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DATING REASSEMBLED COLLAGEN FROM FOSSIL BONES

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ABSTRACT. Insoluble bone collagen is one of the most common materials used for high-resolution radiocarbon (^{14}C) dating. Unfortunately, in some bones, poor preservation of the insoluble collagen excludes the possibility of dating. During the burial of the bone the collagen sometimes degrades into peptides. These peptides are soluble in the acid used to dissolve the bone mineral. It is known that under appropriate conditions, collagen has the ability to self-assemble. Here we exploit this capability and present a method for reassembling the soluble collagen peptides in archaeological bones and dating them. We treated the acid fraction generated during the demineralization of the bone by desalting and neutralizing the solution by dialysis. During the dialysis, the soluble collagen peptides reassemble and precipitate in the dialysis bag. We used FTIR spectroscopy to determine that the precipitated material is indeed collagen. The ^{14}C dates obtained from the reassembled collagen were compared to the dates of "standard" insoluble collagen, extracted in parallel from the same bone. Although there are some divergences of the dates, 3 out of 10 samples could have been dated only by the reassembled collagen. This shows that collagen peptides reassembly can be a valuable tool for dating bones with little or no insoluble collagen.

KEYWORDS: AMS dating, collagen, collagen reassembly, degradation, FTIR spectroscopy.

INTRODUCTION

Bones are among the most important materials found in archaeological sites. In addition to the fascinating archaeozoological information that can be obtained from the study of fossil bone assemblages, the bone material itself often contains preserved organic material. The major organic component of modern bone is type I collagen, and it is also the major organic component in relatively well-preserved fossil bones. Type I collagen is therefore a very important material for high-resolution radiocarbon (^{14}C) dating. The material dated is the insoluble collagen itself or individual amino acids derived from collagen (Stafford et al. 1982; McCullagh et al. 2010). The vast majority of the methods for dating collagen are variations of the method proposed by Longin (Longin 1971), which is based on the analysis of the acid insoluble residue that remains after the dissolution of the bone inorganic phase with acid (Brown et al. 1988; Law and Hedges 1989; Hedges and van Klinken 1992; Taylor 1992). The major component of this acid insoluble fraction is collagen, and the procedures used for removing all other contaminants take advantage of the insolubility of this fraction.

Some fossil bones, however, do not contain an acid insoluble fraction after the mineral has been dissolved in acid. The absence of this fraction has been ascribed to various environmental factors such as temperature, soil pH, microbial activities and more (Hedges and Millard 1995; Hedges 2002; Collins et al. 2002b; Jans et al. 2004). One approach for dating these bones could be the dating of the inorganic bioapatite phase (Haynes 1968; Haas and Banewicz 1980; Cherkinsky 2009; Zazzo and Saliège 2011). As the carbonate in apatite can exchange during the burial, the question of mineral integrity is crucial. Therefore, there is a need to ensure that what is dated is indeed the pristine carbonate. In a recent comprehensive review by Zazzo and Saliège (Zazzo and Saliège 2011) on ^{14}C dating of bioapatites it was suggested that bioapatite is a reliable material to date only in the case of calcined bones or for skeletal remains in arid environments.

Another approach exploits other organic constituents of the bone besides collagen as the so-called non-collagenous soluble proteins that are often preserved even when the

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collagen insoluble fraction has degraded (Gillespie 1989; Collins et al. 2002a). The non-collagenous proteins that have been ^{14}C dated after purification include osteocalcin, osteonectin and other phosphoproteins, proteoglycans and glycoproteins (Termine 1988; Gillespie 1989; Weiner and Bar-Yosef 1990; Ajie et al. 1990; Tuross and Stathoplos 1993). Some of the results are inconsistent (Ajie et al. 1990; Brandt et al. 2002) and the analytical complexity of the methods involved has prevented the dating of these proteins from becoming routine in most ^{14}C laboratories. Here we present a different approach to dating bone collagen that is based on the ability of collagen peptides to self-assemble from solution (Gross and Kirk 1958).

The collagen molecular structure is composed of three polypeptide α-strands that wrap around each other to form a triple helix (Katz and Li 1973; Fraser et al. 1979).The triple helices self-assemble into fibrils by formation of inter- and intra-molecular bonds, including covalent cross-links between α-chains (Otsubo et al. 1992; Kadler et al. 1996). When the collagen is partially degraded it loses this triple helical conformation and unfolds (Collins et al. 1995). This unfolding or "gelatinization" makes it soluble. But, it is well known that under appropriate conditions, namely ambient temperature and physiological pH and ionic strength, these "disassembled" collagen peptides can spontaneously reassemble into collagen fibrils (Gross and Kirk 1958; Fessler 1974). Different mechanisms that facilitate this self-assembly process have been proposed (Wood and Keech 1960; Wood 1960; Fessler 1974; Holmes et al. 1986), based on the common assumption that collagen peptides themselves possess the "internal parameters" (Ramachandran and Sasisekharan 1968; Fraser et al. 1979) that are necessary for self-assembly (Helseth and Veis 1981).

So while all the methods for collagen extraction deal with the acid insoluble residue left after demineralization of the bone, we focus here on the soluble peptides derived from the breakdown of the collagen chains that are left in the resulting acid soluble fraction. It has been shown that many fossil bones do contain soluble peptides of collagen (Elster et al. 1991).

The strategy we use after mineral dissolution in acid is to slowly desalt and neutralize this fraction by dialysis against water at ambient temperature. Under these conditions soluble collagen peptides can interact with each other and self-assemble into fibrils. This process can be visualized by turbidity of the supernatant (Zhu and Kaufman 2014) and the formation of a precipitate in the dialysis bags. In the paper by Brock et al. (2013), it was shown that the final acid insoluble "collagen" product, extracted using different extraction procedures, is in fact an aggregate of collagen with other both organic and inorganic substances. Ideally, this self-assembly process should exclude many of the other molecules in the acid soluble phase, including contaminating molecules of a different age. We monitor the products obtained using mainly infrared spectroscopy, as the spectra can reveal both the presence of collagen and some of its contaminants (DeNiro and Weiner 1988; D'Elia et al. 2007; Weiner 2010). The FTIR sensitivity to contaminations is very much depends on the specific contaminant and the sharpness of its peaks. Therefore, it might be difficult to quantify it based only on the peak heights. For the particular application presented in this paper, the most important contaminants are probably humic substances and non-collagenous proteins. Both of these have a broad absorption around 1050 cm^{-1}, a region in which pure collagen does not absorb.

MATERIALS AND METHODS

Bone Samples

The bones analyzed in this study came from five different sites in Israel. Archaeological and geological information such as the type of bone, burial environment and estimated

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Table 1 List of bones analyzed. For every bone sample, represented by the laboratory number (RTD), the type of the bone, burial environment and the expected archaeological period are specified.

RTD#	Type	Burial environment	Expected archaeological period/ estimated age
8588	Human (tibia)	Limestone/clay	Early Chalcolithic ca. 5th millennium BC
8754	Human (femur)	Limestone, Judea Desert cave	Chalcolithic period 4300–3300 BC
8796	Bovine (compact bone)	Ashy layer, calcite, and clay	Early Bronze II 3100–2900 BC
8803	Human (femur)	Quartz and clay stratum	11th to 8th centuries BC
8833	Human (femur)	Quartz and clay stratum	11th to 8th centuries BC
8795	Human (femur)	Quartz and clay stratum	11th to 8th centuries BC
8823	Human (femur)	Quartz and clay stratum	11th to 8th centuries BC
8794	Human (femur)	Quartz and clay stratum	11th to 8th centuries BC
8824	Human (femur)	Quartz and clay stratum	11th to 8th centuries BC
8719	Human (arm)	Aragonite and calcite	2nd century BC up to 1st century AD

archaeological periods are summarized in Table 1. Samples with thick cortical bone were preferentially selected. Bones collected had different appearances. Mostly they were light in color with the sediments included in the inside. They were mostly grey or beige and very friable.

FOURIER TRANSFORM INFRARED SPECTROMETRY (FTIR)

A few milligrams of sample were homogenized and powdered in an agate mortar and pestle. A small amount (about 100 micrograms) of the powder was mixed with FTIR grade KBr and pressed into a 5-mm pellet using a Specac hydraulic press. The homogenization of the sample and mixing with KBr are done for production of a well-homogenized pellet for the analysis and elimination of possible optical artifacts, which can arise when large particles are analyzed. The measurements were carried out on a Nicolet 380 spectrometer. Infrared spectra were obtained at 4 cm^{-1} resolution for 32 scans.

Prescreening of Bones

At this stage of method development, the final amounts of the reassembled collagen are still unpredictable. For a preliminary, rough approximation of the amount of bone powder needed for extraction of both insoluble and soluble fractions of collagen, we used a prescreening procedure.

The bones were analyzed for the mineral crystallinity based on their "splitting factor" calculated from the height and separation of the phosphate absorption peaks at 603 cm^{-1} and 565 cm^{-1} in the infrared spectrum of the bone powder (Weiner and Bar-Yosef 1990; Wright and Schwarcz 1996). The splitting factor provides information regarding the size of the crystals and the atomic order of the lattice. The splitting factor serves as a proxy for general bone preservation.

Collagen Extraction

After the prescreening the bones were pretreated according to the procedure presented in Yizhaq et al. (2005) and Boaretto et al. (2009). Each bone was ground to a fine powder with an agate mortar and pestle. Depending on the initial amount of bone, varying amounts of 1N HCl

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(100–400 mL) were added stepwise until the bone mineral was dissolved. As the starting weights of the bone were relatively high we used 1N HCl for demineralization. The relatively high acid concentration was necessary to keep the resulting solution volumes small enough for continued processing.

After complete demineralization of the bone the sample was centrifuged for 3 min at 3000 rpm. The pellet (insoluble fraction collagen, IF) and the supernatant (soluble fraction collagen, SF) were separated and from hereon, each fraction was treated separately.

Acid Insoluble Collagen—IF

The cleaning procedure for the collagen samples chosen for dating was based on the AAA technique (De Vries and Barendsen 1954). The pellet was washed with distilled water and centrifuged until the suspension reached pH 7. Then the pellet was resuspended in 7 mL of 0.1% NaOH for 15 min and centrifuged again for 7 min at 3000 rpm. If the resulting supernatant after rising is still colored, this decontamination step needs to be repeated. The clear and colorless supernatant was removed and the pellet was washed with distilled water to pH 7. Finally the atmospheric CO_2 adsorbed during the alkali treatment was removed by adding 10 mL of 1N HCl for 30 min and washing the pellet until the supernatant reached pH 3. Gelatinization was achieved by heating the pellet in an acid solution pH 3 to 70°C for 20 hr (Law and Hedges 1989). The solution was then filtered through a polyethylene filter (Ezee-filter®) and then by ultrafiltration (Vivaspin 20). The filtrate was lyophilized (HetoLyoLab 3000) to produce dry collagen (Brown et al. 1988).

Acid Soluble Collagen—SF

The supernatant collected during the demineralization of the bone was centrifuged again for 3 min at 3000 rpm and then filtered through a polyethylene filter (Ezee-filter®), to ensure removal of small particles such as clay and quartz. Spectra/Por®3 dialysis bags with molecular cutoff of 3.5 kD that is suitable for retaining both the collagen molecule and its peptides, were used for dialysis of the resulting solution against water. We chose dialysis tubing with no glycerin as preservative. Prior to use the bags were soaked in distilled water for an hour and then rinsed thoroughly. The solution was placed in the bag, which was closed with dialysis tubing Spectra/Por® clamps. The dialysis bag was then placed in a 3-L glass beaker containing distilled water that was continuously stirred. The water was changed after 1 and 3 hr, and then after 15 hr. At that stage the pH of the solution was 5–6. Sodium azide (0.01%) was added to the solution to prevent bacterial growth. After about 6 hr the sodium azide solution was changed again to distilled water and left overnight. The final pH of the solution was 6–7. The content of the dialysis bag with usually visible precipitation was then centrifuged for 10 min at 3500 rpm and the pellet was subsequently lyophilized (HetoLyoLab 3000) to produce dry reassembled collagen (SF).

Stable Isotope Analysis

Stable isotope analysis was performed on both IF and SF of 3 bone samples. Collagen samples of 0.50–0.55 mg were weighed into tin foil capsules (Elemental Microanalysis Ltd. 5 × 3.5 mm #D1015). Sample $\delta^{13}C$ values were determined with an elemental analyzer (Carlo Erba 1108) linked to a continuous flow isotope ratio mass spectrometer (Optima, Micromass, UK).

Target Preparation and ^{14}C Measurement

Collagen samples were weighed into prebaked (1 hr at 900°C) quartz tubes and sealed with 200 mg CuO in a vacuum line. The combustion to CO_2 was done at 900°C for 3 hr.

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The reduction from CO_2 to graphite was performed in the presence of H_2 using 2 mg of Fe as a catalyston a laboratory graphitization line. Prior to reduction the Fe catalyst was activated at 400°C with H_2. The graphitization process was carried out at 600°C, with continuous removal of water formed during the process by a Peltieri cooling system, for 12 hr. The resulting graphite was then pressed at 180 psi into an aluminum cathode and its ^{14}C content was measured at the DANGOOR Research Accelerator Mass Spectrometry Laboratory at the Weizmann Institute. All the ^{14}C dates were corrected for isotopic fractionation based on the stable carbon isotope ratio ($\delta^{13}C$ value) and reported in pMC in accordance with the established international convention (Stuiver and Polach 1977).

RESULTS

The main aim of this study was to examine the feasibility of using reassembled acid soluble collagen peptides for dating. It was therefore important to date some samples using both the "conventional AAA method" namely the acid insoluble collagen (IF), as well as using the re-assembled soluble collagen (SF). Ten different bones were analyzed for this purpose. The same bone powder was used for the extraction of the two different fractions of collagen.

The splitting factor values calculated for the bones ranged from 2.9 to 4.6 and are presented in Table 2. Only one archaeological bone, RTD 8754, falls in the range of 2.5–3.1 that is reported for fresh bones (i.e. well-preserved bone) (Weiner and Bar-Yosef 1990; Stiner et al. 1995; Sillen and Parkington 1996). Three of the bones, RTD 8803, 8823, and 8794, had splitting factors higher than 4, suggesting greatly degraded bone mineral (Weiner and Bar-Yosef 1990; Smith et al. 2007). Based on the notion that we are dealing with poorly preserved bones, we used the maximum amount of bone powder available for collagen extraction, namely between 6.4 and 27.1 g. The ^{14}C results obtained, presented both as pMC and as ^{14}C yr BP with corresponding standard deviations, are summarized in Table 2.

Table 2 Summary of the ^{14}C results obtained for the 10 analyzed bones. ^{14}C results are given as pMC and as ^{14}C yr BP with corresponding standard deviations. For every bone sample, represented by the laboratory number (RTD), the initial weight of the bone powder, the splitting factor, and weight percentage of the resulting collagen both from the acid insoluble and soluble fractions are given.

			Acid insoluble collagen					Acid soluble collagen				
RTD#	Mass of bone powder (g)	Splitting factor	% collagen	pMC	± 1σ	^{14}C yr BP	± 1σ	% collagen	pMC	± 1σ	^{14}C yr BP	± 1σ
8588	6.7	3.4	0.39	44.0	0.2	6600	40	0.02	47.0	0.2	6060	30
8754	10.2	2.9	2.38	50.3	0.2	5520	35	0.80	50.3	0.2	5515	35
8796	22.8	3.2	0.01	58.9	0.2	4250	25	0.04	58.7	0.2	4280	30
8803	23.4	4.2	0.13	70.7	0.3	2785	35	0.02	71.7	0.2	2665	25
8795	12.9	3.6	2.19	72.1	0.2	2630	25	0.04	72.6	0.2	2570	25
8833	17.1	3.4	0.96	72.9	0.2	2635	25	0.03	72.0	0.2	2635	25
8823	23.0	4.2	0.01	76.7	0.2	2215	25	0.01	74.4	0.2	2370	25
8794	27.1	4.6	0.00	—	—	—	—	0.02	73.0	0.3	2530	30
8824	26.0	3.8	0.00	—	—	—	—	0.05	74.0	0.2	2420	25
8719	6.4	3.9	0.00	—	—	—	—	0.07	74.9	0.2	2325	25

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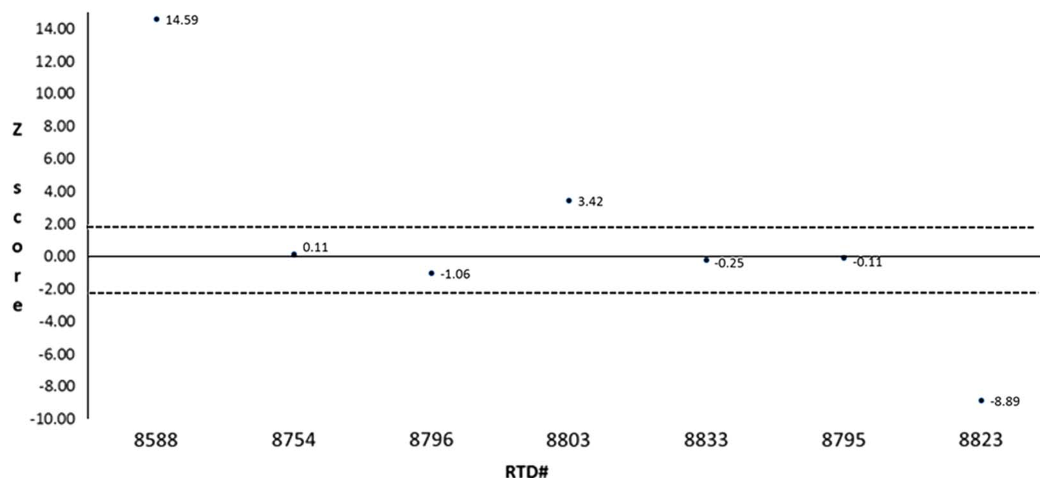

Figure 1 Z-score values for the 7 pairs of insoluble and soluble fraction collagens analyzed. The Z-score of 2.0 standard deviations is highlighted by dotted line.

Taking into account the error in the age of the insoluble and reassembled fractions, most of the samples are in good agreement. The age of the soluble fraction collagen is sometimes younger and sometimes older than the insoluble fraction collagen and there is no clear correlation with the age of the sample. It should be noted that 3 out of 10 samples, RTD 8794, 8824, and 8719, have results only for the soluble fractions as there was no collagen in the insoluble fractions.

For evaluating the reliability of the results obtained from the soluble fractions we carried out the Z-score test to compare the results of the acid soluble fraction to the standard insoluble fraction results (Figure 1). The Z-score of 2 standard deviations is highlighted. Two samples RTD 8588 and RTD 8823 have extremely high and low Z-scores. Three samples: RTD 8754, 8833, and 8795 are in the range of 1 standard deviation, sample RTD 8796 has Z-score of –1.06 and sample RTD 8803 has Z-score of 3.42.

To confirm the presence of collagen and to assess its quality, we monitored the extraction process using FTIR spectroscopy. Figure 2 shows representative spectra of the acid insoluble fraction collagen (b) and the soluble fraction collagen (c) for sample RTD8754 in comparison to a spectrum of a fresh pure collagen (a). This sample (RTD 8754) produced a good agreement between the ^{14}C dates obtained for both collagen fractions. Both spectra (b) and (c) show the characteristic peaks of collagen, namely 1645, 1545, 1450, and 1239 cm^{-1} representing Amide I and II, proline peak sand the Amide III, respectively. These peaks show the presence of well-preserved collagen. Note that the spectrum of the acid soluble fraction collagen has stronger C-H group peaks (2919, and 2850 cm^{-1}) than in the spectrum of the acid insoluble fraction. This may indicate the presence of organic substances in the acid soluble fraction other than collagen.

Figure 3 shows spectra of the acid insoluble fraction collagen (a) and the soluble reassembled fraction (b) for sample RTD 8588. There is a clear difference in pMC between the two fractions (Z score = 14.59). Both spectra show the characteristic peaks of collagen as described above. Remnants of quartz (1,080, 797, and 779 cm^{-1}) and clay (1,036, 526, and 469 cm^{-1}) are also present, but there is no clear indication from the FTIR spectra showing the presence of exogenous organic material that could explain the discrepancy between the two fractions.

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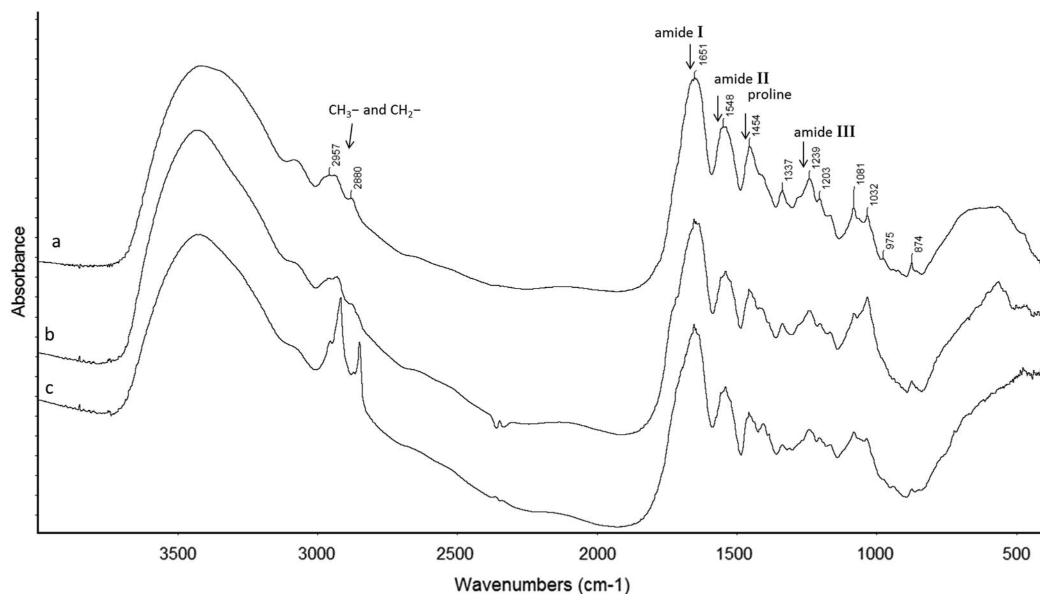

Figure 2 FTIR of (a) pure, fresh bone collagen; (b) acid insoluble fraction of sample RTD 8754; and (c) acid soluble fraction collagen of sample RTD 8754.

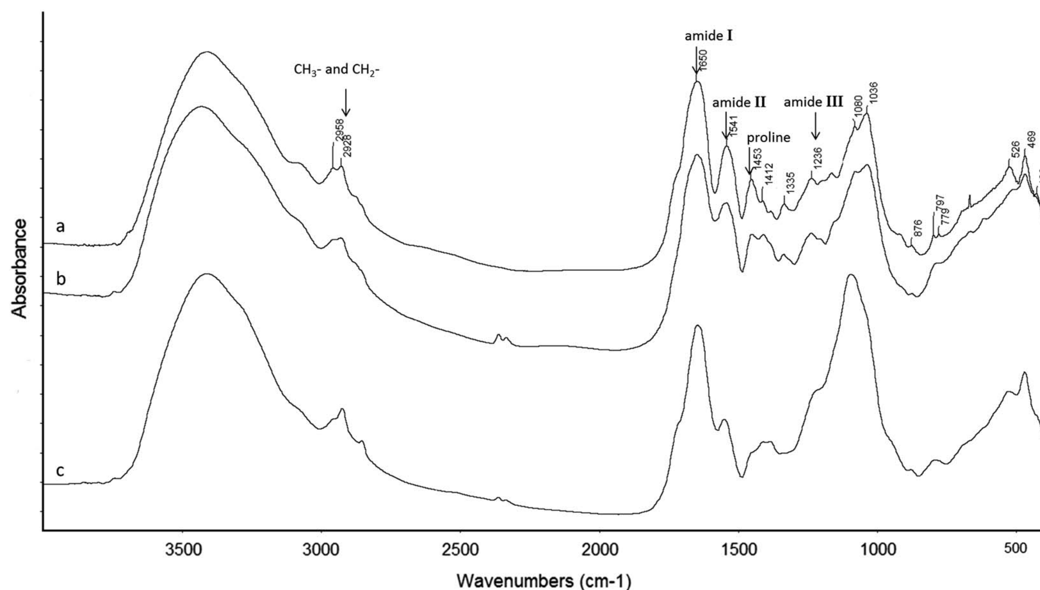

Figure 3 FTIR of (a) acid insoluble fraction of sample RTD 8588; (b) acid soluble fraction collagen of sample RTD 8588; and (c) acid soluble fraction collagen of sample RTD 8794.

Figure 3 also shows the spectrum of the soluble reassembled fraction for sample RTD 8794. This bone sample yielded only a soluble fraction. This sample has a strong Amide I peak and a weak Amide II peak, it exhibits strong C-H group peaks as well. It may indicate presence of other organic material besides collagen as for instance non-collagenous proteins which

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Table 3 $\delta^{13}C$ values obtained for IF and SF of 3 analyzed bones. The results are given with corresponding standard deviations.

RTD#		$\delta^{13}C$
8754	IF	–21.35 ± 0.04
	SF	–22.50 ± 0.04
8795	IF	–21.11 ± 0.04
	SF	–21.55 ± 0.04
8833	IF	–20.90 ± 0.04
	SF	–21.63 ± 0.04

do have a strong polysaccharide component. Additional peaks can be assigned to quartz and clay minerals (as described above) that were not removed during the procedure.

Additional characterization of the soluble collagen fractions in comparison to the insoluble collagen fractions was done by measuring the $\delta^{13}C$ of both fractions for 3 bones. The results obtained presented in Table 3.

The obtained $\delta^{13}C$ values are in the range expected for human collagen (Van Der Merwe and Vogel 1978; Ambrose et al. 1997; Richards and Hedges 1999). Although the values for the SF are somewhat lighter, the minor divergence suggests that there is no significant contamination from external source of carbon.

DISCUSSION

Here we present a method for extracting and dating collagen using the reassembled acid soluble fraction of archaeological bones. We rely on the ability of the collagen peptides to reassemble, and in this way we can harvest the reassembled fraction simply by centrifugation. Furthermore, we assume that the reassembly process excludes many of the contaminants that are also present in the acid extract.

To validate the method, we simultaneously extracted both insoluble and soluble collagen fractions from the same starting bone powder. As the method for extraction of acid insoluble collagen is known and well-validated, we regard the ^{14}C concentration of this fraction as the “gold standard” to which we compare the results of the reassembled soluble fraction. Overall, 10 bones were dated. For the 7 samples that yielded both insoluble and soluble collagen, a comparison was possible. The fractions in 2 samples were significantly different; in one RTD 8588, the soluble fraction is much younger than the insoluble fraction and in the other, RTD 8823, it is much older. Besides these two soluble fractions all other ^{14}C results obtained were in good agreement with the expected archaeological age. We also note that in the 5 samples where the ^{14}C concentrations are comparable in both fractions, the deviations are both negative and positive indicating that there is no inherent bias due to methodological issues. At this stage of the research, whether or not there is a correlation between IF and SF weight percentages in the same bone is not yet clear. Interestingly the best agreement or lower z-score is found for the samples with the IF percentage above or very close to 1%.

Three of the samples could be dated only by the soluble fraction collagen. We see this as proof of concept, namely that in some cases when the bone insoluble fraction collagen is degraded, the collagen peptides in the soluble fraction can be reassembled and used for dating. All of the samples that had only soluble collagen fraction yielded ages in the expected archaeological-chronological frame.

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We demonstrate here that FTIR can be used to monitor the presence and the quality of the collagen fractions obtained. As the FTIR is not always sensitive or indicative enough regarding possible organic contamination in the sample we suggest using additional quality criteria in the future, such as stable isotope measurements of carbon and nitrogen as a routine part of the procedure. One interesting possibility that needs to be developed in future is that if these quality controls show that the reassembled fraction is contaminated, it is possible to dissolve the fraction again in acid and by using the same procedure allow it to reassemble. This second reassembly process is expected to remove most if not all the remaining contaminants.

CONCLUSIONS

Here we present a method for dating bones in which the normally used acid insoluble collagen fraction is absent. We take advantage of the ability of collagen peptides to reassemble and then harvest the reassembled fraction by centrifugation. Five out of the 7 bones tested by comparing acid insoluble and soluble collagen fractions yielded comparable results. Furthermore, 3 of the 10 bones tested did not have any preserved acid insoluble fractions, but did produce reassembled collagen fractions, demonstrating that this new method for dating bones may prove invaluable for poorly preserved bones.

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