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ORIGINAL PAPER

Impact of modern cattle feeding practices on milk fatty acid stable carbon isotope compositions emphasise the need for caution in selecting reference animal tissues and products for archaeological investigations

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Abstract Degraded animal fats, characterised by the presence of palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) fatty acids and related glycerolipids are the most common class of preserved lipids in organic residues trapped in the porous clay matrix of archaeological ceramic vessels. The ubiquitous presence of fatty acids in animal fats and plant oils precludes identification of fat types by the solely molecular composition of residues. Hence, animal fats are identified by determining their fatty acyl lipid distributions and stable carbon ($\delta^{13}C$) values allowing distinctions to be drawn between non-ruminant and ruminant, and dairy and adipose fats. The $\Delta^{13}C$ proxy (= $\delta^{13}C_{18:0}$ - $\delta^{13}C_{16:0}$) originally proposed in the 1990s by Evershed and co-workers was based on modern reference fats sampled from animals raised in Britain on C_3 plant diets. Further analyses on adipose and dairy fats from ruminants grazing in a wide range of isoscapes have shown that the $\Delta^{13}C$ proxy can be applied in mixed C_3/C_4 environments, such as in Africa. Here we show, however, through the investigation of milk fats, how the $\Delta^{13}C$ proxy can be perturbed when animals are reared on modern diets, specifically maize silage. It is thus shown that extreme care has to be taken when choosing modern reference fats for archaeological studies, and especially that insecurely sourced animal fats should be excluded from such databases.

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Introduction

The most common class of lipids extracted from archaeological potsherds are degraded animal fats, recognisable by the presence in high temperature-gas chromatography (HT-GC) profiles of triacylglycerols (TAGs) and their degradation products, namely: diacylglycreols (DAGs), monoacylglycerols (MAGs) and free fatty acids (Evershed et al., 1990, 2002a). The routine identification of the fat source based on comparison of the molecular distributions, e.g. TAG and fatty acid compositions, with modern reference fats is complicated due to changes in composition brought about by vessel use and burial (Evershed, 2008). Thus, in the 1990s, Evershed and co-workers began to explore the use of compound-specific stable isotopic techniques to provenance these degraded archaeological animal fats by determining the stable carbon isotopic composition ($\delta^{13}C$ values) of the two major fatty acids $C_{16:0}$ and $C_{18:0}$, namely palmitic and stearic acids (Evershed et al., 1994, 1997).

The development of any biomolecular or stable isotope proxy for reconstructing past environments or processes requires measurements of reference collections of organisms from contemporary or past environments of known provenance. Hence, the determination of the origins of ancient fats based on their stable carbon isotope compositions required comparison of the compound-specific $\delta^{13}C$ values of palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids of archaeological fats with the same compounds in modern reference fats. The founding study highlighted differences in the $\delta^{13}C$ values of $C_{16:0}$ and $C_{18:0}$ fatty acids from different sources of terrestrial animals (non-ruminant and ruminant) and different fat types (adipose

and dairy fats; Dudd and Evershed, 1998; Copley et al., 2003). The $\delta^{13}C$ values of the major saturated fatty acids in modern reference fats were interpreted as reflecting fundamental differences in the metabolism and physiology of ruminant and non-ruminant animals and in the biosynthesis of fats in different tissues, i.e. adipose versus mammary, suggesting that dairy and adipose fats could be distinguished based on the difference in their $\delta^{13}C_{18:0}$ values (Copley et al., 2003; Mukherjee et al., 2005). However, this relationship is complicated by the fact that $\delta^{13}C$ values of fatty acids reflect dietary $\delta^{13}C$ values, which vary e.g. according to the photosynthetic pathway (C_3 and C_4) of plants. Therefore, the $\Delta^{13}C$ ($=\delta^{13}C_{18:0} - \delta^{13}C_{16:0}$) proxy was introduced to remove the environmental component of the $\delta^{13}C$ variation common to both the $C_{16:0}$ and $C_{18:0}$ fatty acids, leaving only the variations attributable to the metabolic and physiological processes in the animals (Evershed et al., 2002b; Dunne et al., 2012).

The reference fats analysed in the studies of Copley et al. (2003) and Dunne et al. (2012) were carefully sampled from animals raised on diets isotopically similar to those that would have existed in prehistory, i.e. grass pastures and fodder. However, a number of recent studies have derived $\delta^{13}C$ values of fatty acids from meat or dairy products sourced from markets, animals raised on unknown diets and potentially exposed to modern practices (e.g. Spangenberg et al., 2006; Gregg et al., 2009). Researchers have argued that these collections are required to account for regional variations; however, exposure of the modern diets, e.g. silages, where that natural balance of macronutrients are affected and their carbon isotopic relationships are lost, is likely to introduce confounding factors. Here, we present the results of molecular and isotopic analyses of fatty acids from milk obtained from cattle reared on three types of diet, ranging from a pure C_3 natural diet (pastures and fodder) to pure silage (grass and maize).

Materials and methods

Samples

The study compares milk from either cattle raised on a strict C_3 pasture diet (diet A; $n = 8$; reported in Dudd and Evershed, 1998; and Copley et al., 2003), with milk sampled on two occasions from the Langford Farm (University of Bristol, UK) herd: (i) in April 2009, when cattle were feeding on a mixed grass/maize silage (C_3/C_4) overnight, while grazing on grass swards (predominately *Lolium perenne*; C_3) during the day (diet B; $n = 10$) and (ii) in January 2010, when cattle were housed and feeding entirely on the same mixed grass/maize silage (C_3/C_4; diet C; $n = 10$).

Lipid analyses

Lipid analyses were performed using established protocols described in detail in other publications (Dudd et al., 1998; Copley et al., 2003; Evershed et al., 2008). The animal fats were freeze-dried and a portion (few mg) extracted with a mixture of chloroform/methanol (2 × 3 mL, 2:1 *v/v*) by sonication (2 × 20 min). The solvent was evaporated to dryness under a gentle stream of nitrogen, and the total lipid extract (TLE) stored in a refrigerator until required for further analysis. The TLE was weighed, and a mixture of chloroform/methanol (2:1 *v/v*) added to give a 1 mg mL^{-1} solution. An aliquot (the equivalent of 1 mg of fat) was treated with $NaOH/H_2O$ (9:1 *w/v*) in methanol (5 % *v/v*; 70 °C, 1 h). Following neutralisation, lipids were extracted in dichloromethane (3 × 3 mL) and excess solvent evaporated under a gentle stream of nitrogen. Fatty acid methyl esters (FAME) were prepared by reaction with BF_3-methanol (14 % w/v; 70 °C, 1 h). FAME derivatives were extracted with dichloromethane (3 × 2 mL), and the solvent removed with a gentle stream of nitrogen. FAME derivatives were then re-dissolved into hexane for analysis by gas chromatography (GC) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

The $\delta^{13}C$ values obtained for the modern reference fats were not adjusted for post-industrial revolution effects of fossil fuel burning (Friedli et al., 1986) and are thus not directly comparable to $\delta^{13}C$ values from archaeological fats.

Results

Fatty acid composition of dairy fats

The major fatty acids in cattle milk are $C_{16:0}$ and $C_{18:1}$, with a lower proportion of $C_{18:0}$, and characteristic short-chain fatty acids (C_2 to C_{14}; Jensen et al., 1962). The main fatty acid composition of dairy fats from cattle fed on diets B and C are presented in Table 1. The percentage of $C_{14:0}$ and $C_{18:0}$ fatty acids of the total fatty acids do not vary from diet B to diet C. However, the percentage of the $C_{16:0}$ fatty acid of the total fatty acids increased (*t* test, p value = 0.02) while the abundance of the unsaturated $C_{18:n}$ fatty acids decreased (*t* test, p value = 0.02) significantly in dairy fats from cattle raised on diet B to diet C. Furthermore, a significant increase in the $C_{16:0}/C_{18:0}$ ratio (*t* test p value = 0.04) is observed between dairy fats from cattle raised on diet B and C. Several studies have shown previously an increase in the $C_{16:0}$ fatty acid content of cattle milk when the diet is changed from grass to silage (e.g. Elgersma et al., 2004).

Isotopic composition of dairy fats

The mean $\delta^{13}C_{16:0}$ values of dairy fats raised on a pure C_3 grass diet A (Copley et al., 2003), diet B (~20 dry matter weight % of

Table 1 Composition (% of the total fatty acids) of some individual fatty acids and ratios of fatty acids, for dairy fats from cattle raised on diet B (fresh grass (C_3) and a mixed grass/maize (C_3/C_4) silage; n = 9, one sample was considered as outlier) and diet C (mixed grass/maize (C_3/C_4) silage; n = 10)

	Diet B		Diet C		p value	Significance
	%	s.d	%	s.d		
$C_{14:0}$	7.8	2.5	8.9	1.9	0.28	NS
$C_{16:0}$	32.4	2.6	36.7	4.6	0.02	*
$C_{18:0}$	15.3	2.3	14.2	2.0	0.29	NS
$C_{18:n}$ (total)	35.7	4.7	30.1	4.6	0.02	*
$C_{18:n}/C_{18:0}$	2.40	0.60	2.16	0.46	0.46	NS
$C_{16:0}/C_{18:0}$	2.17	0.43	2.63	0.46	0.04	*

Note: datasets are compared using statistical tests that are currently usually used for this type of study, although it should be noted that the inferences are limited as the data is compositional

Significance of the measurements between means at *NS* not significant

*p value <0.05 (t tests for all but for $C_{18:n}/C_{18:0}$ ratio—Kruskall-Wallis test)

maize silage) and diet C (~30 % dry matter weight % of maize silage) are −30.2, −26.9 and −26.2 ‰, respectively, while the mean $\delta^{13}C_{18:0}$ values are −35.2, −30.9 and −28.9 ‰, respectively (Figs. 1, 2). $\delta^{13}C_{18:0}$ values become more enriched in ^{13}C when the proportion of C_4 plants in the diet is increased (Tukey HSD tests, p value <0.0001 for all three comparisons). $\delta^{13}C_{16:0}$

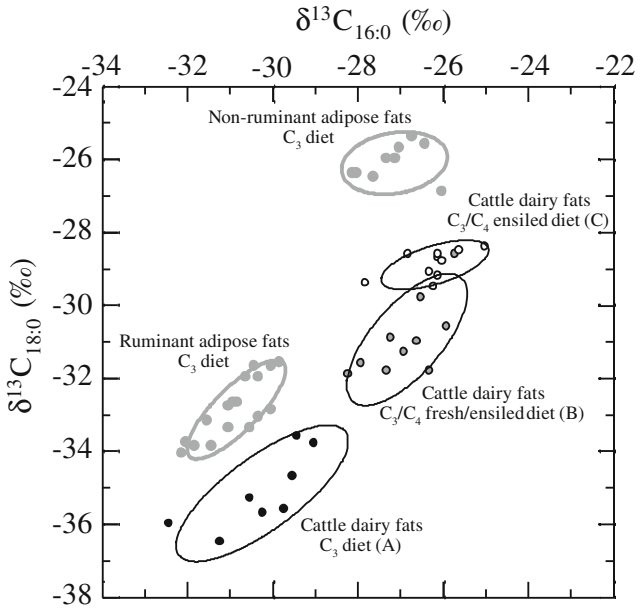

Fig. 1 $\delta^{13}C$ values for $C_{16:0}$ and $C_{18:0}$ fatty acids from milk of cattle raised in the UK on a C_3 diet (*black dots*, diet A; Copley et al., 2003); fresh grass (C_3) and a mixed grass/maize (C_3/C_4) silage (*grey dots*, diet B, this study) and a mixed grass/maize (C_3/C_4) silage (*white dots*, diet C, this study). For comparison, $\delta^{13}C$ values for $C_{16:0}$ and $C_{18:0}$ fatty acids from ruminant adipose fats (cattle and sheep) and non-ruminant adipose fats (pigs) in *grey* (Copley et al., 2003). Confidence ellipses are 1 sigma CI.

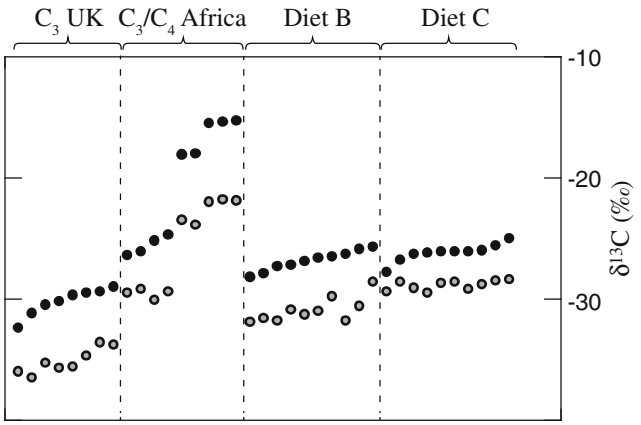

Fig. 2 $\delta^{13}C$ values for $C_{16:0}$ (*black dots*) and $C_{18:0}$ (*grey dots*) fatty acids (in order of increasing $\delta^{13}C_{16:0}$ values) from milk of cattle raised on (i) C_3 UK: a C_3 diet in the UK (Copley et al., 2003); (ii) C_3/C_4 Africa: mixed C_3/C_4 diets in Kenya (Dunne et al., 2012); (iii) diet B: fresh grass (C_3) and mixed grass/maize (C_3/C_4) silage in the UK (this study) and (iv) diet C: mixed grass/maize (C_3/C_4) silage in the UK (this study)

values are more enriched in ^{13}C when cattle are fed on diets B or C compared to diet A (Tukey HSD test, p value <0.0001 for the two comparisons). However, differences in the $\delta^{13}C_{16:0}$ values are less significant when cattle are raised on diets B or C (Tukey HSD test, p value = 0.2458), probably because of small sample sizes.

The $\Delta^{13}C$ values of dairy fats from cattle raised on diet B range between −5.5 and −3.0 ‰ (mean −4.1 ‰) show no significant difference to the $\Delta^{13}C$ values of dairy fats from cattle raised on a pure C_3 diet (Tukey HSD test, p value = 0.2146). In contrast, $\Delta^{13}C$ values of dairy fats from cattle raised on diet C range between −3.4 and −1.6 ‰ (mean −2.7 ‰) and are significantly higher compared to those of cattle raised on C_3 fresh pasture (Tukey HSD test, p value <0.0001; Fig. 3).

In order to assess the influence of C_4 plant component of the silage on the $\Delta^{13}C$ values of cattle milk, milk from cattle raised in the UK on diets ranging from C_3 plants to C_3/C_4 silage (diets A, B and C) were compared to milk from cattle raised on natural pastures of mixed C_3 and C_4 plants in Kenya (n = 9). Dairy fats from cattle from Kenya exhibit $\delta^{13}C_{16:0}$ values ranging from −26.4 to −15.3 ‰ (mean −20.5 ‰) and $\delta^{13}C_{18:0}$ values ranged from −30.1 to −21.8 ‰ (mean −25.7 ‰; Dunne et al., 2012; Fig. 1a). They are thus on average ca. 10 ‰ more enriched in ^{13}C than dairy fats from cattle raised on a C_3 diet in the UK (Copley et al., 2003), confirming the influence of C_4 plants in their diet (Fig. 2).

Dairy fats from cattle from Kenya exhibit $\Delta^{13}C$ values ranging from −3.1 to −6.6 ‰ (mean −5.2 ‰; Dunne et al., 2012; Fig. 1b) show no evidence of differing (t test, p value = 0.6054) from the milk from cattle raised on a pure C_3 diet in the UK (Fig. 3; Copley et al., 2003). No changes in the $\Delta^{13}C$ values of milk fats from cattle raised on a diet comprising fresh C_4 plants are thus observed, suggesting that the presence of fresh C_4 plants in the animal's diet does not affect

$\Delta^{13}C$ values. The increase in the $\Delta^{13}C$ values of dairy fats from cattle raised on diet C (mixture of maize/grass silage) is thus likely to be linked to modern feeding practices.

Discussion

As expected, the $\delta^{13}C$ values of $C_{16:0}$ and $C_{18:0}$ fatty acids extracted from cattle milk are more enriched in ^{13}C when cattle are raised on diet containing increasing amounts of C_4 plant matter (maize). While C_3 plants fix atmospheric CO_2 using the ribulose biphosphate carboxylase (RuBisCO) catalysed Calvin cycle (Calvin and Benson, 1948) and discriminate against $^{13}CO_2$ (Boutton, 1991), C_4 plants fix atmospheric CO_2 with the Hatch-Slack pathway (Hatch and Slack, 1966) which discriminates less against $^{13}CO_2$ (Boutton, 1991). Bulk $\delta^{13}C$ values of C_3 and C_4 plants thus range from −34 to −24 ‰ and −19 to −6 ‰, respectively (Smith and Epstein, 1971). Hence, the $\delta^{13}C$ values of the fatty acids and carbohydrates are more enriched in C_4 plants compared to C_3 plants. The carbohydrates and fatty acids are then incorporated into the body fats of the consumer animals, leading to enriched $\delta^{13}C_{16:0}$ and $\delta^{13}C_{18:0}$ values of synthesised milk.

The difference between the raw $\delta^{13}C$ values of $C_{18:0}$ and $C_{16:0}$ fatty acids (namely $\Delta^{13}C$ values) in milk from cattle raised on a pure C_3 diet range between −5.9 and −3.6 ‰. Furthermore, milk from cattle raised traditionally on pastures comprising C_3 and C_4 plants in Africa display $\Delta^{13}C$ (= $\delta^{13}C_{18:0}$ - $\delta^{13}C_{16:0}$) values similar to that observed in cattle raised on C_3 diet in Britain (Fig. 3). This difference in the isotopic composition of the two main fatty acids reflects the different sources for the $C_{16:0}$ and $C_{18:0}$ fatty acids in milk. In fact, while $C_{16:0}$ fatty acids are biosynthesised from carbohydrates and fatty acids from the diet, $C_{18:0}$ fatty acid in milk has

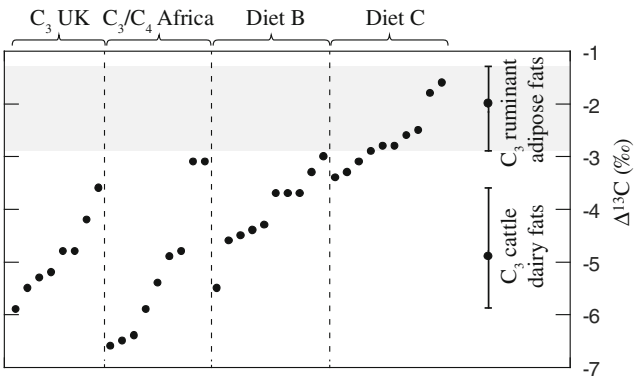

Fig. 3 $\Delta^{13}C$ (= $\delta^{13}C_{18:0}$ - $\delta^{13}C_{16:0}$) values (in order of increasing $\Delta^{13}C$ values) from milk of cattle raised on (i) C_3 UK: a C_3 diet in the UK (Copley et al., 2003); (ii) C_3/C_4 Africa: mixed C_3/C_4 diets in Kenya (Dunne et al., 2012); (iii) diet B: fresh grass (C_3) and mixed grass/maize (C_3/C_4) silage in the UK (this study) and (iv) diet C: mixed grass/maize (C_3/C_4) silage in the UK (this study). Ranges and mean values of C_3 ruminant (cattle and sheep) adipose fats and C_3 cattle dairy fats from the UK (Copley et al., 2003) for comparison

two distinct contributions. As the mammary gland is unable to biosynthesise the $C_{18:0}$ fatty acid, it is sourced from: (i) the remobilisation of $C_{18:0}$ fatty acids from the carcass depot fats and (ii) the direct incorporation of $C_{18:0}$ from the diet, after rumen biohydrogenation of $C_{18:n}$ fatty acids from dietary plants (Fig. 4a; Copley et al., 2003). The difference in the isotopic composition of these two pools relates to the 8 ‰ difference between carbohydrates and fatty acids in plants when pyruvate is decarboxylated to acetylCoA (DeNiro and Epstein, 1977) with the mass balance leading to the difference of ca. 3 ‰ evident between the $C_{18:0}$ fatty acids from carcass and dairy fats (Copley et al., 2003).

The consistent $\Delta^{13}C$ values arise because the carbon isotopic difference between fatty acids and carbohydrates (ca. 8 ‰) is common to all plants, no matter what photosynthetic fixation mechanism is used. The difference is controlled by fractionation resulting from the pyruvate dehydrogenase enzyme complex involved in the decarboxylation of the pyruvate in forming acetyl CoA (DeNiro and Epstein, 1977). Given the latter and the fact metabolism of ruminant mammals is consistent no matter what region of the world the animal comes from, the arithmetic transformation from raw $\delta^{13}C$ values to $\Delta^{13}C$ values is valid in removing the influence of varying proportions of C_3 and C_4 plants in ruminant forages (Mukherjee et al., 2005; Dunne et al., 2012). While this model was devised on the basis of fresh C_3 plant consumption, as discussed above, this difference of ca. 3 ‰ holds for cattle, or any other ruminant animal, raised on any combination of

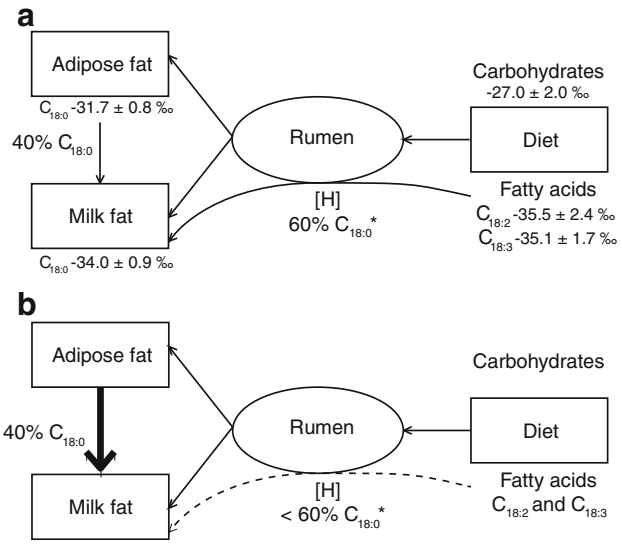

Fig. 4 Routing of dietary fatty acids and carbohydrates in the rumen, adipose tissue and mammary gland of the ruminant animal. **a** When fed on traditional diets, approximately 60 % of the $C_{18:0}$ fatty acid in milk appears to be directly incorporated from the diet, after the biohydrogenation of unsaturated fatty acids (e.g. $C_{18:3}$) in the rumen (after Copley et al., 2003). **b** When fed on high starch/high free oil, <60 % of the $C_{18:0}$ fatty acid in milk is directly incorporated from the diet, while >40 % is remobilized from the adipose fat, leading to $\delta^{13}C_{18:0}$ values in milk similar to those observed in adipose fats

traditional C_3 and C_4 pastures. This is confirmed here by the similarity between $\Delta^{13}C$ values of milk from cattle raised on a pure C_3 diet (diet A) or the C_3/C_4 diets from Africa.

In contrast, however, milk from cattle raised on a mixture of grass/maize silage exhibits $\Delta^{13}C$ higher values ranging from −3.4 to −1.6 ‰ (Fig. 3). The carbon isotopic compositions of the main saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) are thus more similar by *ca.* 2 ‰, compared to the same fatty acids from cattle grazing pasture (C_3 in the UK and C_3/C_4 in Kenya), suggesting that the $C_{16:0}$ and $C_{18:0}$ fatty acids have a more similar metabolic source than when cattle are grazing on herbage.

Changes in the biohydrogenation of polyunsaturated fatty acids in the rumen is known to happen when high starch and high free oil diets are fed to cattle, inducing milk fat depression through a downregulation of de novo synthesised fatty acids in the mammary gland. Remobilisation of $C_{18:0}$ fatty acids from the adipose fats to milk fats is thus increased when diets fed to cattle are high in starch or free oil (Peterson et al., 2003), leading to the carbon isotopic composition of $C_{18:0}$ fatty acids from milk fats being more comparable to that of $C_{18:0}$ fatty acids from the adipose fats (Fig. 4b). The difference between the $\delta^{13}C$ values of $C_{18:0}$ and $C_{16:0}$ fatty acids in milk is thus diminished, leading to less negative $\Delta^{13}C$ values.

Conclusions

The results presented herein lead to the following conclusions and recommendations:

(i) Feeding high starch and high concentrate/low forage diets to cattle alters the $\delta^{13}C$ values of $C_{18:0}$ fatty acids (and thus the $\Delta^{13}C$ values) of their milk by affecting pathways of fatty acids biosynthesis. In prehistory, cattle were very unlikely to have been fed such diets as high starch or oil rich produce are likely to have been kept for human consumption. Furthermore, human food waste would likely have been fed to pigs.

(ii) Any modern feeding practices, in which the biochemical and isotopic relationships that exist in natural plants are lost, may affect other species and other products (e.g. cattle and deer adipose fats). Hence, animals raised using modern manipulated or formulated feeds, i.e. silage and concentrates, must be avoided when building a reference dataset to compare with archaeological animal fats.

(iii) In our experience, establishing the validity of reference fats requires explicit conversations with the actual farmers to establish the feeds they employ during the entire annual feeding cycle.

(iv) The label 'organic' does not offer any guarantee in terms of comparability to prehistoric farming practices as a modern 'organic' cattle diet will still include silages and formulated feeds, which negate the use of animals raised on such diets for interpreting the origins of archaeological animal fat residues.

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