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Collagen extraction with assistance of microwave irradiation

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Abstract (Deutsch)

Für das Erreichen des besseren Wissens über unsere Vergangenheit und des Verstehens der Schlüsselaspekte über menschliche Geschichte, ist es unentbehrlich eine korrekte und zuverlässige Zeitleiste zu haben. Unsere nächsten Vorfahren lebten in der Zeitperiode namens Jungpaläolithikum (50,000-12,000 BP). Radiokarbondatierung ermöglicht uns in der Vergangenheit lebendes Material von heutigen Zeiten bis ~50,000 Jahre *before present* (BP) zu datieren. Es misst das radioaktive Isotop ^{14}C mit Beschleuniger-Massenspektrometrie. Für archäologische Datierung sind vor allem die Knochen wegen ihres Kollageninhalts sehr attraktiv. Kollagen ist das am häufigsten vorkommende Protein in Säugetieren und macht etwa ~25 % des Gesamtproteingewichts in menschlichen Knochen aus. Klassische Methoden, die sich mit Extraktion von Kollagen Typ 1 auseinandersetzen, erfordern mehrere Stunden bis Tage. Diese Arbeit fokussiert sich auf die Beschleunigung vom Extraktionsprozess mit Verwendung von Mikrowellenbestrahlung. In einer Mikrowelle ist die elektromagnetische Energie in die thermale Energie umgewandelt. Das verursacht schnelle Erhitzung von dem Material. Knochenfragmente um die 250 mg untergingen zwei 30 Minuten langen Runden von Mikrowellen-assistierter Extraktion (MAE) bei 130°C in geschlossenen XAD Reagenzgläsern, die mit MilliQ^{TM} Wasser gefüllt waren. Danach wurde Ultrafiltration von dem extrahierten Kollagen durchgeführt. Wir erhielten ausreichende Menge von Kollagen aus archäologischen Knochen mit MAE in einer Stunde. Analyse von stabilen Isotopen zeigte, dass die MAE Ergebnisse identisch zu den routinemäßig analysierten Proben waren. MAE Paläolithikum ^{14}C Alter waren jünger als diejenigen, die mit routinemäßiger Vorbehandlung detektiert wurden. Weitere Reinigung der Probe, wie XAD, könnte noch notwendig werden. Archäologisches Kollagen, das bei 130°C extrahiert wurde, ist sehr gut aufbewahrt und kann für andere Zwecke genutzt werden. Neben der Extraktionsgeschwindigkeit ist ein weiterer Vorteil der MAE, dass das Aussehen der Proben weitgehend unverändert bleibt. Schließlich macht der Verzicht auf saure oder alkalische Lösungen die Extraktion zu einem umweltfreundlichen Verfahren.

Abstract (English)

For achieving better knowledge about our past and understanding of the key aspects of the human story, it is indispensable to have a proper and reliable timeline. Our nearest ancestors lived during a period called Upper Paleolithic (50,000-12,000 BP). Radiocarbon dating enables us to date once living material from today until ~50,000 years before present (BP). It measures the radioisotope ^{14}C by accelerator mass spectrometry. For archeological dating, mainly bones are attractive due to their collagenous content. Collagen is the most abundant protein in mammals and comprises ~20-25% of the total protein weight in human bones. Classical approaches dealing with the extraction of type 1 collagen from archeological bones take several hours to days. This thesis is focused on speeding up the extraction process by using microwave irradiation. In a microwave, electromagnetic energy is converted into thermal energy, which causes rapid heating of the material. Bone fragments around 250 mg underwent two 30 minute long rounds of microwave-assisted extraction (MAE) in closed XAD tubes filled with MilliQ^{TM} water at 130°C. Afterwards, ultrafiltration of the extracted collagen was performed. We obtained a sufficient amount of collagen from archeological bones with MAE in one hour. Stable isotopes analyses showed that results for MAE were identical to routinely analyzed samples. MAE Paleolithic ^{14}C ages were younger than the ages detected by routine pretreatment. Further purification such as XAD might be necessary. Archeological collagen extracted at 130°C is well-preserved and can be used for other purposes. Besides the extraction speed, an additional advantage of MAE is the appearance of the samples is largely unaltered. Finally, the lack of acidic or alkaline solutions makes the extraction a green process.

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1. Introduction

Humans have an innate interest in their past, and where they came from. Archaeology allows us to understand key aspects of the human story, but as in any historical science, it is crucial that we have a proper and reliable timeline. Dating is, therefore, very important. Our nearest ancestors, Neanderthals and Denisovans, span from the last ~450,000 years, but towards their extinction, fall into the period called of the Early Upper Paleolithic (50,000-35,000 BP)[1]. Radiocarbon dating, which relies on the measurement of the radioisotope ^{14}C, enables us to date once living material from today until ~50,000 years before present (BP)[2]. The ^{14}C isotope is formed through the actions of energetic cosmic rays which impact the Earth´s troposphere, where they cause a range of nuclei to be formed from these collisions. Neutrons from these impacts cause a change in the nucleus of ^{14}N, liberating a proton and forming ^{14}C. This then forms $^{14}CO_2$ by oxidation and this carbon dioxide enters the biosphere via photosynthesis and thereby enters other living organisms through the food chain. Once an organism dies, ^{14}C decays back into ^{14}N[3]. One half-life of ^{14}C is given at 5730 +/- 40 years[4], although the initial half-life measurement, the so-called Libby half-life (5568±35 years) is still used for historical reasons. After ten half-lives have elapsed, there is no more radioactive carbon to measure, so ~50,000 years is the limit of the technique. ^{14}C is measured by accelerator mass spectrometry (AMS)[5] and through an age equation, radiocarbon dates are calculated based on the residual amount of radiocarbon remaining. In bones, which are commonly used in dating in archaeology, collagen is the main fraction targeted. Human and animal bones consist of 70% of inorganic minerals, mainly hydroxyapatite. The remaining part is composed of bone cells and organic extracellular matrix, mainly built from collagenous proteins. The most dominant one is type I collagen[6]. It is the most abundant protein in the animal kingdom. It degrades over time, however, in post-depositional contexts. Proper conditions of the place, where the bone is deposited, are crucial for the collagen preservation. There is a range in the preservation conditions of collagen. In tropical environment there is much quicker degradation of the protein. This is mainly due to higher temperature and rainfall, which creates a warm humid environment and higher microbial activity. A dry and cool environment is more beneficial[2,7,8]. This factor has to be considered before performing collagen extraction on precious bones, because it influences the amount of preserved collagen. Getting to the point of dating analysis takes a lot of time. Every

collagen extraction begins with pretreatment of the material, which takes several days to perform and comprises a series of chemical steps. In my thesis, I focused my research on accelerating this process using microwave-assisted extraction (MAE) of type I collagen.

1.1. Type 1 collagen

Collagen is the most represented protein in all animals and makes up approximately 20-25% of the total protein weight in human bones. Altogether, there are 28 types of collagen. The most abundant is type 1 collagen. A single collagen molecule consists of a right-handed twisted triple helix, which is made from three polypeptide alpha chains. These are connected and stabilized with hydrogen bonds[9]. Many collagen molecules together build a collagen fiber. The hydrogen bonds could break by exposure to high heat. It remains unclear, how high this temperature should be, when we only want to extract the maximum of collagen, but we not intend to destroy it. One key consideration for us when using microwave technologies to extract collagen is the temperature setting. Previous works have used very low temperatures, while doing microwave assisted extraction, however these are uniformly studies on modern collagen samples. Zhang et al. (2023), for example, extracted collagen from modern bovine hide at 37°C[7]. Li et al (2010) performed the MAE of bovine tendon collagen in acetic acid solution at 40°C[10]. They both used this temperature since it is close to the denaturation temperature of collagen. León-Mancilla et al. (2016) found out with their thermal analysis that type I collagen denatures at 75–85 °C[11]. Luftensteiner et al. (2025) extracted well-preserved collagen from archeological bones in hot water at 90°C[12]. However, in general, there are no papers talking about collagen extraction and its denaturation from bones.

My research is focusing on the extraction of collagen from archeological bones, which are sometimes thousands of years old. The three polypeptide chains in the collagen structure have so-called cross-links between them. These are covalent bonds between collagen molecules, which become much more stable with age. This means, that the samples needs much higher temperatures to denature it. Another influence of the collagen thermal stability is in its amino acids composition. The hydrogen bonds between the three polypeptide chains are situated between the amino acids proline and hydroxyproline. Depending on the amount of these two amino acids in the collagen, higher temperatures

could be required[13]. However, it is already known that collagen will survive at 90°C in a hot water extraction (see section 1.3.). We intend to continue to increase the temperature to speed up the reaction.

1.1.2. Contamination and cleaning

An important factor in dealing with collagen with a focus on subsequent radiocarbon dating is contamination. Possible contamination may occur in any stage of dealing with the material, during excavation or in a museum or laboratory. For instance, humic acids infiltrated in a bone matrix, manipulation with the material with glue or markers in museum or contact of the cleaned samples with modern carbon sources such as skin. These influences must be considered or eliminated before starting the dating process, since they might influence the values of stable isotopes or an accurate radiocarbon age[14]. Otherwise, they would provide misleading results[15]. To ensure that contamination is reduced to a minimal level, it is crucial that pretreatment chemistry is performed. Treating bone collagen using methods such as ultrafiltration, ought to be applied. An ultrafilter separates the molecules based on their molecular weight. In radiocarbon dating of bone, we usually use 30,000 Dalton ultrafilters, the rationale being that these will isolate the long polypeptide alpha chains from the collagen which weight 95-100,000 Daltons. Heavier proteins and polypeptides that are undergraded stay above the filter, while molecules with a lower molecular weight will flow through the filter to the bottom. The material that passes through the ultrafilter consists, it has been suggested, of possible contaminants such as salts and insoluble particles from the environment, as well as degraded collagen[8]. This must be considered, because bad preserved samples with broken up collagen would get lost during the ultrafiltration[14]. However, small amounts of contamination (sometimes microgram sized amounts) derived from the laboratory while treating the sample must be taken into account and cannot be avoided[16]. One of the main collagen quality indicators is the C:N atomic ratio. This can inform us about possible contamination, degradation, or low content of collagen. The Oxford Radiocarbon Accelerator Unit (ORAU) defines an acceptable C:N atomic ratio range as 3,1-3,5. Values outside this range could be a sign of contamination with additional carbon. Another important parameter is the $\delta^{13}C$ value. It should be between -19 and -22‰ for mammalian bones from a C3-diet pathway. Sometimes more negative values might suggest contamination of the sample, although this has to be a contaminant in a high

concentration[17]. On the other hand, $\delta^{15}N$ has much wider range, namely 2-12‰. Those values are strongly influenced by nutrition of the animal or a human[17]. Another important parameter is the percentage carbon in a combusted collagen. This should be around 45 wt % C in normal well-preserved collagen samples (Higham, pers.comm.).

1.2. Routine radiocarbon pretreatment of bone

The commonly used method for extracting collagen from archaeological bones in the Higham-Lab at the University of Vienna is based around the "Longin" collagen extraction method, with additional purification steps[18]. The extraction follows three main treatment steps: with acid, base, and again with acid (ABA). The first acid treatment with diluted HCl aims to solublize the inorganic hydroxyapatite from the bone and demineralise it. It also removes sediments and contaminants. This leaves the raw collagen. The second wash with dilute NaOH is intended to remove humic and fulvic acids. Last acid treatment dissolves the modern carbon that may have been absorbed from the atmosphere during the base wash. Between each step, rinses with ultrapure MilliQ^{TM} water are done[19]. MilliQ^{TM} is ultrapurified deionized water and contain extremely low level of organic carbon[20]. The treated sample is then mixed with pH3 water and placed into an oven for gelatinization at 75°C for 20 hours. This process releases the triple helices and untwists the collagen polypeptide chains. Following this the gelatin samples are additionally cleaned with Ezee filters and ultrafilters. After freeze-drying we obtain purified collagen.

This method was standardized at the ORAU. Over the past decades it has been used in many laboratories for thousands of extractions. Following pretreatment, AMS dating is undertaken. Samples of pretreated collagen are placed into tiny aluminum tin capsules. They are combusted and CO_2 from the reaction is purified and then graphitized using standard methods. In the accelerator, carbon ions are directly measured[19]. In the last 30 years, this technique has helped us improve our knowledge of the late period of human evolution, especially Neanderthals and *Homo sapiens*. Now we can say with greater precision in what periods they lived and whether there was any contact between them[21]. A long period of use, improvements and accurate dating results has ensured a reliable collagen extraction method. However, this method has two main disadvantages. The first one is the duration. All the steps described below take together three to five days to

perform. This often requires some advance planning and is not effective. The second one is unavoidable destructive character of the procedure. It is not possible to recover any used sample to their original state. Because of these two factors, new non-destructive approaches are being developed.

1.3. Hot water extraction

Recently, a new method has been proposed for extracting collagen from archaeological and paleontological bones and teeth. It comprises a non-destructive water based approach[12]. In contrast to the routine destructive method, this technique was developed to enable radiocarbon dating while maintaining the original form and structure of the used sample. It now enables the dating of precious or rare fossils without destroying them. Its principle lies in heating of the sample in hot water at 90°C for several hours. The longer the extraction time is, the higher are the yields. However, to receive a sufficient collagen yield for further purification and AMS dating, the used samples have to be well enough preserved to have some soluble collagen in them. With this method, it was possible to extract about half of the ordinary collagen amount that is normally extracted with the routine ABA extraction. In initial tests it was shown that ^{14}C ages coming from hot water extraction were in agreement with the ^{14}C ages that one would obtain with the routine destructive method. Both underwent purification steps with ultrafiltration. Another advantage is that the temperature of the water ensures the preservation of DNA. It also does not change morphological features of the researched object. The fact that there is no requirement for chemicals during the whole process presents a green and ecological procedure[12]. An approach with such features is novel.

1.4. Microwave assisted extraction

As I mentioned above, collagen extraction is an involved process. A general aim is to maximize the efficiency of the extraction process, and one means to do this is to accelerate the chemical reaction by increasing the temperature. One solution could be a microwave-based approach. Microwave is characterized by a rapid initial heating up of the material. In a microwave, electromagnetic energy is converted into thermal energy. Polar molecules in the material interact by rapidly rubbing against each other, creating thermal energy[22,23]. In the collagen extraction process with the microwave, the high temperature causes denaturation

of the collagen. This means it comes first to decalcification to isolate the collagen fibers. The second step is gelatinization, so the helical structure unravels and we get the single polypeptide chains. This process does not require many resources, as is usual with the established methods (e.g. acid and base by ABA extraction). It represents a green and a low-energy way of extraction, without additional material needed or unnecessary waste produced[24].

When it comes to the integrity of the sample, MAE is less destructive than the conventional destructive method. Regarding of the sample size, it is necessary to cut a small piece of the material. Unlike that method, this used piece stays preserved in its original form after the MAE. Although the bone structure persists unchanged, there is a remaining question about DNA preservation after the treatment with high temperature. The DNA strands degrade by breaking its covalent bonds or by deamination of cytosine. Karni et al. (2016) found that DNA begins to degrade at 130°C and is destroyed at 190°C[25]. Lozano-Peral et al. (2021) studied DNA degradation in human teeth. They claim DNA can stand temperature until 100°C without any damage. At 200°C and higher, however, the DNA was destroyed[26]. With our MAE analysis, we are aware that the temperatures approach the point where they can have an influence on DNA damage, because, as we show below, we performed the extraction maximum at 130°C. The effect of high temperature on DNA has to be considered before treating the sample. We would suggest that one way of avoiding this would be to perform the DNA analysis before the MAE.

Microwave-assisted extraction in general is not a new term in the scientific field. It is widely used in botany (i.g, for polyphenols or inositols extraction)[27,28,29], the food industry (for antioxidants extraction from Macroalgae or for vanillin extraction)[30,31] and chemical engineering[32,33]. Liu et al. (2019) used MAE to extract rabbit skin gelatin, to make the process more efficient in terms of time, yield and gel properties[34]. It is widely used for collagen extraction due to gelatin production, because it speeds up the gelatinization. For instance, Feng et al. (2022) gelatinized collagen from a fish skin much more rapidly, then with the conventional acid-alkali method[24]. To my knowledge, the first scientific paper which used only MAE (without any other supplemental method) to extract type I collagen was Zhang et al. (2023)[7]. In contrast to my research, they extracted collagen from modern bovine hide and not from archaeological bones. For their extraction, a very low temperature

was used, only 33-35°C. Additionally, their experiment took 8 hours to achieve good collagen yields. In contrast, I will experiment with much more rapid methods to ensure a more efficient collagen extraction from ancient bones.

2. Aim of the thesis

We know it is possible to extract collagen using minimally-destructive methods, and avoid significant destruction of bone or teeth. Our question is, can this be done faster? There are two well-established methods for collagen extraction in our C14 lab. The first one is the routine radiocarbon pretreatment of bone (referring to ABA extraction), which takes approximately three days to perform. In this case, the sub-sample of bone taken for dating is always completely destroyed, without any possibility of returning to its original condition. For a deeper explanation of this method please, see section 4.2. and the attached Oxford protocol (Fig. 1), which forms the basis for this method. The second one (and the newest one) is the non-destructive hot water method. Its principle lies in heating a bone in water at 90 °C for 9 hours. During this time, soluble collagen is extracted, and the material remains untouched and undamaged. Besides archeological bones, this method is applicable for various types of material with a collagenous content, including teeth, skin (keratin), or leather. Both of these procedures have one important thing in common, they are time intensiv. It takes from 9 hours to 3 days, to perform the extraction. I intend to test whether it would be possible to speed up the collagen extraction using microwave irradiation. It is hypothesized that this procedure could significantly reduce extraction duration by several hours. The benefits are obvious; with a reduction time one could process more samples and reduce analyses costs. This method could be even more effective than the current routine pretreatment. To test the effectiveness of the MAE, I will compare the collagen yield of the new method with the standard destructive and non-destructive procedures. As a very last step, all the samples will be dated and analysed for their stable isotopes analysis to test the reliability of the MAE measurements. This is the first time that microwave-assisted collagen extraction has been attempted in radiocarbon dating in archeology.

3. Materials

3.1. Kara-Bom

For establishing microwave settings, one large piece of bone was used, so settings could be tested repeated multiple times. The chosen sample came from Kara-Bom, Russia (Fig. 2). It is a bone of unknown animal origin, but it was not classified as a human bone. It was dated twice, one result was 38641±436BP (VIE-1945) and the second was 36290±420 BP (VIE-1984). We are not sure why there is a difference in the results, since they were both from the same extracted collagen. The Hollis backgrounds used in the correction were very similar and ranged from ~49-52,000 BP. The original weight of the bone was 24 944 mg. All samples were prepared in the drilling room of the Higham Lab, with all standard cleaning procedures to avoid any potential contamination with modern carbon. This includes sterile gloves, cleaning of the tools (such as tweezers or a saw) by air abrasion with sandblaster or with MilliQ^{TM} water. I cleaned all working surfaces with MilliQ^{TM} as well and then covered them with aluminum foil. The surface of the bone used for sampling was cleaned with the sandblaster too. From the one original bone were sawn small cuts with a weight of 150-300 mg with a small electric saw.

Figure 2

Bone from Kara-Bom, used for experimenting.

Source: own photography.

3.2. Archeological samples

Once the most optimal MAE settings were established and working in our favor, further samples were analyzed. Table 1 shows the list of the samples that were used in these tests. They come from a range of different environments and time periods. Kara-Bom is in the Siberian Altai. Zaragizh is a Bronze Age site in the Russian Caucasus. Isturitz is a Paleolithic site in southwestern France. Tsak Pumakos and Kiowa are from tropical Papua New Guinea. Some of them were classified as human bones, other have unknown origin. All of them are archaeological samples, and they have been previously dated. In addition, I also analyzed radiocarbon lab standards (Hollis and VIRI). Hollis is a mammoth pelvis bone (*Mammuthus primigenius*) from Yukon, Canada[35], and it is around 700,000 years old[36]. VIRI is a whale bone and is 8,400 years old[37]. They are commonly used in the Higham-Lab as control samples for modern carbon contamination by destructive collagen extraction. We are highly confident in their age because they have been dated countless times, so we are using them in every batch we want to date. If the results diverge from those expected, it would be a clear sign for us that contamination must have occurred during pretreatment and therefore we cannot take the determined age of the new samples as completely accurate.

Table 1
Archeological samples for collagen extraction

Origin	R-number	Country of origin	Age (BP)
Mammoth	R00001.188	Hollis	>50,000
	R00001.189	Hollis	>50,000
	R00001.190	Hollis	>50,000
Unknown	R01340.8	Kara-Bom	38641±436/36290±420
	R01340.9	Kara-Bom	38641±436/36290±420
	R01340.10	Kara-Bom	38641±436/36290±420
Whale	R00040.78	VIRI standard	8400
Mammoth	R00017.3	Dent mammoth	11000
Unknown	R01297.2	Isturitz	34535±650
Unknown	R01299.2	Isturitz	35100±650
Human	R00600.2	Kiowa	3210±15
Human	R00602.2	Kiowa	6030±20
Human	R00615.2	Kiowa	400±20
Human	R00182.2	Kiowa	3209±28
Human	R00670.5	Zaragizh	4910±33
Unknown	R00537.2	Tsak Pumakos	Failed low yield

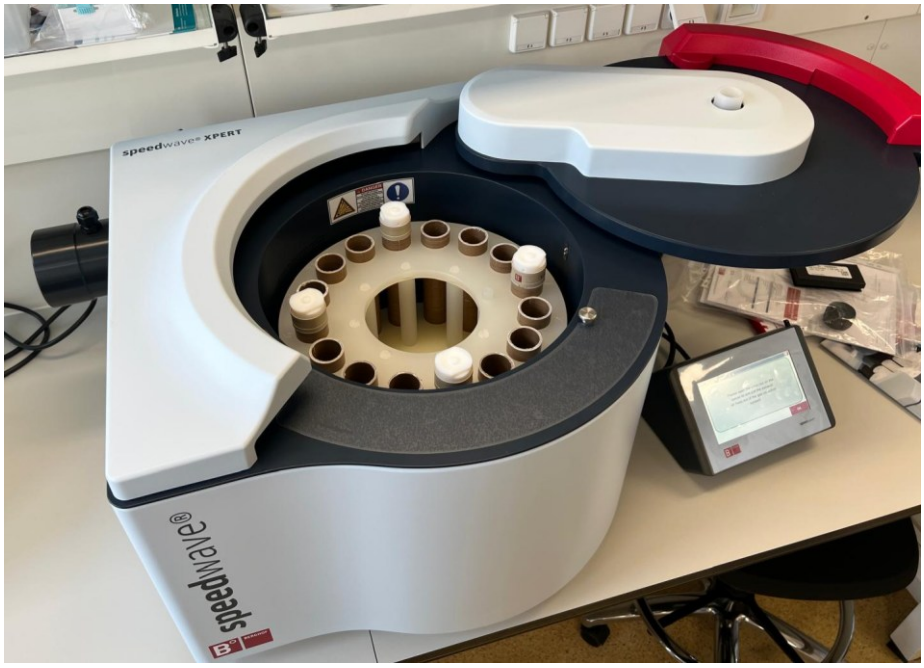
4. Methods

4.1. Microwave assisted extraction

The microwave instrument we used is the "Speedwave XPERT" model from Berghof Products + Instruments GmbH (Fig. 3)[38]. It enables measurement of various parameters, including pressure and temperature. Before performing each experiment, the following parameters had to be set: ramp time (the time it takes for the device to heat up to the desired temperature), hold time, temperature, and time for the instrument to cool down. All settings and also the process of the extraction can be followed on a digital screen, where the instrument can be monitored and the data stored. Data can be transferred to a computer using a USB stick and then viewed using the application "Datarecord Wiever". Another important element that needed to be tested is a type of tube to be used. I tested three types. First, borosilicate tubes (12 ml), commonly used in the Higham Lab for the routine extraction (ABA extraction). These were tested with and without borosilicate glass lids. Second; borosilicate XAD tubes with screwtop caps (9 ml). Third; quartz tubes (12 ml). These should have better thermal conductivity than borosilicate.

Figure 3

Microwave unit used for extraction.



Source: own photography.

For the most efficient collagen extraction it is crucial to establish reliable settings for the microwave unit. This is important to eliminate potential disadvantageous effects, which could cause carbon contamination or lower collagen yields, and therefore influence the final radiocarbon date. For instance, it might be a contamination of the sample with modern carbon through microwave vessels, destruction of the collagen by too high temperature, or boiling out/evaporation of the water out of the tube. The goal in these tests was not to extract the maximum amount of collagen from the bone. The main aim was to obtain enough collagen for purification (e.g. ultrafiltration) and subsequent radiocarbon dating. In the experimental phase of this dissertation, I aimed to establish the most reliable protocols in the light of three main parameters: temperature, extraction duration, and the composition of the sample tubes. These three factors directly influence the amount of extracted collagen. All of them were tested step by step in multiple rounds of experiments.

In each experiment, samples were placed in tubes filled with 5ml of ultrapure MilliQ^{TM} water. I put them into the microwave vessels, which contain 15ml of MilliQ^{TM} water (exceptions are noted in the relevant sections). Kara-Bom samples used for experimenting were labeled with the abbreviation “KB” and corresponding number. For each round of tests I noted the applied settings and tube types. After every extraction, I took the bone particles out of the tubes and froze them in a freezer for 24 hours. Then I transferred the samples into a freeze-dryer for 48 hours. Freeze-drying is a process where a frozen sample is put under the vacuum and the solid state changes into gas state, skipping the liquid stage[40] resulting in dried collagen. Collagen dried in this way can be returned to its previous wet state by rehydration.

The SPSS software (version 25) was used for statistical analysis of the obtained data. We chose the value of $p < 0,05$ as statistically significant. The software OxCal online was used for the statistical comparison of the ^{14}C dates acquired by MAE and by the routine extraction.

4.1.1. Experiments 1 – 6

Several experiments were performed to establish the most reliable settings for the microwave as described above. In the first, we observed that 30 minutes is sufficient for collagen extraction in the microwave. We also found that increasing temperature results in

higher collagen yields. In a second experiment two main problems were observed. First were the temperature fluctuations. These were about 10 degrees lower in the vessels than the set temperature. Second, the water boiled out of the tubes while doing the extraction at 130°C. To overcome this, in experiment 3, we added more vessels for a better balance and additionally changed the ultrapure water for ordinary tap water, to stabilize the settings and solve the problems. We used glass lids on every tube in a fourth experiment. Temperature fluctuations and water boiling over remained a problem here as well. The manufacturer informed us that, while the temperature setting represented the maximum temperature that could be reached, we should expected variation of the measured temperature down to 10% below the instrument setting. We concluded that differences of about 10-15°C cannot be avoided but must be carefully monitored. In experiment 5 we added more water to prevent boiling out of water. The probable reason for this was lifting of the lid due to pressure during the extraction. Experiment 6 was pivotal, because we used closed XAD tubes with screw cups for the first time. They resisted the pressure during the MAE and maintained a stable temperature. Additionally, quartz tubes were tested. Their better thermal conductivity was not proven in our experiment and water boiled out as in previous experiments at the beginning. The testing process is described in detail in the Supplementary Material section (Experiment 1-6). Following these sets of experiments, we were able to establish optimized settings. These include temperature, time, type of tubes and number of blanks. Finally, we analyzed a series of archeological samples to test whether the method we had developed could be applied to previously dated samples.

4.1.2. Experiment 7

In this experiment, three rounds were done. First at 120°C for 30 min, second two at 130°C, one for 30 min and one for 60 min. In each round, two samples and 14 blanks were used. This extraction was performed in closed XAD tubes. They were screwed on with matching caps. There was a slight doubt whether they would be able to resist the pressure. In the end, the tubes fulfilled the expectations. They functioned properly and there was no problem with the pressure. Since there was no boiling or evaporation of water from the tubes into the vessels, data from this experiment demonstrated the most reliable results. We can be sure that all extracted collagen remained in the closed tube.

4.1.3. Archeological samples

After establishing accurate settings in the initial set of experiments, we could proceed to the selection of various archeological bones for collagen extraction (Table 1). The samples for analysis were given an R-number (reference number in the Higham Lab database). Small bone pieces with weight of 167-300 mg were sawn. I used the XAD tubes with screw tops. As a first step, I filled all 16 vessels with 15 ml of MilliQ^{TM} water and the tubes with 5 ml of MilliQ^{TM}. I placed the bone samples into the tubes (Fig. 4). I let them heat to 60°C for 30 minutes, to remove any absorbed dirt particles, humics and soils. Then I decanted the water and set it to onside. I added new MilliQ^{TM} water to the samples. This process was done in two rounds as a multiple extraction. This tends to be more efficient than one long extraction. In each round, 8 samples and 8 blanks were used. The samples were heated on 130°C for 30 minutes. Afterwards, the water was stored in labeled tubes and the further extraction continued another 30 minutes with new water. This was stored in other labeled tubes as well. Both fractions of the samples were freeze-dried and weighed out.

4.1.3.1. Preparation of the samples for dating

Before samples with sufficient collagen could be dated, they require post-extraction purification. Usually this is achieved with ultrafiltration (UF) for an accurate and reliable radiocarbon age[39]. Only samples with collagen amounts higher than 8 mg are suitable for UF (Table 2). Samples with lower amounts may result in no sufficient yield for dating due to the high proportion of collagen that is lost. There is always a degree of collagen loss following ultrafiltration but it does not matter if a person does ABA extraction or MAE; it is always essential pass the obtained collagen through a filter[8].

First, I rehydrated the collagen with MilliQ^{TM} water and used an Ezee filters to remove insoluble residues such as bigger dirt particles, loose soil material or bone residuals. I cleaned the ultrafilter membranes with MilliQ^{TM}, because the glycerol humectants can lead to potential contamination with modern carbon if unremoved[39]. Following this, I placed the collagen solution in the ultrafilter and centrifuged the solution (see Bone pretreatment protocol (Fig. 1)). After filtration, the samples were placed into a freezer for 24 hours and then into a freeze-dryer for 48 hours. After this procedure we obtained freeze-dried purified collagen. Collagen was weighed out into two tin capsules: one for stable isotope analysis, a

second for AMS dating. Exact weights are summarized in Table 2. The majority of the samples were radiocarbon dated in the Keck AMS Laboratory, University of California, Irvine[47] or the VERA facility at the University of Vienna. Stable isotope analysis was done at the Queen's University of Belfast.

Table 2
Collagen yields and weighed amount in tins

R-number	Country	Previous collagen yield (mg)	Collagen yield after UF (mg)	AMS tin (mg)	Stable isotope tin (mg)
R00001.188	Hollis	16,34	9,80	2,5	1,01
R00001.189	Hollis	27,40	20,79	2,85	1,00
R00001.190	Hollis	26,38	17,47	2,68	1,04
R01340.9	Kara-Bom	11,54	5,93	2,41	1,02
R01340.10	Kara-Bom	13,58	6,76	2,6	1,07
R00040.78	VIRI standard	22,44	15,29	2,47	1,05
R01299.2	Isturitz	8,01	3,26	2,36	0,79

4.1.3.2. Crushed samples

Because we gained much lower collagen yields from some archeological samples than we initially expected, I decided to test whether analyzing small chunks or bone powder would be more successful. So I used a steel pestle and mortar (Fig. 5) and the same samples that were already microwaved (Table 8) to test whether they would give better yields when they are crushed. In this experiment, only the standards, Hollis and VIRI, were new samples of bones. The extraction was undertakenat130°C as a multiple extraction (30 + 30 minutes; same principle as in section 4.1.2.). Extracts were cleaned with Ezee-filters to remove all bone residues and then freeze-dried.

4.2. Routine radiocarbon pretreatment

When applying new forms of analysis, it is always advisable to compare the results with standard verified methods, to assess the differences. For this, I extracted collagen from the same Kara-Bom bone with the standard procedure, to be able to compare the results with the MAE results. The method used was:

1. Demineralization with acid:

We added 0,6 M HCl (diluted with MilliQ™ water) to the crushed sample (Fig. 6). We observed some bubbling, as usual, following this, due to the demineralization. Additional reactivity is due to the powdery structure of the crushed bone, because the acid has a larger surface area. Three acid washes were applied, with 2 hours rest time. We decanted the acid each time. After the third wash, the sample was left overnight. In the end, the inorganic hydroxyapatite of the bone was removed.

2. Base-acid washes:

To bring the pH value back to the neutral level, we applied MilliQ™ water rinses and then added 0,1M NaOH solution to remove humic contaminants and salts from the sample for 30 minutes. To achieve neutral pH-value again, we did three water rinses. Further 0,5M acid washes with 0,5M HCl were performed, with another three water rinses. In the end, we adjusted the pH to 3 prior to gelatinization.

3. Gelatinization:

We then performed a gelatinization by heating the samples at 75 °C for 20 hours in the oven. This brings the three polypeptide chains of the collagen structure into solution.

4. Ezee filtering and Ultrafiltration:

Ezee filters were cleaned by soaking up in the MilliQ™ water and then ultrasonicating for 20 minutes. They were used to mechanically separate bigger dirt particles from the rest of the sample. I then ultrafiltered the samples. First, glycerol from the membrane of the ultrafilters was cleaned and removed with multiple MilliQ™ washes. Soluble collagen samples were centrifuged until 1 ml level of the sample remaining, so all particles smaller than 30 kDa were removed by the filter.

5. Freeze-drying:

The sample was frozen for 24 hours and placed into the freeze-dryer for 48 hours. Solid and dense dried white collagen was visible. I weighed out the collagen and calculated the percentage yield. 45,23 mg of collagen was extracted from the 544 mg sample, which makes up 8,31%. This is therefore a very well-preserved bone.

5. Results

5.1. Experiment 7

The results of this experiment were highly encouraging. The closed XAD tubes with screw cups worked very efficiently. They handled the pressure during microwaving at 130°C.The most significant result was that we were able to extract sufficient amount of collagen, mainly in KB. 43 and KB. 44 (Table 3). They both underwent extractions for 60 minutes at 130°C. We observed higher yields than in previous experiments, both in milligrams of collagen extracted and percentage yield. We obtained more than 12 mg of collagen (4,8-5,6%) (Fig. 7). In earlier experiments (see Supplementary materials), values after 60 minutes were in general smaller due principally, I think, to water boiling out from the tubes, which resulted in major loss of collagen. Table 4 shows a comparison of the yields of open Ezee filter tubes and closed XAD tubes (Fig. 8). However, when doing the extraction for 30 minutes, it does not make any difference, if open or closed tubes are used. Yields of KB. 39 - 42 were very similar to the yields of previous samples done for 30 minutes, probably because the water was not boiling out with earlier tests (Table 5, Fig. 9).

Table 3
Results of the first extraction in XAD tubes.

Sample	Sample weight (mg)	Temperature (°C)	Actual temperature (°C)	Time (min)	Collagen yield (mg)	Collagen yield (%)
KB. 39	179	120	120	30	4,13	2,30
KB. 40	148	120	110	30	4,66	3,14
KB. 41	231	130	120	30	7,84	3,39
KB. 42	253	130	130	30	6,76	2,67
KB. 43	254	130	130	60	12,24	4,81
KB. 44	224	130	130	60	12,70	5,67

Table 4
Yields after 60 minutes extraction on 130°C. The main difference in the yields is made by sample loss due to boiling over.

	Collagen yield (mg)	Collagen yield (%)
KB.17*	7,03	3,46
KB.18*	7,08	4,02
KB.25*	3,62	2,12
KB.26*	5,73	2,97
KB.33*	7,77	4,20
KB.34*	4,07	2,09
KB.43***	12,24	4,81
KB.44***	12,70	5,67

* Ezee filter tubes
*** XAD tubes

Figure 8

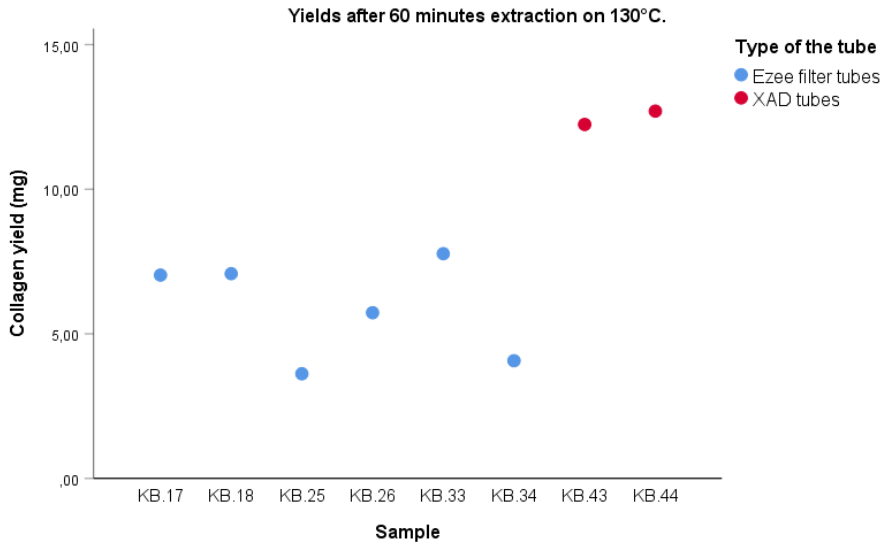
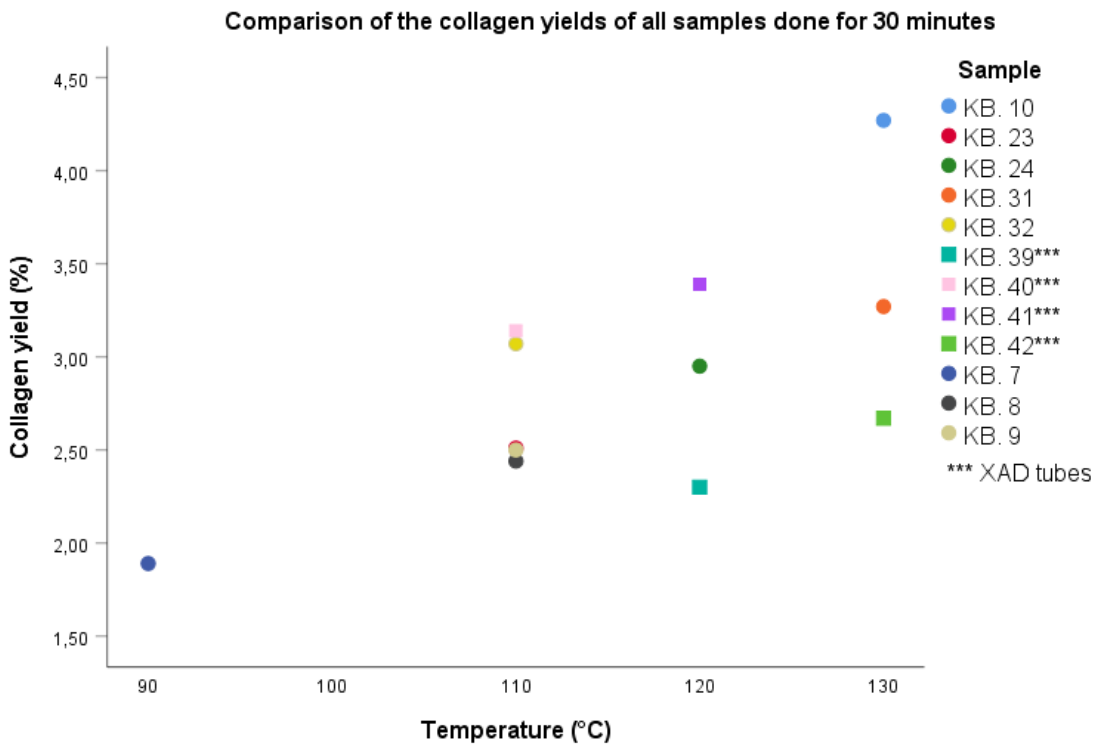


Table 5
Comparison of the collagen yields of all samples treated for 30 minutes.

Sample	Temperature (°C)	Collagen yield (%)
KB. 7	90	1,89
KB. 8	110	2,44
KB. 9	110	2,50
KB. 10	130	4,27
KB. 23	110	2,51
KB. 24	120	2,95
KB. 31	130	3,27
KB. 32	110	3,07
KB. 39***	120	2,30
KB. 40***	110	3,14
KB. 41***	120	3,39
KB. 42***	130	2,67

*** XAD tubes

Figure 9



5.2. Experiment 8

Collagen yields of Hollis and VIRI standards were as high as we hypothetically predicted based on previous destructively performed extractions (Table 6). From the standards, we were able to extract equivalent collagen amount as with the routine treatment method (Table 7). We obtained much less collagen from Zaragisk (R00670.5) and some of the Kiowa samples (R00600.2, R00615.2) than with the destructive method previously applied. The Kara-Bom samples provided about half of the amount of collagen compared to the destructive yield. In general, we obtained less collagen nearly in every sample than we did with the destructive method. Since many yields were not sufficient, some samples were crushed and the MAE was performed with them again (Table 8). The new yields were higher or the same as previous MAE collagen yields (Table 7, Fig. 10). We can see the biggest difference in Kiowa R00600.2 (1,60 vs. 3,45 %) or in the “Dent mammoth” sample (1,26 vs. 2,17 %).

Table 6
Results of collagen extraction of archeological samples performed as a multiple extraction. Set temperature: 130°C

R-number	Weight of the sample (mg)	Measured temperature (°C)	Time (min)	Collagen yield (mg)	Total together (mg)	Collagen yield (%)	Total together (%)
R00001.188	217	120	30	8,41	16,34	3,88	7,53
		120	30	7,93		3,65	
R00001.189	300	130	30	15,6	27,4	5,2	9,13
		125	30	11,8		3,93	
R00001.190	269	125	30	16,55	26,38	6,15	9,8
		130	30	9,83		3,65	
R01340.8	175	130	30	4,11	7,59	2,35	4,34
		120	30	3,48		1,99	
R01340.9	269	130	30	6,32	11,54	2,35	4,29
		120	30	5,22		1,94	
R01340.10	213	130	30	6,65	13,58	3,12	6,37
		130	30	6,93		3,25	
R00040.78	174	130	30	14,58	22,44	8,38	12,9
		120	30	7,86		4,52	
R00017.3	300	130	30	0,86	3,8	0,29	1,26
		125	30	2,92		0,97	
R01297.2	193	130	30	0,89	1,7	0,46	0,87
		125	30	0,81		0,41	
R01299.2	239	130	30	4,28	8,01	1,79	3,35
		115	30	3,73		1,56	
R00600.2	211	130	30	1,73	3,39	0,81	1,6
		130	30	1,66		0,79	
R00602.2	174	125	30	0,37	0,99	0,21	0,57
		130	30	0,62		0,36	
R00615.2	248	125	30	0,37	1,14	0,15	0,46
		130	30	0,77		0,31	
R00182.2	167	115	30	0,56	1,52	0,34	0,91
		120	30	0,96		0,57	
R00670.5	201	130	30	0,66	1,85	0,33	0,92
		130	30	1,19		0,59	
R00537.2	225	130	30	Failed	Failed	Failed	Failed
		125	30	Failed		Failed	

Table 7
Comparison of yields obtained with MAE and destructive method.

R-number	Country of origin	MAE yield (%)	MAE yield of crushed samples (%)	Destructive yield (%)
R00001.188	Hollis	7,53		9,60
R00001.189	Hollis	9,13		
R00001.190	Hollis	9,80		
R01340.8	Kara-Bom	4,34		8,30
R01340.9	Kara-Bom	4,29		
R01340.10	Kara-Bom	6,37		
R00040.78	VIRI standard	12,90		16,20
R00017.3	Dent mammoth	1,26	2,17	4,15
R01297.2	Isturitz	0,87		1,20
R01299.2	Isturitz	3,35		4,90
R00600.2	Kiowa	1,60	3,45	10,30
R00602.2	Kiowa	0,57	0,59	1,10
R00615.2	Kiowa	0,46	1,38	14,90
R00182.2	Kiowa	0,91	1,92	2,20
R00670.5	Zaragizh	0,92	1,58	12,00
R00537.2	Tsak Pumakos	Failed		0,10

Figure 10

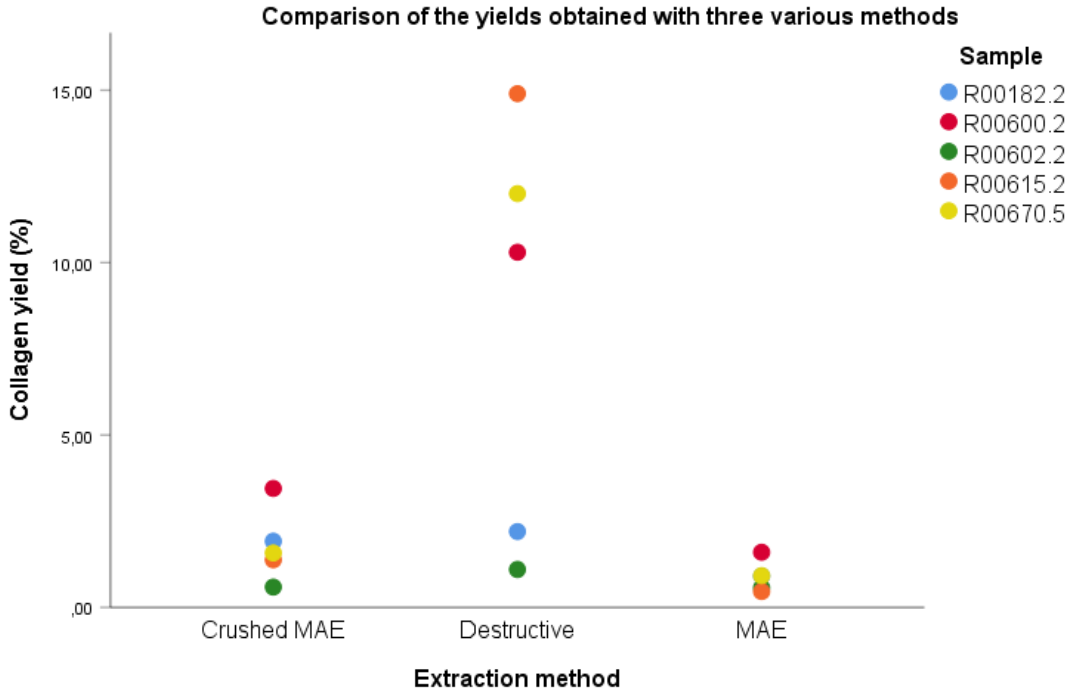


Table 8
Collagen yields of crushed archeological samples. The samples were sequentially treated for 30 min in two rounds. Set temperature: 130°C.

R-number	Country	Weight of the sample (mg)	Measured temperature (°C)	Collagen yield (mg)	Total together (mg)	Collagen yield (%)	Total together (%)
R00001.192	Hollis	233	130	16,79	25,27	7,20	10,84
R00001.192.1			120	8,48		3,64	
R00040.79	VIRI standard	243	130	18,35	25,79	7,55	10,64
R00040.79.1			110	7,44		3,06	
R00017.3.2	Dent mammoth	290	120	2,74	6,31	0,94	2,17
R00017.3.3			120	3,57		1,23	
R00600.2.2	Kiowa	199	130	2,22	6,86	1,12	3,45
R00600.2.3			130	4,64		2,33	
R00602.2.2	Kiowa	165	125	0,57	0,97	0,35	0,59
R00602.2.3			120	0,4		0,24	
R00615.2.2	Kiowa	241	130	2,14	3,31	0,89	1,38
R00615.2.3			110	1,17		0,49	
R00182.2.2	Kiowa	163	130	1,17	3,11	0,72	1,92
R00182.2.3			130	1,94		1,20	
R00670.5.2	Zaragizh	187	130	0,91	2,95	0,49	1,58
R00670.5.3			130	2,04		1,09	

5.2.1. Dating and stable isotopes analysis

All analyzed archeological samples were previously dated after the routine extraction. MAE collagen >8 mg in weight was radiocarbon dated. We compared the new MAE results with their expected ages (Table 9).The results for the standard Hollis samples show how much modern radiocarbon we introduced to our samples. The results span 46-48.3 ka BP which is marginally higher than for the routine method, suggesting a low contamination level with modern carbon. The VIRI MAE result is statistically indistinguishable from the routine average age (8360±25 vs. 8400 BP). The Kara-Bom results are difficult to interpret. The routine method gave divergent ages, as mentioned above. The MAE gave two different ages as well, one was 33.5 ka BP and the second 35.1 ka BP. The MAE age of the Isturitz sample is about 5000 years younger than it should be (29830±250 vs. 35100±650 BP).

Table 9
^{14}C ages of ultrafiltered archeological samples.

R-number	Country	Known age	MAE age
R00001.188	Hollis	>50,000	47260±180
R00001.189	Hollis	>50,000	48440±220
R00001.190	Hollis	>50,000	46100±190
R01340.9	Kara-Bom	38641±436/36290±420	33470±380
R01340.10	Kara-Bom	38641±436/36290±420	35110±470
R00040.78	VIRI	8400	8360±25
R01299.2	Isturitz	35100±650	29830±250

We obtained stable isotopes measurements (δ^{13}C and δ^{15}N values) and compared these with the results obtained using the routine collagen extraction method in the Higham Lab. For the VIRI standard whale bone, the routine results average is -16.9‰ for carbon and 12.1‰ for nitrogen, with an uncertainty of ±0.2 and 0.3 respectively. For %C the average is 44.3%. Our results for VIRI were -16.7‰, 12.0‰ and 44,6%, respectively. The C:N ratio was 3,15. The results are therefore in good agreement with the routine method and compare well with expected values for collagen[17].

For the Hollis bone in the Higham Lab, the isotopes average is -20.6‰ for carbon and 6.7‰ for nitrogen. The results we obtained using MAE are on average -20,7‰ and 6,6‰, respectively. The C:N ratios ranged between 3,15-3,3, which falls within the acceptable range of 2.9—3.5 (Higham, pers.comm.). All of these values are consistent with expected values.

The C:N atomic ratio of the Isturitz sample obtained by routine extraction is 3,2. Carbon isotopes are -20,9‰ and nitrogen isotopes are 6,2‰. Percentage carbon is 40,9%. MAE detected results are very similar: -20,8‰, 5,3‰, 44,7%, respectively. C:N atomic ratio is 3,3. The Kara-Bom MAE results are in average 19,3‰ for carbon and 5,2‰ for nitrogen. Percentage carbon refers to 43,4% and C:N atomic ratio to 3,3 in average. Again these values are consistent with collagen values[17].

5.3. Statistical testing

We tested the effect of temperature on the collagen yields (in %) in the samples extracted for 30 minutes with a simple linear regression. This revealed that temperature has a significant effect on collagen yield (*p = 0,022*; Table 10). The R-Square value is 0,425 and explains 42,5% of the variance (Table 11).

Table 10
Results of the simple linear regression.

ANOVA[a]

Model		Sum of squares	df	Mean of the squares	F	Sig.
1	Regression	1,822	1	1,822	7,394	,022[b]
	Non-standardized residues	2,464	10	0,246		
	In total	4,286	11			

a. Dependent variable: Collagen yield
b. Influencing variables: (constant), Temperature

Table 11
Results of the simple linear regression.

Model summary

Model	R	R-Square	Corrected R-squared	Standard error of the estimator
1	,652[a]	0,425	0,368	0,49641

a. Influencing variables: (constant), Temperature
b. Dependent variable: Collagen yield

To explore this further, we also divided the results into two independent groups (Table 12). The first contained results with extraction temperatures 130°C and 120°C. The second contained data points lower than 120°C. We concluded there is a difference between the two means and a significant positive effect of temperature on collagen yields. The two-sided significance is 0,080 and 0,066; divided by 2, they both fall under *p < 0,05* (Table 13).

Table 12
Results of the independent samples t-test.

Group statistics					
	Temperature	N	Mean	Standard deviation	Standard error of the mean
Collagen yield	High	7	3,1314	0,62227	0,23519
	Low	5	2,496	0,44309	0,19816

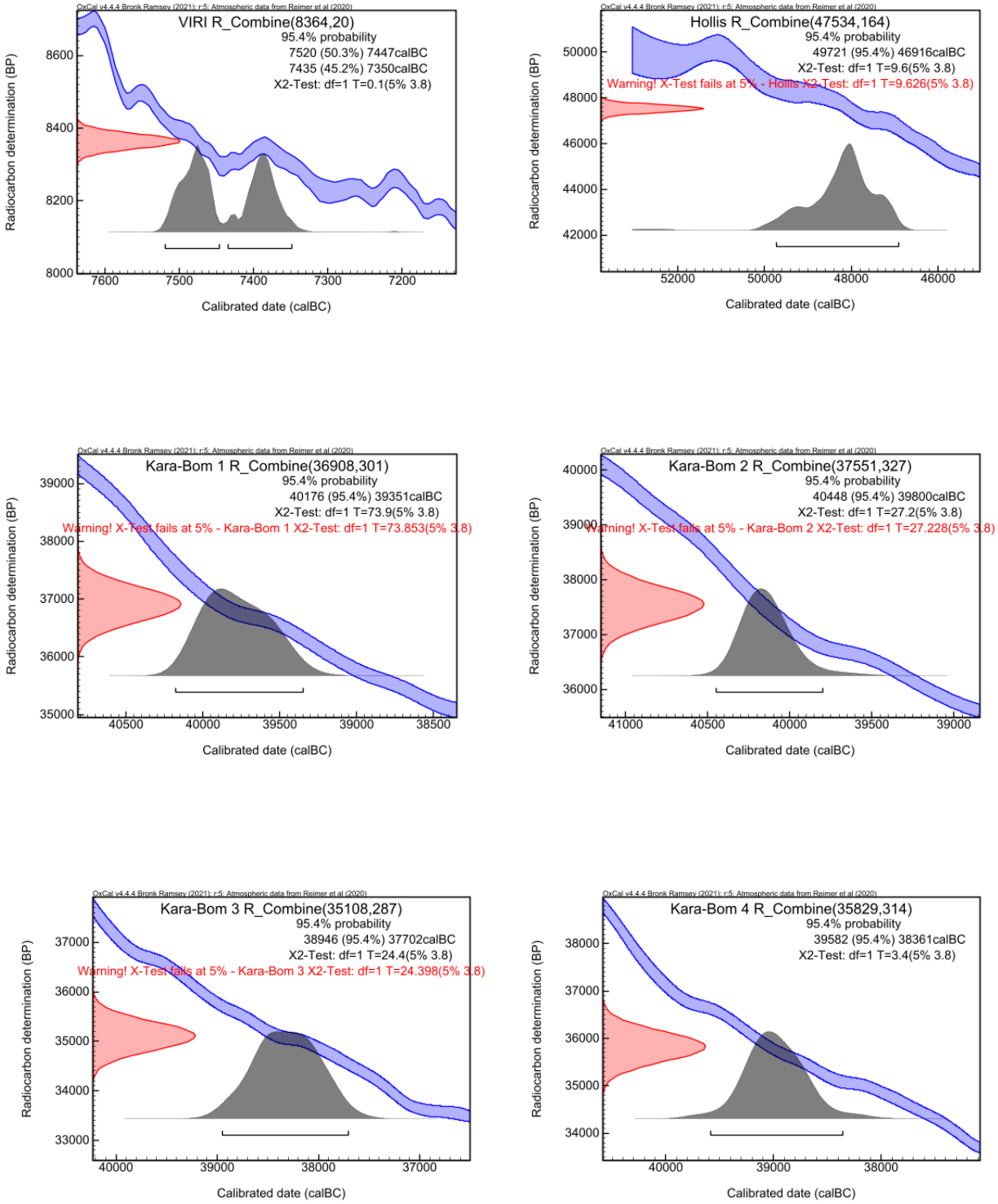
Table 13
Results of the independent samples t-test.

Independent sample t-test										
		Levene's test for equality of variances		T-test for mean equality						
									95% confidence interval of the difference	
		F	Significance	T	df	Sig. (2-sided)	Mean difference	Standard error of the difference	Lower	Upper
Collagen yield	Variances are equal	0,626	0,447	1,946	10	0,08	0,63543	0,32647	-0,09199	1,36284
	Variances are not equal			2,066	9,99	0,066	0,63543	0,30754	-0,04991	1,32076

As described above, some of the ^{14}C dates of archeological samples differ from each other. The given T-value 3,8 corresponds to the 5% significance. All dates with higher T-values are statistically different (Table 14, Fig. 11). There are two exceptions, where the dates are in good statistical agreement. First is the VIRI sample. Second; the youngest Kara-Bom destructive result is in agreement with the oldest MAE result. More work is needed to understand why there is this variation.

Figure 11

OxCal plots showing differences in radiocarbon dates of the archeological samples. ^{14}C ages extracted by MAE and by routine pretreatment were compared.



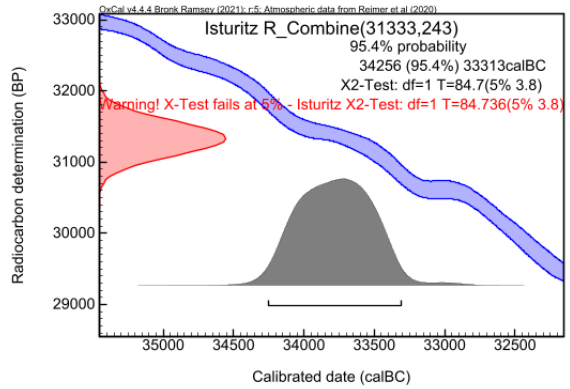


Table 14

OxCal table shows statistical results of radiocarbon dates of the archeological samples.

^{14}C ages extracted by MAE and by routine pretreatment were compared.

Name	Unmodelled (BC/AD)				
	from_68.3	to_68.3	from_95.4	to_95.4	*T value (Chi2=3.8)
Isturitz	-34050	-33510	-34260	-33310	**84,7**
VIRI	-7510	-7370	-7520	-7340	0,1
Kara-Bom 4	-39270	-38720	-39590	-38360	3,4
Kara-Bom 3	-38640	-37970	-38950	-37700	**24,4**
Kara-Bom 2	-40320	-40010	-40450	-39800	**27,2**
Kara-Bom 1	-40040	-39570	-40180	-39350	**73,8**
Hollis	-48670	-47230	-49730	-46910	**9,6**

*T-value 3,8 corresponds to the 5% significance

6. Discussion

In this thesis, I have presented a new method for collagen extraction with the aim of subsequently radiocarbon dating the extracted collagenous material. We were able to successfully extract collagen from archeological bones with the assistance of microwave method. The major benefit of this approach is a reduction in the pretreatment and extraction time by several hours. We obtained enough collagen for ^{14}C dating within one hour. Compared with the routine bone pretreatment and the non-destructive hot water extraction, MAE is the most efficient extraction process (see sections 1.2. and 1.3.).

We could not demonstrate the accuracy of ^{14}C ages by comparison with the dates obtained by routine pretreatment methods. The only date in the good statistical agreement was the one coming from VIRI the whale bone. The MAE age of Isturitz sample appear to be ~3000-5000 years younger. The same applies for the Kara-Bom sample, although the youngest destructive date overlapped statistically with the oldest MAE extracted result. One reason for this could be insufficient removal of contaminants from the bone fragment which came with the MAE treated soluble fraction, since we were not using any acids or alkaline solutions for cleaning of the material. To avoid this problem in the future, as a possible solution we suggest perhaps applying XAD treatments to the extracted collagen. As expected, stable isotopes analysis did not detect any significant contamination and all values were within the normal range[17].

It is crucial that the most optimal parameters are investigated if one is to make effective use of MAE. These parameters include glassware material, heating ramp times, hold times, pressure and temperature. One of the most important factors for accomplishing successful results was the discovery of suitable tubes. We found that it was important for the tubes to be closed tightly; otherwise water inside of them boiled out and mixed with the outside water. This caused the loss of collagen and has obvious implications for contamination effects. It was the major complication during the development of this method. We explored many setting options in the microwave unit, to be able to use open tubes or ones that were covered without sealing. This was unsuccessful and tightened lids on XAD tubes had to be applied. One could ask why we did not use the XAD tubes at the beginning. XAD tubes have specific form and size (Fig. 12). Their upper part is very narrow, so it is complicated to pull out the sample out of them and could result in crushing of the wet bone. Therefore, we

tried to avoid this. However, this limits the microwave practical usage. We suggest a bespoke quartz tube with a screw top would be the way forward.

Figure 12

"XAD tubes" with screw caps used for MAE in the Higham-Lab.

Flinn Scientific. (2026). *Test Tubes with Screw Caps, 20 x 150 mm, 30 mL*. [Online image]. https://www.flinnsci.com/test-tubes-with-screw-caps-20-x-150-mm-30-ml/gp9158/?srsltid=AfmBOoo5kl4rMdZBx6R-rfUCFOFoBUnxcT4Tk-d1D5EhQoekjdIQ0Khb

Due to relatively small and narrow microwave containers, only collagen from small bone fragments or cut pieces could be used in the extraction process, as mentioned above. Using bigger vessels would be beneficial for larger samples or even for the whole bones or artifacts. This would mean a new dual non-destructive and timesaving approach. Traditionally, the treatment of samples for radiocarbon dating has been highly destructive. Bone fragments are often destroyed due to crushing and the subsequent chemical treatment[19]. Therefore, the MAE represents an important intermediate step in future research.

For radiocarbon dating, only around 2,5 mg collagen is required. However, the starting quantity of bone has to be higher because collagen is often present in low amounts (down to ~0.5-1.0% weight). Due to additional purification of the sample after the pretreatment (such as ultraftiltration), a certain amount of collagen is always lost. It means we have to concentrate on achieving of higher yields. Mainly in bad preserved samples it could be a problem to extract sufficient collagen amount. In our case, one of the solutions was

performing a multiple extraction. It includes two or three separate rounds of experiments using the same sample with an ever-new solvent. The sample was first extracted in the MilliQ^{TM} water. The water with soluble collagen was stored. The same sample was extracted again in fresh new water. Compared to a single extraction, the advantage of the multiple extractions lies in enlarging of the extractive power of the solvent[41]. This is due to using more portions of the solvent during the process. In future research, it could be beneficial to perform three or four rounds of the extraction to receive higher collagen yields. However, it would be questionable, if it then fulfills the main intent of the original idea. That is, to achieve more effective and quicker extraction than with conventional methods.

This leads to another important factor that ensures higher collagen yields in shorter timeframe, mainly the temperature. For a chemical reaction to take place, collision of the reactant molecules is needed. Such interaction is possible because of the kinetic energy of the molecules. If we increase the temperature, the average kinetic energy raises, therefore the chemical reaction will be accelerated[42]. We performed our final experiment with archeological samples at 130°C. This temperature turned out as the most optimal setting. It distinguishes MAE from the hot water extraction, where was the extraction done at only 90°C, so its duration was significantly longer[12]. Zhang et al. (2023) performed their collagen extraction from bovine hide for 8 hours, but at very low temperature of 35 °C[7]. In contrast, we raised the temperature significantly to ensure lower extraction time. This is the main difference between my research and other papers on this topic[10,11,12].

The simple linear regression showed a significant effect of the temperature on collagen yields. However, we can explain only 42,5% of the collagen yields with the temperature (R^2 = 0,425). This low value indicates that the yields are not explainable only by the temperature, and there are other influence variables too. One of them could be the type of tube used. Not every extraction was performed in the XAD tubes, and water boiling out influences low yields. Nevertheless, the effect of the temperature on collagen yields was confirmed with the independent samples t-test. Overall, it is not very favorable to perform a statistical test with such a limited range of data. We have undertaken a few yields at 30, 60, 90 or 120 minutes. At the same time, these were extracted in two types of tubes which significantly influenced the collagen yield. In each group, only small amounts of values are present. It is not possible to use data from the extraction done on archeological samples, since the yields

there were very small due to degraded collagen, and not because of the applied temperature. This would bias the statistical result. Together with the large variation of affecting factors it makes a complicated situation for a statistical testing. For these reasons, we chose to perform only a test on the experimental samples extracted for 30 minutes.

We did not try any experiments at higher temperatures, so shifting temperature at 140 °C (or higher) would be valuable to test. Potentially, it could bring higher yields in a shorter time or at least in the same time period as in our experiments. However, in this case tests for collagen preservation would be necessary, since we do not have any research papers talking about collagen undergoing such high temperature during its extraction. We would suggest applying so-called collagen preservation assessment methods. They include analysis of C:N atomic ratio or the percentage amount of carbon and nitrogen in the sample[43]. Other prescreening methods for collagen preservation, for example, FTIR spectroscopy[44] or the non-destructive NIR spectroscopy[45], would also be useful to apply before treatment. Analysis of amino acid composition by a high-performance liquid chromatography (HPLC) could be useful too[48]. These methods could point to collagen alterations. Additionally, we are moving near the temperature limit of DNA degradation. It would be beneficial to examine DNA before and after microwaving samples at 130 °C, whether the temperature causes any damage to the DNA. As I already mentioned in the section 1.4., the used and also the suggested temperature by us is in a sensitive range for DNA damageability, since several sources refer to 130°C as the start point of the DNA degradation[25,26].

In this thesis, I also encountered poorly preserved samples. R00600.2 and R00615.2, both coming from Kiowa, showed significantly lower yields after MAE compared to the routine pretreatment method. The yields may be affected by the fact that they come from a humid and warm environment; Papua New Guinea. The tropical environment negatively influences collagen preservation, because it may lead to the gelatinization of the collagen. This means a break of the bonds in the protein structure and thus contributing to microbial activity with subsequent degradation[46]. So collagen in those samples may have degraded over a short period of time and become denatured. One hypothesis is that it is not possible to extract it anymore, and therefore the extraction method is not relevant. The samples are already at the limit of being dateable. Another factor, such as alkali soil residues on the bone, could

have also played a role. It might result in collagen alteration and stabilization of the bioapatite, which results in more stable hydroxyapatite[46].

The Zaragizh sample (R00670.5) has also much lower yield compared to the destructively extracted sample. Unlike Kiowa samples, Zaragizh comes from colder parts of Russia. One reason for the low yield could be the good preservation state of the inorganic hydroxyapatite. We cannot exclude the possibility that this also happened because of the alkali pH in the soil, as already described above. In such case, it is difficult to get intact collagen from the bone only with hot water. Since we are not using any acid during MAE as by routine pretreatment, it does not come to demineralization of the inorganic part of the bone, which makes achieving of higher collagen yields more problematic, since the collagen is not easily accessible.

Whether the MAE collagen amount is sufficient, depends strongly on its intended use. Ideally, purification of the extracted collagen afterwards is essential. Methods such as ultrafiltration or XAD2 resin chromatography are necessary. It is crucial to remove contaminants regardless of the extraction method[8,19,21]. However, in poor preserved samples, where only small amount of collagen is possible to obtain, it represents a problem. During ultrafiltration, a certain amount of collagen is always lost. Smaller yields compared to the destructive method sometimes mean that additional ultrafiltration is not possible. Using bigger samples presents one of the possible solutions, since our samples were always around 200 milligrams. Normally, 500 mg is used by routine pretreatment. This could ensure higher yields by MAE.

Nevertheless, even small yields are meaningful for researchers. We were able to extract collagen from denatured Kiowa samples. After that, we took the already microwaved bone pieces, crushed them and microwaved them again. We obtained more collagen in all cases, then when they were uncrushed. This indicates that it is also possible to extract collagen from poor preserved samples while using MAE. We can hypothesize, if the samples were crushed already at the beginning in the first extraction, they could give significantly larger collagen yields.

The major limitation in my experimental work was the temperature fluctuation of the microwave system. The microwave was not able to reach the set temperature in some of

the vessels. For instance, instead of 130°C, some of the containers reached only 115°C. Unfortunately, this cannot be avoided for technical reasons. However, this fact can be a major complication in the collagen extraction from rare samples, if it cannot be performed repeatedly. In my thesis, I demonstrated that the ideal temperature for a rapid and efficient extraction is 130°C. A lower temperature in a combination with the same time period would mean lower yields. However, it is necessary to say that the temperature can be controlled because of the digital display connected to the microwave, so one remains informed about the real extraction temperature and can thus plan the next research steps.

7. Conclusions

- It remains undisputed we can extract collagen from archeological bones with the microwave assistance much quicker and thus effectively, and replace a 3 day long extraction with only a one hour long process.
- Stable isotope analysis showed MAE as reliable and unaffected by contamination with exogenous carbon, when proper cleaning techniques such as ultrafiltration are applied.
- Radiocarbon ages coming from MAE were younger than they should in some instances. Further purification such as XAD might be necessary.
- Archeological collagen extracted at 130°C is well-preserved and can be used for other purposes.
- Using only ultrapure water and no acids or alkali solutions presents a green extraction method.

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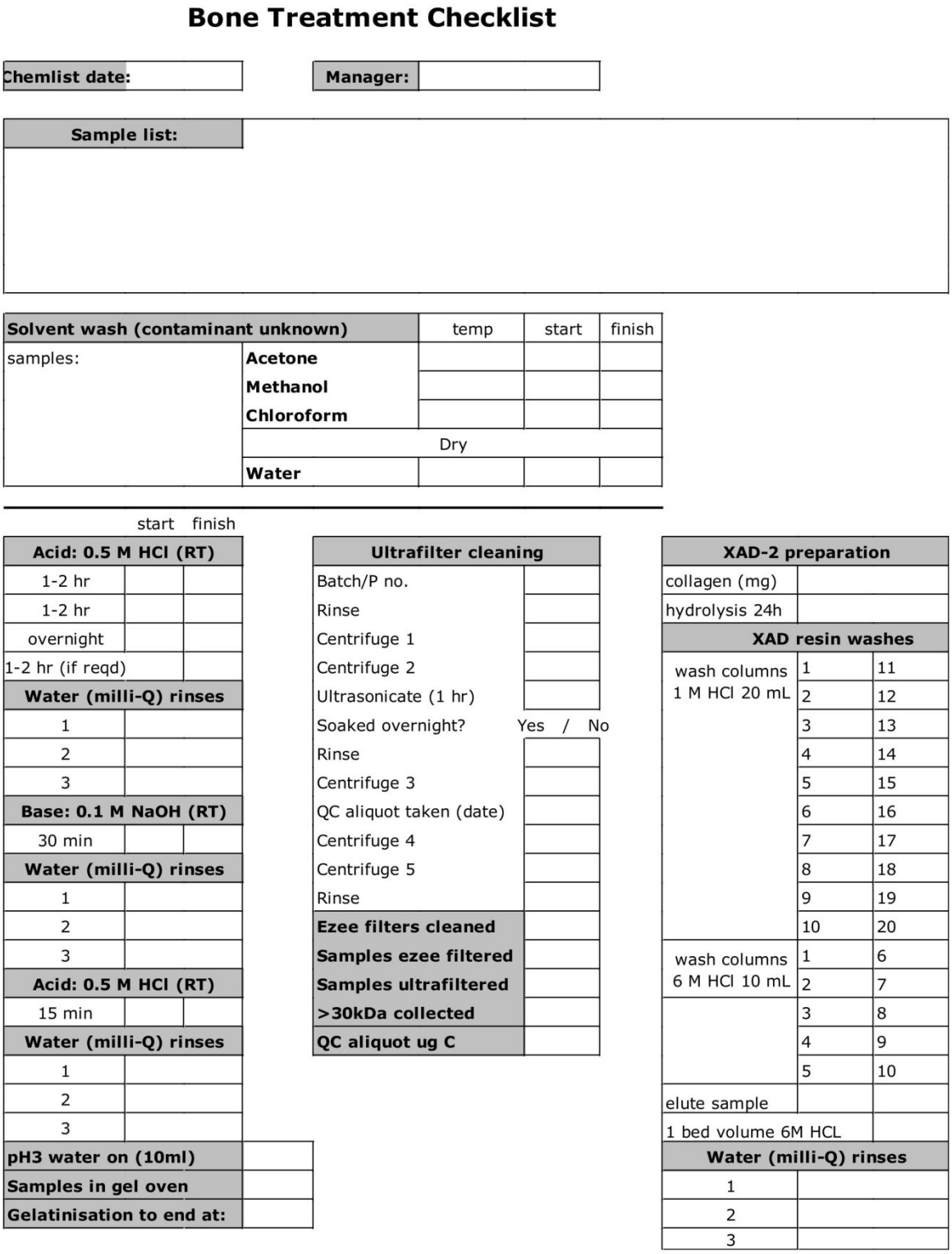
10. Figures

Figure 1

Bone pretreatment protocol used in the Higham-lab.



Bone Treatment Checklist

Chemlist date:		Manager:	

Sample list:	

Solvent wash (contaminant unknown)		temp	start	finish
samples:	**Acetone**			
	Methanol			
	Chloroform			
	Dry			
	Water			

	start	finish
Acid: 0.5 M HCl (RT)		
1-2 hr		
1-2 hr		
overnight		
1-2 hr (if reqd)		
Water (milli-Q) rinses		
1		
2		
3		
Base: 0.1 M NaOH (RT)		
30 min		
Water (milli-Q) rinses		
1		
2		
3		
Acid: 0.5 M HCl (RT)		
15 min		
Water (milli-Q) rinses		
1		
2		
3		
pH3 water on (10ml)		
Samples in gel oven		
Gelatinisation to end at:		

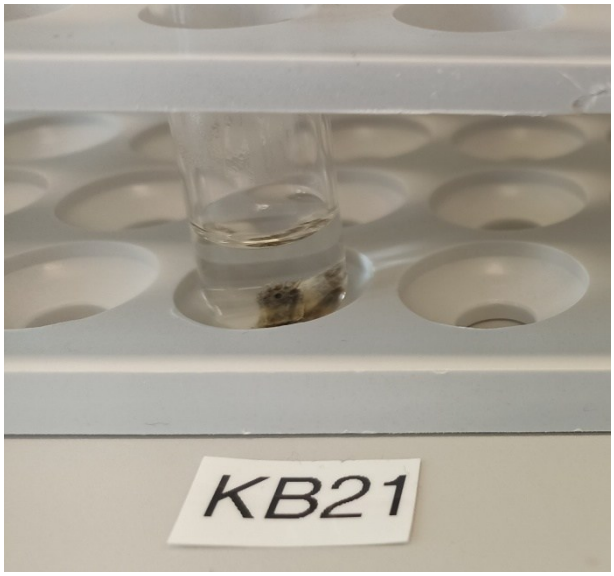
Ultrafilter cleaning	
Batch/P no.	
Rinse	
Centrifuge 1	
Centrifuge 2	
Ultrasonicate (1 hr)	
Soaked overnight? Yes / No	
Rinse	
Centrifuge 3	
QC aliquot taken (date)	
Centrifuge 4	
Centrifuge 5	
Rinse	
Ezee filters cleaned	
Samples ezee filtered	
Samples ultrafiltered	
>30kDa collected	
QC aliquot ug C	

XAD-2 preparation		
collagen (mg)		
hydrolysis 24h		
XAD resin washes		
wash columns 1 M HCl 20 mL	1	11
	2	12
	3	13
	4	14
	5	15
	6	16
	7	17
	8	18
	9	19
	10	20
wash columns 6 M HCl 10 mL	1	6
	2	7
	3	8
	4	9
	5	10
elute sample		
1 bed volume 6M HCL		
Water (milli-Q) rinses		
1		
2		
3		

Source: Higham-lab.

Figure 4

Sample in MilliQ water after the extraction.



Source: own photography.

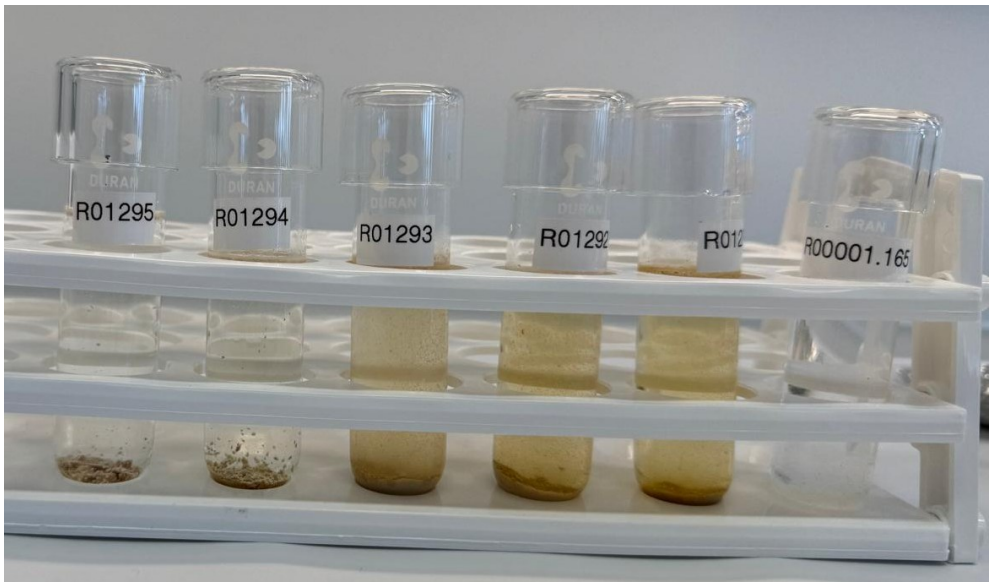
Figure 5

Crushing: preparation of samples before extraction.

Source: own photography.

Figure 6

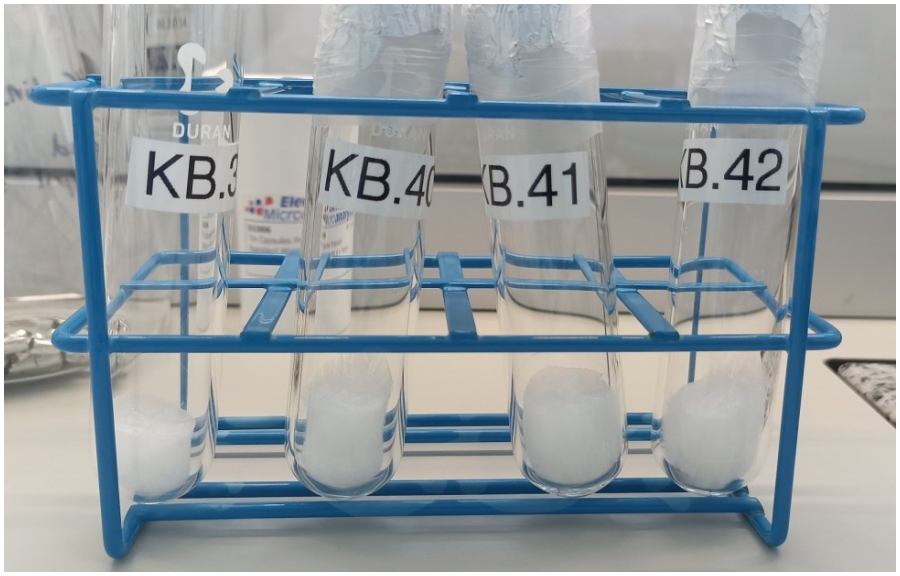
Samples in acid during ABA extraction.



Source: own photography.

Figure 7

Extracted and dried collagen.



Source: own photography.

11. Supplementary material

Experiment 1

The first experiment is documented in the Table S15. Small pieces from the Kara-Bom bone with the weight of 94-194 mg were cut. I used so called "Ezee filter" borosilicate tubes. They are conventionally used in the Higham-Lab for Ezee filtering of the samples and they do not contaminate the samples with modern carbon. Four rounds were performed, each of them with one vessel with a sample and three vessels with blanks. Blanks consist of MilliQ™ water. This ensures the same ion ratio in every vessel, so the microwave heating is not influenced by them and remains same in all vessels. The chosen four containers were distributed symmetrically in the microwave. Each round was 30 minutes long. The ramp time was set at 5-7 min and the cooling break at 10 minutes. The temperature was increased gradually in each round. It was 90 °C in the first one, 110 in second and third round, and 130 in the fourth. It has to be noted, in the third round was the temperature set on 120 °C, but the microwave did not reach that point. First series of tests was successful and we managed to extract collagen from the archeological bone by microwave assistance. However, the yields were not sufficient for further collagen processing, for example for future ultrafiltration. A one can see higher yields corresponding to rising temperature.

Table S15
Results of the first experimental microwave-assisted collagen extraction done for 30 minutes. Sawn fragments of the Kara-Bom bone were used.

Sample	Weight of the sample (mg)	Temperature (°C)	Power (%)	Collagen yield (mg)	Collagen yield (%)
KB.7	107	90	50	2,03	1,89
KB.8	194	110	60	4,74	2,44
KB.9	146	110	60	3,65	2,50
KB.10	94	130	60	4,02	4,27

Experiment 2

In this experiment, two major temperature values of our interest were tested. The first one is 90°C, because the same temperature was used by the hot water collagen extraction[12]. Comparing the results of these two methods could be significant. The second one, 130 °C, was suggested by us as a maximal extraction temperature for this testing. Again, Ezee filter tubes without any lids were used. The experiment was conducted with four vessels, in this

scenario with two samples and two blanks. It was performed for 60, 90 and 120 minutes with samples of 137-203 mg. However, in this testing cycle temperature fluctuations occurred very often. In Table S16 and Table S17 are summarized all unwanted temperature changes and details about the experiment and its results. Temperature was often 10 degrees lower than set. But, as we are working on the lower limit of temperature control, the fluctuations of 10-15°C cannot be avoided due to technical issues of the microwave unit. Even bigger problem occurred while testing on 130°C. We could see low amount of water in the tubes in the end of the test. The amount was half as less as at the beginning. Rest of the water was in the vessel. It is not clear if we can talk about evaporation of the water or about boiling out. In two samples (KB.19 and KB.20) was no water left in the tubes anymore. It is interesting that there was no problem in experiment for 60 and 120 minutes, but there was no water left after 90 minutes. Due to this complication, I tested the water directly in the vessel for the collagen presence. This would bring evidence about water boiling out. Another theory is that it evaporated and the condensation got out from the tube. After freeze-drying the samples, we found out very small amount of collagen in the outside water. This cannot explain with certainty if we should speak here about evaporation or boiling over. However, the extracted collagen had adequate dense structure. It was similar to the ones extracted by the routine extraction method. The yields were the highest with the temperature of 130°C.

Table S16
Results of the second experiment done at 90°C. Unexpected temperature fluctuations are noted.

Sample	Weight of the sample (mg)	Measured temperature (°C)	Time (min)	Collagen yield (mg)	Collagen yield (%)
KB. 11	147	90	60	3,45	2,35
KB. 12	149	90, gradually declining to 70	60	2,96	1,99
KB. 13	137	90; 90	60+30	4,28	3,12
KB. 14	180	80-85; ~80	60+30	4,04	2,24
KB. 15	169	90; 70-60	60+60	3,64	2,15
KB. 16	171	90; 90	60+60	5,58	3,26

Table
S17
Results of the second experiment done at 130°C. Unexpected temperature fluctuations are noted.

Sample	Weight of the sample (mg)	Measured temperature (°C)	Time (min)	Collagen yield (mg)	Collagen yield (%)
KB. 17	203	130, gradually declining to 105	60	7,03	3,46
KB. 18	176	~120-115	60	7,08	4,02
KB. 19	150	~110; 130	30+60	0,12	0,08
KB. 20	188	113-102; 130	30+60	0,08	0,04
KB. 21	154	130; 130	60+60	6,62	4,30
KB. 22	164	110; 100	60+60	6,95	4,24

Experiment 3

Due to the evaporation problem and the ongoing requirement for a higher temperature (130°C), tests without any sample were done for optimization of the microwave settings. Another persistent complication was the temperature fluctuation, when the set temperature was often 10-15° lower. All the mentioned tests were done at 90°C. As a first possible solution we added more containers. Altogether 16 vessels were used. Second, we changed the ultrapure MilliQ^{TM} water in the outside area of the container for a tap water, because temperature also depends on the polarity of the water and its ionic concentration. As previously, we used borosilicate tubes 5 ml of MilliQ^{TM} water inside and 15 ml of tap water outside. We increases ramp time to 10 min and lowered the power on 35%. The temperature 90 °C turned out to be accurate. There was no visible evaporation. We assumed that borosilicate absorbs microwave radiation, so this might explain why water evaporates, because borosilicate is not transparent so the sensor is blind. Quartz tubes might be necessary to change this.

Experiment 4

In this experiment 16 vessels were used; 2 with samples and 14 with blanks. This should ensure more balanced layout of the vessels, so more constant temperature of the vessels should be guaranteed. As in previous experiments, fragments sawn from Kara-Bom bone were used. Every tube had a glass lid on. There was 5 ml MilliQ^{TM} inside the glass tube, 15 ml MilliQ^{TM} outside in the vessel. Ramp time was set on 10 minutes, cooling time on 7 minutes. Temperature was set at 130°C. The actual temperature of the vessels, where our samples were placed, was during this extraction always around 120 or 100 °C. The results are summarized in the Table S18. We can speculate that the real temperature inside the tube could be actually around 130°C, because water boiling over could happen only if the temperature inside of the tube is higher than outside of the tube. However, this remains a speculation and we cannot claim with certainty that the extraction was done on 130°C. It seems like samples with bones have more temperature fluctuations. Interestingly, future experiments did not show those fluctuations in vessels with the sample, so the reason remains unclear for us. We concluded that temperature deviations from 10 to 15°C cannot be avoided. Slower heating and reduced power did not help to prevent the evaporation. We observed lower amount of water in all blanks and in our samples; in average there was around 3 ml from the original 5 ml left. We also hypothesized the reason for the evaporation could be leakage on top of the vessel. It turned out to be a false assumption. There were many vessels with large temperature changes, but they were well-tightened. Nevertheless, it was still possible for us to extract the collagen in a microwave at the lower temperature.

Table S18
Results of the fourth experiment performed at 130°C. Temperature changes are noted.

Sample	Weight of the sample (mg)	Measured temperature (°C)	Time (min)	Collagen yield (mg)	Collagen yield (%)
KB.23	167	122-105	30	4,19	2,51
KB.24	173	120	30	5,10	2,95
KB.25	171	126-96	60	3,62	2,12
KB.26	193	120	60	5,73	2,97
KB.27	175	120-93	90	6,14	3,51
KB.28	176	120	90	6,87	3,90
KB.29	133	122-100	120	4,25	3,19
KB.30	214	125-100	120	8,55	4,00

Experiment 5

For the next experiment, we suggested to add more water. We used 10 ml MilliQ^{TM} inside the borosilicate tubes with glass lids and 15 ml MilliQ^{TM} outside. We loaded all 16 vessels: 2 with samples, 14 blanks. Ramp time was set for 10 minutes, cooling for 10 minutes and temperature at 130 °C. However, increased water amount in the tubes did not help. More water than in previous experiment disappeared from the tubes. In this case, duration of the extraction does not matter; the lost amount of water was always around 5 ml. While opening the vessels, the clinking glass was heard. The reason for this could be pushing of the glass lid up because of the pressure during the extraction and then water could come out easily. Outside water was taken out to test collagen presence, which was also confirmed (Table S19).

Table S19
Results of the fifth experiments done at 130°C. Changes of the temperature and amount of the lost collagen due to water boiling out in the vessels are noted.

Sample	Weight of the sample (mg)	Measured temperature (°C)	Time (min)	Lost collagen (mg/%)	Collagen yield (mg)	Collagen yield (%)
KB. 31	159	130	30	x	5,20	3,27
KB. 32	250	110	30	x	7,68	3,07
KB. 33	185	125	60	0,31/0,17	7,77	4,20
KB. 34	194	110	60	x	4,07	2,09
KB. 35	150	120	90	x	4,49	2,99
KB. 36	220	120	90	0,13/0,06	8,18	3,72
KB. 37	187	130	120	0,31/0,17	8,22	4,40
KB. 38	169	110	120	0,01/0,006	7,97	4,72

Experiment 6

We tested the so-called XAD borosilicate tubes with screw cups, because the opened tubes, where the lids were only laid, did not meet our requirements for safe and successful extraction. I filled them with 5 ml of MilliQ^{TM} water and I put 15 ml of MilliQ^{TM} outside into the vessels. The test was done without samples in three rounds for 30 minutes, at 110°C, 120°C and 130°C. Our main intention was to test, if the closed tube with a screw cup can handle the pressure during the microwave session at the given temperature. If so, further collagen extraction could proceed with bone samples. However, the results were satisfactory. There was no damage on the XAD tubes after 30 min on 110°C. The caps were

preserved untouched without any traces of looseness. After 120°C were some caps tilted vertically, indicating possible weak pressure opening. After 130°C on 30 min we observed the same situation. We assume from our experiment that closed XAD tubes are suitable and reliable for our tests. The temperature was stable in all three rounds. There were no significant deviations from the set 130°C.

Additionally, we tested the quartz tubes covered with borosilicate glass lids, but these were not screwable. The experiment was done at 130°C for 60 minutes. We intend to test, if the borosilicate material of the tubes was responsible for the water boiling over. We expected water would stay in the tubes, because quartz has better thermal conductivity then the borosilicate. Interestingly, water was boiling out the same way as in previous experiments. From the original 5 ml of water only 3-4 ml was left. Another unexpected event was worsen temperature fluctuation. In 12 of 16 vessels the temperature did not reach the set 130°C.