# Archaeological bone lipids as palaeodietary markers

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<td>Date Submitted by the Author:</td>
<td>n/a</td>
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<td>Complete List of Authors:</td>
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## Abstract

**RATIONALE**

Stable isotope analysis of archaeological and fossil bone samples can provide important insights into past environments, ecologies and diets. Previous studies have focused on stable carbon and nitrogen isotopes in bone collagen, or carbon isotopes in bone mineral (bioapatite). Carbon isotope analysis of lipids from archaeological bone has received much less attention, partly due to the lack of suitable methodologies allowing sufficient recovery of compounds for structural and isotopic characterisation. Here we show that lipids can be easily and reliably recovered from archaeological bone using a modified protocol, and that these provide complementary dietary information to other bone components.

**METHODS**

Human and animal bones were obtained from a variety of archaeological contexts. Lipids were sequentially extracted using solvent extraction (dichloromethane/methanol), followed by acidified methanol extraction (methanol/H2SO4). Lipids were analysed by gas chromatography-mass spectrometry (GC/MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS).

**RESULTS**

Our results show that appreciable amounts of endogenous lipid can be recovered from archaeological bone. Importantly, a comparison between compound specific and bulk collagen isotopic data shows that archaeological bone lipids reflect dietary input and can be used to distinguish between marine and terrestrial consumers, as well as between C3 and C4 plant consumers. Furthermore, the presence of essential fatty
acids directly incorporated from diet to bone may provide additional palaeodietary information.

CONCLUSION
Our findings suggest that archaeological bone lipids are a hitherto untapped resource of dietary information that offer further insights in addition to those gained from other isotopic analyses of bone.
Title: *Archaeological bone lipids as palaeodietary markers*

Short title: lipids as dietary markers

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RESULTS

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**Keywords**: Archaeology, bone lipid, palaeodiet, GC/MS, GC/C/IRMS, EA/IRMS

**Introduction**

Bone is an invaluable resource for the reconstruction of palaeodiets, with stable isotope analysis of bulk collagen, amino acids, and apatite providing important insights into different aspects of past diets of both animals and humans [1–5]. The distribution and isotopic composition of lipids in ancient skeletal tissues can theoretically provide additional complementary palaeodietary information [6–12], but so far lipids have been under-utilised in studies of past diet.

Lipid in modern bone originates from marrow adipose tissue (MAT), blood, and bone-forming cells (osteocytes) [13]. Dietary lipids are incorporated into a consumer’s tissue in a predictable manner [14], and the distributions of tissue lipids, notably fatty acids, are widely used within food-web studies in modern settings [15]. In addition, analysis of the stable carbon isotopes present in lipids may provide complementary dietary information to that obtained from more widely studied organic molecules, such as collagen. Firstly, lipids turnover much faster compared to bone collagen and mineral, and therefore reflect diet over a shorter period [16–18]. Secondly, experiments have shown that collagen is preferentially synthesised from dietary protein, whereas lipids represent whole diet (i.e. a combination of protein, lipids and carbohydrates) or dietary lipids [19–21]. Thirdly, the stable carbon isotope (δ¹³C) values of lipids (notably mid-chain length n-alkanoic acids) extracted from archaeological artefacts have already been widely measured to investigate their use. Inferences are normally made by comparison with modern authentic reference fats [22]. Obtaining comparative isotope measurements of these compounds directly from associated archaeological faunal remains would allow for a more precise elucidation of artefact use.

Although lipids have long been recognised as a source of dietary information, there are doubts concerning their preservation and authenticity in bone samples that have been extensively exposed to the burial environment. Absorbed fat-rich marrow is likely to be susceptible to leaching by ground water, microbial degradation, and removal prior to burial through boiling or cooking. Indeed, fatty acids, one of the most abundant classes of lipids in modern tissues, are rarely recovered by solvent extraction in all but arid environments [7]. For this reason, research has generally focused on more hydrophobic compounds, such as steroids, which are more resistant to oxidation and ground water leaching [6]. Lipids present in bone cells are protected within the bone lamellae and are expected to be less diagnostically altered, less prone to contamination with soil lipids and therefore more promising for palaeodietary studies. Nevertheless, these molecules are likely to be more difficult to extract with conventional solvent extraction. Additional endogenous polar lipids may also form strong electrostatic interactions with the bone mineral phase or be directly hydrogen bonded with the mineral surface. The removal of either cellular or such structurally-bound lipid from bone requires a re-evaluation of the extraction methods.
Here we evaluate a novel approach using acidified methanol to partly demineralise bone whilst simultaneously extracting and derivatising any fatty acyl components for analysis by gas chromatography mass spectrometry (GC/MS) and gas chromatography combustion mass spectrometry (GC/C/IRMS).

**Experimental**

**Sample selection**

Human (n=10) and faunal (n=11) bone samples, dating from ~6,000 to 500 calibrated years before present were obtained from a variety of coastal and inland sites from Europe and South America, in order to capture a range of environments and C3/C4 terrestrial, and marine subsistence strategies (Tab. 1).

**Collagen extraction**

For most of samples the bulk collagen stable isotope data was already available (Tab. 1) and details of the methods can be found in previous studies [23-27]. Briefly collagen was extracted using a modified Longin method [28]. Bone (0.5-1 g) was coarsely ground and demineralised (0.6 M HCl, 4°C, 3-12 days), samples were rinsed with distilled water and then gelatinised (pH3 [0.001M] HCl, 80°C, 48 h). The supernatant containing the collagen was filtered (30 kDa, Amicon® Ultra-4 Centrifugal Filter Units, Millipore, Billerica, MA, USA), frozen, and lyophilised.

**Lipid extraction**

Between 0.3 and 0.9 g of mechanically cleaned bone was ground to a coarse powder with a pestle and mortar. Lipids were then extracted from individual bone samples via one of two methods:

*Method 1 – Acidified methanol extraction*

Lipids from two human (ABM08 and GBM24/04) and two bovine (GBC2 and GBC4) bone samples were extracted with a one-step, direct extraction and methylation [22,29]. In short, after adding 4 mL of methanol, samples were ultra-sonicated for 15 minutes. Subsequently, 800 µL of H$_2$SO$_4$ was added and the samples were heated at 70°C for 4 hours. Samples were then centrifuged (850 x g) for 5 minutes. Supernatant was then extracted with hexane (3 x 2 mL) and neutralised with K$_2$CO$_3$. Extracts were then dried under a gentle stream of N$_2$. 10 µL of internal standard (0.2 µg µL$^{-1}$ tetratriacontane or 1.0 µg µL$^{-1}$ hexatriacontane) was added to each sample before further analysis by GC/MS and GC/C/IRMS.

*Method 2 – Solvent extraction followed by acidified methanol extraction*

Lipids from all 21 bone samples were solvent extracted with dichloromethane/methanol (2/1 vol/vol; 3 x 2 mL), by ultra-sonicating for 15 minutes and then centrifuging (850 x g) for 10 minutes. The supernatant was removed, discarded and the bone powder was dried completely under gentle stream of N$_2$. The same procedure, outlined in method 1 (acidified methanol extraction), was then applied to the remaining bone powder.

**Instrumentation**

*Elemental Analysis – Isotope Ratio Mass Spectrometry (EA/IRMS)*
Collagen samples (1 mg) were analysed in duplicate or triplicate by EA/IRMS in a Sercon GSL analyser coupled to a Sercon 20-22 Mass Spectrometer (Sercon, Crewe, UK) at the University of York, or a Roboprep Combustion Device coupled to a Europa 20/20 Mass Spectrometer (PDZ-Europa, Crewe, UK) at the University of Bradford. For all these instruments the analytical error, calculated from repeated measurements of each sample and measurements of the bovine control from multiple extracts, was <0.2‰ (1σ). The results from the analysis are reported per mil (‰) relative to an international standard (V/PDB). Collagen standards were exchanged between laboratories to ensure instrument consistency.

Gas Chromatography – Mass Spectrometry (GC/MS)

GC/MS was carried out on all samples using an Agilent 7890A Series chromatograph attached to an Agilent 5975 C Inert XL mass-selective detector with a quadrupole mass analyser (Agilent technologies, Cheadle, Cheshire, UK). A splitless injector was used and maintained at 300°C. The carrier gas used was helium, and inlet/column head-pressure was constant. The GC column was inserted directly into the ion source of the mass spectrometer. The ionisation energy of the mass spectrometer was 70 eV and spectra were obtained by scanning between m/z 50 and 800. Two different columns phases were used. General screening was performed using a DB-5ms (5%/phenyl)/methylpolysiloxane column (30 m x 0.250 mm x 0.25 µm; J&W Scientific, Folsom, CA, USA). The temperature for this column was set at 50°C for 2 minutes, then raised by 10°C min\(^{-1}\) until 325°C was reached, where it was held for 15 minutes. A second more polar column (DB-23, (50%/Cyanopropyl)/methylpolysiloxane column, 60 m x 0.25 mm x 0.25 µm; J&W Scientific, Folsom, CA, USA), was used for a better resolution of isoprenoid fatty acids. The temperature was set at 50°C for 2 minutes, then raised by 10°C min\(^{-1}\) until 100°C, then raised at 4°C min\(^{-1}\) until reaching 250°C where it was held for 20 minutes.

Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry (GC/C/IRMS)

A procedure identical to that described by Craig et al. [30] was followed. Carbon stable isotopes were determined on two fatty acid methyl esters: methyl palmitate (C\(_{16:0}\)) and methyl stearate (C\(_{18:0}\)) in each extract using a Delta V Advantage IRMS Thermo Fisher linked to a Trace Ultra gas chromatograph Thermo Fisher with a ConFlo IV interface (Cu/Ni combustion reactor held at 1000°C; Thermo Fisher, Bremen, Germany). All samples were diluted with hexane and subsequently 1 µL of each sample was injected into a DB5 fused-silica column. The temperature was set for 1 minute at 45°C, and raised by 6°C min\(^{-1}\) until 295°C was reached, at which it stayed for 15 minutes. The carrier gas was ultra-high purity grade helium with a flow rate of 1.4 mL min\(^{-1}\). Eluted products were combusted to CO\(_2\) and ionized in the mass spectrometer by electron impact. Ion intensities of m/z 44, 45, and 46 were monitored in order to automatically compute the \(^{13}\)C/\(^{12}\)C ratio of each peak in the extracts. Computations were made with Isodat 3.0 Gas Isotope Ratio MS Software (version 3.0; ThermoFisher Scientific) and were based on comparisons with a standard reference gas (CO\(_2\)) of known isotopic composition that was repeatedly measured. The results from the analysis are reported in parts per mil (‰) relative to an international standard (V-PDB). Replicate measurements of each sample and a mixture of fames fatty acid methyl esters (FAMES) with \(\delta^{13}\)C values traceable to international standards were used to determine instrument precision (<0.3‰) and accuracy (<0.5‰). Values were also corrected subsequent to analysis to account for the methylation of the carboxyl group that occurs during acid extraction. Corrections were based on comparisons with a standard mixture of C\(_{16:0}\) and C\(_{18:0}\) fatty acids of known isotopic composition processed in each batch as a sample.
Results and discussion

Stable carbon and nitrogen isotopic composition of bulk collagen

Based on the isotope values of the bulk collagen and other archaeological data, humans were assigned to a variety of dietary categories including marine, terrestrial C_{3}, terrestrial C_{4}, and terrestrial C_{3}/marine mixed (Fig. 1). Human remains with a marine protein diet exhibited average δ^{13}C and δ^{15}N values of -11.3‰ and +16.6‰. Individuals with a terrestrial diet based on C_{3} plants and C_{3} plant fed animals provided average δ^{13}C and δ^{15}N values of -20.2‰ and +11.2‰. One individual had δ^{13}C and δ^{15}N values of -16.4‰ and +11.0‰, which is attributable to C_{4} plant-derived carbon in the diet [24]. Humans with a mixed diet including terrestrial based C_{3} plants and marine resources had average δ^{13}C and δ^{15}N values of -17.5‰ and +12.1‰ respectively.

The δ^{13}C and δ^{15}N of marine fish (*Pogonias cromis*) and sea mammals (Pinnipedia, Cetacea) had values ranging from -9.8‰ to -11.8‰ and +12.6‰ to +19.5‰ respectively. The δ^{13}C and δ^{15}N of terrestrial faunal remains from C_{3} plant ecosystems, including ruminants (*Bos taurus*, *Capreolus capreolus*), monogastric herbivores (*Agouti pacu*, *Alouatta* sp.), and omnivores (*Sus scrofa*), had values ranging from -20.1‰ to -22.9‰ and +3.5‰ to +7.9‰. Finally, the δ^{13}C and δ^{15}N of one bovine sample (GBC4) had values of -14.3‰ and +7.4‰ respectively, with the relatively elevated δ^{13}C value attributed to C_{4} plants in its diet [24].

Comparison of lipid extraction methods

Two human (ABM08 and GBM24/04) and two bovine (*Bos taurus*) bone samples (GBC2 and GBC4) were extracted using both acidified methanol (method 1) and sequential solvent-acidified methanol extraction (method 2). There were major differences observed in the distribution, quantity and isotopic composition of lipids extracted with the two methods (Tab. 1, SI1).

Lipid extracts of the two bovine bones obtained via method 1 were very similar in nature, mainly containing saturated fatty acids with even carbon numbers (between C_{12:0}-C_{24:0}), cholesterol derivatives, lesser concentrations of monounsaturated fatty acids with even carbon numbers (between C_{16:1}-C_{24:1}), and linoleic acid (C_{18:2}) (Fig. 2A). Numerous phthalates (plasticizer contaminants; P) were present in the method 1 extracts. The extracts of bovine bone obtained via method 2 were also similar to each other and similar to the extracts of method 1. Both contained saturated fatty acids with even carbon numbers (between C_{14:0}-C_{24:0}), cholesterol derivatives, monounsaturated fatty acids with even carbon numbers (between C_{18:1} and C_{24:1}), and linoleic acid (C_{18:2}) (Fig. 2B). The major difference between method 1 and method 2 extracts was the lack of plastic contaminants in the latter, therefore these contaminants were efficiently removed by solvent wash prior to acid-extraction.

Human bone lipids extracted via method 1 were also similar in nature, containing saturated fatty acids with even carbon numbers (C_{12:0}-C_{24:0}), monounsaturated fatty acids (C_{18:1}, C_{22:1}, C_{24:1}), linoleic acid (C_{18:2}), and cholesterol derivatives. Like the bovine bones extracted with method 1, phthalates were also present in the human bones extracted with method, most likely reflecting their conservational history. The same samples extracted with method 2 had similar lipid profiles, containing saturated fatty acids (C_{16:0}-C_{24:0}), monounsaturated fatty acids (C_{18:1}}
and C_{24:1}, linoleic acid (C_{18:2}), and cholesterol derivatives. Similar to the cattle bone samples, plastic contamination was absent in the human bone lipid extracts obtained via method 2.

The GC/MS results presented here demonstrate that method 2 removed contaminants from archaeological bone samples, whilst extracting a similar range of lipids to method 1. However, much lower overall lipid concentrations were obtained with method 2. For example, the total lipid yield of GBC2 was 82 µg.g\(^{-1}\) with method 1, and only 31 µg.g\(^{-1}\) for method 2. Additionally, yields of C_{16:0} and C_{18:0} dropped from 20 and 18 µg.g\(^{-1}\) to 5 and 4 µg.g\(^{-1}\) respectively.

GC/IRMS analysis showed that isotopic values of C_{16:0} and C_{18:0} were greatly affected by the method used for lipid extraction (Fig. 3A-B). Based on bulk collagen isotope data, GBC2 bovine sample was assigned to the ‘C\(_3\) diet’ category, and had a δ\(^{13}\)C value that was lower by ~6‰ relative to the C\(_4\) bovine sample (GBC4). Additionally, the δ\(^{13}\)C value of human bulk collagen from ABM08 (C\(_3\) diet) was ~2‰ lower relative to the GBM2404 value, attributed to a greater C\(_4\) diet in the latter. The δ\(^{13}\)C values of C_{16:0} and C_{18:0} obtained from method 1 did not reflect bulk collagen data; the two bovine samples (GBC2, GBC4) had nearly identical δ\(^{13}\)C values, as did the two human extracts (ABM08, GBM24/04). In contrast, using method 2 the δ\(^{13}\)C value of GBC4 was higher by ~6‰ compared to GBC2. Similarly, the δ\(^{13}\)C value of GBM2404 was higher by ~2‰ compared to ABM08. These differences are consistent with the offset observed in bulk collagen δ\(^{13}\)C values.

These results highlight the efficiency of sequential extraction (method 2) for obtaining reliable archaeological bone lipid extracts. It is likely that exogenous lipids were removed by extraction with organic solvents leaving endogenous lipids present in bone cells (osteocytes) or bound to the mineral matrix. Although solvent extraction probably also removed some endogenous lipid, an adequate amount remained for subsequent biomolecular and isotopic analyses. Another, albeit less likely, explanation is that lipids soluble in organic solvents were derived from either a different fraction of diet or foods consumed over a different period of time to both collagen and the cellular/bound lipid fraction.

Based on these results, the remainder of the samples analysed in this study were extracted with method 2.

**The distribution of lipids in archaeological bone**

The lipid extracts from all archaeological bones analysed in this study were similar in composition (Fig. 4, SI1). In general, the extracts contained saturated fatty acids (primarily C\(_{14:0}\), C\(_{16:0}\), and C\(_{18:0}\)), lesser concentrations of monounsaturated fatty acids (primarily C\(_{18:1}\)), and some products of cholesterol oxidation (primarily cholesta-3,5-diene). In some samples, linoleic acid (C\(_{18:2}\)) was present in low quantities, and in one case hexadecadienoic acid (C\(_{16:2}\)) was present.

Traces of phytanic and/or pristanic acid were present in all of the samples analysed. The synthesis of phytanic acid from phytol occurs in abundance in ruminant animals and marine ecosystems, as phytol is made bioavailable by gut-bacteria in the former, and by zooplankton in the latter [31–33]. As other animals cannot separate phytol from chlorophyll, the presence of phytanic and pristanic acid is normally attributed to direct incorporation from the consumption of meat and/or fish and/or dairy products [31,33,34].
Although these isoprenoid fatty acids may be present at low abundance in the burial environment, their persistence in the bone samples following solvent extraction indicates that they are most likely endogenous and directly derived from diet, as previously reported in other studies of exceptionally well preserved soft and skeletal tissues [8]. The presence of isoprenoid fatty acids in bone up to 6,000 years old is encouraging and warrants further study.

Fatty acids stable carbon isotope values

All of the lipid extracts obtained via method 2 yielded sufficient amounts of C\textsubscript{16:0} and C\textsubscript{18:0} for GC/C/IRMS analysis (Tab. 1).

The δ\textsuperscript{13}C values of C\textsubscript{16:0} and C\textsubscript{18:0} across all samples ranged from -20.1‰ to -29.4‰, and -21.2‰ to -31.5‰ respectively, with clear separation between marine and terrestrial fauna, and between terrestrial C\textsubscript{3} and C\textsubscript{4} consumers. Similar isotopic differences were identified in humans according to their bulk collagen isotope values (Fig. 5).

Importantly, the humans with mixed terrestrial C\textsubscript{3}/marine diets plot towards the middle of the range of isotopic values obtained here, and fall between humans with dominant marine and C\textsubscript{3} terrestrial diets.

Nevertheless, two seals had C\textsubscript{16:0} and C\textsubscript{18:0} depleted in \textsuperscript{13}C relative to the other marine organisms, plotting among the C\textsubscript{3} consumers, and did not correlate with the bulk collagen isotope data (Tab. 1). This is unlikely to be the result of contamination, since both seals come from a marine shell midden site (Oronsay, Western Scotland) where contamination with lipids from the depositional matrix would be expected to elevate δ\textsuperscript{13}C values. While the seal values appear to be outliers in the data presented here, similar δ\textsuperscript{13}C values of bulk lipids from modern seals have been reported previously [35,36].

As expected, the fatty acids were consistently depleted in \textsuperscript{13}C relative to bulk collagen values from the same samples (Fig. 6). Such \textsuperscript{13}C depletion reflects fundamental differences between the biosynthesis of collagen and fatty acids. Both C\textsubscript{16:0} and C\textsubscript{18:0} fatty acids can either be directly routed from diet or biosynthesised \textit{de novo} from all dietary macronutrients. However, a major kinetic isotope effect in lipid biosynthesis occurs through the oxidative decarboxylation of pyruvate to acetyl Co-A resulting in a depletion in \textsuperscript{13}C [37].

Interestingly, the offset between collagen and lipids (Δ\textsuperscript{13}C\textsubscript{FA-COLL}) is not consistent and ranges from ~6‰ to ~18‰. This variation may reflect isotopic differences in dietary macronutrients, however Δ\textsuperscript{13}C\textsubscript{FA-COLL} offset in marine fauna is consistently larger than in terrestrial animals. The offset in terrestrial C\textsubscript{3} animals (6.0‰ to 11.4‰) was consistent with the expected value for individuals with diets high in carbohydrates [19,20], where non-essential amino acids and fatty acids are at least in part \textit{de novo} synthesised from this source. The larger Δ\textsuperscript{13}C\textsubscript{FA-COLL} observed in the marine organisms (i.e. >13‰) may be due to the fact that the pool of dietary carbon in fish and marine mammals is derived from a greater amount of lipid compared to terrestrial herbivores, which then becomes further depleted in \textsuperscript{13}C during subsequent lipogenesis. More work to determine the isotopic differences between essential and non-essential fatty and amino acids is needed to understand the degree of routing versus biosynthesis of macronutrients in marine organisms.
Conclusions

By using a modified two-step protocol, we show that a greater range of lipids than previously encountered can be reliably recovered from archaeological bone. The extraction of appreciable amounts of endogenous fatty acids from bone buried even in temperate environments is particularly promising since these compounds can be traced isotopically to dietary macronutrients or directly incorporated from known dietary sources. In addition, δ\(^{13}\)C values of fatty acids associated with artefacts have been widely measured in ancient contexts providing the opportunity to compare data. New avenues for research on bone fatty acids include the comparison with bulk collagen and single amino acids to identify the source and relative contribution of dietary protein, lipids and carbohydrates with more accuracy. Furthermore, as lipids turn-over much faster in bone in comparison to collagen, changes in diet through life may also be studied using this approach.

Acknowledgments

The authors want to thank Paulo DeBlasis, Levy Figuti, Veronica Wesolowski, Sabine Eggers, Claudia Regina Plens, Alison Sheridan, Sören H. Andersen, Kurt Gron, Antonio Hernández Pardos, Joan Cardona, the Museu Arqueològic de Gandía, the National Museum of Scotland and the Instituto de Patrimônio Histórico e Artístico Nacional (IPHAN) for providing materials and permissions for analysis. This research was partially funded by the EU Marie-Curie Action: “Intra-European fellowships for career development” (Coastal resources and South American hunter-gatherers: biochemical perspectives from Brazilian sambaquis - COREBRAS, Ref. 273734); FAPESP (Sambaquis e Paisagem, Ref. 11038-0); AHRC (Changing Scientific and Cultural Perspectives on Human-Chicken Interactions, Ref. AH/L006979/1); NERC (Finding the Mesolithic: Accessing Biomolecular Information from Bone Fragments, Ref.NE/K500987/1). The authors thank the anonymous reviewers for their comments on the manuscript.

References


Table 1. Stable carbon isotope values of extracted lipids (palmitic and stearic acid) using method 1(*) and method 2. Bulk collagen stable carbon and nitrogen isotopic composition of human and faunal remains.

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<td>6000 cal BP</td>
<td>Agouti paca</td>
<td>Terrestrial C₃</td>
<td>16.8</td>
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</tr>
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<td>37A</td>
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<td>6000 cal BP</td>
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<td>Terrestrial C₃</td>
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<td>Havnø</td>
<td>4000 cal BP</td>
<td>Mesolithic/Neolithic</td>
<td>Capreolus capreolus</td>
<td>Terrestrial C₃</td>
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<td>Munlee[27]</td>
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<td>ABM08*</td>
<td>Spain</td>
<td>Almacabra de</td>
<td>1015-12b</td>
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<td>-27.3</td>
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<td>Almacabra de</td>
<td>1015-12b</td>
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<td>-20.1</td>
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<td>1515-16b</td>
<td>Bos taurus</td>
<td>Terrestrial C₃</td>
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<td>-31.5</td>
<td>-20.1</td>
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<td>Alexander et al.</td>
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<tr>
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<td>Benipeixcar</td>
<td>1515-16b</td>
<td>Bos taurus</td>
<td>Terrestrial C₃</td>
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<td>-28.3</td>
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<td>Alexander et al.</td>
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<td>-26.3</td>
<td>-16.4</td>
<td>+11.0</td>
<td>Alexander et al.</td>
</tr>
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</table>
Supporting information 1 (SI1) - Composition of lipid extracts. Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; DUFA, diunsaturated fatty acid; br, branched fatty acid. Method 1 (*).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Taxa</th>
<th>Site</th>
<th>Lipids extracted</th>
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<tbody>
<tr>
<td>CC10502</td>
<td>Pinnipedia</td>
<td>Cnoc Coig, Oronsay</td>
<td>SFA(C14:0,18:0, C20:0, C22:0, C24:0, C26:0), MUFA(C16:1, C18:1, C20:1, C22:1), DUFA(C18:2), br(C15-17), cholesta/3,5-diene, phytanic, pristanic</td>
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<td>Pinnipedia</td>
<td>Cnoc Coig, Oronsay</td>
<td>SFA(C14:0,18:0, C20:0, C22:0, C24:0), MUFA(C16:1, C18:1, C24:1), DUFA(C16:2, C18:2), br(C15-17), cholesta/3,5-diene, phytanic, pristanic</td>
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<tr>
<td>CRB21</td>
<td>Pogonias crassus</td>
<td>Jabuticabeira II</td>
<td>SFA(C14:0,18:0, C20:0, C22:0, C24:0), MUFA(C18:1, C24:1), br(C15-17), phytanic, pristanic</td>
</tr>
<tr>
<td>CRB26</td>
<td>Cotoca</td>
<td>Jabuticabeira II</td>
<td>SFA(C14:0,18:0, C20:0, C22:0, C24:0), MUFA(C18:1, br(C15-18), phytanic, pristanic</td>
</tr>
<tr>
<td>104A-L1.85</td>
<td>Homo sapiens</td>
<td>Jabuticabeira II</td>
<td>SFA(C14:0,18:0, C20:0, C24:0), MUFA(C16:5, C18:1, br(C15-17), cholesta/3,5-diene, phytanic, pristanic</td>
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<td>Havana</td>
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<tr>
<td>AEE/OL1</td>
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<td>Bjørnsholm</td>
<td>SFA(C14:0,24:0, C20:0, MUFA(C16:1, C18:1, C24:1), DUFA(C18:2), br(C15-18), cholesta/3,5-diene, cholest/5/ene/(3/methoxy), phytanic, pristanic</td>
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<td>Homo sapiens</td>
<td>Moraes</td>
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<td>ABM08*</td>
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<td>Almacabra</td>
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<td>Benipeixcar</td>
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</table>

* Indicates samples from the same site.

Method 1: A combination of gas chromatography (GC) and mass spectrometry (MS) was used to identify the fatty acid composition of the lipid extracts.
Figure captions

Fig. 1. Bulk collagen $\delta^{13}C$ and $\delta^{15}N$ values for the humans and faunal samples in this study. For details on assigning the samples by diet see Table 1 and references therein.

Fig. 2. Comparison of partial gas chromatogram of a bovine (GBC2) bone lipid extract using method 1 (A) and 2 (B). Abbreviations: $C_{n:x}$ indicates a fatty acid with $n$ carbon atoms and $x$ double bond; $P$ indicates contaminant phthalate; IS indicates internal standard.

Fig. 3. Comparison of stable carbon isotope composition of palmitic and stearic fatty acid from acidified methanol (method 1; A) and sequential solvent- acidified methanol extraction (method 2; B) of bovine (GBC2, GBC4) and human (ABM08, GBM24/04) bones.

Fig. 4. Partial gas chromatogram of a human (1.1/2-T10) bone lipid extract. Inset shows partial mass spectrum of phytanic acid characterized by $m/z$ ions 101, 171, and 326. Abbreviations: $C_{n:x}$ indicates fatty acid with $n$ carbon atoms and $x$ double bonds; br represents branched fatty acid with $n$ carbon atoms; IS indicates internal standard (2 $\mu$g of tetratriacontane).

Fig. 5. Plot of $\delta^{13}C$ values of $C_{16:0}$ and $C_{18:0}$ fatty acids from all samples analysed in this study extracted with method 2.

Fig. 6. Offset between $\delta^{13}C$ values of $C_{16:0}$ and $C_{18:0}$ fatty acids and collagen ($\Delta^{13}C_{FA-Coll}$).
A \[ \delta^{13}C_{\text{cell}} \%_{\text{V-PDB}} \]

B \[ \delta^{13}C_{\text{cell}} \%_{\text{V-PDB}} \]

Method 1

Method 2

204x94mm (300 x 300 DPI)
Fauna

Humans

358x113mm (300 x 300 DPI)