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Comparison of common maceration techniques to prepare porcine bone for fluorescence analysis using alternative light sources (ALS)

Catherine Maidment [a,b,*], Anna Williams [b]

[a] School of Applied Sciences, University of Huddersfield, Huddersfield, HD1 3DH, UK
[b] School of Natural Sciences, University of Central Lancashire, Preston PR1 2HE, UK

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ABSTRACT

Objectives: Investigating the impact of three common maceration techniques on the collagen content and autofluorescence of porcine bone, to ascertain the most suitable preparation method for bone undergoing ALS analysis.
Materials and methods: Hot water (80°C), biological washing powder (55°C), and enzymatic (55°C) maceration were used to prepare thirty porcine ribs (*Sus scrofa domesticus*) (n=10). Ribs were photographed before and after maceration using blue light (Crime-Lite 2, 450nm), coupled with an orange camera filter. Thermogravimetric analysis was used to quantify collagen content, and a bespoke computer program: The Osteo-Fluorescence Calculator (OFC) was used to quantify bone fluorescence.
Results: Ribs macerated in hot water exhibited homogenous fluorescence and produced a 5.5% average increase in fluorescence levels (n=10, s.d.=9.36, p=0.012) alongside a 11.2% loss in collagen content (n=10, s.d.=0.09, p=0.023). Biological washing powder was destructive to bone surfaces and produced an average collagen loss of 22.9% (n=10, s.d.=0.05, p= <0.001), while fluorescence was augmented (54.49%) and inconsistent (n=10, s.d.=27.46, p=0.180). Enzymatic maceration produced an average increase in fluorescence of 23.2% (n=10, s.d.=23.72, p=0.180), with a mostly consistent appearance except for some dark patches, and experienced a 19.5% loss in collagen content (n=10, s.d.=0.09, p=0.001).
Conclusions: Hot water maceration produced fluorescence results comparable to fresh bone with little impact on bone collagen and provides a suitable preparation technique for osseous ALS examination. Biological washing powder was destructive to bone collagen and produced exaggerated, inconsistent fluorescence and therefore should be avoided. Enzymatic maceration was the fastest method but requires an optimised formulation.

Introduction

Alternative light sources (ALS) are a staple of crime scene investigation, harnessing the properties of autofluorescence to reveal latent biological evidence such as semen and saliva [1,2], with great success demonstrated particularly at shorter wavelengths (approx. 400-500nm) [3]. Autofluorescence is generated through molecular excitation of a substrate through exposure to selected wavelengths of light [4] resulting in the rapid emission of longer wavelengths [2,5,6]. When observed through coloured camera filters or eyewear this fluorescence naturally enhances the contrast between the subject and its background, making latent evidence more visible [7], allowing for easy observation and documentation [8]. This process is non-destructive, non-contact, and produces an immediate response with no permanent effects as the fluorescence disperses once the light source is removed [6,8]. This preserves the integrity and structure of the evidence [1]; both of which are integral for effective crime scene analysis and anthropological assessment [9,10].

The taphonomic environment can permanently alter the morphological characteristics of bone, through weathering, scavenging, and fragmentation [11]. These changes make identification and differentiation challenging [12], especially for police investigators who may not be trained in osteology. Overlooking skeletal elements reduces the volume of potential forensic evidence but can also jeopardise victim identification, by limiting the data available for constructing an osteological profile [13]. Stimulating bone fluorescence could make bone more visible and provide a means of preliminary identification. It is theorised that the proteins within biological material are ultimately responsible

* Corresponding author at: School of Natural Sciences, University of Central Lancashire, Preston PR1 2HE, UK.
E-mail addresses: camaidment@uclan.ac.uk (C. Maidment), awilliams34@uclan.ac.uk (A. Williams).

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for the production of fluorescence [14], and as protein constitutes 30-35% of bone volume [15–17], of which approximately 90% is collagen [18–20], ALS offers a potential new approach for the detection of bone at the scene [21–23] and in the laboratory [24–26]

Many of the ALS bone fluorescence studies in current literature focus on burned bone and how thermal destruction of the proteinaceous organic content leads to a decrease in fluorescence [20,27,28]. However, the natural fluorescence of unaltered bone in forensic contexts has rarely been discussed. Brown and Christensen (2018) [21] successfully demonstrated how ALS could be used to locate surface deposited bone, but used samples that had been previously prepared using warm water maceration. Other studies exploring method development including optimal wavelength and filter combination [23,25], distance from ALS to specimen [24], and influence of time and the deposition environment [29] provide valuable insight into how collagen can denature over time, and how blue light (approximately 450nm) coupled with an orange or yellow barrier filter is the most effective for visualising bone fluorescence [24,25,29]. However, these studies also used de-fleshed bone, prepared using a variety of techniques, including being dried, heat treated, boiled, and macerated with warm water [24,25,29], but do not include technical specifications such as temperature or duration, or discuss whether the preparation method may have influenced the fluorescence results seen.

Maceration is a common procedure utilised in forensic anthropology to prepare skeletal remains for examination, by removing residual soft tissue and exposing the key morphological landmarks and skeletal structures required to provide an osteological profile and assist investigators with identification [30,31]. Maceration techniques can be categorised into six types based on the mechanism of tissue removal including: mechanical, bacterial, chemical, enzymatic, invertebrate, and cooking [32], as many use the application of heat, chemicals, or insects [33–35], to remove the soft tissue, leaving clean, dry bone for skeletal analysis and subsequent storage [36]. Methodologies are often born from inherited institutional practice or specific casework experience [33,37], and so parameters vary greatly between institutions and across literature [38].

Aqueous techniques are particularly common, with submersion in hot or boiling water and biological washing powder being the most popular [39–41]. These methods are cheap and easy to carry out, and biological washing powder contains high concentrations of enzymes and cleaning ingredients, making it very effective at removing tissue, grease, and any discolouration from bone [33,37,42,43], with the accompanying heat expediting results [39]. However, literature suggests that this powerful combination can compromise the cortical structure of bone, making it brittle, leading to splitting and fraying [32,39,41,44]. Historically, it was thought that the highly reactive hydroxyapatite [45] and the more vulnerable organic content of bone could become altered irreversibly when exposed to high temperatures [35,40]. Gent et al (2023) found that prolonged high temperature maceration with liquid detergent (a methodology favoured by human taphonomy facilities) up to 87 °C, was highly damaging to the biomolecular integrity of bovine bones and diminished the opportunity for proteomic investigations [46]. However, other studies, primarily investigating the preservation of DNA during maceration, have hypothesised that destructive thermal alterations to bone may not occur until temperatures reach over 100°C [36, 47], and specifically that shorter durations of heat exposure may not be as detrimental as first suggested [41]. However continuous exposure to extreme heat or 'overcooking' should be avoided [30,48,49]. This is illustrated by the success seen by Steadman et al (2006) and King and Birch (2015) using microwaving maceration, where bursts of heat were used to macerate porcine ribs [39]. Similarly, targeted chemical maceration using chosen enzymes in controlled quantities is cited as a faster and gentler maceration approach [33,41,44], reportedly retaining the bone structure and intricate morphological features [50]. The inclusion of targeted enzymes such as protease to break down proteins and lipase to dissolve lipids eliminates the need for inclusion of destructive additives, such as bleaching agents, therefore limiting corrosion to cortical bone and the bone matrix [39,44].

Currently, there are no studies directly evaluating the impact of maceration on bone fluorescence produced via ALS analysis. Consequently, it is difficult to interpret whether currently published results represent the natural fluorescence of bone or are the product of the maceration process. This lack of data potentially undermines current and future understandings of ALS bone fluorescence. To address this deficit, here we examine the effect of three of the most popular maceration techniques on bone fluorescence; hot water maceration at 80°C [30,34,39], warm water at 50°C with biological washing powder [37,39, 41], and enzymatic maceration using a lipase and protease solution at 55°C [41,44]. Photography was used to record the bone fluorescence, which was subsequently quantified using bespoke C++ computer software, the Osteo-Fluorescence Calculator (OFC) designed specifically for this research. Quantification of collagen was conducted using thermogravimetric analysis with subsequent statistical analyses, focusing on evaluating the impact of maceration on bone fluorescence and collagen preservation. All three methods were considered simple and required limited space and equipment.

Materials and methods

Materials

Thirty porcine ribs (*Sus scrofa domesticus*) suitable for human consumption were obtained fresh from a local butcher, and frozen at -18°C until use. The ribs were purchased as individual bones rather than in racks to make sure that the bones did not originate from the same pig, therefore ensuring population variability within the dataset. This research project was granted ethical approval by the Animal Welfare and Ethics Review Body (AWERB), reference number: RE/21/06, and received Research Programme Approval (RPA). All porcine bones purchased for this project were originally destined for human consumption and therefore were not killed specifically for this project.

Sample preparation

Ten ribs were allocated to each maceration method. Prior to experimentation, they were defrosted overnight in the refrigerator (2°C), and then de-fleshed manually using a PM40 scalpel, to remove excess soft tissue, eliminating the need to replenish the solutions during maceration. All ribs were weighed and then photographed in natural light prior to maceration.

Maceration

The porcine ribs were macerated in groups of five to ensure complete exposure to the maceration solution and to prevent overcrowding within the containers.

1 Hot water maceration: Two batches of five ribs were heated in 1L of tap water using a Morphy Richards 48709 stainless steel 3.5L slow cooker set to high (Fig. 1). The water was brought to temperature of 80°C, measured using a digital thermometer, before the ribs were added.
2 Biological washing powder and warm water maceration: Two batches of five ribs were macerated in a 6.2L stainless-steel stockpot (Fig. 2) containing 50g of Surf Tropical Lily & Ylang Ylang biological washing powder™ in 1L of tap water heated to 50°C using a Fisherbrand™ AREX Stirring Hotplate. Once the water reached 50°C (measured using a digital thermometer), the washing powder was added, stirred to ensure even distribution, and then the ribs added.
3 Enzyme maceration: Two batches of five ribs were macerated in a solution comprising of 20ml Novozymes Savinase® liquid protease and 20ml Novozymes Lipex® liquid lipase in 1L of tap water heated

Fig. 1. Slow cooker used for hot water maceration (80°C).

Fig. 2. Stockpot used for washing powder (50°C), and enzymatic maceration (55°C).

in a 6.2L stainless-steel stockpot (Fig. 2) to 55°C (measured using a digital thermometer) using a Fisherbrand™ AREX Hotplate. Enzymes were obtained from the National Centre for Biotechnology Education – University of Reading.

During maceration the ribs were initially observed every 30mins until nearing completion when observations were increased to every 10mins, and then 5mins to ensure maximum maceration but to prevent overcooking. Maceration was considered complete once all grease was removed, and any remaining tissue could be lifted off easily. Throughout maceration, the lids of the slow cooker and stockpot were kept in place to help maintain temperature and to limit evaporation. The temperature was recorded using a digital thermometer and once the maceration process was complete, the ribs were removed from the water and rinsed with cold deionised water to halt any further changes. The ribs were then left to dry overnight at room temperature before fluorescence photography the next day.

Fluorescence photography

Each bone was photographed using a Foster and Freeman single LED blue Crime-Lite 2 forensic light source, with a wavelength of 420 - 470nm (peak of 450nm) and a Nikon D3100 Digital Single Lens Reflex (DSLR) camera fitted with an AF-S Nikkor 18-55mm lens attached to a Calumet tripod. The distance from the camera to the specimen was set at 25cm with a camera zoom of 40mm to ensure full coverage of the specimen whilst limiting background interference. The light source was positioned in line with the camera at 25cm with the camera set to automatic camera settings. The default UV filter was removed from the camera to maximise fluorescence emission and capture, and to ensure the most representative fluorescence images possible.

During photography, each bone was housed in a 32L black box (Really Useful Storage Ltd) and placed on a background of 300 white 10mm polystyrene spheres to act as a control, enhance contrast and to limit reflections from the black plastic box [25]. Each specimen was firstly photographed under natural light with a scale, followed by the ALS with an orange barrier filter fitted to the camera lens. A 1.6 x 2.1m sheet of black photography cloth covering the box to limit interference from natural light (Fig. 3).

Fluorescence quantification

Fluorescence intensity was quantified using a bespoke C++ computer program: the "Osteo-Fluorescence Calculator" (OFC), specifically

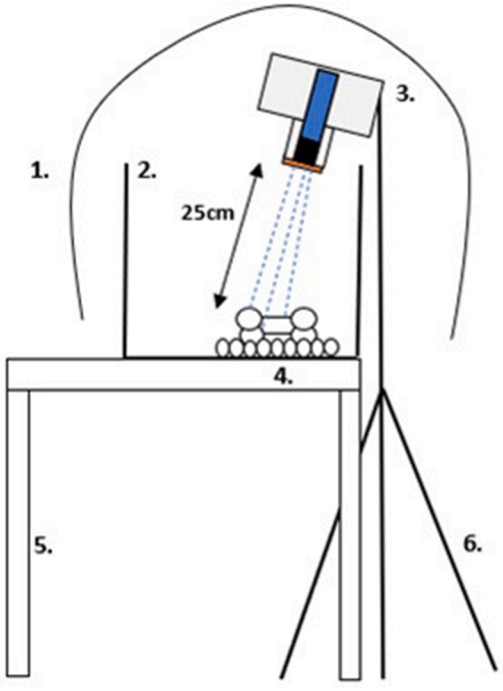

Fig. 3. Diagram of fluorescence photography equipment configuration.

created for this study, designed by the author, and created by software engineer Liam MacDonald. Firstly, each 6000 x 4000 jpeg photograph was converted to grayscale using a weighted equation known as the "luminosity method" [51], which converts the red, green, and blue (RGB) values of each pixel to a singular grayscale value ranging from 0 (black) to 255 (white) using the following equation:

$$(0.299x\mathbf{R}) + (0.587x\mathbf{G}) + (0.114x\mathbf{B})[51]$$

A weighted equation was used in preference to the "averaged" RGB method used in other studies [25,29], as a weighted ratio is thought to better represent how the human eye interprets colour, with the luminosity method being the most commonly used [51,52]. The grayscale image was then overlaid with a grid comprised of 300 x 300-pixel squares labelled with the average grayscale value of the 90,000 pixels in each square, alongside coordinates to aid selection of regions of interest (ROI). This calculated grayscale value is the calculated fluorescence as it measures the brightness of the fluorescence seen. Three adjacent grid squares corresponding to the central anterior aspect of each rib were selected as ROIs to measure the bone fluorescence levels. These sites were chosen as they represent the location where the bone is most exposed, the light distribution is the most consistent, and the locations correspond to where the bone was scraped for thermogravimetric collagen analysis. Three photographs of each specimen were used, and an overall fluorescence average calculated. An example output from the OFC can be seen in Fig. 4.

Collagen quantification

Collagen quantification was conducted using thermogravimetric analysis (TGA), using a Mettler Toledo TGA machine 1 and complimentary Star analysis TGA software to quantify changes in mass before and after maceration. 5mg of bone was scraped from the central, anterior surface of each rib onto clean filter paper using a size 10 scalpel before being transferred to 2ml plastic tubes. The scrapings were frozen (-18°C) to limit bacterial growth and defrosted overnight in the refrigerator (2°C) before processing. Each sample was processed in individual alumina 70μl crucibles in a Nitrogen atmosphere at a flow rate of 50ml per minute, with an insertion temperature of 25°C increasing 10°C per minute to a final temperature of 800°C. Resulting peaks were analysed using the integrated Star analysis software. As collagen is thought to comprise approximately 90% of the organic component of bone [18–20] 90% of the organic volume result was used as a calculated value for collagen content.

Histograms were produced using IBM SPSS Statistics version 23 statistical software to test the distribution of the fluorescence, collagen, and bone mass datasets and determine subsequent statistical analyses. The fluorescence data produced by each maceration technique was shown to not be normally distributed therefore non-parametric, Wilcoxon sign-rank tests, was conducted. The collagen and bone mass datasets were determined to be normally distributed, therefore parametric paired sampled student t-tests were performed.

Results

All maceration techniques succeeded in complete removal of observable residual tissue from the porcine ribs. Overall, the washing powder maceration took the longest to reach completion at 3 hours and 30 minutes; hot water maceration took 2 hours and 55 minutes, and the enzymatic approach took the shortest time, at 1 hour and 15 minutes. Mass loss from the ribs ranged from 12.6% to 19% (mean 15%) as shown

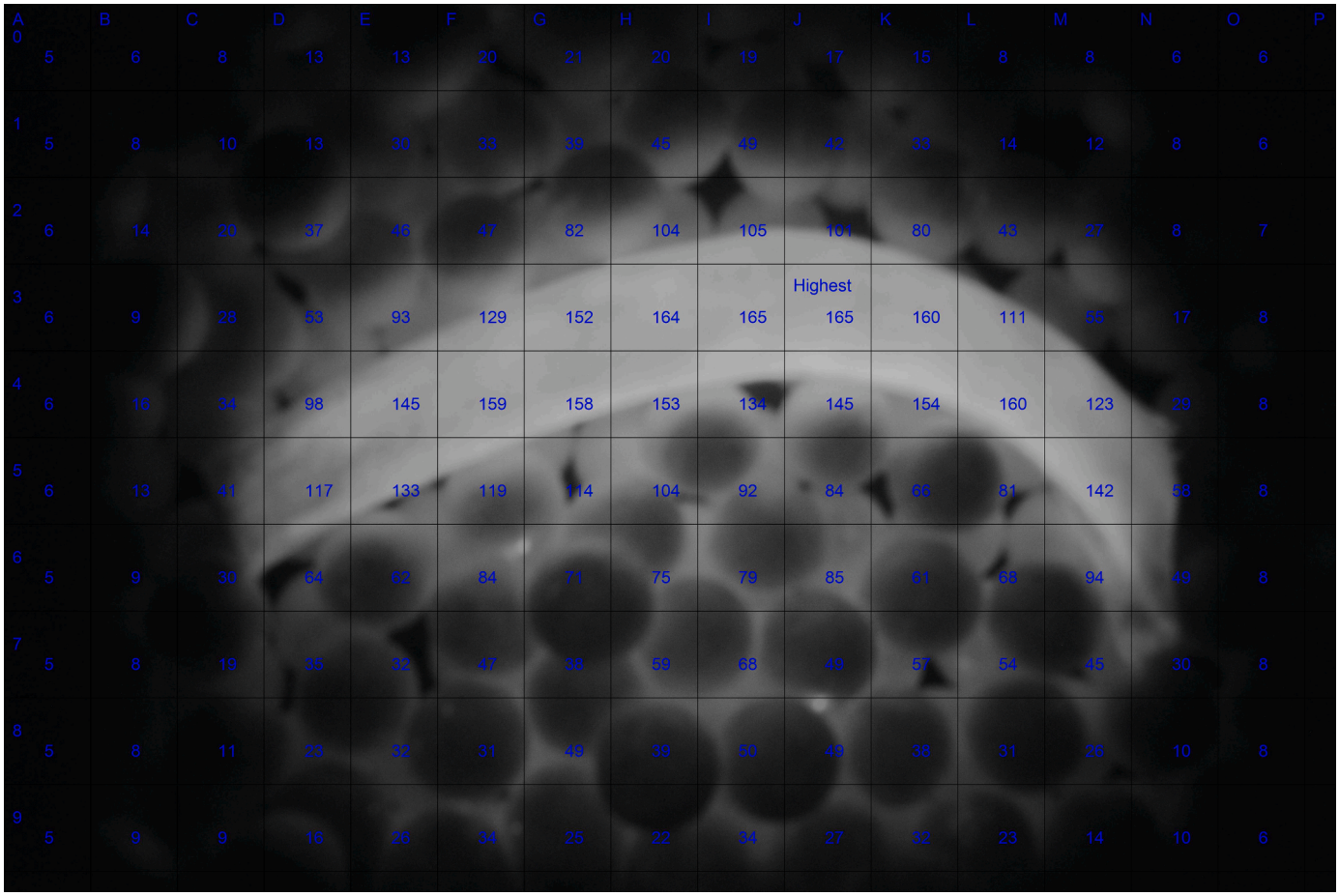

Fig. 4. A final grayscale image of a porcine rib (Sus scrofa domesticus) processed using the Osteo-Fluorescence Calculator (OFC).

in Table 1 below.

Ribs macerated in hot water produced a smooth, clean finish to the bone's surface (Fig. 5) and generated consistent and homogenous fluorescence results across the length of the bone (Fig. 6) An average post maceration fluorescence increase of 5.5% (n=10, s.d.=9.36, p=0.012) was seen across the dataset. Of the three maceration techniques examined, hot water maceration was found to be the least detrimental to bone collagen levels, exhibiting an average loss of 0.9mg (n=10, s.d.=0.09, p=0.023). Biological washing powder maceration was the most destructive to bone, giving the ribs a bleached appearance with observable evidence of splitting, fraying, and cracking to the bone's surface (Fig. 7) as well as producing the greatest average decrease in collagen content of 0.19 mg (n=10, s.d.=0.05, p= <0.001). However, maceration with biological washing powder also produced the greatest average increase in observed fluorescence of 54.49% (n=10, s.d.=27.46, p=0.180) when compared to the fresh values. However, the observed fluorescence was inconsistent, having an irregular, patchy appearance on each rib's surface (Fig. 8). Enzymatic maceration produced an average increase of 23.2% in observed fluorescence (n=10, s.d.=23.72, p=0.180), which was largely homogeneous across the diaphysis of the ribs, however some pinkish discolouration was also noted. Dark patches located towards the epiphyses (Fig. 9) caused inconsistency in fluorescence values (Fig. 10). On average, the ribs macerated using enzymes experienced a 19.5% loss in collagen content at 0.15mg (n=10, s.d.=0.09, p=0.001).

Fig. 5. Example of final bone appearance of porcine ribs macerated using hot water (80°C).

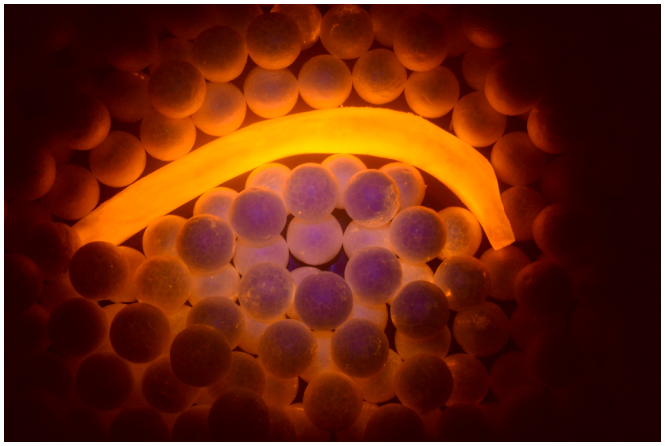

Fig. 6. Example fluorescence results of porcine rib prepared using hot water maceration (80°C).

Discussion

Hot water maceration was the most successful technique for preparation of bone for ALS analysis as it affected the collagen content the least and produced the most consistent fluorescence across the length of the bone. Only a small increase in measured fluorescence levels (n=10, s.d.=9.36, p=0.012) and small decrease in collagen content was seen when compared to the fresh values prior to maceration (n=10, s.d.=0.09, p=0.023). This small increase in fluorescence after maceration is consistent with the successful removal of residual soft tissue and periosteum, as soft tissue does not fluoresce under these wavelengths. Furthermore, the hot water maceration fluorescence values were very similar to the fluorescence results obtained from the fresh bone prior to maceration (fresh = 164.6, macerated = 173.1) and therefore the most representative of natural bone fluorescence. This result suggests that hot water maceration can be used to remove residual tissue to facilitate osteological examination and to clean bone for storage, without impacting the integrity of the bone or any subsequent ALS analysis, especially if some manual de-fleshing has already been conducted. The maceration process took less than three hours, which supports discussion in the literature that shorter exposure to heat, particularly below 100°C, is not as damaging to bone as previously thought [41,47,53]. However, it not possible to comment on how longer durations of maceration would impact collagen content, as this would need to be explored further in future research.

Conversely, the biological washing powder maceration took the longest time to process and led to the greatest increase in fluorescence levels and greatest loss of collagen content from the porcine ribs. This loss is likely due to the corrosive bleaching agents combined with aggressive concentrations of enzymes in the biological washing powder damaging the bone surface and structure. Bleach is known to degrade the surface layers of bone [40], which could explain the irregular patchy appearance seen in the experimental bones. This chemical composition not only damaged the bone but possibly artificially whitened some areas

Table 1
Summary of changes in average bone mass, calculated collagen levels, and quantified fluorescence for each examined maceration technique.

Maceration method	Average Mass (g)			Average Fluorescence			Average Collagen (mg) (90% of organic content)		
	Fresh	Mac.	Change	Fresh	Mac.	Change	Fresh	Mac.	Change
Hot water (80°C) N = 10	21.67	18.50	3.17 (-14.7%) s.d.=0.83	164.6	173.1	8.51 (+5.5%) s.d.=9.36	0.78	0.68	0.09 (-11.2%) s.d.=0.09
Bio. washing powder (50°C) N = 10	16.77	14.61	2.16 (-12.6%) s.d.=1.02	116.7	171.2	54.49 (+49.3%) s.d.=27.46	0.84	0.65	0.19 (-22.9%) s.d.=0.05
Enzymatic (55°C) N= 10	16.59	13.36	3.23 (-19%) s.d.=1.84	136.5	154.1	17.52 (+23.2%) s.d.=23.72	0.77	0.62	0.15 (-19.5%) s.d.=0.09

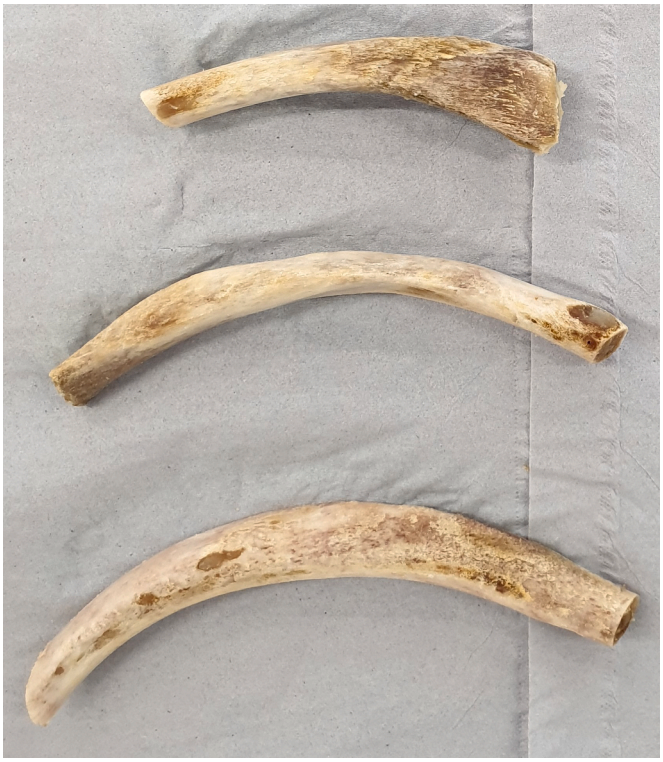

Fig. 7. Example of final bone appearance of porcine ribs macerated using biological washing powder (50°C).

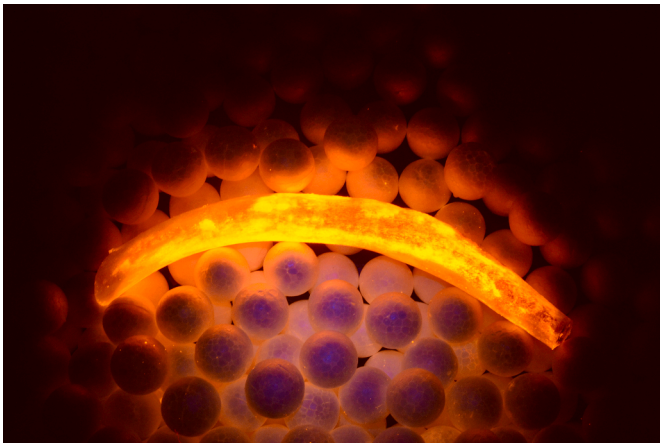

Fig. 8. Example fluorescence results of porcine rib prepared using biological washing powder maceration (50°C).

of the bone's surface. Some optical brighteners contain in biological washing powders are thought to exhibit fluorescent properties [38], therefore this may have contributed to the grossly amplified but inconsistent fluorescence results.

The patchy appearance of the fluorescence could be attributed to natural variation in bone composition due to the anatomical region of the rib i.e., closer to the spine or closer to the sternum would mean differences in porosity and the ratio of trabecular and cortical bone [54]. This may have influenced the absorption rate and distribution of the maceration solution throughout the rib. However, this inconsistency was not seen in either the hot water or the enzymatic maceration methods examined. As the water temperature in the washing powder maceration method was kept relatively low, it is most likely that the chemical composition of the washing powder was the driving force

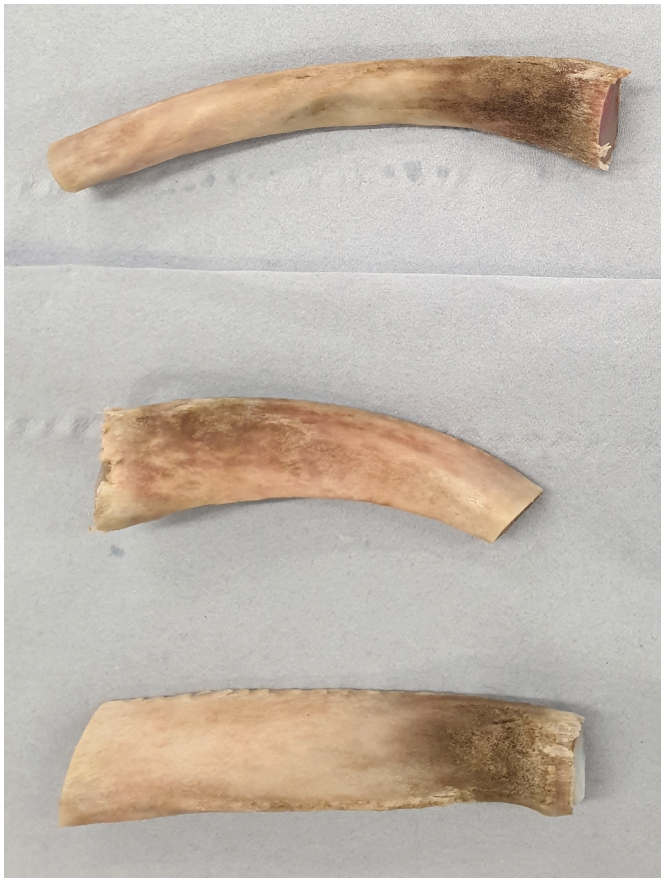

Fig. 9. Example of final bone appearance of porcine ribs macerated using the enzymatic technique (55°C).

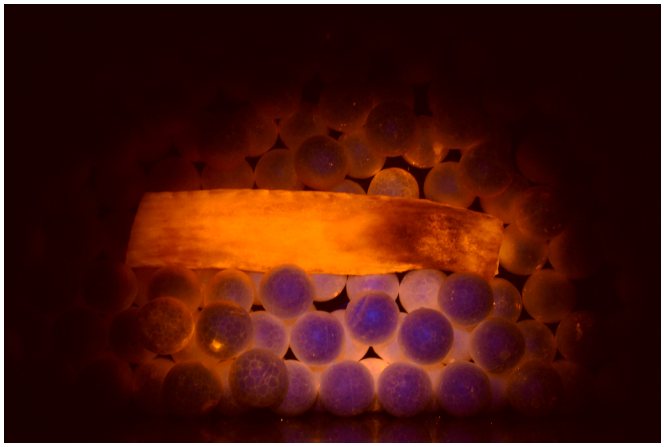

Fig. 10. Example fluorescence results of porcine rib prepared using enzymatic maceration (55°C).

behind these damaging changes rather than the heat. This is supported by the absence of bone surface corrosion, seen after the hot water maceration at 80°C.

The purely enzymatic approach was more gentle on the structure of the bone than the washing powder maceration but more destructive than the hot water technique, although no visual damage to the bone's surface was noted. The ratio of enzymes used for this technique was informed by current literature [41,44], which focused on maceration of whole mice, fleshed porcine rib, and sections of human rib, therefore was not specific to partially de-fleshed porcine bone. It is likely that the

pinkish discolouration and dark patches featured on these specimens were the result of undissolved lipids, as current literature suggests that during enzymatic maceration, lipids can disperse and settle into the external structure of the bone [31,44]. Unfortunately, it was not possible to make direct comparisons with the specific enzymes or their concentrations of the washing powder as exact concentrations are proprietary knowledge and subsequently unavailable.

However, based on the results of both techniques, it is apparent that enzymes alone, without bleaching agents and brighteners, are less destructive to bone than biological washing powder. In addition to this, the enzymatic approach was by far the fastest technique for macerating bone, which could prove very beneficial if a lot of samples are required quickly. However, hot water maceration produced results much more demonstrative of untreated, natural bone and did not require enzymes, which incurred additional expense and storage requirements, therefore hot water maceration was considered the most successful preparation technique and best suited to prepare bone for ALS analysis.

Conclusions

We have established that the most successful maceration technique for forensic anthropology and ALS purposes was hot water maceration, as it is a cheap, simple, and effective methodology that produces clean bones which behave in a very similar way to natural, unaltered bone. We have shown quantitatively for the first time that it has minimal impact on collagen, and bones macerated by this method exhibit consistent fluorescence. Conversely, the industry-favoured method of biological washing powder substantially decreased the collagen content of the bone ($p = <0.001$), and was destructive to the bone surface, creating patchy, irregular, and damaged areas of bone that led to correspondingly uneven and artificially augmented fluorescence. Enzymatic maceration provided the fastest results but had a noticeable impact on collagen levels ($p=0.001$), with pinkish discolouration and dark accumulations on the bone surface most likely due to incomplete hydrolysis of lipids. It is hypothesised that this is a result of a suboptimal lipase and protease ratio, and concentration adjustments would be needed to ensure complete dissolution of lipids, however further investigation is required. In summary, hot water maceration was identified as a suitable preparation technique for bone undergoing ALS examination, whereas biological washing powder maceration techniques should be avoided entirely. Enzyme maceration poses promising benefits with regards to timescales but would require methodological investigation and development. We have shown for the first time that the chosen maceration technique can have a substantial effect on collagen content in porcine bone, which in turn affects the fluorescence of the bone under ALS analysis, potentially creating misleading ALS results. This effect has not been noted before in research regarding the use of ALS for detecting bone in different contexts.

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Author contributions

First author was responsible for the conception and design of the study, the acquisition, analysis, and interpretation of data, and the primary writing of the final article. Second author provided guidance and expertise in relation to study design and data interpretation, and critical feedback and manuscript contributions. Both authors approved the article for submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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