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A comparative study of commercially available, minimally invasive, sampling methods on Early Neolithic humeri analysed via palaeoproteomics

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ABSTRACT

Due to methodological advances in the archaeological sciences, an increasing number of archaeological specimens undergo destructive sampling. However, the preservation of cultural heritage is a primary concern. This leads to a dilemma between accessing sample material and obtaining sufficient information for a meaningful analytical outcome. Ideally, sampling a specimen would preserve the object for further macro, micro, and molecular analyses. For palaeoproteomics, a number of minimally invasive sampling approaches have been proposed, representing different benefits and limitations. There have been studies comparing a selection of these protocols, however, these have focused on specimens from a homogenous preservation environment using Zooarchaeology by Mass Spectrometry (ZooMS). Here we expand on earlier work by extending the comparison to specimens from two highly different preservation environments through both ZooMS and liquid-chromatography tandem mass spectrometry (LC-MS/MS). We compare five sampling approaches and seven extraction protocols in total, on 10 *Bos* sp. humeri from the Early Neolithic site of La Draga, Spain, utilising MALDI-ToF MS and LC-MS/MS to generate proteomic output, while assessing protocol invasiveness using microscopy and 3D imaging. Five humeri originate from Sector A, which is mostly related to dry, terrestrial preservation conditions, while the other five humeri stem from Sector B, which is characterised by its phreatic/aquatic preservation conditions. We show that there is a significant difference in protein recovery and taxonomic specificity between the sampling techniques applied, as well as between burial conditions. Additionally, various surface modifications were observed depending on the specific sampling technique applied. It is therefore essential to assess protein preservation for each sedimentological context within an archaeological site before performing extensive sampling, as protein preservation can be highly inter- and intra-site-specific.

1. Introduction

Bone objects constitute a significant component of the archaeological record, both in abundance as well as in scientific value. In archaeology, bone, antler, and ivory have in their raw and modified forms been subject to countless studies from various perspectives and disciplines. Traditional zooarchaeological approaches and traceological analyses play a crucial role in identifying anthropic and non-anthropic traces, skeletal element, taxonomy, and taphonomics providing information on subsistence economy and animal exploitation (Kveiborg, 2008; Manning et al., 2013), clarifying functions of objects (Daujeard et al., 2014; Diego et al., 2018), manufacturing processes (Farbstein, 2013; Wild et al., 2022), domestication processes (Rowley-Conwy, 2013; Saña, 2013), and ecological questions (Arriaza et al., 2016), among others. Such analyses

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require no destructive sampling. This stands in opposition to DNA, isotope, lipid, and proteomic analyses, where sampling to various degrees is inevitable. Nevertheless, these approaches are widely used to obtain information that is otherwise inaccessible (Armitage et al., 2023).

Appropriate sampling strategies are crucial in the collaboration with institutions storing and curating archaeological collections. Circumspection in the world of bone material has mainly been revolving around hominins and precious bone artefacts in regards to sampling, but lately more attention towards faunal assemblages has been brought into light (Pálsdóttir et al., 2019). The notion that the archaeological record as a whole is not an infinite resource calls for a more elaborate dialogue between researcher and curator, where sampling method, documentation and traceability (writing, photos, and/or 3D scans), data sharing, and consultation with specialists should be incorporated into sampling strategies in order to better ensure the integrity of the archaeological record while producing new knowledge (Pálsdóttir et al., 2019; Spyrou et al., 2022).

Owing to the destructive nature of sampling, and archaeological artefacts being increasingly sampled (Dekker et al., 2021; Mannermaa et al., 2022; Rey-Iglesia et al., 2023), it is paramount that a proper sampling strategy and workflow are applied, which are ideally based on empirical data. Comparative studies on, among others, extraction methods (Gilbert et al., 2024a; Mylopotamitaki et al., 2023), digestion durations (Le Meillour et al., 2024), and protease choice (Fagernäs et al., 2024; Lanigan et al., 2020) have already shown that such research has the potential to significantly improve workflows and establish best practices without compromising the scientific output. As the study of ancient proteins is a fast growing field allowing for taxonomic identifications (Brandt and Mannering, 2021; Runge et al., 2021), establishing dietary practices (Hendy et al., 2018), and exploring phylogenetic relations (Chen et al., 2019) of ancient bone specimens we chose to focus on palaeoproteomics. Building on previous studies investigating different aspects of sampling methods for palaeoproteomics (Evans et al., 2023; Sinet-Mathiot et al., 2021), we compare five commercially available sampling approaches, two of which are followed by two different protein extraction procedures. Each resulting protein extract is analysed using both Zooarchaeology by Mass Spectrometry (ZooMS) (Buckley et al., 2009) and full proteome analysis using liquid-chromatography tandem mass spectrometry (LC-MS/MS).

This study shows that preservation plays an important role in which sampling approaches can be utilised for obtaining satisfactory taxonomic identification as well as other proteomic data. For bone material where protein preservation is adequate, minimally invasive techniques may be useful and will not reduce bone volume visibly, though surface alterations may complicate use-wear analysis. However, for material with poor protein preservation, more invasive techniques may be useful for taxonomic identification as well as recovering various protein groups, though compromising future micro- and macro analyses.

2. Material and methods

2.1. Specimen selection

We selected 10 bone specimens, morphologically identified as humeri from *Bos* sp., from the Early Neolithic site of La Draga (5300-4900 cal BCE) (42°07′35.4″N 2°45′30.6″E, coordinate for centre of Sector A, Fig. 1) (Andreaki et al., 2022). La Draga is located on a lake margin, causing large differences in preservation conditions across the site. To date, four sectors have been excavated; A, B, C, and D. In general the upper layers consist of dark clays with disturbances of modern intrusions due to recent agricultural practices. Below, travertine blocks form a sequence between clayey layers, followed deeper down by a layer of collapsed wooden structures whose preservation is of differential stages caused by water table variation. Above the base layer, which consists of carbonate sands, a level of dark, organic sediment is present (Palomo et al., 2014). Sectors B and D consist of phreatic/aquatic levels, while

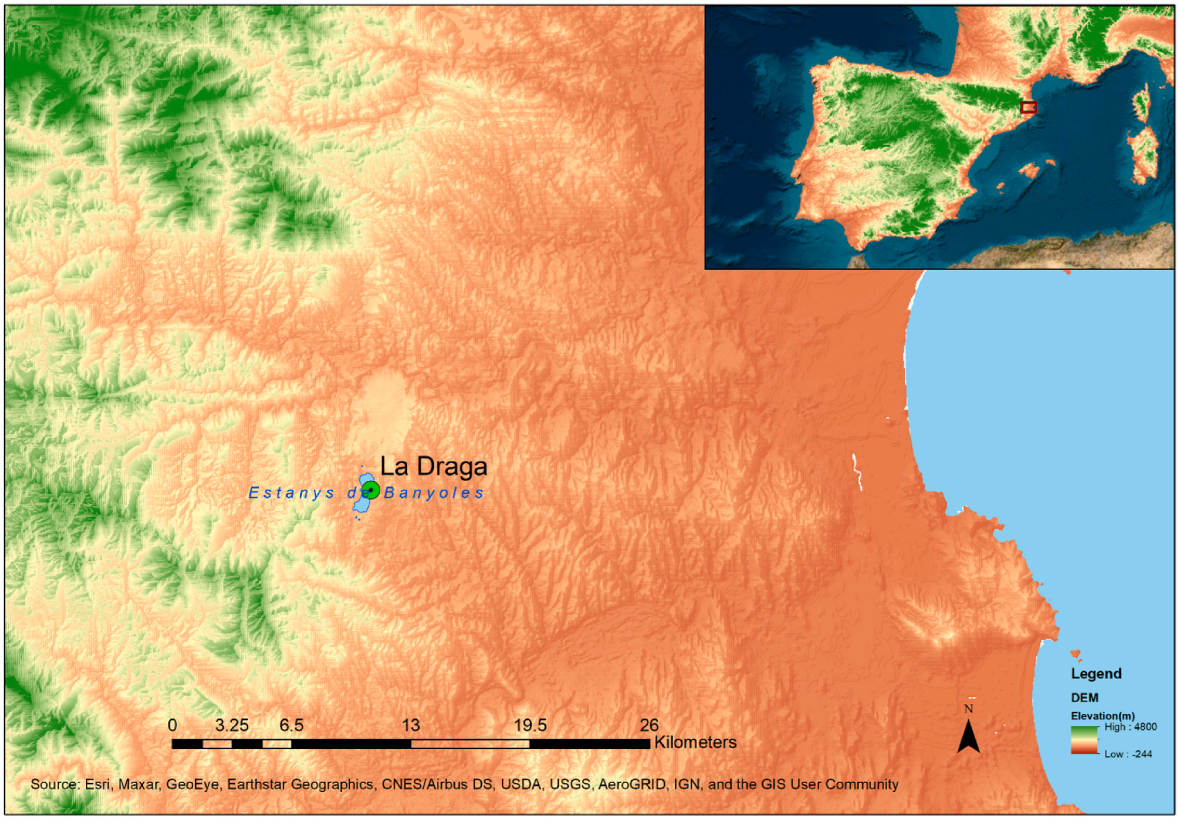

Fig. 1. Map displaying the location of the site of La Draga.

Sector C is fully aquatic. Sector A is considered the most dry/terrestrial sector, with archaeological levels being approximately 70 cm above phreatic conditions (Antolín et al., 2014; Saña et al., 2014).

From these, we sampled five bone specimens from Sector A (terrestrial) and five bone specimens from Sector B (waterlogged). Since all sectors are contemporaneous with each other, this allowed us to conduct sampling on specimens from the same species, skeletal element, site, and timeframe, while simultaneously enabling a comparison of two intrasite environmental extremities.

2.2. Experimental design

Based on the existing literature on sampling strategies for proteomic analysis, we selected five sampling approaches that are commercially accessible. These are 1. obtaining a bone chip, 2. acid etching of the bone surfaces, inspired by acid etching of dental enamel surfaces (Rebay-Salisbury et al., 2020; Stewart et al., 2016), 3. using an eraser on the bone surface (Fiddyment et al., 2015; Sinet-Mathiot et al., 2021), 4. using a polishing film on the bone surface (Kirby et al., 2020), and 5. membrane box storage (Martisius et al., 2020). As a number of previous studies already compared the plastic bag protocol to other minimally invasive protocols and consistently found it to underperform (Evans et al., 2023; Martisius et al., 2020; McGrath et al., 2019), this approach was excluded. As for tape based protocols, using dermatology-grade skin tape (Multari et al., 2022) or ethylene vinyl acetate (EVA) film (Righetti et al., 2020), at the time of this study design there were doubts regarding their ability to extract ancient proteins and limits in regards to the commercial accessibility, though recently several additional applications have been published (Cucina et al., 2022; Fabrizi et al., 2023; Pittalà et al., 2023).

Furthermore, for two of the sampling methods two extraction methods were performed. First, the bone chip sampling allowed for a comparison of an extraction method without using a demineralisation step (AmBic protocol) (van Doorn et al., 2011) and one including a preceding demineralisation step before incubation in ammonium-bicarbonate (HCl protocol) (Buckley et al., 2009). Second, as the acid etching protocol was originally designed for dental enamel surfaces (Stewart et al., 2016), some modifications to this protocol were necessary before application to bone surfaces to allow for subsequent protein digestion. The acid etch solution was therefore divided equally to allow for a direct comparison of, firstly, neutralising the acid chemically (neutralisation protocol) and, secondly, neutralisation with a filter-aided sample preparation protocol (FASP protocol) (Wiśniewski et al., 2009), the latter involving a direct buffer exchange. As a result, our five sampling approaches resulted in a total of seven extraction methods being compared (Fig. 2).

Both before and after sampling, all ten specimens were 3D scanned with an Artec Space Spider, with a 0.05 mm accuracy and a 0.1 mm resolution, and edited using *Artec Studio 16 Professional* v.16.0.8.2 (Artec3D, 2021) for subtracting the before and after sampling scans from each other. Additionally, the specimens were photographed with a digital microscope (Levenhuk DTX TV) using the *PortableCaptureHD software* v.2.3 (Celestron, 2016). The 3D scans cover the total surface area of all the specimens, while the microscopy images were limited to three zones for each specimen: Zone 1 (acid etching), Zone 2 (polishing film), and Zone 3 (eraser). No microscopy images of the bone chips were taken, as this represents a more destructive approach. Although bone fragments were used for the membrane box approach, this method should be considered non-invasive as various sizes of membrane boxes exist allowing whole bone objects to be enclosed while ensuring their

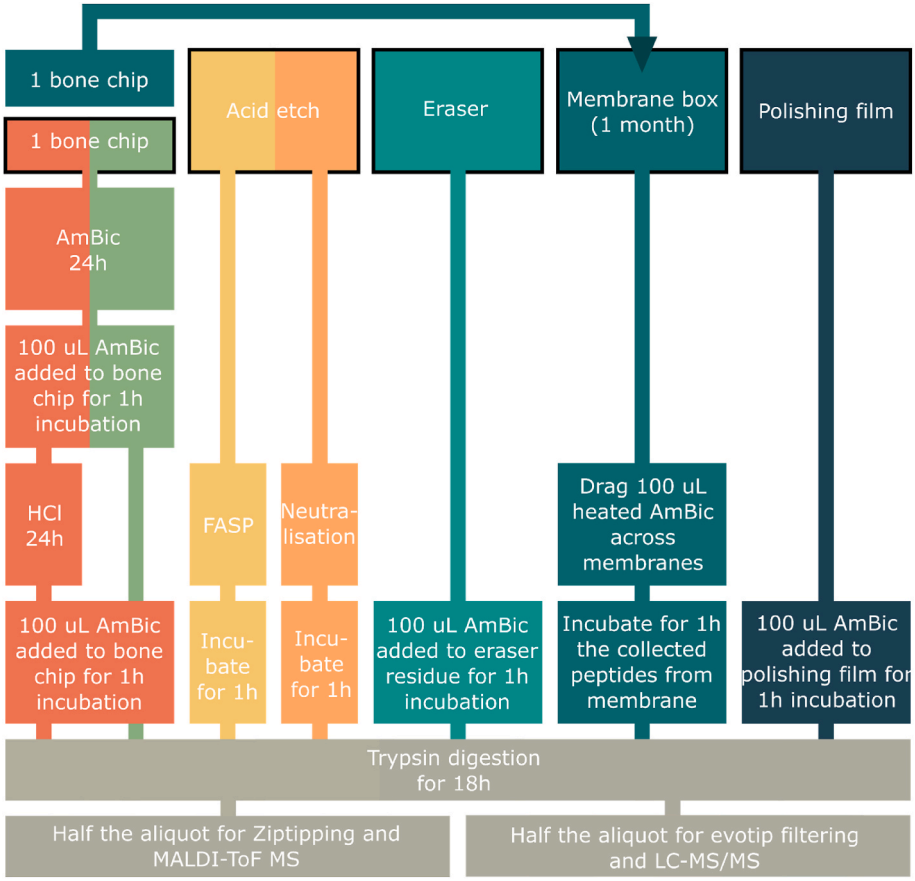

Fig. 2. Simplified workflow of the comparative sampling and protein extraction. Each bone specimen was sampled through five sampling methods (indicated by black outlines) and a total of seven extraction methods.

integrity.

Each sample followed a specific extraction protocol up to trypsin digestion (SI A), subsequent to which all extractions followed the same protocol (Fig. 2). More specifically, the gelatinised samples, consisting of 100 μL 0.05 M NH_4HCO_3 (ammonium bicarbonate, hereafter AmBic), were combined with 0.8 μg trypsin (Promega, #V115A) and digested for 18 h at 37 °C using a heating block (Thermal Shake *lite*, VWR*)*. Subsequently, samples were centrifuged for 1 min at 10,000 rpm before adding 2 μL of 0.13 M CF_3CO_2H (Trifluoroacetic acid, hereafter TFA) to stop the digestion. The samples were split into two aliquots of 52 μL, one for MALDI-ToF MS and another for LC-MS/MS.

2.3. MALDI-ToF MS and associated data analysis

In accordance with Welker et al. (2016) aliquots for MALDI-ToF were purified and desalted using C18 ZipTips (Thermo Fisher) and spotted on an MTP 384 target MALDI plate ground steel BC (Bruker) in triplicates. Spots comprised of 1 μL eluted peptides and 1 μL α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution. Mass spectra were acquired using a Bruker UltrafleXtreme MALDI-ToF in reflector mode set to a laser intensity of 50–70% and a mass range of 800–4000 Da. All peptide masses below 650 Da were suppressed. Each sample was externally calibrated against an adjacent spot containing a mixture of six peptides (des-Arg1 Bradykin m/z = 904.681, Angiotensin I m/z = 1295.685, Glu1-Fibrino-peptide B m/z = 1750.677, ACTH (1–17 clip) m/z = 2093.086, ACTH (18–39 clip) m/z = 2465.198 and ACTH (7–38 clip) m/z = 3657.929).

ZooMS triplicate spectra were merged (Le Meillour et al., 2024) and processed using *MALDIquant v.1.22.1* (Gibb, 2023a) and *MALDIquantForeign v.0.14* (Gibb, 2023b) via the software R *v.4.2.3* (R Core Team, 2023). Selected peaks were manually matched to published biomarkers (Presslee, 2020) in order to obtain taxonomic identifications. Glutamine deamidation values were calculated through the Betacalc3 package (Wilson et al., 2012).

2.4. LC-MS/MS and associated data analysis

LC-MS/MS data generation followed a two-step approach involving both Data-Dependent Acquisition (DDA) data generation and Data-Independent Acquisition (DIA) data generation. We generated a single pooled DDA library taking 10 μL of each sample. The purpose of a pooled sample was to facilitate the creation of a custom spectral library covering the contents of all the individual samples (Barkovits et al., 2020). All aliquots for LC-MS/MS analysis were then processed through Evotip filtering. Evotips with C18 membranes (EV-2011, Evosep) were rinsed, conditioned, and equilibrated following Evosep's preparation protocol (Evosep, 2022), which consisted of priming the Evotips in 100% acetonitrile and soaking in isopropanol. Subsequently, the single Evotip injections were washed with 0.1% formic acid, and 10 μL of the aliquot was loaded followed by a washing step, and kept wet for subsequent LC-MS/MS analysis. For more details see SI A.

Additionally, the Spectronaut output was processed with the library DIA version of SPIN (Rüther et al., 2022) to test how well this alternative approach to proteomic taxonomic identification handles data obtained from minimally invasive sampling protocols. SPIN analysis was performed using a slightly modified version of the original R script against the same bone protein database used in the initial SPIN publication (Rüther et al., 2022). For the final taxonomic identification of SPIN, we used the output of the "fine grouping" step, which combines peptide biomarkers with the general sequence database search.

2.5. Statistical analyses

MALDI-ToF MS proteomic analysis was largely conducted via R *v.4.2.3* (R Core Team, 2023) using MALDIquant *v.1.22.2* (Gibb and Strimmer, 2012), MALDIquantForeign *v.0.14.1* (Gibb, 2023b), tidyverse *v.2.0.0* (Wickham et al., 2019), janitor *v.2.2.0* (Firke, 2023), stringr *v.1.5.1* (Wickham, 2023), xlsx *v.0.6.5* (Dragulescu, 2020), and gsubfn *v.0.7* (Grothendieck, 2018), while LC-MS/MS analysis was mostly conducted following (Rüther et al., 2022).

Statistical analyses were calculated using two-way ANOVA (Type III) and post-hoc analysis with Tukey's test through car *v.3.1-2* (Fox and Weisberg, 2019) and MASS *v.7.3-60.0.1* (Venables and Ripley, 2002). Dunn's test was performed through FSA *v.0.9.5* (Ogle et al., 2023) and dunn.test *v.1.3.5* (Dinno, 2024). The Kruskal-Wallis rank sum test was done using stats *v.4.1.1* (R Core Team, 2023). Normal distribution of residuals was measured using the Shapiro-Wilk normality test (Shapiro and Wilk, 1965) and homogeneity of the variances was conducted by Levene's test (Levene, 1960).

As will be apparent in Section *3. Results*, the acid etch (FASP) protocol is not performing at a satisfactory level. Therefore, it was decided to exclude acid etch (FASP) from all statistical tests, though it will appear throughout all figures.

3. Results

3.1. Microscopy and 3D scanning

Microscopic images (SI Fig. B1-8) and 3D scans were taken prior to and after sampling in order to assess the invasiveness of the sampling methods. In relation to the microscopy images, almost the entirety of the surface area shown have been affected by the sampling approaches, exceptions being some corners. Among the two more visually destructive methods, bone chips for the AmBic followed by HCl extraction protocol remove the largest surface volume in our study, approx. 20–40 mm^3. Such removal will at the fresh breakage point expose unseen inner bone surface, easy to distinguish from bone surface which has been exposed to outer parameters for much longer. Secondly, acid etching (Zone 1) visibly modifies the surface exposing deeper layers within the bone. Accounting for the two darkest shades of blue in Zone 1 from the 3D scans (SI Fig. B9), 0.4 mm ± 0.07 SD (Sector A) and 0.37 mm ± 0.06 SD (Sector B) surface depth was absent after acid etching. All zones exposed to acid etching show either a gradual reduction in bone volume and discolouring from the center of application and outwards (e.g. Fig. 3), or bone flakes breaking off in an oblique manner (Fig. 4).

The three remaining sampling techniques, polishing film, eraser, and membrane box, appeared comparably less visibly destructive. No before and after images were taken of the bone chips placed in membrane boxes, however, these would show no signs of surface modifications other than already loose particles possibly being detached from the bone specimen. The polishing film technique often left minor traces of polish or scratches, though being difficult to detect in a few cases with the resolution used in this study. Green micro-grit residue could often be observed in cracks and folds on the bone surface. The eraser technique mainly seemed to remove loose particles such as dirt and sediment as well as leaving eraser residues in cracks and folds across the affected bone area.

In general, the membrane box, polishing film, and eraser sampling approaches keep the bone volume intact compared with acid etching and bone chip extraction. However, the polishing film and eraser often leave residues behind and will modify the surface on a microscopic level (Sinet-Mathiot et al., 2021).

3.2. MALDI-ToF MS

We generated 70 extracts for MALDI-ToF MS through seven different extraction methods. For Sector B, all extracts could be assigned to the taxonomic level of *Bos/Bison*, with the exception of extracts treated with the acid etch (FASP) protocol, where no identifications could be assigned (Fig. 5). As the ZooMS analysis was conducted prior to LC-MS/MS analysis it was decided to exclude acid etch (FASP) from further analysis and statistical comparisons. However, the extraction method is

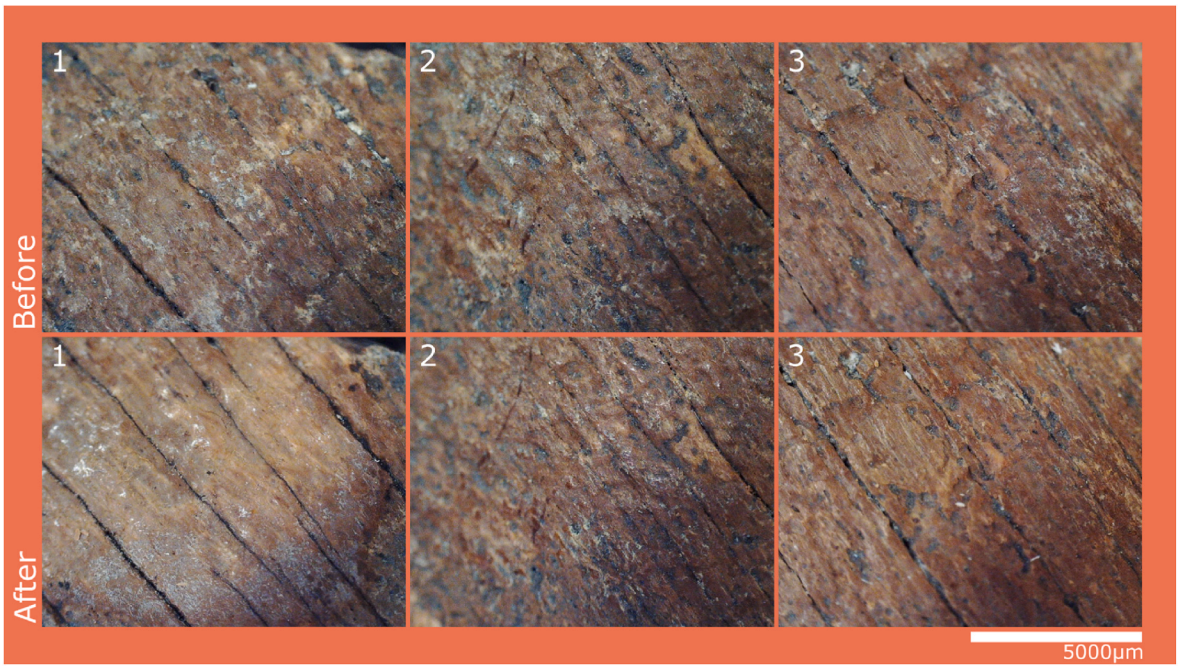

Fig. 3. Before and after microscopy images of the three zones of Specimen 63, Sector A. Zone 1 = acid etch, Zone 2 = polishing film, Zone 3 = eraser.

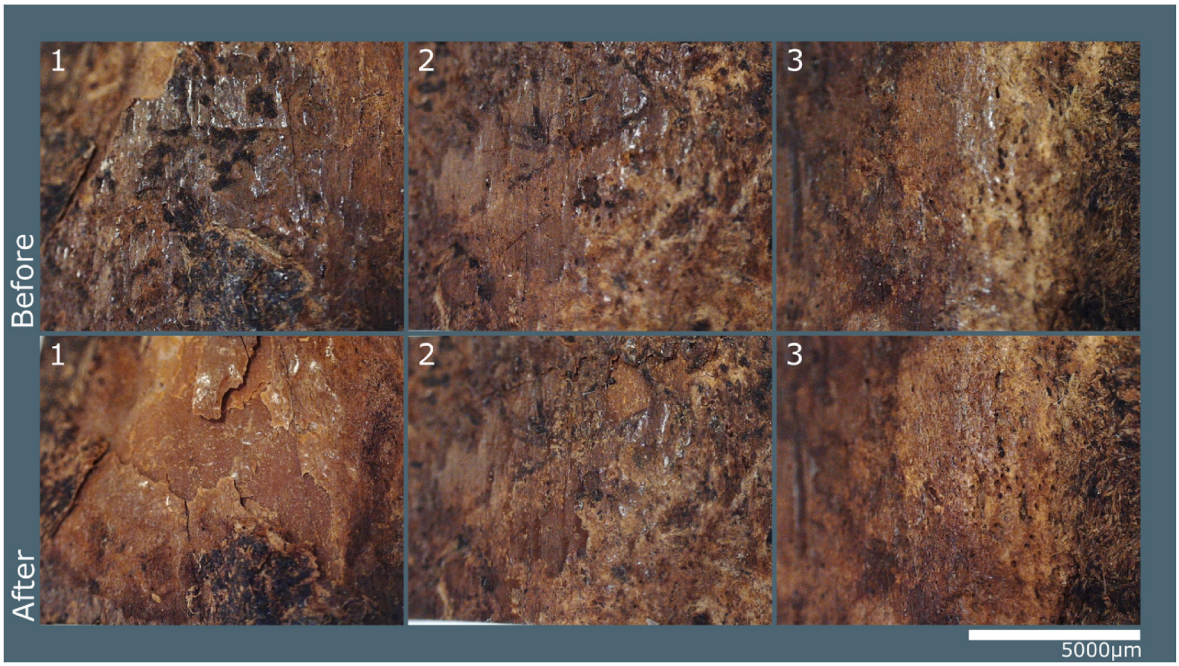

Fig. 4. Before and after microscopy images of the three zones of Specimen 59, Sector B. Zone 1 = acid etch, Zone 2 = polishing film, Zone 3 = eraser.

still shown in figures and tables for completeness. *Bos/Bison* would be the most precise taxonomic attribution possible using the 9 most common peptide markers for taxonomic assignment in our study, indicating great success for all sampling and extraction methods for Sector B. As to Sector A, only nine out of 35 extracts resulted in an assignment to *Bos/Bison*, four extracts could be identified to a less specific taxonomic unit, and the majority were not identifiable at all. Across both sectors the HCl protocol was the most successful in obtaining precise taxonomic identifications (9/10 extractions).

Within Sector A, the HCl method provides the most peptide markers by far, counting in average 7.6 ± 1.67 SD out of nine, while the other sampling approaches perform less well: 4.2 ± 3.49 SD for acid etch (neutralisation), 3.8 ± 3.56 SD for AmBic, 3.0 ± 1.73 SD for membrane box, 2.2 ± 2.39 for eraser, and 1.4 ± 0.89 SD for polishing film (Fig. 6). Yet, all sampling approaches seem to be performing uniformly on extracts from Sector B, with the exception of acid etch (neutralisation) identifying the lowest amount of peptide markers (6.2 ± 1.09 SD). Otherwise, the remaining approaches identify almost all peptide markers (8.8 ± 0.22 SD).

Both sampling method and sector as well as their interaction significantly affect the number of peptide markers identified (ANOVA Type III: $F = 7.862$, $p = 0.001$). Out of 66 possible combinations, 30

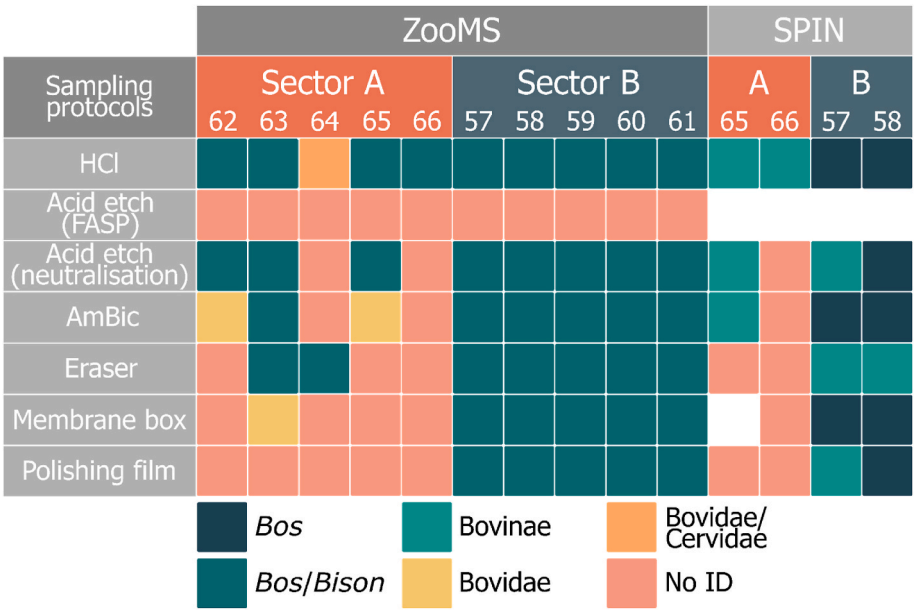

Fig. 5. The taxonomic level of identification achieved for each specimen and for all seven extraction approaches tested. The taxonomic levels from lowest to highest are *Bos* sp., *Bos/Bison*, Bovinae, Bovidae, Bovidae/Cervidae, and No ID. The individual sample ID and sector are written above, while the sampling methods are seen to the left. Note that only specimens 65, 66, 57, and 58 were studied using SPIN, while all specimens were studied using ZooMS. The blank cells indicate where no SPIN analysis was performed.

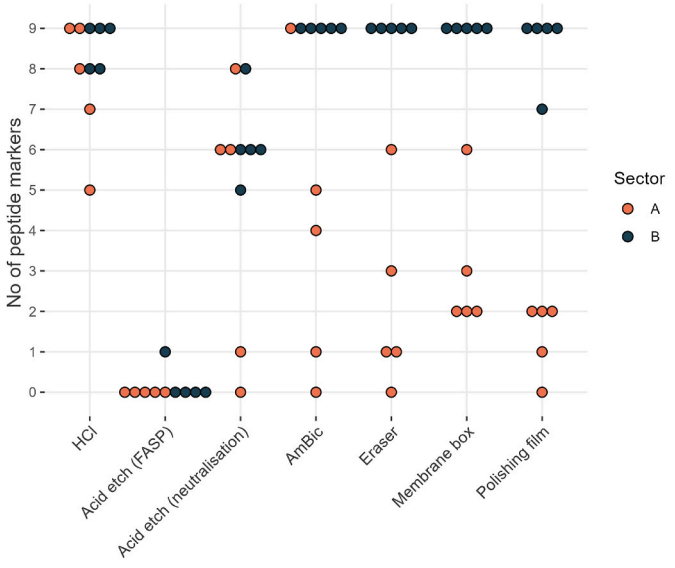

Fig. 6. Number of peptide markers recovered across sampling methods. A maximum of 9 peptide markers could be observed.

showed a statistically significant difference, almost always related to sector variance, except for six combinations. Three of these are all Sector A combinations, where the HCl method performs significantly better than the minimally invasive sampling protocols of eraser (Tukey, $p = 0.003$), membrane box (Tukey, $p = 0.001$), and polishing film (Tukey, $p = 0.001$).

Similarly, we determined the total number of monoisotopic peaks in each ZooMS spectrum (SI Fig. B10). Here, we find that sector variance is the most important factor, as significantly more monoisotopic peaks can be observed across all sampling approaches from Sector B compared with Sector A (ANOVA Type III: $F = 44.441$, $p = 0.001$). Based on the Tukey's post hoc test, only 17 out of 66 pair combinations showed significant differences based on the interaction between the sampling approach and sector, whereof 13 are related to sector variance. In general 10 out of 17 of these pair combinations have the HCl protocol as at least one of the components.

Deamidation values from COL1α1 508–519 and COL1α1 934–963 (Wilson et al., 2012) can be seen in SI Fig. B11. Generally more deamidation values could be determined from Sector B samples compared with Sector A.

The MALDI-ToF MS data, both.txt triplicates and.msd merged files have been uploaded to the repository Zenodo (European Organization for Nuclear Research, 2013) (https://doi.org/10.5281/zenodo.11098529).

3.3. LC-MS/MS

Apart from using the minimally invasive sampling protocols for ZooMS we also tested their ability to extract proteins for LC-MS/MS analysis. Due to the higher costs of LC-MS/MS analysis we analysed a subset of specimens, two from Sector A (specimens 65 and 66) and two from Sector B (specimens 57 and 58). For each of these specimens we processed an extract obtained with the HCl, AmBic, acid etch (neutralisation), eraser, membrane box (only specimens 57, 58, and 66), and the polishing film protocol. Moreover, the LC-MS/MS data, as well as Spectronaut output, have been deposited to the ProteomeXchange Consortium repository via the PRIDE (Perez-Riverol et al., 2022) with the accession identifier PXD050661.

3.3.1. MS/MS. From DDA to DIA

For the LC-MS/MS analysis we employed a library-based DIA workflow. In general DIA analysis is considered to be more reproducible than DDA analysis (Li et al., 2021; Zhang et al., 2020), but its advantages rely heavily on the spectral library used. Although it is possible to perform library-free DIA analysis, this is usually outperformed by library-based DIA. In order to use DIA to its greatest utility we created a spectral library of a pooled aliquot of all the samples with DDA (SI Table B1). In total 27 proteins were identified with a "Protein existence" score of one in the pooled sample, of which 26 were identified during the search against the *Bos taurus* proteome. Only one protein was identified during the search against the *Ovis aries* reference proteome.

3.3.2. Proteome abundance and diversity

As described above, the Spectronaut protein identifications were filtered in order to exclude likely contaminants and an overview of all remaining protein and peptide identifications can be found in SI D. In total we detected eight distinct contaminant protein groups in our extraction blank, which were trypsin, three varieties of human skin keratin and four entries of collagen. The contaminant collagen protein groups consisted of multiple entries from the TrEMBL database of *Bos taurus* and/or *Ovis aries* collagen. As the minimally invasive sampling protocols extract the protein from the very surface of the sample, we expected to find a larger degree of contamination in their results. To test this assumption we calculated the ratio of the intensities of the eight contaminant proteins to the total protein intensity (Fig. 7B). The data shows that for all extractions at least half of the total protein intensity is contributed by contaminant proteins. For the minimally invasive extractions the fraction of contaminant intensity is around 0.8 at a minimum. The high fraction of contaminant protein intensities highlights the small amount of ancient protein that can be extracted with the minimally invasive sampling protocols. This is also reflected in a lower number of identified protein groups (Fig. 7A). The destructive protocols, led by the HCl approach, provided the most protein group identifications for each sample, followed by either the AmBic or acid etch (neutralisation) protocol. However, all the minimally invasive protocols still manage to extract proteins for the Sector B samples. For Sector A, of the minimally invasive protocols only the eraser and polishing film obtained protein and only for sample 65.

Apart from substantial differences in the number of protein groups identified in each sample, there are also differences in the intensity of the recovered proteins (Fig. 8A). A Kruskal Wallis test shows that there are significant differences in the total intensity of each sample between the different sampling methods ($p = 0.001$). A post-hoc Dunn's test reveals that the groups that differ significantly are the acid etch (neutralisation) and membrane box (p adjusted = 0.002), HCl and membrane box (p adjusted = 0.001) and HCl and polishing film (p adjusted = 0.001).

Fig. 8B also highlights an interesting pattern in the intensity of the detected proteins. For all collagens the polishing film yields the highest intensity out of the minimally invasive sampling protocols, exceptions being collagen type V alpha 1 where no minimally invasive protocol was able to extract the protein and collagen type II alpha 2 where the eraser protocol extracted the highest intensity. In general, the minimally invasive sampling protocols yield lower intensities than the destructive methods. As for the non-collagenous proteins (NCPs), osteocalcin is considered to be the most abundant bone NCP (Nielsen-Marsh et al., 2005; Smith et al., 2021), yet it does not seem any more abundant than other NCPs, such as alpha-2-HS-glycoprotein, osteomodulin and prothrombin, for example. A similar pattern has been observed in other palaeoproteomic bone studies (Procopio et al., 2021; Sawafuji et al., 2017; Schroeter et al., 2016). However, along with SPARC, these four seem to be the only NCPs that were detected in at least two of the minimally invasive protocols.

These results indicate that in terms of abundance and diversity of the proteome, the destructive protocols still outperform the minimally invasive protocols, though the differences between the destructive and

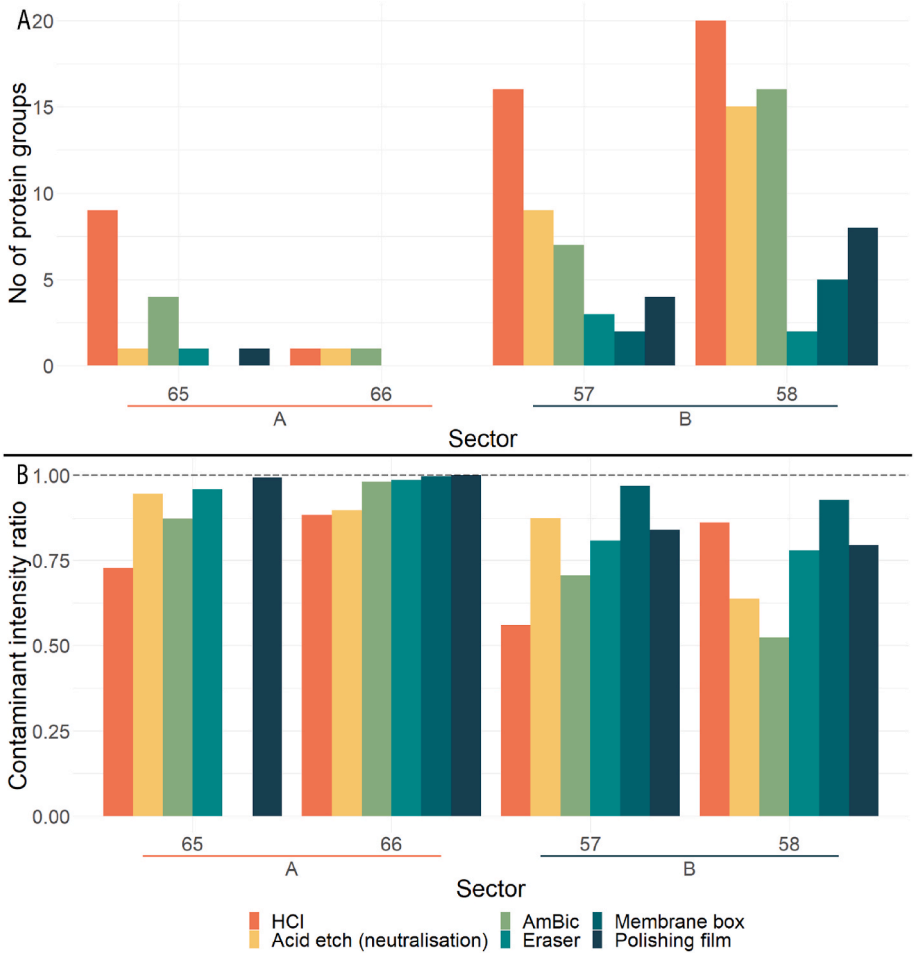

Fig. 7. DIA-identified proteome composition. A) number of protein groups identified, per sample and method, B) the ratio of the sum of all contaminant proteins to the total protein intensity in the sample. A value of 1 signifies an identified proteome completely composed of contaminants.

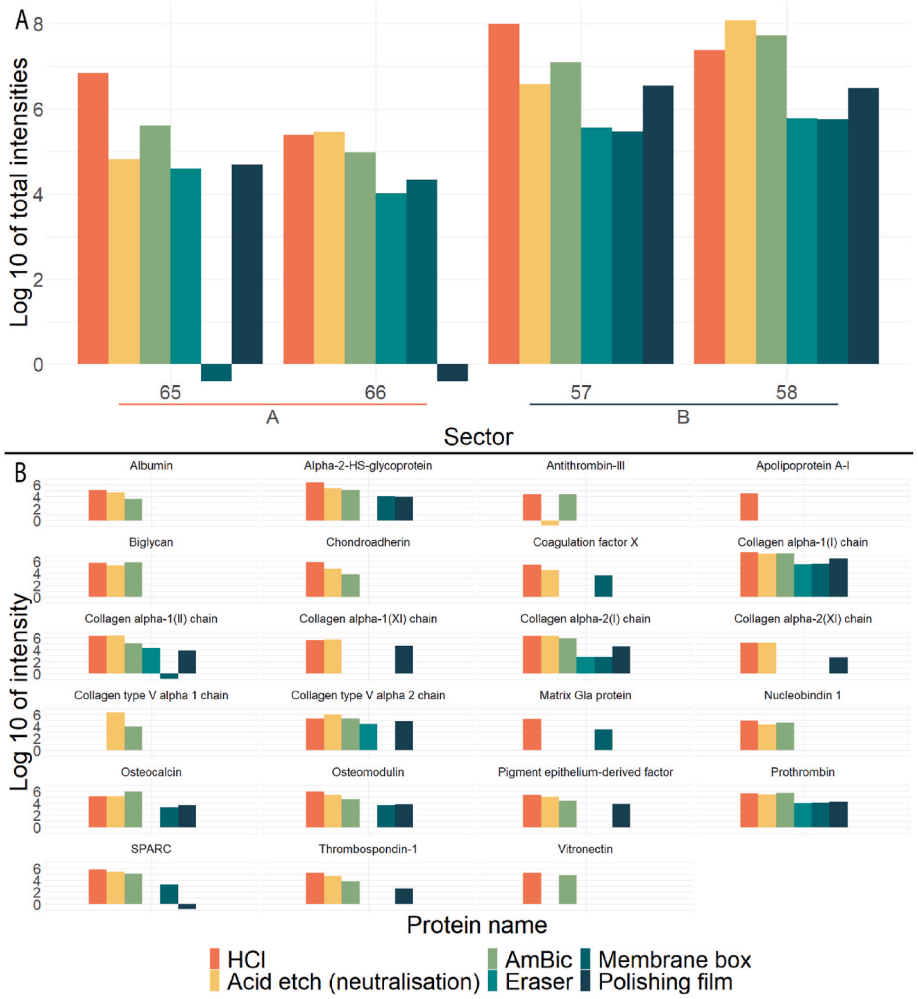

Fig. 8. Observed intensities. A) Log10 scaled sum of all observed non-contaminant proteins per sector and sample. B) Log10 scaled intensities of all the observed NCPs and collagen alpha-1(I) and collagen alpha-2(I), per sample and method.

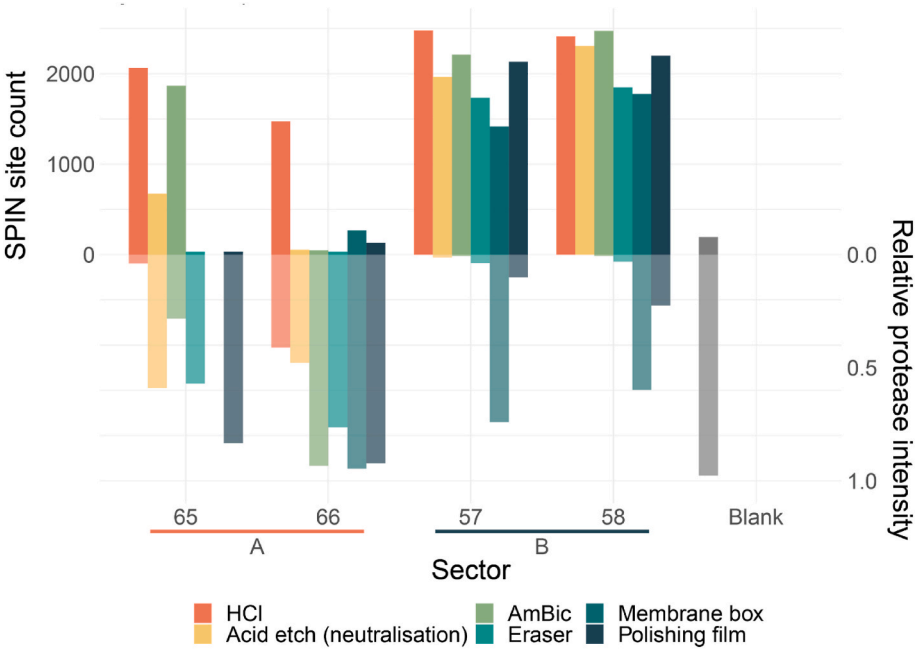

Fig. 9. SPIN site count (top) and relative protease intensity (bottom).

minimally invasive protocols are not always statistically significant.

3.3.3. SPIN analysis

The second aim of the LC-MS/MS analysis was to compare the specificity of the taxonomic identifications obtained via LC-MS/MS to the ZooMS identifications (SI E). To this end, we processed the LC-MS/MS samples with SPIN (Rüther et al., 2022). Of the extracts analysed, seven could not be taxonomically identified, which were the eraser, membrane box, and polishing film samples of Sector A, as well as one of the AmBic and acid etch extracts of sample 66. An additional eight extracts could be identified to the level of Bovinae, while the remaining eight extracts could be identified up to the level of *Bos* sp. (Fig. 5). To further investigate the robustness of the taxonomic identifications, we compared the site count, which is the count of amino acid identifications matching the taxonomic identification, and the relative protease intensity of the SPIN samples (Fig. 9). The relative protease intensity is the intensity of trypsin divided by the total protein intensity observed in the sample. The lower the relative protease intensity, the greater the expected abundance of other proteins present in the extract. For many of the extracts, these two metrics display an inverse relationship, indicating that an extraction of low endogenous protein abundance is limited in its use for taxonomic identification. However, the membrane box extracts of Sector B are an exception to this pattern. Although the membrane box only extracts limited amounts of protein, the protein it extracts is suitable for taxonomic identification. No differences were detected between sampling methods (Kruskal Wallis, $p = 0.357$), however, as there are very few observations per sampling method any statistical results must be interpreted with caution.

By applying SPIN we were able to provide a taxonomic identification using all tested methods for the Sector B specimens. The excellent performance of the minimally invasive protocols for Sector B is the opposite of their performance for Sector A. Only one AmBic, one acid etch (neutralisation) and the HCl extracts yielded a taxonomic identification for these samples, all of which were to the level of Bovinae (Fig. 5). These results suggest that the SPIN analysis of minimally invasive protocols will only work well in well preserved contexts.

4. Discussion

The key aims of this study were to evaluate the proteomic outcome of seven sampling protocols and their degree of invasiveness. To this end we analysed 10 bones with MALDI-ToF MS and four with LC-MS/MS analysis in combination with 3D scans and microscopy from two different environment contexts at the Early Neolithic site of La Draga.

It is evident that the environment of the material's deposition context affects the proteomic output regardless of sampling approach or palaeoproteomic analysis, which is also evident in other studies (Wadsworth et al., 2017). It was possible to taxonomically identify all samples from Sector B (waterlogged environment) to the lowest possible taxonomic level achievable with ZooMS, *Bos/Bison*. Similarly, SPIN could assign all samples to either Bovinae (subfamily) or *Bos* sp. (genus). This stands in opposition to the low success rate observed in Sector A. Here, most samples were not given any taxonomic identification, except for the HCl protocol where almost all samples could be determined to the lowest taxonomic level possible via both ZooMS and SPIN. The same sector difference persists in the number of peptide markers and monoisotopic peaks observed in the ZooMS spectra, as well as in diversity and abundance of the proteome measured by LC-MS/MS. These results highlight the magnitude of both the effect that the archaeological context has on protein recovery, as well as of the heterogeneity of preservation conditions within the same site.

Though SPIN has the potential to distinguish between *Bos* sp. and *Bison* sp., allowing for more specific taxonomic identifications than ZooMS, our results show that for the Sector A samples and the acid etch (neutralisation), eraser, and one of the polishing film extracts of Sector B, SPIN is less specific than ZooMS. Though LC-MS/MS is more sensitive than MALDI-ToF MS, ZooMS may be better suited for low protein concentration samples as it relies on a set of spectral fingerprint biomarkers opposed to SPIN which requires well resolved MS2 fragment ion spectra (Buckley et al., 2009; Rüther et al., 2022). A notable exception to this pattern is posed by the results of the membrane box, which was the second poorest performing approach in terms of number of identified proteins and had the lowest SPIN site counts, but nevertheless yielded more specific identifications for Sector B than the acid etch (neutralisation), eraser, and polishing film protocols. Interestingly, the better performance of ZooMS over SPIN contrasts with the previous application of SPIN to Palaeolithic Portuguese sites, which showed no cases of ZooMS providing more specific taxonomic identifications than SPIN. Here, each extract had the same amount of peptides for SPIN and ZooMS (Rüther et al., 2022). Though the Portuguese samples were treated using another protocol than in our study, other studies conducted on Late Pleistocene specimens indicate that the HCl and AmBic protocols may be better suited extraction protocols for SPIN when dealing with poorly preserved specimens (Mylopotamitaki et al., 2023, 2024). Additionally, ZooMS outperforming SPIN could partly be explained by the different MS intrumentation's sensitivity to high salt concentrations, though this is conjecture. Nevertheless, the ZooMS and SPIN results from this study are not a one time occurrence. Other specimens from La Draga, selected for both SPIN and ZooMS analysis, had more specific taxonomic identifications within the ZooMS analysed samples than for the SPIN samples as well (Le Meillour et al., 2024).

Furthermore, we wanted to assess the invasiveness of the various sampling protocols as well as their ability to extract proteins. Studies using archaeological enamel acid etching have been successful in extracting protein and macroscopically the method seems to be minimally invasive (Stewart et al., 2016), but a recent study suggested that acid etching may be more invasive than previously thought (Brůžek et al., 2024). Their results indicated a 10% loss of enamel and a 2% loss of dentine in the affected area. Additionally, microcracks in the enamel surface widened after acid etching (Brůžek et al., 2024). The damage caused by acid etching to the bone samples in this study is even more apparent as the outer layer of the bone has been completely removed. Therefore, for bone, the acid etch protocol should not be considered minimally invasive. Among the minimally invasive approaches included in this study, we observed no substantial changes in bone volume, although similar to previous studies we did note microscopic surface alterations caused by the eraser and polishing film (Kirby et al., 2020; Sinet-Mathiot et al., 2021) as well as residue leftovers. These surface alterations may complicate future use-wear analysis. The membrane box protocol is the closest approach to a truly non-destructive sampling approach, though it is a lengthy and more expensive procedure.

Regarding their ability to extract proteins, the minimally invasive protocols and the acid etch (neutralisation) protocol are outperformed by the conventional HCl and AmBic protocols both in the number of proteins and their intensity as shown by the LC-MS/MS results. However, the ZooMS results, accounting for peptide markers and the specificity of taxonomic identification, are more complicated. The HCl approach performs well on all the samples regardless of preservation, while the AmBic and acid etch (neutralisation) protocols place themselves with the minimally invasive protocols (eraser, membrane box, and polishing film). These protocols perform just as well as the HCl protocol on the Sector B samples, but fail to do so on the Sector A samples, though the acid etch (neutralisation) appears to extract fewer peptide markers from Sector B in comparison to all other sampling approaches.

In this context, the eraser protocol seems to have worked better in our study than in other studies, as it performs similarly to the polishing film and the HCl and AmBic protocols for Sector B, while in other studies it often performs less effectively than both the polishing film and more destructive protocols (Evans et al., 2023; McGrath et al., 2019; Sinet-Mathiot et al., 2021). The ability of the polishing film approach to extract protein is well-attested in several other studies (Gilbert et al.,

2024b; Kirby et al., 2020), but has not often been compared to conventional destructive sampling. In the cases where it has been compared to destructive protocols it often recovers fewer peptide markers, but is still able to obtain taxonomic identifications in the vast majority of cases (Evans et al., 2023). However, conducting two separate scrapings utilising polishing film on the same area and then processing the second used polishing film may enhance chances of increased protein extraction (Gilbert et al., 2024b). Only one study has utilised the membrane box approach thus far, where it succeeded in taxonomic identification of four out of five samples (Martisius et al., 2020). In general, our results match the observation from previous experiments, where destructive protocols often recover more protein and yield more precise taxonomic identifications, but that minimally invasive protocols are frequently able to extract protein and produce taxonomic identifications as well.

We therefore recommend, when working with precious bone material, using one or several minimally invasive protocols on a small selection of specimens in cooperation with the curator or responsible person for the collection, weighing assets and liabilities of the sampling techniques. Considering the impact of intra-site variability in protein preservation, as evident in this study, the environmental and sedimentary conditions should be taken into account. Through minimally invasive ZooMS analysis the preservation state can be quickly and relatively cheaply investigated, without damaging the material, before assessing whether it is beneficial to continue using minimally invasive techniques or if it might be necessary to look at alternative and more invasive sampling methods. If more destructive sampling approaches are necessary, our study displays that the HCl protocol outperforms the AmBic and acid etch (neutralisation) protocols, and is thus the most adequate approach in this regard.

5. Conclusions

Here, we compared commercially available minimally invasive sampling techniques and their performance in extracting proteins from archaeological bone material. We found that the minimally invasive approaches, including the eraser, membrane box, and polishing film protocols, obtain sufficient taxonomic identifications through ZooMS (MALDI-ToF MS) and SPIN (LC-MS/MS) when there is an expectation of good collagen preservation. However, the minimally invasive protocols are outperformed by the conventional HCl approach on less well-preserved specimens.

We therefore advise that in sampling precious bone material, it is beneficial to process a small subset, taking into account intra-site variability, with minimally invasive sampling techniques through ZooMS to assess the preservation of the site. This enables the possibility to assess whether a continuation of sampling with minimally invasive sampling techniques (and which) can be executed, or whether only more invasive protocols should be considered in collaboration with the collection or excavation responsible personnel.

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CRediT authorship contribution statement

Jakob Hansen: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Joannes Dekker:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gaudry Troché:** Writing – review & editing, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Zandra Fagernäs:** Writing – review & editing, Validation, Software, Formal analysis. **Jesper V. Olsen:** Resources. **Maria Saña Seguí:** Writing – review & editing, Supervision, Resources, Formal analysis. **Frido Welker:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Formal analysis, Data curation.

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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Appendix A. Supplementary data

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