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Review

Proteomics in forensics: from source attribution to reconstruction of events

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ABSTRACT

Proteomics has become an increasingly valuable tool in analytical sciences, including the field of forensic science. Initially introduced for body fluid identification, its applications have since expanded significantly. This literature review, spanning primarily over two decades (2004–2024), traces the steady evolution of proteomics within forensic science, with a particular focus on its potential for source attribution and event reconstruction. Here, we explore the potential of proteomics for what we refer to as "ultimate event reconstruction." This reflects the dual role proteomics can play in forensic investigations, addressing early-stage questions related to source attribution, as well as later-stage questions concerning the reconstruction of events and timelines. To illustrate this potential, we highlight three case studies where proteomics has been successfully applied, while also briefly discussing the challenges encountered in implementing such a novel approach within a forensic framework. Additionally, we examine broader challenges that could hinder the adoption of proteomics in forensics, particularly those related to its relevance, complex data analysis, and the legal admissibility of proteomic evidence in court. We propose a set of guidelines aimed at preserving evidentiary integrity and supporting the responsible integration of proteomics into forensic practice. In conclusion, we emphasize that proteomics in forensic science should not be regarded as a standalone research discipline. Its true strength lies in how it can complement and integrate seamlessly with established disciplines such as genomics and toxicology to name a few, enhancing the depth and breadth of forensic trace analysis. Moreover, we highlight how proteomics can contribute to ultimate forensic reconstruction- not in isolation, but as part of a broader multimodal framework that integrates proteomics with techniques like DNA profiling, isotope analysis, and toxicological screening, to provide a more comprehensive understanding of forensic events.

Abbreviations: AI, Artificial intelligence; ANSI, American national standards institute; ASB, Academy standards board; BFI, Body fluid identification; COVID-19, Coronavirus disease 2019; CRF, Cardiorespiratory fitness; DDA, Data dependent acquisition; DIA, Data independent acquisition; DNA, Deoxyribonucleic acid; ePMI, Estimation of PMI; ESI, Electrospray ionisation; ELISA, Enzyme-linked immunosorbent assay; ENFSI, European network of forensic science institutes; ETD, Electron transfer dissociation; GC, Gas chromatography; GE, Gel electrophoresis; GVP, Genetically variant peptide; HCD, High-energy collision dissociation; HS, Headspace; ISO, International organization for standardization; iTRAQ, Isobaric tag for relative and absolute quantification; LC, Liquid chromatography; LIMS, Laboratory information management system; LOD, Limit of detection; LOQ, Limit of quantification; MALDI, Matrix assisted laser desorption/ionization; ML, Machine learning; mRNA, Messenger ribonucleic acid; MS, Mass spectrometry; nsSNP, Non-synonymous single nucleotide polymorphism; PAGE, Polyacrylamide gel electrophoresis; PCA, Principal component analysis; PCR, Polymerase chain reaction; PMI, Post-mortem interval; PRIDE, Proteomics identification database; PSM, Peptide spectrum match; PTM, Post translational modification; Q, Quadrupole; RNA, Ribonucleic acid; RPLC, Reversed-phase liquid chromatography; RT, Reverse transcription; SAP, Single amino-acid polymorphism; SDS, Sodium dodecyl sulphate; SP3, Single-pot, solid-phase-enhanced sample preparation; SPE, Solid phase extraction; STR, Short tandem repeat; S-TRAP, Suspension trap; SWGTOX, Scientific Working Group for Forensic Toxicology; TOF, Time-of-flight; TRL, Technology readiness level; TSD, Time since deposition; ZooMS, Zooarcheology by mass spectrometry.

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1. Introduction

The field of proteomics studies the whole proteome or the sum of all proteins in an organism, cell, tissue, or biofluid. This results in a wealth of information about the expressed protein and in some cases their modifications under given conditions [1]. Over the past two decades (2004–2024), approximately 236,100 publications have addressed proteomics, of which around 17,100 specifically focus on forensic applications. This represents 7.2 % of the literature on proteomics, indicating that it is a point of significant interest in the forensic field. The graph (Fig. 1) below depicts a clear upward trend in the number of forensic proteomics publications over the past two decades, suggesting a growing interest in applying proteomic techniques within forensic investigations. The notable increase in the number of publications may be a result of advancements in analytical technology, improved data analysis methods, and an overall recognition of the potential for proteomics to enhance forensic casework. The stabilization or slight decline in forensic proteomics publications in the last few years (2022–2024) may reflect temporary disruptions due to the COVID-19 pandemic, as laboratory-based research slowed worldwide, but could also stem from researchers reassessing the practical and legal hurdles that still hinder routine adoption of proteomic methods in forensic casework.

The pie chart below (Fig. 2) illustrates the primary areas of focus in forensic proteomics research. The largest segment (35.7 %) relates to inferring donor characteristics, such as age and biological sex, while the second-largest portion (32.0 %) centers on body fluid identification. Interestingly, time-based categories such as estimation of time since deposition (TSD) of a trace (25.0 %) and post-mortem interval (PMI) (6.6 %) together account for 31.6 % of the research. When combined, these two categories reflect a major interest in reconstructing events and determining timelines, a need driven by the lack of any existing technique capable of precisely establishing either the PMI or when a trace was deposited at the crime scene.

Investigating the applicability of MS-based proteomics in forensic science is gaining attention for its potential to enhance and complement traditional forensic analyses. However, current forensic DNA practices often overlook the potential of proteins, likely because DNA analysis is already well-established, thoroughly validated, and highly effective at addressing most forensic questions, reducing the need to explore the potential utilization of other biomolecules.

Forensic DNA analysis can provide powerful evidence in criminal investigations, when comparing a DNA profile recovered from the crime scene to profiles in a DNA database or from a suspect, extremely large likelihood ratios can be generated. For its application in donor identification and its evidential strength, DNA is prioritized over other biomolecules in biological (trace) evidence. A comparison of the applicability of DNA and proteins in different facets of forensic science is shown in Table 1. Proteomics can also be advantageous in the field of biological trace analysis, primarily due to the abundance and stability of proteins when compared to DNA. As proteins are present in larger quantities and are more resilient to various external factors, they can be detected in samples where DNA may be degraded or insufficient for analysis [2]. The analysis of proteins is crucial in determining some toxins in toxicology cases, for example, assessing proteins in the identification of venoms or determining toxic plant proteins such as Ricin. Moreover, the reference DNA profile might not be in the DNA database and the suspect could be unknown, leaving traditional STR profiling to be of limited value in such a criminal investigation. Most standard protocols involve the removal of proteins prior to purification and analysis of DNA, which inhibits the incorporation of proteomics in trace analysis. To combat this problem, the exploration of methods for the simultaneous extraction of DNA and proteins presents a promising development as these approaches allow for multiple analyses to be conducted in parallel with the same sample [3]. Nonetheless, the significant potential of proteomics in forensic applications is not fully well understood, resulting in this robust analytical technique being underexplored and under-utilized within the field. This gap can be attributed to several challenges, including the need for highly specialized expertise, high cost of equipment(s), and the development of protocols designed for specific forensic inquiries [4].

Implementing proteomic approaches in forensics would contribute to two of the five interdisciplinary themes outlined in the Dutch Forensic Research Agenda (2023) [46], namely the themes of ‘finding, recovering, and following trace evidence’ and ‘ultimate forensic

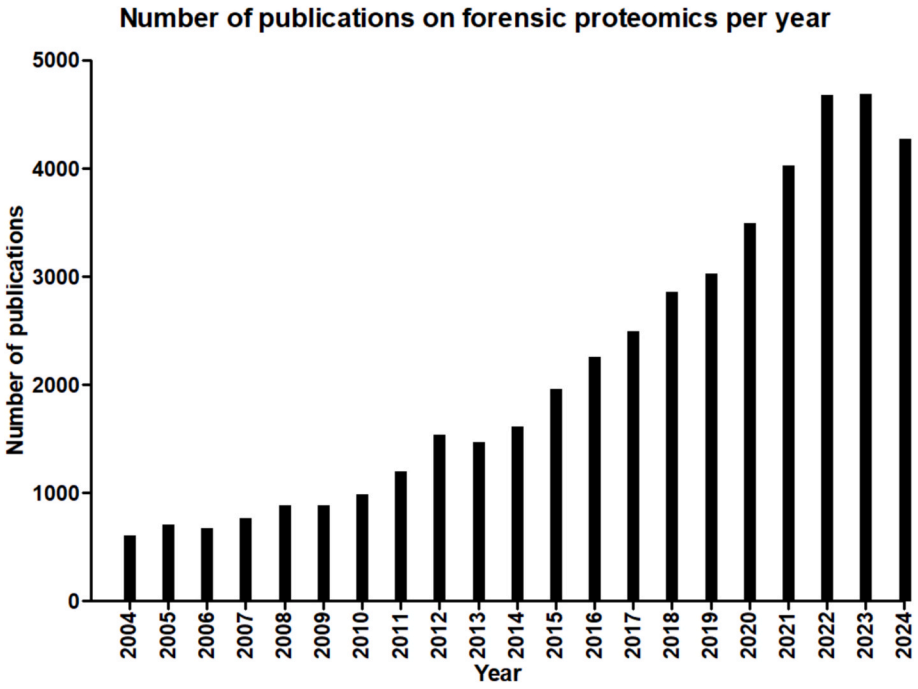

Fig. 1. Publication trend in forensic proteomics spanning the past two decades (2004–2024), based on the number of results returned by a Google Scholar search using the query “forensic proteomics”.

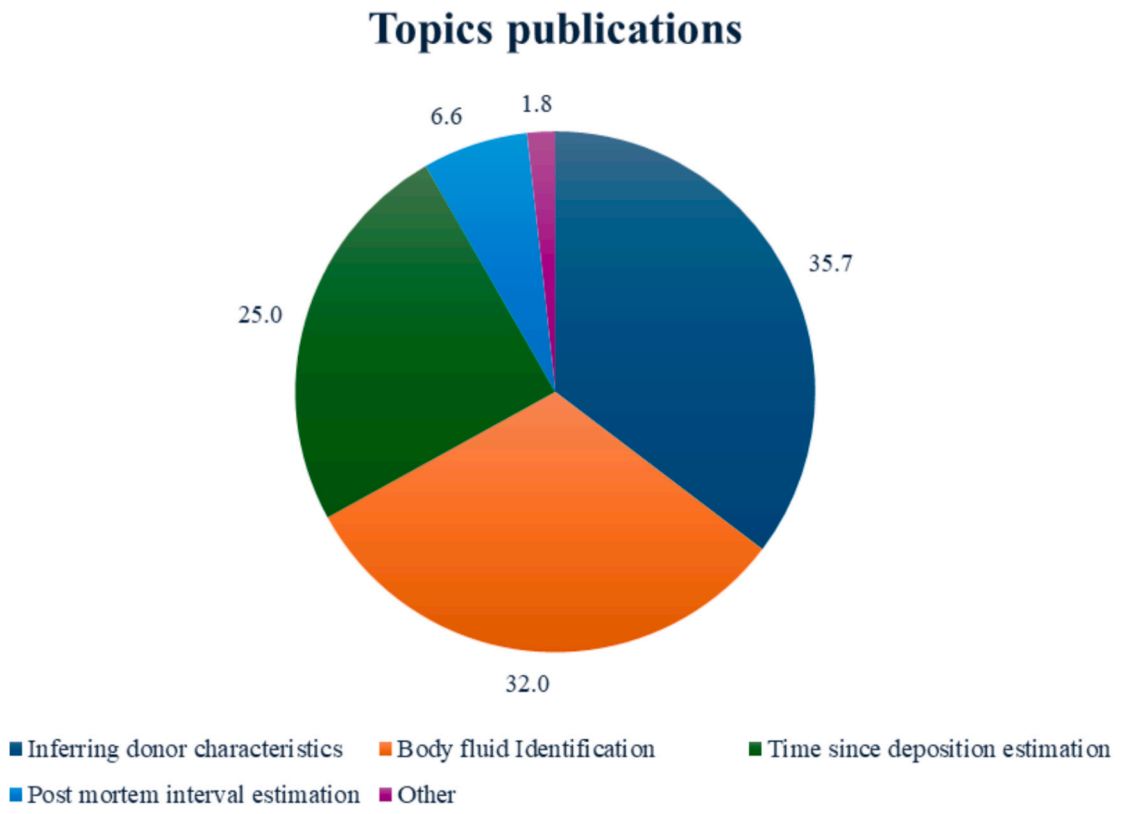

Fig. 2. Distribution of key research areas in forensic proteomics based on published literature. Publications were categorized using the following keyword queries in Google Scholar: "forensic proteomics AND body fluid identification" (BFI); "forensic proteomics AND time since deposition" (TSD); "forensic proteomics AND post mortem interval" (PMI); "forensic proteomics AND (age OR fuming OR alcohol OR sex OR smoke OR fitness OR obesity)" (Donor characteristics); and a residual "Other" category for results not captured by the above.

Table 1
Applicability and technology readiness level (TRL) of DNA and protein characterization in answering different inquiries in forensic biological trace analysis. According to the Dutch Ministry of Economic Affairs, the TRLs range from 1 to 9, and can be summarized in four phases: discovery (levels 1–3), development (levels 4–6), demonstration (levels 7 & 8), and deployment (level 9) [5]. In a forensic context, level 1 is fundamental research and level 9 denotes that the innovation is validated and accepted by the judicial system by being admissible as evidence to court or being accepted as an investigative method to be used by crime scene investigators.

Category	Area of research	Examples of specific inquiries	DNA		Proteins	
			Applicable	TRL	Applicable	TRL
Source attribution	Donor characteristics	Donor's biological sex?	Yes	9	Yes	7 [6]
		Donor's geographical origin?	Yes	9 [7,8]	Yes	4 [9,10]
		Donor's age?	Yes	8 [11–13]	Yes	4 [14,15]
		Donor characteristics from low DNA samples like hair and bone	Yes	9 [16]	Yes	5 [10,17]
	Donor identification	Source of the trace, i.e., who?	Yes	9	Yes	4 [10,17]
	Species identification	Is the trace left by a human?	Yes	9 [18]	Yes	9 [19–23]
Reconstruction	Body fluid identification	Identifying the trace, i.e., what?	Yes	9 [24–26]	Yes	9 [27–35]
	Timeline reconstruction	Post-mortem interval (PMI)	Yes	4 [36–38]	Yes	3 [39]
		Time since deposition (TSD)	Yes	4 [40]	Yes	4 [41–43]
	Cause of death	Identifying toxins	Yes	4 [44,45]	Yes	9 [21]

reconstruction'. As mentioned previously, when recovering biological trace evidence with degraded DNA, or dealing with complex matrices such as bone, it may be possible to continue following the trace by gaining forensically relevant information from proteins in the sample. Criminal investigations revolve around reconstructing the events at the crime scene. The ultimate reconstruction is an ideal where one could determine the individuals involved, the actions carried out at the crime scene and can estimate the sequence of those actions within a known

timeline. Proteomics has attempted to infer donor characteristics from biological material, providing more information on who was at the crime scene. Furthermore, research has focused on recreating the sequence and timeline of events to decipher what happened at the crime scene and when. Combining proteomics with other disciplines can provide additional insights in forensic investigations for a fuller picture to aid forensic reconstruction. This is evidenced by several case examples detailed in this review.

The purpose of this literature review is to examine the emerging role of proteomics in forensic science, particularly its potential advantages in source attribution and reconstruction of events and timelines. Since there is often a limited amount of trace evidence available, a method that is rapid, sensitive, specific, minimally invasive, and compatible with existing techniques can be of great added value. This review can also be seen as an updated and extended follow-up to the review by Keane *et al.* [47], building on their foundational work by further illustrating the translational nature of forensic proteomics. We do this by presenting three case examples where proteomics has been practically applied within a forensic investigation. An important addition in this review is the discussion of the remaining challenges and steps needed to facilitate the broader adoption and acceptance of proteomics within forensic science, including standardization, validation frameworks, and legal admissibility guidelines.

2. Fundamentals of MS-based proteomics in forensics

2.1. Integration of proteomics with mass spectrometry

Mass spectrometry has become a cornerstone in proteomics due to its sensitivity and specificity which help identify and quantify proteins in complex biological samples. Before the widespread integration of MS in proteomics, more 'traditional' methods were used that focused on protein separation and characterization, but they were less powerful than MS in terms of specificity and sensitivity. The most common technique, which is still widely used for separation of proteins is two-dimensional gel electrophoresis (2D-GE). The most popular form of this technique being SDS-PAGE, where the proteins are separated first by their isoelectric point and then based on their molecular weight [48]. Following protein separation by GE, proteins were characterized using Western Blotting, which made use of antibody-based detection for identification of specific proteins [49]. As for sequencing a protein or peptide, the most common and well-known method was the Edman degradation method [50]. These traditional methods paved the way for slightly more powerful techniques, such as liquid chromatography, which were used for protein purification, separation, and identification. The coupling of MS to chromatographic techniques is highly desirable as it incorporates the physical separation capabilities of chromatography and the analytical/detection capability of the mass spectrometer [51].

Breakthrough advancements in the MS field, like the development of soft ionization techniques (ESI and MALDI) and mass analyzers (Time-of-flight (TOF), Orbitrap, and Quadrupole), have increased the accuracy and resolution enabling the detailed analysis of proteomes. Improvement in sample preparation methods, including protein digestion (both in gel and in solution), selective enrichment of certain proteins and peptides of interest, and various clean up protocols (e.g., SPE, SP3 S-Trap) are a few key reasons why MS has effectively been integrated with proteomics.

The development of sophisticated software and algorithms (e.g., compareMS2, Andromeda, MSFragger, and X!Tandem) to search MS/MS data against protein databases aid in protein identification. Furthermore, the manufacturing of superior synthetic enzymes such as Trypsin Gold/Platinum and Trypsin/LysC enhance sample processing and subsequently, peptide identification. These enzymes are free of chymotryptic activity, possess autoproteolytic resistance, have no (animal) contaminants, and are highly specific, which reduces the number of miscleavages thereby reducing the false discovery rate [52].

2.2. Bottom-up proteomics vs top-down proteomics

Forensic analysis typically employs the bottom-up proteomics approach, primarily because it is well-established and much simpler compared to its top-down counterpart [53]. In bottom-up proteomics, proteins in a sample are digested with proteases to generate smaller peptides, which are then analyzed in an MS/MS instrument. Each peptide's mass-to-charge ratio (m/z) is determined, and the predicted sequence can be used to identify proteins present in the sample. One of the biggest advantages of this approach is that upon enzymatic digestion, a protein can produce many peptides that are useful in both identification and characterization of that protein. For example, if a protein can theoretically produce 50 peptides that can be analyzed by MS, only a few of those peptides are needed to demonstrate the presence of that protein. This is because peptides can be efficiently separated using reversed-phase liquid chromatography (RPLC), tend to ionize well, and fragment in a predictable way. This feature makes bottom-up proteomics robust and high-throughput, enabling the identification and quantification of thousands of proteins in complex samples.

In contrast, top-down proteomics analyses intact proteins by introducing them directly into the mass spectrometer. This technique provides detailed information on post-translational modifications (PTMs) and sequence variants, which are often difficult to resolve using peptide-based methods. By examining both the whole protein and its fragment ions, top-down approaches offer better comprehensive structural insights that might otherwise be missed. Since proteins are analyzed in their native state without chemical modification, this method reduces the number of experimental artifacts.

However, successfully applying top-down proteomics often requires advanced separation techniques prior to mass spectrometric analysis, which are still evolving for whole proteins. As a result, top-down proteomics has not yet seen widespread implementation. Nevertheless, its ability to provide extensive information regarding protein structure, especially PTMs and sequence variants, makes it especially promising for time since deposition (TSD) and post-mortem interval (PMI) studies in forensic science.

As aforementioned, the bottom-up approach is more frequently performed in the forensic analysis of proteins. Many of these proteomic studies employ data-dependent acquisition (DDA) for data collection [3]. This entails selecting a narrow mass-to-charge ratio (m/z) range, in between which a predetermined number of precursor ions can pass through the mass analyzer to be fragmented in the collision cell. The precursor ion(s) is typically selected based on its abundance and/or charge state, discriminating against low-abundance proteins (that may be forensically valuable). Furthermore, if the number of precursor selection cycles does not return the desired number of precursor ions chosen by the analyst, precursor selection becomes stochastic, and the reproducibility is jeopardized [55]. Due to interferences stemming from co-eluting peptides, this biased approach is susceptible to false positive and false negative identifications using database searches [56]. This is particularly harmful to forensic investigations where it would be necessary to analyze unknown samples but is a powerful way to improve sensitivity in targeted studies. The resulting product scans or spectra are relatively simple since they pertain to fragments of specific analytes in the sample and are consequently more easily interpreted. A less biased, more reproducible acquisition method is data-independent acquisition (DIA). DIA describes the simultaneous fragmentation and analysis of all sample components within a selected m/z range, improving sample coverage. Moreover, its specificity is especially advantageous in the assessment of unknown complex samples, where it may be crucial to distinguish between isobaric peptides [56]. This comprehensive and more accurate representation of the sample proteome is harder to interpret but becomes more accessible with the development and advancement of relevant computational tools.

3. Applications of proteomics for ultimate forensic reconstruction

Over the last few decades, MS-based proteomics has grown tremendously. Proteomics first made its impact in the forensic field when it was applied in the identification and differentiation of body fluids by Steendam *et al.* [27]. One of the significant reasons for the recent popularity of this technique, strictly from a forensic viewpoint, is that proteins are intrinsically more stable and more abundantly available than DNA [57–59]. This means that there is still a possibility that a trace contains enough proteins to gather significant information when DNA is too degraded for further analysis with PCR. The idea behind the evolving field of forensic proteomics is not that it can compete with DNA analysis, but rather, when DNA analysis fails to provide answers to certain questions, proteomics can offer a complementary technique. Proteomics provides valuable information in cases where DNA analysis leads to no significant matches, by revealing certain lifestyle attributes of the donor such as smoking [60], consumption of alcohol, and drugs [61–63]. This information can help narrow the suspect pool, and aid in streamlining investigations by allowing informed decisions to be made at an earlier stage. In addition, proteomics can help distinguish between a healthy proteome and a proteome that is altered due to the result of a chemical attack [64].

As outlined in the TRL table (Table 1), proteomics shows promise in answering questions both at the initial and more advanced phases of an investigation. On one hand, it holds promise for early-stage applications such as donor profiling and inferring various donor characteristics. While these applications are still in their infancy and less developed compared to established methods like DNA analysis, they demonstrate the growing capabilities of proteomics. On the other hand, proteomics may also play a role in later-stage forensic questions, such as estimating timelines and postmortem intervals (PMIs), areas where no gold-standard techniques currently exist. Although this may seem ambitious, given proteomics' early developmental stage within the forensic field, it is not unreasonable to suggest that it could eventually contribute to answering questions both at the source attribution and event reconstruction levels, this could be considered the "ultimate reconstruction" in forensic investigations. However, it is important to emphasize that proteomics is not a standalone solution capable of addressing every forensic question. Rather, its true potential lies in integration with other relevant forensic disciplines, such as genomics, toxicology etc., to provide multi-dimensional insight. The concept of ultimate forensic reconstruction should therefore be viewed as a future goal enabled by the synergistic use of complementary methods, with proteomics representing a promising, but partial, contributor. To keep this review clear and focused, we have grouped proteomics applications under two main categories:

1. **Source attribution**, the process of identifying the origin of a trace or sample. It can involve matching a biological trace such as blood, saliva, or semen to a donor, or involve the chemical profiling of illicit drugs and explosives. This helps investigators uncover important connections and narrow the search area in relation to people or places of interest.
2. **The reconstruction of events** is crucial in not only clarifying when traces were deposited but also understanding the circumstances governing it. This allows investigators to either form new leads or streamline ongoing investigative analysis. For instance, discovering both blood and semen at a crime scene may indicate a potential sexual assault but if investigators are able to estimate when the blood trace was deposited and if it turns out to be older than the semen stain then it could be disregarded, thereby altering the course of the investigation. The dating of traces can indicate which traces are relevant to the reconstruction of events at the crime scene, potentially placing a donor not only in space but also in time. Otherwise, reconstructions of the events at the crime scene allow investigators to pose research questions that can influence how the trace is subsequently analyzed. Given that trace evidence is often limited, effectively identifying, prioritizing, and analyzing traces is essential for maximizing investigative outcomes.

In some cases, it is possible that the findings for questions concerning source attribution can also contribute to reconstructing the events at the crime scene. For example, in determining the source of a biological trace, an individual is placed at the scene of the crime. So, these categories are not to be considered in total isolation.

3.1. Proteomics for source attribution

The proteome is dynamic and reflects the state of a cell, tissue, or an organism. Thus, it can be used for identifying disease biomarkers for diagnostics and therapeutics. From a forensic perspective, the proteome can shed light on multiple features that can be used to gain insights on source attribution. These factors can be indicative of a person's age, biological sex, cardiorespiratory fitness, conditions of obesity, and can even shed light on the smoking status of an individual. This type of information can narrow the suspect pool and provide investigative leads in the absence of direct DNA matches.

3.1.1. Donor profiling

Although the following research studies were not designed with forensic objectives in mind, they provide a solid foundation for future investigations focused on source attribution and donor profiling. Currently, DNA analysis is the method of choice for donor profiling. Ideally in the future, the integration of proteomic approaches with DNA analysis could maximize the amount of information obtained from a single trace. Thus, allowing forensic scientists to gather investigative information about both source attribution and event reconstruction from a single analysis.

In 2010, Fleissig *et al.* embarked on a study to understand gender and age-related variations in the oral fluid proteome. They highlighted specific proteins, including *β-2 microglobulin* and *Calgranulin A*, which were markedly higher in females, and noted distinct age-dependent expression changes in proteins like *Prolactin inducible protein* and *Cystatin SN* [65]. Nagaraj *et al.*, explored the urinary proteome, revealing *Human Serum Albumin (HSA)* as the most abundant in the core urinary proteome, their study also highlighted that the urinary proteomes of different individuals were clearly distinguishable [66]. The direct detection of peptides and small proteins in fingermarks using MALDI-MS profiling can be utilized in estimating the sex of a donor with an accuracy between 67.5 and 85 %. The most discriminating biomarkers identified by Ferguson *et al.* are SSL-29 and LEK-45 for male donors and DCD-1L for female donors [67]. Fingermarks are ubiquitous in nature, and as such to have a method for sex determination offering both chemical and morphological information with minimal destruction of the sample is highly advantageous for forensic investigations.

Tanaka *et al.*'s study on plasma proteins and chronological age found a notable correlation with the protein *GDF15* and age, alongside sex-specific associations for proteins, half of which were sex hormones, showing a stronger correlation with age in women [15]. Robbins *et al.* in 2021 shed light on cardiorespiratory fitness (CRF) through plasma protein profiles, identifying proteins like *Extracellular matrix protein 1 (ECM1)*, *Decay accelerated factor (DAF)*, *Tetranectin (TN)*, *Apolipoprotein F (APOF)*, and *Lipase K (LIPK)* as indicators of CRF [68].

The link between obesity and specific proteomic profiles was further explored in 2021 by Kresoja *et al.*, who observed a distinctive proteomic signature in obese individuals with heart failure [69]. Earlier, in 2016, Geyer *et al.* had documented the impact of sustained weight loss on the plasma proteome, noting significant changes in proteins, predominantly within the *Apolipoprotein* family, which reflects an individual's physiological health status and is associated with cardiovascular disease risks [70].

Several studies, including one by Sinha *et al.* in 2021, have demonstrated distinct differences in the salivary proteome among smokers and non-smokers. By asking smokers to smoke their regular brand of cigarettes and asking non-smokers to 'smoke' sham or unlit cigarettes, the impact of smoking on the salivary proteome was revealed. This study highlighted the upregulation of specific proteins in smokers, notably *Fibrinogen alpha*, *Cystatin-A*, and *Serum Amyloid A (SAA)* as shown in Fig. 4 [71].

As evidenced by these studies, many of the lifestyle or demographic characteristics of donors in these studies are examined in isolation, and over a range of different biological matrices. Considering donor characteristics separately, or independently, can give an oversimplified or unrealistic view of trends witnessed or how informative or selective biomarkers are for a specific characteristic. Similar studies should be conducted with forensic objectives at their core. This would mean that future efforts focus on the biological matrices most frequently encountered in forensic casework (such as blood, fingermarks, semen, vaginal fluid, and saliva) and focus on the frequently asked questions to narrow a pool of suspects. For example, inferring the donor's sex, geographical origin, or blood group type would be more forensically relevant or more effective in narrowing the suspect pool than inferring their caffeine consumption, nicotine consumption, or recent weight loss. Moreover, future studies should concern the sample types that are most problematic for individualization using DNA analysis (e.g., hair and fingermarks). This would ensure that the inference of donor characteristics using the proteome of different sample types is most effective for answering forensic enquiries at the investigative phase.

3.1.2. *Proteomics for body fluid classification and identification*

In recent years there has been a shift in focus from trace individualization, to incorporating the context [72] of a particular case to trace analysis. This elevates interpretations from source to activity level, allowing for a broader understanding of the crime scene and the crime committed. In addition to tactical or contextual information, insights on the composition of a stain can help investigators and scientists make informed decisions about subsequent downstream processes. Body fluid identification (BFI) and organ tissue identification are two key aspects in forensic investigations of unknown biological trace evidence. Preliminary indicative tests must be sensitive, reliable, and reproducible. Due to aforementioned reasons, the "classical" tests are often being complemented by more modern, accurate, versatile, and efficient methods. These confirmatory methods must provide information on the composition of a trace (single source or mixture) but also provide comprehensive information regarding the donor species. Given the nature of trace evidence, non-destructive analytical methods that require a minimal amount of sample are preferred, thereby ensuring the integrity of the trace for further analyses. A method fitting most of these requirements is MS-based proteomics.

One of the earliest applications of MS-based proteomics in forensic science was the identification and classification of biological matrices. Since then, advances in this field have led to significantly higher sensitivity and improved analytical capabilities [27]. In the field of BFI, most detection and identification pertain to the "big five", blood (both peripheral and menstrual), saliva, semen, vaginal fluid, and urine (summarized in Table 2) [72]. Once potential body fluid-specific protein markers have been identified, based on a literature survey or by

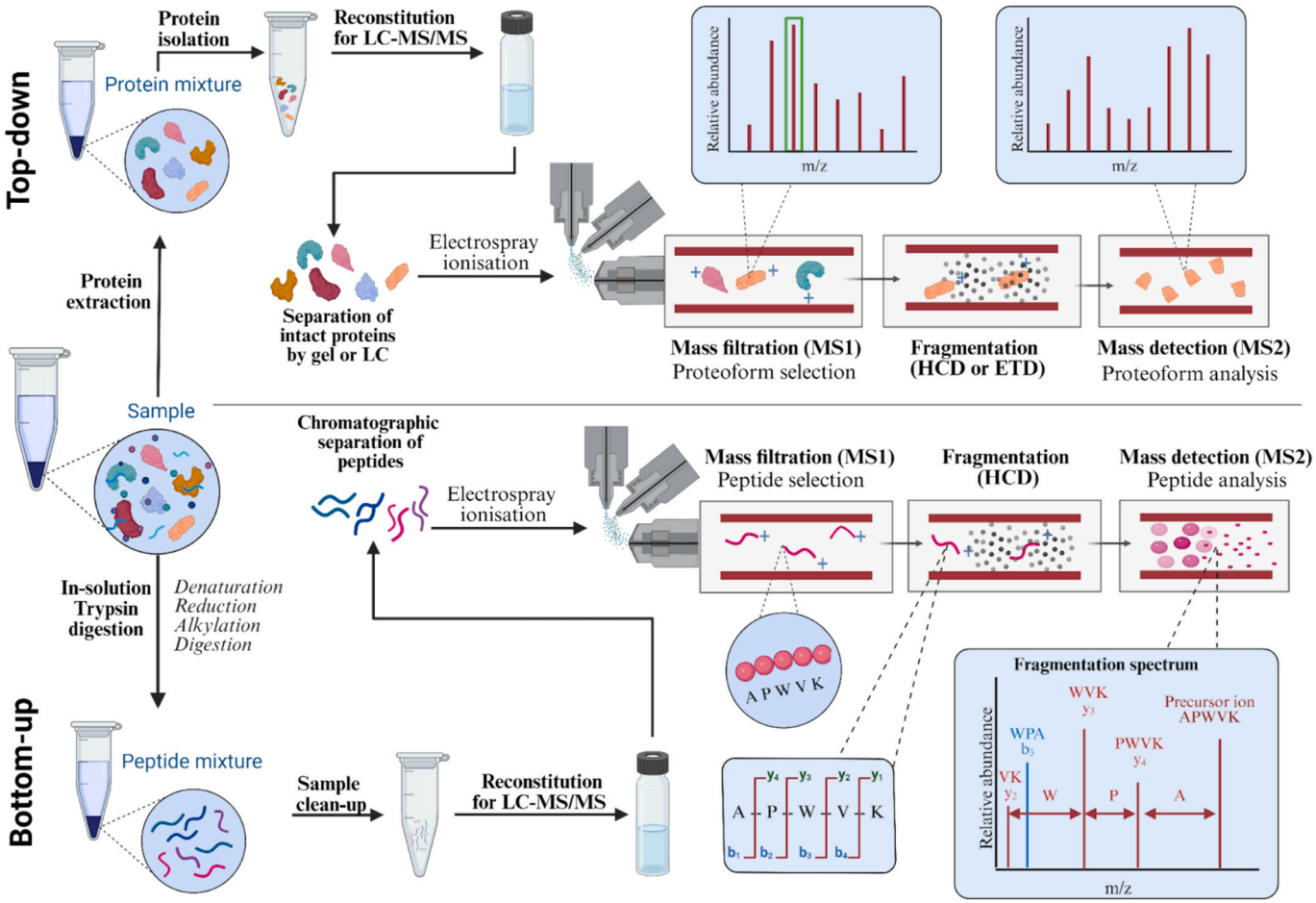

Fig. 3. Schematic comparing the top-down and bottom-up proteomic approaches. (Extended and [], Dupree [], and Neagu [] adapted from Parker 35354).

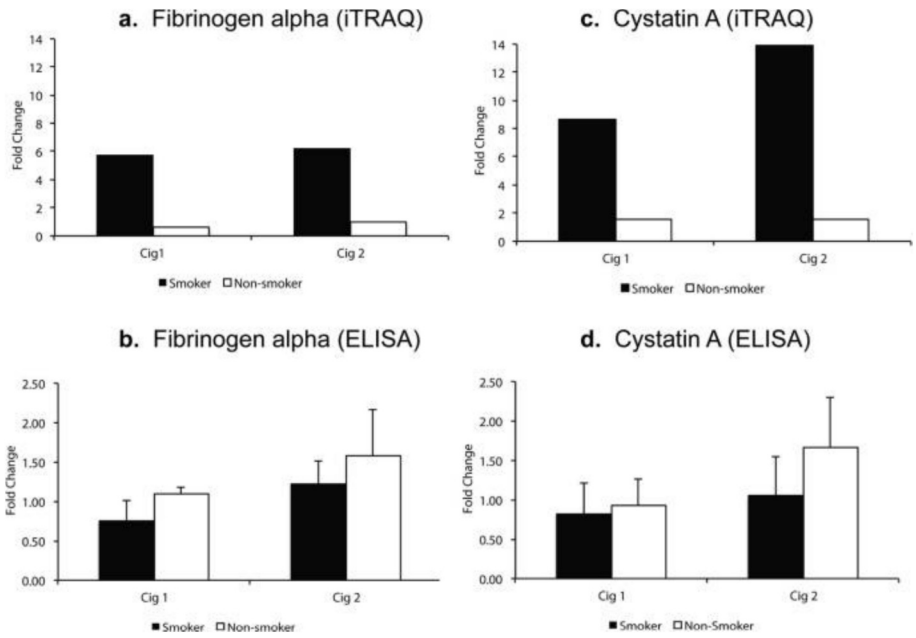

Fig. 4. Salivary fibrinogen alpha and cystatin A levels following iTRAQ analysis and validation by ELISA in smokers vs non-smokers (Extended and []. adapted from Sinha et al. 71)

experimental analysis, MS-based analysis can facilitate the detection of the protein when it is present at a low abundance, in complex matrices, or in the presence of highly abundant non-target molecules. This detection of bodily fluids can assist in reconstructing events that occurred at the scene of the crime, for example, with the detection of human peripheral blood, there was an incident at the crime scene that led to bloodshed. This is not to say that the trace is necessarily crime-related but offers investigators more insight into what could have occurred at the location of interest.

It is important to understand that there is no single marker, or specific protein that is selective for one type of body fluid. For instance, proteins commonly used to identify blood traces, like *Haemoglobin subunit beta (HBB)* and *Haemoglobin subunit alpha 1* and *2 (HBA1 & HBA2),* are commonly present in saliva, urine, and vaginal fluid. Since these proteins are not exclusive to blood, using a combination of markers would be a more effective identification strategy. For example, in the case of identification of blood traces, instead of solely relying on *HBB*, *HBA1* and *HBA2*, incorporating additional markers such as *Solute carrier family 4 anion exchanger member 1 (SLC4A1)* and *Spectrin alpha chain, erythrocytic 1 (SPTA1)* can improve the accuracy of the identification, thereby increasing the likelihood of drawing correct conclusions [3]. Given below is a compilation of protein markers commonly used for identifying different body fluids (Table 2). This compilation conveys the markers that are commonly found and reported for BFI across different research groups leading to an accurate and robust identification of body fluids in forensic science.

Broad screening strategies are typically preferred in forensic science, as they save time and resources while meeting the high demand for case processing [34]. There has been a shift towards developing multiplex assays that can identify multiple body fluids and tissue origin in a single analysis. Screening for multiple biological fluids simultaneously may also allow for stains to be assigned as mixtures more rapidly, again offering more insight into the events that occurred at the crime scene. Moreover, analysts can develop better-informed trace evidence assessment strategies. Efforts have also focused on ensuring these assays are compatible with existing DNA analysis workflows, thereby minimizing both sampling and the consumption of trace evidence [73].

Current advances in the field of MS-based proteomics have incorporated the use of sophisticated technologies like machine learning (ML) for the prediction of tissue and cell types, as recently shown by Claeys *et al.* in 2023 [35]. They utilized a random forest model to analyze proteomics data from 183 public datasets available in PRIDE [74]. Their model, which classified cell and tissue type based on protein abundances, achieved remarkable prediction accuracies – 98 % for tissue types and 99 % for cell types. Such an approach is particularly valuable for identifying patterns in large and complex proteomic datasets or while dealing with unknown samples. This is a significant step forward, offering a powerful tool for researchers to extract meaningful insights from complex proteomics data.

3.1.3. *Individualization in mixtures*

When dealing with a biological trace or a mixture of biological traces from a crime scene, questions arise such as what types of biological matrices are present and what their respective origins are. Currently, messenger RNA (mRNA) profiling via endpoint reverse transcription polymerase chain reaction (RT-PCR) addresses the former, albeit with limitations of cost, specificity, and relative instability in degraded samples [75,76]. The latter question, concerning the origin of each biological trace in a mixture, is typically resolved through DNA profiling. While individualization of biological traces from mixed samples was feasible primarily when mixtures involved a single male and female donor or required differential extraction, advances in probabilistic genotyping now allow deconvolution of complex mixtures, enabling individualization even in multi-person or same-sex mixtures, though interpretation remains challenging in highly degraded or low-template samples [77]. A proteomic-based strategy holds promise to mitigate these limitations of both mRNA and DNA profiling mixtures of biological traces: proteomics has shown to be able to answer the forensically valuable question what biological trace(s) a (degraded) sample contains and has demonstrated the ability to individualize hair shafts and more recently bone protein and fingermarks, whilst proteins exhibit greater chemical stability than DNA and RNA, and only a single analysis is necessary to answer both questions [15,63–66,68–71,75,76,78,79]. This alternative proteomics-based methodology entails leveraging single amino-acid polymorphisms (SAPs) in proteins induced by single non-synonymous single nucleotide polymorphisms (nsSNPs), thereby potentially narrowing down a pool of suspects: proteins encapsulate genetic information in the form of SAPs,

Table 2
An overview of the proteins per body fluid reported in literature. The six body fluids seminal fluid, saliva, peripheral blood, menstrual blood, vaginal fluid and urine and the corresponding proteins found after LC-MS analysis in seven different studies (extended and []

Body fluid	Protein/ Biomarker	Yang et al. (2012) [28]	Van Steendam et al. (2013) [27]	Yang et al. (2013) [29]	Kamanna et al. (2016) [30]	Legg et al. (2016)[31]	Legg et al. (2017) [32]	Zhao et al. (2018) [33]
Seminal fluid	Semenogelin-2		●	●	●	●	●	●
	Semenogelin-1		●	●	●	●	●	●
	Prostatic acid phosphatase		●	●		●	●	●
	Prostate-specific antigen		●	●	●	●	●	
	Mucin-6			●				
	Beta-microseminoprotein			●				
	Glycodelin			●		●	●	
	Cysteine-rich secretory protein 1			●				
	Epididymal secretory protein E1					●		
	Prolactin-inducible protein					●		
Saliva	Alpha-amylase 1		●	●	●			●
	Submaxillary gland androgen-regulated protein 3B			●		●	●	
	Protein LEG1 homolog			●				
	Cystatin-SA			●		●	●	
	Cystatin-D					●	●	
	Cystatin-SN							●
	Histatin-1			●		●	●	
	Statherin					●	●	
	Prostasin							●
	Mucin-7							●
	Alpha-amylase 2B				●			
	Pancreatic alpha-amylase				●			
	Salivary acidic proline-rich phosphoprotein 1/2					●		
Peripheral blood	Haemoglobin subunit beta		●	●	●	●	●	●
	Haemoglobin subunit alpha		●	●	●		●	●
	Band 3 anion transport protein			●				
	Spectrin alpha chain, erythrocytic 1			●				
	Alpha-1-antitrypsin					●	●	●
	Clusterin							●
	Complement C3					●	●	●
	Hemopexin					●	●	
	Haemoglobin subunit delta				●			
Menstrual blood	Fascin	●						
	Orexin A	●						
	Plexin A1	●						
	Plexin D1	●						
	Padocan	●						
Vaginal fluid	Cornulin		●			●	●	●
	IgGFc-binding protein					●	●	
	Involucrin		●				●	●
	Ly6/PLAUR domain-containing protein 3					●	●	
	Matrix metalloproteinase-9					●	●	
	Neutrophil gelatinase-associated lipocalin					●	●	
	Periplakin					●	●	
	Suprabasin					●	●	
	Vimentin					●	●	
	Mucin-5B						●	
	Cornifin-A		●					●
	Cornifin-B							
	Involucrin					●		
Urine	Osteopontin					●	●	●
	Uromodulin		●		●	●	●	●
	Kininogen-1							●
	Protein AMBP		●					●

adapted from Parker et al.3):

resulting from these nsSNPs. Utilizing proteomics to initially identify biological traces (in a mixture) using biological trace specific biomarkers and to subsequently detect biological trace specific genetically variant peptides (GVPs) containing SAPs holds large potential.

3.2. Proteomics for the reconstruction of events

3.2.1. Proteomics for time since deposition estimations

The need to answer questions at an activity level has gained importance in the forensic field. This is particularly relevant in cases where the defendant does not dispute that they are the source of the trace, but states that they deposited the trace innocently, typically before a crime occurred. The evaluation of propositions at activity level helps in testing and differentiating presented scenarios. In this section we will focus on the estimation of the age of a trace. Understanding the chronological sequence of events at a crime scene provides a more comprehensive narrative of the incident. It goes beyond who was involved, placing them in space and time in crime reconstructions. This depicts a clearer picture of what, how, and when specific activities occurred.

Blood is one of the most encountered body fluids at crime scenes and is primarily analyzed to answer identity-related questions (*is this blood?*), class-related questions about the origin (*what type of blood is this? Is it human, and if so, is it menstrual or peripheral blood?*) and ultimately individualize the trace (*who is the donor of the trace?*). However, to better understand if and how the bloodstain relates to an alleged crime, additional forensic investigation at activity level is required including temporal analysis (*when and how was this blood stain created?*). Despite significant research efforts utilizing both physical and chemical methods, estimating the time since deposition (TSD) remains one of the most complex problems in the field, with no method yet providing a definitive solution. TSD involves determining the degradation of (certain components of) a trace, an intricate process influenced by various external factors, including but not limited to temperature, humidity, rainfall, exposure to light, and the presence of microbes. Estimating TSD can assist in assessing the relevance of a biological trace, by not only helping establish a time frame but also connecting a suspect to the time frame, thereby addressing critical questions at activity level.

Schneider *et al.* conducted a proof-of-concept study to demonstrate the applicability of MS-based bottom-up proteomics for estimating the TSD of dried blood spots [42]. They were able to successfully detect and quantify around 3000 peptides from approximately 400 proteins without the need to remove highly abundant proteins found in blood such as albumin and haemoglobin. By plotting the data on a PCA graph (Fig. 5), clustering indicated that environmental conditions accelerated protein aging in dried blood spots. They further observed that the oxidation of certain peptides was linked to these age-related changes, underlining the importance of studying post-translational modifications (PTMs) over time. Although this study was set up as a proof of concept, it contributes to a growing body of evidence supporting the versatility of MS-based proteomics, and its potential in forming timelines necessary to answer forensic questions.

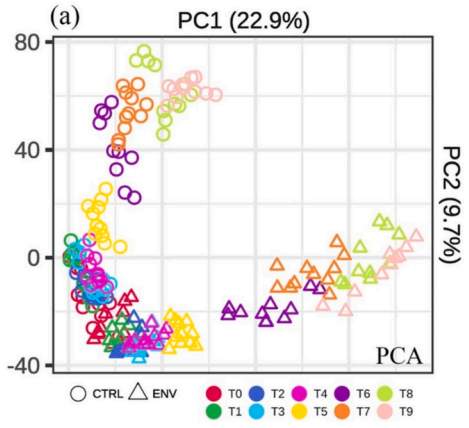

Fig. 5. Principal component analysis (PCA) plot of both ctrl (O) and environmental (env Δ) samples illustrating sample clustering and increasing class separation towards later time points for both storage conditions throughout the entire time series (T0-T9) (Extended and [].
adapted from Schneider et al. 42)

The aging of fingermarks was monitored over a 16-day period by S. Oonk *et al.* [43] using bottom-up proteomics. A fingermark proteome of 52 proteins was recorded where four keratins (namely, K2C1, K22E, K1C9, and K1C10) and dermcidin exhibited distinct responses during aging. Using the normalized levels of abundance of each biomarker over the 16 days the keratins were seen to increase as time elapsed and dermcidin, conversely, decreased with time. To gain more insights for forensic applications it is necessary to monitor the proteome over a longer timeframe, with fingermarks from more donors, under different environmental conditions.

To ensure their reliability, comprehensive stability studies are essential to evaluate the influence of individual environmental conditions on these protein markers. An "ideal" marker must change with time but not be strongly impacted by varying environmental conditions such as temperature, humidity, light exposure and various other physical conditions. Without thorough validation and evidence of stability, markers cannot be incorporated into aging studies. As previously mentioned, it is necessary to use a combination of protein markers to make a robust identification of biological traces. Similarly, estimating the time since a trace was deposited should be performed using the ratio of two or more markers in the trace [80].

An additional challenge lies in the difficulty of replicating real-life forensic scenarios. It is practically impossible to account for all possible physical conditions under which biological traces might be found, limiting the ability to test markers under a wide range of realistic settings. Furthermore, understanding the transfer, persistence, and recovery of these biomarkers is critical, as these factors also influence their suitability for TSD estimation. Finally, standardization and validation of the methods used to collect, analyze, and interpret the samples is necessary for the identification of aging biomarkers and the subsequent use of TSD estimations in casework.

3.2.2. Proteomics for post-mortem interval estimations

Like TSD, estimating the time since death, or the post-mortem interval (PMI), is a highly debated and researched topic in the field of forensic science and yet there is no universal method that can be routinely employed due to the lack of applicability and accuracy [81]. Accurately determining the time since death would offer invaluable information to investigators, answering a key question in forensic casework involving human remains. This could narrow the field of suspects or be used in conjunction with other contextual information to assemble a series of possible events surrounding a crime. Currently, PMI is estimated using techniques such as, measuring the rectal temperature [82], observing changes in body temperatures [83], examining the stages of rigor mortis [84], and studying the surroundings to obtain more entomological knowledge [85]. Such methods are no longer applicable when the tissues are heavily decomposed and thus are not suited to estimate longer PMIs.

Some common limitations of these techniques are their subjectivity and how easily they are influenced by internal and external factors. Other methods have been based on measuring the concentration of potassium and urea in vitreous humor in combination with statistical methods to predict PMI [86]. There is no standardized method for estimating the PMI since decomposition is a complex process governed by many conditions and parameters including body size, age, pre-existing pathologies, physical traumas, and some environmental parameters such as temperature, humidity, soil composition, and burial conditions (burial depth, accessibility for scavengers and so on) [87].

Studies have explored if the amount of preserved DNA in tissues and bones of the cadaver could shed some light on the PMI, but it has been found that the depth of burial, soil geochemistry, and various other environmental factors significantly affect the quality of the DNA recovered, leaving room for the development of novel research techniques [88,89].

In addition to techniques such as histological [90], immunohistochemical [91], and Western blot-based methods [92], proteomics has emerged as a promising technique for the estimation of the PMI. This technique requires the understanding and analysis of proteomic alterations, including the degradation of the bone proteome at various stages of bone decomposition [93], differential degradation patterns of inter/intramuscular [94,95] and skeletal proteins [96], as well as the post-mortem proteolytic profiles of vital organs, such as the liver and pancreas [97]. Swift categorized the early post-mortem period as the 'soft tissue phase of decomposition' and the late post-mortem period as 'skeletonization and alterations to the bony matrix' [98]. Several studies have explored PMI estimation using proteomics for both the early and late PMI stages, leveraging changes in the proteome of soft tissue and bony tissue, respectively. Zissler *et al.* extensively reviewed 36 protein-based PMI estimation studies, including 6 MS-based proteomic studies [39]. Table 3 summarizes these and other studies, detailing their design, protein markers, and applicable time frame. These investigations into proteomic dynamics offer a more comprehensive understanding of the changes that occur post-mortem providing a more accurate method for PMI estimation.

Most research in this area provides an initial understanding of post-mortem protein degradation in various specimens. Further investigation and validation are necessary to fully comprehend the role environmental factors and burial conditions play on the post-mortem proteome, and subsequently the PMI estimation. Most of these studies have made use of rat or porcine models, raising concerns of the applicability of these results for homo sapiens. One of the most prominent challenges in the field of PMI estimation is the limited availability of human specimens for research due to the scarcity of taphonomical sites and ethical concerns regarding such research and the use of human donor material for forensic purposes. For these reasons, the PMI estimation is not yet employed in routine forensic analysis, but proteomics-based research and methods show potential in tackling some of these challenges.

4. Case studies and success stories

In recent years, proteomics has proven valuable in forensic investigations, particularly when traditional methods fall short. To illustrate its broad applicability, we highlight three case studies spanning from sexual assault to suspicious deaths that demonstrate how proteomics can offer potentially crucial insights when other techniques fail. These examples are not exhaustive but underscore how proteomics is gradually being incorporated into the field and is more than just an analytical method limited to body fluid identification

Case 1 [19,21]**:** The case in reference is a complex forensic investigation in Vancouver in May 2014, where a two-year-old child tragically died under mysterious circumstances. Upon initial investigation, it was suspected that the babysitter abused the child, but the nature of bruising found on the child suggested otherwise. After further investigation, it was discovered that the babysitter kept exotic, venomous snakes at home. The nature of bruising found on the child was consistent with that of a snake bite. Unable to obtain a warrant for the babysitter's house, investigators sought help from the local scientific community. A biochemist from the University of British Columbia analyzed the child's blood and urine samples. By focusing on the non-human peptides and comparing them to known snake venom peptides in a database, he was able to conclude beyond reasonable doubt, the toxins in the child's blood matched those of a venomous snake, in particular to that of a rattlesnake. This finding significantly changed the direction of the investigation.

Case 2 [108]**:** In the mentioned case, in Italy, proteomics played a significant role in a rape case where traditional forensic methods could not provide much clarity due to the minimal trace(s) available for analyses. The incident allegedly occurred in the defendant's car, which was cleaned after, leaving behind minimal traces of evidence. The victim claimed there was no consent, due to her vomiting and subsequently losing consciousness, while the defendant stated there was prior consent. Proteomic analysis was employed to analyze the residual traces present in the car. Despite the challenges posed by the limited sample size and quality, the results obtained were significant. The results indicated the presence of proteins that are quite commonly found in saliva, gastric fluid, and partially digested food. These findings favored the victim's statement, providing crucial insights to investigators of the case.

Case 3 [109]: 20 years after a boy was reported missing in the Shandong Province of China, a suspect confessed to murdering the boy and burying the body in his garden alongside waste that included animal bones. The body was moved, and after many years could not be located. All that could be retrieved from the suspect's garden were 19 bone fragments, most of them less than 2 cm in size and undiagnostic in anthropology. This case highlights the value of a combined proteomic and DNA strategy, where zooarchaeology by mass spectrometry (ZooMS) was used to screen 19 bone fragments, identifying one piece as human bone. Thereafter, the kinship between the victim and the parents of the missing child was confirmed via DNA methods such as next-generation and Sanger sequencing.

With these cases we would like to highlight that proteomics is a versatile technique that is gradually moving from the lab to the scene and just like any other technique, it has its own challenges.

For instance, in the first case [21], a key obstacle was the need to manually review 111 peptides flagged by the algorithm as snake-specific, some of which differed from human peptides by just a single amino acid. To ensure accurate identification, investigators compared the sample to a reference venom sample and conducted confirmatory testing via ELISA at an external laboratory. As noted by the biochemist, who identified snake proteins in the victim's blood, reptilian DNA alone would not have been sufficient to confirm the bite, but it would have only suggested that the child had come in contact with the snake [19].

Similarly in the second case [108], the investigation faced significant analytical challenges due to the complex nature of the sample, which consisted of trace amounts likely containing a mixture of saliva, gastric fluid, intestinal fluid, and various food components each potentially contributing interfering proteins. Additionally, there were no established proteomic biomarkers for such a matrix. To address this, a multi-method toxicological approach was employed, including GC–MS, LC-(QqQ)-MS/MS, and HS-GC–MS, followed by proteomic analysis using a separate LC-(Q-Orbitrap)-MS/MS system. Notably, 20 days had passed between the alleged sexual assault and the initiation of toxicological consultation, further complicating the analysis. Despite these difficulties, proteomic analysis of vomit offered valuable potential: it could either provide corroborative evidence or help rule out certain investigative hypotheses.

In conclusion, the use of multiple techniques in parallel and comparison with known standards ensured that the findings could be admissible to court.

5. Integration with other forensic disciplines

MS-based proteomics is a dynamic field, enhanced by efforts made to improve MS resolution, accuracy, and speed, constantly creating new opportunities for forensic analysis. These advancements enable the qualification and quantification of proteins even in the most minute and challenging samples, offering a better understanding of protein structure, variants, and modifications. This enhanced perspective opens new avenues in forensic science, from estimating the time of death and time of deposition of traces, to discerning specific donor characteristics, such

Table 3
An overview of proteomics-based early and late PMI estimation studies including the biological matrix, specimen and sample size, estimation of PMI (ePMI), protein markers, and their change in abundance over time (extended and []

	Author and year	Biological matrix	Specimen and sample size	Researched time frame	Protein marker(s)	Change	Result possibilities
Early PMI	Finehout et al. (2006) [99]	Cerebrospinal fluid	Human (n = 7)	1.5–9.5 hpm	ENOA MDHM PRDX2 COF GRHPR HDHD1 PSB2	Increase Increase Increase Increase Increase Increase Increase	ePMI between 1.5 and 9.5 h
	Marrone et al. (2023) [100]	Skeletal muscle	Pig (n = 3)	0–24 hpm	eEF1A2 eEF2 GPS1 MURC IPO5 SERBP1 COX7B SOD2 MAO	Decrease Decrease Decrease Decrease Decrease Increase Increase Increase Increase	ePMI between 0 and 24 h
	Choi et al. (2019) [101]	Skeletal muscle	Human (n = 3),Rat (n = 20),Mouse (n = 10)	0–96 hpm	DESM eEF1A2 GAPDH VINC	Loss + degradation products Loss Decrease Degradation products	ePMI between 0 and 96 h
	Battistini et al. (2023) [102]	Skeletal muscle	Pig (n = 3)	0–120 hpm	ATP2A2 PDLIM7 TPM1	Decrease Decrease Decrease	ePMI between 0 and 120 h
	Li et al. (2017) [103]	Liver	Animal (n = 36), Human (n = 24 + 4)	0–144 hpm	Full proteome	Decrease in MALDI-MSI ion signal	ePMI between 0 and 144 h
	Li et al. (2017) [104]	Skeletal muscle	Animal (n = 4)	0–144 hpm	Full proteome	Decrease in MALDI-MSI ion signal	ePMI between 0 and 144 h
	Nolan et al. (2020) [105]	Decomposition fluid	Pig (n = 16)	2–10 dpm in summer, 6–34 dpm in winter	HBA peptide HBB peptides ENOB peptide KCRB peptide LDHA peptides	Increase Increase + decrease Increase + decrease Increase Increase + decrease	–
Late PMI	Procopio et al. (2018) [93]	Bone	Pig (n = 4)	1–6 mopm	ALDOA ANXA1 ENOB HBA HBBHPT KCRM MHY6 MYH2 TPIS TRFE TRFL	Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease	ePMI between 1 and 6 months
	Prieto-Bonete et al. (2019) [106]	Bone	Human (n = 40)	5–20 ypm	AHSG ANXA2R AREG CILP COL10A1 COMP CTC1 CTNNB DLX5 ENPP1 INSIG2 LMNA MAF MUC15 PCOLCE SLC26A2 SOX6 SUCO ZCCHC12	Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss Loss	Differentiate between PMI of 5–12 and 13–20 years
	Pérez-Martínez et al. (2017) [107]	Bone	Human (n = 40)	5–47 ypm	COL	Decrease	Differentiate between PMI of < 20 and > 20 years

adapted from Zissler et al.39):

as biological sex, smoking habits, or physical condition, through detailed protein profiles.

MS-based proteomics can be merged with the workflow of various forensic disciplines, particularly genomics and toxicology, to provide additional information in complex criminal investigations. The integration with genomics, for example, faces the challenge of standard DNA/RNA extraction kits employing Proteinase K, which is known to degrade secondary and tertiary protein structures, potentially hindering simultaneous proteomic and genomic analysis, especially if samples are of a low volume, degraded, or contain mixtures of body fluids. Parker *et al.* [110] recently demonstrated that semen samples digested with Proteinase K following a DNA isolation protocol, resulted in informative peptides that can be utilized in the identification of Semenogelin-1 in the digest and the subsequent classification of the body fluid as semen. Addressing this challenge further, Kranes *et al.*, introduced a novel protocol that replaces Proteinase K with Trypsin and substitutes SDS with ProteaseMax, a mass spectrometry-compatible detergent [73]. This approach allows for the concurrent extraction of DNA and proteins, segregating DNA on a molecular weight filter membrane while proteins remain in the filtrate. Compared to traditional methods using Proteinase K, this co-extraction technique not only conserves trace evidence but also improves DNA typing results for single thumb prints, enabling the simultaneous investigation of DNA and protein-specific markers, genetic variations in proteins (GVPs), and post-translational modifications (PTMs). Comparing the yield of DNA after extraction with Trypsin and Proteinase K should be investigated for more sample types that are frequently encountered in forensic casework. As recently as January 2025, Zaarour *et al.* [111], demonstrated the possibility for source attribution of DNA extraction waste following extraction of DNA without Proteinase K. Furthermore, Eychner *et al.* [112], compared Proteinase K to other proteases to assess their extraction of DNA from different sample types. For 50 μL blood samples, Proteinase K generally performed worse than Bromelain and Papain, with average DNA recovery values of 32.4 ng, 40.2 ng, and 59.1 ng, respectively. However, Proteinase K still offers sufficient DNA for PCR analysis, thus remaining the workhorse of the forensic DNA extraction methods.

Integrated "omics" approaches are being applied in the fields of food science and cancer research to simultaneously assess the results concerning the genome, transcriptome, and proteome. Such methods are developed for "scarce or irreplaceable samples," for example tumor biopsies in clinical studies. This description is similar to how biological traces are described in a forensic setting. Yin Bo *et al.* [113] proposed a DNA extraction using TRIzol reagent, which did not interfere with RNA and protein extraction procedures. High-quality DNA was extracted from animal tissue, sufficient for subsequent restriction endonuclease digestion and quantitative PCR. To make integrating these workflows more accessible and applicable in a laboratory, commercial kits are available. Mathieson *et al.* [114] compared the performance of two commercially available kits, TriplePrep (GE Healthcare) and AllPrep (Qiagen), in their coordinated analysis of DNA, RNA, and proteins from fresh-frozen rat liver samples. Unfortunately, extraction performance by the kits was suboptimal compared to the single "gold-standard" extraction methods as the kits compromised the yield and quality of the extract. If the performance of such commercial kits improves over time, with the proteolytic reagents not significantly affecting the amount of DNA available for PCR-STR analysis, a simultaneous extraction of both DNA and proteins would assist in omics integration in the forensic field.

Pairing forensic toxicology with proteomics offers a comprehensive methodology that enhances the detection and analysis of toxins in biological traces. This is especially true for proteinaceous toxins such as ricin and venoms. Ricin is a remarkably toxic protein originating from the castor plant and can be employed as a bioweapon. It has been detected using enzyme-linked immunosorbent assays (ELISA), handheld lateral flow immunochromatographic devices, microarrays, surface plasmon resonance, polymerase chain reaction, and mass spectrometry [115]. In 2011, Kanamori-Kataoka *et al.* described a method to determine ricin using lactose-immobilized monolithic silica extraction followed by tryptic digestion and analysis with LC/MS [116]. This method closely resembles the bottom-up proteomic workflow shown in Fig. 3. Venomics is the field of analyzing proteins for the identification of toxins [117]. This definition overlaps with the purpose of toxicology in answering forensic inquiries. As highlighted by Case 1 [19] above, with the international (clandestine) exportation of exotic animals, it is relevant to include such protein markers in toxicology screenings even in countries where native wildlife is not lethally venomous. Merging proteomics and forensic toxicology for simultaneous toxin screening seems feasible as protein precipitation is already being executed in the pretreatment of toxicological samples. Many parallels can be drawn between the two workflows, including the instrumentation, analytical methods, and approach to data analysis, as illustrated in Fig. 6.

The union of toxicology and proteomics allows toxicologists not only to identify the presence of toxic substances but also to examine their effects on the proteome, potentially providing information on the effects of such toxins on affected persons. Such insights offer a deeper understanding of the physiological responses elicited by these substances. For instance, despite chlorine being highly irritant, reactive, and toxic, it is one of the most used industrial cleaners worldwide. Traditional biomarkers such as 3-chlorotyrosine and 3,5-dichlorotyrosine used in biomedical analysis are not very specific, as they are also elevated in individuals with certain inflammatory diseases, and not just those exposed to chlorine. One such study carried out by De Bruin-Hoegée *et al.*, investigated the effect of chlorine exposure on the proteome [64]. Their study aimed at refining chlorine exposure detection by identifying site-specific chlorinated peptides in human blood plasma as a more definitive indicator of external chlorine exposure. Using advanced mass spectrometry techniques, they were able to identify 50 potential biomarkers, particularly focusing on certain peptides that show promise for distinguishing between endogenous and exogenous chlorine exposure.

Proteomics may assist in providing contextual information and investigative leads when paired with forensic pathology and toxicology to determine the composition of an individual's last meal. As previously mentioned, some toxins are proteins and may be found in the gastric contents. Otherwise, understanding the composition of the last meal offers valuable information about an individual's lifestyle, socioeconomic status, and dietary preferences, all of which can contribute to reconstructing the circumstances surrounding their demise. Moreover, discrepancies between the reported last meal and the actual findings can raise red flags, prompting further investigations into potential foul play or negligence [118–120]. It can also provide context regarding energy intake and physical exertion, aiding in the reconstruction of events leading up to the demise of a victim [121]. A study conducted by Pieri *et al.* on last meal identification, sheds light on the pivotal role of proteomic analysis in forensic investigations [122]. The study utilized proteomics to characterize the gastric contents of a deceased individual, revealing detailed insights into his final meal composition. Contrary to initial reports, the proteomic analysis revealed a diverse array of proteins derived from both endogenous gastric proteins and ingested food items. Aside from characteristic endogenous proteins found in the gastric environment, proteins originating from the ingested meal, such as various bovine milk-derived proteins (including casein families and whey proteins) and wheat protein components, were identified. The analysis uncovered that the individual had consumed a meal comprising milk and (toasted) bread, indicative of a typical Italian breakfast. This discovery contradicted previous assertions by medical personnel, prompting further inquiries by legal authorities to ascertain facts and potential liabilities.

Biological toxins, particularly protein-based ones, represent a diverse group of compounds that share features with both biological and chemical threat agents. Their wide molecular weight ranges from under 1,000 Da to over 150 kDa which adds to the complexity of their detection and characterization. These agents pose significant challenges due to their high potency, unpredictable nature, and potential for deliberate

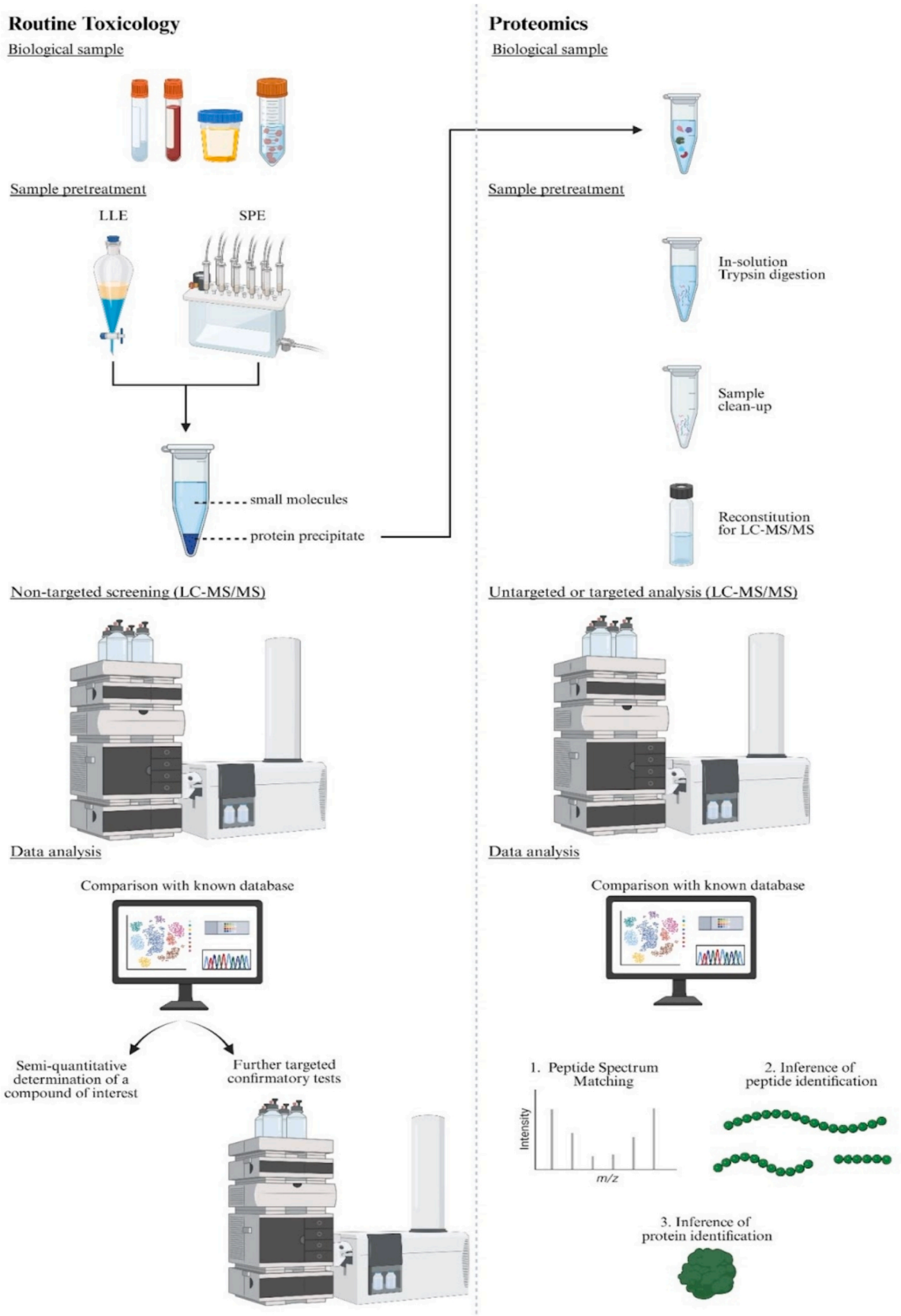

Fig. 6. Schematic of a proteomics workflow integrated into a forensic toxicology workflow.

release through various contaminated matrices such as food, water, aerosols, or postal delivery systems, as seen in incidents like the 2001 anthrax attacks and subsequent ricin cases [123]. Proteomic analysis offers critical insights in cases involving infectious disease outbreaks, biothreats, or suspicious contamination. By identifying unique microbial proteins, it can reveal strain-specific traits, laboratory cultivation history, or toxin production details that may not be evident from DNA analysis alone [124]. Differences between microbial profiles found at a scene and those expected based on known sources can raise suspicions of tampering, biocrime, or accidental release, prompting further forensic investigation. As highlighted by Merkley *et al.* [125], both targeted and untargeted mass spectrometry approaches are instrumental in identifying threat agents, for instance, mass spectrometry analyses have been used to distinguish *Bacillus anthracis* from closely related non-pathogenic species by detecting unique protein signatures, aiding in the investigation of bioterrorism cases. Similarly, in the field of anti-doping, proteomics has been used to detect protein-based doping agents such as recombinant erythropoietin and growth hormone through their unique peptide fingerprints [126,127]. In the context of food safety, bacterial toxins, both endotoxins and exotoxins, pose an ongoing public health risk, especially in an increasingly globalized supply chain where surveillance and mitigation strategies are still evolving. In recent years, "omics" technologies have become essential in addressing these challenges. While bacterial genome sequencing remains a cornerstone of routine risk assessment, proteomics particularly LC-MS and LC-MS/MS has gained traction for rapid bacterial subspecies differentiation, secretome analysis, and as a robust alternative to traditional immunoassays [128,129]. These proteomic approaches not only enhance detection capabilities but also support the development of comprehensive datasets on secreted proteins and toxins. Mapping these molecular networks offers valuable insight into host-pathogen interactions, ultimately advancing both forensic investigations and public health responses [130].

Proteomics has become an indispensable tool in wildlife forensics, particularly in case of species identification, a core requirement in many forensic scenarios, including the enforcement of wildlife protection laws, customs and border inspection, and investigations of illegal trade and food fraud [3]. For example, as evidenced by Cucina *et al.* [20], MS-based proteomics offers the prospect of more objective species classification in studying animal hairs and tissues. One widely adopted approach in this context is ZooMS, which uses MALDI-ToF MS to identify species from collagen peptides, even in morphologically unrecognizable samples like powdered bone, ivory, or keratin even in the absence of DNA [2,131–135]. ZooMS operates on the principle that evolutionary proximity yields overlapping peptide profiles, while more divergent species present distinct biomarker combinations [136–139]. This is invaluable for the identification of biological material from protected animals that may be present in confiscated products such as medicinal powders. Although protocols from bioarchaeology and paleoproteomics are being adapted for forensic purposes, wildlife forensic investigations impose specific demands, such as robust chain-of-custody documentation, courtroom admissibility, and rapid turnaround times. Nevertheless, the capacity of proteomics to provide accurate species identification from trace and degraded samples holds great promise for combating wildlife crime and supporting legal proceedings where traditional methods fall short.

The expanding role of MS proteomics is laying the groundwork for more advanced and integrated forensic analyses. By connecting proteomics with fields such as genomics, toxicology, food safety, surveillance, and wildlife forensics, the discipline is broadening its investigative reach, pushing the boundaries of what is possible in forensic investigation and evidence analysis.

6. Challenges and limitations

The integration of MS-based proteomics within forensic laboratories is met with significant technical and operational obstacles. These include the necessity for tailored methods compatible with existing mass spectrometry instruments and the complexities of incorporating a multidisciplinary approach, which necessitates specialized software and a high degree of expertise. Despite the proven efficacy of proteomics for identifying body fluids, there is an urgent need for extensive research on its application in real-world scenarios, especially regarding the factors of transfer, persistence, prevalence, and recovery in various environments. While in controlled studies the experimental design is set to vary only those features of interest, in a criminal investigation several relevant parameters can vary simultaneously and in an uncontrolled and unknown manner. Moreover, the forensic analysis of proteins is hampered by the absence of direct sequencing or replication methods for proteins like those used for DNA and RNA, highlighting a gap in the analytical toolkit that requires innovative solutions.

One of the most common scientific challenges in proteomics is the lack of a well-defined "ground truth." Currently, there is no clear reference for what a healthy human proteome should look like, making it difficult to distinguish what changes are caused by normal biological variation and those that result from exposure to external conditions. This issue is especially problematic when estimating the time since deposition of a trace. Once a sample leaves the body, its proteome continues to change, and it can be difficult to distinguish between these alterations being due to the passage of time or simply the transition from an internal to an external environment. To that end it is important to discover proteome markers that show a specific selectivity towards a feature of interest but are robust to other parameters. This will also possibly require the consideration of markers behaving relative to each other.

Upon admission to the forensic laboratory, biological trace evidence may be an unknown sample. This introduces a challenge in accurately quantifying the proteins present in the proteomic analysis using tandem MS. To achieve accurate quantitation of proteins in a sample, it is necessary to include labelled peptide markers in the analysis. However, appropriate candidates cannot be included during the initial screening of unknown samples.

One of the biggest hurdles in a proteomics experiment lies in data analysis which mostly focuses on identifying the proteins present in a sample. This process involves two steps: first, matching the observed spectra to a peptide sequence (a peptide-spectrum match, or PSM), and second, assigning the matched PSMs or peptide sequences to their corresponding proteins. Both steps rely heavily on reference databases. Such databases are of a substantial size, which increases the likelihood of ambiguous matches. This can result in the detection of proteins that are not truly present in the sample, so-called "false positives." In a forensic context, the presence of false positives can be especially problematic and potentially (and literally) misleading. Moreover, the analysis of data varies between laboratories with some considering one peptide sufficient for the identification of a protein [140], and others opting for a threshold of two [141] or three [142] peptides for the same identification. For proteomics to be more widely implemented in forensics it is necessary for these thresholds and protocols to be standardized across institutions. Another major issue is the sheer volume of data generated in proteomic experiments [143]. Vendor raw files can exceed 100 GB per case, far beyond the capacity of many forensic LIMS systems originally designed for much smaller STR files. Future guidelines must account for the long-term storage and management of large-scale proteomics data.

One potential reason proteomics has yet to gain widespread adoption in forensic practice is its comparison to established gold-standard techniques like DNA analysis, which are already deeply embedded in legal systems and forensic workflows. This comparison often overshadows proteomics, making it harder for the technique to gain traction. Additionally, its complexity, particularly in sample preparation and data analysis can be a barrier, as it requires specialized expertise and is often time-consuming. As a result, some researchers question whether it's

worth pursuing, especially when many forensic questions can already be addressed using existing methods. Moving forward, it will be essential not only to showcase specific applications where proteomics adds value but also to emphasize its complementary role alongside traditional forensic techniques. While current advancements, including AI, may not yet allow for individualization of forensic traces based on the proteome, as is possible with DNA, the potential has been recognized by Heaton and Fierro-Monti *et al.* [144,145]. That said, full individualization is less crucial when proteomics is mainly used to generate new investigative leads.

6.1. Ethics and legal admissibility

As aforementioned, proteomics can be implemented in forensic casework to create new investigative leads where traditional methods fall short, such as identifying donors in low DNA samples such as hair, or performing activity-based analysis. Integrating proteomics with forensic science for activity-based analysis and source attribution requires further research and development to achieve the validation necessary to withstand judicial scrutiny.

Ethical guidelines, as with all research, should be strictly adhered to. The risks of handling human biological materials are mitigated by implementing responsible procurement, storage, and data management. Responsible procurement includes collecting samples from volunteers who provide informed consent. It would be necessary for the sampling to occur under known conditions, including any details about the participant's lifestyle or health that can affect the proteome (e.g., smoking habits and diseases). Data management is understood to include safe processing, storing, and sharing of data. Responsible data management includes the anonymization of data, which is of particular importance in proteomics, where common practice is to deposit found peptide sequences in public repositories such as PRIDE [74]. It is possible that biases are introduced using open-source databases. These databases may unevenly represent demographics in the population, such as genders and ethnicities, impacting the relevance and reliability of findings in forensic investigations. To reduce biases in forensic research it is also necessary to choose a participant pool that is representative of the respective population in a given country or region where the crime occurred.

As highlighted by Girod *et al.* [146], avoiding inconsistencies in the handling of proteomic evidence in court requires the use of validated, standardized methods across institutions. For proteomic techniques to be legally admissible under the Daubert criteria, they must achieve broad acceptance within the relevant scientific disciplines. Additionally, the analysis of forensic evidence should be conducted in ISO 17025-accredited laboratories, ensuring that procedures are reliable, reproducible, and meet internationally recognized quality standards.

Currently, there are no standardized guidelines developed for forensic proteomics by official governing bodies. As a result, forensic proteomics must rely on broader, general validation frameworks. However, applicable guidelines do exist, such as those set by the Scientific Working Group for Forensic Toxicology (SWGTOX). For instance, this provides validation criteria for LC-MS platforms, including assessment of accuracy, precision, selectivity, sensitivity, linearity, carryover, and matrix effects. These validation steps can be directly mapped onto proteomic workflows for establishing key metrics such as limits of detection (LOD) and quantification (LOQ) for peptide biomarkers. Additionally, the ANSI/ASB 098-2023 [147] (serves as an update to the guidelines set by SWGTOX) outlines specific requirements for mass spectrometry in forensic toxicology, including parameters for signal-to-noise ratio, mass accuracy windows, and isotopic pattern matching. These well-defined standards offer a foundation that can be adapted for forensic proteomics.

The next critical step would be for the forensic proteomics community to collaboratively develop a set of clear, well-considered guidelines that align with existing forensic toxicology standards. Once formulated, these guidelines should be subjected to review and consensus by the broader scientific and forensic community. If these new standards mirror those already accepted by organizations such as ENFSI, SWGTOX, and ANSI/ASB, there should be no substantive barrier to the legal admissibility of forensic proteomics in court.

One of the major challenges in developing and maintaining forensic proteomics guidelines is the rapid evolution of mass spectrometry instruments, data acquisition methods, and bioinformatics software for analyzing proteomic data. While most guidelines are updated only every 5–10 years, advancements in instrumentation, workflows, and software occur much more frequently. As a result, current guidelines may quickly become outdated and fail to reflect the impact of new technologies on data acquisition, analysis, and interpretation. Unlike toxicology where the biological matrices are most commonly blood and urine and the most standardized tests are for alcohol and narcotics, in proteomics, evidence can originate from a wide variety of complex biological matrices, including hair, bone, latent fingerprints, and decomposed tissue. Each of these sample types requires unique extraction and cleanup protocols, yet the overarching guidelines must remain coherent and applicable across all use cases. For example, recent optimization studies on bone samples have shown that simply switching the demineralization reagent can reduce artificial deamidation rates by half, underscoring that a universal, "one-size-fits-all" protocol is not feasible [148]. Under the Daubert standard, the criteria for assessing the admissibility of expert testimony is the known or potential error rate of the method [149]. This evaluates the reliability of the technique based on how consistently it produces accurate results. In proteomics, this involves modeling false discovery rates, likelihood ratios, and measurement uncertainty across thousands of spectra, a complex task that is still in development and not yet refined enough to define clear numerical thresholds [150]. Gittelson *et al.* have very eloquently provided a "Practical Guide for the Formulation of Propositions in the Bayesian Approach to DNA Evidence Interpretation in an Adversarial Environment," a similar approach can and must be used for the progression of forensic proteomics [79].

In summary, the main challenges arise from proteomics being both highly data-intensive and matrix-diverse, making it especially difficult to develop and implement universally accepted guidelines.

7. Conclusion

Although the use of MS-based proteomics in forensic science is under-explored and under-utilized, it has shown significant potential, particularly for source attribution and reconstruction of events. Proteins as biomolecules offer slight advantages over DNA due to their abundance, and stability, making them resilient to various external factors. The resilience of these biomolecules enables the analysis of degraded traces, which can be crucial in forensic investigations. Despite this technique not being suitable for every scenario, we highlight three success stories in forensic casework to emphasize its promise. These examples also emphasize the interdisciplinary nature of forensic science, which often draws on multiple branches of knowledge to answer questions in a legal context. In the same way, forensic proteomics offers versatility: it can identify perpetrators in sexual assault cases, detect toxins in suspicious deaths, and even uncover which animal or plant species were used to create 600-year-old garments.

The integration of proteomics with other forensic disciplines, such as genomics, toxicology, venomics, and wildlife forensics creates synergies that can aid complex case investigations. Breakthroughs in protocols for sample treatment such as the ability to use samples even after Proteinase K treatment and the simultaneous extraction of DNA and proteins from a single trace can significantly enhance the depth and comprehensiveness of forensic analyses. Future advancements may reveal lifestyle attributes of the donor, such as alcohol and drug consumption, aiding in narrowing down suspect pools and streamlining investigations. However, integrating proteomics into forensic laboratories currently faces significant technical, operational and legal challenges, including the need for

specialized methods compatible with existing protocols and mass spectrometry instruments, tailored software and algorithms, and highly specialized expertise. Ethical and legal frameworks must address privacy concerns and ensure compliance with regulations. Addressing these challenges through continued research and technological advancements is crucial for fully recognizing the potential of proteomics in forensic science.

To conclude, this review proposes that proteomics has the potential to contribute meaningfully to what we refer to as *ultimate forensic reconstruction*. While this concept may initially appear ambitious, our intent is not to position proteomics as a standalone discipline within forensic science. Rather, its true value lies in its ability to complement and integrate with various established disciplines, as illustrated through the different cases. Proteomics as a complementary tool can enhance the resolution and depth of forensic trace interpretation. By embedding proteomics within a multidisciplinary framework, we can move toward a more holistic approach to forensic trace investigation.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT3.5 and 4 for editing, proofreading, and paraphrasing sections of written text, all with the purpose of increasing readability. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Shirin Alex: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Thomas P. Shehata:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Andreea Iris Gergely:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Marcel de Puit:** Supervision.

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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