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Distinguishing wild ruminant lipids by gas chromatography/combustion/isotope ratio mass spectrometry

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RATIONALE: The carbon isotopic characterisation of ruminant lipids associated with ceramic vessels has been crucial for elucidating the origins and changing nature of pastoral economies. $\delta^{13}C$ values of fatty acids extracted from potsherds are commonly compared with those from the dairy and carcass fats of modern domesticated animals to determine vessel use. However, the processing of wild ruminant products in pottery, such as deer, is rarely considered despite the presence of several different species on many prehistoric sites. To address this issue, the carbon isotope range of fatty acids from a number of red deer (*Cervus elaphus*) tissues, a species commonly encountered in the European archaeological record, was investigated.
METHODS: Lipids were extracted from 10 modern red deer tissues obtained from the Slowinski National Park (Poland). Fatty acids were fractionated, methylated and analysed by gas chromatography/combustion/isotope ratio mass spectrometry (GCCIRMS). The $\delta^{13}C$ values of *n*-octadecanoic acid and *n*-hexadecanoic acid, and the difference between these values ($\Delta^{13}C$), were compared with those from previously published ruminant fats.
RESULTS: Nine of the ten deer carcass fats measured have $\Delta^{13}C$ values of less than −3.3‰, the threshold previously used for classifying dairy products. Despite considerable overlap, dairy fats from domesticated ruminants with $\Delta^{13}C$ values less than −4.3‰ are still distinguishable.
CONCLUSIONS: The finding has implications for evaluating pottery use and early pastoralism. The processing of deer tissues and our revised criteria should be considered, especially where there is other archaeological evidence for their consumption. Copyright © 2012 John Wiley & Sons, Ltd.

The analysis of lipids associated with prehistoric pottery has led to major new insights into the transition to farming[1] and the nature of early pastoral economies,[2–5] and is now an established method in archaeological research. Data from lipid residues are particularly important for identifying dairying in the archaeological record and have been pivotal for defining the emergence of dairying in western Eurasia[3] and North Africa.[5] As lipid compounds are lost and/or become highly modified through exposure to the burial environment, these studies have relied almost entirely on carbon isotopic characterisation of the medium length $C_{16:0}$ and $C_{18:0}$ *n*-alkanoic acids to determine vessel use.[6] These fatty acids, present in all animal fats, are readily extractable from unglazed ceramic cooking pots up to at least 9000 years old. Although not diagnostic in themselves, their carbon isotopic characteristics are reliably preserved allowing comparison with modern authentic reference fats to distinguish different animals products from ruminant, non-ruminant, freshwater and marine species.

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Differences in the biosynthesis of fatty acids in ruminant and non-ruminant tissues are reflected in their individual $^{13}C/^{12}C$ ratios ($\delta^{13}C$ values) providing the basis for discrimination. These isotopic differences can be reliably measured using gas chromatography/combustion/isotope ratio mass spectrometry (GCCIRMS). All animals *de novo* synthesize both *n*-hexadecanoic ($C_{16:0}$) and *n*-octadecanoic ($C_{18:0}$) acids, mainly from dietary carbohydrate, and also absorb these components directly from diet. However, ruminants vary from mono-gastric animals in the precursors used for fatty acid biosynthesis,[7] and by extensive absorption of $C_{18:0}$ directly from the rumen following biohydrogenation of principal unsaturated fatty acids present in their forage (e.g. $C_{18:2}$, $C_{18:3}$). As plant fatty acids are depleted in ^{13}C compared with plant carbohydrates, the $C_{18:0}$ component in ruminant tissues is measurably ^{13}C-depleted compared with $C_{16:0}$. Unlike other ruminant tissues, the lactating mammary gland is unable to *de novo* synthesise $C_{18:0}$[8] and therefore an even greater proportion of this acid is routed directly from the rumen, leading to a further depletion in ^{13}C of $C_{18:0}$ relative to $C_{16:0}$ in milk and all other dairy products.

To test this empirically, the difference in $\delta^{13}C$ values of $C_{18:0}$ and $C_{16:0}$ have been measured using GCCIRMS in 26 authentic ruminant depot fats from domesticated cattle (*Bos taurus*) and sheep (*Ovis aries*) reared in the UK on a C_3 pasture

(Table 1[4,9]). As expected, the $\delta^{13}C$ values of $C_{18:0}$ in dairy fats were significantly lower than those of the adipose fats (a difference between means = 2.2‰; t(24) = −6.2, P = <0.001). There were no significant differences in the distribution of $\delta^{13}C$ values for either fatty acid in either class of fat (i.e. adipose and dairy) between the cattle and sheep (Mann–Whitney P = >0.05 for all tests). From these data, a difference between the $\delta^{13}C$ value of each fatty acid ($\Delta^{13}C = \delta^{13}C_{C18:0} - \delta^{13}C_{C16:0}$) of less than −3.3‰ (mean = −4.8‰) has been used as a criterion to identify ruminant dairy (Table 1), while $\Delta^{13}C$ values between −1 and −3.3‰ (mean = −2.1‰) are used to distinguish other ruminant fats which are likely to have derived from the animal's carcass (Table 1[2–4]). Similar studies of modern domesticated ruminant fats obtained from animals with different dietary regimes, including C_4 plants such as sorghum and maize, from the UK, India and Germany (Table 1[1,10]) have corroborated these findings. Whilst differential enrichment in the ^{13}C content of fatty acids has been measured in processed (heated and heavily fermented) dairy products,[11,12] none of the authentic domesticated ruminant adipose fats analysed so far have $\Delta^{13}C$ values < −3.3‰; thus the criterion for identifying dairy products appears to be sound.

The processing of wild ruminants, such as deer (*Cervidae* spp.), in pottery vessels has been rarely considered. As many studies have focused on the European and south-west Asian Neolithic period,[2–4,11] which followed the introduction of both domesticated animals and pottery, it seemed logical to associate pottery use with animal husbandry and to focus on distinguishing domesticated animal products. In certain contexts, for example the UK, this is justified as common domesticated animals (e.g. cattle, sheep, goat, pig) often dominate the Neolithic faunal assemblages suggesting that the hunting of wild animals (e.g. deer, aurochs) was at most a minor economic activity (e.g.[13,14]). However, more recently, lipid analysis[1] and a more general re-assessment of hunter-gatherer pottery use[15] has questioned the association of early pottery with domestication and farming, and many also accept significant regional variation in the uptake of domesticated resources.[16–18] In addition, aurochs and wild deer continued to be widely hunted throughout Europe in the later prehistoric and historic periods, and other genera of wild ruminant (e.g. numerous Antilopinae) were formerly widespread in other parts of the world, including regions where early adoption of dairying has been argued from lipid residues in ceramics.[5,19] This necessitates greater consideration of potential isotopic ranges for wild animals, including wild ruminants.

A previous study of adipose fats from seven red deer (*Cervus elaphus*) raised on unimproved pasture in the UK showed a much greater fatty acid isotopic variability than expected,[9] with one individual plotting within the range of reference milk fats ($\Delta^{13}C$ = −5.2‰). If this finding is confirmed, vessels might have been wrongly designated as having been used for processing dairy products, with potentially huge implications for understanding the origins of pastoralism. Currently, the evidence of an overlap between deer adipose and ruminant milk is tenuous. In the study of Evershed *et al.*[9] all but one deer had $\Delta^{13}C$ values within the range of ruminant carcass fats and similar values have also been measured in a further study of two deer from a Swiss forest.[11]

Table 1. Summary of isotopic measurements of *n*-hexadecanoic ($C_{16:0}$) and *n*-octadecanoic ($C_{18:0}$) acids from modern authentic ruminant products. To facilitate comparison with archaeological data, all isotope measurements have been adjusted for the effects of post-industrial carbon[22]

Description	*n*	Details	Tissue of origin	Mean $\delta^{13}C$ values (‰)		mean $\Delta^{13}C$ values (‰)		$\Delta^{13}C$ values, min, max and interquartile ranges (‰)					Ref
				$C_{16:0}$	$C_{18:0}$	mean	2σ	Min	Q_2	Median	Q_3	Max	
Ruminant milk	9	UK; C3 pasture; 7 cows, 2 ewes	Mammary	−29.2	−34.0	−4.8	1.4	−5.9	−5.3	−4.8	−4.4	−3.6	Copley *et al.*[4]
Ruminant milk	9	UK, Germany, India; C3 and C4 pasture and supplements; 7 cows, 1 ewe, 1 goat	Mammary	−26.5	−31.4	−4.9	2.6	−6.5	−6.0	−4.6	−3.9	−3.3	Craig *et al.*[1,10]
Ruminant carcass	16	UK; C3 pasture; 4 cows, 12 sheep	Muscle, renal	−29.6	−31.7	−2.1	0.9	−3.0	−2.4	−2.0	−1.8	−1.3	Copley *et al.*[4]
Ruminant carcass	7	UK; C3 pasture; red deer	Muscle, renal	−29.7	−32.2	−2.5	3.2	−5.2	−3.2	−2.8	−1.6	−0.3	Evershed *et al.*[9]
Ruminant carcass	10	Poland; C3 pasture; red deer	Bone, periosteum	−29.4	−33.0	−3.6	0.4	−4.3	−3.8	−3.6	−3.6	−2.7	this study

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Here we report on the carbon isotope range of fatty acids extracted from the tissues of ten wild deer from northern Europe. Unlike previous measurements of deer, these animals were recovered from their natural habitat providing optimal comparison with the archaeological data.

EXPERIMENTAL

Ten samples of mandibular deer bone were obtained from the Slowinski National Park in northern Poland between 1986 and 2002. Habitats within the park have been little affected by human activity. The animals foraged on mainly C_3 grasses and forbs in a range of different habitats, from open meadows to forest (for details, see Stevens *et al.*[20]). Collagen had previously been extracted from sub-samples of each deer bone and analysed by isotope ratio mass spectrometry (IRMS).[20] Separate sub-samples that had been cleaned but not subjected to any lipid removal were used in all subsequent procedures. Lipids were extracted using a modified Bligh and Dyer extraction.[21] Briefly, bone samples (200 mg) were pulverised under liquid nitrogen and extracted (×3) with methanol (1 mL), dichloromethane (2 mL) with sonication steps between the additions of each solvent. The solvent was transferred to a clean tube and aqueous KCl (2 mL; 0.88%) and KCl (0.88%)/MeOH (2 mL; 1:1 v/v) were added, with mixing and removal of the aqueous phase following the addition of each reagent. The remaining solvent was removed under N_2 to leave a total lipid extract (TLE). An aliquot of each TLE was saponified with methanolic NaOH (4 mL; 0.5 M in methanol/water at 9:1 v/v; 1 h; 70°C) to liberate free fatty acids (FFAs). Following removal of neutral lipids, the solution was acidified with HCl (200 µL; 6 M) and the FFAs were extracted with hexane (3 × 1 mL) and dried under N_2. The FFAs were methylated, along with a standard mixture of high-purity $C_{18:0}$ and $C_{16:0}$ fatty acids (Sigma-Aldrich, Poole, UK) of known isotopic composition, using BF_3-MeOH (100 µL; 14% w/v; 1 h; 70 °C) to prepare fatty acid methyl esters (FAMEs) which were extracted with hexane (3 × 1 mL) and dried under N_2.

FAMEs from each of the bone samples were analysed by gas chromatography/mass spectrometry (GCMS) and GCIRMS. GCMS analysis was performed using an Agilent 7890A Series gas chromatograph connected to an Agilent 5975 C Inert XL mass-selective detector with a quadrupole mass analyser (Agilent Technologies, Cheadle, Cheshire, UK). The splitless injector and interface were maintained at 300°C and 340 °C respectively. Helium was the carrier gas at constant inlet pressure, and the GC column was inserted directly into the ion source of the mass spectrometer. The ionisation energy was 70 eV and spectra were obtained by scanning between *m/z* 50 and 800. All samples were analysed using a DB5-ms (5%-phenyl)-methylpolysiloxane column (30 m × 0.32 mm × 0.25 µm; J&W Scientific, Folsom, CA, USA). The temperature program was 2 min at 50°C, 10°C min^{-1} to 325°C and 15 min at 325°C.

The stable carbon isotopic compositions of individual lipids were determined at least in duplicate using a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher, Bremen, Germany) linked to a Trace Ultra gas chromatograph (Thermo Fisher) with a ConFlo IV interface (Cu/Ni combustion reactor held at 1000 °C; Thermo Fisher). Samples were diluted in hexane and 1 µL was injected onto a DB5 fused-silica column (30 m × 0.25 mm × 0.25 µm; J&W Scientific). The temperature program was 1 min at 45°C, 6°C min^{-1} to 295°C and 15 min at 295°C. Ultra high purity grade helium was used as the carrier gas (at a flow rate of 1.4 mL min^{-1}). The ion intensities of *m/z* 44, 45, and 46 were monitored and the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios of each sample peak were automatically computed (Isodat version 3.0; Thermo Fisher) by comparison with a standard reference CO_2 gas of known isotopic composition, which was repeatedly measured with each sample.

All results are reported in per mil (‰) relative to VPDB international standard. Replicate measurements of each sample and a mixture of FAMEs with $\delta^{13}C$ values traceable to international standards (Indiana F8 standard; obtained from Arndt Schimmelmann, Indiana University, Bloomington, IN, USA) were used to determine instrument precision (<0.3‰) and accuracy (<0.5‰). The values of unknown samples were corrected for methylation by comparison with standard $C_{18:0}$ and $C_{16:0}$ fatty acids of known isotopic composition, which were methylated with each batch of samples using identical reagents and procedures. The data were further corrected for the burning of fossil fuels[22] to allow comparison with archaeological data.

RESULTS AND DISCUSSION

The GCCIRMS data for $C_{16:0}$ and $C_{18:0}$ acids from the red deer from Poland are reported in Supplementary Table S1 (see Supporting Information) and compared with the range and means for other ruminant products, where the values have been reported (see Table 1). The $\Delta^{13}C$ values for individual deer samples are plotted against their $\delta^{13}C_{C16:0}$ values in Fig. 1 and compared with those from carcasses and milk fats of domesticated ruminants raised on C3 pasture in the UK.[4] GCMS analysis of each sample following saponification showed that the deer bone samples have very similar free fatty acid distributions to other domesticated and wild ruminant tissues.[23] $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ acids predominate, with minor amounts of shorter chain saturated fatty acids (<$C_{12:0}$) and branched odd-chain fatty acids (e.g., $C_{15:0br}$; $C_{17:0br}$). The major fatty acids were adequately separated for GCCIRMS analysis, as shown in Fig. 2.

The different products (milk, deer fat, domesticated ruminant fat) are distinguished by their $\Delta^{13}C$ values (Kruskal-Wallis H (2) = 29.9; P = <0.01) plotted in Fig. 1, due to a much greater difference in $\delta^{13}C_{C18:0}$ than in $\delta^{13}C_{C16:0}$. All the ruminant animal products with predominately C_3 pasture diets from northern Europe have similar means and ranges of $\delta^{13}C_{C16:0}$ values, presumably because this component is largely derived from the same source (C_3 pasture carbohydrate) growing under similar conditions, i.e. at approximately the same altitude and latitude. Conversely, the extent of $C_{18:0}$ acid routing is variable in the different animals and tissues. As a result, the mean $\delta^{13}C_{C18:0}$ value is significantly lower in ruminant milk than in deer carcass fat, which in turn is lower than in sheep and cow carcass fats. Nine of the ten deer carcass fats measured have $\Delta^{13}C$ values less than −3.3‰, the threshold previously used for classifying dairy products. However, unlike dairy products, in this study no deer fats have values < −4.3‰, suggesting that the approach is still useful for investigating dairying albeit with revised criteria.

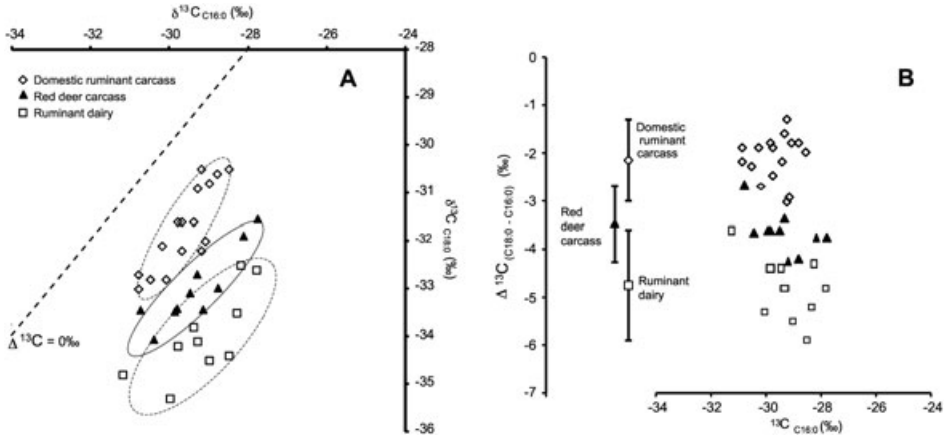

Figure 1. (A) Plot of $\delta^{13}C$ values of the major unsaturated fatty acids extracted from different ruminant fats from northern Europe. Confidence ellipses ($P = 0.684$) are shown for each class of fat. (B) Plot of the difference in $\delta^{13}C$ values ($\Delta^{13}C$) between the $C_{18:0}$ and $C_{16:0}$ fatty acids. Also shown are the median and ranges in $\Delta^{13}C$. All data are from animals fed exclusively C_3 diets. The ruminant dairy and carcass fats are from Copley *et al.*[4] and sheep and cattle reared in the UK. The wild ruminants are from Polish red deer analysed in this study. To facilitate comparison with archaeological data, all isotope measurements have been adjusted for the effects of post-industrial carbon.[22]

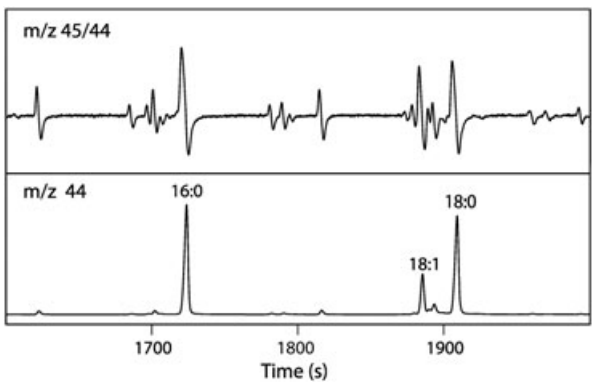

Figure 2. Partial *m/z* 45/44 and *m/z* 44 ion chromatograms obtained by GCCIRMS of fatty acids from a typical modern deer tissue. Fatty acids were analysed as their methyl ester derivatives. Sample: M/PF/B/20/41.

The difference between the $\Delta^{13}C$ values in the carcass fats of deer and other ruminants is intriguing and provides a means to distinguish the former especially in pre-dairying economies and prior to domestication. Little work has been done on lipogenesis specifically in wild ruminants but the difference might be due to (a) a greater difference between carbohydrate and lipid $\delta^{13}C$ values in the deer's diet than in other ruminants; (b) differences in the degree of *de novo* synthesis or routing in different ruminant carcass tissues; or (c) a greater extent of $C_{18:0}$ routing in deer than in other ruminants.

Although deer forage more selectively than bovids,[24] it is unlikely that (a) is significant, given that the diets of both the Slowinski deer and the UK domesticated bovids will have been dominated by C_3 carbohydrate. Furthermore, any systematic isotopic differences associated with CO_2 assimilation, due to the canopy effect,[25] latitude or altitude, would be expected to act on both plant carbohydrate and lipid. The extent of fatty routing and synthesis in different tissues (b) should be considered since the measurements in this study are of bone and previous measurements were of deer muscle and renal tissues.[9,11] However, this is unlikely to explain the observed isotopic differences given that, other than the lactating mammary gland, adipocytes are the only significant site of fatty acid synthesis in ruminants.[7] Thus bone adipocyte lipid synthesis should follow similar biosynthetic pathways to those in all other tissues. Greater routing of $C_{18:0}$ directly from diet in the deer tissues than in domesticated ruminants (c) is a more likely explanation. Despite their broadly similar tissue lipid composition, wild ruminants are substantially leaner than modern domesticated animals[23] and therefore *de novo* synthesised fatty acids from adipose tissue might make a lesser contribution to the animal's total pool of fatty acids. As a consequence, dietary fatty acids would be expected to make a greater contribution to the adipose tissue of wild ruminants compared to domesticates. If this were the case, a greater relative amount of the $C_{18:0}$ acid would be incorporated directly from the diet, as this is the major fatty acid to be absorbed from the rumen following hydrogenation of unsaturated dietary lipids. More experimental work on the lipogenesis of wild ruminants is needed to clarify but increased dietary routing as the result of fat content might explain the $\Delta^{13}C$ differences between the deer raised in deer parks[9] and the truly wild, and presumably leaner, deer from the Slowinski National Park analysed in this study (Table 1). There is also 'suggestive' evidence of reduced fatty acid elongase activity, the enzyme needed for fatty acid *de novo* synthesis, in adipose tissue from north American red deer compared with other ruminants,[23] which may lead to further enhanced routing of the $C_{18:0}$ acid from the diet.

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CONCLUSIONS

The analysis of fatty acids in wild ruminant tissues by GCCIRMS establishes new criteria for distinguishing different ruminant fats. We have established that wild ruminant carcass fats have a wide range of $\Delta^{13}C$ values, between −2.7‰ and −4.3‰, whereas the maximum reported value for ruminant dairy fats is −3.3‰ and the minimum for domesticated ruminant adipose fats −3.0‰ (Table 1). These findings have important implications for the interpretation of ceramic use in the past and our understanding of the nature of early pastoral economies. For example, a significant number (>50%) of Early Neolithic ceramic samples from the UK, central and northern Europe and NW Anatolia designated as dairy (e.g.[1–4]) could be re-classified as deer in light of the new evidence. However, the rationale for doing this would be somewhat weak, as in many cases the pots analysed were deposited almost exclusively with domesticated animal remains. In other cases where wild ruminants are found in the Neolithic faunal assemblage (e.g.[1]), the interpretation of dairy products in some vessels could be questioned. However, even with some re-classification, our findings do not necessarily contradict the wider and important interpretation of early Neolithic dairying in these regions, as many samples have $\Delta^{13}C$ values below −4.3‰, which currently can only be explained by processing ruminant milk. In a small number of cases, the triacylglycerol distributions are also consistent with a dairy origin.[26] Nevertheless it is recommended that isotopic data from lipids on archaeological pottery are always used in conjunction with the analysis of faunal remains. Secondly, further analysis of fatty acids from different species of wild ruminants, such as European and Mesopotamian fallow deer (*Dama dama*/*Dama mesopotamica*), roe deer (*Capreolus capreolus*) and wild bovids, such as gazelle (e.g. *Gazella subgutturosa*), in their natural habitat should be undertaken to extend the reference range. Finally, other mass spectrometric approaches, such as soft-ionisation mass spectrometry techniques, e.g.[27], should be employed to further classify the acylglycerol component of remnant ruminant lipids in pottery. Despite the complications in interpreting isotopic signatures from pottery residues, as outlined here, accurate interpretation of strategies for wild and domesticated animal exploitation can still be achieved provided that data from chemical analysis of pottery and faunal remains are integrated within the broader archaeological context of the site and its palaeoecological setting.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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