

## 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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## 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.

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## 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](#); [Frischia 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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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  $^{14}\text{C}$  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  $^{14}\text{C}$  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 pseudo-morphs 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  $\text{CO}_2$  and  $\text{N}_2$  and analyzed using a Fisons NA1500NC elemental analyzer/Finnigan Delta Plus isotope ratio mass spectrometer combination. Replicate measurement errors on known standards were approximately  $\pm 0.1\%$  for  $\delta^{13}\text{C}$  and  $0.2\%$  for  $\delta^{15}\text{N}$ . Accelerator mass spectrometry (AMS) dating is then performed on graphitized  $\text{CO}_2$  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 re-dissolved 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 asphaltene that have a strong tendency to combine into

macroscopic deposits (Mullins et al., 2007; Goual, 2012). This was confirmed by radiocarbon dates carried out on aliquots of the original collagen, plus the lyophilized <30 kDa filtrate and >30 kDa retentate from the ultrafiltration (Table A.1). The low molecular weight fractions were systematically younger than the unfiltered collagen by up to 400 years, while the few >30 kDa samples that yielded sufficient material for dating were several thousand years older. Our subsequent efforts therefore focused on maximizing the yield of an uncontaminated low (or intermediate) molecular weight collagen fraction, and on simplifying and shortening the extraction procedure.

### 3.3. Additional test samples

We selected nine bone specimens of unknown age from the Project 23 site for systematic protocol development, plus six samples from an earlier excavation (Pit 91) that had been dated by Friscia et al. (2008) using total amino acid extracts prepared by strong acid hydrolysis and purified using an XAD resin (Stafford et al., 1988, 1991). While time consuming, that method is an established technique for AMS  $^{14}\text{C}$  dating of poorly preserved bone or bone that has been conserved or contaminated (Stafford et al., 1988, 1991; Minami and Nakamura, 2000, 2004, 2005; O'Keefe et al., 2009); we were keen to compare results from our new ultrafiltration method against dates obtained using the well-established XAD technique. We also obtained stable isotope and elemental (%C and %N) data for six XAD amino acid extracts that had been previously dated by Friscia et al. (2008), and we compared isotopic results on ultrafiltered and non-ultrafiltered fractions using five ultrafiltered samples from specimens that were previously isotopically analyzed by Coltrain et al. (2004).

With each batch of samples tested, we ran aliquots of  $^{14}\text{C}$ -dead Beaufort Sea Whale (age 60–70 kyr; Tom Stafford, pers. comm.), an in-house whalebone standard ( $^{14}\text{C}$  age  $8350 \pm 50$  BP), and a cow bone from a late 19th century archaeological site (Brendan Cullerton, pers. comm.) that was used to monitor dead carbon background from (for example) solvent retention. We considered soaking an aliquot of the modern bone in tar or a tar/solvent mix to create an additional test sample, but did not implement that for the present study, as it is uncertain how well such a blank would simulate the effects of tens of kyr of immersion in tar at RLB.

### 3.4. Investigated parameters for purification of RLB collagen

#### 3.4.1. Ultrafiltration

It has been shown that provided proper cleaning procedures are followed, ultrafiltration is highly effective at removing contaminants and purifying bone collagen for radiocarbon dating, and it is used as a standard method in several  $^{14}\text{C}$  laboratories (Brown et al., 1988; Higham et al., 2006; Brock et al., 2007; Beaumont et al., 2010; Brock et al., 2013). Here we test a combination of three ultrafilters with different molecular weight cut-offs: 30 kDa, 10 kDa, 3 kDa, for their ability to separate hydrocarbons from bone gelatin.

#### 3.4.2. Gelatinization temperature

Piez (1984) quotes the melting point of pure mammalian collagen at approximately  $58^\circ\text{C}$  (Piez, 1984) and >30 kDa collagen yields for normal bone peak at around that temperature (Brown et al., 1988; Beaumont et al., 2010). For this method development we examined how five different gelatinization temperatures:  $60^\circ\text{C}$ ,  $70^\circ\text{C}$ ,  $75^\circ\text{C}$ ,  $80^\circ\text{C}$ ,  $85^\circ\text{C}$  at  $\sim 14$  h influenced the quality (color, texture) and quantity (collagen yield %) of recovered collagen from the RLB specimens.

#### 3.4.3. Solvent washes

The standard procedure for the removal of hydrocarbons from bone is based on solvent extraction, e.g. with a Soxhlet apparatus (Ho et al., 1969; Marcus and Berger, 1984; Fox-Dobbs et al., 2006) with different combinations of solvents used for the different protocols. Here we tested the effects of three different conditions and how they influenced the removal of asphalt:

1. No solvent treatment (bone directly demineralized and gelatinized).
2. Sonication in 2:1 toluene/methanol (4–6 h), followed by sonication in methanol (1 h), and Milli-Q water (1 h).
3. A protocol based on Coltrain et al. (2004): bone samples were soaked for 48 h in 2:1 toluene/methanol (replacing the solution after 24 h), followed by 24 h of Soxhlet extraction in 2:1 toluene/methanol.

#### 3.4.4. Crushed vs. powdered bone

In contrast to whole bone chunks, crushed bone fragments are easier to work with and have a faster time of demineralization. If the fragments are too finely powdered they can be lost during liquid transfer and pipetting, but the greater surface area of powdered vs. crushed bone can potentially result in more effective solvent extraction of petroleum products. Here we tested if improved  $^{14}\text{C}$  ages and greater tar removal could be obtained from powdered vs. crushed bone.

#### 3.4.5. NaOH treatment

Some collagen extraction protocols recommend the use of NaOH for the removal of humic acids (Arslanov and Svezhentsev, 1993; Minami et al., 2004), but care is needed when using NaOH as it can dissolve the protein and result in decreased yield or loss of sample, and in our laboratory this step is only used if the collagen is darkly stained. We initially considered humic contamination to be a second-order problem at RLB, and since Longin gelatinization was specifically designed to discriminate against humics (Longin, 1971) and free humic acids and other smaller contaminants are removed by ultrafiltration, our initial tests did not include a base treatment. However, our results on bone carbonate (see Section 4.6 below) suggested that RLB bones were significantly affected by contact with groundwater, and we therefore tested whether the additional step of treating demineralized bone with 0.1 N NaOH for 1 h resulted in improved  $^{14}\text{C}$  ages and isotopic results.

### 3.5. Carbonates

Bone carbonate radiocarbon results are widely considered unreliable compared to dates on bone collagen since they are far more susceptible to diagenetic alteration, especially due to groundwater contamination (Tamer and Pearson, 1965; Hassan et al., 1977). Limited numbers of carbonates were previously measured for radiocarbon dates in RLB samples, and the results were considered unsuccessful (Marcus and Berger, 1984). However, we decided to investigate if accurate radiocarbon dates could be obtained from a subset of these bones ( $n = 5$ ) given the possibility that the same tar/asphalt which protected the collagen from degradation may have also acted to seal off the bone carbonate from contact and exchange with groundwater. If so, the RLB tar pits would be a unique burial environment where carbonates could provide useful chronological information.

## 4. Results

### 4.1. Tar and bitumen

Untreated RLB tar and bitumen samples produced  $\delta^{13}\text{C}$  values of  $-23.4\text{‰}$  and  $-23.1\text{‰}$ , respectively, and  $^{14}\text{C}$  ages of  $50,030 \pm 300$  BP

(radiocarbon years Before Present, i.e. before AD 1950) and  $48,130 \pm 240$  BP (Table 1). These are “raw” radiocarbon ages (without background subtraction) and are slightly younger than typical lab backgrounds of 50–55 kyr BP measured on chemically cleaned wood and coal. Since the tar and bitumen are composed of “radiocarbon dead” petroleum products they should have infinite  $^{14}\text{C}$  ages, and the measured radiocarbon ages likely reflect small inclusions of younger organic fragments and soil carbon trapped in the tar.

#### 4.2. Preliminary tests

Radiocarbon results on the collagen prepared for the Coltrain et al. (2004) study are shown in Table A.1 and summarized in Table 2. Yields for the >30 kDa fraction were low, likely due to the very high gelatinization temperature of 120 °C, but the few samples that yielded sufficient material for dating gave  $^{14}\text{C}$  ages between 1800 and 4500 years older than the original unfiltered extracts. Conversely, the <30 kDa fractions were systematically younger than the unfiltered collagen, by up to 410 radiocarbon years. The dilution effect from the  $^{14}\text{C}$ -free tar in the unfiltered collagen (i.e. the fraction of the carbon contributed by the tar) is simply  $f = \Delta T/\tau$  where  $\Delta T$  is the age difference between the <30 kDa and unfiltered collagen and  $\tau$  is the Libby mean  $^{14}\text{C}$  lifetime of 8033 years (Libby, 1955). (For these initial tests we did not use a second ultrafiltration to reject low molecular weight contaminants, but given the appearance of the solutions we assumed that the <30 kDa fraction was essentially hydrocarbon free). Based on this analysis, the tar content of the unfiltered collagen that had already been subjected to prolonged and rigorous decontamination by solvent extraction, ranged from zero to 5.1% with a mean of  $3.1 \pm 1.6\%$ .

#### 4.3. Gelatinization temperature and molecular weight cutoffs

For the first attempt, the standard UC Irvine collagen extraction protocol (gelatinization at 60 °C) was used except for two key modifications. First, the samples were solvent soaked and Soxhleted (by L. G. at the University of Kansas) using the protocol of Coltrain et al. (2004) and were then shipped to UC Irvine for collagen extraction. Second, an additional 10 kDa ultrafiltration step was added. This produced three different collagen fractions: >30 kDa, 10–30 kDa, and <10 kDa.

Usually, the >30 kDa fraction containing the least diagenetically altered collagen is selected for isotopic analysis and radiocarbon dating, but the preliminary tests showed that this fraction was heavily contaminated with asphalt. While producing the most material (>5 mg), the >30 kDa fractions from the 60 °C gelatinization were very dark brown to black. The 10–30 kDa fractions typically produced only 1–2 mg of collagen, but the product was fluffy and white, indicating that it was relatively free of hydrocarbon contamination. The <10 kDa fractions consisted of an amber or yellow goeey/sticky substance that smelled like tar/asphalt.

Based on these results, we increased the gelatinization temperature to 85 °C, hoping that this would break up the collagen fibrils sufficiently to increase the 10–30 kDa yield while leaving most of the asphalt in the >30 kDa fraction. Two samples (#11 and #13) did yield enough 10–30 kDa collagen for both isotopic

analysis and radiocarbon dating. The atomic C:N ratios of these samples were 3.18 (#11) and 3.20 (#13) and the %C ( $\approx 42$ ) and %N ( $\approx 15$ ) were within the range of modern collagen values (Ambrose, 1990; van Klinken, 1999), suggesting that the collagen was essentially contaminant free and suitable for isotopic analysis and radiocarbon dating (Table A.2). However, most of the yield consisted of a goeey/sticky amber mass in the <10 kDa fraction, indicating that the temperature of gelatinization (85 °C) was too high.

Further tests showed that a gelatinization temperature of 70 °C was too low (the majority of the collagen was brown and black in appearance and located in the >30 kDa fraction) and that 80 °C was too high (two out of five samples #13 & #21 produced good 10–30 kDa collagen yields and C:N ratios (Table A.2) but the majority of the material from the other three was in the <10 kDa fraction). We therefore adopted a gelatinization temperature of 75 °C. In addition, we shifted the lower molecular weight cut-off to >3 kDa to maximize the yield of the middle fraction while maintaining at least some discrimination against small (hygroscopic) polypeptides and low molecular weight contaminants (soil humics, small fragments of tar residues, etc.). The 75 °C gelatinization temperature worked well for all but two specimens (#14 and #19) that produced almost no satisfactory collagen in any of the tests; (Table A.2). The collagen in the 3–30 kDa fractions isolated at 75 °C had  $^{13}\text{C}$ -enriched values compared to the >30 kDa and <3 kDa fractions (consistent with less contamination from isotopically lighter tar; Table 1) and excellent atomic C:N ratios (3.2–3.4) indicative of well preserved and uncontaminated collagen (Table A.2; see representative examples in Figs. 1a and 2a).

#### 4.4. Solvent washes

The studies reported above were made on bones subjected to prolonged solvent soaking and Soxhlet extraction using the protocol of Coltrain et al. (2004). We tested whether similar results could be obtained by simply sonicating RLB specimens in suitable solvent mixtures. Following Coltrain et al. (2004), we used a 2:1 toluene/methanol solution: toluene for its effectiveness in removing aromatics (“like dissolves like”) and methanol to facilitate the subsequent removal of the toluene. Five crushed bone samples (12,15,17,20,21; Table A.2) were sonicated repeatedly until the solution remained almost clear (typically, four to six 1 h cycles with the solvent replaced every hour), followed by sonication in methanol (1 h) and Milli-Q water (1 h). Cold tap water was circulated through a plastic hose in the sonicator bath to keep the temperature around 45 °C, well below the 58 °C melting point of collagen. The samples were gelatinized at 75 °C and the 3–30 kDa collagen fraction was collected and analyzed. All five bones yielded fluffy white collagen with C:N ratios between 3.2 and 3.4 and collagen yields sufficient for  $^{14}\text{C}$  and stable isotope analysis. In addition, the radiocarbon ages were similar to those of the Soxhleted bones, and samples 20 and 21 had radiocarbon ages similar to values reported previously for XAD-treated aliquots (Frischia et al., 2008) (see representative examples in Fig. 3a,b).

Given these results, we checked if the petroleum products could be removed by ultrafiltration alone, by processing the same five bone samples without any solvent extraction. Four out of the five bones yielded fluffy white 3–30 kDa collagen with C:N ratios between 3.2 and 3.4, but there was slightly more variability in the  $\delta^{13}\text{C}$  values and radiocarbon dates compared to the solvent-treated bones (Table A.2). Comparison of the radiocarbon ages of the “no solvent washed” bones to the dates of Frischia et al. (2008) showed mixed results. Sample 20 produced a  $^{14}\text{C}$  age of  $27,180 \pm 250$  BP that was indistinguishable from the  $27,350 \pm 120$  BP date in Frischia et al. (2008) (Fig. 3a), but the  $28,600 \pm 300$  BP result for sample 21 was marginally older than the published age of  $28,170 \pm 160$  BP (Fig. 3b).

**Table 1**  
 $^{14}\text{C}$  and isotopic results for the tar and bitumen samples from Rancho La Brea.

UCIAMMS	Sample	$\delta^{13}\text{C}$ (‰)	%C	$^{14}\text{C}$ age (BP)	$\pm$
108898	Bitumen 1	−23.1	23.6	50,030	300
	Bitumen 2	−23.1	31.5		
108897	Tar 1	−23.4	67.7	48,130	240
	Tar 2	−23.3	73.5		

**Table 2**  
Summary of all paired radiocarbon and isotopic results from Rancho La Brea. Mean  $\pm$  SD results are reported for isotopic values and collagen quality control indicators where samples have been measured three or more times. Samples denoted with a), b), c) or d) indicate different bones that are believed to represent same individual animal.

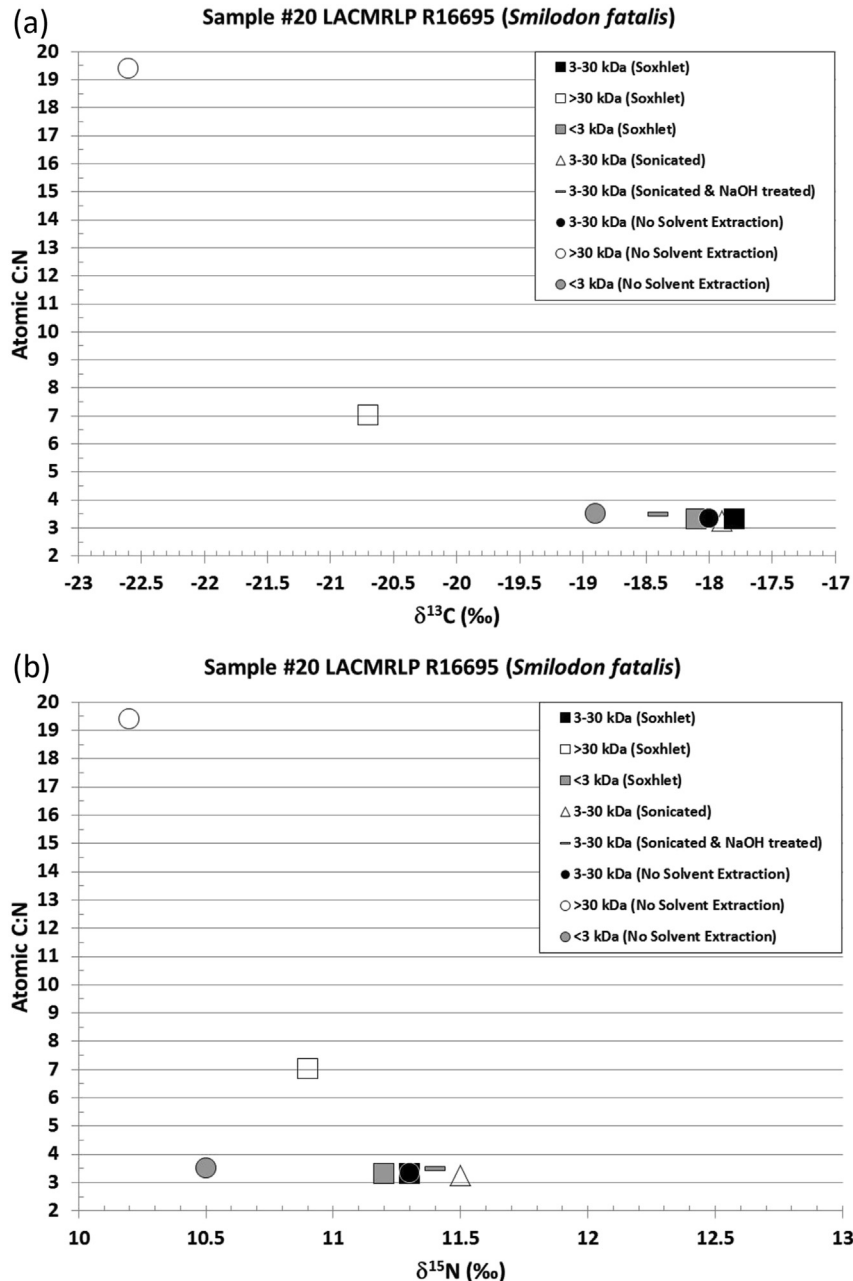
Sample# & Museum Catalogue#	Species	Pit	Collagen fraction analyzed	Collagen yield (%)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	Atomic C:N	Uncalibrated $^{14}\text{C}$ age (BP)	$\pm$
1a LACMHC Z1078; 1b LACMHC 60247	<i>Mammut americanum</i>	3	<30 kDa	14.0 <sup>a</sup>	-20.0 <sup>a</sup>	4.8 <sup>a</sup>	43.7 <sup>a</sup>	14.8 <sup>a</sup>	3.46 <sup>a</sup>	14,035	30
2a LACMHC 85217; 2b LACMHC 85216; 2c LACMHC 85219	<i>Mammut americanum</i>	61/67	<30 kDa	14.7 $\pm$ 1.2 <sup>a</sup>	-20.7 $\pm$ 0.2 <sup>a</sup>	4.3 $\pm$ 0.1 <sup>a</sup>	42.2 $\pm$ 1.1 <sup>a</sup>	14.3 $\pm$ 0.1 <sup>a</sup>	3.45 $\pm$ 0.13 <sup>a</sup>	12,030	40
3 LACMHC 35353	<i>Smilodon fatalis</i>	3	<30 kDa	12.0 <sup>a</sup>	-19.0 <sup>a</sup>	11.9 <sup>a</sup>	45.6 <sup>a</sup>	15.5 <sup>a</sup>	3.44 <sup>a</sup>	12,645	40
4 LACMHC 35915	<i>Smilodon fatalis</i>	67	<30 kDa	12.7 <sup>a</sup>	-19.1 <sup>a</sup>	12.7 <sup>a</sup>	45.4 <sup>a</sup>	15.3 <sup>a</sup>	3.47 <sup>a</sup>	11,335	35
5 LACMRLP R17668	<i>Smilodon fatalis</i>	91	<30 kDa	7.4 <sup>a</sup>	-18.2 <sup>a</sup>	11.7 <sup>a</sup>	45.4 <sup>a</sup>	15.0 <sup>a</sup>	3.54 <sup>a</sup>	26,370	200
6 LACMHC X7098	<i>Panthera atrox</i>	61	<30 kDa	21.1 <sup>a</sup>	-18.6 <sup>a</sup>	12.3 <sup>a</sup>	40.7 <sup>a</sup>	15.2 <sup>a</sup>	3.13 <sup>a</sup>	11,895	35
7 LACMHC X7117	<i>Panthera atrox</i>	67	<30 kDa	18.6 <sup>a</sup>	-19.2 <sup>a</sup>	12.4 <sup>a</sup>	41.4 <sup>a</sup>	15.0 <sup>a</sup>	3.22 <sup>a</sup>	12,225	35
8 LACMHC K3569	<i>Smilodon fatalis</i>	61/67	3–30 kDa	4.8 $\pm$ 2.6	-19.4 $\pm$ 0.1	12.3 $\pm$ 0.2	41.9 $\pm$ 0.8	15.1 $\pm$ 0.4	3.24 $\pm$ 0.04	12,515	25
9 LACMHC K3593	<i>Smilodon fatalis</i>	61/67	3–30 kDa	6.4 $\pm$ 2.7	-18.7 $\pm$ 0.1	12.1 $\pm$ 0.3	42.1 $\pm$ 1.6	15.3 $\pm$ 0.6	3.22 $\pm$ 0.04	11,895	30
10 LACMP23-1316	<i>Mammuthus columbi</i>	Project 23-11	3–30 kDa	3.1	-19.4	9.4	42.3	15.3	3.24	36,770	750
11 LACMP23-967	<i>Smilodon fatalis</i>	Project 23-1	3–30 kDa	4.4 $\pm$ 2.3	-18.0 $\pm$ 0.1	11.2 $\pm$ 0.2	42.5 $\pm$ 0.8	15.6 $\pm$ 0.3	3.19 $\pm$ 0.04	34,270	540
12 LACMP23-2294	<i>Canis dirus</i>	Project 23-1	3–30 kDa	6.3 $\pm$ 2.0	-18.0 $\pm$ 0.1	12.0 $\pm$ 0.1	42.5 $\pm$ 0.8	15.2 $\pm$ 0.3	3.26 $\pm$ 0.07	34,660	220
13a LACMP23-4096; 13b LACMP23-693; 13c LACMP 23-659	<i>Panthera atrox</i>	Project 23-1	3–30 kDa	5.2 $\pm$ 2.1	-18.4 $\pm$ 0.1	8.3 $\pm$ 0.4	42.9 $\pm$ 0.4	15.5 $\pm$ 0.2	3.23 $\pm$ 0.03	34,740	390
14 LACMP23-5001	<i>Mammut americanum</i>	Project 23-14	None	None	None	None	None	None	None	None	None
15 LACMP23-5002	<i>Bison antiquus</i>	Project 23-14	3–30 kDa	3.4 $\pm$ 0.8	-19.0 $\pm$ 0.2	11.1 $\pm$ 0.1	42.6 $\pm$ 0.6	14.8 $\pm$ 0.2	3.36 $\pm$ 0.02	>47,770	NA
16 LACMP23-5003	<i>Camelops hesternus</i>	Project 23-5B	3–30 kDa	7.3	-19.7	9.0	43.2	15.4	3.27	42,600	1700
17 LACMP23-6184	<i>Canis dirus</i>	Project 23-14	3–30 kDa	2.6 $\pm$ 1.0	-16.6 $\pm$ 0.0	12.2 $\pm$ 0.0	42.4 $\pm$ 0.5	14.7 $\pm$ 0.2	3.35 $\pm$ 0.04	42,500	1900
18 LACMP23-6185	<i>Smilodon fatalis</i>	Project 23-7B	3–30 kDa	4.1	-17.9	12.6	42.1	15.3	3.22	40,390	970
19 LACMRLP R15406 <sup>b</sup>	<i>Canis dirus</i>	91	None	None	None	None	None	None	None	None	None
20 LACMRLP R16695	<i>Smilodon fatalis</i>	91	3–30 kDa	3.8 $\pm$ 1.8	-18.0 $\pm$ 0.2	11.4 $\pm$ 0.1	42.5 $\pm$ 1.6	15.0 $\pm$ 0.3	3.31 $\pm$ 0.1	27,280	230
21 LACMRLP R34802	<i>Smilodon fatalis</i>	91	3–30 kDa	5.8 $\pm$ 1.4	-18.2 $\pm$ 0.1	12.2 $\pm$ 0.2	42.6 $\pm$ 0.6	15.2 $\pm$ 0.1	3.26 $\pm$ 0.04	28,200	280
22 LACMRLP R42645	<i>Smilodon fatalis</i>	91	3–30 kDa	3.8	-18.8	10.7	40.1	14.2	3.30	40,660	960
23 LACMRLP R49462	<i>Canis dirus</i>	91	3–30 kDa	4.4	-18.5	11.4	30.6	10.6	3.36	41,300	1200
24 LACMRLP R14493	<i>Canis dirus</i>	91	3–30 kDa	6.4	-18.9	11.6	42.2	15.2	3.24	14,280	60
25 LACMP23-1619	<i>Canis dirus</i>	Project 23-1	3–30 kDa	3.9	-18.1	8.8	41.7	15.0	3.25	33,380	800
26 LACMP23-11369	<i>Canis dirus</i>	Project 23-1	3–30 kDa	6.9	-19.0	11.0	44.5	14.8	3.50	36,690	890
27 LACMP23-12101	<i>Canis dirus</i>	Project 23-14	3–30 kDa	1.7	-16.5	11.9	42.9	15.0	3.34	>45,200	NA
28 LACMRLP R27398	<i>Canis dirus</i>	91	3–30 kDa	3.6	-18.9	9.9	42.4	14.9	3.32	24,830	210
29 LACMRLP R24956	<i>Canis dirus</i>	91	3–30 kDa	6.5	-18.3 $\pm$ 0.1	9.9 $\pm$ 0.2	42.5 $\pm$ 0.5	15.1 $\pm$ 0.3	3.28 $\pm$ 0.03	27,500	280
30 LACMHC H1908	<i>Canis dirus</i>	60	3–30 kDa	4.2	-18.2 $\pm$ 0.1	10.4 $\pm$ 0.1	43.2 $\pm$ 0.3	15.1 $\pm$ 0.3	3.32 $\pm$ 0.04	25,230	220
31 LACMHC H1789	<i>Canis dirus</i>	67	3–30 kDa	8.2	-19.3 $\pm$ 0.1	9.3 $\pm$ 0.2	44.2 $\pm$ 0.1	15.1 $\pm$ 0.1	3.41 $\pm$ 0.02	12,605	40
32 LACMHC X7113	<i>Panthera atrox</i>	61	3–30 kDa	5.7	-19.1 $\pm$ 0.1	9.2 $\pm$ 0.2	43.0 $\pm$ 0.6	15.2 $\pm$ 0.1	3.30 $\pm$ 0.02	11,580	40
33a LACMHC 123491	<i>Nothrotheriops shastensis</i>	91 (caved)	XAD Amino Acids	?	-19.6	9.4	28.3	10.5	3.14	28,290	230
33b LACMRLP R36388	<i>Nothrotheriops shastensis</i>	91	XAD Amino Acids	?	-19.6	9.6	27.4	10.2	3.14	28,590 <sup>d</sup>	240 <sup>d</sup>
33c LACMRLP R38722	<i>Nothrotheriops shastensis</i>	91	XAD Amino Acids	?	-19.2	8.8	27.5	10.2	3.16	28,530 <sup>d</sup>	240 <sup>d</sup>
33d LACMRLP R22385	<i>Nothrotheriops shastensis</i>	91	XAD Amino Acids	?	-19.8	9.4	29.0	10.5	3.21	28,350 <sup>d</sup>	240 <sup>d</sup>
Mean results for sample#33					-19.6 $\pm$ 0.3	9.3 $\pm$ 0.3	28.1 $\pm$ 0.8	10.4 $\pm$ 0.2	3.16 $\pm$ 0.03	28,440	120
34a LACMRLP R22811 <sup>c</sup>	<i>Puma concolor</i>	91	XAD Amino Acids	?	-18.8	11.9	28.8	10.6	3.17	28,650 <sup>d</sup>	250 <sup>d</sup>
34b LACMRLP R54033	<i>Puma concolor</i>	91	XAD Amino Acids	?	-18.8	12.0	29.6	10.8	3.19	28,650 <sup>d</sup>	240 <sup>d</sup>
Mean results for sample#34					-18.8	12.0	29.2	10.7	3.18	28,650	170
35 LACMHC H1872	<i>Canis dirus</i>	61	3–30 kDa	3.1	-19.3	9.7	43.1	14.9	3.38	11,820	30
36 LACMP23-12102	<i>Smilodon fatalis</i>	Project 23-14	3–30 kDa	2.2	-16.6	12.5	42.4	14.9	3.34	43,200	1100
37 LACMHC Y5504	<i>Bison antiquus</i>	36	3–30 kDa	2.5	-19.9	9.1	43.4	15.0	3.38	29,150	200
38 LACMP23-15027	<i>Canis dirus</i>	Project 23-13	3–30 kDa	5.7	-18.7	10.7	43.3	15.3	3.31	32,340	300
39 LACMP23-15023	<i>Canis dirus</i>	Project 23-1	3–30 kDa	5.7	-18.9	10.7	42.7	15.0	3.33	35,400	420
40 LACMP23-15065	<i>Smilodon fatalis</i>	Project 23-1	3–30 kDa	5.4	-18.9	10.7	43.3	15.4	3.28	35,000	400

<sup>a</sup> Results from Coltrain et al. (2004).

<sup>b</sup> Note: Sample was mislabeled in Friscia et al. (2008) as R15408 on page 37, but the correct catalog number is R15406.

<sup>c</sup> Note: Sample was mislabeled in Friscia et al. (2008) as R11704 on page 37, but the correct catalog number is R22811.

<sup>d</sup>  $^{14}\text{C}$  ages from Friscia et al. (2008).



**Fig. 1.** a – Atomic C:N vs.  $\delta^{13}\text{C}$  values for ultrafiltered bone collagen fractions from sample #20 (*Smilodon fatalis*) subjected to different solvent extraction procedures. 1b – Atomic C:N vs.  $\delta^{15}\text{N}$  values for ultrafiltered bone collagen fractions from sample #20 (*Smilodon fatalis*) subjected to different solvent extraction procedures.

More telling was the fact that sample 21 had a  $^{13}\text{C}$ -depleted value relative to that for a solvent-treated aliquot ( $-18.9$  vs.  $-18.1\text{‰}$ ), an elevated C:N ratio of 3.8, and a tan color, indicating that it was contaminated with hydrocarbons. This suggests that while the majority of the collagen can be isolated from the asphalt by ultrafiltration, some form of solvent extraction is indeed necessary to produce reliable isotopic and radiocarbon data.

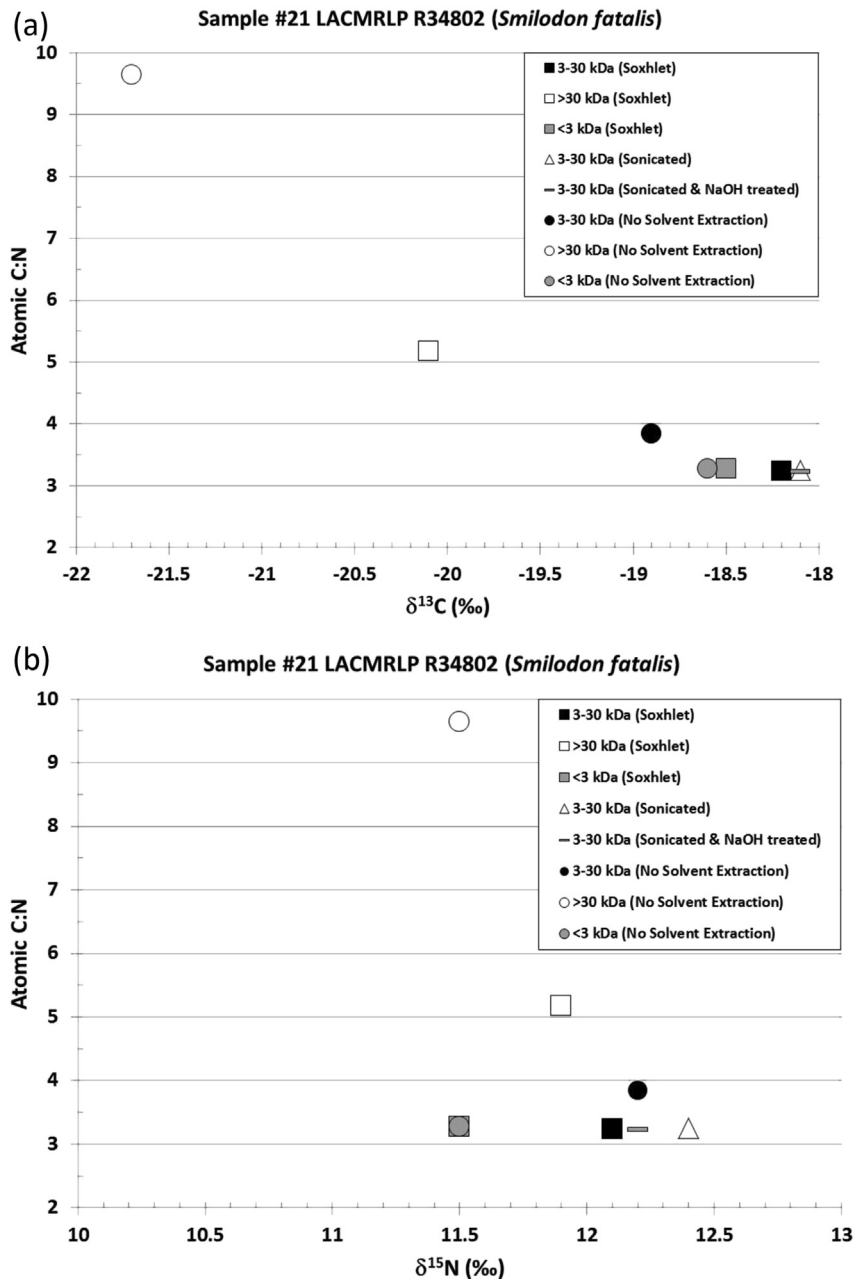
#### 4.5. Crushed vs. powdered bone

We compared the removal of tar from crushed vs. finely powdered bone. Four bones (samples 8,9,12,17) were selected and  $\sim 150$  mg of each was crushed to 1–2 mm pieces with a hammer, while another  $\sim 150$  mg was ground to a fine powder with a mortar and pestle. All were then treated with the 2:1 toluene/methanol

sonication and gelatinization/ultrafiltration protocols described above. One powdered sample (#17) was lost during collagen extraction, which attests to the difficulties of handling finely divided samples. The remaining three samples (8,9,12) showed no significant differences between the crushed vs. powdered samples in terms of isotopic results or radiocarbon dating (Table A.2). Furthermore, a sample of crushed bone subjected to the sonication sequence, that was dried, ground, and subsequently re-sonicated, produced no additional color in the solvent solution, indicating that the initial tar removal from the crushed bone was effective.

#### 4.6. NaOH treatment

The results of a NaOH treatment of five samples (8,9,12,20,21) after demineralization showed no offsets in  $^{14}\text{C}$  ages or isotopic



**Fig. 2.** a – Atomic C:N vs.  $\delta^{13}\text{C}$  values for ultrafiltered bone collagen fractions from sample #21 (*Smilodon fatalis*) subjected to different solvent extraction procedures. 2b – Atomic C:N vs.  $\delta^{15}\text{N}$  values for ultrafiltered bone collagen fractions from sample #21 (*Smilodon fatalis*) subjected to different solvent extraction procedures.

differences compared to the other procedures (e.g. Fig. 3a,b; Table A.2) and this step was omitted from the final protocol design.

#### 4.7. Carbonates

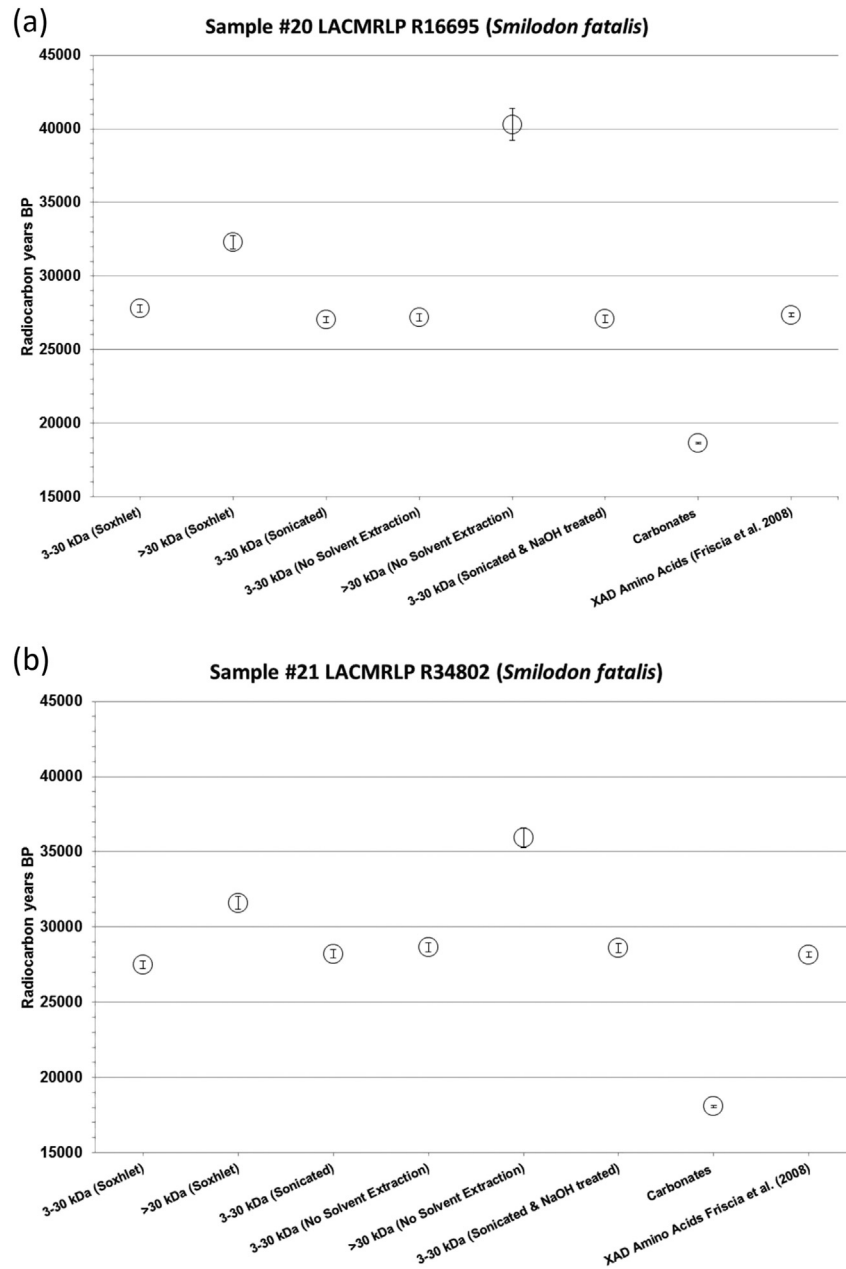
$\text{CO}_2$  samples for  $^{14}\text{C}$  dating were extracted from bone carbonate by hydrolyzing ~150 mg samples of crushed bone with 85% phosphoric acid in septum sealed vials according to standard carbonate processing procedures (UCI AMS Facility, 2011). All five bones (samples 9,10,15,20,21) produced carbonate radiocarbon dates that were significantly younger in comparison to the collagen radiocarbon dates (e.g. Fig. 3a,b; Table A.2). This indicates that there was  $\text{CO}_2$  exchange between the bone carbonate and the environment, probably due to exchange with river water that periodically pooled and ran through the various tar seeps of the area (Marcus

and Berger, 1984; Stock and Harris, 1992). Whatever the mechanism, our results support those of Marcus and Berger (1984), and show that carbonates should not be used for radiocarbon dates at the RLB site.

## 5. Discussion

### 5.1. Findings and recommendations for the ultrafiltration protocol

The protocol developed here is based on the selection of a 3–30 kDa molecular weight fraction that is least affected by tar contamination and/or is most effectively purified by ultrafiltration, but our tests showed that some form of preliminary solvent treatment to remove most of the contaminating hydrocarbons is still required. Soxhlet extraction is typically used for this initial step,



**Fig. 3.** a – Bone collagen radiocarbon dates BP from sample #20 (*Smilodon fatalis*) plotted by solvent extraction and ultrafiltered collagen fraction. Also shown are carbonate and XAD amino acid radiocarbon ages (Frischia et al., 2008) for comparison. b – Bone collagen radiocarbon dates BP from sample #21 (*Smilodon fatalis*) plotted by solvent extraction and ultrafiltered collagen fraction. Also shown are carbonate and XAD amino acid radiocarbon ages (Frischia et al., 2008) for comparison.

but is cumbersome, costly, and time consuming. Our tests showed that sonication in a 2:1 toluene/methanol solution is equally effective; and this is a relatively simple procedure that can be completed within a single working day. Surprisingly, the particle size of the raw bone (crushed vs. finely powdered) did not significantly affect the efficiency of the solvent extractions, and we recommend that for ease of handling the bone be crushed to 1–2 mm pieces rather than powdered.

We found that a gelatinization temperature of 75 °C for ~14 h produced the best results for the RLB bones in terms of collagen quality (based on color, texture, 3–30 kDa collagen yield, %C, %N, C:N ratios; Table A.2). We hypothesize that any solvent extraction technique for removing the hydrocarbons is only effective up to a point: small molecules are dissolved but at least some large

aggregates, formed from colloidal asphaltene or other tar constituents (Mullins et al., 2007; Goual, 2012) are not significantly affected by the solvents and remain within the bone. Using a higher than normal gelatinization temperature allows well preserved collagen molecules to be broken down to sizes <30 kDa but evidently leaves most residual ultra-large hydrocarbon aggregates essentially intact. This is a delicate balancing act, since if the temperature is too high, then both the collagen and the tar residues will be degraded and pass through the ultrafilter. If however, the temperature is too low, then very little collagen breakdown will occur and the collagen will remain in the >30 kDa fraction with the petroleum contaminants. The second ultrafiltration step, using a 3 kDa filter, removes low molecular weight proteinaceous material, at least some fraction of any soil humic contamination not



eliminated previously as insoluble residue in the gelatinization step, and any small hydrocarbon fragments such as free asphaltenes (MW ~1 kDa; Mullins et al., 2007) lost from the large aggregates during gelatinization.

While this use of two ultrafilters results in decreased collagen yields (typically 1–9% in the 3–30 kDa fraction), sufficient amounts remained to permit multiple isotopic and radiocarbon measurements. The collagen produced was characterized by atomic C:N ratios between 3.2 and 3.5 and %C ( $42.0 \pm 2\%$ ) and %N ( $15.0 \pm 1\%$ ) values close to modern bone (Ambrose, 1990; van Klinken, 1999), consistent with minimal residual contamination by hydrocarbons. Thus far, we have only applied the dual ultrafiltration method to bones from RLB, though we see no reason why it should not be effective for samples from other hydrocarbon seeps. However, given our present limited understanding of why the technique is successful at purifying the collagen, why the temperature of 75 °C is optimal for RLB, and the complexities of asphaltene and petroleum resin chemistry, it is possible that bones from other deposits may require different gelatinization temperatures and/or ultrafiltration cut-offs. Our technique may serve as an initial guide on how to best approach these samples in the new future.

### 5.2. Collagen quality indicators as a check of $^{14}\text{C}$ ages and isotopic ratios

Current practices at many radiocarbon facilities require that stable isotope measurements be carried out on all bone collagen samples, and that the %C, %N, and C:N ratios be examined before a bone date is reported. Boundaries set by DeNiro (1985) of 2.9–3.6 for acceptable atomic C:N ratios for stable isotope studies are also cited in the radiocarbon literature (e.g. van Klinken, 1999; Higham et al., 2006; Brock et al., 2007; Talamo and Richards, 2011), but given the high level of petroleum contamination at the RLB site we propose to define a much narrower window. In our experience, atomic C:N ratios for the >30 kDa fraction of ultrafiltered collagen from “normal” (hydrocarbon-free) bone are almost invariably within the range 3.15–3.45, and the protocol developed here produced collagen from the 3–30 kDa fractions of RLB bones with ratios of 3.2–3.5. If collagen C:N ratios fall outside of this narrow range (especially to higher values) then caution should be employed in the interpretation of the radiocarbon dates, as petroleum contaminants are likely present and will potentially skew the ages older.

The requirements for stable isotope measurements are less critical, but care is still required. In particular, collagen  $\delta^{13}\text{C}$  values from RLB with C:N ratios in excess of 3.6 should be interpreted with caution as the samples are probably contaminated with  $^{13}\text{C}$ -

depleted hydrocarbons (e.g. tar  $\delta^{13}\text{C} = -23.4\%$ ; bitumen  $\delta^{13}\text{C} = -23.1\%$ ; Table 1). This point is illustrated by sample 21 (Table A.2; Fig. 2a). A 3–30 kDa collagen sample that was not solvent treated (UCIAMS 112123) produced an atomic C:N ratio of 3.8 and a  $\delta^{13}\text{C}$  value of  $-18.9\%$ , whereas an aliquot that was solvent-sonicated (UCIAMS 114098) yielded a C:N ratio of 3.3 and a  $\delta^{13}\text{C}$  of  $-18.1\%$ . Thus,  $\delta^{13}\text{C}$  values with atomic C:N ratios in excess of 3.6 could be depleted as much as 0.5–1% from the true values.

In the stable isotope study of Coltrain et al. (2004), 23 out of 143 samples had C:N ratios between 3.6 and 4.0, and it is likely that some of the  $\delta^{13}\text{C}$  values in that subset are  $^{13}\text{C}$ -depleted due to asphalt contamination. However, as Coltrain et al. (2004) pointed out, their  $\delta^{13}\text{C}$  values are not correlated with the C:N ratios, suggesting that any overall effect is rather small. Furthermore, our  $^{14}\text{C}$  measurements on collagen from ten bones prepared for that study and on the <30 kDa fraction of aliquots subsequently ultrafiltered by us (see Section 4.2) show differences consistent with a  $^{14}\text{C}$ -free carbon (tar) contamination of just  $3.1 \pm 1.6\%$  in the non-ultrafiltered samples (Table A.1). For collagen  $\delta^{13}\text{C}$  values in the range  $-18$  to  $-20\%$ , a 3% contribution from tar/asphalt at  $-23\%$  would shift the measured  $\delta^{13}\text{C}$  by just 0.1–0.2%. This was confirmed by isotopically analyzing five ultrafiltered samples from individuals that had been previously measured by Coltrain et al. (2004) (Table 3). While the ultrafiltration method improved the C:N ratios of all five specimens, the  $\delta^{13}\text{C}$  results were  $^{13}\text{C}$ -enriched by at most 0.3% when the C:N ratio decreased from 3.6 to 3.3 (sample #30; Table 3). This confirms that the carbon stable isotope ratios are less sensitive to hydrocarbon contamination compared to radiocarbon ages, and that samples with C:N ratios between 2.9 and 3.6 (DeNiro, 1985) yield  $\delta^{13}\text{C}$  results close to the true values. Thus, the inferences on paleodiet based on the  $\delta^{13}\text{C}$  data in Coltrain et al. (2004) are not seriously affected, and the major conclusions of the study remain intact.

In contrast,  $\delta^{15}\text{N}$  results should not be influenced by asphalt contamination because the tar and bitumen contain negligible amounts of nitrogen. However, comparison of the  $\delta^{15}\text{N}$  results for the 3–30 kDa collagen fractions (measured in triplicate) with the results reported by Coltrain et al. (2004) show a  $^{15}\text{N}$ -enrichment (0.2–0.5%) in four out of the five samples (Table 3). This offset is far smaller than interspecies or intra-species differences or trophic level enrichments, but is nevertheless greater than expected based on the precision of the mass spectrometer measurements (0.2%).

### 5.3. Ultrafiltration vs. XAD resin

Radiocarbon ages for samples 19–24 from this study were determined previously (Frischia et al., 2008) on total amino acid

**Table 3**

Comparison of the isotopic and collagen quality results between the present study (measured in triplicate except #28; duplicate) and Coltrain et al. (2004). The 3–30 kDa collagen fraction shows improved C:N ratios for all samples.

Sample#, Museum#, species	Study	Collagen fraction analyzed	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	Atomic C:N
28; LACMRLP R27398; ( <i>Canis dirus</i> )	Present	3–30 kDa	-18.9	9.9	42.4	14.9	3.32
28; LACMRLP R27398; ( <i>Canis dirus</i> )	Coltrain et al., 2004	Unfiltered collagen extract	-18.8	9.7	37.0	12.5	3.45
Difference			-0.1	+0.2	+5.4	+2.4	-0.13
29; LACMRLP R24956; ( <i>Canis dirus</i> )	Present	3–30 kDa	$-18.3 \pm 0.1$	$9.9 \pm 0.2$	$42.5 \pm 0.5$	$15.1 \pm 0.3$	$3.28 \pm 0.03$
29; LACMRLP R24956; ( <i>Canis dirus</i> )	Coltrain et al., 2004	Unfiltered collagen extract	-18.2	9.9	45.1	15.4	3.42
Difference			-0.1	+0.0	-2.6	-0.3	-0.14
30; LACMHC H1908; ( <i>Canis dirus</i> )	Present	3–30 kDa	$-18.2 \pm 0.1$	$10.4 \pm 0.1$	$43.2 \pm 0.3$	$15.1 \pm 0.3$	$3.32 \pm 0.04$
30; LACMHC H1908; ( <i>Canis dirus</i> )	Coltrain et al., 2004	Unfiltered collagen extract	-18.5	10.0	40.4	13.2	3.58
Difference			+0.3	+0.4	+2.8	+1.9	-0.26
31; LACMHC H1789; ( <i>Canis dirus</i> )	Present	3–30 kDa	$-19.3 \pm 0.1$	$9.3 \pm 0.2$	$44.2 \pm 0.1$	$15.1 \pm 0.1$	$3.41 \pm 0.02$
31; LACMHC H1789; ( <i>Canis dirus</i> )	Coltrain et al., 2004	Unfiltered collagen extract	-19.1	8.9	45.4	15.4	3.44
Difference			-0.2	+0.4	-1.2	-0.3	-0.03
32; LACMHC X7113; ( <i>Panthera atrox</i> )	Present	3–30 kDa	$-19.1 \pm 0.1$	$9.2 \pm 0.2$	$43.0 \pm 0.6$	$15.2 \pm 0.1$	$3.30 \pm 0.02$
32; LACMHC X7113; ( <i>Panthera atrox</i> )	Coltrain et al., 2004	Unfiltered collagen extract	-19.3	8.7	43.2	14.0	3.60
Difference			+0.2	+0.5	-0.2	+1.2	-0.30

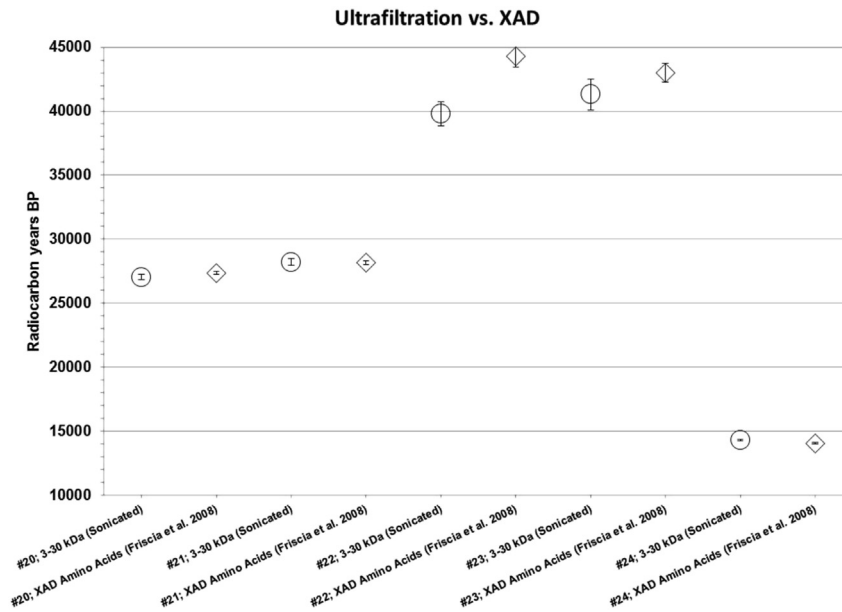


Fig. 4. Comparison of sonicated 3–30 kDa bone collagen vs. XAD amino acids (Frischia et al., 2008) radiocarbon ages BP for samples 20–24.

extracts purified using the XAD method (Stafford et al., 1988, 1991). We were unable to obtain useful amounts of good quality collagen from sample 19, but samples 20–24 all produced excellent collagen using the ultrafiltration technique (Table A.2). The  $^{14}\text{C}$  ages for samples 20 and 21 are almost identical to those published in Frischia et al. (2008), but samples 22 and 23 have ultrafiltration ages that are younger than those obtained with XAD by 4 and 2 standard deviations respectively; and the ultrafiltration age for sample 24 is 4 standard deviations older than the XAD result (Fig. 4). Which of these values are closer to the true ages is unclear: given that petroleum is radiocarbon-dead, incomplete tar removal would result in older radiocarbon dates, while contaminants such as soil humics are more likely to be young. Ultrafiltration by definition cannot remove contaminants that are covalently crosslinked to the collagen, though this process is commonly associated with brown coloration of the collagen and this was not observed in the 3–30 kDa fractions. Since Frischia et al. (2008) did not measure stable isotope or elemental data we cannot directly compare the extract quality between the two protocols, but we did measure such data for six other extracts from that study (samples 33–34) and all yielded acceptable C:N ratios as expected (Table 2).

Overall, the tests showed no systematic bias between the two methods, suggesting that the ultrafiltration method is comparable in terms of accuracy to the established total amino acid extraction/XAD resin purification technique. The latter is a preferred sample preparation method for poorly preserved or highly degraded bones (Stafford et al., 1988, 1991; Minami and Nakamura, 2000, 2004, 2005), and this technique produced a date from sample 19, where the ultrafiltration method was unable to yield sufficient 3–30 kDa collagen. Thus, for RLB samples with poor collagen preservation, the XAD resin protocol should be used for isotopic analysis and radiocarbon dating. However, given the ease of sample preparation with the ultrafiltration method, we recommend it as an effective, faster and simpler alternative to the XAD protocol for well-preserved bones from RLB.

#### 5.4. Findings of $^{14}\text{C}$ ages paired with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values

Of several stable isotope studies published for RLB fauna (Coltrain et al., 2004; Chamberlain et al., 2005; Fox-Dobbs et al., 2006; Bump

et al., 2007), only one has involved direct measurements of stable isotopes and radiocarbon dates from the same bones: Fox-Dobbs et al. (2006) examined the diets of several bird species recovered at RLB, and eight California Condors (*Gymnogyps californianus*) were radiocarbon dated and analyzed for C, N and H stable isotope ratios. The bulk of the past research at RLB has relied heavily upon assigning the ages of specimens based upon the average  $^{14}\text{C}$ -dates from the specific pits where they were excavated (e.g. Coltrain et al., 2004; Chamberlain et al., 2005; Bump et al., 2007; Prothero et al., 2012). While these average  $^{14}\text{C}$ -pit ages may allow the detection of gross temporal trends, bones from a single pit can have enormous age variations (e.g. Pit 91 14–44 kyr; Frischia et al., 2008), which can obscure or obliterate any meaningful patterns. This was also the case for wood specimens from juniper trees as noted in Ward et al., 2005. Thus, given the unique nature of the bone assemblages at RLB where there is no well-defined stratigraphy (Woodward and Marcus, 1973; Stock and Harris, 1992; Frischia et al., 2008; O’Keefe et al., 2009), stable isotope results must be directly paired with radiocarbon dates to elucidate the possible influences of diet, migration, or climate on RLB fauna over millennial and shorter timescales. Here we present 38 sets of carbon and nitrogen stable isotope results combined with paired radiocarbon ages (believed to be derived from 38 separate individuals) of different species from the RLB site and examine possible temporal trends.

The sabertoothed cats (*Smilodon fatalis*) display  $^{13}\text{C}$  and  $^{15}\text{N}$ -enriched values consistent with their classification as top level carnivores, and the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  results plot nearly identical to results from sabertoothed cats isotopically analyzed by Coltrain et al. (2004) (Table 2; Fig. 5). However, a single individual shows a  $^{13}\text{C}$ -enriched result ( $-16.6\text{‰}$ ) that stands out from the population and was  $^{14}\text{C}$ -dated to  $43,200 \pm 1100$  BP. This represents the oldest *Smilodon fatalis* of this study and could suggest that there was something unique about the diet ( $\text{C}_4$  or marine foods) of this individual or that it was influenced by climatic conditions (c.f. the discussion of dire wolf results below). Coltrain et al. (2004) and Bump et al. (2007) observed a trend to  $^{13}\text{C}$ -depletions in some (but not all) RLB plant and animal species since the Last Glacial Maximum. The *Smilodon* results presented here are consistent with such a trend (Fig. 6a), though the sample size ( $n = 12$ ) is too small to draw firm conclusions.

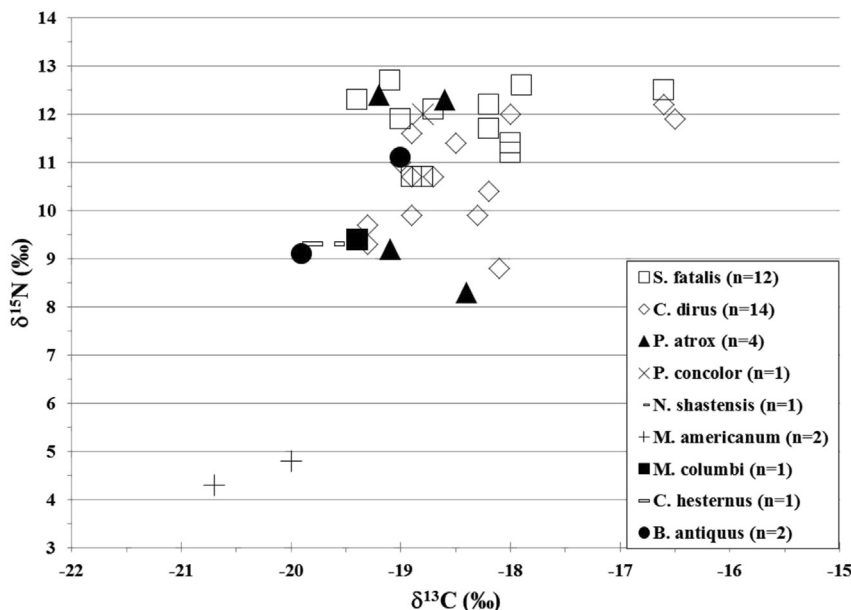


Fig. 5.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  results from all of the individual species from the Rancho La Brea site.

The 14 dire wolves (*Canis dirus*) studied here display similar isotopic values to those presented by Coltrain et al. (2004) (Table 2; Fig. 5). Interestingly, two individuals have significantly  $^{13}\text{C}$ -enriched results ( $-16.5\text{‰}$ ) that set them apart from the main population cluster. Coltrain et al. (2004) also found dire wolves and sabertoothed cats with  $^{13}\text{C}$ -enriched values and proposed that nutritional stress could lead to increased trophic level  $\delta^{13}\text{C}$  fractionation between diet and bone collagen, but while this does affect  $\delta^{15}\text{N}$  values (e.g. Fuller et al., 2005), evidence for a corresponding  $\delta^{13}\text{C}$  shift has not been found. It seems more likely that these sabertoothed cats and dire wolves (Fig. 5) had a unique  $^{13}\text{C}$ -enriched diet, though collagen from prey species recovered at RLB shows no correspondingly large enrichment (Coltrain et al., 2004; Feranec et al., 2009) and the local vegetation was overwhelmingly  $^{13}\text{C}$ -depleted  $\text{C}_3$  species (References in Coltrain et al., 2004). Alternatively, given that RLB was just 20–25 km from the ocean in the late Pleistocene, it may have been within the range of a population of sabertoothed cats and dire wolves adapted to shoreline scavenging of  $^{13}\text{C}$ -enriched fish and marine mammals. Similar foraging behavior, and movement over tens of km within a single night, is reported for modern African lions in Namibia (Desert Lion Conservation, 2013). Evidence of marine food consumption was found in bird species from RLB, and Fox-Dobbs et al. (2006) argued that marine food resources likely acted as an important dietary buffer that permitted the survival of the California Condors across the Pleistocene–Holocene extinction boundary. It is also possible that these  $^{13}\text{C}$ -enriched individuals may have migrated to the RLB site from habitats that contained more  $^{13}\text{C}$ -enriched  $\text{C}_4$  vegetation, or perhaps reflect temporal  $\delta^{13}\text{C}$  changes driven by variations in local climate and ecology. In support of this latter possibility is the fact that these dire wolves and the sabertoothed cat are the oldest carnivores analyzed with ages in excess of 40,000 years BP (Table 2; Fig. 6a). It remains to be determined if other  $^{13}\text{C}$ -enriched carnivores have radiocarbon ages that cluster near the limit of radiocarbon dating or are distributed more uniformly in time.

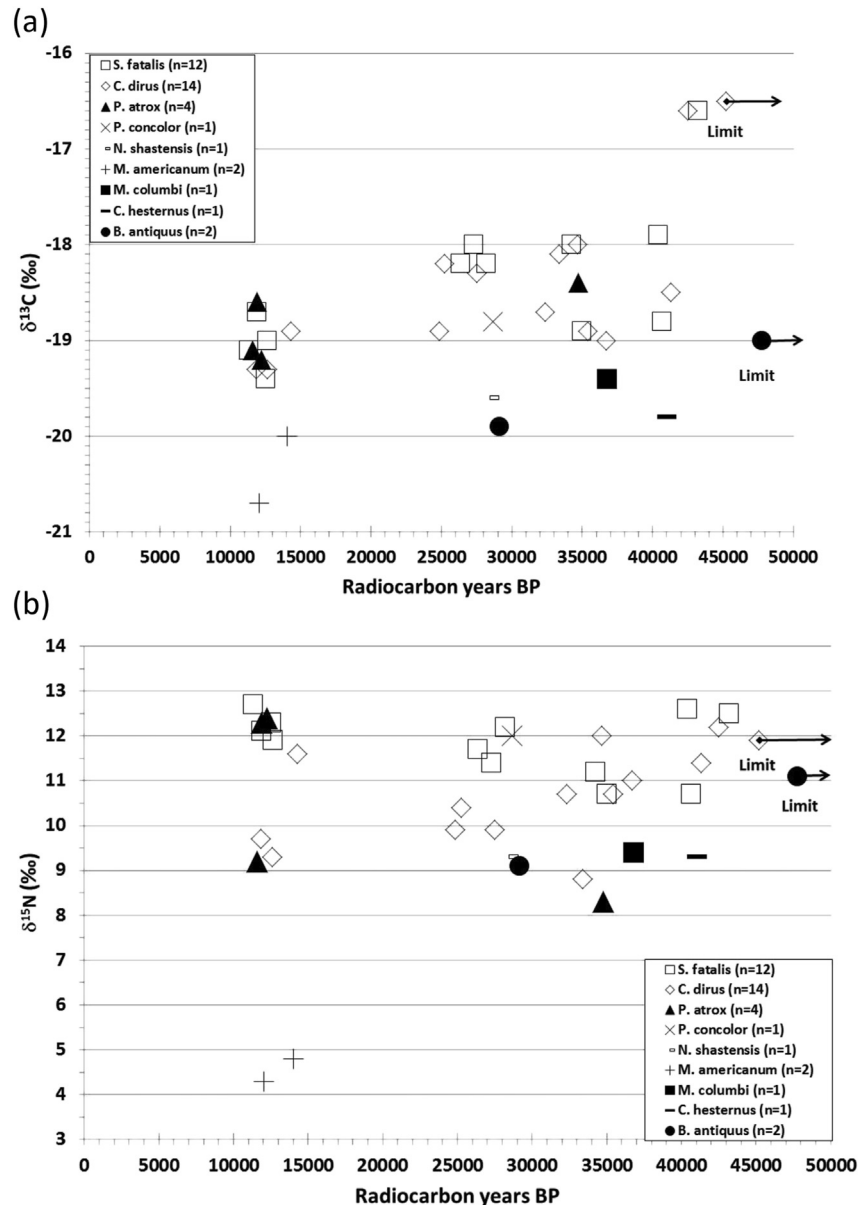
The four American lions (*Panthera atrox*) examined here have similar  $\delta^{13}\text{C}$  but two have low  $\delta^{15}\text{N}$  values (8.3 and 9.2‰) and the two other individuals display values nearly a full trophic level higher (12.3 and 12.4‰; Table 2; Fig. 4), suggesting that there were

two populations of American lions at RLB that focused their hunting efforts on different prey species or ecological niches. One of the  $^{15}\text{N}$ -depleted lions dates to  $34,740 \pm 390$  BP and the other dates to  $11,580 \pm 40$  BP (Fig. 6b). Comparing these measurement to the isotopic results of Coltrain et al. (2004) (without regard to time resolution) and assuming a  $\delta^{15}\text{N}$  trophic level effect of 3–4‰, we speculate that the two lions with values over 12‰ were mainly eating bison (*Bison antiquus*), camels (*Camelops hesternus*), or Shasta ground sloths (*Nothrotheriops shastensis*), while the  $^{15}\text{N}$ -depleted lions primarily preyed on horses (*Equus occidentalis*) and/or mastodons (*Mammuthus americanum*).

Two mountain lion (*Puma concolor*) bone specimens previously radiocarbon dated by Friscia et al. (2008) but thought to represent a single individual were isotopically analyzed and found to be similar to the sabertoothed cats (Table 2; Fig. 5). In addition, four Shasta ground sloth (*Nothrotheriops shastensis*) bone specimens (three previously reported by Friscia et al., 2008) that were all believed to be from a single individual were measured for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, and the results were similar to the bison (*Bison antiquus*) and camels (*Camelops hesternus*) in Coltrain et al. (2004). Five mastodon bone specimens (*Mammuthus americanum*) (likely representing two individuals) with isotopic results previously reported by Coltrain et al. (2004) were radiocarbon dated and yielded ages of  $12,030 \pm 40$  and  $14,035 \pm 30$  BP, respectively.

A single *Camelops hesternus* from this study has  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  isotopic results similar to those reported in Coltrain et al. (2004) (Table 2; Fig. 5). Two *Bison antiquus* were analyzed and one has an extremely elevated  $\delta^{15}\text{N}$  value (11.1‰), such that it could pass for a top-level carnivore (Table 2; Fig. 5). Since Coltrain et al. (2004) measured two other bison with  $\delta^{15}\text{N}$  values  $>11\text{‰}$ , this seems to be a real result. Coltrain et al. (2004) suggested that high  $\delta^{15}\text{N}$  values were probably not a faunal response to physiological water stress (Sealy et al., 1987) but might be the result of  $\delta^{15}\text{N}$  shifts in vegetation adapted to a more arid climate in southern California. The  $^{15}\text{N}$ -enriched bison presented here dated to a limit ( $>47$  kyr BP) so the true age is unknown, but this elevated  $\delta^{15}\text{N}$  value could be a characteristic of a specific time period/climate.

A notable outcome of the Project 23 excavations was the recovery of a nearly complete male mammoth (*Mammuthus columbi*) skeleton, the first relatively complete mammoth skeleton found at



**Fig. 6.** a –  $\delta^{13}\text{C}$  vs. radiocarbon age BP for the individual species studied from the Rancho La Brea site. Note that the carnivores aged >40 kyr BP have  $^{13}\text{C}$ -enriched values. b –  $\delta^{15}\text{N}$  vs. radiocarbon age BP for the individual species studied from the Rancho La Brea site. Note that the *Panthera atrox* individuals show a large range of  $\delta^{15}\text{N}$  results.

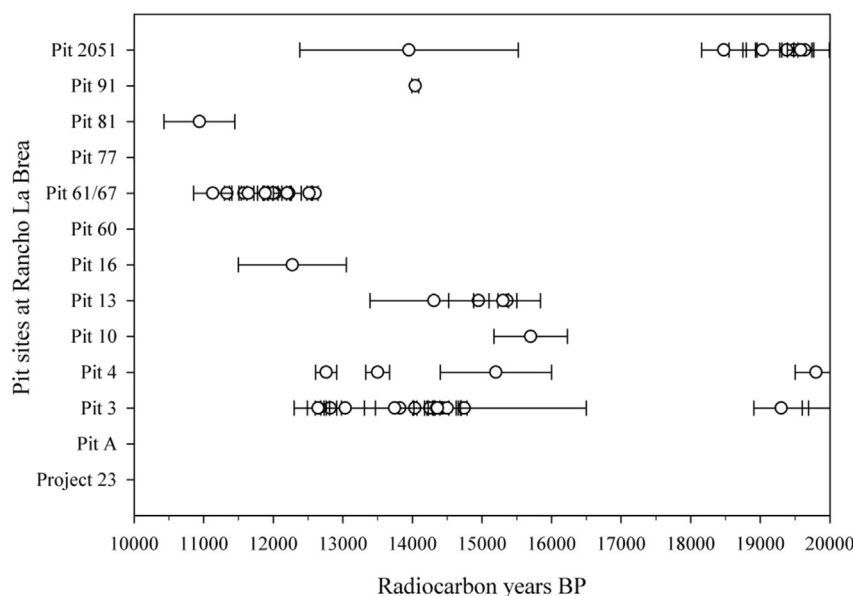
RLB. This individual has a  $^{14}\text{C}$  age of  $36,770 \pm 750$  BP and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of  $-19.4\text{‰}$  and  $9.4\text{‰}$ , respectively (Table 2; Figs. 5 and 6a,b), similar to those of mammoths recovered from the Alaska/Yukon and Siberian regions (e.g. Fox-Dobbs et al., 2008; Metcalfe et al., 2010; Szpak et al., 2010). Comparison of these isotopic values to the results of Coltrain et al. (2004), suggest that the diet of this individual was similar to those of the camels and bison, but not to that of the more closely related mastodons.

Finally, the bone  $^{14}\text{C}$  ages from O'Keefe et al. (2009) plus those presented here suggest that the RLB tar pits have been active entrapment zones for large animals during much of the past >50 kyr. However, the results from 10 to 20 kyr BP (excluding carbonate dates) (Fig. 7) show an extended period ( $^{14}\text{C}$  ages  $\approx 16$ –18.5 kyr BP) that included the peak of the Last Glacial Maximum (LGM) where faunal  $^{14}\text{C}$  dates are absent (Marcus and Berger, 1984). This could suggest that temperatures were cool enough (e.g. Heusser, 1998) to allow the tar to remain solid year round ( $<18$  °C; Holden et al., 2013) and/or that the deposits were buried under

sediment during this interval. Additionally, there is the potential for reduced sizes of faunal populations in the vicinity of RLB during the LGM, and that this could have contributed to this gap. More specifically, Ward et al. (2005) inferred from isotopic studies of vegetation recovered from RLB that unprecedented carbon starvation due to low glacial  $\text{CO}_2$  levels could have negatively impacted plant productivity and hence resource availability for herbivores. Significant numbers of additional bone  $^{14}\text{C}$  dates from other RLB pits will be required to determine whether the quiescence in fauna entrapment during the LGM was truly site-wide and to investigate whether similar hiatuses may have occurred during other periods within the last 50 kyr.

## 6. Conclusions

We have developed and tested a simple new protocol to isolate collagen for radiocarbon dating and isotopic analysis from tar-



**Fig. 7.** All known radiocarbon ages BP sorted by individual pit sites at Rancho La Brea from 10 to 20 kyr (Data from O'Keefe et al., 2009 and this study). Note that no bone collagen  $^{14}\text{C}$ -dates have been found between  $\approx 16$  and 18.5 kyr BP, and that this age range includes the peak of the Last Glacial Maximum.

impregnated bones from the RLB tar pits. This method can be summarized as follows:

1. Crush bones to 1–2 mm fragments.
2. Sonicate bone fragments repeatedly in 2:1 toluene/methanol solution until clear, sonicate in methanol (1 h), sonicate in Milli-Q water (1 h). Keep temperature of sonication bath  $\leq 45^\circ\text{C}$ .
3. Demineralize bone fragments in 0.5 N HCl for 24–36 h at room temperature.
4. Rinse collagen pseudomorph with Milli-Q water to  $\text{pH} > 3$ .
5. Gelatinize in 0.01 N HCl at  $75^\circ\text{C}$  for  $\sim 14$  h.
6. Ultrafilter at 30 kDa.
7. Discard the  $>30$  kDa fraction; ultrafilter the  $<30$  kDa fraction at 3 kDa.
8. Discard the  $<3$  kDa fraction; lyophilize the 3–30 kDa fraction.
9. Record % collagen yield and check collagen quality ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , %C, %N, and C:N).
10. If collagen is of good quality, process for radiocarbon dating.

This ultrafiltration method produced high quality 3–30 kDa collagen from 38 out of the 40 specimens studied, with yields ranging from 1 to 9% of the initial bone weight. Collagen color and texture, and elevated  $\delta^{13}\text{C}$  values compared to other fractions, were all consistent with minimal hydrocarbon contamination, and the elemental and C:N ratio results were typical of those obtained from well preserved uncontaminated bone.  $^{14}\text{C}$  ages for five of the bones tested had already been determined for XAD-purified amino acid extracts: in four out of the five cases, the ultrafiltration method produced radiocarbon ages in agreement with or slightly younger than the published dates. These results suggest that ultrafiltration is a simple, fast, and effective alternative to XAD for radiocarbon sample preparation of well-preserved RLB specimens, and probably for similar material from other tar and asphalt seeps as well (e.g. Cuba (Suarez and Olson, 2003), Venezuela (Prevosti and Rincón, 2007), Ecuador (Lindsey, 2011), Peru (Campbell, 1975) or Ukraine (Kuc et al., 2012)).

The 38 individual animal specimens presented here represent the largest published terrestrial animal  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values from

RLB that are directly linked with radiocarbon dates. Most previous isotopic studies of RLB fauna focused exclusively on either stable isotopes or radiocarbon dating. Thus, it has been difficult to establish links between specific or unique isotopic results and age, since the bones recovered from RLB lack a well-defined stratigraphic context. The small sample size of this investigation prevented the study of detailed temporal trends, but we observed several unusual results:  $^{13}\text{C}$ -enriched values (possibly  $\text{C}_4$  derived or marine dietary protein) in *C. dirus* and *Smilodon fatalis* older than 40 kyr BP, a  $^{15}\text{N}$ -enriched value in *Bison antiquus*  $>47$  kyr, and  $^{15}\text{N}$ -depleted values in *P. atrox*. Whether the RLB fauna that gave rise to these isotopic signatures were migrants to southern California, or whether the isotopic outliers reflect unusual dietary patterns within the region or temporal changes in climate and ecology, is presently unclear and invites further work. The simple collagen extraction protocol developed here will facilitate the much larger stable isotope and radiocarbon studies required to understand how and why these isotopically unique individuals differ from the populations as a whole.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.quageo.2014.03.002>.

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