

# Bone Proteomics In Forensic Investigations: Advances, Challenges, And Methodological Innovations

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## Abstract

Similarly to their forensic application, for which bone remains are an invaluable source of biological information, traditional analytical techniques, mainly DNA analysis, frequently lacks the sensitivity to overstep the challenges with aged or environmentally challenged material. Bone proteomics – the mass analysis of proteins preserved in skeletal tissue – has become a powerful complementary tool, taking advantage of the longer-term durability of proteins in comparison with nucleic acids. Here, we have consolidated the state of the art of forensic bone proteomics concentrating on important developments, persisting challenges and major methodological refinements. Bone proteome analyses for PMI, age-and sex determination and species determination have shown a rapid progress focusing on the analysis for certain post-translational modifications of proteins (e.g. Biglycan deamidation) or protein markers (e.g. Fetuin-A, Amelogenin, COL1A2). Nevertheless, some challenges exist for the application of the field. These challenges include the intricate interplay of diagenetic factors affecting bone composition, and of diverse environmental conditions (temperature, pH, humidity, burial context) and recycling of skeletal proteins on survival, exogenous proteome mixtures, and endogenous proteome signals, biological inherent variability among individual organisms, and robust bioinformatic approaches for analysis of often low-abundant or modified peptides. Moreover, inter-comparison and implementation of these methods are difficult due to the absence of standardized, validated protocols among different laboratories. Recent methodological developments, like improved protein extraction protocols (S-Trap workflow for instance), novel MS acquisition strategies (as data independent acquisition approach DIA) and complex bioinformatics pipelines, are attempting to overcome these issues. Future work, including biomarker validation, pathway elucidation, contamination control, workflow optimization and forensic protein databases work are required to translate the potential of bone proteomics to practical and robust tools for forensic investigations.

**Keywords:** Forensic Science; Bone Proteomics; Mass Spectrometry; Post-Mortem Interval (PMI); Diagenesis; Method Validation; Forensic Identification.

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## INTRODUCTION

Skeletal remains are commonly encountered in the context of forensic science and represent a permanent record of biological information, which is invaluable in the identification of unknown individuals, recovery of information regarding the circumstances surrounding death, and the reconstruction of events of the past. Due to its mineralized nature, bone often remains long after the decomposition of soft tissue and thus becomes a major focus in forensic and archaeological contexts [1]. Bone has been analyzed forensically based on morphology and, in recent years, through DNA. However, DNA can be extremely sensitive to degradation caused by a range of environmental variables such as changing temperature, humidity, pH, and microbial activity, and analyses from aged or poorly preserved skeletal remains typically return inadequate or uninformative results [2,3,4]. This limitation has prompted the development of new molecular methods that can recover informative data from difficult samples.

Proteomics, which is involved in investigation of all proteins produced by an organism or tissue, is a promising field. Proteins, especially those that are entrained within the bone or tightly bound with mineral components, are more stable and long-lived on taphonomy scenarios than is DNA [1, 4, 6, 7]. This increased persistence of proteins is exploited in the field of forensic bone proteomics, in which modern mass spectrometry-based methodologies are used to detect and measure proteins and their post-translational modifications (PTMs) from skeletal remains [1, 12]. This protein-level information may carry significant implications regarding the time since death (post-mortem interval, PMI), the age-at-death

(AAD) of an individual, biological sex, species of origin, and might also offer a snapshot genotypic information based on genetically variant peptides (GVPs) [1, 9, 10, 7, 12]. Thus, bone proteomics is a valuable add-on (or even an alternative) to the established forensic expertise, especially when DNA work is unsuccessful [5, 12, 14]. This review seeks to present a detailed summary of the current state of bone proteomics in forensics, detailing the major achievements that have been accomplished, assessing limitations and challenges in present approaches, reviewing recent methodological developments for addressing these, as well as an outlook for research and application.

### **Materials and Methods of Forensic Bone Proteomics**

The successful application of proteomics to problematic forensic bone material involves highly standardized and meticulously followed methodologies ranging from the reception of the sample to the interpretation of data. Although the individual protocols can differ based on the experimental question, sample state, or lab facilities, a number of basic steps and approaches are frequently used, as well as new advances to increase sensitivity, reproducibility, and throughput [2, 4, 8].

#### **Sample Preparation and Pre-treatment**

Forensic bone samples tend to be contaminated with soil, soft tissue remains or other environmental clutter. Preparation typically starts with careful mechanical cleaning of the surfaces to remove exogenous material, and wash with mild abrasives or extensive rinses to decontaminate surfaces with minimal removal of potentially informative outer bone and introduction of new materials. After which, the bone is generally crushed or comminuted to powder in order to maximize the accessible surface area for chemical treatments [4, 8]. One approach to achieve this is using the cryogenic grinding technique, or aseptic drilling in order to reduce the amount of heat transfer to bone tissue that may result in protein denaturation or in the formation of artificial modifications [6, 8].

#### **Protein Extraction**

The removal of proteins from the dense mineralized matrix of bone is a crucial and frequently difficult process. The main constituent of the bone is hydroxyapatite, which can bind a variety of proteins. Accordingly, demineralization is typically performed to release the proteome [4, 8]. The process of demineralization is generally done gently, often with ethylenediaminetetraacetic acid (EDTA) at a neutral or slightly basic pH to retain protein structure, although acidic techniques have also been used [14, 8]. Proteins are then solubilized by a variety of lysis buffers after demineralization - most often lysis buffers are formulated with strong denaturants such as urea or SDS and reducing agents (e.g. DTT) which break disulfide bonds then titrated with alkylating agents (e.g. iodoacetamide) which prevent the covalent reformation of those bonds [4, 8].

Various procedures for aiding removal, digestions, and peptide cleanup are available. Conventional procedures frequently included filter-aided sample preparation (FASP) or in-solution digestion with subsequent peptide purification by solid-phase extraction, i.e., C18 cartridges or in-StageTip (iST) based approaches, for example ZipTips [14, 8]. Though powerful, such approaches are often time-consuming and at risk for loss of sample during the transfer steps, and do not lend themselves easily to scaling up for high-throughput analysis [8]. Even more recently, suspension trapping (S-Trap) protocols have been modified and refined for forensic bone proteomics. S-Trap protocols rely on a porous quartz matrix in a spin column format that enables protein capture by centrifugation (spinning), washing, on-matrix proteolysis (usually using trypsin) and elution of peptides in a single tube, thereby decreasing manipulation, reducing loss of material, and increasing the reproducibility, while potentially shortening the processing time [8, 15]. Optimization studies have most typically sought to compare alternative lysis buffers (e.g., levels of SDS) for S-Trap technology to achieve higher levels of protein recovery, while minimizing artificially induced PTMs (e.g., deamidation) [8].

#### **Mass Spectrometry Analysis**

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the workhorse method by which the complex peptide mixtures derived from bone protein extracts are analyzed [1, 4]. It is normal that peptides are separated by their hydrophobicity using reversed-phase liquid chromatography and then ionized (often employing electrospray ionization, ESI) and introduced into the mass spectrometer.

There are two principle strategies for data acquisition; data-dependent acquisition (DDA) and data-independent acquisition (DIA). The mass spectrometer in DDA scans the incoming peptides and isolates and fragments the most abundant species (typically top N) for MS/MS spectra analysis in a cycle. Although successful in the routine detection of highly abundant proteins, DDA still suffers from a considerably stochastic selection which results in a large number of missing values for less abundant peptides in

different runs [8, 3]. Much more aggressively, DIA and/or DIA cuts every single peptide at every given mass-to charge ( $m/z$ ) window throughout the entire chromatographic run. This leads to more challenging MS/MS spectra, but more complete and reproducible quantification, especially for low-abundance proteins, although it requires more advanced data analysis approaches [8,15,3]. Recent work indicated that DIA, in combination with optimized extraction strategies such as S-Trap, provides for more sensitive and more reproducible analyses in the context of forensic bone proteomics [8, 15].

#### **Data Analysis and Interpretation**

The raw MS data need to be computationally processed in order to match them to peptides and to deduce the presence and abundance of proteins. Typical pipelines for processing are to search the MS/MS spectra against a protein sequence database (eg, UniProtKB/Swiss-Prot) with e.g., SEQUEST, Mascot, or MaxQuant [10, 4]. Such searches compare observed spectra to spectra of theoretical peptides generated from sequences from a sequence database, taking into account possible post-translational modifications (PTMs; e.g., deamidation, oxidation, phosphorylation) and enzyme cleavage specificity (e.g., trypsin). For species discrimination, a focused search using species-specific protein reference databases, such as COLIA2, would allow a 'protein barcoding' strategy [10].

For DIA-based analyses similar but not the same applications are used (e.g., Spectronaut [11], DIANN or OpenSWATH) and these are mostly dependent on the use of spectral libraries that have been created from previous DDA-based libraries of spectra or created in silico in order to make use of these complex DIA-based summaries to identify and quantify the peptides [8].

The bioinformatic analysis is of paramount importance, in order to filter the obtained results on the basis of the confidence scores (e.g., the False Discovery Rate, FDR), to quantify the proteins (label free quantification is very frequent), to find statistical differences between groups of samples, and to search for particular biomarkers related to PMI, AAD, the sex (e.g., the peptides of the amelogenin) or the species [6, 9, 7, 4]. Dealing with PTMs, discriminating between endogenous proteins and microbial contaminants and compensating for diagenetic changes remain major bioinformatic obstacles [6,1,12].

#### **Quality Control and Standardization**

Quality control Quality control is essential because MS is sensitive porous and insane require sample preparation and often begins to conduct analysis on detective and possibly contaminated forensic samples. These may involve separate laboratory locations and reagents, processing of blank controls with samples to track background contamination, use of standardized methodologies, or in certain instances the use of internal standards. Method validation and standard operating procedure development are key continuing lines of development to secure the desired level of reliability and inter-laboratory conformity necessary for forensic use [1, 8, 15]. Optimization publications that compare various extraction and acquisition protocols, e.g. those of Gent et al. [8,15], are important milestones to this standardization.

### **RESULTS:**

Several important results, of great forensic concern, have already been obtained by implementing proteomic analysis on skeletal remains. The most significant reported successes and established applications are summarised in this section from the reviewed literature.

#### **Post-Mortem Interval (PMI) Estimation**

Proteomics has shown a great potential for the estimation of the PMI from skeletal samples, as it provides molecular markers that undergo a predictable pattern of change in time. Time-dependent degradation patterns of several bone proteins have been identified in studies [15]. Certain post-translational modifications (PTMs), such as the deamidation of asparagine and glutamine residues in Biglycan, have demonstrated a measurable link with increasing PMI in controlled and simulated forensic scenarios [5, 9]. Studies of decayed human remains that decomposed under natural conditions have also supported protein degradation and modification patterns as PMI indicators, and have shown that environmental factors affect the rate and progress of protein decay [6]. Reduction in the abundance of specific non-collagenous proteins, that are possibly derived from those of blood or muscle which were once associated with bone, has also been detected and related to PMI [9].

#### **Age-at-Death (AAD) Estimation**

In addition to the conventional morphologic approaches, bone proteomics is a possible source of molecular biomarkers for AAD estimation. The protein Fetuin-A, also known to be involved in bone mineralization, has repeatedly been found in different stainings in other studies and might have its absolute concentration or find own Object cts of modification also a reference to the real biologic age of

the individual at time of death [5, 9]. Currently, other aspects of bone proteomic profile are still being explored in connection to changes associated with aging [6].

### **Biological Sex Determination**

Proteomics offers an extremely accurate sex determination technique, especially, when the amelogenin protein isoforms (AMELX and AMELY) are investigated from tooth enamel [7]. The enamel's toughness guards these peptides to a point where 15 sex-specific peptides permit the sexed determination even when the DNA has degraded to alarming low quantities. Sex estimation based on amelogenin with proteomic technology has shown a high rate of success when compared with osteological and low-coverage genome analysis in comparative studies of archaeological remains, particularly fragmentary or subadult individuals [7].

### **Species Identification**

The identification of human and non-human bones is a basic issue in forensic practice, proteomics clearly provides a number of advantages compared to the morphological approach, particularly in the case of fragmented bones. Species-specific differences in amino acid sequences and in particular those of the common collagen proteins are stable markers. One notable outcome is the establishment of "protein barcoding" based on Collagen Type 1 Alpha 2 (COL1A2) [10]. Taking advantage of species-specific peptide sequences in COL1A2 via regular shotgun proteomics and customized database searching, it has been demonstrated that precise and automated species identification can be made in a wide variety of vertebrate taxa [10].

### **Individual Identification**

In its infancy and in need of further refinement, bone proteomics demonstrates a strong potential for individual identification through the evaluation of genetically variant peptides (GVPs) [11, 14]. They are composed of amino acid variability based on a person's unique genetic code – a possible DNA-independent identification pathway (although issues do exist in the ability to get sufficient coverage of the proteome and data robustness in the data-analysis) [14].

## **DISCUSSION**

The successful use of proteomic analysis on ancient remains, as reported in the Results section, is a major step forward in forensic science. It provides molecular information complementary to, or in some cases competitive with, standard osteological and genomic approaches, in particular in cases involving degraded specimens or the analysis of difficult samples [3, 4, 5]. The intrinsic stability of proteins, some even of structural nature such as collagen or stabilized by incorporation into the hydroxyapatite matrix, commonly lead to their prolonged post-fossilization preservation even within less favorable taphonomy records or environments with fragmented DNA to a degree that is then not of use [4, 6, 7, 1]. While the promise is evident, for bone proteomics to become established in casework represents a considerable hurdle. This commentary elaborates on the implications of this in the face of the prominent challenges, methodological advances, and future directions at play in the field.

### **Challenges and Limitations**

Despite the positive results described above, there are several practical limitations preventing the regular use of bone proteomics in forensic practice.

**Diagenesis and environmental effects:** "Bone" is not an inert material; it is subjected to a complex series of post-mortem, climate induced diagenetic changes [1, 12]. Soil pH, temperature variation, moisture concentration, bacterial activity, and burial type (i.e., inhumation or entombment) seriously affect protein presence [6, 1, 12]. Acidic conditions can solubilize the mineral matrix and alkaline conditions can speed up the degradation of collagen [1]. Protein burial patterns are further complicated by waterlogging, redox potential, and freeze-thaw cycles [1]. Comparisons of inhumed versus entombed! remains! have revealed differential preservation of certain no collagenous proteins, underscoring the importance of considering the burial environment when interpreting proteomic data [12]. As chemical leaching, microbial degradation, and mineral recrystallization are interrelated, it may be challenging to predict how this interaction affects protein preservation and how it is incorporated into quantitative data, such as used for PMI measurement [1].

**Contamination:** Forensic samples are inherently susceptible to contamination from multiple sources: environmental ( eg, soil microbiota, fungi), handling at retrieval and during analysis, and cross-contamination in the laboratory [4, 13]. Exogenous microbial proteins may dominate the native bone proteome, thereby confounding data analysis and results, and potentially resulting in the

misidentification of biomarker or wrong species identification [4]. True endogenous protein identification from contaminants involves sensitive experimental techniques (i.e., stringent washing conditions, background controls) and complicated computational filtering methods [4].

**Degradation/Modification of Proteins:** Endogenous proteins are subject to degradation and modification processes after death, which are both enzymatic (autolysis, microbial activity) and non-enzymatic (e.g., hydrolysis, oxidation, deamidation) [5, 6, 9, 1]. Although some modifications (e.g. deamidation) are being considered as PMI markers, uncontrolled degradation creates decreases in protein yield and sequence coverage, and identification is difficult, particularly for low-level proteins or variable regions required for individualization [14]. In addition, technical artefacts such as heat or pH extremes during extraction leading to deamidation might give biased information and confuse biological relevant PTMs crucial for PMI or AAD estimation [13, 8]. Knowledge of these degradation pathways is important for a proper interpretation [15].

**Biological Variability:** Humans exhibit variability in bone composition and proteome as a result of age, sex, and health conditions, and genetics [6]. So, one may assume (or not) that BMD (bone mineral density), as an example, had indeed been linked to muscle mass in relation to protein preservation and you just, then, added another wrinkle to all when somebody wants to compare people, still looking for a common biomarker for traits as difference as PMI or AAD [6]. Knowledge of this intrinsic variation is essential to inform the development of robust predictive models relevant for different populations.

**Standardization and Validation:** The main challenge is the unavailability of standardized protocols in different laboratories [5, 8, 13]. Not only do differences in sample preparation, extraction techniques, MS parameters, data analysis software and databases exist, but the comparisons between results are extremely challenging and validation is found to be complicated. The development of standard operating procedures, reference materials, and proficiency testing programs is necessary to support and ease the transfer of bone proteomics from research laboratories into accredited forensic facilities [5,13,8].

**Complexity of the data analysis:** Proteomic experiments are generating large and complex dataset. Determination of proteins/peptides that occur at low abundance, quantification of changes therein, interpretation of complex PTM patterns, and separation of signal from noise (including contaminants) require bioinformatics expertise and sound statistical methods [3, 4, 8]. The implementation of easy to use and validated software pipelines specifically for the forensic arena is emerging and required to ensure widespread adoption [8].

### **Methodological Innovations**

Cutting-edge approaches are being engineered by researchers to overcome these obstacles.

**Optimized Extraction Protocols** Given the limitations of traditional protocols, substantial work has been devoted to developing optimized protein extraction methods from bone. The adaptation and validation of S-Trap have been a major breakthrough with increased protein recovery, lower sample handling, improved reproducibility and feasibility for more high-throughput than either filter-aided or in-solution digestion with tip-based cleanup [8, 15]. Lysis buffers are carefully chosen and digestion conditions are optimized in order to achieve maximal digestion combined with minimal artificial PTMs [8].

**Advanced techniques in mass spectrometry:** The transition to DDA versus Data-Independent Acquisition (DIA) offers a potential for improvement over 429 DDA [8, 11, 15]. DIA yields deeper proteome/peptidome coverage, enhances quantitative accuracy and precision (particularly for low-abundance peptides), and decreases missingness by donors. Although specific data analysis software is necessary, the advantages of DIA are becoming more and more appealing in the field of forensic test for both high sensitivity and reliable quantitation for the determination of biomarkers and GVAs [8, 15].

**Bioinformatics and Data Analysis Software:** Advanced algorithms and software are available for the processing of complex forensic proteomic data. These include new tools for refined PTM determination, improved differentiation of endogenous vs. contaminant proteins, effective label-free quantification, and the application of machine learning methods for constructing predictive models for PMI or AAD by complex protein patterns [4, 8]. The further creation of targeted spectral libraries for bone proteins and their common contaminants will also improve the accuracy of the data analysis[8].

### **Future Directions**

Forensic bone proteomics is a fast developing field with great possibilities that still remain unexploited. There needs to be a focus on the follow up work in the future. Certainly, large independent validation studies on the vast number of well-characterized human remains from multiple environmental contexts over the past century will be necessary to validate the reliability and applicability of putative biomarkers

for PMI, AAD, and other traits across populations and taphonomy occurrences [6, 4]. Secondly, sustained efforts to decipher the basic processes of protein degradation and diagenesis in bone across different environments are necessary in order to improve the interpretation of proteomic signatures and the accuracy of predictive models [6, 1, 12]. Thirdly, the implementation of standardized and validated protocols in the collection, preparation, analysis, and data interpretation is key for inter-laboratory comparability and for eventual forensic casework introduction [5,8,13]. Fourth, creating comprehensive, curated protein sequence databases and spectral libraries (conditional on forensic application) relevant to bone (as well as well recognized contaminants of the bone samples and no less relevant PTMs) would greatly enhance the statistical confidence of the data analysis [8]. Finally, the investigation of the complementarity among proteomics and other forensic aspects like anthropology, taphonomy and other 'omics' approaches (of which the former may be more applicable to the young YF victim studied here) is expected to provide the largest breakthrough in the field of forensic identification and reconstruction [11, 7]. When these areas are covered, bone proteomics will become an increasingly essential technique for the forensic scientist.

## CONCLUSION

The forensic bone proteome has conclusively shown its status as a game-changer in the toolbox for medico-legal inquiries, providing precious molecular information that remains accessible when classical DNA typing fails due to extensive degradation. This review has demonstrated substantial advancements in this aspect, when we consider how protein analysis of bone is being used to answer difficult forensic questions: (1) estimation of PMI (2) age at death (3) biological sex (4) species (and potentially individual) identification. Protein biomarkers and particular variations in protein post-translations are now identified as relevant factors that are opening possibilities thanks to the improved sensitivity of mass spectrometry as well as the better development of data analysis pipelines.

Yet the way to widespread use is fraught with challenges that need to be addressed as a matter of scientific rigor. The confounding influence of environmental diagenesis, pervasive microbial contamination, biological variation, and complexities of protein degradation pathways all provide challenges for the veracity and interpretation of proteomic data in skeletal remains. Overcoming these challenges require a collective focus on method standardization, including optimized and validated protocols for sample preparation (eg S-Trap-based) and analysis (DIA-MS-based), as well as development of robust bioinformatics tools that can cope with increasingly more complex data and differentiate between signal and noise.

Finally, the future of forensic osteo proteomics will depend on continued basic research elucidating degradation processes, large-scale validation studies across different contexts, the development of SOPs and reference materials, and forensic protein databases. When these needs are met, bone proteomics can evolve from a highly promising area of research into a validated, robust, and indispensable part of the forensic scientist's armamentarium, greatly increasing our capacity to exploit critical skeletal evidence for justice and historical reconstruction.

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