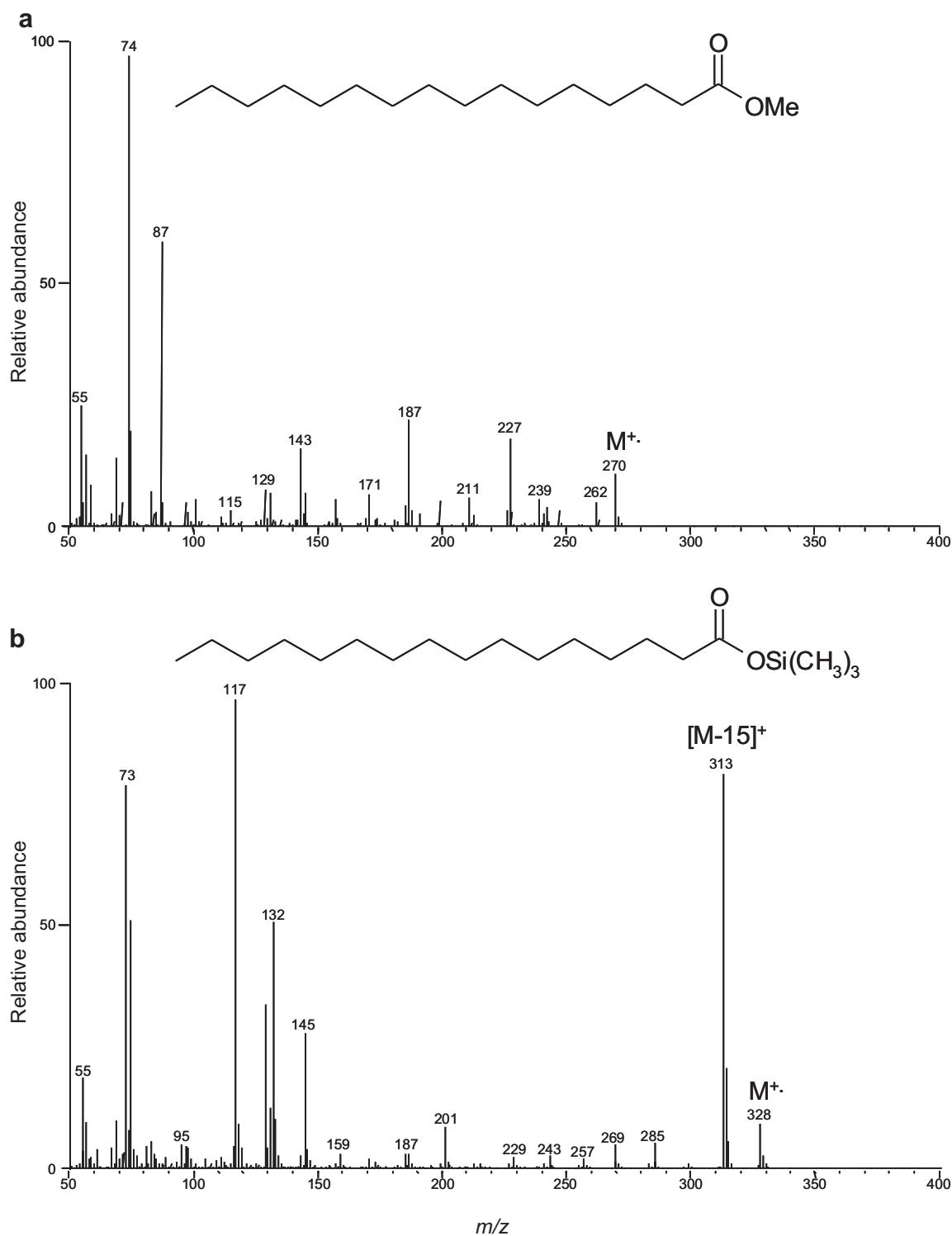


FIGURE 10. Mass spectrum of palmitic acid as (a) methylated derivative and (b) trimethylsilylated derivative obtained by electron ionization (70 eV) with a quadrupole mass spectrometer.

retention times with those of commercial reference compounds and by their EI mass spectra (Fig. 10).

Despite the susceptibility of unsaturated fatty acids to oxidation, monounsaturated fatty acids with 18 carbon atoms are frequently present in the lipid extracts (e.g., Evershed, Heron, & Goad, 1990; Dudd, Evershed, & Gibson, 1999; Copley et al.,

2005a; Spangenberg, Jacomet, & Schibler, 2006). Determination of the double-bond position based on the mass spectra of DMDS derivatives has revealed the existence of two kinds of distributions: in some samples, a single monounsaturated fatty acid, Z-9-octadecenoic acid, was identified, whereas other residues display a more complex composition characterized by

a mixture of positional isomers of octadecenoic acid (C_{18:1}) with a double bond located at the 9, 11, 13, 14, 15, and 16-positions (Fig. 3; Evershed et al., 1997b; Mottram et al., 1999). It has been established that this difference reflects the origin of the fats: in ruminant animals, a biohydrogenation of dietary fats occurs in the rumen, that leads to the formation of several positional C_{18:1} isomers, whereas in non-ruminant monogastric animals, such as pigs, a single isomer is encountered (Enser, 1991; Evershed et al., 1997b).

Furthermore, minor straight and branched *iso*- and *anteiso*-fatty acids with 15 and 17 carbon atoms are encountered (Evershed, Charters, & Quye, 1995; Evershed et al., 1997b, 1999; Dudd, Evershed, & Gibson, 1999; Regert et al., 2001b; Spangenberg, 2004). These fatty acids are known to be formed in the gut by bacterial synthesis, and thus might be assigned to ruminant animal fats (Christie, 1981; Dudd, Evershed, & Gibson, 1999).

The criteria based on fatty acids features, summarized in Table 2, are now commonly used for the study of fats from terrestrial animals.

Fresh fats derived from marine animals are characterized by a set of common criteria including (i) long-chain polyunsaturated fatty acids that contain more than 20 carbon atoms, (ii) palmitic acid is the most prominent saturated fatty acid, and (iii) specific isoprenoid fatty acids (Hilditch & Williams, 1964; Ackman & Hooper, 1968; Malins & Wekell, 1970; Morgan et al., 1984; Patrick, de Koning, & Smith, 1985; Hansel et al., 2004). These biomarkers undergo chemical and bacterial transformations during the use of vessels in which they are preserved or during post-depositional burial. Polyunsaturated fatty acids in particular are highly susceptible to degradation and have thus never been encountered in archeological samples (see below for the details of the degradation pathways, Morgan et al., 1984; Patrick, de Koning, & Smith, 1985). Some of these polyunsaturated acids give rise to the formation of specific degradation markers in the form of cyclic compounds (Hansel et al., 2004) that are discussed below. Nevertheless, the presence of saturated and monounsaturated fatty acids with an even carbon number that range from C₂₀ to C₂₄, together with a high proportion of palmitic acid and minor amounts of myristic and stearic acids, are generally

TABLE 2. Summary of molecular and isotopic criteria established by HT-GC-MS, nano-ESI-MS/MS, HPLC-APCI-MS, and GC-C-IRMS to distinguish animal fats in archeological ceramic vessels

	Triacylglycerols	Fatty acids	Other constituents	δ ¹³ C and Δ ¹³ C signature
NON-RUMINANT ANIMALS	Porcine adipose fats - Narrow distribution C ₄₄ to C ₅₄ with low abundance of C ₄₄ , C ₄₆ and C ₅₄ - Rich in tripalmitin - Palmitic acid preferentially located in the 2-position (P:S ratio in <i>sn</i> -2 position is of ≈ 95:5)	- C _{16:0} more abundant than C _{18:0} - Absence of minor odd carbon number fatty acids - Monounsaturated fatty acid: only a single isomer Z-9-octadecenoic acid	- Odd-numbered ketones ranging from C ₂₉ to C ₃₅ , maximising at C ₃₃ - Monounsaturated ketones with 33 and 35 carbon atoms - Result from condensation of fatty acids during heating animal fats - Free or bound oxidised fatty acids (diacids, hydroxy-acids), Fig. 11	- Palmitic and stearic acids enriched in ¹³ C compared to ruminant fats (Fig. 18) - Δ ¹³ C > -1 ‰ (Fig. 21, D)
ADIPOSE FATS FROM RUMINANT ANIMALS	Cattle adipose fats - Distribution from C ₄₂ to C ₅₄ - P:S ratio in <i>sn</i> -2 position is of ≈ 60:40 Goat adipose fats - Distribution from C ₄₄ (trace) to C ₅₄ - P:S ratio in <i>sn</i> -2 position is of ≈ 60:40 Sheep adipose fats - Distribution from C ₄₄ (trace) to C ₅₄ - P:S ratio in <i>sn</i> -2 position is of ≈ 60:40	- C _{16:0} less abundant than C _{18:0} - Low amount of straight carbon chain with odd carbon number, specifically C _{15:0} and C _{17:0} - Low amount of branched-chain alkanolic acid (C _{15:0} and C _{17:0}) - Mixture of isomers of octadecenoic acid (double bond at 9, 11, 13, 14, 15 and 16-positions)	- Same ketones as for porcine adipose fats	- Δ ¹³ C from -3 to -1 ‰ (Fig. 21, D)
DAIRY PRODUCTS OF RUMINANT ANIMALS	Cow milk - Large distribution from C ₄₀ to C ₅₄ - in all triacylglycerols, C _{10:0} present at lower abundance than in goat milk Sheep milk - Large distribution from C ₄₀ to C ₅₄ Goat milk - Large distribution from C ₄₀ to C ₅₄ - in all triacylglycerols, C _{10:0} present at higher abundance than in cow milk	- Same fatty acids as for adipose fats of ruminant animals	- Same ketones as for porcine adipose fats	- C _{18:0} depleted in ¹³ C in comparison with adipose animal fats - Δ ¹³ C < 3.3 ‰
AQUATIC RESOURCES	Marine fish Not preserved Freshwater fish Not preserved	- Palmitic acid more abundant than stearic acid - Long chain fatty acids with more than 18 carbon atoms - Triunsaturated fatty acids (usually not preserved in archaeological contexts) - Same acids as for marine fish	- Presence of isoprenoid acids (phytanic acid = 3,7,11,15-tetramethylhexacosanoic acid and 4,8,12-trimethyltridecanoic acid = 4,8,12-TMDT) at low abundance - Series of isomers containing 16, 18 and 20 carbon atoms of ω-(<i>o</i> -alkylphenyl) alkanolic acids (cyclic compounds) with a wide range of positional isomers formed by degradation of triunsaturated fatty acids - Same constituents as for marine fish	- Palmitic and stearic acids are isotopically enriched in ¹³ C compared to those of terrestrial animals, even though they plot not far from adipose fats of domestic pigs (Fig. 18) - freshwater fishes are depleted in ¹³ C for both C _{16:0} and C _{18:0} fatty as compared to marine resources

considered as deriving from marine resources (Morgan et al., 1984; Patrick, de Koning, & Smith, 1985; Copley et al., 2004). Among the monosaturated fatty acids, gadoleic acid (eicoseinoic acid, C_{20:1}), behenic acid (docosenoic acid, C_{22:1}), or/and nervonic acid (tetracosenoic acid, C_{24:1}) indicate a marine origin (Patrick, de Koning, & Smith, 1985). The ratio of the abundances between some fatty acids has been used to assess the origin of the marine resources, for example, the C_{16:0} to C_{18:0} ratio, and the ratio between oleic acid (C_{18:1} ω9) and vaccenic acid (C_{18:1} ω7), were calculated (Patrick, de Koning, & Smith, 1985). Once again, these data have to be used very cautiously, and must always be accompanied by other criteria, due to the possible modifications that occur in fatty acids during burial and human activities, for example, mixing of various commodities.

Other biomarkers, namely isoprenoid acids such as phytanic acid (3,7,11,15-tetramethylhexacosanoic acid), and 4,8,12-trimethyltridecanoic acid (4,8,12-TMDT) have recently been identified at low concentrations in various archeological samples (Copley et al., 2004; Hansel et al., 2004; Craig et al., 2007b). The latter components are characteristic lipid biomarkers of marine resources (Ackman & Hooper, 1968; Hansel et al., 2004), which combination with other resources offer considerable potential to detect the presence of marine substances in the archeological record.

Beyond the biomarkers discussed in this section, various degradation markers that might be related to specific biomolecular constituents have also been identified in degraded archeological animal fats. These components and the mechanisms of their formation are presented below.

E. Transformations of Animal Fats in Ceramic Vessels

While some biomarkers of animal fats might survive over archeological time scales in specific contexts of preservation, in most cases, they are transformed through a series of degradation processes that modify the initial composition of the various commodities processed or stored in the vessels. Degradative reactions include hydrolysis, oxidation, polymerization, condensation, cyclization or microbial degradation (Evershed, 2008a). Combinations of these reactions lead to degraded lipid signals, from which it is often possible to assess the biomolecular precursors and to understand the ways in which they were degraded through natural decay processes and/or human activities.

At the beginning of the 1990s, when it became possible to analyze intact TAGs, one of the questions addressed concerned the modes of evolution and alteration of the initial profile of these biomarkers. By combining analysis of modern fresh fats, aged samples, and archeological extracts, several mechanisms have been highlighted (Evershed et al., 1992a,b, 2002; Heron & Evershed, 1993; Evershed, Charters, & Quye, 1995; Dudd & Evershed, 1998; Dudd, Regert, & Evershed, 1998; Copley et al., 2003; Evershed, 2008b). One of the main reactions that affects the distribution of TAGs results in the partial or complete chemical and/or enzymatic hydrolysis (Evershed et al., 1992a,b). The hydrolysis products are fatty acids, and MAGs, and DAGs. To understand TAG alterations, cooking experiments were performed followed by aerobic natural aging with replica ceramic vessels (Evershed, Charters, & Quye, 1995; Dudd & Evershed, 1998; Dudd, Regert, & Evershed, 1998). In this way, TAG distributions similar to those obtained from the total lipid extract of ancient

ceramic vessels were observed. Selective decay of the short-chain acyl moieties was evident, particularly in milk fats that contain short-chain fatty acids in the range C₄–C₁₂, which constitute up to 20% of all the fatty acid moieties comprising TAGs (Copley et al., 2003). This phenomenon has been shown to be responsible for the transformation of the TAGs profile of dairy products towards a distribution that resembled that of adipose fats (Fig. 8; Dudd & Evershed, 1998).

The reason why short-chain acyl moieties are more susceptible to hydrolysis than their long-chain counterparts is still under discussion. The preferential position of short-chain fatty acids on the *sn*-3 position could explain this phenomenon because the reduced steric hindrance at this position will lead to a more efficient hydrolysis process (Copley et al., 2003). Moreover, the short-chain fatty acids released by hydrolysis are rarely identified in archeological samples because they are relatively water soluble and are thus easily lost through leaching in the burial environment (Dudd & Evershed, 1998; Copley et al., 2003). The question surrounding the microbial or chemical origin of the hydrolysis was also addressed, and the results showed that this phenomenon was microbially mediated (Dudd, Regert, & Evershed, 1998).

Oxidation processes produce further lipid degradation markers. Unsaturated fatty carboxylic acids are precursors of short-chain dicarboxylic acids, hydroxy- and dihydroxy carboxylic acids that might be preserved either as free compounds or in a “bound” form (Gülaçar, Buchs, & Susini, 1989; Evershed, 1990; Regert et al., 1998). Despite extensive analyses of organic residues in ancient ceramic vessels, expected oxidation products anticipated to form during food preparation were detected and identified in 1998 for the first time (Regert et al., 1998). Oxygenated fatty acid derivatives began to be encountered when research focused on dry and warm contexts or, conversely, on waterlogged environments (Fig. 11). For this latter case, lipid oxidation products accompanied by saturated carboxylic fatty acids were released after saponification, and showed their presence as esters bound into the ceramic fabric or the carbonized residues (Regert et al., 1998; Copley et al., 2005e; Craig et al., 2007a).

A series of homologous α,ω-dicarboxylic acids, that ranged from C₅–C₇ to C₁₂–C₁₃, with azelaic acid the main component, were observed in ceramic vessels from one of the sites of Chalain (waterlogged site, France) and the Egyptian site of Qasr Ibrîm. The mass spectra of their bis-TMS derivatives display characteristic fragment ions that correspond to [M–15]⁺ and [M–131]⁺; the latter is only observed for diacids that contain more than five carbon atoms. The fragment ion at [M–131]⁺ results from scission of the C(α)–C(β) bond (McCloskey, 1969; Regert et al., 1998). The formation of these dicarboxylic acids arises through oxidative reactions at the double-bond of unsaturated fatty acids via a variety of processes described elsewhere (Evershed et al., 1992a,b; Regert et al., 1998). The common predominance of azelaic acid implies the precursor fatty acid bare a double-bond at the 9-position. Another homologous series of oxygenated fatty acids released after alkaline hydrolysis consisted of ω-hydroxy even-numbered saturated carboxylic acids that range from C₈ to C₁₂, and maximized at C₈ (Regert et al., 1998; Regert et al., 2001b). Although an M⁺ is generally not observed in the mass spectra of these aliphatic hydroxy fatty acids, the molecular weight might be established by the presence of an abundant [M–31]⁺ ion related to the loss of the 'OCH₃ radical (Eglinton, Hunneman, & McCormick, 1968). Intense ions at [M–15]⁺ and

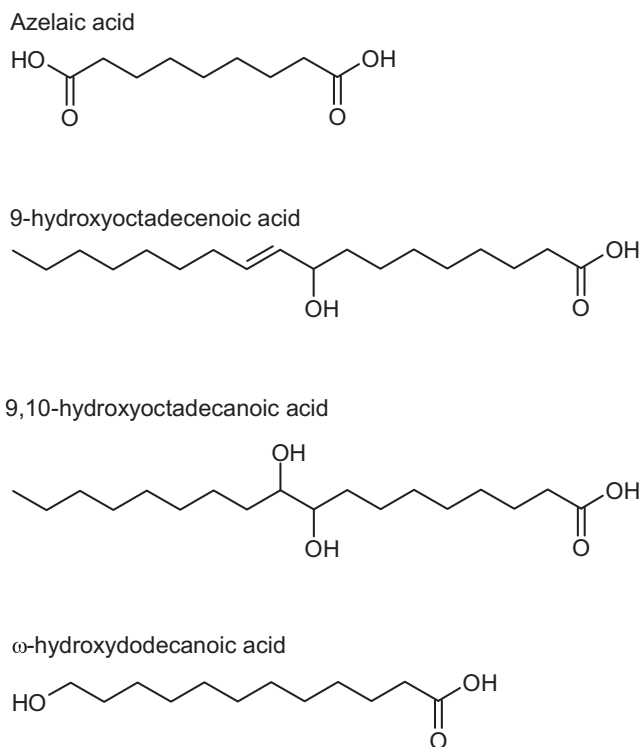


FIGURE 11. Main oxidative products encountered in the saponified residue of an archeological residue sampled on a potsherd preserved in a lacustrine environment. From Regert et al. (1998).

$[M-47]^+$ are also characteristic of ω -hydroxy fatty acids (Eglinton, Hunneman, & McCormick, 1968; Gülaçar, Buchs, & Susini, 1989; Regert et al., 1998).

Further oxidized fatty acids identified as “bound” components included 9- and 10-hydroxyoctadecanoic acids which also form through oxidation of oleic acid followed by dehydration (Fig. 12). The main fragments observed in their mass spectra arise from a facile cleavage on either side of the carbon atom bearing the trimethylsilyloxy group (Eglinton, Hunneman, & Douraghi-Zadeh, 1968; Regert et al., 1998; Berstan et al., 2004). Other degradation markers observed include unsaturated dihydroxy-carboxylic acids (9,10-dihydroxy-octadecanoic acids). A last class of degradation markers of fatty acids, oxo-octadecanoic acids, have to be mentioned. They were not found in ceramic vessels but in white waxy substances generally called “bog butter” and preserved in peat bogs from northern Europe (Thornton, Morgan, & Celoria, 1970; Berstan et al., 2004). Particularly, 10-oxo-octadecanoic acid was identified by its mass spectrum that displays a characteristic peak at m/z 215 resulting from β -cleavage and less abundant fragments at $[M-15]^+$ and $[M]^+$ (Berstan et al., 2004).

Cholesterol is also subjected to oxidation reactions that produce various alteration products, by reaction at the C_7 position (Evershed et al., 1992a,b). A series of hydroxy-, oxo-, and epoxy-derivatives of cholesterol might be formed by this way, particularly during heating of the fats in the ceramic vessels but also by natural decay.

Odd-numbered mid-chain ketones that ranged $C_{29}-C_{35}$, and maximized at C_{33} , are unusual molecular constituents that were identified in various ceramic vessels in which animal fats were

processed (Evershed et al., 1995; Raven et al., 1997; Dudd, Evershed, & Gibson, 1999; Evershed et al., 2002; Craig et al., 2007a; Mirabaud, Rolando, & Regert, 2007). Their mass spectra allowed identification of nonacosan-15-one, triacontan-14-one, triacontan-15-one, hentriacontan-16-one, dotriacontan-15-one, dotriacontan-16-one, tritriacontan-16-one, tetratriacontan-17-one, and pentatriacontan-18-one (Evershed et al., 1999, 2002). Monounsaturated ketones with 33 and 35 carbon atoms were also detected (Evershed et al., 1995).

Although such compounds are usually associated with higher plant leaf waxes (Walton, 1990), their carbon number range, the position of carbonyl group, relative abundances of fatty acids and ketones, and the close examination of $\delta^{13}C$ values of mid-chain ketones and fatty acids present in a same vessel, confirmed that ketone formation results from a condensation of fatty acids, as shown in Figure 13 (Evershed et al., 1995; Evershed et al., 1999, 2002; Raven et al., 1997). This thermally induced phenomenon occurs when animal fats are submitted to substantial heating—as demonstrated by experiments performed on replica vessels (Raven et al., 1997).

In pottery vessels from coastal sites of different periods and countries (Southern Brazil, South Africa and north-west Europe), several unusual ω -(*o*-alkylphenyl) alkanolic acids were reported (Hansel et al., 2004; Copley et al., 2004). The mass spectra of the methyl ester derivatives of these cyclic compounds allowed identification of a series of isomers that contained 16, 18, and 20 carbon atoms, with a wide range of positional isomers (Hansel et al., 2004). Their mass spectra exhibit intense ions at m/z 105 (base peak) and m/z 91, that respectively correspond to $C_8H_9^+$ and $C_7H_7^+$ ions, as illustrated in Figure 14 (Hansel et al., 2004). The M^+ ion at m/z 262 (C_{16}), 290 (C_{18}), and 318 (C_{20}) is also observed as in the mass spectrum of the methyl ester derivatives (Hansel et al., 2004).

These ω -(*o*-alkylphenyl) alkanolic acids might be formed by degradation of triunsaturated fatty acids after a multi-step alteration pathway that begins with the alkali isomerization of a triunsaturated fatty acid, probably catalyzed by clays present in the pottery (Hansel et al., 2004; Evershed et al., 2008a). The second step consists of 1,5-hydrogen shift to form a conjugated triene system. Following this reaction, two modes of transformation might occur. The first one consists of a series of three reactions: namely, *E/Z* isomerization followed by an intramolecular Diels–Alder (IMDA) reaction, and aromatization to form conjugated cyclic products. Alternatively a 1,7-hydrogen shift can occur followed by an intramolecular Diels–Alder reaction and a step of aromatization (Hansel et al., 2004; Evershed et al., 2008a; Fig. 15).

The formation of cyclic products from C_{16} , C_{18} , C_{20} , and C_{22} triunsaturated acids, together with the presence of isoprenoid marine biomarkers and archeological evidence of fish consumption led to the conclusion that the ω -(*o*-alkylphenyl) alkanolic acids could be undeniably considered as biodegradation markers of marine products (Hansel et al., 2004; Copley et al., 2004).

In some samples, C_{20} cyclic acids isomers were also detected as traces (Copley et al., 2004). Such constituents were usually encountered together with isoprenoid acids (4,8,12-TMTD and phytanic acid) known to be marine biomarkers, as previously discussed.

All molecular criteria useful to discriminate animal fats are summarized in Table 2.

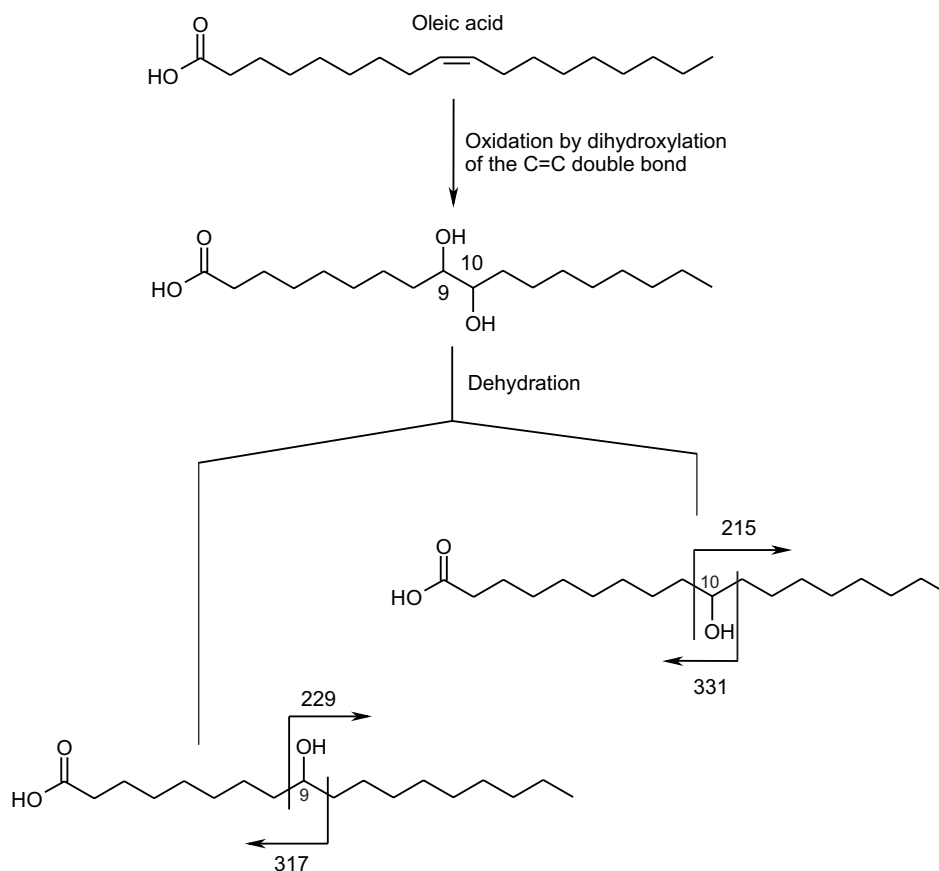


FIGURE 12. Degradation pathway of oleic acid leading to the formation of hydroxy-fatty acids. The number on the hydroxy-acids correspond to the characteristic fragments obtained by EI-MS on TMS-derivatives. From Regert et al. (1998).

III. COMBINING MOLECULAR AND ISOTOPIC COMPOSITIONS

The detection and identification of lipid biomarkers represents powerful means to investigate the nature, origin, and transformations of organic residues preserved in ancient pottery. Nevertheless, this only constitutes one aspect of the information contained in organic residues preserved at archeological sites. During the 1980s, determinations of the stable carbon isotopes appeared as additional criteria to identify the commodities processed in ancient vessels (Hastorf & DeNiro, 1985; DeNiro, 1987).

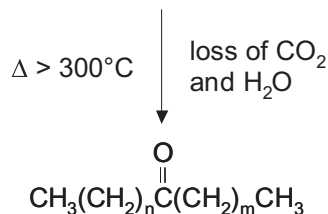
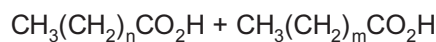
Food residues burnt onto pottery vessels were submitted to determination of bulk stable carbon analysis, mainly to distinguish C₃ from C₄ plant contributions in American vessels (Hastorf & DeNiro, 1985; DeNiro, 1987). However, the results obtained from the bulk isotope analysis are difficult to interpret due to the mixing of several substances and to degradative phenomena.

During the 1990s, Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC-C-IRMS), a technique developed at the end of the 1970s (Matthews & Hayes, 1978), began to be applied in the field of archeology to determine $\delta^{13}\text{C}$ values of individual components of lipid extracts from potsherds (Evershed et al., 1994). This method opened up considerable opportunities to precisely identify a wide range of

substances, making it possible to distinguish subcutaneous animal fats from dairy products and to assess the specific origin of certain fats (Evershed et al., 1997b; Stott et al., 1997; Dudd & Evershed, 1998; Dudd et al., 1999; Evershed et al., 2002; Copley et al., 2003, 2005a,b,c,d; Spangenberg, 2004; Craig et al., 2005a; Spangenberg, Jacomet, & Schibler, 2006; Evershed et al., 2008b; Spangenberg et al., 2008). In all of these articles, the results were systematically obtained by combining molecular and carbon isotopic compositions of biomarkers and degradation markers identified in absorbed and/or charred surface residues.

The possibility to extract and identify representative compounds that are unequivocally linked to their animal source has also valuable potential to date archeological pottery by compound-specific ^{14}C analysis of preserved lipids (Stott et al., 2001, 2003; Berstan et al., 2008).

In this section, I first provide a general background of isotopic signatures, and focus on the tracer potential of isotopic characteristics. I then discuss the advantages of compound-specific carbon isotopic analysis versus bulk analysis, and describe the specific requirements of the technique. The last sections detail the criteria that have been established to distinguish animal fats based on isotopic compositions, and presents the innovative studies that allow direct radiocarbon dating of individual fatty acids isolated from archeological lipid extracts.



with

- $n = 13$ and $m = 13$ for nonacosan-15-one (C_{29})
- $n = 12$ and $m = 15$ for triacontan-14-one (C_{30})
- $n = 13$ and $m = 14$ for triacontan-15-one (C_{30})
- $n = 14$ and $m = 14$ for hentriacontan-17-one (C_{31})
- $n = 13$ and $m = 16$ for dotriacontan-15-one (C_{32})
- $n = 14$ and $m = 15$ for dotriacontan-16-one (C_{32})
- $n = 14$ and $m = 16$ for tritriacontan-16-one (C_{33})
- $n = 15$ and $m = 16$ for tetratriacontan-17-one (C_{34})
- $n = 16$ and $m = 16$ for pentatriacontan-18-one (C_{35})

FIGURE 13. Pathway of formation of long chain ketones by condensation of fatty acids during heating animal fats. IMDA = intramolecular Diels–Alder reaction. From Evershed et al. (Evershed et al., 1995, 2002).

A. General Background to Isotopic Signatures

Stable isotopes of carbon are valuable tracers of the nature and origin of various natural substances preserved in the archaeological record. Differences in stable isotope compositions reflect differences in the reaction rates for the isotopes of an element (DeNiro, 1987). The incorporation of carbon into a living organism through various biochemical pathways leads to products in which the ratios between the stable isotopes of a given element are slightly different from those of the reactants. This phenomenon, known as isotopic fractionation, is rather small for reactions involving carbon (Van der Merwe, 1982;

DeNiro, 1987). The ratios of stable isotopes are thus generally expressed relative to a universal standard by a specific notation created by geochemists to produce numbers with magnitudes that are easy to use (Bocherens, 1997):

$$\delta^E\text{C} = \frac{R_{\text{sample}}}{R_{\text{standard}} - 1} \times 1,000$$

In this formula, E corresponds to 13, and $R = {}^{13}\text{C}/{}^{12}\text{C}$. The data obtained are expressed as ‰, that is, per mil.

Organic residues preserved in ancient pottery originate from the plant and animal substances processed in the vessels. Establishing accurate relationships between isotopic characteristics of organic matter and natural resources is far from simple. Indeed, isotope ratios of carbon reflect (i) the primary sources of carbon, (ii) the metabolic pathways of living organisms used to incorporate their diet in their body, and (iii) the environmental conditions (Spangenberg, 2004 and references therein). Furthermore, in the case of archeological samples, one must not forget that isotopic fractionation might occur during the processing of the commodities or might result from diagenetic alteration. Last, several substances might have been mixed in a same container to make the isotopic data particularly difficult to decipher.

Interpreting $\delta^{13}\text{C}$ values obtained from the analysis of charred or absorbed residues in pottery vessels relies on the foundations established in the 1970s based on isotopic characteristics of a wide range of plants and animals.

Although this review is focused on animal fats, the isotopic story necessarily begins with knowledge of plants as producers at the basis of the trophic chain, from which are derived the isotopic signatures of all the animals, whether they are herbivores or carnivores.

Plants can be classified into two main groups, C_3 and C_4 plants, and a third one (CAM plants, i.e., Crassulean Acid Metabolism) that depend on the biochemical pathway involved in the fixation of atmospheric CO_2 during photosynthesis (Hastorf & DeNiro, 1985; DeNiro, 1987; Bocherens, 1997; Spangenberg, 2004). C_3 plants use the Calvin cycle to incorporate the carbon from atmospheric CO_2 . This mode of incorporation leads to the formation of a first reaction intermediate that contains three carbon atoms. During this transformation cycle, “most of the fractionation occurs in the enzymatic carboxylation of CO_2 by ribulose-1,5-biphosphate carboxylase” (Deines, 1980; Spangenberg, 2004). Cereals and most of the plants that grow

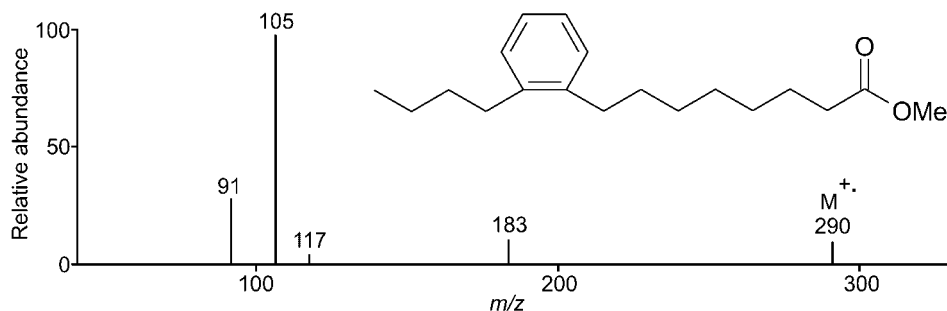


FIGURE 14. Mass spectrum of a C_{18} ω -(*o*-alkylphenyl) alkanonic acid (as methyl ester derivative) derived from a $\text{C}_{20.3}$ precursor. Reproduced from Craig et al. (2007b) with permission from Wiley-Blackwell, copyright 2007.

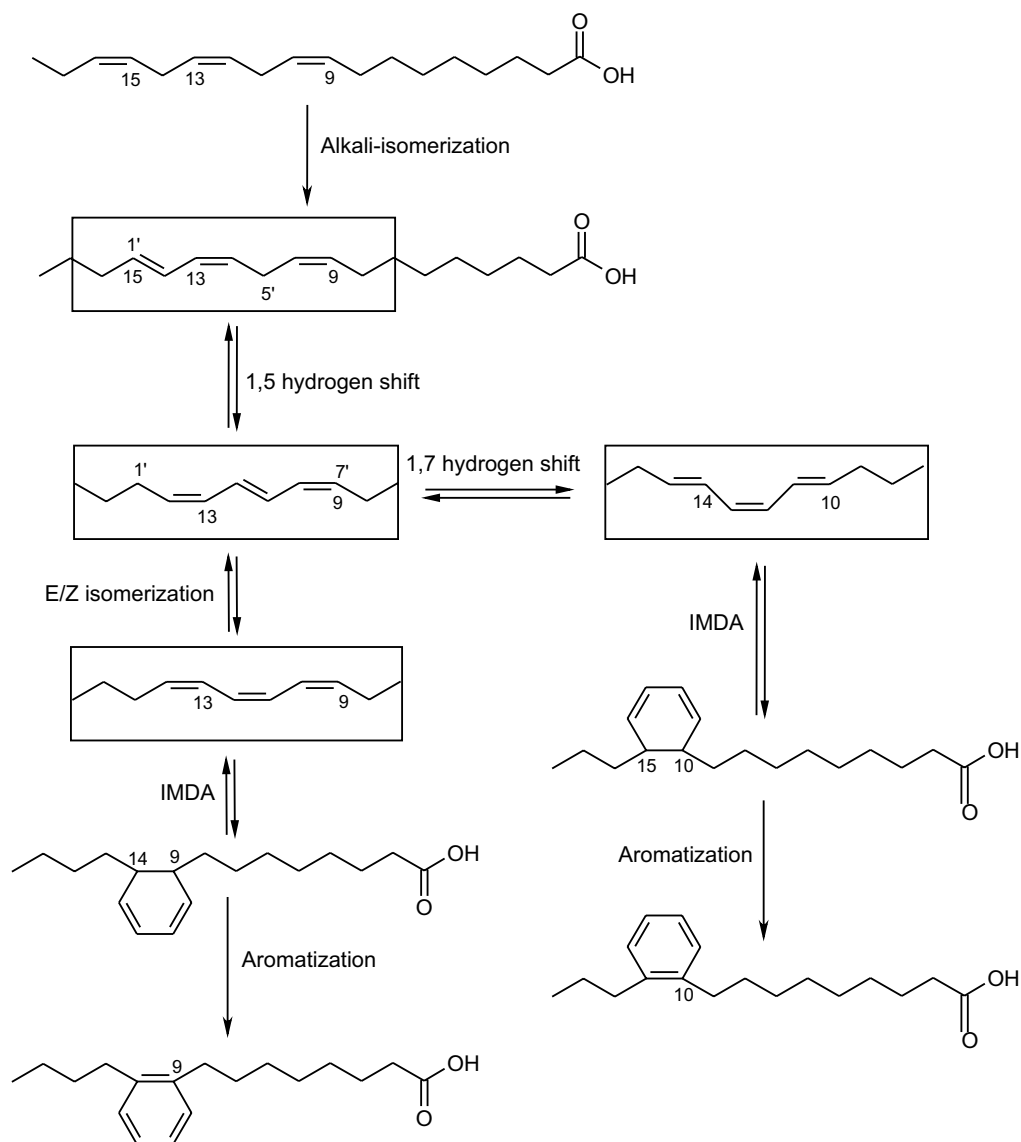


FIGURE 15. Formation pathways of cyclic compounds issued from the degradation of triunsaturated fatty acids of marine origin. From Hansel et al. (2004) and Evershed et al. (2008a). With permission from Elsevier, copyright 2004.

in temperate regions belong to this group of plants. The biosynthesis of biomass by C_4 plants relies on the Hatch-Stack cycle in which the first reaction intermediate contains four carbon atoms (Deines, 1980; Spangenberg, 2004). Several plants of warm regions such as maize, sugarcane, and sorghum belong to this category (O'Leary, 1981; Bocherens, 1997). CAM plants use the two kinds of metabolisms that depend on the circumstances (O'Leary, 1981). The mean $\delta^{13}\text{C}$ values for these three groups of terrestrial plants are $-27.1 \pm 2.0\%$ for C_3 plants and $-13.1 \pm 1.2\%$ for C_4 plants (O'Leary, 1981; Bocherens, 1997; Spangenberg, 2004). $\delta^{13}\text{C}$ values of CAM plants have a bimodal distribution centered on -33% and -11% (O'Leary, 1981; Bocherens, 1997).

Because carbon isotopic fractionation between the tissues of the consumer and its diet is very small, from 1‰ to 2‰ (DeNiro & Epstein, 1978), $\delta^{13}\text{C}$ values of animals are directly linked to

those of plants consumed by the herbivores at the beginning of the trophic chain.

Stable isotopes are also useful to discriminate between terrestrial and marine organisms (Schoeninger & DeNiro, 1984; DeNiro, 1987). Marine plants, that use dissolved bicarbonate as carbon source, have mean $\delta^{13}\text{C}$ values of -20% , which also correspond to those of marine animals (DeNiro, 1987; Bocherens, 1997).

It must be noted that all of the $\delta^{13}\text{C}$ values presented in this section are for plants and animals based on bulk organic matter. However, values are known to vary in relation to several factors, including environmental conditions, kinds of tissues and biochemical fractionations, physiology, source of primary carbon used, and diet (see, e.g., DeNiro & Epstein, 1978; Ambrose & DeNiro, 1986; Heaton et al., 1986; Sealy et al., 1987; Fizet et al., 1995).

B. Bulk Versus Single Compounds

The first investigations of the isotopic characteristics in charred visible or absorbed organic residues in ceramic vessels focused on bulk carbon and nitrogen determinations (Hastorf & DeNiro, 1985; DeNiro, 1987). They mainly concerned plant residues in America. Determinations of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the bulk of residues provide mean values for all the compounds present in the potsherds that include lipids, proteins, and polysaccharides. All of these biochemical fractions are known to exhibit various isotopic values dependent on the sources from which they derive and their mechanisms of incorporation (DeNiro & Epstein, 1978). Although they provide valuable and general overviews of the commodities preserved in the vessels, more specific information might be obtained by determining the isotopic values of individual biomarkers (Evershed et al., 1994, 1997b; Dudd & Evershed, 1998). The development of a new technique of isotope ratio monitoring gas chromatography–mass spectrometry (irm GC–MS), also called CG–C-IRMS at the end of the 1970s (Matthews & Hayes, 1978), and applied in the field of environmental chemistry in the 1980s, allows isotope ratios of individual compounds in complex mixtures to be determined. This method was first applied in the field of archeology by Evershed and co-workers to study C_3 plant leaf wax components in pottery vessels (Evershed et al., 1994); it was thereafter used to discriminate animal fats, in combination with compositional information obtained from GC–MS. This approach is now used by several teams, and has greatly enhanced the studies of amorphous organic materials preserved in the archeological record.

By combining isotopic values from organic matter from ancient vessels with molecular studies, it is theoretically possible (i) to assess the terrestrial or marine origins of the resources studied, (ii) differentiate C_3 from C_4 plants, (iii) distinguish fats of ruminant and non-ruminant animals, and (iv) discriminate subcutaneous animal fats from dairy products.

C. Specificities of Isotopic Values in Archeological Materials

A comparison between isotopic ratios measured for contemporary reference materials and those from archeological samples must be performed cautiously because of the post-industrial revolution effect (Evershed et al., 1994). Carbon dioxide emitted to the atmosphere by combustion of fossil fuel and biomass during industrial activities has a $\delta^{13}\text{C}$ value of approximately -25‰ that is significantly lower than the present $\delta^{13}\text{C}$ value of atmospheric CO_2 in rural areas which is $\sim 7\text{‰}$ (Friedli et al., 1986; DeNiro, 1987). Atmospheric CO_2 fixed by plants during the photosynthetic process is thus depleted in ^{13}C since the middle of the 19th century. In other words, atmospheric CO_2 prior to the post-industrial revolution contained a higher proportion of ^{13}C than in present time. Accordingly, a correction of approximately 1.2‰ is generally applied to values acquired for contemporary reference materials to allow comparison with those obtained from archeological samples (Peng et al., 1983; Dudd & Evershed, 1998).

Furthermore the methyl group introduced into each fatty acid during derivatization contributes to the isotopic composition of the FAME. A correction must be done to obtain the true $\delta^{13}\text{C}$ value of each fatty acid. Because little or no fractionation occurs

during this type of derivatization (Rieley, 1994), the following mass balance equation is used (Woodbury et al., 1995; Abraham, Hesse, & Pelz, 1998):

$$\delta^{13}\text{C}_{\text{FA}} = \frac{(C_n + 1)\delta^{13}\text{C}_{\text{FAME}} - \delta^{13}\text{C}_{\text{MeOH}}}{C_n}$$

where $\delta^{13}\text{C}_{\text{FA}}$ corresponds to the $\delta^{13}\text{C}$ value of the underivatized fatty acid; C_n is the number of carbon atoms in the fatty acid; $\delta^{13}\text{C}_{\text{FAME}}$ is the $\delta^{13}\text{C}$ value of the fatty acid methyl ester, and $\delta^{13}\text{C}_{\text{MeOH}}$ is the $\delta^{13}\text{C}$ value of the methanol used for the methylation.

Second, the use of isotopic values to identify the kind of animal fats processed in ancient pottery vessels relies on a comparison between $\delta^{13}\text{C}$ values acquired from archeological samples and those of modern reference materials. Because the isotopic signatures of animal tissues depends on their diet, it is important to obtain fats from modern animals with known diets ideally similar to that of animals of the periods and regions investigated. Particularly, for the Old World, modern reference animals must be reared on C_3 plants such as grasses and cereals for which $\delta^{13}\text{C}$ values are known (Evershed et al., 1997b; Copley et al., 2003). Generally speaking, one must be very cautious when modern reference materials are not well-described or were simply purchased in foodstores or markets without any knowledge or control of the trophic chain of the animals investigated.

Last, $\delta^{13}\text{C}$ values might be affected by diagenetic alteration and/or food processing. These processes should be well-understood before interpreting isotopic values of natural resources preserved in ceramic vessels. Several authors describe laboratory experiments and provide comparison between contemporary and archeological samples with the aim to understand the effects of transformation on isotopic signatures (Hastorf & DeNiro, 1985; DeNiro, Schoeninger, & Hastorf, 1985; DeNiro, 1987; Marino & De Niro, 1987; Evershed et al., 1994, 1999, 2002; Spangenberg, Jacomet, & Schibler, 2006; Spangenberg et al., 2008).

First experiments that aimed to assess the influence of thermal effect on isotopic values during cooking consisted of boiling, roasting, and burning modern food plants. The measure of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values before and after these operations showed a shift of no more than $2\text{--}3\text{‰}$ between raw and carbonized modern commodities in a random way (DeNiro, 1987). This shift might be explained by a differential alteration of the various biochemical compounds present in the samples—namely carbohydrates, proteins, and lipids (Evershed et al., 1994). It was also demonstrated that plants cooked in pottery vessels retain their isotopic identities during burning (Hastorf & DeNiro, 1985). All these experiments, based on the measure of stable carbon and nitrogen isotopes in the bulk organic matter, tend to show that $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of animal and plant products are not substantially altered during cooking.

Furthermore, by the determination of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in archeological carbonized and uncarbonized plants identified to the species level by their morphology, it was established that the isotopic record of carbonized plants is not modified by diagenetic process, whereas isotopic values of uncarbonized plants are more likely to be affected by burial (DeNiro, 1987). Complementary experiments in which modern carbonized and uncarbonized plants were incubated with active soil microbes helped to explain this observation: it indeed appeared that carbonized plants, probably because they do not contain a high level of nutrient, are

TABLE 3. Data showing the very stable $\delta^{13}\text{C}$ values of fatty acids extracted from a modern ovine adipose fat before and after degradation experiment for 100 days at 45°C

Incubation time T_{days} (incubation conditions)	$\delta^{13}\text{C}$ values (‰)			
	$\text{C}_{14:0}$	$\text{C}_{16:0}$	$\text{C}_{18:0}$	$\text{C}_{18:1}$
T_0 (pre-incubation)	-28.4	-29.8	-31.8	-30.4
T_{100} (oxic)	-28.1	-29.5	-31.9	-30.1
T_{100} (anoxic)	-28.8	-29.5	-32.3	-31.0

Evershed et al. (1999).

less sensitive to bacterial alteration than the uncarbonized ones (DeNiro, 1987).

After these pioneer investigations, further experiments tend to understand more specifically the isotopic signatures of lipids preserved in ancient potsherds by using the isotopic values of individual molecular compounds (Evershed et al., 1994, 1997b, 1999, 2002; Spangenberg, Jacomet, & Schibler, 2006; Spangenberg et al., 2008).

The effect of boiling on the isotopic signature of the bulk organic matter and individual fatty acids of modern milk samples was recently discussed (Spangenberg, Jacomet, & Schibler, 2006; Spangenberg et al., 2008). Among the variations that occurred, it was generally observed that stearic and oleic acids are enriched in ^{13}C during heating. This was explained by cleavage of carbon-carbon bonds and loss of ^{12}C -rich moieties (Spangenberg et al., 2008). However, it was surprising that changes in the $\delta^{13}\text{C}$ values for the bulk organic matter and in the main fatty acids were different for the different fatty acids and milks investigated (from cow, goat, or sheep). Further experiments would thus be necessary to fully understand the alteration pathways and their influence on isotopic values during cooking (Spangenberg et al., 2008).

Other experiments focused on $\delta^{13}\text{C}$ values of the main fatty acids preserved in the archeological record (myristic, palmitic, stearic, and oleic acids) that were determined before and after different degradation experiments in oxic and anoxic environments to understand the effect of burial on $\delta^{13}\text{C}$ values (Evershed et al., 1995; Evershed et al., 1999, 2002). The results obtained clearly showed that the $\delta^{13}\text{C}$ values remain unchanged after this process (Evershed et al., 1999, 2002; Evershed, 2008a,b; Table 3), probably due to the fact that no carbon-carbon bonds are broken in the compounds that survive (Evershed et al., 1994).

Most of these experiments showed that $\delta^{13}\text{C}$ values determined for bulk organic matter, but more particularly for individual lipids, are robust criteria to determine the classes of commodities processed in pottery vessels, and of particular value to discriminate key types of animal fats at archeological sites. The use of carbon isotopic signatures are enhanced further when used in conjunction with biomarker structures and distribution (Evershed et al., 2008b and references therein).

D. Specific Requirements of the GC-C-IRMS Technique

The GC-C-IRMS technique was developed in the 1970s (Matthews & Hayes, 1978). It is based on the connection in series of a gas chromatograph, a combustion oven, and a mass spectrometer equipped with a magnetic sector. This on-line

system presents considerable advantages compared to previous conventional off-line approaches. It provides the opportunity to measure continuously $^{13}\text{C}/^{12}\text{C}$ ratios at natural abundance levels of individual compounds chromatographically resolved from complex mixtures of volatile components (Matthews & Hayes, 1978).

The combustion step converts the separated molecular species into CO_2 . This conversion then allows comparison of isotopic abundance of a standard and samples of identical molecular structures (Evershed et al., 1994). This combustion step that occurs between the gas chromatograph and the mass spectrometer is probably the most critical operation to be performed to obtain reliable data (Matthews & Hayes, 1978). Obtaining quantitative combustion is of prime importance to avoid isotopic fractionation that would affect the carbon isotope ratios obtained. The oven must be maintained at a high enough temperature, and a constant flow of helium maintained through the combustion system. Matthews and Hayes (1978) showed that cupric oxide was an appropriate catalyst, and it is now currently employed, sometimes associated with platinum. A trap placed after the combustion oven removes combustion products that are not of interest, particularly water, to prevent them to reach the source of the mass spectrometer (Matthews & Hayes, 1978; Merritt et al., 1995). It is important to eliminate water from the eluent stream entering the MS ion source to prevent the formation of HCO_2^+ that has the same nominal mass as $^{13}\text{CO}_2$ and affect recorded $^{13}\text{C}/^{12}\text{C}$ ratios.

Although GC-C-IRMS is suitable to study complex molecular mixtures, some prerequisites are essential to obtain accurate $\delta^{13}\text{C}$ values. The samples must be free of sulfur, which can be readily removed with copper filings (Evershed et al., 1994). At least 100 ng of each compound were needed to obtain optimal $\delta^{13}\text{C}$ values in the 1990s (Evershed et al., 1994), but new generations of modern instruments are now much more sensitive (Evershed, 2009). $\delta^{13}\text{C}$ values can only be obtained from peaks that baseline resolved by gas chromatography (Matthews & Hayes, 1978; Evershed et al., 1994). Sample preparation is thus an essential step to obtain sufficiently purified materials for analysis of archeological samples. A solvent extraction yields the lipid fraction from organic residues, as previously described above. An aliquot of the total lipid extract is saponified to release fatty acids that are converted to fatty acid methyl esters FAMES (Dudd, Evershed, & Gibson, 1999; Mottram et al., 1999; Copley et al., 2005a,b,c,d; Craig et al., 2005a,b; Spangenberg, Jacomet, & Schibler, 2006). This saponification step affords simple chromatograms in which the main peaks are methylated fatty acids, among which palmitic and stearic acids are generally the main components in archeological materials.

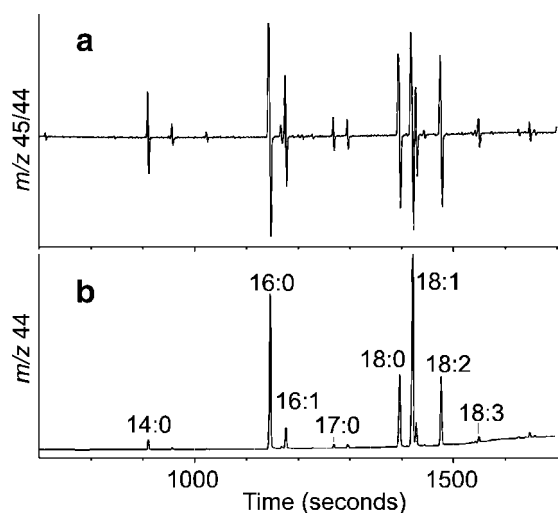


FIGURE 16. Chromatograms obtained by GC-C-IRMS analysis of the CO₂ generated by the combustion of the fatty acids analyzed as their methyl esters, from modern adipose fat of pig. **a:** Chromatogram of the ion at *m/z* 44 and **(b)** instantaneous ratios of the *m/z* 44 and 45 ion currents. From Evershed et al., 2002. With permission from American Chemical Society, copyright 2002.

GC separations use two main kinds of stationary phases. For reference samples that usually contain polyunsaturated fatty acids, a polar coating such as polyethylene glycol or cyanopropyl polysiloxane is currently used, whereas an apolar dimethylpolysiloxane film is preferred in the case of archeological samples since these samples are free of polyunsaturated fatty acids due to degradation during burial (Mottram et al., 1999).

To obtain accurate and reproducible $\delta^{13}\text{C}$ values, duplicate or triplicate injections are performed for each sample (Mottram et al., 1999; Craig et al., 2005a,b). Repeatability and the precision of the GC-C-IRMS system, and also chromatographic and combustion oven performance are regularly assessed by injection of reference mixtures of known isotopic composition (Spangenberg, 2004). Replicate samples of reference CO₂ gas are introduced at the beginning and end of each analysis for standardization purposes (Mottram et al., 1999).

The data generated by GC-C-IRMS analyses appear as chromatograms of *m/z* 44 and instantaneous ratios of the *m/z* 44 and 45 ion currents (Evershed et al., 1994; Mottram et al., 1999, Fig. 16).

The precision of $\delta^{13}\text{C}$ values is typically $\pm 0.3\text{‰}$ or better (Merritt & Hayes, 1994).

$\delta^{13}\text{C}$ values are expressed relative to the international standard PDB, which is a belemnite fossil from the Pee Dee formation of South Carolina. However, this fossil has been exhausted, and the VPDB (Vienna-PDB) standard (*Belleminitella americana*) is now used (Coplen, 1995; McCarroll & Loader, 2004).

E. Criteria to Discriminate Animal Fats by GC-C-IRMS

Most of the molecular analyses performed on organic residues from ancient ceramic vessels show that animal fats were exploited widely in the past from at least the Neolithic period (see, e.g., Evershed et al., 1992a,b, 1997b, 1999, 2002, 2008b; Charters et al., 1993, 1995, 1997; Heron & Evershed, 1993;

Regert et al., 2001b; Dudd et al., 1999; Regert, 2007a,b). When TAGs are well-preserved, hypothesis may be drawn to assess the species from which animal fats are derived and the type of substance processed in the vessels, namely dairy or carcass products (see Section II). However, in most cases, determining the nature and origin of the fats preserved in pottery containers is a difficult task due to their alteration during vessel use and burial. From its earliest applications to archeology, GC-C-IRMS appeared to provide complementary and robust information that allowed more precise and complete assignments of the nature of commodities exploited in pottery vessels (Evershed et al., 1994, 1997b, 2002, 2008b; Dudd & Evershed, 1998).

Investigations of the $\delta^{13}\text{C}$ values of C_{16:0} and C_{18:0} fatty acids extracted from the fats of modern animals raised on controlled C₃ diets showed that a clear distinction could be made between adipose fats of ruminant (cattle, sheep, and goat) and those of non-ruminant, that is, porcine animals (Evershed et al., 1997b; Dudd, Evershed, & Gibson, 1999; Mottram et al., 1999; Copley et al., 2003; Mukherjee et al., 2007; Mukherjee, Gibson, & Evershed, 2008). The graph plotting the $\delta^{13}\text{C}$ values for the C_{18:0} versus C_{16:0} fatty acids from modern animals indicates that the two groups are well separated by a difference of ca. 4‰ for palmitic acid and ca. 7‰ for stearic acid (Fig. 17, Mukherjee et al., 2007). Plant lipids are assimilated by ruminant species after substantial transformation in the rumen involving triacylglycerol hydrolysis and biohydrogenation of the unsaturated fatty acids, while dietary lipids undergo less complex transformation, mainly hydrolysis, in monogastric animals (Harfoot, 1981; Spangenberg, Jacomet, & Schibler, 2006). Therefore, the incorporation of lipids by ruminant and non-ruminant animals differs due to their specific digestive systems. Furthermore, both animals produce fatty acids in adipose fats by *de novo* synthesis. However, the carbon source is not the same for the two groups of animals: ruminants including cow, goat and sheep incorporate carbon from acetate to biosynthesize palmitic and stearic acids whereas porcines use carbon from both acetate and glucose ($\delta^{13}\text{C}_{\text{acetate}} < \delta^{13}\text{C}_{\text{glucose}}$) to produce the same fatty acids (Vernon, 1981). These differences in metabolic and digestive physiologies of these two categories of animals explain why their adipose fats may be discriminated by their carbon isotope signatures (Mukherjee et al., 2007). Although all reports that present $\delta^{13}\text{C}$ values for C_{16:0} and C_{18:0} acids clearly distinguish ruminant from non-ruminant adipose fats based on plots of $\delta^{13}\text{C}_{18:0}$ values versus $\delta^{13}\text{C}_{16:0}$ values, these are not absolute values. For example, data recently published by the team of Spangenberg for pigs (Spangenberg, Jacomet, & Schibler, 2006) appear depleted in ¹³C for both C_{16:0} and C_{18:0} compared to that published earlier Evershed and co-workers (Copley et al., 2003); these differences likely arise from variations in the diet of the animals used as modern references.

Another category of fat of considerable importance in the field of archeology is that derived from dairy products (Dudd & Evershed, 1998). Determining the chronology of dairy product exploitation and establishing the intensity of production and use are indeed major and complex tasks in studies of the evolution of Neolithic economies (Bogucki, 1982; Sherratt, 1983; Bogucki, 1984, 1986; Dudd & Evershed, 1998; Copley et al., 2003, 2005a,b,c,d; Craig et al., 2005a; Vigne & Helmer, 2007; Evershed et al., 2008b; Spangenberg et al., 2008).

During analyses of lipid extracts from several archeological pottery vessels, it was noticed that ruminant animal fats could be

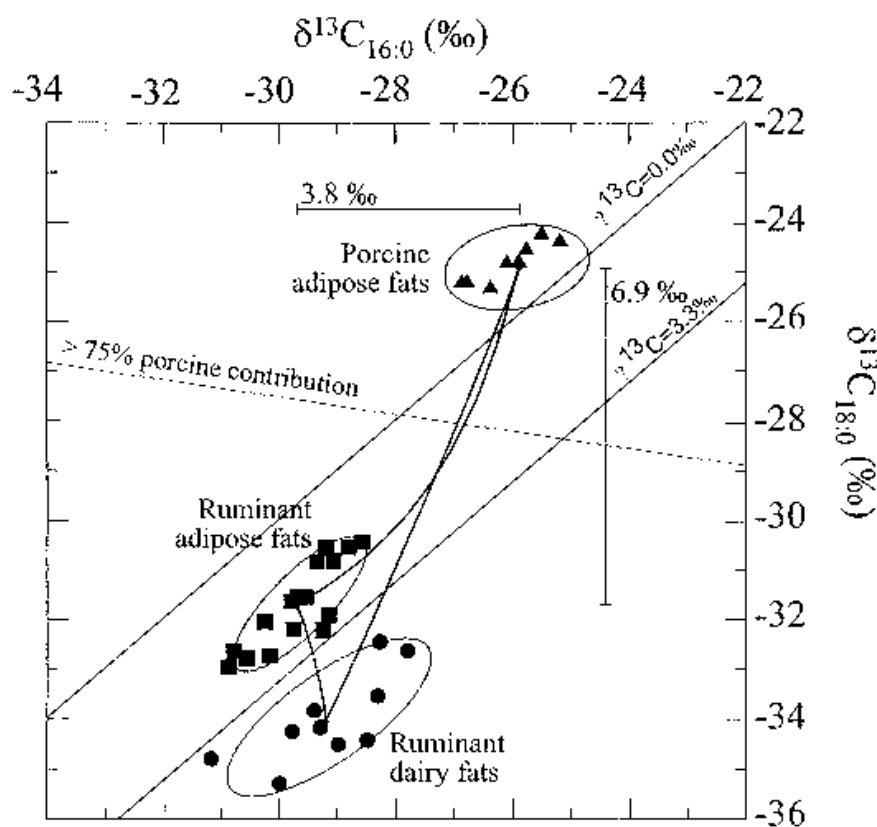


FIGURE 17. Graph plotting $\delta^{13}\text{C}$ values for stearic versus palmitic acid (as methyl esters) of various modern animals reared on C_3 diet. From Mukherjee et al., 2007. The confidence ellipses displayed on the graph were calculated for the $\delta^{13}\text{C}$ values of domestic animals (Copley et al., 2003). The curves linking the ellipses of non-ruminant fats to those of other products correspond to mixture of several substances (Copley et al., 2003). Reproduced from Mukherjee et al. (2007) with permission from Antiquity, copyright 2007.

separated into two groups: one being depleted in ^{13}C for stearic acid (Fig. 17, Dudd & Evershed, 1998). Comparison of these archeological data with those obtained on modern reference fats indicated that the lower $\delta^{13}\text{C}_{18:0}$ values correlated with those of ruminant milk fats (Dudd & Evershed, 1998). The mean difference of $\approx 2.3\text{‰}$ observed between $\delta^{13}\text{C}_{18:0}$ values of adipose and dairy fats is related to two main differences in the production of these substances (Copley et al., 2003). First, the dietary precursor compounds used to produce stearic acid are not the same in milk and adipose fats; second the biosynthesis of $\text{C}_{18:0}$ fatty acid in milk as adipose fats arises from distinct biochemical pathways that lead to a specific difference in their carbon isotope values (Dudd & Evershed, 1998; Copley et al., 2003).

In adipose fats, stearic acid originates in part from dietary lipids and to a significant extent from *de novo* synthesis from acetate, derived mainly from dietary carbohydrates (Dudd & Evershed, 1998). In contrast, the mammary gland is unable to biosynthesize $\text{C}_{18:0}$ fatty acid (Copley et al., 2003). In milk, this compound derives from the dietary lipids, mainly $\text{C}_{18:2}$ and $\text{C}_{18:3}$, that are biohydrogenated by bacterial reduction in the rumen, with a proportion also coming from mobilization of fatty acids from subcutaneous animal fats (Dudd & Evershed, 1998; Copley et al., 2003). During lactation, dietary fatty acids are thus routed

preferentially to milk. This differential routing results in the shift of $\delta^{13}\text{C}_{18:0}$ values between the two categories of fats (Dudd & Evershed, 1998; Evershed et al., 2002; Copley et al., 2003). Furthermore, a study of the $\delta^{13}\text{C}$ values of unsaturated fatty acids and carbohydrates of plants consumed by ruminant animals provided mean values of -36.3‰ and -28.2‰ , respectively (Copley et al., 2003; Dungait, Docherty, & Evershed, 2008). This difference in $\delta^{13}\text{C}$ values of the major dietary classes of biochemicals also contributes to the more depleted $\delta^{13}\text{C}_{18:0}$ values of milk fat compared with those of adipose fat of animals reared on the same diet (Copley et al., 2003).

Unlike $\text{C}_{18:0}$, palmitic acid is derived from a combination of dietary lipids and *de novo* biosynthesis in adipose fats but originates mainly from *de novo* synthesis of acetate from carbohydrates in the mammary gland for milk fats (Dudd & Evershed, 1998). Table 4 summarizes the differences of origin and biosynthesis of palmitic and stearic acids in milk fats and adipose fats.

Therefore, the determination of $\delta^{13}\text{C}$ values on palmitic and stearic acids of several reference modern fats sampled from animals raised on the same controlled diet of C_3 plants, similar to that consumed by ancient animals, provided graphs from which the fats of the main domesticated animals of prehistoric Britain could be distinguished (Fig. 17). $\Delta^{13}\text{C}$ values, corresponding to

TABLE 4. Summary of the origins of C_{16:0} and C_{18:0} fatty acids in milk and adipose fats of ruminant animals (Dudd & Evershed, 1998; Evershed et al., 2002; Copley et al., 2003)

	Origin in adipose fats	Origin in milk
C _{16:0}	Derives from a combination of dietary lipids and <i>de novo</i> synthesis of acetate and thus from both dietary carbohydrates and lipids	Derived mainly from <i>de novo</i> synthesis from acetate (derived mainly from dietary carbohydrates) in the mammary gland and thus mainly derives from dietary carbohydrates
C _{18:0}	Derives from a combination of dietary lipids and <i>de novo</i> synthesis of acetate and, to a significant extent, from <i>de novo</i> synthesis from acetate and thus from both dietary carbohydrates and lipids	Derived in part from biodehydrogenation in the rumen of dietary unsaturated fatty acids (mainly C _{18:1} , C _{18:2} and C _{18:3}) and <i>de novo</i> synthesized fatty acids (non mammary)

$\delta^{13}\text{C}_{18:0} - \delta^{13}\text{C}_{16:0}$, were also calculated to further distinguish dairy products from adipose fats (Copley et al., 2003, 2005a) since $\Delta^{13}\text{C}$ values of milk fats are always less than -3.3‰ which is lower than the value for adipose fats (Fig. 17, Copley et al., 2003).

More recently, new data were obtained on modern reference fats from Switzerland (Spangenberg, 2004; Spangenberg, Jacomet, & Schibler, 2006). $\delta^{13}\text{C}$ values obtained on dairy products exhibit values that range on a larger scale than data previously published (Dudd & Evershed, 1998; Dudd et al., 1999; Evershed et al., 2002; Copley et al., 2003). $\delta^{13}\text{C}$ values were also determined for the adipose fat of young animals, including calf and lamb, still suckled (Spangenberg, 2004; Spangenberg, Jacomet, & Schibler, 2006). Interestingly, these fats cluster in the range of monogastric animals. The use of glucose from mother's milk as carbon source for fatty acid biosynthesis by young animals likely explains why their adipose fat plots near porcine fats that derive from acetate and glucose carbon source but not in the range of adult ruminant animals that mainly use acetate as carbon source for fatty acid biosynthesis (Spangenberg, Jacomet, & Schibler, 2006). Since only a single lamb and calf were investigated, further analyses of lipids from adipose fats of young animals for which the diet of the mother is well controlled are necessary to confirm these preliminary observations.

Although domestic animals were an important source of fats and proteins during the Neolithic period, they were not the only species to be consumed. Wild animals and aquatic resources were also exploited. Despite the difficulty in controlling the diets of modern wild animals, carbon isotope values have been reported for wild boar and deer (Fig. 18, Spangenberg, 2004; Spangenberg, Jacomet, & Schibler, 2006; Craig et al., 2007b). Nevertheless, due to the low number of samples studied, further investigations are necessary to assess the systematics of the $\delta^{13}\text{C}$ values for this category of animals.

The question of preservation of lipids in ancient pottery vessels was recently addressed with the aim at understanding the evolution of food consumption in northern Europe at the beginning of Neolithic period (Craig et al., 2007b). Before analyzing archeological materials, the authors determined $\delta^{13}\text{C}$ values for palmitic and stearic acids of a range of marine (two samples) and freshwater (pike, perch, and carp) fish. Marine fats were shown to be isotopically enriched in ^{13}C compared to those of terrestrial animals, even though they plot not far from adipose

fats of domestic pigs (Fig. 18, Craig et al., 2007b). Furthermore the data obtained provide evidence for differences between marine and freshwater fishes, these latter being depleted in ^{13}C for both C_{16:0} and C_{18:0} fatty as compared to marine resources (Fig. 18, Craig et al., 2007b). As for wild animals, further analyses are now needed on such commodities to assess whether systematic variations exist in the $\delta^{13}\text{C}$ values depending on species and environments (sea water, lakes, rivers, etc.).

The isotopic criteria established by GC-C-IRMS, together with molecular information useful for distinguishing animal fats, are presented in Table 2.

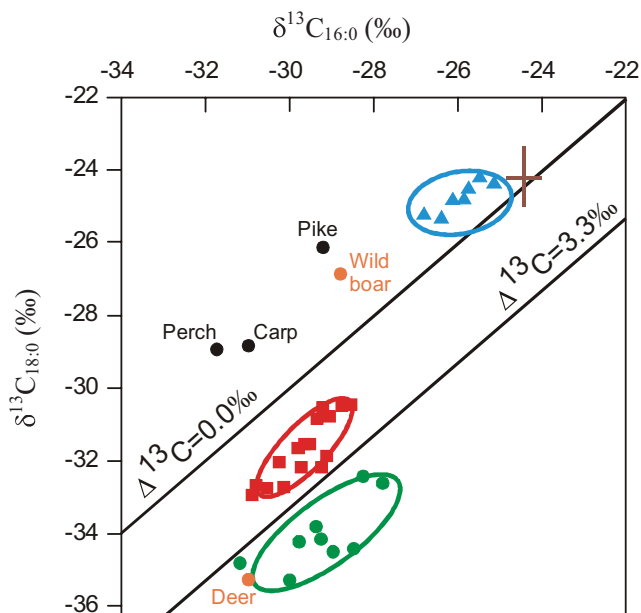


FIGURE 18. Plot of the $\delta^{13}\text{C}$ values obtained on reference wild and domestic animals by the teams of Evershed (from Copley et al., 2003) and Craig (from Craig et al., 2007b). Data in blue = porcine fats (Copley et al., 2003); in red = ruminant adipose fats (Copley et al., 2003); in green = ruminant milk fats (Copley et al., 2003). Data in orange = wild species (Craig et al., 2007b); brown crosses = marine fish (Craig et al., 2007b). Reproduced from Copley et al. (2003) with permission from National Academy of Science, copyright 2003. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

F. Archeological Implications of Combining Molecular and Isotopic Information

Detailed studies that combine fatty acid and sterol compositions, distribution of TAGs (when they are preserved), and $\delta^{13}\text{C}$ values of palmitic and stearic fatty acids, constitute a robust way to discriminate several categories of animal fats in ceramic vessels as a mean for studying pottery function, the economies of ancient societies and the evolution of the exploitation of specific products over time (Evershed et al., 2008b).

The combined use of molecular and isotopic information has allowed the study of porcine exploitation in Great Britain during ancient times (Evershed et al., 1997b; Mukherjee et al., 2007; Mukherjee, Gibson, & Evershed, 2008), reconstruction of the history of dairy products and adipose ruminant fats during the Neolithic period from the Near East to western Europe (Dudd & Evershed, 1998; Dudd, Evershed, & Gibson, 1999; Evershed et al., 2002; Copley et al., 2003, 2005a,b,c,d; Craig et al., 2005a,b; Evershed et al., 2008b; Spangenberg et al., 2008), and investigation of marine lipids at Neolithic sites of northern Europe (Craig et al., 2007b) and in the southern hemisphere (Copley et al., 2004; Hansel et al., 2004; Evershed et al., 2008a). Mixtures of several fats or of fats with other products have also been revealed (Charters et al., 1995; Dudd & Evershed, 1999; Copley et al., 2005a,b,c,d; Mukherjee et al., 2007; Mukherjee, Gibson, & Evershed, 2008). Additionally, questions related to pottery function have been addressed by establishing relationships between the nature of fats preserved and the types of vessel from which they derived (Evershed et al., 1997b; Mottram et al., 1999).

1. Porcine Adipose Fats

The first attempts to distinguish animal fats based on their carbon isotopic and biomarker composition centered on the study of

medieval ceramic vessels, that display two distinct shapes, referred to as lamps and “dripping dishes” (medieval site from the Causeway Lane excavation, Leicester, UK; Evershed et al., 1997b; Mottram et al., 1999). A series of potsherds sampled from three lamps and four “dripping dishes” were investigated. Significant concentrations of saturated fatty acids were encountered in each sample although TAGs were poorly preserved (Mottram et al., 1999). The $\delta^{13}\text{C}$ values of the palmitic and stearic acids clearly indicated that these two types of vessels contained two kinds of animal fats (Fig. 19). The fatty acids in the lamps were depleted in ^{13}C as compared to the “dripping dishes”; the latter clustering near the reference adipose porcine fats while the former plot close to the values obtained for modern non ruminant fats (Evershed et al., 1997b; Mottram et al., 1999). Significantly, the $\delta^{13}\text{C}$ values were consistent with the biomarker compositions determined by GC–MS, since the suite of fatty acids identified in lamps was consistent with ruminant fats while those determined in “dripping dishes” were characteristic of monogastric animals (Table 5). These findings supported the first hypothesis based on pictorial evidence. More specifically, it was confirmed that ruminant tallow was used as fuel in medieval lamps while the porcine fats determined in the “dripping dishes” confirmed the role of these vessels to collect pig fat during spit-roasting and exclude the re-use of their contents as lamp fuel (Mottram et al., 1999).

Although porcine adipose fats are not the substances most commonly identified in archeological ceramic vessels (Mukherjee et al., 2007), preliminary results obtained on Neolithic Grooved Ware pottery of United Kingdom tend to show that they contained significant amount of these products (Evershed et al., 1997b; Dudd, Gibson, & Evershed, 1999). These findings led to the specific study of pig product processing in a wide range of vessels from both Grooved Ware and non-Grooved Ware cultures (Neolithic period) to understand the role of pigs exploitation in

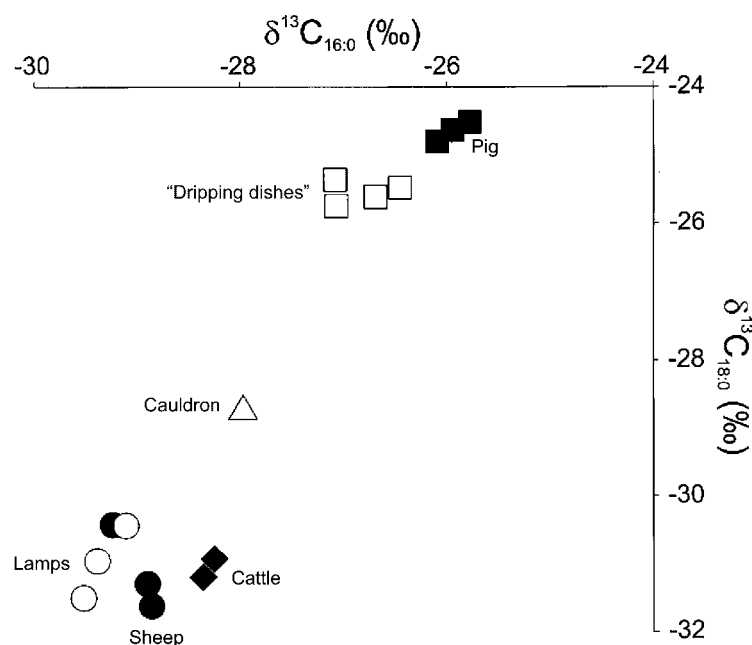


FIGURE 19. Plot showing the $\delta^{13}\text{C}$ values of modern reference animal fats and archeological samples from different shapes of vessels of British medieval site. From Evershed et al. (1997b) and Mottram et al. (1999). With permission from Elsevier, copyright 1999.

TABLE 5. Molecular and isotopic criteria used to discriminate ruminant from non-ruminant fats in two types of medieval ceramic vessels

Analytical data	Lamps	Dripping dishes
$\delta^{13}\text{C}$ values	consistent with ruminant fats (see Fig. 20)	consistent with non-ruminant (porcine) adipose fats (see Fig. 20)
Saturated even-numbered fatty acids	$\text{C}_{18:0}$ more abundant than $\text{C}_{16:0}$	$\text{C}_{16:0}$ more abundant than $\text{C}_{18:0}$
Fatty acids with odd carbon number	odd carbon numbered compounds, particularly $\text{C}_{15:0}$, $\text{C}_{17:0}$ and $\text{C}_{19:0}$ present in higher abundance than in dripping dishes	low abundance or absence of these compounds
Unsaturated fatty acids (identified as DMDS derivatives)	series of different positional isomers of octadecenoic acid, the double-bond being located at 9, 11, 13, 14, 15 and 16-positions	only a single isomer Z-9-octadecenoic acid

Summarized from Evershed et al. (1997b) and Mottram et al. (1999).

Neolithic economies of prehistoric Britain (Mukherjee et al., 2007; Mukherjee, Gibson, & Evershed, 2008). A total of 385 potsherds was selected, including 222 samples from 11 Grooved Ware settlements and 163 samples from 5 non-Grooved Ware sites (Mukherjee et al., 2007; Mukherjee, Gibson, & Evershed, 2008). Lipids were shown to be preserved in a higher number of Grooved Ware vessels, that is, 58–70%, than non-Grooved Ware vessels, that is, 36% (Mukherjee et al., 2007; Mukherjee, Gibson, & Evershed, 2008). In most cases both molecular and isotopic information were consistent with the presence of degraded animal fats. The plots of $\delta^{13}\text{C}$ values for $\text{C}_{18:0}$ versus $\text{C}_{16:0}$, as well as the $\Delta^{13}\text{C}$ values, are consistent with the presence of dairy fats, ruminant adipose fats and porcine lipids (Fig. 20; Mukherjee et al., 2007). Values from a series of vessels also plot between the modern reference fats, indicating mixtures of fats from different origins. To assess the proportions of pig fats in these mixtures, a mixing model plotting $\delta^{13}\text{C}$ values for each fatty acid of reference fats against the percentage of different fats in the mixture was developed (Woodbury et al., 1995; Evershed et al., 2002; Copley et al., 2005b; Mukherjee et al., 2007; Fig. 17). By using this model, it was demonstrated that pig lipids were processed in 16% of the Grooved Ware vessels and that half of these only contained porcine products. In contrast, only four extracts of the non-Grooved Ware pottery were dominated by the presence of non-ruminant adipose fats, the others being dedicated to the exploitation of ruminant products (Mukherjee et al., 2007). These results confirm the preliminary studies on Grooved Ware and Peterborough Ware vessels (Evershed et al., 1997a; Dudd, Evershed, & Gibson, 1999). In case of Grooved Ware vessels, triacylglycerols were well-preserved in half of the extracts in which significant amount of lipids were detected. For half of the samples for which triacylglycerols were preserved, their distribution was consistent with the identification based upon compound-specific $\delta^{13}\text{C}$ values showing a good agreement between molecular and isotopic information (Mukherjee, Gibson, & Evershed, 2008).

It should be noted that in some cases, $\delta^{13}\text{C}$ values obtained on archeological samples cluster near the range of porcine lipids

but exhibit values slightly depleted in ^{13}C (Evershed et al., 1997b; Mottram et al., 1999; Fig. 19). This shift in $\delta^{13}\text{C}$ values between archeological and reference substances probably reflects the incorporation of carbon from dietary sources depleted in ^{13}C such as nut kernels and whey (Dudd, Evershed, & Gibson, 1999); the latter substance, which is a by-product of cheese-making, is known to be consumed by pigs in several societies (Dudd, Evershed, & Gibson, 1999).

All these data indicate that the incidence of porcine products is higher in Grooved Ware vessels than in other ceramic containers, even though other ruminant products were also processed in these vessels (Mukherjee, Gibson, & Evershed, 2008). Furthermore, it appears that pig lipids are very rare in Bronze and Iron Age ceramic vessels (Fig. 20). The occurrence of porcine fats in Grooved Ware vessels was compared to bone assemblages from the sites investigated to study the relationships between these two types of artifacts. Table 6 shows that these data are strongly correlated even though differences due to differential alteration of cultural practices may occur (Mukherjee, Gibson, & Evershed, 2008). Such results are of prime importance in the case of sites where bones are not preserved since faunal information might then be obtained from molecular and stable isotope proxies based on preserved fats in ceramic vessels. The detailed study of occurrence of porcine fats in different Grooved Ware sites showed that these substances were predominantly exploited in non-domestic/ceremonial versus domestic sites and that they were mainly encountered in most recent southern sites (Mukherjee, Gibson, & Evershed, 2008; Table 7).

This may give evidence for the specific exploitation of pigs for “large scale feasting events” or for “special significance of pig to the Grooved Ware people, allowing them to be consumed only at certain places or by specific people” (Mukherjee, Gibson, & Evershed, 2008).

2. Ruminant Adipose Fats and Dairy Products

The detailed study of stable carbon isotope values and their interpretation with the mixing model showed that the presence of

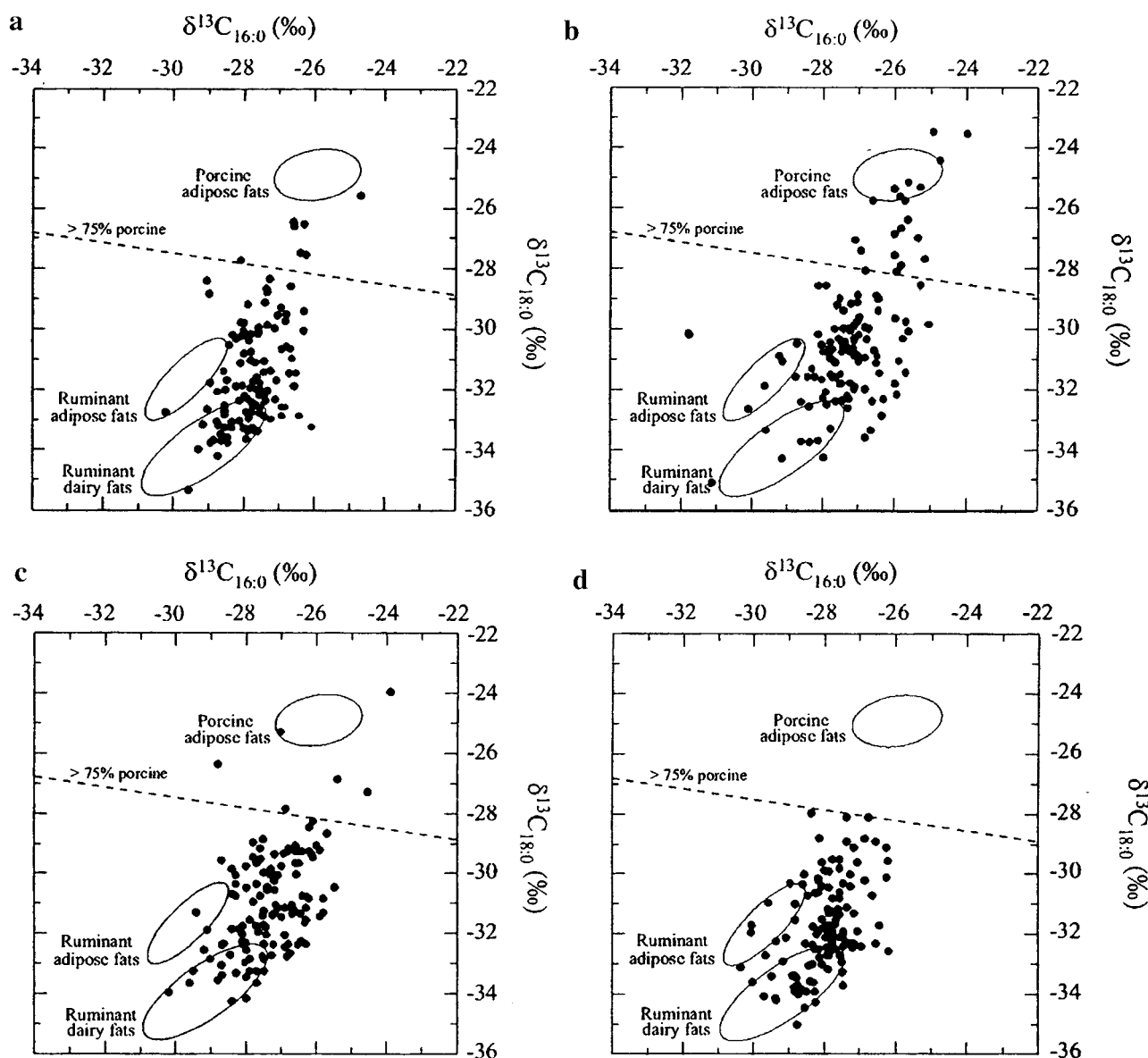


FIGURE 20. Plot showing the $\delta^{13}\text{C}$ values obtained from several British sites: (a) non-Grooved Ware neolithic sites, (b) Grooved Ware; (c) Bronze Age, and (d) Iron Age. Reproduced from Mukherjee et al. (2007) with permission from Antiquity, copyright 2007.

non-ruminant adipose fats in ancient ceramic vessels has likely been under-estimated due to mixing with other fats. Nevertheless, ruminant lipids still remain the most common animal fats encountered in pottery vessels, even in Grooved Ware (Fig. 21; Dudd & Evershed, 1998; Dudd, Evershed, & Gibson, 1999; Evershed et al., 2002; Copley et al., 2003, 2005a,b,c,d; Spangenberg, 2004; Craig et al., 2005a,b; Spangenberg, Jacomet, & Schibler, 2006; Evershed et al., 2008b; Spangenberg et al., 2008). Ruminant adipose fats were also evidenced in the formulation of a Roman cosmetic together with other organic and inorganic ingredients (Evershed et al., 2004).

Ruminant lipids may arise from two distinct sources: subcutaneous fats and dairy products, the latter being of considerable importance to understand domestication process and economies of Neolithic societies (Sherratt, 1983; Bogucki,

1986; Vigne & Helmer, 2007; Evershed et al., 2008b). The exact timing of dairying has been long debated since the introduction of the “Secondary Products Revolution” theory in the 1980s by Sherratt (Sherratt, 1981, 1983). In this scenario, milk would have been exploited only 2,000–4,000 years after the beginning of domestication of cattle, sheep and goats, during the 4th millennium BC in the Near East and the 3rd millennium BC in Europe. This hypothesis relied on several arguments that are beyond the scope of this review but have been recently summarized by Vigne and Helmer (2007). An alternative theory supports an earlier use of dairy products rapidly after or even during the process of domestication (Bogucki, 1982, 1984; Vigne & Helmer, 2007). Whether dairying occurred concomitantly with emergence of animal domestication or a few millennia afterwards is still discussed. Before the development of biomolecular

TABLE 6. Grooved Ware British Neolithic sites in which the results obtained on lipid residues in ceramic vessels were compared with those issued from faunal assemblages

	Durrington Walls		West Kennet		Wyke Down		Skara Brae	
	Bones, %	Animal fats, %	Bones, %	Animal fats, %	Bones, %	Animal fats, %	Bones, %	Animal fats, %
Pigs	63	38	73	67	15	33	0.4	3
Ruminants	28		22		82		97	94
Others	9		4		3		3	

The first line corresponds to the name of the sites. The columns “Animal fats, %” indicate the percentage of pottery containers that contained predominantly non-ruminant carcass fats. The columns “Bones, %” present the percentage calculated on the number of bones, except for the site of Durrington Walls for which it is the Minimum Number of Individuals (MNI). Modified from Mukherjee, Gibson, and Evershed (2008).

and isotopic methods, assumptions relating to dairy product exploitation relied on pictorial records (milking scenes), faunal remains and specific items supposed to be dedicated to dairy products processing, such as perforated vessels. The possibility of extracting lipids from ancient ceramic vessels and distinguishing animal fats using a combination of molecular and isotopic criteria opened up new avenues to investigate the chronology of dairying in Europe and the Near East (Copley et al., 2003, 2005a,b,c,d; Craig et al., 2005a,b; Evershed et al., 2008b).

Up to now a wide range of pottery vessels derived from Europe and the Near East and dating from the Neolithic period to the Iron Age have been analyzed. The most recent results obtained show the story of dairying dates to the beginning of Neolithic in the Near East (Evershed et al., 2008b). The most ancient dairy products were attested in northwestern Anatolia at sites dated from the seventh millennium BC in approximately 10% of the vessels investigated at six sites of this region (Evershed et al., 2008b). Additionally, cattle bones were present in higher abundances than in faunal assemblages of sites from other areas, indicating a positive correlation between cattle husbandry and dairying (Evershed et al., 2008b). Dairy lipids were also identified in sites of other regions of Central Europe and Near East but to a lesser extent (Craig et al., 2005b; Evershed et al., 2008b), providing evidence for regional variation (Evershed et al., 2008b). It thus appears that dairying was an important

component of Neolithic economies at least as early as the emergence of pottery vessel production.

For more recent periods including the late Neolithic, Bronze Age and Iron Age, dairy products have been identified at sites in Switzerland and Germany (Spangenberg, 2004; Spangenberg, Jacomet, & Schibler, 2006; Spangenberg et al., 2008), France (Regert et al., 1999, 2001b, 2003a; Mirabaud, Rolando, & Regert, 2007; Regert, 2007a,b) and United Kingdom (Copley et al., 2005a,b,c,d; Craig et al., 2005a,b). The most extensive studies concern ceramic vessels from United Kingdom; these studies showed that “dairying was an integral component of agricultural practices, suggesting that it was introduced, as a ‘package,’ to Britain from the Continent along with other farming methods” (Copley et al., 2005d).

The early use of milk raises the question of its relationship to lactose intolerance in humans (Copley et al., 2005d; Evershed et al., 2008b). Lactose, that is the main carbohydrate of milk, can be digested, that is, broken down to monosaccharides, by the lactase enzyme. While this enzyme is present in all of us during infancy, some modern populations present lactase deficiency during adulthood and cannot digest milk sugars. This is the case in Chinese and South-eastern Asian people for example (Copley et al., 2005d). This lactase deficiency was sometimes used to explain that milk could not have been exploited at the very beginnings of the Neolithic period when people were unable to transform milk into derivative products such as butter, cheese,

TABLE 7. Relation between the site status and the incidence of pig fats in ceramic vessels at Grooved Ware Neolithic British sites

	Non-domestic / ceremonial site	Domestic site
General characteristics of the site	"Sites with henges or enclosures and little evidence of domestic occupation"	Sites with pits, middens and house structures as predominant features
Incidence of porcine fats	42 extracts submitted to isotopic analysis Incidence of pig fats in 40 % of cases	86 extracts submitted to isotopic analysis Incidence of pig fats in 3 % of cases

Modified from Mukherjee, Gibson, and Evershed (2008).

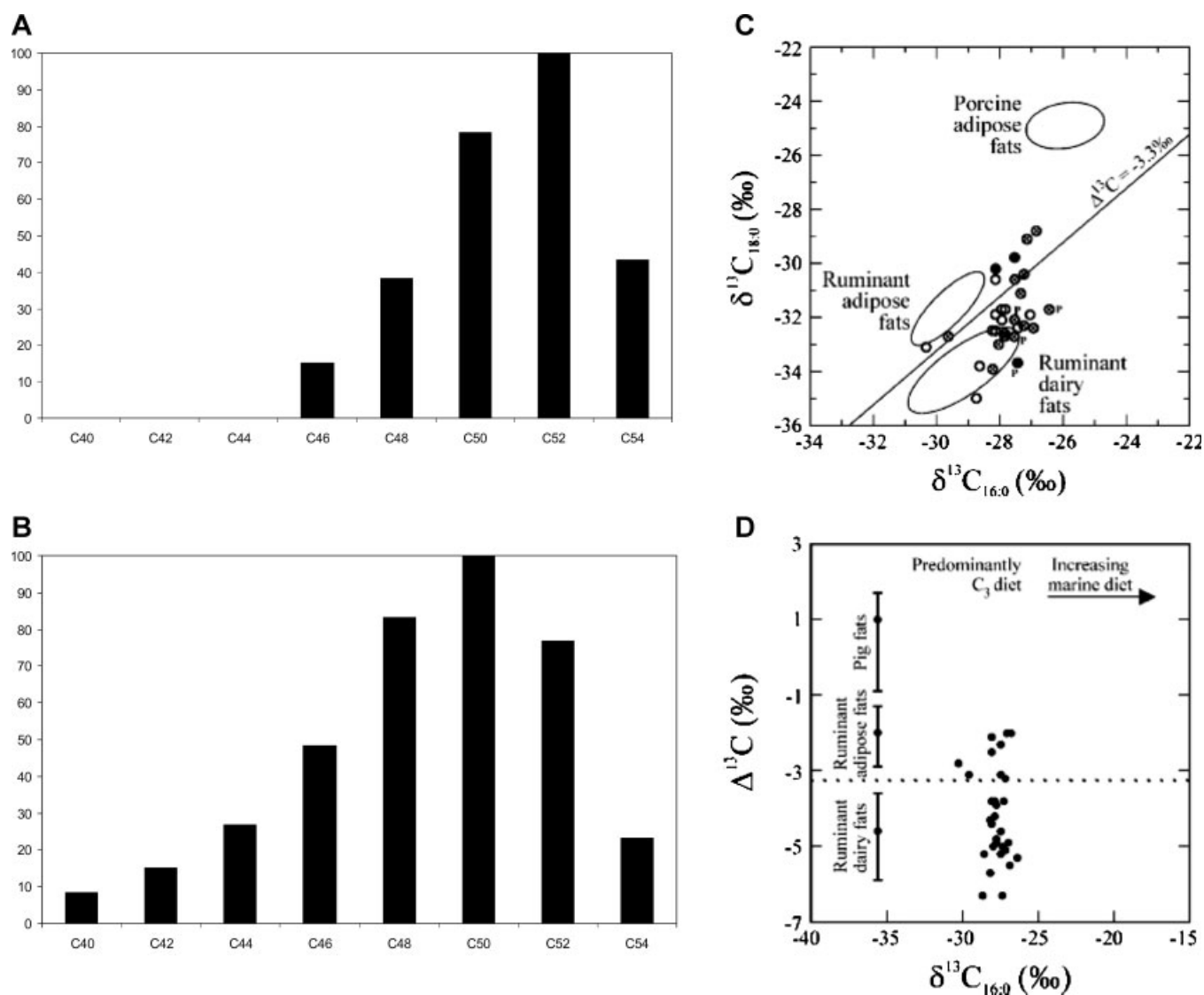


FIGURE 21. Molecular and isotopic criteria used for determining the presence of dairy products in ancient ceramic vessels. These data concern the British Iron Age site of Danebury Hillfort. **A:** Narrow triacylglycerol distribution corresponding to an unknown animal fat. **B:** Wide distribution of triacylglycerols typical of degraded dairy fat. **C:** $\delta^{13}C$ values obtained on samples from Danebury Hillfort vessels, and **D:** $\Delta^{13}C$ values obtained on the same vessels. From Copley et al. (2005c). With permission from Elsevier, copyright 2005.

and yogurt, that are more easily digested than milk. However, experiments performed on replica vessels indicated that milk is much faster degraded than butter (Table 8), probably because of the presence of proteins and carbohydrates that are excellent nutrients for soil microorganisms (Copley et al., 2005a). In contrast, butter is composed of pure lipids and thus does not represent a suitable substrate for microbial growth (Copley et al., 2005a). Based on these results, the authors concluded that the accumulation of dairy fats in several ancient ceramic vessels may derive from “the processing of large amount of butter fats” (Copley et al., 2005a). Furthermore, it should be noted that dairy products are sometimes identified in ceramic vessels which bear sooting traces, indicating that food preparations were cooked in the vessels by heating. Whether dairy substances were mixed

with other substances or not, this provides evidence for the processing of milk products for various purposes, including sterilization, production of sour milk, curds and whey, butter or cheese (Craig et al., 2005a). Together these findings imply that lactase deficiency probably did not prevent the exploitation of dairy resources if milk was transformed into dairy derivatives (Evershed et al., 2008b).

The last question to be addressed in relation with the identification of dairy fats in pottery vessels concerns the purpose for which milk or its derivatives were used. Although these products have high food value, they may also have been used for other properties. Particularly, milk is known to be a very efficient sealant that decreases the porosity of ceramic vessels (Rice, 1987). Nevertheless, this hypothesis may be rejected

for several reasons (Copley et al., 2005c; Craig et al., 2005a,b): (i) most of the studies have shown that animal fats accumulate near the rim; (ii) lipids are generally highly concentrated in the inner part of the pottery than near the outer surface; (iii) high degree of preservation of dairy fats tend to indicate that dairy derivatives, rather than milk, were processed in the vessels. Indeed, if milk had been used as a sealant, lipids would probably have been much less well-preserved, and when preserved, they would have been uniformly distributed across the walls of the vessels.

It may thus be assumed that dairy products encountered in ceramic vessels provide evidence for the processing and/or consumption of food preparations. Surprisingly, no correlation could be established between the shape of a vessel and its dairy fat content but it was observed that dairy fats were present in smaller pots or in vessels with smaller rim diameter than those containing ruminant adipose lipids (Copley et al., 2005a,b).

Viewed together, these findings have significantly modified our knowledge of the history and the chronological scale of dairying. Indeed, instead of a simple model beginning by animal domestication and followed a few millennia later by dairy exploitation, it now appears that dairying was an integral component of the economy of Early Neolithic societies, even though regional variations may be dependent on environmental conditions and cultural choices (Evershed et al., 2008b). Faunal assemblages, that give evidence for dairy herds of sheep and goats as early as the early 8th millennium BC in the Near East support this hypothesis and some authors even think that dairying could have partly driven the domestication process (Vigne & Helmer, 2007).

3. Horse Fats

Geochemical characteristics of mare's milk versus horse carcass fats have been recently established to address specific archeological questions related to early horse domestication processes (Outram et al., 2009). By using three complementary and independent approaches, an interdisciplinary team demonstrated that horses were first domesticated in the Kazakh steppe in the mid-fourth millennium B.C., within the Botai culture (Outram et al., 2009). This assertion relied on (i) the use of metrical analyses, (ii) the study of pathological feature of faunal assemblages, and (iii) organic residue analysis of ceramic vessels. Because this review is centered on methods of mass spectrometry, I will only present the results obtained on organic residues preserved in potsherds.

Before the analysis of archeological samples, $\delta^{13}\text{C}$ values were acquired on modern equine adipose and milk fats from Kazakh animals fed on the natural steppe vegetation (Outram et al., 2009). The comparison of the data obtained on equine species with those already available for other animals showed that equine fats all plot in the same area, which is distinct from that of other animals (Fig. 22A, Outram et al., 2009). Nevertheless, $\delta^{13}\text{C}$ values are not efficient to discriminate mare's milk from horse carcass fats. It was thus necessary to look for another proxy to overcome this problem. Compound-specific deuterium isotope (δD) analysis was then carried out to discriminate milk and subcutaneous horse fats, and it was shown that the δD values obtained for these two types of horse products were distributed in two distinct clusters (Fig. 22B, Outram et al., 2009). The difference of δD values between milk and carcass fat is related to the variation of δD values of summer and winter precipitation that differ by $>100\%$ in intercontinental regions such as the Kazakh steppe (Rozanski, Araguas-Araguas, & Gonfiantini, 1993). Adipose fats integrate water and dietary δD signal of all year (Hobson, Atwell, & Wassenaar, 1999), while mares only produce milk in summer to feed their foal, which explains the difference of δD signals between equine milk and carcass fats (Outram et al., 2009). The determination of $\delta^{13}\text{C}$ and δD values was then performed on palmitic and stearic acids extracted from Botai ceramic vessels. The $\delta^{13}\text{C}$ values were consistent with the predominance of horse fats (Fig. 22C, Outram et al., 2009). The δD values exhibit two distinct clusters (Fig. 22D, Outram et al., 2009), which indicates that two types of equine products were processed in Botai cooking vessels and gives evidence for the presence of mare's milk in five of the vessels investigated. The higher δD values obtained on archeological samples attributed to mare's milk compared to those of modern reference milk fats are explained by climate conditions that were more arid during prehistory than now (Kremenetski, Tarasov, & Cherkinsky, 1997; Tarasov, Jolly, & Kaplan, 1997).

This first promising study on horse products provided new proxy to study animal fats. Furthermore the identification of mare's milk demonstrated that at least some of the horses of the Kazakh steppe were domesticated as early as 3500 B.C. (Outram et al., 2009).

4. Aquatic Resources

The first works dealing with marine resources preserved in the archeological record were performed in the 1970s and 1980s (Morgan et al., 1984; Patrick, de Koning, & Smith, 1985;

TABLE 8. Table showing the differences observed in lipid degradation depending on their nature (milk or butter)

	Milk	Butter
Fat content at day 0	1 mg / g	70 mg / g
Fat content after 25 days of degradation experiment	TAGs completely degraded, leaving only traces of fatty acids	still contained high concentration of TAGs that had not altered their distribution significantly
Fat content after 1 year of degradation experiment	characteristic loss of lower carbon number TAGs	about 20 % of the original fat present

Data extracted from Copley et al. (2005a).

Morgan, Edwards, & Pepper, 1992). These studies relied on the identification of a single class of biomarkers (fatty acids) by chromatographic methods and allowed to draw the first hypothesis about the exploitation of marine resources at different coastal archeological sites.

More recently, the discovery of new degradation markers and the use of $\delta^{13}\text{C}$ values supplied interesting results for different archeological sites from southern hemisphere (Copley

et al., 2004; Hansel et al., 2004; Evershed et al., 2008a) and from northern Europe (Craig et al., 2007b).

To assess the preservation of marine resources, series of ceramic potsherds were selected at Brazilian and South African coastal sites, in which the exploitation of marine resources was attested by abundances of faunal remains, such as fish bones, mollusca shells or marine bone elements (Copley et al., 2004; Hansel et al., 2004). One site, dated to ca. 10th century AD, was

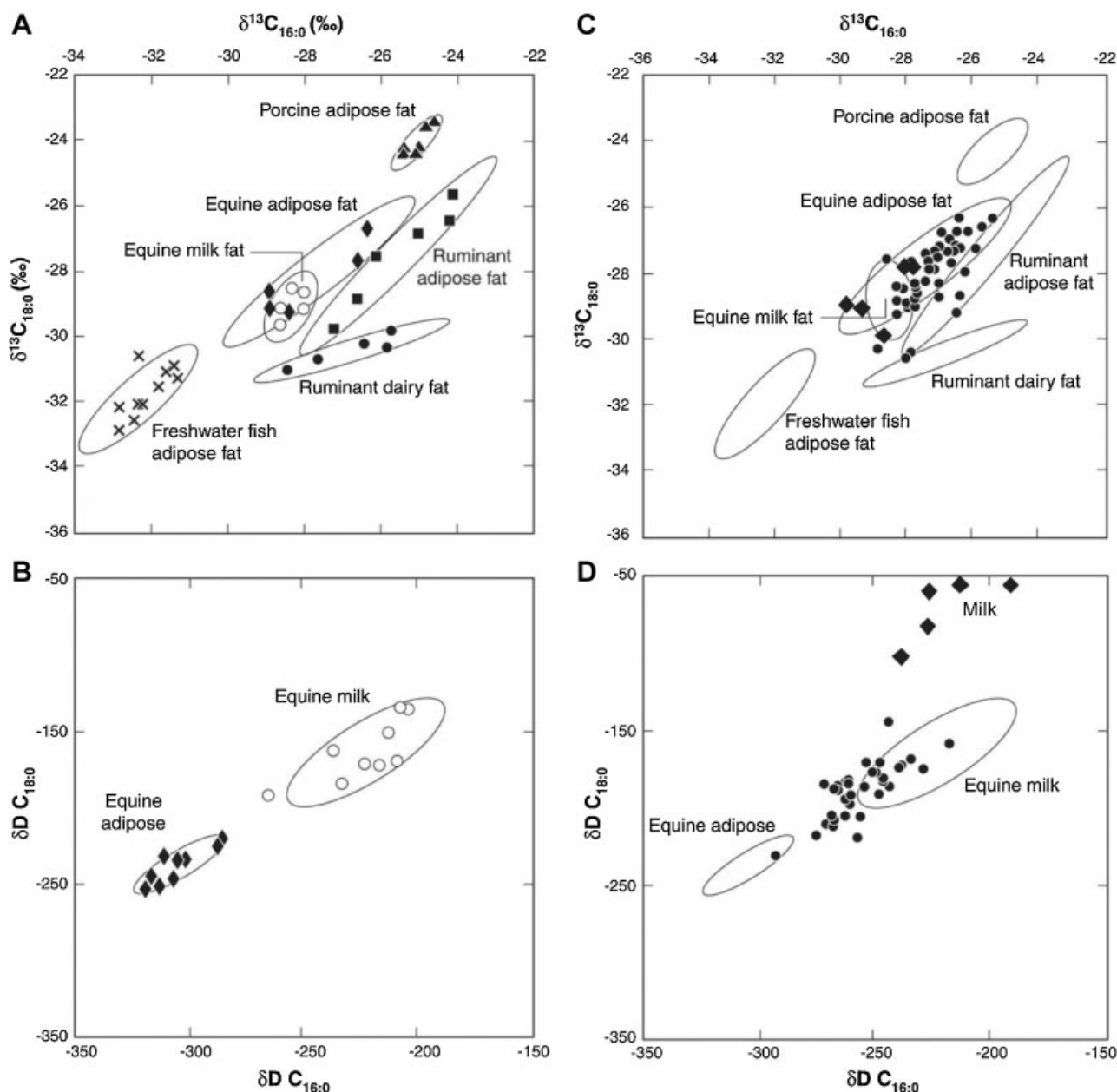


FIGURE 22. Plots of the $\delta^{13}\text{C}$ and δD values obtained on modern reference animal fats (A and B) and archeological samples from Kazakhstan (C and D). Reproduced from Outram et al. (2009) with permission from The American Association for the Advancement of Science, copyright 2009. (<http://www.sciencemag.org/cgi/content/abstract/323/5919/1332>). [Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, this material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher.]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

located on Santa Catarina Island in Brazil; the other one being situated in South Africa, ca. 150 km north of Cape Town. These latter contained two types of ceramic vessels, spouted wares that date to the mid- and late millennium AD, and lugged wares (second millennium). Fourteen of the vessels investigated at the Brazilian site yielded uncommon assemblages of biomolecular markers (long chain fatty acids, isoprenoid fatty acids, including phytanic acid, and ω -(*o*-alkylphenyl)alkanoic acids with 16–20 carbon atoms), that provided evidence for the processing of marine-derived products in pottery containers (Hansel et al., 2004). Following the preliminary work carried out by Patrick and collaborators at the archeological site from Kasteelberg D East in South Africa on organic residues that adhered to fragments of pottery (Patrick, de Koning, & Smith, 1985), Copley et al. (2004) sampled 20 potsherds probably from spouted wares in archeological deposits corresponding to occupations dated from the 8th to the 11th century AD. The combination of $\delta^{13}\text{C}$ values and detailed compositional analysis by GC–MS suggested that these vessels contained marine resources (Copley et al., 2004). These data allowed to revisit previous hypotheses related to the use of spouted wares that were first supposed to be associated with dairying.

Concerning northern Europe, a series of pottery samples originating from several sites in Denmark and Belgium were investigated (Craig et al., 2007b) to understand the evolution of aquatic resources exploitation during the transition Mesolithic–Neolithic, a crucial transition in the history of humanity (Evershed, 2007; Tresset & Vigne, 2007). The association of biomarkers from aquatic species with fatty acids enriched in ^{13}C that plot near the reference marine fish clearly indicates that

during the Mesolithic period at the site of Tybrind Vig in Denmark, marine resources were processed in ceramic vessels (Fig. 23; Craig et al., 2007b). At other sites, lipid biomarkers were also encountered but the $\delta^{13}\text{C}$ values of $\text{C}_{16:0}$ and $\text{C}_{18:0}$ appeared to be lighter than in Mesolithic vessels (Fig. 23; Craig et al., 2007b). These data most likely indicate mixtures of fats from terrestrial and aquatic animals. As in case of dairying, these new findings are modifying our ideas of the transition between Mesolithic and Neolithic periods. Indeed, in the common scenario, it is supposed that marine resources were replaced, at least at a significant level, by pastoral and agricultural products during the 4th millennium BC in northern Europe (Craig et al., 2007b). Therefore, the identification of aquatic resources in Neolithic ceramic vessels tends to show that “aquatic resources were still consumed after the transition to agriculture, and thus that Early Neolithic pottery had a broader range of use” than previously expected (Craig et al., 2007b).

These recent results, obtained on sites of varied periods and regions, demonstrate the importance of enlarging the range of natural substances studied in ceramic vessels. Investigations on aquatic resources indeed helped not only to assess pottery function as shown for a coastal South African site but also to address key archeological questions in relation with the neolithization processes.

G. New Developments for Radiocarbon Dating of Individual Fatty Acids

The possibility of using organic matter from ceramic vessels for ^{14}C dating was first explored during the 1980s and 1990s (Evin,

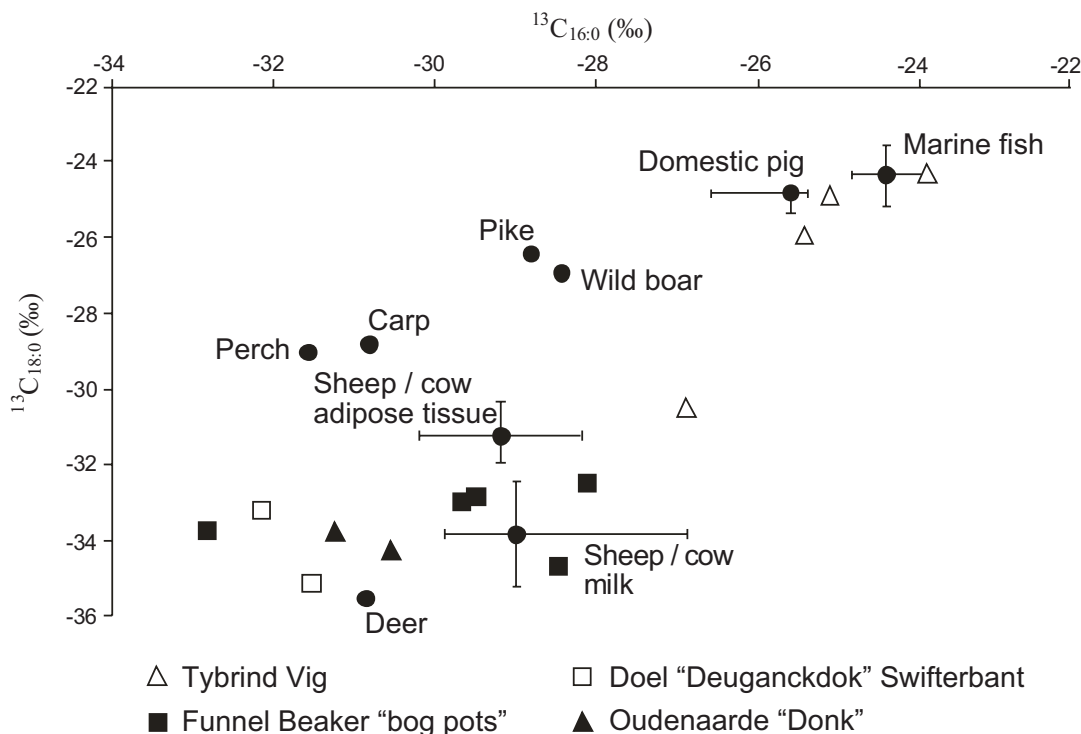


FIGURE 23. Plot of the $\delta^{13}\text{C}$ values obtained on archeological samples from northern European sites (see sites indicated at the bottom of the figure) and reference data indicated with black circle, some of them being extracted from Dudd et al. (1999) and Craig et al. (2007b). Reproduced from Craig et al. (2007b) with permission from Wiley-Blackwell, copyright 2007.

Gabasion, & Lefebvre, 1989; Hedges, Tiemei, & Housley, 1992; Kolic, 1995). Carbon contained in pottery vessels is derived from various sources of different ages including, residual geological organic material naturally present in clay, organic temper added to clay, condensed soot formed during firing, natural substances related to vessel use, and post-burial organic contamination (Stott et al., 2001). A variety of methods was used to discriminate these different origins of carbon but none of them was effective to provide accurate ^{14}C dates (Stott et al., 2001).

Lipids extracted from ceramic vessels represent new organic archives susceptible to be radiocarbon dated (Stott et al., 2001, 2003; Berstan et al., 2008). The dating of lipid individual compounds isolated by preparative capillary gas chromatography (PCGC) from sedimentary matrices by high precision accelerator mass spectrometry (AMS) demonstrated the feasibility of compound-specific ^{14}C dating approach when sufficient concentrations of matter are available (Eglinton et al., 1996).

Measuring ^{14}C content directly on lipid individual compounds previously extracted from pottery vessels has several advantages: (i) this allows to obtain ^{14}C ages for carbon that originates from the products processed in the vessels, (ii) the dates obtained are unambiguously related to the event of pottery use (Berstan et al., 2008), and (iii) lipids are known for their fast turnover rates, which guarantees the obtention of ages close the date of the death of the organisms (Stott et al., 2003; Berstan et al., 2008).

The most challenging tasks were to isolate individual components from ceramic containers in sufficient amount, and to obtain acceptable controls and blanks in the AMS dating procedure (Stott et al., 2003). Because palmitic and stearic acids are the most abundant components preserved in archeological pottery, it was decided to focus on these molecular markers.

The PCGC protocol was first developed and the reproducibility of retention times of the fatty acids during repetitive trapping sequences typically of $>100\text{ h}$ and $>100\text{ GC}$ sequences was established (Stott et al., 2003). Appropriate analytical controls showed that the contribution of chromatographic stationary phase ("column bleed") and the percentage of modern ^{14}C in solvents could be neglected (Stott et al., 2003). Correction factors were introduced to account for the effect of the addition of a methyl carbon through derivatization and for isotopic fractionation effects (Stott et al., 2003).

The protocol was then tested on a range of ceramic vessels selected for their high content in lipids ($>300\ \mu\text{g g}^{-1}$) and their well dated archeological context. ^{14}C ages obtained were found to be in good agreement with the dates expected (Stott et al., 2001, 2003). Differences between ^{14}C content of palmitic and stearic acids were nevertheless observed, the former providing slightly more modern date; although difficult to explain, this difference might arise from migration of palmitic acid in soil due to its higher abundance and mobility compared to stearic acid (Stott et al., 2001, 2003). More recent investigations on Early Neolithic artifacts from Great Britain provided dates for both $\text{C}_{16:0}$ and $\text{C}_{18:0}$ in total agreement with ages obtained by dendrochronology (Berstan et al., 2008).

The combination of specific sample treatments to isolate and concentrate fatty acids and of high precision AMS analysis thus appears as a promising method to establish direct dating of the use of ancient pottery vessels and illustrates a new field of application of mass spectrometry.

IV. SOFT IONIZATION METHODS

Soft ionization methods are of great interest to elucidate the molecular composition of natural substances in the field of food, agriculture and adulteration (Smyth, 1999; Futrell, 2000; Siciliano et al., 2000; Cech & Enke, 2001). These methods are of particular value to study animal fats that are composed of high molecular weight compounds such as DAGs, and TAGs (Gresti et al., 1993; Byrdwell & Emken, 1995; Laakso, 1996; Mottram & Evershed, 1996; Mu, Sillen, & Hoy, 2000; Byrdwell, 2001; Han & Gross, 2001; Hvattum, 2001; Andrikopoulos, 2002; Laakso, 2002; Burchgraber, Ulberth, & Anklam, 2004; Momchilova et al., 2004; McAnoy, Wu, & Murphy, 2005; Leskinen, Suomela, & Kallio, 2007; Molquentin, 2007; Picariello, Sacchi, & Addeo, 2007). Nevertheless, few reports have focused on the use of electrospray (ESI) and atmospheric pressure chemical ionization (APCI) to unravel the molecular compositions of fatty substances of animal origin preserved in the archeological record (Kimpe, Jacobs, & Maelkens, 2001, 2002; Evershed et al., 2002; Kimpe et al., 2004; Mirabaud, 2007; Mirabaud, Rolando, & Regert, 2007; Romanus et al., 2007). This may be explained by several reasons. First of all, the primary components of animal fats, triacylglycerols, are sensitive to degradation processes and are not always preserved in archeological remains. Secondly, the complexity of the original fats combined with the extent of alteration and possible mixing with other commodities in ancient pottery vessels produces very complex molecular mixtures that require extensive purification and pre-fractionation before ESI- or APCI-MS analysis (Mottram & Evershed, 2001; Dugo et al., 2005). Finally, the ion yield of triacylglycerol species is often very low and a supplementary step of preparation, such as cation addition, is necessary (Hsu & Turk, 1999; Gamero-Castaño & De la Mora, 2000; Charles et al., 2001; Afonso et al., 2005; Mirabaud, Rolando, & Regert, 2007).

Despite these difficulties, recent investigations have shown that structural information can be obtained from TAGs by optimizing sample preparation and analytical conditions (Kimpe, Jacobs, & Maelkens, 2001; Mirabaud, Rolando, & Regert, 2007). Long-chain esters of beeswax have also been studied by such strategies but this is beyond the scope of this review and will thus not be discussed further (Garnier et al., 2002; Kimpe, Jacobs, & Waelkens, 2002). It should be noted that in all cases, APCI- or ESI-MS analyses are always preceded by GC-MS analysis necessary to assess the quantity of lipids still preserved and their degree of preservation as the basis to select samples that still contain amounts of DAGs and TAGs.

This section focuses only on articles dedicated to the study of TAGs in archeological samples to show how MS soft ionization techniques have been applied to discriminate complex and degraded fatty materials.

A. Gaining Structural Information from TAGs in Archeological Fats

Fresh animal fats contain $>95\%$ of TAGs (Gurr & Harwood, 1991) that are composed of glycerol esterified with three fatty acids. To determine the exact structure of TAGs, the following information is required:

- (i) the position of each fatty acid at position *sn*-1, *sn*-2, or *sn*-3 on the glycerol backbone;

- (ii) the carbon chain length of each fatty acid involved in the TAGs molecular species;
- (iii) the number and positions of unsaturations in the fatty acyl moieties;
- (iv) the *cis* or *trans* configurations of the double bonds in the fatty acyl moieties.

Depending on their nature (milk or adipose fat) and origin (cattle, sheep, or goat, etc.), the TAGs may display different structural characteristics according to one or more of the above criteria.

Prior to 2000, all investigations of archeological lipids relied on the use of GC or HT GC, usually coupled with mass spectrometry, using electron ionization. Although this remains a valuable method to distinguish animal fats from other products such as tars, resins or waxes, it is ineffective in providing comprehensive structural information concerning the position of each fatty acid on the glycerol backbone. EI-MS spectra of TAGs, dominated by a single fragment resulting from the loss of a fatty acyl radical RCOO[•] (Fig. 7), only allow to draw conclusions concerning distributions of the TAGs in terms of their acyl carbon number and degree of unsaturation (Figs. 5 and 8).

However, the use of ESI- and APCI-MS allow hitherto unobtainable insights into the structures of TAGs preserved in degraded fats of archeological origin (Kimpe, Jacobs, & Waelkens, 2002; Mirabaud, Rolando, & Regert, 2007; Romanus et al., 2007).

B. Triacylglycerol Investigation Using LC-APCI-MS Methodologies

The application of a soft ionization method in the study of TAGs in the field of archeology appeared in 2001 and employed LC-APCI-MS (Kimpe, Jacobs, & Waelkens, 2001). The approach adopted was based on earlier studies of modern fats and oils with the same methodology (Mottram, Woodbury, & Evershed, 1997; Mu, Sillen, & Hoy, 2000). Although initially investigating olive oil residues, it was further exploited to detect animal fats to specify their origin (Kimpe, Jacobs, & Waelkens, 2002; Kimpe et al., 2004).

Before the analysis of archeological samples, standard mixtures were analyzed by LC-APCI-MS with a reversed-phase column (Supelcosil LC-18 type, 25 cm × 4.6 mm, Supelco). To optimize resolution and sensitivity, several solvents were used for the LC separation, including propionitrile, a gradient of methanol/isopropanol and a gradient of methanol/acetonitrile (Kimpe, Jacobs, & Waelkens, 2001). The sensitivity was maximized by using the gradient methanol/isopropanol while the other gradient, corresponding to a combination of methanol with acetonitrile, was shown to be required to improve the resolution of the peaks that correspond to the different TAG species investigated. Due to the low concentrations of these species in archeological samples, it was decided to optimize analyses for sensitivity, rather than resolution.

Analyses were performed with a Finnigan LCQ DECA MS with a vaporizer temperature of 500°C, a capillary temperature of 225°C and a corona current of 5 μA. Mass spectra were acquired in the range *m/z* 422–1000 with a scan time of 0.2 s (Kimpe, Jacobs, & Waelkens, 2001). The interpretation of TAGs mass spectra relied on the first investigations performed on commercial compounds by APCI-MS (Mottram & Evershed, 1996).

Mass spectra of TAG species are characterized by intense [M + H]⁺ and DAG fragment ions [MH-RCOOH]⁺ ions (Mottram & Evershed, 1996; Mottram, Woodbury, & Evershed, 1997; Mu, Sillen, & Hoy, 2000; Kimpe, Jacobs, & Waelkens, 2001; Laakso, 2002). Interestingly, the fatty acyl moieties in the *sn*-1 and *sn*-3 positions give rise to more abundant [M-RCOO]⁺ ions than those that are in the *sn*-2 position; the loss of a [M-RCOO]⁺ ion in the *sn*-2 position is indeed energetically less favorable than that in the *sn*-1 or *sn*-3 position (Mottram & Evershed, 1996). Thus, by determining the ratio of the [M-RCOO]⁺ fragments, it is possible to study the regioisomeric structure of the TAGs. For instance it has been shown that palmitic acid is preferentially located in the 2-position in non-ruminant fats compared to ruminant lipids; P:S ratio (for palmitic:stearic acids ratio) in *sn*-2 position is of ≈60:40 in ruminant adipose fats and of ≈95:5 in non-ruminant adipose fats (Evershed et al., 2002).

Following these investigations on commercial standards and modern fats, series of archeological samples from ancient pottery vessels of the site of Sagalassos (Turkey), dated back from the mid-Imperial to the early Byzantine period (6th century AD to 7th century AD), were studied with a mobile phase consisting of a gradient of methanol and isopropanol with a flow-rate of 0.8 mL min⁻¹ (Kimpe, Jacobs, & Waelkens, 2001). Among the substances identified by this method, a vegetable oil and beeswax have to be mentioned (Kimpe, Jacobs, & Waelkens, 2001, 2002). More in the scope of this review, a series of TAGs typical of animal fats were determined (Kimpe, Jacobs, & Waelkens, 2002). The high proportion of saturated TAGs versus unsaturated components led the authors to conclude that these biomarkers derived predominantly from animal fats (Fig. 24, Kimpe, Jacobs, & Waelkens, 2002). When possible, by determining the ratio between [M-RCOO]⁺ fragment ions, the position of the fatty acids on the glycerol backbone was determined. Furthermore, the distribution of saturated TAGs, less susceptible to alteration than the unsaturated moieties, was studied in both modern reference fats and archeological samples by using the relative intensity of the protonated molecular ions (Fig. 25; Kimpe, Jacobs, & Waelkens, 2001, 2002; Romanus et al., 2007). For instance, the fact that ovine fats contain high relative abundances of tristearin while porcine lipids are rich in tripalmitin and that the latter are characterized by high proportions of palmitic acid in the *sn*-2 position (Al-Rashood et al., 1996), were used to discriminate archeological samples (Evershed et al., 2002).

C. Distinguishing Animal Fats by Nano-ESI-MS and ESI-MS/MS Device

A recent development has been the elucidation of the structure of TAGs with nano-ESI-MS and ESI-MS/MS. A major advantage of this methodology is its enhanced sensitivity which overcomes one of the major difficulties raised by low abundance of TAGs in complex archeological materials. The approach was successfully applied to a series of modern reference fats and Neolithic archeological samples (Mirabaud, Rolando, & Regert, 2007).

The first step consisted of establishing an efficient way to separate the TAGs from other well-ionized lipids. This was performed by fractionation of the TLE on silica solid-phase extraction (SPE) cartridges (Mirabaud, Rolando, & Regert, 2007). By eluting with *n*-hexane, dichloromethane, and mixtures of dichloromethane and methanol (2:1, v/v and then

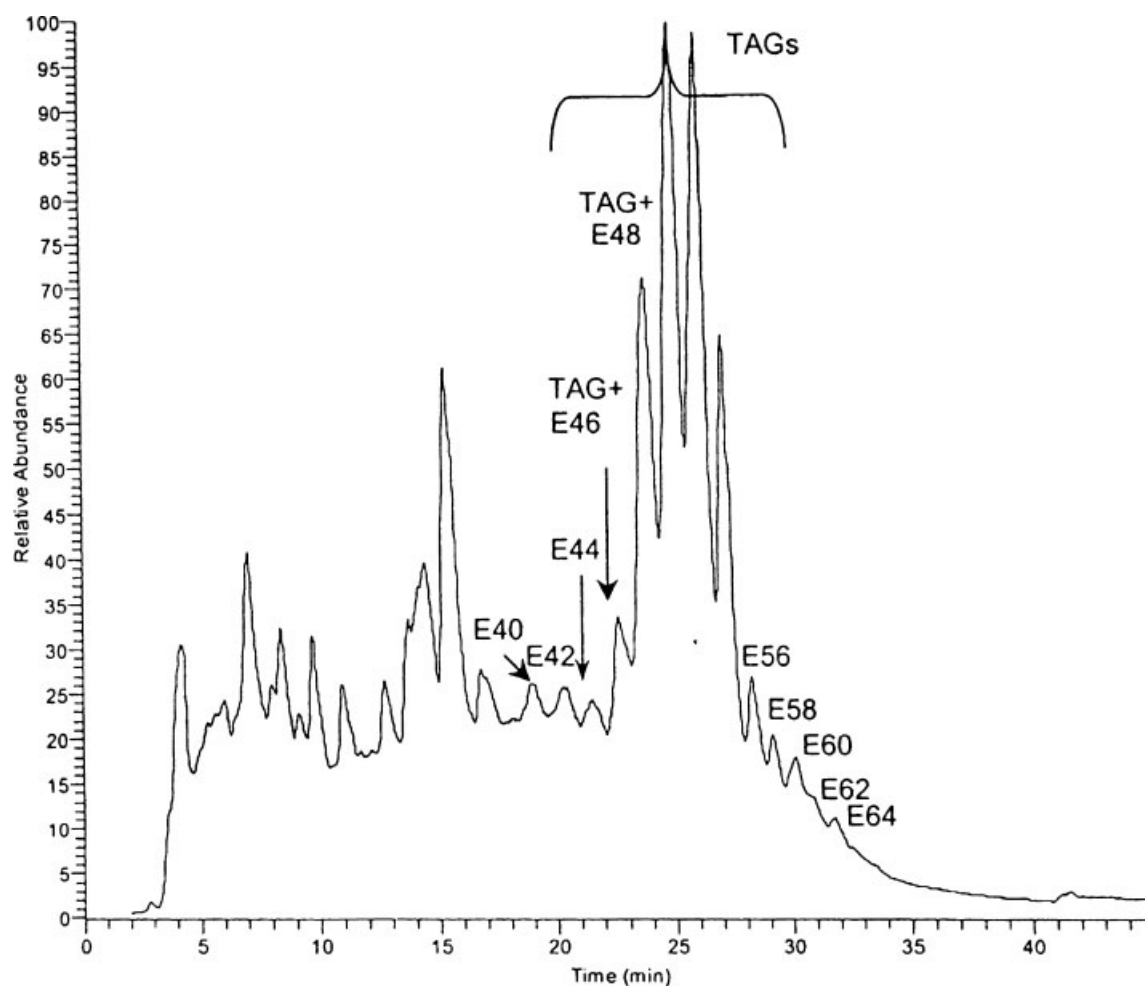


FIGURE 24. HPLC-APCI-MS profile of an archeological sample from the site of Sagalassos (Turkey) showing a mixture of wax monoesters (E_x with x from 40 to 48) and diesters (E_x with x ranging between 56 and 64) and triacylglycerols from animal fats. From Kimpe, Jacobs, and Waelkens (2002). With permission from Elsevier, copyright 2002.

1:1, v/v), the TAGs eluted in the second fraction, fully resolved from more readily ionized fatty acids, which eluted in the last fraction.

To improve the ionization of TAGs, lithium chloride was added as a “doping” agent, resulting in the TAGs being ionized as their lithium adducts $[M + Li]^+$ (Hsu & Turk, 1999). The lithiated solution obtained was analyzed after infusion into the source of the MS with application of high voltage of 800 V in both nano-ESI-MS and nano-ESI-MS/MS modes with an MS equipped with a Q-q-TOF analyzer (Mirabaud, Rolando, & Regert, 2007).

Because no step of liquid chromatography preceded the ESI analysis, the spectra obtained correspond to the sum of the spectra of all the individual TAGs present in the sample investigated. The spectra exhibit ions that arise from the loss of the fatty acyl moieties $[M + Li-RCOOH]^+$ and $[M + Li-RCOOLi]^+$ and acylium ions, RCO^+ , derived from the latter (Fig. 26; Mirabaud, Rolando, & Regert, 2007). Series of ions regularly spaced by 28 a.u. show the distribution of TAGs in the samples. The detection in modern milk samples of TAGs with an acyl carbon

number of 24 for the first time, proves the high sensitivity of the method. A further advantage of lithium salts “doping” is the very high stability of the lithiated adducts, which limits in-source random fragmentation (Hsu & Turk, 1999; Mirabaud, Rolando, & Regert, 2007). Each $[M + Li]^+$ TAG corresponds to a group of TAGs characterized by the same acyl carbon number and thus to different triacylglycerol isomers. To establish criteria to discriminate animal fats, tandem MS experiments were carried out (Fig. 26), allowing graphs that plot the relative intensity of the ion corresponding to the loss of a specific fatty acyl moiety in the MS/MS spectrum against the number of carbon atoms for each series of isomers, to be obtained (Fig. 27; Mirabaud, Rolando, & Regert, 2007). By comparing the data obtained from reference fats to those derived from archeological samples, new criteria were established to differentiate animal fats. Importantly, sheep and cow adipose fats could be distinguished on the basis of the fatty acyl moiety distributions in TAGs with acyl carbon numbers of 44 and 46 (Fig. 27). Additionally, cow and goat milks differed in the relative abundance of $C_{10:0}$ which was consistently higher in goat than in cow milk (Fig. 28). Such results were then used to

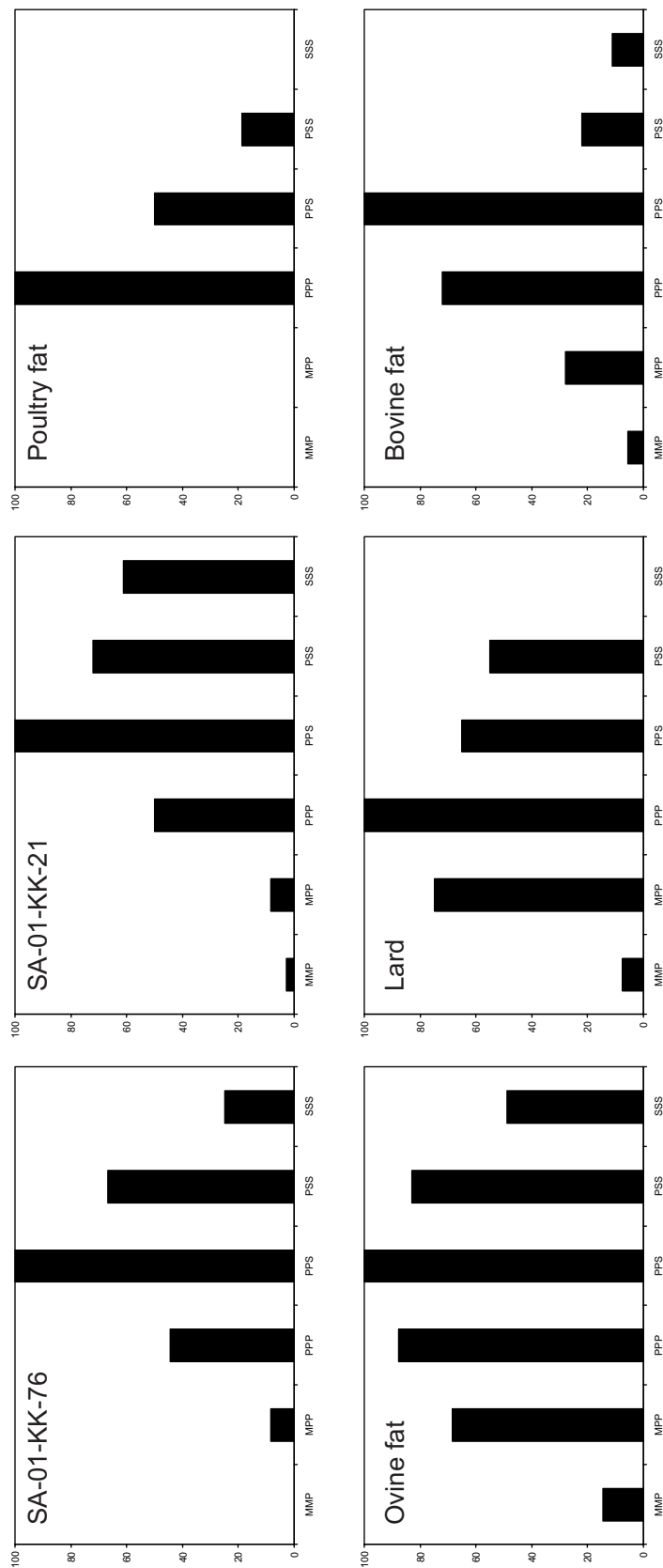


FIGURE 25. Distribution of the main triacylglycerols in modern reference animal fats and archeological samples from the site of Sagalassos. From Kimpe, Jacobs, and Waelkens (2002). With permission from Elsevier, copyright 2002.

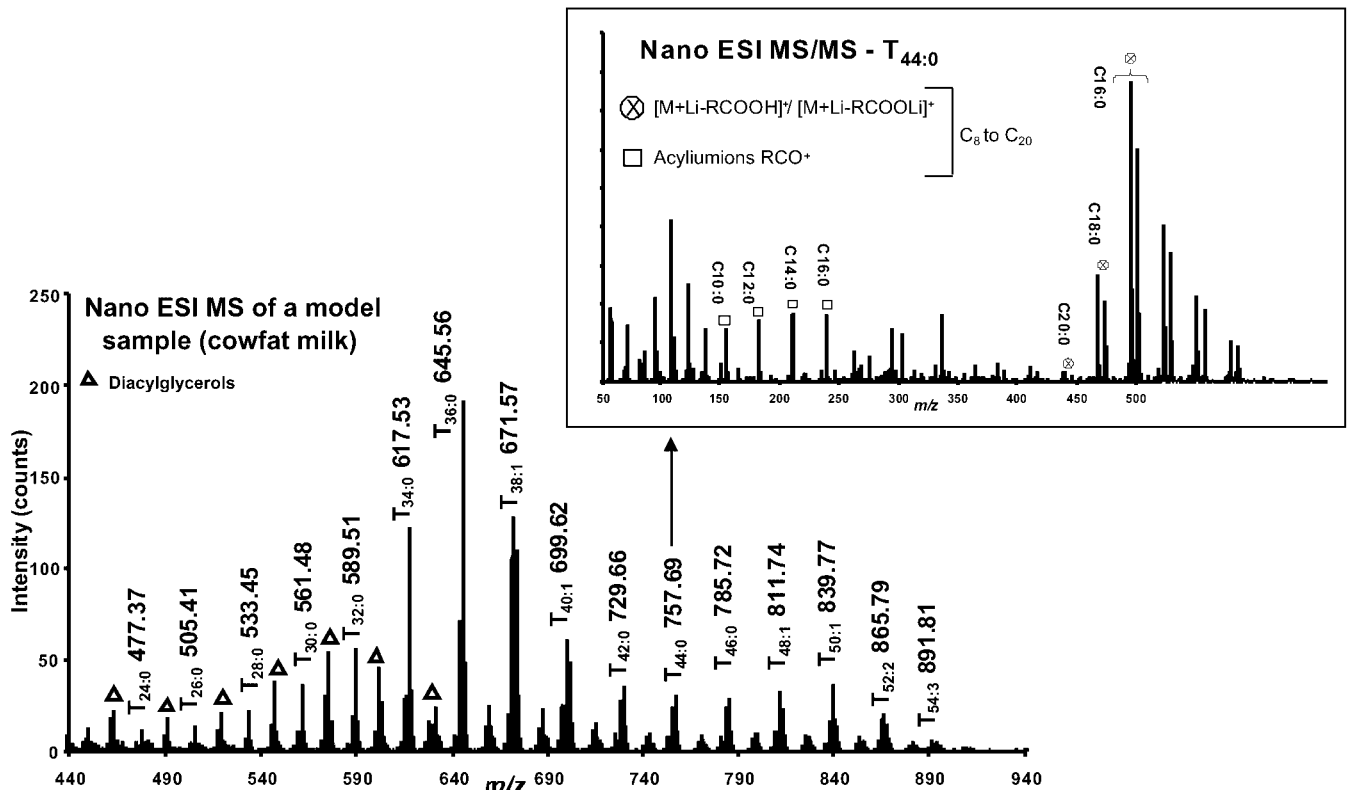


FIGURE 26. Mass spectrum obtained by nano-ESI-MS of a model sample of cow fat milk and mass spectrum obtained by nano-ESI-MS/MS of the triacylglycerol $T_{44:0}$. Adapted from Mirabaud, Rolando, and Regert (2007) and from Regert (2009).

successfully identify animal fats in Neolithic ceramic vessels, as discussed below.

D. Application of TAG Compositions to Distinguish the Origins of Archeological Animal Fats

Using the range of criteria discussed above, it was possible to investigate the possible presence of goat milk, cow milk and

sheep carcass fat in Neolithic pottery from the site of Clairvaux XIV (Mirabaud, 2007; Mirabaud, Rolando, & Regert, 2007), opening new perspectives to study the evolution of dairying and exploitation of domesticated animals over time. Furthermore, the application of LC-APCI-MS to pottery residues from Sagalassos in Turkey confirmed the presence of ruminant animal fat based on the high abundance of palmitic acid and the low abundance of palmitic acid in the *sn*-2 position in the TAGs (Kimpfe, Jacobs, &

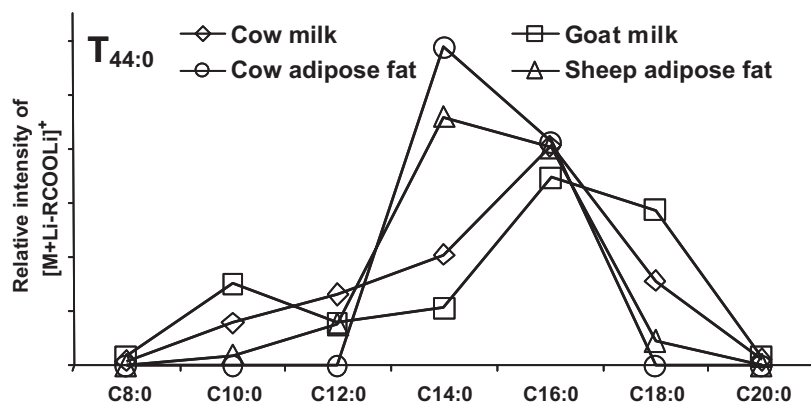


FIGURE 27. Plot obtained by nano-ESI-MS/MS analyses of modern reference animal fats of the relative intensity of RCOOLi losses against the corresponding fatty acid for the groups of triacylglycerols with an acyl carbon number of 44. Reproduced from Mirabaud, Rolando, and Regert, (2007) with permission from American Chemical Society, copyright 2007.

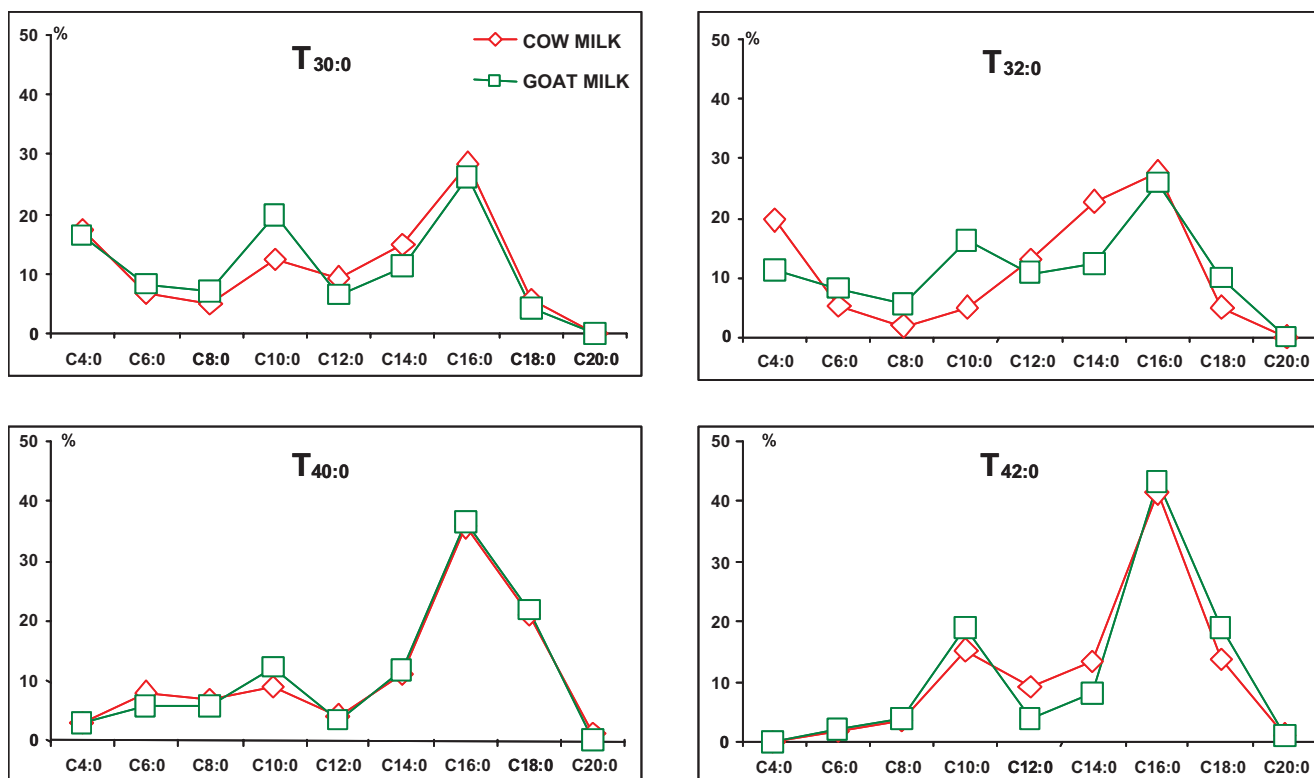


FIGURE 28. Graphs plotting the relative intensity of the fatty acid RCOOLi losses against their CN for T_{26:0}, T_{32:0}, T_{40:0}, and T_{42:0} for cow milk (red) and goat milk (green). From Mirabaud, Rolando, and Regert (2007), supplementary information. Reproduced from Mirabaud, Rolando, & Regert (2007) with permission from American Chemical Society, copyright 2007. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Waelkens, 2002). By combining data obtained by GC–MS and LC–APCI–MS and comparing the content of pottery vessels with their shape, it was possible to begin to draw conclusions concerning the uses of these ancient containers (Kimpe et al., 2004).

Hence, nano-ESI–MS and nano-ESI–MS/MS analyses, as LC–APCI–MS, appear to be a valuable tools to gain structural information on TAGs to assess their origin in archeological ceramic vessels.

V. CONCLUSIONS

Since the 1990s, MS has being increasingly used to investigate molecular composition and isotopic signature of archeological materials. In particular, amorphous organic residues in ceramic vessels have been shown to be mostly derived from degraded animal fats based on the distribution of fatty acids, sterols, MAGs, DAGs, and TAGs. Determining the nature and origin of such lipids was however a great challenge because they reflect a range of complex transformations and mixtures and have undergone series of alteration processes. Nevertheless, the use of the biomarker approach by HT GC–MS combined with carbon isotopic analysis of individual fatty acid via GC–C–IRMS, opened up the possibility to discriminate ruminant from non-ruminant fats and to detect dairy products in pottery vessels dating to Early Neolithic period (Table 2). Aquatic resources

were also highlighted by this combination of MS approaches (Table 2). When molecular information and $\delta^{13}\text{C}$ values of individual fatty acids are ineffective to distinguish different animal substances, such as carcass horse fats and mare’s milk, new proxies have to be established. The determination of δD values is then a new innovative method to go further in the discrimination of certain animal fats (Outram et al., 2009). The determination of ^{14}C ages of individual compounds by accelerator mass spectrometry (AMS) analysis (Stott et al., 2001, 2003; Berstan et al., 2008) is also now emerging, providing a new opportunity to exploit all the informative potential preserved in ancient biomolecular markers.

In addition, the recent application of soft ionization techniques such as atmospheric pressure chemical ionization and electrospray, has been shown to be effective to elucidate the structure of TAGs preserved over archeological timescales (Table 2). For organic residues in which TAGs are preserved in sufficient amounts, analytical protocols were developed to maximize the ion yields to enable the detection of TAGs. In particular, nano-ESI–MS applied to lithiated fractions of TAGs provided mass spectra for intact TAG molecular species in the archeological record, with MS/MS analysis supplying information on the distribution of fatty acyl moieties for each TAG molecular species (Mirabaud, Rolando, & Regert, 2007). These distributions helped to establish further criteria to distinguish ruminant animal fats (dairy products and carcass fats but also cow, sheep and goat products).

The future challenge for the use of MS in archeology is to enlarge the range of biomarkers and stable isotopes investigated. Today, new approaches based on proteomics and study of stable isotopes of nitrogen provide a solution to exploit the proteinaceous signal that might be preserved over archeological timescales (Simpson et al., 1999; Solazzo et al., 2008). LC-MS/MS methods will also be of great interest to investigate new biomarkers preserved in low amount (Guasch-Jané et al., 2004, 2006a,b; Evershed, 2008a).

Last, the combination of different methods including soft ionization techniques, isotope approach, and ^{14}C dating of individual fatty acids, has now to be more widely used to obtain comprehensive information on the various chemical families preserved in archeological remains (Romanus et al., 2007; Berstan et al., 2008).

VI. ABBREVIATIONS

AMS	accelerator mass spectrometry
APCI	atmospheric pressure chemical ionization
DAG	diacylglycerols
DMDS	dimethyl disulfide
ESI	electrospray
GC	gas chromatography
GC-C-IRMS	gas chromatography-combustion-isotope ratio mass spectrometry
GC-MS	gas chromatography-mass spectrometry
HT GC-MS	high temperature gas chromatography-mass spectrometry
MAG	monoacylglycerols
PCGC	preparative capillary gas chromatography
Py GC-MS	pyrolysis gas chromatography-mass spectrometry
TAG	triacylglycerols
TLE	total lipid extract

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