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Steroidal biomarker analysis of a 14,000 years old putative human coprolite from Paisley Cave, Oregon

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ABSTRACT

Lipid components of a putative human coprolite sample from the Paleoindian site of Paisley Cave, Oregon (12,300 14C yr BP) were analyzed using GC/MS to explore its origin in light of controversial data obtained from mitochondrial DNA, cross-immunoelectrophoresis, trisodium phosphate rehydration, and micromorphology analyses. Results show the predominant presence of 5β-stigmastanol, the biomarker of herbivore fecal matter, supporting micromorphological identification of the coprolite as herbivore. This study highlights the potential of the biomarker approach in coprolite studies devoted to research on the first presence of humans in North America.

1. Introduction

The time when humans spread to the Western hemisphere remains an open question. Although some claims have been made for pre-Clovis sites in North (e.g., Adovasio et al., 1999; Gilbert et al., 2008; Falk, 2004; Jenkins et al., 2012) and South America (Dillehay, 2000; Guidon, 1989; Waters and Stafford, 2007), the putative earliest sites that are typically mentioned are still controversial (Fiedel, 2000; Kelly, 2003). The first clear evidence of occupation appears much later and is related to the Clovis complex −11.000 to 10.800 14C yr BP (Waters and Stafford, 2007).

Paisley Cave in south-central Oregon is commonly cited as the earliest evidence of humans in North America. The genetic data obtained from 12,300 yr BP radiocarbon-dated paleofecal remains (coprolites) has been interpreted as proof of a pre-Clovis occupation of North America (Gilbert et al., 2008; Jenkins et al., 2012) associated with evidence of technologically different industries of uncertain authorship (Eren et al., 2013). However, there is no consensus as to whether all the claimed coprolites are indeed human. Some doubts are based on micromorphological and micro-FTIR analysis of specimen 1374-5/5D-31-2 (Goldberg et al., 2009), and the ambiguous detection in those of Native American mitochondrial DNA (Poinar et al., 2009).

The origin of the oldest specimen (1374-5/5D-31-2) has been hotly debated (Gilbert et al., 2008, 2009; Goldberg et al., 2009; Poinar et al., 2009; Rasmussen et al., 2009; Jenkins et al., 2012). The coprolite has been directly radiocarbon-dated to 12,400 +− 60 ^{14}C yr BP by Beta Analytic, and to 12,275 +− 55 by Oxford University, with both reporting substantially different δ^{13}C values −18.4 per mil and −16.6 per mil, respectively. This discrepancy has been interpreted as too great to correspond to the same dated material (Poinar et al., 2009). Poinar and coworkers point out that the δ^{13}C of the coprolite matches that of grasslands. Since humans are normally omnivorous, these values are inconsistent with an omnivorous diet and therefore suggest that the coprolite is not human in origin. The DNA analysis suggests a Native American origin on the basis of the presence of Haplogroup B2. In

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addition, other human (non-Native American), and canid sequences were detected with generic mammalian 16S mtDNA primers. Some studies cast doubts on the reliability of molecular studies from feces due to the possibility of contamination (Hofreiter et al., 2010). And at Paisley Cave, although contamination by modern haplogroups was detected in all the paleofecal specimens, the source of such contamination was never clearly identified (Gilbert et al., 2009; Rasmussen et al., 2009).

In addition, the results of cross-immunoelectrophoresis (CIEP) analysis did not reveal human antigens for sample 1374-5/5D-31-2 (Gilbert et al., 2008). Moreover, the micromorphological and Fourier transform infrared spectroscopy (FTIR) analyses carried out by Goldberg et al. (2009) are incompatible with a human origin of the specimen and more consistent with herbivore fecal excreta. In order to distinguish between a human vs. herbivore origin, Rasmussen et al. (2009) present genetic data showing that the putative endogenous DNA in this sample is such because it is highly fragmented and that the presence of modern human and *Bos taurus* sequence should be attributed to laboratory contamination, since the cow was not present in the Americas before European contact. Moreover, they show the presence of DNA from local plants, such as grasses and conifers (Rasmussen et al., 2009).

Fecal biomarkers have proved be an increasingly valuable tool in the identification of the likely source of fecal matter. This approach has successfully been applied in other fields, including organic geochemistry (Pratt et al., 2008), archaeological chemistry (Baeten et al., 2012; Birk et al., 2011; Bull e al., 1999a,b, 2001, 2002, 2003, 2005; D'Anjou et al., 2012; Evershed et al., 1997; Gill et al., 2009; Gulaçar et al., 1990; Lin et al., 1978; Shillito et al., 2011; Van Geel et al., 2008, 2011), environmental science (Cordeiro et al., 2008; Glaser and Birk, 2012), biochemistry (Rawlins et al., 2007), geomicrobiology (Veiga et al., 2005), and water research (Isobe et al., 2002; Leeming et al., 1996, 1997; Saim et al., 2009).

The biomarker analysis approach to coprolites provides direct evidence of the digestive system and diet of the source and is strongly called for in order to assess the origin of the fecal deposit (Baeten et al., 2012; Gill et al., 2009; Gulaçar et al., 1990; Lin et al., 1978; Linseele et al., 2013; Shahack-Gross, 2011; Shillito et al., 2011; Van Geel et al., 2008, 2011). Here we present a biochemical study from a subsample of the specimen 1374-5/5D-31-2 aimed at distinguishing between omnivore and ruminant material toward archaeological interpretation.

The present study targets sterols and stanols, lipid compounds known to be relatively stable across organic cycling processes (Floate, 1970; Hunt, 1977; Mackenzie et al., 1982; Peters et al., 2005). The use of stanols as biomarkers is possible because such products are uniquely formed during the metabolic reduction of cholesterol and phytosterols in the intestinal tract of most higher mammals. Their scarcity in natural environments and the biomarker specificity of different fecal sources can be a useful tool in tracing the origin of fecal deposits. In addition, relative proportions are indicative of mammal dietary preferences (Bethell et al., 1994; Bull et al., 1999a; Van Geel et al., 2008, 2011).

2. Materials and methods

2.1. Sample preparation and analytical procedures

A subsample (2.70 mg) was taken from the coprolite specimen 1374-5/5D-31-2 stored in a plastic bag. Samples of modern sheep stool were collected and analyzed using the same weight in order to reflect the reliability of this method with such small amounts. This was the only sample we could analyze. We did not have access to the surrounding matrix, which would shed some light on potential contamination. The normal procedure would involve more steps including sample workup/purification and separation or addition of internal standards. However, we decided to avoid potential risks of contamination or losing the sample due to its small size. Hence, we did not analyze bile acids, which have proved to be a highly diagnostic fecal marker but entail a more difficult procedure. We considered that the diagnostic capability of the 5β-stanols is high enough to distinguish between human and herbivore species.

To test the utility of the lipid biomarker approach to coprolite analysis, lipids were extracted using a modified version of the lipid extraction of Bligh and Dyer (1959). Briefly, all samples were air dried and ultrasonically extracted twice with chloroform (CHCl)/methanol (MeOH) (2:1 v/v) at 40 °C for 45 min to obtain a total lipid extract.

Derivatization was carried out by heating (70 °C for 1 h) the dried total lipid extract with a mixture of 50 μL pyridine with 50 μL of N,O-bis(trimethylsilyl)triofluoroacetamide containing 1% trimethylchlorosilane solution (BSTFA + 1% TMCS; Sigma) (Bull et al., 2001, 2003). Excess derivatization agent and pyridine were removed under a gentle stream of nitrogen. Samples were then diluted with an appropriate volume of hexane prior to GC/MS analysis.

2.2. Gas chromatography–mass spectrometry (GC/MS)

GC/MS analysis was conducted using an Agilent Technologies 6890N coupled to an Agilent 5973 quadrupole mass selective detector, with a DB5-ms non polar capillary column (5%-methyl phenyl polysiloxane, 0.25 mm of internal diameter, 30 m length and 0.25 μm phase thickness).

The analysis was carried out using full scan, with the following parameters, 5 μL injected via automatic liquid sampler; the oven was temperature programmed with an initial isothermal of 2 min at 40 °C followed by an increase to 350 °C at 10 °C per minute followed by a final isothermal at this temperature of 15 min. Helium was used as carrier gas and held at a constant flow at 2.0 ml per minute throughout the analysis. Compounds were identified based on mass fragmentation spectra and GC retention times (e.g., Bethell et al., 1994; Evershed et al., 1997; Bull et al., 1999a, 2001, 2002, 2003).

3. Results and discussion

Fig. 1 depicts a GC profile of the total lipid extract obtained from specimen 1374-5/5D-31-2 represented as total ion current (TIC) and three partial chromatograms recording the TIC and occurrence of diagnostic ions arising from specific sterol fragments. The distribution of sterols observed is dominated by 5β-stigmastanol (24-ethyl-5β-cholestan-3β-ol) followed by its sterol precursor sitosterol (24-ethylcholest-5-en-3β-ol) with lower amounts of cholesterol and its stanol product, coprostanol (5β-cholestan-3β-ol). The stanols (*m*/*z* 215) represent more than 69% of the total sterol fraction.

The 5β-stanols are the product of their Δ^5-sterol precursors reduced by the enteric bacteria in the gut of higher mammals and then excreted in their feces (Hatcher and McGillivary, 1979; Murtaugh and Bunch, 1967). Herbivores produce a higher relative proportion of 5β-campestanol and 5β-stigmastanol by biohydrogenation of campesterol and sitosterol (Δ^5-sterols), respectively (Nichols et al., 1996). The sterol distribution dominated by 5β-stigmastanol has been previously used as a proxy indicator of manure (Bull et al., 1998, 1999a, 2002; Evershed et al., 1997).

The low occurrence of cholesterol and coprostanol is not consistent with a human origin, since coprostanol is the major 5β-stanol in human feces, about 60% of the total sterol content (Bethell et al., 1994; Leeming et al., 1984; Shah et al., 2007). Coprostanol is formed by microbial hydrogenation of its cholesterol precursor (Δ^5-