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COLLAGEN EXTRACTION AND STABLE ISOTOPE ANALYSIS OF SMALL VERTEBRATE BONES: A COMPARATIVE APPROACH

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ABSTRACT. Bone remains of small vertebrate fossils provide valuable information for paleoenvironmental and paleoclimatic reconstructions. However, direct radiocarbon dating of small vertebrates remains challenging as the extraction of sufficient good quality collagen is required. The efficiency of eight collagen extraction protocols was tested on seven samples, representative of different ages and burial environments, including both macro and small vertebrate taxa. First, the samples were prescreened using attenuated total reflectance–Fourier transform infrared spectroscopy (ATR-FTIR) to quantify collagen content in archaeological bones, revealing that one should be discarded for ^{14}C dating. Then, the quantity of protein extracted (yield) and collagen integrity were checked using conventional elemental analysis. The results show that one protocol was not able to accurately extract collagen from the samples. A soft HCl-based protocol seems more appropriate for the pretreatment of archaeological small mammal bones, whereas a harsher protocol might be more efficient to extract a higher amount of collagen from large mammals as well as amphibian bones. The influence of the tested protocols on carbon and nitrogen isotope values was also investigated. The results showed that isotopic variability, when existing, is related to the interindividual differences rather than the different protocols.

KEYWORDS: bone, collagen preservation, ATR-FTIR, isotopic analysis, small vertebrates, archaeozoology.

INTRODUCTION

Small vertebrates provide valuable information necessary to reconstruct the paleoenvironment and paleoclimate of a specific region (Chaimanee et al. 1993; Bona et al. 2009; Rofes et al. 2015). As specific conditions are needed for them to survive and as they do not travel far from their living areas, microfaunal species and changes in their communities over time give important clues about local vegetation variations and, as a consequence, about climate changes. Finding paleontological material from commensal species may also indicate the presence of human communities nearby. Furthermore, as small vertebrates have a high rate of mutation, are usually abundant (compared to macrofaunal species) in archaeological and paleontological sites, and as the biochronology of many taxa is relatively well known, they can be excellent biochronological markers to date stratigraphic and archaeological units corresponding to relatively short periods of time (e.g. Cuenca-Bescós et al. 2010). Possible applications include the reconstruction of paleoenvironmental changes in a given region, the reconstruction of recolonization processes of different species, and the understanding of the impact of human pressure on small vertebrate populations, as it induces fragmentation of ecosystems, anthropization of landscapes, destruction of territories, and introduction of alien species (Kotsakis and Barisone 2000; Flynn and Wessels 2013; Rofes et al. 2015; Royer 2016). Isotopic analysis of small mammals may also be used as a proxy of continental climate changes (Navarro et al. 2004; Commendador and Finney 2016). However, for recent periods like the latest Pleistocene and Holocene, where the human pressure is progressively increasing, obtaining fine chronological frameworks of these changes through direct radiocarbon dating of small vertebrate bones is required to obtain high-resolution paleoenvironmental reconstructions and to understand complex historical-biogeographical processes like the post-glacial recolonization of several species. Dating of bone microsamples (including rodents; Soricomorpha, e.g. shrews; Erinaceidae, e.g. hedgehogs; bats; amphibians; and reptiles) has

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scarcely been tried because of two main challenges: (1) the insufficient amount of collagen for ^{14}C dating given the limited amount of material (e.g. 20–30 mg for a shrew hemimandible) and (2) the use of a suitable extraction protocol to ensure an efficient recovery of the limited amount of collagen from the bone.

Small vertebrate bones are more sensitive to alterations both because of digestion by predators and postburial diagenesis (Dauphin and Denys 1992; Dauphin et al. 1999). Moreover, the small size of small vertebrate samples yields a higher specific area (surface to volume ratio) than macrovertebrates, leading to higher rates of interaction with the surrounding sedimentary environment. In the particular case of fish, investigation on the chemistry of the bones and studies of archaeological material also revealed a lower degree of collagen preservation than for other bones coming from the same sites, probably due to the lower calcification of several parts of the skeleton (rather cartilaginous than bony). This lower mineralization also facilitates the penetration of contaminants from the sediment (Szpak 2011).

Regardless of the protocol chosen, pretreatment steps and further measurements are time-consuming, labor-intensive, and expensive. That is the reason why, in addition to a careful visual preliminary examination, samples may be tested for nitrogen and carbon content prior to ^{14}C dating to assess if the sample has sufficient collagen preservation. Nondestructive alternatives to isotope ratio mass spectrometry (IRMS) have been proposed using Raman spectroscopy (Pestle et al. 2015) or a combination of X-ray microtomography and ion beam analysis (Beck et al. 2012). More recently, attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectroscopy has been shown to give reliable results within a few minutes for a very small amount of sample (<1 mg) and can thus be used to discard poorly preserved specimens (Lebon et al. 2016).

The amount of carbon that can currently be extracted from microsamples can narrowly reach the detection limit (1 mg of carbon) for ^{14}C measurement using conventional AMS. Currently, AMS ^{14}C dating of collagen from bones usually requires between 300–600 mg of archaeological material, depending on the level of preservation of the protein. Therefore, collagen extraction protocols (yields) must be optimized, but the quality of the collagen extracted must also be assessed as the presence of any exogenous carbonaceous contaminant, either coming from the sediment or from lab processing of the sample, would significantly bias the resulting date for small bone samples. A large diversity of collagen extraction protocols can be found in the literature. Most of them follow the well-established acid/alkali/acid (AAA) procedure (Longin 1971; Arslanov and Svezhentsev 1993), but acid, temperature, and duration of exposure may change for the first acid treatment; the alkali step may be avoided (or its duration limited); and the last acid step, known as gelatinization, may also vary in duration, temperature, and pH (Brown et al. 1988; Semal and Orban 1995; Brock et al. 2013). This treatment may be performed on crushed bones directly but a grinding (and sometimes a sieving) step may be added. Following the AAA treatment, purification and/or concentration steps are often added and different choices have been made for material and pore size of the filters. The addition of the ultrafiltration step has led to controversies regarding its efficiency (Hüls et al. 2009; Fülop et al. 2013). Differences in quantity and quality of collagen in extracts between the different protocols have previously been investigated on macrovertebrate bones (Jørkov et al. 2007; Caputo et al. 2012; Cleland et al. 2012; Brock et al. 2013; Fülöp et al. 2013; Minami et al. 2013; Sealy et al. 2014) but not on small vertebrate bones except fish (Szpak 2011; Keaveney and Reimer 2012).

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Only a few laboratories have tried to date small vertebrate bones. Modern rat bones (*Rattus norvegicus*) from Tokaanu Bay on the volcanic lake of Taupo were dated by the Rafter Radiocarbon Lab AMS facility (Lower Hutt, New Zealand) to investigate the influence of diet on their ^{14}C age (Beavan-Athfield et al. 2001). Ancient bones were also dated by different labs (ORAU laboratory, AMS facility Tucson, and Kiel AMS) on Holocene samples coming from temperate to semiarid areas (Wilmshurst et al. 2008; Alcover et al. 2009; Rando et al. 2014) and on older samples from cold climate sites (Heaton and Grady 2003; Martinkova et al. 2013; Palkopoulou et al. 2016).

In this study, eight representative collagen extraction protocols were tested on both modern and archaeological small vertebrate bones and on macromammal bones as control, prescreened upstream for collagen preservation using ATR-FTIR spectroscopy. Yields were calculated and compared and collagen "quality" was assessed using the C:N criteria as well as the influence of the protocols chosen on isotopic $\delta^{13}C$ and $\delta^{15}N$ results.

EXPERIMENTAL

Sample Selection

Seven types of samples were selected including two macromammal bones, with the remaining five being subsampled from assemblages of small vertebrate bones (Table 1). In the case of small vertebrates, only one type of bone and species was selected when possible. If not, only samples corresponding at least to the same family (for mammals) or to the same suborder (for amphibians) were kept. Selections and species identification of the bones were achieved in collaboration with archaeozoologist colleagues. Macrovertebrates bones were sampled using a diamond-tipped Dremel grinder in order to obtain 3 to 8 g of sample. For small vertebrates, several bones from the same context were gathered to reach a mass of about 5 g. Bones of juveniles or showing digestion marks were discarded. When still present, teeth were removed from hemimandibles. Modern small vertebrate bones were sifted from the rejection pellet of a barn owl (*Tyto alba*), a class 1 predator according to Andrews' classification (Andrews and Cook 1990). They did not show heavy digestion marks and can thus be considered as modern reference material for small vertebrate bones. A subsample of VIRI E (*Mammuthus* sp.) was chosen as the macromammal archaeological bone reference. Good climatic and chronological

Table 1 Taxonomical, geographical, and chronological contexts of the test samples.

Sample code	Taxon	Type of bone	Origin	Expected age or calibrated range
P77	*Ovis aries*	Hemimandible	Egiin Gol (Mongolia)	Modern
VIRI E*	*Mammuthus* sp.	Pelvis	Yukon territory (Canada)	39,305 ± 121 BP
PEL	Muridae	Hemimandible	Thaon (France)	Modern
CAR	*Rattus norvegicus*	Long bones	Limoges (France)	AD 1870–1880
BOU_{rod}	*Mus musculus*	Hemimandible	Bourges (France)	350–270 BC
BOU_{amph}	*Bufo* (*bufo* and *calamita*) and *Rana*	Long bones		
SAN 7	*Chiroptera*	Long bones	Marie-Galante (West Indies)	11,860 ± 60 BP

*Intercalibration sample.

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coverage were ensured with samples coming from tropical and temperate environments, and ranging in age from ~40,000 BP to Modern.

ATR-FTIR Analysis

Prior to collagen extraction, bone micro-subsamples were prescreened using FTIR to assess the preservation of collagen, following the procedure described in Lebon et al. (2016). Briefly, FTIR spectra were performed in ATR mode [using a Golden Gate™ Single Reflection Diamond ATR accessory (Specac, France) with KRS-5 lens] on a Vertex 70 FTIR spectrometer (Bruker Optics, France). ATR-FTIR spectra were obtained by the accumulation of 128 scans in the wavenumber range 4000–400 cm^{-1} with a spectral resolution of 2 cm^{-1}. The heterogeneity of each microsample was considered by performing several measurements (up to six), on several grains of powdered bone (<1 mg). OPUS software (Bruker Optics, France) was used to draw linear baselines and measure the amide I and phosphate $\nu_3(PO_4)$ bands, between 1710–1590 and 1110–940 cm^{-1}, respectively (for the latter, baseline correction was applied between 1160 and 890 cm^{-1}). Nitrogen and collagen contents of the bones were then estimated from the amide I/PO_4 ratio using equations reported in Lebon et al. (2016) taking standard deviations into account:

$$\%\ \mathrm{N\ (wt)} = 20.6\,\mathrm{amide\ I}/PO_4 + 0.31 \text{ and } \%\ \mathrm{Collagen\ (wt)} = 113.13\,\mathrm{amide\ I}/PO_4 + 1.69.$$

Extraction Protocols

Following physical cleaning with a diamond drill, macromammal bones were coarsely crushed into fragments. If required (protocols F–H, see below), cleaned crushed bones were ground using an agate pestle and mortar and sieved until the size of the particles obtained ranged between 0.3 and 0.7 mm. The collagen of each prepared bone subsample (150 mg) was then extracted according to eight different protocols, available in the scientific literature (Longin 1970; Brown et al. 1988; Tuross et al. 1988; Bocherens et al. 1991; Semal and Orban 1995; Beaumont et al. 2010; Brock et al. 2010, 2013; Tuross 2012; Stafford 2014; Waters et al. 2015). A detailed description of each protocol is given in the following and a summary is given in Table 2. Protocols A–B can be considered as "soft," protocols C–E involve the use of ultrafilters, protocol F is our in-house protocol for ^{14}C dating and isotope analysis, and protocols G–H can be considered as "harsh." Solutions were freshly prepared with ultrapure Milli-Q™ water and volumes were adapted to the amount of sample. Unless stated otherwise, each step of the protocols was performed at room temperature. For protocols A and B, glass filters were required and a specific filtration kit was designed. Sintered glass vacuum filtration devices (porosity P5 corresponding to 1.6-µm maximum pore size) were obtained from Ellipse (France). For protocols C–E, Sartorius Vivaspin 15™ 30 kDa MWCO ultrafilters were purchased from Dutscher (France) and precleaned following Brock et al. (2007) and Beaumont et al. (2010). HDPE Ezee-filters™ were purchased from Elkay (UK) and cleaned by ultrasonication (20 min) following Brock et al. (2007). For protocol G, Spectra/Por 2 MWCO 12–14 kDa dialysis tubing regenerated cellulose membranes were purchased from Spectrumlabs (France) and precleaned according to the manufacturer's instructions.

Protocol A (after Tuross et al. 1988; Tuross 2012)

Small bone chunks are immersed in 0.5M EDTA solution (pH 7.4) until complete decalcification (with a visual and "mechanical" check every day, on weekdays). The solution is replaced every 4–5 days. The remaining pale yellow transparent phantom is rinsed 15 times including one time overnight (to remove EDTA). Extraction is performed with HCl pH 5 at 100°C for 16 hr followed by glass filtering.

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Table 2 Summary of the collagen extraction protocols.

	Crushing	Demineralization step		Decontamination step	Solubilization step			Purification steps	
Protocol	Size	Agent	Duration	Duration	pH	Temperature	Duration	Filtration (pore size)	UF /dialysis
A	Small chunks	EDTA 0.5 M	Weeks	No	5	100°C	16 hr	Glass (1.6 µm)	No
B	5–10 mm	HCl 0.2 M (4°C)	2–4 days	Yes (4°C) 2–4 days	1	90°C	5–60 min	Glass (1.6 µm)	No
C	Chunks (10–60 mg)	HCl 0.25 M	Several days	No	2	58°C	16 hr	Ezee™ (45–90 µm)	UF* (30 kDa)
D	0.5–2 mm	HCl 0.5 M	24–36 hr	(Yes) 15–60 min	2	60°C	16 hr	No	UF* (30 kDa)
E	Coarsely ground chunks	HCl 0.5 M	24 hr	30 min	3	75°C	20 hr	Ezee™ (45–90 µm)	UF* (30 kDa)
F	Powder 0.3–0.7 mm	HCl 1 M	20 min	20 hr	2	100°C	17 hr	MF-Millipore (5 µm)	No
G	Powder >0.3 mm	HCl 2 M	20 min	No	1	95°C	50 min	Whatman (0.45 µm)	Dialysis (12–14 kDa)
H	Powder >0.3 mm	HCl 2 M	20 min	No	3	90°C	25 hr	No	No

*UF = ultrafiltration. Ultrafiltration and dialysis membrane cutoff are given in parentheses.

Protocol B (after Stafford 2014; Waters et al. 2015)

Bone shards (5–10 mm) are immersed in 0.2M HCl at 4°C for 2 to 4 days (visual and mechanical check). The solution is replaced three times a day. The remaining phantom is rinsed with Milli-Q water and immersed in 0.1M NaOH at 4°C for 2 to 4 days (visual check: coloration of the solution), then rinsed with 0.2M HCl, and then Milli-Q water. Extraction is performed in 0.06M HCl at 90°C for up to 1 hr. Solubilization is checked every 5 min and followed by glass filtering.

Protocol C (after Brown et al. 1988)

Coarsely ground bone chunks (~10–60 mg) are immersed in 0.25M HCl for several days until complete decalcification (visual check). The remaining phantom is rinsed with Milli-Q water and extraction is performed in HCl pH 2 at 58°C for 16 hr. Purification is performed using Ezee-filters and collagen is concentrated via ultrafiltration: the sample is spun for 20 min at 3000 rpm followed by the remaining volume check. This process is repeated until 0.5–1 mL remaining volume.

Protocol D (after Beaumont et al. 2010)

Coarsely ground bone chunks (~0.5–2 mm) are immersed in 0.5M HCl for 24 to 36 hr until complete decalcification (visual check). The remaining phantom is rinsed with Milli-Q water. If required, the sample is treated with 0.1M NaOH for up to 1 hr. The treatment is stopped after 15 min if no coloration of the solution is observed. The remaining phantom is rinsed with 0.1M HCl, then Milli-Q water and extraction is performed in HCl pH 2 at 60°C overnight. Solubilized collagen is concentrated via ultrafiltration: the sample is spun twice, then diluted, and finally spun twice more.

Protocol E (after Brock et al. 2010, 2013)

Coarsely ground bone chunks are immersed in 0.5M HCl for 24 hr. The solution is replaced three times. The remaining phantom is rinsed with Milli-Q water and treated with 0.1M NaOH for 30 min. The remaining phantom is rinsed with 0.5M HCl for 15 min, then Milli-Q water and extraction is performed in HCl pH 3 at 75°C for 20 hr. Acid-insoluble residues are removed via filtration through Ezee-filters. Collagen is then concentrated via ultrafiltration. The sample is spun for 20 to 40 min at 3000 rpm until 0.5–1 mL remains in the ultrafilter.

Protocol F (after Bocherens et al. 1991)

Coarse bone powder (0.3–0.7 mm) is immersed in 1M HCl for 20 min under continuous stirring. The solution is then filtered on MF-Millipore™ membranes (mixed cellulose ester membranes of 5.0-µm pore size from Fisher Scientific, France) while Milli-Q water rinsing is performed. The acid-insoluble residues are then immersed in 0.1M NaOH for 20 hr. The solution is then filtered again on MF-Millipore™ while Milli-Q water rinsing is performed. The alkali-insoluble residues are immersed in HCl pH 2 and extraction is performed at 100°C for 17 hr. The final solution is filtered on MF-Millipore membranes before being collected.

Protocol G (after Semal and Orban 1995)

Coarse bone powder (>0.3 mm) is immersed in 2M HCl for 20 min. The solution is then diluted to perform the collagen extraction in 0.2M HCl at 95°C for 50 min. The final solution is filtered

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on Whatman® nitrocellulose membrane filters of 0.45-µm pore size from Sigma-Aldrich (France), before being collected and dialyzed against bidistilled water until 1 mL remains in the membrane (overnight to several days). The aqueous dialysis solution is replaced after 1 hr the first time, then once a day.

Protocol H (after Longin 1970)

Coarse bone powder (>0.3 mm) is immersed in 2M HCl for 20 min with sporadic manual stirring. The solution is then discarded and the residues diluted to reach pH 3 to perform the collagen extraction at 90°C for 25 hr. The final supernatant is collected after 10 min centrifugation at 3000 rpm.

At the end of each processing, supernatants were pipetted out and freeze-dried for at least 48 hr. The final extract was weighted quickly to calculate the collagen extraction yield for each sample and each protocol.

Elemental Analysis and Isotopic Ratio Mass Spectrometry (IRMS)

Carbon (%C) and nitrogen (%N) contents as well as C:N ratios were calculated to evaluate collagen preservation. About 0.3 to 0.5 mg of extracted collagen were weighed and packed in precleaned tin capsules. Stable isotopic measurements were performed with a Thermo Scientific EA Flash 2000 coupled to a Delta V Advantage isotopic mass spectrometer. Isotopic values of all samples were measured relative to a laboratory standard of alanine. A reproducibility of 0.3 wt% for N and 0.6 wt% for C was estimated based on this standard. Carbon and nitrogen isotopes values are reported relative to the VPDB and AIR, respectively. Analytical precision is $\pm 0.2‰$ (2σ) for $\delta^{13}C$ and $\delta^{15}N$.

RESULTS AND DISCUSSION

Upstream FTIR Analysis

Examples of ATR-FTIR spectra from both modern and archaeological bone samples studied are shown in Figure 1. Modern bones are characterized by intense organic bands: clearly visible amide I (at 1640 cm^{-1}) but also amide II, amide III, amide A and B bands (at 1545, 1240, 3285, and 3075 cm^{-1}, respectively) can be distinguished. On the contrary, archaeological bones show weaker or no organic bands but an increase in the intensity of mineral bands, as previously described for fossils (Farre et al. 2014): carbonate ν_2 and ν_3 (CO_3) bands (at 875 and 1410 cm^{-1}, respectively) and phosphate ν_3 and ν_4 (PO_4) bands (at 1010 and 555 cm^{-1}, respectively). Amide I/PO_4 ratios are reported for each sample in Table 3 and vary between 0.01 and 0.20. The highest values are calculated for the modern sheep (P77) and the mammoth (VIRI E), whereas the lowest value corresponds to a bat from a tropical environment (SAN 7).

Nitrogen contents were estimated from these ratios based on Lebon et al. (2016) and are shown in Table 3. They allow to classify bone samples according to their preservation state (Stafford et al. 1988), as shown in Figure 2. Logically, modern bones (P77 and PEL) as well as VIRI E mammoth fall into the highest range, with a N wt% content between 3.5 and 4.1, with the maximum reported being 4.4 for modern fresh bone (Bocherens et al. 2005). Most of the other archaeological small vertebrate bones belong to the well-preserved class except the bat bone from a tropical climate (SAN 7), which exhibits a very low nitrogen content below the 0.7% threshold defined by Brock et al. (2012). It is also noteworthy that within the same site (Bourges), bone samples from amphibians (BOU_{amph}) are better preserved than rodent bones (BOU_{rod}). As far as we know, information about the collagen content in amphibian bones and

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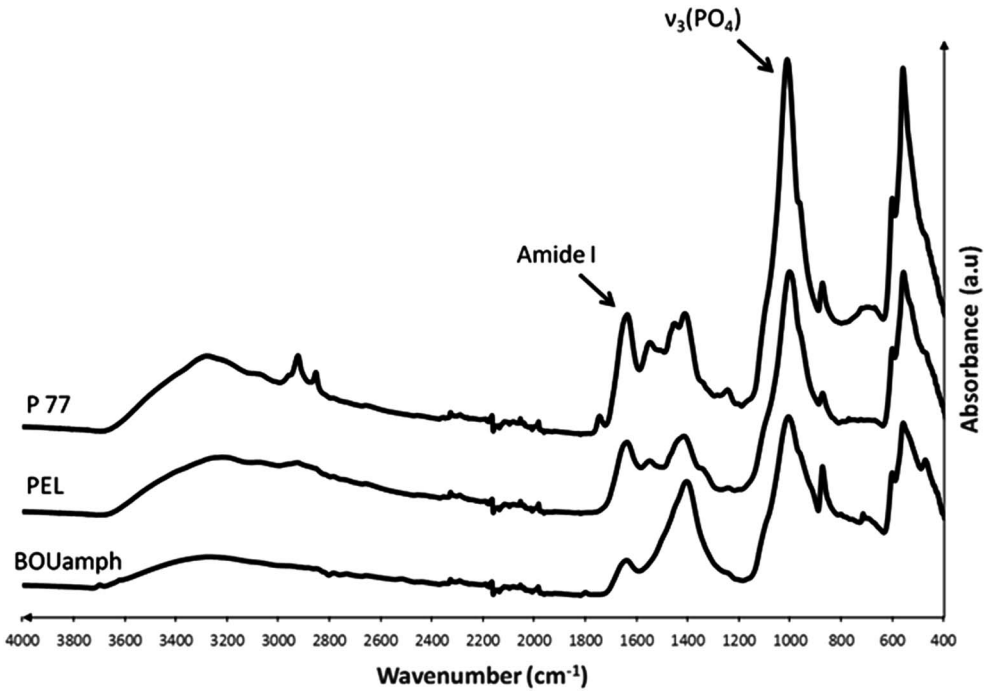

Figure 1 Prescreening example of ATR-FTIR spectra of test bone samples: modern macromammal bone (sheep P77), modern small mammal (rodent PEL), and archaeological amphibian (BOU_{amph}). The two bands of interest for collagen preservation study [$\nu_3(PO_4)$ mineral and amide I organic bands] are indicated by arrows. Samples are ranked according to the intensity of the amide I band.

possible discrepancies according to individual ages is not currently available. Thus, our observation is at the present time difficult to interpret. Estimation of the collagen content deduced from the amide I/PO_4 ratio as proposed in Lebon et al. (2016) is also shown in Figure 2.

ATR-FTIR spectra also offer the possibility of detecting exogenous compounds in bone. The list of exogenous compounds identified in our samples is given in Table 3. The spectra of buried samples (i.e. all the samples except the modern sheep bone P77) show contamination bands corresponding, for example, to carbonates (calcite) and aluminosilicates (kaolinite) coming from the soil in which the bones were found. This information could be further used to monitor the pretreatment of bone during the first acid step (e.g. increasing the duration of decalcification if large amounts of calcite is detected) and the second alkali step (e.g. avoiding this step if no exogenous alkali-soluble compound is found). It could also indicate the need to perform a preliminary chemical cleaning step (e.g. using acetone to remove conservatives). Moreover, it is worth noting that the detection limit in the bone-calcite mixture has been estimated from ATR-FTIR spectra as 2.5 wt% of calcite (Dal Sasso 2015).

Yields: Quantity of Collagen Extracted

Collagen extraction yields vary from zero to 24.7% and are summarized in Table 4. For clarity and to enable a direct comparison of the protocol efficiencies, a normalized yield was calculated and is reported in Table 4. It corresponds to the ratio of the final measured collagen over the median value of the collagen content (wt%) calculated using FTIR analysis (as reported in Figure 2). For most of the protocols, modern and well-preserved VIRI E macromammal bones

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Table 3 ATR-FTIR data analysis: measurement of the amide I/PO_4 ratio, calculated bone nitrogen content (wt%) and exogenous compounds identified in the bone sample. For a given sample, the different lines correspond to different analyses on different grains of the bone sample.

Sample	Amide I/PO_4 ratio	Calculated N_{bone} (wt%)	Exogenous compounds
P77	0.18	3.99	None
VIRI E	0.17	3.81	None
	0.2	4.34	None
	0.16	3.56	Quartz
	0.17	3.77	None
PEL	0.16	3.59	Unidentified (695 and 755 cm^{-1})
	0.17	3.74	Carbonates
	0.17	3.73	Carbonates
CAR	0.13	3.01	None
	0.16	3.61	None
	0.14	3.12	Kaolinite
	0.16	3.58	Kaolinite
	0.15	3.38	Kaolinite
BOU_{rod}	0.05	1.29	Calcite and kaolinite
	0.07	1.67	Kaolinite (and calcite)
BOU_{amph}	0.07	1.77	Calcite
	0.08	1.86	Calcite
	0.1	2.29	Calcite
	0.07	1.8	Calcite
SAN 7	0.01	0.6	Unidentified (1575 cm^{-1})

show logically the highest yield. Within the exception of VIRI E and BOU_{amph} samples, the soft EDTA protocol A always shows lower normalized yields. This surprising result, also observed for the modern sheep bone (P77), suggests that this procedure is not able to efficiently extract collagen from the bones. This could be due to an incomplete demineralization as this step is stopped by visual and mechanical examination by the experimenter. Results for protocol G are variable and lead to lower normalized yields, probably due to the harsh solubilization conditions. The soft protocol B seems to be the most suitable to recover the highest quantity of collagen from rodent bones, whereas the harsher protocol F seems best suited to amphibian bones. This would suggest a higher mineralization of the amphibian bone samples studied. None of the protocols were able to extract enough collagen from the bat sample (SAN7).

It can finally be noted that FTIR prescreening was able to give a reliable estimation of the expected extractible collagen content of bones, but sometimes underestimated this content as shown by normalized yield above 1 (Table 4). As mentioned in Lebon et al. (2016), limitations in the estimation may exist. The presence of N-containing contaminants may lead to an underestimation of the nitrogen content within the sample. Another limitation should also be underlined, even if unusual: measurement of the amide I/PO_4 ratio may be disturbed if another band overlaps the two absorption bands of interest. In this case, identification of the compounds followed by deconvolution of the bands, though a little more delicate and time consuming, could be considered.

Elemental Analysis: Quality of Extracted Collagen

To check the quality of collagen extracted and to assess its integrity in the different samples, $\%C_{collagen}$, $\%N_{collagen}$, and C:N ratios were measured. The results are shown in Table 4.

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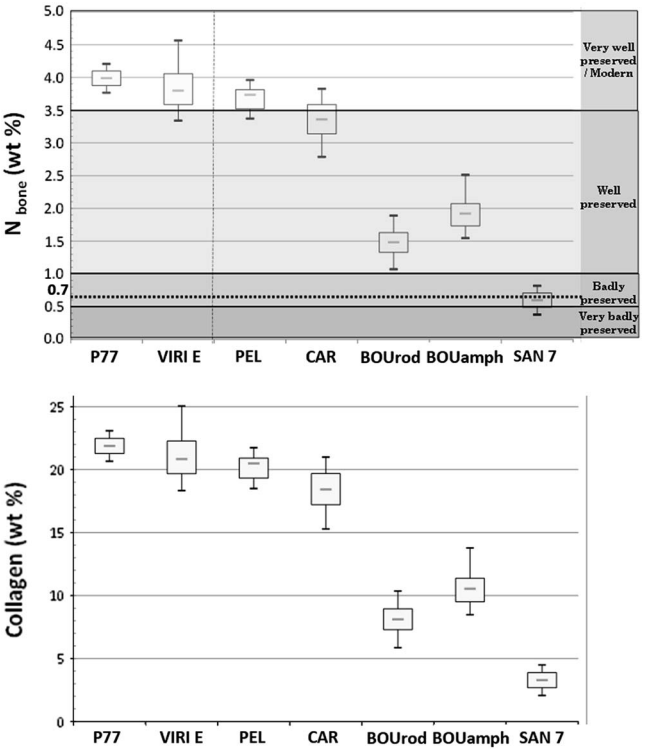

Figure 2 Box plot of the nitrogen (wt%) and collagen content (wt%) for modern and archaeological bone samples, calculated from FTIR amide I/PO_4 ratios estimations, using Lebon's calibration (Lebon et al. 2016). The dotted line indicates the 0.7% N (wt) threshold defined for possible ^{14}C dating of Paleolithic to Bronze Age bones (72% chance) using ORAU's protocol with ultrafiltration (Brock et al. 2012). Shaded areas delineate the threshold of preservation state classification stated by Stafford et al. (1988).

Apart from protocol G, no significant difference was detected in $\%C_{collagen}$ between protocols with values ranging from 37.0 to 45.2 for all the samples but SAN 7. In the case of BOU_{rod}, protocol G leads to lower %C wt, even below the 30% limit for accurate collagen integrity (DeNiro 1985; Ambrose 1990; van Klinken 1999). None of the extract from SAN 7 shows evidence of collagen preservation, confirming the results of FTIR prescreening. The same trends are observed for comparison of weight percentage of nitrogen: low (<10% wt) or no %N content was detected for samples extracted with protocol G. All samples but SAN 7 contained about 15% nitrogen, whatever the protocol considered. Therefore, the use of an alkali step (B, D, E, and F) or of an ultrafiltration step (C, D, and E) does not seem to affect %C and %N, confirming previous observations on very well-preserved human bones by Jørkov et al. (2007). Similarly, grinding (protocols F, G, and H) does not seem to affect the results either (Schoeninger et al. 1989).

C:N ratios fall within the 2.9 and 3.6 limit defined for modern and archaeological bones (DeNiro 1985; van Klinken 1999), indicating that bones that passed other tests (FTIR, yield, %C wt, and/or %N wt) are suitable for ^{14}C dating. Moreover, the C:N ratios obtained for VIRI E (between 3.20 and 3.24) are consistent with published values (Minami et al. 2013). With the

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Table 4 Final collagen yield and stable isotope results for the different protocols applied on each sample. Normalized yield corresponds to the ratio of the final measured collagen over the median value of the collagen content (wt%) calculated via FTIR analysis (as reported in Figure 2). * Indicates that the sample contained insufficient nitrogen and could not give a valid isotopic measurement. ** Indicates that the sample contained insufficient carbon and nitrogen and could not give valid isotopic measurements.

Sample	Protocol	Yield (%)	Normalized yield	C %	N %	C:N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
P77	A	3.4	0.16	41.6	14.4	3.37	–21.5	7.4
	B	17.2	0.79	4.1	—*	—*	–23.1	—*
	C	19.3	0.88	43.9	15.5	3.3	–21.4	7
	D	20.4	0.93	44.1	15.2	3.38	–21.5	6.9
	E	16.1	0.74	45.2	16.3	3.22	–21.2	6.9
	F	24.7	1.13	43	15.4	3.25	–21.6	6.9
	G	8.1	0.37	33.2	—*	—*	–22.4	—*
	H	13.3	0.61	44.4	15.3	3.39	–21.7	7
VIRI E	A	20.8	1	43.5	15.7	3.24	–21.4	6.6
	B	20.5	0.98	39.9	14.6	3.18	–21.4	6.6
	C	17.3	0.83	44.1	15.9	3.24	–21.2	6.5
	D	12.8	0.61	39.5	14.4	3.21	–21.2	6.6
	E	17.6	0.84	44.6	16.1	3.24	–21.2	6.5
	F	19.8	0.95	43	15.7	3.2	–21.3	6.6
	G	5.8	0.28	32	—*	—*	–21.7	—*
	H	17	0.82	41.4	14.9	3.25	–21.4	6.6
PEL	A	6.5	0.32	44.7	16	3.26	–22.5	3.3
	B	16	0.78	21	—*	—*	–23.2	—*
	C	11.4	0.56	43.6	15.6	3.26	–24	4.1
	D	9.1	0.44	44.5	15.9	3.27	–24.1	4
	E	10.3	0.5	42.5	15.2	3.26	–22.6	3.3
	F	13	0.63	40.8	14.9	3.18	–24.1	4
	G	11.7	0.57	36.1	12	3.5	–24.6	4.1
	H	10.8	0.53	40.5	14.2	3.32	–24.2	4.2
PEL_{long}	A	7.4	0.36	41.8	15.1	3.22	–23.9	3.7
	B	16	0.78	39.4	14.2	3.25	–24.2	4.3
	C	12.6	0.61	42.3	15.1	3.27	–23.9	3.9
	D	9.1	0.44	—**	—**	—**	—**	—**
	E	14.3	0.7	41.9	14.6	3.34	–24.2	3.7
	F	13.3	0.65	41	14.8	3.23	–24	3.8
	G	1.8	0.09	27.1	—*	—*	–25.8	—*
	H	11.7	0.57	41.3	14.9	3.23	–23.8	4.1
CAR	A	8.9	0.48	42.6	15.2	3.26	–21.4	8.8
	B	18.4	1	39.5	14.4	3.19	–19.8	8.3
	C	12.3	0.67	42.4	15.2	3.26	–21	9.1
	D	11.4	0.62	42.1	15.1	3.25	–20	8.8
	E	12.4	0.67	42.8	15.4	3.25	–19.8	7.8
	F	11.9	0.65	41.6	15.3	3.16	–19.9	8.6
	G	6.4	0.35	34.8	—*	—*	–20.5	—*
	H	10.7	1.32	40.5	14.6	3.25	–20	8.6

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possible exception of a slight increase in C:N ratio for BOU samples prepared according to protocol A, no significant difference between protocols was observed in our study. The use of EDTA in protocol A was shown to give slightly lower C:N ratios (VIRI E C:N ratio is 0.3 lower in Tuross 2012) but no significant difference was observed in our study. A lack of influence of ultrafiltration on C:N ratios was also noted in several studies performed on modern (Jørkov et al. 2007) and ancient (Brock et al. 2013) bones.

IRMS: Influence of Extraction Protocols on Isotopic Results

Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic values are reported in Table 2 and Figure 3. As expected, all isotopic values correspond to terrestrial species with $\delta^{15}N$ value below 12‰ and $\delta^{13}C$ below –17‰ and to non-carnivorous diet (Bocherens and Drucker 2003; Britton et al. 2008). For macrovertebrate bones, reproducibility of $\delta^{13}C$ and $\delta^{15}N$ values is within the analytical error (±0.2‰). As in Jørkov et al. (2007), most of the variability is for carbon, while nitrogen isotopic values remain constant. Moreover, for mammoth sample VIRI E, isotopic values are consistent with previously published ones (Minami et al. 2013).

On the contrary, an important isotopic variability was observed between protocols for small vertebrate samples. This is particularly significant for $\delta^{13}C$ values, which showed a 2‰ variability. As such differences may lead to differences in dietary interpretation, understanding the origin of these discrepancies is important. This could indicate that, unlike macromammal bones, pretreatment protocols have an influence on the isotopic results for small vertebrate collagen. Contrary to Jørkov et al. (2007), we did not find any influence of NaOH or ultrafiltration on $\delta^{15}N$ values. No influence of the choices made during the first acid step (HCl or EDTA, demineralization duration, acid strength) was noted, as previously reported for macromammals (Pestle 2010; Tuross 2012; Sealy et al. 2014). Nevertheless, as no influence of the protocols has been observed on macromammals and as collagen is as well preserved in small

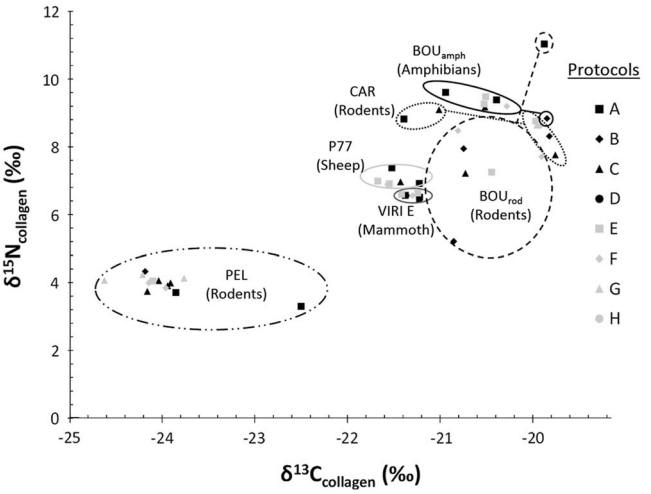

Figure 3 Carbon ($\delta^{13}C_{collagen}$) and nitrogen ($\delta^{15}N_{collagen}$) isotope compositions of the collagen extracted from the bone test samples according to the different protocols. Each symbol corresponds to one protocol. Black and gray symbols correspond to the softer and harsher protocols, respectively. For greater clarity, points corresponding to the same sample are circled (either by solid or dotted lines).

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vertebrate bones (same range of values of %C and %N as macrovertebrate bones), the observed variability cannot be related to the protocol chosen. Another interpretation may be proposed. Due to their small size, we had to use a different bone each time for each protocol. Therefore, the isotopic variability could in fact reflect interindividual differences in diet for these small vertebrates. DeNiro and Schoeninger (1983) estimated that interindividual variability in $\delta^{13}C$ values could reach 2‰. A similar variability (2‰) was observed for an assemblage of *Cavia porcellus* bones from Peru (Finucane et al. 2006). This variability may be linked to annual variability in plant $\delta^{13}C$ values, differences in fractionation, and access to different foodstuffs even for individuals from the same taxa and coming from the same site. This result shows that analyzing individual bones from small vertebrates can provide information regarding the dietary variability within a given population.

Finally, it is noteworthy that modern rodent samples (PEL) exhibited the most negative $\delta^{13}C$ values (about –3‰ between BOU_{rod} and PEL). This could reflect the influence of the Suess effect affecting the isotopic composition of the atmospheric CO_2 and ingested plants for the past 150 yr (Suess 1955; Francey et al. 1999).

CONCLUSIONS

This study compared the quality and quantity of small vertebrate collagen extracted using eight different protocols. On these types of samples that may undergo differential and important diagenesis, we confirm the efficiency of the FTIR prescreening method to select the samples that are best suited for ^{14}C dating. Our comparative approach demonstrated that protocol B (after Stafford 2014; Waters et al. 2015), a soft protocol involving slow demineralization at low temperature, is the most suitable for rodent bone samples as it allows to recover the highest amount of material (>75% for micromammals) from archaeological bones while preserving the collagen integrity and isotopic values by efficiently removing nitrogen-rich contaminants. Even if this protocol is time consuming (demineralization can take up to 4 days), it is not more complicated than the conventional protocols and is relatively inexpensive as neither disposable filters nor ultrafilters are required. We also demonstrated that neither grinding nor ultrafiltration or the presence of an alkali step in the chosen protocol influence the elemental composition of the extracted collagen. Finally, we did not find any influence of the extraction protocol on the isotopic values of collagen extracted from macromammals. The differences observed for small vertebrate bones are most likely due to individual differences in dietary preferences. The conclusions drawn from our experiment will be validated soon with ^{14}C dates on a larger sample set of small vertebrate bones from archaeological assemblages.

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