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Archaeological bone lipids as palaeodietary markers

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Keywords:	Archaeology, bone lipid, palaeodiet, GC/MS, GC/C/IRMS, EA/IRMS
Abstract:	<p>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.</p> <p>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).</p> <p>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</p>

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	<p>acids directly incorporated from diet to bone may provide additional palaeodietary information.</p> <p>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.</p>

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25 14
26 15 RATIONALE

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28 16 Stable isotope analysis of archaeological and fossil bone samples can provide important
29 17 insights into past environments, ecologies and diets. Previous studies have focused on stable
30 18 carbon and nitrogen isotopes in bone collagen, or carbon isotopes in bone mineral
31 19 (bioapatite). Carbon isotope analysis of lipids from archaeological bone has received much
32 20 less attention, partly due to the lack of suitable methodologies allowing sufficient recovery of
33 21 compounds for structural and isotopic characterisation. Here we show that lipids can be easily
34 22 and reliably recovered from archaeological bone using a modified protocol, and that these
35 23 provide complementary dietary information to other bone components.

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38 24 METHODS

39 25 Human and animal bones were obtained from a variety of archaeological contexts. Lipids
40 26 were sequentially extracted using solvent extraction (dichloromethane/methanol), followed
41 27 by acidified methanol extraction (methanol/H₂SO₄). Lipids were analysed by gas
42 28 chromatography-mass spectrometry (GC/MS) and gas chromatography-combustion-isotope
43 29 ratio mass spectrometry (GC/C/IRMS).

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46 30 RESULTS

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48 31 Our results show that appreciable amounts of endogenous lipid can be recovered from
49 32 archaeological bone. Importantly, a comparison between compound specific and bulk
50 33 collagen isotopic data shows that archaeological bone lipids reflect dietary input and can be
51 34 used to distinguish between marine and terrestrial consumers, as well as between C₃ and C₄
52 35 plant consumers. Furthermore, the presence of essential fatty acids directly incorporated from
53 36 diet to bone may provide additional palaeodietary information.

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56 37 CONCLUSION

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3 38 Our findings suggest that archaeological bone lipids are a hitherto untapped resource of
4 39 dietary information that offer further insights in addition to those gained from other isotopic
5 40 analyses of bone.

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7 41
8 42 **Keywords:** Archaeology, bone lipid, palaeodiet, GC/MS, GC/C/IRMS, EA/IRMS
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11 44 **Introduction**

12 45 Bone is an invaluable resource for the reconstruction of palaeodiets, with stable isotope
13 46 analysis of bulk collagen, amino acids, and apatite providing important insights into different
14 47 aspects of past diets of both animals and humans [1–5]. The distribution and isotopic
15 48 composition of lipids in ancient skeletal tissues can theoretically provide additional
16 49 complementary palaeodietary information [6–12], but so far lipids have been under-utilised in
17 50 studies of past diet.

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21 51 Lipid in modern bone originates from marrow adipose tissue (MAT), blood, and bone-
22 52 forming cells (osteocytes) [13]. Dietary lipids are incorporated into a consumer's tissue in a
23 53 predictable manner [14], and the distributions of tissue lipids, notably fatty acids, are widely
24 54 used within food-web studies in modern settings [15]. In addition, analysis of the stable
25 55 carbon isotopes present in lipids may provide complementary dietary information to that
26 56 obtained from more widely studied organic molecules, such as collagen. Firstly, lipids
27 57 turnover much faster compared to bone collagen and mineral, and therefore reflect diet over a
28 58 shorter period [16–18]. Secondly, experiments have shown that collagen is preferentially
29 59 synthesised from dietary protein, whereas lipids represent whole diet (i.e. a combination of
30 60 protein, lipids and carbohydrates) or dietary lipids [19–21]. Thirdly, the stable carbon isotope
31 61 ($\delta^{13}\text{C}$) values of lipids (notably mid-chain length *n*-alkanoic acids) extracted from
32 62 archaeological artefacts have already been widely measured to investigate their use.
33 63 Inferences are normally made by comparison with modern authentic reference fats [22].
34 64 Obtaining comparative isotope measurements of these compounds directly from associated
35 65 archaeological faunal remains would allow for a more precise elucidation of artefact use.

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40 66 Although lipids have long been recognised as a source of dietary information, there are
41 67 doubts concerning their preservation and authenticity in bone samples that have been
42 68 extensively exposed to the burial environment. Absorbed fat-rich marrow is likely to be
43 69 susceptible to leaching by ground water, microbial degradation, and removal prior to burial
44 70 through boiling or cooking. Indeed, fatty acids, one of the most abundant classes of lipids in
45 71 modern tissues, are rarely recovered by solvent extraction in all but arid environments [7].
46 72 For this reason, research has generally focused on more hydrophobic compounds, such as
47 73 steroids, which are more resistant to oxidation and ground water leaching [6]. Lipids present
48 74 in bone cells are protected within the bone lamellae and are expected to be less diagenetically
49 75 altered, less prone to contamination with soil lipids and therefore more promising for
50 76 palaeodietary studies. Nevertheless, these molecules are likely to be more difficult to extract
51 77 with conventional solvent extraction. Additional endogenous polar lipids may also form
52 78 strong electrostatic interactions with the bone mineral phase or be directly hydrogen bonded
53 79 with the mineral surface. The removal of either cellular or such structurally-bound lipid from
54 80 bone requires a re-evaluation of the extraction methods.

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3 81 Here we evaluate a novel approach using acidified methanol to partly demineralise bone
4 82 whilst simultaneously extracting and derivatising any fatty acyl components for analysis by
5 83 gas chromatography mass spectrometry (GC/MS) and gas chromatography combustion mass
6 84 spectrometry (GC/C/IRMS).

85 **Experimental**

86 **Sample selection**

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

91 **Collagen extraction**

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

99

100 **Lipid extraction**

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

104 *Method 1 – Acidified methanol extraction*

105 Lipids from two human (ABM08 and GBM24/04) and two bovine (GBC2 and GBC4) bone
106 samples were extracted with a one-step, direct extraction and methylation [22,29]. In short,
107 after adding 4 mL of methanol, samples were ultra-sonicated for 15 minutes. Subsequently,
108 800 μL of H_2SO_4 was added and the samples were heated at 70°C for 4 hours. Samples were
109 then centrifuged (850 x g) for 5 minutes. Supernatant was then extracted with hexane (3 x 2
110 mL) and neutralised with K_2CO_3 . Extracts were then dried under a gentle stream of N_2 . 10 μL
111 of internal standard (0.2 $\mu\text{g } \mu\text{L}^{-1}$ tetratriacontane or 1.0 $\mu\text{g } \mu\text{L}^{-1}$ hexatriacontane) was added
112 to each sample before further analysis by GC/MS and GC/C/IRMS.

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

114 Lipids from all 21 bone samples were solvent extracted with dichloromethane/methanol (2/1
115 vol/vol; 3 x 2 mL), by ultra-sonicating for 15 minutes and then centrifuging (850 x g) for 10
116 minutes. The supernatant was removed, discarded and the bone powder was dried completely
117 under gentle stream of N_2 . The same procedure, outlined in method 1 (acidified methanol
118 extraction), was then applied to the remaining bone powder.

119 **Instrumentation**

120 *Elemental Analysis – Isotope Ratio Mass Spectrometry (EA/IRMS)*

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3 121 Collagen samples (1 mg) were analysed in duplicate or triplicate by EA/IRMS in a Sercon
4 122 GSL analyser coupled to a Sercon 20-22 Mass Spectrometer (Sercon, Crewe, UK) at the
5 123 University of York, or a Roboprep Combustion Device coupled to a Europa 20-20 Mass
6 124 Spectrometer (PDZ-Europa, Crewe, UK) at the University of Bradford. For all these
7
8 125 instruments the analytical error, calculated from repeated measurements of each sample and
9 126 measurements of the bovine control from multiple extracts, was $<0.2\%$ (1σ). The results
10 127 from the analysis are reported per mil (‰) relative to an international standard (V-PDB).
11 128 Collagen standards were exchanged between laboratories to ensure instrument consistency.

12
13 129 *Gas Chromatography – Mass Spectrometry (GC/MS)*

14
15 130 GC/MS was carried out on all samples using an Agilent 7890A Series chromatograph
16 131 attached to an Agilent 5975 C Inert XL mass-selective detector with a quadrupole mass
17 132 analyser (Agilent technologies, Cheshire, UK). A splitless injector was used and
18 133 maintained at 300°C. The carrier gas used was helium, and inlet/column head-pressure was
19 134 constant. The GC column was inserted directly into the ion source of the mass spectrometer.
20 135 The ionisation energy of the mass spectrometer was 70 eV and spectra were obtained by
21 136 scanning between m/z 50 and 800. Two different columns phases were used. General
22 137 screening was performed using a DB-5ms (5%-phenyl)-methylpolysiloxane column (30 m x
23 138 0.250 mm x 0.25 μm ; J&W Scientific, Folsom, CA, USA). The temperature for this column
24 139 was set at 50°C for 2 minutes, then raised by 10°C min^{-1} until 325°C was reached, where it
25 140 was held for 15 minutes. A second more polar column (DB-23, (50%-Cyanopropyl)-
26 141 methylpolysiloxane column, 60 m x 0.25 mm x 0.25 μm ; J&W Scientific, Folsom, CA,
27 142 USA), was used for a better resolution of isoprenoid fatty acids. The temperature was set at
28 143 50°C for 2 minutes, then raised by 10°C min^{-1} until 100°C, then raised at 4°C min^{-1} until
29 144 reaching 250°C where it was held for 20 minutes.

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34 145 *Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry (GC/C/IRMS)*

35 146 A procedure identical to that described by Craig et al. [30] was followed. Carbon stable
36 147 isotopes were determined on two fatty acid methyl esters; methyl palmitate ($\text{C}_{16:0}$) and
37 148 methyl stearate ($\text{C}_{18:0}$) in each extract using a Delta V Advantage IRMS Thermo Fisher
38 149 linked to a Trace Ultra gas chromatograph Thermo Fisher with a ConFlo IV interface (Cu/Ni
39 150 combustion reactor held at 1000°C; Thermo Fisher, Bremen, Germany). All samples were
40 151 diluted with hexane and subsequently 1 μL of each sample was injected into a DB5 fused-
41 152 silica column. The temperature was set for 1 minute at 45°C, and raised by 6°C min^{-1} until
42 153 295°C was reached, at which it stayed for 15 minutes. The carrier gas was ultra-high purity
43 154 grade helium with a flow rate of 1.4 mL min^{-1} . Eluted products were combusted to CO_2 and
44 155 ionized in the mass spectrometer by electron impact. Ion intensities of m/z 44, 45, and 46
45 156 were monitored in order to automatically compute the $^{13}\text{C}/^{12}\text{C}$ ratio of each peak in the
46 157 extracts. Computations were made with Isodat 3.0 Gas Isotope Ratio MS Software (version
47 158 3.0; ThermoFisher Scientific) and were based on comparisons with a standard reference gas
48 159 (CO_2) of known isotopic composition that was repeatedly measured. The results from the
49 160 analysis are reported in parts per mil (‰) relative to an international standard (V-PDB).
50 161 Replicate measurements of each sample and a mixture of fames fatty acid methyl esters
51 162 (FAMEs) with $\delta^{13}\text{C}$ values traceable to international standards were used to determine
52 163 instrument precision ($<0.3\%$) and accuracy ($<0.5\%$). Values were also corrected subsequent
53 164 to analysis to account for the methylation of the carboxyl group that occurs during acid
54 165 extraction. Corrections were based on comparisons with a standard mixture of $\text{C}_{16:0}$ and $\text{C}_{18:0}$
55 166 fatty acids of known isotopic composition processed in each batch as a sample.

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5 168 **Results and discussion**6 169 *Stable carbon and nitrogen isotopic composition of bulk collagen*

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8 170 Based on the isotope values of the bulk collagen and other archaeological data, humans were
9 171 assigned to a variety of dietary categories including marine, terrestrial C₃, terrestrial C₄, and
10 172 terrestrial C₃/marine mixed (Fig. 1). Human remains with a marine protein diet exhibited
11 173 average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of -11.3‰ and +16.6‰. Individuals with a terrestrial diet based
12 174 on C₃ plants and C₃ plant fed animals provided average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of -20.2‰ and
13 175 +11.2‰. One individual had $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of -16.4‰ and +11.0‰, which is
14 176 attributable to C₄ plant-derived carbon in the diet [24]. Humans with a mixed diet including
15 177 terrestrial based C₃ plants and marine resources had average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of -17.5‰
16 178 and +12.1‰ respectively.

17 179 The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of marine fish (*Pogonias cromis*) and sea mammals (Pinnipedia, Cetacea)
18 180 had values ranging from -9.8‰ to -11.8‰ and +12.6‰ to +19.5‰ respectively. The $\delta^{13}\text{C}$
19 181 and $\delta^{15}\text{N}$ of terrestrial faunal remains from C₃ plant ecosystems, including ruminants (*Bos*
20 182 *taurus*, *Capreolus capreolus*), monogastric herbivores (*Agouti paca*, *Alouatta* sp.), and
21 183 omnivores (*Sus scrofa*), had values ranging from -20.1‰ to -22.9‰ and +3.5‰ to +7.9‰.
22 184 Finally, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of one bovine sample (GBC4) had values of -14.3‰ and +7.4‰
23 185 respectively, with the relatively elevated $\delta^{13}\text{C}$ value attributed to C₄ plants in its diet [24].

24 186 *Comparison of lipid extraction methods*

25 187 Two human (ABM08 and GBM24/04) and two bovine (*Bos taurus*) bone samples (GBC2
26 188 and GBC4) were extracted using both acidified methanol (method 1) and sequential solvent-
27 189 acidified methanol extraction (method 2). There were major differences observed in the
28 190 distribution, quantity and isotopic composition of lipids extracted with the two methods (Tab.
29 191 1, S11).

30 192 Lipid extracts of the two bovine bones obtained via method 1 were very similar in nature,
31 193 mainly containing saturated fatty acids with even carbon numbers (between C_{12:0}-C_{24:0}),
32 194 cholesterol derivatives, lesser concentrations of monounsaturated fatty acids with even
33 195 carbon numbers (between C_{16:1}-C_{24:1}), and linoleic acid (C_{18:2}) (Fig. 2A). Numerous
34 196 phthalates (plasticizer contaminants; P) were present in the method 1 extracts. The extracts of
35 197 bovine bone obtained via method 2 were also similar to each other and similar to the extracts
36 198 of method 1. Both contained saturated fatty acids with even carbon numbers (between C_{14:0}-
37 199 C_{24:0}), cholesterol derivatives, monounsaturated fatty acids with even carbon numbers
38 200 (between C_{18:1} and C_{24:1}), and linoleic acid (C_{18:2}) (Fig. 2B). The major difference between
39 201 method 1 and method 2 extracts was the lack of plastic contaminants in the latter, therefore
40 202 these contaminants were efficiently removed by solvent wash prior to acid-extraction.

41 203 Human bone lipids extracted via method 1 were also similar in nature, containing saturated
42 204 fatty acids with even carbon numbers (C_{12:0}-C_{24:0}), monounsaturated fatty acids (C_{18:1}, C_{22:1},
43 205 C_{24:1}), linoleic acid (C_{18:2}), and cholesterol derivatives. Like the bovine bones extracted with
44 206 method 1, phthalate were also present in the human bones extracted with method, most likely
45 207 reflecting their conservational history. The same samples extracted with method 2 had similar
46 208 lipid profiles, containing saturated fatty acids (C_{16:0}-C_{24:0}), monounsaturated fatty acids (C_{18:1}

209 and C_{24:1}), linoleic acid (C_{18:2}), and cholesterol derivatives. Similar to the cattle bone samples,
210 plastic contamination was absent in the human bone lipid extracts obtained via method 2.

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

217 GC/C/IRMS analysis showed that isotopic values of C_{16:0} and C_{18:0} were greatly affected by
218 the method used for lipid extraction (Fig. 3A-B). Based on bulk collagen isotope data, GBC2
219 bovine sample was assigned to the 'C₃ diet' category, and had a δ¹³C value that was lower by
220 ~6‰ relative to the C₄ bovine sample (GBC4). Additionally, the δ¹³C value of human bulk
221 collagen from ABM08 (C₃ diet) was ~2‰ lower relative to the GBM2404 value, attributed to
222 a greater C₄ diet in the latter. The δ¹³C values of C_{16:0} and C_{18:0} obtained from method 1 did
223 not reflect bulk collagen data; the two bovine samples (GBC2, GBC4) had nearly identical
224 δ¹³C values, as did the two human extracts (ABM08, GBM24/04). In contrast, using method
225 2 the δ¹³C value of GBC4 was higher by ~6‰ compared to GBC2. Similarly, the δ¹³C value
226 of GBM2404 was higher by ~2‰ compared to ABM08. These differences are consistent with
227 the offset observed in bulk collagen δ¹³C values.

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

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

238 *The distribution of lipids in archaeological bone*

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

245 Traces of phytanic and/or pristanic acid were present in all of the samples analysed. The
246 synthesis of phytanic acid from phytol occurs in abundance in ruminant animals and marine
247 ecosystems, as phytol is made bioavailable by gut-bacteria in the former, and by zooplankton
248 in the latter [31–33]. As other animals cannot separate phytol from chlorophyll, the presence
249 of phytanic and pristanic acid is normally attributed to direct incorporation from the
250 consumption of meat and/or fish and/or dairy products [31,33,34].

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3 251 Although these isoprenoid fatty acids may be present at low abundance in the burial
4 252 environment, their persistence in the bone samples following solvent extraction indicates that
5 253 they are most likely endogenous and directly derived from diet, as previously reported in
6 254 other studies of exceptionally well preserved soft and skeletal tissues [8]. The presence of
7 255 isoprenoid fatty acids in bone up to 6,000 years old is encouraging and warrants further
8 256 study.

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10 257

11 258 *Fatty acids stable carbon isotope values*

12
13 259 All of the lipid extracts obtained via method 2 yielded sufficient amounts of C_{16:0} and C_{18:0}
14 260 for GC/C/IRMS analysis (Tab. 1).

15
16
17 261 The $\delta^{13}\text{C}$ values of C_{16:0} and C_{18:0} across all samples ranged from -20.1‰ to -29.4‰, and -
18 262 21.2‰ to -31.5‰ respectively, with clear separation between marine and terrestrial fauna,
19 263 and between terrestrial C₃ and C₄ consumers. Similar isotopic differences were identified in
20 264 humans according to their bulk collagen isotope values (Fig. 5).

21
22
23 265 Importantly, the humans with mixed terrestrial C₃/marine diets plot towards the middle of the
24 266 range of isotopic values obtained here, and fall between humans with dominant marine and
25 267 C₃ terrestrial diets.

26
27 268 Nevertheless, two seals had C_{16:0} and C_{18:0} depleted in ^{13}C relative to the other marine
28 269 organisms, plotting among the C₃ consumers, and did not correlate with the bulk collagen
29 270 isotope data (Tab. 1). This is unlikely to be the result of contamination, since both seals come
30 271 from a marine shell midden site (Oronsay, Western Scotland) where contamination with
31 272 lipids from the depositional matrix would be expected to elevate $\delta^{13}\text{C}$ values. While the seal
32 273 values appear to be outliers in the data presented here, similar $\delta^{13}\text{C}$ values of bulk lipids from
33 274 modern seals have been reported previously [35,36].

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35
36 275 As expected, the fatty acids were consistently depleted in ^{13}C relative to bulk collagen values
37 276 from the same samples (Fig. 6). Such ^{13}C depletion reflects fundamental differences between
38 277 the biosynthesis of collagen and fatty acids. Both C_{16:0} and C_{18:0} fatty acids can either be
39 278 directly routed from diet or biosynthesised *de novo* from all dietary macronutrients. However,
40 279 a major kinetic isotope effect in lipid biosynthesis occurs through the oxidative
41 280 decarboxylation of pyruvate to acetyl Co-A resulting in a depletion in ^{13}C [37].

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43
44 281 Interestingly, the offset between collagen and lipids ($\Delta^{13}\text{C}_{\text{FA-COLL}}$) is not consistent and
45 282 ranges from ~6‰ to ~18‰. This variation may reflect isotopic differences in dietary
46 283 macronutrients, however $\Delta^{13}\text{C}_{\text{FA-COLL}}$ offset in marine fauna is consistently larger than in
47 284 terrestrial animals. The offset in terrestrial C₃ animals (6.0‰ to 11.4‰) was consistent with
48 285 the expected value for individuals with diets high in carbohydrates [19,20], where non-
49 286 essential amino acids and fatty acids are at least in part *de novo* synthesised from this source.
50 287 The larger $\Delta^{13}\text{C}_{\text{FA-COLL}}$ observed in the marine organisms (i.e. >13‰) may be due to the fact
51 288 that the pool of dietary carbon in fish and marine mammals is derived from a greater amount
52 289 of lipid compared to terrestrial herbivores, which then becomes further depleted in ^{13}C during
53 290 subsequent lipogenesis. More work to determine the isotopic differences between essential
54 291 and non-essential fatty and amino acids is needed to understand the degree of routing versus
55 292 biosynthesis of macronutrients in marine organisms.

1
2
3 293 **Conclusions**

4 294 By using a modified two-step protocol, we show that a greater range of lipids than previously
5 295 encountered can be reliably recovered from archaeological bone. The extraction of
6 296 appreciable amounts of endogenous fatty acids from bone buried even in temperate
7 297 environments is particularly promising since these compounds can be traced isotopically to
8 298 dietary macronutrients or directly incorporated from known dietary sources. In addition, $\delta^{13}\text{C}$
9 299 values of fatty acids associated with artefacts have been widely measured in ancient contexts
10 300 providing the opportunity to compare data. New avenues for research on bone fatty acids
11 301 include the comparison with bulk collagen and single amino acids to identify the source and
12 302 relative contribution of dietary protein, lipids and carbohydrates with more accuracy.
13 303 Furthermore, as lipids turn-over much faster in bone in comparison to collagen, changes in
14 304 diet through life may also be studied using this approach.

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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.

Samples	Country	Site	Aprox. Age	Taxa	Inferred diet	Lipid conc. (ug/g ¹)	$\delta^{13}\text{C}_{16:0}$	$\delta^{13}\text{C}_{18:0}$	$\delta^{13}\text{C}_{\text{COLL}}\%$	$\delta^{15}\text{N}_{\text{COLL}}\%$	Reference
CC10502	UK	Cnoc Coig	5500 cal BP	Pinnipedia	Marine	24.9	-28.1	-29.7	-11.6	+18.8	This study
CC10420	UK	Cnoc Coig	5500 cal BP	Pinnipedia	Marine	35.9	-27.5	-29.2	-11.8	+19.5	This study
CRB 21	Brazil	Jabuticabeira II	3000 cal BP	<i>Pogonias cromis</i>	Marine	4.9	-24.1	-24.7	-9.8	+12.6	Colonese et al. ^[23]
CRB 26	Brazil	Jabuticabeira II	3000 cal BP	Cetacea	Marine	7.7	-24.2	-25.9	-10.8	+16.1	Colonese et al. ^[23]
104A-L1.85	Brazil	Jabuticabeira II	3000 cal BP	<i>Homo sapiens</i>	Marine	9.2	-23.5	-23.3	-11.2	+18.8	Colonese et al. ^[23]
1.1/2-T10	Brazil	Jabuticabeira II	3000 cal BP	<i>Homo sapiens</i>	Marine	24.3	-20.1	-21.2	-11.2	+17.6	Colonese et al. ^[23]
OTD	Denmark	Havnø	5600 cal BP	<i>Homo sapiens</i>	Marine	8.3	-25.2	-25.5	-11.5	+13.3	This study
AEEEE/OL1	Denmark	Bjørnsholm	4000 cal BP	<i>Halichoerus grypus</i>	Marine	20.6	-24.1	-24.2	-10.4	+13.8	Craig et al. ^[25]
CRB 34	Brazil	Moraes	6000 cal BP	<i>Agouti paca</i>	Terrestrial C ₃	16.8	-27.9	-29.7	-20.8	+7.9	Colonese et al. ^[23]
CRB 36	Brazil	Moraes	6000 cal BP	<i>Alouatta sp.</i>	Terrestrial C ₃	9.2	-27.5	-28.4	-21.5	+7.9	Colonese et al. ^[23]
37A	Brazil	Moraes	6000 cal BP	<i>Homo sapiens</i>	Terrestrial C ₃	16.9	-27.3	-28.9	-21.0	+10.3	Colonese et al. ^[23]
9A	Brazil	Moraes	6000 cal BP	<i>Homo sapiens</i>	Terrestrial C ₃	19.6	-27.6	-28.9	-20.8	+11.4	Colonese et al. ^[23]
HSU	Denmark	Havnø	Mesolithic/Neolithic	<i>Capreolus capreolus</i>	Terrestrial C ₃	13.3	-29.2	-29.7	-22.9	+3.5	Gron ^[26]
ABM08*	Spain	Almacabra de Albarracín	10 th -12 th	<i>Homo sapiens</i>	Terrestrial C ₃	52.9	-26.6	-27.0	-18.9	+11.9	Mundee ^[27]
ABM08	Spain	Almacabra de Albarracín	10 th -12 th	<i>Homo sapiens</i>	Terrestrial C ₃	26.4	-25.6	-27.3	-18.9	+11.9	Mundee ^[27]
CC17050	UK	Cnoc Coig	5500	<i>Sus scrofa</i>	Terrestrial C ₃	24.5	-28.0	-28.3	-21.0	+4.6	This study
GBC2*	Spain	Benipeixcar	15 th -16 th	<i>Bos taurus</i>	Terrestrial C ₃	81.7	-27.7	-28.5	-20.1	+5.8	Alexander et al. ^[24]
GBC2	Spain	Benipeixcar	15 th -16 th	<i>Bos taurus</i>	Terrestrial C ₃	31.3	-29.4	-31.5	-20.1	+5.8	Alexander et al. ^[24]
XPG	Denmark	Havnø	4000 cal BP	<i>Homo sapiens</i>	Terrestrial C ₃ /marine	7.0	-26.0	-26.7	-17.9	+12.5	This study
24A-L2	Brazil	Jabuticabeira II	3000 cal BP	<i>Homo sapiens</i>	Terrestrial C ₃ /marine	24.1	-24.8	-26.6	-16.2	+12.7	Colonese et al. ^[23]
17C-L2	Brazil	Jabuticabeira II	3000 cal BP	<i>Homo sapiens</i>	Terrestrial C ₃ /marine	59.8	-23.8	-27.1	-18.4	+11.1	Colonese et al. ^[23]
GBC4*	Spain	Benipeixcar	15 th -16 th	<i>Bos taurus</i>	Terrestrial C ₄	201.1	-28.7	-28.9	-14.3	+7.4	Alexander et al. ^[24]
GBC4	Spain	Benipeixcar	15 th -16 th	<i>Bos taurus</i>	Terrestrial C ₄	11.5	-25.3	-27.4	-14.3	+7.4	Alexander et al. ^[24]
GBM2404*	Spain	Benipeixcar	15 th -16 th	<i>Homo sapiens</i>	Terrestrial C ₄	110.4	-26.7	-26.3	-16.4	+11.0	Alexander et al. ^[24]
GBM2404	Spain	Benipeixcar	15 th -16 th	<i>Homo sapiens</i>	Terrestrial C ₄	30.9	-22.9	-25.1	-16.4	+11.0	Alexander et al. ^[24]

Supporting information 1 (S11) - Composition of lipid extracts. Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; DUFA, diunsaturated fatty acid; br, branched fatty acid. Method 1 (*).

Samples	Taxa	Site	Lipids extracted
CC10502	Pinnipedia	Cnoc Coig, Oronsay	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
CC10420	Pinnipedia	Cnoc Coig, Oronsay	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, pristanic
CRB21	<i>Pogonias cromis</i>	Jabuticabeira II	SFA(C14:0-18:0, C20:0, C22:0, C24:0), MUFA(C18:1, C24:1), br (C15-17), phytanic, pristanic
CRB26	Cetacea	Jabuticabeira II	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C18:1), br (C15-18), phytanic, pristanic
104A-L1.85	<i>Homo sapiens</i>	Jabuticabeira II	SFA(C14:0-18:0, C20:0, C22:0, C24:0), br (C15-17), phytanic, pristanic
1.1.2-T10	<i>Homo sapiens</i>	Jabuticabeira II	SFA(C12:0-20:0, C22:0-C24:0), MUFA(C16:1, C18:1), br (C15-17), cholesta-3,5-diene, phytanic, pristanic
OTD	<i>Homo sapiens</i>	Havno	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), br (C15-17), cholesta-3,5-diene, phytanic, pristanic
AEEE/OL1	Björnsholm	Björnsholm	SFA(C14:0-24:0, C26:0), MUFA(C16:1, C18:1, C24:1), DUFA(C18:2), br (C15-18), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), pristanic
CRB34	<i>Agouti paca</i>	Moraes	SFA(C14:0-18:0, C20:0, C22:0, C24:0), MUFA(C16:1, C18:1, C24:1), br (C15-17), phytanic, pristanic
CRB36	<i>Alouatta sp.</i>	Moraes	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), br (C15-18), pristanic
37A	<i>Homo sapiens</i>	Moraes	SFA(C12:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C22:1), br (C15-17), DA(C13), phytanic, pristanic
9A	<i>Homo sapiens</i>	Moraes	SFA(C12:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C22:1, C24:1), br (C15, 16), DA(C9), cholesta-3,5-diene, phytanic, pristanic
HSU	<i>Capreolus capreolus</i>	Havno	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), DUFA(C18:2), br (C15-18), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), phytanic, pristanic
ABM08*	<i>Homo sapiens</i>	Almacabra	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C22:1, C24:1), DUFA(C18:2), br (C15-17), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, phytanic
ABM08	<i>Homo sapiens</i>	Almacabra	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C22:1, C24:1), DUFA(C18:2), br (C15-17), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, phytanic
CC17050	<i>Sus scrofa</i>	Cnoc Coig, Oronsay	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1), DUFA(C18:2), br (C15-17), cholesta-3,5-diene, phytanic, pristanic
GBC2*	<i>Bos taurus</i>	Alqueria de Benipeixcar	SFA(C12:0, C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C22:1, C24:1), DUFA(C18:2), br (C15-18), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, phytanic, pristanic
GBC2	<i>Bos taurus</i>	Alqueria de Benipeixcar	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C22:1, C24:1), DUFA(C18:2), br (C15-18), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, phytanic, pristanic
XPG	<i>Homo sapiens</i>	Havno	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), br (C15-17), cholesta-3,5-diene
24A-L2	<i>Homo sapiens</i>	Jabuticabeira II	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), DUFA(C18:2), br (C15-17), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), phytanic, pristanic
17C-L2	<i>Homo sapiens</i>	Jabuticabeira II	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), br (C15-17), cholesta-3,5-diene, cholest-5-ene- (3-methoxy)
GBC4*	<i>Bos taurus</i>	Alqueria de Benipeixcar	SFA(C14:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), DUFA(C18:2), br (C15-18), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, phytanic, pristanic
GBC4	<i>Bos taurus</i>	Alqueria de Benipeixcar	SFA(C15:0-18:0, C20:0, C22:0-C24:0), MUFA(C16:1, C18:1, C24:1), DUFA(C18:2), br (C15-18), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, phytanic, pristanic
GBM24/04	<i>Homo sapiens</i>	Benipeixcar	SFA(C12:0, C14:0-18:0, C20:0, C22:0, C24:0), MUFA(C18:1, C20:1, C22:1, C24:1), DUFA(C18:2), br (C15, 16), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, pristanic
GBM2404*	<i>Homo sapiens</i>	Benipeixcar	SFA(C12:0, C14:0-18:0, C20:0, C22:0, C24:0), MUFA(C18:1, C20:1, C22:1, C24:1), DUFA(C18:2), br (C15, 16), cholesta-3,5-diene, cholest-5-ene- (3-methoxy), cholesterol, pristanic

Figure captions

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

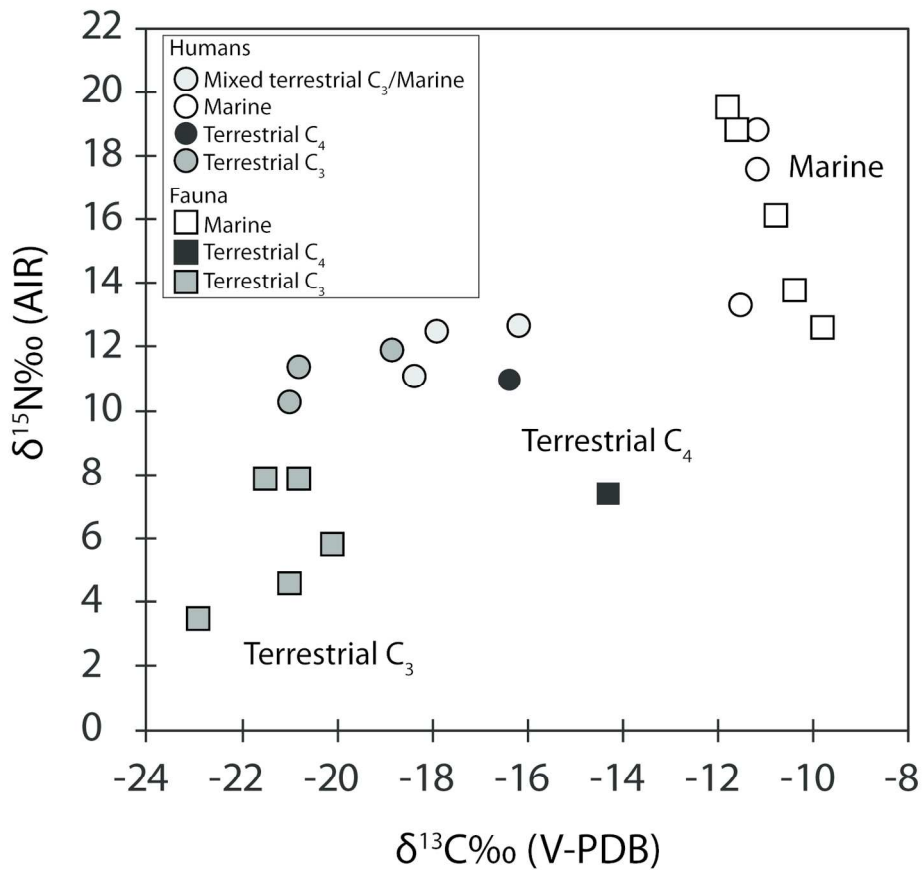
Fig. 2. Comparison of partial gas chromatogram of a bovine (GBC2) bone lipid extract using method 1 (A) and 2 (B). Abbreviations: $\text{C}_{n:x}$ indicates a fatty acid with n carbon atoms and x double bond; P indicates contaminant phthalate; IS indicates internal standard.

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: $\text{C}_{n:x}$ 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 μg of tetratriacontane).

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

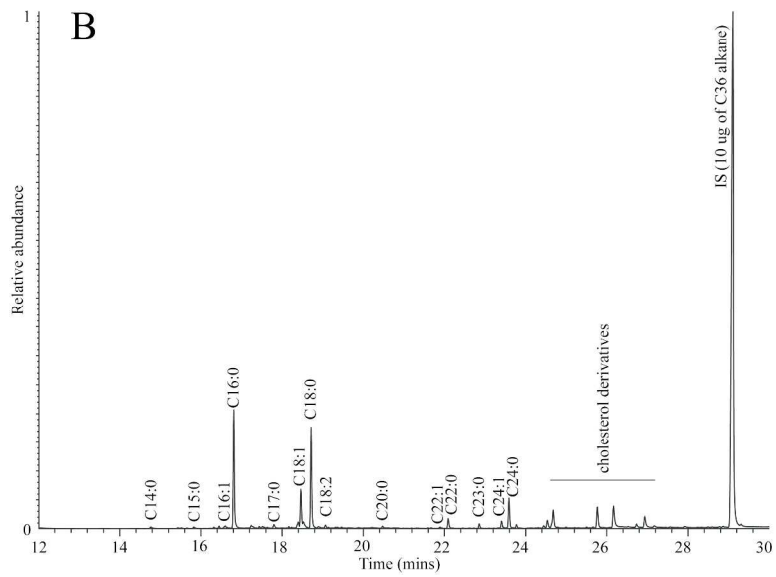
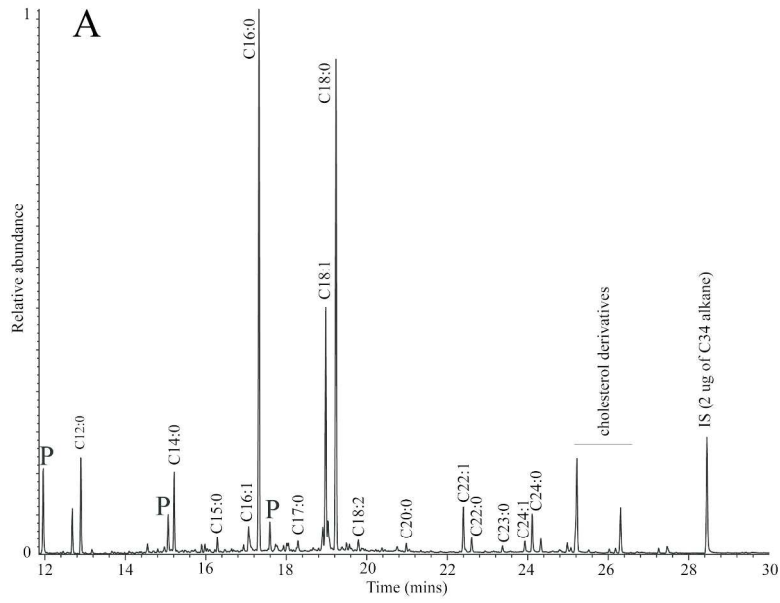
Fig. 6. Offset between $\delta^{13}\text{C}$ values of $\text{C}_{16:0}$ and $\text{C}_{18:0}$ fatty acids and collagen ($\Delta^{13}\text{C}_{\text{FA-Coll}}$).



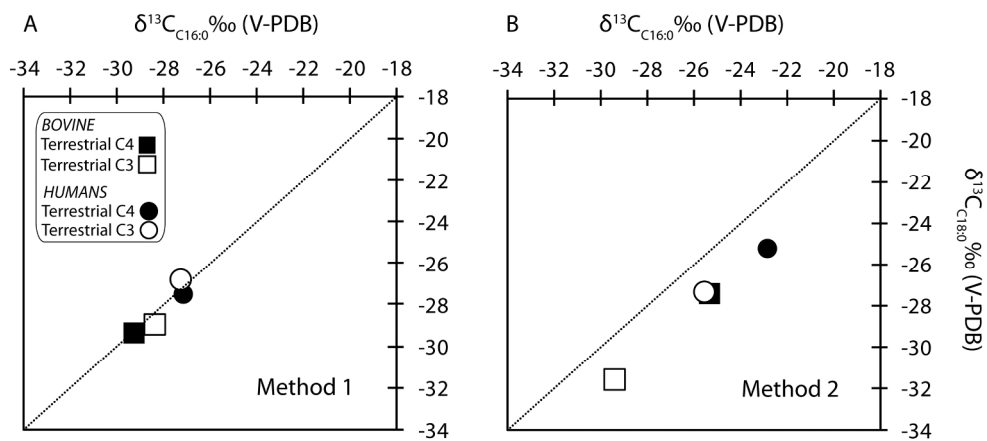
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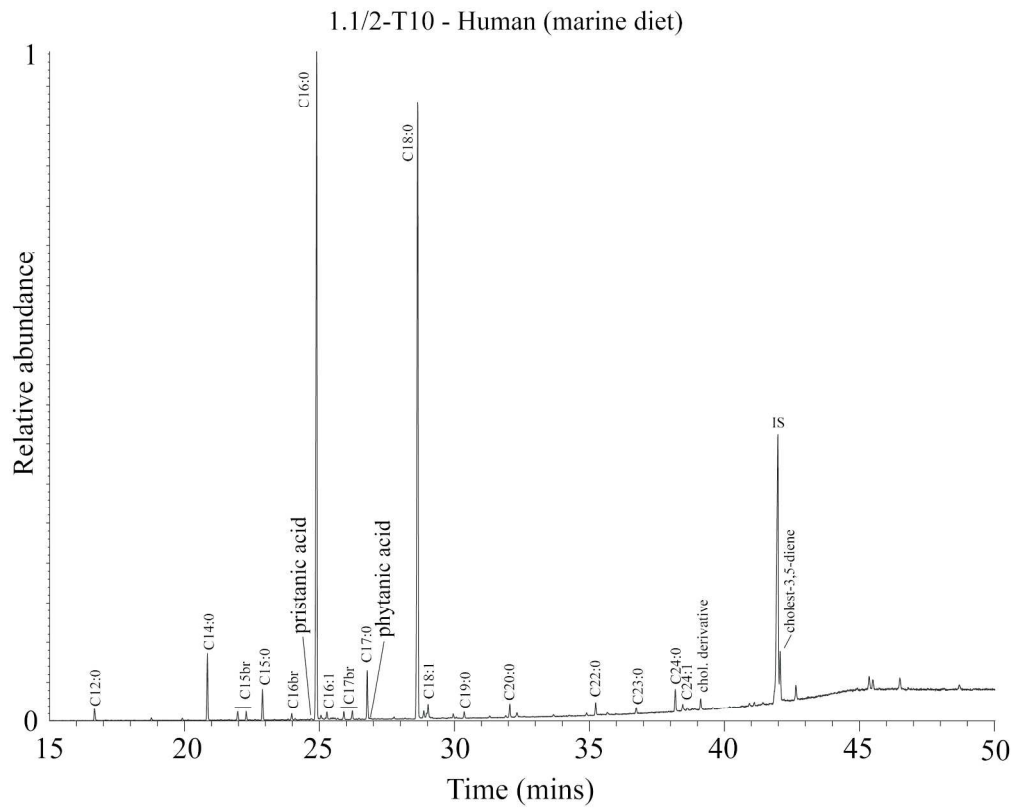
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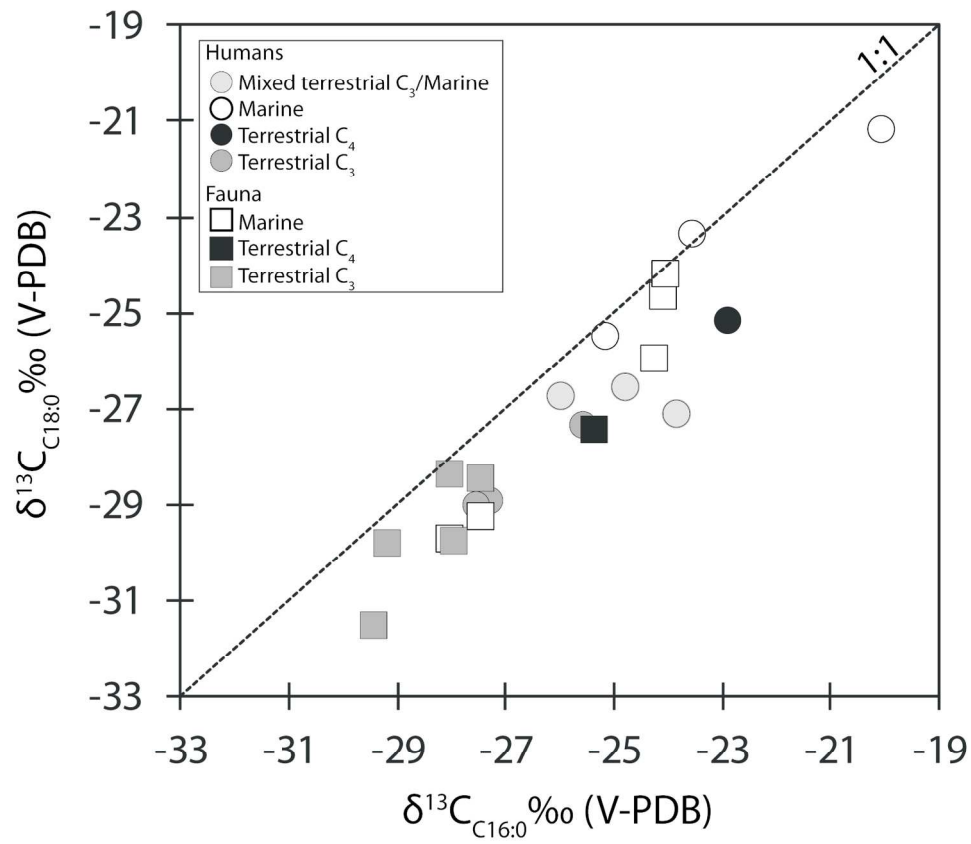
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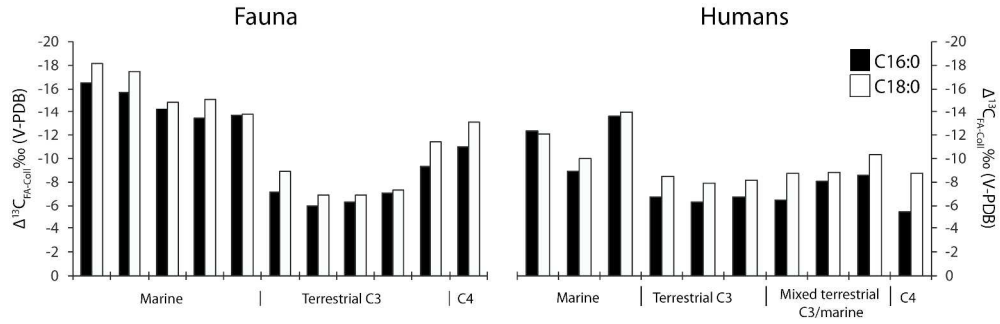
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