Cholesterol as a New Source of Palaeodietary Information: Experimental Approaches and Archaeological Applications

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Cholesterol and its diagenetic congeners have been detected in a wide range of human and animal bones dating to the Mesolithic period. Using isotope ratio monitoring-gas chromatography/mass spectrometry (irm-GC/MS), it is possible to determine accurately compound specific δ¹³C values of preserved cholesterol with high precision (± 0.4‰). The δ¹³C value of cholesterol provides information complementary to that derived from collagen and apatite stable isotopes for use in palaeodietary studies. Potential advantages of using cholesterol are that: (1) the δ¹³C value will be biased towards the original isotopic composition of the carbohydrates and fats in the diet, (2) its δ¹³C value will retain isotopic integrity, i.e., will not be diagenetically altered, provided its carbon skeleton is characterized, and (3) that its relatively rapid rate of turnover provides a means of investigating carbon cycling on a shorter time scale than collagen. Laboratory animal feeding experiments have shown that cholesterol faithfully derives its isotopic signature from the diet and has a faster turnover rate than collagen. This paper reviews aspects of the methodologies employed in recording δ¹³C values from cholesterol preserved in skeletal materials and presents new results obtained from modern and archaeological humans, and studies of experimental animals.

Keywords: CHOLESTEROL, STABLE ISOTOPES, δ¹³C VALUES, COMPOUND SPECIFIC, LABORATORY ANIMAL FEEDING EXPERIMENTS, PALAEODIET.

Introduction

Palaeodietary reconstruction was revolutionized in the 1970s with the introduction of stable isotope analysis of carbon and nitrogen. Important information regarding dietary behaviour in the past has included comparisons of: (1) protein versus carbohydrate consumption (Lubell et al., 1994; Ambrose et al., 1997); (2) consumption of marine versus terrestrial foods (Tauber, 1981); and (3) consumption of C₃ versus C₄ photosynthetically derived food (Bender, Baerreis & Steventon, 1981). Studies have focused on both the inorganic, e.g., biomineral constituents of bone and teeth, and organic components, e.g., collagenous proteins and amino acids preserved in archaeological remains. Collagen comprises a complex mixture of several different amino acids, mainly glycine (33%), proline (12%) and hydroxyproline (10%) with glutamate, aspartate, alanine, serine and valine in lower proportions, each having different and sometimes variable δ¹³C and δ¹⁵N isotopic compositions (Tuross, Fogel & Hare, 1988). Biogenic apatite (hydroxyapatite, Ca₁₀(PO₄)₆(OH,F)₂) is the dominant inorganic mineral phase in bones and tooth dentine and enamel (Koch, Fogel & Tuross, 1994). Both bioapatite (δ¹³C) and collagen (δ¹³C and δ¹⁵N) isotopic analyses are routinely utilized in palaeodietary studies with notable successes.
Until very recently, the lipids present in ancient skeletal materials had not been considered as a source of palaeodietary information even though their existence in biomineralized tissues had been suggested. Das, Doberenz & Wyckoff (1967) and Everts, Doberenz & Wyckoff (1968) reported the presence of lipids in fossil bone and teeth based on the results of thin-layer chromatographic and packed column gas chromatographic analyses. Ambrose (1990) presented the first carbon isotope analysis of bulk lipids from modern and archaeological animal bone. More recent work using gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) (Evershed et al., 1995) confirmed the presence of lipids in a wide variety of bones (animal and human) from differing geographical locations and archaeological contexts. The discovery of cholesterol and its diagenetic congeners in appreciable concentrations (2–50 μg/g dry wt of bone) in each bone analysed led us to consider the use of the δ13C content of cholesterol as an additional source of palaeodietary information. Cholesterol was found to be the most abundant constituent of the bone lipid extracts in the majority of cases. Cholesterol is not naturally present in plants and soils, therefore in archaeological bones and teeth it must be derived from (1) the remnants of the original blood-borne lipid (in the case of vascular bones); (2) the fat component of bone marrow that would have been present at the time of death of the individual; or (3) a component of the cellular lipids present in bone-forming cells. Other biomarkers preserved in bone lipid extracts, such as those derived from saprotrophic organisms, have been described in detail in previous publications (Evershed et al., 1995; Stott & Evershed, 1996).

Compound-specific Stable Isotope Analyses

The majority of palaeodietary research carried out to date has concentrated on the δ13C and δ15N analyses of bulk organic and inorganic matter, e.g., collagen and bioapatite, recovered from skeletal or dental remains. This has proven to be a valuable source of information providing bulk isotopic signatures representing dietary averages of the common biomolecular classes. Surprisingly few studies, however, have been carried out at the molecular level. This potentially valuable source of stable isotopic information should provide a better understanding of the isotopic relationship between diet and tissues and minimize problems of exogenous contamination in the case of archaeological remains. The molecular species used to represent a specific biomolecular class must, however, be carefully chosen, since a single amino acid is not a favourable substitute for whole collagen. However, the isotopic analysis (δ13C and δ15N) of a suite of individual amino acids (essential and non-essential) may provide more precise information concerning carbon and nitrogen sourcing and routing from the diet to tissues.

Indeed, Hare et al. (1991) separated individual amino acids from collagen purified from laboratory-reared and wild modern animals using cation exchange chromatography. Off-line combustion followed by isotope ratio mass spectrometry revealed consistent patterns of carbon and nitrogen isotopic fractionations between different amino acids, e.g., the isotopic patterns of eight major amino acids isolated from modern and fossil whales were shown to be almost identical. Deviations from this pattern could therefore potentially indicate dietary stress, contamination from exogenous proteins or unusual dietary constituents. Additionally, the most abundant collagenous amino acids (proline and hydroxyproline) may provide a source of isotopic palaeodietary information in samples containing contaminated or degraded collagen (Koch, Fogel & Tuross, 1994). Hence, isotopic values of individual amino acids provide potentially useful criteria for testing the indigeneity of fossil proteins such as collagen (Tuross et al., 1988).

The opportunity now exists for increasing the specificity of stable isotope ratio studies (δ13C and δ15N) in the field of palaeodiet by the use of on-line isotope ratio monitoring-gas chromatography/mass spectrometry (irm-GC/MS; Hayes et al., 1990; Macko, Engel & Qian, 1994). This important technological breakthrough has provided new opportunities to measure the δ13C (and δ15N) content of specific compounds, e.g., amino acids, carbohydrates, lipids, etc. Cholesterol and its diagenetic congeners preserved in ancient bone and teeth are also obvious candidates for irm-GC/MS analyses and potentially provide an additional source of δ13C information for the purposes of palaeodietary or palaeoecological reconstruction (Stott & Evershed, 1996; Stott, Evershed & Tuross, 1997). The attraction of using cholesterol as a source of δ13C information is that we can: (1) confirm its structure unambiguously by conventional GC/MS analysis; (2) be completely assured on structural grounds that it must be endogenous to a specific inhumation and has not derived from the burial environment; (3) be sure that its isotopic composition has not been affected by diagenetic processes; and (4) the biochemical knowledge of the biosynthetic origins of cholesterol provides valuable information on the catabolic energy sources of the diet, i.e., carbohydrate/lipid and to a lesser extent protein. The latter point is discussed in further detail below.

Biochemical Considerations in Palaeodietary Reconstruction

Dietary interpretation based on collagen and apatite isotopic data from archaeological samples is an area of ongoing interest, specifically with respect to the relationship between the diet and tissue synthesis. Studies have addressed the question of whether specific dietary biochemicals are routed to respective tissues, e.g.,
proteins to collagen and non-proteins to apatite carbonate (Chisholm, Nelson & Schwarcz, 1982; Krueger & Sullivan, 1984; Klepinger & Mintel, 1986; Lee-Thorp, Sealy & van der Merwe, 1989; Schwarcz, 1991) or whether carbon from several biochemical sources is scrambled prior to re-synthesis (Schoeninger, 1989). Animal feeding experiments (DeNiro & Epstein, 1977, 1978, 1981; Ambrose & Norr, 1993; Tieszen & Fagre, 1993) have utilized stable isotopes to gain valuable insights into the specific carbon and nitrogen fluxes from formulated diets into the body tissues of laboratory animals. The general consensus is that dietary protein contributes greatly to the biosynthesis of amino acids during collagen formation. One analysis suggests that the essential amino acids from the diet contribute c. 22% of the carbon atoms in collagen, yet the remaining 78% of carbon atoms from the non-essential amino acids can be derived from all dietary sources (Schwarcz, 1991). The minimum amount of routing of carbon from diet to tissue protein is conventionally thought to depend on the proportions of essential amino acids (those that cannot be synthesized by the consumer and must be absorbed from the diet) in the consumer tissue protein (Schwarcz, 1991). Essential amino acids comprise 17-8% of the carbon atoms in collagen (Klepinger & Mintel, 1986; Schwarcz, 1991; Ambrose, 1993), which defines the minimum amount of routing from dietary protein to collagen. In theory, the remainder can be obtained from neosynthesis using carbon from lipids and carbohydrates. However, a new tripartite division of amino acids into indispensable (essential), dispensable (non-essential) and conditionally indispensable (Young & El-Khoury, 1995), suggests protein routing should be much higher. Conditionally indispensable amino acids include: (1) those with essential amino acid precursors (2-5% of collagen carbon), (2) those whose synthesis is moderated by dietary supply, and (3) those whose rates of synthesis are insufficient to meet increased metabolic demand under stress. Because of the metabolic energy costs of neogenesis, deamination and transamination of amino acids, it is more efficient to use dietary sources of non-essential amino acids when available. Essential amino acids require five to 10 enzymes for neosynthesis. Glycine, proline and hydroxyproline are the most abundant amino acids in collagen (comprising c. 46% of its carbon atoms). Although not essential, each requires four enzymes for neosynthesis; truly non-essential amino acids each require only one (Rodwell, 1996). The expected amount of routing of carbon from dietary protein to tissue protein should be about 65% when the diet supplies an excess of each amino acid. This estimate of routing (Ambrose et al., 1997) is consistent with the results of the controlled diet experiments by Ambrose & Norr (1993) and Tieszen & Fagre (1993).

Cholesterol is a component of all mammalian cell membranes and a precursor of steroid hormones and bile acids. Although most mammalian tissues are able to biosynthesize their own cholesterol, the majority of active synthesis occurs in the liver and small intestine (Sabine, 1977; Myant, 1981). In humans it has been estimated that the amount of cholesterol that the body can synthesize in a day is at least twice that of the dietary cholesterol intake in a typical Western European diet. Approximately half of the dietary cholesterol is absorbed, the remainder being excreted. Hence, dietary cholesterol contributes to approximately 20% of the total body cholesterol of an individual, i.e., the majority of tissue cholesterol is derived via biosynthesis (exceptionally high intakes of cholesterol occur in high-protein diets). Acetyl-CoA is a common metabolite formed in the body by the catabolism of dietary carbohydrates, lipids and proteins (Voet & Voet, 1995; Stryer, 1988). However, cholesterol is thought to be biosynthesized from the acetyl-CoA derived predominantly from dietary carbohydrates and lipids (Sabine, 1977) therefore the isotopic composition of cholesterol should theoretically be biased towards these two dietary components.

Thus the rationale behind our work was to utilize the δ13C value of cholesterol as a palaeodiagnostic indicator to be ultimately employed in conjunction with collagen and apatite stable isotope data. The main objective was to apply irm-GC/MS to access isotopic information from skeletal remains that had not been possible in previous studies using bulk stable isotope approaches. In this paper our analytical protocol is described and several applications involving the use of cholesterol as a carrier of a stable carbon isotope signal are discussed.

In our studies on modern heterotrophs (laboratory animal feeding experiments) we address the questions of: (1) whether cholesterol inherits the isotopic signature from the diet? (2) whether specific dietary bio-chemicals influence the δ13C value of cholesterol more than others? and (3) what turnover rates are observed in the 13C content of cholesterol during dietary changes? Studies of modern human bone lipids have provided an indication of how the isotopic composition of cholesterol reflects both biosynthesis and uptake from the diet. The results discussed herein support the concept of using the δ13C value of cholesterol preserved in archaeological bone and teeth as a palaeodiagnostic indicator. New results are presented for archaeological populations which serve to highlight the utility of cholesterol as a sensitive indicator of dietary preference.

**Analytical Methods**

Two methods were used to sample either modern and ancient skeletal members. Modern bone, taken from controlled laboratory animal feeding experiments, was sampled by initially removing hair and muscle using a scalpel, prior to crushing and extraction (Evershed et al., 1995; Stott et al., 1997; Jim et al., in press). Total lipid extracts were saponified in 2 ml of 0.5 M
methanolic NaOH, heated in a water bath at 70°C for 1 h, cooled to ambient temperature and acidified to pH 3 with 1 M HCl. Lipids were then extracted into 2 × 5 ml hexane, the supernatant being removed each time under nitrogen. Neutral and acid fractions were isolated from the saponified lipid extracts (10–20 mg) using amino propyl (NH2 sorbent) solid phase extraction (SPE) cartridges (6 ml, 500 mg, ACCU-BOND; J+W Scientific) conditioned with hexane. Neutral lipids were separated from the fatty acids and phospholipids by elution with chloroform/2-propanol (2:1, v/v, 9 ml). Fatty acids were eluted with 2% acetic acid in diethyl ether, evaporated under nitrogen and stored at 4°C. Prior to GC analysis, fatty acids were derivatized to their corresponding methyl esters with boron trifluoride in methanol at 70°C. Following the addition of double distilled water and diethyl ether, the supernatant was then transferred to a screw capped vial and stored at −20°C until required for GC, GC/MS and irm-GC/MS analysis.

For archaeological samples, sections of bone and whole teeth were analysed. Exogenous lipids from adhering soil or post-excavational contamination were removed from the surfaces of the bones and teeth by abrasion. Following grinding, the powdered samples were weighed (2–3 g) and extracted with chloroform/methanol (2:1, v/v) by ultrasonication (4×30 min). The lipid extraction was performed both manually or using semi-automated methods (Stott & Evershed, 1996). Prior to extraction, 20 μg (1 mg/ml) of either 5α(H)-cholesterol or 6-tetratriacontane (Aldrich Chemical Company) were added as internal standards for quantitative measurements. The use of laboratory blanks is a routine procedure but is essential to monitor for laboratory or procedural contamination since cholesterol is a component of human skin lipids.

Following centrifugation (1800 rpm, 45 min), the supernatants containing the lipid extract were decanted and evaporated to dryness under a stream of nitrogen. Aliquots (typically one-fifth) were then transferred to vials (2 ml) in a minimum of solvent, evaporated, and stored at 4°C until required for analysis. In certain samples, cholesterol and cholesteryl esters were isolated from the total lipid extract using thin-layer chromatography (TLC). Separations were achieved on 0.25 mm silica gel (Merck Kieselgel Grade 60 G) plates using dichloromethane as the developer. Plates were previously developed in ethyl acetate to remove contamination, and activated at 110°C for 4 h before use. After development, the plates were dried and visualized using Rhodamine 6 G and UV light. The cholesterol and cholesteryl ester bands were removed from the plate and eluted from the silica using dichloromethane. After rotary evaporation, the lipid extracts were reconstituted in solvent (chloroform) and approximately one-quarter of the total extract removed for further analysis. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% v/v trimethylchlorosilane (Sigma Chemical Co.; 30 μl) was added to each extract, heated at 70°C (c. 1 h). After evaporation under a stream of nitrogen, the trimethylsilylated extract was diluted with an appropriate volume of solvent prior to analysis by gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS) and isotope ratio monitoring-gas chromatography/mass spectrometry (irm-GC/MS).

Fully automated GC analyses were carried out on a Hewlett Packard 5890 Series II gas chromatograph fitted with a fused silica capillary column (30 m × 0.25 mm i.d.) coated with a 5% phenyl methyl silicone stationary phase (HP-5, 0.25 μm film thickness). Samples were diluted in dichloromethane and introduced by on-column injection using a Hewlett Packard 7673 automatic sampler. Following an isothermal hold at 50°C (2 min), the oven temperature was increased to 250°C at 10°C/min and then to 325°C (10 min) at 4°C/min. Hydrogen was used as carrier gas and flame ionization detection (FID) was used to monitor the column effluent. HT-GC was carried out as above, using a fused-silica capillary column (15 m × 0.25 mm i.d.) coated with a dimethyl polysiloxane stationary phase (DB-1, 0.1 μm film thickness). Following an isothermal hold at 50°C (2 min) the temperature was increased to 350°C (20 min) at 10°C/min. Data were analysed on an Opus V PC using HP Chemstation software.

HT-GC/MS analyses were carried out using a Finnigan 4500 quadrupole mass spectrometer (electron voltage, 35 eV; filament current, 0.35 mA; electron multiplier, 2 kV; source temperature, 280°C) interfaced to a Carlo Erba HRGC 5160 Mega series gas chromatograph. The GC was fitted with a fused-silica capillary column (15 m × 0.25 mm i.d.) coated with a dimethyl polysiloxane stationary phase (DB-1, 0.1 μm film thickness). The temperature programme was the same as that described above (cf. HT-GC). Hydrogen was used as carrier gas. Data were acquired and processed using an INGOS data system. Peak assignments were made by coinjection of authentic standards and by comparison of mass spectra and retention times.

Both electron ionization (EI) and chemical ionization (CI) modes were used in the GC/MS analyses of the cholesteryl esters isolated from the bone total lipid extracts. The latter is a soft ionization technique, providing diagnostic ions indicative of the molecular weight of the intact cholesteryl ester and the nature of the sterol and fatty acid moieties. Chemical ionization spectra were obtained using ammonia (BOC micrographic grade) as the reagent gas. The indicated reagent gas pressure was 0.5 mTorr. Other GC/MS parameters were as described above.

Automated irm-GC/MS analyses were performed using a Varian 3500 gas chromatograph coupled to a Finnigan MAT DELTA-S isotope ratio monitoring-mass spectrometer via a Finnigan MAT combustion interface. The GC column used was as described above for the GC/MS analyses. Helium was used as carrier gas and the mass spectrometer source pressure was
The temperature programme was as follows: after an isothermal hold at 50°C (2 min), the temperature was increased from 50 to 250°C at 10°C/min, then to 300°C at 4°C/min, and finally held at 300°C for 25 min. All δ\(^{13}\)C values quoted are means of triplicate analyses.

### Results and Discussion

**Laboratory animal feeding experiments**

A laboratory animal feeding experiment (Hare et al., 1991) in which pigs were raised on pure 100% C\(_3\) and 100% C\(_4\) plant diets provided the opportunity to carry out a detailed study of the \(^{13}\)C content of individual lipids present in an animal’s diet and bone tissue (Stott et al., 1997). Such assessments provide insights into the routing of carbon from the diet to the tissues of large heterotrophic animals and allow us to determine whether cholesterol is of use in archaeological studies.

The isotopic relationship between dietary composition and a specific tissue type is complicated by a variety of factors such as tissue turnover rates, differences in biosynthetic pathways and nutritional status. Deconvolution of these factors in wild organisms is impossible since their diets are ill-defined. Feeding experiments in which pigs were raised on pure C\(_3\) and C\(_4\) cereal diets showed that bone cholesterol inherited the \(^{13}\)C isotopic signature of the respective diets, taking into account the isotopic fractionation occurring during lipid biosynthesis (Stott et al., 1997). Domestic pigs raised on pure C\(_3\) (77.5% barley, δ\(^{13}\)C = −25.3‰) and C\(_4\) (67% ground yellow corn, δ\(^{13}\)C = −11.3‰) diets were sacrificed at 90 kg weight, after reaching a similar physiological status (Hare et al., 1991). Figure 1 shows the δ\(^{13}\)C measurements of cholesterol plotted against the collagen and the bulk dietary components determined in an earlier investigation (Hare et al., 1991). It is evident that the δ\(^{13}\)C values for cholesterol and collagen reflect the overall isotopic composition of the dietary carbon pool.

Much consideration has been given as to whether or not dietary carbon is routed or scrambled prior to incorporation into body tissues (Ambrose & Norr, 1993; Tieszen & Fagre, 1993). The observed differences between the isotopic composition of collagen relative to the diet have been modelled (Krueger & Sullivan, 1984; Lee-Thorp et al., 1989). These models suggest that collagen is enriched in \(^{13}\)C by 5‰ from the diet, when plant biochemicals are consumed then assimilated by herbivores. The corresponding lipids will be slightly depleted from the diet and will be approximately 5‰ more negative than the collagen δ\(^{13}\)C value (Tieszen & Fagre, 1993). The experimental animals in this study showed similar isotopic patterns for both C\(_3\) and C\(_4\) animals, although the \(^{13}\)C enrichment in both collagen values was not as great as 5‰. The isotopic composition (δ\(^{13}\)C) of cholesterol isolated from the C\(_3\) and C\(_4\) pig bone is significantly depleted in \(^{13}\)C relative to that of the bone collagen. Interestingly, the cholesterol–collagen spacing (δ\(^{13}\)C cholesterol–δ\(^{13}\)C collagen) is similar to
proposed 5% depletion; $\Delta^{13}C_{\text{cholesterol-collagen}}$ spacings of 4.5 and of 5.2% were observed for the C$_3$- and C$_4$-fed animals, respectively. Depletion of lipid $^{13}C$ relative to collagen relates to the combined effects of: (1) the different biosynthetic pathways of collagen versus lipids; (2) routing of different carbon pools from the diet into the protein and lipid; and (3) isotopic effects associated with assimilation of different biochemical components by an organism.

Individual fatty acids were isolated from the diet and bone tissue of the pigs (Stott et al., 1997). The $\Delta^{13}C$ values of individual fatty acids and cholesterol strongly correlated with both bulk diet $^{13}C$ values and those of specific dietary lipids. Significantly, the non-essential fatty acids were depleted by an average of 3.5% from the bulk $^{13}C$ values of the diet. This is consistent with the homogenizing effect of carbon routing through acetate which is the precursor in fatty acid and cholesterol biosynthesis. Thus, fatty acids, cholesterol and collagen all accurately reflect the C$_3$ and C$_4$ composition of the respective diets. Interestingly, cholesterol was depleted by 3.7 and 1.4‰ relative to the respective C$_{16:0}$ fatty acid in the animals raised on the C$_3$ and C$_4$ diets. The $\Delta^{13}C$ value of the C$_{16:0}$ fatty acid derives from acetate which consists of one methyl and one carboxyl group. These are thought to be isotopically distinct due to an isotope effect associated with the pyruvate dehydrogenase complex which gives rise to an alternating pattern of depletion in the carboxyl carbon positions of acetate (Monson & Hayes, 1980). Differences appear to arise in eukaryotes through a "reversal" in the methyl/carboxyl groups, leading to the depletion in the $^{13}C$ content of the methyl group in acetate (Monson & Hayes, 1982). Cholesterol, deriving from C$_3$ isoprene units containing a ratio of 3 methyl to 2 carboxyl carbon atoms, comprises 15 methyl and 12 carboxyl carbons that derive from acetate. The reverse fractionation between methyl and carboxyl carbons in eukaryotes would lead to the depletion of cholesterol relative to the C$_{16:0}$ fatty acids (1:1 methyl:carboxyl; Monson & Hayes, 1982).

Work is currently being carried out in our laboratory to further investigate the routing and synthesis of dietary constituents by monitoring the stable isotope composition of individual lipids from laboratory rats raised on isotopically controlled diets consisting of differing proportions of C$_3$, C$_4$ and marine protein, and C$_3$ and C$_4$ energy components (Ambrose & Norr, 1993). This particular study is providing us with the opportunity to gain insights into, amongst other things, the turnover rate of cholesterol in muscle and bone. Early indications from a study in which rats were raised on monotonous C$_3$ diets for 91 days and then switched to pure C$_4$ showed that a 7.3‰ enrichment in the $\Delta^{13}C$ of cholesterol occurred after only 40 days cf. Table 1 (Jim et al., in press). Significantly, the enrichment in the $\Delta^{13}C$ value for bone collagen and carbonate were only 0.7 and 1.0‰, respectively, during the same period of time which reflects their slower turnover rate and hence the long-term integrated dietary signal represented by their stable isotope values. Using this information we have the first evidence that the isotopic value of cholesterol is very sensitive to dietary change due to its quicker turnover time and is therefore of potential value in interpreting subtle dietary changes in archaeological populations over a much shorter time scale compared with collagen and apatite.

### Modern human surgical tissue

A study using surgical hip joint tissue (North Americans) was performed in an attempt to gain insights into the isotopic variations likely to exist between collagen and cholesterol in individuals in a modern human population. This work, to our knowledge, was the first time compound specific stable isotopes of individual modern human lipids ($\Delta^{13}C$ values of fatty acids and cholesterol) had been used in conjunction with the $\Delta^{13}C$ (and $\Delta^{15}N$) values of bone collagen and individual amino acids. Modern North American Type I collagen showed $\Delta^{13}C$ values indicative of mixed C$_3$/C$_4$ diet, with a greater input of C$_4$ carbon sources, e.g., cane sugar and corn-fed meat. Cholesterol-collagen spacings for six individuals, aged between 55 and 89 years, yielded values of between 3.4 and 5.8‰ (Tuross, Fogel, Stott & Evershed, unpubl. data). Trends in the isotopic composition of individual fatty acids were similar to those of the previous study carried out on the experimental pigs; however, cholesterol $\Delta^{13}C$ values were similar to those of the C$_{16:0}$ fatty acid presumably due to the combination of metabolic fractionation and dietary input (Figure 2). Interestingly, the essential C$_{18:2}$ fatty acid in each human reflected a contribution from predominantly C$_3$ vegetable oils. The range of $\Delta^{13}C$ values for the various tissue components potentially provides further information on their rate of turnover. For example, the range of values observed for collagen was 1-6‰. Significantly, the ranges for the synthesized lipids (cholesterol, C$_{16:0}$ and C$_{18:1}$ fatty acids) were 3.2, 3.7

<table>
<thead>
<tr>
<th>Feeding experiments†</th>
<th>$\Delta^{13}C$ bone collagen (‰)</th>
<th>$\Delta^{13}C$ bone carbonate (‰)</th>
<th>$\Delta^{13}C$ muscle cholesterol (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet A (pure C$_3$ control)</td>
<td>-20.0</td>
<td>-14.0</td>
<td>-29.1</td>
</tr>
<tr>
<td>Day 131</td>
<td>-18.9</td>
<td>-13.2</td>
<td>-21.8</td>
</tr>
<tr>
<td>Diet H (pure C$_4$ control)</td>
<td>-8.0</td>
<td>-3.2</td>
<td>-15.7</td>
</tr>
</tbody>
</table>

†Diets A and H are control animals consuming pure C$_3$ and C$_4$ diets. Day 131 corresponds to an animal consuming a pure C$_3$ diet from birth then switched to a pure C$_4$ diet at 91 days and then sacrificed at 131 days. The change in diet is detected as a 7.3‰ enrichment in the $\Delta^{13}C$ content of the cholesterol after only 40 days of feeding on the pure C$_4$ diet.
and 5·1‰, respectively. The wider ranges of δ^{13}C values observed for the lipids compared with those of the collagen support the contention that lipids will reflect short-term variations in diet due to their faster rates of turnover and/or that metabolic smoothing is a more pronounced factor in the isotopic composition of collagen.

Archaeological Populations

Figure 3 shows typical partial HT-GC profiles of the total lipid extracts recovered from femoral sections of varying date and geographical location. The extracts are dominated by cholesterol (c. 18·5 min retention time), identified unambiguously by GC/MS (m/z 368, M^+ 458, [M-TMSOH]). The most dominant degradation product is cholest-5-en-3β-ol-7-one indicating oxidizing conditions pre- or post-excavation. Cholesteryl fatty acyl esters were confirmed unambiguously (m/z 368; [M-RCO_2H]^+) eluting at longer retention times (~25–28 min) than cholesterol, by electron impact HT-GC/MS analysis.

Homogeneity of cholesterol δ^{13}C values and stability of the cholesterol molecule

The above results based on modern human and experimental animals were essential to underpin the isotope analysis of cholesterol in bones and teeth from archaeological populations. However, a major methodological concern was whether cholesterol was isotopically homogeneous: (1) throughout a skeletal member; (2) within different teeth from around a jaw; and (3) throughout different skeletal members of individuals. To assess the isotopic homogeneity of cholesterol in bone, we carried out multiple sampling of sections of bone taken at 3 cm intervals along the length of an intact femur (excavated from Barton-on-Humber, U.K.). The δ^{13}C values along the femur sections provided evidence that the cholesterol signature was isotopically homogeneous along the bone. Statistical analysis of the scatter showed that the precision of the technique was better than ± 0·4‰. Student’s t-test confirmed that there were no significant differences in the δ^{13}C values of cholesterol, confirming the use of irm-GC/MS as a highly reproducible analytical tool for the accurate determination of the δ^{13}C values of individual lipids preserved in archaeological bones (Stott & Evershed, 1996; Stott, Evershed & Tuross, 1997). The δ^{13}C values of cholesterol in teeth around a jaw were determined and isotopic homogeneity in both the upper and lower jaw was again very reproducible (Figure 4).

Skeletal members from five individuals, including rib, vertebral arch, radius, femur, pelvis, cranium and teeth were also measured. Isotopic differences between skeletal members were found to be relatively insignificant (± 1·0‰). Based on our observations, femurs and teeth were selected as our preferred sources of cholesterol δ^{13}C information since: (1) teeth were found to contain the greatest concentrations of cholesterol on a dry weight basis; (2) femurs and teeth are the most often recovered skeletal remains found at archaeological sites; and (3) the femur is the largest bone in the human skeleton, providing sufficient opportunities for sampling for isotopic analysis without causing...
interference to other types of skeletal investigations. These analyses served to demonstrate the simplicity and reliability of accessing palaeodietary information from archaeological bones (and teeth) using a single biomolecule such as cholesterol.

Isotopic variations between two Mediaeval populations

The rationale behind this study was to demonstrate the potential of using cholesterol as a complementary source of isotopic information for use in palaeodietary studies, in addition to the well-established use of bone collagen and bioapatite. The δ¹³C and δ¹⁵N isotopes of collagen should provide an indication of the life time input of carbon and nitrogen into the diets of ancient individuals, whereas the δ¹³C value of cholesterol should provide isotopic information relating specifically to the carbohydrate/lipid (energy) component of the diet. Our main focus has been to measure the isotopic composition of cholesterol (δ¹³C) and collagen (δ¹³C and δ¹⁵N) recovered from the skeletal remains of two ancient English populations. A Mediaeval coastal population from Barton-on-Humber (Lincolnshire, U.K.) and a Mediaeval inland population from Abingdon Vineyard (Oxfordshire, U.K.) were sampled, providing individuals whose major dietary intake would have been predominantly C₃ cultigens, terrestrial protein and marine foods. The aim of this study was to determine whether or not the δ¹³C value of cholesterol could be used to provide evidence of subtle dietary differences during a period of time in which we do not expect to see drastically different dietary subsistence between the two communities. Figure 5 shows a plot of the δ¹³C values measured for bone cholesterol recovered from different individuals recovered from these two locations. The distribution of cholesterol δ¹³C values from femurs for the Barton-on-Humber population maximizes at c. −21·4‰, whereas that of the Abingdon population maximizes at c. −23·4‰. Statistical analyses showed that the 1·7‰ variation between the mean cholesterol δ¹³C values from the two populations is significant (6·1 s.e.; P ≤ 0·001), therefore isotopically distinguishing the two communities. It is apparent from the isotopic data summarized in Figure 5, that significant differences in the δ¹³C values of cholesterol exist between some individuals, which must reflect the varied consumption of dietary lipid and carbohydrate metabolic variations within the individuals of the two populations. The δ¹³C values of cholesterol from individuals from Barton-on-Humber show...
a mean δ¹³C value of −22.2‰ (n=50, variance=0.7, σ=0.8, s.e. of mean=0.1), however, several individuals plot to the extremities c. −26‰ and c. −20‰. Similarly, the individuals from Abingdon Vineyard also exhibit this range of δ¹³C values, however, the mean δ¹³C of cholesterol plots at −23.9‰ (n=27, variance=2.3, σ=1.5, s.e. of mean=0.3). No such variation was seen in the δ¹³C values of collagen from the same individuals (see Figure 5). Collagen δ¹³C values measured on the Abingdon population show a mean of −19.0‰ (variance=0.9, σ=0.9, s.e. of mean=0.3) while that of the Barton-on-Humber population plotted at −18.9‰ (variance=0.2, σ=0.4, s.e. of mean=0.1), a difference of 0.1‰. Statistical analysis of the data revealed that the difference between the mean collagen δ¹³C values of the inland and coastal populations was insignificant (2.3 s.e.; 0.05>P>0.01) indicating no variation between the average collagen δ¹³C content of the two communities.

We can, at present, only speculate in the interpretation of the observed differences in the cholesterol δ¹³C values between the two populations. As previously mentioned, the δ¹³C value of cholesterol predominantly reflects input from dietary carbohydrate and lipids. Marine foods are, however, primarily protein by weight. The following possibilities may therefore exist as to why there is such variation in the δ¹³C values of the cholesterol between the coastal and inland populations and subsequently within a single population. Since collagen reflects mainly the carbon
isotopic composition of the dietary protein, the absence of differences in mean collagen $\delta^{13}C$ values between Abingdon Vineyard and Barton-on-Humber suggests that the isotopic composition of dietary protein was basically the same for both populations. Conversely, since consumer tissue cholesterol preferentially reflects the isotopic composition of the mainly non-protein macronutrients, the difference in mean cholesterol $\delta^{13}C$ values between the populations must reflect either differences in the isotopic composition of the dietary lipids and/or carbohydrate.

Additionally, if the isotopic signature of cholesterol is very sensitive to changes in the lipid/carbohydrate content of the diet, as observed in the rat turnover experiment, it could be predicted that the observed cholesterol $\delta^{13}C$ values may offer a short-term reflection of differing inputs of the lipid/carbohydrate into the diets of the individuals. As an example, if an individual consumed a diet consisting of a typical C3 terrestrial/marine protein diet (with associated carbohydrate and lipids) over a period of 10 years then the collagen $\delta^{13}C$ value over that 10-year period would not change dramatically. However, during that 10-year period the cholesterol $\delta^{13}C$ value would surely fluctuate according to the specific input of dietary carbohydrate and lipids (relating to the terrestrial and/or marine source) due to the faster turnover rate of this component. The controlled dietary turnover experiment in which C3-fed rats were switched to a C4 diet may help to exemplify this point, e.g., rats at the end of the turnover experiment largely had a C3 collagen signature yet the cholesterol signature was C4. Could not a similar type of scenario be feasible for a human population? The higher mean $\delta^{13}C$ value obtained for the cholesterol from the Barton population may suggest a temporary bias toward a consumption of marine foods which would not be immediately reflected in their collagen $\delta^{13}C$ value due to its slower turnover time. Likewise, the majority of the inland population perhaps reflects a bias towards terrestrial C3-derived carbohydrate and lipid consumption, based on the observation that cholesterol was isotopically depleted in $\delta^{13}C$ by up to 6‰ in some individuals. These latter observations emphasize the potential of using cholesterol as a palaeodietary indicator as its isotopic signature appears to record the short-term changes in diet which are not recorded by the collagen.

**High-marine diets and prehistoric humans**

Figure 5 also shows the cholesterol $\delta^{13}C$ values measured for several individuals whose diet comprised a high input of marine food. The most enriched $\delta^{13}C$ value plots at $-19‰$ and was obtained from a prehistoric native North American who originated from a population known ethnographically to be seal hunters. Two individuals from a Mesolithic coastal European site, whose diet consisted of a large proportion of marine shellfish, showed cholesterol $\delta^{13}C$ values of approximately $-20‰$. These results are very encouraging with respect to the cholesterol values measured for the two English populations since they
provide us with the first insight into the cholesterol \( ^{13}C \) value from individuals consuming high-marine protein/lipid diets.

**Conclusions**

The development of a methodology to measure compound-specific \( ^{13}C \) values of cholesterol preserved in skeletal tissues using irm-GC/MS, provides a new source of palaeodietary information to be used in conjunction with collagen and apatite stable isotope data. The current status of our research in this area can be summarized as follows:

1. The use of experimental animals raised on isotope-different diets has shown that cholesterol faithfully records the isotopic signature of dietary biochemicals and that the \( ^{13}C \) values are biased towards the original isotopic composition of the dietary carbohydrates and fats c. 70\% of whole diet.
2. We have the first evidence that the \( ^{13}C \) value of cholesterol is sensitive to dietary changes due to its quicker turnover rate and would therefore be of great value in interpreting subtle dietary changes in archaeological populations.
3. The use of human surgical tissue (hip bone) sampled from individuals in a modern population showed that isotopic variation exists between collagen and cholesterol and that the lipids appear to reflect short-term variations in the diet.
4. The \( ^{13}C \) value of cholesterol in archaeological skeletal remains has been shown to be homogeneous throughout a skeletal member (femur), between different teeth from around a jaw and between different skeletal members of an individual.
5. Comparison of the cholesterol \( ^{13}C \) values recovered from two ancient English populations (inland and coastal) appear to indicate subtle differences in consumption of terrestrial and marine foods which seem not to be reflected in their collagen \( ^{13}C \) values alone.
6. Archaeological information and cholesterol \( ^{13}C \) values measured for prehistoric humans appearing to consume high-marine diets provided corroboration evidence for the apparent consumption of marine foods by individuals from the Mediaeval English coastal population.

Our future objective is to further develop these various areas of palaeodietary research, ultimately aiming to combine isotopic information from cholesterol and collagen to produce a high-fidelity approach to palaeodietary analysis.

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**References**


