Quaternary Geochronology 22 (2014) 85–98

Contents lists available at ScienceDirect

Quaternary Geochronology

journal homepage: www.elsevier.com/locate/quageo

Research paper

Ultrafiltration for asphalt removal from bone collagen for radiocarbon dating and isotopic analysis of Pleistocene fauna at the tar pits of Rancho La Brea, Los Angeles, California

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ARTICLE INFO

Article history:
Received 21 October 2013
Received in revised form
5 March 2014
Accepted 15 March 2014
Available online 26 March 2014

Keywords:
Palaeoecology
Megafauna
Extinction
Palaeodietary reconstruction
Climate change

ABSTRACT

A novel protocol to purify bone collagen for radiocarbon dating and stable isotope ratio analysis from asphalt-impregnated skeletal remains stored in the George C. Page Museum of La Brea Discoveries (Los Angeles, California) is presented. This simple technique requires that bones be crushed (1–2 mm), sonicated in a 2:1 toluene/methanol solution, and gelatinized at 75 °C overnight to break down collagen strands for ultrafiltration. However, here the traditional protocol of ultrafiltration is reversed, and the high molecular weight fraction (>30 kDa) contains mainly the asphalt (too big to pass through the filter), while the lower molecular weight fraction (<30 kDa) contains the collagen. A second ultrafiltration (>3 kDa) is then performed on the <30 kDa fraction to remove lower molecular weight contaminants such as hydrocarbons and humic acids. The middle fraction (3–30 kDa) is freeze dried and produces collagen with excellent atomic C:N ratios between 3.2 and 3.5. The steps involved in the design of the protocol will be discussed in detail, and the first isotopic results and radiocarbon dates from the Project 23 site will be presented. In addition, the largest compilation of carbon and nitrogen isotopic results directly paired with radiocarbon ages on bone collagen from 38 land mammals found at the Rancho La Brea site are presented. Finally, while this protocol was specifically designed to extract collagen from samples at the Rancho La Brea site, it is likely that it can be applied to other localities (e.g. Cuba, Ecuador, Peru, Venezuela, etc.) where bones have been impregnated with petroleum.

1. Introduction

The tar pits of Rancho La Brea (RLB) located in Los Angeles, California contain one of the largest concentrations of floral and faunal remains from the late Pleistocene and provide one of the most detailed and complete pictures of North America at the end of the most recent glacial period (for reviews see, Marcus and Berger, 1984; Quinn, 1992; Stock and Harris, 1992; Harris, 2001; Ward et al., 2005; Friscia et al., 2008; Gerhart et al., 2012). The fossils were preserved in open asphalt seeps that pooled and accumulated at surface localities and acted as episodic traps that captured plants, insects, reptiles, birds and mammals. Over the last century, excavations at this location have recovered millions of specimens that range in age from >50,000 years to modern, with each deposit suspected of recording a variable timeframe of accumulation (O'Keefe et al., 2009). Most of the vertebrate skeletal remains are exceptionally well preserved and contain close to modern amounts of collagen as they were immersed and impregnated with asphalt shortly after death and display little evidence of weathering (Spencer et al., 2003; Coltrain et al., 2004; Holden et al., 2013). However, this asphalt now poses a significant challenge to remove from bone collagen for accurate radiocarbon dating and palaeodietary reconstruction using stable isotope analysis.

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http://dx.doi.org/10.1016/j.quageo.2014.03.002
1871-1014/

Methods developed to purify collagen from petroleum-saturated bones at RLB are summarized by O'Keefe et al. (2009) (for details, see Ho et al., 1969; Marcus and Berger, 1984; Coltrain et al., 2004; Fox-Dobbs et al., 2006; Friscia et al., 2008). Briefly, all of these techniques involve a combination of solvent washes (e.g. Soxhlet treatment) followed by the isolation of the bone collagen or amino acids under different hydrolysis conditions (time, temperature, acid strength, etc). While these protocols have been successfully applied, the removal of asphalt is still considered difficult and time consuming, and the resultant extracts may still be contaminated with residual hydrocarbons. This is likely one reason why relatively few radiocarbon and isotopic results from the RLB collections have been published (Ho et al., 1969; Marcus and Berger, 1984; Coltrain et al., 2004; Chamberlain et al., 2005; Fox-Dobbs et al., 2006; Bump et al., 2007; Friscia et al., 2008; O'Keefe et al., 2009). For example, in a pioneering study Coltrain et al. (2004) measured carbon and nitrogen stable isotope ratios in 143 faunal bone samples, but 23 of these produced atomic C:N ratios >3.6 that fell outside the accepted range of 2.9–3.6 for stable isotope ratio analysis (DeNiro, 1985; Ambrose, 1990), suggesting that residual tar contamination was present. This is unlikely to greatly alter the interpretation of the stable isotope results (see Discussion 5.2), but the presence of even small amounts of hydrocarbon contamination can introduce significant errors into calculated ^{14}C ages. If the RLB deposits are to be accurately radiocarbon dated and analyzed for stable isotopes on a large scale, a rapid and effective method for the removal of the asphalt from bone collagen is required.

Here we describe a novel protocol for collagen purification from asphalt-impregnated skeletal remains recovered from the RLB tar pits of Los Angeles, California. A total of 38 individual land animals are attributed with ^{14}C ages directly paired with carbon and nitrogen isotopic results from a number of pit localities including a recently discovered area of excavation known as Project 23 ($n = 16$). A subset of these specimens was intensively studied for method development. The processes involved in the design of the protocol will be discussed in detail and the first radiocarbon dates and stable isotope measurements from the Project 23 site will be presented, including the first radiocarbon date for a RLB mammoth (*Mammuthus columbi*).

2. Project 23 at the George C. Page Museum of La Brea Discoveries

In 2006, during construction of a new underground parking structure at the Los Angeles County Museum of Art, 16 previously unknown asphalt-encased fossil deposits were discovered and also the semi-articulated, largely complete skeleton of an adult male mammoth. As a complete in-situ excavation of the fossils was not financially feasible, 23 wooden boxes were built around large blocks of sediment that were removed intact for subsequent detailed excavation (hence "Project 23"). Detailed descriptions of earlier excavations, the geology, and the fossils recovered at the RLB tar pits can be found in Marcus and Berger (1984), Quinn (1992), Stock and Harris (1992) and Friscia et al. (2008).

3. Methods

3.1. Standard collagen extraction procedure at UC Irvine

At the UC Irvine, Keck Carbon Cycle AMS Laboratory the standard method for isolation of bone collagen for radiocarbon dating and stable isotope analysis (Beaumont et al., 2010) is based on the protocol of Longin (1971), modified by Brown et al. (1988) to include ultrafiltration. Bone is sectioned and cleaned using a handheld Dremel rotary tool and samples of ~150 mg are cleaned, crushed to small pieces (1–2 mm), and demineralized in 0.5 N HCl for 24–36 h at room temperature. The resultant collagen pseudomorphs are rinsed with Milli-Q water to pH > 3, and gelatinized in a 0.01 N HCl solution at 60 °C overnight. The gelatin solutions are concentrated using pre-cleaned (Beaumont et al., 2010) 30 kDa Centriprep ultrafilters (Fisher Scientific). Samples are ultrafiltered twice, diluted with Milli-Q water to reduce salt content, and then ultrafiltered twice more (RCF 1500 g, 20 min each time). The concentrated (≈1 ml) high molecular weight fractions >30 kDa are frozen with liquid nitrogen and lyophilized overnight in a vacuum centrifuge. Collagen quality is checked (C:N ratio, %C, %N) and stable isotope ratios are analyzed by placing ~0.7 mg of collagen in tin capsules which are combusted to CO_2 and N_2 and analyzed using a Fisons NA1500NC elemental analyzer/Finnigan Delta Plus isotope ratio mass spectrometer combination. Replicate measurement errors on known standards were approximately ±0.1‰ for δ^{13}C and 0.2‰ for δ^{15}N. Accelerator mass spectrometry (AMS) dating is then performed on graphitized CO_2 derived from 2 mg of collagen using a National Electrostatics Corporation 0.5 MV 1.5SDH-1 Pelletron with a 60 sample modified MC-SNICS ion source (Southon and Santos, 2004). All unknowns are run with oxalic acid standards (OX1), known age bone standards, modern (19th century cow) and "radiocarbon dead" blanks (Beaufort Sea whale, 60–70 kyr), that are prepared in the same manner as the unknowns.

Given the unique nature of the asphalt-impregnated bone specimens from RLB, this protocol required significant modification. In addition to the work of Longin (1971) and Brown et al. (1988), the publications of Marcus and Berger (1984) and Coltrain et al. (2004) also guided the development of this method.

3.2. Preliminary tests

Initial tests were performed on ten bones believed to represent seven individual animals from several previous excavations at RLB (Pits, 3, 61, 67 and 91) using collagen samples extracted for an earlier stable isotope study by Coltrain et al. (2004). These had undergone a prolonged 3-stage solvent extraction procedure to remove tar. Briefly, bone plugs removed from adult skeletal elements with a coring drill were subjected to two successive 24-hr soaks in 2:1 toluene/methanol, followed by 24 h of Soxhlet extraction with the same solvent mix. The plugs were then demineralized in 0.6 N HCl, extracted with 5% KOH to remove humics, and lyophilized. The demineralized bone plugs then underwent a second 48-hr solvent soak, followed by vacuum drying, gelatinization at pH 3 for 24 h at 120 °C, and lyophilization.

Since the second toluene/methanol wash of the original procedure had produced at least some color in the solvent indicating that residual tar was being removed, a further 48-hr soak was carried out but no solvent coloration was observed. However, when we redissolved the collagen in water all ten samples yielded rich golden gelatin solutions, suggesting that residual tar was still present. In at least some "normal" bone (uncontaminated by hydrocarbons), color in gelatin solutions is associated with low molecular weight contaminants and can be effectively removed by isolating a >30 kDa fraction by ultrafiltration, and this technique was therefore applied to the RLB bones. The results were dramatic and completely unexpected: all ten samples yielded clear or very pale gold <30 kDa filtrate, while the high molecular weight fractions, that would normally be selected for dating, were invariably dark brown or completely black.

It was clear from these results that the great majority of the residual tar/asphalt contamination not removed by the solvent treatment was composed of very large molecules, or more likely, of large aggregates of smaller tar constituents such as colloidal asphaltenes that have a strong tendency to combine into