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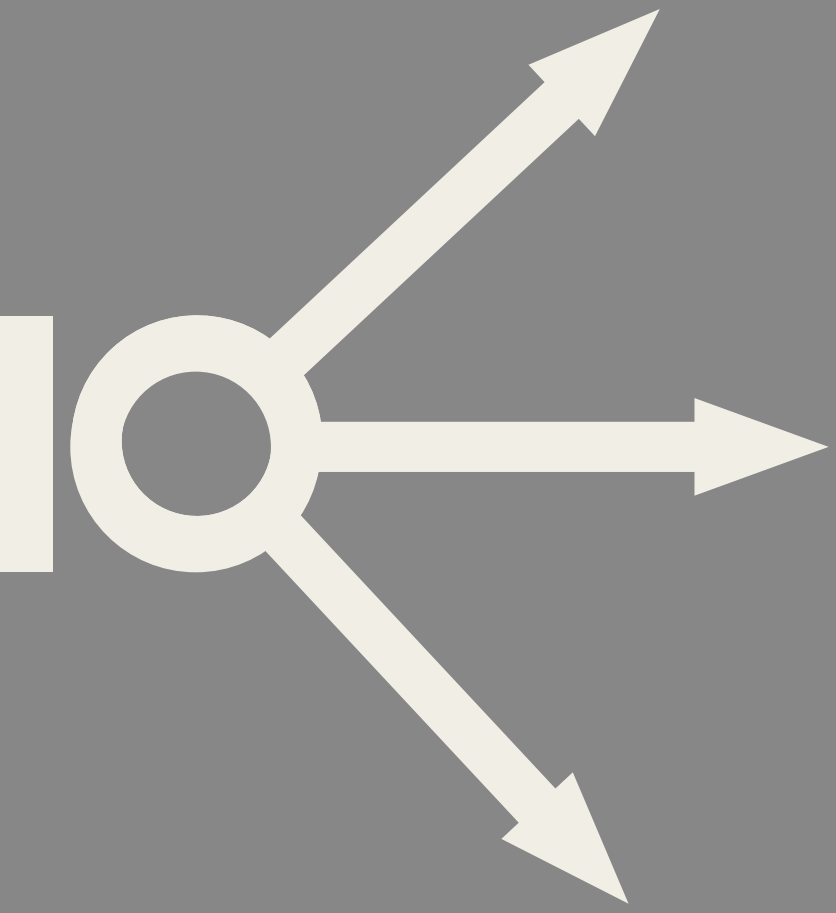
METHODOLOGY & ARCHAEOMETRY

Zagreb, 1st – 2nd December 2022



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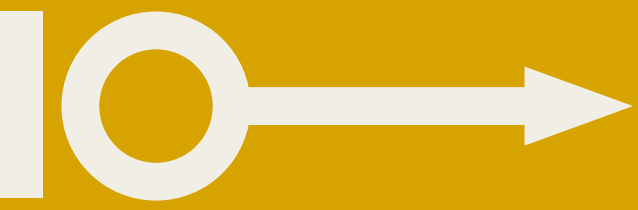

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Filling in the blanks: the application of palaeoproteomics in faunal analysis

Lia Vidas, Sara Silvestrini, Federico Lugli, Matteo Romandini, Cristina Real, Siniša Radović, Ivor Janković, Stefano Benazzi

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Lia Vidas
Ivor Janković
Centre for Applied Bioanthropology
Institute for Anthropological Research
Gajeva 32, Zagreb, Croatia
lia.vidas@inantro.hr
ivor.jankovic@inantro.hr

Sara Silvestrini
Matteo Romandini
Stefano Benazzi
Department for the Cultural Heritage
University of Bologna
Via degli Ariani 1, Ravenna, Italy
sara.silvestrini6@unibo.it
matteo.romandini@unibo.it
stefano.benazzi@unibo.it

Federico Lugli
Department of Chemical and Geological Sciences,
University of Modena and Reggio Emilia
Via Giuseppe Campi 103, 41125 Modena, Italy
federico.lugli@unimore.it

Cristina Real
Department of Prehistory,
Archaeology and Ancient History
University of València
Avda. Blasco Ibáñez, 28, València, Spain
cristina.real@uv.es

Siniša Radović
Institute for Quaternary Paleontology and Geology
Croatian Academy of Sciences and Arts
Ante Kovačića 5, Zagreb, Croatia
sradovic@hazu.hr

Faunal assemblages from archaeological contexts often consist of highly fragmented osseous remains which can be a limiting factor for the purposes of standard archaeozoological analysis. Therefore, efforts to further improve our knowledge of human—animal relationships in the past have been intensified in the last few decades. Apart from the already well-established ancient DNA analyses, in the past ten years, we have seen a rise in the use of palaeoproteomics in archaeology. Proteomic studies range from exploring whole proteomes of tissue or substrate (e.g., bone, enamel, shell) to detecting peptides to identify the taxon from the bone specimen. The latter can be achieved through Zooarchaeology by Mass Spectrometry (ZooMS), a technique of peptide mass fingerprinting (PMF), that aims at discriminating tissue rich in collagen type I from a taxonomic point of view. Collagen is a protein less prone to decay owing to its high abundance in osseous tissues and its arrangement into a highly stable triple helix making it suitable for ancient samples. ZooMS is fast, cost-effective, and is being explored and improved rapidly making it an accessory tool for improving and complementing morphological faunal analysis. Its effectiveness varies based on the taphonomic processes and the age of the sample itself but, generally, it has a significantly higher success rate than the DNA analysis. Here, we present the basic principles, history, possibilities, and limitations of proteomic studies in archaeology as well as preliminary results of the analysis conducted on the Late Pleistocene sites in Istria, Croatia, the first of its kind in the region.

Keywords: *palaeoproteomics, ZooMS, faunal analysis, Late Pleistocene, Istria*

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Introduction

In the attempt to accurately reconstruct life in the past, archaeology has become an inherently interdisciplinary science. Therefore, the field of biomolecular analyses, such as stable isotope, ancient DNA, lipids, and proteomic studies, is becoming crucial in archaeological research projects (van Doorn 2012; Cappellini et al. 2018). These analyses can give us insight into nutritional habits, trophic niches changes, migrations, and other forms of human adaptations in all periods of the past (van Doorn 2012). In the last ten to fifteen years, the study of ancient proteins has shown great potential as a rich source of information. Proteins are building blocks of all life and their robust morphology and abundance in various tissues are making their decomposition much slower in relation to, for example, the DNA molecule (Warinner et al. 2022). The rise and advancements in palaeoproteomics have been swift and today this type of research is being used in the fields of biology, palaeontology, palaeoecology, and archaeology, to name a few (Hendy 2021; Warinner et al. 2022).

In archaeology, its appeal is mostly recognized in the studies of prehistory. Archaeological contexts are notoriously rich in biomineralized tissues, such as bones and teeth, but sometimes also egg shells, skin, and keratinous tissues (van Doorn 2012; Buckley 2018a). The most abundant and common protein in vertebrates is collagen with most of its mass being stored in bones (Buckley 2018b; Shoulders & Raines 2009). Therefore, collagen is the most commonly targeted protein in proteomic studies which are cost-effective, relatively simple, and have a high success rate (Buckley 2018a). These are mostly being used to complement standard analysis, as those assemblages are often highly fragmented and therefore the success of "classical" archaeozoological methods can be limited. It is a fast-evolving field that will certainly result in more analyses and, consequently, in more precise results in the near future (Hendy et al. 2018).

A brief history of palaeoproteomics

The beginnings of what became the field of ancient proteomics is in the 1950s when the first amino acids have been found in the fossils of Devonian fishes, Jurassic and Cretaceous dinosaurs and Oligocene and Pliocene horses (Abelson 1954, 1956; Warinner et al. 2022). The first few decades of research were focused on amino acids, immunological approaches and the efforts to sequence ancient proteins (Buckley 2018a). Nevertheless, the real, rapid evolution of palaeoproteomics begins at the start of this millennium, with the first successful sequencing of the non-collagenous protein osteocalcin from the 53-thousand-year old bison (*Bison priscus*) bone (Ostrom et al. 2000). Later on, a few collagen peptides have been (debatably) identified in a dinosaur fossil dating to 68 million years ago (Asara et al. 2007). Still, the first significant use of ancient proteins in the field of archaeology was the taxonomic identification of faunal remains based on collagen peptide mass fingerprinting (Buckley et al. 2009). Since then, techniques and protocols for the successful extraction and differentiation of collagen peptides have started to develop. It was hoped these would broaden our understanding of human-animal relationships, ecological conditions of the past, and phylogenetic connections (Cappellini et al. 2018). In the last 13 years, proteomic analyses have successfully been applied for distinguishing sheep (*Ovis aries*) and goat (*Capra hircus*) bones (Buckley et al. 2010), a non-destructive protocol for collagen extraction has been developed (van Doorn et al. 2011), glutamine deamidation in collagen peptides was correlated with the thermal age of the samples (van Doorn et al. 2012), hominin fossils were identified in Pleistocene faunal assemblages (Brown et al. 2016; Welker et al. 2016; Douka et al. 2019), a bone ring from a Denmark Neolithic site was determined as red deer (*Cervus elaphus*) (Jensen et al. 2020), and many faunal assemblages have been broadened in at least their NISP, if not with new species using peptide mass fingerprinting (Welker et al. 2015; Sinet-Mathiot et al. 2019; Pothier-Bouchard et al. 2020; Silvestrini et al. 2022a, 2022b; Paladugu et al. 2023; Ruebens et al. 2023). Also, methods exploring other, non-collagenous proteins, such as amelogenin from the tooth enamel for sexing of human remains, have been developed (Stewart et al. 2017; Lugli et al. 2019, 2020).

Basic principles and application in archaeology

Proteome is the entire set of proteins in a single organism and *palaeoproteomics* is a discipline studying ancient proteins and proteomes, mostly in archaeological and fossil samples (van Doorn 2012; Warinner et al. 2022). Due to its abundance and stability, proteins can survive in biomineralized tissues for long periods of time and are a source of genetic information that are valuable for reconstructing phylogenetic relationships (Buckley 2018a). The advances in palaeoproteomics were made possible by the development of mass spectrometry and especially by advances in soft ionization methods and protein sequencing (van Doorn 2012; Buckley 2018a). Today, the MALDI-TOF MS (*Matrix Assisted Laser Desorp-*

tion/Ionization- Time of Flight Mass Spectrometer) and the LC-MS/MS (*Liquid Chromatography-Tandem Mass Spectrometry*) are most commonly used to detect single amino acid polymorphisms (SAPs) that cause protein sequence variation (Welker 2018). This variation allows taxonomic and phylogenetic analysis of ancient proteins (Welker 2018).

Generally, there are two main approaches to studying proteomes. On one hand, top-down proteomics analyses proteins in their unchanged form to gain information on post-translational modifications, isoforms, and other valuable information (Cappellini et al. 2018). This is a computationally complicated and, for now, high-end resource approach and is, therefore, not used for ancient samples that often (Cappellini et al. 2018). In archaeology, the second approach, known as bottom-up, is most commonly used as it identifies the protein sequence (Cappellini et al. 2018). Proteomes and proteins are more easily studied as smaller fractions, so most protocols use proteinases to digest the proteins into peptides that are up to 20 amino acids long (van Doorn et al. 2011).

Even though there are many different types of protein in vertebrates, the ones that can be found in bones and teeth are of special interest for archaeological purposes. The most abundant protein is collagen type 1 (COL1), a structural, fibrous molecule making 1/3 of all proteins in human organism (Shoulders & Raines 2009). Its water resistance and mineralized bone environment make its decay slower and is therefore a suitable candidate for palaeoproteomic investigations (van Doorn 2012; Buckley 2018a). In addition, due to its triple-helix structure, it is a highly stable molecule (Shoulders & Raines 2009). Apart from collagen, other proteins of significance for archaeological analysis include e.g. osteocalcin, albumin, and amelogenin (van Doorn 2012; Stewart et al. 2017; Cappellini et al. 2018).

Zooarchaeology by mass spectrometry (ZooMS)

As discussed previously, faunal assemblages are very often composed of a high percentage of bone fragments, drastically limiting their taxonomic identifications through the morphological approach. To overcome this, the so-called zooarchaeology by mass spectrometry, or ZooMS, was developed. It aims at discriminating tissues rich in COL1 from a taxonomic point, based on peptide mass fingerprinting (PMF) approach (Welker 2018). Namely, it measures the mass of taxon-specific peptides (markers) in the extract and is most often successful up to a genus level of determination (van Doorn 2012; Wang et al. 2021). It depends heavily on the available reference databases as the resulting MS spectra need to be inspected and compared to identify the sample. Today, it is mostly used for determinations of mammal bones, but the databases for birds, fish, reptiles and amphibians are being developed (Buckley 2018b; Richter et al. 2021). This is a fast and cost-effective method that is developing rapidly, suitable for complementing faunal analysis (e.g. Silvestrini et al. 2022a, b) but could also be a large-scale screening tool for identifying hominin fossils in large, fragmented osteological assemblages (Warinner et al. 2022). Also, it is a minimally destructive approach, given the fact that 10-30 mg bone chip usually yields enough collagen for successful analysis (Wang et al. 2021). It was shown that protocols including demineralization phase, usually using hydrochloric acid, have the highest success rate (Wang et al. 2021), but there are non-destructive protocols that can also yield accurate results (van Doorn 2012).

Preliminary results of palaeoproteomic analyses of faunal assemblages from Istria, Croatia

Apart from analyses carried out on the assemblages from layer G from the Vindija cave (Devièse et al. 2017), whose primary goal was the identification of new hominin remains, so far there has been no attempt to implement palaeoproteomics to osteological assemblages from Croatian sites. As a part of the PREHISTRIA project (HRZZ IP-2019-04-7821), there are ongoing excavations of two Late Pleistocene sites in Istria: Abri Kontija 002 and Ljubićeva pećina. ZooMS analyses for both, but also for the samples from the Romualdova pećina in the same region (Fig. 1), are underway and therefore represent the first attempt at systematically identifying fragmentary faunal assemblages, to help reconstruct the palaeoenvironment and subsistence strategies of hunter-gatherer groups that inhabited this region during different periods in the Late Pleistocene.

Ljubićeva pećina

Ljubićeva pećina is a vast cave located in southern Istria, with a wide entrance at the bottom of a karstic sinkhole, composed of two big chambers on two levels (Percan et al. 2020) (Fig. 2). Excavation of this site began in 2008 and the layers assigned to the Epigravettian period of the Upper Palaeolithic have been found in the side chamber of the upper level (Percan et al. 2009). Even though the sedimentation was slow, and layers with the evidence of

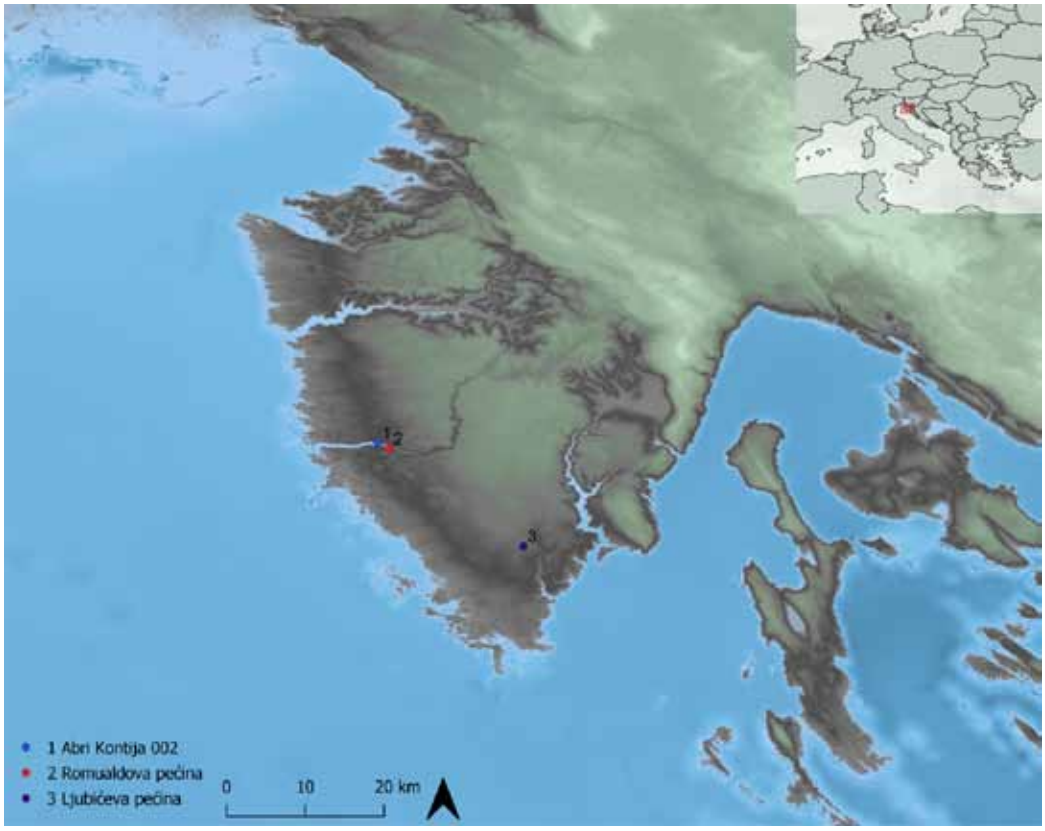


Figure1. Geographic position of the sites mentioned in the text. (Created by the authors).

Figure 2. The inside of Ljubićeva pećina. (Photo by the authors).

Pleistocene human activities are not too thick, they are rich in bone and lithic material, attesting to intense human occupation at different times.

Preservation of the osseous material is exceptionally good and traces of human activities, such as cutmarks, fractures and thermal alterations, are common. Due to the abundance of bone material, archaeozoological analyses are still ongoing, but we were able to identify medium and large herbivores, such as aurochs or bisons (*Bos/Bison*), horses (*Equus ferus*) and red deer (*Cervus elaphus*). On the other hand, remains of small carnivores, such as foxes (*Vulpes vulpes*), badgers (*Meles meles*) and pine martens (*Martes martes*) were also present (Percan et al. 2020). Still, the fragmentation of this assemblage is high which makes it a good candidate for the use of palaeoproteomic approach, and around 100 bones were sampled for ZooMS analysis. The available results are in accordance with the morphological analysis, showing a big prevalence of large bovids (Fig. 3). It also confirmed remarkably good preservation of bone samples, as none of them failed to provide identification on a scale lower than subfamily or genus. Future work using ZooMS on this assemblage will help us to better understand human occupation and exploitation of animal sources in the region during the Late Upper Palaeolithic.

Abri Kontija 002

Abri Kontija 002 is a rockshelter on the northern slopes of the Lim channel on the western coast of the Istrian peninsula (Janković et al. 2022) (Fig. 4). The trench, positioned close to the rock wall, in front of the cave entrance now filled with sediment, yielded an almost 2-meter-deep cultural sequence dating to Early/Middle Upper Palaeolithic period.

The layers are extraordinarily rich with archaeological material (lithics, bones, shells, ochre) suggesting the intense use of this area overlooking a river gorge during the Late Pleistocene. Faunal assemblage at this site is heavily fragmented and morphological analysis is therefore very limited. Nevertheless, remains of horses, red deer, hares (*Lepus europaeus* or *Lepus timidus*) and cave bears (*Ursus spelaeus vel ingressus*) have been identified so far. ZooMS analysis has been performed on around 100 unidentified bone fragments from the whole archaeological sequence of the site. For now, it seems that the uppermost layers do not have enough collagen preserved for successful determinations. On the other hand, samples from the rest of the sequence, from approximately layer 4 to layer 9, have produced enough collagen, and taxa like horses, red deer and large bovids have been identified (Fig. 5).

Figure 3. An example of the sample from Ljubićeva pećina, defined as Bos/Bison. Created by the authors

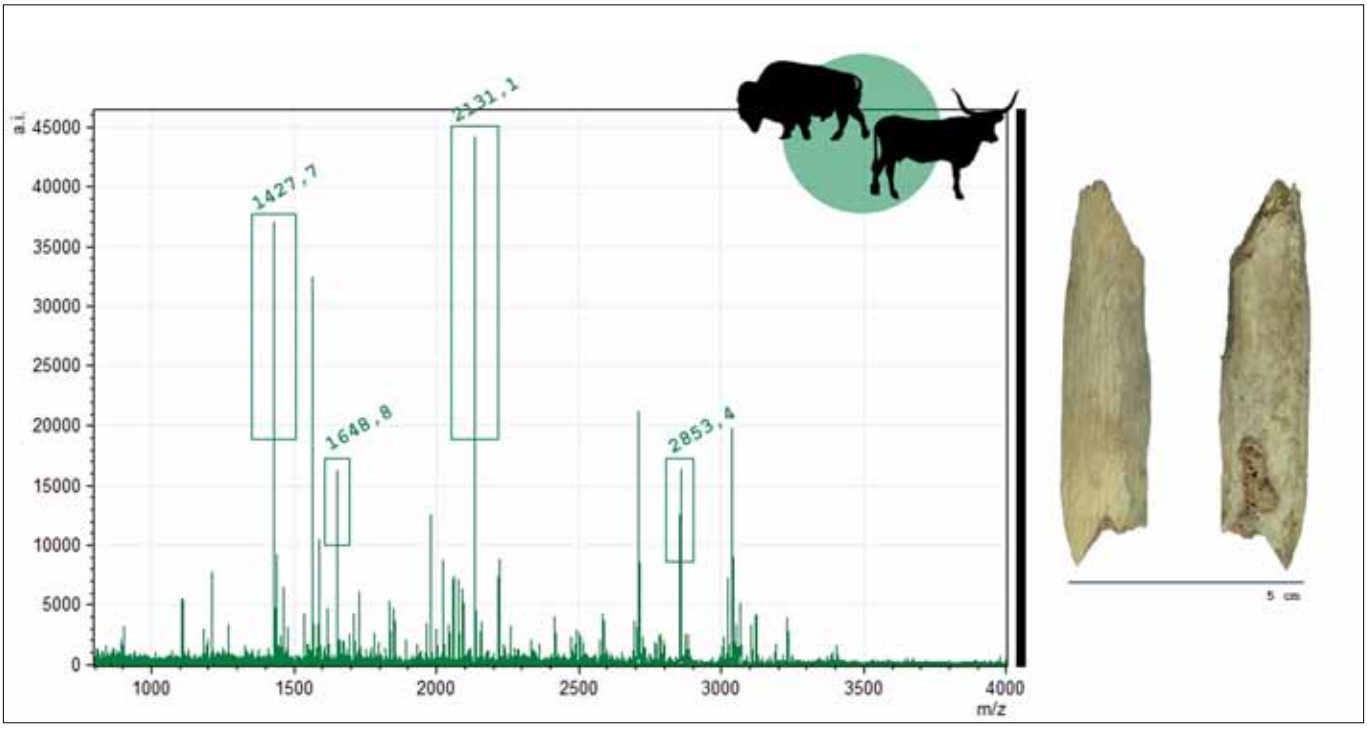
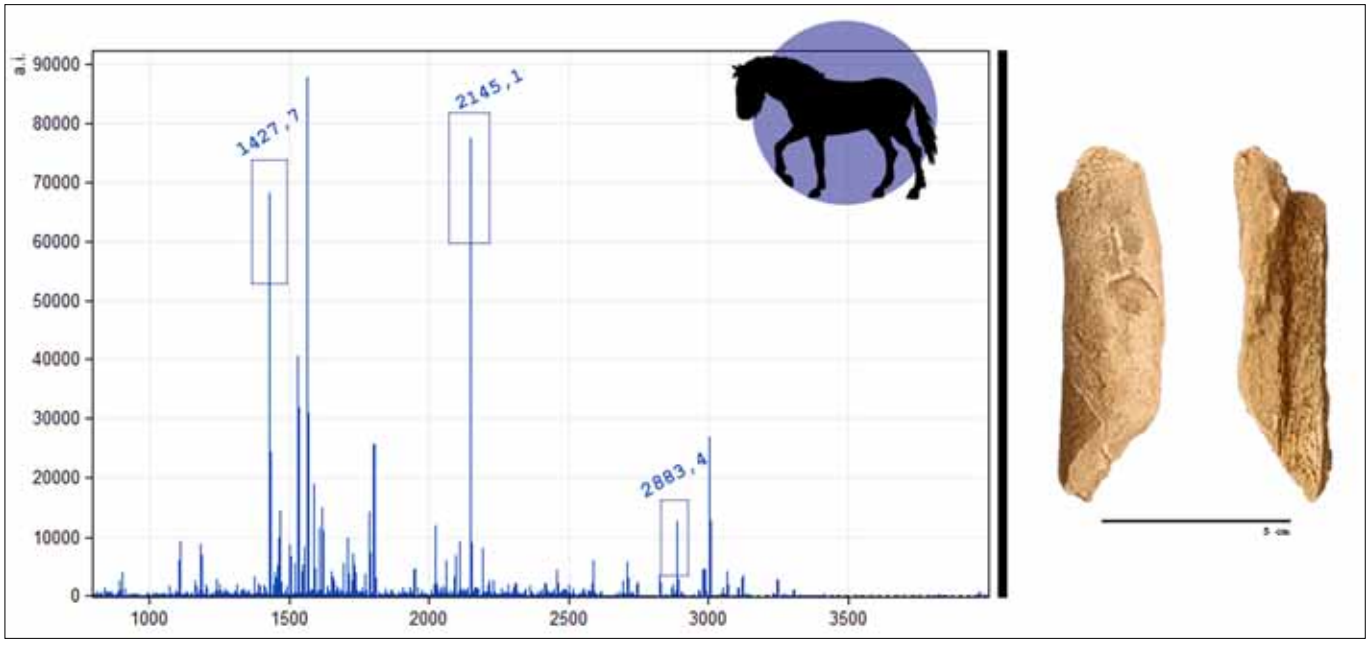


Figure 4. The site of Abri Kontija 002. (Photo by the authors).

Figure 5. An example of the sample from Abri Kontija 002, defined as Equus sp. (Created by the authors).

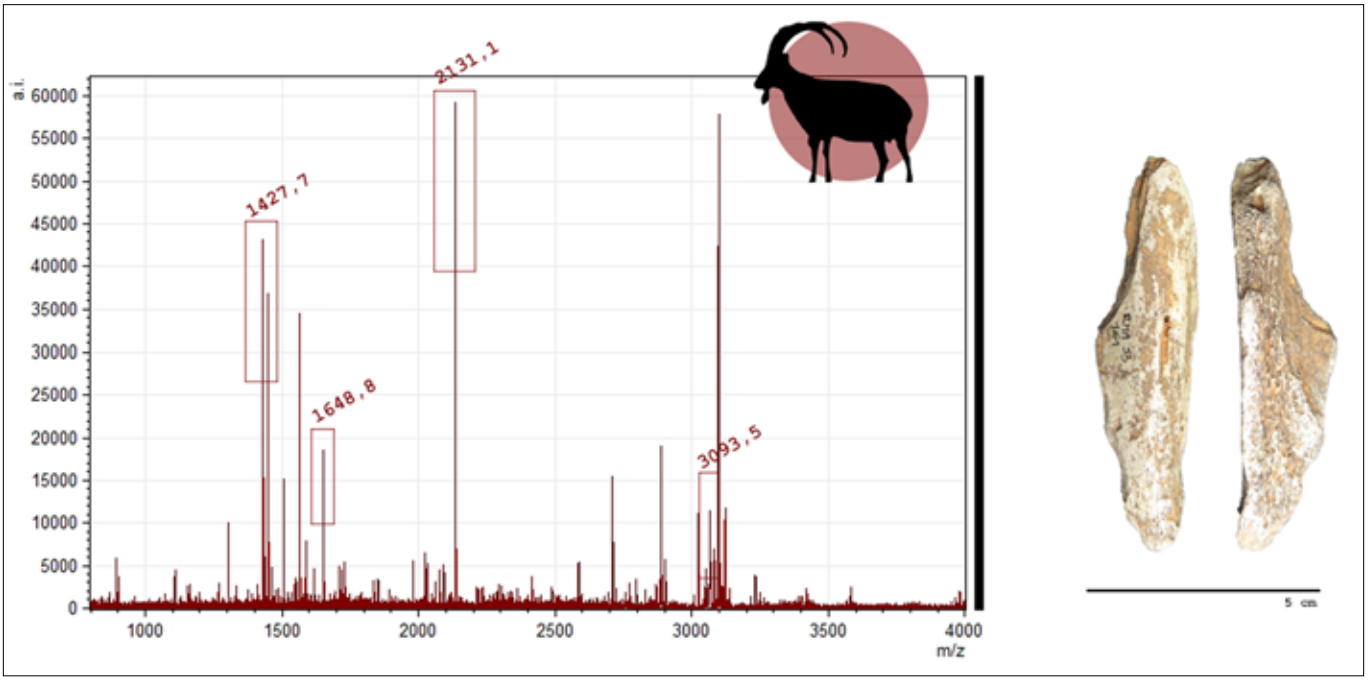


Romualdova pećina

Romualdova pećina is located in close vicinity of the Abri Kontija site, on the opposite (southern) slope of the Lim Channel. It is a cave of simple morphology, with a long hall that branches out into several smaller chambers (Janković et al. 2016) (Fig. 6). The stratigraphic sequence, testifies to episodes of sporadic human occupation during both Middle and Upper Palaeolithic, as well as later prehistoric periods (Bronze and Iron Age, see Janković et al. 2015).

Figure 6. The inside of Romualdova pećina. (Photo by the authors).

Figure 7. An example of the sample from Romualdova pećina, defined as Capra ibex. (Created by the authors).



Still, the primary visitors to the site were animals, mostly bears, that used the cave as a hibernation den. Nevertheless, lithic material is present at the site, as well as the faunal assemblage rich in bears, ibex (*Capra ibex*) and red deer. For the purposes of ZooMS identification, 35 bone fragments were sampled from the Middle Palaeolithic layers. Even though the state of preservation seemed to be poorer at this site, with common manganese coating, 33 samples were successfully identified to a subfamily or genus level, with bears and ibexes being the most common ones (Fig.7). Yet, further analyses are needed, but the current results are optimistic even for

this site with different, more complicated post-depositional conditions. Also, analysis of sediment DNA from the site is in progress and already yielded results, confirming the presence of several different Pleistocene taxa.

Discussion and conclusion

In general, ancient biomolecules are an invaluable source of information about the past. In particular, much work that used the biomolecular approach has been done on issues on modern human origins and various archaic human groups, demography, species extinction, exploitation and domestication of plants and animals, phylogenetic relationships of extinct species, palaeoclimate and palaeoenvironmental reconstruction, and so on (Cappellini et al. 2018). While being the most valuable in terms of information gaining, the DNA molecule is also very prone to decay and analyses are often complicated and expensive. On the other hand, analyses of ancient proteins have been proven to be cost-effective and often more successful. In addition, ancient proteins could contribute to our understanding of phylogenetic relationships in geographic regions where the DNA survival is poor (Cappellini et al. 2018). Furthermore, palaeoproteomic analyses can be combined with other collagen-based approaches, such as radiocarbon dating and stable isotope analysis, which minimizes the destruction of the sample (Hendy 2021). These approaches can thus have an impact on some of the long-lasting questions relating to archaeological science, such as the timing and nature of water-resource exploitation or raw-material choices in regard to the worked-bone industry (Buckley 2018b). Future proteomic analyses on different types of materials and from different contexts will allow a better understanding of the agents preventing or speeding up the degradation of proteins (Hendy 2021). Current evidence suggests that stable, cold and anaerobic environments are more likely to preserve collagen, than hot, highly-seasonal and humid conditions (Warinner et al. 2022).

Particularly useful for taxonomic identification of large assemblages of fragmented collagen-rich archaeological materials is the so-called zooarchaeology by mass spectrometry (ZooMS). Having the ability to discern taxa based on peptide mass fingerprints is being used more and more among researchers of all periods, but especially prehistory. In less than 15 years since its implementation, ZooMS has found its niche in the research of faunal assemblages from mid-tempered Late Pleistocene European sites, such as Les Cottès (Welker et al. 2015), Grotte du Renne (Welker et al. 2016), Grotta di Fumane (Sinet-Mathiot et al. 2019), Uluzzo C, Riparo del Broion (Silvestrini et al. 2022a, 2022b), Salzgitter-Lebenstedt (Ruebens et al. 2023) and so on. Also, the efforts to further improve, standardize and optimize analytical protocols and interpretation of the results are continuous. For example, a non-destructive protocol for collagen extraction has been developed only two years after the first successful application of PMFs for taxonomic identification (van Doorn et al. 2011). Still, there is a need for more in-depth sequencing of collagen type 1 in the hope of identifying new variations among more closely related species, as well as improving data for classes of birds (Aves) and fish (Pisces) (Richter et al. 2022). The lack of centralized, accessible, and complete databases of all known peptide markers is a somewhat limiting factor, making the work slower and more prone to mistakes (Brown et al. 2021). Because of the fact that the rate of changes in protein sequence is slower and less frequent compared to that in genes, proteins have a smaller resolution potential (Warinner et al. 2022). Therefore, species that diverged from one another less than five million years ago, at the moment cannot be differentiated using peptide fingerprinting (Buckley 2018b). Nevertheless, the dating of the observed sequence, site formation processes, and information on palaeoenvironment can oftentimes help eliminate some of the taxa (e.g. comparing the Pleistocene and Holocene samples). Still, methods like ZooMS stand out as a valuable approach and its fast development will most likely result in better precision of the results in the upcoming years. In order to maximize efficiency of these, and other methods, field and laboratory work needs to be optimized and the communication among researchers prompt and clear. Reporting on the protocols and materials used in specific analysis is still uneven and should be made obligatory when publishing the results (Hendy 2021). In the end, it is crucial to combine the results of ZooMS with the results of morphological analyses of faunal remains in order to better understand the context of the site. It should be mentioned that ZooMS cannot increase categories like the MNI (Minimal Number of Individuals) and the MNE (Minimal Number of Elements), but it can increase the NISP (Number of Identified Specimens).

Even though still relatively new, and not without limitations, palaeoproteomic approaches are successfully being implemented as an archaeological accessory tool (Buckley 2018a). The chemical and biological characteristics of proteins make them one of the more robust biomolecules with high survival rates for hundreds of

thousands of years. Therefore, their use in the realm of prehistory does not come as a surprise. For the Palaeolithic, they could have a big impact on reconstructing environmental conditions, subsistence strategies, mobility patterns, and so on. Their high compatibility with morphological archaeozoological, but also palaeogenomic analyses, makes them one of the most promising fields of interdisciplinary research. At the moment, no systematic efforts were performed to implement methods like ZooMS in southeastern Europe. Therefore, we hope that the preliminary results presented in this paper will contribute to the understanding of major issues of the Pleistocene research in the region, and that they soon will become a standard in the archaeological analysis of faunal material. The examples listed here prove that faunal assemblages from Croatian sites, at least those in the region of Istria, have a substantial amount of collagen preserved which enables the taxonomic identification up to the genus level for most samples.

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